HUMAN IMMUNITY TO THE MENINGOCOCCUS

III. Preparation and Immunochemical Properties of the Group A, Group B, and Group C Meningococcal Polysaccharides*

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The species *Neisseria meningitides* has been subdivided by serological methods and the present classification includes Group A, B, C, and D (1). These groups, historically, have caused the majority of meningococcal disease. Furthermore, several additional groups have been described by Slaterus and named X, Y, and Z (2). Evans et al. (3) have described several other serogroups, one of them called Bo. This group is of particular interest since it is very commonly isolated from the nasopharynges of military recruits but only rarely has caused disease. Evans et al. (3) suspected that group Bo was related to the group Y organisms described by Slaterus (2). Branche[1] has recently isolated the polysaccharides responsible for the group specificity of group Bo and group Y organisms, and by serological means has shown that these are indistinguishable.

Relatively little information has been available concerning the immunochemistry of the group-specific meningococcal antigens. The best studied is the group C antigen which was shown by Watson et al. (4) to be mainly a polymer of sialic acid. Scherp and Rake (5) and Kabat et al. (6) have isolated the group A antigen and shown that it contained both phosphorus and nitrogen. Liu et al.[2] have shown that the group A polysaccharide consists mainly of *N*-acetyl mannosamine phosphate which is partially *O*-acetylated. Menzel and Rake (7) found that group B meningococci contained a polysaccharide-peptide complex characteristic of that group. The serological properties resided mainly in the polysaccharide moiety which could be isolated only after tryptic digestion of the antigen.

The present report will describe a new method for the isolation of the menin-

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[1] Branche, W. C. Personal communication.

[2] Liu, T. Y., J. K. Jönsen, and E. C. Gotschlich. Manuscript in preparation.

gococcal group-specific polysaccharides. With this procedure, it was possible to produce highly purified group A and group C polysaccharides having mean molecular weights in excess of 100,000. Furthermore, when this method was applied to group B meningococci, a protein-free polysaccharide was isolated without tryptic digestion. This polysaccharide consisted mainly of sialic acid, but was serologically distinct from the group C antigen. It is not known whether this newly isolated polysaccharide is immunologically related to the polysaccharide-peptide complex which Menzel and Rake isolated (7).

Materials and Methods

Meningococcal Strains.—The majority of the strains used came from the departmental collection. Strains M1027, M2092, M963, M1054, and M1628 came from the collection of Dr. Branham (1) and were kindly donated by Dr. M. Pittman. All lots of group A polysaccharide were prepared from cultures of strain A1, isolated from spinal fluid in Germany in 1965. All lots of C polysaccharide were prepared from strain C11 which was also isolated from spinal fluid in Germany in 1965. Three lots of group B polysaccharide were prepared from cultures of strain B11. This organism was isolated from the spinal fluid during an illness that occurred in 1966 in a member of this laboratory. One lot of group B polysaccharide was prepared from cultures of M2092, a strain isolated in 1948 in Boston, Mass., from spinal fluid (1).

Media and Cultural Conditions.—Mass cultures were performed in the casamino acid medium described by Watson et al. (8). Certified casamino acids were substituted for technical grade material (Difco Laboratories, Detroit, Mich.). Other fluid media used were the defined medium described by Frantz (9) and a medium which consisted of a 1/5 dilution of medium 199 and a 1/10 dilution of the casamino acid medium described by Watson et al. (8).

The organisms, which were preserved either in lyophile or by freezing in a medium consisting of 5% w/v bovine serum albumin and 5% w/v monosodium glutamate (10), were streaked onto Mueller-Hinton agar (11) and incubated at 37°C in a candle jar. The organisms were taken from the agar with sterile cotton swabs, suspended in 50 ml of fluid medium in a 250 ml conical flask, and incubated at 37°C for 3 hr on a rotary platform shaker revolving at about 120 rpm. 5 ml of this culture was transferred into another 50 ml aliquot of fluid medium and allowed to incubate as described for 4 hr. This culture was poured into either 500 ml of media in a 2000 ml conical flask or 1000 ml of media in a 4000 ml flask which were incubated as described until ready for harvesting. The doubling time of meningococci grown under these conditions was approximately 1 hr and the final bacterial density was about 2×10^9 organisms/ml. Many attempts were made to grow meningococci in 30 liter lots of media contained in 40 liter carboys. The inoculum consisted of 1000 ml of an actively growing culture, and aeration was provided by bubbling filtered air. Except in one instance, the organisms grew poorly or not at all under these conditions.

Chemical Reagents.—Technical grade hexadecyl trimethylammonium bromide (Cetavlon) (Eastman Chemical Corp., Rochester, N. Y.) and chloroform N.F. (Mallinckrodt Chemical Works, St. Louis, Mo.) were used. All other chemicals employed were reagent grade.

Preparation of Group-Specific Meningococcal Polysaccharides.—The group-specific polysaccharides do not remain cell-associated in a fully grown culture, and it is therefore necessary to isolate the polysaccharides from the culture fluid. The method of isolation developed in this study employs the cationic detergent Cetavlon to rapidly precipitate the polysaccharides from the whole culture. The group A and C polysaccharides are negatively charged and are therefore precipitated by Cetavlon. This description applies to the preparation of polysaccharide from 20 to 30 liters of culture.

