STRUCTURAL STUDIES ON SOME CAPSULAR ANTIGENS FROM *ESCHERICHIA COLI* AND *KLEBSIELLA*

THESIS

Submitted in Fulfilment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

by

ANDREW NIXON ANDERSON

School of Pharmaceutical Sciences

Rhodes University

Grahamstown, 6140

South Africa

December 1987

ACKNOWLEDGEMENTS

The author would like to express his sincere gratitude for the assistance rendered by the following people during the course of this doctoral study:

Professor H. Parolis for his supervision, encouragement, and support of the work in this thesis over the past four years; Dr L.A.S. Parolis for her assistance with the laboratory work performed for this thesis; Mr D.L. Morley for his valuable technical support; Mr I. Antonowitz, National Chemical Research Laboratory, Pretoria, for recording the nuclear magnetic resonance spectra; Professor A.M. Stephen, University of Cape Town, for the use of the Micromass mass spectrometer; Mr A.W. Sonemann, Rhodes University, for his assistance with mass spectrometric analyses; Professor S. Stirm, Max Planck Institute for Immunobiology, Freiburg-Zahringen, for the sample of *Klebsiella* Ø5; Drs I. and F. Ørskov, Statens Seruminstitut, Copenhagen, for cultures of the bacteria and the immunoelectrophoresis of *Klebsiella* K39 polysaccharide; Mr P.L. Hackland for the isolation of *Klebsiella* Ø69; Miss S.D. Mee for her contribution to the bacteriophage degradation of *Klebsiella* K30 polysaccharide; Ms H.J. Kew for the preparation of the manuscript; Dr B.D. Glass and Mr E. Meyer for proof-reading the manuscript; and Mrs E. Grant for the preparation of the graphical figures.

The author would also like to thank the Council for Scientific and Industrial Research, Pretoria for the financial support, and Rhodes University for the post-graduate bursary (1984).

TABLE OF CONTENTS

ABSTRACT

A review of the structural studies of bacterial capsular polysaccharides (K-antigens) from *Escherichia coli* and *Klebsiella*, and of the trends in modern chemical and instrumental techniques available for the analysis of carbohydrate material is presented. The structural elucidations of the capsular polysaccharides from *E. coli* K37 and K55, and *Klebsiella* K39 are reported with comments on the novelty and possible immunological significance of the structures. The usefulness of the bacteriophage degradation technique has been emphasized using the polysaccharides from *E. coli* K55, and *Klebsiella* K30 and K39 to demonstrate the scope of the reaction.

1. INTRODUCTION

1. INTRODUCTION

The use of extracellular bacterial polysaccharides is becoming more widespread as new applications are found within industrial and medical fields. These polysaccharides are beginning to replace the role of natural gums, such as tragacanth, pectin and agar, and synthetic polymers; xanthan gum, for example, is extensively used in the petroleum oil industry. The production of bacterial polysaccharides has several advantages over that of gums from other natural sources. The large-scale growth of bacteria is industrially a simple and convenient process which may be performed anywhere geographically, a greater control over the growth conditions for the bacteria is possible, and the end product will have more uniform batch-to-batch properties. In addition, extracellular bacterial polysaccharides provide a wide variety of polymers with novel physicochemical characteristics and hence potential uses.

A systematic programme to elucidate the primary structures of the extracellular polysaccharides produced by encapsulated strains of *Escherichia coli* and *Klebsiella* has been in progress over the last two decades. These polysaccharides form part of the antigenic character (the K-antigen) of the pathogenic or potentially pathogenic bacteria within these species and thus their structural elucidation will have obvious benefits to the field of immunology and the pharmaceutical industry. It has already been shown that an effective vaccine can be produced by combining purified K-antigen polysaccharide with a protein carrier. The elucidation of the structure of K-antigens will also provide insight into the response of mammalian immune systems to antigenic stimuli.

Of the above bacterial species, *Klebsiella* is more pathogenic and tends to produce copious amounts of extracellular polysaccharide material, and has thus been more extensively studied than *E. coli*. This thesis represents the transition period between the completion of the *Klebsiella* K-antigen structural studies and the beginning of the studies on *E. coli*, with the structures of *Klebsiella* K39, and *E. coli* K37 and K55 polysaccharides being reported. The application of enzymatic degradation processes using bacteriophages for the production of oligosaccharides has been shown to be a viable and convenient technique which may be applicable elsewhere. Modern instrumental techniques for the analysis of oligosaccharides, for

example high-performance liquid chromatography, mass spectrometry, and nuclear magnetic resonance spectroscopy, are discussed with comments on their potential future applications to carbohydrate structural analyses.

2. THE ENTEROBACTERIAL CAPSULE

2. THE ENTEROBACTERIAL CAPSULE

2.1 ANATOMY AND FUNCTION OF THE CAPSULE

The Enterobacteriaceae are a family of Gram-negative, non-sporeforming rods which may be motile, *e.g. Escherichia coli*, or non-motile, *e.g. Klebsiella*. The anatomy of the Gram-negative bacterium[1] (Fig. 1) has been established by electron microscopy which shows the cytoplasm surrounded by a cytoplasmic and an outer membrane. These membranes are separated by a peptidoglycan layer consisting of linear glycan strands, alternating ß-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid units, cross-linked with tetrapeptide chains. This peptidoglycan forms a rigid network which is responsible for the shape and integrity of the cell. Specific somatic antigens, for example O-antigens, are attached to the outer membrane and in some genera a discrete capsule or an extracellular slime is produced around the bacterium. The capsule and slime are composed of acidic polysaccharides and form a hydrophilic, mucilagenous envelope covering the bacterial surface. It is this capsular polysaccharide that has been designated as the K-antigen.

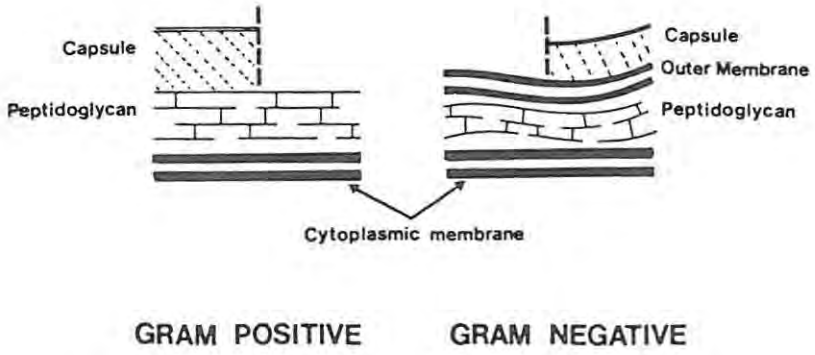


Fig. 1. The cell surface of Gram-positive and Gram-negative bacteria.[2]

The function of the bacterial capsule has not as yet been fully clarified. Loss of capsular production may result from repeated propagation on synthetic growth media, showing that the capsule is not necessary for *in vitro* survival. The capsule may provide survival advantages in feral microbial life by, for example, cementing freshwater bacteria together or

facilitating adhesion of marine bacteria to solid surfaces.[3] The virulence of pathogenic bacteria is related to some extent to the amount of capsule produced however, some non-pathogenic bacteria are encapsulated. The capsule, therefore, is not just a physical, protective barrier; the chemical nature appears to have some effect on the pathogenicity of the bacterium. This is exemplified within the *E. coli* species where serotypes K1, K2, K3, K5, K12, and K13 are the most common strains causing urinary tract infections.[4] In pathogenic bacteria, the major role of the capsule appears to be that of defending the microbe against the host's immune system. There are two aspects to this defence; firstly the capsule masks the somatic antigens, effectively suppressing the host's immune response to these antigens, and secondly provides a surface that has a low immunoactivating potential. Full advantage of this latter aspect is taken by *E. coli* K5 where, by molecular mimicry, the capsule is composed of a disaccharide repeating unit closely resembling desulpho-heparin, a precursor of the blood anticoagulant heparin.[2] The body will recognize the K5 antigen as "self" and thus not activate its immune system.

Within Nature there are a wide variety of capsular materials produced by bacteria, for example about eighty serologically different K-antigens have been identified within the *Klebsiella* genus.[5,6] A systematic study of these various polysaccharides will provide insight into the role of the capsule, the response of the host's immune system and the virulence of the bacterium, and facilitate the production of synthetic vaccines for prophylactic therapy.

2.2 THE *KLEBSIELLA* CAPSULAR ANTIGENS

The *Klebsiella* genus are opportunistic pathogens causing diseases such as cystitis, septicaemia, and pneumonia in man. Two major antigens, O- and K-serotypes, have been implicated in these disease states. Twelve serologically different O-group antigens have been recognized[7] however, structural analysis indicates that there are only nine chemically different O-specific polysaccharides.[8] Eighty different polysaccharides had been qualitatively analyzed within the K-antigen series by 1971[9,10] and since these analyses three additional antigens, K81,[11] K82,[5,12] and K83,[6] have been recognized. Ørskov[5] has proposed that five of the

previously assigned antigens, K73 (a strain of *Enterobacter aerogenes*), K75, K76, K77, and K78, be deleted however, according to Heidelberger *et al.*,[13] K75 and K76 are chemically distinct capsular polysaccharides.

The structures of fifty-four K-antigens reported up to 1983 have been reviewed by Kenne and Lindberg,[14] and since this review about nine structures remain unreported. The K-antigen is made up of discrete repeating units comprising of between three[15,16] and nine[17] monosaccharide residues, the composition and diversity of which have been reviewed by Parolis.[18] Of the reported structures, K30 (ref. 19) and K33 (ref. 20) are serologically distinct and contain a 3,4-(1-carboxyethylidene)-galactopyranosyl residue but vary chemically only in the degree of *O*-acetyl substitution present (about 30% of the K30 repeating units are substituted whereas all of those in K33 contain an acetate group). K69 (ref. 21) is similar to K30/K33 except that in this case the pyruvate ketal is linked to the 4 and 6 positions of the pendant galactopyranosyl residue, again with about 30% *O*-acetyl substitution of the repeating units. Some reported structures are similar to those within the *E. coli* species, for example *Klebsiella* K20,[22] K57,[23] and K63 (ref. 24) are identical to *E. coli* K30,[25] K36,[26] and K42,[27] respectively. *Klebsiella* K16 (ref. 28) differs from *E. coli* K28 (ref. 29) only in that 70% of the latter's repeating units are *O*-acetylated whereas the former antigen contains no *O*-acetyl groups. The site of *O*-acetylation varies between the structures for *Klebsiella* K5 and *E. coli* K55, otherwise these antigens are the same (see Section 5.3).

Immunochemical studies into the cross-reactions of the capsular antigens from *Klebsiella* have been carried out by Heidelberger and co-workers.[13,30-33] The results of these studies have shown the serological importance of such functional groups as pyruvate and acetate, and have been able to confirm and to predict the presence of certain immunodominant sites within polysaccharide structures.

2.3 THE *ESCHERICHIA COLI* CAPSULAR ANTIGENS

E. coli are saprophytic commensals making up the predominant aerobic constituent of normal colonic flora. Under abnormal conditions, such as debilitation, immunosuppression, or

trauma, strains of *E. coli* can cause disease, for example urinary tract infections, neonatal meningitis, and diarrhoea. Classically the serology of *E. coli* comprises H, O, and K antigens.[34] The H-antigens are the flagellate antigens, sixty of which have been recognized. These H-antigens are thermolabile proteins and they may or may not be present in the bacterial strain. The O-antigens, present in all strains, are thermostable lipopolysaccharides, one hundred and sixty-seven distinct antigens having been recorded. These two antigens allow *E. coli* to be categorized into O:H serotypes.

Many strains of *E. coli* have capsular or K-antigens. Ørskov *et al.*[4] reported seventy-six different K-antigens, two of which, K88 and K99, were proteins and the remainder, K1-57, K62, K74, K82-84, K87, K92-98, and K100-103, acidic polysaccharides composed of distinct repeating units. The missing K-numbers have not been demonstrated as special K-antigens independent of the O-antigens. Prior to the above report, the K-antigens had been subtyped as A, B, or L depending upon their bacterial agglutination reaction. However, because of difficulties in defining these subtypes, Ørskov *et al.*[4] proposed the discontinuation of this nomenclature and the restriction of K-antigen classification to polysaccharide K-antigens and protein K-antigens. Two broad categories of polysaccharide K-antigens, based upon genetic considerations, were noted;[35] those found in combination with the O8, O9, O20, and O101 antigens and, due to association with core-lipid A, could be considered as K-lipopolysaccharides, and those polysaccharide K-antigens with O-groups other than O8, O9, O20, and O101. Formal definition of these subgroups was not proposed. Phospholipid substitution of the K-antigens tends to cause micellar aggregation within isolated material.[36] The biological role of the lipid is unclear, with suggestions that it serves to maintain the polysaccharide in close association with the outer bacterial membrane or that the polysaccharides are synthesized on a phospholipid carrier.[37] Since the report by Ørskov *et al.*,[4] the two proteinaceous antigens have been reclassified as fimbrial antigens, F_2 and F_3,[34] and two of the polysaccharides, K21 and K82, deleted from the antigenic test strains.[38,39]

Of the established capsular antigens, the structures of about one half have been reported. Reviews by Kenne and Lindberg,[14] and Stanley[26] cover the reports up until 1986, and in

addition structures for K9,[40] K34,[41] K36,[26] K37 (this thesis), K43,[42] K44,[43] K51,[44] K55 (this thesis), and K95 (ref. 45) have been published. Reviews of these structures[14,18,26] have illustrated the variety of monosaccharide residues and non-carbohydrate components found within the K-antigens, the diversity being greater than that in the *Klebsiella* genus. Novel aspects, within the *E. coli* species, of the above unreviewed structures are that K9 contains a 4-linked neuraminic acid moiety, the K43 antigen does not have an acidic functional group, K44 contains both *N*-acetylgalactosamine and *N*-acetylglucosamine residues, K51 is a polymer of *O*-acetylated *N*-acetylglucosamine phosphate, and that the K95 antigen contains a furanosidic 3-deoxy-D-*manno*-2-octulosonic acid (KDO) residue.

Similar structures for various antigens have been reported, for example *E. coli* K7 and K56 have identical structures.[46] Serotypes K13, K20, and K23 consist of the same basic repeating unit; K23 is non-acetylated, and K13 and K23 are partially acetylated at position 4 of the KDO residue and position 5 of the ribosyl residue, respectively.[47] The *E. coli* K100 antigen is structurally related to and cross-reacts with the *Haemophilus influenzae* type b capsular antigen.[48] Similarities between *E. coli* and *Klebsiella* antigens have already been discussed (see Section 2.2).

3. CHEMICAL ANALYSIS OF POLYSACCHARIDES

3. CHEMICAL ANALYSIS OF POLYSACCHARIDES

With improvements in technology, emphasis on the chemical analysis of polysaccharides has shifted towards the production of oligosaccharides that are analyzed instrumentally by, for example, mass spectrometry or nuclear magnetic resonance spectroscopy. This shift has encouraged the development of more specific and controlled depolymerization techniques in which the least information about the polysaccharide is lost during the experiment (see Section 3.2). There still remains, however, a need for the chemical analysis of monosaccharide residues, usually by derivatization, to support the oligosaccharide analyses. The derivatives used have been extensively reviewed and thus only a brief commentary will be presented here, more attention being given to specific degradation procedures in current use.

3.1 STANDARD CHEMICAL ANALYSES

3.1.1 Monosaccharide Composition

The initial step in the structural elucidation of carbohydrate material involves the determination of the monosaccharide composition, *i.e.* to obtain the Total Sugar Ratio (TSR). Different classes of sugars undergo specific colour reactions based upon their ability to form furfural derivatives which complex with other sugars or with organic substances.[49] Although these reactions are used in the analyses of carbohydrates, for example the phenol-sulfuric acid reaction,[50] they are not specific enough for the routine quantification of monosaccharide residues. Chromatographic separation, after hydrolysis of the poly- or oligo-saccharide, has become the method of choice for rapid and facile analyses. Using paper chromatography (p.c.), Partridge[51,52] showed that mixtures of stereoisomeric monosaccharides could be separated by judicious choice of solvent and conditions. Since then p.c. has been used to separate numerous sugars including monosaccharides, oligosaccharides, alditols, and methyl glycosides, with various solvents and developing techniques for the chromatograms having been established.[53] The time needed for and the inaccuracies involved in quantitative use of p.c. have restricted this technique to qualitative analyses and preparative separations of sugars.

The introduction of gas-liquid chromatography (g.l.c.) in 1958[54] revolutionized the analysis of sugars, allowing the rapid, accurate quantification of small amounts of material. The high resolving power of g.l.c. has the disadvantage that the chromatographed sample must conform to certain inherent requirements;[55] the material must be suitably volatile and stable at the operating temperature of the gas chromatograph and must be separated by but not adsorbed onto the column or packing material. Sugars are not volatile enough to be analyzed directly and thus must be quantitatively derivatized prior to analysis. These derivatives have been reviewed by Bishop,[56] Dutton,[57,58] Drozd,[55] and Laker.[59]

Derivatives of methyl glycosides, such as acetates, trifluoroacetates, and methyl and trimethylsilyl ethers, form up to four derivatives per monosaccharide residue due to anomers in the pyranose and furanose forms. The resultant multiple peaks in the chromatogram cause a reduction in the resolution and sensitivity of the analysis, and inaccuracies in quantitative measurements. To overcome these difficulties, acyclic derivatives giving single peaks were investigated. Gunner and co-workers[60,61] were able to separate a wide range of alditol acetates but were unable to resolve the glucitol and galactitol derivatives. This problem was solved by Swardeker *et al.*,[62] who used ECNSS-M as the stationary phase and achieved good separation of the common alditol acetates. Since this improvement, analysis using alditol acetates has become an established technique, with procedural refinements being proposed by Blakeney *et al.*[63] and Dasgupta *et al.*[64] The trimethylsilyl ethers of alditols, introduced by Sweeley and co-workers,[65,66] although easily formed, could not be adequately separated and were susceptible to hydrolysis and thus have been superseded in use by the acetates. Other derivatives of alditols, for example trifluoroacetates,[67] have been investigated.

One drawback to the use of alditol derivatives is that different sugars, for example arabinose and lyxose, may give rise to the same alditol when reduced. To circumvent this problem, derivatives that label C1 were developed. Nitriles, formed *via* the dehydration of oximes, have proved to be very versatile derivatives. Lance and Jones[68] introduced acetylated nitriles in their study on methyl ethers of D-xylose and the use of these derivatives was extended by Dmitriev *et al.*[69] and Varma and co-workers[70,71] to the analysis of

polysaccharides. The peracetylated aldononitriles (PAAN's) formed by this derivatization technique are easily separated by g.l.c., identify the position of the aldehyde group in the original glycose, and give readily interpretable mass spectra.[72] Chen and McGinnis[73] reported the preparation of PAAN's using 1-methylimidazole as a catalyst and solvent, and established a rapid and convenient method for derivatization.[74] It has been shown that by-products are formed in significant amounts during the preparation of the PAAN derivatives of glucose and galactose, casting doubts upon their quantitative use[75] however, it is generally accepted that the analytical data from these derivatives are representative of the carbohydrate material analyzed. Morrison[76] made use of both PAAN and alditol acetate derivatives to determine the degree of polymerization of oligosaccharides. After initial reduction of the saccharide to give the oligosaccharide alditol, derivatization *via* the PAAN's procedure produces an alditol acetate from the original reducing end sugar and PAAN's from the other sugar residues, these derivatives being readily distinguished by g.l.c.

