BBA 75340

SEPARATION AND PROPERTIES OF OUTER AND CYTOPLASMIC MEMBRANES IN *ESCHERICHIA COLI*

TAKASHI MIURA AND SHOJI MIZUSHIMA
Institute of Applied Microbiology, University of Tokyo, Tokyo (Japan)
(Received June 26th, 1969)

SUMMARY

Two kinds of membrane were resolved when spheroplast membranes of *Escherichia coli* K12 were dialyzed against EDTA and then subjected to linear sucrose gradient centrifugation.

The less dense band, which is known to be composed mainly of lipoprotein, has a "unit membrane" structure and contains a relatively large amount of ATPase (EC 3.6.1.3) and components of the electron-transfer system. The denser one contained a relatively large amount of carbohydrate and NADH dehydrogenase (EC 1.6.99.3) without any other respiratory components, it has a complex-coiled structure similar to the outer membranes described by BIRDSELL AND COTA-ROBLES[1]. This membrane fraction was found—by immunological studies—to be located on the outside of the cell surface.

INTRODUCTION

The surface layer of Gram-negative bacteria is known to consist of three layers, *viz.*, a cytoplasmic membrane, a mucopeptide layer and an outer cell wall[2]. The mucopeptide layer can be digested and removed by the action of lysozyme and EDTA, and the spherical body called the "spheroplast" is obtained[3]. The spheroplasts so formed still have two component membranes in their envelope[1]. Spheroplast membranes obtained by osmotic shock of the spheroplast would therefore be a mixture of outer and cytoplasmic membranes, and hitherto it has not been possible to separate these. For the biochemical characterization of outer and cytoplasmic membranes, the resolution of these two entities from the spheroplast membrane fraction is necessary.

In a previous paper[4], we have shown that this separation could be performed by sucrose density gradient centrifugation. The present paper concerns the morphological and immunological characteristics of these two fractions.

MATERIALS AND METHODS

Medium and culture condition. Escherichia coli K12 (Met$^-$, F$^-$, λ^-) was used throughout the work. 10 ml of the cell culture, aerobically incubated overnight at 37°

in the glycerol medium according to FRASER AND JERREL[5] was inoculated into 1 l of the medium in a 5-l flask and incubated with shaking. Exponentially growing cells (about 400 mg dry cells/l culture) were harvested by centrifugation and washed once with cold distilled water.

Preparation of spheroplasts and spheroplast membrane subfractions. Spheroplasts were prepared by a modification of the method of REPASKE[6] in the presence of sucrose. About 400 mg of cell dry matter were suspended in 30 mM Tris–HCl (pH 8.0) containing 0.54 M sucrose and 1.3 mM EDTA (the pH of the EDTA was adjusted to 8.0 with NaOH). To this suspension lysozyme (Esai Chem. Co., Tokyo) was added to a final concentration of 200 μg/ml. The suspension was incubated at 30° for 60 min. The spheroplasts thus formed were collected by centrifugation at 25000 $\times$ *g* for 20 min, and caused to burst at 4° in about 50 ml of 5 mM $MgCl_2$ solution. This was vigorously shaken by hand in an ice bath until almost all the spheroplasts were burst. The burst spheroplast suspension was centrifuged at 25000 $\times$ *g* for 20 min, washed twice with about 180 ml of 5 mM $MgCl_2$ solution, suspended in 10 ml of cold 34 mM EDTA (pH 7.0) and dialyzed twice against 500 ml of 34 mM EDTA (pH 7.0) at 4°. Dialyzed preparations were centrifuged at 60000 $\times$ *g* for 60 min and the precipitates were suspended in distilled water. The yield of spheroplast membrane was 24–27 % of the cell dry weight. For a better separation of outer and cytoplasmic membranes it was necessary to prepare spheroplasts which were easily burst by osmotic shock; otherwise a clear-cut separation cannot be achieved.

Subfractionation of EDTA-dialyzed membranes. The procedure for the separation of the two types of membranes was the same as described previously[4].

Chemical analysis. Protein was determined by the method of LOWRY *et al.*[7].

