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Studies of Polysaccharide Fractions from the Lipopolysaccharide of *Pseudomonas aeruginosa* N.C.T.C. 1999

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Two polymeric water-soluble fractions were isolated by gel filtration after mild acid hydrolysis of the lipopolysaccharide from *Pseudomonas aeruginosa* N.C.T.C. 1999. The fraction of higher molecular weight retained the O-antigenic specificity of the lipopolysaccharide and may be 'side-chain' material. This fraction was rich in N (about 10%) and gave several basic amino compounds on acid hydrolysis; fucosamine (at least 2.8%, w/w) was the only specific component identified. The fraction of lower molecular weight was a phosphorylated polysaccharide apparently corresponding to 'core' material. The major components of this fraction and their approximate molar proportions were: glucose (3–4); rhamnose (1); heptose (2); 3-deoxy-2-octulonic acid (1); galactosamine (1); alanine (1–1.5); phosphorus (6–7). In the intact lipopolysaccharide this fraction was probably linked to lipid A via a second residue of 3-deoxy-2-octulonic acid, and probably also contained additional phosphate residues and ethanolamine. The residues of 3-deoxy-2-octulonic acid were apparently substituted in the C-4 or C-5 position, and the phosphorylated heptose residues in the C-3 position. The rhamnose was mainly 2-substituted, though a little 3-substitution was detected. The glucose residues were either unsubstituted or 6-substituted. Four neutral oligosaccharides were produced by partial acid hydrolysis and were characterized by chemical, enzymic, chromatographic and mass-spectrometric methods of analysis. The structures assigned were: Glc*p*α1–6Glc; Glc*p*β1–2Rha; Rha*p*α1-6Glc; Glc*p*β1–2Rha*p*α1–6Glc. The galactosamine was substituted in the C-3 or C-4 position, the attachment of alanine was indicated, and evidence that the amino sugar linked the glucose–rhamnose region to the 'inner core' was obtained.

Although several studies of lipopolysaccharides from *Pseudomonas aeruginosa* have been reported, little detailed information about the architecture and structures of these macromolecules is available. However, the use of mild hydrolysis with 1% (v/v) acetic acid (Fensom & Meadow, 1970) has permitted the isolation of relatively undegraded lipid A and polysaccharide fractions and opened the way to structural studies. Thus the structure of the chloroform-soluble lipid A and the composition of water-soluble low-molecular-weight hydrolysis products from the lipopolysaccharide of *P. aeruginosa* N.C.T.C. 1999 have been described in previous reports from this laboratory (Drewry *et al.*, 1971, 1972*a*, 1973). Two water-soluble fractions of higher molecular weight (coded P1 and P2) have also been isolated by Sephadex chromatography (Drewry *et al.*, 1971). Fraction P2 was rich in phosphorus and carbohydrate and appeared to correspond to a 'core polysaccharide' (Fensom & Meadow, 1970; Ikeda & Egami, 1973; Chester *et al.*, 1973; Wilkinson & Galbraith, 1975). The results of further studies of this fraction and of a preliminary characterization of fraction P1 are presented in this paper. A brief account of part of the work has been given (Drewry *et al.*, 1972*b*).

Materials and Methods

Materials

Alkaline phosphatase (EC 3.1.3.1) was type 1 from calf intestinal mucosa (Sigma Chemical Co., St. Louis, Mo., U.S.A.). Phosphodiesterase (EC 3.1.4.1) from *Crotalus adamanteus* venom was obtained from Sigma and Boehringer-Mannheim G.m.b.H., Mannheim, Germany. Yeast α-glucosidase (EC 3.2.1.20) was from Sigma and almond β-glucosidase (EC 3.2.1.21) from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; suspensions of both enzymes for quantitative analytical purposes were obtained from Boehringer. Reagents for the determination of D-glucose by using glucose oxidase (EC 1.1.3.4) were from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and Boehringer. The following materials were received as gifts: laminaribiose (Glc*p*β1–3Glc), from Dr. J. E.

McCormick; the hepta-acetate of scillabiose (Glc$p\beta$1–4Rha), from Dr. G. G. S. Dutton; lipopolysaccharide from *Klebsiella* O group 10, from Dr. W. Nimmich; the O-specific polysaccharide from *Serratia marcescens* Bizio, from Dr. P. Alaupovic. Nigerose (Glc$p\alpha$1–3Glc) was obtained by partial acid hydrolysis (Stacey & Webber, 1962) of nigeran (Koch–Light); other disaccharides of glucose were obtained from Koch–Light or Sigma. Rutinose (Rha$p$1–6Glc) was obtained from rutin (Sigma) by the method of Zemplén & Gerecs (1938). NaB^2H_4 was from The British Oxygen Co. Ltd., London S.W.19, U.K.

Methods

Growth of bacteria and preparation of cell walls. Cells of *P. aeruginosa* N.C.T.C. 1999 were grown for 16h at 37°C in nutrient broth no. 2 (20litres; Oxoid Ltd., London E.C.4, U.K.) in a stirred fermenter (Fermentation Design Inc., Durham, Pa., U.S.A.), from a 1% inoculum of a 24h-broth culture. Aeration was at 20litre/min; polyglycol P-2000 (1ml; The Dow Chemical Co., Midland, Mich., U.S.A.) was added to prevent foaming. Cells (60–100g wet wt./batch) were collected by using a refrigerated continuous centrifuge (Sharples), and walls were isolated and purified as described by Wilkinson (1968).

Isolation and fractionation of lipopolysaccharide. Each batch of walls was checked for purity and for consistency of composition by electron microscopy, i.r. spectroscopy, quantitative analysis and qualitative analysis. Batches of walls were then combined and loosely bound lipids were extracted. Lipopolysaccharide was extracted from the defatted walls by a single treatment with aq. 45% (w/v) phenol for 15min at 68°C, and was purified by the method of Key *et al.* (1970).

Lipopolysaccharide was split into lipid A and partly degraded polysaccharide by mild hydrolysis with 1% (v/v) acetic acid at 100°C (Drewry *et al.*, 1973). The water-soluble products were fractionated by chromatography on Sephadex G-75 or G-50 (Pharmacia, Uppsala, Sweden) as described by Drewry *et al.* (1971).

Analytical methods. Methods for the determination of total P, P_i, N, amino compounds, total fatty acids (expressed as hexadecanoic acid), total carbohydrate (expressed as glucose), D-glucose, rhamnose, heptose and 3-deoxy-2-octulonic acid were those used by Wilkinson *et al.* (1973). In some cases, N was also determined by elemental combustion analysis and 3-deoxy-2-octulonic acid by the semicarbazide method (Dröge *et al.*, 1970). Glucose and rhamnose were also determined by autoanalysis (Hough *et al.*, 1972). Alditol acetates were prepared by a modification of the method of Sawardeker *et al.* (1965) and were determined by g.l.c. with column I (see under 'G.l.c.').

Gel filtration. Fractionations of polysaccharides and their degradation products were carried out with various grades of Sephadex. Fractions were eluted with pyridine–acetic acid buffer (pH5.4) (Schmidt *et al.*, 1969) and were analysed, as necessary, for P and total carbohydrate. Materials were recovered from appropriately pooled fractions by freeze-drying.

Anion-exchange chromatography. Fractionation of phosphates in hydrolysates of lipopolysaccharide was carried out by using DEAE-cellulose (Whatman DE-32, CO_3^{2-} form). Fractions were eluted first with water and then by application of a linear gradient of aq. $(NH_4)_2CO_3$ (0–0.15M), generated with a Dialagrad (Instrumentation Specialities Co., Lincoln, Neb., U.S.A.).