Other derivatives for the g.l.c. analysis of sugars have been studied, for example pertrimethylsilylated aldononitriles,[77] pertrimethylsilylated and peracetylated derivatives of methyloximes,[78,79] of deoxy(methoxyamino)alditols,[80] and of diethyl dithioacetals,[81,82] and *n*-butyl boronates.[83] These compounds have been developed for classes of sugars where alditol acetates and PAAN's have proved inadequate. Uronic acid residues are generally analyzed after reduction to the neutral hexose, while ketoses and amino sugars are suitably quantified using the peracetylated oximes (PAKO's)[84] and methyloximes,[85,86] respectively.

High-performance liquid chromatography (h.p.l.c.) has become an attractive new technique for analyzing sugar residues, offering the advantages of rapid quantitative analysis combined with minimum sample preparation[87] and, depending upon the detection system used, derivatization of the sugars can be avoided (see Section 4.1.2). Reports have been published concerning the analysis of neutral monosaccharides,[88,89] uronic acids,[90] and amino sugars.[91] A further advantage of h.p.l.c. is that it may be extended to the analysis of oligosaccharides.

3.1.2 Determination of the Absolute Configuration

The assignment of the D or L configuration to sugar residues can be achieved by polarimetry,[92] circular dichroism,[93] or enzymatic methods,[94,95] however g.l.c. provides the most convenient means for this determination. Lochmüller and Souter[96] reviewed the theory and conceptual models for the resolution of enantiomers by g.l.c. Applying these concepts, racemic mixtures of sugars may be resolved either by converting the enantiomers into diastereomers using a chiral reagent, with separation on a non-chiral stationary phase, or by separation using a chiral stationary phase, the former method being the more suitable for routine analyses. Chiral alcohols, for example (-)-2-butanol[97,98] and (+)-2-octanol,[99] have been used to produce diastereomeric glycosides, each monosaccharide forming up to four derivatives. This method has similar disadvantages to the cyclic derivatives discussed earlier (Section 3.1.1) and thus acyclic derivatives such as dithioacetals,[100] (-)-menthyloximes,[101] and (-)-bornyloximes,[102] where each enantiomer produces two peaks, have been investigated. The separation of isomeric mixtures of trifluoroacetylated sugars or their methyl glycosides, achieved by Kŏnig and co-workers[103,104] on a chiral stationary phase, has the disadvantages of cost and multiple peak analysis. H.p.l.c. has also been applied to the resolution of enantiomers.[105]

3.1.3 Methylation Analysis

Methylation analysis[106] is the most widely used chemical technique for the determination of linkage positions within carbohydrate material. The technique involves the etherification (methylation) of all free hydroxyl groups in the sample, followed by hydrolysis and analysis of the released methylated monosaccharides in which the positions of hydroxylation represent the linkage sites in the original material. The methylated monosaccharides are conveniently analyzed as the alditol acetates using g.l.c. and gas-liquid chromatography-mass spectrometry (g.l.c.-m.s.)[107] (see Section 4.2) although other derivatives may be used, for example acetylated methyl glycosides.[108] Complete methylation of the sample is essential for a meaningful analysis, the extent of etherification being monitored by methoxyl (Zeisel) analysis, by infrared

spectroscopy, or by using triphenylmethane as an indicator.[109] The results obtained by methylation analysis will also give information concerning the nature of branch-points and end-groups within the molecule and the number of residues in naturally occurring repeating units.

Methylation usually involves alkoxide formation under basic conditions with subsequent nucleophilic attack on the alkylating agent. Since Purdie and Irvine[110] first successfully permethylated α-methylglucoside in 1903, numerous methylation reactions have been proposed, with progress in the technique having been well reviewed.[111,112] The most significant recent development was the introduction by Hakomori in 1964 of the methylsulfinyl anion (sodium dimsyl) as the alkoxylating base, the reaction being performed under anhydrous conditions in dimethylsulfoxide (DMSO) with methyl iodide as the alkylating agent.[113] This method generally achieves complete methylation in one step and enables small quantities of material (0.2-2 mg) to be analyzed however, solubility in DMSO is a prerequisite. Improvements to the Hakomori reaction have entailed the use of dimsyl salts that are more easily and safely prepared and which give rise to fewer peaks of non-carbohydrate origin during g.l.c. analysis, for example the potassium[114,115] and lithium[116,117] salts, and the use of 1,1,3,3-tetramethylurea to increase solubility in DMSO by relaxing hydrogen bonding.[118] Procedural improvements have been proposed by Waeghe *et al.*,[119] who analyzed microgram amounts of carbohydrate using reversed-phase chromatography to purify the permethylated samples, and by Harris *et al.*,[120] who conducted a "one-pot" reaction with optimization of the reaction steps.

Repeated Hakomori methylation may cause ß-elimination reactions in susceptible uronic esters[121] and has been reported to produce *C*-methylation at *N*-methylacetamido groups.[122] Ciucanu and Kerek,[123] in their study on the role of the dimsyl anion during methylation, concluded that hydroxyl or hydride ions were more important bases than dimsyl and therefore proposed the use of a powdered hydroxide in DMSO and methyl iodide, claiming that this would produce a cleaner, more rapid reaction and give higher yields. This method has been applied by Larson *et al.*[124] to the analysis of glycosphingolipids and, with modifications, by Isogai *et al.*[125] to the analysis of cell-wall polysaccharides.

Under alkaline methylating conditions, base-labile substituents, for example acetyl groups, may migrate or be cleaved from carbohydrate material. To overcome this problem, Gros and co-workers[126,127] used diazomethane in dichloromethane with boron trifluoride etherate as the catalyst for the methylation of acetylated monosaccharides. The acetyl groups were stable under these acidic conditions and the methylation of primary and secondary hydroxyl groups was achieved however, the reaction is limited due to the nature of the solvent and the presence of the Lewis acid. The use of methyl trifluoromethanesulfonate (methyl triflate) in dichloromethane with a proton scavenger (a weak, sterically hindered base) was proposed as an alternative route to achieve methylation where base-labile groups are present,[128] the technique being improved by the use of trimethyl phosphate as the solvent.[129] The position of *O*-acylation may also be determined by the procedure proposed by de Belder and Norrman[130] whereby the free hydroxyl groups in a sample are acetalated using methyl vinyl ether and an acid catalyst; subsequent methylation under basic conditions and acid hydrolysis produces a mixture of sugars and methylated sugars in which methoxyl groups mark the positions of original *O*-acylation.

The established techniques for methylation analysis have the disadvantage of subjecting the sample to prolonged acid hydrolysis with concomitant degradation and of being unable to distinguish between, for example, 4-linked aldopyranosyl and 5-linked aldofuranosyl residues. The publication in 1982 by Rolf and Gray of the reductive-cleavage reaction for glycosides[131] introduced a new concept in methylation analyses where the regiospecific, ionic hydrogenation of glycosidic carbon-oxygen bonds in permethylated saccharides *via* a cyclic oxonium ion intermediate gives a mixture of anhydroalditols which may be acetylated *in situ* and analyzed by g.l.c. and g.l.c.-m.s. The reaction is performed using triethylsilane in trifluoroacetic acid with either boron trifluoride etherate or trimethylsilyl trifluoromethanesulfonate as the catalyst. This reaction has numerous advantages: (1) the glycosyl linkage positions and ring form for each residue in the sample can be determined, (2) selective, partial reductive-depolymerization giving sequencing information is possible depending upon the catalyst and reaction conditions used,[132] and (3) the procedure provides a rapid, less laborious, one-pot

reaction that can be performed on microgram amounts of material giving virtually quantitative yields. Attempts to use deuteriotriethylsilane to label the configuration at C1 and thereby obtain anomeric data were unsuccessful.[133] Artifacts may be formed during the reaction however, these can be minimized by using anhydrous conditions[134] and the correct choice of catalyst.[135] Extensive gas-liquid chromatographic and mass-spectrometric reference data for anhydroalditols have been published[136] and the reaction has been successfully applied to D-mannans,[137] D-fructans,[138] and D-glucans.[139] In a study on 2-acetamido-2-deoxy-D-glucopyranosyl residues,[140] it was noted that the glycosidic carbon-oxygen bond of the amino sugar residues was resistant to reductive cleavage but that β-linked residues were selectively hydrolyzed while the bond of α-linked residues was stabilized during the reaction, thus providing a means for the chemical determination of their anomeric configuration and for possible sequencing information.

3.2 SPECIFIC DEGRADATIONS

The chemical analyses described in Section 3.1 provide information concerning the TSR and linkage patterns within a polysaccharide however, these techniques cannot readily be used to obtain anomeric and sequencing information. Anomeric data can be obtained by using chemical reactions, for example oxidation with chromium trioxide,[141] or by using specific enzymatic cleavages[142] although the information is more usually gained by instrumental techniques (see later). To determine the glycosyl sequence, the polysaccharide is partially depolymerized under controlled conditions, producing oligosaccharide fragments which are then structurally characterized. It is often possible to deduce the glycosyl sequence within the original material by "piecing together" the oligosaccharide structures thus obtained.

The chemical techniques for the degradation of polysaccharides may be classified according to their general reaction types: (1) acid degradations, including partial acid hydrolysis and acetolysis, (2) oxidative reactions, for example the Smith degradation which incorporates periodate oxidation, (3) deamination reactions, and (4) ß-elimination reactions. In addition to these chemical means, enzymatic methods have been used to achieve selective

depolymerization, for example bacteriophage degradations. All these degradative techniques take advantage of specific structural features within the polysaccharide, for example the ß-position to the carboxyl group of the glycosidic linkage in 4-linked hexopyranuronate rings, and may provide more than just sequencing information, as in the case of periodate oxidation where the determination of the number of vicinal hydroxyl groups complements the methylation analysis data. However, the chemical reactions tend to use conditions where labile substituents are cleaved, with a resultant loss of information, and where low yields of oligosaccharides result. The enzymatic methods use less stringent conditions and provide more control over the depolymerization, and thus give greater yields of oligosaccharide fragments with the labile substituents intact.

The established chemical degradative procedures have been extensively reviewed by Lindberg and co-authors[111,143] and elsewhere,[144-149] and thus will not be discussed here however, recently developed techniques such as hydrofluorinolysis and bacteriophage depolymerization warrant detailed commentary.

3.2.1 Hydrofluorinolysis

In 1977, Mort and Lamport,[150] while studying the deglycosylation of glycoproteins, noted that anhydrous hydrogen fluoride (HF) facilely cleaved the linkages of neutral and acidic sugars and that more severe conditions were needed to cleave the *O*-glycosidic linkage of amino sugars, and thus proposed the use of this reagent for the solvolysis of carbohydrate material. Mort and Bauer[151] introduced two new methods for oligosaccharide production during their study on the capsular and extracellular polysaccharides of *Rhizobium japonicum*, firstly using lithium in ethylenediamine to cleave galacturonic acid residues (see Section 3.2.2) and secondly extending the use of HF to achieve partial hydrofluorinolysis. These workers found that at -23° HF rapidly cleaves α-glycosidic linkages of neutral sugars but the β-glycosidic, uronic acid, and ester bonds remain intact. In a later study done at -40°, Mort *et al.*[152] were able to selectively cleave α-glucopyranosyl residues in preference to α-manno- and α-

galacto-pyranosyl residues, thereby establishing hydrofluorinolysis as a viable alternative method for the production of oligosaccharide fragments from polysaccharides.[153]

Hydrofluorinolysis has been applied by Knirel *et al.*[154] in their investigation of *Pseudomonas aeruginosa* O:3a,d,e lipopolysaccharide, which was found to contain a novel acidic amino sugar, and by Bosso *et al.*[155] to the preparation of ß-(1 → 4)-linked 2-acetamido-2-deoxy-D-glucopyranosyl oligosaccharides, where almost quantitative yields with no apparent *N*-de-acetylation or *N,O*-acyl migration were reported when the reaction was performed at 20°. Mort and co-workers used HF to identify the acyl substitution patterns in the extracellular polysaccharides of two *Rhizobium* species,[156] reporting de-esterification and acyl migration at elevated temperatures.[157]

3.2.2 Lithium/Ethylenediamine Degradation

Since the introduction by Mort and Bauer[151] of lithium dissolved in ethylenediamine for the cleavage of galacturonic acid residues, Albersheim and co-workers[158] have developed the reaction to enable the selective cleavage of underivatized glycosyluronic acid containing carbohydrates to produce oligosaccharide fragments. In their detailed study using model compounds and complex carbohydrates, Albersheim and co-workers found that this reagent cleaved at the linkage sites of uronic acids, methyl glycosides, methyl esters, and pyruvate ketals. Neutral sugars were not cleaved during the reaction, although reducing sugars were converted to the corresponding alditols.

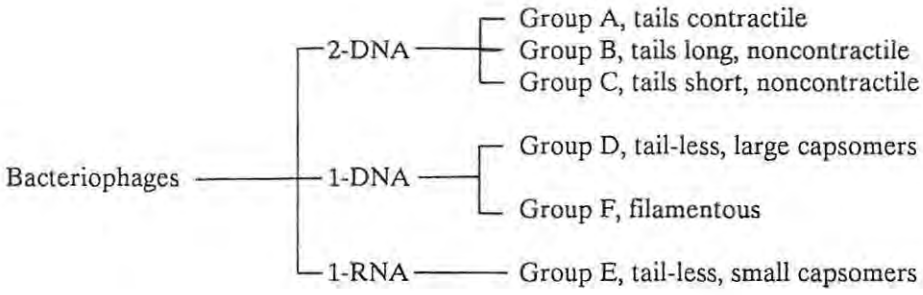
The degradation involves the reaction of an anhydrous solution of the carbohydrate sample (~2 mg) in ethylenediamine with lithium metal at 10-20° for one hour. Quenching of the reaction with water and removal of the solvent and lithium ions by coevaporation with toluene and ion-exchange chromatography, respectively, yields one or more oligosaccharide products which are conveniently separated by gel-permeation chromatography. The mechanism for the reaction was investigated using 2-hydroxyhexanoic acid and involves the cleavage of the bond between the ring oxygen and C5 of pyranosyl residues *via* an α-elimination with a concomitant loss of the C1 oxygen to the aglycone.[158]

The linkage sites, but not the anomeric nature of the uronic acid residue, proved to be important to the type of degradative product recovered. The glycosidic linkage of the uronic acid was cleaved in all cases, as was the glycosyl linkage to the uronic acid when situated at C2 or C4. With 3-linked residues, the glycosyl linkage was not cleaved, resulting in a modified alditol being produced at the reducing end of the fragment, for example a 3-linked D-galactopyranosyluronic acid residue forms 2-deoxy-L-lyxohexitol linked in the 4-position. The modified oligosaccharide alditol products not only provide an insight into the mechanism of the lithium/ethylenediamine degradation but also give confirmatory evidence of the linkage pattern on uronic acid residues.

Lithium/ethylenediamine degradation provides a rapid and convenient method for the selective fragmentation of uronic acid-containing polysaccharides, giving yields of greater than 80% where the carbohydrate material is readily soluble in ethylenediamine. This technique promises to become of major importance to the analysis of complex carbohydrate structures.

3.2.3 Bacteriophage Degradation

Viruses are autonomous microbes that are obligately parasitic upon a host organism, subjecting the host to infection or antibiosis. Where the host is a bacterium, the virus is termed a bacteriophage (phage) and generally shows a high degree of bacterial strain specificity. Bradley[159] has reviewed the molecular structures and infective processes of bacteriophages, and has proposed a system of classification based upon the morphological type and nucleic acid content of the virion (Scheme I). The natural habitat of bacteriophages is, of course, closely related to that of their hosts. Solid matter produces higher concentrations and a greater variety of bacteriophages than do liquid sources because of the rapid distribution and resultant lysis of susceptible strains within the latter environment. Thus, for example, the bacteriophages that infect enteric bacteria are readily found in faeces and sewage water (an artificial dispersion of human faeces and other solid wastes), providing that the host strain is present within the population.



Scheme I. Classification of bacteriophages.[159]

Adams and Park,[160,161] while studying the properties of the bacteriophage Kp active against *Klebsiella pneumoniae* type 2, noted that two hydrolytic enzymes were produced by the virion, a phage-bound and a soluble depolymerase. Both enzymes seem to be produced under the control of the bacteriophage genome, the soluble form being freely diffusible and responsible for producing haloes around phage plaques in bacterial lawns. Characterization of the purified soluble enzyme showed that it was capable of hydrolyzing the polysaccharide capsule from *Klebsiella pneumoniae* type 2 however, it did not affect the viability of the micro-organism which was still susceptible to bacteriophage infection, indicating that the enzyme substrate was not the same as the receptor site needed for bacteriophage replication.[162] The phage-bound enzyme had the same enzymatic activity as the soluble form and, for both enzymes, the hydrolytic products were shown to be large oligosaccharides containing about 1% reducing sugars. Similar results were reported by Humphries,[163] Ecklund and Wyss,[164] and Bartell *et al.*[165]

Sutherland and Wilkinson[166,167] studied the polysaccharide depolymerases induced by the bacteriophage infection of *Klebsiella aerogenes* type 54 [A3(Sl)]. Digestion of the capsular material from this bacterium using partially purified enzymes and subsequent isolation of the degradative products showed that the polysaccharide was cleaved into oligosaccharide units corresponding to the tetrasaccharide repeating unit of the polymer and the dimer thereof. These oligosaccharides contained *O*-acetyl substituents, indicating that labile groups were not removed during the degradation. The enzyme was characterized as an endofucosidase and

shown to be specific for the exopolysaccharides produced by *K. aerogenes* A3 and A3(Sl). Although Sutherland[166] reported that the induced enzyme was active against alkali- or acid-treated polysaccharides, Yurewicz and co-workers[168] in their study on the endogalactosidase induced from *Aerobacter aerogenes* showed that significant chemical changes in the substrate, such as removal of a pendant side-chain, considerably reduced enzymatic activity.

The morphology of *E. coli* bacteriophages was investigated by Stirm and Freund-Mölbert[169] using electron microscopy. Of the twelve phages studied, one was shown to belong to Bradley's morphological group A while the rest belonged to group C. All the bacteriophages possessed spikes that were linked to the capsid either directly or *via* a base plate and that were probably responsible for the recognition and depolymerization of the capsular polysaccharide. Rieger-Hug and Stirm[170] isolated fifty bacteriophages using the serological test strains for *Klebsiella* K antigens and showed that 36 belonged to Bradley's group C, 11 to group B, and 3 to group A. These viruses were usually host specific and, by studying their depolymerization reactions, these workers were able to conclude that (1) most cleavages occur on either side of a negatively charged sugar unit but reducing uronic acid residues are not produced, (2) the reducing sugar is often substituted at position 3, (3) ß-linkages are the more frequently hydrolyzed glycosidic bond, and (4) where a polysaccharide is degraded by more than one enzyme, the same glycosidic bond is split. Cleavage at sterically hindered sugar residues is also favoured, especially for enzymes having lyase activity (see later).

