The Cell Walls of *Pseudomonas aeruginosa*

GENERAL COMPOSITION

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1. Cell walls of *Pseudomonas aeruginosa* were prepared and analysed. 2. Separate preparations were found to be reproducible, e.g. the phosphorus contents of all batches lay in the range 2.0–2.1%. 3. Ninhydrin-positive compounds in hydrolysates accounted for 43–46% of the cell wall and 79–87% of the nitrogen of the cell wall. Examination of the results for individual ninhydrin-positive compounds showed that 5–15% of the cell wall was murein and about 30% protein. 4. Trypsin treatment of crude cell-wall preparations preferentially liberated arginine, lysine and leucine, but it was not clear whether these arose from cell-wall or cytoplasmic proteins.

When intact cells of Gram-negative bacteria are treated with aq. 45% (w/w) phenol at 65–68° and the mixture is cooled to room temperature, lipopolysaccharide and nucleic acid can be isolated from the aqueous phase and protein and lipid from the phenolic phase (Westphal, Lüderitz & Bister, 1952), and insoluble material (mainly murein; Weidel & Pelzer, 1964) remains as a residue insoluble in either phase.

This technique has yielded valuable information about the outer structures of bacteria, but when working with whole cells it is not possible to be precise about the source of the fractions, since these may originate from different parts of the cell. This limitation of the technique would be overcome by applying the extraction procedure to isolated cell walls of bacteria, and in fact Wardlaw (1963) has used this method to study the cell walls of two strains of *Escherichia coli*. To further our knowledge of the cell walls of *Pseudomonas aeruginosa* (Gray & Wilkinson, 1965a,b), we thought that it would be profitable to obtain at least a preliminary fractionation of the cell walls by using aqueous phenol. Several batches of cell-wall material were needed and it was therefore essential to ensure that various cell-wall preparations were reproducible. The results obtained from the elemental analysis of nitrogen and phosphorus, and from the quantitative estimation of amino acids and amino sugars in hydrolysates or the cell walls, have been particularly valuable in this respect, although infrared spectroscopy and electron microscopy have also given useful checks on purity and reproducibility of cell-wall material. This paper presents the results of

the quantitative determinations that give the general composition of the cell walls of the strain of *P. aeruginosa* being studied.

MATERIALS AND METHODS

Growth of organism and preparation of cell walls. The strain of *Pseudomonas aeruginosa* used was N.C.T.C. 1999. The organism was grown and the cell walls were prepared by the methods of Salton & Horne (1951) and Cummins & Harris (1956), as described by Gray & Wilkinson (1965a). The reproducibility of the cell-wall preparations was checked by quantitative analysis for nitrogen, phosphorus and individual ninhydrin-positive species. Electron micrographs (magnification $\times 15000$) showed that the preparations were free from contaminating material.

As a further check on reproducibility, infrared spectra of individual batches of cell walls were recorded in the range 650–4000 cm^{-1} with a Unicam SP.100 spectrophotometer equipped with grating and fore prism (NaCl). Samples (1mg.) were dispersed in anhydrous KBr (100mg.) and examined as pressed disks. Spectra were qualitatively very similar, but to make more accurate comparisons, particularly in cases in which the base lines had different slopes, the extinctions for all the peaks and the more prominent shoulders were obtained. These were then expressed relative to the extinction at 1660 cm^{-1} (amide-I band) as unity, and the values compared for different batches of cell walls.

Ultraviolet spectra of aqueous suspensions (0.1mg./ml.) of cell walls were obtained with a Unicam SP.500 spectrophotometer.

In one experiment, the suspension of whole cells was heated at 100° for 5 min. before disintegration, to destroy any lytic enzymes. In another instance, treatment of the crude cell-wall suspension with trypsin and ribonuclease was omitted. All three types of cell walls were hydrolysed and the percentages of ninhydrin-positive species were compared.

Quantitative methods of analysis. Phosphorus was estimated by the method of Allen (1940) and nitrogen by a

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modification of the method of Umbreit, Burris & Stauffer (1957). Anthrone determinations for sugars were carried out on unhydrolysed samples, as described by Trevelyan & Harrison (1952). The protein content was estimated by the biuret method of Gornall, Bardawill & David (1949) with bovine albumin as standard.

