

## Isolation and Characterization of Membranes from a Hydrocarbon-Oxidizing *Acinetobacter* sp.

CHRISTINA C. L. SCOTT, R. A. MAKULA, AND W. R. FINNERTY\*

*Department of Microbiology and Biochemistry, University of Georgia, Athens, Georgia 30602*

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Membranes were isolated and purified from nutrient broth-yeast extract- and hexadecane-grown cells of *Acinetobacter* sp. strain HO1-N. Two membrane fractions were isolated from nutrient broth-yeast extract-grown cells, the cytoplasmic membrane and the outer membrane. In addition to these two membrane fractions, a unique membrane fraction was isolated from hexadecane-grown cells (band 1) and characterized as a lipid-rich, low-density membrane containing high concentrations of hexadecane. The outer membrane preparations of *Acinetobacter*, obtained from nutrient broth-yeast extract- and hexadecane-grown cells, exhibited a low ratio of lipid phosphorus to protein and contained phospholipase activity and 2-keto-3-deoxyoctulosonic acid. Phosphatidic acid cytidyltransferase, adenosine triphosphatase, and reduced nicotinamide adenine dinucleotide oxidase were recovered almost exclusively in the cytoplasmic membrane fractions. The cytoplasmic membrane fractions contained 20 to 25 polypeptide species on sodium dodecyl sulfate-polyacrylamide gels, and the outer membrane fractions contained 15 to 20 polypeptide species. A major polypeptide species with an apparent molecular weight of approximately 42,000 to 44,000 was found for all outer membrane fractions. The buoyant densities of the cytoplasmic membrane fractions and the outer membrane fractions were closely similar, necessitating their separation by differential centrifugation. Band 1 of hexadecane-grown cells had a ratio of lipid phosphorus to protein that was almost twice that of the cytoplasmic membrane and a correspondingly low buoyant density (1.086 g/cm<sup>3</sup>). Enzyme activities associated with band 1 were identical to those associated with the cytoplasmic membrane. The electrophoretic banding pattern of band 1 was essentially identical to the banding pattern of the cytoplasmic membrane. The phospholipid and neutral lipid compositions of the isolated membrane fractions were determined as qualitatively similar, with significant quantitative differences. The ultrastructure characteristics of the respective membrane fractions were examined by the negative-stain technique.

The cell envelope of gram-negative bacteria represents a complex structure composed of an outer membrane, a rigid intermediate layer designated peptidoglycan, and a cytoplasmic membrane. The development of procedures for the isolation of these complex structures individually and in relative states of purity has been slow to emerge. The isolation of peptidoglycan relatively free of the other envelope components has been obtained through the use of boiling 4% sodium dodecyl sulfate (SDS) (43). More recently, suitable, but not totally satisfactory, procedures have been developed for the separation of the outer membrane from the cytoplasmic membrane of several gram-negative bacteria (12, 22, 23, 27, 29, 35). The outer membrane, unique to gram-negative bacteria, exhibits a typical "unit membrane" structure,

which appears convoluted in thin sections or wrinkled in negative strains of whole cells (10). A specific lipoprotein has been shown to covalently link the peptidoglycan to the outer membrane (4). The lipid portion of the lipoprotein was proposed to exhibit hydrophobic interactions with outer membrane phospholipids (36). The bacterial cytoplasmic membrane is a trilaminar structure consisting of protein and lipid, which exhibits characteristics similar to those described for other biological membranes. Cellular functions that have been ascribed to the cytoplasmic membrane include selective permeation and active transport, electron transport, oxidative phosphorylation, and the biosynthesis of phospholipids, peptidoglycan, and proteins (34).

Other complex intracellular membrane sys-

tems have been observed for a variety of bacteria. Mesosomes possess long-standing recognition although their function remains obscure (31). Complex intracytoplasmic membrane systems have been described for specialized groups of microorganisms, which include the methane-oxidizing bacteria (6, 7), the autotrophic-nitrifying bacteria (42), the photosynthetic *Thiorhodaceae* and *Athiorhodaceae* (25), the nitrogen-fixing *Azotobacter* (28, 30), *Escherichia coli* strain O11a (44), and *Gluconobacter oxydans* (2, 5). Recently, intracytoplasmic membrane development in the alkane-oxidizing microorganism *Acinetobacter* sp. HO1-N was reported (15, 16).

A limited number of reports have appeared describing the isolation of intracellular membranes from gram-negative bacteria. The intracellular membranes of the photosynthetic bacterium *Rhodospseudomonas capsulata* have been isolated and their purity has been assessed on the basis of bacteriochlorophyll content (25). The intracellular membranes of *E. coli* O11a have been isolated, employing their size and unique ultrastructure characteristics (11).

The hydrocarbon-oxidizing bacterium *Acinetobacter* sp. HO1-N offers a unique system in which to study the structure and function of specific bacterial membrane systems and their relationship to alkane oxidation. These studies are directed to the development of techniques for the isolation and chemical characterization of the various membrane systems in this organism after growth on hydrocarbon and nonhydrocarbon substrates.

#### MATERIALS AND METHODS

**Bacterial strain.** *Acinetobacter* species HO1-N (3, 13), formerly the HO1-N strain of *Micrococcus cerificans* (39), was used for all experiments. The bacterium was maintained at  $-20^{\circ}\text{C}$  in 15% glycerol from cultures pregrown in nutrient broth-yeast extract (NBYE).

**Culture conditions.** The organism was grown in a mineral salts medium with the following composition (in grams per liter):  $(\text{NH}_4)_2\text{SO}_4$ , 2;  $\text{KH}_2\text{PO}_4$ , 4;  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 4;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.001; and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.001, pH 7.8. This was supplemented with either presterilized *n*-hexadecane (Humphrey Chemical Co., New Haven, Conn.) to a final concentration of 0.3% (vol/vol) or with 0.8% nutrient broth (Difco) plus 0.5% yeast extract (Difco). Starter cultures (100 ml) in NBYE were inoculated from the glycerol stock cultures and used to inoculate hexadecane starter cultures. Cultures were grown on a gyratory shaker at  $28^{\circ}\text{C}$  and harvested during the midexponential growth phase. Growth was followed with a Klett-Summerson colorimeter equipped with a 540-nm filter.

