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The Effect of Ethylenediaminetetra-acetate on *Pseudomonas alcaligenes* and the Composition of the Bacterial Cell Wall

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1. EDTA in borate buffer has a marked bactericidal effect on *Pseudomonas alcaligenes*, which is more sensitive than *Pseudomonas aeruginosa*. The bactericidal effect is accompanied by solubilization of lipopolysaccharide and release of intracellular solutes. These effects are more pronounced at pH 9.2 than 7.1. 2. Cell walls of *P. alcaligenes* were prepared and from them were obtained the readily extracted lipids and the fractions given by treatment with aqueous phenol. 3. The cell walls and the above components were analysed and results are compared with those for *P. aeruginosa*. 4. Lipopolysaccharide obtained by treatment of cell walls with aqueous phenol is contaminated with glycosaminopeptide to a variable extent. 5. The lipopolysaccharide contains less neutral sugar but more phosphorus than the lipopolysaccharide of *P. aeruginosa*; fucosamine is not a component of the lipopolysaccharide of *P. alcaligenes*.

EDTA has a toxic effect on the Gram-negative organism *Pseudomonas aeruginosa* (MacGregor & Elliker, 1958; Gray & Wilkinson, 1965*a*; Eagon & Carson, 1965; Wilkinson, 1967), and a correlation exists (Gray & Wilkinson, 1965*a*) between the metal chelate stability constants for EDTA and related polyaminocarboxylic acids and the bactericidal activities of the compounds against the organism. In support of the view that chelation of metal ions is involved in the toxic action, it has been shown (Eagon & Carson, 1965; Asbell & Eagon, 1966*a*,*b*) that multivalent ions are important in maintaining the structural integrity of the cell wall of *P. aeruginosa*, and that cations are solubilized (Eagon & Carson, 1965) on treatment of cells with EDTA. It was suggested that the toxic action of EDTA on cells of *P. aeruginosa* resulted from a lytic action (Gray & Wilkinson, 1965*a*; Eagon & Carson, 1965) on the cell wall of the bacterium, and this view was substantiated by studies of the effect of EDTA on the isolated cell walls of a number of Gram-negative organisms (Gray & Wilkinson, 1965*b*). At pH 9.2 a high proportion of the carbohydrate and phosphorus of the cell walls of *P. aeruginosa* was solubilized by EDTA, apparently as lipopolysaccharide. Cox & Eagon (1968) and Rogers, Gilleland & Eagon (1969) confirmed this, and it has been reported (Leive, 1965; Leive, Shovlin & Mergenhagen, 1968) that lipopolysaccharide was solubilized by EDTA treatment of *Escherichia coli*, although in this case the cells remained viable. Except when used together with organic cations (Wolin, 1966; Goldschmidt & Wyss, 1967; Voss, 1967), EDTA does not seem to be highly toxic towards Gram-negative bacteria other than pseudomonads (Gray & Wilkinson, 1965*a*; Wilkinson, 1967), and Wilkinson (1967, 1968) has shown that species of *Pseudomonas* that are resistant to EDTA have cell walls that lack the structural features of cell walls of EDTA-sensitive pseudomonads, and from which lipopolysaccharide is not solubilized by treatment with EDTA.

To investigate the probable involvement of lipopolysaccharide in the bactericidal action of EDTA and to establish whether the cell wall and in particular the lipopolysaccharide of *P. aeruginosa* possess unique structural features, *P. aeruginosa* (N.C.T.C. 1999) has been studied in these laboratories (Clarke, Gray & Reaveley, 1967*a*,*b*,*c*; Roberts, Gray & Wilkinson, 1967; Fensom & Gray, 1969). To find whether similar results are obtained for another EDTA-sensitive pseudomonad, studies have now been made of the cell wall of *Pseudomonas alcaligenes*.

MATERIALS AND METHODS

Growth of organism and preparation of cell walls. The organism used was *Pseudomonas alcaligenes*, a laboratory strain (BR 1/2) kindly provided by Reckitt and Sons Ltd., Kingston-upon-Hull, U.K. Cells used for investigation of lysis by EDTA were grown for 24 h at 37°C on slopes of Nutrient Agar (Oxoid). The slopes were inoculated with cultures grown for 24 h at 37°C in Nutrient Broth no. 2 (Oxoid). Cells were washed from the slopes

with buffer (see below) and the suspension was passed through a sinter (no. 1 porosity). The filtrate was centrifuged (12000g for 15min at 18°C) and the cells were resuspended in the original volume of buffer. After repeating the centrifugation the cells were suspended in sufficient buffer to give a final concentration of cells of approx. 10mg dry wt./ml. The dry weight of cells was checked by weighing the residues obtained by evaporating to dryness (24h at 105°C) equal volumes of the cell suspension and buffer solution.

Cells used for preparing cell walls were grown for 24h at 37°C in a culture vessel (Bio-Kulture Assembly, Fermentation Design Inc., Durham, Pa., U.S.A.) in Nutrient Broth no. 2 (Oxoid). Cells (approx. 75g wet wt.) were removed from the medium (20 litres) by continuous centrifugation and suspended mechanically in water. After centrifugation (12000g for 15min at 18°C) the cells were resuspended in ion-depleted water (150ml). Cell walls were prepared according to the methods of Salton & Horne (1951) and Cummins & Harris (1956) as described by Gray & Wilkinson (1965*a*), except that disintegration was carried out for 2min in a Braun MSK homogenizer (B. Braun, Melsungen, Germany). The reproducibility of individual batches of cell walls was checked by quantitative analysis for phosphorus, nitrogen, carbohydrate and ninhydrin-positive compounds, by i.r. spectroscopy in the range 650–4000cm^{-1}, by u.v. spectroscopy of aqueous suspensions (0.1mg/ml) and by electron microscopy.

