Location of *O*-Acetyl Groups in the *Shigella flexneri* Types 3c and 4b Lipopolysaccharides

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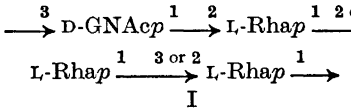
The structures of the lipopolysaccharides (LPS) of *Shigella flexneri* proposed by Simmons [1] have recently been revised.[2] It was suggested that all serotypes contain the same basic repeating unit. Only a partial structure for this unit (I) was given, assignment of a complete structure must await further studies. Structural variation is provided by terminal D-glucose residues, which may be linked to one or several positions in this basic repeating unit, and by *O*-acetyl groups which may occupy different positions in the sugar residues.



I

LPS from some serotypes contain a high percentage of *O*-acetyl groups. In the present communication studies on two such LPS from serotypes 3c and 4b are reported. According to serological classification [3] these serotypes contain the type antigens III and IV, respectively, and both contain the group antigen 6. Polysaccharides (PS) prepared from the LPS by mild acid hydrolysis [4,5] inhibited the group 6 system. Removal of *O*-acetyl groups by mild alkaline hydrolysis, however, resulted in complete loss of the group 6 specificity.[6]

The 3c PS contained L-rhamnose, D-glucose, 2-acetamido-2-deoxy-D-glucose and *O*-acetyl in the relative molar proportions 2.5 : 0.17 : 1.0 : 1.1 as determined by conventional methods. The corresponding figures for 4b were 2.2 : 1.1 : 1.0 : 1.1.

The sugar compositions of the PS were also determined by acid hydrolysis (0.25 M sulphuric acid, 15 h, 100°), reduction of the sugars in the hydrolysate to alditols which were analysed, as their acetates, by gas-liquid chromatography (GLC).[7] The identities of the components were confirmed by mass spectrometry (MS).[8] L-Rhamnose, D-galactose, D-glucose, a heptose and 2-acetamido-2-deoxy-D-glucose, in the molar proportions 3.6 : 0.12 : 0.53 : 0.53 : 1.0 (3c) and 4.3 : 0 : 2.0 : 0.38 : 1.0 (4b), respectively, were found. Hydrolysis of the 2-acetamido-2-deoxy-D-glucoside linkages is incomplete because of the simultaneous hydrolysis of the *N*-acetyl group which renders the 2-amino-2-deoxy-D-glucosidic linkages more resistant to hydrolysis. Further, the quantitative analysis of 2-acetamido-2-deoxy-D-glucose derivatives by GLC is difficult, and for these reasons the sugar analyses are not very accurate.

Methylation analyses [9–11] of the two PS (Table 1) gave results similar to those observed for other *S. flexneri* LPS.[2] In addition to the ethers listed in Table 1, the alditol acetates derived from 2-deoxy-2-*N*-(methyl-acetamido)-4,6-di-*O*-methyl-D-glucose and 2-acetamido-2-deoxy-4,6-di-*O*-methyl-D-glucose (from 3c and 4b) and 2-deoxy-2-*N*-(methyl-acetamido)-4-*O*-methyl-D-glucose and 2-acetamido-2-deoxy-4-*O*-methyl-D-glucose (only from 4b) were identified as their alditol acetates. For reasons discussed in Ref. 2 no quantitative

Table 1. Methyl ethers from the hydrolysates of methylated *Shigella flexneri* polysaccharides.

Sugar	T_R [a]	Mol % in serotype 3c	3c [b]	4b
2,3,4-Rha [c]	0.49	2.1	1.8	2.1
3,4-Rha	0.80	55.5	23.0	58.1
2,4-Rha	0.94	40.0	40.0 [d]	24.3
2,3,4,6-Glc	1.00	2.4	–	15.6
4,6-GNAc [e]	–	+	–	+
4-GNAc [e]	–	–	–	+

[a] Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an OS-138 column. [b] Methyl ethers from the hydrolysate of the methylated and reduced polysaccharide. [c] 2,3,4-Rha = 2,3,4-tri-*O*-methyl-L-rhamnose, *etc.* [d] It is assumed that the percentage of 2,4-Rha is not affected by the $LiAlH_4$ reduction. [e] These components were separated on a Dexsil 300 column.

analysis of these components was attempted. The mass spectra of the components listed in Table 1 were in agreement with the assigned structures.

The fully methylated PS from 3c was reduced with lithium aluminium hydride,[2] hydrolysed and the sugars in the hydrolysate analysed as their alditol acetates by GLC-MS.[11] The *N*-methyl-acetamido group in the GNAc moiety is reduced to an *N*-ethyl *N*-methyl group by this treatment, and the glycosidic linkages of the modified 2-amino-2-deoxy-D-glucose residues become more resistant to acid hydrolysis. Consequently, this residue, and the sugar to which it is linked, are absent in the methylation analysis (Table 1) whereby it may be adduced that the 2-acetamido-2-deoxy-D-glucose residue is linked to the 2-position of an L-rhamnose residue in the original PS.

Although the stoichiometry of the analyses is not very good, the combined evidence supports the revised structure (I) of the basic repeating unit. In the 3c PS, this residue is essentially devoid of terminal D-glucose residues. In the 4b PS a high percentage of the 2-acetamido-2-deoxy-D-glucose residues were substituted at the 6-position by D-glucopyranose residues.

