Somatic Antigens of *Shigella*

Structural Investigation on the O-Specific Polysaccharide Chain of *Shigella Dysenteriae* Type 1 Lipopolysaccharide

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The O-specific polysaccharide obtained from the lipopolysaccharide of *Shigella dysenteriae* type 1 (*Shigella shiga*) by mild acid hydrolysis followed by fractionation on Sephadex G-50 was found to be identical to that described by Morgan’s group and was composed of L-rhamnose, D-galactose and N-acetyl-D-glucosamine in a ratio 2:1:1. On the basis of methylation analysis data the polysaccharide was proved to be a linear chain of monosaccharide residues in pyranose forms substituted at position 3, except for that of galactose substituted at position 2.

Selective cleavage, based on the N-deacetylation reaction of the polymer, together with determination of linkage configurations by chromic anhydride oxidation showed that the O-specific polysaccharide is built up of repeating tetrasaccharide units whose proposed structure is given below

\[-3)-\alpha-L-Rhap(1-3)-\alpha-L-Rhap(1-2)-\beta-D-Galp(1-3)-\alpha-D-GlcNAcp(1-\]

where Rhap = rhamnopyranose, Galp = galactopyranose, and GlcNAcp = N-acetyl-glucosamine.

The present findings confirmed the considerations of Heidelberger on the substitution patterns of L-rhamnose and D-galactose residues from the results of serological studies.

*Shigella dysenteriae* type 1 (*Sh. shiga*) is the most investigated from ten serotypes of *Shigella dysenteriae* subgroup and its O-specific polysaccharide was found to be composed of L-rhamnose, D-galactose and N-acetyl-D-glucosamine residues [1,2]. Moreover, as follows from the data of Heidelberger and coworkers [3] on serological relationship between this polysaccharide and two K-specific polysaccharides from *Pneumococcus* types 2 and 6, it was proposed that rhamnose and galactose residues in the *Sh. dysenteriae* polysaccharide were substituted at positions 3 and 2 respectively. In spite of these findings, the complete chemical structure of the antigenic polysaccharide from *Sh. dysenteriae* type 1 has not previously been established.

In the course of systematic structural investigations on *Sh. dysenteriae* lipopolysaccharides, we have performed the chemotyping of all ten lipopolysaccharides of this subgroup [4], determined the structures of polysaccharide chains of serotypes 3 [5,6] and 6 [7], and discovered a new type of acidic monosaccharides [8] which were found to be the components of these O-antigens.

The present paper describes data on the chemical structure of the oligosaccharide repeating unit of the O-specific polysaccharide chain of *Sh. dysenteriae* type 1 lipopolysaccharide.

MATERIALS AND METHODS

**Paper Chromatography, Electrophoresis and Gel Filtration**

Descending paper chromatography was performed on Filtrak FN-11 paper using ethyl acetate/acetic acid/formic acid/water (18/3/1/4, v/v/v/v). Paper electrophoresis was carried out with 0.025 M pyridine/acetate buffer pH 4.5 at 28 V/cm for 90 min. Oligosaccharides were detected with alkaline silver nitrate after a brief treatment with potassium periodate solution. Gel-filtration chromatography was carried out with Sephadex G-50 in pyridine/acetate buffer...
(column 55 x 3.7 cm), and Biogel P-2 in water at 65 °C (column 100 x 2.5 cm), elution curves being recorded with a Technicon sugar analyzer SC-2.

Gas-Liquid Chromatography and Mass Spectrometry

Gas chromatography was accomplished with a Pye Unicam series 104 (model 64) instrument with a flame-ionisation detector on a glass column (90 x 0.4 cm) packed with 3% ECNSS-M on Gas-Chrom Q (100–120 mesh, column A), and on a stainless-steel column (150 x 0.4 cm) packed with 3% SE-30 on Diatomite CQ (100–120 mesh, column B). The nitrogen flow-rate was 45 ml/min.

Mass spectrometry combined with chromatography was performed on a Varian instrument GNOM 111 with the use of column packed with 10% SE-30. Mass spectra were measured with a Varian CH-6 instrument with a direct probe inlet and an ionization potential of 70 eV.

