# AN OUTER MEMBRANE PROTEIN OF *NEISSERIA MENINGITIDIS* GROUP B RESPONSIBLE FOR SEROTYPE SPECIFICITY*

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*Neisseria meningitidis*, like most gram-negative eubacteria is bounded by a cell envelope consisting of an inner or cytoplasmic membrane, an intermediate peptidoglycan layer, and an outer membrane. The outer membrane is immunologically the most important structure because it is this portion of the organism that interacts with the host defense mechanisms. There are two main groups of meningococcal cell surface antigens responsible for the production of protective antibodies, the polysaccharide group-specific antigens (23, 24) and protein serotype antigens (9, 40).

Whereas effective polysaccharide vaccines have been developed for meningococcal groups A and C (1, 14, 15, 16, 28, 32), attempts at production of an effective group B polysaccharide vaccine have failed (42). The protein antigens of group B must therefore be further investigated for their potential as effective immunizing agents. Protection against meningococcal infection appears to be clearly associated with the presence of bactericidal antibody (12, 13, 32). The polysaccharide antigens of groups A and C strongly elicit bactericidal antisera, while group B polysaccharide elicits little bactericidal antibody (22, 40). The majority of the bactericidal antibody against group B appears to be directed against the protein serotype antigens (6, 22, 40). It is now known that the serotype antigens are shared among strains of all major meningococcal groups (22).

Earlier studies on the serotype antigens by Frasch and Chapman (9) indicated that these antigens were most likely high molecular weight complexes containing protein and that they were extractable by mild acid or saline at 100°C. The cellular location of the serotype antigen was assumed to be on the cell surface because the bactericidal activity directed against the serotype antigen was complement mediated. Presence of an immunologically identifiable protein component in the meningococcal cell envelope provided a useful tool for the examination of the cell envelope proteins. The outer membrane proteins of several gram-negative bacteria have been examined by polyacrylamide gel electrophoresis (19, 20, 30, 33, 34). In most instances there were relatively few

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major proteins present in the outer membrane of which one or two were predominant. The isolation techniques that have been used for gram-negative cell envelope proteins such as those of Schnaitman (34) or Moldow et al. (27) and for the serotype antigen (7) are not gentle methods. One of the purposes of these investigations was to develop milder methods for the extraction and isolation of outer membrane proteins such that the isolated proteins would retain the immunological characteristics of the native cellular proteins.

The present report will describe investigations concerning the nature of the serotype antigen and its anatomic origin. The serotype antigen proved to be a very high molecular weight lipoprotein-lipopolysaccharide complex. The serotype antigen is located on the surface of the outer membrane. Furthermore, evidence will be provided that, in the case of type 2, the major outer membrane protein contains the serotype antigen determinant. The isolated serotype antigen was an effective immunogen producing high titered type-specific bactericidal antisera in rabbits.

## *Materials and Methods*

*Bacterial Strains and Growth Conditions.*—The group B meningococcal strains used for this investigation were characterized by Frasch and Chapman (6). Trypticase soy agar (TSY agar)[1] (Baltimore Biological Laboratories, Cockeysville, Md.) containing 1% yeast extract (Difco Laboratories, Detroit, Mich.) was the plating medium used for these studies. Large masses of cells were obtained using an 80 liter Biogen Fermenter (American Sterilizer Co., Erie, Pa.). 40 liters of trypticase soy broth (TSB; Difco Laboratories, Detroit, Mich.) were placed in the Biogen, sterilized 15 min at 125°C, quickly cooled, and inoculated with 2 liters of an overnight growth in TSB. Over 200 g wet weight of cells were routinely harvested 5–6 h later using a Sharples centrifuge (Sharples-Stokes Div., Pennwalt Corp., Warminster, Pa.).

*Isolation of Serotype Antigen.*—Cells grown overnight in TSB or those obtained from the fermenter were washed once with 0.15 M NaCl and resuspended in 200 ml 0.2 M LiCl/10 g wet weight cells. They were extracted at 45°C for 2 h. Glass beads (6-mm diameter) were added to maintain a uniform cell suspension during agitation on a gyratory shaker. The extract was obtained by centrifugation at 12,000 *g* at 4°C for 20 min. The extract was then centrifuged 30,000 *g* at 4°C for 40 min to pellet any large membraneous material.

The serotype antigen (STA) could be obtained from the 30,000 *g* supernate by either gel filtration on Sepharose 6B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) or by ultracentrifugation. For recovery by centrifugation the 30,000 *g* supernate was centrifuged at 100,000 *g* for 2 h. The resulting pellet was washed twice in distilled water and repelleted by centrifugation at 100,000 *g*, then resuspended in either distilled water or saline, and finally centrifuged at 2,000 *g* for 15 min to sediment any debris. To the opalescent STA solution $NaN_3$ was added to a concentration of 0.02% (wt/vol). If the STA was to be obtained by gel filtration the 30,000 *g* supernate was adjusted to 1 mM $MgCl_2$ and treated with approximately 10 μg/ml each of bovine pancreatic deoxyribonuclease and ribonuclease (Worthington Biochemical Corp., Freehold, N. J.) for 15 min at 37°C. The nuclease-treated 30,000 *g* supernate was dialyzed against 18 liters of distilled water for 24 h at 4°C, concentrated on a Diaflo PM-10 membrane (Amicon Corp., Lexington, Mass.), and then chromatographed on Sepharose 6B

[1] *Abbreviations used in this paper:* SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; STA, serotype antigen; TSB, trypticase soy broth; TSY agar, trypticase soy agar.

equilibrated with 0.15 NaCl in 0.01 M tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.5 (Tris-saline buffer). The STA eluted in the void volume.

