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STRUCTURAL STUDIES ON *Klebsiella* TYPE 61 CAPSULAR POLYSACCHARIDE

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ABSTRACT

The capsular polysaccharide from *Klebsiella* Type 61 was found to contain D-galactose, D-glucose, D-mannose, and D-glucuronic acid in the ratios 1:2:1:1. Acid hydrolysis of the polysaccharide gave one aldobiouronic acid, whose structure was established. Methylation analysis of the polysaccharide provided information about the linkages in the polysaccharide. The polysaccharide is composed of a pentasaccharide repeating unit for which structures are proposed.

INTRODUCTION

Nimmich[1] has isolated the type-specific capsular polysaccharide from *Klebsiella* Type 61. It was reported that, on acid hydrolysis, the polysaccharide gave galactose, glucose, glucuronic acid, and a trace of mannose. We now report on some structural studies of this polysaccharide.

RESULTS AND DISCUSSION

The capsular polysaccharide from *Klebsiella* Type 61 (K-61) was purified by passing a solution of it through a column of Sephadex G-100. The major peak contained the polysaccharide (86% of the crude material), having $[\alpha]_D^{25}$ +56° (*c* 0.3, water). There were two small peaks of impurities, one of which appeared before, and the other after, the major peak for K-61.

Hydrolysis of K-61 with 0.5M sulfuric acid for 6 h at 100° gave a mixture; paper chromatography (both solvent systems *A* and *B*) of this revealed the presence of galactose and glucose, and traces of mannose and glucuronic acid. Paper chromato-

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graphy in solvent system *A* gave, in addition to these sugars, a very prominent spot having $R_{Lactose}$ 1.03 and a minor spot having $R_{Lactose}$ 0.78; in solvent system *B*, the mobility of these components was almost zero. The extent of movement of these components in solvent systems *A* and *B* suggested that these are, respectively, an aldobiouronic acid and an aldotriouronic acid. The acidic and neutral components were separated from the hydrolysis mixture by means of a column of Dowex-1 X4 (OAc^-). From the acidic part, aldobio- and aldotrio-uronic acids were isolaedt by preparative, paper chromatography.

The neutral fraction from the Dowex-1 X4 column was analyzed as its alditol acetates by g.l.c., which revealed the ratios of galactose:glucose:mannose to be 2.8:4.8:1. The neutral fraction had $[\alpha]_D^{25}$ +53° (*c* 0.5, water); as the calculated value of $[\alpha]_D^{25}$ for a mixture of D-galactose, D-glucose, and D-mannose in the ratios of 2.8:4.8:1 is +56°, this showed that all of the sugar components in the K-61 polysaccharide are D sugars. Hydrolysis of K-61 polysaccharide with 0.5M sulfuric acid for 20 h gave D-galactose, D-glucose, and D-mannose in the ratios 2.1:4.1:1: this indicated that K-61 contains D-galactose and D-glucose residues in the ratio 1:2,and that the aldobiouronic acid part had been hydrolyzed to the extent of ~50% in 20 h.

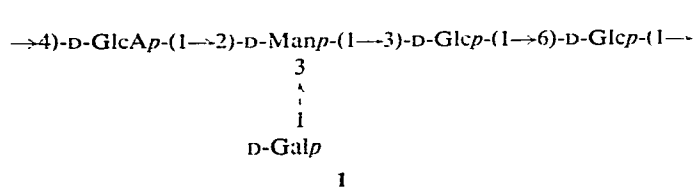
Acid hydrolysis of the aldobiouronic acid with 0.5M sulfuric acid for 20 h gave D-mannose as the only neutral sugar (as observed in g.l.c. of its alditol acetate); this sugar might have come from the "aglycon" part of the aldobiouronic acid. The aldobiouronic acid was converted into the corresponding neutral disaccharide by reducing the carboxyl group with diborane[2]. On acid hydrolysis, the reduced material gave D-glucose and D-mannose in the ratio 1.2:1 on analysis as their alditol acetates by g.l.c.; this result strongly indicated that the aldobiouronic acid was GlcA → Man. Methylation[3] of the aldobiouronic acid gave a fully methylated product that, on hydrolysis, gave a g.l.c. peak (as alditol acetate) of 3,4,6-tri-*O*-methyl-D-mannose. In another experiment, the permethylated aldobiouronic acid was reduced with lithium aluminum hydride, and the product was hydrolyzed; the products, as the alditol acetates, gave signals for 3,4,6-tri-*O*-methyl-D-mannose and 2,3,4-tri-*O*-methyl-D-glucose (in g.l.c.) in the ratio of 1:1; this finding, together with the fact that the aldobiouronic acid has $[\alpha]_D^{25}$ +14°, confirmed that the aldobiouronic acid is *O*-(β-D-glucopyranosyluronic acid)-(1→2)-D-mannopyranose.