Paper chromatography. The qualitative complement of amino compounds (see Table 2) and sugars in the cell wall was established by paper chromatography as described by Gray & Wilkinson (1965b). The presence of *O*-phosphoryl-ethanolamine was confirmed with the solvent system propan-1-ol-aq. NH_3 (sp.gr. 0.88)-water (6:3:1, by vol.) and the molybdate- HClO_4 reagent of Hanes & Isherwood (1949).

Estimation of ninhydrin-positive compounds. Cell walls (5 mg.) were hydrolysed (under N_2) with redistilled constant-boiling HCl (1 ml.) in a sealed tube at $105 \pm 0.5^\circ$ for 2, 5, 16 and 30 hr. The hydrolysate was evaporated to dryness *in vacuo* over P_2O_5 and KOH . The residue was dissolved in a little ion-depleted water and again evaporated. After repeating the process once more, the residue was dissolved in hot ion-depleted water and the solution passed quantitatively through a sinter (no. 3 porosity). The solution was again evaporated to dryness. The residue was finally dissolved in 5 ml. of 0.1N- HCl containing norleucine ($0.08865 \mu\text{mole/ml.}$), which served as an internal standard for ion-exchange chromatography. Samples (1 ml.) of this solution were taken for analysis of ninhydrin-positive components with an automatic amino acid analyser (Technicon Instruments Co. Ltd., Chertsey, Surrey). Results from duplicate hydrolysates agreed to within $\pm 4\%$, compared with $\pm 3\%$ for duplicate estimations on a standard solution of amino acids.

The results were used to plot concentration-time curves, from which could be obtained rates of release or destruction for all the principal ninhydrin-positive components present in hydrolysates of the cell wall. These curves were extrapolated to infinite or zero time to give estimates of the absolute amounts of amino compounds present in the cell wall.

Estimation of fatty acid content. Samples of cell walls (approx. 10 mg.) were hydrolysed with redistilled constant-boiling HCl (2 ml.) at $105 \pm 0.5^\circ$ for 4 hr. (Salton, 1953). The cooled hydrolysates were extracted five times with diethyl ether (total volume 25 ml.). The ether extracts were evaporated with a filtered stream of N_2 , and the residues dried *in vacuo* over P_2O_5 . The dried products were dissolved in anhydrous chloroform, and the volume was made up to 75 ml. Volumes (5 ml.) of these solutions were used to estimate the fatty acid content of the cell walls by the colorimetric method of Duncombe (1963).

RESULTS

General. We consider first cell walls prepared in the normal manner, i.e. without heating the whole-cell suspension but including the trypsin-ribonuclease treatment of the crude cell-wall material. Simple analyses of such cell walls are given in Table 1, and it should be emphasized that, in the first four analyses in the Table, all batches gave results within the ranges quoted. The phosphorus content of the cell walls is slightly lower than that

(2.3%) of the walls prepared by Gray & Wilkinson (1965b) from the same strain of *P. aeruginosa*. The infrared spectrum of a typical batch of cell walls compares closely with that recorded by Gray & Wilkinson (1965b). The extinctions of the principal bands relative to that of the amide-I band at 1660 cm^{-1} did not vary by more than 10% from batch to batch, confirming that the cell-wall preparations were reproducible. Examination of the ultraviolet spectra of aqueous suspensions of cell walls showed that preparations were free from nucleic acids.

When examined under the electron microscope, preparations of cell walls were seen to be homogeneous and free from cell debris, although individual cell walls contained regions of varying electron density. As the cell walls were examined in transmission, it was not certain whether the regions of high electron density were located on the surface or inside the cell wall.

Ninhydrin-positive components of the cell wall. Cell walls were hydrolysed with constant-boiling hydrochloric acid at 105° for various times, and these experiments were repeated on a separate batch of cell-wall material to ascertain that rates of release and destruction of ninhydrin-positive species did not differ from one batch to another. Amino acids other than leucine, isoleucine, valine, tyrosine, proline, methionine and possibly cystine were completely liberated from the cell wall by hydrolysis for 16 hr., and were not significantly destroyed on treatment with acid for a further 14 hr. Leucine, isoleucine and valine were liberated only very slowly from the cell wall, and the last two amino acids were probably not fully released after hydrolysis for 30 hr. Some variable destruction of tyrosine, proline and methionine was observed. The cause of this was not established, but there are reports (Hill, 1965) that these amino acids are destroyed appreciably on acid hydrolysis.

