ISOLATION OF COLITOSE-CONTAINING OLIGOSACCHARIDES FROM THE CELL WALL LIPOPOLYSACCHARIDE OF ESCHERICHIA COLI. [1]

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The cell walls of Gram-negative bacteria contain a lipopolysaccharide (LPS) which in the case of E. coli 0111-B_4 contains fatty acids, phosphorus, hexosamine, 3-deoxyoctulosonate, 0-phosphorylethanolamine, heptose, glucose, galactose and colitose (3,6-dideoxy-L-xylohexose). Using a UDP-gal-4-epimerase-less mutant which produces incomplete LPS, biosynthetic studies suggested that the sugar constituents of the polymer were transferred sequentially in the order; galactose, glucose, N-acetyl glucosamine, and colitose (Edstrom and Heath, 1964). Precise structural determinations on the antigenic polysaccharide require a procedure which permits the isolation of colitose-containing oligosaccharides from the polymer. The extreme acid-lability of the glycosidically bound colitose precluded the isolation of colitose-containing oligosaccharides by partial acid hydrolysis. However, by utilizing the method of Goldstein et al. (1959), it has now been possible to isolate, in substantial yield, colitose-containing oligosaccharides from the LPS of E. coli 0111-B_4. Although complete structural details of the oligosaccharides have not yet been established, the results of preliminary structural analyses are outlined in Figure 1.

The origin of threitol from carbons 3-6 of Gal was established as follows: (a) the degradation procedure totally destroys Gal with the formation of an equivalent amount of threitol in the product; (b) the degraded product of LPS isolated from a UDP-gal-4-epimerase-less mutant of E. coli 0111-B_4 (Elbein and Heath, 1965) that was grown on U-C^{14}-Gal contained threitol as the only detectable radioactive component, the specific activity (6,750 cpm/μmole) of which was approximately 4/6 of that of Gal (11,000 cpm/μmole) isolated from the untreated polymer; (c) when the preceding experiment was performed with 1-C^{14}-Gal, Gal was the only radioactive component of the LPS and, after degradation, threitol was unlabeled.

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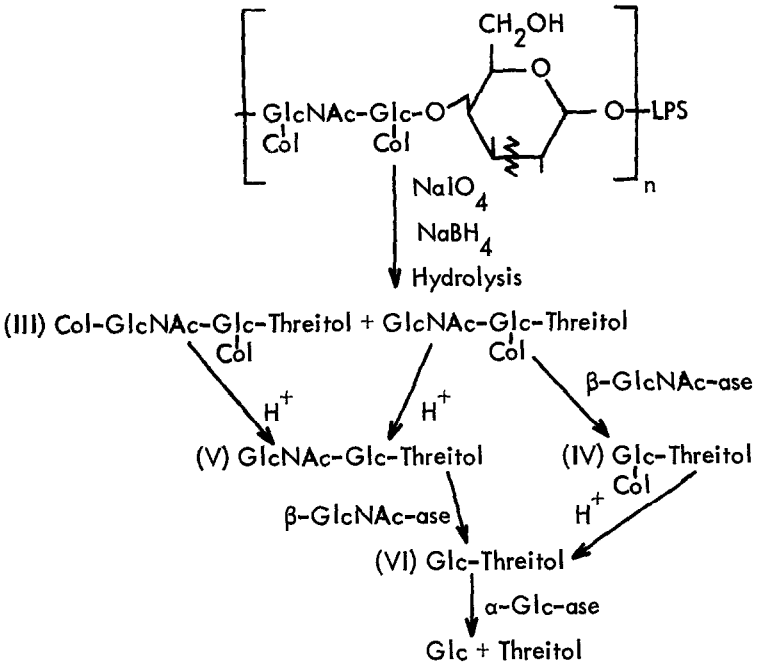

Figure 1: Summary of oligosaccharide degradation

Material and Methods---All bound sugars except colitose were determined after hydrolysis in 2 N HCl for 2 hours. Colitose was determined after hydrolysis of the sample in 0.2 N H_2SO_4 for 20 minutes at 100°. Individual sugars were determined quantitatively as previously described (Elbein and Heath, 1965). Threitol[3] was isolated by paper chromatography (Solvent A) and determined by the method of Korn (1959). The values presented for threitol are given as ratios compared to glucose isolated from the same chromatogram. Descending chromatography was performed with Whatman No. 1 paper. The following solvent systems were used: A, propane-2-ol:water (8:2); B, butane-1-ol:pyridine:water (4:4:2); C, butane-1-ol saturated with water; D, butane-1-ol:propane-2-ol:water (1:7:2); E, ethyl acetate:pyridine:water (2:1:2). Sugars on chromatograms were detected with alkaline silver nitrate (Trevelyan et al., 1950) or periodate-benzidine (Gordon et al., 1956).

