The Immunochemistry of *Shigella flexneri* O-Antigens

THE BIOCHEMICAL BASIS OF SMOOTH TO ROUGH MUTATION

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1. Smooth to rough mutation has the same biochemical basis in *Shigella* as in *Salmonella*. It is the result of enzyme defects blocking the incorporation of the O-specific side chains that characterize the smooth lipopolysaccharide with the consequent exposure of the underlying basal structures that determine 'rough'-specificity. 2. The *Shigella flexneri* basal structure resembles its *Salmonella* analogue in that it has the same qualitative sugar composition, and enzyme defects in its biosynthetic pathway give rise to 'rough'-lipopolysaccharides that are indistinguishable from those of *Salmonella* chemotypes Ra, Rb, Rc and Rd. However, the *Salmonella* and *Shigella* basal structures are not identical as judged by quantitative analysis and the absence of serological cross-reaction. 3. The *Sh. flexneri* basal structure side chain has been isolated and characterized as an α -N-acetylglucosaminyl-(1 $\rightarrow$ 4)-galactosyl-(1 $\rightarrow$ 3)-glucose sequence with α -glucosyl radicals substituted on the 3- and 4-positions of the galactose and glucose respectively. The different sugar types in this side chain are incorporated into the growing molecule in the same order as in *Salmonella*, which explains why the enzyme defects associated with smooth to rough mutation produce the same series of R-chemotypes from both genera. The terminal α -glucosyl and α -N-acetylglucosaminyl-(1 $\rightarrow$ 4)-galactosyl residues of the *Sh. flexneri* basal structure are sufficiently different from the terminal α -galactosyl and α -N-acetylglucosaminylglucosyl residues of the *Salmonella* analogue that they offer an explanation for the absence of serological cross-reaction between these two basal structures.

A recent review of *Salmonella* O-antigens (Lüderitz, Staub & Westphal, 1966) shows that the lipopolysaccharide components of these complex polymers contain two distinct regions, namely a basal structure which is common to all *Salmonella* and O-specific side chains which differ structurally from serogroup to serogroup. Moreover, smooth (S) to rough (R) mutation results when single enzyme defects in the biosynthetic pathway of the S-lipopolysaccharide prevent the incorporation of the O-specific side chains, with the consequent exposure of underlying R-specific determinants in the basal structure. Though a comparatively large number of specific enzyme lesions may give rise to R-mutants, two distinct mechanisms of smooth to rough mutation can be recognized. In the first type, which depends on the loss of a side-chain synthetase or transferase, there is a total absence of O-specific side chains but the basal structure is completed normally. In the second type, which depends on the loss of a basal synthetase or transferase, the biosynthesis of the O-specific side chains proceeds normally but these cannot be transferred to the incomplete basal structure which lacks the appro-

priate acceptor. Comparative studies of serially related lipopolysaccharides from *Salmonella* R-mutants of both the above types have not only led to the elucidation of the biochemical basis of smooth to rough mutation in this genus, but have also contributed to our knowledge of the sugar sequences in their common basal structure.

In the genus *Shigella* no comparable analysis of a series of R-lipopolysaccharides has been made hitherto, and in no instance has the fine structure of an R-lipopolysaccharide been elucidated. Nevertheless, two observations suggest that the biochemical basis of smooth to rough mutation in *Shigella* may be similar to that in *Salmonella*. The first of these is to be found in the pioneer studies of Davies, Morgan & Record (1955) and Davies (1957) who demonstrated that an R-mutant of *Shigella shigae* lacked the rhamnose that is present in the S-lipopolysaccharide of that species, and the second is the more recent observation (Simmons, 1966) that the *Shigella flexneri* lipopolysaccharides resemble the analogous *Salmonella* substances in that they also possess two regions. Their common basal structure, though not necessarily identical with its *Salmonella*

counterpart, contains the same 'basal sugars' (aldosephosphate, 3-deoxy-2-oxo-octonate, O-phosphorylethanolamine, D-galactose, D-glucose and N-acetyl-D-glucosamine) whereas their O-specific side chains contain a number of repeating units composed of D-glucose, N-acetyl-D-glucosamine and L-rhamnose in a structural sequence that is probably unique to each serotype. With such a marked similarity in the gross molecular structure of *Salmonella* and *Shigella* lipopolysaccharides it is probable that the mechanism of smooth to rough mutation is the same in both genera, though the particular enzyme defects are probably not identical. The present study of a series of *Sh. flexneri* R-mutants was undertaken to test this hypothesis and to elucidate the fine chemistry of the *Sh. flexneri* basal structure.

