THE STRUCTURAL ELUCIDATION OF THE CAPSULAR ANTIGEN OF KLEBSIELLA SEROTYPE K69

THESIS

Submitted in fulfilment of the

requirements for the degree of

MASTER OF SCIENCE

of Rhodes University

by

PETER LINTON HACKLAND

January 1987

ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to the following people:

Professor H. Parolis, Rhodes University, for his dedicated supervision and interest during the period spent in preparation of this thesis;

Dr. L. A. S. Parolis for valuable assistance in laboratory procedures;

Mr. D. Morley for helpful technical assistance;

Dr. J. Grundy and Mr S. M. R. Stanley for proof reading the manuscript;

Mrs. C. M. Lunn for help with the typing of the manuscript;

Drs. F. and I. Ørskov, Statens Serum Institut, Copenhagen, for a sample of Klebsiella K69;

the C. S. I. R., Pretoria, for financial support and for the use of their n.m.r. facilities;

Rhodes University, for post-graduate bursaries during 1985 and 1986;

the mass spectrometry unit of the University of Cape Town, for the use of their facility.

TABLE OF CONTENTS PAGE

INTRODUCTION

THE BACTERIAL CAPSULE

Figure 1 shows the differences in the cell walls of gram-positive and gram-negative bacteria together with the location of their capsules.

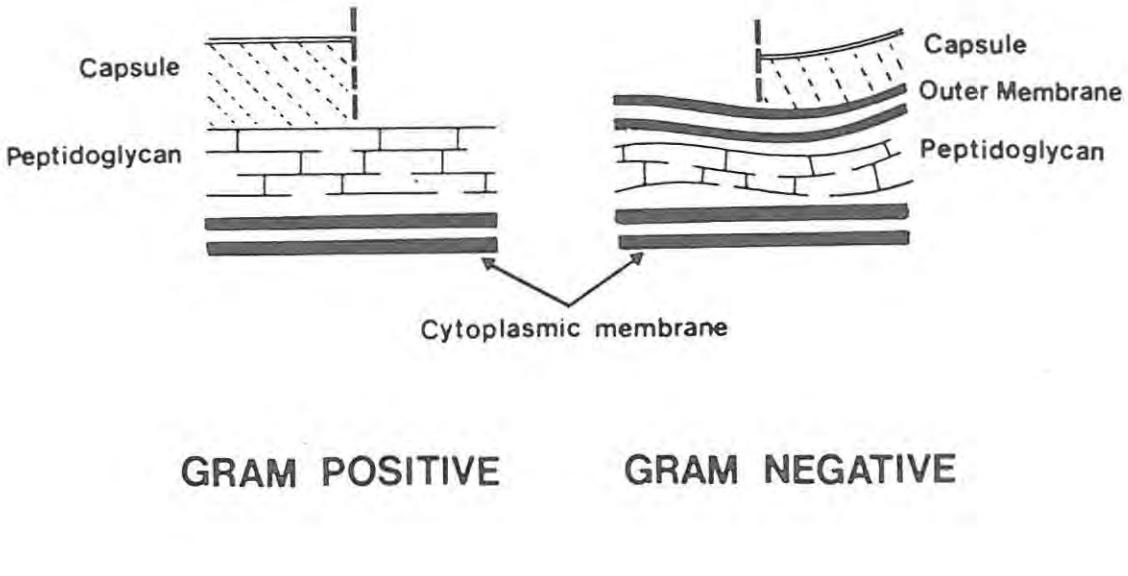


Fig. 1.[3] Location of carbohydrates in the cell wall of gram-negative and gram-positive bacteria.

Bacterial capsules are exocellular polysaccharides located on the cell walls of both gram-positive and, more commonly, gram-negative bacteria. An important group of capsular gram-positive bacteria, are the Pneumococci[1], which have been the subject of many serological studies. Encapsulated gram-negative bacteria, include members of the Enterobacteriaceae, notably Klebsiella, Proteus, Shigella, Escherichia and Enterobacter.

The capsule is not essential for growth or survival of the bacterial cell because noncapsulated mutants can be isolated. Although the role of the capsule has not been accurately determined, it suffices to say that when the bacteria are pathogenic, it serves to enhance virulence and protect the cell from phagocytosis. This has been shown in the Pneumococci where the non-capsulated variant is unable to cause progressive illness in test animals unless injected in an overwhelming dose[2].

The capsules are attached to the bacterial cell surface through a close association with the cell wall and are only removed by vigorous shaking or alkali extraction. They are immunogenic, labelled the "K" or Kapsel antigen and are biosynthetically related to the O-antigen (lipopolysaccharides) and peptidoglycans[3] (Fig.1), all having lipid-linked saccharides as intermediates.

The capsular polysaccharides are heteropolymers made up of various neutral hexoses, uronic acids, 6-deoxyhexoses, amino-sugars and, rarely, pentoses. E.coli K1 which possesses a capsule consisting of the homopolysaccharide of N-acetylneuraminic acid (sialic acid)[4-5] is an exception. Unusual components of capsules include phospholipids (phosphatidic acids)[6], amino acids[7-8] and 3-deoxy-D-manno-octulosonic acid (KDO)[9]. The large number of different components give rise to many variations in polymer structure. Certain exocellular polysaccharides are of commercial importance[10], some being used as vaccines[11] (meningococcal polysaccharide vaccines).

The bacterial capsule is thought to play a part in the disease process, therefore structural studies on capsular polysaccharides of two members of the Enterobacteriaceae, Klebsiella and Escherichia, are

being conducted in our laboratory in order to determine the absolute chemical structure of these bacterial antigens. Bacteria of the genus Klebsiella, include the species K.ozaenae, K.rhinoschleromatis and K.pneumoniae, which are opportunistic pathogens causing respiratory and urinary tract infections in man. E.coli is saprophytic within the intestine and only under exceptional circumstances causes diarrhoea and urinary tract infections (cystitis).

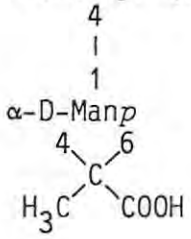
Structural studies have recently concentrated on strains of E.coli as the majority of Klebsiella capsular polysaccharides have been studied. The work presented in this thesis, however, covers the capsular polysaccharide of a strain of Klebsiella serotype K69 and forms part of a continuing programme, involving the structural elucidation of capsular antigens.

1. THE KLEBSIELLA SERIES OF CAPSULAR (K) ANTIGENS

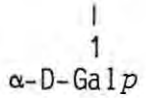
The family Enterobacteriaceae, which includes the genus Klebsiella, is an important group of bacteria found in the intestinal tract. Species of Klebsiella are gram-negative rods, some having an exocellular polysaccharide capsule; these capsules are complex polysaccharides made up of oligosaccharide repeating units which comprise the capsular (or K) antigens.

The existence of more than eighty different Klebsiella K antigens has been reported[12-13]; the chemical structures of more than sixty of these have been elucidated. The majority of these structures have been reviewed[14], with the following having subsequently been established :

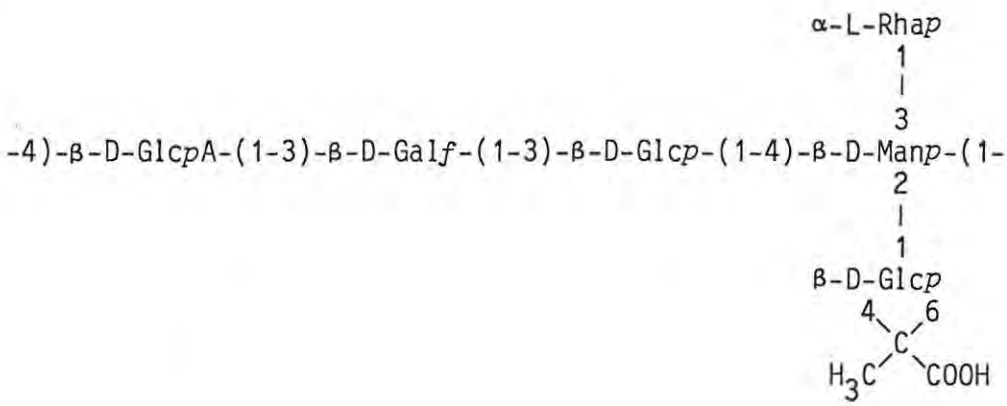
K3[15] -2)-α-D-Gal*p*A-(1-3)-α-D-Man*p*-(1-2)-α-D-Man*p*-(1-3)-β-D-Gal*p*-(1-



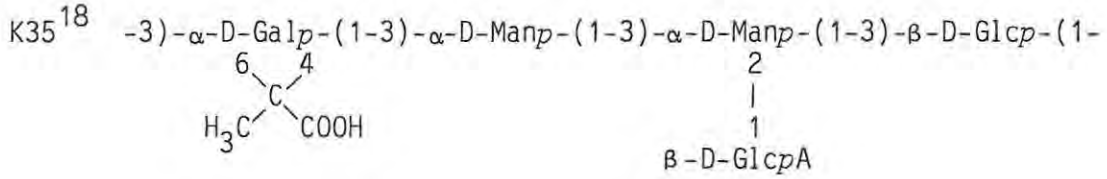
K10[16] -4)-β-D-Glc*p*A-(1-2)-α-D-Man*p*-(1-2)-β-D-Gal*p*-(1-6)-α-D-Glc*p*-(1-4)-β-D-Gal*p*-(1-



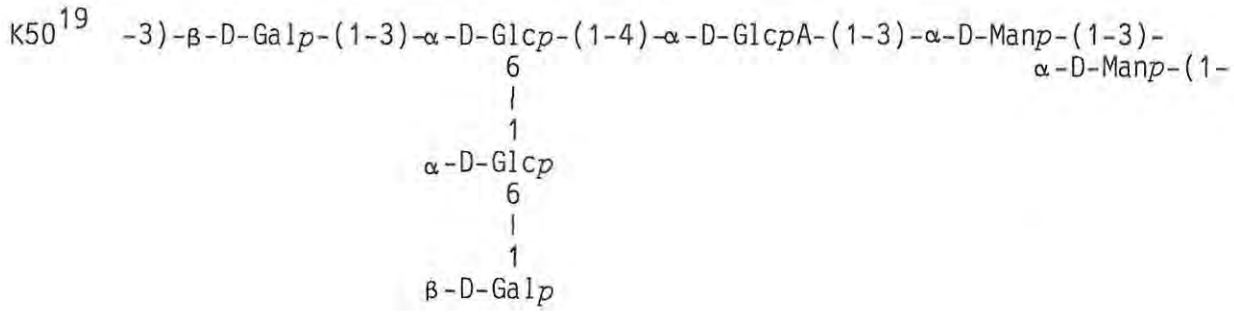
K14[17]



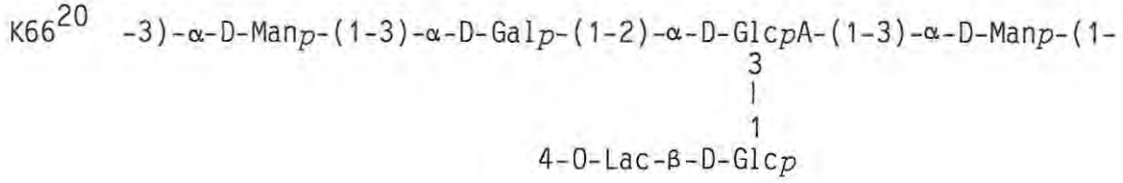
K35[18]



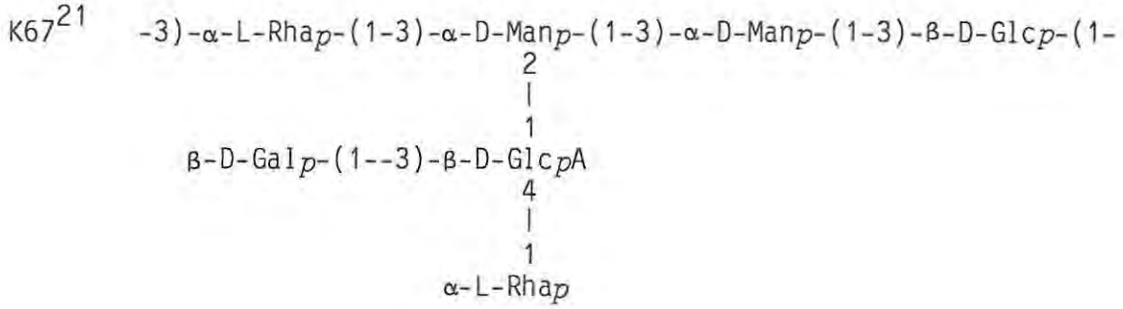
K50[19]



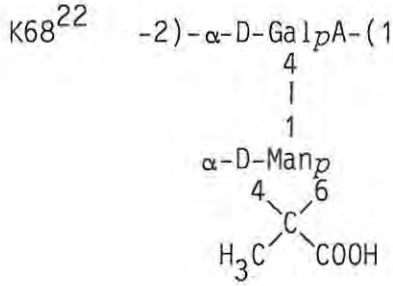
K66[20]



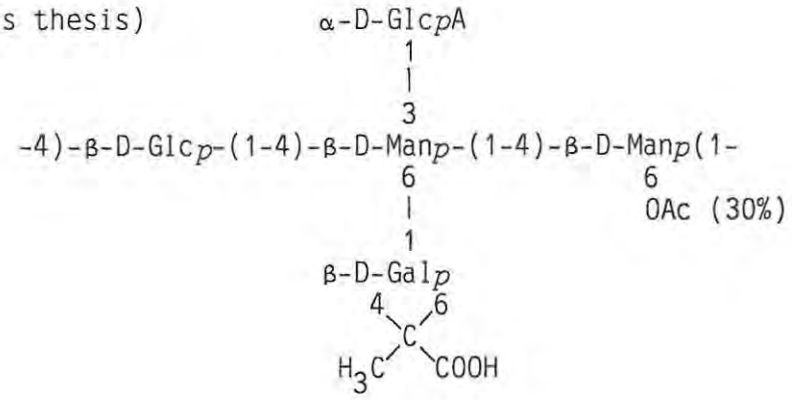
K67[21]



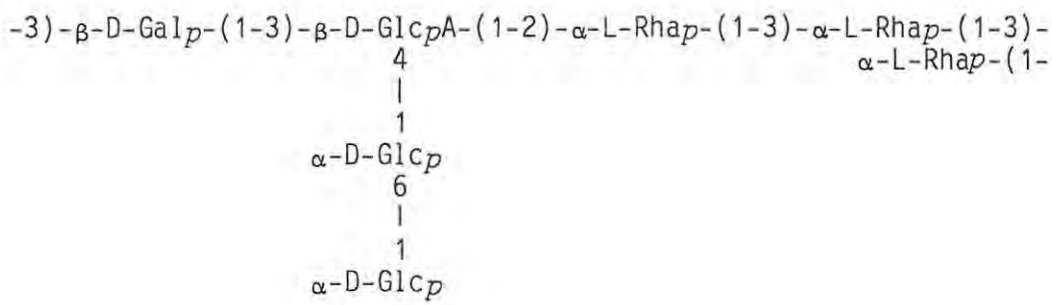
K68[22]



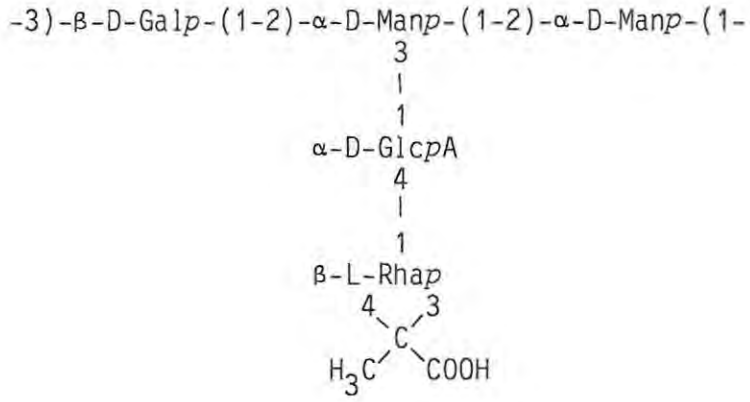
K69 (this thesis)



K79[23]



K80[24]



K82[25]

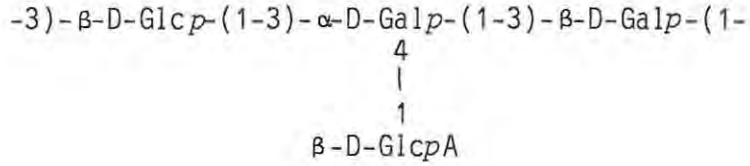


Fig.2 Repeat units of recently published structures.

The existence of strains K1 through K84 have been reported. Ørskov and Fife-Ashbury[13] found that the capsular polysaccharide of Aerobacter aerogenes was labelled K73 and K75, K76, K77 and K78 were identical to previously established structures, therefore these strains were deleted and a new strain, K83, was reported. Preliminary investigations conducted by Nimmich[12] on KLEBSIELLA types 1 to 72 showed their capsular antigens to be made up of sugars from the following :

Neutral sugars - D-glucose
D-galactose
D-mannose

Acidic sugars - D-glucuronic acid
D-galacturonic acid

6-Deoxyhexoses - L-fucose
L-rhamnose

The Klebsiella capsular polysaccharides are acidic, often containing glucuronic acid, but galacturonic acid does occur in a few cases, while other polymers have a pyruvate acetal in addition to a uronic acid. In the K32[26], 56[27] and 72[28] polysaccharides the pyruvic acid acetal is the sole acidic component. The O-acetyl groups present in K2[29], K24[30], K30[31], K33[32], K44[33], K49[34], K54[35], K55[36], K58[37], K59[38], K64[39] and K69 (this thesis) are of serological importance. This was clearly demonstrated by Lindberg et al.[31] who showed that K30 and K33 are distinct serological types although they differ chemically only in the extent of O-acetylation.

Some less common sugars and sugar components have been reported. The capsule of K37[40] has a terminal 4-O-[(S)-carboxyethyl]-D-glucuronic acid group, K22[41] is thought to have a terminal 4-deoxy-

threo-hex-4-enosyluronic acid, K66[20] has a terminal 4-O-[(R)-1-carboxyethyl]-D-glucose unit, while K38[42], contains a 3-deoxy-L-glyceropentulosonic acid. The majority of monosaccharides involved are in the pyranose form with only K12[43], K14[17] and K41[44] having furanose residues (viz D-Galactofuranose).

It can thus be seen that there exists a wide diversity of chemical structure within the series of Klebsiella capsular antigens. Serological cross reactions between these polysaccharides and antisera have been conducted[45-49] allowing correlations to be made between chemical structure and antigenic character.

2. ISOLATION AND PURIFICATION OF CAPSULAR POLYSACCHARIDES

Before structural studies can be carried out on capsular polysaccharides the material must be isolated in a suitably pure form. Moreover, isolation procedures must not modify the polymer in any way. The general approach is to extract the polymer from the culture by solubilisation, then purify it by means of chromatography or selective precipitation, the latter being the most successful and widely used method.

The process of selective precipitation involves conversion of the required polysaccharide to an insoluble complex with, for example, aqueous cetyltrimethylammonium bromide (CTAB). This forms insoluble complexes with acidic but not neutral polysaccharides (O-antigen)[50], thus providing an efficient method for selectively isolating acidic polysaccharides. This approach is routinely used in our laboratory and is essentially that described by Dutton and Okutani[51].

The bacteria are grown on a suitable solid agar medium and after incubation the cells are harvested and the capsular material is extracted into a 1% w/v aqueous solution of phenol. The resulting suspension is centrifuged and the supernatant decanted from the cells and poured into 95% ethanol to precipitate crude polysaccharide material. The precipitate, after dissolution in water, is treated with CTAB to isolate the acidic polysaccharide. The polysaccharide/CTAB complex is collected and then decomplexed by dissolution in 3M sodium chloride; addition of ethanol precipitates the free polysaccharide. This material is further purified by dialysis.

An alternative method which may be used to separate acidic from neutral polysaccharides, is ion-exchange chromatography, for example

on DEAE-Sephadex or -Sepharose. Lindberg et al.[52] have successfully applied this method in the isolation of capsular polysaccharides.

PROCEDURES USED IN THE STRUCTURAL ELUCIDATION OF POLYSACCHARIDES

PROCEDURES USED IN THE STRUCTURAL ELUCIDATION OF POLYSACCHARIDES

3. CHEMICAL METHODS USED IN THE STRUCTURAL ELUCIDATION OF BACTERIAL POLYSACCHARIDES

3.1 Monosaccharide composition analysis

The initial step in structural studies of complex polysaccharides is the qualitative and quantitative determination of the monosaccharides making up the polymer. Usually, the polysaccharide is hydrolysed and the resulting sugars are identified by means of paper chromatography or gas liquid chromatography of the suitably derivatised sugars. Quantitation (total sugar ratios) is achieved by g.l.c. of the sugar derivatives from a hydrolysate and a methanolysate.

Hydrolysis of polysaccharides in an acidic medium is accompanied by some degradation of the free sugar units[53-55], thus a compromise must be reached regarding the choice of acid and the concentration used in order to get optimum hydrolysis with minimal degradation. Acid labile substituents (O-acetyl and pyruvate acetals) are lost during acid hydrolysis, but fortunately, can be quantitated by ^{1}H-nuclear magnetic resonance (n.m.r.) spectroscopy. A variety of acids have been used for the hydrolysis step, these include aqueous hydrochloric, aqueous sulphuric and aqueous trifluoroacetic acid (TFA)[56]. A study has shown that TFA and sulphuric acid are the acids of choice[57] as both these acids cause less degradation than others. TFA, however, has the added advantage of being volatile and therefore can be easily removed from the reaction products. Aqueous TFA (2M) is routinely used to effect complete hydrolysis, while at lower concentrations it is used in partial hydrolysis studies[57].

It must be emphasized that, under given conditions, different glycosidic linkages are hydrolysed at different rates. Most bacterial polysaccharides that have been studied contain uronic acids, whose glycosidic linkages are more resistant to acid hydrolysis than those of neutral sugars[58]. In order to facilitate hydrolysis, it is necessary to reduce the uronic acid units to their parent sugars prior to acid treatment. The methods for uronic acid reduction have been outlined by Aspinall[59]. In this laboratory the polysaccharide is methanolysed in refluxing 3% methanolic hydrogen chloride forming oligo- and mono-saccharides as methyl glycosides. During this process carboxyl groups are all converted to the methyl esters which are reduced in dry methanol with sodium borohydride. The hydrolysis can now be completed using 2M TFA. A highly recommended method for reducing uronic acid residues is that of Conrad and Taylor[60], involving the treatment of an aqueous solution of the acidic polysaccharide with a water soluble carbodiimide, followed by reduction with sodium borohydride.

3.2 Methylation analysis

Methylation analysis forms a very important procedure in the structural elucidation of polysaccharides. This procedure was originally laborious and time consuming[61], however, modern procedures[62] enable the complete analysis to be done within a normal working day. The general procedure involves etherification (methylation) of all the free hydroxyl groups on the sugar residues of the poly- or oligo -saccharide. Subsequent acid hydrolysis of the permethylated material will generate hydroxyl groups on carbons

formerly involved in the glycosidic linkages allowing the assignment of linkage positions to the various sugars by identification of the methylation positions of these products. This is achieved by g.l.c.-mass spectrometry of the derived permethylated sugars.

The unambiguous location of the monosaccharide linkage positions relies upon complete methylation of the polysaccharide. To ensure that this has occurred a methoxyl determination can be carried out. More frequently however, infra red spectroscopy is utilised to detect any remaining OH groups.

Of the available methylation procedures, the method of choice is that of Hakomori[63]. This involves the dissolution of the poly- or oligo-saccharide in dimethylsulphoxide (DMSO), treatment with sodium methylsulphinylmethanide converting OH groups to alkoxides and reaction with methyl iodide to effect the methylation at the alkoxides. This method has largely replaced the earlier methods of Purdie and Irvine[64] (methyl iodide and silver oxide) and Haworth[61] (dimethyl sulphate and aqueous sodium hydroxide); the latter was the standard method prior to the Hakomori procedure [53]. The method of Kuhn and coworkers[65], who modified the Purdie method, involves the use of N,N-dimethylformamide (DMF) or DMSO as solvent, silver oxide as base, and methyl iodide as alkylating agent.

Remethylation of partially methylated polymers by the Hakomori method is to be avoided to prevent the loss of uronic acid substituents. Uronic acids are esterified during the Hakomori methylation. These uronic esters are susceptible to base catalysed β-eliminations with subsequent degradation. Fortunately the esterification only occurs after etherification of the more nucleophillic alkoxides when the base concentration is too low for

degradation to occur. In order to avoid β-elimination the Kuhn method[65] has been used to complete the methylation of partially methylated material, for example, after reduction of uronyl esters in methylated poly- or oligo-saccharides with lithium aluminium hydride[66].

Incomplete methylation is usually due to incomplete dissolution of the polysaccharide in DMSO. This can be overcome by ultrasonication and heating to 60° or by the modified Hakomori procedure described by Narui et al.[67]. In this procedure a 1:1 mixture of 1,1,3,3-tetramethylurea and DMSO is used. The urea serves to relax inter- and intra-molecular hydrogen bonds affording better dissolution of the polymer. Recently, the Hakomori technique has been further modified by the use of potassium dimsyl[68] in place of sodium dimsyl[69]; potassium dimsyl has the advantages of ease of preparation and of producing fewer artefacts on g.l.c. analysis of the permethylated monosaccharide derivatives.

Pyruvate acetals are stable under the alkaline conditions of the Hakomori methylation, while O-acetyl substituents are removed. Methods have been developed to prevent this from occurring[70-71], for example, De Belder and Norrman[72] have developed a very useful chemical method (using methyl vinyl ether) to locate O-acetyl substituents in polysaccharides. Modern n.m.r. spectroscopy is also useful in the location of these base labile substituents.

3.3. Specific degradations of polysaccharides

3.3.1. Graded acid hydrolysis

Partial depolymerisation of polysaccharides by acid forms oligosaccharides which can be isolated by preparative paper or gel permeation chromatography. Partial hydrolysis, the subject of several review articles[73-74, 55], provides valuable sequencing information once the resulting oligosaccharides have been characterised by n.m.r. spectroscopy and methylation analysis.

Many polysaccharides have glycosidic linkages which do not differ significantly in their lability to acid hydrolysis. Fortunately, the bacterial polysaccharides have glycosidic linkages of differing acid lability, for example, furanose and deoxy- sugar linkages are weaker than those of uronic acid and 2-amino-2-deoxyhexose. Uronic acids are commonly encountered with bacterial exocellular polysaccharides, hence by carefully controlling the reaction, predominantly aldobio-, aldotrio-, aldotetrao- and higher uronic acids will be formed[75]. Even where all the glycosidic linkages are hydrolysed at the same rate, the polymer can be chemically modified prior to partial hydrolysis[76] to induce selective fragmentation. However, a disadvantage of partial hydrolysis is that complex mixtures of oligosaccharides are formed, resulting in low yields of the pure oligosaccharides.

The depolymerisation is generally carried out in aqueous 0.5M TFA, though non-aqueous conditions can be employed, including: acetolysis (acetic anhydride, acetic acid and sulphuric acid)[77]; hydrofluorinolysis (liquid hydrogen fluoride at low temperatures); methanolysis and mercaptolysis. The non-aqueous methods afford selective cleavage of different glycosidic bonds to those hydrolysed

by aqueous TFA, producing different oligosaccharides and, hence, additional structural information, e.g. acetolysis cleaves 1-6 linkages more readily than aqueous TFA[78]. Selective cleavage has also been effected by Nilsson et al.[79] who stabilized glycosidic bonds to acid hydrolysis by O-trifluoroacetylation.

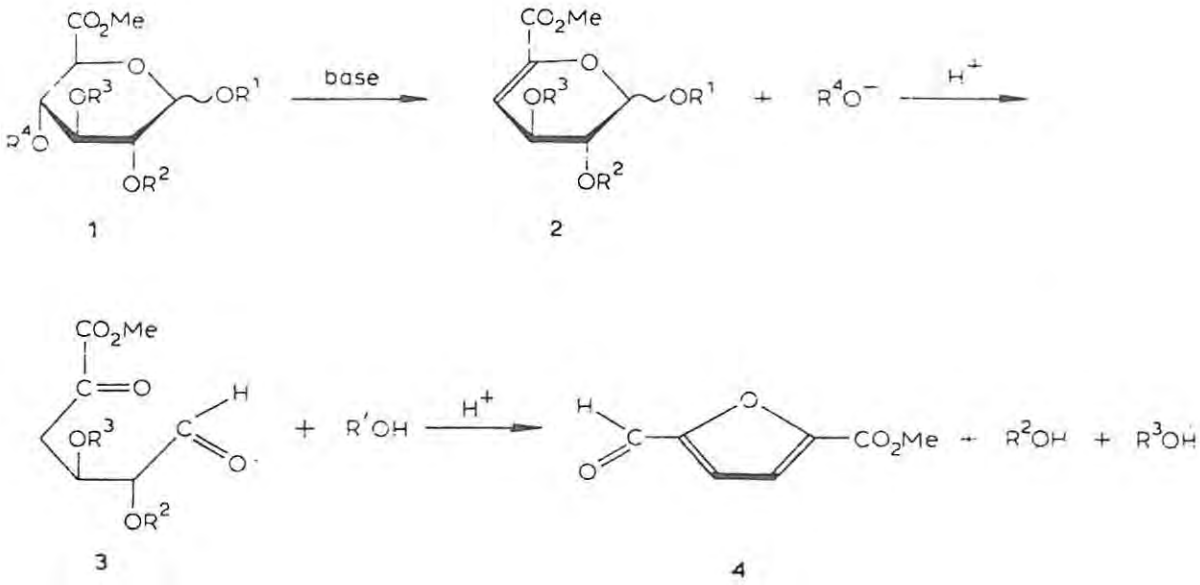
Pyruvate is an acid labile substituent which can be selectively cleaved by acid, revealing its linkage positions which are identified by methylation analysis[39].

3.3.2. β-Elimination

Selective degradation of methylated polysaccharides containing uronic acids reveals the linkage position of the uronic acid and provides useful sequencing information. Lindberg et al.[80] demonstrated this by treating a methylated polysaccharide with a strong base (dimsyl).

The 4-O-substituted uronic acid has a good leaving group β to the electron withdrawing carboxyl. The methoxyl or a sugar group attached to the 4- position is β-eliminated as methanol or saccharide on treatment with base, forming an unsaturated uronyl ester. Subsequent mild acid hydrolysis cleaves the glycosidic bond, releasing the uronyl ester which then undergoes further degradation (see Scheme 1, Fig.3). Where the uronic acid is "in chain" oligomers are formed and where terminal or pendant a modified polymer results. On re-etherification and analysis of the products, the linkage position of the uronic acid and some sequencing data are ascertained.

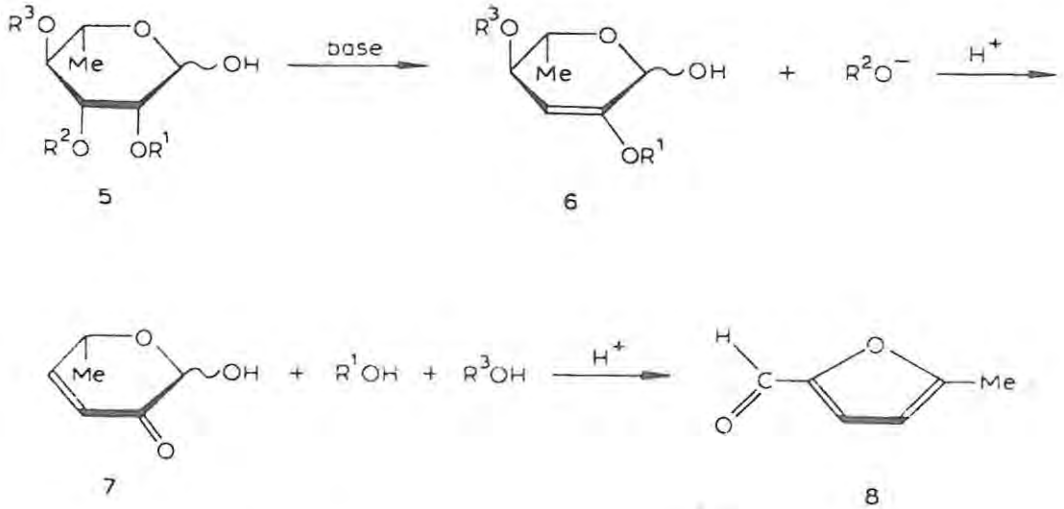
Lindberg and Lönngren[81] later published a more detailed account of the above procedure. Where the 4-O-substituent is a sugar residue,



R^1 = sugar residue

R^2, R^3, R^4 = Me or sugar residue

SCHEME 1



R^1, R^2, R^3 = Me or sugar residue

SCHEME 2

FIG.3[14] β-Elimination reactions

it too is degraded on prolonged treatment with base (Scheme 2, Fig.3) providing further useful structural information.

These β-elimination reactions have been reviewed[82] by Kiss, and later updated by Lindberg et al.[73] concentrating on their application to structural carbohydrate chemistry.

Aspinall and Rosell[83] have since modified the procedure of Lindberg [80]. They showed that the treatment of methylated polysaccharides with a strong base, under the conditions described by Lindberg, results in complete degradation of the uronic acid, making the acid hydrolysis step unnecessary. Accordingly, the Aspinall/Rosell modification is generally utilised. This takes the form of a single operation whereby the methylated acidic polymer is treated with a base and then directly alkylated with trideuteriomethyl iodide or with ethyl iodide to tag the site where the uronic acid was linked. A further advantage of this method is that it prevents the secondary degradations (Scheme 2, Fig.3) which occur using the Lindberg method. The secondary reactions involve the reducing-sugar products of the initial base catalysed degradation; substituents at O-3 of these reducing sugars, methoxyl or glycosyl, are beta to an aldehyde carbonyl and are eliminated on prolonged exposure to base to yield an unsaturated product which undergoes further degradation upon treatment with acid.

It is interesting to note that only limited degradation by β-elimination of 4-O-substituted uronic acids occurs during Hakomori methylation, although degradations have been reported[84]. It is postulated that the base is rapidly decomposed on addition of the methyl iodide (section 3.2).

3.3.3. Periodate oxidation

Periodate oxidation of polysaccharides, first utilised in 1928[85], remains an important analytical technique. Inevitably the procedure has been modified to optimize its usefulness in carbohydrate structural analysis. It is based on the oxidative cleavage of 1,2-diol or 1,2,3-triol groups[86] to form two aldehyde groups and, in the latter case, one molar portion of formic acid; one and two molar proportions of periodate are consumed, respectively. The method has been reviewed[87] and the dialdehyde products have been the subject of further discussion[88].

Structural information, including nature and proportion of the glycosidic bonds present in a polysaccharide, is obtained by quantification of periodate consumed, formic acid generated and the proportion of intact sugar units. Various methods of quantitation are used[86], principally spectrophotometric and titrimetric analysis. Modifications to the oxidation procedure, such as periodate oxidation followed by reduction and hydrolysis, (or by variations of this sequence) enable the controlled degradation of polysaccharides[89] (Fig.4). The resulting dialdehydes exist as cyclic acetals[90] which are stable to further oxidation and to acid hydrolysis. However, by reduction of these dialdehydes to the corresponding alcohols, acetal formation is prevented, allowing further oxidation and selective acid hydrolysis of the products. This procedure is known as the Smith degradation[86] and is often utilised because the acyclic acetals (polyalcohols) are more readily hydrolysed than the remaining sugars, allowing the controlled degradation of polysaccharides.

