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# The Immunochemistry of *Salmonella* Chemotype VI O-Antigens

## THE STRUCTURE OF OLIGOSACCHARIDES FROM *SALMONELLA* GROUP G (O 13, 22) LIPOPOLYSACCHARIDES

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1. Lipopolysaccharides have been isolated from 'smooth' (S) strains of *Salmonella friedenau* and *Salmonella poona* by the phenol–water method and purified in the preparative ultracentrifuge. 2. These lipopolysaccharides are serologically indistinguishable and on partial acid 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 β-galactosidase on the isolated oligosaccharides indicate that the O-specific side chains of these lipopolysaccharides have a repeating pentasaccharide unit that is β-D-galactosyl-(1→3)-*N*-acetylgalactosaminyl-(1→3)-*N*-acetylgalactosaminyl-(1→4)-L-fucose with a D-glucose residue bound at an undetermined point on this structure. 4. Two oligosaccharides, a glucosylgalactose and an *N*-acetylglucosaminylglucose, have also been isolated and these seem to be identical with oligosaccharides obtained from 'rough' (R) *Salmonella* lipopolysaccharides. These findings are in accordance with the view that *Salmonella* S-lipopolysaccharides have a core that consists of R-lipopolysaccharide.

The *Salmonella* lipopolysaccharides have been divided into 16 chemotypes according to their monosaccharide constituents (Kauffmann, Lüderitz, Stierlin & Westphal, 1960). The basal sugars of chemotype I (heptose, 3-deoxy-2-oxo-octonate, galactose, glucose and glucosamine) are common to all chemotypes, and the antigens of chemotypes II–XVI contain additional 'special sugars' such as ribose, mannose, *N*-acetylgalactosamine, fucose, rhamnose, colitose, abequose, paratose and tyvelose, which occur singly or together in a pattern that is unique for each chemotype. The occurrence of the basal sugars in all these substances, the discovery that the lipopolysaccharides obtained from most R-mutants belong to chemotype I (Kauffmann, Krüger, Lüderitz & Westphal, 1961) and the demonstration of underlying 'rough' (R) serological specificity in 'smooth' (S) lipopolysaccharides after the removal of 'special sugars' by mild hydrolysis (Westphal & Lüderitz, 1961; Lüderitz, Beckmann & Westphal, 1964) suggest that the S-lipopolysaccharides of all 16 chemotypes probably have a common core to which determinant O-specific side chains are attached. It is believed that this core is identical with the R-lipopolysaccharide of mutants belonging to R serogroup RII, which fail to synthesize the determinant side chains of the corresponding S-lipopolysaccharide through lack of a specific synthetase or transferase (Nikaido, Nikaido, Subbaiah & Stocker, 1964; Lüderitz *et al.* 1965). The study of different *Salmonella* R-mutants has provided information about the mechanism of core synthesis (Osborn, Rosen, Rothfield, Zeleznick & Horecker, 1964). By contrast, little is known of the presumably more complex pathways required to synthesize the numerous serologically distinct O-specific side chains of all known *Salmonella* species. Despite the serological complexity of these antigenic determinants, there could be structural relationships between the different S-lipopolysaccharides if steps in their biosynthetic pathways were shared. The present investigation was undertaken to discover if such structural similarities exist and to provide, if possible, direct chemical evidence for the presence of a 'rough' core in S-lipopolysaccharides.

The S-lipopolysaccharides of *Salmonella* chemotype VI, which contains serogroups G, N, and U, seemed to provide a suitable model for these studies. The present paper is concerned with the structure of *Salmonella* group G lipopolysaccharides.

## MATERIALS AND METHODS

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

*Chromatography.* This was carried out on Whatman no. 1 paper with the following solvents: I, butan-1-ol–pyridine–water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951); II, butan-1-ol–acetic acid–water (4:1:5, by vol.) (Partridge, 1948); III, ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.) as developing phase with ethyl acetate–pyridine–water (40:11:6, by vol.) to saturate the atmosphere (Fischer & Dörfel, 1955). The following identifying reagents were used: alkaline $AgNO_3$ (Trevelyan, Procter & Harrison, 1950); aniline phthalate (Partridge, 1949); ninhydrin (Consden, Gordon & Martin, 1944); *p*-dimethylaminobenzaldehyde (Cardini & Leloir, 1957).

