Studies on the Chemical Basis of the Phage Conversion of O-Antigens in the E-Group *Salmonellae**

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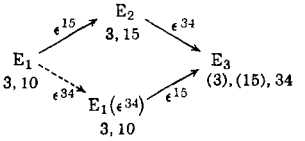
The effects of two temperate bacteriophages, ϵ^{15} and ϵ^{34}, on the chemical structure of the polysaccharide O-antigens of the E-group *Salmonellae* are described. It is shown that the basic antigen structure contains a galactosyl-mannosyl-rhamnosyl sequence. In the organism that carries neither phage the galactosyl-mannose linkage has an α configuration. After infection with ϵ^{15}, however, the galactosyl-mannose bond has been shown to have the β-glycosidic configuration. The second phage, ϵ^{34}, brings about the attachment of glucose to the β-D-galactosyl residue of the polysaccharide. Since this β-D-galactosyl residue is present only in cells that carry ϵ^{15}, a chemical explanation is provided for the observation that ϵ^{34} cannot affect the antigenic structure of an organism without the simultaneous presence in the cell of the ϵ^{15} genome. Possible biosynthetic schemes for the polysaccharides are discussed.

The metabolic relationship between a virus and the cell it infects is a subject of increasing interest. When the virus is a temperate bacteriophage the interaction between the cell and prophage may continue for many generations and the prophage may take part in the direction of the biosynthetic activities of the cell. Lederberg (1955) has proposed the term "lysogenic conversion" to define cell characteristics that are determined exclusively by temperate bacteriophages. Although the distinction between lysogenic or phage conversion and transduction has become less and less clearcut, it is still of fundamental interest and importance that certain cell characteristics are always associated with the presence of a bacteriophage (Barksdale, 1959).

In the genus *Salmonella* there are several cases known where a temperate phage will change the properties of the bacterial polysaccharides or O-antigens that serve as the basis for the serologic classification of this group of organisms. Specifically, in the E-group *Salmonellae* two temperate phages, ϵ^{15} and ϵ^{34}, may be present, and the structural change brought about by each phage in the O-antigen leads to a modification of the antigenic and phage receptor properties of the cell (Iseki and Sakai, 1953; Uetake, 1956; Uetake *et al.*, 1955, 1958).

The relationship may be summarized as follows:



The numbers represent the antigenic components of the polysaccharide fraction of the cell wall as defined by Kauffmann (1958). An E_1 organism with antigenic factors 3 and 10 is converted to an organism with components 3 and 15 by phage ϵ^{15}. The E_2 organism is converted to E_3 by phage ϵ^{34} and antigenic factor 34 is added. If E_1 is infected with ϵ^{34} by an indirect method, there is no change in its antigenic structure (Uetake and Hagiwara, 1960).

Recent work by Staub (1960) and Westphal and co-workers (Westphal, 1960; Kauffmann *et al.*, 1960) has furnished evidence that all of the *Salmonella* polysaccharides are composed of a basic glycosidic structure to which are attached short oligosaccharide side-chains. These side-chains are believed to be responsible for the antigenic properties of the polymer. The interesting biological interrelationship between the E-group *Salmonellae* and ϵ-phages and the general formulation of the nature of the *Salmonella* polysaccharides by Staub and Westphal has led us to undertake a study of the chemical and enzymatic basis of the conversion phenomenon in the E *Salmonella* group. The present paper deals with the chemical nature of the changes brought about by ϵ^{15} and ϵ^{34} in the structure of the cell polysaccharides. The biological and serologic properties of the system are described and discussed in the papers by Uetake and Hagiwara (1961), Uetake and Uchida (1959), and Uetake *et al.* (1955, 1958).

A tentative structure for the E-group antigenic determinants is shown in Figure 1. This structure gives a composite picture of the three antigens 3, 10; 3, 15; and (3), (15), 34, and thus summarizes the changes brought about by ϵ^{15} and ϵ^{34}.

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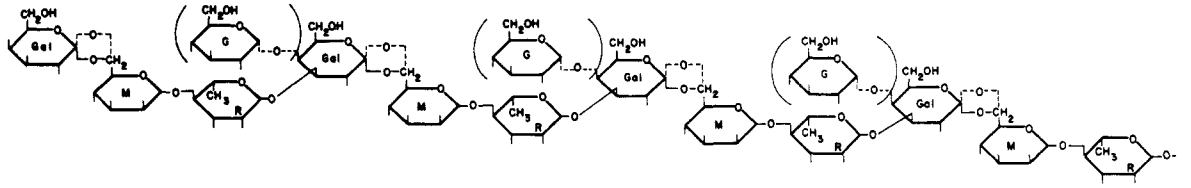

FIG. 1.—Composite structure of the three E-group antigenic side-chains. Antigen 3,10 is represented by the solid lines showing α-D-galactosyl residues (└O—). In antigen 3,15 the galactosyl residues are present as the β-anomers and are shown by the dotted lines (┌C┈┈). Antigen (3),(15),34 contains the β-D-galactosyl residues and in addition has the α-D-glucosyl residues shown in parentheses.

MATERIAL AND METHODS

Salmonella anatum strain 1 (A_1) has O-antigens 3, 10 and H antigens e, h; 1, 6. Lysogenic derivatives were made by infecting strain A_1 with phages ϵ^{15} and ϵ^{34}; organisms thus obtained are denoted as A_1 (ϵ^{15}) and A_1 (ϵ^{15} ϵ^{34}). The former has O-antigens 3, 15, the latter O-antigens (3), (15), 34. Organism A_1 (ϵ^{34}) with O-antigens 3, 10 and phage ϵ^{34} was obtained by the method of Uetake and Hagiwara (1961).

All of the chemical and serologic studies were carried out with lipopolysaccharide prepared essentially by the method of Westphal *et al.* (1952). Cells were grown for about 40 hours at 37° on a tryptone-yeast extract agar (Bertani, 1951)[1] in large baking dishes. The cells were washed from the agar with isotonic sodium chloride, centrifuged, washed with saline, dried with acetone, and stored in a vacuum over $CaCl_2$. The cells were extracted with phenol-water (1:1) at 68° and the combined aqueous extracts were dialyzed, precipitated with acetone, and freed from degraded nucleic acid by ultracentrifugation (for details see Westphal *et al.*, 1952). Approximately half of the lipopolysaccharide is sedimented in the ultracentrifuge in 75 minutes at 140,000 × *g*. The highly polydisperse nature of lipopolysaccharide was confirmed by an examination of the dialyzed phenol extract on Sephadex G-75. Although a large amount of material came directly through the column, the polysaccharide fraction tailed and heavily overlapped the fairly sharp nucleic acid peak even though the size of the nucleic acid fragments had been reduced by a pretreatment with RNase and DNase. All of the experiments reported in this paper were carried out with high-molecular-weight material prepared by the ultracentrifugation technique.

For unexplained reasons, the yield of lipopolysaccharide is much lower from nonlysogenic bacteria. The yield of material from A_1 is only 10–20% of that from A_1 (ϵ^{15}), A_1 (ϵ^{34}), or A_1 (ϵ^{15} ϵ^{34}). The same difference was found with preparations of *S. anatum* 293 and *S. anatum* 293 (ϵ^{15}) provided by Dr. E. S. Lennox. It has not been determined whether the difference is in the amount of polysaccharide, extractability, or sedimentability. Because of this effect most of the studies on antigen 3, 10 were carried out with material extracted from strain A_1 (ϵ^{34}) rather than from A_1. As noted in the results, some of the results on antigen 3, 10 prepared from A_1 (ϵ^{34}) were checked with the small amount of material available from A_1.

Much of the work was carried out with C^{14}-labeled lipopolysaccharide, prepared as follows: C^{14}-labeled sucrose was prepared with *Canna indica* leaves by the usual procedure (Abraham and Hassid, 1957). The alcohol extract from the radioactive leaves was treated with invertase and passed successively through 3 × 1 cm columns of Dowex 50(H^+) and Dowex 1(HCO_3^-). The final effluent contained 2 μmoles/ml each of glucose and fructose. Both sugars had a specific activity of approximately 15 μC/μmole. At least 99% of the radioactivity was accounted for as glucose and fructose when checked by paper chromatography. The glucose-fructose solution was sterilized and added to the yeast extract–tryptone broth or agar to a final concentration of 0.1–1% by volume depending on the specific activity needed in the product. Lipopolysaccharide prepared in this way contained 5–20% of the added C^{14}. As discussed below, each of the monosaccharide units of the polysaccharide had the same specific activity in such preparations.

