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ISOLATION, SOLUBILIZATION AND REAGGREGATION OF OUTER MEMBRANE OF *ESCHERICHIA COLI*

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SUMMARY

Cytoplasmic (inner) and outer membranes of *Escherichia coli* K-12 were isolated with fair separation from each other, and their chemical, biological and morphological properties were compared. The outer membrane isolated was composed of protein, phospholipid and lipopolysaccharide as major high molecular weight components in a ratio of 100:82:34 (by wt), and was solubilized in 1% sodium dodecyl sulfate without any sediments. In polyacrylamide disc gel electrophoresis with the sodium dodecyl sulfate-solubilized outer membrane, six proteins were found to be major. Removal of sodium dodecyl sulfate from the sodium dodecyl sulfate-solubilized outer membrane by dialysis induced a self-assembly to form a membrane structure which has similar properties in chemical composition, density and morphology to those of the original outer membrane.

INTRODUCTION

Gram-negative bacteria, such as *Escherichia coli*, have a multi-layered structure in their cell envelope[1,2]. Under the electron microscope, two kinds of membrane having a so-called unit membrane structure (dark–light–dark) can be observed and are designated as inner (cytoplasmic) and outer membranes. The physiological properties of bacterial envelope have been investigated in relation to DNA replication, cell growth, cell division, transport, energy metabolism, actions of bacteriocin and phage, and so on. However, the relationship between structure and function is scarcely known. Our interest lies in the study of the biogenesis of the surface layer of bacterial cells. In this paper, we describe the characteristics of both inner and outer membranes, and report on the solubilization and reaggregation of the outer membrane.

MATERIALS AND METHODS

Organism and cultivation

Escherichia coli K-12 Ya-2 (F^-, *met*$^-$, λ^-) was cultured in a Fraser–Jerrel medium[3] at 37 °C with shaking.

Preparation of inner (cytoplasmic) and outer membranes

Preparation of inner and outer membranes was conducted by employing the method developed by Miura and Mizushima[4] in this laboratory. This method includes the spheroplast-inducing process described below. Cells in the log phase of growth (approx. 1.5 g wet wt of cells per l medium) were collected by centrifugation, washed once and suspended in deionized water. The cell suspension, equivalent to 10 mg dry cells/ml, and all reagents (described below) were cooled in ice–water prior to use. Additions were performed in the following order: to the cell suspension (53.0 ml) in a 300-ml Erlenmeyer flask were added 30.0 ml of 10^{-1} M Tris–HCl buffer (pH 7.0), 27.0 ml of 2 M sucrose, 5.0 ml of 1% EDTA (pH 7.0), and 5.0 ml of lysozyme (Eizai, Tokyo) (EC 3.2.1.17) solution (5.0 mg/ml). The flask was then transferred from the ice bath to a hot water bath to warm the contents rapidly to 30 °C; incubation for 1 h at 30 °C was then carried out. During the incubation, almost all cells were converted to spheroplasts with release of outer membrane from the cells into the medium. The mixture was cooled to 4 °C and centrifuged at $18000 \times g$ for 10 min. The supernatant fraction contained outer membrane, periplasmic materials and a small amount of cytoplasmic materials, the pellet fraction contained spheroplasts. The supernatant fraction was centrifuged at $87000 \times g$ for 1 h to isolate the outer membrane fraction as pellet, and the spheroplast fraction was shocked in 300 ml of cold $5 \cdot 10^{-3}$ M $MgCl_2$ solution containing 100 μg of deoxyribonuclease (Sigma) and homogenized for the induction of complete burst. The resultant homogenate was centrifuged at $25000 \times g$ for 20 min to isolate the inner membrane fraction as a pellet. After washing with 300 ml of $5 \cdot 10^{-3}$ M $MgCl_2$ solution, both crude inner and outer membrane fractions were separately suspended in 5 ml of 1% EDTA solution (pH 7.0) and dialyzed overnight against 250 ml of the same EDTA solution at 4 °C. The membranes thus obtained were designated as crude inner and outer membranes. Purified membranes were prepared as described below.

The crude membrane was centrifuged at $25000 \times g$ for 20 min, and the pellets obtained were resuspended in distilled water and centrifuged at $105000 \times g$ for 1 h. The collected pellets were suspended in 0.8 ml of 10% sucrose solution (approx. 20 mg protein/ml), and charged onto a 4.7-ml 35–50% linear sucrose gradient and centrifuged at $130000 \times g$ for 4 h in a swinging rotor Hitachi Model 55-P centrifuge. After the centrifugation, the outer and inner membranes were predominantly situated at the heavy and light positions, respectively. Membrane fraction was isolated by dropwise sampling from the bottom of the centrifuge tube which was punctured with a pin. Membrane fraction was occasionally taken from the tube by using a J-shaped pipette. The membranes thus isolated were designated as purified outer and inner membranes in this paper.