Preparation of Lipopolysaccharide---LPS was isolated from washed cell wall preparations of E. coli 0111-B_4 as previously described (Elbein and Heath, 1965). The product was converted to a more soluble form by treatment with alkali prior to degradation. One gram of LPS was

3. Authentic D-threitol was a gift from Dr. N. K. Richtmyer of the National Institutes of Health.

dissolved in 200 ml of 0.2 N NaOH and heated at 60° for 30 min. After adjusting the solution to pH 5 with glacial acetic acid, precipitation with 6 volumes of ethanol yielded 790 mg of powder which contained 85% of the colitose originally present in the untreated LPS.

Degradation of LPS---To 410 mg of alkali-treated LPS (Fraction I) in 50 ml of water (adjusted to pH 6.8) were added 60 ml of 0.1 M sodium periodate. After 16 hours in the dark at 25°, 3.35 ml (60 mmoles) of ethylene glycol were added to destroy the excess periodate. The solution was cooled to 4°, 4.3 g (120 mmoles) of sodium borohydride were added, and the mixture was maintained at 4° for 2 hours. The solution was then permitted to stand at room temperature for an additional 2 hours. Acetone (20 ml) was added to destroy the remaining sodium borohydride. The solution was dialyzed for 3 days against 100 volumes of distilled water; the water was changed twice daily. The dialyzed material was concentrated to 3 ml in vacuo and adjusted to pH 2.0 with 2 N H_2SO_4. After heating at 100° for 20 minutes, the solution was centrifuged to remove the slight precipitate, adjusted to pH 9 with NH_4OH and applied to a column (4.3 x 53 cm) of Sephadex[4] G-25. Two colitose-containing fractions were eluted with 0.015 N NH_4OH. Free colitose (96 µmoles) emerged from the column between 660 ml and 740 ml of eluate. A peak of material containing bound colitose (123 µmoles) was eluted between 500 ml and 640 ml; these fractions were pooled (Fraction II) and concentrated to 3 ml. Paper chromatography of an acid hydrolysate of Fraction II in solvent A and B indicated the presence of colitose, glucosamine, glucose, and threitol. The identity of the threitol was confirmed by chromatography on borate treated paper with solvent F (Cabib et al., 1953). R_{glc} values were: glycerol, 3.86; erythritol, 1.98; threitol, 1.50; and the polyol from the oligosaccharide, 1.48. Quantitative analysis of Fraction II (Table I) indicated that all of the constituents except colitose were present in equimolar quantities; the values obtained for colitose in this fraction were consistently higher than the other sugars, suggesting that the fraction was composed of a mixture of oligosaccharides. However, this mixture was not resolved by paper chromatography of Fraction II in solvents A through E. As indicated below, further study of Fraction II showed that this material actually consisted of a mixture of a tetrasaccharide (Col, GlcNAc, Glc, threitol) and a pentasaccharide (Col_2, GlcNAc, Glc, threitol).

β-N-Acetyl glucosaminidase Treatment of Fraction II---A portion of Fraction II (41 µmoles colitose) was treated with β-N-acetyl glucosaminidase (Findlay and Levvy, 1960-kindly supplied by Dr. H. Heymann, Ciba Pharmaceutical Co., Summit, N.J.). In 20 hours at

4. Pharmacia Fine Chemicals Inc., New Market, N.J.

37°, 70% of the N-acetyl glucosamine was released. Addition of more enzyme resulted in no further liberation of N-acetyl glucosamine. The incubation mixture was applied to a column (1.5 x 110 cm) of Bio-Gel P-2[5] and the column was developed with water (Figure 2). The colitose-containing material was eluted from the column in two distinct peaks: Fraction III, contained 16 μmoles of bound colitose and Fraction IV contained 23 μmoles of bound colitose. Another fraction eluted from the column contained 22 μmoles of free N-acetyl glucosamine. The compositions of Fractions III and IV (Table I) and the elution pattern of these compounds from the gel, indicated that Fraction III was a pentasaccharide containing two moles of colitose, and one mole each of N-acetyl glucosamine, glucose and threitol. Since the N-acetyl glucosamine in the pentasaccharide was resistant to β-N-acetyl glucosaminidase treatment, it was proposed that one colitose was attached to the N-acetyl glucosamine in this oligosaccharide.

