The Purification and Chemical Composition of the Lipopolysaccharide of *Pseudomonas alcaligenes*

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1. A method for obtaining lipopolysaccharide free from glycosaminopeptide from isolated cell walls of *Pseudomonas alcaligenes* is discussed. 2. About 70–75% of the lipopolysaccharide and 86–90% of the isolated lipid A have been accounted for in terms of identifiable components. 3. Hydrolysates of lipid A contain mainly inorganic phosphate, glucosamine, *O*-phosphorylglucosamine and fatty acids (dodecanoic acid, dodec-2-enoic acid, 3-hydroxydodecanoic acid and 3-hydroxydodecanoic acid), of which the last is the main *N*-acylating acid of the glucosamine backbone. 4. Material corresponding to the polysaccharide moiety of the lipopolysaccharide is extensively degraded. 5. Solubilization of the lipopolysaccharide by using sodium deoxycholate appreciably affects the chemical composition of the material.

Treatment of acetone-dried whole cells of *Pseudomonas aeruginosa* with aqueous phenol yields lipopolysaccharide that is contaminated with nucleic acids (2–7%) (Fensom & Gray, 1969); similar treatment of cell walls isolated from *P. aeruginosa* (Clarke, Gray & Reaveley, 1967c; Fensom & Gray, 1969) and from *P. alcaligenes* (Key, Gray & Wilkinson, 1970) gives lipopolysaccharides containing variable amounts of glycosaminopeptide. The presence of these contaminants complicates interpretation of analytical results obtained in studies of the composition and structure of these lipopolysaccharides.

Methods of purifying the lipopolysaccharide obtained from cell walls of *P. alcaligenes* have now been investigated, and complete removal of the glycosaminopeptide has been achieved by centrifugation of the aqueous phases obtained from the treatments with aqueous phenol. Analysis of the pure lipopolysaccharide has been carried out, and the results are compared with those obtained in earlier studies (Clarke *et al.* 1967c; Fensom & Gray, 1969) of the lipopolysaccharide of *P. aeruginosa*.

MATERIALS AND METHODS

Growth of organism and preparation of cell walls. The organism used was *Pseudomonas alcaligenes*, a laboratory strain (BR 1/2) kindly provided by Reckitt and Sons Ltd., Kingston-upon-Hull, U.K. Cells were grown and cell walls prepared as described previously (Key *et al.* 1970).

Isolation and purification of lipopolysaccharide. Lipopolysaccharide was extracted by treatment of defatted

cell walls with aq. 45% (w/v) phenol. The procedure was that described by Clarke, Gray & Reaveley (1967b), but before dialysis, the aqueous layers from the two extractions were separately centrifuged (20000*g* for 1 h at 4°C) and the supernatant solutions removed from the glycosaminopeptide. Phenol was removed from the solutions by dialysis against ion-depleted water. On freeze-drying of the non-diffusible material, samples of lipopolysaccharide designated AqI and AqII were obtained and shown to be free from glycosaminopeptide by automatic analysis of hydrolysates for amino compounds.

Treatment of lipopolysaccharide with sodium deoxycholate. Samples of freeze-dried lipopolysaccharide (approx. 30 mg) were solubilized by the method of Ribí *et al.* (1966) by treating suspensions in 0.05 M-tris-HCl buffer, pH 8 (5 ml) with aq. 4% (w/v) sodium deoxycholate (5 ml). Immediate dissolution of the lipopolysaccharide occurred. This procedure was applied first to samples of lipopolysaccharide that were contaminated with glycosaminopeptide (Key *et al.* 1970). The solution was centrifuged (20000*g* for 1 h at 4°C) and the supernatant solution withdrawn from the sediment. The sediment was suspended in water and freeze-dried; this material was mainly glycosaminopeptide. The supernatant solution was dialysed for 3 days against 0.05 M-tris-HCl buffer, pH 8, and for 3 days against ion-depleted water. The material in the non-diffusible residue was freeze-dried and found to be free from glycosaminopeptide.

The procedure was then applied to lipopolysaccharide that had been obtained free from glycosaminopeptide by centrifugation of the aqueous phases from the treatment of cell walls with aqueous phenol. The lipopolysaccharide was re-isolated as described above, the centrifugation being omitted.

