Studies on the Meningococcal Polysaccharides

II. COMPOSITION AND CHEMICAL PROPERTIES OF THE GROUP B AND GROUP C POLYSACCHARIDE*

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SUMMARY

Both Group B and C meningococcal polysaccharides were shown to be pure homopolymers of sialic acid. The two polysaccharides are, however, quite different chemically and immunologically. Acetyl determinations indicated that the C-polysaccharide contains both N- and O-acetyl groups, whereas the B-polysaccharide contains N-acetyl but not O-acetyl groups.

Acid-catalyzed methanolysis in anhydrous methanolic HCl at 65° showed that the C-polysaccharide is much more susceptible to the cleavage than the B-polysaccharide. Under the conditions used, only 12% of the B-polysaccharide was hydrolyzed while complete hydrolysis of the C-polysaccharide was achieved in 22 hours. On the other hand, treatment of the C-polysaccharide with two preparations of neuraminidase (Vibrio cholerae and Clostridium perfringens) resulted in the release of less than 2 to 3% of free sialic acid. The B-polysaccharide was completely hydrolyzed by the enzymes under identical conditions. Possible implications of these observations on the structures of these two polysaccharides are discussed.

A new procedure for the determination of sialic acid as methyl ester-methyl glycoside of neuraminic acid (methyl-(methyl D-neuraminid)ate) or as methyl glycoside of neuraminic acid (methoxy neuraminic acid) on the amino acid analyzer has been developed and is discussed.

The Group C meningococcal polysaccharide was first isolated by Watson and Scherp (2) and was shown by Watson, Marinetti, and Scherp (3) to be a polymer consisting primarily of sialic acid. Gotschlich, Liu, and Artenstein (4) developed a procedure using cetavlon to isolate the group-specific meningococcal polysaccharides. The advantage of this method is that the Group C polysaccharide isolated is of higher molecular weight and is immunogenic in man (5). Recently Artenstein et al. (6) have shown that immunization with this polysaccharide is able to prevent Group C meningococcal meningitis among young adults. It, therefore, is imperative to study the chemistry of this antigen in detail.

By the use of the cetavlon procedure it has been possible to isolate a protein-free polysaccharide from Group B meningococci (4). Inasmuch as this material may well be developed as a vaccine for human use, knowledge concerning its chemistry is important.

The present report describes investigations which have shown that both the B- and C-polysaccharides are homopolymers of sialic acid, but that they are chemically and immunologically distinct.

EXPERIMENTAL PROCEDURE

Materials

p-Toluenesulfonic acid, reagent grade, was purchased from the J. T. Baker Chemical Company, Phillipsburg, New Jersey, and recrystallized, chloride free as described by Liu and Chang (7). Colominic acid was a gift of Dr. W. Goebel, Rockefeller University. Crystalline sample of neuraminic acid β-methyl ketoside was a gift from Dr. F. Wirtz-Peitz, Carbohydrate Research Laboratory, Massachusetts General Hospital, Boston. α- and β-Methoxy N-acetylneuraminic acid methyl esters were a gift from Dr. V. N. Reinhold, Massachusetts Institute of Technology, Boston. N-Acetylneuraminic acid (Lot 301601) was obtained from Pierce Chemical Company, Rockford, Illinois. Neuraminidase (Vibrio cholerae strain 24) and neuraminidase (influenza virus No. 8120) were obtained from the Mann Research Laboratory, New York, New York. Neuraminidase (Clostridium perfringens) was obtained from Pierce Chemical Company.

Preparation of Group B and Group C Meningococcal Polysaccharides

Groups B and C polysaccharides used for the present study were prepared by the procedure using cetavlon described previously (4).
Analytical Methods

Sodium and calcium content of the polysaccharides was determined by atomic adsorption with a Perkin-Elmer model 303 adsorption spectrophotometer. For the analysis of carbon, hydrogen, and nitrogen, a Perkin-Elmer model 450 gas chromatography machine was used. Phosphorus was determined by the method of Chen, Toribara, and Warner (8) with slight modifications (9). Sialic acid was determined by the modified Ehrlich procedure of Werner and Odin (10) and by the thiobarbituric acid assay procedure of Warren (11). Reducing groups were determined by a modified (12) Park-Johnson procedure (13) and by the sodium borohydride procedure (9). N-Glycolyl group content of the polysaccharides were determined by a modification (14) of the method of Klenk and Uhlenbruck (15).

Moisture Determination

Moisture content was determined by the Karl-Fisher titration method (16).

Acetyl Determination

Four different methods were used for the determination of acetyl groups present in the polysaccharides.

Chromic Acid Procedure (17)—Samples containing 0.03 to 0.05 µmole of acetyl were placed into a 25-ml round bottom flask which was attached to a reflux condenser. Chromic acid solution, 2.0 ml, prepared by mixing 4 parts of 5 N chromic acid and 1 part of concentrated H₂SO₄ were added to the flask and the mixture was heated in an oil bath maintained at 170 ± 10°. Two drops of concentrated H₂SO₄ were placed at the glass joint between the condenser and the flask to check for the tightness and to seal the joint. The reaction was allowed to proceed for 30 min during which period the condenser was maintained with efficient cooling.

