Isolation and Characterization of a Native Cell Wall Complex from *Neisseria meningitidis*

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A cell wall complex has been isolated by gentle methods from both the medium supernatant fluid and whole organisms of *Neisseria meningitidis* cultures. The two types of preparations have been shown to be essentially identical on the basis of chemical composition, electron microscopy, and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). Four major components were identified in the complex: group-specific polysaccharide (4 to 10%), protein (45 to 65%), lipopolysaccharide (10 to 25%), and lipid (15 to 30%). The whole complex was found to be immunogenic in rabbits and to elicit production of antibody directed against the protein, the group-specific polysaccharide, and the lipopolysaccharide components. The isolated protein component was also found to be immunogenic in rabbits and to elicit production of serotype-specific antibody. The protein component was found to produce a band pattern in SDS-PAGE that is simple, reproducible, and strain dependent. The lipopolysaccharide component was found to have chemical and biological properties characteristic of bacterial endotoxin. We propose that this complex is representative of the outer trilaminar membrane of the meningococcal cell envelope in its native state.

Three major classes of meningococcal cell surface antigens have been described: serogroup-specific polysaccharides, serotype-specific proteins, and lipopolysaccharides. The most widely accepted serological classification for *Neisseria meningitidis* is based on the group-specific polysaccharide as expressed in bacterial agglutination tests (10). The polysaccharides of the major serogroups have been purified and analyzed both chemically and serologically (7, 15, 16). More recently, Roberts (27), Gold and Wyle (6), and Frasch and Chapman (5), by utilizing the bacterial reaction, have demonstrated antigenic diversity within the serogroups. Strain variation within serogroups B and C has also been demonstrated by Kingsbury (12) and Counts et al. (3) by means of bacteriocin reactions. These serotype antigens are not group-specific but have been found to be shared across serogroup lines (Kasper et al., Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 89, 1972) and have been shown in several cases to be protein in nature (Wyle and Kasper, Bacteriol. Proc., p. 99, 1971, and Frasch and Chapman, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 89, 1972). The presence of antilipopolysaccharide antibody in antisera to whole meningococci has been demonstrated by Merengehagen et al. (20), but the role of lipopolysaccharide (LPS) antigens in the serotyping schemes that have been reported is not clear. Little is known about this class of antigen in *N. meningitidis*.

The total antigenic structure of a given strain must be a composite of these three classes of antigens plus any which remain unidentified. To understand the complete antigenic makeup and diversity of *N. meningitidis* strains, it is essential to understand the immunochemical properties of each class of antigen separately as well as any immunochemical properties arising from the association and interaction of the different classes of antigen. Such an understanding is important in relation to microbial structure, serological classification, and development of effective vaccines against the organism.

Two prerequisites for conducting the immunochemical studies needed to gain this understanding are (i) the development of procedures for the isolation, identification, and purification of the separate classes of antigen and (ii) isolation of subcellular structures which contain the different antigens in their native association and configuration. Adequate methods have been described for the preparation of purified, group-
specific polysaccharide and lipopolysaccharide (7, 40), but adequate methods for obtaining purified, type-specific protein antigens have not been described.

Lipopolysaccharide-lipid-protein complexes have been isolated by gentle methods from *Escherichia coli* (13, 14, 30) and *Pseudomonas aeruginosa* (29). It has been proposed that such complexes are representative of the in situ form of native endotoxin (29). It is evident from the work of Rake and Sherp (24) and Menzel and Rake (18) that such a complex also exists in *N. meningitidis*.

In this report we describe the isolation from *N. meningitidis* of a cell wall complex consisting of protein, lipid, lipopolysaccharide, and group-specific polysaccharide. We have partially characterized this complex, as a whole as well as its individual components, and propose that it is representative of the outer cell wall layer in its native state.

**MATERIALS AND METHODS**

**Bacterial strains.** The strains of *N. meningitidis* were from the culture collection of the Department of Bacterial Diseases, Walter Reed Army Institute of Research. Cultures were preserved in the lyophilized state and used within one to six passages of original isolation. The strain used in the experiments described in this paper was the group B strain 99M, unless otherwise indicated.

**Media and growth conditions.** Lyophilized organisms were rehydrated with sterile water and grown overnight on BYE agar (Baltimore Biological Laboratories, Cockeysville, Md.) in a CO₂ incubator at 35°C. A modification (fourfold increase in sodium phosphate and deletion of cystine; Maloney et al., *in preparation*) of the Casamino Acid medium of Watson and Sherp (37) was used for all liquid cultures. Bulk liquid cultures were inoculated from liquid starter cultures and grown either as 250-ml cultures in 1-liter Erlenmeyer flasks or as 11-liter cultures in a Microfem fermentor (New Brunswick Scientific, Inc., New Brunswick, N.J.). Cultures were grown at 36 to 37°C for 15 to 21 hr. Labeling of cultures with ¹⁴C was accomplished by addition of 5 mCi of ¹⁴C-sodium acetate per liter (New England Nuclear, Boston, Mass.) to the medium at the time of inoculation.