Up until 1981, the degradation of capsular polysaccharides using viral enzymes required a rigorous purification of the bacteriophage particles by, for example, polyethylene glycol sedimentation.[171] However, when the objective is to use the bacteriophage as a depolymerizing reagent rather than to characterize the viral particle, a less stringent purification step can be justified. Dutton *et al.*[172] investigated the experimental conditions involved in bacteriophage degradations and reported four simplified procedures for this technique. These workers found that in general 10^{13} plaque-forming units (PFU) were needed to depolymerize one gram of capsular material and that the type of oligosaccharide material

recovered depended on the concentration of the polysaccharide, high concentrations producing mainly the oligosaccharide corresponding to the repeating unit of the polymer and lower concentrations producing oligomers of the repeating unit.

The enzyme carried by a bacteriophage may have deacetylase, glycanase, or lyase activity however, it is specifically the endoglycanase and lyase enzymes that are useful in bacteriophage degradations. In addition to the review by Parolis,[18] degradations using endoglycanase enzymes have been reported for *Klebsiella* K18,[173] K19,[174] K20, K24,[175] K25,[176] K26,[177] K30 (this thesis), K39(this thesis),[178] and K44 (ref. 179) and for *E. coli* K13, K20, K23,[180] K27,[181] K28,[182] and K30.[25] Interestingly, the degradation of the *E. coli* K27 polysaccharide was achieved using enzymes induced from *Klebsiella aerogenes* A3(Sl),[181] the *E. coli* K13 and K23 capsules were degraded by *E. coli* Ø20,[180] and the *E. coli* K30 polysaccharide was degraded using *Klebsiella* Ø20.[25] Lyase activity, involving a ß-elimination reaction on a uronic acid unit to form a terminal non-reducing hex-4-enuronic acid residue, has been reported for *Klebsiella* K5,[183] K14,[18] and K64,[184,185] and for *E. coli* K55 (this thesis). In all these cases the substrate contains a sterically hindered mannopyranosyl unit (tri- or tetra-substituted) attached to the 4 position of a glucopyranuronosyl unit, a situation that would favour the β-elimination reaction.

Bacteriophage degradation has become one of the methods of choice for the elucidation of bacterial capsular polysaccharide structures. This technique combines convenience and high yields with the isolation of oligosaccharides containing acid- or alkali-labile substituents like acetate,[179] formate,[186] and pyruvate.[187] In addition, bacteriophages provide a source of specific enzymes that may be used in other fields, for example an endo-*N*-acetylneuraminidase enzyme induced from *E. coli* K1 has been applied to the hydrolysis of neural membrane glycoconjugates.[188]

4. INSTRUMENTAL TECHNIQUES USED IN CARBOHYDRATE ANALYSIS

4. INSTRUMENTAL TECHNIQUES USED IN CARBOHYDRATE ANALYSIS

In recent years there has been a trend in the structural elucidation of carbohydrates towards the use of instrumental techniques whereby oligosaccharides are analyzed directly. This trend has been facilitated by technological improvements that have allowed the development of newer techniques and experiments in which micro- to milli-gram amounts of material are needed, post-analytical recovery of the sample is possible, a greater amount of information concerning the oligosaccharide is gained per experiment, and time is saved. The ultimate technique would provide the complete *de novo* elucidation of oligosaccharide structures using one experiment, an objective which nuclear magnetic resonance spectroscopy seems the most likely to achieve. The emphasis of the following discussion has been placed upon the newer instrumental techniques since the older, established techniques have been well reviewed in the literature and thus require little comment.

4.1 CHROMATOGRAPHIC METHODS

4.1.1 Gas-liquid Chromatography

The chromatographic separation of volatile substances using a column containing a stationary liquid phase and a mobile gas phase was first achieved by Martin and co-workers.[189,190] The chromatographic process is based upon the repeated distribution of components between the stationary and mobile phases, each effective distribution being termed a "theoretical-plate". The efficiency of the system will depend upon the total number of theoretical-plates, measured either as the height equivalent to a theoretical-plate (HETP) or as the number of plates per unit length of column, and upon the separation of the components per theoretical-plate, which is determined primarily by the gas-liquid partition coefficients. The parameters influencing the efficiency of packed columns were investigated by van Deemter *et al.*[191] and have been reviewed by Bishop.[56] The analogous theory applying to open tubular (capillary) columns has been discussed by Rooney[192] who has identified six first-order parameters that affect the resolution and the time necessary for separation. These parameters may be divided into either column characteristics, *viz.* length, internal diameter (i.d.), and film

thickness, or operational parameters, *viz.* temperature, type of carrier gas, and carrier gas velocity.

Since the first reported separation of permethylated methyl glycosides,[54] g.l.c. has been rapidly and widely applied in the field of carbohydrate analysis.[56-58] Packed columns, usually with an i.d. of about four millimetres and a length of one to two metres, were used with a variety of polymeric stationary phases, for example hydrocarbons (Apiezon M, Apiezon L), silicones (SE-30, OV225), polyesters (DEGS), polyglycols (Carbowax 20M), and mixed stationary phases, coated onto support material. Various derivatives (see Section 3.1) were separated using these columns and the results indicated that the polarity rather than the structure of the stationary phase is important for efficient separation.

In 1957, Martin predicted the development of capillary g.l.c. columns and in the following year Golay produced the first such column from a coated length of Tygon tubing (approximately 0.25 mm x 50 m).[193] Although capillary columns showed a greater efficiency than packed columns, the development of this technique in the following years was slow due to problems in column technology, low sensitivity of the detection systems, difficulties in quantitative analyses, and the state of the instrumentation in general. However, technological advances, including the development of flame-ionization detectors, electronic integrators, and thermostable columns, have facilitated the rapid expansion of this field over the past fifteen years. Comparison of the performances of modern capillary and packed columns (Table I) shows the former to have several advantages: they provide more efficient separations, require a smaller sample size, and give a greater signal-to-noise ratio.[194]

Different types of capillary columns have been produced, for example wall coated open tubular (WCOT) columns have the stationary phase deposited directly onto the capillary surface, porous layer open tubular (PLOT) columns have the coating deposited onto a chemically modified surface, support coated open tubular (SCOT) columns are coated with a mixture of a finely divided solid support and the stationary phase, and bonded-phase columns have the stationary phase chemically bonded to the surface. The bonded-phase columns have proved to be advantageous because they are more thermostable and therefore there is less

column "bleeding" of the stationary phase, phase stripping can be prevented, the peaks are sharper, and the columns can be washed with solvent.[195] The capillary tubing may be made from either glass or fused-silica, the latter being better since it is less reactive, more flexible, and has a higher mechanical strength. Wide-bore (0.5 mm i.d.), thick film (5-8 μm) capillary columns have been produced that combine the advantages of capillary columns with the larger sample sizes used in packed columns and allow tandem analyses like gas-liquid chromatography - Fourier transform infrared spectroscopy (g.l.c. - F.t.i.r.) to be performed.[196]

			WCOT	PACKED
Length	L	m	25	2.5
Efficiency +	N		79000	3500
Max.Sample Quantity	Qc	μg	1 + +	100
Peak Area	A		669	669
Capacity factor	k		5	60
Retention Time	t	s	750	750 + + +
He Velocity	v	cm/s	20	20.3
He Flow	F	mL/min	0.9	14
Peak Height	h		100	21
Signal-to-Noise	S/N		500	4.9
Relative S/N			102	1

\+ Calculated
\+ + Defined
\+ + + Adjusted

Table I. Comparison of capillary and packed columns.[194]

The application of capillary g.l.c. to carbohydrate analyses began two decades ago[197] however, it has only been in the last eight years that the use has become widespread. Bradbury *et al.*[198] have reported the separation of nine trimethylsilylated alditols on a fused-silica column coated with OV101. Thirteen common alditol acetates were separated by Holzer and co-workers[199] on a WCOT column coated with a 0.2% chiral stationary phase consisting of 90% *N*-propionyl-L-valine-*tert*.-butylamide polysiloxane and 10% Witconal LA 23 (surfactant), and these workers were able to extend their analyses to include amino sugar derivatives.[200] Alditol acetates have also been separated on a glass capillary column coated with OV275[201] and on fused-silica columns coated with FFAP[202] and BP-75 (bonded-phase OV275).[203]

Thirty-one partially methylated alditol acetates from various parent sugars were separated on a Silar-10C SCOT column.[204] All the possible di-, tri-, and tetra-*O*-methyl derivatives from the pyranose forms of mannose, glucose, and galactose were analyzed as the alditol acetate derivatives by Barreto-Bergter *et al.*[205] on an OV17 and an OV225 column. Geyer *et al.*[206] reported the separation of thirty *O*-methylated hexitol and 2-deoxy-2-(*N*-methyl)-acetamidohexitol acetates using glass WCOT columns with Silar 9CP, Dexsil 410, SE-30, or OV101 as the stationary phase. These workers found that the methylated hexitols were optimally separated on the most polar (Silar) column and that the amino sugar derivatives were best separated on either Dexsil 410 or OV101. Lomax and Conchie[207] were able to separate fifty partially methylated alditol acetates on a glass SP-1000 WCOT column and, by using 2,3,4,6-tetra-*O*-methyl-D-glucitol and monomethyl-L-inositol as combined internal standards, were able to accurately identify the derivatives by computer.

The detectors used to monitor components eluting from capillary columns must have a sensitivity compatible with the efficiency of the column. Initially thermal conductivity detectors (TCD's) were used but these have been superseded by the more sensitive flame-ionization detectors (FID's) which are now routinely used in capillary g.l.c. More selective monitors have been developed for the detection of specific compounds, for example electron-capture detectors (ECD's) are used to monitor trifluoroacetyl derivatives of monosaccharides[208] and flame-photometric detectors can specifically monitor phosphorus- or halogen-containing compounds. For quantitative analyses, the linearity of the detector response must be determined. Sweet *et al.*[209] investigated the various response-factor theories as applied to partially methylated and partially ethylated alditol acetates and found that calculations based upon the effective carbon response (e.c.r.) theory formulated by Ackman[210] gave the closest correlation to the observed responses. Molar response factors suffice for the routine analysis of carbohydrates containing repeating units however, where more detailed structures exist, the actual response factors should be calculated using model compounds.

Identification of unknown components of a mixture may be made by comparing the g.l.c. retention times to those of known samples run under the same conditions or by comparing

relative retention times based upon some standard. Identification by these means is not always reliable and thus it is preferable to use a second, complementary technique to positively identify the unknown components. The most common technique used in tandem with g.l.c. is mass spectrometry (see Section 4.2), this method (*i.e.* g.l.c.-m.s.) providing unequivocal identification of sample components.

4.1.2 High-Performance Liquid Chromatography

High-performance liquid chromatography (h.p.l.c.) makes use of several chromatographic processes, for example gel-permeation, ion-exchange, and partition chromatography involving either a normal-phase or a reverse-phase process has been used with aqueous or organic solvents. It is this flexibility that has made the technique widely applicable to carbohydrate chemistry. By comparison with g.l.c., h.p.l.c. has several advantages: the compounds are not exposed to excessive heat, less sample clean-up is required, derivatization is usually not necessary, the analysis is rapid, and preparative chromatography is more easily accomplished. The high chromatographic efficiency of capillary g.l.c. is, however, not currently available from conventional h.p.l.c.

In 1974, Belue and McGinnis[211] reported the use of "high-pressure" liquid chromatography for the separation of water-soluble wood polysaccharides from *Larix occidentalis* and *Pinus taeda* using laboratory packed columns of Bio-Glas or Bio-Gel with water as the solvent. In the same year, Belue[212] showed that liquid chromatography could be used to identify polyhydric alcohols produced during Smith degradations, the separation being achieved on a silica gel (Porasil A) column with a ternary organic solvent. Linden and Lawhead[87] were able to separate mono-, di-, and tri-saccharides on a micro Bondapak column eluted with aqueous acetonitrile, obtaining component resolution comparable to packed-column g.l.c. Since these initial applications, h.p.l.c. has been widely used in the analysis of carbohydrate materials, for example McGinnis has separated a variety of substituted carbohydrates on gel-permeation columns,[213] reverse-phase h.p.l.c. using a micro Bondapak C_{18} column has been applied to partially methylated sugars,[214] and a bonded-amine (Chromosorb-NH_2) column has been used to

separate oligosaccharides (up to d.p. 20) from hydrolyzed inulin.[215] Reviews on the technique have been published by McGinnis and Fang,[216] and Verhaar and Kuster.[217]

The detection of carbohydrate components eluting from the column has been a problem with h.p.l.c. Initially, differential refractometer detectors were used but these have the disadvantages of non-specificity and inadequate sensitivity. Ultraviolet (u.v.) detectors were used at a wavelength of about 200 nm however, underivatized carbohydrate material does not significantly absorb radiation at this wavelength and the wavelength is not specific for carbohydrates. Pre-column derivatization involving perbenzoylation was introduced,[218] enabling the u.v. detection of the benzoate ester chromophore and creating an improvement in sensitivity of up to 100-fold over that of the refractive index detectors. This technique is of limited use since benzoylation produces multiple derivatives from single sugars and requires the use of an organic solvent as the eluent. A recent approach to pre-column labelling has been proposed by Her *et al.*[219] whereby fluorescent *N*-(2-pyridinyl)-glycosylamine derivatives are prepared from reducing sugars by condensation with 2-aminopyridine, h.p.l.c. detection then being achieved spectrofluorimetically. This method has the advantages that the derivatives are stable and chromatographically well resolved. The original material can be regenerated by weak acid hydrolysis and, since derivatization tags the reducing end sugar, it aids subsequent mass spectrometric studies. Post-column derivatization of reducing sugars was investigated by Honda *et al.*[220] using 2-cyanoacetamide and subsequent fluorimetric detection. These workers achieved a lower limit of detection of about 2 nmol (with a signal-to-noise ratio of 2). When 2,4-pentanedione and formaldehyde were used for the post-column fluorescent derivatization of hexosamines, a lower limit of about 200 pmol was obtained.[91] Electrochemical detection has been reported to achieve even greater sensitivities, for example a lower limit of about 20 pmol was reached using the 2-cyanoacetamido derivatives,[221] and may be performed on underivatized carbohydrates.[222,223] Low angle laser light scattering has also been used for h.p.l.c. detection.[224]

One of the most useful analytical tools in carbohydrate chemistry is the tandem analysis of mixtures by h.p.l.c. and m.s. (l.c. - m.s.), the technique having been recently reviewed by

Covey *et al.*[225] This technique provides not only a sensitive detector for the h.p.l.c. system but also structural data about the eluting components. Spellman *et al.*[226] have used this technique for the structural analysis of a heptasaccharide isolated from the plant cell-wall, pectic polysaccharide rhamnogalacturonan II. During per-*O*-(trideuteriomethyl)ation of the heptasaccharide-alditol, partial degradation occurred resulting in a mixture of per-*O*-(trideuteriomethyl)ated oligosaccharides and oligosaccharide-alditols. This mixture was separated by h.p.l.c., the elution being monitored by chemical ionization (c.i.)-m.s. Santikarn *et al.*[227] reported the use of a moving polyimide belt as an h.p.l.c. - f.a.b. - m.s. interface and applied the technique to the analysis of oligosaccharides isolated from sheep urine. The interface does not involve the addition of a liquid matrix and therefore gives good signal-to-noise ratios at lower masses. Recently thermospray liquid chromatography-mass spectrometry has been reported for the separation and identification of alditols, mono-, and di-saccharides, with a lower detection limit of about 50 pmol.[228]

4.1.3 Gel-Permeation Chromatography

Since its introduction in 1956,[229] gel-permeation chromatography (g.p.c.) has been developed into one of the most useful techniques for the separation and molecular mass determination of carbohydrate materials. The chromatographic process involves the partitioning of solute molecules between a mobile eluent and a stationary liquid phase held within a gel matrix. The solute molecules penetrate the gel matrix through pores; the smaller the molecule the further into the matrix it can penetrate and thus the longer it takes to elute from the column. By definition, V_o and V_i are the volumes external to and inside the gel matrix, respectively. Since only a proportion of V_i is available to a solute of a given molecular size, the elution volume, V_e, may be expressed as

$$V_e = V_o + K_d.V_i , \qquad (1)$$

where K_d is the partition coefficient and is dependent upon the nature of the solute, the eluent, and the gel. For chromatography involving solely permeation processes, K_d and V_e may take on the values

$$0 \leqslant K_d \leqslant 1 \text{, and} \tag{2}$$

$$V_o \leqslant V_e \leqslant V_o + V_i \text{.} \tag{3}$$

These variables have, however, been found in some cases to exceed their theoretical upper limits, indicating that solute-matrix adsorption effects may also occur. These adsorption effects can be minimized by using an eluent containing an electrolyte, which reduces any ionic interactions between the solute and the gel matrix.[230]

The separation of two solutes, ΔV_e, is governed by the expression

$$\Delta V_e = V_i . \Delta K_d \text{,} \tag{4}$$

ΔK_d being the difference between the individual partition coefficients. Theoretically by increasing V_i, *i.e.* increasing the pore size of the gel, better separation would be achieved however, in practice it is ΔK_d that is the more important factor. The value of K_d depends, *inter alia*, upon the pore size of the gel such that it will equal zero for large molecules that are totally excluded from the gel matrix and approach unity for small molecules (see equation (2)). Separation of these small molecules may fail to occur if ΔK_d is not large enough. The molecular mass of structurally similar solute molecules is generally taken as representative of the molecular size and thus a gel of defined porosity will have a fractionation range, usually expressed in terms of molecular masses, over which it is able to separate components of a mixture. The smaller the porosity of the gel, the smaller the fractionation range and the lower the limits of the fractionation range.

Churms[231] has reviewed the relationships that have been proposed for the gel chromatographic variables. Although no single relationship has yet been established for all the variables, Anderson and Stoddart[232] were able to derive the expression

$$V_e = -b \log M + c \text{,} \tag{5}$$

which correlates the elution volume with the log of the molecular mass (M). This simple linear expression, with b and c as constants, has been found to hold true over the fractionation range of a given column provided that structurally similar solute molecules are

being compared and has been widely used to establish calibration curves for the determination of unknown molecular masses.

The versatility of g.p.c. lies in the range of experimental parameters that may be varied; the type of gel, porosity, column size, eluent, and flow-rate are all chosen to suit a specific situation. Review articles covering these parameters have been published by Churms[231] and others.[233,234] Detection of carbohydrate components eluting from the column may be achieved by continuous monitoring of the eluate using refractive index or u.v. detectors, by quantitative colourimetric analyses either on fractions of the eluate[235] or with fully automatic analyzers,[236] and by gravimetric analyses.