*High-voltage electrophoresis.* This was carried out on 2043 bmgl paper (Schleicher und Schüll, Dassel, Germany) according to the method of Kickhöfen & Westphal (1952), with the following buffers: A, pyridine–acetic acid–water (5:2:43, by vol.), pH 5·3; B, 0·05 M-sodium tetraborate, pH 9·2. With buffer A the electrophoresis was run at 3000 v and 120 mA for 1 hr., and with buffer B at 3000 v and 40 mA for 2 hr. Oligosaccharides eluted from buffer B contained borate, which was removed by passing the eluate through an Amberlite IR-120 column (1·5 cm. × 0·6 cm. diam.). The effluent was dried *in vacuo*, 200 $\mu$l. of methanol added and the solution evaporated to dryness on a 100° water bath. The heating in methanol was repeated twice. The product was dissolved in a small volume of water and stored at −20°.

*Preparation of oligosaccharides.* Oligosaccharides were isolated from 300 mg. samples of *S. friedenau* and *S. poona* lipopolysaccharides by hydrolysing in 1% (w/v) concentrations with N-$H_2SO_4$ at 100° for 25 min. After the hydrolysate had been diluted with 3 vol. of water, neutralization with Amberlite IR-410 ($HCO_3^-$ form) was carried out, the product being concentrated by vacuum-distillation to approx. 1 ml. The charged oligosaccharides were separated by high-voltage electrophoresis on a preparative scale in buffer A. The electrophoretograms were allowed to dry in air at room temperature, as heating causes degradation of oligosaccharides containing basic amino sugars. The oligosaccharides were recovered by elution with water, or with 0·01 N-HCl for basic fractions, after their positions had been localized with the aid of ultraviolet light and guide strips stained with alkaline $AgNO_3$ reagent. The electrophoretic fractions were designated E1–E7 from the anode to the cathode. The preparative electrophoresis was repeated until single spots were obtained in the homogeneity test. Fraction E1, which proved to be 3-deoxy-2-oxo-octonate, migrated to the anode. Fraction E2 contained all the electrophoretically neutral fragments of the partial acid hydrolysate of the lipopolysaccharide, including free glucose, galactose, fucose, *N*-acetylhexosamines and neutral oligosaccharides. Fractions E3, E4 etc. migrated to the cathode and proved to be oligosaccharides containing at least one unsubstituted amino group. Fraction E7 was a mixture of free hexosamines. The neutral oligosaccharides in fraction E2 were separated by paper chromatography on a preparative scale for 5 days in solvent I. After elution, the resulting fractions (designated C1–C5 in order of increasing $R_{Glc}$ values) were rechromatographed if necessary in solvent I or II and finally concentrated *in vacuo* until their concentration lay in the range 0·001–0·01 M. The areas of free galactose and glucose on these preparative chromatograms were also eluted and examined by high-voltage electrophoresis in borate buffer B to detect oligosaccharides with the same chromatographic mobilities as these two hexoses. No oligosaccharides were found in the glucose eluate, but a component (designated C6) was found in the galactose fraction and was recovered by preparative electrophoresis in borate buffer. The oligosaccharide solutions were stored at −20°.

*Hydrolysis of lipopolysaccharides and oligosaccharides.* For the analysis of amino sugars in lipopolysaccharides and oligosaccharides, hydrolysis was carried out in sealed ampoules for 12 hr. at 100° with 4 N-HCl. Samples with an expected content of 10–50 m$\mu$moles of hexosamine were rapidly dried *in vacuo* to remove the acid. For the analysis of neutral sugars, hydrolysis was carried out in sealed ampoules for 4 hr. at 100° with N-$H_2SO_4$. The hydrolysate was diluted by the addition of 4 vol. of water and neutralized with Amberlite IR-410 ($HCO_3^-$ form) before samples were dried *in vacuo* for microanalysis.

Formic acid hydrolysis was carried out with selected oligosaccharides by a method devised by R. Kuhn & H. Egge (personal communication) to ensure complete hydrolysis with the minimum amount of destruction of the resulting sugar components. A 0·1 $\mu$mole sample of oligosaccharide was hydrolysed in 100 $\mu$l. of 80% (v/v) formic acid at 100° for 40 hr. The product was dried *in vacuo*, taken up in 100 $\mu$l. of 0·05 N-HCl and heated at 100° for 4 hr. to remove formyl esters. Then 100 $\mu$l. of water was added and the solution neutralized with Amberlite IR-410 ($HCO_3^-$ form).

