Biochem. J. (1972) **126**, 395–407
Printed in Great Britain

Further Studies of the Chemical Composition of the Lipopolysaccharide of *Pseudomonas aeruginosa*

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(*Received* 16 *September* 1971)

1. Qualitative and quantitative analytical results for the lipopolysaccharide from acetone-dried cells of *Pseudomonas aeruginosa* (N.C.T.C. 1999) are presented and possible contamination of the material with nucleic acid was further examined. 2. Additional sugars detected (only in large-scale hydrolysates) were mannose and arabinose; traces of spermidine and putrescine were also found. 3. The heptose component is L-glycero-D-manno-heptose. 4. The thiobarbituric acid-positive component is a 3-deoxy-2-octulonic acid, of which only 35–40% links lipid A to the polysaccharide. This linkage is not broken by hydrolysis with acetic acid up to 0.08M. 5. Liberation of lipid A required hydrolysis with 0.1M-hydrochloric acid, which substantially degraded the polysaccharide moiety. 6. Fractions obtained from the degraded polysaccharide by high-voltage electrophoresis were examined; in these, the alanine/galactosamine molar ratio is approx. 1. 7. Hydrazinolysis of whole lipopolysaccharide showed that at least 40% of the alanine is in amide linkage, possibly with galactosamine. 8. Lipid A, solubilized by alkaline methanolysis was fractionated; most of the phosphorus of the higher-molecular-weight fractions was released as P_i by a phosphomonoesterase. 9. Hydrazinolysis of lipid A destroyed approx. 80% of the glucosamine, and glycosidically linked glucosamine oligosaccharides could not be isolated.

In earlier studies of the lipopolysaccharide isolated from acetone-dried cells of the Gram-negative organism *Pseudomonas aeruginosa*, Fensom & Gray (1969) established certain points of similarity and difference compared with the lipopolysaccharides of the Enterobacteriaceae studied by other workers. The lipid A moiety was fairly typical, consisting of *N*-3-hydroxydodecanoylglucosamine residues carrying *O*-acyl groups derived mainly from 2-hydroxydodecanoic acid, 3-hydroxydecanoic acid, dodecanoic acid and hexadecanoic acid; 3-hydroxytetradecanoic acid was not, however, detected. The main points of difference were the relatively high phosphorus content (4.3%) and the low sugar content (16–17%) of the lipopolysaccharide and the fact that only about 20% of the polysaccharide moiety was released by treatments of the lipopolysaccharide with dilute acetic acid (Osborn, 1963; Taylor *et al.*, 1966). More vigorous hydrolysis with dilute sulphuric acid was needed for full release of lipid A and resulted in extensive degradation of the polysaccharide and release of P_i. Identifiable components of hydrolysates of the degraded polysaccharide were glucose, rhamnose, galactosamine, fucosamine and alanine; only traces of glucosamine were detected. The presence of heptose and of 3-deoxy-2-octulonic acid was inferred from colorimetric determinations of these sugars, but was not confirmed chromatographically. The polysaccharide moiety apparently contained unidentified nitrogenous components; these were concentrated in the portion of the polysaccharide remaining bound to lipid A after treatment of the lipopolysaccharide with dilute acetic acid.

The present paper reports further studies of the lipopolysaccharide obtained from the same source. Among the results described are the identification of two further sugars (arabinose and mannose) present in small amount, the isolation and identification of 3-deoxy-2-octulonic acid, the isolation and characterization of the heptose and the preliminary fractionation of the polysaccharide by high-voltage electrophoresis.

Materials and Methods

Preparation of acetone-dried whole cells

Whole cells of *P. aeruginosa* (N.C.T.C. 1999) were supplied by the Microbiological Research Establishment, Porton, Wilts., U.K. Cells were stored as a powder after they had been dehydrated with acetone and washed with diethyl ether (Mackie & McCartney, 1960).

Isolation of lipopolysaccharide

Acetone-dried cells were treated twice with aqueous phenol and the combined aqueous extracts freed from phenol by dialysis (Fensom & Gray, 1969). The non-diffusible residue was adjusted to a concentration

of 0.4% and all the lipopolysaccharide was sedimented by centrifugation in an MSE Superspeed 40 ultracentrifuge (55000*g* for 1h at 20°C); most of the nucleic acids remained in the supernatant fluid. The crude lipopolysaccharide was suspended in water and incubated with ox pancreatic ribonuclease (BDH Chemicals Ltd., Poole, Dorset, U.K.) for 16h at 37°C (Fensom & Gray, 1969). The mixture was centrifuged (105000*g* for 1h at 20°C) and the sediment washed by centrifugation and freeze-dried.

Methods of quantitative analysis

Nitrogen, total carbohydrate, pentose, heptose, methyl pentose, 3-deoxy-2-octulonic acid, fatty acids and nucleic acids were determined colorimetrically as described by Fensom & Gray (1969). Total phosphorus was determined by the method of Chen *et al.* (1956) and by a modification of the method of Bartlett (1959); these methods, without the initial digestions with acid, were also used to determine P_i. D-Glucose was determined by using glucose oxidase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). Samples were first hydrolysed with 1M-HCl for 4h at 105°C, and the hydrolysates neutralized and deionized by using ion-exchange resins (Lambkin *et al.*, 1966).

After hydrolysis of samples of lipopolysaccharide and fractions therefrom with constant-boiling (6.1M) HCl at 105°C for 4h (Fensom & Gray, 1969), amino acids and amino sugars were measured by using an automatic analyser (Technicon Instruments Co. Ltd., Chertsey, Surrey, U.K.).

Determinations of total acetyl content were made by Alfred Bernhardt Analytical Laboratories, Elbach über Engelskirchen, West Germany.

Chromatographic methods

Analytical paper chromatography was carried out with Whatman no. 1 paper and preparative paper chromatography with water-washed Whatman no. 1 or 3MM chromatography paper, depending on the size of sample. T.l.c. was carried out with plates of Kieselgel G or H (E. Merck A.-G., Darmstadt, West Germany) or MN-300 cellulose (Macherey, Nagel and Co., Düren, West Germany).

The following solvent systems were employed: A, ethyl acetate – pyridine – water (5:2:5, by vol., upper layer); B, pyridine–butan-1-ol–water (5:9:4, by vol.); C, ethyl acetate – pyridine – water – acetic acid (5:5:3:1, by vol.); D, pyridine – butan-1-ol – water (4:6:3, by vol.); E, butan-1-ol – acetic acid – water (3:1:1, by vol.); F, propan-2-ol–aq. 0.1M-NH_3 (17:4, v/v) (used only for ascending chromatography); G, ethanol – acetic acid – water (80:1:9, by vol.); H, butan-1-ol – pyridine–aq. 0.1M-HCl (5:3:2, by vol.); I, acetone – water (19:1, v/v); J, butan-1-ol – ethanol – aq. 0.1M-HCl (1:10:5, by vol.); K, butan-1-ol – ethyl acetate – propan-2-ol – acetic acid – water (7:20:12:7:6, by vol.).

