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Distribution of Lipids in the Wall and Cytoplasmic Membrane Subfractions of the Cell Envelope of *Escherichia coli*

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Cell wall and membrane subfractions of the cell envelope of *Escherichia coli* have been isolated by a procedure involving particle electrophoresis and sucrose gradient density centrifugation. The lipid content of each fraction has been investigated. The individual phospholipids of both fractions are quantitatively similar except that the proportion of lysophosphatidylethanolamine is greater in the wall than in the membrane. Fatty acid analysis of the phospholipids of each fraction revealed that the wall phospholipids contain a greater proportion of palmitic acid. Coenzyme Q is almost exclusively localized in the cell membrane.

Present evidence indicates that the cell envelope of *Escherichia coli* contains two membranous components, the cell wall and the cytoplasmic membrane (15). To pursue questions dealing with compositional differences in these two membranes, as well as the mechanism of assembly of them, it was necessary to develop convenient techniques for their separation. Recently, suitable procedures for this separation have been developed (13, 17). Thus, Schnaitman was able to show marked compositional differences in the membranous wall and the cytoplasmic membrane fraction: the wall was relatively rich in lipopolysaccharide, whereas the cytoplasmic membrane contained succinic dehydrogenase and other respiratory enzymes (17). In addition, marked qualitative differences were observed in the protein composition of the wall and the membrane. However, prior to this study little or no information was available on the lipid composition of the two fractions. The primary aim of this investigation was to define the composition of the phospholipids of the wall and the membrane subfractions of the cell envelope.

MATERIALS AND METHODS

Materials. Coenzyme Q was purchased from Sigma Chemical Co., St. Louis, Mo. Phosphatidylethanolamine and cardiolipin were purchased from Serdary Research Laboratories, London, Ontario, Canada. Phosphatidylglycerol was purchased from Smith, Klein, and French Laboratories, Philadelphia, Pa.

Lysophosphatidylethanolamine was prepared by treatment of phosphatidylethanolamine with snake venom phospholipase A as described by Long and Penny (11).

Analytical Procedures. Protein was determined by the method of Lowry et al. (5) using bovine serum albumin as the standard. Total lipid was extracted from samples of each of the three fractions, whole envelope, membrane, and wall, by the method of Garbus et al. (6). Neutral lipid was separated from phospholipid by loading the total extract on a column (5 by 1 cm) of silicic acid packed in chloroform and eluting with 15 ml of chloroform. Phospholipids were eluted with 15 ml of chloroform-methanol-water (1:1:0.2). Qualitative analyses of phospholipid fractions were performed on silicic acid-impregnated paper developed at 22 C for 12 to 20 hr in diisobutylketone-acetic acid-water (40:30:3) and by thin-layer chromatography on Silica Gel H (0.4 mm thick) containing 1 mM sodium tetraborate and developed with chloroform-methanol-water (95:35:5). Phospholipids were identified in these systems by comparison with authentic standards. For quantitative analysis of phospholipids, approximately 350 μmoles of lipid phosphorus was applied to the thin-layer plates, and after development the phospholipids were detected and assayed as described previously (19).

For fatty acid analysis, samples of each phospholipid fraction were deacylated by alkaline hydrolysis and the fatty acids were methylated with diazomethane. Analysis of the fatty acid esters was carried out at 175 C on a 6-ft column (ca. 1.8 m) of 15% diethyleneglycol succinate on chromosorb P by using

an E.I.R. gas chromatograph equipped with an ionization detector. Total fatty acids, including β-hydroxymyristic acid, were isolated by direct alkaline hydrolysis of the envelope and its subfractions (14) and analyzed by gas chromatography. Standard β-hydroxymyristic acid was provided by John Law, University of Chicago.

Coenzyme Q was isolated from the neutral lipid fraction by thin-layer chromatography on Silica Gel G plates (0.4 mm thick) developed in light petroleum (bp = 37–50 C)-chloroform (1:4, v/v) as described by Cox and Gibson (4). The area coinciding in mobility to authentic coenzyme Q_{10} was scraped from the plate and the coenzyme Q eluted with developing solvent. After evaporation to dryness with nitrogen, the residue was dissolved in ethanol and the coenzyme Q content was estimated from the difference in absorbance at 275 nm before and after reduction with KBH_4 (8, 9).

