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The Immunochemistry of *Shigella flexneri* Lipopolysaccharides

A QUANTITATIVE ANALYSIS OF THEIR MONOSACCHARIDE CONSTITUENTS

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1. The lipopolysaccharides of a representative selection of *Shigella flexneri* serotypes all contain the same constituents as *Salmonella* chemotype VII, namely, aldoheptose phosphate, 3-deoxy-2-oxo-octonate, O-phosphorylethanolamine, D-galactose, D-glucose, *N*-acetyl-D-glucosamine and L-rhamnose. 2. The presence of all the *Salmonella* basal sugars in *Sh. flexneri* lipopolysaccharides is consistent with the view that the latter contain a basal structure or core which is similar to the common basal structure of *Salmonella* lipopolysaccharides. 3. Although the *Sh. flexneri* lipopolysaccharides belong to one chemotype, there appear to be quantitative differences in the composition of their O-specific side chains. The repeating units of *Sh. flexneri* serotypes 1a, 2a, 3a, 4a, and variant X contain D-glucose, *N*-acetyl-D-glucosamine and L-rhamnose in the proportions 1:1:2 respectively. The analogous repeating units of serotypes 5a and 6 contain an additional mole of D-glucose and D-galactose respectively and that of variant Y 1 mole of D-glucose less.

There is now much evidence, recently reviewed by Lüderitz, Staub & Westphal (1966), to support the view that the *Salmonella* lipopolysaccharides share a common basal structure or core to which side chains carrying the different O-specific factors are attached. The basal sugars of the core, aldoheptose phosphate, 3-deoxy-2-oxo-octonate, *O*-phosphorylethanolamine, D-galactose, D-glucose and *N*-acetyl-D-glucosamine, are therefore found in all *Salmonella* lipopolysaccharides whereas the 'special' (side chain) sugars associated with O-specificity differ from chemotype to chemotype (Kauffmann, Lüderitz, Stierlin & Westphal, 1960). The more recent demonstration of the same structural sequence in the core of *Escherichia coli* and *Salmonella* lipopolysaccharides (Edstrom & Heath, 1964) and the finding of the *Salmonella* basal sugars in *Arizona* and *E. coli* serotypes (Westphal, Kauffmann, Lüderitz & Stierlin, 1960) indicate the probable presence of a similar basal structure in all Enterobacteriaceae lipopolysaccharides. In the *Sh. flexneri* that form the subject of this paper, the presence of all the basal sugars in a single lipopolysaccharide has yet to be demonstrated but reports of aldoheptose phosphate in *Sh. flexneri* serotype 3 (Slein & Schnell, 1953) and of *O*-phosphorylethanolamine in an unspecified *Sh. flexneri* serotype (Grollman & Osborn, 1964) suggest that the lipopolysaccharides of this genus may also possess a basal structure similar to that of the *Salmonella*.

The present study of eight representative *Sh. flexneri* lipopolysaccharides was undertaken to determine their basal sugar composition and to analyse quantitatively all their sugar constituents including those in the O-specific side chains. Earlier studies (Goebel, Binkley & Perlman, 1945; Slein & Schnell, 1953; Simmons, 1957, 1962) have demonstrated glucose, *N*-acetylglucosamine and rhamnose as the major components of this group of polysaccharides.

MATERIALS AND METHODS

Lipopolysaccharides. The lipopolysaccharides were extracted from acetone-dried cultures of *Shigella flexneri* by the phenol–water method and purified in the ultracentrifuge as described by Kauffmann *et al.* (1960). The N.C.T.C. strain numbers and serotypes of the eight smooth *Sh. flexneri* cultures used are given in Table 1 together with the weights extracted and the yields of lipopolysaccharide obtained.

Chromatography. This was carried out by the descending method on Whatman no. 1 paper with the following solvents: I, butan-1-ol–acetic acid–water (4:1:5, by vol.) (Partridge, 1948); II, butan-1-ol–pyridine–water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951); III, ethyl acetate–acetic acid–water (3:1:3, by vol.) (Jermyn & Isherwood, 1949). Alkaline $AgNO_3$ (Trevelyan, Procter & Harrison,

Table 1. *Yield of lipopolysaccharides extracted from some representative* Shigella flexneri *serotypes by the phenol–water method*

Shigella flexneri serotype	N.C.T.C. strain no.	Dry wt. extracted (g.)	Yield of lipopolysaccharide (g.)	Yield of lipopolysaccharide (% dry wt. of bacteria)
1a	3	100	2·29	2·29
2a	4833	85	0·27	0·32
3a	8205	30	0·24	0·80
4a	8206	42	0·81	1·93
5a	8201	50	0·26	0·52
6	8207	30	0·77	2·56
Variant X	2245	36	0·40	1·11
Variant Y	4839	13	0·28	2·15

1950), naphtharesorcinol (Partridge, 1948), the acid–molybdate reagent of Hanes & Isherwood (1949) and ninhydrin (Consden, Gordon & Martin, 1944) were used as identifying reagents both in chromatography and high-voltage electrophoresis.

