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A MEMBRANE FRACTION OBTAINED BY DISINTEGRATION OF CELLS OF *PSEUDOMONAS AERUGINOSA*

G. W. GRAY AND P. F. THURMAN
Department of Chemistry, The University, Hull, Yorkshire (Great Britain)

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SUMMARY

1. Supernatant fluids from the preparation of cell walls of *Pseudomonas aeruginosa* by Mickle disintegration have been examined for the presence of membrane material.

2. After separation of cell-wall material (17 000 × *g* for 30 min), further centrifugation (40 000 × *g* for 30 min) of the supernatant fluids gave a small particle fraction. The yield of washed, freeze-dried material was 15–20 mg/100 mg of cell wall.

3. Analysis for nitrogen, phosphorus, and anthrone sugar showed that the product was quite reproducible and different to cell wall. 3 of the 5 preparations were free from peptidoglycan and RNA. The main constituents were protein (about 52%) and readily extractable lipid (38%), together with smaller amounts of bound lipid (7%) and carbohydrate (2.3%).

4. The small particle fraction was therefore essentially lipoprotein and appeared to be a membrane fraction from *P. aeruginosa*.

INTRODUCTION

Recent studies of the action of EDTA upon whole cells and cell-wall preparations of *Pseudomonas aeruginosa*[1,2] have interested us in the membrane of the organism. The present work was undertaken to establish whether a small particle fraction obtained from supernatant fluids resulting from the preparation of cell walls of the organism, corresponded in composition to protoplast membranes and small particle fractions isolated from other organisms in general and from *P. aeruginosa* in particular.

Protoplast membranes may be prepared from Gram-positive organisms by dissolution of the outer cell wall with lysozyme[3]. In the case of *Staphylococcus aureus*, an autolytic enzyme has been shown[4] to break open the cell wall, and the protoplast membranes so obtained have the same enzymic composition[5] as a small fraction obtained[6] from whole cells of the organism by disintegration. Analytical data are available for the protoplast membranes of several Gram-positive organisms, *e.g.*,

Staph. aureus[6], *Micrococcus lysodeikticus*[7,8], *Bacillus megaterium*[9–11], *Bacillus licheniformis*[8], *Sarcina lutea*[8,12], *Streptococcus faecalis*[13], *Strep. pyogenes* and its derived L form[14], and Group A streptococci[15].

Fewer analytical data are available for envelopes of Gram-negative organisms, mainly because protoplasts are more difficult to obtain. However, combined action of EDTA and lysozyme[16,17] has yielded envelopes or cellular fractions of *P. aeruginosa*[18,19] and *P. fluorescens*[20], and a small particle fraction resulting from disruption of cells of *Azotobacter agilis*[21] has been examined.

For comparison with the results obtained in the present work, the percentage compositions of a number of membrane preparations and cellular fractions are summarised in Table I. In two cases, *P. aeruginosa*[19] and *P. fluorescens*[20], analytical results were quoted only for protein, RNA and DNA, and expressed either as percentages of the dry weight of cells[19] or as percentages of the results obtained for the suspensions of ruptured envelopes. It has not therefore been possible to include results in Table I comparable with the other data.

TABLE I

PERCENTAGE COMPOSITIONS OF MEMBRANE PREPARATIONS AND CELLULAR FRACTIONS FOR A RANGE OF MICROORGANISMS

Results are given as percentages of the dry weight of the preparation, except for Group A streptococci for which the preparations contain about 5% of water. In several cases, average results are quoted for two or more similar preparations, or for preparations obtained either under different conditions or from microorganisms grown in different environments. The two analyses presented for Group A streptococci represent protoplasts (A) and membrane from disrupted cells (B). Dash, no figures quoted.