Electron microscopy. Immediately after preparation the membrane fractions were fixed with 1 % OsO_4 according to the method of KELLENBERGER AND RYTER[8]. Fixation was performed for 18 h in the cold (0–4°); dehydration took place in a graded series of ethanol; and the embedding material was butyl and methylmethacrylate (7:3, v/v). Sections were cut on a Porter–Blum MT-2 ultramicrotome with a glass knife and were stained with uranyl acetate[9] and alkaline $Pb(OH)_2$ (ref. 10).

The membrane fractions were also examined by negative staining with 2 % phosphotungstic acid neutralized with KOH according to the method of BRENNER AND HORNE[11]. Electron micrographs were obtained with a Hitachi HU-11B electron microscope.

Preparation and properties of antisera to intact cells. Cells growing exponentially in the medium according to FRASER AND JERREL[5] were harvested and treated with 0.5 % formalin at 30° for 1 h. The cells were recovered by centrifugation, washed three times with 0.85 % NaCl and suspended in the same solution to make the concentration of cells 1 mg/ml. Formalin-treated cells were injected into young rabbits. Serum specimens were obtained from all rabbits prior to immunization. Each rabbit was given 5 intravenous injections of 0.5, 1.0, 2.0 and 4.0 ml of antigens at intervals of 3 days. One week after injection, they were test-bled; the blood was allowed to clot, and was incubated at 37° for 1 h and then at 4° for 15 h. The sera were separated by centrifugation, then pooled and stored at −20°. The individual antisera were titered by a tube precipitin test, and preparations showing titers of 1:1200 or more were used. When the antisera were fractionated with $(NH_4)_2SO_4$, the specific activities of the γ-globulin fraction showed a 20- to 30-fold increase in the agglutination test with

respect to both formalin-treated cells and band C_3. This fraction was further fractionated on a DEAE-cellulose column by stepwise elution with potassium phosphate buffer (pH 8.0) according to SOBER AND PETERSON[12]. After 1.2 ml of γ-globulin fraction (24 mg protein, having a titer of 1:3200 per mg protein) had been applied in 40 mM of potassium phosphate buffer (pH 8.0), the column (2.2 cm × 40 cm) was eluted successively with 200 ml of 40 mM and 400 mM potassium phosphate buffer (pH 8.0) at a flow rate of 9 ml/h at 4°. The protein content and agglutination activity of 10-ml fractions were measured. Before applying tests, subfractionated membranes as well as formalin-treated cells and antisera were dialyzed against 50 mM phosphate buffer (pH 7.0) and 0.1 ml of the cells or membranes were mixed and incubated at 37° for 2 h, and then at 4° for 1 day. After that, the degree of agglutination was estimated visually.

Quantitative absorption of the antibodies by formalin-treated cells. Specific absorption of the antibodies was carried out in tubes by adding various amounts of formalin-treated cells (1.5 ml containing 1–40 mg of cells, dry wt.) to 250 μg protein of the γ-globulin fraction (0.3 ml having a titer of not less than 1:4800/mg protein). The reaction mixture was stirred gently at 37° for 2 h and at 4° for 18 h. Agglutinated bacteria were removed by centrifugation and 0.2 ml of each supernatant fluid was used to assay the agglutination titers for both formalin-treated cells and band C_3 (see previous paper[4]).

RESULTS

Electron microscopy

Two types of membranes were seen in thin sections of the spheroplast membrane. One, which was thought to be a cytoplasmic membrane, was large and had granular attachments. The other, by contrast, was smaller in size, had a complex-coiled structure and was more easily stained with alkaline $Pb(OH)_2$.

Typical electron micrographs of subfractionated membranes are shown in Figs. 1–5. The membrane from band A_3 (see previous paper[4]) was composed largely of vesicles (Fig. 1) and it had a unit membrane structure with a width of about 60–70 Å (Fig. 2). The structure of the membrane from band C_3, on the other hand, was smaller and had a membrane of a complex-coiled structure with a width of about 80–100 Å (Fig. 4). The membrane obtained in band C_3 was more easily stainable with alkaline $Pb(OH)_2$.

With negative staining, membranes of band A_3 (Fig. 3) were seen to be composed of irregular torn fragments. The surface of this membrane were closely packed with fine granules (less than 40 Å in diameter). In some parts larger granules with a diameter of about 100 Å could be seen.

On the other hand, the membranes of band C_3 have different features from those of band A_3: a circular periphery and rather uniform shape. Surfaces of this membrane showed an amorphous rather than granulated profile (Fig. 5).