Ion-Exchange Chromatography

Hexosamine analysis was carried out on a column (27 x 0.9 cm) packed with Chromex UA-8 resin and fitted in an amino-acid analyzer: BC-200 (Biocal). The column was eluted with 0.35 M sodium citrate buffer pH 5.28 at a flow rate of 80 ml/h.

Neutral sugar anion-exchange chromatography was accomplished with the Technicon SC-2 system on a column (25 x 0.6 cm) packed with anion-exchange resin DAx4 (Durrum, USA) in 0.5 M sodium borate buffer pH 9.0 at 55 °C and at an elution rate of 60 ml/h.

Miscellaneous Methods

Infrared spectra were measured with a UR-10 Karl Zeiss spectrometer in potassium bromide pellets. Proton magnetic resonance spectrum of the polysaccharide was recorded using a Varian XL-100 instrument at 90 °C in deuterium oxide with an external standard. Optical rotations were determined with a Perkin Elmer polarimeter model 141. Solutions were concentrated in vacuo at 40 °C.

Isolation of Polysaccharide

The lipopolysaccharide was extracted from dry cells of *Sh. dysenteriae* type 1, strain 1362, with aqueous phenol according to the standard procedure [9]. The lipid-A-free polysaccharide was prepared from lipopolysaccharide (600 mg) by hydrolysis with 1% acetic acid (60 ml, 1.5 h, 100 °C) and then separated into a high-molecular-weight fraction (150 mg) and an oligosaccharide (core) fraction (170 mg) by gel-filtration chromatography on the Sephadex G-50 column.

Serological Tests

Passive haemagglutination tests and inhibition of passive haemagglutination reaction were carried out at a successive dilution of antiserum containing two haemagglutinating units with sheep erythrocytes, sensitised by lipopolysaccharide, and antiserum against the live and autoclaved culture [4].

Determination of Monosaccharide Composition

Quantitative determination of neutral monosaccharides was performed with the Technicon analyzer after acid hydrolysis of polysaccharide (0.5 mg) with 2 M hydrochloric acid at 100 °C for 3 h followed by evaporation and drying of the residue in vacuo over sodium hydroxide.

Hexosamine was determined in the hydrolysate of the polysaccharide (0.5 mg) with 4 M hydrochloric acid at 100 °C for 4 h.

Simultaneous quantitative determination of neutral sugars and hexosamines by gas-liquid chromatography was performed as described previously [5,10].

Methylation Analysis

The polysaccharide (10 mg) was methylated with methyl iodide in the presence of methyl sulphynil anion [11]. The analysis and identification of partially methylated hexosamine in the form of methyl hexosaminide and neutral sugars in the form of alditol acetates were performed as described previously [5] with the use of the data of Björndal *et al.* [12] on fragmentation patterns of methylated alditol acetates.

N-Deacetylation and Selective Cleavage of Polysaccharide

The polysaccharide (140 mg) was dried in vacuo over phosphoric anhydride at 70 °C and then heated with anhydrous hydrazine (2 ml) containing hydrazine sulphate (200 mg) in a sealed tube for 36 h at 105 °C. Hydrazine was removed by evaporation, and the residue was dried over sulphuric acid in vacuo and was then purified by gel-filtration on the Sephadex G-50 column. Fractions eluted within the void volume of the column were pooled and freeze-dried to give the N-deacetylated polysaccharide (130 mg).

A portion of the N-deacetylated polysaccharide (5 mg) was hydrolysed with 2 M hydrochloric acid (0.5 ml) for 3 h at 100 °C, evaporated to dryness and the product was fractionated by paper electrophoresis.
A solution of N-deacetylated polysaccharide (25 mg) in water (0.5 ml) was treated successively with a 5% aqueous solution of sodium nitrite (1.5 ml) and with 33% acetic acid (1.5 ml) and then kept for 40 min at room temperature. The mixture was passed through a column of KU-2 (H+) resin and then freeze-dried, and the residue was reduced with sodium borodeuteride (10 mg) in water (1 ml). After routine treatment, the product was subjected to preparative paper chromatography.