After the purity of each culture was established by examination of a Gram-stained smear, 10% Cetavlon was added to a final concentration of 0.10% w/v. The precipitate was collected by centrifugation in an International centrifuge model B 20 (International Equipment Co., Boston, Mass.), (20,000 *g* for 5 min), or in a Sharples super centrifuge, the supernate being discarded. The precipitate was thoroughly homogenized with 150 ml of water in an Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.), and centrifuged for 10 min at 13,000 *g*.

Dissociation of the Detergent-Polysaccharide Complex.—The precipitate was extracted three or four times with 100 ml aliquots of 0.9 M calcium chloride in the Omnimixer and centrifuged for 15 min at 13,000 *g* after each extraction.

Removal of Nucleic Acids.—Absolute ethanol was added to a final concentration of 25% v/v to the pooled slightly cloudy or opalescent supernates of the calcium chloride extractions. A fibrous precipitate which formed immediately, and which consisted of nucleic acid, was removed by spooling onto a glass rod. The remaining suspension was placed at 4°C for 3 hr and centrifuged at 2°C at 13,000 *g* for 15 min. This yielded a perfectly clear supernate to which absolute ethanol was added to a final concentration of 80% v/v, causing the precipitation of the polysaccharide. However, the detergent and the calcium chloride remained in solution, as they are freely soluble in ethanol. This suspension was centrifuged for 10 min at 2000 g and the precipitate was washed three times with absolute ethanol, twice with acetone, twice with diethyl ether, and dried *in vacuo*. In some instances centrifugation for 10 min at 13,000 *g* was necessary to completely sediment the precipitate during the ethanol and acetone washes.

Removal of Proteins.—The crude polysaccharide was dissolved in 120 ml of 0.1 M sodium acetate pH 6.8, yielding an opalescent solution which was clarified by centrifugation for 2 hr at 100,000 *g* in a model L or model L2 Spinco ultracentrifuge (Beckman Instrument Co., Fullerton, Calif.). The clear supernate was drawn off with a Pasteur pipette and homogenized at 0°C for 30 min in an Omnimixer operating at 65 v with chloroform and butanol (5:1). The resulting emulsion was centrifuged for 10 min at 13,000 *g* and the clear aqueous layer was drawn off and again homogenized for 1 hr. After six cycles of homogenization with chloroform-containing butanol, chloroform alone was used for several further cycles. This procedure was discontinued when the "cake" forming at the interface after centrifugation was almost negligible.[3]

Final Purification.—The polysaccharide was precipitated by the addition of four volumes of ethanol and collected by centrifugation for 10 min at 2000 *g*. The precipitate was dissolved in 30 ml of saturated sodium acetate pH 7.0 and reprecipitated with four volumes of ethanol. The material was dissolved in 30 ml of water and centrifuged for 2 hr at 100,000 *g*. The polysaccharide contained in the clear supernate was precipitated by addition of four volumes of ethanol. The precipitate was collected by centrifugation for 10 min at 2000 *g*, washed three times with 150 ml aliquots of absolute ethanol, twice with acetone, and vacuum dried.

Analytical Methods.—The protein content in the different lots of polysaccharide was determined by the method of Lowry et al. (12) using bovine serum albumin as a standard. The nucleic acid content was determined by the ultraviolet absorbancy at 260 mμ using $E^{0.1\%}_{1cm}$ of 20.0. Total nitrogen was determined by the method of Kjeldahl (13). The method of Chen, Toribara, and Warner (14) was used for the determination of phosphorus. The acetyl content was determined by the method of Inglis (15) and also by the chromic acid oxidation procedure[2] (16). Sialic acid content in chromatographic eluates was measured by the method

[3] Some preparations were further purified by adding to them 2/3 volume of saturated cupric acetate, pH 5.0. The solution was allowed to stand for 10 min at room temperature and was then centrifuged for 15 min at 13,000 *g*. The polysaccharide was precipitated with ethanol and converted to the sodium salt by dissolving it in saturated sodium acetate and reprecipitating it with ethanol repeatedly until the blue color was lost.

of Svennerholm (17). The anthrone method of Mokrasch (18) was used to estimate the amount of dextran in gel filtration eluates. Sodium and calcium content of the polysaccharides were determined by atomic absorption using a Perkin-Elmer model 303 atomic absorption spectrophotometer.

Ion Exchange Chromatography.—The amino acid and amino sugar content of acid hydrolysates were determined by ion exchange chromatography with the automatic recording equipment described by Spackman, Stein, and Moore (19). Samples of the polysaccharides (about 10 mg) were dissolved in 10 ml of water containing 1 μmole each of norleucine and α-amino-β-guanidopropionic acid used as internal standards. An aliquot of 1.0 ml of this solution was mixed with 1.0 ml of 12 N HCl and hydrolyzed in sealed evacuated glass tubes for varying durations at 110°C. The hydrolysates were evaporated to dryness in a rotary evaporator at 40°C and the residues were dissolved in 30 ml of the pH 2.2 buffer used with the amino acid analyzer. The solutions were filtered through Millipore membranes and 1.0 ml portions were analyzed.