Gel filtration was done on columns of Sephadex G-50 (Pharmacia, Uppsala, Sweden) and ion-exchange chromatography on Whatman DE 23 DEAE-cellulose (W. and R. Balston Ltd., Maidstone, Kent, U.K.).

Gas–liquid chromatography

Fatty acids were identified and relative amounts determined by g.l.c. of the methyl esters prepared from the free acids by using a solution of BF_3 (14%, w/v) in methanol (Applied Science Laboratories Inc., State College, Pa., U.S.A.) as described by Metcalfe & Schmitz (1961) or with an excess of diazomethane in diethyl ether containing methanol (10%, w/v). Free acids were released from samples by hydrolysis with constant-boiling (6.1M) HCl for 4h at 105°C and extracted by shaking the cold hydrolysates with 3×5ml of chloroform. Columns of Apiezon L (at 220°C) and of polydiethyleneglycol succinate (at 180°C) were used; relative amounts of acids were best determined from results obtained with the polar column.

Neutral sugars were identified qualitatively by g.l.c. of the trimethylsilyl derivatives and the alditol acetates. Trimethylsilyl derivatives were prepared from solutions of the sugars (200μg) in dry *NN*-dimethylformamide (60μl) by treatment with bis-(trimethylsilyl)acetamide (40μl). Separation was obtained with a column of silicone gum (SE-52) at 160°C. Alditol acetates were prepared from the neutral sugars (200μg) by the method of Sawardeker *et al.* (1965) as modified by Björndal *et al.* (1967). The acetates were analysed by g.l.c. on columns of neopentylglycol sebacate and Apiezon M at 200°C.

Gas–liquid chromatograms were obtained with either Perkin–Elmer F11 or Pye 104 gas-chromatographic apparatus.

High-voltage electrophoresis

This was carried out with Shandon high-voltage SAE-2550 apparatus. Qualitative analysis was carried out with Whatman no. 1 chromatography paper and preparative electrophoresis with water-washed Whatman no. 1 or 3MM chromatography paper. The following buffers were used: (i), pyridine acetate (pH 5.3) prepared from pyridine–acetic acid–water (5:2:43, by vol.); (ii), 0.05M-$Na_2B_4O_7$.

Physical methods

I.r. and u.v. spectra were obtained as described by Fensom & Gray (1969).

Preparation of lipid A and partly degraded polysaccharide

Lipopolysaccharide (50mg) was hydrolysed in a sealed tube with 0.1M-HCl for 30min at 100°C. The cooled hydrolysate was treated with chloroform (3ml) and the layers were separated by low-speed centrifugation. The aqueous layer was removed, leaving any interfacial material in the chloroform. The chloroform layer was washed with 3×3ml of water, and the combined aqueous extracts were shaken with 3×3ml of chloroform; these chloroform washings were discarded. The chloroform extract was evaporated to dryness in a stream of filtered N_2. The residue was again taken up in chloroform, insoluble material was removed by filtration (no. 4 porosity sinter) and the filtrate was evaporated to dryness. The residual lipid A was dried *in vacuo* over P_2O_5.

The combined aqueous phases were freeze-dried to remove HCl and the residual degraded polysaccharide was thoroughly dried *in vacuo* over P_2O_5.

Degradation of heptose to hexose and pentose

The procedure (conversion of the heptose into heptonic acid with IO^-, followed by treatment with H_2O_2–Fe^{3+}) was similar to that described by Bagdian *et al.* (1966). The method was applied to heptose (about 0.5mg) dissolved in deionized water (5μl). A portion of the hexose–pentose mixture (containing about 30μg of hexose) was dissolved in deionized water (2ml) and incubated overnight in an atmosphere of O_2 with D-galactose oxidase (1mg; Worthington Biochemical Corp.). The solution was deionized by using mixed ion-exchange resins (Lambkin *et al.*, 1966) and examined by paper chromatography.

Degradation of 3-deoxy-2-octulonic acid to 2-deoxyheptose

The method was similar to that of Ghalambor *et al.* (1966) and was applied to 3-deoxy-2-octulonic acid (about 5μmol) dissolved in deionized water (0.5ml).

Hydrazinolysis

Hydrazine (95%; Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) was distilled from calcium oxide with toluene as described by Kusama (1957). The distillate was distilled twice more from fresh calcium oxide; the lower layer of the final distillate was used without further treatment. Samples (8–25mg) for hydrazinolysis were dried *in vacuo* over P_2O_5 at 70°C. Anhydrous hydrazine (0.1–0.3ml) was added and the mixture heated for 16h at 105°C. Most of the hydrazine was removed by rotary evaporation at 60°C and remaining traces by drying *in vacuo* over H_2SO_4. The residue was taken up in water, neutralized with 0.1M-HCl and the resulting mixture centrifuged at low speed. The sediment was washed twice by centrifugation and the aqueous supernatant solutions were combined and freeze-dried.

Alkaline methanolysis of lipid A

The procedure of Kasai (1966) as described by Fensom & Gray (1969) was used to solubilize lipid A.

Fractionation of solubilized lipid A

The solubilized lipid was fractionated on a column (2cm×40cm) of Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, Calif., U.S.A.). Elution was carried out with deionized water and the fractions were screened for phosphorus by the method of Bartlett (1959).

Treatment of solubilized lipid A with enzymes

Material corresponding to peaks 1 and 2 obtained on elution of solubilized lipid A from a column of Bio-Gel P-2 was treated with phosphomonoesterase and/or phosphodiesterase. Samples, made up in 0.05M-$(NH_4)_2CO_3$ (1ml), were treated with a solution (0.2ml) of alkaline phosphatase (0.5mg) in 0.05M-$(NH_4)_2CO_3$. The pH of the solution was adjusted to 9.6 by addition of dilute NH_3, and the mixture, under a layer of toluene, was incubated for 16h at 37°C. Treatment of fractions of solubilized lipid A with a mixture of phosphomonoesterase and a snake venom (containing a phosphodiesterase) from *Crotalus adamanteus* was carried out in the same way, but at pH9.2. Both enzymes were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.; the phosphomonoesterase was Sigma type 1 from calf intestinal mucosa. After incubation, the extent of release of P_i was determined.

Results

Preparation of lipopolysaccharide

The crude lipopolysaccharide obtained by ultracentrifuging the aqueous extracts (adjusted to a concentration of 0.4%) from the aqueous phenol extraction procedure contained 6–10% nucleic acid. After treatment with ox pancreatic ribonuclease, the lipopolysaccharide contained only 2–3% nucleic acid. These nucleic acid contents were determined from the u.v. spectra of aqueous suspensions of lipopolysaccharide by comparing the E_{260} and E_{300} values as described by Barker *et al.* (1966). The results compare favourably with those (6–20% and 2–7% respectively) reported by Fensom & Gray (1969), who ultracentrifuged more-concentrated solutions (3%, w/w).