Having identified all the sugar components in polysaccharide K-61 (namely, D-galactose, D-glucose, D-mannose, and D-glucuronic acid), it was necessary to estimate them. The uronic acid was estimated spectrophotometrically by the carbazole method[7], and its proportion was found to be 21.1%. D-Galactose, D-glucose, and D-mannose were determined from carboxyl-reduced K-61 (prepared by reducing acetylated K-61 with diborane[2]). G.l.c. analysis of the alditol acetates prepared from the carboxyl-reduced K-61 showed D-galactose, D-glucose, and D-mannose in the ratios 1:3:1. The same ratios of sugar components were obtained by reducing[8] the polysaccharide with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate, hydrolyzing the reduced K-61, and analyzing as alditol acetates. As the amount of D-glucose as observed here is the combined value for D-glucose and

D-glucuronic acid, the ratios of D-galactose:D-glucose:D-mannose:D-glucuronic acid in the original K-61 must be 1:2:1:1. This result and the results of the hydrolysis experiment on K-61 suggest that the repeating unit of the polysaccharide contains one aldobiouronic acid, one D-galactose, and two D-glucose units.

The polysaccharide K-61 was methylated first by the Hakomori procedure[4] and then by the Kuhn procedure[3]; the absence of hydroxyl absorption in the i.r. spectrum of the product indicated complete methylation. A small part of the permethylated polysaccharide was hydrolyzed for 20 h, and the alditol acetates were prepared from the hydrolyzate. Analysis by g.l.c. showed 2,3,4,6-tetra-*O*-methylgalactose, 2,4,6-tri-*O*-methylglucose, 2,3,4-tri-*O*-methylglucose, and a small proportion of 4,6-di-*O*-methyl-D-mannose. The major part of the permethylated K-61 was reduced[5] with lithium aluminum hydride, the product was hydrolyzed, and the alditol acetates were prepared from the hydrolyzate. Analysis by g.l.c. showed 2,3,4,6-tetra-*O*-methylgalactose, 2,4,6-tri-*O*-methylglucose, 2,3,4-tri-*O*-methylglucose, 4,6-di-*O*-methylmannose, and 2,3-di-*O*-methylglucose in the ratios 1:1:1:1:0.85, and a trace of 2,3,4,6-tetra-*O*-methylglucose. The 2,3-di-*O*-methyl-D-glucose must have come from D-glucuronic acid residues, because there was not even a trace of this component when permethylated K-61 was analyzed before reduction with lithium aluminum hydride. 4,6-Di-*O*-methyl-D-mannose must have come from the aldobiouronic acid. As all of the mannose appeared as the 4,6-di-*O*-methyl derivative, and all of the galactose as the 2,3,4,6-tetra-*O*-methyl derivative, D-galactose units were probably attached to the side chains as nonreducing groups, and these side chains are probably attached to O-3 of D-mannose. Moreover, the proportion of 4,6-di-*O*-methyl-D-mannose was almost doubled after reduction, whereas the proportion of all the trimethylated sugars remained unchanged. Consequently, the only sugar which is involved in aldobiouronic acid formation is the 4,6-di-*O*-methyl-D-mannose moiety. The only two glucose units to be accounted for had appeared as 2,3,4-tri-*O*-methyl-D-glucose and 2,4,6-tri-*O*-methyl-D-glucose.

