The Biosynthesis of Cell Wall Lipopolysaccharide in Escherichia coli

VII. STUDIES ON THE STRUCTURE OF THE O-ANTIGENIC POLYSACCHARIDE*

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SUMMARY

The cell wall lipopolysaccharide of Escherichia coli 0111-B4 was degraded by (a) acid hydrolysis and (b) by treatment with periodate, followed by reduction with sodium borohydride and mild acid hydrolysis. The various oligosaccharides produced by these procedures were isolated and characterized by chemical and enzymatic methods. Procedure a released all of the colitose (3,6-dideoxy-α-xylohexose) as the free sugar along with several oligosaccharides in very low yield, three of which were identified as Glc + GlcNAc, Glc 3 Gal, and GlcNAc β Glc ↔ Gal. The application of Procedure b resulted in the liberation of approximately one-half of the colitose as the free sugar along with a mixture of two colitose-containing oligosaccharides:

Colitose-1 α 4-Glucose-1 β 2-Threitol

Colitose-1 α 6-N-Acetyl glucosamine-1

Pentasaccharide

Colitose-1 α 4-Glucose-1 β 2-Threitol

N-Acetyl glucosamine-1

Tetrasaccharide

Analysis of the partially degraded polymer prior to the acid hydrolysis step indicated that galactose had been totally destroyed by periodate oxidation and that there was a concomitant formation of an equivalent amount of threitol in the product. The origin of threitol from galactose was confirmed by degradation of the lipopolysaccharide isolated from a mutant without UDP-α-galactose 4-epimerase (Escherichia coli J-5) which incorporated 1-14C-galactose or uniformly labeled 14C-galactose directly into the galactose residues of the polymer. Colitose-containing oligosaccharides isolated in the experiment with 1-14C-galactose yielded unlabeled threitol, while the oligosaccharides isolated in the uniformly labeled 14C-galactose experiment yielded radioactive threitol with approximately two-thirds the specific activity of the galactose used.

The cell wall lipopolysaccharides of gram-negative bacteria are antigenic macromolecules composed of a variety of carbohydrates, fatty acids, organic phosphate, and ethanolamine. The structural details of these polymers have been studied extensively. These investigations have been reviewed recently by Luderitz, Staub, and Westphal (2). Escherichia coli 0111-B4 produces a cell wall lipopolysaccharide in which the structure of the lipid and “core” polysaccharide portions of the polymer appear to be very similar, if not identical, to the corresponding portions of lipopolysaccharides isolated from other species of enteric bacteria. However, the O-antigenic portion of the polymer from E. coli composed of α-glucose, β-galactose, N-acetyl-α-glucosamine (2-acetamido-2-deoxy-α-glucose), and colitose (3,6-dideoxy-α-xylohexose), possesses a distinctive structure which can account for its specific antigenic activity. Hereafter, the detailed structure of the O-antigenic polysaccharide of this organism was not known, although Luderitz et al. (3) demonstrated that colitose is terminally located in the polymer and that the colitol groups are the primary O-antigenic determinants of the molecules. Elucidation of the structure of the O-antigenically active portion of these polymers was complicated by the extreme acid-lability of the colitose bonds; thus, the use of classical methods of mild acid hydrolysis to obtain

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low molecular weight oligosaccharides from the polymers was not possible.

This paper describes the isolation and characterization of a series of oligosaccharides obtained by partial acid hydrolysis of the lipopolysaccharide of E. coli 0111-B4, as well as by application of the technique of polysaccharide degradation developed by Goldstein, Hamilton, and Smith (4). The latter procedure provided conditions of degradation that permitted the isolation of oligosaccharides which contained a large proportion of the colitose residues of the polymer. On the basis of structural analyses of these oligosaccharides, we have proposed the following structure for the antigenically-active oligosaccharide subunit of the lipopolysaccharide:

\[
\text{Colitose-1} \xrightarrow{\alpha} 4\text{-Glucose-1} \xrightarrow{\beta} 4\text{-Galactose} ; \text{Colitose-1} \xrightarrow{\alpha} 6\text{-N-Acetyl glucosamine}
\]

**EXPERIMENTAL PROCEDURE**

**Analytical Methods**—For complete sugar analysis of lipopolysaccharide or oligosaccharides, the samples were hydrolyzed in 2 N HCl for 2 hours at 100°; prior to analysis, HCl was removed by distillation under reduced pressure into a trap containing 50% NaOH. Qualitative analysis of the hydrolysates was conducted by chromatography on Whatman No. 1 filter paper. In certain cases mentioned in the text, the paper was first treated with sodium borate as described by Cabib, Leloir, and Cardini (5).