Paper chromatography. Chromatography was carried out with Whatman papers (no. 1 for analytical separations and water-washed no. 1 or 3MM for preparative separations). Solvent systems used were: A, ethyl acetate–pyridine–water (5:2:5, by vol., upper layer); B, ethyl acetate–pyridine–water (8:2:1, by vol.); C, ethyl acetate–pyridine–water (72:20:23, by vol.) (Wang & Alaupovic, 1973); D, ethyl acetate–pyridine–water–acetic acid (5:5:3:1, by vol.); E, butan-1-ol–pyridine–water (6:4:3, by vol.); F, butan-1-ol–acetic acid–water (4:1:5, by vol., upper layer); G. butan-1-ol–ethanol–water (40:11:19, by vol.); H, butan-1-ol–ethanol–water–aq. NH_3 (sp.gr. 0.88) (40:10:49:1, by vol., upper layer). Reducing sugars were detected by using alkaline $AgNO_3$ (Trevelyan *et al.*, 1950) and aniline hydrogen phosphate (Bryson & Mitchell, 1951). Amino compounds were detected by using ninhydrin.

Paper electrophoresis. High-voltage electrophoretograms were run on Whatman papers as for chromatography. Separations were usually carried out at about 37V/cm for 1h with pyridine–acetic acid–water (5:2:43, by vol.; pH5.3) as the buffer system. Electrophoresis in 0.05M-sodium tetraborate at about 50V/cm for 1h was used for the separation of oligosaccharides. Detection reagents in addition to those used for paper chromatograms were the reagent of Hanes & Isherwood (1949) for phosphates and the reagents of Warren (1960) for 3-deoxy-2-octulonic acid.

G.l.c. All separations were carried out with a Pye 104 gas chromatograph and with packed columns containing the following stationary phases: I, 3% (w/w) ECNSS-M; II, 3% (w/w) OV-225; III, 10% (w/w) polyphenyl ether (OS 138); IV, 15% (w/w) poly(butane-1,4-diol succinate); V, 10% (w/w) poly(2,2-dimethylpropane-1,3-diol sebacate); VI, 3% (w/w) SE-52. Peak areas were determined by using a precision integrator (Honeywell Ltd., Brentford, Middx., U.K.).

Partial acid hydrolysis of polysaccharides. Oligosaccharides were obtained by hydrolysis of samples with 0.5M-HCl at 100°C for 30min. Hydrolysates were neutralized with dilute NaOH and fractionated by chromatography on Sephadex G-10, paper electrophoresis and paper chromatography (solvent systems A and E). Neutral oligosaccharides were obtained more simply by deionization of the hydrolysates, followed by preparative paper chromatography (solvent system A). Partial hydrolyses were also carried out by the methods of Painter (1965) and Galanos *et al.* (1969).

Dephosphorylation of polysaccharides. Samples of phosphorylated polysaccharide were dissolved in 0.2M-ammonium acetate (pH4.0) and heated at 100°C for 42h (Baddiley *et al.*, 1957). The dephosphorylated polysaccharide was separated from P_i by chromatography on Sephadex G-15. Alternatively, the phosphorylated polysaccharide was dissolved in cold (0°C) 40% (w/v) HF and the solution was left for 3 days at 4°C. After the addition of ice-cold water, the solution was freeze-dried and the dephosphorylated polysaccharide recovered as described above.

Methylation analysis. Samples of lipopolysaccharide, polysaccharides, oligosaccharides and oligosaccharide alditols were methylated essentially as described by Hellerqvist *et al.* (1968). The methylated products were hydrolysed by the method of Percival (1968, 1971). Samples of the methylated sugars were converted into methyl glycosides by the method of Cadotte *et al.* (1952). Further samples of methylated sugars were reduced overnight with $NaBH_4$ and, after removal of Na^+ with Dowex 50 resin (H^+ form) and boric acid by distillation as trimethyl borate, the methylated alditols were treated with pyridine–acetic anhydride (1:1, v/v) at 100°C for 45min–2h. Products were examined by g.l.c. with columns III and IV (methyl glycosides) or I, II and III (alditol acetates). In some cases the products were also examined by combined g.l.c.–mass spectrometry. Partially methylated sugars for use as reference compounds were isolated by preparative paper chromatography (solvent system G) from hydrolysates of per-*O*-methylated polysaccharides (laminarin, glycogen, lipopolysaccharides from *Klebsiella* O group 10 and *Serratia marcescens* Bizio).

Periodate oxidation. Samples were treated with 0.05M-$NaIO_4$ at 4°C in the dark for 2–6 days. Consumption of $NaIO_4$ was determined by the method of Avigad (1969). Acetaldehyde was determined by the method of Simmons (1969) and formaldehyde either by the chromotropic acid method (Kabat & Mayer, 1961) or by the acetylacetone–NH_3 method (Speck, 1962). Excess of $NaIO_4$ was destroyed by the addition of ethylene glycol and the oxidation products were treated with $NaBH_4$ for 2h at room temperature (20°C). Excess of $NaBH_4$ was destroyed by the addition of dilute acetic acid.

Enzymic hydrolyses. Samples of phosphate esters were treated with alkaline phosphatase as described by Drewry *et al.* (1971). Oligosaccharides were incubated with glucosidases overnight at 37°C under toluene; the release of glucose was determined both by paper chromatography and by using glucose oxidase.

Miscellaneous methods. Methods used for the hydrazinolysis of lipopolysaccharide, *N*-acetylation of amino compounds, and application of the Morgan–Elson reaction have been described (Drewry *et al.*, 1973). Oligosaccharide alditols were converted into their per-*O*-trimethylsilyl ethers by the method of Ohashi & Yamakawa (1973). The oxidation of a per-*O*-acetylated trisaccharide alditol with CrO_3 was carried out under two sets of conditions (Lindberg *et al.*, 1972; Hoffman *et al.*, 1972). The configurations of glucose and rhamnose isolated by paper chromatography were determined by polarimetry (Bendix model 143A) and, for rhamnose, by measurement of the circular dichroism of the alditol acetate (Bebault *et al.*, 1973).

Ultrafiltration was carried out with a cell fitted with a UM-2 membrane and a magnetic stirrer (Amicon, High Wycombe, Bucks., U.K.). I.r. spectra were recorded with a Unicam SP.200 spectrophotometer, and u.v.–visible spectra with a Unicam SP.700 spectrophotometer. Atomic absorption spectra were determined with a Unicam SP.90 spectrophotometer by Dr. N. S. Angus, and electron micrographs were taken with a Siemens Elmiskop 1A instrument by Mr. P. Worthington. Mass spectra were obtained by using an AEI MS902 spectrometer: combined g.l.c.–mass spectrometry was undertaken by the Physico-Chemical Measurements Unit, Aldermaston, Reading, Berks., U.K.

Results

Composition of cell walls

From 11 batches of cells (each from a 20-litre culture), grown in two series, a total of 19.6g of walls was obtained. Analytical checks confirmed that the wall composition was adequately reproducible and similar to that reported for the walls of cells from surface cultures (Fensom & Gray, 1969). Mean values and ranges for quantitative determinations were as follows: P, 2.3% (2.1–2.5%); N, 7.9% (7.7–8.3%); total carbohydrate, 7.2% (6.2–8.6%); total amino compounds, 41.6% (37.4–45.5%). Loosely bound lipids (mainly phosphatidylethanolamine, with phosphatidylglycerol, diphosphatidylglycerol and a little phosphatidylcholine) comprised 21% of the weight of the walls. Lipopolysaccharide, obtained

by a single treatment of the defatted walls with hot aqueous phenol, accounted for 25% of the weight of the whole walls. The total amount of lipopolysaccharide available for structural studieswas 4.9g.

Composition of lipopolysaccharide

The freeze-dried lipopolysaccharide was obtained as white pithy material with a characteristic ribbon-like structure in electron micrographs. The product was free from nucleic acid (no ribose detected, no absorption maximum at 260nm) and peptidoglycan (neither muramic acid nor 2,6-diaminopimelic acid detected), and amino acids other than alanine accounted for less than 2% of the weight of material. Analytical data for specific components of the lipopolysaccharide are given in Table 1, which also contains comparative data for lipopolysaccharide isolated directly from whole cells grown under different conditions (Chester *et al.*, 1972). Although phenotypic variations in the compositions of wall components are known (Chester *et al.*, 1973; Robinson *et al.*, 1974), the data for the two present batches agree well and closely resemble those for the previous preparations of lipopolysaccharide. Some differences in minor components were, however, noted. Thus ethanolamine and *O*-phosphoethanolamine were not detected in the preparations from whole cells (Fensom & Gray, 1969), but mannose and arabinose were trace components in those preparations only (Chester *et al.*, 1972). In addition to the components listed in Table 1, the occurrence in the lipopolysaccharide of unidentified amino compounds (e.g. unknown compound D, eluted by autoanalysis in about the position of ornithine; Fensom & Gray, 1969) was confirmed.

