STRUCTURAL STUDIES OF THE CAPSULAR POLYSACCHARIDES FROM SOME STRAINS OF *KLEBSIELLA* AND *CRYPTOCOCCUS NEOFORMANS* MICRO-ORGANISMS

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DOCTOR OF PHILOSOPHY

by

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CONTENTS

SUMMARY

The structures of the exopolysaccharides from a number of strains of the bacterial genus *Klebsiella* have been investigated and compared. All of these polysaccharides have been shown to be composed of regularly repeated oligosaccharide units containing D-glucuronic acid and three to five hexose residues, with pyruvic acid ketal and O-acetyl groups also present in some of the polysaccharides.

Graded acid hydrolysis, monitored by gel-permeation chromatography, has been used to study the degradation of each polysaccharide to the structurally significant oligosaccharides and fragments of higher molecular weight which are clearly aggregates of these units. In all cases both acidic and neutral oligosaccharides have been isolated in a high degree of purity, which has permitted their characterisation by standard techniques, including partial acid hydrolysis and methylation analysis, and the use of proton magnetic resonance spectroscopy and measurement of optical rotatory power to determine the nature of anomeric linkages.

The polysaccharides from *Klebsiella* K4 and *K.pneumoniae* (*oxytoca* variant) have been shown to have linear tetra- and pentasaccharide repeating units respectively, while the structures of those from serotypes K27 and K64 have been found to be more complex, consisting of branched hexasaccharide repeating units with pyruvic acid ketal-linked to one sugar residue.

In addition, the fungal polysaccharides from two strains of *Cryptococcus neoformans* have been investigated and shown to be chemically equivalent. The evidence obtained from partial acid hydrolysis, methylation analysis and Smith-degradation studies performed on one of these polysaccharides is consistent with a structure in which a linear (1→3)-linked chain of D-mannose residues is substituted at position C-2 by

either D-glucuronic acid or D-xylose. This represents one of the few complete characterisations of a *Cryptococcus* polysaccharide to be achieved up to the present.

Part of the work reported in this thesis has been published, *viz*;

Structural studies on a capsular polysaccharide from *Cryptococcus neoformans* - E.H. Merrifield, A.M. Stephen, and J. van den Ende, *S. Afr. J. Sci.*, 74, 146 (1978).

A C K N O W L E D G E M E N T S

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ABBREVIATIONS

c	concentration
ca.	approximately
c.d.	circular dichroism
cf.	compare
cm	centimetre
eV	electron volt
Fuc	fucose
g	gravity
g	gram
Gal	galactose
g.l.c.	gas-liquid chromatography
Glc	glucose
GlcA	glucuronic acid
h	hour (s)
HMDS	hexamethyldisilizane
Hz	hertz
i.r.	infrared
l	litre
m	metre
M	molar
Man	mannose
mg	milligram
min	minute
ml	millilitre
mm	millimetre
m.s.	mass spectrometry
mol	mole
mol. wt.	molecular weight

$\overline{M}_w$	weight-average molecular weight
µg	microgram
µm	micrometre
n.d.	not determined
nm	nanometre
p	pyranose
p.m.r.	proton magnetic resonance
Rha	rhamnose
r.p.m.	revolutions per minute
TMS	trimethylsilyl
TMSC	trimethylsilyl chloride
u.v.	ultraviolet
V	volts
v/v	volume per volume
wt.	weight
w/w	weight per weight
Xyl	xylose

Abbreviations and symbols omitted from the above list are defined in the text.

INDEX TO FIGURES

INDEX TO TABLES

INDEX TO TABLES (CONTD)

1. INTRODUCTION

1.1 THE OCCURRENCE AND NATURE OF *KLEBSIELLA* MICRO-ORGANISMS

The light microscope reveals two principal forms of eubacteria : more or less spherical organisms known as cocci and cylindrical ones called bacilli. The genus *Klebsiella* belongs to the family *Enterobacteriaceae* which is part of a large heterogeneous group of gram-negative bacilli that naturally inhabit the gastrointestinal tract of man, and are collectively referred to as the enteric bacilli.

In their virulence for man, the enteric bacilli fall into three general categories. The first includes the coliform bacilli, of which *Klebsiella* is a member, the proteus organisms, *Pseudomonas aeruginosa*, and *Alcaligenes faecalis*. These common inhabitants of the normal human gastrointestinal tract are ordinarily non-pathogenic, causing disease (most often of the urinary tract) only under special circumstances. The second group contains the *Salmonellae*, highly invasive pathogens, some of which characteristically give rise to systemic infections such as typhoid fever. The third is composed of the *Shigellae* and *Vibrio cholerae*, relatively noninvasive microbes, which produce the superficial though disabling intestinal lesions of bacillary dysentery and cholera.

None of the enteric bacilli form spores and all are relatively small gram-negative rods, measuring 2 to 3µm in length and 0.4 to 0.6µm in breadth. Strains of *Klebsiella* are characterised by capsule production, which gives rise to large mucoid colonies. These bacterial capsules are loose, gel-like structures which vary widely among strains in thickness, density and adherence to the cell wall. Capsules are most easily demonstrated in India ink suspension, where they are recognised as a clear zone between the opaque medium and the more refractile cell body.

They can also be rendered visible by exposure to specific anti-capsular antibodies which increase their refractility, or by special stains. Capsules in pathogenic bacteria often play a major role in determining virulence because they protect the cells from phagocytosis. A given bacterial species may include strains of different antigenic types which produce immunologically distinct capsules. In the case of *Klebsiella*, approximately 80 different strains have been recognised on the basis of their serological reactions. The substances responsible for the immunological specificity of these capsules are relatively simple acidic polysaccharides which contain regular sequences of sugars. Toennissen[1] was the first worker to draw attention to the serological specificity of *Klebsiella* capsules and to demonstrate the carbohydrate nature of the capsular antigens, while Kauffmann[2] published the first antigenic classification of the *Klebsiella* group based on the recognition of somatic (O) and capsular (K) antigens.

Klebsiella pneumoniae (Friedlander's bacillus) is the most important human pathogen of the *Klebsiella* group. Except for being non-motile, it is otherwise morphologically and metabolically indistinguishable from *Aerobacter aerogenes*, which also belongs to the coliform bacilli. The similarities of all the *Aerobacter* and *Klebsiella* organisms are in fact so great that Kauffmann suggested that they be classified in the same genus, but this suggestion has not generally been adopted because of the striking differences in their pathogenicities.

K. pneumoniae is found in the nose, mouth and intestinal tracts of healthy subjects and is frequently present as a secondary invader in the lungs of patients with bronchietasis or other chronic pulmonary disease. Its invasive properties are much like those of *Pneumococcus* being primarily due to the antiphagocytic effect of its capsule.

Pneumonia caused by *K. pneumoniae* is characterised by the production of thick, gelatinous sputum and frequently leads to the formation of lung abscesses. The high population densities of the *K. pneumoniae* organisms reached in the oedema zones of the active lesions are due to the very large capsules which impede phagocytosis. The destructive action of the unphagocytized organisms on the pulmonary tissue interferes with antimicrobial therapy and often results in chronic lung abscesses requiring surgical re-section. Streptomycin, chloramphenicol and tetracycline antibiotics are all commonly used in the treatment of *Klebsiella* infections.

Like other coliform bacilli *Klebsiella* organisms cause infections of the human urinary tract. In addition, two other *Klebsiella* species have been implicated in chronic inflammatory diseases of the upper respiratory tract. *Klebsiella ozaenae* has been isolated from the noses of patients with ozaena a progressive fetid atrophy of the nasal mucosa, and *Klebsiella rhinoscleromatis* has been cultured from patients with rhinoscleroma, a destructive granuloma of the nose and pharynx. In an examination of 256 *Klebsiella* cultures obtained during a study of the biochemical and serological reactions of *Klebsiella* capsules, Edwards and Fife[3] found that more than half the cultures were isolated from sputum and bronchial exudate or from urine and the genito-urinary tract. Other sources of *Klebsiella* cultures included the lungs and pleura, stools and intestinal content, pus, blood, abscesses, bones and joints.

The *Klebsiella* strains used for the investigations described in this thesis were isolated from patients at Groote Schuur Hospital, Cape Town, or obtained from the National Collection of Type Cultures, London. All strains were serotyped by courtesy of Dr. I. Ørskov (WHO, Copenhagen).

1.2 THE STRUCTURES OF *KLEBSIELLA* CAPSULAR POLYSACCHARIDES

A qualitative study of the chemical constituents of the polysaccharides from some 80 different *Klebsiella* K-serotypes by Nimmich[4,5] has led to the grouping of the capsular polysaccharides according to their component monosaccharides. These different chemotypes are listed in Table 1, from which it can be seen that certain combinations of sugars are unique, whereas others may be found in as many as seventeen strains. In the latter case it is clearly of particular immunochemical interest to determine the primary structures of the polysaccharides from different serotypes within the same chemotype. This is the major objective of the work described in this thesis. Several *Klebsiella* polysaccharides of different chemotypes have been examined and their structures compared with those postulated for other polysaccharides within the appropriate groups.

The majority of *Klebsiella* capsular polysaccharides owe their acidity to D-glucuronic acid, which is found in combination with hexose and deoxyhexose sugars. D-Galacturonic acid is found in a limited number of strains, while the acidity of the polysaccharides from serotypes K32[6], K56[7] and K72[8] is entirely due to the presence of pyruvic acid, which supplements the acid content in many other *Klebsiella* polysaccharides. The structures of the keto-acids present in the polysaccharides from K37[9] and K38[10] have been determined, but that found in the K22 polysaccharide is still under examination[11]. Hexose sugars occurring in *Klebsiella* polysaccharides include D-glucose, D-galactose, D-mannose and the deoxy sugars L-rhamnose and L-fucose.

One of the first *Klebsiella* polysaccharides to be investigated was that of K-type 54. Results of a preliminary study by Aspinall and coworkers[12] in 1956 suggested that the slime polysaccharide, composed of D-glucuronic acid, D-glucose and L-fucose, had a very complex non-repeating structure. However, ten years later a re-examination of this polysaccharide by Sandford and Conrad[13,21], using the much improved methylation methodology of Hakomori[14], showed that the D-glucose, D-glucuronic acid and L-fucose residues were present in a simple molar ratio of 2:1:1 respectively. A sequence analysis, involving radiochemical methods, of the oligosaccharides obtained on partial hydrolysis indicated that the polysaccharide was composed of simple tetrasaccharide repeating units. The regularity of this repeating unit was later demonstrated in a study of the breakdown of the polysaccharide under varying conditions of mild acid hydrolysis[15], in which the structurally significant tetrasaccharide was detected, by gel-permeation chromatography, together with products having higher molecular weights which were clearly aggregates of this oligosaccharide.

The polysaccharide from *Klebsiella* K-type 1, which belongs to the same chemotype as K54, was the subject of a preliminary investigation by Barker and coworkers in 1963[16], in which methylation analysis and periodate oxidation studies indicated that most of the sugars were linked through the 3-position. A more recent study by Lindberg *et al.*[17] has revealed that the polysaccharide has a simple trisaccharide repeating unit consisting of the aldobiouronic acid 4-O-(β-D-glucopyranosyluronic acid)-L-fucose and a residue of D-glucose which is linked through O-3 in the polysaccharide chain. Pyruvic acid is linked as a ketal to the 2- and 3-positions of the D-glucuronic acid residue, which is in turn linked through O-4 in the chain; this provides the first instance of pyruvic acid

TABLE I

KLEBSIELLA CAPSULAR POLYSACCHARIDES (K1 - K83) GROUPED ACCORDING TO CHEMOTYPE

Glucuronic Acid, Galactose, Glucose	8^{P}, 11^{P}, 15, 51, 25, 27^{P}, 78
Glucuronic Acid, Galactose, Mannose	20, 21^{P}, 29^{P}, 42^{P}, 43, 66, 74^{P}, 76
Glucuronic Acid, Galactose, Rhamnose	9, 47, 52, 9*, 81, 83
Glucuronic Acid, Glucose, Mannose	2, 4, 5^{P}, 24
Glucuronic Acid, Glucose, Rhamnose	17, 44, 71
Glucuronic Acid, Glucose, Fucose	1, 54
Glucuronic Acid, Galactose, Glucose, Mannose	10, 28, 39, 50, 59, 61, 62, 77, 7^{P}, 13^{P}, 26^{P}, 30^{P}, 31^{P}, 33^{P}, 35^{P}, 46^{P}, 69^{P}
Glucuronic Acid, Galactose, Glucose, Fucose	16, 58^{P}
Glucuronic Acid, Galactose, Glucose, Rhamnose	18, 19, 23, 41, 79, 12^{P}, 36^{P}, 45^{P}, 55^{P}, 70^{P}, 73^{P}
Glucuronic Acid, Galactose, Mannose, Rhamnose	53, 40, 80^{P}
Glucuronic Acid, Glucose, Mannose, Fucose	6^{P}, 60
Glucuronic Acid, Glucose, Mannose, Rhamnose	64^{P}, 65^{P}
Glucuronic Acid, Galactose, Glucose, Mannose, Fucose	68^{P}
Glucuronic Acid, Galactose, Glucose, Mannose, Rhamnose	14^{P}, 67
Galacturonic Acid, Galactose, Mannose	3^{P}, 49, 57, 75
Galacturonic Acid, Glucose, Rhamnose	34, 48
Galacturonic Acid, Galactose, Fucose, Rhamnose	63
Pyruvic Acid, Glucose, Rhamnose	72
Pyruvic Acid, Galactose, Rhamnose	32
Pyruvic Acid, Galactose, Glucose, Rhamnose	56
Keto Acid, Galactose, Glucose	22, 37, 38

^{P} - Pyruvic acid present in addition

being bound to uronic acid in a *Klebsiella* polysaccharide. Graded acid hydrolysis studies monitored by gel-permeation chromatography[18] have again confirmed the size of the repeating unit, the trisaccharide and oligomers having higher molecular-weights which were multiples of this being plainly evident from the chromatograms obtained.

The regularity of oligosaccharide sequences within complex heteropolysaccharides, although widely accepted, had received experimental confirmation in only a few instances prior to 1966. Since then, however, the primary structures of more than thirty *Klebsiella* capsular polysaccharides have been published, and all reports have indicated structures consisting of regularly repeated oligosaccharide sequences; the polysaccharides are believed to be biosynthesised from such oligosaccharides. The structural patterns of the capsular polysaccharides from *Klebsiella* strains which have been elucidated to date are given in Table 2, from which it is apparent that these glycans differ only by rather limited variations in the polysaccharide skeleton. Because of this basic similarity, *Klebsiella* polysaccharides of known structure have been used in recent years for a variety of comparative studies, by p.m.r. spectroscopy[19,20], on the structural basis for the serological cross-reactions amongst different *Klebsiella* K-antigens[22,23], and between these and surface antigens of other bacteria[2,24-28], as well as investigations of the substrate specificity of bacteriophage-borne enzymes[29-32].

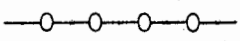
An examination of the *Klebsiella* polysaccharide structures determined thus far indicates that in most cases only a few variations occur within a particular chemotype. The group which contains residues of D-glucuronic acid, D-glucose and D-mannose includes the polysaccharides from *Klebsiella* serotypes K2[19], K4[15], K5[33] and K24[34]. From the

TABLE 2

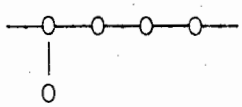
STRUCTURAL PATTERNS OF *KLEBSIELLA* CAPSULAR POLYSACCHARIDES

KEY: X = Uronic acid O = Neutral sugar ; Pyruvate and acetate omitted

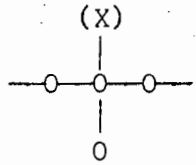
A. Uronic acid absent



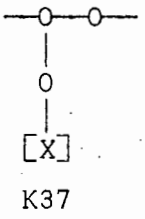
K32, K72



K56



K38



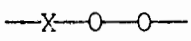
K37

(X) = 3-Deoxy-L-*glycero*-pentulosonic acid

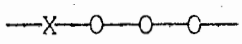
[X] = 4-O-[(S)-1-carboxyethyl]-D-glucuronic acid

B Uronic acid in chain

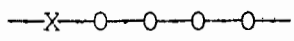
a) linear



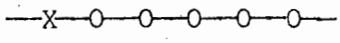
K1, K5



K4

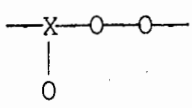


K9*

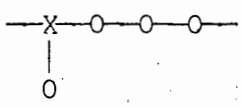


K70, K81

b) branch point on uronic acid



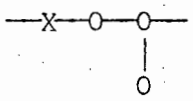
K11, K51, K57



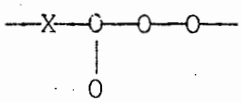
K21, K24

TABLE 2 (Contd.)

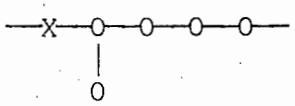
c) branch not on uronic acid



K16, K54

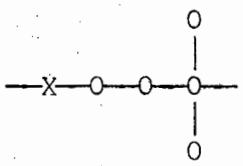


K7, K62



K52

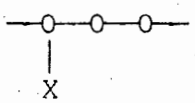
d) double branch not on uronic acid



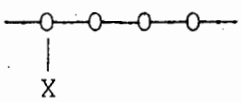
K64

C. Uronic acid in side chain

a) single unit side chain

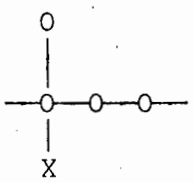


K2, K8

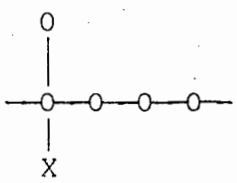


K9, K59

b) two single unit side chains forming a double branch



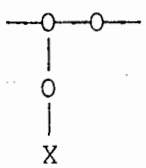
K33



K27

c) two unit side chain

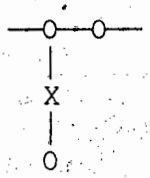
i) uronic acid terminal



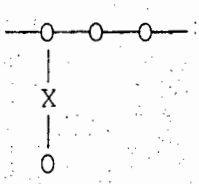
K20, K23, K55, K83

TABLE 2 (Contd.)

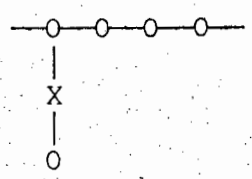
ii) uronic acid non-terminal



K47



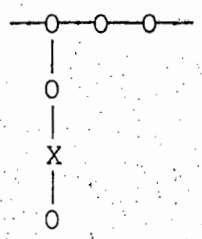
K13



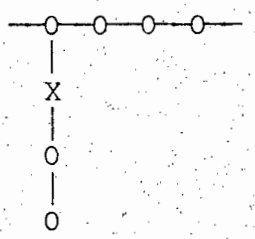
K28, K36

D. Three unit side chain

i) uronic acid non-terminal



K18



K41

primary structures determined for these four polysaccharides it is evident that their repeating units are closely related; this will be discussed in more detail in Section 4.3.

Lindberg and his coworkers have studied, among others, the capsular polysaccharides from *Klebsiella* K-types 9[35], 47[36], 52[37] and 81[38], and also that from *Klebsiella* strain 6412, for which the serotype K83 has been proposed[39]. These polysaccharides all consist of residues of D-glucuronic acid, D-galactose and L-rhamnose. The structures of these polysaccharides will be discussed more fully in Section 4.4.

The above two chemotypes, together with that which includes the polysaccharides from the K1 and K54 serotypes already mentioned, represent small groups where the structures of all the polysaccharides within the particular chemotype are known. There are, however, a number of larger groups where the polysaccharide structures of only a few of the serotypes included have been reported.

The largest chemotype consists of polysaccharides which contain residues of D-glucuronic acid, D-galactose, D-glucose and D-mannose. In the structures which have been determined the D-mannose residues in the polysaccharide chain are linked mainly through the 2- and 3-positions, and in most cases form branch points.

The polysaccharides from K7[40], and K62[41] have single-unit side chains of D-galactose and D-mannose, respectively, attached to a D-mannose residue which is in turn linked through O-2 in the chain. The repeating units of K13[42] and K28[43] each contain a double-unit side chain. In the case of the K28 polysaccharide, the side chain is composed of D-glucuronic acid and D-glucose, the latter being found

in the terminal position. The side chain in the K13 polysaccharide has been characterised as 3,4-O-(1-carboxyethylidene)-D-galactopyranosyl-D-glucuronic acid and is joined to a 4-linked chain residue of D-mannose. Pyruvic acid had previously only been found linked to the 3- and 4-positions of L-rhamnose, the 2- and 3-positions of D-glucuronic acid or the 4- and 6-positions of hexose residues, as in the case of K7 where 4,6-O-(1-carboxyethylidene)-D-glucose forms part of the polysaccharide chain[40]. However, recent studies on the polysaccharide from K33[44], another member of this group, have indicated a branched pentasaccharide repeating sequence in which 3,4-O-(1-carboxyethylidene)-D-galactose is present as a side chain. Within this group the chain residues of D-glucose have been found to be linked through either the 3- or the 4-positions in all structures so far elucidated. The polysaccharide from K28, however, contains a chain residue of D-galactose linked through O-2, a feature which is yet to be found in other *Klebsiella* polysaccharides.

Studies on the polysaccharide from K59[45] have shown the presence of a branched pentasaccharide repeating sequence in which D-glucuronic acid forms a single-unit side chain. The repeating unit also contains O-acetyl groups (linked at the 6-position of some D-mannose residues). It is of interest to note that D-glucuronic acid and D-mannose form the aldobiouronic acid moiety in all these polysaccharides except that from K59, for which the aldobiouronic acid 4-O-(β-D-glucopyranosyluronic acid)-D-galactose has been identified as a structural component.

The capsular polysaccharides of *Klebsiella* serotypes K18[46] and K36[47] are two of a fairly large group known to contain residues of D-glucuronic acid, D-galactose, D-glucose and L-rhamnose. The

polysaccharide from K18 contains a three-unit side chain, a unique feature not so far encountered among *Klebsiella* polysaccharides. The polysaccharides from both serotypes have hexasaccharide repeating units which contain the aldobiouronic acid 2-O-(β-D-glucopyranosyluronic acid)-L-rhamnose.

The two-unit side chain of K36 terminates in 4,6-O-(1-carboxyethylidene)-D-glucose, while D-glucose is found in a similar position in the K18 polysaccharide.

The capsular polysaccharides from serotypes K8[48], K11[49], K27[4] and K51[50], which consist of residues of D-glucuronic acid, D-galactose and D-glucose will be discussed in Section 4.2.

Dutton and Choy have published the structures of the repeating units in the polysaccharides from *Klebsiella* serotypes K20[51] and K21[52], which contain residues of D-glucuronic acid, D-galactose and D-mannose. As reported for other chemotypes, the D-mannose residues in these structures were found to be mainly 2- and 3-linked in the polysaccharide chains. The residues of D-galactose are again linked through O-3 in both repeating sequences, while 4,6-O-(1-carboxyethylidene)-D-galactose forms a side chain in the branched pentasaccharide repeating unit of the K21 polysaccharide. *Klebsiella* K20 has a capsular polysaccharide with a branched tetrasaccharide repeating structure in which D-glucuronic acid terminates a two-unit side chain.

The capsular polysaccharides from serotypes K32[6], K56[7] and K72[8], in which acidity is due entirely to the presence of pyruvic acid, all belong to unique chemotypes. Residues of L-rhamnose are common to all three serotypes and predominate in the structures of the polysaccharides from K32 and K72. These polysaccharides show a marked similarity in that they both contain linear tetrasaccharide repeating

units in which the pyruvic acid is attached to the 3- and 4-positions of an L-rhamnose residue which is in turn linked through O-2 in the polysaccharide chain. The essential difference in the structures is that the K32 polysaccharide contains a residue of D-galactose linked through O-3, while that from K72 contains a D-glucose residue linked in the same position. In the pentasaccharide repeating unit of the polysaccharide K56, the main chain consists of 3-linked residues of D-galactose and D-glucose, with one of the former carrying a terminal unit of L-rhamnose at O-2. The pyruvic acid is present as a 4,6-O-(1-carboxyethylidene)-D-glucose chain unit.

A limited number of *Klebsiella* strains contain D-galacturonic acid in combination with hexose and deoxyhexose residues. The polysaccharide from serotype K57 belongs to a group of four which consist of D-galacturonic acid, D-galactose and D-mannose combined in the form of a branched tetrasaccharide repeating unit. The D-galacturonic acid residue is linked to O-2 of D-mannose in the chain to form the aldobiouronic and carries a further residue of D-mannose at O-4. D-Galacturonic acid is itself linked through O-3 in the polysaccharide chain, as are the residues of D-galactose present[53].

A recent publication by Stirm *et al.*[54] describes the repeating sequence in the polysaccharide from *Klebsiella* serotype K16, one of two strains known to contain residues of D-glucuronic acid, D-galactose, D-glucose and L-fucose. The branched tetrasaccharide includes the aldobiouronic acid 4-O-(β-D-glucopyranosyluronic acid)-L-fucose and a residue of D-glucose linked at O-3 in the chain, with a terminal unit of D-galactose attached to the 4-position of D-glucose. D-Glucuronic acid is linked through O-4 in the polysaccharide chain.

In the polysaccharides from *Klebsiella* serotypes K37[9] and K38[10] the acidity is due to components which have not previously been encountered in studies on natural polysaccharides. Both these serotypes contain residues of D-galactose and D-glucose, as does the polysaccharide from K22 which contains an unknown acidic moiety[11]. The acidity of the K37 polysaccharide is due to the presence of 4-O-[(S)-1-carboxyethyl]-D-glucuronic acid, which is linked to the 6-position of D-glucose to form a two-unit side chain, attached to the polysaccharide chain at O-4 of D-galactose. This D-galactose residue is linked through O-3 in the chain, and the repeating unit is completed by a 4-linked residue of D-glucose. Studies on the K38 polysaccharide have revealed that the acidic component is 3-deoxy-L-*glycero*-pentulosonic acid, which forms part of a pentasaccharide repeating unit. This terminal acid residue is linked to the 3-position of D-galactose, which is joined through O-4 in the chain and carries a second terminal residue of D-glucose, at O-2. The doubly-branched D-galactose residue is followed in the repeating sequence by residues of 6-linked D-glucose and 3-linked D-galactose respectively.

From the above consideration of the repeating units which have been found in capsular polysaccharides from *Klebsiella* K-serotypes it is evident that the structural patterns of the polysaccharides vary only to a limited degree and that certain positions or modes of linkages are definitely preferred by some of the constituent uronic acid, deoxyhexose and hexose residues. These observations have been found to hold true for the repeating units in the polysaccharides from *Klebsiella* serotypes K4, K27, K64 and the capsular polysaccharide from a strain of *Klebsiella pneumoniae* (*oxytoca* variant), which are described in this thesis.

2. GENERAL EXPERIMENTAL CONDITIONS

2. GENERAL EXPERIMENTAL CONDITIONS

2.1 PAPER CHROMATOGRAPHY

Paper chromatography was performed using Whatman No. 1 filter paper and the following solvent systems (all v/v):

(*a*) 1-butanol-acetic acid-water (2:1:1).

(*b*) 1-butanol-ethanol-water (4:1:5, upper phase).

(*c*) 1-butanol-ethanol-water (2:1:1).

(*d*) ethyl acetate-pyridine-water (8:2:1).

(*e*) ethyl acetate-acetic acid-formic acid-water (18:3:1:4).

(*f*) butanone-water azeotrope.

Chromatograms were visualized by heating at 110°C for 5-10 min. after being sprayed with one of the following reagents:

(i) *p*-anisidine hydrochloride in aqueous 1-butanol;

(ii) aqueous ammoniacal silver nitrate;

(iii) *o*-phenylenediamine in aqueous trichloroacetic acid[55].

Chromatograms of methylated sugars were washed with hot water after heating for 10 min.

Preparative paper chromatography was carried out using Whatman No. 3MM filter paper and the solvent system specified. The relevant strips were cut out, and eluted with water for 6h. The aqueous solutions were filtered, concentrated *in vacuo* and finally freeze-dried.

R_{glc} refers to the rate of movement of oligosaccharides relative to D-glucose on Whatman No. 1 chromatographic paper in the solvent system specified.

2.2 GAS-LIQUID CHROMATOGRAPHY

The g.l.c. analysis of trimethylsilylated sugars[56] and derived alditols[57] was conducted with a Beckman GC-4 gas chromatograph with flame ionisation detector, and glass columns (1.80m x 6.25mm O.D.) packed with 3% (w/w) SE-52 on Chromosorb W (80-100 mesh, acid washed), at a flow rate of helium carrier gas of 60ml min^{-1}, isothermally at 140°C. Retention times (T-values) were measured relative to (TMS) 2,3,4,6-tetra--O-methyl-D-galactitol (actual retention *ca.* 5 min). Methyl glycosides[58] were analysed with the same instrument using glass columns (1.80m x 6·25mm O.D.) packed with 14% (w/w) ethylene glycol succinate polyester on Chromosorb W (80-100 mesh, acid washed), at a flow rate of helium carrier gas of 50ml min^{-1}, isothermally at 150°C. Retention times were measured relative to methyl 2,3,4,6-tetra-O-methyl-β-D-glucoside (actual retention, *ca.* 4 minutes).

Analyses of partially methylated alditol acetate mixtures[59] were performed with a Pye 104 gas chromatograph, using glass columns (2m x 4mm O.D.) of (a) 3% ECNSS-M on Gas-Chrom Q (100-120 mesh), at a flow rate of nitrogen carrier gas of 40ml min^{-1}, isothermally at 175°C; (b) 3% OV-225 on Chromosorb W (80-100 mesh, acid washed), isothermally at 170°C; and (c) 3% OV-17 on Chromosorb W (80-100 mesh, acid washed), programmed as indicated. Components were detected by flame ionisation. The quantitative analyses of alditol acetate mixtures were obtained using column (b), isothermally at 190°C[60]. Molar ratios of alditol acetates, and hence the relative molar proportions of sugars in mixtures, were calculated using the detector response factors (R_s - factors) for each sugar. These response factors were determined by calibration of the flame ionisation detector, by injecting several alditol acetate mixtures of known composition into the chromatograph.

Preparative g.l.c. was conducted with the Pye instrument using a glass column (2m x 6mm O.D.) fitted with a 10:1 splitter, and packed with 3% ECNSS-M on Gas-Chrom Q (100-120 mesh), at a flow rate of nitrogen carrier gas of 60ml min^{-1}, isothermally at 190°C. Each fraction collected was examined for purity by re-injection of a sample into column (b).

2.3 GEL-PERMEATION CHROMATOGRAPHY

Analytical-scale gel chromatography was carried out on the agarose gel Sagavac 6F* (60 x 0.9cm column), and on the two polyacrylamide gels Bio-Gel P-300+ (100-120 mesh) (90 x 1.5cm column) and Bio-Gel P-10 (100-120 mesh) (55 x 1.5cm column).

Samples applied to the Sagavac 6F and Bio-Gel P-300 columns were in the concentration range 3-10 mg/ml, but owing to the concentration-dependence[61] of elution volumes on Bio-Gel P-10, the concentration of samples chromatographed on this gel was kept within the range used in the calibration of the column, i.e. 2-3mg/ml. Fractions were eluted from the columns using M sodium chloride[62], and were collected using an Isco Model 270 fraction collector. Samples (1ml) of the column effluent were assayed for carbohydrate by the phenol-sulphuric acid method[63], absorbances at 490nm being measured on a Unicam SP600 spectrophotometer.

Molecular weights corresponding to peaks in the elution diagrams were found from calibration plots relating peak elution volume to log $\overline{M}_w$ (weight-average molecular weight), which were obtained by observing the elution volumes of dextrans of known $\overline{M}_w$ and of sugar standards on the same column[64,65].

* Seravac Laboratories, Cape Town
+ Bio-Rad Laboratories, Richmond, California, (U.S.A.).

Preparative gel-permeation chromatography was performed on a column (112 x 4cm) of Bio-Gel P-2, using water as eluent. The hydrolysates (*ca.* 1g in 10 ml of water) were applied to the column, and fractions (20-50ml) of the effluent were collected and screened for carbohydrate by the phenol-sulphuric acid method.

