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Nuclear magnetic resonance spectra of lipoteichoic acid

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Lipoteichoic acid acids with a range of chemical compositions have been studied using ^{1}H; ^{13}C- and ^{31}P-nuclear magnetic resonance. Proton spectroscopy provided a rapid method for demonstrating whether alanine in a sample is covalently bound to the polyglycerophosphate chains and for monitoring hydrolysis of alanine. The nature of sugar substituents can be determined, with some limitations, from the ^{13}C spectra, and the proportions of glycerol residues substituted by alanine and sugar can be measured. The ^{31}P spectra of lipoteichoic acid provided information about both the degree of substitution and the distribution of the substituent along the polyglycerophosphate chain, except when the substituent was galactose. The polyglycerophosphate chains were shown to undergo rapid internal rotation and no evidence for tertiary structure was found either in the presence or absence of magnesium ions. Magnesium ions exchange rapidly between the bound and free state and the binding constant to lipoteichoic acid of 64 M^{-1} is typical for monophosphates in aqueous solution. There was no evidence that alanine substitution affects the binding constant for magnesium ions.

Introduction

Lipoteichoic acids are a group of structurally related amphiphilic polymers found as cell membrane components in a wide range of Gram-positive bacteria. The hydrophilic portion of the molecule is typically a 1,3-phosphodiester-linked polymer of glycerophosphate, which may be variously substituted at the C-2 position of the glycerol residues with sugars in glycosidic linkage or alanine in ester linkage. The hydrophobic region of the molecule is either a glycolipid or phosphatidylglycolipid linked covalently to the phosphomonoester end of the polymer. The lipidic part is intercalated into the outer half of the bilayer cell membrane and provides a membrane anchor for the molecule. Lipoteichoic acid is also found as a soluble excreted product in cultures of Gram-positive bacteria [1,2].

It has been variously suggested that the function of lipoteichoic acid is sequestration of positive ions like Mg^{2+} [3–5]; modulation of the activity of autolytic enzymes [6] or to serve as carriers for cell wall teichoic acid [7–9]. Furthermore, it has been proposed that the degree of alanine substitution controls functions like ion binding or autolytic activity [3,6]. Knowledge of the precise molecular structure of lipoteichoic acids is also important for an understanding of their immunological activity [2,10,11].

Painstaking chemical investigations have demonstrated unambiguously that, in lipoteichoic acid from *Lactobacillus casei*, alanine is covalently bound to the polymer [3] and is randomly distributed along it [6]. Nuclear magnetic resonance can

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be a convenient tool for answering similar questions, but it has not been extensively applied to the study of lipoteichoic acid. The only reported magnetic resonance study of intact lipoteichoic acid is that for material isolated from *Streptococcus antibioticus* [12], although ^{13}C-NMR has been used to identify hydrolysis fragments from *Bacillus subtilus* var. *niger* lipoteichoic acid [13]. In this work we have examined the ^{1}H-, ^{31}P- and ^{13}C-NMR spectra of a range of lipoteichoic acid samples with differing levels of substitution, and the effect of magnesium on these spectra.

Experimental

Preparation of purified lipoteichoic acid

Purified lipoteichoic acid was prepared by hot phenol extraction [14] of cells from stationary phase cultures, grown with 2% glucose (w/v), as described previously: *Lactobacillus casei* NCTC 6375 [15,16], *Lactobacillus fermentum* NCTC 6991 [16,17], *Streptococcus faecium* ATCC 9790 and *Streptococcus lactis* ATCC 9936 [18]. Crude extracts were purified by repeated chromatography on columns packed with Ultragel AcA22 (LKB, Stockholm, Sweden) [17,18]. All preparations were analysed for phosphorus [14], glucose [19], galactose [20] and D-alanine [21] after acid hydrolysis [17,18].

Nuclear magnetic resonance

NMR spectra were recorded using a Varian XL-200 spectrometer, operating at 200 MHz for protons. Approx. 40 mg of sample were dissolved in 2 ml of $^{2}H_2O$ and the pH adjusted to 6.5. Smaller amounts were used for ^{1}H spectra. Disodium EDTA (final concentration, 10–20 mM) was added to samples used for ^{31}P spectroscopy in order to prevent broadening by paramagnetic cations. The sample was maintained at 30 °C, unless otherwise indicated.

Phosphorus chemical shifts were measured as parts per million (ppm) downfield from external phosphoric acid in a spherical container [22]. Proton chemical shifts were measured relative to ^{2}HHO at 4.815 ppm (20 °C, pH 7.0) and carbon relative to internal methanol at 50.04 ppm.

Results

Composition

The chemical structures of the lipoteichoic acids, which have been reported previously [15,17,18,23], range from one with almost no substituents on the polyglycerophosphate chain, through those with predominantly one substituent (alanine or kojibiose) to ones with mixed substituents. The chemical compositions of the samples used in the present study are summarised in Table I.

