Isolation and Chemical Composition of the Cytoplasmic Membrane of a Gram-Negative Bacterium

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With procedures developed previously in this laboratory, the various layers of the cell wall of a gram-negative bacterium, a marine pseudomonad (ATCC 19855), were removed completely giving rise to true protoplasts. Membranes were isolated from the protoplasts formed. After treatment with ribonuclease, deoxyribonuclease, and washing, the membranes isolated were shown by electron microscopy and chemical analysis to be essentially free from both wall material and cytoplasmic constituents. The membranes gave rise to a single compact band in a sucrose density gradient. All of the lipid and protein were found to be associated in the membrane band. Analysis showed the membranes to contain 30.5% lipid (78% of which was phospholipid), 62.8% protein, and 2% carbohydrate. The predominant phospholipid present was phosphatidylethanolamine with a lesser amount of diphosphatidylglycerol and traces of unidentified compounds.

In most previous attempts to isolate purified and homogenous cytoplasmic membranes from gram-negative bacteria, only limited success has been achieved. For gram-positive bacteria, stabilized protoplasts are formed with facility and exhibit minimal, if any, remaining cell wall components (10, 28, 32). Cell membranes are then readily obtained and purified from the protoplasts of gram-positive cells (28).

The cell wall cytoplasmic membrane complex (cell envelope) of gram-negative bacteria presents a more complicated situation. In addition to an inner cytoplasmic membrane, another outer membrane structure or cell wall double track has been observed (16, 21). A rigid peptidoglycan layer has also been demonstrated between the outer double track layer and the plasma membrane (22). Although osmotically fragile spheroplasts can readily be formed from most gram-negative bacteria (18), at this time there have been no reports from other laboratories of the successful removal of all of the cell wall material from a gram-negative bacterium and the formation of a true protoplast from such an organism.

Previous studies in this laboratory have elucidated a system for the complete removal of all of the cell wall material from a gram-negative marine pseudomonad (7, 8). From the stable, true protoplasts formed by the technique developed, a procedure has now been devised for the isolation and purification of homogeneous cytoplasmic

membranes from the organism. The purity and homogeneity of these cytoplasmic membranes have been established and their chemical composition has been determined.

MATERIALS AND METHODS

Organism. The marine pseudomonad used in this study was originally isolated from a marine clam and is designated as strain B-16. The organism has been classified as a *Pseudomonas* species type IV and is deposited in both the American Type Culture Collection as ATCC 19855 and the National Collection of Marine Bacteria (Aberdeen, Scotland) as NCMB 19.

Medium and culture maintenance. Cells were grown in a liquid medium containing 0.8% nutrient broth (Difco), 0.5% yeast extract (Difco), 0.22 M NaCl, 0.026 M $MgCl_2$, 0.01 M KCl, and 0.1 mM $FeSO_4$ $(NH_4)_2SO_4$. The culture was maintained by monthly transfer on slants of this medium with the addition of 1.5% agar.

Growth conditions. The procedure for growing the cells has been previously described (8). For this work, larger amounts of the exponential-phase cells were required. In the final inoculation step, 40 ml of the culture was added to each of nine 2-liter flasks containing 250 ml of the liquid medium.

Preparation of mureinoplasts and protoplasts. Mureinoplasts and protoplasts were prepared essentially as reported earlier (8), with the following modifications. After harvesting, the cells were washed three times by suspension in and centrifugation from volumes of 0.5 M NaCl equal to one-half the volume of the growth medium. The suspensions were centrifuged at 16,000 × *g* at 4 C. Next, the washed cells were incubated at 25 C

for 30 min on a rotary shaker in 1,200 ml of 0.5 M sucrose. After harvesting, the cells were suspended in 1,200 ml of 0.5 M sucrose and centrifuged immediately. The suspensions in 0.5 M sucrose were centrifuged at 22,000 × g at 4 C. All of the cell wall material except the peptidoglycan layer had now been removed, and the cells in this form have been termed mureinoplasts (8).

