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Studies of the Polysaccharide Fraction from the Lipopolysaccharide of *Pseudomonas alcaligenes*

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Studies of the lipopolysaccharide of *Pseudomonas alcaligenes* strain BR 1/2 were extended to the polysaccharide moiety. The crude polysaccharide, obtained by mild acid hydrolysis of the lipopolysaccharide, was fractionated by gel filtration. The major fraction was the phosphorylated polysaccharide, for which the approximate proportions of residues were; glucose (2), rhamnose (0.7), heptose (2–3), galactosamine (1), alanine (1), 3-deoxy-2-octulonic acid (1), phosphorus (5–6). The heptose was L-*glycero*-D-*manno*-heptose. The minor fractions from gel filtration contained free 3-deoxy-2-octulonic acid, P_i and PP_i. The purified polysaccharide was studied by periodate oxidation, methylation analysis, partial hydrolysis, and dephosphorylation. All the rhamnose and part of the glucose and heptose occur as non-reducing terminal residues. Other glucose residues are 3-substituted, and most heptose residues are esterified with condensed phosphate residues, possibly in the C-4 position. Free heptose and a heptosylglucose were isolated from a partial hydrolysate of the polysaccharide. The location of galactosamine in the polysaccharide was not established, but either the C-3 or C-4 position appears to be substituted and a linkage to alanine was indicated. In its composition, the polysaccharide from *Ps. alcaligenes* resembles core polysaccharides from other pseudomonads: no possible side-chain polysaccharide was detected.

In recent years the structures of polysaccharides derived from the lipopolysaccharides of many Gram-negative bacteria have been determined (Lüderitz *et al.*, 1971). Most studies have dealt with organisms from the family Enterobacteriaceae, and relatively little work has been done with the *Pseudomonas* species.

Pseudomonads are of special interest because of their exceptional sensitivity to EDTA, and *Pseudomonas alcaligenes* is outstanding in this respect (Wilkinson, 1967; Key *et al.*, 1970*a*). The bactericidal action of EDTA involves the extraction of lipopolysaccharide from the cell wall, and comparative studies of other pseudomonads have indicated that sensitivity to EDTA may be related to the composition or structure of the lipopolysaccharide (Gray & Wilkinson, 1965; Wilkinson, 1968, 1970; Wilkinson *et al.*, 1973). The lipopolysaccharides from both *Ps. alcaligenes* and *Pseudomonas aeruginosa* are rich in phosphorus (Fensom & Gray, 1969; Chester *et al.*, 1972; Key *et al.*, 1970*b*), and in the case of *Ps. aeruginosa* this is apparently explained by the presence of condensed phosphates (Drewry *et al.*, 1971, 1972*a*).

The initial studies of the lipopolysaccharide from *Ps. alcaligenes* (Key *et al.*, 1970*b*) were restricted by the extensive degradation that occurred during the hydrolytic separation of lipid A and polysaccharide moieties. In subsequent studies (Drewry *et al.*, 1973) degradation was minimized by hydrolysis of the lipopolysaccharide with 1% acetic acid, and the lipid A fraction was characterized. Studies of the water-soluble hydrolysis products are described in the present paper.

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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.). Inorganic pyrophosphatase (EC 3.6.1.1.), α-glucosidase (EC 3.2.1.20), β-glucosidase (EC 3.2.1.21), hexokinase (EC 2.7.1.1), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), mannose-phosphate isomerase (EC 5.3.1.8) and glucose phosphate isomerase (EC 5.3.1.9) were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, West Germany. Reagents for the determination of D-glucose with glucose oxidase (EC 1.1.3.4) were obtained from C.F. Boehringer und Soehne G.m.b.H. and from Worthington Biochemical Corp., Freehold, N.J., U.S.A., and D-galactose oxidase (EC 1.1.3.9) was obtained from the latter supplier. Laminarin and nigeran, used for the preparation of laminaribiose (Peat *et al.*, 1958) and nigerose (Barker *et al.*, 1957) respectively, and various disaccharides of glucose,

were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Laminarin was also used for the preparation of 2,4,6-tri-*O*-methyl-D-glucose.

Methods

Growth of bacteria, preparation of cell walls, and isolation of lipopolysaccharide. The strain (BR 1/2) of *Ps. alcaligenes* and the methods used were as described by Key *et al.* (1970*a*,*b*) and Drewry *et al.* (1973).

Preparation and fractionation of crude polysaccharide. Lipopolysaccharide (10mg/ml) was hydrolysed with 1% acetic acid at 100°C for 1–1.5h, and the precipitated lipid A was removed as described by Drewry *et al.* (1973). The aqueous solution of crude polysaccharide was filtered (glass sinter no. 4 porosity) and freeze-dried. The product was fractionated by chromatography on Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) with pyridine–acetic acid buffer, pH5.4 (Schmidt *et al.*, 1969), as eluent. The course of elution was monitored with a conductivity analyser (LKB-Produkter AB, Stockholm, Sweden) and by analysis of fractions for total carbohydrate and phosphorus.

Analytical methods. Unless stated otherwise, total carbohydrate was determined by the phenol–H_2SO_4 method (Dubois *et al.*, 1956); the anthrone method (Trevelyan & Harrison, 1952) was used occasionally. Methods for the determination of D-glucose, D-mannose, rhamnose, heptose, 3-deoxy-2-octulonic acid, fatty acids, nitrogen, phosphorus and P_i were those used by Wilkinson *et al.* (1973). Heptose was also determined by the method of Wright & Rebers (1972) in some cases, and both glucose and rhamnose were also determined by autoanalysis (Hough *et al.*, 1972). Amino compounds were determined by using an automatic analyser (Technicon Instruments Co. Ltd., Basingstoke, Hants., U.K. or The Locarte Co., London E.C.3, U.K.) after hydrolysis of samples with 6.1M-HCl at 105°C for 4h: a single borate-containing buffer (slightly modified from that described by Bella & Kim, 1970) was sometimes used for accelerated separations of alanine and galactosamine.