^{1}H spectra

Proton spectra of lipoteichoic acid in water are able to provide useful qualitative information about the type of substitution of the polyglycerophosphate chains and the nature of the lipids. In water lipoteichoic acid aggregates as micelles [2,23], and it was therefore not surprising to find that restricted molecular motion caused broadening of many of the peaks in the spectrum of lipoteichoic acid from *L. fermentum* (Fig. 1). Only signals from the alanyl substituents were sufficiently sharp for proton-proton spin splitting to be resolved.

Assignment of the proton spectra was achieved by comparison with the spectra of chemical analogues [24,25] and the observed chemical shifts are reported in Table II.

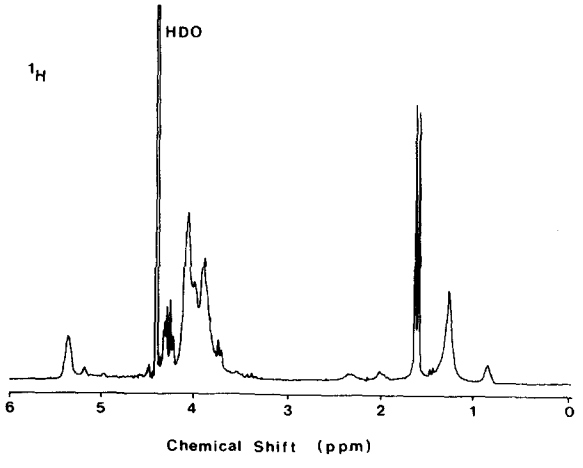

Fig. 1. Proton NMR spectrum of lipoteichoic acid from *L. fermentum* in $^{2}H_2O$ at 60 °C. The spectrum was referenced to the ^{2}HHO signal at 4.428 ppm.

TABLE I

CHEMICAL COMPOSITION OF LIPOTEICHOIC ACIDS

Strain	Mole ratios				Substituent	Ref.
	phosphate	glucose [a]	galactose [a]	alanine		
L. casei NCTC 6375	1.00	0.08	0.04	0.06	alanine	15
L. fermentum, NCTC 6991	1.00	0.06	0.12	0.46	alanine, α-galactose, α-Gal-(1,2)-Glc	17
S. faecium, ATCC 9790 (batch A) [b]	1.00	1.02	0.00	–	alanine, kojibiose	23
S. faecium, ATCC 9790 (batch B)	1.00	1.50	0.00	0.25	alanine, kojibiose	23
S. lactis, ATCC 9936 (batch C)	1.00	0.12	0.46	0.14	alanine, α-galactose	18
S. lactis, ATCC 9936 (batch D)	1.00	0.12	0.78	0.16	alanine, α-galactose	18

[a] Glucose and galactose occur as part of the glycolipid as well as on the polyglycerophosphate chains.
[b] Variations in composition are due to changes in culture conditions and composition of the medium.

Resonances for covalently bound alanine occurred slightly downfield from those for free alanine. In the spectrum of partially hydrolysed lipoteichoic acid from *L. fermentum*, one could distinguish clearly the methyl signal for free alanine at 1.48 ppm from that for covalently bound alanine at 1.64 ppm. The methyl doublet for bound alanine was coupled to a quartet at 4.31 ppm as shown by proton decoupling at either frequency. The doublet at 1.48 ppm in the spectrum of partially hydrolysed lipoteichoic acid was decoupled by irradiation near 4.3 ppm, but the methine resonance itself was obscured. The chemical shift for the methyl protons and the approximate chemical shift for the methine proton agreed with those for free alanine and the resonances increased in intensity as hydrolysis proceeded.

TABLE II

CHEMICAL SHIFTS FOR PROTONS IN LIPOTEICHOIC ACID FROM *L. FERMENTUM*

Assignment	Chemical shift (ppm)
Lipid:	
CH_3	0.89
$(CH_2)_n$	1.30
CH_2-C-CO	2.04
CH_2-CO	2.36
cyclopropyl	0.07
	0.70
Glyceryl groups:	
CH_2 and CH	3.94–4.12
CH-O-Ala	5.40
Alanyl groups:	
CH_3	1.64
CH	4.31

Many sugar resonances occur in the region between 3.0 and 4.5 ppm, where the peaks assigned to glycerol protons also appear. The presence of sugar substituents, therefore, effectively prevents quantitative determination of the degree of substitution using proton NMR spectroscopy. For lipoteichoic acid samples without significant amounts of sugar, the extent of alanine substitution was estimated from the ratio of the area of the doublet at 1.64 ppm (counted as three protons) to the sum of the areas of the peaks at 3.94, 4.12 and 5.40 ppm (counted as five protons). The proton spectrum for lipoteichoic acid from *L. fermentum* gave a value of 45 ± 5 for the percentage of glycerol groups with an alanine substituent. This agreed well with the value of 46% obtained by chemical analysis and with an independent estimate of of 51 ± 4% obtained from the ^{31}P-NMR spectroscopy. The reliability of areas obtained from proton spectra was affected by the difficulty of performing reproducible phasing of spectra containing broad lines.

