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Studies of Lipid A Fractions from the Lipopolysaccharides of *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes*

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Lipid A fractions from *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes* have similar compositions and structural features. By means of hydrazinolysis of the parent lipopolysaccharides and partial hydrolysis of the deacylation products, it was established that both lipids are derived from the β-(1→6)-linked disaccharide of glucosamine. Phosphorylated derivatives of the disaccharide from *Ps. aeruginosa* were also characterized. The lipids differ mainly in the absence of hexadecanoic acid and 2-hydroxydodecanoic acid from the lipid from *Ps. alcaligenes*. Evidence that in *Ps. aeruginosa* these acids are ester-linked to residues of 3-hydroxyalkanoic acids (including 3-hydroxydecanoic acid) was obtained. Heterogeneity of lipid A fractions was indicated by t.l.c., and by gel filtration of de-*O*-acylation products from mild alkaline methanolysis of the lipids.

Although lipopolysaccharides from different Gram-negative bacteria seem to have basically similar architectures, the polysaccharide fractions differ considerably in composition and structure (Lüderitz *et al.*, 1971). By contrast, the lipid A fractions are generally very similar (Rietschel *et al.*, 1972). With a few exceptions so far reported (Adams *et al.*, 1968, 1969, 1970; Wilkinson *et al.*, 1973), the lipids are derived from a di- or poly-glucosamine backbone to which phosphate (ester-linked) and fatty acid (ester- and amide-linked) residues are attached. The fatty acid components characteristically include 3-hydroxyalkanoic acids. In the lipid A of *Salmonella*, the backbone is the β-(1→6)-linked disaccharide of D-glucosamine, and carries phosphate residues in the 4- and 1′-positions (Gmeiner *et al.*, 1969, 1971). The possibility that such units are joined by phosphodiester or pyrophosphate bridges to give more complex structures cannot yet be excluded (Gmeiner *et al.*, 1969; Rietschel *et al.*, 1972). Evidence for different glycosidic linkages and higher oligosaccharides of glucosamine in lipid A fractions from bacteria in other genera has also been obtained (Adams & Singh, 1969, 1970*a*,*b*; Adams, 1971).

Although lipid A fractions have been isolated from a wide variety of bacteria, structural studies have almost entirely been confined to enterobacterial lipopolysaccharides. In previous studies of lipopolysaccharides from *Pseudomonas aeruginosa* (Fensom & Gray, 1969; Chester *et al.*, 1972) and *Pseudomonas alcaligenes* (Key *et al.*, 1970), the compositions of the lipid A fractions were determined, but the only firm structural conclusion was that both lipids were derived from *N*-3-hydroxydodecanoylglucosamine. The present paper describes the results of further studies of these lipids.

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.). β-*N*-Acetylglucosaminidase (EC 3.2.1.30) from rat epididymis and α- and β-*N*-acetylglucosaminidases from *Patella vulgata* were gifts from Dr. O. Lüderitz, Max Planck Institut für Immunbiologie, Freiburg, West Germany. Chitinase (EC 3.2.1.14) was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. *NN′*-Diacetylchitobiose was prepared by treatment of chitin, dispersed in 0.03M-ammonium acetate buffer (pH 5.8), with chitinase for 9h at 37°C, followed by preparative paper chromatography (solvent system E). Chitobiose and higher oligosaccharides were prepared by partial acidic hydrolysis of chitosan (Horowitz *et al.*, 1957) and separation of the products by preparative paper chromatography (solvent system C). Glucosaminol hydrochloride was prepared by borohydride reduction (Crimmin, 1957) of *N*-acetylglucosamine, hydrolysis (4M-HCl for 2h under reflux) of the reduction product, and crystallization from aqueous methanol.

Methods

Growth of bacteria and preparation of cell walls. Cells of *Ps. aeruginosa* (N.C.T.C. 1999) and *Ps. alcaligenes* (laboratory strain BR 1/2) were grown at

37°C in Nutrient Broth no. 2 (Oxoid Ltd., London E.C.4, U.K.) for 16h and 24h respectively, and the cell walls were isolated and purified as described previously (Key *et al.*, 1970; Drewry *et al.*, 1971).

Preparation of lipopolysaccharide and lipid A. Lipopolysaccharide was extracted from defatted cell walls by treatment with hot aqueous phenol as described by Key *et al.* (1970). To cleave lipopolysaccharides into lipid A and polysaccharide fractions, they were hydrolysed with 1% acetic acid at 100°C for 1–1½h. The hydrolysates were mixed with chloroform to dissolve precipitated lipid A, and the chloroform extracts washed with water, filtered (glass sinter no. 4 porosity), and dried by rotary evaporation. The residues of lipid A were further dried *in vacuo* over P_2O_5.

Analytical methods. Total phosphorus was determined by the method of Bartlett (1959) and P_i by the method of Parvin & Smith (1969). Amino compounds were determined by using an automatic amino acid analyser (Technicon Instruments Co. Ltd., Basingstoke, Hants., U.K.) after hydrolysis of samples with 6.1M-HCl at 105°C for 4h. Total fatty acids (expressed as hexadecanoic acid) were determined by the method of Itaya & Ui (1965) after hydrolysis under the same conditions. Fatty acid compositions were determined by quantitative g.l.c., by using a precision integrator (Honeywell Ltd., Brentford, Middlesex, U.K.).

