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THE OUTER MEMBRANE OF *PROTEUS MIRABILIS*

I. ISOLATION AND CHARACTERIZATION OF THE OUTER AND CYTOPLASMIC MEMBRANE FRACTIONS

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SUMMARY

1. The crude envelope preparation obtained by sonication of *Proteus mirabilis* cells in the presence of lysozyme was separated into outer and cytoplasmic membrane fractions by sucrose density gradient centrifugation. The outer membrane fraction accounted for about two thirds of the dry weight of the envelope preparation.

2. In thin sections, the outer and cytoplasmic membrane fractions were shown to consist of vesicles bounded by a single trilaminar membrane, but those of the outer membrane were considerably smaller and were frequently open, forming C-shaped structures. The cytoplasmic membrane vesicles were cleaved by freeze fracturing to expose fracture faces studded with particles, while the outer membrane fragments resisted cleavage.

3. The outer membrane fraction consisted of protein (~40 %), lipopolysaccharide (~36 %) and lipid (~18 %) and had a density of about 1.22 g/cm^3. The cytoplasmic membrane fraction consisted mostly of protein (~56 %) and lipid (~38 %), had a density of about 1.16 g/cm^3, and contained almost all the NADH oxidase, succinate and D-lactate dehydrogenase activities of the crude envelope preparation.

4. Electrophoresis in polyacrylamide gels containing sodium dodecylsulfate revealed over 20 polypeptide bands in the cytoplasmic membrane fraction and only 6–7 in the outer membrane fraction. The outer membrane electrophorogram was dominated by a major band (mol. wt 40 000) which was resolved into two bands when electrophoresed in an acidic gel system. Amino acid analysis revealed a higher content of polar amino acids in the protein moiety of the outer membrane.

INTRODUCTION

The envelope of Gram-negative bacteria is a complex structure which consists of an outer membrane, an intermediate layer composed of peptidoglycan, and an inner, cytoplasmic membrane [1]. The peptidoglycan or rigid layer was the first to be

isolated relatively free of the other envelope components, making use of its resistance to solubilization in boiling 4 % sodium dodecylsulfate [2, 3]. Much greater difficulties were encountered in the resolution of the outer and cytoplasmic membranes. Only recently suitable, but still not entirely satisfactory, procedures for the separation of the two membrane types have been developed, based on the higher density of the outer membrane due to its high carbohydrate content. Envelope preparations obtained by lysis of lysozyme-EDTA spheroplasts [4–6] or by disruption of whole cells in the French pressure cell [7] are subjected to sucrose density gradient centrifugation. Of the two or more bands discernible in the gradient, the heaviest one represents a fraction highly enriched in outer membranes while the lightest band is highly enriched in cytoplasmic membranes. The intermediate bands, if present, consist of fragments of outer and cytoplasmic membranes which are "fused" or attached together [6, 7].

Chemical analysis of the outer and cytoplasmic membrane fractions isolated from several Gram-negative bacteria [4–8] showed the cytoplasmic membrane fraction to consist almost entirely of protein and phospholipids, the usual constituents of microbial cytoplasmic membranes [9], while the outer membrane contained in addition to protein and phospholipids also large quantities of lipopolysaccharide. The presence of lipopolysaccharide as a major membrane component is a unique feature of the outer membrane of Gram-negative bacteria [1]. Elucidation of the molecular organization of this membrane is therefore of particular interest. The suggestion that a bilayer made of interdigitated lipopolysaccharide and phospholipid molecules forms the backbone of the outer membrane is supported by model membrane studies [10–12], but so far has little support from studies carried out on the outer membrane itself. Moreover, the association of the outer membrane proteins with the phospholipid and lipopolysaccharide components, and the disposition of the various proteins in the membrane are subjects which need clarification. Elucidation of the molecular organization of the outer membrane may also throw more light on its function. It has been suggested that the outer membrane constitutes a penetration barrier, preventing large molecules from entering or leaving the cell [13]. Yet, the outer membrane appears to be much more permeable to small molecules than the cytoplasmic membrane, and to lack active transport systems [6, 12]. Our investigations have, therefore, been directed towards the elucidation of the molecular organization of the components of the outer membrane of Gram-negative bacteria, employing physical measures of membrane fluidity, disposition of membrane components by specific labels, and reconstitution techniques.

The present communication, which is the first of a series, is concerned with the isolation of the outer and cytoplasmic membrane fractions of *Proteus mirabilis* and their general ultrastructural and chemical characteristics. The following paper [14] provides information on the phospholipid component of these membranes and on the physical state of their hydrophobic core as measured by electron paramagnetic resonance (EPR) spectroscopy.

MATERIALS AND METHODS

Organism and growth conditions. Proteus mirabilis strain 19 was kindly provided by Professor H. H. Martin (Technische Hochschule, Darmstadt, Germany). The organ-

isms were grown in 2-1 vols of Nutrient Broth (Difco), supplemented with 10 μCi of uniformly labeled [^{14}C]glycerol (19.5 Ci/mol). The flasks were inoculated with a logarithmic culture at an inoculum level of 10 % (v/v) and were incubated with vigorous shaking at 37 °C for 2–3 h until an absorbance of 0.7 at 600 nm was reached. The organisms were then harvested by centrifugation at 10 000 $\times g$ for 10 min at 4 °C.