Fensom & Gray (1969) attempted to decrease

nucleic acid contamination of crude lipopolysaccharide by a variety of methods, but all except the enzymic treatment were unsuccessful. The following additional techniques were tried in the present work. Acetone-dried cells were treated with 0.25M-trichloroacetic acid at 2–4°C before treatment with aqueous phenol (O'Neill & Todd, 1961); the lipopolysaccharide contained 9–11% nucleic acid and was analytically different from that obtained by the normal procedure. Crude lipopolysaccharide was treated with diethylene glycol for 2h at 37°C and then at room temperature for 24h (Morgan & Partridge, 1940), but preferential solubilization of lipopolysaccharide was not obtained.

Nowotny (1966) and Nowotny *et al.* (1966) have shown that endotoxic preparations from a number of Gram-negative organisms may be fractionated by means of ion-exchange chromatography, and in a number of cases, lipopolysaccharide was separated from contaminating nucleic acid. Moreover, contaminating nucleic acid has been removed from an acidic *Pneumococcus* type II polysaccharide by using ion-exchange chromatography (Barker *et al.*, 1966). Suspensions of crude lipopolysaccharide were therefore applied to columns of DEAE-cellulose and eluted with either (*a*) a linear gradient of salt (0–1M-NaCl or LiCl) in tris–HCl buffer (0.05M, pH7.54) or (*b*) a pH gradient applied in two parts: (1) water-saturated $NaHCO_3$ and (2) saturated $NaHCO_3$–2M-NaOH. In (*a*) only 35% of the lipopolysaccharide was recovered from the column in two fractions, which were contaminated with nucleic acid to the same extent as the original lipopolysaccharide. Attempts to improve the recovery by eluting the column with a linear salt gradient (0–1M-NaCl) in aq. 7M-urea were unsuccessful. In (*b*) all the material applied to the column was recovered in five fractions, but no separation of nucleic acid was obtained. These results may indicate heterogeneity of the lipopolysaccharide, but the increasing pH in (*b*) may have caused gradual degradation of lipopolysaccharide bound to the column. In an effort to overcome some of the difficulties associated with insolubility of the lipopolysaccharide, some of these experiments were repeated with lipopolysaccharide that had been solubilized by treatment with sodium deoxycholate according to the procedure of Ribi *et al.* (1966), but these were abandoned when it was found (Key *et al.*, 1970) that the chemical composition of the lipopolysaccharide of *Pseudomonas alcaligenes* was altered by similar treatment with the bile salt. The only satisfactory means of decreasing the nucleic acid content of the crude lipopolysaccharide was, as found by Fensom & Gray (1969), by digestion with ribonuclease.

Purity of the lipopolysaccharide

The degree of contamination of the final lipopolysaccharide with nucleic acid may be overestimated by the u.v.-spectrophotometric method. The u.v. spectrum of a suspension of lipopolysaccharide did not have a maximum at 260nm, and the difference in the E_{260} and E_{300} values may simply reflect a small difference in the value of the background absorption for the lipopolysaccharide itself. Moreover, in subsequent investigations of lipopolysaccharide hydrolysates by various forms of chromatography and by high-voltage electrophoresis, no ribose was detected to substantiate the presence of nucleic acid. It is reasonable to conclude therefore that 2–3% represents the upper limit of possible nucleic acid contamination. Traces of protein amino acids (total $\gtrsim$1.9%) were also detected.

Table 1. *Percentages of amino compounds in six batches of lipopolysaccharide from P. aeruginosa*

Hydrolyses were carried out for 4h at 105°C with constant-boiling (6.1M) HCl and samples were analysed automatically by a ninhydrin method. The results were corrected where necessary for slow release or decomposition. Results are expressed as percentages of the free compounds. +, Measureable amount detected.

	Lower and upper limits of amounts for six batches of lipopolysaccharide (%, w/w)	Average amount (%, w/w)
O-Phosphorylglucosamine*	1.70–2.23	2.04
Alanine	1.15–1.34	1.27
Glucosamine	3.30–4.32	3.79
Galactosamine	2.11–2.70	2.45
Fucosamine	0.57–1.00	0.82
Ammonia	0.83–1.50	1.13
Unknown D (Fensom & Gray, 1969)	+	+
Other amino compounds	0.69–1.90	1.17

* The amount of this component was calculated by using the colour yield for a standard of glucosamine 6-phosphate.

Amino compounds of the lipopolysaccharide

Several batches of lipopolysaccharide were analysed for amino compounds with the results shown in Table 1. The results agree well with those obtained by Fensom & Gray (1969). For each batch analysed, only about 50% of the nitrogen was accountable for in terms of identifiable amino compounds and ammonia. Even if contamination by up to 3% of nucleic acid was allowed for, the nitrogen recovery was only about 70%.

On submitting the lipopolysaccharide to high-voltage electrophoresis, small amounts of spermidine and putrescine (Lüderitz *et al.*, 1968) were detected; these are presumably bound to the lipopolysaccharide by ionic linkages. Although these components will make some contribution to the nitrogen content of the lipopolysaccharide, their amounts are small and will not appreciably affect the low nitrogen recovery referred to above. The actual amounts present were too small (<1%) to determine with accuracy, and it was shown that liberation of more spermidine and putrescine did not occur on hydrolysing the lipopolysaccharide with constant-boiling HCl for 4h at 105°C.

Sugars

Samples of lipopolysaccharide were hydrolysed with 0.5M-HCl at 105°C for various periods of time; the hydrolysates were deionized and submitted to paper chromatography in solvent systems A, B and C. After hydrolysis only two sugars were identified, glucose and rhamnose; even after short hydrolysis (2–15min) no dideoxy sugars were detected. Samples of lipopolysaccharide were analysed specifically for D-glucose, and for 6-deoxyhexose by the cysteine–sulphuric acid method of Dische (1955). The spectrum obtained by the latter method indicated that heptose was also present in the lipopolysaccharide. However, as reported previously for *P. aeruginosa* (Fensom & Gray, 1969) and *P. alcaligenes* (Key *et al.*, 1970), paper chromatography of hydrolysates of lipopolysaccharide failed to detect any heptose. G.l.c. of the trimethylsilyl derivatives of the sugars of the lipopolysaccharide of *P. alcaligenes* (Key *et al.*, 1970) and *P. aeruginosa* did, however, show an unidentified component thought to be heptose.