The STA could also be extracted with 0.2 M $CaCl_2$, 0.2 M LiCl, or 0.2 M LiCl with 0.02 M (ethylenedinitrilo)-tetra acetic acid (EDTA). In the case of the LiCl-EDTA extraction, no nuclease treatment was done.

*Preparation of Outer Membrane.*—Spheroplasts of different group B meningococcal strains were obtained by treatment with hen egg lysozyme (Worthington Biochemical Corp.) according to the methods of Hill et al. (18) and Birdsell and Cota-Robles (2). Spheroplast membranes were obtained by rapid 10-fold dilution of the spheroplasts in cold 5 mM $MgCl_2$ followed by treatment with 10 $\mu$g/ml each of deoxyribonuclease and ribonuclease for 15 min at 37°C to reduce viscosity. The membranes were recovered by centrifugation at 30,000 *g* for 40 min, washed once with 0.01 M Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$, and resuspended in 0.01 M Tris-HCl, containing 0.01 M EDTA (Tris-EDTA buffer), pH 7.5. The suspension was centrifuged at 12,000 *g* for 15 min and any sediment discarded. The membranes were then layered onto a 30–60% (wt/wt) continuous sucrose density gradient containing Tris-EDTA buffer and centrifuged at 250,000 *g* in an SW 50.1 swinging bucket rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 16–18 h. The denser, outer membrane banded in the middle of the tube at a density of 1.21 g/cm$^3$ and was recovered either from the top or by puncturing the side of the tube with a hypodermic needle. The outer membrane was then resuspended in the Tris-EDTA buffer and pelleted by centrifugation at 100,000 *g* for 90 min. The pellet was washed in the Tris-EDTA buffer twice and suspended in distilled water containing 0.02% $NaN_3$ (wt/vol).

*Lipopolysaccharide Extraction.*—Approximately 10 g wet weight of cells obtained from overnight growth in TSB were suspended in 100 ml of distilled water followed by addition of 100 ml of 90% liquid phenol. The mixture was placed in a Sorvall Omnimixer (Ivan Sorvall, Inc., Newtown, Conn.) and extracted at 70°C for 20 min, cooled in an ice bath, and centrifuged 2,000 *g* at 4°C for 20 min. The aqueous phase was recovered taking care to avoid the interfacial layer. 100 mg of sodium acetate and 2 vol of cold acetone (−20°C) were added. The precipitate was collected and washed once with cold acetone. The lipopolysaccharide was dissolved in distilled water and dialyzed 24 h against a large volume of distilled water. The lipopolysaccharide was pelleted at 80,000 *g* for 1 h, then resuspended in distilled water, and repelleted several times until no nucleic acid could be detected by absorbance at 260 nm in the supernate. The lipopolysaccharide was resuspended in distilled water, centrifuged at 2,000 *g* for 20 min to remove any debris, and lyophilized.

*Lipid Extraction of the STA.*—The STA was extracted with chloroform methanol (2:1, vol/vol) when protein denaturation was not important. The preferred method of lipid removal was the *n*-butanol delipidation procedure of Morton (29).

*Detergent Solubilization of STA and Outer Membrane.*—For Triton X-100 solubilization, the outer membrane or STA was suspended in 20 mM EDTA, pH 7.5, to a protein concentration of 1–5 mg/ml as determined by the method of Lowry et al. (25). Triton X-100 was added to a concentration of 5% (vol/vol). After incubation for 30–60 min at 23°C the soluble fraction was obtained by centrifugation at 100,000 *g* for 90 min. The detergent was removed by extracting the soluble fraction twice with an equal volume of *n*-butanol at 0°C for 10 min. After centrifugation at 6,000 *g* for 10 min, the upper butanol layer was discarded. After butanol treatment the aqueous phase was dialyzed against distilled water containing 5 mM EDTA. The butanol treatment removed most detectable Triton X-100.

*Radiolabeling of Proteins.*—Proteins were labeled extrinsically with $^{125}I$ by the chloramine-T method of Greenwood et al. (17). Proteins were labeled intrinsically using the uniformly labeled $^3H$ or $^{14}C$ amino acids, L-leucine and L-tyrosine (New England Nuclear, Boston, Mass.). The labeling medium consisted of a modification of the defined medium described by Frantz (5). We modified the medium by replacing the dibasic sodium phosphate with 5 g/liter

of sodium β-glycerophosphate, omitting the glucose, and by adding 0.16% (wt/vol) yeast extract (Difco Laboratories). Approximately 8 μCi of L-[$^3$H]leucine and L-[$^3$H]tyrosine or 4 μCi of L-[$^{14}$C]leucine and L-[$^{14}$C]tyrosine were added to 100 ml of the modified Frantz medium. The medium was then heavily inoculated using a 4–5 h growth from one TSY-agar plate suspended in 0.15 M NaCl. The organisms were grown overnight on a gyratory shaker at 37°C. The labeled STA was isolated from twice washed cells by methods described above.

