BIOCHEMICAL JOURNAL LETTERS

³¹P N.m.r. evidence for the presence of triphosphate residues in lipopolysaccharides from *Pseudomonas aeruginosa*

One of the most striking features of lipopolysaccharides from *Pseudomonas aeruginosa* is their high phosphorus content. The average value for the products from 35 different strains described in the literature is 3.7% phosphorus, while values obtained in the two most extensive surveys (Chester *et al.*, 1973; Wilkinson & Galbraith, 1975) lie in the range 3.6 to 5.6%. In part, these relatively high values are due to the presence in the preparations of substantial proportions of R-form lipopolysaccharides lacking the O-specific side-chain. Additionally, however, it appears that lipopolysaccharides from *P. aeruginosa* may contain ten or more phosphate residues in the lipid A and inner-core regions, compared with six or seven residues in typical enterobacterial lipopolysaccharides. On mild hydrolysis, much of the phosphate in lipopolysaccharides of *P. aeruginosa* is released as P_i, along with small amounts of PP_i and ethanolamine mono-, di-, and triphosphates (Drewry *et al.*, 1971, 1972). Thus the presence of triphosphate residues (or those of higher phosphoric anhydrides) could contribute to the unusually extensive phosphorylation of these lipopolysaccharides.

In a recent study (Horton & Riley, 1981) of seven lipopolysaccharides from *P. aeruginosa*, no evidence for triphosphate residues was obtained by ³¹P n.m.r. spectroscopy. This finding conflicts with our own results for the lipopolysaccharides from strains N.C.T.C. 1999 and N.C.I.B. 8626 using the same technique. Spectra obtained for both products were qualitatively similar, and those for the lipopolysaccharide from strain N.C.T.C. 1999 are shown in Fig. 1. The three discrete signals of similar intensity at low field appear to correspond to phosphomonoester residues. On acidification (Fig. 1*b*), these signals move about 4 p.p.m. upfield from their positions (+3.6, +3.3, and +3.0 p.p.m.) at alkaline pH (Fig. 1*a*). However, the dominant signals, which make up over 75% of the total peak area, are those expected for 'pyrophosphate' residues

(Mühlradt *et al.*, 1977; Lehmann & Rupprecht, 1977; Rosner *et al.*, 1979; Horton & Riley, 1981). At alkaline pH, these signals appear as major, complex multiplets at about -4.6 and -9.7 p.p.m., with approximately equal integrals, and minor peaks between -10 and -11 p.p.m. At acid pH, the signals appear as three large, broad peaks at -9.2, -10.0, and -10.8 p.p.m. and a lesser peak at -12.5 p.p.m. The signal at -4.6 p.p.m., and its upfield shift on acidification, indicate the presence in the lipopolysaccharides of monoesterified pyrophosphate residues, but the possibility that related diesters contribute to the signals at about -10 p.p.m. cannot be excluded. Also, the number, size, and complexity of the 'pyrophosphate' signals indicate the presence of several such residues, although complications could be introduced by microheterogeneity and other factors (e.g. Mühlradt *et al.*, 1977; Rosner *et al.*, 1979; Van Alphen *et al.*, 1980). But, most significantly, the presence in the spectra of a high-field signal at -20.0 p.p.m. (alkaline pH) or -21.2 p.p.m. (acid pH), which is absent from the corresponding spectra for *Salmonella* species and *Escherichia coli*, shows that not all the 'pyrophosphate' signals can have this attribution. The chemical shift of the high-field signal and its relative insensitivity to pH, its triplet appearance, and a comparison with the spectrum of ATP at alkaline pH (signals at -5.1, -10.4, and -21.0 p.p.m.) show that it can be assigned to the central phosphorus of a triphosphate residue. Thus, the results of this ³¹P n.m.r. study support those of the previous degradative studies of these lipopolysaccharides.

The failure of Horton & Riley (1981) to obtain analogous results in their study may be attributed to the use of different strains, cultural conditions, and methods for the isolation of lipopolysaccharides. In particular, it should be noted that the average phosphorus content of their products was only 2.8% (Hanessian *et al.*, 1971; Horton *et al.*, 1977), compared with 4.6% for the lipopolysaccharide from strain N.C.T.C. 1999 (Drewry *et al.*, 1975), and that cold aqueous trichloroacetic acid was used for extraction of the lipopolysaccharides. In addition, however, the conditions used to solubilize the lipopolysaccharides for spectral studies may have been inappropriate. When we used the method of Horton & Riley (1981) with the lipopolysaccharide