High-voltage electrophoresis. This was carried out on 2043 bmgl paper (Schleicher & Schüll, Dassel, Germany) according to the method of Kickhöfen & Westphal (1952) with buffer A: pyridine–acetic acid–water (10:4:86, by vol.), pH 5·3. Electrophoresis was run at 3000 v and 120 mA for 1 hr.

Hydrolysis of lipopolysaccharides. Hydrolysis was carried out in sealed glass ampoules, each containing approx. 3·0 mg. of the lipopolysaccharide under study at 1% (w/v) concentration. Samples for amino sugar analysis were hydrolysed for 12 hr. at 100° with 4 N-HCl. Portions with an expected content of 10–50 mμmoles of hexosamine were rapidly dried *in vacuo* to remove the acid. Samples for the analysis of neutral sugars were hydrolysed for 4 hr. at 100° with 1 N-H_2SO_4. The hydrolysate was diluted with 2 vol. of water and neutralized with Amberlite IR-410 (HCO_3^- form) before drying portions *in vacuo* for micro-analysis. Each portion contained 10–100 mμmoles of the sugar under study. For the determination of 3-deoxy-2-oxo-octonate, hydrolysis was for 10 min. at 100° with 0·25 N-H_2SO_4. Negatively charged oligosaccharides, obtained by the elution of high-voltage electrophoretograms, were hydrolysed for 12 hr. at 100° with 2 N-H_2SO_4.

Quantitative microanalysis. Analyses were performed in 7·5 cm. × 1·0 cm. tubes with Carlsberg constriction micro-pipettes (H. E. Pedersen, Copenhagen, Denmark) as follows: heptose by a modified H_2SO_4–cysteine method (Osborn, 1963); 3-deoxy-2-oxo-octonate by the thio-barbituric acid method of Waravdekar & Saslaw (1959); *O*-phosphorylethanolamine by the dinitrofluorobenzene method (Ghuysen & Strominger, 1963); D-galactose both by the galactose dehydrogenase method (Wallenfels & Kurz, 1962) and the galactose oxidase method (Fischer & Zapf, 1964); D-glucose by the glucose oxidase method (Krüger, Lüderitz, Strominger & Westphal, 1962); L-rhamnose both by the H_2SO_4/cysteine reaction (Dische & Shettles, 1951) and the L-rhamnose isomerase method (Krüger *et al.* 1962); free hexosamine by a modified Morgan–Elson reaction after *N*-acetylation (Strominger, Park & Thompson, 1959); D-glucosamine by the D-glucosamine-6-phosphate *N*-acetylase method (Lüderitz, Simmons, Westphal & Strominger, 1964); *N*-acetylhexosamine by a modified Morgan–Elson reaction (Reissig, Strominger & Leloir, 1955), and phosphate by the method of Lowry, Roberts, Leiner, Wu & Farr (1954). All measurements were made in the Unicam SP.500 spectrophotometer with standard microanalytical accessories. The results of the analyses are given in Table 2.

Materials. The enzymes were obtained or prepared as follows: glucose oxidase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany); galactose oxidase and horse-radish peroxidase (Seravac Laboratories Ltd., Maidenhead, Berks.); galactose dehydrogenase was prepared by the method of Wallenfels & Kurz (1962) from a culture of *Pseudomonas saccharophila* (a product of Ciba, Basle, Switzerland) kindly given by Dr O. Lüderitz, Freiburg, Germany; D-glucosamine 6-phosphate *N*-acetylase was prepared according to Brown (1962) and L-rhamnose isomerase (also kindly given by Dr O. Lüderitz) was prepared by Krüger *et al.* (1962).