2.4 ELECTROPHORESIS

Electrophoresis was carried out for 45 min at 220V using cellulose acetate strips (17.4cm x 2.4cm) in a pyridine : formate buffer (0.1M) at pH 3 Aqueous solutions (2mg/ml) of the polysaccharides were applied to the strips (previously soaked in buffer and partially dried), by means of a Beckman sample applicator. The polysaccharide bands were detected by immersion of the strips in a 10% aqueous solution of Alcian Blue. The distance of migration of each polysaccharide was measured, and the values obtained were in agreement with the known equivalent weights of the polysaccharides[52].

2.5 ULTRACENTRIFUGATION

Ultracentrifugation tests were performed at 60 000 r.p.m. with a Beckman Model L2-65B ultracentrifuge, fitted with a Schlieren analyser. Polysaccharide concentrations in water varied between 2 mg/ml and 10 mg/ml, with 10 mg of sodium chloride added to each solution. The differences in concentration were necessitated by the high viscosity of some solutions, which resulted in the immediate sedimentation of the polysaccharide. Solutions were centrifuged for 30-90 min at 20°C, and the polysaccharide band was photographed when it was approximately one-third of the distance from the top of the cell.

2.6 GENERAL CONDITIONS

Circular dichroism spectra were run on a Jasco JA 40A

spectropolarimeter, using a quartz cell of capacity 0.3 ml, with a path length 0.1 cm. Compounds were dissolved in high-purity acetonitrile and their spectra recorded in the range 250-205 nm[66]. Optical rotations were measured from aqueous solutions (unless otherwise stated) at $20^{\circ}\pm3^{\circ}$ on a Perkin-Elmer Model 141 polarimeter. The infra-red spectra of methylated derivatives (in chloroform) were recorded on a Perkin-Elmer Model 237 spectrophotometer. Proton magnetic resonance spectra were run on Varian XL 100 or Bruker WH-90 instruments. Samples were prepared by dissolving in D_2O after freeze-drying 2-3 times from D_2O solutions. Spectra were recorded at temperatures ranging from 28° to 90° with tetramethylsilane as an external standard. All τ values are given relative to that for internal sodium 4,4-dimethyl-4-silapentanesulphonate, taken as 10.

Mass spectra were recorded on a Varian 311A mass spectrometer at 70 eV. The position of methoxyl substitution was determined using the data of Lindberg and co-workers[67], and where possible by comparison of mass spectra with those of authentic compounds.

Methylations of polysaccharides and oligosaccharides were carried out by the methods of Hakomori[14,19] and Purdie and Irvine[68] Before analysis the methylated products were passed through columns of Sephadex LH-20 with ethanol : chloroform (2:1 v/v) as eluent (methylated polysaccharides), or Merckogel OR-PVA 500 with methanol as eluent (methylated oligosaccharides). All solutions were concentrated on a rotary evaporator *in vacuo* at a bath temperature of $40^{\circ}C$.

Hydrolysis of polysaccharides and oligosaccharides was performed (unless otherwise stated) with 2M trifluoroacetic acid in sealed tubes under nitrogen at $100^{\circ}C$. After hydrolysis, the acid was removed by

co-distillation with methanol. Derived alditols were acetylated with a mixture of pyridine and acetic anhydride (1:1 v/v) in sealed tubes under nitrogen for 2h at 100°C.

2.7 PREPARATION AND PURIFICATION OF POLYSACCHARIDES

2.7.1 *KLEBSIELLA* POLYSACCHARIDES

Klebsiella cultures were grown in a medium containing agar (10g), sucrose (30g), yeast extract (2g), NaCl (2g), KH_2PO_4 (2g), $CaCO_3$ (0.8g) and $MgSO_4.7H_2O$ (0.25g) per litre. The cells were harvested after 48h, treated with 5% aqueous phenol, and poured into absolute ethanol. The resulting precipitate was dissolved in water, centrifuged for 90 min at 27 000*g* and the clear supernatant liquid freeze-dried to yield the crude polysaccharide. This was purified according to the method of Nimmich[4] in the following manner. The polysaccharide was precipitated from aqueous solution with a 10% solution of cetyl trimethylammonium bromide (Cetavlon). After centrifugation for 30 min at 13 000*g*, the precipitate was dissolved in M sodium chloride, diluted with water, centrifuged, and the clear solution poured into four times its own volume of absolute ethanol. The precipitated polysaccharide was redissolved in M sodium chloride and again precipitated with absolute ethanol, before being dissolved in water, dialysed against running tap water for 3 days and freeze-dried. Before analysis the polysaccharides were deionised with IR-120(H^+) resin. The purified polysaccharides obtained in this way were found to contain little or no nitrogen.

2.7.2 *CRYPTOCOCCUS* POLYSACCHARIDES

Two strains of *Cryptococcus neoformans* were isolated from the cerebrospinal fluid of proven cases of meningitis and identified by (a) the typical capsules as seen in India ink preparations; (b) the demonstration of urease activity; and (c) their pathogenicity, which was confirmed by intracerebral inoculation of mice. Cultures were grown on Littman's capsule medium[69], the constituents of which are shown in Table 3, at 37° for 3-4 days depending on the amount of slime produced.

TABLE 3

LITTMAN'S *CRYPTOCOCCUS* CAPSULE MEDIUM

Constituent	g/l	Constituent	g/l
KH_2PO_4	2	Thiamine	0.001
$(NH_4)_2SO_4$	2	Maltose	5
$MgCl_2.6H_2O$	0.2	Sucrose	5
$CaCl_2.2H_2O$	0.02	Sodium glutamate	2
$FeCl_2.4H_2O$	0.04	Washed agar	20
$MnCl_2.4H_2O$	0.0015	Distilled water	1000
$Na_2MoO_4.2H_2O$	0.0015	pH (adjusted with M NaOH)	7.0
Distribute and autoclave at 118°C for 12 min			

Cells were removed from the agar with sterile glass slides and partially suspended in 200 ml of sterile distilled water. The suspension was then immersed in a water bath, allowed to boil for 30 min., and then centrifuged at 10 000*g* until a clear supernatant solution was

obtained (*ca.* 1h). The capsular polysaccharide was isolated by concentrating the aqueous solution, pouring it into absolute ethanol, and purifying the crude material in the same manner as described for the *Klebsiella* polysaccharides in 2.7.1.

2.8 ACID HYDROLYSIS OF *KLEBSIELLA* POLYSACCHARIDES ; MOLECULAR-WEIGHT DISTRIBUTION ANALYSES

Samples (*ca.*100mg) of the polysaccharides from K4, K27 and K64 were heated at 100°C in 0.01M trifluoroacetic acid (25ml) for 48h. At intervals, samples (2ml) of the hydrolysate were withdrawn and after the removal of the acid, the residue in each case was dissolved in water (2ml) and the optical rotation and reducing power were determined [70]. Samples were then concentrated and examined by chromatography on paper and on an appropriate gel [15]. The acid concentration in the bulk solution was then adjusted to 0.1M by the addition of molar trifluoroacetic acid and the heating was continued, with periodic removal, and analysis of samples, for a further 24h. At the end of this period, the remaining solution was evaporated to dryness and the residue was redissolved in 2M trifluoroacetic acid before being heated at 100°C for 18h.

2.9 SUGAR ANALYSES

Polysaccharide samples (100mg) were hydrolysed in 2M trifluoroacetic acid (20ml) at 100°C for 18h. The hydrolysates were reduced with sodium borohydride, acetylated and quantitatively analysed by g.l.c. using column (b). The individual compounds were recovered by preparative g.l.c. and dissolved in acetonitrile and their circular dichroism spectra were recorded.

2.10 METHYLATION ANALYSES

Fully methylated derivatives of poly- and oligosaccharides (10 mg) were hydrolysed with 2M trifluoroacetic acid (2ml) at 100°C for 18h, and the sugars present in the hydrolysate were reduced with sodium borohydride. After acidification and removal of the borate, the residue was dissolved in anhydrous pyridine (1ml) and treated with HMDS (0.2ml) and TMSC (0.1ml) for 5 min. The solution was evaporated to dryness, and the trimethylsilylated derived alditols were extracted from the residue with n-hexane, and quantitatively analysed by g.l.c. with the use of characteristic retention-times and response factors determined for the constituent methylated neutral sugars[57].

In some cases, methylated derivatives were methanolysed with 10% methanolic hydrogen chloride for 18h at 100°C. After neutralisation with silver carbonate, filtration and evaporation to dryness *in vacuo*, a drop of methanol was added to each residue, and the methyl glycosides were qualitatively analysed by g.l.c.

3. THE CALCULATION OF THE SPECIFIC ROTATIONS OF *KLEBSIELLA* CAPSULAR POLYSACCHARIDE USING HUDSON'S RULES OF ISOROTATION

3. THE CALCULATION OF THE SPECIFIC ROTATIONS OF *KLEBSIELLA* CAPSULAR POLYSACCHARIDES USING HUDSON'S RULES OF ISOROTATION

The theoretical values for the specific rotations of a number of *Klebsiella* polysaccharides of known structure were calculated using Hudson's rules of isorotation[71], and compared with the values obtained by experiment. These rules were also used to assign configurations to certain glycosidic linkages in cases where these could not be determined by measurement of optical rotatory power or by proton magnetic resonance spectroscopy. Since *Klebsiella* polysaccharides have been shown to consist of relatively simple repeating units of hexoses and their derivatives, the theoretical value for the molecular rotation can be found, to a first approximation, by adding together the molecular contributions of each of the component, glycosidically bonded sugars in the repeating sequence: i.e. $[M]_{polysaccharide} = \Sigma[M]_{component\ sugars}$.

In order to avoid calculations involving large numbers, the molecular rotations of the methyl glycosides (shown in Table 4) were determined from the formula $[M] = \frac{[\alpha]_D \times M}{100}$, where M is the molecular weight of the glycoside. The specific rotation of the polysaccharide was then calculated from the equation $[\alpha]_D = \frac{[M]_{polysaccharide} \times 100}{M_0}$, where M_0 is the molecular weight of the polysaccharide repeating unit. For the purposes of these calculations it was assumed that the O-acetyl and pyruvic acid ketal groups present in some of the *Klebsiella* polysaccharides, made but little contribution to the total molecular rotation. Specific rotations of the methyl glycosides were obtained from the literature or calculated with the use of Hudson's rules.

TABLE 4

THE SPECIFIC AND MOLECULAR ROTATIONS OF METHYL GLYCOPYRANOSIDES

Methyl Glycoside	Specific Rotation $[\alpha]_D$ (Degrees)	Molecular Rotation [M] (Degrees)
Methyl-α-D-glucoside	+159	+308
Methyl-β-D-glucoside	-34	-66
Methyl-α-D-galactoside	+196	+380
Methyl-β-D-galactoside	+0.4	+2
Methyl-α-D-mannoside	+79	+153
Methyl-β-D-mannoside	-78	-151
Methyl-α-L-rhamnoside	-63	-103
Methyl-β-L-rhamnoside	+88	+144
Methyl-α-L-fucoside	-203[c]	-364
Methyl-β-L-fucoside	-2[c]	-4
Methyl-α-D-xyloside	+144[c]	+236
Methyl-β-D-xyloside	-58[c]	-95
Methyl-α-D-glucuronoside	+145[c]	+300
Methyl-β-D-glucuronoside	-38[c]	-79
Methyl-α-D-galacturonoside	+148[c]	+308
Methyl-β-D-galacturonoside	-23[c]	-45

c = Calculated from the specific rotation of the corresponding free sugar anomer by using Hudson's rules of isorotation.

The differences between the calculated and observed values for the specific rotations of the *Klebsiella* polysaccharides examined are shown in Table 5 .

The effect of temperature on the optical rotation of three

TABLE 5

CALCULATED AND MEASURED VALUES FOR THE SPECIFIC ROTATIONS OF *KLEBSIELLA* CAPSULAR POLYSACCHARIDES

Klebsiella K-Type	$[\alpha]_D^{Calculated}$ (Degrees)	$[\alpha]_D^{Measured}$ (Degrees)	Δ Calculated-Found (Degrees)
1	-91	-85	-6
2	+59	+79	-20
4	+98	+90	+8
5	-48	-45	-3
7	+60	+40	+20
9	-1	-17	+16
13	+44	+45	-1
16	-20	+65	-85
20	+69	+94	-25
21	+111	+130	-19
24	+47	+79	-32
27	+9	+5	+4
28	+48	+47	+1
36	-45	-56	+11
47	-44	-46	+2
54	-30	-28	-2
56	+25	+79	-54
57	+93	+104	-11
59	+20	+26	-6
64	+33	+28	+5
72	-56	-54	-2
81	-53	-52	-1
83	+90	+89	+1

Klebsiella polysaccharides (K1, K7 and K9*) was studied in the range *ca.* 4°C - 90°C. The results shown in Figure 1 indicate that changes in the conformation of these polysaccharides with varying temperatures are minimal; these were therefore not considered in the calculation

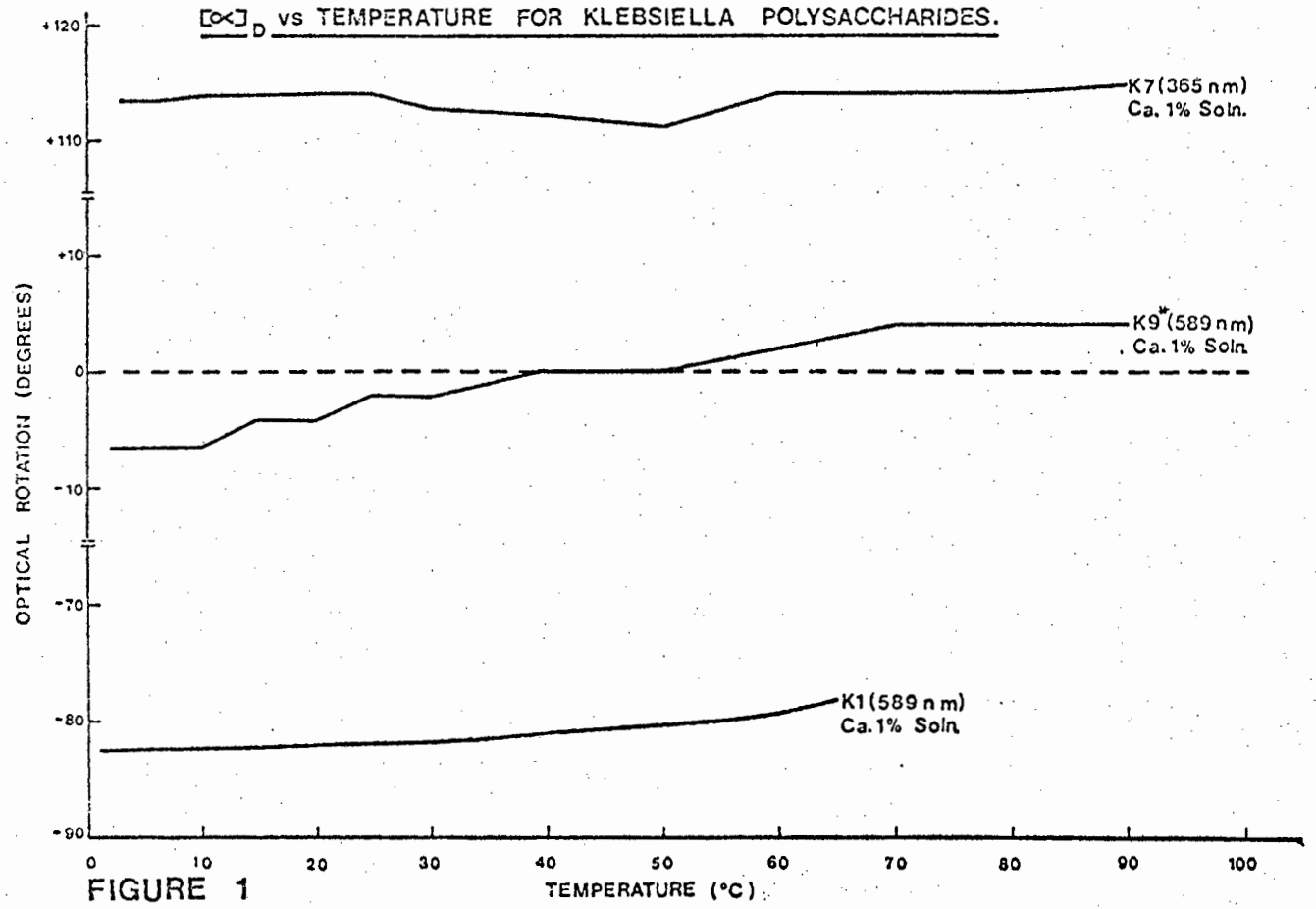
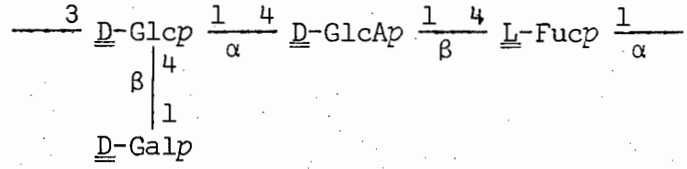


FIGURE 1

The optical rotation-temperature profiles for three *Klebsiella* capsular polysaccharides.

of the theoretical values shown in Table 5.

From the results in Table 5 it is evident that most of the theoretical values are within 20° of those found by experiment, while for more than half of the polysaccharides examined the difference between the observed and calculated values is less than 10°. In two instances, however, much larger differences are found, which suggest either incorrect assignment of the glycosidic linkages within the polysaccharides or experimental errors in the determination of the optical rotations. This is particularly evident in the case of the polysaccharide from *Klebsiella* K16[54], where the calculated and observed values for the specific rotation differ by as much as 85°. According to the structure (I) postulated for this polysaccharide there are two α-glycosidic and two β-glycosidic linkages in the repeating unit.

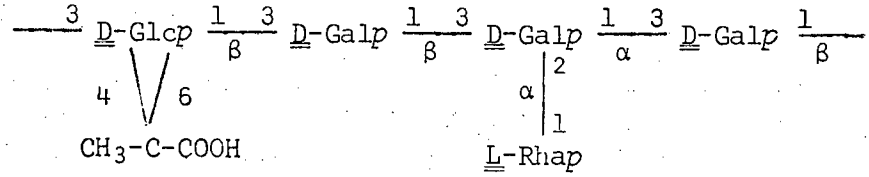


(I)

Table 4 indicates that residues of α-linked L-fucose make an extremely large negative contribution to molecular rotation. This value, together with the contribution from β-D-glucuronic acid, gives a negative total which outweighs the combined positive contribution of the α-D-glucopyranosyl and β-D-galactopyranosyl residues, resulting in a negative calculated value for the specific rotation of the polysaccharide (-20°). The value found by experiment, +65°, not only differs considerably in magnitude from the calculated value but is also of the opposite sign. The observed specific rotation of two other *Klebsiella* polysaccharides, K1 and K54, which also contain residues of α-L-fucose, differ from their calculated values by 6° and 2° respectively, suggesting that the value used for the contribution of this 6-deoxy hexose to the molecular

rotation is correct.

The structure (II) postulated for the polysaccharide from *Klebsiella* K56[7] indicates the presence of two α- and three β-glycosidic linkages within the repeating unit. An examination of the molecular contributions of the individual sugar residues shows that the positive contribution



(II)

made by the α-D-galactopyranosyl residues is greater than that of the combined negative contribution of β-D-glucose and α-L-rhamnose, indicating a positive (+25°) specific rotation for the polysaccharide. The observed rotation, although of the same sign, differs from this value by as much as 54°. It should be noted that by changing certain of the anomeric configurations in the structures postulated for the polysaccharides from *Klebsiella* K16 and K56, values can be obtained for the specific rotations which are closer to those found experimentally. Conversely for those polysaccharides having specific rotations which are within 25° of the theoretical value, changes in postulated anomeric configurations result in differences which are either of the same order of magnitude or much higher than those shown in Table 5.

Hudson's rules have been used in the present work to assign the configuration of a glycosidic linkage involving a terminal residue of L-rhamnose in the polysaccharide from *Klebsiella* K64 and to verify the assignment of the anomeric linkages in the K27 polysaccharide. Lindberg *et al.*[53] used these rules to determine the nature of the glycosidic linkage of the terminal residue of D-mannose found in the polysaccharide from *Klebsiella* K57.

This study indicates the usefulness of Hudson's rules as a supplement to the techniques of optical rotatory power measurement and proton magnetic resonance spectroscopy used to assign configuration of glycosidic linkages.

4. STRUCTURAL STUDIES OF *KLEBSIELLA* POLYSACCHARIDES

4.1 THE CAPSULAR POLYSACCHARIDE FROM *KLEBSIELLA* SEROTYPE K64

4.1.1 INTRODUCTION.

Klebsiella K64 is one of two strains which are known to contain residues of D-glucuronic acid, D-glucose, D-mannose and L-rhamnose. The structure of an acidic polysaccharide from a strain of *Aerobacter aerogenes* N.C.T.C. 8172 (*Klebsiella* type K64) was investigated by Barker and coworkers in 1958[72]. Periodate oxidation studies showed that large numbers of glucose, mannose and glucuronic acid residues were either linked (1→3) or were involved in branching, while the rhamnose was present either as end groups or linked through the 2- or 4- position. The aldobiouronic acid was identified as a glucuronosyl-mannose. A more recent publication[15] has dealt with the molecular-weight distribution for the hydrolysis products from the K64 polysaccharide.

The capsular polysaccharide used for the present study was obtained from a culture (N.C.T.C. 9184), and has the same characteristics as those previously reported[15]. Homogeneity tests carried out by electrophoresis on cellulose acetate strips and by ultracentrifugation indicated that the polysaccharide was monodisperse. The proton magnetic resonance spectrum of the polysaccharide showed two sharp singlets at τ 7.72 and 8.40, characteristic of O-acetyl and pyruvic acid ketal moieties respectively. Integration of the anomeric, acetate, and pyruvate signals indicated one acetyl and one pyruvate group to six sugar units.

The ratios of sugars found by g.l.c. analysis of an hydrolysate of the polysaccharide, using their derived alditol acetates, were rhamnose (1.0), mannose (1.4) and glucose (2.0), in good agreement with

those previously reported. The low proportion of mannose obtained is consistent with a substantial amount of the aldobiouronic acid remaining intact under the conditions of acid hydrolysis used to release the neutral sugars for analysis. Recovery of the alditol acetates by g.l.c. and measurement of their circular dichroism spectra allowed the assignment of the D-configuration to glucose and mannose, and the L-configuration to rhamnose.

4.1.2 GRADED ACID HYDROLYSIS STUDIES

Graded acid hydrolysis studies monitored by paper chromatography have demonstrated that rhamnose and pyruvic acid are the first residues to be released from the polysaccharide structure, followed more slowly by glucose and mannose. Glucuronic acid and several other acidic products of higher molecular weight were detected after prolonged hydrolysis, together with a neutral component having the mobility on paper chromatography of a disaccharide.

The course of mild acid hydrolysis was followed by gel-permeation chromatography[62,64] of samples removed at intervals, and showed a slow decrease in $\overline{M}_w$ during hydrolysis with 0.01M trifluoroacetic acid, as shown in Table 6. The molecular weights of the hydrolysis products were largely within the fractionation range of the agarose gel, even after treatment with 0.01M acid for 4h, and thereafter fractionation on Bio-Gel P-300 was necessary throughout the rest of this phase of hydrolysis (Figure 2).

No significant product became apparent until the hydrolysis was continued in 0.1M trifluoroacetic acid (Table 7); after further hydrolysis under these conditions for 2h a structurally significant product having molecular weight 1100 daltons was present in the hydrolysate to the extent of 53%

TABLE 6

PARTIAL HYDROLYSIS OF *KLEBSIELLA* K64 CAPSULAR POLYSACCHARIDE IN 0.01M TRIFLUOROACETIC ACID

Time (h)	$\overline{M}_w$ (Daltons)	$[\alpha]_D$ [a] (Degrees)
0	1 700 000 [b]	+28
1	520 000	+29
4	140 000	+30
7	29 000	+32
10	17 000	+35
24	6 900	+36
48	4 100	+38

[a] Concentration *c*, 0.40 [b] Single peak (Sagavac 6F)

TABLE 7

FURTHER HYDROLYSIS OF DEGRADED *KLEBSIELLA* K64 CAPSULAR POLYSACCHARIDE IN 0.1M TRIFLUOROACETIC ACID

Time (h)	$[\alpha]_D$ [a] (Degrees)
0	+38
2	+48
7	+49
24	+50

[a] Concentration *c*, 0.36

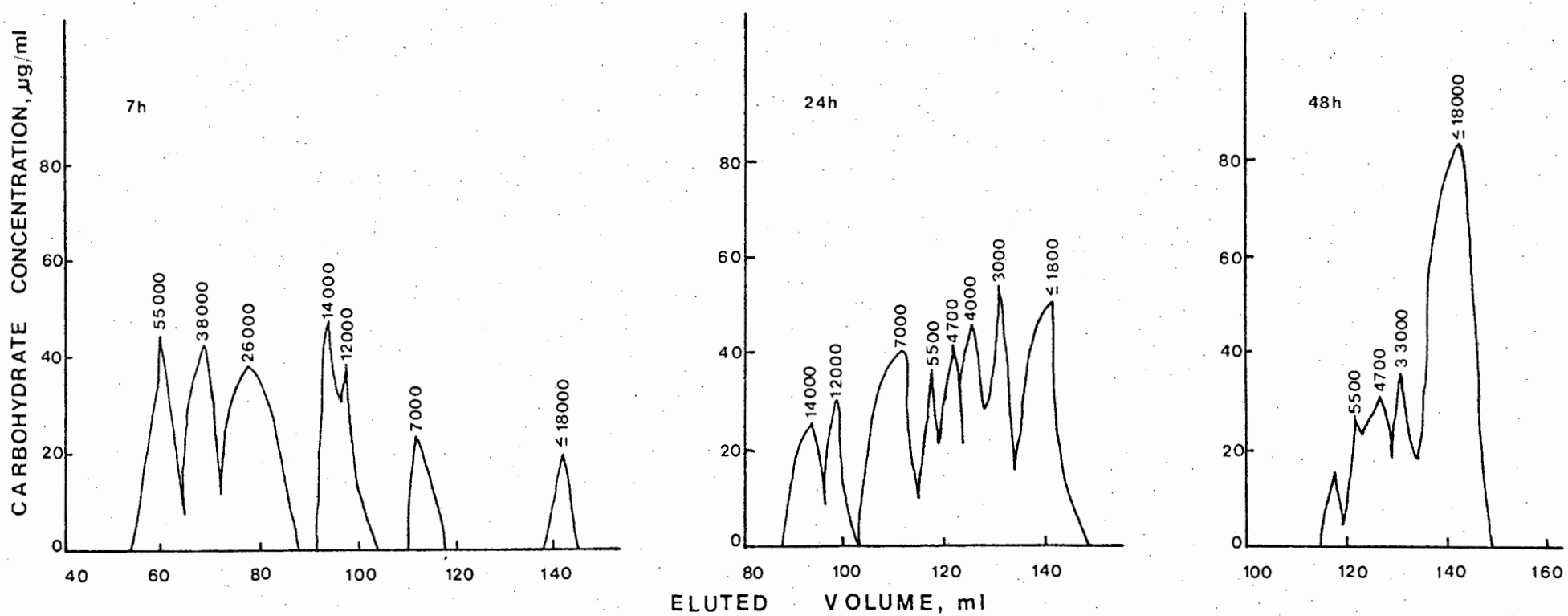


FIGURE 2 Bio-Gel P-300 elution patterns of hydrolysates (0.01M trifluoroacetic acid, 100°, 7-48h) of *Klebsiella* K64 capsular polysaccharide.

by weight (Figure 3). Several neutral and acidic oligosaccharides appeared when the hydrolysis was continued in 0.1M acid. The gradual degradation of the component having molecular weight 1100 daltons to lower oligosaccharides as this step proceeded was demonstrated both by the elution patterns on Bio-Gel P-10 (Figure 3) and by paper chromatography. An aldotriouronic acid showing some resistance to further hydrolysis, and an aldobiouronic acid that survived treatment even with 2M trifluoroacetic acid, were the main breakdown products detected during the final stages of hydrolysis with 0.1M acid.

4.1.3 PARTIAL ACID HYDROLYSIS; ISOLATION AND IDENTIFICATION OF OLIGOSACCHARIDES

Partial acid hydrolysis was used to generate these structurally significant oligosaccharides on a preparative scale. The fractions separated by gel-permeation chromatography were purified by preparative paper chromatography to yield components which gave single peaks at molecular weights 1100, 930, 520 and 350 daltons when examined by gel-permeation chromatography on Bio-Gel P-10. The main physical and chemical properties of these components are shown in Table 8 . The component having molecular weight 350 daltons was found to contain the aldobiouronic acid 3-O-(α-D-glucopyranosyluronic acid)-D-mannose(II), and a mixture (I) of 4-O-β-D-glucopyranosyl-D-mannose and 2-O-β-D-glucopyranosyl-D-mannose in the ratio 3:1. The neutral disaccharide mixture was separated into its components by g.l.c. of the trimethylsilyl derivatives and the positions of linkage were determined by mass spectrometry according to the method of Haverkamp *et al.*[73]. These results were confirmed by methylation analysis of the mixture.

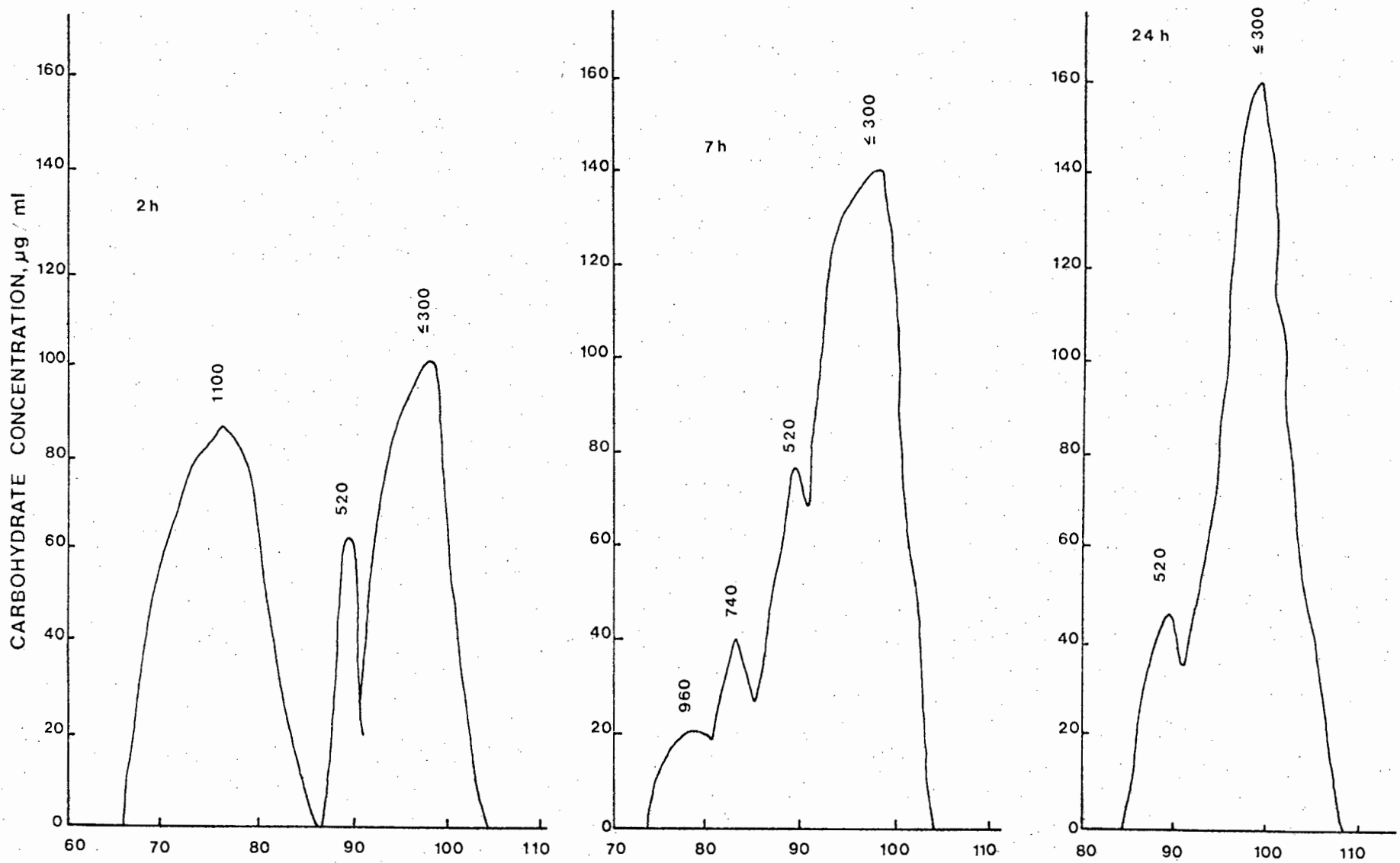


FIGURE 3

Bio-Gel P-10 elution patterns on further hydrolysis (0.1M trifluoroacetic acid, 100°, 2-24h) of *Klebsiella* K64 capsular polysaccharide.