Organism	*Ref.*	*Lipid*	*Protein*	*Hexosamine*	*Hexose*	*DNA*	*RNA*	*N*	*P*
Gram-positive									
Staph. aureus	6	22.5	41**	about 3	1.7	0.5	2.4	9.5	cc
M. lysodeikticus	7	28	52.5*	2.7	15.6–18.8	Traces		8.4	1.2
	8	22.7	68***	—	§§	—	2.3	—	0.8
B. licheniformis	8	28	75***	—	§§	—	0.8	—	—
B. megaterium	9	18.5	66.7***	<0.7§	4.8	0.1–0.2	1.3	10.5	1.5
	10	25	70††	<0.1§	0.8	Variable		11.8°	0.97°
	11	6–9	70–85***	—	1.5	<1.5	10–15	—	—
Sarc. lutea	8	23.3	57***	—	§§	—	5.4	—	0.6
	12	28.9	39.8†	9.6	—	2.4	1.2	12.8	—
Strep. faecalis	13	32.1	45.5††	—	—	2.7		8.5	2.9
Strep. pyogenes	14	15.3	68	—	1.7	—	—	—	—
L form	14	35.6	59	—	7.9	—	—	—	—
Group A streptococci	15 (A)	25	68.1*	0.2	2.1	—	2.0	10.9	1.1
	(B)	19.5	66.9*	0.5	2.2	—	0.6	10.7	0.5
Gram-negative									
P. aeruginosa	18	35.1	60.2*	—	1.39	—	0.5	9.6	—
A. agilis	21	19.5	75*** or †	—	—	1.0	9.4	10.6	2.2

* Per cent N × 6.25.
** Non-lipid Kjeldahl N × 6.2.
*** Biuret method.
† Method of LOWRY *et al.*[22].
†† Based on analyses for individual amino acids.
§ No diaminopimelic acid present or <0.1% diaminopimeltic acid.
§§ Sugars analysed qualitatively.
° Average results for chloroform extracted-membranes.
cc 8% of polyolphosphoric acid.

The figures in Table I show that the preparations consist mainly of lipid and protein, together with smaller, variable amounts of hexosamine, hexose, and nucleic acid. The protoplast membrane of *M. lysodeikticus*[7] has an unusually high percentage of hexose, of which mannose is an important constituent. SALTON AND FREER[8] also qualitatively identified mannose (together with glucose, galactose and ribose) in their membrane preparations from the same organism and from *Sarc. lutea*, but in the case of *B. licheniformis*, only glucose and galactose were detected. Glucose was however the most abundant sugar present in all the membranes studied by SALTON AND FREER[8]. For the various preparations in Table I, the nitrogen contents range from 8.4 to 12.8% and the phosphorus contents from 0.5 to 2.9%.

MATERIALS AND METHODS

Bacteria and growth conditions

Cells of *P. aeruginosa* (NCTC 1999) were grown on a medium of TGE agar–Davis agar–water (26:3:71) at 37° for 16 h.

Disintegration of cells and isolation of membrane material

After washing off the slopes, whole cells were harvested by low-speed centrifugation, washed with ion-depleted water and disintegrated with ballotini beads (grade 12) in a Mickle disintegrator for 10 min. The suspension of ruptured cells was passed through a glass sinter (No. 1 porosity) to remove the beads, and centrifuged, first at 5000 × *g* for 15 min to deposit whole cells, and then at 17 000 × *g* for 30 min to deposit cell walls. The supernatant fluid was then treated in different ways to establish the best means of isolating a reproducible small particle fraction.

(a) To remove residual cell-wall material, further centrifugation at 17 000 × *g* was carried out for 1 h. The supernatant fluid was then centrifuged at 40 000 × *g* for 1 h and at 105 000 × *g* for 1 h. The two deposits were collected, and separately resuspended in ion-depleted water, dialysed for 48 h against ion-depleted water, and freeze dried. The resulting products contained only a small amount of lipid and were contaminated with nucleic acid.

(b) Procedure (a) was again used to remove residual cell-wall material, and three fractions were obtained by centrifuging the supernatant fluid successively at 25 000, 40 000 and 105 000 × *g* for 1 h in each instance. The separate fractions were washed twice with 1 M NaCl and 4 times with ion-depleted water, and then freeze dried. Although these preparations contained no nucleic acid, the yields were very small, and analytical results were inconsistent from one experiment to another.

These procedures therefore gave products which were either irreproducible or which differed in composition to that expected of a membrane preparation. The material deposited in the preliminary spins (17 000 × *g* for 1 h) to remove residual cell-wall material was reddish-brown in colour and unlike cell wall, and this suggested that membrane might be only slightly less dense than cell wall and might be deposited more slowly together with less dense cell-wall material.