Immunological properties

The γ-globulin fraction which was obtained from antisera by $(NH_4)_2SO_4$ precipitation, was applied to a DEAE-cellulose column. The elution pattern (Fig. 6) represents three main protein peaks. Agglutination activity for formalin-fixed cells

was tested with each fraction, and the activity was found to parallel the three protein peaks closely. Subfractionated membranes were also tested for agglutination. As shown in Table I, bands C_3 and B_2 were reacted with the fractions corresponding to the three protein peaks. Band C_3 material reacted much better than that of band B_2. Band A_3 did not react with the antibody. Absorption tests were performed to test the possibility that band C_3 was immunologically identical with the outer cell surface.

The γ-globulin fraction was incubated with various amounts of cells. After removing the cells by centrifugation, the antibodies remaining in solution were measured by the agglutination test. As shown in Fig. 7, the activity of antibodies did not alter during the procedure and the residual quantity of antibodies to cell surface

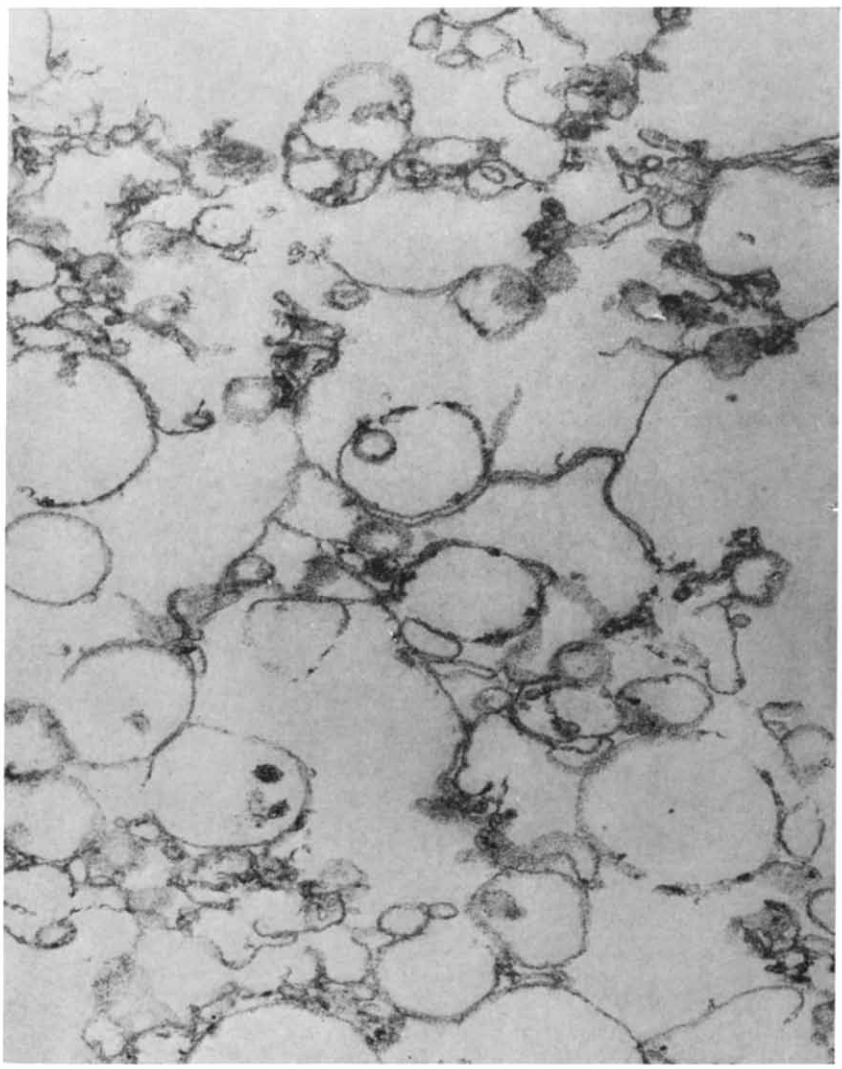

Fig. 1. A typical thin section of subfractionated membrane, band A_3: × 48 000.

material was parallel to the amount of antibody to band C_3. These facts suggest that there is at least one antibody which reacts with both cell surface and band C_3.