The results suggest that the polysaccharide contains a pentasaccharide repeating unit consisting of one D-galactose, one D-mannose, one D-glucuronic acid, and two D-glucose residues. The results also give information on the positions through which the sugar residues are linked. From these structural data, together with the information about the structure of the aldobiouronic acid, it is possible to suggest twelve feasible structures for the repeating units of polysaccharide K-61. Structures **1** to **6** represent six alternatives. Six more structures may be written by interchanging the position of the two D-glucose units.



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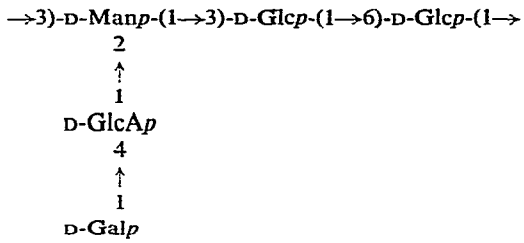
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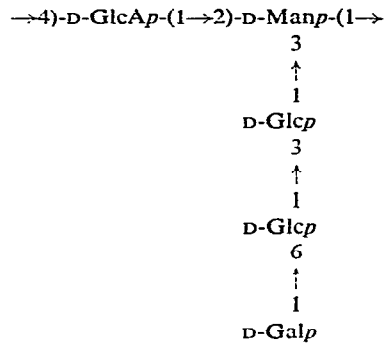
4
1
D-Galp

2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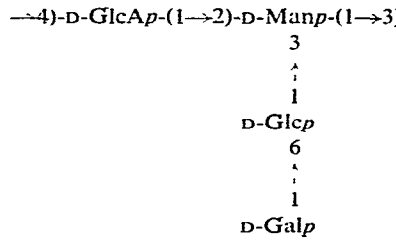
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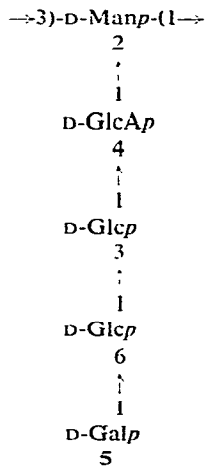
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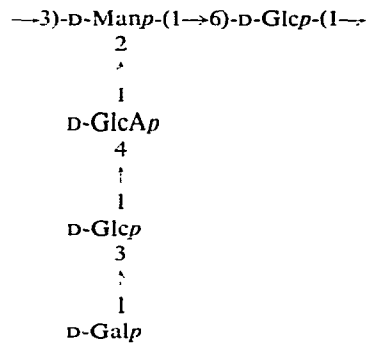
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4
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D-Glcp
3
1
D-Galp

6

EXPERIMENTAL

Materials and methods. — 4,6-Di-*O*-methyl-D-mannose and 2,4,6-tri-*O*-methyl-D-glucose were synthesized by the authors in this laboratory (IACS).

Paper chromatography was performed on Whatman No. 1 paper. Solvent systems (v/v) used were (*A*) 9:2:2 ethyl acetate–acetic acid–water and (*B*) 8:2:1 ethyl acetate–pyridine–water. All solvents were distilled before use, and all evaporations were conducted at 50°, unless otherwise stated.

Gas–liquid chromatography was performed with a Hewlett–Packard Model 5731A gas chromatograph. The columns used were (*A*) a glass column (1.83 m × 6 mm) packed with 3% of ECNSS-M on GasChrom Q (100–120 mesh), and (*B*) a glass column (1.83 m × 6 mm) packed with 5% of OV-225 on GasChrom Q (100–120 mesh). All of the g.l.c. peaks, except the one from 3,4,6-tri-*O*-methyl-D-mannose (whose retention time is the same as that of 2,4,6-tri-*O*-methyl-D-glucose) were confirmed by direct comparison with authentic samples. Retention times for partially methylated alditol acetates were measured with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as unity.