*Polyacrylamide Gel Electrophoresis.*—Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on radioactive samples in 6-mm × 120-mm glass tubes using the method of Weber and Osborn (39). The samples were heated for 1 min in 1% SDS (wt/vol) and 1% (vol/vol) 2-mercaptoethanol before electrophoresis. After electrophoresis the gels were cut into 1-mm sections with a Gilson Autogel Slicer (Gilson Medical Electronics, Inc., Middleton, Wis.). The $^{125}$I-labeled fractions were counted in a Packard gamma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The $^{14}$C- or $^3$H-labeled fractions were counted in a Packard Tri-Carb scintillation spectrometer using Hydromix scintillation cocktail (York Research Corporation, New Hyde Park, N. Y.).

To compare different preparations of several closely related proteins the slab gel apparatus of Maizel (26) with a gel thickness of 1.5 mm was used. The gel and other conditions remained the same as for the tube gels. The following molecular weight markers were used: bovine serum albumin, egg white albumin, pepsin, trypsin, and myoglobin. After electrophoresis the gel slabs were fixed and the SDS leached out using 5% TCA. They were then stained with a 1:20 dilution of 1% aqueous Coomassie Brilliant Blue (Sigma Chemical Co., St. Louis, Mo.) in methanol-acetic acid-water (5:1:4) for 5–8 h, and destained in 7% acetic acid containing 5% methanol.

*Antisera.*—The type-specific antisera used in these studies were prepared as described by Frasch and Chapman (6, 7). Hyperimmune sera were also prepared by thrice weekly intravenous injections of $5 \times 10^8$ viable organisms over a period of several months with rest periods of 1 wk after 3 wk of injections. The isolated STA was used to prepare antisera in large New Zealand red rabbits. 1 mg of STA in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.) was injected into several subcutaneous sites over the scapular region. The rabbits were bled from the ear artery 4 and 6 wk later at which time a second 1 mg dose was given in Freund's incomplete adjuvant. The rabbits were bled 1 and 2 wk later.

*Serological Methods.*—Antisera were tested for direct bactericidal activity by the microassay previously described (6). Agar gel double-diffusion, capillary precipitin, immunoelectrophoretic methods (7, 9) and the bactericidal inhibition technique (8) were also used.

## RESULTS

*Purification and Immunological Properties of the Isolated STA.*—A clear understanding of the term STA is essential to the subsequent development of this paper. It denotes the high molecular weight antigen extractable into aqueous solutions. The STAs contain the antigenic determinant(s) that confer type specificity which was originally determined by the direct bactericidal assay using different group B antisera (6). The extracted STA is characterized by a distinctive curved precipitin band in agar gel double diffusion close to the antigen well that occurs only with the homologous serotype antiserum (7). Frasch and Chapman (7, 9) found that the STAs were extractable from group B meningococcal cells by heating for short periods of time at 100°C in weak acids or saline. Some STAs were found more heat labile and 100°C tended to destroy all STAs when extraction times were extended. A gentler extraction

method was therefore developed. The STAs of all strains tested could be extracted by heating the cells for 2–3 h at 45°C in 0.2 M solutions of lithium chloride, sodium chloride, or calcium chloride. Different extraction methods were compared for yield of $^{14}C$ intrinsically labeled STA (Table I).

For most of these studies, the STAs were extracted by either 0.2 M $LiCl_2$ or 0.2 M $CaCl_2$ . The LiCl extract was found most suitable for gel filtration studies. The $CaCl_2$ extract contained more STA than the other extracts; however, concentration of $CaCl_2$ extracts resulted in precipitation of the STA along with many other proteins, apparently as a result of reaggregation of the membrane components. The STA was recovered from unconcentrated $CaCl_2$ extracts by ultracentrifugation.

TABLE I

*Extraction of $^{14}C$-Labeled STA by Different Methods at 45°C for 2 h*

| Extraction method | CPM extracted | |
|---|---|---|
| | Percent of total incorporated* | Percent as STA‡ |
| 0.2 M LiCl | 6.5 | 23.6 |
| 0.2 M LiCl + 20 mM EDTA | 6.5 | 27.2 |
| 0.2 M NaCl | 7.0 | 21.0 |
| 0.1 M Tris-saline pH 7.5 | 7.9 | 20.9 |
| 0.2 M $CaCl_2$ | 6.7 | 40.7 |

*The total amount of $^{14}C$-labeled amino acids incorporated was determined on a sample of resuspended cell pellet after washing the cells twice.

‡Percent of total counts extracted sedimented at 100,000 *g*.

When salt extracts of several serotype strains were chromatographed on Sepharose 6B in Tris-saline buffer two distinct protein peaks were resolved; one at the void volume and the other near the bed volume. The void volume peak contained the STA as determined by agar gel double diffusion against hyperimmune whole cell antiserum (Fig. 1 A). The STA formed a single precipitin line close to the antigen well. The remainder of antigenic material eluted in the second Sepharose 6B peak. Based on the Sepharose 6B elution data, the estimated mol wt of the STA was >4,000,000 daltons.

The large size of the STA made possible the use of differential centrifugation to isolate the STA. A LiCl extract was centrifuged at 12,000 *g* twice to sediment intact cells. The 12,000 *g* supernate was centrifuged at 30,000 *g* to pellet large membrane fragments. The 30,000 *g* supernate was centrifuged at 100,000 *g* for 2 h and the STA was recovered as a transparent, brownish, gel-like pellet. The STA was dissolved in either saline or distilled water producing an opalescent solution. When the 100,000 *g* supernate and the resuspended STA pellet were tested against hyperimmune antisera by agar gel double diffusion (Fig. 1 A),

the STA line formed only against the 100,000 *g* pellet material and formed a line of identity with that isolated by chromotography on Sepharose 6B.