MATERIALS AND METHODS

Isolation of rough (R) mutants. Ten typical R-mutants were isolated from aging cultures of ten representative smooth *Sh. flexneri* serotypes by the method of Arkwright (1921). As judged by their colonial appearance, sedimentation in broth culture and agglutinability in solutions of NaCl and CaCl₂, all remained stable throughout the period of study. The N.C.T.C. strain numbers of the smooth *Sh. flexneri* serotypes used to isolate the R-mutants were: 1a (9950), 1b (8517), 2a (8519), 3a (8520), 4a (9725), 4b (8522), 5 (8524), 6 (9729), variant X (8527) and variant Y (9951). In this paper smooth and rough forms are differentiated by appending 'S' and 'R' respectively to the serotype designation.

Mass-culture of *Sh. flexneri* smooth and rough forms. The bacteriological purity of the smooth organisms was checked before and after mass-culture by morphological, cultural, biochemical and serological methods. The purity of the rough cultures was checked in the same way except that the serological tests were replaced by electrolyte agglutination reactions. Each organism was grown for 24–36 hr. at 37° in 5–10 l. of ox-heart infusion broth enriched with 0.1% D-glucose; aeration was achieved by vigorous shaking. The organisms were harvested by centrifugation at 5000g for 20 min., washed three times in 0.9% NaCl, resuspended in a small volume of distilled water, killed by the addition of 10 vol. of acetone at 0° and washed twice in acetone before drying. The yields were 1.4–5.0 g. dry wt. of bacteria.

A large quantity of *Sh. flexneri* serotype 4b-R was required for the isolation and characterization of oligosaccharides from its lipopolysaccharide. This organism was grown by courtesy of Dr C. E. Gordon Smith at the Microbiological Research Establishment, Porton, Wilts., on C.C.Y. agar as previously described (Simmons, 1962). The yield was 177 g.

Extraction of lipopolysaccharides and analysis of ultracentrifuge supernatants after the sedimentation of lipopolysaccharides. The lipopolysaccharides were extracted from the acetone-dried smooth and rough *Sh. flexneri* by the phenol-water method and purified in the ultracentrifuge (Kauffmann, Lüderitz, Stierlin & Westphal, 1960). In this

method of purification, the high-molecular-weight O-antigen is ultracentrifuged three times at 105 000g for 4 hr. to separate it from ribonucleic acid and non-specific glucans, which remain in the three supernatants (LI, LII and LIII fractions). The O-specific side chains of R-mutants with a basal enzyme defect continue to be synthesized and these are found as relatively low-molecular-weight polymers in the LI fraction (Beckmann, Subbaiah & Stocker, 1964). Chromatographic analysis of the LI fraction from each R-mutant was therefore performed after hydrolysis, to demonstrate the presence or absence of O-specific side-chain sugars.

The yields of S- and R-lipopolysaccharide were 18–83 mg.

Preparation of oligosaccharides. Oligosaccharides were prepared from 363 mg. of *Sh. flexneri* serotype 4b-R lipopolysaccharide by partial acid hydrolysis and their structures determined by borohydride reduction, periodate oxidation and enzymic hydrolysis by methods previously described (Simmons, Lüderitz & Westphal, 1965a,b). The conditions for complete and partial acid hydrolysis of lipopolysaccharides and oligosaccharides were also detailed in these papers.

High-voltage electrophoresis. This was carried out with the Locarte mark IV apparatus with the following buffers: A, pyridine-acetic acid-water (5:2:500, by vol.), pH 5.3, and B, 0.05 M-sodium tetraborate, pH 9.2, as described by Efron (1960). With buffer A, electrophoresis was run at 10 000 v and 60 ma for 40 min. on Whatman no. 3MM paper, and with buffer B, at 6000 v and 30 ma for 1 hr. on Whatman no. 1 paper.