G.p.c. has several advantages: separations are rapid and efficient; the molecular masses of sample components may be determined on suitably calibrated columns, the calibration of which remain stable over long periods of time; quantitative analyses are possible;[237] little initial sample preparation is necessary; water or a volatile buffer may be used as the eluent; no column regeneration is required; and labile materials are not affected by the process.

4.2 MASS SPECTROMETRY

Mass spectrometry (m.s.), involving the identification of fragments produced from ionized molecules, has become one of the most versatile instrumental techniques available to the analytical chemist. In the analysis of carbohydrate structures, the combination of chromatography (g.l.c. or h.p.l.c.) and m.s.[225,238] provides complementary structural data concerning the components of derivatized samples (see Section 3.1). Electron impact-mass spectrometry cannot readily distinguish between diastereoisomeric sugar derivatives, for example 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-glucitol and -galactitol will produce very similar mass spectra however, these compounds can be separated and identified by g.l.c. Conversely, g.l.c. will not separate the above derivatives from their corresponding 3,5-di-*O*-methyl analogues but differentiation is possible from the mass spectra, if the derivatives have been appropriately labelled (at C1 by borodeuteride reduction of the parent hexose). M.s. may also be used to gauge chromatographic efficiency by testing for the coelution of peaks. Several

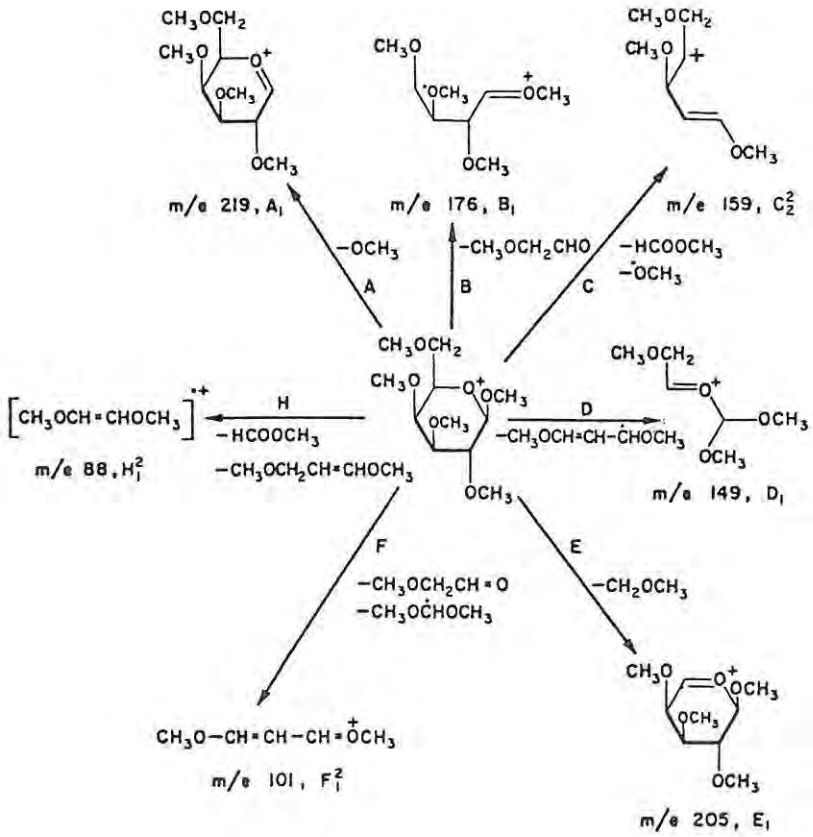
reviews covering the applications of m.s. to carbohydrate chemistry have been published.[106,107,238-241]

The major factor influencing the information content of mass spectrometric studies is the type of ionization technique used. The conventional method of ionization by electron impact (e.i.) has been comprehensively studied however, this technique produces extensive fragmentation of the high-energy, ion-radicle species formed, with a concomitant loss of information concerning the molecular ion, and needs suitably volatile and thermally stable compounds. "Softer" ionization techniques, such as chemical ionization (c.i.) and fast atom bombardment (f.a.b.), have become increasingly more popular; they are able to furnish information about the molecular ion and major fragments derived therefrom, large oligosaccharides and small polysaccharides may be analyzed, and derivatization may not be necessary in some cases. Other ionization techniques have been used in carbohydrate chemistry, for example field ionization (f.i.),[242] field desorption (f.d.),[243] and collision activation,[244,245] but they are not in such widespread use as those mentioned above.

4.2.1 Electron Impact-Mass Spectrometry

Electron impact-mass spectrometry (e.i.-m.s.) was used by Finan *et al.*[246] in 1958 to estimate the bond dissociation energies of α- and ß-methyl glucopyranoside and several disaccharides. The need for physical methods to analyze the structure of carbohydrates prompted further investigation of this technique. Biemann and co-workers applied the technique to the analysis of peracetylated monosaccharides[247] and the acetates of partially methylated monosaccharides[248] while, at the same time, Kochetkov and co-workers investigated the mass spectra of permethylated methyl glycosides.[249,250] Since these investigations, e.i.-m.s. has rapidly developed such that it is now possible to differentiate between pyranose, furanose, and acyclic derivatives, to distinguish the aldose and ketose sugars, to identify uronic acids[251,252] and deoxy,[253] amino,[254] and unusual sugars,[255] to determine the positions of glycosyl substitution, and to provide sequencing information about oligosaccharides.

Kochetkov *et al.*,[249] noting the absence of a molecular ion peak, suggested that rapid and extensive fragmentation of methylated methyl glycosides proceeded simultaneously along several pathways. These workers assumed that the ring oxygen was the preferred site of ionization for these compounds and proposed a series of fragmentation pathways to support their findings. Kochetkov and Chizhov[250] extended this work to include partially methylated sugar derivatives and, by specific labelling with trideuteriomethyl groups, were able to study in detail the pathways involved in the fragmentation. Nomenclature for the ions was proposed; related series of ions from a common origin were denoted by a capital letter (A, B, C, ...), subscript numerals denoted the number of steps needed to form the fragment from the molecular ion, and superscript numerals denoted the ordinal number of isomeric ions (Scheme II).[250] The m.s. of the methyl glycoside derivatives has been reviewed by Kochetkov and Chizhov.[238,239]



Scheme II. Fragmentation pathways for methylated methyl hexopyranosides.[250]

The mass spectra of acyclic derivatives are characterized by relatively simple fragmentations, this simplification being ascribed to the reduced influence of stereochemistry and to the absence of ring forms within these compounds. Golovkina *et al.*[256] studied the fragmentation of alditol acetates and were able to identify two basic pathways: (1) the cleavage of the carbon chain in the molecular ion to form primary fragments and (2) the loss of acetic acid (60 m.u.) and ketene (42 m.u.) from the primary fragments to form secondary fragments. The spectra of isomeric alditol acetates were found to be very similar. Jansson and Lindberg[257] rationalized the formation of fragments having even mass numbers from alditol acetates on the basis of an initial loss of acetaldehyde (44 m.u.) from the molecular ion, with subsequent losses of acetic anhydride (102 m.u.), acetic acid, and ketene. These even mass numbered fragments become insignificant in the mass spectra of partially methylated alditol acetates due to preferential fragmentation sites (see below).

Svensson and co-workers[258-261] investigated the mass spectra of partially methylated alditol acetates because of the importance of these derivatives to structural carbohydrate chemistry (see Section 3.1.3). In accordance with the work on alditol acetates, these workers found that several generalizations may be made concerning the fragmentation of the partially methylated derivatives.[106] Stereoisomers give very similar mass spectra and cannot be unambiguously identified. Primary fragments are formed as described above however, preferential fragmentation is observed between adjacent methoxylated carbon atoms, with either fragment carrying the positive charge. Fragmentation between an acetoxylated and a methoxylated carbon atom will occur rather than between two acetoxylated carbon atoms, and the methoxylated carbon will carry the positive charge. An exception to these rules is the fission of the C2-C3 bond of 1,2-di-*O*-methyl alditol acetates to give a fragment of *m/z* 89 in which the positive charge may be stabilized by two methoxyl groups. Secondary fragments are formed from the primary fragments by the loss of acetic acid, ketene, methanol (32 m.u.), or formaldehyde (30 m.u.).

Other carbohydrate derivatives used for mass spectrometric studies include trimethylsilylated methyl glycosides[262-264] and alditols,[265] peracetylated aldononitrile derivatives of monosaccharides[72,266] and partially methylated monosaccharides,[267-269] peracetylated oxime derivatives of ketoses,[84] partially ethylated alditol acetates,[270] alditol trifluoroacetates,[271] and dithioacetals.[272]

From studies on the trimethylsilyl ethers of disaccharides[262] and disaccharide alditols,[273] it was found that e.i.-m.s. could be used to analyze oligosaccharide structures. The alditol derivatives, especially when reduced with borodeuteride, improved the analytical procedure by simplifying the mass spectral interpretation and by producing a single derivative that could be chromatographed by g.l.c. Kärkkäinen analyzed 21 permethylated trisaccharides by g.l.c.-m.s. as their methyl glycoside[274] and alditol[275] derivatives, and was able to elucidate the following structural information: (1) although the molecular ion was not always present, the molecular mass of the derivatives could be calculated from related peaks, (2) the glycosyl sequence was obtained if the molecular masses of the constituent sugar residues varied, (3) chain branching could be shown, and (4) the glycosidic linkage position could be determined, although prior knowledge of the sugar composition was necessary in some cases. The anomeric configuration of the glycosidic bonds was found to have little influence on the fragmentation patterns, varying only the relative intensity of some peaks.

Since these studies, e.i.-m.s. of oligosaccharides has been widely used as a method for structural analysis. The nomenclature for fragment identification, as proposed by Kochetkov and Chizhov,[250] has been extended to cover the fragments produced from oligosaccharides;[251] prefixed lower-case letters (a, b, c, ...), which label the sugar residues starting from the non-reducing terminus, are used such that the first letter denotes the sugar residue from which the fragment originates and the subsequent letters designate the unchanged parts of the molecule. Sharp and Albersheim,[276] in their study on linear ß-D-glucopyrano-oligosaccharide-alditols, reported the presence of a J_o fragment (*m/z* 310 in borodeuteride reduced samples) and found the presence of this ion and the absence of a J_1 fragment (*m/z* 324) to be indicative of 3-linked glucopyranosyl residues. Banoub *et al.*[255] examined the mass spectra of

the peracetylated methyl glycosides and peracetylated alditols of 2-amino-2,4-dideoxy- and 3-amino-3,4-dideoxy-DL-pentopyranoses by e.i.- and c.i.-m.s. The methyl glycosides of a range of pyruvalated monosaccharides, derived from twelve bacterial polysaccharides, were examined as the pertrimethylsilylated derivatives and were shown to give characteristic mass spectra.[277] E.i.- and c.i.-m.s. have been used to determine the position and extent of labelling in ^{18}O- and ^{13}C-containing permethylated alditols.[278]

4.2.2 Chemical Ionization - Mass Spectrometry

Chemical ionization - mass spectrometry (c.i.-m.s.) is a soft ionization technique in which the ions are formed by ion-molecule reactions. The technique involves the electron bombardment of a mixture of a reagent gas and the sample ($\sim 10^3$:1 ratio), producing essentially ions from the reagent gas. It is the subsequent reactions of these ions that produce the ionized sample molecules and hence the c.i. mass spectrum. The ions formed by the reagent gas react either as proton donors (Brönsted-Lowry acids) or as hydride acceptors (Lewis acids), and produce, for example when using methane, the pseudomolecular ions $(M + 1)^+$ and $(M - 1)^+$ (M = molecular mass), respectively. The extent of the fragmentation of these even-electron pseudomolecular ions will depend upon the acid strength of the ions from the reagent gas; stronger acids will cause the ion-molecule reactions to be more exothermic and therefore more fragmentation will occur. The fragmentation will not be as extensive as with e.i.-m.s. since the amount of excess energy (2-3 eV) transferred to the molecular ion species during ionization and used in the fragmentation process is some 40-60% less than during the e.i. process (5 eV). Munson[279] has reviewed the effects of the operational parameters upon c.i. mass spectra and has reported that the source temperature will vary the relative abundance of the sample ions produced.

C.i.-m.s. produces easily interpretable mass spectra since most of the fragmentation processes consist of simple, eliminative rearrangement reactions of the functional groups. These processes are dependent upon the type of reagent gas used and the sample analyzed. Methane, isobutane, and ammonia have been used as reagent gases, the first two producing

more fragmentation than the last. Although methane has been used for the analysis of carbohydrates,[280] isobutane and ammonia, used either separately or in combination, have been more widely applied. Horton *et al.*[281] used c.i. (C_4H_{10})- and c.i. (NH_4)-m.s. to analyze a wide variety of carbohydrate materials and were able to make several generalizations: (1) use of the milder ionization process involved with ammonia is indicated for samples where the molecular mass is of primary concern, (2) functional groups, especially isopropylidene and acetate, exert a strong effect on the modes of fragmentation and are preferentially cleaved, (3) carbon atoms with more than one heteroatom attached are favoured sites for fragmentation, (4) large molecules that volatilize intact appear to favour the generation of intact capture ions, and (5) skeletal fragmentation rarely occurs.

Dougherty *et al.*[282] applied c.i.-m.s. to 22 peracetylated oligosaccharides (di- to penta-saccharides) using a mixture of ammonia and isobutane (2:1) and found that the data acquired complements the structural information gained from e.i.-m.s. Pseudomolecular ions for the pentasaccharide derivatives were not observed. McNeil and Albersheim[283] were able to differentiate many stereoisomeric partially methylated alditol acetates having the same substitution pattern by the relative intensities of the (M + 1), (M + 1 - 32), and (M + 1 - 60) peaks and were able to identify some derivatives that could not be distinguished by e.i.-m.s. For this differentiation, isobutane was found to be a better reagent gas than methane. C.i. (C_4H_{10})-m.s. has been used to study peracetylated and partially methylated peracetylated aldononitrile derivatives[284] and trimethylsilylated sialic acids[285] while Seymour and co-workers have applied c.i. (NH_4)-m.s. to peracetylated aldononitriles[72] and peracetylated oxime derivatives of ketoses.[84] Application of c.i.-m.s. to structural studies has been reported by Hancock *et al.*[286] and Tanaka *et al.*[287]

4.2.3 Fast Atom Bombardment - Mass Spectrometry

Until recently, mass spectrometric studies on many biologically important compounds were impossible because the polar nature and thermal instability of the materials precluded volatilization. Mass spectrometric techniques involving desorption of ions (*e.g.* f.d.-m.s.) and

spluttering of ions (*e.g.* static secondary ion-mass spectrometry, s.s.i.-m.s.) were investigated to overcome this need in the established techniques for the sample to be in the volatilized form prior to ionization. Fast atom bombardment - mass spectrometry (f.a.b.-m.s.) was developed from s.s.i.-m.s. by the replacement of the primary ion beam with a fast atom beam of argon,[288,289] this adaptation circumventing the problem of surface charging, with the consequential loss of the secondary ion mass spectrum, experienced in s.s.i.-m.s.

F.a.b.-m.s. involves the bombardment of the sample, held within a suitable matrix (usually glycerol with carbohydrates) on a copper or stainless steel target, with a beam of argon or xenon atoms having 7-8 keV of energy. This bombardment will produce both positive, $(M+H)^+$, and negative, $(M-H)^-$, pseudomolecular ions that will then fragment to give the f.a.b. mass spectrum which may be recorded in either polarity. The addition of certain salts, for example sodium chloride, to the sample stabilizes the molecular species formed as the cationic capture ion, in this case $(M+Na)^+$, and thereby enhances the appearance potential of the pseudomolecular ion peak. F.a.b.-m.s. is applicable to polar compounds that are either ionic or predisposed to become so however, until recently nonpolar compounds were unresponsive to this technique. Bartmess and Phillips[290] have developed what is termed electrochemically assisted fast atom bombardment-mass spectrometry (e.f.a.b.-m.s.) in which a potential difference ($\sim$15V) is applied to the sample in a glycerol matrix and have reported a 1000-fold increase in the signals for the pseudomolecular ions from 1-bromohexadecane when a potential difference of greater than 2V is applied. This technique will increase the variety of materials that can be analyzed by f.a.b.-m.s.

F.a.b.-m.s. has several advantages:[291] (1) small (0.1 - 5 μg), easily prepared samples are used, (2) the ionization may be conducted at ambient temperatures, precludes the need for sample volatilization, and produces non-thermally induced, structurally significant fragmentations, (3) the mass spectrum contains information concerning the molecular mass of the material and has an effective mass range of up to about 4000 m.u., (4) either polarity mode may be used to record the mass spectrum, both providing good pseudomolecular ion sensitivity, and (5) the information obtained complements that from e.i.- and c.i.-m.s.

F.a.b.-m.s. is ideally suited to the analysis of carbohydrate materials and has been widely applied by Dell and co-workers,[226,292-296] and others.[297,298] Fragmentations usually consist of the sequential cleavage of monosaccharide residues *via* an A_1-type process (see Scheme II) and provide useful sequencing information. Initially underivatized samples were analyzed however, it was noted that while the 6-*O*-methylglucose polysaccharide (MGP) from *Mycobacterium smegmatis* gave excellent mass spectral data,[292] the cyclic glucans from *Rhizobia* gave relatively poor data.[294] This phenomenon was attributed to the partial *O*-methylation of MGP causing decreased H-bonding interactions with the matrix and thus facilitating desorption and fragmentation. Based on this conclusion, derivatization strategies were developed to obtain both the molecular mass and the glycosyl sequence of samples.[299] The molecular mass can be obtained from underivatized samples (5-10 μg) or peracetylated samples (0.1-5 μg) if below 3000 m.u., whereas for samples of a greater molecular mass, permethylation is recommended provided that labile functional groups are not present. The full sequence for oligosaccharides (d.p. ∿10-11) can be obtained from the combined results of the peracetylated and permethylated derivatives, while the permethylated derivatives provide some sequencing information for larger oligosaccharides.