*Quantitative microanalytical methods.* Analyses were performed in 7·5 cm. × 1·0 cm. tubes by using Lang–Levy constriction pipettes as follows: D-glucose with glucose-oxidase reagent (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany); D-galactose by the galactose-dehydrogenase method (Wallenfels & Kurz, 1962); L-fucose with L-fucose isomerase (Krüger, Lüderitz, Strominger & Westphal, 1962); glucose, galactose and fucose in many cases also by a micro-modification of the cysteine–$H_2SO_4$ reaction with unhydrolysed samples of the lipopolysaccharide or oligosaccharide (Dische & Shettles, 1951); heptose by a modified cysteine–$H_2SO_4$ method (Osborn, 1963); 3-deoxy-2-oxo-octonate by the thiobarbituric acid method (Waravdekar & Saslaw, 1959); free hexosamines by a modified Morgan–Elson reaction after *N*-acetylation (Strominger, Park & Thompson, 1959); D-glucosamine by a specific enzymic method (Lüderitz, Simmons, Westphal & Strominger, 1964) that allows galactosamine to be calculated by difference; *N*-acetylhexosamines by a modified Morgan–Elson reaction (Reissig, Strominger & Leloir, 1956).

*Morgan–Elson reaction on oligosaccharides.* The modified Morgan–Elson reaction was also performed on the intact oligosaccharides containing amino sugars. It has been shown that 4-*O*-substituted *N*-acetylhexosamines react weakly or not at all in this test, that substituents in the 6-position do not influence the amount of colour developed and that 3-*O*-substituted derivatives give increased chromogen formation (see Foster & Horton, 1959). With the 3-*O*-substituted derivatives the substituent is liberated during heating in tetraborate. With the 6-*O*-substituted derivatives no elimination of the substituent occurs so that chromogen derivatives are formed. These reactions were studied quantitatively with the methods described above to determine the proportions of colour developed by oligosaccharides before and after hydrolysis.

*Borohydride reduction.* To 0·1–0·5 $\mu$mole of oligosaccharide in 50 $\mu$l. of water, 10 $\mu$l. of $NaBH_4$ (2·3 mg. in 100 $\mu$l.

of 0·1 N-KOH) was added. The tubes were left in the dark at room temperature for 16 hr. Then 2 N-acetic acid was added until the pH was about 4·0 (about 10 $\mu$l.). The electrolytes in the reaction mixture were removed by high-voltage electrophoresis in buffer A. The sugar composition of the reduced oligosaccharide was analysed qualitatively by chromatography in solvents I and III and quantitatively by the microanalytical methods.

*Periodate oxidation.* The periodate reactions were set up at 0°. To about 0·2 $\mu$mole of the oligosaccharide (or control sugar) in 250 $\mu$l. of water, 80 $\mu$l. of 0·025 M-$NaIO_4$ was added. The reaction was allowed to proceed at 0–4° in the dark and the products were determined quantitatively at convenient periods from 30 min. to 1 week after the addition of the periodate. Reduction of periodate was determined spectrophotometrically at 225 m$\mu$ by the method of Rammler & Rabinowitz (1962); formic acid (about 0·01 $\mu$mole) by a micro-modification of the formyltetrahydrofolate-synthetase method (Rammler & Rabinowitz, 1962); formaldehyde by the chromotropic acid method (Kabat & Mayer, 1961); acetaldehyde by the *p*-hydroxybiphenyl method (Kabat & Mayer, 1961). In some cases the acetaldehyde formed (about 0·03 $\mu$mole) was separated from formaldehyde by passing a stream of air for 1 hr. through the periodate reaction mixture into the identifying reagent, which contained 150 $\mu$l. of water, 20 $\mu$l. of 12% (w/v) $CuSO_4,5H_2O$, 1·0 ml. of conc. $H_2SO_4$ and 20 $\mu$l. of *p*-hydroxybiphenyl (15 mg./ml. in 0·5 N-NaOH). The acetaldehyde was determined spectrophotometrically at 570 m$\mu$. This is a semi-quantitative micro-modification of the method of Neidig & Hess (1952). With oligosaccharide C2 the periodate-treated compound was analysed for unaltered sugar components. This experiment was set up as described above with 1 $\mu$mole of fraction C2. The action of periodate was stopped after 24 hr. by adding 150 $\mu$l. of 0·1 M-lead acetate. After 5 min. at room temperature the precipitate was separated by centrifuging at 2000*g* for 5 min., and a further 30 $\mu$l. of 0·1 M-lead acetate was added to ensure that the precipitation of $IO_4^-$ and $IO_3^-$ was complete. The reaction mixture (about 2·0 ml.) was passed through a column of Amberlite IR-120 (4·0 cm. × 0·8 cm. bore) and washed out with 6·0 ml. of water. After centrifuging at 2000*g* for 5 min. the clear neutral supernatant was dried *in vacuo*. The product was hydrolysed and analysed as described above.

The action of periodate on the intact lipopolysaccharide of *S. friedenau* was studied by analysing the hexose composition before and after oxidation and dialysis by using the methods described above.