(c) The method finally adopted omitted the preliminary spin at 17 000 × *g*, and the supernatant fluid from the cell-wall preparation was centrifuged at 40 000 × *g* for 30 min. The deposit was washed once with ion-depleted water, twice with 1 M

NaCl, and 4 times with ion-depleted water. After each of the centrifugations in the earlier of these washing stages, residual cell-wall material was separated from the deposits and rejected by carefully washing off the upper layer of 'membrane'. In the last two washes, no denser cell-wall material was visible on centrifugation, and the preparation was finally freeze dried and stored *in vacuo* (over P_2O_5) at 4°.

This procedure was reliable and was used to prepare 5 batches of membrane, various analytical methods showing that the preparations were reproducible and different to cell wall. The average yield of membrane was 66 mg (extreme limits 55–83 mg) in experiments yielding on average 370 mg of cell wall. These yields correspond to 15–20 mg of membrane per 100 mg of cell wall.

Analytical methods

The phosphorus content of the preparations was determined by the method of ALLEN[23], and the nitrogen content by a modification of the method of UMBREIT, BURRIS AND STAUFFER[24].

Samples were hydrolysed with 1 M H_2SO_4 for 2 h at 105°. After neutralisation of acid by $Ba(OH)_2$ and removal of insoluble materials, the supernatant fluids were evaporated to dryness over P_2O_5. The residues were taken up in ion-depleted water and the solutions examined by one-dimensional, descending paper chromatography using the solvent system pyridine–ethyl acetate–acetic acid–water (5:5:1:3, by vol.). The papers were dried and treated with $AgNO_3$ (ref. 25). Only glucose was detected, and this was estimated by the anthrone method[26]. Total hexosamine was determined quantitatively by the method of CESSI AND PILIEGO[27], and the results expressed as glucosamine hydrochloride.

Hydrolysates (redistilled, constant-boiling HCl for 16 h at 105°) were examined qualitatively for amino acids and other ninhydrin-positive components by two-dimensional chromatography on Whatman No. 1 paper using *sec.*-butanol–88% formic acid–water (75:15:10, by vol.), followed by phenol–water–5 M ammonia (80:20:0.3, by wt.). Spots were detected using ninhydrin. The presence or absence of α,ε-diaminopimelic acid in the hydrolysates was checked by descending paper chromatography using as solvent a mixture of methanol–water–10 M HCl–pyridine (32:7:1:4, by vol.)[28].

For the quantitative estimation of amino acids, amino sugars and other ninhydrin-positive components in a hydrolysate of a given preparation, a sample (5 mg) was hydrolysed using redistilled constant-boiling HCl (1 ml) in a sealed tube at 105±0.5° for 16 h. 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 this process, 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 0.1 M HCl containing a given weight of norleucine (0.08865 μmole/ml), which served as an internal standard for ion-exchange chromatography. A volume (1 ml) of this solution was used for the estimation of ninhydrin-positive components using an automatic amino acid analyser (Technicon Instruments, Chertsey, Surrey). A standard solution containing a wide range of amino acids, amino sugars *etc.* was similarly analysed to obtain colour yields for each component. These were based on a standard value of 50.6 for norleucine. From the chromatogram obtained for a given hydrolysate, the peak area for each component was calculated, and these

areas were corrected by standardising the chromatogram, *i.e.*, by multiplying the areas by the colour yield for norleucine/50.6. The values for the amino compounds were then in terms of the standard values and could be used to calculate the percentage of each amino compound in the sample, allowing for the loss of water during peptide bond formation. Results from duplicate hydrolysates agreed within $\pm 4\%$, compared with $\pm 3\%$ for duplicate estimations on standard solutions of amino acids.

Since certain amino acids are released slowly on hydrolysis, whilst amino sugars are extensively destroyed on treatment with strong acids, it was necessary to correct the results for slow release and decomposition of the components. This was possible using results obtained earlier by REAVELEY, who plotted concentration *vs.* time curves for the ninhydrin-positive components of the cell wall of *P. aeruginosa*, by analysing hydrolysates after 2, $5\frac{1}{2}$, 16 and 30 h (see ref. 29).

