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IN VITRO REASSEMBLY OF THE MEMBRANOUS VESICLE FROM *ESCHERICHIA COLI* OUTER MEMBRANE COMPONENTS

ROLE OF INDIVIDUAL COMPONENTS AND MAGNESIUM IONS IN REASSEMBLY

KENZO NAKAMURA and SHOJI MIZUSHIMA*

Department of Agricultural Chemistry, Nagoya University, Chikusa-ku, Nagoya, 464 (Japan)

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SUMMARY

A method was developed for the reassembly of membranous vesicle from the sodium deoxycholate-dissociated outer membrane components of *Escherichia coli*. The removal of the detergent by dialysis and the presence of Mg^{2+} were essential for the reassembly.

Membrane protein alone did not form any membranous structure. Closed membranous vesicles similar to the native outer membrane were reassembled only when protein was mixed with both lipopolysaccharide and phospholipid in deoxycholate solution and subsequently dialyzed. The membrane showed a distinct trilaminar structure with a center-to-center distance between two dark lines of 53 Å, which is a characteristic of the native outer membrane. This characteristic trilaminar structure was shown to be due to the presence of lipopolysaccharide. Phospholipd was required for the vesicularization of membrane. Lipopolysaccharide and/or phospholipid formed a membranous structure in the absence of protein, while the morphology of their negatively stained sample was quite different from that of the native outer membrane unless the outer membrane protein was added to the reassembly mixture.

The protein from the cytoplasmic membrane was unable to reform membranous vesicle with lipopolysaccharide and phospholipid, indicating that the reassembly system discriminated outer membrane proteins from cytoplasmic membrane proteins.

INTRODUCTION

The outer membrane, constituting the outermost layer of the envelope of *Escherichia coli* and other gram-negative bacteria, has several unique features in its structure and function compared to the cytoplasmic membrane. The outer and cytoplasmic membranes appear quite different when viewed in the electron microscope by

* To whom correspondence should be sent.

negative-staining [1, 2], thin-sectioning [1, 3–6] and freeze-etching [4, 7] techniques. The cytoplasmic membrane has many important functions, such as the synthesis of macromolecules, oxidative phosphorylation and active transport. On the other hand, the function of the outer membrane is still somewhat obscure. While the outer membrane has been suggested to act as a penetration barrier for certain substances such as antibiotics, dyes and macromolecules [8], the membrane appears to be highly permeable for small, hydrophilic molecules [9, 10]. The outer membrane is also unique in that it contains a large amount of lipopolysaccharides, and the protein composition of the outer membrane is different from that of the cytoplasmic membrane [2, 3, 11]. Furthermore, we have reported that a large portion of proteins in the outer membrane was β-structured polypeptides, while this conformation was found less, if at all, in the cytoplasmic membrane [12, 13].

In spite of these unique features, only a little information is available at present on the role of individual components in the structure and function of the outer membrane, and the mechanism of assembly of the outer membrane. The elucidation of the interactions between components of the outer membrane is expected to shed light on these problems. In the present study, the role of individual components of the outer membrane in the in vitro reassembly of membranous vesicles was examined.

MATERIALS AND METHODS

Chemicals

Sodium deoxycholate was obtained from Yoneyama Chemicals Co., Tokyo. [*carboxyl*-^{14}C]Deoxycholic acid with a specific activity of 47.3 mCi/mmol was obtained from ICN, Irvine, Calif. All other chemicals were obtained from the sources described previously [2].

Bacterial growth and membrane preparation

Escherichia coli YA21 used in the previous papers [2, 12, 13] was grown in the modified Fraser and Jerrel's medium which contained per liter: $Na_2HPO_4 \cdot 12H_2O$ 26.5 g; KH_2PO_4 4.5 g; NH_4Cl 1 g; casamino acids (Difco) 5 g; glucose 10 g; gelatin 10 mg; $MgSO_4 \cdot 7H_2O$ 0.3 g; $CaCl_2 \cdot 2H_2O$ 22 mg; $FeSO_4 \cdot 7H_2O$ 1 mg. For the labelling of phospholipids, [2-^{3}H]glycerol (30 μCi/11 μmol/liter) was added to the medium. Outer and cytoplasmic membranes were prepared as described previously [2]. The outer membrane I was used in the present study.

Preparation of lipopolysaccharide

Lipopolysaccharide was prepared from the strain based on the method of Yuasa et al. [14]. Cells harvested from 4 l of culture were washed twice, suspended in 55 ml of water and extracted twice with an equal volume of 90 % phenol at 65 °C for 5 min. The phenol phase was extracted again with hot water. Aqueous phases (approx. 120 ml) were combined, centrifuged at 12 000 $\times g$ for 10 min to remove non-extracted residue, and dialyzed for 16 h against 3 changes of 3 l each of water at 4 °C. Materials inside the dialysis bag were concentrated to 20 ml in vacuo, sedimented by centrifugation at 105 000 $\times g$ for 90 min, and treated with RNAase (0.4 mg RNAase in 16 ml of 25 mM Tris · HCl (pH 7.6)) at 55 °C for 2 h. Lipopolysaccharide was recovered and washed at least 3 times with water by centrifugation. The purified lipopolysaccharide

preparation which showed no absorption peak around 260 nm was stored in water at −80 °C.

Preparation of phospholipid

The extraction of phospholipid from cells was carried out as described previously [2]. The extract was washed with 0.2 vol of chloroform/methanol/water (3 : 48 : 47) containing 0.05 M KCl [15]. Phospholipid was stored in N_2 in a small volume of chloroform/methanol (2 : 1) at −80 °C.

Solubilization of outer membrane with sodium deoxycholate

The outer membrane preparation (15–20 mg protein/ml of water) stored at −80 °C was thawed and solubilized with 1 % (w/v) sodium deoxycholate in 10 mM Tris · HCl (pH 8.0 at 37 °C)/0.2 M NaCl/1 mM EDTA solution. The final protein concentration was 2 mg/ml. The mixture was incubated at 37 °C for 45 min and centrifuged in a Hitachi RP40 rotor at 105 000 × *g* for 1 h at 15–20 °C. The resultant supernatant solution was used as deoxycholate-solubilized outer membrane. The resultant precipitate was re-extracted twice with the deoxycholate/Tris/NaCl/EDTA solution. The final precipitate was suspended in the same solution and used as deoxycholate-insoluble protein residue. Deoxycholate-insoluble protein residue was stored at −80 °C prior to use.

Sephadex G-200 gel filtration of deoxycholate-solubilized membrane

The deoxycholate-solubilized outer membrane (30 ml) containing 40 mg of protein was concentrated to 5 ml in a Collodion bag (Sartorius, Göttingen, G.F.R.), and fractionated on a Sephadex G-200 column (2.2 × 85 cm) equilibrated with 0.25 % sodium deoxycholate/10 mM Tris · HCl (pH 8.0 at 20 °C)/0.2 M NaCl/1 mM EDTA/0.02 % NaN_3. A gel filtration was carried out with the same buffer at a flow rate of 6 ml per h at 20–25 °C. The protein fractions in eluates were concentrated in the collodion bag to a final concentration of 2 mg/ml, and stored at −80 °C.

Reassembly of membranes

For the reassembly of membranes from dissolved membrane preparations, a 2 ml aliquot of samples was successively dialyzed against 1 l each of following buffers; (1) 5 mM Tris · HCl (pH 7.9)/0.02 % NaN_3 for 7 h, (2) the same buffer for 10 h, (3) 5 mM $MgCl_2$/5 mM Tris · HCl (pH 7.9)/0.02 % NaN_3 for 7 h, (4) the same buffer as (1) for 7 h, (5) the same buffer for 10 h. All the dialysis were carried out at 20–25 °C in a cellophane tubing (Visking Co., Chicago; wall thickness 0.0008 inch; inflated diameter 5/8 inch). The dialysis without $MgCl_2$ in the dialysis buffer (3) will be referred as the dialysis in the absence of Mg^{2+}.

Reassembly of membranes from individual components of outer membrane

The reassembly of membranes from protein, lipopolysaccharide and phospholipid was carried out as follows: Phospholipid (2 mg) was dried in vacuo under N_2 and dissolved in 1 ml of 1 % sodium deoxycholate/10 mM Tris : HCl (pH 8.0)/0.2 M NaCl/1 mM EDTA solution at 37 °C for 45 min. Lipopolysaccharide was dissolved in the same solution. Both solutions were centrifuged at 105 000 × *g* for 1 h to remove a small amount of insoluble materials before use. Solutions were mixed with mem-

brane protein under the same conditions to give protein/lipopolysaccharide/phospholipid ratio of 4 : 2 : 1 in weight. The mixture was successively dialyzed in the presence of Mg^{2+}, as described above, unless otherwise indicated. After the dialysis, materials in the dialysis bag were sedimented by centrifugation at 105 000 $\times g$ for 45 min, and suspended in an aliquot of water.

Analytical methods

Protein content was determined by the method of Lowry et al. [16] with bovine serum albumin as a standard. The amount of lipopolysaccharide was estimated from 2-keto-3-deoxyoctonic acid content which was assayed with thiobarbituric acid as described by Osborn et al. [3]. The purified lipopolysaccharide was used as a standard. Organic phosphorus in phospholipid was assayed by the method of Bartlett [17], and the amount of phospholipid was calculated by assuming 25 μg of phospholipid per μg of lipid/phosphorus. The relative amount of phospholipid in membrane preparations was estimated from the amount of radioactive glycerol. Radioactivity measurements were carried out in 10 ml of Bray's scintillation fluid with Packard 3320 Tri-Carb scintillation spectrometer.