The salt extracts contain five to six distinct antigens in addition to the STA as determined by immunoelectrophoresis. As shown in Fig. 1 A, these other antigens are retarded by gel filtration on Sepharose 6B and fail to sediment at 100,000 *g*.

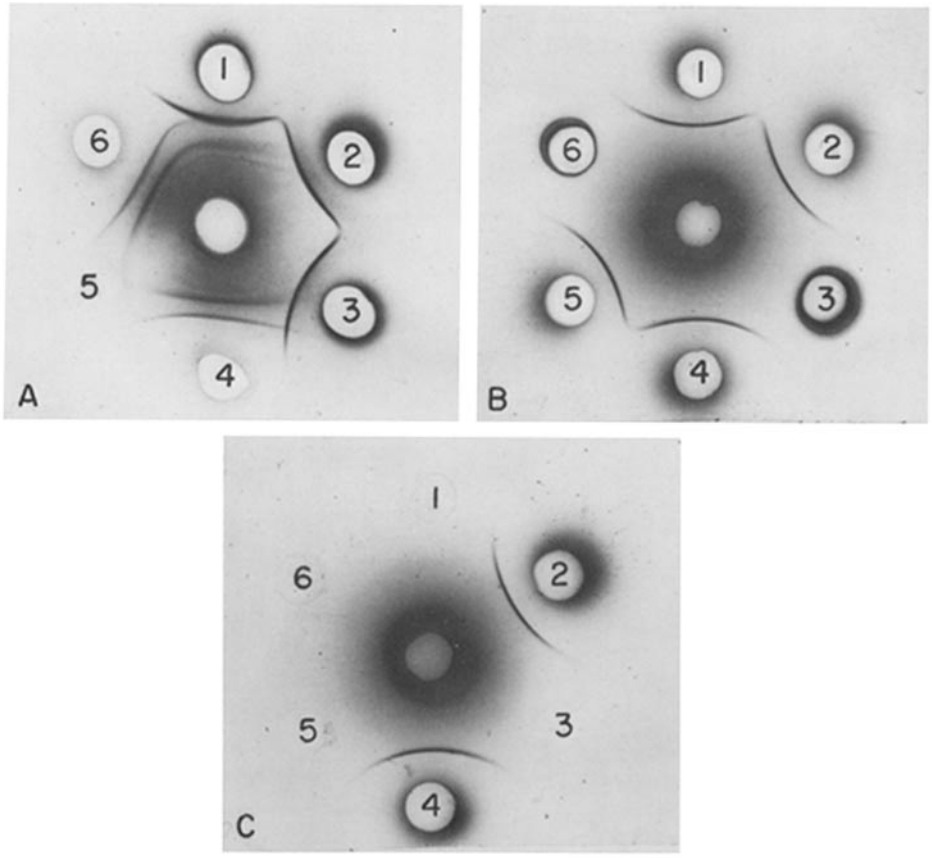


FIG. 1. (A) Isolation of the type 2 STA from a lithium chloride extract. Center well contains whole-cell hyperimmune type 2 antiserum. Peripheral wells contain (1) 0.2 M lithium chloride extract; (2) first fraction eluting in void volume of Sepharose 6B column; (3) 100,000 *g* pellet; (4) 100,000 *g* supernate of extract; (5) blank; and (6) second fraction eluting near bed volume of Sepharose 6B column. (B) The center well contains an antiserum prepared against the isolated type 2 STA. Peripheral wells contain: (1 and 4) purified type 2 STA complex; (2 and 5) lithium chloride extract of the type 2 strain M986; and (3 and 6) the 100,000 *g* supernate of the lithium chloride extract. (C) The center well contains an antiserum prepared against the isolated type 2 STA. Peripheral wells contain lithium chloride extracts of group B strains: (1) M1080 (T1); (2) S-946 (T2); (3) S-3032 (T12); (4) 62VI (T2); (5) B35 (T6); and (6) B17 (T8).

The high molecular weight STA is responsible for the type specificity obtained in the serological typing of group B meningococci by precipitin methods. To show this, saline extracts and the purified STAs from four different strains were tested against two hyperimmune type 2 antisera and against their homologous antisera (Table II). Both hyperimmune sera reacted only with the homologous type 2 antigen of strain M986.

The STA was tested for ability to inhibit the bactericidal activity of a whole-cell type 2 serotyping serum in the bactericidal inhibition technique, and inhibited at a protein concentration of 21 μg/ml. Thus the purification procedures did not impair either the type-specific precipitin or bactericidal inhibitory property of the STA.

TABLE II

*Capillary Precipitin Activity of Two Hyperimmune Serotype 2 Antisera Against Saline Extracts and Purified STAs*

| Strain | Serotype | Antigen | Activity of antiserum | |
|---|---|---|---|---|
| | | | J526R | 881 |
| M986 | 2 | Extract | 4+* | 4+ |
| | | STA | 4+ | 4+ |
| M1080 | 1 | Extract | 4+ | 4+ |
| | | STA | −‡ | + |
| M981 | 4 | Extract | 2+ | 3+ |
| | | STA | − | − |
| M992 | 5 | Extract | 4+ | 4+ |
| | | STA | − | + |

*Strength of precipitin reaction from 1+ (weak) to 4+ (strong).