After cooling, the contents of the flask were quantitatively transferred to a micro-Kjeldahl distillation apparatus with the aid of 10-ml rinse of H₂O. Saturated K₂SO₄, 2.0 ml, was added to the solution and the acetic acid was distilled for titration in the usual manner.

Toluene-sulfonic Acid Procedure—A modification of the Eleck and Harte (18) procedure was used. Samples of polysaccharide (4 to 5 mg) were hydrolyzed with 2.0 ml of a 25% solution (w/v) of p-toluenesulfonic acid in sealed heavy wall glass tubes (Corning 9890, 18 × 150 mm) at 110° for 2 hours. The content of the tubes were quantitatively transferred to a micro-Kjeldahl distillation apparatus with the aid of rinses (2 × 2.0 ml) of water. During the sealing and opening of the glass tubes the tubes were kept frozen in a Dry Ice-acetone bath to prevent the escape of acetic acid. The acetic acid was distilled for titration with 0.01 N NaOH in the usual manner.

NH₄OH Procedure—The method of Hestrin (19) was used for the reaction of O-acetyl groups with hydroxylamine in alkali to form hydroxamic acid. The hydroxamic acid formed was measured by the formation of a colored complex with Fe³⁺ in acid solution. A standard of β-D-glucose pentaacetate was run simultaneously and the quantity of O-acetyl in the sample was calculated by comparison with the standard curve obtained from the analyses of β-D-glucose pentaacetate.

Hydrolysis with 0.01 N NaOH (20)—The procedure is described in the previous paper (9).

Ion Exchange Procedure

The amino acid and amino sugar content of acid hydrolysates was determined by ion exchange chromatography with the automatic recording equipment described by Spackman, Stein, and Moore (21).

For the determination of amino acids and acid-stable amino sugars, samples of polysaccharides were subjected to 6 N HCl hydrolysis. Polysaccharide samples, 3 to 5 mg, were dissolved in 10.0 ml of H₂O containing 1.0 mole each of norleucine and α-amino-β-guanidopropionic acid used as internal standards. A 1.0 ml aliquot of this solution was mixed with an equal amount of 12 N HCl and hydrolyzed in a sealed evacuated glass tube at 110° for 2, 6, 12, and 24 hours. The hydrolysates were evaporated to dryness in a rotary evaporator at 40° and the residues were dissolved in 3.0 ml of the pH 2.2 buffer used with the amino acid analyzer. The solutions were millipore filtered and 1.0 ml portions were added to the 7- and 60-cm columns of the amino acid analyzer with the aid of a sample injector (Chromatronix model SV-8031). The recoveries of amino acids and amino sugars found on the chromatogram for each hydrolysis were calculated by reference to the internal standards norleucine and α-amino-β-guanidopropionic acid present in the sample.

Methanolysis with Anhydrous Methanolic p-Toluenesulfonic Acid

Anhydrous p-toluenesulfonic acid, recrystallized and purified chloride free (7), was used for this purpose. The concentration of p-toluenesulfonic acid used was 1.0 N in anhydrous methanol. Polysaccharide samples and α- and β-methoxy neuraminic acid methyl ester (2 to 3 mg) were dissolved in 2.5 ml of 1 N p-toluenesulfonic acid in anhydrous methanol and 0.5 ml aliquots were distributed into four glass tubes (Kimble 45066A, 1 × 150 mm) equipped with Teflon-coated screw caps. The tubes were flushed with a stream of nitrogen, sealed with the cap, and immersed into a heating block (Exacta-Heat, model 216, Techt; Laboratory Instruments, Piqua, New Jersey, hole depth 50 mm) maintained at 65 ± 0.1° for 4, 8, 16, and 24 hours. At the end of methanolysis, the tubes were added with a short section of Tygon to the condenser of a rotary evaporator which can be operated with the condenser axis at a downward tilt of about 30°. The methanol is removed in about 20 min at 40°. Alternatively, the solvent can be removed by evaporation in a stream of nitrogen at 40°.

The product of the methanolysis, methyl[(methyl]-n-neuraminic acid (Compound II, Fig. 1), was analyzed as neuraminic acid methyl glycoside (Compound III, Fig. 1) after saponification. For this purpose the methanolysate was treated with 1.10 ml of a 0.5 N NaOH for 60 min at 25° (pH should be 12 to 13). The solutions were quantitatively transferred to a 5.0 ml volumetric flask and made to volume with water. One-milliliter aliquots were used for analysis on the 60-cm column of the amino acid analyzer with the pH 3.25 buffer as eluent. The amino acid analyzer constant for methoxy neuraminic acid determined with an authentic crystalline preparation was 5.12 for an instrument for which the aspartic acid constant is 8.84. The elution volume of methoxy neuraminic acid and aspartic acid are 40 and 65 ml, respectively, on this instrument.

