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Biochemical Studies of the Smooth-Rough Mutation in *Salmonella minnesota*

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ABSTRACT

LÜDERITZ, O. (Max-Planck-Institut für Immunbiologie, Frieburg, Germany), H. J. RISSE, H. SCHULTE-HOLTHAUSEN, J. L. STROMINGER, I. W. SUTHERLAND, AND O. WESTPHAL. Biochemical studies of the smooth-rough mutation in *Salmonella minnesota*. J. Bacteriol. **89**:343–354. 1965.—A comparative study of the O antigen from the smooth strain of *Salmonella minnesota* and of the two R antigens derived from two rough forms of *S. minnesota* (strains R 60 and R 345) has been carried out. The O-specific polysaccharide of the smooth form is composed of heptose, galactose, glucose, glucosamine, galactosamine, and ketodeoxyoctanoate (KDO). R 60 polysaccharide contains KDO, heptose, galactose, glucose, and glucosamine, whereas the R 345 polysaccharide contains only KDO, heptose, galactose, and glucose. Serologically, R 345 and R 60 polysaccharides belong to serogroups R I and R II, respectively. Enzymatic studies revealed that the acetylgalactosamine-synthesizing enzyme, uridine diphosphate-*N*-acetylglucosamine-4-epimerase, is present in wild-type and R 345 cells but is absent from R 60 cells. Two distinct polysaccharides were obtained from the R 345 cells: a polysaccharide derived from the R antigen (lipopolysaccharide) containing no galactosamine and exerting R specificity, and a soluble polysaccharide containing galactosamine and exerting O specificity. The structure of O and R antigens is discussed, together with the general significance of the results for the biosynthesis of the O antigens of the genus *Salmonella*.

The O antigens of the genus *Salmonella* have been studied extensively (Davies, 1960; Staub and Westphal, Bull. Soc. Chim. Biol., *in press*). They may be obtained as heteropolysaccharides of high molecular weight bound to lipids or proteins, or to both. Chemical analysis has revealed a close relationship between structure and serological specificity of the polysaccharide component of the O antigens. Different sugars forming long side chains in the highly branched polysaccharides represent the determinant structures of the antigen (Staub and Westphal, *in press*). As well as these "specific sugars," conferring O specificity, the O antigens contain "basal sugars," common to all the O antigens of *Salmonella* species. These basal sugars are: 2-keto-3-deoxy-octonic acid (KDO: Heath and Ghalambor, 1963), heptose, galactose, glucose, and glucosamine.

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Salmonella R mutants are devoid of O specificity (Kauffmann et al., 1961). They contain a lipopolysaccharide which is physicochemically similar to the O-specific lipopolysaccharides. The lipopolysaccharides isolated from *Salmonella* R mutants, obtained by Kauffmann from a number of *Salmonella* O forms of different serotype, have already been analyzed (Lüderitz et al., 1960; Kauffmann et al., 1961). It was shown that R lipopolysaccharides contained only the basal sugars, and were, therefore, qualitatively similar to the lipopolysaccharide of smooth chemotype I. Serologically, these lipopolysaccharides were classified into two R serogroups, designated R I and R II (Beckmann, Lüderitz, and Westphal, 1964). R specificity could be shown to be present in O antigens, especially when they were degraded by mild acid hydrolysis (Lüderitz, Beckmann, and Westphal, 1964). From these and other results, it was postulated that *Salmonella* O antigens might contain a common core polysaccharide, identical with R polysaccharide and composed of the basal sugars (Lüderitz et al., 1960; Kauffmann et al., 1961). It was suggested

that the specific side chains were bound to this core. In R mutants, the synthesis of the specific sugars (in the form of their nucleoside diphospho-derivatives) or their transfer to polysaccharide would be blocked as a result of the mutation, and, hence, the biosynthesis of the O antigen would stop at the level of the R I or R II polysaccharide.

F. Kauffmann (Statens Serum Institute, Copenhagen, Denmark) isolated from *S. minnesota* S form (strain S99) two R mutants, designated R 60 and R 345. These two mutants were serologically disinct. This paper describes the results of chemical, serological, and enzymatic analyses of the S form of *S. minnesota* and of these two R mutants.

MATERIALS AND METHODS

Microorganisms and antisera. Cultures of *S. minnesota* S 99 (O21, O26) and *S. minnesota* R 60 and R 345 were provided by F. Kauffmann. The two R forms had the same flagellar antigens and the same fermentation characteristics as the smooth form. Antisera against these three organisms were also provided by F. Kauffmann.

Mass cultures of the bacteria were grown on agar (Kauffmann et al., 1960), or in liquid medium (Fromme et al., 1958). To obtain small quantities of bacteria for the isolation of enzymes, 100 ml of inoculum were taken from a fully grown overnight culture in Difco antibiotic medium 3, and added to 1 liter of the same medium. The culture was shaken rapidly at 37 C until half-maximal growth was reached (about 90 min). The cells were cooled to 4 C, harvested by centrifugation, and washed once with water (yield, about 3 g of wet sediment).

