Studies on the Structure of Lipopolysaccharides of *Salmonella minnesota* and *Salmonella typhimurium* R Strains

By I. W. SUTHERLAND,* O. LÜDERITZ AND O. WESTPHAL
Max-Planck-Institut für Immunbiologie, Freiburg, Germany

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1. The composition of the lipopolysaccharides and the corresponding lipid-free polysaccharides from four R-mutants of *Salmonella* has been studied. All the lipopolysaccharides, from R I and R II serotypes contained D-glucose, D-galactose, heptose, N-acetylglucosamine and 3-deoxy-2-oxo-octonate. The polysaccharide obtained from the R II lipopolysaccharides also contained all these sugars. The polysaccharides from R I lipopolysaccharides lacked N-acetylglucosamine. 2. From partial hydrolysates of the lipopolysaccharides, a number of oligosaccharides have been isolated and partially characterized. Oligosaccharides containing N-acetylglucosamine or glucosamine were obtained only from R II lipopolysaccharides. Several oligosaccharides composed of glucose and galactose were common to R I and R II preparations. 3. A structural unit, based on the oligosaccharides found, is proposed for the R II lipopolysaccharide. It contains the sequence: α -N-acetylglucosaminyl- α -glucosyl- α -galactosyl-glucosyl.... A second α -galactosyl residue is bound to position 6 of the last glucosyl group. The complete unit is believed to be attached to a polyheptose phosphate backbone in the R II antigen. 4. The R I lipopolysaccharide of *Salmonella minnesota* contains an analogous structure lacking the terminal N-acetylglucosamine residue. 5. A basal structure common to the lipopolysaccharides of several *Salmonella* species is proposed.

Salmonella R ('rough') mutants, like the S ('smooth') strains from which they are derived, contain lipopolysaccharides extractable by the phenol-water technique (Kauffmann, Krüger, Lüderitz & Westphal, 1961). In several of their biological and physicochemical properties they are similar to those of the wild type. They are distinct, however, in their serological specificity (Kauffmann, 1961). In contrast with the great variety of specificities exerted by the O antigens of *Salmonella* species, only a few R serotypes are known. Most of the R antigens isolated from the R mutants of many *Salmonella* species show serological cross-reaction. The R lipopolysaccharides are also chemically simpler than are those of the S strains (Kauffmann *et al.* 1961). Thus the O antigens contain 'specific' sugars determining O-specificity, together with certain 'basal' sugars found in the lipopolysaccharides of all *Salmonella* species (Kauffmann, Lüderitz, Stierlin & Westphal, 1960). The R antigens contain only these 'basal' sugars, the 'specific' sugars being totally absent (Kauffmann *et al.* 1961).

It has been postulated either that R mutants are

unable to synthesize the 'specific' sugars of the O-serospecific side chains found in the S lipopolysaccharides, or that they are unable to transfer these sugars to form the complete O antigen. Thus it is believed that the *Salmonella* R mutants, because of the absence of certain specific enzymes, synthesize an incomplete O antigen, namely the R antigen. In the original, wild type, this R antigen represents a 'basal' or 'core' polysaccharide to which the specific side chains are attached to form the complete O antigen (Lüderitz, Kauffmann, Stierlin & Westphal, 1960).

The present paper describes studies on the chemical nature of the polysaccharides obtained from two R mutants of *Salmonella minnesota* and two R mutants of *Salmonella typhimurium*, with a view to determining what similarities, if any, exist in the basal polysaccharides of these organisms.

MATERIALS AND METHODS

Micro-organisms and methods of culture. The two *Salmonella minnesota* R strains, R 60 and R 345, were those used by Lüderitz *et al.* (1965). The three *Salmonella typhimurium* strains, TV 157, TV 166 and TV 208, were obtained from Dr B. A. D. Stocker, The Lister Institute, London. Their serological and chemical properties had been studied

* On leave from the Bacteriology Department, Edinburgh University.

by Beckmann, Subbaiah & Stocker (1964b). Strains R345 and TV166 are of the RI serotype and strains R60, TV157 and TV208 are RII mutants. One *S. inverness* RII mutant and one *S. poona* RII mutant were also examined (Beckmann, Lüderitz & Westphal, 1964a).

Cultures were mostly grown on nutrient agar or on Bacto Eugone agar. With *S. minnesota* R345, one batch of cells was grown in Difco Penassay liquid medium.

Preparation of lipopolysaccharides and polysaccharides. Lipopolysaccharides were extracted from all strains by a modification of the phenol-water method of Westphal, Lüderitz & Bister (1952). In this, the extraction mixture was heated for only 5 min. (Lüderitz *et al.* 1964). Lipid-free polysaccharide (200 mg.) was prepared from the lipopolysaccharides by hydrolysis with 20 ml. of 1% (v/v) acetic acid at 100° for 60 min. The precipitated lipid A was removed by low-speed centrifugation and the supernatant was dialysed for 24 hr. against water and freeze-dried.

Preparation of partial acid hydrolysates. Approximately 1.0 g. of each lipopolysaccharide preparation was dissolved in 100 ml. of water. An equal volume of 2N-H₂SO₄ was added and hydrolysis carried out in stoppered glass tubes at 100° for 30 min. After the hydrolysate had been allowed to cool, all lipid A precipitated was removed by low-speed centrifugation. The supernatant solution was diluted with 2 vol. of water and neutralized with Amberlite IRA-410 (HCO₃⁻ form). The supernatant solution was then filtered to remove resin and pervacuated until the volume was reduced to about 2.0 ml. Any precipitate appearing at this stage was removed by centrifugation.

