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Studies on the antigenic structure of *Shigella*

VII. Structure of the O-specific side chains of the lipopolysaccharides from strains 2 (serotype 1b) and 1290/63 (variant Y)

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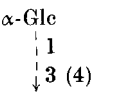
From the partial hydrolysates of the lipopolysaccharides from strains 2 (*Sh. flexneri* 1b) and 1290/63 (*Sh. flexneri* var. Y) we isolated in both cases a Rha-Rha-disaccharide. After trimethylsilylation, gas liquid chromatographic purification and mass spectrometry we could show that the rhamnose molecules of both disaccharides were linked to each other by a 1,3-glycosidic bond. As we could estimate the other linkages in the O-specific side chains of these lipopolysaccharides in recent studies, we are now able to present the following structures for the repeating units:



strain 2 (1b) -GlcNAc-(1,2)-Rha-(1,3)9Rha-(1,6)-
strain 1290/63 (var. Y) -GlcNAc-(1,2)-Rha-(1,3)-Rha-(1,6)-

In addition, we found in the lipopolysaccharide of strain 1290/63 rather big quantities of a poly-α-(1,4)-glucan.

Each repeating unit of the O-specific side chains of *Shigella flexneri* lipopolysaccharides consists of a basic chain of GlcNAc–Rha–Rha. With the exception of variant Y the repeating units of all *Sh. flexneri*-serotypes in addition contain D-glucose, bound in form of short monosaccharidic secondary side chains to one of the L-rhamnoses or to the N-acetyl-D-glucosamine. The serotypes of *Sh. flexneri* differ in the manner of linkage either of the main chain sugars or of the additionally bound glucose (Simmons 1969).

Using the method of permethylation, total hydrolysis, separation of the resulting mixture of partially methylated monosaccharides, and identifying each component by means of mass spectrometry we recently found (Beer and Seltmann 1972, Seltmann and Beer 1972) that one of the two rhamnoses is bound by a 1,2-, the other by a 1,3-linkage and the N-acetylglucosamine by a 1,6-linkage in the case of serotype 1b and variant Y. In addition, a great percentage (>50%) of the repeating units of the serotype 1b lipopolysaccharides contains a glucose molecule, bound in 1,3- or 1,4-position to the N-acetylglucosamine. In order to be able to decide whether the structure of the main chain of the repeating units is

$$\xrightarrow{6} \text{GlcNAc} \xrightarrow{1\ 3} \text{Rha} \xrightarrow{1\ 2} \text{Rha} \xrightarrow{1} \quad \text{or} \quad \xrightarrow{6} \text{GlcNAc} \xrightarrow{1\ 2} \text{Rha} \xrightarrow{1\ 3} \text{Rha} \xrightarrow{1}$$

we now isolated the disaccharide Rha–Rha from the partially hydrolyzed lipopolysaccharides and estimated the linkage between the two rhamnose molecules.

Material and methods

Bacteria and lipopolysaccharides: The origin of the strains tested (1290/63 — variant Y — and 2 — serotype 1b —), their mass cultivation and the isolation and purification of the lipopolysaccharides were described earlier (SELTMANN and BEER 1971).

Production of the polysaccharides and partial hydrolyses: The polysaccharide fraction was produced by heating a 1% solution of the lipopolysaccharides in 1 N acetic acid to 100 °C for 1.5 h (FREEMAN 1942). Precipitated lipid A was removed by centrifugation. The supernatant was lyophilized. About 200 mg polysaccharide from strain 1290 and about 300 mg polysaccharide from strain 2 were subjected to partial hydrolysis in a 1% solution in 0.5 N H_2SO_4 at 100 °C. After 20 min the solutions were cooled, neutralized with Dowex 1 × 8 (HCO_3^-) and evaporated under reduced pressure to 3 ml.

Isolation of the disaccharide fraction by gel chromatography: The gel chromatographic separation of the hydrolysis mixture was performed on a Sephadex G-10 column (l 67 cm, ⌀ 2.2 cm, v_t 250 ml, v_0 90 ml). The column was eluted with pyridine: acetic acid: water = 4:10:986 (pH 5.2) at a flow rate of 20 ml/h. The carbohydrate content of each fraction (~2.5 ml) was determined by means of the phenol/sulfuric acid reaction (DUBOIS *et al.* 1951). The approximative elution volume v_e of the disaccharide fraction was calculated via the known relationship between the K_{av}-values ($K_{av} = (v_e - v_0)/(v_t - v_0)$, see for instance FISCHER 1969) and the molecular weight.

The peak-forming material in this region was collected, concentrated in vacuo and lyophilized.

