STRUCTURAL INVESTIGATION OF
BACTERIAL CAPSULAR POLYSACCHARIDES

by

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Eighty strains of *Klebsiella* bacteria have been isolated and serotyped according to their capsular polysaccharide antigens (K antigens). A number of research groups have taken part in an extensive program to determine the chemical structures of these polysaccharide antigens. As part of this continuing program, this thesis includes a structural investigation of the capsular polysaccharide of *Klebsiella* K39.

An acidic capsular polysaccharide was isolated from *Klebsiella* K39 and a partial hydrolysis study was conducted. An acidic pentasaccharide was isolated from the hydrolysate and studied by $^1$H- and $^{13}$C-n.m.r., mass spectrometry and methylation analyses. This oligosaccharide was assigned the following structure:

\[
\text{GlcA}^{12}\beta\text{Man}^{4}\alpha\text{GlcA}^{12}\beta\text{Man}^{3}\alpha\text{Glc-OH}
\]

The relationship of this oligosaccharide to the polysaccharide will be discussed with reference to n.m.r. and methylation analysis studies.

Similar studies are being conducted to determine the structures of the *E.coli* capsular polysaccharide antigens. This thesis includes a preliminary study of *E.coli* K26.
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Special appreciation is extended to Rani Theeparajah for typing this thesis.
For readers not familiar with carbohydrate nomenclature, an explanation of terms, reproduced from the M. Sc. thesis of T.E. Folkman, has been included in Appendix V.
I. INTRODUCTION
I. INTRODUCTION

A polysaccharide is a polymeric substance composed of monosaccharides joined together by glycosidic linkages. These carbohydrate polymers are ubiquitous in nature and have a number of important biological functions. Many polysaccharides have commercial value and their role in industry continues to expand. Researchers are pursuing structural studies in the hope that the chemical basis for physical and biological properties may be better understood.

Natural polysaccharides show a wide range of relative complexity with significant variations in such features as the number of different sugars, the presence or absence of non-carbohydrate substituents, and the degree of branching. There is variety in the degree of structural regularity with which organisms produce polysaccharides; for example, some bacteria synthesize complex polymers with precisely defined repeating units, while plant gums have more random structures. Listed below are several polysaccharides from representative sources. This list is by no means exhaustive but it does highlight some important features of polysaccharide structures.

Cellulose:

\[
\text{Glc} \left( \frac{1}{\beta} \left( 4 \text{ Glc} \frac{1}{\beta} \right)_n \right) \text{Glc-OH}
\]

This glucose homopolymer is the main component of plant cell walls. Its importance in the textile and paper products industries is well known.
Amylose:

\[ \text{Glc} \frac{1}{\alpha} \left( \frac{4}{\text{Glc}} \frac{1}{\alpha} \right)_n \text{Glc-OH} \]

Amylose is an important component of starch and is a food storage material in plants. Even though it is a glucose homopolymer like cellulose, its \( \alpha 1—4 \) linkages are much more easily hydrolysed than the \( \beta 1—4 \) linkages of cellulose.\(^1\)

Heparin:

The polysaccharide shown above was isolated from beef lung tissue and consists of alternating residues of \( \alpha \)-L-idopyranuronic acid 2-sulfate and 2-deoxy-2-sulfamino-\( \alpha \)-D-glucopyranose 6-sulfate. Heparin is a structural component of some animal tissues.\(^2\)

Gum Tragacanth:

\[
\begin{array}{ccccccc}
\text{GalA} & \frac{1}{\alpha} & \text{GalA} & \frac{1}{\alpha} & \text{GalA} & \frac{1}{\alpha} & \text{GalA} \\
\beta & 4 & \beta & 3 & \beta & 3 & \beta \\
\text{Xyl} & 1 & \text{Xyl} & 1 & \text{Xyl} & 2 & \text{Gal} \\
\alpha & 1 & \alpha & 1 & \alpha & 1 & \\
\text{Fuc} & & & & & & \\
\end{array}
\]
Many trees and shrubs exude complex acidic polysaccharides or gums. Because of their complexity it is only possible to propose partial or average structures.

Capsular Polysaccharide from *Klebsiella* K21:

\[
\begin{array}{c}
\text{GlcA} \quad \frac{3}{\alpha} \\
n \\
\text{Man} \quad \frac{1}{\alpha} \\
\text{Man} \quad \frac{2}{\alpha} \\
\text{Gal} \quad \frac{3}{\alpha} \\
\text{Gal} \quad \frac{1}{\beta} \\
\text{CH}_3 - \text{C} - \text{CO}_2\text{H}
\end{array}
\]

Many species of bacteria produce acidic polysaccharides that are relatively complex but have regular repeating units. The concept of precisely defined oligosaccharide repeating units in bacterial polysaccharides is now well established by nuclear magnetic resonance, partial hydrolysis and phage degradation studies.

This thesis includes structural studies of capsular polysaccharides from strains of *Klebsiella* and *Escherichia coli* bacteria. The relationship of the capsular polysaccharide to the rest of the bacterial cell is given in Figure 1. The capsular polysaccharide has the same chemical composition as the exocellular slime that is exuded by the bacteria. It is also known as the K antigen, an antigen being a substance that induces the production of antibodies. (The term K antigen has a precise immunological definition but it is usually a capsular polysaccharide).

The *Klebsiella* are Gram-negative bacteria of the family Enterobacteriaceae, the genus *Klebsiella*, and the species *Klebsiella*
pneumoniae, Klebsiella ozaenae and Klebsiella rhinoscleromatis, Klebsiella may cause infections of the respiratory and urinary tracts. Approximately eighty strains, as distinguished by their K antigens, have been isolated and serotyped. Heidelberger has studied the immunochemical reactions of the Klebsiella capsular polysaccharides. These polysaccharides were reacted with antibodies prepared using the Pneumococcal polysaccharides, many of which have known chemical structures. The degree of precipitation was measured and conclusions were drawn concerning the relationship between chemical structure and immunochemical reactivity. A number of research groups have embarked on structural studies of the Klebsiella capsular polysaccharides in order to complete the understanding of this work.

Nimmich has performed qualitative analyses of the sugars present in the Klebsiella capsular polysaccharides. His results are tabulated in Appendix I. Along with acidic sugars (e.g., GlcA, GalA) and neutral sugars (e.g., Glc, Gal, Man, Fuc, Rha), pyruvate, acetyl, and formyl substituents may also be present. All of these polysaccharides contain either an acidic sugar or a pyruvic acid substituent. Di Fabio has tabulated the known structures of Klebsiella capsular polysaccharides.

The Escherichia coli (E. coli) belong to the family Enterobacteriaceae, the genus Escherichia and the species E. coli. E. coli was first isolated by Theodor Escherich from feces in 1885. Most strains of E. coli are harmless and occur naturally in the large bowel of humans and other vertebrates. Some strains are pathogenic and cause infectious diarrhea, an affliction that contributes to
Figure 1. Cell wall structure in Gram-negative bacteria.
infant mortality in underdeveloped areas of the world. 

Ørskov and coworkers have reviewed the serology, chemistry and genetics of the O and K antigens of the E. coli. The K antigens consist almost exclusively of acid polysaccharides, although K88 is known to be a protein. Many structures have been published, including one that contains 10 sugars in a repeating unit (K85). The review article mentioned above contains the qualitative sugar analyses of many of the K antigens. Along with acidic, amino, and neutral sugars, threonine (K54) and pyruvate may also be present. Structural studies of the K antigens continue in order to further the understanding of the chemical basis for immunology.

The significance of continued studies of bacterial polysaccharides goes beyond the immediate applications to bacterial immunology. The extensive study of bacterial polysaccharides has provided a rich supply of oligosaccharides by partial hydrolysis and through the use of phage-induced enzymes. A wide range of spectroscopic and chromatographic techniques have been applied to these compounds. Studies of biologically active oligosaccharides have contributed greatly to the understanding of important biomedical problems such as blood-group immunology. As chemists develop their understanding of the spectroscopic and chromatographic properties of these compounds, it is more probable that advanced analytical techniques will be skilfully applied to complex problems involving carbohydrates.

Sections II and III complete the introduction to this thesis. Section II describes the "Chromatographic and Instrumental Methods" that are used in the study of polysaccharides and other carbohydrates.
Section III, entitled "Polysaccharide Structural Methods," includes methods of obtaining oligosaccharides and derivatised monosaccharides that provide information about the polysaccharides from which they are derived.
II CHROMATOGRAPHIC AND INSTRUMENTAL METHODS
II CHROMATOGRAPHIC AND INSTRUMENTAL METHODS

II.1 Paper Chromatography

Paper chromatography was introduced in 1944 by Consden and coworkers who used it to separate amino acids. In 1947 Partridge applied this method to sugars. The arrangement of fibres in chromatography paper provides a series of channels through which a solvent may flow by capillary action. The paper consists of highly ordered regions called crystallites and areas of lesser order called the amorphous regions. It is in these amorphous regions that bound water acts as a stationary liquid phase. The solute is partitioned between this cellulose-water complex and the mobile solvent as it travels through the paper. The paper may be irrigated by the descending, ascending, horizontal or radial methods. The descending mode of paper chromatography was used in the present study.\(^\text{15}\)

Paper chromatography is a simple and effective method for the qualitative analysis and isolation of mono- and oligosaccharides in polysaccharide hydrolysates. Excellent separations may be obtained by a judicious choice of solvent system. In this study, the following solvent systems were used for the purposes given.

Solvent A: Ethyl acetate-acetic acid-formic acid-water (18:3:1:4). This solvent system gives relatively rapid development of acidic and neutral mono- and oligosaccharides. The resolution obtained is not sufficient for the separation of some sugars.

Solvent B: 1-Butanol-acetic acid-water (2:1:1). This solvent system is used to develop acidic and neutral mono- and oligosaccharides.
Development is slower than with Solvent A, but the resolution of oligosaccharides is increased. This system is used for preparative separations of oligosaccharides.

Solvent C: Ethyl acetate - pyridine - water (8:2:1). This solvent system gives excellent resolution of neutral monosaccharides; glucose, galactose and mannose can be separated and identified with this system. Acidic sugars remain essentially at the origin.

Solvent D: 1-Butanol - ethanol - water (4:1:5) (upper phase). On mixing the above solvents, two phases appear and the upper phase is used for paper chromatography. This solvent system gives excellent separation of partially methylated sugars. This method complements gas liquid chromatography in the identification of the partially methylated sugars obtained during methylation analyses of polysaccharides.

A number of methods are available for visualising the chromatograms. The method is chosen according to the circumstances. The following methods were used in this study.

Method A: The chromatogram is successively dipped in solutions of silver nitrate, sodium hydroxide and sodium thiosulfate. This method is very sensitive and is usually applied to reducing sugars.

Method B: p-Anisidine hydrochloride spray. Although this method is less sensitive than Method A, the colours of the spots obtained may give useful information. Dutton and Merrifield used p-anisidine hydrochloride to visualise a series of acylated oligosaccharides obtained from a polysaccharide hydrolysate. They showed that the colours varied dramatically according to the presence or absence of acetate and formate. This method is also used to visualise partially
methylated sugars. Again the colours obtained are significant and imply structural features. This method is usually applied only to reducing sugars but it may visualise such sugars as sucrose that have very labile glycosidic bonds.

The chromatographic mobilities of sugars are usually reported as R values, that is the mobility relative to an appropriate standard. R values have been published for a number of mono- and oligosaccharides in a variety of solvent systems.\textsuperscript{16} R values have also been published for a large number of partially methylated sugars.\textsuperscript{18}

II.2 Gel Chromatography

Gel chromatography (also known as gel permeation chromatography, exclusion chromatography, or molecular sieve chromatography) separates molecules according to their molecular size. The typical resin is a cross-linked polymer with a particular range of pore sizes. During the chromatographic experiment, molecules that are larger than the pores pass directly through the column without entering the resin. Smaller molecules are retained to a degree dependent on their ability to enter the porous resin. If an appropriate resin is chosen for a particular mixture, excellent separations may be obtained.\textsuperscript{19}

Gel chromatography has been used extensively for analytical and preparative separations of carbohydrates. In polysaccharide studies it has been used to isolate oligosaccharides from partially hydrolysed polymers. The resins chosen were designed for elution with aqueous solvents. During a partial hydrolysis study of \textit{Klebsiella} K53, Paulin was able to separate di-, tri-, tetra- and pentasaccharides using the
During a phage degradation study of \textit{Klebsiella} K60, Di Fabio separated saccharides containing 7, 14 and 21 sugars using Bio-Gel P-4.\textsuperscript{14}

Gel chromatography may also be used to determine the approximate molecular weight of a polymer. Molecular size and shape determine the degree of retention on the column. Thus if the molecular weight of a polysaccharide is to be measured, the column must be calibrated with polymers of similar composition and structure. Gel chromatography gives polysaccharide molecular weights that agree to within 5-10\% of values obtained by other methods.\textsuperscript{19} Churms and Stephen used this method to determine molecular weight distribution during partial hydrolysis studies of \textit{Klebsiella} polysaccharides.\textsuperscript{6}

Sephadex LH-20 is a lipophilic resin that was designed for elution with organic solvents. In this study permethylated polysaccharides were purified on LH-20 during methylation analyses.

\textbf{II.3 Gas Liquid Chromatography}

During structural studies of poly- and oligosaccharides, it is necessary to separate, identify and quantify the sugars obtained after total hydrolysis. It is also necessary to perform similar analyses of the partially methylated sugars obtained from hydrolysates of permethylated polymers and oligomers. Gas liquid chromatography (g.l.c.) is an effective and popular method for accomplishing these objectives.