Chromatography. This was carried out by the descending method on Whatman no. 4 paper with the following solvents: I, ethyl acetate-acetic acid-water (3:1:3, by vol.) (Jermyn & Isherwood, 1949); II, butan-1-ol-acetic acid-water (4:1:5, by vol.) (Partridge, 1948). Alkaline AgNO₃ (Trevelyan, Procter & Harrison, 1950) and ninhydrin (Consden, Gordon & Martin, 1944) were used as identifying reagents.

Quantitative microanalysis. Analyses were performed in 7.5 cm. × 1.0 cm. tubes with Carlsberg constriction micropipettes (H. E. Pedersen, Copenhagen, Denmark) as follows: heptose by a modified H₂SO₄-cysteine method (Osborn, 1963), with D-glycero-L-manno-heptose phosphate as the standard sugar; 3-deoxy-2-oxo-octonate by the thiobarbituric acid method of Weissbach & Hurwitz (1959); O-phosphorylethanolamine by the 1-fluoro-2,4-dinitrobenzene method (Ghuysen & Strominger, 1963); D-galactose by the galactose oxidase method (Fischer & Zapf, 1964); D-glucose by the glucose oxidase method (Krüger, Lüderitz, Strominger & Westphal, 1962); L-rhamnose by the H₂SO₄-cysteine reaction (Dische & Shettles, 1951); free hexosamine by a modified Morgan-Elson reaction after N-acetylation (Strominger, Park & Thompson, 1959) with glucosamine as the standard sugar. As a portion of the N-acetylglucosamine in each lipopolysaccharide is derived from its lipid A moiety, the amount in each lipopolysaccharide component was determined by analysing lipid A-free polysaccharides prepared by mild acetic acid hydrolysis according to the method of Boivin & Mesrobian (1934). Earlier studies have shown that the hexosamine in the *Sh. flexneri* polysaccharides is N-acetylated (Simmons, 1962) and that all of it can be accounted for as glucosamine (Simmons, 1966). Each test contained 10–50 μmoles of the sugar under analysis.

Measurements were made in a Unicam SP. 500 or a Carl Zeiss PMQII spectrophotometer with standard microanalytical accessories.

RESULTS

Analysis and chemotyping of Sh. flexneri lipopolysaccharides

The analyses of the ten *Sh. flexneri* S-lipopolysaccharides used in this study are so similar to one another that one representative type only is included in Table 1 for comparison with the R-lipopolysaccharides. By contrast, the ten R-lipopolysaccharides can be divided into four distinct groups or chemotypes on the basis of their qualitative sugar composition. As all the lipopolysaccharides of any one chemotype were found to have the same quantitative sugar composition, only one representative member from each R-chemotype is shown in Table 1. Compared with the S-lipopolysaccharides from the parent smooth forms, those from the rough mutants are characterized by the loss of rhamnose but many also lack one or more of the so-called basal sugars. Compared with the lipopolysaccharide of serotype 4b-R, which contains all the basal sugars (aldoheptose phosphate, galactose, glucose and *N*-acetylglucosamine), type 4a-R lacks *N*-acetylglucosamine, type 1a-R lacks *N*-acetylglucosamine and galactose and type 3a-R lacks *N*-acetylglucosamine, galactose and glucose. Thus the *Sh. flexneri* R-lipopolysaccharides (Table 2) belong to four R-chemotypes that are qualitatively indistinguishable from *Salmonella* R-chemotypes Ra, Rb, Rc and Rd (Lüderitz *et al.* 1966) in that they contain the following sugars: Rd, aldoheptose; Rc, aldoheptose + glucose; Rb, aldoheptose + glucose + galactose; Ra, aldoheptose + glucose + galactose + *N*-acetylglucosamine. These four chemotypes form a series in which each member differs from its precursor by the addition of a single sugar type, namely glucose, galactose and *N*-acetylglucosamine respectively. These findings are consistent with the view that the biosynthesis of the basal structure commences with the polyheptose phosphate 'backbone' of chemotype Rd and proceeds by the sequential addition of glucose, galactose and *N*-acetylglucosamine in that order to the growing side chain of the basal structure. However, the finding of 3 moles of glucose per 2 moles of aldoheptose phosphate in chemotype Ra lipopolysaccharides (Table 2) reveals the presence of additional glucose residues which may be bound to this sequence as secondary side chains. These lipopolysaccharides also contain 3-deoxy-2-oxo-octonate and *O*-phosphorylethanolamine. In the *Salmonella* lipopolysaccharides the former is believed to act as a 'bridge' between the lipid A and polysaccharide components of the complex (Osborn, 1963) and the *O*-phosphorylethanolamine may be bound to the

heptose residues of the 'backbone' (Grollman & Osborn, 1964).