Lysis of cells by EDTA. All glassware was cleaned by the method of Waring & Werkman (1942). Metal ions, other than Na^+, were removed from solutions by passage down a column of Amberlite IR-120 (Na^+ form) ion-exchange resin. The buffers used to prepare cell suspensions and test solutions of EDTA were: pH 9.2, 0.05M-$Na_2B_4O_7$; pH 7.1, 0.2M-$Na_2B_4O_7$–H_3BO_3. EDTA test solutions were prepared by dissolving AnalaR EDTA in the form of the dihydrate of its disodium salt in borate buffer, pH 7.1 or 9.2, to give a 1.0% (w/v) solution (34mM), expressed as the free acid. Cells were treated with EDTA by adding to a given volume of buffered cell suspension one-tenth of this volume of 1.0% EDTA solution, and to a second, equal, volume of buffered cell suspension one-tenth of this volume of buffer solution was added. After being kept for 1h at room temperature, a portion (1ml) was removed from each mixture and viable counts were determined at dilutions of 10^5, 10^7 and 10^8. The remainder of each mixture was centrifuged (12000g for 15min at 18°C), and the cell-free supernatants so obtained are referred to in the Results section as Test and Control. EDTA and low-molecular-weight solutes were separated from high-molecular-weight solutes in the supernatant fluids by using a column of Sephadex G-25 (Pharmacia, Uppsala, Sweden) and eluting the material with 0.05M-NaCl.

Isolation of the loosely bound lipids of the cell wall. Loosely bound lipids were extracted from cell walls (approx. 300mg) by stirring with chloroform–methanol (2:1, v/v) (30ml) for 1h at room temperature. The suspension was filtered through a glass sinter (no. 4 porosity) and the residue was washed with chloroform–methanol (2:1, v/v). The residue was removed from the sinter and the extraction repeated. The combined filtrates and washings were evaporated to dryness in a rotary evaporator; the lipids were dried *in vacuo* over P_2O_5.

Fractionation of the loosely bound lipids. The lipids were fractionated on a silicic acid column according to the method of Lis, Tinoco & Okey (1961). Fractions were collected in tared tubes and the solvent was removed by evaporation by using a stream of filtered N_2. The residual lipid fractions were dried *in vacuo* over P_2O_5.

Treatment of defatted cell walls with aqueous phenol. Defatted cell walls were treated with hot aq. 45% (w/v) phenol by the method described by Clarke *et al.* (1967*b*). Two treatments with aqueous phenol were carried out, the aqueous phases from each treatment being kept separate and designated AqI and AqII; the insoluble residue was designated R. The combined phenolic phases gave two fractions, one insoluble in methanol (PhMP) and one soluble in methanol (PhMS).

Methods of quantitative analysis. Phosphorus was determined by the method of Chen, Toribara & Warner (1956) and nitrogen by a modification of the method of Umbreit, Burris & Stauffer (1964). Determinations of carbohydrate were carried out with anthrone on unhydrolysed samples as described by Trevelyan & Harrison (1952). Fatty acids were determined by the method of Itaya & Ui (1965) after hydrolysis of the samples at 105°C for 4h with constant-boiling (6.1M) HCl (Salton, 1953), followed by three extractions of the fatty acids into chloroform (5ml in each case). After hydrolysis under the same conditions, amino sugars were determined by the method of Cessi & Piliego (1960); amino sugars were also determined by autoanalysis, as described below. Ribose was determined by the method of Mejbaum (1939) as described by Umbreit, Burris & Stauffer (1957), and DNA by the method of Ceriotti (1952). EDTA was determined by the method of Kratochvil & White (1965).

Ninhydrin-positive components of the cell wall were identified and determined by using an automatic amino acid analyser (Technicon Instruments Co. Ltd., Chertsey, Surrey, U.K.) and the procedure described by Clarke *et al.* (1967*a*). Samples were first hydrolysed in constant-boiling (6.1M) HCl for 16h at 105°C.

Methods of qualitative analysis. The normal techniques of paper chromatography were followed with Whatman no. 1 paper, or, for the identification of amino acids, Whatman no. 20 paper. Thin-layer plates were prepared in the usual way with Kieselgel G or H (E. Merck A.-G., Darmstadt, Germany) and activated immediately before use by heating for 1h at 120°C. The following solvent systems were employed: *a*, ethyl acetate–pyridine–water (5:2:5, by vol., upper layer); *b*, butan-2-ol–aq. 88% (w/v) formic acid–water (15:3:2, by vol.) followed by phenol–water–aq. 5M-NH_3 (80:20:0.3, by vol.) in the second dimension; *c*, ethyl acetate–pyridine–water–acetic acid (5:5:3:1, by vol.); *d*, chloroform–methanol–water (65:25:4, by vol.); *e*, di-isopropyl ether–acetic acid (24:1, v/v) followed by light petroleum (b.p. 40–60°C)–diethyl ether–acetic acid (90:10:1, by vol.); *f*, butan-1-ol–diethylene glycol–water (4:1:1, by vol.); *g*, chloroform–methanol–aq. 7M-NH_3 (65:25:4, by vol.). The conditions of hydrolysis of samples before chromatography depended on the compounds under investigation. The occurrence of choline in hydrolysates of the loosely bound lipids was established as follows. The lipids were hydrolysed with constant-boiling (6.1M) HCl for 16h at 105°C; HCl was removed by repeatedly evaporating the hydrolysate and

redissolving the residue in ion-depleted water. The solution was filtered through a sinter (no. 4 porosity) and the filtrate evaporated to dryness. The residue was dissolved in water and submitted to ascending paper chromatography in solvent system *f*. The presence of choline chloride was shown by spraying the dried paper with a solution of $K_3Fe(CN)_6$–$CoCl_2$ (Dawson, Elliott & Jones, 1962) and also by using the method of Levine & Chargaff (1951). For the detection of neutral sugars and amino sugars cell walls were hydrolysed in M-H_2SO_4 for 2h at 105°C. The hydrolysates were neutralized with $BaCO_3$ and the $BaSO_4$ was removed by centrifugation. Amino acids were detected chromatographically after hydrolysis of cell walls in constant-boiling (6.1M) HCl for 16h at 105°C; HCl was removed by repeatedly drying the hydrolysate and redissolving the residue in water. Reducing sugars were detected with alkaline $AgNO_3$ (Trevelyan, Procter & Harrison, 1950), and amino sugars and amino acids with a solution of ninhydrin in butan-1-ol (Cramer, 1955). Thin-layer plates were developed with iodine vapour, periodate followed by treatment with Schiff reagent, ninhydrin in butan-1-ol, the reagent of Dittmer & Lester (1965) for phosphorus or the modified H_2SO_4 spray of Zimiński & Borowski (1966).