Isolation of membrane fractions. Total envelope fraction was obtained by sonic treatment of cells in the presence of lysozyme. The cell pellet obtained from a 2-1 culture was suspended in 100 ml of cold 0.75 M sucrose containing 10 mM Tris · HCl buffer, pH 7.5, and lysozyme (100 μg/ml). After 2 min of incubation at 4 °C the suspension was diluted 1 : 3 with deionized water and 50-ml volumes were sonicated in Raytheon model DF 101 sonic oscillator at 0.9 Amps for 3 min. The sonicated material was incubated at 4 °C for 30 min and then centrifuged, first at 1200 $\times g$ for 20 min to remove unbroken cells, then at 37 000 $\times g$ for 1 h to collect the envelope fraction. The envelope fraction was washed twice with a cold solution of 0.25 M sucrose containing 3.3 mM Tris · HCl buffer, pH 7.5. The washed envelope preparation was suspended in a solution of 25 % (w/v) sucrose and 1 ml containing about 6 mg of protein was layered over 11 ml of a 45–60 % linear sucrose gradient. The gradients were centrifuged at 38 000 rev/min in a SW 41 rotor of a Spinco ultracentrifuge for 18 h at 4 °C. Two membrane bands were clearly visible. These were collected with a coarse needle connected to a peristaltic pump. The collected membrane bands were diluted 1 : 4 with deionized water, sedimented by centrifugation at 45 000 rev/min for 1 h in a Ti 60 rotor of a Spinco ultracentrifuge, and washed once with cold water. The washed membrane preparations were resuspended in cold water and kept in −20 °C until used.

Lipid extraction. Lipids were extracted from freeze-dried cytoplasmic and outer membrane fractions by two successive extractions with chloroform/methanol (2 : 1), the first at 45 °C for 2 h and the second at room temperature overnight. The extracts were combined, washed according to Folch et al. [15] and traces of water were removed by incubating the extracts with anhydrous Na_2SO_4 for 24 h in the cold. The Na_2SO_4 granules were removed by filtration through an ultrafine sintered glass filter and the extract was evaporated to dryness under N_2 and weighed.

Extraction of lipopolysaccharide. Lipopolysaccharide was extracted from whole cells or membrane preparations with phenol/water according to Westphal et al. [16]. The water phase was dialyzed for 24 h against deionized water and the lipopolysaccharide was collected by centrifugation at 40 000 rev/min for 1 h in a Ti 60 rotor of a Spinco ultracentrifuge. To purify the lipopolysaccharide, the sedimented material was suspended in 0.01 M Tris/acetate buffer, pH 7.5, containing 5 mM $MgSO_4$ and 10 μg each of ribonuclease (5 × cryst., Sigma) and deoxyribonuclease (DNAase I from beef pancreas, Sigma) per mg of crude lipopolysaccharide. Incubation was carried out at room temperature for 24 h in the presence of 1 mM sodium azide. Pronase (1 μg/mg lipopolysaccharide) was then added and incubation was continued at room temperature for 24 h. The material was then centrifuged at 40 000 rev/min for 1 h and the sedimented lipopolysaccharide was freeze dried and weighed. The absorbance at 260 nm of the purified lipopolysaccharide was less than 1 % of that shown by the crude lipopolysaccharide preparation. 2-Keto-3-deoxyoctonoic acid determinations were performed throughout the

procedure to correct for lipopolysaccharide losses, using the ammonium salt of purified 2-keto-3-deoxyoctonoic acid as standard (kindly provided by Dr O. Lüderitz, Freiburg, Germany).

Analytical procedures. Protein was determined according to Lowry et al. [17]. Glucosamine was determined by the modification of the Elson-Morgan method [18] and 2-keto-3-deoxyoctonate by the thiobarbituric acid method followed by the extraction of the chromogen with cyclohexanone as described by Osborn et al. [6]. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer using dioxane-toluene scintillation liquor [19].

Enzyme assays. NADH oxidase activity in the cytoplasmic and outer membrane preparations was measured spectrophotometrically by determining the rate of decrease in absorbance at 340 nm on addition of NADH to a final concentration of 0.12 mM to a reaction mixture containing 50 mM Tris · HCl, pH 7.5; 0.2 mM dithiothreitol, and 0.1–0.2 mg membrane protein incubated at room temperature. Succinate and D-lactate dehydrogenase activities were determined as described by Osborn et al. [6].

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of the cytoplasmic and outer membrane was performed in the presence of sodium dodecylsulfate as described by Weber and Osborn [20] and modified by Amar et al. [21]. The molecular weight of the membrane polypeptides was estimated by comparing to a calibration curve prepared with bovine serum albumin, ovalbumin, chymotrypsinogen, and lysozyme. Electrophoretic analysis of the membrane proteins was also carried out by a modification of the Takayama technique (Razin and Rottem, 1967) using polyacrylamide gels containing 7.5 % acrylamide, 35 % acetic acid and 5 M urea. The membranes were solubilized in a phenol/acetic acid/water mixture (2 : 1 : 0.5, w/v/v). Densitometer tracings of the stained gels were made in a Kipp and Zonen Densitometer model DD2.

