

Lipopolysaccharides of *Salmonella* T Mutants

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The composition of lipopolysaccharides derived from various *Salmonella* T forms was studied. All T1-form lipopolysaccharides examined contained 14 to 22% each of both D-galactose and pentose in addition to 4 to 9% each of ketodeoxyoctonic acid, heptose, D-glucosamine, and D-glucose. The pentose was identified as D-ribose. The T2-form lipopolysaccharide examined did not contain a significant amount of pentose, nor more than the usual amounts of D-galactose. Periodate oxidation of T1 (lipo) polysaccharides followed by NaBH₄ reduction revealed that ribose was almost quantitatively protected, galactose was destroyed, and threitol and mannose were newly formed. The latter two products probably originated from 4-linked galactose and heptose, respectively. Ribose and galactose were found in specific precipitates of T1 lipopolysaccharide with anti-T1 antiserum but were not found in specific precipitates of alkali-treated T1 lipopolysaccharide and of Freeman degraded polysaccharide with anti-T1 serum. Ribose and galactose are present in these degraded preparations in the form of nondialyzable polymers. The T1-form mutant lipopolysaccharides lacked the O-specific sugars constituting the side-chains in the wild-type antigens. They did not produce the soluble O-specific haptenic polysaccharide known to be accumulated in RI strains. With these properties, T1 lipopolysaccharides resemble RII lipopolysaccharides. Like RII degraded polysaccharides, T1-degraded polysaccharides also contained glucosamine. Furthermore, strong cross-reactions were found to exist between T1 and RII lipopolysaccharides in both hemagglutination inhibition assays and in precipitation tests. It is proposed that T1 lipopolysaccharides represent RII lipopolysaccharides to which polymers consisting of ribose and galactose are attached.

Two types of *Salmonella* mutants have been described which are intermediate between S and R forms. These are the T forms, first described in 1956 by Kauffmann (15), and the semirough (SR) forms studied by Naide, Nikaido, Makela, and Stocker (30). The SR forms are known to produce lipopolysaccharides which contain the basal core sugars and, in addition, small amounts of those sugars which are specific for the O antigen of the parent strain; hence the name semirough (SR). Members of the group of T mutants were first found in natural sources at different places in Europe and gained attention because they could not be typed by the *Salmonella* O antisera then available (15). These morphologically smooth strains were studied at the Inter-

national *Salmonella* Center in Copenhagen by Kauffmann (15), who recognized them by their flagellar antigens and their fermentation properties as having originated from *S. paratyphi* B and *S. typhimurium* strains. The serological O specificity of these strains, however, was replaced by a new specificity, which was identical in both, but distinct from known O and R specificities. Although the new antigen exhibited general properties common to O antigens, Kauffmann decided to call it T1 antigen, "as this antigen is characteristic for a new form" (15). Kauffmann recognized that T forms may be unstable and frequently undergo mutation to R forms; hence the designation as transient (T) forms.

Later, T1 forms from other *Salmonella* serotypes, i.e., groups B, E, G, and others (17, 36), were found. A second T form, T2, was recognized, but until now it was found only as a mutant from *S. bareilly* (16). Recently, Schlosshardt has succeeded in isolating T forms from *Salmonella* O forms in the laboratory (36, 37), making it feasible to study these mutants in relation to the parent strains. This paper describes our first results on

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the chemistry and immunochemistry of lipopolysaccharide preparations from several T-form organisms.

MATERIALS AND METHODS

Microorganisms. *S. typhimurium* T1, *S. paratyphi* B T1, and *S. bareilly* T2 were grown by F. Kauffmann, Statens Seruminstitut, Copenhagen, Denmark (26); *S. friedenaui* T1 and *S. dessau* T1 were grown by J. Schlosshardt, Zentrallaboratorium f. Bakterielle Darminfektionen, Potsdam, Germany; and *S. friedenaui* was also grown in our laboratories. The bacteria were grown on agar (20, 34) and checked for purity by agglutination reactions and morphology. A strain of *Klebsiella pneumoniae* which was used for the preparation of ribokinase was obtained from the Hygiene Institute of the University, Freiburg, Germany.

Isolation of lipopolysaccharides. These were prepared by the phenol water method as described previously (44). Degraded polysaccharides were obtained from lipopolysaccharide (27) or from bacteria according to the method of Freeman (12, see also 39).

Antisera. Antisera against *S. invernensis* RII mutant and *S. minnesota* RI mutant were provided by F. Kauffmann (3). The antisera against *S. minnesota* R60 and *S. friedenaui* T1 were prepared in white rabbits by giving three intravenous injections in the first week (0.1, 0.2, and 0.4 ml) followed by three injections in the second week (0.4 ml each). Six days after the last injection, the animals were bled. The antisera were stored at -20°C without preservative. Bacteria grown on meat extract agar in Roux bottles were used as antigens for immunization. They were suspended in saline, heated for 2 hr at 100°C , and washed three times with saline. The density of the suspension was brought to 4×10^{10} cells per ml, and it was stored at 4°C .

Serological methods. Hemagglutination inhibition assays (3) and quantitative precipitation tests (27) were performed as previously described. For analysis, precipitates, after three washings, were dissolved in 800 μl of the Folin reagent (containing NaOH, Na_2CO_3 , sodium tartrate, and copper) (24). A 100- μl amount of the solution was used for ribose determination according to Brown (6). To the rest of the solution, phenol reagent was added for the determination of protein and the extinction was read at 750 $\mu\mu$ (24). Bovine serum albumin was used as reference. Occasionally, separate tubes were set up and the total precipitate was analyzed for galactose by the method of Dische (8).

Analytical methods. Samples (10 mg/ml) were hydrolyzed at 100°C in sealed glass tubes. Hexoses were determined on samples after 4-hr hydrolysis in 1 N H_2SO_4 followed by addition of three volumes of water and neutralization with Amberlite IRA-410- HCO_3^- resin. Glucose and galactose were determined with glucose oxidase and galactose oxidase reagents coupled with peroxidase and dianisidine (22) or *o*-cresol (10), respectively. Hexosamines were measured on samples dried over NaOH under vacuum after 10-hr hydrolysis in 4 N HCl, using the micro method

of Strominger, Park, and Thompson (40). D-Glucosamine was also assayed enzymatically (28). Heptose was estimated on nonhydrolyzed material by the cysteine- H_2SO_4 method of Dische as modified by Osborn (31). Ribose was estimated by reaction with orcinol according to Brown (6), and uronic acid was detected by the carbazole method of Dische according to Ashwell (1). The procedure of Waravdekar and Saslaw (43) as modified by Heath (*personal communication*) was used to measure 2-keto-3-deoxyoctonate (KDO), and phosphorus was measured by the procedure of Lowry et al. (23). Nucleic acid was estimated from 260 $\mu\mu$ absorption, by using a yeast ribonucleic acid standard.

Sugars were identified by paper chromatography (Whatman no. 1 and 4 paper) or by thin-layer chromatography (cellulose MN 300 g, Macherrey and Nagel, Düren, Germany) using solvent systems (i) *n*-butanol-pyridine-water (6:4:3, v/v); (ii) *n*-butanol-glacial acetic acid-water (5:1:2, v/v); and (iii) the ethyl acetate-pyridine-acetic acid-water (5:5:1:3, v/v) system of Fischer and Dorfel (9).

Sugars were stained with silver nitrate, *p*-anisidine-HCl, or aniline phthalate, and amino sugars and amino acids were also detected with ninhydrin. High-voltage electrophoresis was performed as described by Kickhofen and Westphal (21). Buffer A was pyridine-acetic acid-water (100:40:860), pH 5.3; and buffer B was hydrated sodium molybdate (25 g) in water (1,200 ml) adjusted to pH 5.1 with sulfuric acid (5).

Periodate oxidation and NaBH_4 reduction. Fifteen mg of (lipo) polysaccharide in 5.5 ml of 0.0165 M NaIO_4 was incubated at room temperature in the dark. Periodate consumption was followed by measuring the absorption of samples (6.7 μl of the mixture in 1 ml of water) at 225 $\mu\mu$ (33). After 24 hr no further uptake of periodate was observed and the excess of periodate was destroyed by the addition of 200 μl of glycol. The samples were left for 30 min at 22°C , and 7 ml of 0.1 M NaBH_4 was added to reduce the aldehyde groups formed. After 2 hr, 2 ml of acetone was added to destroy the excess NaBH_4 , and the mixture was dialyzed for 3 days. The oxidized and reduced polysaccharide was lyophilized.