Gas–liquid chromatography. Fatty acids were identified and relative amounts estimated by g.l.c. of their methyl esters by using a Perkin–Elmer F11 chromatographic apparatus. Separation was carried out on both polar (polyethylene glycol succinate at 180°C) and non-polar (Apiezon L at 220°C) columns. The free fatty acids separated from the loosely bound lipids were methylated by using a solution of BF_3 (14%, w/v) in methanol (Applied Science Laboratories Inc., State College, Pa., U.S.A.) as described by Metcalfe & Schmitz (1961). The bound fatty acids of the phospholipids were converted into methyl esters, without preliminary hydrolysis, by using the same procedure but with a heating time of 10min.

Physical methods. U.v. spectra were obtained with a Unicam SP.700 recording spectrophotometer. Extinctions in the u.v. were also measured manually with a Unicam SP.500 spectrophotometer equipped with 1cm silica cells. The same instrument was used with 1cm glass cells to measure extinctions in the visible region of the spectrum. I.r. spectra were recorded with either a Unicam SP.100 or SP.200 spectrophotometer; samples were examined as pressed discs (approx. 1mg of sample in approx. 100mg of KCl). Cell walls were examined by electron microscopy with a Siemens Elmiskop 1A instrument. Samples (approx. 1mg) were suspended in ion-depleted water (10ml); a drop of suspension was transferred to a carbon film and the water was removed by evaporation.

RESULTS

Bactericidal activity of EDTA against P. alcaligenes. Cells were exposed for 1h at room temperature to a solution of EDTA in borate buffer, pH 7.1 or 9.2 (final concentration of EDTA, 31mM). At pH 7.1, with an initial cell suspension containing 10.2mg dry wt. of cells/ml, the decrease in viable count was 88%, and at pH 9.2, with an initial cell suspension containing 10.0mg dry wt. of cells/ml, the decrease in viable count was 99.995%; both decreases are relative to controls in buffer alone. The results confirm the marked sensitivity of *P. alcaligenes* to solutions of EDTA, the bactericidal activity being more pronounced at pH 9.2.

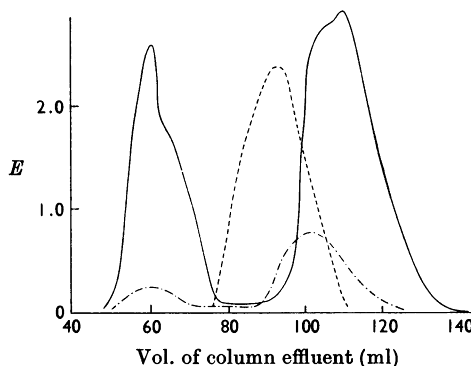

Fig. 1. Fractionation of supernatant fluids obtained by treatment of *P. alcaligenes* with borate buffer, pH 9.2 (control), and with EDTA in borate buffer, pH 9.2 (test). Fractions (2ml) were collected from the column (Sephadex G-25) by elution with 0.05M-NaCl; these were analysed for phosphorus and, in the test experiment, for phosphorus and EDTA. ——, Test phosphorus; –·–·, control phosphorus; ----, EDTA.

Quantitative determination of solutes in supernatant solutions. The solutes in supernatant solutions obtained after exposure of cells to solutions of EDTA in borate buffer, pH 7.1 or 9.2, were separated into high-molecular-weight and low-molecular-weight fractions by gel filtration (Sephadex G-25). Each fraction (2ml) from the column was analysed for EDTA and phosphorus, and the separation achieved for a supernatant solution obtained after exposure of cells to EDTA at pH 9.2 is illustrated in Fig. 1; a clear separation of high-molecular-weight solutes containing phosphorus from both EDTA and low-molecular-weight solutes containing phosphorus was obtained. Recovery of EDTA was 100% of that used in the initial treatment of cells. Fractions corresponding to the high-molecular-weight and low-molecular-weight material containing phosphorus were separately combined, yielding, from each supernatant solution, a high-molecular-weight and a low-molecular-weight pool. Analyses for cell solutes were carried out on each of these solutions and on the original unfractionated supernatant solution. Analyses therefore fall into three categories, those carried out on (*a*) the original supernatant solution, and those carried out on (*b*) the high-molecular-weight and (*c*) the low-molecular-weight fractions resulting from gel filtration of the supernatant solutions. Results for categories (*a*) and (*b*) are given in Tables

Table 1. *Analytical results for supernatant fluids before gel filtration*

Cells of *P. alcaligenes* were exposed to 31mM-EDTA in borate buffer, pH 7.1 or 9.2. Control experiments were conducted using only borate buffer, pH 7.1 or 9.2. Centrifugation (12000*g* for 15min) gave the supernatant fluids. Where two sets of values appear in a column, the actual experimental result is out of the range of the method and is followed in parenthesis by the estimated lower limit of accuracy of the method.