Isolation of the lipid component of the lipopolysaccharide. Lipopolysaccharide (approx. 50 mg) was hydrolysed by heating for 30 min in a boiling-water bath with 0.1 M-HCl

(3ml) in a sealed tube. Chloroform (3ml) was added to the cooled hydrolysate and the mixture shaken. After low-speed centrifugation, the aqueous layer was removed and the chloroform solution washed twice with ion-depleted water. The combined aqueous solutions were shaken three times with chloroform (5ml in each case), and the chloroform solutions were combined. The aqueous solution was evaporated to dryness in a rotary evaporator and the residual polysaccharide was dried *in vacuo* over P_2O_5 . The chloroform solution was passed through a glass sinter (no. 4 porosity) and the solvent removed from the filtrate under a stream of N_2 . The residual lipid was dried *in vacuo* over P_2O_5 .

Methods of quantitative analysis. Methods used for the determination of phosphorus, nitrogen, anthrone sugar and fatty acids have been given previously (Key *et al.* 1970). Amino acids and amino sugars were identified and determined by using an automatic analyser (Technicon Instruments Co. Ltd., Chertsey, Surrey, U.K.); samples were hydrolysed at 105°C for 4 h with constant-boiling (6.1M) HCl, and the procedure was that described by Clarke, Gray & Reaveley (1967a). 3-Deoxy-2-octulonic acid was determined by the method of Weissbach & Hurwitz (1959), and heptose by the cysteine- H_2SO_4 method (Dische, 1955), in both cases by using modifications and data given by Osborn (1963).

Methods of qualitative analysis. Paper chromatography was carried out with unwashed Whatman no. 1 paper and t.l.c. with plates (thickness 0.5mm) prepared from either aqueous or alkaline (0.05M- Na_2CO_3) slurries of silica gel G (E. Merck A.-G., Darmstadt, Germany). The solvent systems used were: *a*, ethyl acetate-pyridine-water (5:2:5, by vol., upper layer); *b*, butan-1-ol-ethanol-water-aq. NH_3 (sp.gr. 0.88) (40:10:49:1, by vol., upper layer); *c*, chloroform-methanol-water-acetic acid (50:25:4:8, by vol.); *d*, chloroform; *e*, chloroform-methanol-water (65:25:4, by vol.); *f*, di-isopropyl ether-acetic acid (24:1, v/v) followed by light petroleum (b.p. 40–60°C)-diethyl ether-acetic acid (90:10:1, by vol.). High-voltage electrophoresis was carried out with standard apparatus (Shandon Scientific Co. Ltd., London N.W.10, U.K.); Whatman no. 1 paper was used and the buffer was 0.05M- $Na_2B_4O_7$.

Samples of lipopolysaccharide were hydrolysed in screw-capped tubes at 105°C for 4 h with 0.5M-HCl. Hydrolysates were neutralized with Dowex 2 resin (X8; 20–50 mesh; bicarbonate form); the resin was removed by filtration and the hydrolysate evaporated to dryness *in vacuo*. The residue was dissolved in water and the solution de-ionized as described by Lambkin, Ward & Walborg (1966). The eluate was evaporated to dryness *in vacuo*; solutions of the residue were used to establish the complement of neutral sugars by descending paper chromatography (solvent systems *a* and *b*) and high-voltage electrophoresis. Paper chromatograms were developed by the $AgNO_3$ -NaOH method of Trevelyan, Procter & Harrison (1950); paper electrophoretograms (2000 V for 90 min) were developed similarly, but with the modification of Frahn & Mills (1959).

Lipid A preparations were examined by t.l.c. with solvent systems *c*, *d*, *e* and *f*. With systems *c*, *d* or *e*, spots were revealed with the H_2SO_4 spray of Zimiński & Borowski (1966) and with system *f*, with I_2 vapour followed by the H_2SO_4 spray. Spots were also detected with

ninhydrin in butan-1-ol and with the phosphorus reagent of Dittmer & Lester (1965).

Gas-liquid chromatography. (1) Fatty acids. Fatty acids were released from samples of lipopolysaccharide or lipid A (approx. 7mg) by hydrolysis at 105°C for 4 h with constant-boiling (6.1M) HCl (1ml) (Salton, 1953); the hydrolysates were extracted three times with light petroleum (b.p. 40–60°C; 2ml in each case) and the extracts evaporated to dryness in a stream of N_2 . The residual acids were esterified by treatment with an excess of diazomethane in diethyl ether containing methanol (10%, w/v). The solution was slowly evaporated to dryness by warming at 30°C. In some cases, the mixture of free acids was separated into hydroxy acids and non-hydroxy fatty acids by the method of O'Brien & Rouser (1964) before these were converted into the methyl esters. The fatty acids were identified and relative amounts determined by g.l.c. of the methyl esters by using a Perkin-Elmer F11 gas-chromatographic apparatus; both polar (polyethylene glycol succinate at 180°C) and non-polar (Apiezon L at 220°C) columns were used. The relative amounts of the acids were best determined from results obtained with the polar column.

