The Chemical Composition of the Lipopolysaccharide of *Pseudomonas aeruginosa*

By A. H. FENSOM* AND G. W. GRAY
Department of Chemistry, University of Hull

(Received 24 February 1969)

1. Lipopolysaccharide was isolated from both cell walls and acetone-dried whole cells of *Pseudomonas aeruginosa* (N.C.T.C. 1999). 2. Closely similar products are obtained, although that from whole cells cannot be completely freed from small amounts (2–7%) of residual nucleic acids. 3. The lipid moiety (23–33%) has a similar amino sugar backbone to that of lipids of enterobacterial lipopolysaccharides, but contains different hydroxy acids (2- and 3-hydroxydodecanoic acid and 3-hydroxydecanoic acid). 3-Hydroxytetradecanoic acid is absent, and 3-hydroxydodecanoic acid is the main *N*-acylating acid. No clear evidence permitting a distinction between the possibilities that phosphodiester or glycosidic linkages exist between the glucosamine residues was obtained. 4. Identifiable sugars (glucose, rhamnose, 3-deoxy-2-octulonic acid and heptose) account for less than 20% of the lipopolysaccharide, and alanine, galactosamine and fucosamine are apparently components of the polysaccharide moiety. 5. The polysaccharide moiety is unusual in that it is not readily obtained from the lipopolysaccharide by treatment with dilute acetic acid, which does, however, solubilize much of the phosphorus of the lipopolysaccharide. 6. The 'polysaccharide' fraction (approx. 21%) obtained by treatment with dilute acetic acid contains only a small proportion of the total polysaccharide components, and in one case only 45% of the fraction was accountable for in terms of identifiable components. 7. Evidence suggests that unidentified nitrogenous components are concentrated in the residual material after removal of both the lipid and the 'polysaccharide' fraction from the lipopolysaccharide.

Interest in these laboratories in the lipopolysaccharide of the Gram-negative organism *Pseudomonas aeruginosa* arose from the observation (Gray & Wilkinson, 1965*a,b*; G. W. Gray & J. W. Payne, unpublished work) that solubilization of this component of the cell occurs at alkaline pH during the bactericidal action of solutions of EDTA on whole cells and on exposure of isolated cell walls to solutions of EDTA. The lipopolysaccharide of *P. aeruginosa*, together with some glycosaminopeptide, may be isolated by fractionation of cell walls (Clarke, Gray & Reaveley, 1967*a,b,c*) by using aqueous phenol, and preliminary studies showed that the lipid and polysaccharide parts are qualitatively similar to the corresponding materials obtained from other Gram-negative organisms; the heptose content was, however, much smaller than that reported (Taylor, Knox & Work, 1966; Sutherland & Wilkinson, 1966) for some lipopolysaccharides.

The present paper describes further studies of the

isolation, purification and composition of the lipopolysaccharide of *P. aeruginosa*. In an attempt to obtain larger quantities of lipopolysaccharide, and because it has been shown (Roberts, Gray & Wilkinson, 1967) that substantial losses of cell lipopolysaccharide occur during the isolation of cell walls, the use in the earlier stages of the work of cell walls as the source of lipopolysaccharide gave way to the use of acetone-dried whole cells, which were kindly supplied by the Microbiological Research Establishment, Porton, Wilts.

MATERIALS AND METHODS

Preparation of cell walls and acetone-dried whole cells. The strain of *P. aeruginosa* used was N.C.T.C. 1999. Cells were grown and cell walls prepared by the method of Salton & Horne (1951) and Cummins & Harris (1956) as described by Gray & Wilkinson (1965*a*). The reproducibility of individual batches of cell walls was checked by quantitative analysis for phosphorus, nitrogen and ninhydrin-positive compounds, by i.r. spectroscopy in the range 650–4000 cm.⁻¹ and by u.v. spectroscopy of aqueous suspensions (0.1 mg./ml.). Electron

* Present address: Department of Biochemistry, University College London, Gower Street, London W.C.1.

microscopy of the preparations showed them to be free of contaminating material.

Large quantities of whole cells that had been grown on a tryptone medium were supplied by the Microbiological Research Establishment, Porton, Wilts. The cells were dehydrated with acetone (Mackie & McCartney, 1960) before isolation of the lipopolysaccharide.

Isolation of the lipopolysaccharide. Both cell walls and whole cells were used as a source of lipopolysaccharide. The lipopolysaccharide was isolated from cell walls by using hot aq. 45% (w/v) phenol by the method described by Clarke *et al.* (1967*b*), with the exception that loosely bound lipids were first removed from the cell walls. This was achieved by shaking the walls with chloroform-methanol (2:1, v/v) for 2 hr. at room temperature. The residual walls were collected after filtration (no. 4 sinter), subjected to two similar treatments and dried *in vacuo* before treatment with aqueous phenol. Two treatments with aqueous phenol were carried out, and the materials obtained from the aqueous phases of each treatment were kept separate (designated AqI and AqII respectively).

The method used for the extraction of lipopolysaccharide from whole cells was essentially that of Westphal & Jann (1965), but an additional procedure involving treatment of the crude lipopolysaccharide with ribonuclease was included to facilitate removal of contaminating nucleic acid. Acetone-dried cells (7 g.) were suspended in water (122 ml.) and heated to 65–68°, with stirring. An equal volume of aq. 90% (w/v) phenol, pre-heated to the same temperature, was added, and the mixture was stirred at 65° for 15 min. The mixture was cooled in ice and centrifuged (2000*g* for 30 min. at 10°), and the layers were separated. The phenolic layer and insoluble residue were extracted a second time with water (122 ml.) under identical conditions. The aqueous layers were then combined and dialysed against ion-depleted water to remove phenol. The non-diffusible residue was concentrated to about 50 ml., and a solution of ribonuclease (15 mg.) dissolved in 0.05 M-tris-HCl buffer, pH 7.7 (50 ml.), was added. The mixture was incubated at 37° for 16 hr. and then concentrated to 25 ml. The resulting solution was centrifuged in an MSE Superspeed 40 ultra-centrifuge (80000*g* for 8 hr. at 18°) to deposit the lipopolysaccharide. Two distinct layers were obtained: a lower hard gel-like pellet (F₁), and a less firmly sedimented upper gel (F₂). Each layer was separately resuspended and submitted to three further centrifugations (105000*g* for 3 hr. at 18°); the lipopolysaccharide preparations were suspended separately in a minimum quantity of water and freeze-dried. Analytical work revealed no differences between preparations F₁ and F₂, which are regarded as different batches of the same lipopolysaccharide, differing possibly in the degree of aggregation.