Methanolysis with Anhydrous Methanolic-HCl

Anhydrous methanolic HCl was prepared by bubbling dry HCl which had passed through a H₂SO₄ tower into anhydrous...
methanol. The concentration of HCl in methanol was adjusted to 1.0 N. Polysaccharide samples and α- and β-methoxy N-acetylneuraminic acid methyl esters (2 to 3 mg) were dissolved in 1.0 ml of H2O and 200-μl aliquots were placed into four glass tubes (12 × 150 mm) equipped with Teflon-coated screw caps. The contents of the tubes were dried over P2O5 in a vacuum. Methanolyzes of the samples were performed with 1.0 ml of the 1 N methanolic HCl for 4, 8, 16, and 24 hours at 65° as described above. The methanolysate was then exposed to 5.0 ml of 0.01 N NaOH for 60 min at 25°. One-milliliter aliquots were used for analyses on the 60-cm column of the amino acid analyzer as described in the previous section.

**Gas Chromatography**

Ninhydrin-negative components of the polysaccharides were determined by gas chromatography on a stainless steel column, approximately 10 feet in length and 0.125 inch in diameter, packed with Chromsorb W coated with 3% OV-17 polymer. The column was equilibrated at 120° in a Perkin-Elmer model 900 gas chromatograph equipped with a flame ionization detector. The procedure used was the one described by Reinhold et al. (22) which utilizes anhydrous methanolic HCl for the cleavage of the polysaccharide and analyses of the trimethylsilylated carbohydrate derivatives on the column of the gas chromatograph.

**Periodate Oxidation**

Periodate oxidations and formaldehyde determination were performed on the polysaccharides essentially according to the method of Suzuki and Strominger (23) and as described by Liu et al. (9).

**Digestion of Group B and Group C Polysaccharides with Neuraminidase**

Group B, and C-polysaccharide, de-O-acetylated C-polysaccharide, and partially hydrolyzed C-polysaccharide were treated with neuraminidases from V. cholerae, C. perfringens, and influenza virus, at 37° essentially according to the method of Casidy, Jourdian, and Roseman (24). Thiobarbituric acid assays were performed on aliquots of the digestion mixture to measure the release of free sialic acid.

**Optical Rotation**

Optical rotations of the polysaccharides were measured with a Zeiss photoelectric precision polarimeter capable of an accuracy of ±0.005°.

**Isolation of N-Acetylneuraminic Acid from Group B Polysaccharide**

A sample of B-polysaccharide (50 mg) was dissolved in water (5 ml) and 0.09 N NaOH was added to the solution until the pH remained constant at 11.0 to hydrolyze any internal ester which
might exist (25). The pH was then reduced to 5.0 with 0.1 M HCl. The volume was brought to 20 ml with 0.1 M acetate buffer, pH 4.9. C. perfringens neuraminidase, 2 ml, was added and the solution was incubated at 37°C for 72 hours. Thiobarbituric acid assay performed on an aliquot of the incubation mixture indicated 38 mg of free sialic acid was present. The enzymic hydrolysate was adjusted to pH 8 to 9 with 1 M NH₄OH and placed on a Dowex 1-X8 (formate form, 100 to 200 mesh) column (2 x 24 cm). The column was washed with 100 ml of HzO and then eluted with dilute formic acid by gradient technique. A 500-ml Erlenmeyer flask containing 0.05 M formic acid served as the mixing vessel. To this was gradually added 2.0 M formic acid and 6.0-ml fractions of the eluate were collected. Analyses were performed for sialic acid on 200+1 aliquots from each tube with the Ehrlich reagent according to the method of Werner and Odin (10). Fractions 330 to 402 ml contained N-acetylneuraminic acid, 22 mg. The peak was dried by lyophilization and the resulting material was crystallized by dissolving in 0.5 ml of H₂O and diluting with 2.5 ml of glacial acetic acid as described by McGuire and Binkley (25). Decomposition point, 184-186°C.

**C₃H₃N₄O₇**
- Calculated: C 42.74, H 6.19, N 4.53
- Found: C 42.51, H 6.35, N 4.07

This material was further characterized by infrared spectroanalysis (Fig. 2).

**Preparation of De-O-Acetylated C-Polysaccharide**

C-Polysaccharide (33 mg) was treated with 10.0 ml of 0.02 N NaOH for 22 hours at 25°C. The solution was neutralized with 1.0 ml of 0.2 N acetic acid, dialyzed extensively against water to remove salt, and lyophilized. The de-O-acetylated C-poly saccharide (25 mg) prepared in this manner contains less than 0.12 residue of O-acetyl group per residue of sialic acid present in the polymer (see Table IV) and was used for the studies of its susceptibility to enzymic hydrolysis with neuraminidases.

**Partial Hydrolysis of C-Polysaccharide**

C-Polysaccharide has weak reducing properties when tested with the Park-Johnson reagent. This may be caused by the presence of a free reducing group on one end of the polymer chain. Upon heating C-poly saccharide in water between pH 3 to 5, hydrolysis occurs, and the amount may be estimated by measuring the increase in reducing value. A 0.1% solution of C-poly saccharide in water was heated at 100°C for 30, 60, and 90 min. In Fig. 3 is shown a hydrolysis curve of the C-poly saccharide under these conditions.