Deacylation of lipopolysaccharide by hydrazinolysis. Fatty acid residues (ester- and amide-linked) were removed from lipopolysaccharide by treatment with anhydrous hydrazine at 100°C for 10h (Gmeiner *et al.*, 1969). Hydrazine was removed by evaporation and by drying the residue *in vacuo* over conc. H_2SO_4. Water-soluble products were isolated by dispersing the residue in water, neutralizing with 0.1M-HCl, extracting fatty acid hydrazides with chloroform, and filtering off insoluble products (glass sinter no. 4 porosity). Low-molecular-weight solutes were removed by ultrafiltration or Sephadex chromatography, and the high-molecular-weight solutes were freeze-dried. This material was hydrolysed (0.1M-HCl at 100°C for 30min, or 1% acetic acid at 100°C for 70min), and the hydrolysate examined for products derived from lipid A.

De-O-acylation of lipid A by mild alkaline methanolysis. The following treatment was used for the solubilization of lipid A from *Ps. alcaligenes*. Lipid A (about 20mg) was dissolved in chloroform (6ml) and 0.2M-KOH in methanol (6ml) was added. After incubation of the solution at 37°C for up to 80min, chloroform (6ml) and ethyl formate (40μl) were added, and incubation was continued for 5min. Water-soluble products were isolated by mixing the solution with deionized water (4.5ml), separating the two phases by centrifugation, and collecting the upper phase. The lower phase was washed (three times) with fresh upper-phase solvent, and the washings were combined with the original upper phase, which was then freeze-dried. Mild alkaline methanolysis of the lipids from both pseudomonads was also carried out by the method of Rietschel *et al.* (1972).

Fractionation by molecular size. Ultrafiltration was carried out in a cell (Amicon, High Wycombe, Bucks., U.K.) fitted with a UM-05 membrane (cut-off at mol.wt. about 500) and a magnetic stirrer.

Gel filtration was done by using various grades (G-15, G-25, G-50) of Sephadex (Pharmacia, Uppsala, Sweden). Elution was normally carried out with pyridine–acetic acid buffer (pH5.4) (Schmidt *et al.*, 1969); 20% (v/v) methanol in water was used for chromatography of de-*O*-acylated lipid A on Sephadex G-25. Fractions of eluate were analysed, as necessary, for phosphorus and total carbohydrate (Dubois *et al.*, 1956).

Thin-layer chromatography. Samples of lipid A were examined by t.l.c. on layers prepared from a slurry of Bio-Sil A (grain size 2–10μm, without binder, Bio-Rad Laboratories, Richmond, Calif., U.S.A.) in 0.05M-sodium phosphate buffer (pH7.8) (B. A. Key, personal communication) with solvent system A [chloroform–methanol–water (65:25:4, by vol.)] or B [chloroform–methanol–water (65:16:2, by vol.)]. Chromatograms were sprayed with the reagent of Dittmer & Lester (1964) and then heated at 200°C, or were treated with Cl_2 and then sprayed with starch–KI reagent (Roberts, 1966).

Separations of fatty esters derived from acidic hydrolysates of lipids were carried out on plates of silica gel G (E. Merck A.-G., Darmstadt, Germany) by using dichloromethane as solvent and the reagent of Zimiński & Borowski (1966) for detection of spots.

N-Acetylglucosamine and *N*-acetylated glucosamine disaccharides were also separated on silica gel G with solvent systems C [ethyl acetate–pyridine–water–acetic acid (5:5:3:1, by vol.)] and D [propan-1-ol–water (7:1, v/v)]. Spots were again detected with the reagent of Zimiński & Borowski (1966).

Paper chromatography and electrophoresis. Paper chromatography and high-voltage electrophoresis were carried out with Whatman papers (no. 1 for analytical separations and washed no. 1 or 3MM for preparative separations). Solvent systems used were: C, as described above; E, butan-1-ol–acetic acid–water (3:1:1, by vol.); F, ethyl acetate–pyridine–water (5:2:5, by vol., upper layer). Paper electrophoresis was carried out with pyridine–acetic acid–water (5:2:43, by vol.; pH5.3) as the buffer system. Spots were detected with ninhydrin, the reagent of Hanes & Isherwood (1949) for phosphates, and the reagents of Trevelyan *et al.* (1950) for reducing compounds.

Gas–liquid chromatography. Fatty acids released by acidic hydrolysis of samples were esterified with diazomethane in diethyl ether containing methanol

(10%, v/v). The esters were identified and determined by g.l.c. on columns of polydiethyleneglycol succinate (at 150° or 165°C) and Apiezon L (at 200° or 220°C) with a Perkin–Elmer F11 gas chromatograph.

Enzymic hydrolyses. Solutions of phosphate esters in 0.05M-$(NH_4)_2CO_3$ (pH 9.6) were covered by toluene and incubated with alkaline phosphatase for 16h at 37°C. *N*-Acetylated glucosamine disaccharides were dissolved in a buffer (pH 4.3) containing sodium citrate and NaCl (both 0.04M) and an *N*-acetylglucosaminidase; the solutions were covered by toluene and incubated for 48h at 37°C.

