c.1

STRUCTURAL INVESTIGATIONS AND BACTERIOPHAGE DEGRADATIONS OF KLEBSIELLA CAPSULAR POLYSACCHARIDES

by

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ABSTRACT

Seventy-seven serologically different strains of Klebsiella are known. The capsular polysaccharides which these bacteria produce are antigenic, and in order to understand the chemical basis of serological differentiation the structural investigation of the capsular polysaccharides has been undertaken. To date, fifty six structures have been determined.

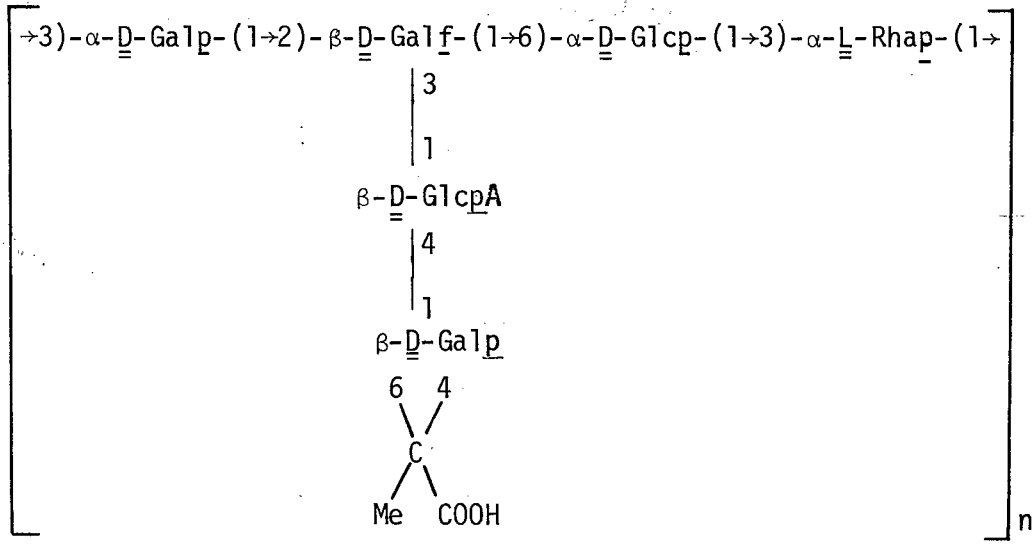
The structures of the capsular antigens isolated from serotypes K12 and K58 are presented here, along with confirmative data for the structure of K23 and a nuclear magnetic resonance investigation of K70 and its specific degradation products.

An efficient means of isolating large quantities of the single repeating units of the Klebsiella polysaccharides using glycanase enzymes, borne and utilized by specific bacteriophage, is demonstrated. Klebsiella K21 polysaccharide has been degraded using a highly purified bacteriophage (β-galactopyranosidase activity), while Klebsiella K12 and Klebsiella K41(which have similar structures) have both been degraded using a crude solution of bacteriophage specific for Klebsiella K12 (β-galactofuranosidase activity), and results compared.

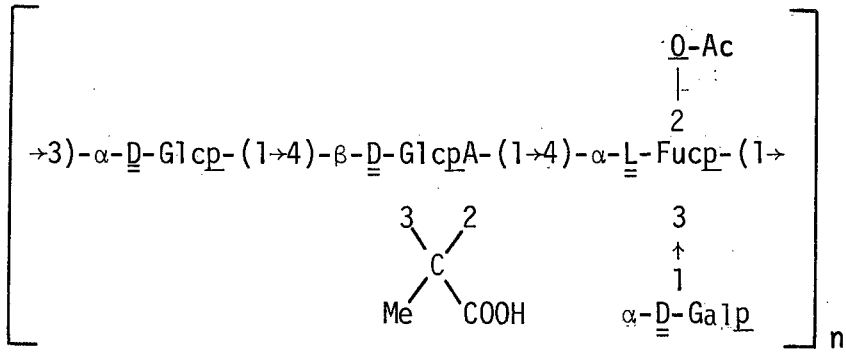
A preliminary investigation of the use of high pressure liquid chromatography in the structural investigation of hetero-polysaccharides is included, along with appendices containing

compilations of the structures and structural patterns of the Klebsiella capsular polysaccharides determined to date.

K12



K58



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The stimulating direction of Professor G.G.S. Dutton and the cheerful support of my colleagues, in particular José Di Fabio, are gratefully acknowledged. I wish to thank Robert St.-Pierre for the illustrations, Dr. E.H. Merrifield for proof reading, and Celine Gunawardene for typing this thesis.

I am grateful to MacMillan Bloedel for the award of a graduate scholarship (1977-1978).

PREFACE

This thesis has been written in the context of the activities of our research group which are concerned with the determination of the primary structure, along with specific degradations, of carbohydrate antigens. Many of the methods used in this field are considered as standard, and so have been discussed only briefly.

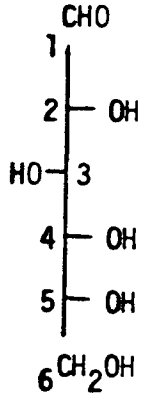
However, in the last few years there have been considerable advances in instrumental techniques and there are now sufficient applications to polysaccharide chemistry reported in the literature to warrant a review of these methods. Therefore, a more detailed account of the use of nuclear magnetic resonance spectroscopy (both ^{1}H and ^{13}C) in the study of polysaccharides is presented here. It is proposed that future theses emanating from this group will similarily review gas-liquid chromatography, mass spectrometry, and high performance liquid chromatography; these techniques are, accordingly, not given special treatment here.

In Appendix I I have revised the list of known structures of Klebsiella polysaccharides along with literature references, compiled by Keith Mackie (Ph.D. 1977). Bacteriophage attack sites, optical rotations, and x-ray crystallography references have also been included. In his M.Sc. thesis (1980) Marcel Paulin classified the known Klebsiella structures according to their structural patterns. This has been revised and included in Appendix II with kind permission.

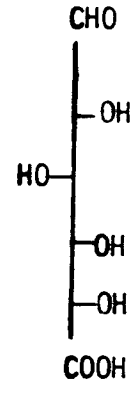
In the Introduction I have attempted to present my work in a historical context and to show its relevance to current immunochemical research, although the main body of work is primarily chemical in nature.

As has been the practice with other theses presented by members of our group an explanation of carbohydrate nomenclature[+] is now offered to familiarize readers who are not acquainted with the field.‡

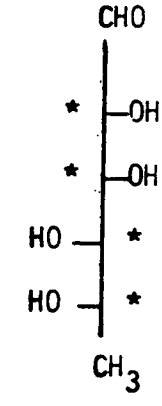
Fischer projection formulae are used to represent the acyclic modification of sugars. Some examples are shown below. Numbering commences from the carbonyl group at the top of the chain (I). Note that D-glucuronic acid (II) differs from D-glucose (I) only



D-glucose
(I)



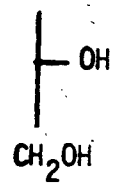
D-glucuronic acid
(II)



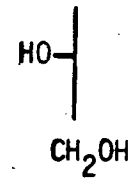
L-rhamnose
(III)

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

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

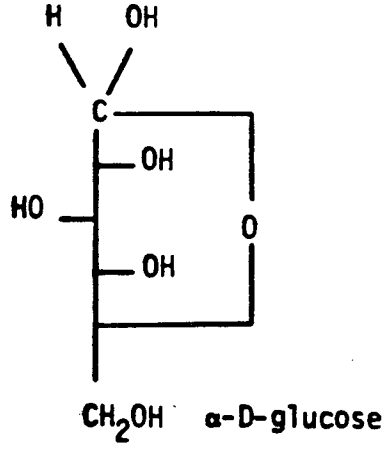


D-series

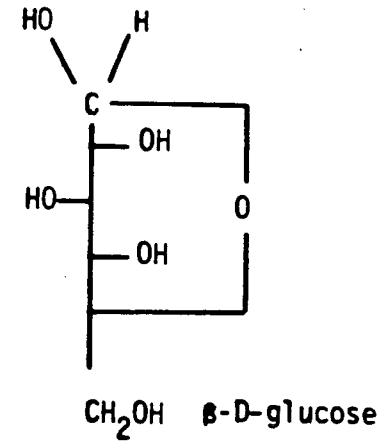


L-series

Physical and chemical evidence indicates that, in fact, these six-carbon polyhydroxyaldehydes exist in a cyclic form. The ring closure occurs by nucleophilic attack of the oxygen atom at C-5 on the aldehydic carbon atom, generating a new chiral (anomeric) center at C-1. This results in two anomers, represented below



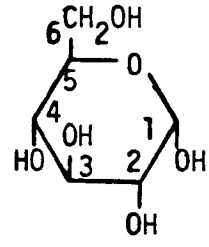
α-D-glucose
(IV)



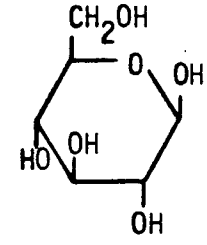
β-D-glucose
(V)

in the Tollens formulae. It should be noted that C-1 is unique in having two attached oxygen atoms, formally making it a hemiacetal carbon.

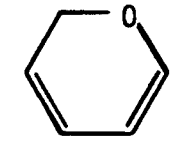
Since the Tollens formulae have obvious limitations with their unequal bond lengths, Haworth developed a perspective method of looking at the six-membered ring (VI and VII). This improvement recognizes that the ring oxygen atom lies behind the carbon chain and that bond lengths are approximately equal.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



α-D-glucopyranose
(VI)

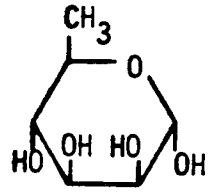


β-D-glucopyranose
(VII)

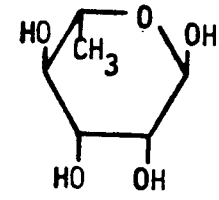


pyran
(VIII)

which he related to such rings at the heterocyclic compound pyran (VIII) and named them pyranoses. Note that hydroxyl groups not involved in ring formation on the right in Fischer and Tollens formulae point down in the Haworth projections and those on the left point up. Similarly, for aldopyranoses, the group on C-5 points up for D(IX) and down for the L enantiomer (X). It follows, then, that when sugar residues are attached there are two possible configurations, an α - or a β-pyranoside, for each linkage.

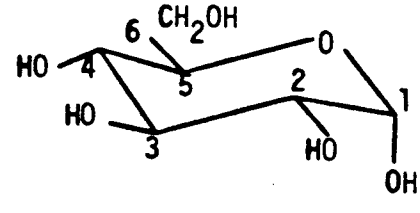


α-D-rhamnopyranose
(IX)



α-L-rhamnopyranose
(X)

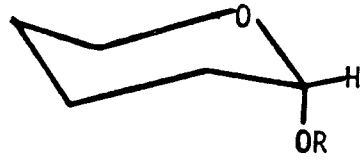
The true conformation of pyranoid carbohydrates is related to the chair form of cyclohexane. X-ray diffraction analysis has shown that a hexose, such as α-D-glucose (XI), consists of a puckered, six-membered, oxygen-containing carbon ring with hydroxyl substituents at C-1 through C-4, and a hydroxymethyl group at C-5. All substituents on the ring, except for that at C-1, are equatorial.



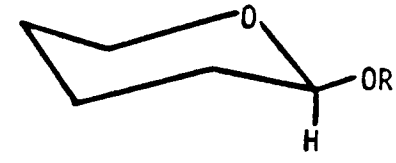
(XI)

Two isomers (anomers) are possible in relation to the anomeric center (C-1), depending on whether a substituent is axial

(α-anomer; XII) or equatorial (β-anomer; XIII), where R = hydrogen, for monosaccharides, and R = another sugar residue, for di-, oligo-, and polysaccharides. Since H-1 is in different chemical environment for the two anomers, nuclear magnetic resonance spectroscopy can easily distinguish between them and, thereby, provides invaluable assistance in assigning anomeric configurations.



(XII)



(XIII)

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

+ The Carbohydrates. Chemistry and Biochemistry Vol. II B. (Eds. W. Pigman and D. Horton). Academic Press. New York. 809-834 (1972)

‡ Reproduced with the kind permission of T.E. Folkman from his M.Sc. thesis entitled "Structural Studies on Klebsiella Capsular Polysaccharides", University of British Columbia, April 1979.

I. INTRODUCTION

I. INTRODUCTION

Polysaccharides are ubiquitous - they are by far the most abundant biopolymers on earth.[1] Those such as cellulose [2,3] and gum arabic [4,5] have been recognized and used by man for centuries. More recently the exopolysaccharide produce by Xanthomonas juglandis[6] has become of economic value in enhanced oil recovery systems [7], and alginate [8,9], obtained from certain species of marine algae is commercially important as a food additive. [10]

Research interests had, for many years, centered on plant polysaccharides, and later, on mucopolysaccharides of higher animals,[11] but the comparatively recent realization that microbial polysaccharides are composed of regular repeating units, along with the fact that they play an important role in fundamental research on the immune reaction,[12-15] have prompted the systematic investigation of the structures of the exopolysaccharides of various families.

Microbial polysaccharides [16] are located on the cell surface and are, therefore, of importance in the recognition and immune response of a higher organism to microbial infection. The polysaccharides are either an integral part of the cell wall - lipopolysaccharide (LPS) or occur as a slime or capsule as in the case of Pneumococcus, Escherichia coli, and Klebsiella (see Fig. 1).

"Capsular" polysaccharide is considered to have a definite boundary and to remain adherent to the cell wall when suspended in

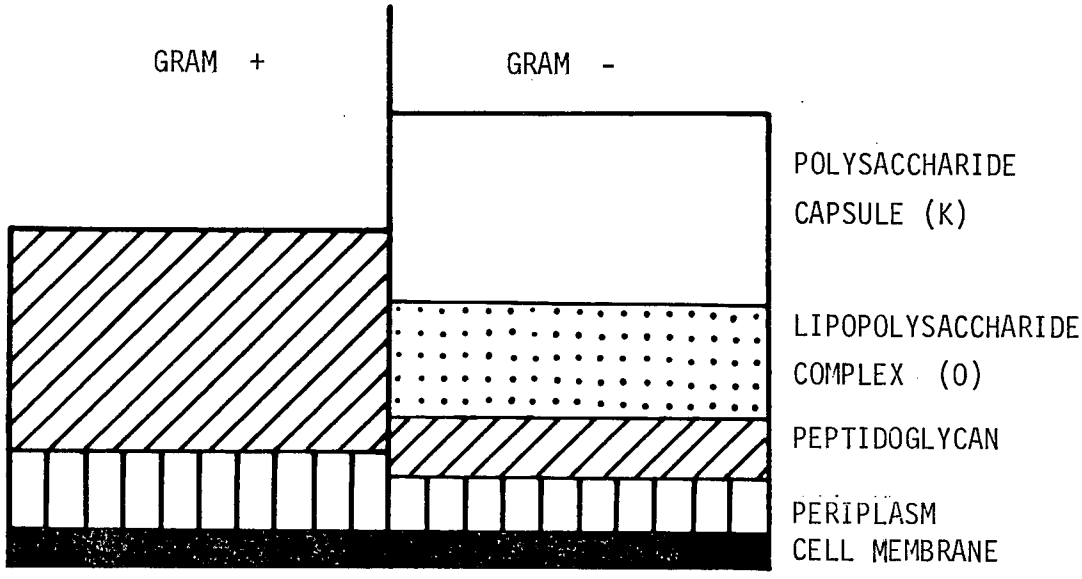


Figure 1 Diagramatic representation of the bacterial cell envelope

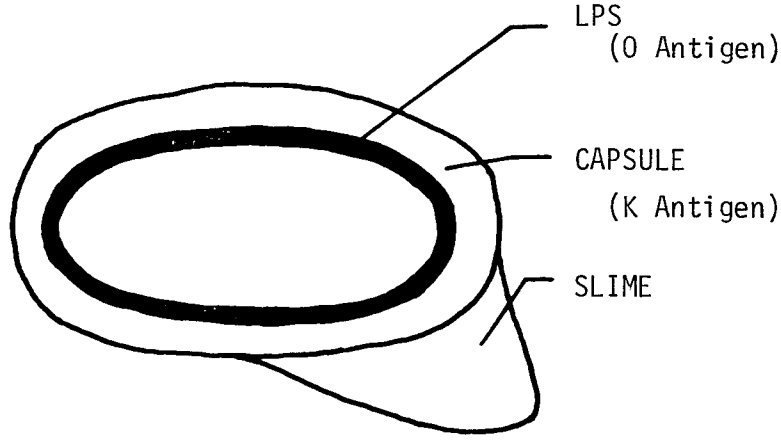


Figure 2 Extracellular polysaccharide.

water. The term "slime" is used to indicate a network of carbohydrate fibres not distinctly associated with any one bacterium. Dudman and Wilkinson found that capsular and slime polysaccharides are identical in chemical composition. [17,18]. Due to the high water content of the capsule (99%) only wet preparations, such as India ink films, can give accurate information about the size and shape of the capsule or slime. When water is removed from capsules of Klebsiella bacteria, by means of ethanol, for visualization by electron microscopy, the individual fibrils of the capsule collapse on one another to form thick projections. Occasionally there is some evidence for peripheral linking of fibres. This would fit well the idea of a well-defined capsular edge (see Fig. 2).

Practically all bacterial capsules consist of polysaccharides and are associated with pathogenic micro-organisms. The genus Klebsiella is composed of Gram-negative, nonmotile bacteria, of the family Enterobacteriaceae and the tribe Klebsielleae [19,20]. Isolants are identified and classified by biochemical reactions into three species: K. pneumoniae, K. ozaenae, and K. rhinoschleromatis. Two distinct antigens are present in encapsulated strains of Klebsiella, one in the capsule (K antigen) and the other in the soma, (see Fig. 1). The capsular antigen was shown to be carbohydrate in nature by Toenniessen [21,22] in 1914. Since most Klebsiella bacteria are heavily encapsulated the O-antigen is completely shielded (see Figs. 1 and 2). Consequently, serological classification is based solely on their capsular K-antigens. To date seventy seven serologically different

capsules have been delineated [23,24].

Many of these micro-organisms are found in healthy carriers in the upper respiratory, intestinal, and genito-urinary tracts [25]. Their pathogenicity to man is well known although some strains are not toxic. Acute infection in the lung occasionally mimics pneumococcal lobar pneumonia. The chronic form resembles tuberculosis. Klebsiella pneumonia K-types 1, 2, 7 and 8 are the commonest invaders and are responsible for approximately three per cent of all bacterial pneumonias, occurring when host-resistance is impaired, for example, in alcoholics[26]. Almost all strains of K. ozaenae are members of K type 4, and are associated with ozena, a fetid, catarrhal condition of the nose. Infections due to K. rhinoschleromatis occur rarely in North America. Klebsiella resists many antimicrobic drugs. Streptomycin and chloramphenicol have proved of value in therapy, but the proportion of resistant strains, due to mutations, is increasing steadily.

One of the most outstanding features of bacterial polysaccharides to become apparent of late is that they are composed of regular repeating units [27], as shown by molecular weight distribution studies [28], and more recently by nuclear magnetic resonance spectroscopy [29]. All are heteropolysaccharides and have proved to be an almost inexhaustible source of novel oligosaccharides and sugars. Nimmich [30,31] has reported the qualitative composition of the Klebsielle K-types and has also classified them into chemotypes [32] (see Table 1).

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

K82 has been added but its qualitative composition is not yet known.[24]

P- Pyruvic acid present in addition

Note: K9 and K9*, see Appendices I and II

TABLE 1 : Klebsiella capsular polysaccharides (K1-K83)
Quantitative analysis and chemotype grouping.

All the capsules are acidic, due to the presence of either glucuronic acid, galacturonic acid or a keto acid. In addition pyruvic acid, linked as a ketal may be present and occasionally is the only acidic component (see Table 1). The hexoses D-glucose, D-galactose and D-mannose usually occur in the pyranose from - the furanosyl form of D-galactose occurs in K12 [33] and K 41[34]. In some strains the 6-deoxyhexoses L-rhamnose and L-fucose are found; non-carbohydrate O-acetyl and O-formyl groups may also occur.

To date structures have been proposed for fifty five Klebsiella polysaccharides. Various structural patterns (linear, branched, comb-like, etc.) have emerged. These have been tabulated by Paulin [35] (see Appendix II). The number of sugars per repeating unit varies from three [36] to seven [34].Polysaccharides are capable of greater diversity per unit structure than other types of macromolecules and so it is not surprising, then, that serological testing has denoted seventy seven different types.

The quantity of capsular polysaccharide produced by organisms has been found to be dependant on culture conditions. For optimal production a low nitrogen content of the medium is essential[37]. Little is known about the function of microbial extracellular polysaccharides, in contrast to the wealth of detailed knowledge of their composition and structure. This imbalance has been attributed [38] to the early discovery that the antigenic specificity of bacterial cells was determined by their outermost components -. invariably polysaccharides.

The great majority of polysaccharide - producing micro-organisms appear to be unable to depolymerize or to utilise their own extracellular polysaccharides as carbon sources. The following functions have, however, been proposed by Dudman.[38].

(a) Virulence - protection against serum bactericidal factors and phagocytosis

(b) Protection against predation

(c) Protection against desiccation

(d) Adhesion in aqueous environments

(e) Role in dental cariogenesis

(f) Role in ionic interactions

(g) Role as general barriers

(h) Role in enzyme reactions

(i) Role in silicon metabolism

The first mentioned will be dealt with later.

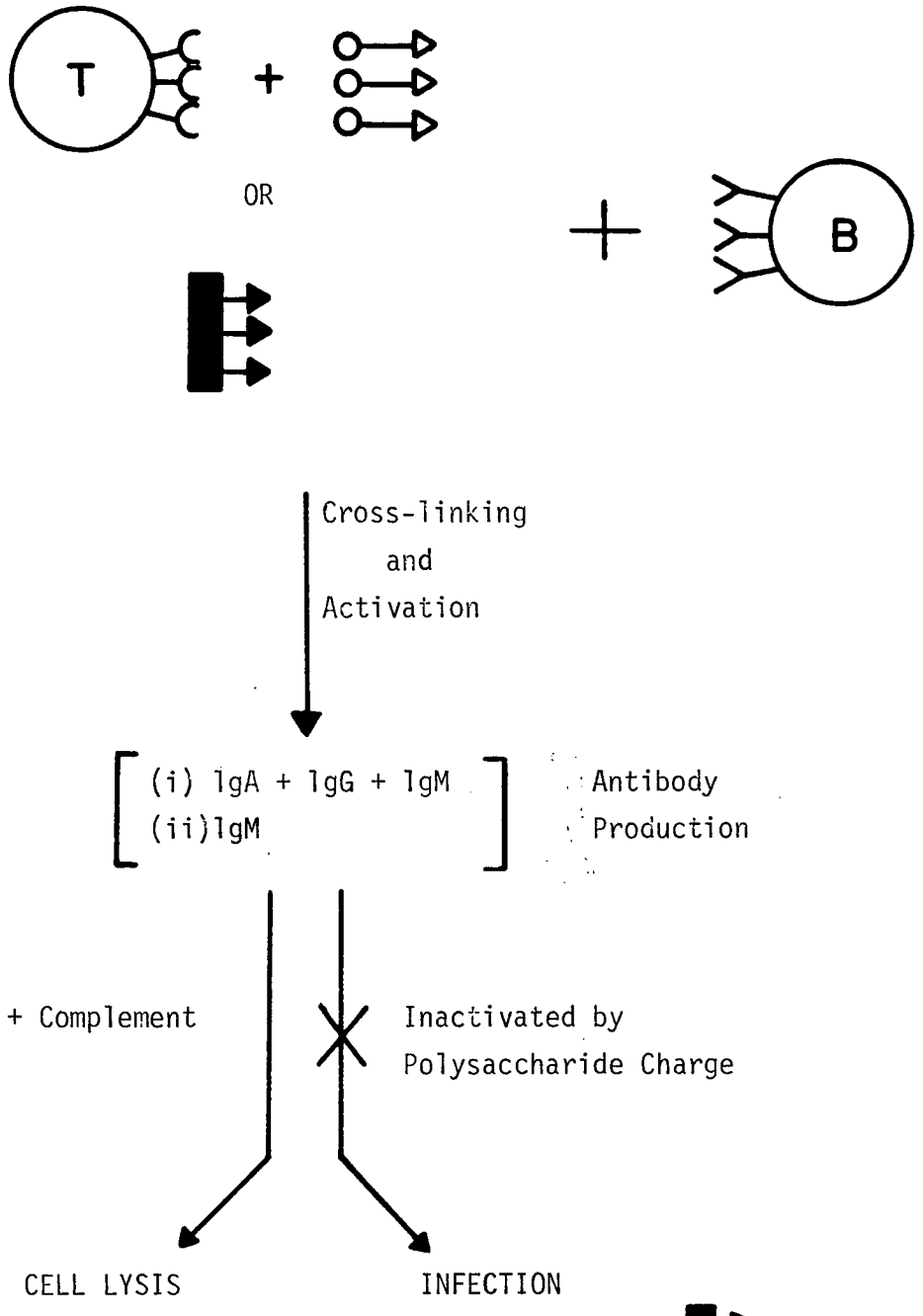
The past few years have seen a notable advance in the study of polysaccharide secondary and tertiary structure, both in solutions and gels[39] and by x-ray diffraction. Progress in the latter field has been made mainly due to the development of improved crystallization techniques by Atkins[40] and the concomitant development of sophisticated computer programmes for analysing the diffraction data obtained. In his review of 1979 Atkins [41] offers stereochemical models for eight Klebsiella serotypes - K5, K8, K9, K16, K25, K54, K57 and K63. In the last mentioned case the x-ray diffraction results help differentiate between two proposed structures.

In dealing with bacterial polysaccharides as antigens [42-44], two aspects have to be considered. One is their capacity to induce the formation of antibodies in mammals i.e. their IMMUNOGENICITY, and the other is their reactivity with antibodies, i.e. their ANTIGENIC SPECIFICITY. Our present knowledge in this field is based to a large extent on the pioneering work of Heidelberger and coworkers at the Rockerfeller Institute [45-47] and of Kabat et al. [48]. The former initiated quantitative studies in immunochemistry, while it is largely the work of the latter from which the concept of the antibody combining site was developed.

The aim of the immunochemical analysis of polysaccharide antigens, which combines serological and chemical studies, is to define oligosaccharide structures within the polysaccharide as chemical expression of its immunological character. According to a proposition of Staub and Heidelberger [49] the sugar unit that contributes most to the serological specificity is termed the IMMUNODOMINANT sugar. It may be terminal or within the main chain. Not only the nature of the sugar unit, its substituents (if any) and the anomeric configuration of its linkage, but also the position to which it is linked may greatly influence the antigenic expression of the determinant. In acidic polysaccharides, such as those of Klebsiella, the charged constituents are often immunodominant sugars or part of antigenic determinants [50]. Due to their repetitive structure, bacterial polysaccharides have the same antigenic determinants expressed many times over (see Fig. 3).

Recent developments in immunology [51] may offer an explanation of the antigenic properties of these polysaccharides. It is postulated that for the production of antibodies to any immunogen certain cells of the host's immune system have to co-operate (see Scheme 1). The most important cells are the T-lymphocytes (Thymus derived) which recognize and concentrate the antigen and present it to the B-lymphocytes (bone-marrow derived), which, after further differentiation to plasma cells, produce antibody molecules. Polysaccharides are T-cell independent and also activate the alternate complement pathway. They may be so since their antigenic determinants are close enough together and also numerous enough to react effectively with B-cells. It is worth noting that the antibodies produced are almost exclusively of the Ig M type. However, when the antigen is an oligosaccharide linked to a protein carrier (see later), then, T-cell co-operation is required and the antibodies subsequently induced are Ig G and Ig A as well as Ig M.

In many polysaccharides from different organisms identical oligosaccharide regions are found. As a consequence of this the same determinant is recognised by its homologous antibody in different polysaccharides. Therefore these polysaccharides, no matter in what organism they are produced, are immunologically related; they cross-react serologically [52] (see Figs. 3, 4).



Scheme 1 Antibody production and the bactericidal reaction.

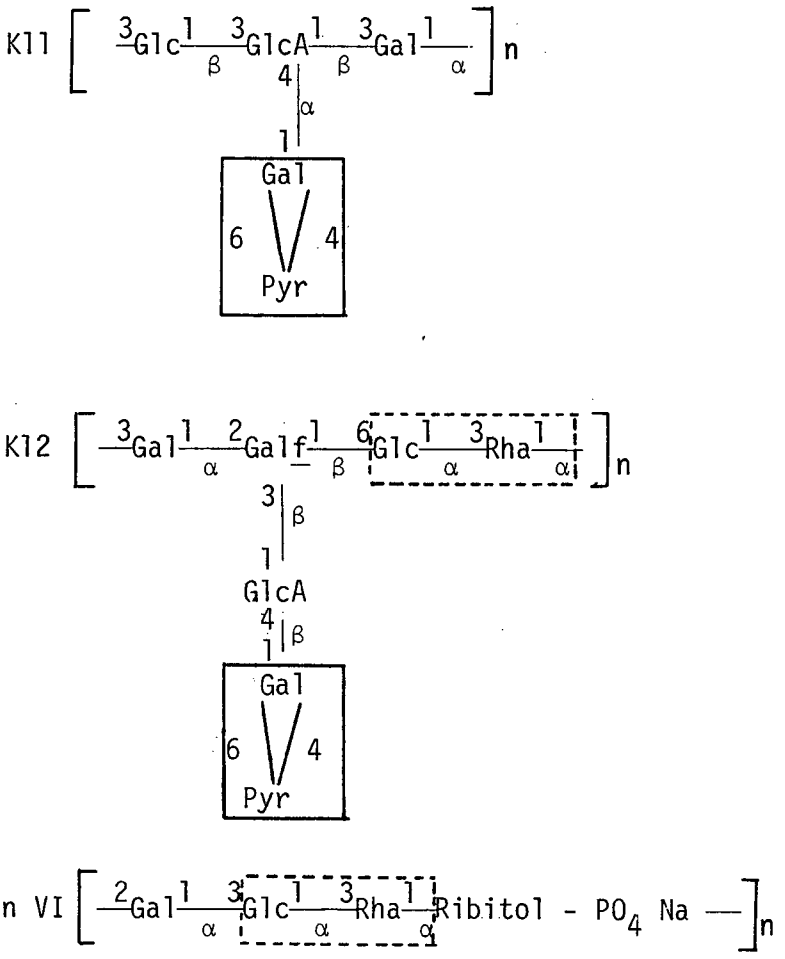


Figure 3 Klebsiella K12 capsular polysaccharide cross-reacts with anti-serum to Klebsiella K11 and also with anti-Pneumococcus VI

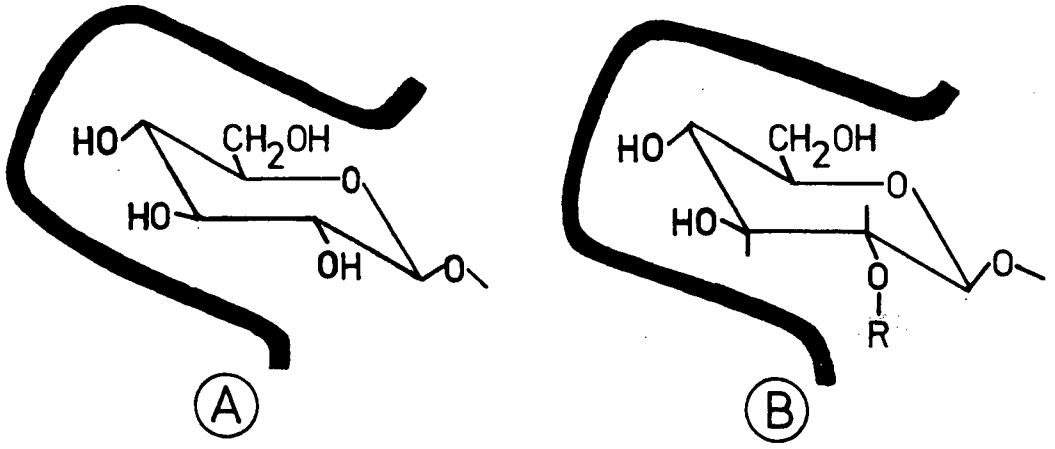


Figure 4 Different polysaccharides containing terminal and 2-substituted sugars may cross-react. The shaded area represents the antibody combining site.

Recently Heidelberger and colleagues [53-56] have examined cross-reactions between the capsular polysaccharides of Klebsiella and pneumococcus and subsequently made elegant predictions on substructures of Klebsiella polysaccharides.

Most of the work to date has involved heterogeneous antibodies, that is to say, sera containing antibody populations, that although specific for one antigen, are made up of differing molecular species of immunoglobulins. These may bind the same antigenic grouping (hapten)* in different ways, or different immunoglobulins may bind small or large sections of the respective

* A hapten is defined as a small molecule, which, by itself, cannot stimulate antibody synthesis but will combine with antibody once formed.

pattern on the polysaccharide chain.

A new potential in polysaccharide immunochemistry is provided by homogeneous immunoglobulins that bind carbohydrate polymers[57]. Here, the specific immunoglobulin - hapten interaction can be characterized in detail. Thus the discipline of carbohydrate chemistry can make a real contribution to the elucidation of the structure of immunoglobulins. In return, carbohydrate chemistry may find a tool that can increasingly be applied to the unraveling of its own unsolved problems in the structural analysis of polysaccharides.

The fate of a host which is invaded by micro-organisms depends on the effectiveness of its defence mechanisms. To a large extent these are directed against the surface of the micro-organism. The host defences may be inhibited thus enabling the micro-organisms to multiply to such an extent as to eventually damage or kill the host. This phenomenon is called VIRULENCE.

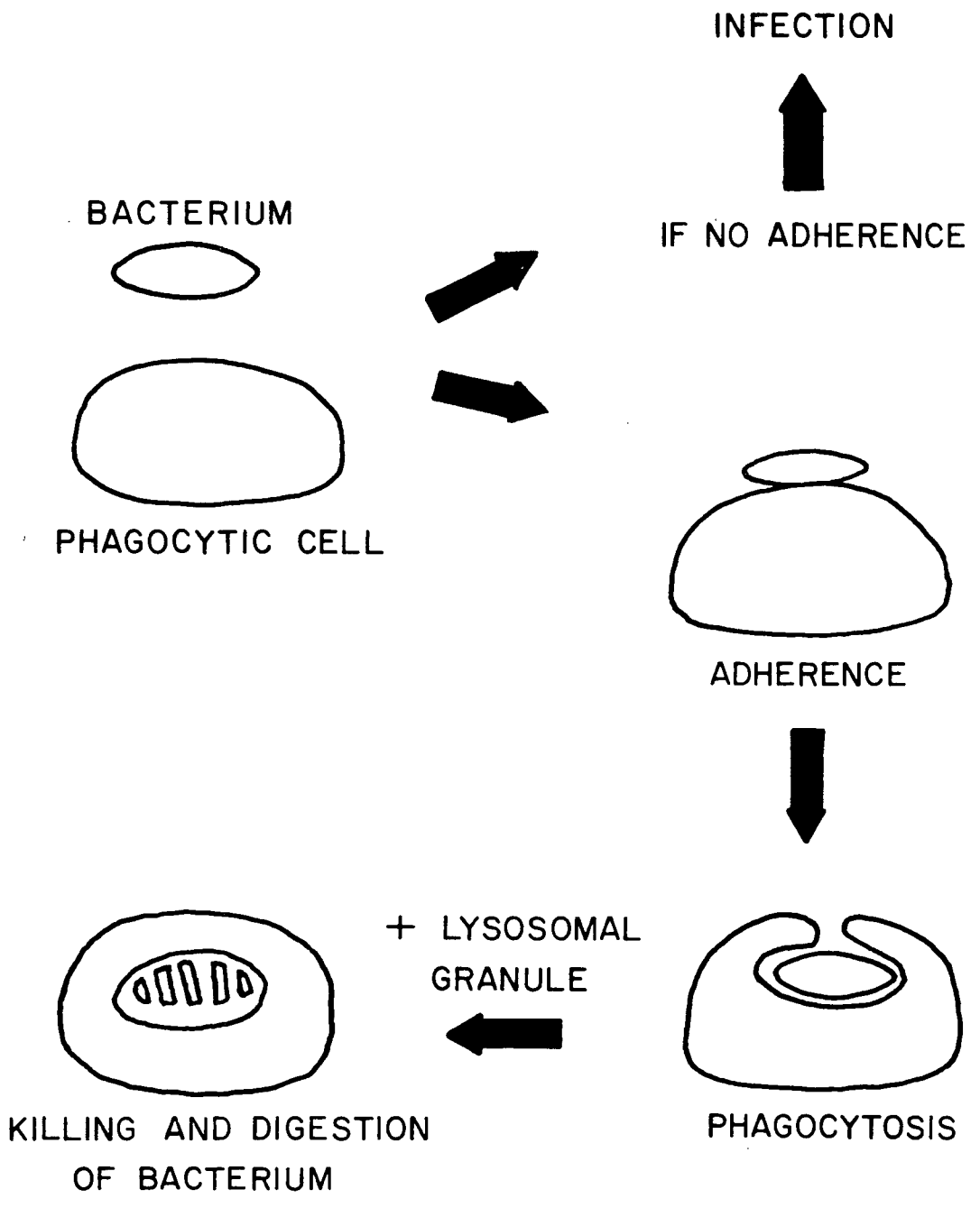
The relationship between virulence and capsulation has been long known (Bordet 1897) [58]. The virulence of an organism does not depend only on the presence of a capsule and the presence of a capsule does not always confer virulence on all strains of a pathogenic species, but it has been shown that capsulated, virulent strains of pathogenic bacteria generally become less virulent when they lose their capsules.

The best understood defence reactions with which microbial polysaccharides are known to interact are those that

involve primarily circulating antibody and the complement system, viz., the bactericidal reaction and phagocytosis. In the former, the interaction of serum antibody with its antigenic determinant activates the complement system (see Scheme 1). Practically all capsular polysaccharides are acidic and they inactivate complement, probably through their charge[59]. A part of the complement system is also involved in phagocytosis - the engulfment and killing of bacteria by macrophages and leukocytes (see Scheme 2). The antiphagocytic effect of capsular polysaccharides is probably not specific, charge and viscosity of the capsular material being prerequisites.

It has been shown that anti-polysaccharide antibodies are protective against certain infections. Heidelberger[60-62] immunized humans with pneumococcal polysaccharides, providing a significant degree of protection in an epidemic in which the corresponding pneumococci were involved. Protective immunization may be performed not only with bacterial vaccines and isolated polysaccharides, but also with artificial antigens, thus avoiding the possible toxic effects of bacteria.

The task of producing artificial antigens in large quantities is indeed arduous. The fact that monosaccharides are multifunctional and may be linked in either an α or a β configuration presents an enormous challenge to the synthetic carbohydrate chemist. Consequently, only a very small proportion of the possible number of disaccharides has been synthesised[63], and although synthetic procedures are progressing rapidly[64] only a limited



Scheme 2 Phagocytosis.

number of synthetic oligosaccharides has been realised [65,66].

An alternative, then, is to look to the possibility of obtaining oligosaccharides from the degration of polysaccharides. In 1969 a method was described [67] for the hydrolysis of polysaccharides, according to which the oligosaccharides, once formed, are separated from the hydrolysis mixture by dialysis, and are thus protected from further degradation. This technique has been used by Himmelspach et al [68] in 1973 to obtain the tetrasaccharide repeating unit of Salmonella illinois which was then coupled to protein to obtain an artificial antigen. However the procedure suffers from the fact that acid hydrolysis is non-specific and many different oligosaccharides are produced, and subsequent separation may prove difficult.

To obtain hydrolysis of polysaccharides at specific points attention has been turned by Stirm et al [69-71] to depolymerization with the aid of bacteriophage glycanases. By this method heteropolysaccharides consisting of repeating units may be specifically cleaved either by the whole bacteriophage, or the purified enzyme, to yield a homologous series of the single repeating unit and multiples thereof. It has been pointed out by Dutton [72] that this is the only method of obtaining an oligosaccharide with an intact acid labile pyruvate group.

To obtain the immunogen the haptenic oligosaccharide is coupled to an immunologically efficient carrier protein. Goebel and Avery's method [73,74] of preparing the conjugates via aminophenyl

glycosidation of the reducing sugar end groups was extensively used and was not improved upon for about three decades, although its application was almost exclusively restricted to mono- and disaccharides. Newer methods of coupling [75-77] have no such limitation and appear to be suited to virtually any reducing oligosaccharide and also to any chain length.

Microbial polysaccharides have also shown potential in the area of cancer research [78]. It has been known for more than a century that human malignant growths sometimes undergo regression following an acute, bacterial infection [79]. Since then extensive studies have been made on noncytotoxic and host-mediated antitumor polysaccharides from various sources. It has been reported [80,81] that polysaccharide complexes from Klebsiella, among others, were active against solid tumors. Many questions remain unanswered and the role of the polysaccharides as immunopotentiators is being especially debated.

In Chapter II the methodology of the structural analysis of polysaccharides is examined. Advances in chemistry are invariably aided by progress in instrumentation. Recent developments are discussed, then, with special reference to n.m.r. spectroscopy. To date the application of high performance liquid chromatography to carbohydrate analysis has been limited. Therefore, this technique is examined in Chapter V and its potential as a tool in the structural elucidation of polysaccharides and in obtaining large quantities of oligosaccharides is examined.

If continued advances are to be made in the area of immunochemistry it is clear that co-operation between the chemist and the immunologist is essential. For the latter to understand complex immunological reactions, well-classified antigens are required; and if the field is to expand to include large scale vaccination then, non-toxic immunogens are needed. These two areas -- structure determination of polysaccharides and their degradation by bacteriophage - - are examined in Chapters III and IV of this thesis.

II METHODOLOGY OF STRUCTURAL ANALYSIS OF POLYSACCHARIDES

II. METHODOLOGY OF STRUCTURAL ANALYSIS OF POLYSACCHARIDES

To determine the complete structure of a polysaccharide the constituent sugars must be identified, and their ratio, substituents, sequence, linkage pattern and linkage configuration ascertained. The chemistry of polysaccharides has been reviewed by Aspinall and Stephen [82] and Aspinall [83]. Recent theses from our group (Paulin 1980, Folkman 1979 and Mackie 1977) have surveyed the methodology of structural determination of polysaccharides. In the following section the most widely used techniques are discussed briefly, with special regard to ^{1}H and ^{13}C n.m.r. spectroscopy.

II. 1. Isolation and Purification

Samples of Klebsiella bacteria of specified serotype and strain number were received as stab cultures from Dr. Ida Ørskov (Copenhagen). Bacterial cultures were streaked on agar plates at 37°C until large, individual capsular colonies were obtained. Bacteria were grown by innoculation of beef-extract medium with a single colony for 3h at 37°, with shaking and subsequent incubation of this liquid culture on a tray of sucrose - yeast extract - agar for three days. The lawn of capsular bacteria produced was harvested by scraping from the agar surface, and the bacteria destroyed with a 1%. phenol solution. The polysaccharide was isolated by ultra-centrifugation of this solution. The viscous honey-coloured supernatant was precipitated into ethanol. The precipitate was dissolved in water and

was treated with CETAVLON[84] (cetyltrimethylammonium bromide) and centrifuged to isolate only the acidic polysaccharide. The CETAVLON-polysaccharide complex was dissolved in 4M sodium chloride, precipitated into ethanol, redissolved in water, and dialyzed against running tap-water for two days. The polysaccharide was isolated, as a styrofoam-like material, by lyophilization, and was shown to be homogeneous by electrophoresis on cellulose acetate strips and by nuclear magnetic resonance spectroscopy. (see App. IV).

II. 2. Separation Techniques

To obtain information on the constituent sugars of the polysaccharide itself and of its degradation products, the latter must be separated, purified and then checked for homogeneity. Separation is obtained by the established technique of paper chromatography, by gel chromatography and paper electrophoresis and by newer techniques such as high pressure liquid chromatography.

II. 2. 1. Paper chromatography (p.c.)[85,86]

The great value of this technique lies in it's ability to separate mixtures of monosaccharides and oligosaccharides simply, accurately and without derivatization, by employing different solvent systems. For analytical analysis very small amounts of material are needed, while the technique may also be employed on a preparative level. Acidic components may be distinguished from neutral components by using different solvent systems.

Paper chromatography is used for identifying constituents

either as the free sugars or as alditols [87] from hydrolysis of the native polysaccharide and of its degradation products. Methylated sugars and oligomers may also be identified [88]. P.c. is also used for monitoring controlled hydrolysis of the polysaccharide and for checking the composition of fractions obtained from gel chromatography of mixtures from partial hydrolysis and periodate oxidation. When pure oligomers are not obtained by gel chromatography preparative paper chromatography may be employed. Although tedious and time consuming, good results may be obtained, as in the analysis of K58 polysaccharide, where large quantities of the aldobi -, tri-, and tetrauronic acids were obtained. Sugars are detected with either (i) $AgNO_3$/ ethanol--NaOH / $Na_2S_2O_3$ or with (ii) p-anisidine hydrochloride in aqueous 1- butanol followed by heating at 110^0 for 5 min.[90]

II. 2. 2. Paper electrophoresis (p.e.)[86,91,92]

Large acidic oligomers move very slowly on paper chromatography. Paper electrophoresis, however provides an alternative convenient method for their examination. Good separations can be achieved in a number of hours.

Electrophoresis involves the migration of charged substances in a conducting solution under the influence of an electric field. Buffer pH conditions are chosen so that the materials to be separated exist in a charged state. The usual coolant used is kerosene. P.e. may be employed both analytically and preparatively. Sugars are detected as for paper chromatography [89,90].

II. 2. 3. Gel chromatography (g.c.)

Gel chromatography originated in 1956 with the work of Lathe and Ruthven[93], who achieved some degree of separation of tri -, di - and monosaccharides on a column of potato starch. The field has been reviewed by Churms [94]. Also known as gel filtration, gel-permeation chromatography, or molecular-sieve chromatography it is based on the decreasing permeability of the three dimensional network of a swollen gel to molecules of increasing size. As the order of elution of a series of similar substances from a gel column is governed largely by molecular weight, gel chromatography provides a means of determining molecular weights of polymers.

This technique has been used extensively for the separation of products of partial hydrolysis and periodate oxidation. Both SEPHADEX and BIO-GEL gels have been used in this study. In cases when the molecular exclusion limit of two different gels (eg. G-15 and P-2) is the same, the elution profile and the order of elution may not necessarily be the same, and this may be used to advantage to obtain a clean separation. The particle size grade (superfine to coarse) should also be taken into account in choosing a gel. BIO-GEL has the advantage of being resistant to bacterial contamination since it is a synthetic material. Wet sephadex (derived by crosslinking dextran) should be stored in a solution of 0.1% NaN_3.

The material is eluted with distilled water, or preferably with a buffer eg. water - pyridine - acetic acid 1000: 10: 4. Carbohydrate fractions from the column are first localized using the Molisch test [95]. To determine the elution profile a quantitative colorimetric technique such as the phenol-sulfuric assay [96] may be used, or individual fractions may be lyophilized and weighed. P.c. and p.e. are used to investigate the composition of fractions. Where homogeneity is not achieved, purification may be obtained with preperative p.c. or p.e.

Molecular - weight distribution studies of acid hydrolysis products from Klebsiella K54 exopolysaccharide by Churms and Stephen on BIO-GEL P-10 gave evidence for repeating units in the structure [28a,b].

Sephadex LH-20 (G-25 with most of the -OH groups alkylated) may be used successfully to purify large molecular weight carbohydrate material that is soluble in organic solvent, eg. permethylated or peracetylated polysaccharide.

II. 3. Instrumentation

II. 3. 1. Gas-liquid chromatography (g.l.c.)

Extensive reviews of the applications of g.l.c. to carbohydrates have been published by Dutton (1973 and 1975)[97,98]. Most carbohydrates are not sufficiently volatile to be used for g.l.c. and they must therefore be converted into volatile compounds.

The fact that each monosaccharide may give more than one peak owing to the formation of anomeric derivatives has led to a search for means to eliminate this complication. The latter problems may be surmounted by reduction to the alditol or by conversion to the corresponding nitrile. Direct acetylation then yields volatile derivatives.

Identification of the constituent sugars of an oligosaccharide or polysaccharide is achieved by hydrolysis, reduction, acetylation and g.l.c. analysis. SP-2340 (75% cyanopropyl silicone) is the stationary phase of choice for analysis of alditol acetates (see Fig. 5). ECNSS-M (ethylenesuccinate - cyanoethylsilicone copolymer) may also be used, but the maximum operating temperature is quite low, and tailing of peaks may occur.

To analyze mixtures from methylation analysis (see later) OV-225 (25% phenyl, 25% cyanopropyl, methyl silicone) (see Fig. 6) and OV-17 (50% phenyl, methyl silicone) along with ECNSS-M are used most commonly. A column packed with 0.3% to 0.4% OV-225 on Chromosorb surface modified with high molecular - weight polyethylene glycol has been developed recently [99] to achieve a higher operating temperature, giving shorter retention times and negligible column bleeding, hence constant retention parameters. OV-225 has also been used to separate di- and trisaccharide derivatives [100]. OV-1 (methyl silicone) is used for the analysis of permethylated oligosaccharides.

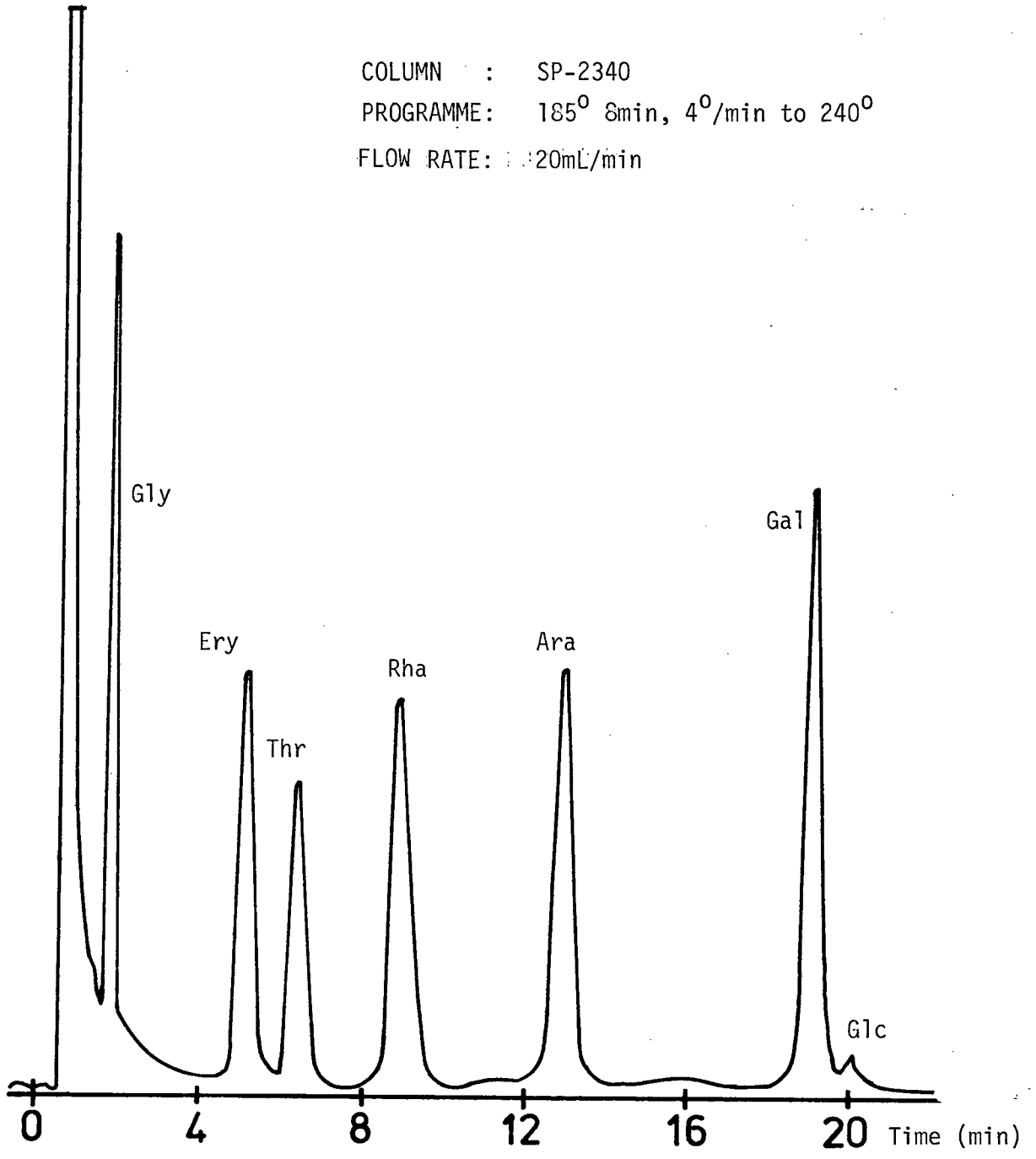


Figure 5 G.l.c. separation of alditol acetates from periodate oxidation of Klebsiella K12

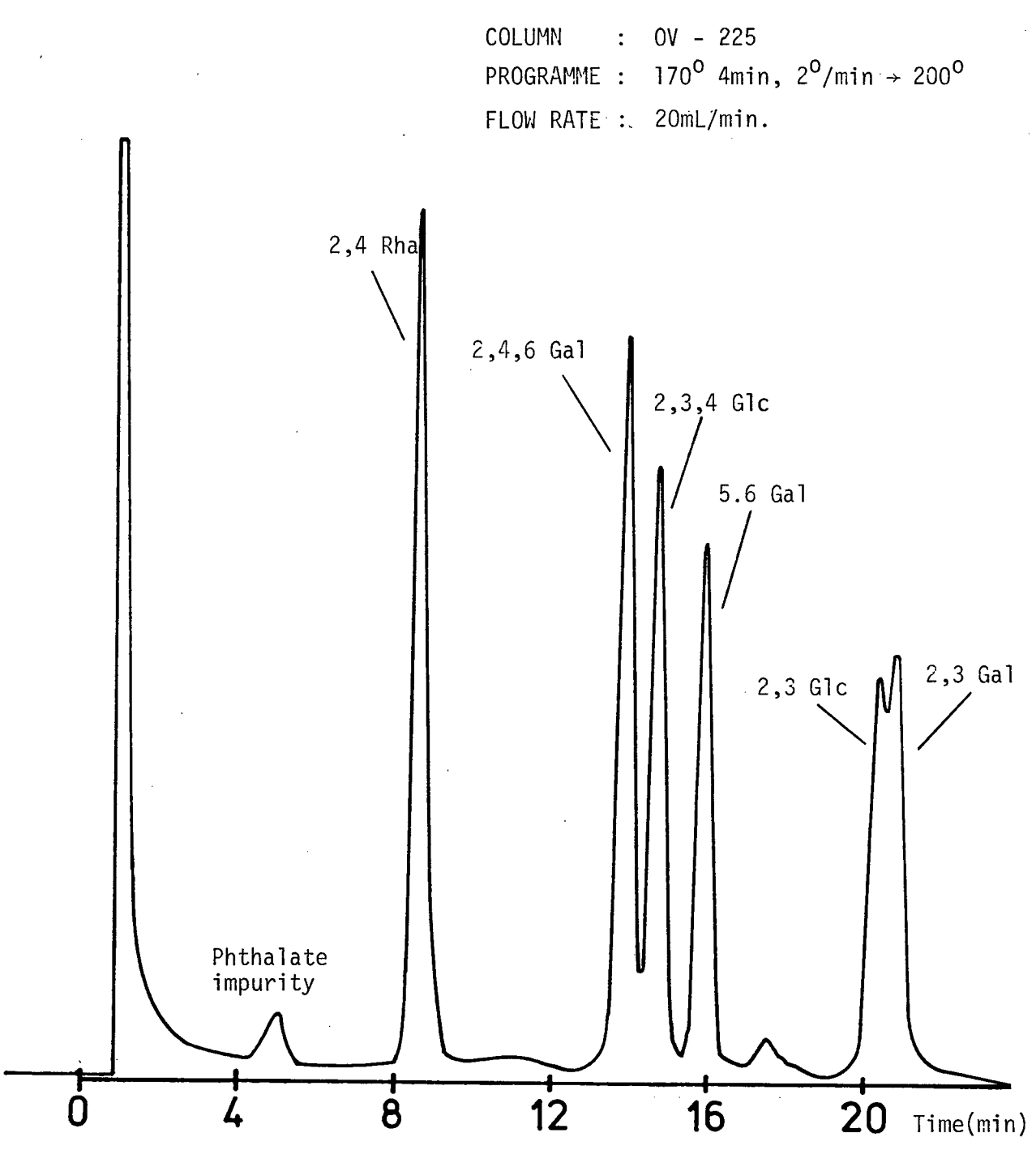


Figure 6 G.l.c. separation of products of methylation analysis of Klebsiella K12

When difficulty is encountered in separating permethylated alditol acetates the trimethylsilyl (TMS) ether derivatives [101] may be used to advantage, employing SE-52 (5% phenyl : methyl silicone) as stationary phase. The ease with which the original material may be recovered following derivatization makes the use of TMS derivatives attractive where the amounts of material are limited.