N-*Acetylation of deacetylated oligosaccharides.* The possible relationship between the C (neutral) and E (basic) series of oligosaccharides was elucidated by *N*-acetylation of the E fractions and chromatography of the products in solvent I or III with the C fractions. The acetylation was carried out according to the method of Strominger *et al.* (1959). High-voltage electrophoresis in buffer A was used to eliminate the electrolytes.

$\beta$-*Galactosidase experiments.* The tests were set up as follows. First 10 $\mu$l. of 0·05 M-tris–HCl buffer, pH 7·7, and 100 $\mu$l. of $\beta$-galactosidase solution (50000 units as defined by Wallenfels & Kurz, 1962) were added to 0·1–0·3 $\mu$mole of oligosaccharide that had been dried *in vacuo*. After the reagents had been mixed, 10 $\mu$l. of toluene was added and, without mixing, the tests were incubated at 37° overnight. The amount of galactose split by the enzyme was measured by the dehydrogenase method (Wallenfels & Kurz, 1962).

*Complement-fixation inhibition studies.* The specific inhibition of antigen–antibody reactions by saccharides of known structure was measured quantitatively in complement-fixation tests, according to the method of Wasserman & Levine (1961), by using serogroup G lipopolysaccharides and their homologous antisera.

## RESULTS

The two *Salmonella* group G lipopolysaccharides used in this study were extracted from *S. friedenau* and *S. poona* strains that have the same O-antigenic structure (O 13, 22). Their chemical structure also seems to be very similar, if not actually identical, since the same series of oligosaccharides was obtained from both lipopolysaccharides by partial acid hydrolysis. Therefore only one representative set of results for each pair of oligosaccharides is presented here.

*Analysis of sugars in* Salmonella *group G lipopolysaccharides.* On quantitative microanalysis the composition (%, w/w) of sugars present in *S. friedenau* lipopolysaccharide was as follows: glucose, 5·9; galactose, 11·6; *N*-acetylglucosamine, 8·1; *N*-acetylgalactosamine, 27·7; fucose, 8·7; heptose phosphate, 3·1; 3-deoxy-2-oxo-octonate, 5·0. The carbohydrate content of this lipopolysaccharide is therefore about 70% (w/w). The amount of lipid was not determined. From their reactions with the specific enzymes used in the microanalysis, the hexoses and glucosamine are of the D-configuration. The fucose is present as L-fucose.

*Homogeneity of oligosaccharides.* All the isolated oligosaccharides appear homogeneous by the criteria of chromatography in solvents I and III and electrophoresis in buffers A and B. Despite these appearances of homogeneity, the analytical results provide strong evidence that fraction C6 is a mixture of two disaccharides that proved inseparable by the methods used. The structures proposed for these two saccharides (redesignated C6a and C6b) therefore depend on analytical and borohydride reduction studies of these two substances in mixture.

The $R_{Glc}$ values of the isolated 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 C4 and E6.* Microanalysis of oligosaccharides C4 and E6 (Table 1) showed that these two substances contain galactosamine and galactose in the ratio 2:1. These findings, together with the chromatographic behaviour of fraction C4 and the electrophoretic mobility of fraction E6 in buffer A, are consistent with the view that both are trisaccharides. The *N*-acetylation of fraction E6 gives a product that is chromatographically