Because of their low volatilities carbohydrates must be derivatised before they can be analysed by g.l.c. Sweeley and
coworkers\textsuperscript{21} made a significant contribution to this work when in 1963 they reported the successful g.l.c. analysis of carbohydrates as their trimethylsilyl (TMS) ethers. TMS derivatives are prepared rapidly at room temperature in a solution containing pyridine, hexamethyldisilazane and trimethylchlorosilane. They applied this method to a number of monosaccharides and oligosaccharides, up to a tetrasaccharide. This method has been used extensively to analyse mixtures of sugars derived from biological material.\textsuperscript{22}

Other cyclic derivatives of monosaccharides include acetates and trifluoroacetates. Methyl glycosides have been analysed as acetates, trifluoroacetates and methyl ethers. Chromatograms of cyclic derivatives of hexoses and pentoses are complicated by the occurrence of α and β anomers and pyranose and furanose ring forms. This problem has been remedied by the introduction of acyclic derivatives that give only one peak on a chromatogram. The removal of the anomeric centre is usually achieved by reduction to the alditol with sodium borohydride. Alditols have been analysed as TMS ethers but acetates give better resolution if an appropriate liquid phase is chosen. Alditols are readily acetylated by heating at 100° for 20 minutes in 1:1 pyridine–acetic anhydride.\textsuperscript{23}

Anomeric centres may also be removed by conversion of aldoses to aldononitriles. Lance and Jones\textsuperscript{24} prepared peracetylated aldononitriles (PAANs) of sugars, via their oximes, by treating them with hydroxylamine hydrochloride in pyridine at 90° for one hour, followed by addition of acetic anhydride and heating for another hour. Recently Chen and McGinnis\textsuperscript{25} reported that PAAN derivatives could be prepared
in 10 minutes using 1-methylimidazole as a solvent catalyst instead of pyridine. They also reported excellent g.l.c. separations of common sugars as PAANs.

Because PAANs are quickly and simply prepared, and are readily separated, it had appeared that they could replace alditol acetates as the derivatives of choice for the analysis of polysaccharide hydrolysates. A recent study, however, has shown that when the derivatisation procedure of Lance and Jones is used, a significant amount of a cyclic derivative is produced. For glucose, the actual yield of the PAAN derivative is only 70%. No similar side-reactions have yet been reported for the method of Chen and McGinnis, but a thorough scrutiny of this method is required.

Partially methylated sugars have been analysed as acetates and TMS ethers, and as the acetates and TMS ethers of methyl glycosides. Again chromatograms are simplified by using methods that remove the anomeric centre. Aldononitrile acetates give one g.l.c. peak, are readily separated, and have interpretable mass spectra. Alditol acetates, however, have gained the widest popularity. Partially methylated alditol acetates (PMAAs) give one peak on the chromatogram and show sufficient separation in most cases. They have been studied extensively by g.l.c./mass spectrometry, and an exhaustive summary of relative retention times and standard mass spectra has been published. Recently a computer program has been developed for the identification of PMAAs using data obtained by g.l.c./mass spectrometry.

Several liquid phases were used during the present structural study. SP-2340 was used for the separation of alditol acetates. Both
PMAAs and PAANs were separated on OV-225. HIEFF-1B (diethylene glycol succinate) was used to separate PMAAs. 1,2,5-Tri-O-acetyl-3,4,6-tri-O-methylmannitol and 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylglucitol are not separated on OV-225 or ECNSS-M, but on HIEFF-1B they are separated to a degree sufficient for identification by g.l.c./mass spectrometry.

II.4 High-Performance Liquid Chromatography (h.p.l.c.)

This analytical technique was not used in this structural study but a discussion is included here because of its potential in more advanced approaches to carbohydrate analysis. Several columns are available for the separation of underivatised mono- and oligosaccharides. H.p.l.c. has been recommended as a method for routine carbohydrate analysis because one is able to avoid the derivatisation step required by g.l.c.

In polysaccharide studies h.p.l.c. has been used most creatively to separate alkylated carbohydrates. Lindberg and coworkers used h.p.l.c. to isolate a fully methylated oligosaccharide during a uronic acid degradation study. Albersheim and coworkers have developed a method for the analysis of the partial hydrolysis products of permethylated polysaccharides. After the hydrolysis step, the mixture of partially methylated oligosaccharides is reduced and ethylated, thus derivatising all free hydroxyl groups. The hydrolysate is then analysed by reversed-phase h.p.l.c. with a differential refractometer as a detector. Analytical separations have been reported with 20 μg of starting material, and preparative separations with 10 mg.

A recent paper reports the use of h.p.l.c./mass spectrometry in
the analysis of derivatised oligosaccharides.  

II.5  Ion-exchange Chromatography

DEAE-Sephadex, a weakly basic anion exchanger, may be used to purify polysaccharides or to check their purity. An automated ion-exchange system is available for the analysis of neutral monosaccharides. During partial hydrolysis studies of polysaccharides, the separation procedure is simplified by an initial separation of neutral and acidic sugars. A suitable resin for this purpose is Bio-Rad AG 1-X2 (formate form), which has quarternary ammonium active sites. After application to the column, neutrals are eluted with distilled water. Acidics are subsequently eluted with 10% formic acid.  

II.6  Silica Gel Chromatography

Underivatised sugars can be separated by silica gel thin layer chromatography. In polysaccharide structural studies, silica gel chromatography (thin layer and column) is most useful in the isolation of derivatised oligosaccharides. Mackie used silica gel column chromatography to separate two tetrasaccharides, as the permethylated alditols, that could not be separated by gel permeation chromatography. Choy used thin layer chromatography to isolate partially methylated oligosaccharides from a permethylated polysaccharide hydrolysate. Van Halbeck and coworkers used short silica gel columns to purify permethylated oligosaccharide alditols for mass spectrometry. The Hakomori methylation is the standard method by which oligosaccharides are derivatised for mass spectrometry. The present study showed that
oligosaccharides methylated by this procedure should be purified by thin layer or column chromatography before mass spectrometric analysis.

II.7 Mass Spectrometry

Mass spectrometry (m.s.) is one of the most powerful tools available to the structural carbohydrate chemist, especially when it is combined with g.l.c. Because carbohydrates have low volatilities and low thermal stabilities, mass spectral analyses are usually performed with volatile derivatives. When ionized by electron impact (e.i.) the molecular ions of carbohydrate derivatives are weak or non-existent. Field ionization, field desorption and chemical ionization are more likely to give the molecular ion, but these techniques have the disadvantage of being "non-routine" in most laboratories.

During polysaccharide studies, mass spectral analyses are usually performed on partially methylated alditol acetates and permethylated oligosaccharides. The salient features of their mass spectra will now be discussed.

a) Partially methylated alditol acetates (PMAAs):

The mass spectra of various monosaccharide derivatives have been studied. These derivatives include permethylated glycosides, alditol acetates and alditol trifluoroacetates. PMAAs are the products of the methylation analysis of poly- and oligosaccharides (see Section III.3). G.l.c./m.s. of PMAAs has become a routine method in structural carbohydrate chemistry. 40,41

When a PMAA is ionized by electron impact, the molecular ion
is not observed. The degree of methylation is implied by the g.l.c. retention time and the substitution pattern is deduced from the mass spectrum. In this case stereoisomers are not differentiated by m.s. Figure 2 illustrates how m.s. can be used to distinguish a 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylhexitol from a 1,2,5-tri-O-acetyl-3,4,6-tri-O-methylhexitol. Note that in the first case m/e 117 is very strong, but in the second case it is not observed.28

Primary fragments are formed by fission between carbon atoms in the alditol chain. The following scheme demonstrates the relative importance of various modes of fission along the alditol chain.

(a) is favoured over (b); (a) and (b) are favoured over (c) and (d). Secondary fragments are formed from the primary fragments by the elimination of such compounds as formaldehyde, methanol, ketene and acetic acid.40
Figure 2. Mass spectrometry of partially methylated alditol acetates
(b) Permethylated oligosaccharides:

Oligosaccharides have been studied extensively, both as permethylated methyl glycosides and as permethylated alditols, and it has been demonstrated that mass spectrometry can be used to determine the sugar sequence and linkage positions. Kochetkov and Chizhov, followed by Kovacik and coworkers, developed a nomenclature for the fragment ions of permethylated glycosides. Kovacik and coworkers studied an aldotriouronic acid by m.s., and their conclusions were used to interpret the mass spectrum of an oligosaccharide isolated from Klebsiella K39. Figure 3 illustrates some of their more important results, specifically the origins of the A and J series of fragments. These fragments provide information about the sequence of sugars in the oligosaccharide.

Karkkainen studied 21 trisaccharides by g.l.c./m.s., as the permethylated methyl glycosides and as the permethylated alditols. His papers give retention times and detailed analyses of the mass spectra. He showed that the linkage position could be determined by mass spectrometry.

Two recent papers demonstrate the use of mass spectrometry in the structure determination of naturally occurring oligosaccharides. During a study of hog submaxillary glycoproteins, Van Halbeek and coworkers isolated a series of oligosaccharides, up to a pentasaccharide, containing N-acetyl-galactosamine, galactose, fucose, and N-glycoloyl-neuraminic acid. These oligosaccharides were analysed by m.s. as the permethylated oligosaccharide alditols, and the spectra obtained were interpreted in detail. In another recent paper, Aman and coworkers...
Figure 3. Mass spectral study of a permethylated aldotriouronic acid
used h.p.l.c./m.s. with chemical ionization to analyse fully alkylated oligosaccharides obtained during a partial hydrolysis study of a permethylated polysaccharide.

II.8 Nuclear Magnetic Resonance (n.m.r.)

$^{13}\text{C}$ and $^1\text{H}$ nuclear magnetic resonance have been used extensively and effectively in the structure determination of poly- and oligosaccharides. The fact that Klebsiella capsular polysaccharides give interpretable n.m.r. spectra is evidence that they have regular repeating sequences of sugars. N.m.r. spectroscopy is especially useful in determining the number of sugars in this repeating unit and in determining the anomeric configuration of glycosidic linkages.

a) $^1\text{H}$ Nuclear magnetic resonance.

Derivatised poly- and oligosaccharides have been analysed in non-aqueous solvents, but in polysaccharide studies good spectra have been obtained using deuterium oxide, without chemical derivatisation. Carbohydrates to be studied are repeatedly dissolved in deuterium oxide and freeze-dried to exchange all hydroxylic and acidic protons for deuterons. A residual HOD signal is always observed however, and it may mask some important anomeric proton signals. The HOD signal may be shifted upfield out of the anomeric region by recording the spectrum at high temperature (90-95°C). When polysaccharide spectra are run at high temperature the viscosity is reduced and the resolution is increased due to the corresponding increase in $T_2$. The viscosity of polysaccharides may also be reduced by a very
mild acid hydrolysis, but this method must be used with caution because acid-labile substituents may be removed.

The major features of poly- and oligosaccharide $^1H$-n.m.r. spectra will now be discussed, beginning with those signals that appear at high field.

i) 6-Deoxyaldohexoses (e.g., Rha and Fuc) give methyl proton signals at approximately $\delta 1.3$ with $J_{5,6} = 6$ Hz (doublet). If a 6-deoxyaldohexose is the reducing sugar of an oligosaccharide, the methyl signal is split according to the anomeric ratio.

ii) Non-carbohydrate substituents containing methyl groups give distinctive signals at relatively high field. Pyruvate acetals give a methyl signal at $\delta 1.6$ to $\delta 2.1$, with the chemical shift being dependent of the stereochemistry. Acetyl groups give a methyl signal at approximately $\delta 2.2$.

iii) Ring protons and H-6 of aldohexoses give signals that appear in the region $\delta 3.0$ to $\delta 4.5$. These signals may be completely assigned, but for poly- and oligosaccharides this area of the spectrum is often too complex for complete rationalisation. Some ring proton resonances occur significantly downfield of the others. H-2 of a 2-linked mannose may appear as far downfield as $\delta 4.2$, and H-5 of galacturonic acid may appear as far downfield as $\delta 4.85$.

iv) The anomeric protons resonate in the region $\delta 4.5$ to $\delta 5.5$. These
signals provide information about the anomeric configuration of glycosidic linkages, the number of sugars in the repeating unit of a polysaccharide, and the degree of polymerisation of an oligosaccharide. The signals in this region have been empirically divided at $\delta 5.0$: $\beta$ signals, in general, appear upfield of this point and $\alpha$ signals downfield. This division, however, is not absolute because $H-1$ of $\beta$-galactofuranose has been observed at $\delta 5.13$. In oligosaccharides the reducing end anomeric protons are usually distinguishable because they integrate to less than one proton, although reducing mannose may exist almost completely in the $\alpha$ form.

The $H-1-H-2$ coupling constants ($J_{1,2}$) must be taken into account when assigning anomeric signals. According to Karplus, the magnitude of the coupling constant between two hydrogens on adjacent carbons is dependent on their dihedral angle $\theta$: $J_{1,2}$ passes through maxima at $\theta = 0^\circ$ and $180^\circ$ and a minimum at $\theta = 90^\circ$. An example of the usefulness of this relationship is illustrated in Figure 4.

v) A formate substituent has been identified by a signal at $\delta 5.9$.

b) $^{13}$C-Nuclear magnetic resonance.

Because of the low natural abundance of $^{13}$C, and the low solubilities of polysaccharides, $^{13}$C-n.m.r. studies of polysaccharides have been aided by the introduction of pulsed Fourier transform techniques. Polysaccharide samples are dissolved in deuterium oxide for locking purposes, and spectra may be recorded at elevated temperature to reduce the viscosity. Spectra are simplified through
Figure 4. Comparison of $J_{1,2}$ for β-D-Glucopyranose and β-D-Mannopyranose.
the application of broad band proton decoupling.

This technique also enhances the signal intensity via the nuclear Overhauser effect (n.O.e.). The n.O.e. is not uniform over all carbons in the sample, and integration is therefore less useful than in $^1$H-n.m.r. spectroscopy. The spectra obtained under these conditions show better resolution than $^1$H-n.m.r. spectra.

