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STRUCTURAL STUDIES ON THE HEXOSE REGION OF THE LIPOPOLYSACCHARIDE FROM *Escherichia coli* C

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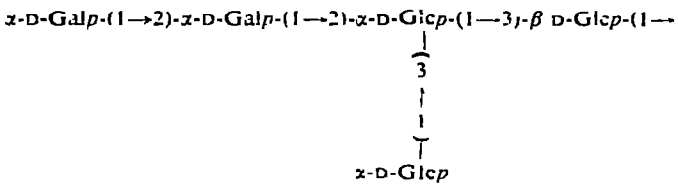
ABSTRACT

Escherichia coli C is an R-strain, and hence its lipopolysaccharide consists only of lipid A joined to a basal core. Intact core-polysaccharides have been prepared from this strain, and from mutants of the same strain defective in various stages of core biosynthesis. Using sugar and methylation analyses, and chemical and enzymic degradations, the hexose region of the core of the parent strain has been shown to be a pentasaccharide for which the following structure is proposed



INTRODUCTION

Lipopolysaccharides from Gram-negative bacteria are composed sequentially of a lipid part (lipid A), a basal core, and an O-antigenic side-chain. The lipid moiety seems to be the same or similar for all bacteria belonging to the family Enterobacteriaceae[1], whereas O-antigenic side-chains vary in structure and are specific for the different serotypes. The nature of the core region differs in lipopolysaccharides from different genera, and variations in core structures have also been observed in lipopolysaccharides from different strains of *Escherichia coli*. A detailed knowledge of core structures is important in understanding the sensitivity of different strains to bacteriophages and may also give additional genetic information. We now report structural studies on the hexose region of the lipopolysaccharide from the R-strain *E. coli* C.

RESULTS AND DISCUSSION

E coli C is a wild type R-form, and the S-form from which it derives is unknown In this study, lipopolysaccharides were prepared, by the method of Galanos[2], from *E coli* C and the defective mutants C/5, C/15, and C/62 The latter were kindly supplied by Dr N. G. Godson[3].

Core regions were prepared from the lipopolysaccharides by mild hydrolysis with acid, and will subsequently be referred to as polysaccharides (this term may not be adequate for these rather small polymers, but will be used for the sake of consistency) Sugars obtained on acid hydrolysis of these polysaccharides were analysed by g l c of their alditol acetates (Table I) and were shown to be D-galactose, D-glucose, and L-*glycero*-D-*manno*-heptose Identifications were based on relative retention times, and absolute configurations were assumed to be the same as those previously observed for related polysaccharides 2-Acetamido-2-deoxy-D-glucose was not found, and the presence of this sugar was also precluded by n m r spectroscopy of the intact polysaccharides, in which no signals for acetyl protons were observed Sugar analysis with an internal standard showed that the sugars present in the *E coli* C polysaccharide accounted for 62% by weight of the material Analyses also showed phosphorus (4.49%) and nitrogen (0 69%).

TABLE I

SUGAR ANALYSES OF POLYSACCHARIDES FROM DIFFERENT MUTANTS OF *E coli* C

Sugars	*C*	*C/5*	*C/15*	*C/62*
D-Gal	30	14	19	32
D-Glc	44	37	53	35
Heptose	26	49	28	33

TABLE II

METHYLATION ANALYSES OF ORIGINAL AND MODIFIED POLYSACCHARIDES FROM DIFFERENT MUTANTS OF *E coli* C

Sugars	T[b]	*C*	*C*'[c]	*C*"[d]	*C*"'[e]	*C/5*	*C/15*	*C/62*	*C/62*'[f]
2,3,4,6-Glc[a]	1 00	21	24	36	15	48	13	5	45
2,3,4,6-Gal	1 16	14	4	5	21	12	23	22	4
3,4,6-Glc	1 72	3	0	0	6	5	17	23	0
2,4 6-Glc	1 76	20	42	47[g]	9	14	32	27	46
3,4,6-Gal	1 98	21	7	0	23	14	0	23	2
4 6-Glc	3 00	22	23	12	25	7	14	2	4

[a]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc [b]Retention times of the derived alditol acetates relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an SP-1000 glass-capillary column at 220 [c]After oxidation, first with D-galactose oxidase and subsequently with hypoiodite [d]The material treated as in [c], and then subjected to uronic acid degradation [e]After acetylation and oxidation with CrO_3-AcOH [f]After treatment with α-D-galactosidase [g]Approximately 40% of this ether was trideuteriomethylated at C-2

Methylation analyses of polysaccharides from the various strains were performed as previously described, with identification of the methylated sugars as their alditol acetates by g l c–m s [4]. In addition to the derivatives listed in Table II, 2,3,4,6,7-penta- and 2,4,6,7-tetra-*O*-methyl-L-*glycero*-D-*manno*-heptose were observed. It is evident from the data in Tables I and II that simple ratios of methyl ether components, indicative of a homogeneous sample, were obtained only for the C and the C/62 polysaccharides.