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

N.T., Not tested; C.A., not determined quantitatively, chromatographically absent; * chromatographic evidence only. $GalNH_2$, galactosamine; $GlcNH_2$, 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 | $10^6 \times$ Electrophoretic mobility in buffer A ($cm.^2 v^{-1} sec.^{-1}$) | $10^{-2} \times$ Monosaccharide content ($\mu$moles/10 $\mu$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 | 0·7 | 5 | 0·18 | 0·0 | 2·7 | 0·0 | 1·3 | 1·2 | 1·0 | Pentasaccharide | $GalNH_2$, Gal, Glc, Fuc | N.T. | E3 acetylated |
| C2 | 5·2 | 38 | 0·35 | 0·0 | 26·3 | (0·9) | 11·9 | (0·3) | 10·0 | Tetrasaccharide | $GalNH_2$, Gal, Fuc | Fucose | E4 acetylated |
| C3 | 0·3 | 2 | 0·49 | 0·0 | (0·4) | 0·0 | 1·0 | 1·3 | 0·0 | Disaccharide | Gal, Glc | Galactose* | — |
| C4 | 0·5 | 3 | 0·58 | 0·0 | 4·4 | (0·2) | 2·1 | (0·3) | 0·0 | Trisaccharide | $GalNH_2$, Gal | Galactosamine | E6 acetylated |
| C5 | 0·3 | 2 | 0·68 | 0·0 | (0·1) | 2·4 | 0·0 | 2·0 | (0·1) | Disaccharide | $GlcNH_2$, Glc | Glucose | — |
| C6a | 1·2 | 9 | 0·87 | 0·0 | 12·8 | (0·1) | C.A. | C.A. | 0·0 | Disaccharide | $GalNH_2$, | Galactosamine | E5 acetylated |
| C6b | 0·8 | 6 | 0·87 | 0·0 | 4·6 | (0·1) | C.A. | C.A. | 4·6 | Disaccharide | $GalNH_2$, Fuc | Fucose | — |
| E3 | 1·8 | 14 | 0·08 | 3·9 | 4·6 | (0·1) | 2·5 | 2·1 | 2·2 | Pentasaccharide | $GalNH_2$, Gal, Glc, Fuc | Fucose* | C1 deacetylated |
| E4 | 1·7 | 13 | 0·19 | 4·8 | 3·8 | (0·2) | 1·5 | (0·2) | 1·6 | Tetrasaccharide | $GalNH_2$, Gal, Fuc | Fucose* | C2 deacetylated |
| E5 | 0·7 | 5 | 0·44 | 6·6 | 1·1 | (0·1) | 0·0 | 0·0 | 0·0 | Disaccharide | $GalNH_2$ | N.T. | C6 deacetylated |
| E6 | 0·3 | 3 | 0·27 | 7·1 | 1·0 | 0·0 | 0·5 | (0·1) | (0·1) | Trisaccharide | $GalNH_2$, Gal | Galactosamine* | C4 deacetylated |

indistinguishable from fraction C4. Thus fraction E6 seems to be a deacetylated form of fraction C4 that has arisen during partial acid hydrolysis of the parent lipopolysaccharide. On borohydride reduction the galactosamine content of a sample of fraction C4 fell from 100 to 44 m$\mu$moles, which indicates that half the galactosamine is bound as the reducing end group of the trisaccharide. The Morgan–Elson reaction according to the method of Reissig *et al.* (1956) on intact oligosaccharide C4 was strongly positive, the colour yield of the unhydrolysed trisaccharide being about 1·9 times that given by the same quantity of fraction C4 after complete hydrolysis. This finding indicates that both *N*-acetylgalactosamine residues are probably substituted in the 3-position. When fraction C4 was reduced with sodium borohydride the Morgan–Elson reaction of the product was negative. After treatment with $\beta$-galactosidase 46·5 m$\mu$moles of galactose were obtained from 44·5 m$\mu$moles of oligosaccharide C4. These results confirm that the galactose is terminal and bound $\beta$-glycosidically. A possible structure for oligosaccharide C4 on the basis of the above evidence is $\beta$-D-galactosyl-(1→3)-*N*-acetylgalactosaminyl-(1→3)-*N*-acetylgalactosamine.

*Oligosaccharides C2 and E4.* Microanalysis of oligosaccharides C2 and E4 indicates that these two substances contain galactose, galactosamine and fucose in the proportions 1:2:1, so that they are probably tetrasaccharides. The $R_{Glc}$ value of fraction C2 in solvent I and the electrophoretic mobility of fraction E4 in buffer A are consistent with this conclusion. Fraction E4 seems to be fraction C2 deacetylated, since *N*-acetylation of the former yields a product that is chromatographically indistinguishable from fraction C2. On borohydride reduction the fucose was quantitatively reduced and fucitol demonstrated chromatographically in solvents I and III.

Oligosaccharide C2 reacted strongly in the Morgan–Elson reaction, the colour yield of the unhydrolysed saccharide being about 1·1 times that of hydrolysed fraction C2 after *N*-acetylation according to the method of Strominger *et al.* (1959). As both the amino sugars in fraction C2 were assumed to be glycosidically linked, chromogen formation can take place only if the oligosaccharide is degraded during the Morgan–Elson reaction. A sample of fraction C2 was therefore hydrolysed at 100° for 7 min. in 1% (w/w) tetraborate (as in the Morgan–Elson reaction) and the resulting products were analysed chromatographically after the removal of borate by high-voltage electrophoresis in buffer A. Three products detected with the silver nitrate reagent were eluted from the chromatogram. The first was shown by chromatography and quantitative analysis to be undegraded fraction C2. The