The mureinoplasts were converted to protoplasts by the procedure described previously (8). In the present instance, however, the concentration of tris(hydroxymethyl)aminomethane (Tris) in the protoplasting medium was increased to 0.01 M. Protoplast formation was complete as determined by phase-contrast microscopy and terminated after a 10-min incubation period at 25 C with occasional shaking. The protoplasts were harvested by centrifugation at 10,000 × g at 4 C.

Membrane preparation. After removal of the supernatant protoplasting medium, the protoplasts were suspended, by using a Pyrex Ten Broeck tissue homogenizer, in 80 ml of a precooled solution which contained 0.3 M NaCl, 0.05 M MgSO$_4$, 0.01 M KCl, and 0.01 M Tris buffer, pH 7.5 (salts-Tris buffer solution). The suspended protoplast material was then passed a single time through a French pressure cell (American Instrument Co., Inc., Silver Spring, Md.), at a pressure of 15,000 to 16,000 lb/in^2, and added directly to 170 ml more of the precooled salts-Tris buffer solution. Lysozyme (Sigma Chemical Co., 3× crystallized, 30,000 units/mg), 150 µg/ml; deoxyribonuclease (Sigma Chemical Co., beef pancreas, 720 Kunitz units/mg), 50 µg/ml; and ribonuclease (General Biochemicals, 5× crystallized, 48 Kunitz units/mg), 50 µg/ml, were added to the total of 250 ml of the salts-Tris buffer solution containing the broken protoplasts, and the complete mixture was incubated for 15 min at 25 C with shaking.

After the incubation period, a low-speed centrifugation step (1,000 × g for 10 min at 4 C) was carried out to remove any unbroken protoplasts. The low-speed pellet (20 to 25% of the total material) was discarded, and the remaining material in the supernatants was subjected to a high-speed centrifugation step (73,000 × g for 60 min at 3 C). The high-speed pellets obtained were checked for any remaining protoplasts with the phase-contrast microscope, and the material in them was washed six times by successive suspension in 250-ml volumes of precooled salts-Tris buffer solution and centrifugation at 73,000 × g for 30 min at 3 C.

After each centrifugation, it was necessary to use a cell homogenizer to effect suspension. When six washes had been completed, no ultraviolet-absorbing material could be detected in the supernatant fluids upon examination with a recording Unicam spectrophotometer over the range of 220 to 300 nm. For chemical determinations, the washed membranes were suspended in cold glass-distilled water and dialyzed at 4 C against six 3-liter volumes of cold glass-distilled water changed every 30 min. The optical density of the membrane suspension remained constant or showed a very minimal reduction over the dialysis period. The dialyzed membrane suspension was lyophilized and stored in a desiccator.

Electron microscopy. The washed membranes were prefixed by adding to the centrifuged membrane pellet a solution of 0.5% glutaraldehyde in 0.2 M phosphate buffer (pH 6.2) containing 0.3 M NaCl, 0.05 M MgSO$_4$, and 0.01 M KCl. After incubation for 1 hr at 25 C, the prefixative solution was poured off the pellet. The pellet was then immediately homogenized for 5 to 10 sec in liquid agar at 60 C containing the above salts, and solidified agar cores of the homogenized membranes were prepared immediately. The membrane agar cores were then fixed, postfixed, and sectioned, and the sections were stained by using procedures described previously for cells of this organism (31).

Density gradient centrifugation. Membrane samples (0.4 ml, containing 0.5 mg of lyophilized membranes or an equivalent amount of a freshly prepared membrane suspension) were layered over 12.6 ml of a sucrose gradient formed from 6.8 ml of 30% (w/v) sucrose and 5.8 ml of 60% (w/v) sucrose, with both solutions also containing 0.3 M NaCl, 0.05 M MgSO$_4$, 0.01 M KCl, and 50 mM Tris-hydrochloride buffer, pH 7.5. The gradients were centrifuged at 198,500 × g for 60 min in a Beckman model L2-65B ultracentrifuge by using an SW 40 Ti swinging bucket rotor. After centrifugation, the gradients were visually examined, and 0.5-ml fractions were collected with an Isco model 180 density gradient fractionator. The various fractions were then diluted with 0.5 ml of glass-distilled water, and the absorbance of each was measured at 280 nm with a recording Unicam spectrophotometer.