The oligomeric residue obtained after treatment of the C/62 polysaccharide with α-D-galactosidase yielded, on methylation analysis, essentially 2,3,4,6-tetra- and 2,4,6-tri-*O*-methyl-D-glucose (Table II, column C/62′). Treatment of the same polysaccharide with β-D-galactosidase and analysis of subsequently methylated material showed this enzyme to be without effect. These results therefore show that the hexose region in the C/62 polysaccharide residue is a linear tetrasaccharide having the partial structure **1**, the anomeric configurations of the D-glucose residues, as shown in this formula, will be discussed later.

α-D-Gal*p*-(1→2)-α-D-Gal*p*-(1→2)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→

1

As the parent C-polysaccharide should contain the tetrasaccharide residue of C/62 and, according to the methylation analysis, a terminal D-glucopyranosyl group that must be linked to O-3 of the branching D-glucopyranosyl residue, the sequence of sugars in the pentasaccharide residue is as depicted in **2**.

α-D-Gal*p*-(1→2)-α-D-Gal*p*-(1→2)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→

3

α-D-Glc*p*

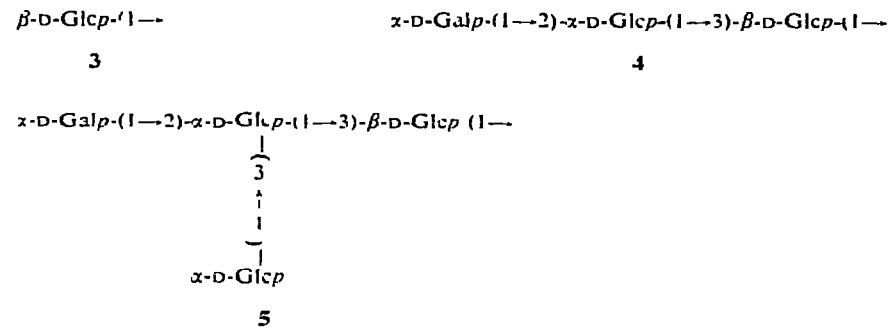
2

Treatment of the C-polysaccharide with either α- or β-D-glucosidase was without effect, and treatment with α-D-galactosidase released only the terminal D-galactose residue. On treatment with D-galactose oxidase, however, both D-galactose residues were oxidized, as subsequent oxidation of the D-*galacto*-hexodialdose residues to uronic acid residues with hypoiodite, followed by sugar analysis, yielded D-galactose and D-glucose in the ratio 8:92. Oxidation of the chain D-galactopyranosyl residue was not unexpected, as 2-*O*-methyl-D-galactose is also known to be oxidized by this enzyme[5]. Methylation analysis of the oxidized material (Table II, column C′) yielded essentially D-glucose derivatives. The material was further modified by a uronic acid degradation[6] which involved methylation followed by sequential treatment with base and acid, when the uronic acid residues were eliminated. The degraded material was remethylated, with trideuteriomethyl iodide as alkylating agent, and hydrolysed.

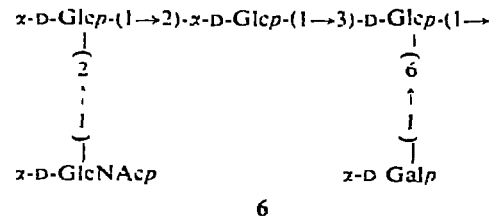
The main components in the hydrolysate (Table II, column C″) were 2,3,4,6-tetra- and 2,4,6-tri-*O*-methyl-D-glucose. About 40% of the latter derivative was trideuteriomethylated at O-2, the position to which the eliminated side-chain had been linked. The result of this degradation therefore supports structure **2**.

