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The Glucosidic Linkages of the *Shigella flexneri* Polysaccharides

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A recent review of the specific polysaccharides of the Gram-negative bacilli (Davies, 1960) shows that the monosaccharide constituents of many of these substances have now been identified and the taxonomic significance of different groups of sugars defined. Comparatively little progress has been made, however, in determining the structure of these complex polysaccharides, so that in most instances it is not yet possible to interpret their serological activity in terms of molecular configuration. Nevertheless, significant advances have been made in the assignment of molecular structures to a few determinant groups of the *Salmonella* O somatic antigens, principally by Staub, Tinelli, Lüderitz & Westphal (1959) and Staub (1960) who used the specific serological-inhibition techniques so successfully employed in related fields by McCarty (1956) and Watkins & Morgan (1952). With the *Shigella flexneri* polysaccharides that form the subject of this paper, the nature of the glucosidic linkages between the constituent hexose units is unknown. Once this information is available, chemical interpretations of type specificity and cross-reactivity in this serologically heterogeneous group of organisms may become possible. The object of the present investigation was to define the type of glucosidic linkages which occur in the specific polysaccharides of the eight common *Sh. flexneri* serotypes. This was achieved by means of infrared spectroscopy and by specifically inhibiting the activity of high-titre *Sh. flexneri* antisera with glucose polymers of known chemical structure.

A necessary prerequisite to a study of the molecular structure of any polysaccharide is the identification of its monosaccharide constituents. Goebel, Binkley & Perlman (1945) identified the amino-

hexose in electrophoretically homogeneous polysaccharides from *Sh. flexneri* Types 1a, 2a, 3 and 6 as glucosamine, and estimated the percentage of this monosaccharide and of the other reducing sugars in hydrolysates of these substances. Slein & Schnell (1952, 1953) and Slein (1952) identified the main hexose constituents of *Sh. flexneri* Type 3 polysaccharide as glucosamine, glucose and rhamnose, but this material also contained 1% (w/w) of organically bound phosphorus in the form of a hexose 6-phosphate and an aldoheptose phosphate. Simmons (1957) demonstrated that the specific polysaccharides from *Sh. flexneri* Types 1a, 2a, 3, 4a, 5 and 6 and Variants x and y all contained glucosamine, glucose and rhamnose. The occurrence of the same hexoses in the polysaccharides of this serologically heterogeneous group is a possible chemical basis for their high degree of cross-reactivity (Simmons, 1958).

MATERIALS AND METHODS

Organisms investigated. The strains of *Sh. flexneri* used in this investigation were as follows, the numbers in parentheses being the N.C.T.C. numbers: Type 1a (3); Type 2a (4833); Type 3 (8205); Type 4a (8206); Type 5 (8201); Type 6 (8207); Variant x (2245); Variant y (4839).

Preparation of mass cultures. The purity of each organism was checked by morphological, biochemical and serological tests and its smoothness confirmed by colonial appearance and salt stability before mass culture was initiated. The organisms were grown, by courtesy of Dr D. W. Henderson, at the Microbiological Research Establishment, Porton, on C.C.Y. agar (Gladstone & Fildes, 1940), containing 0.5% (w/v) of lactose, for 18 hr. at 37°. The harvested material was homogenized, filtered and acetone-dried. The amount in terms of dry weight of bacteria varied from 93 g. with *Sh. flexneri* Variant x to

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223 g. with *Sh. flexneri* Type 1a. Saline suspensions of each serotype standardized to contain 2×10^9 organisms/ml. were agglutinated to titre by the homologous antisera.

Centrifuging. All centrifuging was carried out in a Swedish angle (A. B. Winkel) centrifuge at 5000g for 15 min.

Extraction of bacteria. A sample (100 g.) of each *Sh. flexneri* serotype was extracted by the phenol-water method of Westphal, Lüderitz & Bister (1952) except for *Sh. flexneri* Type 5 and Variants x and y, where the weight used was only 50 g. In this method, which gives a high yield of serologically active material, the main component of the aqueous phase is the specific lipopolysaccharide, but this is invariably contaminated with nucleic acid and, since none of the solvents currently used in the extraction of Gram-negative bacilli is absolutely specific, there is usually a small amount of the undegraded lipopolysaccharide-protein or antigenic complex as well. The nucleic acid was removed by acetone fractionation by the method of Goebel *et al.* (1945), the resulting precipitate being removed by centrifuging. The lipopolysaccharide was precipitated from the supernatant by the addition of 4 vol. of ethanol and recovered by centrifuging. The acetone fractionation was repeated until no nucleic acid remained, the lipopolysaccharide being recovered from the supernatant as described above by ethanol fractionation and subsequent centrifuging.

