INFECTION AND IMMUNITY, Apr. 1972, p. 612-621

Vol. 5, No. 4
Printed in U.S.A.

Characterization of Spheroplast Membranes of *Neisseria meningitidis* Group B

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Received for publication 23 November 1971

Spheroplast membranes (spheroplast envelopes) of strain 2091 of group B *Neisseria meningitidis* were prepared by a procedure that included lysozyme treatment of the cells and osmotic lysis of the resulting spheroplasts. Electron microscopy revealed that the membranes consisted of two unit layers, generally parallel to each other. The membrane preparation migrated as a single component in a 40 to 70% sucrose gradient and consisted of 62% protein, 28% lipid, 9% ribonucleic acid, small amounts of carbohydrate, hexosamine, and deoxyribonucleic acid. When 1 or 10 μg (dry weight) was injected intravenously into rabbits, a mild pyrogenic reaction was elicited. In immunodiffusion tests, immune rabbit serum prepared against spheroplast membranes produced three major precipitin lines, with the homologous antigen solubilized with sodium dodecyl sulfate, and a single line with untreated antigen. The immune serum also reacted with a cell wall antigen, and to a lesser extent with some of the cytoplasmic antigens. Succinate dehydrogenase and reduced nicotinamide adenine dinucleotide (NADH) oxidase activities were found to be associated with the spheroplast membranes. NADH dehydrogenase also was associated with the membranes but was gradually released and recovered in other fractions. Glutamate-oxaloacetate transaminase, glutamate, glucose-6-phosphate, and isocitrate dehydrogenase activities were not found in the membrane preparation. About one-third of these enzymatic activities were recovered in the supernatant fluid after the sedimentation of the spheroplasts and two-thirds were recovered in the cytoplasmic fraction. *N*-acetylneuraminic acid (NAN)-condensing enzyme and cytidine monophosphate-NAN synthesizing enzyme also were identified in this organism. These enzymes were not associated with the membranes and were recovered from extracts from whole cells, spheroplasts, or cells exposed to osmotic shock, as well as from spheroplast supernatant and shock fluids. It is concluded that the spheroplast membranes of the strain of meningococci used in these studies are typical of those recovered from gram-negative bacteria.

Much of the recent interest in *Neisseria meningitidis* has been directed toward the development of a vaccine for the prevention of meningococcal disease. Extracellular polysaccharides derived from the medium of growing cultures of group A and C meningococci stimulate excellent group-specific antibody responses in man (13-15, 24, 25, 34). Group C polysaccharide has been widely used as a vaccine in field trials, and its efficacy in reducing the carrier rate and in preventing meningococcal group C disease has been demonstrated (1, 8, 14).

Unfortunately, the group B polysaccharide has not been shown to be a satisfactory human immunogen, even though the initial difficulty of isolation of polymers of large molecular weight has been overcome (25). This failure may be explained by the fact that, although group B and C polysaccharides are both homopolymers of sialic acid, their chemical compositions and structures are considerably different. Group C polysaccharide contains both *N*- and *O*-acetyl groups and is susceptible to acid-alcohol hydrolysis but is not cleaved by neuraminidase. Group B polysaccharide, however, has only *N*-acetyl groups and is relatively resistant to chemical agents but is depolymerized enzymatically (25). This last property may be responsible for destruction by mammalian neuraminidases.

These studies were prompted by the belief that the development of a vaccine against group B meningococci required a better understanding not only of the polysaccharide but also of other cell structures and constituents. The experiments described here were initial efforts to characterize the cell membranes and to define the location of

some of the enzymatic activities with respect to the membranes, especially those that were involved in sialic acid synthesis (3, 41). Detailed information is available on the properties of protoplast and spheroplast membranes of several gram-positive and gram-negative organisms (20, 36) and on enzymatic activities in the peripheral spaces of some gram-negative bacteria (16, 17). Salton (35) suggested that some of the potentially antigenic components of the membranes are masked in the intact structures. The same may be true of some other fractions, especially those located in the peripheral spaces. The development of methods for the isolation of these components is a necessary step in the determination of their immunogenic value.

The results obtained thus far clearly indicate that the cell membranes and enzymatic activities of group B meningococci can be well characterized by methods successfully employed for *Escherichia coli* and other gram-negative bacteria and provide useful information for several avenues of further investigation.

MATERIALS AND METHODS

Organism and growth conditions. The 2091 strain of group B *N. meningitidis* used in this study has been described (18). The growth from a Mueller-Hinton agar slant (Difco), incubated for 12 to 14 hr at 37 C in a humid atmosphere of 5% CO_2 in air, was suspended in 5 ml of tryptic soy broth (TSB, Difco); 0.5-ml samples were used for the inoculation of two 1-liter flasks, each containing 250 ml of prewarmed TSB. The cultures were shaken for 10 hr at 37 C, diluted 1:3 with fresh, prewarmed TSB, distributed in 250-ml samples in four 1-liter flasks, and incubated for an additional 60 to 90 min until optical densities (OD) of 0.6 to 0.7 at 540 nm were reached.