	Content (μg/ml)			
	pH 9.2		pH 7.1	
	Test	Control	Test	Control
Phosphorus	53.1	7.9	24.0	3.3
Hexose*	105	4	167	16 (47)
Ribose	20.6	Absent	18.1	1.3 (5)
DNA	14.7	4.4	11.2	3.0 (5)
Fatty acids†	121	10 (45)	81	Absent

* Results expressed in μg of glucose/ml.
† Results expressed in μg of hexadecanoic acid/ml.

Table 2. *Analytical results for the pooled high-molecular-weight fractions obtained from supernatant fluids*

Solutes were separated into high-molecular-weight and low-molecular-weight components by elution (0.05M-NaCl) from a column of Sephadex G-25. Where two sets of values appear in a column, the actual experimental result is out of the range of the method and is followed in parenthesis by the estimated lower limit of accuracy of the method. —, Not determined.

	Content (μg/ml of original supernatant fluid)			
	pH 9.2		pH 7.1	
	Test	Control	Test	Control
Phosphorus	13.3	—	8.2	—
Hexose*	87.7	—	123	9 (38)
Ribose	11.5	Absent	7.7	Absent
DNA	10.2	Absent	5 (20)	Absent
Fatty acids†	120	10 (30)	53	Absent
Amino sugars‡	15.7	Absent	7.9	Absent

* Results expressed in μg of glucose/ml.
† Results expressed in μg of hexadecanoic acid/ml.
‡ Results expressed in μg of glucosamine/ml.

1 and 2, together with results from control experiments conducted under identical experimental conditions, but in the absence of EDTA. Comparison of results for test and control experiments (Tables 1 and 2), at both pH 7.1 and 9.2, demonstrates that EDTA has a marked effect on the cells, resulting in the release of high-molecular-weight solutes. The effect is markedly dependent on pH, being greater (except for hexose) under the more alkaline conditions; direct evidence for release of nucleic acids is given by analyses for ribose and DNA. The results for both conditions of pH suggest that considerable damage is caused to the cell wall by EDTA. Results (Table 2) relating to the release of lipopolysaccharide from the cell wall, i.e. the concentrations of amino sugars and fatty acids in the high-molecular-weight fraction, show that more lipopolysaccharide is solubilized by EDTA at pH 9.2 than at pH 7.1, although more hexose was released at pH 7.1. The hexose is associated with high-molecular-weight solutes, and this suggests that hexose-containing polymer other than lipopolysaccharide is released from the cell.

Fewer analyses were carried out on the low-molecular-weight pool. For test and control experiments at pH 7.1 and 9.2, fatty acids were present in low concentration and the results were out of the range of the method. However, the

differences indicated between the concentrations of fatty acids in the low-molecular-weight fractions from test and control experiments at both conditions of pH appeared to be small. Coupled with the absence of amino sugars from all low-molecular-weight fractions, this suggests that no serious degradation of solubilized lipopolysaccharide occurred through the action of EDTA during the release from the cell wall.

Yield and purity of bacterial cell walls. The average yield of freeze-dried cell walls from cells grown in 20 litres of culture medium was 1.5g. Examination of the walls by electron microscopy showed that they were homogeneous and reasonably free from cytoplasmic contamination. Four separate batches of cell walls designated B1–B4 were prepared. The i.r. spectra of the batches were indistinguishable and closely similar to those of other Gram-negative bacterial cell walls (Gray & Wilkinson, 1965*b*). Examination of the u.v. spectra of suspensions of the cell walls confirmed the absence of nucleic acids absorbing at 260nm.

Analysis of the batches of cell walls gave the following results: phosphorus, 1.94–2.3%; nitrogen, 7.4–7.6%; anthrone sugar, 2.8–3.7%.

Analysis of the cell walls for ninhydrin-positive compounds. The amino compound composition of each of the four batches of cell walls was determined by autoanalysis of hydrolysates for ninhydrin-positive species; results are given in Table 3. Amino compounds accounted for 36–45% of the cell wall and contributed 82–90% to the total nitrogen of the cell wall. The qualitative complement of amino compounds was the same for each of the four batches and was confirmed by paper chromatography of cell-wall hydrolysates (solvent

Table 3. *Quantitative analyses for the amino compounds of four batches (B1–B4) of cell walls of* P. alcaligenes

Values for amino acids relate to amino acid residues; other values relate to the free compounds. Results were obtained after hydrolysis of cell walls for 16h at 105°C with constant-boiling (6.1M) HCl and are not corrected for slow release or destruction during hydrolysis. Except for batch B4, valine and galactosamine were unresolved, and results give combined percentages of the two components. +, Present, but no quantitative determination could be made. —, Not determined.

	Amount in cell wall (%, w/w)			
	B1	B2	B3	B4
O-Phosphorylethanolamine	0.36	+	0.07	0.26
Aspartic acid	3.60	4.20	3.20	3.90
Threonine	1.46	1.94	1.35	1.33
Serine	1.58	1.91	1.56	2.10
Muramic acid	1.27	0.80	1.19	0.74
Glutamic acid	4.26	4.92	5.35	4.37
Proline	1.12	1.60	0.97	1.25
Glycine	1.77	2.23	1.67	1.90
Alanine	3.98	4.23	3.84	3.89
Glucosamine	2.51	1.85	2.33	1.96
Valine* } Galactosamine*	1.44	2.55	1.84	2.15
				0.45
Cystine	+	+	+	+
Methionine	0.46	+	0.60	+
Isoleucine	0.68	1.66	1.11	1.36
2,6-Diaminopimelic acid	1.29	0.52	1.55	0.64
Leucine	2.42	3.70	2.42	3.00
Tyrosine	1.71	2.05	1.78	1.72
Phenylalanine	1.35	2.20	1.52	1.72
Ethanolamine	0.42	0.60	0.48	0.36
Ammonia	0.61	1.48	1.11	0.79
Ornithine	+	1.00	0.86	+
Lysine	1.36	2.00	1.46	1.63
Histidine	0.69	0.91	0.60	0.67
Arginine	2.20	2.80	2.05	2.53
Total	36.5	45.2	38.9	38.7
Nitrogen	7.4	7.6	7.5	—
Nitrogen recovery (%)	82.1	86.0	89.8	—

* The presence of valine and galactosamine was confirmed by paper chromatography.

systems *b* and *c*). Batch B2 had a higher protein and lower hexose content than the other batches, and this could be accounted for in terms of variable loss of lipopolysaccharide during cell-wall preparation as reported by Roberts *et al.* (1967) for *P. aeruginosa*.