The quantitative data for the rhamnose content of the lipopolysaccharide (Table 1) were obtained by application of the cysteine–H_2SO_4 reaction to unhydrolysed samples. More recent determinations on deionized hydrolysates both by specific analysis and by autoanalysis have given the following results: rhamnose, 3.6%; molar ratio of glucose/rhamnose, about 2.4:1.0. It appears that the determinations on unhydrolysed samples, which are based on differential extinctions to eliminate interference by glucose, are subject to interference by other components. The identification of glucose as the D isomer was based on the measurement of specific rotation ($[\alpha]_D^{20}+50°$, *c* 1 in water), and on a comparison of determinations made by the phenol–H_2SO_4 and glucose oxidase methods. Similarly, rhamnose was identified as the L isomer by the measurement of its specific rotation ($[\alpha]_D^{20}+9°$, *c* 1 in water) and of the circular dichroism ($\Delta\varepsilon_{213}^{MeCN}$ negative) of the derived rhamnitol penta-acetate (Bebault *et al.*, 1973).

It should be noted that the data for heptose and 3-deoxy-2-octulonic acid, which were obtained by using literature calibrations, are unlikely to be precise (Wright & Rebers, 1972; Charon & Szabó, 1972).

Table 1. *Composition of lipopolysaccharide*

Data in columns A and B refer to lipopolysaccharides isolated from the two series of wall batches. Data in column C refer to lipopolysaccharide isolated from whole cells (Chester *et al.*, 1972). Results for amino compounds were obtained by autoanalysis after hydrolysis (6.1M-HCl at 105°C for 4h) of samples, and are expressed (A and B) as residues of amino compounds, without correction for slow release or destruction during hydrolysis. –, Not determined; + present but not determined.

	Content (%, w/w)		
Lipopolysaccharide ...	A	B	C
P	4.6	4.7	4.6
N	4.6	4.2	3.7
Total carbohydrate	20.4	20.9	20.5
Total fatty acids	18.1	+	20.9
Glucose	10.1	10.8	8.0
Rhamnose	2.3	2.3	2.0
Heptose	4.2	5.1	4.2
3-Deoxy-2-octulonic acid	3.2	3.3	2.8
Glucosamine*	4.63	4.34	4.68
Galactosamine	1.96	2.19	2.21
Fucosamine	0.79	0.78	0.73
Alanine	1.55	1.59	1.01
Ethanolamine*	+	+	–

* Present partly as phosphate ester.

Methylation analysis of lipopolysaccharide

G.l.c. analysis of the methylated alditol acetates from methylated lipopolysaccharide did not readily yield consistent or interpretable results. Chromatograms for material methylated once contained a single major peak apparently corresponding to the derivative from 2,4,6-tri-*O*-methylglucose. On the assumption that methylation was incomplete because of the low solubility of the lipopolysaccharide in dimethyl sulphoxide (e.g. Hämmerling *et al.*, 1971), further samples were methylated twice. Chromatograms then showed significant additional peaks corresponding to the derivatives from 3,4-di-*O*-methylrhamnose, 2,3,4,6-tetra-*O*-methylglucose and 2,3,4-tri-*O*-methylglucose.

The identification of glucose derivatives was based on comparison with reference compounds by using columns I, II and III, and was confirmed by similar comparison of methyl glycosides by using columns III and IV. No reference sample of 3,4-di-*O*-methylrhamnose was available, but the methylated rhamnitol acetate from *P. aeruginosa* had retention times (I, 0.92; II, 0.88) relative to 1,5-di-*O*-acetyl-2,3,4,6-

tetra-*O*-methylglucitol close to literature values (Björndal *et al.*, 1970*b*; Chaudhari *et al.*, 1972). Also, by using a reference mixture of the alditol acetates from 2,3- and 2,4-di-*O*-methylrhamnose derived from the lipopolysaccharide of *Klebsiella* O group 10 (Björndal *et al.*, 1970*a*), it was confirmed that these sugars were not resolved from 2,3,4,6-tetra-*O*-methylglucose by g.l.c. with column I. By using column III at 180°C, the major rhamnose derivative from *P. aeruginosa* (relative retention time 0.75) could be clearly differentiated from 2,3- and 2,4-di-*O*-methylrhamnose (relative retention times 0.81 and 0.90 respectively), but the presence of a little 2,4-di-*O*-methylrhamnose was also detected (less than 15% of the amount of 3,4-di-*O*-methylrhamnose).

Because of uncertainty about the extent of methylation even after two treatments, no reliable quantitative data can be given. Thus, for the lipopolysaccharides described in Table 1, the ratio of peak areas for 2,4,6-tri-*O*-methylglucose to 2,3,4-tri-*O*-methylglucose was 2:1; for more recent batches the ratio was about 1:3. Also the proportion of 3,4-di-*O*-methylrhamnose was less than expected from the analytical data (Table 1), possibly because of selective loss of the relatively volatile derivative and slight inflation of the peak area (column I) for 2,3,4,6-tetra-*O*-methylglucose by the underlying 2,4-di-*O*-methylrhamnose derivative.

Preparation and fractionation of the partly degraded polysaccharide

After treatment of the lipopolysaccharide with 1% (v/v) acetic acid at 100°C for 1–2h, about 70% of the material was recovered as water-soluble products. As the longer period of hydrolysis was unnecessary for the cleavage of lipid A from polysaccharide and seemed only to increase the amount of P_i formed, the period of 1h was used for preparative purposes. Attempts to separate polymeric solutes from low-molecular-weight products by ultrafiltration with a UM-2 membrane (cut-off at mol.wt. about 1000) were unsuccessful, as some polysaccharide passed through the membrane. Experiments with different grades of Sephadex established that G-75 gave satisfactory fractionation and at least partial inclusion of all components (Drewry *et al.*, 1971). Two polymeric fractions were isolated: fraction P1 [V_e (elution volume)/V_0 (void volume) about 1.3] accounted for 15–20% by weight of the lipopolysaccharide, and fraction P2 (V_e/V_0 about 2.1) approx. 40%.

Preliminary studies of fraction P1

By monitoring the eluate during Sephadex chromatography, the presence of fraction P1 was only indicated by small peaks for P- and carbohydrate-containing material (Drewry *et al.*, 1971). Representative analyses (Table 2) confirmed that these and other identifiable components made up only a small part of fraction P1. The outstanding observations are that fraction P1 contained most of the fucosamine of the lipopolysaccharide and was exceptionally rich in N. The occurrence of unidentified nitrogenous components in the lipopolysaccharide of *P. aeruginosa* N.C.T.C. 1999 has been diagnosed (Fensom & Gray, 1969). A check on the u.v. spectrum of fraction P1 showed that residual pyridinium salts (from chromatography) could not explain the high N content, and atomic absorption spectrophotometry revealed only traces of calcium and magnesium.

Table 2. *Composition of fraction P1 from the total polysaccharide*

Results for amino compounds were obtained by autoanalysis after hydrolysis (6.1M-HCl at 105°C for 4h) of samples, and are expressed as residues of amino compounds, without correction for slow release or destruction during hydrolysis. Values for N were determined both by combustion and by nesslerization (result in parentheses).