To determine the degree of polymerization of an oligosaccharide and to identify the reducing sugar the aldononitrile method of Morrison (see later) is used [102]. Aldononitriles give sharp single peaks on g.l.c. and may be analyzed with OV-17 (see Fig. 7), OV-225 or ECNSS-M.

Publicatons by Albersheim et al [103,104] and Lindberg et al [122] list the relative retention times for a large number of partially methylated alditol acetates. Identification of unknowns is achieved by consideration of the relative retention time, by co-chromatography with an authentic sample, and by g.l.c. - m.s. (see later). Where uncertainty still exists the melting point (m.p.) of the sample (from preparative g.l.c.) may provide identification (if the sample is crystalline). To determine the parent hexose, demethylation [105] and reacetylation gives a crystalline alditol hexaacetate which may be identified by m.p. or by g.l.c. retention time. Samples collected by preparative g.l.c. are used also for mass spectral studies and for circular dichroism measurements (see later).

Partially ethylated, partially methylated alditol acetates

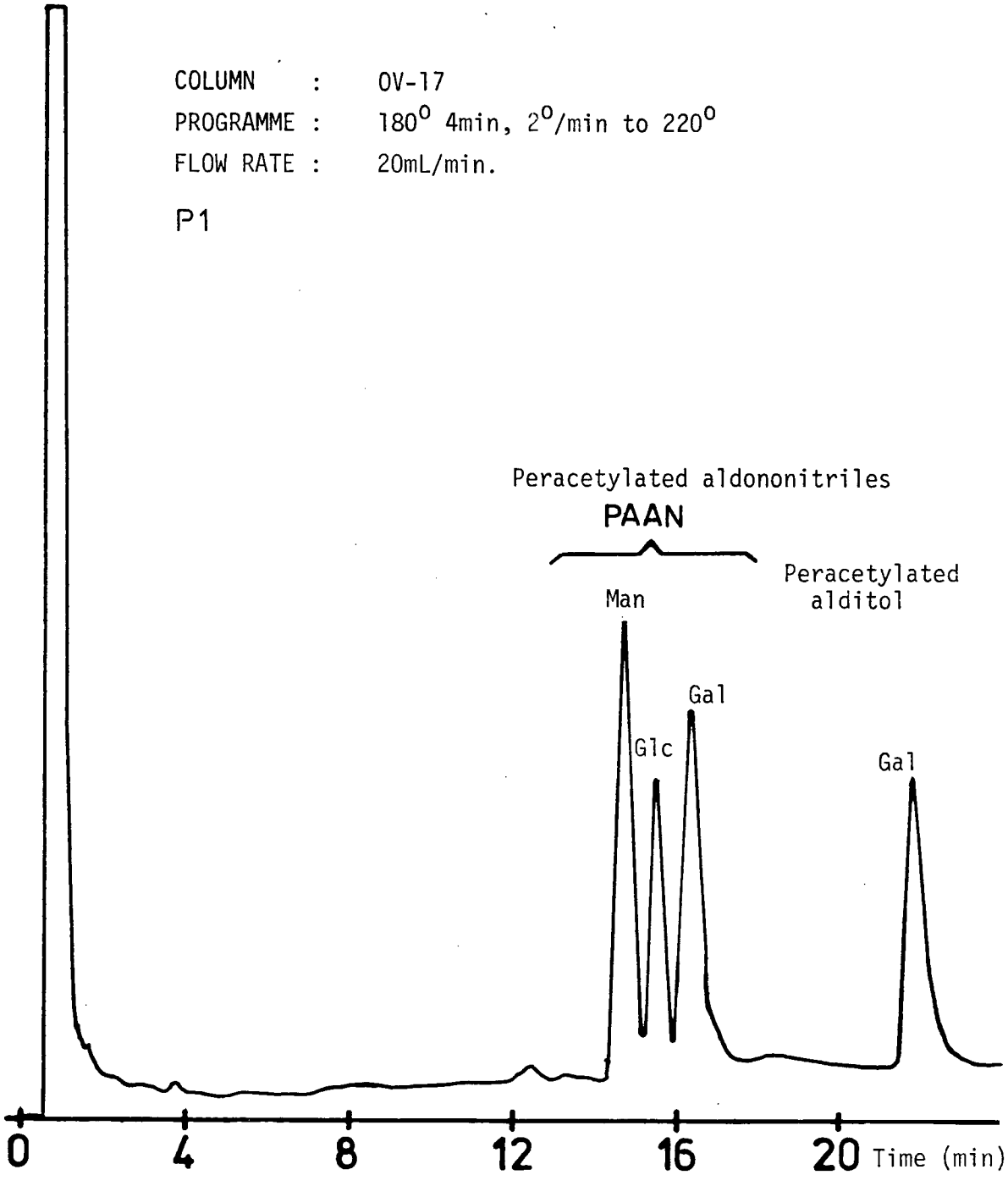


Figure 7 Degree of polymerization of product of bacteriophage degradation of Klebsiella K21, using g.l.c.

COLUMN : ECNSS-M
PROGRAMME : 165° 4min, 2°/min to 195°
FLOW RATE : 20mL/min.

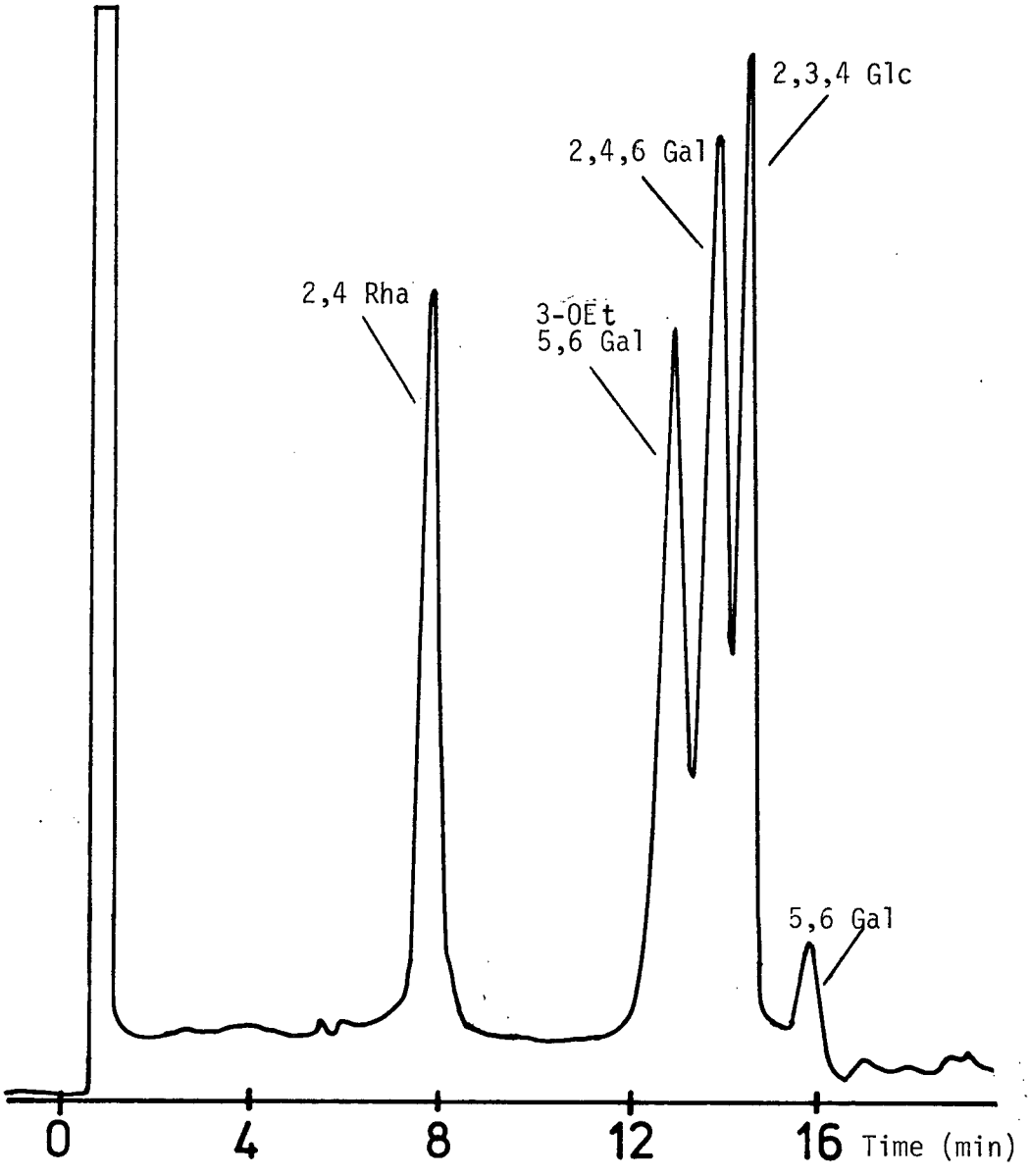


Figure 8 G.l.c. separation of products of uronic acid degradation of Klebsiella K12.

are obtained from uronic acid degradation (see later). When compared to a methyl group the ethyl group is less polar and hence, with polar liquid phases, components containing ethyl groups tend to travel more quickly than their methyl analogues (see Fig.8). Conversely, with nonpolar liquid phases retention times are longer. Mass spectrometry is invaluable in identification of partially ethylated derivatives (see later).

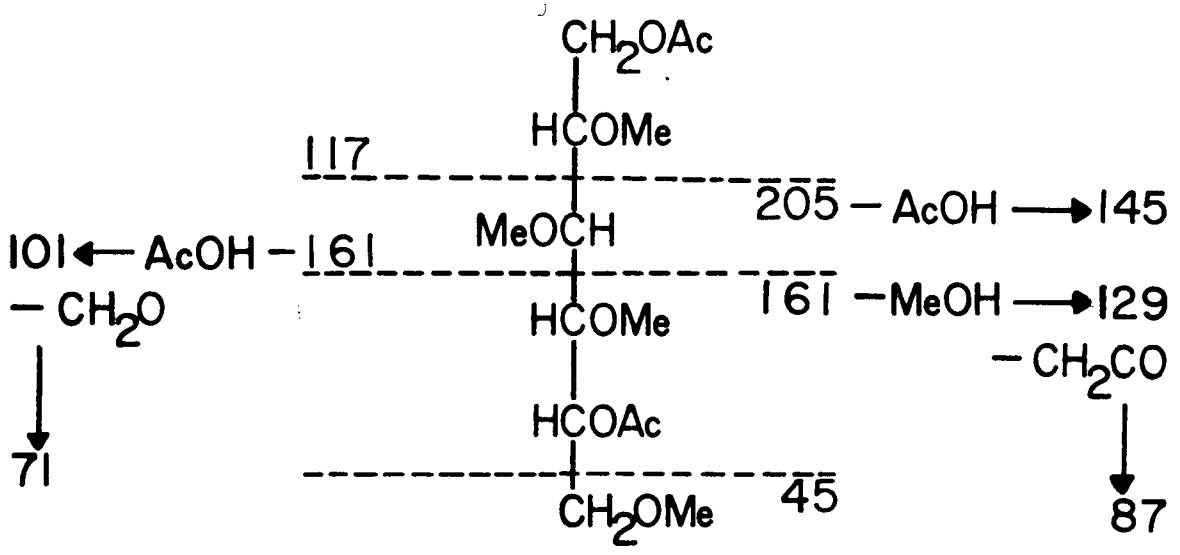
For quantification of peaks molar response factors [106] are taken into consideration.

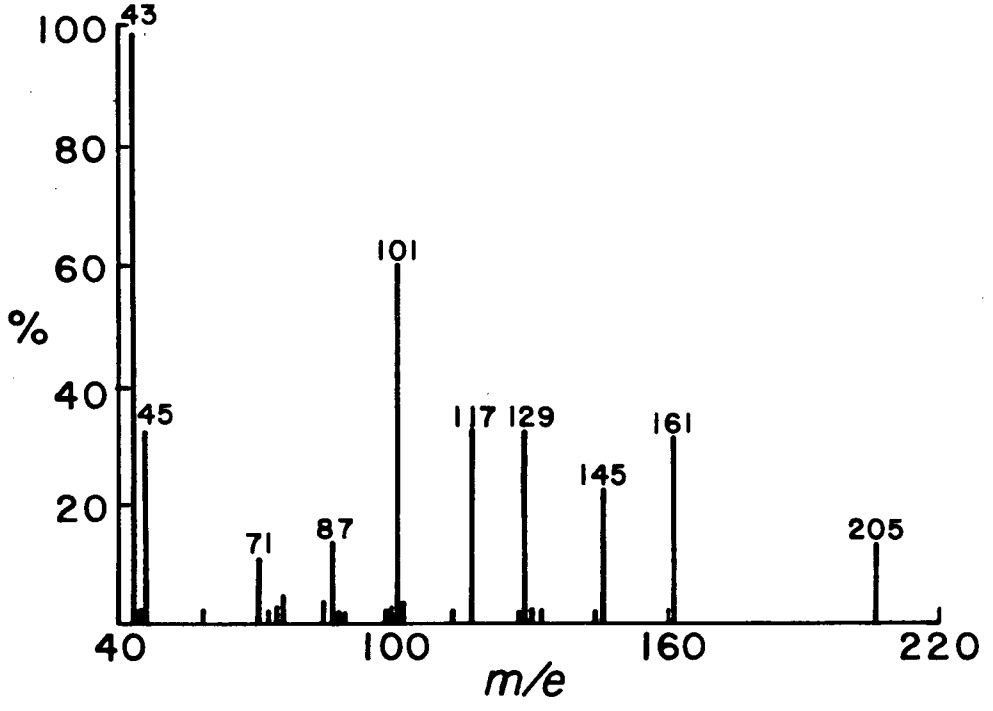
It should be noted that phthalic esters (used extensively as plasticisers) may be encountered as contaminants in g.l.c. analyses [107]. However, mass spectral data differentiate the contaminant from sugar derivatives.

II. 3. 2. Mass Spectrometry (m.s.)

The mass spectral method was first applied to carbohydrate derivatives in 1958 by Reed and coworkers [108], and since then, it has become an important and versatile technique in carbohydrate chemistry. Lönngren and Svensson reviewed the field in 1974 [109]. In this study m.s. has been used in the analysis of partially methylated/ethylated alditol acetates in order to assign substitution patterns (see later, Scheme 3), and in the analysis of oligosaccharides to determine the sequence of sugars.

Mass spectra can be recorded by using any one of several





Scheme 3 Primary and Secondary Fragmentation Pattern and Mass Spectrum of 1,5-Di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol

different systems of instrumentation. The inlet system can be either a hot reservoir inlet, a direct probe inlet, or a g.l.c. inlet. The latter has become increasingly important in the investigation of complex mixtures. Most underivatized mono- and oligosaccharides are thermally unstable and non-volatile and therefore must be converted into volatile derivatives for spectral (m.s.) analysis. Stereoisomers of carbohydrate derivatives give similar mass spectra, and the small differences in peak intensity sometimes observed do not generally allow an unambiguous assignment of configuration. However, consideration of g.l.c. retention time along with mass spectral data aids in assignment.

Carbohydrate derivatives give weak or no molecular ions on electron impact (e.i.) mass spectrometry. Molecular weights may more easily be determined by field ionization (f.i.)[110,111], field desorbtion (f.d.)[112-116], or chemical ionization (c.i.)[117-120] techniques. The latter two techniques are particularily useful with free sugars and derivatives of low volatility. In this study mass spectra were obtained by combined g.l.c. - m.s. in the e.i. mode at 70eV for the partially methylated/ethylated alditol acetate mixtures, and using a direct probe inlet in the e.i. mode at 70 ev for a permethylated oligosaccharide.

Considerable data [121,122] are now available on the fragmentation pattern of partially methylated alditol acetates, and a bank of standard mass spectra is maintained in our laboratory.

As an example most of the signals in the mass spectrum of 1,5-di-O-acetyl- 2,3,4,6 - tetra -O- methyl-D-glucitol can be accounted for from primary and secondary fragmentation (see Scheme 4).

Vliegenthart and coworkers have shown that there are characteristic differences between the fragmentation patterns of hexopyranosides and hexofuranosides [123].

Aldononitrile acetates (which have the advantage of molecular asymmetry) are suitable for analysis by g.l.c.- m.s. and give characteristic mass spectra that are easy to interpret [119,124].

Oligosaccharides have been examined by e.i.-m.s. as their acetate [125,126], TMS, [127-130] and methyl ether [131-134] derivatives, the last proving most expedient. The nomenclature used by Chizov and Kochetkov [135] for the different fragmentation series of permethylated glycosides, modified, as suggested by Kovacik and coworkers [136], is now standard. In this work a trisaccharide glyceride was obtained on periodate oxidation of K12. The sequence of sugars was readily ascertained by direct probe e.i.-m.s. of its permethylated derivative since the constituent moieties were, fortuitously, a hexose, a deoxyhexose, a pentose and glycerol (see later).

Mass spectra obtained from permethylated di- and tri-saccharides by the f.i. technique show strong molecular ions;[110,111] the fragments observed make it possible to determine the nature of the constituent monosaccharides but not the inter-sugar linkages.

However, conversion of the reducing sugar to the corresponding alditol with a deuterated hydride distinguishes between 3- or 4- and 2- or 5- linkages respectively.

Partial hydrolysis of methylated polysaccharides, followed by reduction and remethylation with trideuteriomethyl iodide gives a mixture of oligosaccharide alditols that can be analyzed by g.l.c. - m.s. The CD_3 groups occupy positions to which a sugar residue is linked in the original polysaccharide. This technique has been used in structural studies of the lipopolysaccharide from Klebsiella O-group 9 [137].

Mass spectrometry has become increasingly significant in the structural elucidation of biologically important glycoconjugates since quantities of material are very limited. Egge and coworkers[138-142] have made many contributions to this rapidly developing area of carbohydrate chemistry.

II. 3. 3. Polarimetry

Assignment of the anomeric configuration of a specific glycosidic linkage in a poly- or oligosaccharide may be accomplished by nuclear magnetic resonance spectroscopy (see later). However, ambiguities may arise for some sugars, depending on the linkage pattern and ring size. In these instances polarimetry, using Hudson's rules of isorotation may be employed to advantage.[143,144] Since Klebsiella polysaccharides have been shown to consist of

repeating units of hexoses and their derivatives the theoretical value for the molecular rotation can be found, to a first approximation, by summation of the molecular contribution of each component. It is assumed that the O-acetyl and pyruvic acid ketal groups present make negligible contributions to the total molecular rotation.

Application of Hudson's rules gives information only on the overall molecular rotation of the poly - or oligosaccharide. However, the individual glycosidic linkage configurations may be deduced if the rotations of a series of oligomers, eg. di-,tri-, and tetrasaccharides from the repeating unit are observed.

The specific rotation of the polysaccharide is calculated from the equation

$$[\alpha]_D = \frac{[M]_{PS} \times 100}{M_o}$$

where $[M]_{PS}$ is the summation of the individual rotations and M_o is the molecular weight of the repeating unit.

The experimental specific rotation is

$$[\alpha]_D = \frac{\alpha_D \times 100}{l \times c}$$

where α_D is the polarmeter reading

l is the cell length in dm,

and c is the concentration in g/100 ml.

Merrifield [145] has compared the theoretical and observed values for a number of Klebsiella polysaccharides and found very little discrepancy in most instances. He also found that the effect of temperature change is negligible. His compilation is included in Appendix 1.

II. 3. 4. Circular dichroism (c.d.)[146]

The configuration (D or L) of a sugar may be determined conveniently by c.d. measurements at 213 nm on alditol acetates or their methylated derivatives, where the acetoxy group acts as a chromophore [147]. The method is well suited to analysis of samples obtained by preparative g.l.c., since only milligram quantities of material are required. Configurational assignments are made after comparison with data from authentic samples.

In the case of D-glucose and D-galactose specific oxidases are available (Worthington Biochemical Corporation). This method was employed in the investigation of K58 to determine that the galactose was of the D-configuration, since the c.d. method is inapplicable to the meso-galactitol hexaacetate.

II. 3. 5. Nuclear Magnetic Resonance Spectroscopy

Both ^{1}H and ^{13}C n.m.r. spectroscopy have been used extensively in this work. The fact that interpretable spectra can be obtained by these two methods on polysaccharides with molecular

weights of the order of 10^6 indicates that the structures have regular repeating units. Spectra of the native and permethylated polysaccharides and of degradation products were analyzed, indicating that information from the two techniques is very often complementary.

II. 3. 5. 1. ^{1}H. n.m.r. spectroscopy

Proton magnetic resonance is now firmly established as the most widely used technique for the structural, configurational, and conformational analysis of carbohydrates and their derivatives. The observations of Arnold and coworkers [148] and Gutowski and Hoffman [149] that the chemical shift of a proton depends on the precise chemical environment of that proton were made in 1951. However, it was not until 1957 that the first ^{1}H n.m.r. spectra of carbohydrates were reported by Lemieux and coworkers [150]. In a classic paper in 1958 [151] these authors showed the effects of configuration and conformation on the chemical shifts and coupling constants of acetylated sugars. This work was extended to free sugars in D_2O solution by Lenz and Heeschen [152] in 1961 and the observation that glycosidic linkage protons resonate downfield of the ring protons was made in 1963 by van der Veen [153] who also succeeded in correlating the splittings, observed for the anomeric hydrogen atoms, with the glycoside configuration. The first review of the field appeared in 1964 by Hall [154].

Progress in the intervening years has been rapid [155] due to (a) the development of superconducting solenoid spectrometers with higher magnetic fields and resonance frequencies (b) the improved performance of radio - frequency circuits (c) the development of

the Fourier - transform n.m.r. method and (d) decoupling and multinuclear abilities along with (e) advancements in data systems eg. multitasking and queuing capabilities. To date many reviews of this ever expanding field have been published [82, 83, 156-160].

The technique, however, suffers from a number of limitations inherent with increase in molecular size and complexity, in particular when attention is turned to polysaccharides; solutions to these problems have been proposed by Hall [159]. More recently, homonuclear two-dimensional J n.m.r. has been used [161] to simplify complex spectra of mono - and disaccharides, by separating the effects of chemical shifts and scalar coupling.

The ^{1}H n.m.r. spectrum of a polysaccharide provides information on the number of sugars present in the repeating unit and indicates the presence of 6-deoxysugars [162]. O-Acetyl, O-formyl [163a] and 1-carboxy-ethylidene acetal substituents are also recognised [163b,c]. The anomeric nature of the linkages (α or β) may be differentiated for both pyranosyl [164] and furanosyl [165] sugars, from consideration of the chemical shift (δ) along with the value of the coupling constant (J) between H-1 and H-2($J_{1,2}$).

Spectrum No. 10 (App. III) shows the presence of a 6-deoxy sugar, a 1-carboxyethylidene, and an O-acetyl group in equimolar proportions, along with signals attributable to four anomeric centres : three α(δ>5 ppm, $J_{1,2}$<4) and one β(δ<5 ppm, $J_{1,2}$>6) linked pyranoses. Care should however, be exercised when furanosyl sugars are present (from g.l.c. - m.s. data). Spectrum No. 1 shows the anomeric signal for the

β-galactofuranosyl unit at δ 5.13 (δ>5). Ring protons may resonate in the so-called "anomeric region", depending on the ring size and linkage pattern of the sugar [166-168]. Spectrum No. 1 shows H-2 and H-3 of the galactofuranosyl unit at δ 4.3-4.5 ppm [169,170].

Garegg and coworkers [171] have demonstrated that the differences in chemical shifts obtained for stereoisomeric pairs of acetalic CH_3 groups from pyruvic acid [172] and related acetals are of sufficient magnitude to make possible the unequivocal determination of the stereochemistry of the acetals.

For oligosaccharides, the degree of polymerization may be calculated from the ratio of the integral of the anomeric protons from the reducing sugar (α and β) to those from the non-reducing sugars (see Spectrum No. 26)

High resolution n.m.r. spectra contain, in addition to chemical shift values, coupling constants and area integrations, two further sets of nuclear parameters: the spin-spin (T_2) and spin-lattice (T_1) relaxation times [173-175]. Since the latter show a number of stereospecific dependencies they provide a useful basis for configurational assignments. Another very useful application of relaxation studies lies in the removal of the unwanted residual peaks of deuterated solvents - in this study of HOD - when high - temperature facilities are unavailable, (see Spectrum No. 3).

The literature contains few ^1H n.m.r. investigations of bacterial polysaccharides.+ These assignments are made after

+see, however, references in Appendix I from Dutton et al and Joseleau et al and reference 176 from Perlin et al.

consideration of the spectra of the degradation products eg. the backbone polymer, and di-, tri-, and tetrasaccharides, or of the methyl glycosides of monosaccharides. De Bruyn et al have refined and extended the increment rules of Lemieux [177] following analysis of parameters obtained in the 300-MHz ^1H - n.m.r. spectra of D-glucose, D-mannose, D-galactose, and their methyl glycosides,[178] of the glucose disaccharides,[179] and of rhamnose, the methyl glycoside and a disaccharide [180]. The ^1H n.m.r. spectra of glucobioses and glucotrioses [181] and of various di- and trisaccharides containing L-rhamnose [182] have been described. Perlin has published 220 MHz spectra of some mucopolysaccharides [176].

^1H n.m.r. examination of carbohydrate derivatives soluble in organic solvents have advantages over the use of aqueous solutions: there is no need for proton exchange, if the hydroxyl groups are derivatized and no residual HOD peak, therefore ambient temperature is sufficient and also solvent effects may be employed to advantage. Vliegenthart and coworkers have described the complete interpretation of ^1H n.m.r. spectra of solutions of permethylated α- and β- D-glucose[183] and galactose [184] and of mannose and of the 6-deoxy analogues of mannose, glucose and galactose [185], (with a view to use in methylation analysis) and of permethylated disaccharides [186]. The chemical shifts of anomeric protons of the methyl ethers of various disaccharides were reported in 1972 by Minnikin [187] but the complete interpretation of ^1H n.m.r. spectra of permethylated oligo - and polysaccharides is yet to be achieved some valuable information may be provided. For

example in the structural analysis of Klebsiella K12 comparison of the spectra of the permethylated polysaccharide and of the β-elimination product (see later), indicates that the two side-chain sugars removed were β-linked.

Stephen et al have used lanthanide shift reagents in ^{1}H n.m.r. studies on fully methylated aldohexopyranosides and their 6-deoxy analogues [188] and on the permethyl ethers of galactose [189].

TMS derivatives have been used to determine the number of hydroxyl groups in a molecule [190] and to determine the configuration of the glycosidic linkages in an oligosaccharide [191].

The solvent of choice for ^{1}H n.m.r. spectroscopy of underivatized poly- and oligosaccharides is D_2O. To eliminate interference in the spectrum by the numerous hydroxyl groups present, a number of exchanges are made with 99.7% D_2O, followed by lyophilization and heating under vacuum. The sample is then dissolved in 99.9% D_2O and any residual HOD is shifted away from the anomeric region (to δ 4.18 ,90°) when the spectrum is run at elevated temperature. Alternatively, a relaxation type (T_1) experiment may be performed whereby the HOD signal is nulled. This may be especially successful with non-viscous oligosaccharides. Sample sizes of polysaccharides are generally of the order of 1-2%, while larger samples may be employed with oligosaccharides. If the polymeric sample is extremely viscous partial depolymerization with acid may improve the sharpness of the spectrum.

Because samples are of low concentration spectra are generally run in the FT mode. Where sample size is minimal a 5mm sample tube with a cylindrical semi-micro volume cavity may be used to advantage.

Spectra are usually run with an internal standard of acetone. However, initial spectra of an unknown polysaccharide should be run without acetone, since a substituent O-acetyl group may be masked by the standard. Acetone has the advantages of being volatile (hence it may be readily removed from the sample) and its chemical shift is virtually unaffected by variations in temperature.

Stephen et al have recommended caution in the use of some reference systems for ^{1}H n.m.r. spectroscopy of carbohydrates [192].

II. 3. 5. 2. ^{13}C n.m.r. spectroscopy

The proliferation of studies on the ^{13}C spectra of carbohydrates during the past few years attests to the fact that ^{13}C n.m.r. is acquiring a status, not only as a useful adjunct to ^{1}H n.m.r. spectroscopy, but one characterized by its own unique contributions. As in ^{1}H n.m.r. progress in instrumentation has been rapid, with concomitant increase in the number of applications : structural elucidation, configurational and conformational analyses, detection of impurities, and analysis of mixtures [193,194]. The field has been reviewed by Perlin [195,196].

Not surprisingly glucose has been one of the earliest and most extensively studied of the carbohydrates [197-200]. Previous

assignments of natural abundance ^{13}C n.m.r. chemical shifts of mono- and disaccharides have been re-evaluated by use of a differential isotope (DIS) technique [201], Gorin [202] has assigned the ^{13}C signals of the more common sugars and their methyl glycosides - the latter proving most expedient in approximating the chemical shifts of glycosidic carbons in oligo- and polysaccharides. Chizhov et al [203] here extended these data to include all the methyl ethers of methyl (methyl α-D-glucopyranosid) uronates, since uronic acid residues are frequently encountered in naturally occuring polysaccharides.

Vliegenthart and coworkers have interpreted ^{13}C spectra of permethylated α- and β-glucopyranoses [183], galactopyranoses [184] mannopyranoses and 6- deoxy sugars [185] with a view to interpreting methylation analysis data (see later), in conjunction with ^{1}H n.m.r. data.

The effect of O-alkylation on the ^{13}C n.m.r. spectra of methyl pentafuranosides has been determined by Gorin [204] and by Ishido [205]. Vignon has studied the effect of the trichloroacetyl group on glucopyranose and gentiobiose [206,207], and Seymour et al have examined the ^{13}C spectra of compounds containing the β - fructofuranosyl group [208].

^{13}C n.m.r. data of monosaccharides and their derivatives have been extended to di-, oligo-, and polysaccharides. Usui et al have studied the glucobioses [209], and glucotrioses and glucans [210]. Di - and trisaccharides containing galactose have been examined by Cox et al [211], and those containing glucose, galactose and

rhamnose by Laffitte et al [212] and Colson and King [213]. Hough et al [214] have extended their analysis of permethylated carbohydrates to include disaccharides.

Gagnaire et al [215] determined the spectrum of α-cellobiose octaacetate and compared it to the spectrum of cellulose triacetate. These data have been extended by Capon et al [216] to include spectra of peracetylated α-cellotriose, α-cellotetraose and α-cellopentaose

In 1971 Dorman and Roberts [217] demonstrated the applicability of ^{13}C- N.M.R. spectroscopy to the study of oligosaccharides. Boyd and Turvey [218] have identified spectra of oligosaccharides derived from alginic acid, and Kochetkov et al [219] have applied the technique in the structural study of complex oligosaccharides.

Perlin has reviewed the characterization of carbohydrate polymers by ^{13}C n.m.r. spectroscopy. As expected, homopolymers have been among the first carbohydrate polymers to be studied. As early as 1970 Dorman and Roberts [217] extended a ^{13}C survey of oligosaccharides to include a brief investigation of amylose and cellulose acetate. In 1973 Jennings and Smith [220] determined the composition and sequence of a glucan containing mixed linkages by ^{13}C n.m.r. and the following year [221] used the technique to assign completely two cyclodextrins and several linear glucans by comparison with spectra of glucobioses and glucotrioses.

In 1975 Gorin [222] commented on the methodology of assigning signals of a mannan containing alternate (1→3) and (1→4) linked

β - D - mannopyranose residues. The 12 signals were assigned by preparation of D - mannans from specifically deuterated D-glucoses and observation of α - and β - deuterium isotope - effects. Dextrans [223a,b], and levans [223c] have been studied by Seymour et al, as have galactomannans by Grasdalen and Painter[224]. Glycosidically substituted and free C-5 groups have been distinguished, and different types of linkages have been identified by Joseleau et al. [225] in a ^{13}C study of two arabinans. Methyl and acetyl substituent effects on ^{13}C chemical shifts have been determined on α - and β - (1→3) and (1→4) linked polysaccharides by Gagnaire et al [226].

As in ^{1}H n.m.r., very few ^{13}C n.m.r. spectral data of heteropolysaccharides have been published. Perlin et al [227] in 1972 gave evidence for a biose repeating unit for heparin using ^{13}C n.m.r. and in 1979 [227b] made a conformational study of the polymer. Variations with respect to the presence and location of sulphate groups in agar and some carrageenans were shown in a study by Hamer et al [228]. The spectrum of specifically labelled (^{13}C)nigeran - a regular, alternating copolysaccharide having differences in ^{13}C signal intensities - has been assigned by Bobbit et al[229].

In 1977 Dutton et al[230] delineated the diagnostic potential of ^{13}C n.m.r. spectroscopy in the structural elucidation of Klebsiella polysaccharides composed of three to six sugar residues and carrying O-acetyl and 1-carboxyethylidene substituents. Since then many Klebsiella polysaccharides have been characterized, using ^{13}C n.m.r. spectroscopy by our group. Assignments have been made on the basis of spectral data

obtained from degradation products of the polysaccharides, from methyl glycosides, and from synthesized oligosaccharides.[231]

Routine ^{13}C spectra are run with complete proton decoupling. However, valuable information may be obtained from the coupled spectrum. A smaller ^{13}C-1-H-1 coupling (161Hz) is found for the β (equatorial) anomer of glucose than for the α(axial) anomer (169 Hz)[198]. For nuclei other than the anomeric centre the magnitude of ^{1}J is substantially smaller, and there is an overall tendency for direct coupling to decrease with an increase in shielding of the ^{13}C nucleus. In 1979 Friebolin et al [232] used ^{1}J (C-1, H) coupling constants to identify the anomeric configuration of some polysaccharides and their methyl derivatives. To gain further insight into the architectureof the gel network of some branched β (1→3) linked glucans Saito et al [233] made a ^{13}C n.m.r. study of sodium hydroxide - induced conformation changes.

^{13}C 2-D J spectroscopy has been used to facilitate the measurement of ^{1}H - ^{13}C couplings in spectra of oligosaccharides.[234a]

Bock and Hall [234b] in 1975 showed the practical relevance of T_1 measurements in obtaining efficient F.T. ^{13}C spectra : the time interval between successive 90^{o} pulses should be no less than five T_1 periods to prevent saturation of resonances. Coupling and T_1 experiments provide insight into the microdynamics of the motion of carbohydrate molecules in solution. T_1 values for polysaccharides have been reported for bovine nasal cartilage [235] and a gel-forming glucan [233b], giving information on the molecular motion and the overall conformation respectively. T_1 measurements on branched - chain polysaccharides

made by Gorin and Mazurek[236] show that these values can be useful in distinguishing resonances of side-chains from those of the main chain.

^{13}C spectra of underivatized carbohydrates are generally run in 50% D_2O to give a deuterium lock and with acetone as internal standard. Chemical shifts of the ^{13}C nuclei of carbohydrates and derivatives encompass most regions of the 200 pp, range covered by organic compounds. Simplification of the spectrum by proton decoupling, together with the resolution of less than 0.1 ppm afforded by present instruments usually ensures an excellent overall separation of resonance signals even for highly complex molecules or mixtures. Peak areas, which are highly sensitive to the relaxation properties of the various ^{13}C nuclei, and to the extent of Overhauser enhancement, may be used if comparisons of the integrated intensities are based on signals representing the same class of carbon (such as the anomeric centres in a polysaccharide). Spectra should be run at elevated temperature, when possible, to reduce line broadening.[196]

Resonances of simple sugars are distinguishable for the most part in terms of carbonyls from uronic acids and pyruvic acid (180 ± 6 ppm), anomeric carbons (100 ± 8 ppm), secondary carbons (75 ± 5 ppm), primary carbons (65 ± 5 ppm), methyl groups from O-acetyl substituents (30 ± 3 ppm) and from 6-deoxy sugars and pyruvate substituents (20 ± 5 ppm). Variations within each class are associated with changes in ring size, configuration, conformation and substitution.

For example in the spectrum of K12 (No.2) the signal due to the β - galactofuranosyl residue appears at 108.39 ppm while that

due to the β - galactopyranosyl residue appears at 106.99 ppm, and the signal due to the α - galactopyranosyl residue occurs at 99.43 ppm, Resonances due to the anomeric carbons at reducing centres occur upfield of the corresponding glycosidic centres. For example in the disaccharide $\underset{\sim}{1}$ from K12 (No.6) the signal due to β - galactose occurs at 96.98 ppm and that due to the α anomer at 93.01 ppm.

II. 4. Techniques of Structure Determination

II. 4. 1. Characterization of component sugars.

Klebsiella polysaccharides are heteropolysaccharides, made up of regular repeating units. To identify the constituent sugars, and to determine the ratios, various techniques are employed. The most informative first analysis is made with paper chromatography; the polysaccharide is hydrolyzed completely with acid and, using various solvent systems, hexoses, 6-deoxy hexoses and acidic sugars are identified. G.l.c. analysis of the corresponding alditol acetates is used to determine the quantitative composition. It is of prime importance, then, to hydrolyze all the glycosidic linkages, and to minimize degradation of monosaccharides.

The rates of hydrolysis of interglycosidic linkages vary greatly [237]; 6-deoxy sugars and furanosyl bonds hydrolyze easily, while uronic acid residues are most resistant. This problem is overcome with a technique developed in this laboratory.[238]. Treatment of the polysaccharide with methanolic hydrogen chloride cleaves most glycosidic bonds, leaving some uronosyl linkages intact.

At the same time the methyl ester of the uronic acid is formed and can be reduced using sodium borohydride [239,240] in anhydrous methanol. Subsequent hydrolysis with 2M trifluoroacetic acid (TFA) ensures complete hydrolysis.

Theoretically, for each free sugar five forms are possible (α - and β - pyranoses, α - and β - furanoses, and linear). Reduction, then, of C-1 to the alcohol, simplifies the situation, and subsequent acetylation yields volatile derivatives for g.l.c. analysis.

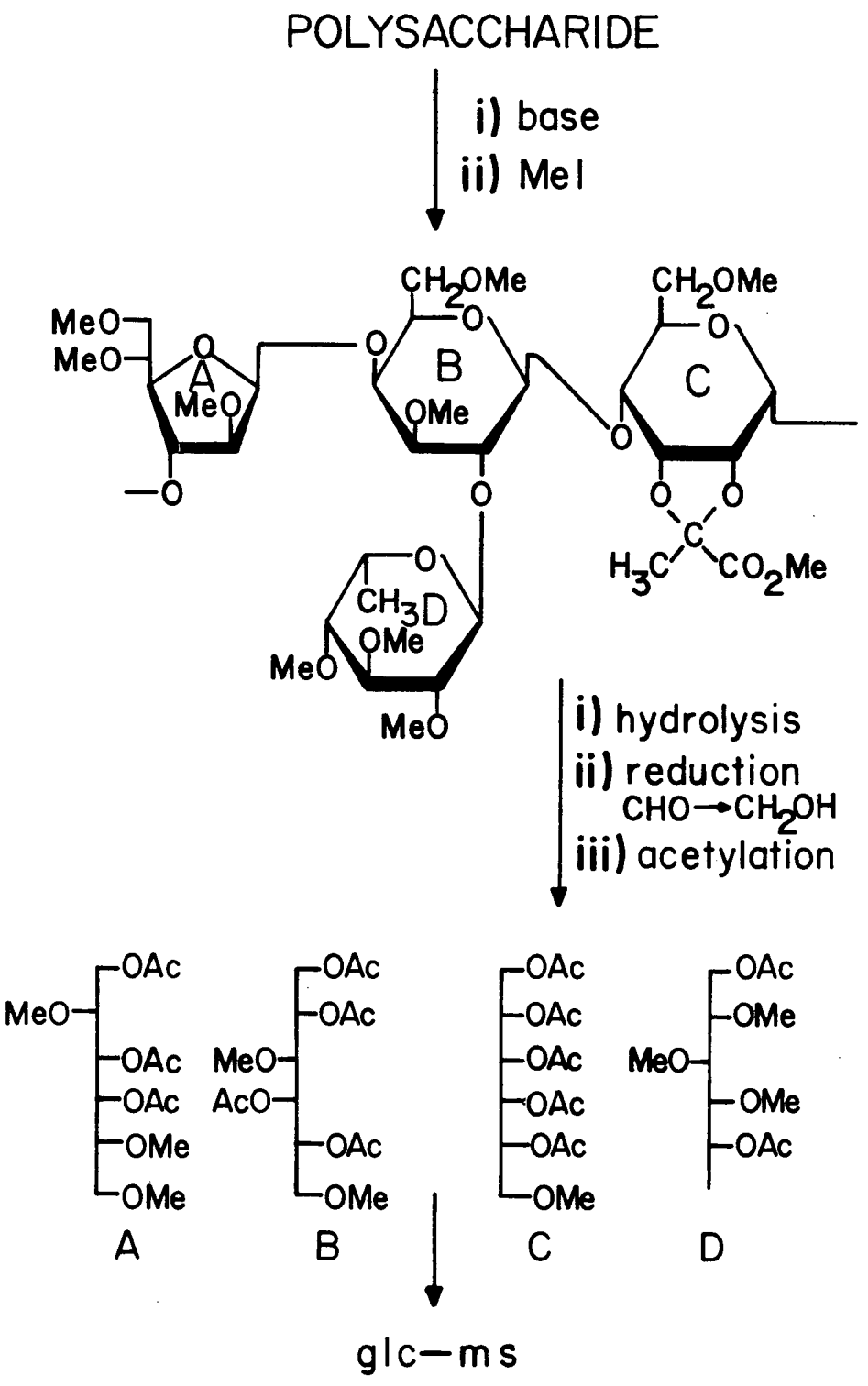
The use of HPLC to determine the quantitative composition of the polysaccharide is investigated in Section V.

As evinced in Sec. II.3.5. ^{1}H and ^{13}C n.m.r. spectroscopy also give information on the constituent sugars, and their substituents (if any), along with the relative numbers of α - and β - linkages.

Constituent analysis of degradation products of the polysaccharide is performed in a similar manner.

II. 4. 2. Methylation analysis.[241]

This technique has proved invaluable for the elucidation of ring size and of the position of linkage between the sugar residues. In addition the number of sugars in the repeating unit may be ascertained, along with the identity of the terminal unit(s), branching unit(s) and the position of base-stable substituents (eg. pyruvic acid ketal), but not generally, of the base-labile O-acetyl group (see Scheme 4 and Table 2).



Scheme 4 Methylation Analysis of a Polysaccharide.

METHYLATION ANALYSIS

Methylation pattern is identified by:

(a) Retention time on g.l.c.
(b) Mass-spectrum from g.l.c. - m.s.

Methylation analysis gives information on:

(i) number of sugars per repeating unit.
(ii) ring size.
(iii) linkage positions and location.
(iv) pyruvate substitution.

Hexose	6-Deoxy Hexose		Location
OMe_4	OMe_3	:	terminal
OMe_3	OMe_2	:	in-chain
OMe_2	OMe_1	:	terminal + pyruvate, or branch
OMe_1	-	:	in-chain + pyruvate. or doubly branched

Methylation at position 5 indicates a furanose sugar

TABLE 2 METHYLATION ANALYSIS OF A POLYSACCHARIDE.

YEAR	NAME	SOLV.	BASE.	ME.REAGENT
1903	Purdie[242]	CH_3I	Ag_2O	CH_3I
1915	Haworth[243]	$(CH_3)_2CO$	$NaOH/H_2O$	Me_2SO_4
1926	Menzies[244]	H_2O/CH_3I	TlOH	CH_3I
1934	Muskat[245]	NH_3	K/Na	CH_3I
1955	Kuhn[246]	$HCON(CH_3)_2$	Ag_2O	CH_3I
1964	Hakomori[247]	$(CH_3)_2SO$	CH_3SOCH_2Na	CH_3I
1966	Gros[248]	CH_2Cl_2	BF_3	CH_2N_2
1975	Arnap[249]	CH_2Cl_2	K_2CO_3	$CF_3SO_3CH_3$
1980	Prehm[250]	$(CH_3O)_3PO$	2,6DTBP	$CF_3SO_3CH_3$
1980	Finne[251]	$(CH_3)_2SO$	K^tBuO	CH_3I

TABLE 3 METHYLATION PROCEDURES

The procedure involves treatment of the polysaccharide, in solution, with base and a methylating agent. Table 3 indicates various procedures. The most versatile method is that developed by Hakomori[247,252]. Usually complete etherification is realized with one treatment (as evinced by infra red (i.r.) spectroscopy). If this is not the case complete methylation can be achieved by a subsequent Purdie [242] reaction since a second Hakomori treatment would result in β - elimination (see later) if the polysaccharide is acidic.

To deduce the identity of the acidic sugar the methyl ester is reduced with lithium aluminum hydride in oxolane (tetrahydrofuran) and then re-methylated.

Methylated material is recovered either by dialysis followed by lyophilization in the case of polysaccharides, or by extraction with chloroform for oligomers. Subsequent hydrolysis, reduction of the resulting sugars to the alditols, and acetylation yields volatile derivatives for g.l.c. - m.s. analysis. Comparison of data from the native, the uronic acid reduced and the depyruvulated materials yields valuable information (see Tables 6, 8).

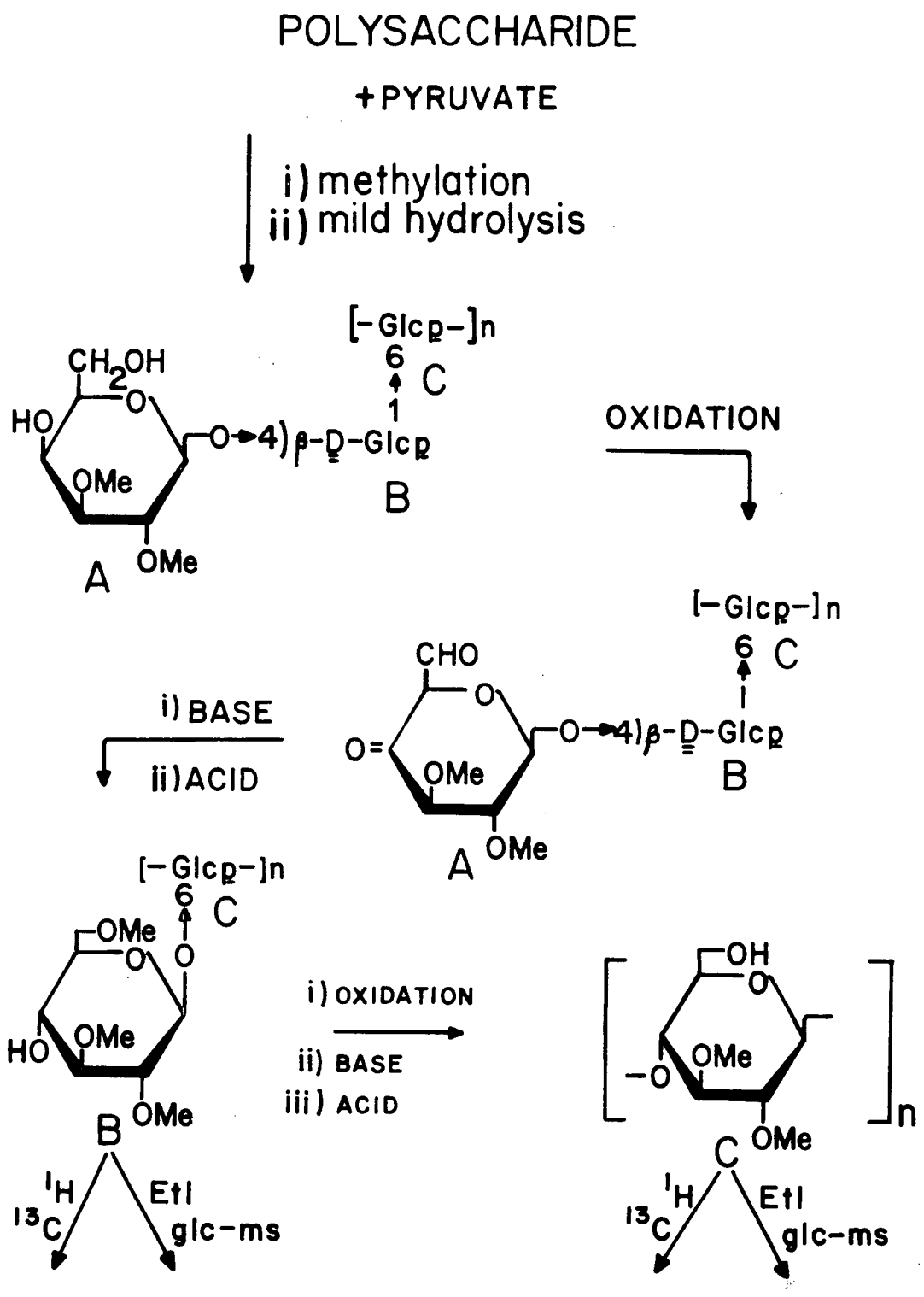
Various stationary phases may be used in the g.l.c. analysis (see Section II.3.1.) of partially methylated alditol acetates. Identification is made by consideration of relative retention times, co-chromatography with authentic samples, and mass-spectral data.

II. 4. 3. Oxidation

Two main types of oxidation are used in structural investigations of polysaccharides . The classical periodate reaction [253-256] cleaves the carbon-carbon bond between vicinal diols, while reaction of a selected alcohol group [257] with either trifluoroacetic acid, or chlorine, along with dimethyl sulfoxide and triethylamine gives a product which can then be subjected to a base-catalyzed degradation (see Fig.12).

The product of the former is a polyaldehyde, which is then reduced with sodium borohydride.The total hydrolysis product of the derived polyol may be examined qualitatively by paper chromatography or quantitatively by g.l.c. [258], after derivatization to the alditol acetates. Alternatively, a mild Smith hydrolysis [259,260], whereby only the true acetal linkages are cleaved and the glycosidic linkages are left intact, gives glycosides of mono- or oligosaccharides. These products, after separation and purification, are investigated by ^{1}H and ^{13}C n.m.r. spectroscopy, and methylation and m.s. analysis of the intact methylated oligomer to give information on the sequence and linkage patterns of residues. A selective oxidation [261], using periodate, under controlled conditions, has been used to advantage, for example to oxidize preferentially a terminal residue [262].

The second type of oxidation has only been employed quite recently in structural investigations of polysaccharides. Selected alcohol groups may be derived, for example, [263] by mild hydrolysis of a methylated polysaccharide to remove only the pyruvic acid ketal.



Scheme 5 Selective Oxidation and Degradation.