The *O*-acetyl groups in the two PS were located using the method devised by de Belder and Norrman.[12] This involves treatment with methyl vinyl ether and an acidic catalyst, thus protecting all the hydroxyl groups as acetals. On subsequent methylation by the Hakomori method [9,10] the *O*-acyl groups are replaced by *O*-methyl groups. Hydrolysis of the modified polysaccharide yields a mixture of sugars and partially methylated sugars which is analysed, as the alditol acetates, by GLC-MS.[11] The results of these analyses are given in Table 2. It is evident that both serotype 3c and 4b PS are acetylated essentially in the 2-position of L-rhamnose residues, and that this moiety is responsible for the group 6 specificity.

The 2-*O*-acetyl groups could, according to the evidence available, only be linked to the 3-linked L-rhamnose residue in (I). No *O*-acetyl groups were detected on D-glucose residues. It was not possible to establish the presence of *O*-acetyl substituents on the 2-acetamido-2-deoxy-D-glucose residues. The agreement between the *O*-acetyl analysis and the aforementioned *O*-methyl substitution analysis is, however, reasonably good, and this possibility therefore seems unlikely.

Table 2. Neutral sugars obtained after substitution of the alkali-labile groups by *O*-methyl groups.

Sugar	T_R [a]	Mol % in serotype 3c	Mol % in serotype 4b
2-Rha [c]	1.52	21.7	17.0
4-Rha	1.72	—	2.0
3-Rha	1.94	—	1.2
Rha	2.2	64.0	44.6
Glc	9.5 [b]	14.2	35.2

[a] Retention times of the corresponding alditol acetates on an ECNSS-M column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol at 170°; [b] at 190°. [c] 2-Rha = 2-mono-*O*-methyl-L-rhamnose, *etc.*

Experimental. Shigella flexneri strain 3298 of serotype 3c (III, 6) (stock collection of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland) and standard strain of serotype 4b (IV, 6) (Dysentery Reference Laboratory, London) were used.

LPS were isolated from the acetone dried bacteria by extraction with phenol–water [13] and purified by chromatography on Sepharose 2 B.[14] PS were prepared from the LPS by hydrolysis with 1 % acetic acid at 100° for 1.5 h [4] and purified by chromatography on Sephadex G-50.[4]

The presence of group 6 specificity in the PS was demonstrated by means of the complement fixation inhibition micromethod.[15]

General methods and procedures for sugar and methylation analysis were essentially the same as in the previous study of *Shigella flexneri* LPS.[2]

D-Glucose,[16] 2-amino-2-deoxy-D-glucose,[17] L-rhamnose [18] and *O*-acetyl [19] were also determined by conventional methods.

Location of O-*acyl groups in the polysaccharides.* Strong absorptions at 1735 cm^{-1} (KBr) were observed in the IR spectra of the PS, indicating the presence of *O*-acyl groups.

The PS (3 mg) were dissolved in dimethyl sulphoxide (2 ml), methyl vinyl ether (condensed at −35°, 2 ml) and toluene-*p*-sulphonic acid (5 mg) were added and the mixture was kept at 15° for 3.5 h. Excess methyl vinyl ether was flushed away by passing a gentle stream of nitrogen through the solution, which then was added to the top of a Sephadex LH-20 column (35 × 2.5 cm). The column was irrigated with anhydrous acetone and the eluates were monitored polarimetrically. The acetalated product was collected and concentrated to a

light-yellow syrup. This material was methylated as previously described and hydrolysed, and the methylated sugars were analysed, as their alditol acetates, by GLC-MS.

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A Reaction Calorimeter

Some Modifications of a Previous System

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A reaction calorimeter of the isothermal jacket type used at this laboratory has been described earlier.[1] The calorimeter has now been in operation for almost five years and the experience gained during this time has led to some modifications of the system. The main principles in the construction have, however, been retained.

The calorimeter. The new calorimeter is shown in Fig. 1 and is very similar to the one described earlier.[1] The most important changes can be summarized as follows: (a) The mechanical design is much more rugged. (b) The parts included in the reaction vessel, *i.e.* the thermistor, the heater and the stirrer, can all easily be exchanged. (c) The addition capillary tube has been furnished with a spring-loaded valve. This valve provides protection against diffusion, *i.e.* it prevents undesirable reactions in the contact area between the reactant in the capillary tube and the solution in the inner vessel. (d) The transmission between the motor and stirrer (with new precision bearings) is improved by a cog-wheel-cogbelt transmission. (e) The capacity of the heat-exchanger is substantially increased. Solution can be added through the heat-exchanger at a rate of 1 cm^3/min during 5 min at a temperature difference of 15 K, without any noticeable heat transfer from the titrant to the solution.

The thermistor circuit and the Wheatstone bridge. The thermistor circuit has been changed in order to allow adjustments of the thermistor current. This is done by the connection of a potentiometer and a standard resistance in series with the bridge. The current is determined by measuring the potential drop over the standard resistance with a digital voltmeter (see below). It is important that this adjustment can be made with good precision as the current causes self-heating in the thermistor. The resistance of the thermistor changes substantially with temperature