Preparation of spheroplast membranes and cell-free extracts. Cells from 1 liter of culture medium were harvested by centrifugation at 16,000 × *g* for 10 min, washed twice in 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 7.5, resuspended in 200 ml of 0.15 M Tris-hydrochloride containing 20% sucrose (w/v), and placed in a shaking incubator at 37 C. Disodium ethylenediaminetetraacetate (EDTA) and crystalline egg white lysozyme (Calbiochem) with final concentration of 5 mM and 100 μg/ml, respectively, were added. Spheroplast formation, virtually complete in 60 to 75 min, was determined by the reduction in OD at 540 nm from approximately 0.45 to 0.02 of samples diluted 1:10 in distilled water. The spheroplasts were harvested by centrifugation at 13,000 × *g* for 20 min and lysed by quick suspension in cold 0.02 M Tris-hydrochloride containing 0.01 M $MgCl_2$ (Tris-Mg), aided by a 40-ml Dounce tissue grinder (Kontes Glass Co., Vineland, N.J.), and rapid dilution to 300 ml in this fluid. Crystalline deoxyribonuclease (Calbiochem) was added to a final concentration of 100 μg/ml, and the mixture was incubated at 37 C for 15 min with occasional gentle agitation. The flask was quickly chilled in an ice-water bath, and all subsequent steps were carried out at 0 to 4 C. The spheroplast membranes were harvested by centrifugation at 20,000 × *g* for 30 min, washed four times with Tris-Mg, and resuspended in 15 ml of the same buffer. These and subsequent resuspension were made with the aid of 7-ml Dounce homogenizers. The membrane suspension, in 5-ml samples, was layered on 25 ml of 70% sucrose (prepared in Tris-Mg) and centrifuged for 90 min at 24,500 rev/min in an SW25.1 rotor of a Spinco model L ultracentrifuge. The band which remained at the top of the sucrose was removed, diluted with Tris-Mg, and sedimented by centrifugation at 30,000 × *g* for 40 min. It was resuspended in Tris-Mg, washed twice, resuspended in 10 ml of the same buffer, dispensed into 1-ml samples, and stored at −70 C.

Spheroplast supernatant fluid, when needed, was dialyzed for 24 hr at 4 C in two changes of Tris-Mg, concentrated to 25 ml with a Diaflo filtration apparatus (Amicon Corp., Lexington, Mass.) equipped with a UM-10 membrane filter, lyophilized to dryness, reconstituted to 5 or 10 ml in distilled water, and stored in 1-ml samples at −70 C. The cytoplasmic fraction obtained from the osmotic lysis of the spheroplasts was similarly concentrated without prior dialysis.

Crude cell extracts were prepared with a French pressure cell (American Instrument Co., Silver Spring, Md.) as previously described by Hill (18). Unbroken cells were eliminated by three cycles of centrifugation at 3,000 × *g* for 5 min. Purified cell walls were extracted by using the procedure of Vedros and Hill (40).

The osmotic shock procedure was carried out as described by Neu and Heppel (33) and Heppel (16, 17). The shock fluid was concentrated by lyophilization.

Electron microscopy. Sedimented *N. meningitidis* spheroplast membranes were fixed for 2 hr at 4 C in a 3% solution of glutaraldehyde in 0.065 M potassium phosphate buffer (*p*H 7.2) containing 0.45 mM $CaCl_2$, washed twice with phosphate buffer containing 0.2 M sucrose, fixed in 0.5% osmium tetroxide for 2 hr at 4 C, washed, and exposed to 0.5% uranyl acetate for 2 hr at room temperature. The pellets were embedded in Epoxy resin (32), sectioned with a Porter-Blum microtome (Sorvall Co.) equipped with a diamond knife, stained with both uranyl acetate and lead acetate, and examined in a Siemens Elmiskop IA electron microscope.

Density gradient centrifugation. A continuous 40 to 70% (w/v) sucrose gradient was overlayered with 1 ml of spheroplast membrane suspension and centrifuged for 4 hr at 24,500 rev/min at 4 C in an SW25.1 Spinco rotor. The tubes were pierced from the bottom with a Buchler piercing unit (Buchler Instruments, Fort Lee, N.J.), and 0.6-ml fractions were collected and diluted to 2 ml with Tris-Mg buffer. Their absorbance was determined at 280 nm.

