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Isolation and Characterization of Outer and Inner Membranes of *Selenomonas ruminantium*: Lipid Compositions

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The isolation procedure and characterization of the outer and inner membranes from *Selenomonas ruminantium* cells, a strictly anaerobic bacterium, are described. The metabolic fate of [^{14}C]decanoate incorporated into the outer and inner membranes was examined. The percent distribution of radioactivities in the outer and inner membranes was about 40 and 50% of the total incorporated activity, respectively. Approximately 47% of the radioactivity incorporated into the outer membrane was recovered in the phospholipid fraction, and the remaining radioactivity was found in both aqueous and phenol layers when the outer membrane was treated with phenol-water. In contrast to [^{14}C]decanoate, the percent distribution of [^{3}H]glycerol in the outer and inner membranes was about 25 and 70% of the total incorporated activity, respectively. Most of the assimilated ^{3}H was located in the phospholipid fraction of both membranes. However, no significant label was detected in either the protein or cell wall fraction. The following observations were made concerning lipid compositions in the outer and inner membranes by chemical and isotopic analyses. (i) The outer and inner membranes contained no detectable phosphatidyl glycerol or cardiolipin. (ii) A prominent radioactive compound, designated band III lipid, was found mainly in the outer membrane as a major radioactive spot when cells were grown with [^{14}C]decanoate. This lipid contained phosphorus, 2-keto-3-deoxyoctulosonic acid and 3-OH fatty acid but no detectable glycerol. This lipid was identified tentatively to be 2-keto-3-deoxyoctulosonic acid-lipid A. (iii) Although the ubiquity of phosphatidyl ethanolamine plasmalogen in both outer and inner membranes was confirmed, the occurrence of the molecular species of phosphatidyl ethanolamine plasmalogen was quite different in the outer and inner membranes.

The cell envelope of gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium* consists of two distinct membranes: the inner (cytoplasmic) membrane and the outer membrane that is located outside the peptidoglycan layer. Both membranes contain proteins and phospholipids. In addition, the outer membrane contains lipopolysaccharides (LPS) (see reference 20). Recently, it has been confirmed that peptidoglycan and LPS are closely associated with some of the outer membrane proteins, and this relationship is considered to be essential for the formation of the outer membrane (see references 8 and 18). Most of the information comes from the studies with the *Enterobacteriaceae,* and little work on the outer and inner membranes of strictly anaerobic, gram-negative bacteria is available. Kanegasaki and Takahashi reported that *Selenomonas ruminantium* subsp. *lactilytica,* which is a strictly anaerobic, gram-negative strain isolated from sheep rumen, required a normal saturated volatile fatty acid for its normal growth in a glucose medium; however, no such obligate requirement of fatty acid was observed when the cells were grown in a lactate medium (16). In addition, we noted that *S. ruminantium* contained plasmalogen and glyceryl ether as components of the phospholipids (11, 16). Further studies showed that [^{14}C]valerate or [^{14}C]caproate was easily incorporated into fatty acid, fatty aldehyde, and fatty alcohol moieties of the phospholipids and that the labeled fatty acid was also incorporated into a fatty acid moiety of the lipid A component of LPS of this organism (10-13). In a previous paper, we described the characterization of glycolipid whose basic structure was identified to be β-glucosaminyl-1,6-glucosamine of the lipid A component of LPS (12, 14). In the course of these studies our interest was focused on phospholipids and lipid A, which are components of the cell envelope of this strain. However, all of the information described above was obtained by the analysis of intact cells or the crude cellular fraction of the strain, since in previous studies we did not isolate separately the outer and inner membranes.

In the present paper, we describe the isolation

procedure and the characterization of the outer and inner membranes of *S. ruminantium*.

MATERIALS AND METHODS

Bacterial strains. *S. ruminantium* subsp. *lactilytica*, described in an earlier paper (16), was used in the present study. *E. coli* XA7012 (F⁻ Δ*lac galE*), kindly given by J. R. Beckwith, was also used. A spontaneous mutant, which contains only 2-keto-3-deoxyoctulosonic acid (KDO) residues in the saccharide portion of LPS of *E. coli* K-12, was isolated from strain XA7012 by selection with phage C21 (1).

Media and culture conditions. *S. ruminantium* was grown in 2 liters of a yeast extract-glucose medium supplemented with 100 μCi of sodium *n*-[1-^{14}C]decanoate at the final concentration of 0.01%. When lactate was used as an energy source, 2 liters of a yeast extract-biotin medium was used. The details of the composition of these media were described previously (16). *S. ruminantium* was grown at 37°C under anaerobic conditions (16).

Preparation of [^{3}H]glycerol- or [^{32}P]phosphate-labeled cells. The yeast extract-glucose medium (100 ml) containing sodium *n*-decanoate, and 2 mCi of [2-^{3}H]glycerol (300 nmol) was inoculated with 1 ml of an overnight culture of *S. ruminantium* and incubated at 37°C under anaerobic conditions for 4 h. The cells were collected by centrifugation, washed once with the yeast extract-glucose medium containing *n*-decanoate, and finally suspended into 100 ml of fresh medium which had the identical composition as the previous medium, except that it did not contain labeled glycerol. After 1 h of incubation at 37°C under anaerobic conditions, cells were collected and used for the isolation of the outer and inner membranes. [^{32}P]phosphate-labeled cells of *S. ruminantium* were prepared by growing cells to mid-exponential phase in 20 ml of the yeast extract-glucose medium containing *n*-decanoate and 0.1 mCi of [^{32}P]phosphate. [^{32}P]phosphate-labeled cells of *E. coli* were also prepared by growing cells with shaking vigorously to mid-exponential phase in 5 ml of nutrient broth (pH 7.2) containing peptone (1%; Difco Laboratories, Detroit, Mich.), yeast extract (0.3%; Difco), NaCl (0.3%), and 0.1 mCi of [^{32}P]phosphate.

