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Outer Membranes of Gram-Negative Bacteria

XIX. Isolation from *Pseudomonas aeruginosa* PAO1 and Use in Reconstitution and Definition of the Permeability Barrier

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A method for separating the outer and inner membranes of *Pseudomonas aeruginosa* PAO1 in the absence of added ethylenediaminetetraacetic acid was devised. The method yields two outer membrane fractions which show the same protein pattern on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, but differ substantially in their relative contents of phospholipids. One of these outer membrane fractions and the inner membrane fraction are less than 4% cross-contaminated, as judged by the content of typical inner and outer membrane markers. The outer membrane contains four major protein bands with apparent molecular weights of 37,000, 35,000, 21,000, and 17,000. Vesicles reconstituted from lipopolysaccharide and phospholipids were impermeable to all saccharides included in the vesicles during vesicle formation. When the vesicles contained outer membrane proteins, they fully retained only those saccharides of greater than 9,000 molecular weight, suggesting that the exclusion limit of the outer membrane of *P. aeruginosa* for saccharides is substantially larger than the figure (500 to 600 daltons) obtained for certain enteric bacteria. The advantages and potential disadvantages of having an outer membrane with a higher exclusion limit for hydrophilic substances are discussed.

Gram-negative bacteria have two cell envelope membranes separated by a single sheet of peptidoglycan. The inner or cytoplasmic membrane contains all known active transport systems and many of the cell envelope enzymes. The outer membrane is distinguished by a unique component lipopolysaccharide (LPS) and a unique set of proteins (H. Nikaido and T. Nakae, Adv. Microb. Physiol., in press).

It has been demonstrated that the outer membrane of the enteric bacteria *Escherichia coli* K-12 and B, *Salmonella typhimurium*, *Proteus mirabilis*, and *Proteus morganii* constitutes a permeability barrier for hydrophilic substances of greater than 550 to 650 daltons (6, 22, 25). This exclusion limit is reflected by vesicles reconstituted from the individual components of outer membranes (19, 25) and has been shown to be due to certain proteins named porins (20, 21, 25). These porins vary from strain to strain, but are, generally speaking, major outer membrane polypeptides of around 35,000 to 40,000 daltons.

In contrast, studies with *Pseudomonas aeruginosa* revealed that significant levels of larger saccharides, which were excluded by the outer membranes of enteric bacteria, diffused into plasmolyzed *P. aeruginosa* cells (6). In this paper we study this problem using reconstituted outer membrane vesicles. At the commencement of this study, the techniques available for isolating the outer membrane of *P. aeruginosa* (4, 35) were unsatisfactory, possibly due to the extreme sensitivity of the cell and its outer membrane to ethylenediaminetetraacetic acid (EDTA) (9, 29, 30), a chelator widely used in the preparation of purified outer and inner membranes. Therefore a method was devised which allowed separation of the outer and inner membrane in the absence of EDTA.

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MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* PAO1 was a gift from A. J. Clark. *S. typhimurium* LT2M1 was a previously described (22) rough mutant of strain LT2.

Medium and culture conditions. The medium used was tryptone (0.8%, wt/vol)-yeast extract (0.5%, wt/vol)-NaCl (0.5%, wt/vol). Overnight cultures, grown with shaking at 37°C, were diluted 10- to 20-fold into fresh medium and grown with vigorous shaking at 37°C to an optical density at 650 nm of 1.0 as measured with a Coleman 124 spectrophotometer.

Chemical and radioactive compounds. The chemicals used were of the highest grade commercially available, with the exception of sodium dodecyl sulfate (SDS), which in accordance with the method used was of a lower grade (16). [^{14}C]sucrose was obtained from

New England Nuclear (Boston, Mass.). [^{3}H]raffinose, [^{3}H]stachyose, and [^{3}H]verbacose were prepared by G. M. Decad and have been previously described (6). [^{3}H]dextran of 50,000 daltons was purified by gel filtration of 1 mCi of [*methoxy*-^{3}H]dextran from New England Nuclear (NET-427B, lot no. 940-179, 250 μCi/mg) on a BioGel A 0.5-m column using 0.5 M $(NH_4)HCO_3$ as a column buffer. The $(NH_4)HCO_3$ was removed by lyophilization, and the dextran was maintained at −20°C in 3 mM sodium azide. [^{3}H]dextrans of lower molecular weights were obtained by gel filtration of [*methoxy*-^{3}H]dextran from New England Nuclear (NET-427A, lot no. 622-252, 0.33 mCi/mg) on a BioGel P30 column (100 to 200 mesh, 1.2 by 50 cm). The column buffer used was 0.5 M $(NH_4)HCO_3$. The column was standardized as described in the legend to Fig. 1. The fractionation led to the recovery of [^{3}H]-dextran fractions with average molecular weights of 16,500, 15,000, 12,500, 10,000, 8,500, and 6,700. Under identical conditions, [*methoxy*-^{3}H]inulin from ICN (lot no. 858586, 680 μCi/mg) was fractionated on the same column, resulting in [^{3}H]inulin fractions of average molecular weights of 7,400, 5,200, 3,600, 2,500, and 1,700.