Chemical analyses. Protein was determined by the method of Lowry et al. (26) using Dade Lab-Trol (American Hospital Supply Corp., Miami, Fla.) as a standard. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were extracted by the method of

Schneider (38), except that chloroform-methanol (2:1, v/v) was used for extraction of lipids instead of 95% ethanol. Deoxyribose and ribose were determined by the diphenylamine (23) and orcinol (27) methods, respectively. Carbohydrate was determined by the α-naphthol reaction (42). Total extractable lipid was determined by the method of Bligh and Dyer (5), as modified by Kates (21). For the determination of hexosamine, samples were hydrolyzed with 4 N HCl for 4 hr at 100 C, and the HCl was removed by evaporation with flowing nitrogen under vacuum. The dried residue was reconstituted with distilled water and assayed by the method of Cessi and Piliego (6), with glucosamine hydrochloride used as the standard.

Dry weights. The membrane preparations were dried at 60 C to a constant weight. The weights of buffer controls dried under the same conditions were subtracted.

Immunodiffusion. Rabbit antiserum to spheroplast membranes was prepared by the procedure of Fukui et al. (12). Immunodiffusion tests were performed with plastic templates mounted on standard microscope slides covered with 0.75% agarose as described by Fink et al. (10). The slides were incubated 16 to 18 hr in a humidified chamber at room temperature. After removal of the templates, the slides were rinsed in 0.9% NaCl, and the precipitin lines were photographed with a Cordis immunodiffusion camera (Cordis Laboratories, Miami, Fla.).

Animal toxicity. The pyrogenicity of the membrane preparation was tested by injecting rabbits intravenously with samples diluted in 0.5-ml volumes of pyrogen-free saline and by periodic monitoring of their rectal temperatures. Control groups received *Escherichia coli* lipopolysaccharide (Difco) or physiological saline.

Enzyme assays. Dehydrogenase, oxidase, and transaminase activities were assayed by measurement of changes in absorbance of oxidized and reduced nicotinamide adenine dinucleotides (NAD and NADH, respectively) and nicotinamide adenine dinucleotide phosphate (NADP) at 340 nm or 2,6-dichlorophenol indophenol (DCPIP) at 600 nm in a Beckman Kintrac VIIA or Acta III spectrophotometer. The assays were performed at room temperature (23 C) with cuvettes containing total volumes of 3 ml consisting of the reagents listed below, 0.1 ml of enzyme preparation, and, unless otherwise indicated, 0.05 M potassium phosphate buffer, *p*H 7.4. Glutamate, glucose-6-phosphate, and isocitrate dehydrogenase reactions were started by the addition of substrate and the other reactions by the addition of the enzyme preparation.

The following reagents were used: for glutamate dehydrogenase, NADP (0.3 μmole) and potassium glutamate (20 μmoles); for glucose-6-phosphate or isocitrate dehyrogenase, $MgCl_2$ (10 μmoles), NADP (0.3 μmole), and glucose-6-phosphate (20 μmoles) or trisodium isocitrate (10 μmoles); for glutamate-oxaloacetate transaminase, 2.8 ml of GOT reagent (GOT Stat-Pack, Calbiochem) and 3 μmoles of NaCN; for NADH oxidase (9), NADH (0.375 μmole); for succinate dehydrogenase (9, 22), DCPIP (0.09 μmole), NaCN (3 μmoles), and succinate (40 μmoles), followed by 1.3 μmoles of phenazine methosulfate 10 min later; for NADH dehydrogenase (2), NaCN (3 μmoles), DCPIP (0.09 μmole), and NADH (0.125 μmole).

The rate of the reaction was calculated during the period of linear change of OD, usually between 20 and 40 sec. For calculation of activities, the values employed were: $E^{mM}_{340nm} = 6.22$ for NADH or NADPH and $E^{M}_{600nm} = 18.8$ for DCPIP. One unit of enzyme activity is defined as the amount required to oxidize or reduce 1 μmole of substrate per hour.

Enzymatic activities involved in sialic acid biosynthesis, namely, *N*-acetylneuraminic acid (NAN)-condensing enzyme and cytidine-5'-monophosphate-NAN (CMP-NAN)-synthesizing enzymes, were assayed by the procedures of Blacklow and Warren (3, 41).

The reaction mixtures (0.5 ml) for the NAN-condensing enzyme contained 0.2 ml of enzyme (diluted in Tris-Mg), 1.25 μmoles each of *N*-acetyl-D-mannosamine and phosphoenol pyruvic acid, 12.5 μmoles of reduced glutathione (GSH), and 2.5 μmoles of $MnCl_2$. The various components were prepared immediately before use in 0.25 M Tris-acetate buffer, *p*H 8.3.