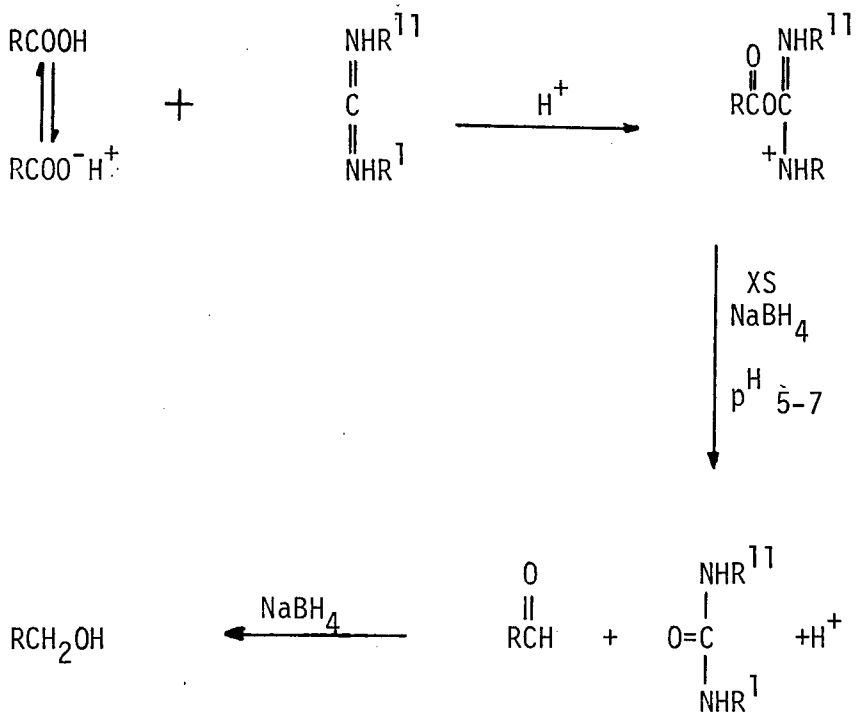
Oxidation gives a residue containing ketone/aldehyde functionalities which may then be degraded with base (see Scheme 5). Another residue with a free hydroxyl group is then exposed in the chain and the series of reactions may be repeated. The newly exposed hydroxyl groups may be labelled with either EtI or CD_3I. ^1H and ^{13}C n.m.r. spectroscopic analysis along with g.l.c. - m.s. analysis gives information on the anomeric nature of linkages and the linkage patterns of the products.

In the investigation of K12 a trisaccharide glyceride was obtained by periodate oxidation, while in K58 the terminal galactose was similarly oxidized, leaving the intact polymeric backbone. A selective functional group oxidation was not used in this study.

II. 4. 4. Reduction

It may be deemed expedient to perform reactions on the uronic - acid reduced polysaccharide, for example to alter hydrolysis patterns or to facilitate periodate oxidation. A technique developed recently by Taylor and Conrad [264] involves the use of water-soluble carbodiimides and sodium borohydride (see Scheme 6). The product is recovered by dialysis and lyophilization. Two treatments may be necessary to achieve complete reduction .

During the course of methylation analysis the uronic ester must be reduced. This may be achieved either with lithium aluminium hydride or with calcium borohydride[265] in tetrahydrofuran (THF). To transform the reducing sugar of a methylated oligosaccharide to the alditol, sodium borohydride in THF: ethanol (1:1) may be used, and to reduce free



Scheme 6 Reduction of Carboxylic acid in aqueous solution using a carbodiimide reagent.

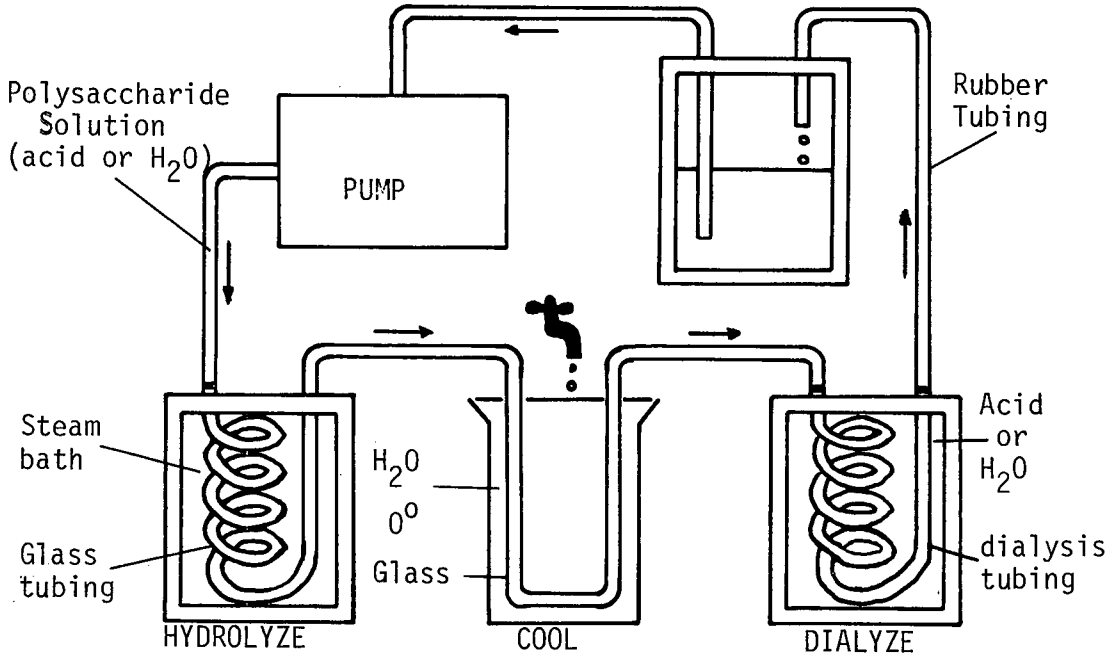


Figure 9 Partial Hydrolysis Apparatus

sugars to the corresponding alditols sodium borohydride in water is used. In the analysis of the constituent sugars the methyl ester of the uronic acid is reduced with sodium borohydride in anhydrous methanol.

II. 4. 5. Base-catalyzed degradation

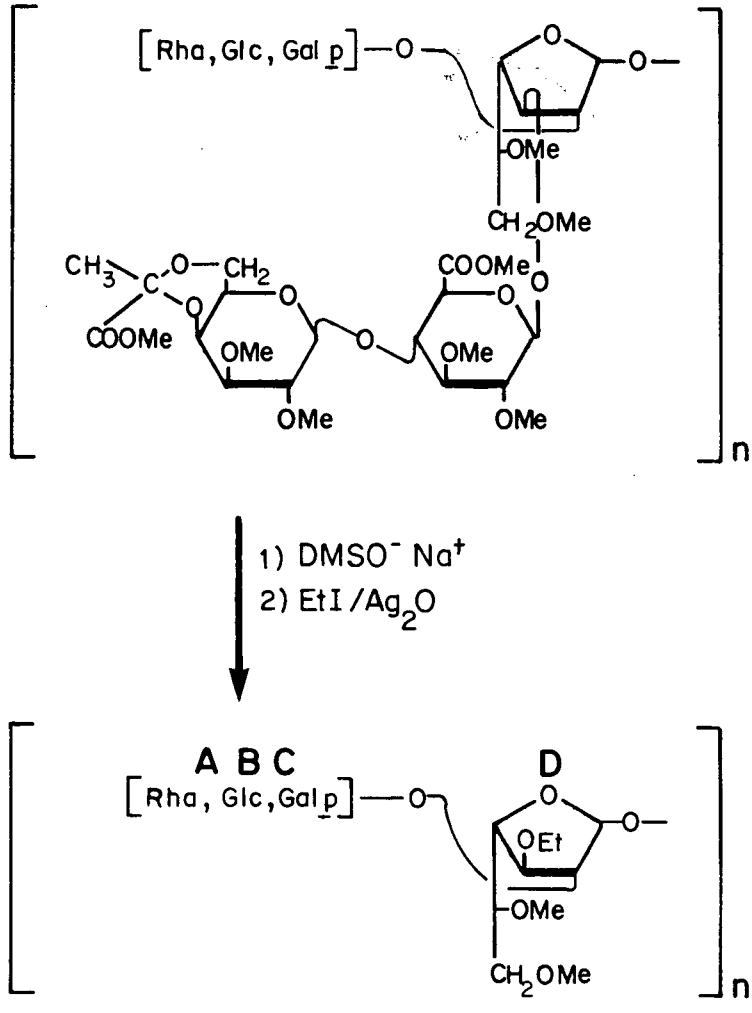
The specific degradation of polysaccharides has been an area of keen interest recently [266,267]. In this study a base-catalyzed β - elimination [268-271] reaction was used in the structural investigations of both K12 and K58. The reaction is performed on the methylated polysaccharide to achieve degradation of the uronic acid residue (see Scheme 7) and hence to determine its location (whether in the backbone or side-chain) and its point of attachment.

The base used is the methylsulfinyl anion. Joseleau [272] has suggested the use of the potassium instead of the sodium counter-ion. The point of attachment of the uronic acid residue is then directly labelled with ethyl iodide [273]. The reaction product is characterized by ^{1}H and ^{13}C n.m.r. spectroscopy and by g.l.c. - m.s. analysis.

II. 4. 6. Partial Hydrolysis [274]

Isolation of fragments from partial hydrolysis is a major key to elucidating the sequence of sugars in the polysaccharide and also to making assignments in the ^{1}H and ^{13}C n.m.r. spectra.

Capon[237] has reviewed the first order rate constants for the acid catalyzed hydrolysis of the glycosides and these data may be



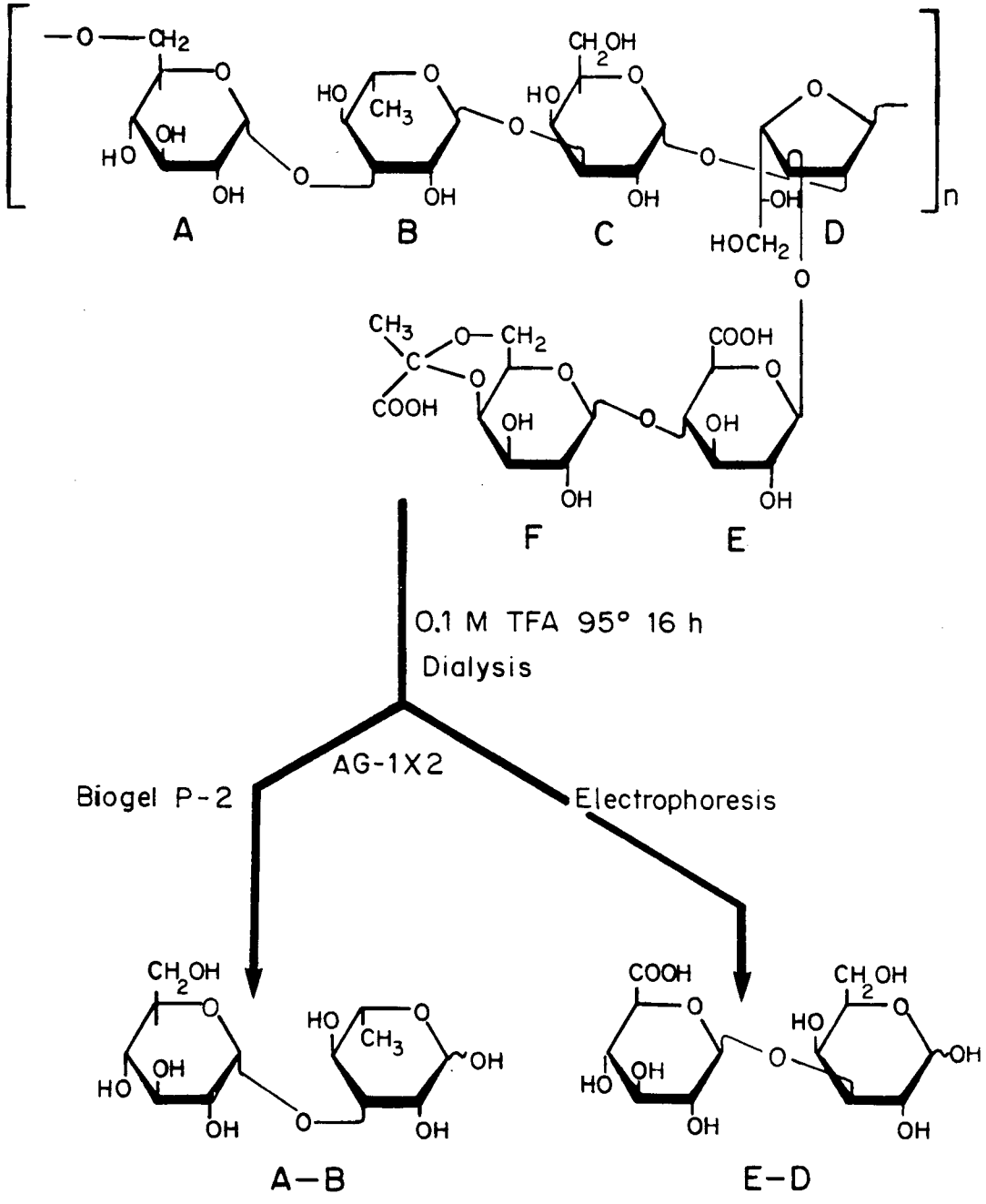
Scheme 7 Base-catalysed degradation of Klebsiella K12

extended to polysaccharides. By varying acid type and concentration, along with temperature and the reaction time, an optimal yield of oligosaccharides may be obtained.

The following generalisation may be made;furanosidic and deoxy sugars are more labile than the corresponding pyranosidic hexoses, which are in turn more labile than uronic acid residues. To minimise further degradation of oligosaccharides, once formed, an apparatus similar to that described by Galanos et al [67] (see Fig. 9) was employed in the investigation of K12. After separation and purification (see Scheme 8) a neutral and an acidic dissachar ide were characterized by n.m.r. spectroscopy.

In the investigation of K58 the aldobi -, aldotri-and aldotetraouronic acids were obtained. Characterization of each, incrementally, by ^{1}H and ^{13}C n.m.r. spectroscopy permitted the assignment of the spectra of the intact polysaccharide.

To demonstrate conclusively the linkage positions of the pyruvic acid ketal of K58, a very mild acid hydrolysis was performed on the native polysaccharide in which the ketal was removed (as evinced by ^{1}H n.m.r. spectroscopy). Subsequent methylation analysis (see Table 8) indicated the positions of attachment. In addition a mild hydrolysis was performed on the fully methylated material in order to remove the pyruvate ketal , and the product was re-methylated. G.l.c. analysis of the product verified the positions of linkage (see Table 8).



Scheme 8 Partial hydrolysis and purification of *Klebsiella* K12

II. 4. 7. Location of O-acetyl group

An O-acetyl substituent in the polysaccharide may be detected by ^{1}H and ^{13}C n.m.r. spectroscopy. The ratio of the integration of the O-acetyl CH_3 peak to the peaks in the anomeric region will indicate if substitution occurs on every repeating unit. A sharp n.m.r. peak indicates that the group occurs at a discrete position in each repeating unit, (see Spectrum No. 21).

The O-acetyl group may be located by the method of de Belder and Norman[275]. With this procedure all the free hydroxyl groups are blocked with methyl vinyl ether. The base labile O-acetyl group is removed and replaced with a stable methyl group. The protecting groups are removed with acid and the product is analyzed by g.l.c. - m.s., as the alditol acetates of the constituent sugars.

Problems arise if (a) all the free hydroxyl groups are not protected (methylation at more than one position), (b) the O-acetyl group is not removed with base (no methylation) or (c) the O-acetyl group is removed at the protecting stage and replaced with methyl vinyl ether (no methylation).

An alternative is to methylate the native polysaccharide under conditions which do not remove the O-acetyl group. Prehm[250] has described a procedure (see Table 3),using trimethylphosphate as solvent (less electron - donor activity), 2, 6 di-(tert- butyl) pyridine as proton scavenger,and trifluoro-methanesulfonate as methylating agent.

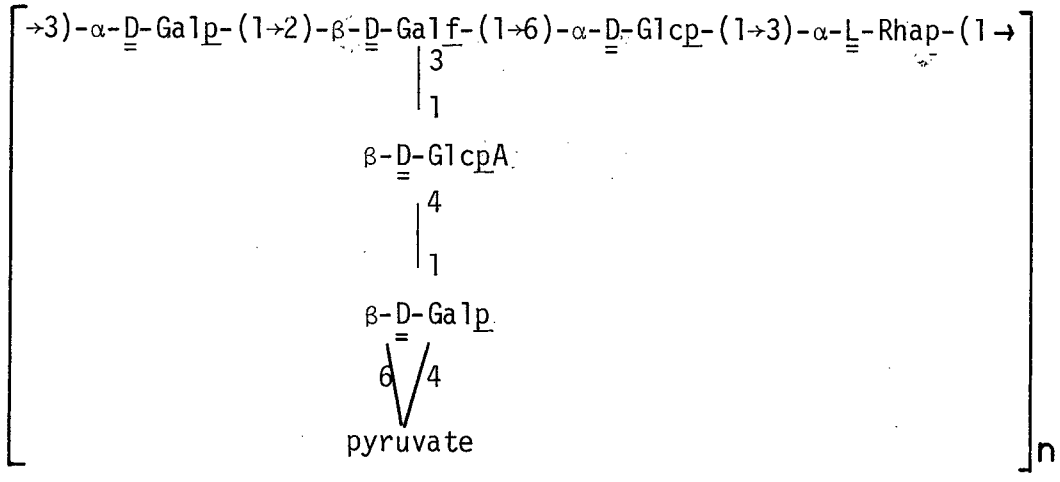
In this work the O-acetyl substituent in Klebsiella K58 polysaccharide was located by comparing g.l.c. - m.s. data from the analysis, by the method of de Belder and Norman, [275] of samples of the native and de-acetylated polysaccharide.

III. STRUCTURAL INVESTIGATION OF *KLEBSIELLA* CAPSULAR POLYSACCHARIDE SEROTYPES K12, K58, K23 and K70

III. I. STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE OF KLEBSIELLA SEROTYPE K12.

ABSTRACT

Klebsiella K12 capsular polysaccharide has been investigated by the techniques of methylation, Smith degradation - periodate oxidation, uronic acid degradation and partial hydrolysis, in conjunction with ^{1}H-n.m.r. spectroscopy at 100 and 220 HMz and ^{13}C-n.m.r. spectroscopy at 20 MHz. The structure has been found to consist of the hexasaccharide repeating unit shown, having a D-galactofuranosyl unit at the branch point. A galactofuranosyl residue has only previously been found, in this series, in the polysaccharide from Klebsiella K41.



III. I. I. Introduction

The genus Klebsiella has been classified by Ørskov [23] into approximately 80 serotypes, based on their antigenic, capsular polysaccharides. Nimmich [30,31] has analyzed qualitatively the polysaccharide from each strain; K12 was found to contain glucose,

galactose, rhamnose, glucuronic acid and pyruvic acid. As part of our continuing investigation of the relationship between primary, chemical structure and immunological activity, we now report on the elucidation of the structure of the K12 polysaccharide.

This structure is in agreement with the predictions made by Heidelberger and coworkers, based on the cross-reactions of the polysaccharide with anti-pneumococcal and anti-Klebsiella sera, of the occurrence of a 1,3-α linked L-rhamnosyl residue [55] and of a (non-reducing) 4,6-O-(1-carboxyethylidene)-D-galactosyl group [54] in the repeating unit.

III. 1. 2. Results and discussion

Composition and n.m.r. spectra

Klebsiella K12 bacteria were grown on an agar medium, and the capsular polysaccharide isolated was purified by one precipitation with Cetavlon. As described in Sec. II.1. the product had $[\alpha]_D$ + 24.2°.

Paper chromatography of an acid hydrolyzate of the polysaccharide showed the presence of glucose, galactose, glucuronic acid and rhamnose. Carboxyl-reduced K12 polysaccharide was hydrolyzed, and the presence of glucose, galactose and rhamnose in the ratio of 2:3:1 was determined by gas-liquid chromatography (g.l.c.) of their alditol acetates (see Table 4). Rhamnose was shown to be of the L configuration and glucose of the D configuration by circular dichroism (c.d.) measurements of the derived alditol acetates[147]. Subsequently arabinitol pentaacetate was similarly shown to have the L configuration,

TABLE 4

G.L.C. ANALYSIS OF NATIVE AND PERIODATE OXIDIZED[a] POLYSACCHARIDES.

Sugars (as alditol acetates)	T[b] Column C[c] (SP 2340)	Mole % I[d]	Mole % II[d]
Glycerol	0.09		11.7[e]
Erythritol	0.26		12.4[e]
Threitol	0.33		9.9[e]
Rhamnose	0.45	17.3	21.8
Arabinose	0.65		22.5
Galactose	0.95	51.7	21.7
Glucose	1.00	31.0	-

[a] On carboxyl-reduced polysaccharide.

[b] Retention time relative to glucitol hexaacetate.

[c] Programmed at 180° for 8 min, and then 4° per min to 240°.

[d] I, native polysaccharide, uronic acid reduced.

II, periodate oxidation, reduction, and total hydrolysis of the carboxyl reduced polysaccharide.

[e] Some loss of volatile components during derivatization.

indicating that the galactofuranose unit from which it was derived by loss of C-6 on oxidation has the D configuration. An acid hydrolyzate of the polysaccharide gave a positive reaction with D-galactostat reagent,* thus confirming the D configuration of the galactose.

The 220 MHz, ^{1}H-n.m.r. spectrum of the polysaccharide, after mild hydrolysis to lower the viscosity, showed a sharp singlet at δ 1.66 indicative of a 1-carboxyethylidene group. This signal was present in a 1:1 ratio with a doublet at δ 1.34 attributable to the methyl group of rhamnose [162,164]. Six discernible signals were observed in the anomeric region, at δ5.22 (1H, $J_{1,2}$ weak), δ5.16 (1H, $J_{1,2}$ 3Hz), δ5.13 (2H $J_{1,2}$ 2Hz), δ 4.66 (1H $J_{1,2}$ 8Hz), δ 4.48 (1H $J_{1,2}$ 6Hz). The ^{13}C-n.m.r. spectrum of the polysaccharide [202,231] (150 mg/2 ml) showed high field peaks at 17.57 p.p.m. (rhamnose CH_3) and 22.13 p.p.m. (pyruvate CH_3). In the anomeric region five signals in the ratio 1:1:2:1:1 were seen at 108.39, 106.99, 102.64, 99.43 and 97.18 p.p.m. Interpretation of these p.m.r. and ^{13}C-n.m.r. data initially caused some difficulty, as the p.m.r. data suggested four α-linked and two β-linked residues, while ^{13}C-n.m.r. data indicated three α-linked and three β-linked residues. This problem was resolved when the methylation data showed the presence of a furanosyl sugar (see later).

The signals at 63.85 p.p.m. and 61.77 p.p.m. were assigned to the C-6 carbon atoms of hexoses and the signal at 66.44 p.p.m. to a C-6 carbon involved in a linkage. Full assignments are shown in Table 5.

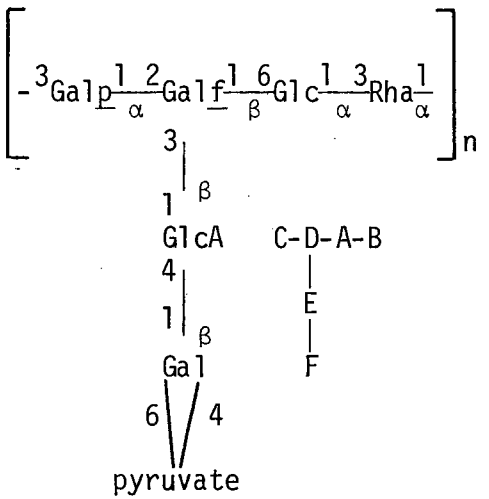
* A colorimetric enzymic reagent (Worthington Biochem.Co.)

TABLE 5

N.M.R. DATA FOR Klebsiella K12 CAPSULAR POLYSACCHARIDE AND THE DERIVED OLIGOSACCHARIDES.

Compound[a]	^{1}H-n.m.r. data				^{13}C-n.m.r. data	
	δ [b]	$J_{1,2}$ (Hz)[c]	Integral (H)	Assignment[d]	p.p.m.[e]	Assignment[f]
	5.30	2	0.6	α-Gal~OH	104.46	β-GlcA
GlcA $\frac{1\ 3}{\beta}$ Gal~OH (1)	4.76	7.5	1	GlcA	96.98	β-Gal~OH
	4.65	8	0.4	β-Gal~OH	93.01	α-Gal~OH
E-D					61.79	C-6 of Gal
	5.15	s	0.6	α-Rha~OH	96.41	α-Glc[g]
Glc $\frac{1\ 3}{\alpha}$ Rha~OH (2)	5.10	b	0.6	α-Glc	96.13	α-Glc[g]
	5.08	b	0.4	α-Glc	94.52	α+β-Rha~OH
A-B	4.88	s	0.4	β-Rha~OH	61.12	C-6 of Glc
	1.30	6 ($J_{5,6}$)	3	CH_3 of Rha	17.76	CH_3 of Rha
	5.19	1	1	α-Rha	106.93	Ara
Rha $\frac{1\ 3}{\alpha}$ Gal $\frac{1\ 2}{\alpha}$ Ara $\frac{1\ 1}{\alpha}$ glycerol (3)	5.10	2	1	α-Ara	103.10	Rha
	5.05	1	1	α-Gal	99.14	Gal
B-C-D-A	1.30	4	3	CH_3 of Rha	17.46	CH_3 of Rha

TABLE 5 Contd...



				186.47	C-6 of β-GlcA
5.22	s	1	α-Rha	108.39	β-Galf
5.16	3	1	α-Glc	106.99	β-Galp
5.13	2	2	α-Galp	102.64	α-Rha + β-GlcA
5.13	2		β-Galf	99.43	α-Gal
4.66	8	1	β-GlcA	97.18	α-Glc
4.48	6	1	β-Galp	85.71	C-2 Galf
4.3-4.5	b	2	H-2, H-3 β-Galf	84.24	C-3 of β-Galf
1.66	s	3	CH_3 of acetal	83.00	C-4 GlcA
1.34	6	3	CH_3 of Rha	66.44	C-6 of α-Glc
				63.85	C-6 of β-Galf
				61.77	C-6 of α-Gal
				22.13	CH_3 of acetal
				17.57	CH_3 of Rha

[a] For the origin of compounds 1 - 3, see text. See Appendix III for reproductions of the spectra.

[b] Chemical shift relative to internal acetone; δ2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.).

[c] Key: b = broad, unable to assign accurate coupling constant, s= singlet .

[d] For example, α-Gal = Proton on C-1 of α-linked D - Gal residue.

[e] Chemical shift in p.p.m. downfield from Me_4Si, relative to internal acetone; 31.07 p.p.m. downfield from D.S.S.

[f] As for [d], but for anomeric ^{13}C nuclei.

[g] This glycosidic atom resonates as two doublets, because of the anomeric equilibrium of the reducing unit.

Methylation of original polysaccharide

Methylation [241,242,247] of K12 polysaccharide, followed by reduction of the uronic ester, hydrolysis, derivatization as alditol acetates, and g.l.c. - m.s. analysis [103,121] indicated that K12 is composed of a hexasaccharide repeating unit with five pyranose sugars and one furanose, namely, galactofuranose, which constitutes a branch point (see Table 6). These data also indicate that the (1-carboxyethylidene) group is linked to O-4 and O-6 of a (terminal) galactopyranosyl group. Analysis of a re-methylated sample of the reduced product showed the formation of 2,3,6-tri-O-methylglucose and the disappearance of the 2,3-di-O-methylether, thus establishing that the uronic acid is glucuronic acid.

Base-catalyzed degradation

To determine the location of the glucuronic acid, the methylated polysaccharide was subjected to a base-catalyzed degradation, and was then directly ethylated[273]. The isolation of a polymeric, degraded product indicates that the uronic acid is in the side chain (see Scheme 7). On hydrolysis, and derivatization, for g.l.c. -m.s., the compounds shown in Table 6 were obtained, indicating that the glucuronic acid is attached to O-3 of the galactofuranosyl unit, and that the only other sugar in the side chain is a 4,6-O-(1-carboxyethylidene)-D-galactose group. The ^{1}H - n.m.r. spectrum indicated the absence of two β-linkage signals in the anomeric region attributable to the sugars of the side chain.

TABLE 6

METHYLATION ANALYSIS OF NATIVE, AND DEGRADED, Klebsiella K12 CAPSULAR POLYSACCHARIDE

Methylated sugars[a] (as alditol acetates)	T[b] Column A[c] (OV-225)	T[b] Column B[d] (ECNSS-M)	Mole %[e] I[f]	II	III
2,4-Rha	1.00	1.00	20.02	18.17	22.48
2,4,6-Gal	1.88	1.55	21.66	21.88	26.46
2,3,4-Glc	2.05	1.63	16.96		25.24
2,3,6-Glc	2.05	1.63		31.10	
5,6-Gal	2.28	1.75	15.88	13.11	
2,3-Glc	3.30	2.31	13.38		
2,3-Gal	3.42	2.40	12.10	15.74	
3,5,6-Gal[g]		1.46			25.82

[a] 2,4-Rha = 1,3,5-tri-O-acetyl-2,4-di-O-methyl-L-rhaminitol etc.

[b] Retention time relative to that of the alditol acetate derivative of 2,4-Rha.

[c] Programme: 180° for 4 min and then 2° per min to 200°.

[d] Programme: 165° for 4 min, and then 2° per min to 200°.

[e] Values corrected by using effective carbon response factors[106].

[f] I original polysaccharide methylated and uronic ester reduced, column B. II as in I but remethylated, column A. III after uronic acid degradation and ethylation.

[g] 1,2,4-Tri-O-acetyl-3-O-ethyl-5,6-di-O-methyl-galactitol.

Partial hydrolysis

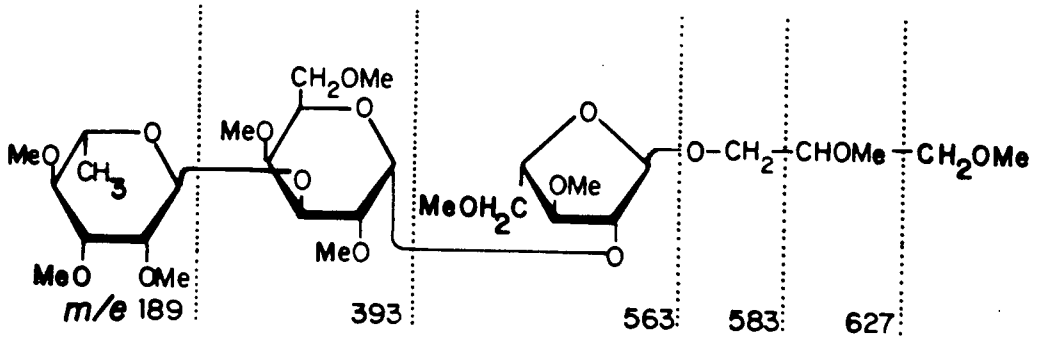
A sample of K12 polysaccharide in the free-acid form was hydrolyzed for 10h with 0.1M trifluoroacetic acid in an apparatus similar to that described by Galanos and colleagues[67], yielding a mixture of oligomers and monosaccharides, which was separated with AG-1 X2 ion exchange resin into acidic and neutral fractions (see Scheme 8). Preparative, paper electrophoresis of the acidic fraction gave an aldobiouronic acid (1) which, after hydrolysis and paper chromatography, was shown to consist of glucuronic acid and galactose. ^{1}H- and ^{13}C-n.m.r. spectroscopy indicated that the reducing galactose was now in the pyranose form and verified that the side chain linkage is β (see Table 5). The structure of the aldobiouronic acid (E-D 1) is thus β-D-GlcpA-(1→3)-D-Galp-OH. Separation of the mixture of neutral oligomers by gel chromatography (Bio-gel P-2), followed by purification on paper chromatography, gave a disaccharide (A-B 2) which, after hydrolysis and paper chromatography, was shown to consist of glucose and rhamnose. The ^{1}H- and ^{13}C- n.m.r. spectra are consistent with the structure α-D-Glcp-(1→3)-L-Rhap-OH.

Periodate oxidation of the carboxyl-reduced polysaccharide

To determine the sequence of the sugars in the backbone of the polymer, the carboxyl-reduced polysaccharide [264] was oxidized with periodate. After 90h. the consumption of oxidant [256] was 5.4 mol per mol of repeating unit. The theoretical consumption is 5 mol if the (1-carboxyethylidene) group remains intact; the higher consumption indicates loss of some of the ketal groups. Total hydrolysis of

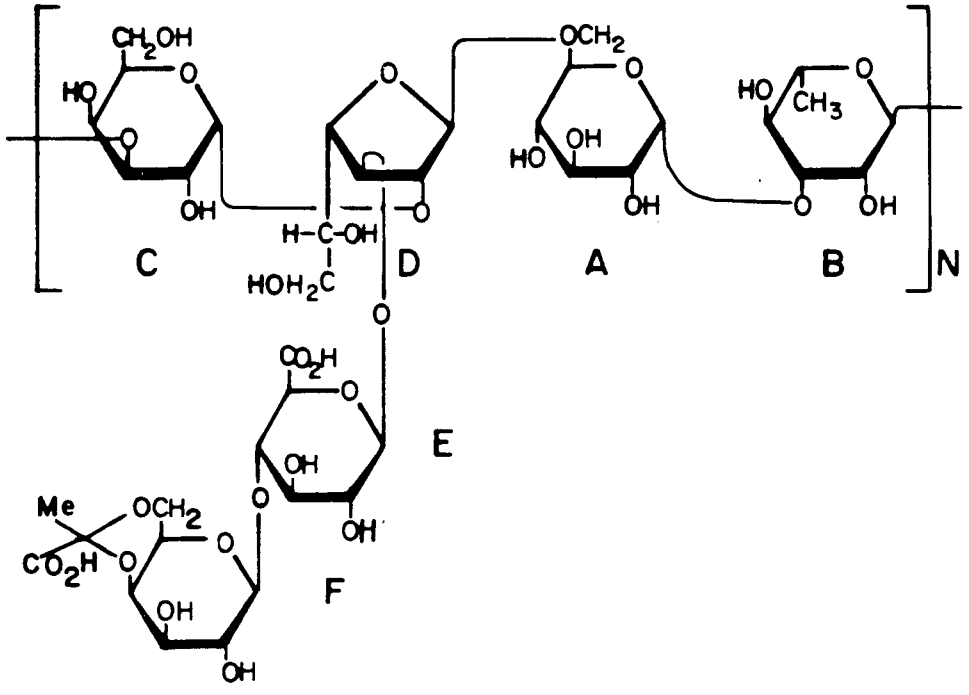
the polyol obtained after sodium borohydride reduction, followed by derivatization as the alditol acetates, gave the g.l.c. separation shown in Fig. 5 (see Table 4). The low proportion of threitol is consistent with some loss of the pyruvic acid ketal . Smith hydrolysis [253] of the polyol, followed by sodium borohydride reduction, yielded a mixture of oligosaccharides which was separated by gel chromatography. Oligomer 3 was obtained pure by preparative paper chromatography and was shown to consist of rhamnose, galactose, arabinose, and glycerol in equal proportions. ^{1}H- and ^{13}C-n.m.r. spectra were in agreement with these data (see Table 5). To determine the sequence of sugars in 3, the oligosaccharide was permethylated by the Purdie method[242] and the product purified by g.l.c. on OV-1 and examined by electron-impact, mass spectrometry. Detection of peaks at m/e 189 and 393, among others, indicated that the deoxyhexose is linked to the hexose, not to the pentose. The source of some pertinent fragments is illustrated below. The anomeric nature of the linkages was determined by ^{1}H- and ^{13}C-n.m.r. spectroscopy. Oligomer (B-C-D-A 3) is thus established as having the structure

α-L-Rhap-(1→3)-α-D-Galp-(1→2)-α-L-Araf-(1→1)-glycerol



III. 1. 3 Conclusion

It thus follows that Klebsiella K12 capsular polysaccharide had the following structure.



After the realization, from the methylation g.l.c.-m.s. data, that a furanosyl residue was present, the ^{1}H- and ^{13}C-n.m.r. spectra were more easily interpreted. In the former, the β-Galf anomeric signal appears at δ 5.13, the region normally attributed to α-linked pyranoses. In the ^{13}C spectrum, however, the anomeric signal occurs in the unambiguous β-linkage region at 108.39 p.p.m. (see Table 6).

It is interesting to note that the only other Klebsiella polysaccharide reported [169] to have a furanosyl unit, K41 (see App.I) has a very similar structure in which the terminal 4,6-O-(1-carboxyethylidene)-β-D-galactopyranosyl group is replaced by β-D-Glcp-(1→6)-α-D-Glcp-. As expected,no cross-reaction occurs between these two polysaccharides, because the sidechain is usually the immunodominant

group. Cross-reaction does, however, occur with anti-K11[54] which has a 4,6-O-1(1-carboxyethylidene)-α-D-galactopyranose side chain[276] (see App.I) and with anti - Pn-VI [55] which has an in-chain-α-D-Glcp-(1→3)-α-L-Rhap- unit.

III. 1. 4. Experimental

General methods --- Concentrations were carried out under diminished pressure at bath temperatures not exceeding 40°. Paper electrophoresis was performed on a Savant high voltage (5 Kv) system (model LT - 48A) with kerosene as coolant. The buffer used contained pyridine - acetic acid - water (5 : 2 : 743, v/v), pH 5.3. Strips of Whatman No. 1 paper (77 cm x 20 cm) were used for all runs, with application of 25 - 50 mA for 1½ h. Descending paper chromatography was carried out using Whatman No.1 paper. The following solvent system (v/v) were used: (1) freshly prepared 2:1:1 1-butanol -- acetic acid -- water, and (2) 8:2:1 ethyl acetate -- pyridine -- water. Sugars and oligosaccharides were detected, after electrophoresis and after descending, paper chromatography, with an alkaline silver nitrate reagent[24]. Analytical g.l.c. separations were performed with a Hewlett Packard 5700 instrument fitted with dual flame-ionisation detectors. An Infotronics CRS-100 electronic integrator was used to measure peak areas. Separations were performed in stainless-steel columns (1.8 m x 3 mm) with a carrier-gas flow-rate of 20 mL/min. Columns used were (A) 3% of OV-225 on Gas Chrom Q (100-120 mesh); (B) 5% of ECNSS-M on the same support; and (C) 3% of SP-2340 on Supelcoport (100-120 mesh). Analogous columns (1.8 m x 6.3 mm) were used, along

with a column of 5% of OV-1 on Gas Chrom Q (100-120 mesh) for preparative g.l.c. separations. G.l.c.-m.s. was performed with a Micromass 12 instrument fitted with a Watson-Biemann separator. Spectra were recorded at 70 eV with an ionisation current of 100 μA and an ion-source temperature of 200°.

^{1}H n.m.r. spectra were recorded on either a Varian XL-100 instrument at 90°, or a (Nicolet/Oxford Instruments) H-270 at ambient temperature. ^{13}C n.m.r. spectra were recorded on either a Varian CFT-20 or a Bruker WP-80 ^{13}C 20.1 MHz instrument at ambient temperature. Additional spectra were obtained courtesy of Dr. A.A. Grey and A. Lee (^{1}H : HR-220 MHz, 90°) and Dr. Michel Vignon (^{1}H : Cameca 250 MHz, 90° and ^{13}C : Cameca 62.87 MHz, 80°).

Circular dichroism (c.d.) spectra were recorded on a Jasco J20 automatic recording spectropolarimeter with a quartz cell of path length 0.01 cm. Optical rotations were measured at 23 ±2° on a Perkin Elmer model 141 polarimeter, with a 10-cm cell. Infrared spectra were recorded using a Perkin-Elmer 457 spectrophotometer.

Preparation and properties.--- A culture of Klebsiella K12 (313) was obtained from D.I. Ørskov (Copenhagen). The polysaccharide was isolated as described, in section II.1 and showed $[\alpha]_D$ + 24.2° (c 1, water).

Analysis of constituent sugars. --- Methanolysis of a sample (20 mg) of K12 polysaccharide with 3% methanolic hydrogen chloride

and subsequent treatment with sodium borohydride in anhydrous methanol reduced the uronic ester. Hydrolysis with 2M trifluoroacetic acid (TFA) overnight at 95° followed by reduction ($NaBH_4$) and acetylation gave galactitol hexaacetate, glucitol hexaacetate and rhamnitol pentaacetate in the ratio 3:2:1 (column C, programmed at 180° for 8 min and then 4°/min to 240°). Circular dichroism (c.d.) of the latter two components isolated by preparative g.l.c., showed positive and negative curves, respectively, confirming that glucose has the D configuration and rhamnose the L configuration. The configuration of galactose was deduced to be D from the negative c.d. of the pentaacetate of its oxidation product, namely, arabinitol pentaacetate. This was confirmed by the positive action of D - Galactostat (Worthington Biochemical Co.) on the hydrolysis product of the polysaccharide.

Methylation of the native polysaccharide. --- Methylation of K12 polysaccharide under the Hakomori [247] conditions, followed by a Purdie [242] treatment yielded a product that showed no hydroxyl absorption in the i.r. spectrum. This material was reduced overnight with sodium borohydride in oxolane (THF) and ethanol (1:1 v/v). A portion of this product was hydrolyzed with 2M trifluoroacetic acid for 16 h at 95°; the resulting mixture was reduced with sodium borohydride and the product acetylated. G.l.c. - m.s. gave the results shown in Table 6.

Another portion of the material (reduced uronic ester) was remethylated under Purdie conditions for 2 days, and derivatized for g.l.c. - m.s. giving the compounds shown in Table 6.

Uronic acid degradation.[267] --- A solution of carefully dried, methylated polysaccharide (100 mg) and p-toluenesulfonic acid (a trace) in 19:1 dimethyl sulfoxide - 2,2-dimethoxypropane (20 mL) was prepared in a serum vial which was then sealed with a rubber cap. The vial was flushed with nitrogen, and the solution was stirred for 3h. Sodium methylsulphinylmethanide (2M) in dimethyl sulfoxide (10 mL) was then added with the aid of a syringe, and the solution was stirred at room temperature overnight. After external cooling to 10^{o}, ethyl iodide (3 mL) was added slowly using a syringe [16]. The solution was stirred for a further 30 min., excess of ethyl iodide was removed using a rotary evaporator, and the solution was dialyzed overnight against tap water. After lyophilization the product (65 mg) was purified by precipitation with petroleum ether (30^{o}- 60^{o}), yielding 60 mg of polymeric material. Subsequent hydrolysis and derivatization for g.l.c. - m.s. gave the results in Table 6.

Partial hydrolysis --- A sample of Klebsiella K12 polysaccharide was exchanged to the free-acid form with Amberlite IR-120 (H+) ion-exchange resin, and lyophilized. This material (1g) was dissolved in water (100 mL pH 3.2) and was then auto-hydrolyzed on a steam bath for 16 h in an apparatus similar to that described by Galanos et al [267]. Very little hydrolysis occurred; therefore the solution was made 0.1M in TFA, and the reaction continued for a further 16 h. After removal of TFA the products (700 mg) were separated into neutral and acidic fractions by using AG-IX2 ion

exchange resin. Portions (200 mg) of the acidic fraction were separated by gel chromatography on a column (100 x 2.5 cm) of Sephadex G-25, which was irrigated with a buffer (1000:10:4 v/v water -- pyridine -- acetic acid,) at a flow rate of 10 mL/h. Separation of components was poor. Fractions containing components with $\frac{R}{Glc}$ >0.2 (solvent 1) were combined, and separated by preparative, paper electrophoresis. A component $\underset{\sim}{1}$ with $\frac{R}{GlcA}$ 0.69 was obtained pure (30 mg) and was shown on total hydrolysis to consist of glucuronic acid and galactose (E-D). ^{1}H- and ^{13}C-n.m.r. spectroscopy indicated the presence of a β- linked glucuronic acid and a reducing galactose (see Table 5). Portions (200 mg) of the neutral fraction were separated by gel chromatography on a column (100 x 2.5 cm) of Biogel P-2. Irrigation with the same buffer, at a flow rate of 10 mL/h, lyophilization of the fractions, and examination by paper chromatography (solvent 2) revealed that separation was not complete. Purification of a component with $\frac{R}{Glc}$ 0.61 by paper chromatography then yielded compound $\underset{\sim}{2}$ (30 mg) which on hydrolysis was shown to consist of glucose and rhamnose (A-B). ^{1}H- and ^{13}C- n.m.r. spectroscopy indicated the presence of an α-linked glucose and a (reducing) rhamnose (see Table 5).

Periodate oxidation of carboxyl-reduced polysaccharide. --- A sample of the polysaccharide was reduced by the procedure of Taylor and Conrad[264]; two treatments were required in order to achieve complete reduction. Reduced, capsular polysaccharide (200 mg) was

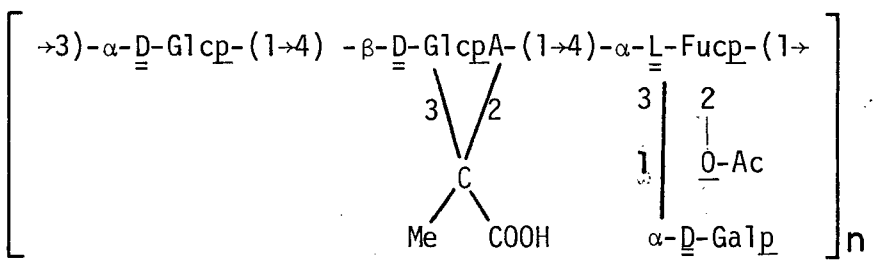
dissolved in water (40 mL) to which 0.1M sodium metaperiodate (40 m L) was then added. The solution was stirred in the dark at 3° and periodate consumption was monitored spectrophotometrically [256]. After three days, consumption had reached 5.4 molecules per repeating unit. Ethylene glycol (10 mL) was then added. After stirring for a further 30 minutes the mixture was dialyzed overnight against running tap water, and the product reduced with sodium borohydride. The polyol was isolated by dialysis and lyophilization.

A portion (5 mg) of the polymeric product was hydrolyzed with 2M TFA overnight at 95°. Paper chromatography (solvent 2) then showed the presence of glycerol, a tetrose, rhamnose, arabinose and galactose. Conversion of the hydrolysis products into the corresponding alditol acetates gave the g.l.c. results shown in Table 4. Smith hydrolysis (0.5M TFA overnight at room temperature) of the polyol gave a mixture which was separated on Biogel P-2. An oligomer (B-C-D-A)$_3$ (R_{Glc} 0.46 solvent 1) was purified by paper chromatography. ^{1}H- and ^{13}C- n.m.r. data are shown in Table 5. The mass spectrum of the permethylated (Purdie [242] method) oligomer showed significant peaks at 627, 583, 553, 540, 527, 467, 393, 375, 361, 290, 289, 273, 272, 260, 259, 217, 189, 187 and 103. Total hydrolysis of the oligomer (2M TFA, 90°, 16 h) gave galactose, rhamnose, arabinose and glycerol by paper chromatography (solvent 2). G.l.c. analysis of the derived alditol acetates showed that the constituents were present in equimolar proportions. The anomeric nature of all the linkages was shown to be α by ^{1}H- and ^{13}C- n.m.r. spectroscopy (see Table 5).

III. 2 STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE OF KLEBSIELLA SEROTYPE K58

ABSTRACT

Klebsiella K58 capsular polysaccharide has been investigated by the techniques of methylation, Smith degradation - periodate oxidation, uronic acid degradation and partial hydrolysis, in conjunction with ^{1}H-n.m.r. spectroscopy at 100 and 220 MHz, and ^{13}C-n.m.r. spectroscopy at 20 MHz. The structure has been found to consist of the tetrasaccharide repeating unit shown, with one O-acetyl group per repeating unit. A uronic acid residue bearing a 1-carboxyethylidene moiety has previously been found, in this series, only in the polysaccharide from Klebsiella K1.



III.2.1. Introduction

The genus Klebsiella has been classified by Ørskov [23] into approximately 80 serotypes, based on their antigenic, capsular polysaccharides. Nimmich [30,31] has qualitatively analyzed the polysaccharide from each strain; K58 was found to contain glucose, galactose, fucose, glucuronic acid and pyruvic acid. In addition, K58 was shown to contain one O-acetyl group per repeating unit. As

part of our continuing investigation of the relationship between primary, chemical structure and immunological activity we now report on the elucidation of the structure of K58.

III.2.2. Results and Discussion

Composition and n.m.r. spectra

Klebsiella K58 bacteria were grown on an agar medium, and the capsular polysaccharide isolated was purified by one precipitation with Cetavlon as described in Sec. II.1. The product had $[\alpha]_D$ + 19.0°.

Paper chromatography of an acid hydrolyzate of the polysaccharide showed the presence of glucose, galactose, glucuronic acid and fucose. Carboxyl-reduced K58 polysaccharide was hydrolyzed, and the presence of glucose, galactose and fucose in the ratio of 2:1:1 was determined by gas-liquid chromatography (g.l.c.) of their alditol acetates. Fucose was shown to be of the L configuration and glucose of the D configuration by circular dichroism (c.d.) measurements of the derived alditol acetates [147]. Galactose was shown to be of the D configuration by the positive reaction of D-galactostat reagent with an acid hydrolyzate of the polysaccharide.

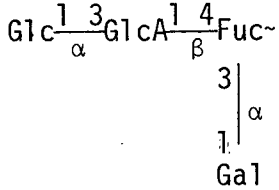
The 220-MHz ^1H - n.m.r. spectrum of the polysaccharide showed sharp singlets at δ2.17 and δ1.64 and a doublet at δ1.33 in the approximate ratio of 1:1:1. These were assigned to methyl groups of O-acetate, 1-carboxyethylidene and fucose, respectively [162-164]. Four discernible signals were observed in the anomeric region, at δ5.45 (1H, $J_{1,2}$ 2Hz), δ5.18 (1H, $J_{1,2}$ 2Hz), δ5.13 (1H, $J_{1,2}$ 2Hz)

TABLE 7

N.M.R. DATA FOR Klebsiella K58 CAPSULAR POLYSACCHARIDE AND THE DERIVED OLIGOSACCHARIDES

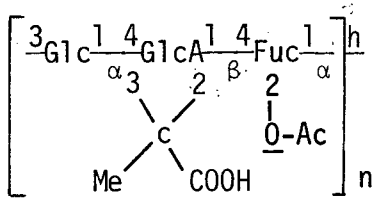
Compound[a]	^{1}H-n.m.r. data				^{13}C-n.m.r. data	
	δ[b]	$J_{1,2}$ (Hz)[c]	Intergral (H)	Assignment[d]	p.p.m.[e]	Assignment[f]
GlcA$\frac{1\ 4}{\beta}$Fuc~OH	5.26	3	.5	α-Fuc~OH	103.91	β-GlcA
	4.59	10	1.7	β-Fuc~OH	97.07	β-Fuc~OH
(1)	4.54	6.5		β-GlcA	93.16	α-Fuc~OH
~	1.33	6.0	3	CH_3 of α-Fuc~OH	23.7	CH_3 of 1-carboxy-ethylidene
	1.29	6.5		CH_3 of β-Fuc~OH	16.3	CH_3 of fucose
	1.64	s	0.3	CH_3 of 1-carboxy-ethylidene		
Glc $\frac{1\ 3}{\alpha}$GlcA$\frac{1\ 4}{\beta}$Fuc~OH	5.46	3.5	1	α-Glc	103.94	β-GlcA
	5.26	3	.6	α-Fuc~OH	99.5	α-Glc
	4.61	7	1.8	β-Fuc~OH	97.16	β-Fuc~OH
	4.57	7		β-GlcA	93.3	α-Fuc~OH
(2)	1.64	s	0.4	CH_3 of 1-carboxy-ethylidene	61.2	C-6 of Glc
~	1.33	6	3	CH_3 of α-Fuc~OH	23.7	CH_3 of 1-carboxy-ethylidene
	1.29	6		CH_3 of β-Fuc~OH	16.25	CH_3 of fucose

TABLE 7 Contd..



(3)

5.45	3.5	.9	α-Glc	104.0	β-GlcA	
5.29	3	1.6	α-Fuc~OH	99.7	α-Gal	
			α-Gal	99.5	α-Glc	
4.60	8	1.7	β-Fuc~OH	97.2	β-Fuc~OH	
4.55	7		β-GlcA	93.3	α-Fuc~OH	
1.64	s	0.4	CH_3 of 1-carboxy-ethylidene	61.07	C-6 of Glc	
				62.59	C-6 of Gal	
1.33	6.5	3	CH_3 of Fuc~OH	23.7	CH_3 of 1-carboxy-ethylidene	
1.29	6.5		CH_3 of Fuc~OH	16.25	CH_3 of fucose	

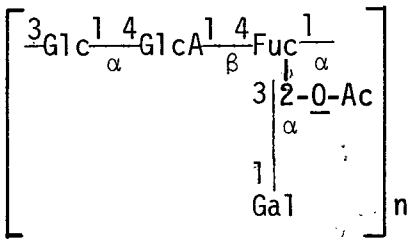


(10%)

(4)

5.44	2	1	α-Glc	104.0	β-GlcA
5.27	b	1	α-Fuc	100.3	α-Fuc
4.57	8	1	β-GlcA	99.7	α-Glc
1.64	s	1	CH_3 of 1-carboxy-ethylidene	61.3	C-6 of Glc
1.33	6	3	CH_3 of fucose	23.5	CH_3 of 1-carboxy-ethylidene
				16.1	CH_3 of fucose

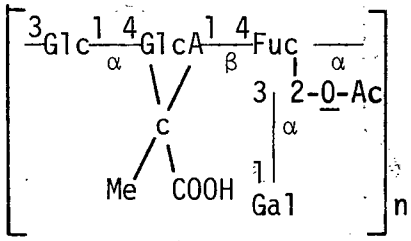
TABLE 7 Contd..