Chromatography of ultracentrifuge supernatants after the sedimentation of Sh. flexneri R-lipopolysaccharides. These supernatants (LI fractions) can be divided into two distinct groups on the basis of their qualitative sugar composition. In the first group (comprising 1a-R, 2a-R, 3a-R, 4a-R, 5-R and variant X-R) the side-chain sugars glucose, *N*-acetylglucosamine and rhamnose were present and appeared to be bound as in the O-specific determinants since they were precipitated strongly with homologous smooth *Sh. flexneri* antiserum. In the second group (comprising 1b-R, 4b-R, 6-R and variant Y-R) these three sugars were absent and no precipitate was obtained with homologous smooth antiserum. Thus the chemotype Ra mutants, which have all the basal constituents, fail to synthesize O-specific material whereas those chemotypes (Rb, Rc and Rd) which lack one or more basal components continue to synthesize O-specific side chains. Mutants 1b-R and variant Y-R are exceptions to this rule and are assumed to be defective in an enzyme system that is essential for the synthesis of both regions of the molecule. All the LI fractions contained large amounts of ribose derived from contaminating ribonucleic acid but none contained the basal sugars galactose and aldoheptose phosphate.

Cross-reactivity of Salmonella and Shigella Ra chemotypes. As the biosynthesis of the *Salmonella* and *Shigella* basal structures takes place by the sequential addition of glucose, galactose and *N*-acetylglucosamine in the same or a closely similar order, it is possible that identical structural sequences are shared by both genera. An attempt was made to demonstrate such sequences by studying the cross-reactivity of chemotype-Ra lipopolysaccharides of one genus with chemotype-Ra antiserum to the other. When tested in the complement-fixation system of Wasserman & Levine (1961), the chemotype-Ra lipopolysaccharide of *Salmonella minnesota* R60 (Sutherland, Lüderitz & Westphal, 1965) failed to react with a high-titre chemotype-Ra antiserum prepared with trichloroacetic acid-extracted antigen from *Sh. flexneri* serotype 4b-R as described by Simmons (1962). The chemotype-Ra lipopolysaccharides of *Sh. flexneri* serotypes 4b-R and 6-R also failed to react with *S. minnesota* R60 antiserum. Thus the *Salmonella* and *Sh. flexneri* Ra chemotypes have no immunodeterminant groups in common.

Inhibition of homologous Sh. flexneri chemotype-Ra system. The inhibitory effect of 0.5 μ mole and 1.0 μ mole of α - and β -methyl derivatives of glucose, galactose and *N*-acetylglucosamine on the reaction of *Sh. flexneri* chemotype-Ra lipopolysaccharide with a homologous antiserum was tested by using the complement-fixation system described by

Table 1. *Quantitative microanalysis of the sugar components of some representative Shigella flexneri S- and R-lipopolysaccharides*

The lipid A moiety of *Sh. flexneri* S-lipopolysaccharides amounts to approximately 30% of the complex (Simmons, 1966), and in R-lipopolysaccharides the lipid content is believed to be higher (Lüderitz *et al.* 1966).

Each figure is a mean of twelve determinations; the maximum error (expressed as a percentage of the value given) is believed to be within the following limits: heptose ± 15 ; 3-deoxy-2-oxo-octonate ± 12 ; O-phosphoryl-ethanolamine ± 8 ; galactose ± 10 ; glucose ± 4 ; N-acetylglucosamine ± 8 ; rhamnose ± 5 . —, The sugar was not detected on chromatographic and quantitative analysis.

Serotype	% of sugar in lipopolysaccharide						
	3-Deoxy-2-oxo-octonate	O-Phosphoryl-ethanolamine	Aldoheptose phosphate	D-Glucose	D-Galactose	N-Acetyl-D-glucosamine*	L-Rhamnose
4b-S	2.4	1.3	6.0	14.7	1.0	15.8	20.1
4b-R	8.6	8.0	13.5	10.6	3.8	5.8	—
4a-R	8.4	7.1	24.6	6.9	7.4	—	—
1a-R	8.7	6.5	20.3	6.8	—	—	—
3a-R	7.8	5.0	23.4	—	—	—	—

* Analysis of lipid A-free polysaccharide.