	Content (%, w/w)
P	0.7
N	7–9 (10.2)
C	42.2
H	5.7
Total carbohydrate	3.8
Glucose	1.8
Rhamnose	1.2
Heptose	1.2
3-Deoxy-2-octulonic acid	Absent
Glucosamine	0.1
Galactosamine	0.7
Fucosamine	2.8
Alanine	0.3

The variability of N analyses (Table 2) may be attributable in part to the difficulty in obtaining complete combustion of fraction P1. Variable values (2–10%) were also obtained for the total carbohydrate content of different batches. This may be explained by partial resolution during chromatography of carbohydrate and N-rich fractions, and the absence of an elution profile for the latter.

Although the high N content of fraction P1 could not be accounted for by identifiable amino acids and amino sugars, several ninhydrin-positive compounds were detected by paper electrophoresis of acid hydrolysates. The most mobile products (P1-8 and P1-9) were galactosamine and fucosamine, with alanine as the major neutral product (P1-1). The unidentified products (P1-2 to P1-7) were all cationic at pH5.3, and all were probably oligomers. Compounds P1-2 (m_{GlcN} 0.19) and P1-5 (m_{GlcN} 0.52) were only trace products, detected after vigorous hydrolysis of fraction P1. Compound P1-3 (m_{GlcN}

0.27) was formed under relatively mild conditions (0.2–1.0M-HCl at 100°C for not more than 1h): under more drastic conditions it was apparently converted into compound P1-4. Optimum conditions for the formation of compound P1-7 (m_{GlcN} 0.85) were hydrolysis with 2–3M-HCl at 100°C for 1h; fucosamine was apparently released during further hydrolysis. Compounds P1-4 (m_{GlcN} 0.39) and P1-6 (m_{GlcN} 0.64) were the most acid-stable products, and the only significant unidentified components of hydrolysates prepared by using 6.1M-HCl at 100°C for 4h or longer. During autoanalysis (Technicon), both were eluted in the ornithine region, corresponding to the unidentified material detected in previous studies. Compounds with the same properties on electrophoresis and autoanalysis as P1-4 and P1-6 have also been detected in high-molecular-weight fractions from the lipopolysaccharides of other strains of *P. aeruginosa* belonging to the same serogroup (Habs group 2B; Wilkinson & Galbraith, 1975). Attempts to identify compounds P1-4 and P1-6 by paper chromatography, ninhydrin degradation and oxidative degradation (Volk *et al.*, 1970) were unsuccessful. Studies (K. C. Symes & S. G. Wilkinson, unpublished work) indicate that they are oligomers with exceptional resistance to acid hydrolysis.

Composition of the phosphorylated polysaccharide (fraction P2)

Analytical data for different batches were very similar, and average values are given in Table 3. Fraction P2 contained almost all of the neutral carbohydrate, galactosamine and alanine of the lipopolysaccharide, much P, a little fucosamine (otherwise specific for fraction P1), and no glucosamine (exclusive to lipid A). Although little 3-deoxy-2-octulonic acid was detected by the thiobarbituric acid method (Table 3), more (5.8%) was detected by the semicarbazide method. No colour was produced in the thiobarbituric acid reaction after treatment of fraction P2 with $NaBH_4$, indicating that the polysaccharide was terminated by a single reducing residue of 3-deoxy-2-octulonic acid. The molar ratio of glucose/rhamnose given by the data in Table 3 was 3.6:1. Corresponding ratios determined by autoanalysis of this and other batches of polysaccharide were in the range 3.1:1 to 3.8:1. Thus the major components of fraction P2 and their approximate molar proportions were as follows: glucose (3–4); rhamnose (1); heptose (1–2); 3-deoxy-2-octulonic acid (1); galactosamine (1); alanine (1–1.5); P (6–7). These results closely resemble those obtained for similar phosphorylated polysaccharides from other strains of *P. aeruginosa* (Fensom & Meadow, 1970; Chester *et al.*, 1973; Wilkinson & Galbraith, 1975).

Table 3. *Composition of the phosphorylated polysaccharide (fraction P2)*

The values given are averages for four batches. Results for amino compounds were obtained by autoanalysis after hydrolysis (6.1M-HCl at 105°C for 4h) of samples, and are expressed as residues of amino compounds, without correction for slow release or destruction during hydrolysis.

	Content (%, w/w)
P	5.7
Total carbohydrate	39.1
Glucose	20.8
Rhamnose	5.3
Heptose	9.9
3-Deoxy-2-octulonic acid	2.0
Galactosamine	4.4
Fucosamine	0.3
Ethanolamine*	0.3
Alanine	2.8

* Mainly as ethanolamine phosphate.

Methylation analysis of the phosphorylated polysaccharide

Results were generally similar to those obtained with the parent lipopolysaccharide. In the initial studies, two methylations were again found to be necessary and more 2,4,6-tri-*O*-methylglucose than 2,3,4-tri-*O*-methylglucose was produced. In more recent work, the polysaccharide was warmed with dimethyl sulphoxide before adding the dimethylsulphinyl reagent. Essentially the same results were then obtained by either one or two methylations, and the ratio of peak area for the derivative from 2,3,4-tri-*O*-methylglucose to that from 2,4,6-tri-*O*-methylglucose was at least 5:1, and the sum of the areas was about equal to that of the peak for 2,3,4,6-tetra-*O*-methylglucose. In this later work, the identities of these sugars and of the 3,4-di-*O*-methylrhamnose and 2,3,4,6-tetra-*O*-methylglucose were confirmed by combined g.l.c.–mass spectrometry (Björndal *et al.*, 1970*b*). By this technique, the presence of a little 2,4-di-*O*-methylrhamnose was substantiated and a little 2,4-di-*O*-methylglucose was detected. The significance of the minor components is not clear, but most or all may be products of undermethylation. In summary, it appears that most glucose residues in the polysaccharide are either unsubstituted or 6-substituted, and the rhamnose is mainly 2-substituted, both sugars being in the pyranoid form.

Periodate oxidation of the phosphorylated polysaccharide

The consumption of periodate was rapid for 2–3h, then continued slowly for several days. After 3 days the recoveries of individual components were as follows: glucose, 40%; rhamnose, 15%; heptose,

0%; galactosamine, 100%; alanine, 47%. Mannose was also detected by paper chromatography of a hydrolysate from the oxidized polysaccharide. After oxidation for 5 days, the recovery of glucose was only 14%, but the results for rhamnose and galactosamine were unchanged. The results of these studies are consistent with those of the methylation analysis described above.

The loss of alanine during periodate oxidation was unexpected, but a similar result was obtained during oxidation of the polysaccharide from *Pseudomonas alcaligenes* (Lomax *et al.*, 1974). These authors suggested that the loss of alanine could be explained by a condensation between its amino group and a carbonyl group generated during the oxidation. Support for this suggestion has been obtained by carrying out the oxidation in the presence of increasing amounts of glycine. The recovery of alanine could be raised thereby to about 95%.

Neutral oligosaccharides from the phosphorylated polysaccharide

Hydrolysis of the polysaccharide with 0.5M-HCl at 100°C for 30min was apparently optimum for the formation of oligosaccharides. In addition to free glucose and rhamnose, three reducing spots were detected by paper chromatography (solvent system A), and further studies showed that one of them corresponded to two products. The characterization of the four oligosaccharides is described below.

Oligosaccharide 1. This compound had R_{Glc} values of about 1.2 in solvent systems A, B, C and E, and contained glucose and rhamnose in equal amounts. It was completely hydrolysed to these sugars by treatment with either acid or β-glucosidase (α-glucosidase had no effect). The destruction of all the rhamnose but no glucose by treatment with $NaBH_4$ confirmed that the oligosaccharide was a β-glucopyranosylrhamnose. It resembled synthetic Glcpβ1–4Rha (Bebault & Dutton, 1972) on paper chromatography in solvent systems A, B, C and E. However, the disaccharides were differentiated by using solvent system F (R_{Glc} values: oligosaccharide 1, 1.1; Glcpβ1–4Rha, 1.0), and by g.l.c. (column VI at 230°C) of the per-*O*-trimethylsilyl alditols (retention times relative to that for per-*O*-trimethylsilylsucrose: oligosaccharide 1, 1.12; Glcpβ1–4Rha, 1.20). The differentiation was confirmed by the formation of acetaldehyde (1 molar proportion) during periodate oxidation of the alditol from oligosaccharide 1. Formaldehyde (molar proportion 0.3–0.7, varying with the experiment and the method of measurement) was also apparently formed during the oxidation, indicating that the oligosaccharide was Glcpβ1–3Rha. However, methylation analysis of the oligosaccharide and studies of the methylated alditol acetates by g.l.c. and mass spectrometry gave only 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol in approximately equal amounts. The mass spectrum for the latter compound contained the expected peaks at *m/e* 131 and 189, but no peak at *m/e* 117 (expected for the derivative from 3-substituted rhamnose). It was concluded therefore that oligosaccharide 1 was Glcβ1–2Rha, and that the detection of formaldehyde was an artifact of overoxidation or evidence of contamination by some component not detected by the methods used.