Chemical analysis

Protein was determined according to the method of Lowry *et al.*[5]. Lipid phosphorus was estimated as orthophosphate in the $HClO_4$ hydrolyzate of a chloroform–methanol (2:1, v/v) extract. Orthophosphate was determined by the method of Allen[6]. For calculation of the amount of phospholipid the factor lipid phosphorus (lipid-P) $\times$ 25.5 was used[7]. 2-Keto-3-deoxyoctonate, a marker for lipopolysaccharide, was determined by the method of Weissbach and Hurwitz[8]. In this paper, it was ascertained that the amount of lipopolysaccharide which was extracted from the cells

with phenol according to the method of Osborn *et al.*[9] corresponds to 2.5% of dry cells, and the amount of ketodeoxyoctonate corresponds to 4.7% of lipopolysaccharide. After removal of total lipid by extraction with chloroform–methanol (2:1, v/v), the defatted membrane was hydrolyzed with 6 M HCl (previously purified by evaporation) at 110 °C for 48 h in a sealed tube, and then amino acids in the hydrolyzate were routinely analyzed by the Shibata-Kagaku, Model A-600, amino acid analyzer.

Assay for enzyme activity

The activity of magnesium-activated ATP phosphohydrolase (Mg-ATPase) (EC 3.6.1.3) was determined in the presence of $5 \cdot 10^{-3}$ M $MgCl_2$. The activity of succinate dehydrogenase (EC 1.3.99.1) was determined from the reduction rate of 2,6-dichloroindophenol as an electron acceptor. Details are given in the footnote to Table I.

Electron microscopic observation

The membrane preparation was fixed with 1% osmic acid (adjusted to pH 6.2 with veronal buffer) according to the method of Kellenberger *et al.*[10]. After dehydration, the membrane preparation was embedded in methacrylate[4]. A thin section was then prepared using a Porterblum ultra-microtome. The section was stained with uranylacetate[11], and observed using a JEM JE-7A electron microscope. Negative staining was performed with 1% phosphotungstate (pH 7.0).

Polyacrylamide disc gel electrophoresis

The behavior of membrane proteins in polyacrylamide disc gel electrophoresis was examined by two entirely different methods. (1) The method of Takayama *et al.*[12]: the membrane preparation was solubilized with a system of phenol–acetic acid–urea (2:1:1, by wt), then electrophoresis was performed using 7.5% polyacrylamide gel and 10% acetic acid as electrode buffer. (2) The method of Weber and Osborn[13]: the membrane preparation was solubilized with a solvent system consisting of 1% sodium dodecyl sulfate, 10^{-1} M sodium phosphate buffer (pH 7.0) and 0.1% mercaptoethanol. Electrophoresis was performed using 5% polyacrylamide gel and, as electrode buffer, 10^{-1} M sodium phosphate buffer (pH 7.0), containing 0.1% sodium dodecyl sulfate.

After electrophoresis, the gels were stained with 0.25% Coomassie brilliant blue and destained with an aqueous solvent containing 15% methanol and 7.5% acetic acid. Protein bands stained were scanned densitometrically by using a Joyce-Loebel Chromoscan densitometer.

Labeling of membrane lipid with [^3H]glycerol

Membrane was prepared in which the lipid was exclusively labeled with tritium by performing the following cultivation. Cells were cultured in the Fraser–Jerrel medium except that 2% glucose containing 10 μg/ml of uniformly labeled [^{3}H]glycerol ($7 \cdot 10^7$ cpm/mmole) was used as carbon source instead of 3% glycerol. After cultivation, approx. 61% of the radioactivity found to be incorporated into log-phase cells were in the membrane fraction (sum of the crude inner and outer membranes) and distribution of the radioactivity in the membrane fraction was found to be 95%

in lipid and 1.2% in lipopolysaccharide. The purified inner and outer membranes showed the same value of $3.36 \cdot 10^4$ cpm/μmole lipid-P for the specific radioactivity per phosphorus in lipid. Therefore, radioactivity was employed as a marker for phospholipid in membranes.

Solubilization of outer membrane with sodium dodecyl sulfate

10% sodium dodecyl sulfate was added to a suspension of the purified outer membrane (5 to 7 mg protein per ml) to give a sodium dodecyl sulfate concentration of 1%, and incubated at 50 °C for 1 h, after which the suspension became entirely clear.