‡In each case the homologous serotype antiserum reacted strongly with the isolated STA.

Antiserum prepared by immunizing rabbits with purified type 2 STA in Freund's complete adjuvant was examined by agar gel double diffusion against the STA and against saline extracts (Fig. 1 B). The anti-STA serum reacted strongly only with the STA. To determine the degree of serotype 2 specificity, the anti-STA serum was tested in agar gel double diffusion against the 0.2 M LiCl extracts from a number of different group B strains (Fig. 1 C). The antiserum reacted only with the homologous type 2 antigen.

The antisera raised against the purified STA were examined for type-specific bactericidal activity in the microbactericidal assay. The homologous bactericidal titer was >1:1,280. The antisera failed to kill beyond a titer of 1:20 any of the heterologous serotype strains tested. It can be concluded that the STA isolated from the salt extracts was highly immunogenic, eliciting high titered type-specific bactericidal antibody.

*Cellular Origin of the STA.*—The fact that antibody to the STA is able to initiate a complement-mediated bactericidal effect strongly suggests that this antigen is located on the surface of the meningococcus, most likely associated with the outer membrane of the cell envelope. Proof that the STA originated from the outer membrane was provided by SDS-PAGE of the STA and of the isolated outer membrane. A slab gel apparatus was used to obtain a precise comparison of the SDS-PAGE patterns of the STA and of the outer membrane. The outer membrane had a relatively simple SDS-PAGE pattern as opposed to the multibanded pattern of the spheroplast membrane (Fig. 2). The outer

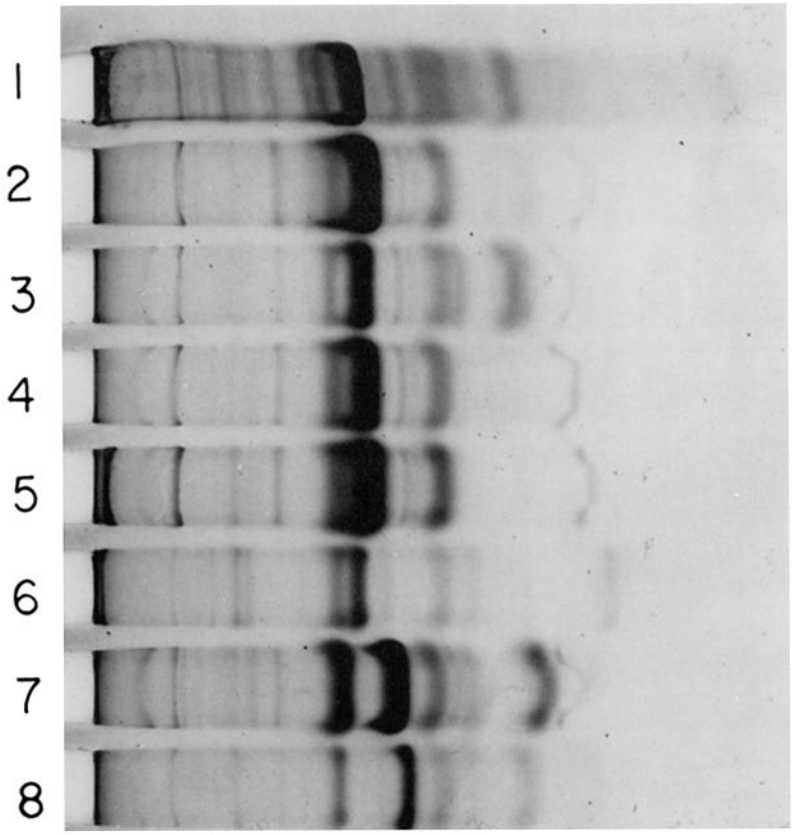


FIG. 2. Comparison of the SDS-PAGE pattern of purified type 2 STA of strain M986 with spheroplast membranes, isolated outer membranes, and STA of other group B strains. The gel patterns represent: (1) spheroplast membranes of M986; (2) outer membranes of M986; (3) STA of M986; (4) STA of B16B6 (T2); (5) STA of 62IV (T2); (6) STA of B37 (nontypable); (7) STA of S-3032 (T12); and (8) STA of B36 (nontypable). The different preparations were solubilized in 1% SDS at 100°C for 1 min and applied to the gel. The gel was stained with 0.025% Coomassie Brilliant Blue after electrophoresis.

membrane has five major protein bands of which one is predominant. By comparison to proteins of known molecular weight, the mol wt of the major bands were determined to be 100,000; 64,000; 50,000; 41,000; and 32,000 daltons. The predominant protein species in both the outer membrane and the STA was the 41,000 dalton protein and represented about 50% of the outer membrane protein. The gel pattern of the STA was almost identical to that of the outer membrane. The STAs from several type 2 strains have been examined by SDS-PAGE and found to be virtually identical to the prototype strain M986 with respect to the major bands (Fig. 2).

The two protein bands associated with all serotype 2 strains thus far examined by SDS-PAGE are the 50,000 and 41,000 dalton proteins. The latter protein is not present in the STAs of other serotypes, but is replaced by proteins of slightly different mobility. The 100,000 dalton protein is common to most group B strains.