Another procedure to obtain outer membranes

When cells were suspended in lysozyme–EDTA solution containing hypertonic sucrose in an ice bath and then warmed to 30°, considerable amounts of fragments were released from cells during incubation at 30° for 60 min. Fragments were isolated and fractionated as follows. Spheroplasts and intact cells were sedimented by centrifugation at 25000 × *g* for 10 min. The supernatants were centrifuged at 60000 × *g* for

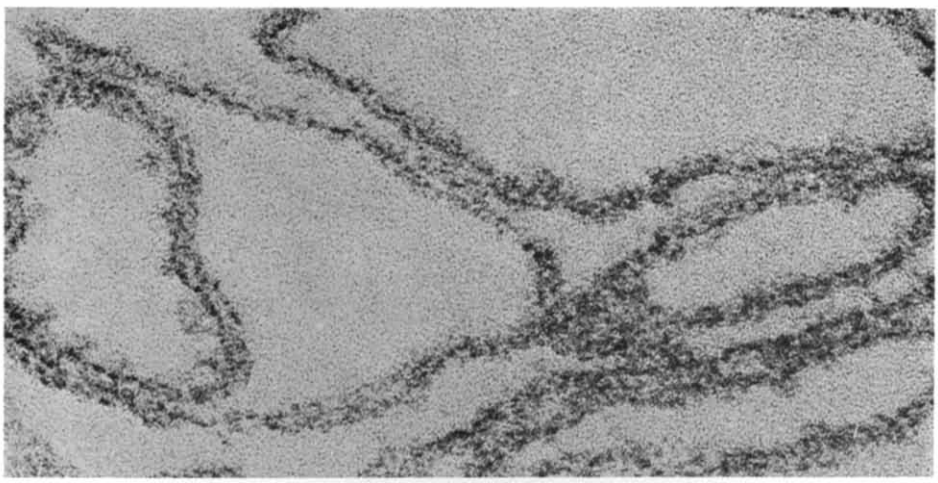

Fig. 2. A thin section of subfractionated membrane, band A_3: × 350000.

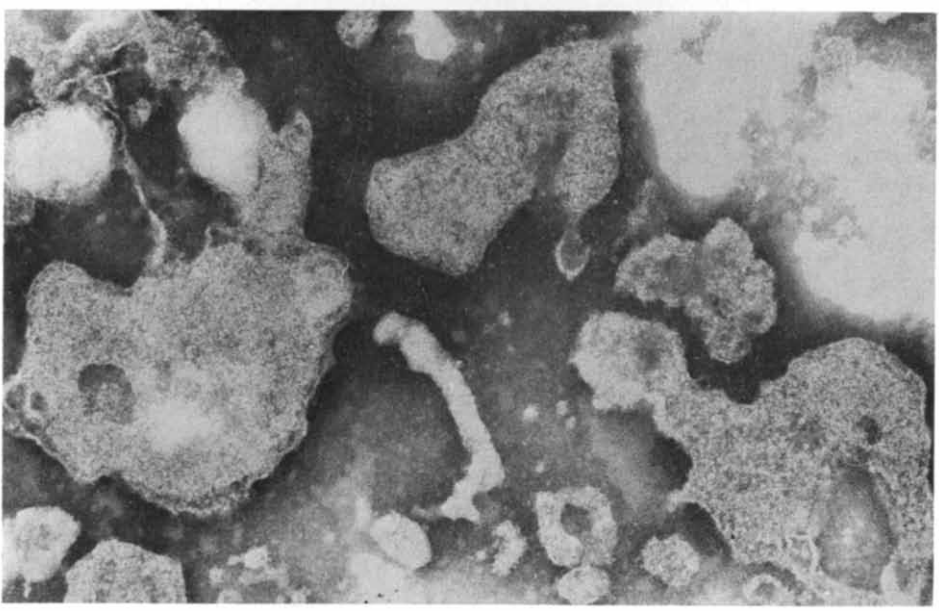

Fig. 3. Negative staining of subfractionated membrane, band A_3; membranes were granular-surfaced: × 124000.

