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Chemical Analysis of the Outer Membrane and Other Layers of the Cell Envelope of *Acinetobacter* sp.

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Chemical analysis of fractions of the cell envelope of *Acinetobacter* sp. strain MJT/F5/199A, prepared by breakage in the French press and removal of plasma membranes, followed by sequential treatment with lysozyme and with papain, confirmed the existence of layers previously identified by electron microscopy. Outside the plasma membrane and periplasmic space, the envelope is composed of (i) a peptidoglycan-containing dense layer, (ii) an intermediate layer, (iii) a lipopolysaccharide-containing outer membrane, and (iv) an ordered array of protein subunits. A small amount of carbohydrate (3%) is found associated with protein in the fraction containing both the surface subunits and the intermediate layer. The papain-treated outer membranes contain 67% protein, 24% lipid, together with 11% lipopolysaccharide, and about 6% of non-lipopolysaccharide hexosamine. Lipid is located only in the papain-treated outer-membrane and is mainly phospholipid: 29% phosphatidyl glycerol, 30% phosphatidyl ethanolamine, and 40% cardiolipin. The principal fatty acid is $C_{18:1}$. Significant amounts of alcohols$_{16:1}$ and alcohols$_{18:1}$, which are found in *Acinetobacter* waxes, were recovered from the outer membrane.

Acinetobacter sp. strain MJT/F5/199A is a gram-negative bacterium with an ordered array of subunits on its outer surface. It is a convenient organism to use for analysis of the layers of the cell envelope since it has proved possible to remove various layers sequentially under relatively mild conditions (36).

The fine structure of the cell envelopes of this *Acinetobacter* has been determined from electron micrographs of thin sections, negatively stained and freeze-etched preparations of whole cells and of envelopes treated to remove the various layers (35, 36). These studies have shown that, outside the plasma membrane and periplasmic space, the layers of the envelope consist of (i) a peptidoglycan-containing dense layer, (ii) an intermediate layer, (iii) an outer membrane, and (iv) an ordered array of surface subunits. A cell wall preparation free of plasma membrane can be made from cells broken in the French press. The electron-dense peptidoglycan layer is removed by incubation with lysozyme. Treatment with papain removes both the intermediate layer and the surface subunits.

The present paper reports the results of chemical analyses of the various fractions of the cell envelope previously examined by electron microscopy.

MATERIALS AND METHODS

The bacteria (*Acinetobacter* sp. strain MJT/F5/199A from the culture collection of M.J.T.) were grown in heart infusion broth (Difco), to which 0.01% $CaCl_2$ was added. Cultures in conical flasks were aerated by shaking at 28 C, and the bacteria were harvested in the late logarithmic phase of growth.

Cell walls free of plasma membrane were prepared from cells broken in a French pressure cell by the procedure previously described (36). The washed cells were suspended in 0.05 M phosphate buffer, pH 7.4, at a protein concentration of 10 to 20 mg/ml and broken by two passages through an Aminco French pressure cell at a pressure of 16,000 lb/in^2. The suspension of broken cells was treated with deoxyribonuclease (5 μg/ml) and ribonuclease (20 μg/ml) in the presence of 5 mM $MgCl_2$ and then centrifuged at 25,000 $\times$ *g* for 10 min at 5 C. This temperature was used for all centrifugation, and during all washing procedures the temperature was between 5 and 10 C.

The pellet was suspended in 20 mosmol phosphate buffer, pH 7.4, and centrifuged at 3,000 × *g* for 5 min to remove unbroken cells. The supernatant fluid was centrifuged at 25,000 × *g* for 10 min, and the pellet was washed three more times with 20 mosmol phosphate buffer, pH 7.4. Contamination with plasma membrane was less than 2% as determined by reduced nicotinamide adenine dinucleotide oxidase activity.

Cell walls were treated with lysozyme at 100 μg/ml in 0.05 M phosphate buffer (pH 7.4) containing 20 mM $MgCl_2$ for 60 min at 20 C with stirring. The suspension was then centrifuged at 25,000 × *g* for 15 min, and the pellet was washed twice with 20 mosmol phosphate buffer, pH 7.0, containing 20 mM $MgCl_2$. Measurement of the content of diaminopimelic acid, described below, showed that this preparation still contained 7.1% of the peptidoglycan present in an equivalent amount of the cell wall preparation.

