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Isolation and Characterization of the Outer Membrane of *Neisseria gonorrhoeae*

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The cell envelope of *Neisseria gonorrhoeae* strain 2686, colonial type 4, was isolated from spheroplasts formed by the action of ethylenediaminetetraacetic acid and lysozyme. Isopycnic centrifugation of osmotically ruptured spheroplasts resolved the cell envelope into two main membrane fractions. Chemical and enzymatic analyses were used to characterize these isolated membranes. Succinic dehydrogenase, reduced nicotinamide adenine dinucleotide oxidase, and D-lactate dehydrogenase were localized in the membrane fraction of buoyant density, $\rho^\circ = 1.141$ g/cm^3. Lipopolysaccharide and over half of the cell envelope protein were associated with the membrane that banded in sucrose at $\rho^\circ = 1.219$ g/cm^3. These fractions were consequently designated cytoplasmic and outer or L-membrane, respectively. Sodium dodecyl sulfate-polyacrylamide electrophoresis of isolated membranes demonstrated the relative simplicity of the protein spectrum of the outer membrane. The majority of the protein in this membrane could be accounted for by proteins of molecular weights 34,500, 22,000, and 11,500. The protein of molecular weight 34,500 accounted for 66% of the total protein of the L-membrane. Isoelectric precipitation at pH 4.6 with 10% acetic acid selectively removed this protein from a 150 mM NaCl in 10 mM tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.4, extract of purified outer membrane. At pH 4.0, the other proteins of the L-membrane were precipitated. It was concluded that the membrane components of the cell envelope of *N. gonorrhoeae* were similar to those of other gram-negative bacteria. The cell envelope fractions described here, in particular the outer membrane, are sufficiently well defined to provide a valuable tool for future biochemical and immunological studies on *N. gonorrhoeae*.

The cell envelope of gram-negative bacteria is an immunochemically complex structure composed of three morphologically distinct layers (7, 20), namely, the cytoplasmic membrane, a rigid peptidoglycan layer, and a second membranous structure, the outer or L-membrane. The outer membrane contains substantial amounts of protein, phospholipid, and lipopolysaccharide (LPS), the class of antigens referred to as the O-antigen. However, very little is known about the location of protein antigens; therefore, the development of methods for the isolation of these membranes is one important step in the determination of the nature of the antigens present in these structures.

The outer surface of certain colonial types of *Neisseria gonorrhoeae* possesses pili which have proved to be antigenic in man and other animals (2, 3). These and other antigenic components of the cell envelope are of interest as agents of potential immunoprophylaxis. Preparation of relatively pure membrane fractions would facilitate this approach.

For this purpose, a technique for the separation of outer membrane from cytoplasmic membrane was necessary. Miura and Mizushima (17) first described the separation of the cell envelope of *Escherichia coli* into two fractions, enriched respectively for cytoplasmic and outer membrane by a technique involving isopycnic sucrose density centrifugation of membranes obtained by spheroplast lysis. More recently, Schnaitman (27) has reported similar results following disruption of *E. coli* in a pressure cell. Similar procedures with modifications were found to be applicable to *N. gonorrhoeae*, permitting rapid reproducible separations of cell envelope components.

MATERIALS AND METHODS

Bacteria and media. Strain 2686, colonial type 4, an isolate of *N. gonorrhoeae* obtained from D. S.

Kellogg, Jr., Center for Disease Control, Atlanta, Ga., was subcultured for 16 h at 35 C in a candle extinction jar on GC agar base supplemented with Isovitalex (Baltimore Biological Laboratories, Cockeysville, Md.). Approximately 7×10^8 bacteria were suspended in 50 ml of modified Frantz' meningococcal medium (10) prepared at 0.16% L-glutamic acid, 0.60% NaCl, 0.25% Na_2HPO_4, 0.125% NH_4Cl, 0.009% KCl, 0.002% L-cysteine hydrochloride, 2% anhydrous dextrose, 0.3% $MgSO_4 \cdot 7H_2O$, and 0.5% dialyzed yeast extract. When cultures were to be intrinsically labeled, approximately 4 μCi of each of the following isotopes was added to 50 ml of medium: uniformly labeled [^{14}C]leucine (460 mCi/mmol) and [^{14}C]tyrosine (304 mCi/mmol), L-[4,5-^{3}H]leucine (5 Ci/mmol), and L-[3,5-^{3}H]tyrosine (48.2 Ci/mmol), all obtained from New England Nuclear, Boston, Mass. Portions of radioactive samples were added to 5 ml of Hydromix liquid scintillation fluid (Yorktown Research, New Hyde Park, N.Y.), and radioactivity was determined in a refrigerated Packard Tri-Carb liquid scintillation counter. Counting efficiencies were 81% for [^{14}C] and 45% for [^{3}H].