Growth of cells on medium containing ¹⁴C-oleic acid. In the last inoculation step in the procedure for cell growth, 9.43 µCi of ¹⁴C-oleic acid (57.8 mCi/mM; Amersham/Searle Corp.) was added to each of two 2-liter flasks containing 250 ml of the growth medium. The membranes were prepared as described above with the volumes of the various solutions reduced in proportion to the amount of growth medium used. Both a freshly prepared membrane suspension and lyophilized membranes were centrifuged in sucrose density gradients. The respective gradients were fractionated as described, and a 0.1-ml sample from each fraction was counted in a Packard Tri-Carb liquid scintillation spectrometer. Samples from these same fractions were also checked for absorbance at 280 nm.

Dry weight determination. Cells washed in 0.5 M NaCl were suspended in 0.5 M NaCl and adjusted to a specific optical density with additional 0.5 M NaCl. Samples of these suspended cells were removed and dried at 80 C. Saline controls were also dried at 80 C. The amount of cells not removed for dry weight determinations was noted, and these cells were subjected to the procedure for membrane preparation. To determine the percentage of the membrane in the cells, the low-speed centrifugation step was omitted to preserve quantitation. The few protoplasts, observed after the initial high-speed centrifugation step, were not seen after the sixth wash because of the use of the cell homogenizer for the resuspensions. At the end of the washing procedure, the membranes were suspended in 0.5 M NaCl, and samples representing specific amounts of cells were withdrawn, dried at 80 C, and weighed. The samples were then ashed at 500 C and weighed. Since the weight of the saline controls remained unchanged, the difference between the weight of the samples at 80 and 500 C was taken as the salt-free dry weight of the samples.

Chemical determinations. Protein was determined by the method of Lowry et al. (17) with lysozyme (Sigma

Chemical Co., 3× crystallized) as the standard. In the sucrose density gradient fractions, protein was taken to be proportional to the amount of 280-nm absorbing material in the fractions.

Free lipid was estimated by using the procedure of Folch et al. (11), and bound lipid was estimated by the method of Huston and Albro (13). Lipid samples were dried at 60 C in tared vessels to a constant weight. Total phospholipid was calculated from percentage of phosphorus, assuming a weight conversion factor of 25 (R.C. Gordon, *unpublished data*). This conversion factor was based on 4.00% phosphorus found in the acetone-insoluble lipids for whole cells of the B-16 marine pseudomonad. The phospholipids were separated by thin-layer chromatography and identified by using procedures described previously (12).

Phosphorus was determined by the method of Allen (1). Hexosamine was analyzed by the technique of Cessi and Piliego (6) by using glucosamine as the standard on samples hydrolyzed for 4 hr at 105 C with 4 N HCl. The method of Dubois et al. (9) was employed for the estimation of nonamino carbohydrate with anhydrous D-glucose as the reference. Nucleic acids were extracted by the method of Schneider (29) with the variation that chloroform-methanol (2:1, v/v) was used instead of 95% ethanol to remove the free lipid present. The diphenylamine (30) and orcinol (30) methods were used to determine deoxyribose and ribose, respectively.

RESULTS

Electron microscopy. The protoplasts produced in this study were examined in thin section by electron microscopy. The protoplasts could be seen to be spherical structures which appeared to be bounded only by their cytoplasmic membrane. They were identical to protoplasts produced from this organism previously. Electron photomicrographs of these protoplasts have been presented in previous publications (7, 8).