Application of the method of WEBB[30] showed that RNA was absent from the preparations, and the low absorbance of aqueous suspensions at 260 mμ showed that no nucleic acid was present.

The infrared absorption spectra of samples were obtained from dispersions of the material in KBr discs, using an SP 100 double-beam spectrophotometer equipped with grating (Unicam Instruments, Cambridge).

Extraction and examination of lipids

The free or loosely bound lipids were extracted by stirring the preparations (30–50 mg) with a mixture of purified chloroform–methanol (2:1, by vol.; 5 ml) for 1 h at room temperature. The residue was collected and washed on a sinter (No. 4 porosity) with the same solvent mixture (10 ml). The washings were combined with the filtrate, and the solvent was removed in a tared bottle using a stream of filtered N_2. The entire procedure was repeated (4 times in all) on the insoluble residue.

The combined lipid extracts (about 10 mg) from one of the preparations were fractionated on silicic acid, using the micro-column method of LIS, TINOCO AND OKEY[31]. The infrared spectra of the whole lipid, of the four separate fractions and of the defatted residue were obtained (see above) for comparison, and these samples were also analysed for phosphorus[23] and, when amounts permitted, for nitrogen[24]. The whole lipid and the defatted residue were analysed for hexose by the anthrone method[26], and for ninhydrin-positive components using the amino acid analyser.

The whole lipid and the phospholipid fraction from the silicic acid chromatography were examined by thin-layer chromatography on silica gel G, using the solvent mixture methanol–chloroform–water (25:65:4, by vol.). Spots were detected by treating the plates first with ninhydrin reagent and then with ammonium molybdate–perchloric acid reagent.

To remove more firmly bound lipid from the defatted residue, a sample was heated under reflux for 30 min with 0.1 M HCl. The mixture was cooled and treated with redistilled chloroform. The chloroform extracts were washed with ion-depleted water, the solvent was evaporated off, and the residual lipid was dried *in vacuo* over P_2O_5 at 4°.

RESULTS

Preliminary analyses of small particle fractions

The small particle fractions from the supernatant fluids of 5 consecutive cell-wall preparations on the same scale were collected and purified. The yields obtained and the results of preliminary analyses, together with corresponding data for cell walls, are summarised in Table II. The diaminopimelic acid content of the cell walls

TABLE II

YIELDS AND ANALYTICAL RESULTS FOR SMALL PARTICLE FRACTIONS AND CELL WALLS

Fraction	*Yield (mg)*	*Analytical results (%)*			
		Diamino-pimelic acid	*N*	*P*	*Hexose*
Batch No.					
1	57.7	None	9.9	1.40	2.1
2	70.0	None	10.1	1.44	2.6
3	65.7	None	9.6	1.38	2.1
4	55.0	<0.2	10.5	1.44	1.8
5	83.1	<0.5	10.2	1.43	2.7
Cell walls	about 370	1.7–1.9	7.6–8.3	2.0–2.1	6.8–7.4

was obtained by automatic amino acid analysis[29], whereas the figures for Batches 4 and 5 were estimated visually by comparing spot intensity with that for a standard amount of diaminopimelic acid on a paper chromatogram. The figures represent upper limits for the diaminopimelic acid contents. The absence of diaminopimelic acid from Batches 1 and 2 was confirmed by automatic amino acid analysis. 3 of the batches (1, 2 and 3) were therefore free from peptidoglycan, and analysed quite consistently for phosphorus, nitrogen and hexose: average results, N, 9.9%; P, 1.41%; hexose, 2.3%. Whilst all 5 preparations were clearly quite different to cell wall, Batches 4 and 5, containing some diaminopimelic acid, were less pure and were probably contaminated with cell-wall material. These two preparations were however used for exploratory analyses, since the other analytical data in Table II suggest a close similarity to Batches 1, 2 and 3.

The infrared spectra of the 5 preparations were closely similar, showing marked absorptions associated with protein (3280, 1660, 1552 and 1245 cm^{-1}) and lipid (2900 and 1750 cm^{-1}). From the relative absorbances of the bands compared with those for cell walls, it was concluded that the small particle fractions contained appreciably more lipid relative to protein.