A standard curve of reducing groups determined versus sialic acid concentration was constructed and this curve was used to determine the degree of hydrolysis of C-poly saccharide. It was
Table I

| Method                      | Glucose acetate | B-Poly saccharide Lot B-4 | C-Poly saccharide Lot C-2 |
|-----------------------------|-----------------|---------------------------|---------------------------|
| Chromic acid digestion     | %               | %                         | %                         |
| Toluenesulfonic acid       | %               | %                         | %                         |
| Basic hydrolysis, 0.01 N NaOH | %             | %                         | %                         |
| NH₄OH                      | %               | %                         | %                         |
| Micromoles of O-acetyl per 100 mg | 0 | 263                      | 275                       |
| Micromoles of O-acetyl per 100 mg | 128 | 148                      | 138                       |

Sialic acid

1. Methanolic HCl: gas chromatography          11.2  30
2. Methanolic HCl: ion exchange column         12.3  42
3. Toluenesulfonic acid: ion exchange column   74.8  85.2 200 293
4. Enzymic hydrolysis                         76.4  82.5 263 283
Acetyl                                      0  0  0  0
Sodium                                      2.5  5.7 100 117
Total recovery                               87.3 87.9
Nitrogen                                     3.84 4.55 274 325

Amino acids and amino sugars were determined by ion exchange chromatography. The values for sialic acid were determined by four different procedures. For the computation of the weight recovery, the value obtained by the toluenesulfonic acid method was used. Acetyl and nitrogen were determined as described in the text. Not included in the total recovery are ninhydrin-negative carbohydrate determined by gas chromatography, total amino acids, and nucleic acids, all of which were present in less than 1% by weight. Moisture was determined by the Carl-Fisher titration procedure.

Table II

| Composition | Weight per 100 mg of sample | Amount per 100 mg of sample |
|-------------|-----------------------------|----------------------------|
| Lot R-4     | Lot R-4                     | Lot R-4                    | Lot R-3                    |
| %           | %                           | %                          | %                          |
| Total recovery                                    | 11.2  30                      |
| Nitrogen                                            2.5  5.7 100 117                     |
| Acetyl                                              0  0  0  0                             |
| Moisture                                            10.0  0
| Sodium                                              2.5  5.7 100 117
| Total recovery                                     87.3 87.9
| Nitrogen                                            3.84 4.55 274 325

Amino acids were determined by ion exchange chromatography. The values for acetyl were determined by four different procedures. For the computation of the weight recovery, the value obtained by the toluenesulfonic acid method was used. Sialic acid and nitrogen were determined as described in the text. Not included in the total recovery are ninhydrin-negative carbohydrate determined by gas chromatography, total amino acids, and nucleic acids, all of which were present in less than 1% by weight. Moisture was determined by the Carl-Fisher titration procedure.

Optical Rotatory Properties and Infrared Spectra—The optical rotation of a 1.09% aqueous solution of the B-poly saccharide is 7.27.

RESULTS

Chemical Composition of Group B Polysaccharide—The results shown in Tables I and II indicate that the B substance prepared by the method of Gotschlich et al. (4) is nearly a pure polymer of sialic acid. The material contains less than 1% of protein or muramylpeptide. Chromatographic analyses of the 6 N HCl hydrolysate of the sample indicated that no amino acids, muramic acids, or other amino sugars were present. Gas chromatography failed to reveal any glucose, galactose, mannose, xylose, fucose, or ribitol (less than 1% by weight). The polysaccharide is also devoid of glycyl group. Acid catalyzed methanolysis of the polysaccharide with 1.0 N HCl in anhydrous methanol followed by the identification of the product by ion exchange chromatography as methyl(methyl n-neuraminidate) or methoxy neuraminic acid methyl ketoside (Compounds II and III in Fig. 1) or by gas chromatography as trimethylsilyl derivative yielded only 11 to 12% of the polysaccharide as sialic acid. However, when the B-polysaccharide was treated with anhydrous methanolic toluenesulfonic acid followed by analysis on the ion exchange column 74.8 to 85.2% by weight was recovered as sialic acid. The Group B polysaccharide could also be hydrolyzed with neuraminidases from V. cholerae and C. perfringens. Thiobarbituric acid assay performed on an aliquot of the incubation mixture indicated that the sialic acid released accounted for 76% to 82% of the weight of the Group B polysaccharide added. The analytical data in Table I also indicate that nearly all the nitrogen in the preparation can be accounted for as sialic acid. The moisture content of the polysaccharide as determined by the Park-Johnson procedure is 7.27.

Chemical Composition of Group C Polysaccharide—the material is free of protein and mucopeptide. By gas chromatographic analyses the C-polysaccharide contained no ninhydrin-negative mono saccharides such as glucose, galactose, mannose, xylose, fucose, or ribitol. The material is also devoid of glycolyl group.