For preparative chromatography, Whatman No. 3MM paper was used unless otherwise specified. In all cases, descending development was used with one of the following solvents: Solvent A, 2-propanol-water (8:2); Solvent D, 1-butanol-pyridine-water (4:4:2); Solvent C, 1-butanol saturated with water; Solvent D, 1-butanol-2-propanol-water (1:7:2); Solvent E, ethyl acetate-acetic acid-water (upper phase, 3:1:3); Solvent F, ethyl acetate-pyridine-water (upper phase, 2:1:2); Solvent G, 1-butanol-pyridine-0.1 N HCl (5:3:2); Solvent H, 1-butanol-ethanol-water (10:1:2). After solvent development, the chromatograms were dried and the location of compounds was detected as follows: reducing sugar, by alkaline silver nitrate (6); polysaccharides and nonreducing oligosaccharides, by the periodate-benzidine stain of Gordon, Thornburg, and Wern (7); N-acetyl hexosamines, by ethanolic NaOH (8); nonreducing oligosaccharides containing colitose which were relatively insensitive to both the silver and the periodate reagents were detected by elution of 1 cm of transverse sections of the chromatogram, followed by the colorimetric determination of colitose in the eluates as described below.

Quantitative methods were: glucose and galactose determined by Glucostat and Galactostat (Worthington); glucosamine by the method of Elson and Morgan (9) as modified by Rosman and Daffner (10); N-acetylg glucosamine by the procedure of Aminoff, Morgan, and Watkins (11); heptose by the method of Osborn (12). The determination of threitol depended on the method of Korn (13). The value obtained in this manner is presented as a ratio in which it is compared to the amount of glucose eluted from the same chromatogram. Colitose was determined by a modification of the method of Cynkin and Ashwell (14). The sample and standards (0.002 to 0.05 μpmole), each in 0.3 ml of 0.2 N H2SO4, were heated for 20 min at 100°. This was found to release all of the colitose from either the oligosaccharides or lipopolysaccharide. The tubes were cooled to 55° and 0.2 ml of 0.05 N NaOH, in 0.25 N H2SO4, was added. After 30 min at 55°, 0.5 ml of 4% NaNO3 in 0.4 N HCl was added and after the yellow color disappeared, 2 ml of 0.6% thiorbituric acid were added. The tubes were heated at 100° for 10 min, cooled, and 0.1 ml of 50% NaOH was added. The absorbance at 544 nm was determined (molar extinction coefficient was 9.8 × 10^4 liter mole-1 cm-1).

**Preparation of Lipopolysaccharide**—Lipopolysaccharide was prepared by the phenol extraction of washed cell wall preparations of E. coli 0111-B4 as previously described (15). In certain cases, the relatively insoluble material obtained in this manner was treated with alkali to increase its solubility prior to further degradation. One gram of lipopolysaccharide was dissolved in 200 ml of 0.2 N NaOH and heated at 60° for 30 min. The pH was then adjusted to 5.0 with glacial acetic acid and 6 volumes of absolute ethanol were added. The suspension was cooled in ice for several hours; the precipitate was collected by centrifugation and washed with 200 ml each of 95% ethanol, absolute ethanol, acetone, and ether. This procedure yielded 790 mg of a white, readily soluble powder which contained 85% of the colitose originally present in the untreated lipopolysaccharide.

**Specific Hydrolytic Enzymes—β-Glucosidase (almond emulsion)** was obtained from Sigma. α-Glucosidase, prepared from yeast, was kindly supplied by Dr. M. J. Osborn (Department of Molecular Biology, Albert Einstein College of Medicine, New York); with maltose as substrate, 0.025 ml of this preparation catalyzed the liberation of 1 μmole of glucose per hour. 2-Acetamido-2-deoxy-β-d-glucosidase was the gift of Dr. H. Heymann (Ciba Pharmaceutical Company). The latter enzyme was isolated from boar epididymis according to the method of Findlay and Levy (16); with p-nitrophenyl-2-acetamido-2-deoxy-β-d-glucoside as substrate, 0.005 ml of this preparation catalyzed the liberation of 1 μmole of N-acetyl glucosamine per 5 min.

**RESULTS**

**Oligosaccharides Produced by Acid Hydrolysis**

Lipopolysaccharide was suspended in 0.5 N HCl and heated at 100° for 30 min. After cooling, the precipitate (lipid A) was removed by centrifugation and the supernatant liquid was neutralized by the addition of an excess of Dowex-3 (free base form). The neutral solution was then concentrated under reduced pressure to a small volume and streaked on Whatman No. 3 MM paper. The chromatograms were developed with Solvent A overnight; then, after drying, guide strips were removed from the edges and stained by the alkaline silver method. This procedure resulted in the separation of the mono-oligosaccharides from a wide band of oligosaccharides. The latter were eluted and rechromatographed with Solvent G. The second chromatography allowed the isolation of three apparently homogeneous oligosaccharides listed in Table I. Each oligosaccharide moved as a single spot on chromatography in the solvents listed. The yields

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1 A weakly basic anion exchanger, Dow Chemical Corporation, Midland, Michigan.
of oligosaccharides indicate that most of the sugars were liberated as free hexoses during the hydrolysis. In addition to these three oligosaccharides, small amounts of slower moving products were observed, but these presumably higher molecular weight oligosaccharides were not characterized.

Analysis of Oligosaccharides-The three oligosaccharides were completely free of colitose as determined by the thiobarbituric acid reaction; aldoheptose was also absent. After hydrolysis in 2 N HCl for 2 hours, paper chromatography in Solvent A revealed the presence of glucose and glucosamine in oligosaccharide A-1, glucose and galactose in A-2, and glucose, galactose and glucosamine in A-3. Quantitative analyses were performed on similar hydrolysates and the results are shown in Table II. In parallel experiments, the oligosaccharides were treated with excess sodium borohydride before hydrolysis in order to destroy the terminal reducing sugar. The data indicate that: A-1 is a glucosyl-glucosamine; A-2 is a glucosylgalactose; and A-3 is a trisaccharide containing 1 mole each of glucose, glucosamine and a reducing terminal galactose.

