Structure of Streptococcal Cell Walls

II. GROUP A BIOSE AND GROUP A TRIOSE FROM C-POLYSACCHARIDE

H. HEYMANN, J. M. MANNIELLO, L. D. ZELEZNICK,* and S. S. BARKULIS

From the Research Department, CIBA Pharmaceutical Company, Summit, New Jersey

(Received for publication, October 31, 1963)

On the basis of evidence presented or cited in a previous article (see Reference 1 for Paper I), the group-specific C-polysaccharide of Group A hemolytic streptococci is a polymer of l-rhamnose and N-acetyl-d-glucosamine in which the methylpentose units occur as 3- and 2,3-substituted, α-linked saccharides with terminal β-linked hexosamine molecules and possibly intracatenary hexosamine. The study gave little information bearing on the composition and length of the side chains attached to the 2-position of the branch-point rhamnose residues.

An attractive scheme to preserve at least some of the hexosamine-rhamnose linkages during partial degradation consists in hydrolysis of de-N-acetylated polysaccharide, because glycosidic linkages are greatly stabilized towards acid hydrolysis by the presence of a positively charged group on carbon atom 2 of the hexosamine. The study gave little information bearing on the composition and length of the side chains attached to the 2-position of the branch-point rhamnose residues.

EXPERIMENTAL PROCEDURE

Materials—Reduced C-polysaccharide was prepared as described earlier (1) and the resulting polysaccharide was quite similar to preparations obtained earlier; data appear in Table I. β-N-Acetylglucosaminidase (4). For enzymic liberation of terminal β-linked GlcNAc, the following procedure is typical. An aliquot containing 0.05 to 0.3 µmole of bound GlcNAc was evaporated to dryness or to a small volume, taken up in 50 to 1000 µl of buffer (2 ml of NaCl, 2 ml of m sodium citrate, pH 4.3, 0.2 ml of 1% bovine serum albumin, and H2O to make 20 ml) and treated with 10 to 50 µl of the enzyme preparation at 37° for a sufficient period of time (3 hours). Aliquots were analyzed directly by the Morgan-Elson reaction with borate or desalted by ion exchange or electrophoresis.

Ion exchange resins employed were Dowex 50W-X8 (200 to 400 mesh), and Amberlite IR-45; usually they were cycled with 4.3, 0.2 ml of 1% bovine serum albumin, and H2O to make 20 ml) and treated with 10 to 50 µl of the enzyme preparation at 37° for a sufficient period of time (3 hours). Aliquots were analyzed directly by the Morgan-Elson reaction with borate or desalted by ion exchange or electrophoresis.

Charcoal-Celite was prepared by acid treatment and dialysis of

* CIBA Fellow in Microbiology.

† We thank Dr. Levvy for a generous gift of hog epididymis N-acetylhexosaminidase. The preparation had a specific activity of 2,126,000 as defined by Findlay and Levvy (4).

‡ The following abbreviations and symbols are used: GlcN and GlcNAc, 2-deoxy-2-amino-n-glucose and its N-acetyl derivative; Rh, l-rhamnose; Glc, d-glucose; Rh-OH, l-rhamnitol; GlcNAc-Rh (Group A biose), 3-O-(2-deoxy-2-acetamido-β-D-glucopyranosyl) l-rhamnose; GlcNAc-Rh-Rh (Group A triose), 3 O 3 O (2-deoxy-2-acetamido-β-D-glucopyranosyl)-α-l-rhamnopyranosyl l-rhamnose; GlcNAc-Rh-OH and GlcNAc-Rh-Rh-OH represent products of borohydride reduction; Mα, Mβ, and Mε, electrophoretic mobilities relative to Glc, Rh, and GlcN, essentially as recommended by Barker, Bourne, and Theander (5). Evaporations were performed on rotary vacuum evaporators with bath temperatures of 40-45° or, in case of small samples, in vacuum desiccators over Ascarite and H2SO4. Occasionally, mineral acid was extracted from small volumes of aqueous solutions by means of 0.2 M di-n-acetyl methyl amine, (n-C10H21)2NCCH3, in chloroform.

Methods—Reductions by NaBH4 were performed at 4° overnight with a concentration of 10 mg per ml of reducing agent in an appropriate volume. The products were freed of sodium by passage over Dowex 50W-X8, and of boric acid by a triple evaporation, in vacuo, with methanol. When necessary, controls were included in which samples suspected of lability to alkaline were exposed to carbonate buffer, pH 9.8, for an equal length of time; in no case did the alkaline alone destroy or remove any part of the saccharides.