Chromatography and electrophoresis. Paper chromatography and high-voltage paper electrophoresis were carried out with Whatman papers (no. 1 for analytical separations, and water-washed no. 1 or no. 3MM for preparative separations). T.l.c. was carried out with layers of MN-300 cellulose (Macherey, Nagel and Co., Düren, West Germany). Solvent systems used were: 1, ethyl acetate–pyridine–water (5:2:5, by vol.; upper phase); 2, acetone–water (19:1, v/v); 3, butan-1-ol–acetic acid–water (4:1:5, by vol.; upper phase); 4, butan-1-ol–acetic acid–water (3:1:1, by vol.); 5, butan-1-ol–pyridine–water (6:4:3, by vol.); 6, butan-1-ol–ethanol–water–aq. NH_3 (sp.gr. 0.88) (40:10:49:1, by vol.; upper phase); 7, dioxan–water–trichloroacetic acid–aq. NH_3 (sp. gr. 0.88) (260:110:20:1, v/v/w/v).

Paper electrophoresis was carried out by using pyridine–acetic acid–water (5:2:43, by vol.; pH5.3) as buffer (12mM-EDTA was included for improved separation of phosphates). Analytical separations (no. 1 paper) were done at about 37V/cm for 1h; preparative separations (no. 3MM paper) required 2–3h at about 18V/cm.

Detection reagents used were; ninhydrin, alkaline $AgNO_3$, periodate–Schiff's reagents, periodate–alkaline $AgNO_3$ (Mühlradt, 1969), aniline phosphate (for reducing sugars), and the Hanes & Isherwood (1949) reagent (for phosphates).

Chromatography of purified polysaccharide on DEAE-cellulose (Whatman DE32, carbonate form) was carried out with a linear gradient of aq. $(NH_4)_2CO_3$ (0–1M), generated by using a Dialagrad (Instrumentation Specialities Co. Inc., Lincoln, Neb., U.S.A.).

Gas–liquid chromatography. Monosaccharides were reduced to alditols with $NaBH_4$ and converted into alditol acetates (Sawardeker *et al.*, 1965) or *O*-trimethylsilyl derivatives (Sweeley *et al.*, 1963). The former derivatives were characterized by g.l.c. on columns containing as stationary phase 3% (w/w) ECNSS-M and the latter derivatives on columns containing 3% (w/w) OV-225 or 3% (w/w) SE-30. Partially methylated monosaccharides were also identified and determined as their alditol acetates by using either ECNSS-M (Björndal *et al.*, 1967*a*) or OV-225 (Lönngren & Pilotti, 1971) as stationary phase. Supporting evidence was obtained by g.l.c. of the methyl glycosides, prepared by the method of Cadotte *et al.* (1952), by using columns containing either 15% (w/w) poly(butane-1,4-diol succinate) or 10% (w/w) polyphenyl ether (OS-138). A Pye 104 chromatograph fitted with a flame-ionization detector was used for all g.l.c. separations.

Mass spectrometry. The mass spectrum of the per-*O*-trimethylsilyl derivative of a heptitol was obtained by using an AEI MS902 spectrometer. Spectra of partially methylated alditol acetates were obtained by combined g.l.c.–mass spectrometry with a Micromass 12B instrument (VG-Micromass Ltd., Winsford, Cheshire, U.K.).

Identification of the heptose component. The methods used were essentially those described by Bagdian *et al.* (1966) and Chester *et al.* (1972). These involve (*a*) conversion of heptose into hexose by cleavage of the exocyclic C-6–C-7 bond (controlled periodate oxidation of lipopolysaccharide, followed by reduction with $NaBH_4$), (*b*) conversion of heptose into hexose by removal of C-1 (Ruff degradation, which also gives partial over-oxidation to pentose). Products were identified by paper chromatography (solvent systems 1 and 2) and optical isomers were

differentiated by the use of enzymes and [in the case of the hexose from (*a*)] by the microchemical method of Clarke & Humphreys (1966).

Dephosphorylation of polysaccharide. Polysaccharide (15mg) was dissolved in 0.2M-ammonium acetate buffer (pH4, 1ml) and heated at 105°C for 42h (Baddiley *et al.*, 1957). The dephosphorylated polysaccharide was separated from buffer and low-molecular-weight products (mainly P_i) by chromatography on Sephadex G-15, with pyridine–acetic acid buffer (pH5.4) as eluent.

Partial hydrolysis of polysaccharide. Preliminary experiments indicated that the maximum yield of oligosaccharides was obtained by hydrolysis with 0.5M-HCl at 100°C for 30min. First acid was removed from such hydrolysates by freeze-drying over KOH and then the products were carefully neutralized with dilute NaOH and fractionated by consecutive paper electrophoresis and chromatography. In later experiments neutral products were more simply isolated by preparative paper chromatography (solvent system 1) after deionization of the hydrolysates.

Periodate oxidation. Samples were oxidized with 50mM-$NaIO_4$ (at least 100% excess) in the dark at 4°C, and the consumption of $NaIO_4$ was determined by the method of Avigad (1969). When oxidation was apparently complete, residual $NaIO_4$ was destroyed with ethylene glycol. The oxidation products were treated overnight with 2% (w/w) $NaBH_4$, the excess of $NaBH_4$ was destroyed with dilute acetic acid and, after drying, boric acid was removed by repeated distillation with methanol.