Electron microscopy

Negative staining was performed with 1 % sodium phosphotungstate (pH 6.2). For thin sectioning, samples were fixed with 1 % OsO_4 in phosphate buffer in the cold for 1 h [18], dehydrated through an ascending series of ethanol and embedded in Epon. Sections were cut on a Porter-Blum MT-1 ultramicrotome and stained with uranyl acetate and lead citrate [19]. Observations were made in a Hitachi HS-9 electron microscope.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate and urea was performed as described previously [2] with 0.1 % *N,N'*-methylenebisacrylanide. Densitometer tracings of stained gels were made with Joyce-Loebl Chromoscan.

RESULTS

Solubilization of outer membrane components with sodium deoxycholate

Fig. 1 shows the electron microscopy of native outer and cytoplasmic membranes. The outer membrane has several characteristic features in its morphology when compared with the cytoplasmic membrane, as reported previously [1]; (i) it appears as an empty closed vesicle with a rather homogeneous size (0.2 μm in diameter as average); (ii) it has a more clearly discernible trilaminar structure with a center-to-center distance between two dark lines of 53 Å (Fig. 1 (b) and Table II). The cytoplasmic membrane, in contrast, has rather obscured trilaminar structure with a center-to-center distance of 41 Å (Fig. 1(c) and Table II). Some outer membrane preparations were contaminated by fibrous structures (Fig. 1(a)), the appearance and size of which resembled those of pili.

The solubilization of outer membrane components with sodium deoxycholate was carried out in the presence of NaCl and EDTA. At the final concentration of 1 %, deoxycholate solubilized protein, lipopolysaccharide and phospholipid to 70, 90 and

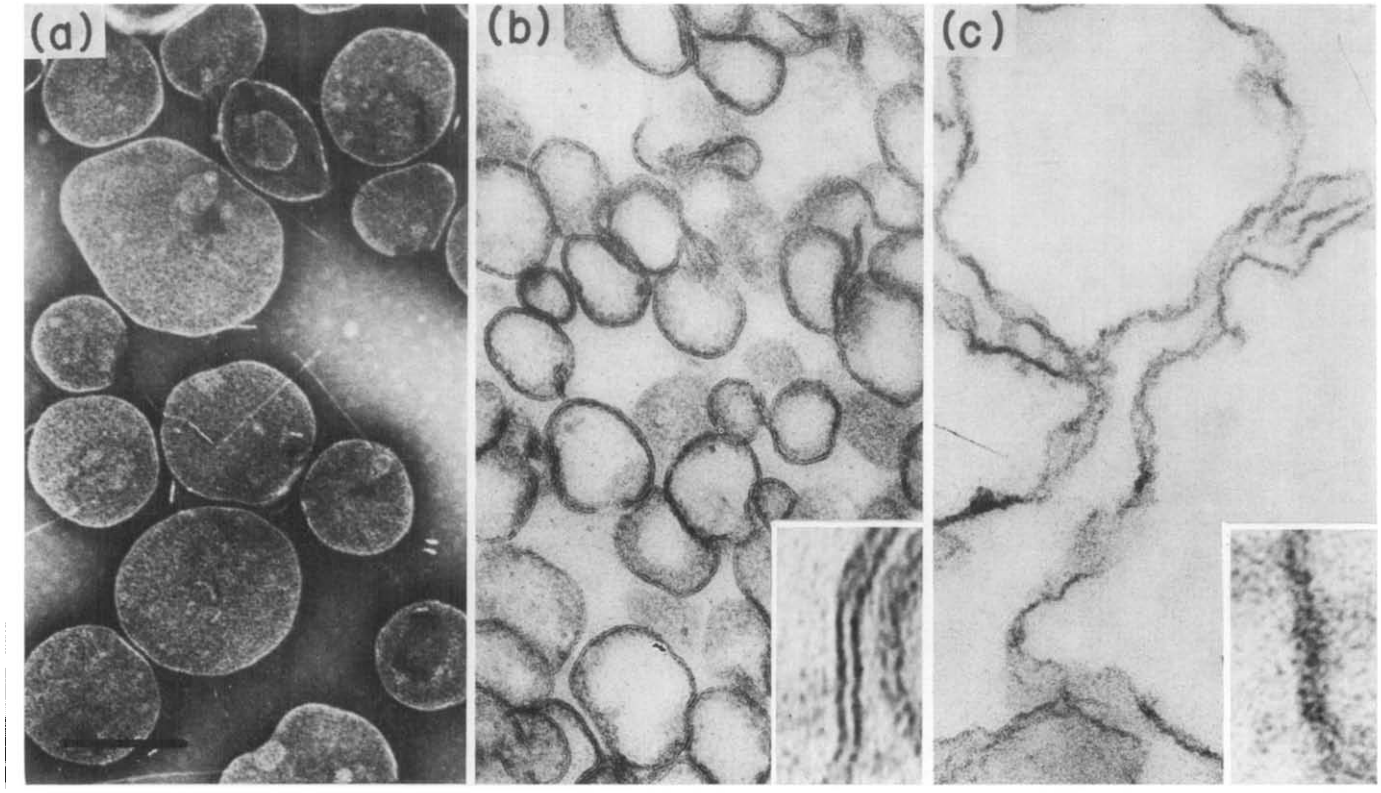


Fig. 1. Electron micrographs of native membranes. (a), Outer membrane, negative staining; (b), outer membrane, thin section; (c) cytoplasmic membrane, thin section. Scale marker indicates 0.2 μm ($\times$ 83 000). Magnification of inserts are $\times$ 393 000.

95 %, respectively. In the absence of NaCl and EDTA, the degree of solubilization was only 20 % for protein. The deoxycholate-insoluble protein residue, which contained only a small amount of lipopolysaccharide and phospholipid (Table I), did not show any membranous structure.

TABLE I

CHEMICAL COMPOSITION OF PREPARATIONS USED IN REASSEMBLY EXPERIMENTS

	Percent of total weight		
	Protein	Lipopolysaccharide	Phospholipid
Native outer membrane	60.6	25.4	13.9
Membrane protein fraction			
Deoxycholate-soluble	99.2	0.8	0
Deoxycholate-insoluble	92.4	4.7	2.9
Lipopolysaccharide	6.4	93.6	0
Phospholipid	6.7	0	93.3

Reassembly of deoxycholate-treated outer membrane

The reassembly of outer membrane-like structure by the removal of deoxycholate was first performed with the deoxycholate-treated total outer membrane or the deoxycholate-solubilized outer membrane. The extent of deoxycholate removal from the deoxycholate-treated total membrane was determined by the use of [^{14}C]deoxycholate. From the initial rate of deoxycholate decrease in the dialysis bag, the half-removal time in the absence of Mg^{2+} was estimated to be 150 min at 20 °C. When Mg^{2+} was present in the dialysis buffer, deoxycholate was not effectively removed out by dialysis, and the reassembly of membrane similar to the native outer membrane was unsuccessful. Therefore, in the reassembly experiments, Mg^{2+} was added to dialysis buffer when deoxycholate concentration inside the dialysis bag was reduced to about 0.01 % by the dialysis for 17 h. After dialysis for 41 h, 99.5 % of deoxycholate was removed from the dialysis bag.

Mg^{2+} was essential for the reassembly of the outer membrane-like structure (Fig. 2). When the deoxycholate-solubilized outer membrane was dialyzed in the absence of Mg^{2+}, the solution remained clear and no material was sedimented by centrifugation at 105 000 $\times g$ for 1 h. The negative staining electron microscopy revealed the presence of structures which appeared either as rods (60 $\times$ 260 Å) or spherules (360 Å in diameter). It was uncertain whether the spherules possess membranous structure or not. On the other hand, when the dialysis was carried out in the presence of Mg^{2+}, 85–95 % of protein, lipopolysaccharide and phospholipid became sedimentable by the centrifugation and the formation of large membranous structure was observed (Fig. 2(d)). The thin section of the precipitates showed the distinct trilaminar structure (Fig. 2(e)), which is a characteristic of the native outer membrane vesicle (Fig. 1(b)).