Culture and growth conditions. The organism used in this study was *E. coli* J-5, a mutant derived from *E. coli* 0111_{B4} which is lacking the enzyme uridine-diphosphate-galactose-4-epimerase. The culture was a gift of Edward Heath. Galactose added to cultures of this mutant is incorporated exclusively into lipopolysaccharide (5, 10). Cultures were grown on minimal salts medium containing 1% succinate as a carbon source (16). The inoculum for the cultures was grown for 16 hr in the above medium supplemented with 20 μg of L-leucine per ml, 20 μg of L-tyrosine per ml, and 2.0 mM D-fucose. The fucose was added to induce the formation of the galactose transport system. Final cultures were grown from a 1.5% inoculum to late log phase (80% of maximal turbidity) in the above medium supplemented with 20 μg of L-leucine per ml, 0.05 μCi of ^{3}H-L-leucine per ml, 20 μg of L-tyrosine per ml, 0.05 μCi of ^{3}H-L-tyrosine per ml, 0.5 mM D-galactose, and 0.016 μCi of ^{14}C-D-galactose per ml. Cultures were grown on a rotary shaker at 37 C, and the doubling time of the cultures under these growth conditions was 1.6 hr.

Breakage of cells and isolation of envelope subfractions. The cultures were harvested and resuspended in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2′-ethane sulfonic acid (HEPES) buffer, *p*H 7.4, and treated with a blendor to remove extraneous cellular material (16). The cells were washed two times with the same buffer and suspended in the same buffer containing approximately 20 μg of ribonuclease per ml and 20 μg of deoxyribonuclease per ml. The cells were broken by passage once through a French pressure cell fitted with a nylon-tipped needle valve. After breakage, the suspension was made 0.1 mM with respect to $MgCl_2$ and was centrifuged at 7,000 × *g* for 10 min to remove unbroken cells. The supernatant fluid was then centrifuged at 200,000 × *g* for 45 min to sediment the envelope fraction. The envelope fraction was washed three times by suspension in electrophoresis chamber buffer (*see below*) and was centrifuged as above. The envelope fraction was then suspended in electrophoresis chamber buffer at a concentration of 20 mg of protein/ml and fractionated on a Brinkmann-DESAGA FF-4 preparative particle electrophorograph essentially as described by Heidrich, et al. (7). The electrophoresis chamber buffer consisted of 10 mM triethanolamine, 10 mM acetic acid, 0.1 mM $MgCl_2$, and 0.33 M sucrose and was adjusted to *p*H 7.4 with NaOH. The electrode buffer consisted of 0.1 M triethanolamine and 0.1 M acetic acid and was adjusted to *p*H 7.4 with NaOH. Electrophoresis was carried out at 4 C at 1 kv and 120 ma. Sample was injected at 1.5 ml/hr, and the chamber flow rate was 1.4 ml per fraction per hr. Peak fractions were pooled and centrifuged at 200,000 × *g* for 1 hr. The pellets were resuspended in HEPES buffer as above, and applied to sucrose gradients. Further details on fractionation are given below. Preparations were frozen prior to analysis.

Succinic dehydrogenase was assayed by a modification of the method of Arrigoni and Singer (1). The enzyme was activated by incubation for 15 min at 23 C in a solution containing 0.05 M phosphate buffer (*p*H 7.6), 40 mM succinate, 1 mM ethylenediaminetetraacetic acid, and 0.5 mM KCN. The reaction was initiated by the addition of 0.06 mM cytochrome *c* and 0.5 mM phenazine methosulfate and was followed spectrophotometrically at 550 nm.

