STRUCTURAL STUDIES OF *KLEBSIELLA* CAPSULAR POLYSACCHARIDES

BY

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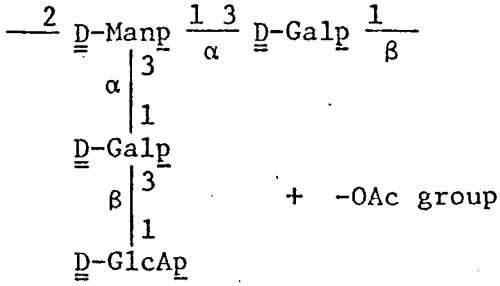
STRUCTURAL STUDIES OF KLEBSIELLA CAPSULAR POLYSACCHARIDES

ABSTRACT

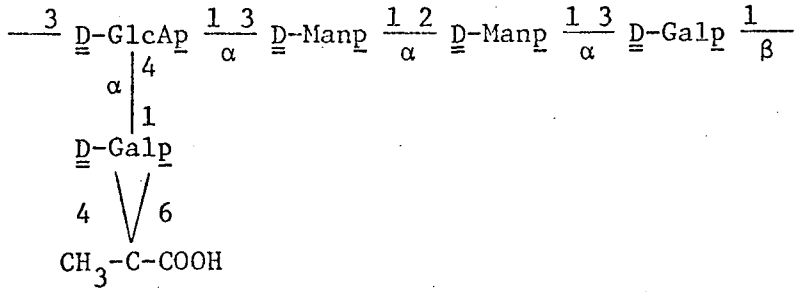
Eighty types of Klebsiella are recognised on the basis of immunochemical tests. Qualitative analyses of the capsular polysaccharides produced by these bacteria have been provided by Nimmich. Nevertheless very few detailed structures are known in contrast to the qualitative data published.

The present investigation deals with the structural studies of capsular polysaccharides from four strains of these bacteria, for example K20, K21, K24 and K56. Besides the classical methods used, new techniques have been used or developed in order to obtain detailed information on the structures. Modern methods used include the circular dichroism (c.d.) for the assignment of the D or L configuration of sugar constituents. Gas-liquid chromatography (g.l.c.) and mass spectrometry (m.s.) were used to analyse the partially methylated sugars which can be characterized further by demethylation and reacetylation to give crystalline derivatives. In the course of our studies, p.m.r. spectroscopy at 95^{o} has been developed and becomes a very powerful tool for the assignment of the anomeric linkages of oligosaccharides and polysaccharides. It has also been found to be an excellent method for the detection and quantitative assay of other functional groups such as O-acetyl or pyruvic acid without degrading the polymer. The application of the above modern techniques together with use of the classical methods affords the detailed structures of K20, K21, K24 and K56, the repeating units of which are as follow:

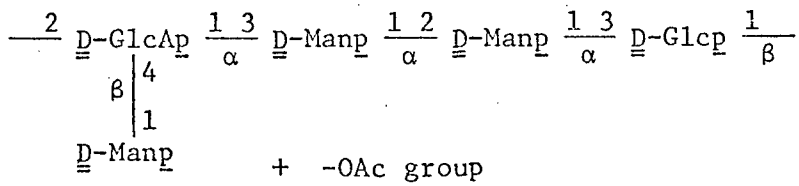
K20



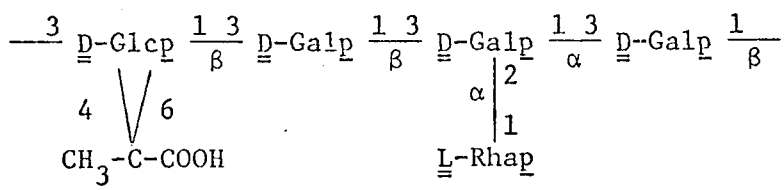
K21



K24



K56



The structure of K20 is the first of these Klebsiella capsular polysaccharides to be encountered with an aldobiouronic acid side chain. The capsular polysaccharides K21 and K24 each contains the same type of aldobiouronic acid with branching on the D-glucuronic acid. The capsular polysaccharide K56 has a L-rhamnose side chain. The acidity of the

polysaccharide is due only to the pyruvic acid present and this constitutes the first investigation of those Klebsiella polysaccharides lacking uronic acid.

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The author wishes to express his sincere gratitude to Dr. G.G.S. Dutton for his guidance, inspiration and assistance. The financial assistance from the Chemistry Department, University of British Columbia and National Research Council of Canada are also gratefully acknowledged.

INTRODUCTION

The polysaccharides of micro-organisms can be divided into three major groups according to their morphological localization: (i) extracellular surface polysaccharides located outside the cell wall and frequently termed capsular polysaccharides, (ii) cell wall polysaccharides, and (iii) somatic or intracellular polysaccharides located inside the cytoplasmic membrane.

The presence or absence of these and the amounts and nature of them will differ widely from organism to organism.

There is a distinction[1] between the cell wall of a bacterium and the capsule, for the latter may be described as a covering layer outside the cell wall. The presence of a capsule is one of the conditions for the virulence of a cell. This layer is usually composed of a single polysaccharide. The capsular polysaccharides give specific agglutination or precipitation reactions with the antisera obtained by the action of the bacteria concerned on animals. These polysaccharides may act as antigens including the production of the appropriate antibody in the animals, the antibodies then conferring immunity against the particular bacterial infection concerned.[2]

The whole cellular structure of some organisms such as Escherichia or Klebsiella may be embedded in a hydrophilic, acidic polysaccharide (often termed capsular or K-antigen) which is loosely bound to the cell surface. Such polysaccharides may sometimes also be found dissolved in the liquid culture filtrate in which the bacteria are grown, hence the term extracellular polysaccharides.

Bacteria of the genus Klebsiella belong to the family Enterobacteriaceae, the chemistry of which has been reviewed by Lüderitz, Wheat and Jann.[3] Approximately 80 types of Klebsiella are known on the basis of immunochemical tests. One of the characteristics of Klebsiella bacteria is the formation of a capsular polysaccharide which is antigenic and the composition of which has been stated to be the same as that of the slime polysaccharide excreted into the medium.[2]

Nimmich[4,5] has reported the qualitative composition of K-types 1 to 80 and has shown that the great majority contain D-glucuronic acid in combination with hexoses such as D-glucose, D-galactose and D-mannose. Many strains contain, in addition, L-rhamnose and a few contain L-fucose. The presence of bound pyruvic acid in Klebsiella was first reported by Wheat, Dorsch and Godoy[6] in the case of K. rhinoscleromatis (type 3) and pyruvic acid has subsequently been found by Wheat and his colleagues to be present in several different species,[7] in fact nearly half of the Klebsiella polysaccharides have been found to contain pyruvic acid. It is interesting to note that all the capsular Klebsiella polysaccharides are acidic in nature, containing either uronic acid or pyruvic acid or both; there are a few containing an unknown ketoacid.

In contrast to the data on the qualitative composition of most Klebsiella capsular polysaccharides there have been few detailed structures published of these carbohydrate polymers. Structures have been given for the capsular polysaccharides of K-types 2,[8] 8,[9] and 54.[10,11] In each of the capsular polysaccharides so far examined the structures may be expressed as multiples of a repeating unit containing three to five sugar units.

To analyze the structures of these Klebsiella polysaccharides full use of the classical and modern techniques has been made.

METHODS USED FOR STRUCTURAL ANALYSIS

The determination of the structure of a polysaccharide may be achieved easily if full use is made of the present knowledge of these substances and the available modern analytical techniques. The following is an outline of the methods used in the present investigation of the structures of the capsular polysaccharides from four strains of Klebsiella:

A. Isolation and Purification

Since it is believed that the slime polysaccharide excreted into the medium of the Klebsiella culture has the same composition[2] as the capsular polysaccharide, the capsular polysaccharide is usually obtained by water extraction.

Detergent cations such as cetyltrimethylammonium react with polyanions to form salts which are very insoluble in water. Neutral polysaccharides do not react except as borate complexes. Cetyltrimethylammonium bromide[12] has therefore been widely employed for purifying Klebsiella polysaccharides most of which are quite acidic.

B. Hydrolysis and Characterization of Sugar Constituents

Before hydrolysis, the purified polysaccharide is characterized by the determination of (i) the optical rotation in water and (ii) the equivalent weight by titration with sodium hydroxide. The individual sugar constituents are obtained by hydrolysis which is usually done with 0.5 M or 1 M sulfuric acid. In recent years, trifluoroacetic acid[13] has been widely used in our laboratory to hydrolyze polysaccharides. In the case of trifluoroacetic acid, advantage has been taken of its easy

removal by evaporation at reduced pressure.

Paper partition chromatography introduced in 1944 for the separation and determination of amino acids, has been highly successful for the separation of sugars. It was demonstrated[14,15] that closely related sugars could be separated from each other provided a suitable solvent or combination of solvents could be selected. Quantitative determination may for instance be accomplished by the phenol-sulfuric acid[16] method. Nowadays, gas liquid chromatography (g.l.c.) has been well developed to the point where it may be generally employed for the qualitative and quantitative analysis of sugars. One advantage of the method is that only small amounts of material are required. The great impact of g.l.c. separation of carbohydrates started in 1963 when Sweeley and coworkers[17] published a paper on the application of trimethylsilyl derivatives of carbohydratesin g.l.c. The discovery that these derivatives were easily formed and that they were volatile revolutionized carbohydrate analysis. A problem is encountered in quantitative analysis of mixtures of sugars, as their trimethylsilyl ethers, due to the overlapping of peaks which complicates the interpretation of the results. Early attempts to analyze sugars as their fully acetylated alditols, where each sugar gives a single peak, were not entirely successful, as the resolution of isomeric substances was often unsatisfactory. However, Sawardeker and coworkers[18] found that improved separation of acetylated alditols could be obtained using a copolymer of ethylene glycol succinate polyester and a nitrile silicone polymer (ECNSS-M) as the stationary phase. One excellent advantage is that g.l.c. of the alditol acetates permits the isolation and characterization of crystalline derivatives of the common hexoses even with semi-

microquantities. The other merit of the method is that each sugar gives a single peak and thus, quantitative evaluation of the chromatogram is considerably facilitated.

C. Configuration of Constituents

The D or L configuration of monosaccharides is usually assigned from the results obtained by measurement of the optical rotation which involves large quantities or alternatively by the action of specific enzymes, if they are available. With the modern development of circular dichroism (c.d.), one can now assign the configuration of monosaccharides using only a semimicroquantity.[19] In the classical method of configuration assignment, the monosaccharides are isolated by paper chromatography and their optical rotations measured. Large quantities are required in order to get an accurate measurement. However, such amounts are not always available in some cases of bacterial polysaccharides. To overcome this difficulty, a semimicroanalytical technique has been developed in our laboratory by measuring the circular dichroism curves of the alditol acetates. The acetoxy group has a very strong absorption at $\sim$ 213 mμ. Thus the c.d. curve may be obtained with less than 1 mg of sample, which is usually isolated via the g.l.c. Identical samples will have identical c.d. curves (i.e., same extinction coefficient and sign) so by comparing c.d. curves of standard samples and unknowns the configuration (D or L) of a monosaccharide may be assigned unambiguously.

D. Methylation Analysis

After purification of the polysaccharide, determination of the composition and characterization of the component sugars, one is faced with the problem of determining (a) the mode of union of various component sugars, (b) the sequential order of the components, and (c) the anomeric nature of the glycosidic linkages uniting the components.

The first problem is solved by subjecting the polysaccharides themselves to methylation analysis. Generally the methylation proceeds normally by treatment with sodium hydride in methyl sulfoxide and methyl iodide,[10,20] a process which gives good methylation within a reasonably short time. The analysis of the partially methylated polysaccharide has been greatly simplified in recent years by three major improvements in experimental techniques. These are (i) the separation of the methylated sugars or their derivatives by g.l.c.,[21] (ii) the determination of the position of methoxyl groups by mass spectrometry (m.s.),[22] and (iii) demethylation and reacetylation of the partially methylated alditol acetates to their parent alditol acetates;[23] discussions of these follow:

(i) G.l.c. of methylated sugars

G.l.c. has become an important method in methylation analysis of polysaccharides, both as an aid in identifying individual methylated sugars and for their quantitative measurement. The methylated sugars usually cannot be separated directly by g.l.c. but have to be transformed into more volatile derivatives such as methyl glycosides. In most cases a methylated sugar gives a mixture of glycosides in fairly constant proportions which sometimes facilitates identification. In more complex

mixtures the multiplicity of peaks becomes a disadvantage, as the quantitative evaluation becomes more difficult. The same problem is encountered in quantitative analysis of mixtures of sugars, as their trimethylsilyl ethers.[16] By reduction of the methylated sugars to alditols a single derivative is obtained from each sugar. The separation of isomeric alditol derivatives (e.g., acetates) was not, however, better than that obtained from isomeric methyl glycoside derivatives until after the introduction of a new phase for g.l.c., ECNSS-M.[17] Columns packed with butanediol succinate (BDS) are also found to be highly effective in separating methylated alditol acetates and in some cases give better resolution than ECNSS-M. For example, 2,4,6- and 3,4,6-tri-O-methyl-D-mannitol acetates are not separated on an ECNSS-M column but are well resolved on a 5% butanediol succinate column.[24]

In situations where the partially methylated alditol acetates are not separated by the above columns (see analysis of capsular polysaccharide of K24 in appendix), satisfactory resolutions have been obtained by using the partially methylated aldose acetates.[25] Although this method causes each compound to give up to four peaks, it is a supplement to the above procedures.

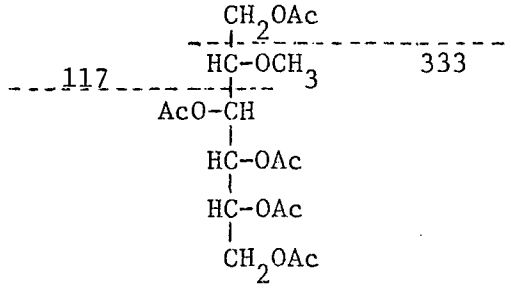
(ii) Mass spectrometry of methylated alditol acetates

Recently, mass spectrometry has become an important tool in carbohydrate chemistry and several groups have studied the mass spectra of various carbohydrates. Heyns and colleagues[26] investigated the mass spectra of fully methylated glycosides, and Kochetkov and coworkers[27] and Samuelson and coworkers[28] examined the mass spectra of the trimethylsilyl

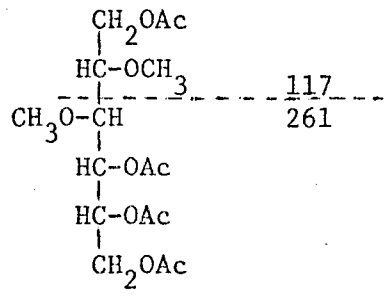
derivatives of partially methylated glycosides. Mass spectrometry of partially methylated alditol acetates has been studied by Lindberg and coworkers[22,29] who made a systematic investigation and made the following generalizations:-

(a) Derivatives with the same substitution pattern (e.g., 2,3,4,6-tetra-O-methyl derivatives of hexitols) give very similar mass spectra, typical of that substitution pattern.

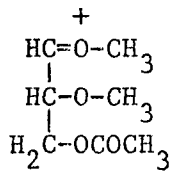
(b) Primary fragments are formed by fission between carbon atoms in the chain. Fission between a methoxylated and an acetoxylated carbon is preferred over fission between two acetoxylated carbons. The alditol acetate derived from 2-O-methyl-D-glucose therefore only gives two main primary fragments.



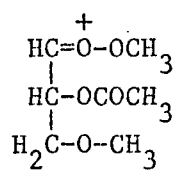
(c) When the molecule contains two adjacent methoxylated carbons, e.g. the alditol acetates derived from 2,3-di-O-methyl-D-glucose, fission between them is preferred over fission between one of these and a neighboring acetoxylated carbon.



(d) Secondary fragments are formed from the primary fragments by single or consecutive loss of acetic acid (m/e 60), ketene (m/e 42), methanol(m/e 32), or formaldehyde (m/e 30). For example, two primary fragments having m/e 161 are expected: F_1 and F_2 (Lindberg's terminology)



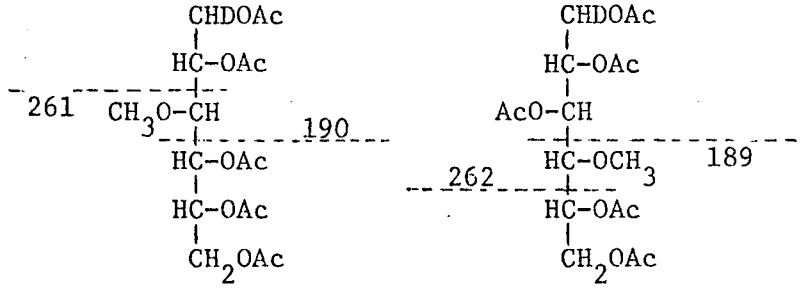
F_1



F_2

Loss of methanol from F_1 gives a secondary fragment having m/e 129, while F_2 gives m/e 101 by loss of acetic acid. Further loss of ketene from m/e 129 gives m/e 87, and loss of formaldehyde from m/e 101 gives m/e 71.

On reduction some pairs of methylated sugars, e.g. a 3-O-methyl and a 4-O-methyl hexose, give an alditol with the same substitution pattern thus providing the same mass spectra. The loss of information brought about by reduction of the sugars to alditols can be avoided if the reduction is performed with sodium deuterioborate; the two alditols then give different mass spectra.



The following table is a summary of the prominent primary peaks of different partially methylated alditol acetates.

Table 1. Primary fragments in the mass spectra of partially methylated sugars in the form of their alditol acetates.

Position of CH_3	m/e														
	45	59	89	117	131	161	175	189	203	205	217	233	261	305	333
Pentoses															
2(4)				x									x		
3								x							
2,3(3,4)				x				x							
2,4				x								x			
2,5	x			x								x			
3,5	x					x		x							
2,3,4				x		x									
2,3,5	x			x		x									
Hexoses															
2(5)				x											x
3(4)								x					x		
6	x														
2,3				x									x		
2,4(3,5)				x				x							
2,5				x											
2,6	x			x											
3,4								x							
3,6	x							x				x			
4,6	x					x							x		
5,6	x		x												x
2,3,4				x		x		x				x			
2,3,5				x								x			
2,3,6	x			x								x			
2,4,6	x			x		x						x			
2,5,6	x		x	x										x	
3,4,6	x					x		x							
2,3,4,6	x			x		x				x					
2,3,5,6	x		x	x						x					
6-Deoxyhexoses															
2				x											
3								x	x						
4					x								x		
2,3				x					x						
2,4				x	x							x			
3,4					x			x							
2,3,4				x	x	x	x								
2,3,5		x		x			x								

Table 1 (continued)

Position of CH_3	m/e														
	45	59	89	117	131	161	175	189	203	205	217	233	261	305	333
3,6-Dideoxyhexoses															
2				x							x				
2,4				x	x		x	x							

(iii) Demethylation and reacetylation of partially methylated alditol acetates

Mass spectrometry has become an important method for the tentative identification of the methylated sugars obtained on methylation analysis of polysaccharides. One advantage of this method is that only small amounts of materials are required. However, isomeric alditol acetates (e.g., 2,4,6-tri-O-methyl-D-galactitol and mannitol acetates) give very similar mass spectra, typical of that substitution pattern. Mass spectrometry cannot distinguish between galactitol and mannitol. Attempts to characterize the partially methylated sugar by demethylation and reacetylation have been made.[23] The partially methylated alditol acetate is demethylated by boron trichloride[30] and reacetylated with pyridine and acetic anhydride. In this case the partially methylated common hexitol acetates (e.g., galactitol, mannitol and glucitol acetates) are converted to hexaacetates which can be isolated via g.l.c. as crystalline derivatives. In the study of K21 capsular polysaccharide, 3,4,6-tri-O-methyl-D-mannose and 2,4,6-tri-O-methyl-D-mannose were individually converted to D-mannitol hexaacetate, m.p. and mixed m.p. 118-121°.[23]

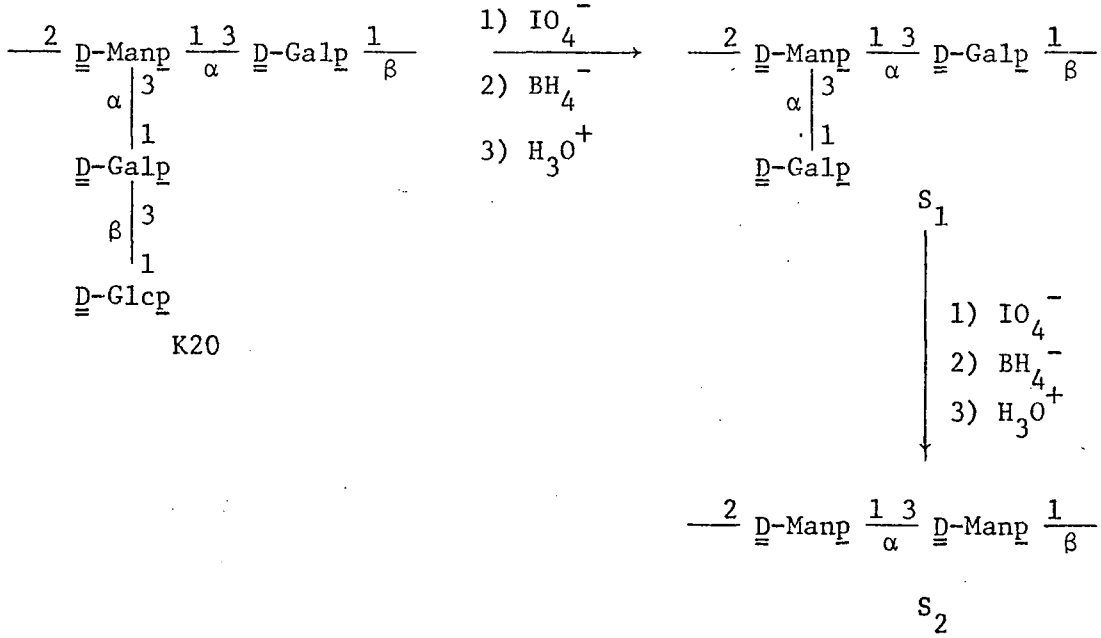
Together with the result obtained by g.l.c.-m.s., the identity of a methylated sugar can be characterized without ambiguity.

