The Immunochemistry of *Salmonella* Chemotype VI O-Antigens

THE STRUCTURE OF OLIGOSACCHARIDES FROM *SALMONELLA* GROUP N (O 30) LIPOPOLYSACCHARIDES

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1. The lipopolysaccharides isolated from 'smooth' (S) strains of *Salmonella godesberg* and *Salmonella urbana* by the phenol-water method were purified in the ultracentrifuge. 2. These lipopolysaccharides have the same O-antigenic structure and on partial hydrolysis the same series of oligosaccharides was obtained in each instance. 3. The results of quantitative microanalysis, borohydride reduction, periodate oxidation, Morgan-Elson reactions and enzymic hydrolysis with α - and β -glucosidases on the isolated oligosaccharides indicated that the O-specific side chains of these S-lipopolysaccharides have a repeating tetrasaccharide unit that is β -D-glucosyl-(1 $\rightarrow$ 3)-N-acetylgalactosaminyl-(1 $\rightarrow$ 4)-L-fucose with a further glucose residue bound at the 4-position on the N-acetylgalactosamine. 4. Another oligosaccharide, a glucosylgalactose, has also been isolated and is indistinguishable from an oligosaccharide isolated from *Salmonella* R-lipopolysaccharides. These findings provide further evidence supporting the view that all *Salmonella* S-lipopolysaccharides have a core consisting of R-lipopolysaccharide.

The division of the genus *Salmonella* into 16 chemotypes according to the monosaccharide constituents of the specific lipopolysaccharides (Kauffmann, Lüderitz, Stierlin & Westphal, 1960) has been of considerable value in revealing chemical relationships between *Salmonella* groups of different O-antigenic structure and has also indicated further chemical studies that might define the molecular structures which determine serological specificity in this complex group of micro-organisms. The present investigations were undertaken to elucidate the molecular structure of a number of *Salmonella* group G, N and U lipopolysaccharides that do not cross-react despite the fact that all belong to chemotype VI and are indistinguishable with respect to their monosaccharide constituents (Kauffmann *et al.* 1960). The results of a structural analysis of *Salmonella* group G lipopolysaccharides have been reported (Simmons, Lüderitz & Westphal, 1965). The present paper is concerned with the structure of *Salmonella* group N lipopolysaccharides.

MATERIALS AND METHODS

Lipopolysaccharides. The lipopolysaccharides were extracted from acetone-dried cultures of *Salmonella godesberg* and *Salmonella urbana* by the phenol-water method and purified in the ultracentrifuge as described by Kauffmann *et al.* (1960).

Isolation of *Salmonella* group N oligosaccharides. The oligosaccharides were prepared from 85 mg. of *S. godesberg* and 200 mg. of *S. urbana* lipopolysaccharides by the hydrolytic, chromatographic and electrophoretic methods described by Simmons *et al.* (1965). On high-voltage electrophoresis each polysaccharide hydrolysate yielded seven fractions designated E1-E7. Fraction E1 migrated to the anode and proved to be indistinguishable from 3-deoxy-2-oxo-octonate. Fraction E2 was electrophoretically neutral and the cathodic fractions, E3-E7, were shown to be saccharides containing basic amino sugars. The neutral fraction E2 contained six oligosaccharides that were separated chromatographically and designated C1-C6. Fraction C6 migrated with galactose in all the chromatographic solvents used. It was separated from galactose by high-voltage electrophoresis in borate buffer.

Structural studies of *Salmonella* group N oligosaccharides. The methods used for the microanalysis, borohydride reduction, periodate oxidation, N-acetylation and Morgan-Elson studies of these oligosaccharides have been described in detail by Simmons *et al.* (1965). Complement-fixation tests were carried out according to the method of Wasserman & Levine (1961) by using antisera kindly given by Professor F. Kauffmann, Copenhagen, and Dr Kampelmacher, Utrecht.

β -Glucosidase experiments. The action of β -glucosidase (Sigma Chemical Co., St Louis, Mo., U.S.A.) on oligosaccharides C1, C5 and E6 was tested as follows: 35 μ l. of water, 20 μ l. of 1.0 M-sodium acetate, pH 5.03, and 100 μ l. of enzyme (1 mg./ml.) were added to 10 μ l. of the oligosaccharide solution containing 20-100 μ moles of substrate. The mixture was incubated at 37° for 20 hr. The glucose

liberated was determined by the glucose-oxidase method (Krüger, Lüderitz, Strominger & Westphal, 1962). Cellobiase, used as a control, yielded 100% of glucose under these conditions.