Periodate oxidations were performed at room temperature in the dark in 0.01 M NaO4 with a 2- to 3-fold excess of the oxidant over the expected consumption. The change in optical density at 225 mµ of samples diluted 1:100 was used as a measure of periodate uptake (6); a pilot experiment showed that, under the conditions employed, an oxidation period of 24 to 30 hours appeared optimal for the disaccharide to be described; hence the length of time was adhered to in further experiments. Excess oxidant was destroyed in defined aliquots with 1 N H2SO4 (0.75 volume) and 1 x sodium arsenite (0.25 volume). Formaldehyde was assayed by conversion to the dihydrolutidine with pentamidine-2,4 and NH2 (7); standard formaldehyde solution was made from commercial formalin, the concentration of which was determined by H2O2 alkaliometry and by dimedon precipitation. For unknown reasons, the chromotropic acid procedure of O'Dea and Gibbons (8) when applied to the periodate oxidation products of rhamnitol gives 10 to 30% more color than anticipated on the basis of formaldehyde formed; acetaldehyde is not the cause of this phenomenon. The Nash method is not subject to this interference.

The aeration procedures variously described (9) to remove acetaldehyde selectively from reaction mixtures were of no avail since we failed in our attempts to obtain reliable transfer of the aldehyde in the serveral pieces of apparatus designed and tested. The hydroxydiphenyl reaction of Eegriwe (10) was used as modified by Tompsett (9) with reduction in volume to 3.65 ml for greater sensitivity. The reaction is subject to interference by CH3O (11) but we found that as long as the ratio of formalddehyde to acetaldehyde does not exceed 1:1, the contribution of the lower aldehyde to absorption at 570 can be effectively corrected, for the optical density difference, O.D.570 - O.D.646, is...
the Morgan-Elson reaction was employed, catalyzed by either the Elson-Morgan reaction (13, 14) and, for N-acetylhexosamine, pH 10, or

\[ \text{proportional to the concentration of acetaldehyde. Pertinent data are included in Table VIII and in Fig. 1.} \]

Rhamnose was measured according to Dicke and Shettles (12). Free hexosamine was determined by suitable versions of the Elson-Morgan reaction (13, 14) and, for N-acetylhexosamine, the Morgan-Elson reaction was employed, catalyzed by either 0.05 \( \text{M} \) borate (15) or 0.05 \( \text{M} \) carbonate (16).

Paper electrophoreses were performed essentially according to Foster (17) on Whatman No. 3MM paper in 0.05 \( \text{M} \) borate, pH 10, or 2 \( \text{N} \) acetic acid.

Descending chromatography was performed with several solvent systems: Solvent A, 1-butanol-acetic acid-water, 62:15:25; Solvent B, pyridine-ethyl acetate-water, 5:12:4; Solvent C, 1-butanol-ethanol-water, 7:1:2; Solvent D, 1-butanol-ethanol-water-ammonia, 70:10:20:1; and Solvent E, 1-butanol-pyridine-water, 2:2:1. When Whatman No. 3MM or heavier paper was employed, the influx of solvent was kept at a slow rate by means of leaders of Whatman No. 1 paper sewn to the heavier sheets. An occasional thin layer chromatogram was obtained on silica gel with Solvent A. For visualization of the spots, alkaline silver nitrate (18), aniline hydrogen phthalate (19), or ninhydrin were used. Elutions of preparative paper chromatograms were routinely done in the descending manner by chromatographic percolation.

Nitrogen was determined by a microgram-Kjeldahl method (20). Infrared spectra were measured on the Perkin-Elmer double beam instrument, and optical rotations were measured with the Rudolph photoelectric polarimeter.1

### RESULTS

**Deacetylation of C-Polysaccharide**—Deacetylation was achieved by treatment at 100° with 0.49 \( \text{n} \) Ba(OH)\(_2\). In a pilot study, 21.22 mg of reduced C-polysaccharide in 2.5 ml of Ba(OH)\(_2\) was placed into sealed vials in 0.5-ml portions which were heated for varying lengths of time and freed of barium by CO\(_2\) and centrifugation. Infrared spectra of the samples in KBr disks were recorded and disappearance of amide absorption was noted after 30 minutes. In another series, the BaCO\(_3\)-supernatants were evaporated to dryness in Conway dishes in vacuo over anhydride and the volatile acid in each was determined by the Conway technique (21). Even though recovery of standard acetate was only 78\%, the method still gave insight into the time-course of liberation of volatile acid; in agreement with the infrared data, acid production slowed markedly after 30 to 45 minutes.