Hydrolysates with 6 N HCl at 110° and subsequent analyses of the hydrolysates on the amino acid analyzer revealed that there were no amino acids or muramic acid in the preparation indicating that the material is free of protein and mucopeptide. By gas chromatographic analyses the C-polysaccharide contained no ninhydrin negative monosaccharides such as glucose, galactose, mannose, xylose, fucose, or ribitol. The material is also devoid of glycolyl group.

Digestion of the C-polysaccharide with neuraminidases resulted in the release of less than 3% of free sialic acid as determined by the thiobarbituric acid test.

Optical Rotatory Properties and Infrared Spectra—The optical rotation of a 1.09% aqueous solution of the B-polysaccharide
Amino acids and amino sugars were determined by ion exchange chromatography. The values for sialic acid were determined by four different procedures. For the calculation of the weight recovery, the value obtained by the toluenesulfonic acid method was used. Acetyl and nitrogen were determined as described in the text. Not included in the total recovery are ninhydrin-negative carbohydrate determined by gas chromatography, total amino acids, and nucleic acids, all of which were present in less than 1% by weight. Moisture was determined by the Carl-Fisher titration procedure.

### Table III

**Chemical composition of meningococcal C-polysaccharides lots C-2 and C-4**

| Composition | Amount per 100 mg of sample | Amount per 100 mg of sample |
|-------------|----------------------------|----------------------------|
|             | Lot C-2                    | Lot C-4                    |
| Starch acida| mg                        | µmoles                     |
| 1. Methanolic HCl: gas chromatography | 77.7 | 267 |
| 2. Methanolic HCl: ion exchange column | 76.24 | 262 |
| 3. Toluensulfonic acid: ion exchange column | 78.5 | 84.9 |
| 4. Enzymic hydrolysis | <2.5 | <3.0 |
| Acetyl      | 5.7                      | 133                       |
| Sodium      | 2.5                      | 109                       |
| Moisture    | 0.4                      | 0                         |
| Total recovery | 96.1 | 94.2 |
| Nitrogen    | 3.81                     | 272                       |

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\[ ^a \text{Calculated as N-acetylneuraminic acid = H}_2\text{O; molecular weight, 291.} \]

\[ ^b \text{Only O-acetyl group is included here. N-Acetyl group is calculated as part of sialic acid. For the calculation of acetyl content, molecular weight, 42 was used.} \]

\[ ^c \text{Dried at 40° over P}_2\text{O}_5 \text{ in a vacuum to constant weight.} \]

\[ ^d \text{Corrected for moisture.} \]

when measured in a 2-dm tube gave a value \([\varepsilon]_D^{25} = 15.3\). A 4.6% aqueous solution of the C-polysaccharide gave a value \([\varepsilon]_D^{25} = -30.7\) and a de-O-acetylated sample of the C-polysaccharide (see text for the preparation) gave a value of \([\varepsilon]_D^{25} = +15.3\) (see Fig. 4). Infrared spectra of Group B and Group C polysaccharide, de-O-acetylated Group C polysaccharide, colominic acid, and N-acetylneuraminic acid were taken in potassium bromide pellets with 1 to 2 mg of the polysaccharide mixed with 150 mg of the salt. The spectra are recorded in Fig. 2.

Red **Reducing Group Analyses**—On reduction with NaBH₄ sialic acid consumes 1 mole of hydrogen, the keto group obviously being reduced to a secondary alcohol group. The amount of reducing groups present in the polysaccharides were determined by the tritiated sodium borohydride method (9). Samples (1 mg/500 µl of H₂O) of the polysaccharides, a standard of NAN¹ (1 mg/500 µl of H₂O) and a blank (H₂O, 500 µl) were treated with 3.5 mg of sodium borohydride (1H) dissolved in 500 µl of 0.1 N NaOH. The reduction was allowed to proceed at room temperature for 18 hours. Glacial acetic acid (600 µl) was added to destroy the excess sodium borohydride. Methanol,

¹ The abbreviation used is: NAN, N-acetylneuraminic acid, or sialic acid.

### Table IV

**Comparison of some properties of groups B and C-polysaccharide and colominic acid**

| Analyses | N-Acetylneuraminic acid | B-Polysaccharide | C-Polysaccharide | De-O-acetylated C-polysaccharide | Colominic acid |
|----------|-------------------------|-----------------|-----------------|---------------------------------|---------------|
| C, percentage | 42.72 | 42.0 | 44.5 | N.D. | 46.08 |
| H, percentage | 6.19 | 5.60 | 6.25 | N.D. | 5.87 |
| N, percentage | 4.54 | 4.55 | 4.10 | N.D. | 4.35 |
| Acetyl, percentage | 13.92 | 10.5 | 18.4 | 14.1 | 14.82 |
| [α]D²⁵ | +15.3 | -30.7 | +17.5 | -5.0⁵ | |
| Reducing group | 1 | 74 | 143 | N.D. | 10⁶ |
| Periodate uptake | 1 hour | 3.1 | 0.02 | 0.19 | N.D. | 0.20 |
| 22 hours | 5.5 | 0.18 | 0.58 | N.D. | N.D. |