Methods of quantitative analysis. Phosphorus was determined by the method of Allen (1940), or by the micro-method of Bartlett (1959). Nitrogen was determined by a modification of the method of Umbreit, Burris & Stauffer (1964). Carbohydrate was determined with anthrone (Trevelyan & Harrison, 1952) and by the phenol-H₂SO₄ reaction (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). D-Glucose was determined with glucose oxidase after hydrolysis of samples at 100° for 4 hr. with M-HCl and neutralization by repeated evaporation *in vacuo*. A control experiment with D-glucose showed that this procedure led to no appreciable loss of the sugar. Heptose was determined by the cysteine-H₂SO₄

method (Dische, 1955) as modified by Osborn (1963). Rhamnose was determined in the same experiment from the difference between E_{402} and E_{415} on the spectrum in cysteine-H₂SO₄. 3-Deoxy-2-octulonic acid was determined by the method of Weissbach & Hurwitz (1959) as modified by Osborn (1963).

Nucleic acid was determined from the u.v. spectra of aqueous suspensions of samples on the assumption that 0.05 mg. of nucleic acid dissolved in water (1 ml.) has $E_{260}^{1\text{cm}}$ 1.0 (Barker, Bick, Brimacombe & Somers, 1966). Ribose was determined by the method of Mejbaum (1939) as described by Umbreit, Burris & Stauffer (1957), and DNA by the method of Ceriotti (1952).

Fatty acids were determined by the method of Itaya & Ui (1965) after hydrolysis of samples at 105° for 4 hr. with constant-boiling (6.1 M) HCl (Salton, 1953), followed by three extractions of the fatty acids with portions (2 ml. in each case) of light petroleum (b.p. 40–60°).

Determination of ninhydrin-positive compounds. Ninhydrin-positive components of the lipopolysaccharide were determined by using an automatic amino acid analyser (Technicon Instruments Co. Ltd., Chertsey, Surrey) as described by Clarke *et al.* (1967*a*). By carrying out hydrolysis for different times from 1 to 16 hr., it was concluded that maximum release of glucosamine and galactosamine was obtained after 4 hr., and subsequent hydrolyses were therefore carried out for this time.

Methods of qualitative analysis. Paper chromatography and t.l.c. were carried out with unwashed Whatman no. 1 paper and t.l.c. plates of silica gel G (E. Merck A.-G., Darmstadt, Germany). The following solvent systems were employed: *a*, ethyl acetate-pyridine-water (5:2:5, by vol., upper layer); *b*, ethyl acetate-pyridine-water-acetic acid (5:5:3:1, by vol.); *c*, propan-1-ol-aq. NH₃ (sp.gr. 0.88)-water (6:3:1, by vol.); *d*, formic acid-butan-2-one-2-methylpropan-2-ol-water (3:6:8:3, by vol.); *e*, chloroform-benzyl alcohol-acetic acid (70:30:3, by vol.).

The conditions of hydrolysis differed for the compounds under investigation. For chromatography of neutral sugars, the soluble oligosaccharides obtained after removal of lipid A from the lipopolysaccharide were hydrolysed for a further 1½ hr. with 0.5 M-H₂SO₄, and the solution was neutralized with BaCO₃. Fucosamine was identified as a component of the lipopolysaccharide after hydrolysis with constant-boiling HCl at 105° for 10 hr. Reducing sugars were detected with alkaline AgNO₃ (Trevelyan, Procter & Harrison, 1950), and amino sugars with a solution of ninhydrin in butan-1-ol (Cramer, 1955) or with the Elson-Morgan reagents (Partridge, 1948).

Physical methods. The i.r. spectra were recorded with a Unicam SP.100 spectrophotometer equipped with grating and fore prism (NaCl). Samples (1 mg.) were dispersed in anhydrous KBr (about 100 mg.) and examined as pressed discs. The u.v. spectra of aqueous suspensions of cell walls or the lipopolysaccharide were obtained with a Unicam SP.500 spectrophotometer.

Cell walls and samples of lipopolysaccharide were examined by electron microscopy with a Siemens Elmiskop I instrument. Specimens were prepared by suspending a portion (1 mg.) in redistilled ion-depleted water (10 ml.), transferring a drop to a carbon grid and evaporating off the water.

Gas-liquid chromatography. Fatty acids were identified and measured by g.l.c. of their methyl esters in a Perkin-

Elmer F11 chromatographic apparatus. Separation was carried out with both polar (polyethylene glycol succinate at 180°) and non-polar (Apiezon L at 220°) columns. Acids were released by hydrolysis of samples with constant-boiling HCl (1 ml.) at 105° for 4 hr., and extracted three times with light petroleum (b.p. 40–60°) (2 ml. in each case). They were methylated with a solution of boron trifluoride (14%, w/v) in methanol (Applied Science Laboratories Inc., State College, Pa., U.S.A.) as described by Metcalfe & Schmitz (1961).

Isolation of the lipid component of the lipopolysaccharide. The method used for isolating the lipid component (lipid A) was based on that of Taylor *et al.* (1966). The lipopolysaccharide (50 mg.) was hydrolysed for 30 min. at 100° with 0.5M-H₂SO₄ in a sealed tube. The precipitated lipid was separated by centrifugation and washed four times with water (1 ml. in each case). The lipid was transferred to a tared tube with a little chloroform; the chloroform was removed under a stream of N₂, and the lipid dried to constant weight *in vacuo* over P₂O₅. Free fatty acids were removed from the lipid by using freshly redistilled ethyl formate as described by Taylor *et al.* (1966).

Alkaline methanolysis of lipid A. Lipid A (6 mg.) was treated with methanolic 0.25M-KOH (2 ml.) at 37° for 16 hr., and insoluble material was removed by centrifugation (Kasai, 1966). The sediment was dissolved in a little water; the solution was neutralized with HCl and dried *in vacuo* over P₂O₅ (lipid A-MP). The methanol-soluble material (lipid A-MS) was acidified with HCl, and the methyl esters and any free fatty acids were extracted with three portions of light petroleum (b.p. 40–60°) (2 ml. in each case). The fatty acids were identified after methylation by g.l.c. Lipid A-MP was hydrolysed with constant-boiling HCl at 105° for 4 hr. to release the residual amide-bound fatty acids. These were extracted and identified as described above.

Determination of N-terminal alanine with 1-fluoro-2,4-dinitrobenzene. The method used was based on that by Fraenkel-Conrat, Harris & Levy (1955). 1-Fluoro-2,4-dinitrobenzene was distilled *in vacuo* before use; diethyl ether (freed from peroxides) was dried over CaCl₂ and distilled. The reaction was carried out with lipopolysaccharide (5 mg.) suspended in water (1 ml.) containing NaHCO₃ (100 mg.); to this a 5% solution (2 ml.) of 1-fluoro-2,4-dinitrobenzene in ethanol was added. The ethereal extracts, containing DNP-alanine, were evaporated to dryness, and the residue was dissolved in acetone (50 µl.). Samples (5 µl.) were examined by t.l.c. in solvent system *c*, all chromatograms being developed in the dark.

Treatment of lipopolysaccharide with carboxypeptidase. Lipopolysaccharide (10 mg.) was dissolved in 0.05M-tris-HCl buffer, pH 7.2 (2 ml.), and a suspension of carboxypeptidase A (0.1 ml.) was added. The mixture was incubated at 23° for 8 hr. with periodic agitation; centrifugation removed insoluble material and the supernatant solution was examined for amino compounds by autoanalysis.