Configuration of Glycosidic Linkages

Determination of the configuration of glycosidic linkages in the polysaccharide was performed by the chromic anhydride oxidation method [13, 14].

RESULTS

Isolation and Characterization of Polysaccharide

The lipopolysaccharide of *Sh. dysenteriae* type 1 was isolated in 4% yield from dry bacterial cells by extraction with hot, aqueous phenol followed by precipitation of nucleic acids with Cetavlon and ultracentrifugation. The high type 1 specificity of the material obtained was confirmed in the passive haemagglutination reaction (titer 51200) and in the inhibition test (active dose 0.4-0.8 pg).

The lipopolysaccharide was hydrolysed with dilute acetic acid to split off lipid A, and the resulting degraded polysaccharide was subjected to chromatography on a Sephadex G-50 column to give two fractions. A polysaccharide fraction was eluted in the void volume of the column; the second fraction was thought to be core oligosaccharide and was not further investigated.

The polysaccharide inhibited the agglutination of erythrocytes in a dose of 3.9-7.8 μg, it had an optical rotation \([\alpha]_D^{20} +88.5^\circ\) (c 0.55 in water) and infrared bands at 1650 and 1560 cm\(^{-1}\) (amide) and 3400 cm\(^{-1}\) (hydroxyl). The proton magnetic resonance spectrum of the polysaccharide contained signals for carbohydrate protons, one N-acetyl group (δ = 2.05 ppm) and two C-methyl groups of 6-deoxyhexose residue (δ = 1.49 ppm) in the form of two overlapping doublets. The data proved the polysaccharide to be neutral and to contain 6-deoxyhexose and N-acetyl-hexosamine residues in the ratio 2:1. After hydrolysis of the polysaccharide, rhamnose (42%), galactose (21%), trace amounts of glucose, and N-acetyl-glucosamine (23%) were identified by ion-exchange technique. The simultaneous determination of neutral sugars and hexosamine by gas-liquid chromatography [5, 10] showed that galactose, rhamnose and glucosamine were present in the ratio 1:1.85:1. The absolute configuration of monosaccharide constituents (L-rhamnose, D-galactose and 2-acetamido-2-deoxy-D-glucose) was evident from the data of Morgan's group [2] who first described the *Sh. dysenteriae* type 1 specific polysaccharide.

Methylation Analysis

The polysaccharide was methylated according to the procedure of Hakomori [11]. A portion of methylated polysaccharide was subjected to methanalysis followed by acetylation and investigation of hexosaminide component by gas chromatography/mass spectrometry. The detected anomers of methyl 2-(N-methyl)acetamido-2-deoxy-3-O-acetyl-4,6-di-O-methyl-glucopyranoside were found to be identical in regard to their retention times and mass spectra with the mixture of authentic anomers obtained from benzyl 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-α-D-glucopyranoside [15] by methylation and subsequent methanalysis (Fig. 1).

Another portion of methylated polysaccharide was hydrolysed, reduced with sodium borodeuteride and investigated after acetylation by gas-chromatography/mass spectrometry. As a result, 2,4-di-O-methyl-rhamnose and 3,4,6-tri-O-methyl-galactose in the
ratio 2:1 were identified as the neutral methylated sugars in the hydrolysate. The results of methylation analysis indicated that the polysaccharide from *Sh. dysenteriae* type 1 was a linear polymer built up of 2-O-substituted galactopyranose, two 3-O-substituted rhamnopyranose and 3-O-substituted N-acetyl-glucosamine residues.

**N-Deacetylation and Selective Cleavage of Polysaccharide**

The polysaccharide was N-deacetylated with anhydrous hydrazine in the presence of hydrazine sulphate. The N-deacetylated polysaccharide was quantitatively recovered from the void volume of the Sephadex G-50 column and the extent of the N-deacetylation, evaluated on the basis of acid hydrolysis data (see below), was found to be not less than 95%.

