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STRUCTURE OF *Klebsiella* TYPE 61 CAPSULAR POLYSACCHARIDE*

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ABSTRACT

An aldotriouronic acid was isolated from the acid hydrolyzate of the polysaccharide from *Klebsiella* Type 61 (K-61), and its structure was established. Degradation of the permethylated polysaccharide with base established the identity of the sugar unit preceding the glucosyluronic acid residue. The modes of linkage and the sequence of different sugar residues were further confirmed by Smith degradation of K-61. The anomeric configurations of the different sugar residues were determined by oxidation of peracetylated native, and carboxyl-reduced, polysaccharides with chromium trioxide. The anomeric configuration of nonreducing D-galactosyl side-chains was further confirmed by enzymic degradation of K-61. Finally, gentiobiose was identified in the partial, acid hydrolyzate of K-61. Based on these results, the structure assigned the repeating unit of K-61 was as follows.

$$\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow$$
$$\begin{array}{c} 3 \\ \uparrow \\ 1 \\ \alpha\text{-D-Gal}p \end{array}$$

INTRODUCTION

There are 81 serologically recognized strains in the genus *Klebsiella*[2,3]. They were differentiated on the basis of their capsular polysaccharides, which behave as antigens. *Klebsiella* Type 61 is one of the approximately twenty strains belonging to the same chemotype, *i.e.*, having the same monosaccharide components. It was, therefore, necessary to determine the structure of each member of the chemotype. Structures of at least five polysaccharides[4–8] of this chemotype have already been reported. In our previous communication[1], we mentioned twelve possible structures for the repeating unit of K-61. We now report further studies on this polysaccharide that have enabled us to select the correct structure for its repeating unit.

*For our first paper on *Klebsiella* Type 61, see ref. 1.

RESULTS AND DISCUSSION

Earlier, we reported[1] that hydrolysis of K-61 with 0.5M sulfuric acid for 6 h at 100° gives an aldobiouronic acid and an aldotriouronic acid, in addition to monosaccharides. The aldobiouronic acid was found to be 2-*O*-(β-D-glucopyranosyluronic acid)-D-mannopyranose.

It was now deemed necessary to study the aldotriouronic acid. On hydrolysis with 0.5M sulfuric acid for 20 h, it gave D-mannose and D-glucose, the proportion of D-glucose being greater than that of D-mannose, as analyzed by g.l.c. (see Table I, column 2). This indicated that the aldotriouronic acid is composed of glucose, mannose, and glucuronic acid. The aldotriouronic acid was converted into the corresponding, neutral trisaccharide by reducing the carboxyl groups[9]. On acid hydrolysis, this neutral trisaccharide gave glucose and mannose in the ratio of 2:1 (see Table I, column 3) by g.l.c. analysis, showing more convincingly that the aldotriouronic acid contains the three sugar components mentioned.

TABLE I

RESULTS OF ACID HYDROLYSIS OF THE ALDOTRIOURONIC ACID AND OF K-61 POLYSACCHARIDE

Sugar (as alditol acetate)	*Mole %*[a]				
	A	*B*	*C*	*D*	*E*
Galactose	—	—	—	15.6	28.8
Glucose	52.8	68.5	26.6	66.4	56.5
Glycerol	—	—	48.3	—	—
Mannose	47.2	31.5	25.1	17.9	14.7

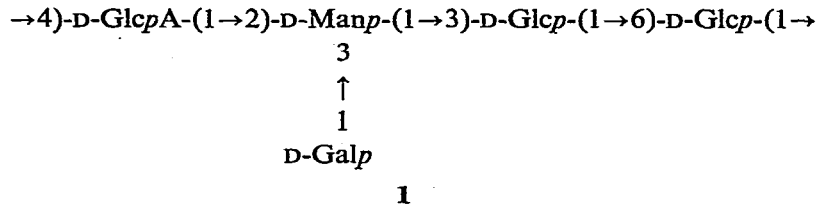
[a]Key: A, Alditol acetates obtained from the aldotriouronic acid; B, alditol acetates obtained from the aldotriouronic acid carboxyl-reduced with diborane; C, alditol acetates obtained from K-61 after periodate oxidation; D, alditol acetates obtained from K-61 after degradation with α-D-galactosidase; and E, alditol acetates obtained from the native polysaccharide.