Dutton and Lam,[300] and Hackland *et al.*[301] have applied f.a.b.-m.s. to the structural analysis of the repeating unit oligosaccharides obtained from the bacteriophage degradation of *Klebsiella* capsular polysaccharides. Dell and Rogers[302] have reported a procedure for the rapid screening of glycoproteins which involves f.a.b.-m.s. analysis of the oligosaccharide portion after peracetylation (trifluoroacetic anhydride/acetic acid) and chloroform extraction. For more detailed structural analysis, the peracetylated oligosaccharides are purified by h.p.l.c. and then either reanalyzed (f.a.b.-m.s.) or permethylated and analyzed by f.a.b.-m.s. and by g.l.c.-m.s. after hydrolysis. Dell and Tiller[303] have developed a method to determine the position and quantification of naturally occurring *O*-acyl substitution in which the oligosaccharide material is perdeuterioacetylated under acidic conditions then analyzed by f.a.b.-m.s. Recent technological advances involving f.a.b.-m.s. include the development of tandem mass spectrometric techniques in an effort to enhance fragmentation, for example

f.a.b.-m.s.-c.i.d.-m.s.,[304,305] and the coupling with chromatographic techniques, for example h.p.l.c.-f.a.b.-m.s. (see Section 4.1.2).[227]

4.3 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Nuclear magnetic resonance (n.m.r.) spectroscopy originated from the nuclear induction experiments reported in 1946 by Bloch *et al.*[306-308] and by Purcell *et al.*[309] However, it was not until about two decades ago with the introduction of pulse methods, Fourier transform (F.t.), and greater magnetic field strengths (superconducting magnets) that n.m.r. spectroscopy developed into what is now the most powerful instrumental technique available for the analysis of molecular structures. These innovations have made computer control of the n.m.r. experiment and data collection essential which in turn has simplified the use of the instrumentation, allowed the performance of more complex experiments, and permitted the accurate measurement of n.m.r. parameters (chemical shift, δ; coupling constant, J; peak area; nuclear Overhauser effects, nOe; and relaxation times, T_1 and T_2). The theory and application of modern pulse methods in high-resolution n.m.r. spectroscopy have been reviewed by, *inter alia*, Benn and Günther,[310] Turner,[311] Morris,[312] and Farrar.[313,314]

N.m.r. spectroscopy was first applied to carbohydrates by Lemieux *et al.*[315,316] who showed that the chemical shifts and coupling constants of the protons in peracetylated sugars were dependent upon the configuration and conformation of the molecule. Since these initial observations, the technique has become extensively used and has been the subject of numerous reviews.[317-322] N.m.r. experiments provide a rapid, convenient, non-destructive means for the direct observation and quantitation of the molecular structure of carbohydrates. Oligosaccharides give well resolved n.m.r. spectra from which the following structural information may be obtained: the determination of the degree of polymerization, the identification of the reducing end sugar, the type and sequence of the non-reducing residues, the presence and location of non-carbohydrate substituents, and conformational and anomeric data. Polysaccharides, which tend to form viscous solutions and have relatively short relaxation times, give less well resolved spectra however, some of the above information may

still be obtained such as the number of residues in a polymeric repeating unit, the anomeric linkages, and the presence of non-carbohydrate substituents.

There has been a natural tendency for the n.m.r. spectroscopist to study carbohydrates in solution and, with most of the work concentrated in this area, rapid advances have been made towards the complete resolution and assignment of n.m.r. signals using 1- and 2-D techniques. Studies of carbohydrates in the solid state have, however, also been performed using ^{13}C cross-polarization magic angle spinning (c.p.-m.a.s.) n.m.r. spectroscopy[323,324] and promise to be a useful adjuvant to high-resolution ^{13}C solution n.m.r. spectroscopy. A complete review of n.m.r. spectroscopy is outside the scope of this thesis and thus the discussion has been limited, with a few exceptions, to those techniques available to our laboratory, emphasizing the usefulness of modern pulse methods.

4.3.1 One-Dimensional Techniques

(i) Proton Nuclear Magnetic Resonance Spectroscopy

The proton (^1H) n.m.r. spectrum of carbohydrates may be divided into three broad regions, *viz.* the anomeric region (δ4.5-5.5), the ring proton region (δ3.2-4.5), and the methyl group region (δ1.2 - 2.3). These regions are not mutually exclusive, for example the H5 proton signal of an α-galacturonic acid may appear in the anomeric region,[325] and signals may occur elsewhere in the spectrum, for example the H4 proton of a ß-hex-4-enuronic acid appears at δ5.83,[183] but they do provide general guidelines for signal assignment. N.m.r. has become the most reliable method for the assignment of anomeric configurations in oligo- and poly-saccharides.[321] As a general rule, signals from δ4.5 to δ5.0 are due to axial H1 protons (*i.e.*ß-linked D-pyranoses) while those from δ5.0 to δ5.5 are due to equational H1 protons (*i.e.* α-linked D-pyranoses). This distinction of the anomeric configurations is not as clear-cut for mannose and rhamnose derivatives but in these cases the coupling constants $J_{H1,H2}$ and especially $J_{H1,C1}$ serve to confirm the assignments. The anomeric signals for furanose conformers appear downfield from their pyranose analogues.

The size of the coupling constants between vicinal protons has been shown by Karplus[326,327] to depend largely upon the dihedral angle (Ø) between the nuclei. The Karplus relationship predicts a large coupling constant for diaxial protons (Ø = 180°, ^3J=9.22 Hz) while gauche (diequatorial and equatorial-axial) protons have a smaller value (Ø = 60°, ^3J=1.72 Hz).[320] This relationship may be practically applied to the assignment of anomeric configurations; where the H2 proton is axial (*e.g.* glucose and galactose), $J_{1,2}$ will be large for ß-anomers (6-8 Hz) and small for α-anomers (3-4 Hz), and where the H2 proton is equatorial (*e.g.* mannose and rhamnose), $J_{1,2}$ will be small for both anomers (0.5-1 Hz).

The analysis of the ring proton signals in 1D spectra is difficult because there is a large overlap of the signals, and first-order analyses of the chemical shifts and coupling constants are only justified if the chemical shift difference between the signals is much greater than the coupling constant, which holds true for the anomeric signals but not necessarily for the rest of the ring protons. Hall[319] has discussed various solutions to this "hidden-resonance" problem including the use of shift reagents and double-resonance experiments however, for facile unambiguous assignments, 2D experiments are more appropriate (see Section 4.3.2).

The methyl group region, besides indicating the presence of 6-deoxy sugars, shows the presence of a number of non-carbohydrate substituents, for example ethylidene[328] acetals, 1-carboxyethylidene (pyruvate)[329,330] ketals, and acetates.[331] Garegg *et al.*[332] studied the configuration of the quaternary carbon atom of pyruvate ketals using ^{1}H-n.m.r. spectroscopy and were able to distinguish between the *R* and *S* configuration by the position of resonance for the methyl group. Applying these results, Garegg *et al.*[333] demonstrated that for the naturally occurring pyruvate ketals found in extracellular bacterial polysaccharides the methyl group is equatorial and the carboxyl group is axial relative to the 1,3-dioxane ring. Di Fabio *et al.*[334] made use of the relative acid lability of pyruvate ketals to help sequence a pyruvalated hexasaccharide obtained from the bacteriophage degradation of the *Klebsiella* K46 polysaccharide. These workers observed the changes in signal resonance positions during a controlled autohydrolysis at 95° of the acid form of the oligosaccharide and were able to identify the sugar residues neighbouring the pyruvalated unit.

The assignment of signals in 1D ^{1}H-n.m.r. spectra has traditionally been done by comparison with the reported literature values for similar compounds. However, it must be emphasized that this technique of "assignment by comparison" may give ambiguous results if not judiciously applied. Several generalizations may be made concerning the proton signals of oligosaccharides: (1) terminal, non-reducing sugars give signals similar to the methyl glycoside derivatives, (2) glycosylation causes a downfield shift of the α-proton signal and may be accompanied by smaller downfield shifts of the ß-proton signals, (3) *O*-acylation will deshield the α-proton and thus cause a significant downfield shift of its signal, and (4) reduction of the reducing end sugar causes a shift in the signal of the anomeric proton adjacent to the alditol residue.

Hall and co-workers have studied proton spin-lattice relaxation times (T_1) of monosaccharides[335,336] and oligosaccharides,[337,338] and have found the values to be dependent upon the orientation of the nucleus. For axial protons, intramolecular dipole-dipole relaxation may occur with other protons that are either vicinal-gauche or 1,3-diaxially orientated. For equational protons, only the former interaction is possible and hence they have longer T_1-values. These stereospecific dependencies imply that the T_1-values may be used for the assignment of anomeric configurations as well as for conformational studies. Hall and Preston[339] investigated the effects of gadolinium ions on the T_1-values of some sugars and showed that the spin-lattice relaxation rate of anomeric protons increases as the concentration of gadolinium ions increases. Bradbury and Collins[340] found that when oligosaccharides are oxidized to produce terminal carboxylic acids, gadolinium ions will bind to the carboxyl group and cause a broadening of the proton resonances and a decrease in the T_1-values for the glycosidic protons. These effects diminish progressively along the oligosaccharide and thus can be used to obtain sequencing information however, this approach does have limitations, for example non-reducing sugars cannot be analyzed.

Recently, Vliegenthart *et al.*[341,342] have developed the concept of "structural-reporter groups" which enables the assignment of a primary structure to oligosaccharides based upon the positions of proton resonance, the coupling constants, and the line widths of the signals

in the spectrum. These workers have applied this concept to the structural determination of carbohydrates derived from glycoproteins.

(ii) Carbon-13 Nuclear Magnetic Resonance Spectroscopy

The low sensitivity of natural abundance carbon-13 (^{13}C) n.m.r. spectroscopy, due to the low isotopic abundance (1,1%) and magnetogyric ratio ($\sim 1/4\ \gamma_H$) of the ^{13}C nucleus, and the complication of ^{13}C-^{1}H coupling restricted the earlier n.m.r. analyses of this nucleus. The advent of broad-band ^{1}H-decoupling and F.t. methods has, however, revolutionized ^{13}C-n.m.r. spectroscopy and allowed the technique to develop rapidly such that it now complements ^{1}H-n.m.r. spectroscopy. Broad-band ^{1}H-decoupling improves the sensitivity of the ^{13}C-n.m.r. experiment by causing ^{13}C-^{1}H coupled signals to collapse to singlets and by generating nuclear Overhauser enhancements although structural information is lost in the process, for example the ^{13}C-^{1}H coupling constants. The nuclear Overhauser effect, with a theoretical maximum enhancement factor (nOef) of 1.988,[343] is not constant for all ^{13}C nuclei present in the compound and therefore integration of the peak areas does not provide an accurate measure of the number of nuclei resonating at specific frequencies. Freeman *et al.*[344] proposed the use of pulse modulation of the ^{1}H-decoupler to eliminate nOe's and thus enable quantitative use of the ^{13}C-n.m.r. signals.

^{13}C-N.m.r. spectroscopy allows the direct observation of the molecular skeleton of organic compounds and provides information concerning non-protonated sites (*e.g.* carbonyl carbons). Spectral resolution is enhanced because of the greater frequency range of the signals, for example in carbohydrates the signals appear from 15 to 180 p.p.m., however, due to long T_1-values for quaternary carbons,[345] care must be taken in choosing the pulse delays for the experiments.

Numerous reviews and data compilations covering the ^{13}C-n.m.r. spectroscopy of carbohydrates have recently appeared in the literature.[346-352] As with ^{1}H-n.m.r. spectra, regions for the ^{13}C-n.m.r. signals may be defined: methyl carbon signals for deoxy sugars, pyruvate, and acetate appear from 15-25 p.p.m.; C6 of unlinked hexoses from 60-65 p.p.m.; ring

carbons from 67-83 p.p.m.; an anomeric region from 90-109 p.p.m.; and carbonyl carbons from 166-180 p.p.m.[18] The anomeric region is of prime importance to the structural chemist and may be further subdivided. Generally the resonances of anomeric carbon atoms having an equatorial proton are upfield from those anomers with an axial proton.[347] Thus with D-pyranoses, the C1 signal for the α-anomer will occur at 90-95 p.p.m. (unlinked) or 98-103 p.p.m. (linked) and for the ß-anomer at 95-98 p.p.m. (unlinked) or 103-106 p.p.m. (linked).[18] Mannosyl and rhamnosyl residues do not adhere to this generalization while the anomeric signals for furanoses appear further downfield than those for pyranoses.

Although most ^{13}C-n.m.r. experiments are performed with ^{1}H-decoupling, the measurement of $^{1}J_{CH}$ values from proton-coupled ^{13}C-n.m.r. spectra can be used to reliably determine the anomeric configuration of glycose units. Bock and co-workers[353,354] have shown that for methyl glycosides with axially orientated H1 protons (*e.g.* ß-D-pyranoses), $J_{C1,H1}$ is about 160 Hz while for the other anomer, $J_{C1,H1}$ is about 170 Hz. This distinction also applies to oligosaccharides. Recently Bock and Pedersen[355] have reported the possibility of determining $^{1}J_{CH}$ values from the ^{13}C satellites of high-field ^{1}H-n.m.r. spectra. Each proton signal has two symmetrically situated ^{13}C satellites of 0.5% intensity which contain information concerning ^{13}C-^{1}H coupling. For anomeric protons, these ^{13}C satellites are found 80-85 Hz from the central proton signal and can be used to determine the anomeric configuration.

Bradbury and Jenkins[352] have published a literature survey of ^{13}C-n.m.r. chemical shift data for numerous mono-, di-, and tri-saccharides that provides a useful base for the assignment of ^{13}C-n.m.r. signals in other oligosaccharides. These workers reported a set of empirical rules for the elucidation of trisaccharide structures of known monosaccharide composition however, these rules could not be extended to higher oligosaccharides without additional chemical shift data. Glycosylation effects, consisting of a strong downfield displácement of the linked carbon atom resonance (α-effect) with a smaller upfield displacement of the signals of adjacent carbon atoms (ß-effect), may be seen from this data compilation however, these shifts must be used with discrimination when assigning resonance signals to structural features.[348]

Garegg and co-workers[332,333] and Gorin *et al.*[356] have reported the use of the three ^{13}C-n.m.r. signals for pyruvate ketal substituents to determine the configuration of the ketal carbon atom while Jansson *et al.*[331] have recorded the n.m.r. chemical shifts of mono-*O*-acetylated methyl α- and ß-D-gluco- and galacto-pyranosides. King-Morris and Serianni[357] studied the ^{13}C-n.m.r. spectra of thirty-six aldoses specifically ^{13}C-enriched at the anomeric carbon atom and, on the basis of the ^{13}C-^{13}C coupling patterns, were able to confirm or reassign the signals for these compounds. These workers proposed empirical rules to correlate the ring configurations and conformations of pyranoses with ^{13}C-n.m.r. chemical shifts and coupling constants. McIntyre and Small[358] reported the development of a computer-based simulation for the prediction of ^{13}C-n.m.r. spectra for monosaccharides. SIMPLE n.m.r. spectroscopy (secondary isotope multiplet n.m.r. spectroscopy of partially labelled entities) involves measuring either ^{1}H- or ^{13}C-n.m.r. spectra under conditions of slow D/H exchange whereby the resonances due to separate isotopomers give rise to defined multiplets for each nucleus. Christofides and co-workers have applied SIMPLE ^{1}H-n.m.r. spectroscopy to the analysis of the intramolecular hydrogen bonding in sucrose[359] and sucrose derivatives[360] while Hoffman *et al.*[361] reported the use of SIMPLE ^{13}C-n.m.r. spectroscopy in the structural analysis of glucodisaccharides.

Different systems for referencing n.m.r. signals have appeared in the literature including internal sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate (TSP), sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) and dioxane, and external carbon disulfide and tetramethylsilane (TMS).[348] To avoid the misinterpretation of spectral data, a standard reference system is clearly indicated.[362] All data reported in this thesis have been measured with acetone as an internal standard (δ2.23 for ^{1}H and 31.07 p.p.m. for ^{13}C) relative to external DSS.

(iii) Multiple Pulse Techniques

Various 1D multiple pulse n.m.r. experiments have been applied to the structural elucidation of carbohydrates. Bax *et al.*[363] demonstrated the use of a modified, selective

INEPT (insensitive nuclei enhanced by polarization transfer) experiment to determine glycosyl sequences. This experiment makes use of long-range heteronuclear scalar couplings, $^3J_{HCOC}$, to transfer polarization across the interglycosidic linkage and can be used for oligosaccharides and for polysaccharides providing that the T_2-values exceed 100 ms. The DEPT (distortionless enhancement by polarization transfer) experiment enables sensitivity enhancement and spectral editing of ^{13}C-n.m.r. spectra to be performed simultaneously and has been used to separate CH, CH_2, and CH_3 subspectra from the spectrum of D-digitoxose.[364] Carver and co-workers have used nOe difference spectroscopy to determine the sequence of *N*-linked oligosaccharides[365] and to confirm the existence of interresidue nOe's.[366] A 1D analogue of the 2D HOHAHA (homonuclear Hartmann-Hahn) experiment has been proposed by Davis and Bax[367] to simplify crowded ^{1}H-n.m.r. spectra. This method, in combination with the *z*-filtering technique, has been used by Subramanian and Bax[368] to obtain pure phase n.m.r. subspectra for a disaccharide residue and has thus enabled the measurement of the homonuclear coupling constants.

4.3.2 Two-Dimensional Techniques

In 1- and 2-D multiple pulse F.t.-n.m.r. spectroscopy, three distinct time periods can be defined during the experiment: a preparation period, t_0; an evolution period, t_1; and a detection period, t_2 (see Fig. 2). In the 1D experiments, the system response to a pulse sequence is recorded as a function of the detection period and it is this single time response, $S(t_2)$, that is Fourier transformed into the frequency domain, $S(F_2)$, to produce the n.m.r. spectrum. Aue *et al.*[369] discussed the addition of another parameter, or "dimension", to this classical form of n.m.r. spectroscopy and showed that this could provide a two-dimensional graphic representation of spectral frequency data. It is from this simple theoretical concept that the field of 2D-n.m.r. spectroscopy has so rapidly expanded.