Optical rotations were determined with a Perkin–Elmer 241 MC spectropolarimeter. Colorimetric estimations were conducted with a Carl Zeiss Model VSU2-P spectrophotometer. The polysaccharide from *Klebsiella* Type 61 (K-61) was isolated by one of us (W.N.); it has already been described[1].

In order to convert an oligo- or poly-saccharide into the alditol acetates of sugar components, the sample was hydrolyzed with 0.5M sulfuric acid for 20 h. The mixture was made neutral with barium carbonate, and filtered, and to the filtrate was added an equal volume of 5% sodium borohydride solution. After 4 h, the excess of borohydride was decomposed with Dowex-50W X8 (H^+) cation-exchange resin, the solution was evaporated to dryness, and the boric acid was removed from the residue as methyl borate. The mixture of alditols was acetylated with acetic anhydride (2 ml) and pyridine (3 ml) for 2 h at 100°.

Purification of the polysaccharide. — The polysaccharide K-61 (23 mg) was put on the top of a column (70 × 2 cm) of Sephadex G-100. The column was eluted with 0.05M ammonium hydrogencarbonate solution (pH 8.0), and 55 fractions (5 ml each) were collected, and analyzed by the phenol–sulfuric acid method[6]. Fractions 11 to 23, containing polysaccharide K-61, were collected and lyophilized; yield 19 mg, $[\alpha]_D^{25}$ +56° (*c* 0.3, water). Two small peaks (impurity; fractions 6 to 9, and 26 to 30) were discarded.

Acid hydrolysis of K-61. — The polysaccharide (25 mg) was hydrolyzed with 0.5M sulfuric acid for 6 h at 100°. The acid was neutralized with barium carbonate, and the solution was treated with Amberlite IR-120 cation-exchange resin, and evaporated to dryness. Paper chromatography of the hydrolyzate, using solvent system *B*, showed galactose, glucose, and a trace of mannose, and a prominent spot whose mobility was almost zero. Paper chromatography of the hydrolyzate using solvent system *A* showed two spots; the major spot had $R_{Lactose}$ 1.03, and the minor

spot had $R_{Lactose}$ 0.78, and there was a trace of glucuronic acid (besides the neutral sugars galactose, glucose, and mannose). The mixture was passed through a column (15 × 1 cm) of Dowex-1 X4 (OAc^-) anion-exchange resin. The neutral sugars were collected by eluting the column with water, and the eluate was evaporated to dryness (9 mg). The acidic sugars were then eluted with 30% acetic acid, and the eluate was evaporated to dryness (12 mg). Alditol acetates were prepared from the neutral fraction (1 mg), and analysis by g.l.c. (column A at 190°) showed galactose, glucose, and mannose in the ratios 2.8:4.8:1. Pure aldobiouronic acid (5 mg) and aldotriouronic acid (1.4 mg) were isolated by preparative paper-chromatography using solvent system *A*. The aldobiouronic acid had $[\alpha]_D^{25}$ +14°. In another experiment, K-61 (1 mg) was hydrolyzed with 0.5M sulfuric acid for 20 h, and the alditol acetates were prepared. G.l.c. analysis (column *A* at 190°) showed galactose, glucose, and mannose in the ratios 2.1:4.1:1.

Hydrolysis of the aldobiouronic acid and its carboxyl-reduced product. — The aldobiouronic acid (0.5 mg) was hydrolyzed with 0.5M sulfuric acid for 20 h. The hydrolysis product was isolated as already described, and analyzed, as the alditol acetate, by g.l.c. (column *A* at 190°). The neutral sugar observed was mannose (and a trace of glucose, probably from an impurity). In another experiment, the aldobiouronic acid (1.5 mg) was acetylated with acetic anhydride and pyridine, and the acetate was reduced with an excess of diborane[2]. The neutral disaccharide thus obtained was hydrolyzed, and the alditol acetates were prepared; analysis by g.l.c. (column *A* at 190°) showed glucose and mannose in the ratio of 1.2:1.