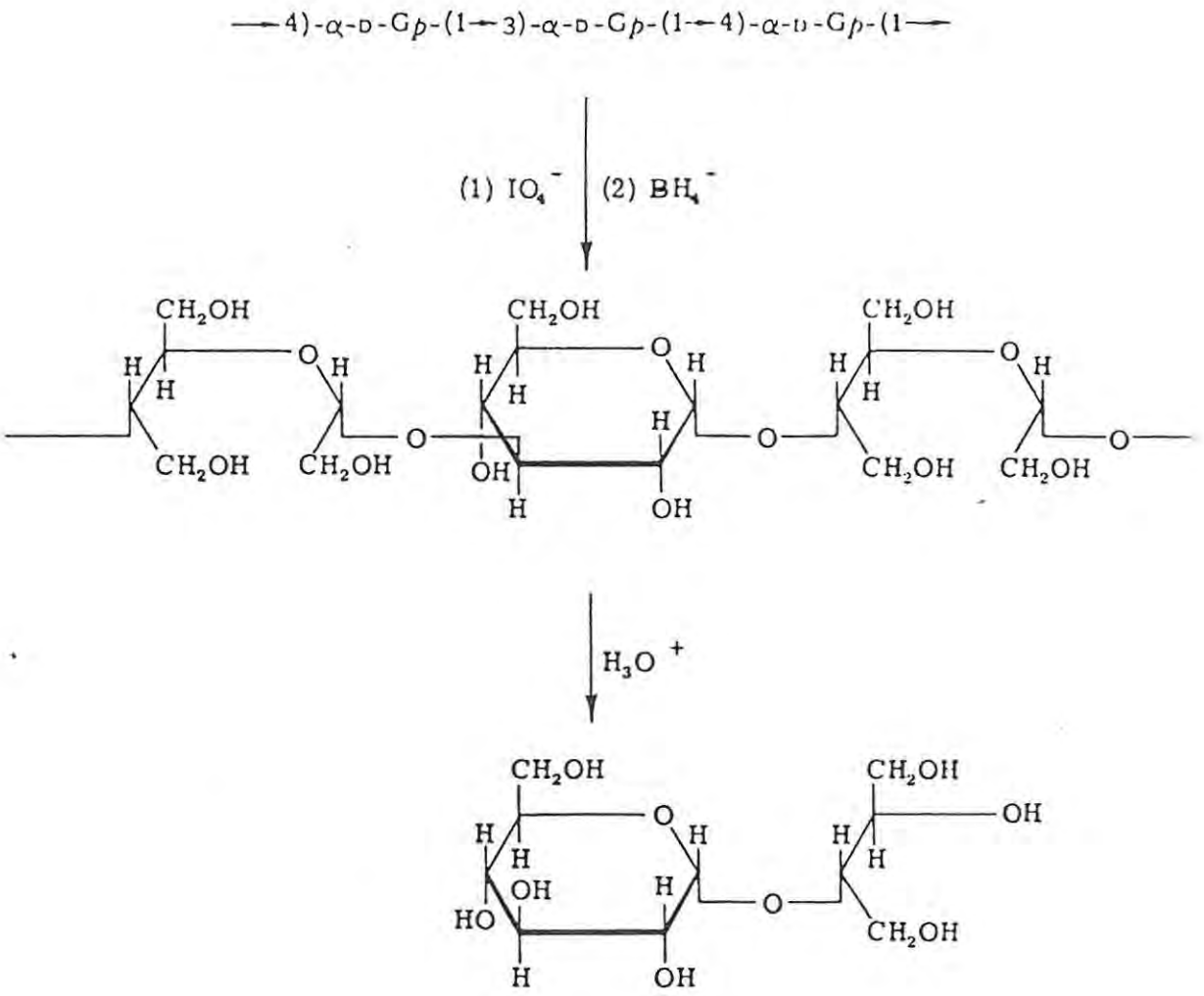


FIG.4[89] THE SMITH DEGRADATION

The advantages of periodate oxidation are:

a) small quantities of material are required,

b) oxidations are quantitative, providing significant structural information,

c) degradations can be controlled and

d) water is used as solvent as carbohydrates are water soluble while periodate/iodate salts are virtually insoluble and are therefore easily removed, allowing facile recovery of the carbohydrate products.

Certain problems associated with periodate oxidation exist and these are primarily due to over- or under-oxidation. Overoxidation is minimised using the reaction conditions described by Bobbit[87], who recommended reactions be carried out in the dark, at low temperature. with pH 3-3.5. Alternatively Painter and coworkers[91] used oxygen free water with added propyl alcohol as a radical scavenger to prevent overoxidation. Reduction of the products, as in the Smith degradation, followed by further periodate oxidation[89], is used to overcome underoxidation. This allows intact sugar residues and the smaller fragments of oxidised sugars (erythritol, glycerol) to be isolated. Analysis of these products provides the researcher with sequencing and linkage information. In addition, the Smith degradation has been modified to overcome problems of underoxidation and to enable selective acid hydrolysis[92-93].

3.4. Monosacchsaccharide configuration analysis

The monosaccharides found in naturally occurring polysaccharides can be either of the D or L configuration and thus, before the structure of a polysaccharide can be considered to have been fully elucidated, it is necessary to determine the absolute configuration of each

component monosaccharide. Several methods have been applied to establish these configurations including polarimetry, circular dichroism[94], enzymic methods[95] and gas-liquid chromatography.

Polarimetry and gas-liquid chromatography are used in this laboratory. The former method involves isolation of the monosaccharides after separation by paper chromatography of the polysaccharide hydrolysate. Each sugar is chiral and has a characteristic specific optical rotation, thus allowing assignment of absolute configuration.

The method of Leontein et al.[96], whereby the absolute configuration of each sugar is assigned by gas-liquid chromatography of its derived glycosides after reaction with a chiral alcohol, is the method of choice. The chiral alcohol used in this laboratory is (-)-2-octanol, the resulting octyl glycosides producing four or five characteristic peaks for each sugar upon g.l.c. analysis (two pyranosides, two furanosides and the straight chain form). The D and L isomers of each sugar give a characteristic chromatogram , enabling the accurate assignment of its absolute configuration. The method is reliable, rapid and only small amounts of material are required.

4. ENZYMIC METHODS

Enzymic methods are playing an ever increasing role in the structural elucidation of polysaccharides. Enzymes in no way entirely replace the chemical methods, involving chemical degradations and methylation analysis but, used together they neatly complement one another. The use of enzymes has several advantages :

(a) Generally they act fairly rapidly and do not require numerous or complex experimental manipulations.

(b) They act specifically on certain glycosidic linkages enabling a polysaccharide in a mixture to be studied. This is especially convenient when difficulties are encountered in the isolation of pure material.

(c) Reaction conditions are mild, leaving labile substituents intact.

(d) Often, only catalytic amounts of enzyme are required.

(e) The enzymes are water soluble and therefore, convenient for the study of polysaccharides in aqueous solution.

(f) The substrate (polysaccharide) does not require chemical modification prior to enzymic hydrolysis and the products are easily isolated in pure form.

(g) Pure enzymes are readily available from commercial sources and are relatively inexpensive.

4.1. Bacteriophage enzymes

The first account of a bacteriophage (normally abbreviated to "phage") was published by F.W. Twort in 1915[97], since this date our knowledge of these bacteria-infecting viral particles has increased considerably. A characteristic of phages which has been exploited by carbohydrate chemists, is their endoglycosidase activity[98]. Phage-mediated enzyme depolymerisation of E.coli and Klebsiella capsular polysaccharides, producing oligosaccharides corresponding to the repeat unit (P1) and multiples thereof (P2 etc.), is now routinely applied in the structural elucidation of these polysaccharides.

A bacterial host "lawn" innoculated with its homologous phage, develops clear zones (plaques) where a phage particle had infected a host cell and replicated[99]. These plaques can be surrounded by "haloes" caused by diffusion of enzymes away from the plaque, resulting in lysis of bacteria in the surrounding region. Stirm et al.[98,99] proved that these enzymes were located in the viral spikes. Bacteriophages carry various enzymes[100], including glycanases[101] and lyases[102], which generally are very specific, catalysing the hydrolysis of a single type of glycosidic linkage and thus making the bacteriophage fairly host specific. For example, Klebsiella bacteriophage No.11[101] was found to cross react with only one out of 81 bacterial exopolysaccharides tested. Higher frequencies of cross reactions have been reported[103], but in these instances certain structural similarities existed between the polysaccharide substrates. Recently, the bacteriophage degradation of the Klebsiella K26 polysaccharide was described[104] in which two similar types of glycosidic bonds in the polymer were hydrolysed.

Normally, bacteriophages hydrolyse one type of glycosidic bond in

a polysaccharide forming oligosaccharides corresponding to one or more repeat units. The repeat unit is isolated in its native form and in some cases is the only method of obtaining a repeat unit carrying acid labile substituents[105]. The repeat unit is suitable for studies of conformation in solution[106] and of the binding properties of immunoglobulins[107], besides being invaluable in the structural elucidation of bacterial exopolysaccharides.

Bacteriophages which infect E.coli and Klebsiella are isolated from sewage and in earlier studies a rigorous purification procedure was adopted[101]. Dutton et al.[108] who used impure phage solutions, simplifying the method, have provided the researcher with a convenient means of isolating the oligosaccharides (P1, P2 etc.) in gram quantities. A solution containing 10^{13} phage particles is generally regarded as sufficient to degrade 1g of polymer.

The repeat units are studied by ^{1}H- and ^{13}C- nuclear magnetic resonance spectroscopy. The oligosaccharides have a reducing sugar and, in addition, many have a pyruvate acetal and/or O-acetyl substituents. These groups influence the n.m.r. spectra producing information aiding in the sequencing of the repeat unit[109]. Analysis by n.m.r. spectroscopy and methylation is often sufficient for a complete structural analysis of the repeat unit, particularly when dealing with a tri- or tetra-saccharide. A more detailed account of the methodology and the chemical and instrumental analyses involved in phage work will be given in section 8.

4.2. Specific glycohydrolases

Various enzymes are commercially available in pure form and can be utilised in structural analysis of polysaccharides. Enzymic activities are based on certain substrate characteristics, including the types of sugars and their sequence, anomeric configuration, solubility, the degree of polymerisation (d.p.) and the extent of branching. These characteristics dictate the susceptibility of the polysaccharide to enzyme hydrolysis and the nature of the hydrolytic products.

Enzymes which have been used are predominantly exo enzymes, hydrolysing the glycosidic linkage of the terminal non-reducing sugars of oligosaccharides isolated from various degradations of the original polysaccharide[110], e.g. α- and β-glucosidase, α- and β-galactosidase. (These enzymes will selectively hydrolyse either an α or β linked, terminal, non-reducing, glucose or galactose residue respectively). The specificity of such enzymes allows the identification of the terminal non-reducing sugar and the nature of its glycosidic linkage. The linkage position of the terminal non-reducing sugar can be determined by methylation analysis of the enzyme modified oligosaccharide.

The mild reaction conditions, and convenience, of enzymic degradation make it a useful method in the study of polysaccharide structures.

5. CHROMATOGRAPHIC TECHNIQUES

Various chromatographic separation procedures are utilised in the structural studies of polysaccharides. In particular, paper chromatography (p.c.), gel permeation chromatography (g.p.c) and gas-liquid chromatography (g.l.c.), which were used in this study, are considered in this review. Other forms of chromatography used in the study of carbohydrates include :

a) Ion-exchange chromatography

This technique has found wide application in carbohydrate chemistry[111]. For example, DEAE Sephadex, an ion-exchange gel, is used in the purification of acidic polysaccharides. Ion-exchange resins, for example Amberlite IR-120 (H^+), are used for purification and exchange of ions in carbohydrate preparations.

b) Paper electrophoresis

Paper electrophoresis overcomes the difficulties encountered in the separation of large, charged polysaccharides using p.c. It is also applicable to other compounds, for example, methylated sugars[112].

c) Thin layer chromatography (t.l.c.)

Silica gel t.l.c. is an alternative method for the qualitative identification of carbohydrates[113].

d) High pressure liquid chromatography (h.p.l.c.)

H.p.l.c. effects the separation of unmodified sugars, eliminating the derivatisation step, and is therefore becoming increasingly important as an analytical technique in carbohydrate chemistry. The method has also been used in the methylation analysis of carbohydrates[114].

5.1. Gas-liquid chromatography

Structural studies of polysaccharides and oligosaccharides require the identification and quantification of their component monosaccharides and g.l.c. is used extensively to achieve these objectives. Dutton has comprehensively reviewed the application of g.l.c. in the carbohydrate field[115-116].

Monosaccharide components of carbohydrates generally have low volatilities and must be derivatised prior to g.l.c. analysis. Sweeley and coworkers[117] effectively separated carbohydrates as their trimethylsilyl ethers (t.m.s.), a technique which led to expansion of the use of g.l.c. in carbohydrate analysis. Cyclic derivatives, however, produce complex chromatograms due to the presence of pyranose and furanose ring forms and their α and β anomers[118]; in some cases the straight chain form is also observed. This problem can be overcome by reduction of the aldose to the alditol or formation of the acyclic nitrile, where each sugar gives a single peak. In practice, the identification and quantification of the constituent sugars from a hydrolysate is achieved by separation of the monosaccharides as their peracetylated aldononitriles (PAANs)[119-120]. In methylation analysis, on the other hand, the partially methylated alditol acetates (PMAAs) are the derivatives of choice[121-123]. Whilst PAANs may be identified by comparing their retention times with those of authentic standards, PMAAs require a combined g.l.c.-mass spectrometry (g.l.c.-m.s.) approach[124-125]. Dmitriev et al.[126] reported g.l.c.-m.s. studies on partially methylated aldononitrile acetates, claiming these derivatives to be more convenient than PMAAs. Various alternative derivatives to PAANs have also been described[115] and, recently, the successful separation of monosaccharides as their alditol acetates[127]

and alditol t.m.s. ethers[123] has been achieved using capillary columns.

Capillary columns, an exciting innovation, are presently used to separate carbohydrate compounds. Fused silica capillary columns are highly inert, flexible and easy to handle. These factors, coupled with the large range of stationary phases available (Fig.5), has led to capillary columns largely replacing packed columns. The columns are either wall-coated open tubular columns (W.C.O.T.), support-coated open tubular columns (S.C.O.T.) or phase-bonded columns. The latter are the most popular because the stationary phase maintains film and thermal stability by being bonded to the column wall, prolonging its lifespan and allowing non-volatile contaminants to be rinsed off the column. Capillary columns effect good separations of compounds previously not well separated by packed columns, providing outstanding reproducibility and separation at substantially higher temperatures with minimal column bleed. In this laboratory phase-bonded silica capillary columns DB-1, DB-17 and DB-225 are used, corresponding to OV-1, OV-17 and OV-225, respectively. Where isomers of PMAAs coelute on OV-17, OV-225 is used[125], and vice versa, as the order in which the compounds emerge is different in each case.

A sensitive flame ionisation detector (FID) is coupled to a recording integrator which quantitates the detector responses. Different compounds produce a different response in the FID and the calculation of molar flame responses should be based upon the effective carbon response[128] for absolute quantitation. This laboratory uses the assumption that response factors of PMAAs are equal on an equal weight basis. Such an approximation is acceptable

because, in our case, we are dealing with repeating units within the polysaccharide where ratios of sugars to each other are more important than absolute quantitation.

SOME RECOMMENDED G.L.C. STATIONARY PHASES

Stationary phase	Composition	Operating temps. (°C)
OV - 101	100% methyl silicone	0 - 300
OV - 1	100% methyl silicone, gum	100 - 350
Apiezon L	Branched aliphatic hydrocarbon mixture	50 - 300
Dexsil 300GC	Carborane-methyl silicone	50 - 400+
OV - 17	50% methyl silicone / 50% phenyl silicone	0 - 375
OV - 210	50% methyl silicone / 50% trifluoropropyl silicone	0 - 275
AN - 600	25% cyanoethyl silicone / 75% methyl silicone	0 - 300
Carbowax 20M	Polyethylene glycol, M.Wt. 15000 - 20 000	60 - 225
EGA	Poly(ethylene glycol adipate)	100 - 200
TCEP	Tris(cyanoethoxy)propane	20 - 175
OV - 225	25% cyanopropyl-25% phenyl-50% methyl silicone	0 - 275

Fig.5

The importance of g.l.c. in carbohydrate chemistry can be illustrated by reference to a few of its other applications. For example, g.l.c. is used to determine the degree of polymerisation of oligo- and poly-saccharides by the method of Morrison[129] (Fig.6). The absolute configuration of the component sugars can be determined by

G.L.C. CHROMATOGRAM OF K69 **P1** ALDITOL, CARBOXYL REDUCED, AS PAANs

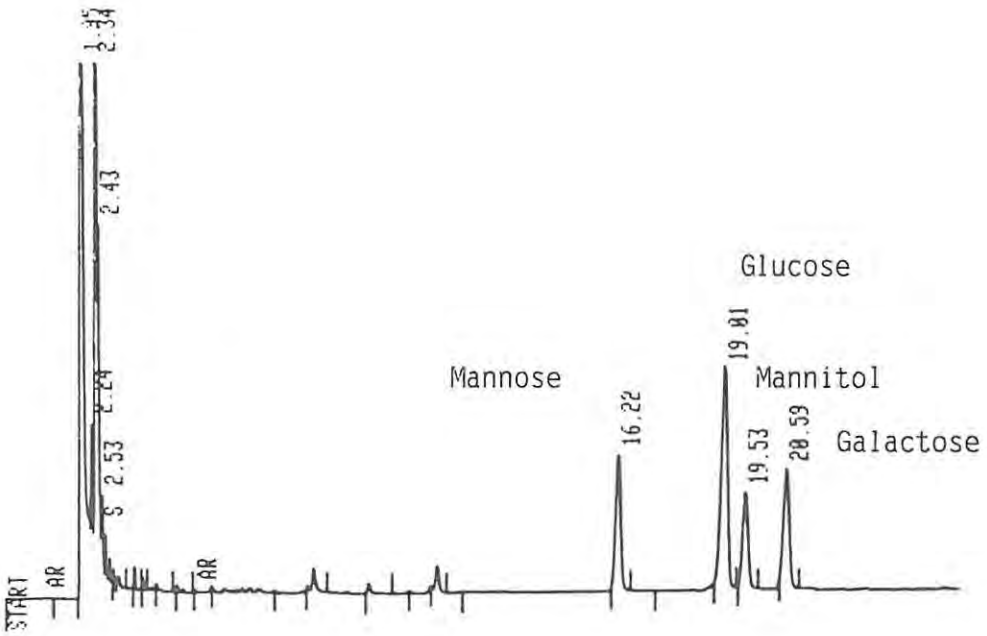


FIG.6 :

g.l.c. of their acetylated octyl glycosides[96]. Gerwig et al.[130] describe a similar method in which they utilise the trimethylsilylated (-)-2-butyl glycosides. Dutton and Gibney[92] have used g.l.c. to monitor the hydrolytic step in the Smith degradation.

5.2. Gel permeation chromatography (g.p.c.)

Gel permeation chromatography, also known as gel filtration or molecular-sieve chromatography, is a simple and reliable separatory technique providing high yields of material. G.p.c. has been described as the decreasing permeability of the 3-D network of a swollen gel to molecules of decreasing molecular size[131]. In g.p.c., gels constitute the stationary phase and have pores of varying size depending on the gel type. The liquid mobile phase carries the solute molecules through the gel. Larger molecules do not enter the pores, moving through the gel fastest, while progressively smaller molecules enter the gel through the pores, spending progressively longer times on the gel column. Molecules are therefore eluted in order of decreasing molecular size.

The types of gel used each have a specific porosity, allowing the separation of substances within a certain range of molecular sizes, that is, each gel type will have a characteristic fractionation range. It follows that the mean molecular sizes fractionated decreases with decreasing pore size. The table below lists some of the gels commonly used in this laboratory.

Type of gel	Fractionation range (mol. wt.) (Dextrans)
Sepharose 4B®	$3x10^5$ - $5x10^6$
Sepharose 4B CL®	$3x10^5$ - $5x10^6$
Sephacryl S400®	$1x10^4$ - $2x10^6$
Sephacryl S500®	$4x10^4$ - $20x10^6$
Bio-Gel P-4®	<4000

Sepharose is prepared from agarose, a neutral, linear polymer made up of alternate D-galactose and 3,6-anhydro-L-galactose residues. Sepharose CL is cross-linked agarose, a gel of similar porosity to Sepharose but structurally more rigid, whilst Sephacryl is a rigid gel consisting of cross-linked allyl dextran. Bio-Gel P is a series of polyacrylamide gels formed by co-polymerisation of acrylamide and N,N'-methylene-bis-acrylamide[132]. The pore size can be varied to give gels with different fractionation ranges. These gels are generally used with aqueous eluents although Sephacryl can also be used with organic solvents. Sephadex LH-20, on the other hand, is often used as a hydrophobic gel in the purification of methylation products, using chloroform as eluent.

The mobile phase (eluent) is chosen to suit the type of compound to be separated. Charged substances require a buffered mobile phase to retard adsorption of solute molecules onto active sites in the gel. However, the pH and ionic strength of the buffer must be such as not to affect the solute in any way. Aqueous sodium chloride is a common eluent, however, if the product is to be lyophilised, volatile buffers such as pyridinium acetate solution are more suitable.

A gel chromatogram is a plot of volume eluted versus

carbohydrate concentration (Fig.7). The elution volume (Ve) is the volume at which the maximum concentration of each component elutes, as determined from the elution diagram. Detection of carbohydrates is achieved using various colour reactions, the phenol-sulphuric acid method[133] being a popular choice.

Some applications of g.p.c. are :

a) Purification of polysaccharides by preparative g.p.c.

b) Separation of oligomers from partial acid hydrolysis and of bacteriophage depolymerisation of polysaccharides. Churms and Stephen[134] have used g.p.c. to monitor the course of acid hydrolysis of the capsular polysaccharides from some strains of Klebsiella.

c) Molecular weight determination, based on the linear correlation between Ve and Log.molecular weight of a series of compounds whose molecular weights fall within the fractionation range of the gel. Typically, a series of dextrans are chromatographed and a calibration curve is constructed for a column (Fig.8). Since polysaccharides have a distribution of molecular weights, Ve for the polysaccharide will be the average molecular weight read off a calibration curve. This is an indirect method of molecular weight determination, weights being determined relative to those of authentic standards.

5.3. Paper chromatography (p.c.)

This technique is simple and requires minute samples of underivatised monosaccharides and oligosaccharides for qualitative determinations. Optimum separation is obtained in a reasonable time period by judicious choice of solvent. Although p.c. has largely been

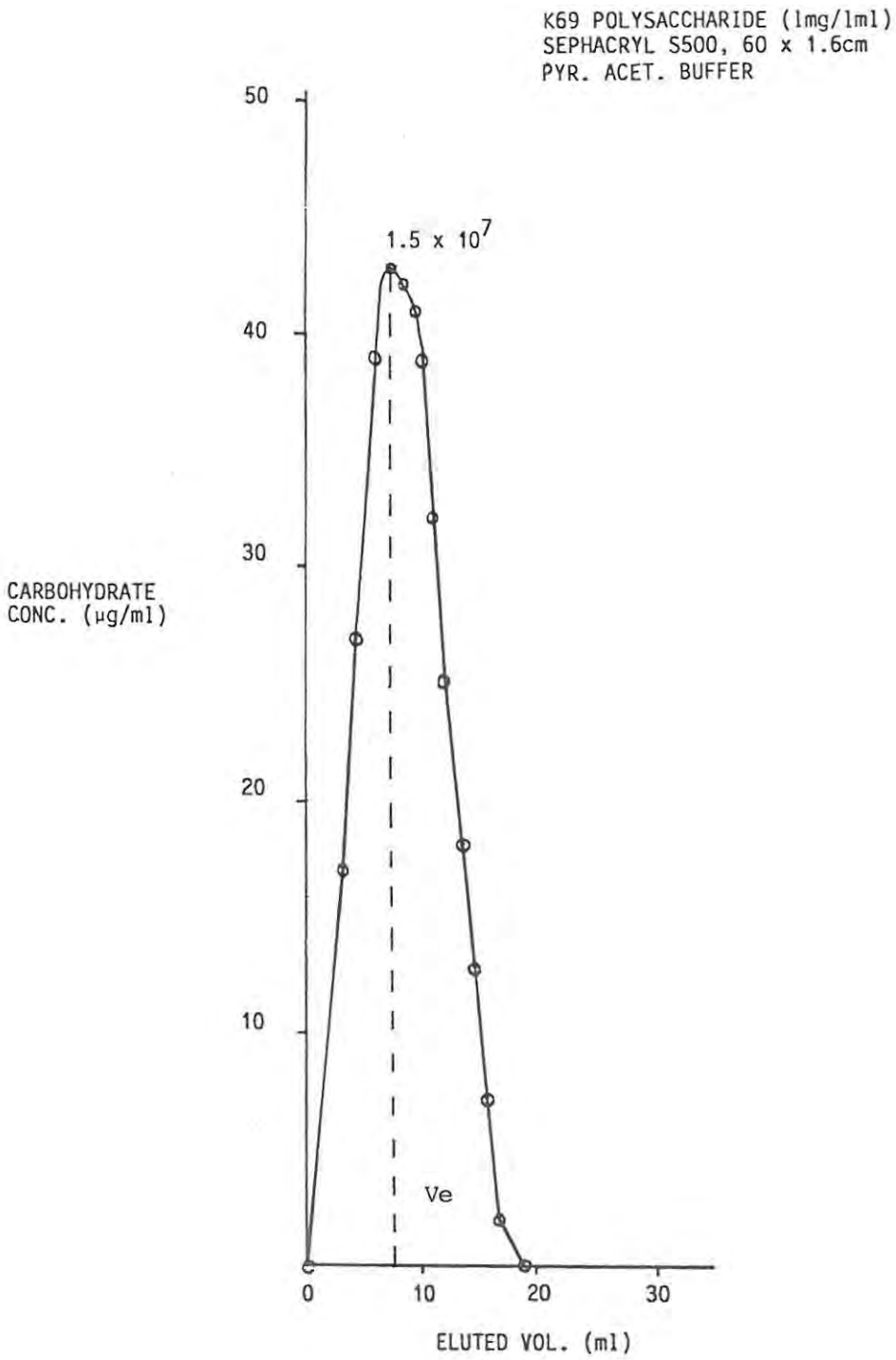

K69 POLYSACCHARIDE (1mg/1ml)
SEPHACRYL S500, 60 x 1.6cm
PYR. ACET. BUFFER
1.5 x 10^7
CARBOHYDRATE
CONC. (µg/ml)
50
40
30
20
10
Ve
0
10
20
30
ELUTED VOL. (ml)

FIG. 7

CALIBRATION CURVE

SEPHAROSE 4B CL (1.6 x 60cm)

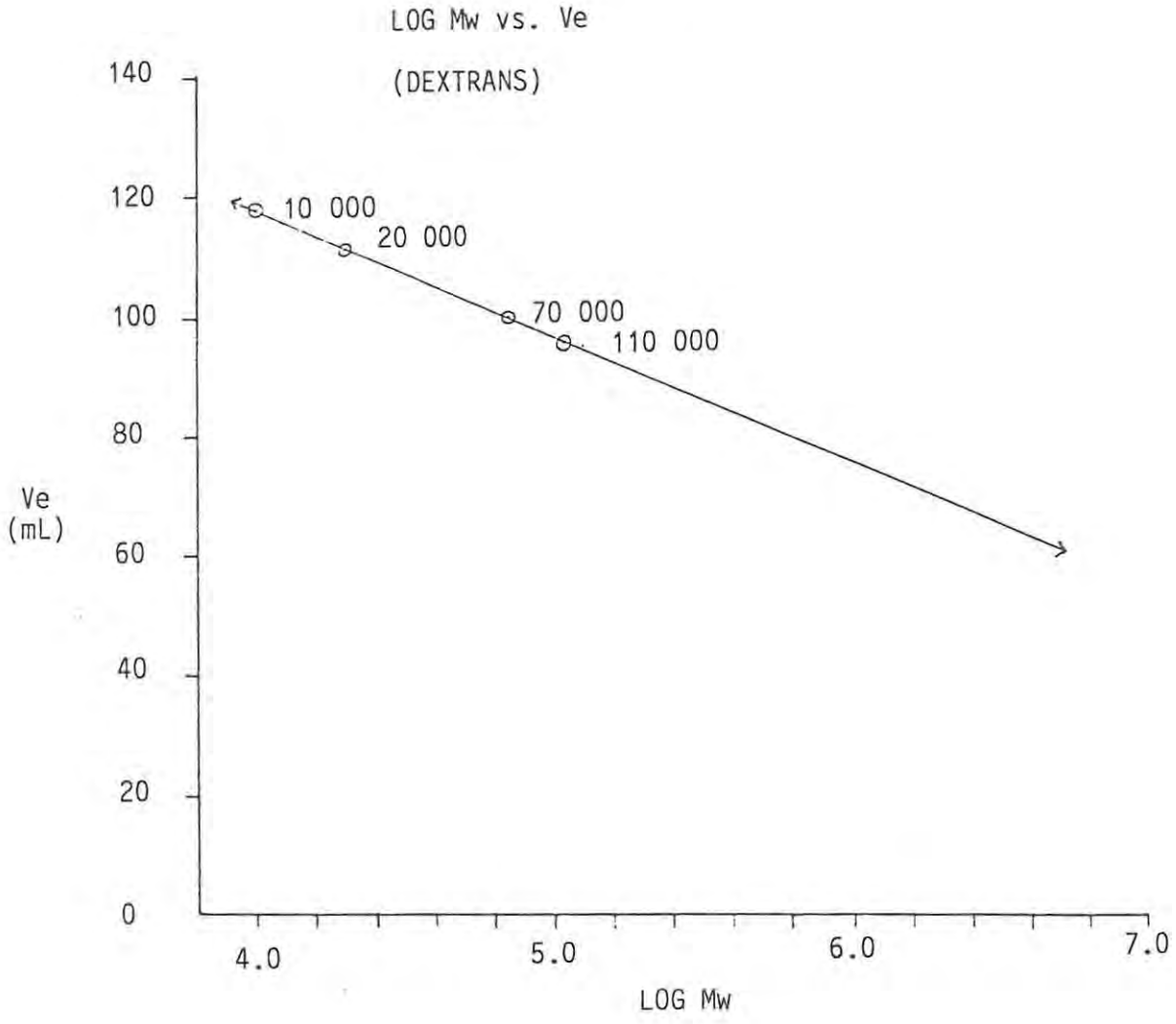


FIG.8

superceded by g.l.c. and g.p.c. in our studies of polysaccharides, it still finds useful applications.

P.c. is used for identifying the components of a polysaccharide hydrolysate, to monitor the graded acid hydrolysis of polysaccharides and to check for oligosaccharides after bacteriophage depolymerisation. Homogeneity of the oligosaccharide fractions isolated from gel permeation chromatography can be determined by p.c. and, where necessary, preparative separations of cochromatographed fractions carried out. Although preparative separations of the products of partial hydrolysis or of periodate oxidation are still carried out by p.c., g.p.c. is the method of choice.

Churms[112] has reviewed the various solvents and detection methods used in p.c. Solvents used during the course of the present work include :

1) 8 : 2 : 1 ethyl acetate - pyridine - water, a basic solvent,

2) 18 : 3 : 1 : 4 ethyl acetate - acetic acid - formic acid - water, an acidic solvent giving good separation of monosaccharides, and,

3) 5 : 1 : 5 : 3 ethyl acetate - acetic acid - pyridine - water, a solvent providing good separation of uronic acids, neutral sugars and amino sugars.

The detection of carbohydrates on paper chromatograms was achieved using one of two methods, either the alkaline silver nitrate/sodium thiosulphate method[135] where reducing sugars show up as brown to black spots, or the periodate/benzidine reagent[136] which leaves white spots on a blue background and is used to detect oligosaccharides and non-reducing sugars.

6. INSTRUMENTAL ANALYSIS

Technological progress in the fields of nuclear magnetic resonance spectroscopy and mass spectrometry (including g.l.c.-m.s.) has made these techniques invaluable to the carbohydrate chemist and are routinely used in the structural elucidation of polysaccharides.

6.1. Nuclear magnetic resonance (n.m.r.) spectroscopy

High resolution n.m.r. spectroscopy has several useful features, it is a non-destructive, quantitative technique and a large amount of data is obtained in a relatively short period of time (no elaborate chemical manipulations are required in the preparation of samples for routine n.m.r. studies).

6.1.1. Proton n.m.r. spectroscopy (^{1}H-n.m.r.)

The routine use of ^{1}H-n.m.r. spectroscopy in the structure elucidation of polysaccharides has been made possible through the introduction of super-conducting magnets[137], which have greatly enhanced the resolution of ^{1}H-n.m.r. spectra, for example, 500 MHz pulsed n.m.r. spectrometers are now available. Carbohydrates were first investigated by n.m.r. spectroscopy by Lemieux et al. in 1958[138]. The many advances made since then have been covered in several review articles[139-142]. The use of ^{1}H-n.m.r. spectroscopy in studies of Klebsiella capsular polysaccharides was first reported by Dutton et al.[143].

Specific types of structural information are obtained :

a) the degree of polymerisation of oligosaccharides,

b) the identity of the monosaccharides and other non-carbohydrate substituents making up the carbohydrate compound and

c) the stereochemical characteristics of the constituents.

A large proportion of this information is obtained from the chemical shifts of the protons linked to the anomeric carbons (the anomeric protons). The chemical shift (δ) is the frequency separation between the reference signal and a proton signal of the compound being analysed.

$$\delta = \frac{\text{frequency separation (in Hz) between the resonance and } Me_4Si \times 10^6}{\text{spectrometer radiofrequency (Hz)}}$$

Tetramethylsilane (TMS) is used as a reference for protons ($\delta=0$), however, since TMS is not water soluble, 4,4-dimethyl-4-silapentane-1-sulphonate (DSS) is used as an internal reference when dealing with aqueous carbohydrate solutions. Acetone is incorporated as an internal reference, producing a signal which is 2.23 p.p.m. downfield from the tetramethylsilane signal.

Deshielding of the anomeric proton by the ring oxygen atom causes the anomeric signals to be well separated from those of the other protons in a carbohydrate compound, occuring well downfield from the rest, in the so called "anomeric region". This part of the spectrum occurs between δ4.5 and δ5.5, with the β- anomeric signals occuring from δ4.5 to δ5.0 and the α- anomeric signals, from δ5.0 to δ5.5. The total sugar ratio in the repeat unit of a polysaccharide can be ascertained from the number of anomeric signals and their relative intensities. The anomeric region is useful for distinguishing furanose sugars from pyranose sugars; signals of sugars in the furanose form appear downfield from those of the pyranose forms[17] (e.g. β-Galf resonates at δ5.3). However, some ring protons also resonate in the anomeric region, including both H-5[144] and H-4[145] of an

α-galacturonic acid, therefore caution must be excercised in the interpretation of these spectra.

Proton spin-spin coupling (J-value), especially between vicinal protons, provide useful structural information. In the present work, we are particularly interested in the coupling ($J_{1,2}$) between H-1 and H-2. This coupling constant depends on the dihedral angle separating the vicinal protons. The value is large (≃8Hz) when the protons are antiparallel (transdiaxial) and relatively small (≃3Hz) when gauche disposed. This information, when taken together with chemical shifts, enables the assignment of the anomeric configuration, as generally, anomeric protons of α-linked sugars resonate from 5.0 to 5.5 p.p.m. (with small $J_{1,2}$ values) whilst those of β-linked sugars resonate from 4.5 to 5.0 p.p.m. (with large $J_{1,2}$ values) (Fig.9).

The accumulation of ^{1}H-n.m.r. data in the literature enables the identification of component sugars through the characteristic chemical shifts and coupling constants ($J_{1,2}$) of their anomeric protons. When one is dealing with oligosaccharides the reducing terminus can be identified through its partial α and β signals (resulting from mutarotation). Furthermore, the degree of polymerisation can be determined from the ratio of the integral of the reducing signal to the integrals of the other anomeric signals.