The major features of $^{13}$C-n.m.r. spectra of poly- and oligo-saccharides will now be discussed, beginning with those signals that appear at relatively high field.

i) The methyl carbon signals of 6-deoxyhexosyl residues appear at approximately 617.

ii) Methyl carbons of acetate and pyruvate substituents resonate at approximately 630 and 620 respectively. The chemical shift of pyruvate is dependent on its stereochemistry.

iii) Primary alcohol carbons appear at approximately 661 unless they are linkage positions, in which case they are shifted downfield 7-10 ppm.

v) Ring carbons, aside from anomeric carbons, appear in the region 665 to 680. Linkage position ring carbons appear at the lower end of this region 675 to 680. For oligosaccharides the reducing sugar's linkage position signal may be split into the anomeric ratio.
v) Anomeric carbons resonate at 693 to 6110 ppm. This region can be empirically divided at 6101, with β signals appearing downfield of 6101 and α signals upfield. The reducing end anomeric carbons of oligosaccharides resonate at 693 to 697.

II.9 Polarimetry and Circular Dichroism

The specific rotation of a sugar is dependent on its absolute configuration (D or L) and its anomeric configuration (α or β). The specific rotation of a compound is given by the following equation:

\[ [\alpha]_\lambda = \frac{\alpha \times 100}{\ell \times c} \]

where \( \alpha \) is the measured rotation, \( \ell \) is the length of the sample cell in decimetres, \( c \) is the concentration of the solution in g/100 ml, and \( \lambda \) is the wavelength of the plane polarised light. The wavelength that is usually used is the sodium D line (589 nm). Hudson's Rules of isorotation \(^{14,69}\) can be used to predict the specific rotations of poly- and oligosaccharides. Savage \(^{53}\) and Merrifield \(^{71}\) have tabulated the predicted and measured values for the \textit{Klebsiella} polysaccharides.

Circular dichroism spectroscopy of alditol acetates, partially methylated alditol acetates, and peracetylated aldononitriles can be used to determine the absolute configuration of sugars. \(^{14,70}\)
III. POLYSACCHARIDE STRUCTURAL METHODS
III. POLYSACCHARIDE STRUCTURAL METHODS

III.1 Isolation and Purification

The methods used by our research group for isolating bacterial capsular polysaccharides have been discussed in other theses. Cultures are grown on agar plates and single colonies are then incubated in a beef-extract broth at 37°C. After several hours the broth becomes turbid and it is then spread on agar trays containing a high sucrose content. After several days the bacterial slime is scraped off of the agar and centrifuged. The supernatant is then precipitated into ethanol, methanol or acetone. The precipitate is dissolved in water and acidic polysaccharides are then precipitated with CETAVLON (cetyltrimethylammonium bromide). This precipitate is dissolved in 2M sodium chloride and precipitated into ethanol or methanol. The polysaccharide precipitate is then dialysed against running water and freeze-dried.

III.2 Sugar Analysis

During the structural analysis of a polysaccharide, the identity of the sugars present and their relative amounts (total sugar ratio) must first be determined. After acid hydrolysis, paper chromatography with Solvent C (see Section II.1), is a simple method of identifying the neutral sugars present. The total sugar ratio is usually determined by g.l.c. analysis of the derivatised sugars.

A number of acids have been used for the hydrolysis step, including aqueous hydrochloric, sulfuric and trifluoroacetic (TFA)
Figure 5. Total sugar ratio reaction sequence
acids. Hough and coworkers studied these three acids and showed that TFA and sulfuric acid give significantly less degradation of sugars. An advantage of TFA over sulfuric acid is its volatility; it is easily removed from the hydrolysate under reduced pressure.

When studying polysaccharides containing uronic acids, it is difficult to obtain an accurate total sugar ratio due to the resistance of uronosyl bonds to acid hydrolysis. In this study of Klebsiella K54, Conrad and coworkers showed that the glucuronosyl bond is hydrolysed much more slowly than the glycosidic bonds of neutral sugars. To overcome this problem, the carboxyl groups of uronic acids are usually reduced to primary alcohols. A disadvantage of such a reduction is that glucuronic acid, for example, is no longer distinguishable from glucose. This problem can be remedied by using a deuterating reducing agent (e.g. sodium borodeuteride) and analysing the hydrolysate by g.l.c./m.s.

Several methods have been developed for the carboxyl reduction of uronic acids. The polysaccharide may be treated with propionic anhydride in pyridine to convert all of the hydroxyl groups to propionate esters. The uronic acid carboxyl groups are then esterified with diazomethane and reduced with lithium borohydride. In another important method, uronic acids are esterified with a carbodiimide and reduced with sodium borohydride.

In the present study, poly- and oligosaccharides were first methanolysed in refluxing 3% methanolic hydrogen chloride. After such a treatment, all carboxyl groups are converted to methyl esters and the product is a mixture of oligo- and monosaccharides as methyl
glycosides. The esters are then reduced by treatment with sodium borohydride in anhydrous methanol. After carboxyl reduction the polysaccharide can be completely depolymerised by hydrolysis with 2M TFA at 95°C for 8 hours.37

The derivatives available for g.l.c. analysis have been discussed in Section II.3. Alditol acetates and peracetylated aldononitriles (PAANs) are the derivatives of choice. The problems associated with PAANs are discussed in Section II.3. Figure 5 illustrates the reaction sequence for the total sugar ratio of a hypothetical polysaccharide.

III.3 Methylation Analysis

The methylation analysis provides more information than any other single method used in the structural analysis of polysaccharides. The steps in the analysis are: the methylation of all hydroxyl groups, acid hydrolysis, and the analysis of the partially methylated sugars released. In the case of acidic polysaccharides, carboxyl reduction may precede the acid hydrolysis; otherwise depolymerisation may be incomplete due to the resistance of uronosyl linkages to acid hydrolysis. The type of information provided by the methylation of a hypothetical polysaccharide is illustrated in Figure 6.

Before the introduction of the Hakomori method in 1964, several procedures were used to methylate polysaccharides. The most important methods will now be discussed. In 1903 Purdie and Irvine reported that sucrose could be methylated using methyl iodide as an alkylating agent and silver oxide as a catalyst. This technique has
\[
\begin{align*}
\text{D-GlcA} & \xrightarrow{1/3} \text{D-Gal} & \xrightarrow{1/4} \text{L-Rha} & \xrightarrow{1/\beta} \\
\beta \text{D-Glc} & \\
\end{align*}
\]

\[
\begin{align*}
& \xrightarrow{1} \text{H}_3\text{CSCH}_2^-/\text{DMSO} \\
& \xrightarrow{2} \text{CH}_3\text{I} \\
& \xrightarrow{\text{LiAlH}_4/\text{THF}} \\
& \xrightarrow{2\text{M TFA}} \\
\end{align*}
\]
Figure 6. Methylation analysis of a hypothetical polysaccharide.
often been used to complement other procedures that have achieved only partial methylation. In 1915 Haworth\textsuperscript{59} reported that carbohydrates could be methylated using dimethyl sulphate in aqueous sodium hydroxide. In 1955 Kuhn showed that the Purdie method could be improved as a method for methylating polysaccharides by using dimethyl formamide as a solvent.\textsuperscript{60}

Today the most important methylation method for poly- and oligosaccharides is that reported by Hakomori in 1964.\textsuperscript{61} He showed that glycolipids and polysaccharides could be completely methylated using methyl iodide as an alkylating agent, the methylsulfinyl carbanion as a base and dimethyl sulfoxide as a solvent. The reaction takes place in two steps:

1. \[ R — \text{OH} + \text{CH}_3 — \text{SO} — \text{CH}_2^- \text{Na}^+ \rightarrow R — \text{O}^- \text{Na}^+ + \text{CH}_3 — \text{SO} — \text{CH}_3 \]

2. \[ R — \text{O}^- \text{Na}^+ + \text{CH}_3\text{I} \rightarrow R — \text{O} — \text{CH}_3 + \text{NaI} \]

Most polysaccharides should be completely methylated in one treatment. Completeness of methylation is indicated by the absence of infrared absorption at 3600 cm\textsuperscript{-1}. Unfortunately this method does not lend itself to successive treatments of polysaccharides containing uronic acids. On esterification, H-5 of a hexuronic acid becomes acidic and the polysaccharide may be degraded in the presence of methylsulfinyl carbanion, especially if the uronic acid is linked at C-4.\textsuperscript{64}

In the present study, the uronic esters of permethylated poly- and oligosaccharides were reduced with lithium aluminum hydride in tetrahydrofuran. Hydrolysis was effected in 2M TFA on a steam bath.
Partially methylated sugars obtained by hydrolysis of permethylated polysaccharides have been separated by column chromatography (cellulose, silica gel, charcoal) and by paper chromatography. Today the most important method for quantitative and qualitative analysis is g.l.c./m.s. of the partially methylated alditol acetate derivatives. Lindberg and coworkers have published a booklet giving the details of the methylation procedure. This publication includes standard g.l.c. retention times and standard mass spectra for partially methylated alditol acetates.

III.4 Partial Hydrolysis

During the structural analysis of a polysaccharide, it is useful to isolate oligosaccharides from the polymer by partial hydrolysis. Data obtained from oligosaccharides (e.g., n.m.r., methylation analyses) can be used to help interpret the corresponding data obtained from the polysaccharide. The sugar sequence of a polysaccharide can be determined by the analysis of a series of oligosaccharides obtained by partial hydrolysis.

Wolfrom and coworkers determined the first-order rate constants for eight glucose disaccharides. They showed that the rate of hydrolysis is dependent on both the linkage position and the anomeric configuration. More important, however, in the study of bacterial polysaccharides, is the resistance of uronosyl bonds to acid hydrolysis. Therefore the hydrolysis of a polysaccharide is not a completely random process, and if proper conditions are chosen certain oligosaccharides will accumulate in the hydrolysate. It is almost always possible to isolate an aldobiouronic acid from an acidic polysaccharide,
but the acid concentration, temperature and reaction time should be chosen to give the maximum yield of higher oligomers. Oligomers up to pentasaccharides have been isolated from *Klebsiella* polysaccharides.\textsuperscript{20,64}

After the hydrolysis step, the isolation of oligosaccharides presents an interesting separation problem. Neutral and acidic components can be separated by ion exchange chromatography. The present study employed Bio-Rad AG 1X-2, a resin with quarternary ammonium active sites. With the resin in the formate form, neutral sugars are eluted with water and acidics with 10\% formic acid. Oligosaccharides may be isolated from each fraction by gel permeation chromatography, paper chromatography,\textsuperscript{14} or paper electrophoresis.\textsuperscript{53} Silica gel chromatography may be used to isolate derivatised oligosaccharides.\textsuperscript{37}

After isolation oligosaccharides can be analysed by the methods of sugar analysis, methylation analysis, n.m.r. and mass spectrometry. Some workers have studied the products obtained by partial hydrolysis of permethylated polysaccharides. Extra information is provided by the location of hydroxyl groups obtained on hydrolysis of glycosidic linkages. Choy used this method in his study of *Klebsiella* K56.\textsuperscript{38} Albersheim\textsuperscript{32,46} and coworkers have developed a method by which a permethylated polysaccharide is partially hydrolysed and the oligosaccharide products are isolated by h.p.l.c.

III.5 Uronic Acid Degradation (β-elimination)

Many bacterial polysaccharides contain hexopyranosyluronic acid residues. If such a polysaccharide is permethylated, the methyl uronâte
residues have a good leaving group at C-4 (either methoxyl or another sugar residue); treatment with the methylsulfinyl anion will cause β-elimination with initial removal of a proton from C-5. Mild acidic hydrolysis results in complete degradation of the uronate residue. This sequence of reactions has been discussed in detail. According to Aspinall the hydrolysis of the uronate residue will occur on treatment with the methylsulfinyl anion. It is this β-elimination reaction that eliminates the possibility of successive Hakomori methylations of uronic acid containing polysaccharides.

Uronic acids in the polysaccharide may be located by labelling (with trideuteriomethyl or ethyl groups) the hydroxyl groups liberated by uronic acid degradation. This method may also be used to isolate alkylated oligosaccharides.

III.6 Periodate Oxidation and Smith Degradation

Periodic acid and its salts oxidatively cleave 1,2-diols to give two aldehydes, and 1,2,3-triols to give two aldehydes and formic acid. Under proper conditions these reactions are considered to be quantitative. By measuring the amount of periodate consumed by a polysaccharide, and by determining which sugars have been oxidised, some of the results of the methylation analysis can be confirmed. The Smith degradation of a polysaccharide (periodate oxidation, sodium borohydride reduction, and controlled acid hydrolysis) can be used to isolate glycosides of mono- and oligosaccharides. Oligosaccharides isolated by this procedure provide information about the sequence of sugars in the polysaccharide.
III.7 Immunochemical Methods

Heidelberger and coworkers have prepared antibodies using, as antigens, the Pneumococcal capsular polysaccharides, many of which have known chemical structures. The degree of reaction between an antigen and antibody is measured by determining the amount of precipitate produced on mixing. Thus it is possible to predict structural features of an unknown polysaccharide, having determined its reactivity with the Pneumococcal anti-sera. Cross reactivity has been determined for many of the *Klebsiella* polysaccharides.11,72
IV A STRUCTURAL INVESTIGATION OF *KLEBSIELLA*

K39 CAPSULAR POLYSACCHARIDE
IV A STRUCTURAL INVESTIGATION OF KLEBSIELLA K39 CAPSULAR POLYSACCHARIDE.

ABSTRACT:

An acidic capsular polysaccharide was isolated from *Klebsiella* K39. This polysaccharide was partially hydrolysed and an unusual pentasaccharide, containing two uronic acid residues, was isolated and assigned the following structure.

\[
\text{GlcA} \quad \begin{array}{c} \beta \\ \frac{2}{3} \end{array} \quad \text{Man} \quad \begin{array}{c} \alpha \\ \frac{4}{5} \end{array} \quad \text{GlcA} \quad \begin{array}{c} \beta \\ \frac{2}{3} \end{array} \quad \text{Man} \quad \begin{array}{c} \alpha \\ \frac{4}{5} \end{array} \quad \text{Glc-OH}
\]

This pentasaccharide was studied by sugar analysis, methylation analysis, \(^1\)H- and \(^{13}\)C-n.m.r., and by mass spectrometry of the permethylated oligosaccharide alditol. The relationship of this oligomer to the polysaccharide will be discussed with reference to methylation and n.m.r. studies.