Enzymes. Glucose oxidase reagent (Glucostat) was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Trypsin and ribonuclease were purchased from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, and deoxyribonuclease was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Carboxypeptidase (suspension) was supplied by the Sigma Chemical Co., St Louis, Mo., U.S.A.

Table 1. *Analytical results for cell walls of Pseudomonas aeruginosa*

Component	Amount in cell wall (%)	
	Present work	Clarke <i>et al.</i> (1967a)
Phosphorus	1.8–2.0	2.0–2.1
Nitrogen	8.0–8.5	7.6–8.3
Anthrone sugar	5.6–6.4	6.8–7.4

RESULTS AND DISCUSSION

General. Several batches of cell walls were prepared, and as shown in Table 1, there were some differences between these preparations and those studied by Clarke *et al.* (1967a). The i.r. spectra of the preparations were, however, indistinguishable from the spectra of the earlier preparations, and in all cases the cell walls were free from nucleic acids as judged by the u.v. spectra of their aqueous suspensions. Autoanalysis of cell-wall hydrolysates for ninhydrin-positive components (Clarke *et al.* 1967a) showed that the preparations were reproducible and comparable with the cell walls examined by Clarke *et al.* (1967a). The contributions of amino compounds to the cell-wall composition (43–46%) and to the total nitrogen of the cell wall (81–83%) were in close agreement with the earlier results.

Two batches of cell walls (A, P content 1.8%; B, P content 2.0%) were fractionated by using aqueous phenol (Clarke *et al.* 1967b,c). The walls were first defatted to remove loosely bound lipids, and each sample was treated twice with aqueous phenol. The aqueous layers yielded two fractions, AqI and AqII (mainly lipopolysaccharide). Since our main interest lay with the lipopolysaccharide, little work was carried out on the other fractions (R, PhMP, PhMS) and the loosely bound lipid; yields and general analytical results for these fractions agreed with previous results (Gray & Wilkinson, 1965b; Clarke *et al.* 1967a,b,c).

Fractions AqI and AqII. The phosphorus contents and analytical results for ninhydrin-positive components for fractions AqI and AqII obtained from the two cell-wall preparations mentioned above are recorded in Table 2. These results demonstrate the presence of both glycosaminopeptide and lipopolysaccharide in fractions AqI and AqII. Clarke *et al.* (1967c) have discussed the contamination of fractions AqI and AqII with glycosaminopeptide in terms of the possible aggregation of this with lipopolysaccharide, but in the present work greater success was achieved in separating glycosaminopeptide from the lipopolysaccharide. Thus the sample of fraction AqI from preparation A (Table 2) contains only 1.8% of glycosaminopeptide and 0.5% of protein, and is a relatively pure sample of

Table 2. *Quantitative analyses for phosphorus and amino compounds of fractions AqI and AqII obtained from cell-wall preparations A (P = 1.8%) and B (P = 2.0%)*

Values for amino acids relate to amino acid residues, but all other values relate to the free compounds. Results are corrected for destruction or slow release during hydrolysis, being based on a hydrolysis time of 16 hr. Except in the case of amino sugars (see the Materials and Methods section), correction factors obtained by Clarke *et al.* (1967a) with cell walls were applied. +, Only a trace detected; ++, measurable amount detected.

	Amount in sample (%)			
	Preparation A		Preparation B	
	Fraction AqI	Fraction AqII	Fraction AqI	Fraction AqII
Unknown (C)*	++	++	++	++
Muramic acid	0.32	1.73	1.74	5.80
Glutamic acid	0.37	1.10	1.40	3.24
Alanine	1.40	2.16	2.33	3.92
Glucosamine	3.80	4.26	4.19	5.89
Galactosamine	2.80	2.60	2.37	2.05
Fucosamine	0.9	0.8	0.7	0.6
2,6-Diaminopimelic acid	0.31	1.21	1.65	4.11
Ethanolamine	1.01	0.40	0.70	0.88
Unknown (D)	+	+	+	0.0
Lysine	0.17	0.22	0.14	0.30
Unknown (E)	++	++	++	++
Other ninhydrin-positive components, including NH ₃	0.82	1.48	0.54	0.85
Total	11.90	15.96	15.76	27.64
Nitrogen	3.9	4.2	4.2	4.6
Phosphorus	4.1	3.5	4.1	2.9
Nitrogen recovery (%) ...	57.8	64.9	70.3	81.7

* Believed to be either *O*-phosphorylethanolamine or *O*-phosphorylglucosamine.

Table 3. *Molar ratios for the components of glycosaminopeptide in fractions AqI and AqII from cell-wall preparations A and B*

	Preparation A		Preparation B	
	Fraction AqI	Fraction AqII	Fraction AqI	Fraction AqII
Muramic acid	1.00	1.00	1.00	1.00
2,6-Diaminopimelic acid	1.41	1.02	1.38	1.03
Glutamic acid	1.51	0.95	1.47	1.03
Alanine	14.29	4.09	4.55	2.32
Glucosamine	16.61	3.44	3.36	1.42

lipopolysaccharide. The degree of contamination by glycosaminopeptide is therefore considered to be merely a reflection of the efficiency of the extraction procedure with aqueous phenol.

The molar ratios for the components of glycosaminopeptide in fractions AqI and AqII from the two cell-wall preparations (*A* and *B*) are given in Table 3. It is clear, particularly from the ratios for fraction AqI of preparation *A*, that alanine and glucosamine are also components of the lipopolysaccharide.

The unknown component E detected in chromatograms from amino acid analysis 125–130 min.

later than ornithine and 45–50 min. later than histidine is almost certainly 2-chloroethylamine, a synthetic sample of which was eluted from the column after the same time. This component is detected in all hydrolysates containing ethanolamine, and may arise from this either directly by nucleophilic attack or through the intermediate ethyleneimine. Autoanalysis of a sample of ethyleneimine gave a peak in the position for 2-chloroethylamine, showing that ring-opening had occurred during chromatography.

As found by Clarke *et al.* (1967c), the nitrogen recoveries (Table 2) for fractions AqI and AqII

were low, and these decreased with increasing lipopolysaccharide content. With appropriate conditions of hydrolysis, good nitrogen recoveries can, however, be obtained for systems containing large amounts of carbohydrate (Dustin, Czajkowska, Moore & Bigwood, 1953; Pusztai & Morgan, 1963). Since low recoveries were still obtained for fractions AqI and AqII, it seems possible that the lipopolysaccharide contains as yet undetected nitrogenous components.

By correcting the combined yields of fractions AqI and AqII for the content of glycosaminopeptide, the percentages of lipopolysaccharide in wall preparations *A* and *B* are calculated as 29.4 and 30.9%. The average value quoted by Clarke *et al.* (1967c) was 31%.