+OAc

(5)

5.35	s	1	α-Glc		
5.25	s	0.9	α-Fuc		
5.16	s	1	α-Gal		
4.53	8	1	β-GlcA		
2.17	s	2	CH_3 of acetate		
1.33	6	3	CH_3 of fucose		



+ O-Ac

(6)

5.45	2	1	α-Glc	104.5	β-GlcA[g]
5.18	b	2	α-Fuc	101.2	α-Fuc
5.13			α-Gal	99.5	α-Glc
4.59	8	1	β-GlcA	97.5	α-Gal
2.17	s	3	CH_3 of acetate	62.5	C-6 of Gal
1.64	s	3	CH_3 of 1-carboxy-ethylidene	61.4	C-6 of Glc
1.33	6	3	CH_3 of fucose	29.98	CH_3 of acetate
				23.4	CH_3 of 1-carboxy-ethylidene
				16.0	CH_3 of fucose

[a] For origin of compounds 1 - 5, see text.* [b] Chemical shift relative to internal acetone; δ2.23 downfield from sodium 4,4-dimethyl-4silapentane-1-sulfonate (D.S.S.). [c] b = broad, unable to assign accurate coupling constant, s-singlet. [d] For example, α-Gal = proton on C-1 of α-linked D-Gal residue. [e] Chemical shift in p.p.m. downfield from Me_4Si, relative to internal acetone; 31.07 p.p.m. downfield from D.S.S. [f] As for [d], but for anomeric ^{13}C nuclei. [g] Spectrum recorded on Brucker WP-80 (20.1 MHz) using 5 mm tube with semi-micro volume cylindrical cavity (6mm x 4.2 mm). [h] 10% of 1-carboxyethylidene remaining after Smith hydrolysis. *See Appendix II for reproductions of the spectra.

and δ4.59 (IH, $J_{1,2}$ 8Hz). The ^{13}C spectrum of the polysaccharide[202,231] showed signals of equal intensity at 16.0 p.p.m. (fucose CH_3), 23.4 p.p.m. (1-carboxyethylidene CH_3) and 29.98 p.p.m. (acetate CH_3). In the anomeric region four signals in the ratio 1:1:1:1 at 104.5, 101.2, 99.5 and 97.5 p.p.m. were observed (see Table 7).

The signals at 61.4 and 62.5 p.p.m. were assigned to the C-6 atoms of hexoses. Both the ^1H n.m.r. and ^{13}C data indicate the presence of three α-linked and one β-linked residues. Assignments were made after n.m.r. spectral investigation of oligosaccharides isolated from partial hydrolysis and periodate oxidation (see later).

Methylation of original polysaccharide

Methylation [242,247] of K58 polysaccharide, followed by reduction of the uronic ester, hydrolysis, derivatization as the alditol acetates, and g.l.c. - m.s. analysis [103,121] indicated that K58 is composed of tetrasaccharide repeating units. The sugars are in the pyranoid form with fucose constituting a branch point (see Table 8). Analysis of a re-methylated sample of the reduced product showed the formation of 6-O-methylglucose, thus establishing that the uronic acid is glucuronic acid. Mild hydrolysis of this remethylated polysaccharide (to remove the 1-carboxyethylidene group), followed by methylation, showed on g.l.c. - m.s. analysis, the formation of 2,3,6-tri-O-methyl glucose indicating that the 1-carboxyethylidene residue is linked at O-2 and O-3 of the glucuronic acid residue; this was confirmed by methylation analysis of a sample of autohydrolyzed native polysaccharide (see Fig. 10).

TABLE 8

METHYLATION ANALYSIS OF NATIVE, AND DEGRADED Klebsiella K58 CAPSULAR POLYSACCHARIDE

Methylated sugars[a] (as alditol acetates)	T[b] Column A[c] (OV-225)	Column B[d] (ECNSS-M)	Mole %[e] I[f]	II	III	IV	V	VI
2,3,4-Fuc	0.80	-				4		
2,3-Fuc	0.82	0.92					30	
4-O Et, 2-Fuc	-	0.9						38
2,4-Fuc	0.89	-	2	2	2	2		
2,3,4,6-Gal	1.00	1.00	28	28	29	23		43
2-Fuc	1.14	1.23	22	23	25	19	4	
2,4,6-Glc	1.31	1.45	25	25	25	26	36	
2,3,6-Glc	1.39	-		3	19			
2,3-Glc	2.05	-	3			21		
6-Glc	2.17	-		19				
Glc	2.78	3.4	20			5	30	19

[a] 2,3,4-Fuc = 1,5-di-O-acetyl-2,3,4-tri-O-methyl-L-fucitol etc.

[b] Retention time relative to that of the alditol acetate derivative of 2,3,4,6-Gal.

[c] Programme: 180° for 4 min and then 2°per min to 200°.

[d] Programme: 160° for 4 min and then 4° per min to 200°.

[e] Values corrected by using effective carbon response factors,[106] and adjusted to the nearest integer.

[f] I, original polysaccharide, methylated and uronic ester reduced. II, as in I, but re-methylated. III, as in II, then hydrolyzed to remove 1-carboxyethylidene moiety and remethylated. IV, auto-hydrolyzed polysaccharide, methylated, and uronic ester reduced. V, Smith degradation product, methylated, and uronic ester reduced. VI, product of uronic acid degradation, and ethylation.

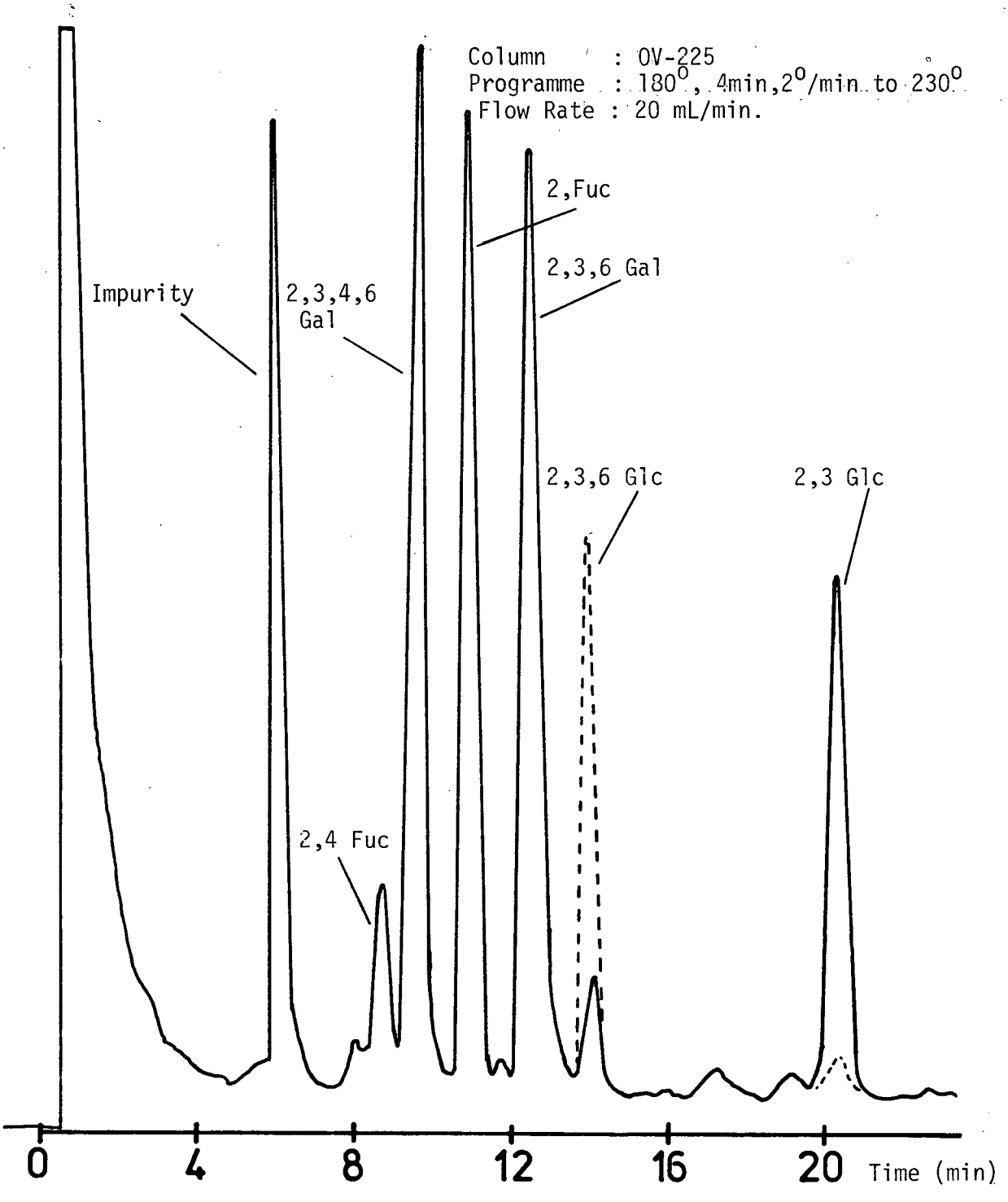


Figure 10 G.l.c. separation of products of methylation analysis of Klebsiella K58

—— = IV, --- = III (see Table 8).

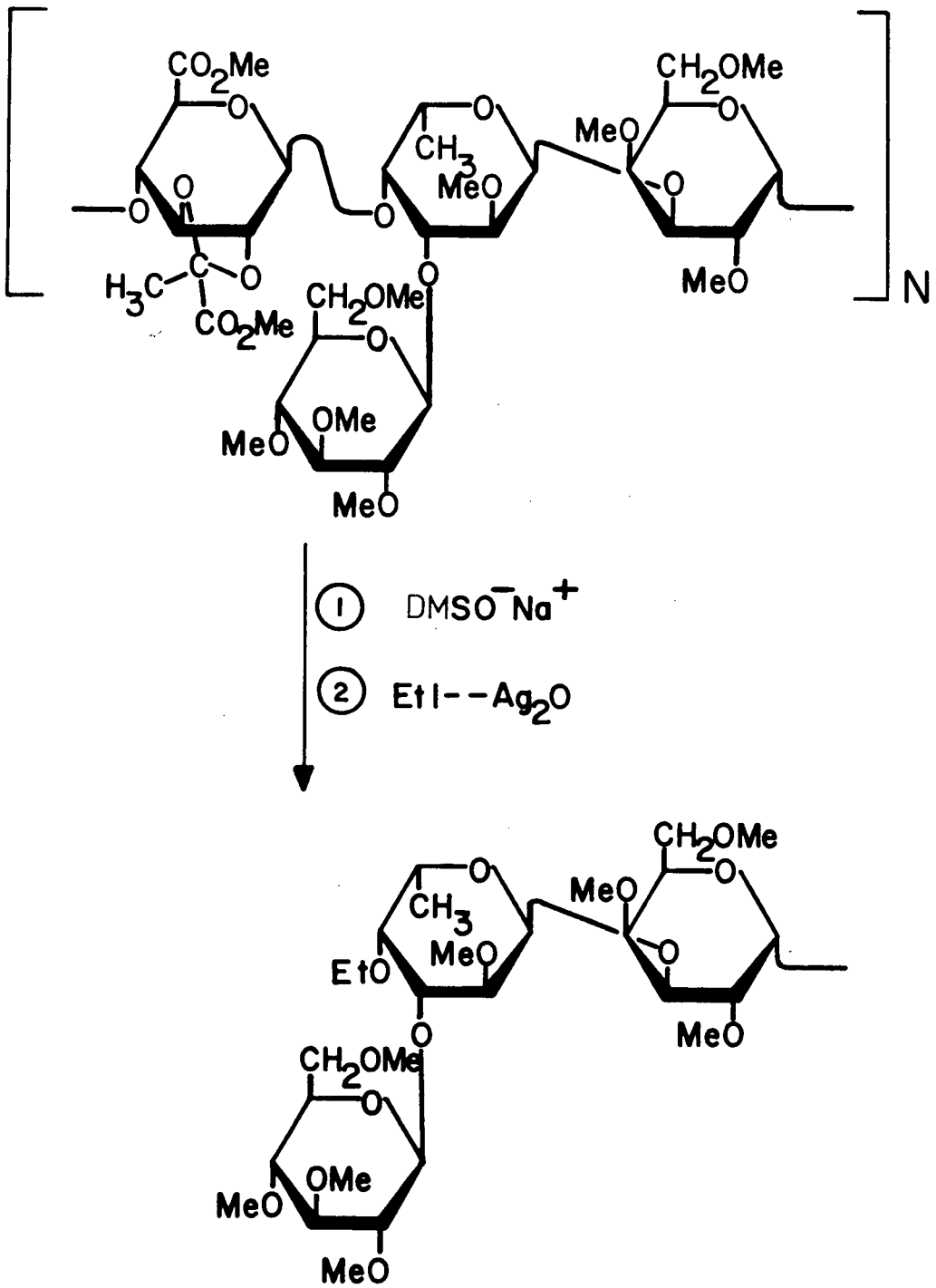
Base-catalyzed degradation

To determine the location of the uronic acid, the methylated polysaccharide was subjected to base-catalyzed degradation, and was then directly ethylated[273]. The isolation of an oligosaccharide indicates that the uronic acid is in the backbone. On hydrolysis, and derivitization, for g.l.c. - m.s., the compounds shown in Table 8 were obtained indicating that glucuronic acid is attached to O-4 of fucose. Loss of some glucose suggests that it is linked to the glucuronic acid in the backbone (see Scheme 9).

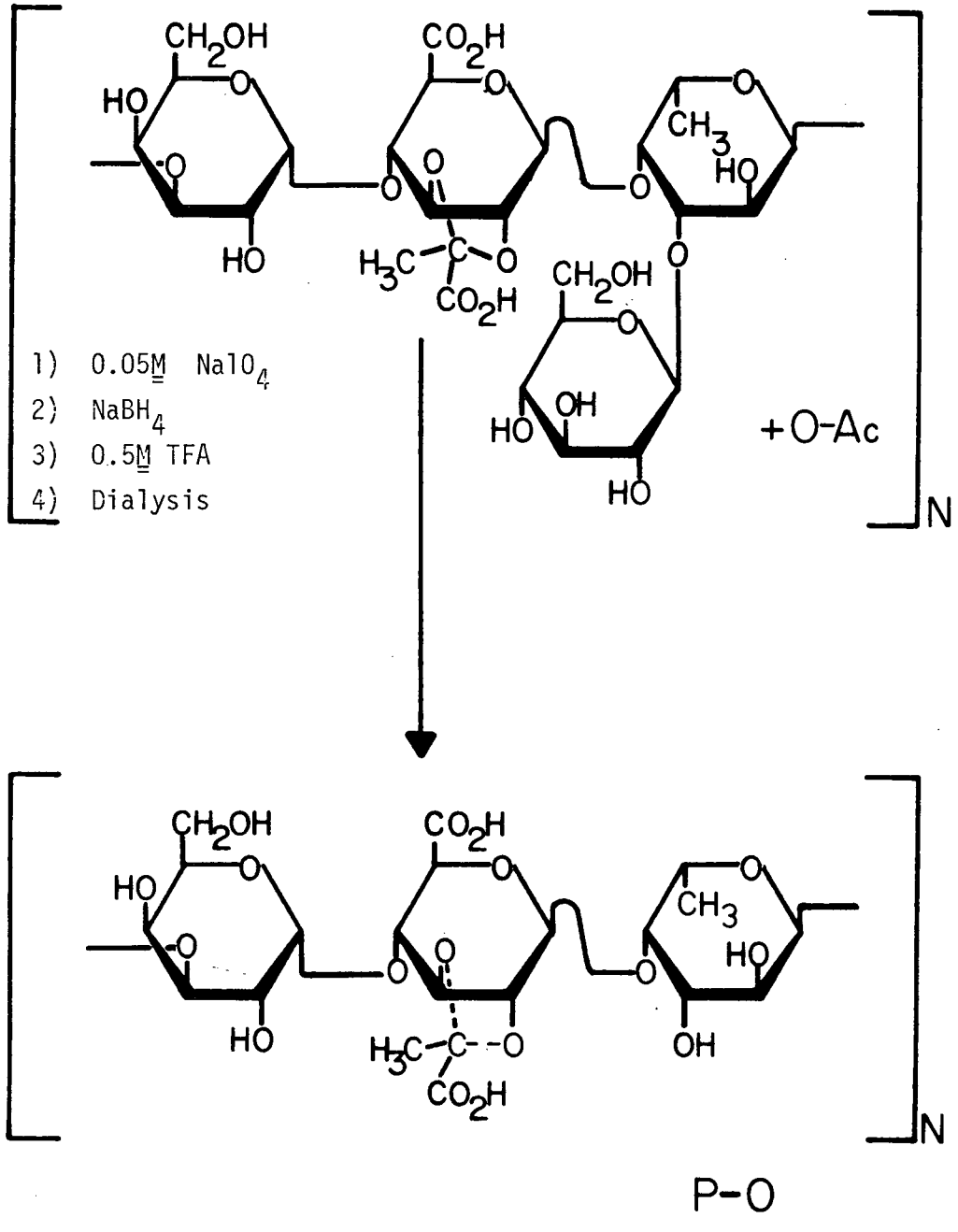
Periodate Oxidation

The native polysaccharide consumed 1.8 moles of periodate per repeating unit [255] in 10 h, yielding, after sodium borohydride reduction and Smith hydrolysis [253] a polymeric product (4). Reduction of the uronic acid followed by total hydrolysis and derivatization for g.l.c. showed the presence of glucose and fucose in the ratio 2:1 ^{1}H and ^{13}C n.m.r. spectroscopy of the Smith - degradation product (4) showed the presence of two α-linked and one β-linked sugars, indicating that the oxidized terminal galactose is α-linked (see Table 7, Scheme 10).

Methylation of a portion of the Smith -degradation product, followed by reduction of the uronic ester, and derivatization for g.l.c. - m.s. , gave the results shown in Table 8 indicating that the side chain galactose is linked to O-3 of fucose.



Scheme 9 Base-catalysed degradation of Klebsiella K58



Scheme 10 Periodate Oxidation of Klebsiella K58

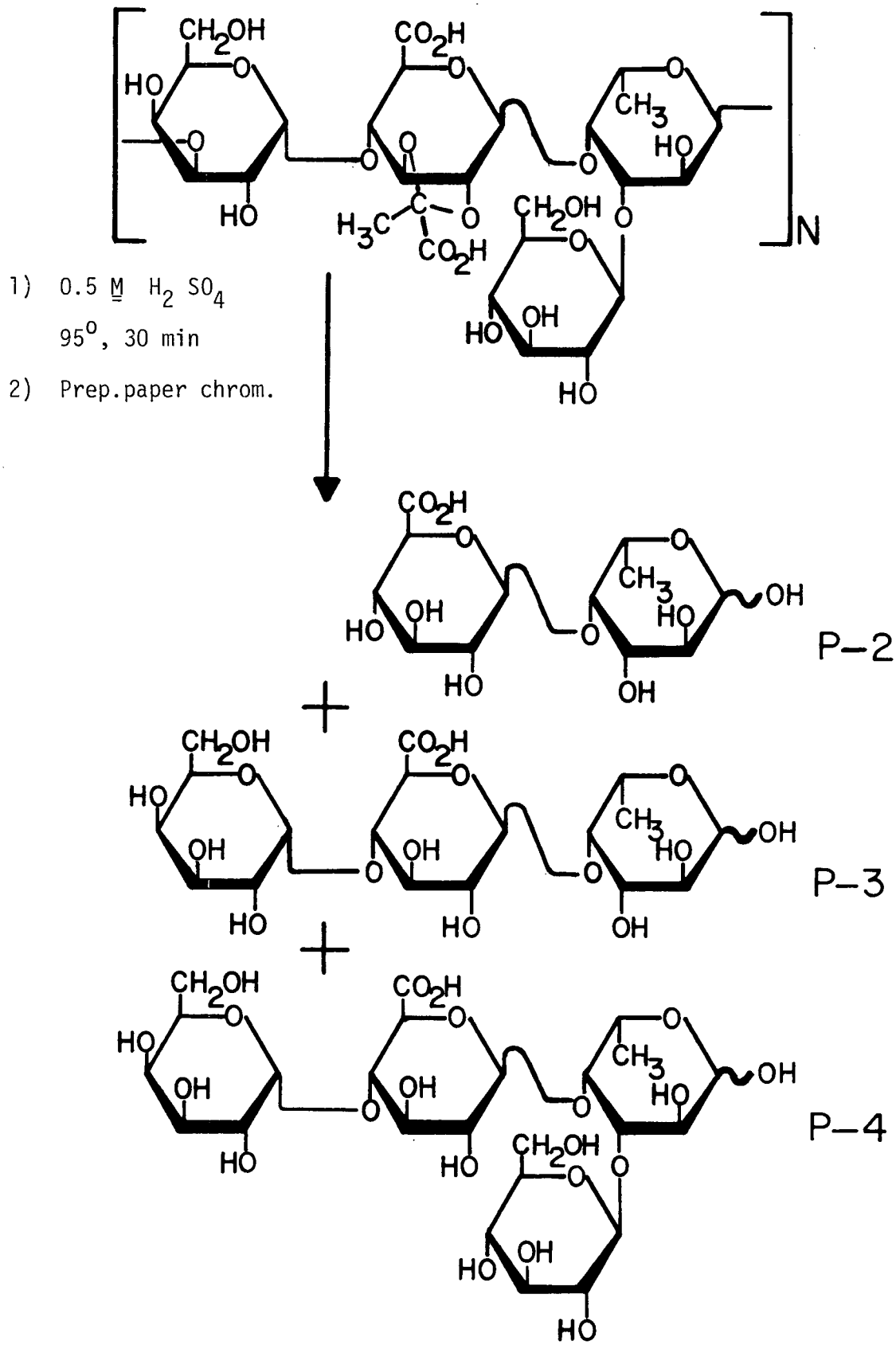
Autohydrolysis

A sample of K58 polysaccharide in the free-acid form was autohydrolyzed at 95° for 3 h and then dialyzed. ^{1}H n.m.r. spectroscopy of the product (5) showed the absence of 1-carboxyethylidene. Methylation of this material followed by reduction of the uronic ester and derivatization for g.l.c. - m.s. gave the results in Table 8 indicating that the 1-carboxyethylidene group is attached to O-2 and O-3 of the glucuronic acid residue.

Partial Hydrolysis

A sample of K58 polysaccharide was hydrolyzed with 0.5M H_2SO_4 at 95° for 30 min yielding a mixture of oligosaccharides. Preparative paper chromatography gave an aldobiouronic acid (1) an aldotriouronic acid (2) and an aldotetraouronic acid (3). ^{1}H and ^{13}C spectroscopic data (see Table 7) indicated that the glucuronic acid is β-linked, and the glucose is α-linked and confirmed that the side chain galactose is α-linked. Comparison of spectral data of (2) and (4) indicated that the fucose is α-linked (see Scheme 11).

Location of the O-acetyl group. --- The ^{1}H n.m.r. spectrum of the native polysaccharide showed a sharp acetate peak at δ 2.17 suggesting that the group appears in a discreet position in each repeating unit. In order to locate the O-acetyl group K58 was treated with methyl vinyl ether in the presence of an acid catalyst, and the product was then subjected to methylation analysis [18]. However complete

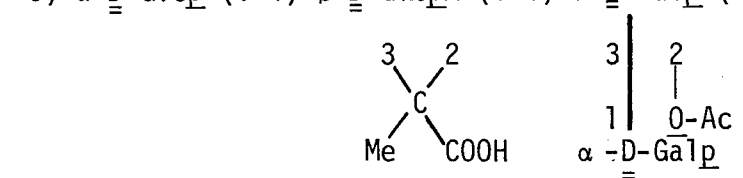


Scheme 11 Partial Hydrolysis of Klebsiella K58

blocking of all OH groups was not realized, but comparison of data from analysis of the native polysaccharide and from a similar analysis of a deacetylated sample indicated that the O-acetyl group was linked to O-2 of fucose.

III. 2.3. Conclusion

It thus follows that Klebsiella K58 capsular polysaccharide has the following structure.



A uronic acid residue bearing a 1-carboxyethylidene group has only previously been found, in this series, in the polysaccharide from Klebsiella K1[277]. (see note P. 102).

III. 2. 4. Experimental

General methods. --- Concentrations were carried out under diminished pressure at bath temperatures not exceeding 40°. The equipment for m.s., n.m.r. spectroscopy, g.l.c., and g.l.c. - m.s. was the same as that used in the investigation of Klebsiella K12 polysaccharide (see Sec. III.1) Paper electrophoresis was performed on a Savant high voltage (5 KV) system (model LT - 48A) with kerosene as coolant. The buffer used contained pyridine - acetic acid - water (5:2:743, v/v) pH 5.3. Strips of Whatman No. 1 paper (77 cm x 20 cm) were used for all runs, with application of 25-50 mA

for 1½ h. For descending paper chromatography the following solvent system (v/v) were used: (1) freshly prepared 2:1:1 1-butanol - acetic acid -- water, (2) 8:2:1 ethyl acetate - pyridine -- water, and (3) 18:3:1:4 ethyl acetate - acetic acid - formic acid - water. Sugars and oligosaccharides were detected with an alkaline silver nitrate reagent [21]. Analytical g.l.c. separations were performed in stainless-steel columns (1.8 x 3 mm) with a carrier-gas flow-rate of 20 mL/min. Columns used were (A) 3% of OV-225 on Gas Chrom Q (100 - 120 mesh); (B) 5% of ECNSS-M on the same support; and (C) 3% of SP-2340 on Supelcoport (100-120 mesh). Analogous columns (1.8 m x 6.3 mm) were used for preparative g.l.c. separations.

Preparation and properties. --- A culture of Klebsiella K58 (636/52) was obtained from Dr. I. Ørskov (Copenhagen). The polysaccharide was isolated as previously described, (see Sec. II.1) and showed $[\alpha]_D$ + 19.0° (c 1, water).

Analysis of constituent sugars. --- Methanolysis of a sample (20 mg) of K58 polysaccharide with 3% methanolic hydrogen chloride and subsequent reduction with sodium borohydride in anhydrous methanol reduced the uronic ester. Hydrolysis with 2M trifluoroacetic acid overnight at 95°, followed by reduction ($NaBH_4$), and acetylation, gave galactitol hexaacetate, glucitol hexaacetate, and fucitol pentaacetate in the ratio 1:2:1 (column C, programmed at 180° for 8 min and then 4°/min to 240°). Circular dichroism (c.d.) of the latter two components isolated by preparative g.l.c., showed

positive and negative c.d. curves respectively, confirming that glucose has the D configuration and fucose the L configuration. Galactose was shown to be of the D configuration by the positive action of D -Galactostat (Worthington Biochemical Company) on the hydrolysis product of the polysaccharide.

Methylation analyses. --- Methylation of K58 polysaccharide under the Hakomori [247] conditions, followed by a Purdie[242] treatment yielded a product that showed no hydroxyl absorption in it's i.r. spectrum. This material was reduced overnight with sodium borohydride in 1:1 (v/v) oxolane (THF) and ethanol. A portion of the product was hydrolyzed with trifluoroacetic acid (2M) for 16 h at 95°, and the mixture was reduced with sodium borohydride and then acetylated. G.l.c. - m.s. gave the results shown in Table 8.

Another portion of the reduced, uronic ester material was re-methylated under the Purdie conditions for 2 days, and derivatized for g.l.c. - m.s. giving the compounds shown in Table 8.

A portion (10 mg) of the re-methylated material was hydrolyzed with 90% formic acid for 30 min at 95° to remove the 1-carboxyethylidene group. Methylation under the Purdie conditions for 2 days, and derivatization for g.l.c. - m.s. gave the results shown in Table 8.

Uronic acid degradation[267] --- A solution of carefully dried, methylated polysaccharide (100 mg) and p-toluenesulfonic acid (a trace) in 19:1

dimethyl sulfoxide and 2,2-dimethoxypropane (20 mL) was prepared in a serum vial which was sealed with a rubber cap. The vial was flushed with dry nitrogen, and the solution was stirred for 3 h. Sodium methylsulphinylmethanide (2M) in methyl sulfoxide (10 mL) was then added with the aid of a syringe, and the solution was stirred overnight at room temperature. After cooling to 10^{o}, ethyl iodide (3 mL) was added slowly, using a syringe [273].

Following the addition of water, the ethylated, degraded product was isolated by partition between chloroform and the aqueous solution. Hydrolysis of the isolated product was performed with 2M trifluoroacetic acid; g.l.c. - m.s. analysis of the alditol acetate derivatives yielded peaks corresponding to 4-O-ethyl-2, O-methylfucose, 2,3,4,6-tetra-O-methylgalactose and 2,4,6-tri-O-methylglucose (see Table 8).

Periodate Oxidation. --- *Klebsiella* K58 capsular polysaccharide (200 mg) was dissolved in water (25 mL), to which a solution (25 mL) of 0.1M sodium metaperiodate was then added. The solution was stirred in the dark at 3^{o} and periodate consumption was monitored (Fleury-Lange method)[255]; after 10 h consumption had reached 1.8 molecules per repeating unit. Ethylene glycol (10 mL) was then added, and, after stirring for a further 30 minutes the mixture was dialyzed overnight against running tap-water, and the product was reduced with sodium borohydride. The polyol was isolated by dialysis and lyophilization. Smith hydrolysis (0.5M TFA overnight

at room temperature) gave a polymeric product. A portion (5 mg) of this material was hydrolyzed (2M TFA overnight at 95°); paper chromatography (solvent 2) then showed the presence of glucose and fucose, and the absence of galactose. Analysis of the constituent sugars of the Smith-degradation product was performed as for the native polysaccharide. G.l.c. analysis showed the presence of glucose and fucose in the ratio 2:1. ^{1}H and ^{13}C n.m.r. spectral data are shown in Table 7.

Methylation analysis of a portion (20 mg) of the Smith-degradation product, as for the native polysaccharide, gave the results shown in Table 8.

Autohydrolysis --- A sample of Klebsiella K58 polysaccharide was exchanged to the free-acid form with Amberlite IR-120 (H^+) ion-exchange resin, and the solution was lyophilized. A portion of this material (100 mg) was dissolved in 5 mL H_2O (pH 3.0) and was introduced to a sealed length of standard cellulose dialysis tubing. This was autohydrolyzed in 100 ml H_2O at 95° for 3 h. Loss of the 1-carboxyethylidene group was shown to be complete by ^{1}H.n.m.r. spectroscopy. Methylation analysis of the product, as described earlier gave the results shown in Table 8.

Partial Hydrolysis --- A sample (300 mg) of the native polysaccharide was hydrolyzed with 0.5M H_2SO_4 for 30 min at 95°. The products were separated by preparative paper chromatography (solvent 3). Compound

1 (35 mg) with R_{Glc} 0.63 and $[\alpha]_D$-20.0° (c 1, water) was shown to be the aldobiouronic acid by ^1H and ^{13}C n.m.r. spectroscopy. Compound 2 (30 mg) with R_{Glc} 0.32 and $[\alpha]_D$ + 16.0° (C 1, water) and compound 3 (10 mg) with R_{Glc} 0.12 and $[\alpha]_D$ + 38.0° (c 0.5, water) were similarly shown to be the aldotriouronic and aldotetraouronic acids respectively (see Table 7).

Deacetylation

Deacetylation was achieved by treatment of a solution of the native polysaccharide (200 mg) in water (25 mL) with excess of sodium borohydride for 3h, at room temperature, with stirring. Dialysis and lyophilization yielded a product with no acetyl peak at δ2.17 in the p.m.r. spectrum.

Location of O-acetyl group

Since complete blocking of the polysaccharide with methyl vinly ether proved difficult, giving small amounts of methylated sugars other than 2-OMe fucose, on g.l.c.-m.s. analysis, the O-acetyl location procedure of de Belder and Norman[275] was performed on both the native polysaccharide, and a deacetylated sample. Reaction conditions were identical in both cases.

The entire reaction was carried out in a sealed vial, flushed with nitrogen. A solution of carefully dried polysaccharide (50 mg) and p- toluenesulfonic acid (20 mg) in dimethylsulfoxide (20 mL) was prepared in a serum vial, which was sealed and flushed with nitrogen.

The solution was frozen (-60^{o}) and methyl vinyl ether (*ca* 3 mL) was introduced from a gas bottle to the reaction vessel *via*. a hypodermic syringe. The solution was brought to room temperature and stirred for 3 hr. Two further portions of methyl vinyl ether were similarly introduced, after which time the reaction mixture had a red/yellow colour. Methylsulfinyl anion in dimethylsulfoxide (3 mL) was introduced and the reaction mixture stirred for a further 30 min. Methyl iodide (2 mL) was added to the cooled mixture which was then stirred for 1h. Dialysis (overnight) and lyophilization gave a mixture of derivatized polysaccharide and polymeric methyl vinyl ether. Pure polysaccharide was eluted froma column of Sephadex LH-20 with methanol.

Since the *O*-acetyl substituent could not be on the glucuronic acid this functionality was not reduced. The polysaccharide was hydrolyzed with trifluoroacetic acid (2M), for 16h at 95^{o}, and the mixture was reduced with sodium borohydride, and then acetylated. The ratio of 2-OMe fucose to fucose of 19 : 1 for the native polysaccharide and 1 : 1 for the deacetylated polysaccharide (g.l.c. - m.s. analysis) indicates that the *O*-acetyl group is attached to O - 2 of fucose.

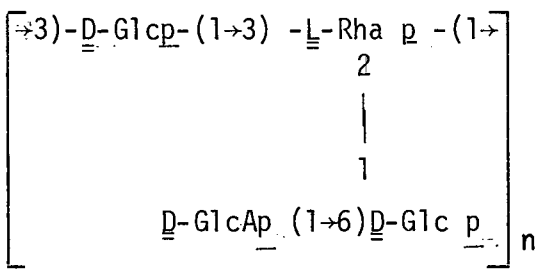
Note added in proof.

Dr. J.M. Fournier (Institut Pasteur, Paris) has shown that, although *Klebsiella* K58 capsular polysaccharide is not virulent for mice, immunization with the polysaccharide does provide protection

against infection with the virulent Klebsiella K1 capsular polysaccharide.[277] This is in agreement with the similarities in the structures of the two polysaccharides (see App.I).

III. 3 CONFIRMATON OF THE STRUCTURE OF KLEBSIELLA K23 CAPSULAR POLYSACCHARIDE.

Structural investigation[278] of Klebsiella K23 polysaccharide by the techniques of methylation analysis and Smith degradation indicated that the structure consisted of a tetrasaccharide repeating unit with a two-sugar side-chain, as shown:



Comparison of ^{1}H n.m.r. spectra of the native polysaccharide and the polysaccharide obtained on Smith degradation [253] (the two side-chain sugars were removed) showed that the glucosyl residue in the backbone was β-linked and that the rhamnosyl residue was α-linked. The anomeric nature of the side-chain residues could not, however, be demonstrated conconclusively.

To obtain this information the methylated polysaccharide was subjected to a β-elimination reaction [267], whereby, only the terminal glucuronic acid residue was degraded. Comparison of the ^{1}H n.m.r. spectra of the methylated polysaccharide and this product demonstrated the absence of a signal in the anomeric region at δ 4.34, corresponding to loss of a β-linked glucuronic acid residue (see Table 9). By deduction the glucosyl residue in the side-chain

TABLE 9

P.M.R. DATA FOR Klebsiella K23 CARSULAR POLYSACCHARIDES

Compound	δ[a]	Integral	Assignment
Methylated,	5.21	1	α-Rha[b]
Native	5.08	1	α-Glc
Polysaccharide.	4.52	1	β-Glc
	4.34	1	β-GlcA
	1.31	3	CH_3 of Rha.
Methylated/ethylated,	5.24	1	α-Rha
degraded	5.10	1	α-Glc
polysaccharide.	4.55	1	β-Glc
	1.31	3	CH_3 of Rha
	1.21	3	CH_3 of ethyl.

[a] Chemical shift relative to internal acetone; δ 2.23 downfield from sodium 4,4-dimethyl -4-silapentane-1-sulfonate (D.S.S.). Spectra were run in $CDCl_3$ at 270MHz and ambient temperature.

[b] α-Rha = proton on C-1 of α-linked L-Rha residue, etc.

TABLE 10

METHYLATION ANALYSIS OF ORIGINAL AND BASE DEGRADED Klebsiella K23 CAPSULAR POLYSACCHARIDE.

Methylated sugars[a] (as alditol acetates)	T[b]	I[c] Mole %[e]	II[d] Mole %[e]
4-Rha	0.91	26.0	34.1
2,3,4,6-Glc[f]	0.72	--	32.4
2,3,4-Glc	1.13	48.1	--
2,4,6-Glc	1.00	25.9	33.5

[a] 4-Rha = 1,2,3,5-tetra-O-acetyl-4-O-methylrhamnitol etc.

[b] Retention time relative to alditol acetate of 2,4,6-tri-O-methyl-D-glucose on OV-225.

[c] I, original polysaccharide, methylated and uronic ester reduced.

[d] II, degraded polymer obtained after β-elimination.

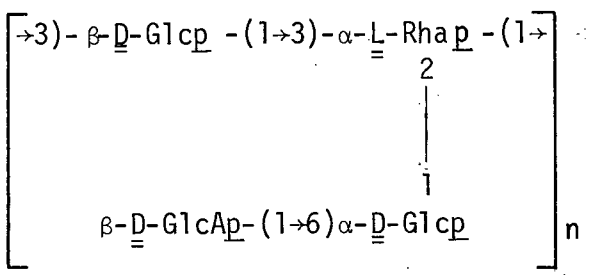
[e] Values are corrected by use of the effective carbon response factors given by Albersheim et al.

[f] 1,5-Di-O-acetyl-6-O-ethyl-2,3,4-tri-O-methylglucitol

is α-linked.

The product was directly ethylated [106], thus labelling the position of attachment of the glucuronic acid residue. Hydrolysis, and g.l.c. - m.s. [103,121] analysis of the ethylated product showed that only the glucuronic acid residue had been removed and verified that it was attached to O-6 of the side-chain glucosyl residue (see Table 10).

It thus follows that the Klebsiella K23 polysaccharide has the structure



The experimental conditions were essentially the same as those used in the investigation of the structure of Klebsiella K12 capsular polysaccharide (see Section III, 1).

III. 4 ^{1}H AND ^{13}C SPECTRAL INVESTIGATION OF Klebsiella K70 CAPSULAR POLYSACCHARIDE

The structure of the capsular polysaccharide of Klebsiella K70 has been shown [279] to consist of a linear hexasaccharide repeating unit having a 1-carboxyethylidene attached to a 2-linked α-L-rhamnosyl residue in every second repeating unit as shown:

$$\left[-{}^{2}\text{Rhap}\underset{\alpha}{1\,4}\text{GlcAp}\underset{\beta}{1\,4}\text{Rhap}\underset{\alpha}{1\,2}\text{Rhap}\;\underset{\alpha}{1\,2}\text{Glcp}\;\underset{\alpha}{1\,3}\;\text{Galp}\;\underset{\beta}{1}\right]_{n}$$

A B C D E F

In that investigation ^{1}H and ^{13}C n.m.r. spectroscopy were used, and some assignments were made. However, the complete assignment of all the signals in the anomeric region proved difficult, since four α-linked residues were present, three of which were due to rhamnosyl residues.

The polysaccharide has since been degraded by Merrifield [298] using bacteriophage, to give an oligosaccharide corresponding to two repeating units. Another specific degradation by Mort [323] whereby the native polysaccharide was cleaved at the uronic acid residue, using lithium in ethylamine, with concomitant loss of that residue, produced a pentasaccharide. ^{1}H and ^{13}C spectral investigations of the penta - and hexasaccharide now allow a more complete assignment of all the signals in the anomeric region of the native polysaccharide.

TABLE 11

N.M.R. DATA FOR Klebsiella K70 POLYSACCHARIDE AND OLIGOSACCHARIDES ISOLATED

Compound[a]	^{1}H-n.m.r. Data				^{13}C n.m.r. Data			
	δ[b]	$J_{1,2}$(Hz)	Integral	Assignment[c]	p.p.m.[d]	Integral	Assignment[e]	
[-A-B-C-D-E-F-]$_n$	5.22	s[f]	1	α Rha C	105.7	1	β Gal	F
	5.10	s	2	α Rha A+D	103.8	1	β GlcA	B
	4.97	s	1	α Glc E	102.9	1	α Rha	C
(1)	4.77	7	1	β Glc E	101.7	1	α Rha	A
	4.55	7	1	β Gal F	100.9	1	α Rha	D
	1.59	s	1.5	CH_3 of acetal	95.7	1	α Glc	E
	1.30	6[g]	9	CH_3 of Rha	62.2	1	C-6 Gal	
					61.3	1	C-6 Glc	
					17.5	3	C-6 Rha	
A-B-C-D-E-F-A^1-B^1-C^1-D^1-E^1-F^1-OH	5.28	2	0.7	α Gal OH.F^1	105.64	1	β Gal	F
	5.23	2	1.8	α Rha C + C^1	103.88	2	β GlcA	B + B^1
(2)	5.10	s	4	α Rha A+A^1+D+D^1	102.79	2	α Rha	C + C^1
	4.98	s	2	α Glc E + E	101.7	1	α Rha	A
	4.80	7	2	β GlcA B+B^1	100.98	2	α Rha	D + D^1
	4.58	4	0.3	β Gal~OH F^1	99.7	1	α Rha	A^1
	4.40	7	1	β Gal F	98.61	1	α Glc	E^1
	1.30	6[g]	9	CH_3 of Rha	97.28	0.6	β Gal~OH	F^1
					95.77	1	α Glc	E
					94.80	0.4	α Gal~OH	F^1
					61.78		C-6 Gal	
					61.36		C-6 Glc	
					17.6		C-6 Rha	

TABLE 11 Contd.

C-D-E-F-A-OH	5.32	2	0.8	α Rha~OH.	A	105.5	1	β-Gal	F
	5.23	2	1	α Rha	C	104.7	0.2[h]	β GlcA	B
(3)	5.10	3	1	α Rha	D	103.1	0.8	α Rha	C
	4.98	2	1	α Glc	E	101.0	1	α Rha	D
	4.80	s	0.2	β Rha~OH	A	95.7	1	α Glc	E
	4.55[(i)]	4	0.4	β Gal	F	93.9	0.6	α Rha~OH	A
	4.58	4				93.6	0.4	β Rha~OH	A
	1.30	6[g]	9	CH_3 of Rha		61.7		C-6 Gal	
						61.2		C-6 Glc	
						17.5		C-6 of Rha	

[a] For structures of (1), (2), (3) see text.

[b] Chemical shift relative to internal acetone; δ2.23 downfield from sodium 4,4-dimethyl-4-silapentane - 1-sulfonate (D.S.S.)

[c] α-Rha=proton on C-1 of α-linked L - Rha residue etc.

[d] Chemical shift in p.p.m., downfield from Me_4Si, relative to internal acetone; 31.07 p.p.m. from D.S.S.

[e] As for [c] but for anomeric ^{13}C nuclei.

[f] S-singlet

[g] Value for $J_{5,6}$. overlapping doublets centred at δ1.30.

[h] A small amount of β-GlcA was not eliminated.

[i] The chemical shift of this proton is affected by the α,β equilibrium of the reducing Rha residue.

Acknowledgments

I wish to thank Dr. I. Ørskov (Copenhagen) for the Klebsiella cultures used, Dr. A.A. Grey (Toronto) for recording the 220 MHz ^{1}H n.m.r. spectra at high temperature, Dr. M. Vignon (Grenoble) for recording ^{13}C spectra at high temperature, and Dr. A. Mort (Charles F. Kettering Research Laboratory, Ohio) for a sample of degraded K70.

The isolation and initial investigation of Klebsiella K12 polysaccharide were performed by Christiane Martel.

IV. BACTERIOPHAGE DEGRADATION OF *Klebsiella* CAPSULAR POLYSACCHARIDES K21, K12 and K41.

IV. 1. Introduction

The field of medicinal microbiology became well - established in the period between 1880 and 1900 with the identification and characterization of many of the causative agents of both human and animal diseases. In 1892, Iwanowski and Beijerinck while independently studying the tobacco mosaic disease, and Pasteur while carrying out studies on rabies, recognised the causative agents to be "filterable substances", which the latter termed "viruses." [280]

Some years later, in 1915 and 1917 virus infections in bacteria were also described. Twort and d'Herelle demonstrated independantly that cultures of bacterial cells could be infected with and destroyed by filterable agents that were subsequently termed "bacteriophages". It was not until the electron microscope was developed that the morphological character of viruses was elucidated. Today, bacteriophages (phages, designated ϕ) are the best characterized and studied group of viruses, since their propagation and manipulation has proven technically much easier than equivalent studies on other types of viruses. [281]

Phages, which are quite different from other virus types, in that they tend to be structurally more complex, are grouped according to the morphological classification of Bradley [282]. (see Fig. 11). For type A (see Fig. 12) the head, which itself is composed of repeating identical protein monomers, has basically

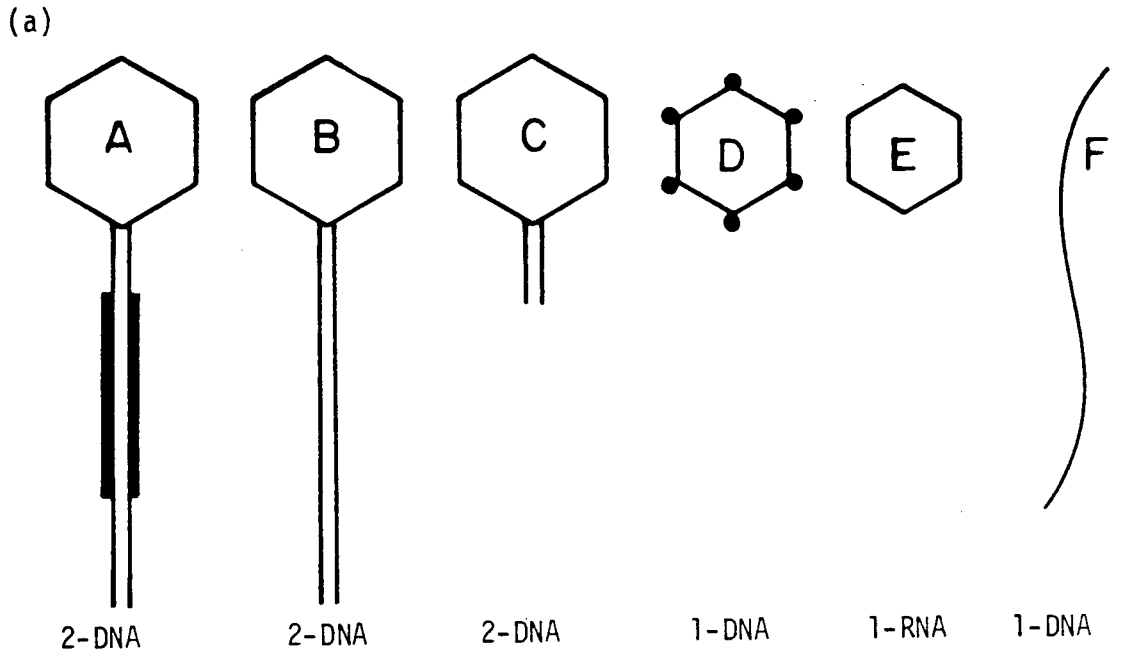


Figure 11 Morphological grouping of phages according to Bradley[282].

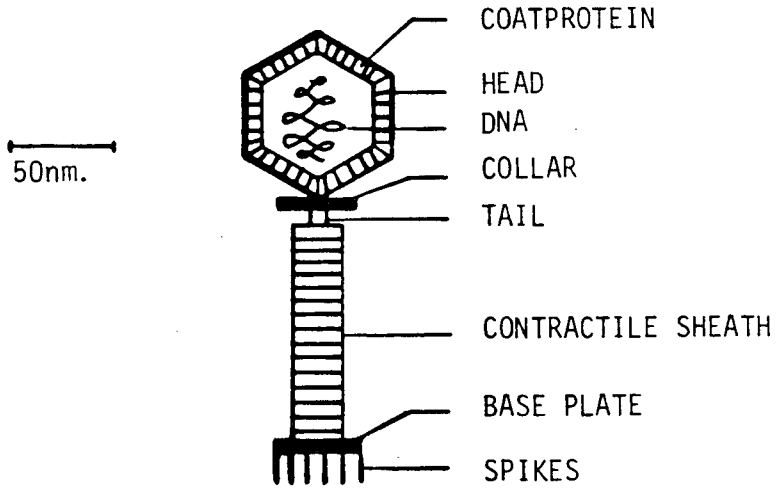


Figure 12 Schematic representation of a Type-A phage particle.

an icosahedral structure and contains doubly stranded DNA. The phage tail consists of a tube which is made up of helically arranged protein molecules, encased by a contractile sheath, also composed of protein monomers. The plate contains small pins to which are attached the tail spikes. [283a]

Type B is similar to type A, but without the contractile sheath. Type C contains a baseplate and spikes, but no tail. Type D consists only of a head, with a capsomere on each apex, type E consists of a head, without capsomeres, and type F is filamentous.

Bacteriophage which lyse encapsulated bacteria often form plaques, surrounded by large haloes that continue to spread after growth has ceased (see App. IV). Within the haloes the bacteria have lost their capsules. It has been long known that,[283b,c] generally, the formation of these haloes is due to the production of enzymes during phage infection. These enzymes diffuse from the plaque and catalyze the hydrolysis of the bacterial capsules. A wide variety of enzymic activities, catalyzing different degradation reactions of host surface, polysaccharides may be associated with bacterial virus particles.[284] So far esterases (saponification of O-acetyl substituents), glycanases, and lyases have been recognized [285a]. It has been shown that the depolymerases responsible for the formation of haloes are in fact free phage spikes [285b,c,d], produced in addition to whole virus, and that the purified spikes exert the same glycosidic activity[286]. The enzymic

activity has been found to be associated with a subunit (m.w. 62,500) of the viral spike (m.w. 155,000).

When a phage particle infects a susceptible host it causes that cell to lyse, with the concomitant release of a characteristic number of newly formed phage particles. The phases of the cycle include the following:

(i) absorption 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 phage protein, and

(iv) phage maturation and release.

The first step occurs by attachment of the phage tail to receptor sites on the bacterial cell. There is considerable evidence that reciprocal charges on the phage tail and on the receptor sites of cells are involved in the formation of electrostatic bonds during attachment.

Phage are relatively easy to isolate from almost any bacterial environment, for example, sewage [287]. Those active on exopolysaccharide producing bacterial strains are generally exopolysaccharide specific; non - capsulate or non - slime producing mutants are resistant to the phages. One common feature of all phage, active on exopolysaccharides, is that the baseplates, as seen in the electron microscope, are provided with spikes, and that no tail fibres are seen. The spikes appear as hollow tubes

(12.5 nm in Klebsiella phage 11). [286] Stirm and Rieger - Hug, [288] employing seventy four serologically different Klebsiella test strains, tested the host range of fifty five Klebsiella bacteriophage. The viral depolymerases proved to be very specific (33 not cross-reacting, 18 cross-reacting with one, 2 with two, 1 with three and 1 with four heterologous polysaccharides. ϕ 12 cross-reacts with K41,[34] which is in agreement with the structure proposed for K12 capsular polysaccharide.

Depolymerization of a polysaccharide using bacteriophage yields products corresponding to a single repeating unit of the polysaccharide, and multiples thereof, with labile O-acetyl[289a] and 1-carboxyethylidene (pyruvate) groups intact. [289b] These may then be used for (a) the preparation of synthetic antigens, [77](b) detailed examination by nuclear magnetic resonance spectroscopy, and (c) the study of conformations in solution. [290] The degradation of Klebsiella K21 capsular polysaccharide using purified ϕ 21 particles, and the degradation of Klebsiella K12 and K41 using a crude ϕ 12 suspension are presented here, and results compared in terms of efficiency of depolymerization and yields of oligosaccharides.

IV. 2. RESULTS

IV. 2. 1. Isolation and purification

Both ϕ 21 and ϕ 12 were isolated from sewage, and stock suspensions in broth were obtained by the confluent lysis method. The bacteriophage were propogated on their host strains, Klebsiella K21

and 12 respectively, to a volume of ∿ 1.5L. (see App.IV) ϕ 21 was purified by precipitation with poly (ethylene glycol) 6000[291], followed by isopycnic centrifugation, and was shown by electron microscopy (see Fig. 15) to belong to Bradley Type B.[282]

IV. 2.2. Conditions of depolymerization

The purified capsular polysaccharide from Klebsiella K21 [292] was dissolved in buffered saline, and the depolymerization was followed viscometrically and by assay of the reducing power [293] (see Fig. 13) which became constant after 24 h.

The depolymerizations of Klebsiella K12 and K41 capsular polysaccharides were carried out in separate, crude, broth suspensions of ϕ 12 particles and followed viscometrically. Although the viscosity of both solutions decreased dramatically during the first 3 h, the reactions were allowed to continue for 48 h.

IV. 2. 3. Purification and analyses of products of depolymerization of K21.

The lyophilized depolymerization mixture was desalted on a column of Sephadex G10. The carbohydrate fraction [95] was lyophilized, redissolved in Tris HCl buffer, and the solution added to a column of DEAE - Sephadex A25. The elution pattern is shown in Fig. 14, where P1 represents the single repeating unit of the polysaccharide, P2 the double repeating unit, and P3 polymeric material. P1 and P2 were separately desalted (Sephadex G10), and examined by ^{1}H n.m.r.