TABLE 8

THE MAIN PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE OLIGOSACCHARIDES FROM *KLEBSIELLA* K64 CAPSULAR POLYSACCHARIDE

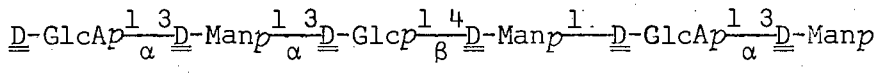
FRACTION	OLIGOMER	$[\alpha]_D$(in H_2O) (Degrees)	R_{glc} (Solvent *a*)	Mol.wt.	Equivalent Weight	Neutral sugars[a] (Molar ratios)	Methylated sugars[b] (Alditol acetates)
I	Neutral disaccharide mixture	-6	0.74	340	-	Glc (1.0) Man (1.0)	2,3,4,6-Glc 2,3,6-Man 3,4,6-Man
II	Aldobiouronic acid	+40	0.74	360	n.d.	Man	2,4,6-Man
III	Aldotriouronic acid	+68	0·42	520	n.d.	Glc (1.0) Man (0.7)	2,4,6-Glc 2,4,6-Man
IV	Acidic oligosaccharide	+64	0.15	930	440	Glc (2.0) Man (2.2)	2,3,4,6-Glc (tr) 2,4,6-Glc 2,4,6-Man 2,3,6-Man 3,6-Man (tr)
V	Acidic oligosaccharide	+59	0.04	1100	530	Glc (2.0) Man (2.7)	2,3,4,6-Glc 2,4,6-Glc 2,4,6-Man 2,3,6-Man (tr) 3,6-Man

[a] By g.l.c. of their derived alditol acetates on column (b).

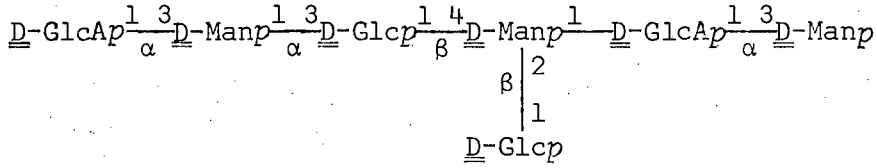
[b] Column (a).

The aldotriouronic acid (III), identified as [O-(α-D-glucopyranosyluronic acid) - (1→3)-O-α-D-mannopyranosyl - (1→3)-D-glucose], yielded the aldobiouronic acid (II) and D-glucose when subjected to mild acid hydrolysis, while the acidic higher oligosaccharides of molecular weights 1100 and 930 daltons were found to contain the same three types of sugar residues.

The assignment of structures (IV) and (V) to these higher oligosaccharides was based primarily on the evidence obtained from equivalent weight determinations and methylation analyses. That D-glucuronic acid residues are located at the non-reducing end of each oligosaccharide was supported by methanolysis studies performed on their respective methylated derivatives, which gave methyl 2,3,4-tri-O-methyl-D-glucuronate in addition to the methylated sugars in Table 8 when analysed by g.l.c.



D-GlcAp (1→3) α D-Manp (1→3) α D-Glcp (1→4) β D-Manp (1→) D-GlcAp (1→3) α D-Manp (IV)



D-GlcAp (1→3) α D-Manp (1→3) α D-Glcp (1→4) β D-Manp (1→) D-GlcAp (1→3) α D-Manp (V)

β (2→1) D-Glcp

The low proportion of 2,3,6-tri-O-methyl-D-mannose found in the hydrolysate of (V) is attributed to the presence of a small amount of (IV) in this fraction, due to incomplete separation of these oligosaccharides by the gel-permeation and paper chromatographic methods used in their isolation. Identification of 2,3,4,6-tetra-O-methyl-D-glucose and 3,6-di-O-methyl-D-mannose in the hydrolysate of the fraction of molecular weight 1100 indicates the branched nature of this oligosaccharide; the presence of traces of these two methylated sugars in the hydrolysate of IV is probably the result of contamination with the oligosaccharide V. Since no residues of L-rhamnose were detected in the hydrolysates of

these oligosaccharides, it is reasonable to assume that they are present as terminal groups in the polysaccharide structure. Comparison of the methylation analysis of the oligosaccharide (V) with that of the original polysaccharide (A) indicates that these terminal residues are attached to O-3 of D-mannose. Loss of the terminal D-glucose residue from this structure (V) then gives rise to the lower oligosaccharide (IV). An examination of the lower oligosaccharides (I - III) isolated from *Klebsiella* K64 polysaccharide supports the assignment of these structures to the components having molecular weights 1100 and 930.

4.1.4 AUTOHYDROLYSIS OF THE ORIGINAL POLYSACCHARIDE (A)

Autohydrolysis of the original polysaccharide (A) yielded pyruvic acid and traces of rhamnose. Proton magnetic resonance spectroscopy of the residual polysaccharide (obtained by dialysis and freeze-drying of the aqueous hydrolysate) showed the absence of pyruvate ketal, while gel-permeation chromatography indicated a high degree of polymerisation ($\overline{M}_w$ 1.5×10^6 daltons). A sample of the autohydrolysed polysaccharide (B) was methylated and the product used for the methylation analysis described in Section 4.1.6.

4.1.5 METHYLATION ANALYSIS OF POLYSACCHARIDE (A)

Fully methylated K64 polysaccharide was hydrolysed for 6h and 18h, and a portion of each hydrolysate was analysed by paper chromatography and by g.l.c. of the trimethylsilylated derived alditols to yield the molar proportions listed in Table 9. The gas chromatogram is shown in Figure 4.

It is evident that the number of branch points in the structure is higher than can be accounted for in terms of the terminal residues of

L-rhamnose found in the hydrolysate, and that the low proportion of 2,4,6-tri-O-methyl-D-mannose in the hydrolysate after 6h is again due to the incomplete hydrolysis of the aldobiouronic acid linkages involving residues of this sugar.

TABLE 9

METHYL ETHERS FROM THE HYDROLYSATES OF METHYLATED *KLEBSIELLA* K64 CAPSULAR POLYSACCHARIDES

Sugar[a]	T[b]	Molar Proportions (%)		
		A[c]	A[d]	B[d]
2,3,4-Rha	0.59	21.4	19.5	17.8
2,3,4,6-Glc	1.01	-	-	18.7
2,4,6-Man	1.19	11.5	18.2	16.6
2,4,6-Glc	1.34	20.8	20.4	20.8
3,6-Man	1.63	-		6.8
2,3-Glc	2.12	24.0	21.0	-
6-Man	2.33	20.8	20.8	19.3

a 2,3,4-Rha = 2,3,4-tri-O-methyl-L-rhamnose *etc.*

b Retention time of the corresponding (TMS) alditol relative to that of 2,3,4,6-tetra-O-methyl-D-galactose on an SE-52 column at 140°C.

c,d Hydrolysates examined after 6h and 18h hydrolysis respectively.

A ORIGINAL POLYSACCHARIDE

B AUTOHYDROLYSED POLYSACCHARIDE

Isolation of 2,4,6-tri-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-mannose indicates that these units represent 3-substituted sugars in the chain, while 2,3-di-O-methyl-D-glucose cannot represent a branch point in the

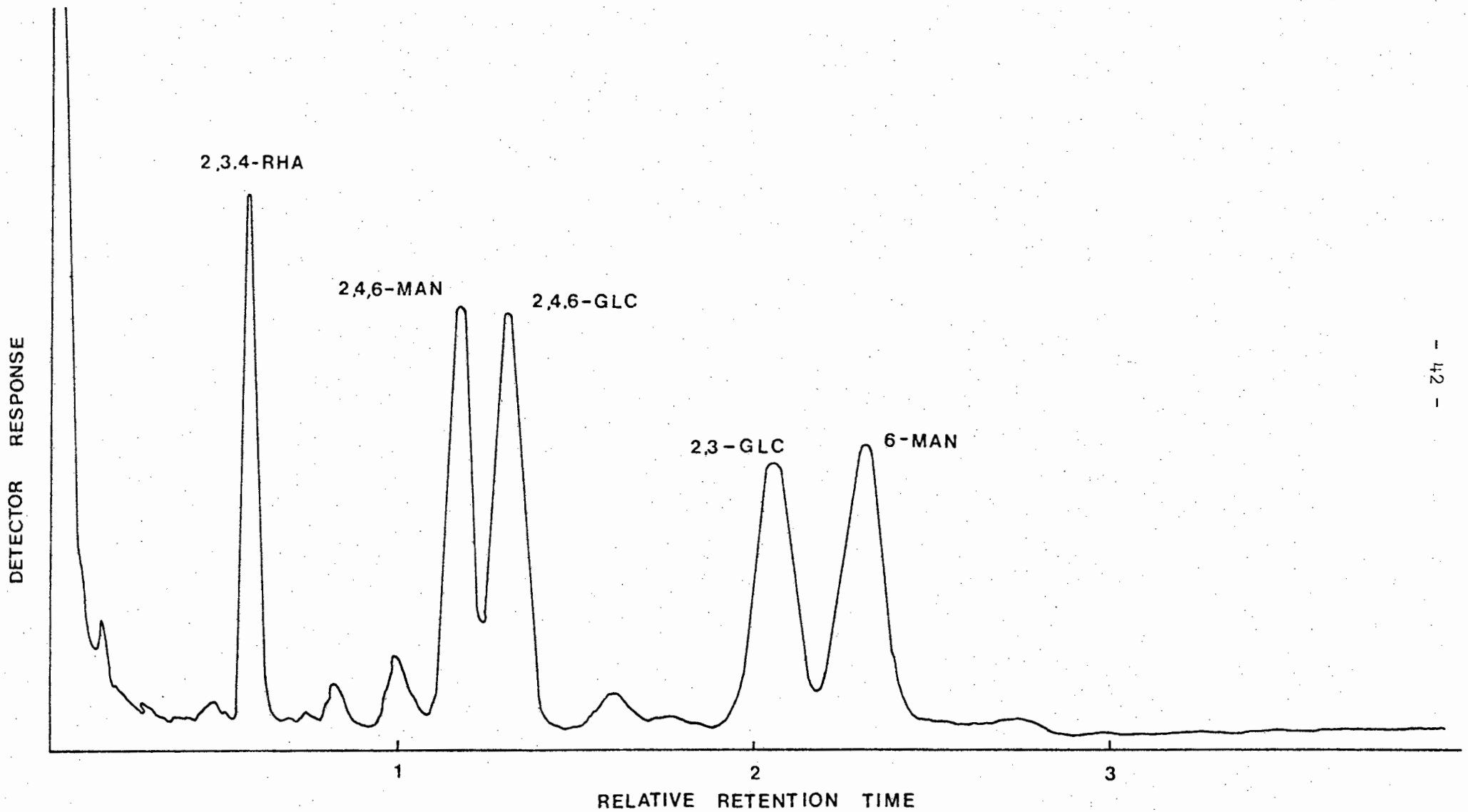


FIGURE 4 G.l.c. trace of the partially methylated (TMS) alditols from fully methylated *Klebsiella* K64 polysaccharide (A).

polysaccharide chain, since no equivalent number of terminal units was found. However, an examination of the structure of the oligosaccharide (V) shows that terminal units of D-glucose are also present in the K64 polysaccharide. The pyruvic acid must therefore be linked to these D-glucose residues as a 4,6-ketal in order to account for the proportion of 2,3-di-O-methyl-D-glucose found in the hydrolysate of (A). Identification of 6-O-methyl-D-mannose indicates that this residue forms a double-branch point in the polysaccharide chain. Since the L-rhamnose residues have been shown to occupy one branch point at O-3 (*cf.* structures IV and V), the 4,6-O-(1-carboxyethylidene)-D-glucose unit must be attached to either O-2 or O-4 of this same D-mannose residue. The identification of 4-linked chain residues of D-mannose in the higher oligosaccharides, however, shows that the second branch point is at O-2.

A large-scale hydrolysate of the methylated K64 polysaccharide was separated into its neutral and acidic components by means of ion-exchangers. The methylated neutral-sugar mixture was separated into its component sugars by cellulose-column chromatography and in some cases these were purified by preparative paper chromatography. 2,3,4-Tri-O-methyl-L-rhamnose, a mixture of 2,4,6-tri-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-mannose (separated by means of preparative g.l.c. as their trimethylsilylated, derived alditols), 2,3-di-O-methyl-D-glucose and 6-O-methyl-D-mannose were isolated in this manner. All the above sugars were characterised by standard techniques, including paper and gas-liquid chromatography, mass spectrometry and proton magnetic resonance spectroscopy.

Methylated acidic sugars were converted into a mixture of the methyl ester methyl glycosides, which were ester-reduced with lithium aluminium hydride[74] and the products hydrolysed. Analysis of the

hydrolysate by paper chromatography and by g.l.c. as their derived alditol acetates showed only 2,4,6-tri-O-methyl-D-mannose and 2,3-di-O-methylglucose to be present in the mixture. Recovery of the glucose derivative followed by de-O-methylation gave glucitol, which was shown to be of the D-configuration by measurement of the c.d. spectrum of its hexaacetate. Since no residues of 2,3-di-O-methyl-D-glucose were found in the methylated acidic sugars before reduction their appearance here must be due to the reduction of the glucuronic acid residues which were present in the original polysaccharide, and which must therefore also be of the D-configuration. These results confirm that the aldobiouronic acid obtained on hydrolysis of the polysaccharide (A) is 3-O-(D-gluco-pyranosyluronic acid)-D-mannose and that the D-glucuronic acid is not in a terminal position or involved in branching.

4.1.6 METHYLATION ANALYSIS OF DEGRADED POLYSACCHARIDE (B)

Methylation analysis of the polysaccharide (B) obtained in the autohydrolysis experiment in Section 4.1.4 (see Table 9 and Figure 5) showed the complete disappearance of 2,3-di-O-methyl-D-glucose, which was replaced by 2,3,4,6-tetra-O-methyl-D-glucose in significant molar proportions; this confirms that the pyruvic acid group must be linked to the 4- and 6-positions of a terminal D-glucose residue in the original polysaccharide (A). The small quantity of 3,6-di-O-methyl-D-mannose found in the hydrolysate arises from the loss of terminal residues of L-rhamnose during the autohydrolysis of the polysaccharide and again shows that these are linked to the 3-position of D-mannose. Since only the 6-position of this D-mannose residue is unsubstituted, the residues of D-glucose in the polysaccharide must be linked to O-2 and O-4 of this same residue in order to produce the neutral disaccharides mentioned in Section 4.1.3.

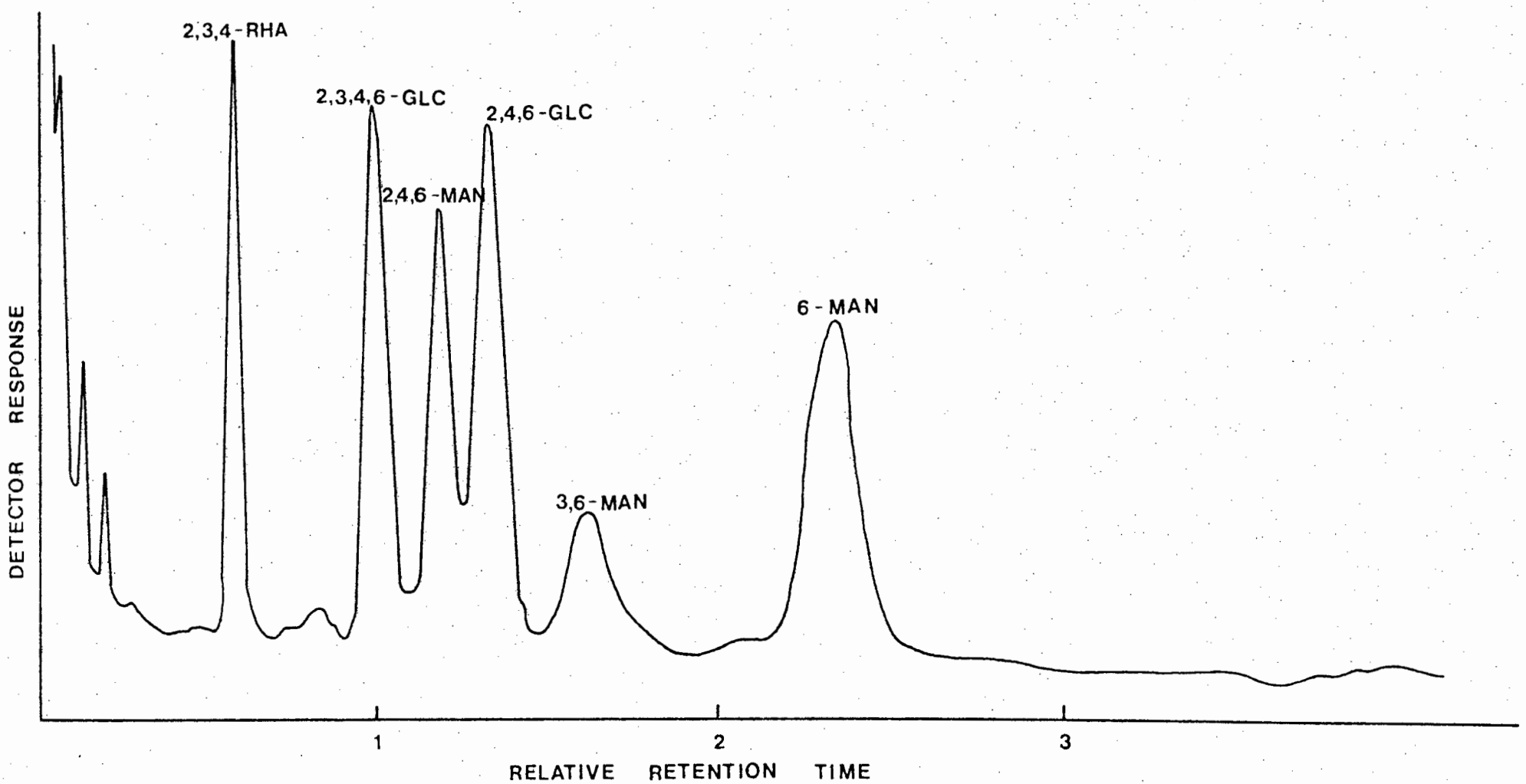


FIGURE 5 G.l.c. trace of the partially methylated (TMS) alditols from fully methylated degraded polysaccharide (B) from *Klebsiella* K64.

4.1.7 REDUCTION OF THE METHYLATED DERIVATIVES OF POLYSACCHARIDES (A) AND (B)

Reduction of the fully methylated K64 capsular polysaccharide gave a product (C), whose i.r. spectrum showed no absorption at 1740 cm^{-1} (which would indicate the presence of methyl ester groups). Hydrolysis of a sample, followed by paper chromatographic analysis of the hydrolysate, failed to reveal any new neutral sugars resulting from the reduction of the D-glucuronic acid residues in the chain. G.l.c. analysis, however, indicated a substantial rise in the proportion of 2,3-di-O-methyl-D-glucose in the hydrolysate, as can be seen from the results given in Table 10 and shown in Figure 6.

TABLE 10

METHYL ETHERS FROM THE HYDROLYSATES OF THE REDUCED METHYLATED DERIVATIVES OF POLYSACCHARIDES (A) AND (B)

Sugar[a]	T[b]	Molar Proportions (%)	
		C[c]	D[c]
2,3,4-Rha	0.59	17.0	13.6
2,3,4,6-Glc	1.01	-	18.5
2,4,6-Man	1.19	17.8	16.9
2,4,6-Glc	1.34	16.8	17.3
3,6-Man	1.63	-	6.8
2,3-Glc	2.12	33.8	11.5
6-Man	2.33	14.6	15.4

[a,b] As for Table 9.

[c] Hydrolysates examined after 6h hydrolysis.

C REDUCED METHYLATED ORIGINAL POLYSACCHARIDE

D REDUCED METHYLATED AUTOHYDROLYSED POLYSACCHARIDE

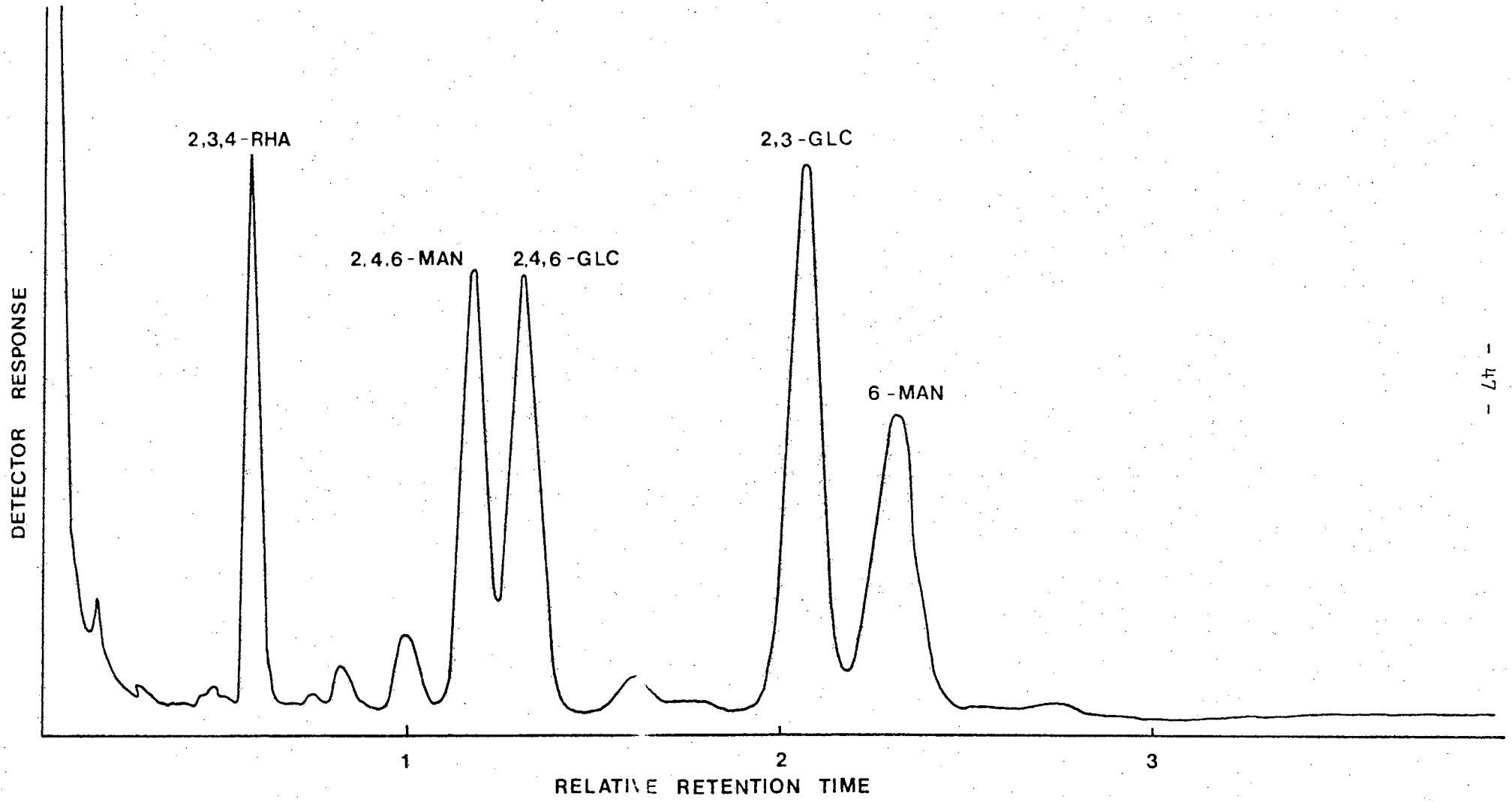


FIGURE 6 G.l.c. trace of the partially methylated (TMS) alditols from the reduced methylated derivative of *Klebsiella* K64 polysaccharide (A).

This increase must therefore be due to the reduction of 4-linked D-glucuronic acid residues within the repeating unit of the polysaccharide. In order to confirm this result, the fully methylated autohydrolysed polysaccharide was reduced with lithium aluminium hydride. The methylation analysis of an hydrolysate of this reduced product (D) shown in Table 10 and Figure 7 , again indicated the presence of a substantial amount of 2,3-di-O-methyl-D-glucose. Since the autohydrolysed polysaccharide (B) contained no such D-glucose residues, their identification here could only have resulted from the reduction of 4-linked D-glucuronic acid residues in the polysaccharide chain.

4.1.8 SMITH DEGRADATION STUDIES ON ORIGINAL POLYSACCHARIDE (A)

The capsular polysaccharide (A) was oxidised with periodate, 0.49 mol per hexose residue being consumed. The polyol obtained by reduction of the periodate-oxidised material with sodium borohydride was hydrolysed at room temperature to give glycolaldehyde and an acidic oligosaccharide which had $\overline{M}_w$ 560 daltons (by gel-permeation chromatography). The p.m.r. spectrum of this oligosaccharide in D_2O showed the presence of one β-D-glycosidic linkage per three anomeric protons, the coupling constant of which allowed its assignment to a residue of D-glucose. Hydrolysis of a sample followed by g.l.c. analysis of the sugars present in the hydrolysate, as their derived alditol acetates, gave D-mannose and D-glucose in the ratio 1.9:1. The i.r. spectrum of the fully methylated oligosaccharide showed absorption at 1720 cm^{-1} indicating the presence of a methyl ester grouping, which disappeared on reduction of the methylated product with lithium aluminium hydride. The reduced methylated oligosaccharide was hydrolysed, and the hydrolysate was analysed by paper chromatography and by g.l.c. of the derived alditol

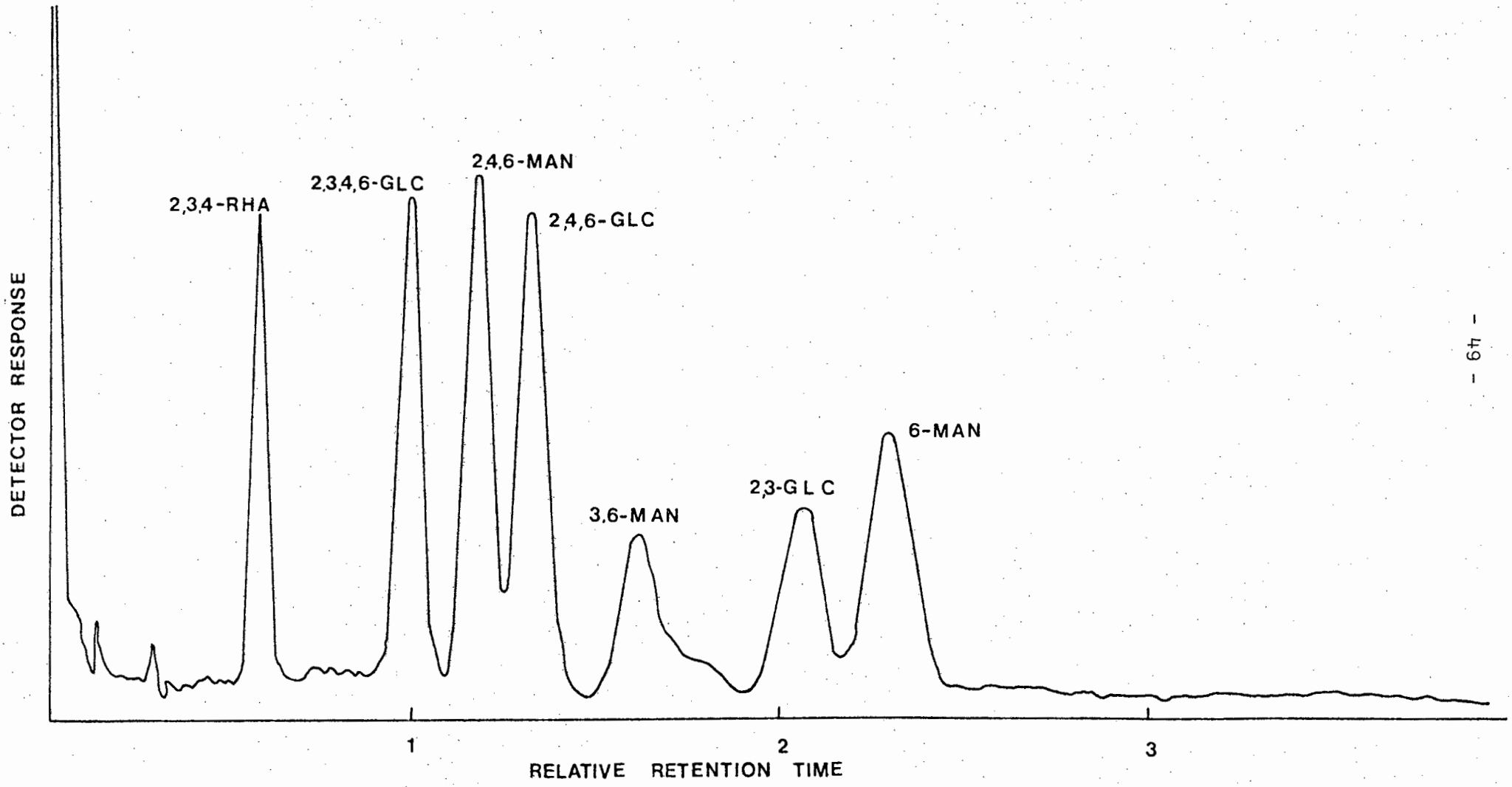


FIGURE 7 G.l.c. trace of the partially methylated (TMS) alditols from the reduced methylated derivative of degraded polysaccharide (B) from *Klebsiella* K64.

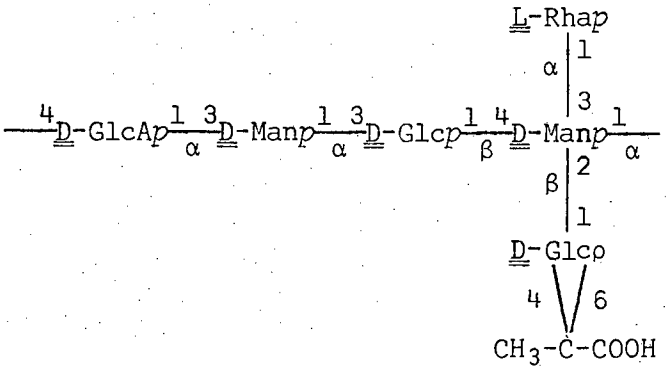
acetates of the component sugars; 2,3,4,6-tetra-O-methyl-D-mannose (1.0), 2,4,6-tri-O-methyl-D-glucose (0.8) and 2,3,6-tri-O-methyl-D-mannose (1.0) were found to be present in the molar ratios shown. The identification of a 4-linked residue of D-mannose in the product obtained by Smith degradation of the K64 polysaccharide further proves that the 4-O-β-D-glucopyranosyl-D-mannose forms part of the chain within the repeating unit, while 2-O-β-D-glucopyranosyl-D-mannose is obtained as a result of the attachment of a terminal unit of D-glucose to the 2-position of the same D-mannose residue. The higher proportion of the (1→4)-linked isomer obtained on mild acid hydrolysis of the polysaccharide is to be expected since the glycosidic bond between the terminal D-glucose unit and D-mannose should be relatively more acid-labile than the bond between the same two sugar residues in the chain of the repeating unit.