Isolation of the outer and inner membranes. Isolation of the outer and inner membranes was carried out essentially as described previously (24). Cells were grown as described above. At mid-exponential phase, cells in 2 liters of culture were harvested by centrifugation at 4°C, washed twice with ice-cold 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.4), and finally suspended into 15 ml of HEPES buffer. Cells were disrupted at 0°C in a French pressure cell at 1,200 kg/cm^{2} in this buffer, containing 0.5 mg each of DNase and RNase. The cells were never exposed to EDTA during the preparation. Total membranes were collected by centrifugation in a Beckman TY65 rotor for 60 min at 65,000 rpm, suspended into 3 ml of HEPES buffer, and divided into two portions. Each was applied to a discontinuous sucrose density gradient (1.6 ml of 2.02 M, 5.6 ml of 1.44 M, and 4 ml of 0.77 M sucrose in this buffer). The gradients were centrifuged in a Beckman SW41 rotor for 18 h at 20,500 rpm. The isolated membrane fractions were washed three times with distilled water. All operations after harvesting of cells were carried out at 0 to 4°C, except as noted. These membrane fractions were stored frozen at −70°C until analysis. A possible contamination of the outer membrane fraction by the inner membrane was checked by assaying for cytochrome b_1 and NADH oxidase.

For preparation of the membranes from [^{3}H]glycerol- or [^{32}P]phosphate-labeled cells, a similar but scaled-down procedure was employed.

Determination of the outer or inner membrane components. (i) Protein. Protein was determined by the method of Lowry et al. (17), with bovine serum albumin as a standard.

(ii) Phospholipids. Phospholipids were extracted by the method of Bligh and Dyer (3) and quantitated by assaying for phosphorus after digestion in H_2SO_4 (2). An average molecular weight of 700 was assumed for the calculation of phospholipid content.

(iii) KDO. KDO content was determined by the thiobarbituric acid method described by Osborn et al. with KDO as a standard (21).

(iv) 3-OH myristic acid. Membrane fraction (10 mg [dry weight]) was hydrolyzed in 2 ml of 4 N HCl at 100°C for 15 h, together with a known amount of an internal standard, 3-OH tridecanoic acid. The hydrolysate was extracted three times with ether, and the fatty acids were converted into methyl esters by treatment with diazomethane. The esters were analyzed by gas chromatography. The amount of methyl 3-OH myristic acid was calculated by comparing it with methyl 3-OH tridecanoic acid. When *S. ruminantium* cells were grown in the glucose medium supplemented with a sufficient amount of decanoate, no 3-OH tridecanoic acid was detected in either the outer or the inner membrane fraction. Therefore, 3-OH tridecanoic acid was suitable as the internal standard in the present study.

(v) Fatty aldehyde and glyceryl ether. The amounts of fatty aldehyde and glyceryl ether in phospholipid fractions were determined by previously described methods (9, 11).

(vi) Cytochrome. Cytochrome was assayed from the difference in optical densities at 429 nm between oxidized and dithionite-reduced preparations (21).

(vii) NADH oxidase. Freshly prepared membrane fractions were used for the enzyme assay of reduced nicotinamide adenine dinucleotide. This assay was carried out by the method of Osborn et al. (21). The rate of decrease in absorbance at 340 nm was measured at 25°C by a Shimadzu UV200 recording spectrophotometer (Kyoto, Japan).

(viii) Peptidoglycan. Membrane fraction (30 mg [dry weight]) was treated with hot 4% sodium dodecyl sulfate (SDS) according to the method of Braun and Rehn (4). The SDS-insoluble fraction was obtained and its dry weight was measured.

Distribution of radioactivities in the outer and inner membranes of *S. ruminantium*. The outer and inner membrane fractions were prepared from [^{14}C]decanoate-labeled or [^{3}H]glycerol-labeled cells. The following two procedures were conducted for the isolation of phospholipid, LPS, protein, and peptidoglycan fractions.

Procedure 1. The outer or inner membrane fraction was extracted with chloroform-methanol-water

by the method of Bligh and Dyer (3). The chloroform layer was removed. The remaining layers (aqueous and interphase) were evaporated to dryness under N_2 gas. The resulting residue was fractionated by phenol-water as follows. To the residue was added 1 ml of distilled water and 1 ml of 90% phenol. After extraction for 10 min at 70°C, the suspension was chilled to 4°C and then centrifuged. The aqueous fractions were combined and dialyzed overnight against 10 liters of distilled water. The remaining fraction (phenol phase plus interphase) was treated once with 2 volumes of acetone and twice with 2 volumes of ethyl ether to remove phenol. The resulting precipitate was treated with 2% SDS at 100°C for 2 min. The SDS-soluble or -insoluble fraction was obtained by ultracentrifugation at 50,000 rpm for 60 min at 20°C.