Amino acid analysis. Amino acid analysis were performed in a Beckmann model 120 B amino acid analyzer. Cytoplasmic and outer membrane preparations were hydrolyzed by heating with 6 M HCl at 110 °C for 22 h. The polarity index of the protein moiety of the cytoplasmic and outer membrane preparations was estimated according to Capaldi and Vanderkooi [23] by determining the sum of the residue mole percentages of polar amino acids, classifying aspartic acid, glutamic acid, lysine, serine, arginine, threonine and histidine as polar residues and the remaining amino acids as nonpolar.

Density gradient analysis. For isopycnic density gradient analysis of the isolated membrane fractions, samples (0.2 ml, 200 μg protein) were layered over 11 ml linear 25–55 % sucrose gradients. The gradients were centrifuged at 38 000 rev/min in the SW 41 rotor of a Spinco ultracentrifuge for 18 h at 4 °C. Fractions (0.04 ml), collected with a coarse needle connected to a peristaltic pump, were assayed for protein and/or radioactivity.

Electron microscopy. For thin sectioning, pellets of whole cells or membrane preparations were fixed at room temperature by the Ryter-Kellenberger procedure [24] for 16 h. The material was then washed in 8 ml of the Ryter-Kellenberger buffer, embedded in agar blocks and post-fixed in a 0.5 % sodium uranyl-acetate solution for 2 h at room temperature. The material was dehydrated and embedded in Epon by the method of Luft [25]. Sections were stained with uranyl acetate and lead citrate [26] and examined in a Phillips EM-300 electron microscope. Freeze fracturing

of membrane preparations suspended in 20 % glycerol was carried out by the method of Moor and Mühlethaler [27] in a Balzers' freeze-etching apparatus (Balzers, Fürstentum, Liechtenstein) as described in detail by Rottem et al. [28].

RESULTS

Isolation of two membrane fractions of *P. mirabilis* strain 19 was achieved by sucrose density gradient centrifugation of the total envelope fraction obtained after lysozyme treatment and sonication of the bacteria. Fig. 1 shows that centrifugation to equilibrium of the total membrane fraction on a 45–60 % sucrose gradient resulted in the separation of two bands, the first one (band I) floated on top of the gradient while the other (band II) sedimented to about one third to one half of the way down the gradient. The material included in each of the bands was collected, washed

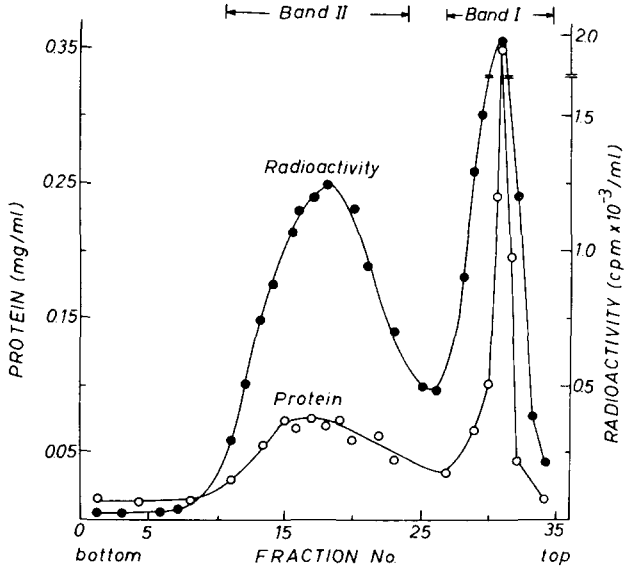

Fig. 1. Sucrose gradient centrifugation of total envelope fraction from *P. mirabilis*. The envelope fraction was obtained from cells grown with [^{14}C]glycerol and run on a linear 45–60 % sucrose gradient.

TABLE I

GROSS CHEMICAL COMPOSITION OF MEMBRANE FRACTIONS OF *P. MIRABILIS*

The data represent the mean of 2–3 determinations carried out on four different batches of membranes. Lipopolysaccharide was determined according to 2-keto-3-deoxyoctonate content.