Chemical procedures. Protein was determined by the method of Lowry et al. (15), using bovine plasma albumin (Metrix Armour Pharmaceutical Co., Chicago, Ill.) as standard. Total hexose, pentose, and methylpentose was determined by the α-napthol reaction as described by Dische (8); uronic acids and amino sugars gave no color. Hexosamine content was established by the modified Elson-Morgan procedure as described by Williams and Chase (34).

Phospholipid analysis was carried out as described by Osborn et al. (23).

LPS content of membranes was determined by measuring the amount of 2-keto-3-deoxyoctonate released from the membranes by hydrolysis with 0.02 N H_2SO_4 at 100 C for 20 min. Portions were removed and analyzed for 2-keto-3-deoxyoctonate by the thiobarbituric acid procedure of Weissbach and Hurwitz (33) as modified by Osborn (22); the absorbance was read at 548 nm. The amount of LPS in the original digest was calculated from a standard curve obtained with purified LPS extracted by the procedure of Osborn et al. (24).

Nucleic acid was determined spectrophotometrically at 260 nm.

Proteins were iodinated with either [^{125}I] or [^{131}I] by the chloramine T procedure (12). Before labeling, sodium azide was removed by overnight dialysis.

Enzyme assays. All enzyme reactions were followed in a Gilford spectrophotometer. Specific activity of enzymes was expressed as micromole of substrate coverted per minute^{-1} per milligram of protein.

Succinic dehydrogenase (EC 1.3.99.1) was measured by coupling the enzyme via phenazine methosulfate to the reduction of cytochrome *c* (1) and following the reaction spectrophotometrically at 550 nm. The reaction mixture was that essentially described by MacGregor and Schnaitman (16). A sample volume of 100 μliters (50 to 75 μg of protein) was added to 2.0 ml of the following reaction mixture: 100 ml of 100 mM sodium phosphate buffer (pH 7.5), 5 ml of 1 M sodium succinate (pH 7.5), 1.5 ml of 100 mM KCN, 20 ml of 10 mM sodium ethylenediaminetetraacetate (EDTA) (pH 7.5), and 85 ml of distilled water. After incubation at 22 C for 20 min, 1.5 ml was transferred to a cuvette, and 20 μliters of cytochrome *c* (10% wt/vol) was added. To start the assay, 5 μliters of phenazine methosulfate (1% wt/vol) was added. D-Lactate dehydrogenase (EC 1.1.1.28) activity was assayed as for succinic dehydrogenase, with 1 ml of 1 M sodium D-lactate instead of succinate in the reaction mixture.

Reduced nicotinamide adenine dinucleotide (NADH) oxidase (EC 1.6.3.1.) activity was measured according to Osborn et al. (23). A sample volume of 50 μliters of membrane suspension containing 20 to 70 μg of protein was used. The rate of decrease in absorbance at 340 nm was measured at 22 C.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed as described by Noltmann et al. (21).

