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Polysaccharides of Type XIX Pneumococcus. III.[1] Chemical Structure of the Repeating Unit

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The oligosaccharide is minimum repeating unit of the type specific polysaccharide, was prepared by the treatments with mild alkali, acid, and then acid phosphatase.

The oligosaccharide, $[\alpha]_D$ +20° (H_2O), which was composed of L-rhamnose, D-glucose, and N-acetyl-D-mannosamine=2:1:1.1.

From the results of periodate oxidation and methylation studies, the most probable structure of the tetrasaccharide was discussed.

In the previous paper,[1] we showed that the type specific polysaccharide, S-XIX, composed of residues of L-rhamnose, D-glucose, N-acetyl-D-mannosamine, and phosphate in an approximate ratio of 2:1:1:1, and acid phosphatase treatment liberated 7.5% of the total phosphate. When the phosphatase-treated S-XIX was hydrolyzed with dilute alkali, its serological activity disappeared and two main oligosaccharides, F1 and F2, were isolated. Both oligosaccharides have approximately the same carbohydrate composition as the original polysaccharide but acid phosphatase-treatment of F2 liberated 64.1% of its phosphate residues. Further successive treatments of this product with acidic and basic ion-exchange resins resulted in its conversion to F1.

These results suggested that the oligosaccharide F1 is a minimum repeating unit of the type specific polysaccharide.

In this communication, chemical structure of the repeating unit, F1, will be discussed.

Preparation of the repeating unit was carried out as follows: S-XIX was treated with 0.1N sodium hydroxide solution at 37° for 48 hr, and the reaction mixture was passed through a column of Amberlite IR-120 (NH_4^+), the eluate and washings were combined, and dialyzed against distilled water. The yielded dialyzable substance was hydrolyzed with 0.1N sulfuric acid at 37° for 24 hr and, after neutralization of the hydrolyzate, it was treated with wheat germ acid phosphatase. The reaction mixture was passed through columns of Amberlite IR-120 (H^+) and IRA-400 (OH^-) resins. The eluate was concentrated *in vacuo* to a syrup and purified by paper chromatography. Thus obtained oligosaccharide, F1, showed $[\alpha]_D$ +20° (c=1.2, H_2O) and its infrared (IR) spectrum is shown in Fig. 1. The absorbance at 1640 and 1550 cm^{-1} seem to suggest that acetamido group exists in F1 same as in S-XIX.

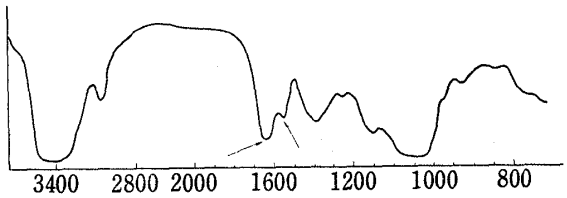


Fig. 1. Infrared Absorption Spectrum of Tetrasaccharide (KBr Tablet)

Acid hydrolysis of F1 gave rhamnose, glucose, and mannosamine. Molar ratio of the component sugars, rhamnose: glucose: N-acetylmannosamine=2:1:1.1, was determined by the methods of Dische[3] and of Blix.[4]

1) Part II: T. Miyazaki and T. Yadomae, *Carbohydrate Res.*, "in press."
2) Location: *600 Kashiwagi 4-chome, Shinjuku-ku, Tokyo.*
3) Z. Dische, *Methods Carbohyd. Chem.*, **1**, 488 (1962).
4) S. Gardell, *Acta Chem. Scand.*, **7**, 207 (1953).

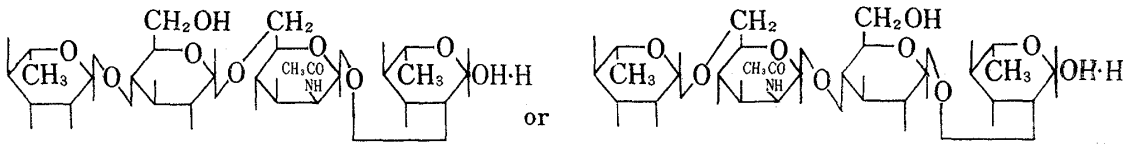
Quantitative estimation of the reducing end of F1 by the Somogyi–Nelson method[5] revealed the respective ratio of the reducing end to monosaccharide unit of 1:0.258 calculated as rhamnose. Therefore, it is certain that the repeating unit, F1, is a tetrasaccharide.

On periodate oxidation of F1, the consumption of periodate[6] per tetrasaccharide was 5.48 (after 24 hr), the value of formic acid[7] liberated from F1 was 1.37 (after 24 hr), that of acetaldehyde[8] was 1.00 (after 24 hr), and that of formaldehyde[9] was 0.235 (after 30 hr). The periodate oxidized F1 was then treated with sodium borohydride followed by mild acid hydrolysis. Thin-layer chromatographic analysis of this hydrolysate revealed the presence of propanediol, glycerol, and erythritol but not rhamnose, glucose, mannosamine, or N-acetyl-mannosamine.