Separation of outer and inner membranes. Four liters of cells grown as described above was harvested by centrifugation. This and all subsequent operations were done at 4°C. The cells were washed with 30 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0) (Tris buffer) and resuspended in 20 ml of 20% (wt/vol) sucrose in Tris buffer containing 1 mg of pancreatic deoxyribonuclease and 1 mg of pancreatic ribonuclease. The cells were then passed twice through a French press at 15,000 lb/in^2, after which 2 ml of hen egg white lysozyme (Calbiochem; 1 mg/ml) was added, followed 10 min later by the addition (as a precaution) of a protease inhibitor, *p*-toluenesulfonylfluoride (to 1 mM). Cell debris was then removed by centrifugation at 1,000 × *g* for 10 min, and the supernatant was decanted and diluted by the addition of 14 ml of Tris buffer. Five to six milliliters were then layered onto a sucrose step gradient containing 1 ml of 70% (wt/vol) sucrose and 6 ml of 15% (wt/vol) sucrose in Tris buffer, and the tubes were centrifuged at 183,000 × *g* in a Beckman SW41 rotor for 1 h. The bottom 2 ml of each gradient was removed, and each 2 ml was applied to a further sucrose density gradient containing steps of 1 ml of 70% (wt/vol) sucrose, 3 ml of 64% (wt/vol) sucrose, 3 ml of 58% (wt/vol) sucrose, and 3 ml of 52% (wt/vol) sucrose in Tris buffer and centrifuged overnight (14 h) at 183,000 × *g* in an SW41 rotor. The four bands observed were removed either by dropwise collection from the bottom or by suction from above, and the individual samples were diluted in distilled water and centrifuged at 177,700 × *g* in a Beckman 60 Ti rotor. The resultant pellets were resuspended in a small amount of distilled water and frozen at −70°C. This procedure is a modification of the method described originally by Schnaitman (33); recently a somewhat similar modification was developed independently by Jones and Osborn (14).

The method of Osborn et al. (28) was used to isolate the outer membrane of *S. typhimurium*.

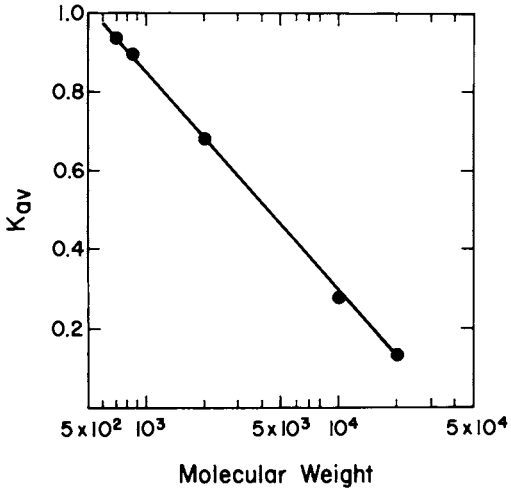

FIG. 1. *Standard curve for the determination of the molecular weights of ^{3}H-saccharides by gel filtration. A column (1.2 by 50 cm) of BioGel P30 (100 to 200 mesh) was calibrated with [^{3}H]stachyose (666 daltons), [^{3}H]verbacose (828 daltons), [^{3}H]inulin (2,000 daltons), Dextran T10 (10,000 daltons), and Dextran T20 (20,000 daltons). The molecular weight of the inulin was determined using a BioGel P2 superfine column, and the running positions of the 10,000- and 20,000-dalton saccharides were determined from a chart of the size distribution of Dextran T10 and T20, as provided by the manufacturer, Pharmacia Fine Chemicals AB (Uppsala, Sweden). The molecular weights of the ^{3}H-inulins and ^{3}H-dextrans were calculated from their K_{av} values (relative running position) on this column (as previously described [22]).*

Polyacrylamide gel electrophoresis. Before electrophoresis of the proteins, the outer membrane samples (OM1, OM2, and M bands) were individually extracted with an equal volume of 88% phenol at 70°C for 10 min, cooled to 4°C, and centrifuged at 5,000 × *g* for 10 min, and the upper aqueous layer (which had been shown to contain no detectable protein) was discarded. The interface and phenol layer were treated once more with distilled water at 70°C, and the aqueous phase was discarded after centrifugation as above; the lower phase was then treated twice with 2 volumes of acetone and once with 2 volumes of ether, with the protein being collected by centrifugation and the supernatants being discarded after each treatment. This procedure, which removes LPS, was shown to improve the straightness of bands on SDS-polyacrylamide slab gel electrophoresis, which was carried out by the method of Lugtenberg et al. (16). Trichloroacetic acid precipitation of the outer membrane protein had the same effect. Neither procedure was required for obtaining straight bands of inner membrane proteins.

Analytical procedures and enzyme assays. The LPS sugars 2-keto-3-deoxyoctonate and L-*glycero*-D-*manno*heptose were assayed as described by Osborn

(27). Protein was assayed by the method of Schacterle and Pollack (32). Lipid phosphate was assayed by the method of Ames and Dubin (1). Succinate dehydrogenase was assayed as described by Kasahara and Anraku (15), and D-lactate dehydrogenase was assayed as described by I. Yamato, Y. Anraku, and M. Ohki (J. Biol. Chem., in press).

Preparation of LPS and phospholipids. Lipids were extracted from *P. aeruginosa* PAO1 by the method of Folch et al. (7) and maintained in chloroform at −20°C. Since it is known that 90% of *P. aeruginosa* lipids are phospholipids (10) and we used phosphate content as an assay of lipid levels, we refer to crude lipids as "phospholipids" in this paper. LPS was extracted from log-phase cells of strain PAO1 by the phenol-water technique, except that the cells were lysed by resuspension in lysozyme (100 μg/ml) and 2 mM EDTA before extraction (13). After removal of the remaining traces of phenol by extensive dialysis and digestion of nucleic acid with nucleases, 5 mM $MgSO_4$ was added to the LPS-containing suspension to facilitate collection by centrifugation. The LPS was maintained as an aqueous suspension at 4°C. LPS concentrations were calculated by assuming that each mole of LPS contained 2 mol of heptose.