On a larger scale, 690 mg of reduced polysaccharide (Table I) was sealed in a 200-ml Kjeldahl flask with 90 ml of 0.44 \( \text{n} \) Ba(OH)\(_2\) immersed into a steam bath for 45 minutes, cooled, and neutralized with CO\(_2\) until the color of phenolphthalein was just discharged. The suspension was warmed on the steam bath for 1 hour to render the barium carbonate filterable, the clear filtrate and washings were concentrated under reduced pressure at 40°C and made up to 50.0 ml. A rhamnose determination showed that 312 mg (79.5\% of the amount initially present) had survived the alkali treatment. Dialysis of a 1-ml portion for 18 hours in the cold against 100 ml of water led to a distribution of 64\% inside versus 36\% outside the bag, in terms of rhamnose.

The deacetylated polysaccharide gave a weak ring test at a concentration of 700 \mu\text{g} per ml of rhamnose with Group A antisem known to respond to 1 \mu\text{g} per ml of the authentic hapten; there was no reaction even at 700 \mu\text{g} per ml with K-variant (22) antisem.

**Partial Hydrolysis of Deacetylated C-Polysaccharide**—A solution of the material described containing 305 mg of rhamnose in 21 ml of 2 \( \text{n} \) HCl was sealed in a Pyrex test tube and heated at 100°C for 30 minutes. This period of time was selected on the basis of a pilot experiment in which aliquots in 2 \( \text{n} \) HCl were heated and examined at intervals for maximal base-associated (i.e. retained by Dowex 50W) rhamnose; in the pilot experiment, about 13\% of all the rhamnose present was so retained after 30 minutes of hydrolysis.

The hydrolysate was passed over a 40-ml column of IR-45 acetate, reduced to 5 ml, made 0.01 \( \text{n} \) in HCl, and passed over a 40-ml column of Dowex 50W-X8 (H\(^+\)) (200 to 400 mesh). The effluent neutral fraction was found to contain 162 mg of rhamnose (53\% of starting material) in monomeric form, and a small quantity of N-acetylglucosamine (approximately 2.5 mg) on examination by column and paper chromatography, and colorimetric analysis.

The basic fraction was eluted from the Dowex 50 column with 50 ml of 2 \( \text{n} \) HCl. The acid was neutralized with solid sodium acetate and the solution was reduced to a small volume in vacuo. Sodium chloride was removed by filtration and addition of ethanol. The filtrates were freed of ethanol in vacuo, adjusted to 10 ml, and acetylated with 0.75 ml of acetic anhydride and 1.34 g of NaHCO\(_3\) for 30 minutes in ice and 30 minutes at room temperature.

### Table I

<table>
<thead>
<tr>
<th>Analyses of reduced C-polysaccharide</th>
<th>Weight from 100 mg</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhamnose</td>
<td>57.0</td>
<td>2.54</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>30.4</td>
<td>1.00</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>2.51</td>
<td>1.31</td>
</tr>
</tbody>
</table>

1 Infrared spectra and optical rotations were determined in the microanalytical laboratory of Mr. L. Dorfman.
showed the presence of GlcNAc and several chromatographically
electrophoresis in borate buffer, and paper chromatography
Morgan-Elson test with borate or carbonate or both, paper
volume. Examination of the fractions by rhamnose analysis,
and analyzed. The rhamnose content was 99 mg (0.603 meq)
GlcNAc-Rh-Rh
GlcN-Rh
GlcNAc-Rh-OH
collected with ethanol concentrations 0, 1, 5, 10, 15, 20% by
water were used as eluants and fractions of 150 to 200 ml were
Celite-545 column, 19 x 57 mm in size; mixtures of ethanol with
under borate catalysis and thus was presumably present in
buffer was employed. By difference, then, 0.347 mmole of
acetylglucosamine was not susceptible to chromogen formation
stoyred as ascertained by separate tests in appropriate carbonate buffer. 
The absence of nonreducible rhamnose is the
fraction contained 33.4 mg (0.155 mmole) of N-acetylglucosa-
0.2
or 32% of the quantity submitted to partial hydrolysis. The
acetylglucosamine basic fraction was applied to a 1:l charcoal-
charcoal gradient elution was applied to six 7-inch sheets of
biose fraction was located by means of guide strips, eluted, passed
in the Morgan-Elson test with borate, but do so with carbonate
(Table VI). Related fractions were pooled in three groups,
and each group was refractionated on smaller charcoal columns
with mixtures of alcohol-0.01 N formic acid (23) but again the
areas of overlap were considerable. The fractions were neutral-
ized by passage over IR-45 (-OH).
Isolation of Group A Biose—A fraction from the small charcoal
columns consisting largely of Group A biose (RglcNAc = 0.85)
was subjected to careful elution from a 13 ml charcoal-Celite
column with a solution of alcohol in 0.001 N formic acid increasing
linearly from 0 to 20% ethanol by volume. Material in the
effluent was located by rhamnose assay, and absence of GlcNAc
was ascertained by excluding fractions giving a Morgan-Elson test with borate. The tubes representing the center position of
the elution peak were pooled, deacidified, and dried to a glass.
A solution (2.205 mg per ml) was found to be homogeneous
chromatographically (Solvent A); it contained 932 µg per ml of
rhamnose, but on borohydride reduction (2.2 mg of saccharide,
1 ml of H2O, 12 mg of NaBH4 overnight at 4°) only 94% of the
rhamnose was found reducible. The acetylglucosamine was
liberated from the reduced disaccharide by Levery's epididymal
β-N-acetylglucosaminidase and estimated by Morgan-Elson test
(borate). The molar ratio of total rhamnose to GlcNAc was
1.05:1.00; whereas, the ratio of reducible rhamnose to GlcNAc
was 0.98:1.00. The absence of nonreducible rhamnose is the
most sensitive criterion available to ascertain the absence of
higher oligosaccharides from preparations of Group A biose.