For the quantitative determination of sialic acid, 2–3 mg samples of group B and group C polysaccharides were dissolved in 1.0 ml of water, 200 μl aliquots were placed into four tubes equipped with Teflon-coated screw caps, and the contents of the tubes were dried over P_2O_5 *in vacuo*. Hydrolysis of the samples was performed at 65°C with 500 μl of methanolic-HCl (3 M) for varying intervals of time. The hydrolysates were freed of methanolic-HCl with a stream of nitrogen directed at the solution and analyzed on the amino acid analyzer. The constant used for the calculation of sialic acid from the chromatogram was obtained by treating an authentic sample of *N*-acetyl-neuraminic acid with methanolic-HCl in the same manner as the group-specific polysaccharides.

Gas Chromatography.—Ninhydrin-negative components were detected by hydrolyzing samples of 1 mg with 2 ml of 0.5 N methanolic-HCl for 22 hr at 65°C. D-mannitol (25 μl of a 50 mg/100 ml solution) was added to the samples as an internal standard. After hydrolysis, the contents of the tube were evaporated to dryness at 65°C under a stream of nitrogen. The hydrolysates were then treated with 2 ml of a mixture of pyridine:trimethylchlorosilane: hexamethyl disilazene (5:1:1.5 v/v) at 25°C. After 30 min, the reagents were removed at 65°C under a stream of nitrogen and the residues were extracted with 2.0 ml of heptane. The heptane layer was centrifuged to remove the insoluble material and was then dried under nitrogen at 65°C. The residue was dissolved in 10 μl of heptane. For gas chromatographic analysis, 1 μl of this sample was injected into the column[2] (20, 21) which was approximately 10 ft in length and 0.125 inch in diameter, and packed with Chromosorb W coated with 3% OV-17 polymer. The column was equilibrated at 120°C in a Perkin-Elmer model 900 gas chromatography machine equipped with a flame-ionization detector.

Gel Filtration.—Gel filtration was performed on Sephadex G-200 (Pharmacia, Inc., Piscataway, N. J.) in a column 1.5 × 80 cm using a 0.1 M tris(hydroxymethyl) aminomethane (Tris)-HCl buffer pH 8.0. The column was calibrated with dextran T 80 ($\bar{M}w$[4] 72,000), dextran T 40 ($\bar{M}w$ 42,000), and blue dextran ($\bar{M}w$ 2,000,000), all obtained from Pharmacia. Volumes of fractions were estimated gravimetrically using tared tubes.

Serological Methods.—A hemagglutination technique utilizing polysaccharide-coated erythrocytes was developed. Hyperimmune rabbit sera were obtained from the departmental collection. Furthermore, Dr. M. Pittman generously donated several sera. Freshly drawn type O Rh-negative human red cells were fixed with glutaraldehyde as described by Bing et al. (22).

Prior to sensitization, the glutaraldehyde-fixed red cells were washed three times with buffer and diluted to a concentration of 2.5%. Equal mounts of the red cell suspension and

[4] $\bar{M}w$, weight average molecular weight.

buffer containing antigen were mixed and placed in a 37°C water bath for 30 min. Optimal sensitization was obtained if the antigen solution added to the red cells contained 20 μg/ml of A antigen, lot A-2, or 2 μg/ml of B antigen, lot B-1, or 20 μg/ml of C antigen, lot C-2. After incubation, the cells were washed three times with buffer and diluted to a red cell concentration of 0.5% with buffer containing 0.05% w/v bovine serum albumin.

Hemagglutination tests were performed by the Microtiter technique employing round bottom plates (Linbro Chemical Co., Inc., New Haven, Conn.) and calibrated loops and droppers (Cooke Engineering Co., Alexandria, Va.). Phosphate-buffered saline, pH 7.28, was prepared from Na_2HPO_4 M/15, KH_2PO_4 M/15, NaCl 8.2% w/v, and water in the proportions 80:20:100:800. Passive hemagglutination tests to detect antibody were performed by first adding 0.05 ml of buffer containing 0.5% w/v of bovine serum albumin to each well. Serial 2-fold dilutions of the antisera were carried out with 0.05 ml loops. The sensitized red cells were then added with a 0.05 ml dropper. Hemagglutination inhibition (HI) tests to measure antigen concentration were performed by adding 0.05 ml of buffer containing 0.5% w/v bovine serum albumin to each well, serially diluting the antigens with 0.05 ml loops and adding 0.025 ml of antiserum diluted in buffer containing 0.5% w/v bovine serum albumin. The antiserum was allowed to interact with the antigen for at least 15 min and excess antibody was then estimated by adding sensitized red cells. The A antiserum CH1 was diluted 1/1000, the B antiserum 880, 1/200, and the C antiserum CH7, 1/1000.

For serogrouping meningococcal isolates by HI test, saline extracts of meningococci were prepared by heavily seeding a plate of Mueller-Hinton agar with the strain of meningococcus to be studied. The organisms were harvested from the plate with a cotton swab after 6 to 8 hr of incubation at 37°C in a candle jar, and were suspended in 1 ml of phosphate-buffered saline. The bacteria were then removed by centrifugation for 10 min at 30,000 *g* and the supernates were stored frozen.