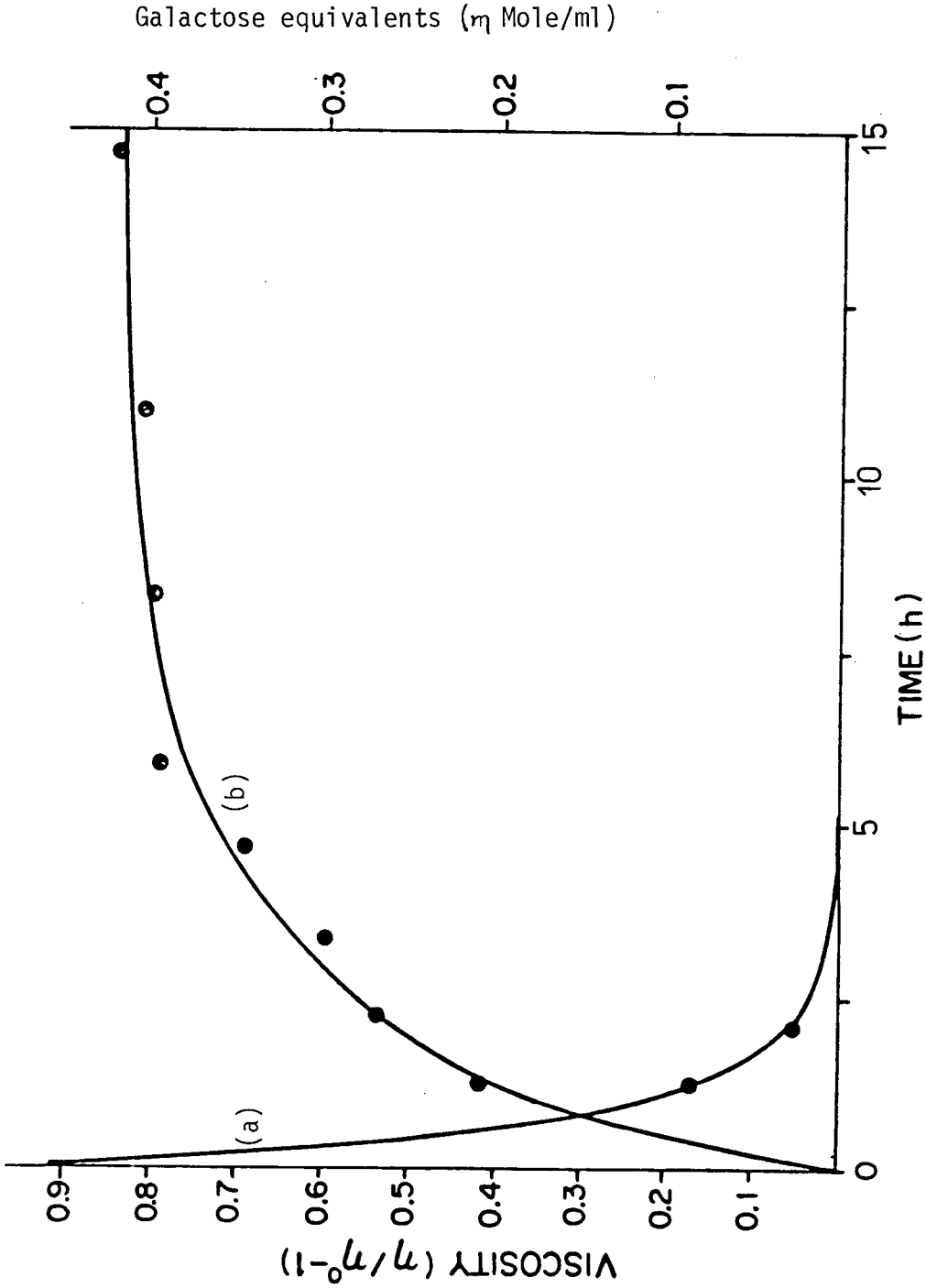


Figure 13 (a) Decrease in viscosity, and (b) increase in reducing power of K21 polysaccharide solution, on incubation with bacteriophage.

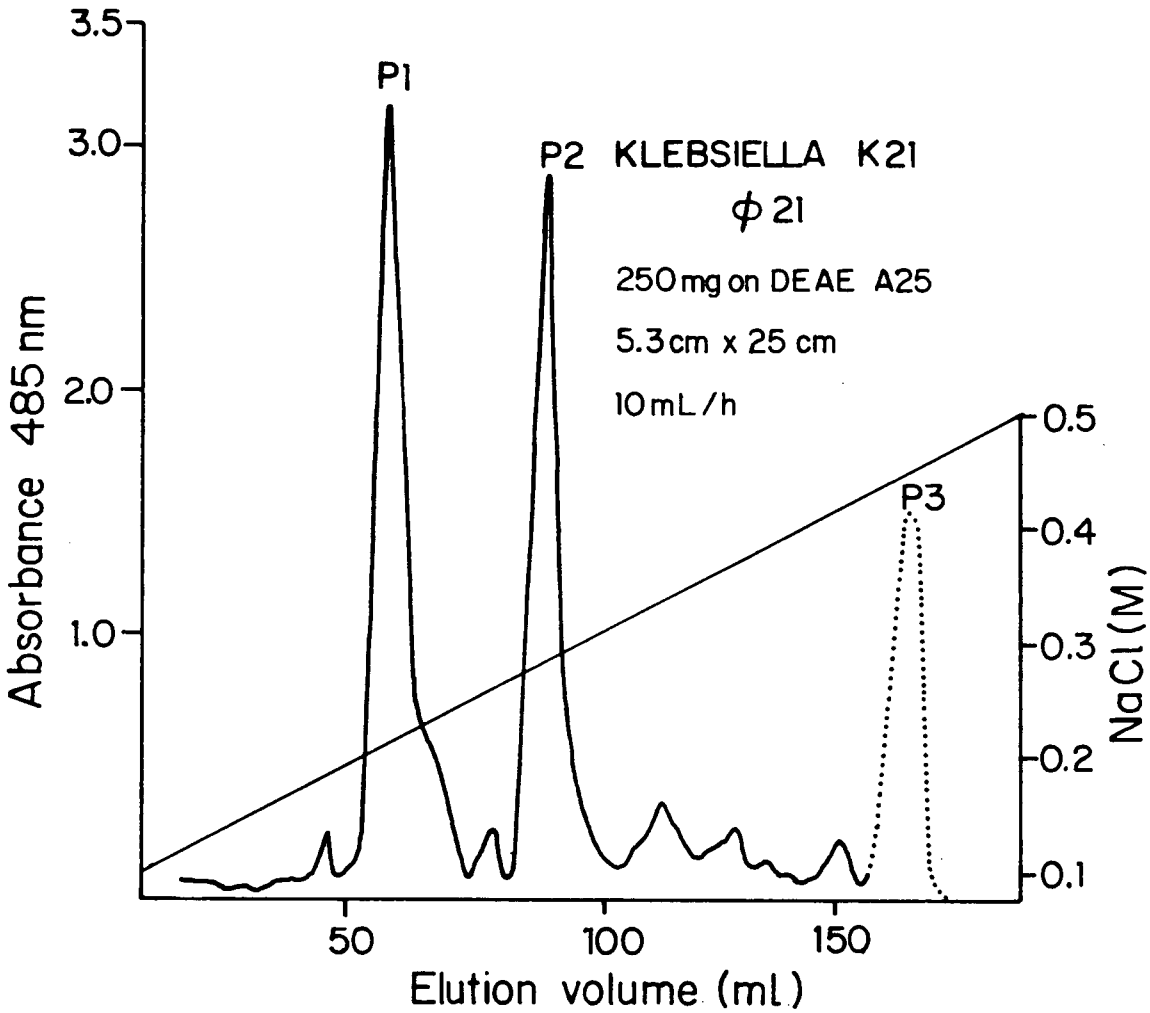


Figure 14 Separation of products of bacteriophage degradation of Klebsiella K21

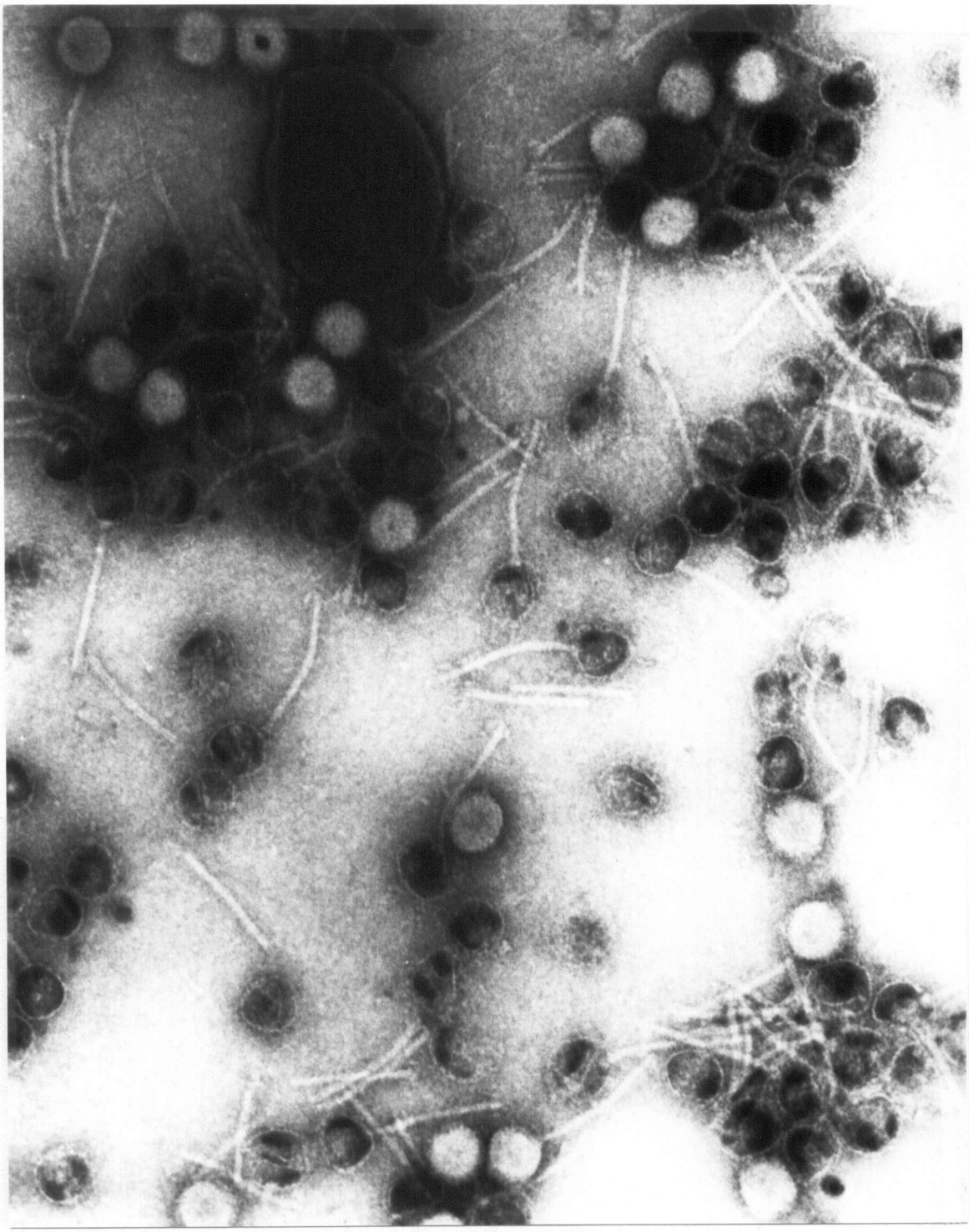


Figure 15 Electron Micrograph of purified K21 bacteriophage, negatively stained. X=122,500 Courtesy of Dr. H. Chanzy C.E.R.M.A.V. (Grenoble). Bradley [282] Type B.

spectroscopy (see Table 12). Several spurious peaks were observed at low field, but, after passage through a column of Amberlite IR-120 (H^+) resin gave the spectra shown in App. III.

^1H and ^{13}C spectra demonstrate that P1 is a hexasaccharide corresponding to one repeating-unit, with galactose as the reducing residue, and P2 is composed of two repeating units. The two oligosaccharides were analyzed by gas-liquid chromatography, using the method of Morrison, [102, 294-297] whereby the ratio of acetylated aldononitrile to acetylated alditol is determined. The results(see Fig. 7, Table 13) confirmed that P1 is a hexasaccharide and that P2 is the dimer. The mobility of P1 in paper chromatography was R_{Glc} 0.045, and in paper electrophoresis, P1 and P2 had R_{GlcA} 0.75 and 0.85, respectively.

IV. 2. 4. Purification and analyses of the products of depolymerization of K12 and K41.

The reaction mixtures were dialyzed (M.W. cutoff = 3,500) against water, and the dialysates were lyophilized. Preparative paper chromatography yielded products free of most broth contaminants. Only baseline carbohydrate spots were observed. Further purification, by passage through a column of Amberlite IR-120 (H^+) resin gave products which were examined by n.m.r. spectroscopy.

The results shown in Table 14 show that the product of depolymerization of K12 polysaccharide with ϕ 12 is a hexasaccharide, corresponding to a single repeating unit of the polysaccharide. This

TABLE 12

N.M.R. DATA FOR *Klebsiella* K21 CAPSULAR POLYSACCHARIDE AND THE OLIGOSACCHARIDES P1 AND P2

Compound	δ[a]	^{1}H- n.m.r. data[b]	
		Integral (H)	Assignment
K21 polysaccharide[292]	5.48	1	α-GlcA
	5.30	2	α-Gal
	5.25		α-Man
	5.08	1	α-Man
	4.88	1	β-Gal
	1.55	3	acetal
Oligosaccharide P1	5.49	1	α-GlcA
	5.34	2.4	α-Gal
	5.30		α-Man
			α-Gal-OH
	5.10	1	α-Man
	4.66	0.6	β-Gal-OH
	1.52	3	acetal
Oligosaccharide P2	5.46	1	α-GlcA
	5.31	2.3	α-Gal
	5.27		α-Man
	5.06	1	α-Gal-OH
	4.87	0.5	β-Gal
	4.64	0.2	β-Gal-OH
	1.53	1.5	acetal

[a] Chemical shift relative to internal acetone; δ2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.)

[b] Chemical shifts are recorded only for anomeric protons and those of the 1-carboxyethylidene acetal.

TABLE 13

DETERMINATION OF DEGREE OF POLYMERIZATION OF P1 AND P2 (K21) AND IDENTIFICATION OF THE REDUCING SUGAR

Acetylated derivative of	Relative Retention time OV-17[a]	Moles %[b] P1	P2
Mannononitrile	0.67	2.0	2.0
Gluconononitrile	0.72	0.91[c]	0.93[c]
Galactononitrile	0.75	0.98	1.5
Galactitol	1.00	0.94	0.47

[a] 3% OV-17 on Gas Chrom Q (100-120 mesh) programmed at 180° for 4 min and then 2°/min to 220°.

[b] Values corrected using molar response factors.

[c] Due to incomplete reduction of uronic acid.

TABLE 14

P.M.R. DATA FOR *Klebsiella* K12 CAPSULAR POLYSACCHARIDE AND THE PHAGE DERIVED OLIGOSACCHARIDE.

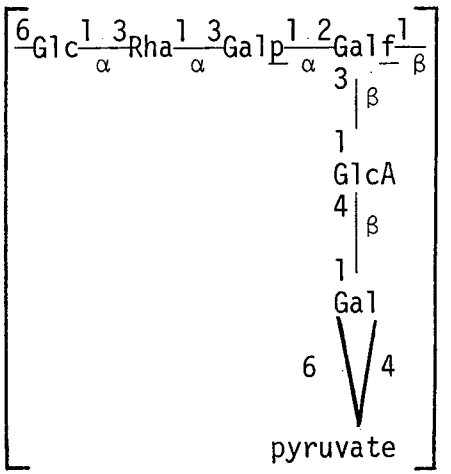
Compound[a]	^{1}H-n.m.r. data			
	δ[b]	$J_{1,2}$ (Hz)	Integral (H)	Assignment[d]
 n	5.22	s	1	α-Rha
	5.16	3	1	α-Glc
	5.13	2	2	α-Galp
	5.13	2		β-Galf
	4.66	8	1	β-GlcA
	4.48	6	1	β-Galp
	4.3-4.5	b	2	H-2, H-3 β-Galf
	1.66	s	3	CH_3 of acetal
	1.34	6	3	CH_3 of Rha

TABLE 14 (Contd..)

Compound[a]	^{1}H-n.m.r. data			
	[b]	$J_{1,2}$ (Hz)	Integral (H)	Assignment[d]
Glc(α1→3)Rha(α1→3)Galp(α1→2)Gal-OH	5.26	b	.6	α-Rha
3 | β	5.20	b	.6	α-Gal-OH
1	5.16	s	.6	α-Glc
GlcA	5.09	b	1	α-Gal
4 | β	4.61	b	1	β-GlcA
P1 1	4.52	8	.4	β-Gal-OH
Gal	4.48	6	(1)	β-Gal
6 4	1.66	b		CH_3 of acetal
pyruvate	1.34	b		CH_3 of Rha

[a] For the origin of compound P1 see text. See Appendix III for reproductions of the spectra

[b] Chemical shift relative to internal acetone; δ2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-

[c] Key: b = broad, unable to assign accurate coupling constant, s = singlet.

[d] For example, α-Gal = Proton on C-1 of α-linked D - Gal residue.

was confirmed by g.l.c. analysis (see above).

In contrast, the product of the depolymerization of K41 polysaccharide with ϕ 12, was shown to be an oligosaccharide corresponding to two repeating units of the polysaccharide. Confirmatory evidence was provided by g.l.c. analysis (see Table 15)

IV. 3. DISCUSSION

THe ultimate aim of our research group, in degrading polysaccharides using bacteriophage, is to obtain pure oligosaccharides, in as high a yield as possible. In purifying the bacteriophage, as for ϕ 21, the volume of the phage suspension is reduced by a factor of 10^2, but with a concomitant loss of $\sim$ 75% of the phage. The efficiency of depolymerization is, however, very high, giving a good yield of the single repeating unit.

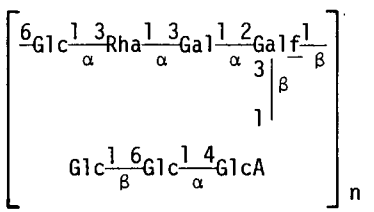
Pure oligosaccharides may also be obtained with a crude solution of bacteriophage, thereby alleviating the time consuming and expensive (CsCl) purifications using ultracentrifuges.

In the degradations of K12 and K41, the yields of the single repeating unit were low (for K41 only the double repeating unit was obtained). This can be improved upon by using a higher titre of crude phage particles. [298]

Dialysis of the products, using tubing with a low exclusion limit, is an efficient method of separating the single and double repeating units from larger material. However, if a

TABLE 15

N.M.R. DATA FOR Klebsiella K41 CAPSULAR POLYSACCHARIDE[34] AND THE PHAGE DERIVED OLIGOSACCHARIDE

Compound[a]	^{1}H-n.m.r. data			^{13}C-n.m.r. data		
	δ[b]	Integral	Assignment[c]	p.p.m.[d]	Integral	Assignment[e]
	5.48	1	α-Glc	109	1	β-Galf
	5.22	1	α-Rha	105.5	1	β-Glc
	5.17	1	α-Glc	104.8	1	β-GlcA
	5.12	2	α-Galp	104.45	1	α-Rha
	5.12		β-Galf	101.55	1	α-Gal
	4.63	1	β-GlcA	101.05	1	α-Glc
	4.52	1	β-Glc	99.5	1	α-Glc
	1.34	3	CH_3 of Rha	19.7	1	CH_3 of Rha
P2	5.48	1	α-Glc	107.2	0.5	β-Galf
	5.24	1.3	α-Rha	103.44	1	β-Glc
n = 2	5.20		α-Gal~OH	102.64	1	β-GlcA
	5.14		α-Glc	101.7	0.5(?)	α-Rha
	5.14	2.5	α-Galp	100.24	1	α-Gal
	5.14		β-Galf	100.10	1	α-Glc
	4.63	1	β-GlcA	99.96	1	α-Glc
	4.52	1.2	β-Glc	97.2	0.2(?)	β-Gal~OH
	4.52		β-Gal~OH	91.10	0.3(?)	α-Gal~OH
	1.34	3	CH_3 of Rha.	17.65		CH_3 of Rha.

[a] For the origin of compound P2, see text. See Appendix III for reproduction of the spectra.

[b] Chemical shift relative to internal acetone; δ2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.)

[c] For example, α-Gal = Proton on C-1 of α-linked D - Gal residue.

[d] Chemical shift in p.p.m. downfield from Me_4Si, relative to internal acetone; 31.07 p.p.m. downfield from D.S.S.

[e] As for [c], but for anomeric ^{13}C nuclei.

crude phage solution in both is employed, then low M.W. material from the broth contaminates the oligosaccharides.

The alternative then, is first to reduce the volume of phage suspension, (by lyophilization, or by rotary evaporation at $< 40^{o}$)[298] and dialysis of this solution to remove broth contaminants. Low M.W. products may then be easily isolated by dialysis, and separated, either by preparative paper chromatography, or by gel permeation chromatography.

IV. 4. EXPERIMENTAL

For all phage work the standard procedures given by Adams[280] were used. ϕ 12 and ϕ 21 were originally isolated from Freiburg sewage, and were propogated on their respective host strains by tube and bottle lyses (see App. IV).

Purification

ϕ 21 lysate, which had a titre of 7.5×10^{11} PFU/mL (1.5L), was centrifuged at 5,000 g (20 min) and made 0.5 M in NaCl. 10% w/v of poly (ethylene glycol) 6000 was added slowly to the supernatant and after storage at 4^{o} for 48 h the phage particles were sedimented at 20,000 (g) (30 min). The supernatant was shown to contain 0.15% of phage.

The pellets were taken up in PBS (20 mL), using a syringe (22g) and the solution was centrifuged at 5,000g (20 min). The supernatant was then centrifuged at 100,000 g for 4 h and the pellets were taken up in a minimum of PBS (4 mL). The concentrated virus-suspension was further purified by isopycnic centrifugation in linear CsCl gradients as follows:

32.3g of CsCl was dissolved in 32.1 mL 0.1% of Tris HCl (ρ = 1.63 g/mL) and 6.5g of CsCl in 31.1 mL (ρ = 1.13 g/mL). The two solutions were mixed using a three channel peristaltic pump into two disposable cellulose nitrate tubes (capacity 38.5 mL) for a Beckman SW 27 rotor. The phage suspension in PBS (2x2mL) was loaded carefully onto each gradient, and was centrifuged at 86,000 g for 1.5 h. An opalescent band (ρ = 1.445 g/mL) was then clearly visible and was withdrawn using a syringe with new needle, and dialyzed against PBS to remove CsCl.

Conditions of depolymerization of K21

Purified capsular polysaccharide (250 mg) from Klebsiella K21 was dissolved in PBS (100 mL) and to this solution was added a total of 2.5×10^{12} plaque forming units (PFU) in 30 mL of PBS. A sample of the reaction mixture was transferred to an Ostwald viscometer which, together with the flask containing the bulk of the solution, was placed in a bath at 37^{o}. The viscosity of the solution was determined periodically and at the same time aliquots were withdrawn and analyzed for reducing power by comparison with

a standard curve based on galactose.[293]

Purification and separation of depolymerized K21 material.

Portions of the crude lyophilized depolymerization mixture (2 x 1.5 g) were desalted using a column of Sephadex G10 (100 cm x 19.5 cm^2). The column was eluted with buffer (water -- pyridine -- glacial acetic acid, 1000:10:4, pH 4.5) at a flow rate of 25 mL/h and carbohydrate material was localized using the Molisch test. This material (220 mg) was then applied to the top of a DEAE - A25 Sephadex column. The column 25 x 5 $cm,^2$ was packed in 0.5M Tris/HCl buffer (pH 7.2) and then equilibrated with 0.025M Tris/HCl buffer; at least 10 column volumes of the latter buffer are required to achieve equlibration as determined by performing conductivity measurements. The material was applied as a solution in 0.025M Tris/HCl(2 mL). The column was eluted (10 mL/h) with 0.025M Tris/HCl (140 mL) and a linear salt gradient (from 0 to 0.35M NaCl) was then begun. Fractions (2 mL) were collected and examined using the phenol-sulfuric acid assay. The elution profile is shown in Fig. 14.

Analyses of depolymerization products

The p.m.r. spectra (XL-100, 90^o) of products P1 and P2 showed several spurious peaks (buffer?) at low field, but these were eliminated by passage through a column of Amberlite IR-120 (H^+) ion - exchange resin. The ^{13}C spectrum of P1 was in agreement with

the ^{1}H n.m.r. spectrum.(see App. III).

The degree of polymerization of the products (the ratio of acetylated aldononitriles to acetylated alditol) was determined using the method of Morrison.[102] The oligosaccharide (5 mg) was reduced with sodium borohydride (excess) for 3 h (reducing sugar converted to alditol). The glucuronic acid was then reduced via. the methyl ester [299] by methanolysis and reduction (see Sec. II.4.1.) The residue was hydrolyzed with trifluoroacetic acid (2M) at 95° for 16 h, and evaporated to dryness. The aldoses were converted to the oxime by heating at 95° for 15 min with 5% hydroxylammonium chloride in pyridine (0.2 mL/mg of aldose). After cooling, acetic anhydride (0.2 mL/mg of aldose) was added and heating was continued for a further 30 min to dehydrate the oxime to the nitrile and to acetylate the free hydroxyl groups. G.l.c. analysis gave the results shown in Figure 7 and Table 13.

Paper electrophoresis was performed on a SAVANT high voltage 5kV) system (model LT-48A) with kerosene as coolant. The buffer used contained pyridine -- acetic acid -- water (5:2:743, v/v), pH 5.3. Strips of Whatman no. 1 paper (77 cm x 20 cm) were used with a current of 100 mA for 4 h. For descending paper chromatography freshly prepared 2:1:1 1 butanol -- acetic acid -- water was used.

Conditions of depolymerization of K12 and K41.

The conditions of depolymerization of K12 and K41 capsular polysaccharides, using the bacteriophage propagated on K12 were identical.

Purified capsular polysaccharide (200 mg) was dissolved in the crude broth suspension (20 mL) of ϕ 12 ($\sim 10^9$ PFU/mL). The reaction mixture was transferred to an Ostwald viscometer which, was placed in a bath at 37^{o}. The viscosity of the solution was determined periodically, and was shown to decrease substantially in both cases, within 3 h.

After 48 h the solution was transferred to a sealed portion of dialysis tubing (M.W. cutoff = 3,500) and dialyzed against three portions (200 mL) of distilled water. The dialysates were reduced to dryness and broth contaminants were removed by preparative paper chromatography using freshly prepared 2:1:1 1-butanol -- acetic acid -- water.

The yield of the single repeating unit of K12 was 10 mgs and of the double repeating unit of K41, 20 mgs.

The degree of polymerization of the products was determined as for the products of K21 ϕ 21.

Acknowledgments

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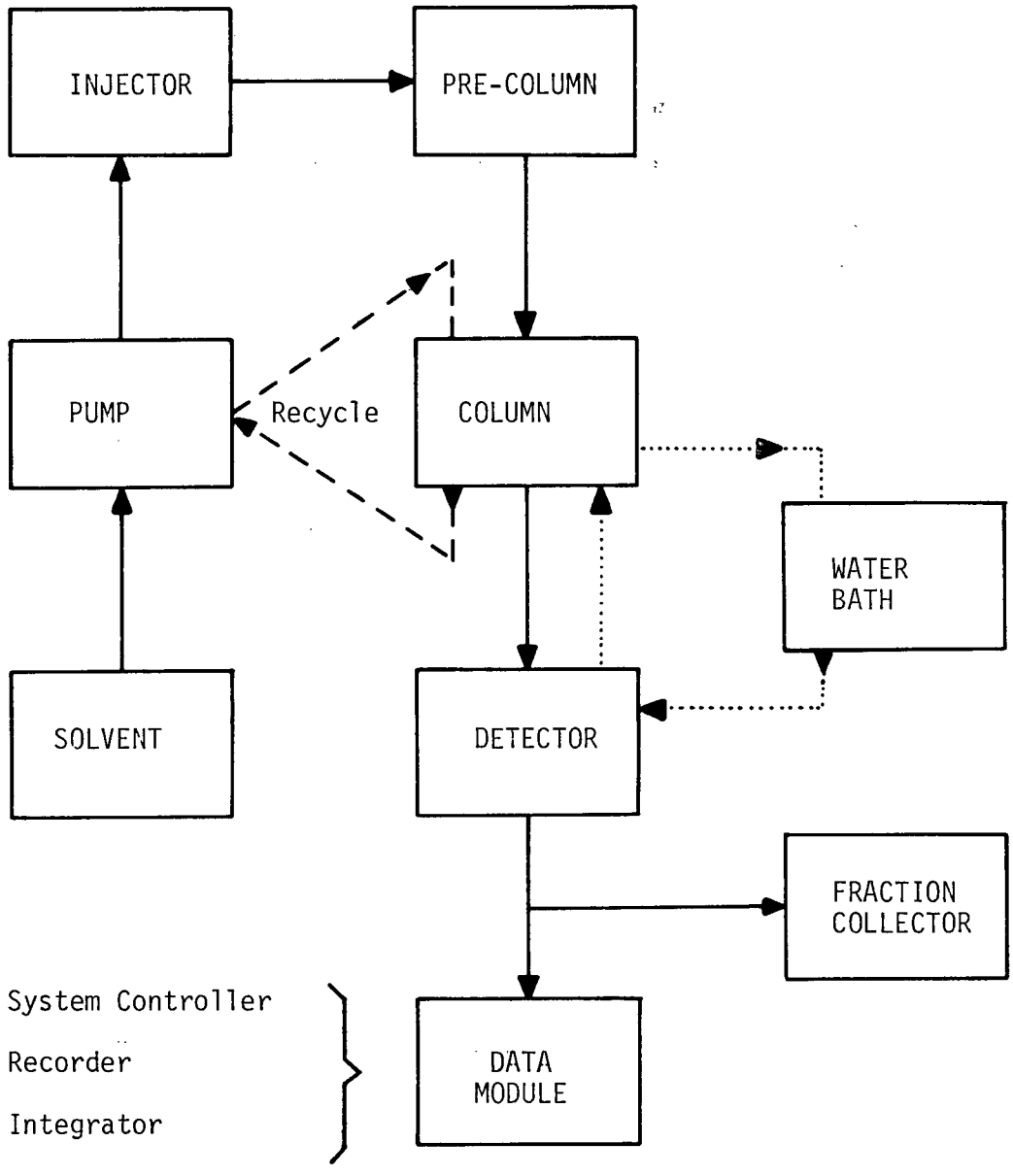
V HIGH PRESSURE LIQUID CHROMATOGRAPHY OF CARBOHYDRATES

V. 1. Introduction

Although classical column liquid chromatography has been an effective separation method since the beginning of this century, it is still characterized by low column efficiencies and long separation times. However, in the last decade it has been recognised that column efficiency and speed of separation could be improved by several orders of magnitude if materials of very small particle size are used as column packings. Such columns give a high theoretical plate number which decreases only slightly with flow velocity. As a result, a high resolving power and speed of separation can be achieved. Since such columns require a high pressure for their operation, this modern version of column liquid chromatography is often called high-pressure liquid chromatography (HPLC).

The fundamental instrumentation necessary for HPLC separations[300] consists of (see Scheme 12) (a) a solvent reservoir, (b) a pump capable of giving flow against moderately high back pressures (6,000 psi) with a recycle capability (c) an injection head (d) a column, fitted with a pre-column, (e) a detector (for carbohydrate analysis, usually a thermostated refractive index detector) and (f) a recorder/integrator/data system.

The basis of chromatographic separation is the distribution (or partition) of sample components between two phases which are immiscible.[301] The interactions between the molecules of the mobile and stationary phases determine the degree of sorption of particular substances and also the effectiveness or selectivity of the separations.



Scheme 12. Block Diagram of Instrumentation for High Performance Liquid Chromatography

Gas chromatography separations are based on vapour pressure. Liquid chromatography separations are based on solubility. With the latter the composition of the mobile phase, along with the type of packing material, is of prime importance in obtaining separation.

Research to date on the application of HPLC to carbohydrate analysis has focused on the quantitative analysis of sugars in foods and beverages [302-304]. The main sugars of interest here are glucose, fructose and sucrose. Since three different types of sugars (hexose, ketose, disaccharide) are involved, the technique has enjoyed some success in routine analysis, thus replacing paper chromatography (which is time consuming) and gas-liquid chromatography (which requires derivatization).

As yet, the application of HPLC in the structural analysis of heteropolysaccharides has been limited [324], although the technique shows much promise. The following areas of applicability are apparent.

On an analyitical level:

(i) Constituent analysis of the native polysaccharide.

(ii) Constituent analysis of the products of Smith degradation.

(iii) To monitor the partial hydrolysis of polysaccharides.

(iv) Analysis of the products of base-catalyzed degradation.

(v) Determination of the molecular weight and the homogeneity of polysaccharides.

On a preparative level:

(vi) To obtain oligosaccharides from partial hydrolysis and from Smith degradation.

(vii) To purify and separate the products of bacteriophage degradation.

(viii) To obtain methylated/ethylated oligosaccharides from based-catalysed degradation (of the methylated polysaccharide).

(ix) To separate methylated oligosaccharides obtained from methylation of the products of partial hydrolysis.

(x) To separate methylated/ethylated oligosaccharides obtained on ethylation of the products of partial hydrolysis of the methylated polysaccharide.

The inherent problems are:

(a) The difficulty of separating closely related compounds e.g. glucose, galactose, and mannose, or erythritol and threitol [for (i) and (ii)].

(b) The presence of acidic sugars - it is not possible to separate these on amine - bonded columns [for (i), (ii), (iii), and (vii)] .

In this study the retention times and molar response factor are measured for (1) the neutral sugars occuring in Klebsiella capsular polysaccharides, (2) some products of Smith degradation (3) a number of disaccharides and a trisaccharide, and the application of HPLC in the analysis of Klebsiella capsular polysaccharides is

discussed.

V. 2. Chromatographic Conditions

The following equipment was used:
Waters Associates ALC 201 liquid chromatograph equipped with M6000A pump, U6K Universal (septumless) injector, R401 differential refractometer, thermostated at 40^{0} using a Brinkmann Instruments Landa K-2/R circulating water bath,and Waters Model 730 Data Module (printer/plotter/integrator).

Column A: Waters Carbohydrate Analysis (10μ) 3.9mm I.D. x 30cm thermostated at 40^{0} with a home-made stainless steel water jacket connected to a circulating water bath.
Column B: Stainless steel 4mm x 24cm, with Swagelok end fittings. The column was slurry packed with 5μ Lichrosorb S160 (Merck) in methanol - water (9:1).

For both columns a small pre-column filled with Lichrosorb S160 was also used.

Solvents: Acetonitrile, HPLC grade (BDH), and water, glass distilled. Eluant, Column A: Acetonitrile water(80:20, 85:15 v/v). Column B was modified with 500 ml acetonitrile : water (4:1) containing 0.1% of HPLC amine modifier I (NATEC, Hamburg, G.F.R.)[305,306] Thereafter the eluant (acetonitrile - water, 4:1 or 9:1 v/v) contained 0.01% amine modifier.

Sugars were dissolved in water, filtered through 0.5 μm Millipore filters, and between 5 and 20 μl of 5% solutions were injected using a 25 μl Waters Gas Tight Syringe.

V. 3. Results and Disscussion

(i) Separation of monosaccharides (Column A and Column B).

It is possible to get baseline separation for different classes of sugars, e.g. rhamnose fructose and glucose (see Table 16, Fig. 16). Separation of the hexoses, mannose, glucose and galactose (See Fig.17) is poor, and because the molar response factors to refractive index of galactose and mannose are low, these peaks appear as shoulders on the glucose.

(ii) Separation of di- and trisaccharides

It is possible to identify mono-,di-and trisaccharides, based on the relative retention times (see Table 16, Fig. 18). Disaccharides with dissimilar structures e.g. cellobiose (Glc $\frac{1\ 4}{\beta}$ Glc) and melibiose (Gal $\frac{1\ 6}{\alpha}$ Glc) give a good separation, whereas disaccharides with closely related structures eg. cellobiose and maltose (Glc $\frac{1\ 4}{\alpha}$ Glc) do not.

Comparison of the change in flow rate from 2 mL/min to 3 mL/min (see Fig. 18) shows that good separation is still obtained with the shorter retention times.

The retention times were longer on Column A than on Column B for the same flow rate and solvent ratio.

TABLE 16

SEPARATION OF MONO- DI- AND TRISACCHARIDES BY HPLC

SUGAR	M.R.F.[b] R.I.	COLUMN A[a] 2 mL/min (80 : 20)[c] Time(mins)	Rel.R.T.[e]	COLUMN B[a] 2 mL/min (80 : 20)[c,d] Time(mins)	Rel. R.T.[e]
Rhamnose	1.4	1.84	0.46	1.5	0.51
Fucose	1.5	2.27	0.56	1.75	0.6
Arabinose	0.7	2.69	0.67	2.05	0.7
Fructose	1.2	3.0	0.75	2.2	0.76
Mannose	0.35	3.8	0.95	2.6	0.9
Glucose	1.0	4.0	1.0	2.8	1.0
Galactose	0.3	4.2	1.05	3.1	1.07
Sucrose	0.53	6.6	1.65	4.3	1.5
Cellibiose	0.53	9.0	2.25	5.5	1.90
Maltose	0.55	9.5	2.37	5.65	1.94
Mellibiose	0.26	13.0	3.25	7.53	2.62
Raffinose	0.34	20.7	5.18	11.33	3.93

[a] For column specifications see Chromatographic conditions (V.2).

[b] Molar response factor to refractive index.

[c] Acetonitri e : water (v/v) [d] With 0.01% amine modifier I added.

[e] Retention time relative to that of glucose.

TABLE 17

SEPARATION OF PRODUCTS OF SMITH DEGRADATION USING HPLC.

SUGAR	M.R.F.[b] R.I.	COLUMN A[a] 2 mL/min (85 : 15)[c] Time(mins)	Rel. R.T.[e]	COLUMN B[a] 2 mL/min (90 : 10)[c,d] Time(mins)	Rel. R.T.[e]
(H_2O)	-	0.86	0.07	0.95	0.08
Ethylene Glycol	-	1.10	0.12	1.15	0.1
Glycerol	0.7	1.88	0.15	1.85	0.15
Rhamnose	1.4	2.6	0.30	3.09	0.25
Erythritol	0.7	2.8	0.32	3.16	0.26
Threitol	0.7	2.8	0.32	3.2	0.27
Arabinose	0.7	4.3	0.51	6.4	0.53
Glucose	1.0	8.36	1.0	12.0	1.0

[a] For column specifications see Chromatographic conditions (V.2).

[b] Molar response factor to refractive index.

[c] Acetonitrile : water (v.v)

[d] With 0.01% amine modifier I added.

[e] retention time relative to that of glucose.

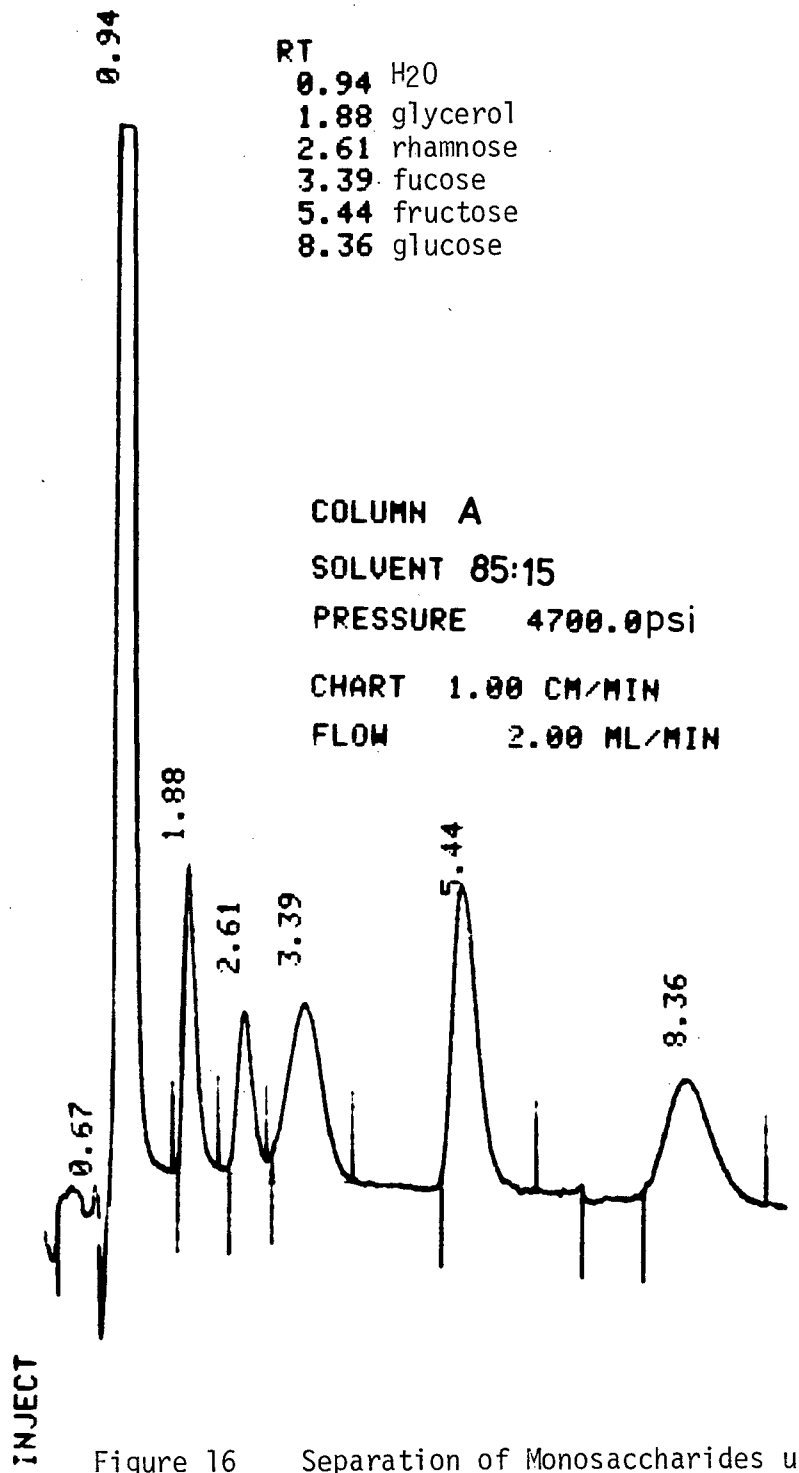


Figure 16 Separation of Monosaccharides using HPLC (Column A)

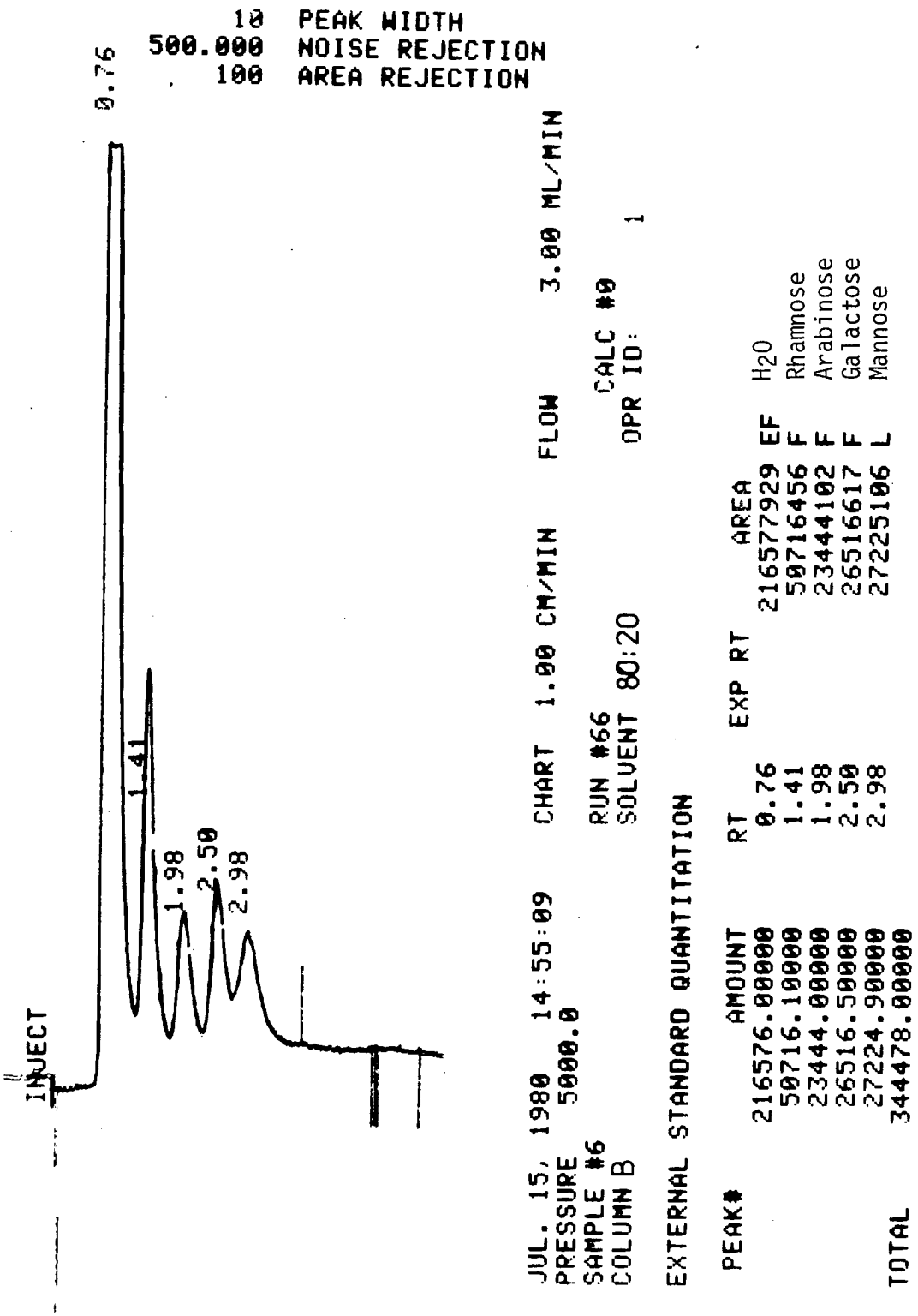


Figure 17 Separation of monosaccharides using HPLC (Column B)

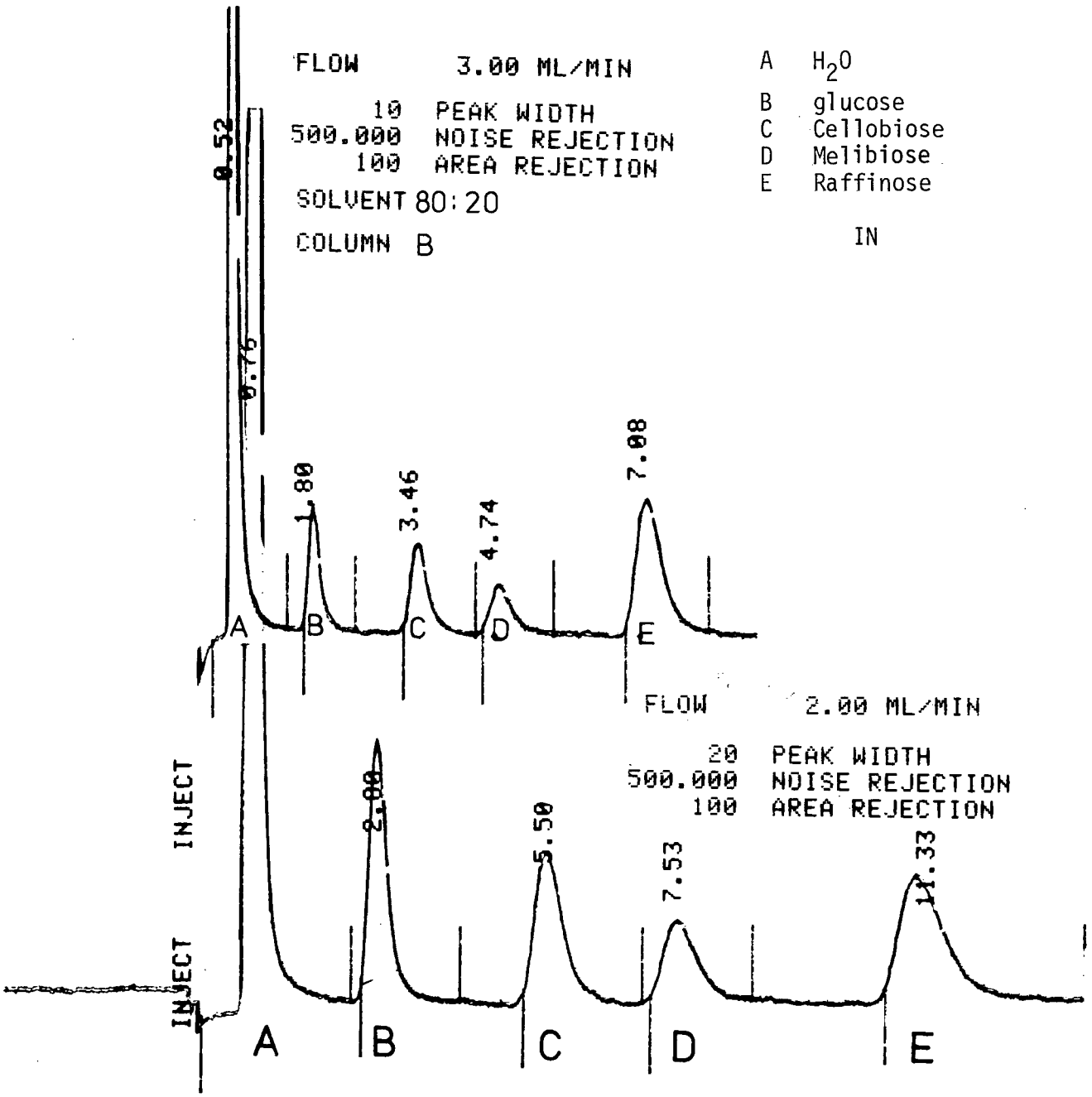
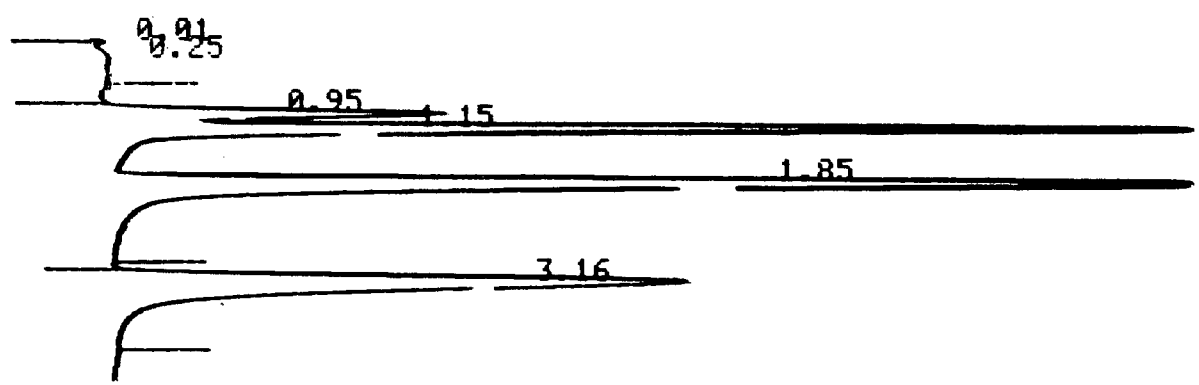


Figure 18 Separation of di- and trisaccharides using HPLC, with different flow-rates.



JUL. 22, 1980 21:37:47 CHART 1.00 CM/MIN FLOW 2.00 ML/MIN
PRESSURE 3000.0
SAMPLE #33 RUN #94 CALC #0
COLUMN B SOLVENT 90:10 OPR ID: 1

EXTERNAL STANDARD QUANTITATION

PEAK#	AMOUNT	RT	AREA		
	148.33700	0.01	148337	EHL	-
	20813.70000	0.95	20813782	F	H_2O
	66082.90000	1.15	66083403	F	ethylene glycol
	95954.70000	1.85	95955426	L	glycerol
	65673.70000	3.16	65674190	L	lrythritol
TOTAL	248673.00000				

Figure 19 Separation of some products of Smith degradation using HPLC.

(iii) Separation of products of Smith degradation.[253,254,259]

Glycerol and erythritol are well separated from each other and from the solvent (H_2O) peak. Ethylene glycol has a very short retention time (see Fig.19) and therefore, samples containing this compound should be injected in a solvent composition identical to the eluant. Erythritol and threitol co-chromatograph (see Table 17) with solvent 85:15 and have very similar retention times for solvent 90:10. The retention time of glucose with solvent 90:10 is relatively long (12 mins.)

V. 4. Conclusion

HPLC has the potential to become an important technique in the structural analysis of heteropolysaccharides. The two columns described here give similar results. Column B has, however, the advantage of being less expensive (since it is home-packed) and also, the separations may be extended to a preparative scale. In this case a larger particle size of silica would have to be used to decrease the back-pressure generated. Column A is available only as a prepacked column suitable for analytical work.

Either column could be used in determining the ratio of sugars in a polysaccharide and in its degradation products. Although the separation of mannose, glucose and galactose is poor, these three sugars do not necessarily occur in the same polysaccharide. Since acidic sugars (uronic acids) may not be applied to the

(amino-bonded) column an indication of the number of sugars in the repeating unit may be obtained as follows

(a) Total hydrolysis of the poly-,.oligosaccharide.[238]

(b) Separation into neutral and acidic fractions (AG-1X2).

(c) Analysis of the neutral sugars on HPLC

(d) Total hydrolysis of the reduced polysaccharide (via. carbodiimide [264]) or reduced oligosaccharide (via. the methyl ester)[238,239]

(e) Analysis of sugars on HPLC

(f) Comparison of data from (c) and (e).

The advantage of this method over gas-liquid chromatography lies in the absence of the need for derivitization.

The major products obtained from partial hydrolysis of an acidic polysaccharide are acidic (aldobi - aldotri - and aldotetrauronic acids). Hence columns A and B could be used only in the analysis of the minor, neutral oligomers obtained. On a preparative level, this technique shows potential in obtaining pure neutral oligosaccharides.

Separation of the products of Smith degradation into neutral and acidic components may give some/all of the following:

(a) neutral: glycerol, erythitol, threitol, ethylene, glycol, oligosaccharides, polysaccharide.

(b) acidic : erythronic acid, threonic acid, oligosaccharides, polysaccharides.

Analysis of (a) on an analytical level would provide useful information, and could be extended to a preparative scale.

V. 5. Alternatives

As denoted above, the main problems encountered in the use of columns A and B are (i) the poor separation of mannose, glucose and galactose and (ii) acidic sugars may not be separated.

To overcome (i) Adam [307] has reported the use of a radially compressed Silica (10 μm) column (Waters Assocs.)[308] which eliminates voids and channels in the packed bed, thereby giving higher efficiencies. Although not quite baseline, the separation of mannose, glucose and galactose are much improved, and results are reproducible.

Barton et al [309] report excellent separation of rhamnose, xylose, arabinose and glucose, and adequate separation of glucose and galactose using a Micromeremetics Microsil amine bonded phase column, in the analysis of hydrolyzates of plant cell wall fiber. Meagher and Furst [310] have used a μ bondapak - carbohydrate (Waters) column with acetonitrile - water (85 : 15) in the analysis of carbohydrates in rat urine.