Vesicle preparation. Reconstitution of outer membrane vesicles was by a technique based on that of Nakae (19, 20, 21). Phospholipids (0.5 μmol) were dried down under a stream of N_2 in disposable borosilicate tubes (Dispo, Scientific Products), and the tubes were placed in an evacuated desiccator for 30 min. LPS (0.08 μmol), with or without outer membrane (125 to 140 μg of protein derived from the OM1 or OM2 band material; see Results), was added to the tube, then blended in a Vortex mixer for 30 s and sonically disrupted in a sonicating water bath ("Bransonic 12," Branson Co., Shelton, Conn.) for 60 s to thoroughly randomize the components. The suspension was then dried down at 45°C under a stream of N_2, and the tubes were placed in a desiccator for 30 min. A 100-μl volume of a solution containing 10 μmol of NaCl, 1 μmol of $MgCl_2$, 0.1 μmol of *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.4), and 2.2×10^5 cpm each of [^{14}C]sucrose and a tritiated saccharide was added, and the vesicle components were resuspended with a spatula, followed by 30 s of blending in a Vortex mixer and 30 s of sonic disruption in a sonicating water bath. The tubes were then incubated in a water bath at 45°C for 30 min, and finally the water bath was turned off and allowed to come slowly (2 h) to room temperature. The contents of the tube were then diluted and collected as described by Nakae (20). Preliminary experiments were performed using another collection method (19); results were essentially identical, however, and the method routinely used had the advantage of being relatively rapid.

Triton X-100 and sodium deoxycholate solubilization. Pooled OM1 and OM2 fractions (final concentration 5 mg/ml; see below for nomenclature) were solubilized in 2% (vol/vol) Triton X-100 or 1% sodium deoxycholate (wt/vol) in Tris buffer containing 2 or 10 mM EDTA. Solubilization was assisted by 2 min of sonic disruption in a sonicating water bath followed by 20 min of incubation at 37°C. Insoluble material was removed by centrifugation in a 60 Ti rotor at 50,000 rpm for 60 min and resuspended in a small amount of water as Triton-EDTA- or deoxycholate-EDTA-insoluble material. Triton-EDTA- or deoxycholate-EDTA-soluble material was recovered from the supernatants by the addition of NaCl to 0.5 M, followed by 2 volumes of ethanol. After overnight incubation at −20°C, the protein precipitate was collected by centrifugation (8,000 × *g*, 10 min) and resuspended in distilled water.

RESULTS

Separation of outer and inner membrane. Four bands were observed after the second sucrose gradient centrifugation, one at each of the original sucrose density steps (see Fig. 2). The IM band was deep red and the OM1 band translucent white, while the OM2 and M bands showed varying degrees of orange coloration. When 3 mM EDTA was present during the membrane preparation, only the three lightest bands were observed, and the heaviest of the three (OM2) was much diminished in size (Fig. 2). Table 1 shows the results of the analysis of the bands. The OM1 and IM bands are highly purified outer and inner membranes, respectively, as judged by the relative amounts of inner membrane marker enzymes (D-lactate dehydrogenase and succinate dehydrogenase [28]) and outer membrane-specific LPS in the two bands. The OM2 band contains a moderate amount of the two inner membrane marker enzymes (12%), and the M band contains a larger amount,

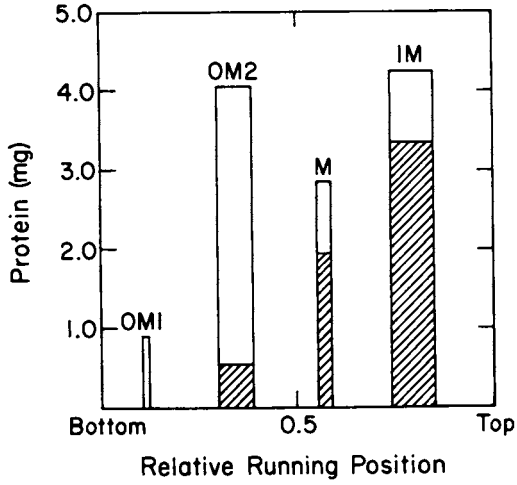

FIG. 2. *Relative running positions of the four visible bands on the second sucrose density gradient (see the text). The size of the visible bands is represented by the width, and the amount of protein by the height, of the bars. The shaded area represents the separation of membranes which were prepared and run in the presence of 3 mM EDTA.*

TABLE 1. *Constituents of the membrane fractions*[a]

Determination	Percent of protein recovered	Activity		KDO[c]	Lipid phosphate[b]	Weight (% of fraction)[c]		
		LDH[d]	SDH[d]			Protein	LPS	Phospholipid
Fraction								
OM1	8.1	0.7	19	248	0.23	38	54	8
OM2	40.6	2.5	56	61	0.46	57	20	23
M	16.7	4.4	97	132	0.65	43	32	25
IM	34.5	19.9	458	7	0.26	79	3	18
Ratio								
OM1:IM		0.03	0.04	36				
OM2:IM		0.12	0.12	9				

[a] The results reported are the averages of three separate membrane separations.
[b] Expressed in micromoles of extractable lipid phosphate per milligram of protein.
[c] Calculated using the formulas: 2-keto-3-deoxyoctonate (KDO) = 2.8% of the weight of LPS (5); lipid phosphorus = 3.5% of the weight of total lipid (here called "phospholipid") (29).
[d] LDH, Lactate dehydrogenase; SDH, succinate dehydrogenase. Activity is expressed as nanomoles of dichloroindolephenol reduced per minute per milligram of protein.
[e] Amount expressed as nanomoles per milligram of protein.

although both bands contain substantial amounts of LPS. When prepared in the presence of EDTA, OM2 and IM were highly cross-contaminated, as judged by the above criteria (results not shown) and by membrane protein patterns (Fig. 3).