The results obtained will show the composition of the polysaccharide, the nature of the building units, how they are joined together, and the number of residues in the average repeating unit. The findings also enable the units at which branching occurs to be designated. However, by themselves, methylation studies on the polysaccharides provide little knowledge concerning the exact sequence of the building units. Methylation results become more diagnostic when considered in conjunction with Smith periodate degradation and partial hydrolysis studies.

E. Smith Periodate Degradation

Non-reducing terminal units in a polysaccharide or (1→6)-linked non-terminal units having three adjacent hydroxyl groups will be cleaved by two molecular proportions of periodate to give one molecular proportion of formic acid. Non-terminal units joined by (1→2) or (1→4) bonds undergo cleavage by one molecular proportion of periodate, but no formic acid is generated. Units which do not possess adjacent hydroxyl groups such as non-terminal units joined by (1→3) bonds or units involved in branching at C_2 or C_4 are not affected by periodate. Thus oxidation of a polysaccharide and quantitative determination of the proportion of the surviving sugar units will give information concerning the nature and proportion of the glycosidic linkages present in the polysaccharide. Smith and coworkers[31] reported that when a sugar residue of a polysaccharide is cleaved by periodate and reduced,

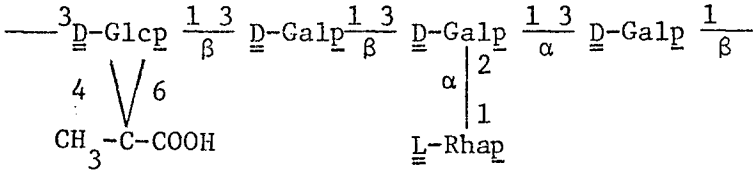
the resulting alcoholic derivative, being a true acetal, is sensitive to mild acid hydrolysis, whereas when a sugar unit which survives cleavage is joined to a unit which is cleaved, the surviving unit appears as a glycoside which is relatively stable to mild acid hydrolysis. Because of the marked difference in stability between true acetals and glycosides, it is now possible to obtain glycosides of mono-, di- and oligosaccharides from a wide variety of polysaccharides after the Smith periodate degradation. The analysis of the structures of these glycosides will throw light on the fine structure of the parent polysaccharides. In our study of the capsular polysaccharide of K20, successive Smith degradations have been used to give a series of degraded polysaccharides. For example, in the original carboxyl reduced polysaccharide, only D-glucose can be removed by the Smith degradation.



In the degraded polysaccharide S_1, only D-galactose in the side chain is susceptible to periodate oxidation. Removal of the D-galactose

side chain will give a new polysaccharide S_2 which in turn gives a galactosyl-glyceraldehyde on further degradation.

For K56 capsular polysaccharide all sugars are resistant to periodate oxidation because of 1→3 linkages, except the L-rhamnose side chain which is linked 1→2 to the main chain.



K56

Instead of using partial hydrolysis to remove the L-rhamnose side chain the Smith degradation has been found to be excellent for the above purpose.

F. Partial Hydrolysis

If the hydrolysis of a polysaccharide is stopped before its completion, oligosaccharides may be isolated. Analysis of the structures of these di-, tri- and higher oligosaccharides will provide evidence for the mode of linkage between them. Partial hydrolysis is usually done by using lower acid concentrations than for complete hydrolysis (e.g. 0.125 M sulfuric acid or 0.5 M TFA) under the ordinary conditions or by using the dialysis apparatus described by Galanos, Lüderitz and Himmelspach.[32] The monosaccharides can be separated by charcoal column chromatography while acidic oligosaccharides can be separated from neutral oligosaccharides by an ion exchange resin (Duolite A4).

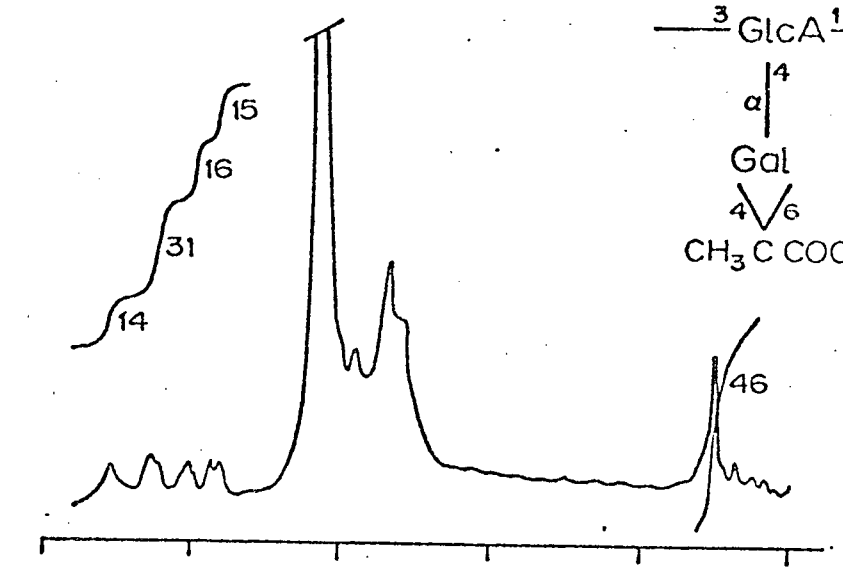
Oligosaccharides up to a D.P. of 4 are separated by paper chromatography. Alternatively, they can be separated by using gel chromatography[33] (e.g. K56 using Sephadex G-15).

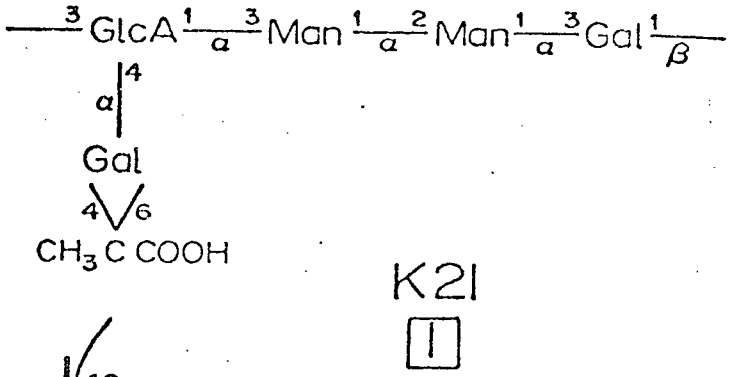
G. Anomeric linkages

Proton magnetic resonance (p.m.r.) spectroscopy which is widely used in carbohydrate chemistry has now been extended to polysaccharide and oligosaccharide chemistry for the assignment of anomeric linkages.[8,34] In general the protons at C-1 of aldopyranoses in deuterium oxide resonate at lower field than the remaining annular protons. Moreover, the anomeric protons in equatorial positions (α-D-configuration) resonate to lower field than those occupying corresponding axial positions (β-D-configuration). In glucose and galactose a trans diaxial interaction between the protons on C-1 and C-2 (β-anomer) results in a spin-spin coupling constant ($J_{1,2}$) which is larger than that given by a gauche interaction (α-anomer).

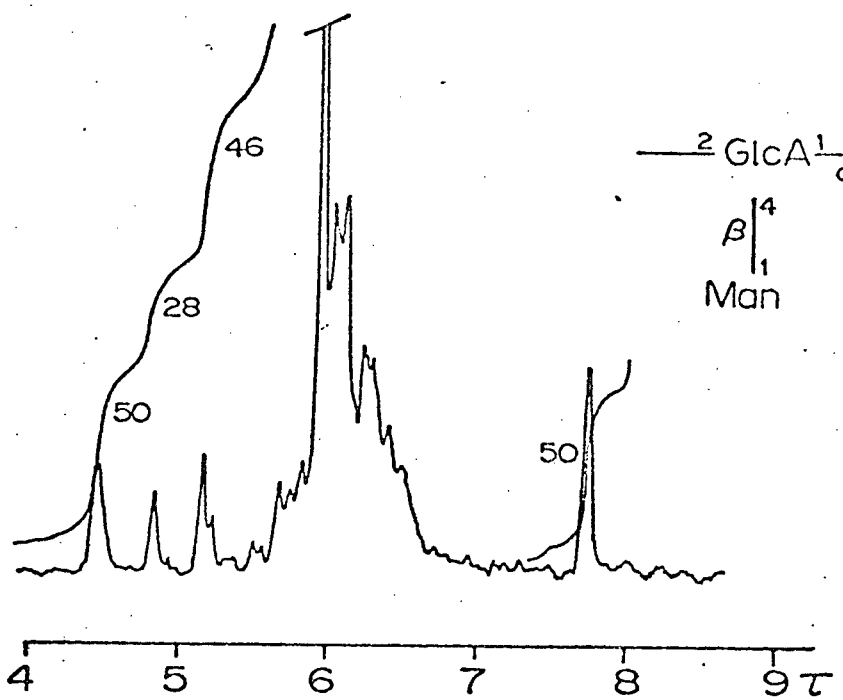
In the case of Klebsiella polysaccharides, which have very high molecular weights, some difficulty was experienced in making qualitative and quantitative determinations of the anomeric protons on account of the large HOD peak present. This peak appears about τ 5-6 and partly covers those regions of the spectrum associated with anomeric and ring protons. The magnitude of the HOD peak was largely due to the necessity of working with solutions of less than 2% concentration because of their viscosity. This same viscosity prevented the solutions being cooled in order to move the HOD peak downfield. It has now been found that excellent p.m.r. spectra may be obtained by dissolving the sodium

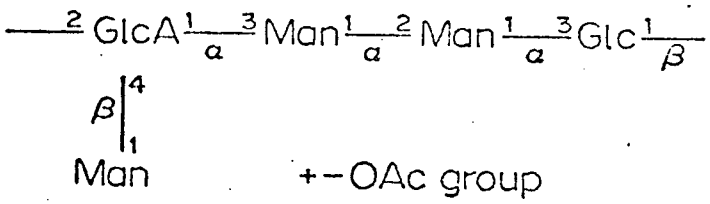
salt of the polysaccharide, after D_2O exchange, in D_2O and running the spectra at 95°.[35,36] In this way the HOD peak is shifted upfield to τ 5.5 leaving the region of the anomeric protons clear.





K21
[1]





+−OAc group

K24
[2]

Figures 1-2
P.m.r. spectra (100 MHz, τ scale) of Klebsiella capsular polysaccharides in D_2O at 95°.
1. K21, 2. K24

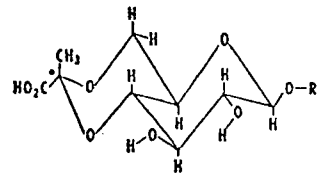
The above figures are p.m.r. spectra of K21 and K24 Klebsiella capsular polysaccharides. In K21, the anomeric signals appear at τ 4.65, (4.9 + 4.95), 5.05 and 5.4 amounting to 1:2:1:1 indicating a pentasaccharide repeating unit. The signal at τ 5.4 shows a distinct doublet with coupling constant of 7 Hz. This is evidence of a β-linkage of the D-galactose moiety, while all the other anomeric signals are at lower field and have small coupling constants (α-linkages). These results are in agreement with those obtained from optical rotation.

In the case of K24, signals at τ 4.6, 4.95 (5.27 + 5.31) in a ratio of 2:1:2 also show a pentasaccharide repeating unit. The signals at τ (5.27 + 5.31) show the presence of a β-linked mannose unit whose coupling constant is very small, together with a β-linked glucose, having a coupling constant of 6 Hz. All other signals are at low field and have small coupling constants indicating they are all α-linkages.

P.m.r. . . . is currently used as a routine screening process from which the polysaccharide samples may be recovered unchanged. Together with the results obtained from spectra of the derived oligosaccharides, the anomeric linkages can be assigned without difficulty.

H. Detection of Other Functional Groups

It is interesting to note that pyruvic acid is present as a 4,6-ketal in more than one third of Klebsiella polysaccharides.



4,6-ketal

4,6-O-(1-carboxyethylidene)-

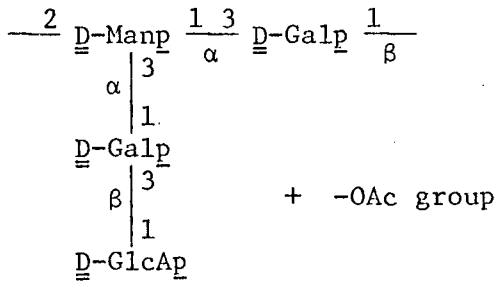
Also present is the less common O-acetyl group, since a few of the sugar hydroxyl groups may be esterified. Pyruvic acid and acetate can be identified by hydrolysing the polysaccharide. Pyruvic acid is then characterized by making the 2,4-dinitrophenylhydrazone[35] derivative while the acetate forms a hydroxamate derivative.[38] The above technique is a destructive method which involves total hydrolysis of the polysaccharide. A non-destructive method was designed in our laboratory again by using the above p.m.r. techniques.[35,36] Pyruvate ketal shows a sharp singlet at τ 8.5 while the O-acetyl is at τ 7.8. Other functional groups such as CH_3 of 6-deoxyhexose can also be ascertained (doublet at τ 8.7). P.m.r. spectroscopy also provides quantitative information on the functional groups present in the polysaccharide.

From the figure of K21, the presence of one pyruvate ketal per five sugar units is indicated. That of K24 shows the presence of an O-acetyl group and the absence of pyruvate. The acetate content corresponds to one O-acetyl group per 7 or 8 sugar units.

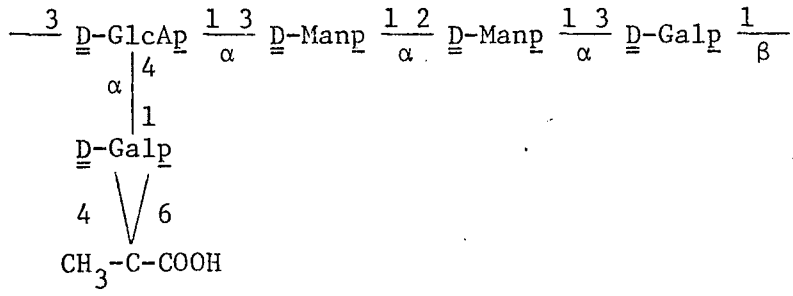
STRUCTURES INVESTIGATED

The object of the present investigation is to isolate and study the structures of several Klebsiella capsular polysaccharides. The capsular polysaccharides studied in this thesis are from Klebsiella K-type 20, K-type 21, K-type 24 and K-type 56 whose structures are given as follows:

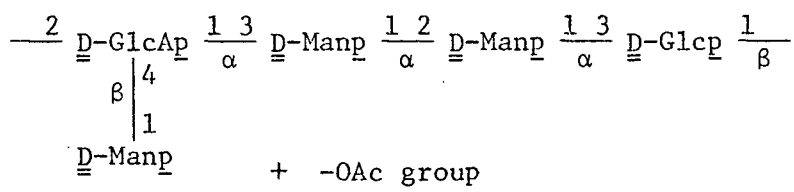
K20



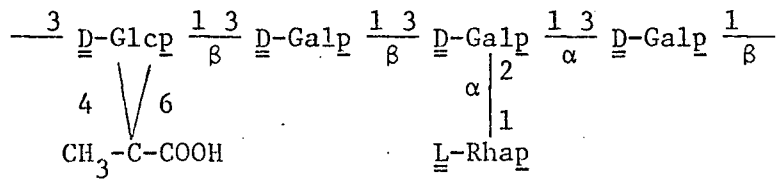
K21



K24



K56



The structures found for K21, K24 and K56 are similar in the sense that they each have a repeating unit of five sugars including a single unit side chain. The structure of K20 is different from the above in that it has a repeat of four sugars including a two unit side chain and is the first investigation of a Klebsiella polysaccharide which contains an aldobiouronic acid side chain. K56 represents the first investigation of these capsular polysaccharides lacking a uronic acid. Structures of the capsular polysaccharides from K-type 20 and 56 are reported in Part I and II of the thesis. The work on the capsular polysaccharides K21 and K24 has already been reported in the Canadian Journal of Chemistry and Xerox copies are included as appendices.

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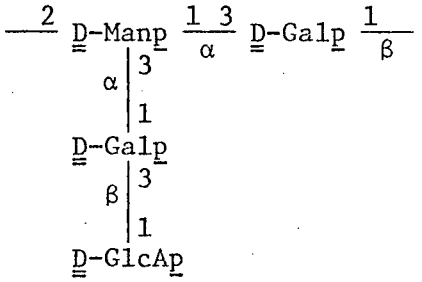
PART I

THE STRUCTURE OF THE CAPSULAR POLYSACCHARIDE FROM *KLEBSIELLA* K-TYPE 20

SUMMARY

Methylation, periodate oxidation and partial hydrolysis studies on the capsular polysaccharide, and on the carboxyl reduced polymer, of Klebsiella K20 show the structure to consist of a repeating unit.



The anomeric linkages were determined by p.m.r. spectroscopy of the carboxyl reduced polysaccharide and periodate degraded polysaccharides. The p.m.r. spectroscopy of the original polysaccharide also showed the presence in the polysaccharide of one O-acetyl group per 8 sugar residues.

DISCUSSION

For all the Klebsiella capsular polysaccharides so far published, the structures are composed of a repeating unit involving a single sugar side chain. The structure of K-type 20 is the first of these capsular polysaccharides to be encountered with an aldobiouronic acid side chain. A preliminary report[1] has appeared which gives other background references.

A culture of Klebsiella K20 (889/50) was obtained from Dr. I. Ørskov, Copenhagen as an agar slant and grown on sucrose yeast-extract agar. Cells were harvested after 3 days, diluted with water containing 1% phenol, and centrifuged at 60,000 x g for 30 minutes. The clear supernatant fluid was poured into ethanol and the product was purified by Cetavlon precipitation.[2] The precipitated acidic polysaccharide was recovered and the small amount of neutral material in the supernatant was discarded.

The polysaccharide purified by Cetavlon, deionization and freeze drying had $[\alpha]_D^{20}$ +94° (c 0.21, water) and a neutralization equivalent (by titration) of 701. One hexuronic acid to three hexose units requires a neutralization equivalent of 661.

The p.m.r. spectrum of a 2% solution of the sodium salt of the polysaccharide in D_2O run at room temperature showed a singlet at τ 7.8 characteristic of O-acetyl and the absence of pyruvate.[3,4] Determination of the acetyl:sugar ratio was hampered by the presence of the large HOD signal due to the high viscosity. However integration of the ring protons and the acetate signal indicated one acetyl group to approximately eight sugar units. The presence of O-acetyl groups was also confirmed by

formation of the hydroxamic ester.[5] The attempt to locate the O-acetyl group using the procedure of de Belder and Norrman[6] was unsuccessful due to the very low yield, probably because of the high molecular weight ($5\text{-}9 \times 10^5$) of the Klebsiella capsular polysaccharide.

Acid hydrolysis of the polysaccharide showed the rapid liberation of an aldobiouronic acid and after 2 hours at 100° with 2 M trifluoroacetic acid (TFA) D-mannose, D-galactose and an aldobiouronic acid were shown by paper chromatography. Separation of the hydrolysate into neutral and acidic fractions, either by ion exchange resin or via the barium salt of the acid, gave an acidic component having $[\alpha]_D^{20}$ +17° (c 0.75, water) and a neutralization equivalent of 349 ($C_{12}H_{20}O_{12}$ requires 356). Analysis of the neutral fraction showed D-mannose and D-galactose in a molar ratio of 1:1 as determined by the gas liquid chromatography (g.l.c.) of the alditol acetates. Collection of the alditol acetates permitted characterization of D-mannitol hexaacetate, m.p. 118-120° and galactitol hexaacetate, m.p. 162-164°. A portion of the aldobiouronic acid was dissolved in 0.3 M acetate buffer (pH 4.8) and β-D-glucuronidase was added, and the solution was incubated at 38° for 6 hours. Paper chromatography showed the formation of D-galactose as the only neutral sugar, together with D-glucuronic acid, and D-glucuronolactone. Similar results were obtained by hydrolysis of the aldobiouronic acid by using 3.5 M hydrochloric acid at 100° for 2 1/2 hours. The configuration of D-galactose was established by D-galactose oxidase, D-glucuronic acid as D-glucose, by D-glucose oxidase and D-mannose by the circular dichroism curve (c.d.) of D-mannitol hexaacetate.[7]

Hydrolysis of the polysaccharide with 0.4 M TFA at 95° for 2 hours

gave the aldobiouronic acid as the only component mobile on paper chromatography, thus suggesting the presence of this unit as a side chain. Methylation of the aldobiouronic acid gave on hydrolysis 2,3,4-tri-O-methyl-D-glucuronic acid and 2,4,6-tri-O-methyl-D-galactose demonstrating a 1→3 linkage.

A portion of the original capsular polysaccharide was partially hydrolyzed at 95° with 0.4 M TFA for 30 hours using the apparatus described by Galanos, Lüderitz and Himmelspach.[8] The dialyzate was evaporated and separated into neutral and acidic fractions. Paper chromatography of the acidic fraction showed the presence of aldobiouronic acid as the major component, with related aldotetrauronic acid together with traces of aldotriouronic acid. The aldobiouronic and aldotetrauronic acid were separated by preparative paper chromatography. Partial hydrolysis of the aldotetrauronic acid after sodium borohydride reduction yielded galactitol, D-mannose and the aldotri- and aldobiouronic acids present in the original hydrolyzate. The analysis of the aldobiouronic acid showed that it had the same characteristics as the aldobiouronic acid isolated previously.

