Biochimica et Biophysica Acta, 375 (1975) 44–53

BBA 76829

ISOLATION AND CHARACTERIZATION OF TWO OUTER MEMBRANE PREPARATIONS FROM *ESCHERICHIA COLI*

SHOJI MIZUSHIMA and HISAMI YAMADA

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

(Received August 8th, 1974)

SUMMARY

A method was developed for releasing specifically a part of outer membrane during spheroplast formation. A highly purified outer membrane (outer membrane I) was obtained from the spheroplast medium by isopycnic sucrose gradient centrifugation. The remaining outer membrane (outer membrane II) and cytoplasmic membrane was also isolated from the spheroplasts by the isopycnic centrifugation.

Two outer membrane preparations were different from the cytoplasmic membrane in protein composition, enzyme localization, phospholipid composition, lipopolysaccharide content and electron micrographs. Although outer membranes I and II were almost the same in various respects, they seemed to be different from each other under electron microscope and in cardiolipin content. It is suggested that the outer membrane I and the outer membrane II, at least a part of the outer membrane II, are integrated in a different fashion in the outer-most layer of *Escherichia coli* cell surface.

INTRODUCTION

The isolation of outer membrane from *Escherichia coli* was first achieved by Miura and Mizushima by a technique involving isopycnic sucrose density gradient centrifugation [1, 2]. During the study it was noticed that a part of the outer membrane was released from cells when reagents for the spheroplast formation was added to cells in cold followed by a quick warming of the mixture to 30 °C [2].

Taking advantage of this specific release of the outer membrane, we have developed a method by which outer membrane of high purity can be prepared quickly. The outer membrane preparation isolated by the method has already been used for some studies [3, 4]. This paper first deals with a precise method of the isolation of the released outer membrane and the outer and cytoplasmic membranes from spheroplasts. General characterization of these membrane preparations with special regard to purity and comparison of two outer membrane preparations is also made in this paper.

MATERIALS AND METHODS

Chemicals

L-[4, 5-$^3H_2$]Leucine (spec. act. 32 Ci/mmole), L-[U-^{14}C]leucine (spec. act. 282 Ci/mole) and [2-^{3}H]glycerol (spec. act. 500 Ci/mole) were purchased from Daiichi Pure Chemicals Co. Deoxyribonuclease I (2300 units/mg) was purchased from Worthington Biochemicals Corporation and egg white lysozyme was a gift of Eizai Co.

Bacterium and media

E. coli YA21 used was a leucine auxotroph mutant derived from *E. coli* K12 (*met*$^-$, *F*$^-$, λ^-) used in the previous study [2]. Cells were grown by reciprocating shaking at 37 °C in the modified Fraser and Jerrel's medium in which the amount of glycerol and polypeptone was one-third of that in the original prescription [5]. Glycerol was replaced by glucose when [2-^{3}H]glycerol was added to the medium for labeling phospholipids. Cells were harvested at the mid exponential phase of growth ($A_{600\ nm}$ was 0.32 after 10-fold dilution).

Analytical procedures

Protein. The method of Lowry et al. [6] was employed with bovine serum albumin as standard.

Lipopolysaccharide. The amount of lipopolysaccharide was estimated from 2-keto-3-deoxyoctonic acid content which was determined as described by Osborn et al. [7]. The purified lipopolysaccharide was prepared from the same bacterial strain by the method of Yuasa et al. [8] and used as standard.

Lipids. The relative amount of lipids was estimated from radioactivity after specific labeling of lipids with radioactive glycerol. Cultures of *E. coli* were grown for at least two generations in 1 l of a glucose medium supplemented with 10 μmoles of [2-^{3}H]glycerol (10 Ci/mole). Over 90 % of the radioactivity was recovered in the chloroform–methanol (2 : 1, by vol.) extract. For the analysis of phospholipid contents, membrane fractions were prepared, pelleted and extracted with 300 vol. of chloroform-methanol (2 : 1, by vol.) for 3 h at room temperature. After the centrifugation, the clear extract was concentrated to dryness in vacuo and taken up in a small volume of chloroform. Phospholipids were separated and identified by two-dimensional thin-layer chromatography on Kieselgel G (Merck Co.) according to the method of Kanemasa et al. [9]. Spots were developed in iodine vapor and marked. Lipids were then extracted twice with 6 ml of chloroform–methanol (2 : 1, by vol.) for 10 min at room temperature and dried. Radioactivity was counted in 10 ml of Bray's solution with Packard 3320 Tri-Carb scintillation spectrometer.