It was concluded that, although cell-wall preparations varied somewhat in composition, this was not reflected in major differences in the lipopolysaccharide obtained from them. However, facilities for preparing cell walls on a large scale were limited, and it was decided to extract lipopolysaccharide from acetone-dried whole cells in order to obtain larger amounts of material. If the lipopolysaccharide agreed in composition with that obtained from cell walls, more detailed structural studies would then be possible.

Isolation of lipopolysaccharide from acetone-dried whole cells. By using the method described by Westphal & Jann (1965), the yield of crude lipopolysaccharide from 7 g. of cells was 9–10%. Ultracentrifugation of aqueous 3% suspensions of this material removed much nucleic acid, but the sedimented lipopolysaccharide still contained this contaminant, varying between 6 and 20% from one extraction to another, as judged by the u.v. spectra. The yield of material was about 1% based on the weight of dry cells, a lower yield than that reported (Westphal & Jann, 1965) from normal forms of Enterobacteriaceae (1.5–2.5%), but comparable with that from rough forms of *Salmonella* (Lüderitz, Staub & Westphal, 1966b). The remaining nucleic acid was very difficult to remove. Treatment with cetyltrimethylammonium bromide (Westphal & Jann, 1965) precipitated about 35% of the material from solution, but the percentage of nucleic acid was unaltered. Experiments with different con-

centrations of cetyltrimethylammonium bromide were unsuccessful, as were attempts to precipitate purer lipopolysaccharide from solution by using ethanol or Mg^{2+} ions (Osborn, Rosen, Rothfield & Horecker, 1962); chromatography on Sephadex G-200 (Barker *et al.* 1966) gave simultaneous elution of lipopolysaccharide and nucleic acid. The quality of the lipopolysaccharide was improved by treatment with ribonuclease. The extraction procedure of Westphal & Jann (1965) was therefore modified by including a digestion with ribonuclease before ultracentrifugation, and in this way the contamination was decreased to 2–7%. More than one treatment with ribonuclease did not further decrease the nucleic acid content, and more recent work in these laboratories has failed to reveal any method for removing small amounts of nucleic acid from the lipopolysaccharide of *P. aeruginosa* (I. R. Chester, unpublished work).

The amount of ribose in the lipopolysaccharide was equivalent to that expected (Norton, Bulmer & Sokatch, 1963) on the basis of the nucleic acid calculated to be present from the u.v. spectra. Apparently DNA was not responsible for the remaining u.v. absorption because treatment of the lipopolysaccharide with deoxyribonuclease caused no detectable decrease in E_{260} . Determination of DNA showed the presence of only 1–2%. It appears therefore that difficulty in removing the last traces of material absorbing at 260 nm. is caused by complex-formation between nucleic acid and lipopolysaccharide.

Analysis of lipopolysaccharide from acetone-dried whole cells. The i.r. spectra of all preparations were closely similar, showing marked absorption in the region 1720 cm^{-1} , indicating large amounts of esterified acids. Electron micrographs of the preparations revealed that they were homogeneous; small areas containing fragmented electron-dense material were only occasionally observed. At high magnification ($\times 70\,000$ – $90\,000$) the entire sample appeared as a tight arrangement of fibres similar to that observed by Taylor *et al.* (1966). The fibrous structure was particularly clear at the edges of the samples, even at low magnification ($\times 24\,000$), and the electron density was obviously greater where the fibres crossed.

Table 4. *Phosphorus and nucleic acid contents of batches of lipopolysaccharide from acetone-dried cells*

The experimentally determined phosphorus percentages were corrected in each case by subtracting the contribution by the phosphorus of contaminating nucleic acid, assuming this to have a phosphorus content of 8.2%.

	Batch no. ...	1	2	3	4	5	6
Nucleic acid (%)		3.0	6.1	4.1	2.3	7.2	5.1
Phosphorus (%)		4.5	4.1	4.7	4.9	5.7	4.3
Corrected phosphorus (%)		4.25	3.6	4.4	4.7	5.1	3.9

Table 5. *Ninhydrin-positive components of batches of lipopolysaccharide*

Hydrolyses were carried out for 4 hr. at 105° with constant-boiling HCl and the results corrected where necessary for slow release or decomposition. Results are expressed as percentages of the free compounds. ++, Measurable amount detected.

Batch no. ...	1	2	3
Unknown (C)	++	++	++
Alanine	1.19	1.18	1.12
Glucosamine	3.65	3.60	3.29
Galactosamine	2.59	2.45	2.13
Fucosamine	1.0	0.9	0.5
Unknown (D)	++	++	++
Other ninhydrin-positive components, including NH ₃	0.60	0.60	1.50

Phosphorus and nucleic acid. The phosphorus and nucleic acid contents for six batches of lipopolysaccharide are summarized in Table 4. The phosphorus values vary considerably, with a maximum deviation of $\pm 1.0\%$ from the mean of 4.7% . Contaminating nucleic acid is partly responsible, and Table 4 includes corrected phosphorus contents for the lipopolysaccharide, assuming the nucleic acid to have a phosphorus content of 8.2% . A smaller maximum deviation of $\pm 0.8\%$ from the mean value of 4.3% is now obtained. Factors other than nucleic acid content must therefore contribute to the variable phosphorus analyses; this is clear from the values for batches 2 and 5.

The mean corrected phosphorus value (4.3%) agrees well with the phosphorus contents (4.1%) for the two fractions AqI in Table 2. The lipopolysaccharide of *P. aeruginosa* therefore has a phosphorus content appreciably higher than that of the lipopolysaccharides of some enterobacteria, but comparable with the phosphorus contents (3.7 – 4.1%) reported by Taylor *et al.* (1966) for lipopolysaccharide preparations from *Escherichia coli* (A.T.C.C. 12408).

Ninhydrin-positive components of lipopolysaccharide (Table 5). Fucosamine (see also Table 2), the unknown component Y found by Clarke *et al.* (1967c) in hydrolysates of fractions AqI and AqII from *P. aeruginosa*, was identified by comparison of chromatograms with those from an authentic sample of fucosamine. Chromatography on paper with solvent system *b* gave identical R_F values, and auto-analysis for amino compounds gave identical elution times. The colours produced when the fucosamine from the lipopolysaccharide and authentic fucosamine were treated separately on paper with ninhydrin or the Elson–Morgan reagents were identical. The stereochemical configuration of the fucosamine has not been established. Maximum release of fucosamine was obtained after hydrolysis for 10 hr., and values for 4 hr. hydrolysates have been corrected. After fucosamine was identified as a constituent of the lipopolysaccharide, the isolation

of DL-fucosamine from *P. aeruginosa* N10 was reported by Suzuki (1969).

Ethanolamine and 2-chloroethylamine, present in fractions AqI and AqII from cell walls, were absent from the lipopolysaccharide from whole cells. The unknown component C in Table 5 therefore cannot be *O*-phosphorylethanolamine, and is most probably an *O*-phosphoryl derivative of an amino sugar. Release of unknown component C was maximal after hydrolysis for 1 hr., its amount decreasing to a trace after hydrolysis for 16 hr. Unknown component D was again present and in rather larger amount than in fractions AqI and AqII from cell walls. Comparison of the results of Table 5 with those of Table 2, particularly for fraction AqI from cell-wall preparation *A*, shows clearly that the same lipopolysaccharide is obtained from acetone-dried whole cells, and again the only amino compound present in major amount, apart from amino sugars, was alanine.