Preparation of polysaccharides. A serologically active polysaccharide was then prepared from the lipopolysaccharide, which still contained small amounts of contaminating lipopolysaccharide-protein. The lipopolysaccharide was taken up in 200 ml. of water, acetic acid was added to a concentration of 1% (w/v) and partial degradation was effected by hydrolysis under reflux for 4 hr. at 100°. The small amount of acid-insoluble precipitate which formed consists of polypeptide from the antigenic complex and was separated by centrifuging. The supernatant was cooled to 0° and extracted with 0.2 vol. of ether three times to remove the phospholipid. The degraded serologically active polysaccharide, which remains in solution, was precipitated with 4 vol. of ethanol and recovered by centrifuging. The ethanol precipitation was repeated twice. The polysaccharide was then dried *in vacuo* over conc. H_2SO_4 and stored at 0°. The final yield of polysaccharide in % (w/w) of dried bacteria was: *Sh. flexneri* Type 1a, 1.1; Type 2a, 1.0; Type 3, 1.3; Type 4a, 1.3; Type 5, 3.5; Type 6, 1.9; Variant x, 4.8; Variant y, 3.1.

Acid hydrolysis of polysaccharides. A sample (50 mg.) of each polysaccharide in a 1% (w/v) solution was hydrolysed under reflux in 0.5N-HCl for 12 hr., de-ionized in a mercury-cathode desalter until free from chloride and sulphate, and then analysed by chromatographic methods.

Chromatography. The descending method was employed. Whatman no. 4 filter paper was used in all experiments. The following were used in the analysis of each polysaccharide: solvents, butan-1-ol-acetic acid-water (4:1:5, by vol.) (Partridge, 1948) and ethyl acetate-acetic acid-water (3:1:3, by vol.) (Jermyn & Isherwood, 1949); identifying reagents, aniline hydrogen oxalate reagent (Horrocks & Manning, 1949), benzidine reagent (Horrocks, 1949), ninhydrin reagent (Consden, Gordon & Martin, 1944) and 3,5-dinitrosalicylic acid reagent (Williams & Bevenue, 1951).

Chromatographic analysis of 500 µg. samples of the

eight *Sh. flexneri* polysaccharides showed that they were not contaminated with free amino acids or monosaccharides. Analysis of 500 µg. samples of the same substances after hydrolysis failed to demonstrate the presence of amino acids, suggesting that these polysaccharides are not contaminated or combined with the polypeptide component of the antigenic complex. The only hexoses demonstrated in the acid hydrolysates of all eight phenol-extracted polysaccharides were glucosamine, glucose and rhamnose.

Chemicals. The dextran used was the commercial product Dextraven (Benger Laboratories Ltd.). The organism used in its production is *Leuconostoc mesenteroides* N.R.R.L. strain 512 (F). The dextran was recovered from solution by fractionation with 4 vol. of ethanol and the precipitate separated by centrifuging. Ethanol fractionation was repeated twice and the final precipitate dried *in vacuo* over conc. H_2SO_4 . The homogeneity of the product was examined in the ultracentrifuge and by electrophoresis.

The author is indebted to Dr D. J. Manners for gifts of pustulan (Lindberg & McPherson, 1954) and of isomaltotriose and isomaltotetraose (Duncan, Manners & Ross, 1956), to Dr J. R. Turvey for gifts of nigerose and isolichenin (Peat, Whelan, Turvey & Morgan, 1961), and to Dr R. Roger for the sample of laminarin.