Another indication that the STA originated from the outer membrane was the recovery of the high molecular weight STA from the isolated outer membranes (Fig. 3). The STA was released spontaneously from the purified outer membrane as a result of prolonged storage at 4°C in aqueous solutions. No precipitin line in agar gel double diffusion was evident when fresh membranes were used. However, the STA could be extracted from recently isolated outer membranes by treatment with 5% Triton X-100 in the presence of 20 mM EDTA at room temperature for 30 min. The STA could also be recovered from the recently isolated membranes by salt extraction, but the yield was quite low.

*Identification of the Serotype-Specific Protein within the STA Complex.*—The

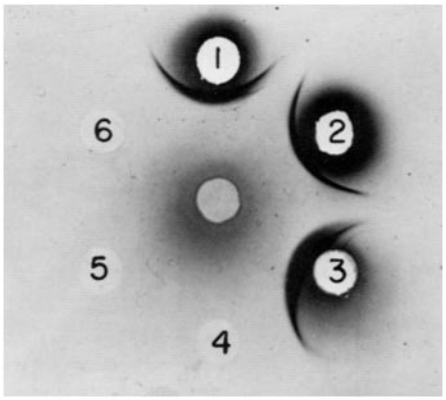


FIG. 3. Presence of type 2 STA derived from isolated outer membranes of strain M986. Center well contains type 2 anti-STA serum. Peripheral wells contain: (1) type 2 STA; (2) 3-mo old outer membrane preparation; (3) fresh outer membranes after treatment with 5% Triton X-100; (4) fresh untreated outer membranes; (5) and hot phenol-water-extracted lipopolysaccharide from strain M986.

STA, based upon SDS-PAGE data, contains one major protein component and approximately four other proteins. The STA also has significant amounts of bound lipopolysaccharide as suggested by its content of 2-Keto-3-deoxyoctonate (manuscript in preparation). Therefore the antigen as recovered from salt extracts is actually a complex moiety derived from the outer membrane. This high molecular weight complex contains the type-specific determinant. Purified lipopolysaccharide does not have type-specific activity (Fig. 3). Each of the prototype strains representing the different serotypes contains a single major type-specific determinant and may contain one or more minor STA determinants (6, 7). For example, the serotype 2 strains may also carry antigens 7 and 10; however, in most of these strains the predominant antigen is the type 2 determinant. Therefore the major antigenic determinant for each serotype is most likely located on a single protein or polypeptide. Based on this assumption, we sought to determine which of the protein components of the STA carries the serotype 2 antigenic determinant.

The relative proportions of the different protein constituents of the high molecular weight type 2 STA is shown in Fig. 4. There are three prominent peaks of which the 41,000 dalton protein is present in the largest amount. Resolution of the STA complex into fractions with different protein composition was accomplished by the following procedure. The STA was treated with 5% Triton X-100 in the presence of 20 mM EDTA for 30 min at room temperature

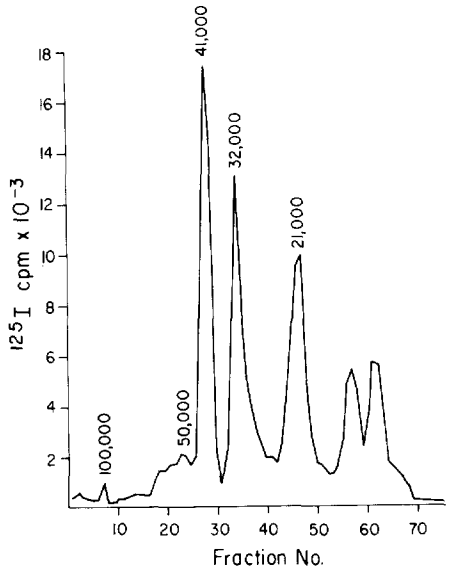


FIG. 4. SDS-PAGE of $^{125}$I-labeled type 2 STA complex. The molecular weights were estimated by comparison with the mobilities of $^{125}$I-labeled standards electrophoresed on separate gels.

and then centrifuged at 150,000 *g* for 2 h to obtain a soluble and an insoluble fraction. The insoluble fraction contained relatively little STA reactivity when examined by agar gel double diffusion and was therefore discarded. The soluble fraction was treated with *n*-butanol to remove the detergent. The Triton X-100 soluble fraction was separated by sucrose gradient density centrifugation into two bands having densities of 1.25 g/cm³ and 1.16 g/cm³ (Fig. 5). These two bands were examined for serotype 2 reactivity using the anti-STA serum and for presence of the other antigens using a whole-cell hyperimmune type 2 serum (Fig. 6). After dissociation of the STA complex by Triton X-100, four anti-

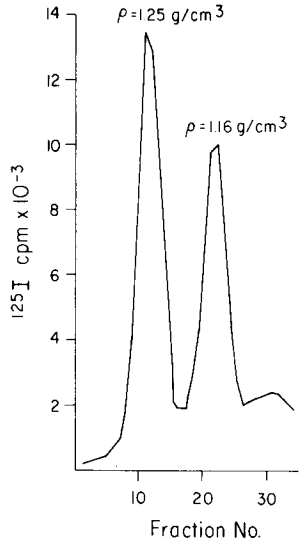


FIG. 5. Sucrose gradient isodensity centrifugation of Triton X-100-dissociated type 2 STA. The STA was treated with 5% Triton X-100 and then most of the detergent was removed by extraction with *n*-butanol in the presence of 50 mM EDTA.

genically distinct fractions were released. However, the type 2 specificity was found almost entirely in the denser fraction as evidenced by the reactions with the type-specific antiserum. In order to determine the relationship between these findings and the protein composition, the two fractions were examined by SDS-PAGE (Fig. 7). The denser band with the serotype 2 reactivity contained the 41,000 dalton protein. The less dense band, devoid of serotype antigen reactivity, did not contain the 41,000 dalton protein, but did contain a significantly higher proportion of the 32,000 dalton protein than the original STA complex. The 21,000 dalton protein was considerably diminished in both fractions while the 28,000 dalton band seen in Fig. 2 is clearly evident in Fig. 7 although not distinguishable in Fig. 4.

