Biochimica et Biophysica Acta 821 (1985) 179–194
Elsevier

BBA 72812

Analytical studies of lipopolysaccharide and its derivatives from *Salmonella minnesota* R595. I. Phosphorus magnetic resonance spectra

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(Received June 17th, 1985)

Key words: Lipopolysaccharide; ^{31}P-NMR; (*S. minnesota*)

The ^{31}P nuclear magnetic resonance spectrum of lipopolysaccharide from *Salmonella minnesota* R595 shows that the pK_a and chemical shift values of the phosphate groups are very sensitive to changes in molecular structure. The presence of 3′ and/or 6′ substituents can be determined from the spectra of the 4′-phosphates and the type of 4′ or 6′ substituent affects the phosphates. When cells are grown in nutrient broth, the 4′ position in the lipopolysaccharide is substituted with as much as 91% aminoarabinose phosphate, with the remaining 9% as monophosphate (with 3′ acyl substituent). When L.B. medium is used, 52% of the 4′ position is substituted with phosphomonoester (with accompanying 3′-acyl group), with smaller amounts of monoester (with no 3′ substituent) and phosphodiester. Phosphate substitution at the 1 position is incomplete when cells are grown in nutrient broth. A detailed analysis is made of the chemical and physical parameters which influence pK_a and chemical shift of phosphates. The conversion of 4′-phosphodiester to 4′-phosphomonoester and 4′-dephospho products with different hydrazine treatments was also studied.

Introduction

Lipopolysaccharide is an important component of the outer membrane of Gram-negative bacteria. Structures resembling the outer membrane have been formed by reconstitution of partially purified components [1–3]. But recent observations have shown that lipopolysaccharide is more than just a structural component of the membrane; specific interactions occur between lipopolysaccharide and proteins, as illustrated by the following observations. (i) The amount of protein in the outer membrane is affected by changes in the structure of the lipopolysaccharide [4–8], and the expression of structural genes for these proteins has been correlated directly with the ability of the protein to bind to host lipopolysaccharide [9]. (ii) Porin proteins also form specific complexes with lipopolysaccharide [10,11] and its presence is necessary for the activity of porins in reconstituted systems [12,13]. (iii) Similar interactions with peripheral proteins have been observed and are thought to be responsible for the specificity of the dicarboxylate uptake system [14] and resistance to antibiotics [15]. If changes in the structure of lipopolysaccharide can regulate the synthesis of proteins via a feedback mechanism, as has been suggested [16], an understanding of the process will require detailed knowledge of the molecular structure.

These observations have reawakened interest in the molecular structure and microheterogeneity of lipopolysaccharide. Phosphorus nuclear magnetic resonance has been used successfully to study the

Abbreviations: KDO, 3-deoxy-D-*manno*-octulosonic acid; SDS, sodium dodecyl sulphate; NMR, nuclear magnetic resonance.

phosphate substitution pattern in material from rough strains of *Salmonella minnesota* and *Escherichia coli* [17–19]. A significant degree of heterogeneity was found in both the lipopolysaccharide and its biosynthetic precursor [20]. Complete specification of the heterogeneity, however, requires determination of the amount of each molecular species. With the assistance of improved resolution we have been able to confirm and extend the earlier assignments, and assess the extent of microheterogeneity.

Lipopolysaccharide from *S. minnesota* R595 contains variable amounts of phosphomonoester and 4-amino-4-deoxy-L-arabinosyl diester in the 4′ position, and either monophosphate or ethanolamine pyrophosphate in the 1 position [20]. Material from *E. coli* K12, strain D31 m4, on the other hand, has varying proportions of 1-phosphate and 1-pyrophosphate, but no diesters [18]. We report here the identification of phosphorus NMR signals from molecules containing all possible combinations of 1 and 4′ substituents and the use of this information to assess the completeness of phosphorylation of lipopolysaccharide from *S. minnesota* R595 and substitution by other substituents, particularly fatty acids.

Materials and Methods

Bacteria and lipopolysaccharide. S. minnesota R595 was grown overnight to stationary phase by shake culture at 37°C in nutrient broth, L.B. medium [21], and yeast-tryptone medium [22]. Unless otherwise stated, the growth medium was nutrient broth. The harvested cells were washed twice with water and dried by repeated washing with acetone. Lipopolysaccharide was extracted with phenol/chloroform/hexane [23] and purified by centrifugation and extensive electrodialysis as described [24]. Lack of contamination by phospholipids was demonstrated by the absence of unsaturated fatty acids. Samples of purified material from the same strain, grown on yeast-peptone medium [22], were generously provided by O. Lüderitz and C. Galanos of the Max Planck Institut für Immunbiologie, Freiburg.

Hydrazine treatment of lipopolysaccharide. 50 mg of lipopolysaccharide were suspended in 1 ml of anhydrous hydrazine and heated for 30 min at 100°C. The tube was cooled in ice and the mixture heated cautiously with excess acetone. After removal of hydrazine by repeated evaporation with acetone under reduced pressure, the residue was extracted three times with acetone. The acetone-insoluble material was purified either by chromatography on a Sephadex G-25 column using 0.1 M pyridinium acetate buffer (pH 5.3) or by ultrafiltration through an Amicon YM10 membrane in an Amicon cell (model M-3) with five changes of water (3 ml).

Analytical methods. Total phosphate, glucosamine, KDO and fatty acid contents were determined as described [25].

^{31}P-nuclear magnetic resonance. ^{31}P-NMR spectra were recorded on a Varian XL-200 spectrometer operating at 80.984 MHz. Chemical shifts were recorded relative to external 85% phoshoric acid in an ideal spherical container. Actual measurements were made using a cylindrical sample of phosphoric acid, which has an apparent chemical shift of 0.74 ppm in a solenoid magnet [26]. The field was locked to the deuterium signal from the ^2H_2O solvent. Numerous checks indicated that with the field so locked, chemical shifts measured relative to the transmitter frequency were accurate to ± 2 Hz, provided that temperature and, to a lesser extent, pH are fixed. The majority of spectra were, therefore, measured relative to the transmitter frequency and corrections for temperature and pH were made with the aid of calibration curves obtained previously.

All samples contained 5–10 mM sodium ethylenediamine tetraacetate. Spectra were obtained at 45°C and the proton decoupler power was kept to a minimum to avoid heating of the sample. Titration curves were fitted to the equation

$$\mathrm{pH} = \mathrm{p}K_a + \frac{1}{n} \log \frac{\sigma - \sigma_{BH}}{\sigma_B - \sigma}$$

where σ is the observed chemical shift, σ_{BH}, σ_B are the chemical shifts for the protonated and deprotonated species, and n is the Hill coefficient [27]. Most of the data could be fitted using a value of unity for n and other values for n were used only when the simpler equation was unsatisfactory. No corrections were made for the effect of ^2H_2O on the electrode.

For quantitative measurements, spectra were obtained using gated decoupling, a pulse angle of 70° and a pulse repetition time of 4 s. A progressive saturation [28] study indicated that the spin-lattice time, T_1, for all peaks in the spectrum of partially deacylated lipopolysaccharide was about 2 s. The relaxation times for unmodified lipopolysaccharide were much shorter. The above conditions are, therefore, a compromise that give good signal-to-noise ratios in 15 h accumulation with little intensity distortion. The similarity of the T_1 values means that the relative areas under the peaks are even less sensitive to the choice of experimental conditions than the absolute values. A check on the effect of shorter pulse repetition times on the relative areas for a representative sample showed that the conditions had been chosen quite conservatively. The areas obtained agreed well with the results of chemical analysis, which are reported in an accompanying paper.

