CAPSULAR ANTIGENS OF GRAM-NEGATIVE BACTERIA

by

ELEONORA ALTMAN

B.Sc., The Hebrew University of Jerusalem, Israel, 1975
M.Sc., The Hebrew University of Jerusalem, Israel, 1977.

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Chemistry)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

AUGUST 1984

© Eleonora Altman, 1984
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of CHEMISTRY

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date Sept 26, 1984
ABSTRACT

Klebsiella, Escherichia coli, Salmonella and Shigella are among the most frequently found pathogenic Enterobacteria. The classification of Salmonella and Shigella relates mainly to the O antigens which are lipopolysaccharides, whereas for Klebsiella and E. coli capsular polysaccharides (K antigens) play an important role.

Approximately eighty serologically different Klebsiella strains are known of which seventy structures have been determined.

The structure of the capsular polysaccharide isolated from Klebsiella serotype K50 is presented here. It is unique among the Klebsiella K antigens in having a 'five-plus-two' repeating unit.

\[
\begin{align*}
\rightarrow 3) &- \beta-D-Gal-(1 \rightarrow 3) - \alpha-D-Glc-(1 \rightarrow 4) - \alpha-D-GlcA-(1 \rightarrow 3) - \alpha-D-Man-(1 \rightarrow 2) - \alpha-D-Man-(1 \rightarrow 6) \\
& + \\
& 1 \\
& \alpha-D-Glc \\
& 6 \\
& + \\
& 1 \\
& \beta-D-Gal
\end{align*}
\]

Klebsiella K50

The K antigens of Escherichia coli can be divided into three groups (A, B, L) on the basis of their thermolability, all of which comprise acidic polysaccharides. The extracellular A antigens of E. coli bear a strong similarity to the K antigens of Klebsiella.
The present investigation describes the isolation and the structural analyses of acidic polysaccharides obtained from *Escherichia coli* 09:K28(A):H- (K28 antigen) and *Escherichia coli* 09:K32(A):H19 (K32 antigen).

\[\rightarrow^3\alpha-D-\text{Glc}-(1\rightarrow 4)\beta-D-\text{GlcA}-(1\rightarrow 4)\alpha-L-\text{Fuc}-(1\rightarrow 2 \text{ and } 3)\]

\[\uparrow\]

\[1\]

\[\beta-D-\text{Gal}\]

*Escherichia coli K28*

\[\text{OAc}\]

\[\uparrow\]

\[2\]

\[\rightarrow^3\alpha-D-\text{Glc}-(1\rightarrow 4)\alpha-L-\text{Rha}-(1\rightarrow 3)\alpha-D-\text{Gal}-(1\rightarrow 3)\]

\[\uparrow\]

\[1\]

\[\beta-D-\text{GlcA}\]

*Escherichia coli K32*

Specific bacteriophage-borne glucanases were utilized to degrade the two *Escherichia coli* capsular polysaccharides. *E. coli* K32 polysaccharide has been degraded using a purified \(\phi\)32 bacteriophage with \(\alpha\)-glucopyranosidase activity, while *E. coli* K28 polysaccharide has been degraded using crude solutions of the bacteriophages \(\phi\)28-1 and \(\phi\)28-2 (both with \(\alpha\)-glucopyranosidase activity), and the results have been compared.
### TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF APPENDICES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF SCHEMES</td>
<td>xiv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xv</td>
</tr>
<tr>
<td>PREFACE</td>
<td>xvi</td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. METHODOLOGY OF STRUCTURAL STUDIES ON POLYSACCHARIDES</td>
<td>22</td>
</tr>
<tr>
<td>II.1 Isolation and purification</td>
<td>23</td>
</tr>
<tr>
<td>II.1.1 <em>Klebsiella</em> polysaccharides</td>
<td>25</td>
</tr>
<tr>
<td>II.1.2 <em>Escherichia coli</em> polysaccharides</td>
<td>26</td>
</tr>
<tr>
<td>II.2 Sugar analysis</td>
<td>26</td>
</tr>
<tr>
<td>II.2.1 Total hydrolysis and methanolysis</td>
<td>26</td>
</tr>
<tr>
<td>II.2.2 Characterizations and quantitation of sugars</td>
<td>28</td>
</tr>
<tr>
<td>II.2.3 Carboxyl reduction of acidic polysaccharides</td>
<td>28</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>II.2.4</td>
<td>Determination of the configuration of the sugars</td>
</tr>
<tr>
<td>II.3</td>
<td>Position of linkage</td>
</tr>
<tr>
<td>II.3.1</td>
<td>Methylation analysis</td>
</tr>
<tr>
<td>II.3.2</td>
<td>Gas-liquid chromatography (g.l.c.)</td>
</tr>
<tr>
<td>II.3.3</td>
<td>Mass-spectrometry (m.s.)</td>
</tr>
<tr>
<td>II.4</td>
<td>Sequencing of sugars</td>
</tr>
<tr>
<td>II.4.1</td>
<td>Partial hydrolysis</td>
</tr>
<tr>
<td>II.4.2</td>
<td>Periodate oxidation and Smith degradation</td>
</tr>
<tr>
<td>II.4.3</td>
<td>Base-catalyzed degradation</td>
</tr>
<tr>
<td>II.5</td>
<td>Determination of linkage</td>
</tr>
<tr>
<td>II.5.1</td>
<td>Optical rotation</td>
</tr>
<tr>
<td>II.5.2</td>
<td>Nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>II.5.2.1</td>
<td>$^1$H-n.m.r. spectroscopy</td>
</tr>
<tr>
<td>II.5.2.2</td>
<td>$^{13}$C-n.m.r. spectroscopy</td>
</tr>
<tr>
<td>II.5.3</td>
<td>Other techniques</td>
</tr>
<tr>
<td>II.5.3.1</td>
<td>Enzymatic hydrolysis</td>
</tr>
<tr>
<td>II.5.3.2</td>
<td>Chromium trioxide oxidation</td>
</tr>
<tr>
<td>II.6</td>
<td>Location of O-acetyl group</td>
</tr>
</tbody>
</table>
III. GENERAL EXPERIMENTAL CONDITIONS ........................................... 76

III.1 Paper chromatography ....................................................... 77
III.2 Gas-liquid chromatography and g.l.c.-mass spectrometry .......... 77
III.3 Gel-permeation chromatography ......................................... 78
III.4 Optical rotation and circular dichroism ................................ 79
III.5 Nuclear magnetic resonance ............................................... 79
III.6 General conditions .......................................................... 80
III.7 Isolation and purification of the polysaccharides .................. 80
   III.7.1 Klebsiella polysaccharides ......................................... 80
   III.7.2 Escherichia coli polysaccharides .................................. 82
III.8 Bacteriophage propagation ................................................ 83
   III.8.1 Tube and flask lysis ................................................. 83
   III.8.2 Large-scale propagation of the bacteriophage .................. 84

IV. STRUCTURAL INVESTIGATION OF Klebsiella SEROTYPE K50
CAPSULAR POLYSACCHARIDE ....................................................... 86

IV.1 Abstract .............................................................................. 87
IV.2 Introduction ......................................................................... 88
IV.3 Results and discussion ....................................................... 88
IV.4 Conclusion ........................................................................... 100
IV.5 Experimental ....................................................................... 101
V. STRUCTURAL INVESTIGATION OF Escherichia coli

CAPSULAR POLYSACCHARIDES

V.1 Structural investigation of Escherichia coli

09:K28(A): H⁻ (K28 antigen) capsular polysaccharide

V.1.1 Abstract

V.1.2 Introduction

V.1.3 Results and Discussion

V.1.4 Conclusion

V.1.5 Experimental

V.2 Structural investigation of Escherichia coli

09:K32(A): H19 (K32 antigen) capsular polysaccharide

V.2.1 Abstract

V.2.2 Introduction

V.2.3 Results and discussion

V.2.4 Conclusion

V.2.5 Experimental
VI. BACTERIOPHAGE DEGRADATION OF *Escherichia coli*

CAPSULAR POLYSACCHARIDES SEROTYPES K28 and K32 .... 151

VI.1 Introduction ........................................... 152
VI.2 Results .................................................. 161
VI.3 Discussion ............................................... 170
VI.4 Experimental ............................................ 174

VII. BIBLIOGRAPHY ............................................. 179
<table>
<thead>
<tr>
<th>Appendix</th>
<th>The known structures of the <em>Escherichia coli</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>O antigens</td>
</tr>
<tr>
<td>II</td>
<td>K antigens</td>
</tr>
<tr>
<td>III</td>
<td>$^1$H and $^{13}$C-n.m.r. spectra</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.1</td>
<td>Klebsiella capsular polysaccharides (Kl-K83). Qualitative analysis and chemotype grouping</td>
</tr>
<tr>
<td>I.2</td>
<td>Schematic representation of the agglutination results on which definitions of A, B, and L antigens are based</td>
</tr>
<tr>
<td>IV.1</td>
<td>N.m.r. data for Klebsiella K50 polysaccharide and derived oligosaccharides</td>
</tr>
<tr>
<td>IV.2</td>
<td>Methylation analysis of Klebsiella K50 polysaccharide and derivatives</td>
</tr>
<tr>
<td>IV.3</td>
<td>Analyses of acidic oligosaccharides from Klebsiella K50 polysaccharide</td>
</tr>
<tr>
<td>V.1</td>
<td>$^1$H-n.m.r. data for Escherichia coli K28 polysaccharide</td>
</tr>
<tr>
<td>V.2</td>
<td>$^{13}$C-n.m.r. data for the native and O-deacetylated E. coli K28 polysaccharide</td>
</tr>
<tr>
<td>V.3</td>
<td>Methylation analysis of Escherichia coli K28 polysaccharide and derived products</td>
</tr>
<tr>
<td>V.4</td>
<td>N.m.r. data for Escherichia coli K28 oligosaccharides derived from partial hydrolysis of the polysaccharide</td>
</tr>
<tr>
<td>V.5</td>
<td>Analysis of the oligosaccharides from partial hydrolysis of Escherichia coli K28 polysaccharide</td>
</tr>
</tbody>
</table>
V.6 N.m.r. data for Escherichia coli K32 native and O-deacetylated polysaccharides ........................................ 137

V.7 Methylation analysis of Escherichia coli K32 polysaccharide and derived products ........................................ 138

VI.1 Methylation analysis and reducing end determination of E. coli K28 oligosaccharide isolated after bacteriophage φ28-1 degradation of E. coli K28 polysaccharide ........................................ 163

VI.2 Determination of the degree of polymerization and the reducing end of E. coli K28 oligosaccharide isolated after bacteriophage φ28-1 degradation of E. coli K28 polysaccharide ........................................ 164

VI.3 Proton assignments (400 MHz) for the oligosaccharides and related compounds generated by bacteriophage depolymerization of the E. coli K32 capsular polysaccharide ........................................ 169

VI.4 Methylation analysis of the reduced fraction II obtained after the separation of the depolymerization products of E. coli K32 polysaccharide ........................................ 171


**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.1</td>
<td>Schematic representation of the Gram-positive and Gram-negative cell wall of bacteria</td>
<td>3</td>
</tr>
<tr>
<td>I.2</td>
<td>Electronmicrograph of a cross-section of <em>E. coli</em> 09:K29 after contrast staining with ruthenium red</td>
<td>5</td>
</tr>
<tr>
<td>I.3</td>
<td>Schematic diagram of the general structure of bacterial lipopolysaccharides (LPS)</td>
<td>13</td>
</tr>
<tr>
<td>I.4</td>
<td>Schematic representation of the structures of N-acetyleneuraminic acid and 2-keto-3-deoxy-D-manno-2-octulosonic acid</td>
<td>15</td>
</tr>
<tr>
<td>I.5</td>
<td>a) <em>E. coli</em> K7 = K56 cross-reacts with antiserum to <em>S. pneumoniae</em> type 3 and type 8. b) <em>E. coli</em> K30 cross-reacts with antiserum to <em>S. pneumoniae</em> type 2</td>
<td>19</td>
</tr>
<tr>
<td>I.6</td>
<td>The structures of capsular polysaccharides of <em>H. influenzae</em> type b and <em>Escherichia coli</em> K100</td>
<td>20</td>
</tr>
<tr>
<td>II.1</td>
<td>Mass spectrum of a uronic acid degradation derivative from <em>Klebsiella</em> K50 polysaccharide (a) compared to the spectrum of a standard derivative (b)</td>
<td>41</td>
</tr>
<tr>
<td>II.2</td>
<td>Common products formed on periodate oxidation, followed by borohydride reduction and hydrolysis of terminal and monosubstituted hexoses</td>
<td>48</td>
</tr>
<tr>
<td>II.3</td>
<td>Sequential periodate oxidation and borohydride reduction of alginate</td>
<td>51</td>
</tr>
<tr>
<td>II.4</td>
<td>Relationship between dihedral angle ($\phi$) and coupling constants for $\alpha$- and $\beta$-D-hexoses</td>
<td>61</td>
</tr>
<tr>
<td>II.5</td>
<td>Schematic representation of different regions in the $^1$H-n.m.r. spectrum of polysaccharides</td>
<td>63</td>
</tr>
<tr>
<td>II.6</td>
<td>The $^1$H-n.m.r. spectra of native (top) and deacetylated (bottom) <em>E. coli</em> K28 capsular polysaccharides</td>
<td>65</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>II.7</td>
<td>The characteristic regions for resonances of carbon atoms belonging to different monosaccharide residues in polysaccharides</td>
<td>68</td>
</tr>
<tr>
<td>II.8</td>
<td>The $^{13}$C-n.m.r. spectrum of deacetylated <em>E. coli</em> K28 capsular polysaccharide</td>
<td>70</td>
</tr>
<tr>
<td>IV.1</td>
<td>Gel-permeation chromatography of the product obtained after selective, partial hydrolysis of <em>Klebsiella</em> K50 polysaccharide</td>
<td>96</td>
</tr>
<tr>
<td>V.1</td>
<td>Partial structure of <em>E. coli</em> K28 polysaccharide</td>
<td>117</td>
</tr>
<tr>
<td>VI.1</td>
<td>Schematic diagram demonstrating the structure of <em>T</em>$_2$ bacteriophage</td>
<td>154</td>
</tr>
<tr>
<td>VI.2</td>
<td>Basic morphological types of bacteriophages with the types of nucleic acid</td>
<td>154</td>
</tr>
<tr>
<td>VI.3</td>
<td>The mechanics of infection by bacteriophage</td>
<td>156</td>
</tr>
<tr>
<td>VI.4</td>
<td>Capsulated <em>E. coli</em> K29 exposed to a m.o.i. (the multiplicity of infection) of 300 phage for 8 min at 37°</td>
<td>158</td>
</tr>
<tr>
<td>VI.5</td>
<td>Separation of the depolymerization products of <em>E. coli</em> K32 by gel-permeation chromatography (<em>Bio-Gel P-4</em>)</td>
<td>167</td>
</tr>
<tr>
<td>VI.6</td>
<td>Molecular weight distribution of fraction II</td>
<td>168</td>
</tr>
<tr>
<td>Scheme</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>II.1</td>
<td>Reduction of carboxylic acid in aqueous solution using carbodiimide reagent</td>
<td>29</td>
</tr>
<tr>
<td>II.2</td>
<td>Methylation analysis of <em>Klebsiella K50</em> polysaccharide</td>
<td>34</td>
</tr>
<tr>
<td>II.3</td>
<td>The mass spectra of the acetates (<em>R</em> = Ac), methyl ethers (<em>R</em> = Me), and trifluoroacetates (<em>R</em> = COCF₃) of alditols. Only primary fragments are shown</td>
<td>39</td>
</tr>
<tr>
<td>II.4</td>
<td>The A-series of fragments for the degradation of a disaccharide methyl glycoside</td>
<td>42</td>
</tr>
<tr>
<td>II.5</td>
<td>Smith degradation of <em>Klebsiella K50</em> capsular polysaccharide</td>
<td>52</td>
</tr>
<tr>
<td>II.6</td>
<td>Uronic acid degradation of <em>Klebsiella K50</em> polysaccharide</td>
<td>56</td>
</tr>
<tr>
<td>II.7</td>
<td>Location of O-acetyl substituents according to the de Belder and Norrman procedure</td>
<td>74</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Professor G.G.S. Dutton for his guidance, encouragement and interest throughout the course of this work.

I wish to thank my colleagues in the laboratory for their support and helpful discussions and Dr. E.H. Merrifield (University of Cape Town, South Africa) for his assistance during the early stages of this work.

Thanks are also due to Dr. S.C. Churms (University of Cape Town, South Africa) for gel-permeation measurements; Dr. S.O. Chan and the staff of the n.m.r. service and Dr. G. Eigendorf and the staff of the mass spectrometry service for their patient assistance.

My special thanks to Dr. B. Lewis (Cornell University) for proof reading of this thesis.

I should also like to thank Rani Theeparajah for typing this thesis.

Finally, my grateful thanks to my husband Boris for his encouragement and moral support.
DEDICATED TO THE MEMORY OF

MY LATE FATHER

ISRAEL KATSIN
PREFACE

The topic of this thesis is concerned with the structure elucidation of bacterial polysaccharides. *Klebsiella* and *Escherichia coli* have many features in common and our laboratory has for several years studied the capsular antigens of *Klebsiella*. Now that the structures of almost all these 80 K strains are known we are embarking on the examination of the capsular polysaccharides of *Escherichia coli*. This thesis therefore, deals mainly with structures of *Escherichia coli* K28 and K32 capsular polysaccharides. The structure of *Klebsiella* K50 polysaccharide was studied first in order to become acquainted with the methodology of the carbohydrate research.

In the Introduction I have attempted to give a concise account of *Escherichia coli* polysaccharides, their biological importance, serological classification and structural diversity.

The Methodology section deals with the standard techniques of structural analysis together with more modern ones, such as the use of nuclear magnetic resonance spectroscopy. Examples from the structural investigation of *Klebsiella* and *Escherichia coli* capsular polysaccharides are chosen to best illustrate the methods used.

Because of the increasing importance of bacteriophage-associated glycanases for the structural analysis of bacterial surface carbohydrates, a chapter which deals with the classification, structure and applications of bacteriophages is included.

Appendix I contains the list of known structures of *Escherichia coli* O antigens. The list of known structures of *Escherichia coli* K antigens along with the literature references is included in Appendix II.
CHAPTER I

INTRODUCTION
I. INTRODUCTION

Natural macromolecules containing carbohydrate units are of widespread occurrence in all living organisms and include (a) polysaccharides as exclusively carbohydrate polymers; (b) glycoproteins, proteoglycans, and peptidoglycans; (c) glycolipids and lipopolysaccharides; (d) teichoic acids and related macromolecules containing phosphodiester-linked oligosaccharide repeating units; and (e) nucleic acids.¹

Commercially, interest in polysaccharides has extended from starch and cellulose in food, pulp and paper industries to natural gums and mucilages.

The usefulness of most commercial polysaccharides is based on their capacity to alter the basic properties of water (e.g. thickening and gelling). Polysaccharides also play an important role in controlling the texture of foods as well as their flavor, appearance and color. They perform as thickening and sizing agents in industrial applications (the textile and paper industries) and as drilling fluids in oil field applications (e.g. xanthan gum).²

Until recently, the biological functions of polysaccharides were thought to be limited to serving as structural polymers and energy reserves. It is now well established that complex carbohydrates play an important role in biological recognition as: receptors for phage and bacteriocins; specific surface antigens; highly specific receptors in eukaryotes for viruses, bacteria, hormones and toxins; and determinants of secreted glycoproteins within the cells. Certain complex carbo-
The envelope of the Gram-positive cell wall

The envelope of the Gram-negative cell wall

hydrates are chemical messengers and are especially important in regulating growth, development, reproduction, and disease resistance in plants.³

The majority of immunologically significant polysaccharides are of microbial origin. A simplified picture of the bacterial cell is shown in Fig. 1.1. Outside the plasma membrane is the cell wall which can be of two general types: one that has an outer membrane over a peptidoglycan layer (Gram-positive cell) and one that lacks the outer membrane but has additional components within the peptidoglycan layer (Gram-negative cell). The main component is lipopolysaccharide, which constitutes 10-15% of the dry cell wall and exerts both immunogenicity and full endotoxicity. The species-specific somatic polysaccharide antigen in the cell wall of Gram-negative bacteria is called the bacterial 0 antigen.

Many bacteria produce extracellular polysaccharides (exopolysaccharides). They may exist in the form of a discrete capsule surrounding the bacterial cell or in the form of a loose slime, unattached to the cell surface.⁴ Capsules "mask" the cell wall 0 antigens and interfere with their serological detection.⁵ They can be recognized by the India ink staining technique or by electron microscopy (see Fig. 1.2). Capsules render bacteria resistant to phagocytosis and to the action of the complement.⁴

This capsular material is also immunogenic and gives rise to specific anti-capsular antibodies which react directly with the encapsulated bacteria.⁵ They were first found in early serological studies of E. coli and the term K antigen (from the German word
Fig I. 2: Electronmicrograph of a cross-section of *E. coli* 09:K29 after contrast staining with ruthenium red. (From ref. 5).
"Kapsel") was introduced in 1945 by Kauffmann. Capsular polysaccharides play an important role in the immune response to bacterial infection due to their location on the outer surface of the bacteria and constitute the principal antigens in most of the pathogenic, Gram-negative and Gram-positive organisms. However, other antigens, such as proteins and lipopolysaccharides (O antigens), can also play a significant role in the human immune-response to bacterial infection.  

The majority of bacterial polysaccharides are heteroglycans composed of oligosaccharide repeating units. This can be shown by molecular weight distribution studies and by nuclear magnetic resonance spectroscopy.  

The extracellular polysaccharides are generally, but not always, acidic. The acidic component is most often a uronic acid, a pyruvic acid acetal, or a phosphoric diester grouping. The capsule creates a simple, physical barrier protecting the underlying surface of the bacteria and for any given pathogenic strain of bacteria, its virulence is directly related to the amount of capsule.  

The nomenclature of bacterial polysaccharides is complicated and is based on the classification of the corresponding bacteria.  

Enterobacteriaceae is a family of Gram-negative, non-sporulating rods, either motile or with peritrichous flagella or non-motile. They grow on ordinary media and ferment glucose rapidly with or without gas production. The family is sub-divided into tribes, genera and species, which are the fundamental units of classification. The system resulted from comparative studies of the biochemical reactions given by
relatively large numbers of cultures of each of the genera. Kauffmann has proposed the following classification of *Enterobacteriaceae*:

<table>
<thead>
<tr>
<th>Familia Enterobacteriaceae</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tribes</strong></td>
</tr>
<tr>
<td>A. Eschericheae</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>B. Klebsielleae</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>C. Proteae</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Knowledge of the chemical structure of bacterial polysaccharides is of great significance for understanding the molecular principles of
their biological activities. Most of the bacteria are pathogenic for man, form small groups of serologically typed species and are convenient for comparative immunochemical research. Purified polysaccharides can be used as human vaccines (e.g. Streptococcus pneumoniae) and a considerable amount of research has been done in this field.\textsuperscript{10}

\textbf{Klebsiella polysaccharides}

The genus \textit{Klebsiella} is composed of three species: \textit{K. pneumoniae}, \textit{K. ozaenae} and \textit{K. rhinoscleromatis}.\textsuperscript{11} Klebsiella cultures were classified by Ørskov on the basis of their K (capsular) and O (somatic) antigens.\textsuperscript{12,13}

\textit{K. pneumoniae} is the most important member of the family. It is found in the respiratory tract of 5\% of normal individuals and is the primary cause of pneumonia in 3\% of all bacterial pneumonias.\textsuperscript{14}

Nimmich has reported the qualitative composition of the approximately 80 different \textit{Klebsiella} K serotypes\textsuperscript{15,16} and has classified them into chemotypes\textsuperscript{17} (see Table I.1).

The structures of seventy \textit{Klebsiella} capsular polysaccharides are known to date. Various structural patterns have emerged. These may be divided into four types: 1) those lacking uronic acid; 2) those where the uronic acid is a component of the main chain; 3) those where the uronic acid is in a side chain; and 4) those with side chains of three units.

A detailed compilation of different \textit{Klebsiella} structures was done by Di Fabio\textsuperscript{18} and is not, therefore, repeated in this thesis. In
TABLE 1.1: *Klebsiella* capsular polysaccharides (K1-K83).

Qualitative analysis and chemotype grouping

<table>
<thead>
<tr>
<th>GlcA</th>
<th>Gal</th>
<th>Glc</th>
<th>8(^P), 11(^P), 15, 25, 27(^P), 51</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcA</td>
<td>Gal</td>
<td>Man</td>
<td>20, 21(^P), 29(^P), 42(^P), 43, 66, 74(^P)</td>
</tr>
<tr>
<td>GlcA</td>
<td>Gal</td>
<td>Rha</td>
<td>9, 9*, 47, 52, 81, 83</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glc</td>
<td>Man</td>
<td>2, 4, 5(^P), 24</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glc</td>
<td>Rha</td>
<td>17, 23, 44, 45, 71</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glc</td>
<td>Fuc</td>
<td>1, 54</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glc</td>
<td>Glc</td>
<td>Man</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glc</td>
<td>Glc</td>
<td>Fuc</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glc</td>
<td>Glc</td>
<td>Rha</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glc</td>
<td>Man</td>
<td>Rha</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glc</td>
<td>Man</td>
<td>Fuc</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glc</td>
<td>Man</td>
<td>Rha</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glc</td>
<td>Glc</td>
<td>Man</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glc</td>
<td>Glc</td>
<td>Man</td>
</tr>
<tr>
<td>GalA</td>
<td>Gal</td>
<td>Man</td>
<td>3(^P), 49, 57</td>
</tr>
<tr>
<td>GalA</td>
<td>Glc</td>
<td>Rha</td>
<td>34, 48</td>
</tr>
<tr>
<td>GalA</td>
<td>Gal</td>
<td>Fuc</td>
<td>63</td>
</tr>
<tr>
<td>PyrA</td>
<td>Glc</td>
<td>Rha</td>
<td>72</td>
</tr>
<tr>
<td>PyrA</td>
<td>Gal</td>
<td>Rha</td>
<td>32</td>
</tr>
<tr>
<td>PyrA</td>
<td>Gal</td>
<td>Glc</td>
<td>Rha</td>
</tr>
<tr>
<td>KetoA</td>
<td>Gal</td>
<td>Glc</td>
<td>22, 37, 38</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GlcA</th>
<th>glucuronic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>GalA</td>
<td>galacturonic acid</td>
</tr>
<tr>
<td>PyrA</td>
<td>pyruvic acid</td>
</tr>
<tr>
<td>KetoA</td>
<td>rare uronic acid</td>
</tr>
<tr>
<td>Glc</td>
<td>glucose</td>
</tr>
</tbody>
</table>

\(^P\) pyruvic acid present in addition
Appendix I and II the list of known structures of *E. coli* O and K antigens is given.

**Escherichia coli polysaccharides**

The organism *Escherichia coli* was first isolated from faeces by Escherich in 1885. It belongs to the family *Enterobacteriaceae* whose normal habitat is the intestinal tract of man and animals. E. coli is often found in human urinary tract infections and is associated with severe infantile diarrhea.

Within the species, many different serotypes are recognized. The serotyping scheme is based on the identification of surface 'K', somatic 'O' and flagella 'H' antigens.

The O antigens are thermostable somatic antigens, resisting heating at 100°, and are not destroyed by alcohol. The term K antigen covers a group of either envelope or capsular antigens which can be divided into three groups (A, B, L) (see Table I.2). Strains which contain the thermolabile L or B antigens do not usually possess morphological capsules, whereas strains with thermostable A antigen are capsulated. The main tests to demonstrate the presence of a K antigen are O inagglutinability of the living bacteria and their agglutination with K sera.

The first antigenic scheme, comprising 25 O antigens, was established by Kauffmann in 1947. Since then, many O antigens have been added and approximately 100 'K', 164 'O' and 56 'H' are currently recognized.
TABLE 1.2: Schematic presentation of the agglutination results on which definitions of A, B, and L antigens are based

<table>
<thead>
<tr>
<th>K type</th>
<th>Antigen preparation</th>
<th>O Serum</th>
<th>OK Serum: Absorbed by culture heated at 100° for 2 h</th>
<th>Unabsorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>Live (or formalin treated)</td>
<td>-^a</td>
<td>+^b</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Boiled (100° for 1 h)</td>
<td>+</td>
<td>-^c</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>Live</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Boiled</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>A</td>
<td>Live</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Boiled</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

a - , Negative or significantly lower than that of boiled culture

b + , Agglutination

c - , No agglutination
**O antigens**

O Antigens consist of three regions: lipid A, an oligosaccharide core, and the O-specific polysaccharide chain (see Fig. I.3).

![Diagram of O antigens](image)

**Fig. I.3:** Schematic diagram of the general structure of bacterial lipopolysaccharides (LPS).

Lipid A (region 1) is buried in the outer membrane of the bacterial cell and is responsible for the general endotoxic properties of lipopolysaccharide.\(^{22}\) It consists of glucosamine, phosphate and fatty acids. The structure of lipid A has been described recently.\(^{23}\)

The core (region 2) is linked to lipid A via a carbohydrate component that is typical for the LPS of Gram-negative bacteria, 2-keto-3-deoxy-D-manno-2-octulosonic acid (KDO). Five different core structures have been described so far. It has been known for a long time that wild-type Enterobacteriaceae growing on agar may undergo a spontaneous S (smooth) $\rightarrow$ R (rough) mutation which is associated with
the disappearance of the O antigen and loss of the pathogenicity.