Methylation analysis. The methylation of lipopolysaccharide and polysaccharides was done by a modification of the method of Hellerqvist *et al.* (1968). Samples (about 5mg, dried *in vacuo* over P_2O_5) were dispersed or dissolved in dry dimethyl sulphoxide (1ml) by sonication for 1h and kept overnight. A solution (1.5ml) containing the dimsyl ion in dimethyl sulphoxide (Conrad, 1972) was added drop-by-drop under N_2, and the mixture was sonicated for 1h and then kept at room temperature for at least 8h. Methyl iodide (0.25ml) was added with cooling, and the mixture was sonicated for 30min. When a single methylation was inadequate, further dimsyl reagent (1.5ml) and methyl iodide (1ml) were used in a second treatment. The final solution was poured into deionized water (40ml) and the salts and reagents were removed by dialysis. The non-diffusible residue was dried by rotary evaporation and then subjected to formolysis with 90% (w/w) formic acid at 105°C for 6h (Percival, 1968, 1971). After fivefold dilution with water, heating was continued for 2h, and the hydrolysate was dried by rotary evaporation. The methylated sugars were converted into alditol acetates or methyl glycosides for further study.

β-Elimination of phosphates. Lipopolysaccharide (4mg) was incubated at 46°C for 2h with 1M-lysine containing 25mM-$NaIO_4$ (0.6ml) (Khym & Uziel, 1968). After the addition of 1M-ethylene glycol (60μl), low-molecular-weight products were isolated by ultrafiltration in a stirred cell (Amicon Corp., High Wycombe, Bucks., U.K.) fitted with a UM-05 membrane (cut-off at mol.wt. approx. 500).

Physical methods. I.r. spectra were recorded with samples dispersed in KCl discs, by using a Unicam SP.200 spectrophotometer, and u.v. spectra were obtained by using a Unicam SP.700 instrument.

Results

Composition of cell walls

Cell walls were isolated from five batches of *Ps. alcaligenes*. Analytical checks on the authenticity and consistency of the walls gave the following results: phosphorus, 2.1–2.5%; nitrogen, 6.8–8.5%; total carbohydrate, 5.3–7.3%; total amino compounds (by autoanalysis), 41–49%; loosely-bound lipids, 20–22%. Glucose and rhamnose were the only neutral sugars detected by paper chromatography, and all batches contained galactosamine (2.3–2.7%) in addition to glucosamine and muramic acid. The i.r. spectra of all batches were indistinguishable and contained the characteristic band at 928cm^{-1} (Gray & Wilkinson, 1965). Thus, although some variations in the relative proportions of wall components were apparent, the data for all batches were acceptably close to those for walls analysed previously (Gray & Wilkinson, 1965; Key *et al.*, 1970*a*).

Composition of lipopolysaccharide

Lipopolysaccharide was extracted from defatted walls by a single treatment with hot aqueous phenol. The yields [about 20% (w/w) of whole walls] and compositions of the products from different wall batches were almost identical. Solutions of the individual products were therefore combined and freeze-dried; analytical data for total lipopolysaccharide are given in Table 1. No contamination by nucleic acid or glycosaminopeptide was detected at this stage, and the amount of protein present was negligible. Qualitatively, the lipopolysaccharide was almost identical with that studied by Key *et al.* (1970*b*), and studies of fatty acid composition (Drewry *et al.*, 1973) confirmed this similarity. However, mannose was detected as a trace component in the earlier preparations of lipopolysaccharide only (Key *et al.*, 1970*b*). In general quantitative agreement between analytical data for the two preparations is satisfactory. The greatest discrepancies were in the values for heptose and 3-deoxy-2-octulonic acid. Literature calibrations were used in both determinations, and the limitations of both

Table 1. *Composition of lipopolysaccharide*

Results for amino compounds were obtained by autoanalysis after hydrolysis (6.1 M-HCl at 105°C for 4h) of lipopolysaccharide, and are expressed as residues of amino compounds, without correction for slow release or destruction during hydrolysis. Values in parentheses are those obtained previously (Key *et al.*, 1970*b*). –, Not determined.

	Content	(%, w/w)
Phosphorus	6.0	(6.7)
Nitrogen	2.0	(2.2)
Total carbohydrate*	21.3	(–)
Glucose	5.3	(–)
Rhamnose	1.9	(–)
Heptose	9.0	(6.8)
3-Deoxy-2-octulonic acid	2.6	(5.7)
Glucosamine	4.2	(4.8)
Glucosamine phosphate(s)	2.1	(1.4)
Galactosamine	2.9	(3.3)
Alanine	1.7	(1.8)
Fatty acids	22.2	(19.8)

* Determined by the phenol–H_2SO_4 method; values obtained by the anthrone method were 14.4 (14.5).

analytical procedures are well known (Wright & Rebers, 1972; Charon & Szabó, 1972, 1973). Corresponding data (S. G. Wilkinson, I. M. Downes & C. L. Kokoszka, unpublished work) for other batches of lipopolysaccharide from *Ps. alcaligenes* are: heptose, 7.6–10.5%; 3-deoxy-2-octulonic acid, 4.0–5.1%.

Although glucose and rhamnose were not determined by Key *et al.* (1970*b*), subsequent analyses of their lipopolysaccharide gave the results: glucose, 6.0%; rhamnose, 1.9%. Closely similar results have been obtained for other batches of lipopolysaccharide both by autoanalysis and by individual determinations on deionized hydrolysates. The average molar ratio of glucose/rhamnose for five batches was 3.1:1.