The outer membranes were suspended in 0.1 M phosphate buffer (pH 6.0) containing 0.6 mg of cysteine per ml, and papain (Sigma London Chemical Co., Kingston upon Thames, Surrey, twice crystallized) was added so that the ratio of papain to membrane protein was 1:50. After incubation at 37 C for 10 min with shaking, the suspension was chilled in an ice bath for 10 min and centrifuged at 39,000 × *g* for 30 min. The pellet was washed twice with 20 mosmol buffer, pH 7.0, containing 20 mM $MgCl_2$.

For chemical analysis, the same preparation was taken sequentially through the various treatments, and samples were removed as follows: one-third after preparation of the whole cell walls, one-third after lysozyme treatment, and the remaining third after papain treatment. All samples were freeze-dried for gravimetric measurement of dry weight. Portions were then taken for determination of protein by Campbell and Sargent's modification of the Lowry method (10), of 3-deoxyoctulsonate (KDO) (5), and of hexosamine (28).

The material remaining after removal of these portions was freeze-dried again, and lipid was extracted by shaking with 13 ml of Pronalys (May & Baker Ltd., Dagenham, England) chloroform-methanol (1:1, vol/vol) at 7 C for 48 h. After addition of a further 5 ml of methanol, the suspension was centrifuged at 6,000 × *g* for 5 min. The pellet was reextracted with a further 5 ml of methanol. The supernatant fractions were combined and used for lipid analysis.

Lipopolysaccharide was extracted from the pellet by incubation with 10 ml of 45% aqueous phenol at 69 C for 10 min (22). The aqueous layer was collected after centrifuging at 6,000 × *g* for 10 min. The phenol layer was re-extracted with a further 5 ml of water. The combined aqueous layers were dialyzed for 24 h at 7 C against water. The extracts were then centrifuged at 100,000 × *g* for 1 h. KDO was found only in the supernatant fluid, not in the pellet. The supernatant fraction was freeze-dried and used for lipopolysaccharide analysis.

Wall fractions and extracted lipopolysaccharide were analyzed for KDO by the thiobarbituric acid method (5). Lipopolysaccharide from *Escherichia coli* 0111-B4 (Difco Laboratories, Detroit, Mich.) was used as standard since it contains 3.3% KDO (13). The chromophore from *E. coli* KDO had an absorption maximum at 543 nm, whereas the absorption maximum for *Acinetobacter* KDO was 552 nm. The identity of the KDO was confirmed by paper chromatography. Hexosamine was determined by the method of Neuhaus and Letzring (28), using glucosamine as standard. Wall fractions and extracted lipopolysaccharide were hydrolyzed with an equal volume of concentrated HCl for 1 h at 120 C. Acid was removed by repeated evaporation with water in a stream of nitrogen, until the pH of the aqueous solution was neutral. Hexose was determined by the method of Loewus (24), heptose was determined by the method of Dische (12), and rhamnose was determined by the method of Gibbons (17).

For fatty acid analysis, a measured amount of trimargarin (about 50 μg per 0.3 mg of extracted lipid) was added as an internal standard. The lipids were methanolyzed with 1 ml of 14% boron trifluoride in methanol (British Drug House Ltd., Poole, Dorset, England) for 10 min over a boiling-water bath in stoppered tubes (27). The sample was then cooled and shaken with 2 ml of petroleum ether (40–60 C) and twice redistilled, and 1 ml of water and the organic layer were collected. The aqueous layer was extracted with a further 2 ml of petroleum ether, and the organic layers were combined and evaporated to dryness. The methyl esters of the fatty acids were analyzed by gas-liquid chromatography on 10% polyethyleneglycol adipate at 190 C and on 10% Apiezon L at 230 C with Pye 104 equipment with a flame ionization detector. Quantitative determination of the amount of each fatty acid was made by comparison with the amount of $C_{17:0}$ from trimargarin.

After addition of trimargarin as internal standard, lipopolysaccharide was hydrolyzed with 2.5 N HCl in 25% methanol at 120 C for 2 h. The fatty acids were extracted with chloroform and methylated with boron trifluoride in methanol (27). Hydroxy acids were identified by comparison of the gas-liquid chromatograms before and after formation of trimethylsilyl derivatives (11).