Membrane protein patterns. Analysis of the proteins by SDS-polyacrylamide gel electrophoresis showed that the patterns of proteins in the OM1 (Fig. 3, gels a and e) and OM2 (Fig. 3, gels b, f, and g) bands were not significantly different, whereas they differed completely from the pattern of proteins in the IM band (Fig. 3, gels d and i). The M band contained all of the outer membrane protein bands in quite high levels (Fig. 3, gels c and h) and contained certain proteins which had the same mobility as inner membrane proteins. Although the relative levels of all outer membrane proteins in the M band seemed to be the same as in the OM1 and OM2 bands, certain inner membrane proteins seemed to be enriched in the M band relative to others. On these gels, 71 inner membrane polypeptides and 29 outer membrane polypeptides of apparent molecular weights between 12,000 and 100,000 (the molecular weight range differentiated on these gels) were observed.

In the inner membrane no protein bands were prominent. However, the outer membrane of *P. aeruginosa* (see for example Fig. 3, gel a), as also observed in other gram-negative organisms (2, 16, 23, 31, 34), contained several protein bands which could justifiably be labeled "major bands." These were bands of 39,000 daltons (39K), 21K, and 17K on long (22-cm) gels. The outer membrane proteins were treated with phenol, a procedure which removed LPS and resulted in sharper and straighter bands on subsequent SDS-polyacrylamide gel electrophoresis. The migration rate of only one band was substantially affected by the removal of LPS by the phenol treatment. The major band of highest molecular weight had an apparent molecular weight of 31.5K before and 39K after phenol treatment; possibly this protein is quite stably associated with LPS in the membrane. It was also noted that removal of LPS by trichloroacetic acid precipitation of the proteins had the same effect, whereas extraction of the membrane with chloroform-methanol (2:1) to remove phospholipids had no effect on mobility of this or any other protein. In 2 of 10 SDS-polyacrylamide gel electrophoresis runs of outer membrane proteins, the 39K protein split into two narrowly spaced bands. When the outer membrane was solubilized in 2% Triton X-100 or 1% deoxycholate in the presence of EDTA, the 39K band (which runs as 37K on the short gel shown) split into major bands of 35K and 37K (Fig. 4). Thus it would appear that there are four major protein bands in this strain, two of which (the 35K and 37K proteins) are possibly quite similar. The band of 44K running above the 37K band in Fig. 3 and 4, although prominent, contained less than 20% of the amount of protein in the major bands and thus is not named among the major bands.

Two further significant observations were made concerning the outer membrane proteins. First, the temperature of solubilization of the outer membrane proteins in SDS prior to application to the gels (whether 37 or 100°C) had no significant effect on the banding pattern, in contrast to all gram-negative enteric organisms studied to date (2, 23, 31, 34). One of the types of protein which migrate differently on SDS-polyacrylamide gels according to the tempera-