Enzyme assays

Succinate dehydrogenase (EC 1.3.99.1) was assayed according to the method described by King using 2,6-dichlorophenolindophenol as electron acceptor in the absence of phenazine methosulfate [10]. Absorbance at 600 nm was determined at 26 °C. Amount of cytochrome *b* was determined from the difference spectra (reduced with dithionite minus untreated) at 429 nm.

Polyacrylamide gel electrophoresis of membrane proteins

About 100 μg of membrane proteins were dissolved in 100 μl of 1 % sodium dodecylsulfate–1 % 2-mercaptoethanol solution and heated either at 50 °C for 30 min or 100 °C for 5 min. Then, 74 mg of solid urea was added to the solution to give the final concentration 8 M. 50 μl of the solution was mixed with 5 μl of 1 % Bromophenol blue and applied onto a gel (0.5 cm × 9.0 cm) containing 8 % acrylamide, 0.13 % *N,N'*-ethylenebisacrylamide, 0.5 % sodium dodecylsulfate, 8 M urea, 0.1 M sodium phosphate buffer (pH 7.2) and 0.6 μl/ml *N,N,N',N'*-tetramethylethylenediamine. The polymerization of gel was initiated with ammonium persulfate (1.2 mg/ml). Electrophoresis was first made at 4 mA per tube for 30 min at room temperature in 0.1 M sodium phosphate (pH 7.2)–0.1 % sodium dodecylsulfate buffer. Then the current was increased to 6 mA per tube and the electrophoresis was continued for 3 h until Bromophenol blue had migrated almost to the bottom of tube. Gels were fixed with 20 % sulfosalicylic acid and stained with Coomassie brilliant blue according to the method of Maizel [11]. The gel System II of Bragg and Hou [12] was also employed.

Electron microscopy of membrane fractions

Samples were negatively stained with 1 % sodium phosphotungstate (pH 6.2) and examined with a Hitachi HS-9 electron microscope.

RESULTS

Isolation of membrane fractions

Bacterial cells in a 1 l culture were quickly harvested by centrifugation in cold (6000 × *g*, 5 min), washed with 80 ml of distilled water and suspended in distilled water to make total volume 35.5 ml. The cell suspension was transferred into a 300 ml flask in ice-water and ice-cold reagents were slowly added into the suspension in the following order; 20 ml of 0.1 M Tris–HCl buffer (pH 8.3 at 4 °C), 18 ml of 2 M sucrose, 3.5 ml of 1 % EDTA (sodium salt, pH 7.0) and 3.5 ml of 0.5 % lysozyme solution. About 5 min were required for the addition of each of the first two reagents and 2 min were required for each of the other reagents. Then the mixture was warmed to 30 °C within 2–3 min in a water bath (40 °C) with gentle shaking and kept at the temperature for 60 min. During the warming, the solution became viscous due to the breakage of a part of the cells. However the viscosity was greatly reduced within 20 min. During the incubation about half of the outer membrane was released from spheroplasts. The released membrane was separated from spheroplasts by the centrifugation at 20 000 × *g* for 15 min, sedimented by high-speed centrifugation (95 000 × *g* 45 min) and suspended in 5 ml of 1 % EDTA (pH 7.0) (Preparation I). The spheroplasts were burst in 80 ml of 5 mM $MgCl_2$ and a membrane fraction was recovered and washed with 5 mM $MgCl_2$ by centrifugation (20 000 × *g*, 20 min) and suspended in 10 ml of 1 % EDTA (pH 7.0) (Preparation II). Both Preparations I and II were dialyzed against 1 l of 1 % EDTA (sodium salt) for 2 h and 15 h successively at 4 °C. During the dialysis, the viscosity of Preparation II increased due to the breakage of contaminated cells. The viscosity was reduced by the treatment in a Teflon glass homogenizer. Membranes from Preparations I and II were recovered by centrifugation (20 000 × *g*, 20 min) and suspended in 1.5 ml and 3 ml of distilled