**Isolation of the native complex.** **Preparation M.** Cultures of meningooccoci grown as described above were centrifuged at 12,000 × *g* for 20 min to pellet the organisms. The culture medium supernatant fluid was decanted, filtered through a membrane filter (0.45 μm pore size, Millipore Corp.) and concentrated 15-fold by ultrafiltration at 4°C by using a Diaflow PM-30 ultrafilter (Amicon Corp., Lexington, Mass.). The concentrate was centrifuged at 12,000 × *g* for 20 min, and the pellet was discarded. Ultra centrifugation of the supernatant fluid at 80,000 × *g* for 2 hr produced a small, gel-like pellet. The supernatant fluid was removed and, in some experiments, further processed as described for preparation CP below. The pelleted material, which is principally native complex, was suspended in water, centrifuged at 8,000 × *g* for 10 min to remove any aggregated cell debris, and repellet by centrifugation at 100,000 × *g* for 2 hr. All centrifugation was done at 5°C.

**Preparation O.** Pelleted organisms were suspended at room temperature in buffer containing 0.05 M sodium phosphate, 0.15 M NaCl, and 0.01 M ethylene diaminetetraacetic acid (EDTA). pH 7.4. The final volume was about one-tenth that of the original culture. The suspension of organisms was incubated at 60°C for 30 min and then subjected to mild shear by twice passing it through a 1-inch (2.5 cm), 23-gauge hypodermic needle by using manual pressure. Orga nisms were pelleted from the suspension by centrifugation twice at 12,000 × *g* for 20 min. The resulting supernatant fluid was then centrifuged at 80,000 × *g* for 2 hr. and the clear, gel-like pellets were suspended in water. The differential centrifugation steps were repeated, and the final product was stored frozen in water dilute buffer.

**Preparation CP.** More high-molecular-weight material was recovered from the 100,000 × *g* supernatant fluid of the concentrated culture medium by addition of hexadecyltrimethylammonium bromide (Cetavlon) to 0.3%. The precipitate which formed was collected by centrifugation and dissolved in 0.9 M CaCl₂. any insoluble material was removed by centrifugation, and four volumes of cold absolute ethanol were added to the supernatant fluid. The resulting precipitate was collected, dissolved in water, and stored at −20°C.

**Fractionation of the native complex.** Lipid was removed from the complex by precipitation of the complex from 0.2 M NaCl with 80% ethanol and by washing the precipitate with chloroform-methanol (2:1, v/v) and then with absolute ethanol. The three supernatant fluids were pooled and reduced to a small volume by evaporation under a stream of air. This procedure removed most of the organic solvents, leaving the lipid in aqueous suspension. This suspension was extracted twice with an equal volume of chloroform-methanol. The organic phase was removed, reduced to a paste by evaporation, and suspended in distilled water. The suspension of lipid was dialyzed against distilled water, lyophilized, and further dried under vacuum over P₂O₅.

Protein was removed from the lipid-extracted complex by suspending the complex in water and adding an equal volume of 50% trichloroacetic acid at room temperature (9). The precipitated protein was removed by centrifugation at 4°C, washed twice with distilled water, and dissolved in water by drop-wise addition of 0.5 M NaOH to pH 10 to 11. The trichloroacetic acid precipitation was repeated, and the final product was dialyzed, lyophilized, and stored under vacuum over P₂O₅. In some experiments, protein was removed by the hot phenol-water method described by Westphal et al. (40) for the extraction of LPS.

The trichloroacetic acid-soluble material, which was predominantly group-specific polysaccharide and LPS, was recovered by slowly neutralizing the trichloroacetic acid in an ice bath and adding absolute
ethanol to 80° v. v. After 2 hr at 4°C, the precipitate was spun out and dissolved in water at neutral pH.

Separation of the group-specific polysaccharide and LPS was achieved by gel filtration at high pH on a 2.5 by 80 cm column of Sephadex G-200. The buffer used to equilibrate the column and to dissolve and elute the sample was composed of 0.05 M glycine-NaOH, 0.5% Triton X100, pH 10.5. Under these conditions, the group-specific polysaccharide, which has been shown to have a molecular weight in excess of 100,000 (15), was eluted from the column as a relatively broad peak at or near the void volume. The LPS, presumably depolymerized, was eluted as a lower-molecular-weight, well-separated second peak. Similar results were obtained with gel filtration on Sepharose 4B in 0.05 M Tris(hydroxymethyl)aminomethane (Tris)-chloride, 0.1% sodium dodecyl sulfate (SDS), pH 7.4.

The sample was recovered by addition of 1 l of volume of 2 N NaCl and four volumes of cold absolute ethanol. After 4 to 8 hr at 4°C, the precipitate was spun out, redissolved in water, dialyzed against three changes of distilled water, and lyophilized.

Sucrose density gradients. Linear sucrose density gradients were prepared from 10° and 60° (w/w) sucrose solutions containing 0.05 M Tris-chloride, pH 7.4. One-half milliliter of 14C-labeled sample was layered on the 4.5-ml gradients, and they were spun for 14 hr at 120,000 X g 4°C. Fractions were obtained by puncturing the bottom of the tubes and collecting drops.