When the deoxycholate-treated total membrane was dialyzed in the absence of Mg^{2+}, membranous structures which was sedimentable by centrifugation appeared in addition to rods (Fig. 2(a)). However, these membranous structure contained only 30 % of the total protein and the appearance was quite different from the native outer

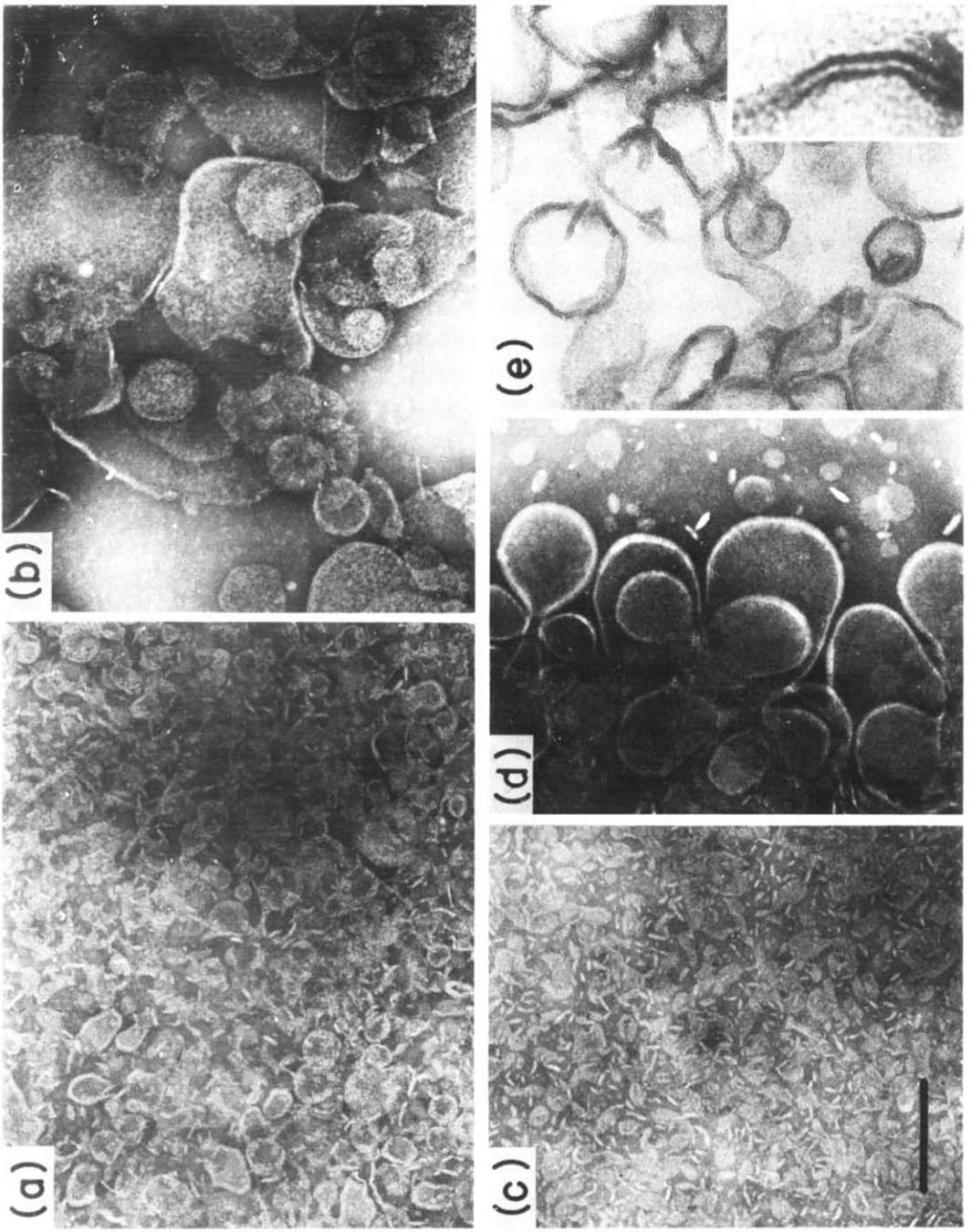


Fig. 2. Effect of Mg^{2+} on reassembly of deoxycholate-treated outer membrane. Deoxycholate-treated total membrane dialyzed in the absence (a) or presence (b) of Mg^{2+}, negative staining. Deoxycholate-solubilized outer membrane dialyzed in the absence (c) or presence (d) of Mg^{2+}, negative staining. (e), same as (d), thin section. Scale marker indicates 0.2 μm ($\times$ 83 000). Magnification of insert is $\times$ 393 000.

membrane vesicle. On the other hand, when deoxycholate-treated total membrane was dialyzed in the presence of Mg^{2+}, almost all of protein, lipopolysaccharide and phospholipid were reassembled into the outer membrane-like structure (Fig. 2(b)).

Preparation of membrane proteins devoid of lipopolysaccharide and phospholipid

In order to examine the role of individual components of the outer membrane in the in vitro reassembly of membranous vesicle, we have prepared both deoxycholate-soluble and deoxycholate-insoluble membrane proteins devoid of lipopolysaccharide and phospholipid. Because the outer membrane preparation contained a considerable amount of lysozyme, it was also desired to prepare membrane proteins free from this enzyme.

Deoxycholate-soluble membrane protein was freed from lipopolysaccharide and phospholipid by gel filtration in the presence of deoxycholate. Fig. 3 shows the gel filtration pattern of the deoxycholate-solubilized outer membrane through a Sephadex G-200 column. Phospholipid was eluted near the full volume of the column in accord with results reported by several other workers on other membranes [20, 21]. Lipopolysaccharide was eluted slightly before phospholipid, consistent with the findings by Ribi et al. [22] and Hannecart-Pokorni et al. [23, 24] that deoxycholate dissociates lipopolysaccharide into the subunit of about 1 S. In Fig. 3, protein was eluted into three peaks. The first two peaks (Fraction A) did not contain any detectable amount of lipopolysaccharide and phospholipid. They were combined and used as the deoxycholate-soluble protein.

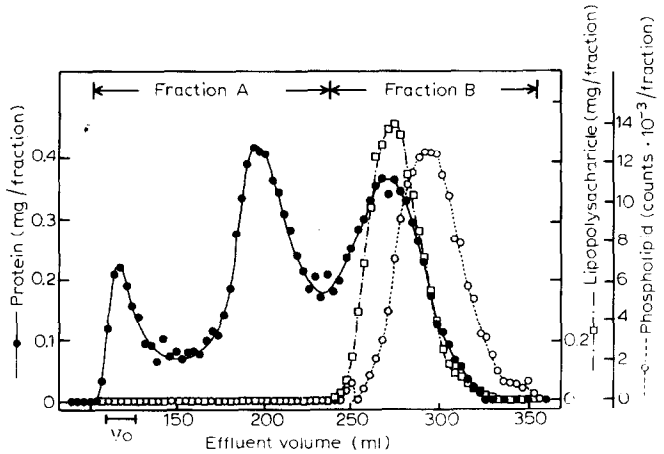


Fig. 3. Sephadex G-200 gel filtration of deoxycholate-solubilized outer membrane. Deoxycholate-solubilized outer membrane (25 mg protein) was applied to column as described in Materials and Methods, and 3.4 ml fractions were collected. Fraction A was used as deoxycholate-soluble protein.

The deoxycholate-insoluble protein residue was also used as a protein source. Although the complete removal of lipopolysaccharide and phospholipid was not achieved by the successive extraction with deoxycholate, the final deoxycholate-insoluble protein residue contained only a small amount of these components as shown in Table I, and showed any membranous structure as mentioned above. Table I also shows the chemical composition of other preparations used in reassembly experiments.