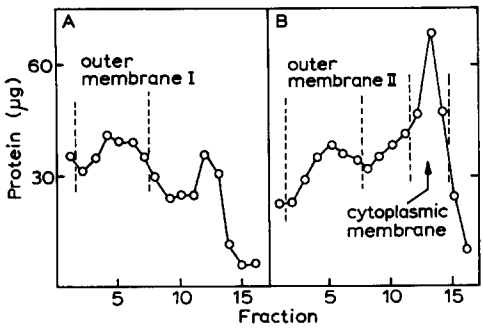

Fig. 1. Separation of outer and cytoplasmic membranes by isopycnic sucrose density gradient centrifugation. EDTA-dialyzed membrane preparations were centrifuged on 35–50 % sucrose gradient at 39 000 rev./min for 4 h at 4 °C in a Hitachi RPS 40 T-2 rotor. 15 drops each were collected from the bottom of tube. 20 μl of each fraction were used for protein determination. A, membrane preparation from Preparation I; B, membrane preparation from Preparation II.

water, respectively. Preparation II was washed once with the EDTA solution before suspending in water.

The isolation and purification of outer and cytoplasmic membranes from these preparations were carried out by an isopycnic sucrose density gradient centrifugation according to the method of Miura and Mizushima [1, 2]. Hitachi RPS 50 or 40 T-2 rotor with 4.4 ml of 35–50 % (w/w) sucrose gradient was used. Samples from Preparations I and II were overlaid on two and four of the gradient tubes, respectively. Centrifugation was made at $125\,000 \times g$ for 4 h at 4 °C. Isopycnic equilibrium had been achieved after 3 h centrifugation. Gradients were fractionated from the bottom of tube. As shown in Fig. 1, the centrifugation resulted in a good separation of two types of membrane at the positions corresponding to outer and cytoplasmic membranes, respectively. The sample from Preparation I was rich in outer membrane, while that from Preparation II contained both outer and cytoplasmic membranes. Fractions were pooled as shown in Fig. 1 and diluted with 2 vol. of distilled water. Then membranes were recovered by centrifugation ($77\,000 \times g$, for 30 min), suspended in small volume of distilled water and used for the following experiments. In this paper, terms outer membranes I and II mean outer membranes derived from Preparations I and II, respectively. Cytoplasmic membrane means that prepared from Preparation II.

Large scale preparation of membranes

For the large scale preparation, a Hitachi RPS 40 T rotor with 10 ml of 35–50 % (w/w) sucrose gradient was employed at $180\,000 \times g$ for 6 h. A sample four times as much as that for a RPS 50 rotor can be applied on a gradient. The separation of membranes was even better than that in a RPS 50 rotor.

Factors influencing separation

Time required for harvesting and washing of bacterial cells seemed to be an important factor for the preparation of good membranes. Prolonged storage of cells in cold often resulted in a failure in the separation. It is recommended to complete the entire procedure from the harvesting to the washing within 60 min. The incubation

in 5–20 mM Mg^{2+} solution at high temperature resulted in the fusion or aggregation of outer and cytoplasmic membranes (Mizushima, S. and Yamada, H., unpublished). Therefore, membrane preparations should be kept in cold especially when they are with Mg^{2+}. Overnight storage of membrane preparations in ice before the isopycnic centrifugation often resulted in better separation of outer and cytoplasmic membranes. The reason is unclear.

Following experiments were performed for characterizing these membrane preparations and for checking cross-contamination.

Electron microscopic observations

The outer membrane I appeared as empty vesicles of rather homogeneous size (0.2 μm in diameter as average) and was resembled to that reported previously [2] (Fig. 2A). On the other hand, the outer membrane II was more heterogeneous (Fig. 2B) and some of them were hardly distinguishable from the cytoplasmic membrane which appeared as large open membrane organella (Fig. 2C). However, as will be discussed later, this heterogeneity is not accounted for by the contamination by the cytoplasmic membrane.