Fraction	Per cent dry weight		
	Protein	Lipid	Lipopolysaccharide
Band I (cytoplasmic membrane)	56.1	38.0	5.3
Band II (outer membrane)	39.8	18.5	29.1

TABLE II

LOCALIZATION OF ENZYMIC ACTIVITIES IN THE CYTOPLASMIC AND OUTER MEMBRANE FRACTIONS OF *P. MIRABILIS*

Preparation	Enzymic activity (per cent of total)*		
	NADH oxidase	Succinate dehydrogenase	D-lactate dehydrogenase
Cytoplasmic membrane (band I)	93.5	98.0	84.0
Outer membrane (band II)	6.5	2.0	16.0

* Per cent of activity detected in the total envelope fraction.

and centrifuged on a 25–55 % sucrose density gradient to determine its exact density. The material of band I yielded a wide and diffuse band of a density ranging from 1.141 to 1.171 g/cm^3, while the material of band II yielded a wide band with a density ranging between 1.210 and 1.240 g/cm^3. Band II exceeded band I in dry weight by a factor of about two. This may be accounted for by the presence of large quantities of lipopolysaccharide in band II as can be seen in Table I. In corroboration with the gravimetric determinations of extracted lipopolysaccharide, band II was found to contain 5–6 times more 2-keto-3-deoxyoctonate and glucosamine than band I. The recovery of all the 2-keto-3-deoxyoctonate and glucosamine in the water phase obtained by the phenol/water extraction of the bands clearly indicates that they originate exclusively from lipopolysaccharide and may thus serve as a measure for lipopolysaccharide content in the membrane fractions. On a dry weight basis 2-keto-3-deoxyoctonate accounted for about 2.3 % of the purified lipopolysaccharide of *P. mirabilis* strain 19. The specific localization of the NADH oxidase and succinate and D-lactate dehydrogenases in band I (Table II) strongly supports its identification with the cytoplasmic membrane fraction since these enzymes are known to be located in the cytoplasmic membrane [6, 29]. Nevertheless, the presence of lipopolysaccharide (about 6 % of the total dry weight) in band I and detection of low levels of the enzymic activities in band II indicate some degree of cross-contamination of the cytoplasmic and outer membrane fractions.

Fig. 2 shows that both the cytoplasmic and outer membrane fractions appeared as empty vesicles of variable size bounded by a single double-track membrane. However, the vesicles composing the outer membrane fraction were much smaller, had a more regular appearance and exhibited most frequently the open C-shaped structures similar to those observed for outer membrane fractions of other enteric bacteria [4, 7, 8]. Freeze fracturing (Fig. 3) confirmed the results obtained by thin sectioning. The outer membrane fragments were sectioned rather than cleaved, exhibiting the characteristic C-shaped structures (Fig. 3A). The cytoplasmic membrane fraction, on the other hand, resembled cytoplasmic membrane preparations of other organisms in showing many concave and convex fracture faces studded with particles [30]. The concentric arrangement of membranes often seen in thin sectioned material (Fig. 2B) can also be observed in freeze fractured preparations of the cytoplasmic membrane fraction (Fig. 3B).