Analysis of the R mutants revealed that they lack the O-specific chain and consist only of the core bound to lipid A.\textsuperscript{23}

Region 3 is the specific O polysaccharide of the LPS of bacterial S forms. It is built up from repeating units of oligosaccharides which may contain up to 6-7 sugars. For a long time all \textit{E. coli} O antigens were thought to contain only neutral polysaccharide chains. More recently, LPS were isolated that contain acidic components such as glycerol phosphate, hexuronic acids, and neuraminic acid.\textsuperscript{22} The structures of the O antigens that have been published until now are given in Appendix I.

**K antigens**

The K antigens are capsular or envelope antigens and can be detected by immunoelectrophoresis. They are all polysaccharides except for two that are proteins (K88 and K99).\textsuperscript{22} Since these capsular polysaccharides are not immunogenic for humans and animals, they will have to be chemically modified to become immunogens. For such studies knowledge of the polysaccharide structures is extremely valuable.\textsuperscript{24} The difficulty in structural investigation of \textit{E. coli} capsular polysaccharides arises from the fact, that unlike for \textit{Klebsiella} polysaccharides, the qualitative composition of the most of the \textit{E. coli} serotypes is not known. This places the researcher in a difficult position, considering the fact that \textit{E. coli} polysaccharides are extremely diverse and contain uncommon and sometimes rare sugars. The
most prominent feature of these capsular polysaccharides is the frequent occurrence of 2-keto-3-deoxy-D-manno-2-octulosonic acid (KDO) and also the occurrence of N-acetylneuraminic acid (NeuNAc or NANA) (see Fig. 1.4). N-Acetylmannosaminuronic acid (ManNAcA) was found in the K7 antigen.²⁵

![Chemical structures](image)

**Fig. 1.4:** Schematic representation of the structures of N-acetylneuraminic acid and 2-keto-3-deoxy-D-manno-2-octulosonic acid (KDO).

A number of K antigens occur exclusively in O groups 08, 09 and 0101.²² The K antigens of these groups are of two types, one with amino sugars and one without. Those devoid of amino sugars have high molecular weights ($3 \times 10^5 - 10^6$ daltons). They are physically heterogeneous and become homogeneous after mild alkali treatment.²⁶ These K antigens (all A antigens and some B antigens) have a very low electrophoretic mobility, form thick and copious capsules and bear a strong resemblance to the K antigens of *Klebsiella.*²⁷ It is noteworthy
that the 08 and 09 antigens are themselves related to *Klebsiella* 0 antigens. Thus the 09 antigen of *E. coli* is identical with the *Klebsiella* 03 antigen, and the *E. coli* 08 antigen is identical with the *Klebsiella* 05 antigen.  

The K antigens of a group that contains amino sugars were thought to be extracellular polysaccharides but they were found to be lipopolysaccharides. *E. coli* with these K antigens contain two cell wall lipopolysaccharides: an acidic one which is termed a K antigen in addition to a neutral one (the 08, 09 or 0101 antigen). The acidic lipopolysaccharides are not capsular (K) antigens in the true sense; they were called thermolabile B antigens in the nomenclature of Kauffmann.

K antigens occurring in 0 groups other than 08, 09 and 0101 are all acidic polysaccharides with rather low molecular weights (below 50,000 daltons) and high electrophoretic mobility. Kauffmann has called them thermolabile L antigens, and they were found in most *Escherichia coli* strains isolated from pathological material. They contain rare sugar constituents, such as N-acetylneuraminic acid or N-acetylmannosaminuronic acid. The K1 antigen, also known as colominic acid, is a poly-N-acetyl neuraminic acid. *K2* is a teichoic acid-like polymer.

Although partial structures have been determined for several *E. coli* polysaccharides, relatively few complete structures have been published. The structures of *E. coli* K42 and *Klebsiella* K63 are identical and cross-react serologically. The *E. coli* K30 and *Klebsiella* K20 antigens are identical, and *E. coli* K100 antigen is structurally
related to the *Haemophilis influenzae* type b capsular antigen. The structures of the *E. coli* K antigens that have been published until now are given in Appendix II.

**Immunology of polysaccharides**

The polysaccharides are true immunoantigens in that they induce an immune response and the generation of specific antibodies. It was shown that only a relatively small portion of a polysaccharide is the major site of antibody specificity and that part is known as the determinant group.

A determinant group may comprise several monosaccharide residues, one of which contributes most to the specificity; that monosaccharide residue is termed the immunodominant sugar. The classical studies of Kabat on an isomaltose oligosaccharide series have shown that the non-reducing terminal residue contributed about 40% and the next two residues together about 60% to the total binding energy. These results indicate that in a linear polysaccharide the immunological specificity resides primarily in the terminal sugar residue and extends along the polysaccharide chain. The situation is different for branched polysaccharides in which the immunodominant sugars are those which are located on the side chains. Certain noncarbohydrate groups may function as antigenic determinants. Jann and Westphal have shown that O-acetyl groups are essential parts of the determinant regions in some *Salmonella* polysaccharides.
Pyruvic acid, attached to a sugar as an acetal was found to be the immunologic determinant in the capsular polysaccharides of *Klebsiella* and *Streptococcus pneumoniae*.\(^{34}\)

Cross-reactions have been used extensively in immunochemical analysis and they can be used to establish the structures of immunodeterminant groups. Heidelberger has used this approach extensively and was able to predict the presence of some structural features before they were verified chemically.\(^{35-38}\) Studies of cross-reactions of more than 60 capsular, type-specific polysaccharides of *Klebsiella* with 26 specific types of antipneumococcal sera permitted the assignment of several structural features such as non-reducing terminal residues of D-glucuronic acid, L-rhamnose, and D-galacturonic acid and some linkages within the polysaccharide chain. The molecular basis of some cross-reactions in which *E. coli* O and K antigens participate has been elucidated\(^{22}\) (see Fig. I.5).

Capsular polysaccharides are important virulence factors in many bacterial infections including those caused by *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Hemophilus influenzae*, *Escherichia coli*, *Salmonella typhosa*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*.\(^4\) The first capsular polysaccharide vaccine arose from the early work on *Streptococcus pneumoniae*.\(^{39}\)

It is important to realize, however, that the immunity received on recovery from infection by encapsulated bacteria differs from that generated by immunization with purified capsular polysaccharide vaccines. In young children polysaccharide vaccines give rise to only IgM antibodies and there is no memory response, whereas protein vaccines
a) $\text{ManNAcA}\, ^{2}\frac{1}{\beta}\text{Glc}\, ^{6}\frac{1}{\beta}\text{OAc}$

$E.\, coli\, K7 = K56$

$\text{GlcA}\, ^{3}\frac{1}{\beta}\text{Glc}\, ^{1}\frac{1}{\beta}$

$S.\, pneumoniae$ type 3

$\text{GlcA}\, ^{4}\frac{1}{\beta}\text{Glc}\, ^{4}\frac{1}{\beta}\text{Glc}\, ^{4}\frac{1}{\alpha}\text{Gal}\, ^{1}\frac{1}{\alpha}$

$S.\, pneumoniae$ type 8

b) $\text{Man}\, ^{2}\frac{1}{\alpha}\text{Gal}\, ^{1}\frac{1}{\beta}$

$E.\, coli\, K30$

$\text{Rha}\, ^{3}\frac{1}{\alpha}\text{Rha}\, ^{3}\frac{1}{\alpha}\text{Rha}\, ^{4}\frac{1}{\beta}\text{Glc}\, ^{1}\frac{1}{\alpha}$

$S.\, pneumoniae$ type 2

Fig. 1.5: a) $E.\, coli\, K7 = K56$ CROSS-REACTS WITH ANTISERUM TO $S.\, pneumoniae$ TYPE 3 AND TYPE 8. b) $E.\, coli\, K30$ CROSS-REACTS WITH ANTISERUM TO $S.\, pneumoniae$ TYPE 2
induce both IgM and IgG antibodies and an immunological memory. There have been several attempts to overcome this problem by conjugating polysaccharides to a protein carrier. This approach has been used also in the development of the vaccine against pathogenic E. coli and the Kl and K5 antigens in particular. Both antigens belong to the most virulent organisms encountered. Meningitis of small children is often caused by N. meningitidis group B and also by E. coli Kl. The capsular antigens of both of these pathogens are identical, both structurally and serologically. A proposal has been made to use a cross-reacting E. coli Kl polysaccharide as an alternative vaccine. E. coli 100 capsular polysaccharide cross-reacts with H. influenzae type b polysaccharide (see Fig. I.6). H. influenza type b may cause several diseases, of which meningitis is the most frequent and more serious health problem. Conjugates were prepared with E. coli K100 capsular polysaccharide because of its structural relatedness to H. influenzae type b. First positive results have been obtained in animal experiments.

\[
\begin{align*}
3 \text{Rib} & \frac{1}{\beta} \text{ribitol} 5 \text{PO}_4 \\
3 \text{Rib} & \frac{1}{\beta} \text{ribitol} 5 \text{PO}_4
\end{align*}
\]

Haemophilus influenzae type b  E. coli K100

Fig. I.6: The structures of capsular polysaccharides of H. influenzae type b and Escherichia coli K100.
A new potential in polysaccharide immunochemistry is provided by homogeneous immunoglobulins that bind carbohydrate polymers. Here, the specific immunoglobulin-hapten interaction can be studied in detail. The oligosaccharides obtained by bacteriophage degradation of bacterial surface carbohydrates may be coupled to the protein carriers serving as immunogens, representative of the corresponding bacterial glycans. Bacteriophage-induced degradation of capsular polysaccharides is discussed in Section VI of this thesis.

Polysaccharides on the surface of a microbial cell are the serological determinants of that organism and therefore represent a highly specific means of identification. Their study by chemical and genetic approaches, as well as the investigation of their biosynthetic pathways, are essential for our understanding of bacterial pathogenicity.

In the course of this thesis, the structures of Klebsiella K50 polysaccharide and two Escherichia coli polysaccharides, serotype K28 and K32, were investigated. Two Escherichia coli capsular polysaccharides (K28 and K32) were degraded by their respective bacteriophages (φ28-1, φ28-2 and φ32) and the degradation products were characterized.
CHAPTER II

METHODOLOGY OF STRUCTURAL STUDIES ON POLYSACCHARIDES
II. METHODOLOGY OF STRUCTURAL STUDIES ON POLYSACCHARIDES

An almost infinite variety of structural types is possible among polysaccharides. Each sugar residue can exist in the pyranose or furanose form, each glycosidic linkage may have the α- or β-configuration and the glycosidic linkage may involve substitution of different hydroxyl groups in an adjacent sugar residue.

Chemical methods of determination of the structure of polysaccharides involve: 1) qualitative and quantitative estimation of the sugar constituents; 2) analysis for removable substituents (O-acetyl, N-acetyl, phosphate, etc.); 3) determination of the linkage configuration; 4) determination of the position of linkage; 5) determination of the sugar sequence.

It is obvious that no one structural method can give answers to all these questions. In the following section an attempt is made to describe each of the known structural methods together with some of its limitations.

II.1 ISOLATION AND PURIFICATION^{44-60}

One of the most important and difficult steps in the investigation of polysaccharides is their purification and fractionation into homogeneous individual polysaccharides.
The simplest extraction methods for polysaccharides are those using water alone at various temperatures. Some polysaccharides can be brought into solution by the use of polar nonaqueous solvents, such as dimethyl sulfoxide for starch and glycogen or N-methylmorpholine N-oxide for cellulose. Dilute alkali has been used for polysaccharide extractions, but one has to be aware of possible base-catalyzed degradation. Extraction of polysaccharides under acidic conditions is normally avoided due to the possible cleavage of glycosidic bonds. In order to obtain the carbohydrate portion of glycoproteins, extensive digestion with protease of low specificity can be performed. Mucopolysaccharides, lipopolysaccharides and nucleic acids can be precipitated from aqueous solution by adding liquid phenol.

The next step involves resolution of a polysaccharide mixture into its components. Methods for fractionation of polysaccharides fall into three broad categories: 1) those based on selective precipitation of polysaccharide themselves (by addition of a water-miscible solvent such as acetone or ethanol) or of their salts (precipitation of acidic polysaccharides with potassium chloride, cupric acetate or sulfate, or with cationic detergents such as cetyltrimethylammonium bromide (Cetavlon)); 2) those based on formation of complexes (use of Fehling solution for mannans, borate for galactomannans, barium hydroxide for gluco- and galactomannans); 3) those based on chromatographic procedures (gel filtration or molecular sieve chromatography, ion-exchange chromatography, affinity chromatography).
The practical problem is to establish the purity of a polysaccharide or rather to demonstrate the absence of heterogeneity by as many independent criteria as possible. This includes demonstration of constancy in chemical composition (based on sugar ratios; spectroscopic examination by nuclear magnetic resonance spectroscopy) and physical properties (optical rotation; molecular weight determination by gel permeation chromatography\textsuperscript{58}; electrophoresis\textsuperscript{59,60}).

II.1.1 *Klebsiella* polysaccharides\textsuperscript{15,61,62}

A culture of *Klebsiella* K50, obtained from Dr. Ida Ørskov, was grown as previously described.\textsuperscript{61,62} Briefly, bacterial culture was streaked on agar plates and grown at 37° until large, individual colonies were obtained. Bacteria were grown by inoculation of beef-extract medium and incubation at 37° until a definite growth was observed (usually 3-5 h). The liquid culture thus obtained was incubated on a tray of sucrose-yeast extract agar for 3 days. The lawn of capsular bacteria produced was harvested and the bacteria were destroyed with 1% phenol solution. The dead cells were spun down by ultra-centrifugation and the polysaccharide was precipitated from the supernatant with ethanol. The precipitate was dissolved in water and treated with Cetavlon (cetyltrimethylammonium bromide) to precipitate the acidic polysaccharide. Further purification involved dissolution in 4M sodium chloride, reprecipitation into ethanol, dissolution in water and dialysis. The dialyzed solution was freeze-dried to yield purified capsular polysaccharide.
II.1.2 *Escherichia coli* polysaccharides

Cultures of *Escherichia coli* K28 and K32 from Dr. I. Ørskov (Copenhagen) were grown on Mueller-Hinton agar with the addition of sodium chloride (5% w/v). Each tray was layered with an actively growing liquid culture of *E. coli* bacteria, obtained by inoculation of Mueller-Hinton broth with a single fresh colony of *E. coli* and further incubation of the resulting solution for 5 h at 37°. The trays were left in the incubator for 5 d. The purification procedure was carried out as previously described for *Klebsiella* polysaccharides.

II.2 SUGAR ANALYSIS

II.2.1 Total hydrolysis and methanolysis

Determination of the chemical composition of a polysaccharide involves an initial acid hydrolysis into constituent monosaccharides. All sugars are, to some extent, degraded by acid. Thus, the conditions of hydrolysis must be carefully chosen and controlled. Dutton\(^\text{63}\) reviewed the advantages and disadvantages in the use of different acids.

Hydrochloric acid is commonly employed for glycoproteins.\(^\text{64}\) Trifluoroacetic acid was first used by Albersheim and co-workers\(^\text{65}\) for hydrolysis of plant cell-wall polysaccharides and it has since been widely used for hydrolysis of other polysaccharides. It is volatile and thus readily removed. Aldose-containing polysaccharides can be completely hydrolyzed with minimum loss of sugar with 2M trifluoroacetic
acid at 100°C for 6-8 h. However, other sugars, such as 2-deoxyaldoses, ketoses including sialic acids, and anhydro sugars are largely destroyed under these conditions. These sugars can be completely released under extremely mild conditions, for example, sialic acid with 0.025-0.05M sulfuric acid at 80° for 1 h. Sialic acids may be stabilized also by methanolysis, giving methyl glycoside methyl esters.

Incomplete hydrolysis is encountered with uronic acids and 2-amino-2-deoxyglycosidic linkages. In the case of uronic acids, that might be of an advantage, since such resistance to hydrolysis permits isolation of uronic acid-containing oligosaccharides as partial hydrolysis products.

The reduction of a hexouronic acid to a hexose residue prior to hydrolysis with a water-soluble carbodiimide and sodium borohydride overcomes this difficulty (see later). A technique, developed in this laboratory, involves use of methanolysis for uronic acid-containing polysaccharides. According to this method, the polysaccharide is first treated with methanolic hydrogen chloride which, together with the cleavage of glycosidic bonds, causes an esterification of uronic acid residues. Treatment with sodium borohydride in anhydrous methanol reduces the uronic esters to the corresponding alcohols. The mixture of methyl glycosides is then hydrolyzed with 2M trifluoroacetic acid (TFA) to give the neutral sugars which are converted into alditol acetates and analyzed by gas-liquid chromatography (g.l.c.).
II.2.2 Characterization and quantitation of sugars

The characterization of the sugars formed on hydrolysis is the starting point in an investigation of the structure of a polysaccharide. The conventional techniques involve paper chromatography, thin layer chromatography and paper electrophoresis. Analyses of certain broad classes of sugars can be performed spectrophotometrically.

Gas-liquid chromatographic methods involve formation of suitable volatile derivatives (see later). Increasingly, high performance liquid chromatography (HPLC) columns are being developed for the separation and quantitative analysis of sugars.

II.2.3 Carboxyl reduction of acidic polysaccharides

The resistance of glycosiduronic acids to complete hydrolysis creates difficulties in compositional and structural analysis of polysaccharides. The best approach is to reduce uronic acids to the corresponding hexose residues and then to carry out the investigation on the carboxyl-reduced polysaccharide. Taylor and Conrad have developed a method in which the acidic polysaccharide in aqueous solution is treated with a water-soluble carbodiimide to give O-acylisoureia, which is then reduced with sodium borohydride. Both stages in this reaction require careful pH control (see Scheme II.1).

The alternative method involves reduction of a permethylated polysaccharide, usually with lithium aluminum hydride in tetrahydrofuran.
Scheme II.1: Reduction of carboxylic acid in aqueous solution using carbodiimide reagent.
or similar solvent. This reagent, however, is not suitable for the reduction of acid groups in unsubstituted polysaccharides because of their insolubility in ether-type solvents. In this case methyl esters may be formed by treatment of the acidic polysaccharide with diazomethane and then reduction of the ester groups using sodium borohydride in aqueous solution.

II.2.4 Determination of the configuration (D or L) of sugars

In general, chromatographic separation methods and spectroscopic analyses do not distinguish between enantiomers. Enantiomeric differentiation of sugars can be achieved on milligram quantities by circular dichroism of alditol acetates or the partially methylated alditol acetates.

Recently, for even smaller amounts of material and for mixtures of sugars, enantiomers have been distinguished by conversion to equilibrium mixtures of glycosides of chiral alcohols (e.g., (+) or (−)-2-butanol or (+)- or (−)-2-octanol), followed by g.l.c. separation of volatile derivatives such as acetate or trimethylsilyl ethers. In this procedure, enantiomeric sugars form diastereomeric mixtures of derivatives whose chromatographic separations provide a characteristic fingerprint.

For a few sugars enzymatic assay can be used for enantiomeric characterization. For example, the enzyme D-glucose oxidase is used for the quantitative assay of D-glucose in a mixture.
II.3 POSITION OF LINKAGE

II.3.1 Methylation analysis

The technique of methylation analysis is routinely employed in the structural characterization of complex carbohydrates as a means to establish the linkage positions of the constituent monosaccharides. This method is based on the ability to separate and characterize the partially methylated monosaccharides generated via hydrolysis of the fully methylated polysaccharide, which is accomplished by combined gas-liquid chromatography/mass spectrometry of their alditol acetate derivatives. The method, however, gives no information on the sequence or the anomeric nature of the linkages in the polysaccharide.

The aim of methylation is to achieve etherification of all free hydroxyl groups in the polysaccharide. In the original procedure used by Haworth this was achieved by repeated reaction with dimethyl sulfate and sodium hydroxide. The partially methylated product obtained was then treated with silver oxide in boiling methyl iodide, according to Purdie and Irvine, to give fully methylated polysaccharide.

Purdie's method was considerably improved by Kuhn and co-workers who used N,N-dimethylformamide as a solvent in conjunction with methyl iodide and silver oxide. The simplest and most convenient method for methylation of polysaccharides was developed by Hakomori. This method was first applied to capsular polysaccharides by Sandford and Conrad. The polysaccharide is treated with the strong base sodium methylsulfinyl methanide (dmsyl sodium) and methyl iodide is subsequently added to
effect methylation. The Hakomori procedure usually gives complete methylation in one step. If this is not the case complete methylation can be achieved using Purdie's method, since a second Hakomori treatment would result in β-elimination if the polysaccharide contains uronic acid. O-Acyl groups present in many polysaccharides and glycoconjugates are completely cleaved under the strong alkaline conditions, but pyruvic acid acetals are stable. Substitution of the carbohydrate residues by O-acyl groups can be determined using the Prehm methylation procedure, where the polysaccharide is dissolved in trimethyl phosphate and then methylated with methyl trifluoromethanesulfonate and 2,6-di-(tert-butyl)pyridine as proton scavenger. If the substrate contains uronic acids these are transformed into methyl esters. Most "undermethylations" are due to incomplete dissolution of a sample. This situation may be improved by careful de-ionization of a polysaccharide (for example, using Amberlite IR-120 (H⁺) resin). Detailed methylation procedures have been published. Recently, potassium methylsulfinyl methanide has been successfully used in Hakomori methylations. Some polysaccharides, such as cellulose, are insoluble in dimethyl sulfoxide (DMSO) alone. Recently, N-methylmorpholine N-oxide (MMNO) has been shown to dissolve polysaccharides, so that the Hakomori methylation can be carried out in MMNO-DMSO mixtures.

The methylated material is recovered by dialysis (polysaccharide) or by partition between water and chloroform (oligosaccharide). The completeness of methylation is checked by i.r. spectroscopy (absence of hydroxyl absorption at 3600 cm⁻¹) or by analysis of the methoxyl content. The subsequent hydrolysis is performed with 2M trifluoroacetic acid on a
steam bath for 16 h. Polysaccharides containing uronic acids may be carboxyl-reduced before (carbodiimide reduction) or after (lithium aluminum hydride) the permethylation step. Scheme II.2 illustrates a typical reaction sequence.

A recent report by Reinhold and co-workers and Gray and co-workers describes a new technique for determining the structure of polysaccharides, that, potentially, has significant advantages over standard methylation analysis. This method involves the ionic hydrogenation of all glycosidic carbon-oxygen bonds in the fully methylated polysaccharide with triethylsilane catalyzed by boron trifluoroetherate. This reductive cleavage affords a series of partially methylated anhydroalditols, which are subsequently acetylated in situ, and analyzed by g.l.c.-m.s.

II.3.2 Gas-liquid chromatography (G.L.C.)

Gas chromatography is a separation process in which the components to be separated are volatilized and distributed between a moving gas phase and a stationary absorbent phase, which may be a solid (gas-solid chromatography), or a liquid (gas-liquid chromatography) adsorbed on an inert support.

The carbohydrate derivatives used in gas-liquid chromatography must be volatile, yet stable at the operating temperature of the column and must not be adsorbed irreversibly on the stationary phase.

Gas-liquid chromatography of carbohydrates was first performed for methylated methyl glycosides. The discovery of trimethylsilyl
Scheme II.2: Methylation Analysis of Klebsiella K50 polysaccharide
derivatives by Sweeley and co-workers\textsuperscript{96} in 1963 has revolutionized the analysis of carbohydrates by gas-liquid chromatography.

Extensive reviews of the applications of g.l.c. to carbohydrate analysis have been published by Dutton.\textsuperscript{63,97}

Although trimethylsilyl derivatives are readily formed and are volatile, their obvious disadvantage is a multiplicity of peaks due to the isomeric forms present at equilibrium. Separations obtained by Jones and co-workers\textsuperscript{98} with complex mixtures of alditol acetates are superior to those obtained for the corresponding mixtures of the trimethylsilyl derivatives. The carbohydrates present in glycoproteins were quantitatively determined by g.l.c. of their alditol acetates\textsuperscript{99} and this procedure is widely used in the analysis of glycoproteins and oligosaccharides. When the mixture is not too complex, analysis of the trimethylsilyl ethers is preferred, since the reaction requires only 5-15 min.\textsuperscript{100} Jeanes and co-workers\textsuperscript{101} investigated several column packings, and found that an organosilicone polyester (ECNSS-M) gives good separation of acetates of common alditols. However, the maximum operating-temperature of this column is rather low (200°) and SP-2340 (75% cyanopropyl silicone) is the stationary phase of choice for analysis of alditol acetates and it was used in this study. To determine the degree of polymerization of an oligosaccharide and to identify the reducing sugar, peracetylated aldonitriles are used.\textsuperscript{102}

Methylation analysis is an important method in structural polysaccharide chemistry. G.l.c. offers the best method for the separation and quantitation of the methylated sugars obtained on hydrolysis of a methylated polysaccharide or glycoconjugate. Partially methylated
alditol acetates have been used extensively in the methylation analysis. One advantage of using alditol acetates is that each aldose derivative will give only one peak on g.l.c. Another advantage of partially methylated alditol acetates is that the quantitation can be performed without the use of response factors (within ±5%) except for the analysis of N-acetamido sugars. The best separations of partially methylated alditol acetates are obtained on medium-polar columns such as ECNSS-M or OV-225 (a silicon polymer containing methyl, phenyl and nitrile groups). OV-225 is usually preferred for routine work because of its thermal stability. A manual on methylation analysis, giving relative retention times on gas-liquid chromatography of partially methylated alditol acetates as well as a collection of computer-drawn bar graph mass spectra of these derivatives has been published. Albersheim and co-workers have reported relative retention times for numerous partially ethylated alditol acetates. They can be used to resolve some of the polysaccharide components that are co-eluted as their partially methylated alditol acetates.

The identification of methylated sugars obtained on methylation analysis by g.l.c. may be confirmed by g.l.c. on other columns and the substitution pattern can be determined unambiguously by g.l.c.-mass spectrometry (see later).

The application of HPLC (high performance liquid chromatography) to carbohydrate analysis was concentrated originally on the quantitative analysis of sugars in food and beverages. Recently, Albersheim and co-workers have applied HPLC to the separation of mixtures of small amounts of peralkylated oligosaccharide alditols. Thus sequencing
complex carbohydrates by formation, separation and characterization of peralkylated oligosaccharide alditols has developed into a powerful method of structural analysis.

II.3.3 Mass spectrometry

Mass spectrometry (m.s.) has become an important and versatile tool in the structural analysis of carbohydrates.

Mass spectrometry is based on the ionization of compounds when they are bombarded with a beam of electrons to form positive molecular ions which subsequently may break down to smaller fragments. The relative peak intensity is proportional to the number of ions of the appropriate m/z (mass-to-charge ratio) value. The number of ions depends on their stability.

Carbohydrate derivatives give weak or no molecular ions on electron impact (e.i.) mass spectrometry. Molecular weights may more readily be determined by field ionization (f.i.), field desorption (f.d.) or chemical ionization (c.i.) techniques.

Since underivatized mono- and oligosaccharides are thermally unstable and practically non-volatile, they are converted into more volatile derivatives such as methyl or trimethylsilyl ethers, acetates, trifluoroacetates or alkylidene derivatives.

The use of combined g.l.c.-mass spectrometry, in which the components from the chromatographic column are introduced directly into the ionization chamber of the mass spectrometer, has led to new methods
for the qualitative and quantitative analysis of the mixtures of methylated sugars. 82

Alditol derivatives exhibit the simplest patterns for fragmentation of carbohydrate molecular ions. The mass spectra of the acetates, methyl ethers, and trifluoroacetates of alditols display primary fragments corresponding to all principal scissions of the compound (see Scheme II.3). 107

A systematic investigation of mass spectra of partially methylated alditol acetates by Lindberg and co-workers has led to the following generalizations. 82

1. Derivatives with the same substitution pattern give very similar mass spectra, typical of that substitution pattern.
2. The base peak of the spectrum is generally m/z 43 (CH₃C — O).
3. Fission between a methoxylated and an acetoxyalted carbon is preferred over fission between two acetoxyalted carbons.
4. When the molecule contains two adjacent methoxylated carbons, fission between them is preferred over fission between one of these and a neighboring acetoxyalted carbon.
5. Secondary fragments are formed from the primary fragments by single or consecutive loss of acetic acid (m/z 60), ketene (m/z 42), methanol (m/z 32), or formaldehyde (m/z 30).

This information was used to identify labelled compounds for which standard spectra were not available. During the structural investigation on Klebsiella K50 capsular polysaccharide the position of
Scheme II.3: The mass spectra of the acetates (R=Ac), methyl ethers (R=Me), and trifluoroacetates (R=COCF₃) of alditols. Only primary fragments are shown.
Linkage between D-glucuronic acid and D-mannose was determined by uronic acid degradation followed by labelling with ethyl iodide. The ethylated, partially methylated alditol acetate (1,5-di-O-acetyl-3-O-ethyl-2,4,6-tri-O-methylmannitol) was obtained and analyzed by means of g.l.c.-m.s. Several masses are shifted by 14 units as illustrated in Figure II.1.

On reduction some pairs of methylated sugars (e.g. a 3-O-methyl- and a 4-O-methyl hexose) give alditols with the same substitution pattern. The loss of information when sugars are reduced to alditols can be readily prevented if the reduction is performed with sodium deuterioborate. This labelling technique, when used during reduction of the uronic acid residues to neutral sugars, permits one to distinguish the neutral sugar residues obtained from other sugar residues present in the same polysaccharide.

G.l.c.-m.s. of partially methylated alditol acetates is now widely used in conjunction with the methylation analysis of polysaccharides and other materials containing carbohydrates. For materials having only one sugar of any one class (such as pentose, hexose, or 6-deoxyhexose), the identification of the components by m.s. is unambiguous.108

If, however, the polysaccharide contains different sugars of the same class an identification of the different methylated sugars can be accomplished only if the relative retention times (T- values) of the relevant alditol acetates are sufficiently different.