Using the method of CESSI AND PILIEGO[27], the small particle fractions were found to contain only a small percentage of hexosamine (0.3–0.45%), expressed as glucosamine hydrochloride. The cell walls of the organism have been shown[29] by ion-exchange chromatography in conjunction with automatic amino acid analysis to contain about 4% of glucosamine together with some galactosamine. Subsequent automatic amino acid analysis of hydrolysates of Batches 1 and 2 did give higher amino sugar contents (about 0.65%), but still much lower than for cell walls.

TABLE III

EXTRACTION OF LIPIDS FROM SMALL PARTICLE PREPARATIONS

Fraction	*Batch 1*	*Batch 2*
Weight of sample treated	35.49 mg	51.66 mg
Yield from 1st extraction	13.72 mg	18.45 mg
Yield from 2nd extraction	0.50 mg	0.78 mg
Yield from 3rd extraction	0.15 mg	0.20 mg
Yield from 4th extraction	0.04 mg	0.06 mg
Total lipid extracted	14.41 mg	19.49 mg
Percentage of lipid extracted	40.6%	37.7%
Weight of residue	21.77 mg	32.28 mg
Recovery	102%	100.8%

The preparations were free from RNA and materials absorbing at 260 mμ. Paper chromatography confirmed the absence of ribose, and glucose was the only monosaccharide detected in hydrolysates of the preparations.

The lipid of the small particle fractions

The lipids were extracted from Batches 1 and 2, with the results shown in Table III. The average percentage of lipid in the two batches was close to 39% compared with only 14–15% of lipid extractable under the same conditions from cell walls of the organism (G. W. GRAY AND J. W. PAYNE, unpublished work). A sample of the whole lipid from Batch 1, having P, 2.71%; N, 1.53%; anthrone sugar, 0.32%, was fractionated using a silicic acid column, giving the results in Table IV. The compositions of the fractions were deduced from infrared spectra, and in the case of Fractions 1, 2 and 3, these conclusions were strengthened by the absence of phosphorus. The aliphatic ester may arise as an artifact; the lipid contains appreciable amounts of fatty acids, and since methanol was used in the extraction procedure, the possible formation of methyl esters cannot be overlooked. The infrared spectrum of Fraction 4 was closely similar to that of a sample of phosphatidylethanolamine, although the nitrogen content (2.14%) was somewhat higher and the phosphorus content (3.11%) appreciably lower than the calculated values for a phosphatidylethanolamine containing 2 palmitoyl residues (N, 2.02%; P, 4.48%). Thin-layer chromatography on Fraction 4 confirmed that the major constituent was phosphatidylethanolamine, and revealed the presence of phosphatidylglycerol and lysophosphatidylethanolamine. A component (ninhydrin-positive and containing phosphorus),

TABLE IV

FRACTIONATION OF WHOLE LIPID (BATCH 1) ON SILICIC ACID*

Fraction	*P (%)*	*Yield (mg)*	*Yield (%)*	*Composition*
1	None	0.77	7.6	Mainly aliphatic ester
2	None	0.11	0.9	Mainly aliphatic ester
3	None	1.68	16.5	Mainly aliphatic acid; some ester
4	3.11	7.61	75	Phospholipid

* Recovery from column, 93%.

giving a spot immediately below phosphatidylethanolamine, was unidentified. Thin-layer chromatography on the whole lipid confirmed these conclusions regarding the compositions of Fractions 1–4.

The infrared spectra of the defatted residues from the lipid extractions showed little absorption in the region 1650–1750 cm^{-1}, confirming removal of most of the lipid. Analysis of the residue from Batch 1 showed a marked increase in the nitrogen content (9.9 to 13.0%), but the phosphorus content (0.52%) was higher than expected. After treatment with 0.1 M HCl at 105° for 4 h, extraction of the residue with chloroform yielded some firmly bound lipid equivalent to 8.8% of the residue. This lipid contained no phosphorus and the infrared spectrum showed that it was mainly aliphatic acid. The phosphorus content of the residue was however reduced to 0.25%.

