JOURNAL OF BACTERIOLOGY, Dec. 1970, p. 1354-1368
Vol. 104, No. 3

Quantitation, Chemical Characteristics, and Ultrastructure of the Three Outer Cell Wall Layers of a Gram-Negative Bacterium

C. W. FORSBERG,[1] J. W. COSTERTON,[2] AND ROBERT A. MACLEOD

Department of Microbiology, Macdonald Campus of McGill University, and Marine Sciences Center, McGill University, Montreal, Quebec, Canada

Received for publication 16 July 1970

The cell wall of the gram-negative marine pseudomonad (American Type Culture Collection 19855) consists of three layers: the loosely bound outer layer, the outer double-track layer, and the underlying layer. These three layers constitute 4.7, 7.9, and 6.1%, respectively, of the dry weight of the whole cells. All three layers contained protein, lipid, and carbohydrate. The loosely bound outer layer and underlying layer were lower in protein and lipid and higher in amino and nonamino carbohydrate than the outer double-track layer. All three layers contained proteins with similar amino acid compositions. Minicell-like forms attached to the ends of cells were separated with and fractionated from the units of loosely bound outer layer. Examination of negatively stained preparations by electron microscopy revealed the loosely bound outer layer to be composed largely of units ranging from 400 to 1000 nm in diameter. The outer double-track layer, by the same technique, appeared as large, usually rounded sheets, each with a distinct rim. Washing this layer changed the gross chemical composition but did not affect the bimolecular leaflet appearance in thin sections. The underlying layer, when negatively stained, appeared to be composed of a heterogeneous mixture of particles differing in size and shape. It was separated by gel filtration into a large fraction with a molecular weight range in excess of 20×10^6 to 40×10^6 and a small fraction with a lower range of molecular weight. The larger fraction contained both protein and hexosamine, whereas the smaller one contained protein and only traces of hexosamine. A cytochrome-like pigment separated with this latter fraction.

Five separate layers have been detected in the cell envelope of a marine pseudomonad. Methods which involved controlling the ion composition of the suspending medium and differential centrifugation have been described which permitted the separation of the outer three layers of the cell envelope from the cells and from each other (9). In this study, the contribution of each of the separated layers to the dry weight of the cells has been determined. Each layer has been analyzed chemically and examined by electron microscopy.

MATERIALS AND METHODS

Culture. The organism used was marine pseudomonad B-16 (American Type Culture Collection 19855). It has been classified by the Torry Research Group (Torry Research Station, Aberdeen, Scotland) as a pseudomonas species, type IV. The methods used to grow the cells and to prepare cell suspensions were the same as previously described (9).

Isolation of cell wall layers. The steps leading to the isolation of the loosely bound outer layer, the minicell-like structures, the outer double-track layer, and the underlying layer, have been presented in detail (9).

Gel filtration. Freshly isolated samples of underlying layer material were dialyzed free of sucrose with deionized water and were concentrated by using a rotary evaporator. The material was applied in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (0.05 M, *p*H 8.0) to a column of Sepharose 2B Gel (Pharmacia Canada Ltd., Montreal) and was eluted with the same buffer. The void volume of the column was determined by using Blue Dextran 2000 (23).

Analytical methods. Free lipid was determined by

[1] Present address: National Institute for Medical Research, Mill Hill, London, N.W.7, England.

[2] Present address: Department of Biology, University of Calgary, Calgary, Alberta, Canada.

extracting lyophilized samples with $CHCl_3$:CH_3OH (2:1, v/v). The extract was washed once with water and then dried to constant weight at 60 C (8). The extracted residue and the water used to wash the extract were combined for the determination of bound lipid (15). Nucleic acids were extracted from lyophilized samples by the method of Schneider (25), with perchloric acid in place of trichloroacetic acid. Nucleic acids were estimated semiquantitatively by referring the 260-nm absorption of the extracts to a previously calibrated curve relating absorption to nucleic acid concentration prepared by using yeast ribonucleic acid (Mann Research Laboratories, New York, N.Y.) as a standard.

For quantitative amino acid analysis of the cell wall layers, the protein components were freed from polysaccharide by treatment with 45% phenol. A 50-mg sample of lyophilized wall fraction was suspended in 25 ml of water at 68 C and was mixed with an equal volume of 90% phenol at the same temperature for 30 min by using an Omni-Mixer (Ivan Sorvall, Inc., Norwalk, Conn.). The mixture was cooled to 4 C and centrifuged, and the water phase was removed. The interfacial fluff and the phenol phase were washed twice with 25-ml volumes of water and then were dialyzed free from phenol and lyophilized. The lyophilized material was hydrolyzed with 6 N HCl in evacuated sealed tubes at 110 C for 22 hr, after which the HCl was removed under vacuum (20). The samples were diluted with 0.2 M sodium citrate buffer (*p*H 2.2) and were analyzed by using a model 120-C amino acid analyzer (Beckman Instruments Inc., Fullerton, Calif.).

Electron microscopy. Material to be examined after embedding and sectioning was prepared by the methods described previously (9), with the exception that the loosely bound outer layer and underlying layer were enrobed in agar prior to the fixation step. Preparations were shadowed by using carbon electrodes and a tightly wound coil of platinum thermocouple wire (0.005 inch) at an angle of approximately 25 to 30° in a Balzers 510M freeze-etching apparatus. Material to be negatively stained was dried on Formvar-coated grids after mixing with an equal volume of a solution of sodium zirconium glycolate made up to contain 2% zirconium oxide and 0.005% sucrose and was adjusted to *p*H 7.1 by the addition of HCl. All preparations were examined by using an AEI EM-6B electron microscope.

RESULTS

Separation of minicells and the loosely bound outer layer. Small spherical structures could be seen attached to the ends of the cells of the marine pseudomonad when the latter was taken directly from the growth medium and examined in wet mounts by phase-contrast microscopy. Figure 1 is an electron photomicrograph of a shadowed preparation which shows the position of one of these structures in relation to the parent cell. The structures remained attached to the cells after the latter were washed with a solution of complete salts (containing 0.2 M NaCl, 0.05 M $MgSO_4$, 0.01 M KCl), but a portion was released during subsequent resuspensions of the cells in 0.5 M NaCl. After release and examination in thin section (Fig. 2), the spherical structures were observed to have an envelope ultrastructure comparable to that of cells of normal size and to contain cytoplasmic material. The structures would thus appear to be minicells similar to those described for other organisms (1, 12, 22).

The minicells and the loosely bound outer-layer material were removed together from the parent cells when the latter were washed by suspension in 0.5 M NaCl and centrifuged at 16,300 $\times$ *g*. It was necessary to remove the minicells from the washings to prevent them from contaminating the loosely bound outer-layer material. This was accomplished by centrifuging the washings at 35,000 $\times$ *g*. This caused the minicells to separate, leaving the loosely bound outer-layer fraction in the supernatant solution. When the supernatant solution was centrifuged at 48,000 $\times$ *g* for 1 hr, no sedimentation could be detected either by the formation of a pellet or by a change in the absorbance of the solution as measured over the range from 210 to 450 nm. If the solution was centrifuged at 73,000 $\times$ *g* for 2 hr, however, a pellet formed and a 57% decrease in absorbance of the solution at 220 nm was observed. Two further centrifugations of the solution under the same conditions decreased the absorbance 73 and 77%, respectively, of that prevailing originally. In contrast, one centrifugation of the original supernatant solution at 158,000 $\times$ *g* for 3 hr resulted in only a 66% reduction in absorbance. Thus, the minicell-free solution of loosely bound outer-layer material was routinely centrifuged twice at 73,000 $\times$ *g* for 2 hr to separate the material into a sedimentable and a nonsedimentable fraction.

The sedimentable fraction of the loosely bound outer layer, when examined in shadowed (Fig. 3) and negatively stained (Fig. 4) preparations, appeared to consist of homogeneous plaquelike units. These ranged from 400 to 1,000 nm in diameter. The morphology of these units appeared the same both before and after dialysis to remove NaCl. The nonsedimentable material was composed of much smaller units which varied widely in size. No flagella-like structures were observed in these preparations. When the loosely bound outer layer was embedded and examined in thin section by electron microscopy, no structure could be detected.