Procedure 2. The outer membrane was directly extracted with phenol-water as described above. After each fraction was dried in a counting vial, the sample was dissolved in 0.5 ml of 2% SDS at 100°C. After the addition of 10 ml of the solution of Bray (5), radioactivity was determined with a Packard 3255 liquid scintillation spectrometer.

Column chromatographic separation. Column chromatographic separation of the LPS fraction was obtained from the outer membrane by treatment of phenol-water without previous extraction of phospholipid. The ^{14}C-labeled LPS fraction was solubilized in 2% SDS at 100°C for 2 min and applied to a Sephacryl S-200 column in 50 mM of Tris-hydrochloride buffer (pH 8.0) containing 2% SDS. The column was eluted with the same buffer at 60°C.

Analysis of phospholipids. Phospholipids were analyzed by thin-layer and ion-exchange paper chromatography.

(i) Silica gel thin-layer chromatography. Chromatography was carried out on a silica gel plate (silica gel 60) as a layer of 0.25 mm. Two solvent systems were used. Solvent A consisted of chloroform, methanol, and water (65:25:4 [vol/vol/vol]) and solvent B contained chloroform, methanol, and acetic acid (65:25:10 [vol/vol/vol]).

(ii) Ion-exchange paper chromatography. Ion-exchange chromatography was done by the method of Shibuya and Maruo (23). Radioactive lipid extracts were applied to Amberlite WB-2 paper and chromatographed, ascending, with diisobutyl ketone-acetic acid-water (8:5:1 [vol/vol/vol]). Radioactive lipids were detected by radioautography or by a Irigaku TRM 1-B radioscanner equipped with a windowless gas flow counter (Tokyo, Japan).

Analysis of water-soluble products formed by hydrolysis of band 1, a main spot of phospholipids. Band 1 phospholipid was hydrolyzed with 1 N HCl for 20 min at 100°C. The hydrolysate was extracted with ether, and water-soluble products were analyzed by a Hitachi LKA-3B amino acid autoanalyzer and by paper electrophoresis. Electrophoresis was carried out on a sheet of Whatman no. 1 filter paper at 2.5 V/cm in 0.05 M ammonia-acetic acid (pH 6.5).

Fatty aldehyde compositions of phosphatidyl ethanolamine plasmalogen. Fatty aldehydes in phosphatidyl ethanolamine plasmalogen were isolated as dinitrophenylhydrazones according to the method of Kamio et al. described previously (10). Fatty aldehydes were regenerated from the hydrazones, reduced to fatty alcohols, and finally acetylated. The acetylated derivatives were analyzed by gas-liquid chromatography.

Gas-liquid chromatography. Gas-liquid chromatography was performed with a Yanagimoto G-8 gas chromatograph (Yanagimoto Seisakusho, Kyoto, Japan) equipped with a hydrogen flame ionization detector. A Diasolid L (60 to 80 mesh) column (3 mm by 2 m) containing 10% poly(diethylene glycol) succinate was employed with N_2 as a carrier gas. Each peak in the effluent was identified by the relative retention time which was obtained with authentic acetylated fatty alcohols. The percentage of each component was calculated from the ratio of the area of each peak to the total area of all peaks.

Polyacrylamide gel electrophoresis. SDS slab gel electrophoresis was performed as described previously (15).

Electron microscopy. Negative staining was performed with 1% sodium phosphotungstate (pH 7.4). Observation was carried out by a Hitachi HU-11A electron microscope with an accelerating voltage of 75 kV.

Materials. Acrylamide and *N,N*-methylenebisacrylamide were from Eastman Kodak Co. *N,N,N',N'*-tetramethylenediamine and ammonium persulfate were from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). SDS was obtained from BDH Chemicals Ltd. (Poole, England). 3-OH tridecanoic acid was synthesized chemically (13). Sodium [1-^{14}C]decanoate and [2-^{3}H]glycerol were purchased from New England Nuclear Corp. [^{32}P]phosphate was from Daiichi Pure Chemicals Co. (Tokyo, Japan). The other chemicals used were of the best grade commercially available.

Sephacryl S-200 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The Silica Gel 60 plate was purchased from E. Merck AG (Darmstadt, Germany). Diasolid L containing 10% poly(diethylene glycol) succinate was from Nihon Kuromato Works, Ltd. (Tokyo, Japan).

RESULTS

Isolation of the outer and inner membranes. Our recent studies have revealed that 10 to 15% of the total lipid components of *S. ruminantium* was released from the cells when they were exposed to EDTA. Therefore, the cells were never exposed to EDTA during the course of the preparation of the outer and inner membranes. The membranes were clearly separated into three bands upon a sucrose gradient centrifugation (Fig. 1A). Band H (fractions 1–6) contained much LPS and was translucent white. Band L (fractions 13–21) contained cytochrome and NADH oxidase (Table 1). Neither cytochrome nor NADH oxidase activity was detected in band M (fractions 7–11). The protein compositions of these three bands were determined by SDS-polyacrylamide gel electropho-