Table 2. *Molar ratios of sugar components in a representative selection of Shigella flexneri R-lipopolysaccharides*

The molar ratios were derived from the analytical data in Table 1; aldoheptose phosphate was taken as 2.0.

Serotype	Aldoheptose phosphate	Glucose	Galactose	N-Acetylglucosamine	Rhamnose	R-chemotype*
4b-R	2.0	2.7	1.0	0.9	—	Ra
4a-R	2.0	0.9	1.0	—	—	Rb
1a-R	2.0	1.1	—	—	—	Rc
3a-R	2.0	—	—	—	—	Rd

* According to the classification of Lüderitz *et al.* (1966). The R-lipopolysaccharides not included in this Table were assigned to the following chemotypes; 6-R to chemotype Ra; variant Y-R to chemotype Rb; 2a-R, 5-R and variant X-R to chemotype Rc; 1b-R to chemotype Rd.

Wasserman & Levine (1961). Inhibitions of 25% and 36% were detected with the lower and higher concentrations of α -methyl-N-acetyl-D-glucosamine respectively but no other significant results were observed. These findings indicate that α -N-acetylglucosaminyl residues play an immunodominant role in the chemotype-Ra lipopolysaccharide.

Analysis of Sh. flexneri basal structure

The isolation of identical R-lipopolysaccharides from R-mutants of different smooth serotypes supports the view that the basal structure is common to all *Sh. flexneri* strains. The common basal structure is probably identical with the R-lipopolysaccharides of serotypes 4b-R and 6-R as these contain all the basal sugars and the mutants from which they were extracted have an O-specific side-chain defect which is presumed not to interfere with the synthesis of the complete basal structure.

The basal structure studies were therefore carried out with *Sh. flexneri* serotype 4b-R lipopolysaccharide. Table 3 summarizes the properties of seven electrophoretically neutral oligosaccharides isolated from a partial acid hydrolysate of this lipopolysaccharide by high-voltage electrophoresis and preparative chromatography as described by Simmons *et al.* (1965a). The probable structures of these oligosaccharides (Scheme 1) were elucidated as follows.

Oligosaccharide C7. Microanalysis and the chromatographic mobility of oligosaccharide C7 are consistent with the view that it is a disaccharide of N-acetylglucosamine and galactose. On borohydride reduction, 0.82 mole of galactose was reduced without any significant loss of N-acetylglucosamine. Thus this oligosaccharide is an N-acetylglucosaminylgalactose. On periodate oxidation, 5.1 moles of periodate were consumed with the production of 2.5 moles of formic acid and 1.1 moles of formaldehyde/mole of oligosaccharide,

Table 3. *Properties of oligosaccharides isolated from the Shigella flexneri basal structure by partial acid hydrolysis*

The oligosaccharides were separated from 363 mg. of *Sh. flexneri* serotype 4b-R lipopolysaccharide. —, The sugar was not detected on chromatographic and quantitative analysis; GlcNAc, *N*-acetylglucosamine.

Oligo-saccharide	Approx. yield (mg.)	% of total oligo-saccharide yield	R_{Glc} in solvent I	Monosaccharide content (10 ⁻² μ mole/100 μ l. of eluate)			Nature of oligosaccharide	Sugar reduced by borohydride
				GlcNAc	Gal	Glc		
C1	0.89	16.6	0.14	12.4	11.4	34.8	Pentasaccharide	Glucose
C2	1.12	21.0	0.21	18.4	18.6	34.8	Tetrasaccharide	Glucose
C3	0.99	18.5	0.26	28.5	28.3	67.2	Tetrasaccharide	Glucose
C4	0.83	15.5	0.41	11.7	11.4	10.5	Trisaccharide	Galactose
C5	0.05	0.9	0.51	—	12.1	12.2	Disaccharide	Galactose
C6	0.52	9.7	0.52	—	—	40.7	Disaccharide	Glucose
C7	0.95	17.8	0.63	16.6	19.6	—	Disaccharide	Galactose

which indicates the absence of 6-*O*-substitution of the terminal reducing galactose.