Aldononitrile acetates are suitable for analysis of sugars by g.l.c. and give characteristic mass spectra that are easy to interpret.
a) 1,5-di-\(\beta\)-acetyl-3-\(\beta\)-ethyl-2,4,6-tri-\(\beta\)-methyl-D-mannitol

b) 1,5-di-\(\beta\)-acetyl-2,3,4,6-tetra-\(\beta\)-methyl-D-mannitol

Fig. II.1: Mass spectrum of a uronic acid degradation derivative from K50 polysaccharide (a) compared to the spectrum of a standard derivative (b).
Partially methylated aldononitrile acetates have been investigated by Kochetkov and co-workers. The mass-spectrometric behavior of per-methylated disaccharides having (1 → 2)-, (1 → 4)-, and (1 → 6)-linked hexose residues has been investigated by Kochetkov and co-workers. It was found that the fragmentation of both moieties of the disaccharide follows principles similar to those for permethylated glycosides. The nomenclature for the different fragmentation series of permethylated glycosides was introduced by Chizhov and Kochetkov and modified later by Kováčik and co-workers. The nomenclature is exemplified for the degradation of a disaccharide methyl glycoside (see Scheme II.4).

![Scheme II.4: The A-series of fragments for the degradation of a disaccharide methyl glycoside](image)

The A-series of fragments serves to establish the molecular weights of the disaccharide and its component sugar residues. The B-series of fragments obtained by degradation of ring b can be used to establish the nature of the linkage between two sugar residues.
The separation and structural analysis of 21 permethylated tri-saccharides by gas-liquid chromatography-mass spectrometry (g.l.c.-m.s.) was described by Kärkkäinen. The position of the glycosidic linkage next to the reducing end of straight-chain trisaccharides can generally be established by m.s., whereas differentiation of (1→6)- and (1→4)-linkages next to the non-reducing end may require previous knowledge of the stereochemistry of the sugar units.

In recent years, m.s. procedures have found a wider application in the structure determination of oligosaccharides and glycolipids having high molecular weights. This has been made possible largely as a result of instrumental developments allowing high sensitivity at high mass, and improvements in sample handling. Electron ionization mass-spectrometry (e.i. m.s.) of these high-mass substances suffers, however, from some limitations, namely, the loss in signal intensity at high mass, which results in the absence of the molecular ion and high-mass sequence ions. The method can be improved by combining e.i. m.s. with the results of field-desorption (f.d.) studies. However, the technical difficulties associated with f.d. m.s. have limited its use. The development of fast-atom-bombardment mass spectrometry (f.a.b.m.s.) has been of considerable importance for carbohydrate-structure elucidation. F.a.b. spectra are relatively easy to acquire, and early reports have shown that f.a.b.m.s. is capable of obtaining both molecular weights and fragment data from small quantities of polar, biological substances, including unmodified carbohydrates and glycolipids.
II.4 SEQUENCING OF SUGARS

II.4.1 Partial hydrolysis

Partial hydrolysis followed by characterization of the product(s) is often used in structural carbohydrate chemistry. The method is of particular value when a polymer contains a limited number of acid-labile glycosidic linkages, which may be cleaved without significant hydrolysis of other glycosidic linkages. It is advisable, therefore, to perform some pilot experiments in order to determine optimal conditions for the partial acid hydrolysis. Partial hydrolysis may also be combined with methylation analysis of the resulting oligosaccharides. Many factors seem to influence the rate of hydrolysis, including the ring size, configuration, conformation, and polarity of the sugar as well as the size and polarity of the aglycon. Hence, it is often impossible to point to a single factor that explains the differences in hydrolysis rates between two glycosides. Capon has reviewed the rate constants for the acid-catalyzed hydrolysis of a large number of glycosides. In general, furanosides are hydrolyzed more readily than pyranosides, deoxyglycopyranosides are more acid labile than glycopyranosides, and aminosugars and uronic acids are relatively resistant to hydrolysis. Graded hydrolysis of uronic acid-containing polysaccharides leads to isolation of acidic disaccharides (aldobiouronic acids) and higher oligosaccharides.

The effect of the type of linkage on the rates of hydrolysis was studied for the D-glucose disaccharides. In all cases, except for
the \((1 \rightarrow 6)\)-linked disaccharides, the \(\alpha\)-D-linkage was more readily hydrolyzed than the \(\beta\)-D-linkage. For different types of linkages in polysaccharides, the rates of hydrolysis seem to parallel the rates of hydrolysis of disaccharides: \((1 \rightarrow 3)\)-linkages are hydrolyzed faster than \((1 \rightarrow 4)\)- and \((1 \rightarrow 2)\)-linkages with \((1 \rightarrow 6)\)-linkages being most resistant. The rate of hydrolysis depends on the location of the linkage within the polysaccharide chain. There is a higher rate of hydrolysis of non-reducing terminal and side-chain bonds, as compared with the main in-chain bonds.\(^{118}\)

A number of alternative procedures may be used for the acid-catalyzed cleavage of glycosidic linkages, including acetolysis,\(^{120}\) methanolysis, and mercaptolysis. The latter two techniques are used mainly in the structural analysis of the sulfated polysaccharides from seaweeds.\(^{121}\)

The usefulness of acetolysis is that it is complementary to acid hydrolysis. In the former, \((1 \rightarrow 6)\)-linkages are the most susceptible to attack; in the latter, they are the least easily ruptured.\(^{120}\) The mixture of mono, di, and higher oligosaccharides formed on partial hydrolysis may be fractionated by a variety of chromatographic procedures, including paper chromatography, gel-permeation chromatography, paper electrophoresis, ion-exchange chromatography of acidic oligosaccharides, gas-liquid chromatography, and high performance liquid chromatography.

Liquid hydrogen fluoride has been successfully used in partial hydrolysis of polysaccharides containing amino sugars. Mort and Lamport\(^{122}\) found that hydrogen fluoride could cleave sugars from
glycoproteins, leaving the peptide moiety intact. They also observed a large difference in the rate of cleavage of glycosidic linkages of amino sugars and neutral sugars in hydrogen fluoride at 0°: the neutral sugar linkages could be broken while leaving those of amino sugars intact.122 Recently, Mort123 reported that in hydrogen fluoride at subzero temperatures, differential cleavage of linkages of neutral and acidic sugars can be obtained, and Knirel et al.124 found differential cleavage of amino sugar linkages at 25°. The method is of particular advantage for production of larger oligosaccharides containing O-acyl groups.125

Another way of isolating oligosaccharides containing acid-labile components, is to use the bacteriophage-induced depolymerization of polysaccharides (see later).

In the present study partial hydrolysis was used in the structural investigations of Klebsiella K50 and Escherichia coli K28 polysaccharides. For K50, di-, tri- and tetrasaccharides were isolated; for E. coli K28, an aldobiouronic acid and neutral disaccharide were isolated.

II.4.2 Periodate oxidation126-138

Glycol-cleaving reagents, especially periodic acid and its salts, and lead tetraacetate have found widespread applications in carbohydrate chemistry. Lead tetraacetate126 had been little used in studies on polysaccharides, because they are insoluble in the solvents generally used for such oxidations.
Treatment of glycol groups with periodic acid and its salts results in cleavage of carbon bonds and the formation of two aldehydic groups, one molecular proportion of periodate being reduced. In general, open chain glycols are most readily oxidized, followed by cyclic cis-glycols; cyclic trans-glycols are more slowly oxidized, or not oxidized at all if fixed in an unfavorable conformation (bicyclic anhydrohexoses). The reactivity may also be affected by the steric effects of neighboring groups.

Smith and co-workers have studied the products obtained from polysaccharides after periodate oxidation, borohydride reduction and hydrolysis. Hexose residues substituted in the 4-position give erythritol or threitol together with glycoaldehyde, while terminal and 6-substituted residues give glyceritol and glycoaldehyde, 2-substituted residues give glyceritol and glycerose, and 3-substituted residues afford the intact hexose (see Fig. II.2).

Where three adjacent hydroxyl groups are present, a double cleavage of the carbon chain occurs with formation of two aldehydic groups, the reduction of two molecular proportions of periodate and the liberation of one molecular proportion of formic acid.

Periodate oxidation studies can be, therefore, used for linkage analysis, determination of degree of polymerization and chain length of polysaccharides (by measuring the proportion of formic acid or formaldehyde released using the oxidation), and degree of branching. Smith and co-workers have given the general procedures used in the oxidation of polysaccharides. The reaction is normally carried out in the dark at 5°, either in distilled water or in buffer within pH 4-5 to avoid acid
<table>
<thead>
<tr>
<th>Terminal and mono-substituted hexoses</th>
<th>Number of molecules of $\ce{IO4^-}$</th>
<th>Products formed after oxidation, $\ce{NaBH4}$ reduction and hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure1" /></td>
<td>2</td>
<td><img src="image2.png" alt="Products1" /></td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure2" /></td>
<td>2</td>
<td><img src="image4.png" alt="Products2" /></td>
</tr>
<tr>
<td><img src="image5.png" alt="Structure3" /></td>
<td>1</td>
<td><img src="image6.png" alt="Products3" /></td>
</tr>
<tr>
<td><img src="image7.png" alt="Structure4" /></td>
<td>0</td>
<td><img src="image8.png" alt="Products4" /></td>
</tr>
<tr>
<td><img src="image9.png" alt="Structure5" /></td>
<td>1</td>
<td><img src="image10.png" alt="Products5" /></td>
</tr>
</tbody>
</table>

**Fig. II.2:** Common products formed on periodate oxidation, followed by borohydride reduction and hydrolysis of terminal and mono-substituted hexoses
hydrolysis and to minimize side-reactions involving non-selective oxidations.

Because of the marked difference in stability between true acetals and glycosides, it is possible by mild acid hydrolysis to cleave the acetal linkages in polyalcohols resulting from periodate oxidation and borohydride reduction of polysaccharides, and to leave any glycosidic linkages intact. This important modification of the periodate oxidation, devised by Smith and his co-workers,\textsuperscript{131} gives valuable structural information on the fine structure of the parent polysaccharide. Depending on the relative location of the periodate-resistant sugar residues, the degradation may result in formation of glycosides of mono- or oligo-saccharides, or degraded polysaccharides, which can be subjected to repeated Smith degradation.

A selectivity in the acid hydrolysis step is sometimes difficult to achieve, since hydrolysis of normal glycosidic bonds might occur together with the removal of cleaved fragments. A modification of the Smith hydrolysis, introduced by Lindberg and co-workers,\textsuperscript{132} involves methylation of the reduced oxidized polysaccharide ("polyalcohol") prior to acid hydrolysis, which ensures complete removal of glycoaldehyde fragments from oxidized residues.

Periodate oxidation is complicated by both under- and over-oxidation. Painter and Larsen\textsuperscript{133,134} have demonstrated that the oxidation is incomplete in certain cases due to the formation of inter-residue hemiacetals between the aldehyde groups of oxidized hexuronic acid residues and the closest hydroxyl groups of unoxidized residues. Those residues protected through the hemiacetal can be exposed to oxidation by first
subjecting them to borohydride reduction (see Fig. II.3). Incomplete oxidation may occur due to electrostatic repulsions between the periodate ions and weakly acidic groups (-COOH) of acid polysaccharides.\textsuperscript{135} This effect is suppressed by adding a salt (0.2M sodium perchlorate).\textsuperscript{135} Over-oxidation\textsuperscript{136} is minimized if the reaction is carried out in the dark, at low temperatures, without great excess of reagent and at pH 3.6-4.5, so that the formyl ester initially formed is not hydrolyzed at a significant rate.

During the present study on the structure of \textit{Escherichia coli} K32 the periodate oxidation conducted on the original polysaccharide at room temperature and in unbuffered sodium metaperiodate (NaIO\textsubscript{4}) yielded a 46\% over-oxidation of 3-linked \(\alpha\)-galactose, due to the partial hydrolysis of the rhamnosyl bond (see experimental). When the oxidation was performed in buffered periodate solution (pH 4.5), 3-linked galactose was recovered quantitatively.

Recently, kinetic studies carried out by Painter and co-workers\textsuperscript{137} have shown that after a limited period of oxidation the \(\beta\)-D-galactopyranosyl side groups in a polysaccharide could be selectively removed by Smith degradation, leaving the 1,4-linked residues in the main chain intact. Numerous possibilities for selective oxidation can be demonstrated by comparison of second-order rate coefficients for periodate oxidation of various methyl glycosides.\textsuperscript{137} Selective oxidation of the terminal \(\alpha\)-rhamnosyl residue was first demonstrated in the structural investigation of \textit{Klebsiella} serotype K17 polysaccharide.\textsuperscript{138}

A typical sequence of periodate oxidation and Smith hydrolysis is shown in Scheme II.5 for \textit{Klebsiella} K50 capsular polysaccharide.
Fig. II.3: Sequential periodate oxidation (0.025 M NaIO₄, 20°) and borohydride reduction of alginate. At the points labelled R, samples were reduced with sodium borohydride and then oxidized further (curves B and C). From ref. 133.
Scheme II.5: Smith degradation of Klebsiella K50 capsular polysaccharide
II.4.3 Base-catalyzed degradation\textsuperscript{139-146}

In general, glycosidic linkages are stable under alkaline conditions. However, base-catalyzed $\beta$-eliminations can be initiated by strongly electron-withdrawing functional groups with consequent cleavage of glycosidic linkages. Groups that may act in this manner include the carbonyl groups of reducing residues, activated hexuronic acid derivatives (esters), carbonyl groups introduced by oxidation at selected sites, and sulfone groups formed in structural modifications.\textsuperscript{139}

Reducing sugars undergo a number of competing reactions when treated with base. The reaction involves base-catalyzed $\beta$-elimination (normally in aqueous solution) with formation of 3-deoxyhex-2-eno-pyranoses. This elimination occurs most easily when there is a 3-O-substituent to provide a good leaving group. The alkaline degradation of polysaccharides is known as a "peeling" reaction.

Base-catalyzed degradations from hexuronic acid residues occur when the uronic acid is esterified and 4-O-substituted and result in the $\beta$-elimination of the 4-O-substituent with the formation of hex-4-eno-pyranosiduronate residues. The most widely used reaction sequence is that developed by Lindberg and co-workers.\textsuperscript{140,141}

The main steps of this degradation are outlined as follows:
The unsaturated product is labile to acids and on mild hydrolysis with acid releases the aglycon \((R_1\text{OH})\) that further yields the furan with the simultaneous release of the substituents at \(O-2\) and \(O-3\). When \(R_4\text{OH}\) is a single residue or chain of sugar residues, a second \(\beta\)-elimination reaction occurs, and the next sugar may be released on the subsequent mild acid treatment. If \(R_4\text{OH}\) is an aldose substituted at \(O-3\), further degradation of the polysaccharide will occur during the treatment with alkali.

In an alternative procedure\(^{143}\) exposed reducing groups are simultaneously protected by acetylation with acetic anhydride if degradation is performed using the organic base 1,5-diazabicyclo[5.4.0]undec-5-ene (DBU).
Later experiments have shown that, under conditions normally used for base degradations, complete loss of uronic acid residues occurs and that the acid hydrolysis is unnecessary.\textsuperscript{144}

The nature of residues released during the degradation is revealed by further alkylation with trideuteriomethyl iodide or ethyl iodide, hydrolysis, and analysis of the resulting sugars, as alditol acetates, by gas-liquid chromatography - mass spectrometry (g.l.c.-m.s.). Comparison of this analysis with the methylation analysis of the original polysaccharide reveals the site of attachment of uronic acid units.

The degradative sequence developed by Svensson and co-workers\textsuperscript{145} involves selective oxidation of the exposed hydroxyl groups in the methylated polysaccharide (either by base-catalyzed uronic acid degradation or by selective hydrolysis of acid-labile glycosidic linkages such as pyruvate), alkaline degradation and mild acid hydrolysis of enol glycosidic bonds. Specific oxidation is effected with chlorine-DMSO.\textsuperscript{146}

Scheme II.6 shows a typical reaction sequence for $\beta$-elimination of \textit{Klebsiella} K50 polysaccharide.

\section*{II.5 DETERMINATION OF LINKAGE}

\subsection*{II.5.1 Optical rotation\textsuperscript{147-149}}

Optical rotation can be used to determine the anomeric configuration of sugar residues in oligo- and polysaccharides.
Scheme II.6: Uronic acid degradation of *Klebsiella* K50 polysaccharide
Although optical activity is one of the physical properties of carbohydrates most often measured, it probably is more complex and less understood than any other. The simplest approach involves application of Van't Hoff's Principle of Optical Superposition,\textsuperscript{147} which proposes the additivity of the rotational contributions of different asymmetric centers in a complex molecule. It was found by Hudson\textsuperscript{148} to be applicable to the determination of configuration at the anomeric center of the free sugars and glycosides. Application of Hudson's Isorotation Rules gives information only on overall molecular rotation of poly- and oligosaccharides. The contributions of O-acyl and pyruvate groups to the total molecular rotation are neglected.

The molecular rotation (M) is defined:

\[
M = \frac{[\alpha] \times \text{M.W.}}{100}
\]

where, \([\alpha]\) - specific rotation
\text{M.W.} - molecular weight

Normally, the optical rotation is measured at the D-line of sodium (589 nm). Using Hudson's Isorotation Rules one can predict specific rotation of oligosaccharides and polysaccharides\textsuperscript{149} using the molecular rotation values of model methyl glycosides.
\[
[a] = \frac{\Sigma M_l \times 100}{\text{M.W.}} 
\]
where, \( \Sigma M_l \) - sum of molecular rotation values of model methyl glycosides

M.W. - molecular weight of polysaccharide.

II.5.2 Nuclear magnetic resonance spectroscopy

II.5.2.1 \(^1\text{H-}n.m.r.\) spectroscopy\(^{150-60}\)

Proton magnetic resonance spectroscopy offers to carbohydrate chemists a method both for determining the configuration of unknown carbohydrates and for ascertaining the conformations of known carbohydrates in solution.\(^{150}\)

The fundamental work of Lemieux and co-workers introduced the successful application of \(^1\text{H-}n.m.r.\) spectroscopy to structural problems in the carbohydrate field.\(^{151}\) An instrumental development of considerable importance to \(p.m.r.\) spectroscopy of carbohydrates has been the introduction of high-resolution magnets based on superconducting solenoids.\(^{152}\) The most significant advance in \(n.m.r.\) spectroscopy since 1964 has been the development of Fourier-transform techniques which afford a large enhancement in sensitivity.\(^{153}\) The use of a high-field spectrometer is especially beneficial for \(^1\text{H-}n.m.r.\) spectroscopy of polysaccharides with regular repeating units. Numerous examples of this
type of application are found in studies on capsular polysaccharides of
*Klebsiella*. These polysaccharides and a variety of fragments prepared
from them (by partial or enzymatic hydrolysis, Smith degradation) were
examined by $^1$H- and $^{13}$C-n.m.r. spectroscopy in combination with chemical
methods, and unique structures were determined.$^{154}$

High-field $^1$H-n.m.r. spectroscopy has been utilized extensively
in determining the structures of glycoproteins.$^{155}$ Two-dimensional
(2-D) homo- and heteronuclear n.m.r. methods have been used as an aid in
the assignment of the proton spectra of oligosaccharide moieties of
glyco-conjugates.$^{156}$

Applications of p.m.r. spectroscopy to problems of carbohydrate
chemistry involve measurement of four parameters.

(i) **Relative intensities of the signals**

The property that, under proper operating conditions, the rela-
tive intensities of absorption signals for different hydrogens are equal
to the relative numbers of the hydrogens producing the signals, has been
particularly important to analytical carbohydrate chemistry.$^{157}$ The
number of anomeric linkages, relative amounts of 6-deoxy sugars,
O-acetyl, N-acetyl and 1-carboxyethylidene substituents can be determin-
ed. For monosaccharides it also permits a rapid quantitative analysis
of the proportions of anomers, including furanose and pyranose forms.

(ii) **Coupling constants**

Nuclear spin-spin coupling constants are designated by J and are
expressed as hertz (Hz). When a first-order spectrum is observed, the
magnitudes of the coupling constants may be determined directly from the spectrum.

The relationship between the vicinal coupling constant (J) and the dihedral angle (\(\phi\)) between protons is given approximately by the Karplus equation.\(^{158}\)

\[
3J(H_1, H_2) = \begin{cases} 
8.5 \cos^2 \phi - 0.28, & 0^\circ < \phi < 90^\circ \\
9.5 \cos^2 \phi - 0.28, & 90^\circ < \phi < 180^\circ 
\end{cases}
\]

The values are maximum when the dihedral angle (\(\phi\)) is 0° or 180°, and minimum when it is 90°.

One of the most important consequences of the Karplus equation is that the order of magnitude of diaxial, axial-equatorial and diequatorial coupling constants (Jaa, Jae and Jee respectively) in a cyclohexane ring chair system can be predicted and is in reasonable agreement with the observed values. This is shown in Fig. II.4. In carbohydrate chemistry, the determination of \(3J(H,H)\) values has been used to establish configuration as well as conformational preferences for pyranose, furanose and acyclic sugars.

(iii) **Chemical shift**

The chemical shifts of protons are strongly dependent upon substitutional, orientational and electronegativity effects of neighboring and distant groups.\(^{157}\) The early observations that the chemical shift of a proton depends on its environment in the molecule was demonstrated in 1958 by Lemieux and co-workers.\(^{151}\) Thus, equatorial
Fig. II.4: Relationship between dihedral angle ($\phi$) and coupling constants for $\alpha$- and $\beta$-D-hexoses

$\beta$-D

$\alpha$-D

$\beta$-D

$\alpha$-D

$J_{12} \approx 7.9 \text{ Hz}$

$J_{12} \approx 1.3 \text{ Hz}$

$R^1 = H$

$R^2 = H, OH$
ring-hydrogen atoms had lower chemical shifts than their axial counterparts. The most important direct shielding effect in carbohydrates is that of the ring-oxygen atom, which causes the characteristic, low-field shift of the anomeric hydrogen atom.\textsuperscript{150}

In the spectrum of an oligosaccharide or a polysaccharide, three main regions can be observed: a) the anomeric region (\(\delta 4.5-5.5\)), b) the ring proton region (\(\delta 3.0-4.5\)) and c) the high field region (\(\delta 1.15-2.5\)) where CH\(_3\) groups of 6-deoxy sugars, pyruvates, O-acetyl, N-acetyl, etc. can be observed (see Fig. II.5).

The configuration at the acetal carbon atom of pyruvic acid acetals present in some extracellular bacterial polysaccharides has been investigated by Garegg and co-workers. The chemical shifts\textsuperscript{159} for the methyl groups of the pyruvic acid acetal differ significantly depending upon whether these groups are axial or equatorial.

The ring protons are usually difficult to assign except for cases where specific protons resonate at lower field (H-5 of glucuronic acid and galacturonic acid, H-5 of fucose, H-2 of mannose, etc.).

Two factors dominate the acquisition of high-resolution \(^1\text{H-n.m.r.}\) spectra of polysaccharides, interference by exchangeable protons (O-H, N-H) and line broadening of signals.\textsuperscript{154} The preparation of aqueous solutions of polysaccharides involves a prior exchange treatment with deuterium oxide (preferably 99.95 atom %) and the use of deuterium oxide as a solvent. Nevertheless a strong peak due to residual water (HOD signal) is often obtained. The chemical shift of the HOD signal at room temperature (\(\delta \sim 4.8\) p.p.m.) interferes with the anomeric region and it is close to the H-1 signal of \(\beta\)-glycopyranosyl residues. By raising the
Fig. II.5: Schematic representation of different regions in the $^1$H-n.m.r. spectrum of polysaccharides
temperature one can displace the HOD signal upfield. There are a number of FT techniques for minimizing the interference by the HOD signal.\textsuperscript{160}

The problem of signal broadening is largely due to the fact that polymer protons have long relaxation times. A substantial enhancement in the quality of most polysaccharide $^1$H spectra can be achieved by using elevated temperatures at $60^\circ$-$90^\circ$ or by performing a very mild hydrolysis in order to reduce viscosity of the sample (however, possible loss of labile groups should be considered). The $^1$H signals of many polysaccharides become appreciably sharper and, when a spectrum is recorded at high field, there is a corresponding enhancement in signal separation.

Some bacterial polysaccharides contain O-acetyl groups which are irregularly distributed along the polymeric chain. This causes certain anomeric signals to be twinned. Such twinning disappears after O-deacetylation of the polysaccharide giving rise to a better resolved spectrum. This is illustrated in Fig. II.6 which shows the $^1$H-n.m.r. spectra of \textit{E. coli} K28 polysaccharide (after autohydrolysis, $95^\circ$, overnight) and the same polysaccharide after O-deacetylation (0.01M NaOH, $23^\circ$, overnight). Both spectra were recorded with acetone as an internal standard ($\delta$ 2.23).

\textbf{II.5.2.2 Carbon-13 n.m.r. spectroscopy\textsuperscript{159,161-172}}

Carbon-13 nuclear magnetic resonance ($^{13}$C-n.m.r.) has proved to be a powerful technique, yielding information on composition, sequence and conformation of the polysaccharides. Using the Fourier transform (FT) method, it allows spectra of polysaccharides to be obtained using
Fig. II.6: The $^1$H-n.m.r. spectra (400 MHz, 95°) of native (top) and deacetylated (bottom) *E. coli* K28 capsular polysaccharides
only their natural abundance $^{13}\text{C}$ atoms; it complements $^1\text{H}-\text{n.m.r.}$ spectroscopy in that it gives better signal separation owing to the wider range of chemical shifts involved.$^{161}$

In many cases, in particular when dealing with complex molecules such as polysaccharides, the amount of information obtainable from $^1\text{H}-\text{n.m.r.}$ spectra is limited compared to that revealed by $^{13}\text{C}-\text{n.m.r.}$. The technique is rapid and nondestructive and can be used on relatively small amounts of material.

Most of the studies to date have been concerned with the proton-decoupled spectra.$^{161}$

However, a proton-coupled spectrum contains information about the $^{13}\text{C}-^1\text{H}$ coupling constants that may be useful for the assignment of anomeric configuration.$^{163}$ In pyranosides CI-H1 coupling $^{1}J[^{13}\text{CH}(1)]$ is larger when H-1 is equatorial (~170 Hz) than when H-1 is axial (~160 Hz). A large number of hexopyranose derivatives and their $^{1}J[^{13}\text{CH}(1)]$ values have been examined by Bock and Pedersen.$^{164,165}$

The method of analysis of $^{13}\text{C}-\text{n.m.r.}$ spectra of polysaccharides is to a large extent based on the comparison of the resonances of the individual carbon atoms of the polysaccharide with those of the previously assigned monosaccharide$^{162,166}$ and oligosaccharide constituents.$^{167,168}$ The chemical shifts of the monosaccharides are similar to those of the monosaccharide units within the polysaccharide except for substituent effects. These effects produced by the attachment of any substituent to a sugar moiety cause an increase in chemical shift of the carbon directly involved in the linkage; this is usually accompanied
by a decrease of smaller magnitude (sometimes an increase) in the chemical shifts of the neighboring β-carbons.161

The sensitivity of carbon-13 chemical shifts towards changes in substitution permits location of such substituents as acetate, malonate, phosphate, or sulfate groups. Introduction of an acyl group onto oxygen causes a small (1.5-4 p.p.m.) downfield shift of the α-carbon atom. However, as O-acylation causes the signal of the β-carbon atom to shift upfield (1-5 p.p.m.), the cumulative effect of several acyl groups may be difficult to predict.162 An alteration in ring size is also accompanied by a change of chemical shifts; thus, furanoses have chemical shifts downfield from those of the pyranoses. Sometimes, an immediate identification may be made when very low-field signals at δ = 107 p.p.m. or more are present, for example, for β-galactofuranoside and α-arabino-furanoside.169

The signals in 13C-n.m.r. spectra of polysaccharides are known to distribute in groups each of which occupies strictly definite regions.

The typical resonance regions useful for the first order analysis of the 13C-n.m.r. spectrum are shown in Fig. II.7.170 They include: a) carbonyl groups from uronic acid, pyruvic acid, N- and O-acetyl substituents (175 ± 6 p.p.m.); b) anomeric carbons (100 ± 8 p.p.m.); c) secondary carbons (75 ± 5 p.p.m.); d) primary carbons (65 ± 5 p.p.m.); e) methyl groups from O-acetyl and N-acetyl substituents (20-28 p.p.m.), pyruvate (18-26 p.p.m.) and 6-deoxy sugars (~15-16 p.p.m.).

The chemical shifts for the R- and S-forms of the pyruvate differ significantly being ~18 p.p.m. for the axial methyl groups and ~26 p.p.m. for the equatorial.159 The anomeric configurations of KDO
Fig. II.7: The characteristic regions for resonances of carbon atoms belonging to different monosaccharide residues in polysaccharides
residues can be established by $^{13}$C-n.m.r. spectroscopy. The C-1 resonance is sensitive to anomeric configuration being $\delta = 174.8$ p.p.m. for the $\beta$-anomeric form and $\delta = 176.5$ p.p.m. for the $\alpha$-anomer. In the case of N-acetylneuraminic acid (NANA) the difference between chemical shifts of C-1 in $\alpha$- and $\beta$-forms is smaller, being $\delta = 175.9$ p.p.m. for the $\beta$-anomeric form and $\delta = 174.6$ p.p.m. for the $\alpha$-anomer.

Quantitative data cannot be satisfactorily obtained from integrated $^{13}$C-n.m.r. spectra, because of saturation phenomena and nuclear Overhauser effects. However, if spectra are measured under suitable conditions and if integrals (or peak heights) of signals from carbon atoms carrying the same number of hydrogen atoms are compared, it is possible to obtain rather accurate information about the relative amounts of components in a mixture.

An important limiting factor in the $^{13}$C-n.m.r. technique is the signal-to-noise ratio obtained in the spectra. A high-field instrument, large sample tubes, and increased concentration of the sample result in a larger signal-to-noise ratio (s/n). If, however, a limited amount of compound is available, it may be advantageous to use a smaller probe-insert. In contrast to $^1$H-n.m.r., particles or undissolved material make very little difference to the quality of the spectra.