The bulk of the Group A biose fraction resulting from the
charcoal gradient elution was applied to six 7-inch sheets of
Whatman No. 5MM paper and irrigated with Solvent A. The
biose fraction was located by means of guide strips, eluted, passed

### TABLE II

<table>
<thead>
<tr>
<th>Analyses of acetylated basic fractions from partial hydrolysis of desacetyl C polysaccharide</th>
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<tbody>
<tr>
<td>Starting material</td>
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<td>-------------------</td>
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<tr>
<td>momoles</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
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<tr>
<td>Total, by borate-catalyzed Morgan-Elson reaction</td>
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<tr>
<td>Free GlcNAc, by borate-catalyzed Morgan-Elson reaction</td>
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<tr>
<td>Carbonate-catalyzed GlcNAc</td>
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<td>Carbonate-catalyzed GlcNAc</td>
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### TABLE III

<table>
<thead>
<tr>
<th>Stoichiometric relationships among constituents of Group A biose and Group A triose and their derivatives</th>
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<tbody>
<tr>
<td>Sugar analyzed</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>GlcNAc-Rh</td>
</tr>
<tr>
<td>GlcNAc-Rh-OH</td>
</tr>
<tr>
<td>GlcN-Rh</td>
</tr>
<tr>
<td>GlcN-Rh-OH</td>
</tr>
<tr>
<td>GlcNAc-Rh-Rh</td>
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<tr>
<td>GlcNAc-Rh-Rh-OH</td>
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</tbody>
</table>

* Rhamnitol was not determined but calculated from the amount of reducible rhamnose submitted to the action of NaBH₄.
† Enzymic is to indicate the use of β-N-acetylglucosaminidase (see "Methods and Materials").
‡ At the pH, temperature, and duration of the NaBH₄ reductions, rhamnose, GlcN, and GlcNAc of the saccharides were not
destroyed as ascertained by separate tests in appropriate carbonate buffer.

slower saccharides; thus clean separation had not been achieved. At this time, we are reporting on two of the slower saccharides, i.e. GlcNAc-Rh, RglcNAc = 0.87, and GlcNAc-Rh-Rh, RglcNAc = 0.72 (Solvent A). For the sake of convenience, they will be referred to as Group A biose and Group A triose, respectively. It is convenient to mention here that both sugars fail to react in the Morgan-Elson test with borate, but do so with carbonate (Table VI).
over short charcoal columns that were rinsed with 90% ethanol, and dried to a hard glass. Repeated dissolution in ethanol, filtration, and drying served to remove most of the inorganic or polymeric contaminants, which tenaciously adhered to the material. Finally 31.8 mg of a hard glass of unknown moisture content resulted which was analyzed for rhamnose before and after reduction with borohydride. The sample contained 10.68 mg of rhamnose indicating that 75% of the sample weight was Group A biose and of this rhamnose at least 99.2% was reducible by borohydride. N-Acetylgulosamine estimated after enzymic hydrolysis of the N-acetylgulosaminylrhamnitol indicated a molar ratio of rhamnose to GlcNAc of 1.01:1.00 (cf. Table III). A periodate pattern obtained with material at this stage appears in Table V.