The modified polysaccharide was treated with hydrochloric acid, and two components containing free amino groups were detected in the hydrolysate in the ratio 2:1. The minor component was identified as glucosamine and its quantity was negligible. The cleavage of the main product with nitrous acid resulted in formation of equal amounts of 2,5-anhydromannose and rhamnose, which were identified by gas-liquid-chromatography of their alditol acetates, thus proving its structure as a disaccharide glucosaminyl-rhamnose (I).
Further, the N-deacetylated polysaccharide was deaminated with sodium nitrite in dilute acetic acid, and gel-filtration chromatography of the reaction product on Biogel P-2 column revealed no polymer but two oligosaccharides (II and III, Scheme 1) in the ratio 1:3. The mixture of oligosaccharides was reduced with sodium borodeuteride and then separated by paper chromatography to give two glycosyl-alditols IV and V respectively. Acid hydrolysis of the more-mobile glycosyl-alditol IV gave rhamnose and galactitol in the ratio 2:1 and this proved that oligosaccharide II was a linear trisaccharide rhamnosyl-rhamnosyl-galactose, for it followed from methylation analysis that the polysaccharide was linear. Acid hydrolysis of glycosyl-alditol V gave two equivalents of rhamnose and equimolar amounts of 2,5-anhydromannitol and galactose, i.e. the same monosaccharides and in the same proportion as in the starting polysaccharide. Thus, oligosaccharide III may represent the modified chemical repeating unit of the specific polysaccharide from *Sh. dysenteriae* type 1. Oligosaccharides II and III are proposed to arise from the N-deacetylated polysaccharide due to the existence of two different routes of the cleavage process (routes A and B).

The formation of two oligosaccharides, one of which was the major product and represented the modified repeating unit of the polysaccharide, and the other shortened by 2,5-anhydromannose residue, was observed and rationalized earlier [6, 7] in terms of β-elimination of carbohydrate chain from 3-O-substituted 2,5-anhydromannose residue under the alkaline conditions of the sodium borohydride reduction [16].

In the case of *Sh. dysenteriae* type 1 oligosaccharides II and III were detected before the borohydride reduction and, therefore, their formation was unequivocally due to the deamination reaction. The oligosaccharide III, representing the modified repeating unit, was formed by route A, whereas the shortened oligosaccharide II was considered to arise by a more complex way (route B) consisting of the intramolecular attack of the carbon atom C-4 on the diazonium cation at C-2 followed by simultaneous ring contraction and conversion of chemical atom C-3 in both the branch chain and hemiacetal function. As a result, the glucosamine residue was transformed into 2-deoxy-2-C-formyl-ribose (VI) and the polysaccharide chain was split on the left from the hexosamine unit. This type of deamination, not leading to the cleavage of the hexosaminide linkages, was observed by Erbing *et al.* [17] on methyl glucosaminides. These authors noticed also that methyl 2-deoxy-2-C-formyl-ribohides, the products of B-type deamination, were very acid-labile. The high lability towards acids exhibited by these glycosides was known to be conditioned by the presence of the second hemiacetal ring, which accelerates 100-fold the hydrolysis rate of glycoside linkage in pyranosides [18]. In the case of furanosides this splitting is expected to proceed even more easily. Since the deamination of the N-deacetylated polysaccharide was performed in an acidic media, it was reasonable to assume that it was accompanied by hydrolytic cleavage, and resulted in formation of oligosaccharide II and 2-deoxy-2-C-formyl-ribose VI. However, the attempts to detect monosaccharide VI by gas chromatography/mass spectrometry of its alditoacetate failed, apparently because of the high lability
of 2-deoxy-2-C-formylpentoses, the chemical properties of which are unknown. The structures of glycosyl-alditols IV and V were proved by mass spectrometry of their acetates and by determination of their monosaccharide composition, the mass numbers of the primary fragments being depicted on Scheme 1.

The fact that deamination cleavage of the N-deacetylated polysaccharide had proceeded by two routes, provided the necessary information on location of galactose and the second rhamnose residues in the native polysaccharide. The formation of trisaccharide II proved by itself that the galactose residue was attached to N-acetylglucosamine, and two rhamnose residues were disposed in succession in the O-specific polysaccharide chain of \textit{Sh. dysenteriae} type 1.