RESULTS AND DISCUSSION:

Isolation and Sugar Analysis

The *Klebsiella* K39 capsular polysaccharide was isolated and purified by the methods described in Section III.1 and in the Experimental section. Molecular weight analysis by gel chromatography (Dr. S.C. Churms, University of Capetown) showed that the product moved as one band with a molecular weight of 560,000. According to
the qualitative analysis of Nimmich (see Appendix 1), this polysaccharide is composed of glucuronic acid, glucose, mannose, and galactose. A sample of the polysaccharide was methanolysed, carboxyl reduced, and hydrolysed. The hydrolysate was analysed by g.l.c. after preparation of the PAAN derivatives, giving Man/Glc/Gal = 1.0/2.2/0.8. The problems associated with PAAN derivatives have been discussed in Section II.3. Hassell used equimolar mixtures of sugars to prepare molar response factors. These response factors were used to correct the data obtained from K39, and therefore the errors associated with PAANs should have been reduced.

**Partial Hydrolysis**

In a preliminary study a sample of K39 polysaccharide (470 mg) was hydrolysed in 1M TFA for 5½ hours on a steam bath. Acidics and neutrals were separated by ion exchange chromatography on Bio-Rad AG 1-X2. The acidic fraction was analysed by gel chromatography on Bio-Gel P-2. The elution profile is illustrated in Figure 7. This chromatogram shows that one acidic component is obtained in excess of all others. A sample of this compound was analysed by 100 MHz ¹H-n.m.r. in deuterium oxide at 95°C. The spectrum gave three α anomeric signals at δ5.20, δ5.26, and δ5.47 and a multiplet of 8 signals between δ4.59 and δ4.73 (see Spectrum 1). A sample of this compound was treated with sodium borodeuteride to convert all reducing sugars to alditols. This reduced sample was studied by 100 MHz ¹H-n.m.r. at 90°C (Spectrum 2) and by 400 MHz ¹H-n.m.r. at ambient temperature
Figure 7. Bio-Gel P-2 Chromatography of Acidic
Fraction obtained by Partial Hydrolysis of K39 Polysaccharide
(see Spectrum 3 and Table 1). The spectra clearly show two signals in the α region (δ5.27 and δ5.37) and two signals in the β region (δ4.51 and δ4.59). This compound, designated A1, is therefore believed to be a pentasaccharide.

In a further study a sample of K39 polysaccharide (700 mg) was hydrolysed in 1M TFA for 7 hours. Acidics were separated from neutrals by ion exchange chromatography on Bio-Rad AG 1-X2. The acidic fraction was chromatographed on paper with Solvent B (see Section II.1) Three bands were extracted from the paper and they were designated A1, A2 and A3. The analysis of these components will now be discussed.

Analysis of A1

This compound, [α]D + 11° (c 0.184, water), had a chromatographic mobility on Whatman No. 1 paper of RGlcn 0.076 (Solvent B). A sample was hydrolysed in 3M TFA for 20 hours on a steam bath. The neutral sugars released were analysed by g.l.c. as the alditol acetate derivatives and the chromatogram gave Man/Glc = 1.56/1. Another sample was methanolysed, carboxyl-reduced, and hydrolysed. The products were analysed by g.l.c. as the alditol acetates giving Glc/Man = 1.47/1.

1H- and 13C-n.m.r. spectra were recorded and the results are given in Table 1. 1H-n.m.r. confirmed that this compound was the previously isolated pentasaccharide. The 400 MHz 1H-n.m.r. spectrum, recorded at ambient temperature, showed six distinct signals in the anomeric region (see Spectrum 4). Three of these signals (δ4.57, δ4.66, δ4.62) are assigned to β anomers on the basis of their chemical
Table 1: N.M.R. STUDY OF Al

A. 400 MHz $^1$H-n.m.r. at ambient temperature; a) Al, b) Al after reduction with NaBD$_4$.

<table>
<thead>
<tr>
<th>Chemical Shift of Anomeric $^1$H's(δ)</th>
<th>$J_{1,2}$ (Hz)</th>
<th>Integral</th>
<th>Number of $^1$H's</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.57</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>β-GlcA</td>
</tr>
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<td>4.62</td>
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<td>1</td>
<td>β-GlcA</td>
</tr>
<tr>
<td>4.66</td>
<td>8</td>
<td>0.55</td>
<td>1</td>
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</tr>
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<td>5.15</td>
<td>singlet</td>
<td></td>
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<td>α-Man</td>
</tr>
<tr>
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<td>5.47</td>
<td>broad singlet</td>
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<td>1</td>
<td>α-Man</td>
</tr>
<tr>
<td>b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.51</td>
<td>8</td>
<td>1</td>
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<td>singlet</td>
<td></td>
<td>1</td>
<td>α-Man</td>
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</table>

B. 200 MHz $^{13}$C-n.m.r. of Al

<table>
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<tr>
<th>Chemical Shift of Anomeric $^{13}$C's(δ)</th>
<th>Integral</th>
<th>Number of $^{13}$C's</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.42</td>
<td>1</td>
<td>α-Glc-OH</td>
</tr>
<tr>
<td>96.74</td>
<td>0.58</td>
<td>1</td>
<td>β-Glc-OH</td>
</tr>
<tr>
<td>100.19</td>
<td>1</td>
<td>1</td>
<td>α-Man</td>
</tr>
<tr>
<td>100.92</td>
<td>1</td>
<td>1</td>
<td>α-Man</td>
</tr>
<tr>
<td>102.31</td>
<td>1</td>
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<td>β-GlcA</td>
</tr>
<tr>
<td>102.98</td>
<td>1</td>
<td>1</td>
<td>β-GlcA</td>
</tr>
</tbody>
</table>
shifts. These signals have relatively large coupling constants (8 Hz) and can therefore be assigned to β-glucopyranosyl or β-glucurono-pyranosyl residues. The signal at 4.62 is assigned to a reducing sugar on the basis of its integral. The signals at 65.15 and 65.47 are assigned to α-mannopyranosyl residues. The signal at 65.23 is assigned to a reducing sugar and the broadening of the signal at 65.47 indicates that it is adjacent to the reducing sugar.

The $^{13}$C-n.m.r. spectrum of Al (see Table 1 and Spectrum 5) shows six signals in the anomeric region, two of which are assigned to the reducing sugar. Five signals between 678.48 and 683.03 are assigned to the linkage position carbons. The signals at 683.03 and 680.46 are assigned to the linkage position carbon of the reducing sugar.

A sample of Al was studied according to the methylation analysis procedure described in Section III. A sample of the oligosaccharide was methylated by the Hakomori procedure, and a portion of the product was hydrolysed and analysed by g.l.c. as the alditol acetate derivatives. The results are given in column I of Table 2 and chromatogram "a" of Figure 8. 3,4,6-Man and 2,4,6-Glc were observed. Another portion of the methylated product was treated with lithium aluminum hydride, hydrolysed and analysed by g.l.c. as the alditol acetate derivatives. The results are given in column II of Table 2 and chromatogram "b" of Figure 8. Two additional peaks (2,3,4-Glc and 2,3-Glc) were observed on the chromatogram, indicating that Al contains two acidic sugars, one of which is at the non-reducing terminus. Analysis by g.l.c./m.s. determined the methylation patterns of the PMAAs. 3,4,6-Man and 2,4,6-Glc were not completely separated by g.l.c., but the mass spectra on
Gas liquid chromatography

Column: HIEFF-1B  Carrier Gas: N₂, 20 mls/min

Temperature Program: 165°C for 8 min, increase by 2°/min to 200°C.

a) PMAAs obtained without carboxyl reduction

b) PMAAs obtained with carboxyl reduction

Mass Spectrum at "A":

<table>
<thead>
<tr>
<th>m/e</th>
<th>% base peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>97</td>
</tr>
<tr>
<td>45</td>
<td>38</td>
</tr>
<tr>
<td>71</td>
<td>19</td>
</tr>
<tr>
<td>87</td>
<td>38</td>
</tr>
<tr>
<td>99</td>
<td>14</td>
</tr>
<tr>
<td>101</td>
<td>16</td>
</tr>
<tr>
<td>129</td>
<td>100</td>
</tr>
<tr>
<td>161</td>
<td>35</td>
</tr>
<tr>
<td>189</td>
<td>24</td>
</tr>
</tbody>
</table>

Mass Spectrum at "B":

<table>
<thead>
<tr>
<th>m/e</th>
<th>% base peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td>45</td>
<td>31</td>
</tr>
<tr>
<td>71</td>
<td>12</td>
</tr>
<tr>
<td>87</td>
<td>14</td>
</tr>
<tr>
<td>101</td>
<td>20</td>
</tr>
<tr>
<td>117</td>
<td>37</td>
</tr>
<tr>
<td>129</td>
<td>35</td>
</tr>
<tr>
<td>161</td>
<td>16</td>
</tr>
</tbody>
</table>

Figure 8. Methylation analysis of K39-A1
Table 2: METHYLATION ANALYSES OF A1 AND A2

<table>
<thead>
<tr>
<th>Partially Methylated Alditol Acetates&lt;sup&gt;a&lt;/sup&gt;</th>
<th>T&lt;sup&gt;b&lt;/sup&gt;</th>
<th>I&lt;sup&gt;d&lt;/sup&gt;</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,3,4,5,6-Man&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.42</td>
<td></td>
<td>10.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,2,4,5,6-Glc&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.43</td>
<td></td>
<td>6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,4,6-Man</td>
<td>1.82</td>
<td>100</td>
<td>94.0</td>
<td>33.5</td>
<td></td>
</tr>
<tr>
<td>2,4,6-Glc</td>
<td>1.87</td>
<td></td>
<td>55.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,3,4-Glc</td>
<td>2.08</td>
<td></td>
<td>23.3</td>
<td></td>
<td>30.4</td>
</tr>
<tr>
<td>2,3-Glc</td>
<td>3.06</td>
<td></td>
<td>21.1</td>
<td></td>
<td>25.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> e.g., 3,4,6-Man = 1,2,5-tri-0-acetyl-3,4,6-tri-0-methylmannitol.
The PMAAs listed are consistent with g.l.c. and mass spectral data.

<sup>b</sup> G.l.c. retention time relative to 2,3,4,6-Glc on 3% HIEFF-1B,
temperature program: 165°C for 8 min, increase 2°/min to 200°C.

<sup>c</sup> Published molar response factors were used where applicable. No
such response factors are available for pentamethylalditols acetates.

<sup>d</sup> I, A1 methylated and hydrolysed without carboxyl reduction;
II, A1 methylated, carboxyl reduced and hydrolysed;
III, A1 reduced with NaBD<sub>4</sub>, methylated and hydrolysed;
IV, A2 reduced with NaBD<sub>4</sub>, methylated, carboxyl reduced and hydrolysed.

<sup>e</sup> Monodeuterated at C-1.
the leading and tailing edges of the peak were sufficiently different to identify both PMAAs (see Figure 8 and Figure 2).

Another sample of Al was treated with sodium borodeuteride to convert the reducing sugar to the alditol. The sample was then methylated, hydrolysed, and analysed by g.l.c./m.s. as the alditol acetates (see column III, Table 2). 1,2,4,5,6-Glc and 3,4,6-Man were observed. No standard spectra of the acetates of pentamethyl hexitols are available but the structure of 1,2,4,5,6-Glc was confirmed by fragment ions at m/e 90 and m/e 206. These ions are assigned the following structures:

\[
\begin{align*}
\ce{CHDOME} & \quad \text{m/e 90} \\
\ce{HCOAc} & \\
\ce{HCOMe} & \\
\ce{CHDOME} & \\
\ce{HC = OMe} & \\
\ce{HC = OMe' & \\
\end{align*}
\]

The mass spectrum is tabulated in the Experimental section. The yield of 1,2,4,5,6-Glc is lower than expected. Other workers have attributed this phenomenon to losses during work-up due to the volatility of pentamethyl sugars and their derivatives. This experiment confirms that the reducing glucose is 3-linked.

The analyses of Al discussed so far have given only partial information about the sugar sequence. Mass spectrometry of permethylated oligosaccharides is an established method for sequencing
Figure 9. Mass spectrum of K39 - Al as the permethylated alditol
Figure 10. Mass spectrum of K39 - Al as the permethylated alditol
oligosaccharides. A sample of Al was treated with sodium borodeuteride to convert the reducing sugar to the alditol. It was then methylated by the Hakomori procedure. A sample of the product was applied to a silica gel thin-layer plate and eluted with ethyl acetate. The permethylated oligomer had $R_f$ 0.36 but a significant amount of material remained at the origin. The product was isolated by preparative t.l.c. and gave an infrared spectrum that confirmed its identity as apermethylated oligosaccharide alditol. In particular absorption maxima were observed at 1750 cm$^{-1}$ (ester carbonyl stretch) and at 1105 cm$^{-1}$, 1080 cm$^{-1}$ (ether stretch). This compound was analysed by electron impact mass spectrometry with an electron beam energy of 20 eV. The mass spectrum is tabulated in Figure 9 and the interpretation is given in Figure 10. The spectrum was interpreted according to the principles described in Section II.7. Other details regarding the mass spectrum of this compound are given in Appendix III.