α -Glucosidase experiments. The α -glucosidase was prepared by the method of Robbins & Uchida (1962). Its action on oligosaccharides C1, C5 and E6 was tested as follows: 38 μ l. of water, 50 μ l. of 0.06 M-sodium phosphate buffer, pH 7.0, and 2 μ l. of α -glucosidase were added to 10 μ l. of oligosaccharide solution containing 25–100 m μ -moles of substrate. The reaction mixture was incubated at 37° and the amount of free glucose assayed after 1, 3, 7, 9 and 20 hr. by the glucose-oxidase method (Krüger *et al.* 1962). Maltose and cellobiase served as positive and negative controls.

RESULTS

The two *Salmonella* group N lipopolysaccharides used in this study were extracted from *S. godesberg* and *S. urbana* strains that have the same O-antigenic structure (O 30). Their chemical structures also seem to be similar, if not identical, since the same series of oligosaccharides was obtained from both lipopolysaccharides by partial acid hydrolysis. As all the experimental evidence indicated that the corresponding oligosaccharides in each series were in fact identical, only one representative set of results for each pair of oligosaccharides is presented here.

Analysis of sugars in *Salmonella* group N lipopolysaccharides. On quantitative microanalysis the sugar composition of *S. godesberg* lipopolysaccharide (in %, w/w) was as follows: glucose, 18.4; galactose, 3.3; *N*-acetylgalactosamine, 14.7; *N*-acetylglucosamine, 5.0; fucose, 8.5; heptose, 2.7; 3-deoxy-2-oxo-octonate, 10. The carbohydrate content of this lipopolysaccharide is therefore about 63% (w/w). The lipid content was not determined. From their reactions with the specific enzymes used in the microanalysis it follows that the hexoses and glucosamine are of D-configuration and the fucose is present as L-fucose.

Homogeneity of oligosaccharides. Oligosaccharides C1, C5, C6, E3, E5 and E6 were homogeneous in chromatographic solvents I and III and on high-voltage electrophoresis in buffers A and B (Simmons *et al.* 1965). Oligosaccharides C2, C3 and C4 could not be completely separated by chromatography but were finally purified by high-voltage electrophoresis in borate buffer. After *N*-acetylation fraction E4 was shown to contain two components, which corresponded to fractions C2 and C4.

The R_{Glc} values of the purified oligosaccharides in solvent I and their electrophoretic mobilities in buffer A are given in Table 1. The yield of each oligosaccharide and its percentage of the total oligosaccharide yield are also given in Table 1.

Oligosaccharides C5 and E6. Chromatography

Table 1. Properties of oligosaccharides obtained from *Salmonella* group N lipopolysaccharide by partial acid hydrolysis

N.T., Not tested; * chromatographic evidence only. GalNH₂, galactosamine; GlcNH₂, glucosamine; Gal, galactose; Glc, glucose; Fuc, fucose. The small amounts of sugar shown in parentheses probably arise from impurities.