Formaldehyde formed via periodate oxidation⁷ | 0.03 | 0.018 | 0.0098 | N.D. | N.D. |

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[^1]: N.D., not determined.
[^2]: Total acetyl.
[^3]: 5% aqueous solution, 2-dm tube [α]D²⁵ (see Barry and Goebel, Reference 28).
[^4]: 1.03% aqueous solution, 2-dm tube [α]D²⁵.
[^5]: 4.67, aqueous solution, 2-dm tube [α]D¹⁵.
[^6]: Determined by the NaBH₄ method as discussed in the text.
[^7]: Taken from McGuire and Binkley (25).
[^8]: Expressed as micromoles of periodate consumed per µmoles of NAN.
[^9]: Expressed as micromoles of formaldehyde formed per µmoles of NAN.

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50 ml, was added and the solution evaporated to dryness in a vacuum. The addition of methanol and the evaporation were repeated an additional four times. The residues were taken up in 1.0 ml of H₂O and aliquots thereof were measured in a Beckman model CPM-100 scintillation counter. Since it was found that even after liberation of the excess of tritium with acetic acid and many subsequent treatments of the residue with methanol to form the volatile methyl borate, a detectable amount of radioactivity still remained; a blank was carried out containing the same amount of NaBH₄ as the sample. This blank correction never exceeded 10% of the tritium incorporated into the sample and in our experience is more satisfactory than purification of the reduced product by column chromatography since in some instances the radioactive contaminant moved along with the reduced products.

A standard curve was constructed with the values obtained from the N-acetylneuraminic acid experiment (Fig. 4). The number of reducing groups present per g of the polysaccharides and the oligosaccharides were computed from the amount of radioactivity incorporated into each sample with the standard curve (Fig. 4). It was found that there is one reducing group per 74 residues of NAN in the B-polysaccharide and the value for the C-polysaccharide is one per 143 residues.

Periodate Oxidation—Table IV and Fig. 5 show the results obtained from the oxidation of colominic acid, N-acetylneuraminic acid, and the Group B and C polysaccharides with periodate. The uptake is less than 0.2 µmoles of periodate in 22
hours per µmole of NAN in Group B polysaccharide and less than 0.04 µmole of formaldehyde per µmole of NAN is produced. The uptake of periodate for the C-polysaccharide is less than 0.6 µmole per µmole of NAN after 22 hours, and the formation of formaldehyde is also less than 0.02 µmole per µmole of NAN. Since the C-polysaccharide was found to be O-acetylated, and that this group may interfere with periodate oxidation, the periodate oxidation was also performed with C-polysaccharide that had been treated with base to remove O-acetyl group. The results were essentially identical with the native material.

**DISCUSSION**

In the early stages of our work, we had used the standard conditions (0.1 N H₂SO₄, 80°, 1 hour, see Reference 26) to liberate sialic acid from our polysaccharides. We soon found out, however, that the results obtained from such studies were misleading and inaccurate. Sialic acid is in general unstable in aqueous acidic conditions (26, 27). The standard mild condition widely used (26) is good perhaps only for the liberation of sialic acid from glycoproteins because of its terminal positions in these molecules. When the method was applied to polysaccharides with molecular weight in excess of 100,000 such as B- and C-polysaccharides, the method failed to yield more than 20% of the sialic acid content in 60 min. Prolonged incubation (3 to 4 hours) resulted in higher recovery of sialic acid (40 to 50%). But the yield never exceeded 55% from both B- and C-polysaccharides. The failure of the “standard” procedure to yield more than 55% of the sialic acid from these polysaccharides is most likely caused by compensating factors; continuous release and destruction of the released sialic acid during the hydrolysis in 0.1 N H₂SO₄ at 80°.

A new procedure for the analysis of sialic acid in polysaccharides has been developed. The method utilizes p-toluene-sulfonic acid instead of HCl as the catalyst for methanolysis and the amino acid analyzer for the quantitative estimation of sialic acid as methoxy neuraminic acid (Compound III, Fig. 1). Under the acid-catalyzed methanolysis condition used, the product is presumably essentially the β anomer (27). However, when authentic samples of α- and β-methoxy neuraminic acid were analyzed on the amino acid analyzer, the two anomers coeluted from the column. The color constants for the two anomers are similar. The method has been applied to several glycoproteins and cell wall carbohydrates and was also found to be successful in the determination of sialic acid content in these samples.