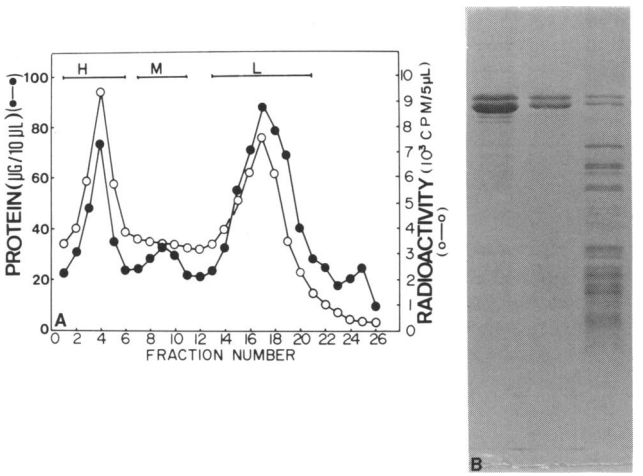

FIG. 1. *(A) Isolation of the outer and inner membranes by sucrose gradient centrifugation. Cells labeled by growing in [^{14}C]decanoate were used for separation of the membranes as described in the text. (B) Protein patterns of fractions indicated in (A) by SDS-polyacrylamide gel electrophoresis.*

TABLE 1. *Quantitative analysis of membranes from S. ruminantium*

Component	Lactate grown[a]		Glucose grown[a]	
	Outer membrane (band H)	Inner membrane (band L)	Outer membrane (band H)	Inner membrane (band L)
Protein	326	796	258	692
Phospholipid	47.9	73.0	37.8	60.6
KDO	8.6	2.1	8.5	2.4
3-OH myristic acid	36.4	7.7	37.4	8.9
Cytochrome	<0.001	0.031	<0.001	0.016
Peptidoglycan	490	33.0	544	43.0
NADH oxidase	<1[b]	48[b]	<1[b]	41[b]

[a] Values represent micrograms per milligram (dry weight) of membrane.

[b] Specific activity (nanomoles per minute per milligram of protein).

resis (Fig. 1B). The band H fraction showed a simple protein profile containing only two major bands. In contrast, the band L fraction consisted of at least 40 bands. These findings indicate that bands H and L correspond to the outer membrane and the inner membranes, respectively. A considerable amount of [^{14}C]decanoate was incorporated into both bands H and L, but no detectable peak of radioactivity was observed in band M, suggesting that bands H and L contained lipid compounds. The outer membrane preparation (band H) was examined for a possible contamination with the inner membrane by assaying the amount of cytochrome b_1 in the outer membrane, and it was found that the contamination was less than 3% for both lactate- and glucose-grown cells. Large amounts of LPS-specific components, 3-OH myristic acid, and KDO were always found in the inner membrane preparation (Table 1), suggesting that the inner membrane preparation was contaminated with the outer membrane.

Quantitative analysis of isolated membrane preparations. Table 1 shows that half of the outer membrane fraction was composed of peptidoglycan. However, this is not merely due to a simple contamination with peptidoglycan but is rather ascribed to the presence of attached peptidoglycan with the outer membrane (see Discussion). As mentioned above, a significant amount of 3-OH myristic acid and KDO and a small amount of peptidoglycan were found in the inner membrane preparation, which might be due to contamination of the outer membrane fragment without the attached peptidoglycan. The amounts of protein and phospholipid per unit weight of the outer or inner membrane preparation were lower in the glucose-grown cells than in the lactate-grown cells. No significant difference was observed in the amounts of KDO and 3-OH myristic acid in the outer membrane fraction between the glucose- and lactate-grown cells.

Electron microscopy of the outer and inner membrane preparations. The outer and inner membrane fractions which were prepared from the lactate- or glucose-grown cells were examined by electron microscopy. As shown in Fig. 2, the outer membrane fraction (band H) prepared from both lactate- and glucose-grown cells contained fragments and vesicles with diameters ranging from 0.1 to 0.25 μm. The inner