Oligosaccharide	Approx. yield (mg.)	% of total oligosaccharide yield	R_{Glc} in solvent I	$10^4 \times$ Electrophoretic mobility in buffer A (cm ² V ⁻¹ sec. ⁻¹)	$10^{-3} \times$ Monosaccharide content (μ moles/10 μ l. of eluate)					Nature of oligosaccharide	Sugar components of oligosaccharides	Sugar reduced by borohydride	Relationship of C and E oligosaccharides
					GalNH ₂	GlcNH ₂	Gal	Glc	Fuc				
C1	2.8	40	0.26	0.0	5.2	(1.0)	(0.8)	9.8	5.5	Tetrasaccharide	GalNH ₂ , Glc, Fuc	Fucose	E3 acetylated
C2	0.7	11	0.44	0.0	5.4	0.0	(0.4)	5.1	4.2	Trisaccharide	GalNH ₂ , Glc, Fuc	Fucose	E4 acetylated
C3	0.2	2	0.48	0.0	(0.1)	(0.1)	2.3	(0.4)	(0.4)	Disaccharide	Gal, Glc	Galactose*	—
C4	0.3	5	0.51	0.0	3.7	(0.1)	(0.2)	3.8	3.5	Trisaccharide	GalNH ₂ , Glc, Fuc	Fucose*	E4 acetylated
C5	0.4	5	0.68	0.0	2.6	0.0	(0.1)	2.4	(0.1)	Disaccharide	GalNH ₂ , Glc	Galactosamine	E6 acetylated
C6	0.7	11	0.87	0.0	4.2	(0.2)	(0.4)	(0.2)	6.4	Disaccharide	GalNH ₂ , Fuc	Fucose	—
E3	1.2	17	0.16	3.9	10.0	(4.0)	(0.6)	18.7	11.3	Tetrasaccharide	GalNH ₂ , Glc, Fuc	N.T.	C1 deacetylated
E4	0.3	5	0.28	5.1	(1.8)	(0.4)	(0.4)	5.5	6.2	Trisaccharide	GalNH ₂ , Glc, Fuc	N.T.	C2 and C4 deacetylated
E5	0.1	1	0.49	6.1	1.5	(0.4)	(0.2)	1.2	(0.2)	Disaccharide	GalNH ₂ , Glc	Galactosamine	—
E6	0.2	3	0.37	7.1	3.1	(0.6)	(0.2)	3.1	(0.3)	Disaccharide	GalNH ₂ , Glc	N.T.	C5 deacetylated

and microanalysis of these oligosaccharides (Table 1) showed that they contained glucose and galactosamine in the ratio 1:1. After *N*-acetylation, fraction E6 proved to be chromatographically indistinguishable from fraction C5. On borohydride reduction the galactosamine content of a solution of fraction C5 fell from 25.6 to 2.0 $\mu\text{moles}/10\mu\text{l}$. with no loss in the glucose content. This result indicates that the *N*-acetylglactosamine residue is at the reducing end of the oligosaccharide. In the Morgan–Elson reaction fraction C5 was strongly positive, the colour yield of the unhydrolysed oligosaccharide being 2.3 times that of the hydrolysed substance. After acetylation fraction E6 also reacted strongly, the colour yield of the unhydrolysed oligosaccharide being 2.5 times that of the hydrolysed substance. These findings suggest that the *N*-acetylglactosamine is probably 3-*O*-substituted (Foster & Horton, 1959). β -Glucosidase hydrolysed 60% of the glucose in fraction E6, whereas no glucose was liberated with α -glucosidase. The above findings taken together suggest that the probable structure of oligosaccharide C5 is β -D-glucosyl-(1 $\rightarrow$ 3)-*N*-acetylglactosamine.

Oligosaccharide E5. On microanalysis fraction E5 contained glucose and galactosamine in the ratio 1:1 and on borohydride treatment the galactosamine was reduced. These findings, together with its electrophoretic mobility in buffer A, indicate that it is a disaccharide. After *N*-acetylation no colour was obtained in the Morgan–Elson reaction, indicating that acetylated oligosaccharide E5 is probably a glucosyl-(1 $\rightarrow$ 4)-*N*-acetylglactosamine.

Oligosaccharide C6. Chromatography and microanalysis revealed the presence of *N*-acetylglactosamine and fucose in the ratio 1:1, indicating that fraction C6 is a disaccharide. On borohydride treatment the fucose was totally reduced and in the Morgan–Elson reaction the colour yield of the unhydrolysed oligosaccharide was low. These findings indicate that this oligosaccharide is an *N*-acetylglactosaminylfucose but provide no evidence as to the nature of the linkage between the two sugar residues.