Carbohydrate concentrations were determined by the phenol–sulfuric acid method (Smith and Montgomery, 1956b). Values on the Klett colorimeter with filter 50 were found to be linear with concentration below readings of 200. The readings with galactose, mannose, and rhamnose are, respectively, 0.64, 0.77, and 0.49 times the reading produced by an equimolar amount of glucose. It is necessary, therefore, to know the composition of an oligosaccharide or polysaccharide before its concentration can be accurately determined by this method. Reducing material was determined by the method of Nelson (1944) and D-glucose with glucose-6-phosphate dehydrogenase. In a 1.5-ml quartz cuvette were placed: 50 μmoles of Tris hydrochloride, pH 7.5; 20 μmoles of $MgCl_2$; 10 μmoles of ATP, 0.5 μmoles of TPN, 5 μg of crystalline hexokinase, and 5 μg of highly purified glucose-6-phosphate dehydrogenase from the Boehringer Co. The neutralized sample and water were present to give a final volume of 1 ml. The amount of TPNH formed did not change after the first few minutes and was stoichiometrically equal to the amount of D-glucose added. The molar extinction coefficient used for TPNH was 6.22×10^3 $M^{-1}cm^{-1}$

[1] The medium was modified by the omission of glucose and by a change of the NaCl concentration from 1% to 0.5%.

at 340 mμ. D-Galactose, D-mannose, and L-rhamnose gave no TPNH under these conditions. Galactose, mannose, and rhamnose were determined by the differential spectrophotometric methods of Dische (1955).

Descending paper chromatography was carried out with 1-butanol, pyridine, water (6:4:3) or other solvents as noted. Sugar spots were detected with $AgNO_3$-NaOH (Block *et al.*, 1958). Methylated sugars were detected on paper with aniline phthalate (Block *et al.*, 1958, p. 181). A chromatogram on which C^{14}-labeled sugars were present was passed through a Nuclear-Chicago strip scanner. The integrated areas on the recording chart were found to be a quantitative measure of the amount of C^{14}-labeled material applied to the paper.

Acid hydrolysis was usually carried out in N H_2SO_4 in a boiling water bath or in 0.4 N H_2SO_4 in a sealed tube at 110°. Samples were neutralized with 0.5 N $Ba(OH)_2$ and concentrated before paper or charcoal chromatography. The charcoal used for chromatography was acid-washed Norite A pretreated with 0.5% stearic acid by the method of Alm (1952). It was mixed with 2 weights of Celite 545 to increase the flow rate.

Periodate oxidation was carried out by the methods outlined by Dyer (1955). Periodate uptake was measured by the standard arsenite iodine procedure and formic acid formation by titration with KOH to a methyl red end point. Methylation was carried out with dimethyl sulfate and sodium hydroxide (Smith and Montgomery, 1960a) followed by treatments with methyl iodide and silver oxide in dimethylformamide (Croon and Lindberg, 1958) and silver oxide in methyl iodide (Purdie and Irvine, 1903). Details of the methylation procedure are given in the text.

β-Galactosidase was prepared from a constitutive mutant of *Escherichia coli* K-12. The fractionation was carried to the dialysis of the 0–40% ammonium sulfate fraction in the procedure of Hogness *et al.* (1955). α-Galactosidase was purified from green coffee beans (Helferich and Vorsatz, 1935). The tannic acid precipitate was purified further by streptomycin fractionation, ammonium sulfate precipitation, and alcohol fractionation at pH 5. Although the specific activity of the final preparation was considerably higher than that of the tannic acid precipitate, the product was still contaminated with traces of β-galactosidase activity and carbohydrate material. α-Glucosidase was prepared from Carling beer yeast. The yeast was frozen with liquid nitrogen and extracted for 3 days at 4° by stirring with an equal volume of 0.05 M K_2HPO_4. After centrifugation the supernatant fluid was treated with RNase (10 μg/ml) for 3 hours at 10°. The solution was then fractionated with solid ammonium sulfate. The fraction precipitating between 55 and 70% saturation was dialyzed overnight at 4° against 0.01 M potassium phosphate, pH 7.2. This fraction was highly active in the hydrolysis of both maltose and methyl-α-D-glucopyranose.

The sugar present at the reducing end of an oligosaccharide was determined either by the borohydride method (Schiffman *et al.*, 1960) or by a semimicro modification of the hypoiodite procedure of Goebel (1927). In the latter procedure 1 mg of oligosaccharide in 0.1 ml of water was treated with 0.066 ml of 0.3 N $BaI_2 \cdot I_2$ solution. During 3 minutes 0.1 ml of 0.5 N $Ba(OH)_2$ was added with shaking. After this preparation had stood for 15 minutes, 0.05 ml of N H_2SO_4 and 0.015 ml of N Na_2SO_4 were added. After centrifugation the supernatant fluid was extracted with ether until colorless. Ether was expelled from the solution by heating briefly at 100°. Finally 0.1 ml of N H_2SO_4 was added and the solution was heated overnight at 110° in a sealed tube. The monosaccharides present were then identified by paper chromatography after neutralization with $Ba(OH)_2$.

Rabbit antisera against O-antigens[2] were prepared by routine methods (Kauffmann, 1954) using *S. anatum* 293 and *S. newington* C_2 and A_1 (ϵ^{15} ϵ^{34}) as immunogens. All sera were heated at 56° for 30 minutes and stored frozen. For preparation of monofactor serum undiluted whole serum was mixed at 50° for 2 hours with heated and washed organisms, and then kept at 3° for 2 days. Anti-34 serum was prepared from anti-A_1 (ϵ^{15} ϵ^{34}) serum by absorbing with A_1 (ϵ^{15}), anti-15 serum from anti–*S. newington* serum by absorbing with A_1, and anti-10 serum from anti–*S. anatum* serum by absorbing with A_1 (ϵ^{15}). After absorption, supernatant fluid was kept at 3° for 1 week to remove any precipitate. Monofactor sera were checked by agglutination, precipitation, and complement-fixation reactions and were stored frozen.

Lipopolysaccharides described above were used for serologic reactions.

The precipitin reaction (Kabat and Mayer, 1948) was carried out by mixing 0.5 ml of 1:2 diluted antiserum containing 0.02% merthiolate with the same volume of C^{14}-lipopolysaccharide solution in ice and incubating at 3° for 42 hours. Precipitates were washed twice with 0.15 M sodium chloride containing 1% bovine serum albumin and then 3 times with 0.15 M sodium chloride. Centrifugation was carried out at 3°. Pellets thus obtained were plated with 0.1 N NH_4OH on planchets for counting radioactivity.

For the purpose of protein measurement in precipitates, washing was continued with 0.15 M sodium chloride alone and the amount of antibody-protein in the precipitate was measured by the method of Lowry *et al.* (1951) with rabbit γ-globulin used as a standard.

To test for the inhibiting capacity of oligosaccharides obtained from partial acid hydrolysis of the lipopolysaccharides, a micro–complement fixation method (Wasserman and Levine, 1960, 1961)[3] was employed. The volume of each reagent was

[2] We would like to express our thanks to Drs. H. Uetake and E. S. Lennox for providing some of the antisera used for this work.

[3] We wish to thank Dr. L. Levine for his help in perfecting the method used in complement-fixation measurements.

scaled up fivefold in the experiments and the total reaction volume was 3.5 ml. Quantities of antiserum and antigen were used to give maximum fixation of complement. Because the original complement (Cappel Laboratories) contained a substance serologically active against lipopolysaccharide (3), (15), 34, complement was absorbed with an equivalent amount of the lipopolysaccharide at 0°. There was no significant decrease in complement activity by such treatment. Sheep red cells were obtained from Probio, Inc., and anti-sheep hemolysin from Carworth Laboratories, Inc. Hemolysis was measured in the Zeiss spectrophotometer at 415 mμ.

Results

Properties of the Polymers.—As mentioned above, the lipopolysaccharide fraction as prepared by the phenol method is highly polydisperse. This might result from the random splitting of a large structure on the bacterial surface by phenol, by aggregation, or by a truly polydisperse nature of the material itself. However, as shown by the experiments below all of the material is active serologically and all of the antigenic determinant groups are present on the same molecule when the lipopolysaccharide is purified by ultracentrifugation. The C^{14}-labeled lipopolysaccharides were used for analysis. As shown in Table I, practically all of the radioactivity of the added lipopolysaccharide was found in the precipitate formed by monofactor or suitable cross-reacting antisera in an excess of antibody or in the equivalence zone. In the earlier studies of several workers (Cohen, 1958; Furth and Landsteiner, 1929; Meyer, 1939; Nakaya and Fukumi, 1953; Staub and Pon, 1956; Stocker *et al.*, 1960) the same conclusions have been reached by serologic methods with bacterial extracts. Recently the findings were confirmed by the quantitative determination of a constituent sugar of the polysaccharide after precipitation with antisera (Luderitz *et al.*, 1960). The claim by Bo and Nava, as reported by Davies (1960), that materials containing antigens 3, 15 and antigen 3 alone can be extracted from *S. newington* we believe to be incorrect. The results could be explained by contamination of monofactor 15 serum with antigen 3, 10.

Table I

Precipitation of C^{14}-Lipopolysaccharides by Monofactor and Cross-Reacting Antibodies

C^{14}-Lipopolysaccharide	Serum	% of Added cpm Recovered in Precipitate
(3),(15),34	anti-34	96–102
(3),(15),34	anti-3,15	94–102
(3),(15),34	anti-3,10	90
3,15	anti-3,10	97
3,10	anti-3,15	103–118
3,15 plus unlabeled (3),(15),34	anti-34	0.5
3,15	anti-34	0.3

Precipitation and counting were carried out as described in Materials and Methods.