The disaccharide D-Glcpβ1–2-L-Rha has been reported as a product of partial hydrolysis of the O-specific polysaccharide from *Serratia marcescens* Bizio (Wang & Alaupovic, 1973). However, this product had an R_{Glc} value (0.66, solvent system C) very different from that of oligosaccharide 1. This discrepancy requires further examination, particularly as methylation analysis (monitored by g.l.c. and mass spectrometry of the alditol acetates) of a sample of the parent polysaccharide given by Dr. Alaupovic [but not from the batch analysed by Wang & Alaupovic (1973)] indicated that the rhamnose residues were in fact 3-substituted. Also, synthetic D-Glcpβ1–2-L-Rha is more mobile than glucose by t.l.c. on cellulose with solvent system E (King & Bishop, 1974).

An attempt to confirm the identity of oligosaccharide 1 by mass spectrometry of the per-*O*-trimethylsilyl derivative of the alditol (Kärkkäinen, 1969) was inconclusive. Although a minor peak at *m/e* 219 (corresponding to cleavage between C-3 and C-4 of the rhamnitol residue) was detected, the potentially diagnostic peak at *m/e* 321 (cleavage between C-2 and C-3) was not. However, cleavage between a glycosidically substituted and a trimethylsiloxylated carbon atom is relatively unimportant (Lönngren & Svensson, 1974).

Oligosaccharide 2. This oligosaccharide had R_{Glc} values slightly higher than those of the most mobile reducing disaccharide of glucose tested (Glcpβ1–3Glc), but equal to those of rutinose (solvent systems A and F). The retention time (1.20, relative to per-*O*-trimethylsilylsucrose) of the per-*O*-trimethylsilyl derivative of the oligosaccharide alditol on g.l.c. (column VI at 200°C) was also equal to that of the rutinose derivative. Both glucose and rhamnose were formed on total acid hydrolysis of the oligosaccharide, but neither glucosidase had any effect. Treatment with $NaBH_4$ caused 80% loss of glucose but no loss of rhamnose, again indicative of a rhamnosylglucose. Although the oligosaccharide seemed to be chromatographically homogeneous, the molar proportions of glucose to rhamnose for different preparations were variable (1:1 to 1:1.8). This variability probably reflects the difficulty of working with small amounts of material (yield about 140μg from 100mg of polysaccharide) near the lower limits of the analytical methods, as no trisaccharide or other

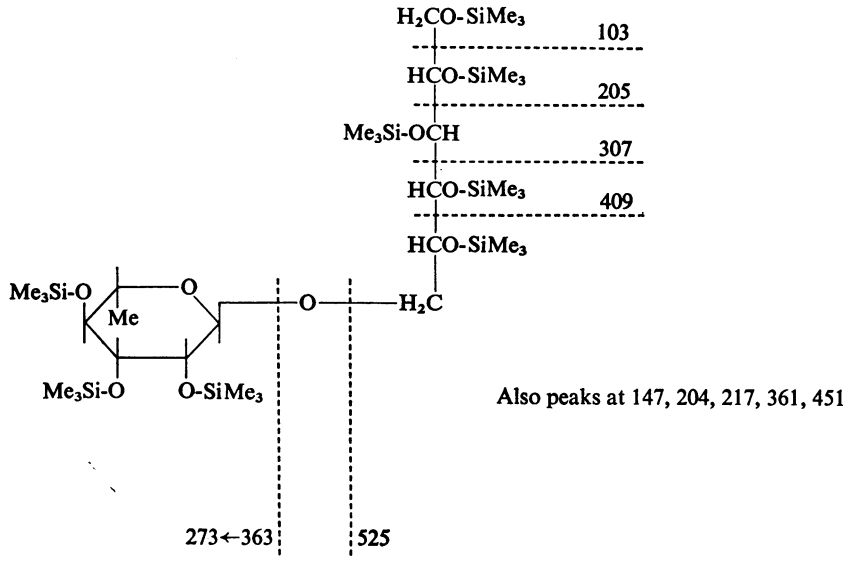

Fig. 1. *Mass-spectrometric fragmentation of the per-O-trimethylsilyl derivative of the alditol from oligosaccharide* 2

disaccharide was detected when the trimethylsilyl derivative of the alditol was examined by g.l.c. on column VI with temperature programming (Wilkinson & Galbraith, 1975). The mass spectrum of the derivative was that expected for a rhamnosylglucitol linked 1–5 or 1–6. Because of the results obtained during methylation analysis of the parent polysaccharide and during the study of oligosaccharide 4 (below), the linkage is represented (Fig. 1) as 1–6, despite the failure to detect a peak at *m/e* 511 (cleavage between C-5 and C-6 of the glucitol residue). The corresponding cleavage for the derivative from isomaltose (Glc*p*α1–6Glc) was also not observed (Larm *et al.*, 1972). Further studies relevant to the structure of oligosaccharide 2 are described below.

Oligosaccharide 3. This oligosaccharide co-chromatographed with oligosaccharide 4 (solvent systems A, B, and C) and was not isolated. However, the combined spots had R_{Glc} values corresponding to those of isomaltose, and treatment of the mixture with α-glucosidase gave glucose and oligosaccharide 4 (β-glucosidase had no significant effect). Paper electrophoresis of the mixture in borate buffer (pH 9.2) and g.l.c. of the per-*O*-trimethylsilyl derivatives of the mixed alditols confirmed the occurrence of isomaltose, and showed that this disaccharide and oligosaccharide 4 were in the proportions of about 1:3.

Oligosaccharide 4. This oligosaccharide was obtained pure by hydrolysis of contaminating isomaltose with α-glucosidase, deionization of the hydrolysate and preparative paper chromatography (solvent system A). It had R_{Glc} values of about 0.45, 0.34 and 0.62 (solvent systems A, B and E, respectively) and m_{Glc} about 0.45 (borate buffer, pH 9.2). The compound resisted hydrolysis by glucosidases, but acid hydrolysis gave glucose and rhamnose in the molar ratio 1.74:1. After treatment of the oligosaccharide with $NaBH_4$, the molar ratio was 0.97:1, indicating a trisaccharide with the sequence Glc–Rha–Glc. Partial acid hydrolysis under the original conditions (0.5 M-HCl at 100°C for 30 min) apparently gave oligosaccharides 1 and 2 (paper chromatography) in addition to monosaccharides and much unchanged material.

Methylation analysis of the oligosaccharide alditol gave 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol, and a mono-*O*-acetyl-penta-*O*-methylglucitol. By using g.l.c. alone (column III) and reference compounds for 6-substituted, 3(4)-substituted, and 2(5)-substituted hexitols (prepared from gentiobiose, cellobiose and sophorose respectively), the mono-*O*-acetyl penta-*O*-methylglucitol could not be conclusively identified. However, the mass spectrum of the compound was that expected for 6-*O*-acetyl-1,2,3,4,5-penta-*O*-methylglucitol (peaks at *m/e* 249, 235, 205, 177, 161, 145, 133, 117, 101, 89 and 45, among others).