^{1}H-N.m.r. spectroscopy allows the identification of non-carbohydrate constituents in polysaccharides. For example O-acetyl groups and pyruvate acetals are common substituents in the Klebsiella capsular polysaccharides, and the methyl protons of these groups have characteristic chemical shifts. Furthermore, the chemical shift values of pyruvate vary with the stereochemistry of the acetal carbon

^{1}H-N.M.R. SPECTRUM OF KLEBSIELLA K69 DE-O-ACETYLATED **P1** (REPEAT UNIT)

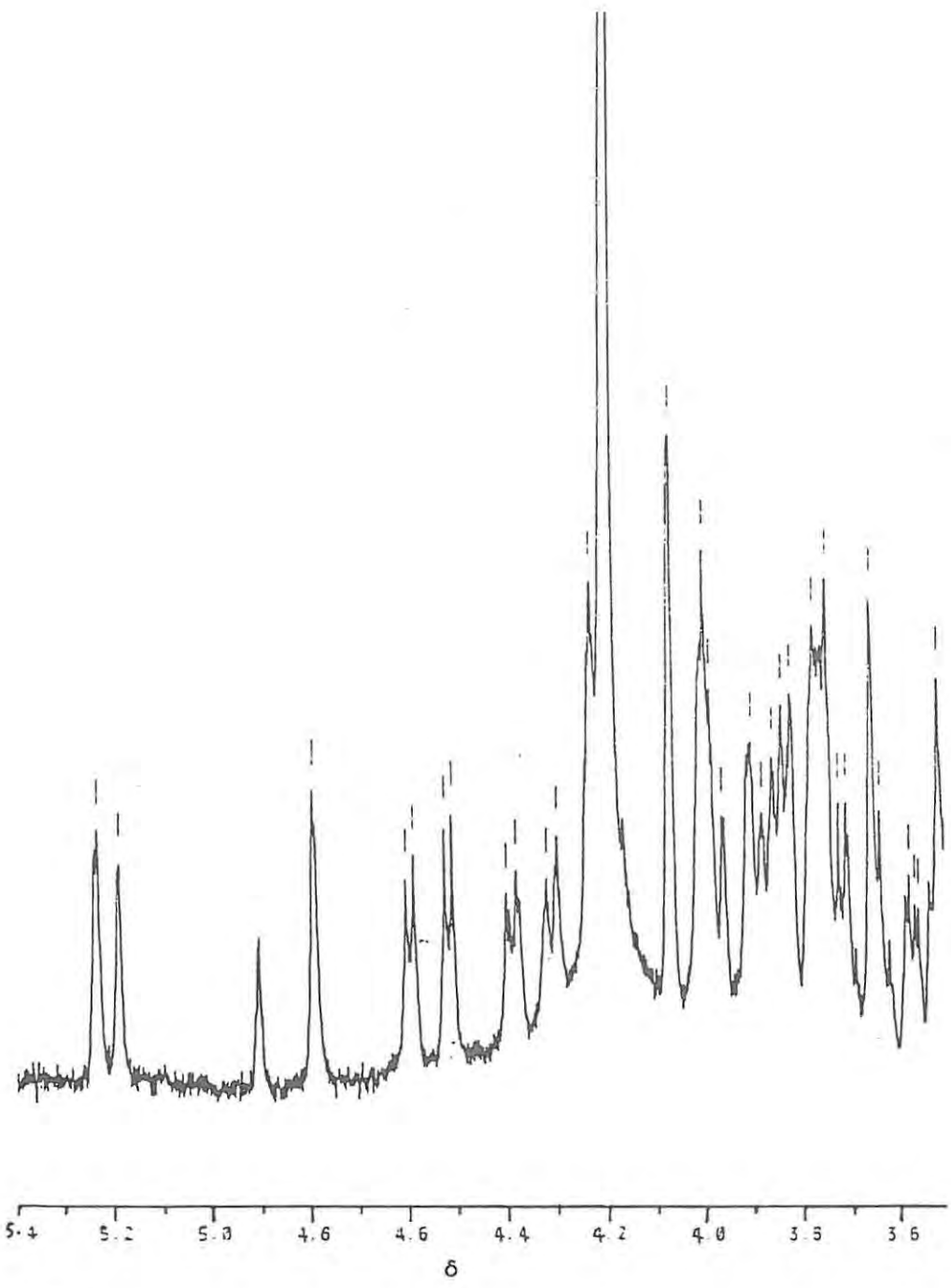


FIG.9

and permit the assignment of the R or S configuration to this group[146].

In practice, n.m.r. studies are performed on solutions of poly- and oligo-saccharides, however, interference from exchangeable protons and line broadening, results in loss of resolution. In order to minimise the problem of exchangeable protons, good quality D_2O (99.95%) is used to exchange hydroxyl protons for deuterons; several exchanges are necessary and D_2O is used as the spectroscopic solvent. Nevertheless, residual water produces a large signal, known as HOD, which resonates at 4.8 p.p.m. when the spectrum is acquired at ambient temperature. Since this signal is in the anomeric region, it can mask the anomeric signals of the sample, but can be shifted upfield by running the spectrum at elevated temperature (80-90°). This treatment can, however, result in the loss of labile substituents (e.g. O-acetyl) and thus structural information, especially if the sample is to be recovered for further analysis. An alternative solution to the problem employs saturation decoupling[147] to eliminate the HOD signal; water protons have longer relaxation times than sugar protons so that by choosing a suitable pulse sequence, the HOD signal can be removed.

Signal broadening, due to the short relaxation times of sugar protons, can be overcome by running the samples at higher temperatures, whilst the use of strong magnetic fields (400-500 MHz) and pulsed Fourier transform n.m.r. spectrometers has overcome line broadening associated with the earlier spectrometers.

For the chemical shifts of polysaccharide and oligosaccharide protons from work presented in this thesis, the reader is referred to sections 7 and 8. It must be stressed that despite the wealth of structural information provided by n.m.r. studies on polysaccharides,

this technique must still be used in conjunction with other physical and chemical procedures.

6.1.2. Carbon-13 n.m.r. spectroscopy

^{13}C-n.m.r. data complements those from ^{1}H-n.m.r. spectra and are often superior due to their resonance signals being distributed over a wider range (200 p.p.m.), giving better resolution. However, the low natural abundance of ^{13}C makes the pulsed Fourier transform technique essential for the acquisition of ^{13}C spectra. Many aspects of ^{13}C-n.m.r. are utilised in the study of complex carbohydrates, for example, ^{1}H-^{13}C and ^{13}C-^{13}C coupled spectra[148]. The use of ^{13}C-n.m.r. spectroscopy in structural studies of the Klebsiella K69 polysaccharide is discussed in section 7. Gorin has given a wider account of the applications of this technique[147] to carbohydrates in general.

Spectra run in the ^{1}H-^{13}C decoupled mode provide sharp singlets for each carbon atom in the compound and utilisation of chemical shift values enables the assignment of these signals and the determination of the anomeric configurations. Just as the anomeric protons resonate downfield of the rest of the ring protons, so do the anomeric carbon atoms in sugars resonate downfield from the other ring carbons. The number of anomeric signals can provide information on the size of the repeat unit (usually, this information is more readily available than from proton spectra because of the better separation of the signals). However, carbon resonances are not easily quantitated because of the differences in nuclear Overhauser effect enhancements.

Assigning sugar residues to anomeric signals can be achieved

^{13}C-N.M.R. SPECTRUM OF K69 OLIGOSACCHARIDE **A2** SHOWING THE ANOMERIC SIGNALS

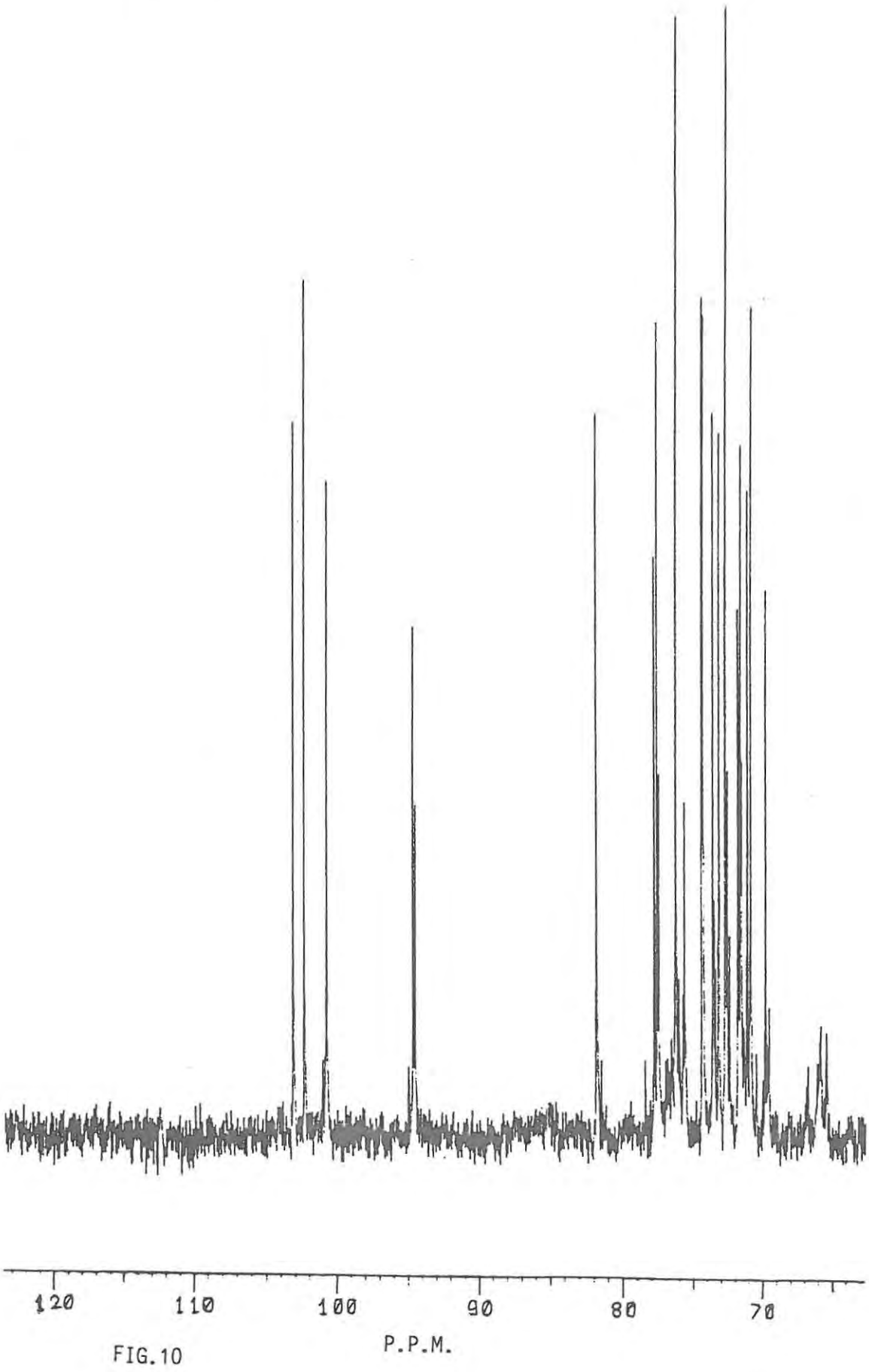


FIG.10

utilising the available data (refs. 15,17,44,147,149,150,151). Pyruvate acetals and O-acetyl groups can be identified by the ^{13}C signals corresponding to their methyl and carboxyl groups (Table 2, pg. 58). Moreover, the chemical shifts of methyl carbons of pyruvate acetals have values which depend on the R and S configuration of the acetal carbon and are thus used to assign configuration[152].

The chemical shifts of anomeric carbons are dependent on anomeric configuration in the same way as those of anomeric protons, with an α-linked anomer resonating upfield of a β-anomer of the same sugar. Furanose sugars have distinct anomeric signals[153], for example, in the study of the capsular polysaccharide isolated from Klebsiella serotype K41[44] it was reported that the C-1 signal of the galactofuranose residue occured considerably downfield from the rest of the anomeric signals. When one is dealing with oligosaccharides, the α and β anomers of the reducing sugar resonate ∿5 p.p.m. (∿95 p.p.m.) upfield from the other anomeric carbons.

6.1.3. Two-dimensional (2D) n.m.r. spectroscopy

Complex molecules such as polysaccharides and oligosaccharides give rise to complex, crowded n.m.r. spectra, with the result that the accurate measurement of n.m.r. parameters and the assignment of resonances to specific nuclei becomes very difficult. Fortunately, new techniques, based on the double Fourier transformation of n.m.r. signals, have been developed, helping the researcher to get around this problem. These techniques are referred to as two-dimensional (or 2D) n.m.r. spectroscopy.

There are two classes of 2D experiments viz. J-resolved and spin correlated experiments. In J-resolved 2D spectra one frequency axis

contains chemical shift information and the other, coupling information, while in spin correlated 2D spectra both axes contain chemical shift data. Each 2D experiment has a closely related 1D experiment, the latter being converted into a 2D experiment by varying the evolution period. The connection between the two axes in 2D spectra is established through spin coupling. Benn et al.[154] have given a detailed account of these modern pulse methods.

J-resolved 2D n.m.r. spectroscopy

This technique finds its most important application in resolution of overlapping multiplets, thus providing the chemical shift and coupling constant parameters of complex molecules. This technique was utilised by Hall and Morris[155] to measure ^{1}H-^{13}C coupling constants in oligosaccharides. Both ^{1}H- and ^{13}C-2D J-resolved spectroscopic techniques have been applied to carbohydrates[156-159]. The ^{1}H-^{1}H and ^{1}H-^{13}C coupling-constants of the anomeric atoms are used to determine the anomeric configurations of the sugar residues in poly- and oligo-saccharides.

Correlated 2D n.m.r. spectroscopy

These experiments provide 2D correlations between homonuclear (e.g. ^{1}H-^{1}H) and between heteronuclear (e.g. ^{1}H-^{13}C) spin systems. The 2D correlations between homonuclear spin systems are known as COSY (J-correlated spectroscopy) and SECSY (spin echo correlated spectroscopy).

a) J-correlated (COSY) experiments provide proton coupling information, where the 1D spectrum lies along the diagonal and off diagonal peaks show coupling correlations (Fig.11).

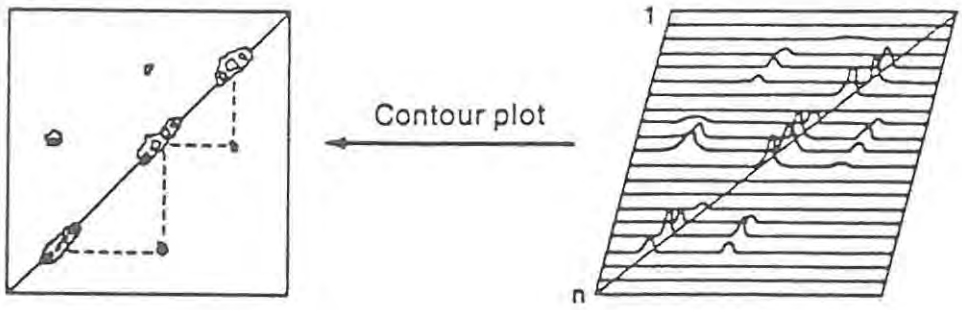


FIG.11 : 2D-COSY N.M.R. SPECTRUM (^{1}H)

2D ^{13}C-correlated experiments show which carbons are connected to each other.

b) 2D heteronuclear correlated spectroscopy has been used to facilitate the unambiguous assignment of anomeric signals in oligosaccharides[155]. In this technique the proton spectrum lies along one axis and the corresponding ^{13}C- spectrum lies along the other axis, the overall spectrum consisting of one signal for each carbon-proton pair in the molecule. (Annexure C, fig.3a). By knowing the carbon assignments, proton assignments can be made on the basis of their correlations, and vice versa. Heteronuclear correlation data was utilised in the assignment of anomeric ^{13}C signals in the work presented in this thesis.

Generally, 2D spectroscopy has the advantages of improved signal to noise ratios, easier determination of coupling constants and the accurate assignment of anomeric signals. However, 1D methods should be used in conjunction with 2D techniques, since this makes the interpretation of 2D spectra far simpler and 1D spectra require less sophisticated pulse transmitters and shorter acquisition times.

Modern n.m.r. spectrometers are equipped (in terms of instrumentation and software) to handle 2D experiments, and therefore 2D-n.m.r. spectroscopy undoubtedly will become a popular technique in the structural analysis of polysaccharides.

6.2. Mass spectrometry of carbohydrates

Mass spectrometry has become an important technique in carbohydrate analysis. Modern instrumentation and inlet techniques have increased the power and versatility of this procedure and its varied uses have been reviewed by Chizhov and Kotchetkov[160]. The method has several advantages: accurate data is rapidly obtained using only small amounts of material (1 μg or less), real data can be distinguished from artefacts, certain chemical manipulations, such as deuterium labelling, can be used for the unambiguous interpretation of results.

Three types of inlet system can be used for the introduction of the sample into the m.s., these are as follows: the hot inlet system (for moderately volatile compounds), the direct probe (for thermally unstable compounds), and the g.l.c.-m.s. interface (which effects the separation of a mixture and the sequential introduction of its components into the m.s.). G.l.c.-m.s. has found greater application in the structural analysis of polysaccharides.

In order to be sufficiently volatile for use in g.l.c.-m.s., carbohydrate compounds need to be derivatised. The various derivatives utilised in m.s. (i.e. monosaccharide glycosides, monosaccharide alditols and other monosaccharide and oligosacharide derivatives) have been reviewed[161]. In the study of bacterial polysaccharides, g.l.c.-m.s. is used in conjunction with methylation analysis[162-163] for the accurate determination of the substitution

patterns of the permethylated monosaccharide components. In this connection partially methylated alditol acetates (PMAA's) appear to be the derivatives of choice .

6.2.1. Electron-impact mass spectrometry (e.i.-m.s.)

Electron-impact ionisation, commonly used in m.s., involves the bombardment of neutral molecules with high energy electrons to cause fragmentations which result in the formation of singly charged ions. These ions are measured in terms of their mass to charge ratios (m/z). The relative abundance of each ion is plotted against the m/z ratio to give a characteristic spectrum and identification of the compound is then accomplished by comparing its fragmentation pattern with those of reference spectra[124]. Stereoisomeric PMAA's (e.g. glucose, galactose and mannose) give similar spectra that are not distinguishable. The fragmentation ions are normally singly charged, thus the m/z ratios provide their molecular weights.

In m.s. two types of fragments are observed - primary fragments and secondary fragments. Primary fragments arise through fission between two carbon atoms of the parent molecule and secondary fragments are formed from primary fragments. With respect to primary fragmentation of PMAA's it is of significance (analytically) that fission between two methoxylated carbons is more common than that between one methoxylated carbon and one acetoxylated carbon, which in turn is more frequent than fission between two acetoxylated carbons. Secondary fragmentations occur by the loss of one or more of the following groups : acetic acid (60 m.u.), ketene (42 m.u.), methanol (32 m.u.), and formaldehyde (30 m.u.). The peaks in the m.s. of 1,5-

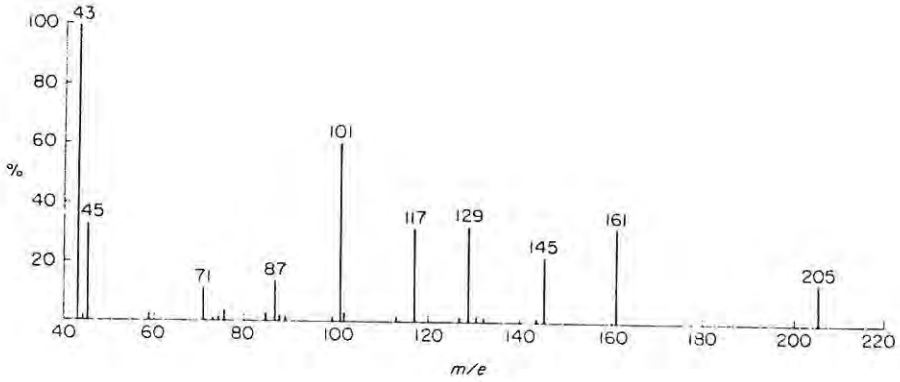
di-O-acetyl-2,3,4,6-tetra-O-methylglucitol (Fig.12) are accounted for using the above information.

Upon reduction of methylated sugars to the alditols, some, for example 3-O-methyl- and 4-O-methyl-hexoses, produce identical mass spectra. This problem may be overcome by carrying out the reduction with sodium borodeuteride, so as to produce deuterium labelled products. With carbohydrates the molecular ion peak cannot be observed using e.i.-m.s. and other techniques such as chemical ionisation (c.i.)[164], field ionisation[165] and field desorption[166], maintain the molecular ion peak. These are therefore used in conjunction with e.i.-m.s.

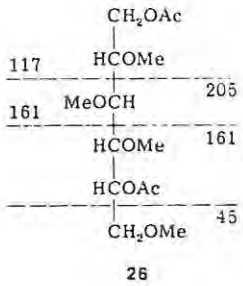
6.2.2. Fast atom bombardment m.s. (f.a.b.-m.s.)

A major drawback of both e.i.-m.s. and c.i.-m.s. (to a lesser extent) is the extensive fragmentation that occurs. Consequently, the mass spectra of high molecular weight compounds suffer from a loss in signal intensity at high m/z values. A method aimed at improving this situation, combines e.i.-m.s. with field desorption (f.d.) studies, and has found application in the molecular weight determination of carbohydrates. Nevertheless, f.d.-m.s. has been replaced for many purposes by f.a.b.-m.s., a relatively new technique.

F.a.b.-m.s. can be performed on unmodified, as well as derivatised oligosaccharides and small polysaccharides. The sample is first dissolved in a suitable solvent (e.g. water, for unmodified material and methanol or chloroform for methylated/acetylated samples) and then added (5μg in 0.5μL) to a drop of glycerol previously placed on the stainless-steel (or copper) target. The target is bombarded

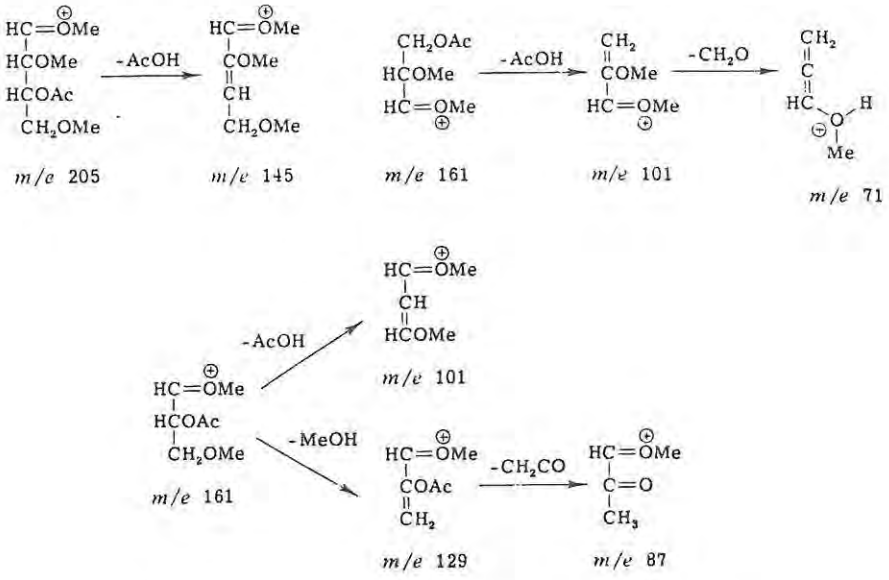


Primary fragmentation:



26

Secondary fragmentation:



161

FIG.12 E.I.-M.S. FRAGMENTATION OF 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol.

with Ar^+ or Xe^+ ions having 2-8 keV of energy. Both positive and negative ions are produced, allowing the recording of both types of spectra and the mass spectra obtained by this method have a high pseudomolecular ion sensitivity, with $(M+H)^+$ in the positive ion spectra and $(M-H)^-$ in the negative. In addition, the spectra shows a wealth of fragmentation information, allowing the sequencing of oligosaccharides and small polysaccharides. Barber et al.[167] demonstrated the superiority of this technique to f.d. studies, this being largely due to the stability of the ions produced by f.a.b.-m.s.

Dell and coworkers[168] studied unmodified, permethylated and acetylated oligosaccharides and glycosphingolipids by f.a.b.-m.s. in order to assess its application in the carbohydrate field. They showed that it is, indeed, applicable to both native and derivatised oligosaccharides, using small samples (1-5 μg). However, because limited fragmentation was obtained with unmodified material, the examination of methylated derivatives was recommended in order to obtain more fragment ions and, hence, enable the sequencing of oligosaccharides. Dr. Dell and her research group are at present conducting f.a.b.-m.s. studies on material isolated from the Klebsiella K69 capsular polysaccharide. Preliminary investigations appear to be promising, and we envisage their results complementing the results presented in this thesis. Dell and Ballou[169] successfully analysed a Mycobacterial O-methyl-D-glucose polysaccharide and lipopolysaccharide by negative ion f.a.b.-m.s. These workers also investigated a polysaccharide-lipid interaction using positive ion f.a.b.-m.s.[170]. The f.a.b.-m.s. technique has even been used to differentiate between underivatised anomeric methyl glycosides[171].

The applications of f.a.b.-m.s. cited above, form only a small

part of the range of this versatile procedure. In conclusion the f.a.b. method of ionisation has the following advantages :

i) A solid is ionised, sometimes at room temperature, in such a manner as to avoid the thermal effects associated with sample volatilisation.

ii) Preparation of the sample is straightforward, particularly when unmodified material is required.

iii) The method operates in the positive or negative ion mode and gives good pseudomolecular ion sensitivity together with structurally significant fragmentations.

iv) Mass spectra can be obtained for molecules of relatively high molecular weight.

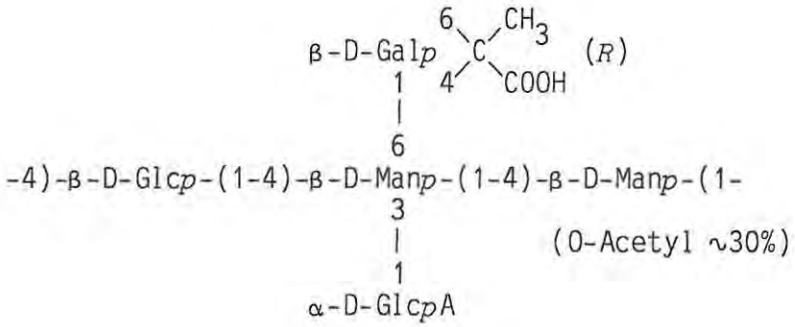
THE EXOCELLULAR POLYSACCHARIDE OF *KLEBSIELLA* SEROTYPE K69

THE EXOCELLULAR POLYSACCHARIDE OF KLEBSIELLA SEROTYPE K69

7. THE STRUCTURAL ELUCIDATION OF THE CAPSULAR POLYSACCHARIDE OF KLEBSIELLA SEROTYPE K69

ABSTRACT

The structure of the capsular polysaccharide (K antigen) of Klebsiella K69 has the pentasaccharide repeating unit shown. Methylation analysis, β-elimination and partial hydrolysis were the principle chemical techniques utilised. N.m.r. spectroscopy (^{1}H- and ^{13}C-) proved invaluable in the determination of the anomeric configurations and the sequence of the sugars making up the polysaccharide.



7.1. Introduction

The concept of a repeating-unit in bacterial capsular polysaccharides has been well established[14]. The Klebsiella K69 capsular polysaccharide has a repeating unit with the qualitative composition D-galactose, D-glucose, D-glucuronic acid and D-mannose. It is therefore one of 18 strains of Klebsiella spp. with capsular polysaccharides having the above monosaccharide composition. Three of these polysaccharides, K30[31], K33[32] and now K69, fall into a subgroup having both 1-carboxyethylidene acetal and O-acetyl

substituents. It is also interesting to note that these three polysaccharides are all made up of a "3+1+1" type repeating-unit .

The elucidation of the chemical structure of Klebsiella K69 exopolysaccharide will now be described.

7.2. Results and discussion

7.2.1. Composition and n.m.r. spectra

The polysaccharide was isolated and purified by the method of Okutani et al.[51] (section 7.4.2). The pure, acidic polysaccharide had $[\alpha]_D$ +54.9 ° and was shown to be homogenous (Mw. 1.5 x 10^7) by gel permeation chromatography (Annexure B, Fig.1) using a column of Sephacryl S500, calibrated with dextrans. Hydrolysis of the native and carboxyl reduced polysaccharide and g.l.c. analysis of the derived peracetylated aldononitriles, gave the results shown in Table 1.

SUGAR ANALYSIS OF NATIVE AND CARBOXYL REDUCED POLYMER

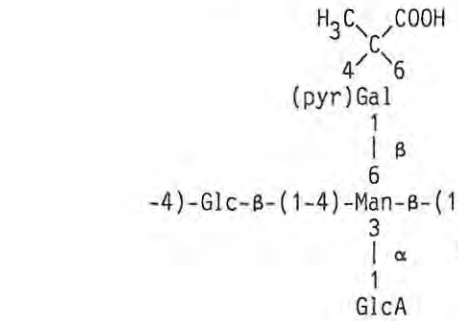
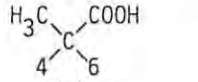
SUGARS(as PAAN's)	MOLAR RATIOS	
	NATIVE	CARBOXYL REDUCED
MAN	1.7	2.0
GLC	1.0	2.0
GAL	1.4	1.4

TABLE 1.

^{1}H- and ^{13}C-n.m.r. spectra of the native polysaccharide were not well resolved due to the poor solubility of the polymer. A fairly well resolved ^{1}H-n.m.r. spectrum was obtained after autohydrolysis

TABLE 2

N.M.R. DATA FOR KLEBSIELLA K69 POLYSACCHARIDE AND DERIVED OLIGOSACCHARIDES

Compound[a]	^{1}H-N.m.r. data					^{13}C-N.m.r. data	
	δ[b] (p.p.m.)	$J_{1,2}$ (Hz)	Integral (No. of H)	Assignment[c]		P.p.m.[d]	Assignment[e]
							
(pyr)Gal	5.19	no	1	GlcA-α-	A		
1							
| β	4.77	no	2	-4-Man-β-	B+C		
6							
-4)-Glc-β-(1-4)-Man-β-(1-4)-Man-β-(1-							
3							
| α (acetate 30%)	4.62	7	1	Gal-β-	D		
1	4.50	7	1	-4-Glc-β-	E		
GlcA	4.47	10	1	H-5 of α-GlcA	H		
K69 Native Polysaccharide	2.16	s	1	CH_3 of O-acetate	F		
	1.48	s	3	CH_3 of pyruvate acetal	G		
Gal	5.18	no	1	GlcA-α-	A	103.84	Glc-β-1-
1							
| β	5.18	no	0.6	-4-Man-α-OH	B	103.13	Gal-β-1-
6							
Glc-β-(1-4)-Man-β-(1-4)-Man-OH	4.90	no	0.4	-4-Man-β-OH	B	102.41	GlcA-α-1-
3							
| α				6			6
1	4.77	no	1	-4-Man-β-	C	100.98	-4-Man-β-1-
GlcA				3			3
A1	4.58	8	1	Gal-β-	D	94.62	-4-Man-α-OH
	4.47	8	1	Glc-β-	E	94.49	-4-Man-β-OH
	4.31	11	1	H-5 of GlcA-α-	F	176.65	COOH of GlcA

Glc-β-(1-4)-Man-β-(1-4)-Man-OH 3 | α 1 GlcA A2	5.19	3.5	1	GlcA-α-	A	176.62	COOH of GlcA
	5.19	no	0.6	-4-Man-α-OH	B	103.13	Glc-β-1-
	4.90	no	0.4	-4-Man-β-OH	B	102.37	GlcA-α-1-
	4.77	3	1	-4-Man-β- 3	C	100.75	3 -4-Man-β-1-
	4.52	8	1	Glc-β-	E	94.64	-4-Man-α-OH
						94.48	-4-Man-β-OH
Glc-β-(1-4)-Man-β-(1-4)-Mannitol 3 | 1 GlcA A2 reduced	5.23	3	1	GlcA-α-	A	173.75	COOH of GlcA
	4.83	no	1	-4-Man-β- 3	C	103.16	Glc-β-1-
						102.22	GlcA-α-1-
	4.51	8	1	Glc-β-	E	100.48	-4-Man-β-1- 3
	4.42	10	1	H-5 of GlcA-α-	H		
GlcA-α-(1-3)Man-β-(1-4)-Man-OH A3	5.29	3	1	GlcA-α-	A		
	5.22	no	0.6	-4-Man-α-OH	B		
	4.94	no	0.4	-4-Man-β-OH	B		
	4.76	3	1	-3-Man-β-	C		
GlcA-α-(1-3)-Man-β-(1-4)-Mannitol A3 reduced	5.29	3	1	GlcA-α-	A		
	4.81	no	1	-3-Man-β-	C		
	4.30	10	1	H-5 of GlcA-α-	H		

[a]For sources of A1, A2 and A3 see text.

[b]Chemical shift relative to acetone, δ 2.23 downfield from sodium 4,4-dimethyl-4-silapentane-1-sulphonate (DSS).

[c]-3-Man-β- refers to a 3-linked mannosyl residue in the β-anomeric configuration.

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

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

s = singlet.

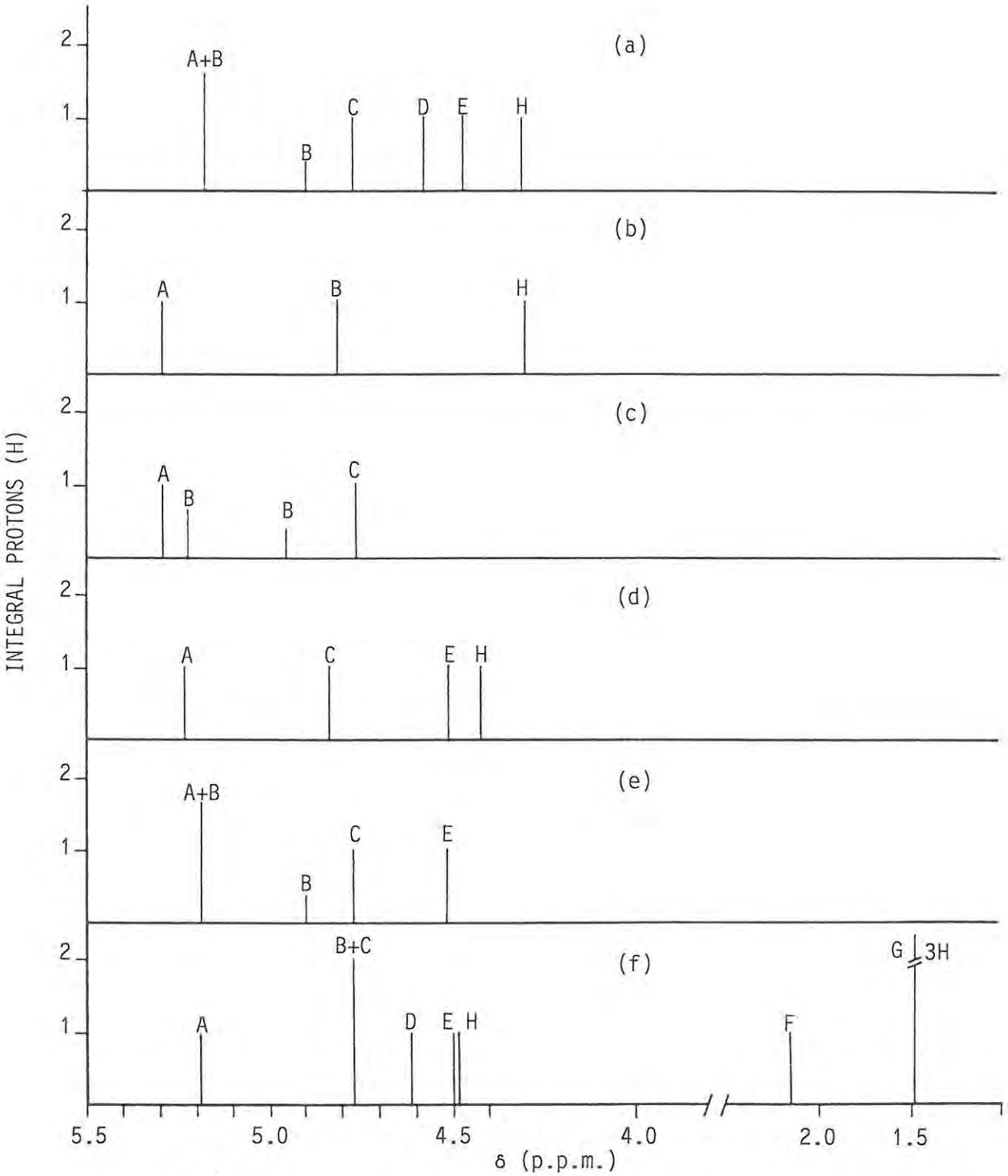


FIG.13 : ^{1}H-N.m.r. spectrum (500MHz) of K69 polysaccharide and derived oligosaccharides.

a) **A1**

b) **A3**-alditol

c) **A3**

d) **A2**-alditol

e) **A2**

f) Autohydrolysed native polysaccharide

(100° ,30 minutes) of the acid form of the polysaccharide. The spectrum of this partially depolymerised polysaccharide [Annexure C, Fig.1(f)], shows five anomeric signals, integrating for six protons. The signal at δ4.77, integrating for two protons, occurs as a singlet in the β-anomeric region, allowing assignment of the β-configuration to two mannose residues. The spectrum contains signals for the methyl groups of both pyruvate (δ1.48) and acetate (δ2.16) (Table 2), their signals integrating for three and one proton respectively. Each repeat unit, therefore, carries a pyruvate acetal, and approximately one out every three repeat units in the autohydrolysed polysaccharide carry an acetate group (Table 2).