Miscellaneous methods. Selective *N*-acetylation was carried out by the method of Strominger *et al.* (1959). The Morgan–Elson reaction, as described by Strominger *et al.* (1959), was applied to *N*-acetylated glucosamine disaccharides: the reaction was repeated after acidic hydrolysis (6.1M-HCl at 105°C for 4h) of the compounds and *N*-acetylation of the glucosamine liberated. Ninhydrin degradation of free glucosamine disaccharide was carried out by the method of Stoffyn (1959). The disaccharide and related compounds were reduced by treatment with 2% (w/v) $NaBH_4$ for 24h at room temperature. Excess of reducing agent was destroyed by the addition of dilute acetic acid, and boric acid was removed by distillation with methanol. Periodate oxidations were carried out with 0.05M-$NaIO_4$ for 2 to 6 days at 4°C in the dark. Mass spectra were obtained with an AEI MS902 spectrometer.

Results

Composition of lipid A fractions

The lipopolysaccharides isolated from cell walls of both species closely resembled those previously obtained from whole cells (*Ps. aeruginosa*; Chester *et al.*, 1972) or cell walls (*Ps. alcaligenes*; Key *et al.*, 1970). To minimize hydrolysis or migration of phosphate and fatty acid residues lipid A fractions were released by mild hydrolysis of the lipopolysaccharides with 1% acetic acid at 100°C for 1–1½h (Fensom & Meadow, 1970; Drewry *et al.*, 1971).

The lipid A fractions were obtained as white waxy solids, insoluble in water but soluble in chloroform. The yields were about 26% (*Ps. aeruginosa*) and about 34% (*Ps. alcaligenes*), compared with previous values of 24.5–31.2% and 27–35%, respectively (Chester *et al.*, 1972; Key *et al.*, 1970). Analyses of the lipids are given in Tables 1 and 2. Agreement with previous results is generally satisfactory, although the low content of 3-hydroxydecanoic acid for the new lipid A from *Ps. alcaligenes* is uncharacteristic (other recent batches gave values of about 17% for this acid). Neither lipid A was contaminated to a significant extent by components of the polysaccharide fraction. Apart from the absence of hexadecanoic acid and 2-hydroxydodecanoic acid from *Ps. alcaligenes*, the two lipids had closely similar compositions. The molar ratio glucosamine:phosphorus was about 1.0:0.7 in both cases, suggesting that most of the glucosamine residues were phosphorylated.

Although lipid A preparations of reproducible composition could generally be obtained, considerable heterogeneity was revealed by t.l.c. A typical chromatogram for lipid A from *Ps. aeruginosa* is shown in Plate 1. A similar but simpler picture was obtained for lipid A from *Ps. alcaligenes*; in the case of *Ps. alcaligenes* about 75% of the material occurred in a single band in the region X indicated (Plate 1). Apart from trace components above region Y, all components detected by t.l.c. contained phosphorus and an –NH– function (determined by treatment with Cl_2 then starch–KI reagent). Some evidence for differences in fatty acid composition has been obtained for sub-fractions of lipid A from *Ps. aeruginosa*. By means of preparative t.l.c., two sub-fractions consisting predominantly of the two major components (Plate 1) in region X and of the three lesser components in region Y, respectively were isolated. Both sub-fractions had fatty acid compositions

Table 1. *Compositions of lipid A fractions*

Results for amino compounds were obtained by autoanalysis after hydrolysis (6.1M-HCl at 105°C for 4h) of lipids, 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; Chester *et al.*, 1972) for lipids released by more drastic hydrolysis of lipopolysaccharides. —, Not determined.

	Content (%, w/w)	
Species ...	*Ps. aeruginosa*	*Ps. alcaligenes*
Phosphorus	2.2 (2.0)	2.3 (2.0)
Glucosamine	12.5 (11.7)	13.1 (11.2)
Glucosamine phosphate(s)	4.5 (6.1)	5.3 (5.1)
Fatty acids	65 (62)	— (76)

Table 2. *Fatty acid compositions of lipid A fractions*

Fatty acids were released by hydrolysis of lipids with 6.1M-HCl at 105°C for 4h. Results are expressed as percentages of the total fatty acids. Values in parentheses are those obtained previously (Chester, 1969; Key *et al.*, 1970) for lipids released by more drastic hydrolysis of lipopolysaccharides.

	Content (%, w/w)	
Species ...	*Ps. aeruginosa*	*Ps. alcaligenes*
Dodecanoic acid	25.2 (23.7)	43.8 (39.6)
Hexadecanoic acid	6.2 (3.8)	0 (0)
Dec-2-enoic acid	3.5 (2.5)	2.0 (0)
Dodec-2-enoic acid	9.4 (7.7)	9.4 (8.9)
2-Hydroxydodecanoic acid	13.9 (19.5)	0 (0)
3-Hydroxydecanoic acid	15.2 (17.2)	9.4 (18.9)
3-Hydroxydodecanoic acid	25.2 (25.6)	32.0 (32.6)
Other acids	1.4 (0)	3.4 (0)

similar to that of whole lipid A, except that the subfraction from X had a lower content (2%) and that from Y a higher content (16%) of hexadecanoic acid. Variations in the hexadecanoic acid content of lipid A preparations from *Ps. aeruginosa* were observed previously (Chester *et al.*, 1972).