After breakage of the protoplasts in the French pressure cell, the membranes obtained were exposed to the action of ribonuclease, deoxyribonuclease, and lysozyme. Ribonuclease was added to aid in the removal of ribosomes from the membranes, deoxyribonuclease was added to reduce the viscosity of the suspension and assist in the removal of deoxyribonucleic acid, and lysozyme was added to solubilize any residual peptidoglycan which might have been left on the outside surface of the protoplasts. Upon examination by electron microscopy, even the low-speed pellet was found to be relatively free from any protoplast-like structures. Occasionally, an unbroken structure was seen, but almost all of the structures noted were vesicles with contaminating cytoplasmic material (Fig. 1).

A high-speed centrifugation step was run on the supernatant fractions from the low-speed centrifugation. The high-speed pellets from this step contained vesicles with contaminating cytoplasmic material (Fig. 2). The material in the

high-speed pellet after six washings in the salts-Tris buffer solution showed no unbroken protoplasts and no particulate contaminating cytoplasmic material (Fig. 3). All of the structures now present were either smooth, gray tangentially sectioned membrane vesicles or equatorially sectioned membrane vesicles with empty centers.

An important point in the preparation of membrane samples for electron microscopy was that it was essential not to store the samples for any length of time at 4 C after prefixation. Such membrane samples would often form myelinic structures similar to the artificial lipid structures reported by Bangham et al. (2).

Density gradient centrifugation. When density gradient centrifugation was run on lyophilized membranes and on an equivalent amount of a freshly prepared suspension of membranes, a similar result was obtained for both (Fig. 4). In each case, the sample migrated as a compact band to the same spot in the gradient. When the gradients containing the similar membrane bands were each fractionated, both the gradient obtained with the lyophilized membranes and the gradient produced with the freshly prepared membranes showed a 280-nm absorption peak at the same position. The 280-nm absorption peak shown in Fig. 5 is representative of both types of membranes. This peak was in fractions which corresponded exactly to the position of the visible compact band on either gradient.

Growth of cells on medium containing ¹⁴C-oleic acid. It was of interest to known if both the lipid and the protein of the cytoplasmic membranes migrated to the same point in the sucrose density gradient. Kahane and Razin (15), using *Mycoplasma laidlawii*, found that ^{3}H-oleic acid was selectively incorporated into cytoplasmic membrane lipids from the growth medium. Cells of our organism were grown on the regular growth medium containing ¹⁴C-oleic acid and membranes were prepared. Both labeled lyophilized membranes and a labeled suspension of freshly prepared membranes were run on gradients similar to those just described, and the various fractions were checked for both absorption at 280 nm and for radioactivity. For both gradients, the same 280-nm absorption peak was again observed. The radioactivity for each gradient was observed in a single peak superimposable upon the 280-nm absorption peak (Fig. 5). The majority of the radioactivity (91%) was extractable as free lipid with chloroform-methanol (2:1, v/v).

Dry weight determination. The membranes were found to constitute 12.3% of the dry weight of the cells on a salt-free dry weight basis.