60 min. The precipitates, which were composed mainly of fragments of cell surfaces, were dialyzed against 34 mM EDTA and applied to a 35–50 % sucrose density gradient. After centrifugation at 140000 × *g* for 4 h, three bands were observed in the centrifuge tube which seemed to correspond to bands A_1, B_1 and C_1 (see Fig. 1 of previous paper[4]) in density and color. The amounts of protein in each band (derived from 250 mg cells, dry wt.) were 1.5, 8.5, and 11.2 mg for the bands A_1, B_1, and C_1, respectively. The main fraction, which corresponded to band C_1, was composed mainly of vesicular membranes identical (electron-microscopically) with the outer membranes shown in Figs. 4 and 5.

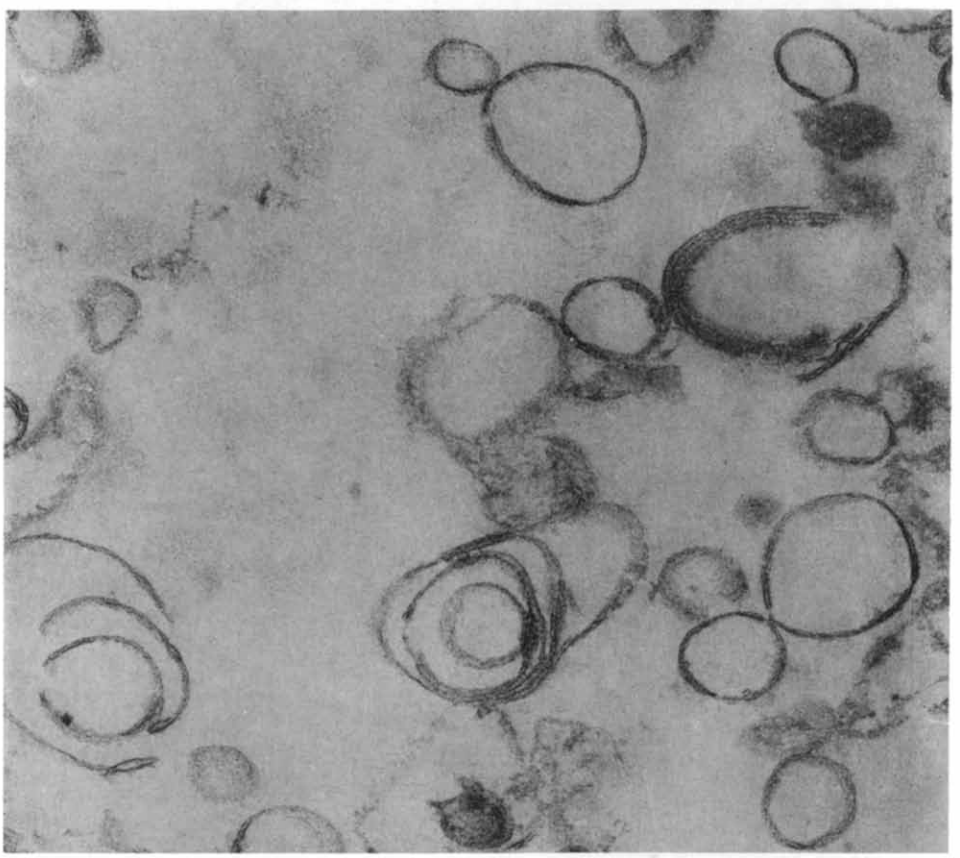

Fig. 4. A typical thin section of subfractionated membrane, band C_3 which was composed largely of complex-coiled structures: × 68000.

DISCUSSION

The spheroplast membrane of *E. coli* K12 was resolved into three subfractions by sucrose density gradient centrifugation. As reported in the previous paper[4], the least dense fraction (band A_3) contained the ATPase activity, components of the electron transfer system, considerable phospholipid but no significant amount of carbohydrate. These chemical and enzymic characteristics were very similar to those