Preparation of antigens and haptens. Dried bacteria were extracted by a modification of the phenol-water method of Westphal, Lüderitz, and Bister (1952), summarized in Fig. 1. The extraction mixture was heated for 5 min instead of 30 min. The combined aqueous layers were dialyzed, concentrated under reduced pressure, and lyophilized. A 3% solution of the material was centrifuged at 100,000 × *g* for 4 hr in a Spinco model L preparative ultracentrifuge. The clear solution, "fraction L1," was lyophilized. The sediment was washed twice with water and lyophilized to give the "lipopolysaccharide." "Degraded polysaccharide" was obtained from lipopolysaccharide by heating with 1% (v/v) acetic acid at 100 C until the lipid A was precipitated (Davies, Morgan, and Record, 1955). After centrifugation, the clear solution was dialyzed and lyophilized. On other occasions, degraded polysaccharide was prepared according to the method of Freeman (1942) with the following modifications. The first and second acetic acid extracts of the bacteria were kept separate. The concentrated extracts, without alcohol or acetic acid fractionation, were

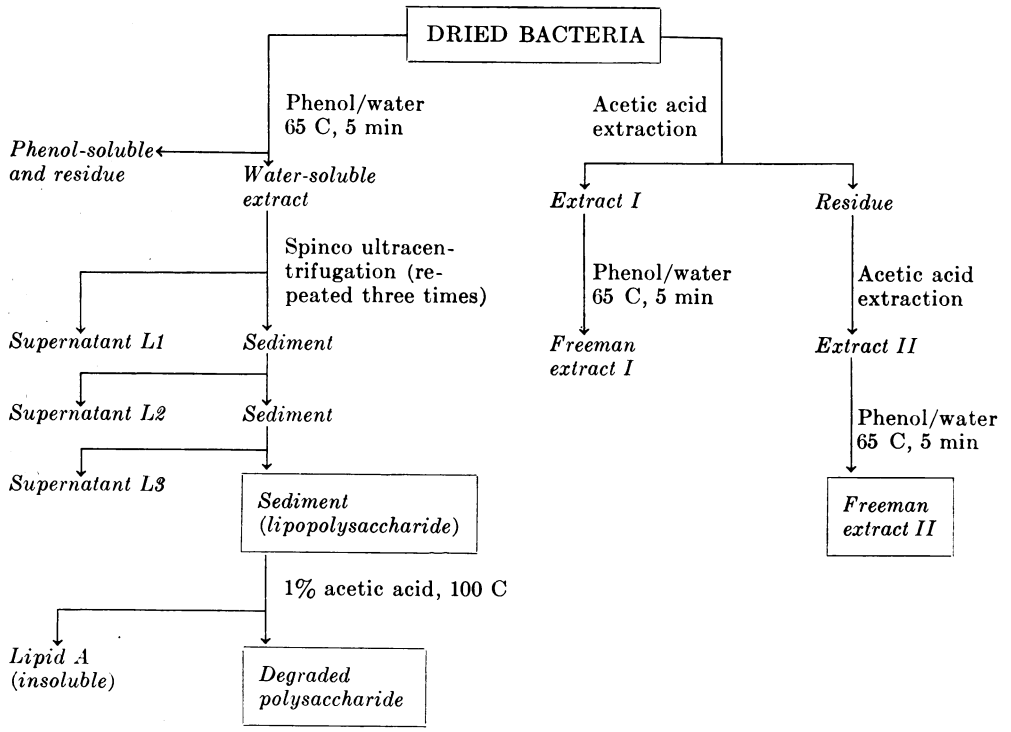


FIG. 1. *Methods used for extraction of bacteria*

treated directly with phenol-water. The aqueous layers were dialyzed, concentrated, and lyophilized. Since the first extract frequently was contaminated with a polyglucose, the second extract was normally used for analysis.

To purify the O-specific polysaccharide of the L1 fraction obtained from R 345 cells, 2 ml of *S. minnesota* O antiserum were added to a solution of 20 mg of R 345 L1 fraction in 2 ml of sodium chloride solution (0.9%, w/v). After allowing the mixture to stand for 1 hr at 37 C, followed by 15 hr at 4 C, the precipitate was removed by centrifugation, washed three times in sodium chloride solution, and suspended in 3 ml of water. The suspension was heated at 100 C for 3 min, and was then extracted with 3 ml of 90% phenol in the usual way. The water-soluble extract was hydrolyzed with 1 N HCl for 4 hr and analyzed by paper chromatography in solvent B.

Analytical methods. Hydrolysis of fractions was performed in sealed tubes. For the liberation of hexoses, 1 N H_2SO_4 at 100 C for 4 hr was used, followed by neutralization with Amberlite IR 410 (HCO_3^- form). Glucose was determined with glucose oxidase reagent or with glucose-6-phosphate dehydrogenase reagent (C. F. Boehringer & Soehne GmbH., Mannheim, Germany). Galactose was determined with galactose dehydrogenase obtained from *Pseudomonas saccharophila* (Wallenfels and Kurz, 1962). Hexosamines were liberated by hydrolysis of the fractions in 4 N HCl at 100 C for 10 hr. The acid was removed under vacuum in the presence of NaOH. Hexosamines were determined by a micromethod (Strominger, Park, and Thompson, 1959). D-Glucosamine was estimated enzymatically (Lüderitz et al., 1964). Heptose was determined on unhydrolyzed material by a modification of the cysteine-H_2SO_4 method (Osborn, 1963). KDO was measured according to Waravdekar and Saslaw (1959), as modified by Heath (*personal communication*), and phosphorus by the method of Lowry et al. (1954).

Sugars were identified by paper chromatography or by thin-layer chromatography on cellulose (cellulose MN 300 g, Macherey a. Nagel, Düren, Germany). The solvents used were: pyridine-butanol-water (4:6:3; solvent A); ethyl acetate-pyridine-acetic acid-water (25:25:5:15; solvent B; Fischer and Dörfel, 1955); and isobutyric acid-1 N NH_4OH (5:3; solvent C).