Chemical determinations. The chemical composition of the cytoplasmic membrane preparation was determined by the procedures described (Table 1).
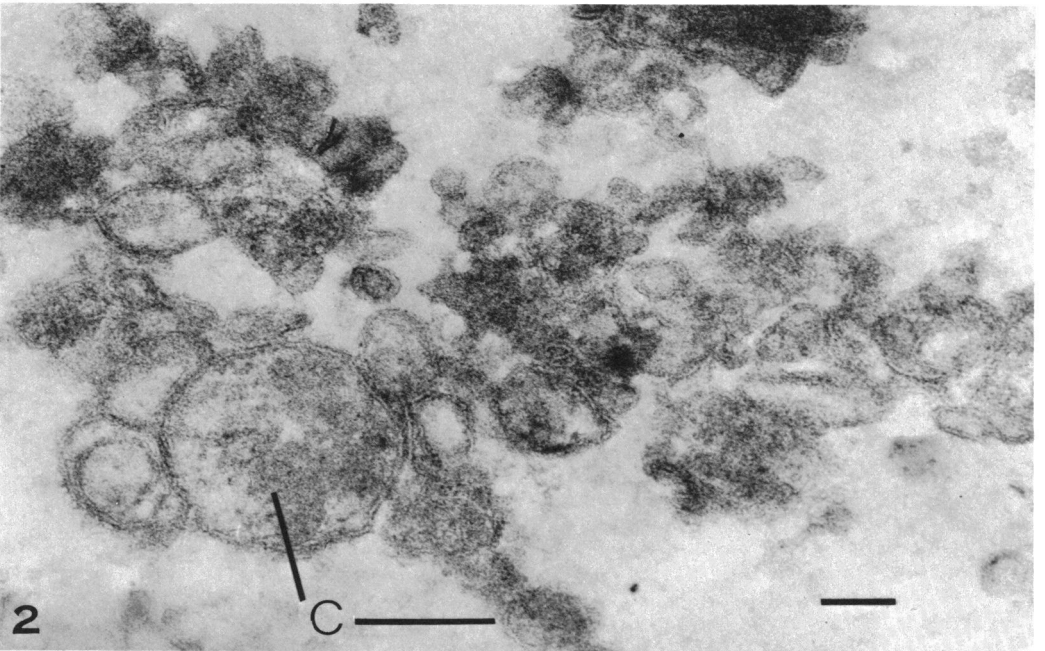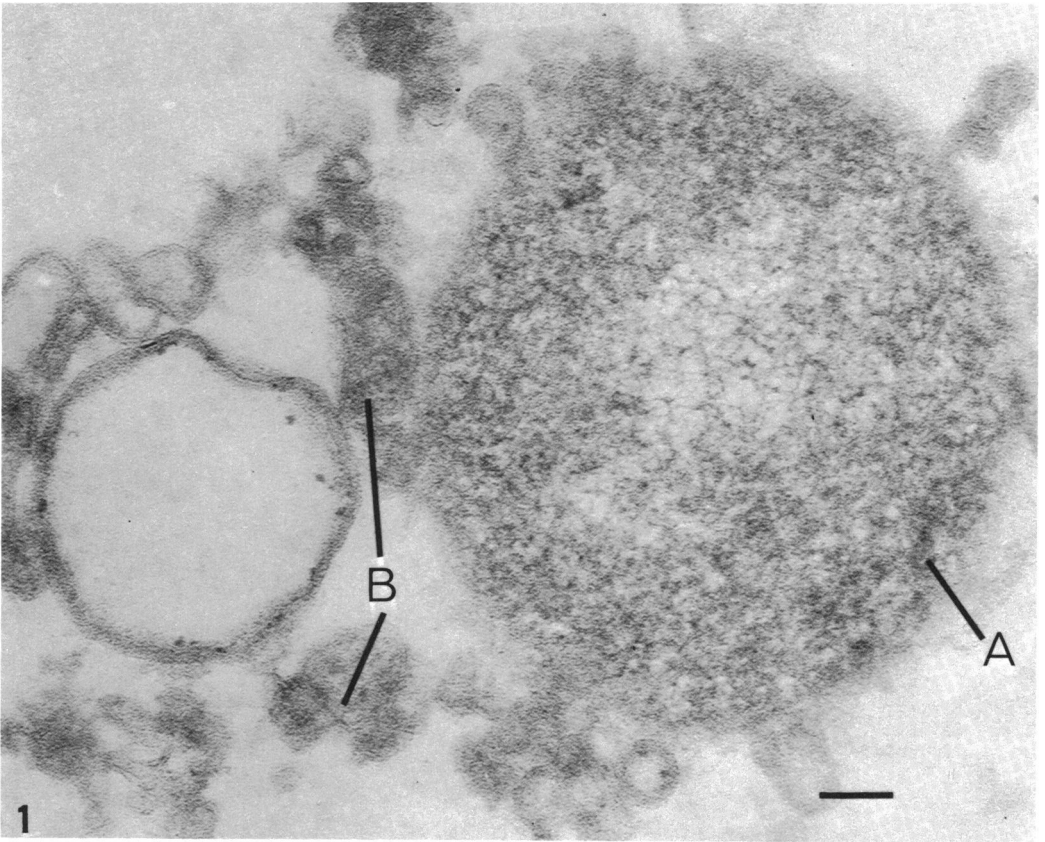
FIG 1. *Electron micrograph of a low-speed pellet after breakage and suspension in deoxyribonuclease, ribonuclease and lysozyme. Note the unbroken structure (A) and the vesicles with contaminating cytoplasmic material (B). The bar represents 0.1 μm.*