Chemical Composition.[4]—The composition of the *Salmonella* lipopolysaccharides has been described in detail by Westphal and co-workers (Westphal, 1960; Kauffmann *et al.*, 1960). The materials consist essentially of a serologically active polysaccharide and a lipid component, lipid A. The latter is partially responsible for the endotoxin activity of the preparation. As shown by Westphal the E-group lipopolysaccharides contain galactose, glucose, mannose, rhamnose, glucosamine, and a heptose. In our preparations from the E-group organisms, galactose, glucose, mannose, and rhamnose were found to account for 60–70% of the dry weight of the material. The glucosamine and heptose are considered not to be part of the antigenic determinants, although it is realized that eventually this assumption may prove to be incorrect. However, these components are present in relatively small amounts and are released only very slowly on acid hydrolysis. Further, these two compounds are present in the lipopolysaccharide of every strain of *Salmonella*, rough or smooth, that has been examined. Therefore, it seems unlikely that they are essential components of the specific determinant structures. It would appear more likely that they are part of the lipid A structure, are present in a nonspecific part of the structure, or are part of the common polysaccharide backbone to which the antigenic side-chains are attached (see below). In any case there is no evidence that they are involved in the antigenic determinants from smooth *Salmonellae*.

The ratios of galactose, glucose, and mannose relative to rhamnose in the E_1, E_2, and E_3 lipopolysaccharides are shown in Table II. The ratio of galactose to mannose to rhamnose is relatively constant. The glucose level needs special comment. The glucose contents of lipopolysaccharides 3,10 and 3,15 were found to depend on the conditions used for growing the organisms. Material prepared from cells grown on liquid tryptone–

Table II

Monosaccharide Compositions Relative to Rhamnose of the E *Salmonella* Lipopolysaccharides

	Lipopolysaccharide		
	3,10	3,15	(3),(15),34
D-Galactose	1.8	1.8	1.9
D-Glucose	0.37	0.25	1.15
D-Mannose	0.9	1.0	0.9
L-Rhamnose	(1.0)	(1.0)	(1.0)

Monosaccharides were determined as described in the text. Samples had been hydrolyzed overnight in sealed tubes at 110° in 0.4 N H_2SO_4 and neutralized with $Ba(OH)_2$. The galactose, mannose, and rhamnose were shown to be D, D, and L, respectively, by measurement of the optical rotations of materials eluted from paper chromatograms. Because of its activity with hexokinase and glucose-6-phosphate dehydrogenase, the glucose must be D-glucose. The mannose was oxidized by glucose oxidase at the same rate as authentic D-mannose.

[4] In the early studies of H. Uetake's laboratory qualitative analyses of Boivin antigens of the E-group *Salmonellae* were carried out (Ise, 1954; Nakagawa, 1954; Sasaki, 1955, 1956).

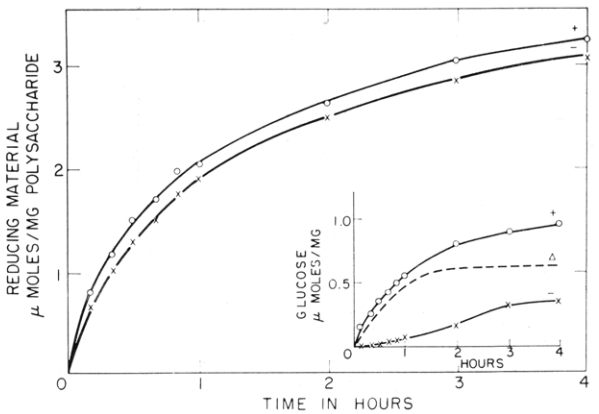

FIG. 2.—Rates of release of copper-reducing material and D-glucose from antigens 3,15 (−) and (3),(15),34 (+). The hydrolysis procedure and assays were carried out as described in the text. The difference between the glucose curves is shown by the dotted line.

yeast extract medium or on agar prepared with mineral salts and galactose contained as much as three times more glucose than cells grown on tryptone–yeast extract agar. The role and consitution of the glucose in the E_1 and E_2 lipopolysaccharide preparations is thus obscure. The possible presence of a contaminating polyglucan in these lipopolysaccharide preparations must be considered seriously. In contrast, glucose appears to be an integral part of antigen (3),(15),34. From the beginning it was noticed that antigen (3),(15),34 contained, in addition to the smaller variable non-acid labile glucose fraction of antigen 3,15, an acid-labile glucose fraction that was approximately equal to the mannose and rhamnose contents of the polysaccharide. Figure 2 shows the release during acid hydrolysis of reducing material and of D-glucose from antigens 3,15 and (3),(15),34. The rates of release of reducing material are practically identical. Antigen 3,15 releases some glucose toward the end of the hydrolysis period. Antigen (3),(15),34 contains this same fraction and in addition a large acid-labile glucose fraction. As can be seen from the difference between the glucose curves, the acid-labile glucose is released without a lag period and with a half-time of hydrolysis of about 25 minutes.

In summary, therefore, the ratios of monosaccharides in the three antigens may be very approximately represented as follows:

	Antigen		
	3,10	3,15	(3),(15),34
Galactose	2	2	2
Glucose	x	x	$x + 1$
Mannose	1	1	1
Rhamnose	1	1	1

The values of x depend on the conditions of growth and may partially be accounted for by polyglucan contamination. The usual values for x range between 0.2 and 0.8.

Products of Partial Acid Hydrolysis.—Paper chromatographic studies showed that the yield of

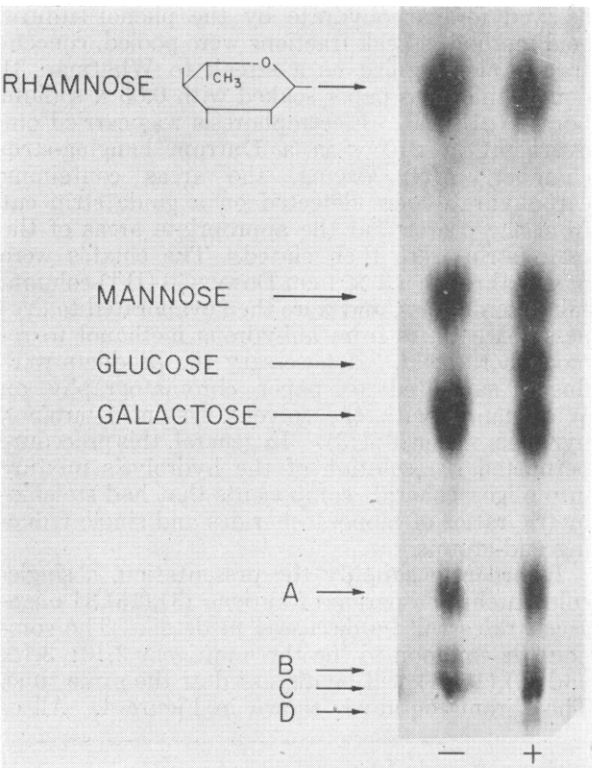

FIG. 3.—Paper chromatogram of 30-minute acid hydrolysates of lipopolysaccharides 3,15 (−) and (3),(15),34 (+). Hydrolysis and chromatography were carried out as described in the text. Spots were developed with $AgNO_3$-NaOH.

low-molecular-weight oligosaccharides was optimal after 20–30 minutes of hydrolysis in N H_2SO_4 at 100°. Preliminary study by paper chromatography in several solvents, two-dimensional chromatography, and borate electrophoresis showed no difference between the oligosaccharide patterns of antigen 3,10 and 3,15. Figure 3 shows the 30-minute hydrolysis pattern for lipopolysaccharides 3,15 and (3),(15),34. Although the patterns are similar, the expected difference in the density of the glucose spot can be seen, as well as the presence of a new oligosaccharide, D, in the acid hydrolysate of antigen (3),(15),34.

A detailed study was carried out with charcoal column fractionation as the primary technique. The surprising result of this work was that all of the major oligosaccharides present after 30 minutes of hydrolysis 0t a single simple sequence of monosaccharides.

The procedure used was as follows. One gram of lipopolysaccharide was hydrolyzed in 50 ml of N H_2SO_4 for 30 minutes in a boiling water bath. After neutralization with $Ba(OH)_2$ and concentration to about 5 ml the solution was applied to a 27 × 170 mm charcoal-Celite column. The column was eluted with a linear gradient prepared by allowing 400 ml of 30% ethanol to run into a mixing chamber containing 400 ml of water. Fractions (about 6 ml) were collected and aliquots were

assayed for carbohydrate by the phenol–sulfuric acid method. Peak fractions were pooled, concentrated, and applied as a streak to Whatman 31 double-thickness paper soaked with 0.05 N sodium borate (pH 9.2). Electrophoresis was carried out overnight at 270 v in a Durrum hanging-strip chamber. After drying, the areas containing carbohydrate were detected on a guide strip cut from the center and the appropriate areas of the main strip were then eluted. The eluates were passed through a 3 × 1 cm Dowex-50 (H^+) column, taken to dryness, and were then evaporated to dryness three times from anhydrous methanol to remove boric acid. If necessary the products were finally subjected to paper chromatography on Whatman 1 with the solvent system 1-butanol, pyridine, water (6:4:3). In general, this procedure permitted a resolution of the hydrolysis mixture into oligosaccharide components that had stoichiometric ratios of monosaccharides and single reducing end-groups.