Paper electrophoresis. This was carried out in the EEL electrophoresis apparatus, with a number of buffers at different pH values, with Whatman no. 1 filter paper. The following buffers were used in the examination of all eight polysaccharides and the purified dextran sample: diethylbarbituric acid-sodium barbitone, pH 8.64 (Flynn & De Mayo, 1951) and sodium dihydrogen phosphate-disodium hydrogen phosphate, pH 7.40 (Block, Durrum & Zweig, 1955). A preparation of the antigenic complex and its polysaccharide component prepared from *Sh. flexneri* Type 1a by trichloroacetic acid extraction (Simmons, 1957) were studied in the above buffers and also in sodium borate-boric acid, pH 9.04 (Block *et al.* 1955) and boric acid-sodium hydroxide, pH 9.97 (Clark, 1928). The pH values were measured with a Cambridge bench pH meter. The weight of antigenic complex or polysaccharide applied to each strip was approx. 1 mg. Electrophoresis was carried out for 6 hr. at 40V/cm. The antigenic complex and all the polysaccharides were identified on the electrophoretic strips by a modified periodic acid-Schiff reaction (Köiw & Grönwall, 1952) since bromophenol blue fails to detect these compounds. After clearing in liquid paraffin the patterns were scanned in an EEL scanner with a Chance glass filter OR2.

Control electrophoretic strips for serological examination were always run under the same experimental conditions as the test. This was done to exclude the possible presence in the test strip of serologically active fractions which could not be identified by the method of Köiw & Grönwall (1952). These control strips were dried in a hot-air oven at 100° for 10 min., cut across into sections 0.5 in. wide and eluted with 1.0 ml. of 0.9% (w/v) sodium chloride at 56° for 4 hr. The eluates were then tested in precipitin tubes by over-floating on the homologous *Sh. flexneri* antisera to determine which section or sections contained serologically active material. In every case the only activity in the control strip corresponded to the zone of the peak on the test strip.

Agar-gel diffusion tests. Tests for the homogeneity of 1% (w/v) solutions of the *Sh. flexneri* polysaccharides were pre-

formed by a modification of the method of Ouchterlony, (1953). The design of the plate is shown in Fig. 1. The wells, which were cut in 1% (w/v) Davis agar containing 0.1% (w/v) of merthiolate, had a capacity of 0.2 ml. Those of the inner ring contained high-titre sera to the eight *Sh. flexneri* serotypes and those of the outer ring the homologous polysaccharides. The centres of the two sets of wells were 1.7 cm. apart. The plate was left at room temperature, the wells were refilled every 24 hr., and the final reading was made after 14 days when the precipitation bands occupied the position shown in Fig. 1.

Polarimetry. The specific rotation of the eight polysaccharides was measured at 20° in a Bellingham-Stanley Model A polarimeter with a 5 cm. cell. Concentrated solutions of these substances tend to be opalescent; this made the determination of the point of uniform illumination difficult. Too dilute solutions on the other hand also lead to inaccurate results because observational errors are multiplied in the determination of the specific rotation. The optimum concentration of polysaccharide was determined for each examination and varied between 0.5% and 1.5% (w/v). The source of illumination was the high-intensity mercury-vapour lamp supplied with this model of polarimeter. The specific rotations for the *Sh. flexneri* polysaccharides are shown in Table 1.

Infrared spectroscopy. The eight polysaccharides and the dextran sample were examined in potassium bromide disks in the Perkin-Elmer Model 137 (Infracord) instrument by Dr D. E. Bethell, Department of Chemistry, Queen's College, Dundee. The weight of polysaccharide in the disk varied from 1.6 mg. for *Sh. flexneri* Type 1a to 4.8 mg. for *Sh. flexneri* Type 4a.

Agglutination and precipitation tests. The techniques used in performing these tests were as described by Simmons (1958).

Fig. 1. Ouchterlony plate showing the number and position of precipitation bands when the *Shigella flexneri* polysaccharides were tested for homogeneity against the homologous antisera. The polysaccharides are in the outer wells and the sera in the inner wells. The designations at the periphery of the plate indicate the serotype of the polysaccharide under test in the opposite pair of wells. The solid lines represent heavy precipitation and the broken lines trace precipitation.