4.1.9 ANOMERIC CONFIGURATIONS OF CONSTITUENT SUGARS.

The modes of linkage of the D-glucuronic acid, D-glucose and D-mannose residues in the polysaccharide were determined by p.m.r. spectroscopy and the measurement of optical rotatory power of the oligosaccharides mentioned in 4.1.3. The terminal residue of L-rhamnose in the repeating unit does not appear in any of these structures. However, the calculated values for the optical rotation of the polysaccharide (A), using Hudson's rules of isorotation[71] and assuming this L-rhamnose residue to be α or β-linked, are $[\alpha]_{589}$ + 33° and $[\alpha]_{589}$ + 57° respectively. The actual value, +28°, agrees much better with the former, which suggests strongly that this residue is α-linked. Furthermore, the increase in optical rotation accompanying the release of L-rhamnose during the initial stages of mild acid hydrolysis indicates also that these residues are α-linked.

4.1.10 POSTULATED STRUCTURE OF THE REPEATING UNIT IN *KLEBSIELLA* K64 CAPSULAR POLYSACCHARIDE

The results of periodate oxidation, methylation analyses and partial acid hydrolysis, together with the identification of a number of structurally significant oligosaccharides, are thus consistent with a doubly-branched hexasaccharide repeating unit (VI) being present in the capsular polysaccharide from *Klebsiella* serotype K64.



(VI)

It is hoped that the X-ray crystallographic methods recently used on other *Klebsiella* polysaccharides[75] can be used to locate the position of the O-acetyl group present in the above unit. However, the low consumption of periodate (the theoretical value for such a repeating unit is 0.58 mol per hexose residue) by the polysaccharide (A) suggests that this group may be in a position where it can prevent attack by periodate which might otherwise occur. Of the periodate-vulnerable residues in the repeating unit (VI), attachment of the O-acetyl group to D-glucuronic acid, the terminal D-glucose or the 3-position of L-rhamnose, would result in a Smith-degraded product different from that obtained in the periodate oxidation studies described in 4.1.8. If the assumption that the O-acetyl group is preventing attack by periodate is, in fact, correct, the location would have to be at O-2 or O-4 of the terminal L-rhamnose residue. Attachment to the chain units of D-glucose or D-mannose would not affect the periodate uptake.

4.1.11 COMPARISON WITH OTHER *KLEBSIELLA* CAPSULAR POLYSACCHARIDES

A number of the structural features found in the capsular polysaccharide from K64 have been reported in other *Klebsiella* polysaccharides. Recent studies[44] have shown that the K33 polysaccharide has a doubly-branched hexasaccharide repeating unit in which a residue of D-mannose is linked through O-4 in the polysaccharide chain, and carries terminal units at O-3 and O-6. Branched hexasaccharide units have also been found in the K18[46], K27, K28[43], K36[47] and K52[37], polysaccharides; of these, only K36 has 4,6-O-(1-carboxyethylidene)-D-glucose as a side chain. Pyruvic acid has also been found attached to 3-linked residues of D-glucose in the polysaccharide chains of K7[40], K27 and K56[7], while the aldobiouronic acid 3-O-(α-D-glucopyranosyluronic acid)-D-mannose forms part of the polysaccharides of K2[19], K4[15], K13[42], K21[52], K24[34] and K33, and is the most common aldobiouronic acid found in the *Klebsiella* polysaccharides examined thus far. The neutral fragment 3-O-α-D-mannopyranosyl-D-glucose is found in the polysaccharides of K7, K24, K28 and K59[45], but not in combination with D-glucuronic acid to form the aldotriouronic acid moiety found in K64. Results obtained from studies on the polysaccharide from *Klebsiella* K4 however, indicate that the same aldotriouronic acid structure occurs here in a linear repeating unit. *Klebsiella* K2[19] contains both the aldobiouronic acid and the neutral disaccharide 4-O-β-D-glucopyranosyl-D-mannose units found in K64, in a tetrasaccharide repeating unit in which the D-glucuronic acid residue occupies a terminal position. Residues of D-glucuronic acid linked through the 4-position have been reported in a number of polysaccharides, but not preceded in the chain by an α-linked residue of D-mannose as found in that from K64.

A preliminary investigation has been performed on the capsular polysaccharide from *Klebsiella* serotype K65, which is the only other strain[4]

known to contain pyruvic acid and glucuronic acid in combination with the neutral sugars found in K64. There are indications that glucose forms part of the aldobiouronic acid moiety, while the equivalent weight (610 by cellulose acetate electrophoresis) is consistent with that of a hexasaccharide repeating unit in which both glucuronic acid and pyruvic acid are present.

The results of the present study on *Klebsiella* K64 are in agreement with the earlier work of Barker *et al.* except for the fact that D-glucuronic acid is neither 3-linked nor is it involved in branching. It is possible, however, that the acid identified in the hydrolysate of the polysaccharide after periodate oxidation in the earlier study by Barker was not in fact glucuronic acid, but the degradation product resulting from periodate oxidation of the 4-linked chain residue of glucuronic acid.

4.1.12 EXPERIMENTAL

4.1.12.1 PROPERTIES OF *KLEBSIELLA* K64 CAPSULAR POLYSACCHARIDE

Purified capsular polysaccharide (A) had $[\alpha]_D^{20}$ +28° (*c* 0.1), N 0.4% and moved as a single component on ultracentrifugation and electrophoresis. The weight-average molecular weight was *ca.* 1.7×10^6 as determined by gel permeation chromatography and the equivalent weight ~ 570 (as acid by titration), in agreement with the values previously reported[15].

The p.m.r. spectrum of a 2% solution of polysaccharide (A) in D_2O showed sharp singlets at τ 7.72 (acetate CH_3) and τ 8.40 (pyruvate CH_3), a doublet at τ 8.62 (L-rhamnose CH_3) and three signals in the anomeric region. Integration of the spectrum indicated a ratio of 1:1:1 for the CH_3 groups and the presence of six anomeric protons.

4.1.12.2 HYDROLYSIS OF POLYSACCHARIDE (A) ; SUGAR ANALYSIS

After hydrolysis of polysaccharide (A), followed by neutralisation of the hydrolysate, paper chromatography (solvents *a* and *d*), showed L-rhamnose, D-mannose, D-glucose, D-glucuronic acid and pyruvic acid (characteristic blue fluorescence under ultraviolet light when sprayed with *p*-anisidine). Analysis of the neutral sugars by g.l.c. of the alditol acetates gave L-rhamnose, D-mannose and D-glucose in the ratio 1.0:1.4:2.0. Preparative g.l.c. of the acetates allowed the recovery of L-rhamnitol pentaacetate as a syrup, and the hexaacetates of D-mannitol and D-glucitol as crystalline compounds. Comparison of the circular dichroism spectra of these derivatives with those of standard samples confirmed the assignment of the D-configuration to glucose and mannose and the L-configuration to rhamnose[66].

4.1.12.3 ACID HYDROLYSIS OF POLYSACCHARIDE (A) ; MOLECULAR-WEIGHT DISTRIBUTION ANALYSIS

The molecular-weight distribution patterns from the graded acid hydrolysis of polysaccharide (A) were obtained by the methods outlined in Section 2.8 and as described in the literature[15].

4.1.12.4 PARTIAL ACID HYDROLYSIS OF POLYSACCHARIDE (A) ; SEPARATION OF OLIGOSACCHARIDES

Polysaccharide (A) (1g) was hydrolysed with 0.01M trifluoroacetic acid (120 ml) at 100° for 48h, and the hydrolysis products were separated by preparative gel-permeation chromatography into four components which gave single peaks at molecular weights 1100, 930, 520 and 350 daltons when examined by gel-permeation chromatography on Bio-Gel P-10. The structures of these oligomers were proved by standard techniques including partial-hydrolysis studies and methylation analysis, as well as the use of p.m.r. spectroscopy and measurement of optical rotatory power to determine the configurations of anomeric linkages. The results obtained are summarised in Table 8. Proportions of neutral sugars were determined by g.l.c. (alditol acetate method) after hydrolysis of the oligosaccharides with 2M trifluoroacetic acid at 100°C for 8h.

The component having molecular weight 350 daltons was shown by paper chromatography (solvent *c*) to be a mixture of an aldobiouronic acid and a neutral fragment having the mobility of a disaccharide. Separation of the mixture by preparative paper chromatography (solvent *c*) gave the compounds I and II. Oligosaccharides I - V were further characterised as follows.

(I) A sample of the disaccharide (20mg) was dissolved in water (2ml) and reduced with sodium borohydride (20mg) for 24h. After acidification

with Amberlite IR-120 (H^+) resin and removal of borate (by distillation with methanol), the product was dissolved in D_2O (2ml) and freeze-dried. The p.m.r. spectrum of this compound (in D_2O) showed a doublet at τ 5.36 (6Hz), indicating a β-D-linkage. Hydrolysis of the reduced product followed by paper chromatography (solvent *d*) gave only D-glucose. Analysis of the neutral compound (by courtesy of Dr. J. P. Kamerling, Utrecht) by g.l.c.-mass spectrometry of the trimethylsilyl derivative indicated that neutral disaccharides with (1→2) and (1→4) type linkages were present, in the ratio 1:3 respectively. Methylation of the mixture followed by hydrolysis and g.l.c. analysis, as the derived alditol acetates (column (a)), of the methylated sugars present in the hydrolysate gave 2,3,4,6-tetra-O-methyl-D-glucose, 2,3,6-tri-O-methyl-D-mannose and 3,4,6-tri-O-methyl-D-mannose.

(II) Methylated aldobiouronic acid on methanolysis and g.l.c. gave methyl glycosides of 2,3,4-tri-O-methyl-D-glucuronate (removed by saponification) and 2,4,6-tri-O-methyl-D-mannose. The identity of the 2,4,6-tri-O-methyl-D-mannose was confirmed by hydrolysis of the methylated aldobiouronic acid and analysis of the hydrolysate by paper chromatography (solvents *b* and *f*), and by the g.l.c. retention time of the trimethylsilylated derived alditol. The p.m.r. spectrum of the aldobiouronic acid in D_2O gave anomeric signals at τ 4.73 (3.5Hz), 4.82 (2Hz) and 5.08 (2Hz), which are in good agreement with those published in the literature for 3-O-(α-D-glucopyranosyluronic acid)-D-mannose[42].

(III) The aldotriouronic acid on reduction with sodium borohydride followed by hydrolysis (2M trifluoroacetic acid, 100°C, 6h) and treatment for 4h with hot molar sodium hydroxide under oxygen yielded D-glucitol (identified by g.l.c. of its hexaacetate). Hydrolysis of

the aldotriouronic acid followed by paper chromatography of the hydrolysate (solvent *a*) gave D-glucose, D-mannose and the aldobiouronic acid II. Methylated aldotriouronic acid on methanolysis and g.l.c. gave methyl glycosides of 2,3,4-tri-O-methyl-D-glucuronate, 2,4,6-tri-O-methyl-D-mannose and 2,4,6-tri-O-methyl-D-glucose. The identity of 2,4,6-tri-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-mannose was confirmed by hydrolysis (2M trifluoroacetic acid, 100°C, 8h) of the methylated aldotriouronic acid and g.l.c. of the trimethylsilyl derivatives of the anomers of the tri-O-methyl sugars. The p.m.r. spectrum in D_2O of the borohydride-reduced aldotriouronic acid showed anomeric signals at τ 4.69 (3Hz) and 4.80 (1.5Hz), consistent with those expected from D-glucuronic acid (α) and D-mannose (α) moieties.

(IV) Hydrolysis of the component having molecular weight 930, followed by paper chromatography of the hydrolysate (solvent *a*), gave D-mannose, D-glucose and the aldobiouronic acid II. The fully methylated derivative of the oligomer was hydrolysed (2M trifluoroacetic acid, 100°C, 18h) and the sugars present in the hydrolysate were analysed by paper chromatography (solvents *b* and *f*) and by g.l.c. of the derived alditol acetates to give 2,3,4,6-tetra-O-methyl-D-glucose, 2,4,6-tri-O-methyl-D-mannose, 2,4,6-tri-O-methyl-D-glucose, 2,3,6-tri-O-methyl-D-mannose and 3,6-di-O-methyl-D-mannose.

(V) Paper chromatography (solvent *a*) of an hydrolysate of the oligomer having molecular weight 1100 revealed the same components as found on hydrolysis of oligosaccharide IV. Methylation, followed by hydrolysis (2M trifluoroacetic acid, 100°C, 18h) and analysis of the sugars present in the hydrolysate by paper chromatography (solvents *b* and *f*) and by g.l.c. of the derived alditol acetates, gave 2,3,4,6-tetra-O-methyl-D-glucose, 2,4,6-tri-O-methyl-D-mannose, 2,4,6-tri-O-methyl-D-glucose,

3,6-di-O-methyl-D-mannose and a small amount of 2,3,6-tri-O-methyl-D-mannose.

Methanolysis and g.l.c. of the fully methylated derivatives of the oligomers having molecular weights 1100 and 930 gave methyl 2,3,4-tri-O-methyl-D-glucuronate in addition to the sugars identified as methylated alditol acetates.

4.1.12.5 AUTOHYDROLYSIS OF POLYSACCHARIDE (A)

Polysaccharide (A) (0.1g) was dissolved in water (initial pH *ca.* 3) and heated at 100°C for 3h. Paper chromatography (solvent *e*) of the concentrated autohydrolysate showed a fast moving spot, chromatographically identical with pyruvic acid and having the same characteristic white fluorescence as the authentic material when sprayed with *o*-phenylenediamine and examined under u.v.[55]. A similar chromatogram, sprayed with *p*-anisidine, showed the presence of L-rhamnose and and a large amount of material at the origin. The autohydrolysate was diluted with water and dialysed against distilled water (1 l) for 24h. The non-dialysable material (17mg) was recovered by freeze-drying of the aqueous solution. The autohydrolysed polysaccharide (B), which had $[\alpha]_D^{22}$ +25° (*c* 0.1), $\overline{M}_w$ 1.5 x 10^6 daltons (by gel-permeation chromatography on agarose gel) and was found to contain no pyruvic acid (as indicated by its p.m.r. spectrum), was used for the methylation studies described in Section 4.1.12.7.

4.1.12.6 METHYLATION ANALYSIS OF POLYSACCHARIDE (A)

A sample (1.26g) of capsular polysaccharide (A) in the acid form was methylated once by Hakomori's method[14] and twice by the method of Purdie and Irvine[68] to give an amorphous product (1.17g), $[\alpha]_D^{20}$ +20°

(*c* 1.1, chloroform), which showed no absorption at 3700-3200 cm^{-1} (OH) and strong absorption at 1740 cm^{-1} (methyl ester CO); pyruvic acid ketal singlet at τ 8.60 (deuteriochloroform solution). The methylated polysaccharide (0.72g) was hydrolysed with 2M triflouroacetic acid at 100°C for 18h, and the neutral sugars (0.36g) produced were separated from the acidic sugars (0.12g) by ion-exchange chromatography on a column of Amberlite IR-45 (OH^-) resin.

The neutral-sugar mixture was applied to a column (65 cm x 4 cm) of cellulose, water-jacketed at 28°, and separated into its components by elution with butanone-water azeotrope. In some cases mixtures were resolved by preparative paper chromatography (solvent *b*). The fractions obtained were characterised as follows:

FRACTION I (19mg), was chromatographically identical with 2,3,4-tri-O-methyl-L-rhamnose. The trimethylsilylated alditol and alditol acetate derivatives were identical in their retention characteristics on g.l.c. with an authentic sample of this methylated sugar.

FRACTION II (90mg), on reduction and trimethylsilylation, gave derivatives of 2,4,6-tri-O-methyl-D-mannose and 2,4,6-tri-O-methyl-D-glucose in the ratio 0.7:1.0. The mixture was separated into its pure components by g.l.c. using a preparative column (2m x 6mm O.D.) packed with 15% SE-52 on Chromosorb W (80-100 mesh), isothermally at 140°C.

FRACTION III (33mg), $[\alpha]_D^{20}$ +39° (*c* 0.3), was indistinguishable from 2,3-di-O-methyl-D-glucose by paper chromatography and by p.m.r. spectroscopy. The g.l.c. retention times of both the trimethylsilylated and alditol acetate derivatives were identical with those of the authentic material.

FRACTION IV (35mg), $[\alpha]_D^{20}$ +8° (*c* 0.1), was characterised as 6-O-methyl-D-mannose by (a) its paper-chromatographic colour and mobility,

(b) its p.m.r. spectrum in D_2O, which showed signals at τ 4.78 (1.5Hz), 5.07 (2Hz) and 6.54, in good agreement with those given in the literature[76] for this compound, and (c) the retention time of its derived alditol acetate in g.l.c.[77].

The identity of each of the above methylated sugars was confirmed by mass spectrometry of the derived alditol acetate.

A sample (20mg) of the fully methylated polysaccharide was hydrolysed with 2M trifluoroacetic acid at 100°C for 18h. Samples were removed after 6h and 18h hydrolysis, reduced, trimethylsilylated and analysed by g.l.c. to give the results shown in Table 9.

The methylated acidic sugars were converted to their methyl ester methyl glycosides by heating with 1% methanolic hydrogen chloride at 100° C for 6h in a sealed tube under nitrogen. After neutralisation with silver carbonate the residue was dissolved in tetrahydrofuran (10ml) and reduced with lithium aluminium hydride (700mg). A sample (30mg) of the reduced product was hydrolysed with 2M trifluoroacetic acid at 100°C for 5h and the sugars present in the hydrolysate were analysed by paper chromatography (solvents *b* and *f*) to give 2,4,6-tri-O-methyl-D-mannose and 2,3-di-O-methylglucose. Quantitative analysis of these sugars by g.l.c. of the trimethylsilylated derived alditols gave 2,4,6-tri-O-methyl-D-mannose and 2,3-di-O-methylglucose in the ratio 1.0:1.6. Reduction, acetylation and preparative g.l.c. gave the individual methylated alditol acetates which were further characterised by mass spectrometry. A sample of the 2,3-di-O-methylglucitol tetraacetate was demethylated (BCl_3)[78] and re-acetylated to give glucitol hexa-acetate, which was shown to have the D-configuration by comparison of its circular dichroism spectrum with that of a standard sample. It appears certain that the origin of the 2,3-di-O-methyl-D-glucose found

as the major component in the hydrolysate of the reduced acidic sugars must be the glucuronic acid present in the original polysaccharide; this acid must accordingly also be of the D-configuration.

4.1.12.7 METHYLATION ANALYSIS OF DEGRADED POLYSACCHARIDE (B)

Polysaccharide (B) (15mg), produced on autohydrolysis of (A), was methylated by Hakomori's method to give a derivative (18mg) which showed no hydroxyl absorption in the i.r. Hydrolysis of the methylated product with 2M trifluoroacetic acid at 100°C for 18h, followed by paper chromatography of the hydrolysate (solvents *b* and *f*) showed the absence of 2,3-di-O-methyl-D-glucose and the appearance of a new spot having the same pink colour and mobility as 2,3,4,6-tetra-O-methyl-D-glucose when sprayed with *p*-anisidine. The hydrolysate was reduced, trimethylsilylated and analysed by g.l.c. to give the results shown in Table 9.

4.1.12.8 REDUCTION OF THE METHYLATED DERIVATIVES OF POLYSACCHARIDES (A) AND (B)

The fully methylated polysaccharide (A) (122mg) was reduced with lithium aluminium hydride (700mg) in tetrahydrofuran (10ml) to give a product having $[\alpha]_D^{20}$ +14° (*c* 0.1, chloroform) whose i.r. spectrum showed no absorption at 1740 cm^{-1} (methyl ester CO). Hydrolysis of a sample with 2M trifluoroacetic acid at 100°C for 8h, followed by paper chromatography (solvent *b*) and g.l.c. analysis of the sugars present in the hydrolysate (as the trimethylsilylated derived alditols), showed a marked increase in the proportion of 2,3-di-O-methyl-D-glucose, as can be seen from Table 10 and Figure 6.

The methylated derivative of polysaccharide (B) (6mg) was

reduced, hydrolysed, and analysed in the same manner to give the results shown in Table 10.

4.1.12.9 SMITH DEGRADATION OF POLYSACCHARIDE (A)

Capsular polysaccharide (A) (0.25g) was dissolved in aqueous sodium metaperiodate (50ml, 0.113M) and allowed to stand in the dark for 4 days, the consumption of periodate (0.49 mol per hexose) being monitored by the arsenite method[79] After this time the reaction was terminated by the addition of ethylene glycol (1.5ml) and the solution was stirred for 2h and then dialysed against running tap water for 3 days. Reduction with sodium borohydride (2g), deionisation with Amberlite IR-120 (H^+) resin, freeze-drying and removal of borate by distillation with methanol yielded the polyalcohol (106mg). This product was dissolved in M trifluoroacetic acid (20ml) and stirred at room temperature for 110h. Gel-permeation chromatography on Bio-Gel P-2 was used to monitor the change in molecular weight during the hydrolysis; this showed that after 24h a single product with $\overline{M}_w$ 560 daltons was generated, and no change in the molecular weight was apparent for samples removed after 48h and 110h. The trifluoroacetic acid was removed by freeze-drying and the residue was dissolved in methanol and examined by paper chromatography (solvent *b*). When sprayed with silver nitrate the paper chromatogram showed the presence of glycolaldehyde and a large amount of slow-moving material near the origin. In order to obtain the pure oligosaccharide, the methanol was removed and the residue extracted with absolute ethanol. The ethanol-insoluble material was dissolved in water and freeze-dried to yield the Smith-degraded product (67mg), which had $[\alpha]_D^{20}$ $+24^o$ (*c* 0.1) and mol. wt. 560 daltons.

This oligosaccharide (5mg) was hydrolysed with 2M trifluoroacetic acid (1ml) at 100°C for 5h. Reduction of the hydrolysate followed by acetylation and g.l.c. analysis of the acetates gave D-glucose and D-mannose in the ratio 1:1.8. The p.m.r. spectrum of the oligosaccharide in D_2O showed signals in the anomeric region at τ 4.78 (1Hz) and 5.14 (6Hz). The integration and coupling constants of the signals allowed their unambiguous assignment to two D-mannose (α) residues and one D-glucose (β) residue.

The Smith-degraded oligosaccharide (18mg) was methylated to give a product (15mg) whose i.r. spectrum showed no absorption in the hydroxyl region and strong absorption at 1720 cm^{-1} due to a methyl ester grouping. Re-examination of the i.r. spectrum of the methylated product after reduction with lithium aluminium hydride (100mg) in tetrahydrofuran (3ml) showed no absorption at 1720 cm^{-1}. The reduced methylated oligosaccharide was hydrolysed with 2M trifluoroacetic acid at 100°C for 8h and the sugars present in the hydrolysate were analysed by paper chromatography (solvents *b* and *f*) and by g.l.c. of the derived alditol acetates to give 2,3,4,6-tetra-O-methyl-D-mannose, 2,4,6-tri-O-methyl-D-glucose and 2,3,6-tri-O-methyl-D-mannose in approximately equal proportions. Preparative g.l.c. permitted the recovery of the acetates which were further characterised by mass spectrometry.

4.2 THE CAPSULAR POLYSACCHARIDE FROM *KLEBSIELLA* SEROTYPE K27

4.2.1 INTRODUCTION

The group of *Klebsiella* K-serotypes which produces capsular polysaccharides consisting of residues of D-glucuronic acid, D-galactose and D-glucose is known to include *Klebsiella* K-type 27 and six other strains. Branched tetrasaccharide repeating units have been found in the polysaccharides from K8[48], K11[49] and K51[50], while the primary structures of those from the remaining serotypes are at present being investigated[11]. In addition to the three types of sugar residue, the *Klebsiella* K27 polysaccharide contains a pyruvic acid moiety, as does the polysaccharide from K11.

Purified *Klebsiella* K27 capsular polysaccharide (A), obtained from a culture (N.C.T.C. 9147), had $[\alpha]_D$ $+5^{\circ}$, $\overline{M}_w$ 9.4×10^5, and was monodisperse according to gel-permeation chromatography and ultracentrifugation. The proton magnetic resonance spectrum of a solution of the polysaccharide showed a sharp singlet at τ 8.48, indicative of pyruvic acid ketal. Integration of the signals due to the anomeric protons and that of the methyl group at τ8.48 showed the presence of one pyruvic acid ketal to six sugar residues; no O-acetyl groups were detected.

The molar proportions of sugars found by g.l.c. analysis (alditol acetate method) of an hydrolysate of the polysaccharide (A) were galactose (2.0) and glucose (3.0). These sugars were shown to be in the D-configuration by the procedures outlined in Section 4.2.9.2.

4.2.2 GRADED ACID HYDROLYSIS

Graded acid hydrolysis, monitored by paper chromatography of the

hydrolysates, showed that glucose and pyruvic acid were the first residues to be released from the polysaccharide structure, followed more slowly by galactose and two neutral disaccharides. Glucuronic acid and several other acidic products of higher molecular weight were then detected, including an aldobiouronic acid chromatographically indistinguishable from 6-O-(β-D-glucopyranosyluronic acid)-D-galactose.

Mild acid hydrolysis, monitored by gel-permeation chromatography of samples removed at intervals, showed that the breakdown of the polysaccharide was slow in comparison with that of most of the other *Klebsiella* polysaccharides that have been examined by this method, the rate being comparable with that for the K64[15] polysaccharide. Gel-permeation chromatography on agarose revealed a slow decrease in $\overline{M}_w$ from 940 000 to 450 000 during the first 4h of hydrolysis in 0.01M acid at 100°; from 24h onward, Bio-Gel P-10 chromatograms (see Tables 11 and 12 and Figures 8 and 9) showed multiple peaks at molecular weight 1000 and integral multiples thereof (up to 10 000), suggesting a repeating unit of about this size (i.e. a hexasaccharide). After hydrolysis for 48h in 0.01M acid, the component having molecular weight 1000 was present in the hydrolysate to the extent of 30% by weight, but 54% of the mixture consisted of material of molecular weight 2000 - 6000 ($\overline{M}_w$ 2700). The products having molecular weights above 1000 disappeared only after further hydrolysis in 0.1M acid for 5h, at which stage much of the hexasaccharide had broken down to lower oligosaccharides (Figure 10), so that its proportion remained at only *ca.* 30% by weight under these conditions.

4.2.3 AUTOHYDROLYSIS OF POLYSACCHARIDE (A)

Autohydrolysis of the polysaccharide (A) yielded pyruvic acid and traces of glucose, which were separated by dialysis from the

TABLE 11

PARTIAL HYDROLYSIS OF *KLEBSIELLA* K27 CAPSULAR POLYSACCHARIDE IN 0.01M TRIFLUOROACETIC ACID

Time (h)	$\overline{M}_w$ (Daltons)	$[\alpha]_D$ [a] (Degrees)
0	940 000 [b]	+ 5
1	720 000	+ 8
4	450 000	+10
7	n.d.	+12
24	4 900	+17
48	2 700	+22

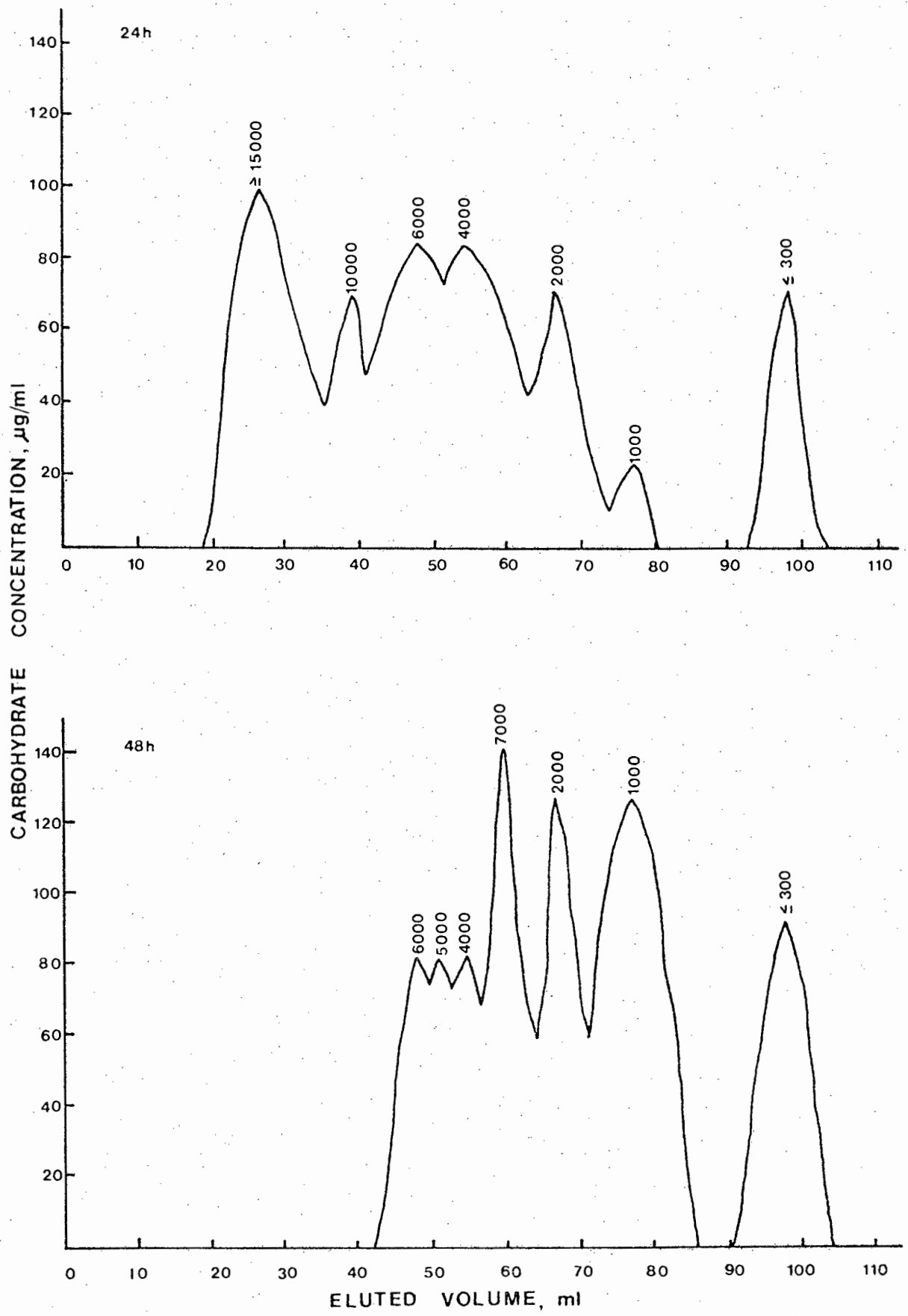
[a] Concentration c, 0.48 [b] Single peak (Sagavac 6F)

TABLE 12

FURTHER HYDROLYSIS OF DEGRADED *KLEBSIELLA* K27 CAPSULAR POLYSACCHARIDE IN 0.1M TRIFLUOROACETIC ACID

Time (h)	$[\alpha]_D$ [a] (Degrees)
0	+22
1	+25
3	+28
5	+29
24	+40

[a] Concentration c, 0.46



FIGURES 8 & 9

Bio-Gel P-10 elution patterns of hydrolysates (0.01M trifluoroacetic acid, 100°, 24-48h) of *Klebsiella* K27 capsular polysaccharide.