Methylation[10] of the aldotriouronic acid gave a fully methylated product. Hydrolysis of the permethylated aldotriouronic acid with 0.5M sulfuric acid gave material that had a single peak in g.l.c., and there was no peak for tetra-*O*-methylglucose. The retention time of this single peak was the same as that of both 3,4,6-tri-*O*-methylmannose and 2,4,6-tri-*O*-methylglucose. (The retention time of 3,4,6-tri-*O*-methylmannose is exactly the same as that of 2,4,6-tri-*O*-methylglucose, in both columns[11] *1* and *2*.) As there was no peak for tetra-*O*-methylglucose, two trimethyl sugars were expected to be formed from the permethylated aldotriouronic acid. Moreover, we observed[1] the presence of 2,4,6-tri-*O*-methylglucose and 4,6-di-*O*-methylmannose during the methylation analysis of K-61. It was, therefore, most probable that this peak represented a mixture of 3,4,6-tri-*O*-methylmannose and 2,4,6-tri-*O*-methylglucose.

In another experiment, permethylated aldotriouronic acid was reduced with lithium aluminum hydride. On g.l.c. analysis, the alditol acetates prepared from the

reduced product gave two signals, namely, one for 2,3,4-tri-*O*-methylglucose, and one for a mixture of 2,4,6-tri-*O*-methylglucose and 3,4,6-tri-*O*-methylmannose, in the ratio of 1 : 2. The 2,3,4-tri-*O*-methylglucose must have arisen from the glucuronic acid present in the aldotriouronic acid. These results showed that the aldotriouronic acid is an *O*-(D-glucopyranosyluronic acid)-(1→2)-*O*-D-mannopyranosyl-(1→3)-D-glucopyranose.

With the general structure of the aldotriouronic acid settled, it became possible to write four structures (disregarding the anomeric configurations of the different sugar residues) for the repeating units of the polysaccharide, namely, **1–4**.



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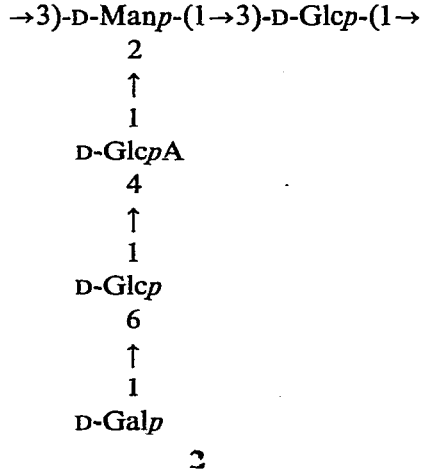

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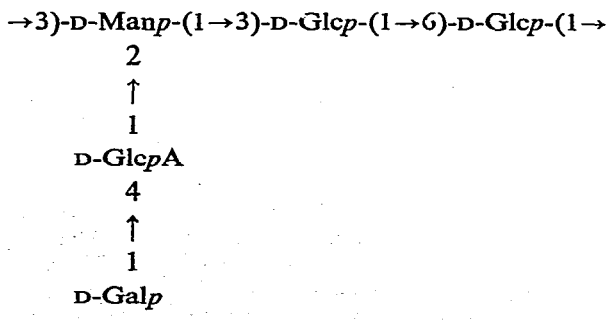

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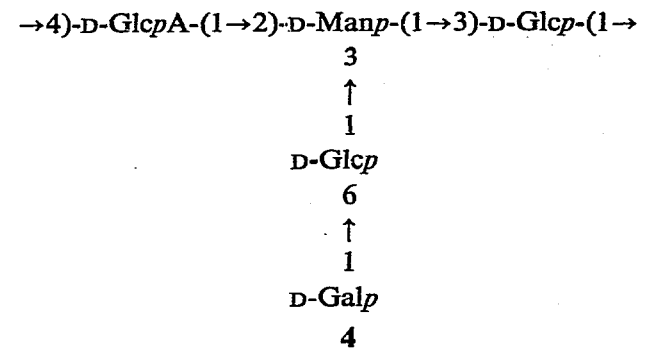

