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Preparation and Crossed Immunoelectrophoretic Analysis of Cytoplasmic and Outer Membrane Fractions from *Neisseria gonorrhoeae*

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Cell envelopes were obtained from lysates of *Neisseria gonorrhoeae*, colony type T1, prepared with lysozyme, ethylenediaminetetraacetate, and Brij 58. This preparation was separated into cytoplasmic (inner) and outer membrane fractions by equilibrium sucrose density gradient centrifugation. The former fraction was 10-fold enriched in L-lactate dehydrogenase activity with respect to the latter. On the basis of buoyant density in sucrose, polypeptide patterns in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and enzymatic activity, these preparations appear similar to cytoplasmic and outer membrane preparations from other gram-negative bacteria. The membrane preparations were analyzed by high-resolution crossed immunoelectrophoretic procedures. This technique permitted the identification of antigens originating from the structural components of the gonococcal cell. Among those found to be cytoplasmic membrane components was the fast-moving antigen which occurs widely in gram-negative bacteria.

The antigens of *Neisseria gonorrhoeae* are of interest for their potential usefulness in serodiagnosis, immunoprophylaxis, and in the investigation of the interaction of the gonococcus with the host and the role of individual antigenic components in the mechanism of pathogenesis. In order to resolve these antigens for analytical purposes, this laboratory has applied crossed immunoelectrophoresis (CIE) to gonococcal antigens (25, 29, 30) and explored the possibility of using these techniques to observe interaction between sera of patients and these bacterial antigens (23).

A complex pattern of immunoprecipitates is generated by analysis of extracts of gonococcal cell envelopes with antiserum raised in rabbits to this structure. Several functional components, including lactate dehydrogenase (LDH) and reduced nicotinamide adenine dinucleotide dehydrogenase, have been identified in this pattern (29). In addition, it would be informative to evaluate the site of origin of these components within the cell. For this purpose, crossed immunoelectrophoretic analysis of preparations of the inner and outer membrane components of the complex cell envelope would permit recognition of those immunoprecipitates formed by antigens which originate from these structures.

Johnston and Gotschlich (11) have obtained cytoplasmic and outer membrane preparations from *Neisseria gonorrhoeae*, colony type T4. The procedures used by these investigators were unsuccessful when applied to cells of T1 colonies in this laboratory (30) and by others (36). This study was undertaken to obtain fractions enriched in cytoplasmic and outer membrane from T1 gonococcal cells and to use these preparations to establish the origin of individual antigens in the gonococcal cell envelope. Moreover, such preparations would also be useful in determining specific binding sites for antibiotics (e.g., β-lactam binding proteins of the inner membranes).

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MATERIALS AND METHODS

Growth of organism. *N. gonorrhoeae* GC9 was grown on New York City Public Health Laboratory medium prepared as described by others (6); antibiotics were not included. Agar plates were inoculated with one to four colonies picked from a plate streaked in order to select T1 colonies. Colonies of this type (T1) conformed to the description of Kellogg et al. (12) and probably correspond to the P+, light/transparent type of Swanson (33, 34). Colony morphology was determined by examination of the plates with transmitted light under a dissecting microscope. These plates were incubated at 37°C in an atmosphere of 95% air-5% CO_2 for 18 to 20 h. The growth from two plates was suspended in 1.5 ml of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and served as the inoculum for 12 fresh agar plates. These plates were incubated for 20 to 22 h.

Preparation of cell extracts. Cells were re-

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Preparation of cell extracts. Cells were recovered from the colonized plates (≥95% T1 colonies) with a rubber spatula and suspended in 0.01 M tris-(hydroxymethyl)aminomethane-hydrochloride (Tris), pH 8.1, and washed once in this buffer. Electron microscopic examination showed these cells to be extensively piliated. A lysis procedure modified from those of Godson and Sinsheimer (8) and Collins and Niederman (4) was employed. In this procedure, the cells (15 g, wet weight) were suspended to 80 ml in 25% (wt/wt) sucrose (special enzyme grade, Schwarz/Mann, Orangeburg, N.Y.) prepared in 0.01 M Tris, pH 8.1. At intervals of 20 min the following were added: 10 ml of lysozyme (6.4 mg/ml in 0.25 M Tris [pH 8.1], EC 3.2.1.17, Worthington Biochemical Corp., Freehold, N.J.); 10 ml of ethylenediaminetetraacetate, disodium salt (EDTA, 20 mg/ml in water); 2 ml of Brij 58 (5% wt/vol in 0.01 M Tris, pH 7.2); 0.2 ml of 4 M $MgCl_2$. A few crystals of deoxyribonuclease I (EC 3.1.4.5, Sigma Chemical Co., St. Louis, Mo.) were added. The lysate was freed from the remaining whole cells and cellular debris by centrifugation at 750 × *g* for 10 min. The lysis procedure was performed at room temperature; all subsequent procedures were performed at 0 to 4°C.