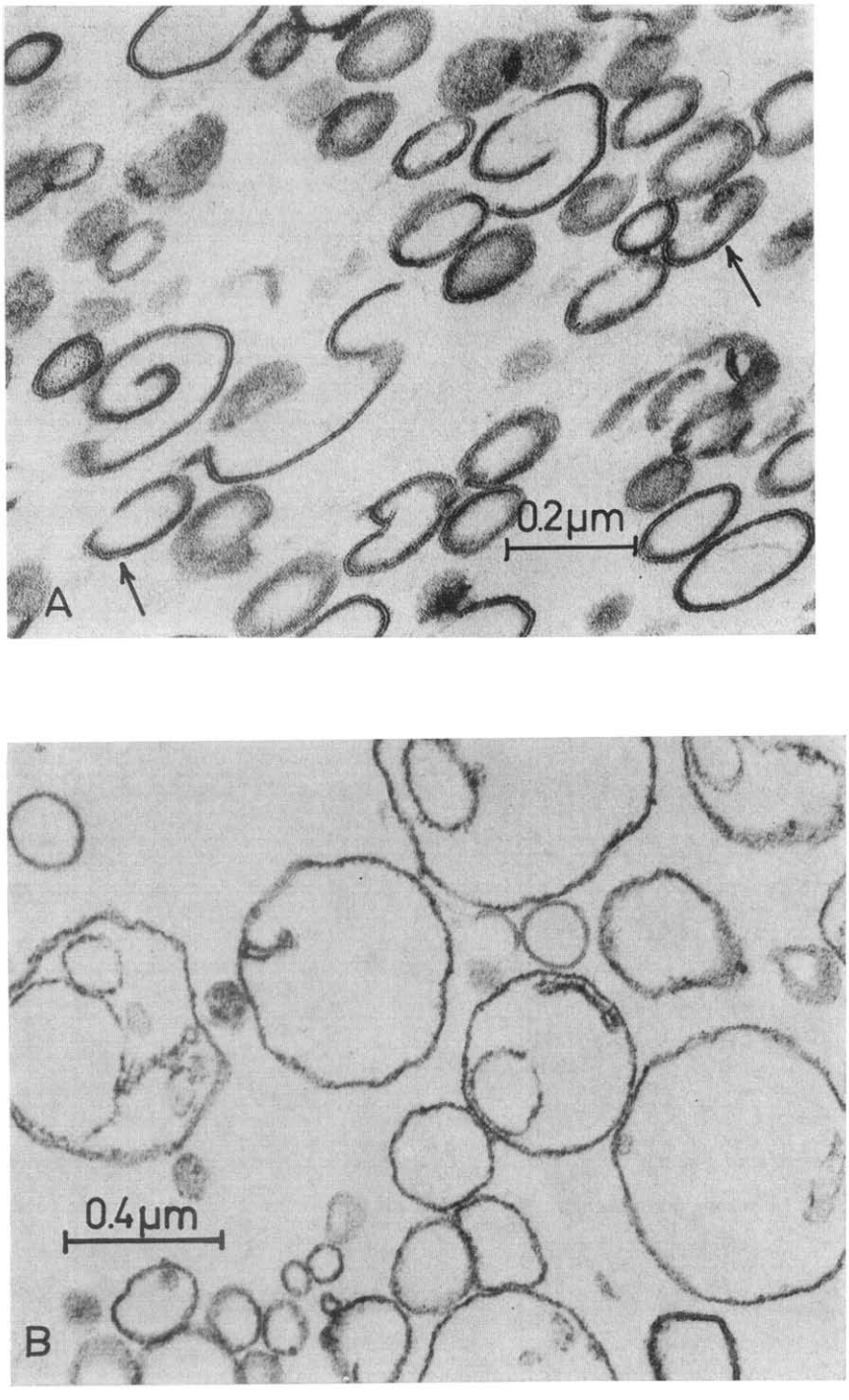

Fig. 2. Thin sections of the outer (A) and cytoplasmic (B) membrane fractions of *P. mirabilis*. C-shaped structures in the outer membrane preparation are marked by arrows.

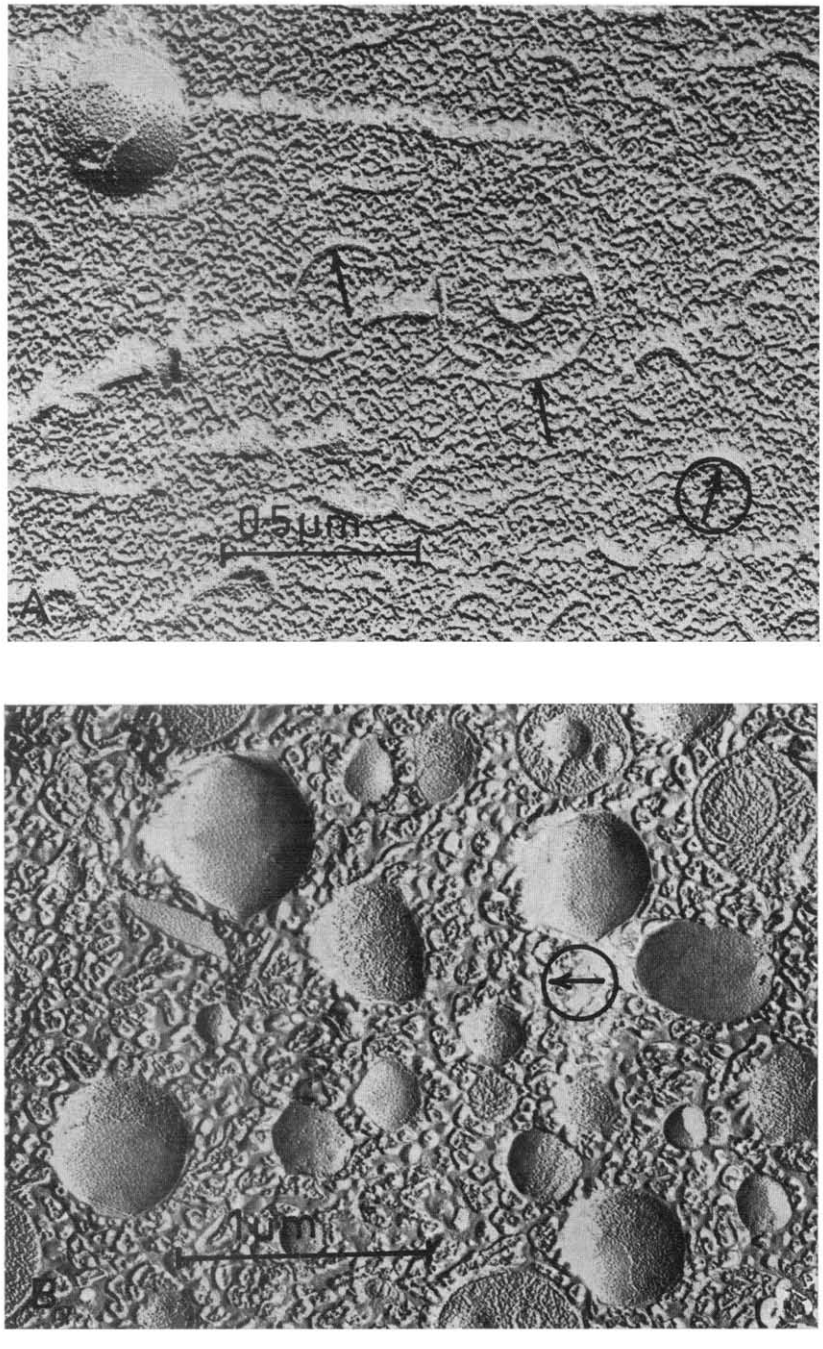

Fig. 3. Replicas of freeze-cleaved outer (A) and cytoplasmic (B) membrane fractions of *P. mirabilis*. Sectioned C-shaped structures (marked by arrows) characterize the outer membrane preparation, while convex and concave fracture faces studded with particles characterize the cytoplasmic membrane preparation. The fractured face on the upper left corner of A probably represents a cytoplasmic membrane contaminant. The circled arrow indicates the direction of shadowing.