Fig. 1. ^{31}P N.m.r. spectra at 162.0 MHz of the lipopolysaccharide from *P. aeruginosa* N.C.T.C. 1999

The lipopolysaccharide (50 mg; Drewry *et al.*, 1975) was added to 2% (w/v) sodium dodecyl sulphate/0.02 M-EDTA in 75% (v/v) $^2\text{H}_2\text{O}$ (2 ml) and dispersed by the addition of triethylamine (25 μl) and sonication. After the spectrum (a) had been recorded, the pH of the solution (apparently 10.7) was adjusted to 4.5 by the addition of 1 M-citric acid and a second spectrum (b) was recorded. The proton-decoupled spectra were obtained by using a Bruker WH-400 spectrometer, with sweep width 10000 Hz, acquisition time 1.6 s, and pulse width 10 μs . The field was locked on the deuterium resonance of the solvent, and 85% orthophosphoric acid was used as an external reference. Spectrum (a) was obtained after 2000 scans and spectrum (b) after 1000 scans.

from *P. aeruginosa* N.C.T.C. 1999, the intensity of the diagnostic high-field signal was much reduced.

I thank the Science Research Council for providing access to the high-field n.m.r. service of the Department of Chemistry, Sheffield University, and Dr. B. E. Mann for obtaining the ^{31}P spectra.

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(Received 20 July 1981)

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0306-3275/81/120833-03\$01.50/1

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The absence of triphosphate or metaphosphate residues from lipopolysaccharides of the seven Fisher immunotypes of *Pseudomonas aeruginosa*

The ^{31}P -n.m.r. spectra of the lipopolysaccharides mentioned in the title have been recorded in a recent report from our laboratory (Horton & Riley, 1981). The spectra provide diagnostic 'fingerprints' of each of the seven different lipopolysaccharides, and these may be useful for classifying unknown samples. Structural information deduced from the spectra included the conclusion that triphosphate and/or metaphosphate residues are essentially absent from these lipopolysaccharides. These products had been isolated for potential clinical use from cell pastes by the Boivin technique, namely extraction with cold trichloroacetic acid (Hanessian *et al.*, 1971) and their phosphorus content averaged about 3% (Horton *et al.*, 1977); details of their structures have been elucidated (Horton *et al.*, 1981).

The preceding letter (Wilkinson, 1981) shows that the lipopolysaccharide from *P. aeruginosa* strain N.C.T.C. 1999, isolated from cells by the Westphal phenol/water extraction procedure and containing 4.6% phosphorus, gives a ^{31}P -n.m.r. spectrum whose broad features correspond to the spectra recorded by us, and the structural interpretations drawn are mostly similar. A major difference, however, is the presence in Wilkinson's spectra of a high-field signal at about 20 p.p.m., for which attribution to the

central phosphorus atom in a triphosphate residue seems clear.

There appears to us no essential difference in the solubilization procedure used by us and that used by Wilkinson (1981); minor variations in our own procedure did not produce notable differences in the observed spectra, and thus we doubt whether this factor contributes significantly to the discrepancy between our results and those of Wilkinson. The important difference appears to relate to the procedure used for isolation of the lipopolysaccharide. Strain N.C.T.C. 1999 of *P. aeruginosa* appears to correspond closely to Fisher immunotype 3, but the Westphal extraction procedure employed by Wilkinson gives a lipopolysaccharide of much higher phosphorus content than our type 3 lipopolysaccharide isolated by the Boivin technique; also the microbial culture conditions may have been different. The previous report (Drewry *et al.*, 1972) of the occurrence of 2-aminoethyl metaphosphate residues in the lipopolysaccharide from strain N.C.T.C. 1999 was not in accord with our results, which ruled out the presence of metaphosphate in the lipopolysaccharide complexes of the seven Fisher immunotypes.

It should be kept in mind that our data are in close accord with ^{31}P -n.m.r. studies on lipopolysaccharide complexes from other Gram-negative bacteria such as *Salmonella typhimurium* and *Escherichia coli* (Rosner *et al.*, 1979; Mühlradt *et al.*, 1977), where the Westphal isolation procedure was employed. The total structural role of phosphorus in bacterial lipopolysaccharide complexes is still open to extensive clarification. Further high-field ^{31}P -n.m.r. studies, including nuclear Overhauser enhancement experiments, of such complexes isolated on a comparative basis by different extraction techniques will be needed to resolve such discrepancies as the present instances, and advance our total understanding of the structures of these molecular complexes.

Work supported by NIH Grant GM-20181.

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(Received 1 September 1981)

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