Specific Enzymatic Hydrolysis of Oligosaccharides—Three portions of A-2 (3.5 μmoles each) were incubated for 24 hours at pH 5.0 (acetate buffer) with either α-glucosidase, β-glucosidase, or no enzyme. At the end of the incubation, 10% of the mixture was spotted on paper and chromatographed in Solvent A. The chromatogram showed that hydrolysis was absent with β-glucosidase or with no enzyme, but the oligosaccharide was completely degraded by α-glucosidase to glucose and galactose. This confirmed the structure of A-2 as glucosylgalactose and demonstrates that the glucoside bond is in the α configuration. A-3 was resistant to treatment with α-glucosidase, which indicates that the glucose was substituted by glucosamine. Confirmation of this was achieved by subjecting A-3 to the action of 2-acetamido-2-deoxy-β-D-glucosidase. This treatment resulted in the liberation of a compound which migrated on paper identically with N-acetyl-α-glucosamine and a compound having an R α of the same as that found for A-2. After treatment with the 2-acetamido-2-deoxy-β-D-glucosidase, the glucose in A-3 was released by treatment with α-glucosidase. No glucose was released from intact A-3 by 2-acetamido-2-deoxy-β-D-glucosidase alone. These studies indicated that A-3 is a trisaccharide of the following type: GlcNAc β-GlcαGal. Further, it is suggested that A-2 (Glc-Gal) is identical with the disaccharide obtained by the removal of N-acetyl-α-glucosamine from A-3.

Periodate Oxidation of A-3—A-3 (1.2 μmoles) was oxidized with NaIO₄ (50 μmoles) at pH 4.5 for 18 hours at 0°C. At the end of the oxidation, 500 μmoles of ethylene glycol were added as a control, an identical mixture was prepared, except that the ethylene glycol was added at the beginning, before the oligosaccharide. After the destruction of the excess periodate, aldehyde groups were reduced by the addition of 1000 μmoles of NaBH₄. After 2 hours, excess borohydride was destroyed with HCl and removed by treatment with HCl-methanol and vacuum distillation of the resulting methyl borate. The oxidized oligosaccharide and its control were then deionized with mixed bed ion exchange resin; hydrolyzed in 2 N HCl for 2 hours, and then analyzed for glucose and glucosamine. The data in Table III show that while 30% of the glucose and 50% of the glucosamine were isolated from the control, both sugars were absent from the material subjected to periodate oxidation. These results suggest that the N-acetyl-α-glucosamine was bound to the glucose at position 2, 4, or 6, since a bond to carbon 3 of the glucose would have rendered the glucose resistant to periodate oxidation.

Isolation of Colitose-containing Oligosaccharides

Degradation of Lipopolysaccharide—Alkali-treated lipopolysaccharide (B-1; 410 mg) was dissolved in 50 ml of water, adjusted to pH 6.8, and 60 ml of 0.1 N NaOH were added. The solution was allowed to stand at room temperature in the dark for 16 hours. Ethylene glycol (3.35 ml, 60 μmoles) was added to destroy the remaining periodate. After cooling to 4°C, 4.3 g (120 mmoles) of NaBH₄ were added and the mixture was maintained at 4°C for 2 hours. The solution was brought to room tem-

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### Table I

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Rα</th>
<th>Solvent A</th>
<th>Solvent B</th>
<th>Solvent H</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-1</td>
<td>0.74</td>
<td>0.64</td>
<td>0.37</td>
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</tr>
<tr>
<td>A-2</td>
<td>0.64</td>
<td>0.50</td>
<td>0.25</td>
<td>4</td>
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</tr>
<tr>
<td>A-3</td>
<td>0.51</td>
<td>0.27</td>
<td>0.08</td>
<td>6</td>
<td></td>
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</tbody>
</table>

* Yield is based on percentage of glucose isolated in the fraction compared to the amount of glucose in the original lipopolysaccharide preparation.

### Table II

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Molar ratio</th>
<th>Before reduction</th>
<th>After reductiona</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Glc</td>
<td>Gal</td>
<td>GlcN</td>
</tr>
<tr>
<td>A-1</td>
<td>1.00</td>
<td>0.00</td>
<td>0.95</td>
</tr>
<tr>
<td>A-2</td>
<td>1.00</td>
<td>1.01 &lt;0.03</td>
<td>1.00</td>
</tr>
<tr>
<td>A-3</td>
<td>1.00</td>
<td>1.04 0.98</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* Reduction was carried out by mixing 1 μmole of oligosaccharide with 10 μmoles of NaBH₄. Total volume was 100 μl. After 1 hour at room temperature, the remaining borohydride was destroyed with 1 drop of concentrated HCl. The borate was then removed by distillation with methanol. The sample was then deionized with mixed bed resin and subjected to hydrolysis and analysis as described in “Experimental Procedure.”