Formation of a new structure from the sodium dodecyl sulfate-solubilized outer membrane

Reaggregation of a membranous structure from the sodium dodecyl sulfate-solubilized outer membrane was performed by removal of sodium dodecyl sulfate as described below. The sodium dodecyl sulfate-solubilized outer membrane was placed in a Visking cellulose tube and dialyzed against 1000 vol. of $5 \cdot 10^{-3}$ mM $MgCl_2$ solution at room temperature for 12 h. At this temperature, crystallization of sodium dodecyl sulfate can be avoided. The dialysis was repeated 4 or more times. Then, successive dialysis was carried out at 4 °C against 50 vol. of 1% EDTA (pH 7.0) solution for 18 h and 1000 vol. of deionized water for 24 h. During the dialysis, the content in the dialysis tube changed from clear to turbid. The precipitate isolated by centrifugation at $100000 \times g$ for 1.5 h was designated as the reaggregated outer membrane in this paper, because the precipitate had a similar structure to that of the original outer membrane (see Results).

RESULTS

Properties of inner (cytoplasmic) and outer membranes

(*i*) *Isolation of inner and outer membranes, and their morphology.* The distribution profile of the crude membranes from the sucrose density gradient centrifugation (see the section Materials and Methods) is presented in Fig. 1. Protein amount and succinate dehydrogenase activity were used as markers for detection of both inner and outer membranes and for inner membrane only, respectively. Fig. 1 indicates that both inner and outer crude membranes consist of one major peak with some contaminant. Therefore, the fractions indicated as CM and OM in the figure were collected as the purified inner and outer membranes by the method described previously.

Morphological properties of the purified membranes were examined by electron microscopic observation. Micrographs of the membranes in thin section (Fig. 2) show the inner membranes as large vesicles of heterogeneous size and shape, and the outer membranes as small vesicles of homogeneous size and shape. In Fig. 3, micrographs of the negatively stained membranes are presented. The inner membrane is an amorphous large vesicle with a ragged edge, and the outer membrane is a homogeneous small vesicle with a rounded edge.

(*ii*) *Chemical and biochemical properties.* The chemical and biochemical characteristics of the purified membranes are summarized in Table I. The chemical composition showed, both inner and outer membranes to consist of mainly three high

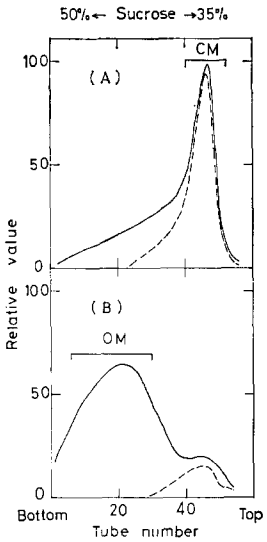

Fig. 1. Distribution profile of crude inner and outer membranes in sucrose density gradient centrifugation. (A), inner membrane; (B), outer membrane. ———, protein; – – –, succinate dehydrogenase activity. After the centrifugation, fractions designated as CM and OM in the figure were isolated as purified inner (cytoplasmic) and outer membranes, respectively. Each tube, 3 drops (approximately 0.12 ml).