**Membrane isolation.** Cells were harvested by

centrifugation at  $13,000 \times g$  for 20 min at  $4^{\circ}\text{C}$ . NBYE-grown cells were washed two times, and hexadecane-grown cells were washed four times with 50 mM tris(hydroxymethyl)aminomethane (Tris)-chloride buffer, pH 7.8. Cells were suspended at a concentration of 1 g (wet cell weight)/20 ml of 50 mM Tris-chloride buffer (pH 7.8) containing 0.6 M sucrose. Lysozyme was added (10 mg/g [wet weight] of cells), and the cell suspension was stirred at  $23^{\circ}\text{C}$  for 30 min. The cell suspension was lysed by adding to 10 volumes of cold distilled water. Deoxyribonuclease (2 mg) was added to decrease the viscosity. After stirring for 90 min at  $4^{\circ}\text{C}$ , the membranes were collected by centrifugation at  $65,000 \times g$  for 2 h. The membrane pellet was washed once in 50 mM phosphate buffer (pH 7.5) and suspended in the same buffer containing 1 mg of deoxyribonuclease and 1 of mg ribonuclease per 10 g of cells (original wet cell weight). After stirring for 30 min at  $4^{\circ}\text{C}$ , the membranes were collected by centrifugation at  $65,000 \times g$  for 2 h, washed two times, and suspended in a small volume of phosphate buffer. The crude membrane suspension was stored at  $4^{\circ}\text{C}$  in the presence of chloramphenicol (20  $\mu\text{g}/\text{ml}$  of membrane) and used within 5 days.

**Sucrose gradient centrifugation.** Discontinuous sucrose gradients were prepared with the following composition: 5 ml of 55% (wt/wt), 10 ml of 41.7% (wt/wt), 10 ml of 24% (wt/wt), and 5 ml of 15% (wt/wt). Crude membranes were layered on top of the gradient and centrifuged at  $51,000 \times g$  in an SW25.1 Beckman rotor for 18 h.

The sucrose gradients were fractionated through an Instrument Specialities Co., Inc. model D density gradient fractionator at a rate of 1.4 ml/min. Fractions of 25 drops (1.2 ml) were collected, and 0.1 ml was taken for protein analysis. The bands of membrane were pooled and used for further analysis.

The buoyant densities of the isolated membrane fractions were estimated by layering on 30-ml linear 25 to 55% (wt/wt) sucrose gradients. The gradients were centrifuged at  $51,000 \times g$  in an SW25.1 Beckman rotor for 18 h. The linearity of the prepared gradient was measured on control gradients under identical conditions with a Bausch & Lomb refractometer.

**Enzyme assays.** Phospholipase activity was determined by measuring the conversion of [ $2\text{-}^3\text{H}$ ]glycerol-labeled phosphatidylethanolamine to water-soluble products. Incubation mixtures contained 100 mM Tris-chloride buffer (pH 8.0), 5 mM  $\text{CaCl}_2$ , 0.2% (vol/vol) Cutscum (Fisher Scientific Co.), 205 nmol of [ $\text{H}^3$ ] phosphatidylethanolamine (112 counts/min per nmol) in a final volume of 0.1 ml. Labeled phosphatidylethanolamine in chloroform-methanol (2:1, vol/vol) was added first, and the solvent was removed under a stream of nitrogen. A mixture of the above reagents (0.05 ml) was added, and the phospholipid was dispersed by vigorous agitation on a vortex mixer. The reaction was initiated by the addition of the membrane fraction (14  $\mu\text{g}$  of protein). After incubation for 20 min at  $37^{\circ}\text{C}$  on a shaking water bath, the reaction was stopped with 1.5 ml of chloroform-methanol (2:1, vol/vol). The monophasic solution was backwashed with 0.5 ml of

0.9% NaCl, and the layers were separated by centrifugation. A portion of the aqueous phase (0.2 ml) was added to 10 ml of Aquasol (New England Nuclear Corp.), and the radioactivity was measured in a Nuclear-Chicago Mark II scintillation spectrometer.

Reduced nicotinamide adenine dinucleotide (NADH) oxidase was assayed by measuring the rate of oxygen consumption over a linear range at 25 C with a Yellow Springs Instrument Co. model 53 biological oxygen monitor. Incubation mixtures contained 50 mM phosphate buffer (pH 7.5), 0.43 mM NADH, and the membrane fraction (0.2 to 1.0 mg of protein) in a final volume of 3.0 ml. The reaction was initiated by the addition of NADH. Observed rates were corrected for endogenous oxygen consumption by subtracting the oxygen consumed, using the above mixture lacking NADH.

Adenosine triphosphatase (ATPase) was assayed by measuring the amount of inorganic phosphate released after incubation of the membrane fractions with adenosine 5'-triphosphate (ATP). Incubation mixtures contained 50 mM Tris-chloride (pH 8.0), 2 mM  $MgCl_2$ , 5 mM  $Na_2ATP$ , and the membrane fraction (150 to 200  $\mu g$  of protein) in a final volume of 1 ml. Membrane fractions were dialyzed overnight at 4 C against 50 mM Tris-chloride, pH 7.5, prior to enzyme analyses. The reaction mixture lacking ATP was preincubated for 1 min at 37 C, and the reaction was started by the addition of ATP. After incubation for 30 min, the reaction was terminated by the addition of 1 ml of cold 10% trichloroacetic acid. The reaction mixtures were centrifuged at  $27,000 \times g$  for 10 min at 4 C to remove precipitated protein. The supernatant (1 ml) was assayed for inorganic phosphate by the method of Fiske and Subbarow (8). The appropriate controls were run to correct for nonenzymatic hydrolysis of ATP.

Phosphatidic acid cytidyl transferase was assayed by measuring the formation of [ $^3H$ ]cytidine diphosphoglyceride after the incubation of membrane fractions with [ $^3H$ ]cytidine triphosphate (New England Nuclear Corp., Boston, Mass.) and phosphatidic acid. The labeled substrate was diluted with unlabeled cold carrier to give an 80 mM stock solution. A buffer-substrate mixture was prepared containing the following reagents at final concentrations: 0.011 M Tris-chloride (pH 8.5), 0.08 M KCl, 0.4% (vol/vol) Cutscum, 0.0125 M  $MgCl_2$ , 0.01 M [ $^3H$ ]cytidine triphosphate, and 0.00312 M phosphatidic acid.  $MgCl_2$  was the last addition made to the reaction mixture.