Results

Hydrazine-treated lipopolysaccharide. The main use of hydrazine in lipopolysaccharide analysis has been for characterization of the fatty acid linkage types [29]. Its particular value in NMR studies is in obtaining a less aggregated preparation [17].

(i) Effect of aggregate size on NMR spectrum. The spectrum of hydrazine-treated lipopolysaccharide dispersed in ^2H_2O contained a mixture of sharp and broader lines. The area under the broad lines was 85% of the total area. The pH dependence of the chemical shifts for the broad lines gave Hill coefficients less than one, indicating negative cooperativity and the formation of aggregates.

Different pK_a values should not, however, affect diesters in the pH range studied, but both sharp and broad components were observed for the diester signal near −2 ppm. The broad component was also unusual in that its chemical shift increased linearly by 0.44 ppm as the pH was changed from 5 to 10. Aggregation therefore produces heterogeneity of chemical shift as well as of pK_a. It may be possible to use this behaviour as a criterion for detecting aggregation.

The sharp monophosphate signals displayed the titration behaviour expected for single-step protonation, while the sharp diester peak was independent of pH. Measured chemical shifts and pK_a values are reported in Table I. The behaviour of the sharp peaks was similar to that of the peaks observed in the presence of surfactant.

The addition of 2% deoxycholate produced much sharper spectra, and the improved resolution revealed previously undetected complexity. Spectra of a fresh sample, and one which has been partially hydrolysed at ph 5.1, are shown in Fig. 1. The three main groups of lines can be assigned as 4′-phosphodiester, 4′-monoester and 1-monoester. In some samples small amounts of pyrophosphate were also observed, but most of the pyrophosphate is removed during hydrazinolysis [25]. The assignments were made using evidence similar to that employed in previous studies [17,18].

(ii) 4′-Diester. The chemical shift of the signal near −2 ppm is constant between pH 4 and 11, and alkaline hydrolysis (at 100°C, pH 12) resulted in the disappearance of this peak. It is thus confirmed as the 4-amino-4-deoxy-L-arabinosyl phosphodiester in the 4′ position, assigned previously in Mühlradt et al. [17].

The same authors used painstaking chemical

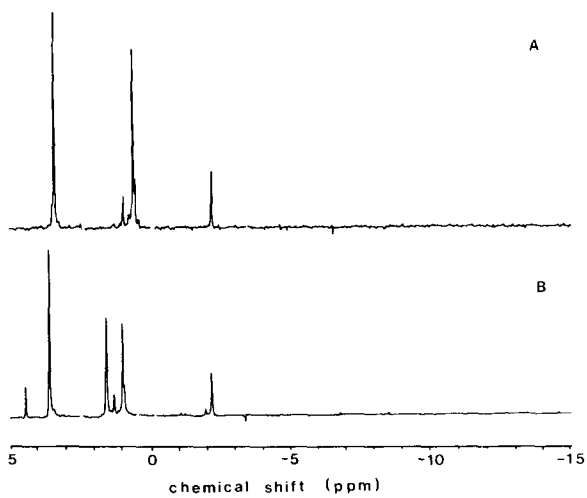

Fig. 1. ^{31}P-NMR spectra of hydrazine-treated lipopolysaccharide from *S. minnesota* R595 at 45°C in ^2H_2O with 3% sodium taurodeoxycholate and 5 mm ethylenediamine tetraacetate; (A) measured at pH 7.7 before hydrolysis; (B) measured at pH 7.9 after partial hydrolysis at 45°C, pH 5.1.

TABLE I

CHEMICAL SHIFT AND pK_a VALUES FOR THE PHOSPHATES OF HYDRAZINE-TREATED LIPOPOLYSACCHARIDE AND ITS DERIVATIVES

Assignment				Chemical shift		pK_a
phosphate resonance	number of KDO groups	other phosphates	surfactant	anion	dianion	
1-Monoester	2	4′-monoester	–	−1.91	2.57	7.4 [a]
1-Monoester	2	4′-monoester	DOC	–	2.28	–
1-Monoester	2	4′-monoester	TDOC	−1.54	2.58	7.40
1-Monoester	2	4′-diester	TDOC	−1.54	2.50	7.44
1-Monoester	2	–	TDOC	−1.47	2.58	7.25
1-Monoester	1	4′-monoester	TDOC	−1.51	2.10	6.95
1-Monoester	1	4′-diester	TDOC	−1.51	2.10	6.93
4′-Monoester	2	1-monoester	–	−0.09	4.07	6.75
4′-Monoester	2	1-monoester	DOC	–	4.00	–
4′-Monoester	2	1-monoester	TDOC	−0.11	4.00	6.75
4′-Monoester	0	1-monoester	TDOC	0.43	4.82	6.50
4′-Diester	2	1-monoester	–	−2.54 (pH 5) −2.10 (pH 10)		
4′-Diester	2	1-monoester	DOC	−2.13		
4′-Diester	2	1-monoester	SDS	−2.39		
4′-Diester	2	1-monoester	TDOC	−2.03		
4′-Diester	1	1-monoester	TDOC	−2.10 (pH 5.5) −1.86 (pH 8.0)		
4′-Diester	0	1 monoester	TDOC	−1.86		
4′-Diester	0	1 monoester	DOC	−2.08		

[a] A Hill coefficient of 0.56 was required to fit the titration curve. All other pK_a values were obtained using $n = 1$.

methods to demonstrate the nature of the diester. We have found that selective proton decoupling can be a convenient and reliable method for checking the identity of the substituent. The diester peak is a poorly resolved triplet with an average splitting of 6.5 ± 0.5 Hz, the poor resolution being caused by a short spin-lattice relaxation time for the H-4′ proton on the glucosamine disaccharide [30]. As demonstrated in Fig. 2, selective proton decoupling shows that the phosphate is coupled to two separate protons with chemical shifts of 5.57 ppm and 3.87 ppm. From the chemical shift of the first proton we deduce that it is an anomeric proton, and because the other coupling is to a single proton C-6′ can be eliminated as a possible site of attachment for the phosphate. In view of the relative instability of aminoarabinose and the resulting difficulties in its analysis [31,32], NMR is the best method for estimating the amount of this substituent, as suggested by Mühlradt et al. [17], and the above procedure can be used to check the nature of the substituent. A further useful test is the phosphorus chemical shift. Glycosidic diester peaks occur about 2 ppm to higher field than other

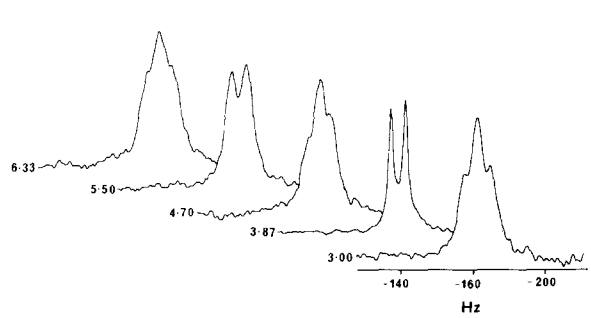

Fig. 2. Selective proton decoupling of the 4′-diester signal in the ^{31}P-NMR spectrum of hydrazine-treated lipopolysaccharide. Other conditions as for Fig. 1(B). The frequency used for proton irradiation, expressed as ppm of the proton resonance frequency, is indicated beside each spectrum.

diesters such as those in polyglycerophosphate [33].