In order to obtain further information regarding the sequence of sugar units, the permethylated polysaccharide was subjected to degradation with base[12,13]. In this degradation, the 4-substituent on a glycosyluronic residue is eliminated, along with the uronic residue. In the base-degradation experiment, separate samples of permethylated K-61 were allowed to react with sodium methoxide for 0.5 h and 2 h, respectively. Both were then made neutral with 50% acetic acid, and the alditol acetates were prepared, and analyzed by g.l.c. G.l.c. of the 0.5-h material showed the presence of 2,3,4,6-tetra-*O*-methylgalactose, 2,4,6-tri-*O*-methylglucose, 2,3,4-tri-*O*-methylglucose, and 4,6-di-*O*-methylmannose in the ratios 4:4:3:4 (see Table II, column 6). When the degradation was allowed to proceed for 2 h, the ratios of these components changed to 20:20:7:20 (see Table II, column 7). Thus, the 2,3,4-tri-*O*-methylglucose had been degraded to the extent of 65% in 2 h, strongly indicating that the parent glucosyl residue precedes the glucosyluronic acid residue in the repeating unit of K-61. It was, therefore, possible to eliminate structures **3** and **4**, leaving only two possibilities for the repeating unit, *viz.*, structures **1** and **2**.

TABLE II

METHYLATION ANALYSIS OF BASE-DEGRADED AND PERIODATE-OXIDIZED K-61 POLYSACCHARIDE

Methylated sugar (as alditol acetate)	*Retention time (minutes)*		*Mole %*[a]				
	Column 1	*Column 2*	*A*	*B*	*C*	*D*	*E*
2,3,4,6-Tetra-*O*-methyl-D-galactose	1.21	1.15	27.0	19.0	23.1	29.0	—
2,3,4,6-Tetra-*O*-methyl-D-mannose	1.00	0.99	—	—	—	—	53.8
2,4,6-Tri-*O*-methyl-D-glucose	1.91	1.71	27.4	21.9	28.2	31.2	46.2
2,3,4-Tri-*O*-methyl-D-glucose	2.41	2.05	28.4	21.0	21.6	10.2	—
2,3-Di-*O*-methyl-D-glucose	5.51	3.78	—	15.9	—	—	—
4,6-Di-*O*-methyl-D-mannose	3.28	2.64	15.2	19.2	27.4	29.7	—

[a]Key: A, Alditol acetates obtained from permethylated K-61 before reduction with lithium aluminum hydride; B, alditol acetates obtained from permethylated K-61 after reduction with lithium aluminum hydride; C, alditol acetates obtained from permethylated K-61 after degradation with sodium methoxide for 0.5 h; D, alditol acetates obtained from permethylated K-61 after degradation with sodium methoxide for 2 h; and E, alditol acetates obtained by methylation of the periodate-oxidized product.

On oxidation of the polysaccharide with sodium periodate[14], followed by treatment of the product with sodium borohydride, a polyol was obtained. Total hydrolysis of this polyol gave mannose, glucose, and glycerol in the ratios of 1:1:2 (see Table I, column 4). The peak for glucose must have arisen from the 3-*O*-substituted glucosyl residue. The presence of mannose was expected, because of its substituents on O-2 and O-3. Thus, the results corroborated the findings of the methylation analysis[1] of K-61. The 4-*O*-substituted glucuronic acid, the 6-*O*-substituted glucose, and the (nonreducing) galactosyl group were completely degraded by periodate.

The polyol obtained on periodate oxidation was hydrolyzed at room temperature, and the product was methylated[10]. G.l.c. analysis of the alditol acetates prepared from the methylated material showed the presence of 2,3,4,6-tetra-*O*-methylmannose and 2,4,6-tri-*O*-methylglucose in the ratio of ~1:1 (see Table II, column 8). These findings showed that periodate oxidation of K-61 produces a disaccharide, namely, *O*-D-mannopyranosyl-(1→3)-D-glucopyranose. This result thus eliminated structure **2**, because periodate oxidation of **2** would give a (1→3)-linked, polymer backbone which, on methylation analysis, would give 2,4,6-tri-*O*-methylmannose and 2,4,6-tri-*O*-methylglucose. It was, therefore, proved that **1** represents the structure of the repeating unit of K-61.