Capsular polysaccharide was methylated by the method of Hakomori[9,10] and hydrolyzed. Analysis of the hydrolysate showed the presence of 2,4,6-tri-O-methyl-D-galactose, 4,6-di-O-methyl-D-mannose, a methylated aldobiouronic acid, and a small quantity of 2,3,4-tri-O-methyl-D-glucuronic acid. The acidic components were isolated using ion exchange resin and hydrolyzed with 3.5 M hydrochloric acid. 2,4,6-Tri-O-methyl-D-galactose was identified as the only neutral sugar, together with 2,3,4-tri-O-methyl-D-glucuronic acid, while the hydrolysis of the

carboxyl reduced acidic component gave 2,3,4-tri-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-galactose.

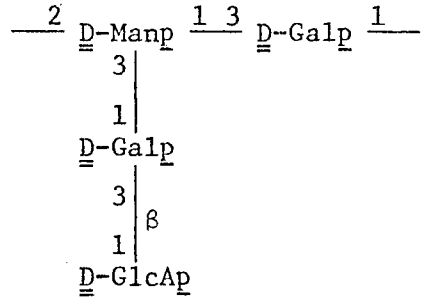
Another sample of the fully methylated polysaccharide was reduced with lithium borohydride and hydrolyzed. Quantitative and qualitative analysis of the derived alditol acetates by gas-liquid chromatography showed the presence of 2,4,6-tri-O-methyl-D-galactose, 2,3,4-tri-O-methyl-D-glucose, and 4,6-di-O-methyl-D-mannose in a molar ratio of 2:1:1. In the above cases the identity of the methylated sugars was confirmed by g.l.c. and mass spectrometry (m.s.)[11,12] of the derived alditol acetates by comparison with standard sugars and demethylation and reacetylation to parent sugar acetates.[13]

These results show that the aldobiouronic acid is 3-O-(β-D-glucopyranosyluronic acid)-D-galactose which is joined to the main chain where the D-galactose units are substituted at position 3, and the D-mannose units at positions 2 and 3.

In order to determine the position of attachment of the aldobiouronic acid side chain to the main chain the degraded polysaccharide obtained from partial hydrolysis as described above was methylated and hydrolyzed. Examination of the hydrolysate showed the presence of 2,3,4,6-tetra-O-methyl-D-mannose, 2,3,4,6-tetra-O-methyl-D-galactose, 3,4,6-tri-O-methyl-D-mannose, 2,4,6-tri-O-methyl-D-galactose and 4,6-di-O-methyl-D-mannose in a ratio of 2:3:9:12:1. The disappearance of 4,6-di-O-methyl-D-mannose and the appearance of 3,4,6-tri-O-methyl-D-mannose indicates that the aldobiouronic acid units are attached to position 3 of D-mannose which in turn must be linked through position 2 in the main chain.

The carboxyl reduced polysaccharide was prepared exactly as described for sapote gum using lithium borohydride in tetrahydrofuran.[14] It was then hydrolyzed and the qualitative and quantitative analysis of the hydrolysate showed the presence of D-galactose, D-glucose and D-mannose in a ratio of 2:1:1. The p.m.r. spectrum run in D_2O at 95° showed signals at τ (4.78 + 4.86), 5.00 and 5.45 in the ratio 2:1:1 (Table 1). The signals at τ 5.45 showed a distinct doublet with a coupling constant of 7 Hz, while that at τ 5.00 gave a broad signal of 6 Hz wide which disappeared when D-glucose was removed by the Smith periodate degradation (discussed later).

The methylation data in conjunction with the results of analysis of the acidic oligosaccharides enable the repeat unit of Klebsiella K20 capsular polysaccharide to be written thus:



Confirmation of the above structure was sought by the Smith periodate degradation procedure.[15] Carboxyl reduced polysaccharide was oxidized with periodate when 0.57 mole per hexose unit was consumed. The derived polyaldehyde after reduction with sodium borohydride was partially hydrolyzed by trifluoroacetic acid at room temperature to give glycerol and a degraded polysaccharide which was recovered by dialysis.

The $[\alpha]_D^{20}$ of the degraded polysaccharide is +129°. P.m.r. of the degraded polysaccharide showed signals at τ 4.60, 4.73 and 5.40 in a ratio of 1:1:1. The signals at τ 5.40 showed a distinct doublet with a coupling constant of 7 Hz, all other anomeric coupling being small. This indicates there are two α-linkages and one β-linkage.

The degraded polysaccharide obtained in the above Smith degradation was then methylated and hydrolyzed. Analysis of the partially methylated sugars showed the presence of 2,3,4,6-tetra-O-methyl-D-galactose, 2,4,6-tri-O-methyl-D-galactose and 4,6-di-O-methyl-D-mannose in a ratio of 1.05:0.9:1. These results show that the degraded polysaccharide consists of a D-galactose unit substituted at position 3 and a D-mannose unit substituted at positions 2 and 3 together with a D-galactose residue present as a side chain.

The degraded polymer was again subjected to a second Smith degradation in which case 0.8 mole of periodate was consumed per hexose unit. After the usual work up as above only glycerol was found together with a new degraded polysaccharide having $[\alpha]_D^{20}$ +57°. P.m.r. showed signals at τ 4.80 and 5.48 in a ratio of 1:1. The signals at τ 5.48 consist of a doublet with coupling constant of 6 Hz while that at τ 4.80 has a very small coupling constant. This shows that the new polysaccharide has one α-linkage and one β-linkage. The new polymer was again methylated and hydrolyzed. The analysis of the partially methylated sugars indicates the presence of 3,4,6-tri-O-methyl-D-mannose and 2,4,6-tri-O-methyl-D-galactose in a ratio of 1:1. This result shows that the new degraded polysaccharide consists of a chain of D-mannose units substituted at position 2 and D-galactose units substituted at position 3.

Together with the results obtained previously, the D-galactose unit which is present as a side chain is joined to position 3 of D-mannose which in turn is linked through position 2 in the main chain.

In order to determine the sequence of the main chain, the new degraded polysaccharide obtained from the above experiment was again subjected to a third Smith degradation. After the usual work up, glycerol and an oligosaccharide were isolated, the oligosaccharide when reduced with sodium borohydride had R_{Gal} 1.05 in solvent B and when hydrolyzed glycerol and D-galactose were obtained in approximately equimolar amount. This finding indicates that the main chain consists of D-galactose units linked (1→3) and these were flanked by (1→2) linked D-mannose units, for example

$$\xrightarrow{2} \text{D-Manp} \xrightarrow[\alpha]{1\ 3} \text{D-Galp} \xrightarrow[\beta]{1}$$

The evidence presented shows clearly that the structure of the capsular polysaccharide of Klebsiella K-type 20 is as given previously. The structure found for K20 is different to those previously reported for K2, K8, K9, K21, K24, and K54 and K56 in that they each have a repeating unit of four or five sugars including a single unit side chain, while that of K20 has a repeat of four sugars including a two unit side chain.

EXPERIMENTAL

General Methods

Paper chromatography was carried out by the descending method using Whatman No. 1 paper and the following solvent systems (v/v): (A) ethyl acetate-acetic acid-formic acid-water (18:3:1:4); (B) ethyl acetate-pyridine-water (4:1:1); (C) 1-butanol-acetic acid-water (2:1:1); (D) butanone-water azeotrope + 1% aqueous ammonia; (E) 1-butanol-ethanol-water (4:1:5). Chromatograms were developed with p-anisidine trichloroacetate spray[16] or with silver nitrate.[17] The clarity of the colors on chromatograms of methylated sugars developed with the former spray is greatly improved by washing the paper under running hot water after heating the chromatogram at 100-110° in the normal manner.

Gas-liquid chromatography was carried out on a F and M model 720 dual column instrument fitted with thermal conductivity detectors. The helium flow was 60-80 ml/min with the following columns: (a) 3% ECNSS-M on Chromosorb W (8 ft x 0.25 in); (b) 5% butanediol succinate on Diatoport S (4 ft x 0.25 in).

Circular dichroism spectra were run on a Jasco J-20 automatic recording spectropolarimeter using a quartz cell with path length 0.1 cm. Optical rotations were measured at 22 $\pm$ 2° on a Perkin Elmer model 141 polarimeter. P.m.r. spectra were run on Varian T60 or XL100 instruments. Samples were prepared by dissolving in D_2O and freeze drying 2 or 3 times before taking spectra in D_2O. Tetramethylsilane was used as an external standard.

Mass spectra, obtained on individual fractions collected[18] from the gas chromatograph, were run on an MS 902 instrument at 70 eV. The

position of methoxyl substitution was determined using the data of Lindberg and coworker,[12] and, where possible, by comparison of mass spectra with those of authentic compounds.

Methylation of polysaccharides and oligosaccharides was carried out by the method of Hakomori.[9,10] Partially methylated alditol acetates were demethylated by reaction with boron trichloride.[19] All solutions were concentrated on a rotary evaporator under reduced pressure at 40°.

D-Glucostat and D-Galactostat reagents were obtained from the Worthington Biochemical Corporation, and β-D-glucuronidase from Sigma Chemical Company.

Isolation and Properties of K20 Capsular Polysaccharide

A culture of Klebsiella K20 was obtained from Dr. Ørskov, Copenhagen and was grown on the following medium for 3 days at 25°: 8 g NaCl, 4 g K_2HPO_4, 1 g $MgSO_4.7H_2O$, 2 g $CaCO_3$, 120 g sucrose and 8 g Bacto Yeast extract in 4 litres of water. The slime was collected after 3 days, diluted with water containing 1% phenol, and centrifuged at 60,000 x g for 30 minutes. The clear supernatant fluid was poured into ethanol, and the recovered polysaccharide was purified by Cetavlon precipitation. The yield of purified polysaccharide was approximately 3 g per 4 l. of medium and two batches were collected and used for the structural studies of the polysaccharide.

The purified polysaccharide was dissolved in distilled water, deionized with Amberlite IR 120 resin, dialyzed and freeze-dried. The product had $[\alpha]_D^{20}$ +94° (c 0.21, H_2O), N 0%. The equivalent weight of the

polysaccharide was found to be 701 as determined by titration with 0.03 M sodium hydroxide.

The above neutralized polysaccharide was lyophilized. The residue was dissolved in D_2O and exchanged twice by lyophilization. The p.m.r. spectrum of a 2% solution in D_2O showed a peak at τ 7.80 indicating the presence of acetyl group.[4] Due to the high viscosity of this solution, the magnitude of the HOD peak was so great that difficulty was encountered in determining a ratio between the anomeric protons and the acetyl group, however, integration of the ring protons and the acetyl group showed there is approximately one acetyl per 8 sugar units.

The presence of acetate was also confirmed by methanolysis of the polysaccharide and identification of the methyl acetate using the hydroxylamine-ferric chloride reagent.[5] Attempted location of the O-acetyl group using the procedure of de Belder and Norrman[6] was unsuccessful due to the extremely low yield involved in the reaction with methyl vinyl ether.

Hydrolysis of the Polysaccharide

Polysaccharide (150 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100° for 2 hours. After evaporation, the hydrolysate was found, by paper chromatography in solvent A, to contain D-mannose, D-galactose and a component with the mobility of an aldobiouronic acid (R_{Gal} 0.26). The hydrolysate was separated into neutral and acidic fractions using ion-exchange resins (Amberlite IR 120 and Duolite A4). The neutral fraction was analyzed and found to contain D-mannose and D-galactose in a 1:1 ratio as determined by the g.l.c. of the alditol acetates (column a). G.l.c. of the acetates allowed recovery of the individual compounds

and identification of D-mannitol hexaacetate, m.p. 118-120°, and galactitol hexaacetate, m.p. 162-164°. A portion of the neutral fraction of the hydrolyzate was tested with D-Galactostat reagent and a positive response confirmed the D-configuration of galactose. The D-configuration of mannose was found by dissolving part of the mannitol hexaacetate collected by g.l.c. in acetonitrile. A positive circular dichroism curve, identical to that given by a standard sample, confirmed the D-configuration of mannose.

The acidic component (60 mg) was found to have $[\alpha]_D^{20}$ +17° (c 0.75, H_2O) and an equivalent weight of 349 when titrated with 0.03 M sodium hydroxide ($C_{12}H_{20}O_{12}$ requires 356). The acidic fraction had R_f 0.16 in solvent C. A portion (20 mg) of the aldobiouronic acid was dissolved in 3 ml of 0.3 M acetate buffer (pH 4.8), 3 mg of β-D-glucuronidase was added, and the solution was incubated at 38° for 6 hours. D-Galactose was found, by paper chromatography in solvent B, to be the only neutral sugar present, whereas the presence of D-galactose, D-glucuronic acid, and D-glucuronolactone was demonstrated in solvent A. The same results were obtained by hydrolysis of the aldobiouronic acid (10 mg) by using 3.5 M hydrochloric acid at 100° for 2.5 hours. The aldobiouronic acid (20 mg) was methylated and hydrolyzed with 3 M hydrochloric acid at 95° for 3.5 hours. Paper chromatography in solvent E showed the presence of 2,4,6-tri-O-methyl-D-galactose (R_f 0.66) and 2,3,4-tri-O-methyl-D-glucuronic acid (R_f 0.22). The identity of 2,4,6-tri-O-methyl-D-galactose was confirmed by g.l.c.-m.s. of the alditol acetate.[12] Another portion of the polysaccharide (20 mg) was hydrolyzed with 0.4M TFA at 95° for 2 hours. The aldobiouronic acid was found to be the only mobile compound on paper chromatography in solvent A.

Partial Hydrolysis of Polysaccharide

K20 polysaccharide (1 g) was partially hydrolyzed with 0.4 M TFA for 30 hours at 95° using the apparatus described by Galanos, Lüderitz and Himmelspach.[8] The degraded polysaccharide in the dialysis sac was kept for the methylation study discussed later. The dialysate was evaporated and separated into neutral and acidic fractions by ion exchange resin. On paper chromatography (solvent C) the acidic fraction was shown to contain an aldobiouronic acid (R_{Gal} 0.45) as the major component together with aldotetrauronic acid (R_{Gal} 0.08) and trace amount of aldotriouronic acid (R_{Gal} 0.30). The acids were separated by paper chromatography (solvent C) and analyzed as follows:-

The aldobiouronic acid (80 mg) was found to have identical characters as the aldobiouronic acid isolated previously. P.m.r. spectrum showed signals at τ 4.75 and τ 5.40 in a ratio of 0.5:1.4 (table 1). The signal at τ 5.40 showed a distinct doublet with a coupling constant of 7 Hz.

The aldotetrauronic acid (50 mg) had an equivalent weight of 650. The acid (10 mg) was hydrolyzed with 1 M TFA at 95° for 1 hour. Paper chromatography (solvent A) showed the presence of D-galactose as the principle monosaccharide with traces of D-mannose and a series of aldobiouronic, aldotriouronic and aldotetrauronic acids which had the same mobilities as the components of the original mixtures. The aldotetrauronic acid (10 mg) was reduced with sodium borohydride and, after removal of the borate, the product was hydrolyzed with 2 M TFA for 1/2 hour. On paper chromatography (solvents A and B), the neutral compound found was galactitol which is confirmed by g.l.c of the alditol acetate,

m.p. 162-164°. Also produced were the same aldobio-, aldotrio- and aldotetrauronic acids (solvent C) described above.

Methylation Analysis of the Polysaccharide

Capsular polysaccharide (90 mg) was methylated by the method of Hakomori[9,10] The product was dialyzed against running water overnight and then extracted with chloroform. Concentration of the chloroform solution yielded a product which showed no absorption at 3600 cm^{-1}. The methylated polysaccharide (20 mg) was hydrolyzed with 2 M TFA at 100° for 2 hours. Analysis of the hydrolysate by paper chromatography (solvent E) showed the presence of 2,4,6-tri-O-methyl-D-galactose (R_f 0.66), 4,6-di-O-methyl-D-mannose (R_f 0.59), a methylated aldobiouronic acid (R_f 0.35), and a small quantity of 2,3,4-tri-O-methyl-D-glucuronic acid (R_f 0.22). The hydrolysate was separated into neutral and acidic fractions using ion exchange resin (Duolite A4). On paper chromatography, the neutral fraction was shown to contain 2,4,6-tri-O-methyl-D-galactose (R_f 0.4, solvent D) and 4,6-di-O-methyl-D-mannose (R_f 0.29, solvent D). It was then reduced with sodium borohydride and after removal of the borate, the product was acetylated in a sealed tube by using pyridine and acetic anhydride (1:1, 1 ml, 95°, 15 min). After removal of the solvents, the alditol acetates were dissolved in a small volume of ethyl acetate and analyzed by g.l.c. on column b at 200° (flow rate 100 ml/min); two peaks were observed.

The component eluted first had a retention time (6.9 min) and mass spectrum identical to authentic 2,4,6-tri-O-methyl-D-galactitol triacetate. The second component had identical characters in retention

time (9.4 min) and mass spectrum as authentic 4,6-di-O-methyl-D-mannitol tetraacetate.* Demethylation and reacetylation of the partially methylated alditol acetates.[13] gave the corresponding galactitol hexaacetate, m.p. 166-169° and D-mannitol hexaacetate, 119-122°.

A portion (4 mg) of the acidic fraction was hydrolyzed with 3.5 M HCl for 2 hours at 100°; after evaporation and neutralization with silver carbonate, the hydrolysate was found to contain 2,4,6-tri-O-methyl-D-galactose (analyzed by g.l.c.-m.s. of the alditol acetate) as the only neutral sugar, and 2,3,4-tri-O-methyl-D-glucuronic acid (R_f 0.22 solvent E). Another portion (5 mg) was methanolyzed with 2% methanolic/HCl and reduced with lithium borohydride. After removal of the borate by IR 120 and evaporations with methanol, the product was hydrolyzed (0.5 M H_2SO_4 for 4 hours). Paper chromatography showed the presence of 2,3,4-tri-O-methyl-D-glucose (R_f 0.62, solvent D) and 2,4,6-tri-O-methyl-D-galactose (R_f 0.40, solvent D). G.l.c. of the alditol acetates showed two peaks at 9.5 min and 8.7 min (column b, 200° flow rate 60 ml/min) which were identical in retention time and mass spectrum to the corresponding authentic alditol acetates.

Another sample of methylated polysaccharide (20 mg) was reduced with lithium borohyride and hydrolyzed. Quantitative analysis of the derived alditol acetates by gas-liquid chromatography (3% ECNSS-M, 180° to 217° at 2°/min) showed the presence of 2,4,6-tri-O-methyl-D-galactose (24.5 min), 2,3,4-tri-O-methyl-D-glucose (26 min), and 4,6-di-O-methyl-D-mannose (29.8 min) in a molar ratio of 2:1:1. The identity of the methylated sugars was again confirmed by mass spectrometry of the derived alditol acetates.

* correct nomenclature: 1,3-di-O-methyl-D-mannitol tetraacetate

Methylation of Degraded Polysaccharide

A portion (120 mg) of the degraded polysaccharide recovered from the partial hydrolysis was methylated and the product was hydrolyzed with 2 M TFA at 100° for 4 hours. After evaporation, the hydrolysate was shown by paper chromatography to contain 3,4,6-tri-O-methyl-D-mannose (R_f 0.58, solvent D) and 2,4,6-tri-O-methyl-D-galactose (R_f 0.38, solvent D) as the major components, together with minor compounds of 2,3,4,6-tetra-O-methyl-D-galactose and D-mannose and faint traces of methylated aldobiouronic acid (solvent E). The hydrolysate was reduced with sodium borohydride, acetylated and injected into column b at 190°. 2,3,4,6-Tetra-O-methyl-D-mannose (retention time 6.5 min), 2,3,4,6-tetra-O-methyl-D-galactose (8.0 min), 3,4,6-tri-O-methyl-D-mannose (12.0 min), 2,4,6-tri-O-methyl-D-galactose (16.0 min) and 4,6-di-O-methyl-D-mannose (18.0 min) was found in a ratio of 2:3:9:12:1. The mass spectrum of 3,4,6-tri-O-methyl-D-mannitol acetate was again identical with an authentic sample.

Reduction of the Capsular Polysaccharide

Capsular polysaccharide (0.5 g) was converted into the methyl ester propionate and reduced with lithium borohyride[14] to give the reduced polysaccharide (0.4 g), $[\alpha]_D^{24}$ +95.5° (c 2, water). The reduced polysaccharide (20 mg) was hydrolyzed with 2 M TFA at 100° for 4 hours. On analysis of the hydrolysate by paper chromatography and g.l.c., D-galactose, D-glucose and D-mannose were shown to be present in a ratio of 2:1:1 as determined by g.l.c. of their alditol acetates.

Galactitol hexaacetate had m.p. 166-169°, D-glucitol hexaacetate had m.p. 96-98° and D-mannitol hexaacetate had m.p. 119-122°. The hydrolysate gave a positive test with D-Glucostat reagent, thus confirming the D-configuration of D-glucuronic acid. The p.m.r. spectrum of the reduced K20 polysaccharide was run in D_2O at 95° and was shown to give anomeric signals at τ (4.78 + 4.86), 5.00 and 5.45 in the ratio of 2:1:1 (Table 1). The signals at τ 5.45 showed a distinct doublet with a coupling constant of 7 Hz, while that at τ 5.00 was 5-6 Hz wide and showed no distinct splitting.