RESULTS

Preparation of the Meningococcal Polysaccharides.—In fully grown cultures, the group A or C–specific polysaccharides are found in great part in the culture supernate, and this may be related to the autolytic properties of this species. Therefore, these polysaccharides have always been isolated from culture fluids concentrated either by ultrafiltration or rotary evaporation[2] (5, 6, 8). Mergenhagen et al. (23) and Liu et al.[2] have shown that group A or C polysaccharides prepared from culture concentrates have molecular weights below 50,000. For reasons which will become apparent in the subsequent report of this series, it was desirable to prepare these polysaccharides with average molecular weights exceeding 100,000. Acting on the assumption that enzymatic depolymerization was occurring during the time necessary to concentrate the culture fluid, a simple method was devised to isolate these polysaccharides from unconcentrated cultures. This procedure depends on the ability of the cationic detergent Cetavlon to precipitate the polyanionic group-specific polysaccharides. Several lots of group A and C polysaccharide were prepared by this method and are described in Table I. Furthermore, when cultures of group B organisms were used as starting material, a polysaccharide which was different from the group A or C substance was isolated by this procedure, and these preparations are also described in Table I.

The purity of the polysaccharides was assayed by estimating to what extent they were contaminated with nucleic acid or with protein. Almost all the group A or C polysaccharide preparations contained less than 1% by weight of either nucleic acid or protein. The purity of the lots of polysaccharide isolated from group B meningococci with the exception of lot B-2 was not as good.

TABLE I

Summary of the Lots of Polysaccharide Prepared by the Method Employing Cetavlon

Conditions of growth								Contamination		Remarks
Lot No.	Strain	Medium	Containers	Age of culture	Total volume	Treated with Cu^{++}	Yield	Nucleic acid	Protein	
				hr	*liter*		*mg*	%	%	
A-1	A1	Cas*	2 and 4 liter flasks	16	16	Yes	179	0.24	Not done	
A-2	A1	Cas	2 and 4 liter flasks	16	20	No	163	0.83	0.88	
A-3	A1	Frantz	40 liter carboy	8	30	Yes	74	2.00	0.79	Medium sterilized by Millipore filtration. Culture lightly contaminated
A-4										Lost as a result of a laboratory accident
A-5	A1	Cas	4 liter flasks	16	30	No	175	0.92	0.35	
C-1	C11	Cas	2 liter flask	16	1					Prepared to tests the feasability of the detergent method for group C cultures
C-2	C11	Cas	2 and 4 liter flasks	16	20	Yes	180	0.60	0.62	
C-3	C11	Cas	40 liter carboy	16	30	Yes	320	0.85	1.22	Only instance in which good growth was obtained in a carboy
C-4	C11	Cas	40 liter carboy	22	30	No	80	0.41	0.31	Poor growth
C-5	C11	Cas	40 liter carboy	22	30	Yes	105	0.18	0.39	Mediocre growth
B-1	B11	Frantz	40 liter carboy	16	30	Yes	24	3.70	1.93	Poor growth
B-2	B11	Cas	2 and 4 liter flasks	16	23	No	418	0.22	0.68	
B-3	M2092	Cas	2 and 4 liter flasks	7	23	No	28	1.40	0.57	
B-4	B11	1/5 199 1/10 Cas	2 and 4 liter flasks	7	23	No	61	4.35	0.30	Polysaccharide precipitated with 0.025% w/v of Cetavlon

* Cas, the casamino acid medium described by Watson et al. (8).

Several lots, as indicated in Table I, were also treated with saturated cupric acetate as described under Materials and Methods. Evidently this step is not essential to obtain polysaccharide essentially free of protein or nucleic acid. Three preparations, lot A-2, lot C-2, and lot B-4, were examined in greater detail.

Chemical Composition of Group A Polysaccharide, Lot A-2.—The results of the analysis on lot A-2 are presented in Table II. The major constituents of this polysaccharide are mannosamine phosphate, acetyl, and sodium. It has

not been possible as yet to measure the moisture content of this polysaccharide accurately because charring of the material occurs at temperatures as low as 65°C. The loss of moisture under high vacuum at room temperature was 4% of the weight. There are more acetyl groups present than there are amino groups indicating that this polysaccharide contains *O*-acetyl groups. Over 90% of the weight of this material can be accounted for as the sodium salt of *N*-acetyl, *O*-acetyl mannosamine phosphate and moisture. The fact that there is almost one residue each of nitrogen and phosphorus per mole of mannosamine

TABLE II

Chemical Composition of Group A Polysaccharide, Lot A-2

Composition	Weight per 100 mg of sample	Amount per 100 mg of sample
	mg	*μmoles*
Mannosamine phosphate*	67.24	279
Acetyl	13.48	321
Sodium	5.66	246
Moisture	4.00	—
Recovery	90.38	—
Calcium	<0.15	<5
Total amino acids	<1.00	<10
Nitrogen	3.77	269
Phosphorus	8.23	266

Amino acids and amino sugars were determined by ion exchange chromatography (19). The value for mannosamine phosphate is corrected for losses due to acid hydrolysis.[2] Acetyl moisture, nitrogen, and phosphorus were determined as described in the text.