Hydrazinolysis of lipopolysaccharides

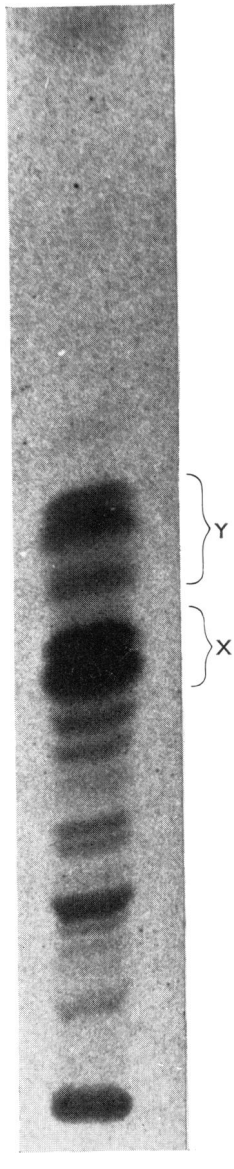
Structural studies of the backbone of lipid A can be simplified by the prior removal of fatty acid residues. However, the lipid A released by acidic hydrolysis of lipopolysaccharides contains reducing glucosamine residues (Burton & Carter, 1964) that are destroyed by hydrazinolysis (Chester *et al.*, 1972). This problem can be overcome by the application of hydrazinolysis to unhydrolysed lipopolysaccharide (Gmeiner *et al.*, 1969).

Lipopolysaccharide from Ps. alcaligenes. Hydrazinolysis converted 70% of the initial glucosamine into water-soluble deacylation products. During ultrafiltration about 20% of the glucosamine and polysaccharide components passed through the membrane filter. The non-diffusible material was hydrolysed with 0.1M-HCl at 100°C for 30min, to break the linkage(s) between the polysaccharide and the deacylation product from lipid A. Again ultrafiltration failed to give a clean separation of the hydrolysis products, so the fractions were combined and treated with alkaline phosphatase. Paper electrophoresis of this hydrolysate revealed the presence of two major cationic reducing amino compounds, of which the less mobile was identified as glucosamine. The second compound (L1) was obtained chromatographically pure by paper electrophoresis and paper chromatography.

The electrophoretic mobility (at pH5.3) and chromatographic mobility (in solvent system C) of compound L1 were those expected for a disaccharide of glucosamine. Glucosamine (about 4% of that in the lipopolysaccharide used) was the only product detected after total acidic hydrolysis of compound L1. By treatment of compound L1 with $NaBH_4$ before hydrolysis, 41% of the glucosamine was reduced to glucosaminol. Experiments with chitobiose (GlcNβ1→4GlcN) confirmed that a disaccharide could readily be differentiated from higher oligosaccharides by paper chromatography, though not by electrophoresis.

A sample of compound L1 was *N*-acetylated and the product treated with *N*-acetyl-D-glucosaminidases. The formation of *N*-acetylglucosamine (identified by t.l.c. of deionized hydrolysates) with β-*N*-acetylglucosaminidase showed that compound L1 was a β-linked disaccharide of D-glucosamine. The position of the linkage was indicated by the application of the Morgan–Elson reaction to *N*-acetylated compound L1. The colour yield was 41% of that obtained after total hydrolysis of compound L1 followed by *N*-acetylation of the glucosamine: expected values for disaccharides linked (1→3), (1→4) and (1→6) are 100–200, 0 and about 60% respectively (Gmeiner *et al.*, 1969). This result indicated that compound L1 was GlcNβ1→6GlcN. Glucosamine was also present in other regions of the paper electrophoretogram, a further 11% of the initial glucosamine being distributed equally between cationic (L2) and anionic (L3) fractions. Fraction L2 contained a reducing, phosphorus-containing amino compound (possibly a disaccharide monophosphate), and chromatography of fraction L3 on Sephadex G-15 revealed the presence of a low-molecular-weight glucosamine derivative (possibly a disaccharide diphosphate) distinct from the phosphorylated polysaccharide component.

Lipopolysaccharide from Ps. aeruginosa. A modified fractionation scheme was applied to the hydrazinolysate from the lipopolysaccharide of *Ps. aeruginosa.*



EXPLANATION OF PLATE 1

Plate 1. *Thin-layer chromatogram of lipid A from Ps. aeruginosa*

The separation was carried out on Bio-Sil A impregnated with 0.05M-sodium phosphate buffer (pH 7.8), with chloroform–methanol–water (65:16:2, by vol.) as solvent system. The chromatogram was sprayed with the reagent of Dittmer & Lester (1964) then heated at 200°C. For other details see the text.