### Table III

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Sugar</th>
<th>Before periodate</th>
<th>After periodate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.73/2.40</td>
<td>0.00/2.40</td>
<td>100</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>1.20/2.40</td>
<td>0.00/2.40</td>
<td>100</td>
</tr>
</tbody>
</table>

* Fraction indicates recovery of sugar from reaction mixture. Final hydrolysis volume was 0.5 ml.

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* Equal parts of Dowex 1-HCO₃⁻ and Dowex 50-H⁺.
Fractior

Analysis of oligosaccharides produced by oxidative degradation

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E. coli J-5 produces an incomplete lipopolysaccharide when tion P-1, the only apparent source of threitol in the Smith deg-

eythritol and glycerol. Considering the composition of Frac-
solvent systems to threitol and was clearly distinguished from

was observed on chromatograms that corresponded in several

B-2, either with galactose oxidase or by paper chromatography,

2 colitose, 1 glucosamine, 1 glucose. Analysis of hydrolysates of

was concentrated under reduced pressure at 30° to 3 ml (B-2).

water; the water was changed twice daily. The dialyzed solution

ysis of the mixture for 3 days against 100 volumes of distilled

was destroyed by the addition of 20 ml of acetone. Low molec-

temperature and, after an additional 2 hours, the residual NaBH₄

was destroyed by the addition of 20 ml of acetone. Low molec-

ular weight products and inorganic salts were removed by dial-

ysis for 3 days against 100 volumes of distilled water; the water was changed twice daily. The dialyzed solution

was concentrated under reduced pressure at 30° to 3 ml (B-2).

Analysis of B-2 (Table IV) indicated a molar composition of

2 colitose, 1 glucoamine, 1 glucose. Analysis of hydrolysates of

B-2, either with galactose oxidase or by paper chromatography, indicated the absence of galactose. However, a new compound was observed on chromatograms that corresponded in several solvent systems to threitol and was clearly distinguished from erythritol and glycerol. Considering the composition of Fraction B-1, the only apparent source of threitol in the Smith deg-
radiation of the polymer would have been from a 4-O-substituted galactose residue. This proposal was confirmed by studies which utilized a mutant organism of E. coli 0111-B₄. This mut-
tant, E. coli J-5 (15), lacks UDP-galactose 4-epimerase and, consequently, it is unable to synthesize UDP-galactose unless provided with preformed galactose in growth media. Further, E. coli J-5 produces an incomplete lipopolysaccharide when grown in the absence of galactose, but, in the presence of galac-
tose, produces lipopolysaccharide which is indistinguishable from that produced by E. coli 0111-B₄. In the case of E. coli J-5, if

1⁴C-galactose is provided in the growth medium, the only radio-
activity in the lipopolysaccharide is found in the galactosyl res-

ides. Two cultures of E. coli J-5 were grown in Trypticase Soy broth (50 ml) containing 25 μmoles of either uniformly labeled ¹⁴C-galactose (11,100 cpm per μmole) or ¹⁴C-galactose (75,000 cpm per μmole). The cells were harvested after 18 hours of growth, and the cell wall lipopolysaccharides were iso-
lated by phenol extraction. Hydrolysis of a portion of the lipopolysaccharides followed by paper chromatography indicated that in each case the only radioactive component was galactose. Each of the lipopolysaccharide preparations was then degraded

as described above to Fraction B-2, which was subjected to hydrolysis in 2 N HCl for 1 hour at 100°. After concentration to dryness, the samples were chromatographed in Solvent B. Scan-
ing of the paper strips for radioactivity indicated that all of the radioactivity in the sample from uniformly labeled ¹⁴C-galactose-
grown cells was located in the position of threitol; no radioactivity was detectable in the area of galactose. In contrast, no signifi-

In this experiment had been absolutely uniform in the distribu-
tion of radioactivity among the 6 carbon atoms, the specific activity of threitol derived from it would have been predicted to be four-sixths of that of the galactose, or 7400 cpm per μmole. Thus, the value obtained for the specific activity of threitol was within 10% of the predicted theoretical value. On the basis of

data, it was concluded that (a) all of the galactose present in the polymer was destroyed by the degradation procedure and (b) threitol found in Fraction B-2 was derived solely from galac-
tose (presumably C-3 through C-6) in the lipopolysaccharide.

\[ \text{Fig. 1. Release of colitose from degraded lipopolysaccharide (B-2) by acid hydrolysis. A sample of Fraction B-1, equivalent to 2.1 μmoles of bound colitose in 5 ml of 0.01 N H}_2\text{SO}_4 \text{ (final pH, 2.4) was heated at 100°; 0.1-ml aliquots were removed at the times indicated, cooled in ice, then analyzed for free colitose. The values given for } k_1 \text{ and } k_2 \text{ are the apparent first order rate constants.} \]