Smith Periodate Degradations and Methylation Studies of the Degraded Polysaccharides

The carboxyl reduced polysaccharide (66 mg) was dissolved in 10 ml water then 20 ml 0.05 M sodium metaperiodate were added. After 66 hours, 0.57 mole of periodate per hexose unit was consumed. Ethylene glycol (1.5 ml) was added and the solution was left at room temperature for 1 hour to destroy excess periodate. The product was then dialyzed overnight against running water. Sodium borohydride (0.5 g) was added and the solution was left overnight. The solution was deionized with Amberlite IR 120, freeze-dried, and the product was distilled with several portions of methanol. The derived polyalcohol was dissolved in 5 ml 0.5 M TFA and left at room temperature overnight. On paper chromatography (solvent A) only glycerol was identified (R_f 0.45). The product was dialyzed against 2 l. of distilled water. The dialysate when evaporated was found to contain glycerol by paper chromatography and g.l.c. (retention time of glycerol triacetate was 6.4 min, 150°, column b).

The degraded polysaccharide remaining in the dialysing sac was freeze-dried. It had $[\alpha]_D^{20}$ +129°. The p.m.r. spectrum of the degraded polysaccharide in D_2O showed signals at τ 4.60, 4.73 and 5.40 in a ratio of 1:1:1. The signals at τ 5.40 showed a doublet with coupling constant of 7 Hz, all other coupling constants being small. The degraded polysaccharide was then methylated by the Hakomori method and hydrolyzed. Paper chromatography showed the presence of 2,3,4,6-tetra-O-methyl-D-galactose (R_f 0.70 solvent D), 2,4,6-tri-O-methyl-D-galactose (R_f 0.40), and 4,6-di-O-methyl-D-mannose (R_f 0.29). The ratio was found to be 1.05:0.9:1 by g.l.c. of the alditol acetates. 2,3,4,6-Tetra-O-methyl-D-galactitol acetate had a retention time of 13 min (column b, 160-200° at 2°/min) and had a mass spectrum identical to an authenic sample.

Another portion of the carboxyl reduced polysaccharide (0.3 g) was oxidized by periodate as described previously to yield 0.2 g of the degraded polysaccharide (S_1). To this degraded polysaccharide S_1, 50 ml of 0.04 M sodium periodate was added. Periodate consumption was found to be 0.8 mole per hexose unit after 2 days. The derived polyaldehyde after reduction with sodium borohydride was partially hydrolyzed with 0.4 M TFA at room temperature overnight. Glycerol was found to be the only component mobile on paper chromatography in solvent A (R_f 0.44). The product was dissolved in 5 ml of water and dialyzed against 1 l. of distilled water. The substance remaining in the dialysis sac was freeze-dried to yield 0.1 g of a new degraded polysaccharide (S_2). The degraded polysaccharide S_2 had $[\alpha]_D^{20}$ +57° (c 0.35, water). P.m.r. of this polysaccharide was run in D_2O at 95° and showed signals at τ 4.80 and 5.48 in a ratio of 1:1. The signals at τ 5.48 consisted of a doublet with a

coupling constant of 6 Hz while that at τ 4.80 had a very small coupling constant. A portion (27 mg) of the degraded polysaccharide S_2 was methylated by the Hakomori method and hydrolyzed (2 M TFA, 100°, 4 hours). Paper chromatography (solvent D) showed the presence of 3,4,6-tri-O-methyl-D-mannose (R_f 0.57) and 2,4,6-tri-O-methyl-D-galactose (R_f 0.38). The ratio was found to be 1:1 by g.l.c. of the alditol acetates. 3,4,6-Tri-O-methyl-D-mannose had an identical retention time (22 min, column b, 180-200° at 2°/min) and mass spectrum to an authentic sample.

The degraded polysaccharide S_2 (43 mg) was further degraded by periodate. After the usual work up, the derived polyalcohol was hydrolyzed with 0.5 M TFA at room temperature for 6 hours. The product was then reduced with sodium borohydride. After removal of the borate, paper chromatography in solvents B and C showed the presence of glycerol (R_{Gal} 4.3, solvent B, R_{Gal} 2.1, solvent C) and an unknown component (R_{Gal} 1.05, solvent B, R_{Gal} 1.2, solvent C). The unknown component was separated by paper chromatography in solvent B, and then hydrolyzed with 2 M TFA for 2 hours. On paper chromatography in solvents A and B, D-galactose and glycerol (R_{Gal} 4.6, solvent A; R_{Gal} 2.4, solvent B) were identified. The product was reduced with sodium borohydride and acetylated. G.l.c. of glycerol triacetate (retention time 8 min, column a, 140-200°) and galactitol hexaacetate (retention time 58 min) were found in approximate equimolar amount.

Table 1. P.m.r. data on Klebsiella K20 capsular polysaccharide and derived polysaccharides and oligosaccharide

Repeating unit of compound	τ value* (coupling constant, Hz)	Ratio of integral	Proton** assignment
$\underline{\quad}^{2}$ Man $\overset{1\ 3}{\underset{\alpha}{\quad}}$ Gal $\overset{1}{\underset{\beta}{\quad}}$ $\alpha\vert^{3}_{1}$ Gal $\beta\vert^{3}_{1}$ Glc	4.78 + 4.86	2	α-Gal $\overset{1\ 3}{\underline{\quad}}$ Man α-Man $\overset{1\ 3}{\underline{\quad}}$ Gal
	5.00 (§)	1	β-Glc $\overset{1\ 3}{\underline{\quad}}$ Gal
	5.45 (7)	1	β-Gal $\overset{1\ 2}{\underline{\quad}}$ Man
$\underline{\quad}^{2}$ Man $\overset{1\ 3}{\underset{\alpha}{\quad}}$ Gal $\overset{1}{\underset{\beta}{\quad}}$ $\alpha\vert^{3}_{1}$ Gal	4.63	1	α-Gal $\overset{1\ 3}{\underline{\quad}}$ Man
	4.73	1	α-Man $\overset{1\ 3}{\underline{\quad}}$ Gal
	5.40 (7)	1	β-Gal $\overset{1\ 2}{\underline{\quad}}$ Man
$\underline{\quad}^{2}$ Man $\overset{1\ 3}{\underset{\alpha}{\quad}}$ Gal $\overset{1}{\underset{\beta}{\quad}}$	4.80	1	α-Man $\overset{1\ 3}{\underline{\quad}}$ Gal
	5.48 (6)	1	β-Gal $\overset{1\ 2}{\underline{\quad}}$ Man
GlcA $\overset{1\ 3}{\underset{\beta}{\quad}}$ Gal-OH	4.75	0.5	α-Gal-OH
	5.40 (7)	1.4	β-Gal-OH
			β-GlcA $\overset{1\ 3}{\underline{\quad}}$ Gal

* Spectra run in D_2O with external tetramethylsilane (τ = 10) at 100 MHz.

** All sugars have D-configuration and are pyranose.

(§) signal is 5-6 Hz wide; shows no distinct splitting.

Table 2. Methyl ethers from the hydrolysis of methylated Klebsiella K20 polysaccharides.

Sugars	Samples*							
	A	B	C	D	E†	F†	G†	H†
2,3,4,6-Tetra-O-methyl-D-mannose					2			
2,3,4,6-Tetra-O-methyl-D-galactose					3		1	
2,4,6-Tri-O-methyl-D-galactose	+	+	+	+	12	2	1	1
3,4,6-Tri-O-methyl-D-mannose					9			1
2,3,4-Tri-O-methyl-D-glucuronic acid		+		+				
2,3,4-Tri-O-methyl-D-glucose			+			1		
4,6-Di-O-methyl-D-mannose	+				1	1	1	

* A, neutral sugars from methylated original K20 polysaccharide;

B, aldobiouronic acid fraction from methylated original K20 polysaccharide;

C, reduced aldobiouronic acid fraction from methylated original K20 polysaccharide;

D, methylated aldobiouronic acid;

E, neutral sugars from methylated residual polysaccharide after partial hydrolysis;

F, sugars from methylated reduced K20 polysaccharide;

G, sugars from 1st Smith degraded polysaccharide;

H, sugars from 2nd Smith degraded polysaccharide.

† Approximate molar ratios.

(+) Signifies present and has no quantitative significance.

Table 3. Diagnostic prominent peaks (m/e) in the mass spectra of acetates of methylated alditols.

Parent sugar	m/e									
	43	45	117	129	145	161	189	205	233	261
2,3,4,6-Me_4Gal	+	+	+	+	+	+		+		
2,4,6-Me_3Gal	+	+	+	+		+			+	
3,4,6-Me_3Man	+	+		+		+	+			
2,3,4-Me_3Glu	+		+	+		+	+		+	
4,6-Me_2Man	+	+	+	+		+				+

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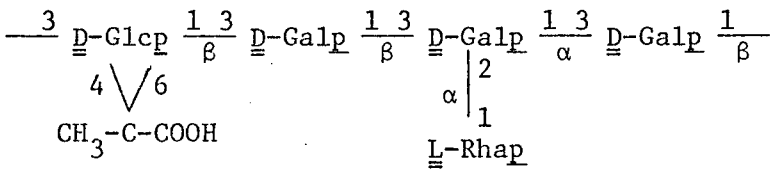
PART II

THE STRUCTURE OF THE CAPSULAR POLYSACCHARIDE FROM *KLEBSIELLA* K-TYPE 56

SUMMARY

Methylation, periodate oxidation and partial hydrolysis studies of the capsular polysaccharide of K56 show the structure to consist of a repeating unit



with a L-rhamnose side chain which joins α(1→2) to the second D-galactose of the main chain. Difficulties have been encountered in assigning the L-rhamnose side chain to one of the three D-galactoses, however, with the isolation of the partially methylated oligosaccharides, this problem has been solved. The anomeric linkages were determined by p.m.r. spectroscopy of isolated oligosaccharides, and in part, by specific enzymes. P.m.r. spectroscopy of the original polysaccharide using the sodium salt in D_2O showed clearly a ratio of one pyruvic acid ketal (CH_3, τ 8.57) to five anomeric protons (τ 4.6 to 5.3), while the ratio of pyruvic acid ketal to L-rhamnose $-CH_3$ is 1:1. Of all the *Klebsiella* capsular polysaccharides so far studied , this is the first investigation of the detailed structure of a *Klebsiella* capsular polysaccharide which does not contain uronic acid.

DISCUSSION

L-Rhamnose has been found present as a constituent in about one-third of the 80 *Klebsiella*[1,2] capsular polysaccharides. Capsular polysaccharide from K-type 56 consists of one L-rhamnose unit per five sugar units and constitutes the first report of these *Klebsiella* polysaccharides lacking uronic acids. The acidity of the polysaccharide is due only to the pyruvic acid present.

The polysaccharide after purification by precipitation with Cetavlon, had $[\alpha]_D^{24}$ +79° (c 0.43, water) and an equivalent weight (by titration) of 908. The p.m.r. spectrum of a 2% solution of the sodium salt of the polysaccharide in D_2O run at 95° showed a sharp singlet at τ 8.57 characteristic of the CH_3 of a pyruvate ketal[3,4] and a doublet at τ 8.73 characteristic of the CH_3 of a 6-deoxyhexose.[4] The ratio of these two signals is 1:1. The anomeric region of the spectrum suggested that the repeating unit consists of five sugar units of which three are linked by β- and two by α-glycosidic bonds.[3,4] Integration of the anomeric and pyruvate signals indicated one pyruvate ketal group to five sugar units.

Acid hydrolysis of the polysaccharide showed the rapid liberation of L-rhamnose, the amount of which reached a maximum after 1/2 hour with only traces of D-galactose and D-glucose. After 4 hours L-rhamnose, D-galactose and D-glucose were proved to be in the ratio of 1.1:3:1. This analysis was carried out on the derived alditol acetates. Samples of these were collected by g.l.c. and measurement of their c.d. spectra confirmed the assignment of the L-configuration of rhamnose, and D-configuration of glucose while the D-configuration of

galactose was assigned by using the alditol acetate of the partially methylated sugar (see later).[5] The presence of L-rhamnose was confirmed by methanolysis of the polysaccharide to give the methyl α-L-rhamnopyranoside which crystallized easily on seeding.[6]

Partial hydrolysis of the polysaccharide gave a series of neutral oligosaccharides which were separated from the monosaccharides by a charcoal column[7,8] and individual components were obtained by Sephadex gel chromatography[9] (G-15). These were shown to be a glucosyl galactose disaccharide, together with related tri- and tetrasaccharides. The structures of these oligosaccharides are given in Table 1 together with the chemical shifts of the anomeric protons which enable the nature of the glycosidic linkages to be determined. The structures given were determined by (a) hydrolysis with acid and enzyme, (b) partial hydrolysis and (c) methylation, as described in the experimental section.

A sample of pure capsular polysaccharide was methylated,[10,11] hydrolyzed and the partially methylated sugars were examined. 2,3,4-Tri-O-methyl-L-rhamnose, 2,4,6-tri-O-methyl-D-galactose, 4,6-di-O-methyl-D-galactose and 2-O-methyl-D-glucose were found in a molar ratio of 0.85:2.2:1:1 which was determined by g.l.c. of the alditol acetates.[12] The identities of the individual partially methylated sugars were determined by comparing with standards using (a) paper chromatography, (b) g.l.c.-m.s. of the alditol acetates[13] and (c) demethylation to the parent sugar alditol acetates.[14]

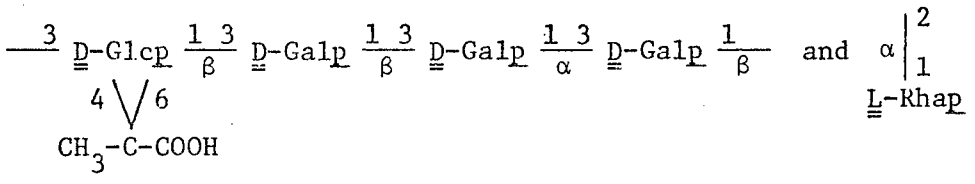
These results show the presence of two D-galactose units substituted at position 3. The isolation of 4,6-di-O-methyl-D-galactose indicates that one of the three galactoses is a branch point. It is substituted at both the

2 and 3 positions, with the L-rhamnose residue present as a side chain. The 2-O-methyl-D-glucose found agrees with the fact that D-glucose is in the main chain substituted at position 3, with pyruvic acid linked to the 4,6-positions as a ketal.

In order to determine the position of attachment of the L-rhamnose units to the main chain a sample of the original polysaccharide was subjected to Smith periodate degradation.[15] The polysaccharide consumed 0.45 mole of periodate per hexose unit after 64 hours at 4°. The derived polyalcohol was partially hydrolyzed with trifluoroacetic acid (TFA) at room temperature to give 1-deoxy glycerol (or 1,2-propanediol) and a residual polysaccharide. The latter was methylated and hydrolyzed to give 2,4,6-tri-O-methyl-D-galactose and 2-O-methyl-D-glucose in a ratio of 3:1 indicating that the L-rhamnose side chains are joined to position 2 of D-galactose, which in turn must be linked through position 3 in the main chain. The p.m.r. spectrum at 95° of a 2% solution of the sodium salt of the above residual polysaccharide also showed a sharp singlet at τ 8.57 indicating that the pyruvic acid ketal[3,4] was resistant to the mild conditions of the Smith periodate degradation. Signals from the anomeric protons appeared at τ 4.75, 5.09 to 5.21 which integrated for 9 +(10 + 20)in comparison to the pyruvic acid methyl signal of 30 (arbitrary units). This established a ratio of one pyruvic acid to four sugar residues. The signal at τ 5.09 was shifted to higher field to τ 5.24 when the pyruvic acid ketal was removed by 0.1 M TFA. The disappearance of one α-anomeric signal indicates that the L-rhamnose is α-linked to the main chain which is consistent with the result obtained from the change in optical rotation

of the polysaccharide [$[\alpha]_D^{20}$ +79° to +97° (c 1.0, water)] as the L-rhamnose side chain is removed.

The methylation data in conjunction with the results of analysis of the oligosaccharides and Smith periodate degradation enable the repeat unit of Klebsiella K56 capsular polysaccharide to be written thus:



The L-rhamnose could be linked (1→2) to either one of the three D-galactose units. It is known that rhamnose linkages[16] and galactose linked (1→3)[17] are very labile to acid hydrolysis. The assignment of the position of the side chain from partial hydrolysis of the original polysaccharide is difficult in the present instance since it is hard to isolate an oligosaccharide containing L-rhamnose, and also it is hard to distinguish between the three D-galactoses as they all have (1→3) linkages. However, by partial hydrolysis of the methylated capsular polysaccharide, this problem has been solved. The fully methylated capsular polysaccharide was hydrolyzed with 90% formic acid for 45 minutes at 70°.[18] On TLC (solvent D) three spots were obtained (R_f 0.78, 0.72 and 0.66) together with 2 faint spots with R_f 0.47 and 0.25-0.30. The component with R_f 0.72 had a mobility identical to 2,3,4-tri-O-methyl-L-rhamnose. A mixture of 2,3,4-tri-O-methyl-L-rhamnose, 2,4,6-tri-O-methyl-D-galactose, 4,6-di-O-methyl-D-galactose and 2-O-methyl-D-glucose was shown by paper chromatography when the minor component with R_f 0.78 was reduced and hydrolyzed. This is probably a

repeating unit of the polysaccharide with 2,4,6-tri-O-methyl-D-galactose at the reducing end which when reduced is not detectable on paper chromatography.

The major component (R_f 0.66) was separated by TLC in solvent D. P.m.r. of this fraction showed a strong signal at τ 8.45 indicating a pyruvate ketal.[3,4] On hydrolysis, 2,4,6-tri-O-methyl-D-galactose, 4,6-di-O-methyl-D-galactose and 2-O-methyl-D-glucose were obtained in a ratio of 1.5:0.8:1 indicating a mixture of oligosaccharides. On reduction with sodium borohydride, this fraction gave two components on TLC with R_f 0.14 and 0.07. The faster component gave on hydrolysis 2,4,6-tri-O-methyl-D-galactitol and 2-O-methyl-D-glucose in a ratio of 1.25:1. On hydrolysis of the slower component, 2,4,6-tri-O-methyl-D-galactose, 4,6-di-O-methyl-D-galactose, 2-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-galactitol were found in a ratio of 1.1:1:1:1.3. When this slow component was methylated and hydrolyzed only 2,3-di-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-galactose were shown by paper chromatography there was no sign of tetramethyl glucose or galactose. These results indicate that the slow component consists of a partially methylated tetrasaccharide with 2,4,6-tri-O-methyl-D-galactose at the reducing end, with 4,6-O-(1-carboxylethylidene)-2-O-methyl-D-glucose at the non-reducing end.

In order to obtain a partially methylated disaccharide, a portion of the above partially hydrolyzed methylated polysaccharide was hydrolyzed for a further period of 4 hours with 0.5 M TFA at 95°. Paper chromatography (solvent E) showed the presence of 2,4,6-tri-O-methyl-D-galactose, 4,6-di-O-methyl-D-galactose, 2-O-methyl-D-glucose and a

new component with R_f 0.79. The new component was separated by TLC (R_f 0.6 solvent D). Hydrolysis and analysis of the partially methylated sugars showed the presence of 2,4,6-tri-O-methyl-D-galactose and 2-O-methyl-D-glucose in a ratio of 1:1. This is good evidence of a partially methylated disaccharide. When the disaccharide was reduced with sodium borohydride and hydrolyzed, 2-O-methyl-D-glucose was shown by paper chromatography, while the g.l.c. of the alditol and aldose acetates showed the presence of 2,4,6-tri-O-methyl-D-galactitol and 2-O-methyl-D-glucose in a ratio of 1.2:1.

Together with the results obtained from the disaccharide, the L-rhamnose side chain could be assigned to the second D-galactose. It was not possible to isolate a partially methylated trisaccharide having 4,6-di-O-methyl-D-galactose as the reducing end probably because this galactose is linked $\alpha(1\rightarrow3)$ which is more stable than the $\beta(1\rightarrow3)$[16] linkage under the above hydrolysis conditions.

The sum of these results firmly establishes the structure written above as representing the repeating unit of the capsular polysaccharide of Klebsiella K56 and this is the first report of the detailed structure of Klebsiella capsular polysaccharide which does not contain uronic acid.

EXPERIMENTAL

General methods are as previously described in Part I.

Isolation and Properties of K56 Capsular Polysaccharide

Klebsiella K56 (3534/51) was grown in the medium as for K20 and the harvested capsular polysaccharide and cells (∿2 l.) were diluted 2-fold and centrifuged at 27,000 r.p.m. at 4° for 30 minutes. The supernatant was collected and poured with stirring into ethanol (5 volumes). The polysaccharide was collected, dissolved in distilled water and purified through precipitation with Cetavlon[19] giving 8 g of pure acidic polysaccharide having $[\alpha]_D^{24}$ +79° (c 0.43, water) as the Na^+ salt. After deionization with Amberlite IR 120 and dialysis, the equivalent weight was found by titration with 0.01 M sodium hydroxide (phenolphthalein) to be 908.

A sample of the Na^+ salt of the polysaccharide was dissolved in D_2O and exchanged twice by lyophilization. The p.m.r. spectrum of the solution (2% in D_2O) was run at 95° and showed a sharp singlet at τ 8.57, a doublet at τ 8.73 and five protons in the range τ 4.6-5.3 (see Table 1). The ratio between the singlet at τ 8.57 and the doublet at τ 8.73 was shown by integration to be 1:1 which in turn was shown to be in the ratio of 1:5 with the anomeric protons.