Sugars and amino sugars were revealed with alkaline silver nitrate and with ninhydrin or Morgan-Elson reagent, respectively.

Serological methods. Precipitation tests in capillary tubes were performed according to the method of Swift, Wilson, and Lancefield (1943). Hemagglutination-inhibition tests were made as described earlier (Beckmann, Lüderitz, and Westphal, 1964). The methods of Wasserman and Levine (1961) were used for complement fixation and complement-fixation inhibition tests.

A quantitative micromethod was used for precipitation. In this procedure, 1 to 100 μg of antigen were mixed with antiserum in a total volume of 300 μliters. After 2 days at 4 C, the precipitate was washed three times with cold saline, and antibody nitrogen was determined with the modified Folin phenol reagent (Lowry et al., 1951). Bovine serum albumin was used as standard (100 μg in 1 ml gave an absorbancy of about 1.4). In precipitation-inhibition tests, the appropriate sugar was incubated with the antiserum for 1 hr at 37 C before the antigen was added.

Preparation of uridine diphospho-N-acetylglucosamine (UDP-GlcNAc)-C^{14} and uridine diphospho-N-acetylgalactosamine (UDP-GalNAc)-C^{14}. UDP-GlcNAc-C^{14}, labeled in the acetyl group (15,000 counts per min per mμmole) was prepared with enzymes obtained from yeast (Glaser and Brown, 1955; Nathenson and Strominger, 1963). This compound was then epimerized to a mixture of UDP-GlcNAc and UDP-GalNAc with a UDP-GlcNAc-epimerase from *Bacillus subtilis* ATCC 9945 (Glaser, 1959). UDP-GlcNAc-C^{14} was then degraded with UDP-GlcNAc-pyrophosphorylase from *Staphylococcus aureus* strain Copenhagen and with phosphomonoesterase (Strominger and Smith, 1959). Finally, UDP-GalNAc-C^{14} (also 15,000 counts per min per μmole) was isolated by paper chromatography in solvent C. After acid hydrolysis and paper chromatography on borate-treated paper (solvent A; Cardini and Leloir, 1957), only GalNAc-C^{14} could be detected by radioautography.

Preparation of enzymatic extracts from S and R cells. Two methods were used. (i) Packed cells (3 g) were suspended in 5 ml of 0.02 M tris(hydroxymethyl)aminomethane (Tris) buffer [pH 7.5, containing 0.002 M ethylenediaminetetraacetic acid (EDTA)]. The suspension, carefully cooled in an ice bath, was disintegrated for 30 sec, four times, in an Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Breisgau, Germany) after the addition of 4.8 g of glass beads (0.88-mm diameter). (ii) Packed cells (3 g) suspended in 10 ml of 0.02 M Tris buffer (pH 7.5) were treated for 20 min in a 10-kc Raytheon sonic oscillator. Results from the two methods were similar. The products were centrifuged at 30,000 × *g* for 10 min. The supernatant fluid was then further centrifuged at 100,000 × *g* for 150 min. The resulting supernatant solution was then used for enzyme studies.

Assays for UDP-GlcNAc-4-epimerase activity. Two methods were used to test strains for the presence of UDP-GlcNAc-4-epimerase. (i) UDP-GlcNAc-C^{14} (3 to 5 mμmoles) was incubated at 37 C in a mixture containing 3.3 μmoles of Tris (pH 8.7), 0.4 μmole of $MgCl_2$, 0.05 μmole of EDTA, 0.02 μmole of nicotinamide adenine dinucleotide, 0.09 μmole of cysteine, and 10 μliters of the enzyme extract in a total volume of 55 μliters. After incubation for 60 min, the samples were heated at 100 C for 3 min and the denatured proteins were removed by centrifugation; 45 μliters of the supernatant fluid were hydrolyzed with 2.7 μliters of 2 N HCl at 100 C for 10 min to liberate the free acetylamino sugars. The hydrolysate was spotted onto borate-impregnated What-

man paper (no. 1) and chromatographed in solvent A. The GlcNAc and GalNAc were thus separated (Cardini and Leloir, 1957). The radioactivity of the dried chromatograms was measured in a Frieseke and Höpfner paper-strip counter, and autoradiograms were prepared. (ii) Nonradioactive UDP-GlcNAc (40 to 50 mμmoles) was incubated for 60 min in the incubation mixture described above, and protein was removed by heat denaturation and centrifugation. After addition of an equal volume of concentrated HCl, hydrolysis was performed for 30 min at 100 C to liberate the free amino sugars in the hydrochloride form. The samples were dried over NaOH and redissolved in about 10 μliters of water. The amino sugars were separated from amino acids and peptides by paper electrophoresis (pyridine-acetic acid-water, 10:4:86) at 3,000 v for 90 min. The amino sugar spots were cut out and eluted with 0.1 N HCl. These solutions were applied to thin-layer chromatoplates and chromatographed in solvent B for 90 min. The plates were dried and rerun for a further 90 min. The amino sugars were detected by staining with ninhydrin. For all serological and chemical tests, Lang-Levy constriction pipettes were used.