Paper chromatography and paper electrophoresis. Charged oligosaccharides and monosaccharides were separated from neutral material in the hydrolysates by paper electrophoresis on MN 214 paper (70 cm. × 15 cm.; Macherey, Nagel and Co., Düren, Germany) with pyridine-acetic acid-water (5:2:43, by vol.) buffer, pH 5.3 (buffer D). A current of 100–120 mA at 3000 v was applied for 1 hr. This same system was used to de-salt the products of enzyme treatment before paper chromatography. Electrophoresis in borate buffer (0.05M-sodium tetraborate, pH 9.1; buffer E) was used to assist in the identification of disaccharides. A current of 30–40 mA was applied at 3000 v for 2 hr, with 2043a paper (Schleicher and Schüll, Dassel, Germany).

The neutral oligosaccharides, eluted from the electrophoretograms in buffer D, were separated and identified with two descending chromatographic solvent systems: pyridine-butanol-water (4:6:3, by vol.; solvent A; Whistler & Conrad, 1954); butanol-acetic acid-water (4:1:5, by vol.; solvent B; Partridge, 1946). The monosaccharides were separated and identified by the solvent system described by Fischer & Dürfel (1955) (ethyl acetate-acetic acid-pyridine-water, 5:1:5:3, by vol.; solvent C). Whatman no. 1 paper was used for all the paper chromatography.

Sugars and oligosaccharides were normally revealed with alkaline AgNO₃. Ninhydrin reagent was used to reveal hexamines and thiobarbituric acid spray reagent (Warren, 1960) for 3-deoxy-2-oxo-octonate. Where it was suspected that oligosaccharides might contain phosphorus, the spray reagent of Hanes & Isherwood (1949) was used.

Nomenclature of oligosaccharides. The oligosaccharides separated by paper electrophoresis in buffer D were numbered E1, E2 etc. respectively from the anode. The neutral material was then subjected to chromatography in

solvent A and the oligosaccharides so obtained were designated C1, C2 etc. in order of increasing mobility. Where chromatography in solvent B showed that these fractions were still complex, they were rechromatographed in this solvent. The oligosaccharides subsequently separated were called C2a, C2b etc. in order of increasing mobility. After elution, oligosaccharide solutions were dried *in vacuo*, and then dissolved in water such that the volume of solution was approximately 100 µl./g. of lipopolysaccharide hydrolysed.

Analytical methods. Hydrolysis of polysaccharides and oligosaccharides was performed in sealed glass tubes with N-H₂SO₄ at 100° for 4 hr. The hydrolysates were then neutralized with Amberlite IRA-410 (HCO₃⁻ form).

D-Glucose was determined with glucose oxidase reagent (Boehringer, Mannheim, Germany). D-Galactose, heptose, total hexosamine, D-glucosamine, 3-deoxy-2-oxo-octonate and phosphorus were determined as described by Lüderitz *et al.* (1964).

Enzymes used in the hydrolysis of oligosaccharides. α-Glucosidase was prepared from brewer's yeast as described by Robbins & Uchida (1962). β-Glucosidase (emulsin) was purchased from Fluka A.-G., Buchs, Switzerland. This preparation contained considerable amounts of α-glucosidase. α-Galactosidase, from green coffee beans, was kindly donated by Dr M. J. Osborn, Yeshiva University, New York, N.Y., U.S.A. β-Galactosidase obtained from an *Escherichia coli* strain (Wallenfels, Zarnitz, Laule, Bender & Keser, 1959) was a gift from Professor K. Wallenfels, Freiburg University, Germany. α-N-Acetylglucosaminidase was prepared from limpets (*Patella vulgata*) (Levy & McAllan, 1963). This preparation also contained large amounts of β-N-Acetylglucosaminidase. β-N-Acetylglucosaminidase was extracted from rat epididymis (Levy & McAllan, 1959). Alkaline phosphatase from *E. coli* cells was purchased from Sigma Chemical Co., St Louis, U.S.A.

After enzymic hydrolysis, quantitative analyses were made for the supposed non-reducing terminal sugar of the oligosaccharide. In all the enzymic experiments controls with the appropriate α- and β-linked disaccharides of known structure were included. For glucosidases these were maltose and cellobiose; for galactosidases, melibiose and lactose; for N-acetylglucosaminidases, α- and β-methyl N-acetylglucosaminide. The conditions used were such that the specific enzyme would cause 90–100% hydrolysis of the substrate.

RESULTS

Chemical composition of the polysaccharides of R strains

Although the lipopolysaccharides of the strains studied contained the same monosaccharide components, they could be classified into two serological groups, RI and RII. Chemical analysis showed that the composition of substances of the same serotype could show some variation. The results for the preparations used in the present study are given in Table 1. Thus the lipopolysaccharide and polysaccharide from *S. minnesota* R345 (RI) had a glucose:galactose ratio approximately 1:2, while in the corresponding preparations from *S.*

Table 1. *Composition of the polysaccharides of Salmonella R strains*

All values are expressed as percentage of dry weight of the lipopolysaccharide or the polysaccharide. —, Not tested.