\textbf{Anomeric Configuration of Glycosidic Linkages}

For determination of the configuration of glycosidic linkages we used a method \cite{13,14} based on chromic anhydride oxidation of acetylated glycosides. Oxidation is known to be ineffective for \(\alpha\)-pyranosides, whereas the residues of \(\beta\)-pyranosides are oxidised into derivatives of aldosulosonic acids. Gas-chromatographic analysis of the oxidized acetylated polysaccharide revealed that all monosaccharide units survived, and thus are linked by \(\alpha\)-glycosidic linkages.

The comparison of the optical rotation values of the polysaccharide and oligosaccharides IV and V to those for methyl \(\alpha\)-D-galactopyranosides and \(\beta\)-D-galactopyranosides confirms the correctness of the chromic anhydride oxidation data (Table 1).

\section*{DISCUSSION}

The O-antigenic lipopolysaccharide of high specificity was isolated from \textit{Sh. dysenteriae} type 1 and split by mild acid hydrolysis to give a polysaccharide and lipid A. The degraded polysaccharide was separated by gel filtration into polysaccharide and oligosaccharide (core) fractions. The high type 1 specificity of the polysaccharide obtained was confirmed by inhibition of the passive haemagglutination reactions with O(K) and O-antisera.

Both the monosaccharide composition and the optical rotation value of the specific polysaccharide corresponded to data reported by Duvies and Morgan \cite{2} for a polysaccharide isolated from \textit{Sh. dysenteriae} type 1 by a different procedure. The polysaccharide, as followed from the proton magnetic resonance spectrum and from determination of its monosaccharide composition, appeared to be a hexosaminoglycan composed of \(\beta\)-rhamnose, \(\alpha\)-galactose and \(N\)-acetylglucosamine in the ratio 2:1:1. Unlike the specific acidic polysaccharides from serotypes 3–10, \textit{i.e.} Large-Saks subgroup and three provisional types, the polysaccharide from \textit{Sh. dysenteriae} type 1 contained no acidic functions.

The structure of the chemical repeating unit for the specific hexosaminoglycan was established by the conventional methods, as described in the previous section, and shown on Scheme 1.

As was shown by Heidelberger \textit{et al.} \cite{3}, the \textit{Sh. dysenteriae} type 1 specific polysaccharide cross-reacted with the K-specific polysaccharides from \textit{Pneumococcus} types 2 and 6, and on the basis of this finding the assumption was made on substitution patterns of galactose and rhamnose residues.

Now that the fine chemical structures of all three type-specific polysaccharides are established, a definite contribution could be made to an understanding of the chemical basis of their immunological relationship. The structures of both specific polysaccharides from \textit{Pneumococcus} types 2 and 6 are presented in Scheme 3.

The hexasaccharide repeating unit of the \textit{Pneumococcus} type 2 polysaccharide represents a chain built up of four monosaccharide residues to which the two-sugar branching is attached \cite{20}. In the linear part there is a section composed of three successively linked 3-0-substituted rhamnose residues. The last residue of \(\beta\)-rhamnose has nothing to do with the serological relationship, for it is linked by a \(\beta\)-glycosidic linkage. The first and the second \(\beta\)-rhamnose residues, though \(\alpha\)-linked, differ from each other since the second lies at the branching point of the polysaccharide chain. Thus, the only monosaccharide residue common with \textit{Sh. dysenteriae} polysaccharide, is considered to be the first \(\alpha\)-l-rhamnopyranosyl residue, and it is thought to be responsible for the serological kinship. However, as follows from the quantitative precipitin theory of Heidelberger and Kendall \cite{21}, cross precipitation is due to the mutual occurrence of multiples of a grouping in common, one would not expect the cross reaction to be extensive, since the end groups are not involved and the only