The mode of linkages of different sugar residues and their sequences having been established, it was necessary to determine their anomeric configurations. The polysaccharide had $[\alpha]_D^{27}$ +56°. It was, therefore, expected that K-61 has both α and β linkages in its repeating unit. The low specific rotation of the aldobiouronic acid strongly indicated that the uronic acid is β-linked. The native polysaccharide (K-61) and the carboxyl-reduced K-61 (having[1] $[\alpha]_D^{27}$ +52°) were acetylated and the peracetates were subjected to oxidation with chromium trioxide[15]. During such an oxidation, only the axially oriented 1-proton is abstracted[16], to yield a 5-hexulosonic acid, thus leading to the disappearance of the sugar residues having β-glycosidic linkages. In both of the foregoing experiments, the amount of glucose decreased very

TABLE III

OXIDATION OF PERACETYLATED K-61, AND CARBOXYL-REDUCED K-61, WITH CHROMIUM TRIOXIDE

Material[a]	*Time of oxidation (hours)*	*Galactose*	*Glucose*	*Mannose*	myo-*Inositol*
A	0	2.7	6.2	1.5	20
	1	1.5	1.3	1.3	20
	2	0.9	0.8	0.9	20
B	0	5.8	17.8	5.5	20
	1	3.2	3.3	3.1	20
	2	2.5	2.7	2.6	20

[a]A, Alditol acetates obtained from peracetylated K-61 oxidized with chromium trioxide; B, alditol acetates obtained from peracetylated, carboxyl-reduced K-61 oxidized with chromium trioxide.

sharply during the first hour, but did not change much in the second hour (see Table III), indicating that one of the two glucosyl residues has an α-glycosidic linkage, and the other, a β. The amounts of mannose and galactose did not change sharply, and this was more prominent in the case of the carboxyl-reduced K-61. It was, therefore, shown that the mannosyl and galactosyl residues have α-glycosidic linkages. It was not, however, possible, from the results of the chromium trioxide experiments, to decide which glucosyl residue is α- and which is β-linked.

The structure of the repeating unit (**1**) of the polysaccharide, which has now been proved, has two glucosyl residues consecutive. Therefore, the neutral disaccharide that could be obtained from the polymer would be either isomaltose or gentiobiose. In order to search for a neutral disaccharide, the polysaccharide was hydrolyzed with 45% formic acid for 2 h, and the neutral and acidic fractions were separated on Dowex-1 X4 (OAc^-) ion-exchange resin. Paper chromatography of the neutral fraction showed a small, but clear, spot for gentiobiose. The identity of the spot was confirmed by comparing its mobility with that of authentic gentiobiose on the same paper chromatogram. Thus, in K-61, the glucosyl residue preceding the glucosyluronic acid residue has the α-anomeric configuration, and that following the mannosyl residue has the β-anomeric configuration.

In order to adduce further information regarding the anomeric configuration of the D-galactosyl groups, the polysaccharide was subjected to degradation with the enzyme[17] α-D-galactosidase and, after removal of the enzyme, the mixture was dialyzed; g.l.c. of the concentrated dialyzate showed only D-galactose. G.l.c. analysis of the dialyzed material retained showed D-galactose, D-glucose, and D-mannose in the ratios of 1:4:1.2 (see Table I, column 5), whereas the alditol acetates prepared from the native polysaccharide under the same conditions showed D-galactose, D-glucose, and D-mannose in the ratios of 2:4:1 (see Table I, column 6). This indicated that >50% of the D-galactosyl groups had been removed by the α-D-galactosidase, further confirming that the D-galactose is α-linked.

We therefore conclude that the structure of the repeating unit of the polysaccharide from *Klebsiella* Type 61 is **5**.

$$\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 2)\text{-}\alpha\text{-D-Man}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow$$

$$\begin{array}{c} 3 \\ \uparrow \\ 1 \\ \alpha\text{-D-Gal}p \end{array}$$

5

EXPERIMENTAL

Materials and methods. — Optical rotations were measured with a Perkin–Elmer Model 241 MC spectropolarimeter. Paper chromatography was performed on Whatman No. 1 and No. 3 papers. Solvent systems (v/v) used were (*A*) 9:2:2 ethyl

acetate–acetic acid–water, (*B*) 8:2:1 ethyl acetate–pyridine–water, and (*C*) 4:1:5 1-butanol–acetic acid–water (upper layer). The spray reagents used were (*a*) alkaline silver nitrate[18], and (*b*) 3% *p*-anisidine hydrochloride in ethanol[19]. All solvents were distilled before use, and all evaporations were conducted at 50°, unless otherwise stated. Small volumes of aqueous solutions were lyophilized. Gas-liquid chromatography (g.l.c.) was performed with a Hewlett–Packard Model 5730 A gas chromatograph having a flame-ionization detector, and glass columns (1.83 m × 6 mm) packed with (*1*) 3% of ECNSS-M on Gas Chrom Q (100–120 mesh), and (*2*) 5% of OV-225 on Gas Chrom Q (100–120 mesh).