Isolation of membrane preparations. The cell extract was centrifuged for 2 h at 50,000 rpm (165,000 × *g*) in a Beckman type 50 Ti rotor (Beckman Instruments, Palo Alto, Calif.) to sediment subcellular particles. Membrane fractions were obtained from the resulting pellet by procedures essentially similar to those of Collins and Niederman (4). Membrane particles were recovered from pooled fractions by centrifugation at 165,000 × *g* for 2 h.

Enzyme assays. L-LDH (lactate: phenazine methosulfate [PMS]oxidoreductase) activity was measured by the reduction of 2,6-dichlorophenol indophenol (DCIP) coupled to PMS. Final concentrations of reagents in the assay mixture were L-lactate, 0.05 M; potassium phosphate buffer (pH 7.6), 0.05 M; KCN, 0.015 M; PMS, 0.002 M; DCIP, 0.0001 M. The decrease in absorbance at 600 nm by DCIP was monitored on a Cary model 15 spectrophotometer.

Electrophoretic procedures. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on slab gels essentially as described by Laemmli and Farve (13). Membrane samples were solubilized by treatment at 100°C for 5 min. Dithiothreitol (0.10 M) and urea (3 M) were incorporated in the solubilization buffer.

The procedures used for CIE have been described in detail (5, 29, 31). Membranes (10 mg of protein per ml) were solubilized by incubation at room temperature in 1% Triton X-100 and 5 mM EDTA or 0.10 M $Ba(SCN)_2$ (final concentrations). After centrifugation at 40,000 × *g* for 30 min, the supernatant fraction was divided into aliquots and stored at −70°C until required for CIE analysis. New Zealand white rabbits were immunized with unfractionated cell envelopes, and serum obtained was purified and stored as described previously (5).

Electron microscopy. Membrane preparations were negatively stained with 2% ammonium molybdate and examined in a Siemens Elmiskop I electron microscope.

Analytical procedures. Protein was determined by the method of Lowry et al. (14) with bovine serum albumin as standard. The orcinol assay for ribonucleic acid (RNA) was performed by the method of Almog and Shirey (1) with yeast RNA (Sigma Chemical Co.) as a standard.

Chemicals. Agarose (type HGT) was obtained from Marine Colloids, Inc. (Rockland, Maine). Triton X-100 was obtained from Research Products International (Elk Grove, Village, Ill.). Brij 58 was a gift of ICI Americas, Inc. (Wilmington, Del.). The *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer and disodium succinate (A grade) were obtained from Calbiochem-Behring Corp. (La Jolla, Calif.). Orcinol, L-lactate (lithium salt), DCIP, and PMS were obtained from the Sigma Chemical Co. Barium thiocyanate was from ICN Pharmaceuticals, Inc. (Plainview, N.Y.).

RESULTS

Preparation of cytoplasmic and outer membrane fractions. Cell extracts were prepared from *N. gonorrhoeae* by Brij 58-mediated lysis of EDTA-lysozyme spheroplasts. Membranes were recovered from such extracts at 165,000 × *g* and resolved into cytoplasmic- and outer membrane-enriched fractions by sucrose density gradient centrifugation (Fig. 1). This procedure resolved a dense (1.22 g/cm^3), copious white band from a less dense (1.11 g/cm^3), translucent amber band which showed a greater activity of LDH. This banding pattern is similar to that observed for outer and cytoplasmic (or inner) membranes, respectively, from other gram-negative bacteria (4, 11, 20, 26).

The supernatant fraction obtained from the 165,000 × *g* centrifugation was analyzed by SDS-PAGE. The polypeptide profile of this fraction was compared with similar fractions obtained from a lysate prepared with one-half the level of detergent and a detergent-free lysate. No difference in the banding patterns was observed (Fig. 2), indicating that membrane components were not solubilized by detergent during preparation of the cell extract.