Reduction and trimethylsilylation of the disaccharide fraction: The disaccharide fraction of the two strains, as obtained by the methods described above, were soluted each in 2 ml borate buffer (pH 8.2) and reduced with 10 mg $NaBH_4$ in 1 ml of the same buffer at 4 °C for 18 h. The solutions were acidified by means of Dowex 50 W × 8 (H^+) and borate was removed by repeated addition and evaporation of methanol. Then the alditols were dried in vacuo over P_2O_5.

The trimethylsilylation was performed in a stoppered thick walled reaction tube containing the alditols dried as described above. Through the stopper 1 ml of absolute pyridine, 200 µl of hexamethyldisilazane and 100 µl of trimethylchlorosilane were added by means of a syringe. The solution was then vigorously shaken and heated to 70 °C for 3 h. After that the solution was evaporated under diminished pressure, the residue soluted in *n*-hexane and filtrated; the filtrate was used for gas liquid chromatographic analysis.

Gas liquid chromatography: We used the model GCHF 18.3 of Fa. W. GIEDE, Berlin, GDR, equipped with flame-ionization detectors. Steel-columns (2 m × 4 mm) filled with packings of 3% SE 30 on Porolith AW (0.1—0.2 mm) were used for separating the trimethylsilyl derivatives. Separations have been performed at 260 °C (column temperature) with argon as carrier gas (40 ml/min).

The trimethylsilyl-oligosaccharide alditols of the isolated disaccharide fractions were soluted in 100 µl of *n*-hexane and 1 µl was injected for gas liquid chromatographic analysis (KÄRKKÄINEN 1969).

After separation by means of g.l.c. [1]) the fractions were collected in a cooling trap cooled to −50 °C.

Mass spectrometry: The model used and the method employed were described in a previous paper (BEER and SELTMANN 1972).

Results

Separation of the mixture resulting from partial hydrolysis of the O-specific polysaccharides

Fig. 1 and 2 show the elution patterns of the gel chromatographic separation of the mixtures resulting from partial hydrolysis of the O-specific polysaccharides of the strains 2 and 1290/63 as described in "Material and Methods".

The effluent could be subdivided into 3 main fractions (see Figs. 1 and 2). The fractions F 1 of both strains contain material of a molecular weight of about

[1]) Abbreviations: g.l.c. = gas liquid chromatography, TMS = trimethylsilyl

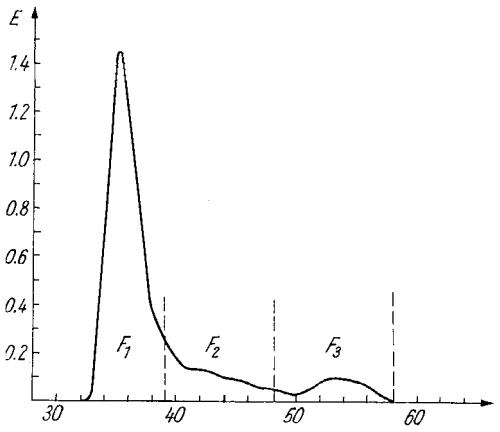


Fig. 1

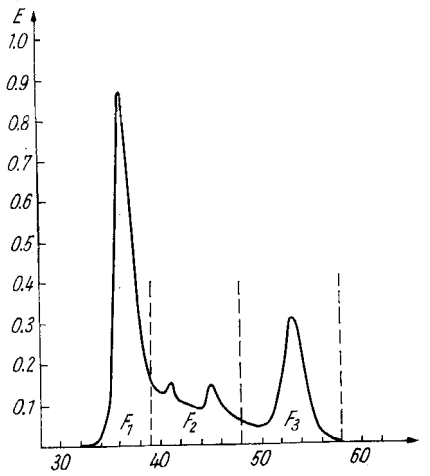


Fig. 2

Fig. 1. Elution pattern of gel chromatographic separation on Sephadex G 10 of the mixture resulting from partial hydrolysis of the O-specific polysaccharides from strain 2. Column 2.2 × 67 cm. Elution with pyridine: acetic acid: water = 4:10:986 (pH 5.2), flow rate 20 ml/h. Determination of the carbohydrate content of the effluent by means of the phenol/sulfuric acid-reaction

Fig. 2. Elution pattern of gel chromatographic separation on Sephadex G 10 of the mixture resulting from partial hydrolysis of the O-specific polysaccharides from strain 1290. Column 2.2 × 67 cm. Elution with pyridine:acetic acid:water = 4:10:986 (pH 5.2), flow rate 20 ml/h. Determination of the carbohydrate content of the effluent by means of the phenol/sulfuric acid-reaction

700 and more, *i.e.* tetrasaccharides and higher saccharides. Fraction F 2 contains di- and trisaccharides, and fraction F 3 mainly monosaccharides. The two fractions F 2 were collected, concentrated in vacuo and lyophilized.