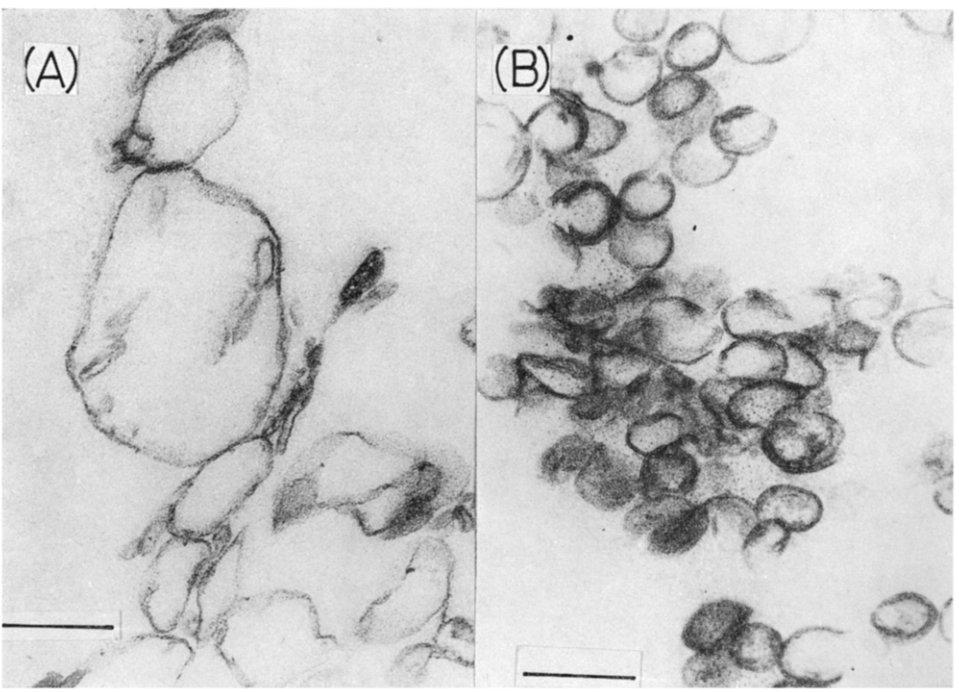

Fig. 2. Micrographs of thin sections of the purified inner and outer membranes. (A), inner membrane; (B), outer membrane. Bar, 0.5 μm.

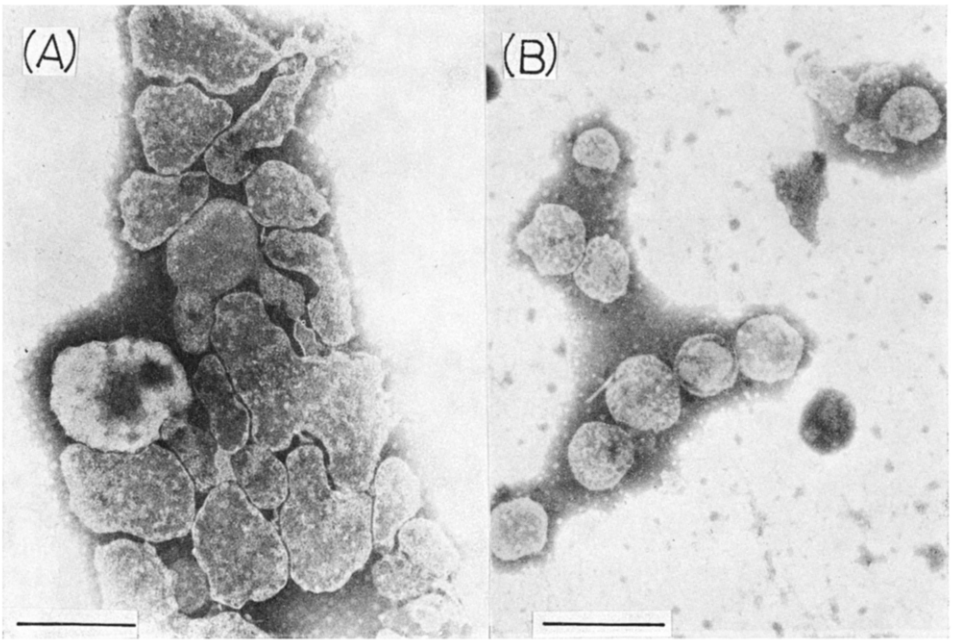

Fig. 3. Micrographs of negatively stained preparations of the purified inner and outer membranes. (A), inner membrane; (B), outer membrane. Bar, 0.5 μm.

TABLE I

CHEMICAL AND BIOCHEMICAL PROPERTIES OF PURIFIED INNER AND OUTER MEMBRANES

Membranes were prepared as shown in Fig. 1.

	Inner membrane (*mg/g dry wt*)	*Outer membrane* (*mg/g dry wt*)
Protein	470	398
Phospholipid	404	328
Lipopolysaccharide	53	136
	nmoles/min per mg protein	*nmoles/min per mg protein*
Mg-activated ATPase*	222	0.0
Succinate dehydrogenase**	106	0.7>

* The reaction mixture contained 40 mM Tris–HCl buffer (pH 7.2), 1 mM $MgCl_2$, 2.5 mM ATP and appropriate amount of enzyme solution. Reaction was at 35 °C. Orthophosphate (P_i) formed was determined.

** The reaction mixture contained 50 mM phosphate buffer (pH 7.6), 1.5 mM cyanide, 20 mM succinate and 0.05 mM dichloroindophenol. Reaction was at 20 °C. Decrease in rate of the absorbance at 600 nm was spectrophotometrically determined. Enzyme activity is given as nmoles P_i formed or 2,6-dichloroindophenol reduced per min per mg protein.