Oligosaccharide C6. This disaccharide, which consisted solely of glucose, was split completely by α -glucosidase, but not to any significant extent by β -glucosidase. It is therefore an α -glucosylglucose. On chromatography it was manifestly different from authentic samples of α -glucosyl-(1 $\rightarrow$ 3)-glucose (nigerose) and α -glucosyl-(1 $\rightarrow$ 6)-glucose (isomaltose) but indistinguishable in solvents I and II from maltose. It is therefore α -glucosyl-(1 $\rightarrow$ 4)-glucose.

Oligosaccharide C5. On chromatography this substance behaved as a disaccharide. Its R_{Glc} value in solvent I was only slightly lower than that of oligosaccharide C6, from which it was completely separated by high-voltage electrophoresis in buffer B. On quantitative analysis glucose and galactose were found to be present in equimolar amounts and 0.98mole of galactose was reduced with borohydride. Of the glucose 35% was liberated on treatment with α -glucosidase compared with only 3% with β -glucosidase. Thus oligosaccharide C5 is most probably an α -glucosylgalactose.

Oligosaccharide C4. On microanalysis oligosaccharide C4 contained equimolar amounts of *N*-acetylglucosamine, galactose and glucose; its chromatographic mobility was also consistent with the view that it was a trisaccharide. Galactose (1.00mole) was reduced by borohydride without any significant loss in the other two components. Partial acid hydrolysis of oligosaccharide C4 resulted in the formation of a disaccharide indistinguishable from oligosaccharide C7, and free glucose. With the isolation of oligosaccharide C5 (α -glucosylgalactose), it seems probable that the glucose residue is substituted on the galactose as shown in Scheme 1. This structure is also favoured by periodate oxidation studies, in which 6.1moles of periodate were

consumed with the formation of 2.7moles of formic acid and 1.0mole of formaldehyde/mole of oligosaccharide. When periodate studies followed borohydride reduction, 1.8moles of formaldehyde/mole of oligosaccharide were liberated, which indicates the absence of 2-*O*- and 6-*O*-substitution of the terminal reducing galactose.

Oligosaccharide C2. On microanalysis oligosaccharide C2 contained *N*-acetylglucosamine, galactose and glucose in the proportions 1:1:2 respectively, so it is probably a tetrasaccharide. Its chromatographic mobility supports this conclusion. On partial acid hydrolysis two oligosaccharides were liberated which were chromatographically indistinguishable from oligosaccharides C6 (maltose) and C7 (*N*-acetylglucosaminylgalactose) and on borohydride reduction 1.00mole of glucose was reduced. Thus the two glucose residues in oligosaccharide C2 are linked as in maltose at the reducing end of the molecule. In the periodate oxidation studies 6.9moles of periodate were used with the production of 2.9moles of formic acid and 1.0mole of formaldehyde/mole of oligosaccharide C2. These findings, which are consistent with the branched structure shown in Scheme 1, also favour 4-*O*-substitution of the galactose and 3-*O*-substitution of the terminal reducing glucose residue. Confirmatory evidence for the branched structure of this oligosaccharide will be presented below. When the oxidized fragment of C2 was recovered and analysed to detect which sugars, if any, were protected it was found that all had been destroyed. As the galactose is unprotected it is most probably not 3-*O*-substituted, a finding which provides further evidence of its 4-*O*-substitution, as 2-*O*- and 6-*O*-substitution are also shown to be absent by the periodate-oxidation studies on borohydride-reduced oligosaccharide C4.

Oligosaccharide C3. This substance contains

N-acetylglucosamine, galactose and glucose in the same proportions as oligosaccharide C2, namely 1:1:2 respectively. These findings and its chromatographic mobility are consistent with the view that it is a tetrasaccharide. On partial acid hydrolysis two oligosaccharides were liberated and these were chromatographically indistinguishable from oligosaccharides C4 and C7. On borohydride reduction 0.90 mole of glucose was reduced with no significant loss in the other two components so that the structural sequence is most probably as shown in Scheme 1. Additional evidence in support of this structure was provided by the periodate-oxidation studies, in which 7.1 moles of periodate were consumed with the production of 3.8 moles of formic acid and 1.0 mole of formaldehyde/mole of oligosaccharide. When the oxidized fragment was analysed for protected sugars, galactose was recovered. This result, which contrasts markedly with that obtained with oligosaccharide C2, indicates 3-*O*-substitution of the galactose with glucose and by a process of elimination provides further evidence that the galactose is also 4-*O*-substituted with the *N*-acetylglucosamine residue, as 2-*O*- and 6-*O*-substitution are shown to be absent by the periodate-oxidation studies on borohydride-reduced C4.