Duplicate reactions were carried out in 40- $\mu l$  volumes of buffer-substrate at 37 C for 15 min, with the reaction started by addition of 2 to 4  $\mu l$  of the membrane fraction (1 to 4  $\mu g$  of protein). The reaction was stopped by the addition of 75  $\mu l$  of 0.3 N HCl in methanol with vigorous mixing. Chloroform (75  $\mu l$ ) was added to each reaction tube, mixed vigorously, and backwashed three times with 200  $\mu l$  of 2 M KCl. The chloroform layer (25  $\mu l$ ) was added to scintillation vials containing 10 ml of Aquasol.

**Polyacrylamide gel electrophoresis.** SDS-polyacrylamide gel electrophoresis on slabs was carried out with an Ortec (model 4200) electrophoresis system employing a pulsed constant power supply

(model 4100). Electrophoresis was carried out as described by Miner (21) with slight modifications. The gels were prepared from a stock solution containing 4 g of acrylamide, 0.1 g of *N,N'*-bismethyleneacrylamide, and 10.0 mg of SDS in 10 ml of water. All gels contained 10% acrylamide and 0.25% bisacrylamide in 0.375 M Tris-sulfate, pH 9.0. A final concentration of 0.4% ammonium persulfate was used for the polymerization of this gel and the cap gel. The stacking gel contained 5% acrylamide and 0.125% bisacrylamide in 0.375 M Tris-sulfate, pH 9.0. The stacking and well gels were polymerized using a final concentration of 0.1% ammonium persulfate. The well gel contained 8% acrylamide and 0.2% bisacrylamide in 0.075 M Tris-sulfate, pH 9.0. The cap gel contained 10% acrylamide and 0.25% bisacrylamide in 0.075 M Tris-sulfate, pH 9.0. The tank electrode buffer consisted of 0.065 M Tris borate, pH 9.0. All chemicals used for polyacrylamide gel electrophoresis were purchased from Canalco (Rockville, Md.).

Membrane samples for electrophoresis (100 to 150  $\mu g$  of protein) were suspended in a solution containing 1% SDS and 1% 2-mercaptoethanol and heated in a boiling water bath for 5 min. To 2 volumes of the membrane suspension, 1 volume of 0.075 M Tris-sulfate, pH 9.0, was added containing bromophenol blue and 80% sucrose.

Electrophoresis was carried out at room temperature using constant voltage (200 V). The tank electrode buffer was chilled (4 C) before each run. The pulse rate (initially set at 75 pulses/s) was increased 75 pulses/s at 10-min intervals to a final rate of 300 pulses/s.

After electrophoresis (1.5 to 2 h), the gel was stained in a solution containing 50% methanol, 10% acetic acid, and 0.25% Coomassie brilliant blue for 4 to 5 h at 60 C in a shaking water bath. The gel was destained in a solution containing 5% methanol and 7% acetic acid at 60 C in a shaking water bath.

The molecular weights of the membrane polypeptides were estimated by extrapolating relative mobilities on a standard curve prepared with bovine serum albumin, egg albumin, and trypsin as standards.

**Extraction of lipids and hexadecane.** The membrane fractions were extracted with chloroform-methanol (2:1, vol/vol) for 30 min at 25 C and backwashed with 0.9% NaCl (9). The chloroform-soluble fraction was dried under nitrogen and dissolved in a known volume of chloroform for further lipid analysis or in a known volume of hexane for the quantitation of hexadecane by gas chromatography.

**Gas chromatography.** Hexadecane was assayed by gas-liquid chromatography with a Packard gas chromatograph, series 7500, using a 20% diethylene glycol succinate column. Operating conditions were: column temperature, 115 C; detector temperature, 190 C; injection temperature, 180 C; outlet temperature, 250 C; argon flow rate, 50 ml/min; and chart speed, 2.5 min/inch (ca. 2.54 cm). Peak areas were determined by multiplication of peak heights by retention time, and the hydrocarbon gas was quantitated by using a reference standard curve.

**Thin-layer chromatography.** (i) Separation of

**phospholipids.** Thin-layer plates (0.4 mm thick) prepared with Silica Gel H containing 1 mM sodium tetraborate were activated at 100 C and used within 30 min. Phospholipids were separated using a solvent system of chloroform-methanol-water (95:35:5, vol/vol/vol). The phospholipids were quantitated by visualizing the individual phospholipids in iodine, subliming the iodine, transferring the silica gel containing specific phospholipids to test tubes, and determining the amount of lipid phosphorus. All samples were done in triplicate with suitable silica gel blanks. Recovery of the lipid phosphorus applied to the plates was greater than 95%.

(ii) **Separation of neutral lipids.** Thin-layer plates prepared with Silica Gel G (0.4 mm thick) were activated at 100 C and used within 30 min. The neutral lipids were separated using a solvent system of petroleum ether-diethyl ether-glacial acetic acid (85:15:1, vol/vol/vol). Lipids were detected by iodine vapors and identified by comparison to know standards of neutral lipids previously identified in this organism (20). To estimate the relative concentrations of the neutral lipids, plates were sprayed with chlorosulfonic acid-acetic acid (1:2, vol/vol) and charred at 130 C. Plates were scanned with an Ortec densitometer model 4310, and the peak areas of the individual neutral lipids were determined by triangulation.

**Lipid phosphorus determination.** Lipid samples were evaporated under a stream of nitrogen and hydrolyzed in 0.5 ml of 70% perchloric acid at 100 C for 60 min. Phosphorus was estimated by the procedure of Bartlett (1) using  $\text{KH}_2\text{PO}_4$  as the standard.

**Protein determination.** Protein was estimated by the method of Lowry et al. (18) using bovine serum albumin as the standard.

**Estimation of KDO.** Membrane fractions (0.2 mg of protein) were suspended in an equal volume (50  $\mu\text{l}$ ) of 0.5 N  $\text{H}_2\text{SO}_4$  and hydrolyzed at 100 C for 8 min to liberate 2-keto-3-deoxyoctulosonic acid (KDO) from lipopolysaccharide (LPS). KDO was estimated by the thiobarbituric acid method (14). The final chromogen was extracted with 1 ml of butanol that contained 5% concentrated HCl. After centrifugation at  $500 \times g$  for 10 min at 4 C, the difference in absorbance between 552 and 508 nm was determined. The amount of KDO per milligram of membrane protein was determined using the micromolar extinction coefficient of 19.

**LPS content.** LPS was extracted with phenol-water from lyophilized whole cells and purified by the modified Westphal and Jann procedure (45).