second product was galactose. Its amount was assayed by galactose dehydrogenase and found to be 46% (w/w) of that present in the original sample. The third product gave a direct Morgan–Elson reaction, i.e. a positive colour reaction with *p*-dimethylaminobenzaldehyde without pretreatment with borate. The colour yield corresponded to 59% of that given by the original sample of fraction C2. No free fucose was found. These results show that, when fraction C2 is treated under the same conditions as prevail in the Morgan–Elson reaction, about 50–60% of it is degraded: fucose at the reducing end of the molecule is destroyed, chromogen is formed and free galactose is liberated. The free galactose is strong evidence that this sugar is terminal at the non-reducing end of the molecule and that it is (1→3)-linked to the next sugar, which is *N*-acetylgalactosamine. Further, when oligosaccharides containing non-reducing amino sugars are exposed to treatment with alkali as in the Morgan–Elson test, the rate of degradation of the reducing sugar depends on the linkage between the penultimate glycosyl residue and the reducing end group (see Foster & Horton, 1959). It has been shown in some examples that (1→3)-linkages split rather rapidly, (1→4)-linkages more slowly and (1→2)-linkages not at all. Thus the fucose in this instance is most probably not 2-*O*-substituted.

On treatment with β-galactosidase for 15hr. under the conditions described above, 66mμmoles of galactose were obtained from 167mμmoles of oligosaccharide C2 (yield: 40%, w/w), and 108mμmoles of galactose were obtained from 183mμmoles of oligosaccharide E4 (yield: 60%, w/w). These results confirm that the galactose is terminal and that it is probably bound as the β-anomer.

In a series of periodate oxidation experiments 1·0mol. of oligosaccharide C2 reduced 4·05mol. of periodate and produced 2·90mol. of formic acid; only traces of formaldehyde and of acetaldehyde could be detected. After 24hr. 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. A possible interpretation of these results is that the terminal non-reducing galactose reduces 2mol. of periodate/mol., yielding 1 mol. of formic acid/mol., and that the fucose at the reducing end reduces 2mol. of periodate/mol., yielding 2mol. of formic acid/mol. The small amounts of formaldehyde and acetaldehyde would be due to overoxidation. If this view is correct then the *N*-acetylgalactosamine residues are protected. An attempt was made to confirm this experimentally by recovering the fragments of oligosaccharide C2 after periodate treatment and determining the amount of amino sugar remaining. In one such experiment 76% of the galactosamine was recovered (making no allowances for experimental losses and overoxidation), which indicates that the *N*-acetylgalactosamine residues are not attacked by periodate. When the monosaccharide composition of *S. friedenau* lipopolysaccharide was analysed before and after periodate oxidation, the percentage of galactosamine fell from 19 to 14 and that of fucose from 8 to 0. These results confirm that the *N*-acetylgalactosamine is largely protected, whereas the fucose is almost completely destroyed. Fucose is therefore not substituted in the 3-position in the lipopolysaccharide. The quantitative periodate oxidation studies of fraction C2, and especially the absence of acetaldehyde formation, favour the view that the *N*-acetylgalactosamine residues are substituted in the 3-position and that the fucose may be 4-*O*-substituted.

On the basis of all the above evidence the structure of oligosaccharide C2 is considered to be β-D-galactosyl-(1→3)-*N*-acetylgalactosaminyl-(1→3)-*N*-acetylgalactosaminyl-(1→4)-L-fucose.

*Oligosaccharides C1 and E3.* On microanalysis oligosaccharides C1 and E3 (Table 1) contained glucose, galactose, galactosamine and fucose in the proportions 1:1:2:1, which indicates that they are probably pentasaccharides. The chromatographic behaviour of fraction C1 and the electrophoretic mobility of fraction E3 support this view. *N*-Acetylation of fraction E3 gave a product that was chromatographically indistinguishable from fraction C1. On borohydride reduction of fraction E3, the fucose was reduced and fucitol detected chromatographically in solvents I and III. In the Morgan–Elson reaction fraction C1 was very weakly positive, the colour yield of the unhydrolysed oligosaccharide being about 0·1 times that given by hydrolysed fraction C1.

Owing to insufficient material further experimental work with these oligosaccharides was not possible.

*Fractions C6 and E5.* On chromatography, fraction C6 behaved as a disaccharide, but on hydrolysis and analysis it contained 174mμmoles of galactosamine and 46mμmoles of fucose/10μl. These results suggested that fraction C6 might be a mixture of 46mμmoles of an *N*-acetylgalactosamine–fucose disaccharide and 64mμmoles of an *N*-acetylgalactosamine–*N*-acetylgalactosamine disaccharide. This view is supported by the borohydride reduction studies on fraction C6 in which the fucose content fell from 46 to 1mμmole (i.e. by 45mμmoles) and the galactosamine content from 174 to 109mμmoles (i.e. by 65mμmoles). The *N*-acetylgalactosaminyl-*N*-acetylgalactosamine was designated fraction C6a and the *N*-acetylgalactosaminylfucose fraction C6b. The colour yield of unhydrolysed fraction C6 in the Morgan–Elson reaction was about 0·9 times that given by the

same quantity of hydrolysed fraction C6. It is assumed that most of the colour is produced by the reaction of fraction C6a and that the two *N*-acetylgalactosamine residues are (1→3)-linked.