Preparation of high-titre antisera for chemical-inhibition studies. The lipopolysaccharides extracted by the phenol method of Westphal *et al.* (1952) are poor antigens whereas the whole antigenic complexes are potent antigens. Antigenic complexes extracted from the above eight *Sh. flexneri* strains by the trichloroacetic acid method of Boivin & Mesrobian (1934) were available from a previous study (Simmons, 1957) and were therefore used in the preparation of the antisera required for the specific chemical-inhibition tests. The eight rabbits used in this group of experiments weighed 2.5 kg. and their sera, before immunization, contained no demonstrable *Sh. flexneri* antibodies when tested by agglutination against whole homologous bacilli and by precipitation against the homologous antigenic complex. Each of the eight rabbits was then immunized against one of the eight *Sh. flexneri* antigenic complexes by three intravenous inoculations of 100 µg. at 3- or 4-day intervals. All the animals were exsanguinated under Nembutal anaesthesia 7-10 days after the last inoculation and the activity of their sera was examined by agglutination and precipitation methods. The sera were preserved by the addition of phenol liquefactum B.P. to a concentration of 0.5% (v/v) and stored at 0°. The titres of these sera varied from 1:3200 to 1:102 400 as measured by tube agglutination, and all precipitated the homologous specific polysaccharide in a dilution of at least 1:10⁶.

Inhibition of high-titre *Shigella flexneri* antisera with polyglucosans of known molecular structure. The capacity of different polyglucosans to inhibit the activity of *Sh. flexneri* antisera was measured by titrating each carbohydrate against a standard agglutinating system. These inhibition tests were carried out with 0.25 ml. of antiserum at a concentration of four times the titre of the serum, 0.5 ml. of inhibitor and 0.25 ml. of a suspension of homologous bacilli containing 4×10^8 organisms/ml. The total volume of each test was therefore 1.0 ml., the final concentration of antiserum equal to titre and the number of homologous bacilli 10⁸/ml. The degree of inhibition was read after the test solution had been kept for 4 hr. at 56° and finally after it had been left overnight at room temperature. The relative inhibiting capacity of the polyglucosans is given in Table 2 in terms of the minimum amount of substance required for inactivation.

RESULTS

Hexosamine

The reaction of Morgan & Elson (1934) for *N*-acetylglucosamine was performed on 1% (w/v) solutions of each polysaccharide (a) before hydrolysis, (b) 10 min. after hydrolysis had commenced and (c) 12 hr. after hydrolysis had commenced. The reaction was positive 10 min. after the commencement of hydrolysis owing to the presence of free *N*-acetylglucosamine in the solution. It was negative before hydrolysis because the reactive aldehydic group of the *N*-acetylglucosamine, necessary for a positive Morgan-Elson reaction, was inactivated by its linkage to the adjacent hexose unit in the parent polysaccharide. The reaction is also negative after prolonged acid

hydrolysis because the *N*-acetylglucosamine is deacetylated. The reaction of Elson & Morgan (1933) for free glucosamine was also performed on 1% (w/v) solutions of these polysaccharides (a) before hydrolysis and (b) after hydrolysis. A negative result was obtained before hydrolysis and a positive after, owing to the liberation of free glucosamine. The glucosamine found in the *Sh. flexneri*-polysaccharide hydrolysates therefore appears to exist in the native polysaccharide in the form of the *N*-acetyl derivative. Confirmatory evidence of the presence of *N*-acetyl radicals in these polysaccharides was provided by infrared spectroscopy.

Homogeneity of polysaccharide preparations

On electrophoresis the direction of migration of all the substances was anodic but their mobility was low. In the pH range investigated all appeared to migrate as single peaks which were fairly sharp and symmetrical. There is thus no electrophoretic evidence of inhomogeneity in any of the materials examined in this way. The actual values of the electrophoretic mobilities in the barbital buffer of Flynn & De Mayo (1951) and the phosphate buffer of Block *et al.* (1955) are given in Table 1.

The polysaccharides were all examined in the Spinco model E analytical ultracentrifuge by Dr C. T. Greenwood, Department of Chemistry, University of Edinburgh. The trichloroacetic acid preparation of *Sh. flexneri* Type 1a polysaccharide was examined by centrifuging for 30 min. at 182 800g at a concentration of 0.5% (w/v) in 0.15M-sodium chloride but the material failed to separate as a peak because of its small particle size. This substance and all the phenol-extracted polysaccharides were therefore examined in the synthetic boundary cell under the experimental conditions described above. After the first attempt at purification the trichloroacetic acid preparation of *Sh. flexneri* Type 1a polysaccharide, the phenol preparations of *Sh. flexneri* Types 3, 5 and Variant x polysaccharides, and the dextran sample, sedimented as one boundary. With *Sh. flexneri* Types 1a, 2a and 4a polysaccharides there was a small amount of a faster-moving component. Trace amounts of a similar substance were present in the Type 6 and Variant y polysaccharides. The fast-moving component appears to be the undegraded lipopolysaccharide used as the starting point in the preparation of the pure polysaccharide because both have comparable sedimentation coefficients. Further, when these samples were repurified as described above, the lipopolysaccharide then appears to have been completely degraded by the acetic acid hydrolysis because the reprocessed polysaccharides sedimented as one boundary in the