The second solution to (i) is the use of an ion - exchange type column, using water or ethanol as the eluant. Lawrence [311] has reported the use of the resins Aminex A-6 Li^+, ZC225 Li^+ and Technicon Type S to achieve separation of mannose, glucose and galactose, but retention times are up to four hours.

Durrum Chemical Co. [312] have reported the separation of mannose, fucose, galactose, xylose and glucose (in that order) on a column of Durrum type DA - X4 (25 cm) in 2.5 hours. Scobell et al [313] report excellent separation of arabinose, galactose, glucose, melibiose and melezitose on Aminex A-5 CA^{++}, at 85^{o} in 32 mins.

In 1975 Linden and Lawhead [314] suggested the use of HPLC to separate oligosaccharides, on a micro Bondapak (Waters) column, and in 1978 Ladisch et al [315] used the ion - exchange resin AG 50W - $X4Ca^{++}$ to separate the oligomers celloheptaose through glucose within 30 min, using water as eluant. These authors have also described [316] a packing procedure and have commented on the theory of rapid liquid chromatography at moderate pressures, using water as eluant.

Belue [317] has used a column of Porasil A to separate polyhydric alcohols (from Smith degradation) using methyl ethyl ketone - water - acetone (85 : 10 : 5) as eluant. McGinnis and Fang [318] have separated substituted carbohydrates on a column of 10 μm silica, Partisil 10 (Whatman) using acetonitrile - water (90 : 10) as eluant.

One of the more interesting applications of HPLC in carbohydrate analysis has been the recent (1980) use of a Dupont Zorbax ™ ODS column by Albersheim and coworkers [320] to separate peralkylated oligosaccharides. These were generated by successive

partial acid hydrolysis, reduction and ethylation of a permethylated, complex carbohydrate. By this technique,the structure of a nonasaccharide derived from xyloglucan, a structural polymer of plant cell-walls, was elucidated.

Doner and Hicks[321] have determined retention times, capacity factors and relative responses to refractive index detection for over forty pentoses,hexoses, di- and trisaccharides, and alditols on two bonded phase silica columns and on a cation (Ca^{++}) exchange column. Scrobell et al [322]describe the preparation and operation of "second generation" silver form cation exchange resin columns that outperform the equivalent calcium form by a factor of two with respect to time, resolution and the number of oligomers seperated.

The seperation of oligosaccharides obtained by phage degradation of Klebsiella K2 polysaccharide was performed by Stirm et al[77] using a μ-Bondapak-NH_2 column with 2% formic acid as eluant.

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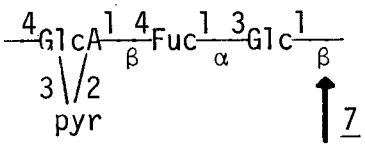
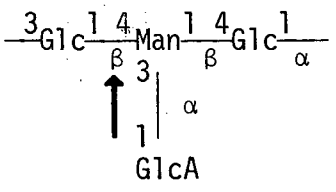
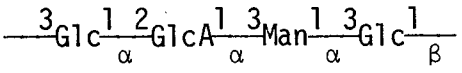
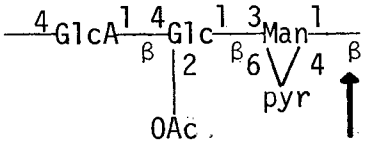
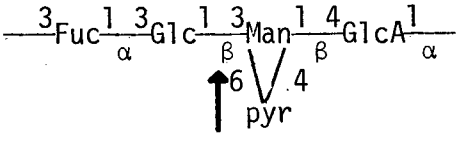
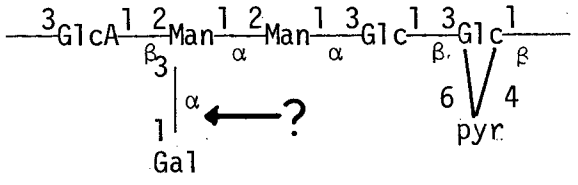
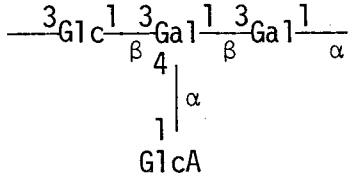
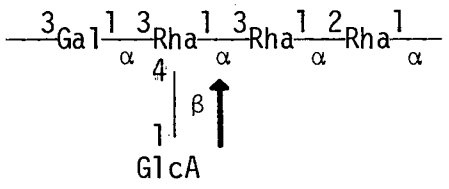
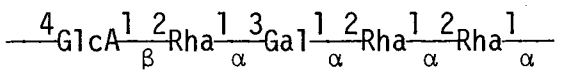
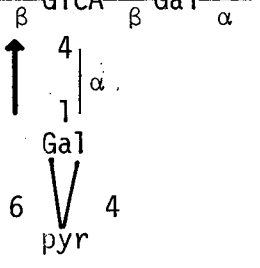
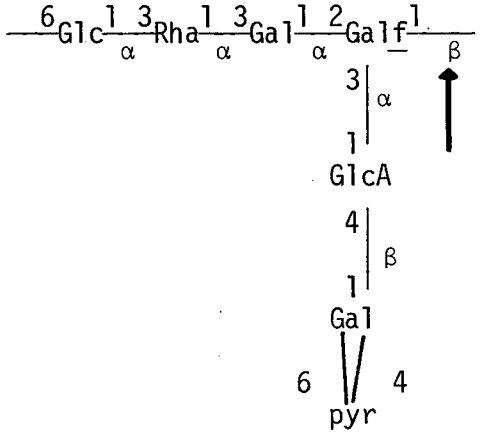
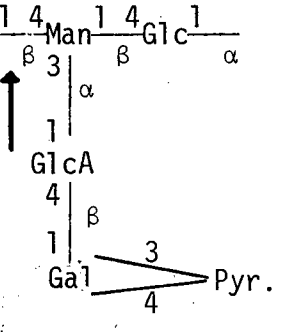
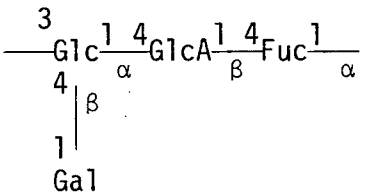
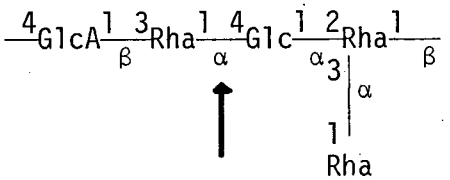
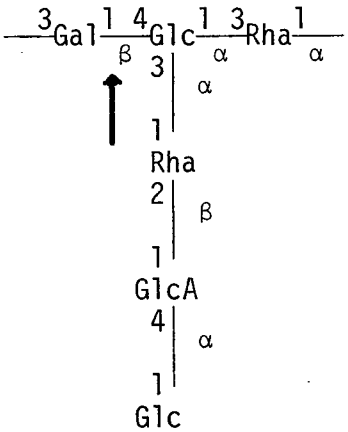
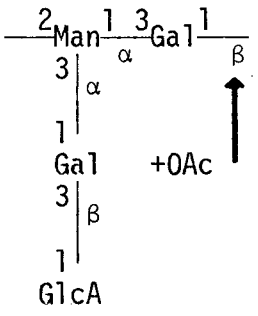
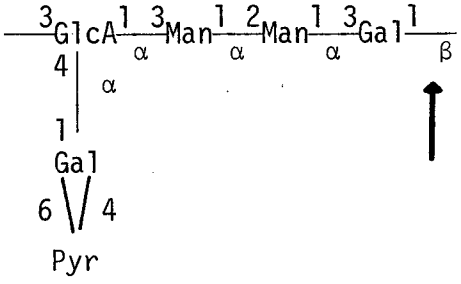
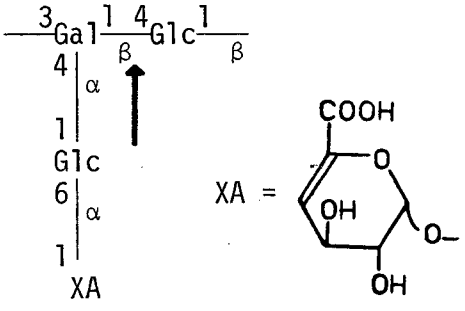
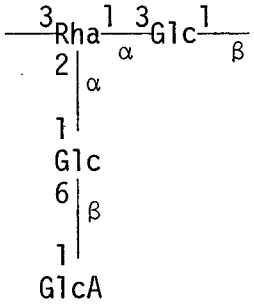
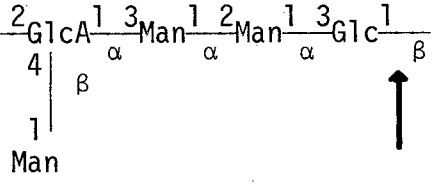
APPENDIX I

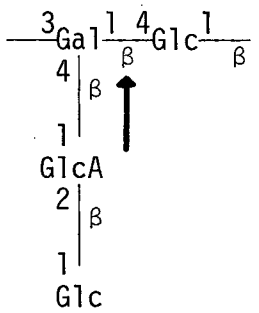
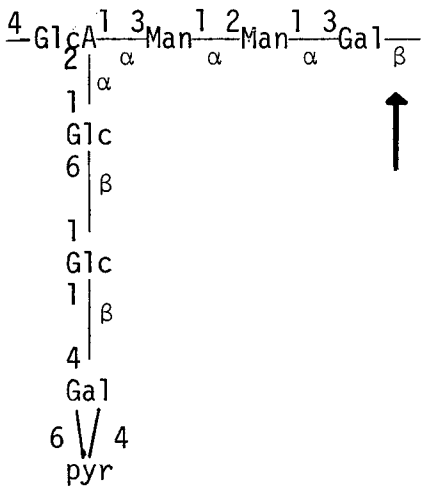
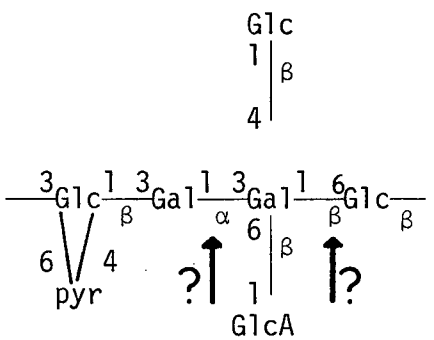
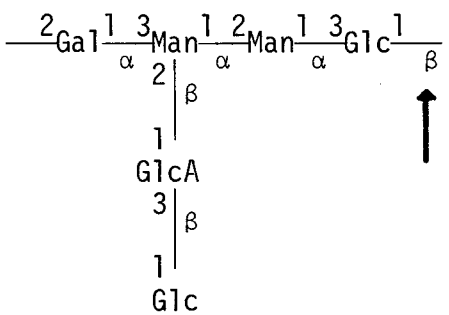
The Known Structures of the *Klebsiella* Capsular Polysaccharides (as of July 1, 1980)

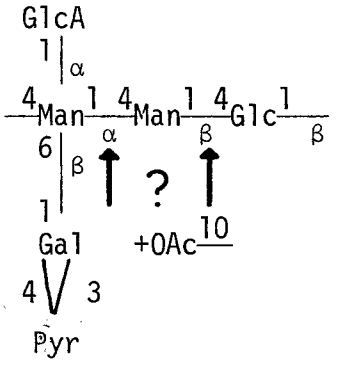
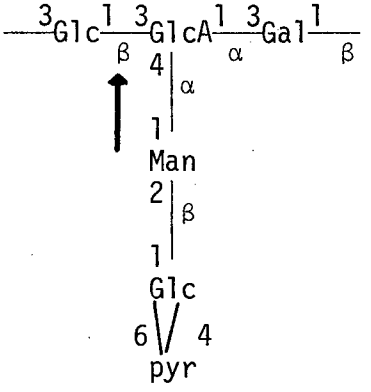
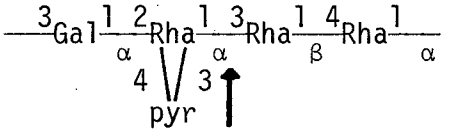
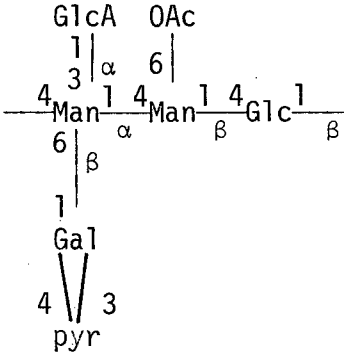
K-type[1] (a)	ϕ[2] (b)	X-Ray[3] (c)	$[\alpha]_D$[4]	Structure[5] References at end
K1			-85°[6]	
K2	+		+79°	
K3				GalA, Gal, Man, Pyr(J) [8]
K4			+90°	
K5		+	-45°	
K6			+46°	
K7			+40°	
K8		+		

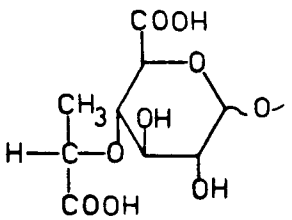
K-type (a)	ϕ (b)	X-Ray (c)	$[\alpha]_D$	Structure
K9[9]		+	-17°	
K9*			-5°	
K10				GlcA, Gal, Glc, Man (C)[8]
K11	+		+106°	
K12	+		+24°	
K13	+		+45°	

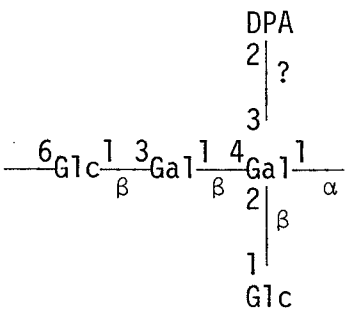
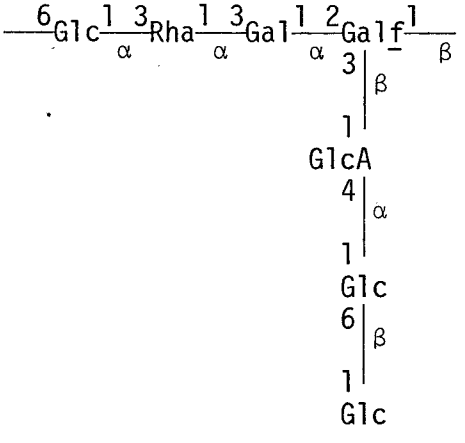
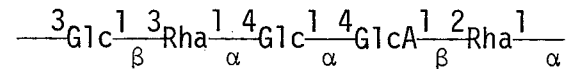
K-type (a)	ϕ (b)	X-Ray (c)	$[\alpha]_D$	Structure
K14				GlcA, Gal, Glc, Man, Rha. (L)[8]
K15				GlcA, Gal, Glc. (S.S.)[8]
K16		+	$+65^o$	
K17			$+30^o$	
K18	+		$+77^o$	
K19				GlcA, Gal, Glc, Rha(J)[8]

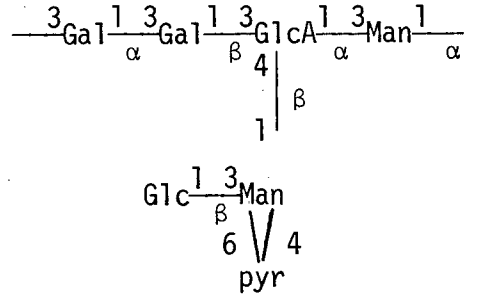
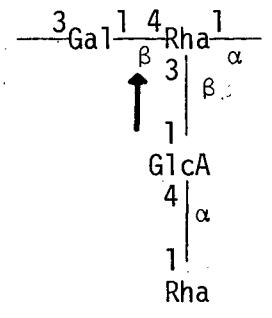
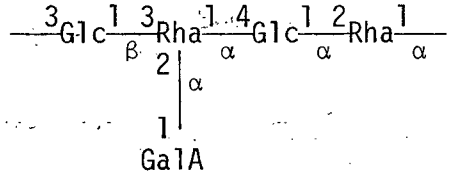
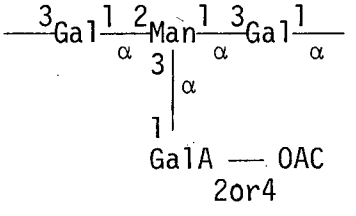
K-type (a)	ϕ (b)	X-Ray (c)	$[\alpha]_D$	Structure
K20	+		+94°	
K21	+		+130°	
K22				
K23			+28°	
K24	+		+79°	

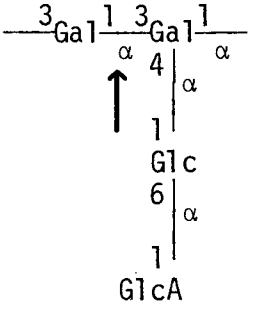
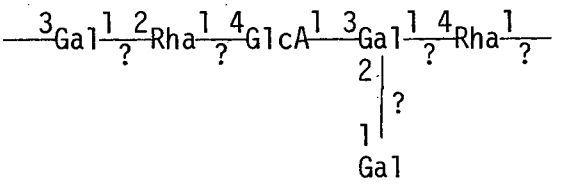
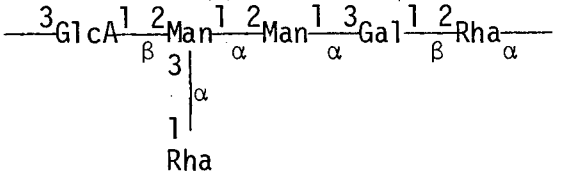
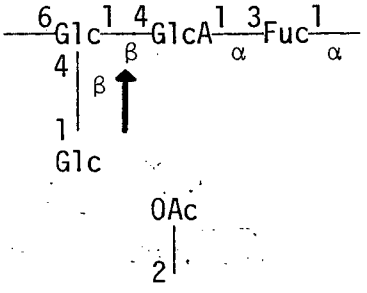
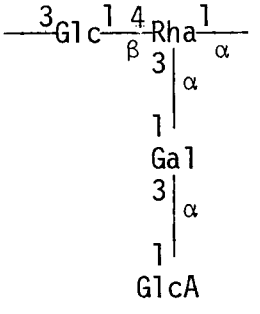
K-type (a)	ϕ (b)	X-Ray (c)	$[\alpha]_D$	Structure
K25		+	-41^o	
K26			$+80^o$	
K27			$+5^o$	
K28			$+47^o$	

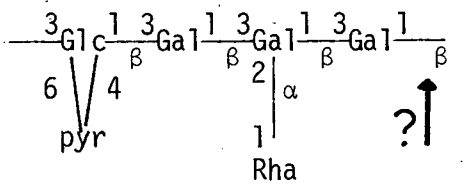
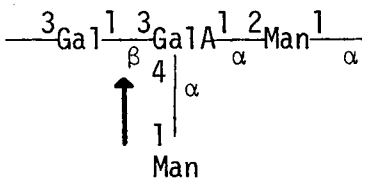
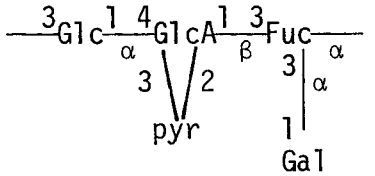
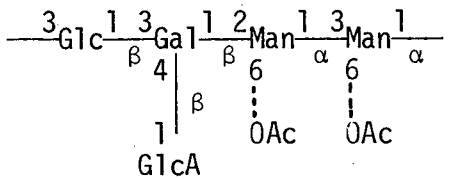
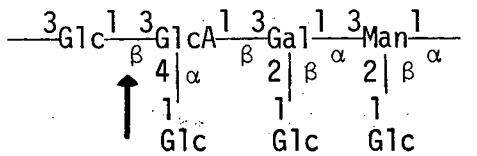
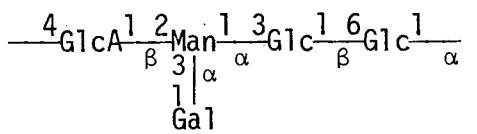
K-type (a)	ϕ (b)	X-Ray (c)	$[\alpha]_D$	Structure
K29				GlcA, Gal, Man, pyr. (N)[8]
K30			+16°	
K31				
K32	+		+113°	
K33			+22°[6]	

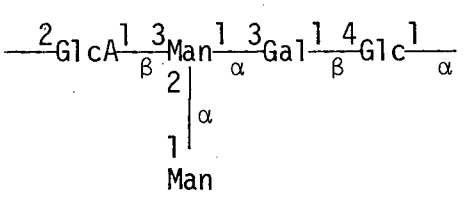
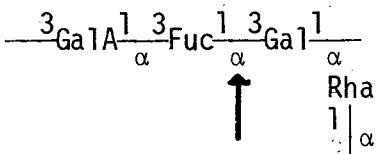
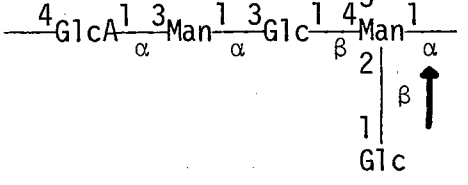
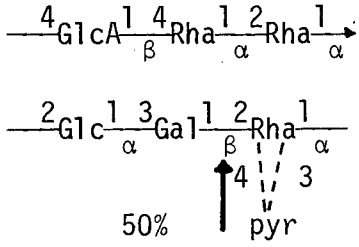
K-type (a)	ϕ (b)	X-Ray (c)	$[\alpha]_D$	Structure
K34			$+21^o$	$\rightarrow 3)\text{Rha}\alpha(1\rightarrow 2)\text{Rha}\alpha(1\rightarrow 3)\text{Glc}\beta(1\rightarrow 3)\text{GalA}\alpha(1\rightarrow 2)\text{Rha}\alpha(1\rightarrow$ $4\vert\alpha$ $1\vert$ Rha
K35				GlcA, Gal, Glc, Man (L)[8]
K36			-56^o	$\rightarrow 3)\text{Gal}\beta(1\rightarrow 3)\text{Rha}\alpha(1\rightarrow 3)\text{Rha}\alpha(1\rightarrow 2)\text{Rha}\alpha(1\rightarrow$ $2\vert\beta$ $1\vert$ GlcA $4\vert\beta$ $1\vert$ Glc $6 \quad\quad 6$ pyr
K37				$\rightarrow 3)\text{Gal}\beta(1\rightarrow 4)\text{Glc}\beta(1\rightarrow$ $4\vert\alpha$ $1\vert$ Glc $6\vert\beta$ $1\vert$ YA YA = 

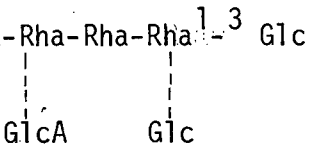
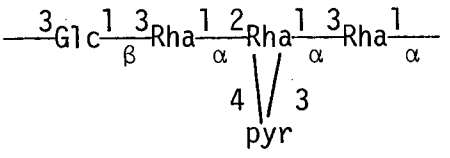
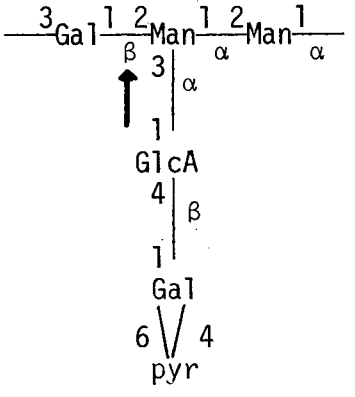
K-type (a)	ϕ (b)	X-Ray (c)	$[\alpha]_D$	Structure
K38			$+28^{o}$ [6]	
				DPA = 3-deoxy-L-glycero-pentulosonic acid
K39				GlcA, Gal, Glc, Man (D) [8]
K40				GlcA, Gal, Man, Rha, Pyr. (C) [8]
K41	+		$+23^{o}$	
K42				GlcA, Gal, Man, pry (N) [8]
K43				GlcA, Gal, Man. (N) [8]
K44			$+4^{o}$	

K-type (a)	ϕ (b)	X-Ray (c)	$[\alpha]_D$	Structure
K45				GlcA, Glc, Rha, pyr. (D)[8]
K46			+116°	
K47			-46°[6]	
K48			+23°	
K49			+152°	

K-type (a)	ϕ (b)	X-Ray (c)	$[\alpha]_D$	Structure
K50				GlcA, Gal, Glc, Man (D)[8]
K51				
K52				
K53			$+2^o$	
K54	+	+	-28^o	
K55			$+90^o$	

K-type (a)	ϕ (b)	X-Ray (c)	$[\alpha]_D$	Structure
K56			+79°	
K57		+	+104°	
K58			+19°	
K59			+26°	
				(dotted lines indicate OAc's not on all residues)
K60	+		+58°	
K61			+56°	

K-type (a)	ϕ (b)	X-Ray (c)	$[\alpha]_D$	Structure
K62			$+60^{o}$ [11]	
K63	+	+	$+133^{o}$	
K64			$+28^{o}$	
K65				GlcA, Glc, Man, Rha, Pyr (A.M.S.)[8]
K66				GlcA, Gal, Man (N)[8]
K67				GlcA, Gal, Glc, Man, Rha (L)[8]
K68				GlcA, Gal, Glc, Man, Fuc, Pyr(L)[8]
K69				GlcA, Glc, Gal, Man, Pyr (I.S.)[8]
K70	+			

K-type (a)	ϕ (b)	X-Ray (c)	$[\alpha]_D$	Structure
K71			-45^o	
K72			-54^o	
K73[12]				≡ Aerobacter aerogenes
K74			$+67^o$	
K75[12]				≡ K68
K76[12]				≡ K46
K77[12]				≡ K39
K78[12]				≡ K15
K79				GlcA, Gal, Glc, Rha (D)[8]

K-type (a)	ϕ (b)	X-Ray (c)	$[\alpha]_D$	Structure
K80				GlcA, Gal, Man, Rha, Pyr.
K81			-52^o	$\rightarrow 2)\text{Rha}\alpha(1\rightarrow 3)\text{Rha}\alpha(1\rightarrow 4)\text{GlcA}\beta(1\rightarrow 2)\text{Rha}\alpha(1\rightarrow 3)\text{Rha}\alpha(1\rightarrow 3)\text{Gal}\beta(1\rightarrow$
K82[12,13]				
K83			$+89^o$ [6]	$\rightarrow 3)\text{Gal}\beta(1\rightarrow 4)\text{Rha}\alpha(1\rightarrow$ 3 1 α Gal 3 α 1 GlcA

Footnotes

1 Serotyping by Ørskov. Structure Ref. (a)

2 Bacteriophage degradation Ref. (b)

3 X-Ray crystallographic study Ref. (c)

4 Rotations at Na-D line except where noted.
Data compiled by E.H. Merrifield.

5 All sugars are D except for rhamnose and fucose which are L

6 Rotation at 578 nm.

7 Bacteriophage attack site. D. Rieger - Hug and S. Stirm.
Virology in press.

8 Under investigation by

J	=	J.P. Joseleau
C	=	A.J. Chakraborty
L	=	B. Lindberg
S.S.	=	S. Stirm
N.	=	W. Nimmich
D.	=	G.G.S. Dutton
A.M.S.	=	A.M. Stephen
I.S.	=	I. Sutherland

9 This serotype has been investigated in two laboratories, and two different structures have been proposed; denoted K9, K9*

10 OAc group located on every third repeating unit.

11 Rotation measured on methylated polysaccharide.

12 G. Ørskov and M.A.Fife - Ashbury
Internat. J. Systematic Bacteriol., 27 386 (1977).

13 No quantitative analysis. Not assigned to a research group.

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APPENDIX II

Structural Patterns of *Klebsiella* Capsular Polysaccharides

Key : X = Uronic Acid 0 = Neutral sugar

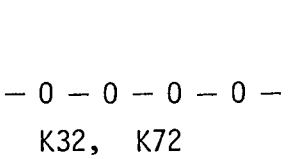
(X) = 3-deoxy-L-glycero-pentulosonic acid

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

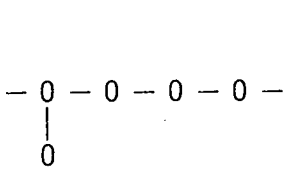
⟨X⟩ = 2R, 3R-hex-4-enopyranosyluronic acid

Pyruvate and acetate omitted

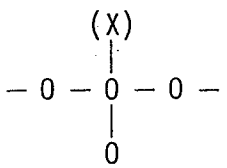
A. Uronic acid absent



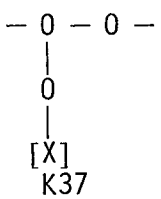
K32, K72



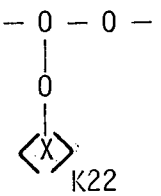
K56



K38



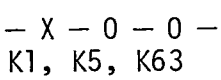
K37



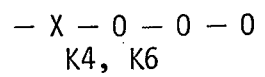
K22

B. Uronic acid in chain

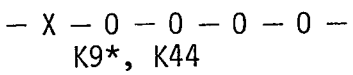
a) linear



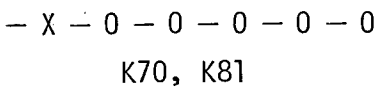
K1, K5, K63



K4, K6



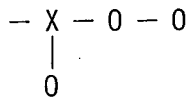
K9*, K44



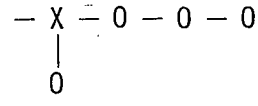
K70, K81

b) branch point on uronic acid

i) single unit side chain

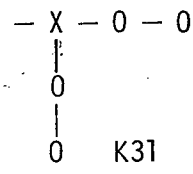


K11, K57

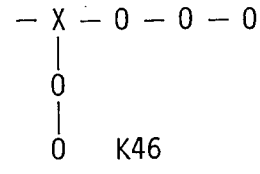


K21, K24

ii) two unit side chain

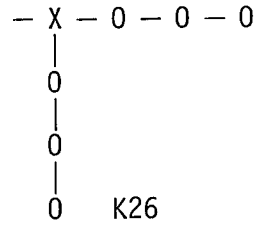


K31



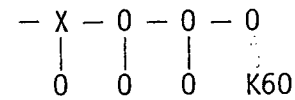
K46

iii) three unit side chain



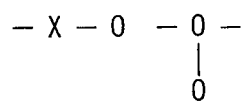
K26

iv) plus branch points on neutral sugars

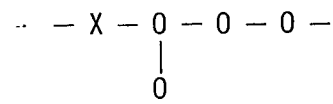


K60

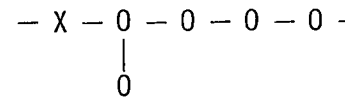
c) branch not on uronic acid



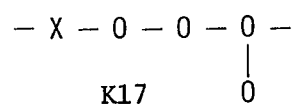
K16, K54



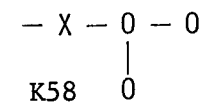
K7, K61, K62



K52, K53

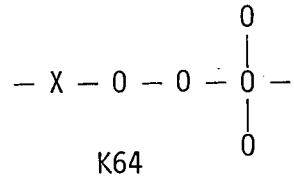


K17



K58

d) double branch not on uronic acid



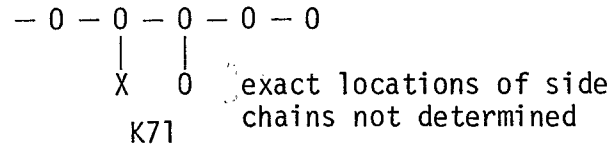
K64

C. Uronic acid in side chain

a) single unit side chain

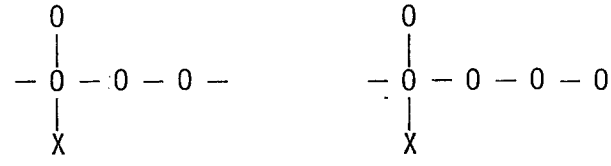
K2, K8, K9, K59

b) two single unit side chains



K71 exact locations of side chains not determined

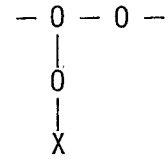
c) two single units side chains forming a double branch



K30, K33 K27

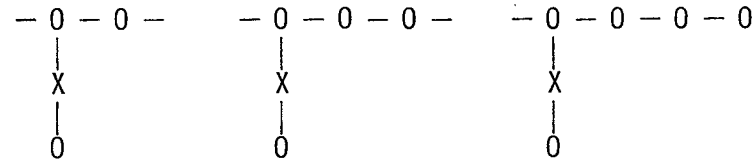
d) two unit side chain

i) uronic acid terminal



K20, K23, K51, K55

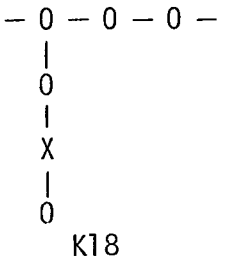
ii) uronic acid non-terminal



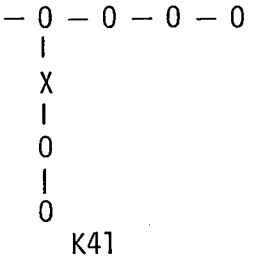
K25, K47 K13, K74 K12, K28, K36

e) Three unit side chain

i) uronic acid non-terminal



K18

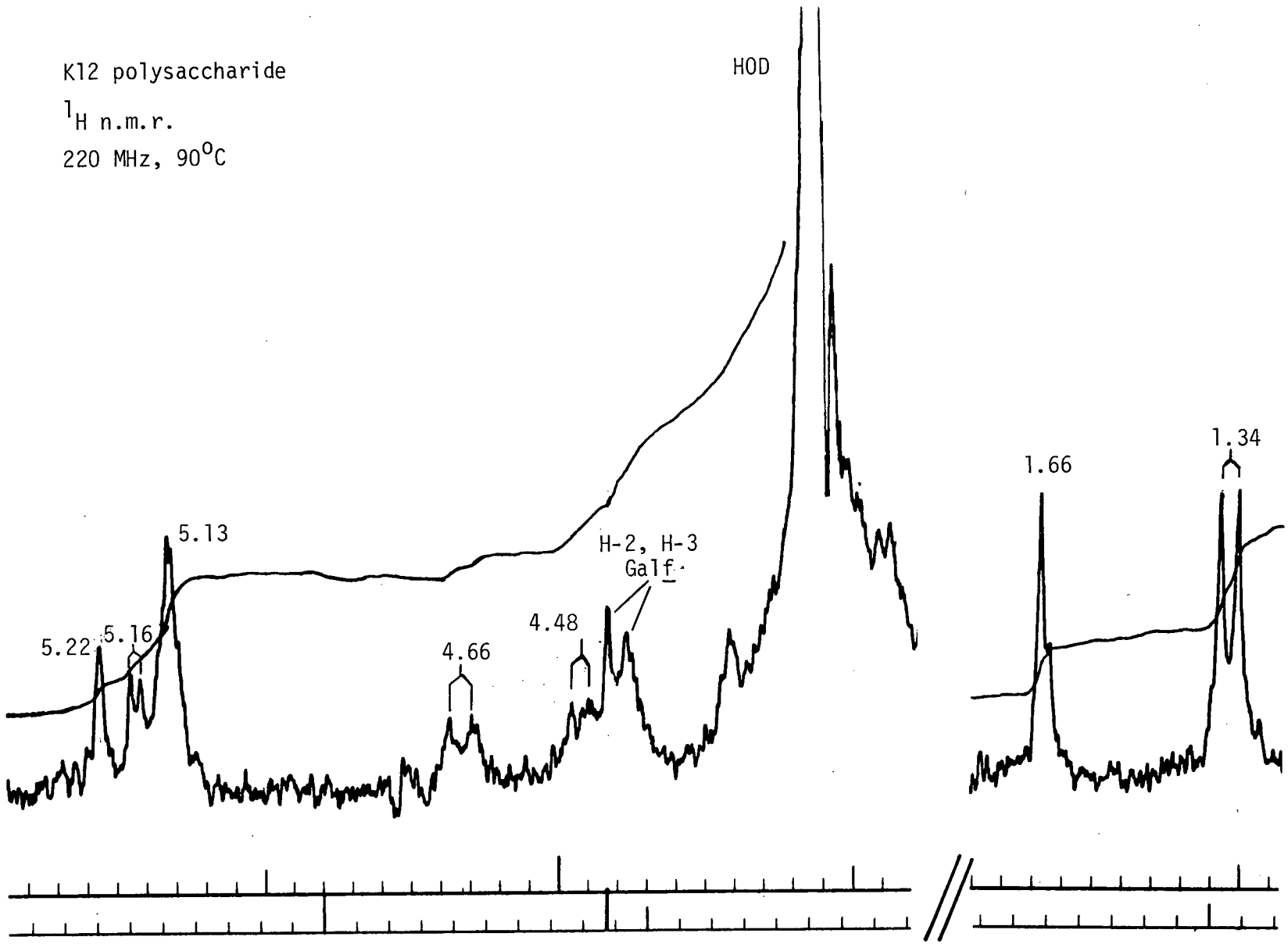


K41

Note: K9 has been investigated in two different laboratories, and two different structures have been proposed. These are denoted K9 and K9*

APPENDIX III

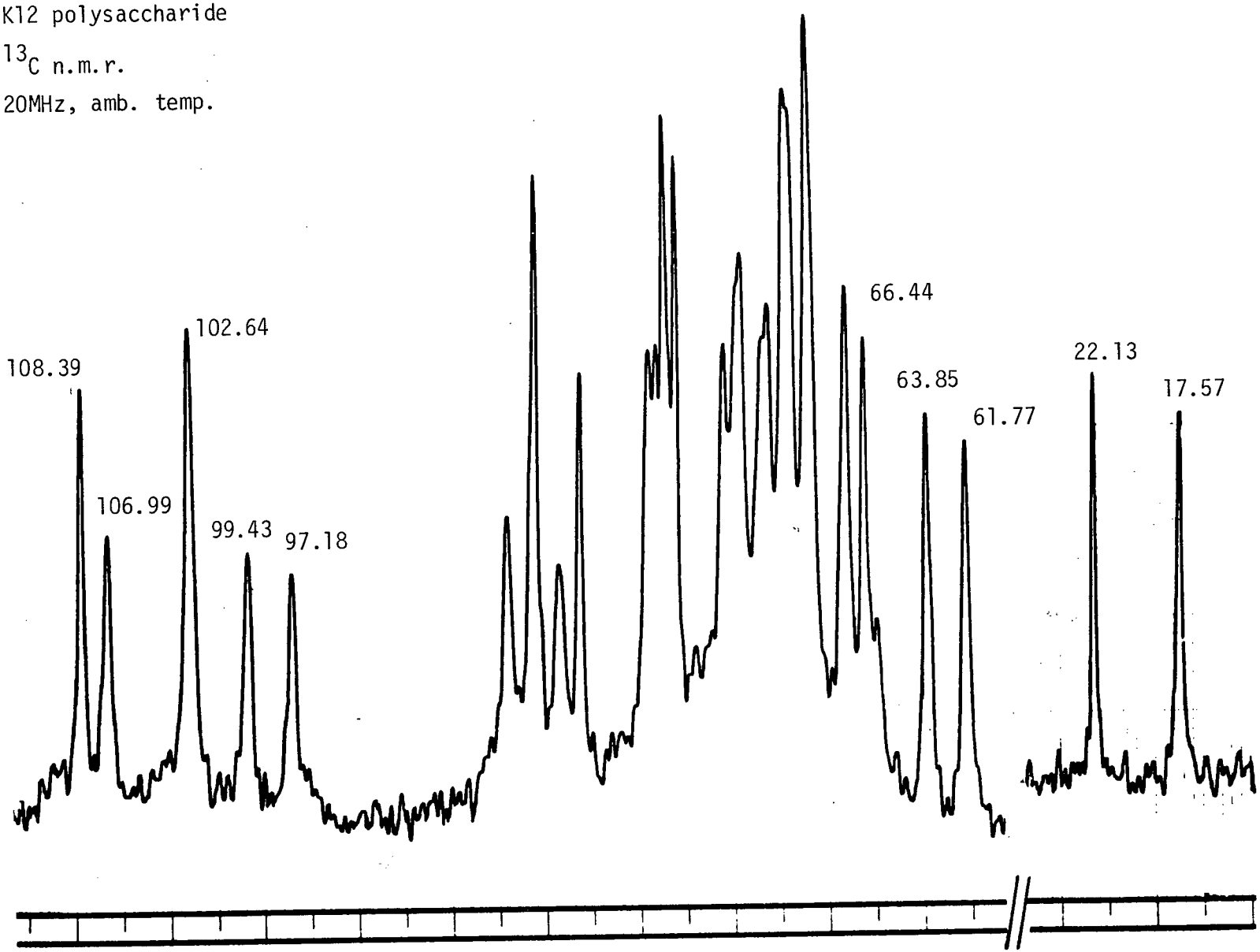
^{1}H and ^{13}C n.m.r. spectra


K12 polysaccharide
^{1}H n.m.r.
220 MHz, 90°C
HOD
5.22
5.16
5.13
4.66
4.48
H-2, H-3
Galf
1.66
1.34
Spectrum No. 1

K12 polysaccharide

^{13}C n.m.r.

20MHz, amb. temp.



Spectrum No. 2

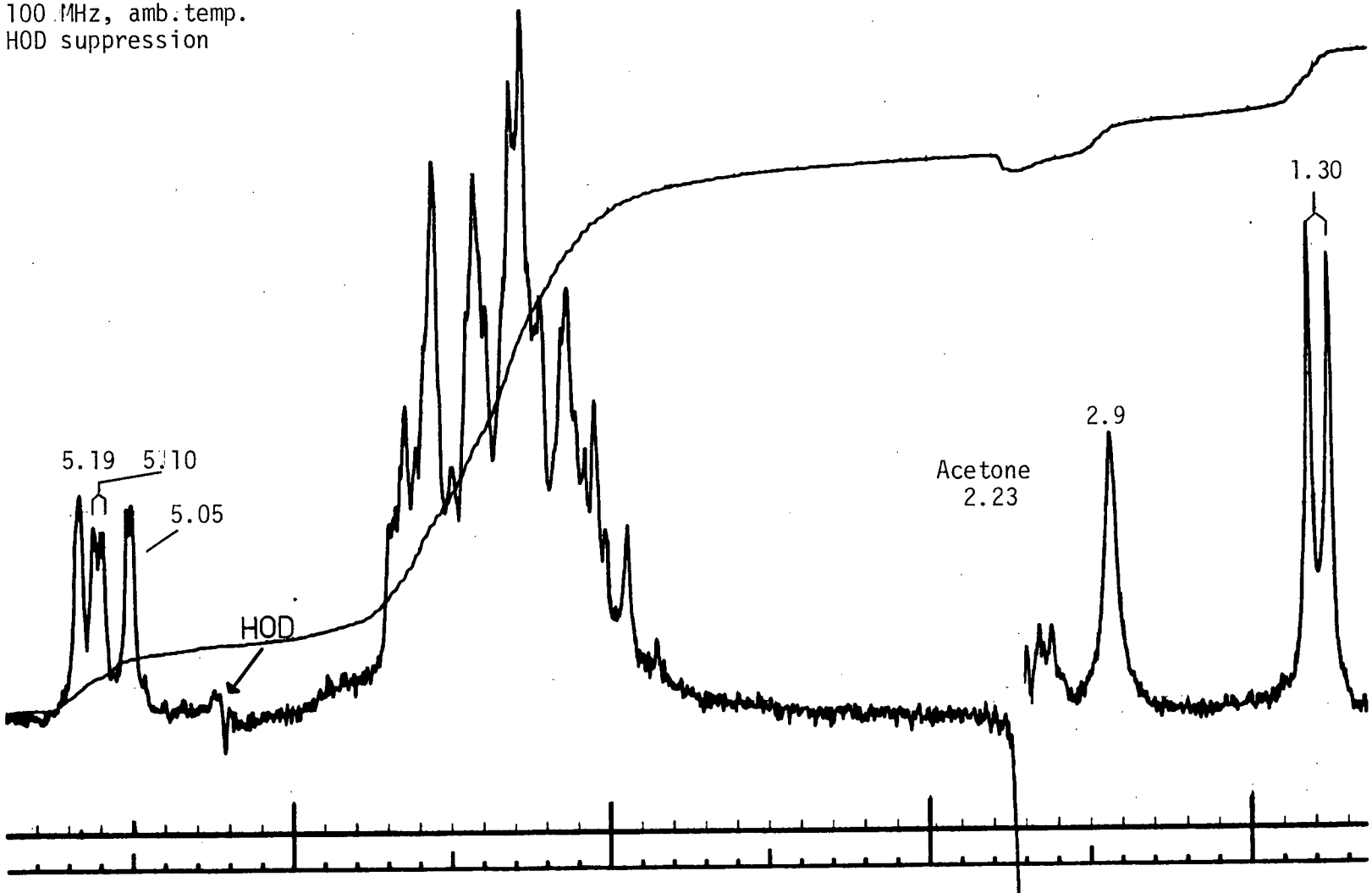
K12 Compund (3)

Rha $\frac{1\ 3}{\alpha}$ Gal $\frac{1\ 2}{\alpha}$ Ara $\frac{1\ 1}{\alpha}$ glycerol

^{1}H n.m.r.

100 MHz, amb.temp.

HOD suppression



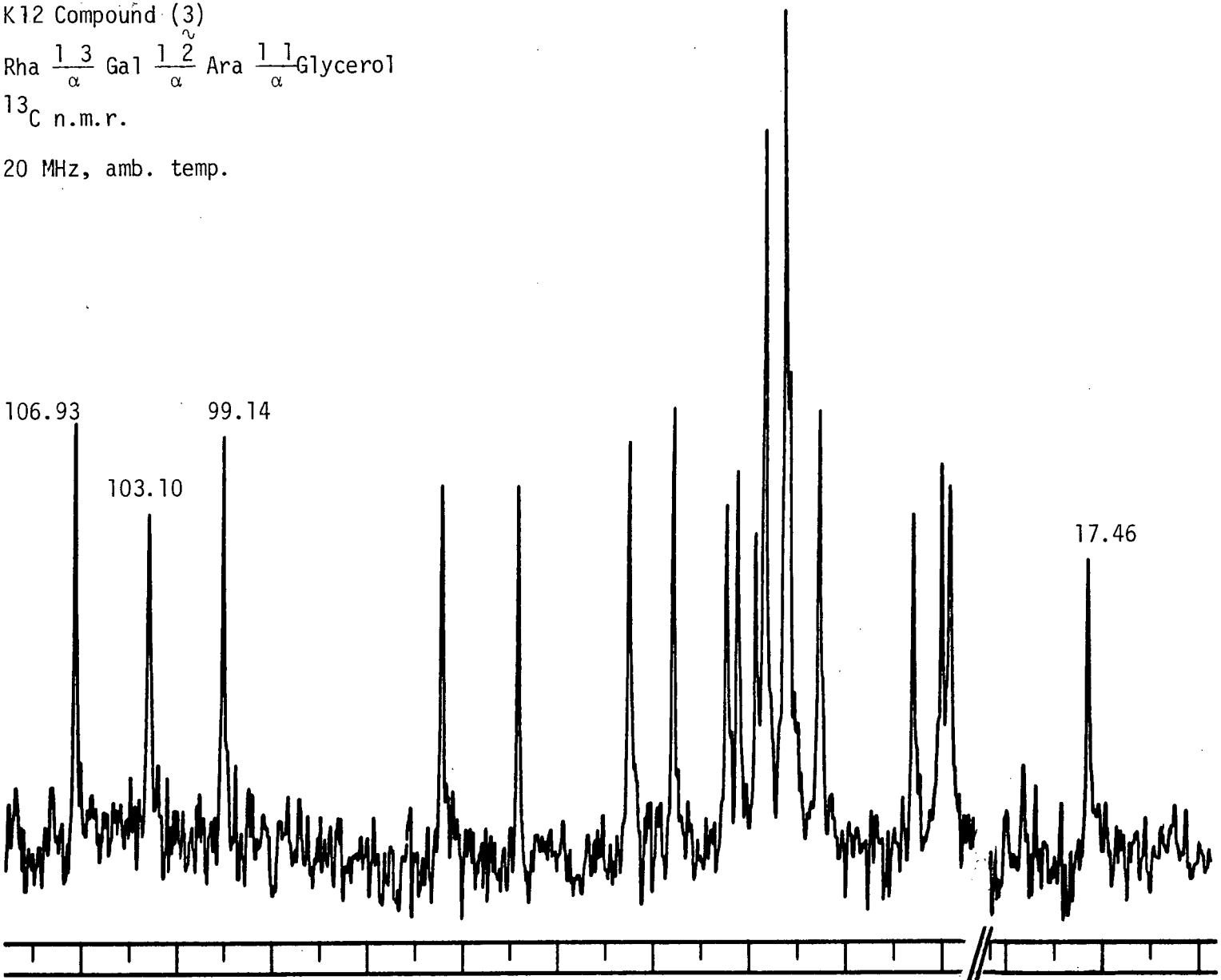
Spectrum No. 3

K12 Compound (3)

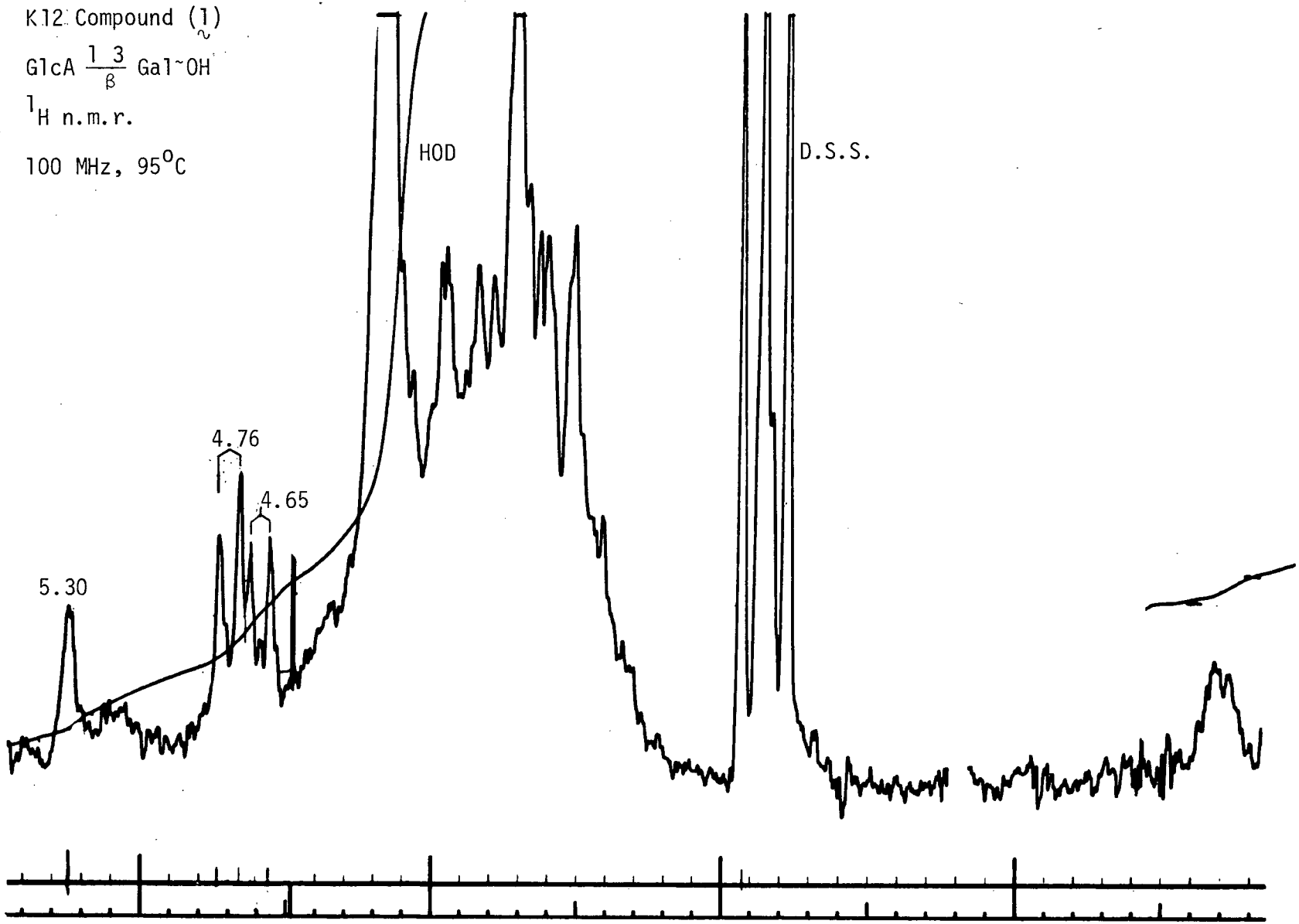
Rha $\frac{1\ 3}{\alpha}$ Gal $\frac{1\ 2}{\alpha}$ Ara $\frac{1\ 1}{\alpha}$ Glycerol

^{13}C n.m.r.