RESULTS AND DISCUSSION

Fractionation of the E. coli envelope. Figure 1 shows the distribution of protein, succinic dehydrogenase, and ^{14}C-galactose after fractionation of the envelope by preparative particle electrophoresis. The cytoplasmic membrane fragments migrate strongly towards the anode, as indicated by the peak of succinic dehydrogenase activity. The cell wall, as indi-

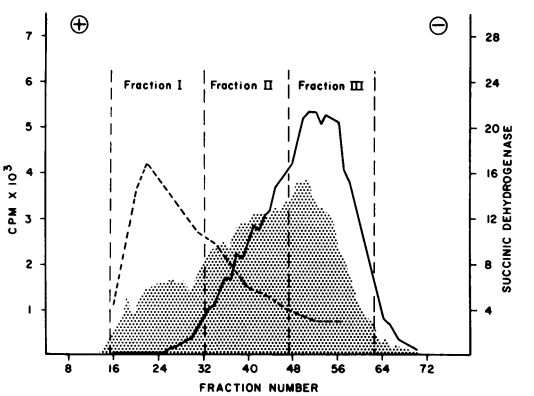

FIG. 1. *Profile of ^{3}H-labeled protein (stippled area), succinic dehydrogenase (dashed line), and ^{14}C-galactose (solid line) after fractionation of the E. coli envelope by preparative particle electrophoresis. The conditions of electrophoresis are indicated in Materials and Methods. A total of 92 fractions were collected across the bottom of the electrophoresis chamber, and the sample injection port was located above fraction 64. Vertical dashed lines indicate the individual fractions which were pooled to give three major fractions, Fraction I (cytoplasmic membrane), Fraction II (intermediate fraction), and Fraction III (cell wall fraction). Succinic dehydrogenase activity is expressed in arbitrary units.*

cated by the ^{14}C-galactose in lipopolysaccharide, shows very little migration. Previous results have indicated that this type of separation is obtained only with envelopes from cells which form a complete lipopolysaccharide. If galactose is omitted from the medium, the cell walls migrate much more strongly towards the anode and a much poorer separation is obtained (C. Schnaitman and H. Heidrich, *in preparation*).

Individual tubes were pooled into three major fractions, as indicated by the dashed lines. Fraction I contained primarily cytoplasmic membrane, Fraction II contained both cytoplasmic membrane and cell wall, and Fraction III contained primarily cell wall. Each of these fractions was then centrifuged in a continuous sucrose gradient (Fig. 2). The fractions from the sucrose gradients which were pooled to obtain the final preparations of cell wall (Fig. 2) and cytoplasmic membrane (Fig. 2) are indicated by the vertical dashed lines. The intermediate fraction (Fraction II, Fig. 1) appeared to contain both cell wall and cytoplasmic membrane, but in addition contained a peak of protein with a density which was intermediate between that of the cell wall and cytoplasmic membrane. This property would be predicted for fragments of cell wall and cytoplasmic membrane which are "fused" or attached together as described by Bayer and Remsen (2) and Schnaitman (18). Examination of this intermediate density fraction by electron microscopy suggests that it is enriched in such fused fragments. Preliminary examination of this intermediate density fraction has failed to demonstrate any unique lipid composition or enrichment of the enzymes of phospholipid biosynthesis, so it has not as yet been possible to demonstrate any physiological significance of these fused fragments. (Further experiments on this fraction are in progress.)

Results on the distribution of ^{14}C-galactose and of succinic dehydrogenase in the envelope subfractions after the particle electrophoresis step and after the sucrose gradient purification are summarized in Table 1. There was little cross contamination of wall and membrane as indicated by the low succinic dehydrogenase activity in the cell wall fraction and the low ^{14}C-galactose label in the cytoplasmic membrane fraction. The combined fractionation on the basis of surface charge (particle electrophoresis) and density yielded much purer fractions than were previously obtained by gradient centrifugation alone, although the results are qualitatively identical (17). Because only the peak tubes of each fraction were combined during

