PRELIMINARY NOTES

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Separation by density gradient centrifugation of two types of membranes from spheroplast membrane of *Escherichia coli* K12

The surface layer of Gram-negative bacteria has been known to consist mainly of three layers, that is, a cytoplasmic membrane, a mucocomplex layer and an outer membrane of cell wall[1]. Spheroplasts, which were formed by the action of lysozyme (EC 3.2.1.17), still have double membranes which correspond to the outer and cytoplasmic membranes of their envelope[2]. Spheroplast membranes obtained by osmotic shock of the spheroplasts, have been thought to be a mixture of outer and cytoplasmic membranes. We have shown that membrane preparations of *Bacillus megaterium* could be separated into their three subfractions by the sucrose-density gradient centrifugation[3,4]. The procedure was applied to *Escherichia coli*. In this report, a separation of outer membrane from cytoplasmic membrane in *E. coli* is presented.

The cells of *E. coli* K12 were grown in FRASER AND JERREL'S[5] medium, harvested at the middle of exponential phase and washed with distilled water. The

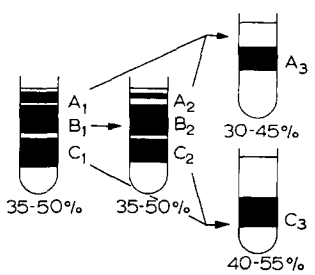


Fig. 1. Schematic representation of separation of membranes by sucrose-density gradient centrifugation. Sucrose solutions of the following linear density gradients were prepared in 6-ml cellulose tubes; (1) 4.4 ml of 0–30% sucrose on 0.2 ml of 50% sucrose (SDG-1), (2) 4.6 ml of 35–50% sucrose (SDG-2), (3) 4.6 ml of 30–45% sucrose (SDG-3), (4) 4.6 ml of 40–55% sucrose (SDG-4). The membrane fractions obtained by an osmotic shock of spheroplasts were homogenized. The membranous preparation (3.6 ml containing about 120 mg membranes) was layered onto SDG-1 in 3 tubes. After centrifugation in an RPS 40 roter (swinging type) of Hitachi 40p Ultracentrifuge at 140000 × *g* for 2 h, membranous fractions were recovered as precipitates. The precipitate was diluted with water to make the sucrose concentration less than 35% and layered onto SDG-2 in 6 tubes. Centrifugation at 140000 × *g* for 4 h yielded visible bands A_1, B_1 and C_1. These three bands were collected by means of a J-shaped pipette. The fractions were diluted with distilled water and centrifuged at 140000 × *g* for 1 h. The precipitates were homogenized in distilled water. The homogenate from band B_1 was then layered onto SDG-2 in 3 tubes and centrifuged at 140000 × *g* for 4 h. Bands A_2, B_2 and C_2 were obtained. Then A_2 was mixed with A_1 and C_2 was mixed with C_1. The mixture of A_1 and A_2 was layered onto SDG-3 (3 tubes) and the mixture of C_1 and C_2 onto SDG-4 (3 tubes). These tubes were centrifuged at 140000 × *g* for 4 h. From the mixture of A_1 and A_2, a single band, A_3, was obtained and a single band, C_3, was obtained from the mixture of C_1 and C_2. Density equilibrium was reached within 4 h in all cases and the gradient pattern was not altered after a 7-h centrifugation. These purified bands A_3, B_2 and C_3 were collected by means of a J-shaped pipette and used for chemical, biochemical and electron-microscopic observations.

spheroplasts were prepared by the method of REPASKE[6]. The spheroplasts were collected by centrifugation, burst at 4° in a 5 mM $MgCl_2$ solution containing a trace amount of deoxyribonuclease (EC 2.7.7.16) and washed twice with cold 5 mM $MgCl_2$ solution. The crude membrane was collected by centrifugation at 25000 × *g* for 20 min, suspended in 3 mM EDTA (pH 7.0) and dialysed against 3 mM EDTA (pH 7.0) at 4° overnight. Dialysed preparations were centrifuged at 60000 × *g* for 1 h. The precipitates were suspended in distilled water and applied to sucrose for density gradient centrifugation. The procedure and results of subfractionation of dialysed membranes by the density gradient centrifugation are shown in Fig. 1. Thus bands A_3, B_2 and C_3 were obtained. The chemical composition of bands A_3, B_2 and C_3 is shown in Table I. Proteins were equally distributed in these fractions. However, lipid content was much higher in band A_3 and lower in band C_3 than in band B_2. On the other hand, carbohydrate was concentrated in band C_3. A ratio of carbohydrate to protein in band C_3 was 4–5 times that in band A_3. Table II shows the distribution of enzymes and cytochromes in these fractions. ATPase (EC 3.6.1.3) and the components of the electron-transport chain were concentrated in band A_3.