It was of interest in connection with the above observations to find that when cells of the marine pseudomonad were washed with complete salts solution and negatively stained, small plaque-like units could be seen in a layer surrounding the

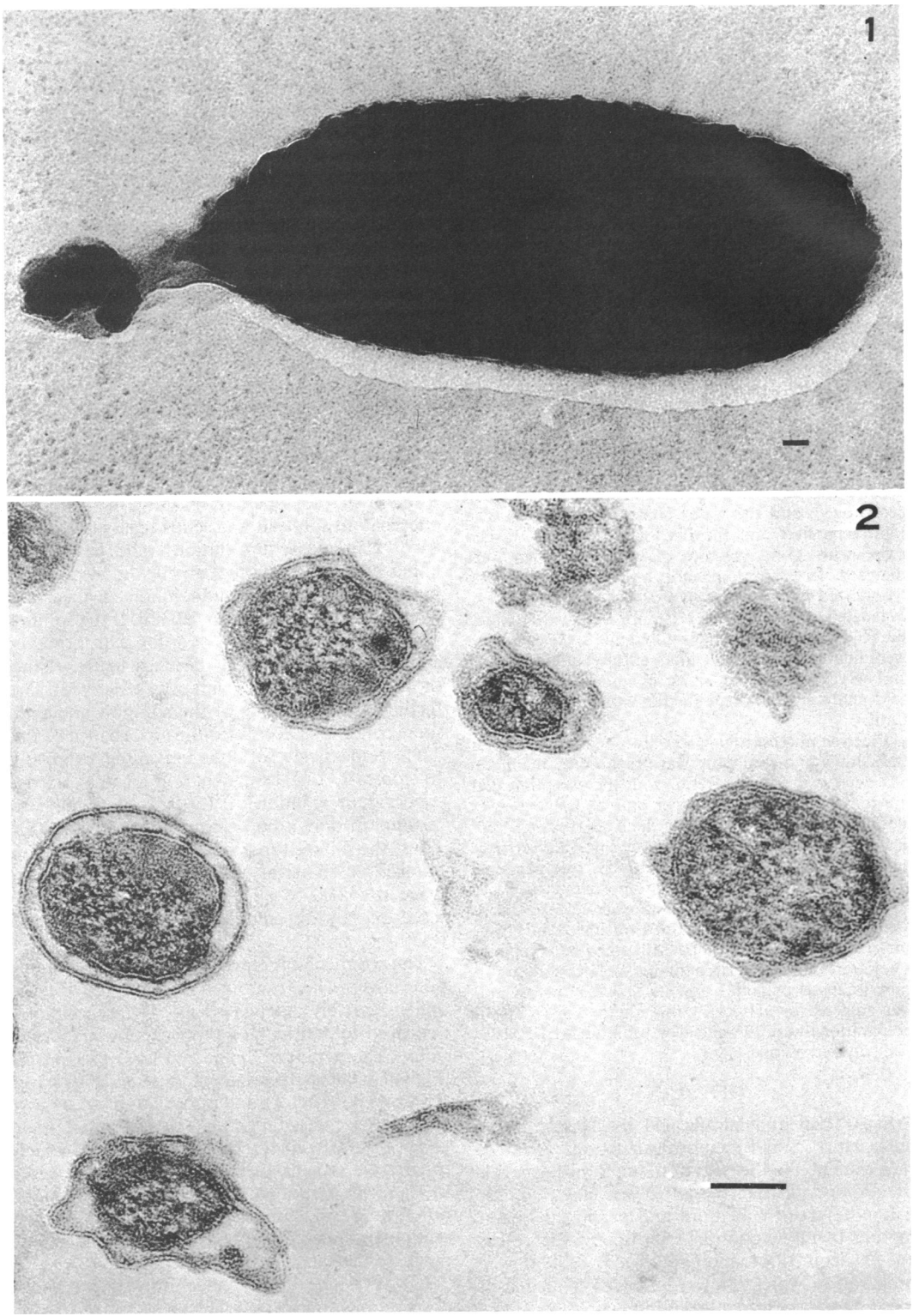
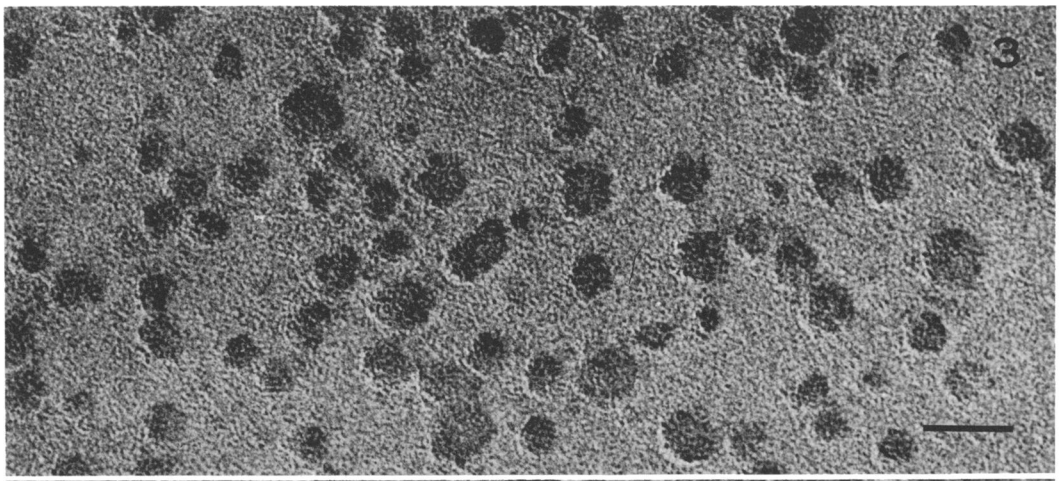


FIG. 1. *Shadowed preparation of the marine pseudomonad B-16 showing minicell attached. Magnification markers on this and subsequent figures indicate 0.1 μm.*

FIG. 2. *Thin section of isolated minicells. Note that each of the minicells is enclosed by an envelope having the inner and outer double-track structure typical of gram-negative bacteria.*



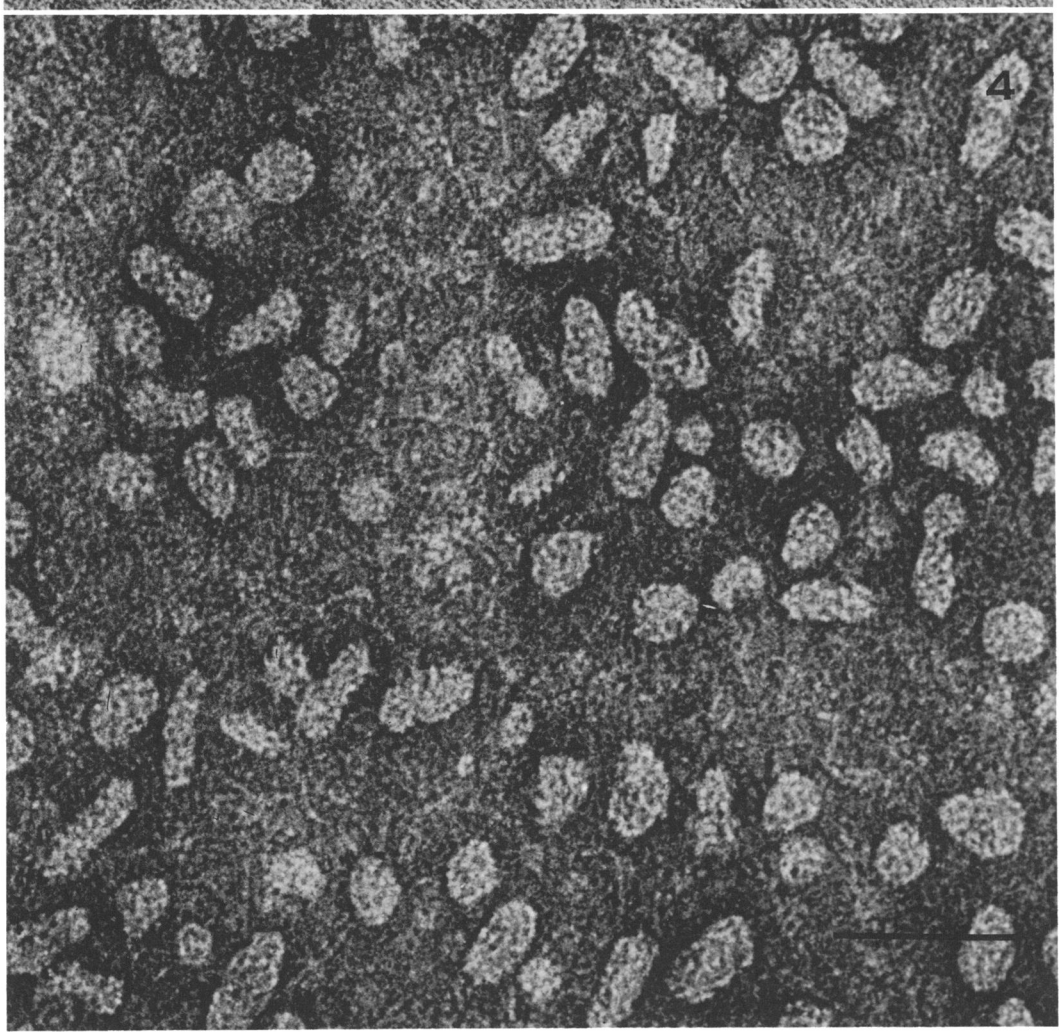


FIG. 3. *Shadowed preparation of the fraction of the loosely bound outer layer sedimentable at 73,000 × g.*