In the earlier studies, heptose was detected and determined, but not identified. Characterization was therefore attempted by chemical degradation. In a control experiment the total complement of neutral sugars was obtained by hydrolysis (1 M-HCl at 105°C for 16h) of the lipopolysaccharide and deionization of the hydrolysate. A similar hydrolysate was prepared from lipopolysaccharide which had been treated briefly with $NaIO_4$ and then with $NaBH_4$. A comparison of the two hydrolysates by paper chromatography (solvent systems 1 and 2) showed that the test mixture contained mannose as an additional component. The sugar was identified as D-mannose by the combined use of hexokinase, mannose phosphate isomerase, glucose phosphate isomerase and glucose 6-phosphate dehydrogenase. This result was confirmed by the formation of a racemate on the addition of L-mannose, diagnosed by the characteristic crystalline form of the osazone (Clarke & Humphreys, 1966). Thus the configurations at C-2 to C-5 in the heptose are the same as in D-mannose. The configuration at C-6 was determined by Ruff degradation of the heptose (about 0.5mg) isolated by hydrolysis (1 M-HCl at 105°C for 16h) of the lipopolysaccharide (50mg), destruction of glucose with glucose oxidase, and preparative paper chromatography (solvent system 2). The products were identified by paper chromatography as galactose and lyxose. The galactose was unaffected by treatment with D-galactose oxidase with conditions under which D-galactose was completely destroyed. This result places the heptose in the L-series and completes its identification as L-glycero-D-mannoheptose. The identity was confirmed by g.l.c. of the heptitol acetate (see below).

Preparation and fractionation of the crude polysaccharide

After treatment of the lipopolysaccharide with 1% acetic acid, about 64% (w/w) was recovered as water-soluble products. These were separated into four fractions (Q1–Q4) by chromatography on Sephadex G-25 (Fig. 1). Fractions Q2 and Q4 were rechromatographed on Sephadex G-50 and G-15, respectively, and gave single, symmetrical peaks in each case. Analytical data for fractions Q1–Q4 are given in Table 2. The trace fraction Q1 [0.4% (w/w) of recovered material] was essentially the same as fraction Q2, but apparently contained a little

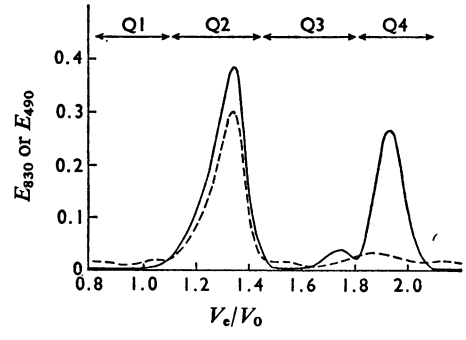

Fig. 1. *Fractionation on Sephadex G-25 of crude polysaccharide*

The material (362mg) was applied to a column (2.5cm × 52cm) of Sephadex G-25. Elution was carried out with pyridine–acetic acid buffer (10ml of pyridine plus 4ml of acetic acid/litre; pH5.4) at a flow rate of 30ml/h. Fractions (4ml) were screened for phosphorus content (——; E_{830}) and carbohydrate content (----; E_{490}). Yields of fractions: Q1, 1.4mg; Q2, 247mg; Q3, 27mg; Q4, 84mg.

Table 2. *Compositions of fractions Q1–Q4 (Fig. 1) from crude polysaccharide*

Results for amino compounds were obtained by autoanalysis after hydrolysis (6.1 M-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. +, Present but not determined.

Fraction ...	Content (%, w/w) Q1	Q2	Q3	Q4
Phosphorus	6.3	6.6	4.4	12.1
P_i	0	0	0.4	12.7
Glucose	12.4	12.3	1.1	0
Rhamnose	4.4	4.3	Trace	0
Heptose	17.7	18.1	2.2	0
3-Deoxy-2-octulonic acid	0.8	1.7	14.8	20.6
Galactosamine	6.1	6.4	Trace	0
Alanine	+	3.5	Trace	Trace
Muramic acid	2.7	0	0	0
Glucosamine	2.0	0	0	0
Glutamic acid	1.5	0	0	0
2,6-Diaminopimelic acid	1.6	0	0	0

glycosaminopeptide (not detected during analysis of the intact lipopolysaccharide). Fraction Q4 [23.4% (w/w) of recovered material] contained P_i and free 3-deoxy-2-octulonic acid: no other significant components were detected. Phosphates and 3-deoxy-2-octulonic acid were also present in fraction Q3 [7.5% (w/w) of recovered material], which was not completely water-soluble after freeze-drying. When freshly isolated, fraction Q3 contained very little P_i: the amount increased during storage, and subsequent examination by paper electrophoresis indicated that this arose through decomposition of PP_i.

The major fraction Q2 [68.7% (w/w) of recovered material] was the purified phosphorylated polysaccharide: it contained all the components of the lipopolysaccharide except glucosamine and fatty acids (both present in lipid A). During chromatography on DEAE-cellulose, the corresponding fraction from a separate hydrolysate was eluted as a single symmetrical peak at a concentration of $(NH_4)_2CO_3$ about 0.15 M. However, when this second hydrolysate was fractionated on Sephadex G-25 with water as eluent, in place of buffer, two polymeric fractions differing in rhamnose content were obtained. The major fraction [V_e (elution volume)/V_0 (void volume) 1.33, the same as for fraction Q2] had twice the rhamnose content of the minor fraction (V_e/V_0 1.65), but in other respects both fractions closely resembled fraction Q2. Although the significance of this finding is not clear, it does suggest heterogeneity in the rhamnose-containing region(s) of the polysaccharide. Autoanalysis of a later batch of polysaccharide (equivalent to fraction Q2) confirmed a glucose/rhamnose ratio of 3:1, as in the parent lipopolysaccharide.