Many aldoheptoses have paper-chromatographic mobilities similar to the hexoses in most solvent systems, and therefore the heptose may have been masked by glucose, the major sugar component of the lipopolysaccharide. Also the heptose may be phosphorylated and therefore require prolonged hydrolysis for its release. A large sample of lipopolysaccharide (250mg) was hydrolysed with 0.25M-H_2SO_4 for 16h at 105°C. The deionized hydrolysate was freed from glucose by treatment with glucose oxidase (Adams *et al.*, 1967). The solution was again deionized and submitted to preparative paper chromatography in solvent system I. Development of guide strips of the chromatogram with aniline phosphate showed that glucose had been destroyed and that another sugar was present, this having a similar mobility to glucose but giving a different colour with aniline phosphate; the colour was identical with that given by several standard heptoses. In addition, two other minor sugar components were detected, with mobilities identical with those of mannose and arabinose and giving the same colours with aniline phosphate as authentic samples of the sugars. These three sugars were eluted separately. The identities of mannose and arabinose were confirmed by paper chromatography in solvent systems A, B and C, by high-voltage electrophoresis in buffer system (ii) and by t.l.c. in solvent systems A with plates of MN-300 cellulose and J and K with plates of Kieselgel H prepared as described by Lato *et al.* (1968).

The third sugar was treated with cysteine–sulphuric acid reagent; the spectrum of the mixture had a maximum at 505nm, indicating that the sugar was in fact the heptose of the lipopolysaccharide. In terms of L-glycero-D-mannoheptose, about 1mg of the unknown heptose was isolated.

By comparing the mobility of the unknown heptose from *P. aeruginosa* with the mobilities of four standard heptoses and those given by Davies (1957) using solvent systems D and I, the identity of the heptose was narrowed to fourteen possibilities. The heptose was then degraded and the mixture of hexose and pentose examined by paper chromatography in solvent systems D and I and by high-voltage electrophoresis in buffer (ii). The sugars detected were galactose and lyxose, which could be produced only from two heptoses, L-glycero-D-mannoheptose and D-glycero-L-mannoheptose; the former would give L-galactose and the latter D-galactose. The galactose formed was not destroyed by D-galactose oxidase which, in a concurrent experiment, completely destroyed the D-galactose in a standard solution of equivalent concentration. The presence of L-galactose therefore confirmed that the heptose of the lipopolysaccharide of *P. aeruginosa* is L-glycero-D-mannoheptose.

A sample of the partly degraded polysaccharide obtained from the lipopolysaccharide by hydrolysis with 0.1M-HCl was treated with sodium metaperiodate and then with sodium borohydride. The hydrolysate of the resulting material (0.25M-H_2SO_4 for 16h at 105°C) was neutralized and compared by paper chromatography in solvent systems A, D and I with a neutralized solution of polysaccharide that had merely been hydrolysed. Oxidation and reduction had clearly increased the amount of mannose present; this is the expected product from L-glycero-D-mannoheptose if this occurs in the lipopoly-

saccharide with free hydroxyl groups at C-6 and C-7, but not at C-5.

The results of g.l.c. of the alditol acetates from the sugars of the lipopolysaccharide were easier to interpret than those obtained with trimethylsilyl derivatives. The two major peaks correspond to glucose and rhamnose, and two minor peaks to mannose and arabinose. A fifth peak occurred after elution of glucose and may correspond to the heptose; this was not, however, confirmed.

Paper chromatography, high-voltage electrophoresis, t.l.c. and g.l.c. failed to detect any ribose in the lipopolysaccharide. No confirmation of the presence of contaminating nucleic acid in the lipopolysaccharide was therefore provided. If ribose is indeed absent, the pentose content (see below) of the lipopolysaccharide must be attributed to the arabinose that has been detected.

General analyses and overall composition of the lipopolysaccharide

The total carbohydrate content of the lipopolysaccharide was determined by both the phenol–sulphuric acid and the anthrone–sulphuric acid methods; the results were 20.5 and 15.5% respectively. Results of analyses for individual sugars were: glucose, 8.0%; rhamnose, 2.0%; heptose, 4.2%; pentose, 1.8%; 3-deoxy-2-octulonic acid, 2.8%; total, 18.8%.

The total fatty acid content of the lipopolysaccharide was 20.9%, as measured by the colorimetric method of Itaya & Ui (1965). The identities of the individual acids and their relative amounts were similar to those reported by Fensom & Gray (1969) except that the amounts of hexadecanoic acid were more variable but always lower; the unknown components (X) and (Y) found by Fensom & Gray (1969) were identified as dec-2-enoic acid and dodec-2-enoic acid respectively. From the relative amounts of the individual acids and their colour yields in the colorimetric method, the true fatty acid content of the lipopolysaccharide was corrected to 17.2%. The phosphorus content of the lipopolysaccharide was 4.6%, the nitrogen content 3.7%, and the acetyl content 8.1%.

From the above analytical results (expressing the phosphorus content of the lipopolysaccharide as orthophosphoric acid, 14.5%) and the average amounts of the amino compounds (Table 1) in the lipopolysaccharide, the total weight recovery for the lipopolysaccharide was about 70%.

Separation of lipid A and partly degraded polysaccharide

After hydrolysis of the lipopolysaccharide with different concentrations of acetic acid (0.01–0.08M) for 30min at 100°C little chloroform-soluble lipid A could be isolated. The lipopolysaccharide was therefore hydrolysed with 0.1M-HCl at 100°C for different periods of time up to 45min and the amounts of chloroform-soluble material were determined. On hydrolysis for 10min, no lipid A was obtained. The optimum release of lipid A was obtained after 30min; the material isolated from the aqueous phase of a hydrolysis carried out under these conditions was designated partly degraded polysaccharide. In a typical case, the percentages of the original lipopolysaccharide isolated as lipid A and polysaccharide were 28 and 64% respectively, a recovery of 92%.

Partly degraded polysaccharide

Analysis showed that the 'polysaccharide' contained practically all the galactosamine, fucosamine, alanine, unknown amino component (Table 1), neutral sugars and 3-deoxy-2-octulonic acid of the lipopolysaccharide. Only small amounts of glucosamine and amino acids were detected, and, as reported by Fensom & Gray (1969), it was not possible to account for all of the nitrogen of the polysaccharide in terms of the amino compounds detected. Treatment of the polysaccharide with an alkaline solution of sodium borohydride (Simmons *et al.*, 1965) did not noticeably change the sugar content (as determined by the anthrone method) or the content of amino compounds, but all the 3-deoxy-2-octulonic acid was destroyed (as determined by the thiobarbituric acid method).

The partly degraded polysaccharide was examined by high-voltage electrophoresis at 40–50V/cm for 1h, in buffer system (i) (Fig. 1).

Five thiobarbituric acid-positive spots were detected (C1 and A1, A2, A3 and A5). Subsequent investigation showed that component A5 corresponded to free 3-deoxy-2-octulonic acid. This was established by eluting the material corresponding to spot A5 from a preparative high-voltage electrophoretogram and purifying this by preparative paper chromatography in solvent system G. The material isolated was then compared with authentic 3-deoxy-2-octulonic acid from a strain of *Escherichia coli*; the materials had identical electrophoretic mobilities in buffer system (i) and identical R_F values on paper chromatography in solvent systems G and H. Further, the deoxyheptoses formed by treatment of component A5 or authentic 3-deoxy-2-octulonic acid with sodium borohydride and then with ceric sulphate had identical R_F values on paper chromatography in solvent systems A and H. The thiobarbituric acid-positive component of the lipopolysaccharide of *P. aeruginosa* was thus a 3-deoxy-2-octulonic acid.