Cleavage of Group B and C polysaccharides with 1 N anhydrous methanolic HCl at 65° for 22 hours (a procedure commonly used for the methanolysis of polysaccharide (22)), followed by conversion of the methyl glycosides of sialic acid to their trimethylsilyl derivative and analyses by gas chromatography gave sialic acid content for the B-polysaccharide as 11.2% (Table II, Lot B-4) and the C-polysaccharide as 77.7% (Table III, Lot C-2). When the products of methanolysis were saponified and analyzed directly on the 60-cm column of the amino acid analyzer, the yield of methoxy neuraminic acid from these polysaccharides was 12.3% for the B-polysaccharide (Table II, Lot B-4) and 76.2% for the C-polysaccharide (Table III, Lot C-2). It appears, therefore, that the analyses of sialic acid after its cleavage from the polysaccharide with

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3 T. Y. Liu and Y. H. Chang, unpublished observations.
anhydrous methanolic HCl by either gas chromatography as the
methoxy neuraminic acid will give almost identical results.
Enzymic hydrolysis of the polysaccharides with neuraminidases
followed by the Warren test on aliquots of the digestion mixture
revealed the release of free sialic acid from the B-polysaccharide
to be 76.4% (Table II, Lot B-4), but from the C-polysaccharide,
the yield of free sialic acid was less than 3% (Table III, Lot
C-2).

The conclusion to be drawn from these studies are as follows:

1. Acid-catalyzed methanolysis of polysaccharide with 1 N
anhydrous methanolic HCl at 65° for 22 hours, sometimes may
not be sufficient to cause the complete release of sialic acid from
the polysaccharide as is shown in the case of Group B poly-
saccharide.

2. Enzymic digestion of polysaccharide with neuraminidases
likewise may not cleave all the sialic acid from the polysac-
charides as is shown in the case of the C-polysaccharide. Evi-
dently, these neuraminidases are not capable of hydrolyzing
certain ketosidic linkages of sialic acids or its O-acetylated
derivative.

3. When anhydrous methanolic 1 N p-toluenesulfonic acid is
used as a catalyst in methanolysis, the release of sialic acid from
the polymer has been consistently higher as is shown here with
the B- and C-polysaccharides and with other sialic acid-con-
taining glycoproteins and cell wall polysaccharides. For
the estimation of sialic acid content in polysaccharides and glyco-
proteins, the advantages of using p-toluenesulfonic acid as a
catalyst for methanolysis, therefore, are 2-fold. First, the
reagent is effective in causing more complete cleavage of sialic
acid from the polysaccharides or the glycoproteins. Second, the
product of methanolysis after removal of solvent, can be analyzed
directly on the ion-exchange column of the amino acid analyzer
without further derivatization as is required for the gas chroma-
tographic procedure. The use of 3 N aqueous p-toluenesulfonic
acid in the hydrolysis of proteins and the quantitative estimation
of tryptophan by the amino acid analyzer has recently been
described.

The results of the present studies indicate that both the B-
and the C-polysaccharides of meningococcus are essentially pure
polymers of sialic acid. The high yield of sialic acid or its
derivative obtained either by methanolysis with toluenesulfonic
acid as catalyst, or by enzymic hydrolysis of the polysaccharides,
and the absence of other carbohydrates, proteins, and nucleic acid
support this conclusion. This concept is supported by the
finding that the infrared absorption spectra of NAN isolated from
B- and C-polysaccharides are similar to an authentic sample of NAN (Fig. 2A). Furthermore, the infrared absorption
spectra of these polysaccharides are quite similar to colo-
mic acid (Fig. 2B), a polymer known to consist mainly of NAN.
Although there was one reducing end, the infrared absorption
spectra failed to react with antisera against B-polysaccharide but
were not separated. The nature of the cross-links that might
exist in the B- and the C-polysaccharides have not been studied.

Comparison of the uptake of periodate by sialic acid and by these
polysaccharides may be instructive (see Table IV and Fig. 5).
If N-acetylneuraminic acid retains the pyranose ring structure
in the polymer as has been suggested by AlcGure and Bunken
(25) for coloaminic acid, then the uptake of less than 0.1 or 0.2
mole of periodate per NAN unit in B- and C-polysaccharides,
respectively, would be best explained by a two to eight ketosidic
linkage between the NAN units in both polysaccharides. Any
other linkage would require the uptake of at least 1 molue per
NAN unit. The formation of less than 0.1 mole of formalde-
hyde per residue of sialic acid present in the polysaccharide even
after 22 hours of periodate oxidation is in agreement with this
proposal. For C-polysaccharide, which is partially O-acetylated,
the same conclusion is valid since the de-O-acetylated poly-
saccharide was similarly unaffected by the treatment with periodate.
The position of the O-acetyl group in the C-polysac-
saccharide remains to be established.

Although B- and C-polysaccharides are both homopolymers of
sialic acid some important differences do exist between the two
polymers. Chemically, the B-polysaccharide is devoid of O-
acyethyl group, while the C-polysaccharide is partially O-acetylated.
As shown in Fig. 6b, the B-polysaccharide is much more re-
sistant to the acid-catalyzed methanolysis in anhydrous HCl
than the C-polysaccharide. The de-O-acetylated C-polysac-
saccharide failed to react with antisera against B-polysaccharide but
retains reactivity toward antisera against C-polysaccharide. This indicates that O-acetylation is not the only difference that