While reasonable estimates of the amount of alanine were obtained, the same was not true for the fatty acids. The length of the lipid chains estimated from the relative areas of the methyl peak at 0.89 ppm and the methylene peak at 1.30 ppm was generally 30–50% too low. The discrepancy is due partly to the lineshapes, which were broad and probably a superposition of Lorentzian shapes [27]. On the other hand, the amount of cyclopropyl fatty acid in lipids of lipo-

amount of cyclopropyl fatty acid in lipids of lipoteichoic acid from *L. casei* was considerably overestimated.

^{13}C spectra

The restricted motion of lipoteichoic acid molecules in micelles does not affect the resolution of ^{13}C spectra as severely as it affects proton spectra. The assignments reported in Table III were again made by comparison with spectra of known compounds and they were supported by determination of the number of protons on each carbon using the INEPT pulse sequence [29].

TABLE III

^{13}C CHEMICAL SHIFTS OF LIPOTEICHOIC ACID

Assignment	Alanine methyl ester	*L. fermentum*	*L. casei*	*S. lactis*	*S. faecium*
Glycerol:					
-$\underline{C}H_2$-CHOH		67.31	67.4	67.39	67.4
-$\underline{C}$HOH		70.52	70.7	70.7	70.6
-$\underline{C}H_2$-CHO-Ala		64.70	64.8	64.75	64.7
-$\underline{C}$HO-Ala		75.27	75.4	75.2	75.3
-$\underline{C}H_2$-CHO-sugar		65.4	–	65.60	66.1
		66.3		66.38	
-$\underline{C}$HO-sugar		–	–	76.28	76.1
Alanine:					
CH_3	16.08	16.37	16.4	16.41	16.5
CH	49.79	49.98	50.1	50.03	50.0
				49.89	
CO	172.27	171.03	–	171.1	171.6
				171.4	
Lipid:					
$(CH_2)_n$		30.66	30.6	30.6	30.8
		35.01	–	35.0	35.4
		25.91	–	26.0	25.6
ω		14.84	15.1	15.0	15.0
$\omega-1$		23.61	23.7	23.7	23.7
$\omega-2$		32.99	32.4	33.1	33.2
-$\underline{C}$-C = C-		28.24	28.2	28.2	28.2
-$\underline{C}$ = $\underline{C}$-		–	–	130.1	130.4
α-D-Galactose:					
C-1		99.01		99.1	
		99.72			
C-2		69.41		69.44	
C-3				70.47	
C-4				70.35	
C-5				72.04	
C-6		62.18		62.20	
α-D-Glucose:					
C-1		98.59		98.88	
				95.8	
				93.5	
C-2				72.6	
C-3				72.6	
				73.9	
C-4				(70.7)	
C-5				72.6	
C-6				61.6	

The glycerol signals were sensitive to the presence of substituents. The assignments reported in Table III were made by comparing samples with different degrees of substitution. For example, the peaks at 64.8 and 75.4 ppm were small in the spectrum of lipoteichoic acid from *L. casei*, but much larger in the spectrum of lipoteichoic acid from *L. fermentum*. The shifts produced by substitution were also found to be consistent with the expected effects of esterification [31]. The resonance for the central carbon moved downfield and the peak for the outer carbons moved upfield as a result of esterification. Thus the glyceryl carbons in lipoteichoic acid from *L. fermentum* give one pair of peaks at 67.3 and 70.5 ppm for residues with no substituent, and a second pair of peaks at 64.7 and 75.3 ppm for residues substituted by alanine (Fig. 2).

The spectra of the two samples from *S. lactis*, which contain significant amounts of galactose, had additional peaks at 65.6, 66.4 and 76.3 ppm (Fig. 3), which were assigned to carbons of the glyceryl residues substituted by galactose. The peaks at 65.6 and 66.4 ppm were shown by the use of the INEPT pulse sequence, to be due to methylene groups. The areas of all three peaks were

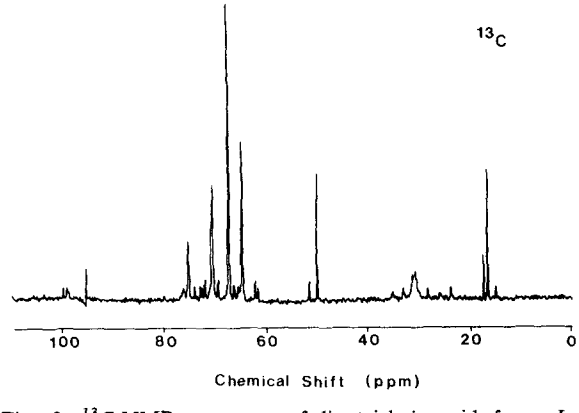

Fig. 2. ^{13}C-NMR spectrum of lipoteichoic acid from *L. fermentum* in ^2H_2O at 20 °C.