In 2D-n.m.r. experiments, the system response is recorded during the detection period as a function of the evolution time to give a two-dimensional array, $S(t_1, t_2)$. This array is then Fourier transformed twice (see Fig. 2) to obtain a two-dimensional frequency spectrum, $S(F_1,$

F_2), which contains information concerning spectral parameters expressed during t_1 and t_2 along individual axes. The 2D-n.m.r. experiments are carefully controlled using specific pulse sequences and decoupler gating such that a limited number of parameters are expressed during each of the time periods. It is this control over the $S(t_1, t_2)$ array data that has enabled the development of so many different experiments and, as stated by Morris, 'their number is limited only by the ingenuity of experimentalists and the patience of journal Editors'.[312]

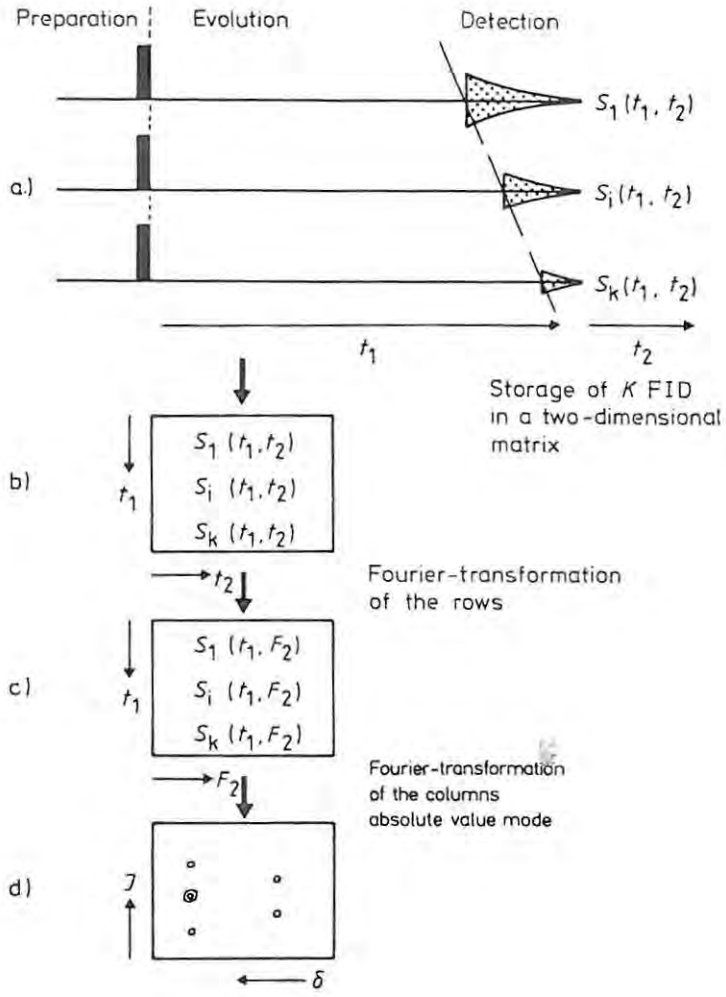


Fig. 2. Data flow in 2D-n.m.r. spectroscopy.[310]

The effects of some pulse sequences on the net macroscopic magnetization of a spin system can be illustrated using the Bloch vector model[307] and the concept of the "rotating frame"[370] however, this treatment is not rigorous and cannot handle the more complex n.m.r. experiments, for example multiple quantum coherence techniques. Sørensen *et al.*[371] have recently proposed a product operator formalism, which is a combination of the simplistic Bloch approach and the rigorous density matrix theory, to describe n.m.r. pulse experiments but even this treatment is restricted to weakly coupled spin systems and to "hard" pulses.[312] Various reviews of the major 2D-n.m.r. techniques and their applications have been published.[310-312]

(i) 2D *J*-Resolved Experiments

Heteronuclear 2D *J*-resolved experiments allow the expression of J_{CH} and δ(C) parameters during the evolution and detection periods, respectively, thus producing a 2D-spectrum of ^{13}C-^{1}H coupling constants on the F_1 axis versus the ^{13}C-chemical shifts on the F_2 axis. The pulse sequence used differs from that of the spin-echo Fourier transform (SEFT) n.m.r. experiment only by having a variable evolution time. The use of the spin-echo sequence ensures that the line width in the F_2 dimension is independent of magnetic field inhomogeneity and corresponds to the natural line width of the signals, thus improving signal resolution. Broad-band decoupling during the preparation period can be used to obtain nuclear Overhauser enhancement.[310]

Hall and Morris[372] applied 2D-*J* spectroscopy to the analysis of raffinose and were able to assign all ^{13}C-chemical shifts and J_{CH} coupling constants. They also assigned the anomeric ^{13}C-signals of the tetrasaccharide repeating unit of *Klebsiella* K32 capsular polysaccharide and found that their results differed from those previously published. Morat *et al.*[373] used a selective heteronuclear 2D-*J* n.m.r. experiment to analyze long-range coupling constants in some mono- and di-saccharides. These workers were able to measure two- and three-bond ^{13}C-^{1}H couplings for the anomeric carbon atoms but found that the technique was limited by carbon signals with short T_2-values.

Homonuclear 2D-*J* resolution is possible for ^{1}H-signals. Since ^{1}H-decoupling is precluded, the spins are coupled during the detection period and thus in the resultant 2D-spectrum, the F_1 axis contains J_{HH} coupling information while the F_2 axis is a function of both δ(H) and J_{HH}. Rotation of the 2D-matrix by 45°, however, produces a spectrum resolving these parameters.[310] Projection along the F_2 axis will give a "^{1}H-decoupled ^{1}H-n.m.r." spectrum and thereby provide an excellent means of solving the hidden-resonance problem experienced in carbohydrate analyses. Hall *et al.*[374] have illustrated the usefulness of this technique in their analysis of mono- and di-saccharide derivatives while Curatolo *et al.*[375] were able to resolve the ^{1}H-signals of α,ß-D-glucose using *J*-resolution on a 500 MHz instrument.

(ii) Heteronuclear 2D Shift Correlated Experiments

Correlated 2D-n.m.r. experiments differ from *J*-resolved 2D experiments in that both dimensions now contain chemical shift information and that an additional time period, the mixing time, is needed between the evolution and detection periods. During this mixing time, "mixing pulses" are used to transfer magnetization between the nuclei under study, *i.e.* to correlate the resonance signals.

Heteronuclear 2D shift correlated (HETCORR) experiments for ^{1}H and ^{13}C nuclei allow δ(H) to evolve during the evolution period and thus eventually to appear on the F_1 axis of the spectrum. During the mixing time, a pulse sequence for polarization transfer correlates the ^{1}H and ^{13}C nuclear spins and also enhances ^{13}C sensitivity. Broad-band ^{1}H-decoupling during the detection period allows δ(C) to be monitored in the t_2 dimension, the 2D spectrum thus revealing chemical shift correlations between ^{1}H and ^{13}C nuclei.[310]

Hall and co-workers[376,377] have reported the analyses of di- and tri-saccharides using HETCORR experiments. These experiments are useful for the structural analyses of carbohydrates because firstly the heteronuclear correlations serve to identify or to confirm signal assignments made by comparison with model compounds (this is especially useful for anomeric signals) and secondly the ^{1}H-signals are spread out over the ^{13}C-n.m.r. range of chemical shifts and thus the resolution of overlapping signals is enhanced. However, one

limitation to these experiments is that connectivities between different ^{13}C-^{1}H systems are not expressed and so complete spin systems cannot be identified. HETCORR experiments are often used in conjunction with their homonuclear equivalent (COSY) because of this limitation.[378,379]

(iii) Homonuclear 2D Shift Correlated Experiments

Homonuclear 2D shift correlated spectroscopy produces a 2D spectrum in which both frequency axes contain the resonances of the same nucleus, for example COSY correlates δ(H). The basis for the COSY experiment is the Jeener sequence of two 90° pulses separated by the evolution period and followed by the detection period.[369] The first pulse generates transverse magnetization while the second pulse has the effect of a mixing pulse and results in an exchange of magnetization between coupled nuclei. The 2D spectrum produced contains "diagonal-peaks" where $F_1 = F_2$ and symmetrically disposed "cross-peaks" which indicate scalar spin-spin couplings. There are phase differences associated with these peaks. From these cross-peaks, it is theoretically possible to trace the connectivities of complete spin systems however, one limitation of COSY is that the couplings must be partially resolved before they can give rise to a cross-peak.[312]

Several variations of the COSY pulse sequence have been proposed. COSY45 uses a 45° mixing pulse and thereby suppresses signals that are near the diagonal and produces cross-peaks that are sensitive to the relative sign of the coupling constant. In the SECSY experiment, the mixing pulse (90°) is applied half-way through the evolution period, causing the F_1 domain to represent differences between the chemical shifts of scalar-coupled nuclei. By adding an additional fixed time delay after each 90° pulse in the Jeener sequence it is possible to get correlations due to long-range couplings. The (multiple-) RELAYED-COSY experiment introduces additional pulse sequences onto the COSY sequence thereby causing consecutive coherence transfer processes to occur.[380] These relayed experiments enable spin systems in complex ^{1}H-n.m.r. spectra to be analyzed. Other variations include phase-sensitive COSY and quantum-filtered (DQF, TQF, and MQF) COSY.

Bernstein *et al.*[381] discussed the application of COSY and SECSY to the assignment of the ^{1}H-n.m.r. signals of carbohydrates, using trideuteriomethyl 2,3,4,6-tetra-*O*-(trideuterioacetyl)-α-D-glucopyranoside as an example. A protocol for the *de novo* sequencing of oligosaccharides by ^{1}H-n.m.r. spectroscopy was proposed by Bernstein and Hall[382] and involved the complete assignment of all signals using 2D-*J* spectroscopy and COSY followed by sequence determination using selective H1 irradiation to measure nOe's. These workers applied the protocol to the analysis of allyl ß-D-galactopyranosyl-1→4-ß-D-glucopyranoside.

Since these early examples, numerous workers have used 2D-n.m.r. shift correlated spectroscopy to establish carbohydrate structures, for example COSY has been used by Jones[383] to identify the tetrasaccharide repeating unit of *Streptococcus pneumoniae* type 23 polysaccharide, by Massiot *et al.*[384] to identify and sequence the sugars in saponins, and by Dua *et al.*[385] to characterize oligosaccharide alditols from glycoproteins. RELAYED-COSY has been applied to the analysis of human meconium glycoproteins,[386] sucrose octa-acetate,[387] and cello-oligosaccharides.[388] Dabrowski *et al.*[389] have used phase-sensitive COSY to study three peracetylated glycosphingolipids and Scarsdale *et al.*[390] have used multiple quantum-filtered COSY to determine the primary structure of an octasaccharide glycolipid from the spermatozoa of bivalves.

(iv) 2D Nuclear Overhauser Effect Spectroscopy

Nuclear Overhauser effects are caused by dipole-dipole interactions between nuclei and are inversely proportional to the sixth power of the internuclear distance. These effects are used in the study of molecular structures because they provide information on the spatial proximity of nuclei. This information complements that obtained from the through-bond connectivities by correlated spectroscopy and may be used to determine the sequence of isolated spin systems. The pulse sequence used for nOe spectroscopy (NOESY) resembles that used for COSY however, it is aimed at detecting any exchange of *z*-magnetization between nuclei during the mixing time, which will then show up as cross-peaks in the 2D spectrum.[312]

Huang *et al.*[391] have reported the use of 2D-*J*-spectroscopy, homonuclear correlated spectroscopy, and NOESY for the complete, unambiguous assignment of the ^{1}H-signals from the trisaccharide repeating unit of the group A streptococcal polysaccharide. Jansson *et al.*,[392] after an initial characterization of the sugar residues, determined the complete structure of the pentasaccharide repeating unit from the *E. coli* O2 lipopolysaccharide by HETCORR, COSY, and NOESY experiments. Jansson *et al.*[393] used NOESY to determine the glycosyl sequences in their study on some *Shigella flexneri* O-polysaccharides.

(v) 2D Homonuclear Hartmann-Hahn Spectroscopy

Recently Davis and Bax[394] proposed a new type of 2D-n.m.r. experiment, 2D homonuclear Hartmann-Hahn (HOHAHA) spectroscopy, to determine homonuclear scalar connectivities in complex molecules based on the same principles as Hartmann-Hahn cross-polarization experiments. During the experiment, homonuclear cross-polarization causes a transfer of net magnetization which results in a phase-sensitive 2D spectrum with all peaks in the near absorption mode. This enhances sensitivity and allows small amounts of sample to be analyzed. The net magnetization transfer will also introduce relayed connectivities and thus enable the determination of spin system sequences. 2D HOHAHA spectroscopy was applied by these workers to the analysis of the icosapeptide alamethicin (1 mg) and by Bax *et al.*[395] to the structural elucidation of desertomycin (16 mg).

(vi) 2D Proton-Detected Heteronuclear Spectroscopy

Lerner and Bax[396] have recently reported the use of three new 2D heteronuclear n.m.r. methods that rely on the indirect observation of ^{13}C nuclei *via* ^{1}H-detection (reverse detection). These new methods have a much greater sensitivity than the conventional ^{13}C-detection methods and thus allow small quantities of carbohydrate material ($\sim$1 mg) to be analyzed, for example Lerner and Bax[396] were able to completely assign the ^{1}H- and ^{13}C-n.m.r. spectra and sequence both anomers (α $\sim$1.2 mg, β $\sim$2.3 mg) of a trisaccharide. These methods determine one-bond (^{13}C-^{1}H), relayed, and interglycosidic long-range connectivities

and, in conjunction with *z*-filtered HOHAHA spectroscopy, allow the structure and conformation of saccharides to be elucidated.

One major problem with the ^{1}H-detected methods is that protons not coupled to ^{13}C nuclei give strong, unwanted signals however, these signals are attenuated either by using a BIRD pulse or by phase cycling and subtraction of alternate scans. The ^{1}H-detected methods have been applied to the structural study of *Haemophilus influenzae* type a capsular polysaccharide (10 mg) by Byrd *et al.*[397]

^{1}H-Detected and HOHAHA spectroscopy promise to be two of the most powerful techniques available to the n.m.r. spectroscopist for the *ab initio* instrumental analysis of complex carbohydrate molecules but they require spectrometer modifications which are not available to this laboratory.

5. STRUCTURAL ELUCIDATION OF CAPSULAR ANTIGENS

5. THE STRUCTURAL STUDIES OF SOME CAPSULAR POLYSACCHARIDES FROM *ESCHERICHIA COLI* AND *KLEBSIELLA*.

5.1 GENERAL EXPERIMENTAL METHODS

Unless otherwise stated, the methods described in this section were those used for the elucidation of the polysaccharide structures in Sections 5.2 - 5.5. Experimental parameters not specified in this Section will be given under the relevant Section.

5.1.1 Instrumental Techniques

Gas-liquid chromatography. - Analytical g.l.c. was performed using a Hewlett-Packard 5890A gas chromatograph, fitted with flame-ionization detectors, and a 3392A recording integrator. A J & W Scientific fused silica DB-225 bonded-phase capillary column (30 m x 0.25 mm) having a film thickness of 0.25 μm was used with helium as the carrier gas and operated isothermally at 205°, 225°, or 230°. Retention times of partially methylated alditol acetates relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol were compared with those reported by Barreto-Bergter *et al.*[205]

Mass spectrometry. - G.l.c.-m.s. was conducted on either a VG Micromass 16F spectrometer with an ionization energy of 40 eV and an ion-source temperature of 170° (Sections 5.2 and 5.4) or a Hewlett-Packard 5988A mass spectrometer with an ionization energy of 70 eV and an ion-source temperature of 200° (Sections 5.3 and 5.5).

Nuclear magnetic resonance spectroscopy. - N.m.r. spectra were recorded with a Bruker WM-500 Ft spectrometer at 30° or at 95°. Samples were deuterium-exchanged by freeze-drying from deuterium oxide solutions and acetone was used as the internal standard, δ2.23 for ^{1}H and 31.07 p.p.m. for ^{13}C, measured against external sodium 4,4-dimethyl-4-silapentane-1-sulfonate. All 2D n.m.r. experiments were performed using standard Bruker software. N.m.r. spectra tabulated and discussed in Sections 5.2 - 5.5 are collated in Sections 7.1 - 7.3.

Gel-permeation chromatography. - G.p.c. was performed using a Pharmacia Fine Chemicals FRAC-100 fraction collector and P-1 peristaltic pump with a constant pressure Mariotte flask

as the eluent reservoir. All fractions collected were analyzed by the phenol-sulfuric acid method[50] using a Beckman model DB spectrophotometer (490 nm). Analytical g.p.c. profiles for polysaccharides were obtained using either a column of Sepharose 4B (1.6 x 60 cm) eluted with M sodium chloride at 15 $mL.h^{-1}$ (Section 5.2) or a column of Sephacryl S500 (1.6 x 65 cm) eluted with 500:5:2 water-pyridine-acetic acid (pyridinium acetate buffer) at 21 $mL.h^{-1}$ (Sections 5.3 and 5.4), and collecting 1 mL fractions. Both columns were calibrated using dextrans. Analytical g.p.c. profiles for crude bacteriophage degradation products were obtained using a column of Bio-Gel P4 (1.6 x 40 cm) eluted with pyridinium acetate buffer at 8.4 $mL.h^{-1}$ (1 mL fractions) while preparative g.p.c. separations of the same material were achieved using a larger column (2.6 x 70 cm) and a faster flow-rate (21 $mL.h^{-1}$, 3 mL fractions).

Other techniques. - Optical rotations of aqueous solutions in a 1-cm cell were measured at 21-23° with a Perkin-Elmer model 141 polarimeter. Infrared spectra were recorded on a Beckman IR8 spectrophotometer for solutions in dry chloroform. Ultracentrifugation (35000 r.p.m., 3 h) was performed using a Beckman L8-80M ultracentrifuge with a type 70.1 Ti rotor.

5.1.2 Chemical Analyses

Isolation of acidic capsular polysaccharide material. - Authentic cultures of the bacterial serotypes were obtained from Dr I. Ørskov (Copenhagen). Nutrient broth was obtained from Difco, nutrient agar from Merck, and agar and Mueller-Hinton agar from Biolab; all culture media were sterilized by autoclaving at 121° for 20 min. The bacterial serotype was plated out on Mueller-Hinton agar and incubated overnight at 37°; this procedure was repeated until single, actively growing colonies were distinguished. These colonies were used for the large-scale growth of the bacteria on trays (6-12, 40 x 60 cm) containing Mueller-Hinton agar (1.5 L) where each tray was inoculated with a 55 mL bacterial culture in nutrient broth (4 h, 37°) and incubated for 36-96 h. The cells and slime were then removed from the surface of the agar, combined, diluted with an equal volume of 2% phenol, and the suspension stirred overnight at 4°. The suspension was ultracentrifuged and the supernatant precipitated into ethanol (5 volumes). The precipitate was recovered (centrifugation), washed (ethanol), and

redissolved in a minimum volume of water. A 5% cetyltrimethylammonium bromide (CTAB) solution was added slowly with stirring to this solution until no further precipitation of the acidic polysaccharide-CTAB complex occurred. This precipitate was collected (centrifugation) and, after washing with water, dissolved in 3M sodium chloride to break the complex. The polysaccharide was then precipitated from this solution using ethanol (5 volumes), isolated (centrifugation), washed (ethanol), and dissolved in a minimum volume of water. This solution, containing the polysaccharide, was exhaustively dialyzed against running water (12-14 000 Mw cut-off; 3 d). Finally the retentate was ultracentrifuged and the supernatant freeze-dried to yield the purified acidic polysaccharide capsular material.