Oligosaccharides C2, C4 and E4. Microanalysis of these oligosaccharides (Table 1) showed that all contained glucose, galactosamine and fucose in the proportions 1:1:1, indicating that they are probably trisaccharides. The chromatographic mobilities of these fractions and the electrophoretic mobility of fraction E4 in buffer A support this view. After *N*-acetylation fraction E4 proved to be a mixture of two trisaccharides that were chromatographically indistinguishable from fractions C2 and C4. Borohydride treatment of fractions C2 and C4 resulted in the reduction of fucose, so that this sugar is at the reducing end of the molecule in both instances. In the Morgan–Elson reaction oligo-

saccharide C2 formed no chromogen, whereas unhydrolysed fraction C4 yielded 0.3 times as much colour as the hydrolysed oligosaccharide. Colour formation with fraction C4 indicates that the linkage between the *N*-acetylglactosamine and the fucose is partially labile under the conditions of the Morgan–Elson reaction. The total absence of colour formation with fraction C2 is probably due to 4-*O*-substitution of the *N*-acetylglactosamine. The structure of oligosaccharide C2 is considered to be glucosyl-(1 $\rightarrow$ 4)-*N*-acetylglactosaminyl-L-fucose. With fraction C4, chromogen formation indicates that the *N*-acetylglactosamine is 3-*O*- or 6-*O*-substituted, but the former seems more probable since glucosyl-(1 $\rightarrow$ 3)-*N*-acetylglactosamine has already been identified among the hydrolysis products of this lipopolysaccharide. The structure of oligosaccharide C4 is considered to be glucosyl-(1 $\rightarrow$ 3)-*N*-acetylglactosaminyl-L-fucose.

Oligosaccharides C1 and E3. The microanalytical results on oligosaccharides C1 and E3 show they contain glucose, *N*-acetylglactosamine and fucose in the proportions 2:1:1, which indicates they are probably tetrasaccharides. The chromatographic mobility of fraction C1 and the electrophoretic mobility of fraction E3 support this view. On *N*-acetylation fraction E3 gave a product that was chromatographically indistinguishable from fraction C1. After borohydride reduction of 55.0 μmoles of fraction C1, the fucose content fell to 1.1 μmoles , whereas the amounts of the other sugars remained unaltered. These findings, together with the chromatographic demonstration of fucitol in the hydrolysate of reduced fraction C1, prove that the reducing end group of this tetrasaccharide is fucose. Fraction C1 and *N*-acetylated fraction E3 gave a negative Morgan–Elson reaction. As the linkage between the *N*-acetylglactosamine and fucose is partially labile under the alkaline conditions of this test, a negative result suggests 4-*O*-substitution of the *N*-acetylglactosamine residue with glucose. This conclusion is supported by the previous isolation of glucosyl-(1 $\rightarrow$ 4)-*N*-acetylglactosamine. With the isolation of β -D-glucosyl-(1 $\rightarrow$ 3)-*N*-acetylglactosamine it seems probable that the second glucose residue in oligosaccharide C1 is β -(1 $\rightarrow$ 3)-linked to the *N*-acetylglactosamine. These findings imply that oligosaccharide C1 is a branched molecule and that its structure is possibly β -D-glucosyl-(1 $\rightarrow$ 3)-*N*-acetylglactosaminyl-L-fucose with a second glucose residue linked to the 4-position of the *N*-acetylglactosamine.

Periodate oxidation was carried out to confirm this structure but the experimental results, though reproducible, proved difficult to interpret. One mol. of oligosaccharide C1 reduced 4.2 mol. of

periodate with the production of 3.0 mol. of formic acid, 0.3 mol. of formaldehyde and no acetaldehyde. After 24 hr. there was a gradual linear increase in the reduction of periodate and the resulting oxidation products owing to overoxidation. The values given above were obtained by extrapolation to zero time. After the treatment of a sample of oligosaccharide C1 with periodate, the monosaccharide composition was redetermined to detect which sugar components, if any, were protected: 92.6% of the galactosamine in the sample was recovered, which indicates that this sugar is virtually unoxidized. This finding is in agreement with the proposed formula. In a similar analysis of the sugar composition of *Salmonella* group N lipopolysaccharide before and after periodate oxidation, the percentage (w/w) of fucose fell from 8 to 0. Fucose is therefore not protected and is therefore not substituted at C-3. Small amounts of chromogen formation with fractions C4 and C6 in the Morgan-Elson reaction contraindicate 2-O-substitution of the fucose, since a linkage of this type should be stable in alkaline conditions (see Foster & Horton, 1959). The absence of acetaldehyde formation in the periodate studies on fraction C1 favours a (1→4)-bond between the *N*-acetylgalactosamine and fucose residues. On the basis of the above evidence the structure of oligosaccharide C1 is considered to be β -D-glucosyl-(1→3)-*N*-acetylgalactosaminyl-(1→4)-L-fucose with a further glucose residue 4-O-substituted on the *N*-acetylgalactosamine.