CMP-NAN-synthesizing enzyme assay mixtures (0.5 ml) contained: enzyme preparation (in Tris-Mg), 0.2 ml; NAN, 0.25 μmole; cytidine triphosphate, 1.25 μmoles; GSH, 6.25 μmoles; and magnesium acetate, 12.5 μmoles. These components were prepared immediately before use in 0.15 M Tris-acetate buffer, *p*H 8.5.

For both reactions, the test vessels were incubated for 1 hr at 37 C. Control vessels contained the same reagents, but were maintained in an ethanol-dry ice bath for 1 hr.

The amounts of NAN formed or combined with CMP were determined by the periodate-resorcinol procedure of Jourdian et al. (19). In the case of the CMP-NAN synthesizing activity, excess NAN was removed by the addition of 0.05 ml of an aqueous solution of 2 M $NaBH_4$, and excess $NaBH_4$ was removed by the addition of 0.05 ml of acetone. After each addition, the tubes were shaken and maintained at room temperature for 7 min. NAN was disassociated from CMP by incubating the tubes at 80 C for 1 hr after the addition of 0.1 ml of 4 N H_2SO_4. One unit of enzyme is defined as the amount required to form, or bind to CMP, 1 μmole of NAN/hr.

RESULTS AND DISCUSSION

Isolation of spheroplast membranes. Procedures for the production of spheroplast membranes (spheroplast envelopes) used in these studies were similar to those described for other gram-negative bacteria (20, 36). Relatively large amounts of lysozyme (100 μg/ml) were required for quantitative spheroplast formation. Results with smaller amounts (60 μg/ml) were not always satisfactory. A crystalline deoxyribonuclease digestion step was introduced to reduce the viscosity of the preparation, but no attempt was made to remove RNA enzymatically. The membranes were