The presence of pyruvic acid was also confirmed by hydrolysis of the polysaccharide and identification of the pyruvic acid by paper chromatography (solvent A) using o-phenylenediamine spray which gave a characteristic fluorescence when examined by u.v.[20]

Hydrolysis of the Polysaccharide

Polysaccharide (50 mg) was hydrolyzed with 2 M TFA at 95° for periods of 1/2 hour, 1 hour, 2 hours, and 4 hours. The liberation of L-rhamnose was found (paper chromatography) to be a maximum at 1/2 hour with no increase on further hydrolysis. After 4 hours, the hydrolysate was evaporated and the ratio of L-rhamnose, D-galactose and D-glucose was found to be 1.1:3:1 by g.l.c. of the alditol acetates (column a); Galactitol hexaacetate had m.p. and mixed m.p. 162-164° and D-glucitol hexaacetate had m.p. and mixed m.p. 98-99°. The configuration of L-rhamnose and D-glucose was determined by measuring the c.d. curves of the alditol acetates.[5] The presence of L-rhamnose was confirmed by methanolysis (3% MeOH/HCl, 2 hours) of the polysaccharide (200 mg) and separation by TLC[6] (R_f 0.4, solvent D) of methyl α-L-rhamnoside which on recrystallization from ethyl acetate had m.p. and mixed m.p. 108-109°.

Methylation

Capsular polysaccharide (1 g) was methylated by the Hakomori procedure.[10,11] The resulting solution was dialyzed against running tap water and extracted 3 times with chloroform. The dried residue was then fractionated by using petroleum ether-chloroform mixture. The fraction soluble in 70:30 petroleum ether-chloroform showed no hydroxyl absorption in the i.r. and was used for analysis.

The fully methylated polysaccharide (85 mg) was hydrolyzed with 2 M TFA at 100° for 2 hours then at 95° for 16 hours. Trifluoroacetic acid was evaporated and the syrup was shown by paper chromatography (solvent D)

to contain four components corresponding to 2,3,4-tri-O-methyl-L-rhamnose, 2,4,6-tri-O-methyl-D-galactose, 4,6-di-O-methyl-D-galactose and 2-O-methyl-D-glucose (R_f values 0.88, 0.42, 0.16, 0.07). The ratio was found to be 0.85:2.2:1:1 as determined by the g.l.c. of the alditol acetates which permitted isolation of 2-O-methyl-D-glucitol pentaacetate as a crystalline derivative having m.p. and mixed m.p. 54-56°.[21] The other three components were confirmed by m.s.[13] of the individual alditol acetates and demethylation (BCl_3) and reacetylation to the parent sugar alditol acetates;[14] m.p. of galactitol hexaacetate 164-167°, m.p. of D-glucitol hexaacetate 99°. The c.d. spectrum of 2,4,6-tri-O-methyl-D-galactitol acetate is identical to a standard sample thus comfirming the D-configuration of D-galactose.

Smith Degradation

Capsular polysaccharide (220 mg) was dissolved in 100 ml 0.025 M sodium metaperiodate. After 64 hours in the dark at 4° 0.45 mole of periodate had been consumed per sugar unit. Following the addition of ethylene glycol, dialysis, reduction with sodium borohydride, dialysis, deionization, lyophilization and removal of borate the product was hydrolyzed (TFA 0.5 M) at room temperature for 8 hours. Paper chromatography in solvent A showed the presence of 1-deoxyglycerol (1,2-propanediol) having identical R_f value (0.80) and retention time (4.2 min on column a at 90°) with authentic standard. The product in 5 ml was dialyzed against 2 l. of distilled water. The residue was freeze dried to yield a degraded polymer (150 mg). A part (70 mg) of

the degraded polysaccharide was methylated (Hakomori procedure) and then hydrolyzed. Paper chromatography (solvents D and E), 2,4,6-tri-O-methyl-D-galactose and 2-O-methyl-D-glucose were shown to be the only two sugars present and had a ratio of 3:1 when determined by g.l.c. of the alditol acetates. The two components were again analyzed by the m.s. of the alditol acetates and m.p. (2-O-methyl-D-glucitol pentaacetate m.p. 54-56°).

The degraded polysaccharide had $[\alpha]_D^{20}$ +97° (c 1.0, water) and 40 mg of the sodium salt of this polysaccharide was exchanged twice with D_2O by lyophilization. The p.m.r. spectrum was run at 95° in D_2O (100%) and showed a singlet at τ 8.57 while the doublet at τ 8.73 was completely absent. The anomeric signals (Table 1) at τ 4.75, 5.09-5.21 were found to be in a ratio of 1:3. The ratio between the CH_3 of pyruvate ketal to the anomeric protons was found to be 3:4 showing one pyruvic acid per four sugar units. The anomeric signal at τ 5.09 was shifted upfield to 5.24 when the pyruvate ketal group was removed by hydrolysis with 0.1 M TFA at 95° for 1 hour.

Partial Hydrolysis of the Polysaccharide

K56 polysaccharide (1 g) was dissolved in trifluoroacetic acid (TFA 50 ml, 0.5 M) and the solution was heated on a steam bath for 1 hour. The solution was evaporated and the monosaccharides were separated from the oligosaccharides by a charcoal column (Darco G60, 10 x 3 cm). Monosaccharides were eluted by distilled water (2 l.) and a series of oligosaccharides (mainly di, tri and tetrasaccharides) was eluted with 20% ethanol-water (1 l.), yield about 0.3 g. The oligosaccharides

(100 mg) were applied to a Sephadex G15 column (110 x 2 cm) and irrigated with water at a flow rate of 4-6 ml per hour. The oligosaccharides were separated into di, tri and tetrasaccharides (R_{Gal} values 0.52, 0.23, 0.16, solvent C) and analyzed as follows:

A. Analysis of disaccharide (40 mg)

P.m.r. (Table 1) of the disaccharide when run in D_2O at 95° showed it to be β-linked. When 10 mg was hydrolyzed with 2 M TFA for 4 hours, D-galactose and D-glucose were found as the only two sugars present in the ratio of approximately 1:1 (as determined by g.l.c. of the alditol acetates). The same ratio was obtained when another portion (10 mg) was dissolved in 5 ml 0.3 M acetate buffer (pH 5.5) to which 10 mg of β-D-glucosidase was added and incubated at 38° for 16 hours. The disaccharide (10 mg) was methylated and hydrolyzed, 2,3,4,6-tetra-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-galactose were found by paper chromatography (solvent D) having R_f 0.80 and 0.40 respectively together with small amounts of tri-O-methyl-D-galactofuranose R_f 0.59 (solvent D) which was not determined. This showed that D-glucose is the non-reducing end. The ratio of 2,3,4,6-tetra-O-methyl-D-glucose to 2,4,6-tri-O-methyl-D-galactose was found to be 1:0.9 as determined by g.l.c. of the alditol acetates.

B. Analysis of the trisaccharide (20 mg)

When the p.m.r. spectrum was run in D_2O at 95°, anomeric protons were obtained at τ 5.29 (Table 1) indicating the sugars are joined by β-linkages. A portion (5 mg) was hydrolyzed by 0.5 M TFA for 1/2 hour

and paper chromatography (solvent C) showed D-galactose, together with the same disaccharide as described above. In order to locate the reducing end, another portion (10 mg) was reduced with sodium borohydride and hydrolyzed (0.5 M TFA, 1 hour) to give galactitol (R_G 0.88 paper chromatography, solvent B) confirmed by g.l.c. of the alditol acetate (m.p. 164-166°) together with the same disaccharide (solvent C) as described above.

C. Analysis of tetrasaccharide (25 mg)

The anomeric linkages were proved by p.m.r. (at 95° in D_2O) to contain one α-linkage and two β-linkages (Table 1). A portion (10 mg) was hydrolyzed by 0.5 M TFA for 1/2 hour. On paper chromatography (solvent C), D-galactose was shown together with the same disaccharide and trisaccharide as described above. Another portion (10 mg) was methylated by Hakomori method and hydrolyzed. 2,3,4,6-Tetra-O-methyl-D-glucose was found by paper chromatography (solvent D, R_f 0.79) together with a major spot of 2,4,6-tri-O-methyl-D-galactose (R_f 0.37). The quantity of the partially methylated sugars was insufficient for g.l.c. analysis.

Partial Hydrolysis of Methylated Capsular Polysaccharide

Methylated capsular polysaccharide (200 mg) was hydrolyzed with 90% formic acid for 45 minutes at 70°. TLC of the hydrolysate in solvent D showed the presence of three spots with R_f 0.78, 0.72 and 0.66 (major), together with 2 faint spots with R_f 0.47 and 0.25-0.30. The component with R_f 0.72 had the same characteristics as 2,3,4-tri-O-methyl-L-rhamnose while the component with R_f 0.78 was reduced and

hydrolyzed, 2,3,4-tri-O-methyl-L-rhamnose, 2,4,6-tri-O-methyl-D-galactose, 4,6-di-O-methyl-D-galactose and 2-O-methyl-D-glucose were shown by paper chromatography to be present.

The hydrolysate (150 mg) was separated by TLC to yield a major component (40 mg) (R_f 0.66, solvent D). P.m.r. of this fraction showed a strong signal at τ 8.45. A small portion (7 mg) was hydrolyzed to give 2,4,6-tri-O-methyl-D-galactose, 4,6-di-O-methyl-D-galactose and 2-O-methyl-D-glucose in a ratio of 1.5:0.8:1 (determined as alditol acetates). On reduction with sodium borohydride, two components were obtained with R_f values 0.14 and 0.07 on TLC in solvent D. The two components were again separated on TLC in solvent D. The faster component (12 mg), on hydrolysis, showed only 2-O-methyl-D-glucose on paper chromatography but when the hydrolysate was acetylated, 2,4,6-tri-O-methyl-D-galactitol acetate and 2-O-methyl-D-glucose acetates were found in a ratio of 1.25:1. When a portion of the slow moving component (10 mg) was hydrolyzed, the following results were obtained:

R_f Paper Chromatography				
Solvent D	Solvent E		T_{min} *	Molar Ratio
0.39	0.61	2,4,6-tri-O-methyl-D-galactose	18(α)+23(β)	1.1
0.14	0.46	4,6-di-O-methyl-D-galactose	26(α)+32(β)	1
0.06	0.35	2-O-methyl-D-glucose	36.5(α)+38(β)	1
not detectable by p-anisidine		2,4,6-tri-O-methyl-D-galactitol	25	1.3

The slower component (5 mg) was methylated by the Hakomori procedure and on hydrolysis showed (solvent D) 2,4,6-tri-O-methyl-D-

* ECNSS-M column program from 170-220° at 2°/min.
T = retention times of sugar acetates.

galactose (R_f 0.38) and 2,3-di-O-methyl-D-glucose (R_f 0.27). There is no evidence of the presence of tetramethyl glucose or galactose. The 2,3-di-O-methyl-D-glucose was confirmed by g.l.c.-m.s. of the alditol acetate (retention time 24 min, 215°, column a).

Another portion (50 mg) of the original hydrolysate was hydrolyzed for a further period of 4 hours with 0.5 M TFA at 95°. By paper chromatography (solvent E), 2,4,6-tri-O-methyl-D-galactose, 4,6-di-O-methyl-D-galactose, and 2-O-methyl-D-glucose were shown together with a new component with R_f 0.79. The new component was separated by TLC (R_f 0.6, solvent D). Yield 15 mg. The new component (5 mg) was hydrolyzed with 2 M TFA at 95° for 16 hours. Paper chromatography (solvent D) showed the presence of 2,4,6-tri-O-methyl-D-galactose (R_f 0.38) and 2-O-methyl-D-glucose (R_f 0.06). The ratio was found to be 1:1 as determined by g.l.c. of the alditol acetates. The rest was reduced with sodium borohydride and hydrolyzed. On paper chromatography only 2-O-methyl-D-glucose was shown (R_f 0.35, solvent E). The hydrolysate was acetylated and g.l.c. showed the presence of 2,4,6-tri-O-methyl-D-galactitol acetate and 2-O-methyl-D-glucose acetates in a ratio of 1.2:1. The retention time of 2,4,6-tri-O-methyl-D-galactitol acetate is 22 min, while that of 2-O-methyl-D-glucose acetates are 36 min (α) and 38 min (β) when column a is programmed from 170-220°.

Table 1. P.m.r. data on Klebsiella K56 capsular polysaccharide and derived oligosaccharides.

Repeating unit of compound	τ-value* (coupling constant, Hz)	Ratio of integrals	Proton** assignment
Glc $\frac{1\ 3}{\beta}$ Gal-OH	4.64	0.5	α-Gal-OH
	5.28 (7)	1.5	β-Gal-OH
			β-Glc $\underline{1\ 3}$ Gal
Glc $\frac{1\ 3}{\beta}$ Gal $\frac{1\ 3}{\beta}$ Gal-OH	4.63	0.5	α-Gal-OH
	5.29 (7)	2.5	β-Gal-OH
			β-Glc $\underline{1\ 3}$ Gal
			β-Gal $\underline{1\ 3}$ Gal
Glc $\frac{1\ 3}{\beta}$ Gal $\frac{1\ 3}{\beta}$ Gal $\frac{1\ 3}{\alpha}$ Gal-OH	4.63	0.5	α-Gal-OH
	4.75	1	α-Gal $\underline{1\ 3}$ Gal
	5.28 (7)	2.6	β-Gal-OH
			β-Glc $\underline{1\ 3}$ Gal
			β-Gal $\underline{1\ 3}$ Gal

Table 1 (continued)

$CH_3CCOONa$ 4,6 3 Glc $\frac{1\ 3}{\beta}$ Gal $\frac{1\ 3}{\beta}$ Gal $\frac{1\ 3}{\alpha}$ Gal $\frac{1}{\beta}$ $\alpha\vert 2$ $\vert 1$ L-Rha	4.76	2	α-L-Rha $\frac{1\ 2}{}$ Gal
			α-Gal $\frac{1\ 3}{}$ Gal
	5.08 (§)	2	β-Glc $\frac{1\ 3}{}$ Gal
			β-Gal $\frac{1\ 3}{}$ Glc
	5.28 (§)	1	β-Gal $\frac{1\ 3}{}$ Gal
	8.57	3	CH_3 of pyruvate ketal
	8.73 (5)	3	CH_3 of L-Rha
$CH_3CCOONa$ 4,6 3 Glc $\frac{1\ 3}{\beta}$ Gal $\frac{1\ 3}{\beta}$ Gal $\frac{1\ 3}{\alpha}$ Gal $\frac{1}{\beta}$	4.75	1	α-Gal $\frac{1\ 3}{}$ Gal
	5.09 - 5.21 (§)	3	β-Gal $\frac{1\ 3}{}$ Glc
			β-Glc $\frac{1\ 3}{}$ Gal
			β-Gal $\frac{1\ 3}{}$ Gal
	8.57	3	CH_3 of pyruvate ketal
3 Glc $\frac{1\ 3}{\beta}$ Gal $\frac{1\ 3}{\beta}$ Gal $\frac{1\ 3}{\alpha}$ Gal $\frac{1}{\beta}$	4.75 (3)	1	α-Gal $\frac{1\ 3}{}$ Gal
	5.24 (5-7)	3	β-Glc $\frac{1\ 3}{}$ Gal
			β-Gal $\frac{1\ 3}{}$ Glc
			β-Gal $\frac{1\ 3}{}$ Gal

* Spectra run in D_2O with external tetramethylsilane (τ = 10) at 100 MHz. ** All sugars have D-configuration except L-rhamnose and all are pyranose. § signals approximately 5-6 Hz wide; shows no distinct splitting.

Table 2. Methyl ethers from the hydrolysis of methylated Klebsiella K56 polysaccharides.

Sugars	Sample*					
	A†	B†	C†	D†	E†	F†
2,3,4,6-Tetra-O-methyl-D-glucose			1	+		
2,3,4-Tri-O-methyl-L-rhamnose	0.85					
2,4,6-Tri-O-methyl-D-galactose	2.2	3	0.9	+	1.1	
4,6-Di-O-methyl-D-galactose	1				1	
2,3-Di-O-methyl-D-glucose						
2-O-Methyl-D-glucose	1	1			1	1
2,4,6-Tri-O-methyl-D-galactitol					1.3	1.2

* A, sugars from methylated original K56 polysaccharide;
B, sugars from methylated Smith periodate degraded polysaccharide;
C, sugars from methylated disaccharide;
D, sugars from methylated tetrasaccharide;
E, sugars from partially methylated tetrasaccharide;
F, sugars from partially methylated disaccharide.

† Approximate molar ratios.

(+) Signifies present and has no quantitative significance.

Table 3. Diagnostic prominent peaks (m/e) in the mass spectra of acetates of methylated alditols.

Parent sugar	m/e											
	43	45	117	129	131	139	161	175	189	233	261	333
2,3,4-Me_3Rha	+		+		+	+	+					
2,4,6-Me_3Gal	+	+	+	+			+			+		
4,6-Me_2Gal	+	+	+	+			+				+	
2,3-Me_2Glu	+		+								+	
2-MeGlu	+		+			+						+

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APPENDIX I

The Structure of the Capsular Polysaccharide from

Klebsiella K-Type 21

Can. J. Chem., 51, 198 (1973)

The Structure of the Capsular Polysaccharide from Klebsiella K-Type 21[1]

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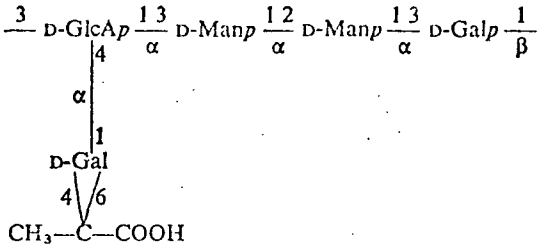
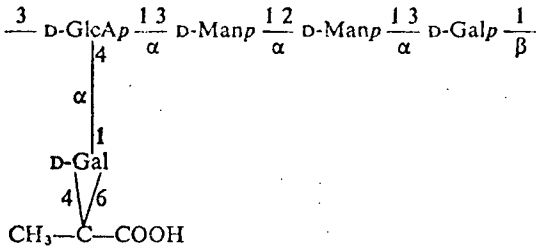
Methylation, periodate oxidation, and partial hydrolysis studies on the capsular polysaccharide, and on the carboxyl reduced polymer, of *Klebsiella* K21 show the structure to consist of a repeating unit.



The anomeric linkages were determined by p.m.r. spectroscopy of isolated oligosaccharides and, in part, by specific enzymes. P.m.r. spectroscopy of the original polysaccharide in methyl sulfoxide-d_6 showed clearly a ratio of one pyruvic acid ketal (CH_3, τ 8.5) to five anomeric protons (τ 4.65–5.40).

Les méthodes de méthylation, oxydation periodique et hydrolyse partielle portées au polysaccharide capsulaire, et au polymère avec le groupe carboxyle réduit, de *Klebsiella* K21 ont démontré que la structure se compose d'une unité qui se répète.



Les liaisons anomères ont été distinguées par la spectroscopie r.m.n. des oligosaccharides isolés et, en partie, par des enzymes spécifiques. La spectroscopie r.m.n. du polysaccharide original dans le méthyl sulfoxide-d_6 a établi le rapport un à cinq entre le cétal de l'acide pyruvique (CH_3, τ 8.5) et les protons anomères (τ 4.65–5.40).

Can. J. Chem., 51, 198 (1973)

Qualitative analyses of the capsular polysaccharides of the 80 serotypes of *Klebsiella* bacteria have been provided by Nimmich (1, 2) who has shown that the great majority contain glucuronic acid in combination with hexose and deoxyhexose sugars. Approximately one half of the polysaccharides also contain pyruvic acid (3) covalently bound (1-carboxyethylidene ketals). Despite the amount of qualitative information available on *Klebsiella* polysaccharides detailed structures are only known for those capsular materials from types K2 (ref. 4), K5 (ref. 5), K8 (ref. 6), K9 (ref. 7), K20 (ref. 8), K54 (refs. 9–11), and three related but untyped species of *Aerobacter aerogenes* (12–14). The structure of the capsular polysaccharide from *Klebsiella* K21 is now reported. A preliminary report has appeared (15) which also gives other background references.

The acidic capsular polysaccharide from *Klebsiella* K21 was purified by one precipitation with Cetavlon and had $[\alpha]_D$ +130°. Gel filtration and electrophoresis showed the material to be homogeneous. In work with other *Klebsiella*

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capsular polysaccharides it has been found that there is an approximate, inverse relationship between the equivalent weight of the polysaccharide and the distance of migration. One may predict that the polysaccharide from *Klebsiella* K62 will have an equivalent weight about 580 (Fig. 1).

The p.m.r. spectrum of a 2% solution of the polysaccharide in D_2O showed a sharp singlet at τ 8.5 indicative of a pyruvic acid ketal (16, 17). Determination of the pyruvic acid:sugar ratio was hampered by the presence of the HOD signal even after several exchanges with D_2O. The HOD peak was shifted downfield by the addition of trifluoroacetic acid or upfield by running the spectrum at 95–100° on the polysaccharide in methyl sulfoxide-d_6. In the latter case integration of the signals due to the anomeric protons and that of the methyl at τ 8.5 showed there was one pyruvic acid ketal to five sugar residues (Fig. 2).

Partial assignment of the anomeric signals may be made using the data of Table 1, which are based on examination of the oligosaccharides obtained in subsequent experiments, and similar data given by Conrad and coworkers (4) and others. These enable one to predict that of the five glycosidic bonds (per repeating unit) only one is of the β-D-configuration (shown subsequently to be β-D-galactose). In later work with other *Klebsiella* capsular polysaccharides (K20, K24) excellent spectra have been obtained by using the sodium salt of the polysaccharide in D_2O at 95° and dispensing with the use of methyl sulfoxide.