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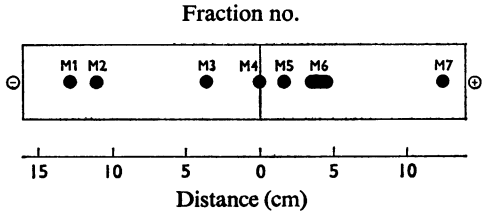

Fig. 1. *Paper electrophoretogram of products obtained by mild acidic hydrolysis of a hydrazinolysate of lipopolysaccharide from Ps. aeruginosa*

The separation was carried out for 1h at about 37V/cm and pH5.3. Spots M1, M2, M3, M5, and M6 reacted with ninhydrin; spots M3, M5, M6, and M7 contained phosphorus; spots M1, M2, M3, and M4 reduced alkaline $AgNO_3$.

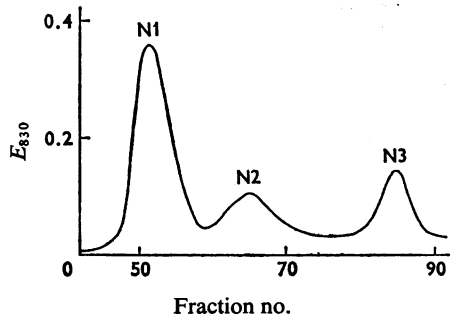

Fig. 2. *Fractionation on Sephadex G-15 of products obtained by mild acidic hydrolysis of a hydrazinolysate of lipopolysaccharide from Ps. aeruginosa*

The material (10mg) was applied to a column (1.8cm×90cm) of Sephadex G-15. Elution was carried out with pyridine–acetic acid buffer (10ml of pyridine+4ml of acetic acid/l; pH5.4) at a flow rate of 10ml/h. Fractions (1ml) were screened for phosphorus content.

By chromatography on Sephadex G-50, the high-molecular-weight material containing phosphorylated polysaccharide and glucosamine (50% of the original amount) was cleanly separated from low-molecular-weight material (consisting mainly of P_i, alanine hydrazide and ethanolamine phosphate). The high-molecular-weight material was then hydrolysed (0.1M-HCl at 100°C for 30min), and the products were isolated by preparative paper electrophoresis (Fig. 1) and paper chromatography (for fractions M1 and M2).

Compound M1 was identified as GlcNβ1→6GlcN by the methods applied to compound L1. In this case the relative colour yield of *N*-acetylated M1 in the Morgan–Elson reaction was 47%. Ninhydrin degradation of compound M1 followed by acidic hydrolysis gave arabinose in addition to glucosamine. Compound M1 accounted for about 18% of the recovered glucosamine.

Compound M2 was free glucosamine, and accounted for 17% of the recovered glucosamine. Compound M3 (m_{GlcN} 0.35) contained most of the recovered glucosamine (33%) and reacted with alkaline $AgNO_3$, ninhydrin, the Hanes–Isherwood reagent for phosphorus, and (after *N*-acetylation) with the Morgan–Elson reagents. Glucosamine was the only amino component of compound M3, and the molar ratio glucosamine:phosphorus was 1.8:1.0. By treatment with alkaline phosphatase, compound M3 was hydrolysed to P_i and the disaccharide M1. Half of the glucosamine in compound M3 was reduced by treatment with $NaBH_4$, and all was destroyed by treatment with $NaIO_4$. However, not all the glucosamine was destroyed when compound M3 was *N*-acetylated before periodate oxidation. Hence compound M3 was probably a monophosphate of GlcNβ1→6GlcN, with the phosphate esterlinked to the 4-position of the non-reducing glucosamine residue (Gmeiner *et al.*, 1969, 1971).

The neutral fraction M4 contained no glucosamine, and will not be discussed further. Fraction M5 contained phosphorus and 20% of the recovered glucosamine, which was released as the monosaccharide by treatment with alkaline phosphatase, and therefore consisted of glucosamine phosphate(s). The remaining glucosamine was present in fraction M6, which also contained the phosphorylated polysaccharide. Although a diphosphate of the glucosamine disaccharide should have a mobility similar to fraction M6 (Gmeiner *et al.*, 1969), attempts to isolate such a component by prolonged paper electrophoresis and by chromatography on Sephadex G-15 were unsuccessful. Fraction M7 was P_i.

In a further attempt to identify a diphosphate of disaccharide M1, the high-molecular-weight material from the hydrazinolysate was subjected to milder hydrolysis (1% acetic acid at 100°C for 70min) and the products were fractionated on Sephadex G-15 (Fig. 2). Most glucosamine (50%) was recovered in fraction N2, and the remainder equally in fractions N1 and N3. Fraction N1 was complex and fraction N3 contained the free glucosamine disaccharide. Fraction N2 contained only glucosamine and phosphorus in the molar ratio 1.0:1.1. It did not reduce alkaline $AgNO_3$ and was unaffected by treatment with $NaBH_4$. Hydrolysis with alkaline phosphatase gave P_i and the disaccharide M1, and hydrolysis with 0.5M-HCl (100°C for 30min) gave P_i, glucosamine and compounds M1 and M3. All the glucosamine was destroyed by periodate oxidation of fraction N2,

but 35% was recovered when oxidation was preceded by *N*-acetylation. On paper electrophoresis fraction N2 had a mobility similar to that of fraction M6. These results are consistent with the identification of fraction N2 as a diphosphate of GlcNβ1→6GlcN, in which the additional phosphate (compared with fraction M3) is a glycosyl phosphate.