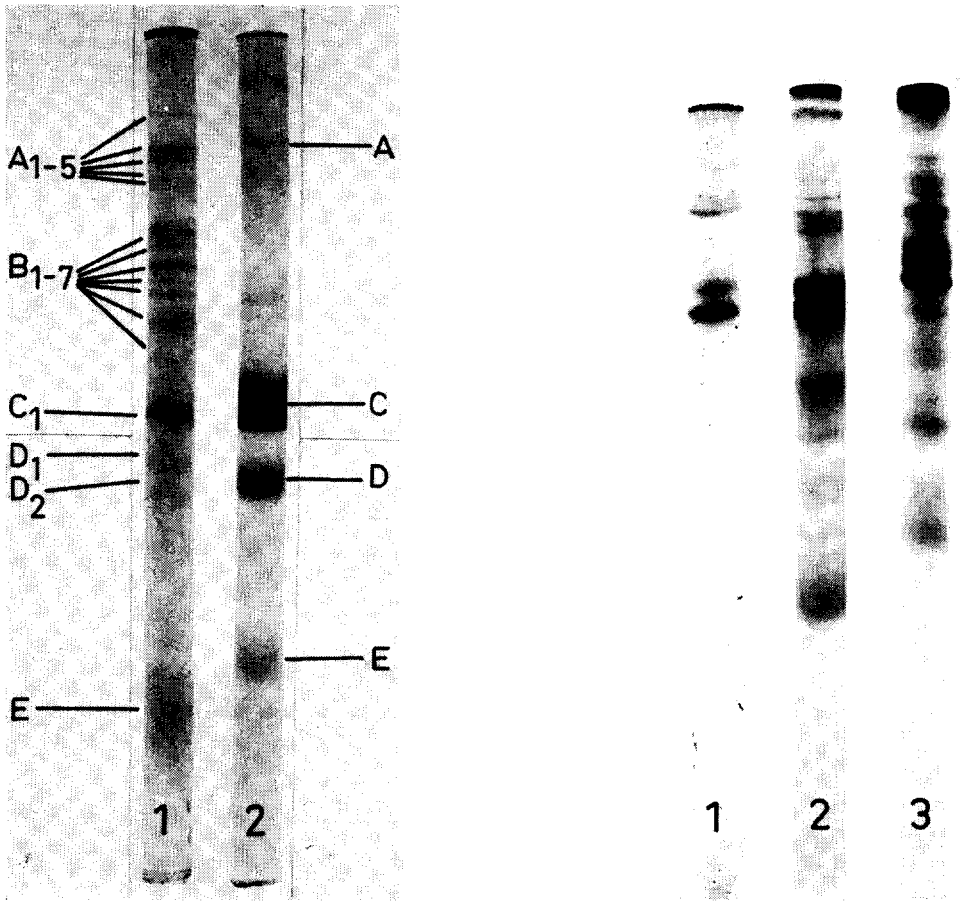

Fig. 4. Electrophoretic patterns and designation of the polypeptides of the cytoplasmic (1) and outer (2) membrane fractions of *P. mirabilis*. Electrophoresis was carried out in polyacrylamide gels containing 0.1 % sodium dodecylsulfate.

Fig. 5. Electrophoretic patterns of the polypeptides of the outer (2) and cytoplasmic (3) membrane fractions of *P. mirabilis* in polyacrylamide gels containing 35 % acetic acid and 5 M urea. Gel 1 shows the pattern of the major band C of the outer membrane (see Fig. 4) eluted with phenol/acetic acid/water (2 : 1 : 0.5, w/v/v) from sodium dodecylsulfate-containing acrylamide gels and run in the acidic gel system.

The electrophoretic patterns of the cytoplasmic and outer membrane fractions in polyacrylamide gels containing sodium dodecylsulfate are shown in Fig. 4. Over 20 polypeptide bands could be detected in the cytoplasmic membrane fraction as against 6–7 bands in the outer membrane fractions. The designation of the major bands and their molecular weights are presented in Table III. It is obvious that, while no dominant bands could be demonstrated in the cytoplasmic membrane fraction, the outer membrane fraction is dominated by a major band having a molecular weight of about 40 000 (band C). According to its staining intensity, band C accounts for about 65 % of the total membrane polypeptides detected on the gels. Reduction of the amount

TABLE III

POLYPEPTIDES OF THE CYTOPLASMIC AND OUTER MEMBRANE FRACTIONS OF *P. MIRABILIS*

Cytoplasmic membrane fraction		Outer membrane fraction	
Polypeptide band	Tentative mol. wt*	Polypeptide band	Tentative mol. wt*
A_1	110 000		
A_2	100 000		
A_3	94 000	A	94 000
A_4	92 000		
A_5	87 000		
B_1	74 000		
B_2	70 000		
B_3	64 000	B_1	64 000
B_4	61 000		
B_5	58 000		
B_6	53 000	B_2	53 000
B_7	47 000		
C_1	39 500	C	40 000
C_2	36 500		
D_1	33 000	D	33 000
D_2	27 500		
E	18 000	E	18 000

* Estimated according to a calibration curve prepared with standard proteins.