Total lipid phosphorus was determined by the Fiske-Subba Row method (14). Lipids were separated by thin-layer chromatography on silica gel G with chloroform-methanol-water (65:25:4, vol/vol/vol) and identified by spraying with acid molybdate (19), periodate-Schiff (33), and ninhydrin, and by comparison with standard lipid markers. For quantitative analysis, the lipids were located with iodine vapor, and the silica was scraped off for measurement of total phosphorus (14) or total lipid (4).

[^{3}H]Diaminopimelic acid (DAP) (25 μCi, 0.125 μmol) was included in 1,250 ml of growth medium. Bacteria were grown, harvested, and fractionated by the normal procedure. The total amount of radioactivity in each fraction was measured by counting samples in a Kennedy scintillation cocktail (East Anglia Chemicals Ltd., Hadleigh, Suffolk, England) (21). The envelope fraction was then hydrolyzed with 6 N HCl for 2 h at 120 C and, after removal of the acid

by repeated evaporation with water in a stream of nitrogen until neutral, chromatographed on thin layers of silica gel G with the solvent *n*-propanol-24% ammonia (67:33, vol/vol) for determination of the proportion of radioactive material in DAP. DAP had an R_f of 0.17; lysine had an R_f of 0.22. Lengths of 0.5 cm of silica were removed from the plate and incubated at room temperature with 0.5 ml of water for 1 h, and the radioactivity was determined by using 10 ml of Kennedy scintillation cocktail.

The methods used for electron microscopy were as described previously (36).

RESULTS

The fine structure of the various fractions has been fully described by Thornley et al. (36) and is illustrated here by electron micrographs of thin sections and negatively-stained preparations of each fraction of the cell envelope which has been examined chemically (Fig. 1-6).

The cell wall preparations contain very little contamination with plasma membrane (36), and in thin sections show the dense layer (d), intermediate layer (i) and outer membrane (om), which appears as a unit membrane (Fig. 1). The regularly arranged subunits are revealed by negative staining and are especially clearly seen at the folded edges of cell wall fragments (Fig. 2, arrow). In freeze-etched preparations the subunits are seen to form the outer surface of the cells (36). The intermediate layer shows no internal structure in thin sections, but negatively-stained preparations of cell walls prepared by disintegration with glass beads have shown that it contains material with a wrinkled appearance which can be removed by papain treatment (35). Cell wall fragments prepared with the French press show only traces of this wrinkled structure (Fig. 2, double arrow) which has presumably been disorganized in the process of rupture.

Outer membranes prepared by lysozyme treatment have lost the dense layer (Fig. 3) but retain the surface subunits (Fig. 4). Amorphous material appears to be attached to both outer and inner surfaces of the curved fragments of membrane (Fig. 3); presumably the material on the inner surface originates from the intermediate layer, but no traces of the wrinkled structure remain on the fragments of outer membrane (Fig. 4).

The papain-treated membranes retain the appearance of unit membranes in thin sections (Fig. 5) and have lost the amorphous material which adheres to the outer membranes. Many of the fragments form closed vesicles. No traces of the surface subunits remain (Fig. 6).

The analysis of the fractions of the cell envelope (Table 1) showed that one of the main cell wall components removed by lysozyme treatment is hexosamine, while the DAP is reduced to 7% of that present initially in the wall. This is consistent with the idea that the dense layer, seen by electron microscopy to be removed by lysozyme treatment, contains the peptidoglycan of the cell envelope. No lipid or hexose is lost during this treatment, and the decrease by one-quarter in the lipopolysaccharide is not yet understood, since this would be expected to be associated with the lipid in the outer membrane. Some protein is also lost at this stage, possibly representing release of material from the intermediate layer.