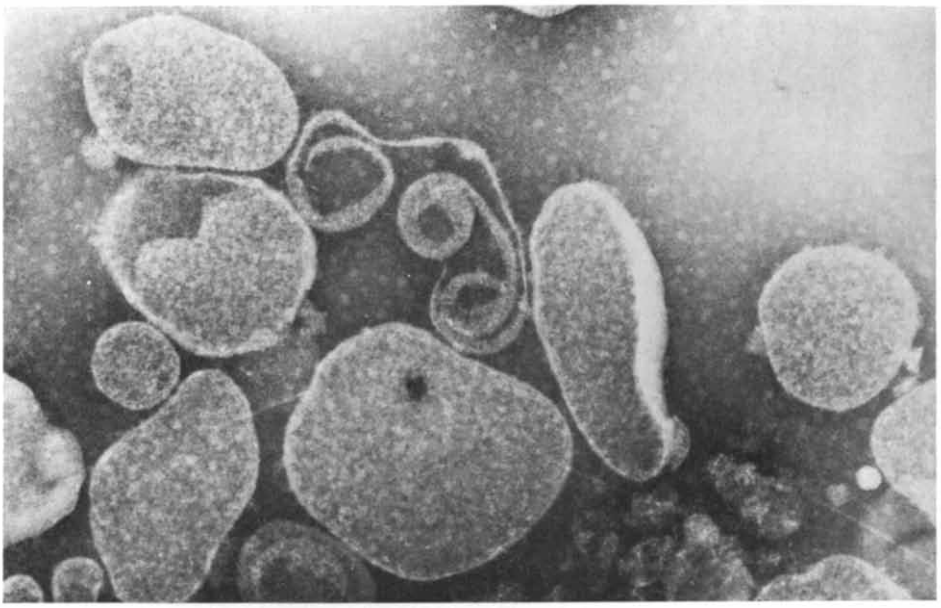

Fig. 5. Negative staining of subfractionated membrane, band C_3; this fraction was composed of fairly regular and uniform membranes: × 120000.

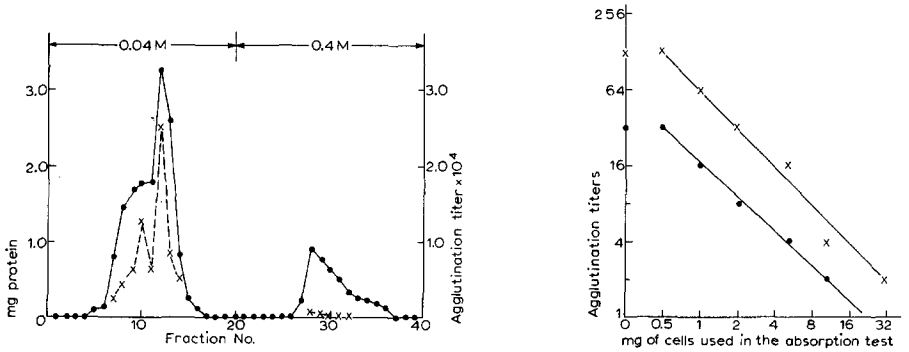

Fig. 6. DEAE-cellulose column chromatography and agglutination test on the γ-globulin fraction. Each fraction (10 ml) was dialyzed against 50 mM phosphate buffer (pH 7.0) and used for determination of protein content and agglutination activity (determined as described in MATERIALS AND METHODS). ●—●, protein content; ×---×, agglutination activity for formalin-treated cells.

Fig. 7. Agglutination activities of the residual antibodies after absorption by formalin-treated cells. The γ-globulin fraction was absorbed by various amounts of formalin-treated cells and the agglutination activities of the unabsorbed antibodies were tested on both the formalin-treated cells and band C_3, as described in MATERIALS AND METHODS. Agglutination titers of the unabsorbed antibodies for formalin-treated cells (●—●) and for band C_3 (×—×).

of the cytoplasmic membranes of Gram-positive bacteria[13]. Band C_3, on the other hand, had only the NADH dehydrogenase activity (as far as we could tell from the present tests) and was richer in carbohydrate and poorer in phospholipid than band A_3.

Electron-microscopic observations revealed that band A_3 was largely composed

of membranous vesicles (Fig. 1, negative staining) and triple-layered structures (Fig. 2, ultrathin sectioning). On the other hand, thin sections of band C_3 indicate that most have become orientated into spherical or complex-coiled structures (Fig. 4). BIRDSELL AND COTA-ROBLES[1] have recently shown that during spheroplast formation, outer membranes of *E. coli* would be broken by EDTA and form complex-coiled structures while cytoplasmic membranes remain intact. Similar observations on the outer membranes of a marine Pseudomonad were reported by COSTERTON *et al.*[14] who showed that the detached outer membrane isolated by centrifugation is composed mainly of spherical or complex-coiled structures.