TABLE II

AMINO ACID COMPOSITION OF INNER AND OUTER MEMBRANES

	Inner membrane (*mole %*)		*Outer membrane* (*mole %*)		*Structural protein*	
					*Neurospora** (*mole %*)	*Beef heart*** (*mole %*)
Lysine	5.9	12.8	5.1	12.7	6.5	4.5
Histidine	1.0		1.7		1.9	3.3
Arginine	5.9		5.9		5.6	5.3
Aspartic acid	17.5	26.1	11.8	21.8	8.8	9.5
Glutamic acid	8.6		10.0		10.2	10.9
Proline	3.4	33.1	4.3	38.3	4.7	4.6
Valine	7.2		7.5		8.4	7.1
Leucine	7.5		9.8		8.4	10.8
Isoleucine	4.4		5.7		5.6	3.0
Phenylalanine	3.6		4.0		3.7	3.8
Tyrosine	1.4		1.5		2.8	3.6
Tryptophan	—		—		1.4	—
Threonine	5.6		5.5		5.6	4.5
Serine	4.4	28.2	5.1	27.6	5.6	6.4
Glycine	11.8		9.6		8.4	7.9
Alanine	10.4		11.1		9.3	13.1
Methionine	0.7		1.1		1.4	1.8
Cystine (half)	0.9		0.7		1.9	—

* Structural protein from mitochondria of *Neurospora*[14].
** Structural protein core protein from mitochondria of beef heart[15].

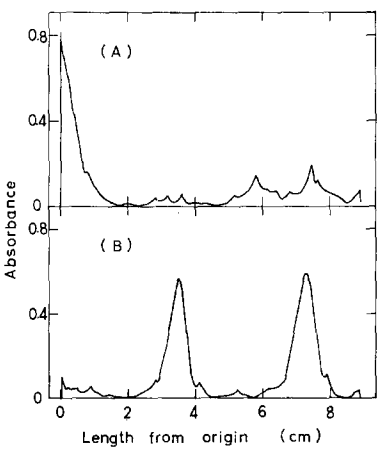

Fig. 4. Behavior of membrane proteins in polyacrylamide disc gel electrophoresis. I. Electrophoresis was performed by the method of Takayama *et al.*[12]. (A), inner membrane; (B), outer membrane. An amount of membrane equivalent to 200 μg of protein was charged. Current was 5 mA per tube (5 by 100 mm).

molecular weight components, protein, phospholipid and lipopolysaccharide, in a ratio of 100:86:11 and 100:82:34 (by wt), respectively. Much Mg–ATPase and succinate dehydrogenase activity was found to be localized in the inner membrane, very little being found in the outer membrane. These properties of the purified membranes indicate that the outer membrane was prepared without contamination of inner membrane. The inner membrane obtained in this study always contained lipopolysaccharide, but we could not estimate whether lipopolysaccharide is an impurity or not.

Amino acid analyses of the purified membranes prepared here are summarized in Table II, those of structural or core proteins from *Neurospora* and beef heart also being given as reference. There is a high similarity between the analysis data of the inner and outer membranes with an exception of aspartic acid content. The structural proteins were also found to have similar values. These findings indicate that neither inner nor outer membranes have a specific amino acid composition.

(*iii*) *Protein composition.* Analysis for protein composition of the membranes was performed by polyacrylamide disc gel electrophoresis. When the method of Takayama *et al.*[12] was employed, the purified inner membrane hardly dissolved in a solvent of phenol–acetic acid–urea, so that the membrane remained at the top of the gel. On the other hand, the outer membrane dissolved well in the solvent and gave two major components in the electrophoresis (Fig. 4). When the method of Weber and Osborn[13] was employed, both of the purified membranes dissolved com-

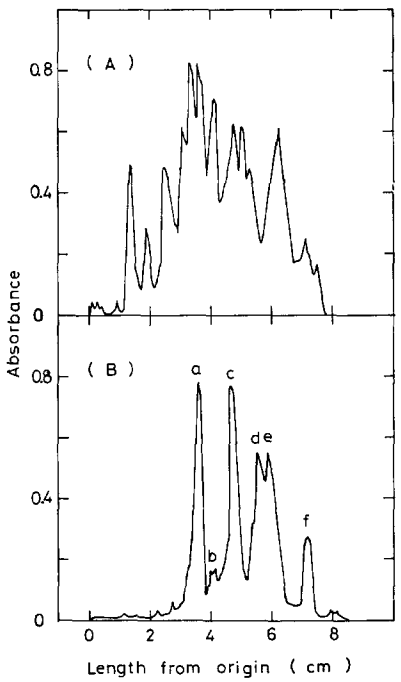

Fig. 5. Behavior of membrane proteins in polyacrylamide disc gel electrophoresis. II. Electrophoresis was performed by the method of Weber and Osborn[13]. (A), inner membrane; (B), outer membrane. An amount of membrane equivalent to 200 μg of protein was charged. Current was 5 mA per tube (5 by 100 mm).

pletely in 1% sodium dodecyl sulfate and developed very well in the electrophoresis. As seen in Fig. 5, more than 20 protein bands were detected as components of the inner membrane. In contrast to this, the outer membrane gave 6 bands (a, b, c, d, e and f in Fig. 5B) with some minor ones. These 6 bands taken together correspond to approximately 90% of the total protein of the outer membrane.