To confirm the partial structure of Glc*p*1–2Rha*p*1–6Glc for oligosaccharide 4, mass spectra were recorded for both the per-*O*-trimethylsilyl and per-*O*-methyl derivatives of the trisaccharide alditol. The former spectrum (Fig. 2) showed peaks at *m/e* 525 and

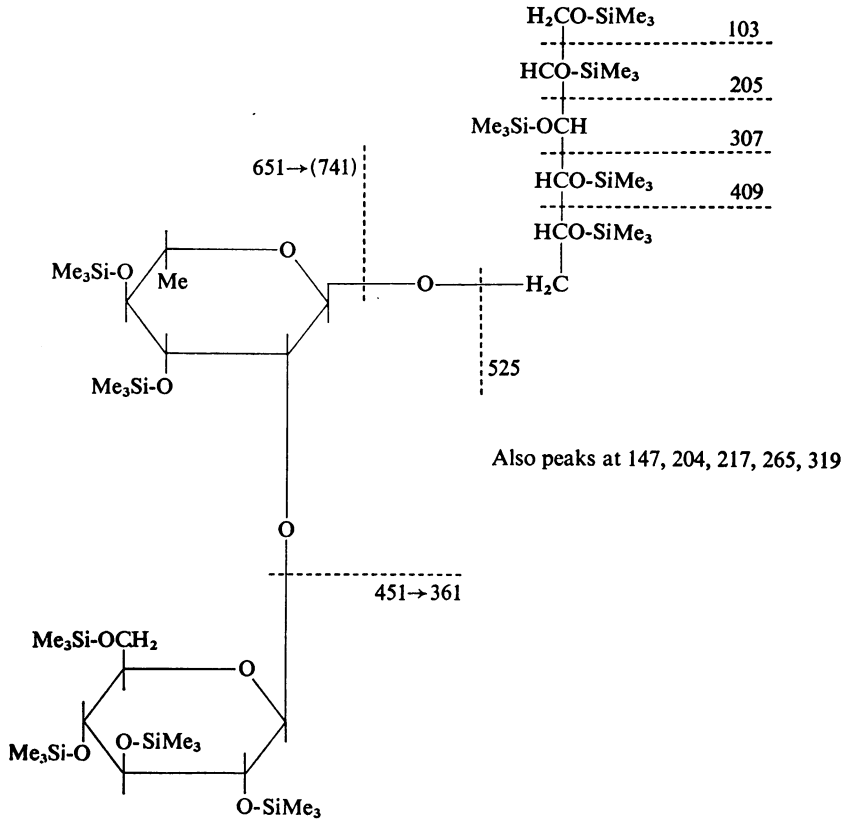

Fig. 2. *Mass-spectrophotometric fragmentation of the per-O-trimethylsilyl derivative of the alditol from oligosaccharide* 4

451 corresponding to the glucitol and glucose residues respectively, and a series of peaks (*m/e* 103, 205, 307 and 409) indicative of substitution of the glucitol residue at C-5 or C-6. As noted for oligosaccharide 2, the possible peak at *m/e* 511 expected for substitution at C-6 was not observed. The peak at *m/e* 651 appears to be derived from the disaccharide residue (Fig. 2).

The mass spectrum of the permethylated alditol (Fig. 3) also supports the postulated structure, although here again the peak (*m/e* 221) expected for cleavage between C-5 and C-6 of the glucitol residue was not prominent. However, the ions at *m/e* 177 and 145 (177−MeOH), and the shifts of these ions to *m/e* 178 and 146 when the alditol was prepared by using NaB^2H_4 in ^2H_2O, are characteristic of a 6-substituted hexitol residue (Kärkkäinen, 1971). The ions at *m/e* 469 and 409, which are also shifted by ^2H labelling, confirm the rhamnosyl substitution of the glucitol residue (Lindberg *et al.*, 1972). Among the ions unaffected by ^2H labelling, those at *m/e* 219 and 187 are diagnostic for the terminal glucose residue, and those at *m/e* 393 and 361 for the glucosyl-rhamnose residue. The prominence of the ion at *m/e* 361 (intensity 16% of the base peak at *m/e* 101 for the labelled compound) is further evidence for substitution of the rhamnose in the 2-position (Kärkkäinen, 1971).

The evidence that oligosaccharide 4 was the precursor of oligosaccharides 1 and 2 indicated that the non-reducing glucose residue was in the β-form, despite the resistance of the linkage to hydrolysis by β-glucosidase. Attempts were made to confirm this and to establish the configuration of the rhamnosidic linkage by oxidation of the peracetylated trisaccharide alditol with CrO_3. The selective oxidation of β-pyranosidic residues was checked by g.l.c. of the alditol acetates derived from the reaction product. When oxidation was carried out with CrO_3 in acetic anhydride at room temperature (Lindberg *et al.*, 1972), no significant change in the amounts of glucose and rhamnose (relative to each other or to xylitol

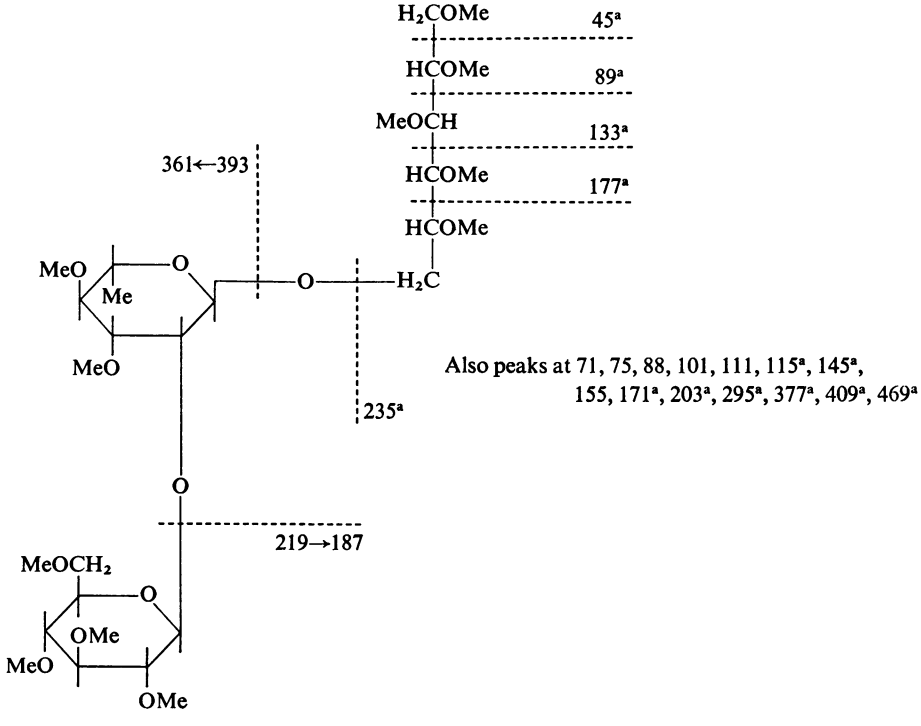

Fig. 3. *Mass-spectrometric fragmentation of the per-O-methyl derivative of the alditol from oligosaccharide* 4

[a] The *m/e* value for the ion was increased by 1 unit when the oligosaccharide was reduced with NaB^2H_4.

penta-acetate used as an internal standard) was detected. However, after more drastic oxidation by sonication with CrO_3 in acetic acid at 50°C for 1h (Hoffman *et al.*, 1972), the glucose/rhamnose ratio changed from 2.71:1 (theoretical value 2:1) to 1.73:1. As the ratios may be inflated by experimental losses of the more volatile rhamnitol penta-acetate, this result is indicative of β-glucosidic and α-rhamnosidic linkages in the trisaccharide. This compound is therefore assigned the structure Glcpβ1–2Rhapα1–6Glc, and oligosaccharide 2 the structure Rhapα1–6Glc. The latter is generally accepted as the structure of rutinose (e.g. Birkofer & Hammes, 1973), although enzymic evidence for a β-linkage has been presented (e.g. Wang & Alaupovic, 1973). The identification of a disaccharide from *Serratia marcescens* Bizio as Rhapβ1–6Glc was based on such evidence (hydrolysis by hesperidinase) and an n.m.r. study, but the compound appeared to be significantly less mobile than rutinose and oligosaccharide 2 on paper chromatography (R_{Glc} value 0.42, solvent system C).