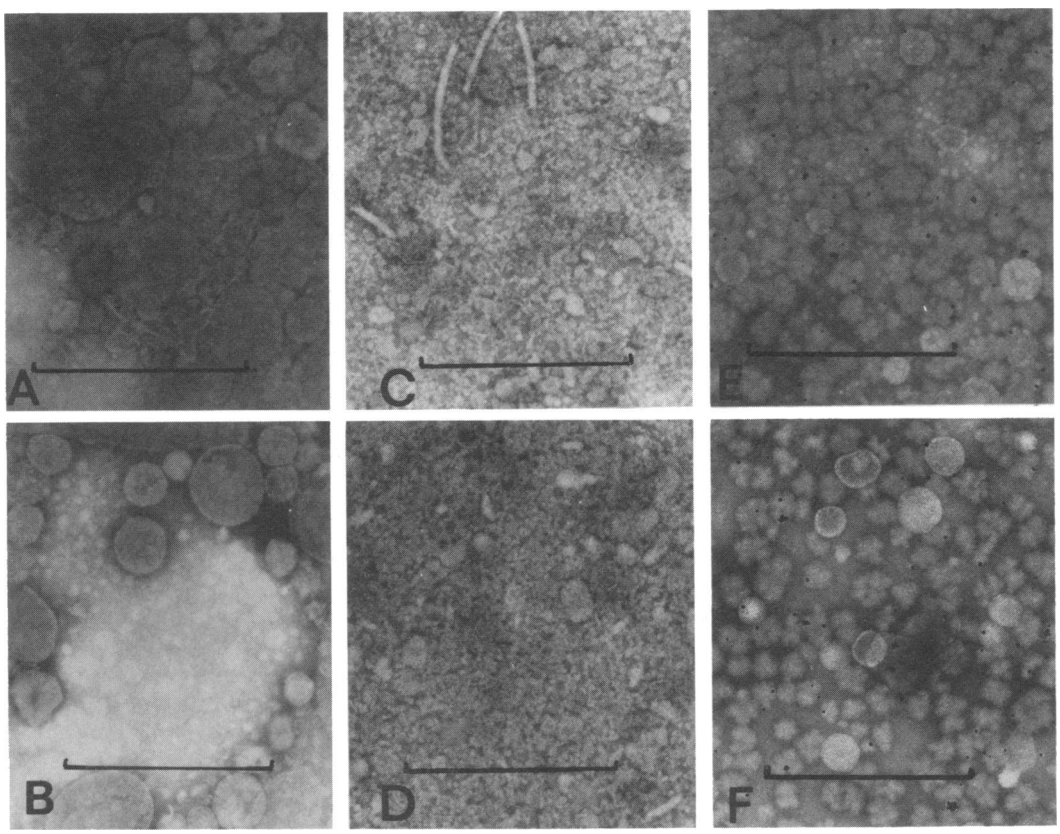

FIG. 2. *Electron micrographs of the membrane preparations from S. ruminantium, which was grown in either lactate (A, C, and E) or glucose (B, D, and F) medium. (A and B) Outer membrane (band H). (C and D) Inner membrane (band L). (E and F) Band M. Magnification bars represent 0.5 μm.*

membrane fraction (band L) contained amorphous fragments of small and various sizes. The band M fraction contained predominantly amorphous particles, which varied morphologically from bands H and L. It is of some interest to note that filament-shaped substances with various lengths but with the same width were observed in the inner membrane fraction prepared from the lactate-grown cells. Similar substances were also seen in the inner membrane fraction from the deflagellated cells (data not shown), the nature of which has not been identified yet.

Distribution of radioactivity in the outer and inner membrane preparations from *S. ruminantium* grown with either [^{14}C]decanoate or [^{3}H]glycerol. Rapid incorporation of the volatile fatty acid into the cellular lipids has already been shown (10, 16). To elucidate the metabolic fate of added fatty acid, the distribution of radioactivities of the incorporated [^{14}C]decanoate and [2-^{3}H]glycerol in the outer and inner membrane preparations of this strain was examined (Tables 2 and 3). The percent incorporation of ^{14}C label into the outer and inner membrane fractions was about 40 and 50%, respectively, of the total incorporated radioactivity. As shown in Table 2A, approximately 47% of the radioactivity incorporated into the outer membrane preparation was recovered in the chloroform layer. When the outer membrane preparation was treated with phenol-water after its phospholipids had been extracted, the percent distribution of radioactivity in the aqueous and phenol layers was 10 and 42%, respectively. When the outer membrane preparation was extracted directly by phenol-water, approximately 28% of radioactivity was detected in the aqueous layer (Table 2B [procedure 2]). Two distinct radioactive peaks were detected when this aqueous (LPS) fraction was applied to SDS-Sephacryl gel chromatography. Furthermore, on SDS-polyacrylamide gel electrophoresis, this aqueous fraction also gave two radioactive bands: one band had an apparent molecular weight of 9,500 and the other band migrated slower than that of phospholipids (data not shown).

In contrast to the outer membrane, 62% of the

radioactivity of the inner membrane preparation was detected in the chloroform layer, and the remaining radioactivity was predominantly found in the phenol layer. When the ^{14}C-labeled compound in the phenol layer of the outer membrane preparation was hydrolyzed by 4 N HCl at 100°C for 5 h, all the ^{14}C counts were recoverable as fatty acids. These results indicate that the ^{14}C-labeled lipid compound in the phenol layer of the outer membrane preparation exists in the cells in a bound form, but not as free phospholipids.

A similar experiment was performed with [2-^{3}H]glycerol-grown cells. The percentage distribution of [^{3}H]glycerol in the outer and inner membrane preparations was about 25 and 70%, respectively, of total radioactivity incorporated. As shown in Table 3, most of the radioactivity was recovered in the chloroform layer, and no significant [^{3}H]glycerol was detectable in the phenol layer. Thus, the profiles of incorporation of [^{3}H]glycerol or [^{14}C]decanoate into the membranes were quite different from each other.

Thin-layer and ion-exchange paper chromatography of the phospholipid fractions from the outer and inner membrane preparations. (i) Silica gel thin-layer chromatography. Thin-layer chromatograms of the phospholipid fraction from *S. ruminantium* and authentic phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), and cardiolipin (CL), which were prepared from *E. coli* K-12, are shown in Fig. 3. Four spots including two major ones (spots I and III) were detected in the phospholipid fraction from the outer membrane preparation of [^{14}C]decanoate-labeled cells. Spot III was a minor component in the inner membrane preparation. To examine the incorpora-

TABLE 2. *Distribution of radioactivity in the outer and inner membrane fractions of S. ruminantium ([1-^{14}C]decanoate-grown cells)*

Fraction	Outer membrane		Inner membrane	
	Total radioactivity (10^4 cpm)	% Distribution	Total radioactivity (10^4 cpm)	% Distribution
A				
Membrane	53.8	100	85.6	100
Chloroform-methanol-water treatment[a]				
Lower chloroform phase	25.1	46.7	53.2	62.1
Upper aqueous phase and interphase	ND[b]	ND	ND	ND
Phenol-water treatment				
Upper aqueous phase	5.4	10.0	2.5	2.9
Lower phenol phase and interphase				
SDS-soluble	22.5	41.8	27.4	32.0
SDS-insoluble	0.07	0.1	0.09	0.11
B				
Membrane	79.5	100		
Phenol-water treatment				
Aqueous phase	22.3	28.1		
Phenol phase and interphase	54.8	68.9		

[a] Extraction was performed by the method of Bligh and Dyer (3).
[b] ND, Not determined.