Fig. II.8 shows the $^{13}$C-n.m.r. spectrum (proton decoupled) of the E. coli K28 deacetylated polysaccharide. Three signals can be observed in the anomeric region corresponding to four anomeric carbons (2 $\beta$-linked and 2 $\alpha$-linked). The signal at 16.1 p.p.m. corresponds to the CH$_3$ group of fucose.
Fig. II.8: The $^{13}$C-n.m.r. spectrum (100.6 MHz, ambient temperature) of deacetylated \textit{E. coli} K28 capsular polysaccharide
II.5.3 Other techniques

II.5.3.1 Enzymatic hydrolysis\textsuperscript{139,173}

All glycosidases are specific for the sugar unit undergoing hydrolysis and for its anomeric configuration. Exoglycosidases act on polysaccharide (or other glycoconjugate) substrates by the removal of non-reducing terminal units. These enzymes, however, can approach only to within a limited distance of other structural features such as other linkages or branch points. Thus, the oligosaccharides, generated by partial hydrolysis or bacteriophage degradation usually give more satisfactory results. Incubation with glycosidases is usually performed in buffer at 37° at the appropriate pH for varying periods of time (sometimes as long as 6-7 days).\textsuperscript{173} In contrast, endo enzymes are not limited by action pattern and can cleave unbranched regions of both external and internal chains.\textsuperscript{139} Highly specific endoglycanases are derived from bacteriophages (see Section VI).

II.5.3.2 Chromium trioxide oxidation\textsuperscript{174-176}

Angyal and James\textsuperscript{174} showed that a fully acetylated aldopyranoside, in which the aglycon occupies an equatorial position in the most stable chair form (generally the $\beta$-anomer) is readily oxidized when treated with chromium trioxide in acetic acid. The anomer with an axial aglycon (generally the $\alpha$-form) is only slowly oxidized.
where, \( R = \) alkyl group or sugar residue

This reaction has been used to determine the anomeric configuration of sugar residues in oligo- and polysaccharides.\(^{175}\) The fully acetylated material and an internal standard are treated with chromium trioxide in acetic acid. Sugar analysis of the original material and the oxidized product show which sugar residues have been oxidized.

The method is valid for gluco-, galacto-, manno-, and xylo- derivatives. However, substitution in oligo- and polysaccharides may alter the conformational equilibrium of \( \alpha \)-fucosyl and \( \alpha \)-rhamnosyl residues thus making them susceptible to oxidation.

In some situations, it may be possible to isolate oligomers after reduction of the oxidized product. This method was used in studies of the *Klebsiella* type 37 capsular polysaccharide.\(^{176}\)

In this study chromium trioxide oxidation was used to determine the anomeric configuration of glucuronic acid in *Escherichia coli* K32 capsular polysaccharide. Sugar analysis performed on the oxidized product showed the disappearance of glucuronic acid thus proving that it was \( \beta \)-linked.
II.6 LOCATION OF O-ACETYL GROUPS

Some polysaccharides with labile O-acetyl groups occur naturally. The removal of such substituents may alter physical and immunological properties of these polysaccharides. Since some of these acetate groups are extremely labile and can be easily removed, they create certain difficulties in obtaining unambiguous evidence for their original location.

The first and simplest method to use is nuclear magnetic resonance spectroscopy (II.5.2). Proton nuclear magnetic resonance of the native acetylated polysaccharide would show a singlet at $\delta = 2.15-2.28$ due to the presence of an O-acetyl group. Presence of the acetate group can cause additional chemical shifts of the neighboring protons; however, the total cumulative effect may be difficult to predict.

$^{13}$C-n.m.r. spectroscopy is more informative in this case, and comparison of the $^{13}$C-n.m.r. spectra of acetylated and deacetylated polysaccharides can often provide very useful information on the O-acetyl group and sometimes unambiguous evidence for its location (for example, when present on C-6 of hexopyranose). Spectrophotometric and gas chromatographic methods can be used for quantitative determination of the O-acetyl groups.

Chemical methods for locating these substituents include periodate oxidation (see Section II.4.2), which in some cases permits identification of sugar residues containing the acetate group. Prehm methylation (see Section II.3.1.1) and the method of de Belder and
Norrman, which involves the conversion of unsubstituted hydroxyl groups to methoxyethylacetals on reaction with methyl vinyl ether (see Scheme II.7), followed by base-catalyzed de-0-acetylation and methylation.

Scheme II.7: Location of O-acetyl substituents according to the de Belder and Norrman procedure

Hydrolysis of the modified polysaccharide then gives sugar derivatives labelled with O-methyl groups at the sites originally occupied by O-acetyl substituents. This method gives the most satisfactory results; however, possibility of "underprotection" should be considered in the case of the polysaccharides. Oligosaccharides bearing O-acetyl groups obtained by bacteriophage-induced degradation of polysaccharides (see
Section VI) or by partial hydrolysis using hydrogen fluoride (see Section II.4.1) give more reliable results.

In this study the position of an O-acetyl group was located using the methyl vinyl ether procedure. The results obtained were further confirmed by $^1$H- and $^{13}$C-n.m.r. findings (for detailed procedure see Section IV).
CHAPTER III

GENERAL EXPERIMENTAL CONDITIONS
III.1 PAPER CHROMATOGRAPHY

Paper chromatography was performed by the descending method using Whatman No. 1 paper and the following solvent systems:

A) ethyl acetate:acetic acid:formic acid:water (18:3:1:4)
B) ethyl acetate:pyridine:water (8:2:1)
C) l-butanol:acetic acid:water (2:1:1)
D) l-butanol:ethanol:water (4:1:5, upper phase)

Chromatograms were developed with alkaline silver nitrate or by heating at 110° for 5-10 min after spraying with p-anisidine hydrochloride in aqueous l-butanol.

Preparative paper chromatography was carried out by the descending method using Whatman 3MM paper and solvent C (unless otherwise stated). The relevant strips were cut out and eluted with water for 6 h. The aqueous solutions were filtered, concentrated and freeze-dried.

III.2 GAS-LIQUID CHROMATOGRAPHY AND G.L.C.-MASS SPECTROMETRY

Analytical g.l.c. separations were performed using a Hewlett-Packard 5700 instrument fitted with dual flame-ionization detectors. Stainless-steel columns (1.8 m x 3 mm) were used with a carrier-gas nitrogen flow-rate of 20 mL/min. The following packing materials and
programs were used (unless otherwise stated): (A) 3% of SP-2340 on Supelcoport (100-120 mesh), programmed from 195° for 4 min, and then at 2°/min to 260°; (B) 5% of ECNSS-M on Gas Chrom Q (100-120 mesh), isothermal at 170°, or programmed from 180° for 4 min, and then at 2°/min to 200°; (C) 3% of OV-225 on Gas Chrom Q (100-120 mesh), isothermal at 170°, or programmed from 180° for 4 min, and then at 2°/min to 230°.

All quantitative data present are based on the average of at least three runs.

Preparative g.l.c. was carried out with a F & M model 720 dual column instrument fitted with thermal conductivity detectors. Stainless-steel columns (1.8 m x 6.3 mm) were used with carrier-gas helium flow-rate of 60 mL/min. The following packing materials and programs were used (unless otherwise stated): (D) 3% of SP-2340 on Supelcoport (100-120 mesh), programmed from 195° to 240° at 2°/min; (E) 3% of OV-225 on Gas Chrom Q (100-120 mesh), programmed from 180° to 230° at 2°/min.

G.l.c.-m.s. analyses were performed with a V.G. Micromass 12 instrument fitted with a Watson-Biemann separator. Spectra were recorded at 70 eV with an ionization current of 100μA and an ion source at 200° (unless otherwise stated).

III.3 GEL-PERMEATION CHROMATOGRAPHY

Preparative gel-permeation chromatography was performed using a column (2.5 x 100 cm) of Bio-Gel P-4 (400 mesh). The concentration of
the sample applied to the column was 100 mg/mL. The column was irrigated with water:pyridine:acetic acid (500:5:2) at a flow rate of 10.5 mL/hour. Fractions of 2 mL were collected, freeze-dried, weighed and an elution profile was obtained.

III.4 OPTICAL ROTATION AND CIRCULAR DICHROISM

Optical rotations were measured on aqueous solutions at 20°±3° on a Perkin-Elmer model 141 polarimeter with a 1 dm cell (5 mL).

Circular dichroism spectra (c.d.) were recorded on a Jasco J-500A automatic recording spectropolarimeter with a quartz cell of 0.3 mL capacity and a path length of 0.1 cm or 0.2 cm. Compounds were dissolved in acetonitrile (spectroscopic grade) and the spectra recorded in the range of 190-260 nm.

III.5 NUCLEAR MAGNETIC RESONANCE

Proton magnetic resonance spectra were recorded on a Varian XL-100, Bruker WP-80, Nicolet-Oxford H-270, or Bruker WH-400 instruments. Spectra were recorded at a temperature of 90°±5° and acetone was used as an internal standard. All values are given relatively to that of internal sodium 4,4-dimethyl-4-silapentane-sulfonate taken as 0. Samples (10-20 mg) were prepared by dissolving in D$_2$O and freeze-drying 2-3 times from D$_2$O solutions. Tubes of 5 mm in diameter were used.

$^{13}$C-n.m.r. spectra were recorded on a Bruker WP-80 or Bruker WH-400 spectrometer at ambient temperature. Samples (30-50 mg) were
dissolved in D₂O and acetone was used as an internal standard. Tubes of 5 or 10 mm in diameter were used.

III.6 GENERAL CONDITIONS

The I.R. spectra of methylated derivatives were recorded on a Perkin-Elmer model 710B spectrophotometer in carbon tetrachloride (spectroscopic grade).

All solutions were concentrated on a rotary evaporator in vacuo at a bath temperature of 40°.

Ion-exchange chromatography for separation of acidic and neutral oligosaccharides was performed on a column (2.0 x 28 cm) of Bio-Rad AG-1-X2 (formate form, 200-400 mesh). The neutral fraction was eluted with water and the acidic with 10% formic acid. Sephadex LH-20 was used for purification of permethylated oligo- and polysaccharides. De-ionizations were carried out with Amberlite IR-120(H⁺) resin.

III.7 ISOLATION AND PURIFICATION OF THE POLYSACCHARIDES

III.7.1 Klebsiella polysaccharides

The following media were used to grow the bacteria.

1. Beef-extract medium ("nutrient broth")
   5 g of Bactopeptone
   3 g Bacto beef extract
   2 g of NaCl
2. Sucrose-yeast extract-agar
   1 L of H₂O
   75 g of sucrose
   5 g of Bacto yeast extract
   37.5 g of agar
   5 g of NaCl
   2.5 g of KH₂PO₄
   0.625 g of MgSO₄·7H₂O
   1.25 g of CaSO₄
   2.5 L of H₂O

A sample of *Klebsiella* bacteria serotype K50 was received as a stab culture from Dr I. Ørskov (Copenhagen). The bacteria were streaked on agar plates and incubated at 37°. An individual colony was inoculated in beef-extract medium and bacteria were grown for 5 h at 37° with continuous shaking. This liquid culture (50 mL) was incubated on a tray (86 cm x 46 cm) of sucrose-yeast extract-agar for 3 d. The lawn of capsular bacteria produced was harvested by scraping from the agar surface, and the bacteria were destroyed with 1% phenol solution. The polysaccharide was separated from the cells by ultracentrifugation (30,000 rpm). The viscous supernatant was precipitated into ethanol (3 volumes). The precipitate was dissolved in the minimum amount of water and reprecipitated with a saturated (10%) solution of Cetavlon (cetyltrimethylammonium bromide). The precipitated acidic polysaccharide was isolated by centrifugation. It was dissolved in 4M NaCl, reprecipitated into ethanol and the precipitate was dissolved in water and dialyzed against running tap water for 2 d. The dialyzed solution
of polysaccharide was ultracentrifuged and the supernatant was freeze-dried.

### III.7.2 Escherichia coli polysaccharides

Stab cultures of *Escherichia coli* bacteria K28 and K32 were obtained from Dr. I. Ørskov (Copenhagen).

The following media were used to grow the bacteria.

1) Bacto Mueller Hinton Broth, dehydrated (Difco)

2) Mueller Hinton agar (BBL) with addition of NaCl 0.5% (w/v).

The streaked plates were incubated at 37° overnight. The bacteria were cultured as described for the *Klebsiella* polysaccharides (see Section III.7.1) and were grown in three small trays (30 x 50 cm) using 1.5 L of Mueller Hinton agar and 5 g NaCl per tray. One tray was filled with water and used as a humidity source.181

Each tray was layered with an actively growing liquid culture of *E. coli* bacteria, obtained by inoculation of 50 mL of Mueller Hinton broth with a single fresh colony of *E. coli* and further incubation of the resulting solution for 5 h at 37°. The trays were left in the incubator for 5 d and then the slime was collected. The purification procedure was carried out as described for the *Klebsiella* polysaccharide.
III.8 BACTERIOPHAGE PROPAGATION

III.8.1 Tube and flask lysis

The bacteriophages \( \Phi 28-1 \), \( \Phi 28-2 \), and \( \Phi 32 \) were isolated from sewage (courtesy of Dr. S. Stirm, Freiburg, Germany) and propagated on their host strains in nutrient broth by tube and flask lysis.

a) Tube lysis. An actively growing bacterial culture of *Escherichia coli* was obtained by successive replatings on agar plates. An actively growing bacterial culture (0.5 mL) was inoculated with 6 x 5 mL of sterile nutrient broth and after 1 h of incubation 0.5 mL of a bacteriophage-containing solution was added to each test tube at 30 min intervals. Continued incubation resulted in gradual clearing of the cloudy solution due to cell lysis. After 4-5 h of incubation a few drops of chloroform were added to each tube to prevent bacterial growth and the contents of the first three and last three test tubes were combined. The bacterial debris was then sedimented by centrifugation and the phage solutions were analyzed by the "plaque assay technique". The technique was based on a series of successive dilutions of phage by transferring 0.3 mL portions of phage to 2.7 mL portions of diluent (nutrient broth), thus making \( 10^{-2} \), \( 10^{-4} \), \( 10^{-6} \) and \( 10^{-8} \) dilutions of original solution. One drop from each dilution was then placed on a bacterial "lawn". (The "lawn" was prepared by inoculation of 2 mL liquid medium with an actively growing colony and incubating this culture for 4-5 h. An agar plate, previously dried in the incubator at
37° was covered with this liquid culture, left for 15 min and the excess of liquid was removed. Incubation for 1 h gave a stable "lawn". The plate was then incubated at 37° overnight. At high phage concentrations (normally dilutions $10^{-4}$, $10^{-5}$, and $10^{-6}$) individual phage particles could not be distinguished, but at suitable dilutions ($10^{-7}$ and $10^{-8}$) individual plaques surrounded by halos (not always) could be easily counted. The titer of the bacteriophage solution was then calculated.

\[
\text{Bacteriophage titer} = \frac{\text{number of plaques} \times \text{dilution}}{\text{drop size}}
\]

(in plaque forming units (P.F.U.) per mL)

b) **Flask lysis.** This technique was essentially the same as described for the tube lysis, except that larger amounts of bacteriophage could be produced. An overnight actively growing culture (5 mL) was added to 6 x 50 mL of nutrient broth and after 1.5 h of incubation 5 mL of bacteriophage solution is added to each flask at 30 min intervals. The procedure was then continued as described for the tube lysis. A flask lysis typically yielded 300 mL of phage solution with a titer of $10^{10}$ P.F.U./mL

### III.8.2 Large-scale propagation of the bacteriophage

Bacteriophage φ32 was propagated in a 14 L fermentor jar, fermentor model Microferm, New Brunswick Scientific. The following
conditions were used: aeration, 5 L.p.m.; agitation, 500 r.p.m.; temperature, 37°. Nutrient broth (10 L) was autoclaved in the fermentor jar for 40 min at 121°. The sterile medium was placed in a fermentor and agitated for 15 min, then it was inoculated with 450 mL of actively growing bacterial culture and stirred for 1 h. To this solution a total of 9.6 x 10^{10} P.F.U. in 400 mL nutrient broth was added. After 2.5 h of incubation 400 mL of CHCl₃ was added to prevent bacterial growth. The bacterial debris was removed by continuous centrifugation using a high-speed Cepa-Schnell Model LE bench-scale centrifuge cooled with tap water and operated at 38,000 r.p.m., and with a flow rate of ~250 mL/min.
CHAPTER IV

STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE

OF *Klebsiella* SEROTYPE K50
IV. STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE OF
Klebsiella SEROTYPE K50.

IV.1 ABSTRACT

The structure of the capsular polysaccharide from Klebsiella K50 has been determined by using the techniques of methylation, periodate oxidation and partial hydrolysis and β-elimination. N.m.r. spectroscopy (1H and 13C) was used to establish the nature of the anomeric linkages. The polysaccharide is comprised of repeating units of the heptasaccharide shown and is the one of 19 capsular polysaccharides that are composed of D-glucuronic acid, D-galactose, D-glucose, and D-mannose residues. It has the only known "five-plus-two" repeating unit; the structure of the polysaccharide from Klebsiella K50 is, therefore, unique in this series.

\[
\begin{align*}
\alpha-D-Glc & \rightarrow 6 \\
\uparrow & \\
\alpha-D-GlcA & \rightarrow (1 \rightarrow 3) \rightarrow \\
\beta-D-Gal & \rightarrow (1 \rightarrow 3) \\
\alpha-D-Man & \rightarrow (1 \rightarrow 4) \\
\alpha-D-Man & \rightarrow (1 \rightarrow 6) \\
\end{align*}
\]
IV.2 INTRODUCTION

The capsular polysaccharide from *Klebsiella* serotype K50 is composed of D-glucuronic acid, D-glucose, D-galactose, and D-mannose residues, and is one of those, from almost twenty strains, having the same qualitative composition. Identity of qualitative analysis, however, bears no relationship to the structural pattern of the polysaccharide, and examination of the capsular material from *Klebsiella* K50 has shown it to have a unique structure in this series. Several capsular polysaccharides based on a heptasaccharide repeating unit are known, but this is the first instance where such a unit is of the "5 + 2" type. Furthermore, in those capsular polysaccharides in which the uronic acid residue is part of the main chain, only single-unit lateral substituents have previously been found. The polysaccharide of serotype K50 is thus, on two counts, unique among the some sixty structures now known in this series. The experimental evidence on which this structure is based is summarized next.

IV.3 RESULTS AND DISCUSSION

The isolation and purification of the polysaccharide were achieved as described in Section III.7.1. The acidic polysaccharide obtained after one precipitation with cetyltrimethylammonium bromide had $[\alpha]_D +102^\circ$, which compares well with the calculated value of $+96^\circ$ using Hudson's Rules of Isorotation. The polysaccharide was
shown to be homogeneous by gel-permeation chromatography with $M_w = 3.2 \times 10^6$ daltons.

Paper chromatography of an acid hydrolyzate of the polysaccharide showed galactose, glucose, glucuronic acid, mannose and an aldobiouronic acid. Acid hydrolysis of carboxyl-reduced polysaccharide and conversion to alditol acetates gave mannose, galactose, and glucose in the molar ratios of 2.0:2.4:3.0. Glucose was proved to be of the D-configuration by circular dichroism (c.d.) measurements made on glucitol hexaacetate. Galactose and glucose were assigned the D configuration from c.d. measurements made on partially methylated derivatives.

The $^1$H-n.m.r. spectrum of partially hydrolyzed polysaccharide was recorded in D$_2$O at 90° with acetone as internal standard (see Appendix III, Spectrum NO. 1). The spectrum exhibits 7 signals in the anomeric region: $\delta$ 5.46 (1H); $\delta$ 5.29 (1H); $\delta$ 5.24 (1H); $\delta$ 5.06 (1H); $\delta$ 5.01 (1H); $\delta$ 4.70 ($J_{1,2}$ 8 Hz, 1H) and $\delta$ 4.49 ($J_{1,2}$ 8 Hz, 1H). From the values of the chemical shifts and coupling constants, 5 $\alpha$- and 2 $\beta$-anomeric linkages were assigned. No deoxy-sugar, $\alpha$-acetyl or pyruvate could be detected.

The $^{13}$C-n.m.r. spectrum confirmed these results (see Appendix III, Spectrum No. 2). Five signals appear in the anomeric region at 104.04; 103.97; 103.17; 101.30 and 99.16 p.p.m. The signals at 104.04 and 99.16 p.p.m. each corresponded to two anomeric carbons.

Precise assignment of the anomeric signals was achieved after comparison of the $^1$H- and $^{13}$C-n.m.r. spectra of the polysaccharide with the corresponding spectra of oligosaccharides obtained after partial
<table>
<thead>
<tr>
<th>Compound</th>
<th>$^1$H-N.m.r. data</th>
<th>$^{13}$C-N.m.r. data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta^b$</td>
<td>$J_{1,2}$ (Hz)</td>
</tr>
<tr>
<td>GlcA$_\alpha$Man-OH</td>
<td>4.93 s</td>
<td>0.4</td>
</tr>
<tr>
<td>A1</td>
<td>5.19 s</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>5.34 2.5</td>
<td>1.0</td>
</tr>
<tr>
<td>GlcA$<em>\alpha$Man$</em>\alpha$Man-OH</td>
<td>4.93 2</td>
<td>0.3</td>
</tr>
<tr>
<td>A2</td>
<td>5.08 2</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>5.32 2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.37 2</td>
<td></td>
</tr>
<tr>
<td>GlcA$<em>\alpha$Man$</em>\alpha$Man$_\alpha$Gal-OH</td>
<td>4.65 7</td>
<td>0.8</td>
</tr>
<tr>
<td>A3</td>
<td>5.08 1</td>
<td>1</td>
</tr>
<tr>
<td>Compound</td>
<td>(^1\text{H-N.m.r. data} )</td>
<td>(^{13}\text{C-N.m.r. data} )</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td></td>
<td>( \Delta^b )</td>
<td>( J_{1,2} )</td>
</tr>
<tr>
<td></td>
<td>(Hz) proton</td>
<td></td>
</tr>
<tr>
<td>5.19</td>
<td>3</td>
<td>3-Gal-( \beta )OH</td>
</tr>
<tr>
<td></td>
<td>2-Man-( \alpha )</td>
<td>GlcA-( \alpha )</td>
</tr>
<tr>
<td>5.31</td>
<td>2-Man-( \alpha )</td>
<td>95.10</td>
</tr>
<tr>
<td></td>
<td>3-Gal-( \alpha )OH</td>
<td>93.04</td>
</tr>
<tr>
<td>Gal( ^1 \beta )Glc( ^1 \alpha )OCH</td>
<td>4.65</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>CHOH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CH(_2)OH</td>
<td></td>
</tr>
<tr>
<td>SML</td>
<td>5.20</td>
<td>4</td>
</tr>
<tr>
<td>( ^3 \text{Gal-} )Glc( ^3 \alpha )Glc( ^3 \alpha )Man-( \alpha )Man-( \alpha )</td>
<td>4.49</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3-Man-( \alpha )</td>
<td>103.97</td>
</tr>
</tbody>
</table>

\(^a\) This compound represents the structure of the polysaccharide. The table lists the \( ^1\text{H-N.m.r. data} \) and \( ^{13}\text{C-N.m.r. data} \) for the polysaccharide, including the chemical shifts (P.p.m.) and the integral assignments for each proton.
<table>
<thead>
<tr>
<th>Compound&lt;sup&gt;a&lt;/sup&gt;</th>
<th>&lt;sup&gt;1&lt;/sup&gt;H-N.m.r. data&lt;sup&gt;b&lt;/sup&gt;</th>
<th>&lt;sup&gt;13&lt;/sup&gt;C-N.m.r. data&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;sup&gt;a&lt;/sup&gt;Integral proton</td>
<td>Assignment&lt;sup&gt;d&lt;/sup&gt; P.p.m.&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.01</td>
<td>1</td>
<td>2-Man&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.06</td>
<td>1</td>
<td>3-Glc&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.24</td>
<td>1</td>
<td>3-Man&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.29</td>
<td>1</td>
<td>6-Glc&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.46</td>
<td>1</td>
<td>4-GlcA&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Degraded K50

<sup>b</sup> Assignment

<sup>c</sup> C-N.m.r. data

<sup>d</sup> H-N.m.r. data

<sup>e</sup> P.p.m.
<table>
<thead>
<tr>
<th>Compound&lt;sup&gt;a&lt;/sup&gt;</th>
<th>&lt;sup&gt;1&lt;/sup&gt;H-N.m.r. data</th>
<th>&lt;sup&gt;13&lt;/sup&gt;C-N.m.r. data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta$&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$J_{1,2}$ (Hz)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> For the source of A1, A2, A3, and SH1, see text. <sup>b</sup> Chemical shift relative to internal acetone; $\delta$ 2.23 downfield from sodium $^4,^4$-dimethyl-$^4$-silapentane-1-sulfonate (DSS). <sup>c</sup> The numerical prefix indicates the position in which the sugar is substituted; the $\alpha$ or $\beta$, the configuration of the glycosidic bond, or the anomere in the case of a (terminal) reducing-sugar residue. Thus $^3$-Gal$\alpha$ refers to the anomeric proton of a 3-linked galactosyl residue in the $\alpha$-anomeric configuration. The absence of a numerical prefix indicates a (terminal) nonreducing group. <sup>d</sup> Chemical shift in p.p.m. downfield from Me$_4$Si, relative to internal acetone; 31.07 p.p.m. downfield from DSS. <sup>e</sup> As in c, but for $^{13}$C nuclei. <sup>f</sup> See text.
hydrolysis and of degraded polysaccharide after selective hydrolysis (see Table IV.1).

**Methylation analysis**

Methylation analysis of the K50 polysaccharide, followed by hydrolysis, derivatization as alditol acetates, and g.l.c.-m.s. analysis, gave the values shown in Table IV.2, column I. These results indicated that the polysaccharide consists of a heptasaccharide repeating unit having a branch on glucose, with galactose as the terminal group. By reduction of the methylated polysaccharide (see column II), the proportion of 2,4,6-tri-O-methylmannose was increased and 2,3-di-O-methylglucose was formed, indicating that glucuronic acid is substituted at O-4, and that it is linked to mannose. Methylation-ethylation analysis of the product obtained by base-catalyzed degradation of the uronic acid showed the presence of 3-O-ethyl-2,4,6-tri-O-methylmannose derived from ethylation at O-3 of the mannose of the aldobiouronic acid (see column III).

**Selective partial hydrolysis**

Treatment of K50 polysaccharide with very dilute acid, and dialysis of the products against distilled water, afforded a non-dialyzable polymeric material and a dialyzate. The dialyzate contained galactose, as was shown by paper chromatography and g.l.c. (as alditol acetate). Examination of the non-dialyzable portion by gel-permeation chromatography showed the polymer to be extensively depolymerized (see Fig. IV.1), and this was attributed to the acid lability of the 3-substituted


TABLE IV.2

METHYLATION ANALYSES OF Klebsiella K50 POLYSACCHARIDE AND DERIVATIVES

<table>
<thead>
<tr>
<th>Methylated sugars (as alditol acetates)</th>
<th>[ T^a ]</th>
<th>Column B(^c) (ECNSS-M)</th>
<th>Mole %(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>I   II   III</td>
</tr>
<tr>
<td>2,3,4,6-Man(^e)</td>
<td>0.87</td>
<td>-</td>
<td>-  16.5</td>
</tr>
<tr>
<td>2,3,4,6-Gal</td>
<td>1.00</td>
<td>19.8</td>
<td>14.5  23.5</td>
</tr>
<tr>
<td>3,4,6-Man</td>
<td>1.42</td>
<td>12.9</td>
<td>10.1</td>
</tr>
<tr>
<td>2,4,6-Man</td>
<td>1.49</td>
<td>8.7</td>
<td>18.4   25.6</td>
</tr>
<tr>
<td>2,4,6-Gal</td>
<td>1.59</td>
<td>15.8</td>
<td>14.5   8.9</td>
</tr>
<tr>
<td>2,3,4-Glc</td>
<td>1.66</td>
<td>26.7</td>
<td>15.2   20.9</td>
</tr>
<tr>
<td>2,4-Glc</td>
<td>2.52</td>
<td>16.0</td>
<td>11.1   4.4</td>
</tr>
<tr>
<td>2,3-Glc</td>
<td>2.59</td>
<td>-</td>
<td>15.5   -</td>
</tr>
</tbody>
</table>

\(^a\) Relative retention time, referred to 2,3,4,6-Gal as 1.00.  
\(^b\) Values are corrected by use of the effective, carbon-response factors given by Albersheim \textit{et al.} \(^{103}\).  
\(^c\) Programmed for 4 min at 160°, and then at 2°/min to 200°.  
\(^d\) I, original polysaccharide; II, carboxyl-reduced polysaccharide; III, uronic acid-degraded polysaccharide.  
\(^e\) 2,3,4,6-Man = 3-O-ethyl-2,4,6-tri-O-methylmannitol, etc.
Fig. IV.1: Gel-permeation chromatography of the product obtained after selective, partial hydrolysis of *Klebsiella* K50 polysaccharide. (Bio-Gel P-300 column 85 x 1.5 cm, M NaCl eluant, flow-rate 3 mL/h). Courtesy of Dr. S.C. Churms, Cape Town, South Africa
β-D-galactopyranosyl unit, even under these mild conditions. The 1H-n.m.r. spectrum of this material lacked one signal (δ 4.7) corresponding to a β-linkage, but integration of the signals for anomeric protons was unsatisfactory due to the depolymerization (see Table IV.1). Methylation analysis of the non-dialyzable material showed that 2,3,4,6-tetra-O-methylglucose largely replaced the corresponding galactose compound.