Isolation of ribokinase and test with ribose. Ribokinase was prepared according to the method of Horecker (14) with the following modifications. *K. pneumoniae* (1.5 g, wet weight) grown on synthetic medium (31) containing D-ribose, was suspended in 15 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris) chloride, pH 7.5. The mixture was treated four times for 30 sec in a homogenizer (Ultraturax) in the presence of glass beads (9 g, 0.08 mm diameter). After each operation the mixture was cooled 30 sec in an ice bath. The homogenate was centrifuged for 20 min at $18,000 \times g$. To eliminate nucleic acids, the supernatant solution was treated successively with a solution of protamine sulfate in water (1%, 0 C; about 20 ml); the precipitate was centrifuged after each addition. Finally, the extract was heated at 60°C for 5 min at pH 5.8, centrifuged, and neutralized as described (14). The extract was stored at -20°C in small portions. Phosphorylation of D-ribose was assayed (14) by incubating 0.25 μmole of sugar, 1.25

μ moles of $MgCl_2$, 2.5 μ moles of adenosine triphosphate, 6.25 μ moles of Tris, pH 7.5, and 100 μ liters of enzyme extract in a total volume of 300 μ liters. After 60 min at 25 C, 40 μ liters of 0.5 M barium acetate and 1 ml of ethyl alcohol were added. Centrifugation of the mixture and washing of the precipitate (two times with 100 μ liters of ethyl alcohol) resulted in a sediment containing ribose-5-phosphate, and a supernatant liquid with unchanged sugars. Portions of the supernatant liquid were concentrated to dryness in a desiccator in vacuo and assayed for pentoses by the orcinol method of Brown (6). The precipitate was treated with two successive portions (100 μ liters) of water, and the extracts were checked for the presence of ribose-phosphate by paper electrophoresis (pH 5.3).

Isolation of ribose from lipopolysaccharides. Lipopolysaccharide hydrolysates (1 N H_2SO_4 , 100 C, 4 hr) were neutralized and subjected to preparative paper chromatography (solvent A, Whatman no. 4 paper). The area containing ribose was eluted, and the eluate was checked by chromatography in solvents A and B for purity of the ribose before assaying samples in the orcinol test and with ribokinase.

Preparation of L-ribose. L-Ribose was obtained by chemical epimerization of L-arabinose (300 mg in 3 ml of anhydrous pyridine at 135 C for 5 hr). The mixture was separated preparatively on paper (solvent A). Staining of guide strips with $Ag^+/NaOH$ revealed the presence of three spots: unchanged arabinose, one spot which migrated like ribose, and one spot ($R_{rib} = 1.06$) which presumably was ribulose. The latter substance did not react with aniline phthalate, but the other two gave the pink color of pentoses. The area migrating the same as ribose ($R = 1$) was eluted, and the identity of the eluted material with ribose was proved by paper chromatography in solvents A and B and by the orcinol assay of Brown (6).

RESULTS

Identification of the pentose from T1 lipopolysaccharide as D-ribose. The occurrence of a pentose as a main constituent in the lipopolysaccharides of *Salmonella* T1 forms has been previously reported (18, 25). The identity of the pentose as ribose was based on paper chromatography. The following experiments were undertaken to determine the configuration of the ribose. Ribokinase, which specifically catalyzes the conversion of D-ribose to D-ribose-5-phosphate (14), was used for this purpose.

Ribose was isolated from a hydrolysate of *S. friedenaui* T1 lipopolysaccharide by paper chromatography, and the amount of ribose eluted from paper was estimated by the orcinol reaction. Samples of standard D-ribose and L-ribose were assayed together with the isolated ribose in the ribokinase test. In the first experiment, ribose-5-phosphate, formed by the action of ribokinase, was precipitated from the incubation mixture by the addition of barium acetate and ethyl alcohol. The supernatant fluid was then tested for un-

changed ribose by the orcinol method (6). As can be seen from Table 1, the amounts of D-ribose and ribose from T1 lipopolysaccharide decreased with time of incubation. After 30 min no ribose could be detected. In contrast, L-ribose was quantitatively recovered in the reaction mixture after 60 min. In another experiment, ribose from T1 was again incubated with adenosine triphosphate and ribokinase. This time, the precipitate formed after addition of barium acetate and ethyl alcohol to the mixture was analyzed by paper electrophoresis at pH 5.3 (buffer A). Simultaneously, several test sugars were treated in the same way with ribokinase. Figure 1 shows a scheme of the electropherogram after staining with $Ag^+/NaOH$. Spots migrating like ribose-5-phosphate formed only from D-ribose and the ribose of T1 lipopolysaccharide. Weak spots of unchanged ma-

TABLE 1. Reaction of ribose from T1 lipopolysaccharide with ribokinase

Time of incubation (min)	Recovery of unchanged ribose ^a (% of initial amount)		
	Ribose from T1	D-Ribose	L-Ribose
0.5	75	65	ND ^b
2	23	40	ND
5	4	7	ND
30	0	0	ND
60	0	0	90

^a Determined by the orcinol reaction in the supernatant fluid of the incubation mixtures after precipitation of ribosephosphate with ethyl alcohol in the presence of barium acetate.

^b Not done.

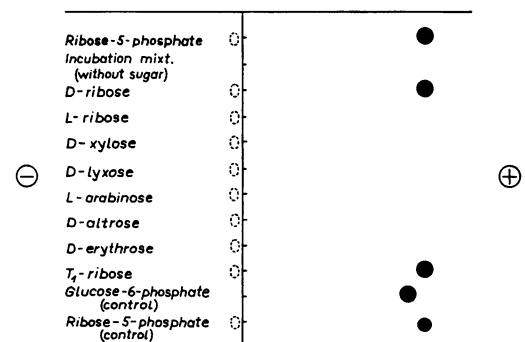


FIG. 1. Paper electropherogram (schematic) demonstrating the conversion of standard D-ribose and ribose isolated from T1 lipopolysaccharide to ribose-5-phosphate by ribokinase in the presence of adenosine triphosphate. The other sugars tested are not converted to sugar phosphates.

terial at neutral position were found with all the other sugars after incubation with ribokinase; these included L-ribose, D-xylose, D-lyxose, L-arabinose, D-altrose, and D-erythrose. This demonstrates the specificity of the enzyme preparation as used, and shows that ribose from T1 lipopolysaccharide has the D configuration.

It has been reported previously (18, 20) that ribose is also a constituent of lipopolysaccharides of *Salmonella* species of group M (antigens 28₁, 28₂), of *S. utrecht* (group 52), and of *S. artis* (group 56). Ribose isolated from *S. tel aviv* and *S. utrecht* lipopolysaccharides and identified chromatographically was treated with adenosine triphosphate and ribokinase. In both hydrolysates, the pentoses were converted to ribose-5-phosphate, showing that in these S-form lipopolysaccharides ribose also occurs in the D configuration.

Specificity of pentose estimation by the orcinol procedure of Brown (6). Because the orcinol reaction for the determination of ribose in T1 (lipo) polysaccharides was used throughout these studies, this method was checked for specificity and possible interference by sugars which are also constituents of T1 lipopolysaccharides.

Shown in Fig. 2 are the absorption curves of T1 lipopolysaccharide, ribose, glucose, galactose, heptose (D-glycero-L-manno-heptose), and KDO (2-keto-3-deoxyoctonate) after heating with the orcinol reagent of Brown. Also included was a hexuronic acid. As expected, pentoses and uronic acids gave an absorption maximum similar to that of ribose.