The chemicals were obtained or prepared as follows: D-glycero-L-mannoheptose was a gift from Dr N. K. Richtmyer (National Institutes of Health, Bethesda, Md., U.S.A.) and penta-acetyl methyl ester of 3-deoxy-2-oxo-octonate was a gift from Professor E. C. Heath (Johns Hopkins University, Baltimore, Md., U.S.A.). Free 3-deoxy-2-oxo-octonate was prepared from this derivative by the method of E. C. Heath (personal communication) as follows: 10 mg. of the derivative was dissolved in 1·0 ml. of methanol; 1·0 ml. of 0·2 N-NaOH was added; after mixing and incubating at 37° for 15 min. the mixture was treated with just sufficient Dowex 50 (H^+ form) resin to adjust it to pH 5–6; the resin was removed by filtration and the solution concentrated to dryness under reduced pressure. The sample was dissolved in water to give a molar concentration of 0·01. The pH should be adjusted to 6·5 if necessary.

All other chemicals, reagents and reference sugars were obtained from British Drug Houses Ltd., Poole, Dorset, and were either AnalaR grade or the highest grade obtainable.

RESULTS

The quantitative results given in Table 2 show that the major constituents of the *Sh. flexneri* lipopolysaccharide hydrolysates are glucose, glucosamine and rhamnose. Galactose, heptose

phosphate, 3-deoxy-2-oxo-octonate and *O*-phosphorylethanolamine are present in smaller amounts. From their reactions with the specific enzymes used in the analyses the glucose, galactose and glucosamine are of the D-configuration and the rhamnose is of the L-configuration. The value obtained for the total free hexosamine in each hydrolysate as measured by the method of Strominger *et al.* (1959) was the same as that obtained for glucosamine by the specific *N*-acetylase technique (Lüderitz *et al.* 1964). The total amino sugar content of each lipopolysaccharide is thus accounted for as glucosamine. This conclusion was confirmed by the chromatographic absence of other hexosamines and by the finding of arabinose only when the hydrolysates were treated with ninhydrin–pyridine buffer (Stoffyn & Jeanloz, 1954), which converts amino sugars into their parent pentoses. There is evidence that the free glucosamine in the *Sh. flexneri* lipopolysaccharide hydrolysates is *N*-acetylated in the native polysaccharides (Simmons, 1962). The values given in Table 2 are therefore expressed in terms of *N*-acetyl-D-glucosamine. The presence of galactose, glucose, rhamnose and *O*-phosphorylethanolamine was also confirmed chromatographically. Chromatographic evidence for the presence of heptose and 3-deoxy-2-oxo-octonate in these hydrolysates is unsatisfactory because the former could not be separated from a galactose/glucose mixture and the latter may be confused with oligosaccharides produced during hydrolysis. As heptose phosphate and 3-deoxy-2-oxo-octonate are negatively charged, their presence was convincingly demonstrated by high-voltage electrophoresis as follows: the electrophoretograms of the hydrolysates showed that the bulk of the material was in the neutral fraction but cathodic and anodic fractions were also present, the former being oligosaccharides containing basic amino sugars. Four anodic fractions (electrophoretic mobilities 0·5, 0·6, 0·8 and 1·1 times that of glucose 6-phosphate in buffer A) could be detected in each lipopolysaccharide hydrolysate and these were designated A1 to A4 in order of increasing electrophoretic mobility. A4 was electrophoretically indistinguishable from authentic 3-deoxy-2-oxo-octonate. On elution, it reacted in the thiobarbituric acid reaction (Waravdekar & Saslaw, 1959) to give a chromogen with a spectrum indistinguishable from authentic 3-deoxy-2-oxo-octonate. A1, A2 and A3 all contained phosphorus on spraying with the reagent of Hanes & Isherwood (1949) and with naphtharesorcinol reagent all gave a purple colour indistinguishable from that of glucose 6-phosphate but readily distinguishable from the bright-blue colour of glucuronic acid. A3, when eluted, hydrolysed and chromatographed, gave a single spot indistinguishable from authentic D-glycero-L-mannoheptose in solvents I and II. This experiment does not differentiate between D-glycero-L-mannoheptose and L-glycero-D-mannoheptose but in all Enterobacteriaceae lipopolysaccharides where the configuration has been determined, it has proved to be the latter. Quantitative analysis of the free phosphate in this fraction gave the aldoheptose:phosphate ratio as 1·00:0·71 under the conditions of the experiment. Thus A3 appears to be L-glycero-D-mannoheptose phosphate or its optical enantiomorph. A1 and A2 also contained heptose as evidence by absorption peaks at 510mμ in the sulphuric acid/cysteine reaction (Dische & Shettles, 1951). Absorption peaks at 410mμ in the same reaction indicated the presence of hexose, which on subsequent chromatography proved to be galactose. A1 and A2 therefore appear to be oligosaccharides containing aldoheptose phosphate and galactose. The ratio of the sugars in these anodic oligosaccharides has not yet been established by quantitative analysis. Pentoses, 3,6-dideoxyhexoses and hexuronic acids have not been found by chromatography among the hydrolysis products of these lipopolysaccharides.