Reduction and trimethylsilylation of the disaccharide fractions and gas liquid chromatography of the resulting mixture

Reduction and trimethylsilylation were performed as described in "Material and Methods". By gas liquid chromatography of the trimethylsilylated di- and trisaccharides we obtained 7 main peaks (see Table 1). A matter of special interest were the peaks with r_{malt}[1]) = 0.37 which we believed to consist of the trimethylsilylated alditols of the rhamnose-rhamnose disaccharide.

The materials of these peaks were collected and used for mass spectrometry.

Mass spectrometry of the trimethylsilylated alditols from the Rha–Rha-disaccharide

Figs. 3 and 4 show the mass spectra of the trimethylsilylated alditols of the two Rha–Rha-disaccharides from the two strains studied.

Of special interest was the mass fragment m/e 363 (indicating the presence of a trimethylsilylated rhamnosyl residue, see Fig. 5). This fragment was found.

[1]) retention of trimethylsilylated maltose under identical conditions

Table 1
Relative retentions of the TMS derivatives of the reduced oligosaccharides obtained from the crude polysaccharides from the strains 1290 and 2 (related to trimethylsilyl maltitol). Gas liquid chromatography was performed in steel columns 2 m × 4 mm with packings of 3% SE 30 on Porolith AW (0.1—0.2 mm). Column temperature 260 °C, argon (40 ml/min) as carrier gas

Peak	PS 1290 Retention	PS 2 Retention
1	0.24	0.24
2	0.37	0.37
3	0.62	0.62
4	0.68	0.68
5	1.00	1.02
6	1.16	1.16
7	1.90	1.82

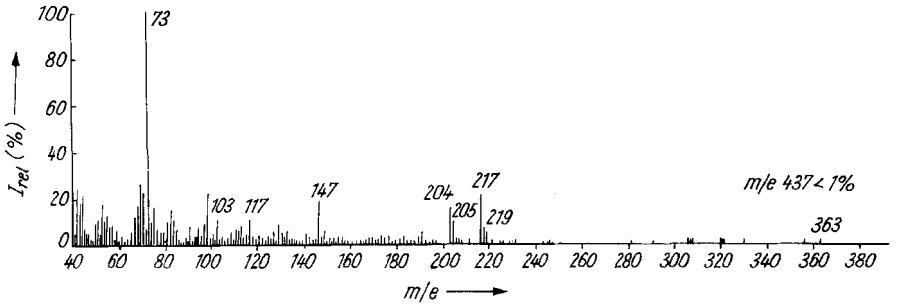


Fig. 3. Mass spectrum of the trimethylsilylated alditol of the presumptive Rha—Rha-disaccharide from strain 2

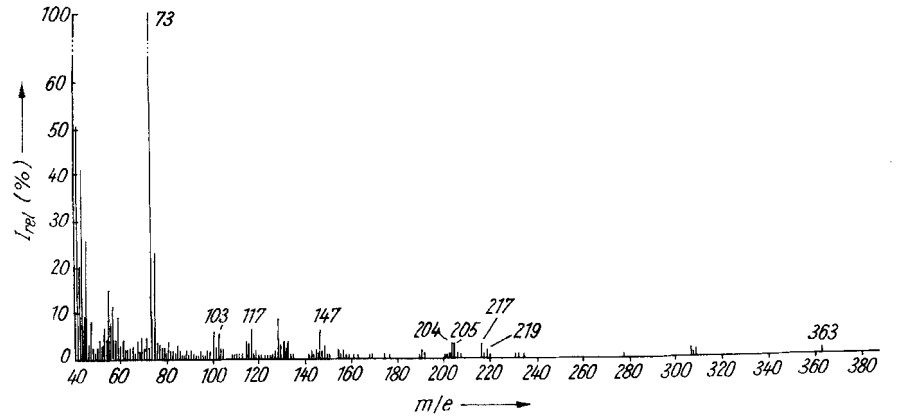


Fig. 4. Mass spectrum of the trimethylsilylated alditol of the presumptive Rha—Rha-disaccharide from strain 1290

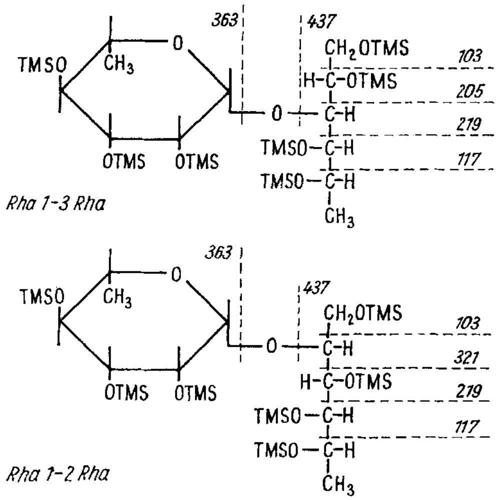


Fig. 5. Mass fragments resulting from the reduced TMS derivative from Rha-1→3-Rha and Rha-1→2-Rha