RESULTS

Chemical analyses of S 99, R 60, and R 345 antigens. The results of analyses performed on these antigens are summarized in Table 1. The sugar composition was determined both in the lipopolysaccharides obtained by phenol-water extraction and in the degraded polysaccharides prepared by the modified Freeman method in the case of R 60, or by degradation of the lipopolysaccharide from R 345 and S 99. Lipid A contains glucosamine as an integral part of the molecule (Westphal et al., 1958). Analysis of the lipopolysaccharides, therefore, showed the presence of glucosamine in all three antigens. That glucosamine is a constituent of the polysaccharide part of the R 60 antigen, and that it is absent from R 345 polysaccharide, could be demonstrated only by the preparation and analysis of the corresponding degraded polysaccharides. Very small amounts of glucosamine found in the R 345 polysaccharide probably originated from traces of contaminating lipid A.

The approximate molar ratios of the sugars are listed in Table 2. S 99 polysaccharide was composed mainly of three sugars, galactose, glucosamine, and galactosamine, which together represented 80% of the sugar components. The R antigen of strain R 60 contained comparatively small amounts of galactose and glucosamine and was essentially devoid of galactosamine. R 345 antigen contained no hexosamines and small amounts of both hexoses. It is thought that the amino sugars are present in the S 99 and R 60 polysaccharides as their *N*-acetylated derivatives, as partial hydrolysates contained appreciable amounts of the corresponding *N*-acetylated derivatives.

Hydrolysates of various fractions from the two R strains were analyzed for the presence of hexosamines by paper chromatography. Although glucosamine and galactosamine were present in the L1 fractions of S 99 and R 345 cells, both of these sugars were absent from the L1 fraction of R 60 (Table 3). R 345 cells, therefore, contain two different polysaccharides, one being the component of the R lipopolysaccharide, lacking galactosamine and glucosamine, and the other, present in the L1 fraction (or the Freeman polysaccharide), containing both hexosamines (Table 3).

Serological studies. Classification of R 60 and R 345 antigens was achieved with precipitation and hemagglutination-inhibition tests (Table 4). Three sera were used: (i) *S. minnesota* O anti-

TABLE 1. *Analyses of Salmonella minnesota S 99, R 60, and R 345 lipopolysaccharides and polysaccharides**

Fraction	Sugar					
	KDO	Heptose	Gal	Glc	GlcN	GalN
Lipopolysaccharide						
S 99	5	2–4	12–15	3–4	9–10	15–19
R 60	5	7	7–9	6	8	0
R 345	5	12	8–9	4	5	0
Polysaccharide						
S 99	2	3	20	5	10	25
R 60†	2	16	16	17	5.5	0
R 345	2	19	19	9	1	0

* Results given in per cent.

† Phosphorus analyses of the R 60 polysaccharide yielded a value of 2.7% or about two phosphorus residues for two heptose or glucose residues.

serum; (ii) *S. minnesota* R 345 antiserum, obtained by immunization with the R 345 mutant [this serum was previously used as the test serum for R serogroup R I (Beckmann, Lüderitz, and Westphal, Z. Physiol. Chem., *in press*)]; and (iii) *S. inverness* R serum, obtained by immunization with an *S. inverness* R strain. [This was used previously as the test serum of the R serogroup R II (Beckmann, Lüderitz, and Westphal, 1964).]

Capillary precipitation tests showed strong reaction between S 99 lipopolysaccharide and *S. minnesota* O antiserum, R 60 lipopolysaccharide and *S. inverness* R antiserum, and R 345 lipopolysaccharide and *S. minnesota* R 345 antiserum. Consequently, the R 60 mutant of *S. minnesota* belongs to serogroup R II, and the R 345 mutant to serogroup R I. This classification is in agreement with an earlier study using hemagglutination-inhibition tests (Beckmann et al., 1964). The R 60 lipopolysaccharide reacted with the R II antiserum at high dilution under these conditions, but not with the R 345 antiserum (R I serum). Conversely, R 345 lipopolysaccharide did not react with R II antiserum, even at high antigen concentration.

TABLE 2. *Approximate molar ratios of sugars in Salmonella minnesota S 99, R 60, and R 345**

Sugar	S 99	R 60	R 345
Heptose	2	2	2
Gal	8	2	2
Glc	2	2	1
GlcN	5	0.7	0
GalN	12	0	0

* Approximate ratios are given relative to glucose taken as 2 for S 99 and R 60 lipopolysaccharide or 1 for R 345 polysaccharide. These ratios are calculated from the data in Table 1. It would be most desirable to express these ratios relative to heptose, but the method for heptose analysis is not sufficiently reliable for this purpose.

TABLE 3. *Analyses of different fractions from Salmonella minnesota R 60 and R 345 mutants for the presence of hexosamines*

Fraction	*S. minnesota* mutant	
	R 60	R 345
Lipopolysaccharide	GN	GN
Degraded polysaccharide from lipopolysaccharide	GN	—
Supernatant fraction L1	(GN)	GN, GalN
Freeman extract II*	GN	GN, GalN

* The Freeman polysaccharide preparation would contain the degraded polysaccharide and the polysaccharide in supernatant fraction L1.