Acidic hydrolysis of the polysaccharide showed,

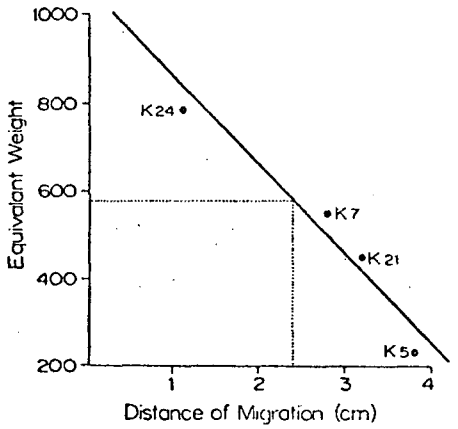


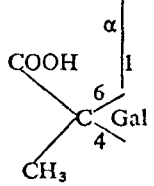
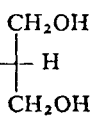
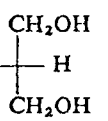
FIG. 1. *Klebsiella* capsular polysaccharides; electrophoresis on cellulose acetate at 300 V for 30 min (pH 8.8, veronal–tris buffer). Distance of migration *vs.* equivalent weight. K62 migrates 2.4 cm indicating an equivalent weight of 580.

by paper chromatography, the presence of D-mannose, D-galactose, D-glucuronic acid, lactone, and an aldobiouronic acid. Separation of the hydrolyzate into neutral and acidic fractions and g.l.c. of the former, both as trimethylsilyl derivatives and as alditol acetates, gave a ratio of D-mannose to D-galactose of 1:1.2. Collection of the alditol acetates permitted characterization of D-mannitol hexaacetate m.p. 118–121° and galactitol hexaacetate, m.p. 162°. The aldobiouronic acid was separated from the acidic fraction by paper chromatography and reduction of the carboxyl function followed by hydrolysis gave D-glucose and D-mannose, further characterized as their alditol acetates. The configuration of D-galactose was established by D-galactose oxidase, D-glucuronic acid as D-glucose by D-glucose oxidase, and D-mannose by the circular dichroism curve of D-mannitol hexaacetate (18).

Autohydrolysis of the polysaccharide gave pyruvic acid (19) characterized as the 2,4-dinitrophenylhydrazone (20). It was considered significant that the aqueous solution showed the presence of D-galactose as the only monosaccharide together with traces of oligosaccharides. The residual polysaccharide, obtained by dialysis and lyophilization of the aqueous hydrolyzate, was saved for subsequent methylation studies discussed later.

A sample of pure capsular polysaccharide was methylated (10, 21) and hydrolyzed to yield a neutral and an acidic fraction after separation on ion-exchange resins. Table 2 (A) gives the neutral sugars obtained which were identified as discussed in the experimental. These results show that D-mannose occurs in the polysaccharide linked in two ways; substituted at position 2 and at position 3. The ratio of 3,4,6-tri-*O*-methyl-D-mannose to the 2,4,6-isomer was 1.7:1, consistent with the concept that part of the mannose remained bound to the uronic acid. Isolation of 2,4,6-tri-*O*-methyl-D-galactose indicates that this unit represents a 3-substituted sugar in the chain. The 2,3-di-*O*-methyl-D-galactose cannot represent a branch point since no corresponding quantity of terminal units was found. The pyruvic acid must therefore be linked to D-galactose as a 4,6-ketal. The acidic fraction of the hydrolyzate was converted to the methyl ester glycoside, reduced and hydrolyzed to give 2,4,6-tri-*O*-methyl-D-mannose and 2-*O*-methyl-D-glucose, Table 2 (B). These results show that the aldobiouronic acid is 3-*O*-(glucopyranosyluronic acid)-D-mannose and that

TABLE 1. P.m.r. data on *Klebsiella* K21 capsular polysaccharide and derived oligosaccharides

Repeating unit or compound	Solvent, temperature (°)	τ-Value* (coupling constant, Hz)	Ratio of integrals	Proton assignment (all sugars have D-configuration)
Original polysaccharide	Me_2SO-d_6 100			
—Gal $\frac{1\,3}{\beta}$ GlcA $\frac{1\,3}{\alpha}$ Man $\frac{1\,2}{\alpha}$ Man $\frac{1\,3}{\alpha}$ Gal—		4.65	1	α-GlcA $\frac{1\,3}{}$ Man
		4.90+4.95	2	α-Man $\frac{1\,3}{}$ Gal α-Gal $\frac{1\,4}{}$ GlcA
		5.05	1	α-Man $\frac{1\,2}{}$ Man
		5.40	1	β-Gal $\frac{1\,3}{}$ GlcA
Aldotetrauronic acid	D_2O 95			
GlcA $\frac{1\,3}{\alpha}$ Man $\frac{1\,2}{\alpha}$ Man $\frac{1\,3}{\alpha}$ Gal—OH		4.75+4.80	2.5	α-GlcA $\frac{1\,3}{}$ Man α-Man $\frac{1\,3}{}$ Gal α-Gal—OH
		5.00	1	α-Man $\frac{1\,2}{}$ Man
		5.47 (8)	0.5	β-Gal—OH
Oligosaccharide from Smith degradation	D_2O 95			
Gal $\frac{1\,3}{\beta}$ GlcA $\frac{1\,3}{\alpha}$ Man $\frac{1}{\alpha}$ 		4.75	0.9	α-GlcA $\frac{1\,3}{}$ Man
		4.95	1.2	α-Man $\frac{1}{}$ 
		5.47 (8)	1	β-Gal $\frac{1\,3}{}$ GlcA

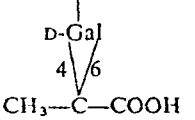
*External tetramethylsilane standard for aqueous solutions, internal for methyl sulfoxide.

the D-glucuronic acid is a branch point in the chain. In the absence of tetramethylhexose as terminal groups the side chain attached to the glucuronic acid must be the 4,6-*O*-(1-carboxyethylidene)-D-galactose units. These methylation data permit the drawing of partial structures such as A and B but do not distinguish between them nor do they establish the position of attachment of the side chains nor the mode of linkage of the glucuronic acid in the main chain.

$\frac{1\,2}{}$ D-Man*p* $\frac{1\,3}{}$ D-Gal*p*— A

—D-Glc*p*A $\frac{1\,3}{}$ D-Man*p* or

$\frac{1\,3}{}$ D-Gal*p* $\frac{1\,2}{}$ D-Man*p*— B



The residual polysaccharide recovered from the

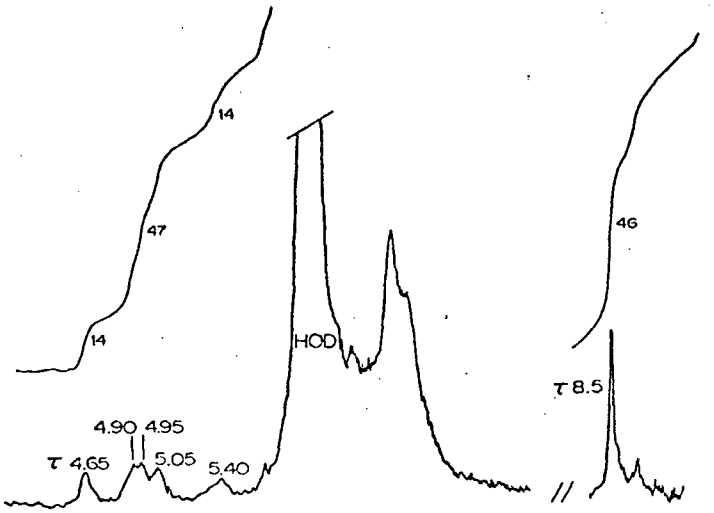


FIG. 2. P.m.r. spectrum of *Klebsiella* K21 capsular polysaccharide in methyl sulfoxide-d_6 at 100°. Tetramethylsilane internal standard (τ 10), 100 MHz.

TABLE 2. Methyl ethers from the hydrolysates of methylated *Klebsiella* K21 polysaccharides

Sugars	Sample*				
	A†	B	C	D	E
2,3,4,6-Tetra-*O*-methyl-D-galactose			3		
2,3,4,6-Tetra-*O*-methyl-D-mannose			1		
2,4,6-Tri-*O*-methyl-D-galactose	1.0		23		0.8
2,4,6-Tri-*O*-methyl-D-mannose	0.6	+	24	+	1.0
3,4,6-Tri-*O*-methyl-D-mannose	1.0		27		1.1
2,3-Di-*O*-methyl-D-galactose	1.0		2		1.0
2,4-Di-*O*-methyl-D-glucose				+	
2,6-Di-*O*-methyl-D-glucose					1.0
2-*O*-Methyl-D-glucose		+			

*A, neutral sugars from methylated original K21 polysaccharide; B, reduced aldobiouronic acid fraction from methylated original K21 polysaccharide; C, neutral sugars from methylated residual polysaccharide after autohydrolysis; D, reduced aldobiouronic acid from methylated residual polysaccharide after autohydrolysis; E, neutral sugars from methylated reduced K21 polysaccharide.

†Approximate molar ratios; (+) signifies present in about equimolecular amounts.

autohydrolysis described above was methylated and hydrolyzed to yield, after separation on ion-exchange columns, neutral and acidic fractions. The composition of the former is shown in Table 2 (C) and the significant feature is the virtually complete disappearance of 2,3-di-*O*-methyl-D-galactose and, incidentally, the very small amounts of tetramethyl sugar produced. Paper chromatography of the acidic fraction showed two components in apparently equal quantities with mobilities corresponding to partially methylated uronic acid (R_f 0.16) and aldobiouronic acid (R_f 0.23). Reduction and hydrolysis of this mixture yielded 2,4,6-tri-*O*-methyl-D-mannose and 2,4-di-*O*-methyl-D-glucose, Table 2 (D). The disappearance of 2,3-di-*O*-methyl-D-galactose in the neutral fraction and the appearance in the acid fraction of 2,4-di-*O*-methyl-D-glucuronic acid is convincing evidence that the 4,6-*O*-(1-carboxyethylidene)-D-galactose units are attached to position 4 of D-glucuronic acid which in turn must be linked through position 3 in the main chain.

The information thus far obtained was confirmed, and more accurate quantitative data were obtained, by examination of the carboxyl-reduced polysaccharide. This was prepared exactly as described for sapote gum using lithium boro-

hydride in tetrahydrofuran (22). This method has given virtually quantitative reduction with this and several related polysaccharides and is now our preferred method of achieving such a conversion.

It should also be noted that this reduced polysaccharide and the methylated polymer described above each showed a characteristic sharp singlet (τ *ca.* 8.6) for the methyl group of the corresponding pyruvic acid derivative. This signal thus affords not only a method for the quantitative estimation of 1-carboxyethylidene ketals but also for checking that the related hydroxyisopropylidene and 1-carbomethoxyethylidene groups survive subsequent chemical transformations. In the polysaccharide only small changes in chemical shift were detected in these methyl signals (16). Hydrolysis of the reduced polysaccharide gave D-mannose, D-galactose, and D-glucose in the ratio of 1:1:0.6 (as alditol acetates).

The reduced polysaccharide was methylated and gave on hydrolysis the sugars in the proportions shown in Table 2 (E). The formation of 2,6-di-*O*-methyl-D-glucose arises from the reduced D-glucuronic acid moiety and the quantitative data are consistent with the proposed partial structure.

A portion of the original capsular polysaccharide was autohydrolyzed at 95° for 3 days (initial p*H* 2.3) using the apparatus described by Galanos *et al.* (23) which circulates the polysaccharide solution through stages of heating, cooling, and dialysis and recycles unhydrolyzed material. This apparatus is a modern improvement on the ideas of Painter (24) and of Perila and Bishop (25) but because of the close control that can be exercized high yields of oligosaccharides may be obtained. As previously noted little, except D-galactose and pyruvic acid, is liberated on autohydrolysis; therefore the polysaccharide solution was made up to 0.125 *M* sulfuric acid and dialyzed against acid of the same concentration. Hydrolysis was continued for a further 3 days at 95°. The dialyzate was neutralized with barium carbonate and separated into neutral and acidic fractions. The latter was separated on paper to give, as the main component, an aldotetrauronic acid with lesser amounts of aldotri- and aldobiouronic acids. Table 1 gives the structure of the aldotetrauronic acid based on the evidence presented in the Experimental. The salient points are that partial hydrolysis of the aldotetrauronic acid gave D-galactose as the main monosaccharide and, similarly, hydrolysis after sodium borohydride reduction yielded galactitol. The other products of the partial hydrolysis corresponded to the aldotri- and aldobiouronic acids isolated. The aldobiouronic acid was resistant to the action of β-D-glucuronidase and gave D-mannose on hydrolysis. Identification of the aldotetrauronic acid enables a decision to be made in favor of structure A. The aldobiouronic acid 3-*O*-(α-D-glucopyranosyluronic acid)-D-mannose has been isolated previously from *Serratia marcescens* (26), Klebsiella K2 (ref. 4), and from *Pseudomonas aeruginosa* (27).

Confirmation of structure A was sought by periodate oxidation of the original capsular polysaccharide. Periodate consumption of 0.36 mol per hexose unit was constant after 3 days and the derived polyalcohol was hydrolyzed by heating an aqueous solution, initial p*H* 2.2, at 95° for 1 h. A separate paper has discussed the wide variety of conditions which has been applied in the hydrolysis step of Smith degradations and has noted that other authors have experienced difficulty in obtaining cleavage of acetal linkages in polymers containing uronic acids (28). Hydrolyses have commonly been carried out with acid strengths of 0.05–0.5 *M* although two groups have recently reported good results with 0.01 *M* acid (13, 29). Johnson and Percival (30) have utilized the acidity of sulfated polysaccharides to effect autohydrolysis of the polyalcohol but the present work appears to be the first instance where the acidity of the uronic acid has been used to achieve hydrolysis of the polyalcohol in a Smith degradation.

Paper chromatographic examination (solvent *A*) of the hydrolyzate showed the presence of (*a*) pyruvic acid, (*b*) glycerol, (*c*) D-threitol, (*d*) D-galactose (traces only), and (*e*) an oligosaccharide which had a mobility (in solvent *E*) intermediate between the aldobi- and aldotriouronic acids previously isolated. The ratio of glycerol to D-threitol was 1.2:1 by g.l.c. of the acetates.

It is pertinent to note here that even under these mild conditions of autohydrolysis the pyruvic acid ketal was hydrolyzed. Similarly, no conditions of partial hydrolysis of the original polysaccharide could be found which would permit the isolation of 4,6-*O*-(1-carboxyethylidene)-D-galactose. Furthermore, an attempt to achieve this by the use of α-galactosidase was unsuccessful since the enzyme was without action on the original polysaccharide. This acid lability

of the pyruvic acid ketal is in direct contrast to the result of Gorin and Ishikawa (16) who were able to hydrolyze the glycoside methyl 4,6-*O*-(1-carboxyethylidene)-α-D-galactoside without cleavage of the ketal. The difference in stability may be accounted for by the conformation of the ketal or by the proximity of the D-glucuronic acid moiety, but no information is as yet available on these points.

The oligosaccharide obtained in the Smith degradation was of sufficient molecular weight to be precipitated when the aqueous solution was poured into ethanol. The p.m.r. spectrum of the reduced oligosaccharide showed the presence of one β-D-linkage per three anomeric protons and incubation of the original oligosaccharide with β-galactosidase yielded D-galactose. When the reduced oligosaccharide was methylated and hydrolyzed examination by paper chromatography in solvent *D* showed 2,3,4,6-tetra-*O*-methyl-D-galactose to be the major neutral sugar with traces of 2,4,6-tri-*O*-methyl-D-mannose. This result was confirmed by g.l.c. Examination in solvent *E* showed also the presence of two acidic components with mobilities identical to 2,4-di-*O*-methyl-D-glucuronic acid and the partially methylated aldobiouronic acid isolated previously from the methylated residual polysaccharide. In neither case was the di-*O*-methylglycerol sought.

The sum of these results firmly establishes structure A as representing the repeating unit of the capsular polysaccharide of *Klebsiella* K21 and demonstrates for the first time the mode of attachment of the pyruvic acid in this genus. The structure found for K21 is similar to those previously reported for K2, K8, K9, K54 (refs. 4, 6, 7, 11) in that they each have a repeating unit of four or five sugars including a single unit side chain. That this is not an invariant rule for *Klebsiella* polysaccharides is shown by the structure of K5 (ref. 5) which has a repeat of three sugars and no side chain and of K20 (ref. 8) which has a repeat of four sugars including a two unit side chain. Full details of these other structures will be published in due course.

Experimental

General Methods

Paper chromatography was carried out by the descending method using Whatman No. 1 paper and the following solvent systems (v/v): (*A*) ethyl acetate – acetic acid – formic acid – water (18:3:1:4); (*B*) ethyl acetate – pyridine – water (4:1:1); (*C*) 1-butanol – acetic acid – water (2:1:1); (*D*) butanone saturated with 1% aqueous ammonia; (*E*) 1-butanol–ethanol–water (4:1:5). Chromatograms were developed with *p*-anisidine trichloroacetate spray (31) or with silver nitrate (32). The clarity of the colors on chromatograms of methylated sugars developed with the former spray is greatly improved by washing the paper under running hot water after heating the chromatogram at 100–110° in the normal manner.

G.l.c. was carried out on an F and M model 720 dual column instrument fitted with thermal conductivity detectors. The helium flow was 60–80 ml/min with the following columns: (*a*) 3% ECNSS-M on Chromosorb W (8 ft × 0.25 in.); (*b*) 5% butanediol succinate on Diatoport S (4 ft × 0.25 in.); (*c*) 8% SE-52 on Diatoport S (8 ft × 0.25 in.).

Circular dichroism spectra were run on a Jasco J-20 automatic recording spectropolarimeter using a quartz cell with path length 0.1 cm. Optical rotations were measured at 23 ± 2° on a Perkin-Elmer model 141 polarimeter. P.m.r. spectra were run on Varian T60 or XL100 instruments. Samples were prepared by dissolving in D_2O and freeze drying 3 or 4 times before dissolving in D_2O or methyl sulfoxide-d_6. Tetramethylsilane was used as a standard, externally for D_2O solutions and internally for methyl sulfoxide-d_6.

Mass spectra, obtained on individual fractions collected (33) from the gas chromatograph, were run on an MS 902 instrument at 70 eV. The position of methoxyl substitution was determined using the data of Lindberg and coworkers (34) and, where possible, by comparison of mass spectra with those of authentic compounds.

Methylations on polysaccharides and oligosaccharides were carried out by the method of Hakomori (9, 21). Partially methylated alditol acetates were demethylated by reaction with boron trichloride (35). All solutions were concentrated on a rotary evaporator *in vacuo* at a bath temperature of 40°.

D-Glucostat and D-Galactostat reagents were obtained from the Worthington Biochemical Corporation, β-D-galactosidase from Koch-Light, and β-D-glucuronidase from Sigma Chemical Company. α-D-Galactosidase was prepared from green coffee beans by the procedure of Clarke *et al.* (36). Sagavac 6F is a product of Seravac Laboratories, Cape Town.

Preparation and Properties of K21 Capsular Polysaccharide

A culture of *Klebsiella* K21 (1702/49) was obtained from Dr. Ørskov, Copenhagen, as an agar slant and was grown on the following medium for 3 days at 25°: 8 g NaCl, 4 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 2 g $CaCO_3$, 120 g sucrose, and 8 g Bacto yeast extract in 4 l of water. Cells were harvested after 3 days, diluted with water containing 1% phenol, and centrifuged at 19 000 g. Capsular polysaccharide was obtained by concentrating the aqueous solution, pouring it into ethanol, and purifying the crude material by Cetavlon precipitation. A sample of the supernatant was shown to contain very little material and therefore this was not further examined. The yield of polysaccharide was approximately 2 g per 4 l of medium. Three batches were pooled and used for the structural studies of the polysaccharide.

Purified polysaccharide (2 g) was dissolved in distilled water (2 l), deionized with Amberlite IR 120 resin, dialyzed, and freeze-dried. The product had $[\alpha]_D$ +130°

(*c* 0.38, water), ash 0.8%, N 0.3%. The equivalent weight of the polysaccharide, determined by titration with 0.01 *M* sodium hydroxide was 455 (calcd. 447). The average molecular weight was *ca.* 4×10^5 as determined by gel permeation chromatography on Sagavac 6F through the courtesy of Dr. S. C. Churms. Electrophoresis at p*H* 3 and 8.8 showed the polysaccharide moved as a single component (migration 3.2 cm in 30 min at 300 V at p*H* 8.8; by courtesy of Dr. P. E. Reid and Miss C. Poder).

The p.m.r. spectrum of a 2% solution of D_2O showed a sharp singlet at τ 8.5 due to the CH_3 of the pyruvic acid ketal group. The relative amount of pyruvic acid was initially obtained by running the spectrum on a sample dissolved in 3 *M* trifluoroacetic acid in D_2O. Integration gave 17–18 units for the CH_3 signal at τ 8.5 and 146 units for the ring protons, excluding anomeric ones. The ratio was confirmed by running the spectrum of a 2% solution of the polysaccharide in methyl sulfoxide-d_6 at 100°. In this case the HOD peak was shifted upfield so that the anomeric proton signals were clear. These were at τ 4.65 (1H); 4.90, 4.95, 5.05 (3H); 5.40 (1H). The ratio of anomeric protons to pyruvate CH_3 was 5:3.

Hydrolysis of the Polysaccharide

Polysaccharide (0.1 g) was hydrolyzed with 0.5 *M* sulfuric acid at 100° for 8 h. Neutralization ($BaCO_3$) followed by paper chromatography (solvents *A* and *B*) showed D-mannose and D-galactose, D-glucuronolactone, D-glucuronic acid, and an aldobiouronic acid. The hydrolyzate was separated into neutral and acidic fractions using ion-exchange resins (Amberlite IR 120 and Duolite A4). The neutral fraction contained D-mannose and D-galactose in approximately equal amounts (1:1.2) as determined by g.l.c. of the trimethylsilyl derivatives (column *c*) and of the alditol acetates (column *a*). G.l.c. of the acetates permitted recovery of the individual compounds and identification of D-mannitol hexaacetate, m.p. and mixed m.p. 118–121°, and galactitol hexaacetate, m.p. 162° (37). A portion of the neutral fraction of the hydrolyzate was tested with D-Galactostat reagent and a positive response confirmed the D-configuration of galactose. Part of the mannitol hexaacetate collected by g.l.c. was recrystallized and dissolved in acetonitrile. A positive circular dichroism curve, identical to that given by a standard sample, confirmed the D-configuration of mannose (18).