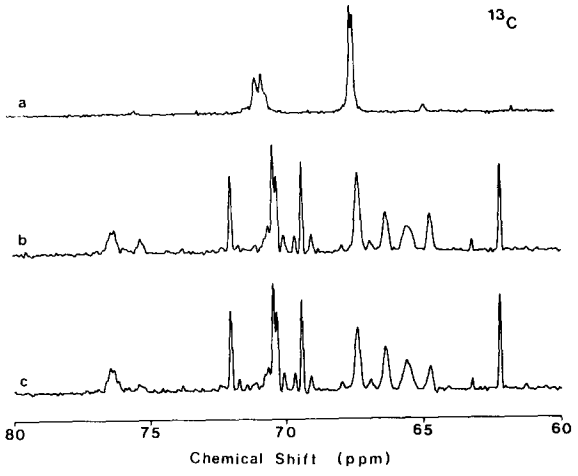

Fig. 3. ^{13}C-NMR spectra of lipoteichoic acid samples in $^{2}H_2O$ at 30 °C. a, *L. casei*; b, *S. lactis*, batch C; c, *S. lactis*, batch D.

approximately equal and it is proposed that the C-1 and C-3 carbons of glycerol are magnetically non-equivalent as a result of the chirality of the galactose substituent. Substitution by kojibiose does not appear to give separate signals for the C-1 and C-3 peaks, but the spectrum of material from *S. faecium* did show broadening of the peak at 66.1 ppm. Lipoteichoic acid from *L. fermentum* also contains a small amount of galactose [17] and small peaks at 65.4 and 66.3 ppm were observed.

In principle, it should be possible to estimate the amount of the various substituents by integration of the corresponding glyceryl peaks in the ^{13}C spectrum. Our spectra were not acquired under the conditions needed for good quantitation, but it has been pointed out [33] that if carbons of similar type are being compared, useful information may still be obtained. The fraction of glycerol residues esterified by alanine and the fraction substituted with either galactose or kojibiose were estimated from carbon NMR spectra and are compared in Table IV with the results of chemical analysis.

TABLE IV

EXTENT OF SUBSTITUTION ESTIMATED BY ^{13}C-NMR

Source of lipoteichoic acid	% substitution			
	^{13}C-NMR		chemical analysis	
	alanine [a]	sugar [b]	alanine	sugar
L. casei	0.05	0	0.06	0
L. fermentum	0.46	0.06	0.46	0.06
S. faecium (A)	< 0.05	0.3	n.d.	0.44
S. faecium (B)	0.28	0.29	0.25	0.71
S. lactis (C)	0.17	0.42	0.14	0.46
S. lactis (D)	0.12	0.49	0.16	0.78

[a] Estimated from the area of the peak at 64.7 ppm divided by the sum of the areas of the peaks at 64.7, 65.5 and 66.3 (or 66.1) and 67.4 ppm (see Table III).

[b] Estimated from the sum of the areas of the peaks at 65.5 and 66.3 (or 66.1) divided by the sum of the areas of the peaks at 64.7, 65.5 and 66.3 (or 66.1) ppm (see Table III).

In four cases agreement is good, but NMR provides a much lower estimate for the extent of substitution for the two samples containing high levels of sugar. The amount of sugar can also be estimated from the intensity of sugar resonances themselves. The area of the C-6 glucose peak in the spectrum of lipoteichoic acid from *S. faecium* and the areas of the C-1, C-5 and C-6 peaks of galactose in the spectrum of material from *S. lactis*, batch D, produced values for the extent of sugar substitution that agreed to within a few percent with estimates based on the relative areas of the glycerol peaks. It must, therefore, be concluded that an estimate based on a ^{13}C-NMR spectrum accurately reflects the degree of substitution of the material producing the spectrum, but that only a sub-population of the sample is observed. Lipoteichoic acid chains with very high levels sugar substitution must preferentially segregate into particles too large for observation by NMR.

Spin-spin coupling

Spin-spin interaction between the glycerol carbons and nearby phosphorus nuclei was clearly evident for lipoteichoic acid with lightly substituted polyglycerophosphate chains. The two bond phosphorus-carbon coupling constant for the CH_2-O doublet was 5.5 ± 0.3 Hz in the spectrum of lipoteichoic acid from *L. casei*. The CH-O signal was a triplet with a 8.4 ± 0.3 Hz separation. Small splittings were also observed in some spectra of lipoteichoic acid from *L. fermentum*, but the resolution was poorer.

The three bond carbon-phosphorus coupling constant was used to estimate the rotamer populations about the C1-O and C3-O bonds [35]. There was a preference for the rotamer in which the

phosphorus and C-2 atoms are *trans*. Unhindered rotation around the carbon-oxygen bond would result in a 33% population of this rotamer, but the value obtained from $^3J_{CH}$ was 53%, using $^3J_{PC}$ (*trans*) = 14.0 Hz and $^3J_{PC}$ (*gauche*) = 2.0 Hz [35]. If smaller values for $^3J_{PC}$ (*trans*) are used, the calculated population of the *trans* rotamer is even higher.