In the ^{13}C-n.m.r. spectrum, the anomeric carbon signals were not well resolved and definitive assignment of the signals could not be made, however, the methyl resonance of the pyruvate acetal, which occurs at 26.0 p.p.m. is well defined and indicates that the acetal carbon has the R-configuration.

7.2.2. Methylation analysis

Methylation of the K69 polysaccharide and analysis of the products by g.l.c.-m.s. of the derived alditol acetates, gave the results shown in Table 3, Column I, while reduction of the methylated polymer gave the results in Column II. The results in Column I show 0.79 moles of 2-O-methylmannose, while after reduction of the uronic acid (Column II), 0.98 moles are obtained. This suggests that the uronic acid is linked to the mannose. The appearance of 2,3,4-tri-O-methylglucose (Column II) means the uronic acid is terminal and the mannose to which it is linked constitutes a double branch point. The 2,3,4-tri-O-methylglucose appears as only 0.55 moles due to incomplete reduction

TABLE 2

METHYLATION ANALYSIS OF KLEBSIELLA K69 POLYSACCHARIDE AND DERIVED POLY- AND OLIGOSACCHARIDES

METHYLATED SUGARS[a] (as alditol acetates)	T[b] (OV-225)	MOLAR RATIOS[c] I	II	III	IV	V	VI
1,2,3,5,6-Man	0.54				0.70	0.36	0.41
2,3,4,6-Glc	1.00				0.84	0.55	
2,3,4,6-Gal	1.11				0.70		
2,4,6-Man	1.60						1.00
2,3,6-Man	1.62	0.92	0.98	1.00			
2,3,6-Glc	1.81	0.98	0.97	0.64			
2,3,4-Glc	1.81		0.55		0.49	0.37	0.51
2,6-Man	2.28					1.00	
2,3-Man	2.73			0.95			
2,3-Gal	3.24	1.00	1.00	0.83			
2-Man	3.90	0.79	0.98		1.00		

[a] 2,3,6-Man = 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol, etc.

[b] Retention time relative to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol, on column DB-225 (J + W fused silica capillary column, 0.25 μm film thickness, 30m x 0.25mm) isothermal at 205° C.

[c] Molar ratios: I, methylated native polysaccharide;
II, methylated, reduced, native polysaccharide;
III, methylated product of uronic acid degradation;
IV, reduced, methylated, reduced, pentasaccharide from partial hydrolysis; V, reduced, methylated, reduced, tetrasaccharide from partial hydrolysis; VI, reduced, methylated, reduced, trisaccharide from partial hydrolysis.

of the uronic acid. The results suggest that the galactose is pyruvylated in positions 4 and 6, forming the second terminal group. The 2-O-methylmannose, carrying the two terminal sugars, plus the 2,3,6-tri-O-methyl -glucose and -mannose (both 4-linked), make up the three "in-chain" sugar units. The data therefore shows that the polysaccharide has a pentasaccharide repeating-unit.

The results in Table 1 therefore also support a pentasaccharide repeating-unit made up of 2 mannose, 1 glucose, 1 glucuronic acid and 1 galactose. These monosaccharides were shown to be all of the D-configuration by g.l.c. analysis of their 2(-)-octyl glycoside acetates[96].

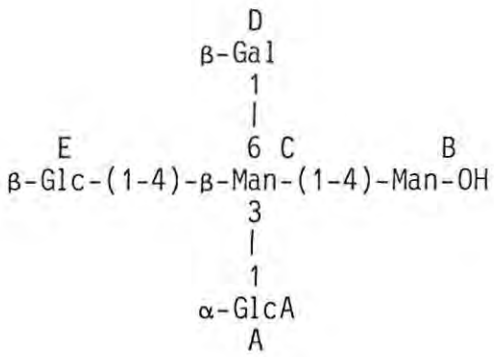
7.2.3. β-elimination (base catalysed degradation)

The methylated polysaccharide was treated with dimsyl anion and directly alkylated with methyl iodide by the method of Aspinall et al.[83]. A polymeric product was obtained, confirming that the glucuronic acid is terminal. Analysis of the product gave the results in Table 3, Column III. The appearance of 2,3-di-O-methylmannose indicates that the glucuronic acid is linked to position 3 of the doubly branched mannose residue.

7.2.4. Partial hydrolysis

The native polysaccharide was subjected to partial depolymerisation with acid and three acidic oligosaccharides were isolated by gel permeation chromatography (Bio-Gel P-2). These correspond to the aldotrio- (A3), aldotetrao- (A2) and aldopentao-uronic acids (A1), respectively.

A1 -



A2 - β-Glc-(1-4)-β-Man-(1-4)-Man-OH
3
1
α-GlcA

A3 - β-GlcA-(1-3)-β-Man-(1-4)-Man-OH

A3 had $[\alpha]_D$ +27° and a d.p. of 3^{129}. Hydrolysis of A3-alditol and g.l.c. analysis of the derived peracetylated aldononitriles gave mannose and mannitol in the ratio 1:1. Carboxyl reduction followed by gl.c analysis gave mannose, glucose and mannitol in the ratio 1:1:1. These results show that A3 is an aldotriouronic acid consisting of two mannose residues and a glucuronic acid, with a mannose as the reducing terminus.

In the ^{1}H-n.m.r. spectrum of A3 (Fig.13) the fractional resonances at δ5.22 (0.6H) and δ4.94 (0.4H) represent the α- and β-pyranose configurations of the terminal reducing mannose residue. The resonance at δ4.76, with a J value of 3Hz, is assigned to the second mannose residue, which is therefore β-linked. This signal is twinned, suggesting that this mannose residue is linked to the terminal reducing mannose which has both the α- and β- configurations. In the

spectrum of A3-alditol (Annexure C, Fig.1f) this twinning is no longer observed, the signal at δ4.81 having sharpened considerably, confirming that the mannose residues are adjacent. The remaining α-anomeric signal at δ5.29 is therefore assigned to the glucuronic acid. In the spectrum of A3-alditol an additional signal at δ4.30 (J 10Hz) is assigned to H-5 of the α-glucuronic acid.

Methylation of A3-alditol followed by carboxyl reduction, hydrolysis and g.l.c. analysis of the permethylated monosaccharides as their derived alditol acetates gave the results in Table 3, Column VI. The appearance of 1,2,3,5,6-penta-O-methylmannose confirms that mannose is the reducing sugar. 2,3,4-Tri-O-methylglucose shows that the glucuronic acid is terminal and 2,4,6-tri-O-methylmannose confirms that the glucuronic acid is linked at position 3 of this central mannose residue.

A2 had $[\alpha]_D$ +32° and a d.p. of 4. A2-alditol was hydrolysed and analysed by g.l.c. analysis and gave mannose, glucose and mannitol in the ratio 1:1:1. Carboxyl reduction followed by g.l.c. analysis of the hydrolysate showed mannose, glucose and mannitol in the ratio 1:2:1. These results show that A2 is an aldotetraouronic acid with mannose as the reducing terminus.

Comparing the ^{1}H-n.m.r. data of A2 (Table 2) and A3, an additional β-anomeric signal at δ4.52 is observed in the spectrum of A2 and is assigned to the glucose residue not present in A3. The remaining signals correspond with those of A3, except that the fractional α-anomeric signal of the reducing mannose (0.6H) overlaps with that of the α-glucuronic acid at δ5.19. Similar twinning due to mutarotation is observed, which again is not present in the spectrum

of A2-alditol (Annexure C, Fig.1d). In this spectrum a signal at δ4.42 (J 10Hz)is assigned to H-5 of the α-glucuronic acid.

The ^{13}C-n.m.r. data for A2 shows fractional resonances at 94.64 and 94.48 p.p.m. attributed to the terminal reducing mannose. Three more anomeric signals are observed which correspond to the signals in the ^{13}C-spectrum of A2-alditol.

Methylation of A2-alditol and g.l.c. analysis of the carboxyl reduced oligosaccharide gave the results in Table 3, Column V. The results are similar to those for A3-alditol (Column VI) with the only differences being the appearance of 2,3,4,6-tetra-O-methylglucose and 2,6-di-O-methylmannose, which means that the glucose residue is terminal and linked at position 4 of the central mannose residue.

A1 had $[\alpha]_D$ +35° with a d.p. of 5. A1 was reduced to the alditol, hydrolysed and analysed by g.l.c to give mannose, glucose, galactose and mannitol in the ratio 1:1:1:1. A1-alditol was then carboxyl reduced and analysed to give mannose, glucose, galactose and mannitol in the ratio 1:2:1:1. These results show A1 to be an aldopentaouronic acid, again with a mannose as the reducing terminus. Comparing the ^{1}H-n.m.r. data of A1 (Table 2 and Fig.13) with that of A2, an additional anomeric signal at δ4.58 is attributed to the β-linked galactose residue. A proton resonating at δ4.31 (J 10Hz) is assigned to H-5 of the α-glucuronic acid. The remaining signals are in agreement with those of A2 and these assignments were used to assign the ^{1}H-n.m.r. signals of the autohydrolysed native polysaccharide (Table 2).

The ^{13}C-n.m.r. spectrum of A1 has two anomeric signals at 94.62 and 94.49 p.p.m. which are assigned to the partial α- and β-configurations of the terminal reducing mannose residue. Four more

anomeric signals downfield of these confirm the pentasaccharide nature of the oligosaccharide. This oligosaccharide therefore represents the repeat unit of the polysaccharide without the pyruvate acetal and acetyl substituents.

A1 alditol was methylated, carboxyl reduced and the hydrolysate analysed by g.l.c. to give the results in Table 3, Column IV. The appearance of 2,3,4,6-tetra-O-methylgalactose confirms that the pyruvate acetal was linked at O-4 and O-6 of the galactose residue in the native polysaccharide and that the galactose occupies a terminal position. Comparing the data for A1 and A2 (Table 3, Columns IV and V respectively), it can be seen that the 2,6-di-O-methylmannose has been replaced by 2-O-methylmannose. This shows that the galactose is linked at position 6 of the central mannose, occupying a branch point. The 2,3,4-tri-O-methylglucose again confirms that the glucuronic acid is terminal, occupying the second branch point on the doubly branched mannose in the polysaccharide.

The methylation data (Table 3) shows the 1,2,3,5,6-penta-O-methylmannose ratio to be considerably depressed and this is attributed to its greater volatility resulting in losses during the work-up procedure. The 2,3,4-tri-O-methylglucose ratio is also depressed due to incomplete reduction of the glucuronic acid residue.

To facilitate assignment of the ^{13}C- anomeric signals of A1 and A2, A1 was subjected to 2D-heteronuclear correlation n.m.r. spectroscopy (Hetcorr). Each carbon-proton pair in the oligosaccharide is represented by one signal in the spectrum. The proton spectrum lies along one axis and the ^{13}C spectrum along the other. Since the anomeric proton signals of A1 have known

ssignments, the corresponding ^{13}C resonances are identified. (Annexure C, Fig.3a). The ^{13}C data of A1 and A2 (Table 2) is shown to complement the corresponding ^{1}H data.

7.3. Conclusion

From the evidence presented, the capsular polysaccharide isolated from Klebsiella K69 is made up of a repeat unit having the structure shown in the abstract and is of the "3+1+1" type. This structure is very similar to those of both K30[31] and K33[32] shown below.

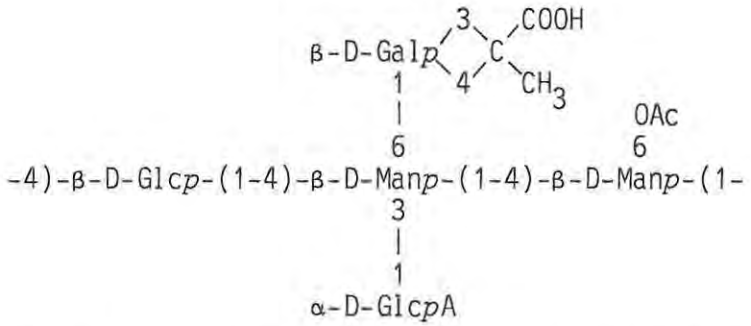


Fig.2 Repeating unit of the capsular polysaccharide of Klebsiella serotypes K30 and K33.

K33 has one acetate group per repeating-unit, while K30 has approximately one acetate group per every three repeating-units. The only major difference in chemical structure of K30/K33 and K69 is that the pyruvate is linked through O-3 and O-4 in both K30 and K33, while it is linked through O-4 and O-6 in K69.

7.4. Experimental

Unless otherwise stated, all solutions were evaporated under diminished pressure at temperatures not exceeding 40°.

7.4.1. General methods

Optical rotations -

The samples, in aqueous solution, were subjected to ultracentrifugation at 35k for 1h, then filtered (8.0 μm Millipore LCWP filter). Their optical rotations were then measured in a 1cm cell at ambient temperature (approx. 23°) with a Perkin-Elmer model 141 polarimeter (sodium lamp).

Chromatography -

For analytical paper chromatography, by the descending method, Whatman No.1 paper and the following solvents were used (v/v):

A. 8 : 2 : 1 ethyl acetate - pyridine - water,

B. 18 : 3 : 1 : 4 ethyl acetate - acetic acid - formic acid - water,

C. 5 : 1 : 5 : 3 ethyl acetate - acetic acid - pyridine - water.

Chromatograms were developed with silver nitrate (I)[135] or periodate/benzidine (II)[136]. Gel permeation chromatography was performed on columns of Sepharose 4B CL and Sephacryl S500 using molar sodium chloride as the eluent. A Hewlett-Packard 5890A gas-liquid chromatograph fitted with a flame ionisation detector was used for analytical g.l.c. separations, with a J & W scientific fused silica, bonded phase (DB-225) capillary column (0.25mm x 30m) with 0.25 μm film thickness. Integration of f.i.d. signal output was achieved using a Hewlett-Packard 3392A recording integrator. A V.G. Micromass 16F

spectrometer was used for g.l.c.-m.s. studies; the spectra were recorded at 40 eV with an ion source temperature of 170°.

^{1}H- and ^{13}C-n.m.r. spectroscopy (Annexure C) -

N.m.r. spectra were recorded on a Bruker W.M. 500 MHz FT spectrometer at ambient temperature (30°), and in some cases at 95° for ^{1}H- spectra. Sample preparation involved hydrogen exchange by dissolution (20 mg) in D_2O (99.7%) and lyophilisation 3 or 4 times, with a final exchange in D_2O 99.96%. Acetone was used as the internal standard (δ2.23 for ^{1}H-n.m.r. and 31.07 p.p.m. for ^{13}C-n.m.r., measured against aqueous 4,4-dimethyl-4-silapentane-1-sulphonate). All ^{13}C-n.m.r spectra were acquired in the proton decoupled mode.

7.4.2. Isolation and purification of the polysaccharide

A sample of Klebsiella K69 was obtained from Dr. I. Ørskov, Copenhagen, and plated out repeatedly on Mueller-Hinton agar (Annexure A), with incubation at 30°. Single capsular colonies (Indian ink test) were transferred to each of six tubes containing 5mL of sterile nutrient broth (Annexure A) and shaken at 37° overnight. The contents of these tubes were transferred to each of six flasks containing 50mL nutrient broth and shaken at 37° until turbid (6-8h). Six stainless steel trays (60 x 40cm) were sterilised and 1.5 litres of autoclaved Mueller-Hinton agar poured aseptically into each tray, forming a 1cm layer of culture medium. The bacterial suspensions were then evenly spread over the agar in each tray and incubated for 4 days at 30°. The cells were removed from the trays (150mL) and an equal volume of 2% phenol added to kill the cells. The suspension was stirred overnight at 4° then ultracentrifuged (35000 r.p.m. on a Beckman L8-

80M ultracentrifuge, rotor type 70ti, approx. 80 000g) for 3h. The total supernatant volume was added to 5 volumes of ethanol, precipitating the crude polysaccharides. The precipitate was removed by centrifugation and washed twice with ethanol, dried, and dissolved in a minimum of water (400mL). 60mL of a 5% cetyltrimethylammonium bromide (CTAB) solution was added to the polysaccharide solution, slowly, with stirring. The precipitate (acidic polysaccharide-CTAB complex) was removed by centrifugation and dissolved in 200mL aqueous 3M sodium chloride to break the complex. The acidic polysaccharide was precipitated into ethanol (5 vols.), removed by centrifugation and dissolved in a minimum of water (250mL). This solution was dialysed (12-14000 Mw cut-off) against running tap water for three days, then ultracentrifuged for 1h at 35k. The supernatant was finally freeze dried, yielding 1.13g of pure capsular polysaccharide. A heat extract was performed on the pelleted cells by suspending them in 250mL 1% phenol and stirring at 60° for 30 minutes. The same work up procedure was followed, yielding 373mg of pure polysaccharide. The heat extracted polymer was kept separate from the rest.

7.4.3. Monosaccharide composition analysis

The purified polysaccharide (5mg) was hydrolysed by refluxing overnight (16h) in aqueous 2M trifluoroacetic acid (TFA) at 100°. The acid was removed by evaporation under deminished pressure and the free monosaccharides were analysed by g.l.c., on a column of OV-225 at 225° (isothermal), as their peracetylated aldononitriles[120]. The polysaccharide (10mg) was methanolysed with 3% methanolic hydrogen chloride, reduced overnight with sodium borohydride in anhydrous

methanol, then hydrolysed with 2M TFA, and the uronic acid was identified by g.l.c. analysis of the derived peracetylated aldononitriles (Table 1).

7.4.4. Methylation analysis

A sample of the K69 polysaccharide in the free acid form (40mg) was methylated by a modified Hakomori procedure[67] using a 1:1 (v/v) mixture of dimethylsulphoxide and 1,1,3,3-tetramethylurea, as solvent. The product was recovered after dialysis and lyophilisation, and was shown to be completely methylated by the absence of hydroxyl absorbtion in the i.r. spectrum. A portion of the methylated polymer (15mg) was methanolysed in 3% methanolic hydrogen chloride (16h, 80°) and neutralised by stirring with silver carbonate. The methylated, methanolysed polymer was split into two portions. One portion was hydrolysed in 2M TFA (16h, 100°), the hydrolysate was reduced with sodium borohydride (1h) in water and the alditols were acetylated with 1:1 (v/v) acetic anhydride/pyridine (1h, 100°). The other portion was reduced with sodium borohydride in dry methanol (room temp., 16h), hydrolysed with 2M TFA (16h, 100°), and was then treated the same as the first portion, and both were analysed as their alditol acetates by g.l.c. and g.l.c.-m.s., giving the results shown in Table 3, Columns I and II.

7.4.5. Uronic acid degradation

A sample of methylated polysaccharide (20mg) was dried in vacuo and dissolved in 19 : 1 dimethylsulphoxide - 2,2-dimethoxypropane (5mL), containing a trace of p-toluene-sulphonic acid (1mg), by stirring,

under nitrogen. The solution was subjected to ultrasonic agitation for 30 minutes, then potassium dimsyl (2M, 3mL) was added and the mixture agitated ultrasonically for a further 30 minutes, then allowed to stand overnight. The solution was cooled on an ice bath, methyl iodide (1mL) added, and stirred for 1h under nitrogen. The methylated, modified polysaccharide was isolated by dialysis (12-14000 Mw cut off) against running tap water for 24h, followed by lyophilisation. The product was hydrolysed with 2M TFA and the free sugars were analysed as described for the methylation analysis, giving the results in Table 3, Column III.

7.4.6. Partial hydrolysis

A preliminary partial hydrolysis was conducted on 20mg of polysaccharide in 0.5M TFA, under reflux (100°). Samples were withdrawn every 30 minutes up to 8h and paper chromatography, using solvent C and reagent I, showed the presence of five oligosaccharides. The process was repeated using three 250mg samples of polysaccharide, hydrolyzing them for 1.0h, 2.5h and 5.0h respectively. The acid was removed by successive evaporations with water and the extent of hydrolysis monitored by p.c. (solvent C, reagent I). Insufficient hydrolysis was obtained under these conditions, therefore each portion was dissolved in 50mL 0.5M TFA and subjected to further hydrolysis (100°, 0.5h).

The acid was removed and each portion was separately applied to a column (65 x 2.6cm) of Bio-Gel P-2, using water as the mobile phase. Separation was poor (Annexure B, Fig.2), therefore fractions which included penta-, tetra- and tri-saccharides were pooled,

rechromatographed and two pure oligosaccharides, A2 (42mg) and A3 (63mg), were isolated. The fractions from this separation corresponding to penta- and larger oligo-saccharides were rechromatographed and a pure oligosaccharide, A1 (20mg), was isolated. The purity of these products was monitored by p.c. using solvent C and reagent I. The material corresponding to oligosaccharides larger than a pentasaccharide (130mg) was dissolved in 0.5M TFA (25mL) and subjected to further partial hydrolysis (100°, 2.0h). A1 (20mg) and A2 (17mg) were isolated.

A1, A2 and A3 had R_{gal} 0.27, 0.39 and 0.56 respectively (17h), using solvent C and reagent I. The d.p. of the oligosaccharides were determined using Morrison's method[129], on A1(3mg), A2(4mg) and A3(3mg) as their alditols ($NaBH_4$). A2(14mg) and A3(20mg) were reduced to the alditols ($NaBD_4$, 1h) and n.m.r. spectra were recorded at ambient temperature and 95° for A1, A2, A2 reduced, A3 and A3 reduced.

A1(9mg), A2(5mg) and A3(6mg) were reduced to the alditols ($NaBD_4$, 1h) and methylated (Hakomori), the products were purified on Sephadex LH-20 (8 x 0.5cm, column), methanolysed in methanolic hydrogen chloride (16h, 80°) and divided into two portions. One portion was hydrolysed (2M TFA, 16h) and derivatised, while the other portion was reduced in anhydrous methanol ($NaBH_4$, 16h), then hydrolysed and derivatised. The resulting alditol acetates were analysed by g.l.c. and g.l.c.-m.s. and gave the results in columns IV, V and VI in Table 3.

8. THE BACTERIOPHAGE-BORNE ENZYME DEPOLYMERISATION OF THE KLEBSIELLA K69 CAPSULAR POLYSACCHARIDE

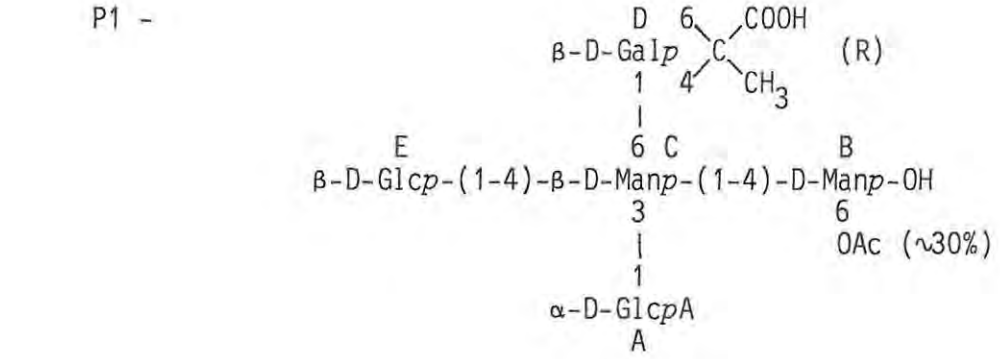
ABSTRACT

The capsular polysaccharide isolated from Klebsiella K69 contains acid labile O-acetyl substituents which cannot be isolated, intact, with oligosaccharides derived by chemical methods. A bacteriophage, which infects Klebsiella K69 bacteria, was isolated from sewage, purified and used to depolymerise the K69 capsular antigen by means of its associated glycanase activity. Two acetylated oligosaccharides were isolated which correspond to the repeat unit (P1) and double repeat unit (P2), respectively. P1 and P2 were fully characterised by n.m.r. spectroscopy and methylation analysis, and were found to have the structures shown in Fig.14.

8.1. Introduction

Bacteriophages are often used to depolymerise capsular polysaccharides, allowing the isolation of high yields of oligosaccharide representing the repeating-unit. The technique is especially useful when the polysaccharide contains acid labile substituents[105], such as acetate and pyruvic acid acetals, making it very difficult to isolate the intact repeating-unit by chemical means. The Klebsiella K69 polysaccharide has been shown to consist of pentasaccharide repeating-units containing both acetate and pyruvate substituents (section 7.). Bacteriophage 69 (θ69) was propagated on, and used to depolymerise the capsular polysaccharide of, Klebsiella K69. It was found to have an endomannosidase activity, catalysing the hydrolysis of -D-Manp-($1\xrightarrow{\beta}4$)-D-Glcp- bonds, and the intact repeating

P1 -


D 6 COOH
β-D-Galp C (R)
1 4 CH3
E 6 C B
β-D-Glcp-(1-4)-β-D-Manp-(1-4)-D-Manp-OH
3 6
OAc (∼30%)
1
α-D-GlcpA
A

P2 -

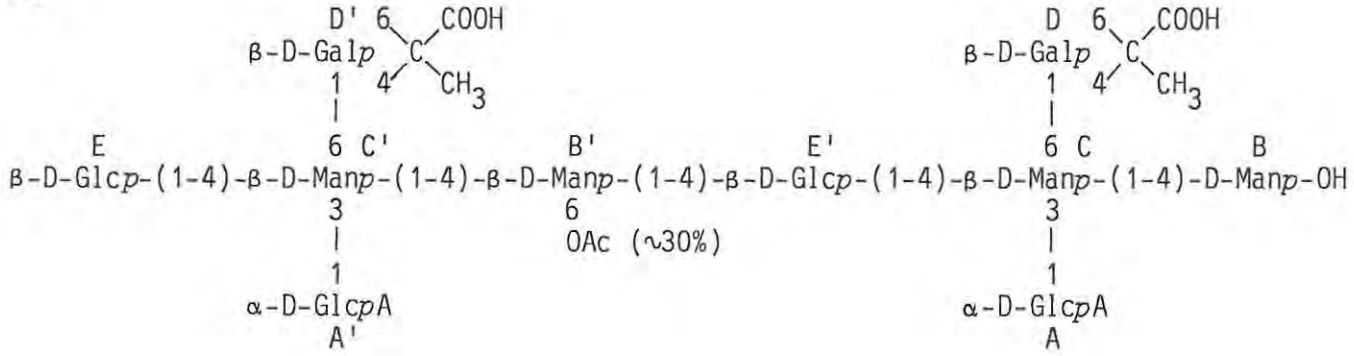

D' 6 COOH
β-D-Galp C
1 4 CH3
D 6 COOH
β-D-Galp C
1 4 CH3
E 6 C' B' E' 6 C B
β-D-Glcp-(1-4)-β-D-Manp-(1-4)-β-D-Manp-(1-4)-β-D-Glcp-(1-4)-β-D-Manp-(1-4)-D-Manp-OH
3 6 3
OAc (∼30%)
1 1
α-D-GlcpA α-D-GlcpA
A' A

Fig.14

unit was isolated. The structural information discussed in section 7 was used together with the following analyses to establish the structure of P1, and to determine the sugar unit to which the acetate is linked and its linkage position.

8.2. Results and discussion

8.2.1. Oligosaccharide composition and n.m.r. spectra

θ69 was isolated from Grahamstown sewage water, purified by successive single plaque pickings and propagated on the host bacteria in nutrient broth. The bacteriophage suspension was added to a solution of purified K69 polysaccharide and incubated with gentle stirring in the presence of a small amount of chloroform. The resulting material was dialysed and the dialysate was applied to a gel column, and two oligosaccharides, P1 and P2, were isolated. P1 and P2 were subjected to n.m.r. spectroscopy and methylation analysis. The degree of polymerisation (d.p.) and identity of the reducing-end sugar was determined for each oligosaccharide using Morrison's method [129] - samples of P1 and P2 were reduced to the alditols, methanolysed, reduced, hydrolysed and the peracetylated aldononitriles formed. On g.l.c. analysis it was established that both P1 and P2 had mannose as the reducing-end and a d.p. of 5 and 10 respectively. P1 and P2 represent a single and double repeat unit of the polysaccharide.

^{1}H-n.m.r. spectroscopy -

^{1}H-N.m.r. spectra were acquired for solutions of P1, P1-alditol and P2 in D_2O, at elevated temperature. Comparing these spectra (Table 4) with that of the native polysaccharide (Table 2), the

TABLE 4

N.M.R. DATA FOR PRODUCTS OF BACTERIOPHAGE DEGRADATION

Compound[a]	^{1}H-N.m.r. data					^{13}C-N.m.r. data	
	δ[b] (p.p.m.)	$J_{1,2}$ (Hz)	Integral (No. of H)	Assignment[c]	Symbol	P.p.m.[d]	Assignment[e]
P1	5.23	3.5	1	GlcA-α-	A	175.08 174.72	COOH of pyruvate, acetate and GlcA
	5.19	1.5	0.40	-4-Man-α-OH	B1	174.24	
	5.175	1.5	0.20	OAc -4-Man-α-OH	B2	103.25	Glc-β-
						103.06	Gal-β-
	4.915	1.5	0.13	OAc -4-Man-β-OH	B2	102.30	GlcA-α-
						101.16	6 (OAc 30%)
	4.905	1.5	0.27	-4-Man-β-OH	B1	101.01 100.94	-4-Man-β-1-4-Man-OH 3
	4.79	no	0.67	6 -4-Man-β-1-4-Man-OH 3	C2	95.73 94.57 94.44	(OAc 30%) -4-Man-OH
	4.74	no	0.33	OAc -4-Man-β-1-4-Man-OH	C1		
	4.60	8	1	H_3C, COOH, C, 6, 4 (pyr)		25.79	CH_3 of pyruvate
						21.03	CH_3 of acetate
				Gal-β-	D	63.95	C-6 of -4-Man-OH 6 OAc
	4.51	7.5	1	Glc-β-	E		
	2.15	s	1	CH_3 of acetate	F	61.89	C-6 of Glc-β-
	1.56	s	3	CH_3 of pyruvate	G	61.33	C-6 of -4-Man-OH
P1 de-O-acetylated	5.23	no	1	GlcA-α-	A	103.32	Glc-β-
	5.19	no	0.6	-4-Man-α-OH	B	103.11	Gal-β-
	4.92	no	0.4	-4-Man-β-OH	B	102.34	GlcA-α-
	4.79	no	1	6 -4-Man-β- 3	C	100.99	Man-β-
						94.62	-4-Man-α-OH
	4.59	8	1	Gal-β- (pyr)	D	94.49	-4-Man-β-OH
					E	25.78	CH_3 of pyruvate
	4.52	8	1	Glc-β-		174.57	COOH of pyruvate
	4.39	10	1	H5 of GlcA-α-	H	173.82	COOH of GlcA
	1.59	s	3	CH_3 of pyruvate	G	61.93	C-6 of Glc-β-
						61.38	C-6 of -4-Man-OH

P1 reduced	5.23	4	1	GlcA-α-	A	174.44	COOH of pyruvate
	4.85	no	1	6 -4-Man-β- 3	C	173.75	COOH of pyruvate
						103.54	Glc-β-
	4.57	8	1	Gal-β- (pyr)	D	103.23	Gal-β- (pyr)
	4.50	7	1	Glc-β-	E	102.28	GlcA-α-
	4.43	10	1	H5 of GlcA-α-	H	100.78	6 -4-Man-β- 3
	1.59	s	3	CH_3 of pyruvate	G		
						25.79	CH_3 of pyruvate
P2	5.24 5.22	4 4	1 1	GlcA-α-	A & A'		
	5.19	2	0.4	-4-Man-α-OH	B1'		
	5.17	2	0.2	-4-Man-α-OH OAc	B2'		
	4.91	no	0.13	-4-Man-β-OH OAc	B2'		
	4.90	no	0.27	-4-Man-β-OH	B1'		
	4.80 4.79 4.78 4.77 4.75 4.74	no	3	6 -4-Man-β- x2 3 & -4-Man-β- x1	B,C & C'		
	4.63 4.60	8 8	1 1	Gal-β- (pyr)	D & D'		
	4.51	8	2	Glc-β- / -4-Glc-β-	E & E'		
	2.16	s	4.5	CH_3 of acetate	F		
	1.56	s	2	CH_3 of pyruvate	G		

[a] For sources of P1 and P2 see text.

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

[c] -4-Glc-β- refers to a 4-linked glucosyl residue in the β-anomeric configuration.

[d] Chemical shift in p.p.m. downfield from Me_4Si relative to internal acetone; 31.07 downfieldd from DSS.

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

s = singlet.

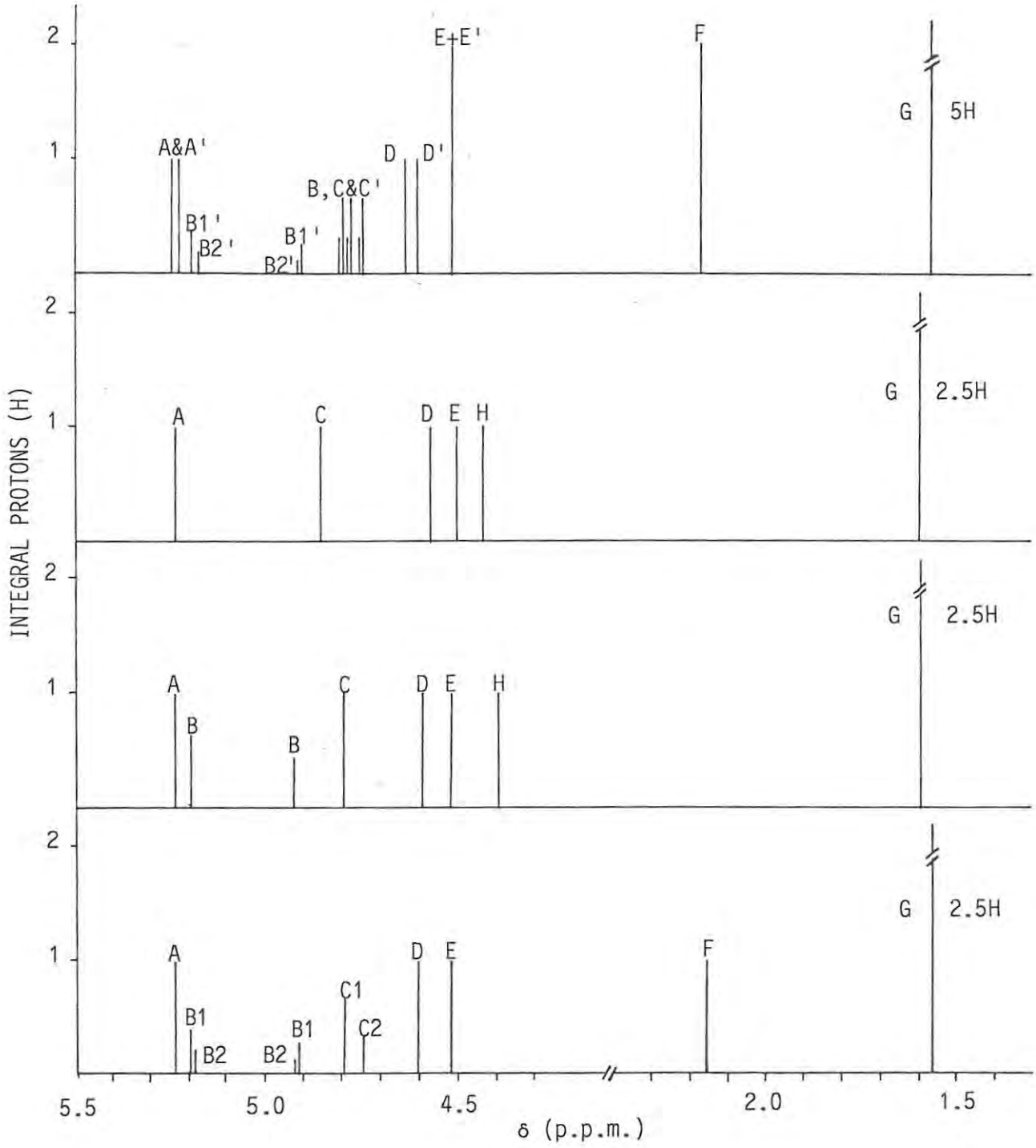


FIG.15 : ^{1}H-N.m.r. spectra of **P1**, **P2** and derived oligosaccharides

a) **P2**

b) **P1**-alditol

c) De-O-acetylated **P1**

d) **P1**

signals for glucuronic acid, glucose, galactose, acetate and pyruvate could be assigned. The methyl group of the pyruvate acetal integrates for 3 protons while that of the O-acetyl substituent integrates for 1 proton. This indicates partial acetylation of the native polysaccharide with approximately one out every three repeat units carrying an acetyl substituent. The ^{1}H-n.m.r. spectrum of P1 (Fig.15), although integrating for 5 anomeric protons, presented interpretation problems arising from extensive twinning of certain signals. Fractional resonances, representing the α- [δ5.19 (0.40H) and δ5.175 (0.20H)] and β- [δ4.915 (0.13H) and δ4.905 (0.27H)] anomeric configurations of the terminal reducing mannose residue, are both split. The β-anomeric signal of the central mannose residue is split δ4.79 (0.67H) and δ4,74 (0.33H) and, furthermore, both these signals are twinned. In effect this spectrum represents two oligosaccharides, one carrying an acetyl substituent and the other without. To ascertain the effect of the acetyl substituent on the chemical shifts observed, a sample of P1 was de-O-acetylated[172] and a ^{1}H-n.m.r. spectrum acquired. The spectrum (Table 4 and Fig.15) shows fractional resonances at δ5.19 (0.6H) and δ4.92 (0.4H) representing the α- and β-anomeric configurations of the terminal reducing mannose residue. The signal at δ4.79 (1H) represents the central β-linked mannose. These signals are no longer split, therefore the signals at δ5.175 (0.20H), δ4.915 (0.13H) and δ4.75 (0.33H) in the spectrum of P1 represent the mannose residues of the acetylated oligosaccharide. Furthermore, the large upfield shift of 0.05 p.p.m. of the resonance representing the central mannose in the acetylated oligosaccharide, indicates that the acetyl substituent is linked to the terminal

reducing mannose, causing its partial α- and β-anomeric resonances to be split, this effect being transmitted to the adjacent mannose residue. The signal at δ4.79 (1H) in the spectrum of P1-de-O-acetylated is twinned. This twinning is no longer observed in the spectrum of P1-alditol, with the signal (δ4.85) sharpening considerably, therefore is attributed to mutarotation of the reducing mannose residue. The spectrum of P1-alditol does show some under reduction of the reducing mannose. The signal at δ4.43 (J 10Hz) is assigned to H-5 of the α-glucuronic acid residue. The ^{1}H- spectrum of P2 was somewhat more complex, the anomeric signals integrating for 10 protons, with some signals extensively twinned. It can be seen that some pyruvate is removed from P1 and P2 during the work-up procedure. Fortunately this did not further complicate the n.m.r. spectra, as only negligible amounts were removed.