(2) Sugars. Neutral sugars (approx. 1mg) obtained from hydrolysates of polysaccharide material or lipopolysaccharide as described above for paper chromatography or electrophoresis were converted into the *O*-trimethylsilyl derivatives (Sweeley, Bentley, Makita & Wells, 1963) by dissolving them in anhydrous pyridine (1ml) and treating the solution with hexamethyldisilazane (0.2ml) and trimethylchlorosilane (0.1ml). The mixture was shaken vigorously (30s) and left for 5 min; approx. 1 μ l of the solution was injected into the gas-chromatographic apparatus equipped with a column of silicone gum (SE-52) at 160°C.

Determination of *O*- and *N*-acyl groups in lipid A. *O*-Acyl groups were cleaved from lipid A by treatment with 0.25M-KOH in methanol at 37°C for 16 h (Kasai, 1966) and *N*-acyl groups by acid hydrolysis of the residual de-*O*-acylated lipid; liberated esters and acids were identified and relative amounts determined by g.l.c. of the methyl esters. The procedure has been described by Fensom & Gray (1969).

Physical methods. Extinctions of solutions in the u.v. or visible regions were measured manually with a Unicam SP.500 spectrophotometer equipped with 1 cm cells of silica or glass, respectively. I.r. spectra were recorded with a Unicam SP.100 spectrophotometer equipped with grating and fore-prism (NaCl); samples (1mg) were dispersed in anhydrous KCl (approx. 100mg) and examined as pressed discs.

Lipopolysaccharide was examined by electron microscopy with a Siemens Elmiskop 1A instrument. Samples (approx. 1mg) were suspended in ion-depleted water (10ml); a drop of suspension was transferred to a carbon film and water removed by evaporation.

RESULTS

Purification and yields of lipopolysaccharide. The two aqueous extracts from the treatment of cell-wall batch B4 (Key *et al.* 1970) of *P. alcaligenes* with aqueous phenol were centrifuged; the sediments

Table 1. *Quantitative results for the amino compounds of the purified lipopolysaccharides (AqI and AqII) of cell-wall batch B4 (Key et al. 1970) of P. alcaligenes*

Values for amino acids relate to amino acid residues; other values relate to the free compounds. Sample AqI was hydrolysed for 4 h at 105°C with constant-boiling (6.1M) HCl, conditions giving maximum release and minimum destruction of amino sugars. Sample AqII was similarly hydrolysed, but for 16 h. Results are not corrected for destruction or slow release.

Lipopolysaccharide...	Amount (% of sample, w/w)	
	AqI	AqII
O-Phosphorylglucosamine*	1.48	0.26
Alanine	1.79	1.78
Glucosamine	5.32	4.65
Galactosamine	3.67	3.04
Muramic acid	Absent	Absent
2,6-Diaminopimelic acid	Absent	Absent
Ammonia	0.95	1.02
Other amino acids	0.69	0.77
Total	13.9	11.52

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

were isolated, hydrolysed at 105°C with constant-boiling (6.1M) hydrochloric acid for 4 h, and the hydrolysates autoanalysed. The amounts of muramic acid and 2,6-diaminopimelic acid confirmed that the materials were largely glycosaminopeptide, and the absence of galactosamine showed that losses of lipopolysaccharide were not involved. The lipopolysaccharides (AqI and AqII) obtained from the supernatant solutions were also hydrolysed and autoanalysed with the results given in Table 1; the absence of muramic acid and 2,6-diaminopimelic acid shows that glycosaminopeptide had been successfully removed. The yields of AqI and AqII were 15.0% and 4.8% respectively, agreeing well with the yields reported by Key *et al.* (1970) for corresponding fractions containing variable amounts of glycosaminopeptide.

Composition of the purified lipopolysaccharide. The following results relate to fraction AqI. The lipopolysaccharide had a high phosphorus content (6.7%), and 95% of the nitrogen content (2.2%) was accounted for in terms of ammonia and ninhydrin-positive compounds (Table 1). These compounds represented 13.9% of the lipopolysaccharide, and again, fucosamine was not detected (Key *et al.* 1970).