* Calculated as mannosamine phosphate — H_2O; molecular weight 241.

phosphate would suggest that the A substance isolated by the present procedure is almost a pure polymer of mannosamine phosphate. It should be noted that the A substance contains less than 1% by weight of all the amino acids combined and is, therefore, free of protein and mucopeptide. Muramic acid is also absent. The polysaccharide was also examined by gas chromatography. The material contains no glucose, galactose, mannose, xylose, fucose, or ribitol.

Chemical Composition of Group C Polysaccharide, Lot C-2.—The results shown in Table III indicate that the C substance prepared by this procedure is nearly a pure polymer of sialic acid. The material contains less than 1% of protein or mucopeptide. Gas chromatography failed to reveal any glucose, galactose, mannose, xylose, fucose, or ribitol. Nearly all the nitrogen in the preparation can be accounted for as sialic acid. In addition to *N*-acetyl, *O*-acetyl groups were also found to be present in the group C polysaccharide. The moisture content was found to be 9.5%. The results of these analyses indicate that the sum of the moisture, sodium, sialic acid, and *O*-acetyl accounts for 94% of

the weight of the material. The sialic acid content was also estimated by gas chromatography yielding the same result as the ion exchange procedure.

The Chemical Composition of the Polysaccharide Isolated from Group B Meningococci.—Using the detergent method, it was possible to isolate a polysaccharide containing mainly sialic acid from group B organisms. Four lots were prepared, three from strain B11 and one from strain M2092. The latter strain is the B neotype proposed by Branham (1). The cultural conditions and yields are described in Table I. The chemical analyses detailed below were performed on lot B-4.

TABLE III

Chemical Composition of Group C Polysaccharide, Lot C-2

Composition	Weight per 100 mg of sample	Amount per 100 mg of sample
	mg	*μmoles*
Sialic acid*	76.24	262
Acetyl‡	5.80	138
Sodium	2.51	109
Moisture	9.50	—
Recovery	94.05	—
Calcium	<0.15	<5
Total amino acids	<1.00	<10
Nitrogen	3.81	272
Phosphorus	0.0	0

Amino acids and amino sugars were determined by ion exchange chromatography (19). The value for sialic acid is corrected for losses due to acid hydrolysis.[2] Acetyl, moisture, nitrogen, and phosphorus were determined as described in the text.

* Calculated as *N*-acetyl neuraminic acid − H_2O; molecular weight 291.

‡ Only *O*-acetyl groups are included here. Total acetyl group was 16.81 or 400 μmoles/100 mg.

A satisfactory procedure for the quantitative determination of the sialic acid content in B substance has not yet been developed. Hydrolytic conditions that were successful in the quantitative determination of sialic acid from the group C polysaccharide failed to release more than 12% of the total sialic acid content from B substance. Hydrolyses with 6 N HCl at 110°C and subsequent analyses of the hydrolysate on the amino acid analyzer revealed the absence of amino acid and muramic acid in the preparation indicating that the material was free of protein and mucopeptide contamination. Gas chromatographic analyses of the B substance indicated the absence of ninhydrin-negative carbohydrates such as glucose, galactose, mannose, xylose, fucose, and ribitol. The material was found to contain 249 μmoles of nitrogen, 240 μmoles of acetyl groups, and 109 μmoles of sodium/100 mg of sample. Based on these results and

a sialic acid content of 66.4% as determined by the colorimetric method of Svennerholm (17), it is tentatively concluded that the B substance contains sialic acid as its major constituent. The material was found to contain 13.5% moisture. Assuming that all the acetyl was derived from *N*-acetyl neuraminic acid, the sum of the moisture, nucleic acid, sodium, and *N*-acetyl neuraminic acid accounted for 90% of the weight of the material.

Accurate quantitative analyses have not been done on lots B-1, B-2, and B-3 but all have been shown to contain more than 65% of sialic acid as measured by the resorcinol method of Svennerholm (17).

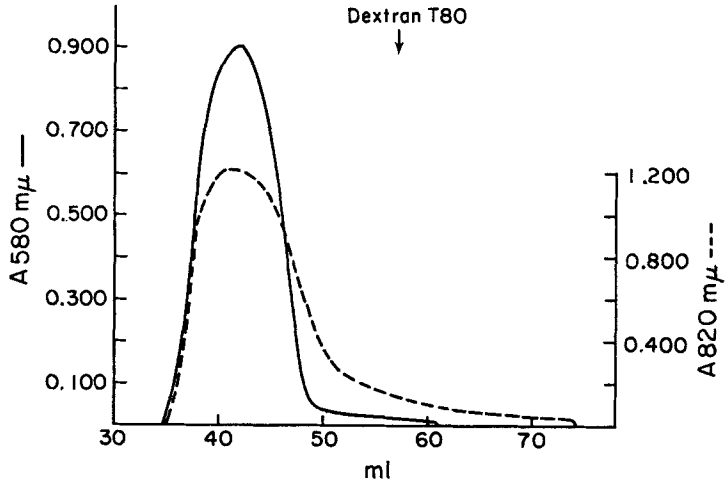

FIG. 1. Gel filtration of group A and C polysaccharide through Sephadex G-200. The solid line is the elution pattern of group C polysaccharide, lot C-2; the dashed line of group A polysaccharide, lot A-2. The eluates were monitored either for sialic acid or for phosphorus. Blue dextran eluted at 41.8 ml and dextran T 80 ($\bar{M}w$ 72,000) eluted at 57.0 ml.