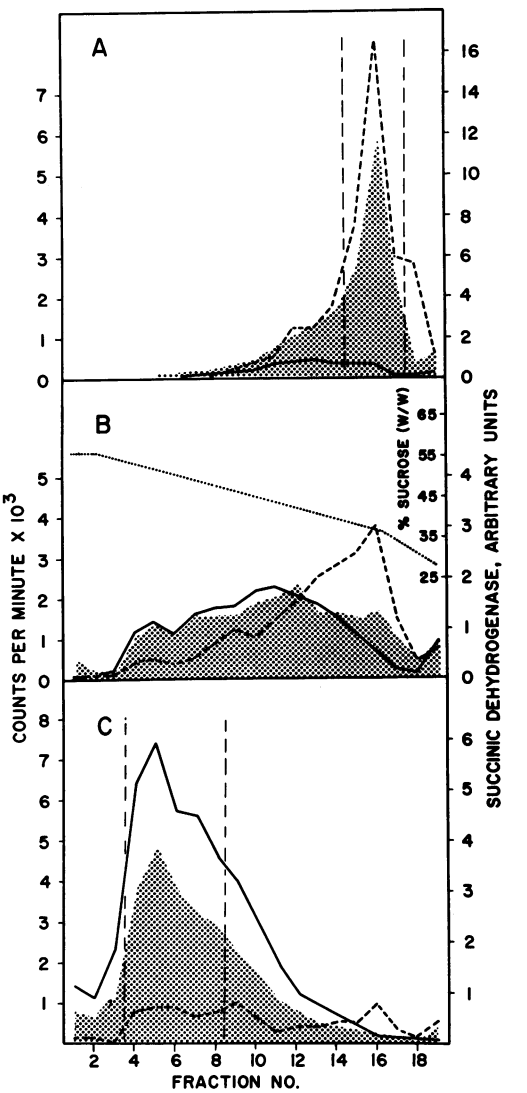

FIG. 2. *Sucrose gradient fractionation of the three major fractions obtained by preparative particle electrophoresis. (A) Cytoplasmic membrane fraction (Fraction I, Fig. 1), (B) intermediate fraction (Fraction II, Fig. 1), (C) cell wall fraction (Fraction III, Fig. 1). Dashed line indicates succinic dehydrogenase, solid line indicates ^{14}C-galactose, and stippled area indicates ^{3}H-labeled protein. The fractions from the particle electrophoresis step were centrifuged at 200,000 × g for 1 hr, and the pellets were resuspended in 10 mM HEPES buffer (pH 7.4) and layered over a 35 to 60% (w/w) sucrose gradient prepared in the same buffer. The gradient was centrifuged for 18 hr at 27,000 rev/min in a Spinco SW 27 rotor. Dotted line in B indicates the sucrose concentration in the gradient. Vertical dashed lines in A and C indicate the fractions which were pooled for the final cytoplasmic membrane and cell wall preparations.*

purification, the final yield of both cell wall and cytoplasmic membrane was about 20% as based on the succinic dehydrogenase and ^{14}C-galactose present in the envelope fraction after breakage in the French pressure cell. However, there was no appreciable loss of total lipid, succinic dehydrogenase activity, or lipopolysaccharide during the fractionation procedure.

In Table 2 a more extensive study of the composition of the purified wall and membrane fraction is shown. Of particular interest was the finding that the distribution of coenzyme Q, a component of the respiratory chain, paralleled that of succinic dehydrogenase; both were almost exclusively found in the inner membrane. Also evident is a marked difference in the ratio of phospholipid to protein in the two fractions, the ratio in the membrane fraction being almost twice that found in the wall. ^{14}C-galactose, in lipopolysaccharide was largely confined to the wall. Total fatty acid analysis of the fractions revealed that β-hydroxymyristic acid, a component thought to be specific for lipopolysaccharide (3), comprised 20% of the envelope fatty acids, 60% of the wall fatty acids, and 8% of the membrane fatty acids. The presence of a small amount of β-hydroxymyristate in the membrane suggests that incomplete lipopolysaccharide precursors may be present in the cytoplasmic membrane.