Chromatography and microanalysis indicate that fraction E5 is a disaccharide containing galactosamine only. After *N*-acetylation it was chromatographically indistinguishable from oligosaccharide C6a in solvent III. Its electrophoretic mobility indicates that one of the residues is probably *N*-acetylated.

From the above evidence oligosaccharide C6a is considered to be *N*-acetylgalactosaminyl-(1→3)-*N*-acetylgalactosamine and oligosaccharide C6b is *N*-acetylgalactosaminylfucose.

*Oligosaccharides C3 and C5.* On chromatography and microanalysis, oligosaccharides C3 and C5 were found to be disaccharides, the former containing glucose and galactose, and the latter containing *N*-acetylglucosamine and glucose. Borohydride reduction studies indicated that the reducing end group in fraction C3 was galactose, and that in fraction C5 was glucose. Further, 100% of the glucose in fraction C3 was split by α-glucosidase. These results indicate that oligosaccharide C3 is an α-D-glucosyl-D-galactose ($R_{Glc}$ 0·49 in solvent I), being different from α-D-glucosyl-(1→4)-D-galactose ($R_{Glc}$ 0·44 in solvent I) and α-D-glucosyl-(1→6)-galactose ($R_{Glc}$ 0·34 in solvent I). Oligosaccharide C5 is an *N*-acetyl-D-glucosaminyl-D-glucose.

*Fraction E1.* Fraction E1 and an authentic sample of 3-deoxy-2-oxo-octonate behaved identically on high-voltage electrophoresis in buffer A, with thiobarbituric acid as identifying reagent. The amount of 3-deoxy-2-oxo-octonate was assayed as described above.

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

*Complement-fixation inhibition.* Of the sugars tested in the homologous *S. friedenau* complement-fixation system, *N*-acetylgalactosamine, *N*-acetylglucosamine, methyl β-D-glucoside and methyl α-D-galactoside gave no significant inhibition. The percentage inhibitions given by glucose, galactose, fucose, methyl α-D-glucoside and methyl β-D-galactoside are given in Table 2. From these results one may conclude that non-reducing α-glucosyl, β-galactosyl and fucosyl groups may play a determinant role in the specificity of *Salmonella* group G (O 13, 22) antigen.

## DISCUSSION

The *Salmonella* group G lipopolysaccharides contain the 'basal sugars' (3-deoxy-2-oxo-octonate, heptose, *N*-acetyl-D-glucosamine, D-galactose, D-glucose) and two 'special sugars', namely *N*-acetylgalactosamine and L-fucose. On partial acid hydrolysis of group G lipopolysaccharides most of the isolated oligosaccharides contained one or both of these 'special sugars'. Their structural relationship is shown in Scheme 1. This series of oligosaccharides indicates that the structural unit of the specific side chain of *Salmonella* group G is most probably a pentasaccharide containing glucose, galactose, *N*-acetylgalactosamine and fucose in the proportions 1:1:2:1. Since the proportions of these sugars in the whole lipopolysaccharide are similar, the

Table 2. *Inhibition of a homologous* S. friedenau (*O* 13, 22) *complement-fixation system with saccharides of known molecular structure*

These complement-fixation inhibition tests were carried out in a standard volume of 1·05 ml., containing: *S. friedenau* (O 13, 22) antiserum, 0·2706 μl.; complement, 0·694 μl.; *S. friedenau* (O 13, 22) lipopolysaccharide, 103 mμg.; amboceptor, 0·1875 μl.; sheep erythrocytes, $1·5 \times 10^7$. N.T., Not tested.

| Inhibiting sugar | Percentage inhibition | | | |
|---|---|---|---|---|
| | 0·5 μmole | 1·0 μmole | 2·5 μmoles | 5·0 μmoles |
| D-Glucose | 3 | 7 | 15 | 25 |
| Methyl α-D-glucoside | 11 | 20 | 26 | 40 |
| Methyl β-D-glucoside | 1 | 0 | N.T. | N.T. |
| D-Galactose | 3 | 6 | 12 | 17 |
| Methyl α-D-galactoside | 1 | 1 | N.T. | N.T. |
| Methyl β-D-galactoside | 7 | 9 | 10 | 12 |
| Methyl α-D-glucoside + methyl β-D-galactoside | 7 | 15 | 43 | 52 |
| Maltose | 9 | 12 | 29 | 41 |
| Lactose | 0 | 0 | 0 | 0 |
| L-Fucose | 5 | 6 | 5 | 8 |
| *N*-Acetylglucosamine | 0 | 0 | 1 | 2 |
| *N*-Acetylgalactosamine | 1 | 0 | 0 | 1 |