As shown previously by Brown (6), pentose, in the absence of uronic acid, is the major component measured by the 650 m μ - 550 m μ difference values (which yielded a linear relationship between absorbancy and concentration). Under these conditions, only uronic acids and KDO interfere. However, their interference in the use

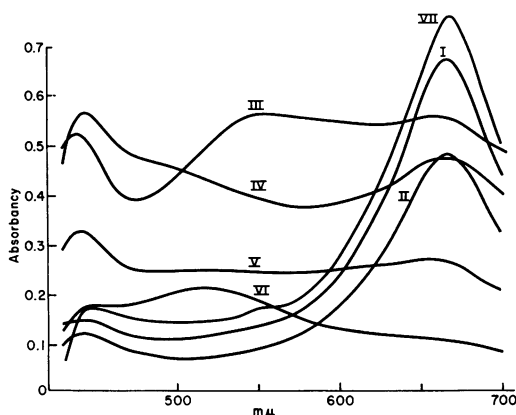


FIG. 2. Spectra obtained with various carbohydrates by the orcinol procedure of Brown (6). I: Ribose, 0.028 μ mole (almost identical spectra were obtained with lyxose, xylose, and arabinose). II: Galacturonic acid, 0.028 μ mole. III: D-Glycero-L-mannoheptose, 0.098 μ mole. IV: Pentaacetyl-2-keto-3-deoxyoctonic acid methylester, 0.12 μ mole (a similar curve was obtained with the free acid). V: Galactose, 0.085 μ mole. VI: Glucose, 0.098 μ mole. VII: T1 lipopolysaccharide of *S. friedenau* (24 μ g).

of this assay of pentose in T1 lipopolysaccharides was ruled out as follows. First, the absence of uronic acids in the T1 lipopolysaccharides was shown independently by tests with the carbazole method (1) and by paper electrophoresis in buffer A. Second, KDO, as calculated from the data shown in Fig. 2, yielded only 3% the color of ribose. Since KDO comprises less than 10% of the T1 lipopolysaccharides (as measured independently by the thiobarbituric acid assay), the contribution by KDO to the orcinol-determined pentose values may be considered quite negligible.

Sugar composition of T1 lipopolysaccharides. Table 2 shows the results of sugar analysis performed with the lipopolysaccharide derived from

TABLE 2. Sugar composition of *Salmonella* group G 13, 22 lipopolysaccharides

I lipopolysaccharide	Sugar in lipopolysaccharide (%)							
	KDO	Heptose	D-Glucosamine ^a	D-Glucose	D-Galactose	Galactosamine	L-Fucose	D-Ribose
<i>S. friedenau</i> S form ^b (wild-type).....	5	3.1	8.1	5.9	11.6	27.7	8.7	1
<i>S. friedenau</i> T1 form.....	3.6	5.3	6.3	4.4	18.7	0	0	22.9
<i>S. poona</i> RII form ^c	4	8.4	9.3	4.9	5.5	0	0	3.0

^a Sum of glucosamine present in lipid A and polysaccharide.

^b Data from Simmons et al. (38).

^c Data from Sutherland et al. (41). *S. friedenau* and *S. poona* differ serologically only in flagellar antigens: d; 1, 6 and z; 1, 6 respectively (17).

S. friedenau T1 and, for comparison, the sugar compositions of the lipopolysaccharides of the corresponding wild-type, *S. friedenau* (38), and of an RII mutant (41) derived from *S. poona* (whose O antigen is closely related serologically and chemically to that of *S. friedenau*). From both mutant lipopolysaccharides, those sugars, namely galactosamine and fucose, which are specific for the parent O antigens are absent. With the exception of ribose and galactose, which occur in high concentration in the T1 lipopolysaccharide, the composition of the T1 lipopolysaccharide is broadly similar to that of *S. poona* RII.

Comparative data obtained from examination of T mutants derived from other *Salmonella* species are shown in Table 3, and it is apparent that all T1 lipopolysaccharides examined contained 14 to 22% each of ribose and galactose. In contrast, the lipopolysaccharide obtained from *S. bareilly* T2 did not contain ribose and no more than usual amounts of galactose.

Since the hexoses were determined with enzymes specific for the D configuration of the sugars, it can be concluded that D-glucose, D-galactose, D-glucosamine, and D-ribose (see above) are the constituents of these lipopolysaccharides. Heptose probably has the structure of L-glycero-D-mannoheptose which is common to all *Salmonella* lipopolysaccharides examined so far (29). Paper and thin-layer chromatography

of hydrolysates of the different lipopolysaccharides did not reveal the presence of sugars other than those assayed in Tables 2 and 3. The lipopolysaccharides show no ultraviolet absorption peak at 260 m μ , indicating the absence of nucleic acids in these preparations.

Degraded polysaccharides derived from T1 lipopolysaccharide. T1 lipopolysaccharide from *S. friedenau* (834 mg) was heated at 100 C for 2.5 hr in 1% acetic acid (84 ml) in a glass-stoppered tube. Lipid A was centrifuged off, washed with 1% acetic acid, and dried (265 mg or 32%). The combined supernatant solution was concentrated in vacuo to 15 ml, and four volumes of absolute ethyl alcohol were added. The (80% ethyl alcohol) precipitate was centrifuged off, washed with 80% ethyl alcohol, acetone, and ether. The yield was 436 mg or 52%. The 80% ethyl alcohol fraction was refractionated with about 80% recovery before being subjected to sugar analyses.

As shown in Table 4 the degraded polysaccharide contained heptose, glucose, galactose, glucosamine, and ribose, i.e., the same sugars which are present in the parent lipopolysaccharide. Only traces of free ribose and galactose were seen on chromatograms of the 80% supernatant fraction. Of special interest was the presence of glucosamine, since glucosamine is also found in degraded polysaccharides of mutants belonging to the class of RII mutants (41), but not in the

TABLE 3. Sugar composition of lipopolysaccharides derived from various *Salmonella* T forms

Lipopolysaccharide	Sugar in lipopolysaccharide (%)					
	KDO	Heptose	D-Glucosamine	D-glucose	D-Galactose	D-Ribose
<i>S. typhimurium</i> T1.....	4.0	7.1	5.9	4.8	21.7	14.9
<i>S. paratyphi</i> B T1.....	4.1	4.2	5.7	6.9	13.9	16.4
<i>S. dessau</i> T1.....	3.8	7.3	5.7	4.6	21.9	14.2
<i>S. friedenau</i> T1 ^a	4.2	7.5	6.2	4.6	17.2	21.0
<i>S. bareilly</i> T2.....	4.9	8.9	6.4	7.0	8.7	0.5

^a This lipopolysaccharide sample had been obtained from a batch of *S. friedenau* T1 cells grown on another occasion than that analyzed in Table 2.

TABLE 4. Sugar composition of degraded polysaccharides obtained from *Salmonella friedenau* T1 lipopolysaccharide and bacteria

Prepn	Sugar in polysaccharide (%)					
	KDO	Heptose	D-Glucosamine (of polysaccharide)	D-Glucose	D-Galactose	D-Ribose
T1 degraded polysaccharide (from lipopolysaccharide).....	5.3	12.3	7.8	7.0	30	21
T1 degraded polysaccharide (from bacteria).....	0.8	9.3	4.0	5.3	32	25

polysaccharides of mutants belonging to other R classes. This finding indicates a structural relationship between T1 and RII antigens.

Degraded (Freeman) polysaccharide derived from S. friedenau T1 cells. T1 degraded polysaccharide was also isolated directly from *S. friedenau* T1 bacteria by the method of Freeman (12) according to Staub (39). A preparation was obtained which again contained high concentrations of ribose and galactose (Table 4) and which also resembled the degraded polysaccharide obtained from lipopolysaccharide with respect to the quantities of the other sugars, except for KDO, which is lower.

Alkali treatment of S. friedenau T1 lipopolysaccharides. In order to check the alkali stability of the pentose linkage, *S. friedenau* T1 lipopolysaccharide was treated with 0.25 N NaOH at 56 C, which is also used to condition lipopolysaccharides for subsequent fixation to cell surfaces (sensitization of erythrocytes to perform hemagglutination, reference 3).

A 26.5-mg amount of T1 lipopolysaccharide was dissolved in 1 ml of water, 1 ml of 0.5 N NaOH was added, and the mixture was held at 56 C for 1 hr. Sodium salts of fatty acids were removed by centrifugation. The clear solution was adjusted to pH 5 by addition of acetic acid, and the polysaccharide was precipitated by addition of four volumes of ethyl alcohol. The precipitate was washed with absolute ethyl alcohol and dried from acetone and ether. The yield was 20 mg or 73%. The ribose and galactose content of the preparation were 21 and 20%, respectively, i.e., 100% yield in these sugars. In a second experiment, 50 mg of lipopolysaccharide was dissolved in 3 ml of 0.3 N NaOH and was left 18 hr at 37 C. These conditions have been suggested for the complete digestion of ribonucleic acid (42). The mixture was centrifuged to remove insoluble material

and the clear supernatant solution was dialyzed for 2 days. Four volumes of alcohol (20 ml), and then 0.5 ml of 1 M sodium acetate were added to the solution. The precipitate which formed was collected, washed with alcohol, and dried from acetone and ether. The yield was 36 mg or 72%. The ribose content of this preparation was 26% (i.e., 100% yield in ribose).