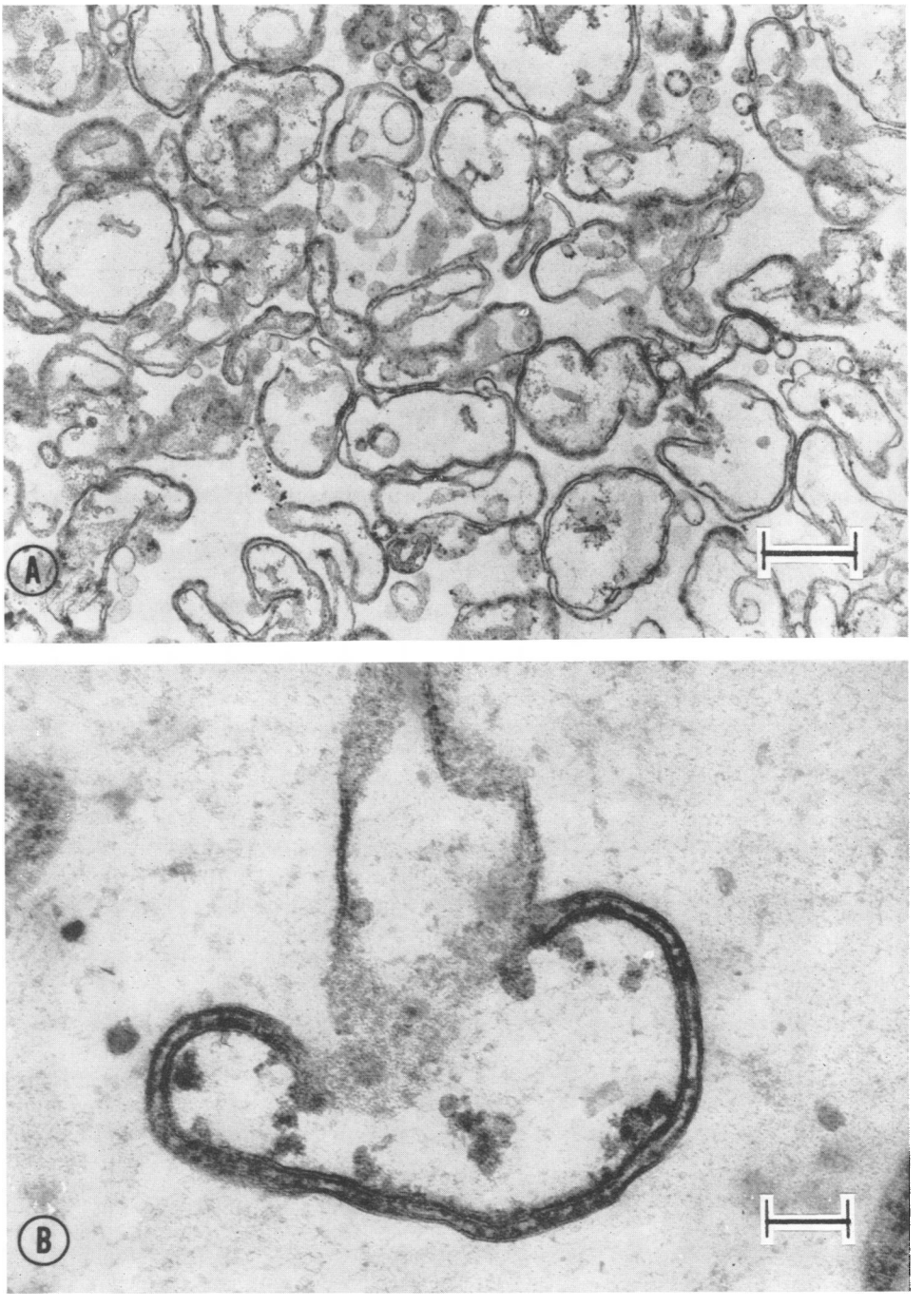

FIG. 1. *Electron micrographs of thin sections of spheroplast membranes. The bar represents 0.5 μm in A and 0.1 μm in B.*

maintained in a relatively high concentration of Mg^{2+} (0.01 M), which also may have contributed to the retention of RNA during the washing procedures (36). Centrifugation of the preparation, over a layer of 70% sucrose, removed about 15 to 25% of the total material that consisted of unlysed spheroplasts and membrane clumps. It is estimated that the final product contained approx-

imately 25% of the total protein of the original suspension of intact cells.

Electron microscopy. In electron micrographs of the spheroplast membranes, it is apparent (lower magnification, Fig. 1A) that most of the nonmembranous material was removed. Some was retained, possibly because extensive enzymatic treatment was avoided. Also apparent is a double unit membrane (Fig. 1B) which was the predominant, if not the exclusive, structure obtained in our experiments. Although a double unit structure is typical of spheroplast membranes or "envelopes" of gram-negative bacteria (36), Kaback (20) reported the isolation of one to six trilaminar membrane layers from certain strains of *E. coli*. However, one strain, *E. coli* ML 308-225, yielded membranes which consisted almost exclusively of single unit layers. Costerton et al. (7) and Martin and MacLeod (29), who used procedures similar to ours, obtained protoplasts and single unit membranes from a marine pseudomonad. It thus appears that, in the strain of *N. meningitidis* used in our experiments, the two layers were not easily separated. However, procedures that have been successful in removing the lipopolysaccharide after the isolation of cell or spheroplast envelopes from other gram-negative bacteria (30, 31, 37) have not yet been applied.

When the sections shown in Fig. 1 were compared with those of whole cells of another strain of *N. meningitidis* (39) or *Veillonella* (4) (our sections of whole cells were entirely comparable and are not shown), the origin of the two layers could be identified. The peptidoglycan layer was presumably digested by lysozyme, leaving the outer membrane and the cytoplasmic membrane separated by space of somewhat variable width. In sections of whole cells, the outer membrane, in striking contrast to the cytoplasmic membrane, appeared to be highly undulated. Some of the undulation of the outer membrane was retained in the spheroplast membranes, but much of it was lost, and the two membranes were often parallel for considerable lengths (Fig. 1).

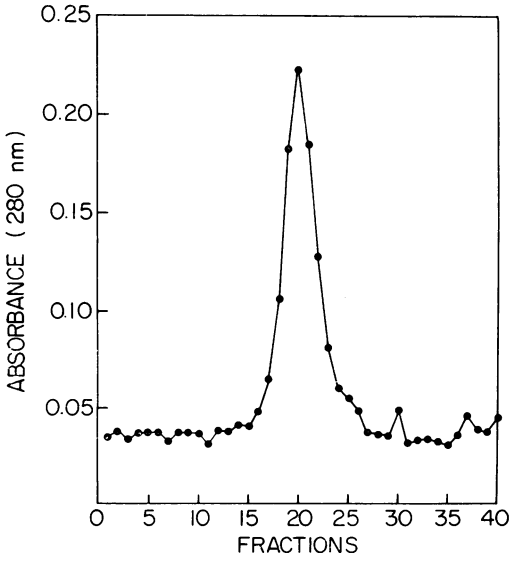

FIG. 2. *Centrifugation of spheroplast membranes, 0.82 mg (dry weight), layered on a 40 to 70% sucrose gradient. Fractions were collected from the bottom of the tube.*

TABLE 1. *Chemical composition of spheroplast membranes*[a]

Constituent	Per cent dry wt (3.6 mg/ml)
Protein	61.9
Lipid	27.5
Nonamino carbohydrate	3.3
Hexosamine	0.9
DNA	0.3
RNA	8.8

[a] Mean of three determinations performed on a pool of 10 preparations.