In order to simplify the presentation, a single-column chromatogram of antigen (3),(15),34 oligosaccharides will be discussed in detail. The components common to the three antigens 3,10; 3,15; and (3),(15),34 will be discussed at the same time. The chromatogram is shown in Figure 4. All of

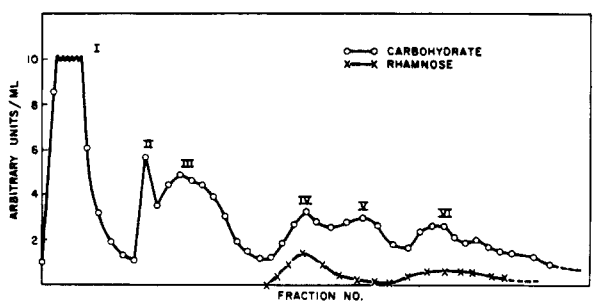

FIG. 4.—Charcoal column chromatogram of a 30-minute acid hydrolysate of lipopolysaccharide (3),(15),34. Details of the procedure and the components present in peaks I-VI are discussed in the text.

the major peaks can be accounted for as the three disaccharides, two trisaccharides, and single tetrasaccharide expected for the single sequence glucosyl-galactosyl-mannosyl-rhamnose.

The compositions of the peaks in Figure 4 are as follows:

PEAK I.—Free galactose, glucose, mannose, and rhamnose are present, as well as a secondary compound that has not been investigated. This minor component is present in all three antigens.

PEAK II.—This sharp peak contains a single component that has an R_F in butanol-pyridine-water identical to that of galactose but is, in fact, a disaccharide. Acid hydrolysis gives mannose and rhamnose. Hypoiodite oxidation destroys the rhamnose without affecting the mannose, so that this disaccharide must be a mannosyl rhamnose. This disaccharide fraction has been obtained from all three antigens.

PEAK III.—Fraction III contains two disaccharides that are easily separated by borate electrophoresis. For the following reasons these disaccharides provided the first indication of the differences among antigens 3,10; 3,15; and (3),(15),34.

Disaccharide IIIa contains equimolar amounts of galactose and mannose, mannose being present at the reducing end. Disaccharide IIIa seemed to be the same in all three antigens until enzymatic studies were carried out. Disaccharide IIIa from antigens 3,15 and (3),(15),34 was completely hydrolyzed to galactose and mannose by β-galactosidase, while that from 3,10 was not split. The disaccharide IIIa from antigen 3,10 was hydrolyzed to the extent of about 80% by α-galactosidase in one experiment, while that from 3,15 and (3),(15),-34 was hydrolyzed only to the extent of about 10%. The less clear-cut results with α-galactosidase can probably be attributed to the lower activity of the enzyme and contamination with traces of β-galactosidase. In any case this result showed the first real difference between antigens 3,10 and 3,15; namely, that the former contains an α-galactosidic linkage and the latter [together with (3),(15),34] contains a β-galactosidic linkage.

Disaccharide IIIb was found only in antigen (3),(15),34. When the unfractionated hydrolysates of the antigens were subjected to paper electrophoresis, disaccharide IIIb could be seen as the only major spot with a mobility lower than that of rhamnose. No corresponding spot appeared in the patterns of antigens 3,10 and 3,15. The disaccharide crystallized readily after borate electrophoresis. It contained equal amounts of glucose and galactose. Galactose was at the reducing end of the disaccharide. The material had 92% of the copper-reducing power of lactose and had a half-time of hydrolysis in N H_2SO_4 of about 25 minutes. There was no detectable hydrolysis by β-glucosidase (commercial emulsin), but there was sluggish hydrolysis by yeast α-glucosidase. Since the 1,2 disaccharide would be expected to have little copper-reducing power and the 1,6 disaccharide is more stable to acid, only 4-*O*-α-D-glucopyranosyl-D-galactose or 3-*O*-α-D-glucopyranosyl-D-galactose were left as possible structures. The optical rotation ($\alpha_D^{20°} = +120°$, $c = 0.25\%$ in H_2O) was close to the value of +140° reported for the α-1,4 disaccharide by Jones and Perry (1957). Subsequent periodate analysis by Whelan was compatible with the structure of disaccharide IIIb as 4-*O*-α-D-glucopyranosyl-D-galactose.[5] This disaccharide

[5] We would like to thank Drs. W. J. Whelan and M. Abdullah for carrying out a periodate oxidation study with borohydride-treated IIIb. The behavior of reduced IIIb in dilute periodate was found to be the same as the behavior of borohydride-reduced maltose. In contrast to the findings with the β-linked disaccharides (Clancy and Whelan, 1959), however, it was found impossible to distinguish between the 1,4 and 1,3 disaccharides by the dilute periodate method. The data do, however, furnish further evidence that the disaccharide could not be linked 1,2 or 1,6. In any case it is felt that the low electrophoretic mobility in borate of IIIb, which is almost identical to the mobilities of maltose and lactose, is strong evidence for the 1,4 rather than the 1,3 glycosidic bond. The 1,3 disaccharide would be expected to have a very substantially greater mobility in borate than that observed for IIIb.

represents a major part of the active center of antigen 34 since, as shown below, it strongly inhibits the monofactor 34–immune system.

In summary, then, peak III from antigen (3),(15),-34 has been shown to contain 4-*O*-α-D-glucopyranosyl-D-galactose and a β-D-galactosyl mannose.

PEAK IV.—The separation between peaks IV, V, and VI was incomplete, but the presence of at least three major components was clear from a monitoring of the fractions by paper chromatography and paper electrophoresis. Although several minor components were present in this region, it was possible to obtain the two major trisaccharides (IV and V) and the major tetrasaccharide (VI) as apparently homogeneous materials by subsequent electrophoresis and paper chromatography.

Peak IV contained galactose, mannose, and rhamnose and appeared to be common to all three antigens. Rhamnose and only rhamnose was destroyed by hypoiodite oxidation or borohydride reduction. The trisaccharide from (3),(15),34 (or 3,15) was split completely by β-galactosidase to give a single spot having the R_F of galactose. Paper electrophoresis gave the expected result that this single spot was a mixture of galactose and the mannosyl rhamnose disaccharide. The trisaccharide from antigen 3,10 was inert to β-galactosidase. When α-galactosidase was used there was substantial hydrolysis of the 3,10 trisaccharide and little hydrolysis of the compound from (3),(15),34 (or 3,15). Again the less clear-cut results with α-galactosidase can probably be accounted for by the lower activity of the enzyme and contamination with β-galactosidase.

PEAK V.—Peak V was found to be unique to antigen (3),(15),34 and to contain galactose, glucose, and mannose. Mannose proved to be at the reducing end of the trisaccharide. Since it was anticipated that the sequence might be glucosyl-galactosyl-mannose, successive enzymatic hydrolysis with α-glucosidase and β-galactosidase seemed to be the best approach. However, α-glucosidase, which acted only sluggishly on disaccharide IIIb, gave no hydrolysis of V at all. As can be seen in Figure 5, however, partial acid hydrolysis gave the two disaccharides IIIa and IIIb. Further, as is seen, treatment of the mixture with β-galactosidase gave galactose, mannose, and intact IIIb. Thus, the sequence *O*-α-D-glucopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl-D-mannose seems justified.

PEAK VI.—The main component of peak VI corresponds to the unique spot D (see Fig. 3) of antigen (3),(15),34. This material has not been investigated in detail but appears to contain galactose, glucose, mannose, and rhamnose, rhamnose being located at the reducing terminal position. There is no reason to believe that this is not the tetrasaccharide that completes the series of expected oligosaccharides. As stated before, an outstanding characteristic of the 30-minute acid hydrolysis mixtures is their relative cleanness. Even though some of the minor components may hold important clues to the total structures there seems little reason to doubt that a large part of the material can be accounted for by the major sequence. Further, the simple changes in the major galactosyl-mannosyl-rhamnose structure are correlated with the changes in antigenic structure and the presence of phages ϵ^{15} and ϵ^{34}.