Acetylated C-polysaccharide was treated with chromium trioxide in acetic acid[7], and the product was subjected to methylation analysis (Table II, column C′). During the oxidation, β-linked D-glucopyranose and D-galactopyranose residues should be oxidized, but the corresponding α-linked sugars should be resistant[7]. Comparing methylation analyses of the original and oxidized material, a decrease in the amount of 2,4,6-tri-*O*-methyl-D-glucose after oxidation is apparent. The results therefore suggest that the chain D-glucose residue in **2** is β-linked and that the other residues are α-linked. The preponderance of α-linkages is also in agreement with the optical rotation, $[\alpha]_{578}$ +112°, of the undegraded C-polysaccharide. In the oxidation with chromium trioxide, the acetylated β-D-glucopyranosyl residue was transformed into a 5-hexulosonate residue and the ester linkage was cleaved during the subsequent methylation. Since no new non-reducing, terminal hexose residue was produced, the position of the D-glucopyranosyl chain-residue in **2** was confirmed.

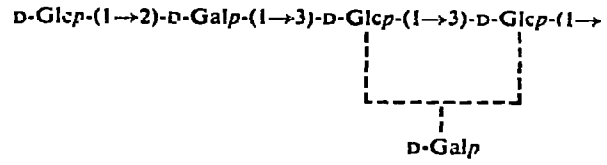
In the light of the results presented above, the sugar and methylation analyses of the C/5 and C/15 polysaccharides may be rationalized by assuming that they are mixtures containing the residues **1**, **2**, **3**, and the residues **4** and **5**, respectively.



Five distinct core-types are known[1] in *E. coli*, and a detailed structure has been determined[8] for one of these (R-2) in which the hexose region has the structure **6**.



E. coli C has a complete core[9] that seems to be identical with the *E. coli* R-1 core, and a partial structure, with the hexose region **7**, has been reported in a review article[1].



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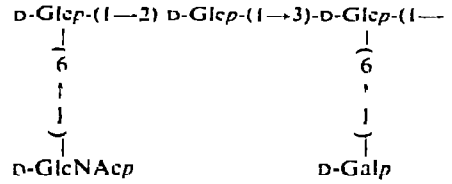
This structure contains the same structural elements as **2**, but in a different sequence A tentative structure **8** has also been proposed for the *E coli* K-12 core[10]



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EXPERIMENTAL

General methods — Bacteria were cultivated in a stirred, 10-litre fermentor (Biotec) under constant aeration at pH 7.0 Concentrations of solutions were performed under diminished pressure at bath temperatures not exceeding 40° Optical rotations were measured with a Perkin–Elmer model 141 polarimeter G.l.c of alditol acetates was performed with a Perkin–Elmer model 990 instrument, equipped with a glass column (200 × 0.3 cm) containing 3% of OV-225 on Gas Chrom Q (100/120 mesh) Peak areas were measured with a Hewlett–Packard 3370-B electronic integrator Separation of partially methylated alditol acetates was achieved by g.l.c with a Hewlett–Packard model 5830-A instrument and a glass-capillary column (25 m × 0.25 mm), wall-coated with SP-1000 (LKB-products) For g.l.c–m.s., a Perkin–Elmer 270 gas chromatograph–mass spectrometer, fitted with an OV-225 S.C.O.T. column, was used Gel filtrations were performed on a Sephadex G-25 column (2.5 × 90 cm), with water as irrigant α-D-Galactosidase was purchased from Boehringer Mannheim Co., D-galactose oxidase from KABI (Stockholm), and all other enzymes from Sigma Chemical Company Methylations were performed as earlier described[4], and methylated polysaccharides were isolated by partition between chloroform and water, and purified on a small column of Sephadex LH-20 irrigated with chloroform–acetone (3:1) The methylated polysaccharides were hydrolysed with 0.25M sulphuric acid for 16 h at 100°, and the sugars analysed[4] as their alditol acetates by g.l.c–m.s.

Preparation of lipopolysaccharides and lipid-free polysaccharides — Lipopolysaccharides from *E coli* C (strain HF 4704) and the spontaneous mutants[3] C/5, C/15, and C/62 were isolated and purified by procedures described earlier[2,11,12] The yield of lipopolysaccharides from the various strains was 1–2% of the bacterial dry-weight

Lipopolysaccharides were treated with aqueous acetic acid (1%,) at 100 for 1 h, to selectively cleave lipid residues which were removed by centrifugation The polysaccharides were purified on a column of Sephadex G-25, freeze-dried, and obtained in yields of 20–40%