Physical, chemical, and pyrogenic properties. The spheroplast membrane preparation migrated through a 40 to 70% sucrose gradient essentially as a single component or a closely related family of components. An example of the 280-nm absorption pattern of the gradient fractions is illustrated in Fig. 2. Although a single peak was obtained in all instances, in some cases the region of elevated absorption was slightly wider, and a small amount of 280-nm absorbing material remained at the top of the gradient.

The chemical composition of the spheroplast membranes is shown in Table 1. The concentrations of the two major components, protein and lipid, were similar to those of other spheroplast or cytoplasmic membranes of gram-negative and gram-positive organisms (11, 20, 29, 36). The RNA content was relatively high, but comparable amounts were reported in several instances in other microorganisms (11, 36). The other components were found in low concentration, as expected. The low hexosamine content indicated that much of the peptidoglycan layer was removed by the lysozyme-EDTA treatment.

In an attempt to determine to what extent endotoxin remained associated with our preparations, portions of spheroplast membranes were injected intravenously into rabbits. A 10-μg dose elicited a fever response similar to, but slightly more prolonged than, a 0.05-μg dose of com-

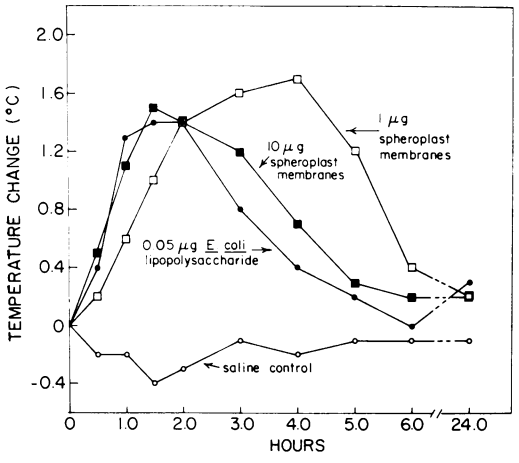

FIG. 3. *Fever response of rabbits after intravenous injections of spheroplast membranes (mean of three rabbits) or control preparations (mean of two rabbits).*

mercial *E. coli* lipopolysaccharide (Fig. 3). The response to a 1-μg dose was elicited with some delay, but fever continued for a somewhat longer period than with the higher dose. Similar results were obtained in two additional, comparable tests (not shown). The fever response was generally longer lasting but less intense than the one elicited by the *E. coli* lipopolysaccharide. Although the significance of the qualitative features of the response is not known, the results are consistent with the morphological evidence (Fig. 1) that our membrane preparations contained lipopolysaccharide and thus represent envelope structures possessing both plasma membranes and external outer membrane.

Antigenic properties. Double-diffusion precipitin reactions occurred between rabbit immune serum to spheroplast membranes and homologous antigen solubilized with three concentrations of sodium dodecyl sulfate (SDS) or used untreated (Fig. 4A). The intensity of the reaction was dependent on the amount of SDS used, but clearest separation of precipitin bands usually was obtained with the intermediate amount of SDS (0.25%, well 2). Three major precipitin bands were obtained in the homologous reaction, but often one or more faint precipitin lines were also seen. When SDS was not used, a single precipitin line was elicited (well 4) which joined the band nearest the antibody well obtained with the antigens solubilized with SDS. The results suggest that a major antigen component of the spheroplast membranes was readily released and diffused rapidly in the agarose gel.

A comparison between the homologous reaction and reactions between the same antiserum and other cell fractions, all solubilized in 0.25% SDS, is shown in Fig. 4A. A strong precipitin reaction was produced with cell wall antigen (wells 2 and 3). This precipitin line joined a