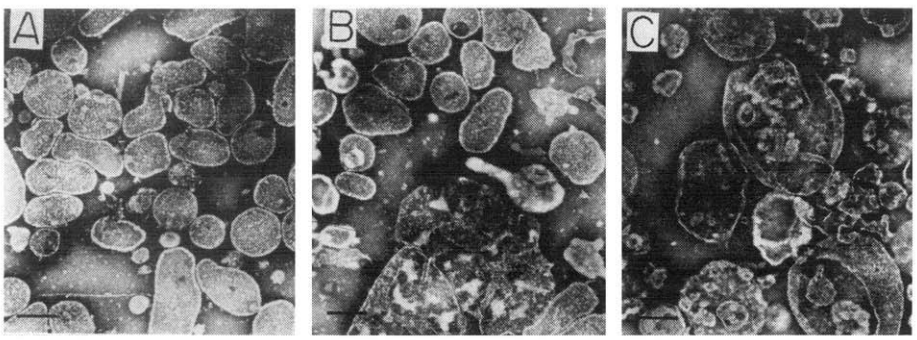

Fig. 2. Electron micrographs of fractionated membranes. A, outer membrane I; B, outer membrane II and C, cytoplasmic membrane. Magnification markers represent 0.2 μm.

Localization of lipopolysaccharides

Fig. 3 shows lipopolysaccharide profiles after the sucrose density centrifugation of membrane preparations. Although almost all of lipopolysaccharides were located in the outer membrane, the cytoplasmic membrane seemed to possess some of lipopolysaccharides (Fig. 3B). Since the contamination by the outer membrane in the cytoplasmic membrane seemed to be insignificant as will be shown in the section of protein composition, such an amount of lipopolysaccharide is hardly accounted for by the contamination of the cytoplasmic membrane by the outer membrane. This might be a precursor of lipopolysaccharide reported by Osborn et al. [13].

Protein composition

The protein composition of membrane fractions was analyzed by polyacrylamide gel electrophoresis in sodium dodecylsulfate–urea (Fig. 4). The outer membrane was composed of a number of well separated bands and no band was observed in some areas where a large number of bands was seen in the cytoplasmic membrane,

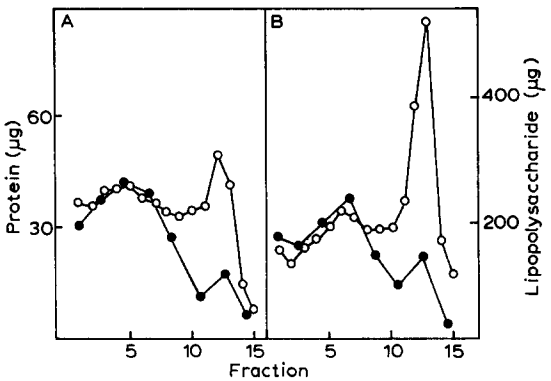

Fig. 3. Lipopolysaccharide profiles in isopycnic sucrose density gradient centrifugation. Conditions of the centrifugation were the same as those shown in Fig. 1. 20 μl of each fraction were used for protein determination. 200 μl each of adjacent two fractions were combined and used for lipopolysaccharide determination, and values were plotted between the fractions. A, membrane preparation from Preparation I; B, membrane preparation from Preparation II. ○, protein; ●, lipopolysaccharide.