On the basis of these data, the following structure is proposed for Al.

$$\text{GlcA} \quad 1 \quad 2 \quad \text{Man} \quad 1 \quad 4 \quad \text{GlcA} \quad 1 \quad 2 \quad \text{Man} \quad 1 \quad 3 \quad \text{Glc-OH}$$

Analysis of A2:

This compound moved slightly faster than Al on Whatman No. 1 paper ($R_{\text{Glc}}$ 0.12, Solvent B). The $^1$H-n.m.r. spectrum (see Table 3 and Spectrum 6) shows that A2 is a tetrasaccharide containing four of the
sugars found in A1, but without the reducing glucose. On reduction with sodium borohydride the signal at 65.35 is removed from the spectrum (see Spectrum 7), indicating that the reducing sugar is mannose. It is interesting to note that 94% of the reducing mannose exists as the α anomer.

The results of the methylation analysis are given in Table 2. A2 was reduced with sodium borodeuteride, methylated, carboxyl reduced, hydrolysed and analysed by g.l.c./m.s. The presence of 1,3,4,5,6-Man confirms that the reducing mannose is 2-linked. Standard mass spectra are not available for the alditol acetates of pentamethyl hexitols but 1,2,4,5,6-Man was identified by fragment ions at m/e 162 and m/e 130. These ions are assigned the following structures:

\[
\begin{align*}
\text{HC} & = \text{OMe} \\
\text{HCOAc} & -\text{MeOH} \\
\text{CHDOMe} & \\
\hline
\text{HC} & = \text{OMe} \\
\text{COAc} & \\
\text{CHD} & \\
\end{align*}
\]

m/e 161 \hspace{2cm} m/e 130

The mass spectrum is tabulated in the Experimental section. 3,4,6-Man, 2,3,4-Glc and 2,3-Glc were also observed as expected.

On the basis of these data, the following structure is proposed for A2:

\[
\text{GlcAp } \frac{1}{2} \text{ Manp } \frac{1}{4} \text{ GlcAp } \frac{1}{2} \text{ Manp-OH}
\]
Table 3: N.M.R. STUDY OF A2

A. 400 MHz $^1$H-n.m.r. at ambient temperature; a) A2, b) A2 after reduction with NaBD$_4$.

<table>
<thead>
<tr>
<th>Chemical Shift (δ)</th>
<th>$J_{1,2}$ (Hz)</th>
<th>Integral Number</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) 4.55</td>
<td>8</td>
<td>1</td>
<td>β-GlcA</td>
</tr>
<tr>
<td>4.60</td>
<td>8</td>
<td>1</td>
<td>β-GlcA</td>
</tr>
<tr>
<td>4.93</td>
<td>singlet</td>
<td>0.06</td>
<td>β-Man-OH</td>
</tr>
<tr>
<td>5.15</td>
<td>singlet</td>
<td>1</td>
<td>α-Man</td>
</tr>
<tr>
<td>5.35</td>
<td>singlet</td>
<td>0.94</td>
<td>α-Man-OH</td>
</tr>
<tr>
<td>b) 4.55</td>
<td>8</td>
<td>1</td>
<td>β-GlcA</td>
</tr>
<tr>
<td>4.59</td>
<td>8</td>
<td>1</td>
<td>β-GlcA</td>
</tr>
<tr>
<td>5.11</td>
<td>singlet</td>
<td>1</td>
<td>α-Man</td>
</tr>
</tbody>
</table>

B. 20 MHz $^{13}$C-n.m.r. of A2

<table>
<thead>
<tr>
<th>Chemical Shift (δ)</th>
<th>Integral Number</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>93.30</td>
<td>~1</td>
<td>α-Man-OH</td>
</tr>
<tr>
<td>100.80</td>
<td>1</td>
<td>α-Man</td>
</tr>
<tr>
<td>102.37</td>
<td>1</td>
<td>β-GlcA</td>
</tr>
<tr>
<td>103.04</td>
<td>1</td>
<td>β-GlcA</td>
</tr>
</tbody>
</table>
Analysis of A3

When irrigated with Solvent B, this compound has a chromatographic mobility of $R_{Glc}^{0.42}$ on Whatman No. 1 paper. A sample was hydrolysed in 2M TFA for 16 hours on a steam bath. A sample of the hydrolysate was chromatographed on paper with Solvent C along with glucose, mannose, and galactose. The chromatogram showed that the neutral sugar released during hydrolysis was galactose.

N.m.r. Study

A sample of K39 capsular polysaccharide was partially depolymerized by hydrolysis in 0.02M TFA on a steam bath for 10 minutes. It was then dialysed against running water and freeze-dried. This sample was analysed by 400 MHz $^1$H-n.m.r. at 90°C (see Spectrum 8).

Figure 11 compares the anomeric proton signals of this polysaccharide with similar signals in the $^1$H-n.m.r. spectrum of the pentasaccharide A1. In the polysaccharide spectrum, the H-1 signals of the $\alpha$-mannopyranosyl residues ($\delta$5.20 and $\delta$5.45) are clearly distinguished. A $\beta$ signal is observed at $\delta$4.95 and a number of unresolved $\beta$ signals appear between $\delta$4.53 and $\delta$4.74. The integral units of the various signals appear below the spectrum. The total number of integral units is 95. Assuming that 14 units account for one sugar residue, then the pentasaccharide A1 accounts for 70 of the 95 integral units attributed to anomeric signals.

The $^1$H-n.m.r. and $^{13}$C-n.m.r. data are tabulated in Table 4. (The sample analysed by $^{13}$C-n.m.r. was partially depolymerised by the
Figure 11. $^1$H-n.m.r. anomeric signals for oligosaccharide Al (top), and K39 capsular polysaccharide (bottom).
Table 4: N.M.R. STUDY OF K39 CAPSULAR POLYSACCHARIDE.

A. 400 MHz $^1$H-n.m.r. at 90°C

<table>
<thead>
<tr>
<th>Chemical Shift of Anomeric $^1$H's(δ)</th>
<th>Integral</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.76-4.53</td>
<td>55</td>
<td>β-GlcA(x2), 3-linked β-Glc</td>
</tr>
<tr>
<td>4.95 ($J_{1,2} = 8$ Hz)</td>
<td>11</td>
<td>β-Glc (terminal)</td>
</tr>
<tr>
<td>5.20</td>
<td>15</td>
<td>α-Man</td>
</tr>
<tr>
<td>5.45</td>
<td>14</td>
<td>α-Man</td>
</tr>
</tbody>
</table>

B. 100.6 MHz $^{13}$C-n.m.r. at 60°C.

<table>
<thead>
<tr>
<th>Chemical Shift of Anomeric $^{13}$C;s(δ)</th>
<th>Integral</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.15</td>
<td>5.3</td>
<td>α-Man</td>
</tr>
<tr>
<td>100.91</td>
<td>7.0</td>
<td>α-Man</td>
</tr>
<tr>
<td>101.30</td>
<td>6.9</td>
<td>3-linked β-Glc</td>
</tr>
<tr>
<td>102.42</td>
<td>6.2</td>
<td>β-GlcA</td>
</tr>
<tr>
<td>102.60</td>
<td>16.5</td>
<td>β-GlcA, β-Glc (terminal)</td>
</tr>
</tbody>
</table>
same method). Not all of the sugar residues indicated by the sugar analysis could be accounted for in the n.m.r. spectra; therefore the assignments are not complete. In particular some of the 8 signals may arise, in part, from galactosyl residues.

A $^1$H-n.m.r. spectrum was also recorded without an initial hydrolysis, and without acetone as a standard (see Spectrum 9). No major differences were observed in the anomeric region of the spectrum. Traces of acetate (δ2.18) and possibly pyruvate (δ1.48) were also observed.

Methylation Analysis

Three methylation analysis experiments were performed: I, K39 methylated and hydrolysed without carboxyl reduction; II, K39 methylated, carboxyl reduced and hydrolysed; III, K39 hydrolysed for 3 hours in 0.02M TFA, followed by dialysis and freeze-drying, methylation, carboxyl reduction and hydrolysis. The products were analysed by g.l.c. and g.l.c./m.s. as the alditol acetate derivatives (see Table 4). These experiments show some similarities to the methylation analysis of Al. In experiment II, 3,4,6-Man, 2,4,6-Glc and 2,3-Glc are observed as in the analysis of Al. 2,3,4,6-Glc is observed in all three cases indicating the presence of terminal glucopyranosyl residues. In experiment III, the relative amounts of 2,3,4,6-Glc and 2-Glc decreased, with a corresponding increase in 2,3-Glc. These results indicate that the side chain containing the terminal glucopyranosyl residue is linked to the 3-position of a 4-linked glucuronopyranosyl residue.
Table 4: METHYLATION ANALYSIS OF KLEBSIELLA K39 CAPSULAR POLYSACCHARIDE.

<table>
<thead>
<tr>
<th>Partially Methylated Alditol Acetates&lt;sup&gt;a&lt;/sup&gt;</th>
<th>T&lt;sup&gt;b&lt;/sup&gt;</th>
<th>I&lt;sup&gt;d&lt;/sup&gt;</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,6-Glc</td>
<td>1.00</td>
<td>28.1</td>
<td>24.6</td>
<td>11.7</td>
</tr>
<tr>
<td>3,4,6-Man</td>
<td>1.82</td>
<td>51.3</td>
<td>38.2</td>
<td>46.6</td>
</tr>
<tr>
<td>2,4,6-Glc</td>
<td>1.87</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,4,6-Gal</td>
<td>2.05</td>
<td>8.5</td>
<td>8.7</td>
<td>8.0</td>
</tr>
<tr>
<td>2,3-Glc&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.06</td>
<td>12.9</td>
<td>25.3</td>
<td></td>
</tr>
<tr>
<td>2-Glc</td>
<td>3.68</td>
<td>12.0</td>
<td>15.6</td>
<td>8.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> e.g., 2,3,4,6-Glc = 1,5-tri-O-acetyl-2,3,4,6-tetra-O-methylglucitol

<sup>b</sup> G.l.c. retention time relative to 2,3,4,6-Glc on 3% HIEFF-1B, temperature program: 165° for 8 min, increase 2°/min to 200°C.

<sup>c</sup> Integrals were corrected with published molar response factors.

<sup>d</sup> I, K39 methylated and hydrolysed without carboxyl reduction; II, K39 methylated, carboxyl reduced and hydrolysed; III, K39 hydrolysed for 2 hours in 0.02M TFA followed by dialysis and freeze-drying, methylation, carboxyl reduction and hydrolysis.

<sup>e</sup> G.l.c./m.s. also detected 2,4-Glc on the leading edge of this peak.
Conclusions

On the basis of the experiments described, it is proposed that the capsular polysaccharide of *Klebsiella* K39 contains the following pentasaccharide unit in its repeating sequence of sugars:

\[
\begin{align*}
4 \text{GlcA}_\beta^1 & 2 \text{ManA}_\alpha^1 4 \text{GlcA}_\beta^1 & 2 \text{ManA}_\alpha^1 3 \text{GlcP}_\beta^1
\end{align*}
\]

These experiments also indicate the presence of a side chain with a terminal β-glucopyranosyl residue. The side chain is attached to the 3-position of one of the glucuronopyranosyl residues. To this date, no other published *Klebsiella* capsular polysaccharide has had two uronic acid residues in the repeating unit of sugars. It was not possible to locate the galactose in the structure of the polysaccharide. Different batches of K39 had different galactose contents, and part or all of the galactose may be present as an impurity.

The immunochemical studies of Heidelberger and Nimmich indicated that K39 shares structural features with K1 and K31. All three structures have a 3-linked β-glucopyranosyl residue. All three structures also have glucuronopyranosyl residues, although the degree of substitution and the anomeric configuration varies. It is probable that these two structural features play a role in the antigen-antibody reaction.
EXPERIMENTAL

**General Methods**

Descending paper chromatography was performed using the solvent systems and visualisation methods described in Section II.1.

G.l.c. analyses were performed on a Hewlett Packard 5710A gas chromatograph fitted with dual flame ionization detectors. G.l.c./m.s. analyses were performed on a Micromass 12 instrument with an electron beam energy of 70 eV.

N.m.r. spectra were recorded on Bruker WP-80, Bruker WH-400 and Varian XL-100 spectrometers. Samples were dissolved in deuterium oxide and acetone was included as a standard.

Mass spectra of a permethylated oligosaccharide were recorded on a Kratos MS50.

**Isolation and Purification**

An authentic culture of *Klebsiella* K39 was obtained from Dr. I. Ørskov, Copenhagen, and grown on five agar trays. Each tray contained agar composed of the following materials: 5g NaCl, 2.5g K$_2$HPO$_4$, 0.62g MgSO$_4$·7H$_2$O, 0.5g CaCO$_3$, 75g sucrose, 5g Bacto yeast extract, 37.5g agar, 2.5L water. After 3 days the bacterial slime was scraped off of the trays and the solution (600 ml) was made 1% in phenol. Insoluble material was removed by ultracentrifugation at 30,000 r.p.m. for 3 hours. The supernatant was mixed with 2.4L of acetone-methanol (3:1) to precipitate the polysaccharide component.
The precipitate was dissolved in water and the acidic polysaccharides were precipitated with 10% Cetavlon. The precipitate was dissolved in 2M sodium chloride (500 ml) and precipitated again by mixing with 2L of acetone-methanol (1250:750). The precipitate was dissolved in water and dialysed against running tap water for 2 days. The contents of the dialysis bag were freeze-dried giving 1.5g of polysaccharide, \([\alpha]_D^{\circ} = -3^\circ\) (c 0.064, water). The molecular weight was determined to be 560,000 by gel chromatography on Sepharose 4B (courtesy of Dr. S.C. Churms, University of Capetown).