Periodate oxidation of purified polysaccharide

Oxidation was apparently complete after treatment of the polysaccharide with $NaIO_4$ for 72h. Analysis showed that essentially all the rhamnose and heptose, but only about half of the glucose, had been destroyed. Galactosamine was virtually unaffected, but only about half of the alanine was recovered. Paper chromatography (solvent system 6) of a hydrolysate of oxidized polysaccharide showed the presence of mannose (it was not detected when oxidation was prolonged to 5 days). Attempts to detect possible products from the degradation of rhamnose residues (propane-1,2-diol from unsubstituted or 2-substituted residues; butane-1,2,3-triol from 4-substituted residues) by paper chromatography were unsuccessful. Glycerol (expected from the degradation of unsubstituted, 2-substituted, or 6-substituted glucose residues) was detected, but not erythritol (expected from 4-substituted glucose residues).

Methylation analysis of purified polysaccharide

Results obtained by methylation analysis of whole lipopolysaccharide and polysaccharide fraction Q2 could not readily be interpreted. Chromatograms of both the alditol acetate and methyl glycoside derivatives of methylated sugars were dominated by peaks for the derivatives of 2,4,6-tri-*O*-methylglucose whereas other peaks were much smaller and variable in size. Such results indicated that methylation was incomplete because of the low solubility of the materials in dimethyl sulphoxide (e.g. Hämmerling *et al.*, 1971). However, more recent batches of polysaccharide could be dissolved completely by storing the sonicated mixture overnight, and closely similar results (Fig. 2 and Table 3) were obtained by either one or two methylations. The retention times of the first three major components (peaks A, B and C, Fig. 2) were the same as those of the alditol acetates from reference 2,3,4-tri-*O*-methylrhamnose, 2,3,4,6-tetra-*O*-methylglucose and 2,4,6-tri-*O*-methylglucose respectively, and the identities of these components were confirmed by combined g.l.c.–mass spectrometry (Björndal *et al.*, 1967*b*, 1970). The diagnosis of unsubstituted residues of glucose and rhamnose and of 3-substituted glucose in the polysaccharide is consistent with the results of periodate oxidation. The relative retention time (2.17) for the component giving peak D (Fig. 2) on the column containing ECNSS-M resembled values (2.14–2.25) obtained for the derivative from a 2,3,4,6,7-penta-*O*-methylheptose from other bacterial lipopolysaccharides that contain L-*glycero*-D-*manno*-heptose (Hellerqvist & Lindberg, 1971; Hämmerling *et al.*, 1971). The derivative from L-*glycero*-D-*manno*-heptose is readily separated from that from D-*glycero*-

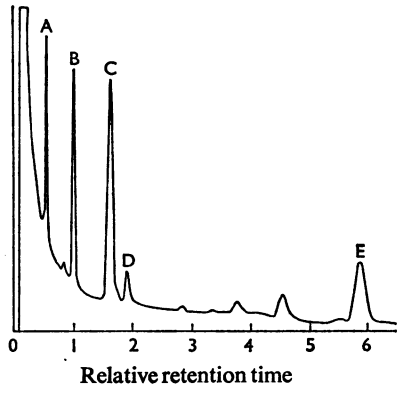

Fig. 2. *Methylation analysis of phosphorylated polysaccharide*

G.l.c. separation on OV-225 at 195°C of partially methylated alditol acetates obtained from methylated polysaccharide.

Table 3. *Methylation analysis of phosphorylated polysaccharide*

Polysaccharide was methylated once and the partially methylated sugars released by acid hydrolysis were analysed as alditol acetates (Fig. 2). Column stationary phases: I, ECNSS-M at 165°C; II, OV-225 at 195°C. –, Not determined.

Peak	Component	Column ...	Retention time* I	Retention time* II	Relative peak area
A.	2,3,4-Tri-*O*-methylrhamnose		0.50	0.53	0.6
B.	2,3,4,6-Tetra-*O*-methylglucose		1.00	1.00	1.0
C.	2,4,6-Tri-*O*-methylglucose		1.91	1.64	2.0
D.	2,3,4,6,7-Penta-*O*-methylheptose		2.17	1.94	0.3
E.	Unidentified		—	5.95	1.9

* Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.

D-*manno*-heptose (Lehmann *et al.*, 1973). The mass spectrum of the component giving peak D contained, among others, peaks at *m/e* 321, 249, 205, 161, and 117, as expected for a 1,5-di-*O*-acetyl-2,3,4,6,7-penta-*O*-methylheptitol (Hellerqvist & Lindberg, 1971). The minor components detected (Fig. 2) could not be identified and the late major component (giving peak E) may not be a carbohydrate (the area of peak E was much less when the polysaccharide was methylated twice). Although the relative size of the peak for the rhamnose derivative was less than expected from the molar ratio rhamnose/glucose of 1:3 for the polysaccharide, this could be explained by preferential loss of the relatively volatile 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol during the work-up.

Table 4. *Composition of dephosphorylated polysaccharide*

Results for amino compounds were obtained by autoanalysis after hydrolysis (6.1 M-HCl at 105°C for 4h) of a sample, and are expressed as residues of amino compounds, without correction for slow release or destruction during hydrolysis. The result in parentheses is dubiously high (see the text for discussion).