These results show that the bulk of ribose in T1 lipopolysaccharides is obviously not linked in phosphodiester linkages.

Analysis of periodate-oxidized T1 (lipo)polysaccharides. Periodate oxidation and reduction of the oxidized products with NaBH₄, followed by dialysis and lyophilization, were performed with the following preparations derived from *S. friedenau* T1: A, lipopolysaccharide; B, alkali-treated lipopolysaccharide; C, degraded polysaccharide derived from lipopolysaccharide (80% ethyl alcohol fraction); and D, degraded polysaccharide derived from bacteria directly. Yields of the final products were 55, 59, 48, and 53%, respectively, compared with the nonoxidized (lipo) polysaccharides A, B, C, and D. The original and oxidized (lipo)polysaccharides were hydrolyzed side-by-side and the sugars estimated by the methods described. The results are summarized in Table 5.

In all preparations checked, KDO, heptose, and galactose were destroyed almost quantitatively. Glucosamine gave variable results, depending on the preparation. This was probably due to the presence or absence of lipid A. Minimal destruction was observed with glucose and no destruction at all with ribose, which seemed to be quantitatively protected from the attack of periodate.

These results were supported when the hydrolysates were analyzed by paper chromatography. Predominant spots were revealed on the

TABLE 5. Sugar composition of *Salmonella friedenau* T1 (lipo) polysaccharides before and after periodate oxidation and reduction with borohydride

Prepn	Per cent of prepn											
	KDO		Heptose		Glucose		Glucosamine		Galactose		Ribose	
	Original	After IO ₄	Original	After IO ₄	Original	After IO ₄	Original	After IO ₄	Original	After IO ₄	Original	After IO ₄
T1 lipopolysaccharide	4.2	1.02	7.5	2.0	4.6	2.2	7.1	6.0	17.2	1.2	21	20
T1 alkali lipopolysaccharide, 60 min, 56 C	5.35	0.7	9.5	1.3	3.9	3.3	7.0	3.9	21	2.5	28	25
T1 degraded polysaccharide from lipopolysaccharide.....	5.3	0.25	12.3	2.1	7.0	5.8	7.8	1.7	30	4.0	21	24
T1 degraded polysaccharide from cells..	0.8	Not done	9.3	1.9	5.3	3.0	4.0	nd	32	7.6	25	30

chromatograms at the position of ribose and glucose. In addition, mannose was identified; this sugar could be expected as a degradation product of L-glycero-D-mannoheptose (11, 2, 7). Furthermore, small amounts of glycerol were detected. When the hydrolysates were subjected to paper electrophoresis with molybdate buffer, B, the presence of threitol (5) was demonstrable. This probably derives from galactose, indicating a 4-linkage for this sugar. No erythritol was detectable.

Comparison of T1 forms with RI and RII mutants. RI mutants synthesize RI-specific lipopolysaccharide and, in addition (and in contrast to RII mutants) O-specific soluble polysaccharide. When the phenol/water procedure is applied to RI mutants, these polysaccharides are found in the soluble supernatant fraction (L1 fraction) after sedimentation of the RI lipopolysaccharide by ultracentrifugation (27, 4). Examination by paper chromatography of the soluble supernatant fractions (L1 fractions) of all the above T forms revealed that these contained no detectable amounts of O-specific sugars in 4 N HCl or 1 N H₂SO₄ hydrolysates, i.e., galactosamine, fucose, or rhamnose. Only glucose, galactose, and ribose were detected in H₂SO₄ hydrolysates. These findings indicate that T1 mutants do not synthesize O-specific polysaccharides, a property which they share with RII mutants.

Hemagglutination inhibition studies. T1 lipopolysaccharides were tested as inhibitors in the hemagglutinating RI/anti-RI and RII/anti-II systems in order to detect possible serological relationships. As seen in Table 6, T1 but not T2 lipopolysaccharides reacted strongly as inhibitors of the RII hemagglutination system (*S. minnesota* RII alkali-treated lipopolysaccharide coated into red cells plus anti-*S. inverness* RII serum). Similarly, *S. minnesota* RII lipopolysaccharide strongly inhibited the T1 system. No cross-reaction was seen between the T lipopolysaccharides and the RI system. The degraded polysaccharide fraction derived from *S. friedenaui* T1 lipopolysaccharide (see Table 6), as well as free ribose and galactose, was also tested. The fact that the degraded polysaccharide was a good inhibitor in the RII system, but showed variable results in the different T1 systems, is of interest, though this behavior cannot be explained satisfactorily. Free ribose, but not galactose, had some inhibitory activity in both the RII and T1 systems.

Table 6 also shows that those S-form lipopolysaccharides which contain ribose did not inhibit the T1 system, thus demonstrating that the determinant groups in these antigens are distinct from those of the T1 antigen.

Analysis of specific precipitates. Figure 3A

shows a typical precipitation curve which was obtained with *S. friedenaui* T1 lipopolysaccharide and homologous antiserum. The precipitates were analyzed for protein by the Folin reaction (24) and for ribose by the orcinol reaction (6). As shown in Fig. 3A, large amounts of ribose were found in the precipitates, which would suggest that ribose is firmly (covalently) linked to the rest of the lipopolysaccharide molecule (see Discussion).

Maximal amounts of antibody protein were precipitated from 10 μ liters of antiserum by about 20 μ g of the lipopolysaccharide containing 20% ribose. Maximal amounts of lipopolysaccharide were precipitated when about 70 μ g of lipopolysaccharide was added to the serum. Under these conditions, about one-third of the lipopolysaccharide added, namely, about 25 μ g, was recovered in the precipitate as calculated from the ribose value. On the other hand, lipopolysaccharide was quantitatively recovered in the precipitate when about 10 μ g was added to the serum. The precipitation curve obtained with *S. friedenaui* T1 lipopolysaccharide and *S. minnesota* RII serum showed a similar slope, although the quantity of antibody precipitated was larger (see Table 7).

When precipitation of T1 serum was performed with alkali-treated lipopolysaccharide, the curves of Fig. 3B were obtained. One of the alkali polysaccharides studied is identical with that described below (i.e., treatment for 60 min at 56 C). The second preparation was treated identically, except that the time of exposure to alkali was 5 min only. The curves show that both products bind only half the amount of antibody compared with the original lipopolysaccharide. However, the amounts of ribose present in the precipitates were greatly diminished. This change in precipitability of ribose was most apparent with the preparation treated for 60 min with alkali, but it was also seen with the preparation which had been in contact with alkali for 5 min only. That ribose was present in the supernatant fluids on the precipitates could be demonstrated with the orcinol method, but the tests could not be performed quantitatively since the serum interfered with the determination.

Similar curves, shown in Fig. 3B, were obtained with alkali-treated T1 lipopolysaccharide and RII serum. As shown in Table 7 also, with this serum only about half of the protein was found in the precipitates, compared with the reaction with untreated T1 lipopolysaccharide. The amount of ribose in the precipitates, though higher than found in the precipitates obtained with T1 serum, was also diminished. Degraded T1 polysaccharide prepared directly from bacterial cells be-

TABLE 6. Hemagglutination inhibition with various (lipo) polysaccharides of *Salmonella* T1, RI, and RII systems^a

Inhibitor	Hemagglutination system			
	<i>S. friedenaui</i> T1	<i>S. dessau</i> T1	<i>S. minnesota</i> RII	<i>S. minnesota</i> RI
Ribose containing S-form lipopolysaccharides				
<i>S. halle</i> (group 28).....	>250			
<i>S. tel aviv</i> (group 28).....	>250			
<i>S. ezra</i> (group 28).....	>250			
<i>S. utrecht</i> (group 52).....	>250			
<i>S. artis</i> (group 56).....	>250			
T1 (Lipo) polysaccharides				
<i>S. typhimurium</i> T1 lipopolysaccharide.....			0.2	>250
<i>S. paratyphi</i> B T1 lipopolysaccharide.....	2	0.05	0.2	>250
<i>S. dessau</i> T1 lipopolysaccharide....	0.5	0.05	0.1	>250
<i>S. friedenaui</i> T1 lipopolysaccharide.....	0.5	0.1	0.1	>250
<i>S. friedenaui</i> alkali lipopolysaccharide.....	3.9	0.3	15	
<i>S. friedenaui</i> degraded polysaccharide, 80% ethyl alcohol fraction.....	>250	63	15	
<i>S. friedenaui</i> degraded polysaccharide from cells.....	>250	0.1	31	
<i>S. friedenaui</i> lipopolysaccharide, IO ₄ /BH ₄ treated.....	2	1	0.1	
<i>S. friedenaui</i> alkali lipopolysaccharide IO ₄ /BH ₄ treated.....	2	1		
<i>S. friedenaui</i> degraded polysaccharide, 80% ethyl alcohol fraction, IO ₄ /BH ₄	>250	>250	>250	
T2 lipopolysaccharide				
<i>S. bareilly</i> T2.....	>250	>250	125	>250
RII lipopolysaccharides				
<i>S. minnesota</i> RII lipopolysaccharide.....	0.5	0.2	0.5	>250
<i>S. typhimurium</i> TV149.....		0.5	2	>250
RI lipopolysaccharide				
<i>S. minnesota</i> RI.....	>250		>250	1
Free sugars				
Ribose.....	9,000		75,000	
Galactose.....	90,000		90,000	