	Glucose	Galactose	Heptose	3-Deoxy-2-oxo-octonate	P	Glucosamine
<i>S. minnesota</i> R345 (RI)						
Lipopolysaccharide	4.0	8.5	12.0	5.0	—	8.0
Polysaccharide	12.1	19.6	15.0	2.0	2.6	0.8
<i>S. typhimurium</i> TV166 (RI)						
Lipopolysaccharide	6.7	6.9	13.7	8.9	—	7.3
Polysaccharide	13.5	13.0	23.7	5.7	3.8	0.6
<i>S. minnesota</i> R60 (RII)						
Lipopolysaccharide	6.0	8.0	7.0	5.0	—	8.0
Polysaccharide	13.0	16.2	21.5	2.0	2.5	5.6
<i>S. typhimurium</i> TV208 (RII)						
Lipopolysaccharide	3.4	14.3	6.0	—	—	5.2
						(Rhamnose 1.8)
Polysaccharide	5.0	20.5	7.3	—	1.5	2.4
						(Rhamnose 2.6)
<i>S. typhimurium</i> TV157 (RII)						
Lipopolysaccharide	5.7	5.9	7.0	—	—	9.3
<i>S. poona</i> RII						
Lipopolysaccharide	4.9	5.5	8.4	—	—	9.3
<i>S. inverness</i> RII						
Lipopolysaccharide	5.0	6.2	9.0	—	—	9.4

typhimurium RI the ratio was about 1:1. Less difference was observed in the other constituents of these polysaccharides. In both the lipopolysaccharides considerable amounts of glucosamine were present, but in the corresponding lipid-free polysaccharides this sugar was essentially absent. Thus in the two RI mutants, glucosamine is present only in the lipid entity of the lipopolysaccharides (Lüderitz *et al.* 1964). In contrast, appreciable amounts of glucosamine were detected in the lipid-free polysaccharides from the two RII mutants, this being a constituent of the RII structure. In lipopolysaccharide and polysaccharide preparations from four RII strains, the glucose:galactose ratio was approximately 1:1. However, in a fifth strain *S. typhimurium* TV208, the ratio was nearly 1:4. An earlier preparation of lipopolysaccharide from this strain contained 6.4% of glucose and 5.5% of galactose (Beckmann *et al.* 1964b). It is thus possible that the bacteria used for our preparation differed from those used earlier.

After a report that treatment with the enzyme α -galactosidase released much of the galactose from the lipid-free polysaccharide of a *S. typhimurium* R mutant (Osborn, Rosen, Rothfield, Zeleznick & Horecker, 1964), we attempted to repeat this with the corresponding preparation from *S. minnesota* R345. We obtained release of 26% of the total galactose present, as determined by the galactose-dehydrogenase assay method. The result was confirmed by subjecting the products of α -galactosidase treatment to chromatography in solvent C. Galactose was the only sugar detected. It thus

seemed that some of the galactose in the polysaccharide of this RI mutant was present as a terminal α -galactosyl residue. No galactose was released from *S. minnesota* R60 polysaccharide by the same treatment. The use of specific enzymic methods for sugar determination indicated that, in all the lipopolysaccharides and polysaccharides examined, glucose, galactose and glucosamine were in the D-configuration and rhamnose was in the L-configuration.

Oligosaccharides isolated from S. minnesota R345 lipopolysaccharide

Negatively charged material. 345/E3. Electrophoresis of partial hydrolysates of lipopolysaccharide from this strain consistently showed the presence of a relatively large amount of material moving to the anode. This fraction stained strongly with thiobarbituric acid reagent and on elution from the paper the substance gave very strong reactions when tested quantitatively for 3-deoxy-2-oxo-octonate. Tests for other sugars were negative. Before and after acid hydrolysis, this fraction moved as a single component in chromatography solvent C, equidistant with a sample of authentic 3-deoxy-2-oxo-octonate. It was therefore assumed that fraction E3 was in fact 3-deoxy-2-oxo-octonate.

345/E4. In partial hydrolysates of some batches of R345 lipopolysaccharide, a small amount of negatively charged material, moving slower than 3-deoxy-2-oxo-octonate in paper electrophoresis in buffer D, was detected. Staining with the reagent

Table 2. *Properties of oligosaccharides isolated from R lipopolysaccharides*

Preparation	R_{Glc}		Yield ($\mu\text{moles/g. of}$ lipopolysaccharide)	Quantitative analysis ($\mu\text{moles/100 }\mu\text{l. of eluate}$)		
	Solvent A	Solvent B		Glucose	Galactose	Other sugars and P
R345 lipopolysaccharide						
C6a	0.70	0.62	0.55	1.09	0	
C5	0.54	0.56	0.39	0.38	0.39	
C4b	0.49	0.51	1.80	1.94	1.80	
C4a	0.44	0.43	0.15	0.59	0	
C3	0.37	0.34	0.16	0.50	0	
C1	0.12	0.15	0.36	0.40	0.50	
C1E	0.20	0.21		0.14	0.05	
E4	0.04	0.40	0.48	0	1.9	Heptose: 4.4 P: 1.2
TV166 lipopolysaccharide						
C3	0.63	0.60	3.66	1.7	1.2	
C2	0.49	0.54	8.0	3.7	2.4	
C1	0.40	0.47	1.08	0.8	0.5	
R60 lipopolysaccharide						
						Glucosamine
C8	0.76	0.72	1.68	1.33	0	1.17
C7	0.66	0.58	1.72	1.37	0	0
C6	0.57	0.59	1.88	1.49	1.14	0
C5	0.51	0.53		0.36	0	0
C4c	0.45	0.49	0.60	0.26	0.25	0
C4b	0.43	0.47	1.85	0.84	0.85	0.78
C4a	0.42	0.34	0.60	0.39	0	0
C3b	0.30	0.25	0.46	0.75	0.40	0.28
C1	0.14	0.13	0.60	0.95	0.58	0.33
E3	0.19	0.25	0.62	0.49	0.49	0.35
TV208 lipopolysaccharide						
C10	0.74	0.69	0.80	0.60	0	0.8
C5b	0.39	0.44	0.35	0.30	0.26	0
C5a	0.34	0.39	0.39	0.34	0.33	0.41
C1	0.08	0.14	0.15	0.21	0.3	0.13
E3	0.18	0.24	0.23	0.16	0.21	0.20