20 MHz, amb. temp.



Spectrum No. 4



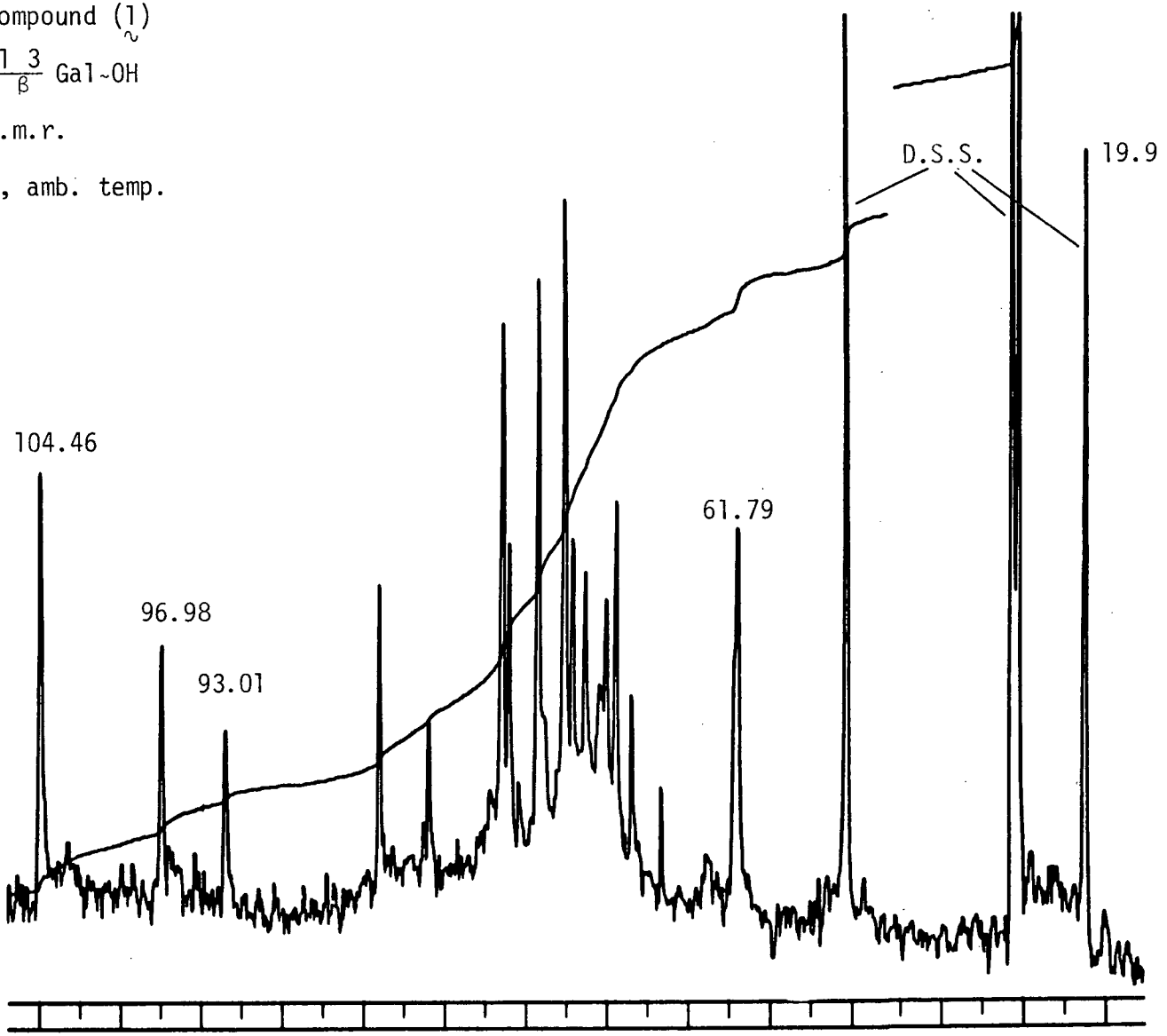
Spectrum No. 5

K-12 Compound (1)

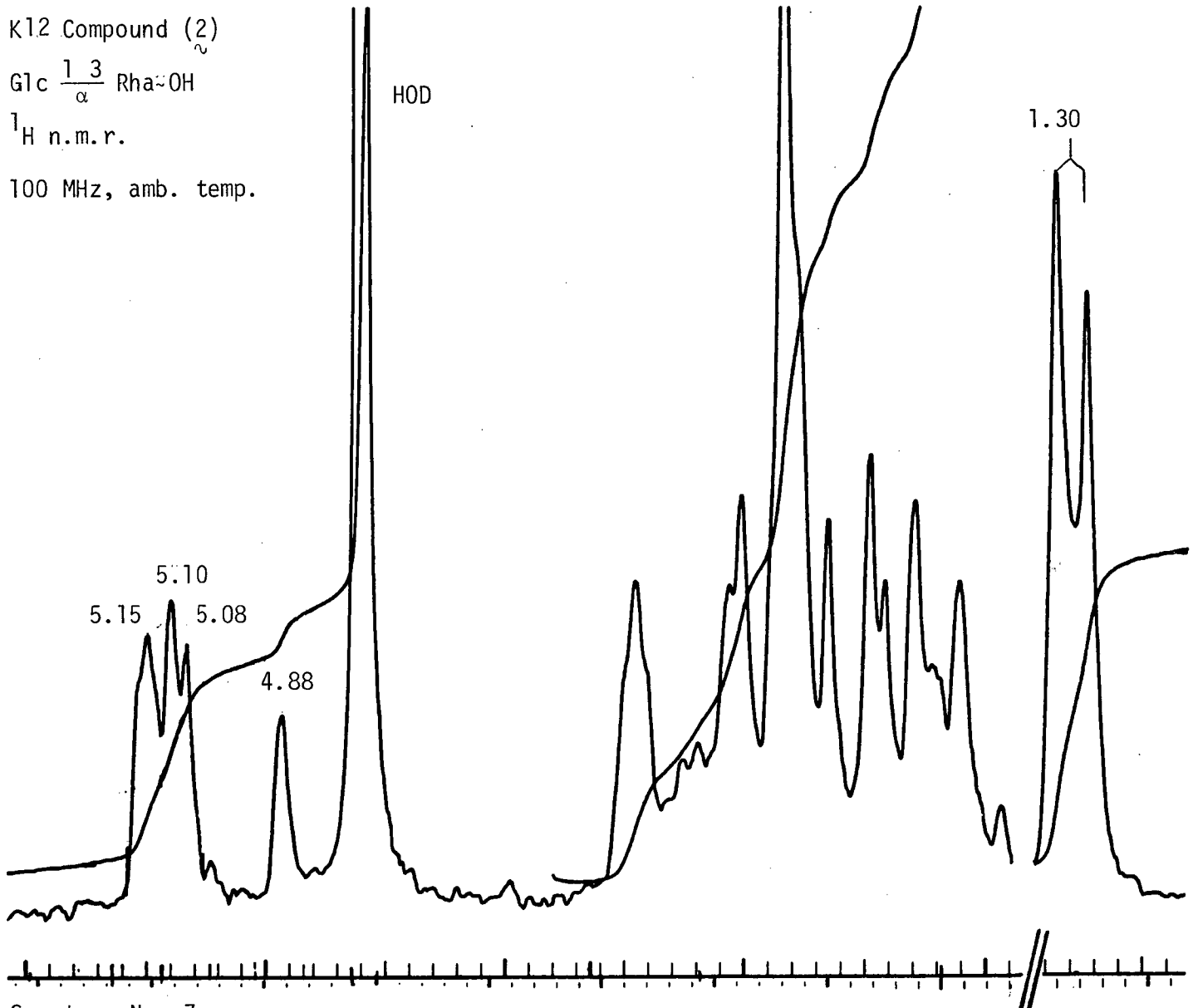
GlcA $\frac{1\ 3}{\beta}$ Gal-OH

^{13}C n.m.r.

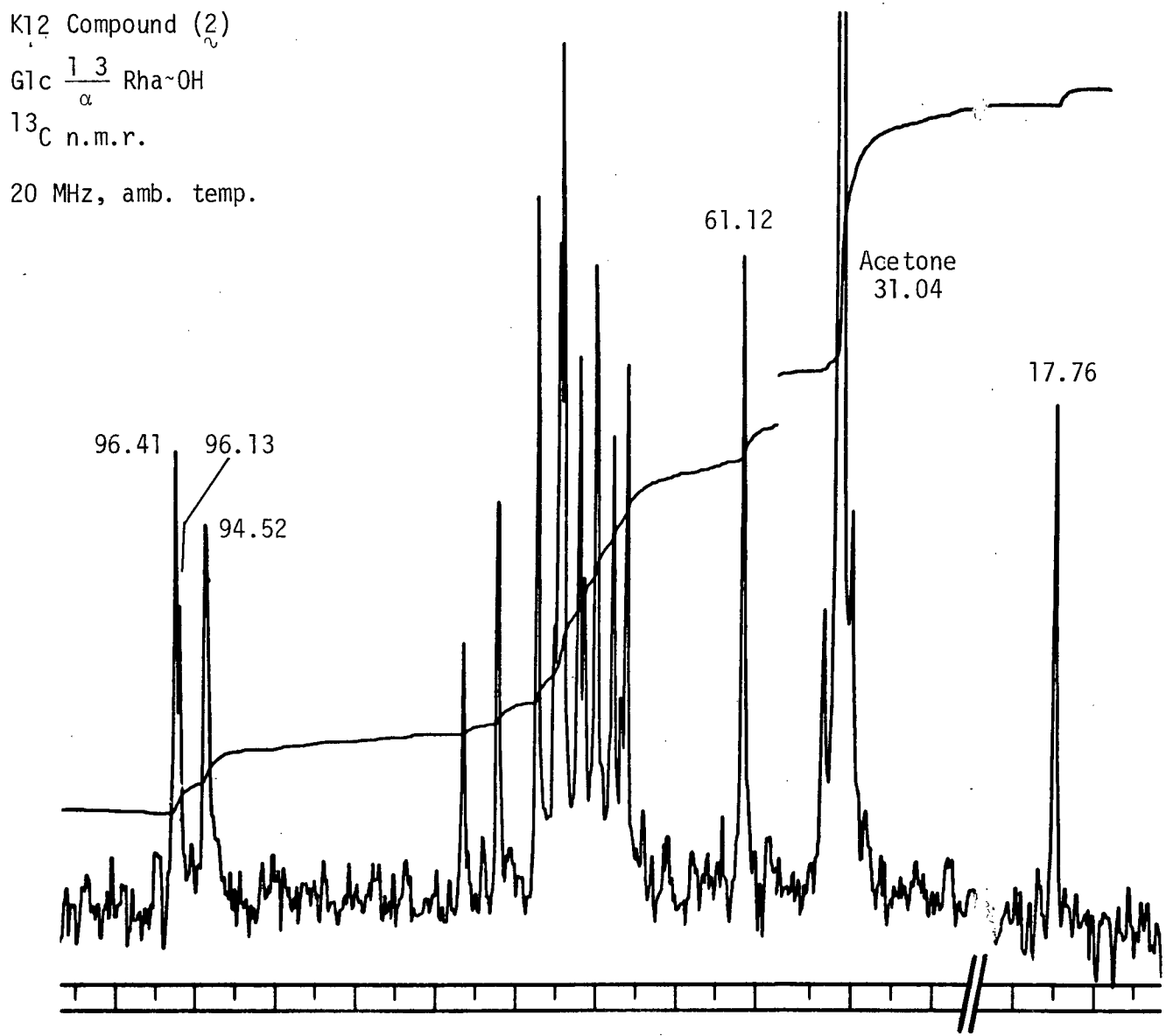
20MHz, amb. temp.



Spectrum No.6



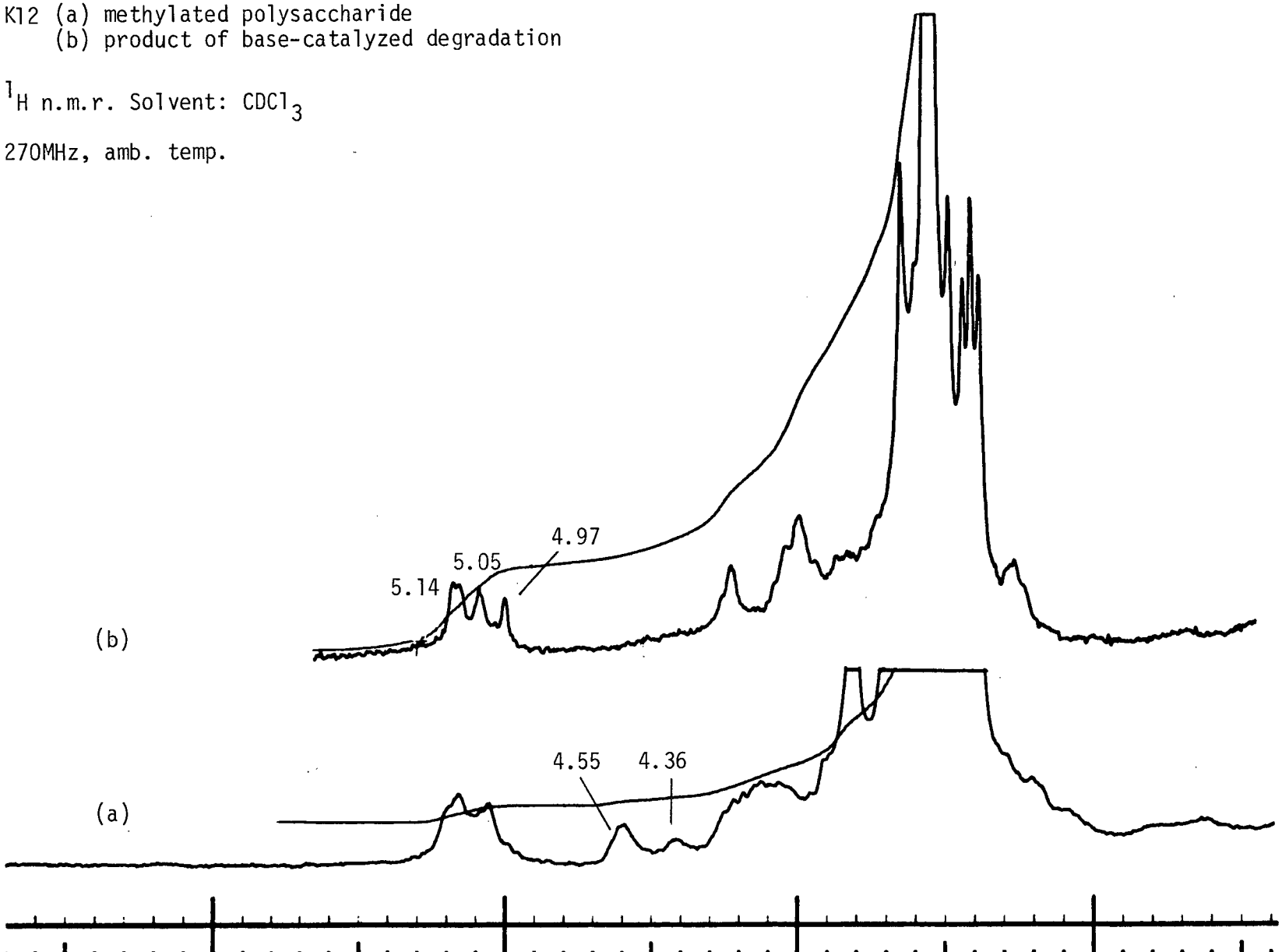
Spectrum No. 7


K12 Compound (2)
Glc 1 3 Rha~OH
α
13C n.m.r.
20 MHz, amb. temp.
61.12
Acetone
31.04
17.76
96.41
96.13
94.52
Spectrum No. 8

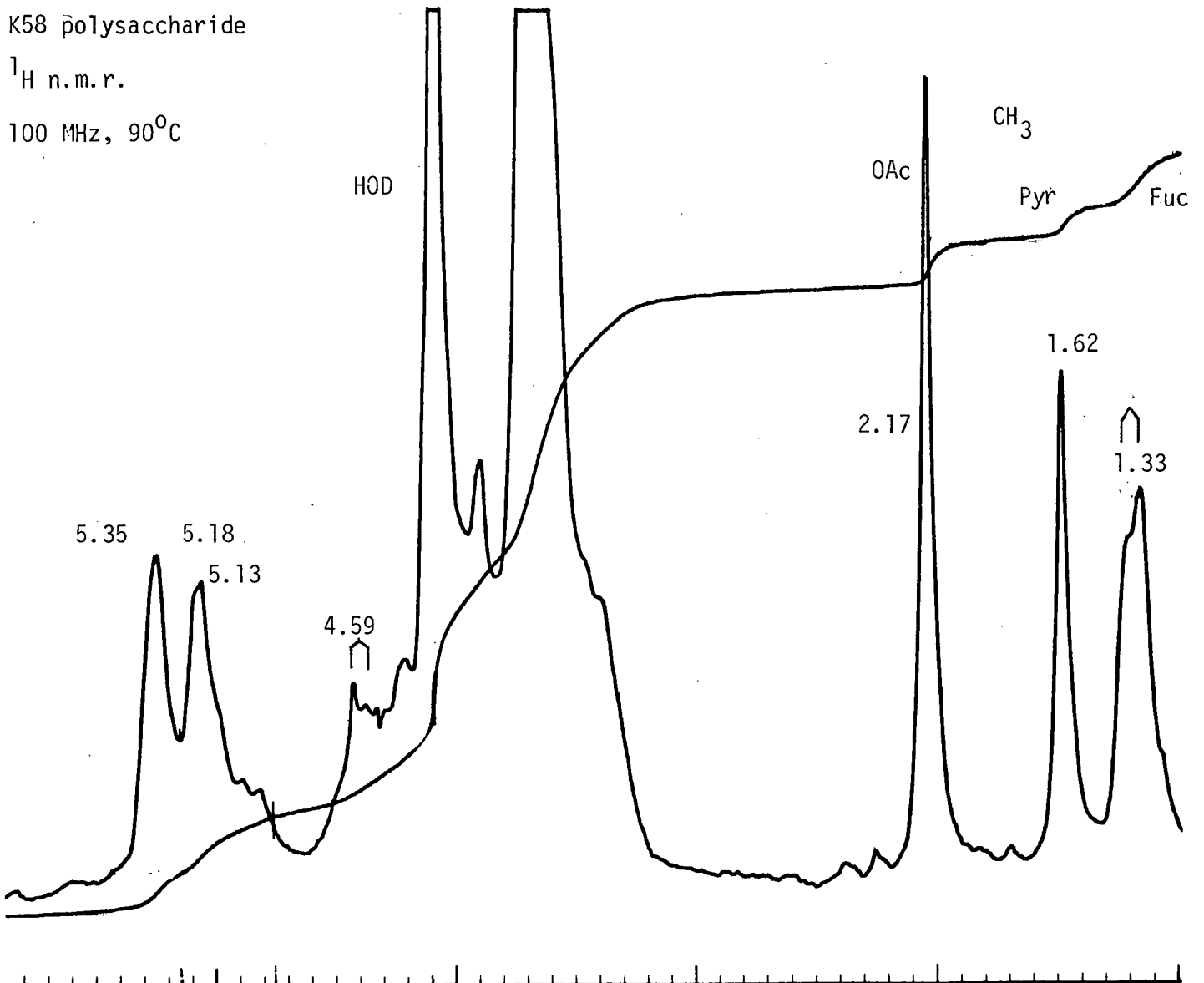
K12 (a) methylated polysaccharide
(b) product of base-catalyzed degradation

^1H n.m.r. Solvent: $CDCl_3$

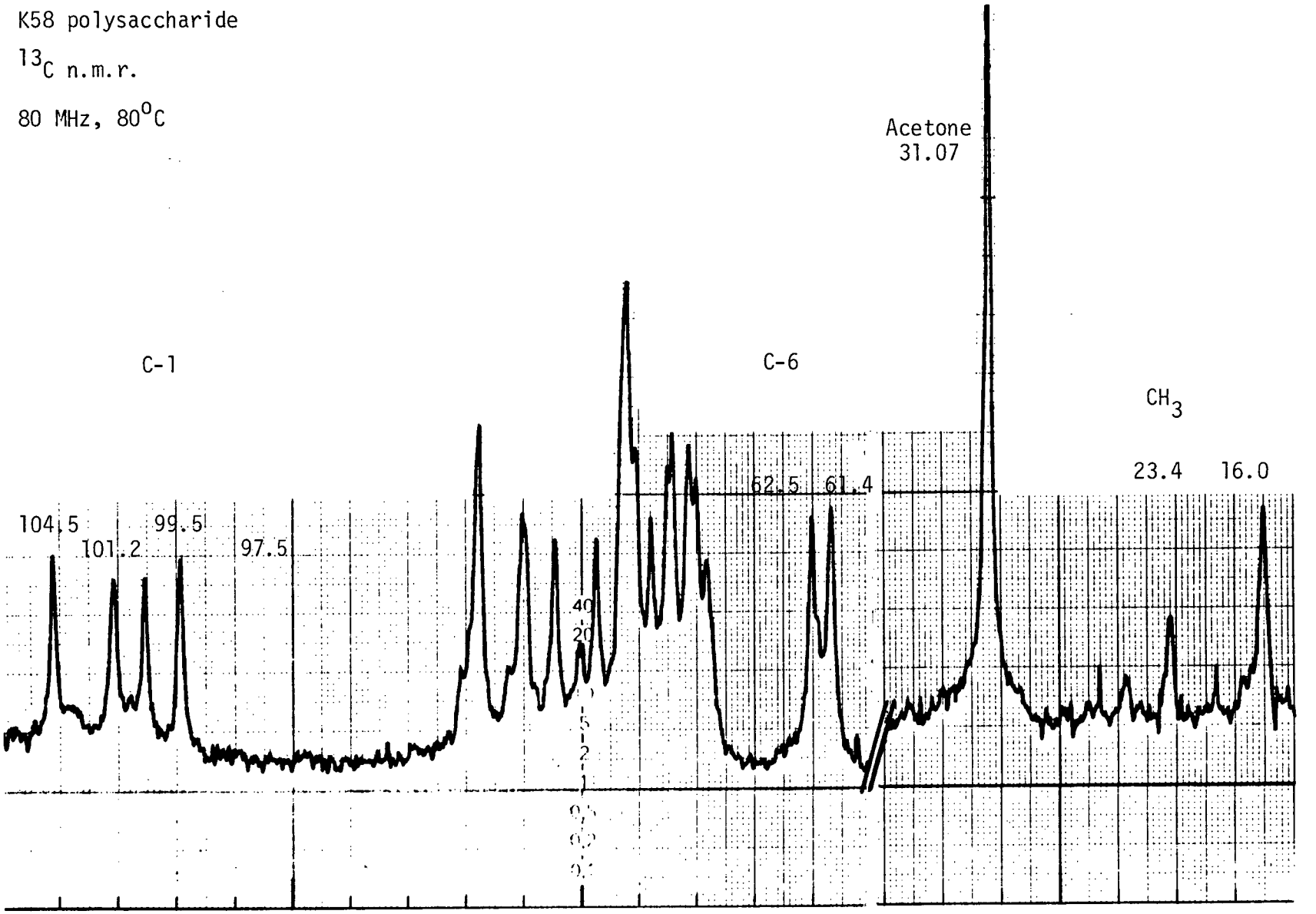
270MHz, amb. temp.

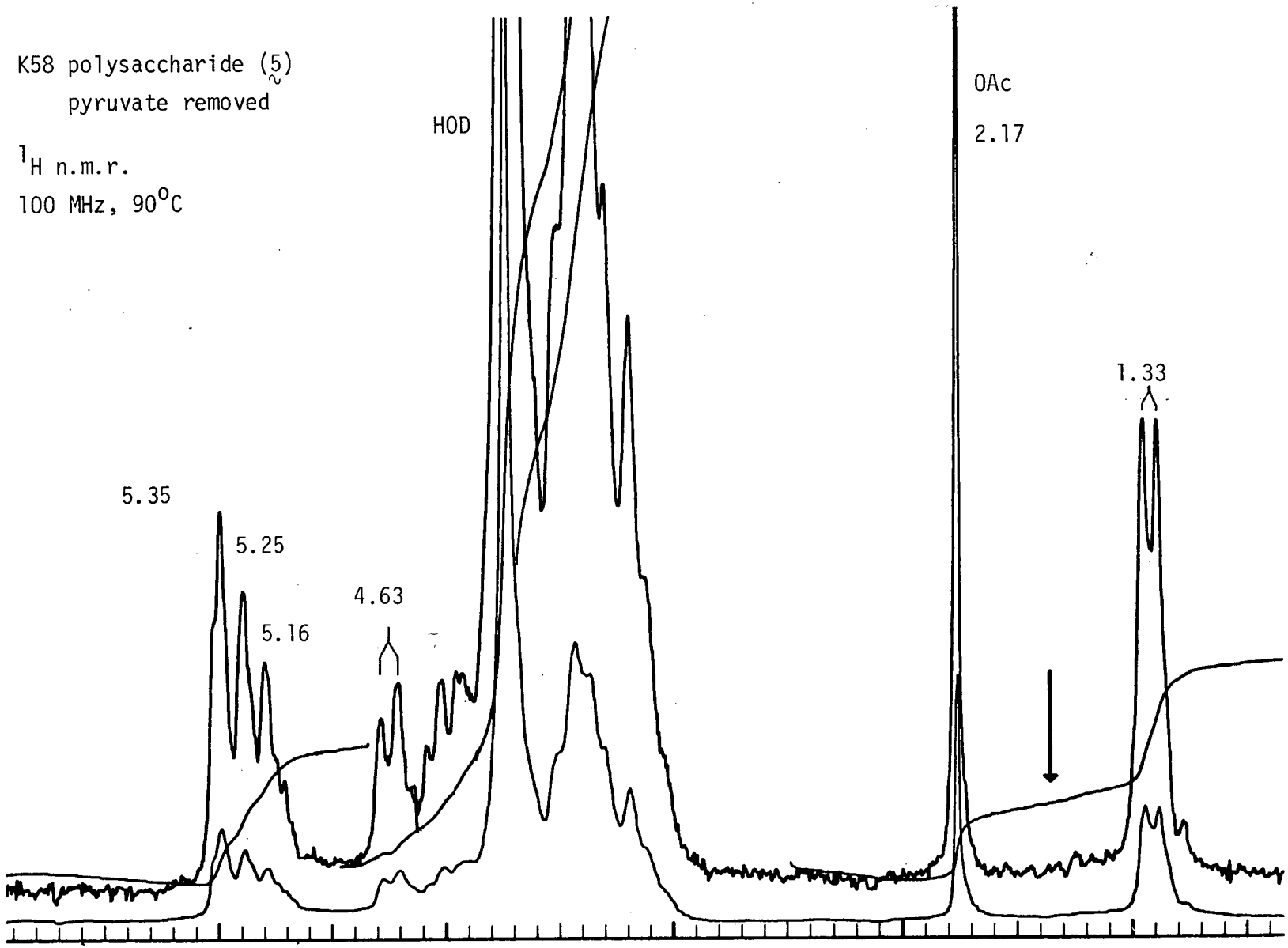


Spectrum No. 9



Spectrum No. 10


K58 polysaccharide
13C n.m.r.
80 MHz, 80°C
Acetone
31.07
C-1
C-6
CH3
104.5
101.2
99.5
97.5
62.5
61.4
23.4
16.0
40
20
Spectrum No. 11



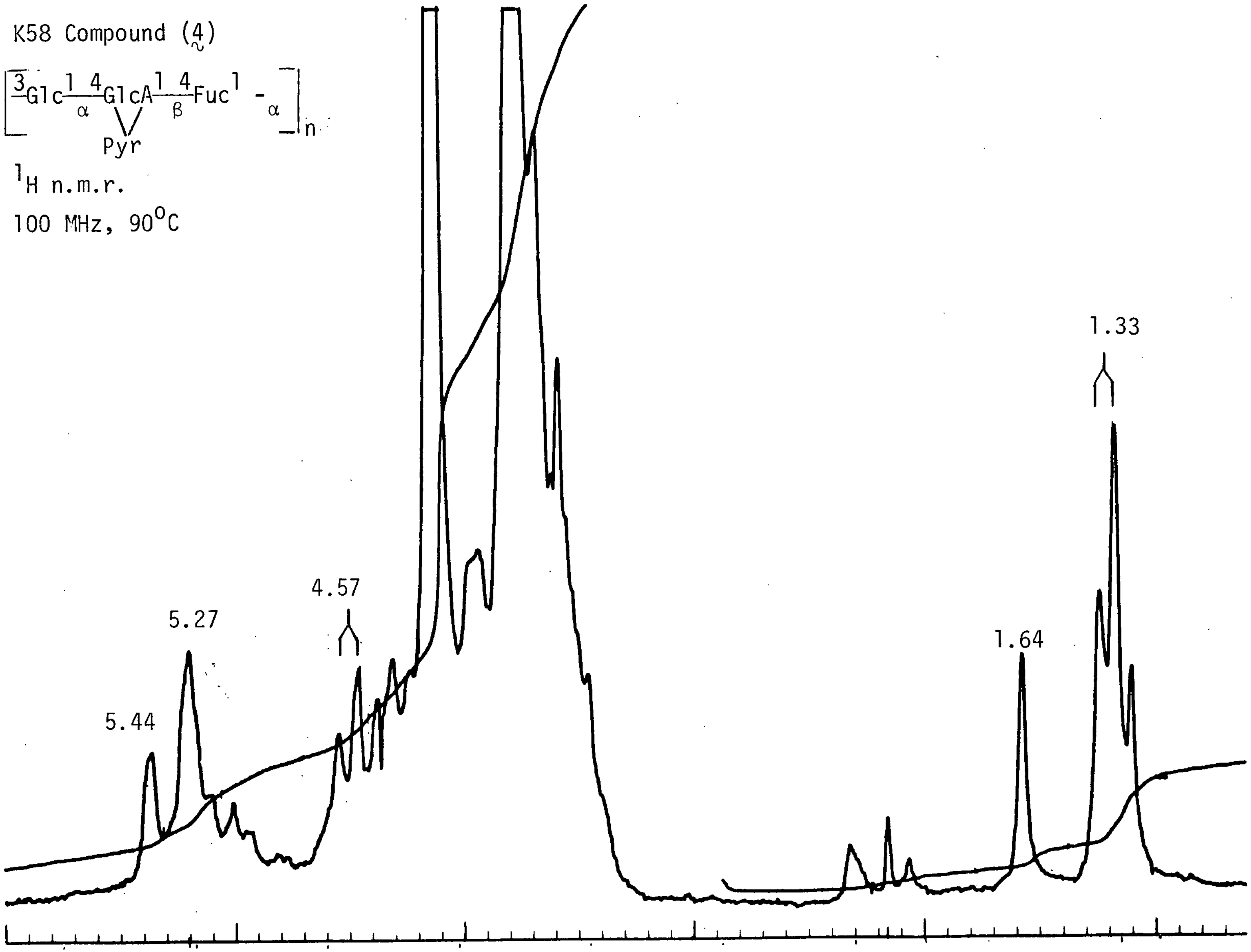
Spectrum No. 12

K58 Compound (4)

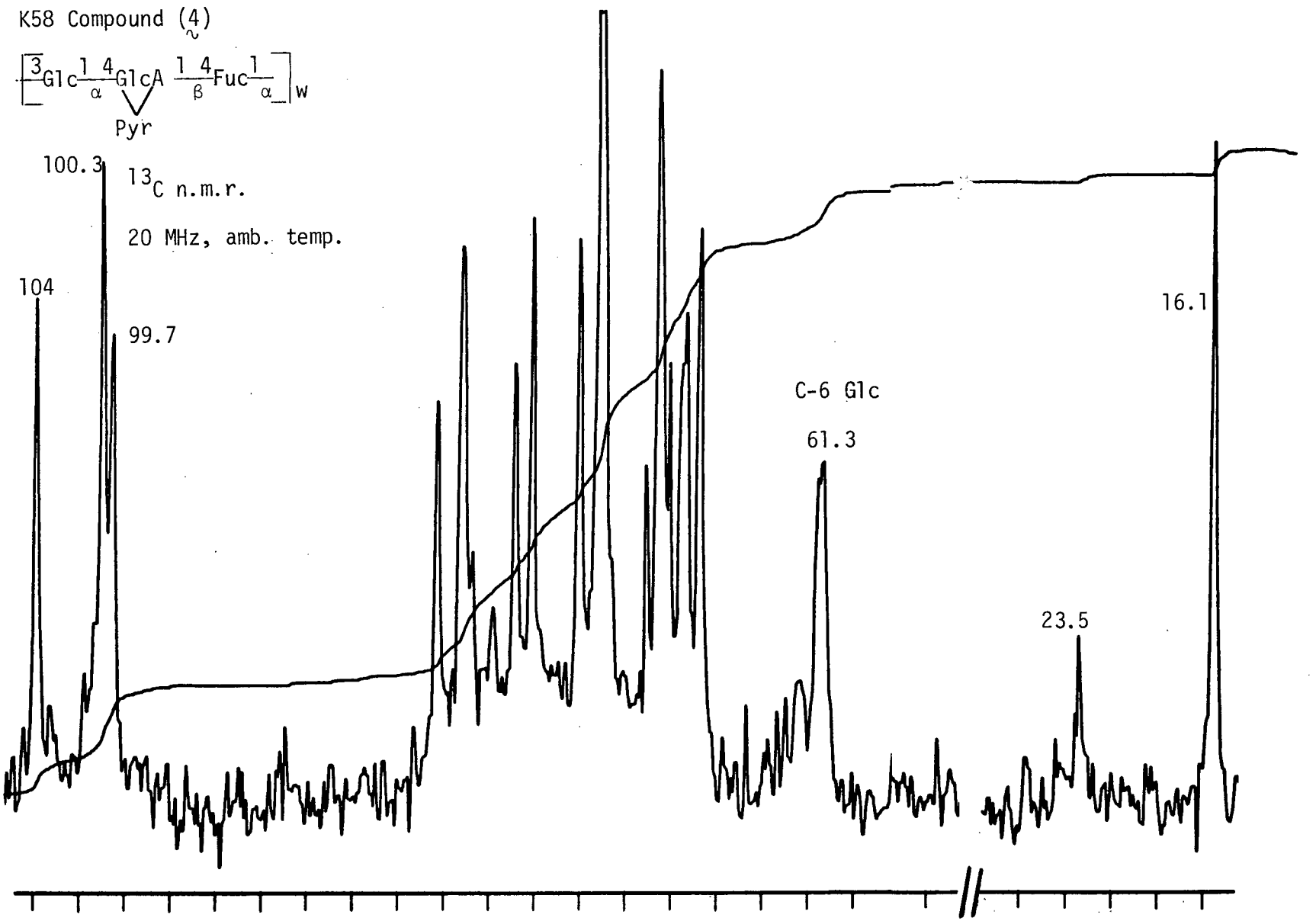
$$\left[\,{}^{3}\text{Glc}\,\frac{1\quad 4}{\alpha}\,\underset{\text{Pyr}}{\text{GlcA}}\,\frac{1\quad 4}{\beta}\,\text{Fuc}^{1}\ -_{\alpha}\right]_n$$

^{1}H n.m.r.

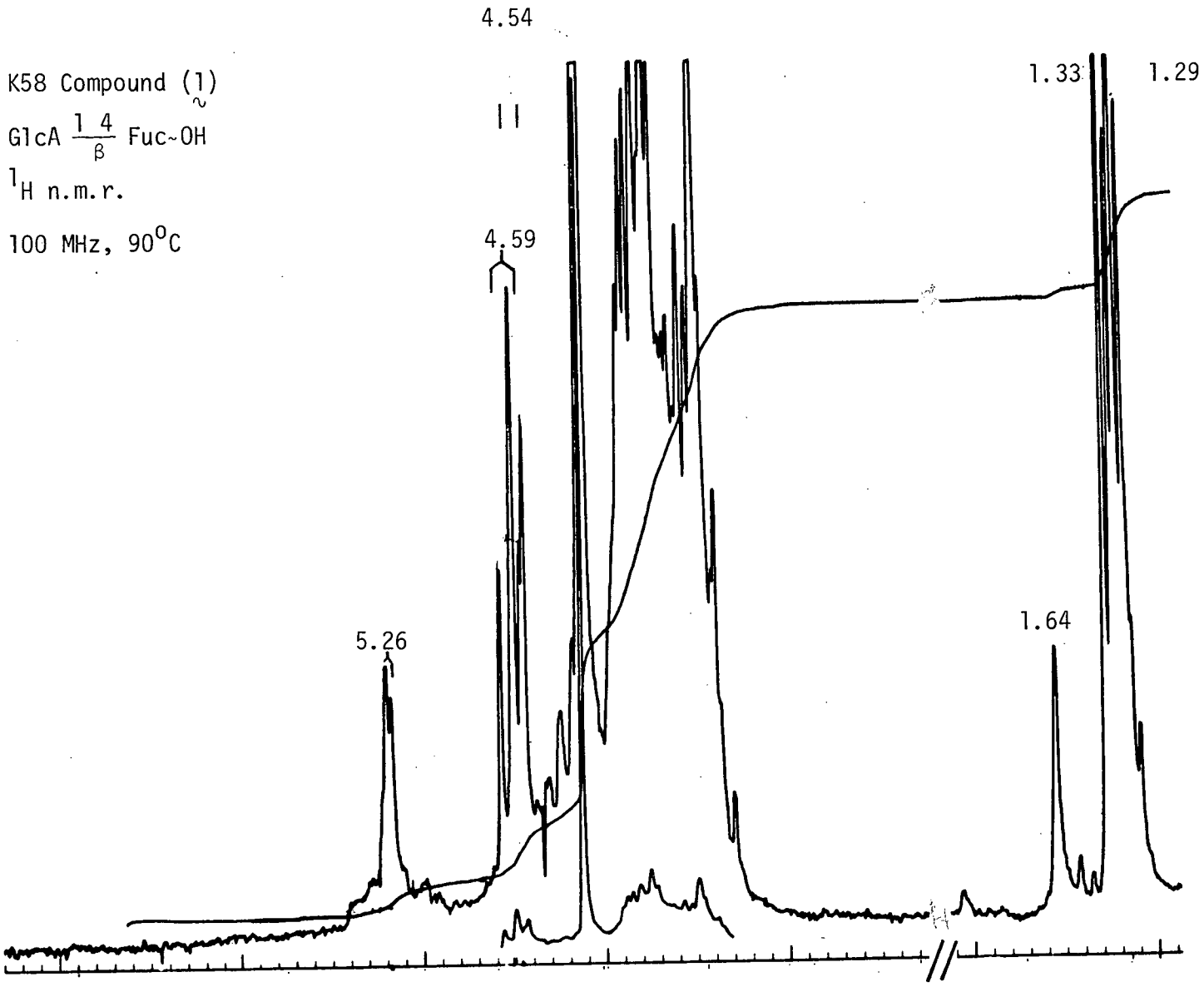
100 MHz, 90°C



Spectrum No. 13



Spectrum No. 14



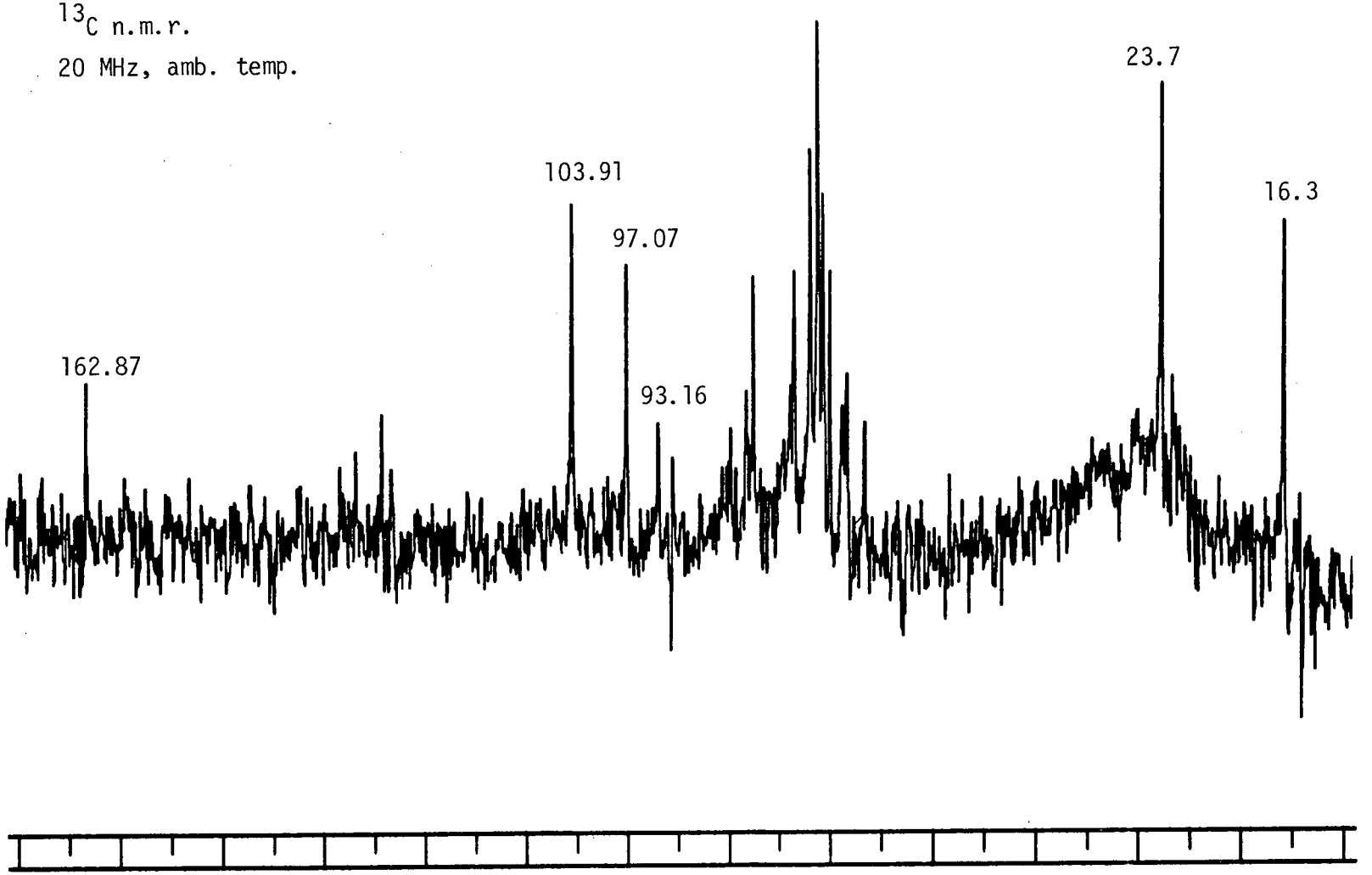
Spectrum No. 15

K58 Compound (1)

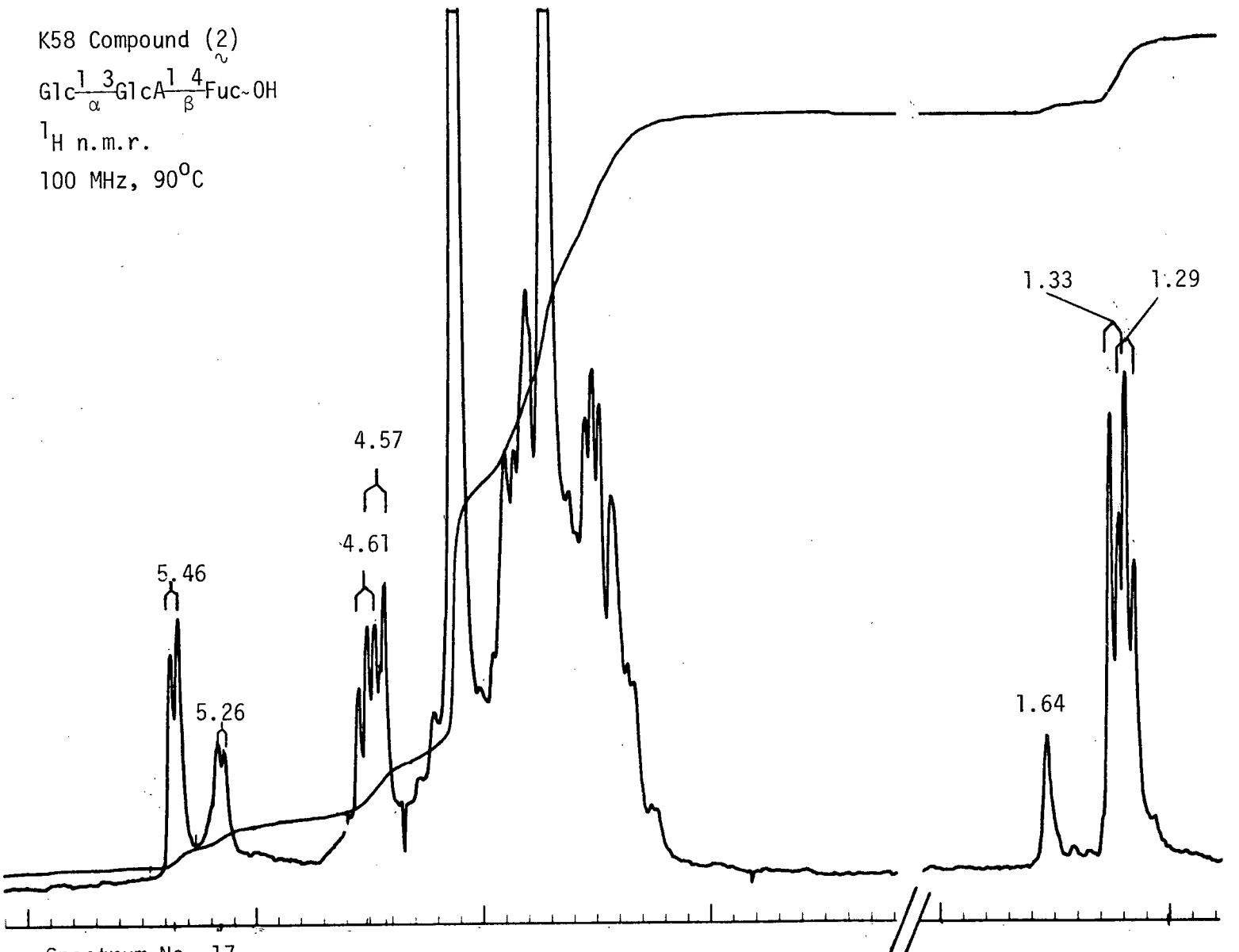
GlcA $\frac{1\ 4}{\beta}$ Fuc~OH

^{13}C n.m.r.

20 MHz, amb. temp.



Spectrum No. 16

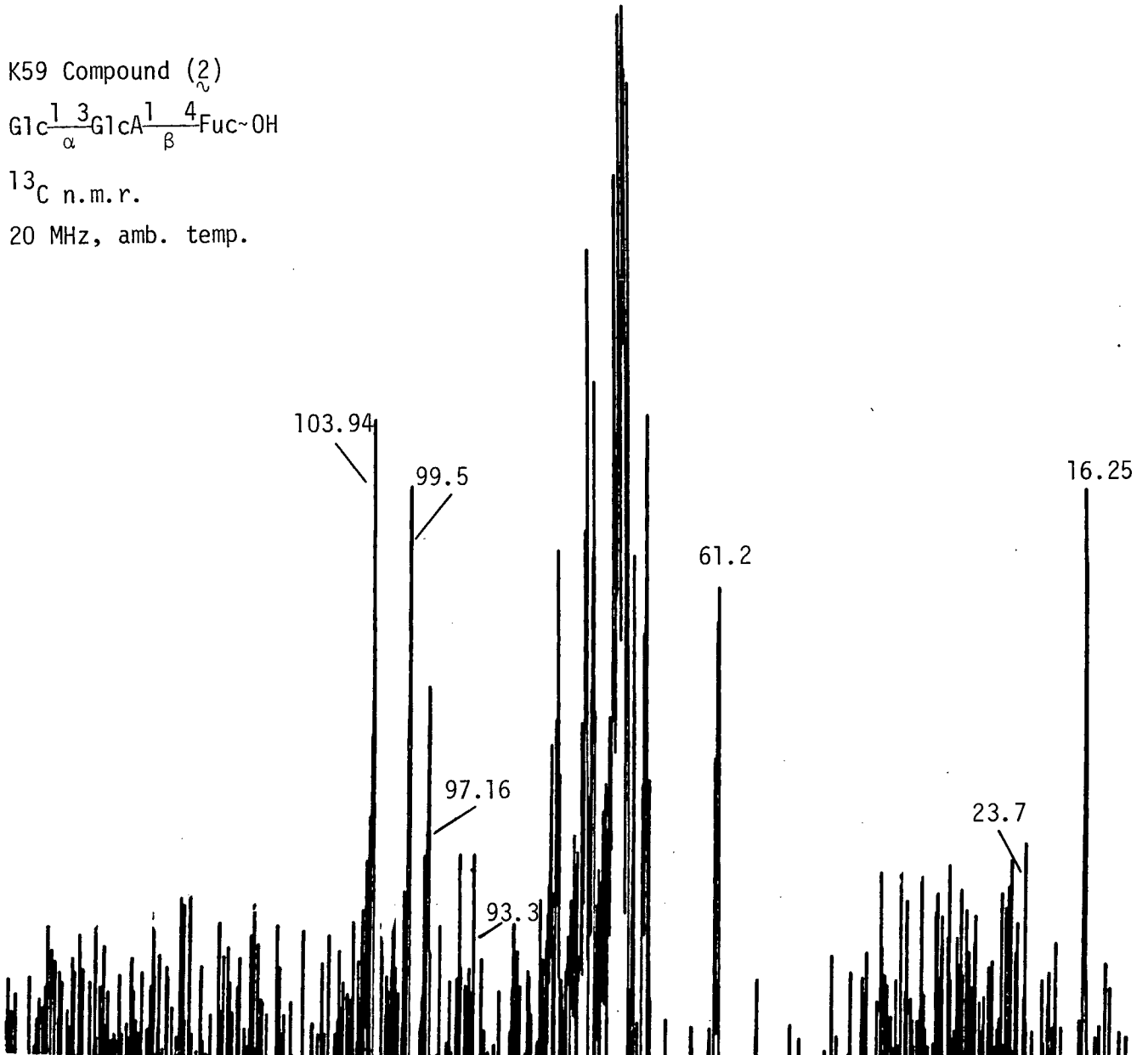

K58 Compound (2)
Glc α1 3 GlcA β1 4 Fuc-OH
1H n.m.r.
100 MHz, 90°C
1.33
1.29
4.57
4.61
5.46
5.26
1.64
Spectrum No. 17

K59 Compound (2)

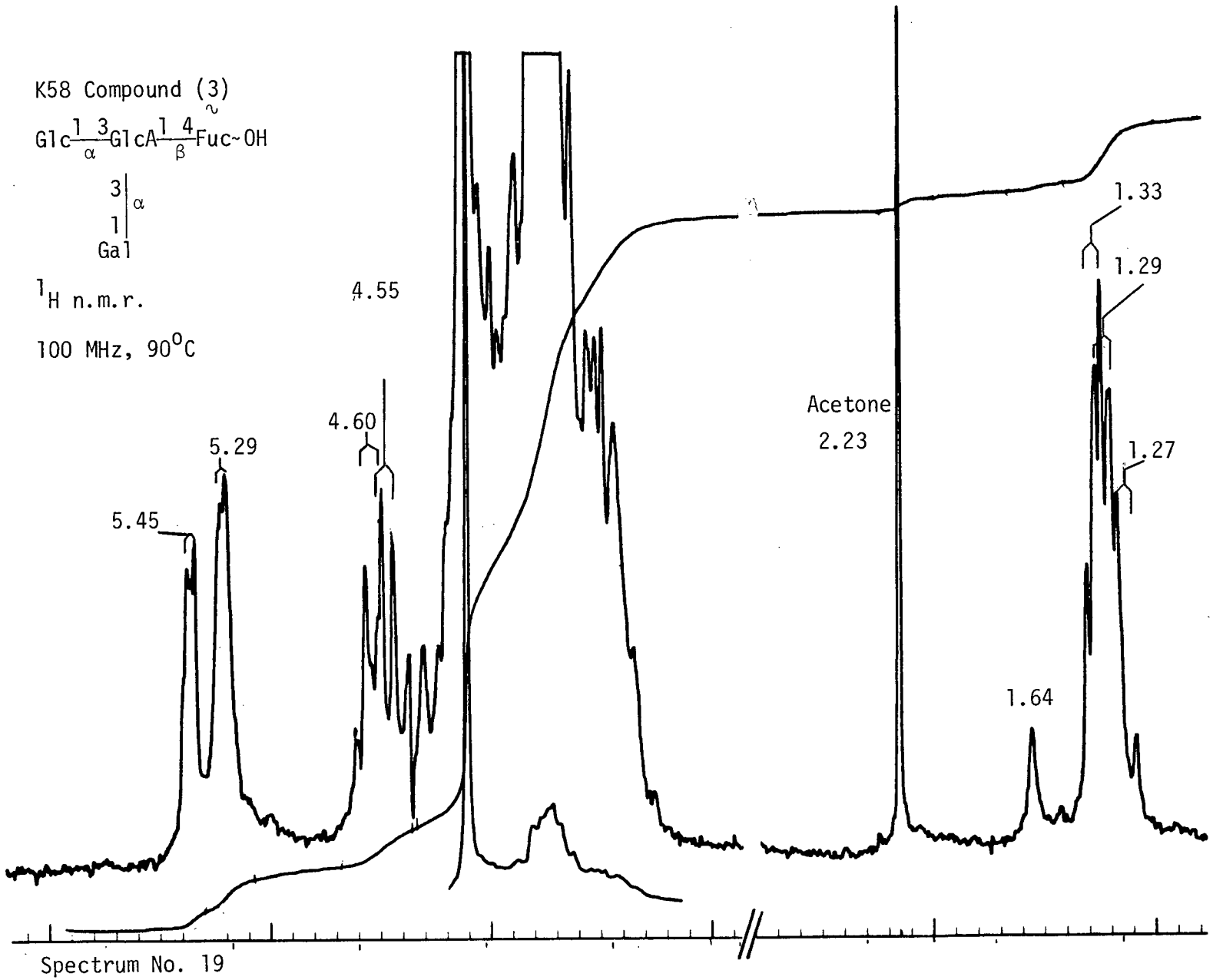
Glc$\underset{\alpha}{1\ 3}$GlcA$\underset{\beta}{1\ 4}$Fuc~OH

^{13}C n.m.r.