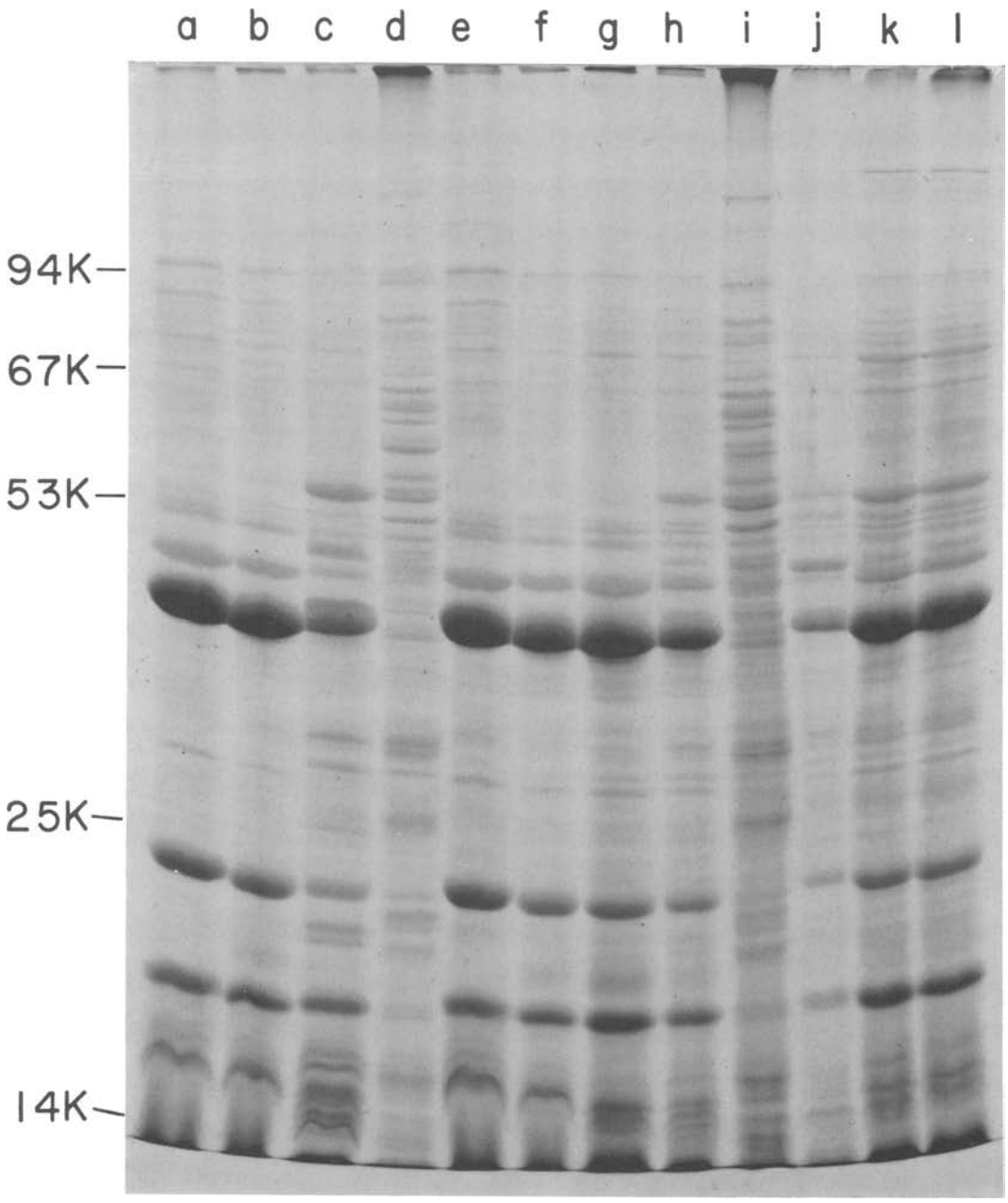

FIG. 3. *SDS-polyacrylamide gel electrophoresis (22-cm gels) of the separated membranes of P. aeruginosa PAO1. The samples in gels a to i were prepared in the absence of EDTA. (a, e) OM1 bands; (b, f, g) OM2 bands; (c, h) M bands; (d, i) IM bands (see Fig. 2 and Table 1 for nomenclature). The samples in gels j to l were prepared in the presence of 3 mM EDTA (see Fig. 2). (j) OM2 band; (k, l) M band. The apparent molecular weights of the proteins were determined using phosphorylase a (94K), bovine serum albumin (67K), glutamic dehydrogenase (53K), α-chymotrypsinogen (25K), and lysozyme (14K) as standards.*

ture of solubilization includes the porins of *S. typhimurium* and *E. coli*, which are responsible for the formation of size-dependent permeability channels. Second, growth in the presence or absence of 0.5% (wt/vol) NaCl had no effect on the major outer membrane protein bands (as it does in *E. coli* and *S. typhimurium*; 11, and J. Smit and H. Nikaido, submitted for publication).

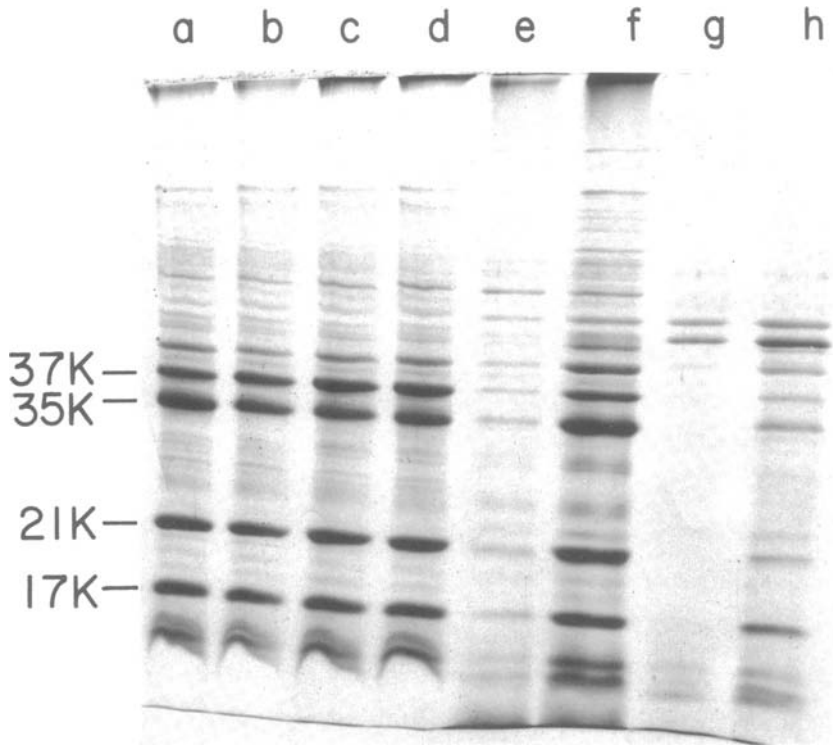

FIG. 4. *SDS-polyacrylamide gel electrophoresis (9-cm gels) of Triton-EDTA-treated and deoxycholate-EDTA-treated outer membranes (pooled OM1 and OM2) of P. aeruginosa PAO1. (a to d) Soluble fractions; (e to h) insoluble fractions. Treatments were: (a, e) 1% sodium deoxycholate + 10 mM EDTA; (b, f) 1% sodium deoxycholate + 2 mM EDTA; (c, g) 2% Triton X-100 + 10 mM EDTA; (d, h) 2% Triton X-100 + 2 mM EDTA. These samples were not extracted with phenol before gel electrophoresis. Standards used to determine the position of the protein bands were as described in the legend to Fig. 2. The major band, which runs at 39K on the long (22-cm) gels shown in Fig. 3 and normally runs at 37K on short (9-cm) gels, has split into two bands of 37K and 35K.*

The presence of NaCl did lead, however, to the induction of a minor band of 25.5K (results not shown).

Reconstitution of outer membrane vesicles. The OM1 and OM2 fractions described above were used as a source of protein for the reconstitution experiments; no significant differences were observed when either one or the other was used. These fractions contributed outer membrane proteins to the reconstitution system, since excess phospholipids and LPS were added. As described below, without these additions neither OM1 nor OM2 formed vesicles enclosing large amounts of the medium. Presumably sonic disruption in the presence of excess LPS and phospholipids caused redistribution of the outer membrane proteins into the phospholipid bilayer of the vesicles. No detergent solubilization was required for this or any subsequent vesicle preparation. Strain PAO1 was used as a source of phospholipids. Thin-layer chromatography in two solvent systems showed that the lipid composition was essentially the same as previously reported for *P. aeruginosa* (10), and that no observable phospholipid degradation occurred during the prolonged incubation with outer membrane fragments needed for the preparation of vesicles.