The addition of Mg^{2+} ions produced no measurable alteration of the spin-spin splitting, indicating that the average conformation around the C1-O and C3-O bonds was unchanged. There was a small change in the chemical shift of the glycerol peaks and in the presence of 100 mM Mg^{2+} the CH_2-O and the CH-O signals moved upfield by 0.07 and 0.17 ppm, respectively.

Molecular motion

Relaxation times and nuclear Overhauser enhancement factors for ^{13}C spectra can provide information about molecular motion [34]. Nuclear Overhauser enhancement values, η, were measured for the glycerol signals of lipoteichoic acid from *L. casei*. The observed values of 1.6 ± 0.1 and 1.7 ± 0.2, are close to the theoretical limit of 1.98 [34]. Similarly, the ratio of the measured spin-lattice relaxation times, T_1, 0.32 ± 0.02 and 0.50 ± 0.07 s for the CH_2-O and CH-O carbons respectively, is close to the factor of two expected if relaxation is caused entirely by dipolar interaction between the carbon nucleus and protons attached directly to it. The correlation time may, therefore, be estimated using the assumption that dipolar interaction is the dominant relaxation mechanism [34]. The value obtained from the T_1 measurements, $7 \cdot 10^{-11}$ s, shows that the segments of the polyglycerophosphate chain are rotating rapidly.

^{31}P spectra

The phosphorus spectra contained a considerable amount of detail and the chemical shifts of the phosphates depended on the substituents on neighbouring glycerol groups. The phosphorus spectrum of the least substituted lipoteichoic acid sample, that from *L. casei*, consisted of a single large peak at 0.96 ppm and small ones at 0.57 and 0.62 ppm. The peak at 0.96 ppm is assigned to phosphates with unsubstituted glycerol on either side. The other signals are produced by phosphates with alanyl ester on one of the adjacent glycerols. The area of the small peaks agreed with that expected from the alanine content of the sample. The difference in chemical shift is due to the different interactions between the chiral alanine and the phosphates on either side of it.

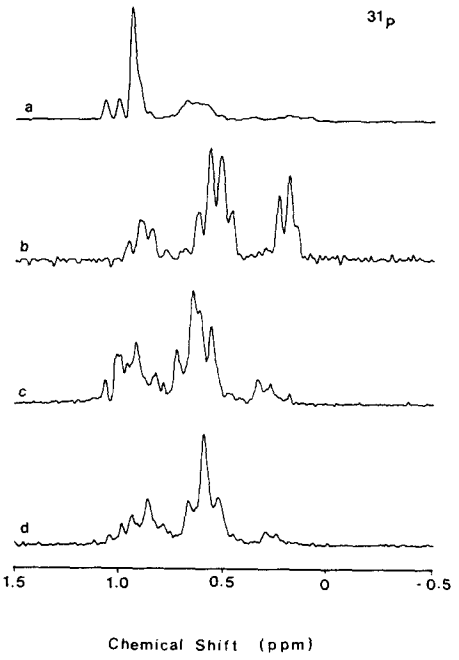

Fig. 4. ^{31}P-NMR spectra of lipoteichoic acid samples in ^2H_2O at 30 °C in the presence of 10 mM EDTA. a, *S. faecium*; b, *L. fermentum*; c, *S. lactis*, batch C and d, *S. lactis*, batch D.

The spectrum of lipoteichoic acid from *L. fermentum*, (Fig. 4), consists of three groups of peaks at 0.91, 0.56 and 0.21 ppm. The new group at 0.21 ppm is presumably due to phosphates with alanine on the glycerols at either side. The assignments were consistent with changes in the relative intensity of each group produced by mild alkaline hydrolysis of the alanyl esters. After complete hydrolysis, only a single large peak from the low field group remained, apart from very small signals at 0.66 and 0.85 ppm due to galactose substitution. The small peaks will be discussed in more detail below.

Closer inspection revealed four peaks in the group at 0.56 ppm and two in each of the other two groups. The relative intensity of the lines within each group varied with degree of alanine

TABLE V

DEGREE OF SUBSTITUTION, α, FROM ^{31}P PEAK AREAS AND COMPARISON OF OBSERVED PEAK AREAS WITH THOSE PREDICTED FOR RANDOM SUBSTITUTION

Strain and batch	α		Peak areas					
	from	from	observed			calculated		
	^{31}P	^{13}C	*A*	*B*	*C*	*A*	*B*	*C*
L. casei	0.05	0.05	0	11	89	0	10	89
L. fermentum	0.51	0.49	24	55	21	26	50	24
S. faecium (A)	0.28	0.3	9	36	56	9	42	49
S. faecium (B)	0.52	0.57	27	50	23	32	49	18
S. lactis (C)	0.37	0.59	11	54	36	–	–	– [a]
S. lactis (D)	0.39	0.61	8	63	29	–	–	– [a]

[a] Galactose substitution causes overlap of the chemical shift ranges of the phosphates with one and two neighbouring substituents. Simple interpretation of the peak areas is not then possible (see text).

substitution, which suggests that the chemical shifts are affected by more distant alanine residues as well.