^a The test was performed according to Beckmann (3). Systems: alkali-treated lipopolysaccharide fixed to erythrocytes. Numbers: minimal amounts of preparation which inhibit hemagglutination.

has like alkali-treated lipopolysaccharide in T1 serum, from which about half of the quantity of antibody was precipitated as by the original lipopolysaccharide. Again, as with alkali lipopolysaccharide, only small amounts of ribose were found in the precipitates.

The determination of galactose in the precipi-

tates of the various systems, though not as accurate as the ribose estimations, gave values which paralleled the amounts of ribose; i.e., high values of galactose in the precipitates of lipopolysaccharides and low values in the precipitates of alkali-treated lipopolysaccharides. In the latter system, however, the galactose values compared

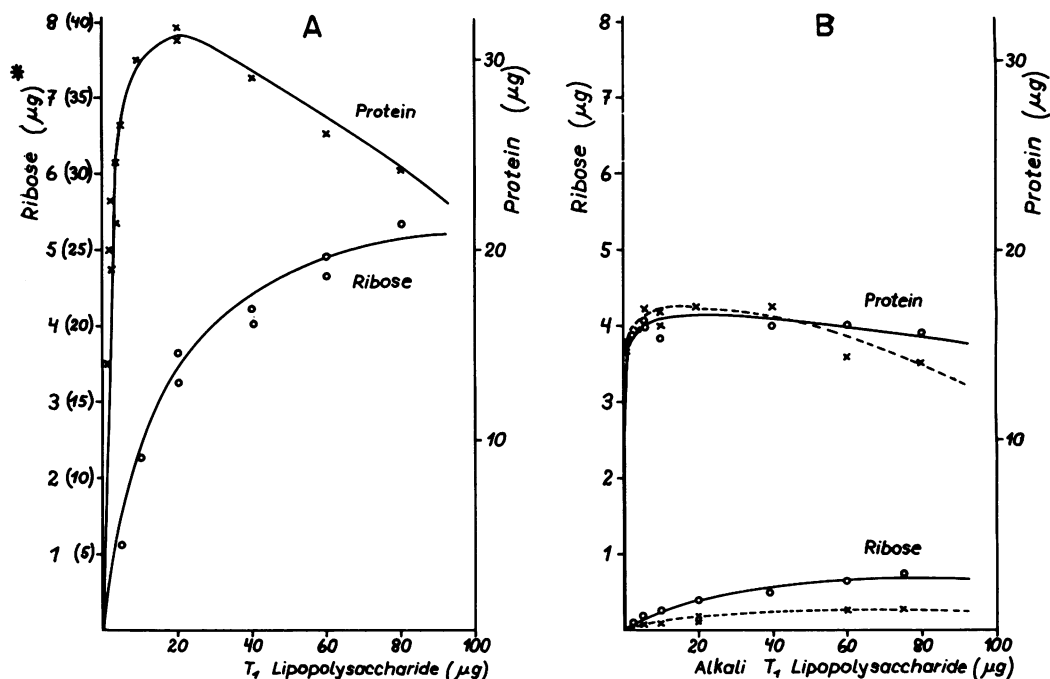


FIG. 3. Precipitation curves with (A) T1 lipopolysaccharide and (B) T1 alkali lipopolysaccharides, treated with 0.25 N NaOH at 56 C for 5 min (solid line) and 60 min (dashed line), respectively. Each tube contained 10 μ liters of *S. friedenaui* T1 antiserum. Values in parentheses, μ g of lipopolysaccharide, calculated from the ribose data.

with those of ribose were relatively higher. This can be explained by the fact that galactose is also a constituent of the core of lipopolysaccharides, while ribose is not. The results described here suggest that even short alkali or acid treatment of T1 lipopolysaccharides results in a release of ribose and galactose. That these sugars are released in the form of large molecules is indicated by the finding that neither ribose nor galactose becomes dialyzable after such treatments of T1 lipopolysaccharides.

As seen from Table 7 the T1 degraded polysaccharide (derived from lipopolysaccharide) was very weakly precipitated by T1 and by RII serum. Ribose was not found in the precipitate. Table 7 also shows the results obtained with the $\text{IO}_4^-/\text{NaBH}_4$ preparations. Oxidized lipopolysaccharide precipitated as much anti-T1 and RII antibody as did the original lipopolysaccharide. Alkali-treated T1 lipopolysaccharide and T1-degraded polysaccharide, after $\text{IO}_4^-/\text{NaBH}_4$ treatment, did not precipitate either in T1 or in RII antiserum. We added to the supernatant fluids (after separating the precipitate by centrifugation), original T1 lipopolysaccharide, and we determined the amount of protein precipitated. There was a striking difference between the re-

actions in T1 and RII serum. The three preparations: degraded polysaccharide (80% ethyl alcohol fraction), degraded polysaccharide (80% ethyl alcohol fraction) $\text{IO}_4^-/\text{NaBH}_4$, and alkali lipopolysaccharide- $\text{IO}_4^-/\text{NaBH}_4$, proved to be strong inhibitors of the system T1 lipopolysaccharide/RII antiserum, but they did not inhibit the system T1 lipopolysaccharide/T1 antiserum. As an example, Fig. 4 demonstrates the results obtained with degraded polysaccharide as inhibitor of the two antisera. At present, the findings cannot be fully explained.

Occurrence of ribose in other Salmonella lipopolysaccharides. It was previously reported that some S-form *Salmonella* lipopolysaccharides contain ribose as a constituent. These include species belonging to groups 28, 52, and 56. These lipopolysaccharides contain from 5 to 7% D-ribose (Table 8), as shown by colorimetry, paper chromatography, and reaction with ribokinase. In other S-form lipopolysaccharides, only 1% orcinol-positive material was found (calculated as ribose). Here, the possibility cannot be excluded that this reaction derives from the presence of small amounts of nucleic acid.

Because of the serological relationship between RII and T1 lipopolysaccharides, it seemed worth-

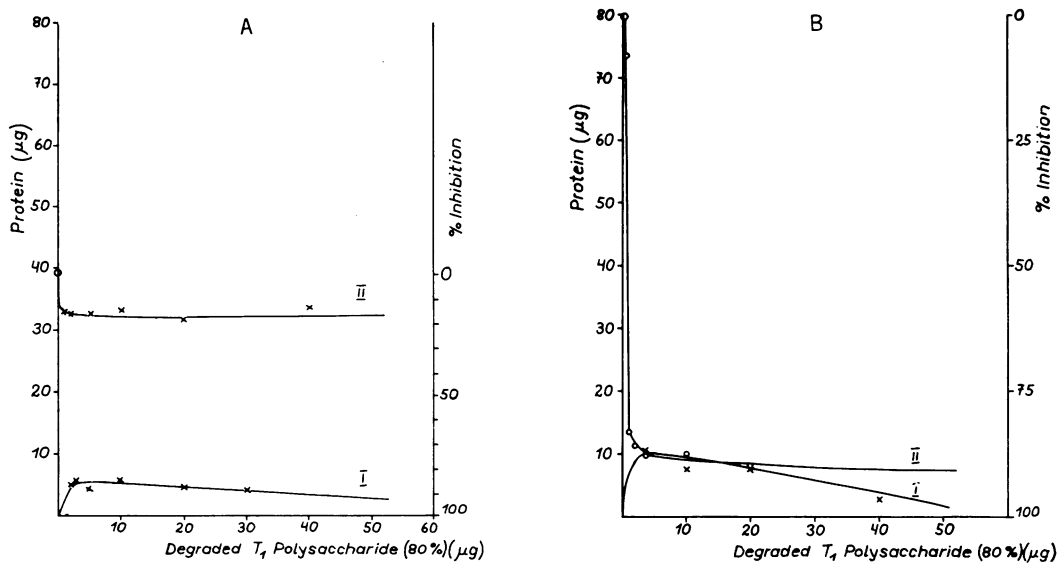


FIG. 4. Inhibition of the precipitation of T1 lipopolysaccharide with T1 and RII antiserum by T1 degraded polysaccharide. Increasing amounts of degraded T1 polysaccharide (80% ethyl alcohol fraction) and 10 µliters of T1 antiserum (Fig. 4A) or RII antiserum (Fig. 4B) were incubated. After 2 days the precipitate was analyzed for protein (curve I). To each supernatant solution 30 µg of T1 lipopolysaccharide was added and the precipitate formed was again tested for protein (curve II).