of Hanes & Isherwood (1949) indicated the presence of phosphorus. Elution and analysis showed the presence of heptose, galactose and phosphorus in proportions about 2:1:1. Sodium borohydride reduced 48% of the heptose. Hydrolysis with β -galactosidase released 70% of the available galactose and alkaline phosphatase treatment liberated 44% of the phosphorus. This compound was very slow-moving in chromatographic solvents A and B (Table 2). These results lead us to suggest that the compound is composed of β -galactosyl-heptose, to which is linked a further heptose molecule and one phosphate residue. The possibility that there is a mixture of compounds inseparable by the methods used, cannot be excluded. Thus the phosphorus might be present in two different linkages.

Positively charged material. Only small amounts of positively charged material were detected on paper electrophoretograms of partial hydrolysates of R345 lipopolysaccharide. The material was

equidistant with glucosamine in buffer D and stained with ninhydrin reagent. It was assumed to be glucosamine originating from the lipid A component of the lipopolysaccharide.

Neutral material. 345/C6a. Hydrolysates of this fraction showed the presence of glucose only. With borohydride, 48% of the glucose was reduced. The oligosaccharide was 95% hydrolysed with α -glucosidase. On chromatography in solvents A, B and C, and on electrophoresis in buffer E it moved as maltose. Thus fraction C6a is probably *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose.

345/C5. This fraction contained glucose and galactose in equal amounts. Borohydride reduction indicated that galactose was the reducing terminal sugar. Of the glucose 65% could be released by α -glucosidase. C5 is therefore an α -glucosyl-galactose disaccharide. The use of diphenylamine reagent (Bailey & Bourne, 1960) showed that the linkage was not (1 $\rightarrow$ 4).

345/C4b. This fraction was present in all R345

preparations and the yield (Table 2) indicated that it was one of the major fractions in the hydrolysate. Paper chromatography of hydrolysates of this oligosaccharide showed that it was composed of glucose and galactose and analysis gave a ratio 1:1:1. After borohydride reduction and hydrolysis, no glucose could be detected but the galactose value remained unchanged, indicating that the substance is probably a galactosylglucose disaccharide. It was extensively hydrolysed with α -galactosidase. Chromatography in solvents A and B, and electrophoresis in buffer E, showed that it moved exactly equidistant with melibiose in each system. It therefore seems probable that C4b is *O*- α -D-galactopyranosyl-(1 $\rightarrow$ 6)-D-glucopyranose.

345/C4a. This substance yielded only glucose on hydrolysis and the glucose content decreased after treatment with borohydride treatment. The ratio of glucose before reduction to glucose after reduction was 3:3:2. Hydrolysis with α -glucosidase liberated 89% of the total glucose as free sugar. Thus the compound may be a trisaccharide with both linkages having the α -configuration. Chromatography in solvent A of the products of enzymic hydrolysis revealed glucose and a spot equidistant with maltose. The oligosaccharide itself has the same R_{GLC} value as maltotriose in this solvent. It thus seems that C4a is *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-*O*- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose. It is possible that small amounts of contaminating material 345/C3 were present. This could be the reason why only 89% of the glucose was liberated enzymically.

345/C3. Like the previous fraction, hydrolysates of C3 revealed glucose as the sole component monosaccharide. A 25% reduction in the glucose content followed treatment with borohydride, so this was probably a tetrasaccharide. Only 26% of the glucose was liberated by a α -glucosidase treatment and β -glucosidase was equally inactive. Chromatography of the products of α -glucosidase hydrolysis revealed traces of materials moving equidistant with maltose and maltotriose in solvent A. This oligosaccharide did not move equidistant with maltotetraose and insufficient material was available to determine its structure further.

345/C2. This fraction was present in very small quantities and paper chromatography of hydrolysates showed that it contained glucose, galactose and galactosamine. The presence of this last sugar indicated that the lipopolysaccharide was contaminated with 'S' material and no further tests were made.

345/C1. This slow-moving oligosaccharide was found to contain glucose and galactose in equal proportions. Borohydride reduced 40% of the glucose, and 44% of the galactose could be liberated with α -galactosidase. The oligosaccharide produced

by α -galactosidase (345/C1E) was shown to be a trisaccharide with glucose as the terminal reducing sugar. It could not be hydrolysed further by α - or β -glucosidase or β -galactosidase. In view of the possible structures of 345/C1 and 345/C1E it is difficult to understand the resistance of both these oligosaccharides to hydrolysis by glucosidases.

Oligosaccharides obtained from S. typhimurium TV 166 lipopolysaccharide

The only charged material obtained from electrophoretograms of partial hydrolysates of this lipopolysaccharide was identified as 3-deoxy-2-oxo-octonate and glucosamine. The neutral material, after elution and chromatography, showed a much simpler pattern than that obtained in the hydrolysates of the other lipopolysaccharides examined, only three oligosaccharides being detected.