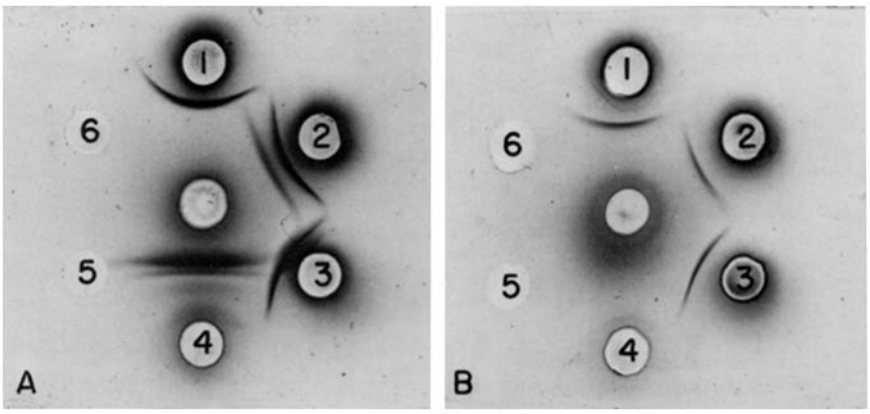


FIG. 6. Localization of the STA reactivity after separation of Triton X-100-dissociated type 2 STA by sucrose gradient isodensity centrifugation (see Fig. 5). Whole-cell hyperimmune type 2 antiserum (A), and anti-STA serum (B) in the center wells were reacted against the following fractions in peripheral wells: (1) STA; (2) Triton X-100-treated STA, unseparated; (3) dense peak from sucrose gradient; (4) light peak from sucrose gradient (concentrated); and (5 and 6) blank.

## DISCUSSION

The STA was originally defined on the basis of strain specificity found in immune bactericidal antisera prepared against several group B strains (6) and group C strains (10). In the case of group B, each serologically distinct strain was assigned a numerical STA designation. The term STA was applied before the physicochemical properties of the antigen were known. The antigens responsible for the type-specific bactericidal activity proved extractable from the cells by either 0.017 M HCl or saline at 100°C (7). However, less severe methods of extraction were required if one wished to study relatively undenatured protein. Our present method is to extract the cells with 0.2 M LiCl or 0.2 M $CaCl_2$ at 45°C for 2 h. The STA is also released at 37°C, but requires 4 h of heating for equivalent release. This mild extraction method does not cause significant lysis of organisms, as revealed by gram stain. Published electron micrographs (43) show that meningococci remain largely intact after similar extraction, but the outer membrane appears to be partially stripped away. Apparent reaggregation of the extracted membrane proteins as evidenced by precipitation often resulted during concentration of the 30,000 *g* supernate of a salt extract or if the extract was dialyzed against distilled water, and was especially evident after $CaCl_2$ extraction. Reaggregation of the membrane material is dependent upon the divalent cation concentration (31) and others working with *Escherichia coli* (3) and Mycoplasma (21) obtained reaggregated membranes after dialysis of solubilized membrane components against a dilute buffer in the presence of divalent cations.

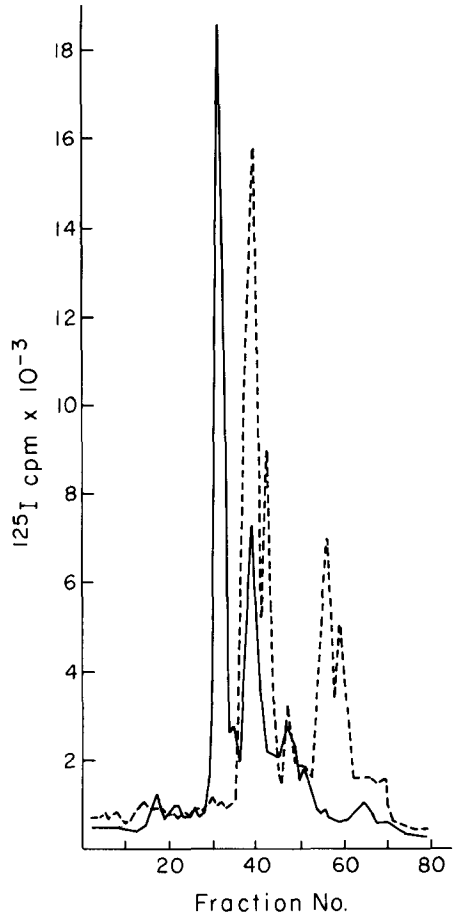


FIG. 7. SDS-PAGE of the dense and light protein fractions recovered from a sucrose gradient after isodensity centrifugation. The STA was labeled with $^{125}$I and then treated with 5% Triton X-100 before application onto the gradient. The dense fraction ($\rho$ = 1.25 g/cm$^3$) is depicted by the solid line and the light fraction ($\rho$ = 1.16 g/cm$^3$) by the broken line.