Studies of phosphate components

Whereas all of the glucose and rhamnose of the phosphorylated polysaccharide was apparently released by acid hydrolysis (2M-HCl at 105°C for 2h), little free heptose was formed, indicating that most or all of the heptose residues were phosphorylated, as in other lipopolysaccharides. Evidence to support this view came from the analysis of polysaccharide oxidized with $NaIO_4$ for 5 days and subsequently treated with $NaBH_4$. Analysis of the residual sugars, by g.l.c. of their alditol acetates, gave a glucose/mannose ratio of 1.0:0.2. When the reaction products were first dephosphorylated by treatment with 40% (w/v) HF at 4°C for 1½ days, the glucose/mannose ratio was 1.0:1.2. As Chester *et al.* (1972) showed, the mannose is derived from residues of L-*glycero*-D-*manno*-heptose in which periodate resistance is conferred either by phosphorylation or by glycosidic substitution in the 3-position. Attempts were therefore made to demonstrate the presence of heptose phosphates in acid hydrolysates from both lipopolysaccharide and phosphorylated polysaccharide.

A sample of polysaccharide P2 was hydrolysed (2M-HCl at 105°C for 2h) and the hydrolysate neutralized (dilute NaOH) and fractionated by chromatography (Sephadex G-15). A small peak (V_e/V_0 1.25) for products containing carbohydrate and P preceded the main peak corresponding to P_i, monosaccharides and NaCl. Paper electrophoresis at pH5.3 showed that the former products included an

anionic component almost as mobile as P_i, which reacted with alkaline $AgNO_3$ and the Hanes–Isherwood (1949) reagent for P, but not with ninhydrin. Analysis of this component gave the molar ratio of heptose/P of about 1:3. Only about one-third of the P was released by treatment with alkaline phosphatase, and about half of the heptose was destroyed by treatment with $NaBH_4$. Although the inference of a phosphorylated disaccharide is only tentative, these results confirm the presence of phosphorylated heptose residues.

In a further study, similar hydrolysates from whole lipopolysaccharide were fractionated either by Sephadex chromatography or by chromatography on DEAE-cellulose. By the former technique the result described above for fraction P2 was confirmed, and the use of a longer column permitted the isolation of a trace product that contained material migrating as an anionic streak (m_{P_i} about 0.5) at pH5.3. This component contained heptose (reducible by $NaBH_4$), P, galactosamine and alanine, but the amount available was too small for further characterization. The result of fractionating an acid hydrolysate from lipopolysaccharide on DEAE-cellulose is shown in Fig. 4. The major P-containing fraction (H1) consisted mainly of P_i, but *O*-phosphoethanolamine was also present, and unidentified material was detected. The latter had a mobility similar to that of glucose 6-phosphate on electrophoresis at pH5.3. After treatment of fraction H1 with alkaline phosphatase and deionization of the hydrolysate, two reducing compounds (R_{Glc} values 1.17 and 0.97, solvent system A) were detected. The more mobile product was not identified, but was neither mannose nor arabinose. The second product corresponded in mobility and colour reactions to free heptose.

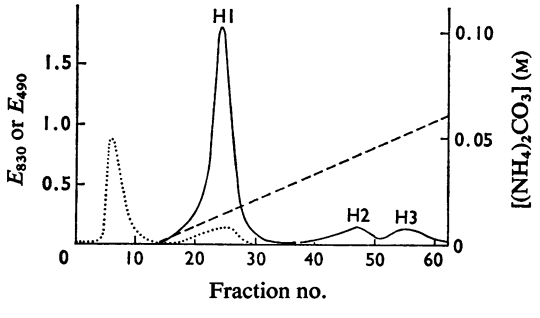

Fig. 4. *DEAE-cellulose chromatography of an acid hydrolysate of lipopolysaccharide*

Lipopolysaccharide (100mg) was hydrolysed with 2M-HCl at 105°C for 2h. After neutralization, water-soluble products were applied to a column (1.8cm×17cm) of DEAE-cellulose (CO_3^{2-} form). Elution was carried out with water (50ml), followed by a linear gradient of $(NH_4)_2CO_3$ (0–0.15M) at a flow rate of 50ml/h (----). Fractions (5ml) were screened for phosphorus content (——, E_{830}) and carbohydrate content (····, E_{490}). For details of fractions H1, H2 and H3 see the text.

In their heptose/P ratios, electrophoretic mobilities and reactions with both $NaBH_4$ and alkaline phosphatase, the components of fractions H2 and H3 (Fig. 4) resembled the more highly phosphorylated heptose derivative already described. Although the basis for their chromatographic resolution is not clear, a difference in the position(s) of phosphorylation is one possibility.

Preparation and study of dephosphorylated polysaccharide

Partial acid hydrolysis of fraction P2 had yielded structural information about the glucose–rhamnose region only. Further attempts to improve the yield and range of oligosaccharides by the methods of Painter (1965) and Galanos *et al.* (1969) were unsuccessful: no significant amounts of fragments containing either heptose or galactosamine were detected. Dephosphorylation of the polysaccharide therefore appeared to be necessary for further progress.

Fraction P2 was resistant to enzymic dephosphorylation: only about 30% of the P was released as P_i by treatment with alkaline phosphatase, and the addition of phosphodiesterase did not lead to further hydrolysis. Dephosphorylation was therefore carried out by chemical methods, treatment with cold aqueous HF (Hämmerling *et al.*, 1971), or prolonged heating at pH4 (Baddiley *et al.*, 1957). Similar products were obtained by either method (Table 4), with little evidence for further degradation of the polysaccharide. In general, dephosphorylation raised the analytical data by about 50%; the rather greater rise for heptose could reflect a greater reactivity of the dephosphorylated residues in the colorimetric analysis.

Methylation analysis of the dephosphorylated polysaccharide gave results generally similar to those for the parent material. The major difference was the appearance of a new peak in the chromatogram of methylated alditol acetates with a retention time (column I) relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol of 4.40. This peak should correspond to a heptose derivative, and the relative retention time matches values reported for the product from a 2,4,6,7-tetra-*O*-methylheptose from other lipopolysaccharides that contain L-*glycero*-D-*manno*-heptose (Hämmerling *et al.*, 1971, 1973; Lehmann *et al.*, 1973). The diagnosis of 3-substituted heptopyranose residues was supported by the results of periodate oxidation. After oxidation for 2 days, the glucose/galactosamine ratio had increased to 1.4:1.0 from the initial 4.2:1.0, and the glucose/mannose

Table 4. *Composition of dephosphorylated polysaccharide*

Data are given for products obtained from phosphorylated polysaccharide (fraction P2) by different methods: A, by treatment with 40% (w/v) HF at 4°C for 3 days; B, by treatment with 0.2M-ammonium acetate (pH4) at 100°C for 42h. Results for amino compounds were obtained by autoanalysis after hydrolysis (6.1M-HCl at 105°C for 4h) of samples, and are expressed as residues of amino compounds, without correction for slow release or destruction during hydrolysis.

	Content (%, w/w)	
	A	B
P	0.2	0
Total carbohydrate	48.0	49.5
Glucose	30.0	28.6
Rhamnose	9.1	9.0
Heptose	21.3	22.4
3-Deoxy-2-octulonic acid	1.7	1.5
Galactosamine	6.4	6.9
Alanine	3.6	3.7

ratio was 1.4:2.4, indicating the presence in the polysaccharide of two 3-substituted heptose residues.