Determination of the Molecular Size of the Meningococcal Polysaccharides.— The molecular size of all the preparations listed in Table I was estimated using Sephadex G-200 gel filtration. The column was calibrated with dextran fractions of known molecular weights. A typical elution profile is shown in Fig. 1 indicating that both the A and the C substance eluted as single peaks in the void volume of the column. The void volume was estimated with blue dextran ($\bar{M}w$ 2,000,000), either by separate chromatography or by cochromatography with the meningococcal polysaccharide. The elution volume of dextran T 80 ($\bar{M}w$ 72,000) is also indicated in Fig. 1. All the other preparations of group A or C polysaccharide listed on Table I behaved the same upon gel filtration indicating that all preparations had molecular weights exceeding 100,000. The elution

profile of the group A polysaccharide tended to be skewed as indicated in the figure.

The molecular size of the four lots of polysaccharide isolated from group B organisms were also compared by gel filtration on Sephadex G-200. The elution patterns of two lots are depicted in Fig. 2.

Lots B-1 and B-3 resembled lot B-4 very closely and were not included in the drawing. There is a marked difference in the molecular size of lot B-2 from the other lots of polysaccharide. Lots B-3 and B-4 were isolated from 7 hr cultures,

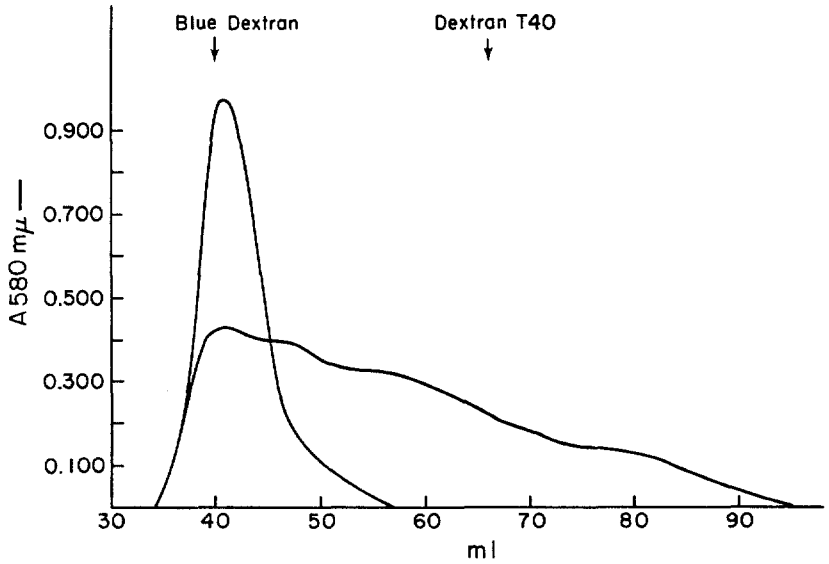

FIG. 2. Gel filtration of group B polysaccharide preparations, lot B-2 and lot B-4. The eluates were monitored for sialic acid. The sharply peaked elution pattern was produced by lot B-4; the broad peak by lot B-2. Blue dextran eluted at 39.8 ml and dextran T 40 ($\bar{M}w$ 42,000) eluted at 66.2 ml.

whereas lot B-2 was isolated from a 16 hr culture. This suggests that the size of this polysaccharide of group B organism is related to the age of the culture. Lot B-1 was isolated from a poorly growing 16 hr culture in a carboy and the relation of the moment of isolation to the growth phase of this culture is not known.

Immunological Properties of the Group-Specific Polysaccharides.—The group A and the group C antigen reacted strongly by capillary precipitation with many homologous meningococcal grouping sera. However, rabbit or horse group B antisera showed only poor precipitation with the group B antigen.

Certain preparations of the three polysaccharides were found to sensitize readily glutaraldehyde-fixed (22) human type O Rh-negative red blood cells.

The sensitized cells were reacted with several rabbit hyperimmune sera and the results are shown in Table IV.

The sera prepared against group A organism (Strain A1 or Strain M1027) reacted with cells sensitized with A polysaccharide but failed to react with

TABLE IV

Passive Hemagglutination Reactions of Rabbit Meningococcal Antisera with Cells Sensitized with the Meningococcal Polysaccharides

Serum No.	Strain used for immunization	Reciprocal hemagglutination titer with red cells sensitized with		
		A polysaccharide	B polysaccharide	C polysaccharide
Group A antisera				
718	M1027	10,000	—*	—
883	M1027	20,000	—	—
598	M1027	20,000	—	—
JE1	M1027	1,280	—	—
BA	M1027	1,280	—	—
H10	?	1,280	—	—
CH1	A1	10,000	—	—
Group B antisera				
CH40	B11	—	1280	—
880	B11	—	640	—
881	B11	20	1280	20
887	B11	—	640	—
IE3	M963	—	160	—
BB	?	—	160	—
CH476	Zeu	40	1280	80
Group C antisera				
BC	M1628	—	—	5120
JE5	M1054	—	—	320
CH7	9 Misc.	—	—	2560
CH601	9 Misc.	—	—	2560
CH568	9 Misc.	—	—	1280
27	C 11	—	—	640
30	C 11	—	—	640

* — indicates that the reciprocal titer was less than 20.

cells sensitized with the B or the C antigen. The antisera prepared against four group C strains again showed group specificity. However, some of the sera obtained from rabbits immunized with group B organisms cross-reacted weakly with the cells sensitized with the A and the C antigens.