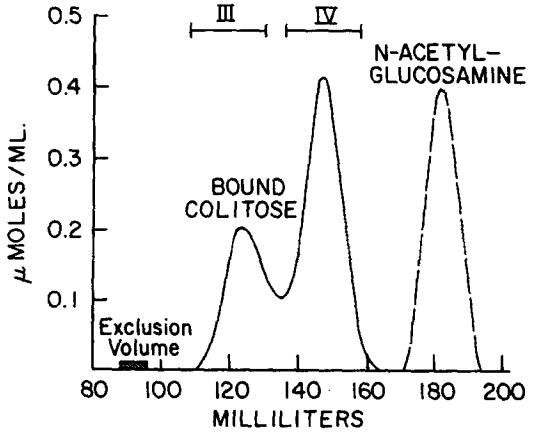

Figure 2: Separation of products of β-N-acetyl glucosaminidase treatment of Fraction II on a column of Bio-Gel P-2. Fraction III, 108 ml through 130 ml; Fraction IV, 136 ml through 158 ml.

Fraction IV contained equal amounts of colitose, glucose and threitol. This trisaccharide was resistant to the action of yeast α-glucosidase (kindly supplied by Dr. M.J. Osborn, Albert Einstein College of Medicine, New York). However, after colitose had been removed by mild acid hydrolysis, all of the glucose was liberated by the enzyme, suggesting that the colitose was linked to glucose in Fraction IV.

5. Bio-Rad Laboratories, Richmond, California.

Mild Acid Hydrolysis of the Oligosaccharide Mixture---Removal of the colitose from 25 μmoles of Fraction II by dilute acid hydrolysis (0.2 N HCl, 15 min., 100°) followed by paper chromatography with solvent A yielded 17 μmoles of Trisaccharide V which contained equimolar amounts of N-acetyl glucosamine, glucose and threitol. The trisaccharide was not cleaved by α-glucosidase.

Isolation of Glucosyl-threitol---Treatment of Fraction V (3.4 μmoles) with β-N-acetyl-glucosaminidase permitted the isolation of 3.0 μmoles of glucosyl-threitol (Fraction VI) by paper chromatography in solvent A. α-Glucosidase catalyzed the complete hydrolysis of the disaccharide to equimolar quantities of glucose and threitol.

Periodate Oxidation Studies---Periodate oxidation resulted in the destruction of N-acetyl glucosamine in III and V, while glucose was destroyed in IV and V, but not in III. These results suggest that colitose is linked at C-6 of N-acetyl glucosamine, and that the glucose

TABLE I

Fraction	Description	Molar Ratio Col	GlcN	Glc	Threitol (Galactose)*	Recovery**
I	Alkali-treated LPS***	1.17	0.95	1.00	(0.46)	100%
II	Tetrasaccharide and Pentasaccharide	1.24	1.00	1.00	1.02	34
III	Pentasaccharide	2.14	0.98	1.00	0.88	14
IV	Trisaccharide	0.99	-	1.00	0.94	20
V	Trisaccharide	-	1.08	1.00	1.04	-
VI	Disaccharide	-	-	1.00	0.90	-

* The value given in parentheses refers to galactose in the intact polymer.
** Recovery from alkali-treated LPS was based on colitose.
*** Ratios for this material reflect the presence of glucose and glucosamine in other portions of the LPS molecule.

residue of the pentasaccharide (III) is substituted at C-2 and C-4, by colitose at one of these positions and colitosyl-N-acetyl glucosamine at the other. Presumably N-acetyl glucosamine is further substituted in intact LPS, since this constituent is resistant to periodate oxidation in the polymer.

Discussion---The primary colitose-containing oligosaccharides (Fraction II) resulting from the degradation of E. coli 0111 LPS by the procedure of Goldstein et al. (1959) were obtained as a mixture of a pentasaccharide and a tetrasaccharide. As shown in Figure I, further chemical and enzymatic analysis of these oligosaccharides indicated that a basic structural unit of the polysaccharide is composed of the trisaccharide, N-acetyl glucosaminyl-1 $\xrightarrow{\beta}$?-glucosyl-1 $\xrightarrow{\alpha}$ 4-galactose; colitose is glycosidically bound to both the acetyl glucosamine and the glucose residues.

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