TABLE 7. Serological precipitation of various fractions derived from *Salmonella friedenau* T1 lipopolysaccharide in antisera from *S. friedenau* T1 and *S. minnesota* RII

Prepn	T1 antiserum		Maximal inhibition caused by prepn	RII antiserum		Maximal inhibition caused by prepn ^d
	Maximal amt of protein precipitated	Maximal amt of ribose precipitated		Maximal amt of protein precipitated	Maximal amt of ribose precipitated	
T1 lipopolysaccharide...	µg 31	µg 5.4 ^c	—	µg 87 ^a	µg 5.2 ^c	% —
T1 lipopolysaccharide ION ₄ /NaBH ₄	36 ^a	Not done	—	85 ^a	Not done	0
Alkali T1 lipopolysaccharide.....	17 ^a	0.3 ^c	—	48 ^a	1.3 ^c	—
Alkali T1 lipopolysaccharide IO ₄ /NaBH ₄	0	—	0	0	—	90
Degraded T1 polysaccharide (80% ethyl alcohol fraction).....	6 ^b	—	18	10 ^b	—	90
Degraded T1 polysaccharide (80% ethyl alcohol fraction) IO ₄ / NaBH ₄	0	—	0	0	—	90
Degraded T1 polysaccharide (derived from cells).....	16 ^b	0.1 ^c	Not done	Not done	Not done	Not done

^a and ^b These amounts of protein were precipitated in those tubes which contained 20 µg and 3 µg, respectively, of preparation.

^c These amounts of ribose were obtained in the precipitate of those tubes which contained 80 µg of preparation.

^d Inhibition was tested by isolating the supernatant liquid of each tube and adding to it 30 µg of T1 original lipopolysaccharide. The precipitate formed was tested with the Folin reaction for protein. T1 and RII serum: 10 µliter per tube.

TABLE 8. Ribose content of various lipopolysaccharides

Chemotype	No. tested	Per cent ribose
O groups 28, 52, 56	3	5 to 7
Other O groups	3	1
T1 mutants	5	15 to 22
<i>S. minnesota</i> RII mutant	5	2 to 5
<i>S. minnesota</i> RII mutant "degraded polysaccharide"	2	3 to 6
<i>S. typhimurium</i> RII mutant	6	0.9 to 1
Other RII mutants	4	1 to 4
<i>S. minnesota</i> RI mutant	2	0.1 to 0.5
<i>S. minnesota</i> Rc mutant ^a	2	0.5
<i>S. minnesota</i> Rd mutant	3	0.2 to 0.4
<i>S. minnesota</i> Re mutant	2	0.2

^a See references 21 and 5.

while to assay further RII lipopolysaccharides, as well as R lipopolysaccharides belonging to other R groups, for the presence of ribose. As shown in Table 8, the RII lipopolysaccharides tested contained pentose in concentrations between 1 and 5%. RI and other R mutant lipopolysaccharides appeared to contain less than 1% of ribose. Ribose was identified by paper chromatography in several RII lipopolysaccharides. It was also shown in several cases of RII lipopolysaccharides that the ribose content was not due to contaminating nucleic acid. These data would indicate that ribose is a constituent sugar in RII lipopolysaccharides.

DISCUSSION

It is generally accepted that *Salmonella* S-form lipopolysaccharides are built up of a common core polysaccharide, constituted by the basal sugars, i.e., L-glycero-D-manno-heptose, KDO, D-galactose, D-glucose, and D-glucosamine, and O-specific chains which are attached to the core and which are distinct in the various O antigens of different *Salmonella* species (29, 32, 25). In the past few years *Salmonella* R mutants belonging to different R classes have been isolated and the respective lipopolysaccharides have been studied (4, 29, 30, 32, 25). These R lipopolysaccharides, without exception, contain less sugar constituents than the lipopolysaccharides of the wild types from which they derived. These and other findings lead to the view that *Salmonella* R mutants are blocked in the synthesis or attachment of one or more of the sugar constituents of their lipo-

polysaccharides and therefore synthesize incomplete lipopolysaccharides. Up to now, no R lipopolysaccharides have been found which contained sugar components not present in the parent lipopolysaccharide (29, 19). The only exception in this rule apparently is the T1 mutants, which contain a sugar in their lipopolysaccharides, namely, D-ribose, which is absent from the lipopolysaccharides of the parent strains. It is noteworthy that this is not a characteristic of T mutants in general, since the T2 lipopolysaccharide derived from *S. bareilly* does not contain ribose, and with respect to its sugar constituents rather resembles R-form lipopolysaccharides.

In addition to ribose now identified as D-ribose, which comprises 14 to 22% of the T1 lipopolysaccharides, equally large amounts of D-galactose are present. Other sugar constituents are heptose (presumably L-glycero-D-manno-heptose), KDO, D-glucose, and D-glucosamine.

We have treated T1 lipopolysaccharide of *S. friedenaui* with hot acetic acid, a procedure known to liberate the two components, lipid A and "degraded polysaccharide." Alternatively, degraded T1 polysaccharide was prepared according to Freeman (12) from whole bacteria. T1 lipopolysaccharide was also subjected to alkali treatment whereby the ester linkages present in lipid A are cleaved. This reaction generally leads to soluble preparations, the "alkali lipopolysaccharides," which carry most of the specificity of the parent O antigen and which are used in the hemagglutination test for sensitization of erythrocytes.

Chemical analyses of the degraded preparations revealed again the presence of large amounts of ribose and galactose in addition to the sugars characteristic for the basal core polysaccharide.

Periodate oxidation, followed by reduction with NaBH₄ of T1 lipopolysaccharide, alkali lipopolysaccharide, and degraded polysaccharides, showed that ribose is not attacked by periodate. Galactose, on the other hand, is destroyed almost quantitatively. Two degradation products, threitol and mannose, have been identified after hydrolysis in larger amounts. Threitol probably is a degradation product of galactose. Its formation would indicate that galactose is linked at C-atom 4 in the nonoxidized preparations. A new sugar, mannose, was identified by paper chromatography. It probably originates from L-glycero-D-manno-hapose by cleavage of the linkage between C₈ and C₇, indicating that the heptose residues are linked at C₈ or form branching points in the lipopolysaccharide (7).

To obtain an insight into the status of ribose and galactose in T1 lipopolysaccharides, i.e., into the question if these sugars are covalently linked to the lipopolysaccharide to form one unique

molecule, we have performed serological precipitation tests, using T1 lipopolysaccharide and specific antisera. In the precipitates formed, we have determined ribose and galactose. It was found that both of these sugars were present in the precipitates obtained with T1 and RII antisera. However, these positive results cannot be taken as a definite proof that ribose and the other sugars form one single molecule. It has been shown that, although an artificial mixture of two serologically different lipopolysaccharides can be separated by serological methods, a mixture of two lipopolysaccharides obtained by extraction of a mixture of the parent bacteria cannot be separated by serological precipitation (M. Sarvas et al., *in press*). These coextracted lipopolysaccharides form mixed aggregates. If, therefore, ribose and galactose formed polymers linked to lipid A to form lipopolysaccharides which would occur in T1 forms in addition to the lipopolysaccharide formed by the other sugars, it would not be possible to separate this mixture by the serological methods mentioned above. The idea of two separate lipopolysaccharides synthesized by T1 mutants, however, seems to us to be improbable. If, on the other hand, ribose and galactose were present in the form of soluble polymers, we would assume that the method of isolation of the lipopolysaccharides from bacteria, which includes centrifugation in the ultracentrifuge, would have resulted in a separation of lipopolysaccharide which is known to be found in the sediment, and the polymers, which would be expected to be found in the supernatant, in analogy to other soluble polysaccharides such as O-specific polysaccharides, capsular specific polysaccharides, glucans, and others, which all are separated from lipopolysaccharides in the ultracentrifuge (4, 44).