The outer membrane preparation contains 85% protein, and over half of its mass is solubilized by papain treatment. The material removed is principally protein, small amounts of lipopolysaccharide, and more carbohydrate than can be accounted for by the lipopolysaccharide. Since the papain treatment removes the surface subunits and probably much of the material of the intermediate layer, it follows that these two layers consist largely of protein,

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FIG. 1 to 6. *Electron micrographs of preparations of fractions of the cell envelope of Acinetobacter sp. strain MJT/F5/199A, all at the same magnification. The scale mark represents 0.1 μm.*

FIG. 1. *A thin section of a preparation of cell walls showing the dense layer (d), which contains peptidoglycan, the intermediate layer (i), and the outer membrane (om), which appears as a unit membrane.*

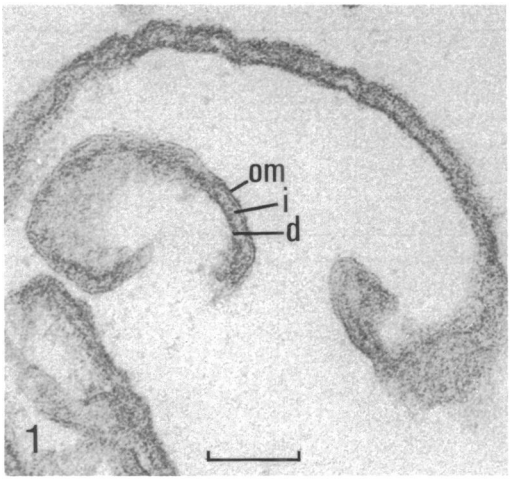
FIG. 2. *The array of surface subunits is visible in a negatively-stained preparation of isolated cell walls. The subunits are particularly clearly seen at folded edges of fragments of the cell wall (arrow). Traces of the wrinkled structure, which is located in the intermediate layer, are visible in some cell wall fragments (double arrow).*

FIG. 3. *A thin section of a preparation of outer membranes shows that the dense layer has been removed from the cell walls by treatment with lysozyme. The outer membrane (om) still has the appearance of a unit membrane, and amorphous material is associated with both the inner and the outer surfaces of the membrane.*

FIG. 4. *The array of surface subunits is clearly visible in a negatively-stained preparation of outer membranes. The wrinkled material is not visible.*

FIG. 5. *A thin section of a preparation of outer membranes after treatment with papain shows that the membranes still have the appearance of unit membranes, but much of the associated amorphous material has been removed. The membrane fragments are smaller than before papain treatment and appear as closed vesicles.*