Isolation of cell envelope components. After 4.5 h of growth, cultures yielded approximately 3×10^8 cells/ml. Cells were harvested by centrifugation at $10,000 \times g$ for 15 min at 4 C. The cell pellets were washed once with tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.8, centrifuged, and then suspended in cold 0.6 M sucrose and 10 mM Tris-hydrochloride buffer, pH 7.8 (20 ml of buffer per 10^{10} cells). Hen egg white lysozyme (EC 3.2.1.17) (Worthington Biochemical Corp., Freehold, N.J.) dissolved in 100 mM Tris-hydrochloride buffer, pH 7.8, was added to a final concentration of 160 U/ml (as defined by Worthington Biochemical Corp.). Within 1 min, 100 mM EDTA in 10 mM Tris-hydrochloride buffer, pH 7.4, was added to a final concentration of 2 mM. The suspension was gently agitated at 30 C for 60 min; spheroplast formation was monitored by reduction in absorbance at 600 nm of 0.5 ml of suspension diluted 1:10 with distilled water. Spheroplasts were harvested by centrifugation at $25,000 \times g$ for 15 min at 4 C and subsequently suspended in 5 ml of 25 mM $MgCl_2$ in 10 mM Tris-hydrochloride buffer, pH 7.0, per 20 ml of original spheroplast solution. Spheroplasts were lysed by diluting the suspension 20-fold in cold 5 mM $MgCl_2$, pH 7.0, and gently stirring for 10 min. The lysed preparation was treated with bovine pancreatic deoxyribonuclease (EC 3.1.4.5.) and bovine pancreatic ribonuclease (EC 2.7.7.16), both obtained from Worthington Biochemical Corp. To 100 ml of lysed suspension, 1,400 U of deoxyribonuclease and 1,500 U of ribonuclease were added, and the suspension was incubated for 30 min at 37 C. Membranes were harvested by centrifugation at $75,000 \times g$ for 25 min, suspended in 5 mM $MgCl_2$ in 10 mM Tris-hydrochloride buffer, pH 7.0, and pelleted as above. The membranes were suspended in 25 mM EDTA in 10 mM Tris-hydrochloride buffer, pH 7.0, and dialyzed for 18 h against the same buffer. Sodium azide was added to a concentration of 0.2% (wt/vol) as a preservative, after it was ascertained that azide did not interfere with the enzymatic analyses.

Suspensions of membranes (1.0 ml containing 15 to 20 mg of protein) were layered over 57% (wt/wt) sucrose dissolved in 5 mM EDTA in 10 mM Tris-

hydrochloride buffer, pH 7.0, and centrifuged in a SW-50.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 7 h at 200,000 × *g*. The interface was retained and diluted 10-fold with 5 mM EDTA in 10 mM Tris-hydrochloride buffer, pH 7.0; membranes were pelleted by centrifugation at 200,000 × *g* for 2 h. Pellets were resuspended in minimal volume of 0.8 M sucrose and 5 mM EDTA in 10 mM Tris-hydrochloride buffer, pH 7.0, to a protein concentration of 10 mg/ml; 500 μliters of sample were applied to a discontinuous gradient prepared by layering sequentially 0.5-ml samples of sucrose solutions ranging in concentration from 60 to 20% (wt/wt). Samples were spun at 250,000 × *g* for 18 h to isopycnic conditions. Tube contents were fractionated by puncturing the bottom of the centrifuge tube and collecting drops; to retrieve banded material, fractions were diluted 1:10 with 10 mM Tris-hydrochloride buffer, pH 7.0, and centrifuged at 250,000 × *g* for 90 min.

Extraction of outer membranes. Isolated outer membranes were extracted with 150 mM NaCl and 0.02% (wt/vol) NaN_3 in 10 mM Tris-hydrochloride buffer, pH 7.4 (TSE buffer). Suspensions of membranes containing 15 mg of protein per ml were incubated with an equal volume of TSE buffer for 2 h at 37 C. After centrifugation at 50,000 × *g* for 30 min, the supernatant fluid was decanted and concentrated on a Diaflo PM-10 membrane (exclusion limit, >10,000; Amicon, Lexington, Mass.). Two milliliters of concentrate was applied to a 1.5 by 30 cm Sepharose 6B (exclusion limit, $>4 \times 10^6$ daltons for globular proteins; Pharmacia, Piscataway, N.J.) column equilibrated in TSE buffer. Fractions eluting at the void volume were pooled and concentrated.