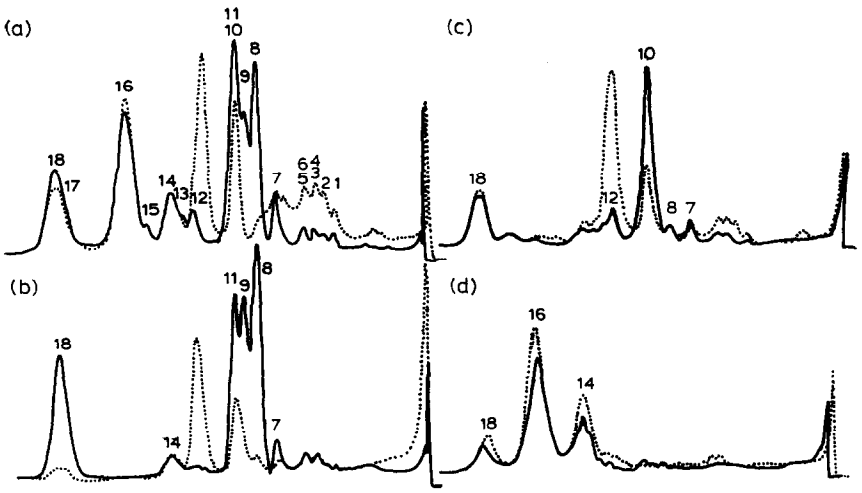


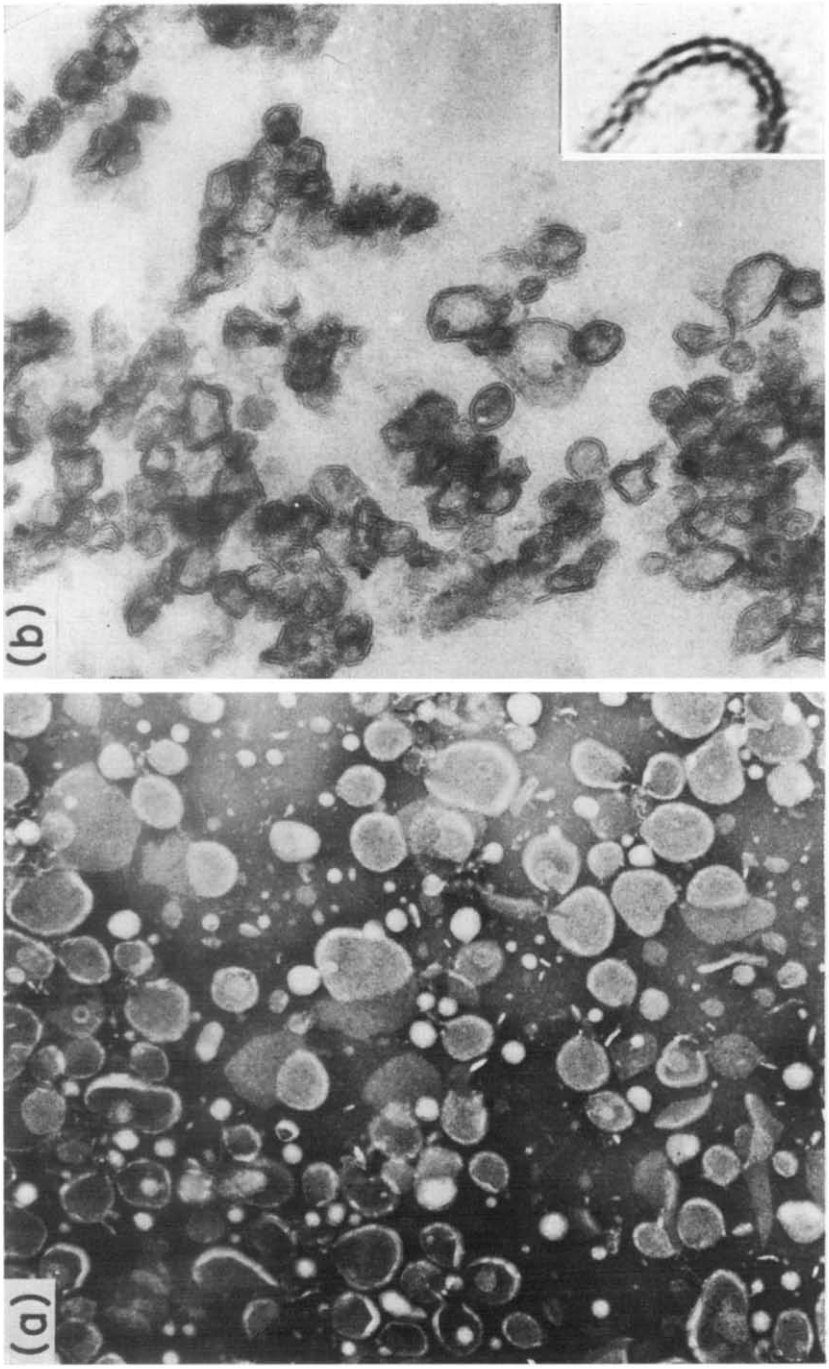
Fig. 4. Scans of sodium dodecyl sulfate-urea polyacrylamide gel electrophoresis of outer membrane fractions. (a), Native outer membrane; (b), deoxycholate-insoluble protein residue; (c), deoxycholate-soluble proteins, Fraction A in Fig. 3; (d), fraction B in Fig. 3. —, samples preheated at 100 °C for 5 min in sodium dodecyl sulfate solution, - - - -, samples preheated at 37 °C for 30 min in sodium dodecyl sulfate solution. Tops of gels are to the right.

Fig. 4 shows the protein composition of these preparations analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and urea [2]. The characterization of proteins in the individual bands will be published elsewhere (Uemura and Mizushima, unpublished). In the present paper, the nomenclature and characterization of individual protein bands by Uemura and Mizushima were referred to. The outer membrane preparation used in this study contained O-2, O-3 and O-9 in a reduced amount (Fig. 4(a)) compared to those used in previous studies [2, 13]. It is known that the addition of glucose and $FeSO_4 \cdot 7H_2O$ in the growth medium resulted in the decrease in the amount of O-9 (Uemura and Mizushima, unpublished) and O-2 and O-3 (Ichihara and Mizushima, unpublished), respectively.

The degree of solubilization with deoxycholate was different in individual bands. Lysozyme (O-16) was completely solubilized and fractionated as a major protein of Fraction B (Fig. 4(d)), making the deoxycholate-insoluble protein residue and the deoxycholate-soluble protein free from the enzyme. Two heat-modifiable major bands (O-8 and O-9) remained almost completely insoluble. O-10 seemed to be more easily solubilized than O-11. Although O-10 and O-11 locate at almost the same position on a gel, these proteins can be distinguished from each other by the difference in their color after staining (Uemura and Mizushima, unpublished). Braun's lipoprotein (O-18) [25] was found in both the deoxycholate-soluble protein and the deoxycholate-insoluble protein residue.

Reassembly of membranous vesicle from molecular components of outer membrane

The reassembly of membrane from molecular components of the outer membrane was achieved by mixing them in deoxycholate solution followed by dialysis in the presence of Mg^{2+}. Mixing of protein, lipopolysaccharide and phospholipid in the


(b)
(a)

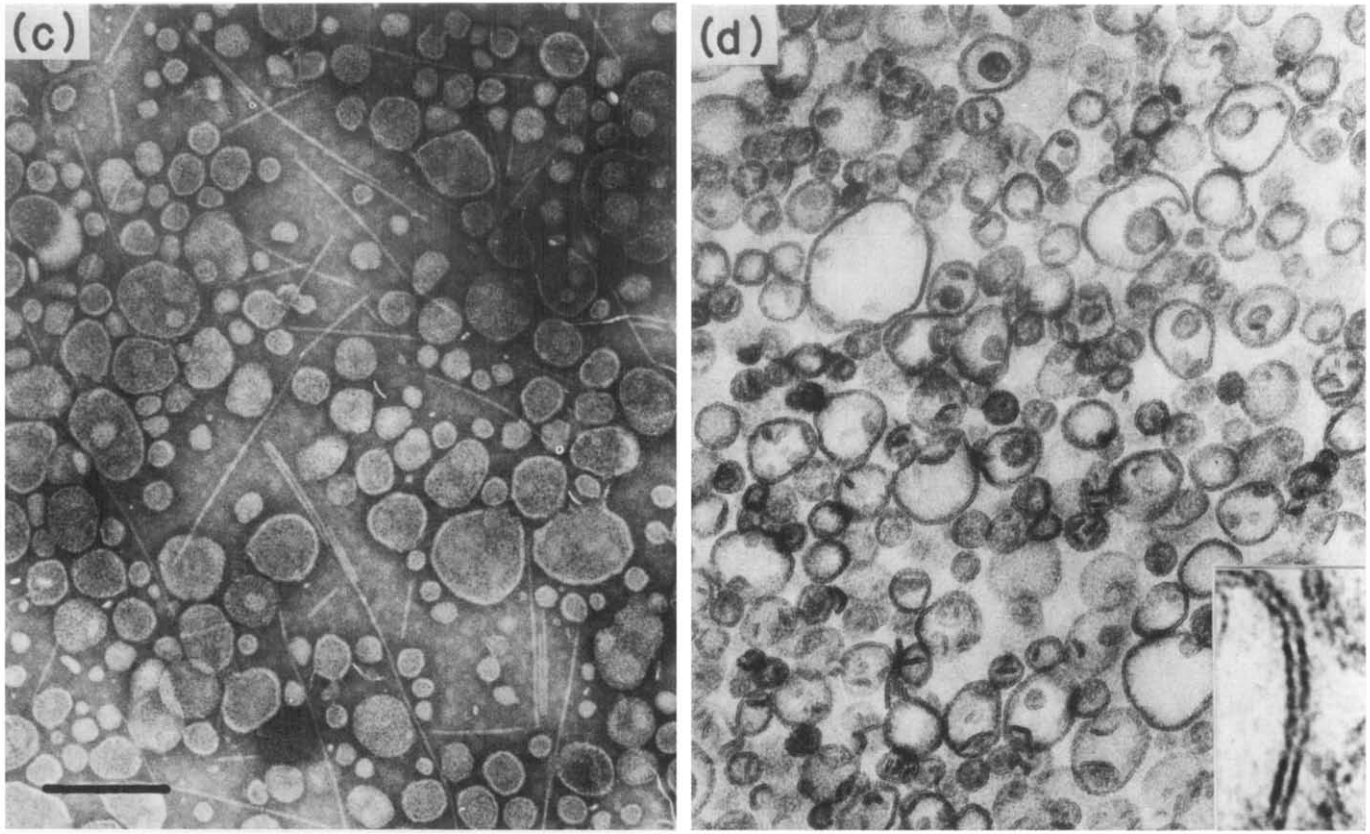


Fig. 5. Electron micrographs of membranous vesicles reassembled with outer membrane protein, lipopolysaccharide and phospholipid. Membranous vesicles were reassembled from either deoxycholate-soluble ((a) and (b)) or deoxycholate-insoluble ((c) and (d)) membrane proteins. (a) and (c), negative staining; (b) and (d), thin section. Scale marker indicates 0.2 μm ($\times$ 83 000). Magnification of inserts are $\times$ 393 000.

weight ratio of 4 : 2 : 1 was always employed. The ratio is those in the native outer membrane.

The reassembly of membranous vesicles from the deoxycholate-soluble protein or the deoxycholate-insoluble protein residue occurred when both lipopolysaccharide and phospholipid were present (Fig. 5). When freshly prepared protein preparations were used, the reassembly of membranous vesicle was quite reproducible. However, unsuccessful results were sometimes obtained with a deoxycholate-soluble protein that had formed some precipitate after storage for a long period at −80 °C. With the deoxycholate-insoluble protein residue, the reassembly was successful even after the prolonged storage of the protein preparation at −80 °C.

Usually larger vesicles were formed with the deoxycholate-insoluble protein residue (360–1600 Å in diameter) (Figs 5(c) and (d)) than with the deoxycholate-soluble proteins (360–1000 Å in diameter) (Figs 5(a) and (b)). With the deoxycholate-insoluble protein residue, most of the reassembled membranes appeared as a single layered vesicle with a distinct trilaminar structure quite similar to that of the native outer membrane vesicle (Fig. 5(d) and Table II). On the other hand, with the deoxycholate-soluble proteins, some of the reassembled membrane incompletely vesicularized and formed amorphous aggregate. The trilaminar structure of these membranes, however, was also quite similar to that of the native outer membrane vesicle (Fig. 5(b) and Table II).