In an attempt at further purification, the material was chromatographed in water-saturated butanol on 3 g of analytical grade Celite holding 3 ml of butanol-saturated water. The column was percolated at the rate of 50 μl per minute, and a total of 30 ml was collected on the grounds of a pilot run with GlcNAc. The disaccharide was located on the extruded column with 1% KMnO₄ in 10% NaOH, sectioned out, and eluted with 25% ethanol. The disaccharide at this stage crystallizes readily soluble in methanol-ethanol-water; sparingly soluble in ether or acetone. The disaccharide was weighed 19.3 mg; much silica was removed by filtering, and drying served to remove most of the inorganic or polymeric contaminants, which tenaciously adhered to the material. Finally 31.8 mg of a hard glass of unknown moisture content proved insurmountable. The crude needles obtained was readily soluble in methanol-ethanol-water; sparingly soluble in ether or acetone. The disaccharide at this stage crystallizes readily readily; e.g. the wide part of a Pasteur pipet used for transfer of an alcoholic solution will soon show formation of microscopic needles; the thick syrup produced by addition of acetone to an aqueous-alcoholic solution will, on standing, solidify to well-shaped needles. However, the crystallization was disappointing because conditions to obtain a defined analytical sample by recrystallizing 15 mg of a highly soluble technical sample were not found; possibly the technical difficulties of recrystallizing 15 mg of a highly soluble substance proved insurmountable. The crude needles obtained

<table>
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<th>Table IV</th>
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<tr>
<td>Chromatographic and electrophoretic migration rates of Group A biose, Group A triose, and derivatives</td>
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<tr>
<td>Migration rates</td>
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<tr>
<td></td>
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<tr>
<td>Paper chromatography</td>
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<tr>
<td>Solvent A</td>
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<td>Solvent B</td>
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<tr>
<td>Solvent C</td>
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<tr>
<td>Solvent D</td>
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<tr>
<td>Solvent E</td>
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<tr>
<td>Thin layer chromatography</td>
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<tr>
<td>Paper electrophoresis</td>
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<tr>
<td>Borate, 0.05 M</td>
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<tr>
<td>Acetic acid, 2 N</td>
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</table>

<sup>*</sup> Tendency to double spot formation.  
<sup>†</sup> On SiO₂; Solvent A; anis-aldehyde spray.

from the syrup had m. p. 145-150° with decomposition; the disaccharide, estimated <i>es</i> its rhamnose content had [α]<sub>D</sub> = -6.7° (c. 0.49 in H₂O). Tables III, IV, and VI list chromatographic, electrophoretic, analytical, and colorimetric data. The periodate pattern obtained at this stage does not differ from that obtained prior to partition column chromatography.

**Isolation of Group A Triose**  Paper-chromatographic fractions containing the bulk of the triasaccharide, Group A triose, were pooled, freed by charcoal passage of the unavoidable extractives originating from the paper and examined for homogeneity by repeated paper chromatography; the quantity obtained at this stage was 7.70 mg or 15 μmoles of GlcNAc-Rh-Rh. An aliquot was found to contain 4.61 μmoles of rhamnose; the Morgan-Elson reaction with borate revealed no acylhexosamine, but with carbonate, color equivalent to 1.61 μmoles of GlcNAc was produced (cf. Tables III and VI). An aliquot containing 0.222 μmole of rhamnose was reduced with borohydride, whereupon the rhamnose content dropped to 0.115 μmole. An aliquot of the reduced trisaccharide solution (GlcNAc-Rh-Rh-OLH) containing 0.055 μmole of rhamnose was evaporated to dryness and treated with 30 μl of 0.011 M Na₂CO₃ at room temperature for 24 hours. NaI₀₄ and NaI₀₃ were removed with PbSO₄ and the supernatant was used for a rhamnose assay, in which 0.047 μmole (85% of the initial value), was found. When a periodate reaction mixture reduced with H₂SO₄ and Na₂S₂O₅ and containing 0.182 μmole per ml of oxidized GlcNAc-Rh-Rh OH was de salted electrolytically, rhamnose was again demonstrable but only to the extent of 0.070 μmole per ml, or 39% of the expected amount; the loss is believed due to diffusion during desalting.