FIG. 4. *Negatively stained preparation of the fraction of the loosely bound outer layer sedimentable at 73,000 × g.*

cells (Fig. 5). If after washing with complete salts the cells were then washed three times with 0.5 M NaCl to remove the loosely bound outer-layer material, negative staining showed that the plaques had disappeared (Fig. 6). The contribution of the loosely bound outer layer to the dry weight of the cells is shown in Table 1. The minicells made only a minor contribution to the cell dry weight. Nearly all the loosely bound outer layer material recovered in the supernatant solutions after suspending the cells four times in 0.5 M NaCl was recovered in the solutions derived from the first three suspensions. Approximately 80% of the material recovered was sedimentable at 73,000 × *g*.

Chemical analysis of the sedimentable fraction (Table 2) showed it to be complex in nature in that it contained carbohydrate, protein, and lipid. The constituents determined accounted for 70% of the dry weight of the material analyzed. The nonsedimentable fraction was less completely analyzed since it accounted for only 20% of the loosely bound outer-layer material. However, it had a protein and carbohydrate composition similar to that of the sedimentable fraction and of a sample of the unfractionated loosely bound outer-layer material. Perchloric acid extracts of these fractions showed no absorption maximum at 260 nm, indicating the absence of nucleic acids.

Outer double-track layer and underlying layer. To isolate the outer double-track layer and the underlying layer, cells from the growth medium were harvested by centrifugation and were washed three times with 0.5 M NaCl to remove the loosely-bound outer layer. The washings were discarded and the cells were suspended three times in fresh portions of 0.5 M sucrose. This caused the outer double-track layer to fragment, releasing underlying-layer material (9). After separation of the cells by centrifugation at 35,000 × *g*, the fragments of the outer double-track layer were separated from the nonsedimenting underlying layer by two centrifugations at 73,000 × *g*, each for 2 hr. This caused a pellet to form and the absorbance of the solution measured at 280 nm to decrease 68%. Three centrifugations of another portion of the same solution at the same gravitational force for intervals of 3 hr decreased the total absorbance of the solution only 71%. A further centrifugation of the latter solution at 144,000 × *g* for 3 hr decreased the absorbance only a further 2%.

The outer double-track fragments recovered from the pellet after centrifugation, when negatively stained, appeared as flattened spherical fragments, each with a distinct rim (Fig. 7). The underlying-layer material present in the supernatant solution after sedimentation of the outer double-track fragments appeared, on negative staining, as granular sheets, rod-shaped structures, and smaller particles (Fig. 8). Flagella-like fragments were rarely seen in preparations of the outer double-track layer, and none was seen in preparations of underlying-layer material. Masses of agglomerated flagella were observed, however, in negatively stained preparations of cells which had been centrifuged from suspension in 0.5 M sucrose.

The contributions of the outer double-track layer and the underlying layer to the dry weight of the cells are shown in Table 3. Of the material recovered from the cells after four suspensions in 0.5 M sucrose, 96% was recovered after the first three suspensions. The supernatant solutions were examined for the release of 260-nm absorbing material from the cells after each suspension. None was detected after the first three suspensions, but some was observed after the fourth. Thus, only the cell wall material released during the first three suspensions was analyzed chemically. The possibility was considered that after the suspensions in 0.5 M sucrose some underlying-layer material might still remain attached to the cells through divalent cation bridges. To test this possibility cells, after removal of the outer three layers, were washed with a solution of 0.5 M ethylenediaminetetraacetic acid. No further release of hexosamine-containing material from the cells was obtained.

Chemical analysis of the outer double-track layer and the underlying layer showed that they were similar in gross chemical composition but differed significantly in the relative proportions of their components (Table 2). The outer double-track layer was higher in protein, lipid, and phosphorus and lower in carbohydrate than the underlying layer. The components determined accounted for 90 and 73%, respectively, of the dry weights of the two layers. It can also be seen (Table 2) that the gross chemical composition and relative proportions of the components of the loosely bound outer layer and of the underlying layer are quite similar. Perchloric acid extracts of these layers displayed no absorption maximum at 260 nm, thus indicating the absence of contaminating nucleic acids.

Amino acid composition of the protein components of the cell wall layers. The protein components obtained by phenol extraction of each of the cell wall layers were analyzed with the aid of an amino acid analyzer. These fractions contained greater than 95% of the protein in the unextracted samples. The results (Table 4) show that the protein component of each of the cell wall layers contained all the amino acids nor-

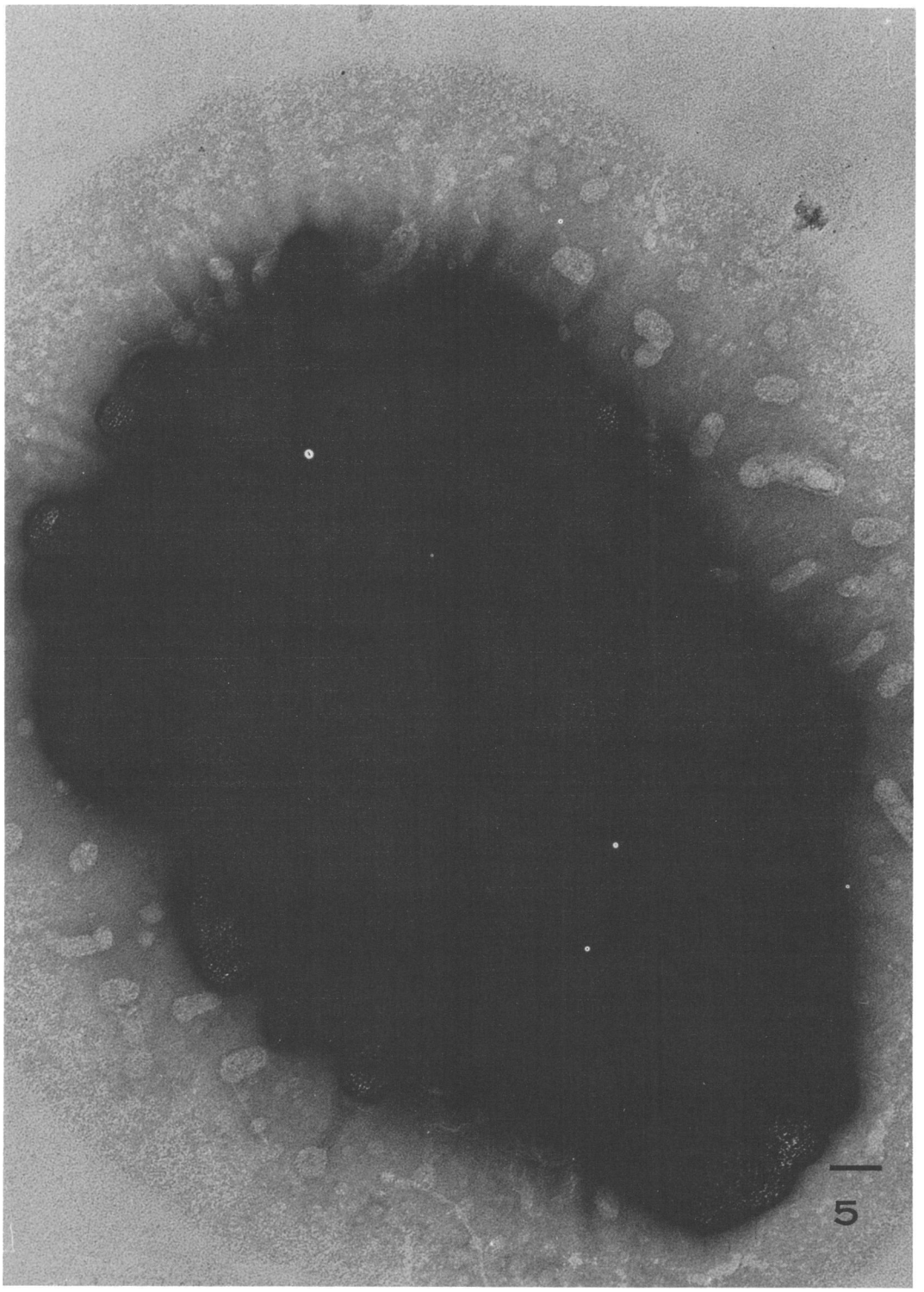


FIG. 5. *Negatively stained preparation of a cell of marine pseudomonad B-16 washed free of medium components with complete salts washing solution. Note the plaquelike structures in the negative stain surrounding the cells. The larger bulbous structures around the cell are salt crystals.*