FIG. 6. *Fragments of papain-treated outer membranes appear smooth in a negatively stained preparation.*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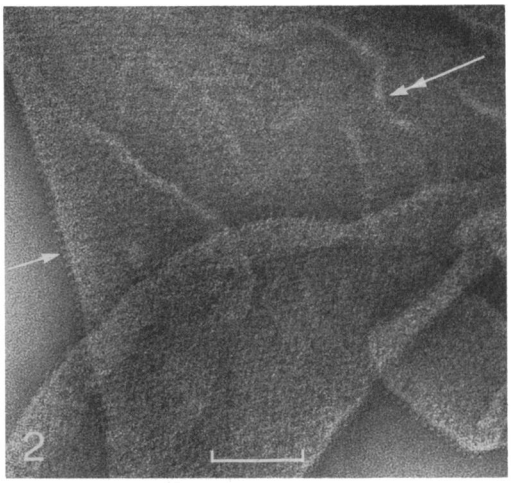
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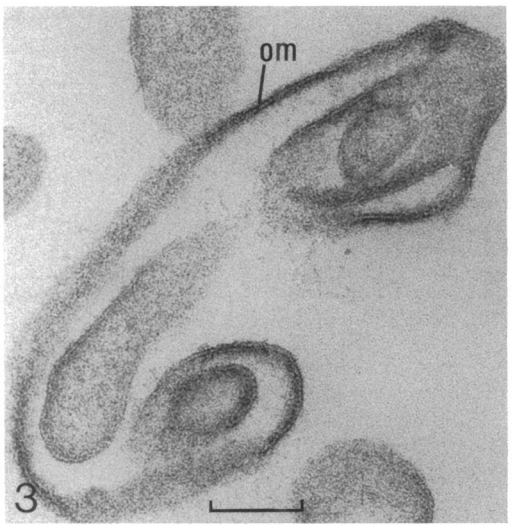
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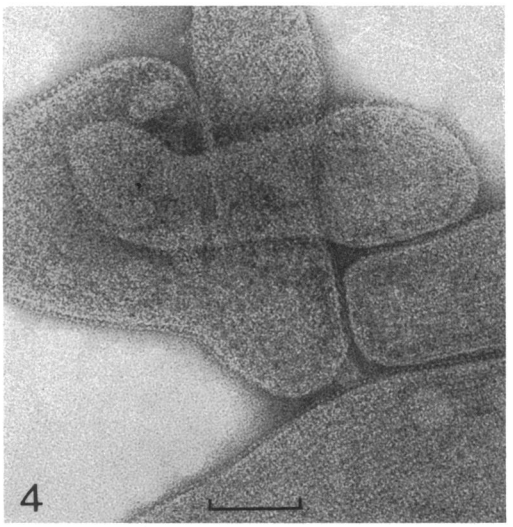
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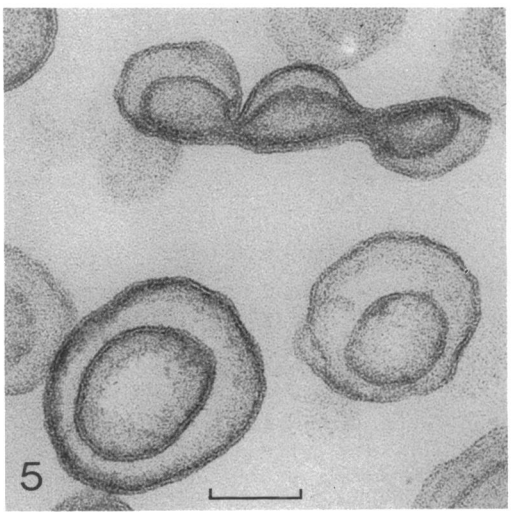
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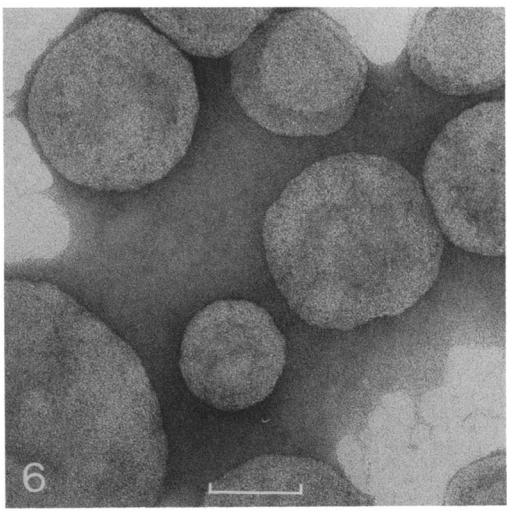
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TABLE 1. *Chemical analysis of cell envelope fractions derived from equal amounts of the cell wall preparation*[a]

Fraction	Dry wt (mg)	Protein (mg)[c]	Lipid		Lipopoly-saccharide[b] (mg)	Hexosa-mine (mg)	Hexose (mg)	DAP[c] (%)
			(mg)[d]	(mg)[e]				
Cell wall	39.0	28.8	2.6	3.25	2.6	3.0	1.35	100
Outer membrane	25.5	21.6	2.8	2.85	1.9	1.0	1.3	7.1
Papain-treated outer membrane	12.5	8.4	2.8	3.1	1.4	0.9	0.75	0.7

[a] Cell walls prepared from 1.5 g (dry wt) of bacteria were divided into three equal portions, two of which were treated further to yield outer membrane and papain-treated outer membrane preparations, in the quantities indicated.

[b] From KDO measurements of total envelope fraction, × 22.5 (see Table 2).

[c] DAP from radioactive measurements.

[d] Gravimetric.

[e] Sum of fatty acids from gas-liquid chromatography, ×1.32.

that they contain about half the protein of the cell wall, and that some of this protein may be glycoprotein. Again no lipid is lost; it is found only in the part of the outer membrane which resists papain treatment.

The final papain-treated outer membrane preparation has a composition in the range normally found for membranes; it contains 67% protein, 24% lipid, and 11% lipopolysaccharide. The hexosamine and also possibly the hexose contents are higher than can be accounted for by the lipopolysaccharide alone (Table 2). The hexosamine does not appear to be present as a constituent of peptidoglycan since only 0.7% of the [^{3}H]DAP of the cell wall remains with the papain-treated outer membrane.

