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

THE INSOLUBLE RESIDUE AND MATERIAL FROM THE AQUEOUS LAYERS

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1. The insoluble residue and material present in the aqueous layers resulting from treatment of cell walls of *Pseudomonas aeruginosa* with aqueous phenol were examined. 2. The products (fractions AqI and AqII) isolated from the aqueous layers from the first and second extractions respectively account for approx. 25% and 12% of the cell wall and consist of both lipopolysaccharide and mureopeptide. 3. The lipid part of the lipopolysaccharide is qualitatively similar to the corresponding material (lipid A) from other Gram-negative organisms, as is the polysaccharide part. 4. The insoluble residue (fraction R) contains sacculi, which also occur in fraction AqII. On hydrolysis, the sacculi yield glucosamine, muramic acid, alanine, glutamic acid and 2,6-diaminopimelic acid, together with small amounts of lysine, and they are therefore similar to the murein sacculi of other Gram-negative organisms. Fraction R also contains substantial amounts of protein, which differs from that obtained from the phenol layer. 5. The possible association or aggregation of lipopolysaccharide, murein and murein sacculi is discussed.

The cell walls of Gram-negative organisms have been found (Salton, 1964) to contain highly antigenic lipopolysaccharides. These macromolecular lipopolysaccharides consist of a central core to which are attached polysaccharide side chains (Lüderitz & Westphal, 1966); variation in composition and structure of the side chains gives rise to antigenic species-specificity. The core of the enterobacterial lipopolysaccharide (Osborn, Rosen, Rothfield, Zeleznick & Horecker, 1964; Grollman & Osborn, 1964; Lüderitz & Westphal, 1966) consists of heptose, *O*-phosphorylethanolamine, 3-deoxy-2-octulonic acid and a lipid designated lipid A (Westphal & Lüderitz, 1954). The lipid is split from the polysaccharide part of the core by mild acid hydrolysis and has been shown (Nowotny, 1961; Burton & Carter, 1964) to be composed of fatty acids, phosphate and D-glucosamine.

Loosely bound lipid and protein are also important components of the cell walls of Gram-negative bacteria, and, when both the lipopolysaccharide and the lipoprotein were removed from the cell wall of *Escherichia coli* (Weidel, Frank & Martin, 1960), a sacculus remained having the shape and dimensions of the cell. The entire sacculus appeared to be constructed from mureopeptides (Weidel & Pelzer, 1964) consisting of muramic acid, glucosamine,

2,6-diaminopimelic acid, alanine and glutamic acid. The results of work by Schocher, Bayley & Watson (1962) and Martin & Frank (1962) suggest that such murein sacculi occur widely in Gram-negative bacteria.

Early investigations by Weidel & Primosigh (1958) indicated that the lipopolysaccharide and the sacculus were associated in the whole cell. However, more recent work (Shands, 1965; Wardlaw, 1963) suggests that only part of the lipopolysaccharide may be so associated, and Wheat, Rollins, Leatherwood & Barnes (1963) have concluded that there is either no link between the lipopolysaccharide and the murein sacculus or that the link is easily broken. The present paper describes preliminary studies of the chemical composition of the sacculus and the lipopolysaccharide of *Pseudomonas aeruginosa*, and discusses the possible relationship between them.

MATERIALS AND METHODS

Growth of organism, preparation of cell walls and treatment of cell walls with aqueous phenol. Experimental details have been given in the preceding papers (Clarke, Gray & Reaveley, 1967a,b) for the preparation of cell walls of *Pseudomonas aeruginosa* (N.C.T.C. 1999) and for the treatment of the walls with aqueous phenol. The cell walls can be separated into five fractions: AqI and AqII (lipopolysaccharide and mureopeptide), R (murein and protein), PhMP (protein) and PhMS (lipid). The compositions of fractions PhMP and

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Scheme 1. Fractionation of cell walls of *P. aeruginosa* with aq. phenol, and of the products, fractions AqI and R.

PhMS have been described in the preceding paper (Clarke *et al.* 1967b).

In the present paper, methods are described for the further fractionation of fractions AqI, AqII and R, and Scheme 1 summarizes the situation and the designation of the fractions.