**Partial hydrolysis**

Partial hydrolysis of the native polysaccharide with acid was followed by separation of the acidic and the neutral fractions by ion-exchange chromatography. The neutral fraction contained monosaccharides. The acidic fraction contained three acidic oligosaccharides. On the basis of paper chromatography, their n.m.r.-spectral data (see Table IV.1), and methylation analysis (see Table IV.3), the structures of these compounds were shown to be as follows.

\[
\begin{align*}
A_1 & \quad \alpha-\text{GlcA-(1→3)}-\text{Man} \\
A_2 & \quad \alpha-\text{GlcA-(1→3)-α-Man-(1→2)}-\text{Man} \\
A_3 & \quad \alpha-\text{GlcA-(1→3)-α-Man-(1→2)-α-Man-(1→3)}-\text{Gal}
\end{align*}
\]

The aldotetraouronic acid (A3) obtained from partial hydrolysis had previously been isolated from other *Klebsiella* capsular polysaccharides, namely K21, K26 and K74.
### TABLE IV.3

ANALYSES OF ACIDIC OLIGOSACCHARIDES FROM *Klebsiella* K50 POLYSACCHARIDE

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Paper chromatography a</th>
<th>Sugar analysis b (molar proportions)</th>
<th>Methylation analysis c (molar proportions)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>GlcA</td>
<td>Man (0.8)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Man</td>
<td>Glc (GlcA) (1)</td>
<td>-</td>
</tr>
<tr>
<td>A2</td>
<td>GlcA</td>
<td>Man (1.5)</td>
<td>2,4,6-Man (0.7) ^d</td>
</tr>
<tr>
<td></td>
<td>Man</td>
<td>Glc (GlcA) (1)</td>
<td>3,4,6-Man (1.0)</td>
</tr>
<tr>
<td>A3</td>
<td>GlcA</td>
<td>Man (1.75)</td>
<td>2,4,6-Gal (1.0)</td>
</tr>
<tr>
<td></td>
<td>Man</td>
<td>Gal (1.2)</td>
<td>2,4,6-Man (0.7) ^d</td>
</tr>
<tr>
<td></td>
<td>Gal</td>
<td>Glc (GlcA) (1)</td>
<td>3,4,6-Man (0.8) ^d</td>
</tr>
</tbody>
</table>

^a Solvents A and B. ^b As alditol acetates. ^c As alditol acetates, neutral sugars only. ^d Ratios are low, due to incomplete hydrolysis of the glucosyluronic linkage.
Periodate oxidation

Periodate oxidation of carboxyl-reduced polysaccharide\textsuperscript{75}, followed by methylation, Smith hydrolysis, remethylation,\textsuperscript{182} and hydrolysis, gave a mixture that was found, by g.l.c. analysis of the alditol acetates, to contain derivatives of 2,3,4,6-tetra-\(\text{O}\)-methylmannose, 2,3,4,6-tetra-\(\text{O}\)-methylgalactose, and 2,4,6-tri-\(\text{O}\)-methylglucose, indicating that the galactose in the main chain is 3-linked to glucose, the branch point. The following structure may, therefore be proposed; in it, the configuration of three linkages has yet to be determined.

\[
\begin{align*}
\rightarrow3\text{-}\beta\text{-Gal} & \rightarrow(1\rightarrow3)\text{-}\beta\text{-Glc} & \rightarrow(1\rightarrow4)\text{-}\alpha\text{-GlcA} & \rightarrow(1\rightarrow3)\text{-}\alpha\text{-Man} & \rightarrow(1\rightarrow2)\text{-}\alpha\text{-Man} & \rightarrow1
\end{align*}
\]

\[
\begin{align*}
\uparrow & \\
?\text{-Glc} & 6 & \uparrow & \\
1 & \beta\text{-Gal}
\end{align*}
\]

Smith degradation of the original K50 polysaccharide gave an oligosaccharide, analysis of which showed it to be

\[
\begin{align*}
\text{COOH} & \\
\text{HCOH} & \\
\text{Gal-(1\rightarrow3)\text{-Glc-OCH}} & \\
\text{CH}_2\text{OH}
\end{align*}
\]

and it's \(1^H\text{-n.m.r.}\) spectrum indicated the presence of one \(\alpha\) and one \(\beta\) linkage. Because the two monosaccharides therein exhibit similar coupl-
ing-constants; no definitive assignment could be made by such spectros-
copy. Incubation with a β-D-galactosidase caused cleavage (α-D-galacto-
sidase, negative) of galactose, thus demonstrating that the in-chain
galactose has the β configuration. Six of the seven anomeric linkages,
including both of the β signals, having been positively assigned, it
follows that the side chain must be attached to the main chain by an
α-glycosidic bond.

IV.4 CONCLUSION

From the sum of these experiments, the complete structure of the
capsular polysaccharide from *Klebsiella* serotype K50 may be written

\[+3)\text{-β-D-Gal}\rightarrow(1\rightarrow3)\text{-α-D-Glc}\rightarrow(1\rightarrow4)\text{-α-D-GlcA}\rightarrow(1\rightarrow3)\text{-α-D-Man}\rightarrow(1\rightarrow6)\text{-α-D-Man}\rightarrow(1\rightarrow6)\text{-α-D-Glc}\rightarrow(1\rightarrow6)\text{-β-D-Gal}\]

It is consistent with the qualitative analysis originally report-
ed by Nimmich. The structure of the K50 polysaccharide is unique
among the *Klebsiella* K antigens in having a "five-plus-two" repeating
unit.
IV.5 EXPERIMENTAL

General methods

The instrumentation used for n.m.r., g.l.c., g.l.c.-m.s. infrared, c.d., and measurements of optical rotation has been described in Section III. Paper chromatography, gas-liquid chromatography, and ion-exchange chromatography were performed as described in Section III.

Preparation and properties of K50 capsular polysaccharide

A culture of Klebsiella K50, obtained from Dr. Ida Ørskov (Copenhagen), was grown as described in Section III.1. The isolated polysaccharide (3.4 g) had \([\alpha]_D^{25} +102° (c 0.095, \text{water})\). The average molecular weight was determined by gel chromatography (courtesy of Dr. S.C. Churms, University of Cape Town, South Africa) to be \(3.2 \times 10^6\) daltons. N.m.r. spectroscopy (\(^1\text{H}\) and \(^{13}\text{C}\)) was performed on the original K50 polysaccharide, but much better spectra were obtained after treatment of the polysaccharide with 0.01M trifluoroacetic acid during 2 h at 95°, in order to lower the viscosity. The principal signals and their assignments for the \(^1\text{H}-\) and \(^{13}\text{C}-\) n.m.r. spectra are recorded in Table IV.1.

Hydrolysis of the polysaccharide

Hydrolysis of a sample (20 mg) of K50 polysaccharide with 2M trifluoroacetic acid (TFA) overnight at 95°, removal of the acid by repeated coevaporation with water, followed by paper chromatography (solvents A and B, see Section III.1), showed the presence of D-mannose,
D-galactose, D-glucose, D-glucuronic acid and an aldobiouronic acid. Hydrolysis of carboxyl-reduced polysaccharide with 2M trifluoroacetic acid overnight at 95°, followed by reduction with NaBH₄ and acetylation with 1:1 acetic anhydride-pyridine afforded the alditol acetates of mannose, galactose, and glucose which were identified by g.l.c. (column A, see Section III.2) and found to be present in the ratios of 2.0:2.4:3.0.

Circular dichroism measurements

Glucose was proved to be of the D-configuration by circular dichroism (c.d.) measurements made on glucitol hexaacetate. Galactose and mannose were assigned D configuration from c.d. measurements made on partially methylated derivatives. Alditol acetates were separated preparatively in column D. Partially methylated alditol acetates were separated in column E. For experimental details see Section III.2 and Section III.4. The following values of \( \Delta c_{213}^{\text{MeCN}} \) were obtained for the acetates of: glucitol, \( +1.3 \); 2,3,4,6-tetra-O-methylgalactitol, \( +0.7 \); and 2,4,6-tri-O-methylmannitol, \( +0.64 \).

Methylation analysis

The capsular polysaccharide (297 mg) in the free-acid form, obtained by passing the sodium salt through a column of Amberlite IR-120 (H⁺) resin, was dissolved in dry dimethyl sulfoxide (20 mL) and methylated by treatment with 10 mL dimethylsulfinyl anion for 4 h, and then with 15 mL methyl iodide for 1 h. The product (316 mg), recovered
after dialysis against tap water, had been completely methylated (no hydroxyl absorption in the i.r. spectrum).

A sample (27.8 mg) of this material was hydrolyzed with 2M trifluoroacetic acid, reduced with sodium borohydride, acetylated with 1:1 acetic anhydride-pyridine, and analyzed as alditol acetates by g.l.c. and g.l.c.-m.s. in columns B and C (see Table IV.2, column I). Carboxyl reduction of the fully methylated polysaccharide (103 mg) with LiAlH₄ in anhydrous oxolane (10 mL) at room temperature overnight, hydrolysis of the product with 2M trifluoroacetic acid, followed by reduction of sugars with sodium borohydride, and acetylation of the alditols with 1:1 acetic anhydride-pyridine gave a mixture of partially methylated alditol acetates which was analyzed by g.l.c. and g.l.c.-m.s. in columns B and C (see Table IV.2, column II).

Uronic acid degradation

A sample (76 mg) of methylated K50 polysaccharide was dried and, together with a trace of p-toluenesulfonic acid, was dissolved in 19:1 dimethyl sulfoxide-2,3-dimethoxypropane (20 mL) under N₂ in a flask that was then sealed. Dimethylsulfinyl anion (5 mL) was added and allowed to react for 16 h at room temperature, when ethyl iodide (7 mL) was added. The solution was stirred for 1 h, and the methylated, degraded product was isolated by partition between water and chloroform. Hydrolysis and g.l.c.-m.s. analysis of the alditol acetate derivatives showed the presence of 3-O-ethyl-2,4,6-tri-O-methylmannose (see Table IV.2, column III).
Selective, partial hydrolysis

A solution of K50 polysaccharide (500 mg) in 0.01M TFA (50 mL) was heated on a steam bath for 12 h. The acid was removed and the product was dialyzed against distilled water (1 L), to afford a polymeric material (250 mg). Paper chromatography of the dialyzable fraction showed galactose, confirmed by g.l.c. as galactitol hexaacetate. Methylation analysis of the polymeric material and g.l.c. (column C) indicated the presence of 2,3,4,6-tetra-O-methylglucose instead of the 2,3,4-tri-O-methylglucose found originally.

Partial hydrolysis

A solution of K50 polysaccharide (710 mg) in 1M TFA (50 mL) was heated for 5 h on a steam bath. After removal of the acid by repeated coevaporation with water, an acidic and a neutral fraction were separated on a column of Bio-Rad AG1-X2 (formate form) ion-exchange resin. The acidic fraction (198 mg) was separated by preparative paper chromatography (solvent C), to give 51.5 mg of a pure aldobiouronic acid (A1), 19.1 mg of a pure aldotriouronic acid (A2), and 27.7 mg of a pure aldotetraouronic acid (A3). Paper chromatography of the neutral fraction showed glucose, galactose and mannose.

The analyses performed on each oligosaccharide were as follows.

(a) Paper chromatography. Acidic oligosaccharides were treated with 2M TFA overnight, and the hydrolyzates were examined by paper chromatography (solvents A and B). (b) Sugar analysis. The hydrolyzates were then reduced with NaBH₄, and the alditols were acetylated with 1:1 acetic anhydride-pyridine. The alditol acetates obtained were analyzed
by g.l.c. in column A. (c) **Methylation analysis.** All methylations were conducted by the method of Hakomori.\(^8\) Dried samples of 5-6 mg were dissolved in 1 mL anhydrous dimethyl sulfoxide, treated with 1 mL dimethylsulfinyl anion for 2 h and then with 2 mL methyl iodide for 1 h. The mixtures were diluted with water and extracted with chloroform (4 x 10 mL). The combined extracts were back extracted with water (3 x 10 mL) and evaporated to dryness under reduced pressure. The products were hydrolyzed with 2M TFA, and analyzed (as alditol acetates) by g.l.c. and g.l.c.-m.s. in columns B and C.

The results obtained for each oligosaccharide are given in Table IV.3, and the n.m.r. data, in Table IV.1.

**Carbodiimide reduction of capsular polysaccharide\(^{75}\)**

A sample of K50 polysaccharide (Na\(^+\) salt, 502 mg) was dissolved in 80 mL water. l-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (CMC, 2.2 g) was added. As the reaction proceeded, the pH was maintained at 4.75 by titration with 0.1 N HCl. The reaction was allowed to proceed for at least two h. When the consumption of hydrochloric acid ceased, an aqueous solution of 2M sodium borohydride (12 g/150 mL H\(_2\)O) was added slowly. The pH rose rapidly to 7.0 and it was maintained between 5-7 by titration with 4M HCl. A drop of 2-octanol (antifoaming agent) was added periodically to control the amount of foam. The reduction was completed in one h, and the reaction mixture was stirred for an additional 30 min. The mixture was dialyzed against tap water during 24 h, concentrated and freeze-dried. A second
treatment was carried out. A total of 412.5 mg of the product was recovered after freeze-drying.

A sample of the reduced polysaccharide (10 mg) was hydrolyzed overnight with 2M trifluoroacetic acid on a steam bath and the sugars in the hydrolyzate were converted into alditol acetates, g.l.c. analysis of which showed mannitol, galactitol and glucitol hexaacetates in a ratio 2.0:2.4:3.0, indicating complete reduction of uronic acid.

**Periodate oxidation of carboxyl-reduced K50 polysaccharide**

Carbodiimide-reduced K50 polysaccharide (46.9 mg) in water (20 mL) was mixed with a solution (5 mL) of 0.1M NaIO₄ and 0.4M NaClO₄, and kept in the dark at 4°. The periodate consumption was followed on 1 mL aliquots by the Fleury-Lange method and reached a plateau after 6 d (7.62 moles of periodate per mole of polysaccharide). Ethylene glycol (10 mL) was added, the polyaldehyde was reduced to the polyalcohol with NaBH₄, the base neutralized with 50% acetic acid, and the solution was dialyzed overnight, and freeze-dried to yield the polyalcohol (50.9 mg) which was methylated by Hakomori procedure. One-third of the methylated product was hydrolyzed with 50% acetic acid for 90 min, and then remethylated. The product was hydrolyzed, and the sugars were characterized as alditol acetates by g.l.c. in column B. The remainder of the material was hydrolyzed with 2M trifluoroacetic acid overnight on a steam bath. Conversion of the partially methylated sugars into alditol acetates and g.l.c. thereof in column B, showed the presence of 2,4,6-tri-O-methylmannose, 2,4,6-tri-O-methylgalactose, and 2,4-di-O-methylglucose in equimolar proportions.
Smith degradation

Polysaccharide (295 mg) in water (100 mL) was mixed with a solution (28 mL) of 0.1M NaIO₄ and 0.4M NaClO₄, and kept in the dark at 4°. After 95 h, ethylene glycol (10 mL) was added. The solution was dialyzed overnight, the polyaldehyde was reduced to the polyalcohol with NaBH₄ (1 g), the base was neutralized with 50% acetic acid, and the solution was dialyzed, and freeze-dried, to yield polyalcohol (258 mg) which was subjected to Smith hydrolysis with 0.5M trifluoroacetic acid for 20 h at room temperature. Paper chromatography (solvent C) of the products showed glycerol and three oligosaccharides. The chromatograms obtained indicated that the Smith hydrolysis was not complete, but 49.6 mg of a pure, acidic oligomer (SH₁) was isolated, \([\alpha]_{D}^{25} +59° (c 0.08, \text{water}) \) and \(R_{\text{Glc}}^o 0.55 \) (solvent C). N.m.r. data are given in Table IV.1. Sugar analysis of the product (by g.l.c., as the alditol acetates) gave galactose and glucose in the ratio of 1:1. Methylation by the Hakomori method gave the partially methylated alditol acetates corresponding to 2,3,4,6-tetra-\(\alpha\)-methylgalactose and 2,4,6-tri-\(\alpha\)-methylglucose.

Enzymatic hydrolysis

The acidic product (SH₁, 2.3 mg) was dissolved in 1 mL of buffer, pH 7.3, and a solution of \(\beta\)-D-galactosidase (1.1 mg, from E. coli, Sigma) in 0.1 mL of the same buffer was added. The mixture was incubated for 3 d at 37°; then, the reaction was terminated by addition of 50% acetic acid. The product, isolated by lyophilization, was examined by paper chromatography (solvent B), which showed the presence of
galactose, the identity of which was confirmed by g.l.c. as galactitol hexaacetate.

In a control experiment, compound SH₁ and melibiose were separately incubated with α-D-galactosidase (pH 4). Galactose was released from melibiose (α-D-galactopyranosyl-(1→6)-D-glucose), but not from SH₁.
CHAPTER V

STRUCTURAL INVESTIGATION OF *Escherichia coli*

CAPSULAR POLYSACCHARIDES
V.1 STRUCTURAL INVESTIGATION OF ESCHERICHIA COLI 09:K28(A):H~
(K28 ANTIGEN) CAPSULAR POLYSACCHARIDE.

V.1.1 ABSTRACT

The structure of the capsular polysaccharide from Escherichia coli 09:K28(A):H~ (K28 antigen) has been determined by using the techniques of methylation, periodate oxidation and partial hydrolysis. N.m.r. spectroscopy (\(^{1}H\) and \(^{13}C\)) was used to establish the nature of the anomeric linkages. Quantitative determination of 0-acetyl groups was done spectrophotometrically. The location of the 0-acetyl group was determined using methyl vinyl ether as a protective reagent.

The polysaccharide is comprised of repeating units of the tetrasaccharide shown (three-plus-one type) with 70% of the fucosyl residues being 0-acetylated at either 0-2 or 0-3.

\[
\begin{align*}
\text{3)} & \alpha-D-Glc-(1\rightarrow4)\beta-D-GlcA-(1\rightarrow4)\alpha-L-Fuc-(1\rightarrow4)\\ & \text{2 or 3} \\
\text{1} & \\
\text{0Ac} & \\
\beta-D-Gal & 
\end{align*}
\]
V.1.2 INTRODUCTION

The K antigens of *E. coli* can be divided into three groups (A, B, L), all of which comprise acidic polysaccharides. Distinctive features of A antigens are that they occur only together with 08 and 09 antigens, that they form thick capsules and that they are free of amino sugars. The extracellular A antigens of *E. coli* bear a strong similarity to the K antigens of *Klebsiella*. The present investigation is thus a logical extension and describes the isolation and structural analysis of an acidic polysaccharide obtained from *E. coli* 09:K28(A):H⁻.

V.1.3 RESULTS AND DISCUSSION

**Isolation and characterization**

A culture of *Escherichia coli* K28 was grown on Mueller Hinton agar and the isolation and purification of the polysaccharide were achieved as described in Section III.7.2. The purified product obtained after Cetavlon precipitation had $[\alpha]_D^{20} -18.2^\circ$ which compares well with the calculated value of $-19.3^\circ$ using Hudson's Rules of Isorotation, and was shown to be heterogeneous by gel-permeation chromatography, containing two components with molecular weight $9 \times 10^6$ daltons (30% by weight) and 450,000 daltons (70% by weight); the average molecular weight $\bar{M}_w$ was $3 \times 10^6$ daltons. The polysaccharide was found to be homogeneous after mild alkali treatment ($\bar{M}_w = 350,000$ daltons). This is a well-known phenomenon for *E. coli* K antigens associated with 0 groups.
08, 09 and 0101. Capsular (K) antigens belonging to this group form very viscous aqueous solutions. Treatment with dilute alkali reduces the viscosity drastically.\textsuperscript{186,187} These findings indicate the presence of inter-chain ester linkages between carboxylic groups of hexuronic acid constituents and hydroxyl groups of sugar rings.\textsuperscript{186,187} The presence of acetate groups could also contribute to the formation of aggregates, since the removal of acetate yields a homogeneous polysaccharide.

The K28 polysaccharide is composed of D-glucose, D-galactose, D-glucuronic acid and L-fucose. It does not contain D-galacturonic acid and D-mannose as was earlier thought.\textsuperscript{188} The presence of glucose, galactose, fucose, glucuronic acid and an aldobiouronic acid in the acid hydrolyzate of the polysaccharide was observed by paper chromatography. Determination of the neutral sugars as the alditol acetates gave fucose, galactose and glucose in the ratios of 0.45:1:0.76. The carboxyl-reduced polysaccharide\textsuperscript{75} gave fucose, galactose and glucose in the ratios of 1:1.1:1.58, indicating that the uronic acid is glucuronic acid.

\textbf{\textsuperscript{1}H-n.m.r. spectroscopy}

The \textsuperscript{1}H-n.m.r. spectrum of the \textit{E. coli} K28 polysaccharide (see Appendix III, spectrum No.) indicated the repeating unit to be a tetrasaccharide and to contain 50\% of \textsubscript{O}-acetyl groups (see Table V.1). The spectrophotometric \textsubscript{O}-acetyl determination showed that 70\% of the polysaccharide was acetylated with one acetyl group per repeat unit.

The spectrum exhibits a signal at $\delta = 1.3$ which arises from the CH\textsubscript{3} group of L-fucose. Two singlets at $\delta 2.15$ and $\delta 2.18$ are due to the
<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>$^1\text{H-N.m.r. data}$</th>
<th>$^1\text{H-N.m.r. data}$</th>
<th>Integral</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta^a$</td>
<td>$J_{1,2}^b$</td>
<td>proton</td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>5.41</td>
<td>b</td>
<td>2.0</td>
<td>${\alpha$-Glc</td>
</tr>
<tr>
<td></td>
<td>4.93</td>
<td>b</td>
<td>1.0</td>
<td>$\alpha$-Fuc</td>
</tr>
<tr>
<td></td>
<td>4.86</td>
<td>b</td>
<td>1.0</td>
<td>$\beta$-GlcA</td>
</tr>
<tr>
<td></td>
<td>4.48</td>
<td>b</td>
<td>1.0</td>
<td>$\beta$-Gal</td>
</tr>
<tr>
<td></td>
<td>4.44</td>
<td>b</td>
<td>1.0</td>
<td>ring proton</td>
</tr>
<tr>
<td></td>
<td>2.18</td>
<td>s</td>
<td>~1</td>
<td>$\text{CH}_3$ of $\alpha$-acetyl</td>
</tr>
<tr>
<td></td>
<td>2.15</td>
<td>s</td>
<td></td>
<td>$\text{CH}_3$ of $\alpha$-Fuc</td>
</tr>
<tr>
<td></td>
<td>1.30</td>
<td>b</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>Native, after</td>
<td>5.41</td>
<td>s</td>
<td>1.0</td>
<td>$\alpha$-Glc</td>
</tr>
<tr>
<td>autohydrolysis</td>
<td>5.39</td>
<td>s</td>
<td>1.0</td>
<td>$\alpha$-Fuc</td>
</tr>
<tr>
<td>(100°, overnight)</td>
<td>4.94</td>
<td>8.0</td>
<td>&gt;1</td>
<td>$\beta$-GlcA</td>
</tr>
<tr>
<td></td>
<td>4.83</td>
<td>8.0</td>
<td></td>
<td>$\beta$-Gal</td>
</tr>
<tr>
<td></td>
<td>4.42</td>
<td>8.0</td>
<td>1.0</td>
<td>ring proton</td>
</tr>
<tr>
<td></td>
<td>2.18</td>
<td>s</td>
<td>1.4</td>
<td>$\text{CH}_3$ of $\alpha$-acetyl</td>
</tr>
<tr>
<td></td>
<td>2.15</td>
<td>s</td>
<td></td>
<td>$\text{CH}_3$ of $\alpha$-Fuc</td>
</tr>
<tr>
<td></td>
<td>1.30</td>
<td>6.0(J$_{5,6}$)</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chemical shift</td>
<td>Multiplicity</td>
<td>Coupling constant (Hz)</td>
<td>Assignments</td>
</tr>
<tr>
<td>-------</td>
<td>----------------</td>
<td>--------------</td>
<td>------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Native, after carbodiimide reduction</td>
<td>5.43</td>
<td>s</td>
<td>2.0</td>
<td>α-Glc</td>
</tr>
<tr>
<td></td>
<td>5.37</td>
<td>s</td>
<td></td>
<td>α-Fuc</td>
</tr>
<tr>
<td></td>
<td>4.95</td>
<td>b</td>
<td>0.76</td>
<td>β-Glc</td>
</tr>
<tr>
<td></td>
<td>4.85</td>
<td>b</td>
<td>2.0</td>
<td>β-Gal</td>
</tr>
<tr>
<td></td>
<td>4.47</td>
<td>b</td>
<td></td>
<td>ring proton</td>
</tr>
<tr>
<td></td>
<td>2.20</td>
<td>s</td>
<td>0.6</td>
<td>CH₃ of O-acetyl</td>
</tr>
<tr>
<td></td>
<td>2.16</td>
<td>s</td>
<td></td>
<td>CH₃ of α-Fuc</td>
</tr>
<tr>
<td></td>
<td>1.31</td>
<td>b</td>
<td>3.0</td>
<td>CH₃ of α-Fuc</td>
</tr>
<tr>
<td>Deacetylated</td>
<td>5.41</td>
<td>s</td>
<td>1.0</td>
<td>α-Glc</td>
</tr>
<tr>
<td></td>
<td>5.39</td>
<td>s</td>
<td>1.0</td>
<td>α-Fuc</td>
</tr>
<tr>
<td></td>
<td>4.83</td>
<td>s</td>
<td>1.0</td>
<td>β-Glc</td>
</tr>
<tr>
<td></td>
<td>4.48</td>
<td>s</td>
<td>1.0</td>
<td>β-Gal</td>
</tr>
<tr>
<td></td>
<td>4.42</td>
<td>s</td>
<td>1.0</td>
<td>ring proton</td>
</tr>
<tr>
<td></td>
<td>1.30</td>
<td>b</td>
<td>3.0</td>
<td>CH₃ of α-Fuc</td>
</tr>
</tbody>
</table>

a Chemical shift relative to internal acetone; δ 2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.). b Key: b = broad, unable to assign accurate coupling constant; s = singlet.
presence of OCOCH₃ groups. The presence of twin signals can be attributed to the location of O-acetyl groups on both O-2 and O-3 positions of fucose. In the spectrum of deacetylated *E. coli* K28 polysaccharide those signals were absent. In the anomeric region four signals were detected. The signal at δ 4.48 represents β-linked galactose. Two signals at δ 4.86 and δ 4.93 belong to the β-glucuronic acid. The twinning is attributed to the presence of O-acetyl groups on the adjacent fucose, since, after deacetylation, it disappeared giving rise to a signal at δ 4.83. A broad signal at δ 5.41 represents the two anomeric protons of α-L-fucose and α-D-glucose. Generally, the ¹H-n.m.r. spectrum was not well resolved due to the extreme viscosity of the solution. The quality of the spectrum was improved after autohydrolysis of the polysaccharide (100°, overnight). A good spectrum was also obtained after deacetylation of the polysaccharide due to the decrease in viscosity.

**¹³C-N.m.r. spectroscopy**

In the ¹³C-n.m.r. spectra of the native and O-deacetylated K28 polysaccharides (see Appendix III, Spectra No. 12) the signals arising from CH₃CO (21.39 p.p.m.) and CH₃C=O (175.62 p.p.m.) were absent in the spectrum of the deacetylated polysaccharide (see Table V.2). The signals for anomeric carbons in the deacetylated polysaccharide were only slightly changed in position (see Table V.2). Carbons 2 and 3 of the fucosyl residue exhibited downfield shifts (Δδ = 2.9 p.p.m. for C-2 and Δδ = 2.67 p.p.m. for C-3) in the native polysaccharide due to the presence of acetate. Once the acetate was removed the signals for C-2
**TABLE V.2**

**$^{13}$C-N.M.R. DATA FOR THE NATIVE AND O-DEACETYLATED *E. COLI* K28 POLYSACCHARIDE**

<table>
<thead>
<tr>
<th>Native polysaccharide</th>
<th>p.p.m.</th>
<th>Assignment $^b$</th>
<th>O-Deacetylated polysaccharide</th>
<th>p.p.m.</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>102.79</td>
<td>C-1 $\beta$-GlcA</td>
<td>103.91</td>
<td>C-1 $\beta$-GlcA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>99.30</td>
<td>C-1 $\beta$-Gal</td>
<td>102.81</td>
<td>C-1 $\beta$-Gal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>99.26</td>
<td>C-1 $\alpha$-Fuc</td>
<td>99.32</td>
<td>C-1 $\alpha$-Fuc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>76.94</td>
<td>C-3 $\beta$-GlcA</td>
<td>77.97</td>
<td>C-3 $\beta$-GlcA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>76.89</td>
<td>C-4 $\alpha$-Fuc</td>
<td>76.88</td>
<td>C-4 $\alpha$-Fuc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75.91</td>
<td>C-5 $\beta$-Gal</td>
<td>75.93</td>
<td>C-5 $\beta$-Gal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>74.34</td>
<td>C-3 $\alpha$-Glc</td>
<td>74.40</td>
<td>C-3 $\alpha$-Glc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>73.74</td>
<td>C-4 $\beta$-GlcA</td>
<td>73.74</td>
<td>C-4 $\beta$-GlcA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>73.46</td>
<td>C-2 $\beta$-GlcA</td>
<td>73.43</td>
<td>C-2 $\beta$-GlcA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>73.33</td>
<td>C-4 $\beta$-Gal</td>
<td>72.21</td>
<td>C-4 $\beta$-Gal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72.27</td>
<td>C-4 $\alpha$-Fuc</td>
<td>72.21</td>
<td>C-4 $\alpha$-Fuc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72.14</td>
<td>C-3 $\alpha$-Fuc</td>
<td>69.47</td>
<td>C-3 $\alpha$-Fuc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>72.10</td>
<td>C-2 $\alpha$-Fuc</td>
<td>69.19</td>
<td>C-2 $\alpha$-Fuc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>69.11</td>
<td>unassigned</td>
<td>67.57</td>
<td>unassigned</td>
<td></td>
<td></td>
</tr>
<tr>
<td>62.28</td>
<td>C-6 $\alpha$-Glc, $\beta$-Gal</td>
<td>62.26</td>
<td>C-6 $\alpha$-Glc, $\beta$-Gal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.39</td>
<td>CH$_3$ of acetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16.02</td>
<td>CH$_3$ of $\alpha$-Fuc</td>
<td>16.12</td>
<td>CH$_3$ of $\alpha$-Fuc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.97</td>
<td></td>
<td>16.06</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Chemical shift in p.p.m. downfield from Me$_4$Si, relative to internal acetone; 31.07 p.p.m. downfield from DSS. $^b$ The assignments were made by comparison with the literature values, see ref. 166.
and C-3 carbons of fucose shifted upfield and are in agreement with the literature values. These data, together with \(^1\text{H-n.m.r.}\) findings, suggest that \(\text{O-}\text{acetyl}\) groups are distributed between \(\text{O-2}\) and \(\text{O-3}\) of the \(\alpha\text{-L-fucosyl}\) residues. The presence of \(\text{O-}\text{acetyl}\) on \(\text{O-3}\) of fucose can explain the splitting of the anomeric signal assigned to \(\beta\text{-D-glucuronic acid}\). A molecular model (Dreiding) shows that only in this case is there a possibility of intermolecular interaction between H-1 of glucuronic acid and the carbonyl group of the acetate (see Fig. V.1).

