The Extraction of Cell Walls of *Pseudomonas aeruginosa* with Aqueous Phenol

MATERIAL FROM THE PHENOL LAYER

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1. A method is described for the fractionation of cell walls of *Pseudomonas aeruginosa* by using aqueous phenol. 2. With this technique, cell walls are quantitatively separated into five fractions: two water-soluble fractions (AqI and AqII), a residual insoluble fraction (R), and two phenol-soluble fractions (PhMP and PhMS). 3. The compositions of fractions PhMP and PhMS are discussed. Fraction PhMP consists almost entirely of protein, which is soluble in aqueous sodium dodecyl sulphate. Analytical ultracentrifugation of the solution yields a single peak, but several components may be resolved by gel electrophoresis. 4. Fraction PhMS consists principally of phospholipid (approx. 44%) and fatty acids (approx. 27%), although smaller amounts (approx. 13%) of protein are present.

The cell walls of Gram-negative bacteria have been found (Salton, 1964) to contain four major components: murein, protein, lipopolysaccharide and lipid. Early investigations by Weidel and co-workers (Weidel & Primosigh, 1958; Weidel, Frank & Martin, 1960) showed that the cell walls of *Escherichia coli* consisted of inner layers of lipopolysaccharide and murein, surrounded by an outer layer of lipoprotein. More recently, Martin (1963) has shown that globules of protein are attached to the murein. Such a structure is in accord with recent electron-microscopic observations of thin sections of Gram-negative cells by Murray, Steed & Elson (1965).

By treatment with aqueous phenol, Wheat, Rollins, Leatherwood & Barnes (1963) were able to separate cells of *Chromobacterium violaceum* into insoluble material and material soluble in the aqueous and phenolic phases. The aqueous phase contained lipopolysaccharide and rRNA, whereas murein occurred in the insoluble residue, and protein in the phenol-soluble fraction. Similar results had been obtained earlier by Westphal, Lüderitz & Bister (1952).

It was thought that such a procedure might prove valuable for the quantitative fractionation of cell walls. This paper describes the phenol extraction technique as applied to cell walls of *Pseudomonas aeruginosa*, and describes the nature of the materials isolated from the phenol layer.

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MATERIALS AND METHODS

Growth of organism and preparation of cell walls. Cells of *Pseudomonas aeruginosa* (N.C.T.C. 1999) were grown and the cell walls prepared as described in the preceding paper (Clarke, Gray & Reaveley, 1967).

Qualitative and quantitative methods of analysis and infrared spectra. Details for most of the methods have been given in the preceding paper (Clarke *et al.* 1967). For the identification of the fatty acid components of fraction PhMS, thin-layer chromatography was carried out by the method of Kaufmann & Makus, as described by Randerath (1963).

Treatment of cell walls with aqueous phenol. Cell walls (approx. 200 mg.) were treated with aq. 45% (w/v) phenol (30 ml.) at 72–74° for 30 min. The mixture was cooled in ice and centrifuged (2000g for 1 hr. at 4°) and the layers were separated. The insoluble residue at the interface was once more treated with aq. phenol, and the layers were again separated. The aqueous layers from the two extractions were kept separate and, after dialysis (3 days at 4°) to remove phenol, freeze-drying gave colourless solids that were designated fractions AqI and AqII.

The insoluble residue was dispersed in ion-depleted water, and the suspension dialysed (3 days at 4°). Freeze-drying of the non-diffusible material gave the residue (fraction R). The two phenolic layers were combined and treated with methanol (150 ml.) at –10° for 24 hr. to precipitate protein (designated fraction PhMP). The supernatant fluid was concentrated by distillation under reduced pressure at 22° and the phenol removed by dialysis (5 days at 4°). Freeze-drying of the non-diffusible material gave a mixture of lipids designated fraction PhMS.

Fractionation of the lipids of fraction PhMS. Fraction PhMS invariably contained protein (approx. 13%), which

was removed by extracting the lipids into chloroform-methanol (2:1, v/v) and passing the mixture through a sinter (no. 3 porosity). The purified lipids were fractionated by the micro-method of Lis, Tinoco & Okey (1961). The lipid mixture (3–7 mg.) was dissolved in light petroleum (b.p. 60–80°) (1 ml.) and the solution applied to the silicic acid column. Four fractions were eluted from the column with the solvents (15 ml. in each case) recommended by Lis *et al.* (1961). All solvents were dried and distilled before use.

Removal of small quantities of lipids from fraction PhMP. Fraction PhMP (approx. 12 mg.) was shaken with chloroform-methanol (2:1, v/v; 10 ml.) at room temperature for 1 hr. Filtration through a sinter (no. 1 porosity) gave a residue on which the extraction procedure was repeated five more times. The residue was finally dried *in vacuo* over P₂O₅ and designated lipid-free fraction PhMP.

Solubilization of lipid-free fraction PhMP. Lipid-free fraction PhMP (10 mg.) was solubilized by shaking with a solution of sodium dodecyl sulphate (0.5%) in 0.05 M-tris-HCl buffer, pH 7.6 (2 ml.). The resulting solution was examined by zone electrophoresis in polyacrylamide gels as described by Davis (1964). The solution was also submitted to analytical ultracentrifugation.