Polyacrylamide gel electrophoresis. Continuous, neutral polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out essentially as described by Weber and Osborne (38). Unless otherwise stated, the gels were 7.5° acrylamide made up in the running buffer, which consisted of 0.05 M sodium phosphate buffer and 0.1% SDS, pH 7.1. Thirty microliters of sample at a concentration of 2 to 10 mg ml was placed in a small tube with 10 ml of 10° SDS and 10 ml of 67° glyc- erol, 0.03% bromophenol blue. The addition of 2-mercaptoethanol to break disulfide bonds was found to have no effect on the band patterns and was, therefore, not routinely used. The samples were then heated to 100°C for 1 min, cooled to room temperature, and 10 to 20 ml of the gels were layered on each gel. Electrophore- 

sis was carried out at 4 ma tube for 3 hr at room temperature. Gels were stained for protein with Coomassie brilliant blue (38) and, in some cases, for carbohydrate with the periodic acid-Schiff reagent (41, 21). Gels to be assayed for radioactivity were placed in a stainless steel trough (Scoopula, Fisher Scientific Co., Pitts- 

burgh, Pa.) and frozen on a block of dry ice. The gels were sliced by pressing against a transverse slicer contain- 

ing razor blades at 1-mm intervals. Each slice was placed in a scintillation vial with 50 ml of water and 8 ml of a solution made up of 100 ml of NCS Solubilizer (Amersham Searle, Arlington Heights, Ill.) and 42 ml of Liquifluor (New England Nuclear) added to 1 liter of toluene. The vials were mixed and then allowed to sit overnight at room temperature before counting.

Electron microscopy. Samples were prepared for electron microscopy by a modification of the method of Ryter and Kellenberger (31) as described by Benjamin J. Veltri (Ph.D. thesis, Catholic University of America, Washington, D.C., 1971). After two wash- 
gings in phosphate buffer, the samples were prefixed in 3° glutaraldehyde in phosphate buffer at 4°C over- 
night. The specimens were then further fixed and stained with 1° osmium tetroxide in Kellenberger Veronal buffer (31) at room temperature overnight. After dehydration and prior to embedding, the specimens were stained with uranyl acetate. Specimens were embedded in Epon 812, and ultrathin sections were cut with a Dupont diamond knife using an LKB ultra- 
microtome. The ultrathin sections were picked up on Formvar-coated, 100-mesh grids, poststained with lead citrate, and examined using an AEI 800 electron microscope.

Analytical methods. Protein was determined by the method of Lowry et al. (17) by using bovine serum albumin as a standard.

Total carbohydrate, exclusive of amino sugars, was determined by the phenol-sulfuric acid method of Dubois et al. (4).

Total hexose was determined by the anthrone reaction as described by Roe (28).

Total LPS-bound 2-keto-3-deoxysugar acid was estimated by the thiobarbituric acid method of Weiss- 
bach and Hurwitz (39) as modified by Osborne (23). The reaction product obtained with purified LPS ab- 
sorbed maximally at 549 nm with no evidence of a second peak at 532 nm due to 2-deoxyyaldose. Sialic acid standards were run and corrections made based on the sialic acid content of the sample as determined by the resorcinol reaction (36).

Heptose was estimated by the cysteine-H2SO4 method as described by Osborne (23). The reaction product obtained with purified LPS was found to have a definite absorption maximum at 505 nm. Estere- 
ed fatty acid was determined by the procedure of Synder and Stephens (35) as modified by Haskins (8).

Sialic acid was determined by the resorcinol method of Swanerholm (36).

Total phosphorus was determined by the method of Chen et al. (2) as modified by Liu et al. (16).

Chicken embryo toxicity test. Tests for toxicity in chicken embryos were carried out as described by Smith and Thomas (34). Serial 10-fold dilutions of samples in 0.1 ml of sterile normal saline were injected onto the lowered chorioallantoic membrane of 10-day- old chicken embryos. Six to ten eggs were injected at each concentration. After closing the injection port with tape, the eggs were incubated in a non-turning tray at 38 ± 0.5°C for 24 hr. At this time, the number of deaths was determined, and 50° lethal end points were calculated by the method of Reed and Muench (25).

Serological methods. Radioactive bactericidal assays were performed as described by Kasper and Wyle (11). Mid-log phase organisms (3 × 108/ml grown in 14C-sodium acetate-enriched Mueller-Hinton broth [Difco]) were washed and suspended in Geys' balanced salt solution (Microbiological Associates, Inc.). One-tenth milliliter of bacterial suspension was mixed with 0.1 ml of antiserum, 0.1 ml of complement from
4-week-old rabbits, and 0.2 ml of Geys balanced salt solution in a total reaction volume of 0.5 ml for 60 min at 37°C. Net percent release of radioactivity due to immune bacteriolysis was calculated from duplicate samplings at zero time and at 60 min and corrected by subtraction of control values. Percentage release was graded on a 1 to 4 scale corresponding to percentage of release above complement controls where 1 equals 5 to 9% release, 2 equals 10 to 14%, 3 equals 15 to 20%, and 4 equals > 20% release. Ten percent net release is equivalent to 50% killing for the 3 × 10^6 organisms/ml in the reaction mixture.