Ninhydrin-positive constituents of small particle fractions and other samples

Paper chromatography detected the wide range of amino acids indicated in the table of quantitative results for ninhydrin-positive species in acid hydrolysates of the preparations (Table V), and visual inspection of spot intensities indicated that aspartic acid, glutamic acid, leucine, isoleucine, phenylalanine, valine, arginine and alanine were the major components. No evidence was obtained by paper chromatography for diaminopimelic acid or muramic acid in Batches 1, 2 and 3, and the

TABLE V

CORRECTED VALUES FOR NINHYDRIN-POSITIVE COMPONENTS IN HYDROLYSATES

Amino compound	*Small particle fraction*				*Residue from Batch 1 after lipid extraction*		*Phospholipid from Batch 1*	
	Batch 1		*Batch 2*					
	%	*moles/100*	%	*moles/100*	%	*moles/100*	%	*moles/100*
Cysteic acid	0.12	0.08	0.09	0.06	0.31	0.21	—	—
Ethanolamine phosphate	0.18	0.13	0.11	0.08	—	—	0.96	0.68
Unknown	—	—	—	—	—	—	0.30	—
Aspartic acid	3.22	2.79	3.97	3.45	4.89	4.25	0.19	0.17
Threonine	2.20	2.17	2.21	2.18	2.98	2.95	0.23	0.22
Serine	1.78	2.04	1.81	2.08	2.84	3.26	0.23	0.26
Glutamic acid	4.20	3.25	4.40	3.41	6.26	4.85	0.23	0.18
Proline	2.81	2.89	1.42	1.46	3.18	3.27	—	—
Glycine	1.95	3.42	2.03	3.56	2.58	4.52	0.15	0.26
Alanine	2.80	3.94	3.13	4.40	4.04	5.68	0.16	0.23
Glucosamine	0.49	0.27	0.55	0.29	0.67	0.37	1.01	0.56
Galactosamine	0.18	0.10	0.11	0.61	0.40	0.22	—	—
Valine	3.60	3.60	3.95	3.95	5.01	5.01	0.09	0.09
Cystine	0.54	0.24	0.44	0.19	0.47	0.21	—	—
Methionine	1.13	0.86	1.24	0.94	3.03	2.31	—	—
Isoleucine	3.06	2.70	3.62	3.20	4.24	3.74	0.08	0.07
Leucine	4.70	4.15	4.76	4.20	5.89	5.08	—	—
Tyrosine	1.80	1.10	2.00	1.23	2.50	1.53	Trace	—
Phenylalanine	2.86	1.94	2.98	2.02	3.64	2.47	Trace	—
Ethanolamine	0.72	1.18	1.16	1.90	Trace	—	2.97	4.86
Ammonia	0.88	5.17	1.16	6.81	0.77	4.52	0.26	1.53
Ornithine	0.36	0.32	0.19	0.17	—	—	0.96	0.84
Lysine	1.99	1.55	2.47	1.93	2.26	1.76	Trace	—
Histidine	1.43	1.04	1.41	1.03	1.83	1.34	Trace	—
Arginine	3.71	2.37	3.68	2.36	4.92	3.15	Trace	—

conclusion that peptidoglycan was absent from these samples was confirmed by automatic analysis for amino compounds.

In Table V are summarised the fully corrected percentages of amino sugars *etc.* and of peptide-linked amino acids for Batches 1 and 2 (small particle fractions), for the defatted residue from Batch 1, and for the phospholipid from Batch 1. These figures have been corrected for slow release of certain amino acids by multiplying the results obtained for 16-h hydrolysates by factors obtained by CLARKE, GRAY AND REAVELEY[29] who hydrolysed samples of cell wall and cell-wall protein of *P. aeruginosa* for different periods of time up to 30 h. Amino sugars are released rapidly and then destroyed on prolonged hydrolysis, and in these cases it is necessary to extrapolate the results for different hydrolysis times back to zero time to obtain a true figure. The factors used to multiply the results for 16-h hydrolysates were: arginine (1.1), histidine (1.43), lysine (1.12), ammonia (1.32), phenylalanine (1.16), leucine (1.13), isoleucine (1.54), valine (1.49), alanine (1.01), glycine (1.04), glutamic acid (1.03), glucosamine (1.34) and galactosamine (1.59). The unknown compound eluted from the column soon after ethanolamine phosphate may be glucosamine phosphate.