TABLE 3. *Distribution of radioactivity in the outer and inner membrane fractions of S. ruminantium ([2-^{3}H]glycerol-grown cells)*

Fraction	Outer membrane		Inner membrane	
	Total radioactivity (10^4 cpm)	% Distribution	Total radioactivity (10^4 cpm)	% Distribution
Membrane	62.0	100	174	100
Chloroform-methanol-water treatment[a]				
Lower chloroform phase	55.0	88.7	156.8	90.1
Upper aqueous phase and interphase	ND[b]	ND	ND	ND
Phenol-water treatment				
Upper aqueous phase	0.15	0.24	0.30	0.17
Lower phenol phase and interphase				
SDS-soluble	0.20	0.32	0.37	0.21
SDS-insoluble	0.04	0.06	0.05	0.03

[a] Extraction was performed by the method of Bligh and Dyer (3).
[b] ND, Not determined.

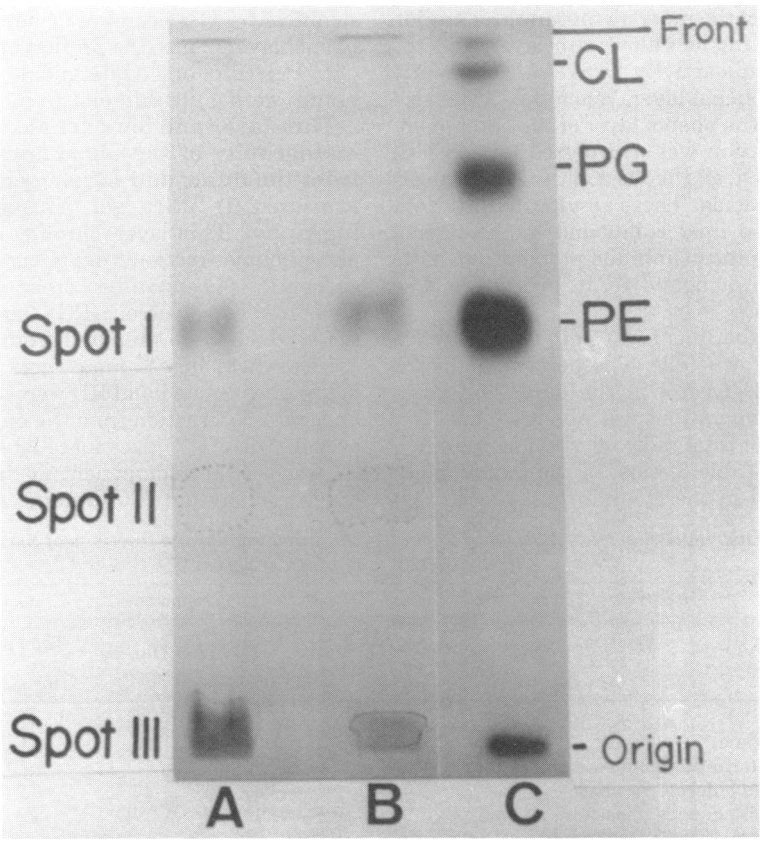

FIG. 3. *Thin-layer chromatograms of the phospholipid fractions from the outer and inner membrane fractions. The spots were detected by spraying 50% H_2SO_4 and heating at 180°C for 10 min. Solvent B system was used. (A) Sample from the outer membrane. (B) Sample from the inner membrane. (C) Sample from the total membranes of the heptose-less mutant of E. coli K-12.*

tion of [^{14}C]decanoate into each spot, a thin-layer chromatography plate was applied to the radioscanner. Approximately 40% of the total radioactivity in the phospholipid fraction from the outer membrane preparation was present in spot I, and about 45% was detected in spot III. In the sample from the inner membrane preparation, about 80% of the total radioactivity in the phospholipid fraction was present in spot I, and 5% or less was found in spot III.

It is of interest to note that neither PG nor CL was detected in either membrane preparation (Fig. 3A and B). A similar result was obtained in the lactate-grown cells. ^{32}P-labeled phospholipids were prepared from the total membrane fraction of *S. ruminantium* cells which were grown in the lactate or glucose medium containing [^{32}P]phosphate and also chromatographed. Neither radioactive PG nor CL was detected in either sample (data not shown).

(ii) Ion-exchange paper chromatography. Paper chromatograms of the ^{14}C-labeled phospholipid fraction and the ^{3}H-labeled phospholipid fraction are shown in Fig. 4. Three radioactive bands were obtained in the membranes of ^{14}C-labeled cells. Bands I, II, and III are considered to correspond to spots I, II, and III, respectively, in Fig. 3. In the outer membrane preparation, the percentage incorporation into bands I, II, and III was about 42, 4, and 47%, respectively. Of the total radioactivity in the phospholipids fraction, 80% or more was found in band I from the inner membrane preparation. The data coincided closely with those obtained by silica gel thin-layer chromatography. [^{3}H]labeled phospholipids which were prepared from the outer membrane fraction of [2-^{3}H]glycerol-labeled cells were also chromatographed on the Amberlite ion-exchange paper. The radioactivity was detected in bands I and II but not in band III (Fig. 4, samples 1 and 2).