The specificity of the serological reactions was further tested by hemagglutination inhibition. The purified antigens were serially diluted in Microtiter plates and appropriate dilutions of rabbit hyperimmune sera directed against

groups A, B, and C were added to each well and allowed to interact with the antigen for at least 15 min. Excess antibody was then estimated by adding appropriately sensitized red cells to each well. Table V indicates the smallest concentration of each of the polysaccharides capable of completely inhibiting passive hemagglutination.

It should be noted that the sensitivity of this reaction depends upon the dilution of antiserum used in the test (see Materials and Methods). The figures in Table V are primarily intended to illustrate that there is some variability between the different lots of polysaccharide which may be related to differences in molecular size of the antigens.

Concentrations as high as 4000 μg/ml of any of these preparations did not inhibit the heterologous reactions. The hemagglutination inhibition test,

TABLE V

Comparison of Polysaccharide Preparations for their Hemagglutination Inhibition Activity

A polysaccharide		B polysaccharide		C polysaccharide	
Lot A-1	0.5*	Lot B-1	0.5	Lot C-2	0.5
Lot A-2	0.5	Lot B-2	10.0	Lot C-3	2.0
Lot A-3	2.5	Lot B-3	5.0	Lot C-4	0.5
Lot A-5	1.0	Lot B-4	1.0	Lot C-5	0.5

* Concentration in μg/ml completely inhibiting passive hemagglutination.

therefore, proved to be a specific and sensitive test, for the presence of the polysaccharide antigens.

The method is also applicable to crude saline extracts of meningococci. 12 group A strains, 61 group B strains, and 25 group C strains were chosen from the collection of the department. The strains came from cases of meningococcal disease and from asymptomatic carriers. Furthermore, the strains originated from all areas of the United States, and certain strains were obtained from Europe and North Africa. Saline extracts of these organisms were prepared as described under Materials and Methods, and these extracts were tested for their ability to inhibit the group A, B, and C passive hemagglutination systems. All group A strains inhibited the A system, but did not inhibit the group B or C systems. All group C strains inhibited the group C hemagglutination systems, but not the heterologous systems. 56 of the extracts of the group B organisms inhibited the group B hemagglutination system without affecting the heterologous systems. The remaining five B strains failed to inhibit. These five strains were reexamined both by the hemagglutination inhibition test and by the slide agglutination method (3). The newly prepared extracts again failed to inhibit the group B hemagglutination reaction. Furthermore, none of these organisms could be typed by slide agglutination as they were either agglutinated

in saline or reacted with several typing sera. Four of these strains were isolates from nasopharyngeal cultures, and one came from a blood culture. It is felt that these strains were untypable meningococci which had been assigned erroneously to group B.

DISCUSSION

The method of purification of the group A and C–specific polysaccharides, which as a first step uses the cationic detergent, Cetavlon, to precipitate the acidic macromolecules from the whole culture, reliably yields highly purified polysaccharide preparations. None of the preparations were more than a few per cent contaminated with nucleic acid or protein (Table I) and in most preparations this contamination was negligible.

The A substance has been shown by Liu et al.[2] to be a polymer of *N*-acetyl mannosamine phosphate which is also *O*-acetylated. The analyses on A polysaccharide lot A-2 indicated that over 90% of the weight of this material could be accounted for by its content of moisture, *O*-acetyl, and the sodium salt of *N*-acetyl mannosamine phosphate. The moisture determination, it should be remembered, is probably an underestimate of the true content of water in this preparation, which could not be determined in the usual manner (see Results). All the other lots, although not analyzed fully, contained more than 8% phosphorus indicating that they resembled lot A-2. The analysis of the C polysaccharide lot C-2 indicated that sialic acid, *O*-acetyl, sodium, and moisture accounted for over 94% of the weight of the material. The other lots of group C polysaccharide were not fully analyzed but all contained over 75% of sialic acid as determined by the resorcinol reaction (17).

This method of purification when applied to cultures of group B organisms yielded a polysaccharide which is chemically and serologically different from the group A or C antigens. In the one instance where a fully grown culture was used as the starting material, the yield of B substance was high (418 mg) and comparable to the yields of group A and C polysaccharides. All lots of B substance gave color reactions typical of sialic acid with the resorcinol reagent (17). Lot B-4 was analyzed more fully. Hydrolysis with methanolic HCl, which releases the sialic acid from the group C polysaccharide would release only 12% sialic acid by weight from the B polymer. Other hydrolytic conditions are presently under investigation. We propose that the B substance is a polymer of *N*-acetyl neuraminic acid which contains no *O*-acetyl groups. The analysis, assuming that all of the acetyl is derived from sialic acid, accounts for 85.7% of the weight of this material. This particular lot of polysaccharide was also known to contain approximately 4.3% of nucleic acid so that over 90% of the weight was accounted for. The other lots of group B polysaccharide have not been analyzed fully except that every lot was shown to contain more than 65% of sialic acid as measured with the resorcinol reagent (17).