Details of the phospholipid composition of the fractions are given in Table 3, which shows that 88% of the lipid from the untreated cell wall is phospholipid. Although the total quantity of lipid remains unaltered after lysozyme and papain treatment, the proportion as phospholipid falls. Traces of a ninhydrin-positive lipid (R_f 0.42), which could be lyso-phosphatidyl ethanolamine, were seen in the papain-treated fraction. This could arise from endogenous phospholipase activity. No lipid hexose was detected. Contamination with protein was determined with a Technicon auto-analyser and found to be 2.6%.

The predominant fatty acid of the outer membrane lipid is $C_{18:1}$ (Table 4). The occurrence of significant amounts of alcohols $C_{16:1}$ and $C_{18:1}$ may be attributed to the waxes found in *Acinetobacter* by Gallagher (16). Contamination with fatty acids from lipopolysaccharide was insignificant, partly because the fatty acid content of lipopolysaccharide was small compared with that of free lipid and partly because the conditions used to extract lipid were too mild to release lipopolysaccharide.

TABLE 2. *Composition of lipopolysaccharide*

Component	Percent dry weight	Molar ratio
KDO[a]	4.45	1.0
Hexose	28	8.1
Heptose	0	0
Rhamnose	0.4	0.1
Hexosamine	9.5	3.4
Total fatty acids	5.75	1.2
Phosphorus	1.5	2.6
Protein	6.1	

[a] 3-Deoxyoctulosonic acid.

Lipopolysaccharide was isolated from the whole, untreated cell wall. It remained in the supernatant fluid after centrifuging at 100,000 × *g* for 1 h and was not precipitable by 25 mM $MgCl_2$. Its composition is shown in Tables 2 and 5. It resembles the lipopolysaccharides of the related microorganisms *Moraxella duplex*, *Acinetobacter* (*Micrococcus*) *calco-aceticus*, and *Neisseria sicca* studied by Adams et al. (1, 3) in having no heptose. *Neisseria perflava* is similar in containing rhamnose, but differs in its content of heptose (2). The predominant fatty acids are β-OH.

DISCUSSION

The most notable chemical characteristic of the cell wall of this strain of *Acinetobacter* is that three-quarters of it consists of protein. Half of this protein occurs in two layers, the intermediate layer between the dense layer and the outer membrane and the array of surface subunits. The occurrence of subunits composed largely of protein on the surface of the outer

TABLE 3. *Phospholipids of cell envelope fractions, derived from equal amounts of the cell wall preparations*[a]

Fraction	Total lipid (mg)	Phospholipid[b] (mg)	Phospholipid composition		
			Phosphatidyl glycerol (%)	Phosphatidyl ethanolamine (%)	Cardiolipin (%)
Cell wall	2.9	2.6	29.4	30.1	40.5
Outer membrane	2.8	2.1	24.9	25.4	49.7
Papain-treated outer membrane	2.9	1.8	24.4	21.2	54.4

[a] Dry weights of each fraction are listed in Table 1.
[b] Lipid phosphorus, ×25.

TABLE 4. *Fatty acid composition of lipids from cell envelope fractions*

Fraction	16:0 (%)	16:1 (%)	16:1[a] Alcohol (%)	17:cyPr (%)	18:0 (%)	18:1 (%)	18:1[a] Alcohol (%)
Cell wall	2.57	7.97	7.05	3.74	4.49	68.58	5.61
Outer membrane	2.16	6.78	11.37	3.73	4.01	71.96	
Papain-treated outer membrane	2.49	8.59	6.64	3.87	4.13	74.28	
R_V PEGA	0.53	0.61	0.63	0.82	1.00	1.15	1.36
R_V Apiezon L	0.42	0.38	0.34	0.53	1.00	0.89	0.82

[a] Gas-liquid chromatography of 16:1 and 18:1 alcohols described by Gallagher (16).

membrane has been described in *Spirillum serpens* (9). The surface layer of a marine pseudomonad, however, contained only 17.5% protein (15) and 40% carbohydrate. Some carbohydrate (0.55 mg) was released from the *Acinetobacter* when the outer membrane was treated with papain; only 0.15 mg of this would be lipopolysaccharide. The solubilized protein may, therefore, contain about 3% carbohydrate. It is not possible at this stage to ascribe it specifically to the intermediate layer or to the ordered subunits on the outer surface.