The hemagglutination assays were done as described by Artenstein et al. (1). New Zealand white rabbits were immunized by giving three intravenous injections per week for 2 weeks. A booster injection was given after 3 weeks, and the rabbits were exsanguinated 1 to 2 weeks after the final injection. Each injection contained 5 μg of antigen in the case of LPS and 50 μg in the case of the proteins. The amount of native complex injected was gradually increased from 10 to 50 μg over the course of the seven injections.

RESULTS

Electron microscopy. The structure and morphology of the native complex and the organisms from which the complex was extracted were investigated by electron microscopy of thin sections. Native complex obtained from the culture medium supernatant fluid (preparation M) and that extracted from the organisms (preparation O) are compared in Fig. 1. The two preparations are very similar in appearance. Both show many closed structures of variable morphology bounded by a single trilaminar membrane. The overall size of the structures in the O preparation (Fig. 1A) is somewhat larger, and some open structures are evident.

In Fig. 2, control organisms from a 15-hr culture are compared with organisms from the same culture after extraction of native complex as described for preparation O. The control organisms (Fig. 2A) exhibit the characteristic multilayered cell envelope of gram-negative bacteria. Two trilaminar membranes are evident: the inner plasma membrane and the outer, endotoxin-containing membrane. Relative to the control organisms, the extracted organisms (Fig. 2B) exhibit an outer trilaminar membrane that in many places has pulled away from the rest of the cell wall. In addition, some of the extracted cells appear to have lost most of the outer trilaminar membrane, but completely disrupted cells are rare. These results suggest that the complex extracted from the organisms predominantly represents fragments of the outer trilaminar membrane, although some contamination by plasma membrane and ribosomes cannot be excluded.

Analysis by sucrose density gradients. The homogeneity of 14C-labeled native complex obtained from the medium supernatant fluid (preparation M) and that extracted from the organisms (preparation O) as described above was investigated by isopycnic centrifugation on sucrose density gradients (Fig. 3). Both preparations yielded a single, relatively broad peak of similar density, but, in the case of the O preparation, the peak had a prominent shoulder which suggested some type of heterogeneity. In Fig. 3, these two preparations are compared to material obtained from the 80,000 × g culture medium supernatant fluid by Cetavlon precipitation (preparation CP). Although the material in the CP preparation had probably not reached equilibrium by the end of the run, it was resolved into two distinct peaks, CP1 and CP2.

Analysis on SDS-polyacrylamide gels. The differences among the three preparations and the apparent heterogeneity of the O and CP preparations were investigated in more detail by SDS-PAGE. Samples were taken from the gradients at positions indicated by the arrows (Fig. 3), and each sample was run on several gels. One set of gels was stained for observation of protein bands (Fig. 4), whereas a second gel of each sample was sliced and assayed for radioactivity (Fig. 5 and 6). It is evident from the stained gels (Fig. 4) that the same protein species are present in each sample that contains a significant amount of protein. (The sample from CP1 contains only a slight amount of protein and none is evident in the CP2 sample.) The distribution of radioactivity in the gels (Fig. 5 and 6) indicates the presence of several nonprotein components labeled at higher specific activity than the protein. The nonprotein component which moves ahead of the marker dye is lipid. This band is absent if the complex is extracted with chloroform-methanol prior to electrophoresis. A second nonprotein component moves just behind the marker dye and ahead of the smallest protein. This component is shown below to be lipopolysaccharide and is present in all of the samples but CP2. A third nonprotein component is the principal constituent present in the CP preparation. It is also present in smaller amounts in the M preparation and is present, but not apparent, in the O preparation. As shown below, this component has the properties of the group-specific polysaccharide. It appears that the CP2 peak consists of soluble, group-specific polysaccharide, whereas the CP1 peak is group-specific polysaccharide complexed with a small amount of protein and LPS. The density heterogeneity seen in the O preparation may be the result of variation in the proportions of the four components present in the complex. No evidence for the presence of a second kind of complex was obtained by these methods although this
FIG. 1. Electron micrographs of thin sections of native cell wall complex from N. meningitidis. A, O preparation; B, M preparation. Bars represent 0.1 μm.
FIG. 2. Electron micrographs of thin sections of N. meningitidis cells. A, Untreated cells from a 15-hr culture; B, cells from the same culture following extraction of native cell wall complex. Bars represent 0.2 μm.
possibility cannot be eliminated. The composition of the M and O preparations as determined by colorimetric assays was very similar. The range of values obtained with several different preparations of both M and O native complex is given in Table 1. The M preparations generally contained slightly more LPS and group-specific polysaccharide and less protein than the O preparations.