Removal of the KDO residues by mild acid hydrolysis resulted in a small downfield shift of the diester signal. The amount of shift depended on the nature of the surfactant used, as shown in Table I.

(iii) 4′-Monoester. The 4′-phosphomonoester resonance was assigned on the basis of its chemical shift range, proton coupling pattern and pH dependence. It has been demonstrated previously that the phosphate is attached to the 4′-carbon in lipopolysaccharide from both *S. minnesota* and *E. coli* K12 [17,18]. The proton-phosphorus coupling constant is smaller than that expected for equal rotamer populations and is pH-dependent [30]. This is consistent with intramolecular hydrogen bonding as discussed below.

(iv) 1-Monoester. In the region where the 1-monophosphate signal is expected, there were three distinct peaks. This splitting has not been reported previously. (When comparing published results care must be taken to correct for the type of spectrometer used if an external phosphoric acid chemical shift standard was used [26].) The assignment was confirmed by selective decoupling, the size of the three- and four-bond proton coupling constants and polarization transfer experiments. These results, and their use in determining the anomeric configuration, have been reported in detail elsewhere [30], and in this report we are concerned only with the similarity between the species.

All three peaks had the same proton-phosphorus splittings with both three- and four-bond couplings clearly visible. The limiting chemical shifts at high and low pH were similar but there were small differences in pK_a values, which produced significant chemical shift differences at intermediate pH values. The three peaks correspond to different 4′ substituents. The intensity of the highest field line, peak a in Fig. 3, is proportional to the amount of aminoarabinose, as measured by the size of the diester signal. Alkaline hydrolysis resulted in loss of both peak a and the diester peak at -2 ppm and a corresponding increase in the intensity of the low field peak, labelled c.

The second peak, b, is assigned to 1-phosphate associated with 4′-monoester. Chemical analysis showed that there are approximately two phosphates per diglucosamine, so peak b should be the major peak when the amount of diester is small, as in Fig. 1(B).

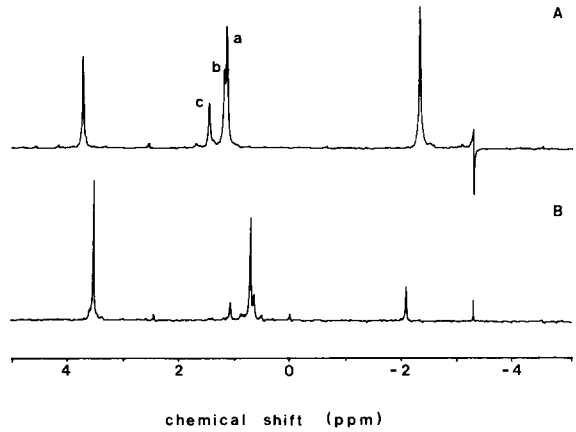

Fig. 3. ^{31}P-NMR spectra of hydrazine-treated lipopolysaccharide from batches containing different amounts of 4′-phosphodiester. (A) High 4′-diester content, pH 8.2. (B) Low 4′-diester content, pH 7.7. Other conditions as for Fig. 1. Peak assignments are (a) 1-phosphate on molecules with 4′-phosphodiester; (b) 1-phosphate with 4′-monoester and (c) 1-phosphate with no 4′ substituent. All molecules had two KDO residues.

The third peak is ascribed to 1-phosphate on molecules with no 4′ substituent. The most direct evident is the increase in this peak when diester is removed by hydrolysis. If the hydrolysis is performed in the NMR tube, the area of the 4′-monoester peak remains unaltered and new peaks are seen near 0 ppm. They were removed when the samples was purified by chromatography or ultrafiltration. Alkaline hydrolysis, therefore, releases aminoarabinosyl phosphate in accordance with expectations based on the known mechanisms of similar alkaline hydrolyses, which are discussed below.

Resolution of the three 1-phosphate peaks allows estimation of the proportions of all five possible phosphate-containing species. It will be shown that the results derived from the NMR spectra agree well with chemical analysis.

The interaction between 1 and 4′ substituents was a little surprising and suggested the possibility of dimer or oligomer formation. No evidence for dimerization was found and the same three peaks were observed with each of the surfactants, deoxycholate, taurodeoxycholate and dodecyl

sulphate. No dependence on concentration, ratio of surfactant to lipopolysaccharide or temperature was detected. The interaction is therefore unlikely to be the result of dimer formation.

If the 1-phosphate pK_a values are affected by the nature of the group at the 4′ position, a reciprocal effect on the 4′-phosphate might be expected. Some heterogeneity of the 4′ signal was found, but the splittings were much smaller. A few Hz downfield from the main 4′ resonance there was a second line, the intensity of which varied qualitatively with the amount of material without anomeric phosphate, as estimated from the fraction of the total area appearing under the 1 peaks and the total phosphate content determined by chemical analysis [25]. In one sample the secondary peak occurred on the high-field rather than the low-field side of the main 4′ signal. The only known difference between this and the other samples was a slightly greater ionic strength. We conclude that there may be a small shift associated with loss of 1-phosphate, but further evidence is required before it can be used diagnostically.

(v) Artificial heterogeneity. Further heterogeneity was produced by progressive hydrolysis in the NMR tube under mildly acidic conditions. Fig. 4 shows an expansion of the 1-phosphate region from Fig. 1(B). Peaks d and e are new species produced by hydrolysis, which also leads to the appearance of a new 4′ peak at 4.5 ppm and a new 4′-diester peak near −2 ppm.

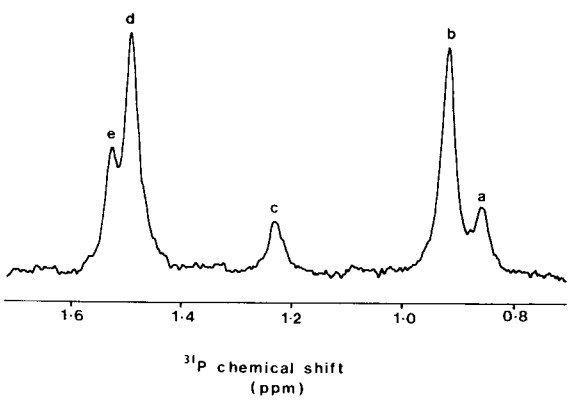

Fig. 4. Expansion of the region of Fig. 1(B) containing the 1-phosphate resonances of partially hydrolysed, hydrazine-treated lipopolysaccharide. Peak assignments are (a), (b), (c) as given in Fig. 3; (d) 1-phosphate with 4′-monophosphate and less than two KDO groups; (e) 1-phosphate with 4′-phosphodiester and less than two KDO groups.

The rate of appearance of the new species was measured from the ^{31}P-NMR peak heights. Hydrazine-treated lipopolysaccharide dispersed in $^{2}H_2O$ with 2% taurodeoxycholate in the presence of 5 mM ethylenediamine tetraacetate was heated to 45°C at pH 5.1. The NMR spectrum was recorded every 4 h for a total of 70 h. Peak heights were measured to an accuracy of ±1% for the monophosphates and ±4% for the smaller diester signals.