![Figure V.1](image)

**Fig. V.1:** Partial structure of *E. coli* K28 polysaccharide

These conclusions were reinforced by the results of periodate oxidation of the native and deacetylated polysaccharides and the positions of the \(\text{O-}\text{acetyl}\) groups were determined by ethylation (see later).

**Methylation analysis**

Methylation of the K28 polysaccharide, followed by hydrolysis, derivatization as alditol acetates, and g.l.c.-m.s. analysis, gave the values shown in Table V.3, column I. These results indicated that the polysaccharide consists of a tetrasaccharide repeating unit having a branch on glucose, with galactose as the terminal group. By reduction
TABLE V.3

METHYLATION ANALYSIS OF *Escherichia coli* K28 POLYSACCHARIDE AND DERIVED PRODUCTS

<table>
<thead>
<tr>
<th>Methylated sugar (as alditol acetate)</th>
<th>Mol %&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2,3,4-Fuc</td>
<td>-</td>
</tr>
<tr>
<td>2,3-Fuc</td>
<td>23</td>
</tr>
<tr>
<td>2,3,4,6-Glc</td>
<td>-</td>
</tr>
<tr>
<td>2,3,4,6-Gal</td>
<td>37</td>
</tr>
<tr>
<td>2,4,6-Glc</td>
<td>-</td>
</tr>
<tr>
<td>2,3,6-Glc</td>
<td>-</td>
</tr>
<tr>
<td>2,6-Glc</td>
<td>40</td>
</tr>
<tr>
<td>2,3-Glc</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> 2,3,4-Fuc = 1,5-di-O-acetyl-2,3,4-tri-O-methylfucitol, etc.  
<sup>b</sup> Values are corrected by use of the effective, carbon-response factors given by Albersheim *et al.*<sup>103</sup> using an OV-225 column, programmed from 180° for 4 min, and then at 2°/min to 230°.  
<sup>c</sup> I, original polysaccharide; II, compounds from LiAlH<sub>4</sub> reduction of methylated *E. coli* K28; III, carbodiimide-reduced polysaccharide; IV, product from Smith degradation of the original polysaccharide; V, product from periodate oxidation of deacetylated polysaccharide.
of the methylated polysaccharide (see Table V.3, column II), the proportion of 2,3-di-0-methylfucose was increased, and 2,3-di-0-methylglucose was formed, indicating that glucuronic acid is linked through O-4, and that it is joined to fucose. Methylation analysis of carbodi-imide-reduced polysaccharide showed the presence of 2,3,6-tri-0-methylglucose, derived from reduction of the carboxyl group of the glucuronic acid (see Table V.3, column III).

**Partial hydrolysis**

Partial hydrolysis of the native polysaccharide with acid was followed by separation of the acidic and the neutral fractions by ion-exchange chromatography. The neutral fraction contained monosaccharides and a disaccharide (N1). The acidic fraction contained an aldobiouronic acid (A1). On the basis of their n.m.r. spectral data (Table V.4) and their methylation analysis (Table V.5), the structures of these compounds were shown to be as follows:

\[ \text{A1} \sim \beta\text{-GlcA-(1\rightarrow4)-Fuc} \]
\[ \text{N1} \sim \beta\text{-Gal-(1\rightarrow4)-Glc} \]

**Periodate oxidation**

Smith degradation of the native polysaccharide, followed by methylation and hydrolysis showed the presence of 2,3,4-tri-0-methylfucose, 2,3,4,6-tetra-0-methylglucose and 2,4,6-tri-0-methylglucose (see Table V.3, column IV). These results show that terminal galactose and the glucuronic acid were completely oxidized thus indicating the absence
### TABLE V.4

**N.M.R. DATA FOR Escherichia coli K28 OLIGOSACCHARIDES DERIVED FROM PARTIAL HYDROLYSIS OF THE POLYSACCHARIDE**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$^1$H-N.m.r. data</th>
<th>$^1^3$C-N.m.r. data</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta^a$</td>
<td>$J_{1,2}$ (Hz)</td>
<td>Integral proton</td>
</tr>
<tr>
<td>GlcA-1(\beta)-Fuc-OH</td>
<td>5.24</td>
<td>2.7</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.63</td>
<td>5.4</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>4.54</td>
<td>8.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>4.29</td>
<td>q</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1.33</td>
<td>6.75</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1.29</td>
<td>6.75</td>
<td>3.0</td>
</tr>
<tr>
<td>Gal(1(\beta)-Glc-OH</td>
<td>5.23</td>
<td>4</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.67</td>
<td>7</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>4.45</td>
<td>7.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

$^a$ Chemical shift relative to internal acetone; δ 2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.).  
$^b$ The numerical prefix indicates the position in which the sugar is substituted; the $\alpha$ or $\beta$, the configuration of the glycosidic bond, or the anomer in the case of a (terminal) reducing-sugar residue.  
$^c$ Chemical shift in p.p.m. downfield from Me$_4$Si, relative to internal acetone; 31.07 p.p.m. downfield from D.S.S.  
$^d$ As in $^c$, but for $^1^3$C nuclei.
TABLE V.5

ANALYSIS OF THE OLIGOSACCHARIDES FROM PARTIAL HYDROLYSIS OF *Escherichia coli* K28 POLYSACCHARIDE

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Sugar analysis</th>
<th>As alditol acetates (molar proportions)</th>
<th>Methylation analysis</th>
<th>As alditol acetates (molar proportions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>Fuc</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Glc(GlcA)</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nl</td>
<td>Gal</td>
<td>1.0</td>
<td>2,3,4,6-Gal</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>Glc</td>
<td>0.9</td>
<td>2,3,6-Glc</td>
<td>1.00</td>
</tr>
</tbody>
</table>
of an O-acetyl group on either of them. The fucosyl linkage was hydrolyzed during the Smith degradation giving rise to 2,3,4,6-tetra-O-methylglucose, proving that fucose and glucose are engaged in a 1→3 linkage. The proportion of 2,3,4-tri-O-methylfucose found indicates that 70% of the fucose survived periodate oxidation. This result suggests that 70% of the fucosyl linkages are O-acetylated at O-2 or O-3 and are thus protected against oxidative degradation.

Periodate oxidation of deacetylated polysaccharide followed by methylation and hydrolysis showed that only 25% of the fucose had survived (see Table V.3, column V). The incomplete oxidation of fucose could be explained on the basis of steric hindrance and possible hemiacetal formation.133

**Quantitative determination of O-acetyl groups**

The percentage of O-acetyl groups present in *E. coli* K28 polysaccharide was determined spectrophotometrically.178 It was found that 72-74% of the native polysaccharide was O-acetylated. The acetate groups could be easily removed by mild alkali treatment. These findings are in a very good agreement with the results obtained after periodate oxidation of the acetylated and deacetylated polysaccharides. Partial removal of O-acetyl groups during the high temperature n.m.r. can be explained on the basis of the lability of the O-acetyl groups located on the fucosyl residues.
Location of O-acetyl groups

O-Acetyl groups in the polysaccharide were located by reaction with methyl vinyl ether and an acidic catalyst, followed by ethylation analysis of the product. It was found that either O-2 or O-3 of the α-L-fucosyl residues is acetylated but no 2,3-di-O-ethylfucose was obtained.

V.1.4 CONCLUSION

From the sum of these experiments the complete structure of the capsular polysaccharide from *Escherichia coli* K28 may be written.

\[
\text{α-D-Glc-(1→4)-β-D-GlcA-(1→4)-α-L-Fuc-(1→4)}
\]

\[
\text{2 or 3}
\]

\[
\text{1 OAc}
\]

\[
\text{β-D-Gal}
\]

This structure closely resembles that of the capsular antigens from *E. coli* K27 and is of the same structural pattern as that of the capsular polysaccharide from *Klebsiella* K54.
\( \alpha-Glc-(1\rightarrow4)-\alpha-GlcA-(1\rightarrow3)-\alpha-Fuc-(1\rightarrow3) \)
\[ \uparrow \]
\[ 1 \]
\[ \alpha-Gal \]

**E. coli K27**

\( \beta-Glc-(1\rightarrow4)-\alpha-GlcA-(1\rightarrow3)-\alpha-Fuc(1\rightarrow4) \)
\[ \uparrow \]
\[ 2 \]
\[ 1 \]
\[ \beta-Glc \]

**Klebsiella K54**
V.1.5 EXPERIMENTAL

General methods

The instrumentation used for infrared and n.m.r. spectroscopy, g.l.c., and g.l.c.-m.s., c.d., and measurements of optical rotation has been described in Section III. Spectrophotometric measurements were made by using a Perkin-Elmer 552A UV/VIS spectrophotometer. Paper chromatography, gas-liquid chromatography, and ion-exchange chromatography were performed as described in Section III.

Preparation and properties of E. coli K28 capsular polysaccharide

A culture of E. coli K28 was obtained from Dr. Ida Ørskov (Copenhagen). The bacteria were first grown on sucrose-rich medium as previously described for Klebsiella capsular polysaccharides. The results were not satisfactory and the yield of polysaccharide was very poor. In order to find suitable growth conditions six different media were tried: (1) trypticase soy agar (BBL); (2) Luria broth (Difco); (3) nutrient broth (Difco); (4) nutrient broth + yeast extract (Difco); (5) beef heart infusion (Difco); (6) Mueller Hinton agar (BBL). Sodium chloride improves the growth of E. coli and was used in preparation of all six media (0.5% w/v). The streaked plates were incubated at 37° overnight. The best results were obtained on Mueller Hinton agar and it was later used as a growth medium with the addition of a small amount of NaCl (0.5% w/v).

The purification procedure was carried out as described in Section III.7.2. Yield: acidic polysaccharide ~ 400 mg, neutral poly-
saccharide ~ 100 mg (per batch). Three different batches of the polysaccharide were grown.

The molecular weight of the polysaccharide was determined by gel-permeation chromatography (courtesy of Dr. S.C. Churms, University of Cape Town, South Africa). The native polysaccharide was shown to be heterogeneous, but became homogeneous after mild alkali treatment ($M_w = 350,000$ daltons). N.m.r. spectroscopy ($^1H$ and $^{13}C$) was performed on the original and deacetylated K28 polysaccharide. The principal signals in the $^1H$- and $^{13}C$- n.m.r. spectra and their assignments are recorded in Tables V.1 and V.2 respectively.

**Deacetylation of polysaccharide**

The polysaccharide was dissolved in 0.01M NaOH and stirred overnight at room temperature. The product was dialyzed against tap water and freeze-dried. The completeness of deacetylation was checked by $^1H$-n.m.r. spectroscopy.

**Hydrolysis of the polysaccharide**

Hydrolysis of a sample (4 mg) of K28 polysaccharide with 2M trifluoroacetic acid (TFA) for 18 h at 95°, removal of the acid by successive evaporations with water, followed by paper chromatography (solvents 1 and 2) showed fucose, glucose, galactose, glucuronic acid, and an aldobiouronic acid. Quantitative sugar analysis of the carboxyl-reduced polysaccharide was performed, and the alditol acetates of fucose, glucose, and galactose were identified by g.l.c. (column A)
and found to be present in the ratios of 1:1:1:1.58. Preparative g.l.c. (column D), followed by measurements of the circular dichroism spectra, showed the glucitol hexaacetate to be of the D configuration, and the fucitol pentaacetate to be of the L configuration. Galactose was assigned the D configuration by the positive action of D-galactose oxidase (Worthington Biochem. Co.) on the hydrolysis product of the polysaccharide.

Methylation analysis

The capsular polysaccharide (60 mg) in the free-acid form, obtained by passing the sodium salt through a column of Amberlite IR-120 (H⁺) resin, was dissolved in dry dimethyl sulfoxide (6 mL) and methylated by the Hakomori procedure. The product, recovered after dialysis against tap water, was not completely methylated (hydroxyl absorption in the i.r. spectrum). It was dissolved in chloroform and subjected to Purdie methylation with methyl iodide and silver oxide. This treatment yielded a fully methylated polysaccharide (57 mg). A portion of this product (5 mg) was hydrolyzed with 2M trifluoroacetic acid, the sugars were reduced with sodium borohydride, the alditols were acetylated with 1:1 acetic anhydride—pyridine, and analyzed by g.l.c. in column C (see Table V.3, column I). Carboxyl reduction of the fully methylated polysaccharide (12.1 mg) with LiAlH₄ in anhydrous oxolane at room temperature overnight, hydrolysis of the product with 2M trifluoroacetic acid, followed by reduction of sugars with sodium-borohydride, and acetylation of the alditols with 1:1 acetic anhydride—pyridine gave a mixture of partially methylated alditol acetates which
was analyzed by g.l.c. and g.l.c.-m.s. in column C (see Table V.3, column I). The neutral polysaccharide obtained by carbodiimide reduction was also subjected to methylation analysis. A sample (9.4 mg) of carbodiimide-reduced polysaccharide was dissolved in dry dimethyl sulfoxide (1 mL) and methylated by treatment with 1 mL dimethylsulfinyl anion for 4 h, and then 2 mL methyl iodide for 1 h. The product recovered after dialysis against tap water, was hydrolyzed with 2M trifluoroacetic acid overnight at 95°, converted into partially methylated alditol acetates and analyzed, with the results shown in Table V.3, column III.

**Carbodiimide reduction of the capsular polysaccharide**

A sample of K28 polysaccharide (Na⁺ salt, 36.2 mg) was dissolved in 20 mL water. l-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (CMC, 0.4 g) was added. As the reaction proceeded, the pH was maintained at 4.75 by titration with 0.1 N HCl. The reaction was allowed to proceed for at least two h. When the consumption of hydrochloric acid ceased, an aqueous solution of 2M sodium borohydride (1 g/15 mL H₂O) was added slowly. The pH rose rapidly to 7.0 and it was maintained between 5-7 by titration with 4M HCl. A drop of 2-octanol (antifoaming agent) was added periodically to control the amount of foam. The reduction was completed in one h. The mixture was dialyzed against tap water during 24 h, concentrated and freeze-dried. A second treatment was carried out. Yield: 32.3 mg. A sample of the reduced polysaccharide (11.1 mg) was hydrolyzed overnight with 2M trifluoro-
acetic acid at 95°, and the hydrolyzate was converted into alditol acetates as before. G.l.c. analysis was conducted in column A.

**Partial hydrolysis**

The K28 polysaccharide (514 mg) was dissolved in 125 mL of 0.01M trifluoroacetic acid (TFA) and the solution was heated for 32 h on a steam bath. After removal of the acid by successive evaporations with water, an acidic and a neutral fraction were separated on a column of Bio-Rad AG1-X2 ion-exchange resin. The acidic fraction was separated by preparative paper chromatography (solvent 3), to give 45.7 mg of a pure aldobiouronic acid (Al). Paper chromatography of the neutral fraction showed fucose, glucose, galactose and a neutral disaccharide (Nl), which was isolated by preparative paper chromatography (solvent 3) to give 21 mg of Nl. P.m.r. spectral data are recorded (Table V.4) for each oligosaccharide and analyses were performed as follows: (a) Sugar analysis. The acidic oligosaccharide was treated with 3% HCl in anhydrous methanol for 18 h on a steam bath. The methyl ester methyl glycoside obtained was reduced with sodium borohydride in anhydrous methanol, followed by hydrolysis with 2M TFA, reduction to the alditols, and acetylation with 1:1 acetic anhydride—pyridine. The alditol acetates obtained were analyzed by g.l.c. in column A. The neutral oligosaccharide was hydrolyzed, and analyzed similarly. (b) Methylation analysis. Compound Nl was methylated by the method of Hakomori and the results given in Table V.5.
Periodate oxidation

A solution of K28 polysaccharide (21.5 mg) in water (10 mL) was mixed with 0.03M NaIO₄ (10 mL) and stirred in the dark at room temperature (23°) for 6 d. After ethylene glycol (2 mL) was added, the polyaldehyde was reduced to the polyalcohol with NaBH₄, the base was neutralized with 50% AcOH, the solution was dialyzed overnight, and freeze-dried to yield the polyalcohol (15 mg). A portion (4.2 mg) was hydrolyzed with 2M TFA overnight on a steam bath and converted into alditol acetates. Analysis by g.l.c. in column A showed fucitol, galactitol and glucitol in the ratios of 0.72:0.10:1. The remainder of the material was treated with 0.5M TFA for 48 h at room temperature. The product (10.7 mg) was methylated by the Hakomori procedure, hydrolyzed with 2M TFA overnight on a steam bath and converted into alditol acetates. G.l.c. analysis, conducted in column B showed the presence of 2,3,4-tri-O-methylfucose, 2,3,4,6-tetra-O-methylglucose and 2,4,6-tri-O-methylglucose in the ratios of 0.70:0.35:0.65. These results indicate that during the Smith degradation partial hydrolysis of the fucosyl linkage has occurred.

Periodate oxidation of deacetylated polysaccharide was performed similarly. A fraction of the polyalcohol product (1.6 mg) was analyzed for constituent sugars and the remainder of the material was methylated by the Hakomori procedure. Conversion of the partially methylated sugars into alditol acetates, and g.l.c. thereof in column B, showed the presence of 2,3-di-O-methylfucose and 2,6-di-O-methylglucose in the ratios 0.25:1.00.
Quantitative determination of O-acetyl groups

O-Acetyl groups were determined spectrophotometrically. The reaction of O-acetyl groups with hydroxylamine in alkali to form hydroxamic acid was employed. The hydroxamic acid was measured by the formation of a colored complex with Fe$^{3+}$ in acid solution. To 1 mL of solution which contained 40 µg of the *E. coli* K28 polysaccharide in 0.001M sodium acetate buffer 2 mL of a freshly prepared 1:1 mixture of 2M hydroxylamine hydrochloride-3.5M NaOH was added. After 2 min at room temperature 1 mL of 12% hydrochloric acid was added followed by 1 mL of 0.37M FeCl$_3$•6H$_2$O in 0.1N HCl. During the addition of each reagent the solution was swirled rapidly. The absorbance at 540 nm of the resulting purple-brown solution was measured in a Perkin-Elmer 552A UV/VIS spectrophotometer. After correcting for non-specific color, the quantity of O-acetyl was calculated from a standard curve prepared using 0.004M acetyl choline chloride in 0.001M sodium acetate pH 4.5.

Location of O-acetyl groups

*E. coli* K28 polysaccharide (17.6 mg) and p-TsOH (5 mg) were dried overnight under vacuum and dissolved in dry dimethyl sulfoxide (10 mL). Methyl vinyl ether (3 mL) was added to the frozen solution, and the reaction mixture was brought to room temperature and allowed to stir for 4 h. Then a second portion of methyl vinyl ether (3 mL) was introduced in a similar manner. The clear, red solution obtained was placed on a Sephadex LH-20 column (58 cm x 1.5 cm) and eluted with acetone (with slight suction). The product was collected and concentrated. Half of the residue was used for ethylation according to the Hakomori
procedure. The product, a dark red-orange oil, was dialyzed (cutoff 3,500) overnight against tap water and extracted with chloroform. The portion of ethylated, methyl vinyl ether protected polysaccharide was hydrolyzed with 2M TFA overnight at 95° and converted into alditol acetates. G.l.c. analysis, conducted in column B (210° isothermal) showed the presence of 2-0-ethylfucose (16.9%), 3-0-ethylfucose (23.8%), fucose (12.7%), galactose (25.1%), and glucose (21.6%). These results were confirmed by g.l.c.-m.s., which was performed on a KRATOS MS80RFA instrument, using DB-225 capillary column (150° for 1 min, and then 10°/min to 210°).
V.2 STRUCTURAL INVESTIGATION OF *Escherichia coli* 09:K32(A):H19 (K32 ANTIGEN) CAPSULAR POLYSACCHARIDE

V.2.1 ABSTRACT

The structure of the capsular polysaccharide from *Escherichia coli* 09:K32(A):H19 (K32 antigen) has been determined by using the techniques of methylation, carboxyl reduction and Smith degradation. The nature of the anomeric linkages was established by using $^1$H- and $^{13}$C-n.m.r. spectroscopy, and was further confirmed by chromium trioxide oxidation of the fully acetylated polysaccharide. The location of β-acetyl groups was determined using methyl vinyl ether as a protective reagent.

The polymer was found to consist of the tetrasaccharide repeating unit shown (three-plus-one type) with half of the L-rhamnosyl residues being β-acetylated at β-2.

$$\begin{align*}
\text{OAc} \\
\uparrow \quad 2 \\
\rightarrow 3\text{-α-D-Glc-(1\rightarrow 4)-α-L-Rha-(1\rightarrow 3)-α-D-Gal-(1→)} \\
\uparrow \quad 3 \\
\uparrow \quad 1 \\
\text{β-D-GlcA}
\end{align*}$$
Escherichia coli serotype K32 is of interest in this series, being a specific host for three different \textit{E. coli} capsular bacteriophages φ28-1, φ31 and φ32. Because of the increasing importance of studies on the substrate specificity of the bacteriophage-borne enzymes, the complete structural analysis of \textit{E. coli} polysaccharide is needed.

Although the composition of the \textit{E. coli} K32 capsule is known, its structure is not. In continuation of our chemical examination of this genus, we now report the primary structure of \textit{Escherichia coli} K32.

RESULTS AND DISCUSSION

A culture of \textit{Escherichia coli} K32 was grown on Mueller Hinton agar, and the acidic polysaccharide was purified by one precipitation with cetyltrimethylammonium bromide. The polysaccharide obtained had $[\alpha]_D^{\text{+}} +80.9^\circ$ which compares well with the calculated value of $+71.5^\circ$ using Hudson's Rules of Isorotation. It was shown to be homogeneous by gel-permeation chromatography with $M_W = 9 \times 10^6$ daltons. Treatment with dilute alkali did not reduce the viscosity of the polysaccharide significantly ($M_W = 6 \times 10^6$ daltons).

Paper chromatography of an acid hydrolyzate of the polysaccharide showed galactose, glucose, glucuronic acid, rhamnose and an aldobiouronic acid. Determination of the neutral sugars as the alditol
acetates gave rhamnose, galactose, and glucose in the ratios of 0.7:1.0:1.5. The carboxyl-reduced polysaccharide gave rhamnose, galactose, and glucose in the ratios of 0.9:1.0:1.9, indicating that the uronic acid is glucuronic acid. The glucose was proved to be of the D and rhamnose to be of the L configuration by circular dichroism measurements made on the alditol acetates. Galactose was assigned the D configuration by the positive action of D-galactose oxidase on the hydrolysis product of the polysaccharide.

N.m.r. spectroscopy

The $^1$H-n.m.r. spectrum of the E. coli K32 polysaccharide (see Appendix III, spectrum No. 20) indicated the repeating unit to be a tetrasaccharide and to contain 0.5 O-acetyl groups. The spectrum exhibits a signal at $\delta = 1.34$ which arises from the CH$_3$ group of L-rhamnose. The signal at $\delta = 2.18$ is due to the presence of COOCH$_3$ groups. In the anomeric region six principal signals can be detected. The signal at $\delta = 4.73$ represents $\beta$-linked glucuronic acid which was further confirmed by chromic acid oxidation of the fully acetylated polysaccharide (see later). The signal at $\delta = 5.52$ belongs to the $\alpha$-rhamnosyl residue bearing an O-acetyl group, and it shifts to $\delta = 5.25$ on the deacetylation of the polysaccharide. This suggests that the O-acetyl group is located on O-2 of the $\alpha$-rhamnosyl residue. The signal at $\delta = 5.11$ belongs probably to $\alpha$-Glc and it remains unchanged after O-deacetylation of the polysaccharide. The twinned signals at $\delta = 5.19$ and $\delta = 5.16$ disappear on deacetylation giving rise to a singlet at $\delta = 5.20$. However, the definitive assignment of $\alpha$-Glc and $\alpha$-Gal cannot be
made on the basis of the $^1$H-n.m.r. spectral data (see Table V.6).

In the $^{13}$C-n.m.r. spectra of the native and O-deacetylated E. coli K32 polysaccharides (see Appendix III, Spectra No. 21 and 23) the signal arising from CH$_3$CO (21.18 p.p.m.) was absent in the spectrum of the deacetylated polysaccharide. The positions of the anomeric signals were changed. Carbon 1 of the $\alpha$-rhamnosyl residue exhibited a definite downfield shift due to the presence of the acetate in the native polysaccharide. However, the unambiguous assignment of the anomeric carbons could not be made (see Table V.6).

**Methylation analysis**

Methylation analysis of the E. coli K32 polysaccharide, followed by hydrolysis, derivatization as alditol acetates, and g.l.c.-m.s. analysis, gave the values shown in Table V.7, column I. These results indicate that the polysaccharide consists of a tetrasaccharide repeating unit having a branch on rhamnose with glucuronic acid as the terminal group. By reduction of the methylated polysaccharide (see column II), the proportion of 2-0-methylrhamnose was increased, and 2,3,4-tri-0-methylglucose was formed, indicating that glucuronic acid is terminal, and that it is linked to rhamnose. Methylation analysis of carbodiimide-reduced polysaccharide$^{75}$ showed the presence of 2,3,4,6-tetra-0-methylglucose, derived from reduction of the carboxylic group of the glucuronic acid (see Table V.7, column III).
TABLE V.6

N.M.R. DATA FOR *Escherichia coli* K32 NATIVE AND O-DEACETYLATED POLYSACCHARIDES

<table>
<thead>
<tr>
<th>Compound</th>
<th>$^1$H-N.m.r. data</th>
<th>$^{13}$C-N.m.r. data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\Delta^\alpha$</td>
<td>Integral (H)</td>
</tr>
<tr>
<td>native</td>
<td>5.52</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>5.25</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>5.19</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>5.16</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>5.11</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>5.05</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>5.02</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>4.73 (J$_1$,2=8Hz)</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>2.18</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>1.34</td>
<td>6.1</td>
</tr>
<tr>
<td>O-deacetylated</td>
<td>5.52</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>5.25</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>5.20</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>5.11</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>4.73 (J$_1$,2=8Hz)</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>2.18</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>1.34</td>
<td>3.2</td>
</tr>
</tbody>
</table>

*a* Chemical shift relative to internal acetone; $\delta$ 2.23 for $^1$H-n.m.r. and 31.07 p.p.m. for $^{13}$C-n.m.r. downfield from sodium 4,4-dimethyl-4-sila-pentane-l-sulfonate (D.S.S.). $b$ The assignment was made by comparison with literature values, see ref. 166. $c$ A definite assignment could not be made; the assignments of $\alpha$-Glc and $\alpha$-Gal are tentative.
### TABLE V.7

**METHYLATION ANALYSIS OF *ESCHERICHIA COLI* K32 POLYSACCHARIDE AND DERIVED PRODUCTS**

| Methylated sugar (as alditol acetate) | T<sup>a</sup> | Column B<sup>c</sup> (ECNSS-M) | Mol %<sup>b</sup> | | | | |
|--------------------------------------|------------|--------------------------------|-----------------|---|---|---|
| 2,3-Rha<sup>e</sup>                  | 0.99       |                                |                 |   |   | 42.7|
| 2,3,4,6-Glc                         | 1.00       |                                |                 |   | 26.5|   |
| 2,3,4,6-Gal                         | 1.24       |                                |                 |   |   | 17.5|
| 2-Rha                               | 1.55       | 32.5                           | 16.4            | 26.5 | 10.3|
| 2,4,6-Glc                           | 1.96       | 53.6                           | 38.7            | 23.5 | 29.3|
| 2,4,6-Gal                           | 2.26       | 13.6                           | 25.7            | 23.5 |   |
| 2,3,4-Glc                           | 2.49       |                                | 19.2            |   |   |   |

<sup>a</sup> Relative retention time, referred to 2,3,4,6-Glc as 1.00. <sup>b</sup> Values are corrected by use of the effective, carbon-response factors given by Albersheim et al.<sup>103</sup> <sup>c</sup> Isothermal; 170°. <sup>d</sup> I, original polysaccharide; II, reduction of uronic ester; III, carbodiimide-reduced polysaccharide; IV, product from Smith degradation. <sup>e</sup> 2,3-Rha = 1,4,5-tri-O-acetyl-2,3-di-O-methylrhamnitol, etc.
Chromium trioxide oxidation

The anomeric nature of the glucuronic acid was confirmed by chromium trioxide oxidation of the fully acetylated polysaccharide, followed by sugar analysis. β-Linked residues should be oxidized under these conditions, but the corresponding α-linked residues should be resistant. Determination, as alditol acetates, of the sugars obtained from the chromium trioxide oxidation gave rhamnose, galactose, and glucose in the molar ratios of 0.9:1.0:0.5 indicating that D-glucuronic acid is β-linked.