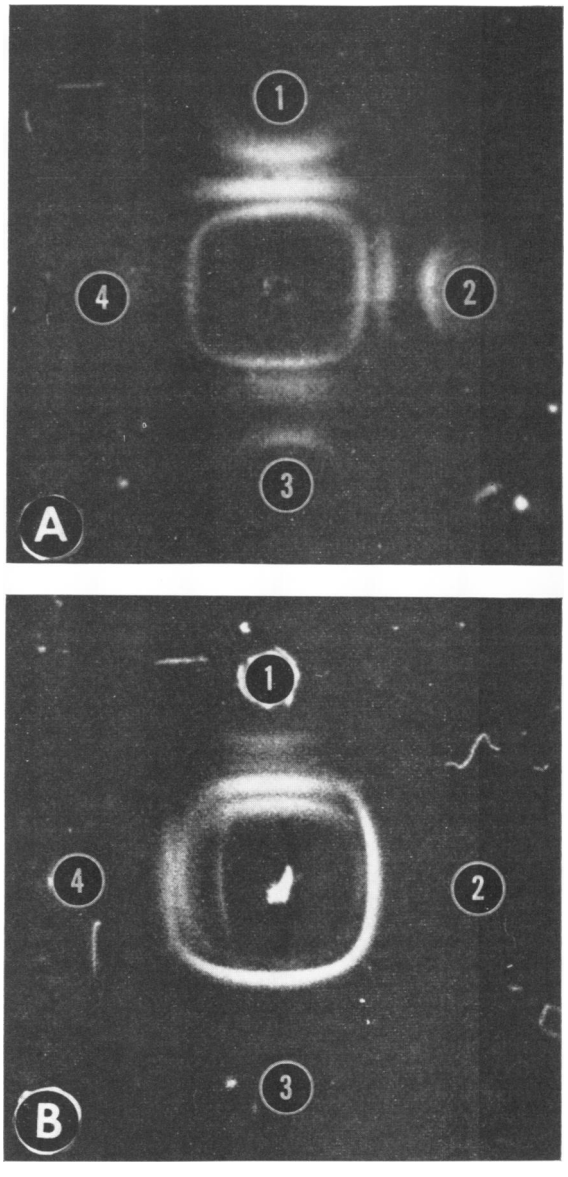

FIG. 4. *Immunodiffusion patterns of rabbit antiserum elicited by spheroplast membranes (in center well) and various cell fractions., A Spheroplast membranes solubilized with 0.5% SDS (well 1), 0.25% SDS (well 2), 0.1% SDS (well 3), and no SDS (well 4). B, Spheroplast membranes (well 1), cell walls (wells 2 and 3), and cytoplasmic fraction (well 4), all solubilized with 0.25% SDS.*

TABLE 2. *Enzymatic activities of cell fractions of Neisseria meningitidis*[a]

Enzymatic activity	Per cent distribution			Total enzyme units[b]
	Spheroplast membranes	Spheroplast supernatant fluid	Cytoplasmic fraction	
Succinate dehydrogenase	100	0	0	46
NADH oxidase	88	0	12	200
NADH dehydrogenase	21	24	55	910
Glutamate-oxaloacetate transaminase	0	39	61	1,110
Glutamate dehydrogenase	0	35	65	200
Glucose-6-phosphate dehydrogenase	0	20	80	1,260
Isocitrate dehydrogenase	0	32	68	210

[a] Data presented are from one experiment, but are representative of data from two additional experiments.

[b] Derived from 2 g (wet weight) of cells.

minor component of the homologous reaction but none of its three major precipitin lines (well 1). Thus, it appears that the spheroplast membrane preparation contained a small amount of cell wall material which was highly antigenic. The cell wall antigen also occasionally elicited a faint precipitin line which joined the most diffusible of the three major precipitin lines formed with spheroplast membranes.

The cytoplasmic fraction (well 4) consisted of the supernatant fluid remaining after the removal of lysed spheroplasts. It was concentrated by membrane filtration and lyophilization but was not subjected to further separation procedures. Two major precipitin lines were elicited in this reaction, one joining a major spheroplast membrane component and one continuing with the cell wall antigen. A third, fainter precipitin line, unrelated to the others, often was seen in close proximity to the antiserum well.

Enzymatic activities of spheroplast membranes and of other cell fractions. Some of the enzymatic activities of the spheroplast membranes are illustrated in Table 2. Also shown are the enzymatic activities of two supernatant fluids collected after sedimentation of the intact spheroplasts and sedimentation of the lysed spheroplasts (cytoplasmic fraction), respectively. EDTA present in the lysozyme-treatment stage possibly contributed to the solubilization of membrane components and therefore may have affected the results presented in Table 2.

Succinate dehydrogenase and NADH oxidase appeared to be closely associated with the spheroplast membranes. NADH dehydrogenase was found in all three fractions, but primarily in the cytoplasmic fraction. This suggests that it was loosely associated with the membranes and was gradually lost during isolation procedures. In fact, NADH dehydrogenase activity was found not only in the supernatant fluids (Table 2) but also in subsequent membrane wash fluids. These results might be correlated with those which indicate that one of the antigenic components of the spheroplast membranes was easily solubilized (Fig. 4A).

Our finding that spheroplast membranes retain, to varying degrees, succinate dehydrogenase, NADH oxidase, and NADH dehydrogenase is in agreement with those of other investigators who have found these three activities in the cytoplasmic membranes of gram-positive (36) and, in some cases, of gram-negative bacteria (11, 37).

Four other enzymatic activities, glutamate-oxaloacetate transaminase, glutamate, glucose-6-phosphate, and isocitrate dehydrogenases, were not associated with the spheroplast membranes. About one-third of these activities were found in the spheroplast supernatant fluid and two-thirds in the cytoplasmic fraction. A similar distribu-

TABLE 3. *Sialic acid-synthesizing activities of cell-free extracts and cell fractions of Neisseria meningitidis*[a]

Enzymatic activity[b]	Cell-free extracts (units)	Cell fractions			
		Spheroplast supernatant fluid (units)	Cytoplasmic fraction (units)	Spheroplast membranes (units)	Total recovery (%)[c]
NAN-condensing enzyme	21.2	3.4	4.6	0.0	38
CMP-NAN-synthesizing enzyme	90.3	4.2	35.6	0.0	44

[a] Cell-free extracts and cell fractions were prepared from identical 1-g (wet weight) samples of cells. Each reaction vessel contained 50 μg of protein. Data are representative of two identical experiments.