All g.l.c. analyses were conducted by first converting the sugars into their corresponding alditol acetates. Retention times of 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. 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 at 100°. The hydrolyzate was made neutral with barium carbonate, the suspension was filtered through a Celite bed, and to the filtrate was added an equal volume of 5% sodium borohydride solution. After 4 h, the solution was decationized with Dowex-50W X8 (H^+) ion-exchange resin, and evaporated to dryness. The boric acid was removed as methyl borate, and the mixture of alditols was acetylated with acetic anhydride (2 mL) and pyridine (3 mL) for 2 h at 100°.

Hydrolysis of aldotriouronic acid and its carboxyl-reduced product. — The aldotriouronic acid (0.5 mg) was hydrolyzed with 0.5M sulfuric acid for 20 h, and the alditol acetates were prepared. The mixture of alditol acetates was analyzed by g.l.c. (column *1* at 195°). In another experiment, the aldotriouronic acid (0.5 mg) was converted into the corresponding neutral trisaccharide according to the method described before[1]. Alditol acetates were prepared from this material, and analyzed by g.l.c. (column *1* at 195°).

Methylation analysis of aldotriouronic acid. — The aldotriouronic acid (1.5 mg) was methylated with methyl iodide and silver oxide as described for the aldobiouronic acid[1] obtained from K-61. Alditol acetates obtained from a portion of the methylated product were analyzed by g.l.c. (column *1* at 155°, and column *2* at 195°). The other portion of the permethylated aldotriouronic acid (1 mg) was reduced with lithium aluminum hydride as described before[1]. Alditol acetates prepared from this material were analyzed by g.l.c. (column *1* at 155°, and column *2* at 195°).

Methylation of K-61. — The polysaccharide (20 mg) was methylated as described before[1]. The permethylated product was purified by passing a solution of it through a column (15 × 1.5 cm) of Sephadex LH-20, with 2:1 chloroform–acetone as the eluant. The eluate was monitored polarimetrically, and the fractions containing the permethylated K-61 were collected, and evaporated to a thick syrup; yield 18.5 mg, $[\alpha]_D^{27}$ +31.5° (*c* 1, chloroform).

Base degradation[20] *of permethylated K-61.* — Carefully dried, permethylated K-61 (8 mg) was dissolved in 10:1:2 dry methanol–2,2-dimethoxypropane–dichloromethane (20 mL). To the solution was added a trace of *p*-toluenesulfonic acid, and

the solution was boiled under reflux for 30 min. A piece of freshly cut sodium (250 mg) was then added to the cooled solution, and the resulting, turbid solution was boiled under reflux. A portion was removed after 0.5 h, and the reaction of the rest was continued for 2 h. After cooling, the pH of the two portions was adjusted to 6.0 by the addition of 50% acetic acid. Water (50 mL) was added to both, and the mixtures were partitioned between water and chloroform. For each, the organic phases were combined, washed with water (2 × 25 mL), dried (anhydrous sodium sulfate), and evaporated to dryness. The two samples thus obtained were separately hydrolyzed, and the alditol acetates were prepared. The mixtures of alditol acetates were analyzed by g.l.c. (Column *1* at 155°, and column *2* at 195°); the results are given in Table II, columns 6 and 7.

Oxidation of K-61 with periodate. — To a 0.05% solution of the polysaccharide (40 mL) was added 0.2M sodium periodate solution (10 mL), and the mixture was kept in the dark for 48 h at 5°. The excess of periodate was decomposed by adding an excess of ethylene glycol (2.5 mL), and the mixture was kept for 3 h and then dialyzed against distilled water. Sodium borohydride (75 mg) was added, and the solution was kept for 4 h at room temperature, decationized with Dowex-50W X8 (H^+) ion-exchange resin, and evaporated to dryness. Boric acid was removed by repeated addition and evaporation of methanol. Alditol acetates prepared from a portion of the polyol thus obtained were analyzed by g.l.c. (column *1* at 190°); the results are summarized in Table I, column 4.