166/C3. The fastest-moving oligosaccharide contained equal amounts of glucose and galactose. All the galactose could be reduced by borohydride and 76% of the glucose was liberated after treatment with α -glucosidase. It thus seems that 166/C3 is a disaccharide with the structure α -glucosylgalactose. However, from its chromatographic properties and electrophoresis in buffer E, it is different from 345/C5.

166/C2. A second fraction containing only glucose and galactose was, like 166/C3, obtained in high yield (Table 2). The ratio of glucose to galactose was 1.5:1 and galactose was found to be the terminal reducing sugar. It proved impossible to split the glycosidic bond with α - or β -glucosidase. Staining of the oligosaccharide with diphenylamine reagent gave a blue colour which is thought to indicate a (1 $\rightarrow$ 4) linkage (Bailey & Bourne, 1960). In solvent A, 166/C2 moved at the same speed as an oligosaccharide isolated from partial hydrolysates of *E. coli* O111 lipopolysaccharide and thought to be *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-galactopyranose (H. Mayer, unpublished work).

166/C1. This oligosaccharide was identical with fraction 345/C4b in all respects tested, i.e. it is probably *O*- α -D-galactopyranosyl-(1 $\rightarrow$ 6)-D-glucopyranose.

Oligosaccharides found in partial hydrolysates of the lipopolysaccharide of S. minnesota R60

60/C8. The fastest-moving neutral oligosaccharide isolated from this preparation yielded glucose and glucosamine on hydrolysis. Analysis showed a ratio 1:1, and all the glucose was destroyed on reduction. As the fraction is neutral, the glucosamine must be substituted. The presence of material moving as *N*-acetylglucosamine in the hydrolysate

of 60/C8 (as well as of the lipopolysaccharide) suggests that 60/C8 is, in fact, *N*-acetylated. The nature of the glycosidic bond was determined with α - and β -*N*-acetylglucosaminidases. Only the α -enzyme liberated free *N*-acetylglucosamine from the oligosaccharide. It is therefore α -*N*-acetylglucosaminyl-D-glucose.

60/C7. An oligosaccharide composed solely of glucose was also found. Half the glucose was reduced by borohydride and α -glucosidase liberated much of the available glucose. On chromatography in solvents A and B, and on electrophoresis in buffer E, it moved as maltose and as 345/C6a. It is therefore *O*- α -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose.

60/C6. Hydrolysates of this fraction contained glucose and galactose in the ratio 1:2:1. All the galactose was reduced by borohydride, indicating that the oligosaccharide is probably a glucosyl-galactose disaccharide. The enzyme α -glucosidase released 58% of the available glucose, indicating that the glucosyl linkage had an α -configuration. It differed in its chromatographic and electrophoretic mobilities from the disaccharides found in TV 166 lipopolysaccharide hydrolysates (166/C3 and 166/C2) but was apparently identical with 345/C5.

60/C5. This oligosaccharide, which was obtained in very small quantities, yielded only glucose on hydrolysis and 33% of this sugar was reduced by borohydride. About 50% of the glucose was released by α -glucosidase. It would seem to be a trisaccharide composed of glucose and differing from maltotriose in its chromatographic behaviour.

60/C4c. This oligosaccharide was identical with 345/C4b and with melibiose in all respects tested. It is therefore *O*- α -D-galactopyranosyl-(1 $\rightarrow$ 6)-D-glucopyranose.

60/C4b. This was composed of glucose, galactose and *N*-acetylglucosamine. The three sugars were present in the molar proportions 1:1:1. All the galactose was reduced by borohydride. Hydrolysis of 0.3 μ mole with *N*-sulphuric acid for 15 min., followed by neutralization and chromatography in solvent B, revealed glucose, galactose, *N*-acetylglucosamine and materials moving equidistant with 60/C6 and 60/C8. It is probable that 60/C4b was the trisaccharide α -*N*-acetylglucosaminyl- α -D-glucosyl-D-galactose. Attempts to release amino sugar with α -*N*-acetylglucosaminidase were unsuccessful and no hydrolysis was obtained with α -glucosidase.

60/C4a. This fraction was identical with 345/C4a and with maltotriose in all respects tested.

60/C3b. Although composed of the same three sugars as 60/C4b, the glucose:galactose:*N*-acetylglucosamine molar proportions were 2:1:1, suggesting that the substance was a tetrasaccharide. Further evidence for this was obtained when 50% of the glucose was reduced by borohydride. Galac-

tose was not released by treatment with α -galactosidase, and α -glucosidase was inactive. Although no free sugar was liberated by α -*N*-acetylglucosaminidase, the most likely structure, deduced from the disaccharides and trisaccharide found, would be α -*N*-acetylglucosaminyl- α -D-glucosyl-D-galactosyl-D-glucose.

60/C1. Hydrolysates of this oligosaccharide contained glucose, galactose and glucosamine in the proportions 2:2:1, and 50% of the glucose was reduced by borohydride. On acid hydrolysis of about 0.3 μ mole of the oligosaccharide in the same way as for 60/C4b, a number of spots, in addition to glucose, galactose and *N*-acetylglucosamine, were detected on chromatography in solvent B: (i) a spot equidistant with 60/C3b, *N*-acetylglucosaminyl-D-glucosyl-D-galactosyl-D-glucose; (ii) a spot moving faster than 60/C3b but slower than the trisaccharide 60/C4b; (iii) a spot equidistant with 60/C4b; (iv) a spot equidistant with 60/C4c and melibiose; (v) a spot equidistant with 60/C8, α -*N*-acetylglucosaminyl-D-glucose. None of the *N*-acetylglucosamine could be liberated by treatment with α -*N*-acetylglucosaminidase, but 60% of the galactose in the oligosaccharide could be liberated by α -galactosidase treatment. When the products of α -galactosidase action were chromatographed in solvent B, two spots were detected, equidistant with galactose and with the tetrasaccharide 60/C3b respectively. From these results we conclude that C1 is a pentasaccharide of possible structure: α -D-galactosyl . . . (α -*N*-acetylglucosaminyl- α -D-glucosyl- α -D-galactosyl-D-glucose).