Solubilization and reaggregation of the outer membrane

As described above, the outer membrane is a structure composed of protein, phospholipid, lipopolysaccharide and some minor component, and is relatively simple in protein composition. Thus, the outer membrane might be suitable for the investigation of the interaction of three major components to form a membranous structure and the mechanism of organization of the outermost layer of the cell surface. To obtain information about morphogenesis of membrane structure, attempts were made to solubilize the outer membrane in sodium dodecyl sulfate solution and to reaggregate a membranous structure from the sodium dodecyl sulfate-solubilized membrane.

(*i*) *Sodium dodecyl sulfate-solubilized outer membrane.* The sodium dodecyl sulfate-solubilized outer membrane prepared here shows the following characteristic properties. (1) No membranous structure was observed at a magnification of 50000 times under the electron microscope with a negatively stained sample. (2) No sediment was obtained after centrifugation at $130000 \times g$ for 4 h in a 35–50% sucrose concentration gradient system containing 1% sodium dodecyl sulfate, but major components, such as protein, lipopolysaccharide and phospholipid were separated by centrifugation at $110000 \times g$ for 31 h in a 5–10% sucrose concentration gradient system con-

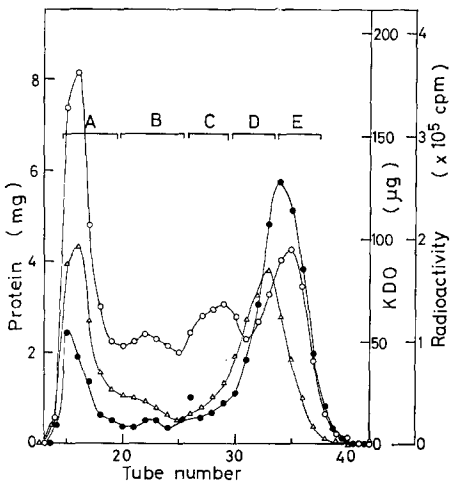

Fig. 6. Sephadex G-150 gel column chromatography of the sodium dodecyl sulfate-solubilized outer membrane. The chromatography was performed as follows. A sample of 8.5 ml of the sodium dodecyl sulfate-solubilized outer membrane (protein, 53 mg; radioactivity (^{3}H), $1.86 \cdot 10^6$ cpm and ketodeoxyoctonate, 0.85 mg) was applied on a column of Sephadex G-150 (3.0 cm $\times$ 63.3 cm, volume 455 ml) which was previously equilibrated with 1% sodium dodecyl sulfate solution. The filtration was conducted with 1% sodium dodecyl sulfate solution at a flow rate of 1.0 ml/min. Each fraction is 10.0 ml. ○—○, protein; ●—●, radioactivity; △—△, ketodeoxyoctonate (KDO).

TABLE III

RECOVERY OF CHEMICAL MARKERS IN REAGGREGATION PROCESS OF SODIUM DODECYL SULFATE-SOLUBILIZED OUTER MEMBRANE

	Chemical markers		
	Protein (%)	*Radioactivity* (^3H %)	*Ketodeoxyoctonate* (%)
Outer membrane before dialysis	100	100	100
Outer membrane after dialysis*	94.5	97.6	96.3
Reaggregated outer membrane	95.4	97.0	97.3
Supernatant fraction** after reaggregation	4.5	2.3	1.2

* Suspension of outer membrane was directly dialyzed according to the procedure for reaggregation of the sodium dodecyl sulfate-solubilized outer membrane.

** After the dialysis, the reaggregated outer membrane was separated by centrifugation (see Materials and Methods). The supernatant obtained was used for analysis.

Fig. 7. Behavior of proteins in the reaggregated outer membrane in polyacrylamide disc gel electrophoresis. Electrophoresis was performed by the method of Weber and Osborn[13]. An amount of the membrane equivalent to 300 μg of protein was applied. Right, original outer membrane; left, reaggregated outer membrane.