The rest of the polyol was hydrolyzed with 0.5M sulfuric acid for 8 h at room temperature. The acid was neutralized with barium carbonate, the suspension was filtered through a Celite bed, and the filtrate was decationized with Amberlite IR-120 (H^+) ion-exchange resin, and lyophilized. The lyophilized material was methylated[10], and the alditol acetates prepared from the methylated product were analyzed by g.l.c. (column *1* at 155° and column *2* at 190°). The results are given in Table II, column 8.

Oxidation of K-61 with chromium trioxide. — A mixture of the original polysaccharide (3.2 mg) and *myo*-inositol (2 mg) was dissolved in formamide (0.5 mL). To this were added acetic anhydride (1 mL) and pyridine (1.5 mL), and the mixture was stirred for 16 h at room temperature, dissolved in chloroform (25 mL), and the solution washed with water (3 × 20 mL), dried (anhydrous sodium sulfate), and evaporated to dryness. The product was reacetylated with acetic anhydride (2 mL) and pyridine (3 mL) for 16 h at room temperature, and the mixture was then evaporated to dryness.

Powdered chromium trioxide (300 mg) was added to a solution of the mixture of acetates of K-61 and *myo*-inositol in glacial acetic acid (3 mL). The resulting mixture was stirred in a water bath at 50°. Aliquots were removed at 0, 1, and 2 h, and immediately diluted with water. Each mixture was partitioned between water and chloroform, and the respective chloroform extracts were combined, dried (anhydrous sodium sulfate), and evaporated to dryness. The samples were deacetylated with 0.2M sodium methoxide for 3 h, and decationized with Amberlite

IR-120 (H^+) ion-exchange resin, and the alditol acetates were prepared in the usual way. The alditol acetates were analyzed by g.l.c. (column *1* at 190°); the results are given in Table III.

Oxidation of carboxyl-reduced K-61 with chromium trioxide. — A mixture of carboxyl-reduced polysaccharide (1.52 mg) and *myo*-inositol (1.1 mg) was acetylated, and the product was oxidized with chromium trioxide in the same way as for the native polysaccharide. The results of the g.l.c. analysis are summarized in Table III.

Degradation of K-61 with enzyme. — To a solution of the polysaccharide (5 mg) in 0.2M sodium acetate buffer (pH 5.0; 1.5 mL) was added an excess (0.5 mL) of α-D-galactosidase (concentration of the enzyme, 5 mg/1 mL; source, *Coffeae arabicae*). A few drops of toluene were added to prevent bacterial growth, and the mixture was incubated for 50 h at 37°. The enzyme was then deactivated by heating for 30 min at 70°. The precipitated enzyme was filtered off through a Celite bed, and the filtrate was dialyzed for two days against distilled water. The dialyzate was collected, and evaporated to dryness. The alditol acetates prepared from this material showed, in g.l.c. analysis, only D-galactose. Alditol acetates prepared from the dialyzed material and from K-61 (2 mg) under the same conditions were analyzed by g.l.c. (column *1* at 190°); the results are given in Table I, columns 5 and 6.

Partial hydrolysis of K-61. — The polysaccharide (18.5 mg) was dissolved in 90% formic acid (5 mL). Water (5 mL) was added, and the mixture was heated for 2 h in a water bath at 100°, cooled, and evaporated to dryness *in vacuo*, and traces of formic acid were removed by repeated addition and evaporation of water. An aqueous solution of the product was treated with Dowex-50W X8 (H^+) ion-exchange resin, and then passed through a column of Dowex-1 X4 (OAc^-) ion-exchange resin. The neutral sugars were collected by eluting the column with water, and the eluate was evaporated to dryness (2.5 mg). The acidic sugars were then eluted with 30% acetic acid, and the eluate was evaporated to dryness (14 mg). Paper chromatography (solvent *C*) of the neutral fraction showed, in addition to galactose and glucose, a spot that corresponded exactly with that of an authentic sample of gentiobiose.

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