TABLE IV

AMINO ACID COMPOSITION OF THE CYTOPLASMIC AND OUTER MEMBRANE FRACTIONS OF *P. MIRABILIS*

Amino acid	Amino acid composition (mol per cent)	
	Cytoplasmic membrane fraction	Outer membrane fraction
Lysine	7.1	12.9
Histidine	1.8	1.9
Arginine	4.3	4.1
Aspartic acid	10.4	15.3
Glutamic acid	10.3	10.3
Threonine	4.9	5.6
Serine	4.1	3.6
Proline	4.4	2.8
Glycine	9.7	10.2
Alanine	10.8	10.6
1/2 Cystine	<0.1	<0.1
Valine	8.3	7.2
Methionine	0.2	0.5
Isoleucine	6.5	4.0
Leucine	11.4	7.2
Tyrosine	0.8	0.9
Phenylalanine	4.9	3.2
Polarity index*	42.9	53.7

* Calculated according to Capaldi and Vanderkooi [23] as described in Materials and Methods.

of protein electrophoresed down to 10 μg per gel [31] did not change the electrophoretic pattern, the major band C still appearing as a single band. The electrophoretic pattern of the outer membrane proteins in the acidic gel system of Takayama et al. [32] was different, however, from that obtained in the dodecylsulfate-containing gels, mainly in showing two major bands moving close to each other (Fig. 5). Moreover, the region of band C eluted from the sodium dodecylsulfate-containing gels with phenol/acetic acid/water (2:1:0.5, w/v/v) was resolved into two bands when run in the acidic gel system, corresponding to the two major bands shown by electrophoresis of whole outer membranes in the acidic gel system (Fig. 5).

The amino acid analysis of the cytoplasmic and outer membrane fractions is presented in Table IV. Most remarkable is the higher content of lysine and aspartic acid and the lower content of leucine and isoleucine in the outer membrane fraction resulting in a higher polarity index of the outer membrane protein fraction. The presence of lysine in the lipopolysaccharide of *P. mirabilis* strain 19 (Gmeiner, J., personal communication) may contribute to the high lysine content of the outer membrane of the organism. Significant amounts of ammonia (12–16 mol per cent) were also found in both membrane preparations suggesting that a considerable fraction of the free carboxylic groups may be amidated, thus the proteins may not really be as strongly acidic as it appears from Table IV. The extremely low levels of 1/2 cystine should be accepted with reservation as they were obtained with the 6 M HCl hydrolysate without prior oxidation to cysteic acid with performic acid [33].

DISCUSSION

The procedure developed by us for the isolation of the outer and cytoplasmic membranes of *Proteus mirabilis* is based on the general principles laid down by Miura and Mizushima [4, 5] and by Osborn et al. [6] for the isolation of the outer and cytoplasmic membranes of Gram-negative bacteria. Yet, one important difference should be stressed: EDTA is not used in any of the steps of our procedure. The procedures of Miura and Mizushima [4, 5] and of Osborn et al. [6] include a step in which the bacteria are transformed into spheroplasts by treatment with EDTA and lysozyme. Osborn et al. [6] stress that good spheroplasting is essential for the successful separation of the membrane fractions. In our case, however, the transformation of *Proteus mirabilis* cells to spheroplasts by EDTA and lysozyme was rather poor, as was also observed by Oltmann and Stouthamer [8]. Nevertheless, as our results show, the failure to produce spheroplasts from *P. mirabilis* did not affect the separation of the cell membranes. It appears that the sonication step included in our procedure suffices to rupture the cell envelope and expose the peptidoglycan layer to lysozyme action. Since the work of Leive [34], many effects of EDTA on the outer membrane of Gram-negative bacteria have been shown. The ability to omit EDTA from our fractionation procedure may thus be considered as an important advantage since the composition of the outer membrane is more likely to remain unaltered if the use of EDTA is avoided.

Another simplification of the Osborn procedure introduced by us is the use of a linear 45–60 % sucrose gradient for the initial separation of the membrane fractions. The cytoplasmic membrane fraction floats on top of this gradient while the outer membrane fraction, the major object of our studies, forms a band at about a

third of the way down the gradient. The outer membrane fraction obtained in this way is relatively free of contamination with cytoplasmic membrane components, as evidenced by the electron microscopical and chemical data, while the cytoplasmic membrane fraction may be less clean than the L_1 fraction of Osborn et al. [6] since it could contain the L_2 and M fractions which consist of cytoplasmic membranes with attached fragments of the outer membrane.