	Content (%, w/w)
Phosphorus	0.5
Glucose	18.8
Rhamnose	1.3
Heptose	(40.3)
3-Deoxy-2-octulonic acid	0.6
Galactosamine	9.5
Alanine	3.7

Preparation and study of dephosphorylated polysaccharide

To obtain more information about heptose residues, the polysaccharide was dephosphorylated by selective hydrolysis of phosphomonoester bonds at pH4. High-molecular-weight carbohydrate was separated from low-molecular-weight products (P_i and free rhamnose) by chromatography on Sephadex G-15. Analytical data (Table 4) for the polymeric material confirmed that almost all of the phosphorus had been removed and showed that much of the rhamnose had also been lost. Similar results (S. G. Wilkinson & C. L. Kokoszka, unpublished work) were obtained by dephosphorylation of the polysaccharide with HF (Hämmerling *et al.*, 1971). As the apparent recovery of heptose (138%) in the polysaccharide dephosphorylated at pH4 was about twice that of other components, the sugar composition of the product was redetermined by g.l.c. of the alditol acetates. By using an appropriate correction factor for heptose (Schmidt *et al.*, 1970), the values were: rhamnose, 3.0%; glucose, 16.4%; heptose, 18.8%. The retention time, relative to that for xylitol penta-acetate, of the heptose derivative was the same as that found for the derivative from L-*glycero*-D-*manno*-heptose (Adams *et al.*, 1967; Schmidt *et al.*, 1970). A minor peak on the chromatogram corresponded to a probable degradation product of the heptose also found by Schmidt *et al.* (1970).

Methylation analysis of the dephosphorylated polysaccharide gave results similar to those described for whole polysaccharide, with the addition of two more components (Table 5). Although facilities for confirmation by g.l.c.–mass spectrometry were not available, the size and relative retention time for peak E′ suggested that it was derived from a tetra-*O*-methylheptose (e.g. Hämmerling *et al.*, 1971). The significance of peak F′ is less clear: if it

Table 5. *Methylation analysis of dephosphorylated polysaccharide*

Polysaccharide was methylated twice and the partially methylated sugars released by acid hydrolysis were analysed as alditol acetates. Column stationary phase: ECNSS-M at 170°C.

Peak	Component	Retention time*	Relative peak area
A'.	2,3,4-Tri-*O*-methylrhamnose	0.49	0.6
B'.	2,3,4,6-Tetra-*O*-methylglucose	1.00	1.4
C'.	2,4,6-Tri-*O*-methylglucose	1.98	2.0
D'.	2,3,4,6,7-Penta-*O*-methylheptose	2.29	0.8
E'.	2,4,6,7-Tetra-*O*-methylheptose?	4.80	3.1
F'.	Unidentified	5.29	0.6

* Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol.

corresponds to the derivative of a di-*O*-methylglucose (Björndal *et al.*, 1967*a*), it could point to a branched polysaccharide or could be an artefact of undermethylation or partial demethylation.

Partial acid hydrolysis of polysaccharide

Phosphorylated polysaccharide was hydrolysed with 0.5 M-HCl at 100°C for 30 min, and neutral and acidic fractions were isolated from the hydrolysate by preparative paper electrophoresis. The fractions were in turn subjected to preparative paper chromatography (solvent systems 1 and 3) and the products were tested for purity by using solvent systems 4 and 5. The two major neutral products were free glucose and rhamnose, but tailing of the 'glucose' spot on paper indicated that an additional component was present. By rechromatography (solvent system 1) a fraction containing mainly the less mobile component was obtained. In the cysteine–H_2SO_4 test for heptose the reaction products from this fraction absorbed strongly with $\lambda_{max.}$ about 505 nm. After treatment of the fraction with $NaBH_4$ and conversion of the alditols into per-*O*-trimethylsilyl derivatives the major product had a retention time of 3.1 relative to that of hexa-*O*-trimethylsilylglucitol (the minor product) by g.l.c. on OV-225 at 150°C. The mass spectrum of the mixed products contained the expected fragment ions (Kärkkäinen & Vihko, 1969), including the high-mass ions at *m/e* 701, 626 and 523 diagnostic for the heptitol derivative. Thus the presence of free heptose in the partial hydrolysate was established.

Apart from monosaccharides, the neutral fraction of the hydrolysate had only one significant component (X) with reasonable chromatographic mobility. On paper chromatograms, component X had R_F values similar to those of the (1→2)-linked glucopyranosylglucoses (sophorose and kojibiose): it could be differentiated from all other glucopyranosylglucoses by using solvent systems 1, 3, and 5. However, component X was unaffected by both α- and β-glucosidase, and analysis showed the presence of heptose (but not rhamnose) in addition to glucose. When heptose was determined by using the method and calibration given by Wright & Rebers (1972) for D-*glycero*-L-*manno*-heptose, the molar ratio of heptose/glucose was 1:1. All of the glucose, but none of the heptose, was destroyed by treatment of component X with $NaBH_4$. Although insufficient material was available for complete characterization, the methylation data for the polysaccharide suggest that component X was a Hep1→3Glc disaccharide. The mass spectrum of the per-*O*-trimethylsilyl derivative of the heptosylglucitol (determined by Dr. J. Kärkkäinen) was consistent with the structure proposed.

Both the neutral and acidic fractions of the total hydrolysate contained products with very low paper chromatographic mobilities. These products were rich in glucose, heptose, galactosamine, and alanine, but contained no rhamnose. These products are likely to be fragments of relatively undegraded polysaccharide.

After hydrolysis of polysaccharide with 2 M-HCl at 105°C for 2 h (as used for the 'total' release of neutral sugars), several reducing, ninhydrin-positive, cationic components were detected by paper electrophoresis at pH 5.3. The major component was free galactosamine. The minor components had the following relative mobilities (m_{GalN} values): B1, 0.82; B2, 0.62; B3, 0.46. Component B1 contained alanine and galactosamine in similar amounts; like alanine, it gave a purple colour immediately in the reaction with ninhydrin. Component B1 is tentatively identified as *N*-alanylgalactosamine. Like galactosamine, components B2 and B3 initially gave a brown colour in the reaction with ninhydrin, and autoanalysis of partially purified component B2 confirmed the presence of galactosamine and alanine (molar ratio about 4:1).