Neutral sugars of the cell wall. Descending paper chromatography (solvent system *a*) of neutralized hydrolysates of the cell wall revealed the presence of only glucose and rhamnose in each of the batches, but other sugars were detected in later work.

Loosely bound lipids of the cell wall. Loosely bound lipids were extracted from the four batches of cell walls with chloroform–methanol (2:1, v/v); the defatted cell walls were used for treatments with aqueous phenol. The results of separating each of three batches of lipid into fatty acid and phospholipid fractions by silicic acid chromatography (Lis *et al.* 1961) are given in Table 4. The low recovery from the silicic acid column with lipid B3L was confirmed on replicate separations. Lipids were applied to the column as solutions in light petroleum, and with lipid B3L an insoluble gelatinous solid was noted. This material was not eluted through the column, and was shown by i.r. spectroscopy to be poly-3-hydroxybutyrate (approx. 20% of the lipid sample), together with a small amount of protein. The other lipid batches did not contain poly-3-hydroxybutyrate. The results for lipid B3L have been corrected for the content of poly-3-hydroxybutyrate and these results are given in the last column in Table 4.

T.l.c. (solvent system *d*) of the whole lipid B1L revealed the presence of phosphatidylethanolamine, apparently as the main phospholipid component, together with a smaller amount of phosphatidylglycerol and some other minor phosphorus-containing components, probably including lysophosphatidylethanolamine and diphosphatidylglycerol.

The phospholipid fractions from lipid batches B2L and B3L were then examined by t.l.c. (solvent system *d*); the presence of phosphatidylethanolamine, phosphatidylglycerol and lysophosphatidylethanolamine was confirmed. The occurrence of a phosphorus-containing spot with an R_F value similar to that of diphosphatidylglycerol was now clear. T.l.c. in solvent system *g* showed that the spot which had been identified as lysophosphatidylethanolamine by using solvent system *d* consisted of two components, lysophosphatidylethanolamine and phosphatidylcholine. Choline was also detected by paper chromatography of an acid hydrolysate.

T.l.c. of the whole lipid B1L by the method of Skipski, Smolowe, Sullivan & Barclay (1965) was also used; solvent system *e* was used, with a layer of 0.25mm in preference to one of 0.5mm. The only detectable components of $R_F > 0$ were free fatty acids. The free fatty acid content of the lipid was assessed quantitatively (*a*) by direct weighing of the fatty acid fractions obtained by column chromatography on silicic acid (54%), (*b*) by colorimetric assay of the free fatty acids in these fractions (46% expressed as hexadecanoic acid) and (*c*) by quantitative removal of the appropriate area of silicic acid from the t.l.c. plate followed by elution and weighing of the fatty acids (40%). Fractionation of the whole lipids was carried out on silicic acid columns. With the elution scheme of Lis *et al.* (1961) only three fractions were obtained. Fractions 2 and 3 consisted entirely of fatty acids, and fraction 4 contained the phospholipids. As shown in Table 4, the recoveries from columns were 81–91%; fraction 3 contained only approx. 12% of the total lipid. The fatty acids were identified as their methyl esters prepared from the free acids of fractions 2 and 3. The fatty acids occurring as *O*-acyl groups in the phospholipid fraction were identified after formation of the methyl esters from fraction 4 by transesterification. The results

Table 4. *Analytical results for the loosely bound lipids of the cell wall of* P. alcaligenes

Results in the column headed B3L (a) were obtained from those for lipid batch B3L after correction for contaminating poly-3-hydroxybutyrate. —, Not determined. Apart from the first line, the values represent percentages of total lipid.

	Amount (% of sample, w/w)			
Lipid batch	B1L	B2L	B3L	B3L(a)
Lipid in cell wall	25.0	24.3	23.3	18.1
Phosphorus	1.25	2.05	2.02	2.56
Nitrogen	0.59	—	—	—
Fatty acids*	54.0	20.4	31.4	38.4
Phospholipid†	37.2	61.1	42.3	51.7
Recovery from silicic acid column	91.2	81.5	73.7	90.1

* Results obtained by direct weighing of fractions from the silicic acid column.

† Results obtained by direct weighing of the phospholipid fraction from the silicic acid column.

Table 5. *Relative amounts of the fatty acids in the loosely bound lipids*

Quantitative g.l.c. results were most readily obtained from columns of polyethylene glycol succinate at 180°C, but similar results were given by columns of Apiezon L at 220°C. With the elution scheme of Lis *et al.* (1961) no material corresponding to fraction 1 was obtained; fractions 2 and 3 from the silicic acid columns consisted entirely of fatty acids and fraction 4 was the phospholipid fraction containing *O*-acyl groups.

	Fatty acid composition (%, w/w)		
	Fraction 2	Fraction 3	Fraction 4
Dodecanoic acid	0.1	0.3	0.4
Tetradecanoic acid	1.0	1.7	1.9
Hexadecanoic acid	38.4	30.3	42.6
Hexadeca-9-enoic acid	14.4	31.9	14.7
Octadecanoic acid	Trace	Trace	Trace
Octadeca-9-enoic acid	42.8	33.3	36.3
Unidentified acids	3.3	2.5	4.1

obtained by g.l.c. are in Table 5 and show that the main acids present both in the free fatty acid fractions 2 and 3 and as *O*-acyl groups in the phospholipid fraction have the same retention times as hexadecanoic acid, octadeca-9-enoic acid and probably hexadeca-9-enoic acid. Some unidentified fatty acids were present in small amounts.