ultracentrifuge and were free from the fast-moving component. With *Sh. flexneri* Type 6 and Variant y polysaccharides no attempt was made to repurify because the yields were low and the losses in reprocessing are considerable.

The sedimentation coefficients, S_{20} , for the *Sh. flexneri* polysaccharides are given in Table 1. Without further measurements a precise estimate of the molecular weights of these degraded polysaccharides cannot be given, but these sedimentation coefficients may be compared with that of 1.7S recorded by Davies, Morgan & Record (1955) for the polysaccharide component of the degraded antigenic complex of *Shigella dysenteriae*. These authors obtained a molecular weight of 26 000 from the results of sedimentation and diffusion measurements. The findings recorded here suggest that the molecular weights of the *Sh. flexneri* polysaccharides are roughly comparable with that of the analogous polysaccharide from *Sh. dysenteriae*.

The results of the agar-gel diffusion tests confirm the findings of the ultracentrifuge investigations that *Sh. flexneri* Types 1a, 2a, 3, 4a, 5 and Variant x polysaccharides show no evidence of inhomogeneity but that the Type 6 and Variant y polysaccharides contain small amounts of a second component.

Optical rotation

The values obtained for the specific rotations of the *Sh. flexneri* polysaccharides are given in Table 1.

Infrared spectra

The infrared spectra of all eight *Sh. flexneri* polysaccharides are so similar that differentiation of the serotypes by this method would not appear to be possible. The absorption spectrum of *Sh. flexneri* Type 1a polysaccharide which is typical of the whole group is given in Fig. 2 with that of dextran for comparison.

The absorption at 1260 cm.⁻¹ was identified as a combination band of N—H deformation and C—N stretching vibrations due to the presence of monosubstituted amide (Cross, 1960). The absorption at 1555 cm.⁻¹ was attributed to N—H vibrations such as occur in monosubstituted amides or *N*-acetyl derivatives (Stacey & Barker, 1960). The occurrence of these two amide bands is consistent with the presence of *N*-acetyl radicals in the *Sh. flexneri* polysaccharides and confirms the results obtained with the Morgan-Elson and Elson-Morgan reactions.

The absorption band at 1382 cm.⁻¹ has been assigned to C—H deformation vibrations in a methyl group attached to a carbon atom (Cross, 1960). The occurrence of this band in these spectra may be accounted for by the methyl radical in the deoxy sugar rhamnose which is a known con-

stituent of all eight *Sh. flexneri* polysaccharides (Simmons, 1957).

The absorption at 813 cm.⁻¹, though weak, was definite and reproducible. It is caused by the phenomenon of 'ring breathing' in glucopyranose configurations (Burket & Badger, 1950).

The absorption at 840 cm.⁻¹ is characteristic of all polyglucosans containing α-glucosidic linkages. Polyglucosans containing β-linkages show absorption at 890 cm.⁻¹ and none at 840 cm.⁻¹ (Barker, Bourne, Stacey & Whiffen, 1954). The total absence of absorption at 890 cm.⁻¹ suggests that these polysaccharides contain no β-glucosidic linkages.

The absorption at 918 cm.⁻¹ is characteristic of α-polyglucosans of the dextran class (α-1→6-linkages). The absence of absorption at 930 cm.⁻¹ and 758 cm.⁻¹ suggests the absence of α-1→4-linkages, and the absence of absorption at 793 cm.⁻¹ suggests that there are no α-1→3-linkages (Barker *et al.* 1954).

The dextran sample has no absorption peaks due to *N*-acetyl and methyl groups but it has a peak at 768 cm.⁻¹ which is characteristic of this polyglucosan.