A further sample (20mg) of dephosphorylated polysaccharide was subjected to partial acid hydrolysis (0.5M-HCl at 100°C for 30min). The neutralized hydrolysate was desalted by brief paper electrophoresis, and the neutral mono- and oligosaccharides were separated from products with low mobility by preparative paper chromatography (solvent system A). The latter products were then fractionated by paper electrophoresis, whereby four ninhydrin-positive components were obtained. The most interesting component had m_{GalN} 0.39 at pH5.3 and contained only glucose (3.9), galactosamine (1.0) and alanine (0.5). Almost 70% of the galactosamine (determined by the Morgan–Elson reaction) was reduced by treatment with $NaBH_4$, and all was destroyed by oxidation by $NaIO_4$. Although too little material was available for characterization, this component provides evidence for direct linkage(s) between glucose and galactosamine. Of the remaining components, one was slightly cationic (m_{GalN} 0.26), one neutral and one slightly anionic (m_{GalN} −0.15) at pH5.3, and all contained heptose and rhamnose in addition to glucose, galactosamine and alanine. In all three cases the galactosamine resisted oxidation with $NaIO_4$.

A single ninhydrin-positive, slightly cationic component was obtained by hydrolysis of dephosphorylated polysaccharide with 0.5M-HCl at 80°C for 5h. Analysis indicated the molar composition: glucose (2.0), rhamnose (trace), heptose (2.0), galactosamine (0.7), alanine (0.8); and again the galactosamine was stable to oxidation with $NaIO_4$. However, after treatment of this component with α-glucosidase (but not β-glucosidase), all the galactosamine was oxidizable, indicating that at least one α-glucopyranose residue was originally attached via the 3- or 4-position.

Studies of the region of lipopolysaccharide containing 3-deoxy-2-octulonic acid residues

On the assumption that the lipopolysaccharide molecule contains a single galactosamine residue, the analytical data (Table 1) also point to a single residue of 3-deoxy-2-octulonic acid. However, the release of free 3-deoxy-2-octulonic acid (Drewry *et al.*, 1971) during the treatment of lipopolysaccharide with acetic acid indicates the presence of at least two consecutive residues of the sugar acid. In fact, the occurrence of a second residue of 3-deoxy-2-octulonic acid, not released by mild hydrolysis and with low reactivity in the thiobarbituric acid test, was diagnosed as the terminal residue of polysaccharide fraction P2. The low reactivity of this residue is indicative of substitution at C-4 or C-5 (Charon & Szabó, 1972). Evidence for similar substitution of the second (releasable) residue was also obtained. Thus no loss of reactivity in the thiobarbituric acid test occurred when either lipopolysaccharide or its water-soluble deacylation product obtained by hydrazinolysis (Drewry *et al.*, 1973) was oxidized with periodate. When oxidized lipopolysaccharide was treated with $NaBH_4$, then hydrolysed with 1% (v/v) acetic acid at 100°C, 3-deoxy-2-octulonic acid was absent from the low-molecular-weight products. However, it was replaced by a more mobile component (*m* 1.18 at pH5.3), presumed to be a 3-deoxy-2-heptulonic acid produced by oxidation between C-7 and C-8.

About 48% of the glucosamine was recovered after oxidation of the deacylated lipopolysaccharide with periodate for 4 days, but no periodate-stable glucosamine derivatives were produced when the deacylation product was hydrolysed with acetic acid under the conditions used to liberate lipid A (Drewry *et al.*, 1973). Thus the periodate resistance of one of the two glucosamine residues of the disaccharide (GlcNβ1–6GlcN) backbone of lipid A in the deacylation product can probably be attributed to ketosidic substitution at C-3.

Studies of fragments containing galactosamine and alanine

Little is yet known about the location and linkage of these amino compounds. As noted above, there are indications that galactosamine may link the 'outer core' region of the polysaccharide (containing glucose and rhamnose) with the 'inner core' region (containing phosphorylated heptose and 3-deoxy-2-octulonic acid). The periodate resistance of galactosamine in

lipopolysaccharide, its deacylation product, and the polysaccharide fraction P2 is indicative of substitution at C-3 or C-4. When oxidized fraction P2 was hydrolysed (0.5M-HCl at 100°C for 30min), two cationic (pH5.3) ninhydrin-positive compounds containing galactosamine were detected by electrophoresis. The more mobile component (m_{GalN} 0.76) had a galactosamine/alanine ratio of 1:1 and the less mobile component (m_{GalN} 0.53) a ratio of 1:2. Several similar unidentified compounds were also detected after hydrolysis (2M-HCl at 105°C for 2h) of unoxidized polysaccharide (Lomax *et al.*, 1974).

Discussion

Sephadex chromatography of the partly degraded polysaccharide from *P. aeruginosa* N.C.T.C. 1999 separated three fractions differing in molecular weight and composition (Drewry *et al.*, 1971, 1972*a*,*b*). The fraction of intermediate molecular weight (P2) was a phosphorylated polysaccharide, apparently corresponding to the 'core polysaccharides' from other lipopolysaccharides. Although the description of this fraction as the 'core polysaccharide' cannot yet be justified in biosynthetic and functional terms, the presence of 3-deoxy-2-octulonic acid and phosphorylated heptose residues, and the isolation of similar fractions from other strains of *P. aeruginosa*, support this description. Thus glucose, rhamnose, galactosamine and alanine are apparently the characteristic components of the outer region of the 'core polysaccharide' in *P. aeruginosa*. Considerable progress has been made towards establishing the structure of this region. In particular, oligosaccharides 3 and 4 could account for all the glucose and rhamnose, and the approximate equivalence of unsubstituted and monosubstituted glucose residues demonstrated by methylation analysis is indicative of microheterogeneity or a branched structure. A partial structure consistent with most of the analytical data is given in Fig. 5. An unsolved problem is the location in the polysaccharide of any fucosamine residues. The corresponding polysaccharides from other strains of *P. aeruginosa* (e.g. Chester *et al.*, 1973; Wilkinson & Galbraith, 1975) and of other *Pseudomonas* species (e.g. Wilkinson *et al.*, 1973; Lomax *et al.*, 1974) may be similar in composition but not necessarily identical in structure. The attachment of additional amino sugars to 'core polysaccharides' from other strains of *P. aeruginosa* has also been reported (Wilkinson & Galbraith, 1975).

In addition to 3-deoxy-2-octulonic acid, the low-molecular-weight fraction from the partly degraded polysaccharide contained much P_i, some PP_i and ethanolamine phosphates (Drewry *et al.*, 1971, 1972*a*). These phosphates were probably derived mainly from the heptose region of the 'core polysaccharide' by the hydrolysis of labile pyrophosphate bonds. This unusually extensive phosphorylation of the lipopolysaccharide seems to be characteristic of *P. aeruginosa* and other EDTA-sensitive pseudomonads (Wilkinson *et al.*, 1973; Wilkinson & Galbraith, 1975). The presence in the 'core polysaccharide' of polyphosphate residues with metal-binding ability could make the outer membrane of the cell envelope exceptionally dependent on stabilization by cations and therefore vulnerable to dissociation by EDTA.

Although there is no direct evidence that the high-molecular-weight fraction (P1) was part of the lipopolysaccharide molecule, there is circumstantial evidence to support this view. Thus the presence of small amounts of the components characteristic of 'core polysaccharide' (Table 3) would be expected for the O-antigenic 'side-chain' polysaccharide with attached 'core'. Also fraction P1 reacted with the homologous O-antiserum (Habs serogroup 2B) (T. L. Pitt, personal communication). Further, similar fractions have been isolated from other strains of *P. aeruginosa* belonging to serogroup 2B (Wilkinson & Galbraith, 1975).

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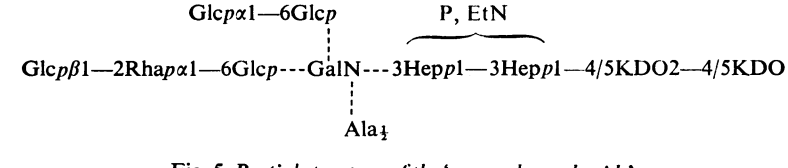

Fig. 5. *Partial structure of the 'core polysaccharide'*

Abbreviations: Hep, L-*glycero*-D-*manno*-heptose; KDO, 3-deoxy-2-octulonic acid; EtN, ethanolamine; P, phosphate.

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