Oligosaccharide C3. On microanalysis oligosaccharide C3 contained equal amounts of glucose and galactose. It behaves chromatographically as a disaccharide and was indistinguishable from oligosaccharide C3 isolated from *Salmonella* group G lipopolysaccharide. On borohydride reduction, chromatographic evidence indicated that the galactose was reduced. Further, 100% of the glucose in fraction C3 was split by α -glucosidase. From these findings the structural sequence of oligosaccharide C3 is α -D-glucosyl-D-galactose.

Fraction E1. Fraction E1 and an authentic sample of 3-deoxy-2-oxo-octonate were indistinguishable when compared chromatographically in solvent I and electrophoretically in buffer A. The amount of 3-deoxy-2-oxo-octonate was assayed by the method of Waravdekar & Saslaw (1959).

Fraction E7. Fraction E7 contained free glucosamine and galactosamine only.

Complement-fixation inhibition. Of the sugars tested in the homologous *S. godesberg* complement-fixation system galactose, methyl α -D-galactoside, methyl β -D-galactoside, methyl α -D-glucoside, *N*-acetylgalactosamine, *N*-acetylglucosamine and fucose gave no inhibition; 0.5, 1.0, 2.5 and 5.0 μ mole amounts of methyl β -D-glucoside gave 3, 7, 10 and 16% inhibition respectively. The same amounts of

glucose gave only 1, 2, 2 and 3% inhibition respectively. These results confirm the presence of β -glucosidic linkages in *Salmonella* group N lipopolysaccharide and indicate that some of these may play a role in specificity. The inactivity of methyl α -D-glucoside in the test system seems to exclude the presence of terminal non-reducing α -glucosyl radicals in the repeating unit of this lipopolysaccharide.

DISCUSSION

The *Salmonella* group N lipopolysaccharides belong to chemotype VI, which contains the 'basal sugars' (heptose, galactose, glucose and glucosamine) and the two 'special sugars', *N*-acetylgalactosamine and fucose (Kauffmann *et al.* 1960). 3-Deoxy-2-oxo-octonate, which is believed to be a constituent of all *Salmonella* lipopolysaccharides, has also been isolated from the *S. godesberg* and *S. urbana* preparations. On partial acid hydrolysis, group N lipopolysaccharide yields a number of oligosaccharides, most of which form a structurally related series that is shown in Scheme 1. This series of oligosaccharides indicates that the structural unit of the specific side chain of *Salmonella* group N lipopolysaccharide is a tetrasaccharide containing glucose, *N*-acetylgalactosamine and fucose in the proportions 2:1:1 respectively. Since the proportions of these sugars in the parent lipopolysaccharide are roughly the same, the bulk of the molecule must lie in the specific side chains, which would therefore seem to be composed of a number of repeating units analogous to those

Scheme 1. Structural relationship of oligosaccharides obtained by partial acid hydrolysis of *Salmonella* group N lipopolysaccharides. Glc, glucosyl; GalNAc, *N*-acetylgalactosaminyl; GalNH₂, *N*-acetylglucosamine; GalNH₂, galactosamine; Fuc, fucose.

proposed for *Salmonella* group E antigens (Robbins & Uchida, 1962) and for *Salmonella* group B and D antigens (Staub & Raynaud, 1964). This repeating unit seems to be oligosaccharide C1. Glucosamine, also found to be a constituent of group N lipopolysaccharides, obviously is not a constituent of the O-specific side chain. It is present as a constituent of lipid A as well as the basal core structure.

An oligosaccharide that does not contain either of the 'special sugars', *N*-acetylgalactosamine and fucose, was also isolated from the 'smooth' (S) lipopolysaccharide of group N. It probably arises from hydrolysis of the underlying basal structure or 'rough' (R) core that is believed to exist in all *Salmonella* S-lipopolysaccharides. It has the structure of an α -D-glucosyl-D-galactose and is indistinguishable from that isolated from the S-lipopolysaccharides of group G and from a number of R-lipopolysaccharides (Sutherland, Lüderitz & Westphal, 1965).

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