Precipitation tests with the L1 fractions and the three antisera revealed that S 99 L1, as expected, reacted with *S. minnesota* O antiserum. R 60 L1 fraction did not react appreciably with any of the antisera. R 345 L1 material, however, showed a strong precipitation reaction with the O antiserum (Table 4). The O-specific material from the R 345 L1 fraction was isolated by specific precipitation with O antiserum. Hydrolysis of

TABLE 4. *Precipitation and inhibition of hemagglutination by various polysaccharides*

Antigen	Serum		
	Salmonella minnesota O	*S. inverness* R 347 (RII serum)	*S. minnesota* R 345 (RI serum)
*Precipitation test**			
S 99 lipopolysaccharide	5	—	—
S 99 L1-fraction	10	—	—
R 60 lipopolysaccharide	—	100	—
R 60 L1-fraction	—	—	—
R 345 lipopolysaccharide	—	—	600
R 345 L1-fraction	300	—	—
Hemagglutination inhibition†			
R 60 lipopolysaccharide		0.5	>250
R 345 lipopolysaccharide		>250	1.0

* For the precipitation tests, the data are recorded as the smallest concentrations of antigen (micrograms per milliliter) which resulted in precipitation or hemagglutination. When no precipitation was observed up to 5,000 μg/ml, this result is indicated by —. For inhibition of hemagglutination, the data are recorded as the lowest concentration (μg/ml) which inhibited.

† Hemagglutination inhibition is recorded for the *S. inverness* R 347 and *S. minnesota* R 345 systems.

this polysaccharide, followed by paper chromatography, revealed the presence of the following sugars: galactosamine, glucosamine, galactose, and traces of glucose. It therefore contained the specific sugars of the *S. minnesota* O antigen.

Two precipitation curves were obtained with *S. minnesota* O antigen and the homologous antiserum (Fig. 2). In one case, precipitation was estimated directly by protein determination. In the other case, the complement-fixation test was used, this being about 1,000 times more sensitive. Inhibition tests performed with these two methods indicated that glucose, galactose, and *N*-acetylgalactosamine were noninhibitory. However, *N*-acetylglucosamine and its glycosides showed marked inhibition (Table 5). In the precipitation test, 20 μmoles of α-methyl-*N*-acetylglucosaminide inhibited to an extent of 20%, whereas, in the complement fixation inhibition test, 1.5 μmoles inhibited to 36%. An α-*N*-acetylglucosaminide appears to be a determinant of the *S. minnesota* O 21 antigen.

UDP-GlcNAc-4-epimerase in the S. minnesota strains. The 30,000 × *g* supernatant fluid obtained after sonic treatment or mechanical disintegration of the bacteria contained particle-bound pyrophosphatase and phosphatase activity. These enzymes degraded UDP-GlcNAc-C^{14} to GlcNAc-C^{14}-phosphate and GlcNAc-C^{14}. These reactions could be readily demonstrated by chromatography in solvent C, which separates these degradation products. These interfering activities were much reduced in the presence of fluoride or phosphate, or both, under conditions

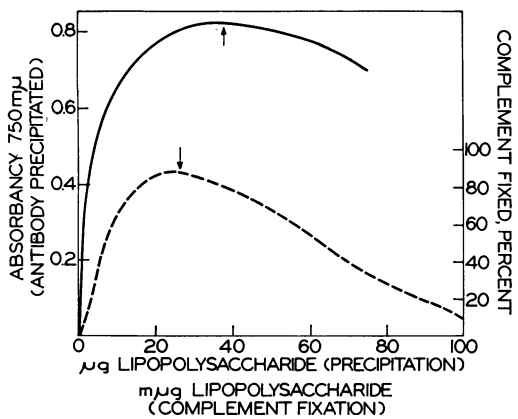


FIG. 2. *Precipitation and complement-fixation curves: Salmonella minnesota O lipopolysaccharide and S. minnesota O serum. Precipitation* (——) *was carried out with 100 μliters of antiserum per tube, and complement fixation* (-----) *with 0.15 μliter of antiserum per tube. The conditions used for inhibition tests are indicated by an arrow.*

TABLE 5. *Inhibition of precipitation and complement fixation in the system Salmonella minnesota O lipopolysaccharide and homologous antiserum by N-acetylglucosamine and its glycosides*

Expt	Amt of sugar per tube	*N*-acetylglucosamine or glycoside			
		Free sugar	α-Methyl-	Mixture of α- and β-benzyl (1:1)	β-Methyl-
	μmoles				
1*	25			31	
	20	15	21		
	10			16	
	5	6	12	10	
	1	0	5	2	
2†	1.5	25	36		18
	0.5	8	10		2
	0.1	0	3		0

* Results of experiment 1 are given in per cent inhibition of precipitation.

† Results of experiment 2 are given in per cent inhibition of complement fixation.

which leave the epimerase activity unaffected. Moreover, the degradation products were virtually absent when the 100,000 × *g* supernatant fluids were used. Such supernatant fluids were therefore used in the following studies.

UDP-GlcNAc-C^{14} was incubated with extracts from the S strain and the two R strains, as described in Materials and Methods. The radioactive nucleotide-linked sugars present after incubation were assayed, by use of a paper-strip counter (method 1 above). The S form and the R 345 enzyme preparations were able to epimerize UDP-GlcNAc, resulting in a mixture of UDP-GlcNAc and UDP-GalNAc. After hydrolysis, these yielded GlcNAc and GalNAc (Fig. 3). The R 60 enzyme preparation, however, had no epimerase activity. In the epimerase-containing strains, 20 to 25% of the original UDP-GlcNAc-C^{14} was converted to UDP-GalNAc-C^{14}. Further experiments indicated that this was the end point of the reaction, and that it was reached after 10 min at 37 C.