[b] Abbreviations: NAN, *N*-acetylneuraminic acid; CMP, cytosine monophosphate.

[c] Units demonstrated with cell-free extracts equal 100.

TABLE 4. *Effect of osmotic shock on the release of sialic acid-synthesizing enzymes*[a]

Determination	Extract from whole untreated cells	Per cent recovery		
		Extract from shocked cells	Shock fluid	Total
Protein	37.4 (mg)	82	17	99
Glucose-6-phosphate dehydrogenase	1,007.0 (units)	72	34	106
NAN-condensing enzyme	21.7 (units)	45	49	94
CMP-NAN-synthesizing enzyme	112.0 (units)	46	8	54

[a] Identical 1-g (wet weight) samples were used for the preparation of cell-free extracts and for fractionation by osmotic shock. Each reaction vessel contained 50 μg of protein. Data are representative of two identical experiments.

tion of glucose-6-phosphate and glutamate dehydrogenases was found in *E. coli* by Malamy and Horecker (28). These enzymes were regarded by the authors as among those that are retained by the spheroplasts.

Of particular interest was the location of the sialic acid-synthesizing enzymes, NAN-condensing enzyme, and CMP-NAN-synthesizing enzyme, previously identified in group C meningococci (3, 41). We demonstrated these two enzymatic activities also in our group B strain (Table 3). The enzymes were detected in whole cell-free extracts, as well as in spheroplast supernatant fluid and cytoplasmic fractions, but not in the washed spheroplast membranes. The activity in whole-cell-free extracts was considerably higher than the combined activity of the two fractions, which suggests that the two enzymes were, in part, inactivated during the relatively long procedures of concentration of the two fractions.

A further attempt was made to determine the location of the two enzymes with respect to the membrane (Table 4). Extracts were prepared from whole cells as in the previous experiment, as well as from an identical sample of cells that had been osmotically shocked (16, 17, 33). The shock fluid was concentrated by lyophilization, and all three preparations were tested for total protein content and for enzymatic activity. The two fractions separated by osmotic shock contained about as much total protein, glucose-6-phosphate dehydrogenase, and NAN-condensing enzyme as the extract from untreated cells. The shock fluid contained about one-sixth of the total protein and one-third of the glucose-6-phosphate dehydrogenase. The concentration of the NAN-condensing enzyme was higher, about half of the total. The results with the CMP-NAN-synthesizing enzyme are more difficult to interpret, because only about half of the activity was recovered in the two fractions and, possibly because of instability under the conditions of concentration, only a low level of activity was found in the shock fluid. Heppel (16, 17) listed enzymes that are released and enzymes that are retained by cells upon osmotic shock or spheroplast formation. If the same criteria are applied to the two sialic acid-synthesizing enzymes, they must be placed, with some reservation, in the category of enzymes that are retained in the cytoplasm.

Relationship of the spheroplast membranes to other cellular components. The morphological, chemical, and enzymatic properties of the spheroplast membranes described above are similar to those of other gram-negative bacteria. When the same isolation procedure is applied to gram-positive organisms, single unit cytoplasmic membranes are usually recovered, but a double unit membrane is the structure generally obtained from gram-negative bacteria (36). However, single unit membranes have been obtained from the gram-negatives with increasing frequency (7, 29, 30, 31, 37), and the application of these methods to meningococci deserves investigation.

It is apparent from these antigenic studies that the spheroplast membranes were not entirely devoid of components of the cell wall and cytoplasm. However, these components were not separated from the membranes by sucrose gradient centrifugation and, thus, they possibly were attached in some manner to the membranes. Conversely, it was evident from antigenic and enzymatic studies that some of the membrane components were gradually lost. Thus, a distinction between functions that are associated with the membranes and those that are not can not be made in every instance and may, in part, depend on the details of the procedure of isolation employed. However, the spheroplast membranes described here are sufficiently well defined to provide a valuable tool for future biochemical and immunological studies.

ACKNOWLEDGMENTS

We are indebted to O. P. Daily and M. H. Powell for the electron microscopy. The excellent technical assistance of R. H. Gwynne is also gratefully acknowledged.

This work was supported by the Bureau of Medicine and

Surgery, Department of the Navy, research task MR041.05.01-0008B6GI and the Office of Naval Research contract NR136-910.

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