The acidic fraction was separated into two parts by paper chromatography (solvent *A*). The slower migrating, major portion had the mobility of an aldobiouronic acid (R_{Gal} 0.39) and was converted into the ester glycoside by refluxing overnight with 3% methanolic hydrogen chloride. The product was dissolved in tetrahydrofuran and reduced by boiling under reflux overnight with lithium aluminum hydride. Hydrolysis of the neutral disaccharide gave approximately equimolar amounts of D-glucose and D-mannose as determined by g.l.c. of the alditol acetates. The configuration of the glucose was confirmed by the D-Glucostat reagent.

A separate portion (1.5 g) of the polysaccharide was dissolved in water (initial p*H* 2–2.3) and heated for 8 h at 95°. Paper chromatography (solvent *A*) of the concentrated solution showed a fast moving spot, chromatographically identical with pyruvic acid (R_f 0.74) and having the same characteristic fluorescence as a standard when sprayed with *o*-phenylenediamine and examined by u.v. (19). The chromatogram also showed D-galactose as the only monosaccharide and traces of slow moving oligomers. The aqueous solution was extracted with ether and the ether extract was treated with 2,4-dinitrophenylhydrazine to yield the derivative of pyruvic acid, m.p. 214–216°, undepressed by an authentic sample (20). The autohydrolysis solution was concentrated to 50 ml and dialyzed against distilled water (2 l). The non-dialyzable material (0.65 g) was recovered by freeze-drying and kept for methylation studies.

Methylation Analysis

Capsular polysaccharide (0.5 g) was methylated to give a product having a methoxyl content of 38.5% and showing no hydroxyl absorption in the i.r. The methoxyl content was not increased by two further treatments with silver oxide and methyl iodide. The fully methylated polysaccharide was dissolved in cold 72% sulfuric acid and after 1 h the solution was diluted to 1 *M* and hydrolyzed at 100° for 8 h. After neutralization and evaporation, the syrupy product was separated into neutral and acidic fractions by ion-exchange resins. The neutral portion was fractionated by cellulose column chromatography (38) using butanone–water azeotrope and the partially methylated sugars were characterized as follows:

Fraction I (80 mg) was shown by paper chromatography, (solvent *D*) to contain two components having R_f 0.57 and 0.55; the faster moving one (*Ia*) gave a brownish yellow spot when sprayed with *p*-anisidine while the slower one (*Ib*) gave a pink color. On demethylation, fraction I was shown by paper chromatography (solvents *A* and *B*) to contain only D-mannose. Fraction I (20 mg) was reduced by sodium borohydride and, after removal of the borate, the product was acetylated in a sealed tube by using pyridine and acetic anhydride (1:1, 2 ml, 100°, 20 min). After evaporation of the solvents, the alditol acetates were dissolved in a small volume of ethyl acetate and analyzed by g.l.c. on column *b* programmed from 150–200° at 2°/min; two peaks were observed.

The component eluted first had a retention time of 17.2 min and mass spectrometry showed it to be a 3,4,6-tri-*O*-methyl hexitol acetate. On demethylation and acetylation, D-mannitol hexaacetate was obtained (g.l.c., m.p., and mixed m.p. 118–121°). The second component had a retention time (19.6 min) identical to that of authentic 2,4,6-tri-*O*-methyl-D-mannitol triacetate. A sample collected from the gas chromatograph had m.p. 65–67° undepressed by authentic material (39) and the identity was further confirmed by the mass spectrum. The ratio of the amount of 3,4,6-isomer to that of the 2,4,6-compound was 1:0.6.

Fraction II (30 mg) was shown by paper chromatography to be a single compound (R_f 0.37, solvent *D*) and on demethylation, D-galactose was obtained. Crystallization of the syrupy sugar from ether gave 2,4,6-tri-*O*-methyl-D-galactose m.p. 103–105° (*lit.* (40) m.p. 102–105°). The derived alditol acetate had a retention time of 21 min (column *a*) and the mass spectrum was consistent with a 2,4,6-tri-*O*-methyl hexitol acetate.

Fraction III (35 mg) was shown by paper chromatography to be a single compound and to correspond to 2,3-di-*O*-methyl-D-galactose (R_f 0.18, solvent *D*). This assignment was confirmed by the mass spectrum of the

alditol acetate which on demethylation and acetylation gave galactitol hexaacetate, m.p. and mixed m.p. 162°.

The acidic fraction was heated under reflux overnight with 2% methanolic hydrogen chloride to give the ester glycoside which was reduced by boiling overnight with lithium borohydride in tetrahydrofuran. The product (20 mg) was hydrolyzed with 0.5 *M* sulfuric acid for 4 h. Paper chromatography showed the presence of 2,4,6-tri-*O*-methyl-D-mannose (R_f 0.5, solvent *D*; 0.80 solvent *E*) and 2-*O*-methyl-D-glucose (R_f 0.07, 0.24). The identities of both partially methylated sugars were confirmed by g.l.c. and mass spectrometry of the alditol acetates. The mannitol derivative crystallized, m.p. 64–66°, and the glucitol derivative was demethylated and acetylated to give glucitol hexaacetate m.p. 92–95°. 2-*O*-Methyl-D-glucitol pentaacetate has been isolated subsequently from *Klebsiella* K7 methylated polysaccharide and has m.p. 56–57°.

Methylation of Degraded Polysaccharide

Degraded polysaccharide (0.5 g), recovered from the autohydrolysis, was methylated, the product was dissolved in 5 ml of 72% sulfuric acid at 0° and then the solution was diluted to 1 *M* and hydrolyzed at 100° for 8 h. After neutralization the hydrolyzate was separated into acidic and neutral fractions. The latter was shown by paper chromatography (solvent *D*) and by g.l.c. of the alditol acetates (column *b*, 160–210° at 2°/min) to contain the sugars listed in Table 2 (C) which also gives their approximate molar ratios.

Paper chromatography of the acidic fraction (solvent *E*) showed two components with R_f 0.16 and 0.23 and in approximately equal amounts. When sprayed with *p*-anisidine trichloroacetate the slower gave the bright red color of a methylated uronic acid and the faster gave a reddish-brown color and was judged to be a partially methylated aldobiouronic acid. A portion of the mixture (20 mg) was converted to the ester glycosides and reduced with lithium borohydride in tetrahydrofuran. Paper chromatography, after acid hydrolysis, showed the presence of 2,4-di-*O*-methyl-D-glucose (R_f 0.18, solvent *D*) as the major component together with 2,4,6-tri-*O*-methyl-D-mannose. The identity of the former was confirmed by g.l.c. – mass spectrometry of the alditol acetate and that of the latter by the crystalline alditol acetate m.p. 64–66°.

Reduction of the Capsular Polysaccharide

Capsular polysaccharide (0.4 g) was converted into the methyl ester propionate and reduced with lithium borohydride as previously described (22) to give the reduced polysaccharide (0.3 g), $[\alpha]_D$ +115° (*c* 0.42, water). The reduced polysaccharide (0.1 g) was hydrolyzed with 0.5 *M* sulfuric acid at 100° for 8 h to give D-mannose, D-glucose, and D-galactose (paper chromatography, solvent *B*). The identities of the sugars were confirmed by g.l.c. of the alditol acetates and collection of the individual fractions afforded D-mannitol hexaacetate m.p. and mixed m.p. 118–120°, galactitol hexaacetate m.p. and mixed m.p. 160–162°, and D-glucitol hexaacetate m.p. and mixed m.p. 92–95°. The ratio of D-mannitol, galactitol, and D-glucitol hexaacetates was 1:1:0.6. The p.m.r. spectrum of reduced K21 polysaccharide in D_2O was shown to contain four anomeric signals at τ 4.54, 4.80, 5.00, and 5.15 in the ratio of 1:2:1:1. All the signals showed small coupling constants except that at τ 5.15 (7 Hz).

The remainder of the reduced polysaccharide (0.2 g) was methylated and hydrolyzed. Table 2 (E) shows the nature and proportion of the methylated sugars found and, in addition to those previously obtained from the methylated original polysaccharide, now included 2,6-di-*O*-methyl-D-glucose which was characterized by g.l.c. – mass spectrometry of its alditol acetate.

Partial Hydrolysis of Polysaccharide

K21 polysaccharide (1 g) was partially hydrolyzed at *pH* 2.3 at 95° for 3 days using an apparatus similar to that described by Galanos and coworkers (23). Since only D-galactose and pyruvic acid were found in the dialyzate the solution was made 0.125 *M* with sulfuric acid and hydrolyzed at 95° for a further 3 days. The dialyzate was neutralized ($BaCO_3$), evaporated, and separated by ion-exchange resins into acidic and neutral fractions. On paper chromatography (solvent *C*) the acidic fraction was shown to contain an aldotetrauronic acid (R_{Gal} 0.2) as the major component together with small amounts of aldobiouronic acid (R_{Gal} 0.70), aldotriouronic acid (R_{Gal} 0.42), and traces of higher oligomers. The acids were separated by paper chromatography (solvent *C*) and analyzed as follows.

The aldotetrauronic acid (50 mg) had an equivalent weight of 660 (NaOH titration, calcd. 680) and $[\alpha]_D$ +117° (*c* 1.2, water) indicating α-linkages. This was confirmed by the p.m.r. spectrum of a 4% solution in D_2O run at 95° (see Table 1). The aldotetrauronic acid (2 mg) was hydrolyzed with 0.5 *M* trifluoroacetic acid at 100° for 1 h. Paper chromatography (solvent *C*) showed the presence of D-galactose as the principle monosaccharide with traces of D-mannose and a series of aldobiouronic, aldotriouronic, and aldotetrauronic acids which had the same mobilities as the components of the original mixture. The aldotetrauronic acid (4 mg) was reduced with sodium borohydride and, after removal of borate, the product was hydrolyzed with 2 *M* trifluoroacetic acid for 2 h. On paper chromatography (solvents *A* and *B*), the neutral compounds found were D-mannose and galactitol. G.l.c. of the alditol acetates of the neutral compounds showed the ratio of galactitol and D-mannitol to be 1:1.3 and permitted characterization of each compound. The acidic sugars were D-glucuronic acid and the aldobiouronic acid.

The aldotetrauronic acid (11 mg) was methylated and then hydrolyzed by 2 *M* trifluoroacetic acid for 2 h. Paper chromatography (solvents *D* and *E*) showed the following partially methylated sugars to be present 3,4,6-tri-*O*-methyl-D-mannose (R_f 0.58, solvent *D*), 2,4,6-tri-*O*-methyl-D-mannose (R_f 0.55), 2,4,6-tri-*O*-methyl-D-galactose (R_f 0.40), 2,3,4-tri-*O*-methyl-D-glucuronic acid (R_f 0.22, solvent *E*), and traces of partially methylated aldobiouronic acid (R_f 0.28, solvent *E*). The identities of the neutral sugars were further characterized as the alditol acetates and the ratio was found to be approximately 1:1:1.

The aldotriouronic acid (R_{Gal} 0.42) had the same mobility on paper as a component obtained by partial hydrolysis of the tetrauronic acid and was not further examined.

The aldobiouronic acid (12 mg) had $[\alpha]_D$ +40° (*c* 0.62, water). A sample (2 mg) was hydrolyzed with 3 *M* hydrochloric acid for 2 h. The only neutral sugar found on paper chromatography (solvent *B*) was D-mannose. A negative result was obtained when the aldobiouronic acid (4 mg) was incubated with β-D-glucuronidase (3 mg) at p*H* 4.8 at 38° overnight. Under the same conditions the enzyme hydrolyzed 3-*O*-(β-D-glucopyranosyluronic acid)-D-galactose (from *Klebsiella* K20, ref. 8) and 6-*O*-(β-D-glucopyranosyluronic acid)-D-galactose.

When a portion (5 mg) of the aldobiouronic acid was methylated and then hydrolyzed by 3.5 *M* hydrochloric acid at 95° for 3 h paper chromatography showed the presence of 2,4,6-tri-*O*-methyl-D-mannose and 2,3,4-tri-*O*-methyl-D-glucuronic acid which had the same mobility as a standard (R_f 0.22, solvent *E*). The mannose derivative was further characterized as the alditol acetate, m.p. and mixed m.p. 64–65°.

Periodate Oxidation

The polysaccharide (0.25 g) was dissolved in aqueous sodium metaperiodate (100 ml, 0.025 *M*) and 0.36 mol of periodate per hexose unit was consumed after 3 days. Sodium borohydride (1 g) was added and the solution was left overnight. The solution was deionized with Amberlite IR 120, freeze-dried, and the product was distilled with several portions of methanol. The polyalcohol was dissolved in water (3 ml) to give a solution of p*H* 2.2 which was heated at 95° for 1 h. On paper chromatography (solvent *A*) the following compounds were identified by comparison with standards: (*a*) pyruvic acid (R_f 0.74), (*b*) glycerol (R_f 0.44), (*c*) D-threitol (R_f 0.30) (erythritol had R_f 0.32), (*d*) D-galactose (in traces only), and (*e*) an oligosaccharide which had a mobility intermediate between the aldobiouronic and the aldotriouronic acids in solvent *E*. The ratio of glycerol to D-threitol was found to be 1.2:1 by acetylation of part of the mixture and g.l.c. (column *b*, 150–180° at 2°/min). A control reaction showed that erythritol tetraacetate crystallized very easily while D-threitol tetraacetate remained as a syrup; both acetates had distinctly different retention times (13.5 and 15 min, respectively).

The oligosaccharide was obtained by precipitation into ethanol, yield 0.1 g. The product was reduced with sodium borohydride and, after removal of borate, the p.m.r. spectrum was run in D_2O (20 mg/5 ml) at 95°. One β-D- and two α-D-linkages were observed (Table 1). The signal at τ 5.47 was a doublet with a coupling constant of 8 Hz. The reduced oligosaccharide (10 ml) was dissolved in 0.5 *M* phosphate buffer (3 ml, p*H* 7.0), β-D-galactosidase (5 mg) was added, and the solution was incubated at 38° overnight. The monosaccharide liberated was D-galactose which was confirmed by g.l.c. of the alditol acetate, m.p. and mixed m.p. 160–162°.

The reduced oligosaccharide (50 mg) was methylated followed by hydrolysis with 2 *M* trifluoroacetic acid for 2 h. Paper chromatography (solvent *D*) showed, by comparison with a standard, that 2,3,4,6-tetra-*O*-methyl-D-galactose (R_f 0.68) was the major neutral sugar with traces of 2,4,6-tri-*O*-methyl-D-mannose. The tetramethylgalactitol acetate also had the same retention time as authentic material (11 min, column *a* at 200°). In solvent *E* the acidic components had the same mobilities as 2,4-di-*O*-methyl-D-glucuronic acid (R_f 0.16) and 3-*O*-(2,4-di-*O*-methyl-α-D-glucopyranosyluronic acid)-2,4,6-tri-*O*-methyl-D-mannose (R_f 0.23) previously isolated from the methylated degraded polysaccharide.

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APPENDIX II

The Structure of the Capsular Polysaccharide of

Klebsiella K-Type 24

Can. J. Chem., in press.

The Structure of the Capsular Polysaccharide of Klebsiella K-type 24

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Received.....

[Quantitative analysis of sugar constituents and partial hydrolysis were done by Dr. A.M. Zanlungo].

[Methylation analysis and periodate degradation were done by Y.M. Choy].

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Methylation, periodate oxidation and partial hydrolysis studies on the capsular polysaccharide, and on the carboxyl reduced polymer, of Klebsiella K24 show the structure to consist of a repeating unit

$$\begin{array}{l} \xrightarrow{2} \text{D-GlcAp} \xrightarrow[\alpha]{1\,3} \text{D-Manp} \xrightarrow[\alpha]{1\,2} \text{D-Manp} \xrightarrow[\alpha]{1\,3} \text{D-Glcp} \xrightarrow[\beta]{1} \\ \quad \beta\big|{}^{4}_{1} \\ \quad \text{D-Manp} \end{array}$$

The anomeric linkages were determined in isolated oligosaccharides by p.m.r. spectroscopy which also showed the presence in the polysaccharide of one O-acetyl group per 7-8 sugar residues. The O-acetyl is tentatively assigned to one of the D-mannose units.

Les méthodes de méthylation, oxydation periodique et hydrolyse partielle portées au polysaccharide capsulaire, et au polymère avec le groupe carboxyle réduit, de Klebsiella K24 ont démontré que la structure se compose d'une unité qui se répéte. Les liaisons anomères ont

$$\begin{array}{l} \xrightarrow{2} \text{D-GlcAp} \xrightarrow[\alpha]{1\,3} \text{D-Manp} \xrightarrow[\alpha]{1\,2} \text{D-Manp} \xrightarrow[\alpha]{1\,3} \text{D-Glcp} \xrightarrow[\beta]{1} \\ \quad \beta\big|{}^{4}_{1} \\ \quad \text{D-Manp} \end{array}$$

été distinguées chez des oligosaccharides par la spectroscopie r.m.n. qui a aussi mis en évidence dans le polysaccharide la présence d'un groupe O-acétyle par rapport à 7-8 sucres. On pense que le groupe O-acétyle est lié à un des restes de D-mannose.

Qualitative analyses of the capsular polysaccharides of the 80 serotypes of Klebsiella bacteria have been provided by Nimmich (1,2). In continuation of our work (3) on the structure of these materials we now report studies on the capsular polysaccharide from Klebsiella K-type 24.

The polysaccharide, purified by precipitation with Cetavlon, moved as a single band on electrophoresis on cellulose acetate and had $[\alpha]_D^{27}$ +79° and an equivalent weight (by titration) of 786. The p.m.r. spectrum of a 2% solution of the sodium salt of the polysaccharide in D_2O run at 95° showed a sharp singlet at τ 7.8 characteristic of O-acetyl and the absence of pyruvate (4,5). The anomeric region of the spectrum suggested that the repeat unit consists of five sugar units of which three are linked by α-D- and two by β-D-glycosidic bonds (5). Integration of the anomeric and acetate signals indicated one acetyl group to seven or eight sugar units. The presence of O-acetyl groups was also confirmed by formation of the hydroxamic ester (6).

Acid hydrolysis of the polysaccharide showed the rapid liberation of D-mannose and after 4 h D-mannose and D-glucose were proved to be the only neutral sugars present, in the ratio of 2.7:1. This analysis was carried out on the derived alditol acetates samples of which were collected by g.l.c. and measurement of their c.d. spectra confirmed the assignment of the D-configuration to both sugars (7).

Partial hydrolysis of the polysaccharide gave a series of acidic oligosaccharides which were separated from the neutral sugars by ion-exchange resins and individual components were obtained by paper chromatography. These were shown to be an aldobiouronic acid together

with related aldotri- and aldotetra-uronic acids. The structures of these oligosaccharides (as their alditols) are given in Table 1 together with the chemical shift of the anomeric proton(s) which enables the nature of the glycosidic linkages to be determined. The structures given were determined by (a) hydrolysis after conversion to the alditol, (b) hydrolysis after carboxyl reduction and (c) methylation as described in the experimental. The aldobiouronic acid 3-O-(α-D-glucopyranosyluronic acid)-D-mannose has been found in Klebsiella K2 and K21 and elsewhere (3).

When a sample of the fully methylated (8,9) polysaccharide was hydrolyzed and the neutral sugars were examined 2,3,4,6-tetra- and 3,4,6-tri-O-methyl-D-mannose and 2,4,6-tri-O-methyl-D-glucose were found together with a small quantity of 2,4,6-tri-O-methyl-D-mannose. An attempt to analyze this mixture quantitatively as alditol acetates was not satisfactory since on columns of ECNSS-M and butanediol succinate the 2,4,6- and 3,4,6-trimethylhexoses were not resolved. On a column of OS 138 these components gave two major peaks poorly resolved and a minor one (ca 9% of the trimethyl fraction) which was identified by its retention time and mass spectrum as 2,4,6-tri-O-methyl-D-mannose. A good separation of the main components was obtained by chromatographing the trimethylsilyl derivatives (10) of the methylated sugars. This was done using three different columns and authentic standards as shown in Table 2. Under these conditions 2,3,4,6-tetra-O-methyl-D-mannose was clearly distinguished from the D-glucose isomer but it was not possible to estimate accurately the small amount of 2,4,6-tri-O-methyl-D-mannose. The ratio of 2,3,4,6-

tetra-O-methyl-D-mannose:3,4,6-tri-O-methyl-D-mannose:2,4,6-tri-O-methyl-D-glucose was 1.1:1:1.

Alternatively it has been found (11) that methylated sugars may be separated conveniently as their acetates. When this method was employed here three main groups of peaks (A; B_1, B_2; C_1, C_2) in approximately equal proportions were obtained; B_2 was present in very small amount and was not examined further. Samples were collected of the other components, deacetylated and examined on paper. Further samples were reduced, acetylated and examined by g.l.c.-m.s. and each of the main components thus obtained was demethylated and converted to the hexitol peracetate. All of these results were consistent with the assignment of peaks A, B and C as 2,3,4,6-tetra-O-methyl-D-mannose (A), 3,4,6-tri-O-methyl-D-mannose (B_1) and 2,4,6-tri-O-methyl-D-glucose (C_1 and C_2). The methylated alditol acetate obtained from B_1 showed an additional minor peak whereas both components C_1 and C_2 gave the same single aditol acetate. This indicates that C_1 and C_2 are the two anomers of the trimethylglucose and suggests that the small amount of 2,4,6-tri-O-methyl-D-mannose has a similar retention time to the 3,4,6-isomer. It may be noted that in this instance the mannose ethers have shorter retention times than the glucose compound but for the 2,3,6-tri-O-methyl derivatives the situation is reversed (11).