The outer membrane proved to be the site of origin of the STA. This was evident from the fact that the outer membrane and STA had almost identical SDS-PAGE patterns. Outer membranes, as isolated from lysozyme-EDTA spheroplasts, do not represent the native outer membrane since EDTA causes release of surface components. Wolf-Watz et al. (41) used lysozyme-EDTA treatment to isolate outer membrane fragments from *E. coli*. We found that during lysozyme-EDTA treatment a significant amount of STA was released into the spheroplasting medium. This could therefore account for the poor yield of STA upon extraction of isolated membranes. The STA was easily

extracted from the intact cells, yet poorly from the recently isolated outer membranes, although known to be present in the outer membrane preparations by SDS-PAGE and immunologically. This suggested that the STA may well exist in two forms, one of which is part of a loosely bound surface component. Devoe and Gilchrist (4) observed that cell wall surface blebs are released by group B meningococci during in vitro growth.

A single major outer membrane protein has been reported for several gram-negative organisms (18, 20, 33). Schnaitman (33) among others reported for *E. coli* a single major outer membrane protein with a mol wt of 42,000 daltons. However, this major protein was later found to be resolvable into three protein bands by the SDS-PAGE method of Bragg and Hou (3, 35, 36). Schnaitman found that these three protein bands represented three different major polypeptides, each of which has a mol wt of about 42,000 daltons (37, 38). The type 2 STA was examined by SDS-PAGE using the method of Weber and Osborn (39) and the SDS-PAGE pattern was almost identical to that of the isolated outer membrane. In both there was a single major protein with a mol wt of approximately 41,000 daltons. This 41,000 dalton major protein band may contain more than one polypeptide; however, we observed no alteration in the gel pattern of the major protein when the STA was subjected to SDS-PAGE using the method of Bragg and Hou (3) or the Tris-glycine method of Maizel (26).

Since the STA pattern in SDS-PAGE is very similar to the outer membrane, we examined the STAs of several different group B strains. In each case, there was one or two major protein bands. The differences in the migration of the major proteins on the SDS gels suggests that each of the antigenically distinct serotypes contain a distinct major outer membrane protein. We examined several serotype 2 strains and found almost identical gel patterns. In addition, recent experiments in our laboratory have shown the type 2 antigen of group C meningococci (22, 43) to be identical on SDS-PAGE to that of the group B type 2 STA. However, when we attempted to serotype strains on the basis of the SDS-PAGE pattern of their STAs the diversity of patterns hindered any simple division of serotypes. Comparison of SDS-PAGE patterns can show whether two or more strains believed related are actually identical. The diversity of proteins found in the outer membranes of different group B meningococcal strains was rather unexpected. Such a diversity of antigens probably is of some protective value to the organism.

Hitherto, whole-cell vaccines have been used for production of serotyping antisera (6, 7, 10). Since crude saline extracts (7) contain several nonspecific antigens as well as the STA, interpretation of capillary precipitin results was difficult in the absence of strong positive reactions because of the minor nonspecific reactions. Also, in the case of group C, it was necessary to remove group-specific antibody due to its interference with the serotyping method (10). Use of the isolated STA in the serotyping assay has eliminated most of the problems arising from nonspecific cross-reactions. In the case of type 2, the

nonspecific cross-reactions have been further minimized by preparing high titered type-specific antisera against the STA.

Isolation of the STA by gentle methods will permit investigations of its use as a meningococcal vaccine. Group B meningococci remain an important cause of meningococcal disease in the United States and abroad, yet no effective group B vaccine exists. The purified group B polysaccharide appears to be nonimmunogenic, whereas the protein STAs are highly immunogenic. The protective antibody against group B infection appears to be directed against the protein antigen. Of the serotypes thus far studied, type 2 is definitely associated with meningococcal disease caused by groups B and C (8, 11). Thus the serotype 2 antigen has potential as a single vaccine, effective against different meningococcal groups.

## SUMMARY

Meningococcal groups B and C have been subdivided into a series of serotypes based upon the antigenic specificity of protein serotype antigens (STA). The purpose of these studies was to obtain the STA by gentle methods and determine its anatomic location in the meningococcal cell. The STA was extracted from group B meningococcal strains by either 0.2 M LiCl or 0.2 M $CaCl_2$ and isolated from the extracts by gel filtration on Sepharose 6B or by pelleting the STA by centrifugation at 100,000 *g*. The isolated STA was a lipoprotein-lipopolysaccharide complex with a mol wt of approximately $4 \times 10^6$ daltons. Antisera prepared against the type 2 STA were bactericidal only for homologous serotype strains. The STA proved to be a constituent of the outer membrane of the cell envelope. This was shown by SDS-polyacrylamide gel electrophoresis (PAGE) of the isolated outer membrane and of the purified STA. The type 2 STA complex contains three principal proteins, one of which is predominant with a mol wt of 41,000 daltons. The type 2 STA was dissociated by Triton X-100 and separated by sucrose gradient isodensity centrifugation into two peaks. The denser peak ($\rho$ = 1.26 g/cm$^3$) contained the majority of the 41,000 dalton major outer membrane protein as shown by SDS-PAGE. This peak also contained the type 2 antigenic determinant. Thus the major outer membrane protein, extracted as part of a lipoprotein-lipopolysaccharide complex, contains the type 2 STA determinant.

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