The occurrence of a large excess of one amino acid, namely alanine, in the lipopolysaccharide was notable, and led us to investigate whether this might be bound in an analogous manner to the ester-bound alanine in teichoic acids of Gram-positive bacteria (Archibald & Baddiley, 1966). Lipopolysaccharide was treated with ethanolic ammonia, and the products of the reaction were investigated for the presence of alanine amide by paper chromatography. A positive result was obtained in one experiment, but in several replicate experiments the amide was not detected. It was concluded that the positive result was spurious, and that the alanine of the lipopolysaccharide was not in ester linkage. This was confirmed by examining the products of the above reaction by automatic amino acid analysis, when alanine amide was not detected. Further, treatment of the lipopolysaccharide with hydroxylamine failed to form any hydroxamic derivative of alanine. It therefore appears that the alanine is present in amide linkage and not in ester linkage.

Treatment of lipopolysaccharide with 1-fluoro-2,4-dinitrobenzene resulted in conversion of 15–20%

Table 6. *Carbohydrate contents (%) of batches of lipopolysaccharide*

Results obtained by the anthrone-H₂SO₄ and phenol-H₂SO₄ methods are expressed in terms of glucose standards. —, Not determined.

	Batch no. ...	1	2	3	4	5	Mean value
Glucose oxidase		9.4	8.8	9.3	9.7	8.6	9.2
Anthrone-H ₂ SO ₄		15.6	14.6	14.6	15.0	—	14.9
Phenol-H ₂ SO ₄		21.6	19.5	20.1	23.2	—	21.1

of the alanine into the DNP derivative, showing that this percentage of the alanine is present in an *N*-terminal position. The remaining alanine is not, however, *C*-terminal, since no alanine was liberated on treatment with carboxypeptidase. The remaining alanine may be present in polyalanyl chains or may be *N*-acylated (Hanessian & Haskell, 1964).

When the contribution of amino compound nitrogen to the total nitrogen of the lipopolysaccharide was calculated, a value of only about 45% was obtained. However, about 25% of the nitrogen must be associated with nucleic acid. The recovery of nitrogen was therefore similar to that for fraction AqI of low glycosaminopeptide content, and suggests again that unidentified nitrogenous components exist in the lipopolysaccharide.

Sugars. Table 6 gives the results for the total carbohydrate contents of different batches of unhydrolysed lipopolysaccharide determined with the anthrone-sulphuric acid and phenol-sulphuric acid methods. Glucose, the principal monosaccharide, was determined with 4 hr. hydrolysates (0.5 M-sulphuric acid) by the glucose oxidase method.

By the Dische reaction, the lipopolysaccharide was found to contain 3.0% of rhamnose (total of glucose and rhamnose, 12–13%). The unspecific nature of the phenol-sulphuric acid method is reflected in the results in Table 6; ribose, present in all preparations in the contaminating nucleic acid, reacts strongly, giving a colour intensity 1.4 times as great as that of an equivalent weight of glucose. Amino sugars do not undergo the reaction, but the heptose of the lipopolysaccharide may interfere. By using the results obtained by Osborn (1963) with L-glycero-D-mannoheptose, the heptose content of the lipopolysaccharide was found to be 1.6–1.8%. However, if the estimation is based on results for D-glycero-D-galactoheptose (Rebers, Heddleston & Rhoades, 1967), the lipopolysaccharide of *P. aeruginosa* could contain up to 3.6% of heptose.

The lipopolysaccharide contained 2.3–2.9% of 3-deoxy-2-octulonic acid, as determined by the thiobarbituric acid reaction. Attempts to detect 3-deoxy-2-octulonic acid in hydrolysates of lipopolysaccharide were, however, unsuccessful. Literature values for this sugar range from 1–2% for lipopolysaccharide of *E. coli* (Taylor *et al.* 1966) to

Table 7. *Percentage composition of the lipopolysaccharide*

Phosphorus (expressed as orthophosphoric acid)	13–18
Ninhydrin-positive components	9–10
Monosaccharides (glucose, rhamnose, heptose and 3-deoxy-2-octulonic acid)	16–17
Fatty acids (expressed as palmitic acid)	13–20
Nucleic acid	2–7
Total	53–72

5–10% for lipopolysaccharide of *Salmonella* of groups G, N and U (Simmons, Lüderitz & Westphal, 1965*a,b,c*).

Results for the analysis of sugars were: glucose, 8.6–9.7%; rhamnose, 3.0%; heptose, 1.6–1.8%; 3-deoxy-2-octulonic acid, 2.3–2.9%; total, 15.5–17.4%.

Fatty acids. Two batches of lipopolysaccharide were hydrolysed and the fatty acids determined colorimetrically. The percentages of fatty acids expressed as palmitic acid were 13.3 and 19.0%; by using the procedure of O'Brien & Rouser (1964), 65–75% of these fatty acids were found to be hydroxy acids. The fraction containing hydroxy acids was examined by g.l.c. and consisted of three acids, which were identified by comparison with reference compounds as 3-hydroxydodecanoic acid, 2-hydroxydodecanoic acid and 3-hydroxydecanoic acid. No 3-hydroxytetradecanoic acid was present. The fatty acid fraction contained mainly lauric acid and palmitic acid; two other components were present in smaller amounts and these were tentatively identified as dec-2-enoic acid and dodec-2-enoic acid.

Percentage composition of the lipopolysaccharide. Table 7 indicates the ranges obtained when analyses were carried out on different batches of lipopolysaccharide; one batch was analysed for all the components listed, and the recovery was 63.9%. Overall recoveries are seldom reported for lipopolysaccharides; incomplete recoveries of 75–85% have, however, been reported particularly for lipopolysaccharides of R mutants of *Salm. minnesota* and *Salm. riuiri* (Lüderitz & Westphal, 1966; Lüderitz *et al.* 1966*b*). The total content of sugars, excluding

Table 8. *Relative amounts of fatty acids in the ethyl formate extract of lipid A and in acid hydrolysates of lipid A*

	Composition of sample (% w/w, of total)			
	Ethyl formate extract	Residual lipid A*	Residual lipid A†	Whole lipid A†
Lauric acid	22.5	14.7	11.8	8.4
3-Hydroxydecanoic acid	1.1	13.5	14.6	14.0
2-Hydroxydodecanoic acid	17.0	16.7	18.5	22.8
Palmitic acid	30.0	9.0	7.8	8.6
3-Hydroxydodecanoic acid	24.0	32.6	35.2	40.2
Unknown (X)‡	0.0	2.2	0.4	0.2
Unknown (Y)§	5.4	10.4	11.7	5.8

* Determined after hydrolysis with 5 M-HCl for 16 hr.