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

The ^{13}C-n.m.r. spectra for P1, de-O-acetylated P1 and P1-alditol were acquired. The ^{1}H- and ^{13}C- data are complementary with similar twinning of the mannose signals. The spectrum of P1 has three C-6 signals at 63.95, 61.89 and 61.33 p.p.m. In the spectrum of de-O-acetylated P1, the signal at 63.95 p.p.m. has disappeared, leaving two C-6 signals at 61.93 and 61.38 p.p.m. These two signals represent unsubstituted C-6 atoms of glucose and the terminal reducing mannose, respectively. Bradbury et al.[153] report a 6-O-substituted β-mannose residue having a C-6 resonating at 63.7 p.p.m. The signal at 63.95 p.p.m. in the spectrum of P1 is therefore attributed to C-6 of the 6-O-acetylated terminal reducing mannose residue. The C-6 substituent results in a downfield shift of its signal, which on de-O-acetylation

moves upfield to coincide with the unsubstituted mannose C-6 signal accounting for its disappearance. The signal for the methyl carbon of the pyruvate acetal at approximately 25.8 p.p.m., and the corresponding proton signal at δ1.59, confirm that the acetal carbon has the R configuration[173]. The anomeric ^{13}C- signals were assigned from the data accumulated from the products of partial hydrolysis (Table 2) and the heteronuclear correlation spectrum (Annexure C, Fig.3a).

8.2.2. Methylation analysis

The alditols of P1 and P2 were methylated by the Hakomori procedure[63], using potassium dimsyl[69] as anion. The results obtained by g.l.c. and g.l.c.-m.s. analysis of the derived alditol acetates are generally similar to those obtained from the carboxyl-reduced polymer (Table 3). The 1,2,3,5,6-penta-O-methylmannitol in P1 and P2 confirms that the reducing-end sugar is the unsubstituted mannose. Trace amounts of 2,3,4,6-tetra-O-methylgalactitol (Table 5) indicate that some pyruvate is removed during the work-up procedure which involves several passages of the phage-degraded material through a column of Amberlite IR-120 (H^+) cation exchange resin. The molar ratios of 2,3,4-tri-O-methylglucitol indicate approximately 40-50% carboxyl reduction.

TABLE 5

METHYLATION ANALYSES OF KLEBSIELLA K69 POLYSACCHARIDE AND OLIGOSACCHARIDES P1 AND P2

METHYLATED SUGARS[a] (as alditol acetates)	T[b] (O.V.-225)	MOLAR RATIOs[c] I	II	III
1,2,3,5,6-Man	0.54		0.57	0.46
2,3,4,6-Glc	1.00		0.82	0.65
2,3,4,6-Gal	1.11		0.18	
2,3,6-Man	1.62	0.98		0.64
2,3,6-Glc	1.81	0.97		0.56
2,3,4-Glc	1.81	0.55	0.36	0.84
2,3-Gal	3.24	1.00	0.75	0.86
2-Man	3.90	0.98	1.00	1.00

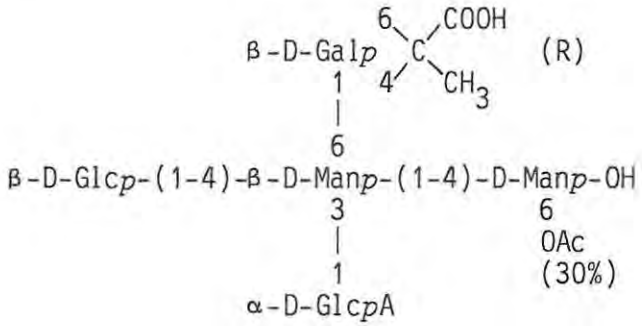
[a] 2,3,6-Glc = 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-glucitol, etc.

[b] Retention time relative to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol, on column DB-225 (J + W fused silica capillary column, 0.25 µm film thickness, 30m x 0.25mm) isothermal at 205° C, except where otherwise stated.

[c] Molar ratios: I, methylated, carboxyl-reduced, native polysaccharide; II, reduced, methylated, carboxyl-reduced P1; III, reduced, methylated, carboxyl-reduced P2.

8.3. Conclusion

The above results confirm that the Klebsiella K69 capsular polysaccharide has a pentasaccharide repeating unit (P1) with the following structure:



The endomannosidase associated with θ69 catalysed the hydrolysis of the -4)-D-Manp-(1$\xrightarrow{\beta}$4)-D-Glcp-(1- glycosidic linkage in the polysaccharide; this is the bond furthest from the double branched mannose. The ^{1}H-n.m.r. data shows that the polysaccharide contains one acetate unit for approximately every three repeat units. It is interesting to note that the acetate is linked to the same sugar as in Klebsiella K30 and K33. It would be an interesting exercise to determine whether θ69 would degrade K30 and K33 polysaccharides, as these polymers have an almost identical structure to that of K69. A study conducted by Rieger-Hug and Stirm[99], using 55 different Klebsiella bacteriophages, showed that only 7 produced oligosaccharides with mannose as the reducing-end sugar. Furthermore, two of these phages, θ5[102] and θ14[151], have been shown to display lyase activity.

8.4. Experimental

The procedures and instrumentation used were generally the same as those described in section 7.4. This discussion will therefore be limited to the additional procedures used in the bacteriophage degradation.

8.4.1. Isolation and purification of θ69

The bacteriophage was isolated from sewage water using an enrichment procedure - 25mL double strength nutrient broth + 5mL Klebsiella K69 nutrient broth suspension + 25mL sewage water (Grahamstown), were incubated in a waterbath shaker (17h, 37°). A 10mL sample was sterilised with chloroform (1mL) and centrifuged (2000 r.p.m.) to remove cellular material. The phage solution was serially diluted (10^{-2} - 10^{-8}) in normal saline, and a drop (0.01mL) from each dilution placed on a freshly seeded bacterial (K69) host "lawn" (Mueller-Hinton agar plate + K69 broth suspension). A single plaque was isolated and propagated in a 5mL nutrient broth/host suspension. This procedure was repeated three times and the final purified θ69 suspension had a titre of 2×10^9 plaque forming units (p.f.u.)/mL. The phage count was increased by successive test tube and small flask lyses, until 600mL of phage suspension (1×10^{10} p.f.u./mL), in nutrient broth, was obtained. This phage suspension was dialysed against running tap water (3500 Mw cut off), then concentrated to 150mL by evaporation under diminished pressure in a rotary evaporator, at a bath temperature not exceeding 35°. This suspension contained approximately 4×10^{12} p.f.u's., which is adequate to depolymerise 500mg of polysaccharide.

8.4.2. Isolation and purification of the oligosaccharides

500Mg of polysaccharide was dissolved in 100mL of water and added to the 150mL phage suspension. Depolymerisation was carried out for 72h at 30° in the presence of chloroform (5mL) to keep the solution sterile. The solution was freeze-dried, then dissolved in 50mL of water and dialysed (6-8000 Mw cut off) against eight 100mL portions of distilled water. Dialysates 3 to 8 were pooled and freeze-dried (A, 260mg), then dissolved in 10mL of water and passed down a column of Amberlite I.R.-120 (H^+) cation exchange resin (4°). This procedure was repeated (3x) and the solution freeze-dried to yield 187mg of material. Dialysates 1,2 and the retentate were combined (B, 306mg) and treated the same as fraction A to yield 200mg of material. A small portion from each fraction (5mg) was separately applied to a column of Bio-Gel P-4 (1.6 x 50cm) and eluted with 5:2:500 pyridine-acetic acid-water (pyridinium acetate buffer, 8mL/h), giving the chromatograms in Annexure B, Fig.3a & b. Fraction A (180mg) was applied to a Bio-Gel P-4 column (2.6 x 50cm) using pyridinium acetate buffer as eluent (20mL/h). P2 and the polymeric material were not well separated (Annexure B, Fig.3c), therefore fraction B (195mg) was applied to a column 2,6 x 70cm affording better separation (Annexure B, Fig.3d). The oligosaccharides were passed through a column of Amberlite IR-120 (H^+) (4°), to remove trace amounts of pyridine, and lyophilised to yield P1 (55mg) and P2 (103mg).

8.4.3. Analysis of P1 and P2

P1 had $[\alpha]_D$ +11° (c 0.90, water) and P2 had $[\alpha]_D$ -8° (c 1.49, water). A sample of P1 (7mg) and P2 (8mg) were dissolved in water (2mL) and

reduced with sodium borohydride (approx. 10 moles per mole reducing sugar) for 1h. P1 and P2 alditols were methanolysed (16h, 80°) in 3% methanolic hydrogen chloride and then divided into two portions. One portion was hydrolysed with 2M TFA (16h, 100°), converted to the peracetylated aldononitriles[120] and analysed by g.l.c. The second portion was reduced with sodium borohydride in anhydrous methanol, hydrolysed with 2M TFA (16h, 100°) and converted to the peracetylated aldononitriles.

P1 and P1-alditol (reduced with sodium borodeuteride in D_2O) and P2 were subjected to 500MHz FT ^1H- and ^{13}C-n.m.r. spectroscopy. Samples were prepared by deuterium exchange (3x with 99.7% D_2O, once with 99.96% D_2O) and spectra were recorded at 95° in 99.996% D_2O (Table 4).

Samples of P1 and P2 (6mg) were reduced to the alditols (sodium borodeuteride in D_2O) and methylated by the Hakomori procedure[63]. The methylated alditols were methanolysed, carboxyl reduced with sodium borohydride, and hydrolysed with 2M TFA (16h, 100°). The hydrolysates were reduced (sodium borohydride in water) and acetylated in a 1:1 mixture of pyridine-acetic anhydride (1h, 100°), giving the results shown in Table 5.

8.4.4. De-O-acetylation

A sample of P1 (15mg) was de-O-acetylated under alkaline conditions using the method of Harada and Amemura[172]. A 10mM aqueous KOH solution was prepared and boiled under reflux (10 minutes) to expel dissolved air. The solution was stoppered with a serum cap and allowed to cool with nitrogen bubbling through the solution. The oligosaccharide was dissolved in 13mL of the KOH solution, under nitrogen, and allowed to

stand for 5h with nitrogen bubbling through the solution (room temp.) The solution was neutralised with 1M acetic acid (ice-bath) and passed through a column of Amberlite IR-120 (H^+) resin at 4°, freeze-dried (yield 10mg) and deuterium exchanged for n.m.r. spectroscopy (Table 4).

REFERENCES

1. M.J. How, J.S. Brimacombe and M. Stacey, Adv. Carbohydr. Chem. Biochem., 19 (1964) 303.
2. D.W. James, J. Preiss and A.D. Elbein, in The Polysaccharides vol. 3, pg. 129. Ed. G.O. Aspinall, Academic Press, N.Y. 1985.
3. E.C. Gotschlich in Bacterial Vaccines pg. 258. Ed. R. Germanier, Academic Press, N.Y. 1984.
4. S.M. Hammond, P.A. Lambert and A.N. Rycroft, in The Bacterial Cell Surface, pg. 57-118. Croom and Helm, 1984.
5. E.D. Mc Guire and J.B. Binkley, Biochemistry, 3 (1964) 247-251.
6. A.M. Schmidt and K. Jann, FEMS Micro. Letters, 14 (1982) 69-74.
7. P. Hoffmann, B. Jann and K. Jann, Carbohydr. Res., 139 (1985) 261- 271.
8. T. Dengler, B. Jann and K. Jann, Carbohydr. res., 150 (1986) 233-240.
9. B. Jann, P. Hoffman and K. Jann, Carbohydr. Res., 120 (1983) 131-141
10. P.A. Sandford and J. Baird, in The Polysaccharides, Vol. 2, pg. 470. Ed. G. O. Aspinall, Academic Press, N. Y. 1983.
11. C.T. Bishop and H.J. Jennings, in The Polysaccharides, Vol. 1, pg. 321. Ed. G. O. Aspinall, Academic Press, N. Y. 1982.
12. W. Nimmich, Z. Med. Mikrobiol. Immunol., 154 (1968) 117-131.
13. J. Ørskov and M.A. Fifeashbury, Int. J. Syst. Bacteriol., 27 (1977) 386-387.
14. B. Lindberg and L. Kenne, in The Polysaccharides, Vol. 2, pg. 287-363. Ed. G. O. Aspinall, Academic Press, N. Y. 1983

15. G.G.S. Dutton, H. Parolis, J.-P. Joseleau and M.-F. Marais, Carbohydr. Res., 149 (1986) 411-423.

16. A.K. Sarkar and N. Roy, Carbohydr. Res., 152 (1986) 205-215.

17. G.G.S. Dutton, H. Parolis and L.A.S. Parolis, Carbohydr. Res., 140 (1985) 263-275.

18. G.G.S.Dutton and A.V.S. Lim, Carbohydr. Res., 145 (1985) 67-80.

19. E. Altman and G.G.S.Dutton, Carbohydr. Res., 118 (1983) 183-194.

20. P.-E. Jansson, B. Lindberg, J. Lönngren and C. Ortega, Carbohydr. Res., 132 (1984) 297-305.

21. G.G.S. Dutton and D.N. Karunaratne, Carbohydr. Res., 119 (1983) 157-169.

22. G.G.S. Dutton, H. Parolis and L.A.S. Parolis, Carbohydr. Res., 152 (1986) 249-259.

23. G.G.S. Dutton and D.N. Karunaratne, Carbohydr. Res., 134 (1984) 103-114.

24. G.G.S. Dutton and A.V.S. Lim, Carbohydr. Res., 123 (1983) 247-257.

25. G.G.S. Dutton and A.V.S. Lim, Carbohydr. Res., 144 (1985) 263-276.

26. J.M. Bebault, G.G.S. Dutton, N.A. Funnel and K.L. Mackie, Carbohydr. Res., 63 (1978) 183-192.

27. Y.-M. Choy and G.G.S. Dutton, Can. J. Chem., 51 (1973) 3021-3026.

28. Y.-M. Choy and G.G.S. Dutton, Can. J. Chem., 52 (1974) 684-687.

29. I.W. Sutherland, J. Gen. Microbiol., 70 (1971) 331-338.

30. Y.-M. Choy, G.G.S. Dutton and A.M. Zanlungo, Can. J. Chem., 51 (1973) 1819-1825.

31. B. Lindberg, F. Lindh, J. Lönngren and I.W. Sutherland, Carbohydr. Res., 76 (1979) 281-284.

32. B. Lindberg, F. Lindh, J. Lönngren and W. Nimmich, Carbohydr. Res., 70 (1979) 135-144.

33. G.G.S. Dutton, T.E. Folkman, Carbohydr. Res., 78 (1980) 305-315.

34. J.-P. Joseleau, Carbohydr. Res., 142 (1985) 85-92.

35. P.A. Sandford and H.E. Conrad, Biochemistry, 5 (1966) 1508-1517.

36. G.M. Bebault and G.G.S. Dutton, Carbohydr. Res., 64 (1978) 199-213.

37. B. Lindberg, J. Lönngren, U. Ruden and W. Nimmich, Carbohydr. Res., 42 (1975) 83-93.

38. E.H. Merrifield and A.M. Stephen, Carbohydr. Res., 74 (1979) 241-257.

39. G.G.S. Dutton and A.V. Savage, Carbohydr. Res., 84 (1980) 297-305.

40. B. Lindberg, B. Lindquist, J. Lönngren and W. Nimmich, Carbohydr. Res., 58 (1977) 443-451.

41. H. Niemann, H. Friebolin and S. Stirm, (Unpublished results).

42. B. Lindberg, K. Samuelson and W. Nimmich, Carbohydr. Res., 30 (1973) 63-70

43. G.G.S. Dutton and A.V. Savage, Carbohydr. Res., 83 (1980) 297-305.

44. J.-P. Joseleau, M. Lapeyre, M. Vignon and G.G.S. Dutton, Carbohydr. Res., 67 (1978) 197-212.

45. A.S. Rao, E.A. Kabat, N.F. Whittaker, B. Nilsson, D.A. Zoph and W. Nimmich, Carbohydr. Res., 116 (1983) 271-276.

46. M. Heidelberger and W. Nimmich, Immunochemistry, 13 (1976) 67-80.

47. M. Heidelberger, W. Nimmich, J. Eriksen, G.G.S. Dutton, S. Stirm and C.T. Fang, Acta. Path. Microbiol. Scand., Sect. C, 83 (1975) 397-405.

48. M. Heidelberger, W. Nimmich, J. Eriksen and S. Stirm, Acta. Path. Mikrobiol. Scand., Sect. B, 86 (1978) 313-320.

49. G.G.S. Dutton and M. Heidelberger, J. Immunol., Vol. 3, No. 3, (1973) 852-859.

50. J.E. Scott, Methods Carbohydr. Chem, 5 (1965) 38-44.

51. K. Okutani and G.G.S. Dutton, Carbohydr. Res., 86 (1980) 259-271.

52. B. Lindberg, B. Lindquist, J. Lönngren and D.A. Powell, Carbodhydr. Res., 78 (1980) 111-117.

53. H. Bouveng and B. Lindberg, Adv. Carbohydr. Chem, 15 (1960) 53-89

54. G.O. Aspinall, in "The Polysacharides" Vol. 1, 36-101, Ed. G.O. Aspinall, Academic Press, N.Y., 1982.

55. G.O. Aspinall, in "The Polysaccharides", Pergamon Press, 1970.

56. G.G.S. Dutton, Adv. Carbohydr. Chem. Biochem, 28 (1973) 11.

57. L. Hough, J.V.S. Jones and P. Wusteman, Carbohydr. Res., 21 (1972) 9.

58. J.N. Be Miller, Adv. Carbohydr. Chem., 22 (1967) 135-149.

59. G.O. Aspinall, Methods Carbohydr. Chem., 82 (1965) 397-399.

60. R.L. Taylor and H.E. Conrad, Biochemistry, 5 (1966) 2808.

61. W.N. Haworth, J. Chem. Soc., 107 (1915) 8.

62. P.J. Harris, R.J. Henry, A.V. Blakeney and B.A. Stone, Carbohydr. Res., 127 (1984) 59-73.

63. S.-I. Hakomori, J. Biochem., 55, No. 2 (1964) - Letters.

64. T. Purdie and J. Irvine, J. Chem. Soc., 83 (1903) 1021.

65. R. Kuhn and H. Egge, Chem. Ber., 96 (1963) 3338-3348.

66. H.O. Bouveng, Acta. Chem. Scand., 15 (1961) 96.

67. T. Narui, K. Takahashi, M. Kobayashi and S. Shibita, Carbohydr. Res., 103 (1982) 293-295.

68. E. Cory and M. Chaykovsky, J. Am. Chem. Soc., 84 (1962) 254.

69. L.R. Phillips and B.R. Fraser, Carbohydr. Res., 90 (1981) 149-151

70. E.G. Gros and S.M. Flematti, Chem. Ind. (London), (1966) 1556-1557.

71. J. Arnap, L. Kenne, B. Lindberg and J. Lönngren, Carbohydr. Res., 44 (1975) C5-C7.

72. A.N. De Belder and B. Norrman, Carbohydr. Res., 8 (1968) 1-6.

73. B. Lindberg, J. Lönngren and S. Svensson, Adv. Carbohydr. Chem Biochem., 31 (1975) 185-240.

74. H.O. Bouveng and B. Lindberg, Adv. Carbohydr. Chem., 15 (1960) 53-89.

75. B. Lindberg, F. Lindh, J. Lönngren and W. Nimmich, Carbohydr. Res., 70 (1979) 135-144.

76. B. Capon, Chem. Reviews, 69 (1969) 407-458.

77. R.D. Guthrie and J.F. Mc Carthy, Adv. Carbohydr. Chem., 22 (1967) 11-23.

78. Y.C. Lee and C.E. Ballou, Biochemistry, 4 (1965) 287-298.

79. B. Nilsson and S. Svensson, Carbohydr. Res., 72 (1979) 183-190.

80. B. Lindberg, J. Lönngren and J.L. Thompson, Carbohydr. Res., 28 (1973) 351-357.

81. B. Lindberg and J. Lönngren, Methods Carbohydr. Chem., 7 (1976) 142-148.

82. J. Kiss, Adv. Carbohydr. Chem., 29 (1974) 229-303.

83. G.O. Aspinall and K.-G. Rosell, Carbohydr. Res, 57 (1977) C23-C26

84. K. Shimizu, Carbohydr. Res., 92 (1981) 65-74.

85. L. Malaprade, C. R. Acad. Sci., Paris, 186 (1928) 382-384.

86. G.W. Hay, B.A. Lewis and F. Smith, Methods Carbohydr. Chem., 5 (1965) 357-360.

87. J.M. Bobbit, Adv. Carbohydr. Chem., 11 (1956) 1-44.

88. R.D. Guthrie, Adv. Carbohydr.Chem., 16 (1961) 105-158.

89. I.J Goldstein, G.W. Hay, B.A. Lewis and F. Smith, Methods Carbohydr. Chem., 5 (1965) 361-370.

90. J.E. Cadotte, G.G.S. Dutton, I.J. Goldstein, B.A. Lewis, F. Smith and J.W. Van Cleve, J. Am. Chem. Soc., 79 (1957) 691.

91. T. Painter and B.Larson, Acta. Chem. Scand., 24 (1970) 813-833.

92. G.G.S. Dutton and K.B.Gibney, Carbohydr. Res., 25 (1972) 99-105.

93. B. Lindberg, J. Lönngren, W. Nimmich and U. Ruden, Acta. Chem. Scand., 27 (1973) 3787-3790.

94. G.M. Bebault, J.M. Berry, Y.-M. Choy, G.G.S. Dutton, N. Funnell, L.D. Hayward and A.M. Stephen, Can. J. Chem., 51 (1973) 324-326.

95. J.N.C. Whyte and J.R. Englar, Carbohydr. Res., 57 (1977) 273-280.

96. K. Leontein, B. Lindberg and J. Lönngren, Carbohydr. Res., 62 (1978) 359-362.

97. J. Douglas, in Bacteriophages, Chapman and Hall, London, 1975.

98. S. Stirm, W. Bessler, F. Fehmel and E. Freund-Molbert, J. Virol, 8 (1971) 343-346.

99. W. Bessler, E. Freund-Molbert, H. Knufferman, C. Rudolf, H. Thurow and S. Stirm, Virology, 56 (1973) 134-151.

100. D. Rieger-Hug and S. Stirm, Virology, 113 (1981) 363-378.

101. H. Thurow, H. Nieman and S. Stirm, Carbohydr. Res, 41 (1975) 257-271.

102. J.E.G. Van Dam, H. Van Halbreek, J.P. Kamerling, J.F.G. Vliegenthart, H. Snippe, M. Jansze and J.M.W. Williers, Carbohydr. Res., 142 (1985) 338-343.

103. H. Nieman, H. Beilharz and S. Stirm, Carbohydr. Res., 60 (1978) 353-366.

104. J.L. Di Fabio, D.N. Karunaratne and G.G.S. Dutton, Carbohydr. Res., 144 (1986) 251-261.

105. G.G.S. Dutton, K.L. Mackie, A.V. Savage, D. Rieger-Hug and S. Stirm, Carbohydr. Res., 84 (1980) 161-170.

106. G.G.S. Dutton, A.V. Savage and M. Vignon, Can. J. Chem., 58 (1980) 2588-2591.

107. C.P.J. Clandemans, Adv. Carbohydr. Chem. Biochem., 31 (1975) 313-346.

108. G.G.S. Dutton, J.L. Di Fabio, D. M. Leek, E.H. Merrifield, J.R. Nunn and A.M. Stephen, Carbohydr. Res., 97 (1981) 127-138.

109. J.L. Di Fabio, G.G.S. Dutton and H. Parolis, Carbohydr. Res., 126 (1984) 261-269.

110. C.J. Bridgen and S.G. Wilkinson, Carbohydr. Res., 145 (1985) 81-87.

111. O. Samuelson, Methods Carbohydr. Chem., 6 (1972) 65-75.

112. S.C. Churms, C.R.C. Handbook of Chromatog., 1982, Eds. G. Zweig and J. Sherma, C.R.C. Press Inc.

113. R.E. Wing and J.N. Be Miller, Methods Carbohydr. Chem., 6 (1972) 42-53.

114. A. Heyraud and P. Salemis, Carbohydr. Res., 107 (1982) 123-129.

115. G.G.S. Dutton, Adv. Carbohydr. Chem. Biochem., 28 (1973) 11-160.

116. G.G.S. Dutton, Adv. Carbohydr. Chem. Biochem., 30 (1974) 9-110.

117. C.C. Sweeley, R. Bentley, M. Makita and W.W. Wells, J. Am. Chem.Soc., 85 (1963) 2497- 2507.

118. P.O. Bethge, C. Holmstrom and S. Juhlin, Svensk Papperstidning, 69 (1966) 60-63.

119. B.W Li, T.W. Cochrane and J.R. Vercelotti, Carbohydr. Res., 59 (197) 567-570.

120. G.D. Mc Ginnis, Carbohydr. Res., 108 (1982) 284-292.

121. H. Björndal, B. Lindberg and S.Svensson, Acta. Chem. Scand., 21 (1967) 1801-1804.

122. H. Parolis and D. Mc Garvie, Carbohydr. Res., 62 (1978) 363-367.

123. A.G.W. Bradbury, D.J. Halliday and D.G. Metcalf, J. Chromatogr., 213 (1981) 146-150.

124. P.-E. Jansson, L. Kenne, H. Liedgren, B. Lindberg and J. Lönngren, Chem. Commun., 8 (1976).

125. E. Barreto-Bergter, L. Hogge and P.A.J. Gorin, Carbohydr. Res., 97 (1981) 147-150.

126. B.A. Dmitriev, L.V. Backinowsky, O.S. Chizhov, B.M. Zolotarev and N.K. Kochetkov, Carbohydr. Res., 19 (1971) 432-435.

127. G. Holzer, J. Oró, S.J. Smith and V.M. Doctor, J. Chromatogr., 194 (1980) 410-415.

128. D.P. Sweet, R.H. Shapiro and P. Albersheim, Carbohydr. Res., 40 (1975) 217-225.

129. I.M. Morrison, J. Chromatog., 108 (1975) 361-364.

130. G.J. Gerwig, J.P. Kamerling and J.F.G. Vliegenthart, Carbohydr. Res., 62 (1978) 349-357.

131. S.C. Churms, Adv. Carbohydr. Chem. Biochem., 25 (1970) 13-51.

132. S.Hjerten, R. Mosbach, Anal. Biochem., 3 (1962) 109.

133. M. Du Bois, K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, Anal. Chem., 28 (1956) 350.

134. S.C. Churms and A.M. Stephen, Carbohydr. Res., 35 (1974) 73-86.

135. W.E. Trevelyn, D.P. Procter and J.S. Harrison, Nature, 166 (1950) 444.

136. H.T. Gordon, W. Thornburg and L.W. Werum, Anal. Chem., 28 (1956) 849-855.

137. A.S. Perlin, Methods Carbohydr. Chem., 7 (1976) 94-100.

138. R.U. Lemieux, R.K. Kullnig, H.J. Bernstein and W.G. Schneider, J. Am. Chem. Soc., 80 (1958) 6098.

139. L.D. Hall, Adv. Carbohydr. Chem., 19 (1964) 51-93.

140. B.Coxon, Adv. Carbohydr. Chem. Biochem., 27 (1972) 7-83.

141. L.D. Hall, in The Carbohydrates, Chem. and Biochem., pg. 1299. Eds. W. Pigman and D. Horton, Academic Press, N.Y. 1980.

142. A.S. Perlin and B. Casu, in The Polysaccharides, vol.1, pg. 133-193. Ed. G.O. Aspinall, Academic Press, N.Y. 1982.

143. Y.-M. Choy, G.G.S. Dutton, A.M. Stephen and M.T. Yang, Anal. Letters, 5 (1972) 675-681.

144. B. Matsuhiro, A.B. Zanlungo and G.G.S. Dutton, Carbohydr. Res., 97 (1981) 11-18.

145. S.M.R. Stanley, M.Sc. Thesis, Rhodes University, 1986.

146. P.J. Garegg, B. Lindberg and I. Kvarnstrom, Carbohydr. Res., 77 (1979) 71-78.

147. P.A.J. Gorin, Adv. Carbohydr. Chem. Biochem., 38 (1981) 13-104.

148. J.L. Marshall, in Carbon-Carbon and Carbon-Proton N.M.R. Couplings, Methods in stereochemical analysis, Vol.2. Ed. A.P. Marchand, Verlag Chemie International, Florida 1983.

149. K. Bock and C. Pedersen, Adv. Carbohydr. Chem. Biochem., 41 (1983) 27-66.

150. K. Bock, C. Pedersen and H. Pedersen, Adv. Carbohydr. Chem.Biochem., 42 (1984) 195-225.

151. L.A.S. Parolis, pHd Thesis, Rhodes University, 1986.

152. P.J. Garegg, P.-E. Jansson, B. Lindberg, F. Lindh, J. Lönngren, I. Kvarnström and W. Nimmich, Carbohydr. Res., 78 (1980) 127-132

153. J.H. Bradbury and G.A. Jenkins, Carbohydr. Res., 126 (1984) 125-126.

154. R. Benn and H. Gunther, Angew. Chem. Int. Engl., 22 (1983) 350-380.

155. G.A. Morris and L.D. Hall, J. Am. Chem. Soc., 103 (1981) 4703-4711.

156. L.D. Hall, S. Sukumar and G.R. Sullivan, J. Chem. Soc. Chem. Com., (1979) 292-294.

157. L.D. Hall, G.A. Morris and S. Sukumar, Carbohydr. Res., 76 (1979) C7-C9.

158. B. Coxon, Anal. Chem., 55 (1983) 2361-2366.

159. L.D. Hall and G.A. Morris, Carbohydr. Res., 82 (1980) 175-184.

160. N.K. Kotchetkov and O.S. Chizhov, Adv. Carbohydr. Chem., 21 (1966) 39-93.

161. J. Lönngren and S. Svensson, Adv. Carbohydr. Chem. Biochem., 29 (1974) 41-106.

162. H. Rauvela, J. Finne, T. Krusius, J. Karkkainen and J. Jarnvelt, Adv. Carbohydr. Chem. Biochem., 38 (1981) 389-416.

163. H. Björndal, C.G. Hellerquist, B. Lindberg and S. Svensson, Angew. Chem. Int. Ed., 9 (1970) 610-619.

164. D. Horton, J.D. Wander and R.L. Foltz, Carbohydr. Res., 36 (1974) 75.

165. H.D. Beckey, Angew. Chem., 81 (1969) 662.

166. H.D. Beckey, Angew. Chem., 87 (1975) 425.

167. M. Barber, R. Bordoli, R. Sedgwick and A. Tyler, J.C.S. Chem. Com., (1981) 325-327.

168. A. Dell, H.R. Morris, H. Egge, H. Von Nicolai and G. Strecker, Carbohydr. Res., 115 (1983) 41-52.

169. A. Dell and C. Ballou, Carbohydr. Res., 120 (1983) 95-111.

170. C. Ballou and A. Dell, Carbohydr. Res., 140 (1985) 139-143.

171. G. Puzo, J.-C. Prome and J.J. Fournie, Carbohydr. Res., 140 (1985) 131-134.

172. A. Amemura, T. Harada, M. Abe and S. Higashi, Carbohydr. Res., 115 (1983) 165-174.

173. P.J. Garegg, P.-E. Jansson, B. Lindberg, F. Lindh, J. Lönngren, I. Kvarnström and W. Nimmich, Carbohydr. Res., 78 (1980) 127-132.

ANNEXURE A

MEDIA

1. Mueller-Hinton agar

	g/l
Meat infusion	5.0
Casein hydrolysate	17.5
Starch	1.5
Agar	14.0

2. Nutrient broth

Bacto peptone	5.0
Bacto beef extract	3.0
Sodium chloride	2.0

The media was sterilised by autoclaving at 121° for 20 minutes.