That UDP-GalNAc-C^{14} and not GalNAc-C^{14} was the end product of the reaction with R 60 and S extracts was shown by isolation of the UDP-*N*-acetylhexosamine mixture by chromatography in solvent C. Only after acid hydrolysis of this mixture were GlcNAc-C^{14} and GalNAc-C^{14} formed. In the reverse direction, when UDP-GalNAc-C^{14} was used as substrate, UDP-GlcNAc-C^{14} was formed after incubation with the extracts from S 99 and R 345 cells, but not with the extract from R 60 cells.

Analogous results were obtained by chroma-

tography of unlabeled hexosamines. In this case, the incubation mixtures containing unlabeled nucleotides were hydrolyzed with strong acid to yield the free amino sugars. These were chromatographed on thin-layer plates, with glucosamine and galactosamine as standards. In the case of enzyme from S 99 and R 345, both hexosamines were obtained; with enzyme from R 60, no galactosamine could be detected.

Attempts to transfer GalNAc-C^{14} from UDP-GalNAc-C^{14} to polysaccharide. Strain R 60 is an epimeraseless strain, similar to UDP-glucose epimeraseless strains in which galactose-C^{14} can be transferred from UDP-galactose-C^{14} to endogenous polysaccharide (see Discussion). Repeated attempts to demonstrate the same phenomenon under the conditions employed in transfer of galactose-C^{14} with enzyme from strain R 60 and UDP-GalNAc-C^{14} as substrate were unsuccessful. No incorporation of GalNAc-C^{14} could be demonstrated. Moreover, addition of various other nucleotide substrates (UDP-glucose, UDP-galactose, UDP-GlcNAc) or of enzyme prepared from the smooth strain S 99 had no effect.

DISCUSSION

Salmonella O antigens (lipopolysaccharides) are branched polysaccharides of high molecular weight, composed of different monosaccharides in various combinations and linked in different ways. The differences in the linkages and the sugar composition provide the chemical basis of serological specificity. The O antigens may contain up to eight different monosaccharides (chemotypes XIV–XVI). At least four sugars are always present in the polysaccharides of *Salmonella* O antigens, namely, KDO, heptose, galactose, and glucose. A fifth sugar, glucosamine, is also a component of the lipid portion of the lipopolysaccharide (Kauffmann et al., 1960). R antigens, isolated from the different *Salmonella* R forms, derived from various S forms, have been shown to contain the same sugars: KDO, heptose, galactose, glucose, and glucosamine (the basal sugars). Comparative studies on polysaccharides derived from *Salmonella* S forms and from the corresponding R mutants led to the hypothesis that the O antigens contain a basal polysaccharide structure composed of the basal sugars, to which the O-specific side chains are attached. The R mutants have in some way lost the capacity to synthesize the O-specific side chains or to transfer them to the basal polysaccharide (Lüderitz et al., 1960).

The O antigen of *S. minnesota* (O 21, O 26) isolated by the phenol-water procedure contains galactosamine, glucosamine, heptose, glucose, and galactose (Tables 1, 2, and 6). After removal of the lipid by acid hydrolysis, the remaining polysaccharide still contains a considerable amount of glucosamine. Moreover, both specific precipitation by homologous antiserum and complement fixation are inhibited by *N*-acetyl-D-glucosamine and not by other sugars. Therefore, *N*-acetyl-D-glucosamine is presumed to represent one of the determinant end sugars in the O antigen. Other data suggest that this determinant may be in the α configuration.

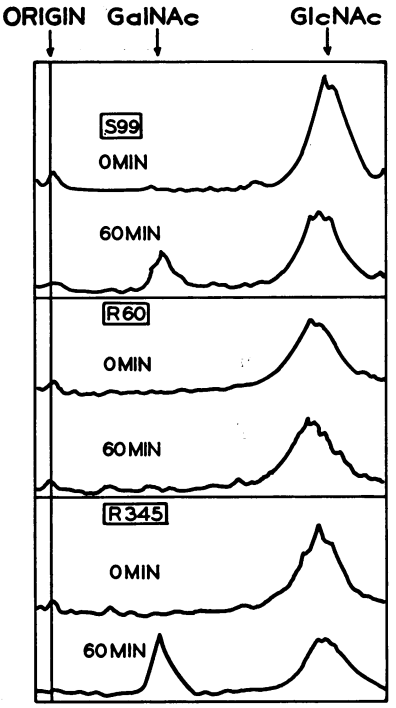


FIG. 3. *UDP-GlcNAc epimerase in Salmonella minnesota S 99, R 60, and R 345. GlcNAc-C^{14} and GalNAc-C^{14} were separated after hydrolysis of reaction mixtures obtained by incubation of UDP-GlcNAc-C^{14} for 0 and 60 min with corresponding enzyme extracts. Chromatography was carried out on borate-treated paper in solvent A. Paper strips were analyzed in a paper-strip counter.*

Strains R 60 and R 345, two R strains from *S. minnesota* S 99, are serologically distinct. R 60 cells were agglutinated by an antiserum to an R strain of *S. typhimurium*. The antibodies responsible were not absorbed by R 345 cells (Kauffmann, *personal communication*). Chemical analysis of the lipopolysaccharides isolated from the R 60 and R 345 mutants showed that both contain only the basal sugars: KDO, heptose, galactose, glucose, and glucosamine (Tables 1, 2, and 6). As in the S lipopolysaccharides, the glucosamine is a constituent of the lipid com-

TABLE 6. *Summary of results obtained from comparative analysis of Salmonella minnesota strains S 99, R 60, and R 345*