Because of the difference in the ratio of phospholipid to protein in the two fractions of the cell envelope, a quantitative and qualita-

TABLE 1. *Distribution of ^{14}C-galactose in lipopolysaccharide (LPS) and succinic dehydrogenase activity in envelope subfractions*

Description of sample	^{14}C-Galactose (LPS) (counts per min × 10^4 per mg of protein)	Succinic dehydrogenase (μmoles per min per mg of protein)
Unfractionated envelope	6.47	1.18
Cytoplasmic membrane fraction after electrophoresis step	1.03	2.69
Cytoplasmic membrane fraction, final preparation from gradient	0.60	2.89
Cell wall fraction after electrophoresis step	9.62	0.67
Cell wall fraction, final preparation from gradient	9.96	0.15

TABLE 2. *Analysis of fractions of the cell envelope*

Component measured[a]	Envelope	Wall	Membrane
SDH[b] (μmoles per min per mg)	1.18	0.15	2.89
Coenzyme Q (nmoles per mg)		<0.4	22.3
Lipid P (μg atoms per mg)	0.609	0.435	0.725
^{14}C-galactose (counts per min × 10^4 per mg)	6.47	9.96	0.60

[a] Results expressed in terms of milligrams of protein of the appropriate fraction.
[b] Succinic dehydrogenase.

TABLE 3. *Phospholipid content of fractions of the cell envelope*[a]

Phospholipid[b]	Envelope	Wall	Membrane
PE	66.3	63.4	78.0
PG	11.3	10.6	11.0
DiPG	10.6	6.3–9.3[c]	6.3
LysoPE	6.3	9.4	<1.0
Unidentified	5.6	7.3–10.3[c]	4.7

[a] Results expressed as the per cent total lipid P. Analyses were performed in sextuplicate, and the values varied ±2%. Recovery of total lipid P from the thin-layer plates was 98 to 102%.
[b] Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; diPG, diphosphatidylglycerol; lysoPE, lysophosphatidylethanolamine.
[c] There was some overlap between DiPG and the unidentified minor component. Therefore, these values are subject to the error indicated.

TABLE 4. *Fatty acid content of the total phospholipids of envelope fractions*[a]

Fraction	Fatty acid[b]							
	14:0	16:0	16:1	17:cy	18:0	18:1	19:cy	Total unidentified
Wall	4.5	40.0	25.5	7.0		18.4	2.9	
Membrane	2.6	29.0	22.5	8.2	2.0	23.0	3.6	7.7

[a] Results expressed as per cent total fatty acids. Analyses were performed in duplicate, and the values varied ±3%. All fatty acids comprising less than 2% are omitted.
[b] Abbreviations: Fatty acid chain length is indicated by numeral before the colon, and degree of unsaturation is indicated by the numeral after the colon; cy represents cyclopropane ring.

tive analysis of the lipids of these fractions was undertaken. The results summarized in Table 3 reveal that the phospholipids in both fractions are qualitatively identical; phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) are the major components. One quantitative difference was observed: the level of lysophosphatidylethanolamine was much higher in the wall than in the membrane, with commensurate differences in the level of PE in the two fractions. However, it should be noted that these differences may not obtain in vivo since preliminary studies indicate the presence of a phospholipase in the crude envelope.

Fatty acid analysis of the total phospholipids of each fraction shown in Table 4 revealed only one noteworthy difference: the proportion of palmitic acid was significantly higher in the phospholipids of the wall than in those of the membrane. As expected, when the fatty acids of the individual phospholipids, PE and PG, were analyzed, similar compositional differences were observed.

In summary, except for the differences noted above, these results reveal that, although the phospholipid content per milligram of cytoplasmic membrane protein is almost twice that of the wall, the phospholipid composition of both is very similar. Recent studies in *E. coli* (White et al., *in press*) as well as in *Salmonella* sp. (Bell et al., *in press*) have shown that, with the possible exception of cytidine diphosphate - diglyceride:L - serine phosphatidyl transferase, the enzymes involved in biosynthesis of the wall and membrane phospholipids are localized in the membrane. Thus, the finding of a relatively symmetric compositional distribution of the phospholipids in the wall and membrane fraction and an asymmetric distribution of enzymes responsible for their synthesis poses interesting questions concerning the mechanism of transfer of phospholipids synthesized in the membrane to the wall.

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