Component A7 was P_i; the extents of release of P_i and of free 3-deoxy-2-octulonic acid from the lipo-

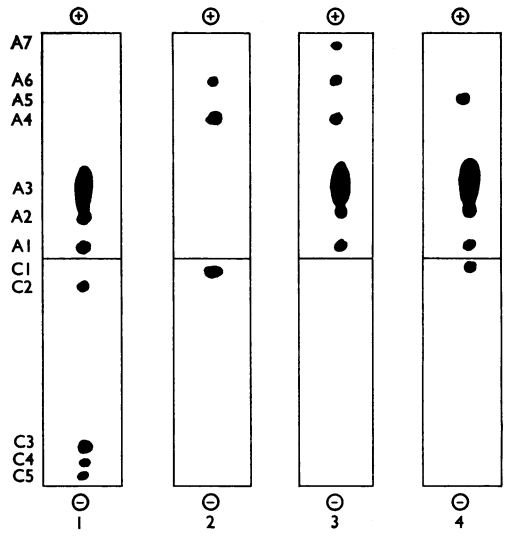

Fig. 1. *Diagrams of paper electrophoretograms of partly degraded polysaccharide*

Electrophoresis was carried out at 40–50 V/cm for 1 h in buffer system (i) and stained to detect: (1) ninhydrin-positive components; (2) silver nitrate-positive components; (3) phosphorus-containing components; (4) thiobarbituric acid-positive components.

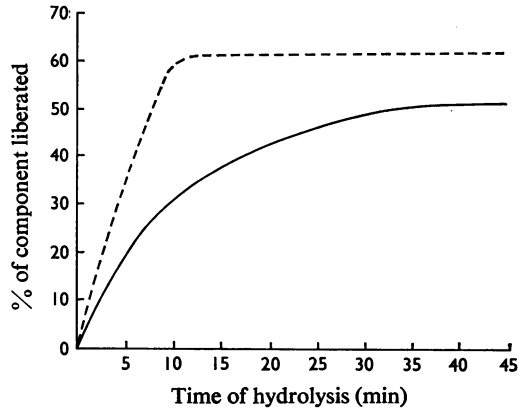

Fig. 2. *Release of P_i and 3-deoxy-2-octulonic acid on hydrolysis of lipopolysaccharide*

Hydrolysis was carried out with 0.1 M-HCl at 100°C. In the case of 3-deoxy-2-octulonic acid, component A5 (Fig. 1) was eluted from electrophoretograms and estimated by the thiobarbituric acid method. The amounts of phosphorus (determined as P_i) (——) and of 3-deoxy-2-octulonic acid (----) are plotted as percentages of component liberated.

polysaccharide with increasing time of hydrolysis in 0.1 M-HCl were then determined (Fig. 2).

After hydrolysis for 10 min, maximum release of 3-deoxy-2-octulonic acid (60–65% of the total amount in the lipopolysaccharide) occurred. Large amounts of free 3-deoxy-2-octulonic acid are therefore liberated before any chloroform-soluble lipid A.

The above results apparently show that considerable degradation of the polysaccharide occurs during release of lipid A by acid hydrolysis. The following results for the other components of the polysaccharide separated by high-voltage electrophoresis (Fig. 1) confirm this view.

Components C1 to C5

Component C1 stained with silver nitrate reagent as well as with thiobarbituric acid reagent; component C2 was only ninhydrin-positive. The closeness of these components on the electrophoretogram made separation difficult and they were eluted together. Paper chromatography (solvent system B) revealed glucose, rhamnose and two other components X($R_{glucose}$ 0.38) and Y($R_{glucose}$ 0.54) stained by silver nitrate reagent; one ninhydrin-positive component was detected. Components X and Y were eluted and after hydrolysis and paper chromatography (solvent system B), component X was found to contain glucose, rhamnose, arabinose and 3-deoxy-2-octulonic acid; component Y contained the same sugars, but only traces of 3-deoxy-2-octulonic acid were present. Only small quantities of components X and Y and of the ninhydrin-positive component were isolated and no further work on them was possible.

Of the other cationic, ninhydrin-positive components, C3 and C4 were isolated and shown to be identical with spermidine and putrescine, both present as minor components of the lipopolysaccharide. No information on the minor component C5 was obtained.

Components A1, A2 and A3

These were the major components of the polysaccharide, A3 alone accounting for approx. 50% of the material isolated after high-voltage electrophoresis. All three components behaved as high-molecular-weight polymers when submitted to chromatography on Sephadex G-50.

Alanine, galactosamine and fucosamine were present in hydrolysates of all three components, and the molar ratios of these in components A1, A2 and A3 are shown in Table 2. For component A1, the molar ratio of the three amino compounds was about the same as for the whole lipopolysaccharide and the

Table 2. *Molar ratios of alanine, galactosamine and fucosamine in whole lipopolysaccharide (LPS), partly degraded polysaccharide (PS) and components A1, A2 and A3 of the partly degraded polysaccharide of P. aeruginosa*

Ratios in whole lipopolysaccharide were calculated from the average amounts of the amino compounds given in Table 1.

	Molar ratio for				
	LPS	PS	A1	A2	A3
Alanine	1.04	0.88	0.83	0.97	0.98
Galactosamine	1.00	1.00	1.00	1.00	1.00
Fucosamine	0.33	0.35	0.39	1.00	0.16

partly degraded polysaccharide. Components A2 and A3 also contained alanine and galactosamine in about the same ratio as that for the lipopolysaccharide and the partly degraded polysaccharide, but component A2 contained a relatively higher and A3 a relatively lower proportion of fucosamine. These molar ratios were unaltered after further purification of components A1, A2 and A3 by high-voltage electrophoresis. This indicates that although fucosamine is an integral part of the lipopolysaccharide, is it not homogeneously distributed throughout the lipopolysaccharide relative to alanine and galactosamine. The unknown amino compound (Table 1) of the lipopolysaccharide was present in components A1, A2 and A3 and occurred in a constant molar ratio to fucosamine, just as the alanine/galactosamine molar ratio was approximately constant. Since bacterial polysaccharides containing fucosamine have been found to yield 4-oxonorleucine when hydrolysed under conditions similar to those used in the preparation of samples of components A1, A2 and A3 for analysis on an automatic analyser (Barry & Roark, 1964), the unknown amino compound was isolated by preparative high-voltage electrophoresis of the material isolated from a hydrolysate of component A2. The unknown compound, however, had chromatographic properties unlike those of 4-oxonorleucine.