**Sugar Analysis**

A sample (20 mg) of K39 capsular polysaccharide was dissolved in water, passed through a column of Amberlite IR-120(H\(^+\)), and freeze-dried. It was then refluxed in 3% methanolic hydrogen chloride for 20 hours. The solution was neutralized with lead carbonate, filtered, concentrated and dried under vacuum. The sample was dissolved in methanol, sodium borohydride was added, and the solution was allowed to sit overnight. The methanol was removed by evaporation and the residue was dissolved in water. The solution was acidified with Amberlite IR-120(H\(^+\)), filtered and concentrated. Borates were removed by codistillation with methanol. The methanolysis and reduction steps were repeated. The sample was then hydrolysed in 2M TFA overnight on a steam bath. PAAN derivatives were prepared by the method of Chen and McGinnis. G.l.c. analysis on 3% OV-225 (isothermal at 220°C) gave Man/Glc/Gal = 1.0/2.2/0.8.
Preparation of Alditol Acetates

In this study, both sugars and partially methylated sugars were analysed by g.l.c. as the alditol acetate derivatives. With minor variations these derivatives were prepared using the following method. Hydrolysates were dissolved in water (2 ml) and 2 drops of 10% NH₃ were added. Sodium borohydride was added and the solution was allowed to sit for 1 - 3 hours. The solution was acidified with IR-120(H⁺), filtered and concentrated by evaporation. Borates were removed by codistillation with methanol. The alditols were acetylated with acetic anhydride-pyridine (1:1) on a steam bath for 30-60 minutes. The residual acetic anhydride was destroyed by adding methanol or ethanol. The solution was concentrated and pyridine was removed by codistillation with water.

Partial Hydrolysis

The K39 capsular polysaccharide was actually prepared several times. This preliminary partial hydrolysis study was actually performed on an earlier batch. A sample of K39 polysaccharide (470 mg) was hydrolysed in 1M TFA (80 ml) for 5½ hours. The hydrolysate was concentrated by evaporation under reduced pressure, and was then applied to a column of Bio-Rad AG 1-X2. Neutral sugars were eluted by washing the column with water. Acidics were eluted with 10% formic acid. The acidic fraction was concentrated and applied to a column (96 x 2.2 cm) of Bio-Gel P-2. The mixture was eluted with water-pyridine-acetic acid (1000:10:4) at a rate of 5 ml/hour and collected in 2.5 ml
fractions. The samples were collected in tared test tubes which were then freeze-dried and reweighed to give the chromatogram in Figure 7.

In a further study K39 polysaccharide (700 mg) was hydrolysed in 1M TFA (90 ml) for 7 hours on a steam bath. The hydrolysate was concentrated and neutrals were separated from acidics as described above. The acidic fraction (290 mg) was applied to Whatman 3MM chromatography paper and irrigated with Solvent B (see Section II.1) for 4 days. Each day the papers were removed from the tank and dried to enhance resolution. Strips were cut from the sides of the papers and visualised by Method A (see Section II.1). Three bands were cut from the paper and extracted with water. The extracts were concentrated and freeze-dried giving A1 (80 mg), A2 (36 mg), and A3 (16 mg).

Analytical paper chromatography showed that A2 was not pure. A2 was applied to Whatman No. 1 paper and irrigated with Solvent B for 4 days with drying each day. A2 (15 mg) was extracted from the chromatogram.

Analysis of A1

A portion (less than 1 mg) of A1 was hydrolysed in 3M TFA on a steam bath for 20 hours. The hydrolysate was concentrated and the neutral sugars released were converted to the alditol acetates which were analysed by g.l.c. on 3% SP-2340 with a nitrogen flow rate of 20 ml/minute. Temperature program: 195°C for 4 min; increase by 2°/minute to 265°C. This analysis gave Man/Glc = 1.56/1.

Another portion (3 mg) of A1 was methanolysed and carboxyl reduced by the method already described, followed by hydrolysis in
2M TFA for 21 hours. The hydrolysate was analysed by g.l.c. on SP-2340 after conversion to the alditol acetates. This analysis gave Glc/Man = 1.47/1.

A sample (12 mg) of A1 was methylated by the Hakomori procedure. The sample was dissolved in DMSO (2 ml) in a sealed flask, which was then evacuated with nitrogen. 2M dimethylsulfinyl sodium (2 ml) was added and the solution was stirred for 50 minutes. The solution was frozen solid and methyl iodide (5 ml) was added, followed by stirring for one hour. The methyl iodide was evaporated off with a stream of nitrogen and water was added. The solution was extracted with chloroform and the combined extracts were extracted with water. The chloroform phase was concentrated and the residual DMSO was removed with a vacuum pump and heat lamp. One third of the residue was hydrolysed in 2M TFA on a steam bath for 17 hours, and the alditol acetates were prepared as previously described. The remainder of the residue was dissolved in tetrahydrofuran, lithium aluminum hydride was added, and the mixture was refluxed for 3 hours. The excess lithium aluminum hydride was destroyed by adding 90% ethanol (10 ml) followed by water (10 ml). The mixture was concentrated to dryness and washed with chloroform. The chloroform washings were concentrated to dryness and hydrolysed in 2M TFA on a steam bath for 12 hours. The hydrolysate was concentrated and alditol acetates were prepared. The partially methylated alditol acetates obtained were analysed by g.l.c. on 3% HIEFF-1B. Temperature program: 165°C for 8 minutes, increase by 2°C/minute to 200°C. G.l.c./m.s. and comparison with standard mass spectra indicated the presence of 3,4,6-Man, 2,4,6-Glc, 2,3,4-Glc and
Another sample (4.3 mg) of Al was dissolved in water, sodium borodeuteride was added, and the solution was allowed to sit for 2½ hours. The solution was acidified with Amberlite IR-120(H⁺) and the borates were removed by codistillation with methanol. The resulting oligosaccharide alditol was methylated by the Hakomori procedure. The product was hydrolysed in 2M TFA for 14 hours on a steam bath. Alditol acetates were prepared and analysed by g.l.c./m.s. 3,4,6-Man was identified by comparison with a standard spectrum. A standard spectrum was not available for 1,2,4,5,6-Glc and the m/e values obtained are therefore listed below (relative abundances in brackets): 43 (100), 44 (24), 45 (88), 46 (16), 58 (34), 59 (28), 60 (40), 71 (24), 75 (30), 85 (18), 87 (18), 88 (16), 89 (20), 90 (66), 100 (16), 101 (80), 114 (16), 206 (22).

For analysis by mass spectrometry, a sample (7.7 mg) of Al was converted to the oligosaccharide alditol by the sodium borodeuteride reduction already described. It was then methylated by the Hakomori procedure and isolated by preparative silica gel t.l.c. An infrared spectrum showed no hydroxyl absorption at 3600 cm⁻¹. Absorption maxima were observed at 2900 cm⁻¹ (C-H stretch), 1750 cm⁻¹ (C = O stretch), and 1105 cm⁻¹, 1080 cm⁻¹ (ether stretch). This permethylated oligosaccharide alditol was analysed by electron impact mass spectrometry with electron beam energies of 20 eV and 70 eV.

Analysis of A2

A2 (7.6 mg) was converted to the oligosaccharide alditol by
the sodium borodeuteride reduction already described. It was then methylated, carboxyl reduced, and hydrolysed by the same methods as Al. The products were analysed by g.l.c./m.s. as the alditol acetates. 3,4,6-Man, 2,3,4-Glc and 2,3-Glc were identified by comparison with standard mass spectra. Analysis of 1,3,4,5,6-Man by g.l.c./m.s. gave ions with the following m/e values (relative abundances in brackets):

43 (100), 45 (89), 46 (46), 59 (30), 71 (19), 72 (30), 73 (19), 75 (24), 87 (14), 88 (32), 89 (31), 101 (51), 102 (25), 103 (25), 114 (13), 130 (55), 133 (14), 141 (43), 162 (34), 187 (14).

Methylation Analysis

A sample (42 mg) of K39 capsular polysaccharide was purified by a further Cetavlon precipitation. The resulting polysaccharide was passed through a column of Amerlite IR-120(H\textsuperscript{+}) to convert it from the sodium salt form to the acid form. The freeze-dried product (35 mg) was dissolved in DMSO (7 ml) under nitrogen with heating to approximately 50°C. 2M dimethylsulfinyl sodium (5 ml) was added and the solution was stirred for 3 hours at room temperature. The solution was frozen solid and methyl iodide (12 ml) was added. The ice bath was removed and the solution was stirred for 40 minutes. Methyl iodide (2 ml) was added and the solution was stirred for another 20 minutes. The residual methyl iodide was removed with a stream of nitrogen, water was added, and the product was dialysed against running water overnight. The contents of the dialysis bag were extracted with chloroform and the combined extracts were concentrated.
and dried. Infrared analysis of the methylated polysaccharide showed no hydroxyl absorption at 3600 cm\(^{-1}\) and methylation was assumed to be complete. The infrared spectrum also showed a strong ester carbonyl absorption at 1750 cm\(^{-1}\). A short column of Sephadex LH-20 (swollen in ethanol) was prepared. The permethylated polysaccharide was dissolved in chloroform, applied to the column and eluted with ethanol-chloroform (2:1). The fast moving material was collected and concentrated. A third of this material was hydrolysed in 2M TFA overnight on a steam bath. Alditol acetates were prepared for g.l.c. analysis. The remaining permethylated polysaccharide was carboxyl reduced with lithium aluminum hydride in tetrahydrofuran for 3 hours refluxing and overnight at room temperature. The carboxyl reduced polymer was isolated from the reaction mixture as described in the analysis of A1. It was then hydrolysed in 2M TFA for 17 hours on a steam bath and alditol acetates were prepared for g.l.c. analysis. Column: 3% HIEFF-1B. Temperature program: 165°C for 8 minutes followed by an increase of 2°/minute to 200°C.

A sample (254 mg) of K39 capsular polysaccharide was hydrolysed in 0.02M TFA for a total of 3 hours on a steam bath. The hydrolysate was dialysed against running water and freeze-dried. A methylation analysis, with carboxyl reduction, was performed as previously described.
V. A PRELIMINARY STRUCTURAL INVESTIGATION
OF E. COLI K26 CAPSULAR POLYSACCHARIDE
ABSTRACT

The capsular polysaccharide of *E. coli* K26 has been isolated and a preliminary structural investigation has been performed. The polysaccharide has been studied by the methods of sugar analysis, methylation analysis, partial hydrolysis and $^1$H-n.m.r.

RESULTS AND DISCUSSION

Isolation and Sugar Analysis

The capsular polysaccharide of *E. coli* K26 was prepared and isolated by the methods described in the Experimental section. This material gave a specific rotation of $[\alpha]_D^{20} -30^\circ$. A molecular weight analysis by gel chromatography (courtesy of Dr. S.C. Churms, University of Capetown) showed that the polysaccharide moved as one band on Sepharose 4B with a molecular weight of $10^7$. According to Ørskov and coworkers, this polysaccharide is composed of rhamnose, galactose and glucuronic acid. The presence of these sugars was confirmed by hydrolysis and paper chromatography as described in the Experimental section. A sample of K26 capsular polysaccharide was methanolysed, carboxyl reduced, hydrolysed and analysed by g.l.c. as the PAAN derivatives. The chromatogram gave Rha/Gal/Glc = 3.0/2.9/1.0.

Methylation Analysis

Two methylation analysis experiments were performed: I, K26
Table 5. METHYLATION ANALYSIS OF *E. COLI* K26 CAPSULAR POLYSACCHARIDE.

<table>
<thead>
<tr>
<th>Partially Methylated Alditol Acetates&lt;sup&gt;a&lt;/sup&gt;</th>
<th>T&lt;sup&gt;b&lt;/sup&gt;</th>
<th>I&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Mole %&lt;sup&gt;c&lt;/sup&gt; I</th>
<th>Mole %&lt;sup&gt;c&lt;/sup&gt; II</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4-Rha</td>
<td>0.48</td>
<td>12.6</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>2,4-Rha</td>
<td>0.94</td>
<td>51.2</td>
<td>54.5</td>
<td></td>
</tr>
<tr>
<td>2,4,6-Gal</td>
<td>1.89</td>
<td>27.2</td>
<td>20.9</td>
<td></td>
</tr>
<tr>
<td>2-Glc</td>
<td>3.66</td>
<td>9.0</td>
<td>16.7</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> e.g., 2,3,4-Rha = 1,5-di-O-acetyl-2,3,4-tri-O-methyrlrhammitol.

<sup>b</sup> G.l.c. retention time relative to 2,3,4,6-Glc on 3% OV-225.
Temperature program: 190°C for 16 minutes, followed by an increase of 2°/minute to 230°C.

<sup>c</sup> Integrals were corrected using published molar response factors.

<sup>d</sup> I, K26 polysaccharide methylated and hydrolysed without carboxyl reduction; II, K26 polysaccharide methylated, carboxyl reduced, and hydrolysed.
polysaccharide methylated and hydrolysed without carboxyl reduction; II, K26 polysaccharide methylated, carboxyl reduced and hydrolysed. The results are given in Table 5.

Both experiments indicate the presence of 3-linked rhamnosyl residues and 3-linked galactosyl residues. The presence of 2-Glc in experiment I cannot be explained given the present knowledge of the polysaccharide. After carboxyl reduction the amount of 2-Glc increases, indicating that the glucuronosyl residues are substituted at the 3 and 4-positions. The nature of the substituents is unknown. Not all of the galactose that was indicated by the sugar analysis could be accounted for in the methylation analysis. It is therefore assumed that some of the galactose is present as an impurity.

**Partial Hydrolysis**

A sample of K26 capsular polysaccharide was hydrolysed in 0.5M TFA on a steam bath for 4 hours. Neutrals and acidics were separated by ion exchange chromatography on Bio-Rad AG 1-X2. The acidic fraction was chromatographed on paper and a compound Al (R_{Glc} 0.79, Solvent B) was isolated. The neutral fraction was chromatographed on paper and a compound N1 (R_{Glc} 1.9, Solvent C) was isolated.