† Determined after hydrolysis with constant-boiling HCl for 4 hr.

‡ Carbon number 11.7 (polyethylene glycol succinate column); possibly dec-2-enoic acid.

§ Carbon number 12.9 (polyethylene glycol succinate column); possibly dodec-2-enoic acid.

glucosamine, which occurs mainly in lipid A, but including galactosamine and fucosamine, is about 20%, appreciably lower than values obtained for lipopolysaccharides of the Enterobacteriaceae, e.g. 48–74% for *Shigella flexneri*.

Lipid A. Lipid A was obtained as a light-brown grease in yields of 23.4–33.0% over four experiments. In two cases free fatty acids were extracted from the lipid by using ethyl formate and amounted to 4.7 and 7.9% of the lipid. The methyl esters of one of these batches of free fatty acids and of the fatty acids liberated by hydrolysis of the two batches of residual lipid after ethyl formate extraction were analysed by g.l.c. (Table 8). The methyl esters of the acids (52.0%, by wt.) liberated from one batch of lipid A that had not been treated with ethyl formate were also analysed (Table 8). The values represent the relative amounts of the constituent fatty acids as determined by the relative areas of the peaks on the chromatograms. The quantitative results were most readily obtained from chromatograms obtained with the polar column (see the Materials and Methods section), except for palmitic acid and 2-hydroxydodecanoic acid, which were unresolved. Results for these acids were obtained with the non-polar column.

The acids are mainly hydroxy acids, although 52.5% of the free fatty acids in the ethyl formate extract are lauric acid and palmitic acid. This shows that the saturated fatty acids are rather more readily cleaved by the hydrolysis procedure used to release lipid A. Colorimetric determination of the fatty acid content of whole lipid A gave 44.2%, expressed as palmitic acid, compared with 52% obtained by direct weighing of the acids released on hydrolysis. The principal fatty acid of lipid A is 3-hydroxydodecanoic acid; 3-hydroxytetradecanoic

acid, a characteristic component of lipid A moieties of enterobacterial lipopolysaccharides, is absent. Hancock & Meadow (1967) have shown that the same three hydroxy acids listed in Table 8 are present in the lipopolysaccharide of *P. aeruginosa* (N.C.T.C. 8602). Further, these acids occur in the bound lipids of *Azotobacter agilis* (Kaneshiro & Marr, 1963), and 2- and 3-hydroxydodecanoic acid occur in the bound lipids of *Rhodocrobium vanneleii* (Park & Berger, 1967). The presence of 3-hydroxydecanoic acid in cultures of *P. aeruginosa* has been reported by Bergström, Theorell & Davide (1946) and Jarvis & Johnson (1949).

The absence of 3-hydroxytetradecanoic acid led us to determine which acid replaced it as the *N*-acylating component of the structure. Lipid A was treated with methanolic potassium hydroxide to cleave *O*-acyl groups (Kasai, 1966; Dawson, 1954); the liberated esters and any free fatty acids (lipid A-MS) were methylated to ensure complete esterification, and the esters identified by g.l.c. The residue was submitted to more vigorous hydrolysis to liberate the remaining fatty acids (lipid A-MP), which were similarly identified. The results (Table 9) show that 3-hydroxydodecanoic acid constituted the main residual acylating group after alkaline hydrolysis, indicating that this is the main amide-bound acid of lipid A. Unknown Y was also concentrated in the acids bound in the residual fraction, suggesting that, if this component is dodec-2-enoic acid, it may be an artifact associated with 3-hydroxydodecanoic acid. The lipid A moiety is therefore similar to the lipid A moieties of lipopolysaccharides of the Enterobacteriaceae in having the main hydroxy acid present in amide linkage.

The i.r. spectrum of lipid A, although generally similar to that obtained by Clarke *et al.* (1965, 1967c),

Table 9. *Fatty acid components of lipid A and the fractions obtained after deacylation*

The compositions were calculated from the relative areas of the peaks. +, Only a small amount detected.

	Composition of sample (%, w/w, of total)		
	Lipid A	Lipid A-MS	Lipid A-MP
Lauric acid	11.8	19.5	0.0
3-Hydroxydecanoic acid	14.6	10.8	9.6
2-Hydroxydodecanoic acid	18.5	64.0	3.3
Palmitic acid	7.8		
3-Hydroxydodecanoic acid	35.2	5.7	76.1
Unknown (X)*	0.4	0.0	0.0
Unknown (Y)*	11.7	+	10.9

* See footnotes in Table 8.

Table 10. *Amino compound composition of lipid A (%)*

The results were obtained after hydrolysis of lipid A for 4 hr. at 105° with constant-boiling HCl. +, Only a small amount detected.

	Prep. no. ... 1	2	3	4
Unknown (C)*	(2.8)	(2.9)	(3.2)	(2.9)
Alanine	0.86	0.90	0.86	1.12
Glucosamine	9.85	9.28	9.74	10.72
Galactosamine	1.66	1.66	1.78	1.70
Fucosamine	0.0	0.0	+	0.0
Protein	0.65	0.79	+	+

* Believed to be an *O*-phosphorylglucosamine; results expressed in terms of glucosamine.

differed in showing less absorption in the region 1000–1250 cm.⁻¹ and in containing fewer maxima in the region 750–1000 cm.⁻¹

The nitrogen content of lipid A was 2.1%, and the phosphorus content was 2.8–3.3%, giving a nitrogen/phosphorus ratio in g.atoms of 3:2. This is rather lower than the value obtained by Burton & Carter (1964) for lipid A obtained from *E. coli* O₁₁₁B₄.

Analysis of lipid A for amino compounds gave the results in Table 10, showing that, although glucosamine was the main component, galactosamine and alanine were also present.

The remarkably constant amounts of the first four components listed in Table 10 suggested that these were all genuine components of lipid A. However, more recent investigations in these laboratories suggest that the alanine and galactosamine arise by contamination of lipid A by degraded portions of the polysaccharide moiety. The constant amounts of the two components would be explained by sedimentation of a constant amount of a degradation product of the polysaccharide with lipid A. The virtual absence of fucosamine and the presence of only a small amount of glucose would be explained

if the degradation products rich in these sugars were more water-soluble and sedimented less efficiently with lipid A.

Amino compounds accounted for 81.4% of the total nitrogen of lipid A, a recovery comparable with that obtained for cell walls, but much higher than for lipopolysaccharide.

The overall weight recovery for lipid A was: fatty acids (determined by weight), 52%; phosphorus (as orthophosphoric acid), 10.1%; amino compounds, 15.9%; glucose, 1.0%; total, 79%. The molar ratios of the components of lipid A were: fatty acid, 3.00; glucosamine, 0.87; galactosamine, 0.11; alanine, 0.14; phosphorus, 1.30. In obtaining the molar ratio for glucosamine, the contribution from unknown component C (Table 10) was included, assuming this to be an *O*-phosphorylglucosamine.