FIG. 2. *Electron micrograph of a pellet obtained by high-speed centrifugation of the supernatant which remained after the low-speed centrifugation which produced the pellet illustrated in Fig. 1. Note the contaminating cytoplasmic material inside the vesicles (C). The bar represents 0.1 μm.*
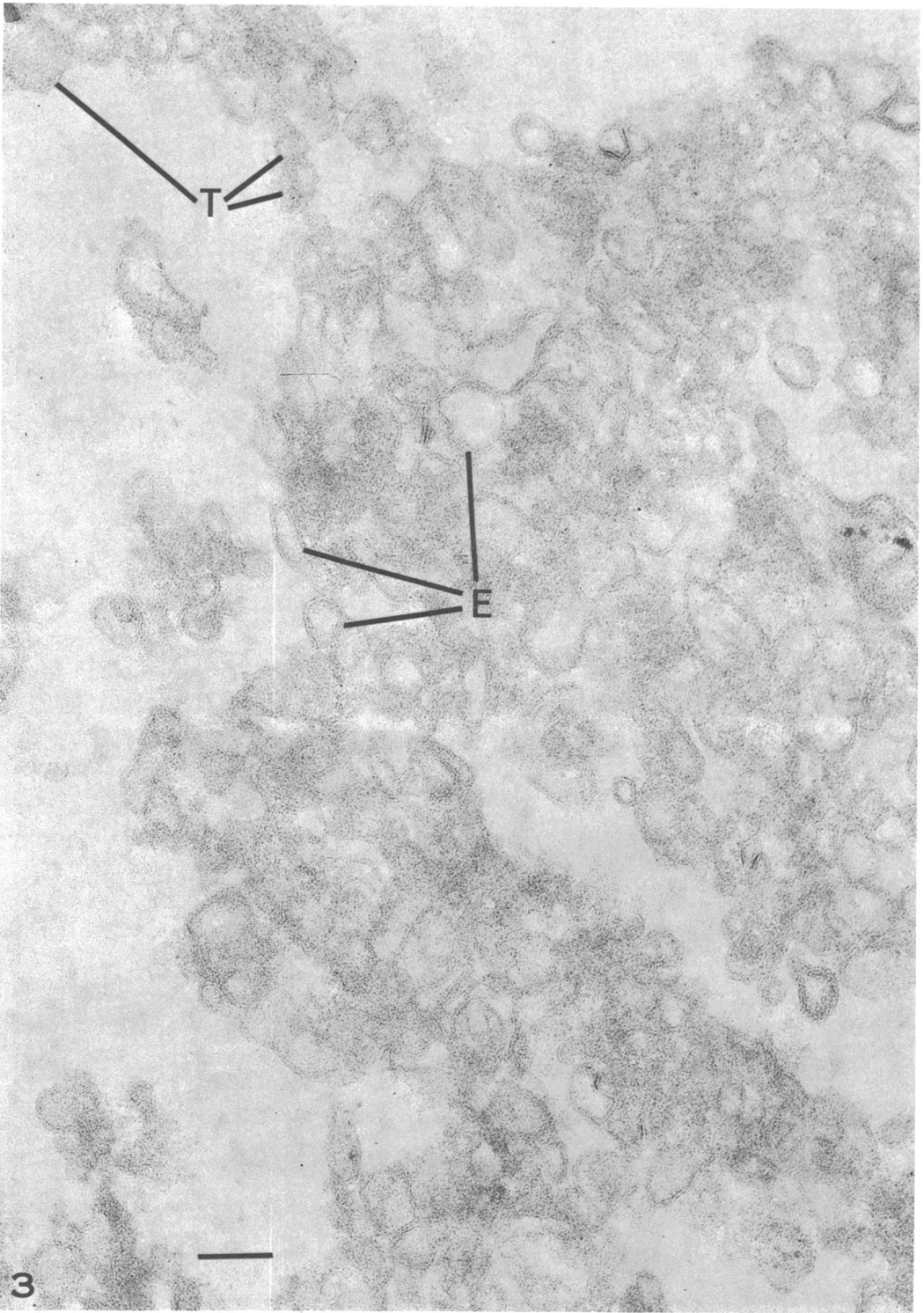
FIG. 3. *Electron micrograph of cytoplasmic membranes after extensive washing in complete salts and Tris buffer. Note the absence of particulate cytoplasmic material, and note that all of the structures seen are either