None of the components alone or in pairs, with the exception of the LPS + phospholipid pair, was able to form closed vesicles capable of retaining large amounts of sucrose, inulin, or dextran (Table 2). Thus the contribution of nonspecific adsorption is minimal. In other experiments, vesicles comprising LPS and phospholipid retained 1.77 ± 0.66% (mean ± standard deviation of 41 results) of the added tritiated saccharides and 1.02 ± 0.47% (mean ± standard deviation of 39 results) of the added [^{14}C]sucrose. The reason for the higher retention of ^{3}H-saccharides than [^{14}C]sucrose is not known, but a similar observation was made by Nakae (19).

When outer membrane fragments were added to the system (complete vesicles), the amount of

TABLE 2. *Requirements for the reconstitution of sucrose-permeable vesicles*

Saccharide[a]	Vesicles[b]	Percent saccharide retained		Ratio, $^3H/^{14}C$
		^3H-saccharide	[^{14}C]sucrose	
Inulin, 2K	Complete	0.47	0.15	3.16
	Lacking OM	2.20	1.50	1.47
	Lacking phospholipid	0	0	
	Lacking LPS	0.02	0.05	
	Phospholipid, LPS, or OM alone	0.006	0.02	
Dextran, 50K	Complete	2.12	0.26	8.41
	Lacking OM	2.52	1.64	1.53
	Phospholipid, LPS, or OM alone	0	0	

[a] The saccharide given is the ^3H-saccharide used in the experiment.

[b] OM, Outer membrane; complete vesicles contained LPS + OM + phospholipids. The results represent the means of between 3 and 13 experiments for each value given; the average standard error was 15%.

[^{14}C]sucrose retained was 0.115 ± 0.073% (mean ± standard deviation of 74 results) of the input, or 11% of the amount retained in the absence of outer membrane proteins. The amount of ^3H-saccharide retained by complete vesicles varied according to the size of the saccharide. As shown in Table 2, only 21% of an inulin fraction of 2,000 daltons was retained (relative to the amount retained in the absence of added outer membrane), whereas 84% of a 50,000-dalton dextran remained in the vesicles. Similar studies were performed using 16 saccharides of varying molecular weights. These studies suggested that the exclusion limit for *P. aeruginosa* is approximately 3,000 to 9,000 daltons (Fig. 5).

As a control, the retention of labeled saccharides was studied using vesicles reconstituted from *P. aeruginosa* strain PAO1 LPS and phospholipids and using unextracted *S. typhimurium* outer membrane as a protein source. The results (Fig. 5) show that these vesicles had an exclusion limit for saccharides of around 600 to 700 daltons. Thus the higher exclusion limit seen with the *P. aeruginosa* system is not an artifact related to the possible "leakiness" or fragility of vesicles containing phospholipids and LPS of *P. aeruginosa*. Furthermore, this result shows that the pore size is determined by the protein and is not influenced by the type of LPS or phospholipid used to construct the vesicles.

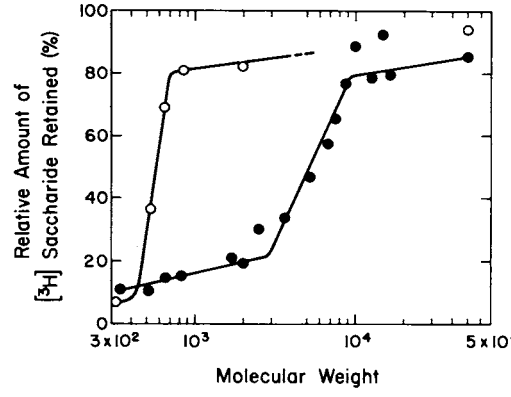

FIG. 5. *Exclusion limit for saccharides in reconstituted outer membrane vesicles of P. aeruginosa PAO1 (●) and S. typhimurium LT2 M1 (○). For each saccharide, control vesicles were formed from P. aeruginosa PAO1 phospholipids and LPS, and the observed retention of saccharides was taken as 100%. The retention in vesicles containing either P. aeruginosa or S. typhimurium outer membrane proteins (complete vesicles) was expressed relative to the retention in control vesicles. Each point on the graph represents between three and six separate control vesicle experiments and between three and nine complete vesicle experiments. Only the means are demonstrated.*

DISCUSSION

In the absence of added EDTA, sucrose density gradient centrifugation of cell envelope preparations of *P. aeruginosa* led to fractionation of the envelope into four bands. From enzyme assays, LPS determinations, and protein compositions, it is clear that the two densest bands (OM1 and OM2) are considerably enriched in outer membrane markers, whereas the least dense band (IM) is substantially purified inner membrane. The M band is also enriched in outer membrane markers, but contains some 20 to 25% contamination by two inner membrane enzymes. In fact all membrane bands are at least 4% contaminated by the typical markers of the other membrane, as has been noted in all separations of the outer and inner membranes of gram-negative organisms to date (see, e.g., 28). There are at least four possible explanations for this phenomenon: (i) certain domains of inner membrane have the same density as outer membrane and vice versa; (ii) French pressure cell

treatment causes the intermembrane migration of certain protein molecules; (iii) membrane fragments of one type become entrapped in the vesicles of the membrane of the other type, or are loosely associated with the other membrane; and (iv) the existence of adhesion sites between outer and inner membranes (3) leads to copurification of portions of the two membranes.