Components A1, A2 and A3 also contained glucose, rhamnose, arabinose and heptose in similar molar proportions to those of the original lipopolysaccharide. As mentioned above, they also contained bound 3-deoxy-2-octulonic acid (reducible by sodium borohydride). Similar treatment of the whole lipopolysaccharide with sodium borohydride did not cause any destruction of 3-deoxy-2-octulonic acid, showing that release of lipid A leaves 35–40% of the 3-deoxy-2-octulonic acid bound at the reducing end of polysaccharide components.

Components A4 and A6

Complete separation of these components from free 3-deoxy-2-octulonic acid (A5) was not easy, and relatively small amounts were available. Both components A4 and A6 contained glucose and arabinose, relatively small amounts of rhamnose, and large amounts of phosphorus.

The various components of the partly degraded polysaccharide discussed above and shown in Fig. 1 accounted for approx. 65–70% of the total polysaccharide.

Mode of linkage of the alanine residue in the lipopolysaccharide

As Table 2 shows, the alanine/galactosamine molar ratio is close to unity in the lipopolysaccharide, the partly degraded polysaccharide, and in components A1, A2 and A3 of the polysaccharide, suggesting that the two compounds are linked. Fensom & Gray (1969) showed that the alanine residue of the lipopolysaccharide was probably not ester linked to the galactosamine, and this has been confirmed by the same method with the whole lipopolysaccharide and component A3. Moreover, no alanine is cleaved from the lipopolysaccharide or component A3 with either aqueous or methanolic potassium hydroxide, conditions that cleave all ester-bound fatty acids from the lipid moiety of the lipopolysaccharide.

The lipopolysaccharide was treated with anhydrous hydrazine to cleave possible amide linkages between the alanine and the galactosamine. The material isolated from the water-soluble fraction of the hydrazinolysate was eluted from a column of Sephadex G-50. The eluate was collected and screened for phosphorus; three peaks were obtained (Fig. 3) and the eluates combined to give the corresponding fractions, two high-molecular-weight and one low-molecular-weight. The molar ratios of amino compounds in the three fractions are given in Table 3. Alanine was in fact cleaved from the lipopolysaccharide, 40% of the total alanine recovered from the column being present as alanine hydrazide in the low-molecular-weight fraction; the presence of the hydrazide was confirmed by paper chromatography (solvent systems E and F) and by high-voltage electrophoresis (buffer system i). The remainder of

the alanine recovered was in the high-molecular-weight fractions. The cleavage of only 40% of the alanine on hydrazinolysis remains unexplained, but incomplete hydrazinolysis is a possible reason. No galactosamine occurred in the low-molecular-weight fraction, suggesting that loss of the alanine does not result in extensive breakdown of the backbone of the lipopolysaccharide.

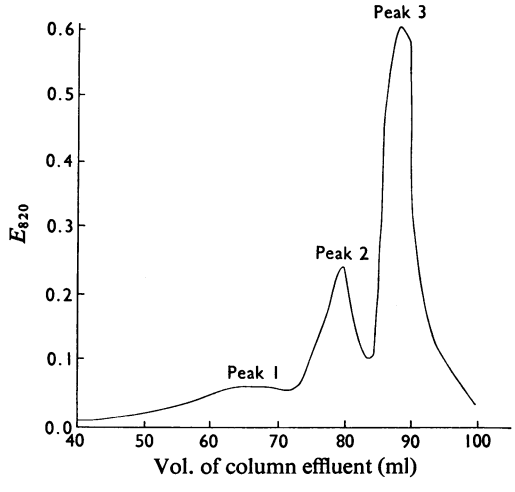

Fig. 3. *Fractionation of a hydrazinolysate of the lipopolysaccharide on Sephadex G-50*

The neutralized water-soluble residue from a hydrazinolysate of 25mg of lipopolysaccharide was dissolved in water (0.5ml), applied to the column (2cm×40cm) of Sephadex G-50 and eluted with deionized water. Fractions were screened for total phosphorus by the method of Chen *et al.* (1956).

Lipid A

Hydrolysis of lipopolysaccharide with 0.1M-HCl for 30min at 100°C gave yields of lipid A from 24.5 to 31.2%. The yields and the i.r. spectra of the preparations agreed closely with those obtained by Fensom & Gray (1969). Hydrolysis of the lipid by the method of Radin (1959) and analysis of the hydrolysate by the anthrone method showed that contamination of the lipid by polysaccharide components was only about 3%. The low percentage of contamination was confirmed by analysis in an autoanalyses of lipid A hydrolysates for amino compounds. Typical results were: *O*-phosphorylglucosamine, 6.54%; glucosamine, 13.00%; galactosamine, trace; fucosamine, 0.25%; alanine, trace; other amino compounds, 0.94%; total of amino compounds, 20.73%. The result for *O*-phosphorylglucosamine is based on the colour yield obtained with a standard of glucosamine 6-phosphate. Alanine, galactosamine and fucosamine, components of the polysaccharide moiety, are therefore present in only small amounts, although occasional preparations were more heavily contaminated. Contamination of the lipid A with polysaccharide components is therefore much smaller than that reported by Fensom & Gray (1969) who obtained the lipid A by more vigorous hydrolysis of lipopolysaccharide with 0.5M-H_2SO_4 for 30min at 100°C and estimated that polysaccharide contamination was in the range 10–20%. Since the polysaccharide is rich in phosphorus, these results offer an explanation of the lower phosphorus content (2.0–2.1%) obtained for lipid A in this work compared with 2.8–3.3% found by Fensom & Gray (1969). The nitrogen content of the lipid was 2.5–2.6%, of which 80% was accountable for by the identifiable amino compounds.

The fatty acids and hydroxy acids liberated from lipid A were the same as those found by Fensom &

Table 3. *Molar ratios of amino compounds relative to alanine for a hydrazinolysate of the lipopolysaccharide and fractions therefrom*

The water-soluble fraction obtained after hydrazinolysis of the lipopolysaccharide and the fractions obtained from this by chromatography on Sephadex G-50 (Fig. 3) were hydrolysed with constant-boiling HCl for 4h at 105°C and the components determined with an autoanalyser. Recoveries after chromatography are given in column 1.

		Molar amounts relative to alanine			
		Water-soluble fraction	Fractions corresponding to		
Amino compound	Recovery (%)		Peak 1	Peak 2	Peak 3
Alanine	86.9	1.00	1.00	1.00	1.00
O-Phosphorylglucosamine	62.9	0.34	0.38	0.59	0.00
Glucosamine	91.8	1.17	2.21	1.71	0.00
Galactosamine	86.1	0.89	1.51	1.41	0.00
Fucosamine	45.9	0.12	0.15	Very small	0.00

Gray (1969). The relative amounts of the acids also agreed with earlier results (Fensom & Gray, 1969; Key *et al.*, 1970), except that the amount of hexadecanoic acid was lower; in some cases only traces of this acid were found. 3-Hydroxydodecanoic acid again constituted the *N*-acylating acid of the lipid, and no 3-hydroxytetradecanoic acid was detected.