The destruction of amino sugars on treatment with strong acids is well known, and muramic acid and glucosamine were found to be quickly liberated from the cell wall, but to be extensively destroyed on hydrolysis with acid for longer periods. Hydrolysis curves for these two amino sugars are shown

Table 1. *Analytical results for cell walls*

Component	Amount in cell wall (%)
Phosphorus	2.0-2.1
Nitrogen	7.6-8.3
Anthrone sugar	6.8-7.4
Protein	57-59
Fatty acid*	15.5

* This estimation was carried out on only one of the batches of cell walls.

Fig. 1. Liberation of glucosamine (▲), muramic acid (○), *O*-phosphorylethanolamine (△) and ammonia (●) from cell walls by acid hydrolysis. Samples were hydrolysed in sealed tubes, under N_2 , with constant-boiling HCl at 105° , for 2, 5½, 16 and 30 hr.

in Fig. 1 (it was not possible to obtain a hydrolysis curve for galactosamine since this amino sugar co-chromatographed with valine on the amino acid analyser). The initial rapid release of ammonia from cell walls (see Fig. 1) was assumed to be complete after 5½ hr., and the amounts of ammonia-producing residues in the cell walls were calculated on this basis. The subsequent slow liberation of ammonia probably arises mainly from the destruction of amino sugars.

Of the other amino compounds, a large amount of ethanolamine was liberated very quickly (within 2 hr.) from the cell wall, and the remainder was liberated only slowly. As this slow liberation was thought to be caused by the destruction of *O*-phosphorylethanolamine, the amounts of ethanolamine present in the cell walls as phosphatidylethanolamine were calculated, assuming complete liberation after 2 hr., and these are the values given in Table 2. A synthetic sample of *O*-phosphorylethanolamine was completely destroyed on treatment with constant-boiling hydrochloric acid at 105° for 30 hr. The recovery of ethanolamine from this sample after hydrolysis was only 50% of the theoretical, and this low recovery may be caused partly by loss by evaporation on drying down the hydrolysate *in vacuo* over potassium hydroxide (Wheeldon, Brinley & Turner, 1962) and partly by destruction by the acid during hydrolysis. However, an unidentified, basic, ninhydrin-positive compound (X) was produced during the hydrolysis of *O*-phosphorylethanolamine, and this compound was present in all hydrolysates of cell-wall material that contained *O*-phosphorylethanolamine. Only one other ninhydrin-positive component (Y) was not identified.

Complete analyses for ninhydrin-positive components of three batches of cell walls prepared in the normal way are given in the first three columns

of Table 2. The results for different batches of cell walls were remarkably reproducible, the largest deviation from the mean value being $\pm 10\%$ for all ninhydrin-positive species with the exception of *O*-phosphorylethanolamine, proline, methionine, isoleucine, ammonia, histidine and ornithine. For the first five of these compounds, the maximum deviations from the mean values were in the range 10–20%, and it was considered that these values showed that the various batches of cell wall were essentially similar. Ornithine, on the other hand, showed a much larger deviation (approx. 50%). Ornithine has been detected in lipids extracted from whole cells (Kates, Adams & Martin, 1964; Houtsmuller & van Deenen, 1963, 1965), and later we found it to be present in the lipid fraction of the cell wall (Clarke, Gray & Reaveley, 1967*a,b*). It seems likely therefore that ornithine may be present as an *O*-ester of phosphatidylglycerol, and, as such esters are labile and may be metabolically active, a large variation in ornithine content of the cell walls may be expected.

The results show that amino compounds account for 43–46% of the cell wall. The amino nitrogen content of each acid hydrolysate of cell walls was calculated from the percentages of amino acids, amino sugars etc. detected. These values were then compared with the total nitrogen content determined by the micro-Kjeldahl method. Typical results are shown in Table 3, and these indicate that at least 79–87% of the nitrogen of cell walls is present as amino nitrogen.