On the basis of the results of the compositional analysis, SDS-PAGE and electron microscopy, we conclude that the M and O preparations of native complex are essentially identical. The two preparations differed only in the relative proportions of the four components in the complex.

**Fractionation of the native complex and identification of the components.** To identify the different components of the native complex and to study their individual properties, it was necessary to fractionate the complex on a preparative scale. When the ^14C-labeled native complex was dis-

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**Fig. 3.** Isopycnic sucrose density gradients (10 to 60% sucrose w/w) of the CP (top), M (center), and O (bottom) preparations of ^14C-labeled cell wall complex. The bottom of the gradients is at the left. The arrows indicate points at which samples were taken for analysis by SDS-PAGE (Fig. 5 and 6).

**Fig. 4.** SDS-PAGE of different preparations of cell wall complex after banding in sucrose density gradients (Fig. 3). The gels were stained for protein with Coomassie brilliant blue. a, M preparation; b, O preparation, high density fraction; c, O preparation, low density fraction; d, CP preparation, peak 1; e, CP preparation, peak 2.
negative bacteria. The lipid fraction is rich in esterified fatty acids and contains 3.3% phosphorus, which suggests the presence of phospholipid. The group-specific polysaccharide fraction is composed almost entirely of sialic acid which is consistent with its identification as the group B polysaccharide (7, 15).

The relative specific radioactivity of each of the four fractions derived from C-labeled native complex is given in Table 3. The specific activity

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**Fig. 5.** Electropherogram of M and O preparations of 14C-labeled cell wall complex. Samples taken from sucrose density gradients (Fig. 3) were resolved by SDS-PAGE. The gels were sliced into 1-mm slices and assayed for radioactivity. The origin of the gels is at the left. A, M preparation; B, O preparation, high density fraction; C, O preparation, low density fraction.
TABLE 1. Composition of the native complex from strain 99M

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage of dry weight of native complexa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>45-65</td>
</tr>
<tr>
<td>Group-specific polysaccharide</td>
<td>4-10</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>10-25</td>
</tr>
<tr>
<td>Lipid</td>
<td>15-30</td>
</tr>
</tbody>
</table>

a Values given represent the range of values obtained for several different M and O preparations.

The presence of any material which stains with Coomassie blue. The protein fraction produces a number of bands which stain with Coomassie blue. The pattern is similar but not identical to that produced by the whole complex. The differences may result from conformational changes caused by exposure to chloroform-methanol and trichloroacetic acid during the fractionation procedure. The presence of a small amount of LPS (about 1%) in the protein fraction is indicated by the

Fig. 6. Electropherograms of two fractions of the CP preparation separated on a sucrose density gradient (Fig. 3). The samples were resolved by SDS-PAGE. Gels were then sliced into 1-mm slices and assayed for radioactivity. The origin of the gels is at the left. a, Peak 1; b, peak 2.

TABLE 2. Chemical analysis of the purified components from 99M native complex

<table>
<thead>
<tr>
<th>Assay</th>
<th>Percentage of dry weight</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td>Protein</td>
<td>86</td>
</tr>
<tr>
<td>Esterified fatty acids</td>
<td>2</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>1</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>0.2</td>
</tr>
<tr>
<td>Heptose</td>
<td>0.1</td>
</tr>
<tr>
<td>Phosphorous</td>
<td>0.9</td>
</tr>
<tr>
<td>2-Keto-3-deoxy sugar acid</td>
<td>0.1</td>
</tr>
</tbody>
</table>

a In this case, only O-esterified fatty acids were determined. The unmodified method of Snyder and Stephens (35) was used.

b ND, Not determined.
Table 3. Relative specific activity of components of the native complex from a \(^{14}\text{C}\)-sodium acetate-labeled culture

<table>
<thead>
<tr>
<th>Component</th>
<th>Relative specific (14\text{C}) radioactivity (^{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>1</td>
</tr>
<tr>
<td>Group-specific polysaccharide</td>
<td>11</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>27</td>
</tr>
<tr>
<td>Lipid</td>
<td>80</td>
</tr>
</tbody>
</table>

\(^{a}\) \(^{14}\text{C}\) counts per minute per microgram of sample. The specific activity of protein is arbitrarily set at 1.

Properties of the protein component. The pattern of protein bands obtained in SDS-PAGE of native complex is reproducible and characteristic of the strain from which it is obtained. The protein band pattern obtained with eight group C strains is shown in Fig. 9. Bactericidal assays have shown these eight strains to be different, but an exact relationship between PAGE pattern and bactericidal serotype of an organism has not been established.

Fig. 7. Electropherograms of the purified components of \(^{14}\text{C}\)-labeled native complex after resolution by SDS-PAGE. The gels were sliced into 1-mm slices and assayed for radioactivity. The origin of the gels is at the left.
The individual protein bands on gels do not stain with the periodic acid-Schiff reagent, and the protein fraction as a whole is low in carbohydrate as determined by the phenol-sulfuric acid reaction. These results suggest that the major proteins are not glycoproteins.