To summarize the results of partial acid hydrolysis, it has been shown that the major oligosac-

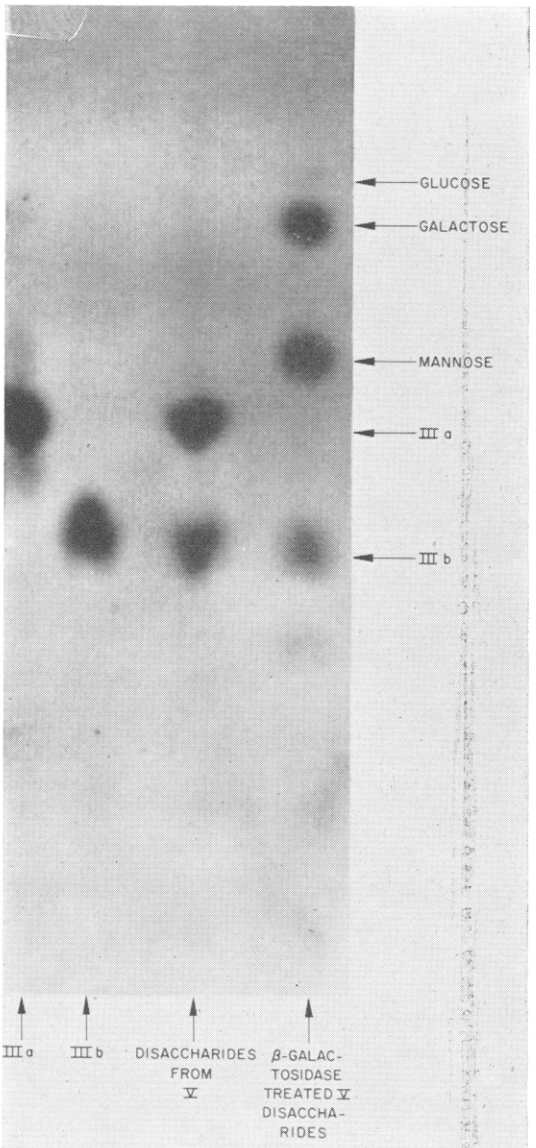

FIG. 5.—Borate electrophoresis pattern of disaccharides derived from peak V before and after treatment with β-galactosidase. The main component of peak V was purified by the general procedure described in the text and was then hydrolyzed in N H_2SO_4 for 10 minutes in a boiling water bath. Paper chromatography showed the presence of mannose, glucose, galactose, a disaccharide area, and unchanged V. The disaccharide area was eluted from the paper and an aliquot of the eluate was treated with β-galactosidase. Electrophoresis was carried out in 0.05 M sodium borate and the paper was sprayed with $AgNO_3$ and NaOH.

charide sequences of the three antigens are as follows:

Antigen	Sequence
3,10	α-D-Galactosyl-mannosyl-rhamnose
3,15	β-D-Galactosyl-mannosyl-rhamnose
(3),(15),34	α-D-Glucosyl-β-D-galactosyl-mannosyl-rhamnose

An explanation is thus provided for the interrelationship between phages ϵ^{15} and ϵ^{34}. In organisms carrying phage ϵ^{15} a galactose moiety is inserted into the polysaccharide in a β rather than an α configuration. Since this β-linked galactose unit is the same galactose moiety glucosylated by cells carrying phage ϵ^{34}, it is seen that the presence of ϵ^{15} is necessary before the converting activity of ϵ^{34} can take effect. In other words, glucosylation can take place only on a β-D-galactosyl–containing precursor, presumably because of the specificity of the enzyme involved in transglucosylation.

Inhibition Analysis.—Serologic inhibition analysis was used to show that the major oligosaccharides discussed above are largely responsible for the antigenic determinants of the intact polysaccharide. The tests were carried out by the micro-complement fixation method. As shown in Table III, 4-*O*-α-D-glucopyranosyl-D-galactose completely inhibited the monofactor 34 immune system, while the monofactor 15 and 10 systems were not inhibited significantly. On the other hand, the β-D-galactosyl-mannose disaccharide and β-D-galactosyl-mannosyl-rhamnose trisaccharide inhibited only the 15 system. The inhibiting properties of the trisaccharide were the same whether it was prepared from antigen 3,15 or from (3),(15),34. The serologic findings thus confirm the assumptions made from chemical evidence that the α-D-glucosyl configuration is largely responsible for the determinant group 34 and that the β-D-galactosyl-mannose unit is the active center of determinant 15. Further evidence for an α-D-glucosyl residue at the active center of antigen 34 was found when it was discovered that monofactor 34 serum cross-reacts with glycogen. The known partial loss (Nagakawa, 1959; Uetake and Hagiwara, 1961) of antigens 3 and 15 that occurs after the addition of antigen 34 by ϵ^{34} may well be caused by the steric effect produced by the α-D-glucosyl residue that is added to part of the active center of antigen 15. Although it is assumed that the α-D-galactosyl-mannosyl-rhamnose sequence may well represent the 10 determinant group, no definitive inhibition results have yet been obtained with this system.

Periodate Oxidation—In contrast to the polysaccharides from A, B, and C group *Salmonellae* studied by Tinelli and Staub (1959), the E-group lipopolysaccharides undergo extensive oxidation by sodium metaperiodate at 3°. Oxidation is about 75% complete after 1 hour, and there is little if any further reduction of periodate after 4 days. Table IV gives the range of values in various experiments for periodate reduction and formic acid formation from lipopolysaccharides 3,10; 3,15; and (3),(15),34. Although there is variation in the data it is clear that well over 1 mole of periodate is reduced per mole of hexose + rhamnose and that almost half a mole of formic acid is formed per mole of monosaccharide.

Two carefully controlled experiments were performed in which the periodate oxidation behaviors of antigens 3,15 and (3),(15),34 were compared under identical conditions and the data were normalized to correct for the extra glucose content of the E_3 material. In these experiments it was found that the (3),(15),34 antigen took up 1.6 and 2.1 more moles of periodate per mole of acid-labile glucose and formed 0.6 and 0.7 more moles of formic acid per mole of acid-labile glucose than antigen 3,15. While these measurements are subject to a number of possible errors and the interpretation

TABLE IV

PERIODATE REDUCTION AND FORMIC ACID FORMATION BY THE E *Salmonella* LIPOPOLYSACCHARIDES

	Lipopolysaccharide 3,10	3,15	(3),(15),34
	(moles/mole monosaccharide)		
Periodate	1.1–1.4	1.1–1.2	1.3–1.5
Formic acid	0.42–0.44	0.35–0.43	0.43–0.48

The experiments were carried out as described in the text. Oxidation was allowed to proceed for 4 days at 0° before analysis.

TABLE III

INHIBITION OF MONOFACTOR 10, 15, AND 34 IMMUNE SYSTEMS BY OLIGOSACCHARIDES

Oligosaccharide	Quantities Added (μmoles)	% Inhibition (3),(15),34 vs. anti 34	% Inhibition 3,15 vs. anti 15	% Inhibition 3,10 vs. anti 10
4-*O*-α-D-Glucopyranosyl-D-galactose	1.0	72	10	9
	1.5	97	13	10
β-D-Galactosyl-mannose	0.74	0	69	0
β-D-Galactosyl-mannosyl-rhamnose from antigen (3),(15),34	0.025	<7	60	3
	0.20[a]	14	99	
β-D-Galactosyl-mannosyl-rhamnose from antigen 3,15	0.028	<6	74	<3
	0.45	<6	104	3

Complement fixation was carried out as described in the text. Controls showed that the oligosaccharides did not cause procomplementary or anticomplementary effects.

$$\% \text{ Inhibition} = \frac{\text{O. D. in the presence of inhibitor} - \text{O. D. of the immune system}}{\text{O. D. of the antibody control} - \text{O. D. of the immune system}} \times 100$$

For the immune systems 10, 15, and 34, the following combinations were employed respectively; anti-10 serum and lipopolysaccharide 3,10; anti-15 serum and lipopolysaccharide 3,15; anti-34 serum and lipopolysaccharide (3),(15),34.

[a] Higher concentrations were not tested because of the small amount of available oligosaccharide.

cannot be taken too seriously, they did at least suggest that the acid-labile glucose of antigen (3),(15),-34 is attached to a position in antigen 3,15 that cannot be oxidized by periodate. This suggestion was initially very puzzling in view of the picture presented by the products of partial acid hydrolysis and clearly showed the necessity for methylation analysis.

The products of periodate oxidation were investigated by paper chromatography after sodium borohydride reduction of the polyaldehyde, dialysis, and complete acid hydrolysis. The patterns obtained in this way with the three antigens were indistinguishable. The principal products found were galactose, glucose, and glycerol. At least 90% of the mannose and rhamnose were destroyed. Not even a trace of threitol or erythritol could be found. These limiting conditions make it possible to estimate roughly the nature of the hexose linkages in the polymer. The considerations are as follows: since the rhamnose is not terminal (see below) and is oxidized by periodate, it must be linked 1,2; 1,4; or 1,5. In any of these cases it would take up only a single mole of periodate and would not give rise to formic acid. Therefore, the periodate uptake by the hexoses can be estimated by subtracting from the total uptake an amount equal to the rhamnose content of the polymer. Since a monosaccharide unit that gives rise to formic acid must take up two moles of periodate, it is clear from the data in Table IV that 70 to 100% of the hexose units that are oxidized must be giving rise to formic acid and must, therefore, be terminal or linked 1,6. Although it is possible that some of the hexose is linked 1,2 or 1,2,6 it seems even more likely that the estimated periodate uptakes may be somewhat high and formic acid yields somewhat low. It is known, for instance, that formic acid is oxidized slowly by periodate. It is possible, then, that all of the hexose that is oxidized by periodate is either terminal or 1,6 linked. This situation is discussed further in connection with the methylation studies.

