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Structural Studies on the O-Specific Side Chains of the Cell Wall Lipopolysaccharides from *Salmonella typhi* and *S. enteritidis*

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The structures of the O-specific side chains of the cell-wall lipopolysaccharide from *S. typhi* (I.S.59) and *S. enteritidis* (I.S.64), serogroup D_1, have been investigated. The positions at which the different sugar residues are linked were determined by methylation analysis of the original lipopolysaccharide and the mutual order of the sugar residues by methylation analysis of a partially hydrolysed product. The position of the *O*-acetyl groups, present in the *S. typhi* lipopolysaccharide, was determined by methylation analysis of a product, in which all free hydroxyl groups had been protected, as acetals, by treatment with methyl vinyl ether. The anomeric nature of some linkages was determined by following the change in optical rotation on acid hydrolysis. As a result of these studies, a structure of the repeating units in the O-specific side-chains is presented. An approximate value for the average number of repeating units in the side chains is also given.

Structural studies on some *Salmonella* serogroup B lipopolysaccharides have been recently reported [1,2] and a general structure for the O-specific side chains was proposed. Methylation analysis provided a valuable tool for investigating the structures of the original lipopolysaccharide, of the partially hydrolysed lipopolysaccharide, and of a material obtained after acetalisation

and deacetylation of the lipopolysaccharide. The partially methylated materials were analysed as their alditol acetates by GLC[3]-mass spectrometry.[4] The present paper reports similar studies on two *Salmonella* serogroup D_1 lipopolysaccharides (*S. typhi* and *S. enteritidis*), both of which contain tyvelose, 3,6-dideoxy-D-*arabino*-hexose.

Isolation of the lipopolysaccharides. The bacteria, *S. typhi* I.S.59 (9, 12) and *S. enteritidis* I.S.64 [(1),9,12_1,(12_2),12_3] were killed by γ-irradiation, disintegrated, the cell wall material collected, and the lipopolysaccharides extracted by the phenol-water method. The presence of O-factors 9,12_2 (*S. typhi*) and 9,12_2 (*S. enteritidis*) was demonstrated, both for the bacteria and for the isolated lipopolysaccharides, by slide agglutination and hemagglutination inhibition tests, respectively.[1] The presence of O-factor 1 in *S. enteritidis* could not be demonstrated.

A two step hydrolysis[1] of the *S. typhi* lipopolysaccharide yielded D-glucose, D-galactose, D-mannose, L-rhamnose, and tyvelose in the relative molar proportions 17:23:21:20:19. The corresponding values for the *S. enteritidis* lipopolysaccharide were 6:27:24:22:21. The sugars were not isolated in the present study but were analysed by GLC[5] as their alditol acetates and their identities confirmed by mass spectrometry.[6] In previous studies,[7] these sugars have been fully identified.

The presence of *O*-acetyl groups in *S. typhi*, not previously observed in *Salmonella* serogroup D_1 lipopolysaccharides, was indicated by absorption in IR at 1738 cm^{-1} and by the presence of a singlet at τ 7.95 in NMR (D_2O). Further confirmation was obtained by methanolysis of the lipopolysaccharide and identification of the methyl acetate formed by GLC and mass spectrometry.

Methylation analysis. The lipopolysaccharide, in dimethylsulphoxide, was methylated by treatment with dimethylsulphinyl sodium and methyl iodide,[8] hydrolysed, the methylated sugars converted into their alditol acetates and analysed by GLC[3]-mass spectrometry.[4] A typical chromatogram (*S. typhi*)