Fractionation of fraction R. A crude fractionation of fraction R was achieved by passing a suspension of fraction R (approx. 1.5 mg./ml.), in ion-depleted water, through a sinter (no. 2 porosity). The solid (fraction RI) remaining on the sinter accounted for 47–57% of fraction R. The solid obtained on freeze-drying the turbid filtrate was termed fraction RS.

Isolation of lipid from fraction AqI. Fraction AqI (approx. 20 mg.) was heated under reflux with 0.1 N-HCl or with N-H₂SO₄ (5 ml. in either case) for 30 min. The mixture was cooled to room temperature and treated with chloroform (4 × 5 ml.). The combined chloroform extracts were washed four times with water, transferred to a tared tube and the solvent was removed at room temperature with a stream of filtered O₂-free N₂. The residual lipids were dried *in vacuo* over P₂O₅ at 4°, and fractionated by the method of Lis, Tinoco & Okey (1961).

Lysozyme digestion of fractions AqI, AqII and R. Lysozyme (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) (4.5 mg.) and a few drops of toluene were added to a suspension of fraction R (15 mg.) in aq. 0.1 M-ammonium acetate (15 ml.). The mixture was then incubated for 24 hr. at 37° with frequent shaking. Insoluble material (fraction R105) was removed by centrifugation (105 000g for 1 hr. at 4°). The supernatant liquid was shaken five times with chloroform to remove lysozyme, concentrated by vacuum

distillation and freeze-dried. The residue (fraction RM) was kept under vacuum over P₂O₅ for 1 week to remove ammonium acetate. The separation of the residue into various components was achieved by descending paper chromatography with the solvent system butan-1-ol-acetic acid-water (3:1:1, by vol.) (Ghuysen & Salton, 1960). The components were identified with ninhydrin and the Morgan-Elson reagent (Salton, 1959).

With fraction AqI or fraction AqII, lysozyme digestion was carried out as described above for fraction R, but centrifugation (105 000g for 1 hr. at 4°) deposited no material. The whole digest was therefore freed from lysozyme, and the soluble material isolated as described for fraction R.

Methods of analysis. In general, these were as described in an accompanying paper (Clarke *et al.* 1967a). Heptose was estimated as described by Osborn (1963), and 3-deoxy-2-octulonic acid, after hydrolysis with 0.02 N-H₂SO₄ for 20 min. at 100°, by the method of Weissbach & Hurwitz (1959) with a final heating time of 20 min. (Osborn, 1963).

RESULTS

Insoluble residue (fraction R). Fraction R accounted for approx. 13% of the cell wall (Clarke *et al.* 1967b). Filtration of an aqueous suspension gave two fractions, insoluble material (fraction RI), equivalent to 47–57% of fraction R, and soluble material (fraction RS). Quantitative results for the ninhydrin-positive components in hydrolysates of fraction RI and of fraction RS are given in Table 1. Assuming murein to consist of glucosamine,

muramic acid, glutamic acid, alanine and 2,6-diaminopimelic acid, the results show that fraction RI contains up to 15.5% of murein and fraction RS up to 36.4% of murein. These are maximum values, since some glutamic acid and alanine are present in protein. It is apparent that murein or mucopeptide is concentrated in fraction RS. From the data in Table 1, fractions RI and RS also contain substantial amounts of protein, together with smaller amounts of lipopolysaccharide and possibly lipid.

Under the electron microscope (Plate 1), at least two kinds of material were clearly visible in fraction R. Much of the material was amorphous, but some was similar in shape to cell walls. These rod-like bodies, which were concentrated in fraction RS, were

thinner and more elongated than cell walls, and on close inspection appeared to consist of two layers: an inner electron-dense layer and an outer electron-transparent layer. Most of the rod-like structures appeared to be solubilized on treatment of fraction R with lysozyme. The resulting mixture was centrifuged at 105 000*g* to give a supernatant fluid and an insoluble residue (fraction R105). This residue consisted mainly of protein, with some lipopolysaccharide and lipid, and was almost completely soluble in a solution of sodium dodecyl sulphate in tris-hydrochloric acid buffer (Clarke *et al.* 1967*b*). After removal of insoluble material, the solution was submitted to analytical ultracentrifugation. The material sedimented as a single peak, which was almost symmetrical, and the sedimentation coefficient (corrected to 20°, but not for buffer salt) was 1.41*s*. This value is different from that (1.95*s*) obtained for the protein from the phenol phase of the cell-wall extraction (Clarke *et al.* 1967*b*).