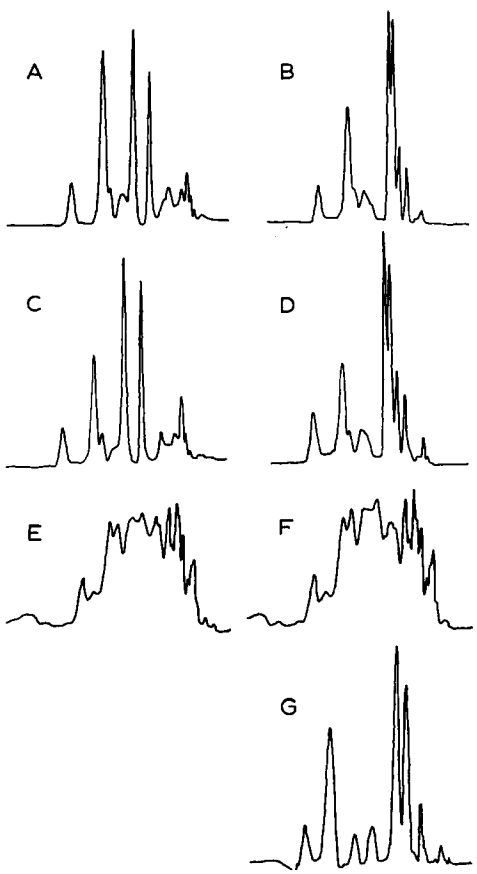

Fig. 4. Scan of polyacrylamide gel electrophoresis of membrane proteins. A, B, and G, outer membrane I; C and D, outer membrane II; E and F, cytoplasmic membrane. Samples in A, C and E had been preheated at 50 °C for 30 min in the presence of sodium dodecylsulfate. Samples in B, D, F and G had been preheated at 100 °C for 5 min in the presence of sodium dodecylsulfate. G, System II of Bragg and Hou. Tops of gels are to the right.

showing that the outer membrane was not contaminated significantly by the cytoplasmic membrane. Upon heating of membrane preparations in sodium dodecylsulfate solution, position and number of bands were changed in outer membranes as observed by other investigators [7, 12, 14, 15], while the heating did not cause any change in the position of bands in the cytoplasmic membrane. This fact indicates that the cytoplasmic membrane was not contaminated significantly by the outer membrane. There was no difference in gel patterns between the outer membranes I and II. The outer membrane II was sometimes slightly contaminated by protein bands of cytoplasmic membrane. Fig. 4 G shows a gel pattern of the outer membrane analyzed by the method of Bragg and Hou [12]. Although the pattern was somewhat different from that in Figs 4B and 4D, the pattern was similar to that of proteins extracted from Triton X-100 treated cell envelopes [12].

Phospholipid composition

We have previously reported that the relative phospholipid content of the outer membrane was appreciably lower than that of the cytoplasmic membrane [1]. The results was confirmed by others [7, 15]. These membranes also differed in phospholipid composition (Table I) as observed by Osborn et al. in *Salmonella typhimurium* [7]. The data in Table I are expressed as percentage of the total ^3H of each membrane preparation recovered in the individual phospholipids and, therefore, do not represent mole fractions. Although the phospholipid composition of the outer and cytoplasmic membranes was qualitatively similar, it was markedly different quantitatively. The cardiolipin content of the outer membrane I was one-fifth to one-sixth that of the cytoplasmic membrane. The outer membrane II showed intermediate values in the cardiolipin content. The phosphatidylglycerol content of outer membranes I and II was slightly lower than that of the cytoplasmic membrane. Although data are not shown, essentially the same result was obtained from one-dimensional thin-layer chromatography using the solvent of chloroform–methanol–acetic acid–water (250 : 74 : 19 : 3, by vol.) [17].

Localization of enzyme and cytochrome

We have previously reported that enzymes related to electron transfer were localized in the cytoplasmic membrane [1]. This view was confirmed by many investi-

TABLE I

DISTRIBUTION OF [2-^3H]GLYCEROL-LABELED PHOSPHOLIPIDS

Membrane preparations were prepared following growth in the presence of [2-^3H]glycerol. Phospholipids were isolated and fractionated as described under Materials and Methods. Values given are expressed as percentage of total ^3H in lipid fraction.