Methylation of F1 using Hakomori method,[10] followed by formic and sulfuric acid hydrolysis was also carried out. The O-methylmonosaccharides so obtained were examined by paper chromatography, thin-layer chromatography, and paper electrophoresis. Tetra-O-methyl-sugar was not detected, and tri-O-methylmonosaccharides were identified as 2,3,4-tri-O-methyl-L-rhamnose and 2,3,6-tri-O-methyl-D-glucose. The values of Rf and M_G of these tri-O-methylmonosaccharides were identical with those of the authentic samples. Di-O-methyl fraction on the thin-layer chromatogram was identified with the authentic sample as 3,4-di-O-methyl-L-rhamnose by paper electrophoresis using 0.026M borate buffer (M_G: 0.31).

From the above results on periodate oxidation and methylation, one of the rhamnose residues exists as C2-linked reducing end which can produce acetaldehyde, glycerol and 3,4-di-O-methyl-L-rhamnose, and the other rhamnose residue should exist as the non-reducing end because it yielded propanediol and 2,3,4-tri-O-methyl-L-rhamnose. Glucose residue in F1 should exist as 1→4 linked pyranose because of yielding erythritol and 2,3,6-tri-O-methyl-D-glucose.

On the other hand, N-acetyl-D-mannosamine in F1 disappeared by the periodate oxidation and, therefore, it is suggested that the sugar residue will be linked C-6 and C-1 which can produce glycerol, but in the case of methylation, reliable methylated amino-sugar could not be detected due to the small quantity of the material used. Therefore, the most probable structure of the repeating unit would be:



The mode of phosphate linkages and total structure of S-XIX will be discussed in a later communication.

Experimental

Methods and Materials——Material: Type S-XIX sample was generously supplied by E.R. Squibb & Son's through the kindness of Prof. M. Heidelberger.

Analytical Method: Paper chromatography (PPC) was carried out by the ascending method on Toyo Roshi No. 50 filter paper and the following solvent systems (v/v):

5) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).
6) L. Malaprade, *Bull. Soc. Chim. France*, **1**, 5, 833 (1934).
7) R.L. Whilstler and J.L. Hickson, *J. Am. Chem. Soc.*, **76**, 1671 (1954).
8) B.F. Folkes, *Analyst*, **78**, 496 (1953).
9) J.F. O'Dea and R.A. Gibbons, *Biochem. J.*, **55**, 580 (1953).
10) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).

A) AcOEt:pyridine:H_2O=10:4:3
B) AcOEt:pyridine:AcOH:H_2O=5:5:1:3
C) $CH_3CH_2CH_2OH$:NH_4OH:H_2O=6:3:1
D) AcOEt:pyridine:EtOH:H_2O=3:2:2:2
E) AcOEt:AcOH:H_2O=9:2:2

Thin-layer chromatography (TLC) was carried out by the ascending method on silica gel plates (Wako Gel B-5), using the following solvent systems (v/v):

F) MeOH:AcOH:H_2O=5:1:2
G) $CH_3(CH_2)_3OH$:EtOH:HCOOH:H_2O=5:5:1:1
H) acetone:EtOH=1:1
I) acetone:C_6H_6=1:1
J) MeOH:acetone:NH_4OH=5:5:1
K) MeOH:AcOEt=1:5

Sugars were detected with the following spray reagents: Alkali silver nitrate,[11] *p*-anisidine hydrochloride,[12] Elson-Morgan reagent,[13] and Molisch reagent.[14]

Preparation of Repeating Unit——Type-specific polysaccharide (200 mg) was hydrolyzed in a 30 ml glass-stoppered flask with 0.1N NaOH (10 ml) at 37° for 48 hr. The reaction mixture was passed through a column of Amberlite IR-120 (NH_4^+) resin and evaporated to a small volume. The solution was dialyzed in Visking tubing against distilled water, and the dialysis was repeated four times. After the outer solution was concentrated to a small volume, the concentrate was examined by TLC. Ascending TLC (in solvent F) revealed one major product which contained phosphate and showed a purple color with Molisch reagent.

The non-dialyzable material in the tube was concentrated to a small volume and EtOH was added to this concentrate. The precipitate that appeared was collected by centrifugation.

The dialyzable fragment after alkali hydrolysis was then hydrolyzed with 0.1N H_2SO_4 at 37° for 24 hr. After neutralization with $BaCO_3$ and filtration, the hydrolysate was passed through a column of Amberlite IR-120 (H^+) resin and the eluate was concentrated to dryness *in vacuo*. TLC in solvent K did not reveal the spot corresponding to the position of glucose or rhamnose.