If the alanine and galactosamine of lipid A arise from contamination by the polysaccharide moiety, then, since lipid A constitutes about 27% of the lipopolysaccharide, a comparison of the results in Tables 5 and 10 suggests that the extent of this contamination is about 20%. Similar calculations based on the glucose content (1.0%) of lipid A and of whole lipopolysaccharide (Table 6) indicate that

this contamination may be about 10%. The average phosphorus contents of lipid A and lipopolysaccharide are 3.0 and 4.7% respectively. Lipid A therefore contributes about 0.8% to the total phosphorus of the lipopolysaccharide, and the phosphorus content of the polysaccharide moiety would be calculated as about 5.3%. If the extent of polysaccharide contamination of lipid A is taken to be about 15%, then this could account for about one-quarter of the phosphorus content of lipid A, and the molar ratio of lipid A phosphorus would be decreased from 1.3 to less than 1.0. Of the 3 mol.prop. of fatty acid, 1.14 mol.prop. is contributed by 3-hydroxydodecanoic acid (the main *N*-acylating acid), and just under 2 mol.prop. by the other acids. This suggests that lipid A is built up from *N*-3-hydroxydodecanoylglucosamine residues, each of which is attached to a phosphate-containing group, such that the glucosamine/phosphorus ratio is about 1.0, and to two other fatty acid residues. If the two fatty acid residues and the phosphate group are in ester linkage with hydroxyl groups of *N*-acylated glucosamine residues, then no hydroxyl groups of the glucosamine residues are available to form glycosidic linkages. In this case the glucosamine residues would have to be linked by phosphodiester bridges. If glycosidic linkages are involved between glucosamine residues, then the hydroxyl group of 1 mol. of hydroxy acid (either *O*- or *N*-acylating)/glucosamine residue must be involved in ester linkage with either 1 mol. of phosphoric acid or 1 mol. of a fatty acid. The presence of substantial amounts of *O*-phosphorylglucosamine in hydrolysates (Table 10) of lipid A suggests that some of the phosphoric acid at least is not esterified by the hydroxy acids, but no clear evidence permitting a distinction between the possibilities that phosphodiester or glycosidic linkages exist between the glucosamine residues has so far been obtained.

Polysaccharide moiety of the lipopolysaccharide. Attempts to obtain the polysaccharide component of the lipopolysaccharide by using modifications of the procedures of Taylor *et al.* (1966) and Osborn (1963) were not successful. By using the former procedure, but with four treatments with acetic acid at 100° instead of only one, the yield of material was only 21.2%, less than 30% of that obtained by Taylor *et al.* (1966). With Osborn's (1963) procedure residual material was sedimented from the supernatant fluid at a lower *g* value to ensure that polysaccharide was not lost, but again the yield was low (21.6%). Analyses were carried out on these 'polysaccharide' fractions and the results compared with average results obtained for whole lipopolysaccharide (Table 11). At the most, only 14% of the carbohydrate of the lipopolysaccharide is solubilized by treatment with acetic acid, contrasting markedly with the result (98%) obtained by

Table 11. *Analytical results (%) for the 'polysaccharides' liberated by dilute acetic acid from whole lipopolysaccharide*

—, Not determined			
Prep. ...	PS*	PS†	Lipopolysaccharide‡
Carbohydrate§	10.6	12.7	19.5–23.2
Glucose	1.8	4.1	8.6–9.7
3-Deoxy-2-octulonic acid	1.0	—	2.3–2.9
Phosphorus	9.8	6.8	4.1–5.7
Nucleic acid	13.2	2.8	2.3–7.2
Amino compounds	3.8	—	8.5–9.0

* 'Polysaccharide' isolated after four treatments (15 min. in each case) with aqueous 0.01 M-acetic acid at 100°, followed by centrifugation at 100000*g* for 2 hr.

† 'Polysaccharide' isolated after four treatments (30 min. in each case) with aqueous 0.01 M-acetic acid at 100°, followed by centrifugation at 15000*g* for 1 hr.

‡ Average results for several lipopolysaccharide preparations.

§ Determined by the phenol-H₂SO₄ method, with glucose standards.

Osborn (1963) for the lipopolysaccharide of *Salm. typhimurium*.

The high phosphorus contents of the 'polysaccharide' fractions were not accounted for by the nucleic acid present. It is possible that treatment with acetic acid splits off phosphate groups from whole lipopolysaccharide and these are recovered in the 'polysaccharide' fraction. Inorganic phosphate was in fact identified in unhydrolysed 'polysaccharide' by t.l.c. on silica gel G (solvent system c). Two other phosphorus-positive spots with *R_F* values 0.35 and 0.51 were also detected, but were not identified.

For one of the 'polysaccharide' preparations, the total weight recovery of identifiable components was only about 45%. All the nitrogen was accounted for in terms of amino compounds and nucleic acid, showing that unknown nitrogenous components of the lipopolysaccharide were not present in this 'polysaccharide' fraction. The remaining material could not be accounted for by t.l.c. of the 'polysaccharide' and various of its hydrolysates. By using silica gel G and solvent system c and 'polysaccharide' that had been hydrolysed with 2M-hydrochloric acid for 3 hr., treatment of t.l.c. plates with Schiff reagent after oxidation with periodate gave spots in the position expected for trace amounts of the known sugars. A more intense spot (*R_F* 0.51) and a weaker spot (*R_F* 0.67) were also detected. The former spot was not glycerol and the component with *R_F* 0.67 gave no colour with thiobarbituric acid reagent. Chromatography of 'polysaccharide' that had been less vigorously hydrolysed also failed to

detect 3-deoxy-2-octulonic acid, and there was no evidence for the presence of ribitol.

The difficulty in preparing the polysaccharide moiety of the lipopolysaccharide of *P. aeruginosa* marks a major difference between this lipopolysaccharide and those of the Enterobacteriaceae. The residual material from treatment of whole lipopolysaccharide with dilute acetic acid was degraded under conditions used to liberate lipid A. Analysis of the material remaining after the lipid A had been separated off showed that it contained alanine, galactosamine and fucosamine in approximately the same amounts as in lipopolysaccharide. Amino compounds and nucleic acid accounted for only 38% of the total nitrogen, indicating that unknown nitrogenous components are concentrated in this fraction. The fraction contained only a trace of glucosamine (the recovery of glucosamine in lipid A was about 80%) and 2.0% of phosphorus, showing again that most of the phosphorus of the lipopolysaccharide is removed by treatment with dilute acetic acid.

Apart from the differences in the fatty acid residues of the lipid A moiety, the major difference between the lipopolysaccharide of *P. aeruginosa* and the lipopolysaccharides of the Enterobacteriaceae therefore seems to reside in the polysaccharide moiety. The material released from *P. aeruginosa* lipopolysaccharide by treatment with dilute acetic acid contains inorganic phosphate, traces of sugars and much unidentified material, and the residual material, after the removal of lipid A, represents some 50% of the whole lipopolysaccharide and may contain substantial amounts of nitrogenous components other than those identified by automatic analysis for ninhydrin-positive systems.