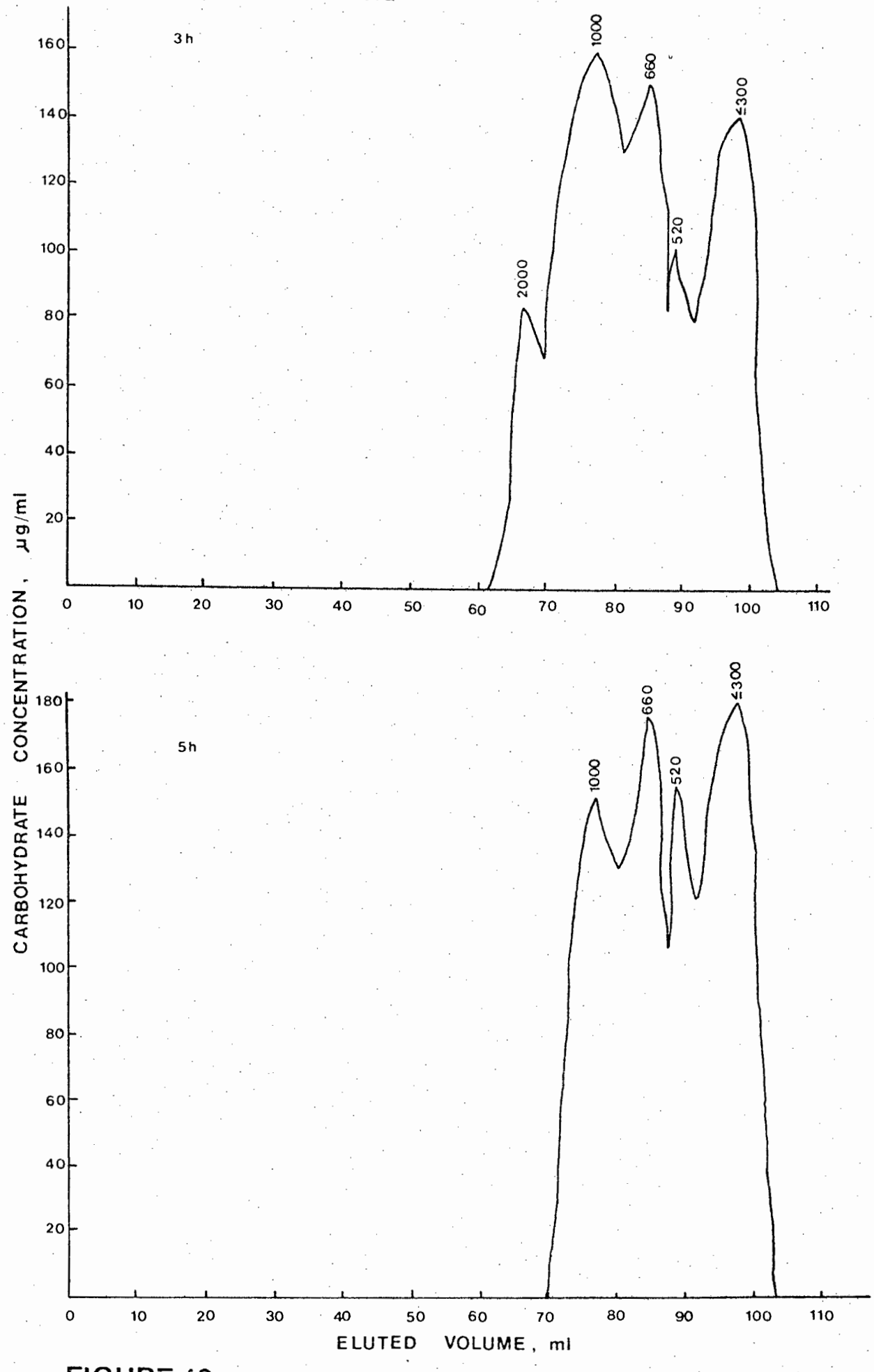


FIGURE 10

Bio-Gel P-10 elution patterns on further hydrolysis (0.1M trifluoroacetic acid, 100°, 3-5h) of *Klebsiella* K27 capsular polysaccharide.

polysaccharide product (B), $[\alpha]_D$ +2°, $\overline{M}_w$ 8.4 x 10^5 (by gel-permeation chromatography), the p.m.r. spectrum of which showed the absence of pyruvic acid ketal. A sample of polysaccharide (B) was methylated and used for the methylation analysis discussed in Section 4.2.5.

4.2.4 METHYLATION ANALYSIS OF POLYSACCHARIDE (A)

Capsular polysaccharide (A) was methylated and a portion hydrolysed, and the sugars present in the hydrolysate were analysed by paper chromatography and by g.l.c. of the trimethylsilylated, derived alditols. The results obtained are shown in Table 13 and Figure 11.

It is evident that the number of branch-points in the structure cannot be accounted for solely by the terminal residues of D-glucose found in this analysis. The identification of 2,4,6-tri-O-methyl-D-galactose and 2,3,4-tri-O-methyl-D-glucose shows that these residues represent 3- and 6-linked sugars in the chain, while the presence of the 2-O-methyl derivatives of both D-galactose and D-glucose indicates a high degree of branching in the polysaccharide (A).

Methanolysis of another portion of the fully methylated polysaccharide followed by g.l.c. analysis of the resulting methyl glycosides showed the presence of 2,3,4-tri-O-methylglucuronate, in addition to methyl glycosides of the neutral sugars given in Table 13, indicating that the glucuronic acid residues in the polysaccharide (A) occupy a terminal position. These acid residues were shown to have the D-configuration by the procedures outlined in Section 4.2.9.7.

TABLE 13

METHYL ETHERS FROM THE HYDROLYSATES OF THE ORIGINAL (A) AND DEGRADED (B) POLYSACCHARIDES FROM *KLEBSIELLA* K27

Sugar[a]	T[b]	Molar Proportions (%)	
		A[c]	B[c]
2,3,4,6-Glc	1.01	19.7	19.3
2,4,6-Gal	1.19	22.5	19.0
2,4,6-Glc	1.34	-	18.3
2,3,4-Glc	1.62	18.7	18.4
2,4-Gal	1.80	-	6.5
2-Gal	2.48	19.4	18.3
2-Glc	2.76	19.7	-

[a] 2,3,4,6-Glc = 2,3,4,6-tetra-O-methyl-D-glucose, *etc.*

[b] Retention time of the corresponding (TMS) alditol relative to that of 2,3,4,6-tetra-O-methyl-D-galactose on an SE-52 column at 140°C.

[c] Hydrolysates examined after 18h hydrolysis.

4.2.5 METHYLATION ANALYSIS OF DEGRADED POLYSACCHARIDE (B)

The fully methylated derivative of polysaccharide (B) was hydrolysed, and a portion of the hydrolysate was analysed by paper chromatography and by g.l.c. of the trimethylsilylated derived alditols to give the results shown in Table 13 and Figure 12. The remainder of the hydrolysate was reduced, the alditols were acetylated, and the acetate mixture was separated into the component methylated alditol acetates[59] by preparative g.l.c.. Mass spectrometry was used to

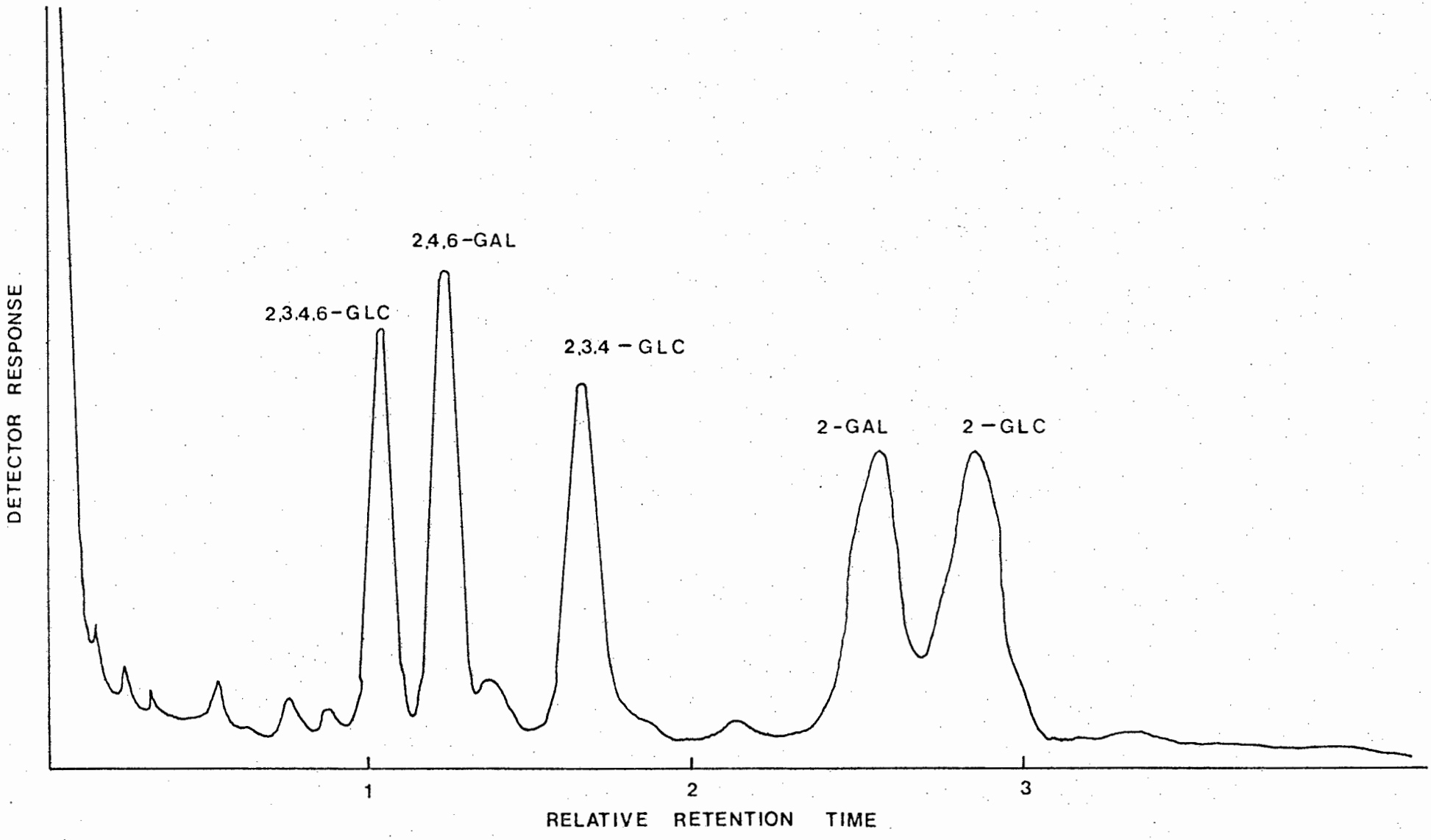


FIGURE 11 G.l.c. trace of the partially methylated (TMS) alditols from fully methylated *Klebsiella* K27 capsular polysaccharide (A).

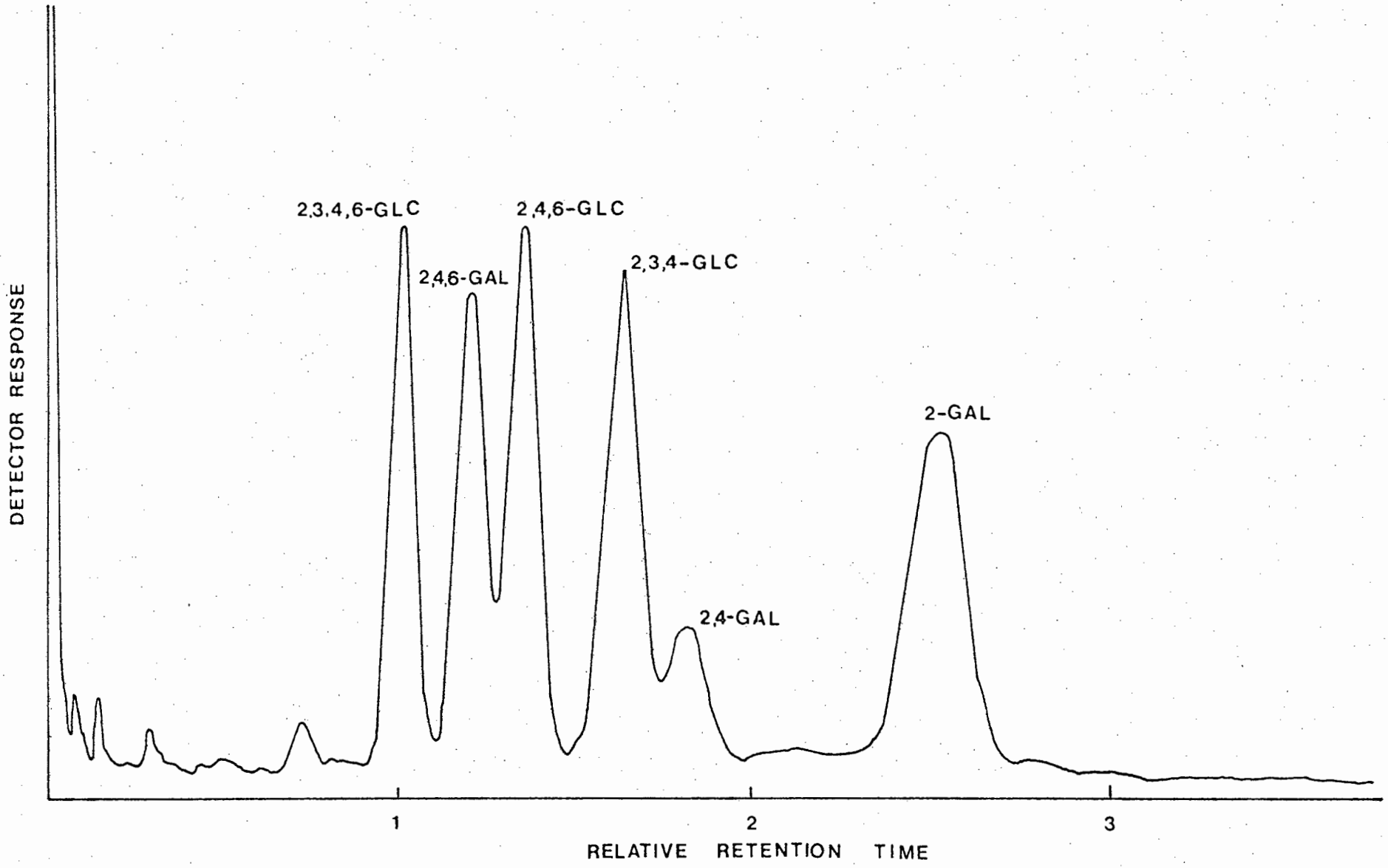


FIGURE 12 G.l.c. trace of the partially methylated (TMS) alditols from fully methylated degraded polysaccharide (B) from *Klebsiella* K27.

confirm the substitution pattern of each methylated alditol acetate.

Identification of 2,4,6-tri-O-methyl-D-glucose in the hydrolysate, and the absence of 2-O-methyl-D-glucose, indicate the removal, during autohydrolysis, of pyruvic acid from the 4- and 6-positions of a D-glucose residue which must be linked through O-3 in the polysaccharide chain. The small amount of 2,4-di-O-methyl-D-galactose in the hydrolysate may be accounted for by the loss of some terminal D-glucose residues during the autohydrolysis, thus affording evidence that these are linked to the 4-position of D-galactose. This same D-galactose residue must also be substituted at O-6 and O-3, in view of the high proportion of 2-O-methyl-D-galactose found in the hydrolysates of both polysaccharides (A) and (B). As indicated by the products of partial hydrolysis (Section 4.2.2), it is probably the terminal unit of D-glucuronic acid which is attached at O-6 of D-galactose to form the aldobiouronic acid moiety.

4.2.6 PARTIAL HYDROLYSIS OF POLYSACCHARIDE (B) ; ISOLATION AND CHARACTERISATION OF OLIGOSACCHARIDES

Hydrolysis of a sample of polysaccharide (B) with 0.1M trifluoroacetic acid at 100°C for 3h yielded a number of oligosaccharides, I - VIII, which were separated by preparative paper chromatography and examined by gel-permeation chromatography on Bio-Gel P-10. Of the acidic oligosaccharides isolated, one fraction contained two components, having molecular weights 3000 (30% by weight) and 2000 respectively; the others each gave a single peak, at molecular weights 1000 (i.e. the structurally significant hexasaccharide), 850, 660, 520 and 360 daltons. The two neutral components were disaccharides (molecular weight 340 daltons), as indicated also by their mobilities on paper chromatography. Where possible the structures of oligosaccharides I - VIII were determined, using standard techniques as described in Section 4.2.9.7; see Table 14.

TABLE 14

ANALYSIS OF THE OLIGOSACCHARIDES FROM *KLEBSIELLA* K27 AUTOHYDROLYSED POLYSACCHARIDE (B)

Fraction	Oligomer	$[\alpha]_D$ (in H_2O) (Degrees)	R_{glc} (Solvent *a*)	Mol.wt. (Daltons)	Neutral Sugars (Molar ratios)	Methylated (TMS) Alditols (Molar ratios)
I	Neutral disaccharide	+ 16	0.73	340	Glc	2,3,4,6-Glc (1.0) 2,4,6-Glc (1.0)
II	Neutral disaccharide	+ 22	0.59	340	Glc (1.0) Gal (1.0)	2,3,4,6-Glc (1.0) 2,4,6-Gal (1.0)
III	Aldobiouronic acid	- 7	0.40	360	Gal	2,3,4-Gal
IV	Aldotriouronic acid	+ 5	0.23	520	Glc (1.0) Gal (0.8)	Indicated that fraction was a mixture
V	Aldotetraouronic acid	+ 4	0.13	660	Glc (2.0) Gal (1.3)	Indicated that fraction was a mixture
VI	Acidic pentasaccharide	+ 16	0.07	850	Glc (2.0) Gal (1.8)	2,3,4,6-Glc (1.0) 2,4,6-Gal (0.8) 2,3,4-Glc (0.7) 2,4-Gal (0.7)

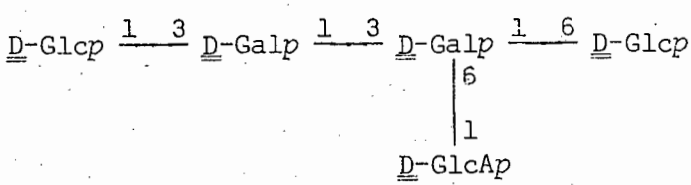
TABLE 14 (CONTD.)

Fraction	Oligomer	$[\alpha]_D$ (in H_2O) (Degrees)	R_{glc} (Solvent *a*)	Mol.wt. (Daltons)	Neutral Sugars (Molar ratios)	Methylated (TMS) Alditols (Molar ratios)
VII	Acidic hexasaccharide	+ 6	0.03	1000	Glc (3.0) Gal (2.5)	2,3,4,6-Glc (1.0) 2,4,6-Gal (1.1) 2,4,6-Glc (0.5) 2,3,4-Glc (0.7) 2,4-Gal (0.4)
VIII	Acidic oligosaccharide	+ 18	0.00	2000 (71%) + 3000 (29%)	Glc (3.0) Gal (2.6)	2,3,4,6-Glc (1.0) 2,4,6-Gal (1.1) 2,4,6-Glc (0.6) 2,3,4-Glc (0.8) 2,4-Gal (0.4) 2-Gal (0.6)

Neutral disaccharide I was indistinguishable on paper chromatography from 3-O-β-D-glucopyranosyl-D-glucose, in three different solvent systems (*a*, *b* and *d*), while the optical rotation and paper-chromatographic mobility of neutral disaccharide II were in good agreement with those given in the literature for 3-O-β-D-glucopyranosyl-D-galactose[80]. The optical rotations of the aldobiouronic acid and of its barium salt ($[\alpha]_D$ -3°) were in accordance with those reported for 6-O-(β-D-glucopyranosyluronic acid)-D-galactose[80]; this structural unit is common in plant gums, but has not been reported previously in work on *Klebsiella* polysaccharides.

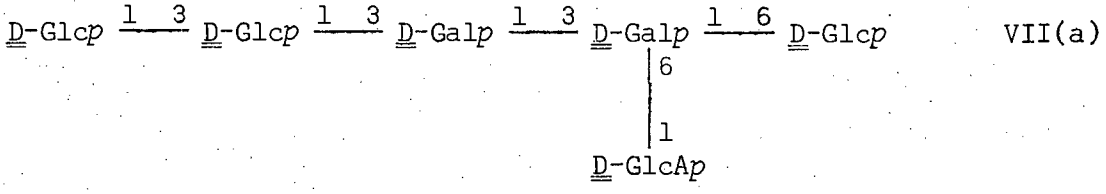
The non-integral value obtained for the molar proportion of D-galactose in the hydrolysates of the acidic oligosaccharides after 8h treatment with 2M trifluoroacetic acid at 100° is consistent with this hexose being part of the aldobiouronic acid. The more vigorous conditions (2M trifluoroacetic acid at 100° for 18h) used in acid hydrolysis of the original K27 polysaccharide (A) gave the neutral sugar ratios reported in Section 4.2.1.

The methylation analyses of the fractions IV and V, of molecular weight 520 and 660 daltons respectively, indicated that these were mixtures of linear and branched oligosaccharides, which were inseparable and could not be fully characterised. Analysis of the acidic pentasaccharide VI shows that it has the structure given below; it is derived from the acidic hexasaccharide VII through the loss of a terminal chain residue of D-glucose.

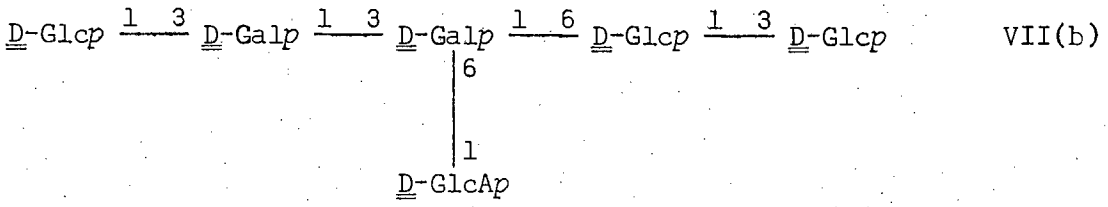


VI

An examination of the methylated sugars obtained on hydrolysis of the fully methylated hexasaccharide VII indicates that there are two possible structures for the oligosaccharide. It is evident that the loss of a D-glucose residue from either of these structures VII(a) or VII(b) would give rise to the acidic pentasaccharide VI. In both cases the D-glucose residue which is lost would have to be linked through O-3 in the original polysaccharide chain.



VII(a)



VII(b)

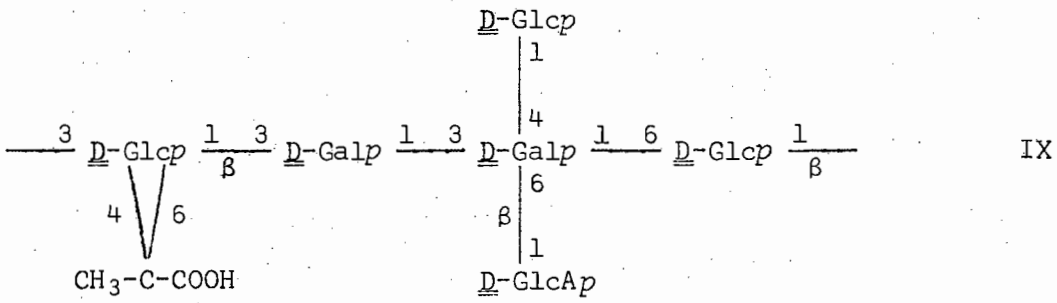
Methylation analysis of the oligosaccharide VIII shows that a large number of the terminal residues of D-glucose, linked to the 4-position of D-galactose in the polysaccharide chain, are not removed under the conditions of acid hydrolysis used to generate the above oligosaccharides. The low proportion of 2,4-di-O-methyl-D-galactose found in the hydrolysates of methylated derivatives of the higher oligosaccharides further confirms that this D-galactose residue is linked to D-glucuronic acid, through O-6, to form the aldobiouronic acid III.

4.2.7 THE REPEATING UNIT IN *KLEBSIELLA* K27 CAPSULAR POLYSACCHARIDE

The methylation analyses show that the repeating unit in the K27 capsular polysaccharide consists of chain residues of 3- and 6-linked D-glucose and of 3-linked D-galactose, to which are attached terminal units of D-glucuronic acid, pyruvic acid and D-glucose. Consideration of these results, together with the oligosaccharides

mentioned in 4.2.6, particularly 3-O-β-D-glucopyranosyl-D-glucose, shows that in order to obtain this neutral disaccharide and 3-O-β-D-glucopyranosyl-D-galactose, the polysaccharide chain must contain the sequence of sugar residues →3)-β-D-Glc*p*-(1→3)-D-Gal*p*-(1→3)-D-Gal*p*-(1→6)-D-Glc*p*-(1→.

The pyruvic acid moiety has been shown to be attached to the only 3-linked D-glucose residue, but the terminal units of D-glucuronic acid and D-glucose could be linked to either one of the D-galactose residues in the chain. However, since neutral disaccharides were obtained with (1→3) linkages between glucose and glucose and between glucose and galactose, but not between galactose and galactose, it would appear that the D-galactose residue carrying the terminal groups occurs directly before the 6-linked glucose residue in the polysaccharide chain. On the basis of the foregoing argument, IX represents the repeating unit for *Klebsiella* K27 polysaccharide:



IX

The nature of certain anomeric linkages in the above repeating unit could not be determined by consideration of the oligosaccharides isolated. The increase in optical rotation accompanying the release of D-glucose during the early stages of mild acid hydrolysis, however, suggests that this terminal unit is β-linked. Proton magnetic resonance spectroscopy of both the original (A) and degraded (B) polysaccharides revealed only one anomeric signal corresponding to an α-D-linkage, the remainder being indicative of β-D-linkages. This α-linkage, therefore, must involve one of the D-galactose residues in the chain, the other being

β-linked. The optical rotation of the polysaccharide ($+5^{\circ}$) is close to the value ($+9^{\circ}$) calculated by application of Hudson's rules of isorotation[71] to the structure as a whole on the basis of the above assignment of the anomeric linkages.

4.2.8 COMPARISON WITH OTHER *KLEBSIELLA* CAPSULAR POLYSACCHARIDES

A number of the structural features found in the capsular polysaccharide from K27 occur also in the branched tetrasaccharide repeating units of the polysaccharides from *Klebsiella* serotypes K8[48], K11[49] and K51[50], which belong to the same chemotype as K27. The aldobiouronic acids from three strains, K8, K11 and K27, contain D-glucuronic acid linked to D-galactose, but the positions and modes of linkage differ, while D-glucose forms part of the aldobiouronic acid in the polysaccharide from K51. The neutral disaccharide 3-O-β-D-glucopyranosyl-D-galactose has been isolated from a partial hydrolysate of the K8 polysaccharide, and pyruvic acid ketal (linked to D-galactose in this case) is found in the repeating unit of the polysaccharide from K11. Chain units of 3-linked D-galactose and D-glucose are common to all four structures and are also found in a large number of other serotypes. The *Klebsiella* K51 polysaccharide shows the unusual feature of a chain D-galactose residue linked through O-4, while residues of D-glucose linked through O-6 have been reported in the polysaccharide chains from K23[46], K38[10], K41[81] and K54[13].

Of the *Klebsiella* polysaccharide structures known, however, that of K64, which has a hexasaccharide repeating unit with a double branch on a residue of D-mannose, is the closest analogue to that of K27, although recent studies have shown that the pentasaccharide repeating unit of the polysaccharide from K33[44] contains a residue of D-mannose which is substituted at O-3, O-4 and O-6. The rate of degradation of both the K27

polysaccharide and that from K64 under conditions of mild acid hydrolysis is comparatively slow; acidic oligosaccharides of fairly high molecular weight (from K27, 1000 and 850 daltons; from K64, 1100 and 930 daltons) are thereby released, together with two neutral disaccharides which have proved crucial in formulating the repeating units. Both polysaccharides contain the structural unit 4,6-O-(1-carboxyethylidene)-D-glucose, which in the case of the K64 polysaccharide forms a side chain. Autohydrolysis of both polysaccharides yields pyruvate-free products having high degree of polymerisation. The only major differences in the structural patterns of the K27 and K64 polysaccharides are the positions of the pyruvic acid and D-glucuronic acid moieties.

4.2.9 EXPERIMENTAL

4.2.9.1 PROPERTIES OF *KLEBSIELLA* K27 CAPSULAR POLYSACCHARIDE

Purified capsular polysaccharide (A) had $[\alpha]_D^{20}$ +5° (*c* 0.7), N 0.4%, and moved as a single component on ultracentrifugation and gel-permeation chromatography on agarose ($\overline{M}_w$ 9.4 x 10^5). The p.m.r. spectrum of a 2% solution of the polysaccharide in D_2O showed a sharp singlet at τ 8.48, due to the CH_3 of the pyruvic acid ketal group, and signals for 6 anomeric protons[20], corresponding to one α-linkage at τ 5.0 (3Hz) and five β-linkages at τ 5.14 - τ 5,62. The chemical shifts and coupling constants of the β-linkages could not be assigned to specific residues due to the close proximity of the signals.

Integration of the spectrum showed a ratio of one pyruvic acid ketal group to six anomeric protons.

4.2.9.2 HYDROLYSIS OF POLYSACCHARIDE (A) ; SUGAR ANALYSIS

Polysaccharide (A) was hydrolysed with acid for 18h. After neutralisation, paper chromatography (solvents *a* and *d*) of the hydrolysate showed D-galactose, D-glucose, D-glucuronic acid, and an aldobiouronic acid. Analysis of the neutral sugars present by g.l.c. of the derived alditol acetates gave the molar ratio of D-galactose to D-glucose as 2.0:3.0. Preparative g.l.c. of the acetates allowed recovery of the individual compounds and identification of D-glucitol hexaacetate by comparison of its circular dichroism spectrum with that of a standard sample. The D-galactose derivative was shown to be achiral by this method. Isolation of D-galactose from the hydrolysate by preparative paper chromatography (solvent *d*), followed by methylation, hydrolysis and reduction with sodium borohydride gave 2,3,4,6-tetra-O-methyl-D-galactitol, (identified by the g.l.c. retention time of the

trimethylsilyl derivative). Acetylation of the methylated alditol gave 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-galactitol, which was purified by preparative g.l.c. and dissolved in acetonitrile. A positive circular dichroism curve, identical to that given by the authentic compound, confirmed the D-configuration of galactose[66].

4.2.9.3 ACID HYDROLYSIS OF POLYSACCHARIDE (A) ; MOLECULAR-WEIGHT DISTRIBUTION ANALYSIS

Graded acid hydrolysis of polysaccharide (A) was performed as described in Section 2.8.

4.2.9.4 AUTOHYDROLYSIS OF POLYSACCHARIDE (A)

Capsular polysaccharide (A) (180mg) was dissolved in distilled water (3ml) and heated in a sealed tube under nitrogen for 3h at 100°C. Paper chromatography (solvent *e*) of the concentrated solution showed a fast-moving spot, chromatographically identical with pyruvic acid and having the same characteristic fluorescence as the authentic material when sprayed with *o*-phenylenediamine and examined under ultraviolet light. Examination of a similar chromatogram sprayed with *p*-anisidine showed the presence of D-glucose and a large amount of material at the origin. The autohydrolysate was diluted with water and dialysed against distilled water (3ℓ) for 24h. The non-dialysable material (132mg) was recovered by freeze-drying of the aqueous solution. The polysaccharide (B) obtained by this process had $[\alpha]_D^{20}$ +2° (*c* 0.5), $\overline{M}_w$ 8.4 x 10^5 daltons (by gel-permeation chromatography) and was found to contain no pyruvic acid (as indicated by its p.m.r. spectrum). A sample of the polysaccharide (B) was methylated (Hakomori method) and used for the methylation studies described in Section 4.2.9.6 below.