Membranes	Total ^3H-glycerol recovered as		
	Phosphatidyl-ethanolamine	Phosphatidyl-glycerol	Cardiolipin
Outer membrane I	85	11.5	2.0
Outer membrane II	78	12.5	8.2
Cytoplasmic membrane	70	16.4	13.1

TABLE II

DISTRIBUTION OF SUCCINATE DEHYDROGENASE AND CYTOCHROME *b* AMONG MEMBRANES

Membranes	Succinate dehydrogenase (nmole/min per mg protein)	Cytochrome *b* ($A_{429\ nm}$/mg protein)
Outer membrane I	0.2	<0.002
Outer membrane II	0.7	0.010
Cytoplasmic membrane	21.0	0.14

gators. As shown in Table II, succinate dehydrogenase and cytochrome *b* were exclusively localized in the cytoplasmic membrane preparation. Judging from the amount of these proteins, the contamination by the cytoplasmic membrane in outer membranes I and II was about 0–1 % and 3–7 %, respectively.

Short-period labeling of outer membranes

The possibility that outer membranes I and II represent a different stage of the membrane formation was examined. Cells which had been prelabeled with [^{14}C]-leucine was labeled with [^{3}H]leucine for one-tenth generation. Then membranes were prepared quickly and analyzed on a sucrose gradient. As shown in Fig. 5, the $^{3}H/^{14}C$ ratio in the outer membrane region was almost the same in Preparations I and II. The result excluded the possibility that one of the outer membrane was the precursor of the other. Fig. 5 also shows that the $^{3}H/^{14}C$ ratio was almost the same throughout the region covering the outer membrane fractions. This also suggests that the heterogeneity in the density of outer membrane preparations was not due to the different stage of the membrane maturation.

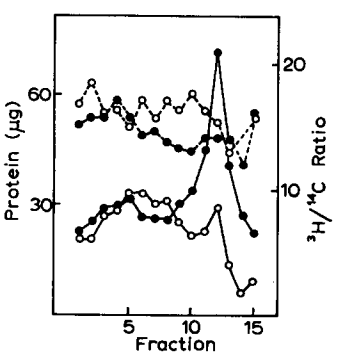

Fig. 5. Short-period labeling of membranes by radioactive leucine. *E. coli* cells were grown for three generations in 1 l of the medium containing 3 μCi of L-[U-^{14}C]leucine. 150 μCi of L-[4,5-$^{3}H_2$]leucine was added when the $A_{600\ nm}$ after 10-fold dilution of the culture reached to 0.29 and the cultivation was continued for one-tenth generation. Membrane preparations were prepared and analyzed on sucrose gradient as described in the text. 5 μl of each fraction were used for protein determination. 50 μl of each fraction were mixed with 50 μl of water and 200 μl of Protosol (New England Nuclear), and heated at 55 °C for 3.5 h, and radioactivity was counted in 10 ml of Bray's solution with Packard 3320 Tri-Carb scintillation spectrometer. ○, membrane preparation from Preparation I; ●, membrane preparation from Preparation II. —, protein; - - -, $^{3}H/^{14}C$ ratio.

DISCUSSION

In the previous paper [1, 2] we have presented a method of isolation of outer and cytoplasmic membranes from *E. coli*. After that Osborn et al. [7] developed a modification of our procedure which appeared to be applicable to other Gram negative enteric bacteria. Recently Wolf-Watz et al. [18] presented another method of isolation of the outer membrane from *E. coli*. The method includes the removal of the outer membrane from plasmolyzed cells by lysozyme–EDTA treatment. In this paper we have made some modification on our previous procedure which is important for preparing a good outer membrane preparation. The procedure described here offers a reproducible method for the isolation of highly purified outer membrane from *E. coli*.