After treatment with mild acid hydrolysis, the dialyzable fragment from alkali hydrolysis was mixed with wheat germ acid phosphatase (2 mg) and dissolved in 0.05M acetate buffer containing $MgCl_2$ (pH 5.6). The solution was kept at 37° for 72 hr and enzymic hydrolysate consecutively passed through columns of Amberlite IR-120 (H^+) and Amberlite IRA-400 (OH^-) resins. The eluate was repeatedly dialyzed in a Visking tubing against distilled water. The combined outer solution was evaporated to dryness *in vacuo*, the remaining solid was dissolved in a small quantity of H_2O and separated by PPC using the solvent system (D). The homogeneity of the oligosaccharide thus obtained was confirmed by TLC in solvent systems (G) and (J).

Properties of the Repeating Unit——This oligosaccharide showed $[\alpha]_D$ +20° (c=1.2, H_2O) and its IR spectrum is shown in Fig. 1.

Acid Hydrolysis——The oligosaccharide (3 mg) was hydrolyzed in a sealed tube with 1N HCl (1.5 ml) at 100° for 12 hr. The acid was removed *in vacuo* and the residue was chromatographed in solvent (B). Glucose, rhamnose, mannosamine, and unidentified component were detected.

Quantitative Estimation of the Component Sugars of Oligosaccharide——a) Mannosamine: A sample of the oligosaccharide (1 mg) was hydrolyzed with 1N HCl (1 ml) at 100° for 11 hr and then the hydrolysate was neutralized with 1N NaOH (1 ml). The amount of mannosamine in 0.5 ml of the neutralized hydrolysate was estimated colorimetrically by the modified method of Blix[4] using N-acetylglucosamine, which was hydrolyzed under same condition with the oligosaccharide, as a standard substance. The content of mannosamine in 0.5 ml of the sample solution calculated as N-acetylglucosamine was 92.6 μg.

b) Neutral Sugars: The content of neutral sugars in 0.2 ml of the sample solution was determined colorimetrically by the method of phenol-H_2SO_4 (Dubois[15]) was 36.2 μg.

c) Ratio of Neutral Sugars[3]: Oligosaccharide (1 mg) was dissolved in H_2O (20 ml), this sample solution (1 ml) was cautiously added to ice-cooled 86% H_2SO_4 (5 ml), and then cooled for 1 min. The mixture was then heated in boiling water bath for 3 min, cooled, 3% cysteine hydrochloride (0.1 ml) was added to this solution and the solution mixed well. After 10 min, the optical densities at 380 and 415 mμ were read. The difference D_{415}–D_{380} is proportional to the concentration of hexose in the solution. Similarly, the content of methyl pentose was determined using the Dische procedure and measuring the optical densities at 390 and 425 mμ. The molar ratio of rhamnose and glucose was 1.99:1.

These results indicated the ratio of rhamnose, glucose, and N-acetylmannosamine to be 2:1:1.1.

11) W.E. Trevelyan, D.P. Protector, and J.S. Harrison, *Nature*, **166**, 444 (1950).
12) L. Hough, J.K.N. Jones, and W.H. Wadman, *J. Chem. Soc.*, 1702 (1950).
13) D. Aminoff and W.T.J. Morgan, *Nature*, **162**, 579 (1948).
14) H. Molisch, *Monatsch.*, **7**, 108 (1886).
15) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

Reducing Power of the Oligosaccharide[5]——Determination of reducing power was carried out as follows: Oligosaccharide (1.23 mg) was dissolved precisely in 20 ml of H_2O, 2 ml of this solution was heated with 2 ml of the copper reagent for 30 min at 100°, and cooled in running water for 3 min. Two ml of the Nelson reagent was added to the solution and the mixture was stirred gently. Absorption of the intense blue color that appeared was read at 500 mμ. Its absorption intensity was 0.19. The control experiment was conducted in the same way using 40 μg of rhamnose and its intensity was 0.24. Therefore, the reducing power of the oligosaccharide revealed the respective ratio of reducing end group to monosaccharide unit of 1.0:0.258, calculated as rhamnose.

Periodate Oxidation——The oligosaccharide (24.4 mg) was dissolved in 10 ml of distilled water and its total volume was made up to 25 ml with 2 ml of 0.22M $NaIO_4$ and distilled water. The mixture was allowed to stand in the dark at room temperature and determinations of the consumption of $NaIO_4$ and the production of HCOOH, HCHO, and CH_3CHO, were carried out with this solution by the procedures of Malaprade,[6] Whilstler,[7] O'Dea and Gibbons,[9] and Folkes.[8]

a) CH_3CHO: The reaction mixture (0.4 ml) was pippetted into the outer chamber of a microdiffusion unit,[16] 2 ml of 1% $NaHSO_3$ was placed in the center chamber, and the unit was kept at room temperature for 5 hr. Then 1 ml of 1% $NaHSO_3$ was transferred by pipette into a test tube and 0.1 ml of 4% $CuSO_4$ was added to the solution. The tube was cooled in an ice bath for 5 min, and then 10 ml of conc. H_2SO_4 was added. The reaction mixture was cooled in an ice bath for 5 min and transferred to a water bath of 27°. After 150 min, the color intensity was read at 570 mμ. Rhamnose was used as the standard solution of acetaldehyde.