It was shown by Eagon and coworkers (9, 30) that spherical units, seen on the concave face of the outer membrane upon freeze fracture, could be 50% extracted by EDTA treatment of cells. These units, when isolated, were relatively high in protein and LPS, but low in phospholipid. Possibly the OM1 fraction, which contains only 8% by weight of phospholipids, represent these units, while the OM2 fraction and at least part of the M fraction represent the rest of the outer membrane. Evidence for this is that the OM1 fraction is lost when membranes are prepared in the presence of 3 mM EDTA (Fig. 2). If this is true, then it is noteworthy that the protein compositions of the OM1 and OM2 fractions are highly similar. One possible explanation for this is that most of the proteins and LPS in the outer membrane of *P. aeruginosa* exist in a domain from which phospholipids are at least partially excluded.

In plasmolyzed cells of *P. aeruginosa*, considerable amounts of saccharides of 4,000 daltons or higher diffused into the periplasm (6). However, the determination of precise exclusion limit was difficult in this system, because of the possibility of outer membrane damage. We therefore turned to the reconstituted vesicle system, which was shown to reflect the exclusion limits of the intact outer membrane in *S. typhimurium* (19) and did not suffer from the "leakiness" caused presumably by the stretching of the outer membrane during plasmolysis of intact cells.

Using either the OM1 or OM2 fraction proteins, vesicles were reconstituted which were able to retain fully only those saccharides with molecular weights greater than 9,000 (Fig. 5). The exclusion limit was defined by a sloping line rather than the theoretically expected abrupt change from permeability at lower molecular weights to impermeability of higher-molecular-weight saccharides. One probable reason for this is that the saccharides were separated by molecular sieving, and the molecular weights defined by the column represent the average rather than the absolute molecular weights. Thus a fraction of, e.g., a nominal molecular weight of 6,000 would be expected to contain some molecules of larger size which would be unable to pass through the pores of reconstituted vesicles.

The exclusion limit of 3,000 to 9,000 daltons for *P. aeruginosa* is much larger than the figure of 550 to 650 daltons reported for both whole cells and reconstituted vesicles of *S. typhimurium* and *E. coli* (6, 19, 22; see also Fig. 5). This raises the question of the possible advantages of possessing larger pores through the outer membrane. *P. aeruginosa* secretes three proteases into the medium (12) and has been shown to possess membrane-bound peptidases (18). Thus the larger pores would permit entry of quite large peptides into the periplasmic space, rendering them susceptible to peptidases, whereas the extracellular proteases may be involved in the initial processing of proteins in the environment. If such a system exists, it would be possible for *P. aeruginosa* to use whole proteins or large polypeptides as carbon and nitrogen sources. Another possible function of such large pores might be to permit the entry of small micelles and hydrophobic solutes surrounded by a "cage" of water molecules, since *P. aeruginosa* is lipolytic and thus may be able to process lipids as an energy source.

There is at least one potential disadvantage to having such large pores. Inhibitory hydrophilic substances of larger molecular weight would have increased access to the periplasm. However, many of the common hydrophilic antibiotics are of about 350 to 500 molecular weight (see ref 24) and would be expected to diffuse through the outer membrane of not only *P. aeruginosa* but also the enteric bacteria. Consequently this would not result in a marked disadvantage for *P. aeruginosa*. *P. aeruginosa* is in fact more resistant to many antibiotics; the increased resistance probably derives from degrading enzymes (26) or from a lack of (or reduction in) inner membrane transport (36) of certain hydrophilic antibiotics. A further method of acquired resistance among natural isolates would involve an alteration in the outer membrane in those cases where the outer membrane constitutes one of the targets of action for the antibiotic (8).

As mentioned in the introduction, the two outer membrane separation methods (4, 35) previously reported were unsatisfactory (R. Hancock, unpublished data). One of these membrane preparations was performed in the presence of EDTA, which can result in the loss of a large portion of the outer membrane (Fig. 2). However, the outer membrane protein profile reported in this study (4) was comparable to ours, although fewer proteins were seen by these authors and we find no prominent bands corresponding to 53K and 56K.

While this paper was in preparation, one other report of the separation of *P. aeruginosa* outer and inner membranes appeared in the literature

(17). However, the very different polyacrylamide gel systems used make comparison difficult. We propose that only provisional molecular weight designations should be used for *P. aeruginosa* outer membrane proteins until functions can be ascribed to these proteins.