Studies of phosphate components

Both the whole lipopolysaccharide and the polysaccharide fraction are notably rich in phosphorus. The release of much P_i and probably PP_i during mild hydrolysis of the lipopolysaccharide with 1% acetic acid suggested that the high phosphorus content could be attributed to the presence of polyphosphates. Under conditions commonly used for the hydrolysis of pyrophosphate bonds (1 M-HCl at 100°C for 7 min), 55% of the lipopolysaccharide phosphorus was released as P_i. Similar treatment of fraction Q2 (which contained 60% of the total lipopolysaccharide phosphorus)

gave 27% of the phosphorus as P_i, but less than 5% P_i was formed by treatment of fraction Q2 (before or after HCl hydrolysis) with alkaline phosphatase.

Evidence that much of the acid-labile phosphorus came from ester-bound pyrophosphate residues was obtained by subjecting oxidized lipopolysaccharide to an amine-catalysed β-elimination reaction (Osborn, 1969; Lehmann *et al.*, 1971). The conditions used were those described by Khym & Uziel (1968), and low-molecular-weight solutes were isolated by ultrafiltration. Paper electrophoresis at pH 5.3 (in the presence of EDTA) and t.l.c. on cellulose (solvent system 7) confirmed the presence of PP_i and a little P_i. Less than 1% of the phosphorus released was initially as P_i, but treatment of the phosphates with inorganic pyrophosphatase gave 82% P_i.

In most lipopolysaccharides, phosphorus is largely attached to heptose residues. A comparison of the results of methylation analyses (Tables 3 and 5) for phosphorylated and dephosphorylated polysaccharide, and the low recovery of heptose in deionized hydrolysates (2 M-HCl at 105°C for 2 h) suggested that this was true also for the lipopolysaccharide of *Ps. alcaligenes*. When fraction Q2 was hydrolysed under the above conditions, only 39% of the phosphorus was released as P_i, and two anionic phosphorus-containing products migrating in the regions expected for sugar mono- and di-phosphates were detected by electrophoresis. After dephosphorylation at pH 4.0, both products gave a spot with the mobility of free heptose on paper chromatography.

Discussion

The lipopolysaccharide of *Ps. alcaligenes* BR 1/2 appears to be constructed on architectural lines similar to those of lipopolysaccharides generally. The lipid-A fraction (Drewry *et al.*, 1973) is typically derived from a phosphorylated disaccharide of glucosamine, and is apparently linked to the polysaccharide fraction via a ketosidic linkage from 3-deoxy-2-octulonic acid. The liberation of much free 3-deoxy-2-octulonic acid during mild hydrolysis to cleave lipid A from the polysaccharide indicates that the lipopolysaccharide of *Ps. alcaligenes* contains clustered residues of the sugar acid, as found in the inner-core regions of other lipopolysaccharides (e.g. Dröge *et al.*, 1970; Hämmerling *et al.*, 1971; Morton & Stewart, 1972). However, no structural information about this region of the lipopolysaccharide was obtained during the present studies.

The heptose component of the lipopolysaccharide of *Ps. alcaligenes*, L-*glycero*-D-*manno*-heptose, is commonly present in the lipopolysaccharides of other bacteria, including strains of *Ps. aeruginosa* (Adams *et al.*, 1967; Chester *et al.*, 1972). Although this heptose is sometimes accompanied by its probable biosynthetic precursor, D-*glycero*-D-*manno*-heptose, the results of studies by Lehmann *et al.* (1973) have indicated that this situation is less common than was indicated by Adams *et al.* (1967). Similar amounts of both isomers were found by Adams *et al.* (1967) in the lipopolysaccharide from one strain of *Ps. aeruginosa*, but only L-*glycero*-D-*manno*-heptose was detected in the product from another strain (Chester *et al.*, 1972). Similarly, g.l.c. of the alditol acetates from the dephosphorylated polysaccharide of *Ps. alcaligenes* revealed only one heptitol hepta-acetate.

Heptose residues in enterobacterial lipopolysaccharides frequently carry residues of orthophosphate or ethanolamine pyrophosphate (e.g. Dröge *et al.*, 1968; Lehmann *et al.*, 1971; Hämmerling *et al.*, 1971, 1973), and a similar situation probably exists in the lipopolysaccharide of *Ps. aeruginosa* (Drewry *et al.*, 1971, 1972*a*). The present results for *Ps. alcaligenes* also indicate that most heptose residues in the polysaccharide are phosphorylated, and that much of the phosphorus occurs in condensed phosphates. In contrast with *Ps. aeruginosa*, the condensed phosphates are not esterified with ethanolamine. The release of free PP_i during mild acid hydrolysis of the lipopolysaccharide could result from cleavage of an additional pyrophosphate bond, and hence point to the presence of triphosphate residues in the intact polysaccharide. On the other hand, the release of PP_i by amine-catalysed β-elimination from oxidized lipopolysaccharide is most readily explained by an ester linkage between PP_i and the C-4 position of a heptose residue with a periodate-labile C-6–C-7 bond. Such a linkage is found for residues of ethanolamine pyrophosphate in lipopolysaccharides of *Salmonella* species (Lehmann *et al.*, 1971; Hämmerling *et al.*, 1973). Residues of ethanolamine pyrophosphate linked to heptose in this way are, however, not readily released by mild acid hydrolysis (Lehmann *et al.*, 1971). The possible presence of acid-labile condensed phosphates in the lipid A fraction as well as in the polysaccharide should not be ignored (Drewry *et al.*, 1972*a*; Lüderitz *et al.*, 1973).