The bulk of the protein is insoluble in water at neutral pH after isolation from the complex as described above. Several of the minor protein bands, however, are fairly soluble under these conditions and can be partially separated from the remainder of the protein on the basis of this solubility difference (Fig. 10).

The insoluble proteins are relatively resistant to dissociation and denaturation as judged by their behavior on SDS-PAGE following various treatments (Fig. 11). Exposure of the proteins to chloroform-methanol and trichloroacetic acid in the process of separating them from the complex causes a shift in the position of several bands relative to their position prior to separation from the complex (Fig. 8 and 10).

Properties of the lipopolysaccharide component. The lipopolysaccharide component of the complex has biological and chemical properties characteristic of gram-negative bacterial endotoxin. The LPS purified from native complex was found to be toxic to chicken embryos with a median lethal dose (LD$_{50}$) of 0.5 µg when injected onto the chorioallantoic membrane (Table 4). The native complex, which contains

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**Fig. 8. SDS-PAGE** of $^{14}$C-labeled native complex (strain 99M) and its purified components. The gels were stained for protein with Coomassie brilliant blue. a, Native complex; b, protein component; c, lipopolysaccharide component; d, group-specific polysaccharide component; e, lipid component.

**Fig. 9. SDS-PAGE** of native complex from eight different group C strains of *N. meningitidis*. Gels were stained for protein with Coomassie brilliant blue. a, 60E (I, IX); b, 1381 (II, IX); c, 126E (III); d, 118V (IV, IX); e, 35E (V); f, 321 (I, IV, VI); g, 89 (VII); h, 1901 (III, VIII); i, a mixture of four molecular weight markers: bovine serum albumin (top band), ovalbumin (second band), chymotrypsinogen A (third band), and ribonuclease (bottom band). Roman numerals in parentheses indicate the serotype antigens that have been identified in each strain on the basis of the bactericidal assay.
about 15% LPS by weight, is nontoxic to chicken embryos at a level of 100 μg; but when the loosely bound lipid is removed from the complex with organic solvents, an LD₅₀ of 2 to 4 μg is obtained. It appears that, in this particular system, the loosely bound lipid is able to mask the toxic effect of the LPS component. When LPS was separated from the complex with 25% trichloroacetic acid and then purified by chromatography on Sephadex G-200 in the presence of Triton X100 at pH 10.5, a purified LPS with an LD₅₀ of 0.4 μg was obtained. Preparations of LPS with the same level of toxicity were obtained by extraction of the complex with hot aqueous phenol. If, however, the purification was carried out by using gel filtration in the presence of SDS, a product was obtained which had an LD₅₀ of 4.8 μg.

The LPS component moves as a single, well-defined band in SDS-PAGE and may be detected either by using radioactive LPS and assaying gel slices for radioactivity or by staining the gel with the periodic acid-Schiff reagent. An experiment was performed to test mobility of LPS in gels varying in total acrylamide concentration from 6 to 18%. A plot of the logarithm of the relative mobility of the center of the LPS band versus total gel concentration was found to be linear and extrapolated at zero gel concentration to the same free mobility as three protein markers (cytochrome c, ribonuclease, and chymotrypsinogen) run under the same conditions. This suggests that the LPS behaves in a manner very similar to proteins in SDS-PAGE. Thus, its $R_f$ in this system is primarily determined by its size and shape rather than its charge. The $R_f$ of LPS shows some concentration dependence in this system and, therefore, a plot of $R_f$ versus LPS concentration extrapolated to zero concentration of LPS has been used to obtain a reproducible $R_f$. This concentration...
TABLE 4. Toxicity of antigen preparations to chicken embryos

<table>
<thead>
<tr>
<th>Preparation</th>
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<tbody>
<tr>
<td>Native complex</td>
</tr>
<tr>
<td>Untreated</td>
</tr>
<tr>
<td>Precipitated with 80% ethanol</td>
</tr>
<tr>
<td>Extracted with chloroform methanol</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Extracted with trichloroacetic acid, chromatographed on G-200 in 0.5% Triton, pH 10.5</td>
</tr>
<tr>
<td>Extracted with trichloroacetic acid, chromatographed on G-200 in 0.1% SDS, pH 7.5</td>
</tr>
<tr>
<td>Extracted with hot aqueous phenol, purified by ultracentrifugation</td>
</tr>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>Lipid</td>
</tr>
</tbody>
</table>

dependence is evident if one compiles the \( R_F \) of the LPS peak in the electropherograms in Fig. 5, 6, 7, and 12. By using well-characterized proteins as molecular size markers, the LPS-SDS complex has been found to have a molecular size nearly identical to that of an SDS-cytochrome \( c \) complex.

Immunogenicity of the whole complex and its components. Most of the important cell surface antigens appear to be associated with the native complex. Native complex was found to be immunogenic in rabbits when 10- to 50-\( \mu \)g injections were given intravenously over a 3-week period. Postimmunization sera from rabbits inoculated with native complex from the group B strain 99M were shown to have at least a 16-fold rise in antibody when tested by the hemagglutination assay by using sheep erythrocytes sensitized with purified group B polysaccharide. The same sera were found to have a 200-fold increase in bactericidal titer against strain 138I, a group C organism which shares protein antigens with 99M. In addition, the presence of anti-LPS antibody in these antisera has been detected in agar immunodiffusion experiments by using purified LPS.