The hexose content (14.5%) of the lipopolysaccharide was determined by the anthrone method. Paper chromatography of neutralized hydrolysates showed that glucose and rhamnose were the pre-

Table 2. *Relative amounts of fatty acids in the purified lipopolysaccharide (AqI) and lipid A*

Samples were hydrolysed at 105°C for 4 h with constant-boiling (6.1M) HCl. The isolated acids were converted into methyl esters and examined by g.l.c. Quantitative g.l.c. results were most readily achieved from columns of polyethylene glycol succinate at 180°C, but similar results were given by columns of Apiezon L at 220°C.

	Fatty acid composition (%, w/w)	
	AqI	Lipid A
Dodec-2-enoic acid	10.5	8.9
Dodecanoic acid	35.0	39.6
3-Hydroxydecanoic acid	20.3	18.9
3-Hydroxydodecanoic acid	34.2	32.6

dominant monosaccharides, but a faint spot corresponding to mannose was also detected. The presence of these three monosaccharides was confirmed by high-voltage electrophoresis of neutralized hydrolysates and by g.l.c. of the *O*-trimethylsilyl derivatives of the neutral sugars obtained by hydrolysis. No evidence for heptose was given by paper chromatography, but an unidentified peak on gas-liquid chromatograms and an unidentified spot ($R_{\text{glucose}} 0.79$) on paper electrophoretograms may be associated with heptose.

The heptose content (6.8%) of the lipopolysaccharide was determined by the Dische (1955) method, data obtained by Osborn (1963) with a sample of L-glycero-D-mannoheptose being used. 3-Deoxy-2-octulonic acid (5.7%) was determined by the thiobarbituric acid reaction.

The fatty acids released from the lipopolysaccharide on hydrolysis were identified and relative amounts determined by g.l.c. of the methyl esters; the results are in the first column of Table 2. The colorimetric method of Itaya & Ui (1965) was used to measure the colour yields for hexadecanoic acid and the four acids listed in Table 2. The method gave the same extinction (1.316 in a 1 cm cell) with 1 μmol of hexadecanoic acid, 0.94 μmol of dodec-2-enoic acid, 1.03 μmol of dodecanoic acid, 0.99 μmol of 3-hydroxydodecanoic acid or 1.29 μmol of 3-hydroxydecanoic acid. From these results and the relative amounts of the acids in Table 2, the extinction measured on application of the method to the mixture of acids obtained from the lipopolysaccharide was used to calculate the total fatty acid content of the lipopolysaccharide. The result was 16.3%; this compares with 19.8% if the result is expressed in terms of hexadecanoic acid, which has a higher molecular weight than any of the constituent acids.

The acids obtained by hydrolysis of the lipopolysaccharide were also fractionated on a column of

silicic acid (O'Brien & Rouser, 1964); and the components of the fractions were identified by g.l.c. of the methyl esters. The hydroxy acid fraction contained 3-hydroxydodecanoic acid and 3-hydroxydodecanoic acid, and the fatty acid fraction contained dodec-2-enoic acid and dodecanoic acid. The results for the various analyses carried out on the purified lipopolysaccharide were: fatty acids, 16.3%; phosphorus (expressed as orthophosphoric acid), 21.2%; ninhydrin-positive components, 13.9%; hexose (anthrone sugar), 14.5%; heptose, 6.8%; 3-deoxy-2-octulonic acid, 5.7%. Bearing in mind that these analyses involve some degree of overlap, the overall weight recovery for the lipopolysaccharide is approx. 70–75%.

The i.r. spectrum of the purified lipopolysaccharide had no unusual features and closely resembled that of the lipopolysaccharide of *P. aeruginosa*. Electron micrographs of the lipopolysaccharide showed that it was homogeneous, and even at low magnification ($\times 24000$), the fibrous structure of the material was clearly visible.

Treatment of lipopolysaccharide with sodium deoxycholate. Solubilization of crude lipopolysaccharide by treatment with aqueous sodium deoxycholate in tris-HCl buffer, pH 8, and centrifugation of the solution made possible removal of contaminating glycosaminopeptide. However, when pure lipopolysaccharide was similarly treated with sodium deoxycholate and the material was reisolated, significant changes in composition were found to have occurred. As the results in Table 3 show, all analytical results with the exception of that for nitrogen were lower for the sodium deoxycholate-treated material. Although contamination of the recovered lipopolysaccharide by deoxycholic acid was shown not to be greater than 3%, the increase in nitrogen content could be explained by the presence of approx. 7% of tris buffer, although this seems unlikely after the prolonged dialysis, and none was detected by autoanalysis. However even if it is assumed that a total of 10% of contaminating material may be present, the large decreases in the percentages of fatty acids and amino compounds recorded in Table 3 are not explained.