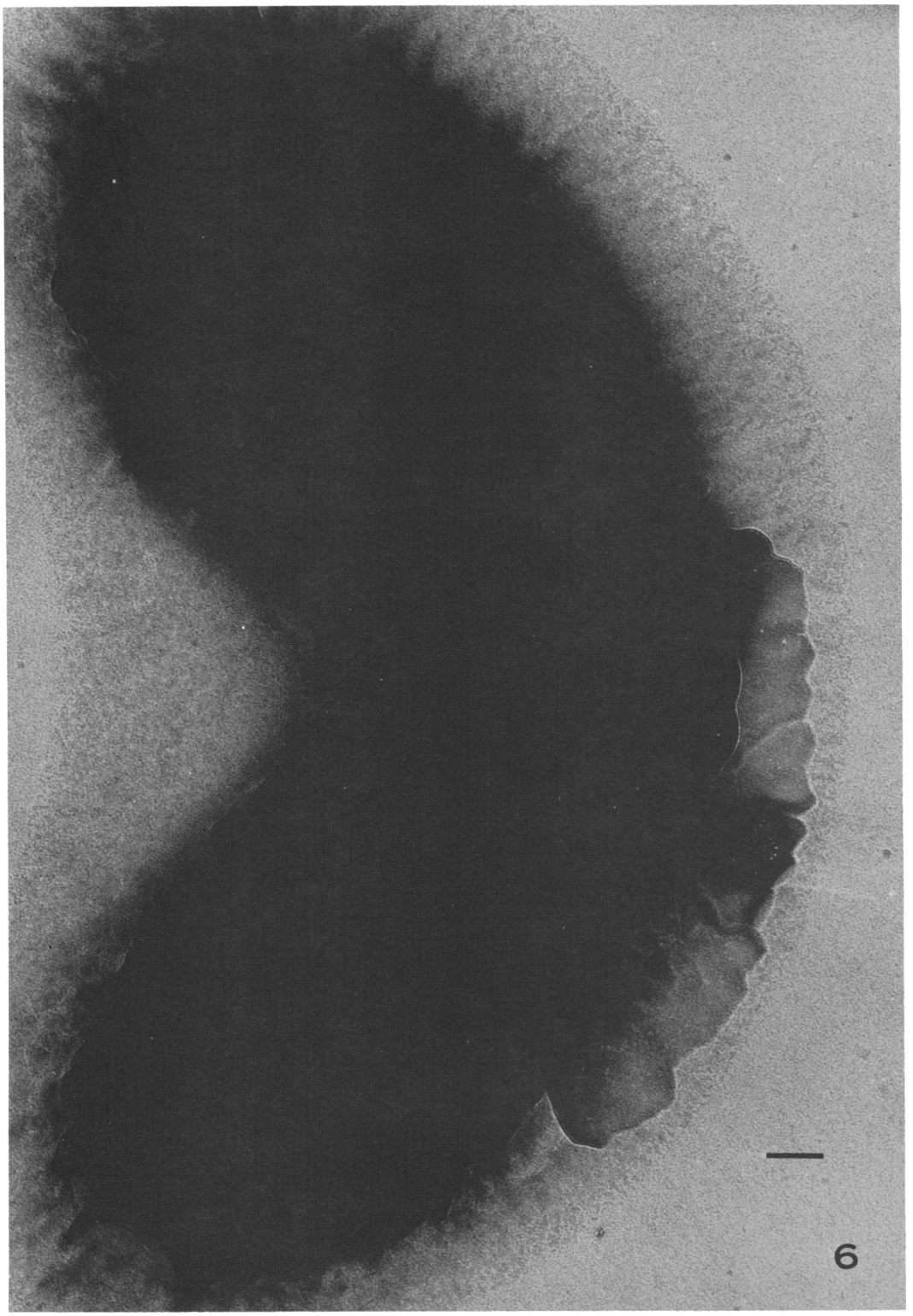


FIG. 6. *Negatively stained preparation of a cell of marine pseudomonad B-16 after washing with 0.5 M NaCl. Note the absence of plaquelike structures in the negative stain surrounding the cell. The structure to the right of the cell is a salt crystal.*

mally found in proteins. No noticeable differences were observed in the concentrations of the amino acids in the various separated layers. All the layers had an excess of acidic over basic amino acids.

Heterogeneity of the outer double-track layer. The results in Table 3 show that most of the outer double-track- and underlying-layer material released by the cells was recovered by successively suspending 0.5 M NaCl-washed cells three times in 0.5 M sucrose. The relative amounts of outer double-track- and underlying-layer material recovered after each of the three suspensions varied (Fig. 9). Furthermore, the outer double-track material released during the first suspension was lower in protein and higher in hexosamine than that released later (Table 5). In contrast, the composition of the underlying-layer material remained relatively constant.

TABLE 1. *Determination of the contribution of minicells and the loosely bound outer layer to the cell dry weight*[a]

Fraction recovered[b]	Per cent of cell dry weight	Per cent of total recovered
Minicells	0.02 ± 0.005	
Loosely bound outer layer		
(a) Amount released by four suspensions in 0.5 M NaCl	4.85 ± 0.1	100
(b) Amount released by three suspensions in 0.5 M NaCl	4.7 ± 0.1	97
(c) Amount of (b) sedimentable at 73,000 × *g*	3.7 ± 0.1	76
(d) Amount of (b) nonsedimentable at 73,000 × *g*	1.0 ± 0.1	21

[a] In this and all subsequent tables, except where indicated, all values reported represent the average and the average deviation of the mean of two determinations.

[b] Based on cells washed free of medium components with complete salts solution.

Stability of the outer double-track material. The outer double-track material released after the first suspension of the cells in 0.5 M sucrose was treated separately from that released on the second suspension in 0.5 M sucrose. The material from each suspension was washed separately by centrifugation from and resuspension in 0.5 M sucrose eight times and in water three times. Determination of the amount of sedimentable material remaining after the 11 washings indicated that a considerable amount of material had been solubilized by the washing procedure (Table 6). This was especially true in the case of the outer double-track material arising from the first suspension of the cells in 0.5 M sucrose. A comparison of the protein and carbohydrate contents of the material before and after washing showed that a higher percentage of carbohydrate (particularly hexosamine) than protein had been removed from the sedimentable material. The residue after washing was considerably enriched with respect to protein. Washed fragments in thin section retained their bimolecular leaflet appearance (Fig. 10) and were not obviously different from unwashed fragments examined previously (6).

Although the analysis of the residue indicated that after 11 washings the fragments of the outer double-track layer had become enriched with respect to protein, analysis of the material released showed that it contained both protein and hexosamine (Table 7). It is also evident that the total amount of protein released was greater than the amount of hexosamine. The ratio of protein

TABLE 2. *Chemical composition of the three outer layers of the cell wall of marine pseudomonad B-16*[a]

Fraction analyzed	Carbohydrate		Protein	Lipid			Phosphorus		Phospholipid[c]
	Hexosamine	Nonamino		Extractable	Bound	Total	Total	Lipid[b]	
Loosely bound outer layer	21.2 ± 0.7	17.8 ± 0.2	17.5 ± 0.7						
Sedimentable	20.1 ± 0.1	17.2 ± 0.2	20.2 ± 0.1	6.6 ± 0.4	4.7 ± 0.3	11.3	1.48 ± 0.02	0.27 ± 0.02	6.9
Nonsedimentable	16.8 ± 0.1	16.9 ± 0.0	15.0 ± 0.0						
Outer double-track layer	9.2 ± 0.7	11.6 ± 0.1	41.5 ± 1.7	21.0 ± 2.0	5.1 ± 0.5	26.1	1.61 ± 0.01	0.70 ± 0.01	17.9
Underlying layer	18.1 ± 1.1	21.0 ± 0.5	25.3 ± 1.3	4.8 ± 0.5	4.0 ± 0.5	8.8	1.05 ± 0.01	0.15 ± 0.01	3.7

[a] Values are expressed as per cent dry weight.