The dense band in the sucrose gradient (Fig. 1) showed a much greater relative absorbance at 235 nm; absorbance at this wavelength is an index of light-scattering (4). The high ratio of absorbance at 260 to 280 nm in the lighter band (Fig. 1, Table 1) and the fact that the dehydrogenase activity did not exactly coincide with the absorbance peak suggested that ribosomal material might be cosedimenting with the cytoplasmic membrane. This banding pattern had been observed previously when such procedures were applied to extracts of *Rhodospirillum rubrum* (4). To test this possibility, we used the orcinol assay procedure to determine the RNA content. The results of this analysis (Table 1) indicate that the cytoplasmic membrane preparation has

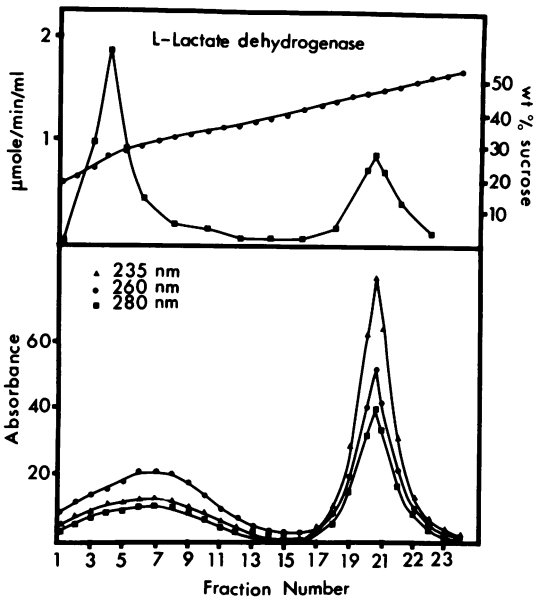

FIG. 1. *Separation of cytoplasmic and outer membranes of N. gonorrhoeae by sucrose density gradient centrifugation. Materials pelleted at 165,000 × g were suspended in 10 mM HEPES buffer and layered on a 25 to 55% (wt/wt) sucrose gradient prepared in the same buffer and centrifuged for 14 h at 27,000 rpm (95,000 × g) in a Beckman SW27 rotor. The absorbance at 235, 260, and 280 nm was determined on diluted fractions on a Beckman DU spectrophotometer. Sucrose concentrations were determined on a Zeiss refractometer. LDH activity was determined as described in the text. Sedimentation is to the right. Cytoplasmic membrane-enriched fractions (3–6) and outer membrane-enriched fractions (20–22) were recovered for analysis.*

more than a threefold greater content of orcinol-reactive material than the outer membrane fraction. The presence of orcinol-reactive material in the latter preparation is probably due to the carbohydrate moieties (22) of lipopolysaccharide.

Rate-zone centrifugation procedures, similar to those used previously (4) to resolve cytoplasmic membrane from ribosomal material with preparations from *R. rubrum*, were not successful when applied to the gonococcal preparations. Centrifugation of the cytoplasmic membrane preparation on gradients of 10 to 40% sucrose (wt/wt) for 1.25 to 4.25 h at 234,000 × *g* or 16.5 h at 189,000 × *g* did not resolve these gonococcal fractions into two bands of material absorbing at 280 nm.

The material banding near the top of these isopycnic sucrose gradients (i.e., light band) was compared with the material recovered from the dense band in order to assess the separation of cytoplasmic and outer membranes by these procedures. The cytoplasmic membrane was found to have a 10-fold-greater content of LDH on the basis of protein (Table 2). This must be considered a minimum, as ribosomal material would contribute to the measurement of protein made on this preparation. Therefore, the light band was highly enriched in this marker of cytoplasmic membrane.