Sugars of the cell wall. Apart from amino sugars (see Table 2), only glucose and rhamnose were detected in cell-wall preparations. Heptose and 3-deoxy-2-octulonic acid were later shown to be present in low concentration in a lipopolysaccharide fraction obtained from the walls.

Fatty acid of the cell wall. A single batch of cell walls was hydrolysed and shown to contain 15.5% of fatty acid (expressed as palmitic acid) (see Table 1).

Other cell-wall preparations. Reports of the deleterious effect of autolytic enzymes during the preparation of cell walls (Weidel, Frank & Leutgeb, 1963; Mandelstam & Rogers, 1959) led to attempts to prepare cell walls that had not been subject to the action of such enzymes. To prevent enzymic action, Weidel and his colleagues either heated an aqueous suspension of cells at 100° for several minutes (Leutgeb & Weidel, 1963) or used the enzyme-inhibiting powers of sodium dodecyl sulphate (Weidel *et al.* 1963). Since sodium dodecyl sulphate is known to solubilize the outer layers of the cell walls of Gram-negative bacteria (Shafa & Salton, 1960) such a procedure was obviously unsuitable from the point of view of our investigations. Enzymes were therefore destroyed by

Table 2. *Quantitative analyses for the amino compounds of some preparations of cell walls*

The 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. The values for valine plus galactosamine have not been corrected.

	Amount in cell wall (%)			Max. variation from mean (%)	Not treated with enzymes
	Batch 1	Batch 2	Batch 3		
O-Phosphorylethanolamine	1.34	1.40	1.15	11	1.18
Aspartic acid	3.61	3.88	4.30	9.4	4.51
Threonine	1.49	(1.5†)	(1.6†)	< 10	1.95
Serine	1.56	(1.8†)	(1.7†)	< 10	1.81
Muramic acid	3.80	3.56	3.59	4.1	2.26
Glutamic acid	4.40	4.67	4.52	3.1	5.67
Proline	0.93	1.06	1.32	20	1.20
Glycine	1.78	1.82	1.82	1.1	1.96
Alanine	3.49	3.73	3.81	5.2	4.62
Glucosamine	4.03	3.77	4.32	6.7	2.93
Valine	1.67	1.82	1.90	7.2	1.93
Galactosamine*	Determined with valine				
Cystine	Trace	Trace	Trace	—	Trace
Methionine	0.41	0.51	0.40	15.7	0.74
Y	Trace	Trace	Trace	—	Trace
Isoleucine	1.20	1.23	1.46	12	1.48
2,6-Diaminopimelic acid	1.80	1.71	1.90	5.5	1.28
Leucine	2.68	2.57	2.59	2.7	3.51
Tyrosine	1.71	1.81	1.97	7.6	1.82
Phenylalanine	1.59	1.64	1.81	7.7	1.83
Ethanolamine	0.48	0.41	(0.5†)	10	0.32
Ammonia	0.56	0.79	0.66	18	0.62
Ornithine	0.20	0.07	0.17	53	0.12
X	Trace	Trace	Trace	—	Trace
Lysine	1.89	1.80	1.87	2.8	2.59
Histidine	0.68	0.61	0.78	13	0.69
Arginine	2.03	1.94	2.20	6.8	3.02
Total	43.3	44.1	45.9		48.0

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

† Values in parentheses are less accurate because of either poor resolution (serine and threonine) or the sloping base line associated with the ammonia plateau (ethanolamine).

Table 3. *Amino nitrogen and total nitrogen content of cell walls*

	Nitrogen content (% of cell wall)			
	Batch 1		Batch 2	
	16	30	16	30
Time of hydrolysis (hr.)				
Calc. amino N	6.25	6.89	6.49	6.67
Total N	7.88	7.88	7.66	7.66
Calc. amino N / Total N × 100	79.3	87.4	84.7	87.1

heating aqueous suspensions of cells at about 100° for 5 min. The yields (about 90 mg.) of cell walls from such preparations were considerably less than those (about 250 mg.) from untreated cells and,

moreover, when examined under the electron microscope, the preparations were found to be heterogeneous and to contain much amorphous material. Such cell walls were unsuitable for our experiments, and attempts to destroy cell enzymes were abandoned. However, in preparing normal batches of cell walls the effects of such enzymes were minimized by carrying out the preparation of cell walls, as far as possible, in the temperature range 0–5°.