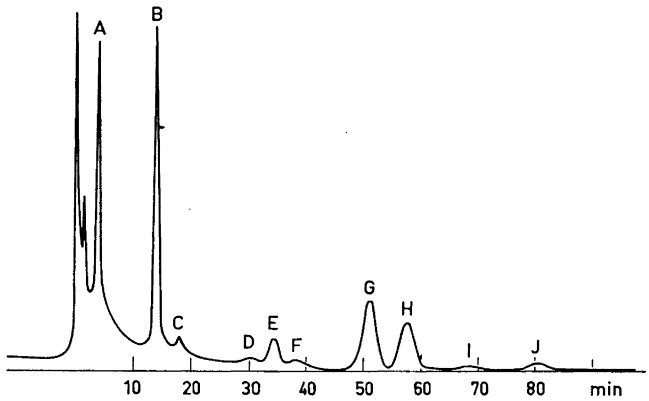

Fig. 1. GLC separation on an ECNSS-M column at 170° of the methylated sugars, as their alditol acetates, obtained from the hydrolysate of the fully methylated *S. typhi* lipopolysaccharide.

Table 1. Methyl ethers from the hydrolysate of methylated lipopolysaccharide.

Sugars	Peak	T^a	mole %[b]	
			S. typhi	*S. enteritidis*
2,4-Di-*O*-methyl-tyvelose	A	0.29	8.0	10.0
2,3-Di-*O*-methyl-L-rhamnose	B	0.98	20.0	22.0
2,3,4,6-Tetra-*O*-methyl-D-glucose	B	1.00	13.5	2.2
2,3,4,6-Tetra-*O*-methyl-D-galactose	C	1.25	0.8	0.5
2,4,6-Tri-*O*-methyl-D-mannose	D	2.08	0.8	1.2
3,4,6-Tri-*O*-methyl-D-glucose	D	2.11	2.1	2.0
2,4,6-Tri-*O*-methyl-D-galactose	E	2.28	8.2	21.7
3,4,6-Tri-*O*-methyl-D-galactose	F	2.50	0.5	1.0
4,6-Di-*O*-methyl-D-mannose	G	3.29	24.5	24.1
2,6-Di-*O*-methyl-D-galactose	H	3.62	16.5	0.5
3,6-Di-*O*-methyl-D-galactose	I	4.30	0.8	1.0
2,4-Di-*O*-methyl-D-glucose	K	5.10	1.0	1.0
2,4-Di-*O*-methyl-D-galactose	L	6.35	—	0.8

[a] Retention times of the corresponding alditol acetates on the ECNSS-M column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

[b] As a considerable amount of 2,4-di-*O*-methyl-tyvelose was lost during the methylation analysis, the mole % of the methylated sugars are given relative to that of 2,3-di-*O*-methyl-L-rhamnose, which it is assumed represehts the mole % of L-rhamnose in the original lipopolysaccharide.

is given in Fig. 1 and the results for the two lipopolysaccharides are summarised in Table 1.

The component in the first peak, A, gave a mass spectrum typical for the alditol acetate of a 3,6-dideoxy-2,4-di-*O*-methyl-hexose and was indistinguishable from that given by the 2,4-di-*O*-methyl-abequose derivative.[1] No authentic 2,4-di-*O*-methyl-tyvelose was available, but as tyvelose is the only 3,6-dideoxy-hexose present in the lipopolysaccharide, the component in peak A must be derived from that sugar.

All the other main components were the same as observed in the methylation of the *S. typhimurium* lipopolysaccharides,[1,2] and were identified by their *T*-values and mass spectra as previously described.

Mass spectra taken at the ascending part of peak D revealed the presence of a 2,4,6-tri-*O*-methyl-hexose derivative. The mass spectrum, taken at the maximum of the peak was, however, typical for a 3,4,6-tri-*O*-methyl-hexose derivative. The former, from its *T*-value (2.08) should be the D-mannose derivative, the latter the D-glucose derivative (*T* 2.11). Mass spectra taken at the descending part of peak F revealed the presence of a low percentage of another 3,4,6-tri-*O*-methyl-hexose derivative. This component (*T* 2.50) was also found in the methylation of *S. typhimurium* LT2 [2] and as the *T*-value differs from those of the D-mannose (1.95) and D-glucose (2.11) derivatives, it must be the D-galactose derivative.