Because the outer membrane was always fractionated into two fractions, outer membranes I and II, by the present procedures, we tried to find out differences between these two membrane fractions. Both membranes were found to be indistinguishable on the following respects: polyacrylamide gel electrophoretic patterns of proteins (Fig. 4), contents of protein and lipopolysaccharides (Fig. 3) and the labeling patterns of membranes (Fig. 5). Infrared spectra of these membranes were also the same [4]. We have found some morphological difference between a part of them as examined under an electron microscope (Fig. 2). The fairly large amount of cytoplasmic membrane-like organelles in the outer membrane II is hardly accounted for by the contamination by the cytoplasmic membrane. This may be the reflection of the difference in the way of attachment of outer membranes to bacterial cell. A difference was also found in the cardiolipin content. As already shown, the contamination by the cytoplasmic membrane in the outer membrane II was 3–7 % on the basis of protein content (Fig. 2) and the phospholipid content of the cytoplasmic membrane is 2.5 times as much as that of the outer membrane [1]. If the outer membrane II is the same as the outer membrane I, the cardiolipin content in the outer membrane II is extimated to be at most 4.2 % of total phospholipid. Since the figure is much less than the cardiolipin content in the outer membrane II (8.2 %), the result suggests that the outer membrane II is different from the outer membrane I in caridiolipin content. Although it is reasonable to conclude that both membrane preparations were derived from the outer-most layer of cellular surface, the outer membrane I and the outer membrane II, at least a part of the outer membrane II, might be integrated in the surface of the bacterial cell in a different fashion. To make the view conclusive, however, more precise experiments must be performed.

The outer membrane I is superior to the outer membrane II and the outer membrane preparation in the previous paper [1, 2] as starting material for the study of outer membrane. It is very pure; the contamination by the cytoplasmic membrane was estimated to be less than 1 % (Table II). It can be prepared easily and quickly from the supernatant solution after the formation of spheroplasts. Furthermore, the content of peptidoglycan in the outer membrane I was only 20 μg per mg protein as estimated from the amount of diaminopimeric acid [4]. The amount may be accounted for the presence of lipoprotein–murein complex [19] in the outer membrane [20, 21, Uemura and Mizushima, unpublished]. The outer membrane preparation reported by Wolf-Watz et al. [18] seems to similar to the outer membrane I in the present paper.

ACKNOWLEDGEMENT

The authors are deeply indebted to Dr Satoru Shimizu for valuable advice on electron microscopy. This work was supported in part by a grant in aid for scientific research from the Ministry of Education 856053 and 838010, and Naito Research Grant 72-121.

REFERENCES

1 Miura, T. and Mizushima, S. (1968) Biochim. Biophys. Acta 150, 159–161
2 Miura, T. and Mizushima, S. (1969) Biochim. Biophys. Acta 193, 268–276
3 Sekizawa, J. and Fukui, S. (1973) Biochim. Biophys. Acta 307, 104–117
4 Nakamura, K., Ostrovsky, D. J., Miyazawa, T. and Mizushima, S. (1974) Biochim. Biophys. Acta 332, 329–335
5 Fraser, D. and Jerrel, E. A. (1953) J. Biol. Chem. 205, 291–295
6 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
7 Osborn, M. J., Gander, J. E., Parisi, E. and Carson, J. (1972) J. Biol. Chem. 247, 3962–3972
8 Yuasa, R., Levinthal, M. and Nikaido, H. (1969) J. Bacteriol. 100, 433–444
9 Kanemasa, Y., Akamatsu, Y. and Nojima, S. (1967) Biochim. Biophys. Acta 144, 382–390
10 King, T. E. (1963) J. Biol. Chem. 238, 4032–4051
11 Maizel, Jr, J. V. (1966) Science 151, 988–990
42 Bragg, P. D. and Hou, C. (1972) Biochim. Biophys. Acta 274, 478–488
13 Osborn, M. J., Gander, J. E. and Parisi, E. (1972) J. Biol. Chem. 247, 3973–3986
14 Inouye, M. and Yee, M. (1973) J. Bacteriol. 113, 304–312
15 Schnaitman, C. A. (1973) Arch. Biochem. Biophys. (1973) 157, 541–552
16 Schnaitman, C. A. (1970) J. Bacteriol. 104, 890–901
17 Mizushima, S., Ishida, M. and Kitahara, K. (1966) J. Biochem. Tokyo 59, 374–381
18 Wolf-Watz, H., Normark, S. and Bloom, G. D. (1973) J. Bacteriol. 115, 1191–1197
19 Hankle, K. and Braun, V. (1973) Eur. J. Biochem. 34, 284–296
20 Bosch, V. and Braun, V. (1973) FEBS Lett. 34, 307–310
21 Lee, N. and Inouye, M. (1973) FEBS Lett. 39, 167–170