The cytoplasmic and outer membranes of

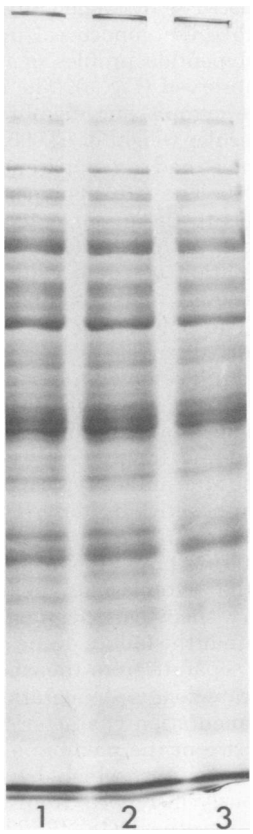

FIG. 2. *SDS-PAGE comparisons of supernatant fractions from 165,000 × g centrifugation from extracts prepared at the following detergent-to-protein ratios: (1) 0, (2) 0.11; (3) 0.22.*

TABLE 1. *RNA content of membrane fractions*

Membrane fraction	Absorbance ratio,[a] 260/280	Specific content[a] of RNA (mg of RNA[b]/mg of protein)
Cytoplasmic	1.83–1.84	0.37–0.44
Outer	1.20	0.13–0.14

[a] Values are the range from determinations on two preparations.

[b] Expressed as yeast RNA equivalents.

TABLE 2. *L-LDH activity of preparations from N. gonorrhoeae*

Prepn	L-LDH sp act (U[a]/mg of protein × 10^{-3})
Cytoplasmic	767
Outer membrane	76
Unresolved cell envelope	140

[a] Micromoles of DCIP reduced per minute.

gram-negative bacteria produce characteristic patterns in SDS-PAGE. When this technique was applied to these gonococcal preparations, distinctive polypeptide profiles of these membranes were observed (Fig. 3). The major polypeptide of the cytoplasmic membrane had an apparent molecular weight of 63,000; that of the outer membrane had an apparent molecular weight of 36,000. The latter polypeptide probably corresponds to the major outer membrane protein observed by others (10, 11, 35). Little cross-contamination of these cytoplasmic and outer membrane fractions could be detected by SDS-PAGE. The polypeptide banding pattern of the unresolved envelope fraction exhibits bands seen in both resolved fractions.

Electron microscopic examination (Fig. 4) of negatively stained preparations revealed that the outer membrane fraction consisted of membranes with the appearance typical of this structure from gram-negative bacteria. The large size of these membrane fragments is consistent with the light-scattering properties of this material. Conversely, the cytoplasmic membrane was present in very small particles and fragments. This suggests that the failure of the cytoplasmic membrane to separate from the ribosomal material during rate-zone sedimentation is attributable to sedimentation characteristics reflecting the small size of the membrane fragments. The pit-like structures, which have been described previously in reports from this laboratory (24) and by others (18, 19, 32), were observed in the outer membrane preparations. Pili were also found in outer membrane preparations. Because they did not appear to be attached to membrane, it is likely that their presence in these fractions is the result of cosedimentation.

CIE studies. To analyze membrane antigens by CIE, it is necessary to disrupt the membrane and solubilize its constituent components. This was accomplished by treatment with either the nonionic detergent Triton X-100 or the chaotropic agent $Ba(SCN)_2$. When Triton disruption was used, EDTA was included in the solubilization mixture because the outer membrane of gram-negative bacteria has been shown to be refractory to detergent dissociation (7, 9, 17, 27) in the absence of this chelator (28). Equal volumes of extracts prepared at equal protein concentrations (10 mg/ml) were compared in CIE. If the solubility of a particular molecular component is independent of differences in total protein recovery, then the distribution of individual antigens may be compared on the basis of volume in these detergent extracts. Therefore, to evaluate the antigenic composition of these preparations, we equalized protein levels before membrane solubilization. The protein content of the extracts of inner and outer and envelope preparations were, respectively, 8.1, 2.5, and 4.1 mg/ml. These differences in protein concentration are due to the differences in the recovery of