ANNEXURE B: GEL PERMEATION CHROMATOGRAMS

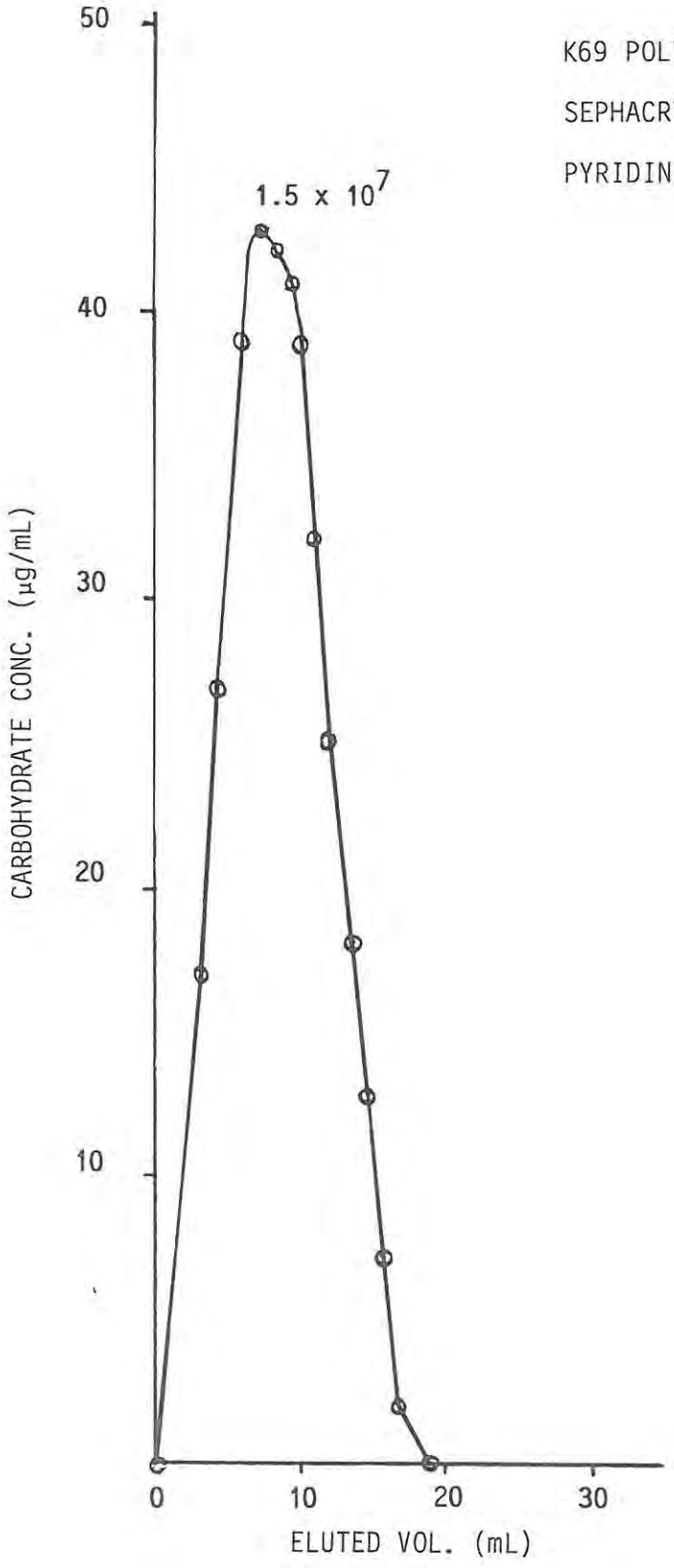

K69 POLYSACCHARIDE
SEPHACRYL S500, 60 x 1cm
PYRIDINIUM ACETATE BUFFER (pH 5.2)
1.5 x 10^7
CARBOHYDRATE CONC. (µg/mL)
50
40
30
20
10
0
10
20
30
ELUTED VOL. (mL)

FIG.1

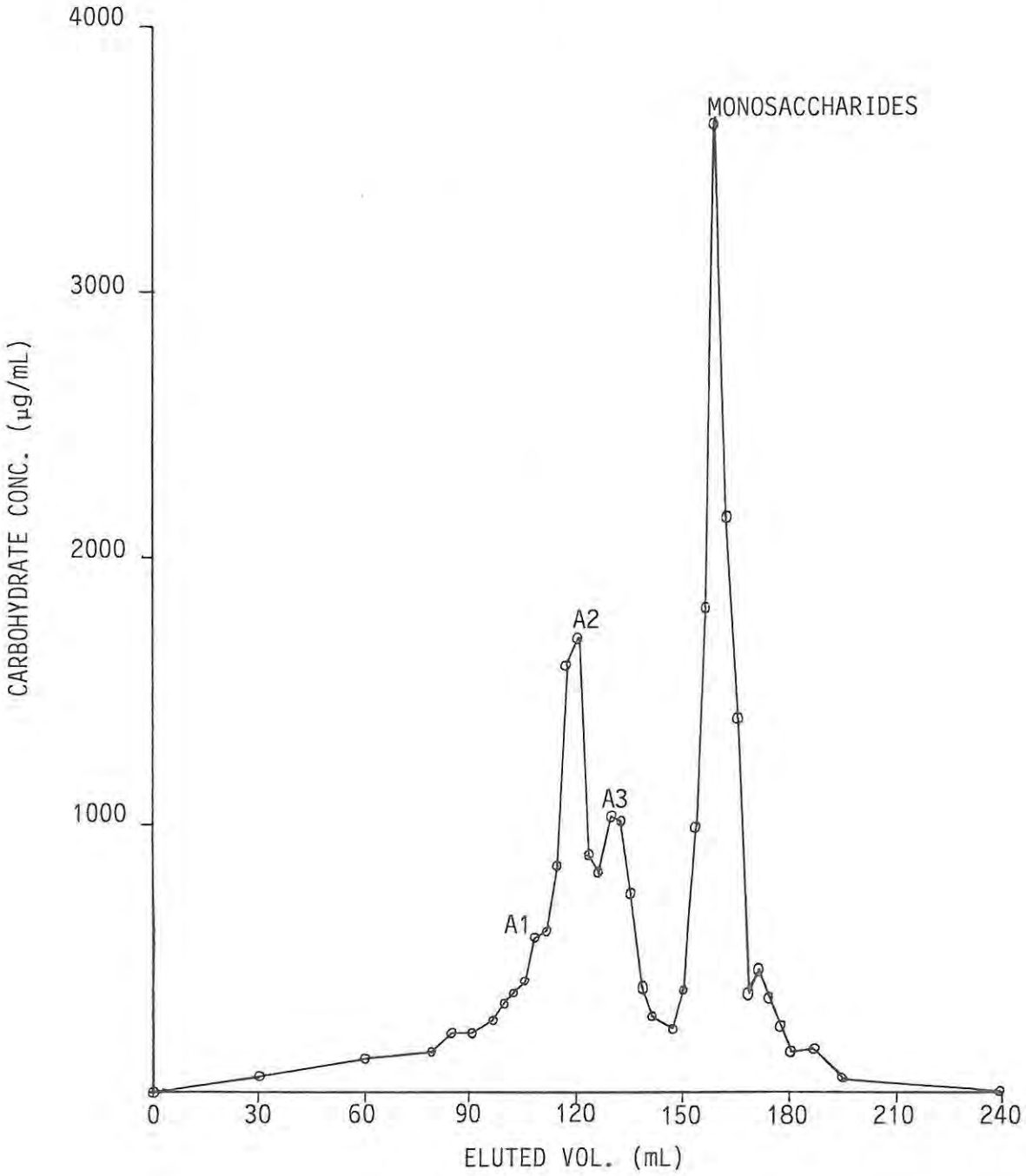

K69 PARTIAL HYDROLYSIS (5.5h)
BIO-GEL P-2, 2.6 x 65cm
WATER (12.5 mL/h)
4000
3000
2000
1000
CARBOHYDRATE CONC. (µg/mL)
MONOSACCHARIDES
A1
A2
A3
0
30
60
90
120
150
180
210
240
ELUTED VOL. (mL)

FIG.2

K69/e69 DEPOLYMERISATION

FRACTION A, 5mg/mL

BIO-GEL P-4, 1.6 x 50cm

PYRIDINIUM ACETATE BUFFER (8mL/h)

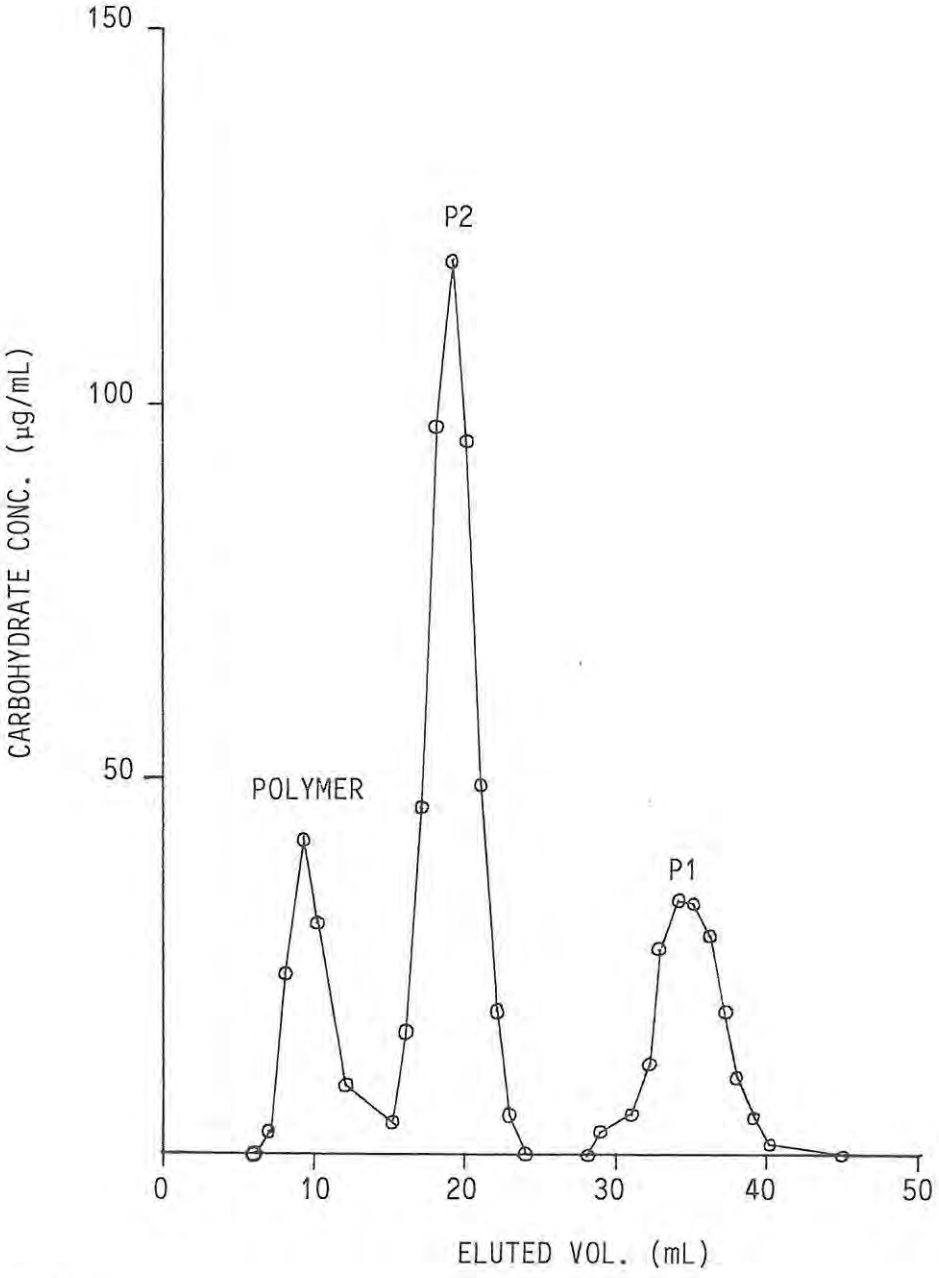


FIG.3a

K69/θ69 DEPOLYMERISATION

FRACTION B, 6mg/mL

BIO-GEL P-4, 1.6 x 50cm

PYRIDINIUM ACETATE BUFFER (8mL/h)

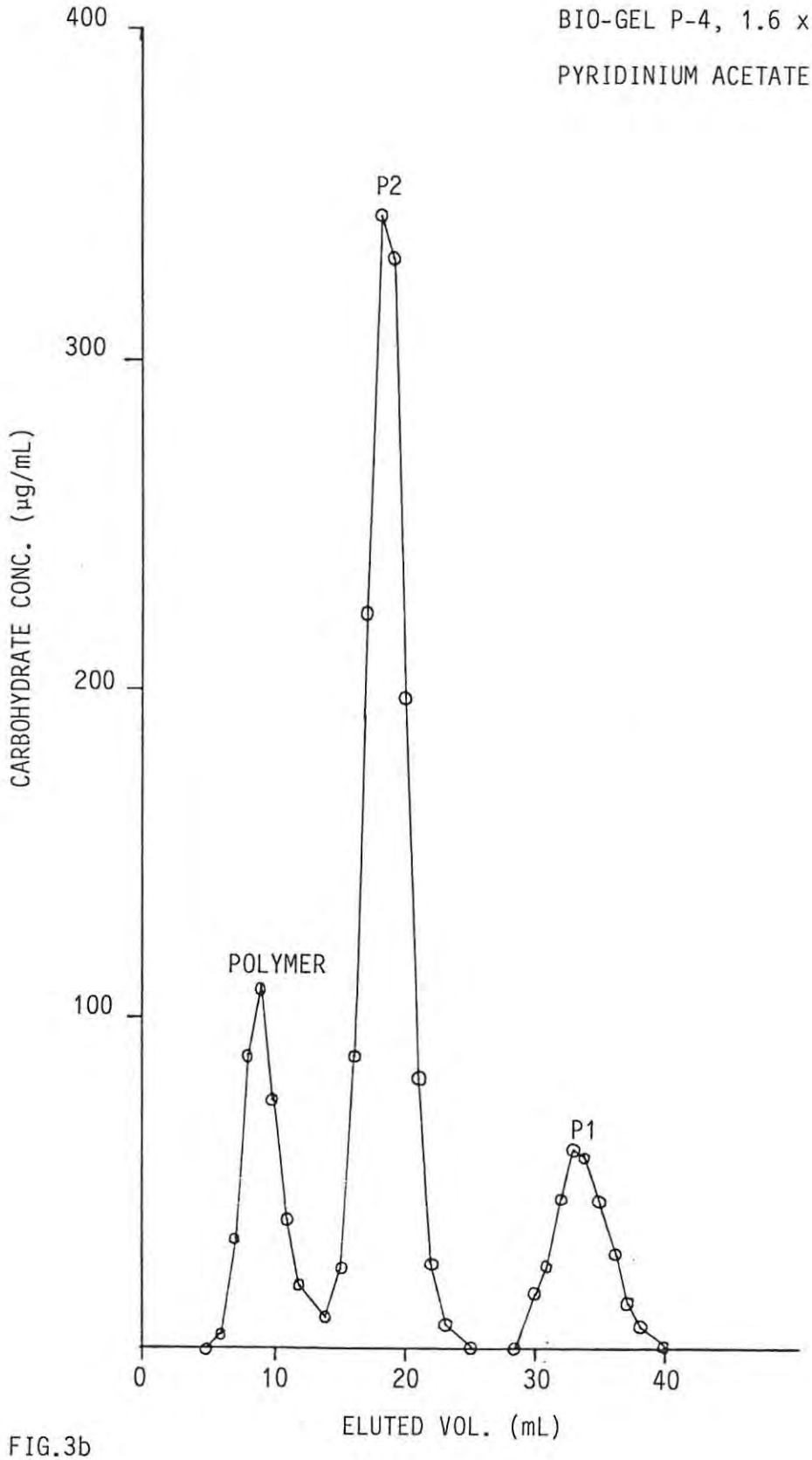


FIG.3b

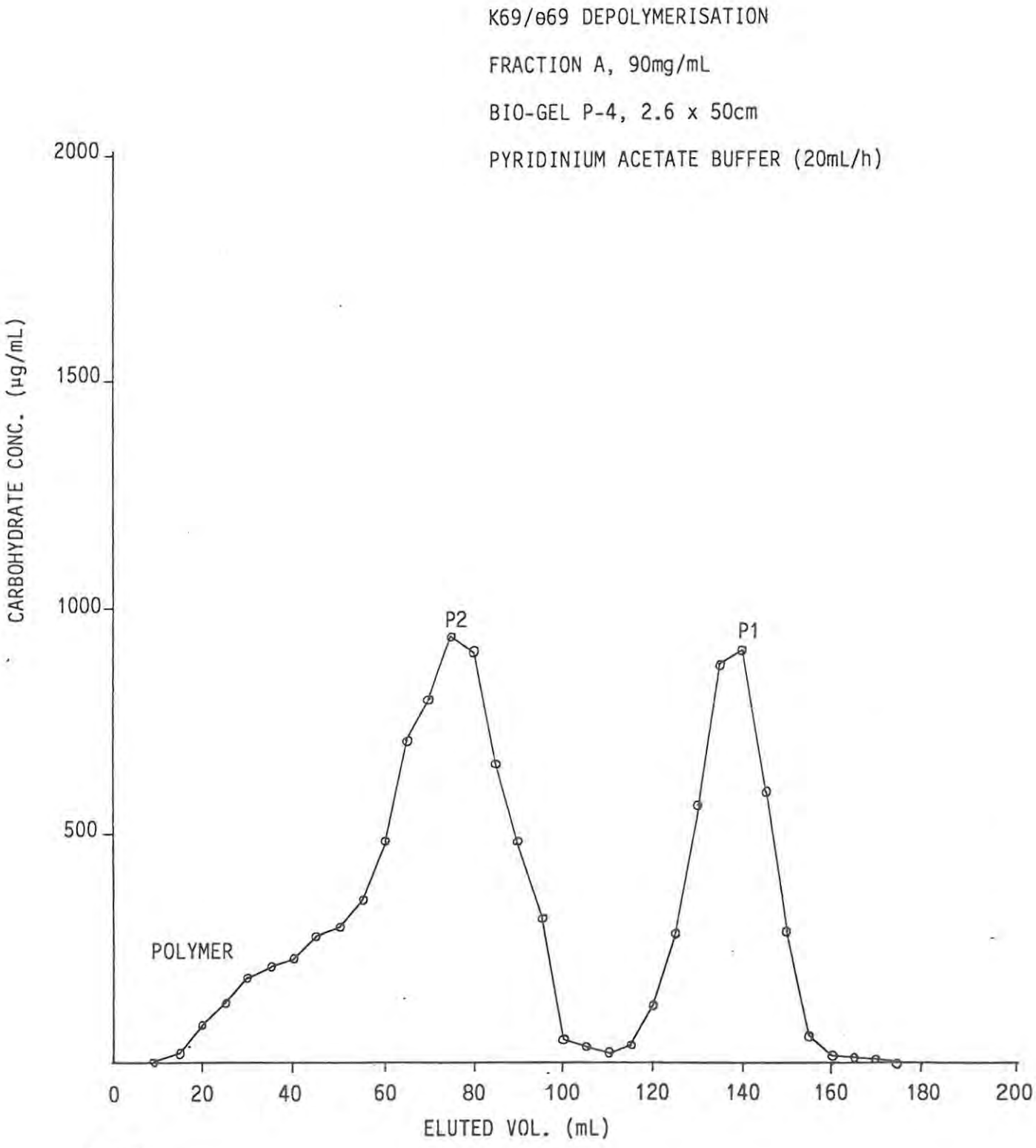

K69/ø69 DEPOLYMERISATION
FRACTION A, 90mg/mL
BIO-GEL P-4, 2.6 x 50cm
PYRIDINIUM ACETATE BUFFER (20mL/h)
CARBOHYDRATE CONC. (µg/mL)
2000
1500
1000
500
P2
P1
POLYMER
0
20
40
60
80
100
120
140
160
180
200
ELUTED VOL. (mL)

FIG.3c

K69/θ69 DEPOLYMERISATION

FRACTION B, 100mg/mL

BIO-GEL P4, 2.6 x 70cm

PYRIDINIUM ACETATE BUFFER (20mL/h)

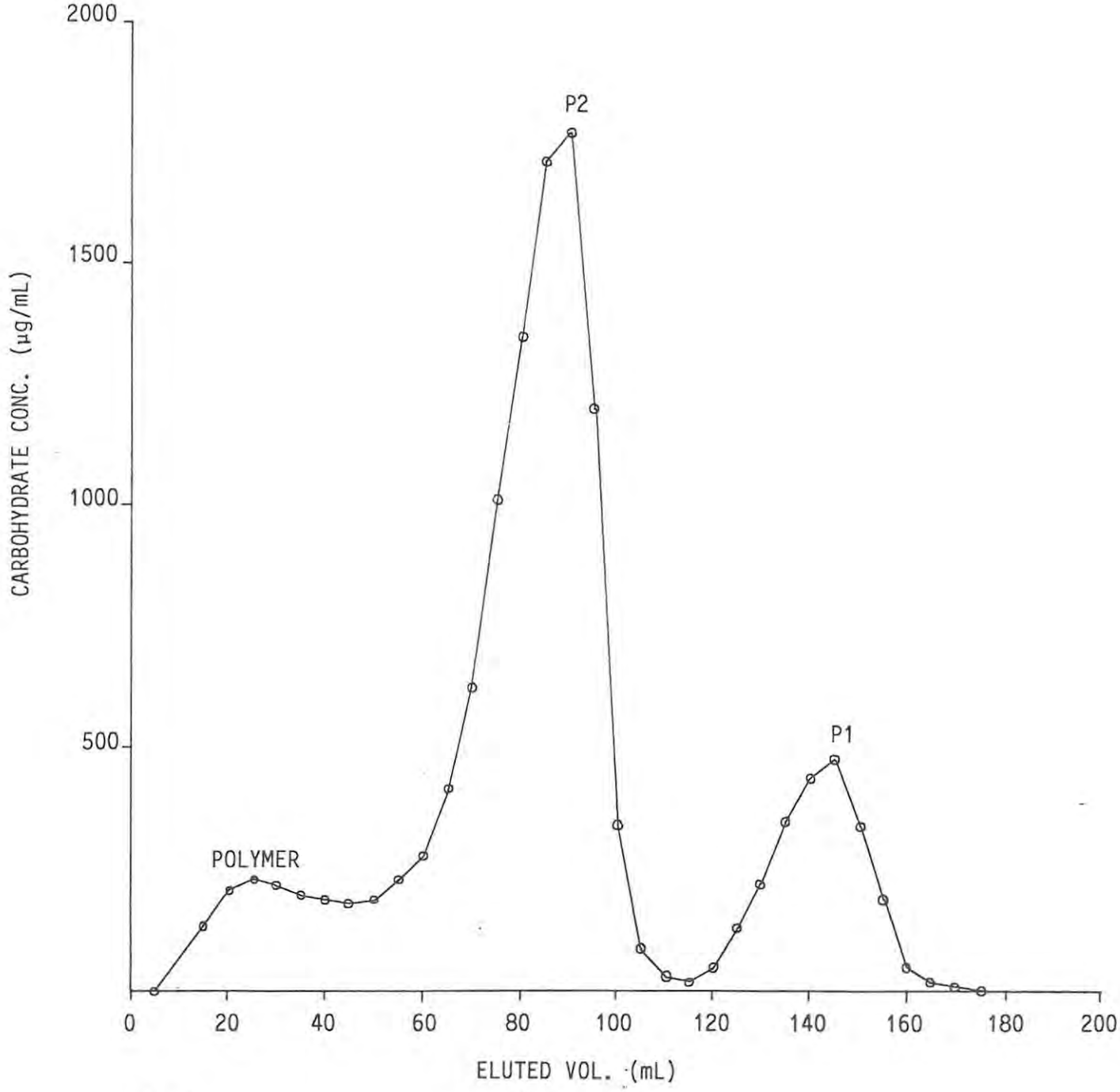


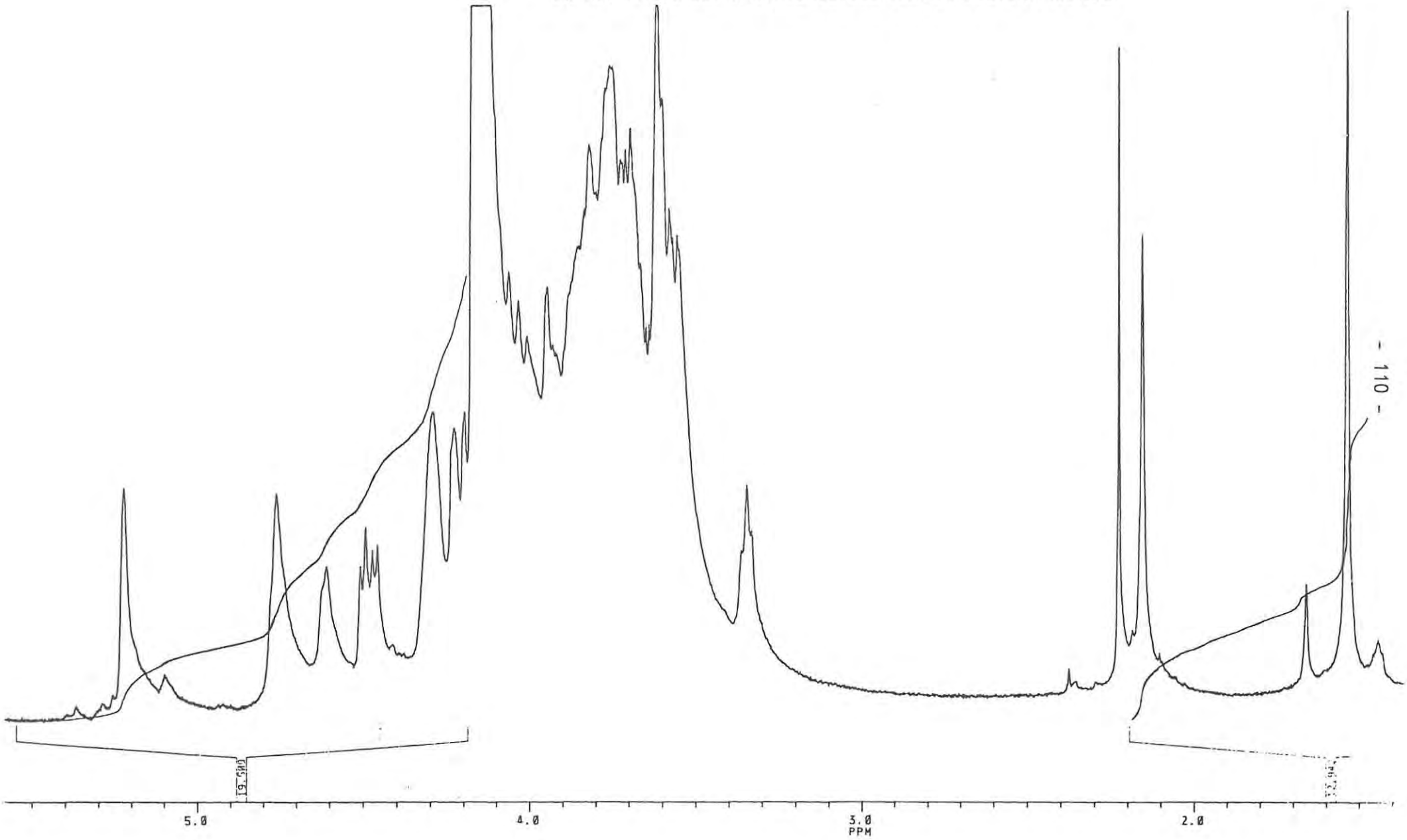
FIG.3d

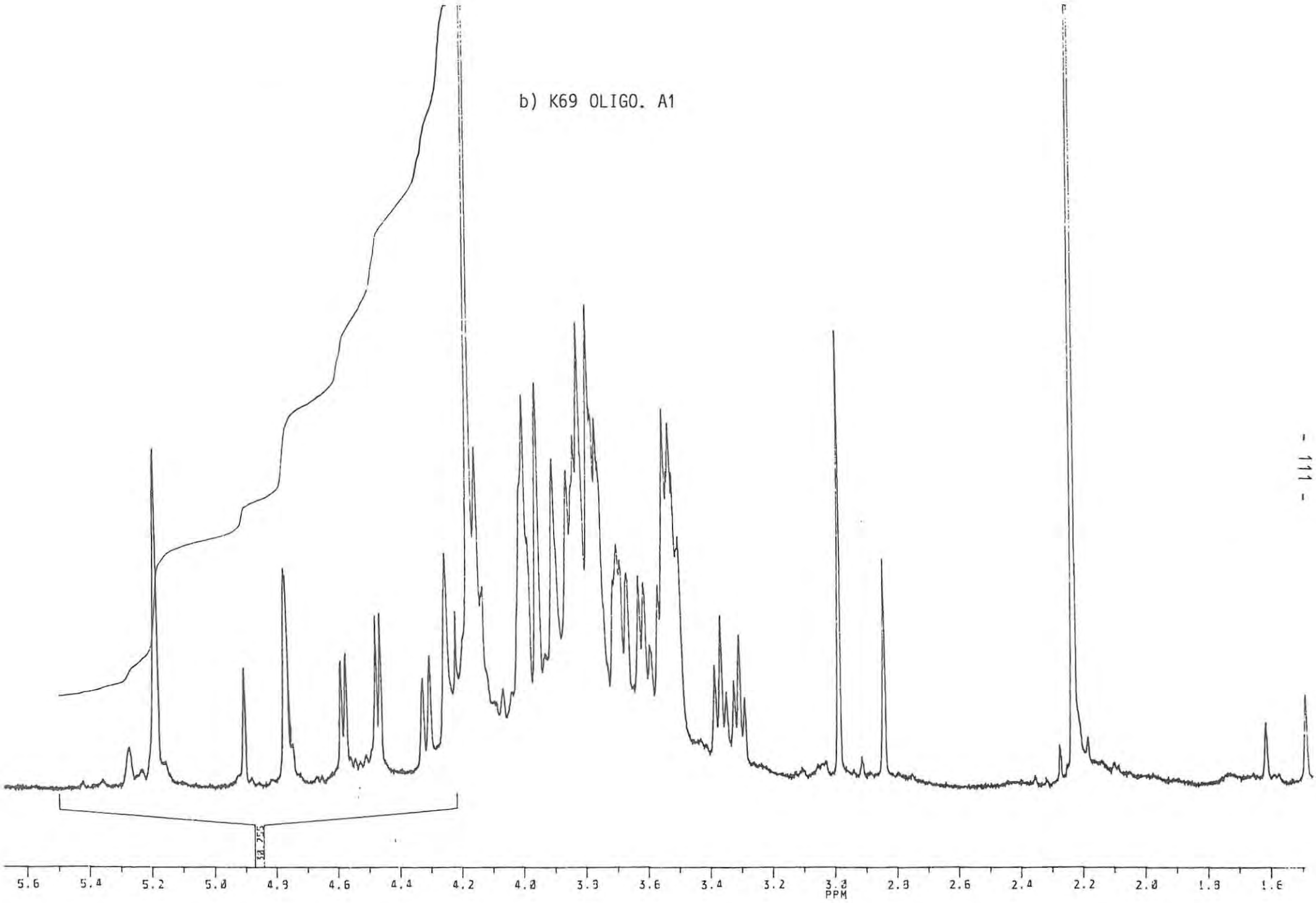
ANNEXURE C

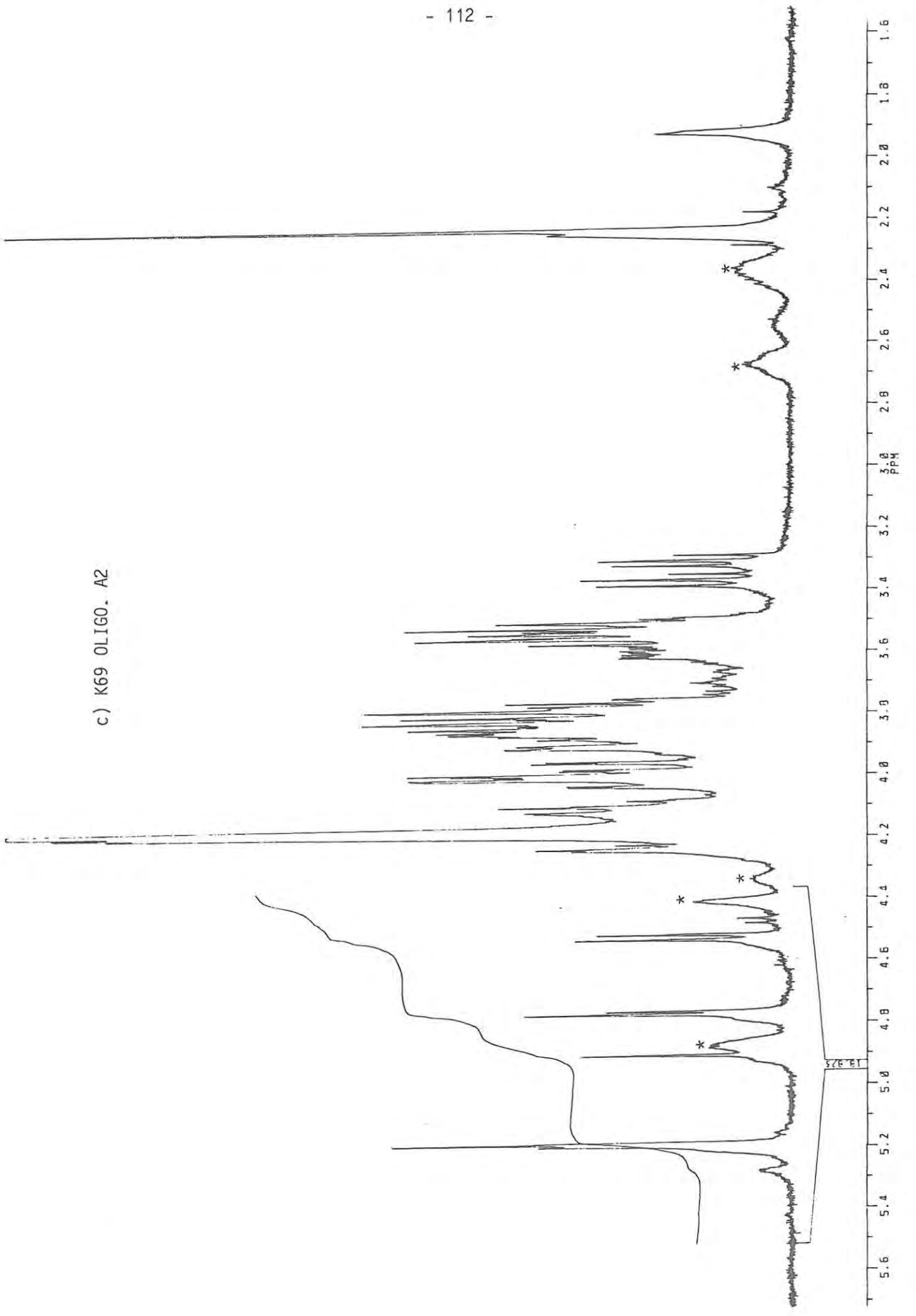
N.M.R. SPECTRA (* = impurities)