The number of moles of $NaIO_4$ consumed per oligosaccharide were as follows: 2.74 (1 hr), 3.77 (3 hr), 5.07 (6 hr), 5.20 (12 hr), and 5.48 (24 hr).

The value of formic acid was 0.15 (1 hr), 0.49 (3 hr), 0.68 (6 hr), 1.10 (12 hr), and 1.37 (24 hr).

Formaldehyde was 0.235 (30 hr) and acetaldehyde was 0.9 (3 hr) and 1.005 (24 hr).

Smith-Type Degradation of the Oligosaccharide——The oligosaccharide (6 mg) was dissolved in H_2O (9.2 ml), 0.22M $NaIO_4$ (0.8 ml) was added to the solution, and the mixture was kept at room temperature in the dark for 12 hr. The solution was passed through columns of Amberlite IRA-400 (OH^-) and Amberlite IR-120 (H^+). The eluate was concentrated to an adequate volume at below 30° *in vacuo*. To this concentrate was added $NaBH_4$ (20 mg) and the mixture was kept for 16 hr at room temperature with stirring. Excess of $NaBH_4$ was decomposed with a few drops of AcOH, the solution was passed through columns of Amberlite IR-120 (H^+) and IRA-400 (OH^-) resins, and the eluate was concentrated to dryness *in vacuo*. The residue was hydrolyzed with 0.1N H_2SO_4 (10 ml) at 100° for 6 hr. The hydrolysate was passed through a column of Amberlite IRA-400 (OH^-) resin, the acid was removed, and the eluate concentrated to a small volume. The concentrate was examined by PPC and TLC using solvent systems (A) and (H). Three distinct spots corresponding to propanediol, glycerol, and erythritol were detected with alkaline silver nitrate reagent.

Another sample (7.8 mg) of the oligosaccharide was oxidized with $NaIO_4$ and the product was reduced with $NaBH_4$ by the same condition as above. The material was hydrolyzed under a more drastic condition (4N HCl) employed in the detection of mannosamine. PPC and TLC of the hydrolysate showed the spots described above.

Methylation of the Oligosaccharide[10]——NaH (200 mg) was mixed with 2 ml of $(CH_3)_2SO$ and the mixture was heated 65—70° for 1 hr with stirring. To this solution was added the oligosaccharide (6 mg) in 2 ml of $(CH_3)_2SO$ with stirring. After 10 min, 4 ml of CH_3I was added dropwise to the reaction mixture with stirring at room temperature and the mixture was stirred for 6 hr. All the procedures were carried out in nitrogen atmosphere. The solution was extracted repeatedly with a mixture of H_2O and $CHCl_3$ (1:1, v/v).

The methylated product thus obtained was methylated again under the above condition. The final product was extracted with ether and the solvent was evaporated *in vacuo*.

Acid Hydrolysis of Methylated Oligosaccharide——The methylated product was hydrolyzed by heating with 90% HCOOH (5 ml) for 1 hr at 100°, HCOOH was removed, and heated with 0.5N H_2SO_4 (10 ml) for further 5 hr. The hydrolysate was neutralized with $BaCO_3$, $BaSO_4$ was filtered off, and the filtrate was concentrated *in vacuo*. The residue was examined by PPC and TLC, and also by paper electrophoresis. The syrup was separated on TLC impregnated with solvent (I). The three distinct spots on the plate corresponded to the tri-O-methyl rhamnose, tri-O-methylglucose, and di-O-methyl rhamnose were detected by anisidine spray and Molisch reagent spray.

The tri-O-methylrhamnose fraction gave a single yellow spot by *p*-anisidine on PPC and TLC, and its *Rf* value was identical with that of an authentic sample of 2,3,4-tri-O-methyl-L-rhamnose.

The tri-O-methylglucose fraction, separated on thin-layer plate, was examined further by paper electrophoresis using 0.026M borate buffer and PPC, and gave a single brown spot corresponding to 2,3,6-tri-O-methyl-D-glucose.

16) E.J. Conwey, "Microdiffusion Analysis and Volumetric Error," Crosby Lockwood and Son Ltd., 1950.

The di-O-methylrhamnose fraction was examined under above condition, showed a single spot (M_G 0.31). The component of M_G 0.31 was identified with that of an authentic sample of 3,4-di-O-methyl-L-rhamnose.

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