Two sets of changes were observed, one occurring at twice the rate of the other. (i) The new pair of glycosidic phosphate signals increased at the expense of the original three peaks, and the 4′-diester peak was converted at the same rate into a new resonance 6 Hz further upfield. In a second, slower, process the 4′-monoester signal was converted into a peak about 1 ppm further downfield. This peak is not visible in Figs. 1 or 4. The peak heights were linear functions of time during the period studied, and the slopes are recorded in Table II. No change was detected in a sample kept at 20°C, pH 5.2, for 24 h.

The shift of the 4′-monoester resonance has been reported previously for *S. minnesota* lipopolysaccharide and *E. coli* de-*O*-acyl lipopolysaccharide [17,18]. It was ascribed to the loss of the KDO residues. In agreement with the results of Rosner et al. [18], we observed that the proton-

TABLE II

RATE OF HYDROLYSIS OF THE PHOSPHATES OF HYDRAZINE-TREATED LIPOPOLYSACCHARIDE AT pH 5.1, 45°C, STARTING WITH PARTIALLY HYDROLYSED MATERIAL

The figures in parenthesis are the estimated uncertainties.

Assignment		Initial Intensity (arbitrary units)	Initial proportion (%)	Rate of change (% per h)
phosphate	KDO groups			
1-Monoester	2	98	61 (1)	−0.21 (2)
1-Monoester	1	62	39 (1)	0.27 (2)
4′-Monoester	2	100	89 (2)	−0.11 (1)
4′-Monoester	0	17	11 (1)	0.11 (1)
4′-Diester	2	13	56 (4)	−0.25 (6)
4′-Diester	1	10	44 (4)	0.25 (6)

phosphorus coupling was greater after hydrolysis, although our value of 8.2 ± 0.1 Hz is slightly larger than that reported previously. The higher temperature used in the present study may make the rotamer populations more nearly equal, thus increasing the coupling constant [30].

The 1-phosphate signals changed at twice the rate of the 4′ peak intensities. The most probable explanation is that the anomeric phosphates are sensitive to the loss of the first KDO residue, but the 4′-phosphate responds only to loss of both. Rosner et al. [18] observed the formation of such an intermediate during mild acid hydrolysis of material from *E. coli*. No further change in the 1-phosphate signal was observed when the second KDO was removed. Checks were made to establish that the new signals were in fact still due to glycosidic phosphates on α-D-*N*-acylglucosamine. The proton-phosphorus coupling constants and the H-1 proton chemical shift were identical to those for the original species. Fig. 5 is a comparison of the chemical shift values of the H-1 protons

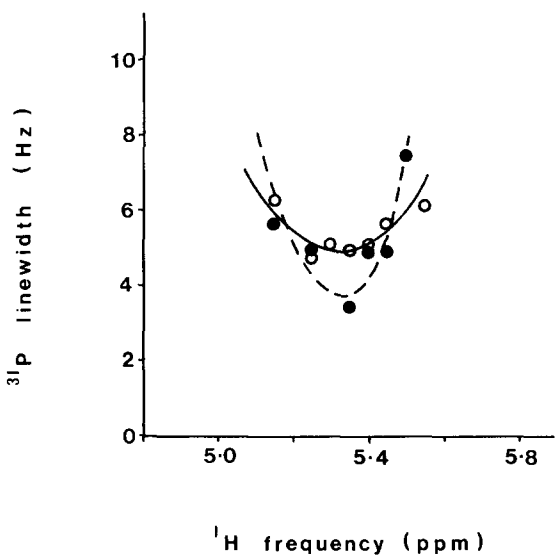

Fig. 5. Verification of the anomeric configuration of the phosphate produced by mild acid hydrolysis of hydrazine-treated lipopolysaccharide. Residual linewidth of 1-phosphate resonances when selectively proton decoupled at low power, as a function of decoupler frequency, is expressed as ppm of the proton resonance frequency. Closed circles: 1-phosphate with 4′-phosphomonoester and two KDO groups. Open circles: 1-phosphate with 4′-phosphomonoester and less than two KDO groups. Other conditions as for Fig. 1(B).

obtained by examination of the effect of selective decoupling on the linewidth of the two phosphate signals. In addition, proton polarization transfer was used to demonstrate that the value of the H-1,H-2 proteon-proton coupling constant is unchanged [30]. Thus the immediate chemical environment of the 1-phosphate has not altered.

Because interaction between KDO at the 6′ position and the phosphate at the anomeric position was unexpected, a search was made for any previously undetected, acid-labile substituent. A 25 mg sample of lipopolysaccharide was suspended in 1 ml of $^{2}H_2O$ at pH 4.7 and the proton NMR spectrum was monitored while heating at 70°C. The sharp peaks that appeared after 60 min were indistinguishable from the spectrum of 3-deoxy-*manno*-octulosonic acid treated similarly. It was concluded that no new molecular fragment containing non-exchangeable protons is released by mild acid hydrolysis.

To test for the possibility of lactone formation, the phosphate signal was examined after heating for 1 h, at pH 11 and 60°C, material from which KDO had been partially removed. No change was detected and lactone formation is thus not the cause of the spectral changes produced by acid treatment.

The 4′-diester also is affected by the loss of the distal KDO residue. This conclusion is based on the rate of appearance of the new diester peak. The signal was unusual in that is was the only sharp diester peak for hydrazinolysed lipopolysaccharide that was pH dependent in the range 5 to 11. In fact, the new peak was distinguishable from the original one only at low pH. Removal of the second KDO group produces a third diester peak at lower field than the other two.

Lipopolysaccharide

(i) General. Linewidths of 3–4 Hz were observed in the spectra of native lipopolysaccharide in the presence of deoxycholate or taurodeoxycholate. Without surfactant or EDTA the peaks were 40 Hz wide [33]. Many of the signals were similar to those seen in the spectra of hydrazinolysed material, as the pK_a and chemical shift values in Table III show. Observation of separate 1-phosphate signals for molecules with mono- or diester at the 4′ position was possible in many

TABLE III

CHEMICAL SHIFT AND pK_a VALUES FOR THE PHOSPHATES OF LIPOPOLYSACCHARIDE AT 45°C IN THE PRESENCE OF 2% SODIUM TAURODEOXYCHOLATE

Assignment			Chemical shift		pK_a
phosphate	KDO groups	3′ ester	anion	dianion	
Before acid hydrolysis:					
1-Monoester	2	+,−	−1.67	2.48	7.45
4′-Monoester	2	+	−0.77	3.22	8.40
4′-Monoester	2	−	0.03	4.27	7.05
4′-Diester	2	+,−	−2.30 (pH 5)		
			−2.09 (pH 10)		
After partial hydrolysis:					
1-Monoester	2	+,−	−1.61	2.46	7.35
1-Monoester	1	+,−	−1.60	2.33	6.95
			−1.50	2.33	6.95
4′-Monoester	2	+	−0.80	3.19	8.21
4′-Monoester	0	+	0.07	4.07	7.15
			0.14	4.13	7.05
4′-Monoester	2	−	0.03	4.19	6.9
4′-Monoester	0	−	0.35	4.86	6.6
4′-Diester	2	+,−	−2.12 (pH 5)		
			−2.06 (pH 10)		
4′-Diester	0	+,−	−2.08 (pH 5)		
			−1.56 (pH 10)		

samples, although the poorer resolution restricted the conditions under which the splitting was seen. Typical separations were 4–6 Hz and the relative intensities were in the same ratio as those for the 4′-mono- and diester, as far as could be determined with the available resolution.