**Electron microscopy.** Negative stains of the various membrane fractions were prepared by charring Formvar-carbon-coated grids with a suspension of the membranes diluted with 1.5% phosphotungstic acid (pH 7.0). After incubation for 1 min at room temperature, excess fluid was removed from the grids with filter paper wicks, and the grids were air dried.

Specimens were examined in a Philips 200 electron microscope operated at 80 kV.

## RESULTS

### Isolation of membranes from *Acinetobac-*

*ter*. Membranes were isolated from NBYE-grown cells for comparative analyses. Discontinuous sucrose gradient centrifugation of the crude membranes obtained from nonhydrocarbon grown cells resulted in the banding pattern shown in Fig. 1. A single band was obtained at a density of 1.182  $\text{g}/\text{cm}^3$ . The band was shown to contain enzymatic activities normally associated with both the outer and cytoplasmic membranes of gram-negative bacteria (Table 1). Differential centrifugation was used to separate the outer and cytoplasmic membrane, the identities of which were determined by enzymatic analysis. Centrifugation of the crude membrane fraction at  $35,000 \times g$  for 1 min resulted in the sedimentation of the cytoplasmic membrane, with the outer membrane remaining in the supernatant. The cytoplasmic membrane was purified further by suspending the pellet and repeating the centrifugation. The outer membrane was recovered from the pooled supernatant fractions by centrifugation at  $65,000 \times g$  for 2 h. Both membrane fractions were analyzed on linear sucrose gradients to determine their buoyant densities. Little difference was found between the densities of the cytoplasmic membrane (1.161  $\text{g}/\text{cm}^3$ ) and the outer membrane (1.147  $\text{g}/\text{cm}^3$ ).

Discontinuous sucrose gradient centrifugation of the crude membranes obtained from hexadecane-grown cells resulted in three bands (Fig. 2). The recovery of membrane protein from the discontinuous gradient was greater than 95% with band 1, band 2, and band 3, comprising 29%, 54%, and 17%, respectively. It was found by enzymatic criteria that outer membrane banded with the cytoplasmic membrane (band 2) at a density of 1.182  $\text{g}/\text{cm}^3$ . Consequently, the outer membrane was isolated by differential centrifugation as described for NBYE outer membrane. The pellet resulting after differential centrifugation was centrifuged through discontinuous sucrose gradients, resulting in band 1 (density, 1.099  $\text{g}/\text{cm}^3$ ). There was no material banding at a density of 1.258  $\text{g}/\text{cm}^3$ . The outer membrane was recovered from the supernatant and separated by discontinuous sucrose gradient centrifugation into band 2 (density, 1.182  $\text{g}/\text{cm}^3$ ) and band 3 (density, 1.258  $\text{g}/\text{cm}^3$ ). Both outer membrane fractions exhibited phospholipase activity but no phosphatidic acid cytidyltransferase activity.

Analysis of the membrane fractions by continuous sucrose gradient centrifugation established their buoyant densities to be 1.086  $\text{g}/\text{cm}^3$  for band 1, 1.138  $\text{g}/\text{cm}^3$  for cytoplasmic membrane (band 2), 1.141  $\text{g}/\text{cm}^3$  for outer membrane (band 2), and 1.258  $\text{g}/\text{cm}^3$  for outer membrane (band 3).

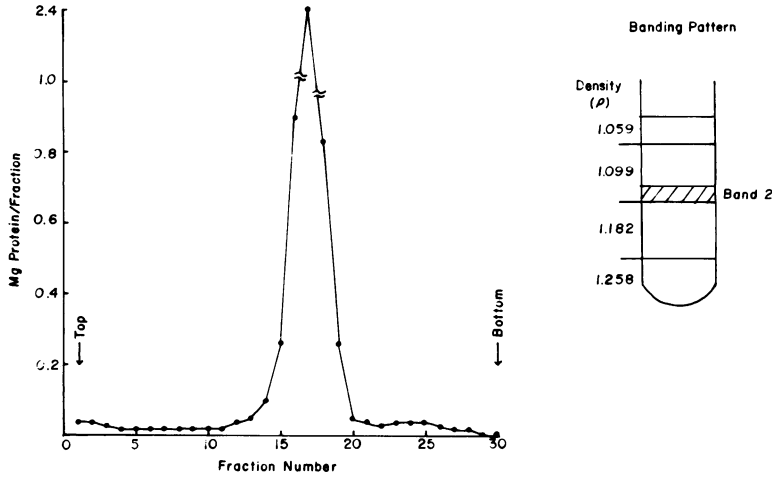


FIG. 1. Discontinuous sucrose gradient centrifugation of crude membranes derived from NBYE-grown cells.

TABLE 1. Enzyme activities of membrane fractions from cells cultured in NBYE and hexadecane

Membrane fraction	Sp act (nmol/min per mg of protein)							
	NBYE membranes				Hexadecane membranes			
	Phospho- lipase	NADH oxidase	ATPase	PACT <sup>a</sup>	Phospho- lipase	NADH oxidase	ATPase	PACT <sup>a</sup>
Crude membrane	1.04	25	17	ND <sup>b</sup>	1.03	ND	ND	2.6
Band 1					0.21	53	18	7.8
Cytoplasmic membrane	0.16	60	22	6.7	0.6	53	21	5.9
Outer membrane	2.71	5	0	0.6	10.50	4	0	1.0

<sup>a</sup> Phosphatidic acid cytidyltransferase specific activity expressed as micromoles per milligram per hour.  
<sup>b</sup> ND, Not determined.

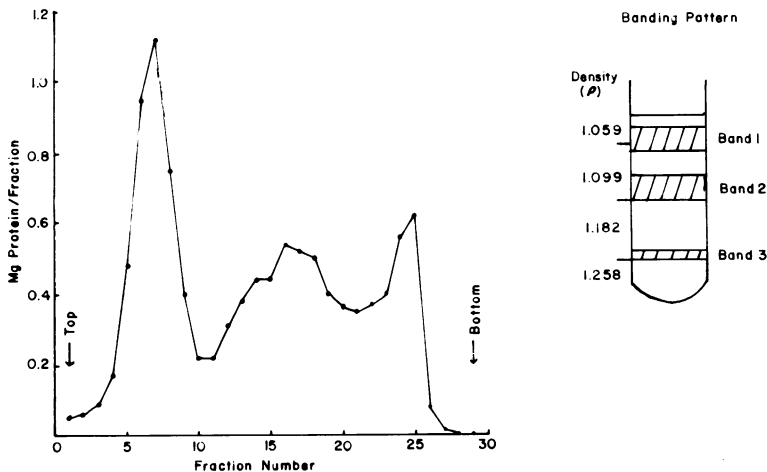


FIG. 2. Discontinuous sucrose gradient centrifugation of crude membranes derived from hexadecane-grown cells.