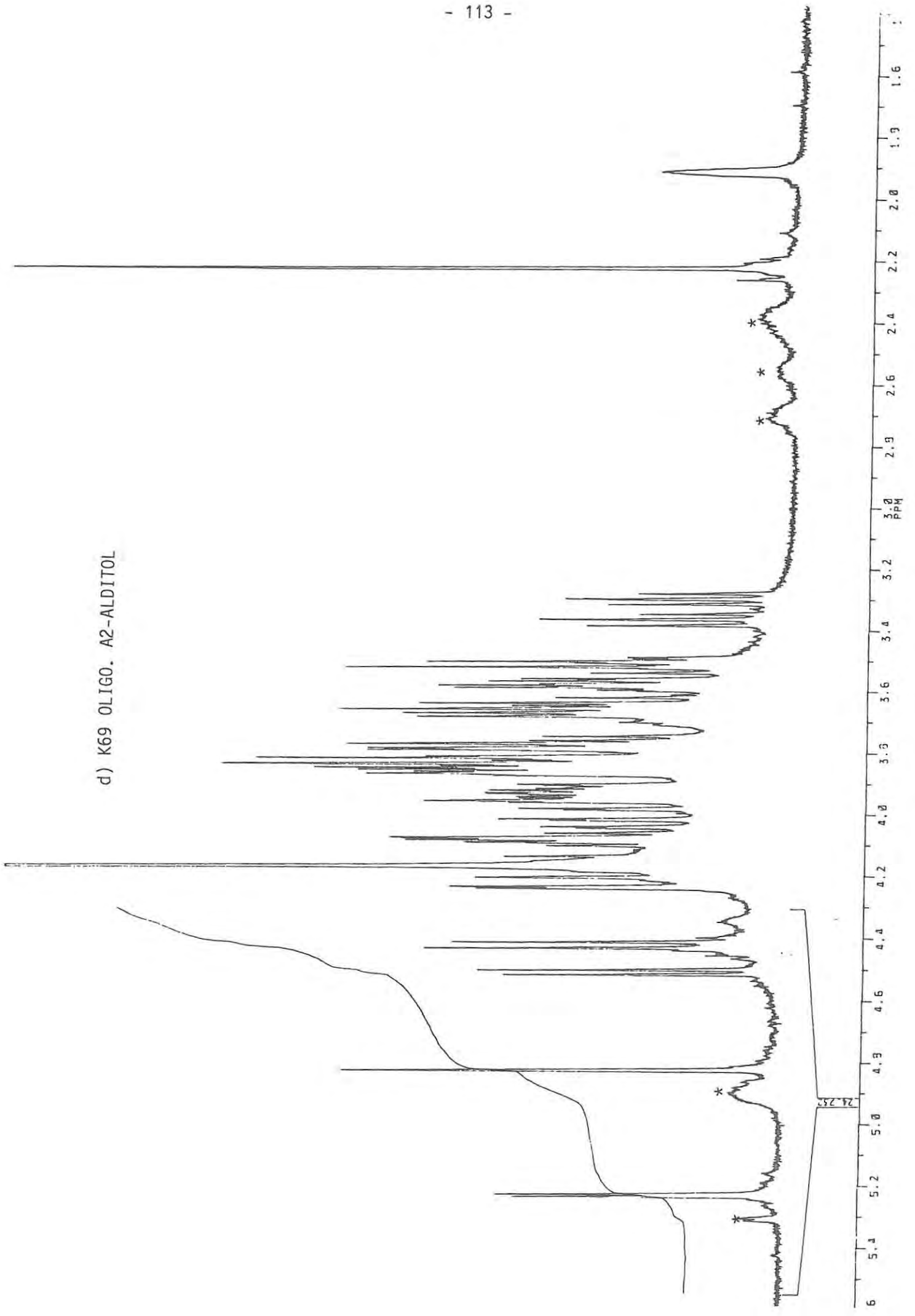
1. ^{1}H-N.m.r. spectra

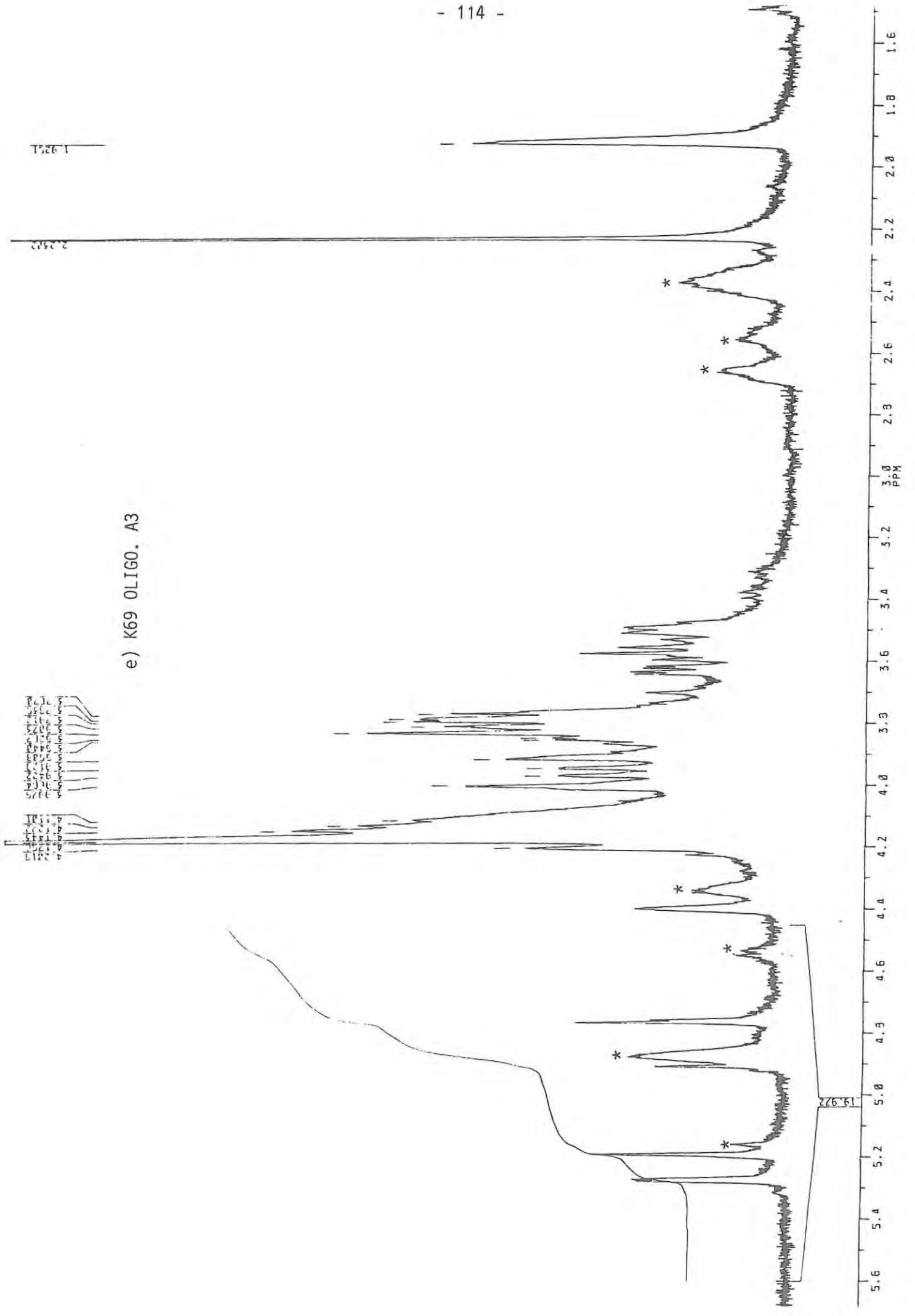
a) K69 PARTIALLY AUTOHYDROLYSED NATIVE POLYSACCHARIDE

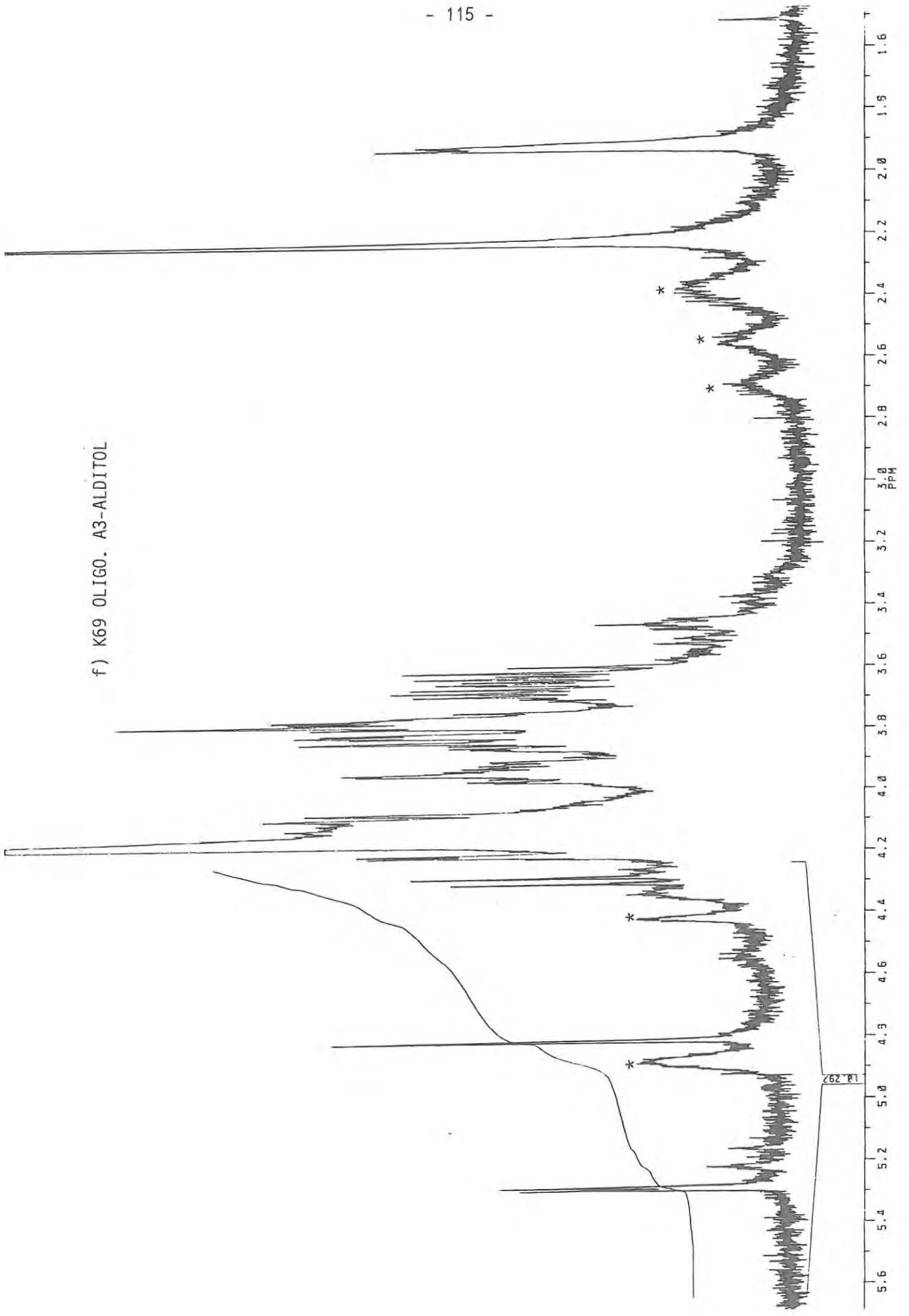



b) K69 OLIGO. A1
10.255
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
PPM
2.8
2.6
2.4
2.2
2.0
1.8
1.6

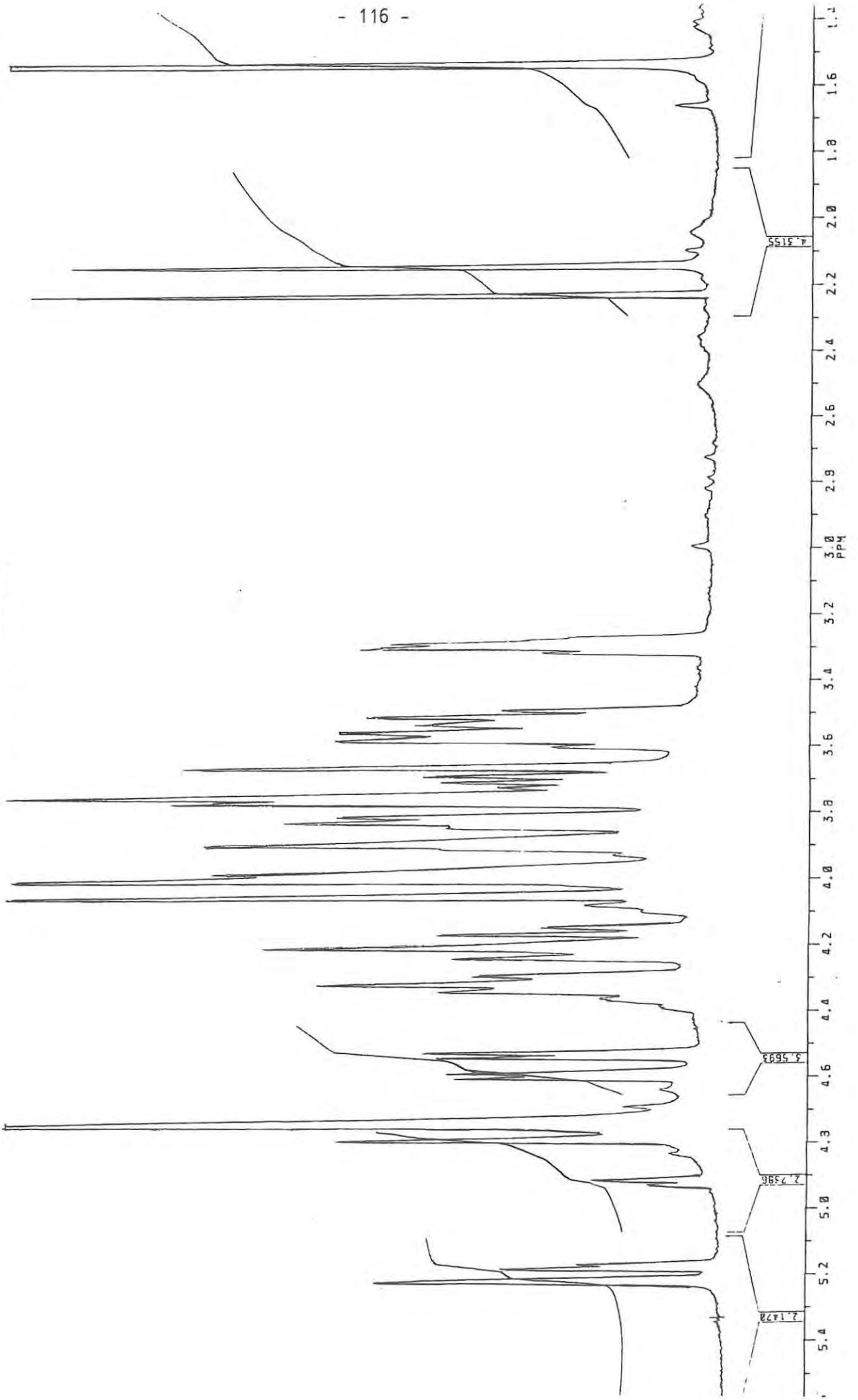

c) K69 OLIGO. A2
13.975
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
PPM


d) K69 OLIGO. A2-ALDITOL
*
*
*
*
*
24.25
6
5.1
5.2
5.0
4.9
4.6
4.1
4.2
4.0
3.8
3.6
3.4
3.2
3.0
PPM
2.9
2.6
2.4
2.2
2.0
1.9
1.6

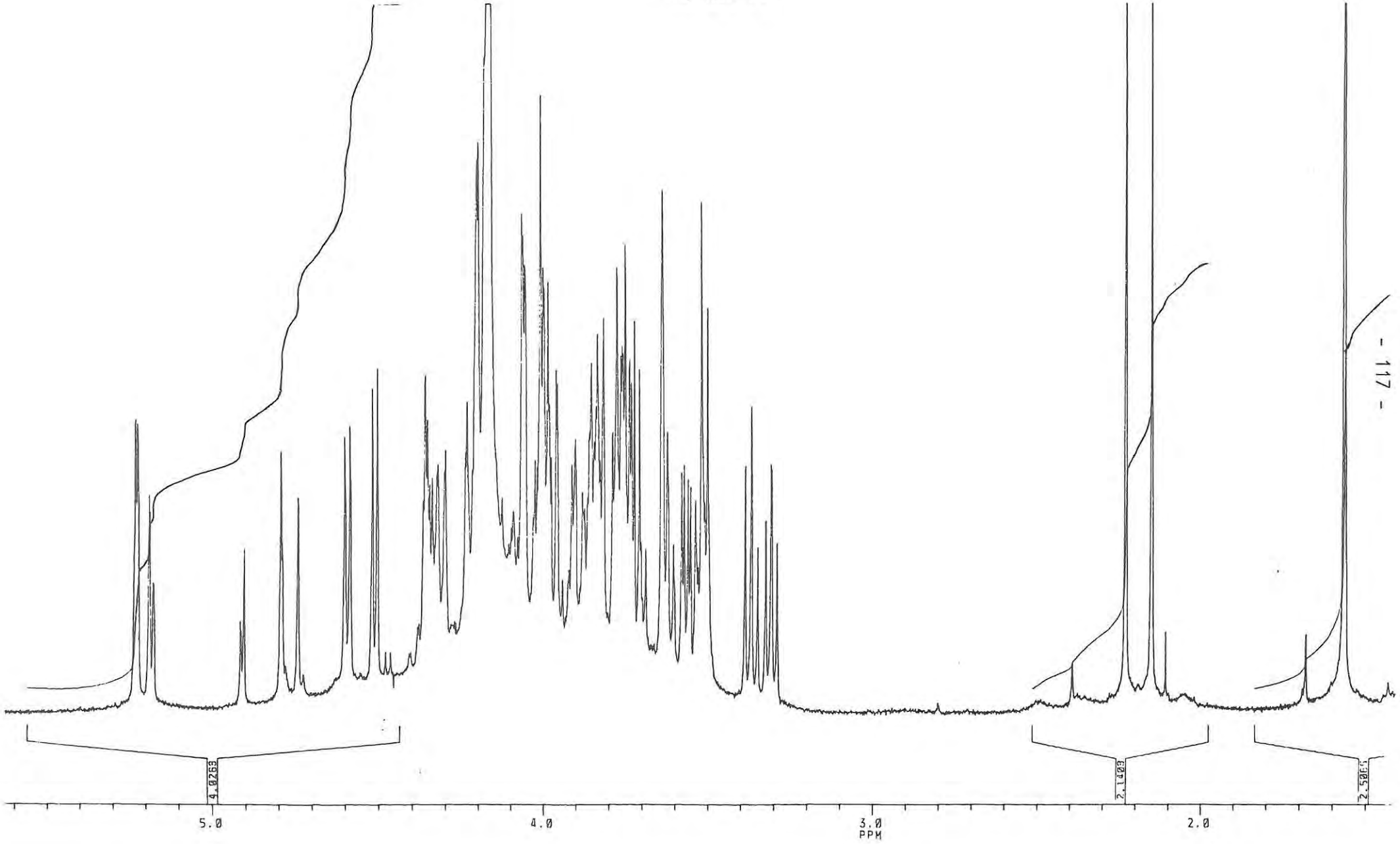

e) K69 OLIGO. A3
PPM


f) K69 OLIGO. A3-ALDITOL
*
*
*
*
*
10.292
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
PPM

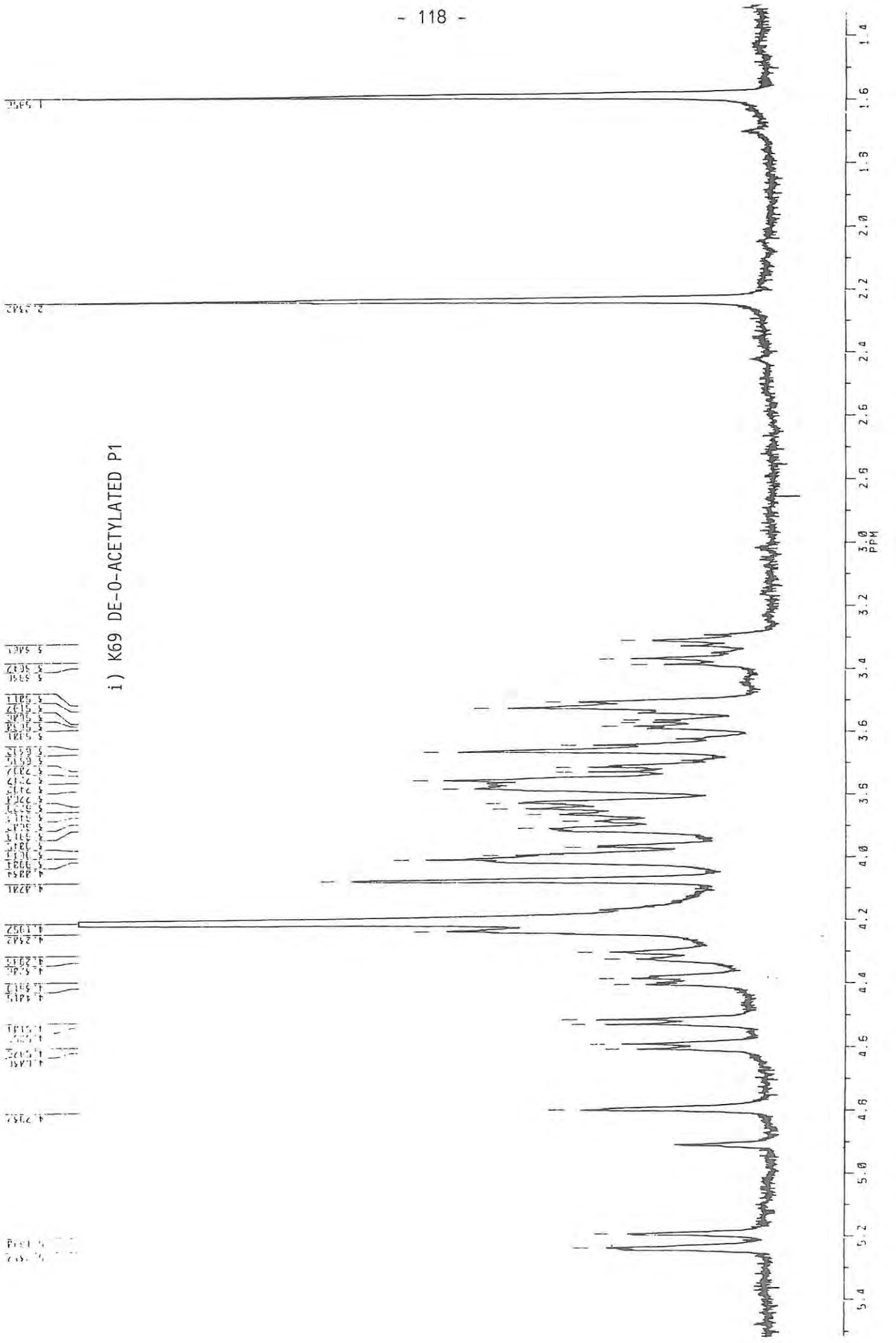
g) K69 P1 (ambient)



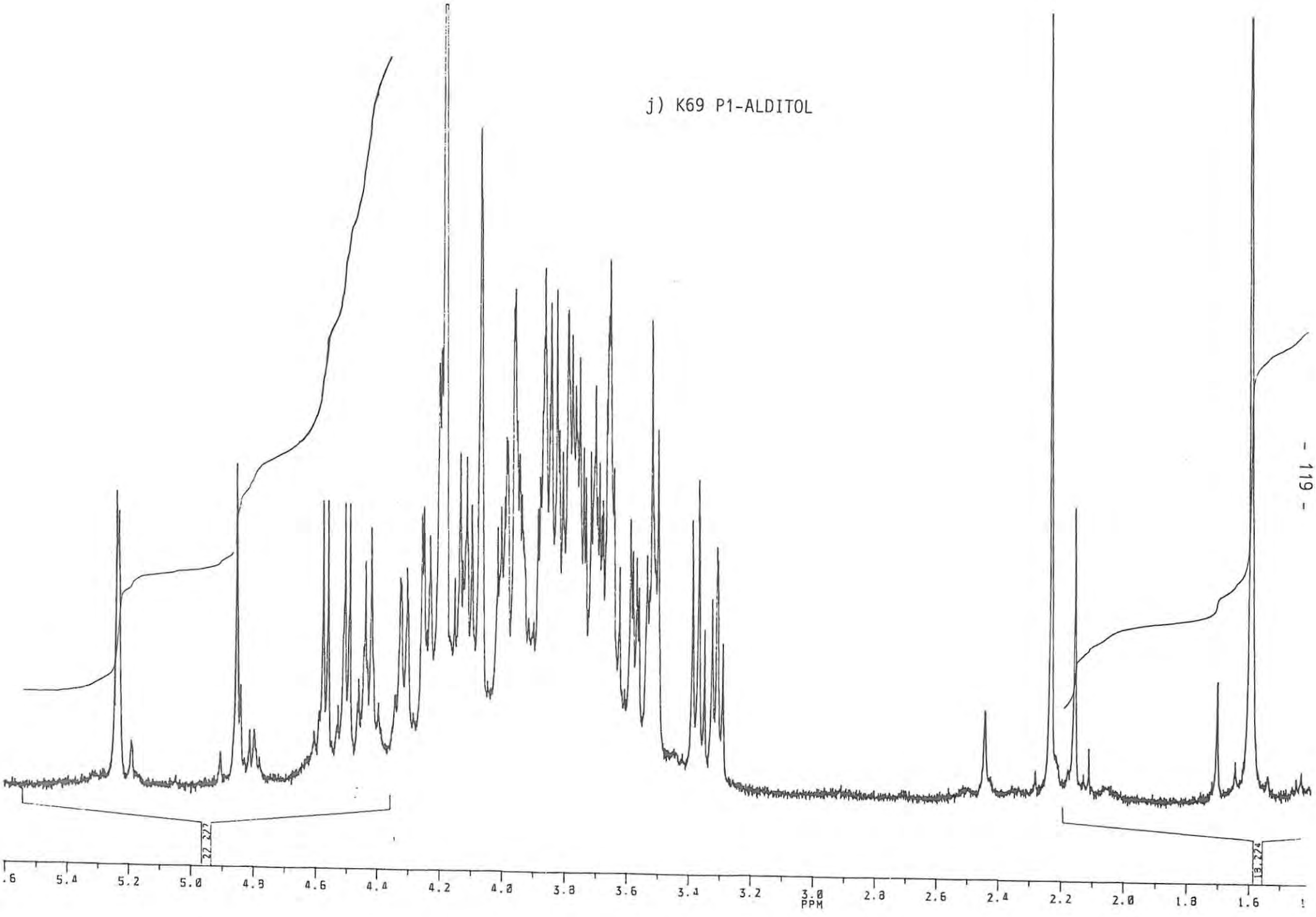
h) K69 P1



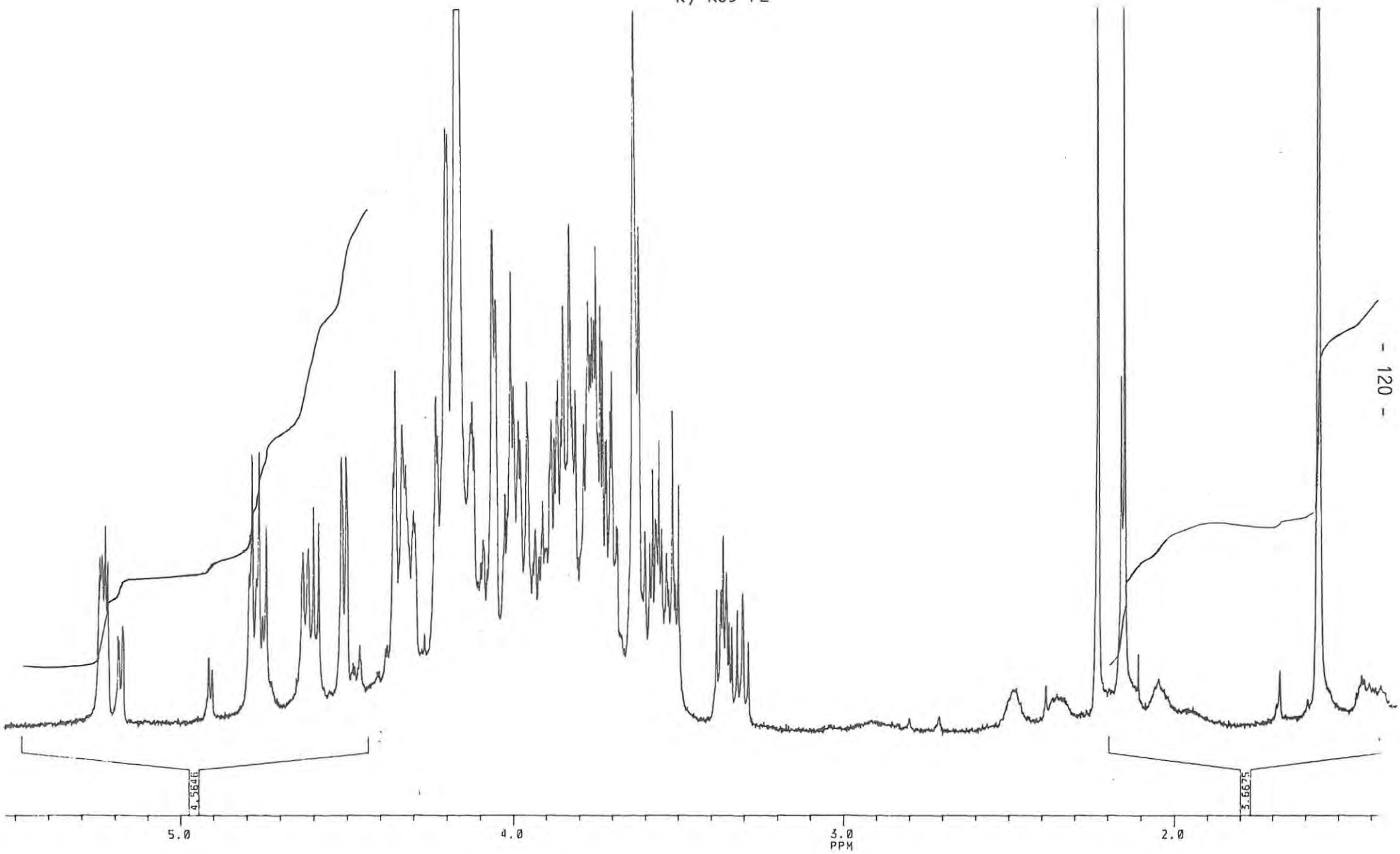
i) K69 DE-O-ACETYLATED P1



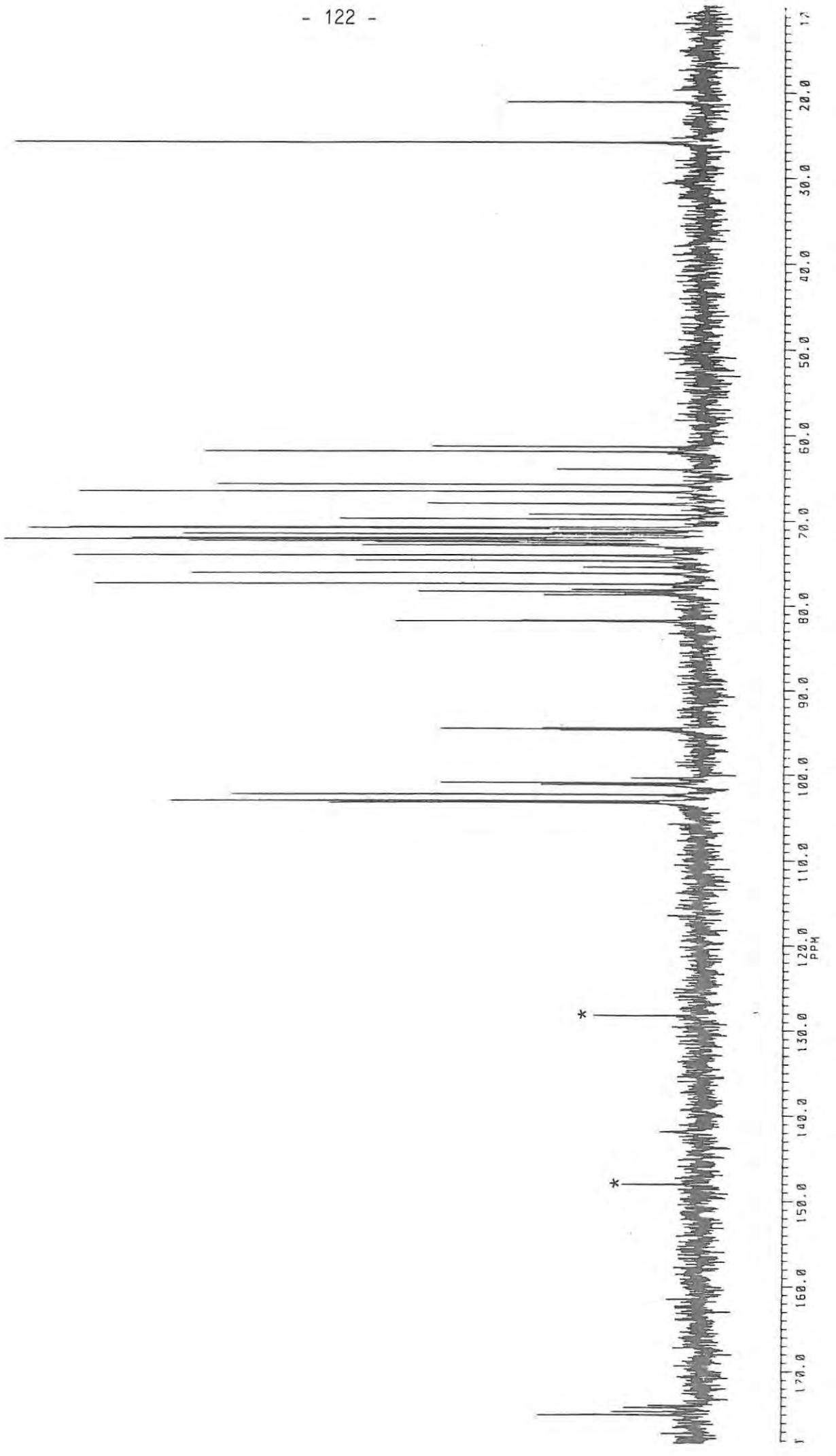
j) K69 P1-ALDITOL



k) K69 P2

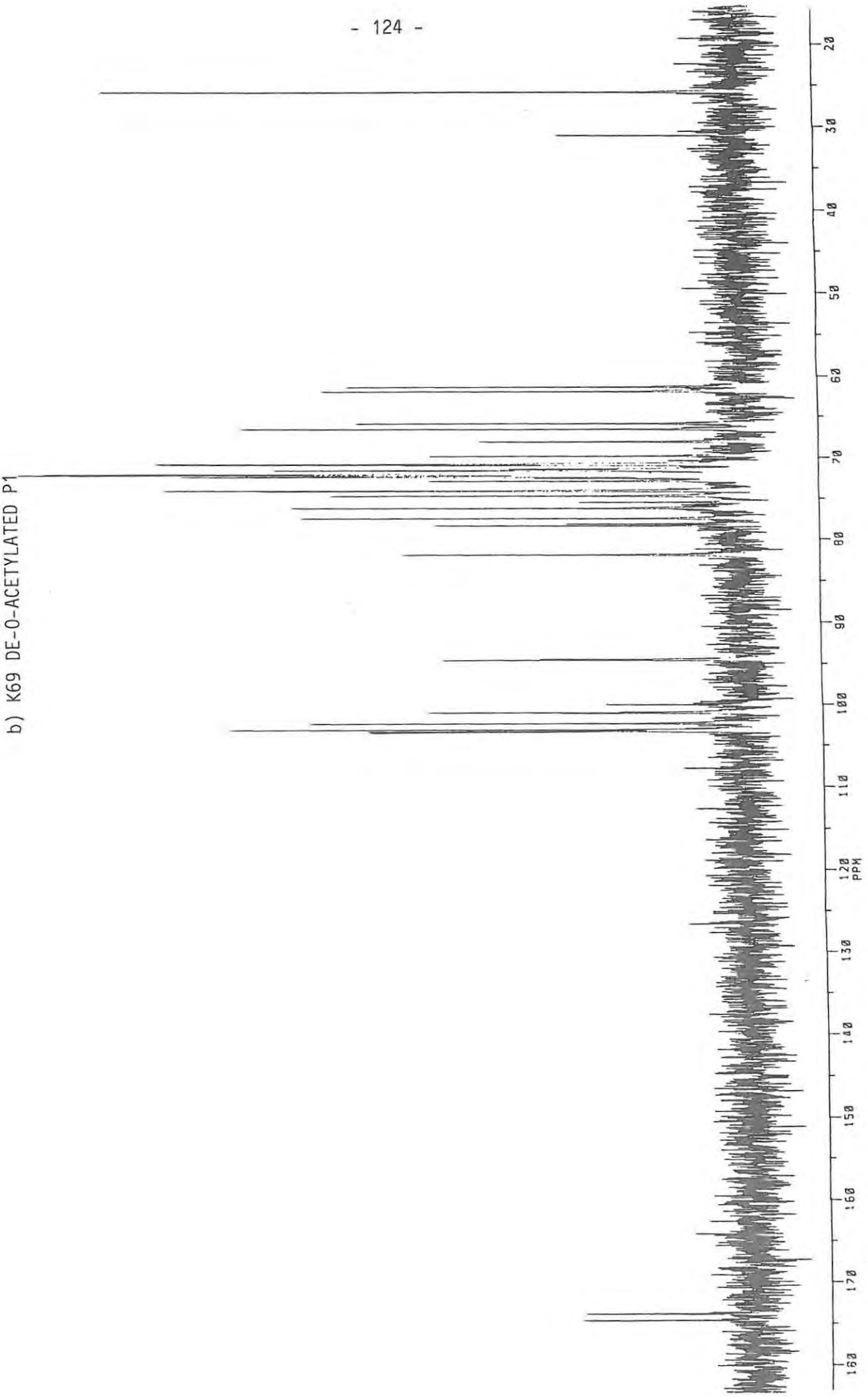


2. ^{13}C-N.m.r. spectra


a) K69 P1
*
*
170.0
160.0
150.0
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
PPM