The methylation analysis of the *S. enteritidis* lipopolysaccharide gave the same methyl ethers (Table 1) as observed for *S. typhi*. In addition to these,

a new component was found, which from its mass spectrum and T-value ((6.35), corresponding to the 2,4-di-*O*-methyl-D-galactose derivative. The corresponding T-values for the D-glucose and D-mannose derivatives are 5.10 and 5.35. It is well separated from the other components and could hardly be overlooked in the structural studies of the other lipopolysaccharides, and thus represents a structural feature of the *S. enteritidis* lipopolysaccharide which is absent in the *S. typhi* and *S. typhimurium* lipopolysaccharides.

The molar percentages of D-glucose, D-galactose, D-mannose, L-rhamnose, and tyvelose, calculated from the methylation analysis, assuming that the molar percentage of L-rhamnose is the same as in the original lipopolysaccharide, are 17:27:25:20:8 (*S. typhi*) and 5:26:25:22:10 (*S. enteritidis*). The agreement between these values and those obtained in the sugar analysis are good, with the exception of tyvelose. It seems resonable to assume, however, that a considerable part of the volatile 2,4-di-*O*-methyl-tyvelose and derivatives was lost during concentration.

As seen from Fig. 1 and Table 1, some components were not well separated by GLC on the ECNSS-M column. The alditol acetates in peak B, derived from 2,3-di-*O*-methyl-L-rhamnose and 2,3,4,6-tetra-*O*-methyl-D-glucose, were resolved on a OS 138 (polyphenyl ether) column (T 0.86 and 1.00, respectively). The absence of the alditol acetate derived from 2,3,4,6-tetra-*O*-methyl-D-mannose (T 1.07), which does not separate from the D-glucitol derivative on the ECNSS-M column, was also demonstrated by GLC on the OS 138 column.

A somewhat better separation of the components in peak D derived from 2,4,6-tri-*O*-methyl-D-mannose (T 2.08) and 3,4,6-tri-*O*-methyl-D-glucose (T 2.11) was obtained when the separation, on the ECNSS-M column, was run at 155°. The estimation of the relative proportions of the components in this peak is, however, rather uncertain.

The lipopolysaccharides were subjected to a mild hydrolysis, which was anticipated to lead to preferential cleavage of all the tyvelosidic residues. The oligo- and polymeric material was recovered and subjected to methylation

Table 2. Methyl ethers from the hydrolysate of methylated, degraded lipopolysaccharide.

Sugars	T^a	mole %[b]	
		S. typhi	*S. enteritidis*
2,4-Di-*O*-methyl-tyvelose	—	—	—
2,3-Di-*O*-methyl-L-rhamnose	0.98	20.0	22.0
2,3,4,6-Tetra-*O*-methyl-D-glucose	1.00	10.4	1.0
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.25	2.0	5.5
3,4,6-Tri-*O*-methyl-D-mannose	1.95	22.6	22.8
2,4,6-Tri-*O*-methyl-D-galactose	2.28	7.0	16.0
2,3,6-Tri-*O*-methyl-D-galactose	2.42	4.0	—
2,6-Di-*O*-methyl-D-galactose	3.62	10.5	—

[a,b] See Table 1.

analysis. The results, given in Table 2, show, both for *S. typhi* and *S. enteritidis* that all the tyvelose residues have disappeared and that all the 4,6,di-*O*-methyl-D-mannose has been replaced by 3,4,6-tri-*O*-methyl-D-mannose. In addition, the relative proportions of D-galactose ethers changed, as will be discussed below.