The two outer membrane fractions were analyzed for differences that might partially explain their centrifugal characteristics. The amounts of glucosamine and KDO were essen-

tially equal (5.3  $\mu\text{g}/\text{mg}$  of membrane protein and 155 pmol/mg of membrane protein, respectively) for the two outer membrane fractions. Phospholipase activity was also equal between

the two membrane fractions (10.0 nmol/min per mg of protein). They also appeared morphologically identical as observed by electron microscopy using the negative-staining technique. Therefore, the two outer membrane fractions were pooled for subsequent analyses.

Enzymatic analyses were used to establish the identity and purity of the membrane fraction at the various stages of isolation (Table 1). NADH oxidase, ATPase, and phosphatidic acid cytidyltransferase were predominantly localized in the cytoplasmic membrane of NBYE- and hexadecane-grown cells and in band 1 of hexadecane-grown cells. Phospholipase activity was mainly localized in the outer membrane of NBYE- and hexadecane-grown cells. A small amount of contamination of the cytoplasmic membrane with the outer membrane was observed, amounting to less than 6%.

**Characterization of isolated membrane fractions.** The various membrane fractions derived from NBYE- and hexadecane-grown cells were examined by electron microscopy using the negative-staining technique. The outer membrane fractions from NBYE (Fig. 4)- and hexadecane (Fig. 7)-grown cells were a homogeneous suspension of small vesicles (0.1  $\mu\text{m}$ ) and open "C"-shaped structures. A regular pattern of subunits was not observed on the outer membrane fragments, as has been reported for a putative *Acinetobacter* sp. (41). The cytoplasmic membrane fractions derived from NBYE (Fig. 3)- and hexadecane (Fig. 6)-grown cells were composed of larger fragments and vesicles of variable size, ranging from 0.1 to 0.5  $\mu\text{m}$ . Band 1 of hexadecane-grown cells (Fig. 5) was similar to the cytoplasmic membrane fractions, except electron transparent vesicles were apparent, averaging 0.2  $\mu\text{m}$  in diameter. These vesicles were identified as the hydrocarbon inclusions previously seen in whole cells (16) and are the subject of a separate report (38).

The chemical composition of the various membrane fractions is shown in Table 2. The ratio of lipid phosphorus to protein of the cytoplasmic membrane from NBYE- and hexadecane-grown cells was twice as high as the ratio of lipid phosphorus to protein of the outer membrane. Band 1 from hydrocarbon-grown cells was a lipid-rich membrane fraction, characterized by a ratio of lipid phosphorus to protein over twice as high as the cytoplasmic membrane.

Analysis of the amount of hexadecane in the various membrane fractions derived from hexadecane-grown cells revealed a high amount associated with band 1. This hexadecane was tightly associated with the membranes since several washes, followed by purification over

sucrose gradients, did not result in the loss of the hydrocarbon. Hydrocarbon inclusions were observed in negative stains of band 1, which may partially account for the presence of the hexadecane. The cytoplasmic membrane fraction contained one-third the amount of hexadecane found in band 1. Hexadecane was not detected in the outer membrane.

The membrane fractions were assayed for KDO, a component of LPS. The KDO was predominantly localized in the outer membrane fractions of NBYE- and hexadecane-grown cells. Smaller amounts of KDO were present in the other membrane fractions. The amount of KDO per milligram of membrane protein in the outer membrane was, however, about 1,000-fold less than reported for the outer membrane fractions of *Salmonella typhimurium* (29). A lower content of LPS has been reported for another species of *Acinetobacter* (40). LPS was extracted and purified from *Acinetobacter* strain HO1-N to determine the amount associated with the outer membrane. NBYE-grown cells contained 0.24% LPS/g (dry cell weight) as compared with 1.37% LPS/g (dry cell weight) for hexadecane-grown cells.

The relative concentrations of phospholipids in the various membrane fractions derived from *Acinetobacter* after growth on NBYE and hexadecane are shown in Table 3. The major phospholipid in all of the membrane fractions was phosphatidylethanolamine. The outer membrane from both NBYE- and hexadecane-grown cells contained a higher concentration of cardiolipin and lysocardiolipin (R. E. Torregrossa, R. A. Makula, and W. R. Finnerty, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, K69, p. 158) than that found in the cytoplasmic membrane. Band 1 of hexadecane-grown cells also contained a high concentration of lysocardiolipin.

The relative concentrations of the neutral lipids in the various membrane fractions are shown in Table 4. Fatty alcohol was the major neutral lipid class in the membrane fractions, being as high as 65 to 70% of the total neutral lipid in membranes from hexadecane-grown cells. Membranes derived from NBYE-grown cells did not contain as much free fatty alcohol as did membranes derived from hexadecane-grown cells but contained more free fatty acid and mono- and diglyceride.

The various membrane fractions were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 8). The cytoplasmic membrane of both NBYE- and hexadecane-grown cells and band 1 contained between 20 and 25 polypeptide bands. Band 1 and the cytoplasmic membrane of hexadecane-grown cells appeared identical, with the