Determination	*S. minnesota* strains		
	S 99	R 60	R 345
Lipopolysaccharides, sugar composition	KDO, heptose Gal, Glc, GlcN (basal sugars) + GalN	Basal sugars	Basal sugars
Polysaccharides derived from lipopolysaccharides, sugar composition	KDO, heptose Gal, Glu, GlcN + GalN	KDO, heptose Gal, Glu, GlcN	KDO, heptose Gal, Glu
Serological specificity of lipopolysaccharides	O 21, O 26	R II	R I
Occurrence of UDP-GlcNAc epimerase	Present	Absent	Present
Occurrence of O-specific polysaccharide in L1-fraction	(Present)	Absent	Present
Defect of the mutant in the synthesis of		O-determinant side chains	Basal structure
Structure of antigens	Basal structure + specific side chains	Complete basal structure	Incomplete basal structure

ponent of the lipopolysaccharides. Marked differences between the two antigens were observed when the corresponding polysaccharides were analyzed. Glucosamine was a constituent of the R 60 polysaccharide, but not of the R 345 polysaccharide.

From the molar ratios of the sugar constituents of the polysaccharides of S 99, R 60, and R 345, it appears that mutation of strain S 99 to R 60 involves a reduction in the proportions of galactose and glucosamine and a total elimination of galactosamine. Mutation of S 99 to R 345 results in a reduction in glucose as well as galactose, and total absence of both glucosamine and galactosamine. The polysaccharide of strain R 345, therefore, appears to be more degraded than that of R 60.

Serological studies, using precipitation and hemagglutination-inhibition methods, revealed that R 60 and R 345 lipopolysaccharides are also serologically distinct and can be allocated to two different R serogroups (Tables 4 and 6). According to the classification of the lipopolysaccharides derived from various *Salmonella* R mutants (Beckmann, Subbaiah, and Stocker, 1964), R 60 lipopolysaccharide belongs to R serogroup R II, and R 345 to serogroup R I.

The results of biochemical analysis show that both the S form and the R 345 mutant contain UDP-GlcNAc-epimerase, whereas this enzyme is absent from R 60 cells. Thus, R 60 cells are unable to synthesize *N*-acetylgalactosamine, and this sugar is absent from the lipopolysaccharide obtained from the cells. [Since UDP-GlcNAc is used in R 60 cells for UDP-GalNAc synthesis, and since only a small amount of GlcNAc is found in the R antigen, accumulation of UDP-GlcNAc within the cells might be expected. Investigation of accumulated nucleotides in S 99 and R 60 cells revealed that, in fact, there was accumulation of UDP-GlcNAc in R 60 cells. UDP-acetylmuramic acid-peptide derivatives also accumulated. These must originate from UDP-GlcNAc, which, because of its high concentration within the cells, may be diverted to these compounds in greater than normal amount (Lüderitz and Strominger, *unpublished data*).] Inability to synthesize galactosamine appears to result also in reduction of the galactose and glucosamine incorporated into the R 60 polysaccharide.

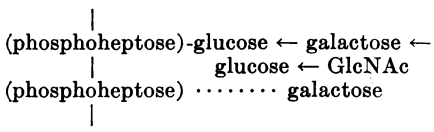
R 345 cells contain UDP-GlcNAc epimerase and are therefore able to synthesize galactosamine, but this sugar is not incorporated into the lipopolysaccharide. The chemical and serological analysis of other cell fractions revealed the presence in R 345 cells of a galactosamine-containing polysaccharide. This polysaccharide is found in the supernatant fluid after ultracentrifugation of the lipopolysaccharide extracts, to-

gether with nucleic acid and other cell components, and is not bound to lipid. It exerts O specificity, without conferring O antigenicity on the R 345 cells. This O-specific polysaccharide was purified by specific precipitation with O 21 antiserum. After isolation of the purified polysaccharide, hydrolysates were shown to contain galactosamine, glucosamine, galactose, and only trace amounts of glucose.

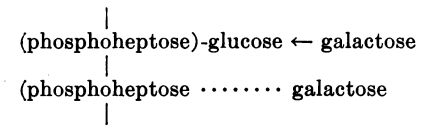
These findings are in accordance with the fact that the polysaccharide extracted from R 60 cells by the Freeman method is identical, with respect to sugar composition, to that obtained by mild hydrolysis of the lipopolysaccharide of R 60. Neither contains galactosamine. The polysaccharide obtained by the latter method from R 345 lipopolysaccharide is also a typical R polysaccharide, containing no galactosamine. The product of Freeman extraction of R 345 cells, however, is a mixture containing at least two polysaccharides: the R-specific and the O-specific polysaccharide, the latter containing galactosamine.

From partial hydrolysates of *S. minnesota* S-lipopolysaccharide, oligosaccharides containing galactose, glucosamine, and galactosamine have been isolated (*unpublished data*), and it is assumed that these oligosaccharides are components of the O-specific side chains. It is also believed that the basal sugars present in the O antigen form the core polysaccharide. Thus, the *S. minnesota* O antigen would be composed of a core structure to which the specific side chains are attached. If this hypothesis is valid, a defect in the synthesis of the specific side chains would lead to a mutant, the antigen of which represents the basal core polysaccharide. This is presumably the case in the R 60 mutant, which has a defect in the synthesis of *N*-acetylgalactosamine, resulting in the absence of the specific side chain (Table 2). Mutation of strain S 99 to R 345 obviously leads to an antigen whose structure is relatively simpler than that of R 60. The defect in this mutant is unknown. Absence of some transferase responsible for the transfer of a sugar in the core polysaccharide is a likely probability. Unless this basal sugar, which may be glucose, and a subsequent one, which may be *N*-acetylglucosamine, are incorporated to complete the basal structure (i.e., the R 60 polysaccharide), the O-specific sugars cannot be transferred. These specific sugars are, however, present in the R 345 mutant and are obviously linked together, since they are found in the cell in the form of material with O specificity.