4.2.9.5 METHYLATION ANALYSIS OF POLYSACCHARIDE (A)

Polysaccharide (A) (50mg) was methylated once by Hakomori's method and twice by the method of Purdie and Irvine to yield a colourless, amorphous product (48mg) which showed no hydroxyl absorption in the i.r.; the continued presence of pyruvic acid ketal in this product was evident from the characteristic singlet at τ 8.60 in the p.m.r. spectrum of a sample dissolved in deuteriochloroform. After hydrolysis of the methylated product in 2M trifluoroacetic acid, paper chromatography of the hydrolysate (solvents *b* and *f*) showed the presence of 2,3,4,6-tetra-O-methyl-D-glucose, 2,3,4-tri-O-methyl-D-glucose, 2,4,6-tri-O-methyl-D-galactose, and a slow-moving component having a colour similar to that of a 2-O-methyl sugar when sprayed with *p*-anisidine. A portion of the hydrolysate was reduced, and the derived alditols were analysed by g.l.c. of the trimethylsilyl derivatives to give the molar proportions shown in Table 13 . The slow-moving component was shown by this method to contain both 2-O-methyl-D-galactose and 2-O-methyl-D-glucose.

A sample of the fully methylated polysaccharide (5mg) was heated with 10% methanolic hydrogen chloride to yield a mixture of methyl glycosides which was analysed by g.l.c. The gas chromatogram showed peaks corresponding to methyl-2,3,4-tri-O-methyl-D-glucuronate (removed by saponification) and the methyl glycosides of 2,3,4,6-tetra-O-methyl-D-glucose, 2,3,4-tri-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-galactose.

4.2.9.6 METHYLATION ANALYSIS OF DEGRADED POLYSACCHARIDE (B)

A sample (20mg) of the fully methylated derivative of polysaccharide (B) was hydrolysed in 2M trifluoroacetic acid. Paper

chromatography of the hydrolysate (solvents *b* and *f*) indicated the presence of the same methylated sugars as were found in the hydrolysate of the methylated derivative of polysaccharide (A), together with a new component, the paper-chromatographic colour and mobility of which were identical with authentic 2,4,6-tri-O-methyl-D-glucose. The mixture of methylated sugars in the hydrolysate was analysed by g.l.c. of the trimethylsilylated derived alditols to give the proportions listed in Table 13 . A further portion of the hydrolysate was reduced and acetylated and the methylated alditol acetates were recovered by preparative g.l.c. The identity of each acetate was confirmed by mass spectrometry.

4.2.9.7 PARTIAL HYDROLYSIS OF POLYSACCHARIDE (B)

Polysaccharide (B) (100mg) was heated in 0.1M trifluoroacetic acid (5ml) for 3h at 100°C. Paper chromatography of the hydrolysate (solvent *a*) showed that a large number of neutral and acidic oligosaccharides were present. The fractions I - VIII were isolated by preparative paper chromatography, and where possible the structures of these oligosaccharides were determined using the techniques of partial acid hydrolysis, methylation analysis, and the measurement of optical rotatory power to assign the configurations of anomeric linkages. The results obtained are shown in Table 14.

The acidic higher oligosaccharides IV - VIII were hydrolysed with 2M trifluoroacetic acid for 8h at 100°C. Paper chromatography of the hydrolysates (solvents *a* and *d*) in each case showed the presence of D-galactose, D-glucose, D-glucuronic acid, and a component having the same mobility as 6-O-(β-D-glucopyranosyluronic acid)-D-galactose. The proportions of the neutral sugars present in the hydrolysates were determined by g.l.c. (alditol acetate method). The lower oligosaccharides I - III were further characterised as follows:

NEUTRAL DISACCHARIDE I. - Methanolysis and g.l.c. analysis of the fully methylated disaccharide gave the methyl glycosides of 2,3,4-6-tetra-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-glucose. The identity of these sugars was confirmed by hydrolysis of the methylated disaccharide, and g.l.c. of the trimethylsilyl derivatives of the anomers of the tetra- and tri-O-methyl sugars.

NEUTRAL DISACCHARIDE II. - Reduction of the disaccharide with sodium borohydride, followed by hydrolysis for 5h, and paper chromatography of the hydrolysate (solvent *d*) gave only D-glucose. The fully methylated disaccharide on methanolysis and g.l.c. gave methyl glycosides of 2,3,4-6-tetra-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-galactose.

ALDOBIOURONIC ACID III. - Methanolysis, followed by g.l.c., of the fully methylated aldobiouronic acid III gave methyl glycosides of methyl 2,3,4-tri-O-methyl-D-glucuronate (removed by saponification) and 2,3,4-tri-O-methyl-D-galactose. Methylated aldobiouronic acid (6mg) was reduced with lithium aluminium hydride (20mg) in tetrahydrofuran (5ml) for 18h[74]. After the addition of aqueous ethanol and evaporation to dryness the residue was extracted with chloroform (3 x 5ml); the extracts were combined and the chloroform was removed to yield a product which was hydrolysed for 4h, after which the sugars present in the hydrolysate were converted to their derived alditol acetates. Analysis by g.l.c. (column (a)) showed approximately equal proportions of 2,3,4-tri-O-methyl-D-glucose and 2,3,4-tri-O-methyl-D-galactose. Recovery of the glucose derivative by preparative g.l.c. followed by demethylation gave glucitol, which was shown to be in the D-configuration by the circular dichroism spectrum of its hexaacetate. Since the 2,3,4-tri-O-methyl-D-glucose was derived from 2,3,4-tri-O-methyl glucuronic

acid, these residues must accordingly be in the D-configuration in the methylated aldobiouronic acid.

The p.m.r. spectrum of the barium salt of the aldobiouronic acid showed anomeric signals at τ 4.78 (3Hz), 5.36 (8Hz) and 5.55 (8Hz), consistent with those expected from D-galactose (α and β) and D-glucuronic acid (β) moieties.

4.3 THE CAPSULAR POLYSACCHARIDE FROM *KLEBSIELLA* SEROTYPE K4

4.3.1 INTRODUCTION

The capsular polysaccharide from *Klebsiella* K4, which contains residues of D-glucuronic acid, D-glucose and D-mannose, was the subject of a note[82] published in 1962. Originally known as *Klebsiella ozaenae* type D, the polysaccharide was reported to contain comparable amounts of galactose and mannose. Nimmich has also noted the presence of[4] galactose in a strain of *Klebsiella* K4. However, subsequent studies have shown[15] that the proportion of galactose varies considerably in the hydrolysates from different preparations of this polysaccharide; this sugar does not, therefore, form part of the repeating unit. *Klebsiella ozaenae* type D was used in a number of inhibition experiments[82] with the individual monosaccharides believed to be present as units in the capsular polysaccharide (the nature of the uronic acid residues in the polysaccharide was unknown at this stage), to investigate the role of the constituent sugars in determining the antigenic specificity of the polysaccharide, but the experiments were inconclusive since none of the sugars caused any inhibition of antibody precipitation. Mild oxidation with periodic acid was found to modify some of the groupings responsible for the antigenic specificity of the polysaccharide, but no qualitative or quantitative differences in composition between the native and oxidised polysaccharides were detected.

In the present investigation the acidic polysaccharide, produced by a strain isolated from a patient having a chronic lung infection, had $[\alpha]_D$ + 90°, $\overline{M}_w$ 2.1 x 10^5 daltons, and was monodisperse according to gel-permeation chromatography, ultracentrifugation and electrophoresis. The proton magnetic resonance spectrum of the polysaccharide indicated that one O-acetyl group was present to four sugar

residues; no pyruvic acid ketal groups were detected. The chemical shifts of the signals in the anomeric region of the spectrum suggested that three of the sugar residues were linked by α-D- and one by β-D-glycosidic bonds.

The ratios of neutral sugars found by g.l.c. analysis (alditol acetate method[60]) of an hydrolysate of the polysaccharide were glucose (2.0) and mannose (0.6), in agreement with those published[15]. The low proportion of mannose is consistent with the incomplete hydrolysis of the aldobiouronic acid linkages under the conditions of acid hydrolysis (2M trifluoroacetic acid, 100°C, 18h) used to release the neutral sugars for analysis. These sugars were shown to have the D-configuration by measurement of the circular dichroism spectra of their respective alditol acetates. Glucuronic acid was shown to have the same configuration by the procedure outlined in Section 4.2.9.7.

4.3.2 GRADED ACID HYDROLYSIS STUDIES

The results of a study[15] of the acid hydrolysis of *Klebsiella* K4 exopolysaccharide are shown in Tables 15 and 16 and Figures 13 and 14. Paper chromatography of the hydrolysate obtained after treatment of the polysaccharide with 0.01M trifluoroacetic acid at 100° for only 3h showed the release of glucose and traces of oligosaccharides having mobilities comparable with those of di- and trisaccharides. The elution patterns obtained on gel-permeation chromatography on Bio-Gel P-300 (for samples removed after 3h and 6h) and Bio-Gel P-10, which showed multiple peaks, most of which occurred at molecular weights approximating closely to integral multiples of 700, and the appearance of a major peak at molecular

TABLE 15

PARTIAL HYDROLYSIS OF *KLEBSIELLA* K4 CAPSULAR POLYSACCHARIDE IN 0.01M TRIFLUOROACETIC ACID

Time (h)	$\overline{M}_w$ (Daltons)	$[\alpha]_D$ [a] (Degrees)
0	210 000 [b]	+90
1	140 000	+87
3	20 000	+80
6	4 800	+72
10	1 600	+63
24	1 200	+55
48	640	+47

[a] Concentration *c.* 0.42 [b] Single peak (Sagavac 6F)

TABLE 16

FURTHER HYDROLYSIS OF DEGRADED *KLEBSIELLA* K4 CAPSULAR POLYSACCHARIDE IN 0.1M TRIFLUOROACETIC ACID

Time (h)	$[\alpha]_D$ [a] (Degrees)
0	+47
2	+41
7	+34
24	+32

[a] Concentration *c.* 0.35

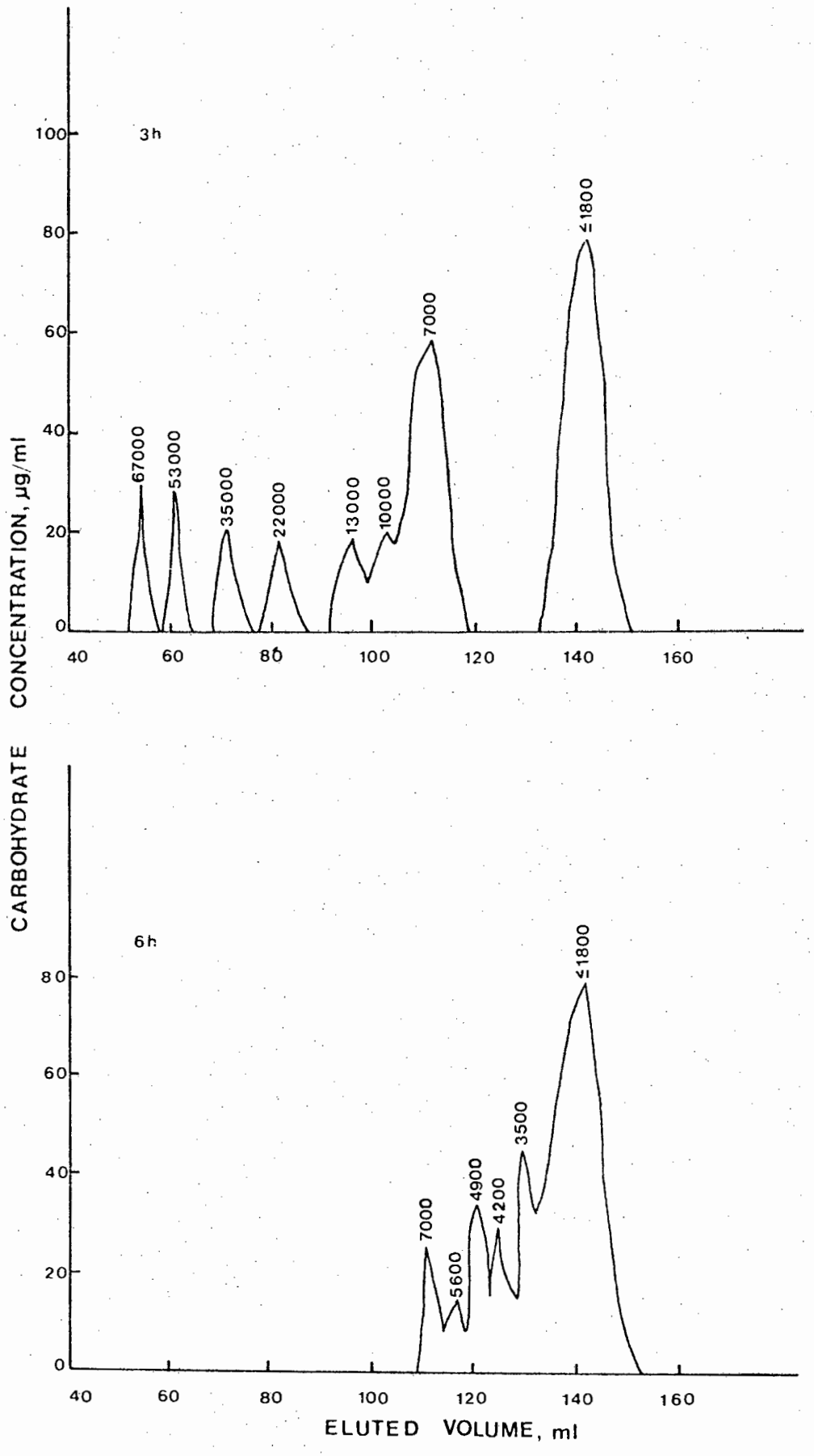


FIGURE 13 Bio-Gel P-300 elution patterns of hydrolysates (0.01M trifluoroacetic acid, 100°, 3-6h) of *Klebsiella* K4 capsular polysaccharide.

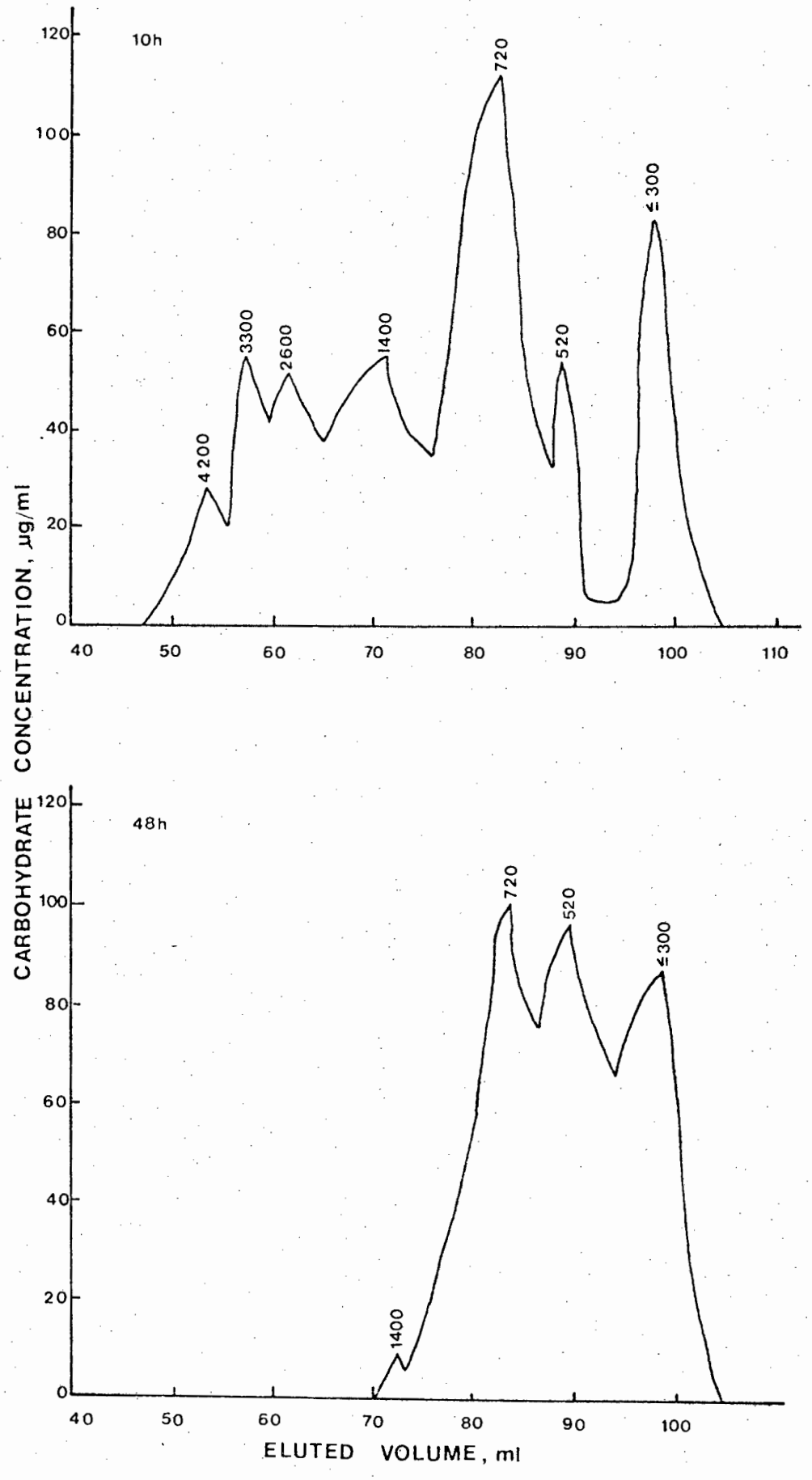


FIGURE 14 Bio-Gel P-10 elution patterns of hydrolysates (0.01M trifluoroacetic acid, 100°, 10-48h) of *Klebsiella* K4 capsular polysaccharide.

weight 720 (36% by weight) in the Bio-Gel P-10 chromatogram of the products of 10h hydrolysis in 0.01M acid (Figure 14) strongly suggested the presence of a tetrasaccharide repeating unit within the polysaccharide. Gel-permeation and paper chromatography indicated that the proportion of the component having molecular weight 720 daltons gradually diminished when the hydrolysis was continued in 0.1M trifluoroacetic acid. After 24h further hydrolysis under these more vigorous conditions, an aldotriouronic acid, showing some resistance to further hydrolysis, and an aldobiouronic acid were the only significant products other than monosaccharides that could be detected in the hydrolysate. A fairly high proportion of the aldobiouronic acid survived further hydrolysis with 2M acid for 18h, as has been reported in similar studies on other *Klebsiella* polysaccharides[15].

4.3.3 METHYLATION ANALYSIS OF THE K4 POLYSACCHARIDE

After methylation of a sample of the capsular polysaccharide and hydrolysis of a portion of the product, the sugars present in the hydrolysate were analysed by paper chromatography, and by g.l.c. as their trimethylsilylated derived alditols. The results, shown in Table 17(A), indicated that residues of D-glucose and D-mannose, linked through O-3 in the polysaccharide chain, were the only neutral sugar components.

TABLE 17

METHYL ETHERS FROM THE HYDROLYSATES OF THE METHYLATED ORIGINAL (A) AND REDUCED METHYLATED (B) POLYSACCHARIDES FROM *KLEBSIELLA* K4

Sugar[a]	T[b]	Molar Proportions (%)	
		A[c]	B[d]
2,4,6-Man	1.19	30.7	26.1
2,4,6-Glc	1.34	69.3	52.6
3,4-Glc	2.13	-	21.3

[a] 2,4,6-Man = 2,4,6-tri-O-methyl-D-mannose *etc.*

[b] Retention time of the corresponding (TMS) alditol relative to that of 2,3,4,6-tetra-O-methyl-D-galactose on an SE-52 column at 140°C.

[c,d] Hydrolysates examined after 18h and 6h hydrolysis respectively.

Methanolysis of another portion of the fully methylated polysaccharide gave, according to g.l.c. analysis, the methyl glycosides of 2,4,6-tri-O-methyl-D-glucose, 2,4,6-tri-O-methyl-D-mannose and two peaks corresponding to either 2,4- or 3,4-di-O-methyl-D-glucuronate. The neutral sugar components were further characterised by g.l.c. as the trimethylsilyl derivatives of the free methylated sugars (Figure 15). From the above methylation analyses it is evident that no branching exists in the polysaccharide structure and that the D-glucuronic acid residues therefore also form part of a linear repeating sequence.

4.3.4 REDUCTION OF METHYLATED K4 POLYSACCHARIDE

Reduction of the methylated polysaccharide with lithium aluminium hydride, followed by hydrolysis and analysis of the sugars

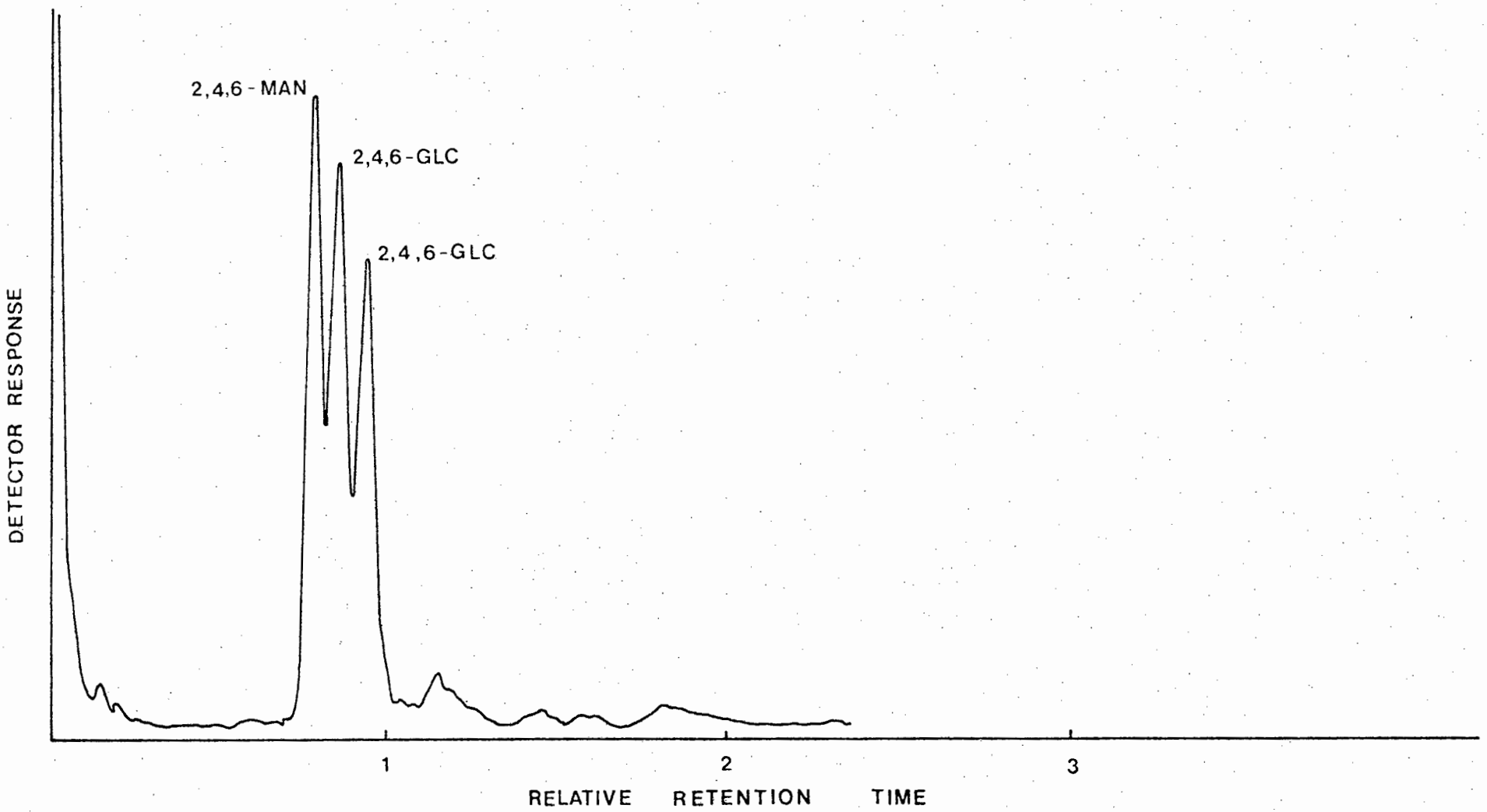


FIGURE 15 G.l.c. trace of the partially methylated (TMS) sugars from fully methylated *Klebsiella* K4 capsular polysaccharide.

present in the hydrolysate by paper chromatography and by g.l.c. as their trimethylsilylated derived alditols gave the results shown in (Table 17(B)). The presence of 3,4-di-O-methyl-D-glucose was confirmed by g.l.c. analysis of the methylated sugars in the hydrolysate as their derived alditol acetates. Since no di-O-methyl derivatives of D-glucose were found in the hydrolysate of the methylated polysaccharide, the appearance of 3,4-di-O-methyl-D-glucose in the hydrolysate of the reduced derivative must result from the reduction of D-glucuronic acid residues that are linked through O-2 in the polysaccharide chain.

The above results indicate that the K4 polysaccharide consists of a linear chain of D-glucose and D-mannose residues linked through O-3 and D-glucuronic acid residues linked through O-2. The actual sequence of sugars in the polysaccharide chain was determined by characterisation of the oligosaccharides obtained from a partial hydrolysate of the polysaccharide, as described below.

4.3.5 PARTIAL HYDROLYSIS OF THE POLYSACCHARIDE; ISOLATION OF OLIGOSACCHARIDES

Mild acid hydrolysis of the polysaccharide with 0.01M trifluoroacetic acid at 100°C for 48h yielded oligosaccharides I - IV, which were separated by gel-permeation chromatography and in some cases purified by preparative paper chromatography. The structure of each oligomer, shown in Table 18, was determined using standard techniques as outlined in Section 4.3.7.

TABLE 18

THE OLIGOSACCHARIDES ISOLATED FROM A PARTIAL HYDROLYSATE OF *KLEBSIELLA* K4 CAPSULAR POLYSACCHARIDE

Oligomer	$[\alpha]_D$ (in H_2O) (Degrees)	Mol. wt. (Daltons)	Neutral Sugars[a] (Molar ratios)	Methylated Sugars[b] (Molar ratios)
Neutral disaccharide I	+19	340	Glc	2,3,4,6-Glc (1.0) 2,4,6-Glc (1.0)
Aldobiouronic acid II	+27	360	Man	2,4,6-Man
Aldotriouronic acid mixture III	+48	520	Glc (1.0) Man (0.7)	2,3,4,6-Glc (1.0) 2,4,6-Man (0.6) 2,4,6-Glc (0.8)
Aldotetraouronic acid IV	+98	720	Glc (2.0) Man (0.8)	2,3,4,6-Glc (0.6) 2,4,6-Man (0.6) 2,4,6-Glc (1.0)

[a] Analysed as alditol acetates on column (b).

[b] Analysed as (TMS) alditols on an SE-52 column at 140°C.

The optical rotation of neutral disaccharide I is in agreement with that given in the literature[80] for 3-O-β-D-glucopyranosyl-D-glucose, i.e. laminaribiose. Oligomer II was indistinguishable on paper chromatography, and also in its optical rotatory dispersion (ORD) and proton magnetic resonance, from the aldobiouronic acid 3-O-(α-D-glucopyranosyluronic acid)-D-mannose isolated from a partial hydrolysate of the polysaccharide from *Klebsiella* K64. Fraction III was found to contain a mixture of two aldotriouronic acids, III(a) and III(b), which are clearly

derived from the aldotetraouronic acid IV through the loss of one or other of the D-glucose residues:

D-Glc*p* $\frac{1\ 2}{\alpha}$ D-GlcA*p* $\frac{1\ 3}{\alpha}$ D-Man*p* III(a)

D-GlcA*p* $\frac{1\ 3}{\alpha}$ D-Man*p* $\frac{1\ 3}{\alpha}$ D-Glc*p* III(b)

D-Glc*p* $\frac{1\ 2}{\alpha}$ D-GlcA*p* $\frac{1\ 3}{\alpha}$ D-Man*p* $\frac{1\ 3}{\alpha}$ D-Glc*p* IV

However, the higher proportion (64%) of the aldotriouronic acid III(a) found in the hydrolysate suggests that the (1→3) glycosidic linkage between D-mannose and D-glucose at the reducing end of the tetraouronic acid is more labile to acid than the (1→2) linkage between D-glucose and D-glucuronic acid at the non-reducing end of the structure. This observation may also be used to explain why a tetrasaccharide having the structure V has not been isolated from the partial hydrolysate of the K4 polysaccharide:

D-GlcA*p* $\frac{1\ 3}{\alpha}$ D-Man*p* $\frac{1\ 3}{\alpha}$ D-Glc*p* $\frac{1\ 3}{\alpha}$ D-Glc*p* V

4.3.6 THE REPEATING UNIT IN THE K4 POLYSACCHARIDE; COMPARISON WITH OTHER *KLEBSIELLA* POLYSACCHARIDES

The evidence presented above shows that the polysaccharide chain must contain the sequence of sugar residues →3)-α-D-Glc*p*-(1→2)-α-D-GlcA*p*-(1→3)-α-D-Man*p*-(1→3)-β-D-Glc*p*-(1→ . Since no branching exists in the polysaccharide structure, this sequence of sugar residues must also represent the tetrasaccharide repeating unit in the K4 capsular polysaccharide. Insufficient material was available to locate the position of the O-acetyl group in the above structure.

The structure of the K4 polysaccharide provides the first

instance encountered in work on *Klebsiella* polysaccharides of a linear tetrasaccharide repeating unit without pyruvic acid attached to it (as in the polysaccharides from K32[6] and K72[8]), although it does have a number of features in common with polysaccharides from other serotypes. The aldobiouronic acid 3-O-(α-D-glucopyranosyluronic acid)-D-mannose forms part of the repeating unit in the polysaccharides of both K2[19] and K24[34], which belong to the same chemotype as K4, and also those of K13[42], K21[52] and K33[44]. In addition the K24 polysaccharide contains chain residues of D-glucose linked through O-3, D-glucuronic acid linked through O-2 and D-mannose residues linked through the 2- and 3-positions; here the pentasaccharide repeating unit is completed by the attachment of a terminal residue of D-mannose to the 4-position of D-glucuronic acid. The fourth member of this chemotype, K5, has a capsular polysaccharide consisting of a linear chain of D-glucuronic acid and D-glucose residues linked through O-4 and D-mannose linked through O-3, combined in the form of a trisaccharide repeating unit; pyruvic acid ketal and O-acetyl groups are attached to D-mannose and D-glucose, respectively. The tetrasaccharide repeating unit of the K2 polysaccharide, which consists of chain residues of D-glucose linked through O-3 and O-4, and D-mannose linked through O-4, differs from the above structures in that the D-glucuronic acid residues occupy terminal positions (at O-3 of D-mannose). Laminaribiose, previously isolated only from a number of plant sources, is also found in the repeating unit of the K27 polysaccharide, while the neutral disaccharide 3-O-α-D-mannopyranosyl-D-glucose has been reported in the structures of the K7[40], K28[43], K59[45] and K64 polysaccharides. Residues of D-glucuronic acid linked through O-2 have also been found in the polysaccharide from K62[41]. In this structure, as in the case of the K4

and K24 repeating units, the D-glucuronic acid is preceded in the polysaccharide chain by a residue of D-glucose.

As mentioned earlier, Joner[82] reported that mild oxidation of the K4 polysaccharide with periodic acid was found to modify some of the groupings responsible for the antigenic specificity of the polysaccharide, but no significant differences were detected between the native and oxidised polysaccharides. However, the uronic acid content of the oxidised polysaccharide was not determined owing to lack of material, although uronic acid was reported to be present in the hydrolysate. From the present study it is evident that periodate oxidation will indeed alter the composition of the K4 polysaccharide by cleavage of the bond between C-3 and C-4 of D-glucuronic acid, thus destroying one of the groups reponsible for the antigenic specificity of the polysaccharide. This observation would explain the marked reduction in the ability of the oxidised polysaccharide to precipitate antibody from homologous immune serum.

4.3.7 EXPERIMENTAL

4.3.7.1 PROPERTIES OF *KLEBSIELLA* K4 CAPSULAR POLYSACCHARIDE

Purified K4 polysaccharide had $[\alpha]_D^{20}$ + 90°, (*c*, 0.2), N 0.2%, and moved as a single component on ultracentrifugation and electrophoresis. The weight-average molecular weight was *ca.* 2.1 x 10^5 according to gel-permeation chromatography and the equivalent weight ~ 710 (as acid by titration), in agreement with the values previously reported[15].