Lipid A. Hydrolysis of purified lipopolysaccharide liberated lipid A, which was isolated as a light-brown grease (P content 2.00%) in yields of 27–35%. The preparations were examined by t.l.c. with several solvent systems including system *e* used by Kasai (1966). The main lipid components moved only a short distance in the form of a streak, and the results suggested that the lipid was fairly homogeneous. Some very minor, faster moving components were detected, one corresponding to non-hydroxy fatty acids. The i.r. spectrum of lipid A was closely similar to that of lipid A of *P.*

Table 3. *Analytical results obtained before and after treatment of purified lipopolysaccharide with sodium deoxycholate*

For the analysis of the ninhydrin-positive components listed below, the conditions of hydrolysis were 4 h at 105°C with constant-boiling (6.1 M) HCl.

	Amount (% of sample, w/w)	
	Purified AqI	Treated AqI
Phosphorus	6.7	5.6
Nitrogen	2.2	2.9
Hexose	14.5	13.9
3-Deoxy-2-octulonic acid	5.7	4.7
Glucosamine	5.32	3.96
Galactosamine	3.67	2.43
O-Phosphorylglucosamine	1.48	1.3
Alanine	1.79	1.52
Ammonia	0.95	1.0
Fatty acids (as hexadecanoic acid)	19.8	11.0

aeruginosa (Clarke, Gray & Reaveley, 1965; Fensom & Gray, 1969).

The lipid preparations contained approx. 2.2% of hexose, indicating some contamination with polysaccharide or unhydrolysed lipopolysaccharide. This was confirmed by autoanalysis of hydrolysates of lipid A for ninhydrin-positive components. Small amounts of galactosamine and alanine, considered to be components of the polysaccharide moiety, were detected. Small amounts of other amino acids were also present and may arise through either contamination by protein or the presence of a peptide unit in the lipid, as reported by Nowotny (1961) and Nowotny, Nowotny & Brigham (1968) for certain species of *Salmonella*. The main components in the hydrolysates were, however, glucosamine and O-phosphorylglucosamine, representing a total glucosamine content of 16.2%. The results of autoanalysis are given in Table 4.

The fatty acids released from lipid A by hydrolysis were identified and relative amounts determined by g.l.c. of the methyl esters. The results are in the second column of Table 2, and agree well with those for the lipopolysaccharide. The relative amounts of 3-hydroxydodecanoic acid and dodec-2-enoic acid varied from one determination to another, but the total amount was nearly constant. The unsaturated acid may therefore be an artifact associated with the hydroxy acid.

The colorimetric method of Itaya & Ui (1965) was used to determine the fatty acid content of lipid A. The results were again calculated by using the relative amounts of the four acids in Table 2 and the extinctions/ μmol of the individual acids. The total fatty acid content of lipid A was in the

Table 4. *Quantitative analyses for the amino compounds of lipid A*

Values for amino acids relate to amino acid residues; other values relate to the free compounds. Results were obtained by autoanalysis after hydrolysis for 4 h at 105°C with constant-boiling (6.1 M) HCl; no other amino compounds in amounts exceeding 0.15% were detected.

	Amount (% of sample, w/w)
<i>O</i> -Phosphorylglucosamine*	5.5
Aspartic acid	0.25
Serine	0.51
Glutamic acid	0.22
Glycine	0.26
Alanine	0.46
Glucosamine	12.4
Galactosamine	0.52
Total	20.12

* The relative colour yield for this component was calculated by using glucosamine 6-phosphate as standard.

range 59–63%. If the results were expressed in terms of hexadecanoic acid, the fatty acid content was considerably overestimated and was in the range 74–78%.

Selective cleavage of *O*- and *N*-acyl groups in lipid A established that the *O*-acylating acids were dodecanoic acid and 3-hydroxydodecanoic acid; very little dodec-2-enoic acid was detected in these acids. The *N*-acylating acids were 3-hydroxydodecanoic acid and most of the dodec-2-enoic acid. This supports the view that the unsaturated acid may be an artifact from the hydroxy acid. The apparent absence of dec-2-enoic acid, a minor component detected in hydrolysates of the lipid and the lipopolysaccharide of *P. aeruginosa* (Fensom & Gray, 1969), is noted. It is possible that this acid is produced in small amounts from the less abundant of the two hydroxy acids of the lipopolysaccharide, and that a combination of its volatility and the small scale on which the experiments were carried out has led to a failure to detect this component.