Solubilization of lipid A from Ps. alcaligenes

The heterogeneity of lipid A revealed by t.l.c. could partly be due to the existence of extended structures, in which acylated disaccharide units are linked together by phosphodiester or pyrophosphate bridges. Although such bridges would probably not survive hydrazinolysis, they might be unaffected by mild alkaline methanolysis. This treatment was therefore used for the solubilization (de-*O*-acylation) of lipid A from *Ps. alcaligenes*.

Lipid dissolved in chloroform (1 vol.) was treated with methanolic 0.2M-KOH (1 vol.) at 37°C, but only about 60% of the lipid phosphorus and glucosamine dissolved in aqueous methanol in preference to chloroform (Fig. 3). Re-treatment of the chloroform-soluble material raised the solubilization only to about 70% overall, indicating that incomplete solubilization was not due solely to the poor solubility of KOH in the reaction mixture.

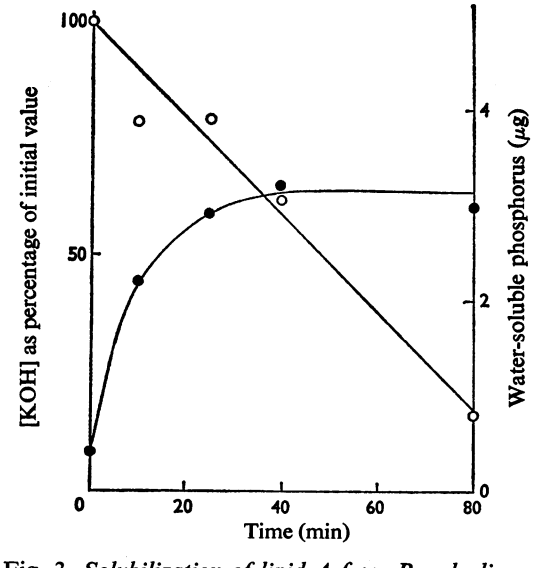

Fig. 3. *Solubilization of lipid A from Ps. alcaligenes by mild alkaline methanolysis*

Samples of lipid A in chloroform (1 vol.) were treated with methanolic 0.2M-KOH (1 vol.) at 37°C. Reaction mixtures were analysed periodically for KOH in solution (○) and for water-soluble phosphorus (●).

Chromatography of the deacylation products on Sephadex G-25 gave a complex elution profile (Fig. 4) but the recoveries of phosphorus and glucosamine were only about 40%. The distributions of components between the fractions obtained are given in Table 3. As found with similar fractions from *Ps. aeruginosa* (Chester *et al.*, 1972), most of the phosphorus in the fractions of solubilized lipid was released by treatment with alkaline phosphatase (Table 3). The presence of reducing glucosamine residues in fractions S1 (16%), S2 (13%) and S4 (31%) was shown by treatment with $NaBH_4$ (insufficient fraction S3 was available for this experiment).

Linkages between fatty acid residues

Recent studies (Rietschel *et al.*, 1972) of lipids from *Salmonella* have shown that ester-bound residues of 3-hydroxytetradecanoic acid are themselves esterified with residues of tetradecanoic acid. Similar studies were undertaken with the lipids from *Ps. aeruginosa* and *Ps. alcaligenes*.

For *Salmonella* lipids, a clue to the occurrence of linked fatty acids was provided by a comparison of acidic and alkaline hydrolysates of lipid A. The greater formation of tetradec-2-enoic acid from 3-hydroxytetradecanoic acid residues under alkaline conditions could be attributed to β-elimination of the tetradecanoyl substituent. In similar experiments

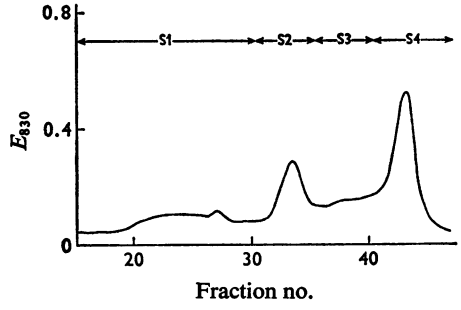

Fig. 4. *Fractionation on Sephadex G-25 of products obtained by mild alkaline methanolysis of lipid A from Ps. alcaligenes*

The water-soluble products from lipid A (16mg) were applied to a column (1.3cm × 53cm) of Sephadex G-25. Elution was carried out with 20% (v/v) methanol at a flow rate of 10ml/h. Fractions (2ml) were screened for phosphorus content, and were then pooled as shown for analysis as described in Table 3.

Table 3. *Analysis of fractions (S1–S4, Fig. 4) of de-O-acylated lipid A from Ps. alcaligenes*

The analysis of fractions for P_i was carried out before and after treatment of samples with alkaline phosphatase. —, Not determined.

Fraction	Total P (μg)	Glucosamine (μg)	Molar ratio GlcN/total P	Total P as P_i Initial (%)	Total P as P_i Final (%)
S1	26.4	185	1.3:1.0	—	86
S2	19.6	138	1.3:1.0	0	67
S3	9.6	85	1.7:1.0	0	96
S4	43.3	303	1.3:1.0	13	63

Table 4. *Acidic and alkaline hydrolysis of lipid A fractions*

Fatty acids were released by hydrolysis of lipids (8–9 mg) at 105°C for 5 h with 4 M-HCl or 4 M-NaOH. Results are expressed as percentages of the total fatty acids.