Very little is known of the structure of the S 99, R 60, and R 345 antigens. Preliminary studies in our laboratory have indicated the presence of four oligosaccharides in partial hydrolysates of R 60 lipopolysaccharide: GlcNAc → glucose, glucose → galactose, GlcNAc → glucose → galactose and GlcNAc → glucose → galactose → glucose. These results, together with analyses of the mutant polysaccharides, lead to the following proposed structure for the basal core polysaccharide present in the R 60 antigen:



The analysis of the R 345 polysaccharide suggests the following structure for this more degraded polysaccharide:



No information is available regarding the attachment of the second galactose residue (indicated by ····).

The results described in this paper are in agreement with earlier findings on R antigens isolated from different *Salmonella* S forms (Kauffmann et al., 1961). It was shown that in the two serological groups into which R strains could be divided, the glucose-galactose ratio in R I lipopolysaccharides was 1:2 (Lüderitz and Westphal, *in press*), whereas in the R II antigens it was approximately 1:1. A number of R mutants of *S. typhimurium* have also been shown to belong to the two serogroups R I and R II (Beckmann et al., 1964). Furthermore, it was shown that the R II mutants were blocked in the synthesis of the O-specific side chain. Some mutants were unable to synthesize rhamnose, and others were presumably unable to transfer rhamnose to polysaccharide (Nikaido et al., 1964). The presence of O-specific material in the supernatant fluids from ultracentrifugation was first observed in the R I mutants of *S. typhimurium* (Beckmann et al., 1964). Preliminary results indicate that partial hydrolysates of R II antigens, other than those of *S. minnesota* R 60, contain oligosaccharides which behave in paper chromatography in the same manner as those obtained from that organism. These studies, using mutants of *S. poona*, *S. inverness*, and *S. typhimurium*, suggest a common structure for these R II antigens. Using another *S. typhimurium* R form, Osborn (*personal communication*) obtained oligosaccharides, probably identical to those indicated above. Thus, it is believed that the R I and R II polysaccharides

represent common intermediate steps in the biosynthesis of *Salmonella* O lipopolysaccharides. The large number of different *Salmonella* O serotypes would, therefore, differ only in the specific side chains attached to a common basal structure.

Mutants other than R I and R II should occur. It should be possible to obtain a double mutant from *S. minnesota* R II. This would be phenotypically a R I mutant but would lack the O-specific polysaccharide. Subbaiah and Stocker (1964) obtained a mutant of this type from *S. typhimurium* by recombination of R I and R II mutants. R mutants distinct from R I and R II, containing different proportions of galactose and glucose, would also be expected to occur. There are indications that such mutants may be included among eight *S. minnesota* R forms isolated by Schlosshardt (1960, 1964).

Other mutations which block the synthesis of the core polysaccharide have been described. The so-called "M" mutant discovered by Murase (1932) and subsequently studied by Nikaido (1961, 1962) has a galactose defect because of the absence of UDP-glucose-4-epimerase. The polysaccharide from this species is composed solely of phosphoheptose and glucose units (Osborn, 1963). Other mutants blocked in UDP-glucose synthesis because of lack of UDP-glucose pyrophosphorylase have been obtained from *Escherichia coli* (Fukasawa, Jokura, and Kurahashi, 1962; Sundararajan, Rapin, and Kalckar, 1962), because of lack of phosphoglucose isomerase from *S. typhimurium* (Fraenkel, Osborn, and Horecker, 1963). They contain phosphoheptose as the only constituent of the R antigen.

Nikaido (1961, 1962) and Osborn et al. (1962) have shown that enzyme preparations from UDP-glucose-epimeraseless mutants readily transfer galactose-C^{14} from UDP-galactose-C^{14} to lipopolysaccharide. This result was anticipated, since the polysaccharide core of this mutant should be synthesized up to the point where galactose is normally added. The subsequent sequential addition of glucose from UDP-glucose and of GlcNAc from UDP-GlcNAc has also been demonstrated (Osborn and D'Ari, 1964; Edstrom and Heath, 1964). The incorporation of each of these sugars is specifically dependent on the presence of the preceding sugar.

Similarly, it might have been expected that the UDP-GlcNAc epimeraseless mutant (R 60) would be primed for the transfer of GalNAc-C^{14} from UDP-GalNAc-C^{14}. Although the failure in the present work to obtain this transfer might have a simple technical explanation, it might also imply that the O-specific side chain is synthesized by mechanisms somewhat different from those of the polysaccharide core, e.g., by some form of simultaneous assembly or by synthesis of and transfer from nucleoside diphosphooligosaccharides (Zeleznick et al., 1964; Nikaido and Nikaido, 1964). These possibilities would also explain the simultaneous deletion of several sugars from the polysaccharide in the mutation from S to R form; and, indeed, the presence of the O-specific L1 polysaccharide, unattached to the lipopolysaccharide, in the R I mutants, lends support to this concept.

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