TABLE II

THICKNESS OF TRILAMINAR STRUCTURES OF NATIVE AND REASSEMBLED MEMBRANES

Electron micrographs of thin section enlarged to × 405 000 were used for the determination of thickness. Values are mean of independent determinations, and ranges are indicated in parenthesis.

Membrane	Center-to-center distance* (Å)	Overall width (Å)	Number of determination	Reference Fig. No.
Native membranes				
Outer membrane	53 (50–57)	90 (82–100)	38	1 (b)
Cytoplasmic membrane	42 (38–45)	75 (68– 83)	8	1 (c)
Membranes reassembled with deoxycholate-soluble protein				
+Lipopolysaccharide +phospholipid	52 (49–57)	84 (75– 93)	30	5 (b)
+Lipopolysaccharide	52 (48–58)	78 (68– 91)	24	7 (c)
+Phospholipid	41 (37–45)	70 (60– 84)	27	7 (e)
Membranes reassembled with deoxycholate-insoluble protein				
+Lipopolysaccharide +phospholipid	51 (45–57)	81 (72– 95)	26	5 (d)
+Lipopolysaccharide	49 (45–55)	78 (68– 90)	37	6 (d)
+Phospholipid	41 (35–45)	67 (62– 75)	15	6 (f)
Membranous lipopolysaccharide	51 (45–58)	76 (70– 90)	38	8 (c)

* Center-to-center distance between two dark lines.

Fibrous structures observed in the outer membrane preparation (Fig. 1(a)) remained insoluble in deoxycholate solution and recovered in membranous vesicle reassembled in the presence of deoxycholate-insoluble protein residue (Fig. 5(c)).

Role of lipopolysaccharide

The reassembly with proteins alone failed to form membranous structure (Figs 6(a), (b) and 7(a)). Although the reassembly with the deoxycholate-insoluble protein residue alone seemed to result in some rearrangement of the protein residue, the appearance was quite different from membranous one (Figs 6(a) and (b)).

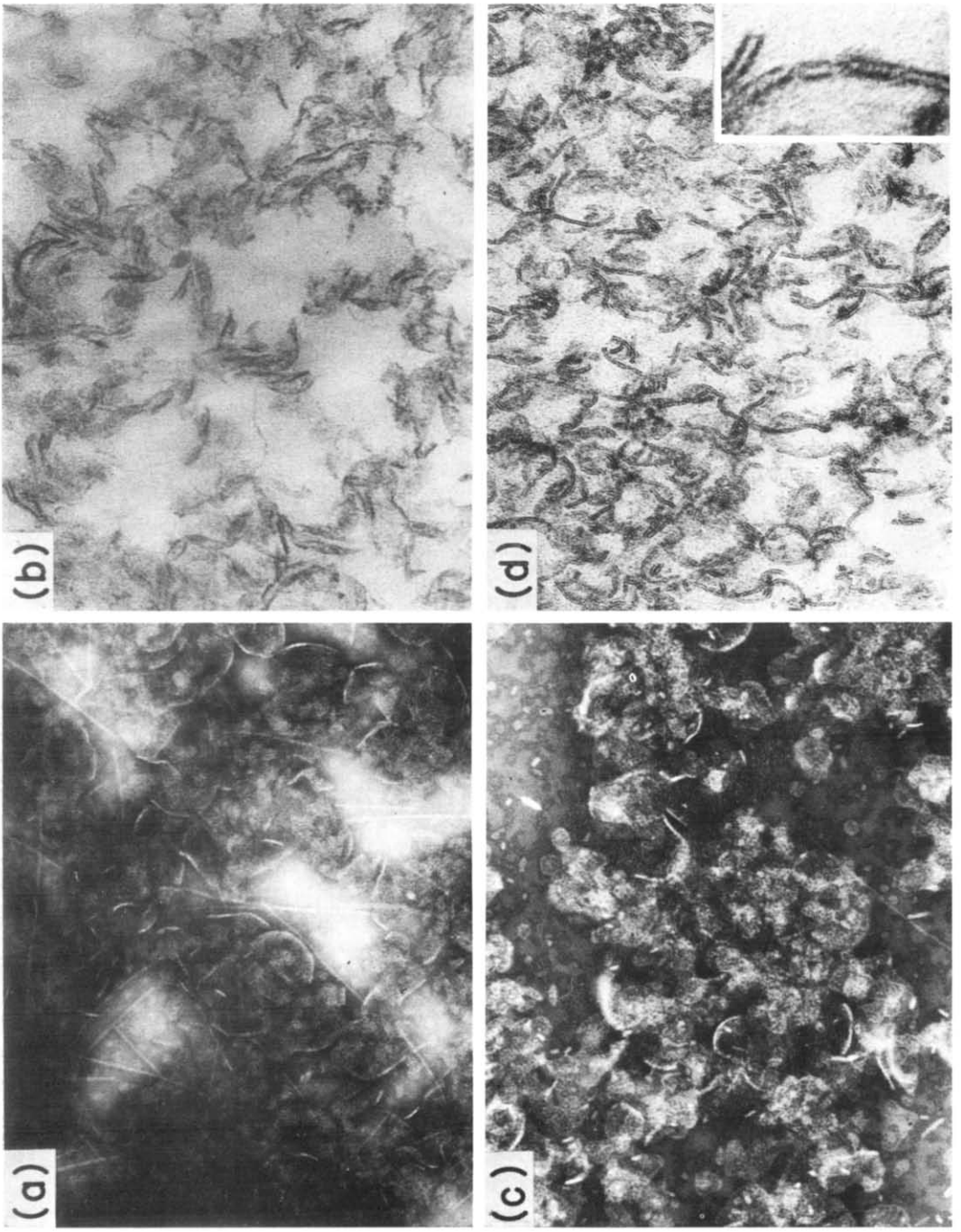
The distinct trilaminar structure similar to that of the native outer membrane vesicle was reformed from either one of these protein preparations when lipopolysaccharide was added to the reassembly mixture (Figs 6(d) and 7(c)). In contrast, when lipopolysaccharide was replaced by phospholipid, the trilaminar structure formed were rather obscure (Figs 6(f) and 7(e)), resembling that of the cytoplasmic membrane (Figs 1(c)).

Figs 8(a) and (b) shows electron microscopy of lipopolysaccharide treated in the reassembly system in the absence and presence of Mg^{2+}. In the absence of Mg^{2+}, long filamentous structure with a width of 120–180 Å were observed (Fig. 8(a)). These filaments had random branchings and resembled to a degree the morphology of lipopolysaccharide extracted from smooth strain of *Salmonella typhimurium* reported by Shands et al. [26]. In contrast, the presence of Mg^{2+} resulted in the formation of membranous structure (Fig. 8(b)). They seemed to be sheet-like rather than vesicular. This membranous lipopolysaccharide showed a distinct trilaminar structure (Fig. 8(c)).

In order to compare the thickness of the trilaminar structures of the native and reassembled membranes, we have measured both the center-to-center distance and overall width of two dark lines. The results are summarized in Table II. The center-to-center distance between two dark lines of the native outer membrane was 53 Å, and was distinctively wider than that of the cytoplasmic membrane (42 Å). The characteristic 53 Å center-to-center distance was observed in the reassembled membranes whenever lipopolysaccharide was present. On the other hand, the membranes reassembled with protein and phospholipid in the absence of lipopolysaccharide showed the center-to-center distance of 41 Å, resembling that of the cytoplasmic membrane. It was concluded from these results that the distinct trilaminar structure with a center-to-center distance of 53 Å observed in osmium-fixed and sectioned outer membrane was due to the presence of lipopolysaccharide.

Role of phospholipid

Although the reassembly of all components of the outer membrane resulted in the formation of vesicular structure, reassembly with protein and lipopolysaccharide in the absence of phospholipid did not result in the formation of vesicular structure. They were either rods, spherule-like (Fig. 7(b)) or amorphous (Fig. 6(c)) under negative staining, while the distinct trilaminar structure was observed in sectioned preparations (Figs 6(d) and 7(c)). In contrast to these, the reassembly with the deoxycholate-insoluble protein residue and phospholipd in the absence of lipopolysaccharide resulted in the formation of membranous vesicle (Figs 6(e) and (f)). Although dimension of these vesicles were similar to those obtained by reassembly


(a)
(b)
(c)
(d)