The p.m.r. spectrum of a 2% solution of the polysaccharide in D_2O showed a sharp singlet at τ 7.70 (acetate CH_3) and signals in the anomeric region at τ 4.60 (3Hz), 4.76 (3Hz), 4.82 (2Hz) and 5.35 (7Hz). Integration of the spectrum indicated a ratio of one O-acetyl group to four anomeric protons.

4.3.7.2 HYDROLYSIS OF THE POLYSACCHARIDE ; SUGAR ANALYSIS

After hydrolysis of the K4 polysaccharide, followed by neutralisation of the hydrolysate, paper chromatography (solvents *a* and *d*), showed the presence of D-glucose, D-mannose and D-glucuronic acid. Analysis of the neutral sugars by g.l.c. of the alditol acetates gave the molar ratio of D-glucose and D-mannose as 2:0.6. Preparative g.l.c. permitted the recovery of the acetates, which were dissolved in acetonitrile and their circular dichroism spectra recorded. Comparison of the c.d. spectra with those of authentic samples confirmed the assignment of the D-configuration to glucose and mannose.

4.3.7.3 ACID HYDROLYSIS OF THE POLYSACCHARIDE ; MOLECULAR-WEIGHT DISTRIBUTION ANALYSIS

The molecular-weight distribution patterns of samples removed during the graded acid hydrolysis of the K4 capsular polysaccharide were

obtained using the methods outlined in Section 2.8 and as described in the literature[15].

4.3.7.4 METHYLATION ANALYSIS OF K4 POLYSACCHARIDE

Capsular polysaccharide (0.25g) was methylated once by Hakomori's method and twice by the method of Purdie and Irvine to give an amorphous product (0.23g), $[\alpha]_D^{20}$ + 72° (*c.* 1.20, chloroform), which showed no hydroxyl absorption in the i.r. The fully methylated polysaccharide (0.15g) was hydrolysed with 2M trifluoroacetic acid at 100°C for 18h. Paper chromatography of the hydrolysate (solvents *b* and *f*) showed the presence of 2,4,6-tri-O-methyl-D-mannose and 2,4,6-tri-O-methyl-D-glucose. A portion of the hydrolysate was trimethylsilylated and the derivatives of the methylated sugars were separated by g.l.c. as shown in Figure 15. A second portion of the hydrolysate was reduced, trimethysilylated and analysed by g.l.c. to give the proportions of sugars shown in Table 17(A). These sugars were further characterised by mass spectrometry of their derived alditol acetates.

Methylated polysaccharide (5mg) was heated with 10% methanolic hydrogen chloride at 100°C for 18h. After neutralisation with silver carbonate and evaporation to dryness, the residue was dissolved in methanol and analysed by g.l.c. to give 2,4,6-tri-O-methyl-D-mannose (T = 4.30), 2,4,6-tri-O-methyl-D-glucose (T = 3.70 and T = 5.55) and either 2,4- or 3,4-di-O-methyl-D-glucuronate (T = 8.90 and T = 11.80).

4.3.7.5 REDUCTION OF METHYLATED K4 POLYSACCHARIDE

Fully methylated K4 polysaccharide (100mg) was dissolved in tetrahydrofuran (10ml) and stirred with lithium aluminium hydride (700mg) for 18h. After the addition of aqueous ethanol, the solution was evaporated to dryness and the residue was extracted with chloroform

(3 x 20ml). The extracts were combined and the chloroform was removed to give a product which showed no absorption at 1740 cm^{-1} (methyl ester CO) in the i.r. spectrum. The reduced product (20mg) was hydrolysed with 2M trifluoroacetic acid at 100°C for 6h. Paper chromatography of the hydrolysate (solvents *b* and *f*) showed the presence of two components. The faster-moving spot had a pink colour, characteristic of a 2,4,6-tri-O-methyl sugar, when sprayed with *p*-anisidine. The slower-moving component had a brownish-yellow colour and a mobility similar to that of 3,4-di-O-methyl-D-glucose. A portion of the hydrolysate was reduced, trimethylsilylated and analysed by g.l.c. to give the proportions of sugars shown in Table 17(B). A further portion was reduced, acetylated and analysed by g.l.c. using column (a).

4.3.7.6 PARTIAL ACID HYDROLYSIS OF K4 POLYSACCHARIDE ; ISOLATION OF OLIGOSACCHARIDES

A sample of the polysaccharide (1.0g) was hydrolysed with 0.01M trifluoroacetic acid (200ml) at 100°C for 48h, and the hydrolysis products were separated by preparative gel-permeation chromatography and in some cases further purified by preparative paper chromatography (solvent *a*). Four components were isolated; these gave single peaks, at molecular weights 720, 520, 360 and 340 daltons, when examined by gel-permeation chromatography on Bio-Gel P-10 and P-2. The structures of these oligomers were proved by the standard techniques including partial-hydrolysis studies and methylation analysis, as well as the use of p.m.r. spectroscopy and measurement of optical rotatory power to determine the configurations of anomeric linkages. The proportions of neutral sugars were determined by g.l.c. of the derived alditol acetates after hydrolysis of the oligosaccharides with 2M trifluoroacetic acid at 100°C for 8h. The main characteristics of each oligosaccharide are

shown in Table 18. These oligomers were further characterised as follows.

I. The neutral disaccharide was indistinguishable on paper chromatography (solvents *a*, *b* and *d*) from authentic 3-O-β-D-glucopyranosyl-D-glucose. Methanolysis of the fully methylated disaccharide (1mg) followed by g.l.c. analysis of the methyl glycosides gave 2,3,4,6-tetra-O-methyl-D-glucose (T = 1.00 and T = 1.42) and 2,4,6-tri-O-methyl-D-glucose (T = 3.70 and 5.52). The fully methylated disaccharide (3mg) was hydrolysed with 2M trifluoroacetic acid at 100°C for 4h. Paper chromatography of the hydrolysate (solvents *b* and *f*) showed two components, both of which had the same pink colour when sprayed with *p*-anisidine. The mobility of the faster-moving spot was identical with that of 2,3,4,6-tetra-O-methyl-D-glucose, while in solvent *f* the slower-moving component separated into two spots, characteristic of 2,4,6-tri-O-methyl-D-glucose.

II. When the fully methylated aldobiouronic acid was methanolysed with 10% methanolic hydrogen chloride for 18h, g.l.c. analysis showed the presence of methyl glycosides of 2,3,4-tri-O-methyl-D-glucuronate and 2,4,6-tri-O-methyl-D-mannose. The identity of 2,4,6-tri-O-methyl-D-mannose was confirmed by hydrolysis of the methylated aldobiouronic acid and g.l.c. of the trimethylsilyl derivatives of the anomers of the tri-O-methyl sugar. The p.m.r. spectrum of the aldobiouronic acid in D_2O gave anomeric signals at τ 4.78 (3.5Hz), 4.88 (2Hz) and 5.12 (2Hz), consistent with the values given in the literature for 3-O-(α-D-glucopyranosyluronic acid)-D-mannose.

III. Hydrolysis (2M trifluoroacetic acid, 100°C, 8h) of a sample (2mg) of the aldotriouronic acid mixture, followed by paper chromatography of the hydrolysate (solvents *a* and *d*), gave D-glucose, D-mannose,

D-glucuronic acid and the aldobiouronic acid II. A sample of the aldotriouronic acid fraction (10mg) was reduced with sodium borohydride for 16h. After acidification with Amberlite IR-120 (H^+) resin and the removal of borate by distillation with methanol, the residue was hydrolysed with 2M trifluoroacetic acid at 100°C for 5h. The hydrolysate was dissolved in molar sodium hydroxide (3ml) and boiled for 4h under oxygen. After deionisation with Amberlite IR-45 (OH^-) and Amberlite IR-120 (H^+) resins, the solution was concentrated, acetylated and analysed by g.l.c.; a molar ratio of 1.0:1.8 was found for D-glucose and D-mannose.

The fully methylated derivative of the aldotriouronic acid mixture was hydrolysed with 2M trifluoroacetic acid at 100°C for 8h. Paper chromatography of the hydrolysate (solvents *b* and *f*) showed the presence of 2,3,4,6-tetra-O-methyl-D-glucose, 2,4,6-tri-O-methyl-D-mannose and 2,4,6-tri-O-methyl-D-glucose. G.l.c. analysis of these sugars as the trimethylsilylated derived alditols gave the proportions shown in Table 18.

Methanolysis and g.l.c. analysis of the methylated aldotriouronic acid mixture (2mg) gave the methyl glycosides of 2,3,4-tri-O-methyl-D-glucuronate, 2,3,4,6-tetra-O-methyl-D-glucose, 2,4,6-tri-O-methyl-D-mannose, 2,4,6-tri-O-methyl-D-glucose and the same two peaks (T = 8.90 and T = 11.80) as were found in the gas chromatogram of the fully methylated K4 polysaccharide.

The p.m.r. spectrum of the aldotriouronic acid mixture in D_2O showed signals in the anomeric region at τ 4.60 (3Hz), 4.76 (3Hz), 4.84 (2Hz), 5.12 (2Hz) and 5.34 (7Hz), consistent with the mixture of aldotriouronic acids III(a) and III(b).

IV. When a sample (3mg) of fraction IV was hydrolysed with 2M trifluoroacetic acid at 100°C for 8h, the sugars detected in the hydrolysate by paper chromatography (solvents *a* and *d*) were D-glucose, D-mannose, D-glucuronic acid and the aldobiouronic acid II. The aldotetraouronic acid (5mg) on reduction with sodium borohydride, followed by hydrolysis (2M trifluoroacetic acid, 100°C, 6h) and treatment for 4h with hot molar sodium hydroxide under oxygen, yielded D-glucitol (identified by g.l.c. of its hexaacetate).

When the fully methylated aldotetraouronic acid (5mg) was hydrolysed with 2M trifluoroacetic acid at 100°C for 18h, the sugars present in the hydrolysate were identified by paper chromatography (solvents *b* and *f*) and by g.l.c. of the trimethylsilylated derived alditols as 2,3,4,6-tetra-O-methyl-D-glucose, 2,4,6-tri-O-methyl-D-mannose and 2,4,6-tri-O-methyl-D-glucose. Methanolysis and g.l.c. of the methylated aldotetraouronic acid gave methyl 2,4- or 3,4-di-O-methyl-D-glucuronate in addition to the above neutral sugars.

The p.m.r. spectrum of the aldotetraouronic acid in D_2O gave anomeric signals at τ 4.60 (3Hz), 4.68 (3Hz), 4.78 (2Hz) and 5.32 (7Hz), consistent with the structure IV.

4.4 CAPSULAR POLYSACCHARIDE FROM A STRAIN OF *KLEBSIELLA PNEUMONIAE* (*OXYTOCA* VARIANT)

4.4.1 INTRODUCTION

The primary structure of the polysaccharide elaborated by the K-antigen from a strain of *Klebsiella* K9, consisting of residues of D-glucuronic acid, D-galactose and L-rhamnose in the ratio 1:1:3, has been determined by Lindberg and coworkers[35]. The present studies were performed on the acidic polysaccharide obtained from a culture of *Klebsiella pneumoniae* (*oxytoca* variant) and serotyped as capsule type 9. Although this polysaccharide was found to contain the same sugar residues as that studied by Lindberg, evidence was obtained which showed that a number of structural differences existed between the polysaccharides from the two strains. In order to avoid confusion, the polysaccharide examined in the present study will be referred to as K9*.

Purified K9* polysaccharide had $[\alpha]_D$ -5°, equivalent weight (as acid) 770, and moved as a single band on electrophoresis on cellulose acetate. Gel-permeation chromatography on agarose gel gave a single peak at $\overline{M}_w$ 1.2 x 10^6 daltons. Proton integration of the p.m.r. spectrum of the polysaccharide indicated a ratio of three 6-deoxy residues to five anomeric protons, suggesting a pentasaccharide repeating unit containing three residues of a deoxy sugar; no pyruvic acid ketal or O-acetyl groups were detected. Paper chromatography of an hydrolysate (2M trifluoroacetic acid, 100°C, 8h) of the polysaccharide showed the presence of glucuronic acid, galactose and rhamnose. Quantitative g.l.c. analysis of the neutral sugars, as alditol acetates, gave the molar proportions of galactose and rhamnose as 1:2.8. The circular dichroism spectrum of the rhamnose derivative indicated that these sugar residues were in the

L-configuration. The D-configuration was assigned to galactose by measurement of the c.d. spectrum of a methylated derivative isolated subsequently.

4.4.2 METHYLATION ANALYSIS OF K9* POLYSACCHARIDE

When a sample of the purified capsular polysaccharide was methylated, hydrolysis of the product gave 3,4-di-O-methyl-L-rhamnose and 2,4,6-tri-O-methyl-D-galactose in the proportions shown in Table 19. These sugars were qualitatively analysed by paper chromatography and by g.l.c. of their derived alditol acetates on columns of OV-17, OV-225 and ECNSS-M. The relative molar proportions of the methylated sugars were determined by g.l.c. analysis of their trimethylsilylated derived alditols.

TABLE 19

METHYL ETHERS FROM THE HYDROLYSATES OF THE METHYLATED (A) AND REDUCED METHYLATED (B) POLYSACCHARIDES FROM *KLEBSIELLA* K9*

Sugar[a]	T[b]	Molar Proportions (%)	
		A[c]	B[d]
3,4-Rha	0.84	74.2	63.2
2,4,6-Gal	1.19	25.8	20.6
2,3-Glc	2.13	-	17.2

[a] 3,4-Rha = 3,4-di-O-methyl-L-rhamnose *etc.*

[b] Retention time of the corresponding (TMS) alditol relative to that of 2,3,4,6-tetra-O-methyl-D-galactose on an SE-52 column at 140°C.

[c,d] Hydrolysate examined after 18h and 6h hydrolysis respectively.

From the results in Column A it is evident that the

polysaccharide chain consists of residues of L-rhamnose linked through O-2 and D-galactose linked through O-3; no evidence of branching was detected, which indicates that the glucuronic acid residues must also form part of the linear chain.

4.4.3 REDUCTION OF THE METHYLATED K9* POLYSACCHARIDE

Reduction of the fully methylated K9* polysaccharide with lithium aluminium hydride followed by hydrolysis with 2M trifluoroacetic acid at 100°C for 6h, and paper chromatography of the hydrolysate, showed the presence of 3,4-di-O-methyl-L-rhamnose, 2,3-di-O-methyl-D-glucose and 2,4,6-tri-O-methyl-D-galactose. Quantitative analysis of the mixture as the trimethylsilylated derived alditols gave the molar proportions listed in Column B of Table 13. The presence of these methylated sugars in the hydrolysate was confirmed by g.l.c. analysis of their derived alditol acetates (Figure 16). Preparative g.l.c. permitted the recovery of the methylated acetates which were further characterised by mass spectrometry. The glucose derivative was de-O-methylated[78] to give glucitol, which was shown to have the D-configuration by comparison of the c.d. spectrum of its hexaacetate with that of a standard sample. Since the original polysaccharide did not contain any residues of D-glucose, the appearance in significant molar proportions of 2,3-di-O-methyl-D-glucose in the hydrolysate of the reduced methylated derivative must result from the reduction of glucuronic acid residues linked through O-4 in the polysaccharide chain. These glucuronic acid residues must accordingly also have the D-configuration.

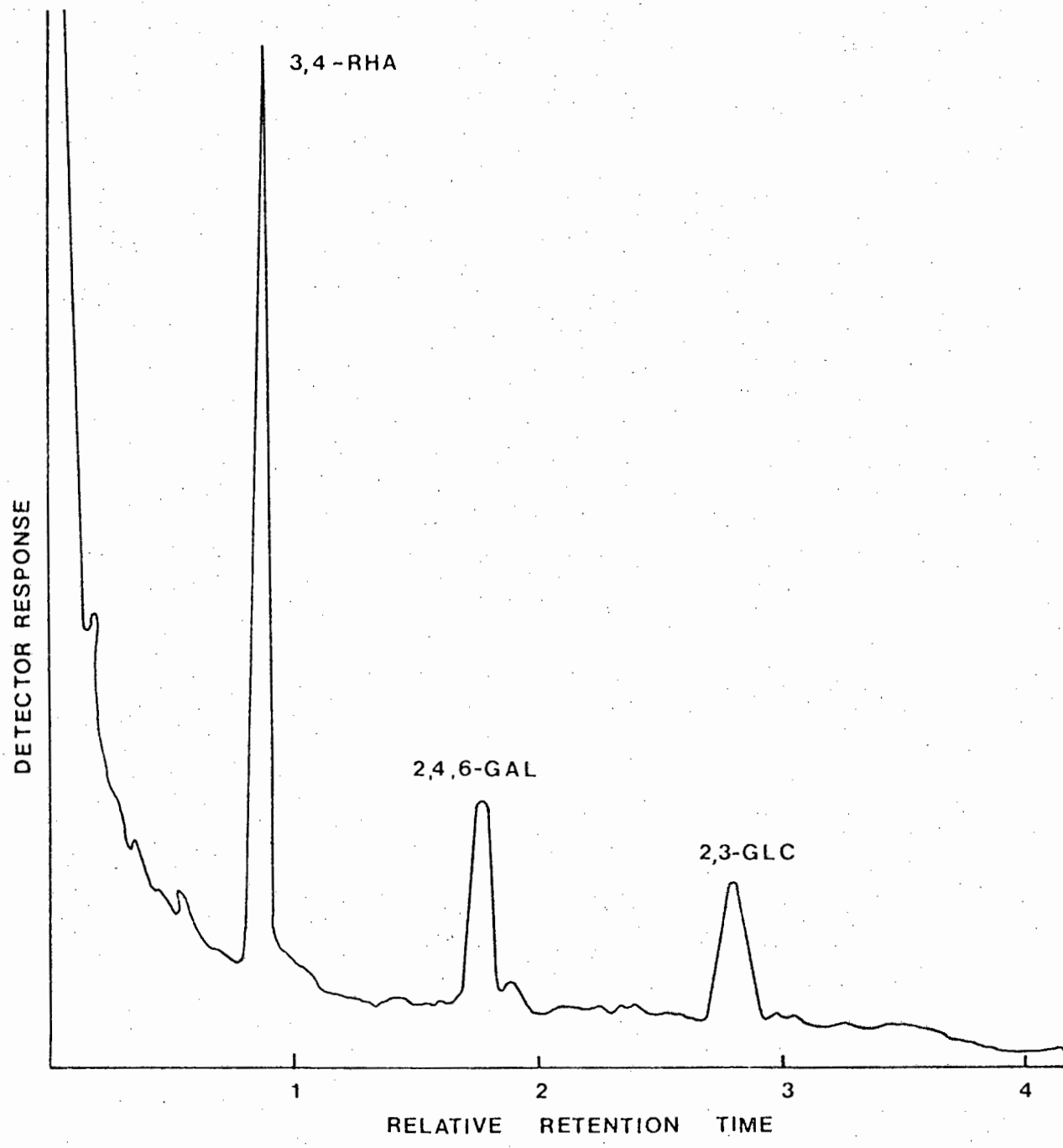


FIGURE 16 G.l.c. trace of the partially methylated alditol acetates from the reduced methylated derivative of *Klebsiella* K9* polysaccharide.

4.4.4 PARTIAL HYDROLYSIS OF K9* POLYSACCHARIDE

Mild acid hydrolysis of the K9* polysaccharide with 0.01M trifluoroacetic acid at 100°C for 5h yielded a component having $[\alpha]_D$ +6°, mol.wt. 800 daltons (by gel-permeation chromatography), and containing residues of D-galactose and L-rhamnose in the same molar proportions as the original polysaccharide. On reduction of the oligosaccharide with sodium borohydride, followed by hydrolysis, paper chromatography of the hydrolysate showed the presence of both D-galactose and L-rhamnose, indicating that a residue of L-rhamnose forms the reducing end of the structure. After methylation of a sample, followed by hydrolysis, the sugars present in the hydrolysate were found by paper chromatography and by g.l.c. as their trimethylsilylated derived alditols to be 2,4,6-tri-O-methyl-D-galactose and 3,4-di-O-methyl-L-rhamnose in the molar ratio 1:3. On methanolysis of the fully methylated oligosaccharide, g.l.c. analysis showed the methyl glycosides of 2,3,4-tri-O-methyl-D-glucuronate, 3,4-di-O-methyl-L-rhamnose and 2,4,6-tri-O-methyl-D-galactose. These results are consistent with a linear pentasaccharide structure in which L-rhamnose and D-glucuronic acid form the reducing and non-reducing ends respectively.

Hydrolysis with 0.1M trifluoroacetic acid at 100° for 18h resulted in the complete degradation of the polysaccharide chain into its component sugars. From previous hydrolysis studies performed on *Klebsiella* polysaccharides[15] it would appear that L-rhamnose must form part of the aldobiouronic acid linkage in the K9* polysaccharide, since aldobiouronic acids containing D-mannose and D-galactose have been shown to survive these, and even stronger, conditions of acid hydrolysis. It is suggested that the methyl group of the 6-deoxy sugar may render the glycosidic bond between that sugar and D-glucuronic

acid more susceptible to acid hydrolysis, resulting in the observed total breakdown of the polysaccharide under relatively mild conditions of hydrolysis. Similar behaviour has been observed in the case of the polysaccharide from *Klebsiella* K1[18], in which L-fucose is linked to D-glucuronic acid. Since methylation analyses have shown that all the L-rhamnose residues in the K9* polysaccharide are linked through O-2, it is reasonable to assume that the aldobiouronic acid portion must be either α or β 2-O-(D-glucopyranosyluronic acid)-L-rhamnose.

4.4.5 THE REPEATING UNIT IN THE K9* POLYSACCHARIDE

The above studies provide sufficient evidence to suggest the presence of a linear pentasaccharide repeating unit in the polysaccharide from *Klebsiella* K9*. From the assumption that the aldobiouronic acid contains L-rhamnose, together with the characterisation of the pentasaccharide isolated from a partial hydrolysate of the polysaccharide, it follows that the D-galactose residue is either one or two L-rhamnose residues removed from D-glucuronic acid in the repeating sequence of sugars. Proton magnetic resonance spectroscopy of the polysaccharide shows the absence of any pyruvic acid ketal or O-acetyl groups. It may be concluded that the capsular polysaccharide from K9* is composed of one of the linear pentasaccharide units given below:

$$\xrightarrow{4}\ \text{D-GlcA}p\ \xrightarrow{1\ 2}\ \text{L-Rha}p\ \xrightarrow{1\ 3}\ \text{D-Gal}p\ \xrightarrow{1\ 2}\ \text{L-Rha}p\ \xrightarrow{1\ 2}\ \text{L-Rha}p\ \xrightarrow{1} \qquad \text{(I)}$$

$$\xrightarrow{4}\ \text{D-GlcA}p\ \xrightarrow{1\ 2}\ \text{L-Rha}p\ \xrightarrow{1\ 2}\ \text{L-Rha}p\ \xrightarrow{1\ 3}\ \text{D-Gal}p\ \xrightarrow{1\ 2}\ \text{L-Rha}p\ \xrightarrow{1} \qquad \text{(II)}$$

Lindberg's structure for the repeating unit of the K9 polysaccharide is shown as (III):

$$\xrightarrow{3}\ \text{D-Gal}p\ \xrightarrow[\alpha]{1\ 3}\ \text{L-Rha}p\ \xrightarrow[\alpha]{1\ 3}\ \text{L-Rha}p\ \xrightarrow[\alpha]{1\ 2}\ \text{L-Rha}p\ \xrightarrow[\alpha]{1} \qquad \text{(III)}$$

$$\text{L-Rha}p\ \beta\left|\begin{matrix}4\\1\end{matrix}\right.\ \text{D-GlcA}p$$

It is evident that neither of the structures (I) and (II) bears a really close resemblance to the branched repeating unit (III) found for the K-antigen from *Klebsiella* type K9. The essential differences between the two polysaccharides (K9 and K9*) can be seen from Table 20.

TABLE 20

THE MAIN PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE *KLEBSIELLA* K9 AND K9* CAPSULAR POLYSACCHARIDES

Polysaccharide	$[\alpha]_{578}$ (Degrees)	Mol. wt. (Daltons)	Neutral Sugars (Molar ratios)	Glucuronic acid (Position)	Methylated Sugars (Molar ratios)
K9 (Stockholm)	-17	-	Rha (2.9) Gal (1.0)	Terminal	3,4-Rha (1.0) 2,4-Rha (1.0) 2-Rha (1.2) 2,4,6-Gal (1.1)
K9* (Cape Town)	-7	1.2×10^6	Rha (2.8) Gal (1.0)	Chain, 4-linked	3,4-Rha (3.0) 2,4,6-Gal (1.0)

4.4.6 COMPARISON WITH OTHER *KLEBSIELLA* POLYSACCHARIDES

A consideration of the results obtained in the present study shows that the polysaccharide from *Klebsiella* K9* belongs to the same chemotype as those of K9, K47[36], K52[37], K81[38] and a *Klebsiella* strain 6412, for which the serotype K83[39] has been proposed. The primary structures of the polysaccharides from these serotypes have been determined, and differ from that found for K9*. The polysaccharide from K81 resembles that of K9* in that it contains a linear (hexasaccharide) repeating unit in which the residues of D-glucuronic acid and D-galactose are also linked through O-4 and O-3 respectively, but differs in that the residues of L-rhamnose

are linked through both the 2- and 3-positions. Branching exists in the polysaccharides of the remaining serotypes, in which both single- (as in K9 and K52) and double-unit side chains (in K47 and K83) have been reported. In all the above serotypes, the chain residues of D-galactose are linked exclusively through O-3, while the hexasaccharide repeating unit of K52 also carries a terminal residue of this sugar. The position of linkage of the L-rhamnose residues appears to vary with each strain, but the mode of linkage is of the α-L-type in all cases. The low optical rotation, together with the chemical shifts of the anomeric signals in the p.m.r. spectrum, suggests that these residues are linked in a similar manner in K9*.

The results obtained in the present study provide the first instance where a single *Klebsiella* serotype has been found to produce two different capsular polysaccharides. The possibility exists, however, that one or other of the strains of K9 examined has been incorrectly serotyped and thus represents a new *Klebsiella* K-type.

A similar case has recently been reported for *Aeromonas salmonicida*, where the lipopolysaccharide endotoxins from two strains were found to be of different chemotypes in spite of the fact that serological differences were minimal[83].

4.4.7 EXPERIMENTAL

4.4.7.1 PROPERTIES OF *KLEBSIELLA* K9* CAPSULAR POLYSACCHARIDE

A culture of *Klebsiella pneumoniae* (*oxytoca* variant) isolated at Groote Schuur Hospital, from a patient suffering from a bronchial infection, was grown. The purified capsular polysaccharide had $[\alpha]_D^{18}$ -5° (*c* 0.2), N 0.3%, and equivalent weight ~ 770 (as acid, by titration). Gel-permeation chromatography gave $\overline{M}_w$ 1.2 x 10^6 (single peak on agarose gel), while electrophoresis showed that the polysaccharide moved as a single component.

The p.m.r. spectrum of a 2% solution of the polysaccharide in D_2O showed a doublet at τ 8.72 (2Hz) and three signals in the range τ 4.60 - 4.90. Integration of the spectrum gave a ratio of three 6-deoxy residues to five anomeric protons.

4.4.7.2 HYDROLYSIS OF THE POLYSACCHARIDE ; SUGAR ANALYSIS

Hydrolysis of the polysaccharide, followed by neutralisation and paper chromatography of the hydrolysate (solvents *a* and *d*), showed the presence of L-rhamnose, D-galactose and D-glucuronic acid. Molar proportions of neutral sugars, as found by g.l.c. analysis of the derived alditol acetates, were D-galactose (1.0) and L-rhamnose (2.8). Preparative g.l.c. of the acetates permitted the recovery of L-rhamnitol pentaacetate (as a syrup) and D-galactitol hexaacetate. Comparison of the circular dichroism spectrum of the L-rhamnitol pentaacetate with that of a standard sample confirmed the assignment of the L-configuration. The D-galactitol hexaacetate was found to be achiral.

4.4.7.3 METHYLATION ANALYSIS OF THE POLYSACCHARIDE

Capsular polysaccharide (136mg) was methylated once by the method of Hakomori to give a colourless, amorphous product (119mg) which showed no hydroxyl absorption in the i.r. and had $[\alpha]_D^{20}$ -19° (*c* 0.5, chloroform). The fully methylated polysaccharide (30mg) was hydrolysed with 2M trifluoroacetic acid at 100°C for 18h. Paper chromatography of the hydrolysate (solvents *b* and *f*) showed the presence of two components. The faster-moving one gave a pink colour when sprayed with *p*-anisidine and was indistinguishable from 2,4,6-tri-O-methyl-D-galactose, while the slower gave a brownish-yellow spot. Reduction of a portion of the hydrolysate with sodium borohydride, followed by acetylation and g.l.c. analysis of the methylated alditol acetates using columns (a), (b) and (c), showed the presence of 2,4,6-tri-O-methyl-D-galactose and 3,4-di-O-methyl-L-rhamnose. Column (c) was programmed at 175°C for 8 min and then at 2° per min to 210°C. A further portion of the hydrolysate was reduced with sodium borohydride and the products were trimethylsilylated; quantitative analysis by g.l.c. gave the molar proportions listed in column A of Table 19.

4.4.7.4 REDUCTION OF METHYLATED K9* POLYSACCHARIDE

Fully methylated polysaccharide (60mg) was dissolved in tetrahydrofuran (5ml) and stirred with lithium aluminium hydride (350mg) for 18h. After the addition of aqueous ethanol and evaporation to dryness the residue was extracted with chloroform (3 x 20ml); the extracts were concentrated and passed through a column of Sephadex LH-20, using ethanol : chloroform (2:1 v/v) as eluent, to give a product (22mg), $[\alpha]_D^{22}$ -15° (*c* 0.3, chloroform), which showed no absorption at 1740 cm^{-1} in the i.r. spectrum (methyl ester CO).

Hydrolysis of the reduced methylated polysaccharide with 2M trifluoroacetic acid at 100°C for 6h, followed by paper chromatography of the hydrolysate (solvents *b* and *f*), gave 2,4,6-tri-O-methyl-D-galactose, 3,4-di-O-methyl-L-rhamnose and 2,3-di-O-methyl-D-glucose. A portion of the hydrolysate was reduced and acetylated and the methylated alditol acetates were analysed by g.l.c. using columns (a) and (b). The identity of each acetate was confirmed by mass spectrometry, after recovery by preparative g.l.c. Demethylation (BCl_3), and reacetylation of the 2,3-di-O-methyl-D-glucitol hexaacetate gave D-glucitol hexaacetate, which was dissolved in acetonitrile and the circular dichroism spectrum recorded. A positive circular dichroism curve, identical with that given by the authentic compound, confirmed the D-configuration of glucose. Since the original K9* polysaccharide did not contain glucose, the origin of the 2,3-di-O-methyl-D-glucose in the reduced methylated polysaccharide must be the glucuronic acid present in the original polysaccharide; this acid must accordingly be in the D-configuration. The c.d. spectrum of 2,4,6-tri-O-methyl-D-galactitol acetate was identical to that of a standard sample, thus confirming the configuration of D-galactose.

The remainder of the hydrolysate was reduced, trimethylsilylated and analysed by g.l.c. to give the proportions of sugars shown in column B of Table 19.

4.4.7.5 PARTIAL HYDROLYSIS OF THE POLYSACCHARIDE

A sample (100mg) of the polysaccharide was hydrolysed with 0.01M trifluoroacetic acid (20ml) at 100°C for 5h. Paper chromatography of the hydrolysate (solvent *a*) showed the presence of a major oligosaccharide component (R_{glc} 0.27) together with L-rhamnose, D-galactose and traces of other oligosaccharides.

The oligosaccharide, isolated by preparative paper chromatography (solvent *a*), had $[\alpha]_D^{20}$ +6° (*c* 0.2) and mol. wt. 800 daltons (by gel-permeation chromatography on Bio-Gel P-2). Hydrolysis with 2M trifluoroacetic acid at 100°C for 8h gave D-galactose and L-rhamnose in the molar ratio 1.0:3.0 (according to g.l.c. analysis as the derived alditol acetates).