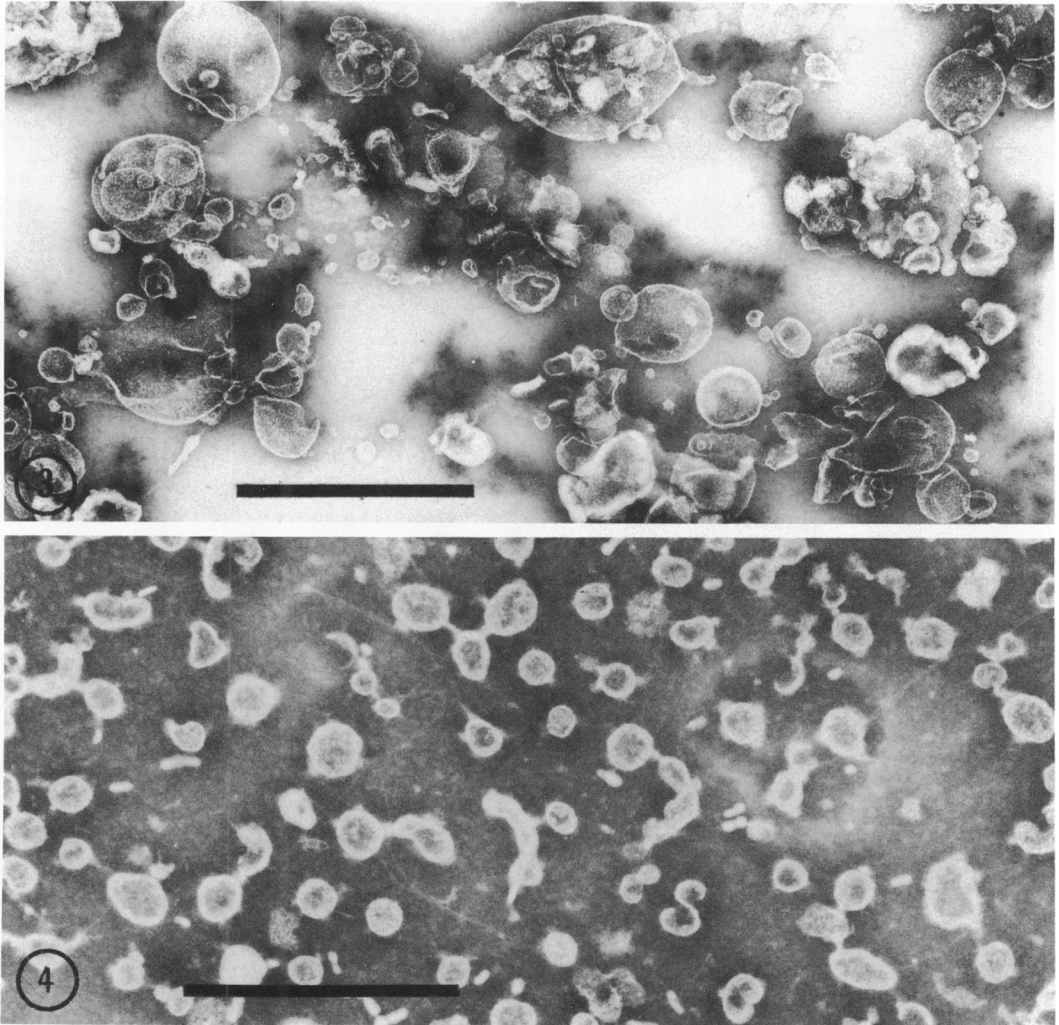


FIG. 3. Negative stain of cytoplasmic membrane derived from NBYE-grown cells. Bar represents 1.0  $\mu\text{m}$ .  
 FIG. 4. Negative stain of outer membrane derived from NBYE-grown cells. Bar represents 0.5  $\mu\text{m}$ .

exception of a greater amount of two polypeptide species in band 1 (arrows, Fig. 8). The outer membrane fractions contained fewer polypeptides (15 to 20) than did the cytoplasmic membrane, especially in the higher molecular weight range. One major protein species predominated in the outer membrane fraction and had an apparent molecular weight of 44,000 for NBYE-grown cells and 42,000 for hexadecane-grown cells.

#### DISCUSSION

The procedure developed for obtaining the membranes of *Acinetobacter* species HO1-N generally follows those described by Miura and Mizushima (22) and Osborn et al. (29), with certain modifications. The major difference was

the absence of ethylenediaminetetraacetate (EDTA) in any of the procedural steps. The treatment of cells suspended in 0.6 M sucrose with lysozyme resulted in excess of 95% cell lysis. Further treatment of the cell preparations by physical or chemical methods was unnecessary for obtaining the successful separation of the membrane fractions. It is stressed that the procedure developed for obtaining the membranes of *Acinetobacter* sp. HO1-N does not require harsh physical or chemical treatments, such as sonic treatment, French pressure cell disruption, or EDTA. The ability to avoid such treatments are considered highly advantageous for securing membrane fractions of unaltered structure or composition.

Lieve (17) has shown that EDTA does effect

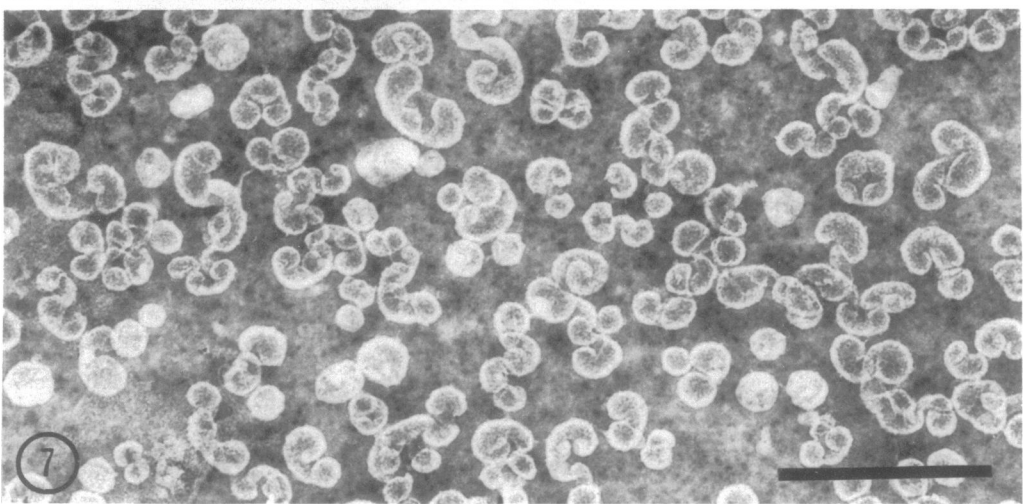
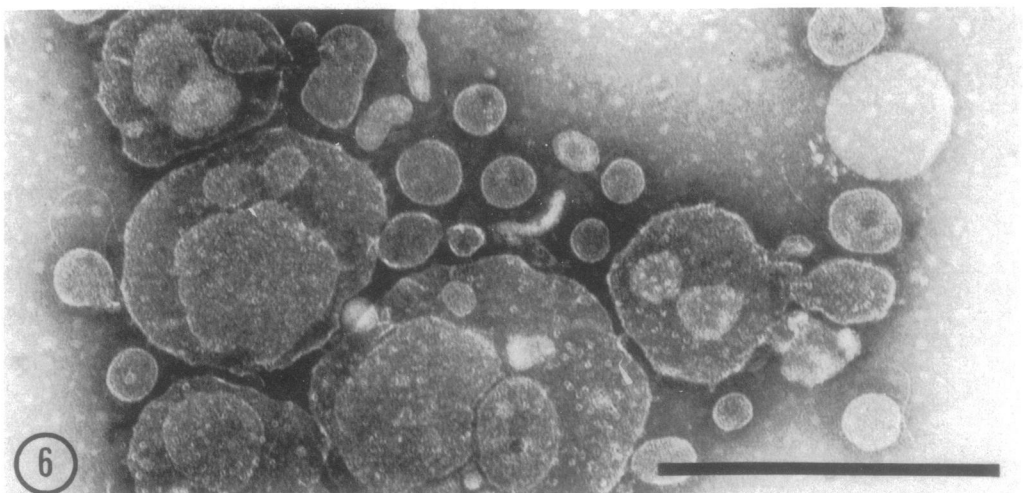
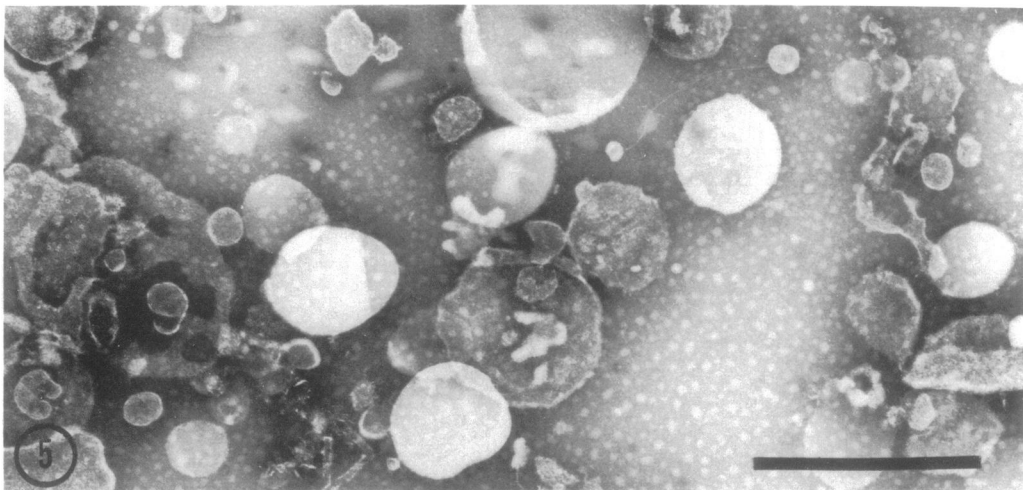