In Table 5 are shown the results of bactericidal tests with antisera made to a variety of 99M antigens. Antiserum to native complex showed bactericidal activity against five meningococcal strains; a pattern identical to that shown by antiserum against whole organisms. Antibody against 99M polysaccharide had a very restricted killing capacity. In spite of exposure to denaturing conditions during isolation of the protein from the complex, both the soluble and insoluble protein fractions were found to be immunogenic in rabbits. The anti-protein antibody had a spectrum of bactericidal activity which was similar but not identical to antiserum against whole organisms or native complex. Purified LPS was only weakly immunogenic.

Comparison of native complex with phenol-water and aqueous ether extracts. The relationship between the native complex and antigen preparations obtained by several other common methods was investigated by SDS-PAGE. The yield of organisms from a small culture of 99M meningococci labeled with \( ^{14} \)C-sodium acetate was divided into three parts, and each part was subjected to a different extraction procedure. The first part was treated in the normal way (preparation O) to obtain native complex. The second part was extracted with hot phenol-water as described by Westphal et al. (40), and a high-molecular-weight fraction was recovered from the aqueous phase by differential centrifugation between 20,000 \( \times \) g and 100,000 \( \times \) g. The third part was extracted with aqueous ether by using a modification of the method of Ribi et al.

TABLE 5. Bactericidal activity of rabbit antiserum to 99M antigens

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Graded killing of strains: ( ^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-99M whole organisms</td>
<td>99M</td>
</tr>
<tr>
<td>Anti-99M native complex</td>
<td>4</td>
</tr>
<tr>
<td>Anti-99M protein component (pH 7 soluble)</td>
<td>4</td>
</tr>
<tr>
<td>Anti-99M protein component (pH 7 insoluble)</td>
<td>4</td>
</tr>
<tr>
<td>Anti-99M group-specific polysaccharide</td>
<td>4</td>
</tr>
<tr>
<td>Anti-99M lipopolysaccharide</td>
<td>2 NT ( ^d )</td>
</tr>
</tbody>
</table>

\( ^a \) Strain 99M: serogroup B, serotype II, III, VI, IX. Strain 138I: serogroup C, serotype II, IX. Strain 60E: serogroup C, serotype I, IX. Strain 35E: serogroup C, serotype V. Strain NE: serogroup B, serotype IV. The numbers represent a graded bactericidal activity where 4 corresponds to 100% killing for 3 \( \times \) 10^8 organisms.

\( ^b \) Immune serum used at a dilution of 1:40.

\( ^c \) 0, No killing above prebleed at any dilution.

\( ^d \) NT, Not tested.
A 60% ethanol precipitate was obtained from the 20,000 \( \times g \) supernatant fluid of the aqueous phase. These three antigen preparations were analyzed in duplicate on SDS-polyacrylamide gels. One set of gels was stained for protein, and the second set was sliced and assayed for radioactivity. The results are shown in Fig. 12 and 13. The phenol-water extract is predominantly LPS, but a small amount of a second nonprotein component is seen near the origin (Fig. 12). No protein bands are visible on the stained gel (Fig. 13). The aqueous ether extract, on the other hand, is principally protein, although a significant amount of LPS and a high-molecular-weight material (presumably nucleic acid or group-specific polysaccharide) are present. Although some proteins are common to both the native complex and the aqueous ether extract, the protein pattern of the latter is much more complex (Fig. 13). The distribution of radioactivity in the gel of native complex was identical to that shown in Fig. 5. Both the phenol-water extract and the aqueous ether extract appear to contain only some of the constituents of the native complex. The aqueous ether extract contains, in addition, constituents not present in the native complex.

**DISCUSSION**

By using gentle methods, we have isolated a cell wall complex from the medium supernatant fluid and from the whole organisms of \( N. meningitidis \) cultures. The two types of preparations have been shown to be essentially identical on the basis of behavior on SDS-PAGE, appearance when examined by electron microscope, and chemical composition.

The cell wall complex (native complex) has been shown to have four major components: group-specific polysaccharide, lipid, protein, and lipopolysaccharide. These components sediment together in repeated washings of the complex and band together on sucrose density gradients. The relative proportion of each component in the complex is not fixed, but can vary within a preparation as was shown by analysis of fractions by SDS-PAGE after isopycnic density gradient centrifugation. The relative amount of each component in the complex is probably also dependent on the strain and the growth conditions. In general, however, the native complex from \( N. meningitidis \) has a high percentage of protein and a low percentage of LPS relative to the composition of similar complexes isolated from \( E. coli \) (13, 14, 30). A complex released from \( P. aeruginosa \) by EDTA (29), however, was found to have a protein content approaching that which we have found for the native complex from meningococcus.