The finding of comparable amounts of all the *Salmonella* basal sugars in *Sh. flexneri* lipopolysaccharides is consistent with the view that both genera have a similar type of basal structure or core. If this basal structure contains approximately equivalent amounts of galactose, glucose and *N*-acetylglucosamine as in the *Salmonella* basal structure (Sutherland, Lüderitz & Westphal, 1965), then the bulk of the glucose and *N*-acetylglucosamine of the *Sh. flexneri* lipopolysaccharides lies in the O-specific side chains and the ratio of the sugars in these side chains may be determined as in Table 3.

In the *Sh. flexneri* serotype 6 lipopolysaccharide the galactose ratio is so high that it appears probable that this sugar is also present in the O-specific side chains. Here it has been assumed that the basal galactose accounts for 1% of the total lipopolysaccharide and that equimolar amounts of glucose and *N*-acetylglucosamine are also of basal origin. The analytical results (Table 2) show that the carbohydrate accounted for in serotype 6 is lower than with other serotypes. This lipopolysaccharide may be more resistant to hydrolysis as larger amounts of oligosaccharide were noted when equivalent weights of all lipopolysaccharide hydrolysates were chromatographed.

DISCUSSION

The eight *Shigella flexneri* lipopolysaccharides used in this study all contain the same constituents, namely, aldoheptose phosphate, 3 deoxy-2-oxo-octonate, *O*-phosphorylethanolamine, D-galactose,

Table 2. *Quantitative analysis of the sugar components of* Shigella flexneri *lipopolysaccharides*

Each figure is a mean of eight or twelve determinations on each lipopolysaccharide hydrolysate. The values given are believed to be accurate within the following limits: heptose ± 0·9; 3-deoxy-2-oxo-octonate ± 1·0; *O*-phosphorylethanolamine ± 0·3; galactose ± 1·2; glucose ± 0·7; *N*-acetylglucosamine ± 1·5; rhamnose ± 1·0.

Lipopolysaccharide from *Shigella flexneri* serotype	Percentage of sugar in lipopolysaccharide							Carbohydrate content of lipopolysaccharide* (%)
	Aldoheptose phosphate	3-Deoxy-2-oxo-octonate	*O*-Phosphoryl-ethanolamine	D-Galactose	D-Glucose	*N*-Acetyl-D-glucosamine	L-Rhamnose	
1a	5·6	2·8	3·5	2·0	12·7	17·9	20·8	65·3
2a	5·6	3·0	3·1	2·3	13·1	16·4	22·2	65·7
3a	4·4	3·1	6·1	2·2	11·0	14·5	17·6	58·9
4a	4·9	1·6	2·4	2·2	12·2	15·9	18·4	57·6
5a	4·6	1·5	4·3	2·1	21·9	17·9	22·1	74·4
6	6·0	2·2	8·7	6·5	6·8	8·6	9·7	48·5
Variant X	5·2	1·6	4·9	2·4	12·0	15·4	17·8	59·3
Variant Y	6·0	1·4	3·9	2·4	5·8	17·3	22·2	59·0

* The precise amount of the lipid A component in each lipopolysaccharide remains undetermined. From unpublished observations made during the ether extraction of acetic acid-degraded antigens (Simmons, 1957) it constitutes approx. 30% of the lipopolysaccharide complex.

Table 3. *Molar ratios of sugars in the specific side chains of* Shigella flexneri *lipopolysaccharides*

The 'percentage of sugar in the O-specific side chains' was derived from the data in Table 2, with the following assumptions. All the galactose and equivalent amounts of glucose and *N*-acetylglucosamine are basal in origin. All the rhamnose lies in the O-specific side chains. In serotype 6 lipopolysaccharide where galactose appears to be a side-chain constituent, basal galactose amounts to 1% of the total lipopolysaccharide. In calculating the molar ratios, rhamnose was taken as 2·00.