Inhibition of agglutination

With a view to confirming the results of infrared spectroscopy with respect to the nature of the glucosidic linkages in the bacterial polysaccharides, a study of the inhibition of high-titre *Sh. flexneri* antisera with polyglucosans of known molecular structure was carried out. The methods used have been described above and the results are shown in Table 2.

The exposure of *Sh. flexneri* antisera to high concentrations of α-1→4-, β-1→3-, β-1→4- and β-1→6-polyglucosans led to no demonstrable inhibition of their activity, but treatment with the α-1→6-polyglucosan dextran produces inhibition. These findings suggest that α-1→6-glucosidic

Table 1. Values for specific rotation, sedimentation coefficient and electrophoretic mobility of some *Shigella flexneri* polysaccharides and dextran

Experimental details are given in the text. The estimated error in the value for $[\alpha]_{5461}^{20}$ is $\pm 5^\circ$. Buffer 1 is the barbital buffer of Flynn & De Mayo (1951). Buffer 2 is the phosphate buffer of Block *et al.* (1955). —, Not measured.

Polysaccharide from <i>Sh. flexneri</i>	Specific rotation [α_{5461}^{20}]	<i>S</i> ₂₀ (s)	10 ⁶ × Electrophoretic mobility (cm. ² v ⁻¹ sec. ⁻¹)	
			Buffer 1	Buffer 2
Type 1a*	—	1.2	3.6	4.4
Type 1a	+28°	0.9	4.0	4.3
Type 2a	+48	1.7	3.8	4.4
Type 3	+42	1.6	3.6	3.6
Type 4a	+37	1.7	3.5	3.4
Type 5	+67	2.0	3.5	3.2
Type 6	+12	1.9	4.4	4.4
Variant x	+26	2.3	3.5	4.3
Variant y	+32	2.6	2.9	3.5
Dextran	+219	—	3.8	4.0

* This *Sh. flexneri* Type 1a sample was extracted by the trichloroacetic acid method.

Fig. 2. Infrared absorption spectra of potassium bromide disks of (a) *Shigella flexneri* Type 1a polysaccharide and (b) dextran.

Table 2. Inhibition of high-titre *Shigella flexneri* antisera with polyglucosans of known chemical structure

Experimental details are given in the text. —, Not tested as insufficient pure chemical was available.

Inhibiting polyglucosan	Predominant linkage	Minimum amount of polyglucosan giving inhibition of antiserum to <i>Sh. flexneri</i> Type (mg./ml.)							
		1a	2a	3	4a	5	6	Var. x	Var. y
Nigerose	α -1 $\rightarrow$ 3	> 1.0	> 1.0	> 1.0	> 1.0	> 1.0	> 1.0	> 1.0	> 1.0
Isolichenin	α -1 $\rightarrow$ 3	1.0	0.3	0.3	0.3	1.0	1.0	0.3	0.5
Laminarin	β -1 $\rightarrow$ 3	> 60.0	> 60.0	> 60.0	> 60.0	> 60.0	> 60.0	> 60.0	> 60.0
Starch	α -1 $\rightarrow$ 4	> 25.0	> 25.0	> 25.0	> 25.0	> 25.0	> 25.0	> 25.0	> 25.0
Glycogen	α -1 $\rightarrow$ 4	> 60.0	> 60.0	> 60.0	> 60.0	> 60.0	> 60.0	> 60.0	> 60.0
Maltose	α -1 $\rightarrow$ 4	> 125.0	> 125.0	> 125.0	> 125.0	> 125.0	> 125.0	> 125.0	> 125.0
Cellobiose	β -1 $\rightarrow$ 4	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0	> 50.0
Dextran	α -1 $\rightarrow$ 6	0.2	0.2	0.1	0.2	0.1	0.2	0.1	0.1
Isomaltotriose	α -1 $\rightarrow$ 6	0.2	—	—	—	—	—	—	—
Isomaltotetraose	α -1 $\rightarrow$ 6	0.2	—	—	—	—	—	—	—
Pustulan	β -1 $\rightarrow$ 6	> 10.0	> 10.0	> 10.0	> 10.0	> 10.0	> 10.0	> 10.0	> 10.0
Gentiobiose	β -1 $\rightarrow$ 6	> 125.0	> 125.0	> 125.0	> 125.0	> 125.0	> 125.0	> 125.0	> 125.0