Sugar composition. - A sample of the polysaccharide (∿3 mg) was hydrolyzed with 2M trifluoroacetic acid (TFA) overnight at 100°. The acid was removed by coevaporation with water under reduced pressure at a bath temperature not exceeding 40°. The released monosaccharides were converted into the peracetylated aldononitrile (PAAN) derivatives by the McGinnis method[74] and analyzed by g.l.c. (225°). To identify uronic acid residues, a sample of the polysaccharide (∿5 mg) was methanolyzed with anhydrous 3% methanolic HCl at 80° for 18-20 h. After removal of the acid ($PbCO_3$), the methanolyzate was carboxyl reduced overnight with sodium borohydride (∿50 mg) in anhydrous methanol and then hydrolyzed (2M TFA, 8 h, 100°). The sugar residues thus produced were derivatized and analyzed as above. Oligosaccharide samples were similarly analyzed however hydrolysis times were shortened. The reducing end sugar of oligosaccharides was determined by the Morrison method[76] involving the reduction of the sample to the oligosaccharide alditol ($NaBH_4$, water), hydrolysis, and subsequent analysis *via* the McGinnis method.[74]

Methylation analysis. - Permethylation of saccharide samples was achieved by the Hakomori method.[113] A dried sample (free acid form; ∿20 mg) was dissolved in DMSO (3 mL) with stirring under nitrogen, methylsulfinyl (dimsyl) anion[115,398] (1.5 mL) was added and the reaction mixture was stirred at room temperature for 1-2 h. The reaction mixture was cooled in ice and methyl iodide (1.5 mL) was slowly added with stirring. After 3-4 h, this solution was then either dialyzed against running water (12-14 000 Mw cut-off) and freeze-dried

(polymeric samples) or extracted with dichloromethane and evaporated (oligomeric samples). The chloroform soluble permethylated product thus obtained was purified by passage down a column of Sephadex LH-20. The extent of etherification was monitored by i.r. spectroscopy. In some cases (Sections 5.2 and 5.3) the methylated product was remethylated by the Kuhn method[399] to ensure complete etherification. The methylated product was dissolved in dimethylformamide (DMF, 0.5 mL), and stirred in the presence of methyl iodide (1.5 mL) and silver oxide (100 mg) at room temperature for 72 h, with two additional amounts of methyl iodide and silver oxide being added during this period. The permethylated product was isolated, after removal of the silver precipitate (centrifugation) and DMF (high vacuum, 70°), and purified as above. A sample of the permethylated product was hydrolyzed (2M TFA, overnight, 100°), reduced ($NaBH_4$, 1-2 h), and acetylated with 1 : 1 pyridine-acetic anhydride (1 h, 100°). A second sample was methanolyzed (18 h, 80°), reduced ($NaBH_4$ in anhydrous methanol, 16 h), hydrolyzed (2M TFA, 18 h, 100°), and similarly derivatized. The partially methylated alditol acetate (PMAA) derivatives were analyzed by g.l.c. (205°) and g.l.c.-m.s. Some oligosaccharides were reduced with sodium borodeuteride (∿40 mg) in deuterium oxide (1 mL) prior to methylation analysis, deuterium oxide being used to ensure maximal deuterium labelling at C1.

Oligosaccharides containing base labile substituents were methylated by the method reported by Prehm.[129] 2,6-Di-*tert.*-butyl-4-methyl pyridine (150 mg) and methyl trifluoromethanesulfonate (150 µL) were added to a solution of the oligosaccharide (∿6 mg) in trimethyl phosphate (1 mL) which was then heated at 50° for 2 h under nitrogen. The methylated product was extracted from this solution using chloroform and purified on a column of Sephadex LH-20. The product was analyzed as described above.

De-O-acetylation. - Oligosaccharides were de-*O*-acetylated under alkaline conditions using the method proposed by Amemura *et al.*[400] A sample of the oligosaccharide (∿15 mg) was dissolved in an oxygen-free 10 mM aqueous potassium hydroxide solution (13 mL) and the reaction left at room temperature for 5 h under nitrogen. The solution was then neutralized with M acetic acid (ice-bath), passed down a column of Amberlite IR-120(H^+) resin at 4°, and freeze-dried to yield the de-*O*-acetylated product.

Determination of absolute configuration. - The method proposed by Leontein *et al.*[99] was used to determine the absolute configuration of monosaccharide residues. A sample (∿6 mg) was carboxyl reduced (as above), hydrolyzed (2M TFA, 8 h, 100°), then dissolved in (-)-2-octanol (0.5 mL) with one drop of TFA and heated in a sealed ampoule at 130° overnight. After concentration to dryness under vacuum at 55°, the residue was acetylated (pyridine-acetic anhydride) and the peracetylated octyl glycosides thus prepared were analyzed by g.l.c. (230°).

Bacteriophage degradation.[172] - The bacteriophage was isolated from sewage, purified by repicking single plaques, and propagated on its host strain in nutrient broth until a titre of about 10^{13} PFU was obtained. The bacteriophage solution (∿500 mL) was purified by dialysis (12-14 000 Mw cut-off) and reduced in volume by evaporation under reduced pressure at 30°. Polysaccharide (∿0.5 g) was added to this solution (∿150 mL) and the reaction mixture was stirred at 30° for 72 h in the presence of chloroform (1 mL). The bacteriophage degraded polysaccharide solution was then freeze-dried and dialyzed (3500 Mw cut-off) against frequently changed distilled water. The diffusates were combined and purified by several passages down an ice-jacketed column of Amberlite IR-120(H^+) resin to give the crude bacteriophage degradation product. The product was analyzed and separated by g.p.c.

General reaction methods. - Hydrolyses were conducted using 2M TFA (∿2 mL) at 100°. Carboxyl reductions were effected after methanolyses by treating the sample in methanol (2 mL) with sodium borohydride (∿50 mg) overnight.

5.2 STRUCTURAL INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE OF *ESCHERICHIA COLI* K37.

5.2.1 Introduction

The structures of about thirty of the seventy-two *E. coli* capsular polysaccharides have been elucidated. Two of these polysaccharides have been reported to contain a 1-carboxyethylidene group (pyruvate ketal); in K29 the ketal is 4,6-linked to the terminal glucopyranosyl residue in the side-chain[401] and in K26 it is 3,4-linked to a pendant rhamnopyranosyl residue.[402] Both K29 and K26 polysaccharides also contain a glucopyranuronosyl residue. Serological cross-reactions have been reported[4] for the K37-K97 and K16-K97 antigens however, no cross-reaction was observed for the K16-K37 antigens. It would therefore be of interest to determine the structures of these antigens to try and rationalize these serological findings on a structural basis.

5.2.2 Results and Discussion

Composition of K37 polysaccharide. - The bacteria were grown on Mueller-Hinton agar containing 2 $g.L^{-1}$ NaCl and the acidic polysaccharide was isolated by precipitation with CTAB. G.p.c. (Fig. 3A) showed the native capsular extract to be polydisperse (three peaks of M_r = 6.3 x 10^6, 4.6 x 10^6, and 2.0 x 10^6). Treatment of this polysaccharide with Amberlite IR-120(H^+) resin yielded a product having a single broad peak (Fig. 3B, M_r = 1.2 x 10^4). Phosphate analysis by the Molybdenum Blue method[403] before and after hydrolysis of the native polysaccharide showed the polymer to contain 1 mol of phosphate per 9 mol of trisaccharide repeating unit (see below). This result suggests that the native polysaccharide exists as short polysaccharide chains substituted by phospholipid, which is in accordance with the report[37] that some *E. coli* K-antigens (K2, K5, K12, and K62) are linked *via* their reducing ends to 1,2-dipalmitoylglycerol by labile phosphodiester bridges. The high relative molecular masses of the native polysaccharide can be ascribed to micellar aggregation which is broken down on treatment with the cation exchange resin. Treatment of polysaccharides with dilute alkali has

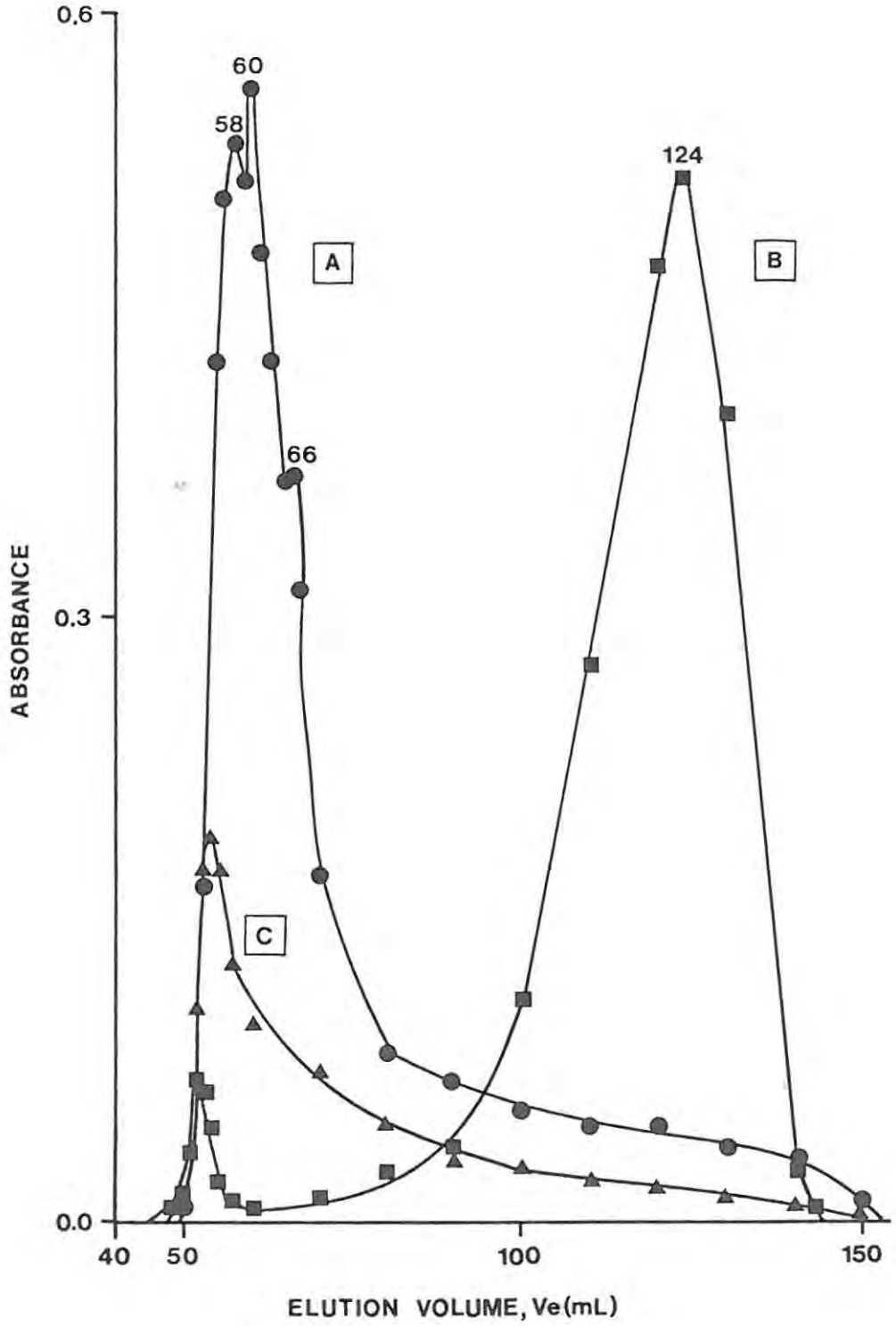


Fig. 3. Gel-permeation chromatography on Sepharose 4B : A, K37 polysaccharide (●); B, K37 polysaccharide after treatment with Amberlite IR-120(H^+) resin (■); and C, alkali-treated (8 h) K37 polysaccharide (▲).

been shown to produce similar deaggregation;[404-407] this did not occur with the K37 polysaccharide (Fig. 3C).

G.l.c. analysis of the PAAN derivatives from an acid hydrolyzate of the polysaccharide, with and without prior carboxyl reduction, showed the polysaccharide to contain glucose and galactose in a molar ratio of 1.0:~2.2 (Table II, columns I and II). The slight excess of galactose may possibly be due to the presence of a galactan contaminant (see Sections 5.3 and

TABLE II

SUGAR ANALYSES OF *E. coli* K37 POLYSACCHARIDE AND DERIVED PRODUCTS

Sugar (as peracetylated aldononitrile)	Molar ratio [a,b]					
	I	II	III	IV	V	VI
Glucose	1.00	1.00	1.00	1.00	1.00	-
Galactose	2.31	2.19	1.60	1.17	-	1.00
Galactitol[c]	-	-	-	1.37	1.32	-

[a]Determined on a capillary DB-225 column at 225°. [b]I, Native K37 polysaccharide; II, carboxyl-reduced K37 polysaccharide; III, Smith-degraded polysaccharide (2); IV, 3-alditol; V, 4-alditol; and VI, 5. [c]Galactitol hexa-acetate.

5.4). The polysaccharide had $[\alpha]_D$ + 105° (water) which indicates a preponderance of α-linkages.[408] This was confirmed by ^{1}H-n.m.r. spectroscopy (Table III) of a partially autohydrolyzed sample of the polysaccharide (1), which indicated, *inter alia*, the presence of two α-linkages (δ5.09 and 5.40) and one ß-linkage (δ4.72, $J_{1,2}$ 7.O Hz), and the presence of one pyruvate ketal per trisaccharide repeating unit. The assignment of the anomeric signals was made by comparing the ^{1}H-n.m.r. spectrum of **1** with those of derived poly- and oligo-saccharides (see below). The ^{13}C-n.m.r. data for **1** substantiated these results (Table III)[349] and, in addition, the signals at 25.83 and 174.10 p.p.m. were assigned to an equatorial methyl

TABLE III

N.M.R. DATA (500 MHz) FOR *E. coli* K37 POLYSACCHARIDE AND DERIVED PRODUCTS

^{1}H-N.m.r. data				^{13}C-N.m.r. data	
δ^a (p.p.m.)	$J_{1,2}{}^b$ (Hz)	Integral (no. of H)	Assignment^c	δ^d (p.p.m.)	Assignment^e
-3Glcβ1-3(4,6-pyr-Galα1-4)Galα1- Autohydrolyzed K37 polysaccharide (**1**)^f					
5.40	4.0	1.0	-3,4Galα1-	174.10	COOH of pyr^g
5.09	n.o.	1.0	4,6-pyr-Galα1-	105.20	-3Glcβ1-
4.72	7.0	1.0	-3Glcβ1-	100.67	4,6-pyr-Galα1-
1.50		3.0	CH_3 of pyr	99.77	-3,4Galα1-
				25.83	CH_3 of pyr
-3Glcβ1-3Galα1- Smith-degraded K37 polysaccharide (**2**) 4 O R R^h = H_a—CH(CH$_2$OH)—*etc.*					
5.42	n.o.	0.2	-3(RO-4)Galα1-		
5.38	n.o.	0.8	-3Galα1-		
4.80	(t)	0.2	H_a		
4.74	7.0	1.0	-3Glcβ1-		
Glcβ1-3(4,6-pyr-Galα1-4)Gal (**3**)					
5.33	n.o.	~0.4	-3,4Galα	176.90	COOH of pyr
5.06	4.0	~0.4	4,6-pyr-Galα1-(4Galα)	105.21	Glcβ1-
				100.74	4,6-pyr-Galα1-
5.05	4.0	~0.6	4,6-pyr-Galα1-(4Galβ)	97.11	-3,4Galβ
				93.07	-3,4Galα
4.68	8.0	~0.6	Glcβ1-(3Galβ)	26.02	CH_3 of pyr
4.66	8.0	~0.4	Glcβ1-(3Galα)		
4.65	8.0	~0.6	-3,4Galβ		
1.48		3.0	CH_3 of pyr		

TABLE III (*continued*)

^{1}H-N.m.r. data				^{13}C-N.m.r. data	
δ[a] (p.p.m.)	$J_{1,2}$[b] (Hz)	Integral (no. of H)	Assignment[c]	δ[d] (p.p.m.)	Assignment[e]
Glcβ1-3(4,6-pyr-Galα1-4)Galactitol (**3**-alditol)					
5.22	~3.0	1.0	4,6-pyr-Galα1-	175.10	COOH of pyr
4.54	8.0	1.0	Glcβ1-	104.68	Glcβ1-
1.56		3.0	CH_3 of pyr	101.33	4,6-pyr-Galα1-
				25.83	CH_3 of pyr
Glcβ1-3Gal (**4**)					
5.30	n.o.	0.3	-3Galα		
4.70	8.0	0.3	Glcβ1-(3Galα)		
4.69	8.0	0.7	Glcβ1-(3Galβ)		
4.63	8.0	0.7	-3Galβ		
4,6-pyr-Gal (**5**)					
5.32	4.0	0.4	4,6-pyr-Galα		
4.62	8.0	0.6	4,6-pyr-Galβ		
1.48		1.8	CH_3 of pyr (β)		
1.46		1.2	CH_3 of pyr (α)		

[a]Chemical shift measured from internal acetone, δ2.23. [b]n.o. = not observed. [c]For example, -3,4Galα1- refers to the anomeric proton of a 3,4-linked galactopyranosyl unit in the α-anomeric configuration. [d]As in *a* with acetone 31.07 p.p.m. [e]As in *c* but for ^{13}C nuclei. [f]For origin of compounds, see text. For n.m.r. spectra, see Sections 7.1-7.3. [g]pyr = 1-carboxyethylidene (pyruvate) ketal. [h]R = remnant from incomplete Smith hydrolysis.

group and an axial carboxyl group of the pyruvate ketal, respectively.[332,333] No other signals for carboxyl groups were observed.

Methylation analysis. - The native polysaccharide was permethylated and the derived alditol acetates analyzed by g.l.c. and g.l.c.-m.s. (Table IV, column I). The results confirm the idea of a trisaccharide repeating unit, establish a galactose branch point, and locate the pyruvate ketal on the other galactose residue.

Periodate oxidation and Smith degradation. - The K37 polysaccharide consumed 0.97 mol of periodate per mol of trisaccharide repeating unit (Fig. 4), thus indicating the presence of a pair of vicinal hydroxyl groups within the repeating unit and supporting the results of the methylation analysis. Smith degradation of the polysaccharide gave a polymeric product (2). The sugar analysis of 2 (Table II, column III) revealed that a galactose residue had been cleaved from the native polysaccharide. Two major anomeric signals at δ4.74 and δ5.38 were observed in the ^{1}H-n.m.r. spectrum of 2, thus allowing the α-signal at δ5.09 in the ^{1}H-n.m.r. spectrum of 1 to be assigned to a galactose residue. The α-signal in the ^{1}H-n.m.r. spectrum of 2 is twinned (δ5.38 (0.8H), δ5.42 (0.2H)) due to the presence of an uncleaved fragment remaining after incomplete Smith hydrolysis; this allows the signal at δ5.40 for 1 to be assigned to the branch point galactose unit. The remaining anomeric signal for 1 (δ4.72) is therefore due to a ß-glucose residue. The triplet at δ4.80 (0.2H) in the ^{1}H-n.m.r. spectrum of 2 can be assigned to the proton of the uncleaved acyclic acetal fragment (see Table III). No n.m.r. signal was observed for a pyruvate ketal in the spectrum of 2. Methylation analysis of 2 (Table IV, column II) complements the ^{1}H-n.m.r. data. The disappearance of the 2,3-di-*O*-methylgalactose derivative and the pyruvate ketal (n.m.r.) from the analyses of 2 indicates that the native polysaccharide contains a terminal 4,6-pyruvalated galactose unit which is cleaved by periodate. The appearance of a 2,4,6-tri-*O*-methylgalactose derivative and a concomitant reduction in the amount of the 2,6-di-*O*-methylgalactose derivative indicates that the oxidized moiety had been attached to the 4-position of the branch point. Incomplete Smith hydrolysis of the oxidized side-chain fragment resulted in the presence of the dimethyl derivative.