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Description</th>
<th>Molar ratio</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Colitose: GlcN: Gic: Threitol: Gal</td>
<td></td>
</tr>
<tr>
<td>B-1</td>
<td>Alkali-treated lipopolysaccharide</td>
<td>1.17: 0.95: 1.00: 0.46</td>
<td>100a</td>
</tr>
<tr>
<td>B-2</td>
<td>Degraded lipopolysaccharide</td>
<td>1.92: 1.04: 1.00: 0.00</td>
<td>98a</td>
</tr>
<tr>
<td>B-3</td>
<td>Pentasaccharide plus tetrasaccharide</td>
<td>1.24: 1.00: 1.00: 1.02</td>
<td>34a</td>
</tr>
<tr>
<td>B-4</td>
<td>Pentasaccharide</td>
<td>2.14: 0.00: 1.00: 0.88</td>
<td>14a</td>
</tr>
<tr>
<td>B-5</td>
<td>Triasaccharide</td>
<td>0.00: 0.00: 1.00: 0.08</td>
<td>20a</td>
</tr>
<tr>
<td>B-6</td>
<td>Triasaccharide</td>
<td>0.00: 1.08: 1.00: 1.04</td>
<td>68a</td>
</tr>
<tr>
<td>B-7</td>
<td>Disaccharide</td>
<td>0.00: 0.00: 1.00: 0.90</td>
<td>88a</td>
</tr>
</tbody>
</table>

\[ a \text{ Based on recovery of colitose from B-1.} \]
\[ b \text{ Based on recovery of glucose from the immediate precursor (B-3).} \]
\[ c \text{ Based on recovery of glucose from B-6.} \]
Partial Acid Hydrolysis of Degraded Polymer—In order to determine optimal conditions for the isolation of colitose-containing oligosaccharides, a portion of B-2 was subjected to hydrolysis in 0.01 N H₂SO₄. Aliquots were removed at intervals and analyzed for free colitose. The results shown in Fig. 1 indicate that colitose is bound to the polymer by two types of bonds which are distinguished by their sensitivities to acid hydrolysis. Further, the two types of colitosyl units are present in nearly equal amounts. Hydrolysis in 0.1 N H₂SO₄ also yielded a diphasic curve, but the initial rate was so rapid that 50% of the colitose was liberated in 2 min. Analysis of the mixtures from the 0.01 N H₂SO₄ hydrolysis showed no free glucose, glucosamine, or N-acetyl glucosamine.

On the basis of the studies described above, the remainder of Fraction B-2 was adjusted to pH 2.0 with 2 N H₂SO₄ and heated at 100° for 20 min. After cooling rapidly, the slight precipitate (which contained no colitose) was removed by centrifugation and the pH was adjusted to 9 with NH₃OH. Again the precipitate (which was free of colitose) was removed and the supernatant solution was applied to a column (4.3 × 53 cm) of Sephadex G-25 (Pharmacia) which had been equilibrated with 0.015 N NH₃OH. Irrigation of the column with 0.015 N NH₃OH resulted in the elution of two fractions which contained colitose (Fig. 2). The last peak, which emerged between 660 and 740 ml of effluent, contained free colitose (96 μmoles). The material which was eluted between 500 and 640 ml contained 123 μmoles of glycosidically-bound colitose (B-3). In addition to the two colitose-containing peaks, a small amount of turbid effluent appeared in the void volume (340 to 419 ml). This material contained all of the phosphorus eluted from the column and was assumed to be the residue of the lipid and core polysaccharide after removal of the α-antigenic polysaccharide portion of the polymer. Preliminary experiments had shown that prior to hydrolysis, degraded lipopolysaccharide (B-2) was also eluted in the void volume. The relative position of elution of B-3 from the column indicates a molecular weight in the range of 500 to 2500. Qualitative analysis of hydrolysates of B-3 by paper chromatography in Solvents A and B revealed the presence of colitose, glucosamine, glucose, and n-threitol. The identity of the threitol was confirmed by its chromatography on borate-treated paper in Solvent F. The R₀₁ values were: glycerol, 3.86; erythritol, 1.96; threitol, 1.50; and polyol from B-3, 1.48. Authentic n-threitol was kindly supplied by Dr. N. K. Richtmyer of the National Institutes of Health. The results of quantitative analysis of Fractions B-1, B-2, and B-3 are shown in Table IV. The molar composition of B-3 indicated an oligosaccharide mixture composed of glucose, glucosamine, threitol, and colitose in a molar ratio of 1:1:1:1.24, respectively. The molar ratios of the sugars indicated that the basic structure may be a trisaccharide containing GlcN(Ac)₂, Glc, and threitol; the nonintegral value for colitose suggested a mixture in which a portion of the basic trisaccharide had one colitose attached and the remainder (20%) possessed two colitosyl units. Paper chromatography of B-3 in Solvents A through E gave no indication of separation of a possible mixture of oligosaccharides; in each case B-3 appeared to be a single colitose-containing compound. However, if B-3 were the proposed mixture, it was thought possible that the oligosaccharide possessing 1 colitosyl unit may be susceptible to degradation by either a glucosidase or a 2-acetamido-2-deoxy-β-D-glucosidase, while the other (with 2 colitosyl residues) would be resistant.

α- and β-Glucosidase Treatment of B-3—Two samples of B-3 (0.3 μmole of colitose each) were incubated separately with 0.025 ml of α-glucosidase and 1 mg of β-glucosidase. The mixtures were buffered at pH 5 with 0.1 M acetate and incubated for 48 hours at 37°. No glucose was liberated in either case. Thus, all of the glucose in B-3 appeared to be protected by colitose or N-acetylglucosamine or both.