TABLE VI

NITROGEN RECOVERIES AND PERCENTAGES OF NINHYDRIN-POSITIVE COMPONENTS

Component	*Small particle fraction*		*Batch 1 after lipid extraction*	*Phospholipid from Batch 1*
	Batch 1	*Batch 2*		
Total of ninhydrin-positive components	46.71	48.89	62.71	7.82
Calculated nitrogen	8.24	9.13	10.46	1.29
Nitrogen (UMBREIT)	10.0	10.15	13.0	2.14
Nitrogen recovery	82.4	90.0	80.5	60.3
Protein*	45.14	46.96	61.64	2.58
Corrected** protein	54.8	52.2	76.6	4.3

* Total percentage of protein amino acids and ammonia.
** Corrected to 100% nitrogen recovery.

Table VI gives figures for the total percentages of ninhydrin-positive components in the four samples, together with the nitrogen percentages calculated from the individual amino acids, amino sugars *etc.* Below these figures are given the percentages of nitrogen obtained by the UMBREIT method and the percentage recoveries of nitrogen. The protein percentages were obtained by summing the percentages of individual protein amino acids and ammonia, and correction to 100% recovery of nitrogen gave the corrected percentages of protein in the samples.

DISCUSSION

The method of isolating the small particle fraction from *P. aeruginosa* gives reproducible results, closely similar products being obtained from the supernatant fluids from 5 consecutive cell-wall preparations. The small particle fractions have a lower phosphorus and a higher nitrogen content than cell walls, and 3 of the preparations contained neither diaminopimelic acid nor muramic acid. Two preparations

containing small amounts of diaminopimelic acid and muramic acid did however analyse consistently for phosphorus, nitrogen and hexose. The amount of readily extractable lipid (39%) obtained from Preparations 1 and 2 was more than twice that obtained from cell walls. The small particle fractions were further distinguished from cell walls by the low hexose content (av. = 2.3%), and by the fact that glucose was the only monosaccharide detected (cell walls also contain rhamnose).

The preparations did not therefore correspond to cell walls, and since cytoplasmic contamination was not significant, further evidence was sought to support the view that these preparations were of the cytoplasmic membrane of *P. aeruginosa*.

The data in Table I show that, in general, membranes isolated from Gram-positive and Gram-negative organisms consist mainly of lipid and protein, together with smaller amounts of hexose. To establish the protein content of the small particle fractions Batches 1 and 2, together with the residue from Batch 1 after lipid extraction and the phospholipid from Batch 1, were analysed quantitatively for ninhydrin-positive components (Table V). However, as the nitrogen recoveries in Table VI indicate, the percentages of ninhydrin-positive components estimated by automatic analysis are probably low. If it is assumed that the non-recoverable nitrogen is present in ninhydrin-positive components, the protein percentage may be corrected to 100% nitrogen recovery. The corrected protein content of the residue from Batch 1 after lipid extraction is 76.6%, or 46.7% of the small particle fraction. The phospholipid from Batch 1 contains 4.3% of protein, equivalent to 3.2% protein in the whole lipid and 1.2% protein in the small particle fraction. The total protein content of Batch 1 is therefore 47.9%. Similarly, the results for Batches 1 and 2 (Tables V and VI) give protein contents of 54.8 and 52.2%, respectively. The protein content of the small particle fractions appears to lie between 48 and 55%, giving an average value of about 52%.

When the residue after removing the readily extracted lipid from Batch 1 had been treated with hot 0.1 M HCl, firmly bound lipid (8.8%) could be extracted. Although the phosphorus content of the residue was reduced from 0.52 to 0.25%, the lipid contained no phosphorus and was mainly fatty acid. Since the lipid A of any cell-wall lipopolysaccharide would not be degraded by 0.1 M HCl (ref. 29), these facts are most simply rationalised by postulating that some phospholipid is too firmly bound to be removed by cold solvent extraction[32]. In support of this, a trace of ethanolamine was detected (Table V) in the residue after removal of the readily extracted lipid. If this firmly bound lipid were dipalmitoylphosphatidylethanolamine, a corrected figure for the firmly bound lipid would be 11.7% of the residue, or 7% of the small particle fraction.