Methylation analysis of the aldobiouronic acid. — The aldobiouronic acid (2 mg) was dissolved in *N*,*N*-dimethylformamide (1.5 ml), silver oxide (0.8 g) and Drierite (0.5 g) were added, the mixture was stirred for 30 min, and then methyl iodide (0.5 ml) was added. Stirring was continued for 30 h. Chloroform (20 ml) was then added while the mixture was vigorously stirred. The solids were filtered off through a Celite bed, and the filtrate was washed with water, dried (anhydrous sodium sulfate), and evaporated to dryness. A portion of this product was hydrolyzed with 0.5M sulfuric acid for 20 h, and the alditol acetates were prepared. Examination by g.l.c. (column *A* at 160°, and column *B* at 195°) showed 3,4,6-tri-*O*-methylmannose. The other portion of the methylated aldobiouronic acid was reduced[5] with lithium aluminum hydride, and the product hydrolyzed; the alditol acetates were prepared, and analyzed by g.l.c. (column *A* at 160°, column *B* at 195°). The results are summarized in Table I.

Preparation of carboxyl-reduced polysaccharide K-61. — Polysaccharide K-61 (25 mg) was dissolved in formamide (1.5 ml), and pyridine (4 ml) and acetic anhydride (2.5 ml) were added. The mixture was stirred for 20 h at room temperature, and evaporated to dryness *in vacuo* at 40°. The product was dissolved in tetrahydrofuran (2 ml), an excess of diborane in the same solvent was added, and the mixture was stirred for 20 h at room temperature. The excess of diborane was decomposed by adding methanol dropwise, very carefully. Methyl borate was removed by repeated addition and evaporation of methanol. The product was deacetylated with sodium methoxide (0.1M), sodium ions were removed with Dowex 50W-X8 cation-exchange

TABLE I

METHYLATION ANALYSIS OF *Klebsiella* TYPE 61 POLYSACCHARIDE AND THE ALDOBIOURONIC ACID THEREFROM

Methylated sugar (as alditol acetate)	*Retention time*		*Mole %*[a]		
	Column A	*Column B*	*I*	*II*	*III*
2,3,4,6-Tetra-*O*-methyl-D-glucose	1.00	1.00	2.10	2.77	—
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.21	1.15	27.00	19.00	—
2,4,6-Tri-*O*-methyl-D-glucose	1.91	1.71	27.40	21.98	—
2,3,4-Tri-*O*-methyl-D-glucose	2.41	2.05	28.40	21.02	50.55
3,4,6-Tri-*O*-methyl-D-mannose	1.91	1.71	—	—	49.45
2,3-Di-*O*-methyl-D-glucose	5.51	3.78	—	15.95	—
4,6-Di-*O*-methyl-D-mannose	3.28	2.64	15.20	19.19	—

[a] I, Alditol acetates from permethylated K-61 before reduction with lithium aluminum hydride; II, alditol acetates from permethylated K-61 after reduction with lithium aluminum hydride; III, alditol acetates from methylated aldobiouronic acid after reduction with lithium aluminum hydride.

resin, and the suspension was filtered. The filtrate was lyophilized, to give carboxyl-reduced K-61 (18 mg) having $[\alpha]_D^{25}$ +52° (*c* 0.2, water). The i.r. spectrum of the compound in a KBr pellet did not show any recognizable peak for a carboxyl stretching-vibration.

In a separate experiment, K-61 (10 mg) was reduced with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate[8]. The reagent (300 mg) was added, with stirring, to a solution of K-61 in water (12 ml). Three drops of cyclohexanol were added as an antifoaming agent, and the pH was kept at 4.75 by addition of 0.01M hydrochloric acid. After 2 h, aqueous 2M sodium borohydride (5 ml) was added during 1 h, and the pH was kept at 7.00 by simultaneous addition of 4M hydrochloric acid. The solution was dialyzed for 24 h against distilled water, and freeze-dried. The procedure was repeated on the same material, to ensure complete reduction of the carboxyl groups.