RESULTS AND DISCUSSION

Treatment of cell walls with aqueous phenol. Results obtained from the treatment of cell walls of *P. aeruginosa* with aqueous phenol are given in Table 1. Although the percentage recoveries show that little cell-wall material was lost during the extractions, a further check was made by calculating the recoveries of phosphorus or nitrogen or both for some of the extractions. These results (Table 2) confirm that no significant amount of a particular component was liberated during the extraction and subsequently lost on dialysis. As shown in Table 1, the combined aqueous extracts constitute on average 36–37% of the cell wall, and the insoluble residue represents on average 13–14% of the wall. The results of analyses of these fractions are not given in the present paper, which sets out to describe the investigations made on the phenol-soluble materials, representing about 43% of the cell wall.

Fraction PhMS. The i.r. spectra of various samples of fraction PhMS indicated that these lipid mixtures contained large amounts of fatty material,

and the high phosphorus content (approx. 2%) of fraction PhMS suggested that much of the lipid was phospholipid. The nitrogen content of fraction PhMS was low (3.3–4.2%), and correspondingly amino compounds (Table 3) accounted for only about 17% of the material. Ethanolamine, serine and ornithine, which may be genuine phospholipid components, comprised about 4% of fraction PhMS. The concentrations of ethanolamine and ornithine were considerably higher than in cell wall (Clarke *et al.* 1967), and serine was the amino acid present in largest amount. The presence of ornithine is noteworthy, for this amino acid has been detected in lipids extracted from whole cells (Kates, Adams & Martin, 1964; Houtsmuller & van Deenen, 1965) and is thought to be present as an *O*-ester of phosphatidylglycerol. No simple sugars were detected in fraction PhMS, indicating the absence of glycolipids (Brundish, Shaw & Baddiley, 1966).

The lipids were extracted from fraction PhMS by treatment with chloroform-methanol and fractionated on silicic acid. The insoluble material was largely protein (cf. Table 3, which shows that approx. 13% of protein amino acids occur in the original fraction PhMS). Allowing for an average of 13% of protein in fraction PhMS, 88–98% of the chloroform-methanol-soluble lipid was recovered from silicic acid columns. About 8% of the soluble lipid was eluted in fraction I and the infrared spectrum indicated that the material was aliphatic ester. This component of the lipid may arise as an artifact, because fraction PhMS contained significant amounts of fatty acids, some of which may have

Table 2. *Phosphorus, nitrogen and weight recoveries after extraction of cell walls with aqueous phenol*

Extraction	Recovery (%)		
	Phosphorus	Nitrogen	Weight
1	—	101	97.1
2	84	92	86.6
3	100	—	98.8

—, Not determined.

Table 1. *Percentages of fractions obtained by treatment of cell walls with aqueous phenol*

Extraction	% of cell wall					Recovery
	Fraction AqI	Fraction AqII	Fraction R	Fraction PhMP	Fraction PhMS	
1	24.8	13.6	12.3	28.3	18.1	97.1
2	20.0	11.2	14.5	21.8	19.1	86.6
3	31.4	11.6	14.6	24.2	17.0	98.8
4	23.5	10.5	12.3	24.0	19.7	90.0
Average	24.9	11.7	13.4	24.5	18.4	93.1

Table 3. *Quantitative analyses for the amino compounds of fraction PhMS and lipid-free fraction PhMP*

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. Separate batches of fraction PhMS and lipid-free fraction PhMP gave very similar analyses, the concentrations of individual amino compounds agreeing in general within $\pm 5\%$.

	% of sample	
	Fraction PhMS	Lipid-free fraction PhMP
<i>O</i> -Phosphorylethanolamine	0.35	0.0
Aspartic acid	0.99	11.0
Threonine	Trace	4.61
Serine	1.50	3.94
Muramic acid	0.0	0.0
Glutamic acid	1.22	8.84
Proline	1.07	3.11
Glycine	0.80	4.98
Alanine	0.99	5.44
Glucosamine	1.21	Trace
Valine	0.77	5.26
Galactosamine	0.0	0.0
Cystine	0.0	0.85
Methionine	Trace	1.54
Isoleucine	0.83	3.11
2,6-Diaminopimelic acid	0.0	Trace
Leucine	1.33	6.99
Tyrosine	0.44	5.11
Phenylalanine	0.66	5.13
Ethanolamine	1.29	0.0
Ammonia	0.17	0.98
Ornithine	0.87	0.0
Lysine	0.99	4.87
Histidine	0.56	2.04
Arginine	0.51	5.55
Total	16.8	83.3

become esterified during the treatment with methanol (Lough, Felinski & Garton, 1962).

About 29–33% of the soluble lipids was distributed between fractions II and III, and this material was shown by infrared spectroscopy to consist mainly of fatty acids. Although the sum of these two fractions was approximately constant, the relative amounts of the two were variable, and it is not certain whether this variation reflects a genuine difference in fatty acid composition from batch to batch of cell walls or was caused mainly by variation in the packing of the silicic acid column. Thin-layer chromatography revealed that both fractions were complex mixtures of saturated and unsaturated acids. Palmitic acid predominated in both fractions, but neither contained any β -hydroxy acids.