In order to obtain a quantitative estimate of the recovery of galactose, glucose, and glycerol, C^{14}-labeled lipopolysaccharide 3,15 was oxidized with excess periodate for 5 days at 3°. The material was reduced with excess borohydride and half of the sample was then dialyzed and hydrolyzed. Since 27% of the C^{14} was lost on dialysis of the polyalcohol, and since it seemed that the loss might be selective, the remainder of the borohydride-treated sample was simply passed through Dowex 50 (H^+) to remove sodium ions and hydrolyzed directly. A control sample of C^{14} antigen untreated by periodate was carried through the same procedure. In the control sample galactose, glucose, mannose, and rhamnose were found to account for 44, 7, 22, and 25%, respectively, of the C^{14}. This result confirms the uniform specific activity of the monosaccharide components of the lipopolysaccharide (*cf.* Table II). In the oxidized-dialyzed and oxidized-undialyzed sample, galactose, glucose, and glycerol were found to account for 35, 10, and 25% (dialyzed) and 32, 7, and 30% (undialyzed), respectively, of the C^{14}. This shows that all of the glucose and 69 to 76% of the galactose is resistant to periodate oxidation. This figure could be somewhat high, however, because of selective loss of C^{14}-labeled materials other than glucose or galactose. In any case, the primary importance of the 1,6 linkage or terminal linkage in the polymer is confirmed by the high yield of glycerol.[6]

Hexose that resists periodate oxidation is presumably linked 1,3 if it is not present at a branching point. Therefore, most of the galactose that is not oxidized by periodate must be 1,3 linked, since the methylation studies (see below) show that only a small fraction of the galactose of antigens 3,10 and 3,15 can be accounted for as branching points. As discussed below it is believed that the 1,3-linked galactose fraction may be the site of attachment of glucose in antigen (3),(15),34. In any case, it is clear that although partial acid hydrolysis showed the acid-labile glucose to be linked 1,4 to a galactose moiety, no threitol was formed after periodate oxidation and borohydride reduction and the patterns of oxidation products obtained seemed identical for the three antigens. These facts argue in favor of the proposition that this glucose is attached to a nonterminal galactose unit of the antigen 3,15 structure.

Methylation Studies.—In order to follow the material during the course of methylation and to simplify the analytical problem, methylation was carried out with C^{14}-labeled materials. In addition, all operations were carried out simultaneously so that all three antigens were treated identically. In the preliminary stages four methylations with dimethylsulfate and sodium hydroxide at room temperature were carried out. Dialysis and lyophilization were used to recover the material between treatments. The materials were then methylated four times with methyl iodide and silver oxide. The first treatment was carried out in *N*,-*N*-dimethylformamide (Croon and Lindberg, 1958) and subsequent treatments used methyl iodide itself as the solvent (Purdie and Irvine, 1903). The final product had a chloroform/water partition coefficient of about 50 and was soluble in diethyl ether but insoluble in petroleum ether. The final recovery of C^{14} was about 20% in each case. The losses seemed to be gradual and were probably mechanical. No methyl group analysis was carried out, since the presence of lipid in the material would make its interpretation difficult.

The methylated lipopolysaccharides were hydrolyzed first in 88% formic acid and then in N H_2SO_4 for 6 hours. After neutralization with $Ba(OH)_2$ samples were spotted or banded on Whatman 1 and

[6] The calculated yield of glycerol is 20–22%. The even higher figure obtained experimentally may be the result of selective loss of materials other than glycerol during the procedure or may be caused by C^{14}-contamination in the glycerol area of the chromatogram.

separated in 2-butanone containing 1% NH_3. Banded sheets were put through the strip counter and the areas obtained from the recording strip were integrated for quantitative measurement. Carbohydrate areas were visualized with aniline phthalate. For larger scale separation the hydrolysates were fractionated on Celite 535 partition columns (Lemieux *et al.*, 1956) with 2-butanone–1% NH_3 as the moving phase and water as the stationary phase. The monosaccharides present in each peak were identified by treatment with HBr followed by chromatography according to the method of Hough *et al.* (1950).

In each case approximately 10% of the C^{14} was present at the origin of the paper chromatogram in an area that did not stain with aniline phthalate. This material was not investigated further and is not included in the subsequent calculations and discussion. Except for a trace of C^{14} at the solvent front, the remaining 90% of the radioactive material all occurred in areas where the radioactivity and staining material coincided exactly. The staining pattern for the three antigens is shown in Figure 6.

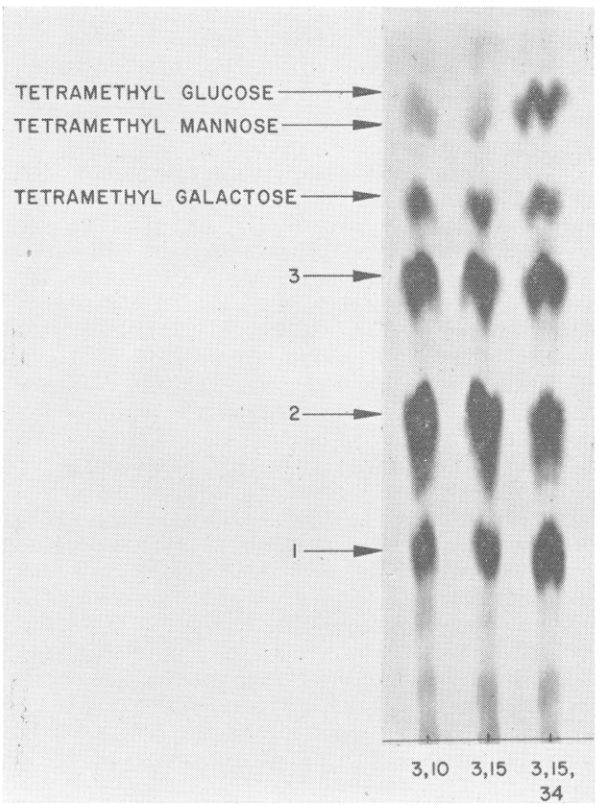

FIG. 6.—Paper chromatogram of methylated derivatives from antigens 3,10; 3,15; and (3),(15),34. The lipopolysaccharides were methylated, hydrolyzed, and chromatographed as described in the text. Spots were visualized with aniline phthalate. The spot slightly below area 2 is methyl red, which was used as an indicator during the neutralization of the hydrolysis mixtures. A comparison of the staining spots with a radioautograph made before staining shows that the lower of the two spots in area 2 is not radioactive or at least has a relatively low radioactivity-to-staining ratio.

Area 1 of antigens 3,10 and 3,15 contained mainly glucose and a trace of galactose, while area 1 of antigen (3),(15),34 contained mainly galactose. Area 2 contained only galactose and area 3 contained mannose and rhamnose in each case. Table V gives the R_F of each area, its radioactivity, and its percentage of total radioactivity. On the assumption that the basic repeating unit of each antigen contained 4 units of mannose and 4 units of rhamnose (see Fig. 1) the figures were all normalized by assigning a value of 8.0 to the mannose-rhamnose area 3. The other figures in the last column, therefore, represent the numbers of other residues in the presumed repeating unit.

The most striking feature of the methylation patterns is the close similarity of the three antigens. No significant difference, either qualitative or quantitative, can be seen between lipopolysaccharides 3,10 and 3,15. This is expected if the only difference between the two antigens is the anomeric configuration of a galactose unit. The major differences between antigens 3,15 and (3),(15),34 are the appearance of tetramethylglucose as a major component in antigen (3),(15),34, a decrease in the trimethylgalactose area, and the appearance of a dimethylgalactose. The order of magnitude of these changes is as expected if the glucose were attached to a singly-substituted galactose unit in the interior of the polymer. Further, there is no difference in tetramethylgalactose contents between the two methylated antigens so that the glucose clearly cannot be attached to what was previously a terminal galactose moiety. As seen above, this result was anticipated by the quantitative and qualitative results of periodate analysis.