20 MHz, amb. temp.



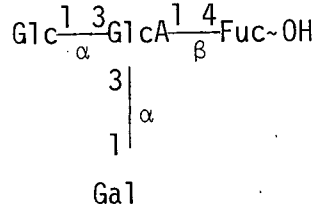
Spectrum No. 18


K58 Compound (3)
Glc α1 3 GlcA β1 4 Fuc~OH
3 α
1
Gal
1H n.m.r.
100 MHz, 90°C
5.45
5.29
4.60
4.55
Acetone
2.23
1.64
1.33
1.29
1.27
Spectrum No. 19

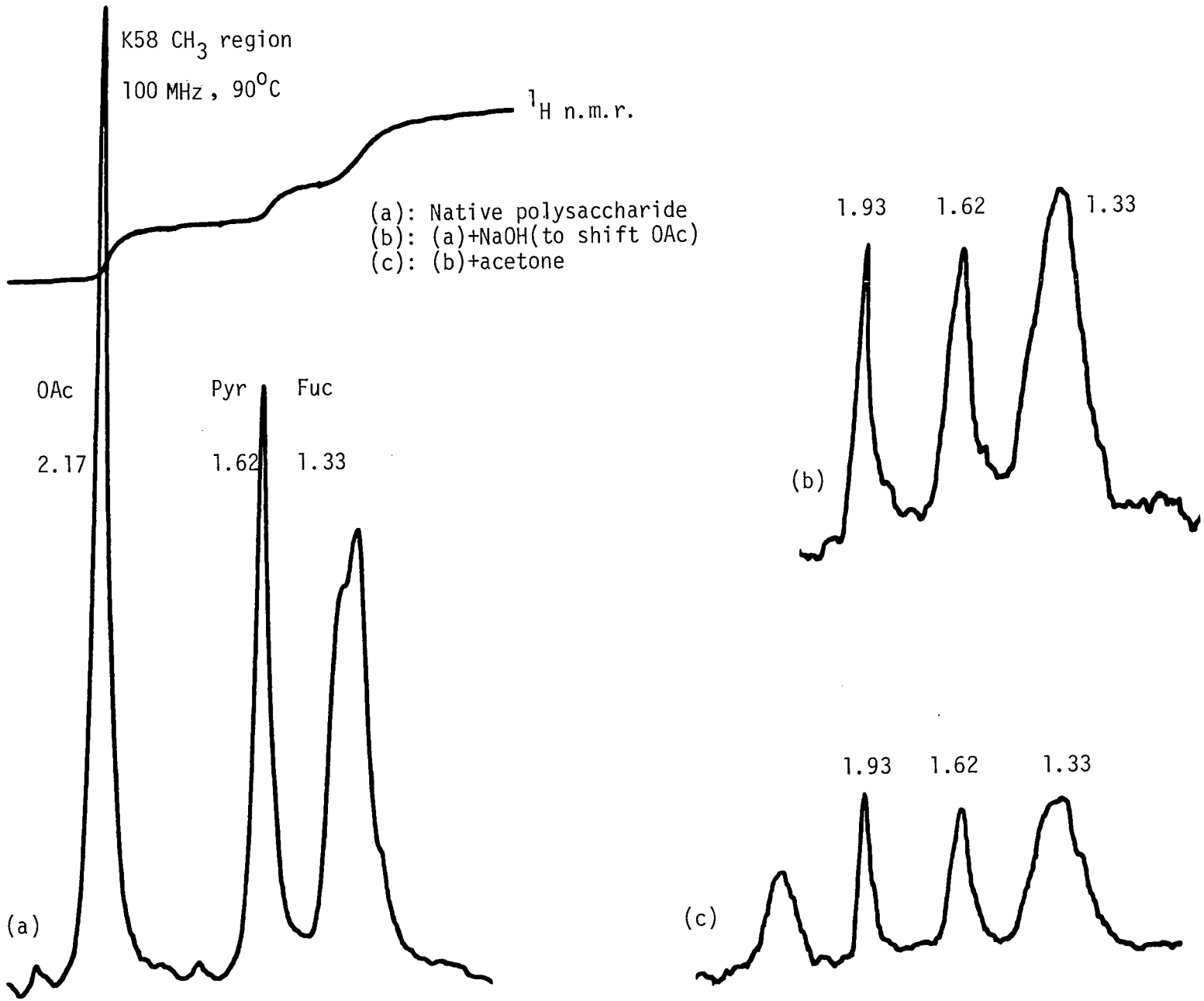
K58 Compound (3)

^{13}C n.m.r.

20-1 MHz, amb. temp.



Spectrum NO.20

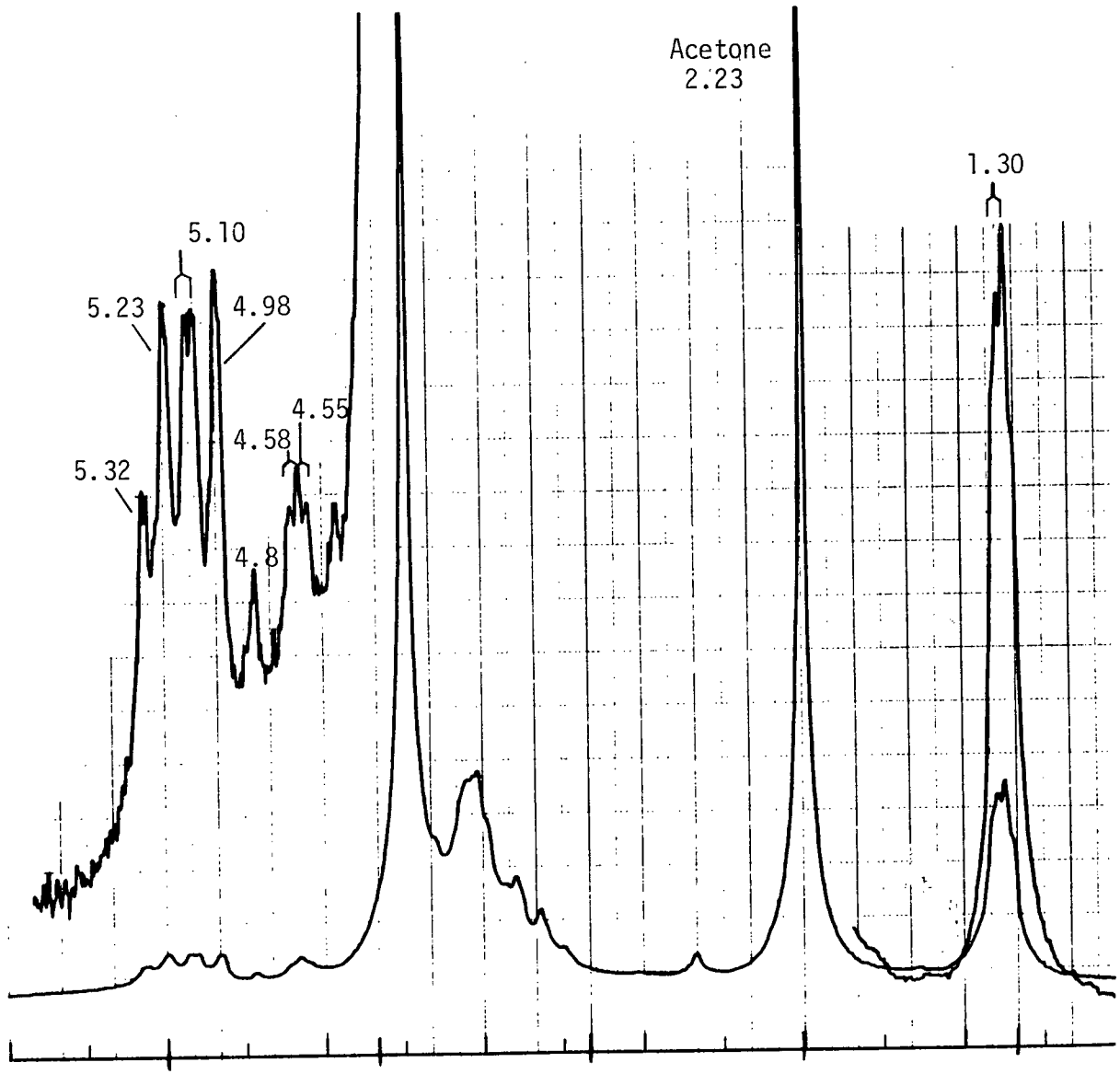


Spectrum No. 21

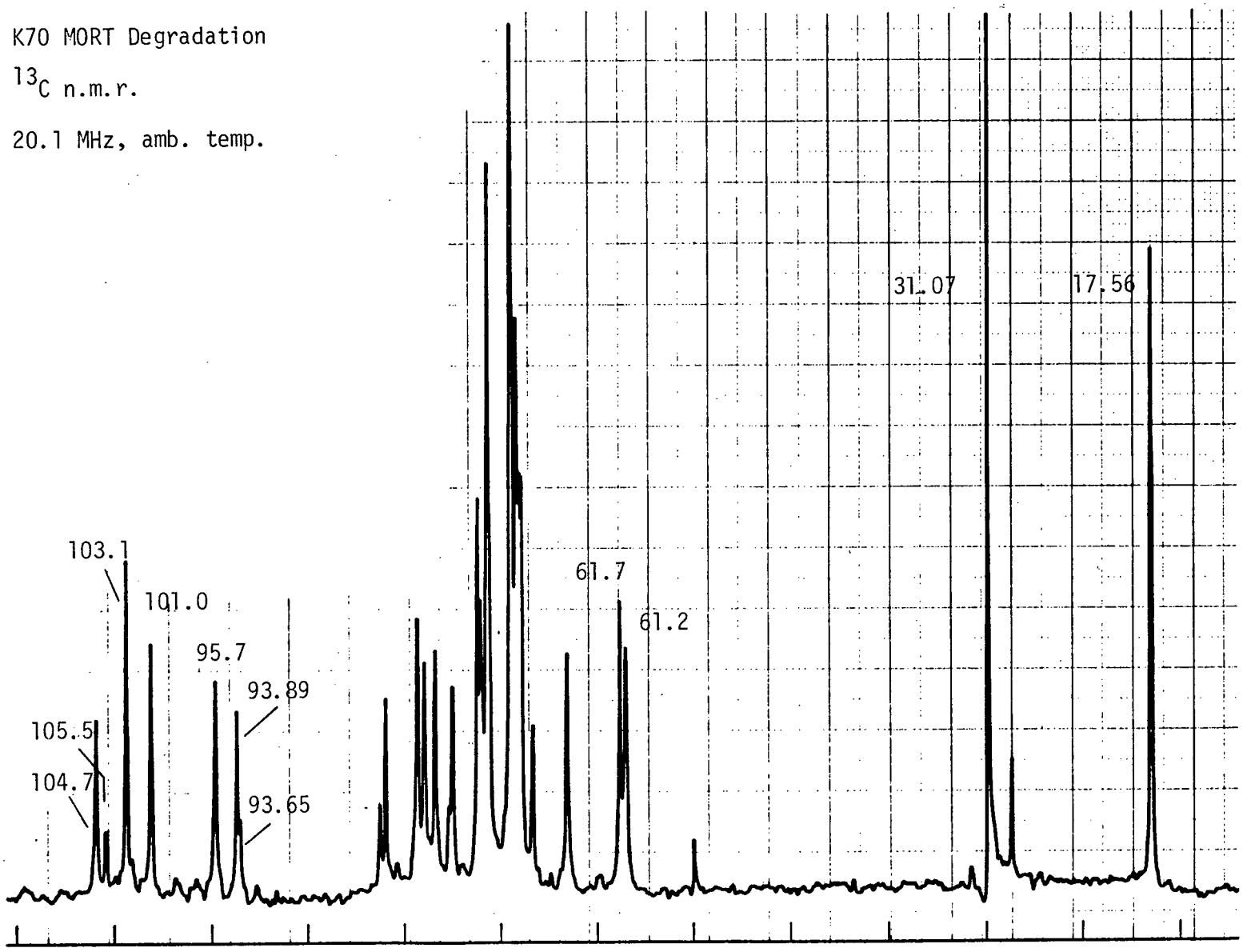
K70 MORT degradation

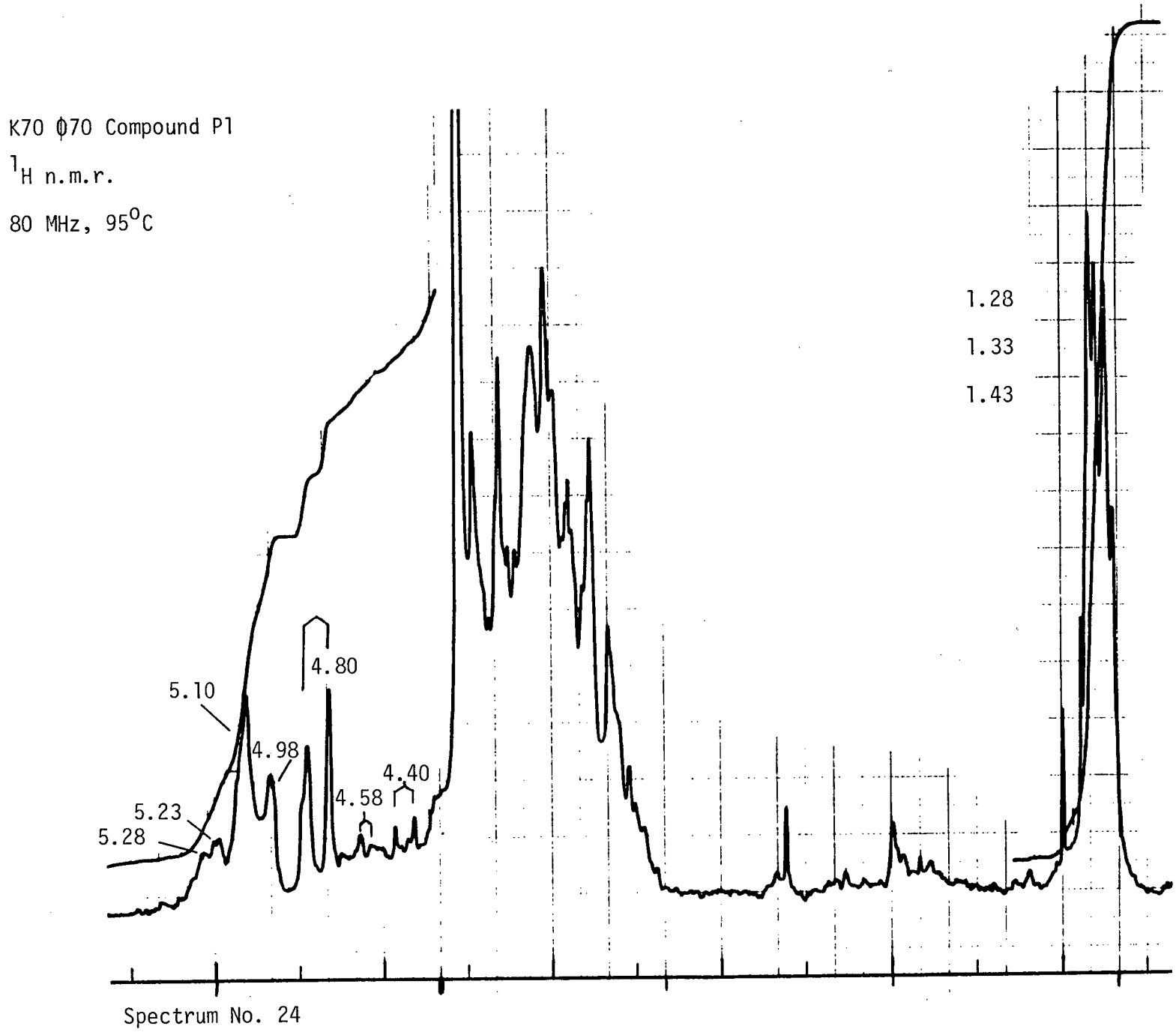
^{1}H n.m.r.

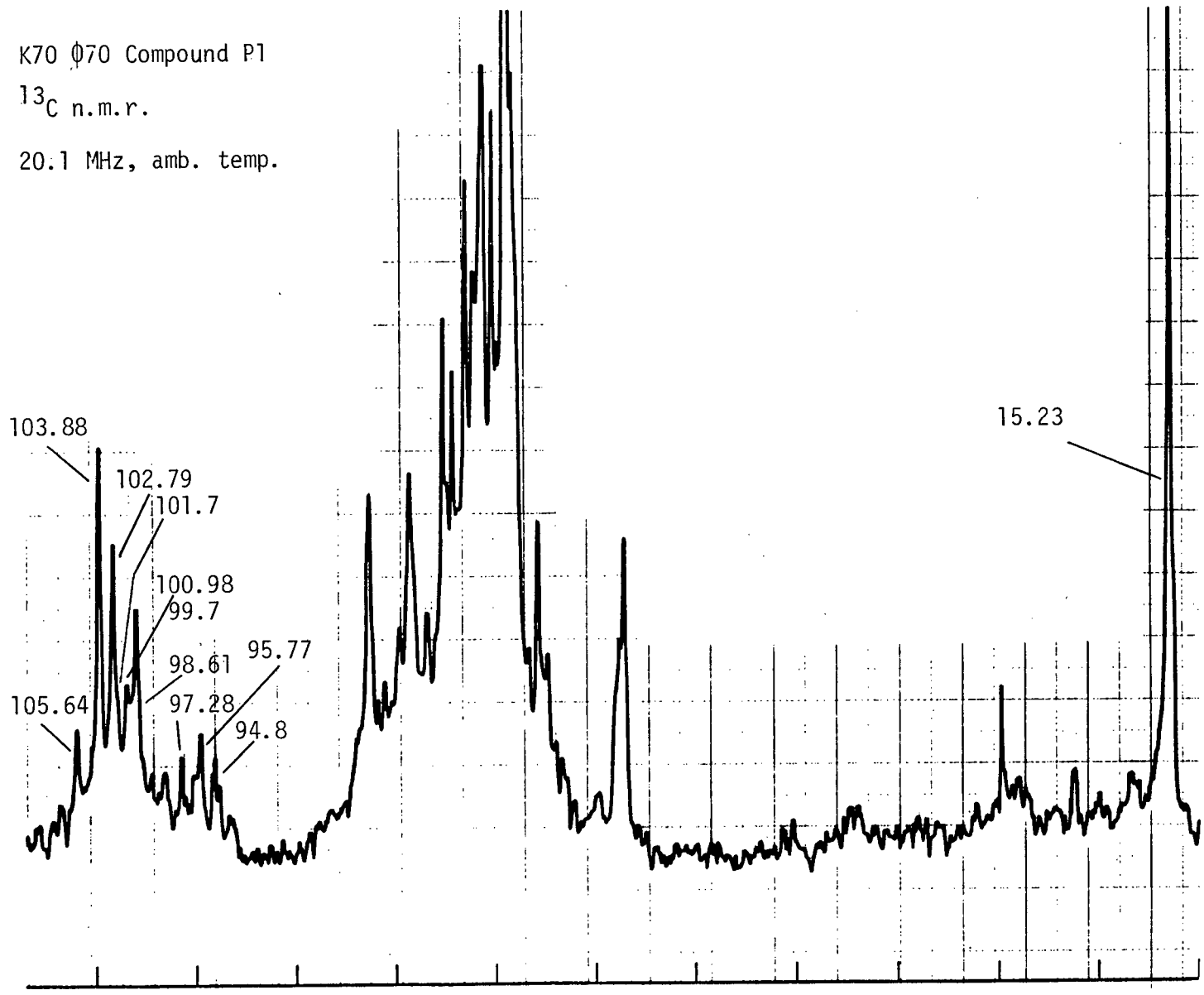
80 MHz, 95°C



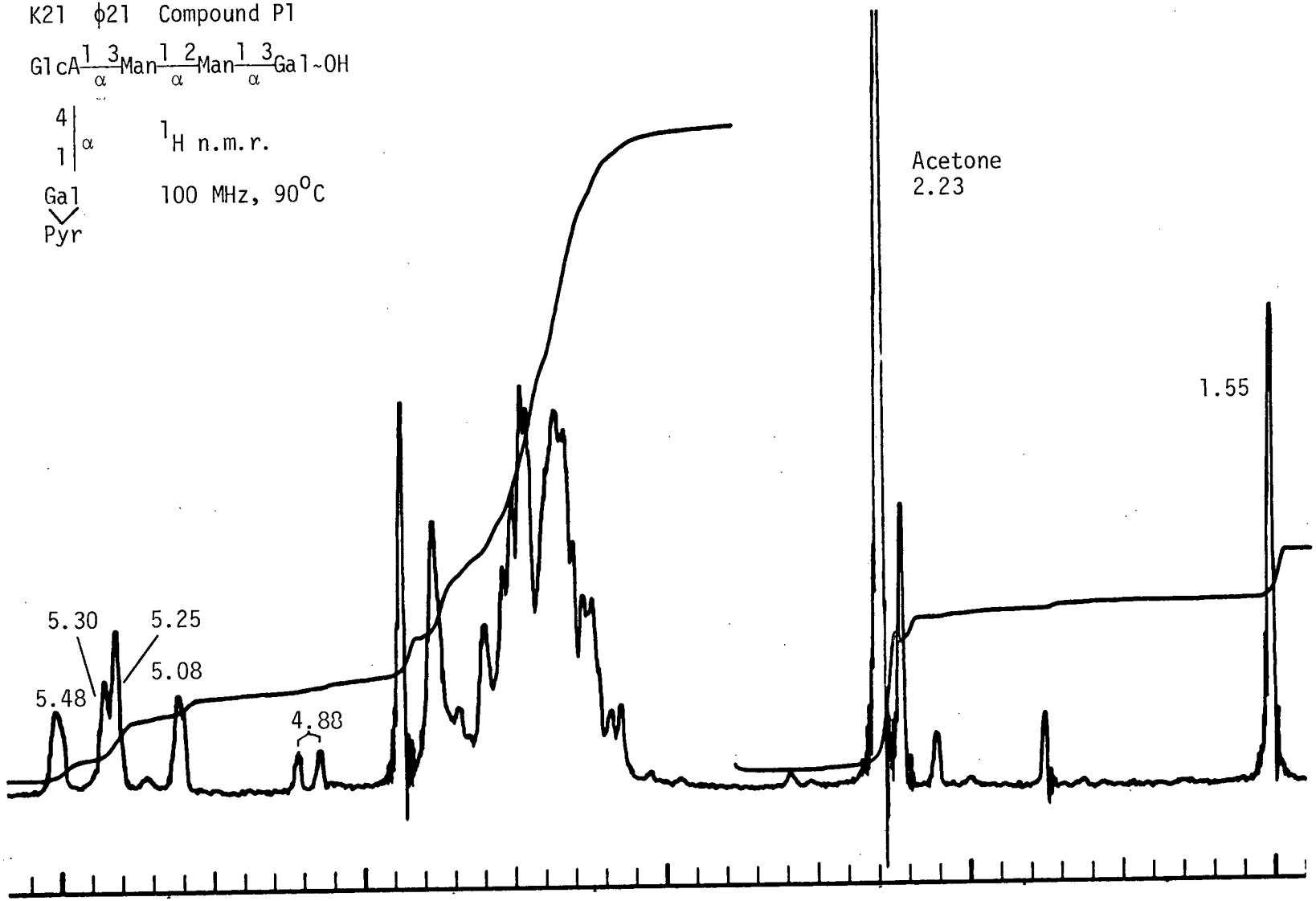
Spectrum No. 22


K70 MORT Degradation
13C n.m.r.
20.1 MHz, amb. temp.
103.1
101.0
95.7
93.89
105.5
104.7
93.65
61.7
61.2
31.07
17.56
Spectrum No. 23


K70 ϕ70 Compound P1
^{1}H n.m.r.
80 MHz, 95°C
1.28
1.33
1.43
4.80
5.10
4.98
4.40
4.58
5.23
5.28
Spectrum No. 24



Spectrum No. 25



Spectrum No. 26

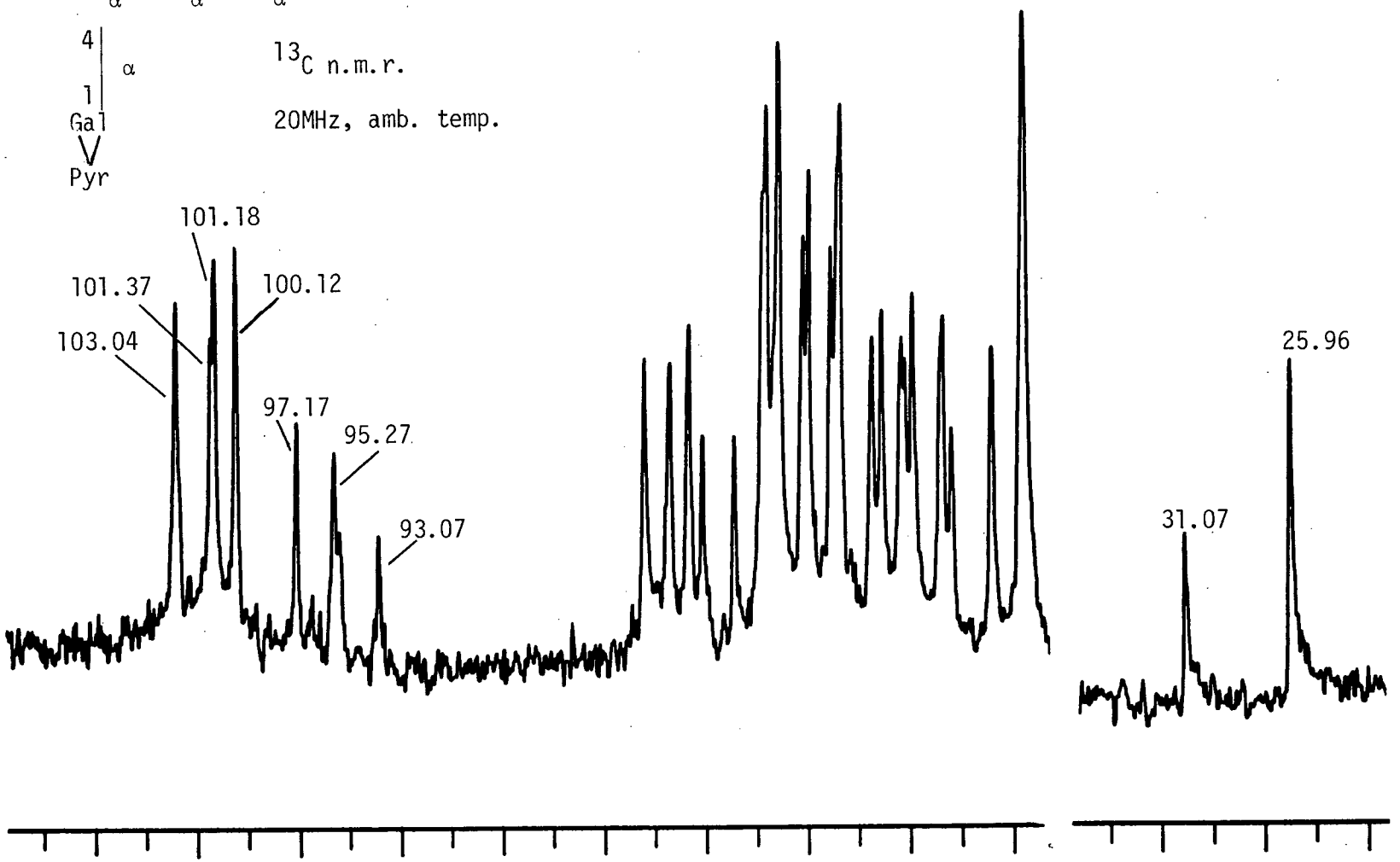
K21 ϕ21 Compound P1

GlcA $\overset{1\ 3}{\underset{\alpha}{-}}$ Man $\overset{1\ 2}{\underset{\alpha}{-}}$ Man $\overset{1\ 3}{\underset{\alpha}{-}}$ Gal ~OH

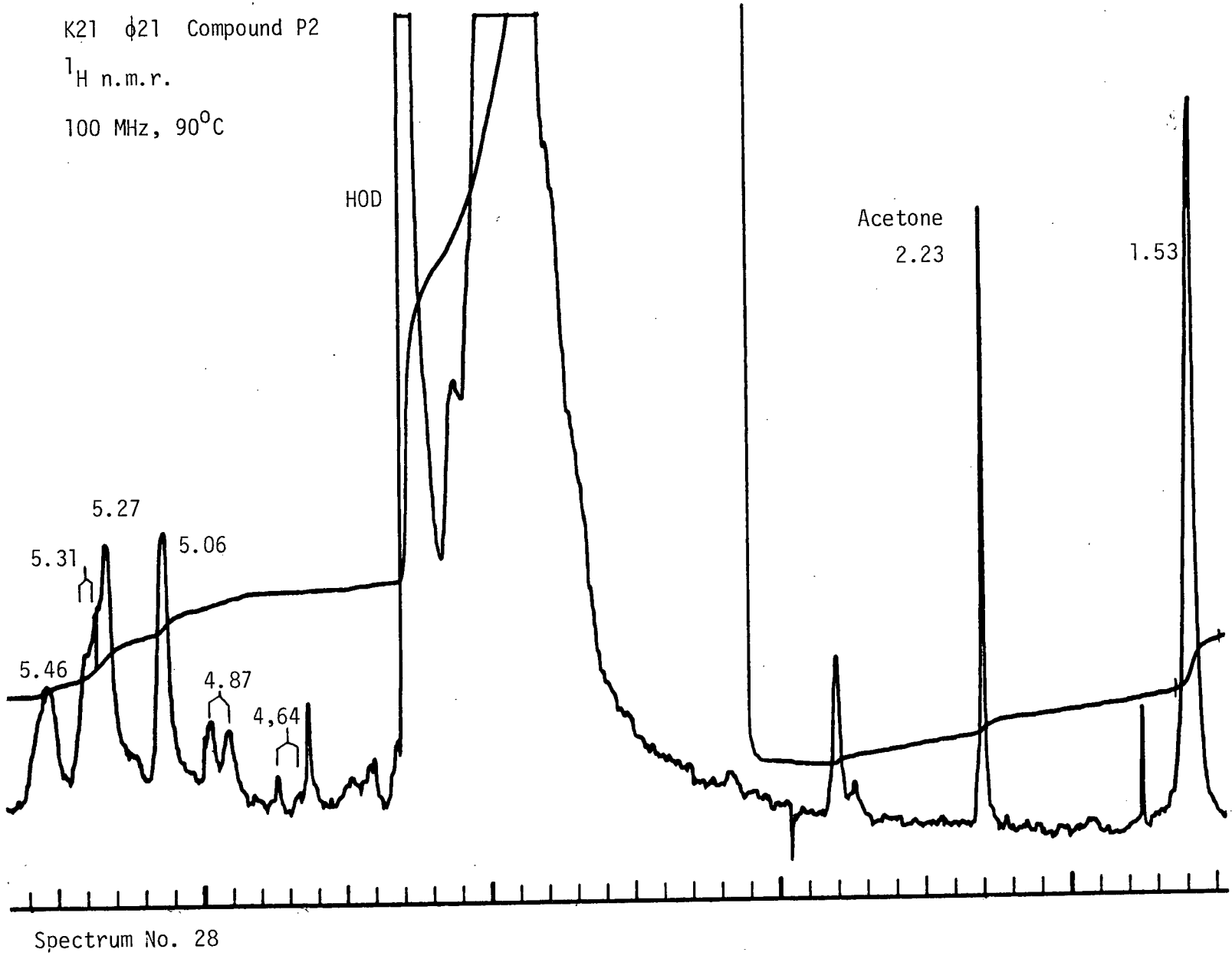
4 | α
1 | Gal
V
Pyr

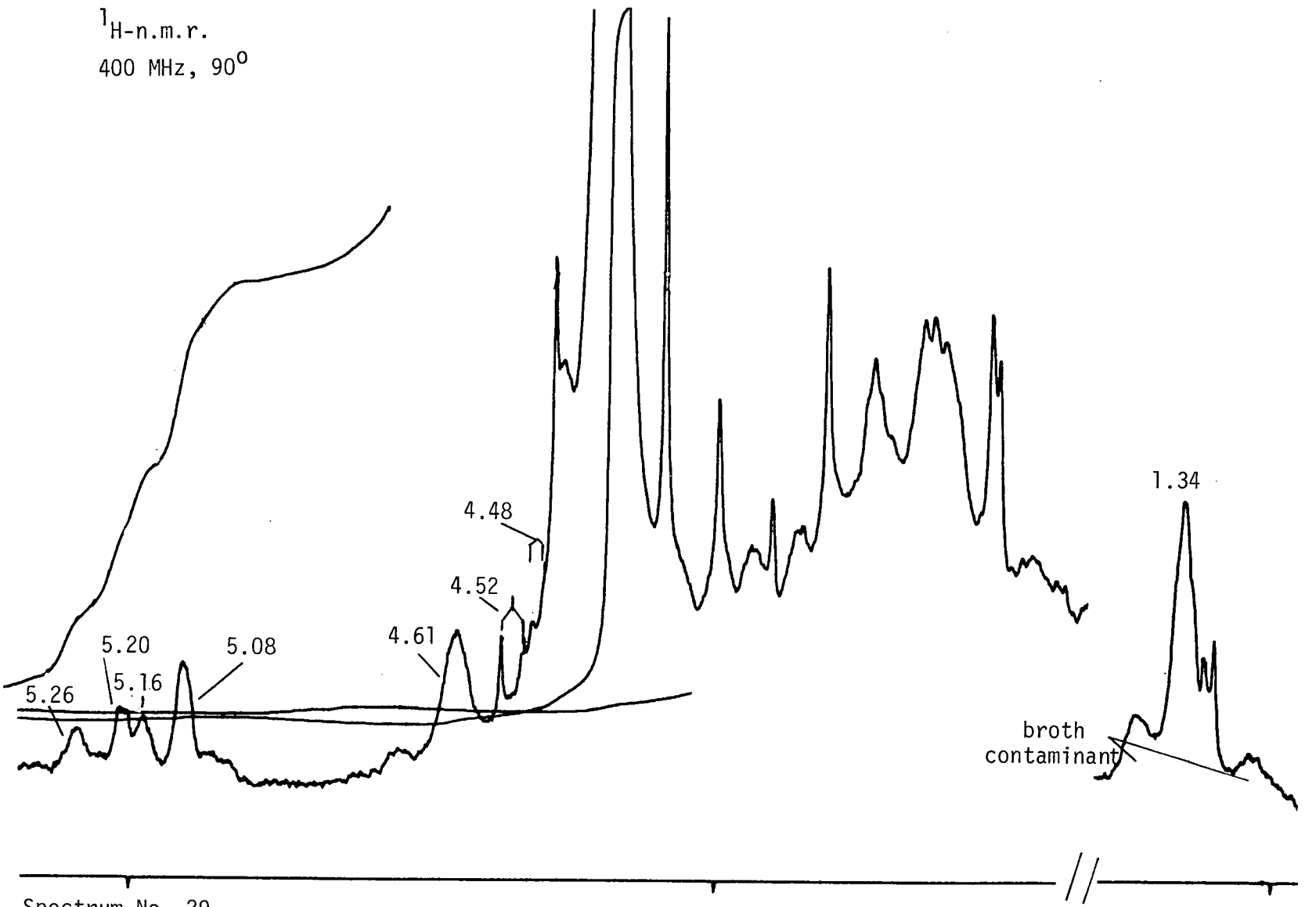
^{13}C n.m.r.

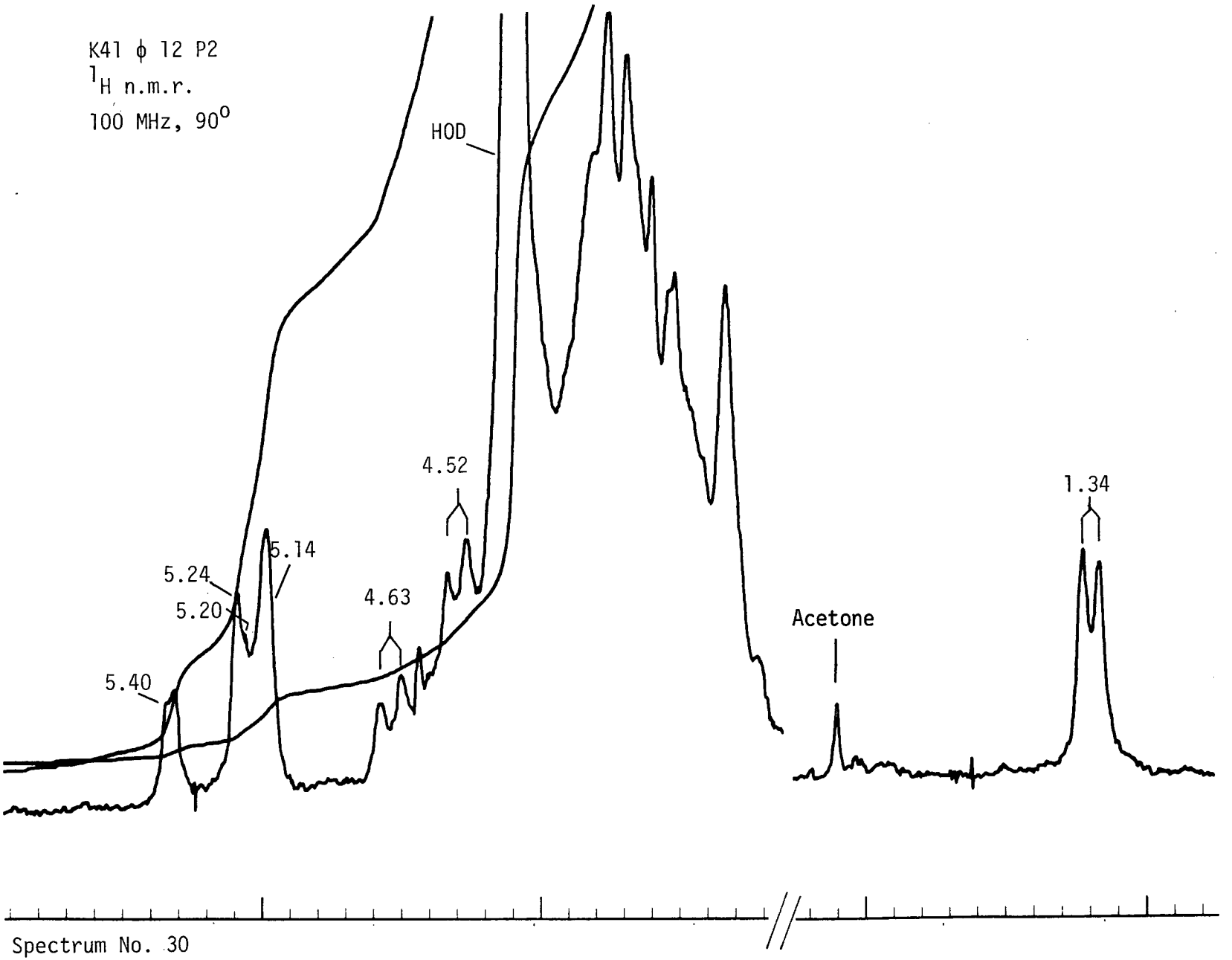
20MHz, amb. temp.

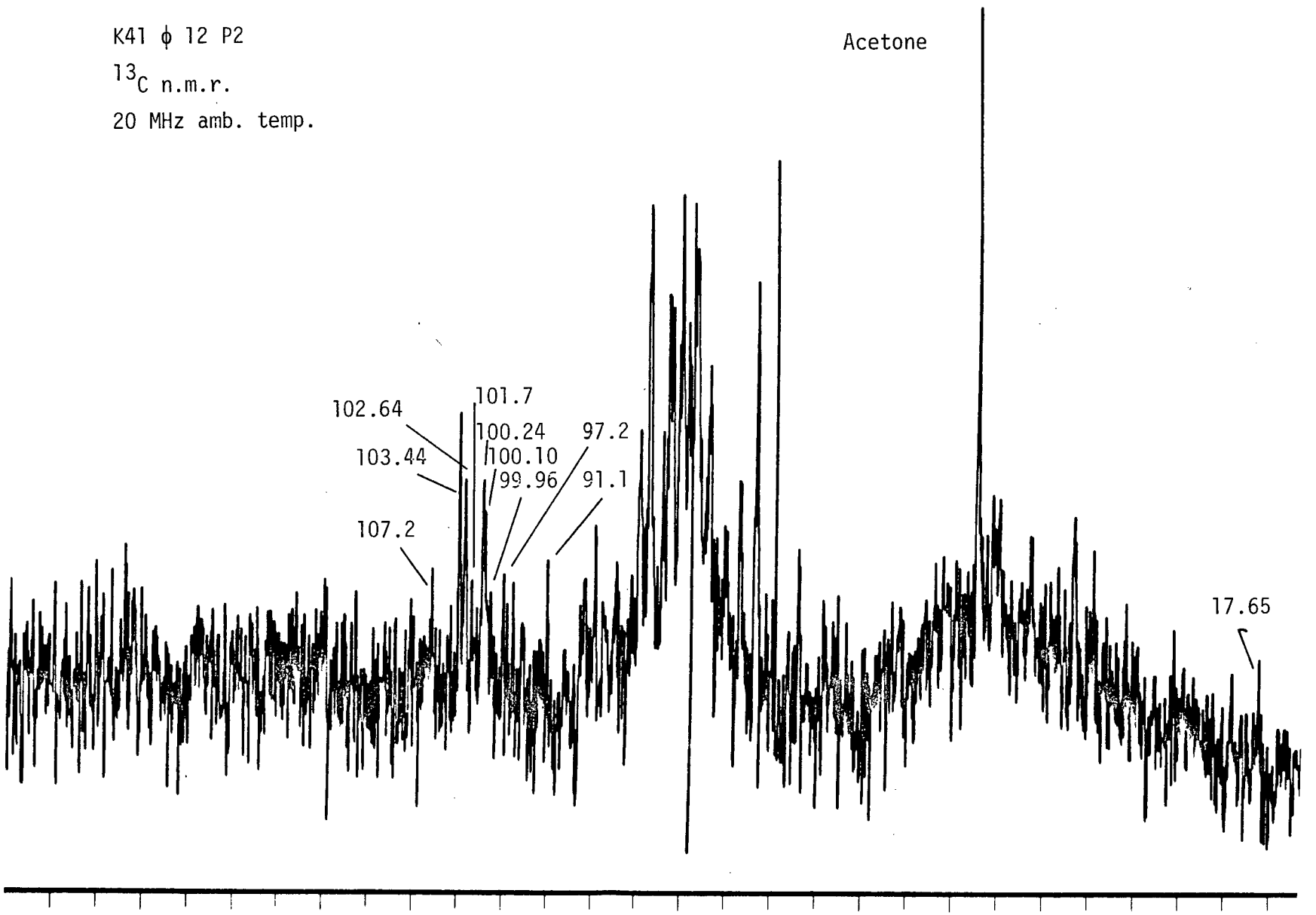


Spectrum No. 27


K21 ϕ21 Compound P2
^{1}H n.m.r.
100 MHz, 90°C
HOD
Acetone
2.23
1.53
5.27
5.31
5.06
5.46
4.87
4,64
Spectrum No. 28


K12 ϕ 12 P1
^{1}H-n.m.r.
400 MHz, 90°
4.48
4.52
4.61
5.20
5.08
5.26
5.16
1.34
broth
contaminant
Spectrum No. 29


K41 ϕ 12 P2
^{1}H n.m.r.
100 MHz, 90°
HOD
4.52
5.14
5.24
5.20
4.63
5.40
Acetone
1.34
Spectrum No. 30



Spectrum No. 31

METHODOLOGY OF BACTERIOPHAGE PROPAGATION AND POLYSACCHARIDE ISOLATION

APP.IV

Media and Buffers

"Standard" liquid broth or medium contained 5g Bactopeptone, 3g Bacto beef extract,and 2g NaCl per litre of water. "Standard" agar plates were made using a solution of "standard"liquid broth to which 15g of agar per litre had been added. 8.5 cm disposable petri plates were used.

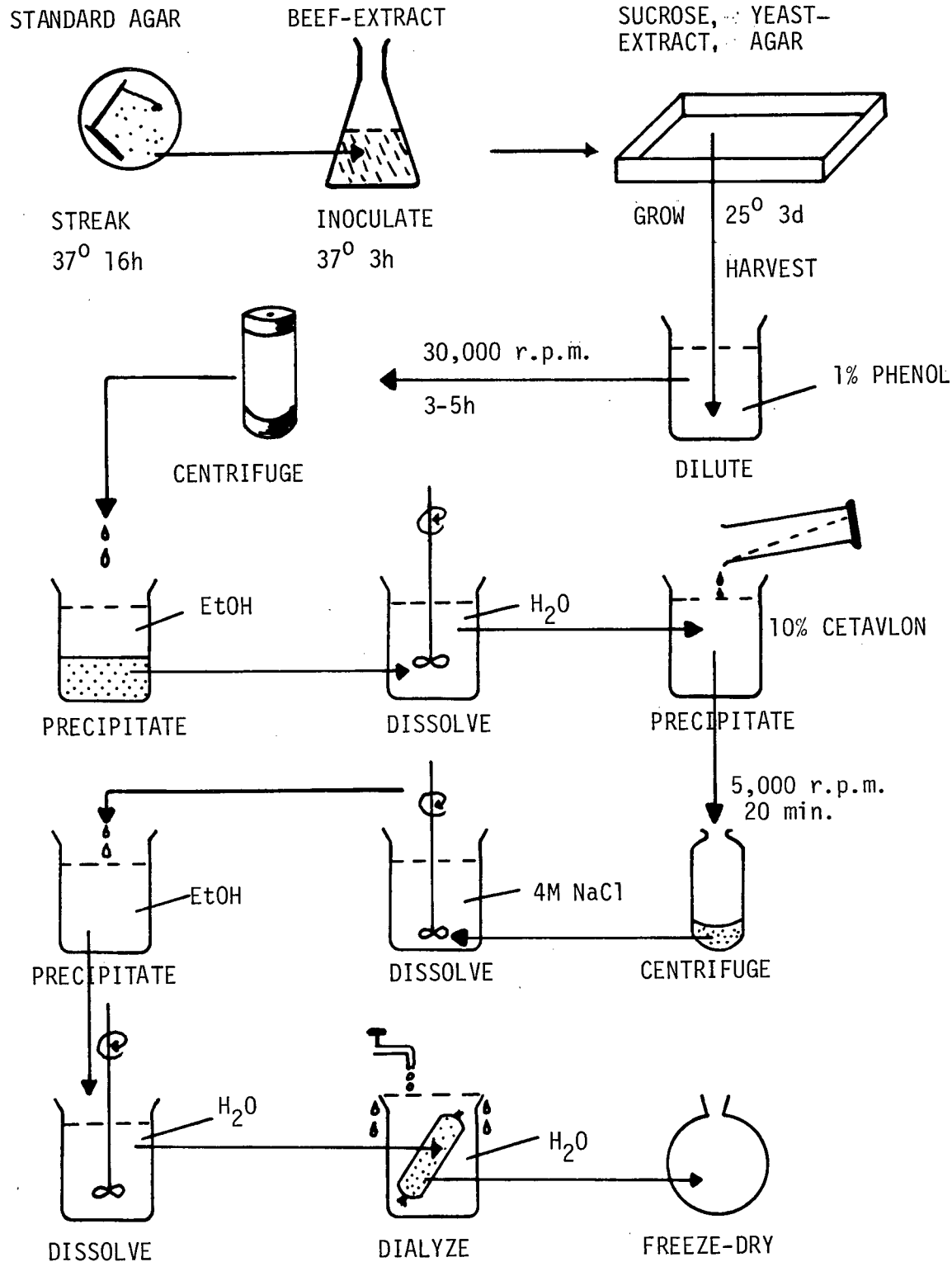
Actively growing cultures of Klebsiella bacteria (50 mL) were grown, in 100cm x 300cm trays, on a medium of sodium chloride (8g), calcium carbonate (2g), sucrose (120g), and Bacto yeast extract (8g) in 2L of water for three days. (See Scheme 13).

Phosphate-buffered saline (PBS) pH=7 was made up using 8.5g NaCl, 1.76g $Na_2HPO_4.H_2O$, and 0.1g KH_2PO_4 in 1L of water.

Tris HCl (pH=7.5) was made up using 82 mL HCl, 12.1g Tris (hydroxymethyl aminomethane),5g NaCl, 1g NH_4Cl in 1L of water.

Corellation of optical density with bacterial count.

A flask of liquid medium was inoculated with a culture of actively growing Klebsiella K21 bacteria and vigorously aerated at 37^{o} . Aliquots were removed at 30 minute intervals, appropriately diluted (10^{-5} to 10^{-8}) with liquid medium and a small quantity (0.1 mL) of the diluted solution was incubated on an agar plate for 12-16 hours. Individual bacterial colonies could then be counted. The bacterial count corellates with optical density.



Scheme 13 Isolation and Purification of Polysaccharide.

Bacteriophage Propogation

(a) Tube lysis. An active bacterial culture of Klebsiella K21 was obtained by successive replatings on agar plates. 7x5 mL of sterile liquid medium was then inoculated with the bacteria by the addition of 0.5 mL of an actively growing liquid K32 bacterial culture. These seven test tubes were incubated at 37^{o} and at 30 minute intervals the tubes were inoculated with 0.5 mL of a solution of liquid medium containing ϕ21. Continued incubation resulted in the first few tubes changing from the cloudy solution associated with actively growing K21 bacteria to a clear solution (lysis). After the last tube had cleared the incubation was continued for 30 minutes and then a few drops of $CHCl_3$ was added to the tube and the mixture was shaken well. A phage "titre" on the solution was performed by successively diluting a small volume (0.1 mL) of the clear liquid with liquid medium and then applying approximately 0.03 mL of these diluations to a 'lawn' of actively growing Klebsiella K21. (The lawn of K21 was prepared by inoculating 2 mL of liquid medium with an actively growing colony of Klebsiella K21 and incubating this culture for 3 hours. An agar plate, previously dried for approximately 1 h in the incubator at 37^{o}, was covered with this liquid culture, left for 5 minutes and then the excess liquid was removed. Incubation for 30 minutes gave a stable 'lawn' of Klebsiella K21.) Individual bacteriophage were observed as clear spots (approximately 0.3 cm in diameter) on the bacterial lawn after incubation for 16 hours. At high phage concentrations individual phage could not be distinguished but at more suitable dilutions,

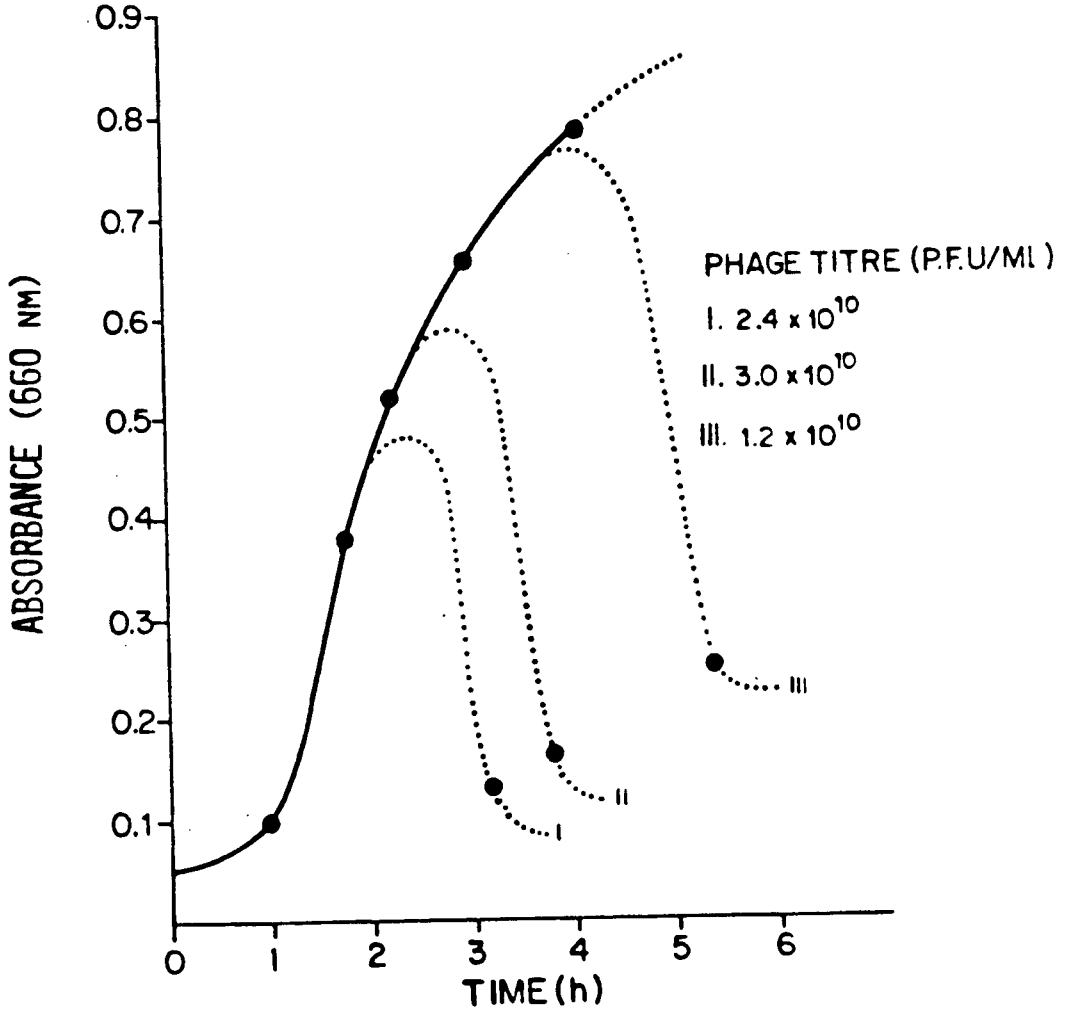


Figure 20 Growth Curve -, and bacteriophage lysis .... of Klebsiella K21 bacteria.

e.g. 10^{-8} to 10^{-10}, individual 'haloes' could be easily counted. As a result of a single tube lysis of this nature an assay yielded 10^9 plaque forming units (PFU) per mL of medium in the last tube to completely clear.

(b) Small flask lysis. This technique is essentially the same as that described for the tube lysis. As larger volumes of liquid medium can be used the overall total of bacteriophage can be increased even though the phage titre per mL. may not be significantly higher. In a typical small flask lysis 50 mL. solutions of K21 cultures were inoculated with 1.5 mL of a phage solution containing 10^9 PFU/mL (from tube lysis). In an analogous manner to that described for the tube lysis, titration of the final flask to completely clear gave a titre of 1.2×10^{10} PFU/mL. 250 mL of an actively growing liquid culture of K32 were vigorously aerated at 37^{o}. A small amount of a silicon antifoam agent (Dow antifoam FG-10 emulsion) was added to each. The optical density of each flask was monitored and at appropriate optical density readings (calculated such that the ratio of total bacteriophage to total bacterial colonies was approximately 3:1) aliquots of liquid phage cultures were added and the optical density monitoring continued. A subsequent drop in optical density indicated lysis had occurred. The results of a typical bottle lysis are shown in Figure 20.

A bottle lysis might typically yield 400 mL of a solution with a titre of 3.0×10^{10} PFU/mL.

(c) One litre flask lysis. In an analogous manner to that described for the bottle lysis three one litre flasks, each containing 600 mL of liquid medium, were inoculated with K bacteria, aerated and incubated to appropriate optical densities, and then bacteriophage solutions were added. A typical result of such a lysis might yield 1400 mL of a phage solution with a titre of $3x10^{10}$ PFU/mL.