Though it was not possible yet to prove definitely that the sugars found in T1 lipopolysaccharides form one molecule, there are no indications against this assumption and several reasons favor it.

When, on the other hand, alkali-treated T1 lipopolysaccharide or acid-extracted degraded polysaccharide was treated with T1 or RII antisera, the precipitates formed did not contain ribose and only small amounts of galactose were found, a result which indicates that in these preparations ribose and most of the galactose are not linked to the polysaccharide formed by the rest of the sugars. If we assume that, in the original T1 lipopolysaccharide, ribose and galactose are linked to the polysaccharide, we have to postulate that these sugars are linked in an alkali- and acid-sensitive linkage and are released after treatment of the lipopolysaccharide

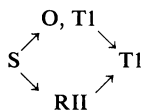
with alkali or acid in form of alcohol-precipitable and nondialyzable polymers of high molecular weight. Alternatively, alkali- and acid-labile substituents (e.g., acetyl groups?) may be present in T1 antigens as serological determinants. This possibility is under investigation. A series of oligosaccharides, obtained by partial hydrolysis of T1 lipopolysaccharide and purified by paper chromatography, all contained ribose and galactose (M. Berst, *unpublished*). Their further investigation will show whether these two sugars occur as mixed polymers.

The performance of hemagglutination inhibition tests has revealed the existence of close serological similarities between *S. friedenaui* T1 and *S. minnesota* RII (lipo) polysaccharides. Strong cross-reactions were also exhibited with T1 lipopolysaccharides derived from other *Salmonella* species (see Table 6) as well as with antisera obtained with other RII and RII-like mutants (E. Ruschmann, *unpublished*). Consequently, cross-absorption of sera with mutants generally resulted also in the disappearance of the homologous antibody as tested in hemagglutination tests. As shown in Table 6, T1 degraded polysaccharides inhibit the RII system and one of the T1 systems tested. Ribose in high concentrations inhibits both T1 and RII systems, while galactose is inactive. The common determinant group in T1 and RII lipopolysaccharides is not known. It must be kept in mind, though, that the hemagglutination tests are performed with red cells coated with alkali-treated lipopolysaccharides. Alkali-labile determinants on the lipopolysaccharides are only detected if they retain their specificity and are also adsorbed to the surface of the red cells. Therefore, if it is true that alkali treatment of T1 lipopolysaccharide splits off a ribose-galactose polymer, the specificity carried by the latter can be expressed in hemagglutination tests only if this polysaccharide is adsorbed to the red cells. Finally, it should be mentioned that RII sera have been obtained which do not cross-react with T1 lipopolysaccharides (E. Ruschmann, *unpublished*). Strong serological cross-reactions between T1 and RII were also observed in precipitation tests, which are shown in Table 7 for the system T1 lipopolysaccharide and RII serum.

When T1 lipopolysaccharide was oxidized with sodium periodate and reduced with NaBH₄ and then tested, unaltered precipitations with T1 and RII serum were found. This shows that the destruction of galactose has no influence on the precipitability of the T1 lipopolysaccharide by specific serum. Alkali T1 lipopolysaccharide and degraded polysaccharide from T1 cells

precipitated about half the amount of T1 and RII antibody as compared with untreated T1 lipopolysaccharide. None of the three products, periodate-treated alkali T1 lipopolysaccharide, degraded T1 polysaccharide, or periodate-treated degraded T1 polysaccharide precipitated in T1 or RII serum. However, these preparations strongly inhibited the precipitation of untreated T1 lipopolysaccharide (as well as RII lipopolysaccharide) in RII serum. On the other hand, these preparations did not inhibit precipitation of the original lipopolysaccharide in T1 serum. At present, the serological results cannot be interpreted. The following similarities exist between T1 and RII mutants. (i) The degraded polysaccharides derived from RII and T1 lipopolysaccharides both contain glucosamine. They therefore represent the intact basal core polysaccharide of *Salmonella* antigens (29). In analogy to RII mutants we would expect that T1 mutants are defective in the synthesis of the O-specific chains present in the wild-type lipopolysaccharides. (ii) This assumption is supported by the finding that both RII and T1 mutants do not synthesize O-specific haptenic polysaccharide as shown by the analyses of the L1 supernatant fractions. (iii) RII lipopolysaccharide may contain up to 5% of D-ribose which does not originate from contaminating nucleic acid and therefore resembles the status of the ribose units found in T1 lipopolysaccharides. (iv) Ribose-containing oligosaccharides have been isolated from RII mutants which inhibit the system RII lipopolysaccharide/anti-RII serum tested in the complement fixation test (25). Obviously ribose units in RII lipopolysaccharide contribute to specificity. (v) T1 and RII lipopolysaccharides show strong serological cross-reactions in different test systems.

From these data we can consider T1 mutants as being double mutants. One defect concerns the biosynthesis of the O-specific chains which are absent in T1 antigens; the second mutation concerns the activation of an enzyme which synthesizes the ribose-galactose polysaccharide. It is possible that RII mutants contain single ribose-galactose units in the molecule which would occur in a polymerized form in T1 antigens under the influence of the activated enzyme (25). On the basis of these considerations the following scheme would show a possible mechanism for the formation of T1 forms from S forms:



O,T1 mutants carrying O and T specificities, or RII forms would be the intermediate mutants. O,T1 mutants have been described by Schlosshardt (36) as derived from *S. weslaco*. O, T1 forms have also been produced by genetic recombination (35). *S. abony* 4, 5, 12, and *S. montevideo* 6, 7, respectively, were crossed with the T1 form of *S. paratyphi* B. Hybrids were isolated which carried the specificities 4,5,12,T1 and 6,7,T1 respectively. It is concluded (35) that in the O,T1 recombinants the defective O locus of the T1 form was replaced by the intact O locus of the respective donor.

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LITERATURE CITED

1. ASHWELL, G. 1957. Colorimetric analysis of sugars, p. 73-105. In S. P. Colowick and N. O. Kaplan [ed.], *Methods in enzymology*, vol. 3. Academic Press, Inc., New York.
2. BAGDIAN, G., W. DROGE, K. KOTELKO, O. LÜDERITZ, AND O. WESTPHAL. 1966. Vorkommen zweier Heptosen in Lipopolysacchariden enterobakterieller Zellwände: L-Glycero- und D-Glycero-D-mannoheptose. *Biochem. Z.* **344**: 197-211.
3. BECKMANN, I., O. LÜDERITZ, AND O. WESTPHAL. 1964. Für Immunchemie der somatischen Antigene von Enterobacteriaceae. IX. Serologische typisierung von *Salmonella*-R-Antigenen. *Biochem. Z.* **339**:401-415.
4. BECKMANN, I., T. V. SUBBAIAH, AND B. A. D. STOCKER. 1964. Rough mutants of *Salmonella typhimurium*. 2. serological and chemical investigations. *Nature* **201**:1299-1301.
5. BOURNE, E. J., D. H. HUTSON, AND H. WEIGEL. 1961. Oligosaccharides in dextran-producing cultures of *Streptococcus bovis*. *Biochem. J.* **79**:549-553.
6. BROWN, A. H. 1946. Determination of pentose in the presence of large quantities of glucose. *Arch. Biochem. Biophys.* **11**:296-278.
7. CHERNIAK, R., AND M. J. OSBORN. 1966. The structure of the heptose-containing backbone of the lipopolysaccharide of *Salmonella typhimurium*. *Federation Proc.* **25**:410.
8. DISCHE, Z., AND L. B. SHETTLES. 1951. A new spectrophotometric test for the detection of methylpentose. *J. Biol. Chem.* **192**:579-582.