The fatty acids are thought to exist as such in the

isolated cell walls and not to be produced during the extraction procedure with aq. phenol, since Gray & Wilkinson (1965) extracted free fatty acids from freshly prepared cell walls with chloroform-methanol at room temperature. The fatty acids may, of course, be liberated from labile lipids during the preparation of the cell walls.

The bulk of the soluble lipids of fraction PhMS (approx. 50–60%) was eluted from the column in fraction IV (phospholipid). The infrared spectrum of this fraction closely resembled that of phosphatidylethanolamine. The absence of large amounts of lecithins and lipids containing sphingosine was inferred from the absence of an absorption maximum at 970 cm^{-1} , and the side peak at 1040 cm^{-1} was characteristic for phospholipids containing ethanolamine (Marinetti & Stotz, 1954). The ratio of the extinctions at 1666 and 1555 cm^{-1} was high compared with that in the spectrum of *L*- α -(dipalmitoyl)phosphatidylethanolamine, and this indicated the presence of other amino compounds. 6*N*-Hydrochloric acid hydrolysates of the phospholipid fraction were in fact found to contain, in addition to ethanolamine, traces of aspartic acid, alanine and glutamic acid, and larger amounts of serine, ornithine and glucosamine.

By using average values, these preliminary analyses of the fractions designated PhMS show that fatty acids (approx. 27%), fatty ester (approx. 6%), phospholipid (approx. 44%) and protein material (approx. 13%) account for 90% of the material.

Fraction PhMP. The nitrogen content (14.8–15.4%) of fraction PhMP was similar to that of a typical protein (15–16%), and protein estimations, with bovine albumin as standard, indicated that the fraction contained 104–112% of protein. This high value merely indicates that the protein of fraction PhMP is more chromogenic than the bovine albumin standard. Fraction PhMP invariably contained small amounts of phosphorus (up to 0.3%), which could be removed by extracting with chloroform-methanol. The material solubilized accounted for 0–10% of fraction PhMP. Only small amounts of this lipid were available, but the infrared spectrum resembled that of the whole lipid fraction PhMS.

Quantitative data for the ninhydrin-positive components in 6*N*-hydrochloric acid hydrolysates of lipid-free fraction PhMP are given in Table 3. Examination of the results shows that amino acids and ammonia account for at least 83% of lipid-free fraction PhMP, and the nitrogen recovery was 92.3%. 2,6-Diaminopimelic acid and glucosamine were present in only trace amounts, showing that murein (or mucopeptides) and lipopolysaccharides were virtually absent. The presence of large amounts of ammonia in the hydrolysates is taken

to indicate that appreciable amounts of asparagine and glutamine are present in lipid-free fraction PhMP. The composition of lipid-free fraction PhMP (Table 3) was similar to that of a protein isolated from the cell envelope of *P. aeruginosa* by Homma & Suzuki (1964).

Fraction PhMP was solubilized by sodium dodecyl sulphate-tris-hydrochloric acid buffer. In the analytical ultracentrifuge, the bulk of the material sedimented as a single peak that was close to symmetrical, though the sample did contain a small amount of very rapidly sedimenting, poly-disperse material. The sedimentation coefficient (corrected to 20°, but not for buffer salt) was 1.95 s. Dialysis of the fraction PhMP-sodium dodecyl sulphate-tris-hydrochloric acid buffer solution against tris-hydrochloric acid buffer followed by analytical ultracentrifugation gave a material with S_{20} (uncorrected for buffer salt) 2.54 s. It seems likely that removal of sodium dodecyl sulphate by dialysis causes the protein to reaggregate.

Results of polyacrylamide-gel electrophoresis of the fraction PhMP-sodium dodecyl sulphate-tris-hydrochloric acid buffer solution were poor owing to non-specific background staining, but nonetheless at least two separate protein bands were obtained. Together, these results indicate the presence in fraction PhMP of two proteins of similar molecular weight, but having different charge properties.

A comparison of the analytical data (Table 3) for fractions PhMS and PhMP (lipid-free) suggests that the protein (approx. 13%) present in fraction PhMS is of a different type from that of fraction PhMP. Leucine, for example, is the principal amino acid (apart from serine, which is present in lipid) of fraction PhMS, whereas leucine is only about two-thirds as abundant as aspartic acid in lipid-free fraction PhMP. More recent work (A. H. Fensom & G. W. Gray, unpublished work) shows that it cannot be assumed that all the serine present in fraction PhMS is associated with phospholipid material, i.e. as phosphatidylserine. Indeed, an excess of serine over that found in cell-wall protein occurs in a lipoprotein fraction that has been isolated from the phenolic phase obtained by carrying out aq.-phenol treatments of cell walls previously defatted with

chloroform-methanol. This lipoprotein fraction may correspond to the insoluble residue obtained in the present work when dissolving fraction PhMS for silicic acid chromatography. A close inspection of the results in Table 3 reveals other differences between the protein of fraction PhMP and the protein of fraction PhMS.

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