Lipid was found only in the portion of the outer membrane which persists after papain treatment. Its constant recovery after both lysozyme and papain treatment demonstrated that there was little loss of outer membrane during processing. Removal of half the lipopolysaccharide, therefore, means either that lipopolysaccharide occurs in other envelope layers as well as the outer membrane, or that some of the lipopolysaccharide is only loosely attached and can be solubilized without disruption of the membrane. Levy and Leive have found evidence that some of the lipopolysaccharide of *Escherichia coli* is more easily liberated by ethylenediaminetetraacetic acid (EDTA) treatment than the rest and that the two pools are in dynamic equilibrium (23).

Peptidoglycan occurred in the dense layer of the cell wall closest to the plasma membrane. Some protein was solubilized when this layer was removed with lysozyme. This could be protein covalently bound to peptidoglycan as described by Braun (7, 8) for *E. coli* or perhaps soluble periplasmic enzymes. About 7% of the peptidoglycan, measured as radioactive DAP, remained with the outer membrane after lysozyme treatment and was only removed by papain.

TABLE 5. *Fatty acid composition of lipopolysaccharide*

Fatty acid	Percent of total
12:0	15.6
12:1	
16:0	5.7
12:βOH	33.0
18:0	3.8
18:1	3.5
14:βOH	29.6

One of the earliest identifications of the various layers of gram-negative bacteria was made by Weidel et al., using *E. coli* B (37). They described the layers as rigid peptidoglycan covered with protein, then lipopolysaccharide, and finally outer lipoprotein. It is now generally agreed that lipopolysaccharide and

lipoprotein are both present at the surface of the outer membrane (18). The occurrence of a rigid, electron-dense peptidoglycan layer adjacent to the plasma membrane is a common feature (25). The layer between peptidoglycan and outer membrane has been identified as globular protein in *E. coli* B (6) and as fibrillar protein in *Chondrococcus columnaris* (31). Forsberg et al. isolated a layer underlying the outer membrane from a marine pseudomonad and found that it was only 25% protein, being rich in carbohydrate (39%) and lipid (9%) (15).

A number of analyses have now been made of the outer layers of other gram-negative bacteria, prepared in a variety of ways. These included lysozyme-EDTA treatment to produce spheroplasts, followed by isopycnic sucrose density gradient centrifugation to separate fragments of outer membrane from plasma membrane (26, 30); breakage of cells in the French press, followed by particle electrophoresis and sucrose density gradient centrifugation to separate cell wall and plasma membrane fragments (38); and incubation of cells under conditions of controlled ion composition, followed by differential centrifugation to isolate fragments of outer membrane (15). The ratio of phospholipid to protein was 0.35 in cell wall fragments of *E. coli* which contained the outer membrane (38), 0.3 in outer membranes of *Salmonella typhimurium* (30) and 0.43 in outer membranes of a marine pseudomonad (15). For comparison, the papain-treated outer membranes of our *Acinetobacter* gave a phospholipid/protein ratio of 0.35. The marine pseudomonad resembled the *Acinetobacter* in having a significant amount of non-phospholipid lipid but differed from it in its high carbohydrate content (39%). More hexose was found in the papain-treated outer membranes of the *Acinetobacter* than could be accounted for by the lipopolysaccharide but the surplus only amounted to 3%. The hexosamine content of the papain-treated preparation was 7% of which only a small fraction is attributable to lipolysaccharide and none to peptidoglycan.

The fatty acid composition of the outer membrane of *Acinetobacter* is largely $C_{18:1}$. It is interesting that a significant amount of the lipid of the outer membrane is the wax described by Gallagher (16). About 12% of the fatty acid fraction was 16:1 and 18:1 alcohol. Combined with acid in a wax this would represent nearly one quarter of the total lipid. Levels of cardiolipin as high as that discovered here have been described for *Streptococcus faecalis* plasma membrane (20). It may reflect the fact that the cells were harvested in late log phase (32) or may vary with the growth medium (see 29). As with most other gram-negative bacteria (34) no evidence was found for glycosyl diglycerides.

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