Total Structures.—Although the methylation analysis is far from complete, the approximate state of the monosaccharide units of the three antigens may be summarized as follows:

MANNOSE AND RHAMNOSE.—The mannose of the antigens can be accounted for as a trimethylmannose. From the results of periodate analysis this would presumably be 2,3,4-trimethylmannose. The rhamnose can be accounted for as a dimethyl rhamnose that has the same R_F as the trimethylmannose. This methylated mannose plus rhamnose spot (area 3 of Fig. 6) accounts for approximately the same percentage of the total radioactivity as the percentage of mannose plus rhamnose in the unmethylated antigens. The rhamnose in the unmethylated polymer is oxidizable by periodate and must, therefore, be linked 1,2; 1,4; or 1,5. Since no propylene glycol was found during the examination of the products of borohydride reduction obtained from periodate-oxidized polysaccharide, the 1,2-linkage seems less likely than 1,4 or 1,5. The finding that rhamnose in the oligosaccharide fractions is always present at the reducing end shows that the rhamnose glycosidic link is rather acid-labile. This may be an indication that the rhamnose is present in the furanose form, since furanosides are known to be more readily hydrolyzed by acid than pyranosides. Thus, the

TABLE V

QUANTITATIVE ANALYSIS OF METHYLATED C^{14}-LIPOPOLYSACCHARIDES

Area	R_F	Radioactivity (Arbitrary Units) 3,10	3,15	(3),(15),34	Radioactivity (%) 3,10	3,15	(3),(15),34	Radioactivity Normalized to Area 3 3,10	3,15	(3),(15),34
Tetramethylglucose / Tetramethylmannose	0.85 / (0.82)	188	252	628	10	9	20	1.74	1.73	4.20
Tetramethylgalactose	0.72	95	144	156	5	5	5	0.88	0.99	1.04
3	0.62	866	1164	1196	45	43	38	(8.0)	(8.0)	(8.0)
2	0.42	574	864	276	30	32	9	5.30	5.90	1.85
1	0.25	200	276	854	10	10	28	1.85	1.90	5.72

Methylation, hydrolysis, and chromatography were carried out as described in the text. The total radioactivity represents integrated areas obtained from the recording chart of the strip scanner. The radioactivity is directly proportional to monosaccharide concentration, since all of the monosaccharides have the same specific activity.

furanose form of rhamnose, linked 1,5 in the intact antigens, must be given serious consideration. There is no reason to believe that either rhamnose or mannose is present in more than one major type of linkage in any of the antigens.

GLUCOSE.—In the case of methylated antigen (3),(15),34, tetramethylglucose is seen to account for 20% of the recovered C^{14} (Table V). In the unmethylated material acid-labile glucose accounts for about 18% of the monosaccharide units. Thus, the glucose for which phage ϵ^{34} is responsible is located largely or completely at terminal nonreducing positions. Since the amounts of tetramethylgalactose are the same in antigens 3,15 and (3),(15),-34 it is clear that the addition of glucose to a terminal galactose residue is not involved. The addition of glucose does, on the other hand, lead to a decrease in the yield of trimethylgalactose and the appearance of a dimethylgalactose derivative. The surprisingly high total of 50% of all monosaccharide units of antigen (3),(15),34 are seen to be accounted for as branching points plus nonreducing terminal groups.

The picture with regard to antigens 3,10 and 3,15 is not quite as clear, and it is possible that part of the radioactivity present in the tetramethylglucose and tetramethylmannose area may represent contamination. The light aniline phthalate staining in this area indicates that there are probably only small amounts of terminal glucose and/or mannose in these two antigens. In any case it is apparent that at most only 1 in 7 monosaccharide units may be present in a nonreducing terminal position. No terminal glucose or mannose in the structures of antigens 3,10 and 3,15 is shown in Figure 1 because of the uncertainty of its presence and the lack of any indication of where such a residue might be located.

GALACTOSE.—Galactose is the major building block of the polymers and occurs at terminal, nonterminal, and branching points. It probably occurs in at least 3 or 4 different types of linkage in the polysaccharides. A considerable amount is presumably present in the basic structure to which the antigenic side-chains are attached (see below).

SUMMARY OF RESULTS

The concept that the *Salmonella* antigens consist of a basic polysaccharide skeleton to which are attached specific antigenic side-chains has been strengthened by the recent finding of Westphal (personal communication) that on brief acid hydrolysis, lipopolysaccharide preparations from smooth organisms give rise to material that cross-reacts with antibodies prepared with rough strains of *Salmonella*. All rough strains of Salmonella are known to form the same "rough" antigen. This R-antigen consists mainly of galactose and glucose (Luderitz *et al.*, 1960). The easiest explanation for these facts would be that the galactose and glucose of the rough antigen form, in fact, a relatively acid-stable framework on which the complex smooth antigens are built. A rough mutant would thus be one that had lost the ability to synthesize the antigenic side-chains through the loss of one of the biosynthetic or condensing enzymes that must be necessary to elaborate these structures. Even though "roughness" is a complicated phenomenon which involves changes in a protein as well as changes in polysaccharides, the explanation above could still apply to roughness as it refers to polysaccharide structure.

In regard to the "backbone" or substructure of the E-group antigens there is little information. However, if the antigenic side-chains of antigens 3,10 and 3,15 are assumed to contain all of the mannose and rhamnose of the antigens, as seems likely, and if the side-chains have a ratio of mannose/rhamnose/galactose of 1:1:1, then the analytical results in Table II show that the substructure would contain galactose and glucose in a ratio of approximately 3 or 4:1. Further, since the methylation of antigens 3,10 and 3,15 gave rise to a dimethyl glucose derivative but little, if any, dimethylgalactose, the glucose units of the substructure would presumably serve as the site for the attachment of the antigenic side-chains. At present, however, the evidence for these proposals can be considered only suggestive. Low-molecular-weight oligosaccharides derived from the substructure were not found in appreciable amounts after partial acid hydrolysis, presumably because of its relatively acid-stable characteristics.

If the idea of a basic galactose-glucose skeleton modified by antigenic side-chains is accepted then the type of structure suggested by the data for the group E antigenic side-chains is that shown in Figure 1. The surprising simplicity of the products of partial acid hydrolysis shows that much of the material in the O antigens of E-group *Salmonellae* can be accounted for by the sequence galactosyl-

mannosyl-rhamnosyl. In antigen 3,10 the galactose is present as the α-anomer and in 3,15 it is linked in the β-configuration. If the yield of tetramethylgalactose is considered, however, it is seen that at least four of these trisaccharide units must be attached end-to-end in the intact antigen. We assume that the inter-unit rhamnosyl-galactose linkages are more acid-labile than the intra-unit bonds. This accounts for the pattern of acid degradation products found. The 1,3-linkage of the galactose is assigned on the basis of the qualitative and quantitative results of periodate oxidation and on studies with larger oligosaccharide derivatives obtained after very brief acid hydrolysis. These oligosaccharides appear to be the hexa- and nonasaccharides predicted from the structure in Figure 1. Both of these oligosaccharides contain the expected amount of periodate-stable galactose, while the mannose and rhamnose are completely destroyed by periodate. The fact that the acid-labile glucose of antigen (3),(15),34 is probably attached to the galactose unit of the galactose-mannose-rhamnose sequence but that this galactose unit cannot be terminal (see periodate and methylation experiments) is further compelling evidence for the type of structure presented. Other formulations are possible, such as . . .-rhamnosyl-X-galactosyl - mannosyl - rhamnosyl - Y - galactosyl- . . . in which X and Y are, for example, short galactose-glucose oligosaccharides, but these structures do not take into account the probable existence of the common acid-stable substructure or the moderately high branching indicated by methylation.

Staub (1960) has pictured the *Salmonella* antigens as consisting of a basic skeleton with side-chains of differing structure, each corresponding to different antigenic determinants. However, it seems possible that if the side-chains contain, for instance, 10–20 monosaccharide units rather than the 1–4 postulated by Staub, all of the side-chains of any given antigen could have similar structures and there would still be enough opportunity for the structural diversity necessary to explain the various smooth determinants present in a given antigen. In other words, all the antigenic determinants not only would be present in the same molecule but would be present on the same side-chain. This implies the further possibility that the side-chains of all smooth *Salmonellae* might, in fact, be based on a single basic plan that is modified more or less extensively in each of the major antigenic groups. In this regard it is interesting to note that Stocker *et al.* (1960) and Tinelli and Staub (1960) have found that the glucosyl-galactosyl-mannosyl-rhamnose sequence constitutes an important part of the antigenic polysaccharide of *Salmonella typhimurium*. Although this is the same order of monosaccharides found in hydrolysates of antigen (3),(15),34, the E_3 *Salmonellae* give no cross-reaction with *S. typhimurium*. The most probable explanation for such a situation would be that the two sequences contain different linkage positions and different anomeric configurations. These ideas are admittedly speculative but, we believe, deserve consideration on the basis of the type of structures discussed above.

Conclusions

The conversion from antigen 3,15 to antigen (3),(15),34 brought about by phage ϵ^{34} will be discussed first.

The most likely explanation for this change is that glucose is added to the 3,15 antigen structure during the course of its biosynthesis. Even though it has been shown that antigen 34 can be detected within a few minutes of the time of phage infection (Hagiwara, 1959; Uetake and Hagiwara, 1961), it seems very unlikely that glucose is added to preformed antigen 3,15 that is already on the cell surface. Such a mechanism would require a biosynthetic type of reaction to take place outside the cell membrane. We have, further, experimental evidence to show that this type of reaction is unlikely.[7] It seems clear that the presence of the phage brings about the change in the antigenic structure during the course of its biosynthesis. This must also apply to the 3,10 to 3,15 conversion by ϵ^{15}.