Two sets of peaks were present in lipopolysaccharide spectra that were absent from the spectra of hydrazine-treated material. One was a group of up to three monoester resonances and the other was the set of pyrophosphate signals at −10 and −12 ppm. For reasons given in the Discussion section, the monophoshates are believed to be 4′ substituents in molecules whose 3′-hydroxyl group is esterified. This assignment is supported by the NMR peak areas in lipopolysaccharide before and after hydrazinolysis. The same peaks in the proton-coupled spectrum appeared as doublets split by 10.0 ± 0.2 Hz. This coupling constants is significantly larger than that found for anomeric phosphates, and for 4′-phosphates adjacent to free hydroxyl groups at the 3′ position. The separation between the three species was typically 16–18 Hz, with little variation with pH. Similar resonances have been reported for lipopolysaccharide from *E. coli* [18].

(ii) Effect of culture conditions. The phosphorylation pattern showed considerable variation with culture conditions, but was reproducible from batch to batch, as shown in Table IV. The proportion of molecules containing aminoarabinose varied from an average of 80%, when grown in nutrient broth, down to 30% for a sample grown in Luria broth. The reasons for the variation are not known, but it appears to be a controllable function of culture conditions.

The proportion of pyrophosphate also varied reproducibly and the amount of pyrophosphoryl diester ranged from 30% to 10%. The proton-coupled spectrum of the pyrophosphate was consistent with that expected for ethanolamine diester [17]. One end of the diester was coupled to a single proton with a spin-spin splitting constant of 8.1 ± 0.3 Hz, which is only slightly higher than the value we found for anomeric monophosphates. At the other end, the second phosphorus nucleus was

TABLE IV

PHOSPHATE COMPOSITION OF LIPOPOLYSACCHARIDE

Values are mole fractions, assuming the total 4′-phosphate content to be 1.00.
N.B., nutrient broth; L.B., Luria broth [20].

Batch number:	1	2	3	4	5	F1 [a]	F2 [a]
Growth medium:	N.B.	N.B.	N.B.	N.B.	N.B.		
4′-Monoester (with 3′-ester)	0.09	0.16	0.22	0.15	0.52	0.40	0.16
4′-Monoester (no 3′-ester)	< 0.02	0.02	0.02	0.05	0.18	0.33	< 0.04
4′-Diester	0.91	0.82	0.76	0.80	0.30	0.27	0.73
1-Monoester	0.37	0.40	0.42	0.57	0.44	0.93	0.61
1-Pyrophosphomonoester	0.27	0.25	0.24	0.20	0.25	0	0
1-Pyrophosphodiester	0.22	0.11	0.12	0.15	0.38	0.08	0.39
Total 1-phosphate	0.86	0.75	0.78	0.92	1.07	1.01	1.00

[a] Samples generously provided by Dr. O. Lüderitz and Dr. C. Galanos, Max Planck Institut für Microbiologie, Freiburg.

coupled to a pair of protons with a coupling constant of 6.8 ± 0.3 Hz. The pyrophosphoryl monoester had a proton doublet splitting of 7.1 ± 0.3 Hz for the phosphorus coupled to the anomeric proton, and no splitting of the signal from the second phosphorus.

One batch of cells grown on yeast-tryptone medium [22] gave lipopolysaccharide containing an extra mol/mol of pyrophosphate. A large part of this was diester that had ^{31}P resonances at -9.9 and -11.0 ppm. The chemical shifts and proton coupling constants indicate that the extra pyrophosphate is joined to a non-anomeric site. The doublet at -11.0 ppm was at lower field than typical anomeric pyrophosphates, but in the middle of the range in which we have observed non-anomeric pyrophosphates on the oligosaccharide from *E. coli* K12 (unpublished data). The proton coupling constant of 10.0 ± 0.5 Hz also suggests non-anomeric substitution. The signal from the second phosphorus nucleus in the new diester was partly obscured, but appeared to be split by a single proton with a coupling constant of 9.0 ± 0.5 Hz. This atypical sample was not examined further.

The proportion of material with free 3′-hydroxyl groups varied from small values when the cells were grown in nutrient broth, to substantial amounts in other media. It should be pointed out, however, that the 4′-diester signal was apparently insensitive to the 3′ substituent. Reliable estimation of the amount of free 3′-hydroxyl is therefore not possible by this method, if the sample contains large amounts of aminoarabinose.

Another interesting aspect of the heterogeneity is the extent of phosphorylation of the 1 position. From Table IV it can be seen that substitution is incomplete for material from cells grown in nutrient broth.

Phosphorylation of the 4′ position is stoichiometric. Only one sample out of nine examined, sample 5 in Table IV, showed any evidence to the contrary. Even then, the presence of 0.1 mol/mol of an undetected diester buried under one of the anomeric phosphate peaks could account for the discrepancy. (Neither the pH dependence of the NMR spectrum nor the phosphorus-glucosamine ratio was determined for this sample.)

It should be emphasized that it is unlikely that results such as those in Table IV could be obtained by chemical analysis, particularly because of the lack of precise assays for aminoarabinose [17] and KDO [25] and because of the quite small changes in glucosamine and phosphate levels which accompany the changes in substitution types.

(iii) Mild acid hydrolysis. When lipopolysaccharide was heated for 30 min at 80°C, pH 4.3, in the presence of 2% taurodeoxycholate and 5 mM EDTA, new peaks appeared in the spectrum, as they did for hydrazine-treated samples. The new 1-phosphate species appeared at a faster rate than the new 4′ peaks, showing the stepwise loss of KDO reported above. Hydrolysis also affected the 4′-diester signal, causing a downfield shift and the

small pH dependence of the chemical shift became greater.

Fig. 6 shows pH titration curves for the chemical shifts of 4′-phosphates in lipopolysaccharide. The change in chemical shift and pK_a value produced by loss of KDO was similar to the changes resulting from removal of the 3′ substituent. Loss of the second substituent produced smaller changes in each case.

(iv) Hydrazine treatment. Identification of the different molecular species by NMR permits us to obtain detailed information about the effects of hydrazine treatment. This is incidental to the present study, but is included here to illustrate the power of the NMR method.

Pyrophosphate, both mono- and diester, was converted almost completely into monophosphate under all conditions used. There was also some destruction of aminoarabinosyl diester. Table V contains the proportions of the molecular species produced by hydrazinolysis of different starting materials, ranging from high to low initial diester content. The composition of the product was obtained from the areas of the ^{31}P-NMR peaks and the analytically determined phosphorus-glucosamine ratio [25]. Increasing destruction of diester is seen as the severity of treatment increases. Loss is negligible after 30 min at 80°C, but almost complete after 3 h at 100°C. After 30 min at 100°C, approx. 15% had been converted into monoester and a further 15% had been cleaved between the phosphate and the glucosamine.