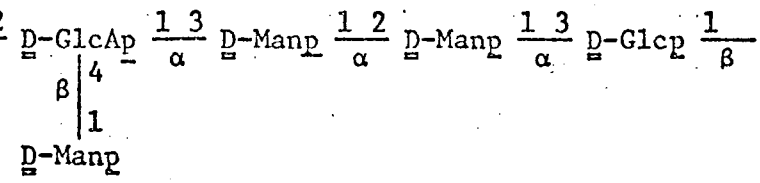
These results show the presence of a D-mannose unit substituted at position 2 and a D-glucose unit substituted at position 3 together with a D-mannose residue present as a side chain. The small amount of 2,4,6-tri-O-methyl-D-mannose undoubtedly arose by partial cleavage

of the aldobiouronic acid linkage.

Another sample of the fully methylated polysaccharide was reduced with lithium borohydride and hydrolyzed. In addition to the methylated sugars described above a new component having the mobility on paper of 3- or 4-O-methyl-D-glucose was obtained. The mixture of methylated sugars was reduced with sodium borodeuteride (12) and the new component was isolated by g.l.c. of the alditol acetates. The mass spectrum clearly identified this component as a 3-O-methyl hexitol. Furthermore the c.d. spectrum of the alditol acetate was identical in sign (negative) with that obtained from authentic 3-O-methyl-D-glucose. This establishes the D-configuration of the glucuronic acid in this polysaccharide (7).

In order to determine the position of attachment of the pendant D-mannose units to the main chain a sample of the original polysaccharide was subjected to mild hydrolysis and the recovered polymeric material was methylated, reduced with lithium borohydride and hydrolyzed. Examination of the hydrolyzate by paper chromatography showed, in addition to the compounds previously obtained, a component having the mobility of 3,4-di-O-methyl-D-glucose. This was confirmed by g.l.c. separation of the alditol acetates and mass spectrometry of the dimethyl fraction together with demethylation and acetylation to give D-glucitol hexaacetate. The ratio of the tri- to dimethyl fraction was 3.2:1. This indicates that the D-mannose side chains are joined to position 4 of D-glucuronic acid which, in turn, must be linked through position 2 in the main chain.

The methylation data in conjunction with the results of analysis of the acidic oligosaccharides enable the repeat unit of Klebsiella K24 capsular polysaccharide to be written thus:



Confirmation of this structure was sought by the Smith degradation procedure (13). Deacetylated polysaccharide was oxidized with periodate when 0.66 mole per hexose unit was consumed. The derived polyalcohol was partially hydrolyzed by trifluoroacetic acid at room temperature to give a mixture of glycerol and an oligosaccharide which was then reduced with sodium borohydride. The latter compound was methylated reduced and hydrolyzed to give 2,3,4,6-tetra- and 3,4-di-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-mannose as expected from the proposed structure; the methylated glycerol derivative was not sought.

An attempt was made to locate the position of the O-acetyl group using the procedure of de Belder and Norrman (14). The polysaccharide was dissolved in methyl sulfoxide and reacted overnight with methyl vinyl ether. Excess ether was removed *in vacuo* and the polysaccharide was treated directly with dimethsulfinyl anion and methyl iodide. When the product was hydrolyzed and the components were examined by paper chromatography a major spot for monosaccharide was obtained together with a faint spot with an R_f greater than all monomethylglucose derivatives and which was therefore assumed to be a monomethylmannose. Analysis

by g.l.c. of the hydrolyzate, as alditol acetates, suggested that there might be two monomethyl mannoses present. Even if the experiment was to be repeated on a larger scale to allow characterization of the monomethyl sugars their identification as mannose derivatives would still not permit any deduction as to which of the three structurally different mannose residues was acetylated in the original polysaccharide. For this reason and because of the low yields obtained this aspect of the structure was not pursued further. The use of methyl vinyl ether as a reagent in locating O-acetyl groups has been applied successfully to lipopolysaccharides (15) which appear to have lower molecular weights than the capsular polysaccharides of Klebsiella (M.W. 5 to 9 x 10^5). This difference in molecular weight may account for the low yield obtained in the present instance; similar difficulties have been experience with other capsular polysaccharides from Klebsiella (16). There is good evidence that these capsular polysaccharides have a true repeating unit and also that where a sugar residue is in the form of a pyruvic acid ketal (1-carboxyethylidene derivative) this feature similarly repeats regularly (5). In the case of partial acetylation the substitution pattern appears to be less well defined.

The evidence presented shows clearly that the structure of the capsular polysaccharide of Klebsiella K-type 24 is as given above with the tentative assignment of some O-acetyl groups on certain of the mannose residues.

EXPERIMENTAL

General methods are as previously described (3).

Isolation and Properties of K24 Capsular Polysaccharide

Klebsiella K24 was grown in the medium (3) as for K21 and the harvested capsular polysaccharide and cells (1 1/2 l) were diluted 5 fold and centrifuged at 27,000 r.p.m. at 20° for 45 minutes. The supernatant was collected and freeze-dried. A 1% aqueous solution of the product (1 vol.) was added with stirring to ethanol (6-7 vol.). The polysaccharide was collected, washed with acetone and air dried. Yield ca. 12 g. The crude product was purified through precipitation with Cetavlon, giving 7 g of pure acidic polysaccharide having $[\alpha]_D^{25}$ +90° (c 0.54, water) as Na^+ salt; $[\alpha]_D^{27}$ +79° (c 0.68, water) after dionization with Amberlite IR 120. Equivalent weight by titration with 0.03 N sodium hydroxide (phenolphthalein) was 786. Addition of excess sodium hydroxide and back titration with hydrochloric acid after stirring under nitrogen for 50 min showed the presence of ca. 4% O-acetyl. Electrophoresis at pH 3 and 8.8 showed the polysaccharide moved as a single component.

Anal. N , 0%; ash, 0%.

A sample of polysaccharide was exactly neutralized and the solution was lyophilized. The residue was dissolved in D_2O and exchanged twice by lyophilization. The p.m.r. spectrum of the solution (ca. 2% in D_2O) was run at 95° and showed a sharp peak at τ 7.8 and five protons in the range τ 4.5-5.5 (see Table 1).

The presence of acetate was also confirmed by methanolysis of the polysaccharide and identification of methyl acetate using the hydroxylamine-ferric chloride reagent (6,16,17).

Hydrolysis of the Polysaccharide

Polysaccharide (25 mg) was dissolved in sulfuric acid (2.5 ml, N) at room temperature (30 min) and the solution was heated on a steam bath for 4 h. The solution was neutralized ($BaCO_3$) and the filtrate was evaporated. On paper chromatography in solvents A and B spots corresponding to D-glucose, D-mannose and D-glucuronic acid were observed. The hydrolyzate was separated into neutral and acidic fractions using ion-exchange resins and the neutral fraction was found to contain D-mannose and D-glucose in an approximate ratio of 2.7:1 as determined by the g.l.c. of the alditol acetates (column a); D-mannitol hexaacetate had m.p. and mixed m.p. 121.5-122° and D-glucitol hexaacetate had m.p. and mixed m.p. 98.5-99°.

Partial Hydrolysis of the Polysaccharide

The polysaccharide (1.0 g) was dissolved in trifluoroacetic acid (TFA, 100 ml, 2M) and the solution was heated on a steam bath for 4 h. The solution was evaporated, dissolved in 5 ml of water and evaporated again. The resulting syrup was separated by ion-exchange resins into neutral and acidic (eluted with 4 M HOAc) fractions.

The latter was concentrated (300 mg) and chromatography in solvent A showed four components: (a) D-glucuronic acid, (b) aldobiouronic acid ($R_{glucose}$ 0.43), (c) aldotriouronic acid ($R_{glucose}$ 0.13) and

(d) aldotetrauronic acid ($R_{glucose}$ 0.04). The syrup was separated by preparative paper chromatography on Whatman 3 mm paper in solvent C for 80 h. Yields: (a) aldobiouronic acid, 130 mg; (b) aldotriouronic acid 50 mg; (c) aldotetrauronic acid 25 mg.

Structural Analysis of Aldobiouronic Acid

(a) Anomeric linkage

A sample of aldobiouronic acid (25 mg) was dissolved in water (1 ml) and reduced with sodium borohydride (25 mg) for 8 h. After workup and removal of borate, the syrup was dissolved in D_2O (2 ml) and freeze-dried. P.m.r. of this product (in D_2O) showed a doublet at τ 4.85, $J \sim 3.5$ Hz, indicating an α-linkage in the disaccharide.

(b) Reducing end

A portion of the above reduced aldobiouronic acid was hydrolyzed with trifluoroacetic acid (2 M) at 100° for 1 h. Uronic acids were removed with anion exchange resin and the neutral fraction was concentrated and acetylated. G.l.c. (column a) gave mannitol hexaacetate m.p. 122°.

(c) Carboxyl reduction and hydrolysis of the disaccharide

Aldobiouronic acid (20 mg) was dissolved in anhydrous methanol (15 ml) containing 1% hydrogen chloride and the solution was left 12 h at room temperature. The solution was neutralized (Ag_2CO_3), centrifuged, and evaporated. The resulting syrup was dissolved in the minimum amount of water, followed by the addition of methanol and the solution was added dropwise over 1 h to a stirred solution of sodium borohydride (25 mg) in a mixture of water (0.5 ml) and methanol (2 ml). The

solution was left 12 h at room temperature and was then carefully neutralized and stirred with Amberlite IR-120 (H^+) resin until the solution was free of Na^+. A portion of the syrup, after removal of borate, was hydrolyzed with trifluoroacetic acid (2 M) at 100° for 1 h and was shown (solvent A) to give D-glucose and D-mannose.

(d) Methylation

Aldobiouronic acid (12 mg) was methylated according to the procedure of Hakomori and was hydrolyzed (HCl, 3.5 N) to give 2,4,6-tri-O-methyl-D-mannose (R_f 0.55, solvent D) and 2,3,4-tri-O-methyl-D-glucuronic acid (R_f 0.22, solvent E). The former compound was also confirmed by g.l.c.-m.s. of the alditol acetate.

Structure of Aldotriouronic Acid

(a) Anomeric linkage

A sample of aldotriouronic acid (20 mg) was reduced and dissolved in D_2O. The signals at τ 4.7, J = 3.5 Hz (from D-glucuronic acid) and τ 5.0, J = 1.5 Hz indicate α-linkages (Table 1).

(b) Reducing end

Hydrolysis of the reduced aldotriouronic acid showed D-mannitol (paper, solvent B and g.l.c.) and the same aldobiouronic acid (paper, solvents A and C).

(c) Methylation

The methyl ester methylglycoside (10 mg) was methylated and on hydrolysis showed (solvent D) two overlapping spots corresponding to 3,4,6- and 2,4,6-tri-O-methyl-D-mannose (R_f 0.58 and 0.55, respectively). G.l.c. of the trimethylsilyl derivatives on column a at 135° and on

column b at 120° confirmed these assignments and showed them to be present in approximately equimolecular amounts.

Structure of Aldotetrauronic Acid

(a) Anomeric linkage

A portion of the aldotetrauronic acid (15 mg) was reduced as for the aldobiouronic acid. The p.m.r. spectrum in D_2O showed the following signals: τ 4.70, J = 3.5 Hz and τ 4.73, J = 3.0 Hz (2H); τ 4.95, J = 1.5 Hz (1H).

(b) Reducing end

A portion of the reduced aldotetrauronic acid was hydrolyzed and paper chromatography (solvent B) showed D-glucitol, confirmed by g.l.c. of the alditol acetate. Also produced were the same aldobi- and aldotriouronic acids (solvent C) described above.

Methylation

Dry polysaccharide (1.7 g) was dissolved in anhydrous methyl sulfoxide (300 ml) under nitrogen (8,9). To this solution methyl sulfoxide anion (26 ml, 2 M) was added and the mixture was stirred for 9 h under nitrogen. Methyl iodide (15 ml) was added at such a rate as to keep the temperature below 20° and the resulting clear solution was stirred for 10 h. The solution was dialyzed against running water for 2 days and concentrated to a syrup which was methylated eight times with Purdie's reagents to give a product showing no absorption at 3600 cm^{-1}, $[\alpha]_D^{20}$+64.5° (c 1.2, chloroform), OMe, 43.2%. Purification on a silica gel column using chloroform-methanol (95:5) raised the methoxyl content to 44.3%.

Methylated polysaccharide (60 mg) was dissolved in trifluoroacetic acid (2 M) and the solution was refluxed for 5 h. Separation on ion-exchange resins gave a neutral fraction which showed on paper chromatography (solvent D) three major components corresponding to 2,3,4,6-tetra- and 3,4,6-tri-O-methyl-D-mannose, 2,4,6-tri-O-methyl-D-glucose (R_f values 0.80, 0.58 and 0.50) and a minor component 2,4,6-tri-O-methyl-D-mannose (R_f 0.55). A portion (10 mg) of the neutral fraction was reduced, acetylated and examined on column a programmed from 170-194° at 2°/min. Two peaks in the ratio of 1:1.8 were observed at 25.1 and 36.6 min. The faster component was tentatively identified by its retention time and mass spectrum as 2,3,4,6-tetra-O-methyl-D-mannose. The mass spectrum of the slower material showed it to be a mixture of 2,4,6- and 3,4,6-trimethylhexoses which also did not resolve on column b. On a column of OS-138 15% on Gas Chrom Q) operated isothermally at 240°. The slower moving fraction was shown to contain one minor and two major components. The minor component crystallized m.p. 63-66° (3) and was further identified as 2,4,6-tri-O-methyl-D-mannitol triacetate* by mass spectrometry; the main peaks were not sufficiently well resolved to permit identification.

Resolution of the trimethylhexose fraction was obtained when the trimethylsilyl derivatives of the neutral sugars were run on columns a, b, and c (samples injected in hexane, see Table 2).

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* Correct nomenclature: 1,3,5-tri-O-methyl-D-mannitol triacetate.

When a portion (40 mg) of the neutral methylated sugars was acetylated (pyridine-acetic anhydride 1:1, 100°, 15 min), extracted with chloroform and injected onto column a at 170° the following peaks were obtained: A (5.8 min); B_1 (8.8), B_2 (10.5); C_1 (16.2), C_2 (17.6). The component B_2 was present in only small amount and this, together with a minor component with a retention time of 7.5 min, was not examined further. Individual fractions were deacetylated (NaOMe, 1 N in MeOH, 25°, 30 min), deionized and examined on paper in solvent D. Components A, B_1, C_1 and C_2 had R_f values of 0.79, 0.57, 0.49 and 0.49. Samples of the individual fractions were also reduced with sodium borohydride, acetylated and examined on column b at 175°. Components A', B_1', C_1' and C_2' were eluted at 7.8, 14.6, 15.8 and 15.8 min, each was examined by mass spectrometry and each was demethylated and the product acetylated. D-Mannitol hexaacetate m.p. 120-122° was obtained from A' and B_1' while C_1' and C_2' yielded D-glucitol hexaacetate m.p. 96-97°.

Reduced Methylated Polysaccharide

Part (100 mg) of the fully methylated polysaccharide was reduced overnight with lithium borohydride in tetrahydrofuran and the product was hydrolyzed by refluxing for 2 h with trifluoroacetic acid (2 M). Paper chromatography in solvent D revealed the presence of an extra

sugar having the mobility (R_f 0.08) of a monomethylhexose which was shown in Solvent E to have the same characteristics as 3- or 4-O-methyl-D-glucose. A portion (30 mg) of the hydrolyzate was reduced with sodium borodeuteride, acetylated and separated on column b. Mass spectrometry of the component having a retention time of 38 min showed it to be a 3-O-methylhexitol and the c.d. spectrum was identical with that from 3-O-methyl-D-glucitol pentaacetate ($\Delta\varepsilon_{213}^{MeCN}$ -0.29).

Methylated Degraded Polysaccharide

Capsular polysaccharide (1 g) was hydrolyzed overnight at 95° with trifluoroacetic acid (0.4 M) using the apparatus of Galanos and co-workers (18). The recovered polysaccharide (200 mg) was methylated, reduced ($LiBH_4$) and hydrolyzed (TFA, 2 M). Paper chromatography showed the presence of a new sugar having the mobility of 3,4-di-O-methyl-D-glucose (R_f 0.26, solvent D; 0.51, solvent E). This was confirmed by the mass spectrum of the dimethyl fraction, separated as alditol acetates on column a, and by conversion of this fraction to D-glucitol hexaacetate m.p. 98°. The c.d. curve of 3,4-di-O-methyl-D-glucitol tetraacetate is weakly negative.

Smith Degradation

Capsular polysaccharide was deacetylated with aqueous sodium hydroxide, dialyzed and lyophilized. The product (130 mg) was dissolved in sodium metaperiodate (20 ml, 0.05 M) and after 3 days in the dark at 4° 0.66 mole of periodate had been consumed per sugar unit. Following

the addition of ethylene glycol, dialysis, reduction with sodium borohydride, dialysis, deionization, lyophilization and removal of borate the product was hydrolyzed (TFA, 0.5 M) at room temperature for 8 h. Paper chromatography in solvent A showed mainly glycerol and an oligosaccharide with traces of glucose. A part of the hydrolyzate was acetylated and gave a peak corresponding to glycerol triacetate (4 min) on column b at 150°. The majority of the hydrolyzate was reduced ($NaBH_4$) and paper chromatography (solvent A) showed one component having $R_{galactose}$ 0.37 and glycerol.

The mixture containing the oligosaccharide was methylated (8,9), reduced and hydrolyzed to give (solvent D) components equivalent to 2,3,4,6-tetra- and 3,4-di-O-methyl-D-glucose (R_f 0.80, 0.28) and 2,4,6-tri-O-methyl-D-mannose (R_f 0.56). This result was confirmed by g.l.c.-.m.s. of the alditol acetates (column a, 160-200° at 2°/min) which had retention times of 14.5, 30 and 21 min, respectively.

Attempted Location of O-Acetyl Group

Capsular polysaccharide (80 mg) was dissolved in methyl sulfoxide (20 ml) and anhydrous p-toluene sulfonic acid (ca. 20 mg) was added. Methyl vinyl ether was passed into the solution until it turned yellow and the reaction was left overnight. Excess methyl vinyl ether was removed on a rotary evaporator and methyl sulfoxide anion was added directly without isolation of the polysaccharide. The solution was shaken for 20 h, methyl iodide (5 ml) was added and the shaking continued for 3 h. The solution was dialyzed against running water, polymeric material formed from methyl vinyl ether was extracted with chloroform

and the aqueous portion was lyophilyzed. Hydrolysis (TFA, 2 M, 100°) showed (solvent E) a major spot for monosaccharides together with a faint spot moving faster than all monomethylglucose derivatives (R_f 0.35). G.l.c. of the alditol acetates gave two small peaks at 21 and 23 min (column b, 190°) in addition to hexitol acetates.

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TABLE 1. P.m.r. data on Klebsiella K24 capsular polysaccharide and derived oligosaccharides.

Repeating unit of compound	τ-Value* (coupling constant, Hz)	Ratio of integrals	Proton assignment (all sugars have D-configuration)
GlcA $\frac{1\ 3}{\alpha}$ Mannitol	4.85 (3.5)	1	α-GlcA $\frac{1\ 3}{}$ Mannitol
GlcA $\frac{1\ 3}{\alpha}$ Man $\frac{1\ 2}{\alpha}$ Mannitol	4.7 (3.5)	1	α-GlcA $\frac{1\ 3}{}$ Man
	5.0 (1.5)	1	α-Man $\frac{1\ 2}{}$ Mannitol
GlcA $\frac{1\ 3}{\alpha}$ Man $\frac{1\ 2}{\alpha}$ Man $\frac{1\ 3}{\alpha}$ Glucitol	4.7 + 4.73	2	α-GlcA + α-Man $\frac{1\ 3}{}$ Man; α-Man $\frac{1\ 3}{}$ Glucitol
	4.95 (1.5)	1	α-Man $\frac{1\ 2}{}$ Man
$\frac{\cdot 2}{}$ GlcA $\frac{1\ 3}{\alpha}$ Man $\frac{1\ 2}{\alpha}$ Man $\frac{1\ 3}{\alpha}$ Glc $\frac{1}{\beta}$ (β 4–1 Man)	4.6	2	α-GlcA + α-Man $\frac{1\ 3}{}$ Man; α-Man $\frac{1\ 3}{}$ Glc
	4.95 (1.5)	1	α-Man $\frac{1\ 2}{}$ Man
	5.27 (1.0)	1	β-Man $\frac{1\ 4}{}$ GlcA
	5.31 (6.0)	1	β-Glc $\frac{1\ 2}{}$ GlcA

* Spectra run in D_2o with external tetramethylsilane (τ= 10) at 100 MHz).

TABLE 2. Per(trimethylsilyl) derivatives of methylated sugars from *Klebsiella* K24 capsular polysaccharide

Compounds	Columns (a) ECNSS-M	(b) BDS	(c) SE-52
	Retention times (min)		
Methylated aldoses of K24	14.4	4.6	17.6
	19.8	7.8	16.6
	21.4	11.3	24.1
3,4,6-Me_3-Man	14.4	4.6	17.6
2,4,6-Me_3-Glc	19.8	7.8	24.1
2,3,4,6-Me_4-Man	21.4	11.3	16.6
2,3,4,6-Me_4-Glc	15.4 (20%)	7.4 (15%)	
	17.0 (80%)	8.2 (85%)	

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