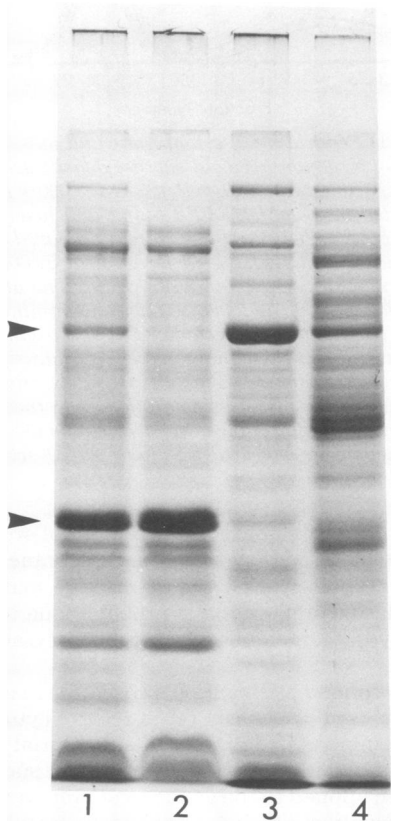

FIG. 3. *SDS-PAGE of fractions obtained from lysates prepared with Brij 58 at a detergent-to-protein ratio of 0.22. (1) Unresolved envelope fraction, 165,000 × g pellet; (2) outer membrane recovered from sucrose gradient; (3) cytoplasmic membrane recovered from sucrose gradient; (4) 165,000 × g supernatant fraction. Molecular weights of major bands of cytoplasmic and outer membranes (arrows) are estimated to be 63,000 and 36,000, respectively, on the basis of migration relative to standards.*