[b] Per cent of total phosphorus present in the extractable lipid.

[c] Per cent of extractable lipid phosphorus times 25.5.

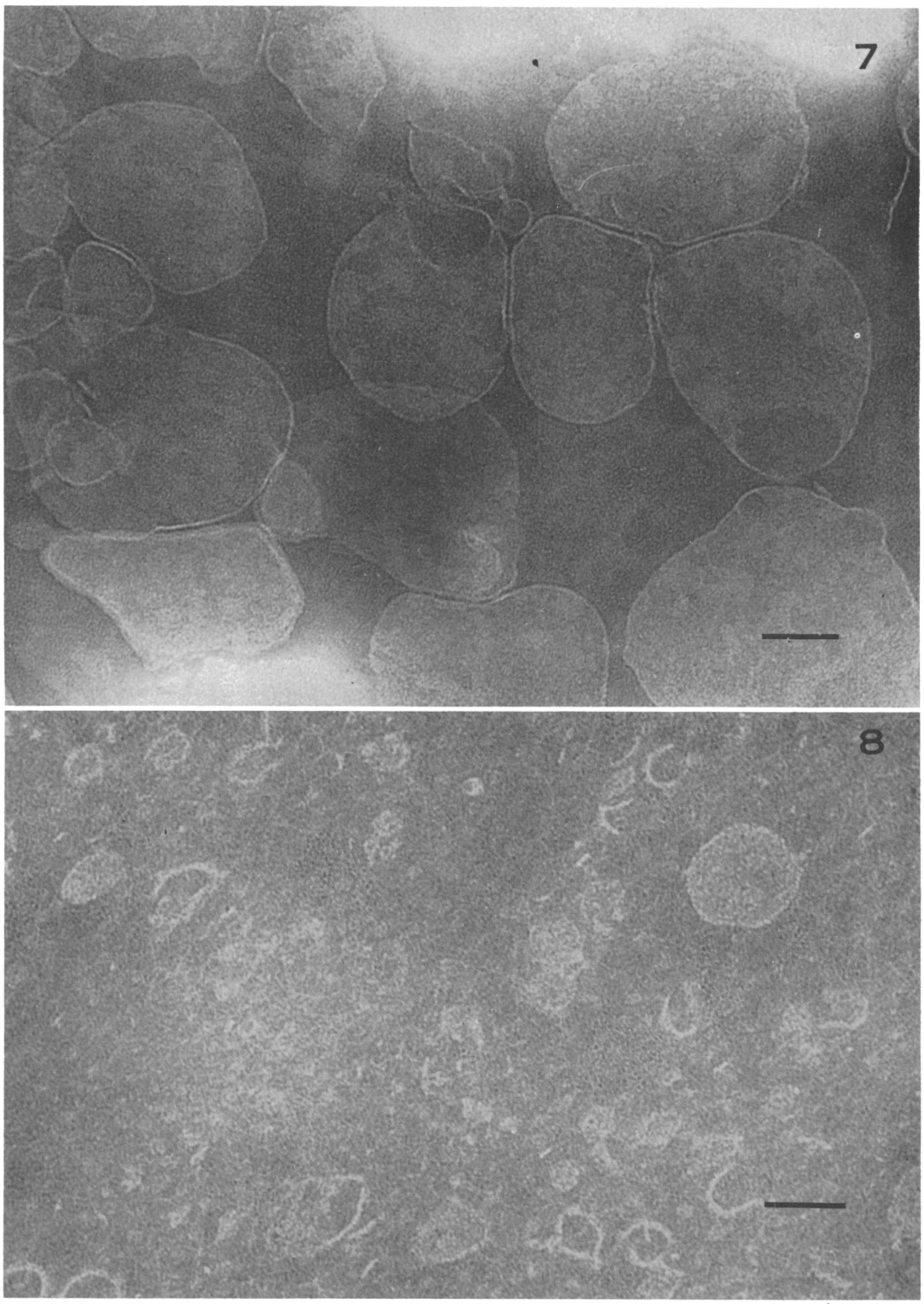


FIG. 7. *Negatively stained preparation of the fragments of the outer double-track layer. Note the distinct rim surrounding each of the flattened spherical fragments.*

FIG. 8. *Negatively stained preparation of underlying-layer material.*

TABLE 3. *Recovery of the outer double-track layer and the underlying layer*

Material released[a]	Per cent of cell dry weight[b]	Per cent of total recovered
(a) By four suspensions in 0.5 M sucrose	15.4 ± 0.3	100
(b) By three suspensions in 0.5 M sucrose	14.7 ± 0.3	96
(c) Amount of (b) which was sedimentable at 73,000 × *g* (outer double-track layer)	8.3 ± 0.1	54
(d) Amount of (b) which was nonsedimentable at 73,000 × *g* (underlying layer)	6.4 ± 0.2	42

[a] From cells which had been freed of the loosely bound outer layer by washing with 0.5 M NaCl.
[b] Based on cells washed with 0.5 M NaCl.

TABLE 4. *Amino acid analysis of the protein components of the three outer layers of the cell wall of marine pseudomonad B-16*[a]

Amino acid	Loosely bound outer layer		Outer double-track layer		Underlying layer	
	μmole/mg	Mole %[b]	μmole/mg	Mole %	μmole/mg	Mole %
Ammonia	0.870		0.873		1.088	
Lysine	0.228	4.17	0.229	3.70	0.281	6.02
Histidine	0.070	1.28	0.071	1.15	0.068	1.46
Arginine	0.206	3.76	0.236	3.81	0.171	3.66
Aspartic	0.878	16.04	0.983	15.87	0.714	15.28
Glutamic	0.460	8.40	0.566	9.14	0.470	10.06
Threonine	0.372	6.80	0.379	6.12	0.277	5.93
Serine	0.514	9.39	0.530	8.56	0.353	7.56
Proline	0.170	3.11	0.228	3.68	0.177	3.79
Glycine	0.504	9.21	0.589	9.51	0.346	7.41
Alanine	0.448	8.20	0.554	8.95	0.448	9.59
Valine	0.318	5.81	0.442	7.14	0.332	7.11
Methionine	0.106	1.94	0.096	1.55	0.096	2.06
Isoleucine	0.256	4.68	0.281	4.54	0.219	4.69
Leucine	0.466	8.51	0.450	7.27	0.354	7.58
Tyrosine	0.230	4.20	0.260	4.20	0.172	3.68
Phenylalanine	0.248	4.53	0.299	4.83	0.193	4.13
Cystine	Trace		Trace		Trace	
Ornithine	Trace		Absent		Absent	
Excess of acidic over basic amino acids[c]	15.23%		16.35%		14.20%	

[a] No corrections have been applied for destruction of sensitive amino acids or for the release of amide nitrogen during acid hydrolysis.
[b] Ammonia was omitted from the calculation of mole % in all cases.
[c] Calculation: (aspartic + glutamic) minus (lysine + histidine + arginine), expressed as a per cent of the sum of the five amino acids.

to hexosamine in the material solubilized can be seen to have increased on successive suspensions of the fragments in the washing solution. Because of the preponderance of protein in the sedimentable material, however, the proportion of protein to hexosamine in this material was increased as a result of the washing.