Ninhydrin-positive components of the loosely bound lipids. Hydrolysis and autoanalysis of both the whole lipid B1L and the phospholipid fraction obtained from lipid B1L showed that glucosamine and galactosamine were absent and that the amounts of protein amino acids were very small. Contamination of the lipids by lipopolysaccharide and protein is therefore not significant. The major component in both cases was ethanolamine: 0.87% by analysis of the whole lipid and 0.77% (expressed as a percentage of the whole lipid) by analysis of the phospholipid fraction. By using a mean value (0.82%) and assuming an ethanolamine content of 8.8% (calculated for dihexadecanoylphosphatidylethanolamine), phosphatidylethanolamine represents approx. 25% of the phospholipid fraction.

Fractionation of cell walls with aqueous phenol. Treatment of defatted cell walls with aq. 45% phenol gave five fractions. Two water-soluble fractions (AqI and AqII) were obtained from the aqueous layers. Phenol-soluble material was separated into methanol-soluble and methanol-insoluble fractions designated PhMS and PhMP respectively. Fraction R was insoluble interfacial material. Table 6 gives quantitative data for the amino compounds in hydrolysates of fractions R, PhMP and PhMS obtained from cell-wall batch B1. Fraction R (P, 0.74%; N, 9.2%), representing 7% of the whole cell wall, consists largely of protein and glycosaminopeptide. Contamination by protein prevents calculation of meaningful molar ratios for alanine and glutamic acid of the glycosaminopeptide. Since galactosamine is present in only trace amount, contamination of R by lipopolysaccharide can be discounted. Therefore all the muramic acid, glucosamine and 2,6-diaminopimelic acid of fraction R must originate from glycosaminopeptide. From the results in Table 6, corrected for destruction of hexosamines during the 16h hydrolysis (Clarke *et al.* 1967*a*), the molar proportions are muramic acid:glucosamine:2,6-diaminopimelic acid, 1.09:1.16:1.0.

Fraction PhMP (P, 0.02%; N, 15.1%) represents 28.1% of the whole cell wall and consists largely of protein, 70% of the fraction being accounted for in terms of amino acids and ammonia. Autoanalysis of cell walls gave a protein content of 36.5%, agreeing reasonably with the above value, bearing in mind that fractions R and PhMS also contain protein. The most abundant amino acids of the protein fraction are glutamic acid, aspartic acid, arginine, alanine and leucine.

Fraction PhMS was obtained in only small amount since the cell walls had been defatted, and represents 3.4% of the whole cell wall. The material contained much protein; the predominant amino acids are the same as for fraction PhMP, but the order of abundance is different. Ethanolamine is a minor component of fraction PhMS and the presence of small amounts of glucosamine and galactosamine indicates that the fraction contained some lipopolysaccharide, possibly of the phenol-soluble type isolated by Hickman & Ashwell (1966). No other analyses were carried out on this fraction.

Yields and elemental analyses for the aqueous fractions, AqI and AqII, from three cell-wall batches are given in Table 7, and Table 8 gives the percentages of amino compounds in these fractions. The fractions consist of lipopolysaccharide contaminated with variable amounts of glycosaminopeptide. The purest sample of lipopolysaccharide, fraction AqI from cell-wall batch B1, contains glucosamine, galactosamine and alanine as major

Table 6. *Quantitative results for the amino compounds of fractions R, PhMP and PhMS obtained by aqueous phenol treatment of defatted cell walls from batch B*1

Values for amino acids relate to amino acid residues; other values relate to the free compounds. Results were obtained after hydrolysis of fractions for 16h at 105°C with constant-boiling (6.1 M) HCl and are not corrected for slow release or destruction during hydrolysis. +, Present, but no quantitative determination could be made. –, Not determined.

	Amount (% of sample, w/w)		
	Fraction R	Fraction PhMP	Fraction PhMS
O-Phosphorylethanolamine	Absent	Absent	Absent
Aspartic acid	1.70	8.14	2.84
Threonine	1.13	3.65	1.78
Serine	1.20	3.36	1.79
Muramic acid	3.31	Absent	Absent
Glutamic acid	4.92	9.20	4.00
Proline	0.98	2.70	1.48
Glycine	1.35	3.95	1.78
Alanine	4.82	5.88	2.94
Glucosamine	3.25	Absent	0.44
Valine	1.45	4.56	1.84
Galactosamine	+	Absent	0.11
Methionine	0.30	0.81	1.32
Isoleucine	1.16	2.44	1.49
2,6-Diaminopimelic acid	3.34	Absent	Absent
Leucine	0.84	5.44	4.02
Tyrosine	1.08	4.54	1.49
Phenylalanine	1.74	3.87	1.97
Ethanolamine	+	Absent	+
Ammonia	0.63	1.08	1.12
Ornithine	0.19	+	0.19
Lysine	0.64	3.21	1.50
Histidine	1.06	1.35	0.78
Arginine	1.42	6.03	2.42
Total	36.5	70.2	35.3
Nitrogen	9.2	15.1	–
Nitrogen recovery (%)	62	81	–

Table 7. *Yields and elemental analyses for fractions AqI and AqII of lipopolysaccharide from batches of cell walls of* P. alcaligenes

—, Not determined.

	Batch B1		Batch B2		Batch B3	
	AqI	AqII	AqI	AqII	AqI	AqII
Yield of lipopolysaccharide fraction (% of cell wall)	18.1	8.8	13.6	4.5	16.2	7.3
Phosphorus (%)	5.6	3.7	5.7	6.0	5.0	—
Nitrogen (%)	2.1	4.0	—	—	—	—

ninhydrin-positive components, and 97% of the total nitrogen is accounted for in terms of amino compounds and ammonia. Except for cell-wall batch B2, fractions AqII were more heavily contaminated by glycosaminopeptide than fractions AqI. For batch B2, both fractions AqI and AqII contained relatively small amounts of glycosaminopeptide; together these represented 18.1% of the whole cell wall. The variable phosphorus analyses for fractions AqI and AqII are partly a reflexion of the different degrees of contamination of the fractions.