TABLE I

CHEMICAL COMPOSITION OF FRACTIONATED MEMBRANES

Data represent mg/g whole cells.

*Material**	*Band* A_3	*Band* B_2	*Band* C_3
Protein	18.3 ± 0.4	18.9 ± 2.9	17.8 ± 0.5
Phospholipid**	9.1 ± 0.4	8.3 ± 0.8	3.4 ± 0.2
Carbohydrate	0.24 ± 0.01	0.67 ± 0.13	1.19 ± 0.12
RNA	0.82 ± 0.01	1.20 ± 0.16	0.58 ± 0.01

* Each was determined as described previously[3].
** Phospholipid = lipid-phosphorus × 25.5.

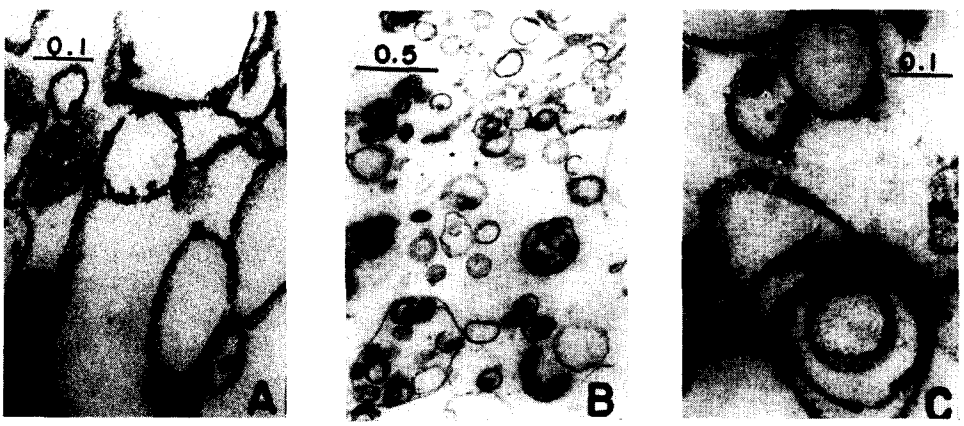
TABLE II

DISTRIBUTION OF ENZYMES AND CYTOCHROMES* AMONG FRACTIONATED MEMBRANES

	Activity (μmoles/mg protein per h, 20°)		
	Band A_3	*Band* B_2	*Band* C_3
ATPase**	2.7 ± 0.3	1.5 ± 0.6	<0.3
NADH dehydrogenase	1.09 ± 0.01	0.88 ± 0.40	0.50 ± 0.04
Succinate dehydrogenase	0.38 ± 0.01	0.37 ± 0.08	<0.02
Cytochrome *a****	0.07 ± 0.01	0.03 ± 0.01	<0.01
Cytochrome *b****	0.18 ± 0.01	0.09 ± 0.01	<0.03

* Each was determined as described previously[3].
** Assay was at 35°.
*** Amounts of cytochromes *a* and *b* were determined from the difference spectra (reduced with dithionite *minus* untreated) at 444 mμ and 426 mμ which correspond to the Soret bands of cytochromes *a* and *b* in *E. coli* K12, respectively, and are expressed as the absorbance at 444 mμ and 426 mμ per mg protein, respectively.

Typical electron micrographs of each thin section of these fractions are shown in Fig. 2. Band A_3 was comprised largely of vesicles with a width of about 75 Å unit membrane. Band C_3, which also showed a unit membrane structure of a width almost equal to band A_3, gave features very different from those of band A_3, that is, a somewhat complex coiled structure which was easily stained by alkaline lead hydroxide. Recently BIRDSELL AND COTA-ROBLES[2] have shown that outer membrane of *E. coli* could be broken by EDTA and formed the complex coiled structure during spheroplast formation. Results of chemical, enzymatic and electron-microscopic observations presented in this paper suggest that band C_3, which is comprised of complex coiled membranes, is mainly composed of the components derived from the outer



Flg. 2. Electron micrographs of fractionated membranes. A. Band A_3. B. Band B_2. C. Band C_3. Electron-microscopic studies were performed as described previously[3].

membrane of the cell wall. On the other hand, cytoplasmic membranes of spheroplasts of *E. coli* seemed to be separated from outer membranes into band A_3 by the density gradient centrifugation. Band B_2 might be a mixture of outer and cytoplasmic membranes and non-membranous materials.

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