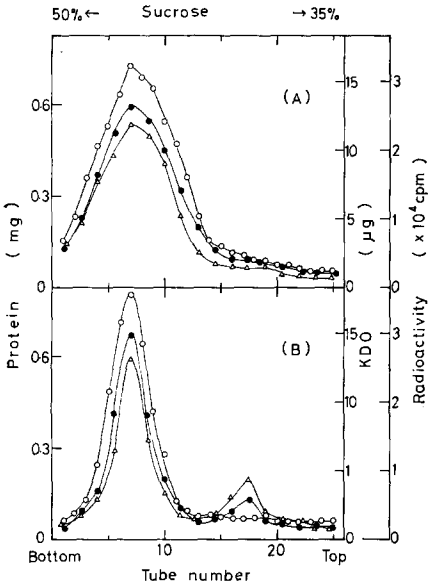

Fig. 8. Distribution profile of the original and reaggregated outer membrane in sucrose density gradient centrifugation. (A), original outer membrane; (B), reaggregated outer membrane. An amount of membrane equivalent to 10 mg of protein was charged in each case. ○—○, protein; ●—●, radioactivity; △—△, ketodeoxyoctonate. Each tube, 6 drops (approximately 0.24 ml).

taining 1% sodium dodecyl sulfate. This finding indicates that the sodium dodecyl sulfate-solubilized membrane is composed of subunits smaller in size than ribosomes. (3) A filtration profile of the sodium dodecyl sulfate-solubilized outer membrane through Sephadex G-150 gel is presented in Fig. 6, protein, radioactivity (^{3}H in lipid) and ketodeoxyoctonate (lipopolysaccharide) have been used as elution marker and complete elution was observed. The values for the composition ratios of protein, radioactivity and ketodeoxyoctonate in the filtration fractions were not in accord with the composition ratio of the outer membrane described below.

These findings indicate that the outer membrane was dissociated to subunits by solubilization with sodium dodecyl sulfate.

(ii) Reaggregation of a new structure from the sodium dodecyl sulfate-solubilized outer membrane. When sodium dodecyl sulfate was removed from sodium dodecyl sulfate-solubilized outer membrane by dialysis as described in Materials and Methods, a new structure (reaggregated outer membrane) was revealed, which could be collected as a precipitate by centrifugation at $100000 \times g$ for 1.5 h. The excellent recovery of three markers in the new structure is shown in Table III. The ratio of the markers (protein, radioactivity and ketodeoxyoctonate) in the structure was 100:3.6:1.7 (mg:10^6 cpm:μg); this is very close to that of the original outer membrane (100: 3.5:1.6). In the polyacrylamide gel disc electrophoresis using the method of Weber *et al.*[13], all protein components in the outer membrane were recovered in the new structure, as seen in Fig. 7. A comparison of the behavior of the original with the new structures on sucrose density-gradient centrifugation is shown in Fig. 8. The new structure clearly gave one major peak and one minor peak; the former was