Determination of the position for the O-*acetyl groups.* The lipopolysaccharide (*S. typhi*) was acetalised by treatment with methyl vinyl ether and deacetylated as previously described.[1,9] The methylated material was hydrolysed, and the resulting sugars reduced, acetylated and analysed by GLC-mass spectrometry. In addition to D-glucose, D-galactose, D-mannose, L-rhamnose, and tyvelose, a new component (*T* glucose 0.64) was observed, which proved on mass spectrometry to be a 2-*O*-methyl-hexose derivative. The molar percentages of the four last sugars were the same as in the sugar analysis, while that of D-glucose had decreased from 17 % to 12 %. This decrease corresponded to the percentage of the new component indicating that it is derived from 2-*O*-methyl-D-glucose. This was further supported by the fact that the new component had the same *T*-value as 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methyl-D-glucitol. The results thus demonstrate that about one third of the D-glucose residues in the lipopolysaccharide carry *O*-acetyl groups in the 2-position and that no other position in the O-specific side chains are acetylated.

Acid hydrolysis of the lipopolysaccharide. A solution of the lipopolysaccharide (*S. typhi*) in 0.05 M sulphuric acid was kept at 80° and the optical rotation followed polarimetrically (Fig. 2) A rapid decrease of $[\alpha]_{578}$ from +102° to

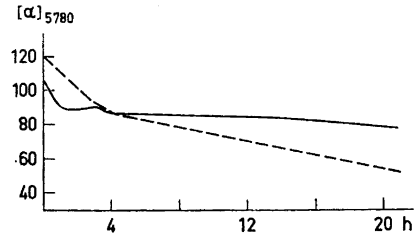

Fig. 2. Optical rotation *versus* time during acid hydrolysis of lipopolysaccharides. *S. typhi* (———), *S. typhimurium* 395 MS (— — —).

+90° in 2 h was observed after which there was little change in optical rotation. A small maximum followed by a slow decrease could, however, be observed. The same pattern was observed for the *S. enteritidis* lipopolysaccharide. For the *S. typhimurium* [1,2] lipopolysaccharide, the first rapid decrease was followed by a slower decrease of the same order of magnitude, and this was interpreted in terms of a fast hydrolysis of α-abequosidic residues, and a slower hydrolysis of β-L-rhamnosidic linkages. The present result similarly reflects the rapid hydrolysis of α-tyvelosidic linkages (decrease in rotation), a slower hydrolysis of α-L-rhamnosidic linkages (increase in rotation) and a still slower hydrolysis of other linkages, if it is assumed that this results in a decrease in optical rotation. The preceding explanation would also account for the appearance of the small maximum.

DISCUSSION

From previous studies[1,11] on the O-specific side chains of *Salmonella* serogroup D_1 polysaccharides, the partial structure given in Fig. 3 has been suggested. O-factor 9 is associated with a tyvelose residue, assumed to be α-linked to C-3 of a D-mannose residue. O-Factor 12 has been divided into three subfactors, one of which appears to be associated with an α-D-glucose residue[12] linked to the 4-position of a D-galactose residue. O-factor 1 is also associated with an α-D-glucose residue, but in this case linked to the 6-position of a D-galactose residue. The structural elements in Fig. 3 have been deduced

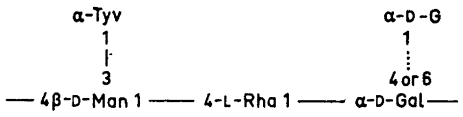

Fig. 3. Previously proposed structure for the O-specific side-chains of group D_1 *Salmonella* lipopolysaccharides.

from partial hydrolysis studies, periodate oxidation studies and immunochemical studies. A previous attempt to study the lipopolysaccharide from *S. typhi* by methylation analysis[13] gave inconclusive results.

From the results of the present study, and in conjunction with previous results, a detailed structure for *Salmonella* serogroup D_1 lipopolysaccharides is proposed (Fig. 4). The structure is based on the evidence given below. As the structure of the lipopolysaccharides from *S. typhi* and *S. enteritidis* are closely related, they will be discussed together.

The pyranosidic nature of all the sugar residues, with exception of the L-rhamnose residues, is evident from the methylation analysis. L-Rhamnofuranose residues may, however, be expected to be much more readily hydrolysed than observed for the L-rhamnose residues in the present study, and it may therefore safely be assumed that these residues are also pyranosidic.