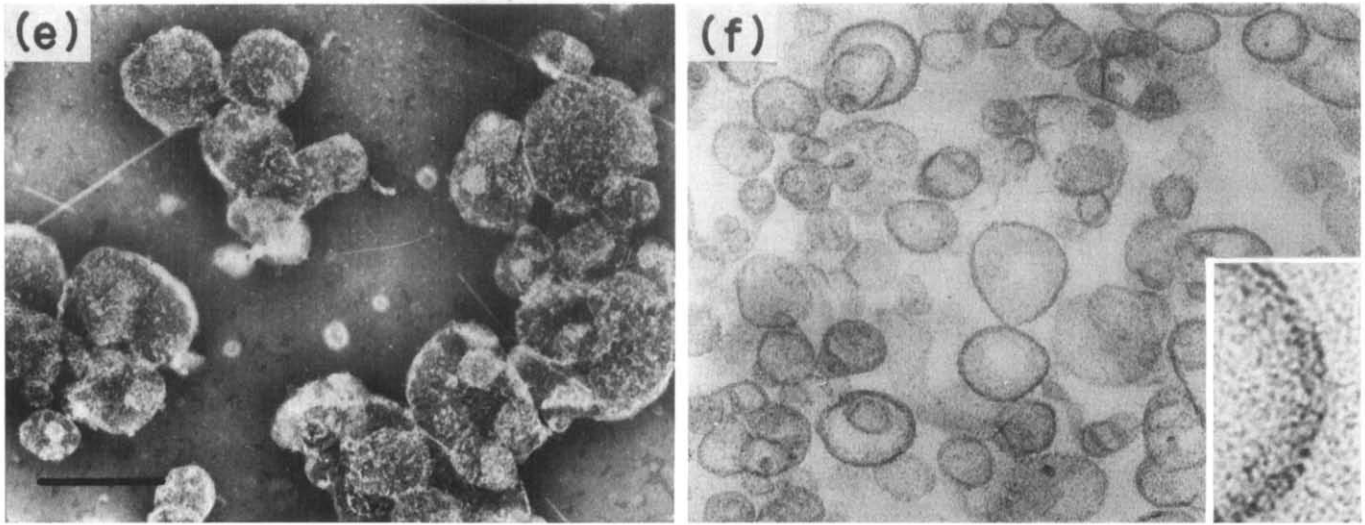
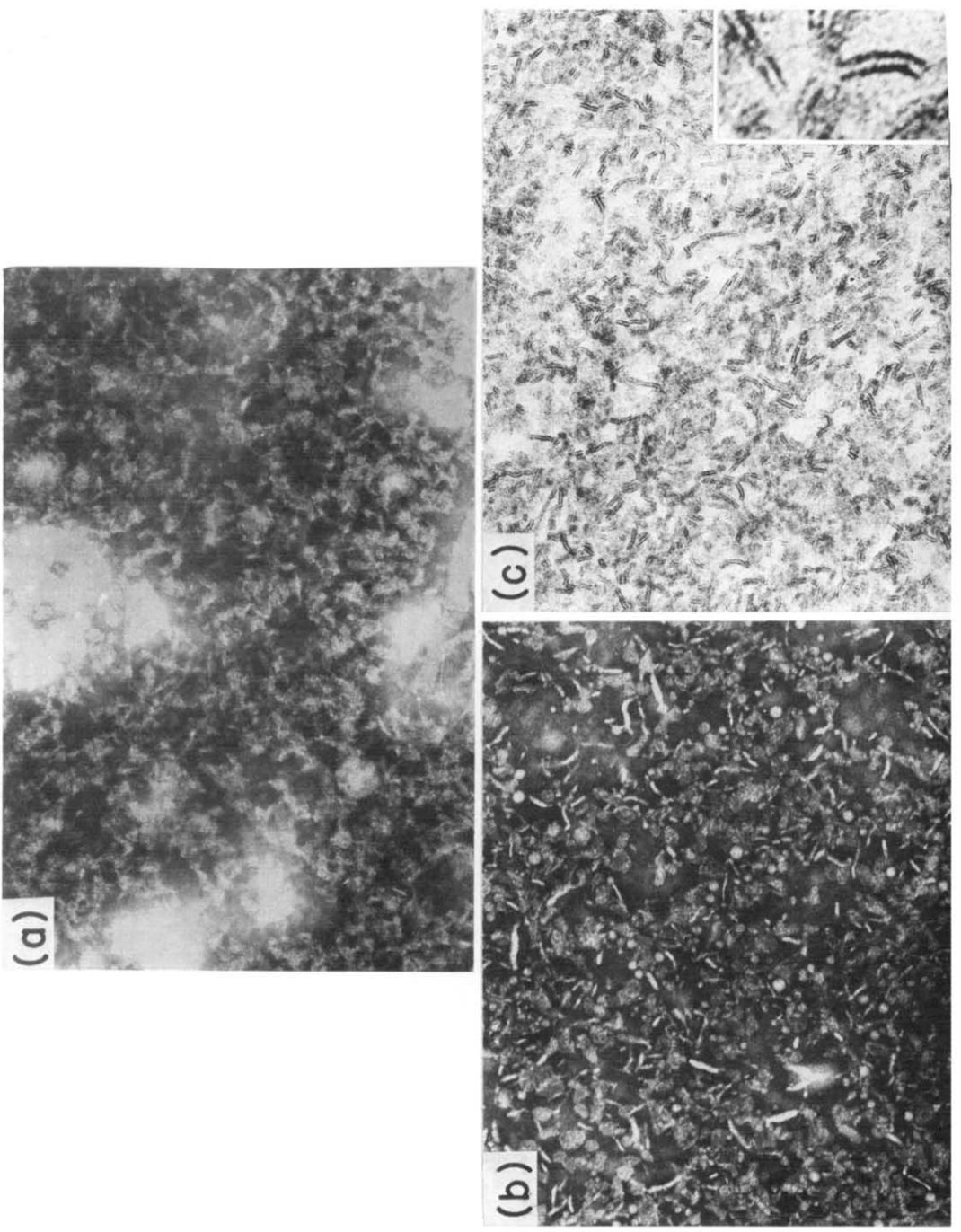


Fig. 6. Reassembly with deoxycholate-insoluble protein residue in the absence and presence of lipopolysaccharide or phospholipid. (a) Protein alone, negative staining; (b), same as (a), thin section; (c), protein plus lipopolysaccharide (2 : 1), negative staining; (d), same as (c), thin section; (e), protein plus phospholipid (4 : 1), negative staining; (f), same as (e), thin section. Scale marker indicates 0.2 μm ($\times$ 83 000). Magnification of inserts are $\times$ 393 000.


(a)
(b)
(c)

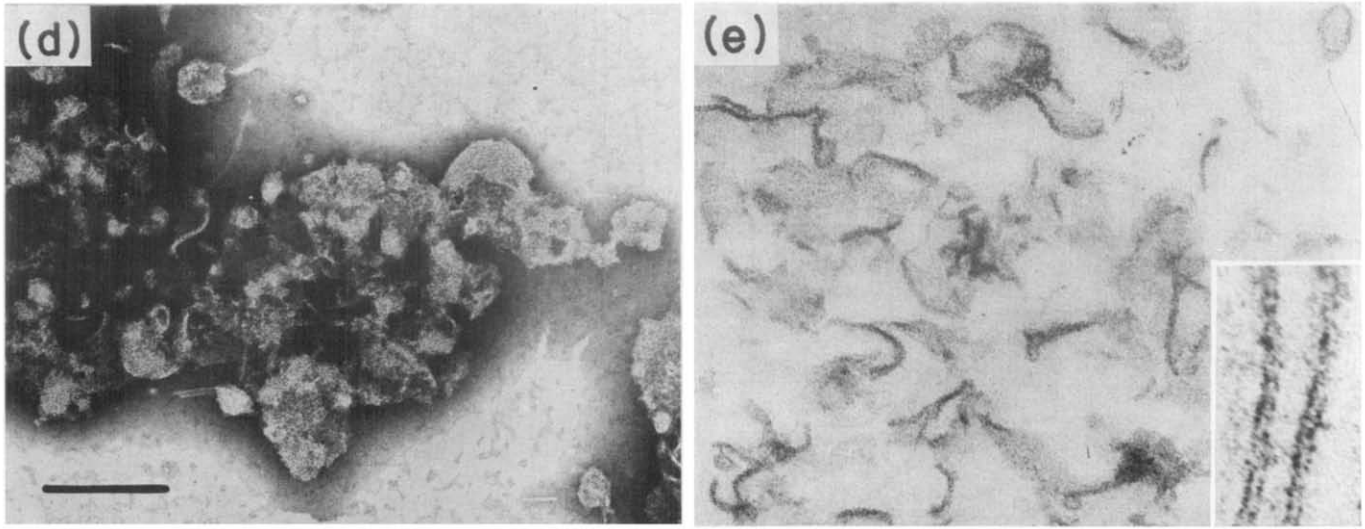
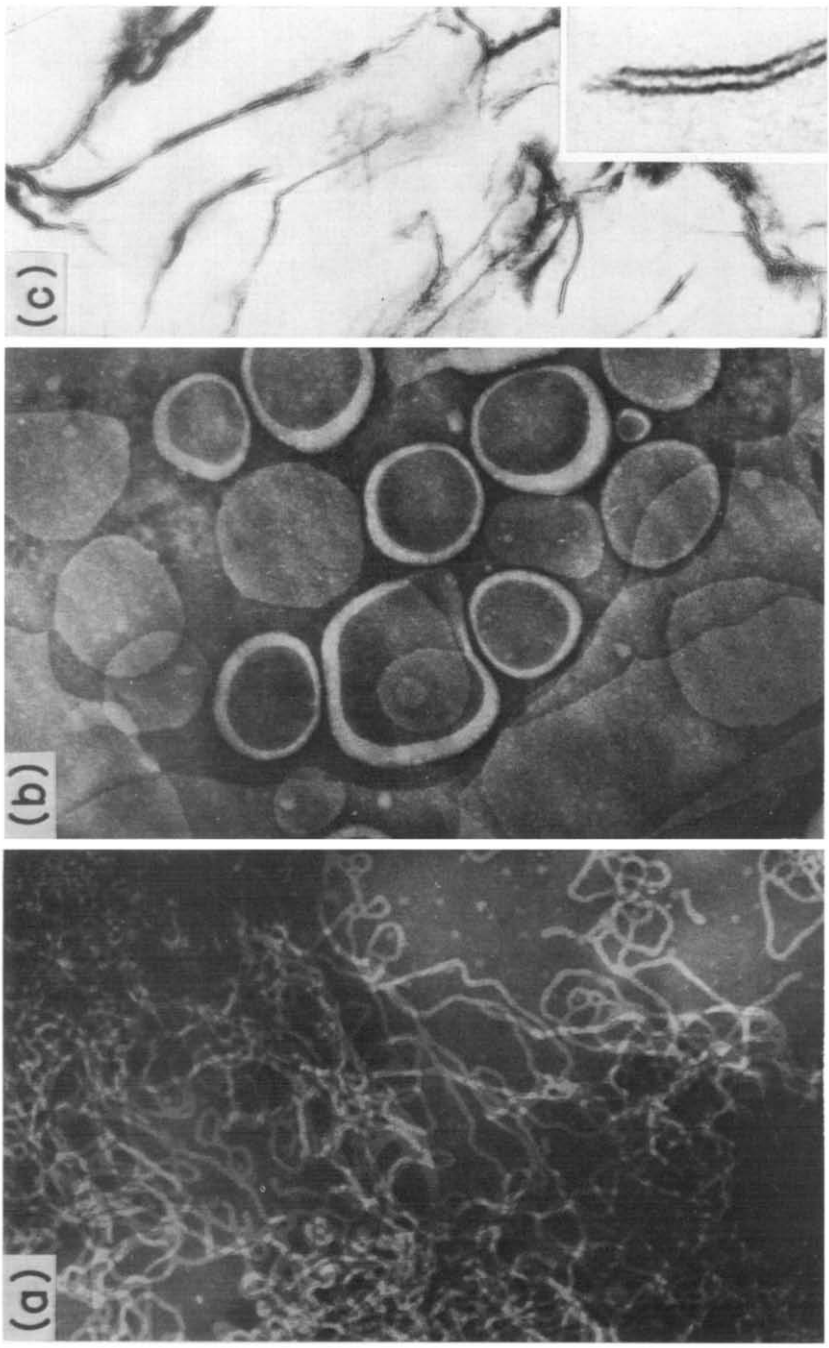