\[ \text{T. Y. Liu, F. T. Dunne, and E. C. Gotschlich, unpublished observations.} \]
exists between the B- and C-polysaccharides. The two polysaccharides also differ in their susceptibility to neuraminidase. Inspection of Fig. 7 indicates that while the B-polysaccharide is almost completely split into monomeric NAN by the hydrolytic action of neuraminidase, the C-polysaccharide and its de-O-acetylated derivative are resistant. Under identical conditions, colominic acid is rapidly and completely hydrolyzed to NAN by the enzyme. From the study of the consumption of alkali before and after saponification and reaction with NH$_2$OH, McGuire and Binkley (25) suggested that in colominic acid some of the carboxylic groups exist as ester linkages with the hydroxyl groups of NAN. It was also noted that the enzyme neuraminidase hydrolyzed the "base-treated" colominic acid much more rapidly than the untreated colominic acid. The data shown in Table I for the acetyl determinations of the B and C polysaccharides seem to rule out the possibility for the existence of "internal ester bond" such as the one proposed to exist in colominic acid. In our experiment, treatment with 0.01 N NaOH followed by distillation and titration will give the value for the volatile acid, acetic acid (the existence of glycoyl group in the B- and C-polysaccharides has been ruled out by the calorimetric test of Klenk and Uhlenbruck (15)) which exist in the C-polysaccharide as O-acetyl groups. Reaction with NH$_2$OH on the other hand could be attributed to the presence of O-acetyl groups, the presence of lactone linkages, or ester linkages, between the carboxyl group of one unit and a hydroxyl group of a neighboring unit. Since our data show that the two values obtained by the 0.01 N NaOH procedure and the NH$_2$OH are nearly identical, it must follow that lactone and ester linkages do not exist in the C-polysaccharide. For the Group B polysaccharide, since the reaction with NH$_2$OH is negative, neither O-acetyl nor an internal ester can exist.

Slowness of the attack by the enzyme neuraminidase on some sialic acid containing glycoprotein has been attributed to the presence of O-acetyl groups substituted on the sialic acid residues (30, 31). Confirmation of this hypothesis was offered in the case of bovine submaxillary mucin when almost complete hydrolysis was obtained after treatment of the mucin with alkali (30, 31). Cassidy et al. (24), however, cautioned against this interpretation for two reasons; first, in their studies with neuraminidase isolated from C. perfringens and from V. cholerae, both enzymes showed no significant difference in the rate of release of NAN from the acetylated and deacetylated substance, and secondly, the effects of alkali on mucins may be much more profound than simple hydrolysis of O-acetyl linkages. In the present studies, the C-polysaccharide was not affected by the enzyme even after it had been treated with base to remove O-acetyl group; hence, the inactivity of the enzyme toward this
polymer of sialic acid is unlikely to be caused by the presence of O-acetyl group.

In order to ascertain whether the resistance of the C-poly saccharide to the hydrolitics action of neuraminidases is the result of its large molecular size, we have prepared a mixture of oligosaccharide from the C-poly saccharide by partial hydrolysis in boiling water (see "Experimental Procedure"). The product was shown to have an average molecular size of 7 to 8 sialic acid units per one reducing group determined by the Park-Johnson procedure. When this preparation was treated with neuraminidase from V. cholerae at pH 5.0 in the presence of serum albumin as described by Cassidy et al. (24), free sialic acid was released to the extent of 11.7% from the oligosaccharide in 24 hours at 37°. Under identical conditions, the release of monomeric sialic acid from the original unheated C-poly saccharide was 1.3% and from the B-poly saccharide was 100%. The results of these experiments would seem to suggest that the inactivity of the neuraminidases toward the C-poly saccharide is not likely the result of the presence of O-acetyl group or to its large molecular size, although smaller fragments of the C-poly saccharide are hydrolyzed more extensively than the one with larger molecular size.

Gottschalk (32) proposed in 1958 that neuraminidase isolated from V. cholerae is an α-ketosidase. Some evidence in support of this hypothesis was offered by McGuire and Binkley (25) when they showed that the enzyme failed to release more than 3% of one form of methyl glycoside of N-acetylneuraminic acid. This form of glycoside was suggested to be in β conformation. Subsequently, Yu and Ledeen (33) conclusively showed that the anomer which is resistant to neuraminidase is the α-ketoside and that the one susceptible to the enzymatic hydrolysis is the α-ketoside. If this proposal is correct, the results presented in this manuscript would suggest that the B-poly saccharide consists of α-ketosidic linkages and that the C-poly saccharide might possibly have β-ketosidic linkages. The existence of β-ketosidic linkages involving sialic acids in poly saccharides or in the glycoprotein have so far not been reported in the literature. It should be emphasized that our suggestion that the C-poly saccharide might involve β-ketosidic linkages is tentative especially in view of the fact that the specificity studies of the neuraminidases were performed on synthetic substrate, N-acetyl neuraminyl methyl ketoside rather than on di- or trisaccharides of sialic and, that it was found that 3% of the β-methyl ketosides were hydrolyzed by the enzyme. It is not known from these studies whether its 3% cleavage is caused by the contamination of the substrate with the α-ketoside or is the result of the intrinsic property of the neuraminidases. Studies are underway in our laboratory to delineate these points.

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