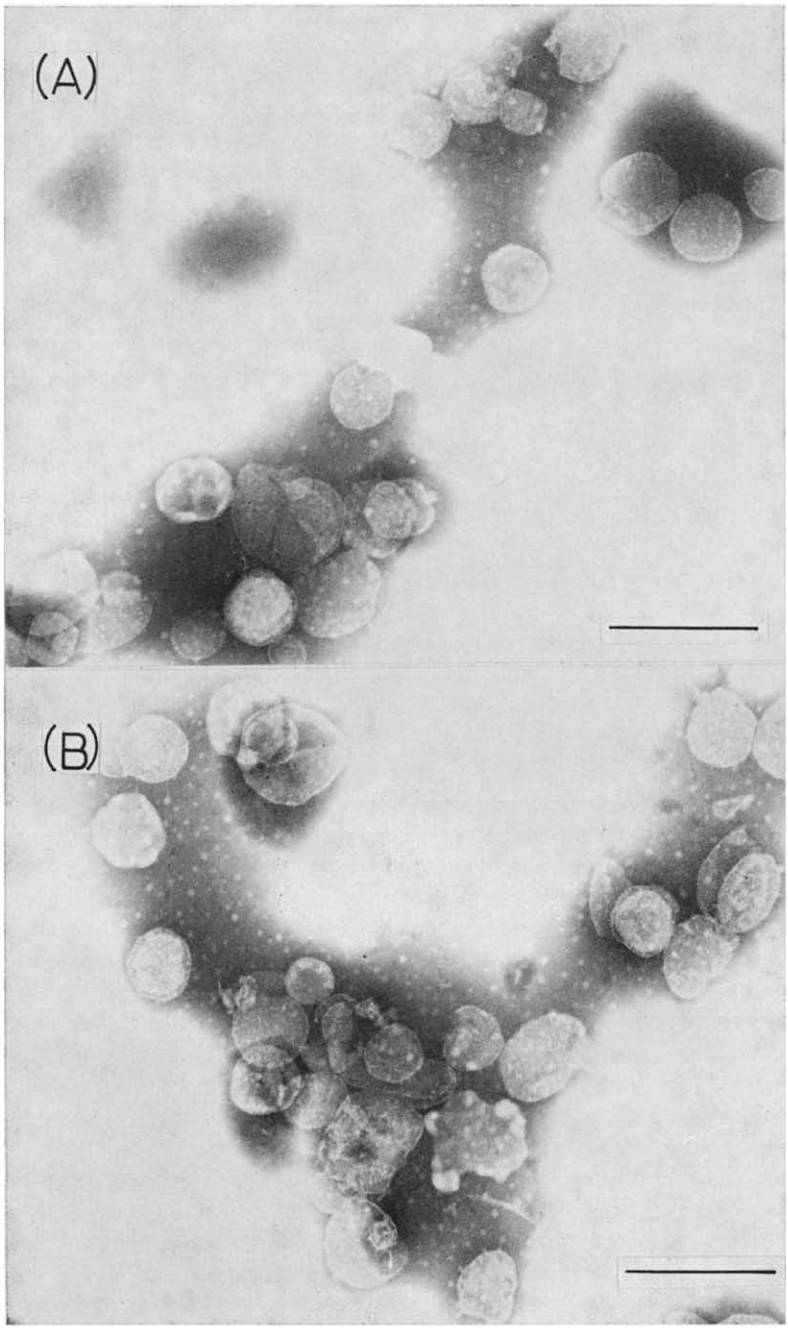

Fig. 9. Micrographs of negatively stained preparations of the original and reaggregated outer membranes. (A), original membrane; (B), reaggregated membrane. Bar 0.5 μm.

very similar in density to the outer membrane. The ratios of the three markers in the major peaks isolated from the original outer membrane and the new structure were 100:3.5:1.6 (with fraction No. 7) and 100:3.6:1.6 (with fraction No. 7), respectively. The minor peak from the new structure was found to be a complex of lipopolysaccharide and phospholipid. In Fig. 9, the electron microscopic appearance of the new structure was compared with that of the original membrane after negative staining with phosphotungstate. As can be seen in the figure, both structures were of circular shape and homogeneous size.

From these findings it might be concluded that a new structure obtained from the sodium dodecyl sulfate-solubilized outer membrane is a reaggregated outer membrane.

DISCUSSION

There are many indications of a multi-layered membrane structure for *E. coli*[1,2]. Miura and Mizushima[4] achieved the preparation of both outer and cytoplasmic membranes, with a unit membrane structure on electron microscopic observation. Costerton *et al.*[1] postulated that the outer cell surface of Gram-negative bacteria such as *E. coli*, comprised a "loosely bound layer" and an "underlying layer" in addition to the "outer double track layer", besides the "peptide glycan layer" and "cytoplasmic membrane" found on chemical analysis of the cells. Nanninga[16] indicated the presence of an "outer lipoprotein–lipopolysaccharide complex" and a "globular protein layer" at the outer surface of *E. coli*.

The outer membrane prepared here also showed the unit membrane structure and was composed of protein, phospholipid and lipopolysaccharide as main components in a weight percentage per membrane of 40, 33 and 14, respectively (Table I). Similar ratios of these components were also observed with the reaggregated outer membrane (41:34:14). The gel filtration fractions of the sodium dodecyl sulfate-solubilized membrane were combined to make five parts, A, B, C, D and E (Fig. 6), and then dialysed to remove sodium dodecyl sulfate. Reaggregation of a new structure which is similar to outer membrane, however, did not occur from each part alone. These findings suggest that all parts might be essential for the reaggregation.

The polyacrylamide disc gel electrophoretic behavior of outer membrane showed it to be composed of a number of small molecular weight peptides. These observations are similar to those described by Schnaitman[17], with some difference in peptide number.

As observed in Fig. 8, the reaggregated outer membrane gave a sharper profile on sucrose density gradient centrifugation than the original outer membrane. However, we have, as yet insufficient results to explain this observation.

In this paper, a self-assembly mechanism has been postulated for the reaggregation of a new membranous structure from the sodium dodecyl sulfate-solubilized outer membrane. However, the reaggregated structure was seen in Fig. 9 to be of circular shape. The rod-like cell form of *E. coli* might be regulated by a peptide glycan, located in the space between cytoplasmic (inner) and outer membranes. If cell form is in fact determined by a peptide glycan, a self-assembly mechanism for morphogenesis of outer membrane is a reasonable postulate, however, no one has yet given any alternative explanation for the orientation of the outer membrane.

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