From the methylation analysis of the original and partially hydrolysed lipopolysaccharide, it is evident that the tyvelose residues are attached to D-mannose residues in the 3-position.

The increase in 2,3,4,6-tetra-*O*-methyl-D-galactose with a corresponding decrease in 2,4,6-tri-*O*-methyl-D-galactose, most pronounced for *S. enteritidis*, and the increase in 2,3,6-tri-*O*-methyl-D-galactose with a corresponding decrease in 2,6,-di-*O*-methyl-D-galactose, observed for *S. typhi*, must be due to the hydrolysis of L-rhamnosidic linkages and demonstrates that the L-rhamnose residues are linked to D-galactose residues in the 3-position. Consequently, if it is accepted that the O-specific side chains are built up of repeating units, the D-galactose residues must be linked through C-2 of the D-mannose residues.

In the *S. typhi* lipopolysaccharide, a considerable part of the D-galactose residues are substituted in the 4-position with D-glucose residues in agreement with the presence of O-factor 12_2.[14]

The low percentages of 2,4-di-*O*-methyl-D-galactose, 2,6-di-*O*-methyl-D-galactose, and 2,3,4,6-tetra-*O*-methyl-D-glucose in the *S. enteritidis* lipo-

polysaccharide indicate that some of the D-galctose residues are substituted in either the 4- or 6-position, with a D-glucose residue. Despite the low percentages, these findings are probably significant. The D-glucose attached at the 4-position is in agreement with the presence of O-factor 12_2, and the D-glucose attached at the 6-position may be due to the presence of O-factor 1, which, however, could not be demonstrated. Further, 2,4-di-*O*-methyl-D-galactose has not been found in methylation analyses of the other lipopolysaccharides which are devoid of O-factor 1.

In the *S. typhi* lipopolysaccharide, part of the terminal D-glucose residues are acetylated in the 2-position. The immunological significance of this structural feature is not known. *O*-Acetyl groups are known to migrate readily, and it is remarkable that they were found only in the 2-position and not in any other position.

The presence of a low percentage of 2,4,6-tri-*O*-methyl-D-mannose indicates that the lipopolysaccharides, and consequently their natural repeating units, are terminated by 3-*O*-tyvelosyl-D-mannose residues. From the percentages of this sugar and of 4,6-di-*O*-methyl-D-mannose, the average number of repeating units in the lipopolysaccharides from *S. typhi* and *S. enteritidis* could therefore be estimated, giving values of 30 and 20, respectively. These figures must, however, be regarded as approaximate, and are higher than the corresponding values for the two *S. typhimurium* strains studied.[1,2] It is probable also significant that the percentages of the typical methylated sugars derived from the basal core *e.g.* 2,3,4,6-tetra-*O*-methyl-D-galactose, were higher for the latter lipopolysaccharides.

From the hydrolysis studies, it may be inferred that the tyvelose residues and the L-rhamnose residues are α-linked. The high optical rotation of the partially hydrolysed product, which should not contain L-rhamnosidic residues, indicated that both the D-galactose and D-mannose residues are α-linked. Partial hydrolysis studies and subsequent isolation of oligosaccharides, however, suggested that whereas the D-galactose residues are α-linked the D-mannosidic linkages are β-linked.[16] According to previous studies.[12,15] the D-glucose residues are α-linked to the 4- or 6-position in D-galactose residues (O-factor 12_2 and 1). In the structure given in Fig. 4 α-D-galactosidic and α-D-glucosidic linkages are assumed but the anomeric nature of the D-mannose residues is not assigned.