tangentially (T) or equatorially (E) sectioned small membrane vesicles. The bar represents 0.1 μm.*
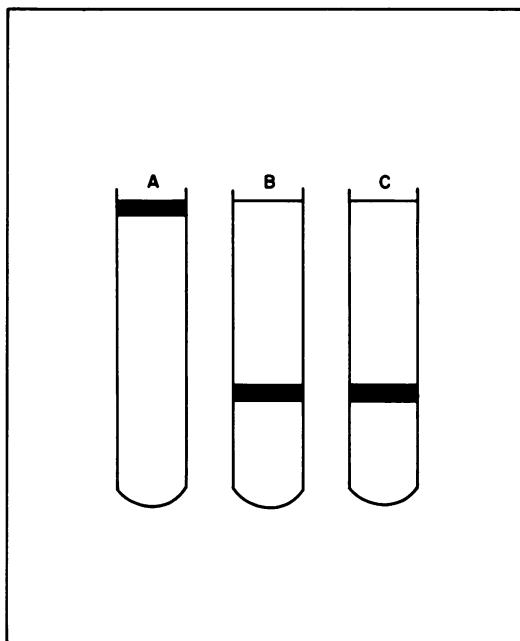
FIG. 4. *Sucrose density gradient analysis of cytoplasmic membrane material. Gradient A aepicts the respective samples after layering on the top of a 30 to 60% sucrose gradient. After centrifugation, 0.5 mg of lyophilized cytoplasmic membrane material (gradient B) and an equivalent amount of freshly prepared suspension of cytoplasmic membranes (gradient C) migrated as shown.*
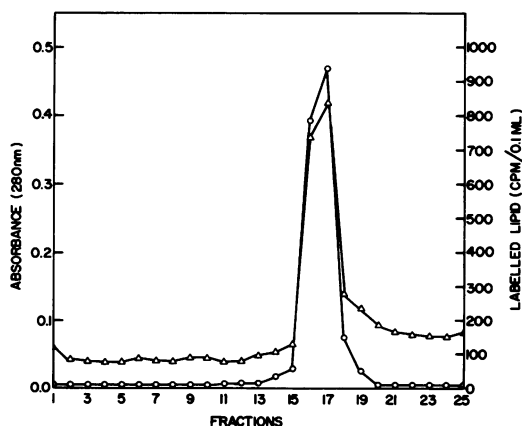