2-Acetamido-2-deoxy-β-D-glucosidase Treatment of B-3—An excess of 2-acetamido-2-deoxy-β-D-glucosidase (0.025 ml) was incubated with 41 μmoles of B-3 for 26 hours at 37°. As shown in Fig. 3, 70% of the N-acetyl-D-glucosamine was released. Addition of more enzyme (10 μl) at 20 hours had no further effect. The incubation mixture was applied to a column (1.5 × 110 cm) of Bio-Gel P-2 (from Bio-Rad). Irrigation of the column with water resulted in elution of three carbohydrate-containing peaks (Fig. 4); the last compound to emerge from the column (160 to 180 ml) was shown to be free N-acetyl-D-glucosamine (18.4 μmoles) by its reactivity in the modified Morgan-Kister reaction (11) and its mobility on chromatography using borate-treated paper with Solvent F. The material had an R₀₁ of 0.09; the corresponding value for N-acetyl-D-galactosamine was 0.61.

The two peaks that emerged from the column before free N-acetylglucosamine both contained glycosidically-bound colitose;
the fractions composing these peaks were pooled (110 to 130 ml, Fraction B-4, and 140 to 160 ml, Fraction B-5). Preliminary analysis, B-4 was rerun on the same column to remove traces of N-acetyl-D-glucosamine. A portion of B-3 (25 pmol based on colitose) was hydrolyzed in 0.2 N HCl for 15 min at 100°. After neutralization with NaOH and treatment with mixed-bed ion exchange resin, the products were separated by paper chromatography on Schleicher and Schuell 589 Green Ribbon paper and developed for 18 hours with Solvent A. Two benzidine periodate-sensitive areas were detected; one component moved as free colitose and the other had an R₆₅₀ of 0.67. Elution yielded 17 pmol of the latter material (B-6) which on analysis was found to contain equivalent amounts of glucose, glucosamine and threitol (Table IV).

**Mild Acid Hydrolysis of B-3**—A portion of B-3 (25 pmol based on colitose) was hydrolyzed in 0.2 N HCl for 15 min at 100°. After neutralization with NaOH and treatment with mixed-bed ion exchange resin, the products were separated by paper chromatography on Schleicher and Schuell 589 Green Ribbon paper and developed for 18 hours with Solvent A. Two benzidine periodate-sensitive areas were detected; one component moved as free colitose and the other had an R₆₅₀ of 0.67. Elution yielded 17 pmol of the latter material (B-6) which on analysis was found to contain equivalent amounts of glucose, glucosamine and threitol (Table IV).

**Enzymatic Degradation of B-6**—Incubation of B-6 (3.4 pmol) with 2-acetamido-2-deoxy-β-D-glucosidase (0.05 ml) for 48 hours at pH 4.4 at room temperature, resulted in the cleavage of the N-acetyl-D-glucosamine from the trisaccharide. Paper chromatography of the products in Solvent B yielded, in addition to N-acetyl-D-glucosamine, 3.0 pmol of a nonreducing substance (B-7) which had an R₆₅₀ of 0.80. Analysis showed that B-7 contained equal amounts of glucose and threitol. Although B-6 was not degraded by α-glucosidase, glucose in the disaccharide B-7 was completely liberated by α-glucosidase treatment with a concomitant release of free threitol. The requirement for the sequential rather than independent degradation by the two enzymes indicates that B-6 is a trisaccharide having the structure GlcNac-Glc-threitol.

**Degradation of Trisaccharide B-5**—Analysis of B-5 indicated 1 mole each of colitose, glucose, and threitol. Since threitol does not have a reducing group, the other two sugars must be bound to this polyol through their reducing groups. In order to determine whether the two sugars were attached to the threitol independently or sequentially, the experiment described in Table V was performed, and it indicated that undegraded B-5 was not susceptible to the action of α-glucosidase. However, after mild hydrolysis, which removed colitose, the glucosyl residue was liberated completely by the enzyme. This pattern of degradation indicated that B-5 was a linear trisaccharide of the type Col-Glc-threitol.

**Periodate Oxidation of B-4, B-5, and B-6**—Samples of the three oligosaccharides (0.5 to 1.0 pmol of each) were treated with 30 pmol of NaIO₄ at pH 4.0 for 16 hours at room temperature. The oxidation was terminated by the addition of 30 pmol of ethylene glycol followed by 150 pmol of NaBH₄. After 2 hours, the remaining borohydride was destroyed by the addition of 0.1 ml of acetone. The samples were then dried, redissolved in 1 ml of water, and treated with an excess of mixed-bed resin. The resin was removed by filtration and washed three times. The washings and supernatant fluids were combined, concentrated to dryness, and the residue was hydrolyzed in 2 ml of 2 N HCl for 2 hours at 100°. The hydrolysates were then analyzed for glucose and glucosamine. As shown in Table VI, the only sugar in any of the oligosaccharides that was significantly resistant to periodate was the glucose in the pentasaccharide B-4. These data suggested that each of the trisaccharides, Col-Glc-threitol (B-5) and GlcNac-Glc-threitol (B-6), possessed a glucosyl residue which was substituted at either position 2 or 4; substitution of glucose at position 3 in either of the oligosaccharides would have rendered the glucose residue resistant to periodate oxidation in both; if position 3 is unsubstituted and either of the substituents on glucose in the pentasaccharide (Fraction B-4) were at