OLIGO P1

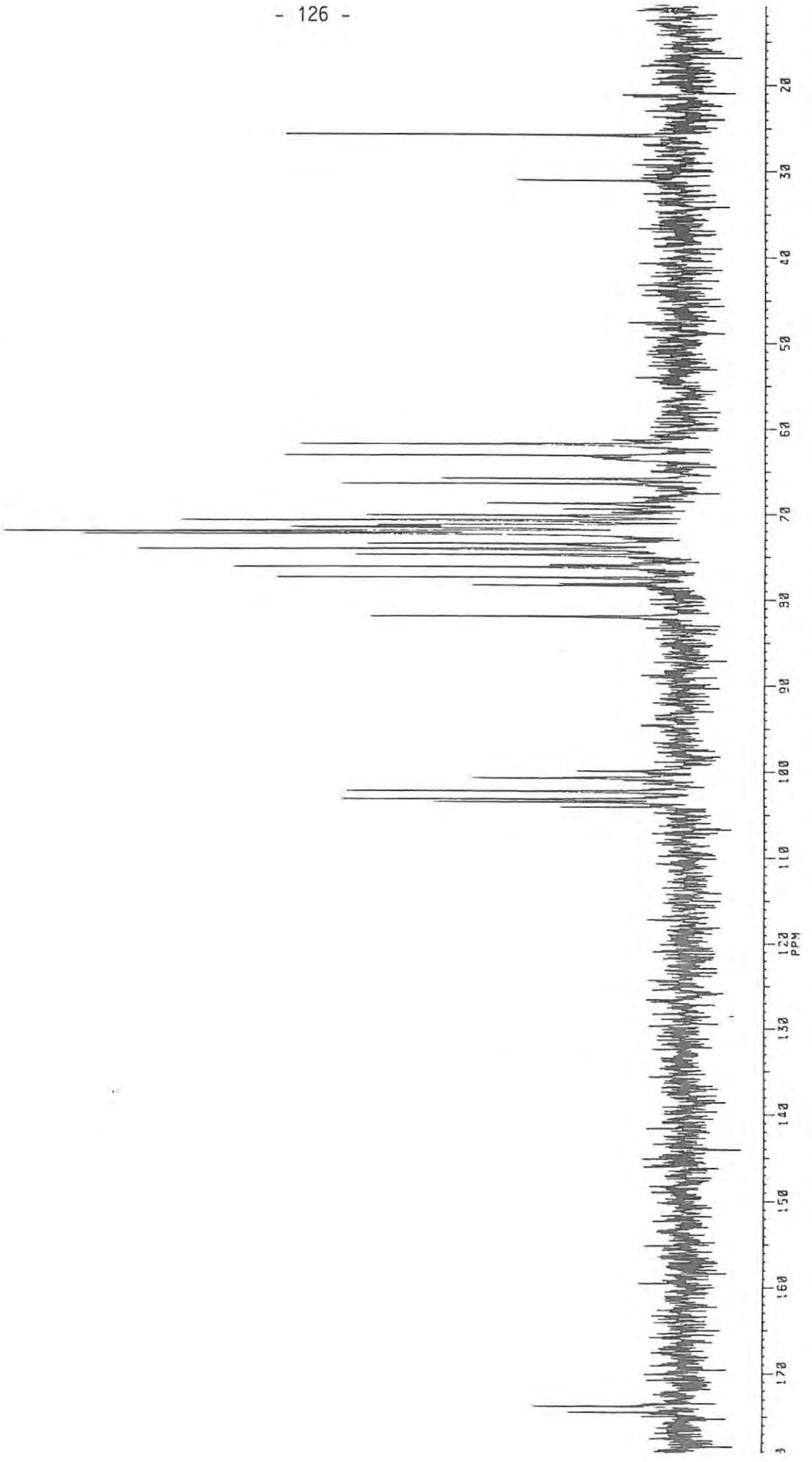
No.	PPM
1	175.01
2	174.65
3	174.18
4	147.99
5	128.19
6	103.25
7	103.06
8	102.30
9	101.16
10	101.01
11	100.94
12	100.37
13	94.73
14	94.57
15	94.44
16	81.88
17	81.76
18	78.71
19	78.50
20	78.31
21	78.07
22	77.42
23	76.18
24	75.45
25	74.71
26	74.11
27	74.04
28	72.92
29	72.87
30	72.55
31	72.41
32	72.21
33	72.12
34	72.03
35	71.63
36	71.56
37	71.39
38	71.26
39	70.97
40	70.91
41	70.79
42	69.81
43	69.27
44	68.00
45	66.60
46	65.76
47	63.95
48	61.89
49	61.33
50	25.79
51	21.03


b) K69 DE-O-ACETYLATED P1
20
30
40
50
60
70
80
90
100
110
120
PPM
130
140
150
160
170
180

K69 DE-O-ACETYLATED P1

#	[illegible]	[illegible]	[illegible]	[illegible]
1	[illegible]	21934.362	174.5144	5.740
2	4406	21360.023	173.8749	4.907
3	9764	12993.196	103.3179	11.391
4	[illegible]	12966.698	103.1071	15.429
5	9824	12870.465	103.3420	13.748
6	9708	12699.932	100.9856	9.559
7	9769	12576.921	100.0079	4.047
8	9302	11699.225	94.6190	8.937
9	9310	11332.506	94.4060	5.949
10	10063	10205.254	81.9447	10.344
11	10307	9954.899	78.3631	9.303
12	10321	9925.112	78.1283	5.264
13	10362	9742.429	77.4698	13.075
14	10438	9587.662	76.2382	13.795
15	10483	9495.209	75.5062	4.949
16	10530	9400.116	74.7468	12.749
17	10566	9326.911	74.1647	14.421
18	10570	9318.772	74.1000	17.316
19	10641	9174.191	72.9504	9.382
20	10663	9129.508	72.5950	4.123
21	10673	9109.109	72.4329	16.990
22	10677	9101.522	72.3725	14.930
23	10687	9080.852	72.2082	20.019
24	10690	9075.295	72.1640	23.459
25	10695	9064.905	72.0806	20.731
26	10720	9014.491	71.6805	11.117
27	10724	9006.275	71.6151	14.019
28	10734	8985.091	71.4467	4.641
29	10760	8931.871	71.0235	10.392
30	10767	8918.514	70.9173	17.249
31	10772	8908.321	70.8362	17.410
32	10833	8784.806	69.8541	9.488
33	10942	8562.164	68.0837	7.467
34	11036	8370.624	66.5606	15.077
35	11079	8283.057	65.8643	11.543
36	11322	7789.230	61.9376	12.329
37	11356	7719.754	61.3851	11.624
38	13230	3907.371	31.0702	5.189
39	13556	3244.151	25.7965	18.846

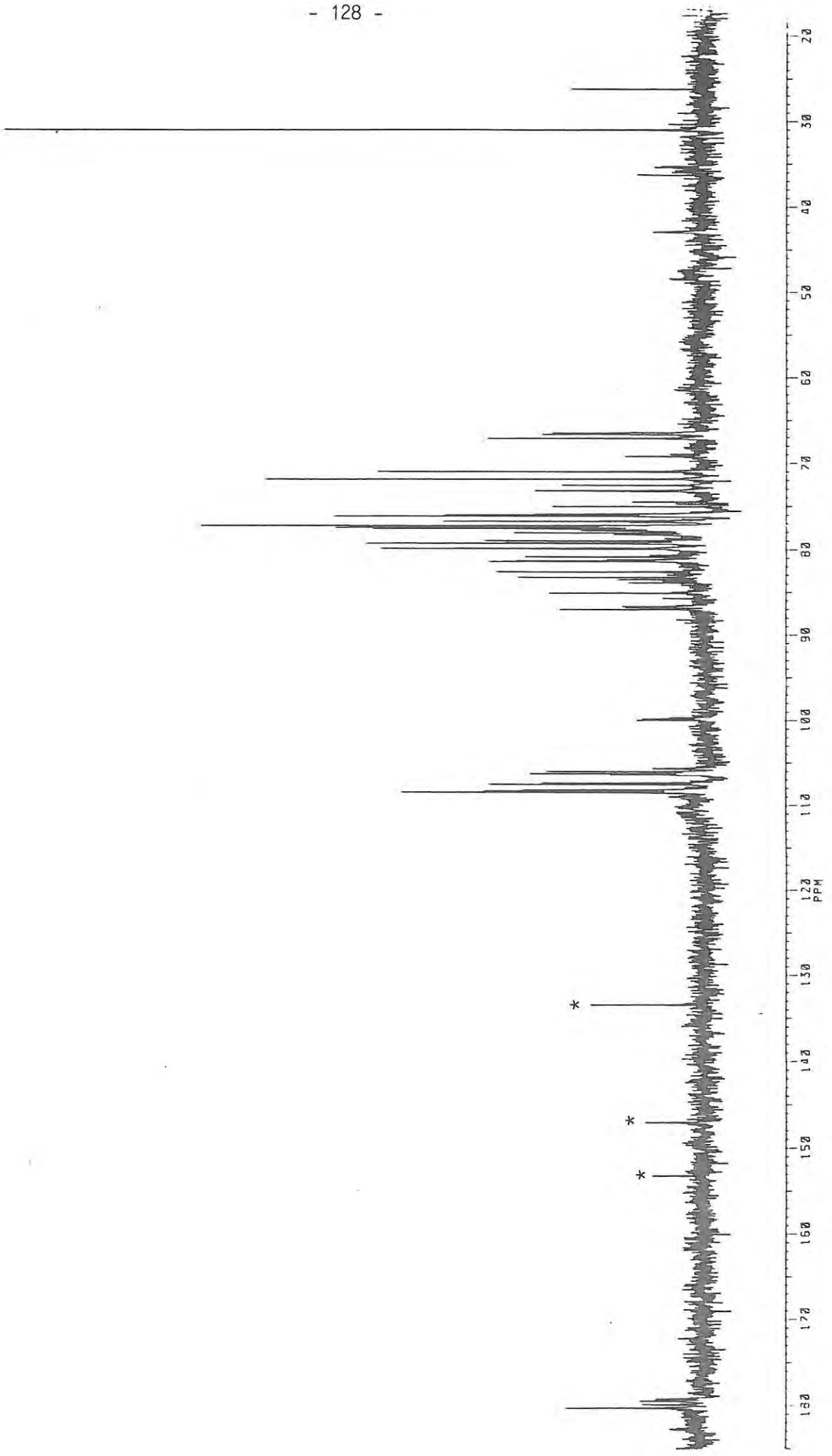
c) K69 P1-ALDITOL



K69 P1-ALDITOL

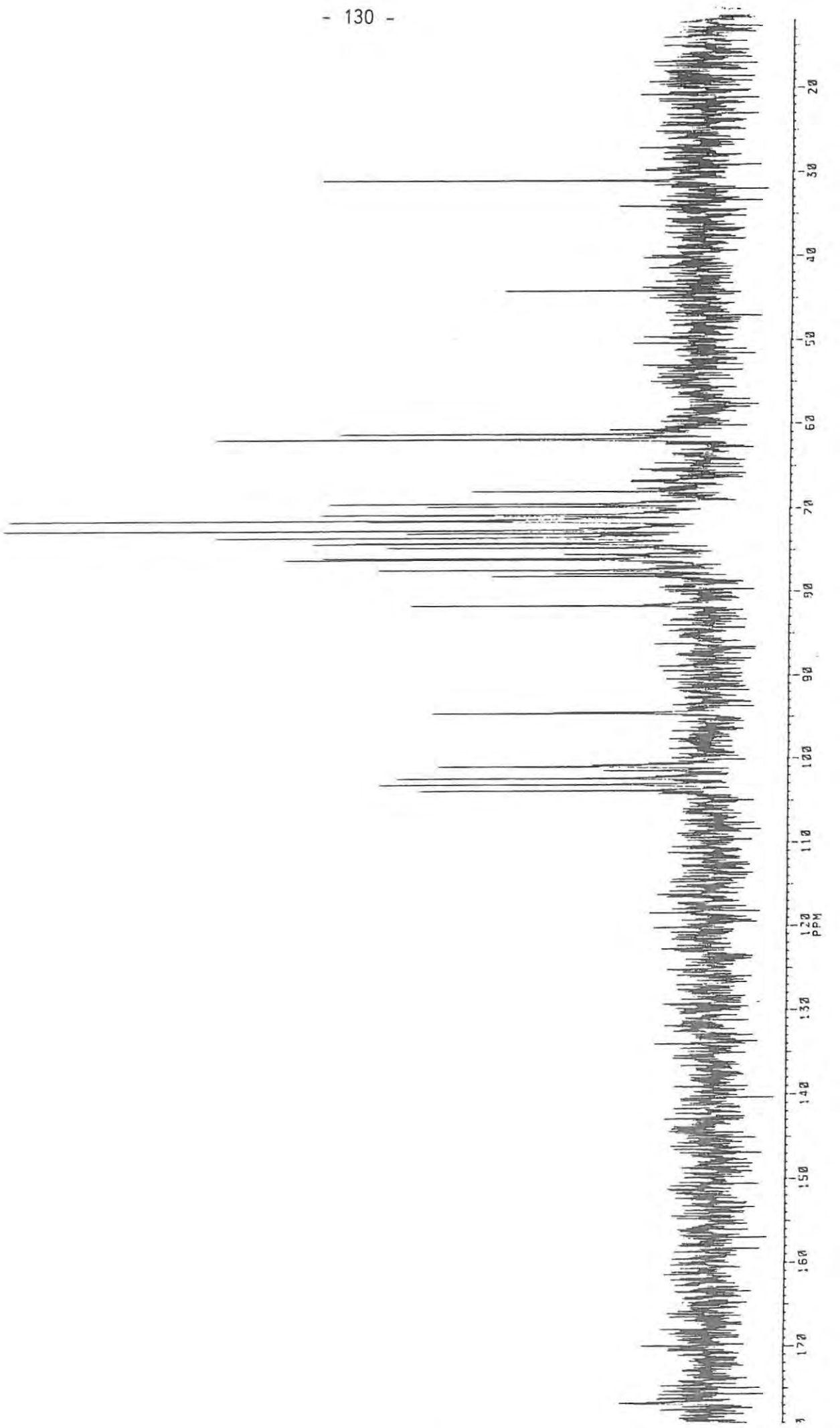
#	[illegible]	[illegible]	[illegible]	[illegible]
1	[illegible]	[illegible]	[illegible]	[illegible]
2	[illegible]	[illegible]	[illegible]	[illegible]
3	[illegible]	[illegible]	[illegible]	[illegible]
4	[illegible]	[illegible]	[illegible]	[illegible]
5	[illegible]	[illegible]	[illegible]	[illegible]
6	[illegible]	[illegible]	[illegible]	[illegible]
7	[illegible]	[illegible]	[illegible]	[illegible]
8	[illegible]	[illegible]	[illegible]	[illegible]
9	[illegible]	[illegible]	[illegible]	[illegible]
10	[illegible]	[illegible]	[illegible]	[illegible]
11	11305	5900.492	78.1944	[illegible]
12	11349	5846.493	77.4490	[illegible]
13	11425	5754.735	76.2532	[illegible]
14	11442	5734.035	[illegible]	[illegible]
15	11513	5647.348	[illegible]	[illegible]
16	11553	5598.229	[illegible]	[illegible]
17	11591	[illegible]	[illegible]	[illegible]
18	11660	5466.393	[illegible]	[illegible]
19	11678	[illegible]	[illegible]	[illegible]
20	11683	5439.483	[illegible]	[illegible]
21	[illegible]	[illegible]	[illegible]	[illegible]
22	11712	5403.888	[illegible]	[illegible]
23	11721	[illegible]	[illegible]	[illegible]
24	11726	[illegible]	[illegible]	[illegible]
25	11760	[illegible]	[illegible]	[illegible]
26	11795	5302.584	[illegible]	[illegible]
27	11843	[illegible]	[illegible]	[illegible]
28	11882	[illegible]	[illegible]	[illegible]
29	12026	[illegible]	[illegible]	[illegible]
30	12063	[illegible]	[illegible]	[illegible]
31	12230	[illegible]	[illegible]	[illegible]
32	12311	[illegible]	[illegible]	[illegible]
33	12345	[illegible]	[illegible]	[illegible]
34	14218	[illegible]	[illegible]	[illegible]
35	14544	[illegible]	[illegible]	[illegible]
36	15820	[illegible]	[illegible]	[illegible]

d) K69 P2



#	[illegible]	FREQUENC	PPM	INTENSIT
1	4220	22677.561	190.3258	3.943
2	4245	22626.731	179.9215	1.679
3	4273	22570.570	179.4749	1.746
4	8274	18498.376	147.0939	1.598
5	7117	16783.689	133.4592	3.140
6	8661	13642.452	108.4810	8.561
7	8671	13622.628	108.3233	6.341
8	8678	13607.009	108.1991	5.341
9	8717	13528.308	107.5733	6.047
10	8728	13506.957	107.4036	4.736
11	8798	13383.176	106.4193	2.727
12	8796	13367.888	106.2977	4.889
13	8811	13337.199	106.0537	4.420
14	9186	12574.632	99.9900	1.850
15	9195	12555.359	99.8367	1.723
16	9984	10951.592	87.0840	4.176
17	9998	10921.933	86.8482	2.148
18	10005	10907.721	86.7352	2.234
19	10103	10709.093	85.1557	4.380
20	10177	10557.947	83.9539	2.048
21	10200	10510.952	83.5802	2.384
22	10216	10478.311	83.3246	5.222
23	10255	10398.492	82.6859	6.172
24	10331	10244.059	81.4579	6.317
25	10337	10233.212	81.3716	5.399
26	10344	10217.842	81.2494	2.711
27	10364	10177.563	80.9291	5.035
28	10423	10056.841	79.9692	9.399
29	10461	9979.412	79.3535	9.706
30	10480	9941.490	79.0520	6.195
31	10526	9847.735	78.3064	2.472
32	10537	9825.583	78.1303	5.348
33	10553	9793.297	77.8736	3.404
34	10565	9768.214	77.6741	9.499
35	10569	9760.565	77.6133	6.123
36	10576	9745.888	77.4966	10.640
37	10578	9742.753	77.4717	10.067
38	10582	9734.467	77.4058	10.082
39	10589	9720.022	77.2909	14.409
40	10613	9670.813	76.8996	2.042
41	10619	9657.865	76.7967	7.652
42	10623	9650.849	76.7409	3.779
43	10657	9582.156	76.1946	10.756
44	10665	9564.983	76.0581	7.367
45	10672	9551.596	75.9516	4.821
46	10726	9441.348	75.0750	4.246
47	10759	9373.790	74.5378	1.953
48	10840	9210.202	73.2370	4.896
49	10879	9130.100	72.6000	3.978
50	10923	9040.058	71.8840	12.621
51	10977	8931.342	71.0195	9.610
52	11087	8706.327	69.2303	2.173
53	11215	8446.165	67.1616	6.082
54	11245	8384.936	66.6747	4.541
55	11254	8367.192	66.5336	4.243
56	13123	4564.174	36.2930	1.832
57	13446	3907.039	31.0677	20.219
58	13741	3307.207	26.2980	3.709

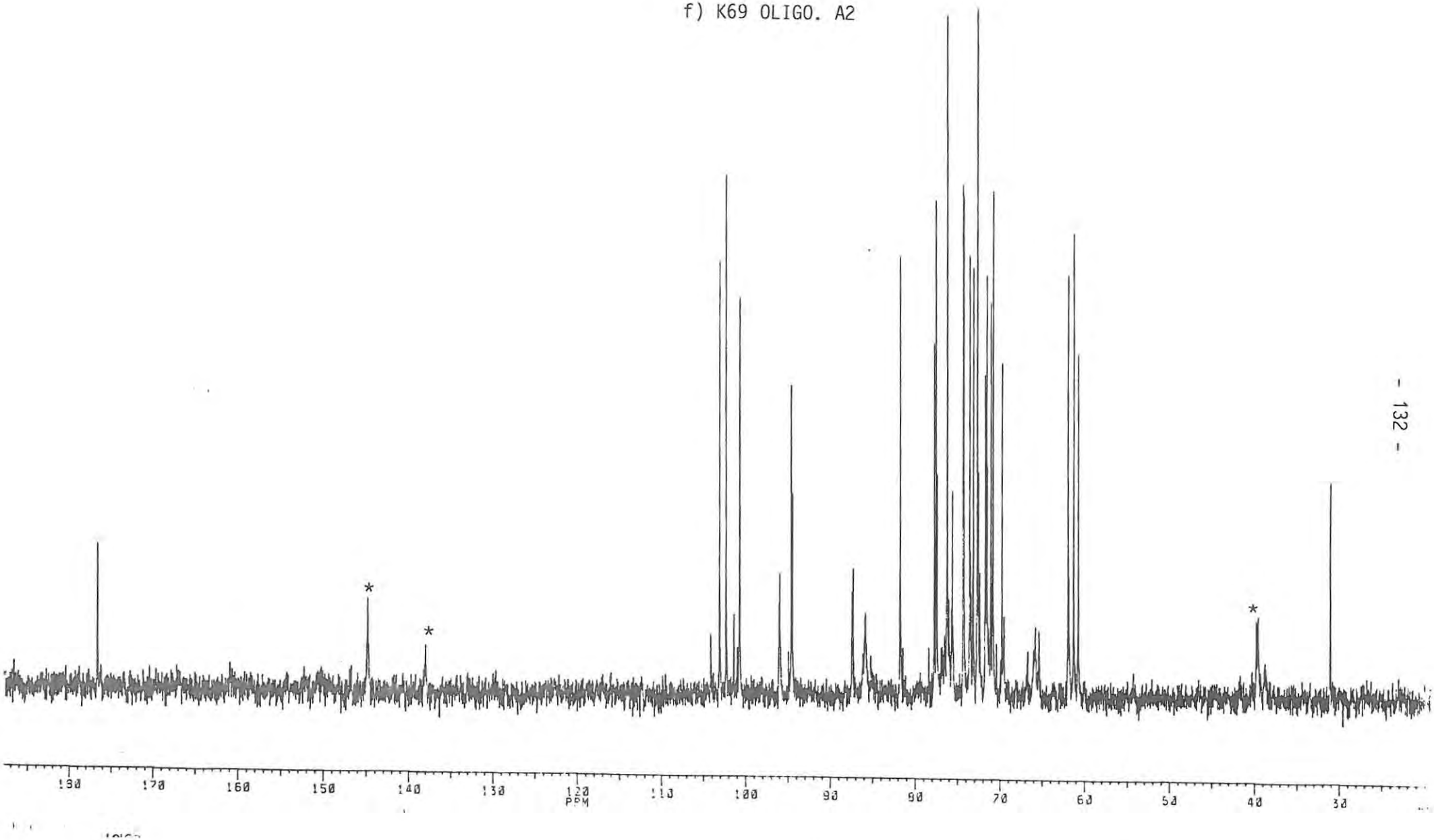
e) K69 OLIGO A1



K69 OLIGO. A1

#	CURSOR	FREQUENCY	PPM	INTENSITY
1	9719	7836.746	103.8410	8.264
2	9763	7782.701	103.1275	9.490
3	9807	7729.042	102.4138	9.064
4	9865	7658.380	101.4842	2.952
5	9896	7621.067	100.9931	7.843
6	10289	7141.200	94.6244	7.995
7	10297	7131.008	94.4896	4.427
8	11085	6169.258	81.7459	8.555
9	11303	5902.776	78.2149	6.372
10	11323	5878.670	77.8954	4.379
11	11349	5847.244	77.4790	9.600
12	11426	5752.649	76.2256	12.507
13	11438	5738.887	76.0432	11.625
14	11469	5701.072	75.5422	4.293
15	11515	5644.588	74.7937	9.354
16	11544	5608.692	74.3181	11.633
17	11553	5598.384	74.1815	10.423
18	11591	5551.800	73.5642	14.480
19	11625	5510.228	73.0134	8.727
20	11645	5485.522	72.6860	20.605
21	11709	5407.367	71.6504	9.931
22	11717	5397.662	71.5218	20.455
23	11752	5354.966	70.9561	8.819
24	11758	5347.475	70.8568	11.340
25	11818	5274.530	69.8903	8.128
26	11838	5250.302	69.5692	11.069
27	11928	5139.908	68.1065	6.905
28	12310	4674.311	61.9371	14.589
29	12348	4627.440	61.3160	10.490
30	12383	4585.407	60.7590	2.962
31	13408	3333.594	44.1719	5.900
32	14030	2574.426	34.1125	2.585
33	14218	2344.778	31.0695	11.263

f) K69 OLIGO. A2

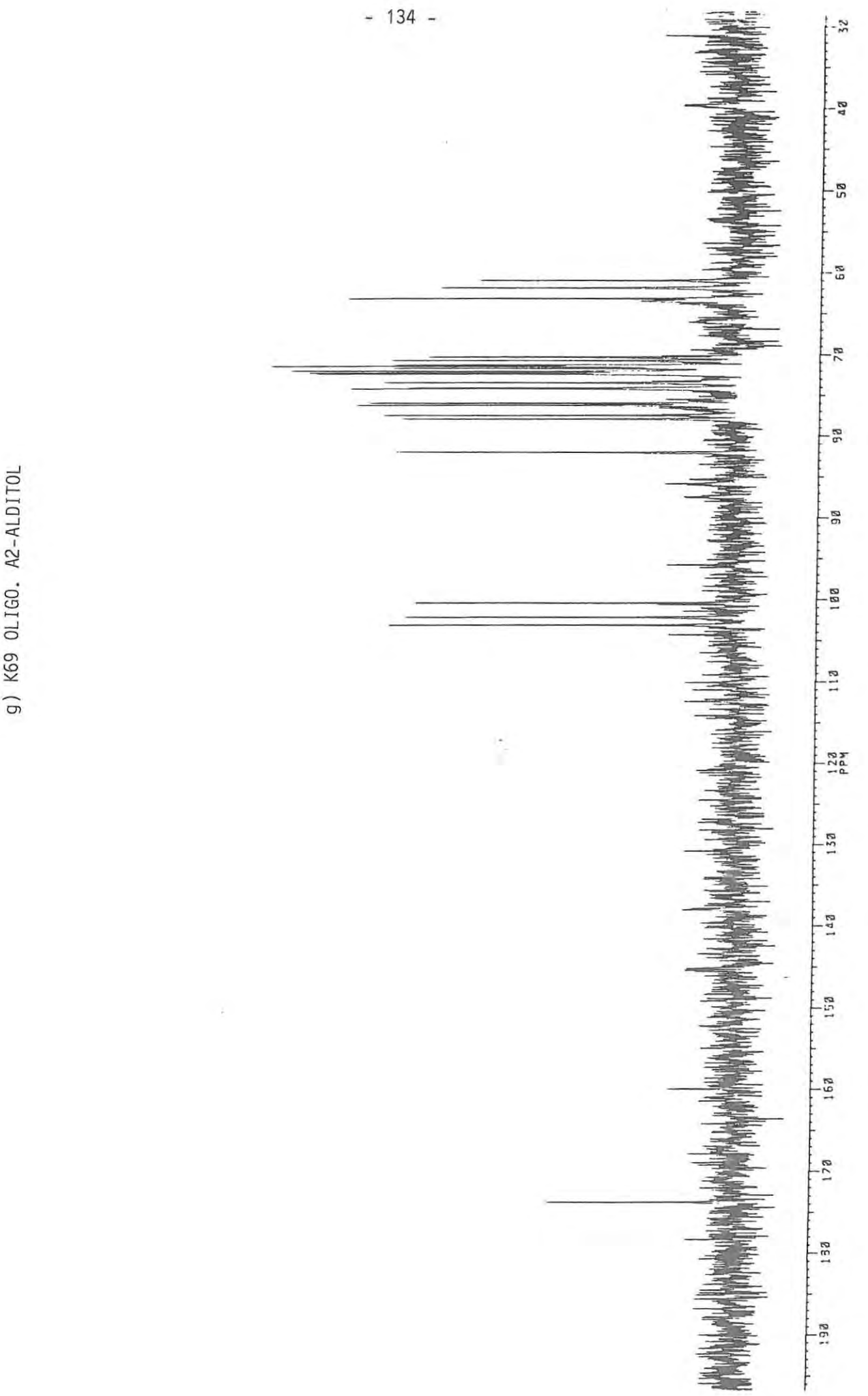


K69 OLIGO. A2

2	4233	22211.367	176.6180	4.037
3	6204	18201.508	144.7328	2.541
4	7621	17353.682	137.9912	1.176
5	8711	13100.743	104.1731	1.627
6	8776	12969.640	103.1306	12.854
7	8823	12873.511	102.3662	14.943
8	8880	12757.801	101.4461	2.178
9	8905	12705.921	101.0336	1.249
10	8923	12670.194	100.7495	11.452
11	9210	12085.481	96.1000	2.556
12	9215	12075.914	96.0240	3.351
13	9282	11940.189	94.9447	1.171
14	9301	11901.318	94.6356	9.000
15	9310	11882.330	94.4846	5.759
16	9744	10999.882	87.4677	2.872
17	9752	10984.195	87.3430	3.698
18	9843	10798.074	85.8630	2.256
19	9882	10718.873	85.2332	1.027
20	10096	10283.873	81.7742	12.815
21	10115	10243.912	81.4565	1.321
22	10306	9856.226	78.3737	1.298
23	10345	9776.997	77.7437	10.181
24	10358	9750.778	77.5352	14.480
25	10366	9734.329	77.4044	6.345
26	10402	9660.900	76.8205	1.332
27	10420	9624.593	76.5318	1.677
28	10438	9587.132	76.2339	20.140
29	10452	9559.624	76.0152	2.754
30	10472	9518.331	75.6869	2.451
31	10477	9508.117	75.6056	5.863
32	10558	9343.879	74.2997	14.903
33	10562	9335.019	74.2292	14.632
34	10605	9247.839	73.5360	12.721
35	10610	9238.192	73.4593	4.174
36	10614	9229.774	73.3923	2.914
37	10631	9194.601	73.1127	12.564
38	10659	9138.348	72.6653	19.969
39	10661	9134.254	72.6328	18.659
40	10668	9119.762	72.5176	6.395
41	10678	9099.244	72.3544	3.507
42	10716	9021.468	71.7360	9.594
43	10728	8998.435	71.5528	12.579
44	10732	8989.821	71.4843	6.614
45	10741	8970.774	71.3328	1.912
46	10758	8936.681	71.0617	11.346
47	10774	8904.297	70.8042	14.654
48	10798	8855.073	70.4128	1.432
49	10827	8796.403	69.9463	.968
50	10838	8774.312	69.7706	9.775
51	10854	8741.299	69.5081	2.227
52	11026	8390.924	66.7221	1.210
53	11083	8274.606	65.7971	1.958
54	11109	8222.612	65.3837	1.807
55	11321	7791.493	61.9556	12.255
56	11361	7709.803	61.3060	13.375
57	11394	7642.205	60.7685	10.029
58	12692	5001.135	39.7675	2.197
59	12705	4975.693	39.5652	2.293
60	12755	4873.975	38.7564	.946
61	13230	3907.359	31.0701	6.231
62	14380	1567.253	12.4623	1.868

3:

g) K69 OLIGO. A2-ALDITOL

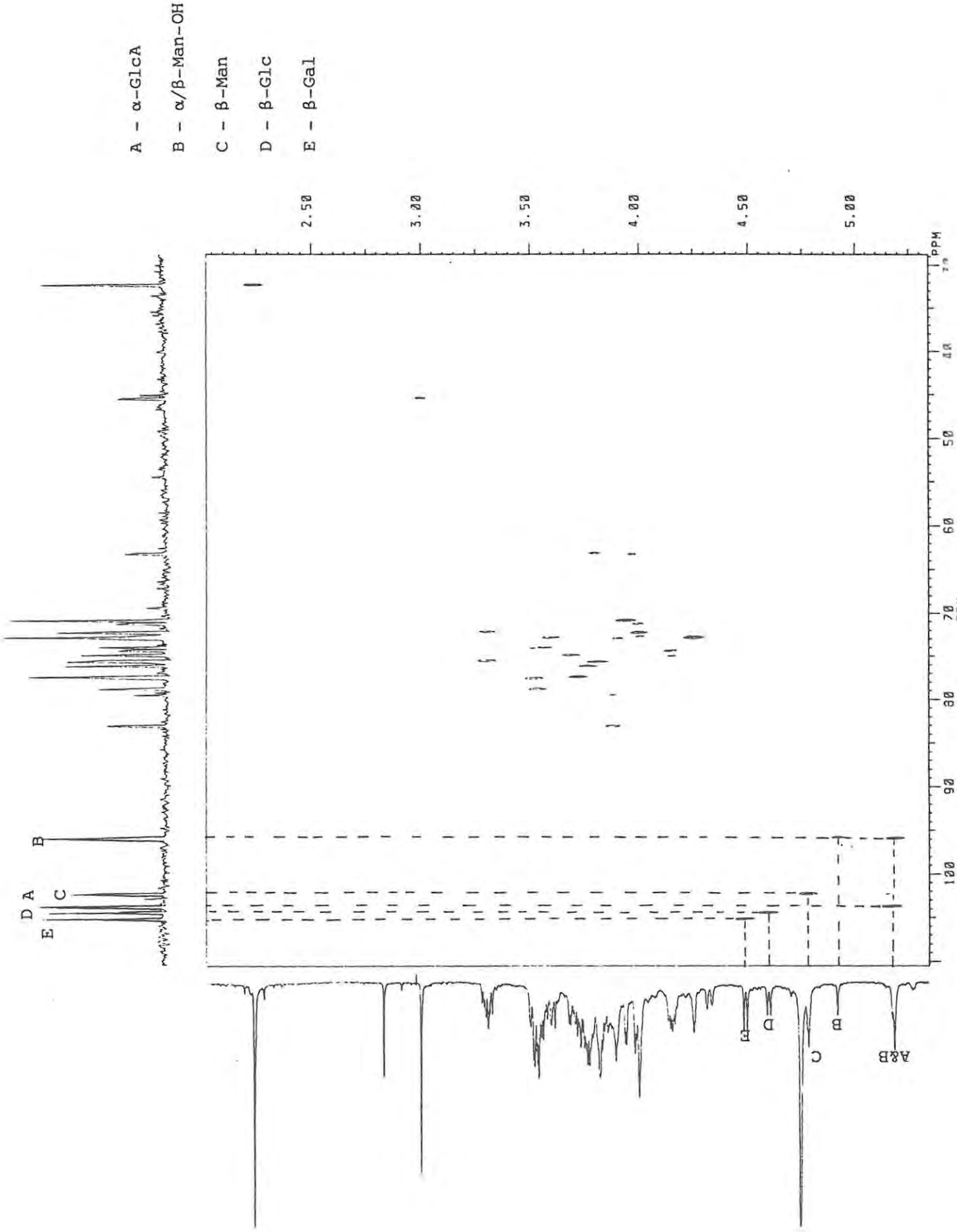


K69 OLIGO. A2-ALDITOL

#	CURSOR	FREQUENCY	PPM	INTENSITY
1	5397	13112.724	173.7504	5.433
2	9761	7785.089	103.1545	10.417
3	9819	7714.299	102.2185	10.077
4	9927	7583.457	100.4449	10.000
5	11067	6191.318	82.0382	10.214
6	11320	5882.556	77.9449	10.034
7	11347	5849.509	77.5080	10.570
8	11425	5754.094	74.2447	11.442
9	11441	5734.396	75.9937	11.169
10	11549	5603.096	74.2439	11.719
11	11556	5593.933	74.1224	10.025
12	11596	5545.581	73.4813	10.584
13	11661	5466.194	72.4299	12.450
14	11670	5455.190	72.2841	12.952
15	11683	5439.412	72.0750	13.396
16	11704	5413.394	71.7303	10.407
17	11719	5395.396	71.4918	10.010
18	11729	5382.991	71.3274	10.393
19	11762	5342.827	70.7952	10.370
20	11788	5311.350	70.3783	9.393
21	12231	4770.695	63.2147	11.915
22	12311	4672.655	61.9151	8.395
23	12368	4603.192	60.9947	7.719

3. HETCORR.

a) K69 OLIGO. A1


A - α-GlcA
B - α/β-Man-OH
C - β-Man
D - β-Glc
E - β-Gal
2.50
3.00
3.50
4.00
4.50
5.00
PPM
D A C B E
A&B C D