The polysaccharides, lots A-2, B-4, and C-2, were also analyzed by gas chromatography and did not contain detectable amounts of glucose, galactose, mannose, fucose, xylose, or ribitol. Examination of acid hydrolysates of these lots of polysaccharide by the automatic ion exchange method of Spackman, Stein, and Moore (19) showed that these lots did not contain any significant amounts of amino acids, glucosamine, or muramic acid, i.e. no appreciable amounts of mucopeptide, when prepared by the detergent methods.

This analysis of the group C polysaccharide differs in one detail from that reported by Watson et al. (4). They found that their preparations of group C polysaccharide contained principally sialic acid but also some hexosamine. We have also prepared group C polysaccharide without the use of detergent by methods similar to the ones described by Watson et al. (8), and our preparations contained appreciable quantities of mucopeptide (approximately 15% by weight). This may have been the source of the hexosamine which Watson et al. (4) have described.

An important property of the group A and C polysaccharides isolated by detergent method is that they are of large molecular weight. In this way, they differ from the polysaccharides prepared from culture concentrates. Mergenhagen et al. (23) have shown that the C antigen prepared by Watson et al. (8) penetrated Sephadex G-75. Liu et al.[2] have shown that both A and C antigens which were prepared from cultures concentrated by rotary evaporation had average molecular weights of less than 50,000. There seems to be no obvious reason why these polysaccharides are intrinsically unstable and probably the depolymerization is enzymatic. The detergent method may circumvent this enzymatic breakdown by the rapidity with which the polysaccharides are isolated from the culture and gotten into organic solvents. Another possibility is that the cationic detergent inactivates the enzymes responsible for the degradation of the polysaccharides.

The molecular size of the four lots of polysaccharide isolated from group B cultures was also examined. Lot B-2, a preparation of polysaccharide originating from a fully grown culture, contained considerable amounts of material less than 100,000 molecular weight (see Fig. 2), suggesting that considerable breakdown had occurred during the 16 hr of incubation. This concept is supported by the observation that polysaccharide derived from 7 hr cultures (end of logarithmic growth phase) such as lot B-3 and B-4 contained little, if any, material of molecular weight less than 100,000 (see Fig. 2).

One might speculate that group B meningococci contain enzymes which are very effective in destroying this antigen, and that this is the reason why this material has not previously been isolated. It was also observed that saline extracts of group B meningococci that had been grown on agar for 18 hr were not always able to inhibit the hemagglutination system, whereas 6 to 8 hr cultures were satisfactory. Extracts of group A and C strains always inhibited regardless of the age of the culture.

Should this sialic acid polymer obtained from group B meningococci be considered the group B specific antigen in the same sense that the A antigen and the C antigen define the strains of meningococci possessing them as belonging to the respective groups? To answer this question, it is necessary to establish that all group B organisms contain this antigen, whereas meningococci of other groups do not contain it. Hemagglutination inhibition tests provide suggestive evidence that all saline extracts of group B meningococci contain this antigen, and that this antigen is not present in group A or group C meningococci as evidenced by the inability of extracts from these organisms to inhibit the group B hemagglutination system.

The specificity of this antigen for group B is further supported inasmuch as none of the hyperimmune sera prepared against group A or group C meningococci react with red cells sensitized with the B antigen. However, antisera made against group B organisms do occasionally react weakly with red cells sensitized with A or C substance, and the reason for this is not known. Two possibilities come immediately to mind. The antigens may be sufficiently similar so that one way serological cross-reactions are possible. Also, it should be noted that *N*-acetyl mannosamine phosphate and sialic acid probably have *N*-acetyl mannosamine as their immediate biosynthetic precursor (24). It is possible, therefore, that all groups of meningococci use a common pathway for the synthesis of the group-specific polysaccharides but with differing final polymerizing enzymes. It is conceivable that the group B organism may be biosynthetically totipotential but makes A and C polysaccharide in amounts insufficient to detect by serological means, but adequate to elicit antibodies when administered to a rabbit. A study of the enzymes used to produce these polysaccharides would clearly indicate whether or not the polysaccharide from group B organisms is metabolically analogous to the A and C antigen.

SUMMARY

The group-specific polysaccharides of group A and group C meningococci have been isolated by a new procedure which employs the cationic detergent Cetavlon to precipitate these polysaccharides from the whole culture. The A and C polysaccharide prepared by this method are noteworthy because they are of high molecular weight. The main constituent of the A polysaccharide is *N*-acetyl, *O*-acetyl mannosamine phosphate; of the C polysaccharide *N*-acetyl, *O*-acetyl neuraminic acid. This purification procedure, when applied to cultures of group B organisms, yields a polysaccharide consisting primarily of *N*-acetyl neuraminic acid.

A passive hemagglutination test developed to measure antibodies to the polysaccharides demonstrated the specificity of these antigens. Using a hemagglutination inhibition test, these antigens were again found to be group-specific, and this test could be used for serogrouping meningococcal isolates.

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