Native complex obtained from both medium and organisms appears to consist primarily of fragments of the outer trilaminar membrane of

![Fig. 12. Electropherograms of two different extracts of the same \( 14C \)-labeled culture of meningococci (strain 99M) after resolution by SDS-PAGE. Gels were sliced into 1-mm slices and assayed for radioactivity. The origin of the gels is at the left. The corresponding electropherogram of native complex (preparation O) from this culture was similar to that shown in Fig. 5. Duplicate gels of the three extracts stained for protein are shown in Fig. 13.](http://iai.asm.org/)
of a significant amount of peptidoglycan which is not dissolved by SDS.

Relative to purified LPS, the native complex has low toxicity in chicken embryos. This is in agreement with the results obtained by Knox et al. (13) with *E. coli*. The lipid and possibly the protein in the complex are apparently able to mask the toxicity of the LPS in this system. In preliminary experiments in our laboratory, we have demonstrated the presence of a weak but reproducible and specific bacteriocin activity associated with native complex from some strains. Other unidentified biological activities are undoubtedly associated with this cell wall complex.

The usefulness of SDS-PAGE in the dissociation and separation of the components of protein-lipid-lipopolysaccharide complexes has been demonstrated by Rothfield and Pearlman-Kothencz (30). We have found the use of SDS-PAGE in conjunction with 14C-sodium acetate-labeled cultures to be a powerful tool for detecting the presence of the various components of the complex and monitoring the effects of various fractionation procedures on the composition of a sample. In this system, the LPS component moves as a single band ahead of all the major proteins. Extrapolation of plots of the logarithm of the relative mobility versus the gel concentration (Ferguson plots) to zero gel concentration have indicated that the LPS component has the same free mobility in the SDS system as several standard proteins of similar size. These results suggest that one might use protein molecular size markers to obtain an estimate of the molecular size of the LPS monomer.

The protein component of the complex is of considerable interest. We have isolated native complex from 30 different strains of meningococcus taken from serogroups A, B, C, Y, 29E, and X and have compared the protein band pattern obtained in SDS-PAGE. The protein band patterns have been found to be reproducible, relatively simple (one to eight major bands), and strain dependent. The way in which these polypeptide chains are associated in the complex is not known, but when milder methods are employed to disaggregate the complex, higher-molecular-weight forms of the proteins are obtained. This suggests that many of the proteins may exist in a multimeric state in the complex.

The proteins are found to be phenol soluble when the complex is extracted with hot phenol-water by the procedure of Westphal et al. (40), but the relationship of these proteins to the phenol-soluble glycoprotein isolated in high yields from the cell envelopes of *E. coli* by Okuda and Weinbaum (22) is unknown. The protein

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Fig. 13. SDS-PAGE of three different extracts from the same culture of 14C-labeled meningococci (strain 99M). Gels were stained for protein with Coomassie brilliant blue. a, Aqueous ether extract; b, native cell wall complex; c, hot phenol-water extract. Duplicate gels assayed for radioactivity are shown in Fig. 12.

The conclusion is based on the following considerations: (i) native complex and the outer trilaminar membrane have a similar appearance when examined by electron microscope; (ii) by this technique the extracted organisms are often seen to have the outer trilaminar membrane loosened or missing, but very few cells are broken open; (iii) the native complex contains LPS, which has been shown by several workers (19, 32) to be located principally in the outer trilaminar membrane of the cell envelope of gram-negative bacteria; (iv) native complex preparations produced a single band on isopycnic sucrose density gradients, suggesting that the preparations were relatively homogeneous; (v) the complex was completely dissociated by 2% SDS as shown by the absence of material with molecular weight greater than several hundred thousand when analyzed by SDS-PAGE and gel filtration. This is inconsistent with the presence
component of the native complex does not appear to consist of glycoprotein as judged by its low content of carbohydrate and failure to stain in the periodic acid-Schiff procedure. Although the bulk of the protein component of the complex is insoluble in water at neutral pH, a specific minor fraction can be separated on the basis of solubility in water at neutral pH. Both the soluble and insoluble fractions have been found to be immunologically active. The more soluble proteins were probably also present in the serotype factor II containing aqueous ether extracts obtained by Wyle and Kasper (Bacteriol. Proc., 1971, p. 99). The soluble and insoluble protein fractions may correspond to the peripheral and integral categories of membrane proteins described by Singer and Nicolson (33).

The observation that nearly the same spectrum of bacterial killing was obtained with anti-protein antisera as with the homologous anti-whole organism antisera suggests that the protein antigens of the native complex are important in bacterial reactions. The few differences observed in the spectrum of killing may reflect either alteration of some protein antigenic determinants by the fractionation procedure or the presence of anti-LPS antibody in the anti-whole organism and anti-native complex antisera, or both. The role of anti-LPS antibody in the bactericidal reactions which form the basis of the serotyping schemes of Gold and Wyle (6) and Frasch et al. (5) remains to be elucidated.

Further studies of the isolated components of the native complex will be required to elucidate their specific roles in the structure and biological functions of the cell wall.

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