FIG. 5. Negative stain of band 1 derived from hexadecane-grown cells. Bar represents 0.5  $\mu\text{m}$ .

FIG. 6. Negative stain of cytoplasmic membrane derived from hexadecane-grown cells. Bar represents 0.5  $\mu\text{m}$ .

FIG. 7. Negative stain of outer membrane derived from hexadecane-grown cells. Bar represents 0.5  $\mu\text{m}$ .



TABLE 2. Chemical composition of membrane fractions from cells cultured in NBYE and hexadecane

Membrane fraction	Lipid phosphorus/protein ( $\mu\text{mol}/\text{mg}$ )		Hexadecane/ protein ( $\mu\text{mol}/\text{mg}$ )	KDO/protein (pmol/mg)	
	NBYE mem- branes	Hexadecane membranes	Hexadecane membranes	NBYE mem- branes	Hexadecane membranes
Crude membranes	0.34	0.33	0.88	ND <sup>a</sup>	ND
Band 1		0.55	1.88		35
Cytoplasmic membrane	0.60	0.30	0.67	14	34
Outer membrane	0.23	0.16	0	390	203

<sup>a</sup> ND, Not determined.

TABLE 3. Relative concentrations of phospholipids in membrane fractions of *Acinetobacter*

Phospholipids	Concn (%) of phospholipids in:				
	Hexadecane-grown cells			NBYE-grown cells	
	Band 1	Cytoplasmic membrane	Outer mem- brane	Cytoplasmic membrane	Outer mem- brane
Phosphatidylethanolamine	50	69	41	70	51
Lysophosphatidylethanolamine	3	3	4	3	3
Cardiolipin	17	6	20	7	19
Lysocardiolipin	11	3	19	10	15
Phosphatidylglycerol	8	13	17	10	11
Unidentified phospholipid	11	6	ND <sup>a</sup>	Trace	Trace

<sup>a</sup> ND, Not detected.

TABLE 4. Relative concentrations of neutral lipids in membrane fractions of *Acinetobacter*

Lipid class <sup>a</sup>	Concn (%) of neutral lipids in:				
	Hexadecane-grown cells			NBYE-grown cells	
	Band 1	Cytoplasmic membrane	Outer mem- brane	Cytoplasmic membrane	Outer mem- brane
Wax ester	11	14	5	15	11
Free fatty acid	3	6	7	14	18
Free fatty alcohol	71	64	69	48	47
Triglyceride	ND <sup>b</sup>	ND	ND	ND	ND
Diglyceride	7	6	9	11	16
Monoglyceride	6	Trace	5	11	9
Unidentified lipid <sup>c</sup>	2	5	4	Trace	Trace

<sup>a</sup> Qualitative and quantitative determinations of the neutral lipids of *Acinetobacter* are described in reference 20.

<sup>b</sup> ND, Not detected.

<sup>c</sup> Unidentified lipid is chromatographically identical in all membrane fractions.

the outer membrane of gram-negative bacteria. Treatment of isolated cell envelopes of *Pseudomonas aeruginosa* with EDTA has been shown to release a protein-LPS complex and phospholipid (32, 33). Other procedures employing sonic treatment or French pressure disruption have resulted in enriched membrane fractions exhibiting characteristic structures (35, 41).

The cytoplasmic and outer membranes of *Acinetobacter* grown on hydrocarbon and nonhydrocarbon substrates, respectively, have been separated with a high degree of purity, as de-

termined by chemical, morphological, and enzymatic criteria. The properties of these membrane fractions are similar to those reported for enriched cytoplasmic and outer membrane fractions of other gram-negative bacteria.