The general appearance of the spectra of lipoteichoic acid samples from *S. faecium* and *S. lactis* was intermediate between those for the two cases discussed so far. All the spectra contained three groups of peaks, each group having a considerable amount of fine structure that defied detailed interpretation. Part of the difficulty is that the phosphate chemical shift changes produced by galactose substitution differ from those caused by alanine. The small peaks at 0.66 and 0.85 ppm in the spectrum of hydrolysed lipoteichoic acid from *L. fermentum* are further downfield and further apart than the peaks for phosphates near alanyl substituents. (Again it is presumably the chirality of the galactose that produces separate signals for phosphates on C-1 and C-3 of the substituted glycerol.)

Distribution of the substituents along the chain

The areas of the three groups of peaks can be used to estimate both the extent of substitution and the distribution of the substituents along the polyglycerophosphate chain. If the areas of the groups are *A*, *B* and *C*, proceeding from high to low field, the overall degree of substitution, α, is given by

$$\alpha = (A + 0.5B)/(A + B + C) \quad (1)$$

for an infinitely long polymer. This estimate is independent of the pattern of substitution along the polymer. If the substituents are arranged randomly, the peak areas should be be in the ratio $\alpha^2 : 2\alpha(1-\alpha) : (1-\alpha)^2$.

The estimates from the ^{31}P spectra obtained for the overall extent of substitution agree well with those obtained from ^{13}C-NMR for all samples except those from *S. lactis*, which contain significant amounts of galactose. The problem is caused by the large difference in chemical shift for phosphates adjacent to galactose substituents. The three groups of peaks, *A*, *B* and *C*, cover the approximate ranges 0 to 0.3, 0.3 to 0.7 and 0.7 to 1.0 ppm. Both resonances resulting from a single alanine substituent (0.57, 0.62 ppm) appear in region B, but the peaks corresponding to a single galactose substituent (0.66, 0.85 ppm) will be found in two different groups, B and C. The phosphorus spectra of the samples from *S. lactis* are not, therefore, amenable to simple interpretation.

For the remaining samples, the areas of the three groups of peaks may be used to decide whether the distribution of the substituents is random. As may be seen from Table V, the substitution pattern in all cases approximates a random distribution.

Phosphorus-proton spin-spin coupling

The complexity of the ^{31}P spectra made observation of proton spin coupling patterns difficult except for samples with relatively simple chemical structures. The main resonance in the proton coupled spectrum of lipoteichoic acid from

L. casei was split into a quintet by the four adjacent protons. This shows that the average rotational conformation is identical on either side of the phosphate groups. The single, well-defined coupling constant indicates that all phosphates have the same average rotational environment. The three bond proton-phosphorus coupling constant of 5.8 ± 0.1 Hz leads to a value of the 64% for the fraction of time spent in the *trans* rotamer. This agrees quite well with the value obtained from $^3J_{CH}$. The parameters used for these estimates were obtained from glycosidic phosphates [35] and any difference between the two estimates may be due to the choice of these parameters.

Addition of Mg^{2+}

Progressive addition of magnesium sulphate to lipoteichoic acid from *L. casei* and *L. fermentum* produced gradual upfield shifts of the phosphate resonances by as much as 48 hz. The observation of a gradual shift rather than the appearance of second resonance demonstrates that the lifetime of the magnesium-phosphate complex is short (< 20 ms).

The fraction of time spent in the complexed state, f, is given by

$$f = \frac{\sigma_0 - \sigma}{\sigma_0 - \sigma_\infty} \qquad (2)$$

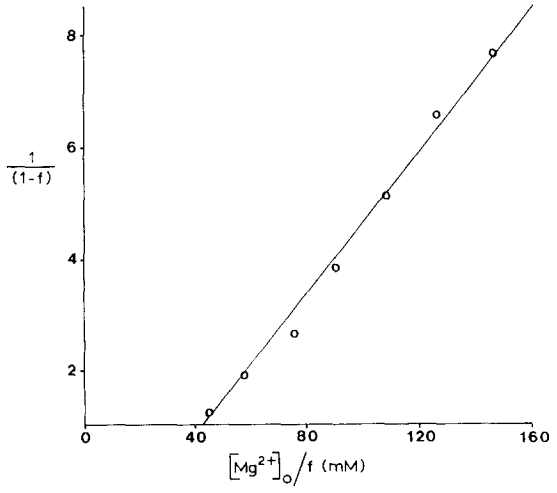

Fig. 5. Dependence of phosphorus chemical shift for lipoteichoic acid from *L. casei* on the concentration of added magnesium.

where σ_0 and σ_∞ are the phosphate chemical shifts in the presence of no magnesium and a large excess. (The quantity f can also be viewed as the fraction of magnesium binding sites occupied at any given time.) In order to obtain the association constant, K, the equilibrium expression must be rearranged in terms of the observed quantities f and M_0, the total concentration of added Mg^{2+} ions. If P_0 is the total phosphate concentration, the concentration of free magnesium is $(M_0 - fP_0)$ and the equilibrium expression becomes

$$1/(1-f) = KM_0/f - KP_0 \qquad (3)$$

A plot of $(1/1-f)$ versus M_0/f for lipoteichoic acid from *L. casei* (Fig. 5) yielded an association constant of 64 M^{-1} and a value of P_0 in agreement with the amount of phosphate present.