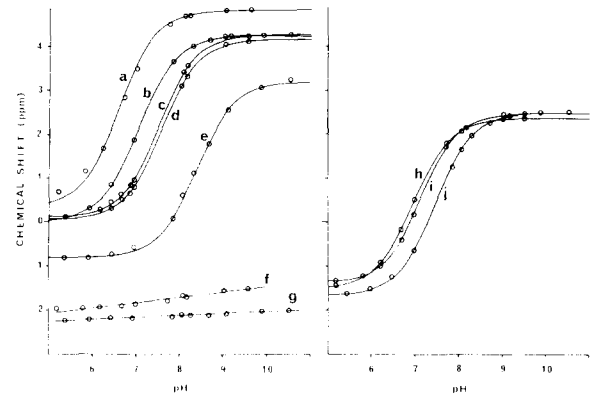

Fig. 6. pH dependence of ^{31}P chemical shifts of the phosphates in lipopolysaccharide. Experimental conditions as for Fig. 1. For clarity, the 4′-phosphate signals have been displayed separately from the 1-phosphate resonances. Assignments are (a) 4′-monoester with no substituent at 3′ or 6′; (b) 4′-monoester with one or more KDO groups at 6′ but no 3′ substituent (the peak was observed in the spectrum of lipopolysaccharide, but the titration curve was measured using hydrazine-treated material); (c), (d) 4′-monoester with 3′-acyl ester and no KDO; (e) 4′-monoester with 3′-acyl ester and one or more KDO groups; (f) 4′-diester with no KDO groups; (g) 4′-diester with one or more KDO groups; (h), (i) 1-monoester with less than two KDO groups and (j) 1-monoester with two KDO groups.

In contrast, alkaline hydrolysis results in the loss of only 4′-phosphate (this work and Ref. 17).

TABLE V

PHOSPHATE SUBSTITUTION PATTERN FOR LIPOPOLYSACCHARIDE, BEFORE AND AFTER HYDRAZINE TREATMENT

Batch	Treatment (°C, min)	Substitution pattern					
		1-monoester:	+	+	+	–	–	–
		4′-monoester:	+	–	–	+	–	–
		4′-diester:	–	+	–	–	+	–
1	nil		0.68	0.28	0.01	0.01	0.03	−0.06
1	100,30		0.71	0.17	0.12	0.02	0.00	– [a]
2	nil		0.16	0.73	0.11	0.00	0.00	– [a]
2	100,30		0.26	0.49	0.20	0.01	0.02	– [a]
3	nil		0.14	0.61	0.00	0.05	0.20	−0.02
3	80,30		0.12	0.69	0.11	0.03	0.13	0.00 [b]
3	100,30		0.28	0.43	0.26	0.02	0.03	−0.02 [b]
3	100,180		0.27	0.09	0.45	0.14	0.05	0.01 [b]

[a] Value was not determined. It is assumed to be zero.

[b] If the random-loss model applies (see text), the values would be 0.02, 0.04 and 0.08.

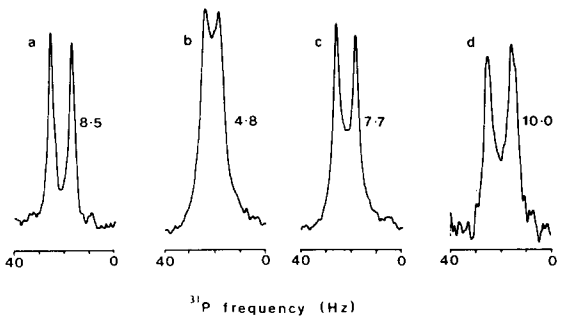

Fig. 7. ^{1}H-^{31}P spin-spin coupling in ^{31}P spectrum of 4′-phosphates in in molecules with (a) two, (b),(c) one, and (d) no free hydroxyl groups at the 3′ and 6′ positions. The numbers beside each peak are the spin-spin coupling constants in Hz. The spectra are from (a) hydrolysed, hydrazine-treated lipopolysaccharide, pH 7.6; (b) hydrazine-treated lipopolysaccharide, pH 9.0; (c) as for (b), pH 5.2; (d) lipopolysaccharide with 3′-acyl ester and one or more KDO groups at 6′, pH 7.4. All other conditions as for Fig. 1

The probable mechanism for the action of alkali involves cyclic diester formation, which has no parallel in hydrazinolysis. The observations are therefore in accord with the expected mechanisms for hydrolysis and hydrazinolysis.

There was no indication that 4′-monoester is affected by hydrazine. Any missing 4′-phosphate can be accounted for by the destruction of diester. The amounts of 4′-phosphate also support the suggestion that the original lipopolysaccharide was completely substituted at this position. Anomeric monosphosphate also appeared to be unaffected by hydrazinolysis. The proportion of 1-phosphate after treatment was sometimes higher, but never lower, than that in the starting material. This may be a consequence of selective loss of material during preparation [25].

No measurable amount of completely dephosphorylated material was recovered, but the only case in which the expected amount was greater than experimental uncertainty was the 3-h treatment. The amount of each species, predicted on the basis of uncorrelated loss of phosphate from the two ends of the molecule, is given in a footnote to Table V. Since phosphate-free material is absent, it is presumably extracted into the acetone during preparation. Only about 60% of the original glucosamine is found in the hydrazinolysis product, and even lower yields have been reported [34].

The ^{31}P spectrum of the sample that had undergone extended hydrazine treatment contained a new peak, not discussed above. We have assignd it to 1-phosphate on molecules that have lost amide-linked fatty acid [25].

The behaviour of the phosphates in lipopolysaccharide is similar to that observed for model compounds. Adenosine diphosphate, α-D-glucose 1-phosphate and D-glucose 6-phosphate were treated with hydrazine for 30 min at 100°C. The consequences were monitored using proton-decoupled and coupled ^{31}P-NMR spectra. Adenosine diphosphate was converted completely into monophosphate and glucose 1-phosphate was unaltered. Glucose 6-phosphate contained 80% unchanged material and 20% of a new species with a slightly different chemical shift. There was, however, no evidence for inorganic phosphate or phosphazide that would indicate dephosphorylation.

Discussion

The pioneering study of Mühlradt et al. [17] demonstrated the general types of phosphate resonances obtained with both native and deacylated lipopolysaccharide from *Salmonella*. Their spectra were measured in the pH range 7.5–8.0, but careful control does not seem to have been carried out. Our results (Fig. 6) indicate considerable movement of resonance positions through this range. The significant conclusion from the present work, using surfactants and close control of pH and ionic strength, is that the phosphorus resonances are so sensitive to the chemical environment that they provide information concerning the structures of the individual lipopolysaccharide molecules, such as the presence or absence of a 3′-acyl group or a KDO in the 6′ position, in addition to the phosphate types in the 1 and 4′ positions. This provides a basis for understanding the heterogeneity of native lipopolysaccharide observed by others [8,17,18] and a powerful tool for assessing lipopolysaccharide structure at the molecular level.

As an illustration of the information readily available, Fig. 8 shows the spectrum of lipopolysaccharide from *S. minnesota*, strain R345 (Rb). The sample was generously supplied by Dr. O. Lüderitz. This strain is believed to produce an almost complete core oligosaccharide, but no

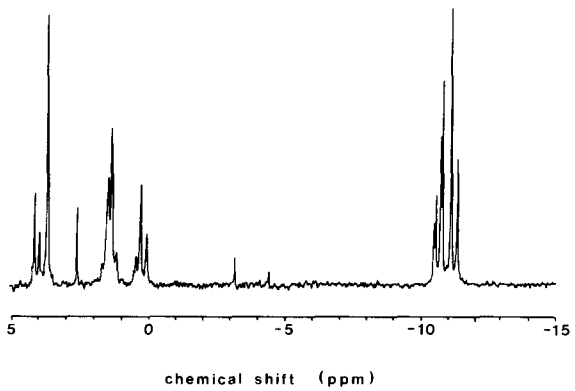

Fig. 8. ^{31}P-NMR spectrum of lipopolysaccharide from *S. minnesota* R345, pH 7.9. Other conditions as for Fig. 1.