**Table V**

<table>
<thead>
<tr>
<th>Periodate oxidation of GlcNAc-Rh-OH, GlcNAc-Rh-Rh-OH, and Rh OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharide oxidized</td>
</tr>
<tr>
<td>NaI₀₄ consumed</td>
</tr>
<tr>
<td>CH₂CHO produced</td>
</tr>
<tr>
<td>CH₃O</td>
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<tr>
<td>CH₃COOH</td>
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**Table VI**

<table>
<thead>
<tr>
<th>Molar extinction coefficients of Morgan-Elson chromophore</th>
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<tbody>
<tr>
<td>Sugar</td>
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<tr>
<td></td>
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<tr>
<td>GlcNAc</td>
</tr>
<tr>
<td>GlcNAc-Rh</td>
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<tr>
<td>GlcNAc-Rh-Rh</td>
</tr>
</tbody>
</table>

* Tendency to double spot formation.  
† On SiO₂; Solvent A; anis-aldehyde spray.
N-Acetylhexosaminidase cleaved GlcNAc-Rh-Rh into two fragments, separable by electrophoresis in borate, of which the slower moved and reacted like GlcNAc, and the faster, rhamnose-containing moiety resembled and is probably identical with a hitherto unreported rhamnoserhamnose occurring in 5% yield under various conditions of partial acid hydrolysis from C-poly-
saccharide, e.g. 0.5 h. Hitherto unreported rhamnosylrhamnose occurring in 5% yield
fragments, separable by electrophoresis in borate, of which the
tography in Solvent D, a homogeneous preparation of the free
negative. Acetylation with acetic anhydride in aqueous bi-
nose could not be detected and the Elson-Morgan test was
reduction, the rhamnose content drops to 50% of the initial
value. Analytical data regarding Group A triose are summarized
in Tables III to VI.

Isolation of GlcN-Rh—In one experiment, the hydrolysate of
decaylated C-poly saccharide was fractionated by elution of the
adsorbed basic compounds from Dowex 50W-X8 with a gradient
of HCl between 0.1 and 0.3 ν. By preparative paper chroma-
tography in Solvent D, a homogeneous preparation of the free
base GlcN-Rh was obtained; analytical details appear in Tables
III and IV. The substance contains rhhamnose and reacts posi-
tively in the Elson-Morgan test; after NaBH4 reduction, rham-
bose could not be detected and the Elson-Morgan test was
negative. Acetylation with acetic anhydride in aqueous bi-
carbonate as described above yielded Group A biose. Hydroly-
sis with aqueous 2 x HCl of GlcN-Rh for periods of from 2 to 8
hours at 100° led to partial destruction of the sugar moieties,
because Elson-Morgan analysis showed decreasing amounts of
hexosamine, and paper chromatograms revealed only feeble spots
for rhamnose.

| Table VII |

| Inhibition of complement fixation by saccharides obtained from or related to C-poly saccharide |

The system used was: 1:16 dilution of rabbit antiserum to
Group A type 14 trypsin-treated streptococcal cell walls (A4),
0.1 ml; formamide-extracted C-polysaccharide giving ring test
at 2 μg per ml (A5), 0.26 μg/0.1 ml, 0.1 ml; complement 1:25, 0.1
ml; sheep erythrocytes 2%; suspension containing a 1:400 sus-
pension of hemolysin, 0.2 ml; isotonic Veronal buffer, pH 7.3,
as needed; inhibitor solution in Veronal buffer, pH 7.3, 0.1 ml.
Appropriate controls showed correct functioning of the comple-
system, and the absence of anticomplementary activity
from the inhibitors or from the polysaccharide alone. The range
of lysis is from 0, no lysis, to 4, complete lysis.

When this preparation was repeated on a larger scale, about
6% of the total rhhamnose appeared in the early eluates from the
Dowex column. The material, though largely GlcN-Rh, was
inhomogeneous as shown by the presence of about 25% of non-
reducible rhhamnose, and by paper chromatography of the acetyled
mixture, which showed the presence of GlcNAc-Rh Rh and
even higher saccharides.

Complement Fixation and its Inhibition—The saccharides
GlcNAc-Rh and GlcNAc-Rh-Rh were examined for their ability to inhibit the reformation of Group A antiserum with the
fungus-specific antigen, the C-polysaccharide. Complement
fixation was used as a measure of antigen-antibody interaction
(25). Table VII lists the results; it is clear that Group A triose
is effective as an inhibitor at about one-tenth the concentration
needed of phenyl β-N-acetylhexosaminide, whereas Group A
biose inhibits at about one-half that concentration.

Discussion

The stabilizing effect exerted by the NH+3 groups of decacety-
lated C-polysaccharide on hexosaminide linkages permitted
about 32% of the total rhhamnose to remain base-linked on partial
hydrolysis. During the preceding deacetylation with hot
baryta, some depolymerization occurred as evidenced by forma-
tion of dialyzable material, but since we were interested in small
fragments, the entire deacetylation product was subjected to
partial hydrolysis, conditions for which were selected so as to
give a maximum of base-associated rhhamnose.