The addition of magnesium ions to lipoteichoic acid from *L. fermentum* produced shifts of all three groups of peaks, accompanied by broadening of the spectral lines. The broadening was not due to paramagnetic impurities because the measured value for the spin-lattice relaxation time (2.4 ± 0.1 s) was much larger than the reciprocal line width, $1/\pi w$ (0.04 s). The magnesium-induced shifts were greatest for phosphates with no adjacent alanines, and least for those with two alanyl neighbours. It can be shown that the ratio of the shift of one peak to that of another is independent of the amount of added magnesium if, and only if, the binding constants are equal (see Appendix). As shown in Table VI, the ratios are similar for quite different amounts of magnesium, so the substituents do not affect binding of magnesium to the phosphate groups.

Phosphate dynamics

The addition of magnesium ions had only small effects on the spin-lattice and spin-spin relaxation times for the phosphate signal of lipoteichoic acid from *L. casei*. The spin-lattice relaxation time, T_1, decreased slightly from 4.66 ± 0.07 s with no magnesium ($f = 0$), to 3.16 ± 0.03 s when $f = 0.75$. The spin-spin relaxation time, T_2, changed by a larger factor, from 0.9 ± 0.1 s to 0.23 ± 0.02 s. All three groups of peaks in the spectrum of lipoteichoic acid from *L. fermentum* behaved simi-

TABLE VI

RATIO OF CHANGES IN ^{31}P CHEMICAL SHIFT OF PHOSPHATE GROUPS IN LIPOTEICHOIC ACID FROM *L. FERMENTUM* PRODUCED BY ADDITION OF Mg^{2+}

A, B, C refer to phosphates with two, one and no adjacent alanine residues.

f (approx.)	$\frac{\sigma_0(A)-\sigma(A)}{\sigma_0(B)-\sigma(B)}$	$\frac{\sigma_0(A)-\sigma(A)}{\sigma_0(C)-\sigma(C)}$
0.3	1.3	3.2
0.6	1.4	2.9

larly. The T_1 and T_2 values in the presence of magnesium ($f = 0.6$), were 2.4 ± 0.1 s and 0.40 ± 0.03 s, respectively.

It was expected that chemical shift anisotropy would be slightly more efficient than proton-phosphorus dipolar interaction at causing spin-lattice relaxation in the magnetic field used [36]. Both interactions give similar estimates for the rotational correlation time based on the observed T_1 value. The nuclear Overhauser enhancement value of 0.42 was used to discriminate between the two different values for the correlation time that are consistent with a given value of T_1 [36]. Assuming that the magnitude for the chemical shift anisotropy is similar to that for phosphodiesters in DNA [36], we estimate that the correlation time is approx. $3 \cdot 10^{-10}$ s. This value is of the same order of magnitude as that obtained for the glycerol carbons from ^{13}C relaxation measurements. Magnesium ions affect the rotational correlation time by less than a factor of two.

The short correlation time leads us to expect that $T_2 \approx T_1$ [36], which clearly it is not. Furthermore, the lines are inhomogeneously broadened; that is the peak-widths are greater than $1/\pi T_2$. Each peak, therefore, consists of overlapping signals with different chemical shifts. Such differences could be produced by heterogeneity in the size of the micelles. If, in addition, the phosphates could move between micelles, the measured T_2 value might be less than T_1.

Discussion

The effects on ^1H and ^{13}C chemical shifts produced by substituents on the glycerol residues are readily understood in terms of normal electronegativity changes, but it is less clear that such effects should extend to phosphorus nuclei, which are much further from the site of substitution. It is possible that hydrogen bonding, which is known to cause changes in phosphate chemical shifts [37,38], occurs between the phosphates and the substituents. Hydrogen bonding could also account for the sensitivity of the chemical shift to more distant substituents. The amount of interaction between a phosphate and an adjacent alanyl ester may depend on the degree of competition with the interaction involving the next phosphate along the chain. This interaction may, in turn, depend on whether the next glycerol is esterified.

There is no evidence from this study for tertiary structure in the polyglycerophosphate chains. The ^{31}P spectrum of lipoteichoic acid from *L. casei* and *L. fermentum* showed (i) that the phosphates were in rapid motion, (ii) the average rotational conformation was the same either side of the phosphate, (iii) all phosphates have the same average rotational conformation, and (iv) all phosphate groups interact equally with magnesium ions. The only observation suggesting any difference between phosphate groups was the increase in inhomogeneous linewidth when magnesium was added to lipoteichoic acid from *L. fermentum*. Because the broadening is inhomogeneous, the different environments must persist for hundreds of milliseconds, but the differences cannot be conformational or the spin-spin coupling would be unresolved. One possible explanation, that has been suggested earlier, is that the aggregates are polydisperse.