Oxidation and uronic acid degradation of the polysaccharide from E coli *C* — The polysaccharide (30 mg), in 0 02M phosphate buffer (pH 7 0, 6 ml), was treated under toluene (0 05 ml) with D-galactose oxidase (160 units) and horseradish peroxidase (3 mg) After 70 h at room temperature, iodine (40 mg) and sodium carbonate (52 mg) in water (8 ml) were added, and the solution was stirred for 4 h and then heated to 90° for 10 min[13] The oxidized polysaccharide (27 mg), after purification on a column of Sephadex G-25 was examined by methylation analysis (Table II, C')

The oxidized and methylated product (10 mg) and toluene-*p*-sulphonic acid (5 mg) were dissolved in methanol (10 ml) containing 2,2-dimethoxypropane (0 5 ml) and the solution was boiled under reflux for 30 min A piece of freshly cut sodium (100 mg) was added and refluxing was continued for 15 min The mixture was neutralised with 50% aqueous acetic acid, the product extracted with dichloromethane, and the extract concentrated to dryness After treatment with 50% aqueous acetic acid (5 ml) at 100° for 1 h and concentration to dryness, methylation analysis of this degraded material was performed (Table II, C') with trideuteriomethyl iodide as alkylating agent

Chromic acid degradation — The polysaccharide (12 mg) from *E coli* C was acetylated by treatment with acetic anhydride (0 5 ml) and pyridine (0 5 ml) in formamide (2 ml) for 16 h, and the reaction was monitored by i.r spectroscopy The product, together with *myo*-inositol hexacetate (3 mg) as an internal standard, was dissolved in acetic acid (0 6 ml), and half of the solution was kept as a control Chromium trioxide (30 mg) was added to the other half, and the mixtures were agitated in an ultrasonic bath for 1 h The solutions were each diluted with water (20 ml) and extracted with chloroform Sugar analysis gave D-glucose and D-galactose in the proportions 1 44 1 00 and 0 80 1 00 for the unoxidized control and the oxidized material, respectively The analysis showed that 100% of the D-galactose in the original sample was recovered after the oxidation

Enzymic degradation — The following enzymes were used α-D-Glucosidase (from yeast) in 0 07M sodium phosphate buffer (pH 6 8), β-D-glucosidase (from almonds) in 0 05M sodium acetate buffer (pH 5 0), α-D-galactosidase (from green coffee-beans) in citric acid–disodium phosphate buffer (McIlvaine[14], pH 6 1), and β-D-galactosidase (from bovine liver) in 0 05M Tris–HCl buffer (pH 7 6) The specificities and activities of the enzymes were tested by using suitable substrates

For enzymic digestion, the polysaccharide (5–15 mg), enzyme (10 mg for β-D-galactosidase, 1 mg for the others), and toluene (0 05 ml) in the appropriate buffer (3 ml) were kept at 37° for 24 h Products were fractionated by chromatography on Sephadex G-25 and subjected to methylation analysis

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REFERENCES

1 K JANN AND O WESTPHAL, in M SELA (Ed), *The Antigens* Vol III Academic Press, New York, 1975 pp 1–125
2 C GALANOS O LUDERITZ AND O WESTPHAL, *Eur J Biochem* , 9 (1969) 245–249
3 G N GODSON *Virology*, 58 (1974) 272–289
4 H BJÖRNDAL, C G HELLERQVIST, B LINDBERG, AND S SVENSSON, *Angew Chem Int Ed Engl* , 9 (1970) 610–619
5 R A SCHLEGEL, C M GERBECK, AND R MONTGOMERY, *Carbohydr Res* , 7 (1968) 193–199
6 B LINDBERG J LONNGREN, AND J L THOMPSON, *Carbohydr Res* , 28 (1973) 351–357
7 J HOFFMAN B LINDBERG, AND S SVENSSON *Acta Chem Scand* , 26 (1972) 661–666
8 G HAMMERLING O LUDERITZ AND O WESTPHAL, *Eur J Biochem* , 22 (1971) 331–344
9 G SCHMIDT, *J Gen Microbiol* , 77 (1973) 151–160
10 P PREHM, S STIRM B JANN K JANN, AND H G BOMAN *Eur J Biochem* , 66 (1976) 369–377
11 C G HELLERQVIST, B LINDBERG, S SVENSSON T HOLME, AND A A LINDBERG *Carbohyd Res* , 8 (1968) 43 55
12 A A LINDBERG AND T HOLME *Acta Pathol Microbiol Scand* , 80 (1972) 751–759
13 J K ROGERS AND N S THOMPSON *Carbohydr Res* 7 (1968) 66–75
14 G GOMORI, *Methods Enzymol* 1 (1955) 138–146