The overall weight recovery for lipid A was: fatty acids, 59–63%; phosphorus (expressed as orthophosphoric acid), 6.3%; glucosamine (including that detected as *O*-phosphorylglucosamine), 16.2%; hexose, 2.2%; other amino compounds and ammonia, 2.5%; total, 86.2–90.2%.

Hydrolysis of lipid A. Hydrolysis of lipid A with *m*- or 2*M*-hydrochloric acid for 3 h at 105°C liberated 43% of the phosphorus as P_i . Hydrolysis with 2*M*-sodium hydroxide under the same conditions released 66% of the phosphorus as P_i , and hydrolysis with *m*-sodium hydroxide for 2 min at 100°C liberated 24% of the total phosphorus as P_i . When the former alkaline hydrolysate was neutralized, evaporated to dryness and the residue hydro-

lysed with 2*M*-hydrochloric acid for 3 h at 105°C, no further inorganic phosphate was released.

Polysaccharide moiety. The water-soluble components obtained after extraction of lipid A from 0.1*M*-hydrochloric acid hydrolysates of lipopolysaccharide were examined. A portion of the acidic solution was repeatedly evaporated to dryness at low temperatures to remove the acid. The residue contained 17% of anthrone sugar; together with the hexose in lipid A, the recovery of anthrone sugar was 80% of that of the whole lipopolysaccharide. The phosphorus content was 7.4%, 54% of this being P_i . Hydrolysis of the material with constant-boiling (6.1*M*) hydrochloric acid followed by autoanalysis showed that galactosamine (4.1%) and alanine (2.3%) were present; no glucosamine was detected. Between 80 and 90% of the galactosamine and alanine of the whole lipopolysaccharide were recovered in this polysaccharide material. The molar ratio of galactosamine to alanine was 1.2:1.0 compared with 1.0:1.0 in the lipopolysaccharide.

The remainder of the acidic solution containing material from the polysaccharide moiety was treated with an anion-exchange resin, and the unadsorbed material was isolated by evaporation of the solution at low temperature. The material contained only 10.8% of anthrone sugar, showing that sugar phosphates were probably removed by the anion-exchange resin. Paper chromatography and paper electrophoresis of solutions of the material showed that glucose, rhamnose and small amounts of mannose were present as free monosaccharides; the predominant monosaccharide was rhamnose. Paper electrophoresis again revealed the unidentified spot (R_{glucose} 0.79), possibly neptose. No free amino sugars were detected.

The material from the neutral solution was further hydrolysed with 0.5*M*-hydrochloric acid for periods of 1.5, 4 and 16 h. Paper chromatography and paper electrophoresis showed that the same components were present, but that rhamnose was not now the predominant monosaccharide. The unidentified spot (R_{glucose} 0.79), detected on paper electrophoretograms with silver nitrate, increased in intensity with length of hydrolysis, and was the most intense spot after hydrolysis for 16 h.

DISCUSSION

The extraction of lipopolysaccharide from cell walls of *P. alcaligenes* (Key *et al.* 1970) and *P. aeruginosa* (Clarke *et al.* 1967c; Fensom & Gray, 1969) has the advantage that the product is not contaminated by nucleic acids, but the disadvantage that contamination by glycosaminopeptide is variable. This difficulty has been overcome by high-speed centrifugation of the aqueous phases obtained

direct from the phenol-extraction procedure. The glycosaminopeptide sediments readily, and the dissolved lipopolysaccharide can be isolated in the usual way. It should be noted that if lipopolysaccharide contaminated by glycosaminopeptide is first isolated and freeze-dried, the solubility characteristics of the lipopolysaccharide are so altered that redissolving and centrifugation do not effect a separation from glycosaminopeptide.

Treatment with solutions of sodium deoxycholate overcomes the diminished solubility of freeze-dried lipopolysaccharide, and centrifugation of the solutions again gives ready sedimentation and separation of contaminating glycosaminopeptide. However, this method markedly affects the chemical composition of the lipopolysaccharide, notably the content of amino sugars and fatty acids.