Fig. 7. Reassembly with deoxycholate-soluble protein in the absence and presence of lipopolysaccharide or phospholipid. (a), Protein alone, negative staining; (b) protein plus lipopolysaccharide (2 : 1), negative staining; (c), same as (b), thin section; (d), protein plus phospholipid (4 : 1), negative staining; (e), same as (d), thin section. Scale marker indicates 0.2 μm ($\times$ 83 000). Magnification of inserts are $\times$ 393 000.


(c)
(b)
(a)

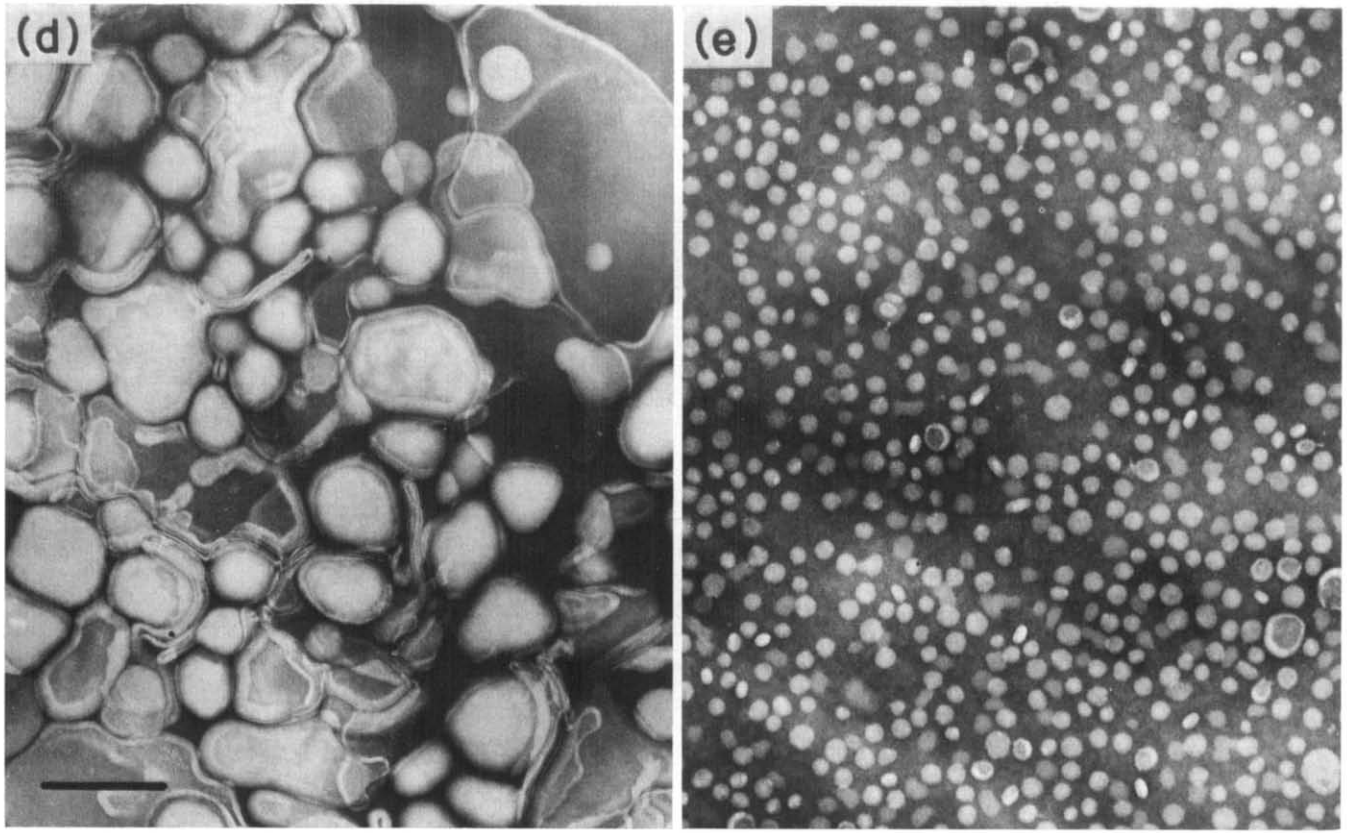


Fig. 8. Reassembly with lipopolysaccharide and/or phospholipid in the absence of membrane proteins. (a), Lipopolysaccharide after dialysis in the absence of Mg^{2+}, negative staining; (b), lipopolysaccharide, negative staining; (c), same as (b), thin section; (d), phospholipid, negative staining; (e), lipopolysaccharide and phospholipid (2 : 1), negative staining. (b), (c), (d) and (e), dialyzed in the presence of Mg^{2+}. Scale marker indicates 0.2 μm ($\times$ 83 000). Magnification of insert is $\times$ 393 000.

with all components, their trilaminar structure was quite different, as mentioned above. The formation of vesicles with deoxycholate-soluble protein and phospholipid had not so far been achieved. The membrane appeared as fragments with an obscure trilaminar structure (Figs 7(d) and (e)).

The morphology of phospholipids in the reassembly system was also studied. It showed characteristic features of multilamellar liposomes with a diameter of 500–2500 Å (Fig. 8(d)). The reassembly with lipopolysaccharide and phospholipid resulted in the formation of various structures depending on the ratio of two components. Fig. 8(e) shows a typical morphology of the assembled structure at the lipopolysaccharide-phospholipid ratio of 2 : 1. They appeared as electron opaque particles with a diameter of 250–700 Å. It was not clear whether the particle had a membranous structure or not.

Finally, it should be noticed that the appearances of the reformed structure with lipopolysaccharide and/or phospholipid were quite different from those of the native outer membrane vesicle or the reassembled membrane from protein, lipopolysaccharide and phospholipid.

Reassembly with cytoplasmic membrane proteins

The specificity of outer membrane proteins in the in vitro reassembly was examined by replacing them with those from the cytoplasmic membrane. Deoxycholate-soluble protein and deoxycholate-insoluble protein residue was prepared from the cytoplasmic membrane by the same procedure used for the outer membrane. Deoxycholate-solubilized protein and phospholipid to 80 and 95 %, respectively. Polyacrylamide gel electrophoresis revealed that the deoxycholate-solubilized protein fraction was essentially free from outer membrane proteins, while the deoxycholate-insoluble protein residue was contaminated by a small amount of heat-modifiable outer membrane proteins.

The reassembly of the deoxycholate-soluble or deoxycholate-insoluble proteins with lipopolysaccharide and phospholipid resulted in the formation of structures quite different from the native outer membrane vesicle and the reassembled membrane with the outer membrane proteins. They appeared as membranous aggregates without any vesicularization. Thin sectioning of the reassembled membrane revealed that they had wide and distinct trilaminar structure, which is characteristic of the membranes containing lipopolysaccharide (data not shown).

DISCUSSION

In the present study, sodium deoxycholate was used to dissolve the outer membrane, since the detergent extensively dissociates lipopolysaccharides into its subunits [22–24]. Its nondenaturating effect on protein [27] and the relative ease of its removal by dialysis [28] also favoured the use of this detergent.

Almost all of lipopolysaccharide and phospholipid, and about 70 % of protein in the outer membrane were solubilized with sodium deoxycholate in the presence of NaCl and EDTA. Gel filtration analysis indicated that these solubilized components dissociated each other (Fig. 3). The remaining insoluble protein was not solubilized by the successive extraction with deoxycholate. Although some differences were found in protein compositions between deoxycholate-soluble and deoxycholate-insoluble pro-

teins (Fig. 4), they were able to be reassembled into membranous vesicle with lipopolysaccharide and phospholipid (Fig. 5).