Heterogeneity of the underlying-layer material. The underlying-layer material was released from the 0.5 M NaCl-washed cells in the largest amount on the first suspension in 0.5 M sucrose (Fig. 9). The protein and hexosamine compositions of the

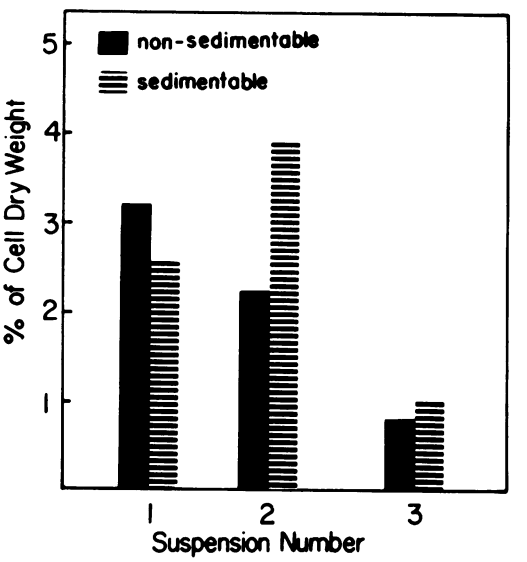


FIG. 9. *Amount of sedimentable (outer double-track layer) and nonsedimentable (underlying layer) material released by cells of the marine pseudomonad on each of three successive suspensions in 0.5 M sucrose. Prior to the suspensions, the cells were washed with 0.5 M NaCl. After the separation of sedimentable and nonsedimentable material, the sedimentable material was washed once by suspension in and centrifugation from a volume of 0.5 M sucrose equivalent to 20% of the volume of the original suspending medium.*

TABLE 5. *Protein and hexosamine composition of outer double-track layer and underlying-layer material released by successive suspension of the cells in 0.5 M sucrose*

Suspension[a]	Outer double-track layer		Underlying layer	
	Protein	Hexosamine	Protein	Hexosamine
	%	%	%	%
1	36.6 ± 0.1	13.5 ± 0.8	27.5 ± 1.3	20.5 ± 1.9
2	46.4 ± 0.2	8.4 ± 0.3	24.8 ± 0.5	20.3 ± 0.3
3	50.5 ± 0.6	6.9 ± 0.1	29.3 ± 0.3	17.1 ± 0.4

[a] Prior to suspension in sucrose, the cells were washed with 0.5 M NaCl.

TABLE 6. *Solubility of the outer double-track layer isolated after the first and after the second suspension of cells in 0.5 M sucrose*

Determination	Outer double-track layer 1[a]		Outer double-track layer 2[a]	
No. of washes	0	11[b]	0	11
Dry weight (mg)	44.5	2.9	57.2	20.7
Per cent remaining		6.5		36.1
Chemical composition[c]				
Protein	36.6 ± 0.1	39.2 ± 0.2	46.4 ± 0.2	52.7 ± 0.2
Hexosamine	13.5 ± 0.8	3.1 ± 0.0	8.4 ± 0.3	3.9 ± 0.3
Nonamino carbohydrate	16.1 ± 0.1	9.3 ± 1.3	11.9 ± 0.1	7.2 ± 0.5
Percent of dry weight accounted for by components determined	66.2	51.6	66.7	63.8
Ratio of protein to hexosamine	2.7	12.5	5.5	13.7

[a] Outer double-track layer 1 was isolated after the first and outer double-track layer 2 after the second suspension in 0.5 M sucrose of cells washed previously with 0.5 M NaCl.

[b] Outer double-track layer fragments were suspended and centrifuged from volumes of fresh 0.5 M sucrose eight times and distilled water three times.

[c] Values are expressed as per cent.

materials from each suspension were similar (Table 5). When the underlying-layer material was applied to a gel filtration column having an exclusion limit for compounds in the molecular-weight range of 20×10^6 to 40×10^6 [for polysaccharides and proteins, respectively (24)], most of the material eluted was recovered in the first peak (Fig. 11), which corresponded to the void volume of the column. Analysis showed that the material collected in this volume contained 61% of the protein and 79% of the hexosamine recovered from the column. The second peak contained 16% of the protein and only 2% of the hexosamine. The remaining protein and hexosamine were distributed in the intermediate fractions in proportion to the absorbance of the eluates at 220 nm. Of the protein and hexosamine applied to the column, 94 and 96%, respectively, were eluted by the buffer system used. When the material which was eluted in the void volume of the column was negatively stained and examined by electron microscopy (Fig. 12), it appeared to be composed of particles which were somewhat more uniform in size than those observed in the unfractionated underlying-layer material (Fig. 8).

A pigment with absorption bands at 412, 520, and 546 nm was released with the underlying-layer material. This pigment was eluted from the gel filtration column with the second peak.

DISCUSSION

When cells of marine pseudomonad B-16 were successively suspended in fresh portions of 0.5 M NaCl, they released a loosely bound outer layer (9). This layer was found to account for 4.7% of the dry weight of the cells. Since 97% of the material released was recovered after the first three of four suspensions in 0.5 M NaCl, it would appear that very little of the loosely bound material remained associated with the cells after the third suspension. Visual support for this contention was obtained when plaquelike units were detected by electron microscopy around negatively stained cells before the cells had been treated with the NaCl solution but not afterwards. Most of the material released (80%) was recovered as sedimentable material which was in the form of plaquelike units. The remainder of the material was morphologically heterogeneous but had a chemical composition similar to that of the more homogeneous fraction and may represent fragmentation products of the homogeneous material.

Chemical analysis of the plaque fraction of the loosely bound outer layer showed that the plaques contained lipid, protein, and carbohydrate. The

TABLE 7. *Change in the composition of the material solubilized as washing of outer double-track layer 2[a] proceeded*

Wash no.	Amounts released into the wash solution		Ratio[b]
	Protein	Hexosamine	
	μg/ml	μg/ml	%
1	237 ± 1.5	93.4 ± 3.4	2.6
2	135.8 ± 0.30	39.1 ± 1.5	3.5
3	86.3 ± 0.8	14.0 ± 0.3	6.2
4	90.3 ± 0.3	13.4 ± 0.1	6.7
5	57.0 ± 0.0	7.6 ± 0.4	7.5
6	52.5 ± 0.1	4.6 ± 0.06	11.4
7	40.3 ± 0.0	4.2 ± 0.4	9.6

[a] *See* Table 6.

[b] Ratio of protein to hexosamine.

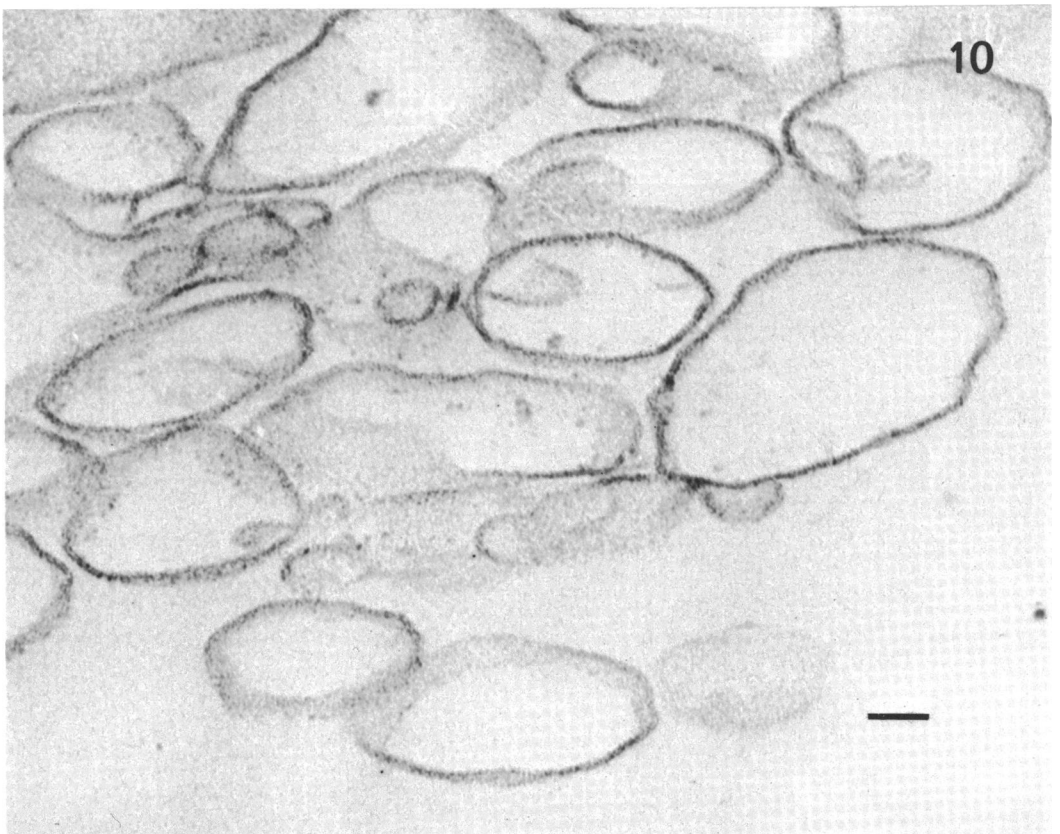


FIG. 10. *Section of outer double-track layer fragments after eight washings with 0.5 M sucrose and three washings with water. Note that the characteristic double-track staining profile has been preserved.*

carbohydrate consisted of both amino and nonamino sugars and the lipid of an appreciable amount of phospholipid.