Polyacrylamide electrophoresis. Membranes and extracts of membranes were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide disc gel electrophoresis by the procedure described by Weber and Osborn (32). A 1% SDS, 10% acrylamide, 0.27% methylene bisacrylamide gel was used throughout. Gels were prepared in glass tubes 6 mm by 150 mm. Samples to be electrophoresed were treated at 100 C for 2 min in a solution of 10 mM sodium phosphate, pH 7.0, 2% (wt/vol) SDS (Biorad Laboratories, Richmond, Calif.), and 2% (wt/vol) 2-mercaptoethanol; after cooling, one-half volume of 0.05% (wt/vol) bromophenol blue in 80% (wt/vol) sucrose was added. After electrophoresis at 5 mA per tube, the gels were sliced in 1-mm sections by a Gilson Autogel slicer (Gilson Medical Electronics, Inc., Middleton, Wisc.) and monitored for radioactivity.

RESULTS

Spheroplasts of *N. gonorrhoeae* were formed by the action of lysozyme on the peptidoglycan layer while cells were in the presence of EDTA. It was found that the ratio of lysozyme to cells was critical for optimal spheroplast formation. "Optimal spheroplast formation" was defined as the conditions existing when 90 to 95% of osmotically balanced spheroplasts would lyse within 15 s upon 1:10 dilution with distilled water. Quantities of lysozyme greater than 200 U/ml resulted in macroscopic agglutination of the cells and spheroplasts of poor quality. When amounts of lysozyme less than 100 U/ml were used, only 10 to 15% of the cells were converted to spheroplasts. At the cell density employed, it was observed that 160 U of lysozyme/ml were sufficient to convert 90 to 95% of the cells to spheroplasts. Addition of EDTA at concentrations in excess of 5 mM resulted in extensive lysis of osmotically balanced cells. Absence of EDTA in the spheroplasting medium decreased the amount of cells converted to spheroplasts by approximately 65%. The optimal EDTA concentration was between 1 to 3 mM; addition of EDTA solution of pH >8.5 resulted in immediate lysis of the culture. In summary, the optimal conditions for spheroplast formation of *N. gonorrhoeae* entailed incubation of cell suspensions 5×10^8 organisms/ml in 0.6 M sucrose and 2 mM EDTA in 10 mM Tris-hydrochloride buffer, pH 7.8, with 160 U of lysozyme/ml.

The membranes derived from lysed spheroplasts used in these studies were free from cytoplasmic contamination as judged by the absence of the soluble cytoplasmic enzyme, glucose-6-phosphate dehydrogenase. Approximately 90% of the glucose-6-phosphate dehydrogenase of intact cells was recovered from the supernatant of lysed spheroplasts. After centrifugation of the initial envelope preparation on a cushion of 57% (wt/wt) sucrose, the buoyant material contained no glucose-6-phosphate dehydrogenase activity within the 2% detection limit of the assay. The material buoyant at 57% (wt/wt) sucrose was unfractionated cell envelope; analyses of this preparation are presented in Table 1.

Isopycnic centrifugation of total membrane fractions resolved the mixture as three visible bands of turbidity: an upper band (A) of buoyant density, $\rho^\circ = 1.141$ g/cm^3, an intermediate band (B) of buoyant density, $\rho^\circ = 1.176$ g/cm^3, and a pronounced bottom band (C) which had a buoyant density of $\rho^\circ = 1.219$ g/cm^3 (Fig. 1). Band A accounted for 18 to 25% of the total protein of the cell envelope; band C contained as much as 58% of the envelope protein (Table 1). Band B ($\rho^\circ = 1.176$ g/cm^3) consisted of unseparated material usually containing less than 19% of the total protein. The amount of this band was determined by several factors, in particular, the ionic strength of the spheroplasting medium. When optimal lysozyme and EDTA concentrations were used, but NaCl was incorporated into the spheroplasting medium, wash buffers, and sucrose gradient

TABLE 1. *Chemical and enzymatic analyses[a] of fractionated membranes of Neisseria gonorrhoeae strain 2686, colonial type 4*