Of the rhhamnose, 79% was recovered in the decacetylation
mixture, and of this amount about one-third remained associated
with hexosamine in the partial hydrolysis. At least 54% of the
initial hexosamine survived the decacylation, and of that por-
tion, at least 70% remained attached to rhhamnose during partial
hydrolysis. These relations are summarized in Table II.

The value for total GlcNAc in Table II is the figure obtained
by carbonate-catalyzed Morgan-Elson reaction, whereas the
value entered there for free GlcNAc was obtained in a borate-
catalyzed reaction. The basis for the interpretation of the re-
results in Table II will be set forth presently.

By a lengthy process of fractionation described in detail in the
preceding section, homogeneous fractions were obtained consist-
ing of GlcN-Rh, GlcNAc-Rh, and GlcNAc-Rh-Rh.

It is noteworthy that gradient elution with hydrochloric acid
from Dowex 50W or with ethanol from charcoal-Celite failed to
effect sharp separation of GlcNAc from GlcNAc-Rh and
GlcNAc-Rh-Rh or of GlcN from GlcN-Rh and its higher
homologues. Paper electrophoresis, likewise, could not readily
distinguish Group A biose from Group A triose (Table II) and
of the various solvent systems used in paper chromatography,
only Partridge solvent (Solvent A) was truly effective. Com-
pared to a hexose disaccharide (lactose, RGlcNAc = 0.39 Solvent
D), GlcNAc-Rh moves far more rapidly, owing no doubt in large
measure to the organophilic C-6 methyl group. The acetylated
sugars (Group A biose, Group A triose) gave no color in the
borate-catalyzed Morgan-Elson reaction, but gave almost quan-
titative yields of Morgan-Elson chromogen when treated with
hot carbonate. Their exact stoichiometry was determined by
rhhamnose assay, and by acetylhexosamine determination
following enzymic cleavage with epichlorhydrin β-N-acetylhexo-
saminidase (4). In the case of Group A triose, the split products
were also separated electrophoretically into GlcNAc and a
rhamnosylrhhamnose that were analyzed separately. In Table

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TABLE VIII

Effect of formaldehyde on estimation of acetaldehyde by p-hydroxydiphenyl

<table>
<thead>
<tr>
<th>Mole ratio of CH$_2$CHO to CH$_3$OH</th>
<th>Relative optical density difference (O.D. 570-646)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>1.00</td>
</tr>
<tr>
<td>1:0.47</td>
<td>1.10</td>
</tr>
<tr>
<td>1:0.94</td>
<td>1.10</td>
</tr>
<tr>
<td>1:1.9</td>
<td>1.26</td>
</tr>
<tr>
<td>1:3.8</td>
<td>1.38</td>
</tr>
<tr>
<td>1:4.7</td>
<td>1.40</td>
</tr>
</tbody>
</table>

Fig. 2. Structures I, II, and III
gives a positive Morgan-Elson test with facility, in which two eliminations of a 3-O-substituent occur. (d) The periodate patterns shown in Table V demonstrate the simultaneous production of formaldehyde and acetaldehyde from the reduction products of Structures I and II. (e) The rhamnose-rhamnose linkage in Structure II is an α-1 → 3 bond on the basis of the methylolation and infrared absorption data reported earlier (1). (f) The GlcNAc rhamnose linkage is of β configuration because it is sensitive to a β-N-acetylhexosaminidase. (g) One-half of the rhamnose in Structure II is nonreducible and also stable to sodium periodate.

Color reactions and periodate patterns cannot usually replace classical tools of structure proof in oligosaccharide chemistry. However, in the present case Elson-Morgan, or Morgan-Elson chromogens are formed from the nonreducing terminals of the saccharides, in good yields, and under defined conditions of basic catalysis. Moreover, treatment of the reduced saccharides with periodate yields a unique combination of oxidation products. The concatenation of the results in the color and the oxidation reactions provides a firm basis for the Structures I, II, and III proposed in Fig. 2.

On electrophoresis in borate at pH 10, Group A triose migrates slightly but definitely faster than Group A biose, contrary to expectations since the number of complexing groups in the two molecules is the same and would lead one to anticipate a smaller charge density for the triose. The most stable conformation for the saccharides has the GlcNAc moiety in the C-1 arrangement, and the rhamnose portion, or portions, exist as 1-C chairs with trans-axial groups at atoms 1 and 2, which avoids the Δ2 instability factor of Reeves (30). Borate complex formation at the reducing terminal requires anomerization to give a cis-glycol grouping with concomitant destabilization. The borate complex so formed is somewhat encumbered by the acetylamino group of the hexosamine moiety in Structure I, as seen on examination of Stuart-Briegleb models. This steric interference is absent in Group A triose (II) where the central rhamnose acts as a spacer, and the observed Δ2 value for the triose may be rationalized as due to the ensuing increased stability of the borate complex.