The structures of the *Salmonella* serogroup B and D_1 lipopolysaccharides seem to be closely related. The main differences are that the former contain abequose residues and the latter tyvelose residues and that, in several sero-

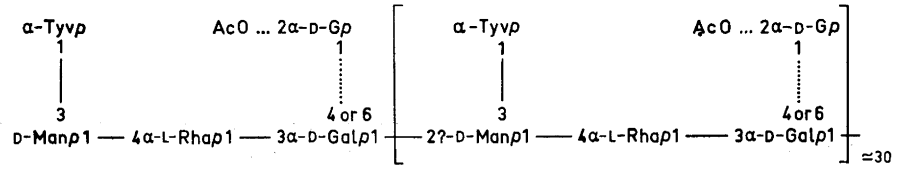

Fig. 4. Proposed structure for the O-specific side-chains of group D_1 *Salmonella* lipopolysaccharides.

group B lipopolysaccharides, the 3,6-dideoxy sugar (abequose) carries *O*-acetyl groups in the 2-position while in the serogroup D_1 lipopolysaccharides, some of the D-glucose residues are acetylated in the 2-position. If, as indicated in the present study, the L-rhamnose and D-mannose residues are β-linked in the B-group but α-linked in the D_1-group, the resemblance becomes more superficial.

EXPERIMENTAL

The experimental methods were the same as used in the similar study of the *Salmonella typhimurium* 395 MS lipopolysaccharide.[1] In order to avoid repetition, only some relevant details are given below. The strains used, *Salmonella typhi* I.S.59 [(9.12) Vi: d:−] and *Salmonella enteritidis* I.S.64 [(1),9,12_1, $(12)_2$, 12_3:gm:−], were obtained from the National Salmonella and Shigella Center, Statens Bakteriologiska Laboratorium.

Preparation of the lipopolysaccharides. The bacteria were killed by irradiation with ^{60}Co γ-rays (12 000 rad/min for 1 h) before disintegration. The cultivation yield was for *S. typhi* I.S.59 13.2 g and for *S. enteritidis* I.S.64 13.7 g (dry weights). The yield of lipopolysaccharide was for *S. typhi* 160 mg and for *S. enteritidis* 290 mg (dry weights).

The lipopolysaccharide preparations were checked in passive hemagglutination inhibition tests using anti O:1, anti O:9 and anti O:12_2 factor sera (Table 3).

Table 3. Passive hemagglutination inhibition of anti O factor serum with *S. typhi* and *S. enteritidis* lipopolysaccharides.

Lipopolysaccharide	Antiserum		
	anti O:1	anti O:9	anti O:12_2
S. typhi	256	1	1
S. enteritidis	256	1	16

Hemagglutination inhibition systems: *S. bredeney* lipopolysaccharide/*S.paratyphi* AO I.S. 256 anti O:1 factor serum, *S. enteritidis* I.S.64 lipopolysaccharide/*S. enteritidis* I.S.64 anti O:9 factor serum and *S. typhimurium* 395 MS lipopolysaccharide/*S. typhi* T4 I.S.58 anti O:12_2 factor serum. Data recorded are the minimal concentrations (μg/ml) of lipopolysaccharide from *S. typhi* and *S. enteritidis* needed for inhibition of the systems.

Preliminary structural studies of the *S. enteritidis* lipopolysaccharide gave inconsistent results, suggesting that it was contaminated with low-molecular weight material, which on hydrolysis yielded D-glucose. It was therefore subjected to dialysis before further structural studies were performed.

O-Acetyl-groups. As mentioned in the general part, IR and NMR indicated the presence of *O*-acetyl groups in the *S. typhi*, but not in the *S. enteritidis* lipopolysaccharide. A more unambiguous identification of these groups was obtained by treating the lipopolysaccharide (10 mg) with 4 % methanolic hydrogen chloride (0.5 ml) at 100° for 1 h and examining the solution by GLC (on a Carbowax column at 40°)-mass spectrometry. This experiment confirmed the presence of *O*-acetyl groups in the *S. typhi* and the virtual absence of such groups in the *S. enteritidis* lipopolysaccharide.

In the experiments performed in order to locate these groups, the step involving deacetylation of the acetalised lipopolysaccharide was omitted, as *O*-acetyl groups are split off during the subsequent methylation.

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