linkages occur in the *Sh. flexneri* polysaccharides and that α -1 $\rightarrow$ 4-, β -1 $\rightarrow$ 3-, β -1 $\rightarrow$ 4- and β -1 $\rightarrow$ 6-linkages are absent. These results are in agreement with those obtained by infrared spectroscopy. The effect of the α -1 $\rightarrow$ 6-linked oligosaccharides isomaltotriose and isomaltotetraose on *Sh. flexneri* Type 1a antiserum is similar to that of dextran and confirms the presence of α -1 $\rightarrow$ 6-linkages in the Type 1a polysaccharide. The inhibition produced by the polyglucosan isolichenin, in which the predominant linkage is α -1 $\rightarrow$ 3, was unexpected because there was no evidence of this type of linkage in the infrared spectra of the *Sh. flexneri* polysaccharides. The α -1 $\rightarrow$ 3-linkages in the native bacterial polysaccharides are either too few to be detected in the infrared spectroscope or hydrolysed during the preparation of the materials for examination. The results obtained with nigerose are probably not significant because the amount of substance available was insufficient to give a suitable range of concentrations for test purposes.

All eight dextran-inhibited *Sh. flexneri* antisera fail to agglutinate not only suspensions of whole homologous bacilli but also suspensions of the other seven serotypes. To show that the inhibiting properties of dextran were not due to some non-specific factor, its effect on high-titre sera to *Salmonella typhi*, *Salmonella paratyphi* B, *Salmonella typhimurium*, *Shigella sonnei* and *Escherichia coli* Type 086 was studied. Attempts to inhibit the activity of these sera with dextran in precisely the same manner as for the *Sh. flexneri* sera led to no reduction in their titres.

DISCUSSION

The specific polysaccharides used in this investigation were obtained by acetic acid degradation of phenol-extracted lipopolysaccharides and must therefore be regarded as degraded substances. Further, studies in the analytical ultracentrifuge show that the particle size of the specific polysaccharide becomes progressively smaller with repeated hydrolysis, suggesting that depolymerization is also taking place. Despite the degraded nature of these products, they are ideal for immunochemical studies because they have been freed from the polypeptide and phospholipid components of the antigenic complex and yet retain the serological specificity and cross-reactivity of the native antigen.

When the standard methods of examining macromolecules for homogeneity were applied to the eight *Sh. flexneri* polysaccharides, no evidence of impurity was found except for the Type 6 and Variant y polysaccharides. The ultracentrifuge shows that these two substances contained traces of the lipopolysaccharide from which they were

prepared, but this did not appear to interfere with their infrared spectroscopy, probably because the lipopolysaccharide is present in such small quantity.

The glucosamine reported in *Sh. flexneri*-polysaccharide hydrolysates (Goebel *et al.* 1945; Slein & Schnell, 1952; Simmons, 1957) appears to occur naturally as the *N*-acetyl derivative because the Morgan-Elson reaction is positive during the early stages of hydrolysis. This finding is confirmed by the occurrence of typical amide absorption bands in the infrared spectra of all these polysaccharides. The hexosamine constituent of the *Sh. flexneri* polysaccharides has thus proved to be identical with the hexosamine of *Sh. dysenteriae* polysaccharide which was characterized as acetylglucosamine by Davies, Morgan & Mosiman (1954). The other hexose constituents are glucose and rhamnose.

The presence of α -1 $\rightarrow$ 6-glucosidic linkages in all these polysaccharides is indicated by the results of infrared spectroscopy and by specific chemical-inhibition tests. The inhibition of all the *Sh. flexneri* sera by isolichenin indicates the presence of α -1 $\rightarrow$ 3-linkages in these polysaccharides, but the absence of infrared absorption at the wavelength characteristic of this type of bond suggests that the number of such linkages is small, or that they have been hydrolysed during the preparation of the polysaccharides.

So far as their glucosidic linkages are concerned the *Sh. flexneri* polysaccharides appear to have much in common with the Class C dextrans. These substances are essentially α -1 $\rightarrow$ 6-polyglucosans with a smaller number of α -1 $\rightarrow$ 3 bonds and, in most cases, an even smaller number of α -1 $\rightarrow$ 4-linkages. The α -1 $\rightarrow$ 3-linkages in these dextrans are cross-links between α -1 $\rightarrow$ 6-linked glucose chains (Lohmar, 1952; Jeanes & Wilham, 1952). At present there is no information about the role of the α -1 $\rightarrow$ 3-linkages in the *Sh. flexneri* polysaccharides but it is possible that they also occur as 1:3:6 branch points.