TABLE I

AGGLUTINATION TEST ON SUBFRACTIONS OBTAINED BY DEAE-CELLULOSE COLUMN CHROMATOGRAPHY

Each 10-ml fraction from DEAE-cellulose column chromatography (Fig. 6) was dialyzed against 50 mM phosphate buffer (pH 7.2) and tested for agglutination activities as described in MATERIALS AND METHODS. +++, agglutinated within 15 min at 37°; ++, agglutinated within 2 h at 37°; +, agglutinated after being incubated at 37° for 2 h and then at 4° for 1 day; ± and −, agglutinated or non-agglutinated after being incubated at 37° for 2 h and then at 4° for 7 days, respectively.

Band	*Reactivity (per 20 μg antibody protein)*														
Fraction No.:	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*28*	*29*	*30*	*31*	*32*	*33*	*34*
A_3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B_2	−	−	±	+	++	++	±	−	−	−	±	−	−	−	−	−
C_3	−	±	+	+	++	+++	+++	±	−	−	−	±	−	−	−	−

Immunological studies on the subfractionated membranes show that the antibody to the intact cell could react only with the membranes isolated in band C_3. The possibility could not be excluded that the antibody to formalin-treated cells could react with certain intracellular constituents. However, the antigen of cells of Gram-negative bacteria are known to be located in the outermost layer of the cell[15], and the parallel reactivity of the antibodies to intact cell and band C_3 would indicate that the antigenic properties of band C_3 material are very similar to those of the outer surface of *E. coli*. The biological, morphological and immunological characteristics of bands A_3 and C_3, described in this paper, indicate that the cytoplasmic and outer membranes of *E. coli* can indeed be separated by density gradient centrifugation after treatment of the spheroplast membranes with fairly high concentrations of EDTA.

These findings may clarify the properties of outer and cytoplasmic membranes and the relationship between them.

ACKNOWLEDGMENTS

The authors express their thanks to Dr. S. Fukui for his interest and to Mr. M. Itho for characterising the strain used in this study. The work was supported in part by the Waksman Foundation of Japan Inc.

REFERENCES

1 D. C. BIRDSELL AND E. H. COTA-ROBLES, *J. Bacteriol.*, 93 (1967) 427.
2 R. G. E. MURRAY, P. STEED AND H. E. ELSON, *Can. J. Microbiol.*, 11 (1965) 547.
3 K. MCQUILLEN, in I. C. GUNSALUS AND R. Y. STANIER, *The Bacteria*, Vol. 1, Academic Press, New York, 1962, p. 261.
4 T. MIURA AND S. MIZUSHIMA, *Biochim. Biophys. Acta*, 150 (1968) 159.
5 D. FRASER AND E. A. JERREL, *J. Biol. Chem.*, 205 (1953) 291.
6 R. REPASKE, *Biochim. Biophys. Acta*, 22 (1956) 189.
7 O. H. LOWRY, N. J. ROSEBROUGH, A. J. FAHR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
8 E. KELLENBERGER AND A. RYTER, *J. Biophys. Biochem. Cytol.*, 4 (1958) 671.
9 M. L. WATSON, *J. Biophys. Biochem. Cytol.*, 4 (1958) 457.
10 G. MILLONIG, *J. Biophys. Biochem. Cytol.*, 11 (1961) 736.
11 S. BRENNER AND R. W. HORNE, *Biochim. Biophys. Acta*, 34 (1959) 103.
12 H. A. SOBER AND E. A. PETERSON, *Federation Proc.*, 17 (1958) 1116.
13 S. MIZUSHIMA, M. ISHIDA AND T. MIURA, *J. Biochem. Tokyo*, 60 (1966) 256.
14 J. W. COSTERTON, C. FORSBERG, T. I. MATULA, F. L. A. BUCKMIRE AND R. A. MACLEOD, *J. Bacteriol.*, 94 (1967) 1764.
15 K. FUKUSHI, F. ARIJI AND J. YAMAGUCHI, *6th Intern. Congr. Electron Microscopy, Kyoto, 1966, Electron Microscopy*, Vol. 2, Maruzen Co., Tokyo, 1966, p. 271.