**Analysis of Al**

Al was analysed by $^1$H- and $^{13}$C-n.m.r. The results are given in Table 6, Spectrum 10 and Spectrum 11. These data indicate that Al is an aldobiouronic acid consisting of αβ-glucuronosyl residue linked
Table 6. N.M.R. STUDY OF Al

A. 400 MHz $^1$H-n.m.r. at 90°C

<table>
<thead>
<tr>
<th>Chemical Shift of Anomeric $^1$H's($\delta$)</th>
<th>$J_{1,2}$ (Hz)</th>
<th>Integral Number of $^1$H's</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.71</td>
<td>8</td>
<td>0.6</td>
<td>$\beta$-GlcA linked to $\alpha$-Rha-OH</td>
</tr>
<tr>
<td>4.73</td>
<td>8</td>
<td>0.4</td>
<td>$\beta$-GlcA linked to $\beta$-Rha-OH</td>
</tr>
<tr>
<td>4.86</td>
<td>singlet</td>
<td>0.4</td>
<td>$\beta$-Rha-OH</td>
</tr>
<tr>
<td>5.12</td>
<td>2</td>
<td>0.6</td>
<td>$\alpha$-Rha-OH</td>
</tr>
</tbody>
</table>

Also:

<table>
<thead>
<tr>
<th>Chemical Shift of Anomer $^{13}$C's($\delta$)</th>
<th>Integral Number of $^{13}$C's</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.30</td>
<td>$7 (J_{5,6})$ 0.6 x 3</td>
<td>$\text{CH}_3$ of $\alpha$-Rha-OH</td>
</tr>
<tr>
<td>1.31</td>
<td>$8 (J_{5,6})$ 0.4 x 3</td>
<td>$\text{CH}_3$ of $\beta$-Rha-OH</td>
</tr>
</tbody>
</table>

B. 20 MHz $^{13}$C-n.m.r. at 35°C

<table>
<thead>
<tr>
<th>Chemical Shift of Anomer $^{13}$C's($\delta$)</th>
<th>Integral Number of $^{13}$C's</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>94.49</td>
<td>0.3</td>
<td>$\beta$-Rha-OH</td>
</tr>
<tr>
<td>94.98</td>
<td>0.7</td>
<td>$\alpha$-Rha-OH</td>
</tr>
<tr>
<td>104.98</td>
<td>1</td>
<td>$\beta$-GlcA</td>
</tr>
</tbody>
</table>

Also:

<table>
<thead>
<tr>
<th>Chemical Shift of Anomer $^{13}$C's($\delta$)</th>
<th>Integral Number of $^{13}$C's</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.54</td>
<td>1</td>
<td>$\text{CH}_3$ of Rha OH</td>
</tr>
<tr>
<td>81.44</td>
<td>0.8</td>
<td>C-3 of $\alpha$-Rha-OH</td>
</tr>
<tr>
<td>83.58</td>
<td>0.2</td>
<td>C-3 of $\beta$-Rha-OH</td>
</tr>
</tbody>
</table>
to a reducing rhamnose. A sample of Al was methanolysed, carboxyl reduced, hydrolysed and converted to the PAAN derivatives. G:1.c. analysis gave Rha/Glc = 1.4/1.

A sample of Al was methylated by the Hakomori procedure and hydrolysed in 2M TFA. Paper chromatography of the hydrolysate showed that the neutral sugar released was a dimethyl rhamnose (R2,3,6-Glc 1.04, Solvent D). The hydrolysate was reduced with sodium borohydride and acetylated. G:1.c. analysis on 3% OV-225 (isothermal at 190°C) gave a retention time of 0.92 relative to 2,3,4,6-Glc. G:1.c./m.s. analysis gave a mass spec-rum that was consistent with 1,3,5-tri-O-acetyl-2,4-di-O-methylrhammitol.28

Al has a specific rotation of [α]D -27° which is consistent with a β-D-glucuronosyl residue linked to a reducing L-rhamnose.37 Al is therefore assigned the following structure:

\[
\text{D-GlcA} \frac{1}{\beta} \text{3 L-Rha-OH}
\]

Analysis of N1

Because of its mobility, N1 was assumed to be a rhamnose containing disaccharide, but it was shown to be unaffected by acid hydrolysis. A 1H-n.m.r. spectrum (Spectrum 12) was recorded at 90°C. The spectrum showed carbohydrate ring proton signals between δ3.2 and δ4.0. Also observed were anomeric proton signals at δ4.58 (J = 7 Hz) and δ5.19 (J = 3 Hz) in the ratio 66:34. The anomeric signals indicate that N1 is a monosaccharide. The most unusual feature of this
spectrum is a group of signals between δ7.61 and δ8.39. NI is therefore assumed to contain an unsaturated moiety. (A $^1$H-n.m.r. spectrum was also recorded at ambient temperature. See Spectrum 13). A sample of the polysaccharide was purified by a further Cetavlon precipitation and a $^1$H-n.m.r. spectrum was recorded. There was no evidence that NI was present and it was assumed to be an impurity.

Nuclear Magnetic Resonance

A $^1$H-n.m.r. spectrum of E. coli K26 capsular polysaccharide was recorded at 90°C (see Spectrum 14). Too little is known about K26 for an adequate interpretation but tentative assignments of anomeric protons have been made. Coupling constants and approximate integrals are given in brackets. A signal at δ5.07 (singlet, 2H's) is assigned to α-rhamnosyl residues. A signal at δ4.87 (singlet, 1H) is assigned to a β-rhamnosyl residue, although the signal is broader than expected. A signal at δ4.72 ($J_{1,2} = 7$ Hz, 1H) is assigned to a β-glucuronosyl residue. A signal at δ1.31 is assigned to the methyl protons of rhamnosyl residues.

Conclusions

Dr. M. Heidelberger of the New York University Medical Center studied the cross-reactions of E. coli K26 with the anti-sera to a number of Pneumococcal K antigens. His results are given in Appendix IV. He attributed some of the cross-reactions to the possible presence of 3-linked L-rhamnosyl residues. The presence of 3-linked
rhamnosyl residues has been confirmed by the methylation analysis, and by the isolation of an aldobiouronic acid by partial hydrolysis.
EXPERIMENTAL

Isolation and Purification

An authentic culture of *E. coli* K26 was incubated in broth and then grown on agar trays for 3 days (the agar was prepared as described in Section IV). The bacterial slime was scraped off of the trays and the solution was made 2% in phenol. Cellular debris was removed by ultracentrifugation at 30,000 r.p.m. for 4 hours. The supernatant (570 ml) was added to methanol (1800 ml) but no precipitate appeared. On addition of acetone (1000 ml), the polysaccharide precipitated out of solution. The acidic capsular polysaccharide was isolated by two Cetavlon precipitations, as described in Section IV. After dialysis and freeze-drying the yield was 660 mg. \([\alpha]_D^{20}\) -50° (c 0.13, water).

A molecular weight analysis by gel chromatography (courtesy of Dr. S.C. Churms, University of Capetown) showed that the polysaccharide moved as one band on Sepharose 4B, and that the molecular weight was 1.7 x 10^7.

Sugar Analysis and Methylation Analysis

A portion (2.5 mg) of K26 capsular polysaccharide was hydrolysed in 2M TFA for 17 hours on a steam bath. The hydrolysate was chromatographed on Whatman No. 1 paper with Solvent C, along with xylose, glucose, mannose, galactose, fucose, and rhamnose as standards. Galactose and rhamnose were observed along with traces of mannose and glucose. Some unidentified materials were also observed. A sample
(11 mg) of polysaccharide was methanolyzed by refluxing in 3% methanolic hydrogen chloride, reduced with sodium borohydride and hydrolysed in 2M TFA as described in Section IV. The hydrolysate was chromatographed on Whatman No. 1 paper with Solvent C. Rhamnose, galactose and a dark spot due to glucose were observed, thus proving that glucuronic acid is present.

The portion of polysaccharide used for the following sugar and methylation analyses was purified by a further Cetavlon precipitation.

A sample (34 mg) of K26 polysaccharide was passed through a column of Amberlite IR-120(H⁺) and freeze-dried. A sample (28 mg) was set aside for methylation analysis and the remainder was refluxed in 3% methanolic hydrogen chloride and reduced with sodium borohydride in methanol as described in Section IV. The methanolyis and reduction were repeated. The products were then hydrolysed in 2M TFA overnight on a steam bath. Water and TFA were removed by evaporation under reduced pressure. PAAN derivatives were prepared by the method of Chen and McGinnis. The integrals were corrected with molar correction factors. G.l.c. analysis on 3% OV-225 (isothermal at 220°C) gave Rha/Gal/Glc = 3.0/2.9/1.0.

A sample (28 mg) of K26 polysaccharide was dissolved in DMSO (5 ml), 2M dimethylsulfinyl sodium (3 ml) was added, and the solution was stirred for one hour. The solution was frozen solid and methyl iodide (5 ml) was added after removal of the dry ice/acetone bath. After stirring for one hour, the residual methyl iodide was removed with a stream of nitrogen. Chloroform and water were added and the mixture was dialysed against running water. The contents of the
dialysis bag were extracted with chloroform, concentrated and purified by chromatography on Sephadex LH-20. (The resin was swelled in chloroform and eluted with chloroform). An infrared spectrum of the product showed no hydroxyl absorption at 3600 cm\(^{-1}\) and a carbonyl absorption at 1740 cm\(^{-1}\). Methylation was assumed to be complete. A third of this material was hydrolysed in 2M TFA for 16 hours on a steam bath and alditol acetates were prepared for g.l.c. analysis. The remaining methylated polysaccharide was carboxyl reduced with lithium aluminum hydride in refluxing tetrahydrofuran for 3 hours. The residual lithium aluminum hydride was destroyed with ethanol. 10% hydrochloric acid was added to dissolve the aluminates, and the solution was extracted with chloroform. After concentrating and drying, an infrared spectrum showed no carbonyl absorption. This material was hydrolysed in 2M TFA overnight on a steam bath and concentrated under reduced pressure. A sample of the hydrolysate was chromatographed on Whatman No. 1 paper with Solvent D and visualised with p-anisidine hydrochloride. The chromatogram indicated the presence of a trimethyl rhamnose (R\(_2,3,6\)-Glc 1.19, green), a dimethyl rhamnose (R\(_2,3,6\)-Glc 1.05, green), a trimethyl galactose (R\(_2,3,6\)-Glc 0.89, pink), and a monomethyl glucose (R\(_2,3,6\)-Glc 0.35, brown). Alditol acetates were prepared and analysed by g.l.c. on 3% OV-225 (190°C for 16 minutes followed by an increase of 2°/minute to 230°C). The PMAAs obtained from the carboxyl reduced product were analysed by g.l.c./m.s. on 3% HIEFF-1B (165°C for 8 minutes followed by an increase of 2°/min to 200°C).
Partial Hydrolysis

A sample (415 mg) of K26 capsular polysaccharide was dissolved in 0.5M TFA (70 ml) and heated on a steam bath for 4 hours. Water and TFA were removed by evaporation under reduced pressure. Neutrals and acidics were separated by ion exchange chromatography on Bio-Rad AG 1-X2 (formate form). The hydrolysate was applied to the column and washed with water (250 ml) to give the neutral fraction (266 mg after concentration and freeze-drying). The column was then washed with 10% formic acid (50 ml), 20% formic acid (50 ml), and 10% formic acid (100 ml) to give the acidic fraction (107 mg after concentration and freeze-drying).

The acidic fraction was chromatographed on Whatman 3MM paper with Solvent B. One component (designated A1) had approximately the same mobility as galactose. This material was extracted from the paper and chromatographed on Whatman No. 1 paper with Solvent C (a basic solvent) overnight, and then for 7 hours with Solvent B (an acidic solvent). Under these conditions A1 moves slower than a neutral monosaccharide, and was therefore assumed to be acidic, but the chromatogram showed that further purification was necessary. A1 was applied to Whatman 3MM paper and irrigated with Solvent C for 19 hours and Solvent B for 16 hours. A1 was extracted from the paper and gave a yield of 22 mg after freeze-drying. $R_{Glc}^0.79$ (Solvent B). $[\alpha]_D^{-27^\circ}$ (c:0.090, water).

A slower moving component was also isolated from the initial chromatogram but $^1$H-n.m.r. showed that it was not pure enough for
unambiguous analysis.

A portion (44 mg) of the neutral fraction was chromatographed on Whatman 3MM paper with Solvent C. A component, designated NI, was isolated from the chromatogram and gave a yield of 15 mg after freeze-drying. \( R_{\text{Glc}} = 1.9 \) (Solvent C). A sample of NI was hydrolysed in 2M TFA on a steam bath overnight. Chromatography on Whatman No. 1 paper with Solvent C showed that NI was not hydrolysed.

Analysis of A1

A sample of A1 (2.5 mg) was methanolysed in 3% methanolic hydrogen chloride, carboxyl reduced with sodium borohydride in methanol, and hydrolysed in 2M TFA overnight on a steam bath. PAAN derivatives were prepared by the method of Chen and McGinnis. Analysis by g.l.c. on 3% OV-225 (isothermal at 220°C) gave \( \text{Rha/Glc} = 1.4/1 \).

Another sample of A1 (7.6 mg) was methylated by the Hakomori procedure (DMSO (0.8 ml), 2M dimethylsulfinyl sodium (0.8 ml), methyl iodide (1 ml)). A portion of the methylated product was hydrolysed in 2M TFA for 17 hours on a steam bath. A sample of the hydrolysate was chromatographed on Whatman No. 1 paper with Solvent D and sprayed with p-anisidine hydrochloride. A green spot appeared at \( R_{2,3,6-\text{Glc}} = 1.04 \) and was assumed to be a dimethyl rhamnose. Two slower moving acidic components were also observed and were bright pink in colour. The hydrolysate was analysed by g.l.c. and g.l.c./m.s. as the alditol acetates. On 3% OV-225 (isothermal at 190°C) a component was observed with a retention time of 0.92 relative to 2,3,4,6-Glc. The mass
spectrum of this compared well with a standard spectrum of a 1,3,5-tri-
0-acetyl-2,4-di-O-methylhexitól.
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VI  BIBLIOGRAPHY

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APPENDIX I: Qualitative Sugar Analysis of the *Klebsiella* Capsular Polysaccharide.