Analysis	Membrane fraction from sucrose gradient			
	Unfractionated[b] cell envelope	Band A ρ° = 1.141 g/cm^3	Band B ρ° = 1.176 g/cm^3	Band C ρ° = 1.219 g/cm^3
Protein (%)	100	18.7-25.6	10.4-18.8	48.6-58.2
Phospholipid (mg/mg of protein)	0.41-0.47	0.55-0.62	0.34-0.39	0.29-0.31
Lipopolysaccharide (mg/mg of protein)	0.96-1.16	0.04-0.06	n.d.[c]	1.19-1.21
Carbohydrate (mg/mg of protein)	0.71-0.78	0.12-0.18	0.42-0.49	0.87-0.92
Hexosamine (mg/mg of protein)	ND	0.01-0.02	<0.01	0.42-0.47
Specific activity[d] of:				
Succinic dehydrogenase	0.54-0.68	5.23-7.41	1.94-2.86	0.05-0.08
D-Lacate dehydrogenase	0.36-0.59	4.99-5.62	3.04-3.89	0.02-0.12
NADH oxidase	0.61-0.74	4.29-4.93	1.08-1.26	0.08-0.15

[a] Figures in table represent range of values determined on a number of different membrane preparations.
[b] Cell envelope buoyant on 57% (wt/wt) sucrose.
[c] ND, Not done.
[d] Specific activity expressed as micromole of substrate converted per minute per milligram of protein.

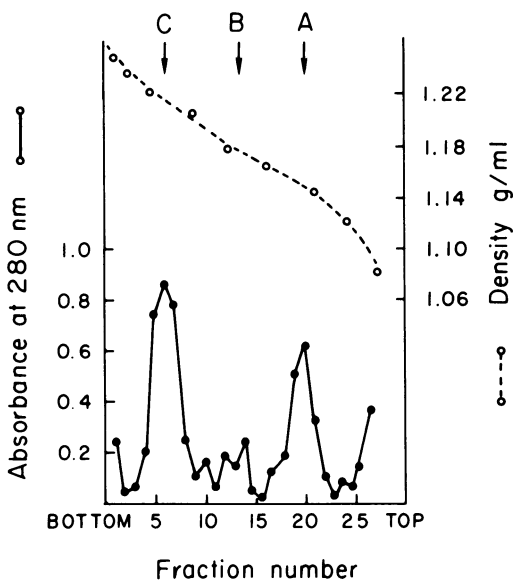

FIG. 1. *Isopycnic sucrose centrifugation of osmotically lysed lysozyme-induced spheroplasts of N. gonorrhoeae, colonial type 4, strain 2686. Cell envelope preparations were layered on a discontinuous sucrose gradient, 60 to 20% (wt/wt), and centrifuged for 18 h at 200,000 × g. Upper case letters, A, B, and C, denote membrane fractions described in Table 1.*

solutions, the increase in material banding at ρ° = 1.176 g/cm^3 was striking. In concentrations of NaCl in excess of 0.1 M, 35% of total radioactivity of intrinsically labeled cell envelope banded at this density. Similar results were obtained with KCl. Optimal resolution of the cell envelope into bands A and C depended on the absence of divalent cations as well. When 5 mM Mg^{2+} was incorporated into the sucrose gradient solution, most material banded in the region of density, 1.18 to 1.19 g/cm^3, and the recovery of bands A and C was minimal. The Mg^{2+} effect could be reversed by the presence of 5 mM EDTA in the gradient medium.

Identification of the membranes resolved by isopycnic centrifugation was achieved by chemical and enzymatic analyses (Table 1). The components of the electron transport system were specifically localized in the A band, ρ° = 1.141 g/cm^3, with some activities detected in the B band, ρ° = 1.176 g/cm^3. The specific activities of succinic dehydrogenase and D-lactate dehydrogenase were found to have increased 10-fold over the unfractionated cell envelope; similarly, the A band was enriched eightfold in NADH oxidase activity. The most dense band, ρ° = 1.219 g/cm^3, was practically free of enzyme activity. The specific activities of succinic dehydrogenase, D-lactate dehydrogenase, and NADH oxidase were 1.0%, 1.3%, and 2.5%, respectively, of the activities present in the A band. Repeated sucrose gradient centrifugation did not reduce this contamination of outer membrane with cytoplasmic membrane.