<table>
<thead>
<tr>
<th>TABLE V</th>
<th>Periodate oxidation of B-4, B-5, and B-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligosaccharide</td>
<td>Amount added*</td>
</tr>
<tr>
<td></td>
<td>Glc</td>
</tr>
<tr>
<td>B-4</td>
<td>0.95</td>
</tr>
<tr>
<td>B-5</td>
<td>1.10</td>
</tr>
<tr>
<td>B-6</td>
<td>0.50</td>
</tr>
</tbody>
</table>

* Based on glucose content. Amount of glucosamine added was: B-4, 0.94 pmol; B-6, 0.54 pmol. See the text for details of procedure.
position 6, periodate oxidation would have destroyed the glucose in this oligosaccharide (see Fig. 5). Further, since the N-acetyl glucosamine residue of the pentasaccharide was susceptible to periodate oxidation, it is probable that the second colitose residue of this oligosaccharide was linked to position 6 of N-acetyl glucosamine.

Therefore, the pentasaccharide possesses a glucosyl residue which is doubly substituted at positions 2 and 4 by a colitose residue in one instance and by a colitose-2-acetamido-2-deoxy-glucosyl residue in the other. In order to determine which of these substituents was linked to the glucose residue at position 2 and which was linked at position 4, the two trisaccharides (B-5 and B-6) were subjected to Smith degradation and the products were identified. Examination of the proposed structures for these two oligosaccharides (see Fig. 5) reveals that this procedure would yield erythritol only from the oligosaccharide that possessed a 4-O-substituted glucosyl residue; the tetratol would be derived from C-3, C-4, C-5, and C-6 of the glucose molecule. Of the identifiable products, one would predict that, in addition to erythritol, glycerol would be obtained from both compounds and intact colitose would be obtained from Fraction B-5. Approximately 1 μmole each of Fractions B-5 and B-6 were dissolved in 2 ml of 0.05 M NaIO₄ at pH 4. After incubation at room temperature for 4 hours in the dark, the solutions were adjusted to pH 8 with NaOH and treated with 0.5 ml of 1 M sodium borohydride. The mixture was placed at room temperature for 30 min and excess borohydride was destroyed by the addition of 1 ml of acetic acid. The solutions were then acidified with HCl and concentrated to dryness under reduced pressure. The samples were dissolved in methanol, acidified with HCl and concentrated to dryness to remove methyl borate; this procedure was repeated six times. The residues were dissolved in water, treated with an excess of mixed-bed ion exchange resin, and hydrolyzed in 2 ml of 0.2 N H₂SO₄ at 100° for 10 min. After cooling, the mixtures were neutralized by treatment with an excess of mixed-bed ion-exchange resin, concentrated to a small volume, and chromatographed on Whatman No. 1 paper for 17 hours in Solvent System A. The results of this experiment, shown in Fig. 6, indicate that erythritol was obtained as a product of degradation of the trisaccharide B-5 (Colitose-Glc-threitol) with no detectable tetratol formed from the trisaccharide B-6 (GlcNAc-Glc-threitol). On the basis of these data, it was concluded that the glucose residue of the pentasaccharide (Fraction B-4) is substituted at position 4 by a colitose group and at position 2 by a colitose-2-acetamido-2-deoxy-glucosyl group. Further evidence to strengthen the proposal that N-acetyl glucosamine is bound to the glucose moiety at position 2 is based on the unusual acid-lability of N-acetyl glucosamine in the trisaccharide B-6 (GlcNAc-Glc-threitol). Thus, hydrolysis of this oligosaccharide in 1 N HCl at 100° for 30 min resulted in the complete liberation of N-acetyl glucosamine; this unusual instability of a 2-acetamido-2-deoxy-glucoside has been shown to be characteristic of those linked to the 2-posi-
The application of this degradation procedure to the cell wall lipopolysaccharide of \textit{E. coli} 0111-B4 resulted in the isolation of a mixture of a pentasaccharide and a tetrasaccharide which contained approximately 40\% of the total polymer-colitose intact. The procedure resulted in the complete destruction of galactose with the concomitant formation of an equimolar quantity of threitol which was terminally located in the isolated oligosaccharides. These data, in addition to the results of the isotope experiment designed to confirm the origin of the threitol (described in the text), indicated that all of the galactose in the native polymer is 4-substituted and completely destroyed by the degradation procedure which results in the formation of an equimolar quantity of threitol in the oligosaccharide products.

While it was not possible to separate the mixture of oligosaccharides produced by degradation of the polymer, the conclusion that this mixture was composed of a pentasaccharide and a tetrasaccharide was made on the basis of the following evidence. Ordinary paper chromatographic and gel filtration procedures gave no indication that more than a single component was present in the preparation. The first suggestion that the preparation contained more than one component was based on the analysis which indicated that colitose was present in the preparation in a quantity somewhat greater than unity relative to the other components present. Subsequently, treatment of this mixture with 2-acetamido-2-deoxy-\(\beta\)-\(D\)-glucosidase yielded the pentasaccharide, (Colitose)\(_2\)-GlcNAc, Glc, threitol (B-4), the trisaccharide, Colitose-Glc-threitol (B-5), and free \(N\)-acytylgalcosamine equivalent to the trisaccharide; these data provided the basis for the conclusion that the original colitose-containing oligosaccharide fraction (B-4) was composed of a mixture of the pentasaccharide and the tetrasaccharide.