	Content (%, w/w)			
Species ...	*Ps. aeruginosa*		*Ps. alcaligenes*	
Hydrolysis ...	HCl	NaOH	HCl	NaOH
Dodecanoic acid	34.2	29.6	59.1	48.7
Hexadecanoic acid	5.7	5.6	0	0
Dec-2-enoic acid	2.6	2.8	4.5	1.6
Dodec-2-enoic acid	8.8	4.8	15.3	7.8
2-Hydroxydodecanoic acid	13.4	16.2	0	0
3-Hydroxydecanoic acid	15.3	15.3	10.1	16.5
3-Hydroxydodecanoic acid	20.0	20.5	11.0	18.5
Unknown A*	0	1.4	0	Trace
Unknown B*	0	3.8	0	6.1

* Carbon numbers (polyester column): unknown A, 11.04; unknown B, 12.68.

with the *Pseudomonas* lipids alkaline hydrolysis did not yield greater amounts of unsaturated fatty acids (Table 4). For *Ps. aeruginosa*, the fatty acid compositions of both acidic and alkaline hydrolysates were similar to that recorded after more drastic acidic hydrolysis (Table 2). Unknowns A and B, found only after alkaline hydrolysis of lipids, were apparently isomerization products from dec-2-enoic acid and dodec-2-enoic acid respectively, since they and other trace components of lipid hydrolysates were also detected after treatment of the pure monoenoic acids with alkali under the same conditions. For *Ps. alcaligenes* lipid, the fatty acid compositions given in Table 4 differ significantly from that given in Table 2; the discrepancy is greatest for 3-hydroxydodecanoic acid (the *N*-acylating acid of lipid A). The lipid from *Ps. alcaligenes*, which has a lower hydroxy acid content than the lipid from *Ps. aeruginosa*, is probably the more hydrophobic and could be more difficult to hydrolyse. However, it was shown by t.l.c. that acidic hydrolysates from both lipids had fatty components other than simple fatty acids. Further studies, (D. T. Drewry, J. A. Lomax, G. W. Gray & S. G. Wilkinson, unpublished work) have shown that these additional components are artifacts produced by heating together 3-hydroxy and non-hydroxy acids under acidic conditions.

Because the extent of dehydration of 3-hydroxyalkanoic acids to alk-2-enoic acids during hydrolysis of lipids could not be determined accurately, the comparison of acidic and alkaline hydrolysates was unreliable. However, the extent of dehydration was unexpectedly high under both conditions. Thus, under the conditions given in Table 4, the dehydration of comparable samples (2 mg) of 3-hydroxydodecanoic acid was only 6.6% (in acid) or 1.1% (in alkali). The extent of dehydration under acidic conditions was not altered by prior esterification, and was lowered to 2.3% by treatment in the presence of hexadecanoic acid (8 mg) and to 4.1% by using a larger sample (10 mg) of the hydroxy acid. Thus, the extensive formation of monoenoic acids during

Table 5. *Mild alkaline methanolysis of lipid A fractions*

Fatty acids were released by treatment of lipids (8–9mg) with methanolic 0.25M-sodium methoxide at 37°C for 10h. Fatty acids released as methyl esters by transesterification were determined by g.l.c.; free fatty acids were then esterified by using diazomethane and the analysis was repeated. Results are expressed as peak areas relative to that for methyl pentadecanoate (internal standard) as 1000. A, Before diazomethane treatment; B, after diazomethane treatment.

	Relative peak area			
Species ...	*Ps. aeruginosa*		*Ps. alcaligenes*	
	A	B	A	B
Dodecanoic acid	841	915	1860	2124
Hexadecanoic acid	Trace	191	0	0
Dec-2-enoic acid	39	35	Trace	Trace
Dodec-2-enoic acid	0	0	0	0
2-Hydroxydodecanoic acid	213	551	0	0
3-Hydroxydecanoic acid	412	474	650	798
3-Hydroxydodecanoic acid	47	65	95	117
Unknown C*	221	209	Trace	Trace
Unknown D*	Trace	Trace	Trace	Trace

* Carbon numbers (polyester column): unknown C, 12.46; unknown D, 14.40.

acidic hydrolysis of lipids cannot be explained readily, but under alkaline conditions the involvement of β-elimination reactions seems very likely.

Mild alkaline methanolysis liberates as methyl esters fatty acids esterified to glucosamine, whereas those esterified to 3-hydroxy fatty acids may be released as free acids and require subsequent esterification to form methyl esters (Rietschel *et al.*, 1972). The results of similar experiments on *Pseudomonas* lipids are given in Table 5. Methyl pentadecanoate was added as an internal standard. Unknowns C and D had retention times identical with those of the major products from treatment of methyl dec-2-enoate and methyl dodec-2-enoate, respectively, with sodium methoxide. These products were expected to be 3-methoxyalkanoic esters, formed by Michael addition of methanol to the monoenoic esters (Rietschel *et al.*, 1972). The identity of the product from methyl dodec-2-enoate was confirmed by mass spectrometry (Ryhage & Stenhagen, 1960).