Carbohydrate, LPS and hexosamine were primarily located in the heavy band, ρ° = 1.219 g/cm^3.

Both membranes dissolve completely in 1% SDS (wt/vol). To distinguish the two membranes from each other a double label experiment was performed. Two cultures of 2686, type 4, were grown in Frantz' medium (10). One culture was grown in the presence of [^{3}H]leucine and [^{3}H]tyrosine enriched medium; the other was supplemented with [^{14}C]leucine

and [^{14}C]tyrosine. The two cultures were harvested, spheroplasts were produced and osmotically lysed, and the envelope fractions were obtained as described above. There was about 14% incorporation of total radioactivity into the cell envelope. Intrinsically labeled [^{14}C]-outer membrane was mixed with [^{3}H]-labeled cytoplasmic membrane and electrophoresed. The disintegrations per minute were normalized by conversion to percentage of total disintegrations per minute, enabling the amount of protein labeled with each isotope to be compared directly. Of 22 proteins resolved on the gel, 13 were derived principally from the cytoplasmic membrane, and five from the outer membrane indicated in Fig. 2 by circled notation above the peaks. One protein which was clearly a component of the outer membrane was number 12. It was found to have a molecular weight of 34,500 by comparison with the migration of proteins of known molecular weight. This protein was the major protein of the cell envelope (Table 2). It accounted for 66% of the labeled material of the outer membrane. In another experiment, this protein accounted for 48% of the total labeled cell envelope protein. It appeared as a single symmetrical peak in SDS-polyacrylamide gel electrophoresis even when the dye marker had migrated 140 mm. The other proteins of the outer membrane accounted for a much lower percentage of the total cell envelope protein. All the proteins resolved on gels had molecular weights of less than 110,000, and the presence or absence of 2-mercaptoethanol had no effect on the electrophoretic profile of the proteins. In most experiments, the amount of protein remaining on top of the gel was from 4 to 7% of the total protein (radioactivity) applied to the gel.

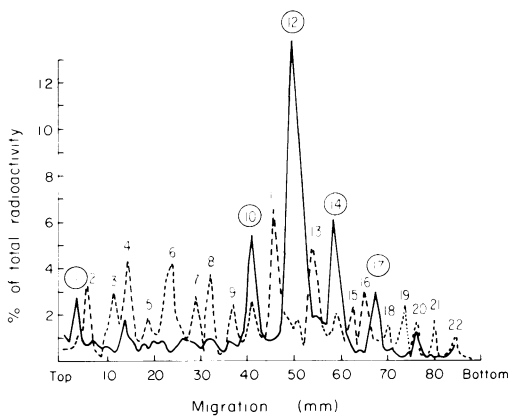

FIG. 2. *SDS-polyacrylamide electrophoresis of intrinsically labeled membrane proteins of N. gonorrhoeae, colonial type 4, strain 2686. Membrane fractions isolated after osmotically lysed spheroplasts were centrifuged to isopycnic conditions. Bacteria were grown in medium supplemented with either [^{3}H]-labeled tyrosine and leucine or [^{14}C]-labeled tyrosine and leucine. Tritium-labeled cytoplasmic membrane (-----) was mixed with [^{14}C]-labeled outer membrane (——) and resolved by disc gel electrophoresis. Circled notation denotes proteins of the outer membrane. Distribution of radioactivity is expressed as a percentage of total radioactivity.*

TABLE 2. *Relative amounts of outer membrane proteins*[a]

Peak no.	Percent of total outer membrane protein
10	10.1
12	66.2
14	11.7
17	7.6

[a] Calculated from data in Fig. 2. The [^{14}C] content in each peak was summed and divided by the total [^{14}C] content in the gel.