A colourless solid (fraction RM), which accounted for 25% of fraction R, was isolated from the supernatant fluid after lysozyme digestion. Acid hydrolysis followed by paper chromatography showed fraction RM to consist of glucosamine, muramic acid, alanine, glutamic acid and 2,6-diaminopimelic acid, although small amounts of other amino acids were present. Paper chromatography of an unhydrolysed sample of fraction RM indicated the presence of a number of ninhydrin-positive components (at least eight), several of which gave positive reactions for *N*-acetyl amino sugars. Material similar to fraction RM has been isolated from Gram-negative bacteria (Weidel & Pelzer, 1964). Thus, by analogy, fraction RM is

Table 1. *Quantitative analyses for the amino compounds of fractions RI and RS*

Values for amino acids refer to amino acid residues, but all other values refer to free compounds. Results are corrected for destruction or slow release during hydrolysis, being based on a hydrolysis time of 16 hr. The values for valine and galactosamine have not been corrected. +, Present, but no quantitative estimate could be made.

	Amount in sample (%)	
	Fraction RI	Fraction RS
<i>O</i> -Phosphorylethanolamine	Trace	Trace
Aspartic acid	5.82	2.83
Threonine	2.97	+
Serine	2.56	+
Muramic acid	1.85	10.9
Glutamic acid	5.69	7.0
Proline	1.65	Trace
Glycine	3.11	1.32
Alanine	4.33	5.74
Glucosamine	2.18	6.87
Valine	2.73	1.54
Galactosamine*	Determined with valine	
Cystine	Trace	0.0
Methionine	1.17	Trace
Y	0.0	Trace
Isoleucine	2.66	1.00
2,6-Diaminopimelic acid	1.48	5.85
Leucine	4.48	1.77
Tyrosine	2.83	1.00
Phenylalanine	3.08	1.01
Ethanolamine	Trace	0.26
Ammonia	0.66	1.34
Ornithine	0.21	0.15
X	0.0	0.0
Lysine	2.76	1.59
Histidine	1.17	0.42
Arginine	2.90	1.20
Total	56.3	51.8

* Although not adequately resolved from valine, the presence of both galactosamine and valine was confirmed by paper chromatography.

Table 2. *Chemical compositions of the fractions AqI and AqII*

+, Present but not estimated quantitatively.

Component	Composition (%)	
	Fraction AqI	Fraction AqII
Amino compounds	21	35-38
Phosphorus (expressed as orthophosphoric acid)	12-14	9-12
Anthrone sugar*	15-18	11-15
Fatty acid	24†	15†
3-Deoxy-2-octulonic acid	2.8†	+
Heptose	2.4†	+
Total	77-82	70-80

* Estimated as glucose.

† Estimated on a single batch of fraction AqI or fraction AqII.

Table 3. *Quantitative analyses for the amino compounds of batches of fractions AqI and AqII*

Values for amino acids refer to amino acid residues, but all other values refer to the free compounds. The samples were hydrolysed for 16 hr. and the results are corrected for destruction or slow release during hydrolysis, by using data obtained with cell walls. The values for galactosamine have not been corrected. The fractions AqI contained only those amino compounds listed below, but both the fractions AqII contained between 0.1 and 0.2%, in each case, of aspartic acid and leucine. +, Present, but no quantitative estimate could be made.