The value of 64 M^{-1} for the association constant with magnesium is close to the value 60 M^{-1} reported for the binding of magnesium to glycerol phosphate, but is two and three orders of magnitude smaller than the association constants for binding to the di- and triphosphates, ADP and ATP [39]. This is further evidence that the phosphates in lipoteichoic acid act independently and not co-operatively.

The absence of a detectable effect of alanine on the magnesium association constant is contrary to the intuitive expectation that alanine should alter the net charge on the polymer [3]. This expectation fails to take into account the ionic strength of

the solution. Mobile counter-ions can eliminate electrostatic interaction between ionic groups within a molecule if the groups are further apart than the Debye radius [40]. For our samples, the calculated Debye radius was 0.34 nm when the fraction of phosphates bound to magnesium was 0.75. This is less than the distance between a phosphate and an alanyl amino group in the fully extended molecule, which is approx. 0.6 nm.

It is of interest to ask whether lipoteichoic acid could increase the rate of Mg^{2+} uptake by the cell. Assuming that the polyglycerophosphate chain adopts a random coil conformation, we can use the random-walk model to calculate the expected molecular radius, R.

$$R = (10/3)^{1/2} n^{1/2} l \tag{4}$$

where n is the number of phosphoglycerol repeat units, and l is the average bond length [40]. The effective concentration of phosphate groups, P_0, within the volume occupied by the random coil is given by

$$P_0 = 17.8 n^{-3/2} \text{ mol/l} \tag{5}$$

This is an upper limit, as the volume of substituents and mutual repulsion between the charged phosphates will lead to a greater volume than predicted by the model. Random walk models generally underestimate molecular size and the NMR experiments showed that there was a preference for extended chain conformations. A lower limit may be obtained by assuming that the phosphates sample uniformly a hemisphere whose radius is equal to the length of the fully extended teichoic acid chain. Then P_0 is given by

$$P_0 = 1.67 n^{-2} \text{ mol/l} \tag{6}$$

For concentrations of Mg^{2+} less than $1/K$, the ratio of bound to free magnesium is equal to KP_0. If $n = 30$, the effective concentration of phosphate of lies between 0.002 M and 0.11 M and the ratio of bound to free magnesium between 0.14 and 7.6. The concentration of bound magnesium is, therefore, little different from that in solution.

This makes it unlikely that lipoteichoic acid assists magnesium uptake and even less likely that the function of alanine substitution is modulation of the magnesium binding power of lipoteichoic acid.

Appendix

The measurement of individual binding constants for magnesium ions to different phosphate groups in lipoteichoic acid requires chemical shift information for a large range of concentrations of magnesium. It is possible, however, to compare binding constants when much less information is available. If there are two binding sites with equilibrium constants K_a and K_b, the fraction of sites occupied can be related to the observed chemical shifts by means of Eqn. 2.

$$f_a = \frac{\sigma_0(A) - \sigma(A)}{\sigma(A) - \sigma_\infty(A)}$$

with a similar equation for f_b. The ratio of magnesium-induced shifts for the two phosphates is then

$$\frac{\sigma_0(A) - \sigma(A)}{\sigma_0(B) - \sigma(B)} = \frac{\sigma_0(A) - \sigma_\infty(A)}{\sigma_0(B) - \sigma_\infty(B)} \cdot \frac{f_a}{f_b} = \Delta \cdot \frac{f_a}{f_b}$$

where Δ is a constant. Using the relationships

$$K_a = \frac{f_a}{(1 - f_a) M}$$

and

$$K_b = \frac{f_b}{(1 - f_b) M}$$

where M is the concentration of unbound magnesium, it can be shown that

$$\frac{f_a}{f_b} = 1 + \left(\frac{K_a}{K_b} - 1\right)(1 - f_a)$$

$$\frac{\sigma_0(A) - \sigma(A)}{\sigma_0(B) - \sigma(B)} = \Delta \cdot \left[1 + \left(\frac{K_a}{K_b} - 1\right)(1 - f_a)\right]$$

Hence the ratio of chemical shifts for two peaks will be independent of the amount of added magnesium, that is independent of f_a, if, and only if, $K_a = K_b$.

The sensitivity of this test can be estimated by calculating the change in the ratio that would occur between $f_a = 0.3$ and $f_a = 0.6$ if $K_a = 2K_b$ or $0.5K_b$.

$\frac{K_a}{K_b}$	$\frac{\sigma_0(A) - \sigma(A)}{\sigma_0(B) - \sigma(B)}$	$\frac{\sigma_0(A) - \sigma(A)}{\sigma_0(C) - \sigma(C)}$
1.0	1.3	3.2
0.5	1.6	3.9
2.0	1.1	2.6

Given that a factor of two is a rather small difference in equilibrium constants, the test is reasonably sensitive.

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