Determination of sugar components in K-61 polysaccharide. — The samples of carboxyl-reduced polysaccharide (1 mg) from the foregoing two experiments were separately hydrolyzed with 0.5M sulfuric acid (4 ml) for 20 h, and the alditol acetates were prepared. Examination by g.l.c. with column *A* at 190° showed galactose, glucose, and mannose in the ratios 1:3:1 (in both experiments); the results are given in Table II. The uronic acid content, estimated by the carbazole method[7], was found to be 21.1%.

Methylation studies on K-61 polysaccharide. — The polysaccharide (20 mg) in methyl sulfoxide (20 ml) was methylated by the Hakomori methylation procedure[4], using methylsulfinyl carbanion (10 ml) and methyl iodide (10 ml). The i.r. spectrum of the methylated product showed a small band for hydroxyl stretching-vibration; the material was therefore remethylated, with methyl iodide and silver oxide[3], and the product then showed practically no hydroxyl band in the i.r. spectrum. A portion

TABLE II

RESULTS OF ACID-CATALYZED HYDROLYSIS OF K-61 AND CARBOXYL-REDUCED K-61 POLYSACCHARIDES

Sugar, as alditol acetate	*Mole %*[a]			
	1	*2*	*3a*	*3b*
Galactose	32.40	29.30	20.03	19.84
Glucose	56.10	56.95	61.20	60.50
Mannose	11.50	13.75	19.50	19.64

[a]Key: 1, Alditol acetates obtained from K-61 polysaccharide hydrolyzed for 6 h; 2, alditol acetates obtained from K-61 hydrolyzed for 20 h; 3a, alditol acetates obtained from K-61 carboxyl-reduced with diborane; 3b, alditol acetates obtained from carboxyl-reduced K-61 [reduced with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate].

(3 mg) of the methylated K-61 was hydrolyzed with 0.5M sulfuric acid for 20 h, and the alditol acetates were prepared, and analyzed by g.l.c. (column *A* at 160°, and column *B* at 195°). The results are summarized in Table I. Another portion (4 mg) of the methylated K-61 was dissolved in a mixture of dichloromethane (8 ml) and ethyl ether (5 ml). To this solution was added an excess of lithium aluminum hydride (40 mg); the mixture was boiled under reflux for 6 h in a hot-water bath, and then kept for 16 h at room temperature. The excess of lithium aluminum hydride was decomposed by adding ethyl acetate and, finally, a few drops of water. The mixture was then made neutral with M phosphoric acid, and filtered through a cotton plug. The filtrate was washed with water (3 × 20 ml), dried (anhydrous sodium sulfate), and evaporated to dryness. The product was hydrolyzed with 90% formic acid for 2 h. The formic acid was evaporated off under diminished pressure, and the product was hydrolyzed with 0.5M sulfuric acid for 20 h. The alditol acetates prepared from this material were analyzed by g.l.c. (column *A* at 160°, and column *B* at 195°); the results are summarized in Table I.

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REFERENCES

1 W. NIMMICH, *Z. Med. Mikrobiol. Immunol.*, 154 (1968) 117–131.
2 N. ROY, W. R. CARROLL, AND C. P. J. GLAUDEMANS, *Carbohydr. Res.*, 12 (1970) 89–96.
3 H. G. WALKER, JR., M. GEE, AND R. M. MCCREADY, *J. Org. Chem.*, 27 (1962) 2100–2102.
4 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–207.
5 B. LINDBERG, J. LÖNNGREN, AND W. NIMMICH, *Carbohydr. Res.*, 23 (1972) 47–55.
6 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Nature*, 168 (1951) 167.
7 E. STUTZ AND H. DEUEL, *Helv. Chim. Acta*, 39 (1956) 2126–2130.
8 R. L. TAYLOR AND H. E. CONRAD, *Biochemistry*, 11 (1972) 1383–1388.