That is the proof that the g.l.c. peak with $r_{malt} = 0.37$ is in fact the trimethylsilylated alditol of a rhamnosyl disaccharide. In order to be able to decide between the two possible linkages 1,2 and 1,3 we needed the mass fragments m/e = 321 typical for 1,2) and 205 (typical for 1,3). Figs. 3 and 4 show considerable quantities of the mass fragment 205, but only very small quantities of the mass fragment 321. The mass fragments m/e 147, 204 and 217 are secondary fragments (DeJongh 1969), which are usually formed in connection with the mass spectrometry of TMS-derivatives of sugars.

Very interesting is the peak $r_{malt} = 1.0$ which appears in the gas liquid chromatogram of the polysaccharide from strain 1290 in rather big quantities. This peak indicates that the lipopolyascсharide of this strain contains rather big quantities of a poly-α-1,4-glucan (see also Beer and Seltmann 1972).

Discussion

In previous studies we found for the O-specific polysaccharide of *Sh. flexneri* serotype 1b and variant Y the following structural possibilities:

$$\mathrm{1b}\left(\overset{6}{\longrightarrow}\underset{}{\overset{\overset{\mathrm{Glc}}{\vdots\,1}}{\downarrow 3\ (4)}}\mathrm{GlcNAc}\overset{1\ 2/3}{\longrightarrow}\mathrm{Rha}\overset{1\ 3/2}{\longrightarrow}\mathrm{Rha}\overset{1}{-}\right)_n$$

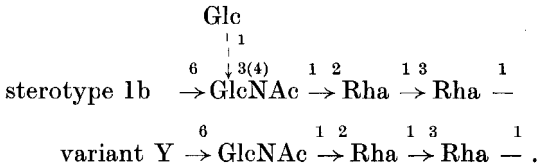
$$\mathrm{Y}\left(\overset{6}{\rightarrow}\mathrm{GlcNAc}\overset{1\ 2/3}{\longrightarrow}\mathrm{Rha}\overset{1\ 3/2}{\longrightarrow}\mathrm{Rha}\overset{1}{-}\right)_n .$$

The dotted line between Glc and GlcNAc in the formula of serotype 1b means that the GlcNAc molecules are only substituted partly by glucose. In addition, variant Y contains a poly-1,4-glucan in its lipopolysaccharide molecule, the function of which is still unknown.

In order to be able to decide whether the linkage between the two rhamnose molecules of both lipopolysaccharides is 1,2 or 1,3 we hydrolyzed the purified

lipopolysaccharides partially, separated the disaccharide fraction from the resulting mixture by means of gel filtration on Sephadex G 10, trimethylsilylated this fraction, separated it by means of gas liquid chromatography and identified the pre-calculated fractions by means of mass spectrometry.

The results of mass spectrometry indicate to a very high degree of certainty that the two rhamnosyl residues in the main chain of the O-specific polysaccharides deriving from both strains are linked to each other by a 1,3-linkage. So the structure of the repeating units is as follows:



The linkage between the two rhamnose molecules in variant Y differs from that found by SIMMONS (1969). This difference may be explained by strain specificities.

In addition, the lipopolysaccharides of variant Y contain a rather high percentage of poly-α-1,4-glucan, the structure of which results from the fact that the gas liquid chromatogram of the trimethylsilyl derivatives of the di- and trisaccharide fraction contains trimethylsilylated maltose.

In recent papers we stated that this polyglucan cannot be separated from the lipopolysaccharide by ultracentrifugation. So we assumed a molecular weight similar to that of the lipopolysaccharide. After FREEMAN-degradation of the lipopolysaccharides the molecular weight of the polyglucan was reduced drastically, so that the separation of polyglucan and polysaccharide was again impossible by means of ultracentrifugation. As it is very improbable that Glc-Glc-linkages should be split off by 1 N CH_3COOH (1 h at 100 °C) to any considerable extent, it may be supposed that the poly-α-1,4-glucan is an integral part of the lipopolysaccharide molecule isolated by the phenol-water method. Therefore, it seems rather probable that both the polyglucan and the specific polysaccharide are bound in the lipopolysaccharide. The fact that the polyglucan shows no serological activity though it is bound in the lipopolysaccharide is not surprising, because poly-α-1,4-glucans are widely distributed in animal bodies.

Some aspects of the relationship between the chemical structure of the polysaccharide chains of the lipopolysaccharides and the serological properties of the *S. flexneri* have been discussed in a separate paper (SELTMANN 1972).

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