The following two structures are consistent with the results obtained so far.

\[ (+3) - \alpha-\text{Glc}(1\rightarrow3 \text{ or } 4) - \alpha-\text{Rha}(1\rightarrow3) - \alpha-\text{Gal}(1\rightarrow4 \text{ or } 3) \]

\[ \uparrow \quad 1 \quad + \text{O-Ac} \]

\[ \beta-\text{GlcA} \]

A

\[ (+3) - \alpha-\text{Gal}(1\rightarrow3 \text{ or } 4) - \alpha-\text{Rha}(1\rightarrow3) - \alpha-\text{Glc}(1\rightarrow4 \text{ or } 3) \]

\[ \uparrow \quad 1 \quad + \text{O-Ac} \]

\[ \beta-\text{GlcA} \]

B
Periodate oxidation

Periodate oxidation of the original *E. coli* K32 polysaccharide followed by hydrolysis and g.l.c. analysis of the alditol acetates gave rhamnose, galactose and glucose in the molar ratios of 1.0:0.5:1.0, indicating that partial hydrolysis of the rhamnosyl bond with further degradation of galactose had occurred. The oxidation was then repeated on the carbodiimide-reduced polysaccharide\(^{75}\) in the presence of 0.1M sodium acetate buffer pH 4.5. G.l.c. analysis, as alditol acetates, of the oxidized and borohydride reduced product gave rhamnose, galactose, and glucose in the ratios of 0.9:1.0:1.0. These results are consistent with the concept that only one terminal residue (glucuronic acid) is oxidized.

Smith degradation

Periodate oxidation of the original polysaccharide, followed by methylation, Smith hydrolysis, remethylation, and hydrolysis, gave a mixture that was found, by g.l.c. analysis of the alditol acetates, to contain derivatives of 2,3-di-\(\beta\)-methylrhamnose, 2,3,4,6-tetra-\(\beta\)-methylgalactose, 2-\(\beta\)-methylrhamnose, and 2,4,6-tri-\(\beta\)-methylglucose (see Table V.7, column IV). These results indicate that the glucose in the main chain is 4-linked to rhamnose, the branch point. The rhamnosyl linkage was hydrolyzed during the Smith hydrolysis giving rise to 2,3,4,6-tetra-\(\beta\)-methylgalactose, proving that the rhamnose in the main chain is linked to the galactose and not the glucose. The unusual instability of the 2,4,6-tri-\(\beta\)-methylgalactose was noticed throughout the structural
investigation of the *E. coli* K32 polysaccharide, especially in the methylation analyses of the original, acidic polysaccharide.

**Location of the O-acetyl group**

Since the complete blocking of the polysaccharide with methyl vinyl ether proved difficult, the O-acetyl location procedure of de Belder and Norrman\(^{180}\) was performed on the oligosaccharide, obtained after bacteriophage depolymerization of the *E. coli* K32 capsular polysaccharide (see Section VI). Ethylation of the protected oligosaccharide, followed by hydrolysis, conversion to the alditol acetates, and g.l.c.-m.s. analysis of the mixture of partially ethylated alditol acetates gave a ratio of 2-O-ethylrhamnose to rhamnose of 4.5:1 indicating that the O-acetyl group is attached to O-2 of rhamnose.

**V.2.4 CONCLUSION**

The sum of these experiments demonstrates that the structure of the capsular polysaccharide from *Escherichia coli* O9:K32(A):H19 is based on the tetrasaccharide repeating unit shown with half of the L-rhamnosyl
residues being O-acetylated at O-2. The structure resembles that of Klebsiella K82; \(^{194}\) it has the same structural pattern ("three-plus-one" type) and a lateral β-D-glucosyluronic acid group as a terminal unit. In common with Klebsiella K55, \(^{195}\) α-L-rhamnose is a branch point with the O-acetyl group present on position O-2 of L-rhamnose. The structural patterns of the two polysaccharides are, however, different.
V.2.5 EXPERIMENTAL

**General methods**

The instrumentation used for n.m.r., g.l.c., infrared, c.d., and measurements of optical rotation has been described in Section III. G.l.c.-m.s. was performed with the NERMAG R10-10 instrument. The capillary columns used were: (F) DB-225, programmed from 195° for 8 min, and then 4°/min to 220°, (G) SE-30, programmed from 70° for 1 min, and then 10°/min to 250°. Paper chromatography, gas-liquid chromatography, and ion-exchange chromatography were performed as described in Section III.

**Preparation and properties of E. coli K32 polysaccharide**

A culture of *Escherichia coli* K32, obtained from Dr. I. Ørskov, (Copenhagen), was grown on Mueller Hinton agar as described in Section III.7.2. Yield: acidic polysaccharide ~660 mg, neutral polysaccharide ~130 mg.

The isolated acidic polysaccharide had $[\alpha]_{D}^{25} +80.9$ (c 0.147, water), and analysis by gel-permeation chromatography (courtesy of Dr. S.C. Churms, Cape Town, South Africa) showed it to be homogeneous, with an average molecular weight of $9 \times 10^6$ daltons. N.m.r. spectroscopy ($^1$H and $^{13}$C) was performed on the original and the deacetylated K32 polysaccharide. The principal signals in the $^1$H- and $^{13}$C-n.m.r. spectra and their assignments are recorded in Table V.6.
Deacetylation of polysaccharide

The polysaccharide was dissolved in 0.01M NaOH and stirred overnight at room temperature. The product was dialyzed against tap water and freeze-dried. The deacetylation was almost complete (as judged from $^1$H-n.m.r. spectrum) and 90% of the O-acetyl groups were removed. Analysis of the deacetylated polysaccharide by gel-permeation chromatography showed it to be homogeneous with $M_w = 6 \times 10^6$ daltons.

Hydrolysis of the polysaccharide

Hydrolysis of a sample (3 mg) of E. coli K32 polysaccharide with 2M trifluoroacetic acid (TFA) for 18 h at 95°, removal of the acid by successive evaporations with water, followed by paper chromatography (solvents A and B), showed galactose, glucose, glucuronic acid, and rhamnose. Neutral sugars were quantitatively determined by g.l.c. as their alditol acetates. The uronic acid was reduced by refluxing a sample (6 mg) of K32 polysaccharide with 3% HCl in methanol (4 mL) overnight, neutralizing the HCl with PbCO$_3$, removing PbCl$_2$, treating the dried product with NaBH$_4$ (50 mg) in anhydrous methanol (4 mL) and stirring overnight. The excess of NaBH$_4$ was neutralized with Amberlite IR-120 (H$^+$) resin, and the boric acid, as methyl borate, was removed by co-evaporation with methanol. The sample was then hydrolyzed with 2M trifluoroacetic acid for 18 h at 95° and the alditol acetates were prepared and identified by g.l.c. (column A). Preparative g.l.c. (column D), followed by measurements of the circular dichroism
spectra, showed the glucitol hexaacetate to be of the D configuration, and the rhamnitol pentaacetate to be of the L configuration.

**Methylation analysis**

The polysaccharide (29.8 mg), converted into the free acid form by passing the sodium salt through a column of Amberlite IR-120 (H+) resin, was dissolved in dry dimethyl sulfoxide (4 mL) and methylated by treatment with 3 mL dimethylsulfinyl anion for 4 h, and then 6 mL of methyl iodide for 1 h. The product, recovered after dialysis against tap water, was not completely methylated (hydroxyl absorption in the i.r. spectrum). It was dissolved in chloroform and subjected to Purdie methylation with methyl iodide and silver oxide. This treatment yielded a fully methylated polysaccharide (15.2 mg). A portion of this product (5 mg) was hydrolyzed with 2M trifluoroacetic acid. The sugars were reduced with sodium borohydride, and the alditols were acetylated with 1:1 acetic anhydride-pyridine, and analyzed by g.l.c. in column B (see Table V.7, column I). Carboxyl reduction of fully methylated polysaccharide (10.2 mg) with LiAlH₄ in anhydrous oxolane (5 mL) at room temperature overnight, hydrolysis of the product with 2M trifluoroacetic acid, followed by reduction of sugars with sodium borohydride, and acetylation of the alditols with 1:1 acetic anhydride-pyridine gave a mixture of partially methylated alditol acetates which was analyzed by g.l.c. (column B) and g.l.c.-m.s. (column F and G). G.l.c. analysis data for the partially methylated alditol acetates are shown in Table V.7, column II. The neutral polysaccharide obtained by carbodiimide reduction was also subjected to methylation analysis. A
sample (8.1 mg) of carbodiimide-reduced polysaccharide was dissolved in dry dimethyl sulfoxide (1.5 mL) and methylated by the treatment with 1.5 mL dimethylsulfinyl anion for 4 h, and then 3 mL methyl iodide for 1 h. The product, recovered after dialysis against tap water, was hydrolyzed with 2M trifluoroacetic acid overnight at 95°, reduced with NaBH₄, and the alditols were acetylated with 1:1 acetic anhydride-pyridine. Subsequent analysis by g.l.c. (column B) and g.l.c.-m.s. (column F) gave the results presented in Table V.7, column III.

**Carbodiimide reduction of capsular polysaccharide**

A sample of *E. coli* K32 polysaccharide (Na⁺ salt, 50.15 mg) was dissolved in 30 mL water (initial pH 5.4). 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (CMC, 0.5 g) was added. As the reaction proceeded, the pH was maintained at 4.75 by titration with 0.1N HCl. The reaction was allowed to proceed for 2 h. An aqueous solution of 2M sodium borohydride (1.3 g/15 mL H₂O) was added slowly. The pH was maintained between 5-7 by titration with 4M HCl. The reduction was completed in one h. The mixture was dialyzed against tap water for two days, concentrated and freeze-dried. Then a second treatment was carried out similarly. A total of 47.2 mg of the product was recovered after freeze-drying.

A sample of the carbodiimide-reduced polysaccharide (2.4 mg) was hydrolyzed overnight with 2M trifluoroacetic acid (TFA) on a steam bath and the hydrolyzate of sugars was converted into alditol acetates, g.l.c. analysis of which (column A) showed rhamnitol pentaacetate,
galactitol hexaacetate and glucitol hexaacetate in ratios of 0.7:1.0:1.70, indicating 70% reduction.

**Chromium trioxide oxidation**

A sample (16.1 mg) of the polysaccharide was dissolved in formamide (5 mL), and treated with acetic anhydride (1 mL) and pyridine (1 mL) overnight at room temperature. The acetylated material (22.9 mg) was recovered by dialysis and freeze-drying, and dissolved in acetic acid (1 mL). The acetic acid solution was treated with chromium trioxide (50 mg) at 50° for 1 h. The material was recovered by partition between chloroform and water. The product was hydrolyzed with 2M trifluoroacetic acid overnight, converted into alditol acetates and analyzed by g.l.c. in column A.

**Periodate oxidation**

A solution of K32 polysaccharide (42.5 mg) in water (10 mL) was mixed with 0.03M NaIO₄ (10 mL) and stirred in the dark at room temperature (23°). The reaction was carried out for 6 d. After ethylene glycol (2 mL) was added to terminate the reaction, the polyaldehyde was reduced to the polyalcohol with NaBH₄, the base was neutralized with 50% acetic acid, and the solution was dialyzed overnight and freeze-dried to yielded the polyalcohol (28.35 mg). A portion (3.4 mg) was hydrolyzed with 2M TFA overnight at 95° and converted into alditol acetates. Analysis by g.l.c. in column A showed rhamnitol, galactitol and glucitol in the ratios of 1.0:0.5:1.0, indicating that partial degradation of the galactose had occurred. Periodate oxidation was then repeated on the
carbodiimide-reduced polysaccharide\textsuperscript{75} using 0.1M sodium acetate buffer and low temperature (4\degree) in order to avoid partial hydrolysis of the rhamnosyl bond and over-oxidation of the galactose.

A sample of the carbodiimide-reduced polysaccharide (15.7 mg) was dissolved in 0.1M sodium acetate buffer (pH 4.5), mixed with 0.015M NaIO\textsubscript{4} (6 mL) and stirred in the dark at 4\degree. Aliquots (0.1 mL) were withdrawn periodically, and diluted 250 times with water. The absorbances\textsuperscript{185} at 223 nm of the resulting solutions were measured in a Perkin-Elmer 552A UV/VIS spectrophotometer. The periodate consumption reached a plateau after ~10 days. Ethylene glycol (2 mL) was added to decompose the excess of periodate, the polyaldehyde was reduced with sodium borohydride, the base was neutralized with 50\% acetic acid, and the solution was dialyzed and lyophilized to yield the polyalcohol. A portion of this polyalcohol (7.2 mg) was hydrolyzed with 2M trifluoroacetic acid overnight at 95\degree and converted into alditol acetates. Analysis by g.l.c. in column A showed rhamnitol, galactose, and glucose in the ratios of 0.9:1.0:1.0.

**Smith degradation**

A sample of the polyalcohol (6.1 mg) obtained after periodate oxidation of the K32 polysaccharide was dissolved in dry dimethyl sulfoxide (3 mL) and methylated by treatment with 2 mL of dimethylsulfinyl anion for 4 h, and then 4 mL of methyl iodide for 1 h. The product was recovered by partition between water and chloroform, and its i.r. spectrum showed complete methylation (no hydroxyl absorption in the i.r. spectrum). It was then subjected to the Smith hydrolysis by treatment
with 50% acetic acid for 90 min at 95°. Acetic acid was removed by co-
evaporation with water and the dry residue was subjected to remethyla-
tion by the Hakomori procedure. The methylated product was hydrolyzed
with 2M trifluoroacetic acid (TFA) overnight at 95°, and the partially
methylated sugars were converted into alditol acetates and analyzed by
g.l.c. in column B (see Table V.7, column IV) and g.l.c.-m.s. in column
E.

**Location of the Q-acetyl group**

A sample (5 mg) of the oligosaccharide obtained after bacterio-
phage degradation of the *E. coli* K32 polysaccharide (fraction II) was
dried together with a trace of p-toluenesulfonic acid and then dissolved
in dry dimethyl sulfoxide (4 mL). Methyl vinyl ether (3 mL) was added
to a frozen solution, and the reaction mixture was brought to 23° and
allowed to stir for 4 h. Then a second portion of methyl vinyl ether
(3 mL) was introduced in a similar manner. The clear red solution was
obtained. It was placed on a Sephadex LH-20 column (16 cm x 2 cm) and
eluted with acetone (with slight suction). The product was concentrated
and the residue was subjected to ethylation. It was dissolved in dry
dimethyl sulfoxide (4 mL) and treated with 2 mL of dimethylsulfinyl
anion for 4 h, and then with 3 mL of ethyl iodide for 1 h. The dark-red
product was extracted with chloroform and purified on a Sephadex LH-20
column (14.5 cm x 2 cm) by elution with methanol. The product, a
dark-red oil, was hydrolyzed with 2M trifluoroacetic acid overnight at
95° and converted into alditol acetates. G.l.c. analysis, conducted in
column A, programmed from 195° for 8 min, and then at 4°/min to 260°,
showed the presence of 2-O-ethylrhamnose (43%), rhamnose (9.6%),
galactose (16.4%), and glucose (30.8%). These results were confirmed by
g.l.c.-m.s. (column F).
CHAPTER VI

BACTERIOPHAGE DEGRADATION OF *Escherichia coli* CAPSULAR POLYSACCHARIDES K28 and K32
VI. BACTERIOPHAGE DEGRADATION OF Escherichia coli CAPSULAR POLYSACCHARIDES K28 and K32.

VI.1 INTRODUCTION

Like many other organisms, bacteria are subject to infection by a range of viruses or virus-like particles which fall naturally into two physiologically separate groups, bacteriophages and bacteriocins. Bacteriophages (φ) are true viruses, infecting their hosts and multiplying within them. The members of the second group differ in that they do not multiply in the cell after infecting it, but only kill it. Bacteriocins may be defined as a natural class of highly specific antibiotics.

The first account of the bacteriophage was published by Twort in 1915. He demonstrated that the cultures of bacterial cells could be infected with and destroyed by filterable agents that were subsequently termed bacteriophages. Two years later, d'Herelle independently isolated a dysentery bacteriophage, characterizing it as an ultramicroscopic parasite of bacteria, and giving it the name "bacteriophage", which means "bacteria-eater".

Although bacteriophages (phages) were the last major group of viruses to be recognized, today they are the best characterized and studied. The reasons for that lie in the fact that propagation and manipulation of phages has proven technically much easier than equivalent studies on the other types of viruses.
Many different strains of phages have been isolated and characterized since they were first demonstrated in 1915. Probably each bacterial strain is susceptible to several different types of phage. Phages are relatively easy to isolate from almost any bacterial environment in which a number of closely related bacterial strains coexist (e.g., the gastrointestinal tract, sewage). If a sample of sewage filtrate is mixed with a growing culture of an enteric bacterium and spread on a plate, the ensuing culture will show growth of the organism on the plate interrupted by small zones of clearing, which are termed plaques. Each plaque represents the propagation of a single phage particle in the growing lawn of bacterial cells and is analogous to an isolated bacterial colony.

Morphologically phages are quite distinct from other virus types in that they tend to be structurally more complex. Bacteriophages have been intensely studied by many different techniques, but one of the most significant contributions to our knowledge of these viruses has been made by electron microscopy. The morphological classification of the bacteriophages was introduced by Bradley.196

The phage head (see Fig. VI.1) contains the virus nucleic acid. In phage the nucleic acid is usually in the form of double-stranded DNA. The head has basically an icosahedral structure. It is composed of repeating identical protein chains and will vary in size according to the strain, approximately 50 nm in diameter. The phage tail is more complex in structure than is the head. A layer of helically arranged protein molecules forms the inner tube of the tail. The tube is encased by the contractile sheath, which extends from the collar to the end
**Fig. VI.1**: Schematic diagram demonstrating the structure of $T_2$ bacteriophage.

**Fig. VI.2**: Basic morphological types of bacteriophages with the types of nucleic acid (from ref. 196).
plate. The sheath plays an important role in the infective process by forcing the phage nucleic acid through the hollow tail into the host bacterium. The plate contains small pins to which are attached six very long, fine tail fibers by which phage particles attach themselves to the cell walls of susceptible host cells.\textsuperscript{197}

The bacterial viruses exhibit a great diversity of forms but it is possible to divide them into six basic morphological types (see Fig. VI.2). The first four groups (with tails) are unique to the bacterial viruses. Groups E and F are different; they resemble many plant, animal, and insect viruses.\textsuperscript{196}

When a phage particle infects a susceptible host it causes that cell to lyse. The phases of the lytic cycle (see Fig. VI.3) include the following:\textsuperscript{197}

(i) adsorption of the phage particles to the susceptible host
(ii) injection of viral DNA (or RNA) into the host
(iii) replication of the phage nucleic acid and synthesis of the phage protein
(iv) phage maturation and release

Adsorption is very host specific and depends on the presence in the bacterial cell wall of precise receptor sites. Adsorption of a phage to its receptor on the cell is, in most instances, followed by penetration of the nucleic acid through the cytoplasmic membrane.\textsuperscript{198}

Bacteriophages active on exopolysaccharide-producing bacterial strains are generally exopolysaccharide specific,\textsuperscript{199} non-capsulate or
Fig. VI.3: The Mechanics of Infection by Bacteriophage

A. Free phage.

B. Phage attaches to cell wall with fibres, base plate in close contact with outer layers of cell wall.

C. Sheath contracts and central core is pushed through the cell wall and DNA transfer begins.

D. Transfer of DNA completed. Phage head is now empty and early events of phage growth cycle begin.

non-slime producing mutants are resistant to the phages. Morphological examination of exopolysaccharide-specific phages has revealed that most of them belong to group C, their base-plates are provided with the spikes and no tail fibres are seen.$^{198}$

Attack of bacteriophages on exopolysaccharide-producing bacteria is often revealed by occurrence of halos around the clear plaques in the lawn of capsulated bacteria.$^{198}$ Within the halo the bacterial lawn is decapsulated. These halos are, at least partly, the result of diffusion of a phage-induced enzyme which hydrolyzes the capsule without killing the bacteria. As shown by Bayer et al.,$^{200}$ such phages can be visualized under the electron microscope on their way from the outer surface of the bacterial capsule to the cell wall underneath (see Fig. VI.4).

The bacteriophage-associated enzymes may be classified according to the type of reaction they catalyze, and according to the genus of the respective bacterial host. Most viral penetrases are hydrolases, but a few lyases have also been found. The hydrolases are either glycanases (glycoside hydrolases) or they are "deacetylases" which cleave off acetyl substituents.$^{201}$ To date, virus-associated enzymes of these types have mainly been studied using phages for bacteria belonging to the family of Enterobacteriaceae, and within the family to the genera Escherichia coli, Klebsiella, Salmonella, Shigella and Proteus.

In an extensive study,$^{202}$ 55 different Klebsiella bacteriophages were tested for their enzymic action on 74 different (acidic) Klebsiella capsular polysaccharides (serotypes K1-K72, K74 and K80). The results may be summarized as follows.$^{202}$
Fig. VI.4: Capsulated *E. coli* K29 exposed to a m.o.i. (the multiplicity of infection) of 300 phage for 8 min at 37°. Virus particles can be seen on the outer membrane surface. One virus particle (upper left corner) has apparently released most of its DNA. (From ref. 200).
(i) The Klebsiella virus-associated glycanases were found to be very specific, 33 cross-reacting with none, 18 with one, two with two, and one each with 3 or 4 of the 73 heterologous polysaccharides;

(ii) In most cases cleavage occurred on either side of the sugar unit carrying the negative charge, but reducing glucuronic acids are not produced;

(iii) Most often, the reducing end sugar formed is substituted at position 3;

(iv) In the majority of cases, β-glycosidic linkages are hydrolyzed;

(v) In most polysaccharides which are acted upon by several phage enzymes, the same glycosidic bonds are split by the different agents.

The bacteriophage-associated glycanases allow the preparative isolation of oligosaccharide fragments. With some acid-labile components, bacteriophage degradation may be the only method for the isolation of repeating unit oligomers. Bacteriophage degradation may be used as a complement to other methods for the partial degradation of bacterial polysaccharides. The large-scale accessibility of these repeating unit oligomers is also of advantage for analyses of nuclear magnetic resonance spectroscopy. When coupled to suitable protein carriers, bacterial surface oligosaccharides of two or more repeating units may serve as immunogens, representative of the corresponding bacterial glycans.

Although a number of highly specific "K bacteriophages" have been found for Escherichia coli capsular strains, the enzymic action of
these phages has been studied to a much lesser extent. The interaction between the capsulated Escherichia coli strain of serotype K29 and a capsule K29-specific bacteriophage has been studied, using virus adsorption kinetics and immunological methods in combination with electron microscopy. Recently, the capsule-degrading enzymic activity of two E. coli bacteriophages (φ92 and φ1.2) has been tested.

The results of the degradation of two Escherichia coli capsular polysaccharides (K28 and K32) with their respective bacteriophages (φ28-1 and φ28-2, and φ32, respectively) are presented here.

VI. RESULTS

Escherichia coli bacteriophages were isolated from sewage (courtesy of Dr. S. Stirm, Freiburg, Germany).

The bacteriophages have been characterized by Stirm and Freund-Müllert and their morphology is known. Phage φ28-1 belongs to Bradley group A, and phages φ28-2 and φ32 belong to Bradley group C. Most of the bacteriophages that are capable of infecting encapsulated Enterobacteriaceae belong to Bradley group C, and bacteriophage-borne enzymatic activity seems to be associated with spike structures.

Depolymerization with bacteriophages φ28-1 and φ28-2

The bacteriophages φ28-1 and φ28-2 were propagated on their host strain Escherichia coli K28 using nutrient broth as a medium. Propagation was continued on an increasing scale until the crude lysates contained a total of ~10^{13} plaque-forming units, an amount sufficient to
degrade one gram of the polysaccharide. The depolymerization of *E. coli* K28 capsular polysaccharide was conducted with the crude solutions of bacteriophages ø28-1 and ø28-2, respectively. It was allowed to proceed for three days, chloroform being added to prevent bacterial growth; the mixture was then concentrated, and the concentrate dialyzed against distilled water. The process of concentration and dialysis was repeated six times, the dialyzates were combined and concentrated. The concentrated dialyzate was treated with ion-exchange resin Amberlite IR-120 (H⁺) three times and freeze-dried.

The mixture of oligosaccharides obtained after depolymerization of *E. coli* K28 polysaccharide with phage ø28-1 was then separated into pure components by preparative paper chromatography. Only base line carbohydrate material was obtained. It was isolated and examined by gel-permeation chromatography (courtesy of Dr. S.C. Churms, Cape Town, South Africa). The results showed that the isolated material was a mixture of high oligosaccharides with the average molecular weight $M_w = 2100$ daltons. The degree of polymerization was determined by Morrison's method and by methylation analysis on the mixture of corresponding oligosaccharide-alditols (see Table VI.1 and Table VI.2). They indicated that the average length of the oligosaccharide is 20 sugars or 5 repeating units and that the glucosyl residue is a reducing sugar. This is in agreement with $^1$H-n.m.r. findings which show the presence of two signals at $\delta = 4.67$ p.p.m. ($J_{1,2} = 8$ Hz) and at $\delta = 5.2$ p.p.m. ($J_{1,2} = 4$ Hz) corresponding to the $\beta$-glucosyl and $\alpha$-glucosyl residues.
TABLE VI.1

METHYLATION ANALYSIS AND THE REDUCING END DETERMINATION OF \textit{E. coli} K28 OLIGOSACCHARIDE ISOLATED AFTER BACTERIOPHAGE \(\phi\)28-1 DEGRADATION OF \textit{E. coli} K28 POLYSACCHARIDE

<table>
<thead>
<tr>
<th>Methylated sugars\textsuperscript{a}</th>
<th>T\textsuperscript{b}</th>
<th>Column B\textsuperscript{b}</th>
<th>Mole %\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>(as alditol)</td>
<td>(ECNSS-M)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,2,5,6-Glc</td>
<td>0.8</td>
<td></td>
<td>15.3\textsuperscript{f}</td>
</tr>
<tr>
<td>2,3-Fuc</td>
<td>1.14</td>
<td></td>
<td>12.9</td>
</tr>
<tr>
<td>2,3,4,6-Gal</td>
<td>1.24</td>
<td></td>
<td>71.7</td>
</tr>
</tbody>
</table>

\textsuperscript{a} 1,2,5,6-Glc = 3,4-di-O-acetyl-1,2,5,6-tetra-O-methylglucitol, etc.

\textsuperscript{b} Relative retention time referred to 2,3,4,6-Glc as 1.00.

\textsuperscript{c} Values are corrected by use of the effective carbon response factors given by Albersheim \textit{et al.}\textsuperscript{103}

\textsuperscript{d} Ratios are low, due to incomplete hydrolysis of the glucosyluronic linkage (2,3-Fuc) and high volatility of the derivative (1,2,5,6-Glc).
TABLE VI.2

DETERMINATION OF THE DEGREE OF POLYMERIZATION AND THE REDUCING END OF
*E. coli* K28 OLIGOSACCHARIDE ISOLATED AFTER BACTERIOPHAGE φ28-1 DEGRADATION
OF *E. coli* K28 POLYSACCHARIDE

<table>
<thead>
<tr>
<th>Peracetylated derivative of Column C (OV-225)</th>
<th>Mole %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucononitrile</td>
<td>0.35</td>
</tr>
<tr>
<td>Glucononitrile</td>
<td>1.00</td>
</tr>
<tr>
<td>Galactononitrile</td>
<td>1.08</td>
</tr>
<tr>
<td>Glucitol</td>
<td>1.20</td>
</tr>
</tbody>
</table>

a Isothermal at 230°.
respectively. Both signals disappeared after reduction with sodium borohydride due to the conversion of the reducing sugar into the alditol.

The molecular weight distribution of the non-dialyzable portion gave a mixture of high oligosaccharides with the average molecular weight $M_w = 4500$. These results indicate that only partial depolymerization had occurred.

Depolymerization of *E. coli* K28 capsular polysaccharide with $\phi 28-2$ gave similar results. Confirmation of the reducing end was obtained by Morrison's method,\textsuperscript{102} whereby the oligosaccharide is reduced to the alditol and after hydrolysis, the free sugars are converted into the peracetylated aldonitriles with the reducing end being converted into the peracetylated alditol. The results showed the presence of glucitol, glucononitrile, galactononitrile and fucononitrile indicating that the glucosyl linkage was cleaved by bacteriophage action.

**Depolymerization with bacteriophage $\phi 32$**

The bacteriophage $\phi 32$ was propagated on its host strain *Escherichia coli* K32 using nutrient broth as a medium.

When the phage concentration reached $\sim 10^{10}$ P.F.U./mL it was further propagated in a fermentor (see Section III.8.2).

Bacteriophage $\phi 32$ was purified by precipitation with polyethylene glycol 6000 (10% w/v).\textsuperscript{213} The depolymerization was carried out in a volatile buffer\textsuperscript{204} for two days in the presence of some chloroform to ensure sterility. The depolymerization mixture was dialyzed against
distilled water overnight. The dialysis was repeated twice more. The
dialyzates were combined, concentrated and freeze-dried. The dialyzable
portion was separated by gel-permeation chromatography on a column of
Bio-Gel P-4. The elution pattern is shown in Fig. VI.5. Three
fractions were obtained. The second fraction was further examined by
gel-permeation chromatography (courtesy of Dr. S.C. Churms, Cape Town,
South Africa) for molecular weight distribution. It showed that the
fraction consisted mainly of an octasaccharide ($M_w = 1600$ daltons) (see
Fig. VI.6).