Several methods for extraction of proteins from the outer membrane utilizing detergents (sodium deoxycholate, Triton X-100) were employed. However, it was found that a modification of the simple method described by Frasch and Chapman (11) was satisfactory. Outer membranes were incubated in 150 mM NaCl in 10 mM Tris-hydrochloride buffer, pH 7.4, for 2 h at 37 C. When the extraction was carried out on intrinsically labeled outer membranes, approximately 46% of the radioactivity was released into the supernatant. All the radioactivity was eluted in the void volume of a Sepharose 6B column equilibrated with the extraction buffer. Addition of acetic acid to pH 4.0 precipitated 86% of the radioactivity in the extract. This precipitate could only be redissolved at a pH >8.0; subsequently, acid precipitates were redissolved in 150 mM NaCl in 10 mM Tris-hydrochloride buffer, pH 8.4. Addition of 2 vol of cold, 90% (vol/vol) acetone precipitated 95% of the radioactivity. After repeated acetone precipitations, the dry material was found to contain 88.7% protein, 3.1% carbohydrate, and 1.7% hexosamine, a composition distinctly different from that of the outer membrane.

A gel electrophoretic pattern of a [^{125}I] extrinsically labeled Tris-saline extract was identical to the electrophoretic profile of a Tris-saline extract of intrinsically labeled outer membrane, indicating that the radioiodination procedure did not introduce artifacts. Addition of 10% (vol/vol) aqueous acetic acid to the iodinated extract caused a pronounced precipitation at

pH 4.6; 63% of the total radioactivity was precipitated. When acid was added to bring the supernatant to pH 4.0, a second precipitate formed, accounting for 31% of the total radioactivity. The sum of both precipitations, namely 94%, approximated the total radioactivity recovered (96%) by a one-step precipitation at pH 4.0. When the acid precipitates were analyzed electrophoretically, the specificity of the reaction was notable. The precipitate obtained at pH 4.6 consisted primarily (72.7%) of the major outer membrane protein (Fig. 3). The supernatant fluid at pH 4.6 was enriched in proteins having molecular weights of 22,000 and 11,500 (Fig. 4). When acetic acid was added to bring the extract to pH 4.0, the precipitate so formed contained the two minor proteins, namely the 22,000- and 11,500-dalton species plus a small amount of 34,500-dalton material.

DISCUSSION

The procedure described above offers a reproducible and useful method for the fractionation of the cell envelope of *N. gonorrhoeae* into cytoplasmic and outer membrane. The results parallel the earlier observations of Miura and Mizushima (17), Schnaitman (27), and Osborn et al. (20), and, more recently, those of Deneke and Colwell (5) and Stinnett et al. (30) on the separation of the membrane of other gram-negative bacteria. The chemical and enzymatic composition of the isolated membranes were similar to those of other gram-negative bacteria (18, 23, 26, 27).

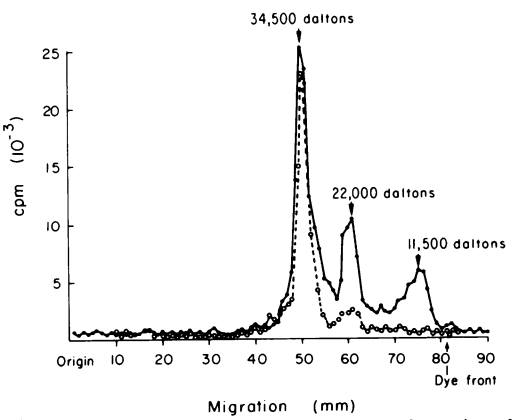

FIG. 3. *SDS-polyacrylamide electrophoresis of acetic acid precipitate at pH 4.6 of 10 mM Tris-saline extract of isolated outer membrane. Extracts were extrinsically labeled with either* [^{125}I] *or* [^{131}I]. *Unfractionated extract labeled with* [^{125}I] (——) *was mixed with* [^{131}I]*-labeled protein acid precipitated at pH 4.6* (-----). *Samples were mixed prior to electrophoretic resolution.*