Oligosaccharide C1. On microanalysis oligosaccharide C1 contained *N*-acetylglucosamine, galactose and glucose in the proportions 1:1:3 respectively and its chromatographic mobility also indicated that it was a pentasaccharide. On treatment with borohydride, 0.91 mole of glucose was reduced without any significant reduction of *N*-acetylglucosamine or galactose. The partial acid hydrolysate of oligosaccharide C1 contained two disaccharides chromatographically indistinguishable from oligosaccharide C6 (maltose) and C7 (*N*-acetylglucosaminylgalactose). A trace of the branched trisaccharide C4 was also present. Thus oligosaccharide C1 probably has the structural sequence shown in Scheme 1. This conclusion is supported by the results of periodate oxidation, in which 7.7 moles of periodate were used with the production of 3.1 moles of formic acid and 1.1 moles of formaldehyde/mole of oligosaccharide. Only galactose was recovered intact from the oxidized fragment after treatment with periodate, which confirms that this sugar is 3-*O*-substituted.

Periodate oxidation of *Sh. flexneri* serotype 4b-R lipopolysaccharide. The action of periodate on intact serotype 4b-R lipopolysaccharide was also studied by analysing the hexose composition before and after oxidation. After periodate treatment of 3.82 mg. of this lipopolysaccharide, 27% of the glucose and 74% of the galactose in the sample were recovered, no allowance being made for losses incurred during manipulation. As 27% of the glucose in the sample is equivalent to 0.81 mole

Scheme 1. Structural relationship of neutral oligosaccharides isolated from *Sh. flexneri* serotype 4b-R lipopolysaccharide by partial acid hydrolysis. GlcNAc, *N*-acetylglucosamine.

(there being three glucose residues in the side chain), these results provide good evidence that the glucose and galactose residues in the primary side chains of this lipopolysaccharide are both protected and therefore most probably 3-*O*-substituted.

Structural analysis of *Sh. flexneri* serotype 4a-R lipopolysaccharide. It was apparent from the results of partial acid hydrolysis of *Sh. flexneri* serotype 4b-R lipopolysaccharide that two important properties of this molecule determined the number and nature of the resulting oligosaccharides. The first of these was the rather marked acid-stability of the sequence *N*-acetylglucosaminylgalactose, which was present in all but two of the isolated oligosaccharides; the second was the marked acid-lability of the sequence galactosylglucose, which could not be isolated from the hydrolysis products in the form of the disaccharide. As attempts to recover this labile structure by weak-acid (0.1 *N*-sulphuric acid) hydrolysis merely increased the yield of those oligosaccharides containing the acid-stable *N*-acetylglucosaminylgalactose sequence, efforts were made to isolate it from *Sh. flexneri* serotype 4a-R lipopolysaccharide, which contains equimolar amounts of galactose and glucose but no *N*-acetylglucosamine (Table 2). However, hydrolysis of this

lipopolysaccharide with weak acid (0.1N-sulphuric acid) for varying periods of time gave 100% of the galactose as free galactose within 10 min., by which time only 2.5% of the glucose had been liberated. These results confirm the extreme acid-lability of the galactosylglucose sequence and indicate that the galactose is the non-reducing end sugar, as it can be selectively split from the molecule without any accompanying release of glucose or oligosaccharides. After periodate oxidation of 1.92 mg. of 4a-R lipopolysaccharide, the oxidized fragment was recovered and analysed for its hexose constituents. The galactose was totally destroyed but 88% of the glucose in the sample could be accounted for if no allowance was made for losses. This finding confirms that the glucose residue in the primary side chain is protected and therefore 3-O-substituted. Thus *Sh. flexneri* 4a-R lipopolysaccharide consists of galactosyl-(1→3)-glucose side chains attached to the polyheptose phosphate 'backbone'. This result, which indicates the existence of a single glucose residue between the galactose unit and the heptose 'backbone', provides strong evidence that the second glucose residue in oligosaccharide C2 (Scheme 1) is present as a side chain and that the branched structure, assigned to that oligosaccharide on the basis of periodate-oxidation studies, is most probably correct.