A sample of the oligosaccharide (2mg) was reduced with sodium borohydride to give a product which on hydrolysis (2M trifluoroacetic acid, 100°C, 4h) and paper chromatography of the hydrolysate (solvent *d*) gave D-galactose and L-rhamnose. The oligosaccharide (4mg) was methylated and a portion of the product was hydrolysed with 2M trifluoroacetic acid at 100°C for 8h. Paper chromatography (solvents *b* and *f*), and g.l.c. analysis, as the trimethylsilylated derived alditols, of the sugars present in the hydrolysate gave 2,4,6-tri-O-methyl-D-galactose and 3,4-di-O-methyl-L-rhamnose in the molar ratio 1.0:2.7. Another portion of the fully methylated oligosaccharide was heated with 10% methanolic hydrogen chloride at 100°C for 10h; g.l.c. analysis of the methyl glycosides produced showed the presence of methyl 2,3,4-tri-O-methyl-D-glucuronate, and glycosides of 2,4,6-tri-O-methyl-D-galactose and 2,4-di-O-methyl-L-rhamnose.

Hydrolysis of the K9* polysaccharide with 0.1M trifluoroacetic acid at 100°C for 18h, followed by paper chromatography of the hydrolysate (solvents *a* and *d*), gave L-rhamnose, D-galactose, and D-glucuronic acid.

5. STUDIES OF THE CAPSULAR POLYSACCHARIDES FROM *CRYPTOCOCCUS NEOFORMANS*

5. STUDIES OF THE CAPSULAR POLYSACCHARIDES FROM *CRYPTOCOCCUS NEOFORMANS*

5.1 INTRODUCTION

Fungal strains of the genus *Cryptococcus* all produce capsules, some of which are much thicker than the enclosed cell. Approximately 12 species have been identified on the basis of antigenic and morphological characteristics, but only one species, *Cryptococcus neoformans*, is pathogenic in man. *Cryptococcus neoformans* has been found to possess an unusual affinity for the central nervous system, presumably due to the presence of vitamins and metabolites in this system that satisfy the nutritional requirements of the organism. These include thiamine, glutamic acid and glutamine, a number of spinal fluid carbohydrates, and essential minerals. Once the yeast has pierced the blood-brain barrier and has seeded the cerebrospinal fluid, it is distributed by the cerebral circulation to all surfaces of the brain and spinal cord. There the organism grows rapidly and forms huge capsules that render it relatively immune to the normal defence mechanisms of the host. As the disease progresses, pressure of the spinal fluid steadily rises, protein content increases, the cells multiply, and the sugar content steadily declines as the yeasts consume the carbohydrates and carbohydrate derivatives of the spinal fluid.

An extensive study of the characteristics of *C. neoformans* has revealed a number of interesting biological phenomena[69]. One already mentioned is the affinity which the organism has for the central nervous system, and in particular the spinal fluid and meninges. Another is that there is a considerable loss of capsular substance suffered by the organism upon transfer from the host body to an artificial culture medium. On the

other hand, intracerebral injection of experimental animals with weakly encapsulated laboratory strains results in the production of huge capsules within the animal brain. A comparison of the capsules produced by *C. neoformans* on artificial culture media with those formed within the body indicates a large difference in size. It appears, therefore, that this organism has found some essential metabolites or optimal conditions in the brain and spinal fluid that provide the stimulus for growth and the synthesis of large capsules. The capsule of *C. neoformans*, its most distinctive feature, is easily visualized in Indian ink suspensions of the cells and is composed of a polysaccharide containing glucuronic acid, mannose and xylose residues.

5.2 COMPARISON OF TWO POLYSACCHARIDES FROM *CRYPTOCOCCUS NEOFORMANS*

In the present study two strains (R and H) of *C. neoformans* were isolated at Groote Schuur Hospital, Cape Town, from the cerebrospinal fluid of patients suffering from culture-proven cases of meningitis. They were identified by the typical capsules produced, as seen in Indian ink preparations on microscopy, their demonstration of urease activity, and their pathogenicity, which was confirmed by intracerebral inoculation of mice. The two strains were grown on Littman's capsule medium[69] and the acidic polysaccharides produced were purified by cetavlon bromide precipitation according to the method of Nimmich[4]. The physical and chemical characteristics of the purified R and H polysaccharides are shown in Table 21.

TABLE 21

PHYSICAL AND CHEMICAL CHARACTERISTICS OF THE R AND H POLYSACCHARIDES FROM *CRYPTOCOCCUS NEOFORMANS*

Polysaccharide	$[\alpha]_D$ (in H_2O) (Degrees)	$\overline{M}_w$ (Daltons)	Neutral Sugars (Mol %)	% Acid [a]	Methylation Analysis [b] (Molar ratios)
H	+5	1.4×10^6	Man (61) Xyl (39)	*Ca.* 15	2,3,4-Xyl (1.8) [c] 2,3,4-GlcA (1.0) 4,6-Man (2.9)
R	+7	1.4×10^6	Man (58) Xyl (42)	*Ca.* 14	2,3,4-Xyl (1.8) 2,3,4-GlcA (1.0) 4,6-Man (2.8)

[a] By i.r. spectroscopy.

[b] As methyl glycosides.

[c] 2,3,4-Xyl = 2,3,4-tri-O-methyl-D-xylose *etc.*

Polysaccharide R, as isolated initially, contained appreciable quantities of bound galactose, and was found to be polymolecular {Mol wts 1.4×10^6 (70% by wt) and 13 000 (30%)} when examined by gel-permeation chromatography on agarose and Bio-Gel P-10. Further purification with cetavlon bromide yielded galactose-free polysaccharide R which was found to be monomolecular. R and H polysaccharides were also found to be monodisperse by the methods of ultracentrifugation and electrophoresis on cellulose acetate.

Mild acid hydrolysis with 0.1M trifluoroacetic acid at 100°C for 24h, monitored by paper chromatography, showed that xylose was the first sugar to be released from the polysaccharides, mannose and glucuronic acid being produced more slowly. More vigorous acid hydrolysis of the polysaccharides with 2M trifluoroacetic acid at 100°C for 18h, followed by g.l.c. analysis as the derived alditol acetates of the sugars present in the hydrolysates, gave the neutral sugar proportions shown in Table 21. Mannose was shown to have the D-configuration by measurement of the c.d. spectrum of mannitol hexaacetate. Glucuronic acid and xylose were found to be of the same configuration by the procedures outlined in Section 5.5.

When samples of the fully methylated polysaccharides were hydrolysed and the sugars present in the hydrolysates analysed by paper chromatography and by g.l.c. of the trimethylsilylated derived alditols, 2,3,4-tri-O-methyl-D-xylose and 4,6-di-O-methyl-D-mannose were the only components detected in both hydrolysates. Methanolysis of the methylated polysaccharides and g.l.c. of the methyl glycosides produced showed the presence of methyl 2,3,4-tri-O-methyl-D-glucuronate in addition to the two neutral sugars (See Table 21).

On the basis of the above analyses the polysaccharides were considered to be identical. Polysaccharide H was subsequently examined

in more detail by the methods of partial acid hydrolysis, methylation analysis and Smith-degradation.

5.3 THE STRUCTURE OF THE POLYSACCHARIDE H

5.3.1 REDUCTION OF THE METHYLATED POLYSACCHARIDE H

Reduction of the fully methylated polysaccharide with lithium aluminium hydride, followed by methanolysis and g.l.c. analysis of the methyl glycosides produced, showed the presence of 2,3,4-tri-O-methyl-D-glucose, 2,3,4-tri-O-methyl-D-xylose and 4,6-di-O-methyl-D-mannose but no 2,3,4-tri-O-methyl-D-glucuronate. Hydrolysis of the methylated polysaccharide, and analysis of the sugars present in the hydrolysate by paper chromatography and by g.l.c. as their derived alditol acetates, also gave 2,3,4-tri-O-methyl-D-glucose, 2,3,4-tri-O-methyl-D-xylose and 4,6-di-O-methyl-D-mannose. The individual compounds were recovered by preparative g.l.c. and their identities confirmed by mass spectrometry. Since the original polysaccharide H did not contain any residues of D-glucose, the appearance of 2,3,4-tri-O-methyl-D-glucose in the hydrolysate of the reduced methylated polysaccharide H must result from the reduction of terminal residues of D-glucuronic acid.

5.3.2 PARTIAL HYDROLYSIS OF THE POLYSACCHARIDE; CHARACTERISATION OF OLIGOSACCHARIDES

Partial acid hydrolysis of the polysaccharide with 2M trifluoroacetic acid at 100°C for 3h yielded oligosaccharides I - IV, which were separated by preparative paper chromatography. The structure of each oligomer was determined using standard techniques, including partial-hydrolysis studies and methylation analysis, and the measurement of optical rotatory power to determine the configurations of anomeric linkages. The results obtained are summarised in Table 22.

TABLE 22

ANALYSIS OF THE OLIGOSACCHARIDES FROM *CRYPTOCOCCUS NEOFORMANS* CAPSULAR POLYSACCHARIDE H

Oligomer	$[\alpha]_D$ (in H_2O) (Degrees)	R_{glc} [a]	Neutral Sugars [b]	Methylated Sugars [c]	
				(TMS) Alditols (Molar ratios)	Methyl Glycosides
Neutral disaccharide I	-8	0.72	Man	2,3,4,6-Man (1.0) 2,4,6-Man (1.0)	2,3,4,6-Man 2,4,6-Man
Aldobiouronic acid II	-24	0.60	Man	3,4,6-Man	3,4,6-Man 2,3,4-GlcA
Aldotriouronic acid III	-5	0.36	Man	3,4,6-Man (0.9) 2,4,6-Man (1.0)	3,4,6-Man 2,4,6-Man 2,3,4-GlcA
Aldotetraouronic acid IV	+8	0.24	Man	3,4,6-Man (0.9) 2,4,6-Man (2.0)	3,4,6-Man 2,4,6-Man 2,3,4-GlcA

[a] Solvent *a*.

[b] As alditol acetates on column (b).

[c] 2,3,4,6-Man = 2,3,4,6-tetra-O-methyl-D-mannose *etc.*

Neutral disaccharide I was identified as 3-O-β-D-manno-pyranosyl-D-mannose from the methylation analysis, and by comparison of its optical rotation with the values obtained for other neutral mannose disaccharides[80]. The aldobiouronic acid II was indistinguishable on paper chromatography (solvents *a*, *b* and *d*) from 2-O-(β-D-glucopyranosyluronic acid)-D-mannose, and its optical rotatory dispersion curve (ORD) was identical with that of the authentic material[140] as shown in Figure 17. Hydrolysis of the oligosaccharides III and IV yielded the aldobiouronic acid II and D-mannose, and the mobilities of these two oligomers on paper chromatography (solvent *a*), suggested that they were aldotriouronic and aldotetraouronic acids, respectively. Methylation analysis of oligosaccharides III and IV showed that they contained both 2- and 3-linked residues of D-mannose. Methylation studies indicated that D-glucuronic acid was located at the non-reducing end of both structures as shown below:

D-GlcA $\frac{1\ \ 2}{\beta}$ D-Man*p* $\frac{1\ \ 3}{\alpha}$ D-Man*p* (III)

D-GlcA $\frac{1\ \ 2}{\beta}$ D-Man*p* $\frac{1\ \ 3}{\alpha}$ D-Man*p* $\frac{1\ \ 3}{\alpha}$ D-Man*p* (IV)

The optical rotations of the aldotriouronic (III) and aldotetraouronic acids (IV) are consistent with the addition of one and two α-(1→3)-linked residues of D-mannose, respectively, to the aldobiouronic acid moiety (II).

5.3.3 SMITH-DEGRADATION STUDIES OF THE CAPSULAR POLYSACCHARIDE H

The polysaccharide H consumed approximately one mole of periodate per sugar residue. Reduction of the oxidised polysaccharide with sodium borohydride, followed by mild acid hydrolysis in molar trifluoroacetic acid at room temperature for 72h, yielded a water-

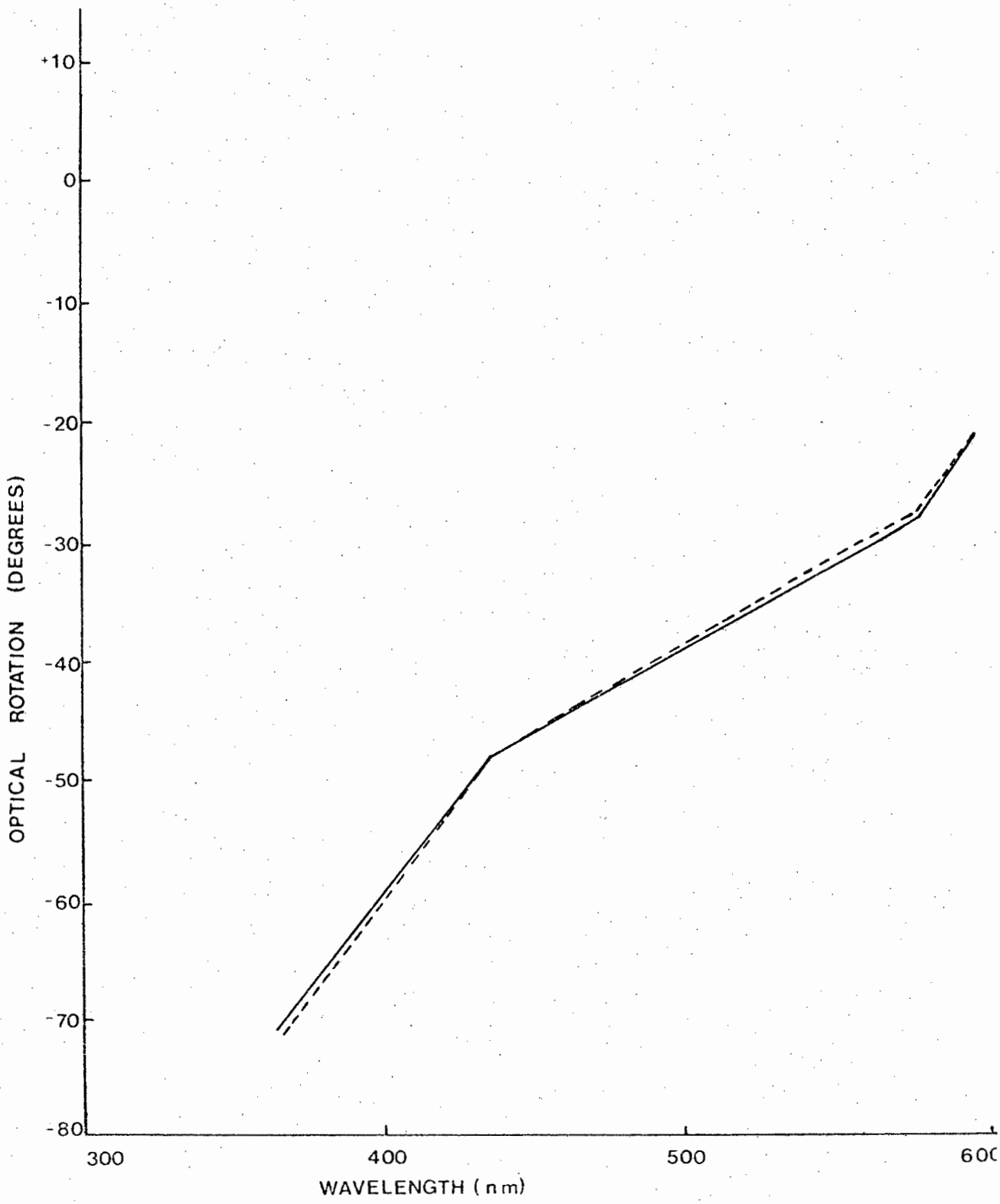


FIGURE 17 The optical rotatory dispersion curves of the aldobiouronic acids isolated from the *Klebsiella* K7 and *Cryptococcus neoformans* H capsular polysaccharides.

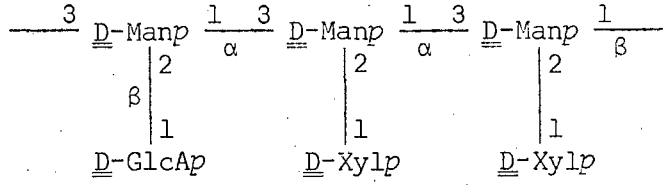
——— *Cryptococcus neoformans*

------- *Klebsiella* K7

insoluble product which was shown by more vigorous acid hydrolysis with 2M trifluoroacetic acid at 100°C for 8h to consist of D-mannose residues only. When the fully methylated derivatives of the Smith-degraded product was hydrolysed and the hydrolysate was analysed by paper chromatography and by g.l.c. (alditol acetate method), only 2,4,6-tri-O-methyl-D-mannose was found to be present. The identity of this sugar was confirmed by mass spectrometry of its alditol acetate, and by g.l.c. of the trimethylsilyl derivatives of the anomers of the tri-O-methyl sugar. These results indicate that all the D-glucuronic acid and D-xylose residues are present as end groups and that the polysaccharide chain is composed of (1→3)-linked residues of D-mannose.

5.3.4 STRUCTURE OF THE REPEATING SEQUENCE IN THE POLYSACCHARIDE H

All the evidence obtained from partial-hydrolysis, methylation analysis and Smith-degradation studies is consistent with a polysaccharide structure (V) in which each of the (1→3)-linked residues of D-mannose in the chain is substituted at O-2 by either D-glucuronic acid or D-xylose. A consideration of the acid content of the polysaccharide H (~15%), together with the proportions of the neutral sugar constituents, indicates that two terminal units of D-xylose are present for every terminal residue of D-glucuronic acid.



(V)

The modes of linkage of the D-glucuronic acid and D-mannose residues in the polysaccharide H were determined by measurement of the

optical rotatory power of the oligosaccharides mentioned earlier. The terminal residues of D-xylose in the repeating sequence do not appear in any of these structures. However, the values calculated for the optical rotation of the polysaccharide H by means of Hudson's rules of isorotation[71] on the assumption that the D-xylose residues are all α-linked or all β-linked, are $[\alpha]_{589}$ + 57° and $[\alpha]_{589}$ -12°, respectively. The actual value, +5°, is much closer to the latter, which seems to suggest that these residues are β-linked.

The R strain from *Cryptococcus neoformans* described earlier has been serotyped by Dr.E.Bennet, USA, as Type A, while the H strain was damaged in transit and could not be typed. However, since the two polysaccharides are chemically identical, they are presumably of the same serotype.

5.4 COMPARISON WITH OTHER POLYSACCHARIDES FROM *CRYPTOCOCCUS NEOFORMANS*

Little has been published concerning the polysaccharides from *C. neoformans*. The only detailed structure has been reported by Miyazaki[84] for an untyped *Cryptococcus* polysaccharide, which differs from polysaccharide H in that it consists of chain residues of D-mannose linked through O-2, each of which is alternately substituted at O-4 by D-xylose or the aldobiouronic acid 4-O-(glucopyranosyluronic acid)-D-mannose. Blandamer and Danishefsky[85] reported that the polysaccharide from Type B consists of a mannan backbone with branches of xylose and glucuronic acid, but the positions of the linkages in the polysaccharide structure have not been determined. The type-specific polysaccharide from *Cryptococcus neoformans* A contains xylose, galactose, mannose and glucuronic acid[86], and has been shown by Rebers *et al.*[87] to consist of a mixture of at least two acidic polysaccharides. Since the polysaccharide H examined in the present study is chemically identical with

the polysaccharide R (serotyped as Type A), and is presumably of the same serotype, it is possible that the mixture examined by Rebers *et al.* includes the polysaccharide H and one or more polysaccharides containing residues of galactose.

5.5 EXPERIMENTAL

5.5.1 CHARACTERISTICS OF THE POLYSACCHARIDES R AND H

The two strains (R and H) were grown and the capsular polysaccharides purified as described in Section 2.7 to yield the acidic polysaccharides, having the characteristics shown in Table 21. The polysaccharide R, which was found by gel-permeation chromatography on agarose and Bio-Gel P-10 to contain two components, was precipitated a second time with cetavlon bromide. The twice-precipitated material gave a single peak on gel-permeation chromatography on agarose gel, as did the polysaccharide H. Each of the two polysaccharides R and H moved as a single component on ultracentrifugation and on electrophoresis.

5.5.2 MILD ACID HYDROLYSIS OF THE POLYSACCHARIDES R AND H

Samples (40mg) of each polysaccharide were hydrolysed with 0.1M trifluoroacetic acid (20ml) at 100°C for 24h. Samples (2ml) of the hydrolysates were withdrawn at intervals and the acid was removed by distillation with methanol. Each residue was dissolved in a few drops of water and analysed by paper chromatography in solvents *a* and *d*.

5.5.3 HYDROLYSIS OF THE POLYSACCHARIDES R AND H ; SUGAR ANALYSES

Each of the polysaccharides (50mg) were hydrolysed with 2M trifluoroacetic acid at 100°C for 18h. Paper chromatography of the hydrolysates (solvents *a* and *d*) showed the presence of D-xylose, D-mannose, D-glucuronic acid and an aldobiouronic acid having the same colour (when sprayed with *p*-anisidine) and mobility as 2-O-(β-D-glucopyranosyluronic acid)-D-mannose. The ratios of neutral sugars in each hydrolysate were determined by g.l.c. analysis of the derived

alditol acetates to give the results shown in Table 21. Preparative g.l.c. of the acetates in each case gave D-mannitol hexaacetate, the circular dichoism spectrum of which was identical with that of a standard sample, and D-xylitol pentaacetate which was found to be achiral by this method. Isolation of samples of xylose from the hydrolysates of the polysaccharides R and H by preparative paper chromatography (solvent *a*) and measurement of their optical rotatory dispersion curves confirmed that these residues were also in the D-configuration.

5.5.4 METHYLATION ANALYSES

Samples (200mg) of the polysaccharides R and H in the acid form were methylated once by Hakomori's method[14] and three times by the method of Purdie and Irvine[68] to give in each case a colourless, amorphous product which showed no hydroxyl absorption in the i.r. spectrum. An estimate of the uronic acid content of each polysaccharide was obtained by measuring the intensities of the absorptions due to the methyl ester carbonyl (1740 cm^{-1}) and methoxyl (1100 cm^{-1}) groups in the methylated derivatives The ratio I_{CO}/I_{OCH_3} can be plotted against the known uronic acid contents of a number of methylated polysaccharides to give a linear graph, from which unknown amounts of uronic acid may be estimated[88].

The fully methylated polysaccharides (20mg) were hydrolysed with 2M trifluoroacetic acid at 100°C for 18h. Paper chromatography (solvents *b* and *f*) and g.l.c. analysis, as the trimethylsilylated derived alditols, of the sugars present in the hydrolysates gave 2,3,4-tri-O-methyl-D-xylose and 4,6-di-O-methyl-D-mannose. Methanolysis, followed by g.l.c. of the mixtures of methylated glycosides produced, gave methyl 2,3,4-tri-O-methyl-D-glucuronate (removed by saponification) and the

methyl glycosides of 2,3,4-tri-O-methyl-D-xylose and 4,6-di-O-methyl-D-mannose. The proportions of each sugar are shown in Table 21.

5.5.5 FURTHER CHARACTERISATION OF POLYSACCHARIDE H

5.5.5.1 REDUCTION OF METHYLATED POLYSACCHARIDE H

Fully methylated polysaccharide H (110mg) was reduced with lithium aluminium hydride (700mg) in tetrahydrofuran (10ml) for 18h. After the addition of aqueous ethanol the solution was evaporated to dryness and extracted with chroloform (3 x 30ml). The extracts were combined and the chloroform was removed to give a product (60mg) which showed no absorption at 1740 cm^{-1} (methyl ester CO). Methanolysis and g.l.c. of the reduced methylated derivative (10mg) gave the methyl glycosides of 2,3,4-tri-O-methyl-D-xylose, 4,6-di-O-methyl-D-mannose and 2,3,4-tri-O-methyl-D-glucose. No peaks corresponding to methyl 2,3,4-tri-O-methyl-D-glucuronate were detected. The reduced methylated polysaccharide (30mg) was hydrolysed with 2M trifluoroacetic acid at 100°C for 8h. Paper chromatography of the hydrolysate (solvents *b* and *f*) showed the presence of a component giving the same colour with *p*-anisidine and having the same mobility as 2,3,4-tri-O-methyl-D-glucose. A portion of the hydrolysate was reduced, acetylated and analysed by g.l.c., using column (a), to give derivatives of 2,3,4-tri-O-methyl-D-xylose, 2,3,4-tri-O-methyl D-glucose and 4,6-di-O-methyl-D-mannose. Preparative g.l.c. allowed the recovery of the individual methylated alditol acetates, which were further characterised by mass spectrometry. The 2,3,4-tri-O-methyl-D-glucitol triacetate was demethylated (BCl_3) and reacetylated to give D-glucitol hexaacetate, which was dissolved in acetonitrile and the circular dichroism spectrum recorded. Comparison of the c.d. spectrum with that of a standard sample confirmed the

D-configuration of glucose. It appears certain that the origin of the 2,3,4-tri-O-methyl-D-glucose found in the hydrolysate of the reduced methylated polysaccharide must be the terminal residues of glucuronic acid present in the original polysaccharide H; this acid must, accordingly, also be in the D-configuration.

5.5.5.2 PARTIAL HYDROLYSIS OF THE POLYSACCHARIDE H ; ISOLATION OF OLIGOSACCHARIDES

Polysaccharide H (100mg) was hydrolysed with 2M trifluoroacetic acid at 100°C for 3h, and the products were separated by preparative paper chromatography (solvent *a*) into four components, each of which was homogeneous on paper chromatography in solvents *a*, *b* and *d*. The characteristics of each component are shown in Table 22. Oligosaccharides I - IV were further characterised as follows:

I. Methylated neutral disaccharide I was hydrolysed with 2M trifluoroacetic acid at 100°C for 4h. Paper chromatography of the hydrolysate (solvents *b* and *f*) showed 2,3,4-6-tetra-O-methyl-D-mannose and 2,4,6-tri-O-methyl-D-mannose. The presence of these sugars in the hydrolysate was confirmed by g.l.c. analysis of the trimethylsilyl derivatives of the anomers of the methylated sugars.

II. Fully methylated aldobiouronic acid II on methanolysis and g.l.c. gave methyl glycosides of 2,3,4-tri-O-methyl-D-glucuronate (removed by saponification) and 3,4,6-tri-O-methyl-D-mannose in the same proportions as were given upon methanolysis of methylated 2-O-(β-D-glucopyranosyluronic acid)-D-mannose. The identity of 3,4,6-tri-O-methyl-D-mannose was confirmed by hydrolysis of the methylated aldobiouronic acid followed by g.l.c. of the trimethylsilyl derivatives of the anomers of the tri-O-methyl sugar, and also by paper chromatography in solvents *b* and *f*.

III. Hydrolysis of the aldotriouronic acid III with 2M trifluoroacetic acid at 100°C for 8h, followed by paper chromatography of the hydrolysate (solvents *a* and *d*), gave D-mannose, the aldobiouronic acid II and a trace of D-glucuronic acid. Fully methylated aldotriouronic acid was hydrolysed with 2M trifluoroacetic acid at 100°C for 18h. Paper chromatography of the hydrolysate (solvents *b* and *f*) showed the presence of 2,4,6-tri-O-methyl-D-mannose and 3,4,6-tri-O-methyl-D-mannose. The identities of these sugars were confirmed by g.l.c. of the trimethylsilyl derivatives of the anomers of the tri-O-methyl sugars. Reduction of the hydrolysate followed by trimethylsilylation and g.l.c. analysis gave the proportions of sugars shown in Table 22.

IV. Results obtained from hydrolysis, paper chromatography and methylation analysis of the aldotetraouronic acid IV, were identical with those for III above, except for the proportion of 2,4,6-tri-O-methyl-D-mannose found on methylation analysis of IV, which showed a marked increase from that yielded by methylated III. Methanolysis and g.l.c. of the methylated derivatives of both III and IV gave methyl 2,3,4-tri-O-methyl-D-glucuronate in addition to the methyl glycosides of the neutral sugars as shown in Table 22.

5.5.5.3 SMITH-DEGRADATION OF POLYSACCHARIDE H

Capsular polysaccharide H (300mg) was dissolved in aqueous sodium metaperiodate (50ml, 0.113M) and allowed to stand in the dark for 3 days, the consumption of periodate (1.12mol per hexose) being monitored by the arsenite method[79]. After this time the reaction was terminated by the addition of ethylene glycol (1ml) and the solution was stirred for 3h and then dialysed against running tap water for 2 days. The solution was concentrated and then reduced with sodium borohydride (1g) for 4 days. After acidification with Amberlite IR-120 (H^+) resin,

the solution was distilled with methanol but was not taken to dryness. The final traces of borate were removed by dialysing the solution against running tap water for 3 days before freeze-drying, which yielded the polyalcohol (200mg). This product was dissolved in M trifluoroacetic acid and stirred at room temperature for 6 days. The trifluoroacetic acid was removed by freeze-drying, the residue was extracted with methanol (3 x 20ml) and the methanol-soluble material was examined by paper chromatography (solvent *b*). When sprayed with silver nitrate the paper chromatogram showed the presence of glycolaldehyde and ethylene glycol.

The methanol-insoluble material, which was found to be almost totally insoluble in water also, was freeze-dried to yield the Smith-degraded product (71mg). After hydrolysis of this product with 2M trifluoroacetic acid at 100°C for 8h, paper chromatography of the hydrolysate (solvents *a* and *d*) showed only D-mannose. A portion of the hydrolysate was reduced, acetylated and analysed by g.l.c. on column (b); only D-mannitol hexaacetate was found.

Owing to the insolubility of the Smith-degraded product the Hakomori methylation procedure was modified as follows. Smith-degraded product (35mg) was suspended in dry dimethyl sulphoxide and heated in an oil bath at 60°C for 90 min. After cooling to room temperature the solution was treated with methylsulphinyl anion (3ml, 1.6M) and stirred for 48h before being cooled in ice water, while methyl iodide (5ml) was slowly added. The solution was diluted with water (10ml) and dialysed against running tap water for 2 days before being extracted with chloroform (3 x 20ml). The extracts were combined and the solvent was removed to give a product (24mg), $[\alpha]_D^{20}$ + 29° (*c* 0.2, chloroform), which showed no hydroxyl absorption in the i.r. Hydrolysis of a

sample (10mg) of the methylated product, followed by paper chromatography (solvents *b* and *f*) and g.l.c. analysis, as the trimethylsilyl derivatives, of the sugars present in the hydrolysate, gave only 2,4,6-tri-O-methyl-D-mannose. Reduction and acetylation of the hydrolysate gave 2,4,6-tri-O-methyl-D-mannitol triacetate, which was isolated by preparative g.l.c. and further characterised by mass spectrometry.

6. CONCLUSION

6. CONCLUSION

The polysaccharides from *Klebsiella* serotypes K4, K9*, K27 and K64 have all been shown to consist of regularly repeating oligosaccharide sequences, consistent with the types of structure found in polysaccharides from other *Klebsiella* strains. Each of the four studied belongs to a different chemotype and shows a structural pattern which has not been previously reported in work on *Klebsiella* polysaccharides. Comparison of the primary structures of the polysaccharides from K4, K9* and K27 with the repeating units found in other serotypes within their respective chemotypes shows only limited variations of the polysaccharide skeleton within each group. The structure of the only other polysaccharide known to contain the same sugar residues as the *Klebsiella* K64 polysaccharide (i.e. K65) has not yet been determined.

A number of methods used for the structural investigation of the *Klebsiella* polysaccharides have been successfully applied to the capsular polysaccharides from two strains of *Cryptococcus neoformans*. These polysaccharides have been shown to be chemically equivalent and are presumably of the same serotype. The elucidation of the structure of the polysaccharide H represents one of the few complete structures which have been reported in work on the polysaccharides from *C. neoformans*.

Hudson's rules have been applied to the calculation of the theoretical values for the optical rotation of a number of *Klebsiella* polysaccharides of known structure. In most cases differences between these values and those obtained by experiment have been found to be minimal.

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