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LITERATURE CITED

1. **Ames, B. N., and D. T. Dubin.** 1960. The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. J. Biol. Chem. **235**:769–775.
2. **Ames, B. N., E. N. Spudich, and H. Nikaido.** 1974. Protein composition of the outer membrane of *Salmonella typhimurium*: effect of lipopolysaccharide mutations. J. Bacteriol. **117**:406–416.
3. **Bayer, M. E.** 1968. Adsorption of bacteriophages to adhesions between wall and membrane of *Escherichia coli*. J. Virol. **2**:346–356.
4. **Booth, B. R., and N. A. C. Curtis.** 1977. Separation of the cytoplasmic and outer membrane of *Pseudomonas aeruginosa* PAO.1. Biochem. Biophys. Res. Commun. **74**:1168–1176.
5. **Chester, I. R., G. W. Gray, and S. G. Wilkinson.** 1972. Further studies on the chemical composition of the lipopolysaccharide of *Pseudomonas aeruginosa*. Biochem. J. **126**:395–407.
6. **Decad, G. M., and H. Nikaido.** 1976. Outer membrane of gram-negative bacteria. XII. Molecular sieving function of cell wall. J. Bacteriol. **128**:325–336.
7. **Folch, J., M. Lees, and G. H. S. Stanley.** 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. **226**:497–509.
8. **Gilleland, H. E., and R. G. E. Murray.** 1976. Ultrastructural study of polymyxin-resistant isolates of *Pseudomonas aeruginosa*. J. Bacteriol. **125**:267–281.
9. **Gilleland, H. E., J. D. Stinnett, I. L. Roth, and R. G. Eagon.** 1973. Freeze etch study of *Pseudomonas aeruginosa*: localization within the cell wall of an ethylenediaminetetraacetate-extractable component. J. Bacteriol. **113**:417–432.
10. **Hancock, I. C., and P. M. Meadow.** 1969. The extractable lipids of *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **187**:366–379.
11. **Hasegawa, Y., H. Yamada, and S. Mizushima.** 1976. Interactions of outer membrane proteins O-8 and O-9 with peptidoglycan sacculus of *Escherichia coli* K-12. J. Biochem. **80**:1401–1409.
12. **Johnson, G. G., J. M. Morris, and R. S. Beck.** 1967. The extracellular proteases from *Pseudomonas aeruginosa* exhibit elastase activity. Can. J. Microbiol. **13**: 711–719.
13. **Johnson, K. G., and M. B. Perry.** 1976. Improved techniques for the preparation of bacterial lipopolysaccharides. Can. J. Microbiol. **22**:29–34.
14. **Jones, N. C., and M. J. Osborn.** 1977. Translocation of phospholipids between the outer and inner membranes of *Salmonella typhimurium*. J. Biol. Chem. **252**: 7405–7412.
15. **Kasahara, M., and Y. Anraku.** 1974. Succinate dehydrogenase of *Escherichia coli* membrane vesicles: activation and properties of the enzyme. J. Biochem. **76**:959–966.
16. **Lugtenberg, B., J. Meyers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the "major outer membrane protein" of *Escherichia coli* K-12 into four bands. FEBS Lett. **58**: 254–258.
17. **Matsushita, K., O. Adachi, E. Shinagawa, and M. Ameyama.** 1978. Isolation and characterization of outer and inner membranes from *Pseudomonas aeruginosa* and the effect of EDTA on the membranes. J. Biochem. **83**:171–181.
18. **Miller, R. V., and J. M. Becker.** 1978. Peptide utilization in *Pseudomonas aeruginosa*: evidence for membrane-associated peptidases. J. Bacteriol. **133**:165–171.
19. **Nakae, T.** 1975. Outer membrane of *Salmonella typhimurium*: reconstitution of sucrose-permeable membrane vesicles. Biochem. Biophys. Res. Commun. **64**:1224–1230.
20. **Nakae, T.** 1976. Outer membrane of *Salmonella typhimurium*: isolation of protein complex that produces transmembrane channels. J. Biol. Chem. **251**: 2176–2178.
21. **Nakae, T.** 1976. Identification of the outer membrane protein of *E. coli* that produces transmembrane channels in reconstituted vesicle membranes. Biochem. Biophys. Res. Commun. **71**:877–884.
22. **Nakae, T., and H. Nikaido.** 1975. Outer membrane as a diffusion barrier in *Salmonella typhimurium*: penetration of oligo- and polysaccharides into isolated outer membrane vesicles and cells with degraded peptidoglycan layer. J. Biol. Chem. **250**:7359–7365.
23. **Nakamura, K., and S. Mizushima.** 1976. Effects of heating in dodecyl sulfate solution on the conformation and electrophoretic mobility of isolated major outer membrane proteins from *Escherichia coli* K-12. J. Biochem. **80**:1411–1422.
24. **Nikaido, H.** 1976. Outer membrane of *Salmonella typhimurium*. Transmembrane diffusion of some hydrophobic substances. Biochim. Biophys. Acta **433**:118–132.
25. **Nixdorff, K., H. Fitzer, J. Gmeiner, and H. H. Martin.** 1977. Reconstitution of model membranes from phospholipid and outer membrane proteins of *Proteus mirabilis*. Role of proteins in the formation of hydrophilic pores and protection of membranes against detergents. Eur. J. Biochem. **81**:63–69.
26. **Nordström, K., and R. B. Sykes.** 1974. Effect of sublethal concentrations of benzylpenicillin on *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **6**:741–746.
27. **Osborn, M. J.** 1963. Studies on the gram negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. Proc. Nat. Acad. Sci. U.S.A. **50**:499–506.
28. **Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*: isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. **247**:3962–3972.
29. **Roberts, N. A., G. W. Gary, and S. G. Wilkinson.** 1970. The bactericidal action of ethylene diaminetetraacetic acid on *Pseudomonas aeruginosa*. Microbios **2**:189–208.
30. **Rogers, S. W., H. E. Gilleland, and R. G. Eagon.** 1969. Characterization of a protein-lipopolysaccharide complex released from cell walls of *Pseudomonas aeruginosa* by ethylenediaminetetraacetic acid. Can. J. Microbiol. **15**:743–748.
31. **Russell, R. R. B.** 1976. Two dimensional SDS-polyacrylamide gel electrophoresis of heat-modifiable outer membrane proteins. Can. J. Microbiol. **22**:83–91.
32. **Schacterle, G. R., and R. L. Pollack.** 1973. A simplified

method for the quantitative assay of small amounts of protein in biologic material. Anal. Biochem. **51**:654–655.
33. **Schnaitman, C. A.** 1970. Protein composition of the cell wall and cytoplasmic membrane of *Escherichia coli*. J. Bacteriol. **104**:890–901.
34. **Schnaitman, C. A.** 1973. Outer membrane protein of *Escherichia coli*. 1. Effect of preparative conditions on the migration of proteins in polyacrylamide gels. Arch. Biochem. Biophys. **157**:541–552.
35. **Stinnett, J. D., and R. G. Eagon.** 1973. Outer (cell wall) membrane proteins of *Pseudomonas aeruginosa*. Can. J. Microbiol. **21**:1834–1841.
36. **Tseng, J. T., and L. E. Bryan.** 1974. The effect of complement and other cell wall reagents on tetracycline and streptomycin resistance in *Pseudomonas aeruginosa*. Can. J. Microbiol. **20**:1101–1107.