While our work was in progress Oltmann and Stouthamer [8] reported the separation of the outer and cytoplasmic membranes of *Proteus mirabilis* strain *PG*. As they failed to transform the bacteria to spheroplasts by EDTA and lysozyme, they employed penicillin for this purpose. Obviously, the use of antibiotic-treated cells may introduce variations in their envelope composition. Moreover, Oltmann and Stouthamer included EDTA and Brij 58 in the solution used for washing and lysing the spheroplasts. Again, these compounds may dissociate and release some membrane components.

The outer membrane fraction of *P. mirabilis* isolated by our procedure differed most markedly from the cytoplasmic membrane fraction when examined by thin sectioning and freeze fracturing. Furthermore, our electron micrographs indicate little cross-contamination of the two membrane types. Most frequently, the outer membrane fraction exhibited the open C-shaped structures, described previously for outer membrane preparations of other bacteria [7, 8, 35]. This shape, which indicates difficulties in vesicularization of the membrane fragments, may suggest some degree of rigidity not found in the cytoplasmic membrane fragments. The resistance of the isolated outer membrane fragments to cleavage by freeze fracturing may also point in the same direction. Schnaitman [7] noted the C-shaped structures in the outer membrane fraction isolated from *E. coli* cells disrupted by sonication without the application of lysozyme. In this case, the peptidoglycan or rigid layer was retained and could actually be seen attached to the outer membrane fragments. Schnaitman proposed, therefore, that the inability of the outer membrane fragments to vesiculate results from the rigidity imparted to them by the attached peptidoglycan layer. Following this reasoning Thornley et al. [35] claim that the digestion of the peptidoglycan attached to outer membrane fragments of *Acinetobacter* sp. contributed more rounded contours to the fragments. This explanation for the C-shaped structures appears untenable in our case since the *P. mirabilis* outer membrane fragments were isolated in the presence of lysozyme and no remnants of the peptidoglycan layer could be observed in our preparations. The suggestion of Henning et al. [36, 37] that some degree of rigidity can be contributed by some other component of the Gram-negative cell envelope besides the peptidoglycan may be of relevance in our case.

The density and chemical data provide further evidence for the successful separation of the outer from the cytoplasmic membrane of *P. mirabilis*. The density of the outer membrane fraction of *P. mirabilis* (1.22 ± 0.02 g/cm^3) is similar to that reported for outer membrane preparations of other Gram-negative bacteria [6, 38]. The high lipopolysaccharide and relatively low phospholipid content are also characteristics of outer membrane fractions of other enteric bacteria [6, 7, 39]. The very low NADH oxidase, lactate and succinate dehydrogenase activities in the outer membrane fraction are also indicative of its successful separation from the cytoplasmic membrane [4–7, 29, 39]. Furthermore, the electrophoretic pattern of the outer

membrane proteins of *P. mirabilis* very much resembles that of other Gram-negative bacteria in exhibiting relatively few polypeptide bands, as compared to the large number of bands shown by the cytoplasmic membrane fraction [6–8, 40]. All this can be taken to indicate that our preparation of the outer membrane of *P. mirabilis* is relatively free of cytoplasmic membrane components.

As was originally shown by Schnaitman [40] with several enteric bacteria, a heavy polypeptide band, having a molecular weight of about 40 000, also dominated the electrophoretic pattern of the outer membrane of *P. mirabilis* in sodium dodecylsulfate-containing polyacrylamide gels. The question of whether this heavy band represents a single protein or more has not been resolved as yet. The difficulty arises from the finding that different patterns are obtained depending on the temperature of solubilization of the outer membrane before electrophoresis [6, 41]. Thus, Inouye and Yee [42] showed that the major single band appears if solubilization is performed at 100 °C, but is replaced by three different bands when solubilization is carried out at 70 °C. In their opinion the major band is an artifact. Ames et al. [31], on the other hand, advocate the use of 100 °C for solubilization, but they also claim that the major band can be resolved into 3–6 different bands on electrophoresis of very small quantities of membrane protein in the slab-gel technique. In our case, a decrease in quantity of the electrophorized sample did not change the electrophoretic picture of the outer membrane of *P. mirabilis*. However, the eluted region of the heavy band could be resolved into two major bands when run in the acidic gel system of Takayama et al. [32] which does not contain sodium dodecylsulfate. Hence, the possibility that the heavy band dominating the electrophoretic pattern of the outer membrane of *P. mirabilis* consists of more than one protein appears plausible.

Another question which can be raised in this context is whether the electrophoretic pattern of the outer membrane proteins of *P. mirabilis* reveals the presence of Braun's lipoprotein. This lipoprotein is covalently linked to the peptidoglycan and may serve to attach it, through its lipid moiety, to the outer membrane [43]. It could be argued that following the digestion of the peptidoglycan layer, this protein will still remain attached to the outer membrane. However, the chances of detecting it in the outer membrane preparations of *P. mirabilis* appear to be rather small since the lipoprotein is either completely absent [44] or present only at the stationary growth phase of this organism [45]. Furthermore, it is doubtful whether, even when present, this protein could be detected in our electrophoretic system, as lysozyme which has a molecular weight about double that of the lipoprotein moves almost as fast as the tracking dye in the gels.

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