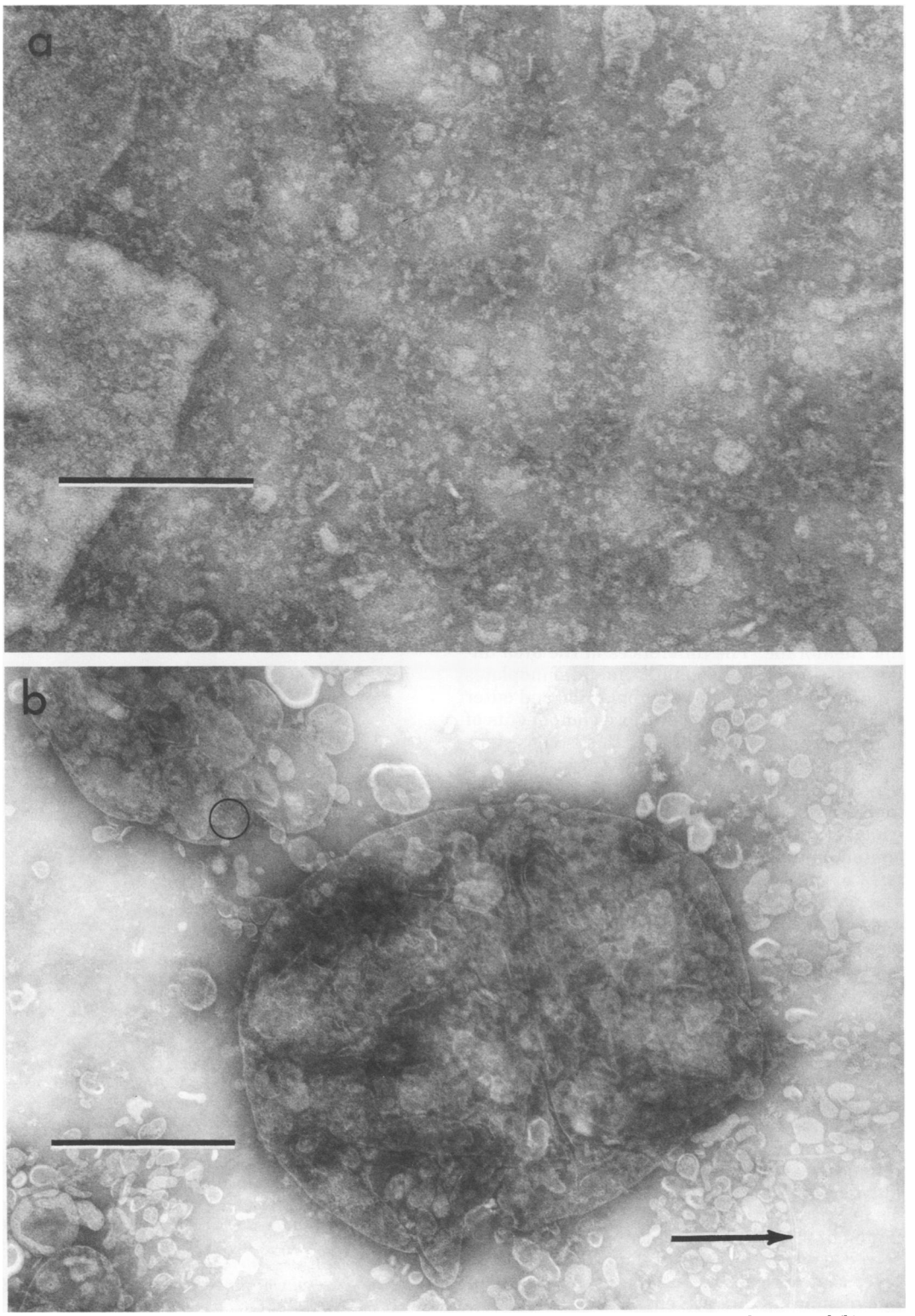

FIG. 4. *Electron micrographs of negatively stained preparations of (a) cytoplasmic membrane and (b) outer membrane. Pili are indicated by the arrow. Typical pits are encircled. Bar, (a) 0.25 μm and (b) 0.50 μm.*

protein in the 40,000 × g supernatant fraction obtained upon centrifugation of the Triton-treated membrane; this reflects both the differential solubility of the outer membrane (27) and the different sedimentation properties of the cytoplasmic and outer membranes.

Analysis of the CIE patterns of Triton extracts of these gonococcal membrane preparations (Fig. 5) indicates the cellular origin of some of the resolved antigens. Quantitative evaluation of these immunoplates is based on the area subtended by the immunoprecipitates. This area (A) is related to the ratio of an antigen (Ag) analyzed to the amount of specific antibody (Ab) present. This relationship is defined by $A = k(\mathrm{Ag}/\mathrm{Ab})$, where k is the area loading constant for a given antigen (21). The value k is in part determined by the electrophoretic mobility of an antigen (37) and therefore is generally greater for more acidic antigens.

On the basis of immunoprecipitate area, some antigens were found to be enriched in the extract of the cytoplasmic membrane (Fig. 5a) and depleted in or absent from the extract of the outer membrane (Fig. 5b). Conversely, some antigens appear to be present in greater quantity in the outer membrane extract, although these antigens are also present in the extract of cytoplasmic membrane. The differences in the area under the immunoprecipitates in immunoplates prepared with extracts of cytoplasmic and outer membranes are greater for those components of high electrophoretic mobility; this is consistent with a greater value of k for these antigens, which appear to be principally cytoplasmic membrane components. This may provide an explanation for the greater apparent degree of contamination of the cytoplasmic membrane extract by antigens of outer membrane origin than vice versa. The SDS-PAGE profiles (Fig. 3) show little evidence of such contamination. It is also possible that the different degrees of cross-contamination observed in CIE and SDS-PAGE may reflect differences in quantitative recovery of some membrane components in extracts prepared with Triton and SDS (S. Alpert and M. R. J. Salton, unpublished data).

Nonetheless these data (Fig. 5) permit recognition of the cellular origin of some antigens (inner versus outer membranes). The fast-moving antigen (FMA, Fig. 5), which appears to be a major cytoplasmic membrane component, is identical with the FMA detected in earlier studies in this laboratory (23, 29, 30). The profile of immunoprecipitates obtained with the unresolved gonococcal cell envelope (Fig. 5c) indicates that antigens of both outer and cytoplasmic membranes are present.