<table>
<thead>
<tr>
<th>Material</th>
<th>([\alpha]_D)</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Polysaccharide \textit{Sh. dysenteriae} type 1</td>
<td>+ 88.50°</td>
<td></td>
</tr>
<tr>
<td>(\alpha)-L-Rhamnopyranosyl(1-3)-(\alpha)-L-rhamno-pyranosyl(1-2)-(\alpha)-D-galactopyranosyl(1-2)-2,5-anhydro-(\alpha)-mannitol, IV</td>
<td>- 49.00°</td>
<td>[19]</td>
</tr>
<tr>
<td>Methyl (\alpha)-L-rhamnopyranoside</td>
<td>- 62.50°</td>
<td>[19]</td>
</tr>
<tr>
<td>Methyl (\beta)-L-rhamnopyranoside</td>
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<tr>
<td>Methyl (\beta)-D-galactopyranoside</td>
<td>- 0.42°</td>
<td>[19]</td>
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\textit{Lipopolysaccharides of Shigella}

Table 1. \textit{Optical rotation data}
\[
\begin{align*}
\text{Pneumococcus type 2} & \\
\text{Pneumococcus type 6} & \\
\end{align*}
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\[
\begin{align*}
\alpha-L-rhamnose residues in common occur within the chains of both polysaccharides. In fact, the relationship between \textit{Shigella} and \textit{Pneumococcus} type 2 was found to be rather weak [3].

The specific polysaccharide from \textit{Pneumococcus} type 6 is built up of linear tetrasaccharide repeating units linked by phosphodiester bonds [22]. This polysaccharide possesses two monosaccharide residues in common with \textit{Shigella} polysaccharide, viz. 3-O-substituted \(\alpha-L\)-rhamnopyranose and 2-O-substituted \(\alpha-D\)-galactopyranose, and the cross reaction was known to be extensive [3]. However, from our point of view, the appearance of an additional common monosaccharide residue also disposed within the linear chain, is hardly likely to cause an extensive increase in the serological relationship. Most likely, the serological relationship between \textit{Sh. dysenteriae} type 1 and \textit{Pneumococcus} type 6 is conditioned not by two separated monosaccharide residues but by substantial analogy in the composition of whole repeating oligosaccharide units, which becomes evident on thorough consideration of the chemical structures of the polysaccharides in question. Indeed, the sequence of monosaccharides \(-2)-\alpha-D\text{-Galp}(1-3)-\alpha-D\text{-GlcNAc}(1-3)-\alpha-L\text{-Rhap}\) from \textit{Pneumococcus} type 6 closely resembles that of \(-2)-\alpha-D\text{-Galp}(1-3)-\alpha-D\text{-GlcNAc}(1-3)-\alpha-L\text{-Rhap}\) from \textit{Sh. dysenteriae} type 1. The only difference is that the \(\alpha-D\)-glucopyranose residue in one polysaccharide is substituted for 2-acetamido-2-deoxy-\(\alpha-D\)-glucose. No doubt, these two monosaccharides exhibit different serological properties, but their stereochemistry and conformation are identical, and therefore it makes the trisaccharide regions mentioned above much more alike than different. Thus, the chemical proximity between trisaccharide blocks of polysaccharide chains is thought most probably to cause the strong serological relationship between \textit{Sh. dysenteriae} type 1 and \textit{Pneumococcus} type 6 polysaccharides.

In conclusion we would like to note that our structural data on \textit{Sh. dysenteriae} type 1 polysaccharide conflict with those of authors from Japan [23] who described the isolation from a partial hydrolysate of the whole lipopolysaccharide the disaccharide identified as \(\beta-D\)-galactosyl(1-2)-D-galactose and a tetrasaccharide composed of two galactoses and two rhamnoses disposed in succession. From these findings it followed that O-specific polysaccharide chain of \textit{Shigella} type 1 lipopolysaccharide contains the sequence of two galactose residues. However, the lack of sufficient chemical information to prove the oligosaccharide structures as well as the hydrolysis of the whole lipopolysaccharide preparation, known to be heterogeneous with respect to carbohydrate components, reduces confidence that the oligosaccharides in question have the proposed structures and originated from O-specific polysaccharide chains.

REFERENCES


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