DISCUSSION

The organism under investigation was a laboratory strain studied by Gray & Wilkinson (1965*a*,*b*) and at that time believed to be a strain of *Alcaligenes faecalis*. However, electron microscopy revealed that the bacterium possessed a polar flagellum (Wilkinson, 1967), and examination of the organism

Table 8. *Quantitative analysis for the amino compounds of water-soluble fractions (AqI and AqII) obtained by aqueous-phenol treatment of defatted cell walls from batches B1, B2 and B3*

Values for amino acids relate to amino acid residues; other values relate to the free compounds. Results were obtained after hydrolysis of samples for 16h at 105°C with constant-boiling (6.1M) HCl, and are not corrected for slow release or destruction during hydrolysis. +, Present, but no quantitative determination could be made. —, Not determined.

	Amount (% of sample, w/w)					
	Batch B1		Batch B2		Batch B3	
	AqI	AqII	AqI	AqII	AqI	AqII
O-Phosphorylglucosamine*	0.30	0.21	0.17	0.24	0.20	0.20
Alanine	1.75	4.20	2.21	1.46	2.57	4.37
Glucosamine	4.60	5.90	4.30	3.54	5.20	6.68
Galactosamine	2.67	1.67	1.96†	2.03†	2.70†	2.50†
Muramic acid	+	3.80	0.70	0.24	1.08	3.90
2,6-Diaminopimelic acid	0.19	3.02	0.99	0.31	1.20	4.00
Ammonia	1.00	0.85	1.40	2.06	0.84	1.27
Other amino compounds	2.51	6.02	4.10	1.20	2.15	4.90
Total	13.0	25.7	15.8	11.1	15.9	27.8
Nitrogen recovery (%)	97	—	—	—	—	—

* The relative colour yield for this component was calculated by using glucosamine 6-phosphate as standard.

† Valine and galactosamine were not resolved, and the components were determined together.

at the laboratories associated with N.C.T.C. confirmed that it was a pseudomonad, with the properties of *P. alcaligenes*. Tests to differentiate between *P. alcaligenes* and *Pseudomonas pseudoalcaligenes* (Stanier, Palleroni & Doudoroff, 1966) were not, however, made.

Gray & Wilkinson (1965*a*,*b*) had demonstrated that EDTA had a direct bactericidal action on the organism, that it was more sensitive to EDTA than was *P. aeruginosa*, and that exposure of isolated cell walls to solutions of EDTA resulted in solubilization of large amounts of phosphorus and carbohydrate. One aim of this work was to confirm that this phosphorus and carbohydrate was associated with high-molecular-weight lipopolysaccharide solubilized in the process of the bactericidal action of EDTA on cells of *P. alcaligenes*. Although the bactericidal action of EDTA on cells of *P. aeruginosa* is extremely rapid, release of cell solutes is more gradual (Gray & Wilkinson, 1965*a*). A time of 1h was therefore chosen for exposure of cells to solutions of EDTA; the extent of bactericidal action of EDTA was determined after the same exposure time, although this was not markedly greater than that after an exposure time of 2min.

Bactericidal action of EDTA on P. alcaligenes. The results confirm the marked sensitivity of *P. alcaligenes* under the conditions used to study release of cell solutes. At pH 9.2 the large decrease in viability was similar to that obtained with *P. aeruginosa*, which, however, at pH 7.1 gave a much smaller decrease in viable count (28%) (N. A. Roberts, G. W. Gray & S. G. Wilkinson, unpublished work). These results confirm that *P. alcaligenes* is more sensitive than *P. aeruginosa* to EDTA when grown under comparable conditions. The results in Tables 1 and 2 show that, under conditions leading to considerable decreases in viability of cells of *P. alcaligenes*, EDTA causes substantial damage to the bacterial cell wall. This is shown by the release of both intracellular nucleic acids and high-molecular-weight lipopolysaccharide from the cell wall. On changing from pH 7.1 to 9.2 a 12% increase in percentage kill of cells is apparently accompanied by an increase of about 50% in the amount of solubilized lipopolysaccharide. This suggests that death of the cell and solubilization of lipopolysaccharide from the cell wall are related processes. Results (N. A. Roberts, G. W. Gray & S. G. Wilkinson, unpublished work) show that, although EDTA has a similar bactericidal effect on cells of *P. aeruginosa* at pH 9.2, much more material is solubilized under both conditions of pH. Further, for *P. aeruginosa*, the increase in percentage kill and in solubilization of lipopolysaccharide on changing from pH 7.1 to 9.2 is almost constant (70–71%). The relationship between release of lipopolysaccharide and death of the cell is not therefore a simple one. A fuller investigation of this aspect has been made (N. A. Roberts, G. W. Gray & S. G. Wilkinson, unpublished work) in relation to the action of EDTA on *P. aeruginosa*.

The present results do, however, demonstrate that EDTA has the same general effects on cells of

P. aeruginosa and *P. alcaligenes*, namely decrease in viability, solubilization of lipopolysaccharide and release of intracellular solutes.

It should be noted that the separation of EDTA and low-molecular-weight solutes from supernatant solutions by gel filtration was employed after unsatisfactory results were obtained by dialysis. In conjunction with N. A. Roberts, the results from both test and control experiments for the removal of EDTA by dialysis were found to be variable; complete removal of EDTA was never achieved, and in most experiments approx. 50% of the EDTA remained in the non-diffusible material. A concentration limit of approx. 0.03% was reached, and below this concentration EDTA failed to diffuse. Similar observations have been made for the dialysis of pyrophosphate (Watson, Pittsley & Jeanes, 1962).