From Scheme 1 it can be seen that the isolated oligosaccharides are degradation products of a single oligosaccharide unit that is identical with oligosaccharide C1. This pentasaccharide is probably identical with the basal-structure side chain because it is the highest oligosaccharide unit isolated and the molar ratios of its three constituents are the same as those in undegraded *Sh. flexneri* serotype 4b-R lipopolysaccharide (Table 1). From a consideration of the oligosaccharide structures in Scheme 1, the periodate-oxidation studies on whole serotype 4b-R and 4a-R lipopolysaccharides and the observation that α -N-acetylglucosaminyl residues play an immunodominant role in chemotype-Ra lipopolysaccharide, the structure of this unit is most probably an α -N-acetylglucosaminyl-(1→4)-galactosyl-(1→3)-glucose sequence, with α -glucosyl residues substituted on the 3- and 4-positions of the galactose and glucose respectively, as shown in Scheme 2.

DISCUSSION

The *Sh. flexneri* lipopolysaccharides contain two distinct regions, namely a basal structure containing aldoheptose phosphate, 3-deoxy-2-oxo-octonate, O-phosphorylethanolamine, D-galactose, D-glucose and N-acetyl-D-glucosamine and O-specific side chains composed of D-glucose, N-acetyl-D-glucosamine and L-rhamnose (Simmons, 1966). On

quantitative microanalysis, the R-lipopolysaccharides that form the subject of this paper show three characteristic features with respect to these side-chain and basal components (Table 1). The first and most striking of these is the loss of the side-chain constituent rhamnose. The second is the relative loss of those sugars which are common to both the side chains and the basal structure, namely glucose and N-acetylglucosamine. The third is the relative increase in the purely basal components aldoheptose phosphate, 3-deoxy-2-oxo-octonate and O-phosphorylethanolamine. These findings taken together provide strong evidence that the *Sh. flexneri* R-lipopolysaccharides are composed of basal sugars only and lack the O-specific side chains that are a feature of the S-lipopolysaccharides. Furthermore, many of the R-mutants are also deficient in one or more of the basal sugars (Table 2) and these mutants generally continue to synthesize O-specific side chains which can be recovered from the ultracentrifuge supernatants after sedimentation of the lipopolysaccharide. They presumably have a defect in a basal synthetase or transferase and cannot bind the O-specific side chains to the incomplete basal structure. By contrast, those R-mutants which contain all the basal sugars in their lipopolysaccharides have no O-specific side chains in the LI fraction and presumably lack a side-chain synthetase or transferase. Thus smooth to rough mutation occurs when an enzyme defect blocks the synthesis or incorporation of the O-specific side chains that characterize the S-lipopolysaccharides, with the consequent exposure of the underlying basal structures that determine R-specificity. The general mechanism of smooth to rough mutation in this genus is therefore the same as in *Salmonella* (Lüderitz *et al.* 1966).

The similarity between these two genera is a much closer one, however. Both contain the same basal sugars (Simmons, 1966) and the *Sh. flexneri* R-lipopolysaccharides can be divided on the basis of their qualitative sugar composition into four R-chemotypes (Table 2) that are indistinguishable from those proposed for *Salmonella* (Lüderitz *et al.* 1966). Despite these close similarities the *Salmonella* and *Shigella* basal structures are not identical. On quantitative analysis the *Salmonella* basal side chain contains two glucose and two galactose units (Sutherland *et al.* 1965), whereas the analogous *Sh. flexneri* structure has three of glucose and one of galactose (Table 2). In addition to these quantitative differences, the absence of serological cross-reaction between Ra-lipopolysaccharides of the one genus and Ra antiserum to the other provides further proof of a structural difference. To elucidate the molecular basis of these similarities and differences in the two basal structures, the detailed

It has been suggested that the common basal structure of the genus *Salmonella* is possibly present in *Escherichia coli* and related genera (Sutherland *et al.* 1965). Although this has now been shown not to be true of *Shigella*, the presence of similar basal structures in these different genera is probably not

the product of chance but indicates fundamental relationships between these different bacterial groups.

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