Layers or structures similar to the plaque-containing, loosely bound outer layer of the marine pseudomonad are found in *Spirillum serpens* (21), and *Acinetobacter* strain 199A (26). The hexagonally packed units in *S. serpens* were characterized as lipoprotein in nature (18). The peglike structures in the *Acinetobacter* strain were removed with proteolytic enzymes and were therefore thought to be protein or attached by protein. Materials released from cells of *Chromobacterium violaceum* by cold, aqueous solvents, and which therefore may have arisen from a location similar to that occupied by the loosely bound outer layer of the marine pseudomonad, were found to contain components characteristic of lipopolysaccharide (4).

Minicells released with the loosely bound outer layer could be separated efficiently by differential centrifugation. Thus, they did not influence either the quantitation or the chemical analysis of the separated layers. Spherical bodies attached to the ends of cells have also been reported in cultures of *Escherichia coli* (1) and *S. serpens* (22).

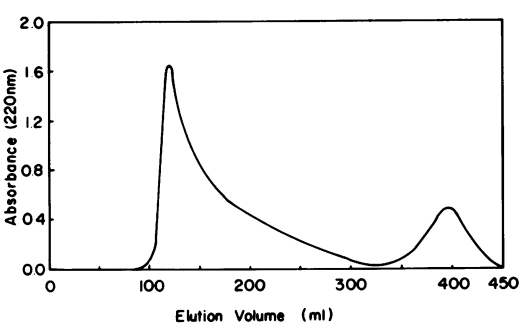


FIG. 11. *Elution profile of the underlying-layer material isolated from marine pseudomonad B-16 as determined by using a column of Sepharose 2B. The void volume of this column was determined to be 120 ml.*

Beneath the loosely bound outer layer of the cell wall of this organism lies the outer double-track layer (9). This layer and the layer immedi-

ately below it (the underlying layer) were released from 0.5 M NaCl-washed cells during successive suspensions in 0.5 M sucrose. The outer double-track layer was found to account for 7.9% and the underlying layer for 6.1% of the dry weight of the whole cells. By incorporating the amounts of each of the separated layers into the model proposed by Forsberg et al. (9), the envelope of this marine pseudomonad can be pictured as shown in Fig. 13.

The outer double-track layer of this organism, like the loosely bound outer layer and the under-

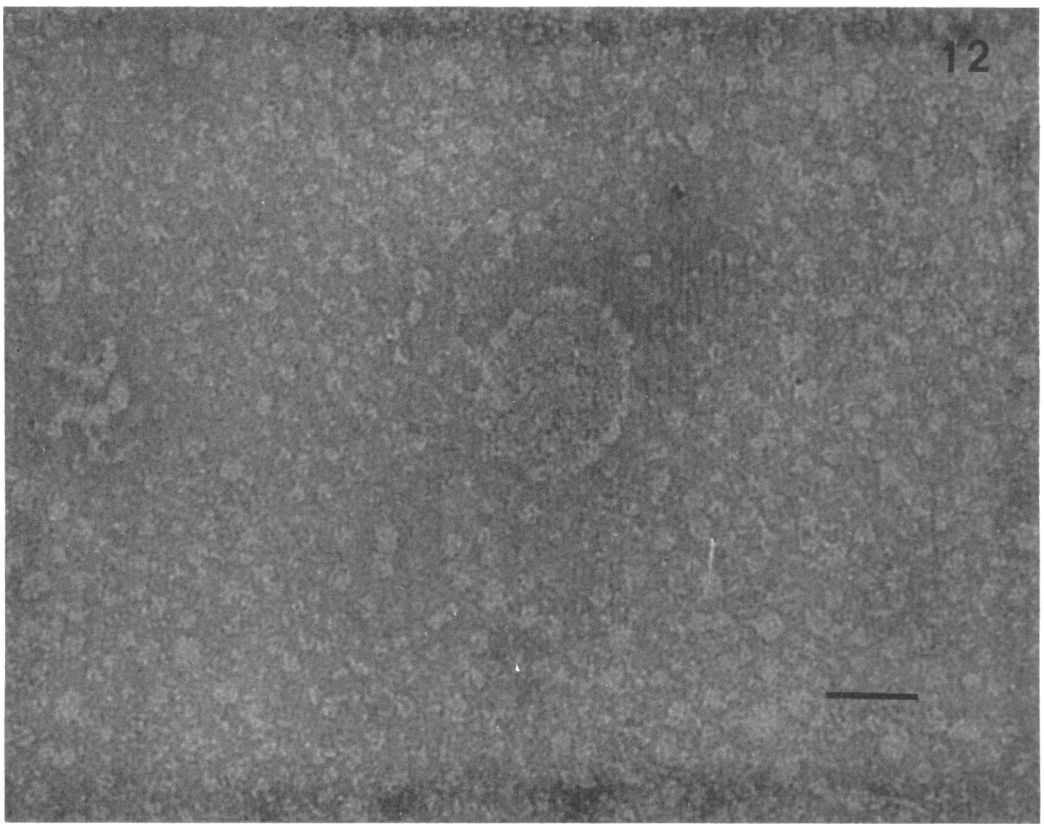


FIG. 12. *Negatively stained preparation of fraction of underlying-layer material eluted in void volume of Sepharose 2B column.*

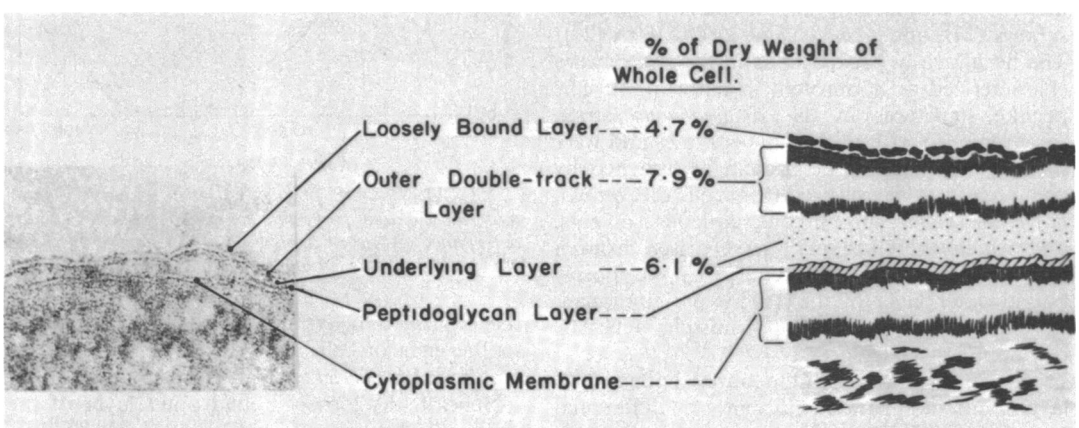


FIG. 13. *Model of the cell envelope of marine pseudomonad B-16 showing the contribution of the three outer layers to the cell dry weight.*

lying layer, contained carbohydrate, lipid, and protein. The protein content of this layer was almost double, and the lipid content double-to-triple that of the other two layers. A large proportion of the lipid was again phospholipid. The protein and hexosamine composition of the fragments of the outer double-track layer released during each of the three successive suspensions of cells in 0.5 M sucrose was found to vary. Since we have detected some clonal variation in this organism, the possibility that the differences in chemical composition of the fragments released might be due to this type of variation in the culture will have to be ruled out before more complicated explanations for the phenomenon are considered.

Subsequent washing of the fragments of the outer double-track layer caused a considerable amount of the sedimentable material to solubilize. Since the material that went into solution was not of constant composition, the solubilized components would not appear to have been simple homogeneous subunits of the outer double-track layer.