Cell wall of P. alcaligenes. As part of a survey of Gram-negative organisms exhibiting a spectrum of sensitivity to EDTA, Gray & Wilkinson (1965*b*) made preliminary investigations of the cell wall of *P. alcaligenes*. The present cell-wall preparations gave indistinguishable i.r. spectra and had the same complement of sugars, amino sugars and amino acids. Quantitative analysis for phosphorus and anthrone sugar gave similar results, and only in the nitrogen content was any appreciable difference noted; cell walls examined in the earlier work contained more nitrogen (8.5%).

Broadly speaking, the results for the cell wall of *P. alcaligenes* were similar to those obtained by Clarke *et al.* (1967*a*) and Fensom & Gray (1969) for the cell wall of *P. aeruginosa*: phosphorus, 1.8–2.1%; nitrogen, 7.6–8.5%; amino compounds (representing 81–83% of the total nitrogen of the cell wall), 43–46%. For both organisms the most abundant amino acids of the cell wall were glutamic acid, aspartic acid, alanine and leucine, and as noted by Gray & Wilkinson (1965*b*) the i.r. spectra of the walls were closely similar. Each spectrum had a weak absorption at approx. $925 cm.^{-1}$ that is absent from the otherwise similar spectra of cell walls of *Escherichia coli* and *Proteus mirabilis*, Gram-negative organisms that are relatively insensitive to the bactericidal action of EDTA. Cell walls of both organisms contained glucosamine, galactosamine, 2,6-diaminopimelic acid and muramic acid, and the only neutral sugars detected by paper chromatography of wall hydrolysates were glucose and rhamnose. Two points of difference did emerge: cell walls of *P. alcaligenes* contain smaller amounts of sugars determined by the anthrone method (2.8–3.7% as compared with 5.6–7.4% for *P. aeruginosa*) and no fucosamine. Otherwise the qualitative complement of ninhydrin-positive components of both cell walls is identical.

Loosely bound lipids of the cell wall of P. alcaligenes. The loosely bound lipids were obtained in yields of 18–25%. The lower yields of lipids (9.5 and 14.2%) obtained from previous batches of walls (Gray & Wilkinson, 1965*b*) may be explained by the less efficient extraction method used and by the different conditions under which the organisms were grown. Similar variations (5.3–26%) have been found for the lipid content of batches of cell walls from *P. aeruginosa* prepared in these laboratories (Gray & Wilkinson, 1965*b*; J. W. Payne & A. H. Fensom, unpublished work). Thus the two cell walls may not differ significantly in lipid content.

Previous results (Gray & Wilkinson, 1965*b*) suggested that the lipids of *P. alcaligenes* might be characterized by a higher content of free fatty acids and a lower content of phospholipids than those of *P. aeruginosa*. Although this observation was also made for batch B1L, the difference was not maintained for batches B2L and B3L. It is well known that the content and composition of bacterial lipids may vary widely unless conditions for growth of the organisms are strictly controlled, and variations similar to those described here have been reported for other cell walls (see e.g. Wardlaw, 1963; Herzberg & Green, 1964).

Studies of the composition of the lipids were made mainly on batch B1L. Autoanalysis of hydrolysates of both the whole lipid and the phospholipid fraction obtained by column chromatography indicated only small amounts of protein amino acids. In contrast with previous results (Gray & Wilkinson, 1965*b*), serine was not a major component of the lipids. Although ethanolamine was the main ninhydrin-positive component of the lipids, perhaps only approx. 25% of the phospholipid fraction was accounted for by phosphatidylethanolamine and lysophosphatidylethanolamine. However, this value may be a considerable underestimate in view of possible losses of ethanolamine during hydrolysis of lipids or drying of hydrolysates (Wheeldon, Brinley & Turner, 1962; Ansell & Hawthorne, 1964), and because the peak corresponding to ethanolamine occurred on a rising baseline of the chromatogram obtained on autoanalysis. Of the remaining phospholipids, diphosphatidylglycerol and phosphatidylglycerol are common bacterial lipids, and phosphatidylcholine has been found as a minor component in several pseudomonads (Ikawa, 1967; S. G. Wilkinson, unpublished work) including *P. aeruginosa* (see e.g. Sinha & Gaby, 1964; Gordon & MacLeod, 1966; Bobo & Eagon, 1968; Randle, Albro & Dittmer, 1969). Although phosphatidylcholine was not detected in earlier studies (Gray & Wilkinson, 1965*b*), a much smaller amount of lipid was then available and analysis by t.l.c. was not done.

The contamination of one batch (B1L) of lipids by poly-3-hydroxybutyrate is noteworthy. Some strains of *P. alcaligenes* have been shown to accumulate this lipid (Stanier *et al.* 1966). On the other hand, *P. aeruginosa* does not have this ability, and the lipid has never been found in preparations of walls from this organism.

Fractionation of the cell walls with aqueous phenol. The present work on *P. alcaligenes* shows that the cell wall contains the same components in about the same amounts as in *P. aeruginosa* (Clarke *et al.* 1967*b*,*c*), although it is possible that the cell wall of *P. alcaligenes* contains rather less lipopolysaccharide. For the lipopolysaccharides of both organisms, glucosamine, galactosamine and alanine are the major ninhydrin-positive components, but fucosamine does not occur in the case of *P. alcaligenes*. The two lipopolysaccharides also differ in the percentage of the total nitrogen accounted for by ammonia and amino compounds. For the lipopolysaccharide of *P. alcaligenes* the nitrogen recovery was 97%, showing that unidentified nitrogen-containing components are not a problem in this instance. With *P. alcaligenes* the phosphorus content of the pure lipopolysaccharide may approach 6%, even higher than for *P. aeruginosa* ($4.3 \pm 0.8\%$; Fensom & Gray, 1969). These phosphorus contents are high compared with those for the lipopolysaccharides of many Gram-negative organisms and may be characteristic of EDTA-sensitive pseudomonads.

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