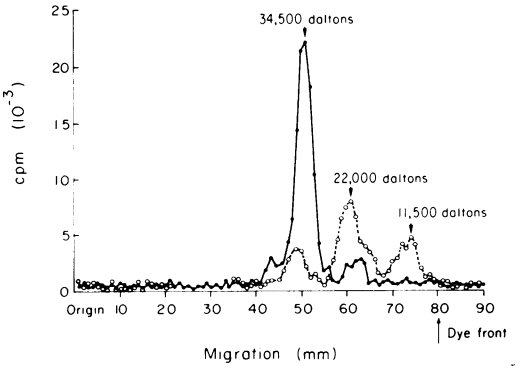

FIG. 4. *SDS-polyacrylamide electrophoresis of acetic acid precipitate of 10 mM Tris-saline extracts of isolated outer membranes. Extracts were extrinsically labeled with either* [^{125}I] *or* [^{131}I]. [^{131}I]*-labeled protein remaining in the supernatant after a pH 4.6 acid precipitation* (-----) *was mixed with* [^{125}I]*-labeled protein acid precipitated at pH 4.6* (——); *the mixture was resolved by disc gel electrophoresis.*

In the preparation of the spheroplasts, the concentration of EDTA was found to be a critical variable; EDTA in excess of 5 mM caused extensive lysis of osmotically balanced cells. This lethal action of EDTA has been attributed to the chelation of divalent metal ions which are required for the structural integrity of the cell envelope (14). Not all gram-negative bacteria are so readily lysed, but a similar phenomenon has been observed with *Pseudomonas aeruginosa* (9). For optimal resolution of gonococcal membranes by equilibrium sucrose density centrifugation, it was imperative to have EDTA present in the gradient medium. When divalent cations were present, most material remained unresolved. High concentrations of Na^+ and K^+ appeared to have the same effect. A similar observation on the influence of Mg^{2+} on the separation of meningococcal cell envelope membranes has been made by Hill et al. (13).

The buoyant densities of the isolated membranes were akin to values found by other investigators for either *E. coli* (17, 27) or *Salmonella typhimurium* (23). The distribution of components of the electron transport system (succinic dehydrogenase, NADH oxidase, and D-lactate dehydrogenase) indicated that the preparations of outer membrane were virtually free of cytoplasmic membrane contamination. SDS-polyacrylamide electrophoresis of the outer membrane indicated a relatively simple but unique protein spectrum. Most interesting was the presence of a single peak accounting for over 60% of outer membrane protein. This protein either represents a single molecular

species or a mixture of proteins so similar in subunit molecular size that the separation techniques used here cannot resolve them. However, others (19, 28, 29, 35), using somewhat different fractionation and electrophoretic techniques, have demonstrated that the major polypeptide of the outer membrane of *E. coli* consists of a complex association of several distinct proteins. Electrophoretic resolution of the outer membrane did not demonstrate the presence of pili which have a subunit molecular weight of 24,000 (3); this is to be expected as the colonial type 4 gonococcus does not carry pili (31). As it has been demonstrated that EDTA can release LPS (4, 14) and protein as an LPS-protein complex (6, 25) from the outer membrane of gram-negative bacteria, it should be noted that the membrane protein spectra described here represents those proteins not solubilized by EDTA.

To prepare materials suitable for immunological analyses, the purified outer membrane was extracted with Tris-saline. This extract contained three proteins of the outer membrane, namely the 34,500-, 22,000-, and 11,500-dalton species. These proteins still existed in an aggregated form; they eluted in the void volume of a Sepharose 6B column. Upon acidification to pH 4.6, a precipitate formed which was markedly enriched with the 34,500-dalton protein species. The mechanism of this purification is not clear. There may be two species of aggregates, one containing primarily the protein of 34,500 daltons, the other the proteins of 22,000 and 11,500 daltons. Alternatively, raising the hydrogen ion concentration may cause dissociation of aggregates containing all three protein species, and, as the pH is lowered further, isoelectric precipitation of the disaggregated proteins results.

On the basis of quantity, location, and its apparent electrophoretic homogeneity, the 34,500-dalton material most likely has immunological significance. The procedures described above were performed to isolate this protein for immunological investigations on the serology of *N. gonorrhoeae*.

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