Reassembly of outer membrane-like vesicles from the deoxycholate-dissociated outer membrane required Mg^{2+} (Fig. 2). DePamphilis [29] also reported the Mg^{2+}-dependent reassembly of membranes from the Triton X-100-EDTA-dissociated outer membrane of *E. coli*. Several lines of evidence have suggested that divalent cation is important for maintaining the structure of lipopolysaccharide: (1) Leive et al. [30] showed that the treatment of *E. coli* cells with EDTA resulted in the release of half of the cellular lipopolysaccharide. (2) Purified lipopolysaccharide was dissociated into smaller subunits upon the treatment with EDTA [29, 31]. (3) The dissociated subunits were reassociated with one another in the presence of Ca^{2+} or Mg^{2+} [29, 31]. (4) In the present study, lipopolysaccharide was reassembled into a filamentous structure in the absence of Mg^{2+}, while it was reassembled into a membranous structure in the presence of Mg^{2+} (Figs 8(a) and (b)). (5) Furthermore, the filamentous lipopolysaccharide was converted to the membranous one by the addition of Mg^{2+} (data not shown).

Reassembly experiments in the present study clearly showed that the distinct and wide trilaminar structure, which is characteristic of the native outer membrane, was due to the presence of lipopolysaccharide as summarized in Table II. Even when lipopolysaccharide alone was reassembled into a membranous structure in the presence of Mg^{2+}, a distinct trilaminar structure quite similar to that of the outer membrane vesicle was observed (Fig. 8(c) and Table II). A similar trilaminar structure was also observed by other authors in osmium-fixed and sectioned preparation of lipopolysaccharides extracted from *Proteus vulgaris* [32] and from *E. coli* B [33]. The fact that both native and reassembled outer membrane vesicles showed quite a similar trilaminar structure to the membranous lipopolysaccharide suggests that lipopolysaccharide molecules are arranged as a bimolecular leaflet in native and reassembled outer membranes. It has been argued from X-ray analyses of lipopolysaccharide [32] and the monolayer and electron microscopic analyses on the interaction of lipopolysaccharide with phospholipid [34], that lipopolysaccharide molecules are arranged as a bimolecular leaflet.

It should be noticed that the distinct trilaminar structure of the native outer membrane vesicle and the membranes reassembled with lipopolysaccharide appeared symmetrical (Figs 1(b), 5(b), (d), 6(d) and 7(c)). On the other hand, the trilaminar structure of the outer membrane in the intact cells appeared rather asymmetrical so as did the outer line stained more densely (Yamada, Nakamura and Mizushima, unpublished), supporting the result obtained by Silva and Sousa [5]. If lipopolysaccharide are responsible for the distinct dark layer in the trilaminar structure as mentioned above, the observation should indicate that lipopolysaccharide locates in the outer face of outer membrane in the intact cells and both outer and inner faces in isolated and reassembled outer membranes. Recently, Mühlradt and Golecki [35] used ferritin-coupled antibody against lipopolysaccharide and showed that the lipopolysaccharide which was originally located on the outer face of outer membrane in the intact cells was reoriented so as to occur on both faces when murein was removed by lysozyme. The localization of lipopolysaccharide in the reassembled membrane must be studied in connection with that in the intact cells.

The reassembly with protein and lipopolysaccharide resulted in the formation

of a trilaminar structure (Figs 6(d) and 7(c)). However, the vesicularization of membrane was observed only when phospholipid was included in the reassembly mixture (Figs 5(b) and (d)). The result indicates that phospholipid is important for keeping the membrane fluid. Spin-labelling studies of Rottem et al. [36] showed that the motion of saturated acylchains of lipopolysaccharide was highly restricted in the delipidized outer membrane of *Proteus mirabilis*. The phospholipid content of the outer membrane is considerably low as compared with that of the cytoplasmic membrane [3, 4]. This may be a reason why the outer membrane was shown to be less fluid than the cytoplasmic membrane [36].

A membrane structure was able to be formed from lipopolysaccharide and/or phospholipid, while the morphology of its negatively stained sample was quite different from that of the native outer membrane vesicle (Figs 8(b), (d) and (e)). On the other hand, the addition of either deoxycholate-soluble or deoxycholate-insoluble proteins to lipopolysaccharide and phospholipid resulted in the reassembly of a membranous vesicle similar to the native outer membrane vesicle (Fig. 5). Although differences were observed in the morphology of reassembled vesicles with them, it has not been clear whether the difference in morphology is caused by the difference in the protein composition or by the difference in the state of proteins (soluble or insoluble).

A significant difference was observed in reassembled membranes, when proteins from the outer membrane were replaced by those from the cytoplasmic membrane. We have previously shown that proteins in the outer membrane are quite different from those in the cytoplasmic membrane not only in their composition but also in their conformation [12, 13]. These results are of great interest when the specificity of membrane assembly is considered. Since there was no qualitative difference in the phospholipid composition between outer and cytoplasmic membranes [2, 3, 36], lipopolysaccharide would play an essential role in the discrimination of proteins of the outer membrane form those of the cytoplasmic membrane in the present reassembly system.

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REFERENCES

1 Miura, T. and Mizushima, S. (1969) Biochim. Biophys. Acta 193, 268–276
2 Mizushima, S. and Yamada, H. (1975) Biochim. Biophys. Acta 375, 44–53
3 Osborn, M. J., Gander, J. E., Parisi, E. and Carson, J. (1972) J. Biol. Chem. 247, 3962–3972
4 Hasin, M., Rottem, S. and Razin, S. (1975) Biochim. Biophys. Acta 375, 381–394
5 Silva, M. T. and Sousa, J. C. F. (1973) J. Bacteriol. 113, 953–962
6 DePetris, S., Karlsbad, G. and Kessel, R. W. I. (1964) J. Gen. Microbiol. 35, 373–382
7 Forge, A., Costerton, J. W. and Kerr, K. A. (1973) J. Bacteriol. 113, 445–451
9 Costerton, J. W., Ingram, J. M. and Cheng, K.-J. (1974) Bacteriol. Rev. 38, 87–110
8 Inouye, M. (1974) Proc. Nat. Acad. Sci. U.S. 71, 2396–2400
10 Decad, G., Nakae, T. and Nikaido, H. (1974) Fed. Proc. 33, 1240
11 Schnaitman, C. A. (1970) J. Bacteriol. 104, 890–901

12 Nakamura, K., Ostrovsky, D. J., Miyazawa, T. and Mizushima, S. (1974) Biochim. Biophys. Acta 332, 329–335
13 Mizushima, S. (1974) Biochem. Biophys. Res. Commun. 61, 1221–1226
14 Yuasa, R., Levinthal, M. and Nikaido, H. (1969) J. Bacteriol. 100, 433–444
15 Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) J. Biol. Chem. 226, 497–509
16 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
17 Bartlett, G. R. (1959) J. Biol. Chem. 234, 466–468
18 Millonig, G. (1962) Fifth International Congress for Electron Microscopy 2, P-8, Academic Press, New York
19 Reynolds, E. S. (1963) J. Cell. Biol. 17, 208–211
20 Ne'eman, Z., Kahane, I., Kovartovsky, J. and Razin, S. (1972) Biochim. Biophys. Acta 266, 255–268
21 Helenius, A. and Simons, K. (1972) J. Biol. Chem. 247, 3656–3661
22 Ribi, E., Anacker, R. L., Brown, R., Haskins, W. T., Malmgren, B., Milner, K. C. and Rudbach, J. A. (1966) J. Bacteriol. 92, 1493–1509
23 Hannecart-Pokorni, E., Dekegel, D., Depuydt, F. and Dirkx, J. (1970) Biochim. Biophys. Acta 201, 155–166
24 Hannecart-Pokorni, E., Dekegel, D. and Depuydt, F. (1973) Eur. J. Biochem. 38, 6–13
25 Braun, V. and Bosch, V. (1972) Proc. Natl. Acad. Sci. U.S. 69, 970–974
26 Shands, Jr, J. W., Graham, J. A. and Nath, K. (1967) J. Mol. Biol. 25, 15–21
27 Makino, S., Reynolds, J. A. and Tanford, C. (1973) J. Biol. Chem. 248, 4926–4932
28 Kagawa, Y. (1974) in Methods in Membrane Biology (Korn, E. D., ed.), Vol. 1, pp. 201–269, Plenum Press, New York
29 DePamphilis, M. L. (1971) J. Bacteriol. 105, 1184–1199
30 Leive, L., Shovlin, V. K. and Mergenhagen, S. E. (1968) J. Biol. Chem. 243, 6384–6391
31 Olins, A. L. and Warner, R. C. (1967) J. Biol. Chem. 242, 4994–5001
32 Burge, R. E. and Draper, J. C. (1967) J. Mol. Biol. 28, 205–210
33 Freer, J. H. and Salton, M. R. J. (1971) in Microbial Toxins (Weinbaum, G., Kadis, S. and Ajl, S. J., eds), Vol. 4, pp. 67–126
34 Rothfield, L. and Horne, R. W. (1967) J. Bacteriol. 93, 1705–1721
35 Mühlradt, P. F. and Golecki, J. R. (1975) Eur. J. Biochem. 51, 343–352
36 Rottem, S., Hasin, M. and Razin, S. (1975) Biochim. Biophys. Acta 375, 395–405