60/E3. One positively charged fraction other than free hexosamine was detected in partial hydrolysates of R 60 lipopolysaccharide. This contained glucose, galactose and glucosamine in approximately equal proportions. The galactose was reduced by borohydride. On acetylation by the procedure used in the determination of total glucosamine (Strominger, Park & Thompson, 1959), the product was found to move equidistant with 60/C4b in solvent B. It was therefore thought that E3 was the trisaccharide α -glucosaminyl- α -D-glucosyl-D-galactose.

Oligosaccharides obtained from partial hydrolysates of S. typhimurium TV 208 lipopolysaccharide

The preparations from this organism contained 3-deoxy-2-oxo-octonate and hexosamine, as did those from all the other lipopolysaccharides studied. One other electrophoretically mobile fraction, moving to the cathode, and large amounts of neutral material were obtained.

208/C10. This oligosaccharide was identical with 60/C8 in all respects tested, i.e. it was apparently α -*N*-acetylglucosaminyl-D-glucose.

208/C2, C7, C8 and C9. These fractions were found to contain galactose, mannose and rhamnose. The presence of these 'specific' sugars indicated that they were derived from contaminating 'S' substance and they were not studied further.

208/C5b. This fraction contained glucose and galactose and it behaved like melibiose in every respect tested.

208/C5a. Hydrolysates of this fraction contained equal quantities of glucose, galactose and *N*-acetylglucosamine. It appeared to be identical with the trisaccharide 60/C4b.

208/C1. This was identical with 60/C in every respect, i.e. it was thought to be a pentasaccharide with possible structure α -D-galactosyl . . . (α -*N*-acetylglucosaminyl- α -D-glucosyl- α -D-galactosyl-D-glucose).

208/E3. The one positively charged oligosaccharide detected contained equal quantities of glucosamine, glucose and galactose. It was apparently identical with 60/E3. After acetylation, it moved in solvent B like 208/C5a and like 60/E3 after acetylation.

*Material from S. typhimurium TV 157 (RII),
S. poona RII and S. invernass RII*

In addition to the preparations from the four bacterial strains which were the main subject of this study, small amounts (50–60 mg.) of lipopolysaccharides from three other RII strains were hydrolysed in parallel with a similar amount of lipopolysaccharide from *S. minnesota* R60. After paper electrophoresis, the neutral fractions were eluted and subjected to paper chromatography in solvent A. Guide strips cut from the four chromatograms showed identical sequences of oligosaccharides. The three major spots were eluted from each chromatogram. After hydrolysis, their monosaccharide constituents were analysed by cellulose thin-layer chromatography in solvent A and by quantitative analysis. The oligosaccharides corresponding in position to 60/C8 all contained *N*-acetylglucosamine and glucose in the ratio 1:1, and those corresponding to 60/C6 contained glucose and galactose in the ratio 1:1. The third group of oligosaccharides, corresponding to 60/C4b, all contained glucose, galactose and *N*-acetylglucosamine in the proportions 1:1:1.

Table 2 summarizes the properties of the oligosaccharides isolated from all the preparations. The R_{glc} values given are those obtained when all oligosaccharides from one preparation were run together. To confirm the tentative identity of oligosaccharides from different preparations, they were always run side by side along with the authentic compound when available.

DISCUSSION

In the genus *Salmonella* the serological specificities of the wild-type (S) strains, the subject of the Kauffmann-White scheme, are exceedingly numerous. The lipopolysaccharides, which can be extracted by the phenol-water technique, account for the heat-stable, O-specificities. However, the R mutants derived during laboratory culture from widely different S strains show a very limited range of serological specificity. On serological examination, most of these R mutants can be allocated to two groups, RI and RII. Similarly, in contrast with the numerous chemotypes of the S polysaccharides, most of the R strains possess lipopolysaccharides of the same chemotype (I), containing only the basal sugars: glucose, galactose, glucosamine, 3-deoxy-2-oxo-octonate and heptose. It has been shown that in the RI lipopolysaccharide of *S. minnesota* R345 the glucosamine is present only in the lipid A entity and is essentially absent from the polysaccharide (Lüderitz *et al.* 1965). Conversely, *S. minnesota* R60, belonging to the serological group RII, contains glucosamine as an integral component of the polysaccharide. It was suggested that the heptose, together with phosphate, forms a 'backbone' to which are attached side chains containing glucose and galactose in the RI lipopolysaccharides, whereas those from the RII mutants also contain glucosamine.

From our analyses of four different R lipopolysaccharides and the corresponding polysaccharides (Table 1), the proportions of glucose, galactose and heptose are such that either the side chains attached to the heptose are very short, possibly containing one glucose and one galactose residue attached to each heptose molecule, or the chains are somewhat longer and not all the heptose molecules possess attached side chains. It is, of course, also possible that the side chains are not uniform in respect to length and to composition.