Identification and characterization of major phospholipids in the outer and inner membrane preparations. (i) Identification

of band I from the outer membrane preparation. Band I was identified as a mixture of PE, phosphatidyl ethanolamine plasmalogen, and alkoxy type (glyceryl ether type) ethanolamine by the following experimental results. (a) This lipid had the identical R_f value as that of authentic PE on a silica gel thin-layer chromatography plate (Fig. 3) and on ion-exchange paper (Fig. 4). (b) The water-soluble product after hydrolysis with HCl was analyzed quantitatively. Glycerol was detected by paper chromatography, and ethanolamine was determined by both paper electrophoresis and amino acid autoanalyzer. The molar ratio of glycerol to ethanolamine was 0.95. (c) Fatty aldehyde was determined quantitatively, and the molar ratio of aldehyde to phosphorus was approximately 0.35. (d) Incorporation of [^{14}C]decanoate into fatty aldehyde, glyceryl ether, and fatty acid moieties of this lipid was examined. The ratio of the incorporation of radioactivity into aldehyde and glyceryl ether moieties to that of the fatty acid

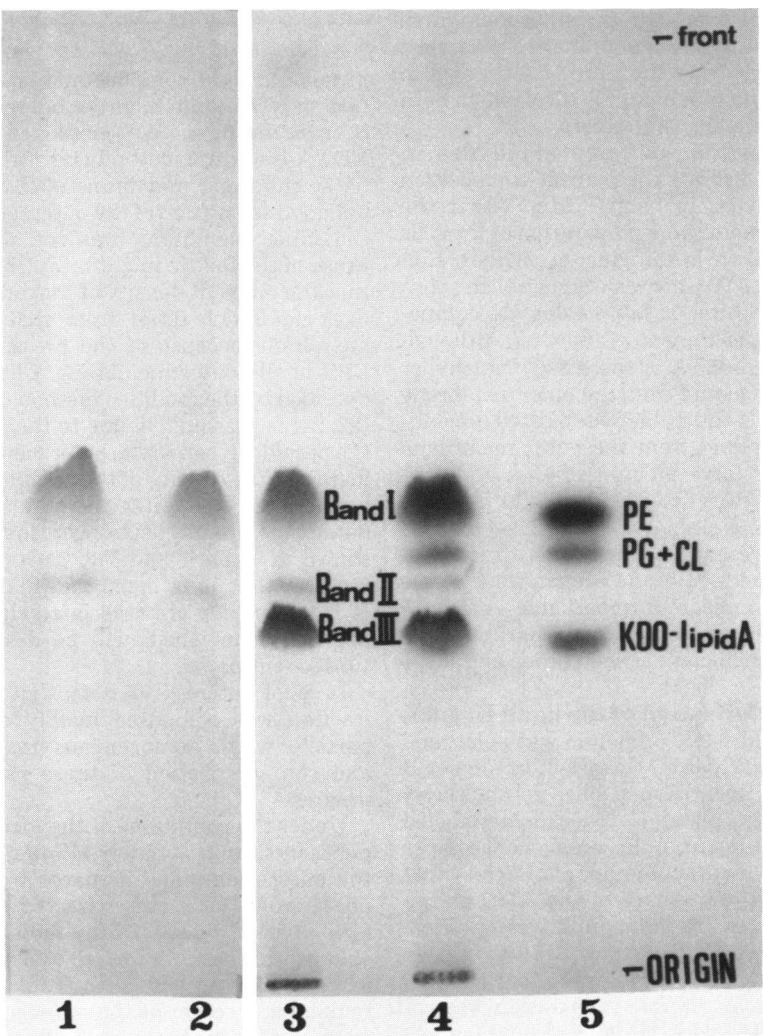

FIG. 4. *Radioautograms of the phospholipid fractions from the outer membrane preparation of S. ruminantium. (1) Sample from the outer membrane of the cells grown in the glucose medium containing [2-^{3}H]glycerol. (2) Sample from the outer membrane of the cells grown in the lactate medium containing [2-^{3}H]glycerol. (3) Sample from the outer membrane of the cells grown in the glucose medium containing [^{14}C]decanoate. (4) Sample 3 plus sample 5. (5) Sample from the total membranes of the heptose-less mutant of E. coli K-12 grown with [^{32}P]phosphate. For preparation of the radioautograms of samples 1 and 2, paper was in contact with medical X-ray film (Fuji Photo Film Co. Ltd., Tokyo, Japan) at room temperature for 9 days. For the radioautogram of other samples, exposure was for 2 days.*

moiety was approximately 0.2 and 0.09, respectively. From these results, the molar ratio of phosphatidyl ethanolamine plasmalogen and glyceryl ether type ethanolamine to PE was calculated at 0.5 and 0.2, respectively.

(ii) Incorporation of labeled decanoate into fatty aldehydes of band I from the outer or inner membrane preparation. ^{14}C-labeled band I was prepared from the outer and inner membrane preparations of [^{14}C]decanoate-labeled cells. The ratio of the incorporation of radioactivity into fatty acid to that into the fatty aldehyde moiety was 4.9 in the outer membrane and 5.0 in the inner membrane, respectively. The results show that the ratio of fatty acid to the aldehyde chain in band I is the same in both the outer and inner membranes.