The authors thank the Medical Research Council for a maintenance grant (A.H.F.) and the Science Research Council for a grant for the purchase of the amino acid analyser. They also thank the Microbiological Research Establishment, Porton, Wilts., for the supply of large quantities of whole cells of *P. aeruginosa*, Dr J. S. Brimacombe, Department of Chemistry, University of Birmingham, for the gift of a sample of fucosamine and Mr P. Worthington, Department of Physics, University of Hull for electron micrographs. They are also grateful to Mr B. Crowder, who prepared a sample of 2-chloroethylamine, and Mr J. Cooper, who synthesized samples of 2- and 3-hydroxydodecanoic acid and 3-hydroxydecanoic acid.

REFERENCES

- Allen, R. J. L. (1940). *Biochem. J.* **34**, 588.
- Archibald, A. R. & Baddiley, J. (1966). *Advanc. Carbohydr. Chem.* **21**, 323.
- Barker, S. A., Bick, S. M., Brimacombe, J. S. & Somers, P. J. (1966). *Carbohydrate Res.* **1**, 393.
- Bartlett, G. R. (1959). *J. biol. Chem.* **234**, 466.
- Bergström, S., Theorell, H. & Davide, H. (1946). *Arch. Biochem.* **10**, 165.
- Burton, A. J. & Carter, H. E. (1964). *Biochemistry*, **3**, 411.
- Cerioti, G. (1952). *J. biol. Chem.* **198**, 297.
- Clarke, K., Gray, G. W. & Reaveley, D. A. (1965). *Nature, Lond.*, **208**, 586.
- Clarke, K., Gray, G. W. & Reaveley, D. A. (1967a). *Biochem. J.* **105**, 749.
- Clarke, K., Gray, G. W. & Reaveley, D. A. (1967b). *Biochem. J.* **105**, 755.
- Clarke, K., Gray, G. W. & Reaveley, D. A. (1967c). *Biochem. J.* **105**, 759.
- Cramer, F. (1955). *Paper Chromatography*, p. 47. Transl. by Richards, L. London: Macmillan and Co. Ltd.
- Cummins, C. S. & Harris, H. (1956). *J. gen. Microbiol.* **14**, 583.
- Dawson, R. M. C. (1954). *Biochim. biophys. Acta*, **14**, 374.
- Dische, Z. (1955). *Meth. biochem. Anal.* **2**, 313.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
- Dustin, J. P., Czajkowska, C., Moore, S. & Bigwood, E. J. (1953). *Analyt. chim. Acta*, **9**, 256.
- Fraenkel-Conrat, H., Harris, J. I. & Levy, A. L. (1955). *Meth. biochem. Anal.* **2**, 359.
- Gray, G. W. & Wilkinson, S. G. (1965a). *J. appl. Bact.* **28**, 153.
- Gray, G. W. & Wilkinson, S. G. (1965b). *J. gen. Microbiol.* **39**, 385.
- Hancock, I. C. & Meadow, P. M. (1967). *J. gen. Microbiol.* **46**, x.
- Hanessian, S. & Haskell, T. H. (1964). *J. biol. Chem.* **239**, 2758.
- Itaya, K. & Ui, M. (1965). *J. Lipid Res.* **6**, 16.
- Jarvis, F. G. & Johnson, M. J. (1949). *J. Amer. chem. Soc.* **71**, 4124.
- Kaneshiro, T. & Marr, A. G. (1963). *Biochim. biophys. Acta*, **70**, 271.
- Kasai, N. (1966). *Ann. N.Y. Acad. Sci.* **133**, 486.
- Lüderitz, O., Galanos, C., Risse, H. J., Ruschmann, E., Schlecht, S., Schmidt, G., Schulte-Holthausen, H., Wheat, R. W., Westphal, O. & Schlosshardt, J. (1966a). *Ann. N.Y. Acad. Sci.* **133**, 349.
- Lüderitz, O., Staub, A. M. & Westphal, O. (1966b). *Bact. Rev.* **30**, 192.
- Lüderitz, O. & Westphal, O. (1966). *Angew. Chem. (int. Ed.)*, **5**, 198.
- Mackie, T. J. & McCartney, J. E. (1960). In *Handbook of Bacteriology*, p. 308. Ed. by Cruickshank, R. Edinburgh and London: E. and L. Livingstone Ltd.
- Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.
- Metcalfe, L. D. & Schmitz, A. A. (1961). *Analyt. Chem.* **33**, 363.
- Norton, J. E., Bulmer, G. S. & Sokatch, J. R. (1963). *Biochim. biophys. Acta*, **78**, 136.
- O'Brien, J. S. & Rouser, G. (1964). *Analyt. Biochem.* **7**, 288.
- Osborn, M. J. (1963). *Proc. nat. Acad. Sci., Wash.*, **50**, 499.
- Osborn, M. J., Rosen, S. M., Rothfield, L. & Horecker, B. L. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1831.
- Park, C. E. & Berger, L. R. (1967). *J. Bact.* **93**, 230.
- Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
- Pusztai, A. & Morgan, W. T. J. (1963). *Biochem. J.* **88**, 546.
- Rebers, P. A., Heddleston, K. L. & Rhoades, K. R. (1967). *J. Bact.* **93**, 7.

- Roberts, N. A., Gray, G. W. & Wilkinson, S. G. (1967). *Biochim. biophys. Acta*, **135**, 1068.
- Salton, M. R. J. (1953). *The Bacterial Cell Wall*, p. 93. Amsterdam: Elsevier Publishing Co.
- Salton, M. R. J. & Horne, R. W. (1951). *Biochim. biophys. Acta*, **7**, 177.
- Simmons, D. A. R., Lüderitz, O. & Westphal, O. (1965a). *Biochem. J.* **97**, 807.
- Simmons, D. A. R., Lüderitz, O. & Westphal, O. (1965b). *Biochem. J.* **97**, 815.
- Simmons, D. A. R., Lüderitz, O. & Westphal, O. (1965c). *Biochem. J.* **97**, 820.
- Sutherland, I. W. & Wilkinson, J. F. (1966). *Biochim. biophys. Acta*, **117**, 261.
- Suzuki, N. (1969). *Biochim. biophys. Acta*, **177**, 371.
- Taylor, A., Knox, K. W. & Work, E. (1966). *Biochem. J.* **99**, 53.
- Trevelyan, W. E. & Harrison, J. S. (1952). *Biochem. J.* **50**, 298.
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques*, 3rd ed., p. 274. Minneapolis: Burgess Publishing Co.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1964). *Manometric Techniques*, 4th ed., p. 208. Minneapolis: Burgess Publishing Co.
- Weissbach, A. & Hurwitz, J. (1959). *J. biol. Chem.* **234**, 705.
- Westphal, O. & Jann, K. (1965). In *Methods in Carbohydrate Chemistry*, vol. 5, p. 83. Ed. by Whistler, R. L. New York: Academic Press Inc.