The average composition of a small particle fraction is summarised in Table VII; about 90% of the material is accounted for by readily extracted lipid and protein. These average results may be compared with the data summarised in Table I, particularly with the results of NORTON, BULMER AND SOKATCH[18] for the protoplast membrane of *P. aeruginosa*. Their analytical results for lipid, hexose, nitrogen and protein are very close to ours; in the last instance, their figure of 60.2% protein must be compared with our figure of 61.9% obtained by multiplying the percentage of nitrogen by 6.25. These results suggest that the small particle fractions obtained in the present work represent the membrane of *P. aeruginosa*, and Batches 1, 2 and 3 have been designated 'purified membrane'. The data in Table VII relate to purified

TABLE VII

AVERAGE COMPOSITION OF A SMALL PARTICLE FRACTION

Component	*Percentage*
Protein	52
Readily extracted lipid*	38
Firmly bound lipid	7
Hexose	2.3
Total	99.3

* 39% less 1% protein in the lipid.

membrane, for which average figures (see Table II) of 9.9% for nitrogen and 1.41% for phosphorus may be quoted.

Since our membrane preparations were obtained by methods involving mechanical disintegration of the cell, it should be noted that electron micrographs revealed no definite shapes of envelopes and indicated that the membranes were highly fragmented.

The qualitative composition (Table IV) of the lipid fraction of the purified membrane does not differ greatly from that of cell wall, and a similar observation has been reported by HANCOCK AND MEADOW[33] in the case of *P. aeruginosa* 8602. Using the percentages of ethanolamine and ethanolamine phosphate (Table V) and correcting to 100% nitrogen recovery, the phospholipid fraction from the membrane contains some 64% of phosphatidylethanolamine, calculated as the dipalmitoyl derivative. Phosphatidylglycerol and lysophosphatidylethanolamine are also components of the phospholipid fraction. Hydrolysates of the phospholipid fraction also contain glucosamine and all of the ornithine of the membrane. If the ornithine occurs as an O-ester[34-36] of phosphatidylglycerol, containing both phosphorus and a free amino group, this may account for the unidentified spot on thin-layer plates immediately below phosphatidylethanolamine. The occurrence of some of the glucosamine of the membrane in the phospholipid fraction suggests the presence of a phospholipid containing this amino sugar, although contamination of the phospholipid with lipid A from cell-wall lipopolysaccharide cannot be ruled out. Hydrolysates of the phospholipid fraction from the membrane also contain small amounts of several amino acids (Table V).

The molar ratios of the amino acids in the protein of the membrane do not correspond to integral values, and the protein is probably heterogeneous. Differences occur between the compositions of the membrane protein and the cell-wall protein[29], there being considerably more methionine, more isoleucine, and a little more arginine in the membrane protein.

The occurrence of glucosamine and galactosamine in the membrane and in the defatted residue from the membrane might suggest that the preparations were contaminated with cell-wall lipopolysaccharide. This is unlikely however, because rhamnose and an unidentified but characteristic ninhydrin-positive component of cell-wall lipopolysaccharide are absent from the membrane preparations and the defatted residue (A. H. FENSOM AND G. W. GRAY, unpublished work). However, neither of these components occur in lipid A, of which about 5% would account for the glucos-

amine present in the membrane. However, free lipid A would be extracted by the solvents used in the lipid extraction, and it is improbable that this accounts for the presence of these amino sugars. It is felt therefore that glucosamine and galactosamine may be genuine components of the membrane of *P. aeruginosa*.

These small particle fractions corresponding to membrane material are therefore obtained at *g* values not greatly in excess of those used to deposit cell walls, and this emphasises the danger that cell-wall preparations may be contaminated with membrane which has been less highly fragmented in the disintegration procedure. Unfortunately, there is no unique component of membrane which can be used as an analytical marker to assess the extent of such possible contamination.

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