After the identification of the same three constituents in all eight *Sh. flexneri* polysaccharides, Simmons (1958) suggested that the serological cross-reactivity of this genus was due to similarities in the molecular structure of its polysaccharides. The precise nature of this chemical similarity has now been more clearly defined by the demonstration of α -1 $\rightarrow$ 6- and α -1 $\rightarrow$ 3-glucosidic linkages in all these substances, and the role of these common structural features in determining cross-reactivity has been established by demonstrating specific inhibition of *Sh. flexneri* antisera with α -1 $\rightarrow$ 6- and α -1 $\rightarrow$ 3-polyglucosans. It should not be assumed, however, that these are the only structural features which these polysaccharides hold in common. It seems probable, on theoretical grounds alone, that

some of them will have other common molecular groupings due to identical glycosidic linkages of their *N*-acetylglucosamine and rhamnose constituents and that these will also give rise to cross-reactivity.

SUMMARY

1. Lipopolysaccharides were extracted from eight *Shigella flexneri* serotypes with aqueous phenol.

2. The lipopolysaccharides were hydrolysed with dilute acetic acid and the specific degraded polysaccharides recovered by ethanol precipitation after removal of traces of insoluble protein by centrifuging and of lipid by ether extraction.

3. The homogeneity of the polysaccharides was studied in the analytical ultracentrifuge and by electrophoretic and serological techniques.

4. The infrared spectra of polysaccharides from the eight types were so similar that differentiation of the serotypes by this method was not possible. All contained absorption peaks which are consistent with the presence of α -1 $\rightarrow$ 6-glucosidic linkages.

5. The inhibition of *Sh. flexneri* antisera with dextran, isomaltotriose and isomaltotetraose confirms the presence of α -1 $\rightarrow$ 6-glucosidic links in these polysaccharides and inhibition of the same sera with isolichenin suggests the probable presence of α -1 $\rightarrow$ 3-glucosidic bonds.

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Some Observations on the Reactive Sulphydryl Groups in Haemoglobin

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The SH groups in a protein are generally described as either 'reactive' or 'unreactive'. The reactive SH groups are readily titrated with heavy-metal reagents and have properties similar to cysteine, whereas the unreactive SH groups in the native protein combine slowly with heavy-metal reagents. In a comparative study of the haemoglobins of various mammalian species, Riggs (1959, 1960) found that the number of Hg^{2+} ions which are bound by the deoxygenated haemoglobins is approximately equal to the number of protons liberated during oxygenation, and he suggested that the reactive SH groups may be the oxygen-linked acidic groups. He also found that the number of protons liberated during oxygenation is inversely related to the body weight of the animal. This implies that there is a relation between the reactive SH group content of the haemoglobin and body weight. However, Benesch & Benesch (1960, 1961) showed that the addition of heavy-metal ions to human haemoglobin does not prevent the liberation of protons during oxygenation. They

concluded that the reactive SH groups are not the oxygen-linked acidic groups.

Allison & Cecil (1958) showed that high values for the SH group content of human haemoglobin are obtained unless precautions are taken to prevent the combination of heavy-metal ions with groups other than SH in the protein. They concluded that the SH groups of human haemoglobin can be estimated by amperometric titration with phenylmercuric hydroxide in the presence of sulphite, with the dropping-mercury electrode as the end-point indicator. Cecil & Snow (1962) showed that the rate of combination of heavy-metal reagents with the unreactive SH groups of human haemoglobin was faster in deoxygenated haemoglobin than in carboxyhaemoglobin. Consequently, carboxyhaemoglobin should be used for the estimation of reactive SH groups to reduce the possibility of heavy-metal reagent combining with unreactive SH groups during the course of the titration. For his comparative study of the reactive SH groups Riggs (1959, 1960) used deoxygenated haemoglobin and did not take precautions against the combination of Hg^{2+} ions with groups other than SH. In the light of the results of Allison & Cecil (1958) and

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