(iii) Composition of fatty aldehydes in band I. Table 4 shows the percent composition of fatty aldehydes in band I from either the outer or inner membrane preparation of the cells which were grown in the glucose medium supplemented with 0.01% *n*-decanoate. In both membranes, the major fatty aldehyde components of this lipid were $C_{14:0}$ and $C_{16:1}$. Although almost equal amounts of these fatty aldehydes were present in band I from the inner membrane preparation, $C_{14:0}$ fatty aldehyde existed predominantly in this lipid from the outer membrane preparation. A large amount of $C_{16:0}$ and $C_{18:1}$ fatty aldehydes was detected in band I from the inner membrane preparation, but such aldehydes were present as minor constituents in band I from the membrane preparation. These results clearly indicate a remarkable difference in the molecular species of phosphatidyl ethanolamine plasmalogen between outer and inner membranes.

(iv) Characterization of the band III compound. The band III compound was polar, and it migrated slightly from the origin in solvent B but stayed in solvent A on a silica gel thin-layer chromatography plate. [^{14}C]decanoate-labeled band III was eluted from the ion-exchange paper by chloroform-methanol-ammonia (20:5:1 [vol/vol/vol]), and the presence of the KDO and 3-OH fatty acid in this compound was certified. These findings suggest that band III is the KDO-lipid A. The ^{14}C-labeled phospholipid fraction from the outer membrane preparation was cochromatographed with ^{32}P-labeled lipid materials from the heptose-less mutant of *E. coli*. Band III was not separated from KDO-lipid A of *E. coli* (Fig. 4, sample 4). From these findings, band III was tentatively identified as KDO-lipid A.

TABLE 4. *Percent composition of fatty aldehydes in phosphatidyl ethanolamine plasmalogen in membranes from cells grown in glucose medium containing n-decanoate*

Carbon no. of fatty aldehydes detected	Membrane prepn (%)	
	Outer	Inner
14:0	75.0	37.3
14:1	4.9	0.1
16:0	5.2	16.7
16:1	11.3	31.3
Unidentified	0.3	1.3
18:1	3.3	13.3

DISCUSSION

The present study clarified the existence of a prominent lipid component, tentatively identified as KDO-lipid A, in the outer membrane of *S. ruminantium*. We employed at first the EDTA-lysozyme method (19) for preparing the outer and inner membrane of *S. ruminantium*, but good separation of the outer membrane from the inner membrane was not achieved. The cause of the failure might be ascribed to the fact that the buoyant density of the outer membrane does not much differ from that of the inner membrane because of the presence of altered LPS in the outer membrane. Clean separation obtained by the modified method of Schnaitman (22, 24) is presumably due to the attachment of the peptidoglycan to the outer membrane, which increases the density of the complex of the outer membrane. Our outer membrane is virtually outer membrane-peptidoglycan fraction, as shown in Table 1 and Fig. 2. Our recent study revealed that outer membrane major proteins of *S. ruminantium* are associated closely with the peptidoglycan which will be described in the subsequent paper.

It is of interest to note that the band M fraction was composed mainly of amorphous particles with a homogeneous size. The isolation and characterization of these particles are in progress.

Under the conditions of the incorporation experiments, approximately 47% of radioactivity of the outer membrane prepared from [^{14}C]decanoate-grown cells was extracted in the chloroform layer (Table 2). Of the radioactivity in the chloroform layer, however, 47% was recovered in band III. As shown in Table 2, when the remaining fraction of the outer membrane was treated with phenol-water after extraction of phospholipids, 10% of the radioactivity of the outer membrane preparation was found in the LPS fraction (upper aqueous fraction). Therefore, one can calculate the approximate percent distribution of the radioactivity of LPS to the total radioactivity incorporated into the outer membrane preparation as (47 × 0.47)% + 10%

= 32%. In contrast, when the outer membrane preparation was extracted directly by phenol-water, 28% of the radioactivity was recovered in the LPS fraction (Table 2B). Thus, the actual value obtained (28%) is close to the calculated value. These findings suggest that there are two species of LPS in this strain. The findings from the SDS-Sephacryl gel and SDS-polyacrylamide gel electrophoresis analyses of the LPS fraction support this possibility. In this connection, the observations of Wober and Alaupović should be noted; they showed that lipid A in *E. coli* and *Serratia marcescens* is bound not only to polysaccharides but also to proteins (25, 26). Further analysis of the LPS fraction of *S. ruminantium* was necessary to confirm this possibility.

Although PG and CL were found to be present widely in animals, plants, and microorganisms, the data presented here demonstrated the absence of PG and CL in both membranes of *S. ruminantium*. It is of interest to investigate the relationship between the absence of PG and CL and strict anaerobiosis. PG is shown to be the precursor of the lipoprotein of Braun (6, 7). Existence of the lipoprotein of Braun in the cell fractions can be explored by examining the incorporation of the [2-^{3}H]glycerol into the protein fraction of the outer membrane and into the peptidoglycan fraction. In *S. ruminantium*, [2-^{3}H]glycerol was not found in the phenol layer (Table 3). In addition, [^{14}C]decanoate and [2-^{3}H]glycerol were not incorporated into the peptidoglycan fraction (Tables 2 and 3, SDS-insoluble fraction). These findings suggest the absence of the lipoprotein component corresponding to lipoprotein of Braun and Rehn in *S. ruminantium* (4). Further studies reported in our accompanying paper (15a) support this hypothesis.

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