Colorimetric determination of the fatty acid content of lipid A gave 62%, based on hexadecanoic acid. If this value is corrected (Key *et al.*, 1970) to allow for the fact that the individual acids give different colour yields the result is 51%. This value agrees closely with the previous value of 52% obtained by weighing (Fensom & Gray, 1969).

The overall weight recovery for lipid A was: fatty acids and hydroxy acids, 51%; phosphorus (expressed as orthophosphoric acid), 6.4%; glucosamine (including that determined as *O*-phosphorylglucosamine), 17.5%; other amino compounds, 1.2%; polysaccharide, 3%; total 79.1%. The phosphorus/glucosamine/fatty acids molar ratio was 0.68:1.00:2.56.

Lipid A was solubilized by alkaline methanolysis and the aqueous fraction eluted with deionized water from a column of Bio-Gel P-2. Screening for phosphorus revealed two major peaks and two very minor peaks (Fig. 4); in some cases peak 3 was not detected and peaks 1 and 2 formed one broad peak. Fractions

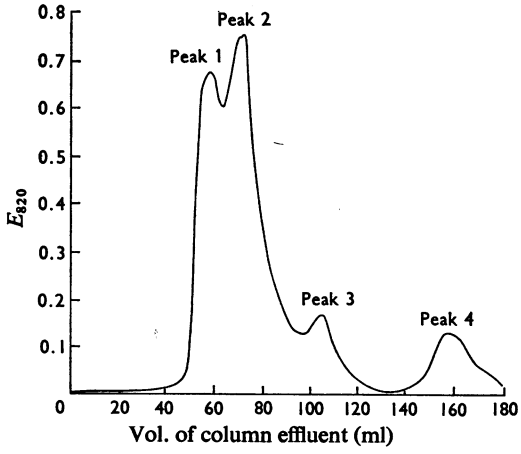

Fig. 4. *Fractionation on Bio-Gel P-2 of lipid A, solubilized by alkaline methanolysis*

The methanolysate from lipid A (8mg) was neutralized with ethyl formate and evaporated to dryness; the residue was dissolved in water (1ml), applied to the column (2cm×40cm) of Bio-Gel P-2 and eluted with deionized water. Fractions were screened for total phosphorus by the method of Chen *et al.* (1956). corresponding to these peaks were obtained; each contained glucosamine bound to one fatty acid only (3-hydroxydodecanoic acid), and the phosphorus/glucosamine molar ratios are given in Table 4.

Further investigation of material corresponding to peaks 3 and 4 was not possible except to show that the phosphorus relating to peak 4 was mainly P_i. Attempts to cleave 3-hydroxydodecanoic acid from fractions 1 and 2 by hydrazinolysis resulted in destruction of most of the glucosamine and a high release of P_i. Fractions 1 and 2 were reduced with sodium borohydride (Simmons *et al.*, 1965) and then hydrazinolysed, but again most of the glucosamine was destroyed.

Fractions 1 and 2 were then incubated with phosphomonoesterase and/or phosphodiesterase. All the phosphorus in fraction 1 and 66% of that in fraction 2 was released as P_i by phosphomonoesterase alone, and inclusion of venom phosphodiesterase in the incubation mixture did not increase the release of P_i.

The glucosamine contents of fractions 1 and 2 were determined after reduction of the materials with sodium borohydride (Simmons *et al.*, 1965). From fraction 1, 62–68% of the glucosamine was recovered and 54–59.5% from fraction 2.

Discussion

The present work on the lipopolysaccharide isolated from acetone-dried cells of *P. aeruginosa* (N.C.T.C. 1999) extends that of Fensom & Gray (1969). Difficulties were again encountered in removing contaminating nucleic acid. Most was, however, removed by ultracentrifugation of the aqueous extracts obtained directly from the treatment of whole cells with aqueous phenol, followed by digestion of the crude lipopolysaccharide with ribonuclease. As measured spectrophotometrically, 2–3% of nucleic acids remained, but this is regarded as an upper limit for the contamination, since no ribose could be detected in hydrolysates of the lipopolysaccharide.

Qualitative and quantitative results for the composition of the lipopolysaccharide confirmed those of Fensom & Gray (1969) and established the presence of dec-2-enoic acid and dodec-2-enoic acid. Small amounts of two additional sugars, arabinose and mannose, were detected. These sugars were separated and identified when preparative paper chromatography was carried out on large-scale hydrolysates of lipopolysaccharide for the isolation and characterization of the heptose. The sugars have not been detected by paper chromatography in small-scale hydrolysates. The amount of pentose determined colorimetrically (1.8%) was presumably attributable to arabinose, since no ribose was detected, although it should be noted that the

Table 4. *Phosphorus/glucosamine molar ratios for fractions* 1–4 *referred to in Fig.* 4

Fractions 1–4 were obtained by chromatography on Bio-Gel P-2 of the water-soluble material from the alkaline methanolysis of lipid A. —, Not determined

Batch	Fraction	Phosphorus/glucosamine molar ratio			
		... 1	2	3	4
1		1.00:0.84	1.00:0.97	—	—
2		1.00:0.95	1.00:0.94	2.00:0.99	1.00:1.20

analytical method used is subject to some interference from other sugars.

The heptose of the lipopolysaccharide has now been isolated and identified as L-glycero-D-manno-heptose. This is the most common heptose found in enterobacterial lipopolysaccharides, and the lipopolysaccharide of *P. aeruginosa* is therefore similar to these in this respect. The thiobarbituric acid-positive component of the lipopolysaccharide has also been isolated and shown to be a 3-deoxy-2-octulonic acid. The presence of the compound in the wall of *P. aeruginosa* was in fact demonstrated during a broad survey of bacterial species (Ellwood, 1970).

The presence of small amounts of spermidine and putrescine in the lipopolysaccharide was established, but the total amount of these was too small to explain the deficiency of nitrogen-containing components reported by Fensom & Gray (1969) in relation to the total nitrogen content of the lipopolysaccharide. This remains a problematical feature of the composition of the lipopolysaccharide. It is not known whether the spermidine and putrescine are genuine components of the lipopolysaccharide or have been absorbed on to the lipopolysaccharide during the extraction procedure. The latter proposal seems feasible because the lipopolysaccharide is anionic, a property that is presumably connected with the large phosphorus content (4.6%) of the material. Spermidine and putrescine were also found in unhydrolysed glycolipid from a heptose-less R mutant of *Salmonella minnesota* (Gmeiner *et al.*, 1969).