Lipopolysaccharide from *Shigella flexneri* serotype	Percentage of sugar in the O-specific side chains				Molar ratios of sugars			
	Galactose	Glucose	*N*-Acetylglucosamine	Rhamnose	Galactose	Glucose	*N*-Acetylglucosamine	Rhamnose
1a	0·0	10·7	15·5	20·8	0·00	0·94	1·13	2·00
2a	0·0	10·8	13·6	22·2	0·00	0·89	0·93	2·00
3a	0·0	8·8	11·9	17·6	0·00	0·92	1·02	2·00
4a	0·0	10·0	13·3	18·4	0·00	1·00	1·10	2·00
5a	0·0	19·8	15·4	22·1	0·00	1·63	1·06	2·00
6	5·5	5·8	7·4	9·7	1·02	1·09	1·16	2·00
Variant X	0·0	9·6	12·5	17·8	0·00	0·99	1·06	2·00
Variant Y	0·0	3·4	14·4	22·2	0·00	0·28	1·00	2·00

D-glucose, *N*-acetyl-D-glucosamine and L-rhamnose. They therefore belong to a single chemotype indistinguishable from *Salmonella* chemotype VII (Kauffmann *et al.* 1960). The occurrence of glucose, *N*-acetylglucosamine and rhamnose as the major constituents of these lipopolysaccharides confirms earlier reports by Slein & Schnell (1953) in respect of *Sh. flexneri* type 3 polysaccharide and of Simmons (1957, 1962) in respect of the degraded haptens of all eight serotypes. Reports of small amounts of galactose in the polysaccharides of serotypes 6 (Efimova, 1959), 2a (Sasaki, 1956) and variant Y (Sasaki, 1957), of aldoheptose phosphate in serotype 3 polysaccharide (Slein & Schnell, 1953) and of *O*-phosphorylethanolamine in a *Sh. flexneri* species (Grollman & Osborn, 1964) are also confirmed and extended to the other types used in this study. 3-Deoxy-2-oxo-octonate, which was first isolated from *E. coli* 0111 (Heath & Ghalambor, 1963) and subsequently shown to be a common constituent of *Salmonella* lipopolysaccharides (see review by Lüderitz *et al.* 1966), has now been demonstrated for the first time in the analogous *Sh. flexneri* preparations. The presence of xylose (Sasaki, 1957), of mannose and xylose (Sasaki, 1957) and of uronic acids (Efimova, 1959) in these lipopolysaccharides has not been confirmed with the techniques described above.

The finding of all the *Salmonella* basal sugars in many *E. coli* (see review by Lüderitz *et al.* 1966) and now in *Sh. flexneri* lipopolysaccharides suggests that many Enterobacteriaceae species have a similar lipopolysaccharide core. However, it should be stressed that the presence of a similar core in all Enterobacteriaceae does not necessarily imply that it is structurally identical from genus to genus. Indeed, comparative studies of the lipopolysaccharides from mutants of *Salmonella typhimurium* and *E. coli* 0111 have already demonstrated that, whereas the sugar sequence is the same in both (Edstrom & Heath, 1964), the nature of the linkage in a glucosylgalactose from the *E. coli* lipopolysaccharide is definitely different from that in the analogous glucosylgalactose from *S. minnesota* RII lipopolysaccharide (Sutherland *et al.* 1965).

The O-specific side chains of the *Sh. flexneri* lipopolysaccharides comprise the greater bulk of the total molecule and are probably composed of a number of repeating units analogous to those proposed for *Salmonella* groups B,D,E,G,N and U (Staub & Raynaud, 1964; Robbins & Uchida, 1962; Simmons, Lüderitz & Westphal, 1965*a*,*b*; Lüderitz, Simmons & Westphal, 1965). Although the *Sh. flexneri* lipopolysaccharides all belong to one chemotype, there appear to be quantitative differences in the composition of their O-specific side chains. The results in Table 3 are consistent with the view that the repeating units of serotypes 1a, 2a, 3a, 4a and variant X contain glucose, *N*-acetylglucosamine and rhamnose in the proportions 1:1:2 respectively. The analogous repeating units of serotypes 5a and 6 appear to contain an additional mole of glucose and galactose respectively while that of variant Y has 1 mole of glucose less. It remains to be seen if these quantitative differences are of significance in the immunochemistry of the determinant groups of the different *Sh. flexneri* serotypes.

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