Analysis of the purified lipopolysaccharide of *P. alcaligenes* has revealed points of similarity and difference compared with the lipopolysaccharide of *P. aeruginosa* (Clarke *et al.* 1967c; Fensom & Gray, 1969). Hydrolysates of both lipopolysaccharides contain glucosamine, *O*-phosphorylglucosamine, galactosamine, alanine, glucose, rhamnose, heptose, 3-deoxy-2-octulonic acid and fatty acids as the major identifiable components. Fucosamine is not, however, a component of the lipopolysaccharide of *P. alcaligenes*, which also contains a simpler range of fatty acids in the lipid moiety. In both cases, 3-hydroxydodecanoic acid is the main *N*-acylating acid in the lipid, but for *P. alcaligenes*, only three other acids were detected, i.e. dodecanoic acid, dodec-2-enoic acid and 3-hydroxydecanoic acid. Additional fatty acids in the lipopolysaccharide of *P. aeruginosa* were 2-hydroxydodecanoic acid, hexadecanoic acid and traces of dec-2-enoic acid. *P. alcaligenes* therefore provides a further example of a lipopolysaccharide having a lipid moiety in which hydroxy acids are major components, but in which there is no 3-hydroxytetradecanoic acid, a characteristic component of lipid A moieties of several enterobacterial lipopolysaccharides.

Comparison of quantitative results for the two lipopolysaccharides shows that they are broadly similar in their contents of ninhydrin-positive components, fatty acids and sugars. It should be noted that the heptose content of the lipopolysaccharide of *P. aeruginosa* quoted by Fensom & Gray (1969) was in error by a factor of two, and should be 3.2–3.6%. The lipopolysaccharide of *P. alcaligenes* therefore contains almost twice as much heptose and 3-deoxy-2-octulonic acid, and moreover, it is significantly richer in phosphorus (6.7%). This is notable, because the phosphorus content ($4.3 \pm 0.8\%$) of the lipopolysaccharide of *P. aeruginosa* is already high compared with the lipopolysaccharides of many Gram-negative organisms. A further point is that almost all the nitrogen

of the lipopolysaccharide of *P. alcaligenes* is accountable for by identifiable ninhydrin-positive components and ammonia released on hydrolysis. In the case of *P. aeruginosa*, the recovery of nitrogen in terms of these components was low, approx. 70%, allowing for contaminating nucleic acids (Fensom & Gray, 1969). The presence of unidentified nitrogenous components, probably associated with the polysaccharide moiety, seemed therefore possible (Fensom & Gray, 1969). No such components appear to be present in the lipopolysaccharide of *P. alcaligenes*.

Although the two lipopolysaccharides are broadly similar, it is apparent that the lipopolysaccharide of *P. alcaligenes*, with its higher phosphorus content, is considerably simpler in overall qualitative composition.

Both lipopolysaccharides yield comparable amounts of lipid A on acid hydrolysis. For lipid A of *P. aeruginosa*, the fatty acid content was determined by weight (52%), and 79% of the lipid was accounted for by fatty acids, phosphorus (as orthophosphoric acid), amino compounds and glucose. For the lipid of *P. alcaligenes*, the weight recovery on a similar basis was 86–90%. The molar ratios of identifiable components of the lipid A preparations are also similar and are given in Table 5.

The molar ratio of total fatty acids to glucosamine (3.45:1) is the same for both lipids, for each of which 3-hydroxydodecanoic acid is the main *N*-acylating acid. If dodec-2-enoic acid is an artifact derived from this hydroxy acid, the molar ratios of *N*-acylating acids to glucosamine are greater than unity in both cases, the discrepancy being larger (1.61:1) in *P. aeruginosa* than in *P. alcaligenes* (1.14:1). However, since some glucosamine must be destroyed during hydrolysis of lipid A, it may be that the ratios obtained are the best approximations to unity that can be expected within the limits of the methods used.

Fensom & Gray (1969) suggested that the true molar proportion of phosphorus in the lipid A of *P. aeruginosa* might be less than 1.30 (see Table 5), because of contamination of the lipid by polysaccharide material. Now the lipopolysaccharide of *P. alcaligenes* contains more phosphorus and the lipid contains less phosphorus than the corresponding materials from *P. aeruginosa*. The polysaccharide of *P. alcaligenes* must therefore be the richer in phosphorus. Since the lipid A of *P. alcaligenes* in fact contained more hexose, the contribution to the phosphorus content stemming from polysaccharide contamination may be even greater than that for *P. aeruginosa*. It is possible therefore that the difference in the molar proportions of phosphorus for the two lipids is even larger than the factor of about two indicated in Table 5.