The purification of lipopolysaccharides after extraction with phenol-water is based on their behaviour in the preparative ultracentrifuge. This technique sediments the lipopolysaccharide as a gel, while most of the contaminating materials, such as nucleic acid and glycogen, remain in the supernatant liquid. Small amounts of these contaminants, however, may be present in the sediment even after two washings with water. In assessing the importance of the oligosaccharides obtained in the partial hydrolysis studies, the origin of these compounds must be considered, as they may be derived from distinct sources: (i) the actual R lipopolysaccharide; (ii) contaminating S material; (iii) other contaminating polysaccharides. There are two possible sources of the S substances. It is known that in RI mutants O-specific material is

found as a haptenic polysaccharide in bacterial extracts (Beckmann *et al.* 1964b; Lüderitz *et al.* 1965). This would seem to be the most likely source of the galactosamine-containing material found in small amounts in the partial hydrolysates of the lipopolysaccharide preparation from *S. minnesota* R 345. Alternatively, the R cultures may partially revert, by back mutation, to the S form. This is especially true of *S. typhimurium* TV 208 (Nikaido, Nikaido, Subbaiah & Stocker, 1964). As there has been no report of S-specific substances in RII cultures, the presence of oligosaccharides containing rhamnose, mannose and galactose in partial hydrolysates of the lipopolysaccharide from *S. typhimurium* TV 208 can apparently only be ascribed to partial reversion of the bacterial culture to the S form. Again, these oligosaccharides were present in relatively small amounts, insufficient to permit analysis and detailed study. Polyglucans of the glycogen type are produced by many species of Enterobacteriaceae (see, e.g., Palmstierna, 1956; Robbins & Uchida, 1962). Because of the physical properties of such substances they are likely to be present as contaminants of lipopolysaccharides prepared by the phenol-water extraction method. This may be the source of the maltose, maltotriose and other α -linked glucose-oligosaccharides found in the *S. minnesota* preparations.

Thus there remain a number of hetero-oligosaccharides which, we consider, originate from the R lipopolysaccharides. The possible structure of these, together with the preparations in which they were found, are shown in Table 3. The most complete series of oligosaccharides was that obtained from the RII mutant, *S. minnesota* R 60. From the disaccharides, trisaccharide and pentasaccharide found, we have formulated the structure (I) for the oligosaccharide 60/C1. As we have no evidence that this pentasaccharide contains

more than two glucose residues, there are theoretically three possible derivations of the disaccharide C4c (melibiose). This may derive from the main chain, where a galactosylglucose unit exists, or the α -galactosyl residue may form a branch attached to either the first or second glucose residue (reading from the right) in the above sequence. We have based our formula on the following considerations. (i) Enzymic hydrolysis of 60/C1 with α -galactosidase led to production of the tetrasaccharide 60/C3b. (ii) When C3b was partially hydrolysed with acid, no melibiose was demonstrable, suggesting that no α -(1 $\rightarrow$ 6)-linked galactose is present in its structure. (iii) Acid hydrolysis of the pentasaccharide C1 led to production of an oligosaccharide chromatographically the same as melibiose. The only possible position of the α -D-galactosyl-(1 $\rightarrow$ 6)- would then be as a branch from the main chain. (iv) If the galactose were attached to the second glucose in the formula, one would expect to find a tetrasaccharide with galactose as the terminal reducing sugar and a trisaccharide with glucose as the terminal reducing sugar, the proportions of *N*-acetylglucosamine:glucose:galactose being 1:1:2 and 1:1:1 respectively. Such oligosaccharides have not been found. It can be concluded that the galactose liberated from the pentasaccharide by the action of α -galactosidase, and thus present in a non-reducing terminal position, is the galactose bound to the 6-position of the first glucose residue in the sequence. The only possible disaccharide derivable from the above structure which we have not found is the galactosylglucose disaccharide from the main chain. However, the absence of this compound from our series may be explicable from the findings of Osborn *et al.* (1964), who transferred galactose from UDP-galactose to the antigen of a mutant of *S. typhimurium*, the polysaccharide of which contained only

Table 3. *Distribution of oligosaccharides found in lipopolysaccharide hydrolysates*

Possible structure of oligosaccharide	<i>S. minnesota</i> R345 (RI)	<i>S. typhimurium</i> TV166 (RI)	<i>S. minnesota</i> R60 (RII)	<i>S. typhimurium</i> TV208 (RII)
α -N-Acetylglucosaminylglucose	—	—	C8	C10
α -D-Glucosyl-D-galactose	—	C3	—	—
α -D-Glucosyl-D-galactose	C5	—	C6	—
Glucosylgalactose	—	C2	—	—
O- α -D-Galactopyranosyl-(1 $\rightarrow$ 6)-D-glucopyranose	C4b	C1	C4c	C5b
α -N-Acetylglucosaminyl- α -glucosylgalactose	—	—	C4b	C5a
α -N-Acetylglucosaminyl- α -glucosyl- α -galactosylglucose	—	—	C3	—
α -D-Galactopyranosyl-(α -D-glucopyranosyl-D-galactosylglucose)	C1	—	—	—
α -D-Galactopyranosyl-(α -N-acetylglucosaminyl- α -D-glucopyranose)	—	—	C1	C1

heptose and glucose. The product obtained after transfer of the galactose contained this sugar as a non-reducing terminal residue bound α -glycosidically to the 3-position of the glucose residues. The α -galactosyl-(1 $\rightarrow$ 3)-linkage was shown to be very acid-labile and it was not possible to obtain the disaccharide, O- α -D-galactopyranosyl-(1 $\rightarrow$ 3)-D-glucose, by partial acid hydrolysis. Thus if the same α -(1 $\rightarrow$ 3)-linkage is present in 60/C1, its lability should lead to preferential formation of oligosaccharides from other parts of the molecule, and in particular C4c, if the structure is as shown.