9. FISCHER, F. G., AND H. DÓRFEL. 1955. Die papierchromatographische Trennung und Bestimmung der Urnsäuren. *Z. Physiol. Chem.* **301**:224-234.
10. FISCHER, W., AND J. ZAPP. 1964. Quantitative Bestimmung der Galaktose mittels Galatoseoxydase aus *Bactyllum dendroides*. I. *Z. Physiol. Chem.* **337**:186-195.
11. FOSTER, A. B., D. A. L. DAVIES, AND M. J. CRUMPTON. 1958. Action of periodate on some polysaccharides containing aldoheptose sugars. *Nature* **181**:412-413.
12. FREEMAN, G. G. 1942. The preparation and properties of a specific polysaccharide from *bactyphosum* Ty₂. *Biochem. J.* **36**:340-355.
13. GHALAMBOR, M. A., E. M. LEVINE, AND E. C. HEATH. 1966. The Biosynthesis of cell wall lipopolysaccharide in *Escherichia coli* III. The isolation and characterization of 3-deoxyoctulosonic acid. *J. Biol. Chem.* **241**:3207-3215.
14. HORECKER, B. L. 1957. Preparation and analysis of ribose-5-phosphate, p. 188-190. In S. P. Colowick and N. O. Kaplan [ed.], *Methods in enzymology*, vol. 3. Academic Press, Inc., New York.
15. KAUFFMANN, F. 1956. A new antigen of *Salmonella paratyphi* B and *Salmonella typhi* murium. *Acta Pathol. Microbiol. Scand.* **39**:299-304.
16. KAUFFMANN, F. 1957. On the T antigen of *Salmonella bareilly*. *Acta Pathol. Microbiol. Scand.* **40**:343-344.
17. KAUFFMANN, F. 1961. Die bakteriologie der *Salmonella* Species. Munksgaard-Kopenhagen, Denmark.
18. KAUFFMANN, F., B. JANN, L. KRUGER, O. LÜDERITZ, AND O. WESTPHAL. 1962. Zur Immunchemie der O-Antigene von Enterobacteriaceae. VIII. Analyse der Zuckerbausteine von Polyxacchariden weiterer *Salmonella*- und *Arizona*-O-Gruppen. *Zentr. Bakteriol. Parasitenk. Abt. I Orig.* **186**:509-516.
19. KAUFFMANN, F., L. KRUGER, O. LÜDERITZ, AND O. WESTPHAL. 1962. Zur Immunchemie der O-Antigene von Enterobacteriaceae. VI. Vergleich der Zuckerbausteine von Polysacchariden aus *Salmonella* S- and R Formen. *Zentr. Bakteriol. Parasitenk. Abt. I Orig.* **182**:57-66.
20. KAUFFMANN, F., O. LÜDERITZ, H. STIERLIN, AND O. WESTPHAL. 1960. Chemie der O-Antigene von Enterobacteriaceae. I. Analyse der Zuckerbausteine von *Salmonella* O-Antigenen. *Zentr. Bakteriol. Parasitenk. Abt. I Orig.* **178**:442-458.
21. KICKHOFEN, B., AND O. WESTPHAL. 1952. Papierelktrophorese bei hohen Spannungen zur Trennung von Peptiden. *Z. Naturforsch.* **7**:655.
22. KRUGER, L., O. LÜDERITZ, J. L. STROMINGER, AND O. WESTPHAL. 1962. Zur Immunchemie der O-Antigene von Enterobacteriaceae. VII. Die Zugehörigkeit von Hexosen und 6-Desoxyhexosen in *Salmonella*-Lipopolysacchariden zur D- bzw. L-Reihe. *Biochem. Z.* **335**:548-558.
23. LOWRY, O. H., N. R. ROBERTS, K. Y. LEINER, M. L. WU, AND A. L. FARR. 1954. The quantitative histochemistry of brain. I. Chemical methods. *J. Biol. Chem.* **207**:1-17.
24. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
25. LÜDERITZ, O., C. GALANOS, H. J. RISSE, E. RUSCHMANN, S. SCHLECHT, G. SCHMIDT, H. SCHULTE-HOLTHAUSEN, R. WHEAT, O. WESTPHAL, AND J. SCHLOSSHARDT. 1966. Structural relationships of *Salmonella* O and R antigens. *Ann. N.Y. Acad. Sci.* **133**:349-374.
26. LÜDERITZ, O., F. KAUFFMANN, H. STIERLIN, AND O. WESTPHAL. 1960. Zur Immunchemie der O-Antigene von Enterobacteriaceae. II. Vergleich der Zuckerbausteine von *Salmonella* S-, T- und R-Formen. *Zentr. Bakteriol. Parasitenk. Abt. I Orig.* **179**:180-186.
27. LÜDERITZ, O., H. R. RISSE, H. SCHULTE-HOLTHAUSEN, J. L. STROMINGER, I. W. SUTHERLAND, AND O. WESTPHAL. 1965. Biochemical studies of the smooth-rough mutation in *Salmonella minnesota*. *J. Bacteriol.* **89**:343-354.
28. LÜDERITZ, O., D. A. R. SIMMONS, J. L. STROMINGER, AND O. WESTPHAL. 1964. A specific microdetermination of glucosamine and the analysis of other hexosamines in the presence of glucosamine. *Anal. Biochem.* **9**:263-271.
29. LÜDERITZ, O., A. M. STAUB, AND O. WESTPHAL. 1966. Immunochimie of O and R antigens of *Salmonella* and related Enterobacteriaceae. *Bacteriol. Rev.* **30**:192-255.
30. NAIDE, Y., H. NIKAIKO, P. H. MAKELA, R. G. WILKINSON, AND B. A. D. STOCKER. 1965. Semirough strains of *Salmonella*. *Proc. Natl. Acad. Sci. U.S.* **53**:147-153.
31. OSBORN, M. J. 1963. Studies on the gram-negative cell wall, I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. U.S.* **50**:499-506.
32. OSBORN, M. J., S. M. ROSEN, L. ROTHFIELD, L. D. ZELEZNICK, AND B. L. HORECKER. 1964. Lipopolysaccharide of the gram-negative cell wall. *Science* **145**:783-789.
33. RAMMLER, D. H., AND J. C. RABINOWITZ. 1962. A procedure for the microdetermination of formic acid in periodate oxidation mixtures. *Anal. Biochem.* **4**:116-123.
34. SCHLECHT, S., AND O. WESTPHAL. 1966. Wachstum und Lipopolysaccharid (O-Antigen)-Gehalt von *Salmonellen* bei züchtung auf Agarnährboden. *Zentr. Bakteriol. Parasitenk. Abt. I Orig.* **200**:241-259.
35. SARVAS, M., AND P. H. MAKELA. 1965. The production, by recombination, of *Salmonella* forms with both T1 and O specificities. *Acta Pathol. Microbiol. Scand.* **65**:654-656.
36. SCHLOSSHARDT, J. 1960. Untersuchungen über die Entstehung von T-Antigenen im S-R-Formenwechsel bei *Salmonellen*. *Zentr. Bakteriol. Parasitenk. Abt. I Orig.* **177**:176-185.
37. SCHLOSSHARDT, J. 1964. Untersuchungen über die

- Entstehung von Mutagenen im Zellstoffwechsel und ihre Rolle im S-R-Formenwechsel bei Salmonellen. Zentr. Bacteriol. Parasitenk. Abt. I Orig. 192:54-66.
38. SIMMONS, D. A. R., O. LÜDERITZ, AND O. WESTPHAL. 1965. The immunochemistry of *Salmonella* chemotype VI O-antigens. Biochem. J. 97:807-814.
 39. STAUB, A. M. 1965. Somatic degraded polysaccharide of gram negative bacteria, p. 93-95. In R. L. Whistler [ed.], Methods in carbohydrate chemistry. Academic Press, Inc., New York.
 40. STROMINGER, J. L., J. T. PARK, AND R. E. THOMPSON. 1959. Composition of the cell wall of *Staphylococcus aureus*: Its relation to the mechanism of action of penicillin. J. Biol. Chem. 234:3263-3268.
 41. SUTHERLAND, J. W., O. LÜDERITZ, AND O. WESTPHAL. 1965. Studies on the structure of lipopolysaccharides of *Salmonella minnesota* and *Salmonella typhimurium* R. strains. Biochem. J. 96:439-448.
 42. VOLKIN, E., AND W. E. COHN. 1954. Estimation of nucleic acids, p. 287-303. D. Glick [Ed.], Methods of biochemical Analysis. Interscience Publishers, Inc., New York.
 43. WARAVDEKAR, V. S., AND L. D. SASLAW. 1959. A sensitive colorimetric method for the estimation of 2-deoxy sugars with the user of the malonaldehyde-thiobarbituric acid reaction. J. Biol. Chem. 234:1945-1950.
 44. WESTPHAL, O., AND K. JANN. 1965. Bacterial lipopolysaccharides, p. 83-91. R. L. Whistler [ed.], Methods in carbohydrate chemistry. Academic Press, Inc., New York.