	Amount in sample (%)			
	Extraction 3		Extraction 4	
	Fraction AqI	Fraction AqII	Fraction AqI	Fraction AqII
<i>O</i> -Phosphorylethanolamine	1.16	2.25	3.68	1.09
Muramic acid	4.03	10.6	3.18	8.37
Glutamic acid	1.99	4.12	1.20	4.20
Alanine	2.66	4.15	2.12	4.48
Glucosamine	6.10	8.96	6.33	7.62
Galactosamine*	2.16	1.80	2.23	1.55
Y	+	+	+	+
2,6-Diaminopimelic acid	2.29	4.93	1.49	6.31
Ethanolamine	+	+	0.47	0.41
Ammonia	0.75	0.58	0.56	0.85
X	+	+	+	+
Lysine	0.20	0.32	0.12	0.35
Total	21.3	37.7	21.4	35.2
Calc. amino N	2.65	3.90	2.36	4.36
Total N	5.16	6.18	4.25	5.61
$\frac{\text{Calc. amino N}}{\text{Total N}} \times 100$	51.3	63.2	55.6	77.7

* Valine was shown to be absent by paper chromatography.

thought to consist of muropeptides, and the rod-like bodies similar in shape to cell walls are thought to be murein sacculi.

Fractions AqI and AqII. It is evident from the data in Tables 2 and 3 that fractions AqI and AqII are chemically similar. The predominant sugars in fractions AqI and AqII were glucose and rhamnose; 3-deoxy-2-octulonic acid and heptose were shown to be present in small amounts in fractions AqI and AqII. Small amounts of three unidentified reducing compounds were also present. These components had mobilities relative to rhamnose of 1.18, 1.34 and 1.90 in the solvent system ethyl acetate-pyridine-water (5:2:5, by vol.). The analytical data for *O*-phosphorylethanolamine (Table 3) are unusual in that this component was sometimes concentrated in fraction AqI and sometimes in fraction AqII. This variation cannot be attributed to variable destruction of *O*-phosphorylethanolamine on acid hydrolysis of fractions AqI and AqII since, for any given extraction, virtually all of the *O*-phosphorylethanolamine of the cell wall appears to be concentrated in fractions AqI and AqII. It is, however, possible that *O*-phosphorylethanolamine is variably cleaved from the lipopolysaccharide by exposure to phenol and that its concentration in fraction AqI or

fraction AqII depends on the extent to which this has occurred during the extraction procedure. Two unidentified ninhydrin-positive components (X and Y; Table 3) present in the cell wall (Clarke *et al.* 1967a) were concentrated in fractions AqI and AqII. Component X appears to be a degradation product of *O*-phosphorylethanolamine formed during acid hydrolysis.

Only 51–56% of the nitrogen of fraction AqI was calculated to be present as amino nitrogen (Table 3), whereas the corresponding values for fraction AqII and cell wall were 63–78% and 79–87% respectively. Nevertheless, no other nitrogen-containing compounds were detected in fraction AqI or fraction AqII. This discrepancy may be due to the high concentrations of sugars in both fractions, especially in fraction AqI. It has been demonstrated (Tamura, Nishihara, Isobe & Matsuno, 1958) that low recoveries of amino acids are obtained when hydrolyses are carried out in the presence of carbohydrates, and our low nitrogen recoveries may well reflect this phenomenon.

The different behaviours of fractions AqI and AqII on centrifugation, coupled with their different appearances under the electron microscope (Plate 2), suggested that they were physically different. Thus

EXPLANATION OF PLATES 1 AND 2

Plate 1. Electron micrograph of the insoluble residue, fraction R. Magnification $\times 10000$.

Plate 2. (a) Electron micrograph of fraction AqI. Magnification $\times 10000$. (b) Electron micrograph of fraction AqII. Magnification $\times 10000$.

fractions AqI and AqII were completely removed from aqueous solution by centrifugation at g values above 20000. At 15000 g for 1 hr., 20% of fraction AqI and about 60% of fraction AqII sedimented to give material similar in analysis to fraction AqII. The supernatant solutions both contained material analytically resembling fraction AqI. Contamination of the lipopolysaccharide fractions AqI and AqII by cell-wall components other than murein or mucopeptide was probably negligible; both fractions, for instance, contained less than 0.5% of typical protein amino acids (i.e. other than those amino acids associated with murein and listed in Table 3).

More conclusive evidence that fractions AqI and AqII were physically different, despite their chemical similarities, came from their examination under the electron microscope. Several preparations were examined; all those of fraction AqII appeared to be similar to the material shown in Plate 2(b), and all those of fraction AqI to that shown in Plate 2(a).