The results (Table 5) for lipid A from *Ps. aeruginosa* confirm that little or no 3-hydroxydodecanoic acid is ester-linked, and show that most or all of the hexadecanoic acid and 2-hydroxydodecanoic acid are ester-linked to a 3-hydroxy acid. The proportions of hexadecanoic acid and 2-hydroxydodecanoic acid released as methyl esters (by transesterification) or free acids (by β-elimination) were rather variable, but the elimination products always predominated. The relatively small increases in the amounts of dodecanoic, 3-hydroxydecanoic, and 3-hydroxydodecanoic esters after treatment of methanolysates with diazomethane were of the order expected from an analysis of lipid A for free fatty acids; thus, these three components are unlikely to be ester-linked to other fatty acid residues. The occurrence in the methanolysates of methyl 3-methoxydecanoate and methyl dec-2-enoate shows that at least some of the 3-hydroxydecanoate residues carry the ester-bound residues of hexadecanoic acid and/or 2-hydroxydodecanoic acid. However, the amounts of methyl 3-methoxydecanoate and methyl dec-2-enoate formed are apparently considerably less than the amounts of hexadecanoic acid and 2-hydroxydodecanoic acid eliminated, suggesting that some residues of the latter acids are linked to the amide-bound residues of 3-hydroxydodecanoic acid.

No clear evidence for linked fatty acids in lipid A from *Ps. alcaligenes* was obtained. The amounts of 3-methoxy esters detected were no greater than could be accounted for by reaction of unsubstituted 3-hydroxy acid residues. Thus, if the increased amounts of methyl esters found after treatment of methanolysates with diazomethane are significant, eliminated acids must have been linked to the amide-bound residues of 3-hydroxydodecanoic acid as suggested to be partly true for *Ps. aeruginosa*.

Discussion

Hydrazinolysis studies have established that the lipid A fractions from *Ps. aeruginosa* and *Ps. alcaligenes* are derived from GlcNβ1→6GlcN, as in

Salmonella (Gmeiner *et al.*, 1969), *Serratia* (Adams & Singh, 1970*a*), and *Selenomonas* (Kamio *et al.*, 1971). Although the isolated yields of the disaccharide were low, this is partly explained by the use of substantial amounts of products in monitoring the fractionation processes, and no evidence for higher oligosaccharides was obtained. The significance of the free glucosamine found in hydrazinolysates is not clear, but it may have been liberated during the hydrolytic cleavage of deacylated lipid A from polysaccharide. The glucosamine disaccharide is phosphorylated to about the same extent in both pseudomonads, and the fragments derived from the hydrazinolysate of lipopolysaccharide from *Ps. aeruginosa* closely resembled those obtained from *Salmonella minnesota* (Gmeiner *et al.*, 1969, 1971).

Lipid A from *Ps. alcaligenes* differs from that from *Ps. aeruginosa* mainly in the absence of hexadecanoic acid and 2-hydroxydodecanoic acid. The fact that these are the fatty acids that are ester-linked to 3-hydroxyalkanoic acids in the lipid from *Ps. aeruginosa* is therefore particularly noteworthy. The lipid A fraction of a lipopolysaccharide is responsible for its endotoxic activity (Lüderitz *et al.*, 1971; Galanos *et al.*, 1971), and there are indications that the degree of endotoxic activity may be determined by the content of acylated hydroxy esters (Rietschel *et al.*, 1972). Further studies will be necessary to determine whether the special importance in clinical bacteriology of *Ps. aeruginosa* can be related to the nature and content of the linked fatty acids of the lipopolysaccharide of this particular pseudomonad.

The general description of lipid A as a glucosamine disaccharide (or oligosaccharide) carrying phosphate and fatty acid residues is widely accepted. Whether such units are linked together to form more complex structures is still a matter for speculation (Gmeiner *et al.*, 1969; Wober & Alaupović, 1971; Rietschel *et al.*, 1972). However, even with the simplest description, structural variations (e.g. in number, nature and position of fatty acid residues, in number and position of phosphate residues) are possible. Similar variations could be introduced by the procedures used for the extraction of lipopolysaccharide and for the separation of lipid A from polysaccharide. The complex mixtures of components detected by t.l.c. could probably be explained in this way. Likewise, the variations in the number of phosphate residues attached to the glucosamine disaccharide in fragments derived from lipid A by hydrazinolysis may to some extent reflect a natural heterogeneity of the lipid.

A further probe of lipid heterogeneity was based on gel filtration of the de-*O*-acylated products obtained by mild alkaline methanolysis (Fig. 4, and Chester *et al.*, 1972). This relatively mild treatment should simplify the situation by eliminating variations due to fatty acid composition and could leave phosphodiester or pyrophosphate bridges intact. The fact that similar molar ratios of glucosamine:phosphorus were obtained for the fractions of solubilized lipids supports the supposition that the fractionations were indeed based on molecular size. However, the failure to obtain clear-cut fractions precluded any precise interpretation of the analytical data for these products.

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