<table>
<thead>
<tr>
<th>Sugar Composition</th>
<th>Positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucuronic Acid, Galactose, Glucose</td>
<td>8&lt;sup&gt;P&lt;/sup&gt;, 11&lt;sup&gt;P&lt;/sup&gt;, 15, 51, 25, 27&lt;sup&gt;P&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucuronic Acid, Galactose, Mannose</td>
<td>20, 21&lt;sup&gt;P&lt;/sup&gt;, 29&lt;sup&gt;P&lt;/sup&gt;, 42&lt;sup&gt;P&lt;/sup&gt;, 43, 66, 74&lt;sup&gt;P&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucuronic Acid, Galactose, Rhamnose</td>
<td>9, 47, 52, 9&lt;sup&gt;*&lt;/sup&gt;, 81, 83</td>
</tr>
<tr>
<td>Glucuronic Acid, Glucose, Mannose</td>
<td>2, 4, 5&lt;sup&gt;P&lt;/sup&gt;, 24</td>
</tr>
<tr>
<td>Glucuronic Acid, Glucose, Rhamnose</td>
<td>17, 44, 71</td>
</tr>
<tr>
<td>Glucuronic Acid, Glucose, Fucose</td>
<td>1, 54</td>
</tr>
<tr>
<td>Glucuronic Acid, Galactose, Glucose, Mannose</td>
<td>10, 28, 39, 50, 59, 61, 62, 7&lt;sup&gt;P&lt;/sup&gt;, 13&lt;sup&gt;P&lt;/sup&gt;, 26&lt;sup&gt;P&lt;/sup&gt;, 30&lt;sup&gt;P&lt;/sup&gt;, 31&lt;sup&gt;P&lt;/sup&gt;, 33&lt;sup&gt;P&lt;/sup&gt;, 35&lt;sup&gt;P&lt;/sup&gt;, 46&lt;sup&gt;P&lt;/sup&gt;, 69&lt;sup&gt;P&lt;/sup&gt;, 60</td>
</tr>
<tr>
<td>Glucuronic Acid, Galactose, Glucose, Fucose</td>
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</tr>
<tr>
<td>Glucuronic Acid, Galactose, Glucose, Rhamnose</td>
<td>18, 19, 23, 41, 79, 12&lt;sup&gt;P&lt;/sup&gt;, 36&lt;sup&gt;P&lt;/sup&gt;, 45&lt;sup&gt;P&lt;/sup&gt;, 55&lt;sup&gt;P&lt;/sup&gt;, 70&lt;sup&gt;P&lt;/sup&gt;</td>
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<tr>
<td>Glucuronic Acid, Galactose, Mannose, Rhamnose</td>
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<tr>
<td>Glucuronic Acid, Glucose, Mannose, Fucose</td>
<td>6&lt;sup&gt;P&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucuronic Acid, Glucose, Mannose, Rhamnose</td>
<td>64&lt;sup&gt;P&lt;/sup&gt;, 65&lt;sup&gt;P&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucuronic Acid, Galactose, Glucose, Mannose, Fucose</td>
<td>68&lt;sup&gt;P&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucuronic Acid, Galactose, Glucose, Mannose, Rhamnose</td>
<td>14&lt;sup&gt;P&lt;/sup&gt;, 67</td>
</tr>
<tr>
<td>Galacturonic Acid, Galactose, Mannose</td>
<td>3&lt;sup&gt;P&lt;/sup&gt;, 49, 57</td>
</tr>
<tr>
<td>Galacturonic Acid, Glucose, Rhamnose</td>
<td>34, 48</td>
</tr>
<tr>
<td>Galacturonic Acid, Galactose, Fucose, Rhamnose</td>
<td>63</td>
</tr>
<tr>
<td>Pyruvic Acid, Glucose, Rhamnose</td>
<td>72</td>
</tr>
<tr>
<td>Pyruvic Acid, Galactose, Rhamnose</td>
<td>32</td>
</tr>
<tr>
<td>Pyruvic Acid, Galactose, Glucose, Rhamnose</td>
<td>56</td>
</tr>
</tbody>
</table>

K82 has been added but its qualitative composition is not yet known.  

P- Pyruvic acid present in addition
APPENDIX II

Nuclear Magnetic Resonance Spectra
Spectrum 1.

100 MHz $^3$H-n.m.r. of K39-A1 at 95°C.
Spectrum 2. 100 MHz $^1$H-n.m.r. of K39-A1(reduced) at 90°C.
Spectrum 3.

400 MHz $^1$H-n.m.r. of K39-A1(reduced)

5.37 5.27

HOD

4.58 4.51
Spectrum 4. 400 MHz $^1$H-n.m.r. of K39-A1
Spectrum 5. $^{13}$C-n.m.r. of K39-A1

Acetone

31.07

102.31, 100.92, 96.74, 92.98
Spectrum b. 400 MHz 'H-n.m.r. of K39-A2

5.15

5.35

4.93

H2O

4.55

4.10

5.0

4.0
Spectrum 7.

400 MHz $^1$H-n.m.r. of K39-A2 (reduced)
Spectrum 7a.

$^{13}$C-n.m.r. of K39-A2

Acetone 31.07

103.04
102.37
100.80
93.30
Spectrum 8. 400 MHz $^1$H-n.m.r. of K39 polysaccharide at 90°C.
Spectrum 9. 400 MHz $^1$H-n.m.r. of K39 polysaccharide (no acetone or depolymerisation).
Recorded at 90°C.
Spectrum 9a. $^{13}$C-n.m.r. of K39 polysaccharide.
Spectrum 10. 400MHz $^1$H-n.m.r. of K26-A1 at 90°C
Spectrum II. $^{13}$C n.m.r. of K26-A1
Spectrum 12.

400 MHz 1H-n.m.r. of K26-N1 at 90°C.
Spectrum 13.

400 MHz $^1$H-n.m.r. of
K26-N1 (ambient temperature).

Acetone
2.23
Figure 14. *E. coli* K26 polysaccharide.
400 MHz $^1$H-n.m.r. at 90°C.
The main purpose for the m.s. analysis of K39-A1 as the permethylated alditol was to distinguish between the following two possible structures:

\[
X: \quad \text{GlcA} \begin{array}{c}
\frac{1}{\beta} \\
\frac{2}{\alpha}
\end{array} \text{Man} \begin{array}{c}
\frac{1}{\beta} \\
\frac{4}{\alpha}
\end{array} \text{GlcA} \begin{array}{c}
\frac{1}{\beta} \\
\frac{2}{\alpha}
\end{array} \text{Man} \begin{array}{c}
\frac{1}{\beta} \\
\frac{3}{\alpha}
\end{array} \text{Glc-OH}
\]

\[
Y: \quad \text{GlcA} \begin{array}{c}
\frac{1}{\beta} \\
\frac{4}{\alpha}
\end{array} \text{GlcA} \begin{array}{c}
\frac{1}{\beta} \\
\frac{2}{\alpha}
\end{array} \text{Man} \begin{array}{c}
\frac{1}{\beta} \\
\frac{2}{\alpha}
\end{array} \text{Man} \begin{array}{c}
\frac{1}{\beta} \\
\frac{3}{\alpha}
\end{array} \text{Glc-OH}
\]

In their study of a permethylated aldtriouronic acid, Kovacik and coworkers showed that the A-series fragments should be intense enough to provide unambiguous sequence information. Structure X gives the following m/e values for the A-series: 437 (ba\textsubscript{1}), 405 (ba\textsubscript{2}), 373 (ba\textsubscript{3}). Structure Y gives the following m/e values for the A-series: 451 (ba\textsubscript{1}), 419 (ba\textsubscript{2}), 387 (ba\textsubscript{3}). A spectrum was recorded with an electron beam energy of 20 eV. The m/e values mentioned above are given in the following list with the relative abundances obtained given in brackets: 373 (46), 387 (18), 405 (100), 419 (52), 437 (14), 451 (18). Therefore:

\[
\frac{\text{Sum of the abundances for structure X}}{\text{Sum of the abundances for structure Y}} = 1.8
\]

A spectrum was recorded with an electron beam energy of 70 eV. The
m/e values mentioned above are given in the following list with the relative abundances given in brackets: 373 (59), 387 (22), 405 (100), 419 (49), 437 (20), 451 (18). Therefore:

\[
\frac{\text{Sum of the abundances for structure } X}{\text{Sum of the abundances for structure } Y} = 2.0
\]

On the basis of these data, structure X is preferred. Further studies of the permethylated alditol of K39 - A1 could include a reduction of the esters to primary alcohols with a deuterating reducing agent, followed by realkylation. M.s. analysis of the product could provide more information regarding the origin of the fragment ions mentioned above, and thus remove any ambiguity in the sugar sequence determination.

Dear Katzir, and Leck:

I have run cross-reactions on K26. These came out as follows: ++ in anti-PhI, +++ in anti-Ph VI, ++, +++ in anti-XXII, XXX, + in anti-3 paratyphi A, - or + in all others tested. Am pretty sure it has non-reducing lateral end-groups of D-gal A, partially hindered, either conformationally or by Ac or some other substituent. If it were 1,2-linked D-gal A there should have been positive in anti-XXV. Reactions in anti-XXIII and XXIV point to such end-groups of L-rham, as well. Most probable, is that both positive spots are due to 1,3-L-rham in tandem, or with XXIII, L-rham as end-groups and 1,4-linked. ++ in anti-III could be due to 1,3-L-rham or 1,2-
1,4-end-groups of B-group, though the last is unlikely because of - in anti-XXIV.

If you send me 5010 units of your purest K28 I'd be glad to put it through the mill unless it is from K 147, which I've done already.

With best wishes and a Merry Christmas and Happy New Year to you all,

Sincerely,

[Signature]
APPENDIX V: An Explanation of Carbohydrate Terminology

In an effort to familiarize readers who do not work in the particular area of organic chemistry to which this thesis refers, the following explanation of terms used is offered.

Fischer projection formulae are used to represent the acyclic modification of sugars. Some examples are shown below. Numbering commences from the carbonyl group at the top of the chain (I). Note that D-glucuronic acid (II) differs from D-glucose (I) only

\[
\begin{align*}
\text{D-glucose (I)} & \quad \text{D-glucuronic acid (II)} & \quad \text{L-rhamnose (III)} \\
1 & \quad \text{CHO} & \quad \text{CHO} & \quad \text{CHO} \\
2 & \quad -\text{OH} & \quad -\text{OH} & \quad * -\text{OH} \\
3 & \quad \text{HO} & \quad \text{HO} & \quad * -\text{OH} \\
4 & \quad -\text{OH} & \quad -\text{OH} & \quad \text{HO} - * \\
5 & \quad -\text{OH} & \quad -\text{OH} & \quad \text{HO} - * \\
6 & \quad \text{CH}_2\text{OH} & \quad \text{COOH} & \quad \text{CH}_3
\end{align*}
\]

in that C-6 is oxidized to a carboxylic acid group. The C-6 of L-rhamnose (III) is part of a methyl group and is referred to also by another common name, 6-deoxy-L-mannose.

There are four chiral centers in these six-carbon chains (marked with asterisks in structure III) making it important to appreciate the spatial arrangement of atoms that is implied by these Fischer representations. To simplify the nomenclature of all the possible isomers (16 for each of
I, II, III), all those having the hydroxyl group at the highest-numbered chiral center (C-5) projecting to the right in the Fischer projection formulae belong to the D-series, and the others to the L-series.

Physical and chemical evidence indicates that, in fact, these six-carbon polyhydroxyaldehydes exist in a cyclic form. The ring closure occurs by nucleophilic attack of the oxygen atom at C-5 on the aldehydic carbon atom, generating a new chiral (anomeric) center at C-1. This results in two anomers, represented below.
in the Tollens formulae. It should be noted that C-1 is unique in having two attached oxygen atoms, formally making it a hemiacetal carbon.

Since the Tollens formulae have obvious limitations with their unequal bond lengths, Haworth developed a perspective method of looking at the six-membered ring (VI and VII). This improvement recognizes that the ring oxygen atom lies behind the carbon chain and that bond lengths are approximately equal. Often in practise regular hexagons are used in Haworth projections,

\[
\begin{align*}
\text{α-D-glucopyranose} & \quad \beta-D-glucopyranose \\
(VI) & \quad (VII) \\
\text{pyran} & \quad (VIII)
\end{align*}
\]

which he related to such rings as the heterocyclic compound pyran (VIII) and named them pyranoses. Note that hydroxyl groups not involved in ring formation on the right in Fischer and Tollens formulae point down in the Haworth projections and those on the left point up. Similarly, for aldopyranoses, the group on C-5 points up for D (IX) and down for the L enantiomer (X), It follows, then, that when sugar residues are attached there are two possible configurations, an α- or a β-pyranoside, for each linkage.
The true conformation of pyranoid carbohydrates is related to the chair form of cyclohexane. X-ray diffraction analysis has shown that a hexose, such as \( \alpha\)-D-glucose (XI), consists of a puckered, six-membered, oxygen-containing carbon ring, with hydroxyl substituents at C-1 through C-4, and a hydroxymethyl group at C-5. All substituents on the ring, except for that at C-1, are equatorial.

Two isomers (anomers) are possible in relation to the anomeric center (C-1), depending on whether a substituent is
axial (α-anomer; XII) or equatorial (β-anomer; XIII), where
R = hydrogen, for monosaccharides, and R = another sugar residue,
for di-, oligo-, and polysaccharides. Since H-1 is in a different
chemical environment for the two anomers, nuclear magnetic
resonance spectroscopy can easily distinguish between them
and, thereby, provides invaluable assistance in assigning
anomeric configurations.

Haworth projections are most useful and will be used in
this thesis, even though they give no indication of three-
dimensional molecular shape. There seems to be little justifi-
cation for the use of formulae which depict states of molecules
as well as structures, when the true states are often unknown
or variable.

- Reproduced from the M.Sc. thesis of T.E. Folkman (University of
  British Columbia, 1979). -