O-antigen [35]. From the integrated spectrum we can readily deduce that molecules in the sample are substituted with monophosphate at the 1 and 4′ positions. The degree of substitution is the same at both sites, and is therefore probably stoichiometric. There is no aminoarabinosyl diester or anomeric pyrophosphate and every molecule has KDO attached. The 3′-hydroxyl is esterified in 49% of the molecules and there is heterogeneity in the substitution pattern. Finally there is exactly one mol/mol of non-anomeric pyrophosphoryl diester.

Factors affecting pK_a

The pK_a values for large multicharged molecules such as lipopolysaccharide are affected by several parameters. The main factors, in approximate order of importance, are (i) intramolecular electrostatic interactions, (ii) the electrostatic field of counter ions, (iii) hydrogen bonding, (iv) molecular conformation and (v) steric inhibition of solvation. In addition, the average charge on the ionized groups may itself be a function of pH, resulting in negative cooperativity [27]. It should also be noted that very small changes in ionization energies can be detected by NMR. The observed difference in pK_a values for α- and β-D-glucose 6-phosphates [33] corresponds to a difference of only 0.14 kJ · mol^{-1} in the Gibb's free energy for ionization.

(i) Electrostatic interactions. The electrostatic model proposed by Kirkwood has been used with some success to account for the observed pK_a values of small multifunctional molecules [36]. The only molecular parameter involved, R, is ostensibly the distance between the charged groups. The charges are placed at the foci of an elipsoidal cavity of low dielectric constant in a continuum of high dielectric constant. The charges are empirically chosen to be 1 Å from the surface.

Electrostatic contributions to pK_a values in the lipopolysaccharide molecule were estimated by graphical interpolation of Tanford's calculations for distances between 3 and 10 Å. Beyond 10 Å, the interaction was assumed to fall off as $1/R$. The approximation is adequate since the quantities are small and the ellipsoidal model is likely to be a less accurate representation when the axis ratio exceeds 6 : 1. Because the molecular conformation of lipopolysaccharide in solution is unknown, we have made no attempt to represent the interatomic distances accurately.

It is clear from Table VI that, while the observed pK_a differences are sometimes of the order of magnitude expected, there is no consistent pattern. The electrostatic model fails to describe the observations. For example, sequential loss of KDO residues should have produced changes that are within a factor of two of each other. Neither the 1- nor the 4′-phosphate conforms to this pattern. Secondly, the increased charge on the 4′-monoes-

TABLE VI

ESTIMATED ELECTROSTATIC CONTRIBUTIONS TO pK_a VALUES FOR THE PHOSPHATES IN DEACYLATED LIPOPOLYSACCHARIDE

The average charge on the 4′-phosphate was taken to be 1.8 at the mid-point of the titration of the 1-phosphate, and the average charge on the 1-phosphate was taken to be 1.2 at the mid-point of the titration of the 4′-phosphate.

Phosphate	Perturbing group	Approximate distance (Å)	Calculated ΔpK_a	Observed ΔpK_a
1-Monoester	4′-Monoester	16	0.34	0.15
	4′-Diester	16	0.19	0.19
	KDO I	16	0.19	< 0.02
	KDO II	< 30	> 0.10	0.45–0.51
4′-Monoester	1-Monoester	16	0.23	< 0.02
	KDO I	8	0.35	0.25
	KDO II	16	0.19	< 0.02

ter compared to that on the diester did not decrease the pK_a of the 1-phosphate as expected. Thirdly, the effect of the 1-phosphate on the 4′-monoester should have been about two-thirds that for the reciprocal interaction, but was in fact very small. Finally, negative cooperativity was observed only for the ionization of molecular aggregates.

The calculations do permit us to dismiss certain arguments. The observation that the 1-phosphate is affected only by the distal KDO, and the 4′-phosphate only by the proximal one, might be taken as evidence that the KDO groups were on different glucosamine rings. Not only is the electrostatic model, on which this reasoning is based, inappropriate, but the calculations show that the effect of the more distant KDO would not be negligible.

We must conclude that other factors are making contributions of equal or greater importance to the pK_a values. While the molecule is too small to be treated formally as a polyelectrolyte with a Gouy-Chapman boundary layer of counter-ions, there may be considerable ionic screening of one group from another. The presence of several negatively charged surfactant molecules may enhance this effect, although the results in Table I demonstrate that surfactant does not affect the pK_a values. The counter-ions lead to an ionic strength effect on the titration behaviour of proteins, but again we noticed no significant change produced by differences in ionic strength. The range of ionic strengths used was admittedly limited, and no systematic variation of ionic strength was performed.

It should be emphasized that we are not proposing tight binding of the counter-ions. Despite their popularity, tight-binding models have been found to be unnecessary for explaining the behaviour of nucleic acids [37]. Octulosonic acids, by virtue of their arrangement of hydroxyl groups, may provide specific binding sites for divalent cations [38], but these are likely to be mobilized significantly by interaction with EDTA.

Sodium ions do exert a small effect on the pK_a of adenosine monophosphate, which can be explained by an equilibrium constant of approx. 3 for the sodium complex [39]. The chemical shift of inorganic phosphate is also ionic strength dependent. At ionic strengths of 0.1, as in the present work, these effects would contribute no more than 0.02 units to the observed pK_a values. For similar reasons, the reported pK_a values were not corrected for the difference between the temperature at which the pH measurement was made and that at which the NMR spectrum was obtained. The correction would be about 0.03 units. The relative pK_a values are of more importance than the absolute values.

(ii) Hydrogen bonding. The possible contribution of intramolecular hydrogen bonding to pK_a values has been invoked for nucleotides [40], and is widely discussed for groups within proteins [41]. For phosphate groups freely exposed to the solvent, the possibility may seem less likely. Intramolecular interactions could, however, affect the entropic part of the free energy of ionization. If the electronegativity of a sugar hydroxyl group differs from that for water, the average hydrogen bonding will be affected, because the hydroxyl group will have a statistical advantage. In other words, it will have a high effective concentration.

Insufficient evidence is available from suitable model compounds to test this suggestion, but the following observations are consistent with it. Glucose 6-phosphate has a pK_a value that is sensitive to the anomeric configuration [33,42]. A possible explanation is that only one anomer is able to form intramolecular hydrogen bonds. Lipoteichoic acid, which contains alanine-substituted polyglycerophosphate, shows shifts of the ^{31}P resonances produced by intramolecular hydrogen bonds [33]. If the chemical shift of the phosphate was affected, the pK_a may be also. Thirdly, glucose 3-phosphate and glucose 4-phosphate have pK_a values 0.45 units lower than those for glucose 1-phosphate and glucose 6-phosphate [43]. The former pair of molecules can form hydrogen-bonded structures with free hydroxyls on either side of the phosphate. The observed pK_a values for lipopolysaccharide can be accounted for by a model in which intramolecular hydrogen bonding is a major contributor (Table III). The greatest change in pK_a for the 4′-phosphate occurs when either the KDO or esters are removed. If the important ester is at the 3′ position, either change would provide a free hydroxyl capable of intramolecular hydrogen bonding with the phosphate. Steric hindrance, or interference with solvation, should produce quite different effects in re-

sponse to removal of 3′ and 6′ substituents. Removal of the second substituent results in a further, but smaller change in pK_a, consistent with the availability of a second free hydroxyl within hydrogen-bonding distance. The properties of the 4′-phosphate in lipopolysaccharide that has a free 3′-hydroxyl are very similar to those for the same species in hydrazinolysed material, indicating that this specific local environment, rather than the overal molecular conformation, determines the observed pK_a.