The outer membrane fractions, composed of small vesicles and C-shaped structures, are similar in appearance to outer membrane preparations of *Escherichia coli* (35), *Proteus mirabilis* (12, 27), and *Acinetobacter* sp. strain MJT/F5/199A (41). Schnaitman (35) has proposed that the inability of the outer membrane to form vesicles is due to the presence of the rigid

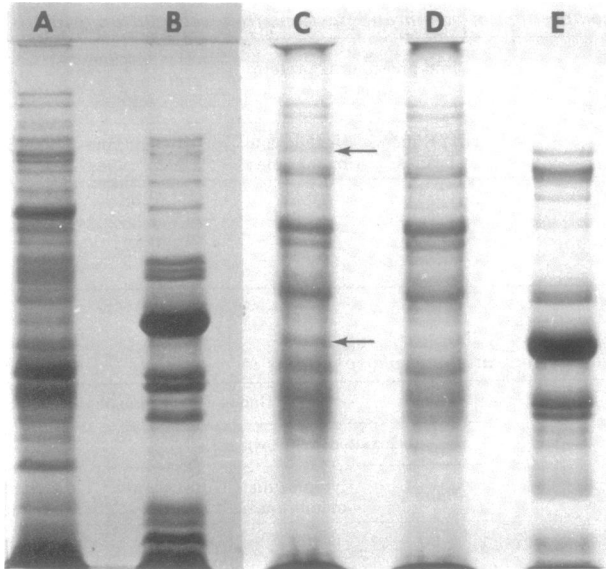


FIG. 8. SDS-polyacrylamide gel electrophoresis of membrane fractions. (A) Cytoplasmic membrane of NBYE-grown cells; (B) outer membrane of NBYE-grown cells; (C) band 1 of hexadecane-grown cells; (D) cytoplasmic membrane of hexadecane-grown cells; (E) outer membrane of hexadecane-grown cells.

peptidoglycan layer. However, in this investigation and in the isolation of outer membranes from *P. mirabilis* (12, 27), lysozyme was used in spheroplast formation, and C-shaped structures were observed in the outer membrane preparations. Oltmann and Stouthamer (27) showed that 95% or more of the mucopeptide layer was lost from the cell envelope of *P. mirabilis* during the lysis procedure. Therefore, factors other than the presence of peptidoglycan may be involved in imparting this characteristic structure to outer membrane fragments.

The relatively low phospholipid content, the presence of KDO, phospholipase activity, and the electrophoretic pattern of the outer membrane proteins were similar to the outer membrane preparations described for other gram-negative bacteria. The outer membrane preparations from both NBYE- and hexadecane-grown cells exhibited approximately 15 to 20 polypeptide species on SDS-polyacrylamide gels. A major protein species with an apparent molecular weight of approximately 40,000 was present in both preparations. This characteristic banding pattern has been observed for outer membrane preparations of *E. coli* (35), *S. typhimurium* (29), and *P. mirabilis* (12, 27). Schnaitman (35) has shown that this major protein species accounts for 70% of the total protein of the outer membrane of *E. coli* O111a. Schnaitman (37) has more recently been able to resolve the 42,000-dalton major protein of *E. coli* O111a

into four distinct protein fractions by ion-exchange chromatography.

A unique difference, compared with outer membrane preparations from other organisms, was found in the outer membrane of *Acinetobacter* grown on both hydrocarbon and nonhydrocarbon substrates, namely, its relatively low buoyant density. The outer membrane obtained from NBYE-grown cells had a buoyant density of 1.147 g/cm<sup>3</sup>, and the outer membranes from hexadecane-grown cells had buoyant densities of 1.141 and 1.25 g/cm<sup>3</sup>. A density of approximately 1.22 g/cm<sup>3</sup> has been reported for outer membrane preparations from *S. typhimurium* (29) and *E. coli* (35). Since the buoyant density of the outer and cytoplasmic membranes of *Acinetobacter* were so similar, differential centrifugation was necessary to obtain their separation. However, a small amount (approximately 15 to 17%) of the total membrane of *Acinetobacter* banded at a density of 1.25 g/cm<sup>3</sup>. Further comparative studies of the two outer membrane fractions obtained are needed to determine the reason for the observed density differences. Apparent differences in the amounts of LPS or peptidoglycan were not obtained for the two outer membrane preparations. Mizushima and Yamada (24) have reported the isolation and characterization of two outer membrane preparations from *E. coli*. These outer membrane fractions differed morphologically when viewed by electron micros-

copy and in their cardiolipin content. The densities of these outer membrane fractions were not determined.

The electron transport enzymes and the phospholipid biosynthetic enzyme phosphatidic acid cytidyltransferase were localized almost exclusively in the cytoplasmic membrane fraction of *Acinetobacter*.

The phospholipids and neutral lipids of all the membrane fractions were qualitatively similar; however, quantitative differences did exist. The major phospholipid in all membrane fractions was phosphatidylethanolamine. This phospholipid has been shown to represent the major phospholipid in the cytoplasmic and outer membranes of *E. coli* (46) and *S. typhimurium* (29). In *E. coli*, the proportion of lysophosphatidylethanolamine was greater in the outer membrane than in the cytoplasmic membrane. In contrast, *Acinetobacter* contained a fairly high amount of lysocardiolipin in the outer membrane. This phospholipid was not reported to be present in the membranes of *E. coli* (46) or *S. typhimurium* (29).

A unique membrane fraction, band 1, was isolated and purified from hexadecane-grown cells of *Acinetobacter*. This low-density membrane fraction, absent in NBYE-grown cells, contained significantly greater amounts of phospholipid and hexadecane than did the cytoplasmic or outer membranes derived from hexadecane-grown cells. The enzymatic activities associated with this membrane fraction were almost identical to those found in the cytoplasmic membrane. The gel electrophoretic pattern of the proteins from band 1 exhibited only minor quantitative differences as compared with the banding pattern of the cytoplasmic membrane. Quantitative differences did exist, however, between the phospholipid content of band 1 and the cytoplasmic membrane.

Band 1 contained significantly greater amounts of phospholipid and hexadecane than did the cytoplasmic membrane. This may be due, in part, to the presence of the hexadecane inclusions and the intracytoplasmic membranes previously observed in hydrocarbon-grown cells. Kennedy and Finnerty (15) have presented evidence for a physical association between the hexadecane inclusions and the intracytoplasmic membranes. The analysis of band 1 through discontinuous and linear sucrose gradients established further support for the hexadecane inclusion-membrane complex. The freeze-etching of hexadecane-grown cells revealed the intracytoplasmic membranes to be smooth surfaced, indicative of a high lipid content. Until more specific chemical, morphologi-

cal, or enzymatic markers are recognized for the intracytoplasmic membrane, the identity of band 1 will remain obscure.

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