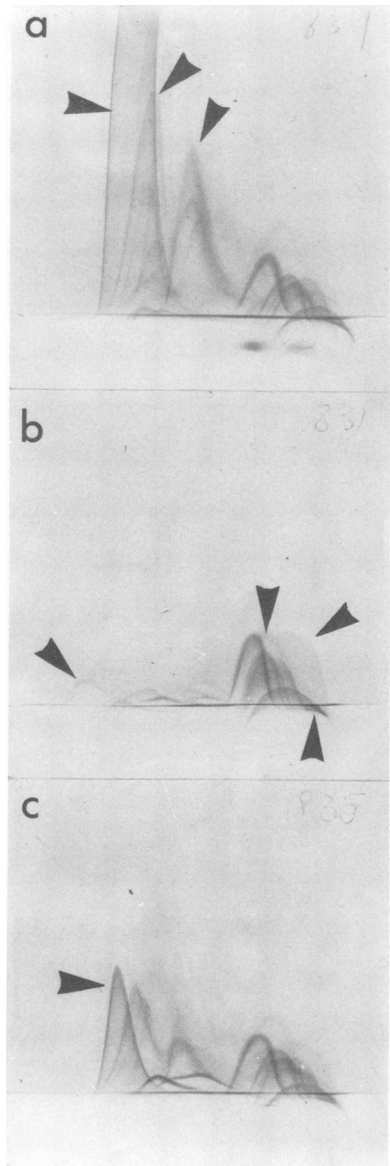

FIG. 5. *CIE of gonococcal membrane preparations. Samples (10 mg of protein per ml) were extracted with Triton and EDTA. Aliquots (5 μl) were applied to origin at lower right, and electrophoresis was conducted for 75 min at 180 V in the first dimension (anode at left) and 14 h at 55 V in the second dimension (anode at top). Top reference gel contains antienvelope immunoglobulins. (a) Cytoplasmic membrane (41 μg of protein); (b) outer membrane (12.5 μg of protein); (c) unresolved cell envelope (20 μg of protein). FMA is indicated by the arrow in (c). Some immunoprecipitates enriched in cytoplasmic membrane and outer membrane preparations are marked by arrows in (a) and (b), respectively.*

A recent study from this laboratory (23) revealed the presence of an antigen which was enriched in $Ba(SCN)_2$ extracts of gonococcal cell envelopes. The sera from patients with gonorrhea were found to react with this antigen. Because of its potential significance, it was of interest to evaluate the intracellular origin of this reactive antigen. In this regard, the $Ba(SCN)_2$ extracts of resolved gonococcal envelope components were prepared and analyzed in CIE. Evaluation of these immunoplates (not shown) revealed that this antigen was present in the extracts of both the cytoplasmic and outer membrane fractions. The determination of the distribution of this antigen in the intact gonococcal cell awaits further studies employing the techniques described here with extracts prepared from cells grown under conditions which result in an increased content of this reactive antigen in the extract.

DISCUSSION

The procedures described in this communication provide a means of obtaining preparations enriched in gonococcal cytoplasmic and outer membranes. These techniques appear to be widely applicable to gram-negative bacteria, as similar procedures have been used successfully to fractionate cell envelopes from *R. rubrum* (4), *Rhodopseudomonas sphaeroides* (3), *Pseudomonas putida* (2), *Escherichia coli* (C. Golenda and R. A. Niederman, personal communication), and *Aeromonas proteolytica* (15). The techniques and results described here will provide the basis for the localization of gonococcal antigens in the intact cell.

In the present study, the detergent Brij 58 was required to obtain a reproducible yield of the cytoplasmic membrane fraction. The detergent-to-membrane protein ratio used in these experiments was 0.22, which is well below the threshold (0.5 to 1) required to effect maximal solubilization of *Micrococcus lysodeikticus* membranes by Triton X-100 (5). Additionally, Brij 58 is less hydrophobic than Triton X-100, making it less effective in membrane solubilization. To evaluate the possibility that membrane components had become solubilized by detergent during the preparation of the cell extract, we examined the polypeptide composition of the soluble fraction (Fig. 2). Comparison of the supernatant fractions obtained from lysates prepared with and without detergent showed no differences. These patterns were distinct from those of the membrane fractions (Fig. 3). These data thus indicate that solubilization of membrane components did not occur. There is, however, evidence of detergent binding. The outer membrane fraction obtained from lysates prepared without detergent banded at a density (1.24 g/cm^3) which was greater than that of outer membrane (1.22 g/cm^3) from Brij-treated lysates. Despite its higher density, the outer membrane from detergent-free preparations appeared to be contaminated to a greater extent with cytoplasmic membrane, as the specific activity of LDH was four to five times greater than that of Brij-treated outer membrane. Similarly, the detergent-free outer membrane showed greater contamination with cytoplasmic membrane antigens in CIE (not shown) when compared to the outer membrane obtained from Brij-treated lysates. It thus appears that Brij treatment of EDTA-lysozyme spheroplasts not only enhances lysis, but also facilitates the separation of cytoplasmic and outer membrane by isopycnic sucrose density gradient centrifugation. It is possible that this effect is a consequence of an alteration of membrane fluidity by bound detergent. The finding (16) that the membranes of T1 gonococci are less fluid than those of T4 gonococci as determined by spin-label electron spin resonance is of interest in this regard.

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