The crude polysaccharides obtained by mild acid hydrolysis of lipopolysaccharides from smooth strains of enterobacteria can be fractionated characteristically into O-antigenic side-chain polysaccharides and core polysaccharides, by gel filtration (e.g. Schmidt *et al.*, 1969). Similar results have been obtained with strains of *Ps. aeruginosa* (Fensom & Meadow, 1970; Drewry *et al.*, 1972*b*; Ikeda & Egami, 1973; Chester *et al.*, 1973). The side-chain polysaccharides are usually detected as the fractions of highest molecular weight. Although the immunochemistry of *Ps. alcaligenes* BR 1/2 has not been studied, gel filtration of the crude polysaccharide (Fig. 1) gave only one polysaccharide fraction (Q2). The presence in this fraction of

phosphorylated heptose residues and some 3-deoxy-2-octulonic acid indicates that this fraction corresponds to the core polysaccharide shorn of certain acid-labile components. The other components of fraction Q2 (glucose, rhamnose, galactosamine and alanine) are commonly found in the core polysaccharides of other representative pseudomonads (Fensom & Meadow, 1970; Drewry *et al.*, 1972*b*; Wilkinson *et al.*, 1973; Ikeda & Egami, 1973; Chester *et al.*, 1973). However, the possibility that fraction Q2 might contain a subfraction (undetected) corresponding to side-chain material cannot be eliminated, as only about 65% (w/w) of fraction Q2 can be accounted for by known components. The results in Table 1 for total and individual carbohydrates indicate that any such subfraction would be unlikely to contain material reacting with phenol–H_2SO_4, and the presence of unidentified nitrogenous components is also unlikely (Key *et al.*, 1970*b*).

On the basis of results obtained, it is not possible to define either the size or the structure of the polysaccharide fraction. The elution volume (V_e) of fraction Q2 from Sephadex G-25 suggested a molecular weight of the order 3000–5000, but no calibration compounds of similar structure were available. The molar proportions for fraction Q2 of glucose (1.7), rhamnose (0.7), heptose (2.2), galactosamine (1.0), alanine (1.2), 3-deoxy-2-octulonic acid (0.2), phosphorus (5.4) do not point to a simple empirical formula, but certain inferences may be drawn. By analogy with other lipopolysaccharides, the polysaccharide fraction from *Ps. alcaligenes* is expected to terminate in a single reducing residue of 3-deoxy-2-octulonic acid. Such residues substituted at C-5 give relatively low colour yields in the reaction with thiobarbituric acid (Dröge *et al.*, 1970; Charon & Szabó, 1972). The actual molar ratio of galactosamine/3-deoxy-2-octulonic acid for fraction Q2 may therefore be 1:1.

The core polysaccharides derived from species of *Escherichia* and *Salmonella* characteristically contain three heptose residues (Hämmerling *et al.*, 1971, 1973 and references cited). These residues occur as a trisaccharide, with two phosphorylated residues in the main chain and an unphosphorylated residue as a branch substituent. Unsubstituted heptose residues have also been detected by methylation analysis of polysaccharides from strains of *Pasteurella pseudotuberculosis* (Hellerqvist *et al.*, 1972*a*,*b*). The analytical data for fraction Q2 and the dephosphorylated polysaccharide suggest that the lipopolysaccharide from *Ps. alcaligenes* also contains two phosphorylated heptose residues, and additional unsubstituted heptose. The latter was detected by methylation analysis of phosphorylated polysaccharide, and probably explains the free heptose found in partial hydrolysates. The presence in these hydrolysates of a heptosylglucose (component X) also indicates that the unsubstituted heptose residue is not part of a heptose trisaccharide such as is found in the lipopolysaccharides discussed above.

Although the presence in the polysaccharide fraction of three glucose residues is suggested by the molar ratio glucose/rhamnose of 3:1 and by the results of methylation analyses (Tables 3 and 5), the molar ratio glucose/galactosamine for all batches of polysaccharide was consistently close to 2:1. Thus, if the polysaccharide contains a single galactosamine residue, not all molecules of polysaccharide can contain rhamnose. As shown by the results of periodate oxidation and methylation analysis, and by the loss of rhamnose residues during dephosphorylation of the polysaccharide, rhamnose occurs only as non-reducing terminal residues. The presence in the polysaccharide of additional terminal residues of glucose and heptose may be attributed either to a branched structure or to a heterogeneous polysaccharide. Some heterogeneity in rhamnose content was also indicated by gel filtration of the crude polysaccharide, and considerable evidence for heterogeneity in core polysaccharides from other bacteria has been accumulated (e.g. Hämmerling *et al.*, 1973).

The location in the polysaccharide of the galactosamine residue(s) has not been established, but the resistance to periodate shows that either the C-3 or C-4 position is substituted (assuming that the sugar occurs as an *N*-acyl derivative). The fact that the alanine/galactosamine ratio for the polysaccharide is near 1:1 and the isolation of an acid-stable cationic component (B1) containing both amino compounds suggest the occurrence in the polysaccharide of *N*-alanylgalactosamine. A similar situation probably exists in the core polysaccharide of *Ps. aeruginosa* N.C.T.C. 1999 (Fensom & Gray, 1969; Chester *et al.*, 1972) from which components corresponding to B1–B3 have been isolated (D. T. Drewry, G. W. Gray & S. G. Wilkinson, unpublished work). The partial loss of alanine during periodate oxidation of fraction Q2 may be attributable to a condensation between its amino group and a carbonyl group generated during oxidation of the polysaccharide.

Thus, although a unique structure cannot yet be written for the polysaccharide from *Ps. alcaligenes*, the analytical data indicate that fraction Q2 has a backbone containing glucose and heptose (2 residues each), and 3-deoxy-2-octulonic acid, galactosamine and alanine (1 residue each). The heptose residues of the backbone are esterified with phosphate and/or pyrophosphate residues. One glucose residue is substituted in the C-3 position, and the second residue may be terminal or substituted in the C-3 position with heptose (or possibly rhamnose). The molecular weight of such a polysaccharide would approach 2000, compared with the value 3000–5000 estimated by gel filtration.

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