The composition of the outer double-track layer of the cell wall of other gram-negative bacteria is not clear. Treatment with 45% phenol is required to remove this layer from the cell walls of enterobacteria. The material extracted can be separated into a phenol-soluble lipoprotein fraction and a water phase containing lipopolysaccharide (13). The relation of these components to one another and to the rigid layer of the cell wall is a matter for speculation. It is generally held that the outer layer of the cell wall of gram-negative bacteria is lipoprotein and the underlying layer is lipopolysaccharide (7, 13). It is of interest, though, that recent studies have shown that *E. coli* K-12 excretes lipid, protein, and carbohydrate as a complex into the medium and that the complex appears to arise from the outer double-track layer of the cell (24).

It has been suggested that lipopolysaccharide is largely responsible for the bimolecular leaflet appearance of the cell walls of *E. coli* in thin section (7, 13). It is of interest in connection with this conclusion that the outer double-track layer fragments of the marine pseudomonad retained their bimolecular leaflet staining characteristics even after the carbohydrate content of the fragments had been appreciably reduced by washing.

The underlying-layer material arose from the electron-transparent region seen in thin sections of the organism, which lies between the outer double-track layer of the cell wall and the cytoplasmic membrane. More precisely, it arises from the space between the outer double-track layer and the peptidoglycan layer which in this organism is very thin and closely associated with the outside surface of the cytoplasmic membrane (9). The peptidoglycan layer is not normally observed by staining thin sections of whole cells of this organism. The transparent region between inner and outer double-track layers of the cell envelope is characteristic of other gram-negative bacteria as well, and has been referred to as the periplasmic space (19). Recent studies indicated that a number of hydrolytic enzymes may be located in this space (11). De Petris detected globular elements in this region in *E. coli* K-12 infected with a bacteriophage. He concluded that these elements which lie immediately beneath the outer double-track layer may be identifiable with the proteic particles revealed by Weidel and coworkers after stripping off the outermost layers of the cell wall of *E. coli* with sodium dodecyl sulfate and phenol (6, 7). Others, on the other hand, have concluded that this same region contains lipopolysaccharide (13, 17).

The underlying-layer material in the marine pseudomonad, when negatively stained, appeared to be composed of a heterogeneous mixture of particles differing in size and shape. Gel filtration chromatography separated the particles into two size groups, those with molecular weights in excess of 20×10^6 to 40×10^6, which accounted for most of the protein and hexosamine in the underlying layer, and those with molecular weights of less than this size range which contained protein and little or no hexosamine. In addition a cytochrome-like pigment with absorption maxima at 412, 520, and 546 nm was liberated with this underlying layer. Fujita and Sato (10) reported that cytochrome C-552 was liberated quantitatively into the surrounding medium when cells of *E. coli* were converted to spheroplasts.

Buckmire and MacLeod obtained a soluble nondialyzable fraction from isolated cell envelopes of this marine pseudomonad when the envelopes were suspended at low salt concentration (2). A similar fraction was isolated when whole cells of the organism were suspended in 0.5 M sucrose (F. L. A. Buckmire, Ph.D. Thesis, McGill University, 1967). When the conditions under which the soluble nondialyzable fraction (NDF fraction) was isolated in these earlier studies are compared with those required to separate the various layers of the cell envelope in the present study (9), it becomes evident that the NDF fraction arose from the underlying layer. This was the conclusion reached by a different means in the earlier work (2).

Amino acid analysis of the protein component of each of the separated cell wall layers showed that the mole percentages of the various amino

acids in each layer were nearly identical, and in all cases there was an apparent excess of acidic over basic amino acids. If any of the carboxyl side chains had been in the form of amides, however, the acidity of the proteins would have been reduced. The amino acid composition of the proteins from the three cell wall layers of this bacterium closely resembled the composition of proteins which were considered to be of cell wall origin from other bacteria (14). Although the amino acid ratios of the latter proteins differed from those of the proteins studied here, they too contained an apparent excess of acidic over basic amino acids.

LITERATURE CITED

1. Adler, H. I., W. D. Fisher, A. Cohen, and A. A. Hardigree. 1967. Miniature *E. coli* cells deficient in DNA. Proc. Nat. Acad. Sci. U.S.A. 57:321–326.
2. Buckmire, F. L. A., and R. A. MacLeod. 1965. Nutrition and metabolism of marine bacteria. XIV. On the mechanism of lysis of a marine bacterium. Can. J. Microbiol. 11:677–691.
3. Buckmire, F. L. A., and R. A. MacLeod. 1970. Penetrability of a marine pseudomonad by inulin, sucrose and glycerol and its relation to the mechanism of lysis. Can. J. Microbiol. 16:75–81.
4. Corpe, W. A., and M. R. J. Salton. 1966. Properties of surface materials released from cells of *Chromobacterium violaceum*. Biochim. Biophys. Acta 124:125–135.
5. Costerton, J. W., C. Forsberg, T. I. Matula, F. L. A. Buckmire, and R. A. MacLeod. 1967. Nutrition and metabolism of marine bacteria. XVI. Formation of protoplasts, spheroplasts, and related forms from a gram-negative marine bacterium. J. Bacteriol. 94:1764–1777.
6. de Petris, S. 1965. Ultrastructure of the cell wall of *Escherichia coli*. J. Ultrastruct. Res. 12:247–262.
7. de Petris, S. 1967. Ultrastructure of the cell wall of *Escherichia coli* and chemical nature of its constituent layers. J. Ultrastruct. Res. 19:45–83.
8. Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497–509.
9. Forsberg, C. W., J. W. Costerton, and R. A. MacLeod. 1970. Separation and localization of cell wall layers of a gram-negative bacterium. J. Bacteriol. 104:1338–1353.
10. Fujita, T., and R. Sato. 1966. Studies on the soluble cytochromes in Enterobacteriaceae. III. Localization of cytochrome C-552 in the surface layer of the cells. J. Biochem. 60:568–577.
11. Heppel, L. A. 1967. Selective release of enzymes from bacteria. Science 156:1451–1455.
12. Hoffman, H., and M. E. Frank. 1963. Time-lapse photomicrography of the formation of a free spherical granule in an *Escherichia coli* cell end. J. Bacteriol. 86:1075–1078.
13. Hofschneider, P. H., and H. H. Martin. 1968. Diversity of surface layers in L-forms of *Proteus mirabilis*. J. Gen. Microbiol. 51:23–33.
14. Homma, J. Y., and N. Suzuki. 1966. The protein moiety of the endotoxin of *Pseudomonas aeruginosa*. Ann. N.Y. Acad. Sci. 133:508–526.
15. Huston, C. W., and P. W. Albro. 1964. Lipids of *Sarcina lutea*. I. Fatty acid composition of the extractable lipids. J. Bacteriol. 88:425–432.
16. Knox, K. W., J. Cullen, and E. Work. 1967. An extracellular lipopolysaccharide-phospholipd-protein complex produced. by *Escherichia coli* grown under lysine-limiting conditions. Biochem. J. 103:192–201.
17. Martin, H. H. 1963. Bacterial protoplasts—a review. J. Theoret. Biol. 5:1–34.
18. Martin, H. H., and H. Frank. 1962. Die Mucopeptidgrundstruktur in der Zellwand gram-negativer Bakterien. Zentrabl. Bakteriol. Abt. 1 (Orig.) 184:306–311.
19. Mitchell, P. 1961. Approaches to the analysis of specific membrane transport, p. 581–603. *In* T. W. Goodwin and O. Lindberg (ed.), Biological structure and function, vol. 2. Academic Press Inc., New York.
20. Moore, S., and W. H. Stein. 1963. Chromatographic determination of amino acids, p. 819–831. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 6. Academic Press Inc., New York.
21. Murray R. G. E. 1963. On the cell wall structure of *Spirillum serpens*. Can. J. Microbiol. 9:381–392.
22. Pease, P. 1956. The gonidia stages in *Spirillum* spp. and *Vibrio* spp. J. Gen. Microbiol. 14:672–675.
23. Pharmacia Technical Bulletin. 1969. Beaded sepharose 2B-4B-6B. Pharmacia (Canada) Ltd., Montreal.
24. Rothfield, L., and M. Pearlman-Kothencz. 1969. Synthesis and assembly of bacterial membrane components. J. Mol. Biol. 44:477–492.
25. Schneider, W. C. 1957. Determination of nucleic acids in tissues by pentose analysis, p. 680–684. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 3. Academic Press Inc., New York.
26. Thornley, M. J., and A. M. Glauert. 1968. Fine structure and radiation resistance in Acinetobacter: studies on a resistant strain. J. Cell Sci. 3:272–294.