S. minnesota R 60 is deficient in the enzyme UDP-N-acetylgalactosamine 4-epimerase (Lüderitz *et al.* 1964). We have therefore concluded that the R antigen of *S. minnesota* R 60 represents the basal core of the corresponding O antigen. This core, according to our results and those of Osborn (1963), would be composed of a backbone of heptose and phosphate to which are bound side chains with the structure of 60/C1.

In *S. minnesota* R 345, the site of mutation which leads to production of the R I antigen is unknown. It has been suggested that the structure of R I antigens should be simpler than that of R II antigens. This has been confirmed. All the glucosamine-free oligosaccharides found in the R 60 hydrolysates were also found in the R 345 hydrolysates. In addition, the tetrasaccharide 345/C1 was obtained. This resembled the pentasaccharide 60/C1 in that about 50% of the galactose could be released by α -galactosidase. Further, one of the products of enzymic treatment of 345/C1 was isolated and shown to be a trisaccharide composed of glucose and galactose in the ratio 2:1. It is concluded that the R 345 antigen contains side chains analogous to the structure of R 60, namely (II). A probable explanation for the absence of this oligosaccharide in R 60 hydrolysates may arise from the relative stability of the α -N-acetylglucosaminyl-D-glucose . . . linkage to acid hydrolysis. The discovery of an oligosaccharide containing galactose, heptose and phosphorus (345/E4) appears to indicate that some

of the galactose present in R 345 polysaccharide is attached directly to the heptose/phosphate core.

The site of mutation in the R II mutant *S. typhimurium* TV 208 involves TDP-rhamnose synthetase. This is analogous to the mutation in *S. minnesota* R 60 in that one of the specific sugars from the O antigen is not synthesized. The oligosaccharides isolated from partial hydrolysates of these two strains were very similar. This would suggest that the basal structures of these two polysaccharides are very similar, if not identical. The site of mutation in the second R I mutant studied is also unknown. The partial hydrolysates from the lipopolysaccharide of this strain, *S. typhimurium* TV 166, contained melibiose. This was to be expected if the basal structure of this species is the same as that of *S. minnesota* and from the results with the *S. typhimurium* R II mutant. As none of the other oligosaccharides obtained from *S. minnesota* R 345 has been detected in *S. typhimurium* TV 166 hydrolysates the biosynthesis may be blocked at an earlier stage in *S. typhimurium* TV 166. The side chains on the lipopolysaccharide of this strain would then comprise galactosylglucose residues bound to the heptose backbone. However, an α -glucosylgalactose disaccharide (166/C3) was obtained which differed in its properties from that detected in *S. minnesota* hydrolysates (e.g. 60/C6). Whether this was derived from a position corresponding to that yielding 60/C6 is uncertain. It is possible that a difference in one linkage such as this might not be reflected in detectable differences in the corresponding trisaccharide and pentasaccharide, by the methods at our disposal. The identity of 166/C2 remains uncertain. Its resistance to both α - and β -glucosidase might indicate that it is O- α -D-glucopyranose-(1 $\rightarrow$ 4)-D-galactopyranose, a disaccharide reported to be extremely resistant to hydrolysis with α -glucosidase (Robbins & Uchida, 1962). The high yield in which this oligosaccharide was obtained seems to preclude the possibility of S material being the source. However, the origin of 166/C2 and C3 remains unresolved. It is possible

that in some mutants deficiency in some locus might lead to the production of sugar sequences in the lipopolysaccharide additional to and differing from those found in the wild type.

Supporting evidence for a common basal structure in *Salmonella* species was obtained from the small amounts of lipopolysaccharides from three other RII strains examined. Unlike the two RII mutants studied in detail, the site of mutation in *S. inверness* RII and *S. poona* RII and *S. typhimurium* TV 157 was not known. However, the finding of oligosaccharides chromatographically and in some cases also chemically similar to those obtained from the four strains forming the main subject of this study, is further indication of a common basal structure. Of particular significance is the result with *S. inверness*, as an antiserum prepared against this RII mutant has been used throughout in the classification of RII mutants. Further, a glucosyl-galactose disaccharide indistinguishable from 60/C6 was obtained during studies on the oligosaccharides obtained from *Salmonella* O antigens of the serological groups G, N and U (D. A. R. Simmons, O. Lüderitz & O. Westphal, unpublished work). From *S. poona* O antigen, a disaccharide composed of glucose and *N*-acetylglucosamine and resembling 60/C8 was isolated. There thus seem to be good grounds for suggesting that many *Salmonella* species possess a common basal structure (III).

The existence of such a structure in *S. typhimurium* has also been proposed by Osborn *et al.* (1964). These workers studied the incorporation of isotopically labelled sugars from nucleotide diphosphate compounds into the lipopolysaccharide of mutants with known defects. They observed the sequential addition of monosaccharides to a basal structure composed of heptose and phosphate. The sequence detected, glucose, galactose, glucose and glucosamine, is that which would be expected if the formula we have derived is correct. Similar results have also been reported for *E. coli* O 111 (Edstrom & Heath, 1964). It is therefore possible that this common basal structure is found not only in members of the genus *Salmonella* but also in *E. coli* and possibly in related genera.

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