FIG. 5. *Migration of cytoplasmic material in the sucrose density gradients. Lyophilized membranes (labeled with ¹⁴C-oleic acid) and freshly prepared membranes (labeled with ¹⁴C-oleic acid) were centrifuged in gradients depicted in Fig. 4. Both gradients were fractionated, and the fractions were examined for 280-nm absorbing material and for radioactivity. Identical curves were obtained for both gradients. Curve 1 (△), absorbance of fractions at 280 nm. Curve 2 (○), distribution of radioactivity. The fractions for each gradient are numbered from the top of the respective gradient.*

TABLE 1. *Chemical composition of the isolated and purified cytoplasmic membranes*

Individual constituents	Per cent of individual constituents
Protein	62.8 ± 0.6%
Lipid	30.5 ± 0.7%
Free	26.9 ± 1.7%
Bound	3.6 ± 1.0%
Phospholipid[a]	23.7 ± 0.8%
Hexosamine	0.7 ± 0.2%
Nonamino carbohydrate	1.3 ± 0.1%
Ribonucleic acid	0.6 ± 0.1%
Deoxyribonucleic acid	0.1 ± 0.0%

[a] All of the phospholipid was found in the free lipid fraction.

It can be seen that the constituents determined accounted for 95% of the dry weight of the cytoplasmic membrane. There was only a negligible amount of hexosamine present, indicating that the cytoplasmic membranes analyzed were essentially free from wall material. Most of the lipid present was phospholipid. In a previous study (12), the phospholipids were extracted from whole cells of this organism and were separated, identified, and determined quantitatively. These studies showed that 70% of the phospholipid present in the cells was phosphatidylethanolamine, 16% was diphosphatidylglycerol, and the remainder consisted of unidentified compounds. Evidence indicates that the phospholipids in the membrane phospholipid fraction are the same compounds that have been detected in the whole cells.

DISCUSSION

Electron microscopy has shown that the protoplasts from which the membranes in this study were prepared were apparently free from wall material, since the spherical protoplasts appeared to be surrounded only by a cytoplasmic membrane. The absence of wall material was confirmed by the chemical analysis of the cytoplasmic membranes which showed that only traces of hexosamine were present. Unpublished observations made in this laboratory indicate that each of the layers of the cell wall of this marine pseudomonad contain appreciable quantities of glucosamine. Electron microscopy has also shown that the cytoplasmic membrane preparations after washing contained no whole cell structures or contaminating cytoplasmic elements. This was also confirmed by chemical analyses which showed that only traces of deoxyribonucleic acid and ribonucleic acid were present.

Evidence that the membrane fraction isolated was homogeneous was obtained by sucrose den-

sity gradient centrifugation. The isolated membranes gave rise to a single compact band in the gradient. Fractionation of the gradient showed that all of the lipid and the 280-nm absorbing material (presumably protein) in the sample were associated in the band. The fact that lyophilized membranes gave rise to a band in the sucrose density gradient in the same position as that obtained with fresh membranes indicates that lyophilization, if it gives rise to changes in the structure of the membrane, does not produce changes detectable by centrifugation in the sucrose density gradient.

Other investigators (3, 5, 14, 26), working with gram-negative bacteria, have developed systems in which cytoplasmic membranes are not completely separated from cell wall material. However, Miura and Mizushima (19, 20) obtained three bands in the sucrose density gradient separation of *Escherichia coli* K-12 cell envelopes: an upper, small band which appeared to be pure cytoplasmic membrane material; an intermediate, large band of cytoplasmic membranes and cell wall material; and a lower, large band of cell wall material. In this step and in the following sucrose density gradient purification step, the *E. coli* cytoplasmic membrane material migrated as a compact band. In this respect, the *E. coli* cytoplasmic membrane fraction resembled the cytoplasmic membrane fraction of the marine pseudomonad reported here. In contrast, cytoplasmic membranes isolated from *Bacillus licheniformis* gave rise to three fractions in a sucrose density gradient (25).

The cytoplasmic membranes of gram-positive bacteria contain 9 to 36% lipid, 41 to 75% protein, and 0.2 to 20% carbohydrate and represent 10 to 20% of the dry weight of the cells (4, 27). The chemical composition of the mycoplasma group also falls within these limits (23, 24). Therefore, this first report of the chemical composition of the cytoplasmic membrane of a gram-negative bacterium shows that, in the case of this organism at least, the composition of its cytoplasmic membrane (30.5% lipid, 62.8% protein, and 2% carbohydrate) is very characteristic of that of the cytoplasmic membranes of other bacteria examined.

ACKNOWLEDGMENTS

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