It was of considerable interest to examine Group A biose and Group A triose for their ability to inhibit the reaction of Group A antiserum with C-polysaccharide. McCarty had found earlier (31) that serological specificity is associated with terminal GlcNAc, and that GlcNAc as well as phenyl β-N-acetylglucosaminidase are inhibitors of the precipitin reaction. Instead of the quantitative precipitin technique, we employed complement fixation as an indicator of antigen-antibody reaction. Wassermann and Levine (25) have described the use of a micro method for complement fixation in determining hapten inhibition of the reaction of pneumococcal polysaccharide with specific antiserum. Gelzer and Kabat4 have found that with dextrins and their antiserum the complement fixation and precipitin assays in general are parallel. As shown in Table VII, Group A triose is by far the most effective inhibitor among known degradation products of the group-specific polysaccharide. Even though determinants are not necessarily terminal groups of haptens, the conclusion appears valid that in C-polysaccharide at least two rhamnose units intervene between the branch-point and the terminal GlcNAc.

The available data on C-polysaccharide may be briefly summarized as follows: (a) the rhamnose to hexosamine ratio is 3:2, but higher ratios are found in the variant strains (22); (b) rhamnose is α-linked; (c) rhamnose is 3- and 2,3-substituted, the ratio being in the vicinity of 1:3 to 1:4; (d) terminal GlcNAc is β-linked; (e) McCarty found a tri- and tetrarhamnose fraction to be more effective than rhamnosylrhamnose in inhibiting the reaction of V-polysaccharide with V-antiserum (31); if we assume that the serologic similarity between V-polysaccharide and hexosaminidase-treated C-polysaccharide from Group A organisms bespeaks a chemical similarity, at least with regard to the determinant side chains, then groups of up to four rhamnose units extend from the branch-points to the terminal GlcNAc units; (f) Estrada-Parra, Heidelberger, and Rebers (32) destroyed all of the GlcNAc but none of the rhamnose by treating C-polysaccharide with periodate, and one-half of the rhamnose became dialyzable after the oxidation. This decrease in molecular size and similar observations made during the methylation study (1) suggest the existence of nonterminal GlcNAc, probably in 1, 6 linkage. Existence of nonterminal GlcNAc agrees with McCarty's (31) report that 50 to 70% but not all of the GlcNAc in various C-polysaccharide preparations is labile to induced bacterial N-acetylhexosaminidase.

At present, then, the existence of side chains incorporating up to four rhamnose units is supported by the evidence. We thus consider that structural elements as depicted in Fig. 3 may occur and recur in C-polysaccharide. The evidence for intracatenary hexosamine is largely indirect, but it appears to indicate that hexosamine plays a part in linking together subunits of the type shown in Fig. 3. However, hexosamine cannot be involved in all of the linkages symbolized by question marks in the figure in view of the periodate data cited (32); these findings suggest the existence of rhamnose homopolymer regions of molecular weight in excess of 5000. Consequently, consideration must also be given to the possibility that accumulations of GlcNAc occur along the backbone or even in side chains, but there is no direct evidence bearing on this point. It is hoped that further experimentation will allow to resolve some of the questions raised.

4 We are indebted to Mrs S. Harelick for assistance in performing the complement fixation assay.

J. Gelzer and E. A. Kabat, personal communication.
SUMMARY

1. The group-specific C-polysaccharide of Group A hemolytic streptococci has been de-N-acetylated and subjected to partial hydrolysis.
2. A fraction containing rhamnose associated with hexosamine was obtained, and from it a di- and a trisaccharide have been isolated.
3. The sugars have been characterized as 3-O-(2-deoxy-2-acetamido-β-D-glucopyranosyl)-L-rhamnose and 3-O-[3-O-(2-deoxy 2-acetamido-β-D-glucopyranosyl)-α-L-rhamnopyranosyl]-L-rhamnose.
4. The triose is a potent inhibitor of the reaction of C-polysaccharide with its specific antiserum, as determined by complement fixation assay.
5. The existence in C-polysaccharide of side chains containing up to four rhamnose units with terminal N-acetylglucosamine is probable. Possible structural subunits have been discussed.

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Structure of Streptococcal Cell Walls: II. GROUP A BIOSE AND GROUP A TRIOSE FROM C-POLYSACCHARIDE
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