Our major efforts recently have been directed toward the detection of a glucose-adding enzyme. The postulated mechanism for the enzymatic process is as follows:

antigen 3,15 + XDPG → antigen (3),(15),34 + XDP

It is assumed that the glucose addition step is the final one in the biosynthetic sequence. In such a case, the 3,15 polysaccharide would react with a nucleotide glucose donor, shown here as X diphosphate glucose, to give the (3),(15),34 polysaccharide and X diphosphate. Other mechanisms such as the condensation of activated oligosaccharides deserve consideration. However, the lack of a glucose unit on the terminal galactose moiety of the side-chain of antigen (3),(15),34 could be explained most simply if the glucose addition were the last step in the biosynthetic sequence. If the (3),(15),-34 antigen were formed by the condensation of an activated glucosyl-galactosyl-mannosyl-rhamnose tetrasaccharide it would be expected that the terminal galactose unit would also carry a glucose residue, which is not the case (see Fig. 1). If the glucose addition were the final step, however, it can be easily imagined that the transferring enzyme might direct the addition of glucose only to the 1,3-linked galactose residues.

In any case it seems likely that the phage genome

[7] Phage ϵ^{34} and C^{14}-glucose were added simultaneously to a culture of A_1 (ϵ^{15}) in broth and incubation was carried out for 30 minutes. The labeling pattern in the monosaccharides was compared to the labeling pattern for control cultures of A_1 (ϵ^{15}) and fully lysogenic A_1 ($\epsilon^{15}\epsilon^{34}$). It was found that the labeling pattern in the freshly infected culture showed a relatively low glucose content similar to that observed with A_1 (ϵ^{15}). An abnormally high glucose incorporation might be expected if glucose had been added to preformed antigen 3,15 after the introduction of ϵ^{34}.

is responsible for the formation of a new and specific glucose-adding enzyme. The possibility cannot be completely dismissed, however, that the glucose addition comes about as a secondary result that stems from a change produced by the phage in the carbohydrate metabolism of the cell. Along these lines we have tested the levels of UDPG pyrophosphorylase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase in extracts of A_1 (ϵ^{15}) and A_1 (ϵ^{15} ϵ^{34}) and found the activities to be identical.

It is interesting that the glucose units added in response to the presence of ϵ^{34} are added to the β-galactosyl unit that has its anomeric configuration determined as the result of the presence of ϵ^{15}. If it is assumed that the glucose-adding enzyme is specific for this β-galactosyl accepting group then the over-all converting scheme for ϵ^{15} and ϵ^{34} discussed above is clear. In the organism E_1 (ϵ^{34}) (see diagram above) the glucose-adding enzyme may be formed but the necessary β-galactosyl acceptor is lacking. Thus, the expression of the effect of ϵ^{34} on the antigenic structure would be prevented.

The conversion from 3,10 to 3,15 produced by ϵ^{15} may be more complex than the 3,15 to (3),(15),-34 conversion. Stated most simply the change involves the attachment of a galactose group to a mannosyl residue in a β rather than an α configuration. Since the organism does not, however, lose the genetic determinants for the 3,10 antigen (Uetake *et al.*, 1955, 1958) the phage gene must take preference or be dominant to the bacterial gene. Whether this dominance operates through a mechanism that involves repression is an open question.

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References

Abraham, S., and Hassid, W. Z. (1957) *in* Methods in Enzymology, vol. IV, Colowick, S. P., and Kaplan, N. O., editors, New York, Academic Press, Inc., p. 489.
Alm, R. S. (1952), *Acta Chem. Scand. 6*, 1186.
Barksdale, L. (1959), *Bacteriol. Rev. 23*, 202.
Bertani, G. (1951), *J. Bacteriol. 62*, 293.
Block, R. J., Durrum, E. L., and Zweig, G. (1958), A Manual of Paper Chromatography and Paper Electrophoresis, New York, Academic Press, Inc.
Clancy, M. J., and Whelan, W. J. (1959), *Chem. and Ind.*, 673.
Cohen, H. H. (1958), *J. Immunol. 81*, 445.
Croon, I., and Lindberg, B. (1958), *Acta Chem. Scand. 12*, 453.
Davies, D. A. L. (1960), *Advances in Carbohydrate Chem. 15*, 271.
Dische, Z. (1955), *Methods of Biochem. Anal. 2*, 313.
Dyer, J. R. (1955), *Methods of Biochem. Anal. 3*, 111.
Furth, J., and Landsteiner, K. (1929), *J. Exp. Med. 49*, 727.
Goebel, W. F. (1927), *J. Biol. Chem. 72*, 809.
Hagiwara, S. (1959), *J. Virol. (Kyoto) 9*, 472.
Helferich, B., and Vorsatz, F. (1935), *Z. Physiol. Chem. Hoppe-Seyler's 237*, 254.
Hogness, D. S., Cohn, M., and Monod, J. (1955), *Biochim. et Biophys. Acta 16*, 99.
Hough, L., Jones, J. K. N., and Wadman, W. H. (1950), *J. Chem. Soc.* 1702.
Ise, T. (1954), *Sapporo Med. J. 5*, 101.
Iseki, S., and Sakai, T. (1953), *Proc. Japan Acad. 29*, 127.
Jones, J. K. N., and Perry, M. B. (1957), *J. Am. Chem. Soc. 79*, 2787.
Kabat, E. A., and Mayer, M. M. (1948), Experimental Immunochemistry, Springfield, Ill., Charles C Thomas.
Kauffmann, F. (1954), Enterobacteriaceae, Copenhagen, Munksgaard.
Kauffmann, F., Luderitz, O., Stierlin, H., and Westphal, O. (1960), *Zentr. Bakteriol. Parasitenk. Abt. I Orig. 178*, 442.
Lederberg, J. (1955), *J. Cellular Comp. Physiol. 45*, 75.
Lemieux, R., Bishop, C., and Pelletier, G. (1956), *Can. J. Chem. 34*, 1365.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Luderitz, O., Kauffmann, F., Stierlin, H., and Westphal, O. (1960), *Zentr. Bakteriol. Parasitenk. Abt. I Orig. 179*, 180.
Luderitz, O., O'Neill, G., and Westphal, O. (1960), *Biochem. Z. 333*, 136.
Meyer, K. (1939), *Ann. inst. Pasteur 62*, 282.
Nakagawa, A. (1959), *Japan J. Bacteriol. 14*, 693.
Nakagawa, T. (1954), *Sapporo Med. J. 5*, 220.
Nakaya, R., and Fukumi, H. (1953), *Jap. J. Med. Sci. and Biol. 6*, 17.
Nelson, N. (1944), *J. Biol. Chem. 153*, 375.
Purdie, J., and Irvine, J. C. (1903), *J. Chem. Soc. 83*, 1020.
Sasaki, T. (1955), *Japan J. Bacteriol. 10*, 997.
Sasaki, T. (1956), *Japan J. Bacteriol. 11*, 11; 107.
Schiffman, G., Kabat, E. A., and Leskowitz, S. (1960), *J. Am. Chem. Soc. 82*, 1122.
Smith, F., and Montgomery, R. (1956a), *Methods of Biochem. Anal. 3*, 163.
Smith, F., and Montgomery, R. (1956b), *Methods of Biochem. Anal. 3*, 182.
Staub, A. M. (1960), *Ann. inst. Pasteur 98*, 814.
Staub, A. M., and Pon, G. (1956), *Ann. inst. Pasteur 90*, 441.
Stocker, B. A. D., Staub, A. M., Tinelli, R., and Kopacka, B. (1960), *Ann. inst. Pasteur 98*, 505.
Tinelli, R., and Staub, A. M. (1959), *Bull. soc. chim. biol. 41*, 1221.
Tinelli, R., and Staub, A. M. (1960), *Bull. soc. chim. biol. 42*, 583.
Uetake, H. (1956) *in* Proc. Intern. Genet. Symposia, Tokyo and Kyoto, p. 645.
Uetake, H., and Hagiwara, S. (1960), *Nature 186*, 261.
Uetake, H., and Hagiwara, S. (1961), *Virology 13*, 500.
Uetake, H., Luria, S. E., and Burrous, J. W. (1958), *Virology 5*, 68.
Uetake, H., Nakagawa, T., and Akiba, T. (1955), *J. Bacteriol. 69*, 571.
Uetake, H., and Uchida, T. (1959), *Virology 9*, 495.
Wasserman, E., and Levine, L. (1960), *Fed. Proc. 19*, 205.
Wasserman, E., and Levine, L. (1961), *J. Immunol. 87*, 290.
Westphal, O. (1960), *Ann. inst. Pasteur 98*, 789.
Westphal, O., Luderitz, O., and Bister, F. (1952), *Z. Naturforsch. 7b*, 148.