Digestion of fractions AqI and AqII with lysozyme rendered both materials much more soluble, and they could not be deposited from solution by prolonged centrifugation at 105000 g . When the materials present in the digests were isolated and examined by paper chromatography [with the solvent system used for fraction RM (butan-1-ol-acetic acid-water, 3:1:1, by vol.)] similar sets of spots were obtained. No spots were produced when untreated solutions of fractions AqI and AqII were examined.

The infrared spectra of fractions AqI and AqII revealed that both contained large amounts of esterified fatty acids. The lipid fraction was obtained from fraction AqI by mild acid hydrolysis followed by extraction into chloroform. With n -sulphuric acid, a mixture of lipids was obtained that was fractionated (Lis *et al.* 1961) on silicic acid (Table 4). A glucosamine-containing lipid (D-lipid AqI) was one of the principal components (fraction IV) of the mixture (Clarke, Gray & Reaveley, 1965). Other lipids were eluted from the column in fractions II and III. Infrared spectroscopy indicated that fraction III consisted mainly of carboxylic acids, but suggested that components other than simple fatty acids were also present, e.g. three absorption peaks occurred in the carbonyl region, one at 1695 cm^{-1} and two in the region 1730–1750 cm^{-1} . Infrared spectroscopy indicated that fraction II consisted mainly of fatty acids.

Hydrolysis of fraction AqI with weaker acid (0.1 N -hydrochloric acid) gave a lipid that was again fractionated. Only small amounts of carboxylic acids were produced and the bulk of the lipid occurred in fraction IV (PD-lipid AqI). The infrared spectra of D-lipid AqI and PD-lipid AqI were

Table 4. *Fractionation of the lipids of fraction AqI with silicic acid*

The lipids eluted by methanol in fraction IV after hydrolyses of fraction AqI with aq. N -H₂SO₄ and aq. 0.1 N -HCl were designated D-lipid AqI and PD-lipid AqI respectively. Fractions II and III were eluted by 4% (v/v) diethyl ether in light petroleum (b.p. 60–80°) and 50% (v/v) diethyl ether in light petroleum (b.p. 60–80°) respectively, and consisted mainly of fatty acids. Fraction III did, however, contain other unidentified components giving carbonyl absorptions in the infrared.

Fraction	Occurrence			
	I	II	III	IV
Method of hydrolysis				
Boiled under reflux with aq. N -H ₂ SO ₄ for 30 min.	—	+	+++	+++
Boiled under reflux with aq. 0.1 N -HCl for 30 min.	—	—	+	++++

qualitatively very similar, differing only in the relative intensities of some of the extinctions; relative to the extinctions in the region 1000–1250 cm^{-1} , PD-lipid AqI absorbed more intensely at 1750 cm^{-1} . This indicates that D-lipid AqI contained more carbohydrate or phosphate or both relative to ester-bound acid. As the total yields of lipids (approx. 11–15% of fraction AqI) from both experiments were approximately equal, and since the residues from the hydrolyses were free from lipid, it is apparent that D-lipid AqI was simply a degraded form of PD-lipid AqI. This degradation appears to involve the splitting off of carboxylic acids from a carbohydrate-phosphorus-containing backbone.

A serious discrepancy exists between the total amount (approx. 11–15%) of lipid and the total amount (approx. 24%) of carboxylic acid (estimated as fatty acid) in fraction AqI. The reason for this discrepancy is not clear, but it is possible that lipid was lost during washing of the chloroform extract with water.

DISCUSSION

The present investigation confirms the views of Weidel and his co-workers (Weidel *et al.* 1960; Weidel & Pelzer, 1964) that murein forms an inner layer of the cell wall of the Gram-negative cell. However, the murein sacculi obtained in the present work appear to be two-layered and to consist of an inner electron-dense layer surrounded by an outer electron-transparent layer (Plate 1). Most of the sacculi are digested by lysozyme, but most of those that do remain appear to lack the

outer electron-transparent layer. The outer layer is therefore assumed to be murein, but the nature of the inner layer, which is less readily digested by lysozyme, is in doubt. The difference in shape between the cell wall and the more elongated murein sacculus suggests that the latter is not exclusively responsible for the shape of the cell wall or the whole cell (Carson & Eagon, 1966).