The unexpected behaviour observed during sequential release of KDO (Table II) is also compatible with this model. The 4′-phosphate would be unaffected until the inner KDO is removed. Furthermore, intramolecular hydrogen bonding between anomeric phosphate and the outer, but not the proximal KDO, is possible. It does not, however, explain the sensitivity of the 1-phosphate to the 4′ substituent. Indirect interaction involving competition between the two phosphates for the KDO is a possible additional postulate to account for this observation. The absence of a reciprocal interaction could also be accomodated. An alternative postulate would be that the molecules exist as head-to-tail dimers.

Hydrogen bonding may affect the pK_a either by altering the electronic structure of the phosphate or by preferentially stabilizing one of the anions. Fig. 6 shows that changes in the molecular structure affect the chemical shifts of both anions by similar amounts, so the effect operates through the former mechanism.

Hydroxyl groups are less acidic than water molecules. They will therefore reduce the probability of transfer of a proton to the phosphate, resulting, in a decreased pK_a. Similarly, they will change the electronic structure of the phosphate in the direction of the less protonated species: that is, to produce a decreased shielding of the phosphorus. Both changes are in the predicted direction.

(iii) Molecular conformation. Chemical modification does seem to affect the molecular conformation. The proton-phosphorus spin-spin couplings for different 4′-monoester species are compared in Fig. 7. The signal assigned to molecules containing both KDO and 3′ ester has the splitting expected for equal population of the rotamers about the C-4′,O bond. The rotamer in which the phosphorus is *trans* to the proton on C-4′ is expected to have a splitting of 24 Hz and the *gauche* conformers 3 Hz [44]. Splittings less than 10 Hz therefore indicate that the *trans* rotamer population is less than 0.33. When the 3′-ester is removed, the population of the *gauche* conformers is increased significantly. We were unfortunately unable to resolve the proton splitting for the species with 3′-ester, but no KDO. Removal of both substituents largely restores unhindered rotation.

The pK_a values for the 4′-phosphate are, however, not correlated with the rotamer populations, so the pK_a is not determined by this aspect of conformation. There is still a possibility that other changes lead, in some unspecified way, to an altered ionization constant. A different average conformation induced by the presence of fatty acid at the 3′ position could affect the pK_a, but the two surfactants, deoxycholate and dodecyl sulphate, are likely to interact with the lipopolysaccharide molecule in different ways. Lipopolysaccharide binds individual deoxycholate molecules to specific regions [46], while sodium dodecyl sulphate probably forms micelles. A dependence of pK_a on surfactant is, therefore, possible. Comparison of our results with those of Rosner et al. [18] leads us to conclude that there is no dependence on surfactant. In addition, loss of KDO would not produce a similar conformational change and a further hypothesis would be required.

(iv) Solvation. The role of solvation is difficult to assess. In proteins, the pK_a values of ionizable groups are altered as a consequence of their location at sites with limited access to solvent [45]. A similar process has been suggested as an explanation for the high pK_a value for the 4′-phosphomonoester associated with 3′-acyl ester [18]. It is difficult to see how lipopolysaccharide associated with a small number of deoxycholate ions [46] would have hindered solvation. It is even less likely in the light of the equal rotamer populations of the phosphate. In a sense, intramolecular hydrogen bonding competes with solvation of the ion. It is in this sense that we believe solvation affects the pK_a values for the phosphates in lipopolysaccharide.

Chemical shifts

The chemical shifts of phosphates are affected

by the electronegativity of the groups to which they are attached [47,48], their state of ionization, conformation [49,50] and possibly hydrogen bonding [51,52]. The chemical shift differences between anomeric and non-anomeric species are electronegativity effects. Examples of monoesters, diesters and pyrophosphates are reported above.

The 4′-monoesters from lipopolysaccharide indicate the relative importance of conformation and hydrogen bonding in determining the chemical shift. The chemical shift changes parallel the pK_a effects, so the arguments are similar to those used earlier. The observed rotamer population do not correlate with the chemical shift values. The chemical shift changes are, however, of similar size to those found as a result of hydrogen bonding in the spectra of polyglycerophosphates [33,53]. Similar shifts are seen in the spectra of glycerophosphates in water [52], as well as for phospholipids in non-aqueous solvents. On the other hand, there may be a small contribution to the chemical shift produced by conformational changes. The heterogeneity of the 4′-phosphate signal associated with 3′-ester is presumably due to the presence of different fatty acids and consequential conformation changes.

The 4′-diesters vary less than the corresponding monoesters and all three factors may be of similar importance. (i) In view of the charge on the KDO, the downfield shift produced by removal of the latter may be an electrostatic effect. (ii) The proton coupling is poorly resolved for the diesters, so we have no direct evidence for changes in its conformation. Several species show linear variation of the chemical shift with pH. The rotamer population of the monoester was similarly a smooth function of pH, whereas sigmoidal behaviour is expected if the effect is a consequence of titration of the phosphate. This may mean that there is a conformational rearrangement that depends on a more general parameter like the average molecular charge. (iii) Hydrogen bonding is also pH-dependent as shown by the behaviour of gels formed by polysaccharides [54], so it could equally well be responsible.

We conclude that loss of KDO from the 6′ position will produce a negligible electronegativity contribution, but conformation or hydrogen bonding changes could produce shifts of about 0.4 ppm. The former suggestion is favoured because the chemical shift was also sensitive to the surfactant used (see Table I).

The dependence of the chemical shift of the 4′-diester signal from aggregated lipopolysaccharide on pH indicates that the average molecular conformation is probably pH-dependent. This may affect the properties of lipopolysaccharide in bilayer membranes.

Heterogeneity

The extent of molecular heterogeneity of lipopolysaccharide from *S. minnesota* R595 is considerable and is affected by culture conditions. Both the phosphate and fatty acid substitution patterns varied. Nevertheless, although the 1-phosphate level could be as low as 75%, there was complete phosphorylation at the 4′ position. This phosphate may be essential for subsequent steps in biosynthesis. Samples with incomplete phosphorylation of the 1 position also had high levels of aminoarabinose, as if there was a need to reduce the overall negative charge on the molecule.

Conclusion

Phosphorus NMR spectra are valuable, not only for determining the types of phosphate substituents in lipopolysaccharide, but also the presence of 3′-fatty ester and 6′-KDO residues in individual molecules. The method is especially valuable as it is independent of individual estimates of glucosamine and phosphorus levels. The results of the present study led to a reappraisal of established analytical methods [25].

An evaluation has been made of the contributions of electrostatic interactions, hydrogen bonding, molecular conformation and solvation to pK_a values of phosphate groups and their influence on NMR resonance positions.

Acknowledgements

This work was partially funded by a grant from the Australian Research Grants Scheme. Mrs. Brigid Roberts provided excellent technical assistance. We are grateful to Drs. Otto Lüderitz and Chris Galanos for generous gifts of lipopolysaccharide.

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