On occasion it has been possible to increase significantly the proportion of the pentasaccharide in the mixture of the pentasaccharides, with the consequent formation of a decreased amount of free colitose. In fact, the large proportion of polymer-colitose that may be isolated as the pentasaccharide mixture would suggest that the antigenic polysacchar-

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Footnote: 1 Dr. Otto Westphal, Max-Planck-Institut für Immunobiologie, Freiburg, Germany, personal communication
ride consists of a high molecular weight polymer composed of repeating units of the pentasaccharide structure. The inability of the d-amino acid to inhibit the precipitation of the d-amino acid indicates that the d-amino acid is not bound to the pentasaccharide in the intact polymer.

Studies on the various oligosaccharides indicated that the N-acetyl glucosamine residue of the pentasaccharide (B-4) was destroyed by treatment with periodate. Since this same N-acetyl hexosamine residue is resistant to liberation by 2-acetamido-2-deoxy-β-D-glucosidase prior to removal of colitose, one must conclude that the labile colitose residue is attached to position 6 of N-acetyl glucosamine. However, since the same N-acetyl glucosamine residue in the intact lipopolysaccharide is resistant to periodate oxidation, it must be proposed that this residue is substituted at positions 3 or 4 in the intact polymer. Indeed, if the antigenic polysaccharide is composed of repeating units of the pentasaccharide isolectin, perhaps the hydroxyl at either C-3 or C-4 of the N-acetyl glucosamine residue provides a site of linkage between the repeating oligosaccharide units.

The position of the glucose residue in the overall structure of the pentasaccharide (B-4) was established on the basis of the following: (a) glucose in B-4 is resistant to periodate oxidation; (b) glucose in each of the tri saccharides, Colitose-Glc-threitol (B-5) and GlcNAc-Glc-threitol (B-6), was destroyed by periodate oxidation; and (c) erythritol was a product of periodate oxidation of Colitose-Glc-threitol but not of GlcNAc-Glc-threitol. A glucose residue substituted only at positions 2 and 4 would give the results obtained in (a) and (b). The linkage of the colitoseyl group at position 4 of glucose was confirmed by the formation of erythritol as a product of periodate oxidation of Colitose-Glc-threitol; thus, position 2 of the glucose residue must provide a site of attachment of the colitoseyl-2-acetamido-2-deoxyglucosyl group.

Assignment of the anomeric configurations of the various glycosidic linkages in the pentasaccharide was accomplished by specific enzymatic methods, except in the case of the colitoses, which were assumed to be α-glycans on the basis of immunochromical evidence of others (3).

Because of the small quantities available, the complete structures of the oligosaccharides isolated from acid hydrolysates of lipopolysaccharide were not determined. However, the structures of A-2 (Glc → Gal) and of A-3 (GlcNAc → Gal → Gal), as far as has been determined, are consistent with the proposal that they may have been derived from the pentasaccharide (B-4) unit of the antigenic polysaccharide. The significance of the disaccharide A-1 (Glc → GlcNAc) in the overall structure remains to be established. This disaccharide was obtained in very low yield from the polymer and no role for this structure is presently known.

Preliminary studies on the relationship between the isolated colitose-containing oligosaccharides and the antigenic determinants of lipopolysaccharide suggest that, indeed, the pentasaccharide (B-4) does represent the antigenically active structural unit of the polymer. Thus, the ability of the individual oligosaccharides to inhibit the precipitin reaction between horse serum prepared against the lipopolysaccharide of E. coli 0111-B4 and the lipopolysaccharide itself are as follows: (Colitose)-GlcNAc-Glc-threitol, potent inhibition; Colitose-Glc-threitol, good inhibition; GlcNAc-Glc-threitol and Glc-threitol, very poor inhibition.4

While studies on the biosynthesis of the O-antigenic polysaccharide of E. coli 0111-B4 are not yet at a definitive stage, it may be assumed that the mechanism for its enzymatic synthesis is analogous to those proposed for other O-antigenic polysaccharides (18, 19); that is, a pre-assembly of the oligosaccharide-repeating unit as a lipid-bound intermediate, followed by polymerization of the oligosaccharide to form the high molecular weight polymer. This general mechanism for the biosynthesis of polysaccharides was first described by Anderson et al. (20) to account for the biosynthesis of the cell wall mucopeptide of gram-positive bacteria. Studies are currently underway in this laboratory to determine the mode of biosynthesis of the antigenic polysaccharide portion of the lipopolysaccharide of E. coli 0111-B4. Previously, we have reported (21) that the “core” portion of the polysaccharide is biosynthesized by the sequential transfer of the various sugar components individually from their respective nucleotide derivatives to the appropriate sites in lipopolysaccharide; these results were consistent with those reported by others (22) relative to the biosynthesis of the “core” region of lipopolysaccharide in species of Salmonella.

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