**Analysis of the depolymerization products of E. coli K32**

polysaccharide

Fractions I, II and III were examined by $^1$H-n.m.r. spectroscopy.
Fraction I contained highly polymeric material, and the spectrum of this
fraction was very similar to that of the native acetylated poly-
saccharide. The presence of an octasaccharide was not obvious from the
$^1$H-n.m.r. spectrum of the second fraction. However, after sodium boro-
hydride reduction of that fraction the $^1$H-n.m.r. spectrum became better
resolved, and was similar to the spectrum of the deacetylated E. _coli_
K32 polysaccharide. The comparison of two spectra did not permit the
assignment of the reducing end (due to the possible overlapping with
other principal signals). Fraction III had a low carbohydrate content
as was judged from its $^1$H-n.m.r. spectrum. It revealed the presence of
an octasaccharide with a low acetate content (30% by n.m.r.). The
$^1$H-n.m.r. data of all three fractions is summarized in Table VI.3.
Fig. VI.5: Separation of the depolymerization products of *E. coli* K32 by gel-permeation chromatography (Bio-Gel P-4)
Molecular weight distribution

<table>
<thead>
<tr>
<th>Mol. wt.</th>
<th>% by wt.</th>
<th>mol. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>4400</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>3300</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>2700</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>1600</td>
<td>58</td>
<td>73</td>
</tr>
</tbody>
</table>

Fig. VI.6: Molecular weight distribution of fraction II (Bio-Gel P-10 column 52 x 1.5 cm, M NaCl eluant, flow-rate 20mL/h). Courtesy of Dr. S.C. Churms, Cape Town, South Africa.
### TABLE VI.3

PROTON ASSIGNMENTS (400 MHz) FOR THE OLIGOSACCHARIDES AND RELATED COMPOUNDS GENERATED IN BACTERIOPHAGE DEPOLYMERIZATION OF THE *E. coli* K32 CAPSULAR POLYSACCHARIDE.

<table>
<thead>
<tr>
<th>Fraction I&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fraction II</th>
<th>Fraction II (R)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Fraction III</th>
<th>Assignment&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Integral</strong> (p.p.m.) (H)</td>
<td><strong>Integral</strong> (p.p.m.) (H)</td>
<td><strong>Integral</strong> (p.p.m.) (H)</td>
<td><strong>Integral</strong> (p.p.m.) (H)</td>
<td><strong>Assignment</strong>&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.51</td>
<td>1.2</td>
<td>5.51</td>
<td>0.6</td>
<td>5.52</td>
</tr>
<tr>
<td>5.45</td>
<td>0.4</td>
<td>5.48</td>
<td>0.4</td>
<td>5.47</td>
</tr>
<tr>
<td>5.24</td>
<td>1.0</td>
<td>5.24</td>
<td>1.0</td>
<td>5.23</td>
</tr>
<tr>
<td>5.18</td>
<td>1.0</td>
<td>5.18</td>
<td>1.0</td>
<td>5.17</td>
</tr>
<tr>
<td>5.10</td>
<td>1.0</td>
<td>5.10</td>
<td>1.0</td>
<td>5.11</td>
</tr>
<tr>
<td>5.05</td>
<td>1.3</td>
<td>5.05</td>
<td>1.3</td>
<td>5.07</td>
</tr>
<tr>
<td>5.03</td>
<td>1.5</td>
<td>5.02</td>
<td>1.5</td>
<td>5.05</td>
</tr>
<tr>
<td>4.72 (8Hz)</td>
<td>2.2</td>
<td>4.71 (8Hz)</td>
<td>2.0</td>
<td>4.66 (8Hz)</td>
</tr>
<tr>
<td>2.19</td>
<td>1.5</td>
<td>2.17 (8Hz)</td>
<td>2.7</td>
<td>2.19 (8Hz)</td>
</tr>
<tr>
<td>1.35</td>
<td>6.0</td>
<td>1.35 (6Hz)</td>
<td>6.0</td>
<td>1.35 (6Hz)</td>
</tr>
</tbody>
</table>

<sup>a</sup> For the source of Fr: I, II and III see text.  
<sup>b</sup> Chemical shift relative to internal acetone; δ 2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS).  
<sup>c</sup> Fraction II after reduction with sodium borohydride.  
<sup>d</sup> A definite assignment could not be made, the assignments of α-Gal and α-Glc are tentative.
Methylation of the reduced fraction II, followed by hydrolysis, derivatization as alditol acetates, and g.l.c.–m.s. analysis gave values shown in Table VI.4.

The data demonstrate that the bacteriophage enzyme is an α-D-glucosidase and that the glucose is present at the reducing end of the oligosaccharide which is comprised of two repeating units. It also shows that the bacteriophage-borne enzyme does not have a "deacetylase" activity, since acetate-bearing oligosaccharides were isolated after the bacteriophage degradation of \textit{E. coli} K32 polysaccharide.

VI.3 DISCUSSION

The main purpose of the bacteriophage work carried out on \textit{Escherichia coli} bacteriophages $\phi$28-1, $\phi$28-2 and $\phi$32 was to obtain oligosaccharides representing subunits of the polysaccharides degraded, with the labile \textit{O}-acetyl substituents present as in the original polysaccharide. These oligosaccharides could be then studied by $^1$H-n.m.r. and $^{13}$C-n.m.r. spectroscopy in order to locate the acetate position. However, this aim has not been achieved.

The bacteriophage degradation of the polysaccharide was carried out using two methods. According to the first method, the bacteriophage was propagated on an increasing scale until a total of $\sim 10^{13}$ P.F.U. was obtained and after dialysis a concentrated crude solution of the bacteriophage was used directly for the depolymerization. This procedure\textsuperscript{208} was developed in our group and gave excellent results with \textit{Klebsiella} bacteriophages. However, use of this method for bacterio-
TABLE VI.4

METHYLATION ANALYSIS OF THE REDUCED FRACTION II OBTAINED AFTER THE
SEPARATION OF THE DEPOLYMERIZATION PRODUCTS OF \textit{E. coli} K32 POLYSACCHARIDE

<table>
<thead>
<tr>
<th>Methylated sugars(^a) (as alditol acetates)</th>
<th>Column B(^b) (ECNSS-M)</th>
<th>Mole %(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2,4,5,6-Glc</td>
<td>0.35</td>
<td>4.9(^d)</td>
</tr>
<tr>
<td>2,4-Rha</td>
<td>1.01</td>
<td>11.9</td>
</tr>
<tr>
<td>2,3,4,6-Gal</td>
<td>1.55</td>
<td>28.6</td>
</tr>
<tr>
<td>2-Rha</td>
<td>1.96</td>
<td>35.5</td>
</tr>
<tr>
<td>2,4,6-Glc</td>
<td>2.26</td>
<td>19.1</td>
</tr>
</tbody>
</table>

\(^a\) 1,2,4,5,6-Glc = 3-O-acetyl-1,2,4,5,6-penta-O-methylglucitol, etc.

\(^b\) Relative retention time referred to 2,3,4,6-Glc as 1.00.

\(^c\) Values are corrected by use of the effective, carbon-response factors given by Albersheim \textit{et al.}\(^{103}\)

\(^d\) Ratios of certain sugars are low, due to incomplete hydrolysis of the glucosyluronic linkage (2,4-Rha) and high volatility of the derivative (1,2,4,5,6-Glc).
phages $\phi 28-1$ and $\phi 28-2$ degradation of _Escherichia coli_ K28 polysaccharide did not give satisfactory results. Only partial depolymerization was achieved. Since these bacteriophages form very small "halos", it is possible that they have low enzymic activity, and do not produce oligosaccharides corresponding to one or two repeating unit(s) of the polysaccharide.

Bearing this in mind, the second method was used for bacteriophage $\phi 32$. It was purified by precipitation with polyethylene glycol 6000 prior to the depolymerization. The degradation was done in "volatile" buffer in order to avoid possible loss of the activity during the depolymerization. The results obtained were more encouraging, and a small amount of the octasaccharide (two repeating units) bearing an $O$-acetyl group was obtained.

All three bacteriophages ($\phi 28-1$, $\phi 28-2$ and $\phi 32$) exhibit $\alpha$-D-glucosidase activity. The bacteriophages $\phi 28-1$ and $\phi 28-2$ cleaved the same glycosyl linkage,

\[ +3)-\alpha-D-Glc-(1\rightarrow 4)-\beta-D-GlcA-(1\rightarrow 4)-\alpha-L-Fuc-(1\rightarrow 4 \]

\[ \uparrow \quad \uparrow \quad 1 \quad \phi 28-1, \quad \phi 28-2 \]

$\beta-D$-Gal

_E. coli_ K28

although morphologically they belong to the different Bradley groups,\textsuperscript{193} $\phi 28-1$ (group A) and $\phi 28-2$ (group C), and different enzymic activity was expected.
In the case of bacteriophage φ32 α-glucosidase activity was quite unexpected, since the rhamnosyl linkage is the most labile one (to acid).

\[ \rightarrow 3)-\alpha-D-Glc-(1\rightarrow \alpha-L-Rha-(1\rightarrow 3)-\alpha-D-Gal-(1\rightarrow \alpha \]

\[ \uparrow \quad \uparrow \]

\[ \phi 32 \quad 1 \]

\[ \beta-D-GlcA \]

**E. coli** K32

Here again the tendency of the bacteriophage to produce a linear structure can be noticed.

Another interesting aspect of this work is the fact that bacteriophages φ28-1 and φ32 give cross-adsorption with the bacterial strain of *Escherichia coli* K32, but not the phage φ28-2.\(^{193}\)

Comparison of the structures of *E. coli* K28 and *E. coli* K32 suggests certain similarity in their structural pattern. Both structures are of a "three-plus-one" type, and their qualitative composition is similar. However, despite these similarities the two structures are different. This suggests that the bacteriophage recognizes only a small portion of the polysaccharide chain. The action of the bacteriophage φ28-1 on the bacterial strain of *Escherichia coli* K32 has yet to be determined.
VI.4 EXPERIMENTAL

The bacteriophages $\phi 28-1$, $\phi 28-2$ and $\phi 32$ were received from Dr. S. Stirm (Freiburg, Germany) and propagated on their host strains. For experimental details see Section III.8. Paper chromatography, gas-liquid chromatography and gel-permeation chromatography were performed as described in Section III. Preparative paper chromatography was performed by the descending method using solvent C (see Section III.1). The instrumentation used for n.m.r., g.l.c. and infrared has been described in Section III. G.l.c.-m.s. was performed on the NERMAG R10-10 instrument using capillary column of DB-225, programmed from 195° for 8 min, and then 4°/min to 220°.

**Incubation of E. coli K28 polysaccharide with bacteriophages**

Purified, capsular polysaccharide (100 mg) from *Escherichia coli* K28 was dissolved in 10 mL of distilled water. A phage $\phi 28-1$ solution containing $2 \times 10^{13}$ P.F.U. was concentrated to a small volume prior to the depolymerization, dialyzed against tap water for three days and concentrated again to 100 mL. A polysaccharide solution was mixed with a phage solution (100 mL) in a nutrient broth, containing a total of $2.0 \times 10^{13}$ plaque-forming units (P.F.U.) and incubated for 48 h at 37°. A significant drop in the viscosity of the original polysaccharide solution was noticed after approximately 3 h of incubation.
Purification and separation of depolymerized material

The resulting crude depolymerization mixture was dialyzed against 250 mL of distilled water. The procedure was repeated six times and the dialysis bag was changed after each time. The dialyzates were combined, concentrated to a small volume on a rotary evaporator and exchanged three times on Amberlite IR-120(H⁺) resin. The resulting solution was freeze-dried. The degradation products were isolated by preparative paper chromatography in solvent C. Only baseline carbohydrate material was obtained (21.7 mg). The content of a dialysis bag was exchanged with Amberlite IR-120(H⁺) ion-exchange resin and freeze-dried. It yielded 92.5 mg of the non-dialyzable material.

In a similar way a degradation of Escherichia coli K28 using crude bacteriophage φ28-2 suspension was done. The bacteriophage degradation was not complete and yielded only high oligomers as was judged by molecular weight distribution of a dialyzable fraction (see Section VI.2).

Purification of the bacteriophage φ32

Bacteriophage φ32 was propagated in a fermentor (see Section III.8.2) to give 9 L of a clear solution with the titer $1.6 \times 10^{10}$ P.F.U./mL. From this preparation 2.5 L was purified by precipitation with polyethylene glycol 6000 in the presence of NaCl (73.1 g, 0.5M). The solution was then placed in a cold room and kept for 42 h at 4°C. A very fine precipitate of a bacteriophage appeared at the bottom of a beaker. It was spun down in a centrifuge at 1400 r.p.m. The precipitate was redissolved in 50 mL of 10 mM (pH 7.1) tris(hydroxy-
methyl)methylamine (Tris) - hydrochloride buffer which contained 10 mM NaCl. In order to remove polyethylene glycol the milky and opalescent phage solution was dialyzed against the same buffer first at room temperature (23°) for 18 h and then at 4° for an additional 24 h.

The Tris-HCl buffer was changed once during the dialysis. The phage solution was then spun at low speed 1200 r.p.m. and assayed. The bacteriophage titer was $2.5 \times 10^{11}$ P.F.U./mL or a total of $1.25 \times 10^{13}$ P.F.U. (for 50 mL of phage solution). The assay of the supernatant gave a titer of $7.2 \times 10^8$ P.F.U./mL. This result shows that the bacteriophage precipitation was not complete and that some activity was still present in the supernatant. The phage was then dialyzed against volatile buffer which contained 0.05M ammonium carbonate and 0.1M ammonium acetate and was adjusted to pH 7.2 with 50% acetic acid.

Depolymerization of the capsular polysaccharide from E. coli K32 by bacteriophage $\phi$32

E. coli K32 polysaccharide (195 mg) was dissolved in 15 mL of a volatile buffer and to this solution a total of $0.85 \times 10^{13}$ P.F.U. in 50 mL of a volatile buffer was added. The mixture was kept for 48 h at 37°, chloroform being added to prevent bacterial growth. The depolymerization mixture was transferred into a dialysis bag and dialyzed against distilled water overnight. The dialysis was repeated twice more, and the dialyzates were combined and freeze-dried. The residue was redissolved in distilled water and freeze-dried again. This process was repeated several times until the weight of the sample
remained unchanged (114.2 mg). The contents of the dialysis bag was freeze-dried separately to yield 260.5 mg.

**Separation of the depolymerized material by gel-permeation chromatography**

The crude dialyzable material (114.2 mg) was placed on a column of Bio-Gel P-4 (400 mesh) and eluted at 10.5 mL/h. Fractions (2 mL each) were collected and freeze-dried. The elution profile is shown in Fig. VI.1 (see Section VI.2). Three fractions of the degradation products were obtained: fraction I - tubes 9-14 (17.4 mg); fraction II - tubes 15-21 (20 mg); fraction III - tubes 22-34 (22.75 mg). Molecular weight distribution of fraction II showed the presence of an octasaccharide, which represented a double repeating unit of *E. coli* K32 polysaccharide (see Section VI.2).

**Determination of the reducing end and the degree of depolymerization**

A sample of oligosaccharide (10 mg) was dissolved in H₂O (5 mL) and to this NaBH₄ (15 mg) was added. After stirring for 2 h, the excess of sodium borohydride was removed by treatment with Amberlite IR-120(H⁺) ion-exchange resin. The dried, reduced oligosaccharide was refluxed in 3% methanolic HCl overnight. After neutralization with Ag₂CO₃, and evaporation of the solvent after centrifugation, the uronic ester was reduced with NaBH₄ in anhydrous methanol (5 mL). The reduced material was hydrolyzed with 2M trifluoroacetic acid on a steam bath overnight and the excess acid was removed by co-evaporations with water. A solu-
tation (0.5 mL) of 5% hydroxylamine hydrochloride in pyridine was then added and the reaction mixture was heated on a steam bath for 15 min. Acetic anhydride (0.5 mL) was added to the cooled solution which was heated on a steam bath for 1 h. The mixture of peracetylated aldononitriles and peracetylated alditol acetates was isolated by partition between water and chloroform. G.l.c. analysis was performed on column C isothermally at 230°.

**Methylation analysis and reducing end determination**

The oligosaccharide (5 mg) was reduced with NaBH₄ prior to methylation by the Hakomori procedure. The reduced material was dissolved in dry dimethyl sulfoxide (2 mL) and methylated by treatment with 1 mL of dimethylsulfinyl anion for 2 h, and then 2 mL of methyl iodide for 1 h. The methylated product was recovered by partition between water and chloroform. It was then hydrolyzed with 2M trifluoroacetic acid on a steam bath overnight, reduced with NaBH₄ and acetylated with 1:1 acetic anhydride-pyridine. A mixture of partially methylated alditol acetates was analyzed by g.l.c. (column B, isothermally 170°) and g.l.c.-m.s. (see Table VI.1 and Table VI.4).
CHAPTER VII

BIBLIOGRAPHY
VII BIBLIOGRAPHY


67 V.N. Reinhold, Methods Enzymol., 25 (1972) 244-249.
76 M. Abdel-Akher and F. Smith, Nature (Lndon), 166 (1950) 1037-1038.
100 V.N. Reinhold, Methods Enzymol., 25 (1972) 244-249.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Author(s)</th>
<th>Title</th>
<th>Journal/Book</th>
<th>Year</th>
<th>Pages</th>
</tr>
</thead>
</table>


191 R.E.W. Hancock, Department of Microbiology, University of British Columbia, personal communication.


190


200 M.E. Bayer, H. Thurow, and M.H. Bayer, Virology, 94 (1979) 95-118.


APPENDIX I

THE KNOWN STRUCTURES OF THE Escherichia coli O Antigens

(as of August 1, 1984)
APPENDIX I

E. coli O antigens

06 \[ \begin{array}{c}
\text{Man}^{3} \\
\text{Man}^{1} \beta \\
\text{Man}^{1} \beta \\
\text{GlcNac}^{1} \alpha \\
\text{GalNac}^{1} \alpha \\
\text{Glc}^{2} \\
\end{array} \]

07 \[ \begin{array}{c}
\text{GlcNac}^{3} \\
\text{Qui}^{3} \alpha \\
\text{4Nac}^{2} \alpha \\
\text{Man}^{1} \alpha \\
\text{Gal}^{1} \beta \\
\text{Rha}^{1} \beta \\
\end{array} \]

Qui 4Nac = 4-acetamido-4,6-dideoxy-D-glucopyranose

08 \[ \begin{array}{c}
\text{Man}^{3} \\
\text{Man}^{2} \alpha \\
\text{Man}^{2} \alpha \\
\text{Man}^{1} \alpha \\
\end{array} \]

09 \[ \begin{array}{c}
\text{Man}^{3} \\
\text{Man}^{1} \alpha \\
\text{Man}^{1} \alpha \\
\text{Man}^{1} \alpha \\
\text{Man}^{1} \alpha \\
\end{array} \]

018ac \[ \begin{array}{c}
\text{Rha}^{2} \\
\text{Gal}^{1} \alpha \\
\text{Glc}^{3} \alpha \\
\text{GlcNac}^{1} \alpha \\
\text{GlcNac}^{1} \alpha \\
\text{Glc}^{1} \beta \\
\end{array} \]
Col=3,6-dideoxy-L-xylopyranose
where Qui$_p^{3N}$ = 3-amino-3,6-dideoxy-β-D-glucopyranose
References


APPENDIX II

THE KNOWN STRUCTURES OF THE \textit{Escherichia coli} K Antigens

(as of August 1, 1984)
APPENDIX II

E. coli K antigens

\[ \frac{8}{\alpha} \text{NANA5Ac} \quad \frac{2}{\alpha} \]

E. coli K1

\[ \begin{array}{c}
- P \quad 4 \text{Gal} \quad \left( \frac{1}{\alpha} \text{Gly} \quad \frac{1(3)}{2n} \right) \\
- (\rightarrow P \quad 5 \text{Galf} \quad \frac{1}{\alpha} \text{Gly} \quad \frac{1(3)}{2n} )_n
\end{array} \]

E. coli K2

\[ \begin{array}{c}
- 4 \text{GlcA} \quad \frac{1}{\beta} \text{GlcNAc} \quad \frac{1}{\alpha}
\end{array} \]

E. coli K5

\[ \begin{array}{c}
- 2 \text{Ribf} \quad \frac{1}{\beta} \text{Ribf} \quad \frac{1}{\beta} \text{KDO} \quad \frac{2}{\alpha} \text{or} \\
- 3 \text{Ribf} \quad \frac{1}{\beta} \text{KDO} \quad \frac{2}{\alpha}
\end{array} \]

E. coli 6a  E. coli K6
\[ \begin{align*}
\text{E. coli K7 and K56} & \\
3 \text{ManNAcA} & \frac{1}{\beta} \text{Glc} \frac{1}{\beta} \\
& \text{OAc}
\end{align*} \]

\[ \begin{align*}
\text{E. coli K12 and K82} & \\
3 \text{Rha} & \frac{1}{\alpha} \text{Rha} \frac{1}{\alpha} \text{KDO} \frac{2}{\beta} \\
& \text{OAc}
\end{align*} \]

\[ \begin{align*}
\text{E. coli K13} & \\
3 \text{Rib} & \frac{1}{\beta} \text{KDO} \frac{2}{\beta} \\
& \text{OAc}
\end{align*} \]

\[ \begin{align*}
\text{E. coli K14} & \\
6 \text{GalNAc} & \frac{1}{\alpha} \text{KDO} \frac{2}{\beta} \\
& \text{OAc}
\end{align*} \]

\[ \begin{align*}
\text{E. coli K15} & \\
4 \text{GlcNAc} & \frac{1}{\alpha} \text{KDO} \frac{2}{\beta}
\end{align*} \]

\[ \begin{align*}
\text{E. coli K20} & \\
3 \text{Rib} & \frac{1}{\beta} \text{KDO} \frac{2}{\beta} \\
& \text{OAc}
\end{align*} \]
-3 Ribf 1-7 KDO 2/β

E. coli K23

-4 Glc 1-4 GlcA 1-3 Fuc 1/α

Gal

E. coli K27

-3 Glc 1-4 GlcA 1-4 Fuc 1/α

4/β

Gal 2 or 3

E. coli K28

-2 Man 1-3 Glc 1-3 GlcA 1-3 Gal 1/α

4/β

Glc 1-2 Man

4/β

PyR

E. coli K29

-2 Man 1-3 Gal 1/β

3/α

GlcA 1-3 Gal

E. coli K30
— Gal $^{1-2}$ Glc $^{1-3}$ GlcA $^{1-4}$ Rha $^{1-2}$ Rha $^1$

**E. coli** K31

\[ \text{GlcA} \]

\[ \text{Gal} \]

\[ \text{Rha} \]

\[ \text{E. coli} \]

**K32**

\[ \text{Glc} \]

\[ \text{Rha} \]

\[ \text{Gal} \]

\[ \text{GlcA} \]

\[ \text{E. coli} \]

**K33**

\[ \text{Glc} \]

\[ \text{GlcA} \]

\[ \text{Fuc} \]

\[ \text{Gal} \]

\[ \text{QAc} \]

\[ \text{pyr} \]

\[ \text{E. coli} \]

**K42**

\[ \text{Gal} \]

\[ \text{GalA} \]

\[ \text{Fuc} \]

\[ \text{E. coli} \]

**K52**

\[ \text{Gal} \]

\[ \text{O} \]

\[ \text{Fru} \]

\[ \text{QAc} \]

\[ \text{OPr} \]

\[ \text{E. coli} \]

**K85**

\[ \text{GlcA} \]

\[ \text{Man} \]

\[ \text{Man} \]

\[ \text{GlcNAc} \]

\[ \text{Man} \]

\[ \text{Man} \]

\[ \text{GlcNAc} \]

\[ \text{Rha} \]

\[ \text{E. coli} \]

**K85**
\[ \text{Glc}^4 \text{A}^3 \beta, \text{FucNAd}^3 \beta, \text{GlcNAd}^3 \beta, \text{Gal}^6 \beta \text{Glc}^1 \beta \text{Glc}^2 \text{OAc} \to \text{E. coli K87} \]

\[ \text{NANA}^8 \text{Ac}^2 \alpha, \text{NANA}^9 \text{Ac}^2 \alpha \to \text{E. coli K92} \]

\[ \text{Rib}^1 \beta \text{Ribitol}^5 \text{O} \to \text{E. coli K100} \]
References


K32  E. Altman, unpublished results.

K33  B.A. Lewis, unpublished results.


APPENDIX III

$^1\text{H}$ AND $^{13}\text{C-n.m.r.}$ SPECTRA
K50 Polysaccharide
$^1$H-n.m.r.
400 MHz, 95°
K50 Polysaccharide

$^{13}\text{C-n.m.r.}$

100.6 MHz, 90°
K50 Degraded Polysaccharide
$^{13}$C-n.m.r.
20.1 MHz, ambient temp.
K50 Polysaccharide Compound A₃

\[ \text{GlcA}^{\alpha} \text{Man}^{\alpha} \text{Man}^{\alpha} \text{Gal}^{-\text{OH}} \]

$^1$H-n.m.r.

100 MHz, 90°
K50 Polysaccharide Compound $\text{A}^3$

\[ \text{GlcA}^{1.3} \text{Man}^{1.2} \text{Man}^{1.3} \text{Gal} \text{OH} \]

$^{13}$C-n.m.r.:

20.1 MHz, ambient temp.
K50 Polysaccharide Compound

GlcA\(\alpha\) Man\(\alpha\) Man\(\alpha\)OH

\(^1\)H-n.m.r.

100 MHz, 90°

Spectrum No. 6
K50 Polysaccharide Compound

\[ \text{GlcA}^{1,3} \text{Man}^{1,2} \text{Man}^\alpha \text{OH} \]

\[ ^{13}C \text{n.m.r.} \]

20.1 MHz, ambient temp.

Spectrum No. 7

acetone

31.07
K50 Polysaccharide Compound A

GlcA\(^{1-3}\)Man\(^{\alpha}-\)OH

\(^1\)H-n.m.r.

100 MHz, 90°
K50 Polysaccharide Compound SH1

\[
\begin{align*}
\text{Gal} & \quad \text{Glc} \\
\beta & \quad \alpha \\
\text{CHOH} & \quad \text{OCH}_2
\end{align*}
\]

$^{13}$C-n.m.r.
400 MHz, ambient temp.
K50 Polysaccharide Compound

\[ \text{Gal}^\beta_1\text{Glc}^\alpha_1\text{OCH} \]

\[ \text{CO}_2\text{H} \]

\[ \text{CHOH} \]

1H-n.m.r.
100.6 MHz, ambient temp.
99.62

Spectrum No. 10

acetone
31.07
E. coli K28 Polysaccharide

$^1$H-n.m.r.

400 MHz, 95°
E. coli K28 Polysaccharide

$^{13}$C-n.m.r.

100.6 MHz, ambient temp.

Spectrum No. 12
E. coli K28 Polysaccharide (after autohydrolysis)

\(^1\)H-n.m.r.

400 MHz, 95°
E. coli K28 Deacetylated Polysaccharide

$^1$H-n.m.r.

400 MHz, 95°

Spectrum No. 14

acetone

2.23

1.29

5.41

5.39

4.83

4.48

4.42
E. coli K28 Deacetylated Polysaccharide

$^{13}$C-n.m.r.

100.6 MHz, ambient temp.

Spectrum No. 15

acetone

31.07

99.32

102.81

103.91

16.05

16.12
E. coli K28 Compound A

\[ \text{GlcA}^{1-4} \text{Fuc}^{-\text{OH}} \]

$^1$H-n.m.r.

270 MHz, ambient temp.
E. coli K28 Compound Al

\[ \text{GlcA}^{1-4} \beta-\text{Fuc-OH} \]

\[ \text{C-}_{13}\text{n.m.r.} \]

20.1 MHz, 95°
E. coli K28 Compound NJ

Gal\(^1\)^\(\beta\) Glc\(^\sim\)OH

\(^1\)H-n.m.r.

400 MHz, ambient temp.
E. coli K32 Polysaccharide

$^1$H-n.m.r.

400 MHz, 95°
E. coli K32 Polysaccharide

$^{13}$C-n.m.r.

100.6 MHz, ambient temp.

Spectrum No. 21
E. coli K32 Deacetylated Polysaccharide

$^1$H-n.m.r.

400 MHz, 95°

Spectrum No. 22

acetone

2.23

2.19

5.52

5.25

5.20

5.11

4.73

1.34

2.0

5.0

4.0
E. coli K32 Deacetylated Polysaccharide

$^{13}$C-n.m.r.

100.6 MHz, ambient temp.

acetone 31.07

18.01
E. coli K28/bacteriophage φ28-1

$^1$H-n.m.r.

400 MHz, 95°

acetone

2.23

1.30
E. coli K28/bacteriophage φ28-1

NaBH₄ reduced

¹H-n.m.r.

400 MHz, 95°

Spectrum No. 25

acetone

2.23

1.30

1.21

2.0

4.0

5.0

5.0
E. coli K32/bacteriophage φ32
Fraction I
$^1$H-n.m.r.
400 MHz, 95°

Spectrum No. 26
acetone
2.19
2.23
1.34
2.16
E. coli K32/bacteriophage φ32
Fraction II
$^1$H-n.m.r.
400 MHz, 95°

Spectrum No. 27
acetone 2.23

1.12
1.35 1.33
2.19
2.17

5.5 5.48 5.44 5.24 5.18 5.15 5.11 5.05 5.02

4.71 4.69

5.0 4.0 2.0
E. coli K32/bacteriophage φ32
Fraction II (R)
$^1$H-n.m.r.
400 MHz, 95°