C1 β-D-Gal-(1→3)-GalNAc-(1→3)-GalNAc-(1→4)-L-Fuc
+ D-Glc
C2 β-D-Gal-(1→3)-GalNAc-(1→3)-GalNAc-(1→4)-L-Fuc
C4 β-D-Gal-(1→3)-GalNAc-(1→3)-GalNAc
C6a GalNAc-(1→3)-GalNAc
C6b GalNAc——→L-Fuc

Scheme 1. Structural relationship of oligosaccharides obtained by partial acid hydrolysis of *Salmonella* group G lipopolysaccharides. Gal-, galactosyl; GalNAc-, *N*-acetylgalactosaminyl; GalNAc, *N*-acetylgalactosamine; Glc, glucose; Fuc, fucose.

bulk of the molecule must lie in the specific side chains that would therefore seem to be composed of a number of repeating units analogous to the structure proposed for *Salmonella* group E antigens (Robbins & Uchida, 1962) and for group B and D antigens (Staub & Raynaud, 1964). The repeating unit seems to be β-D-galactosyl-(1→3)-*N*-acetylgalactosaminyl-(1→3)-*N*-acetylgalactosaminyl-(1→4)-L-fucose with a glucose residue bound at some point on this structure. Though there is no direct evidence to indicate where this glucose is attached, the low colour yield of fraction C1 in the Morgan–Elson reaction compared with the high yield of fraction C2 may suggest that it is bound in such a way that chromogen formation is decreased. This might mean it is linked to the 4-position of the *N*-acetylgalactosamine attached to the fucose, but this must be regarded as open to question. The complement-fixation inhibition results favour an α-linkage for the glucose residue.

*Salmonella* group G lipopolysaccharides are known to possess blood-group H (O) specificity (Springer, Williamson & Brandes, 1961). Using acid hydrolysis, Rege, Painter, Watkins & Morgan (1963) have shown that H (O) substance contained *O*-β-D-galactosyl-(1→3)-β-*N*-acetylglucosaminyl-(1→3)-β-galactosyl-(1→3)-*N*-acetylgalactosamine and *O*-β-D-galactosyl-(1→4)-β-*N*-acetylglucosaminyl-(1→3)-β-galactosyl-(1→3)-*N*-acetylgalactosamine sequences. The oligosaccharides obtained by acid hydrolysis were free of fucose and serologically inactive in inhibition tests (Rege *et al.* 1963). Alkaline hydrolysis of H (O) substance yields a number of fucose-containing serologically active oligosaccharides, the analysis of which indicated that fucose was (1→2)-linked to galactose and most probably (1→4)-linked to *N*-acetylglucosamine (Morgan, 1964; Schiffmann, Kabat & Thompson, 1964). Though no oligosaccharides with terminal non-reducing fucose have been recovered from the *Salmonella* group G lipopolysaccharides, owing to the use of acid for partial hydrolysis, the complement-fixation inhibition studies indicate that a small amount of non-reducing L-fucose is bound to the side chains. If this fucose were (1→2)-linked to galactose, the *Salmonella* group G lipopolysaccharides and blood-group H (O) substance would have a common structural sequence in their specific side chains that would account for the known H (O) specificity of these bacterial polysaccharides.

Two oligosaccharides that do not contain 'special sugars' have been isolated from the S-lipopolysaccharides of group G and these, it is believed, originate from the underlying 'rough' core. The first is α-D-glucosyl-D-galactose, which is indistinguishable from that recovered from a number of R-lipopolysaccharides (Sutherland, Lüderitz & Westphal, 1965). The second is *N*-acetyl-D-glucosaminyl-D-glucose, which has been isolated from RII strains of *S. minnesota*, *S. inverness*, *S. poona* and *S. typhimurium* (Sutherland *et al.* 1965). Both these disaccharides could be hydrolysis products of *N*-acetylglucosaminylglucosylgalactose, which has also been isolated from the above RII strains. Thus there is strong evidence for the earlier idea that the S-lipopolysaccharides contain common structures in their core that are indistinguishable from those present in the lipopolysaccharides of R-mutants.

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