This richness in phosphorus and the low sugar content of the lipopolysaccharide, coupled with the more vigorous conditions of acid hydrolysis required to liberate the lipid A moiety remain the most significant differences compared with normal enterobacterial lipopolysaccharides. More recent work in these laboratories (Drewry *et al.*, 1971) has shown that lipid A is cleaved from the lipopolysaccharide of *P. aeruginosa* by 1% (v/v) acetic acid (0.167M) for 60min at 100°C (cf. Fensom & Meadow, 1970) without extensive degradation of the polysaccharide; in the present work we used concentrations of acetic acid up to 0.08M with no success. Release of lipid A was achieved by heating at 100°C with 0.1M-HCl, the optimum yield being obtained after hydrolysis for 30min. These conditions led to the release of large amounts of P_i (45–50% of the total phosphorus of the lipopolysaccharide) and to substantial degradation of the polysaccharide moiety. The hydrolysis procedure in fact resulted in the release of 60–65% of the total 3-deoxy-2-octulonic acid as the free acid before any lipid A was obtained. It is unlikely therefore that this 3-deoxy-2-octulonic acid is involved directly in the linkage between lipid A and the polysaccharide. After full release of lipid A, the remaining 3-deoxy-2-octulonic acid was found to be attached to components of the polysaccharide as a reducible sugar. Since no 3-deoxy-2-octulonic acid was destroyed on treatment of whole lipopolysaccharide with sodium borohydride, this suggests that 35–40% of the sugar acid is involved in linking lipid A to the polysaccharide. These results are similar to those obtained by Osborn (1963). She concluded that about one-quarter of the total 3-deoxy-2-octulonic acid of the lipopolysaccharide of a strain of *E. coli* served as a link between the lipid A and the polysaccharide. More recent work on *Salmonella* indicates that the linkage is provided by a branched trisaccharide of 3-deoxy-2-octulonic acid, of which two residues are in the main chain (Dröge *et al.*, 1970). The lipopolysaccharide of *P. aeruginosa* appears therefore to share the common feature of enterobacterial lipopolysaccharides (see, e.g., Lüderitz, 1970) of possessing a polyheptose phosphate chain linked via 3-deoxy-2-octulonic acid to lipid A, but for unknown reasons, this bridge is rather less readily cleaved in *P. aeruginosa*.

The high-voltage electrophoresis studies of the polysaccharide released on acid hydrolysis of the lipopolysaccharide confirmed that this was substantially degraded. In the fractions containing galactosamine obtained from the degraded polysaccharide, alanine was always found to be present. The approximate 1:1 galactosamine/alanine molar ratio found by Fensom & Gray (1969) for the whole lipopolysaccharide was maintained in these fractions.

It was shown that 40% of the alanine of the lipopolysaccharide is probably involved in amide linkages involving the carboxyl group of the amino acid and none in ester linkages, and cleavage of this alanine did not liberate free galactosamine. These results suggest that the alanine is not part of a chain of alternate alanine and galactosamine residues, but may be present as side chains on galactosamine residues of the main polysaccharide chain. The failure of hydrazinolysis to cleave all the alanine remains unexplained, except possibly in terms of incomplete reaction with the hydrazine. In this context, the report by Hanessian & Haskell (1964) of a staphylococcal polysaccharide containing amide-linked alanine is of note. The investigations of the fractions of degraded polysaccharide also suggested that the fucosamine is not uniformly distributed throughout the polysaccharide in relation to the alanine and galactosamine.

Only traces of glucosamine and amino acids other than alanine were detected in the polysaccharide. The presence of these is most probably explained by contamination with other cell-wall polymers, or in the case of the glucosamine, by some cross-contamination with lipid A. This contrasts with the lipopolysaccharide of *P. aeruginosa* N.C.T.C. 8602 (Fensom & Meadow, 1970), which contains glucosamine in both the lipid and the polysaccharide moieties.

The phosphorus/glucosamine/fatty acids molar ratio for the lipid A preparations was 0.68:1.00:2.56, in reasonable agreement with 0.72:1.00:3.45 reported by Key *et al.* (1970) for the lipid A of *P. alcaligenes*. The molar ratio reported by Fensom & Gray (1969) for the lipid A of *P. aeruginosa* was however, 1.49:1.00:3.45. The higher ratio of glucosamine to fatty acids is explained by the larger amount of the amino sugar detected in the present lipid preparations. The small molar amount of phosphorus in these preparations is, however, mainly due to their greater purity. Fensom & Gray (1969) noted that their lipid A might be contaminated by at least 10% of the polysaccharide, which is rich in phosphorus, whereas the present lipid A contained no more than 3% of polysaccharide. In accordance with this, the amounts of alanine, galactosamine and fucosamine detected in the present lipid A preparations were much smaller than those found by Fensom & Gray (1969).

From the two highest-molecular-weight fractions (1 and 2) obtained by chromatography of solubilized lipid A on Bio-Gel P-2, most of the phosphorus was liberated as P_i by alkaline phosphatase and none by additional use of a phosphodiesterase. Since these fractions contain most of the phosphorus of the whole lipid A, it is concluded that most of the phosphorus of the lipid occurs as phosphomonoester groups or that phosphodiester linkages were broken during alkaline methanolysis. No evidence for phosphodiester linkages was obtained, but they cannot be ruled out. The nature or environment of the groups containing phosphorus in fractions 1 and 2 was apparently different, since all of the phosphorus of fraction 1, but only 66% of that of fraction 2, was liberated by alkaline phosphatase.

As shown by the results in Table 4, the phosphorus/glucosamine molar ratio in fractions 1 and 2 obtained from solubilized lipid A is approx. 1:1, and in fraction 3 the ratio is approx. 2:1. The only fatty acid remaining after solubilization of lipid A was 3-hydroxydodecanoic acid, and for fractions 1 and 2, the ratio of the amount of this acid to glucosamine was the same as for whole lipid A (approx: 1.1). The backbone unit of the lipid A of *P. aeruginosa* is therefore confirmed as being derived from *N*-3-hydroxydodecanoylglucosamine (Fensom & Gray, 1969). Assuming such units are glycosidically linked (Burton & Carter, 1964; Gmeiner *et al.*, 1969; Adams & Singh, 1969, 1970*a*,*b*) the heterogeneity of the solubilized lipid could involve variations in chain length as well as in extent or position of phosphorylation. However, as about half the glucosamine residues in fractions of the solubilized lipid are reducible by sodium borohydride, it seems likely that the lipid is derived from a glucosamine disaccharide.

Attempts to obtain evidence for glycosidic linkages by the isolation of glucosamine oligosaccharides from hydrazinolysates of lipid A were unsuccessful: most of the glucosamine (approx. 80%) was destroyed during hydrazinolysis. Hydrazinolysis of whole lipopolysaccharide was more successful, but oligosaccharides similar to those obtained by Gmeiner *et al.* (1969) could not be identified with certainty in the fractions obtained by electrophoresis of the hydrazinolysed material.

We thank the Medical Research Council for a maintenance grant (I. R. C.) and a grant for the purchase of equipment. We also thank the Microbiological Research Establishment, Porton, Wilts., U.K., for the supply of large quantities of whole cells of *P. aeruginosa*.

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