Table 5. *Molar ratios of the components of lipid A preparations from P. aeruginosa and P. alcaligenes*

The identities of the components listed by Fensom & Gray (1969) as X and Y have now been confirmed as dec-2-enoic acid and dodec-2-enoic acid, respectively.

	Molar ratio	
	<i>P. aeruginosa</i> (Fensom & Gray, 1969)	<i>P. alcaligenes</i> (Present work)
Phosphorus	1.30	0.63
Glucosamine	0.87	0.87
Dodecanoic acid	0.37	1.20
Hexadecanoic acid	0.24	Absent
3-Hydroxydodecanoic acid	0.36	0.61
2-Hydroxydodecanoic acid	0.62	Absent
3-Hydroxydodecanoic acid	1.03	0.92
Dec-2-enoic acid	0.01	Absent
Dodec-2-enoic acid	0.37	0.27

The results show that both lipids consist largely of *N*-3-hydroxydodecanoylglucosamine residues, each residue carrying on average approx. two other fatty acids as *O*-acyl groups. For the lipid of *P. aeruginosa*, it was argued that, if the molar ratio of phosphorus to glucosamine was considered as unity and the phosphorus was present as phosphomonoester groups, insufficient hydroxyl groups remained per glucosamine residue to link these together glycosidically (Fensom & Gray, 1969). However, for *P. alcaligenes*, the glucosamine/phosphorus ratio is high and may be nearer 2:1, if allowance is made for contaminating polysaccharide. At least partial linkage of the structure through glycosidic linkages would therefore be possible, as in other lipopolysaccharides (Burton & Carter, 1964; Gmeiner, Lüderitz & Westphal, 1969; Malchow, Lüderitz, Kickhöfen, Westphal & Gerisch, 1969; Adams & Singh, 1969, 1970a,b).

The occurrence of phosphodiester bridges in the backbone of lipid A was suggested by Nowotny (1961), but has not yet been proved. However, phosphomonoester groups occurred in the 1- and/or 4'(?) positions of units of glucosaminy- β -1,6-glucosamine from the lipid A of *Salmonella minnesota* (Gmeiner *et al.* 1969), and some of the 6-positions in the glucosamine oligosaccharides (β -1,4-linked) from the lipid A of *Escherichia coli* and *Shigella flexneri* appeared to be similarly substituted (Adams & Singh, 1970b). Our attempts to elucidate the nature of the phosphorus in the lipid A of *P. alcaligenes* also indicate a heterogeneous pattern of substitution. Thus, two-thirds of the phosphorus is labile to alkali, two-thirds of the alkali-labile phosphorus is also labile to acid, and one-third

of the total phosphorus is stable to both acid and alkali. The significance of these results is not yet clear, although acid lability (and alkali stability) is characteristic of glucosamine 1-phosphate (Ramsey & Pizanis, 1965), and alkali-lability may indicate the operation of a β -elimination process (Brown, Hayes & Todd, 1957).

As for the lipopolysaccharide of *P. aeruginosa* (Fensom & Gray, 1969) difficulties were experienced in releasing the polysaccharide moiety from the lipopolysaccharide of *P. alcaligenes*. The material isolated after removal of lipid A from hydrolysates by extraction with chloroform was extensively degraded, as shown by the presence of free monosaccharides and large amounts of P_1 . This observation again focuses attention on the lability of phosphorus, in this case on a considerable proportion of that of the whole lipopolysaccharide. In this connection it is noted that the lipopolysaccharides of *P. aeruginosa* and particularly *P. alcaligenes* have very high phosphorus contents compared with lipopolysaccharides of the Enterobacteriaceae. Moreover, pseudomonads giving phosphorus-rich lipopolysaccharides are particularly sensitive to the antibacterial action of EDTA, and during the toxic process, large quantities of lipopolysaccharide are released into solution (Gray & Wilkinson, 1965; Rogers, Gilleland & Eagon, 1969; Key *et al.* 1970). The possible involvement of phosphate groups in binding the lipopolysaccharide to other cell-wall components, probably through metal ions, provides a further reason for seeking a fuller understanding of the nature of the phosphorus in such lipopolysaccharides. Progress in this direction and in determining the structure of the polysaccharide moieties of the lipopolysaccharides of *P. aeruginosa* and *P. alcaligenes* must, however, await the finding of conditions for the release of the polysaccharides with minimal degradation.

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