Fraction AqI accounts for approx. 25% and fraction AqII for approx. 12% of the cell wall of *P. aeruginosa* (Clarke *et al.* 1967b). The chemical data show that fractions AqI and AqII contain the components of both lipopolysaccharide and murein. Results obtained by centrifugation and electron microscopy indicate that two aggregates of lipopolysaccharide and muropeptide exist, one predominating in fraction AqI and the other in fraction AqII. Fraction AqII is richer in murein components than is fraction AqI (approx. 31% compared with 16%), although these values are overestimates, since some of the glucosamine and alanine occurs in the lipopolysaccharide.

The tendency of lipopolysaccharides to aggregate is well known (Schramm, Westphal & Lüderitz, 1952), and the small particles seen in electron micrographs (Plate 2a) of fraction AqI are believed to be aggregates of lipopolysaccharide to which muropeptides are in some way attached. The appearance of large numbers of sacculi, having the same shape as the murein sacculi of fraction R, in electron micrographs of fraction AqII, is taken (Plate 2b) as evidence that murein sacculi form part of the lipopolysaccharide-muropeptide aggregate of fraction AqII. These sacculi, unlike those of fraction R, were covered with large amounts of electron-dense material, presumably lipopolysaccharide. The much greater electron density of these sacculi compared with cell walls must be a consequence of the extraction procedure. A possible explanation is that, as the extraction proceeds, murein sacculi, possibly having residual lipopolysaccharide on the surface, are liberated, and the lipopolysaccharide and muropeptide, rather than aggregating among themselves as in fraction AqI, are deposited on to murein sacculi. In the first extraction, few of these sacculi would be available, and consequently fraction AqI would be almost free of this kind of material. The aggregation of the lipopolysaccharide and the muropeptide need not necessarily take place during the extraction, and may occur when the aqueous solutions are freeze-dried; the sacculi used as sites for aggregation would then be those that had not centrifuged with fraction R. It is not likely that the lipopolysaccharide and the muropeptide of fraction AqI exist simply as a physical mixture, arising from the overlap of fractions in the phenol extraction procedure, since fraction AqI cannot be deposited

by centrifugation after lysozyme treatment. It should also be pointed out that, if lipopolysaccharide-muropeptide aggregates do exist in fraction AqI, this does not mean necessarily that these exist as such in the cell wall. The cell walls may be subject to the action of autolytic enzymes during preparation and these may free some muropeptide from the murein. The muropeptides would presumably pass into the aqueous phase during the extraction and could then become associated with lipopolysaccharide. The presence of small amounts of lysine in fractions AqI and AqII, coupled with the greater concentration of lysine in fraction AqII, suggests that both lysine and 2,6-diaminopimelic acid may be present in the muropeptides of some Gram-negative organisms.

The lipid component (Clarke *et al.* 1965) of the lipopolysaccharide appears to be similar to the lipid A of the enterobacteria. This similarity probably extends on a qualitative basis to the polysaccharide part, since the common constituents of Gram-negative lipopolysaccharides are heptose, 3-deoxy-2-octulonic acid and *O*-phosphorylethanolamine. However, the heptose concentration in fraction AqI is very much smaller than that reported for some lipopolysaccharides (Taylor, Knox & Work, 1966; Sutherland & Wilkinson, 1966).

The work described in this and preceding papers (Clarke *et al.* 1967a,b) indicates that the cell walls of *P. aeruginosa* (N.C.T.C. 1999) have the general composition: lipopolysaccharide (approx. 31%), murein (approx. 15%), protein (approx. 36%) and lipid (approx. 17%). These values have been calculated by using the average yields (corrected to 100% recovery) for the fractions AqI, AqII, PhMS, PhMP and R obtained on extracting cell walls with aq. phenol, together with results that show that fractions AqI and AqII contain 16% and 31% respectively of murein, that fraction R contains approx. 50% of murein and 50% of protein, and that fraction PhMS contains some 13% of protein.

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