Trypsin was normally used during the purification of crude cell walls to facilitate the removal of cytoplasmic proteins. It was thought that such treatment might remove protein that is present in the cell walls of Gram-negative bacteria, and to investigate this possibility treatment with enzymes was omitted from the preparation of one batch of cell walls. The resulting walls had a higher content

(8.8%) of nitrogen than normal cell walls (7.6–8.3%), and this was reflected in larger concentrations of protein amino acids (Table 2).

DISCUSSION

The above quantitative and qualitative analyses show that the cell walls of *P. aeruginosa* resemble those of other Gram-negative bacteria (Salton, 1964), which consist of murein, protein, lipopolysaccharide and lipid.

Mureins, which are in the form of sacculi surrounding the cells, have been isolated from several Gram-negative organisms (Mandelstam, 1962; Schocher, Bayley & Watson, 1962; Weidel & Pelzer, 1964). These consist of *N*-acetylglucosamine, *N*-acetylmuramic acid, D- and L-alanine, D-glutamic acid and *meso*-2,6-diaminopimelic acid (with possibly traces of other amino acids). All these components were present in the cell walls of *P. aeruginosa*, although an accurate assessment of the amount of murein present in the cell wall, on the basis of the analysis of ninhydrin-positive components, was not possible, since some of the components of murein are present in other cell-wall polymers. It seems likely, however, that the amount is in the range 5–15%.

Assessment of the amounts of protein in the cell walls of Gram-negative bacteria (Collins, 1964; Wardlaw, 1963; Sud & Tyler, 1964) indicates that they contain 55–76% of protein. Results in this range (57–59%) were indeed obtained for the cell walls of *P. aeruginosa*, by using the biuret method, but it is apparent from our estimations of amino acids that the cell walls in fact contain much less protein (about 30%). This discrepancy is partly due to the cell-wall protein being more chromogenic than the bovine albumin used as standard (Clarke *et al.* 1967*a,b*), and the remainder of the discrepancy may be caused by the presence of substances, such as murein, that interfere with the biuret estimation of protein.

The remainder of the cell wall (55–65%) is made up of lipids and lipopolysaccharides that together, on acid hydrolysis, produce fatty acid (expressed as palmitic acid) equivalent to 15–16% of the cell wall. The presence of small amounts of other polymers must not be excluded at this stage.

The attempts to produce cell walls from heat-treated whole cells were disappointing. Within a single batch of such cell walls the analytical results for phosphorus were in the range 2.0–2.2% and those for nitrogen in the range 7.6–9.0%. The results obtained from the quantitative estimation of amino acids on a single batch also showed wide variation, and it was not considered worth while to use such heterogeneous material for further analysis.

The results obtained from the analysis of ninhydrin-positive components in cell walls that had not been treated with trypsin and ribonuclease were of some interest. The molar ratios of protein amino acids (Table 2) relative to glycine were compared with those for cell walls prepared normally, and, although it was found that the values were in general very similar, the protein of untreated cell walls contained about 25% more leucine, 30% more lysine and 40% more arginine than those of normal cell walls.

The last two results were particularly noteworthy, since trypsin is known to hydrolyse proteins at peptide bonds involving the carboxyl groups of lysine and arginine. It is tempting to suggest that the principal effect of trypsin is to liberate either *N*-terminal lysine and arginine or polypeptide fragments enriched in these amino acids. Unfortunately it is not possible to decide whether the enzyme treatment degrades the protein of the cell wall and modifies its structure, or whether omission of the enzyme material results in contamination of cell walls by cytoplasmic material rich in leucine, lysine and arginine.

It is, however, clear that our methods of growing *P. aeruginosa* and isolating cell walls of the organism give a satisfactorily reproducible product suitable for the extended series of investigations of the structure of the cell wall by using the phenol extraction procedure.

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