TABLE IV

METHYLATION ANALYSES OF *E. coli* K37 POLYSACCHARIDE AND DERIVED PRODUCTS

Methylated sugar[a] (as alditol acetate)	T_R[b]	Molar ratio[c,d]				
		I	II	III	IV	V
1,2,4,5,6-Gal	0.52	-	-	-	0.66	-
1,2,5,6-Gal	0.85	-	-	0.87	-	-
2,3,4,6-Glc	1.00	-	-	1.00	1.00	-
1,2,3,5-Gal	1.32	-	-	-	-	1.00
2,4,6-Glc	1.61	1.00	1.00	-	-	-
2,4,6-Gal	1.79	-	0.45	-	-	-
2,6-Gal	2.61	1.08	0.57	-	-	-
2,3-Gal	3.69	1.17	-	0.74	-	-

[a]1,2,4,5,6-Gal = 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylgalactitol, *etc.*; all substitution patterns were confirmed by g.l.c.-m.s. [b]Retention time (T_R) relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol run under the same chromatographic conditions. [c]Determined on a capillary DB-225 column at 205°. [d]I, Native K37 polysaccharide; II, Smith-degraded polysaccharide (**2**); III, 3-alditol; IV, 4-alditol; and V, 5-alditol.

Partial acid hydrolysis. - The products of the partial hydrolysis of native K37 polysaccharide were separated by p.c. (solvent *D*) and yielded four fractions (**3**, **4**, **5**, and **6**). Sugar analysis of **3** and **4** by the Morrison method[76] (Table II, columns IV and V) indicated that these compounds were a trisaccharide (1:2 Glc-Gal) and a disaccharide (1:1 Glc-Gal), respectively, both containing a galactose reducing end. The sugar analysis of **5** showed this compound to contain only galactose (Table II, column VI). Methylation analysis of **3** (Table IV, column III), after reduction ($NaBD_4$) to the alditol, showed that this compound was composed of a branched trisaccharide unit with the galactose reducing end having terminal,

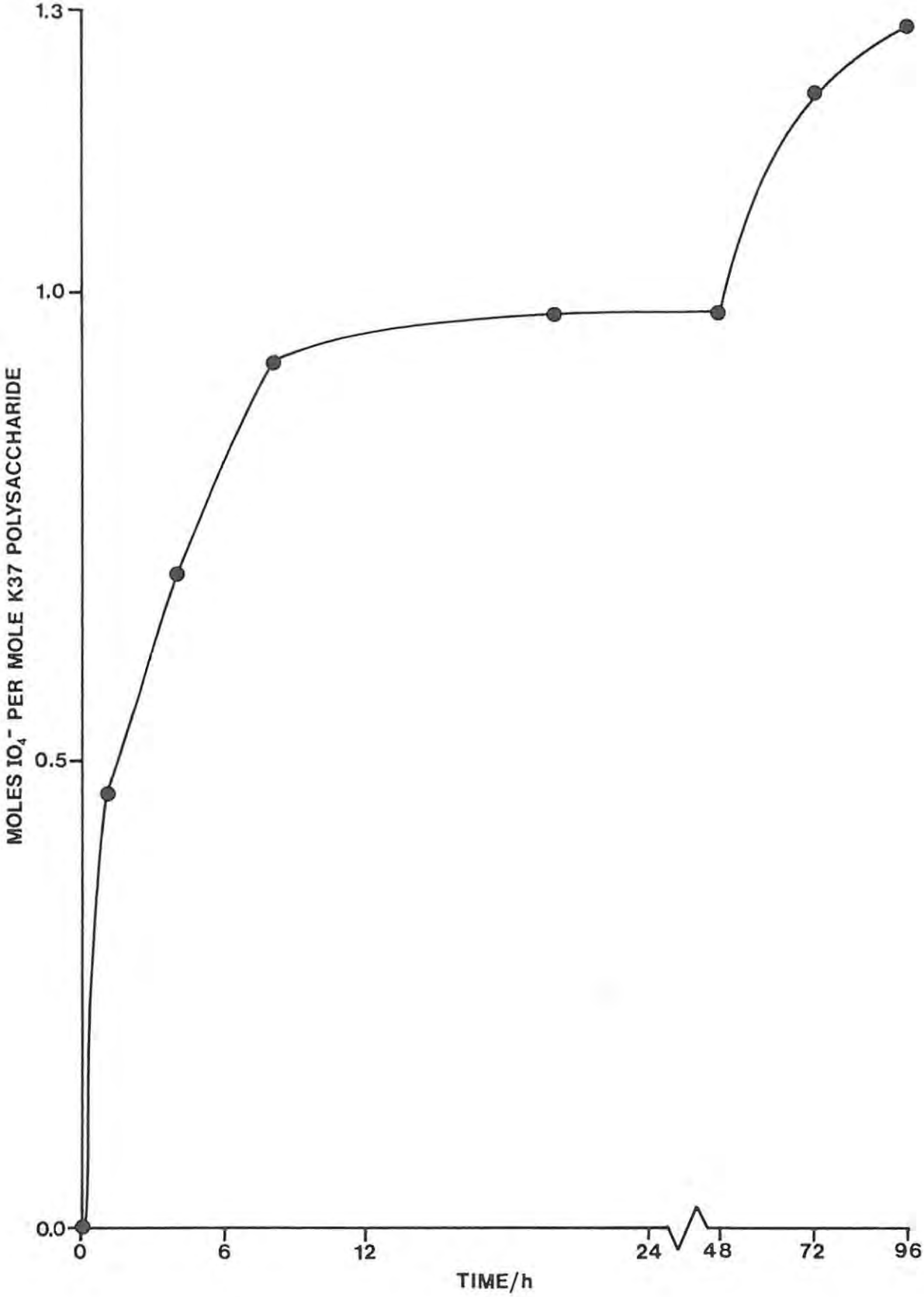
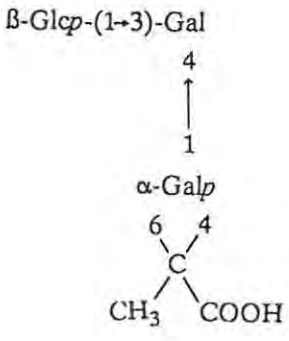


Fig. 4. Periodate absorption curve for K37 polysaccharide.

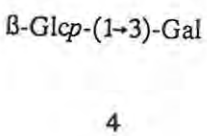
non-reducing substituents (a glucose and possibly a pyruvalated galactose residue) at C3 and C4. Methylation analysis of **4** after $NaBD_4$ reduction (Table IV, column IV) showed this compound to be Glc*p*-(1 → 3)-Gal; the pentamethyl derivative in this analysis is more volatile than the tetramethyl derivative and therefore some is lost during evaporation steps. Reduction of **5** ($NaBH_4$) and subsequent methylation analysis indicated that **5** was a monomeric galactose unit 4,6-disubstituted with non-carbohydrate residues.

^{1}H- and ^{13}C-n.m.r. data for **3-5** are collected in Table III. The ^{1}H-n.m.r. data for **5** indicates that the compound is a pyruvalated galactose sugar. The ^{1}H-n.m.r. data for the disaccharide **4** shows a twinned ß-anomeric signal (1H) for a glucose residue at δ4.69 ($J_{1,2}$ 8.0 Hz, 0.7H) and δ4.70 ($J_{1,2}$ 8.0 Hz, 0.3H), and signals for a reducing galactose sugar at δ4.63 ($J_{1,2}$ 8.0 Hz, 0.7H, ß) and δ5.30 (0.3H, α). ^{1}H- and ^{13}C-n.m.r. data for **3** confirms that the compound is a trisaccharide. The reducing sugar of **3** gives ^{1}H-n.m.r. signals at δ4.65 ($J_{1,2}$ 8.0 Hz, ∿0.6H, ß) and δ5.33 (∿0.4H, α) while the other two sugar residues give twinned signals at δ4.66-4.68 ($J_{1,2}$ 8.0 Hz, ß) and δ5.05-5.06 ($J_{1,2}$ 4.0 Hz, α); the twinning of these signals indicates that both residues are attached to the reducing sugar. The assignments of these signals (Table III) were made by considering the α/ß ratio of the reducing sugar signals for **3** and the ^{1}H-n.m.r. data for **4** and **5**, and show a galactose reducing sugar to which are attached a ß-linked glucose residue and an α-linked pyruvalated galactose sugar. The ^{13}C-n.m.r. data for **3** (Table III) confirm the assignments made for the ^{1}H-n.m.r. data. From the above results, it is possible to corroborate the anomeric assignments made for **1** which were based upon the n.m.r. data for **2**.

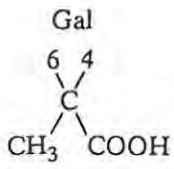
The results from the sugar, methylation, and n.m.r. analyses of **3-5** allow the following structures for these compounds to be proposed.



3



4



5

It is noteworthy that although both anomeric ^{1}H-n.m.r. signals for the residues attached to the galactose reducing end in **3** are twinned due to mutarotation (see Fig. 5), the effect is greater on the ß-glucose signal as would be expected since this residue is closer to the mutarotating anomeric centre than the α-linked pyruvalated galactose residue. Reduction of **3** to the alditol (**3**-alditol, Table III) resulted in a significant downfield shift of the ^{1}H-n.m.r. signal for the α-linked sugar. The pyruvate ketal is assigned the *R*-configuration from the ^{13}C-n.m.r. signals for the methyl and carboxyl groups;[332,333] this pyruvalated galactose unit has been found in numerous biological polysaccharides.[409-411]

Determination of absolute configuration.[99] - From p.c., **6** was identified as a mixture of monosaccharides. These monosaccharides were converted to the acetylated (-)-2-octyl glycosides and analyzed by g.l.c. to determine their absolute configuration; all the sugar residues were shown to have the D-configuration.

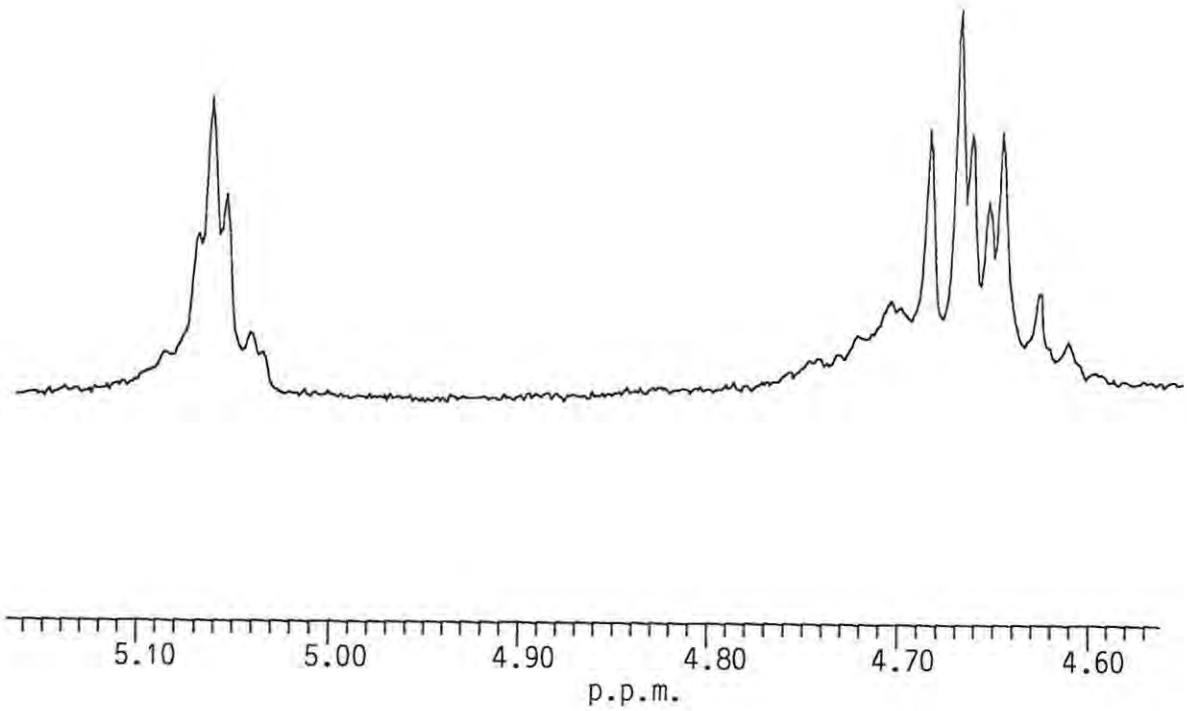
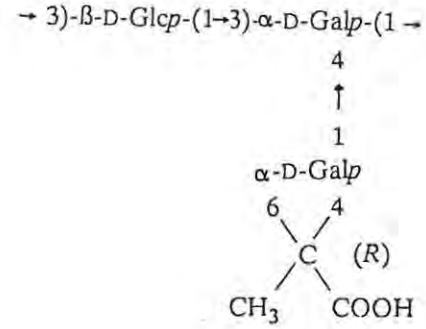


Fig. 5. ^{1}H-N.m.r. anomeric region (expanded spectrum) of **3**.

5.2.3 Conclusion

From the above results, the following structure can be written for the repeating unit of the *E. coli* K37 capsular antigen.



The structure for the K37 polysaccharide is one of three structures reported within the *E. coli* species to contain a pyruvate ketal, the other two being for K26 (ref. 402) and K29.[401] The K37 polysaccharide is, however, the first capsular polysaccharide found to owe its acidity solely to this ketalic structure.

5.2.4 Experimental

General methods. - See Section 5.1. Descending p.c. was performed on Whatman No. 1 paper using the following solvents: *A*, 18:3:1:4 ethyl acetate-acetic acid-formic acid-water; *B*, 40:11:9 1-butanol-ethanol-water; *C*, 8:2:1 ethyl acetate-pyridine-water; and *D*, 5:1:5:3 ethyl acetate-acetic acid-pyridine-water. Chromatographed samples were detected with alkaline silver nitrate.[412]

Preparation and properties of K37 polysaccharide. - An authentic culture of *E. coli* O9:K37 (A 84a) was obtained from Dr I. Ørskov (Copenhagen) and grown (three batches of six trays) on Mueller-Hinton agar containing 2 $g.L^{-1}$ NaCl for 4 d at 37° as described in Section

5.1.2. The acidic capsular polysaccharide was isolated as described, giving a total yield of 2.66 g. G.p.c. was performed on the native polysaccharide (3.0 mg), on the product after passage through Amberlite IR-120(H^+) resin (3.0 mg), and on the base-treated product (0.25M NaOH, 60° for 1, 2, 4, 8 and 12 h) after dialysis (12-14 000 Mw cut-off), using a column of Sepharose 4B and M NaCl as eluent (Fig. 3). The native polysaccharide had $[\alpha]_D$ + 105° (*c* 2.1, water). An aqueous solution of the acid form of the polysaccharide was heated at 90° for 0.5 h and freeze-dried; the autohydrolyzed product (**1**) was examined by ^{1}H- and ^{13}C-n.m.r. spectroscopy (Table III). Phosphate determination was performed by the Molybdenum Blue method on the native polysaccharide (10.6 mg) and on a sample of the polysaccharide (9.0 mg) after hydrolysis in a sealed ampoule (5M H_2SO_4, overnight, 100°) as described by Vogel.[403]

Sugar composition. - The polysaccharide (11.1 mg) was hydrolyzed overnight and the hydrolyzate examined by p.c. (solvents *A* - *C*) which revealed two major products corresponding to glucose and galactose. The hydrolyzate was derivatized (PAAN's) and analyzed as described (Table II, column I). Dried polysaccharide (9.1 mg) was carboxyl reduced and analyzed as described (Table II, column II).

Methylation analysis. - K37 polysaccharide (23.8 mg) in the acid form was methylated once by the Hakomori method (sodium dimsyl)[113,398] and then by the Kuhn method.[399] The product showed no i.r. absorption for hydroxyl but a strong absorption at 1100 cm^{-1} (methoxyl). The permethylated product was hydrolyzed and analyzed as described (Table IV, column I).

Periodate oxidation and Smith degradation. - A solution of K37 polysaccharide (26.4 mg) in water (5 mL) was treated with 0.03M sodium metaperiodate (5 mL) at room temperature in the dark. The uptake of periodate was monitored spectrophotometrically[413] (223 nm). After 48 h, 0.97 mol of periodate had been consumed per mol of trisaccharide repeating unit (Fig. 4). The excess periodate was reduced with ethylene glycol after 96 h and the solution dialyzed against running water (3500 Mw cut-off, 2 d) then freeze-dried. This product was hydrolyzed (0.5M TFA, 0.5 h, 60°), dialyzed, and freeze-dried to give a polymeric product (**2**) which was examined by ^{1}H-n.m.r. spectroscopy (Table III). Sugar analysis of **2** (0.6 mg) as the

PAAN derivatives gave the results in Table II, column III. A sample of 2 (7.2 mg) was methylated by the Hakomori method (sodium dimsyl)[113,398] followed by a Kuhn methylation[399] and the product was analyzed after hydrolysis as described (Table IV, column II).

Partial hydrolysis. - K37 polysaccharide (251 mg) was treated with refluxing 0.5M TFA (50 mL) for 1.5 h at 100°. The acid was removed by coevaporation with water under reduced pressure at a bath temperature not exceeding 40° and p.c. of the residue (solvent *D*) yielded **3** (24.1 mg), $[\alpha]_D$ +94° (*c* 8.2, water); **4** (40.3 mg), $[\alpha]_D$ +21° (*c* 7.2); **5** (33.8 mg), $[\alpha]_D$ +13° (*c* 5.5); and **6** (28.4 mg). Samples of **4** (1.2 mg) and **5** (1.2 mg) were hydrolyzed and analyzed (PAAN's) as described. **3** (1.9 mg), **4** (1.2 mg), and **5** (1.2 mg) were analyzed by the Morrison method[76] after reduction ($NaBH_4$) to the alditol. Results of the sugar analyses are given in Table II, columns IV-VI. 5-Alditol ($NaBH_4$ reduced), and 3- and 4-alditol ($NaBD_4$ reduced) were methylated (Hakomori, sodium dimsyl)[113,398] and analyzed after hydrolysis as described (Table IV, columns III-V). Samples of **3**, **4**, and **5** were analyzed by ^1H- and ^{13}C-n.m.r. spectroscopy (Table III).

Determination of absolute configuration.[99] - A sample of **6** (5.7 mg) was analyzed as the (-)-2-octyl glycosides by g.l.c. (230°) as described (Section 5.1.2).

5.3 STRUCTURAL INVESTIGATION OF *ESCHERICHIA COLI* K55 CAPSULAR POLYSACCHARIDE USING BACTERIOPHAGE DEGRADATION

5.3.1 Introduction

Bacteriophage degradation[172] has become an established technique in the structural elucidation of enterobacterial capsular polysaccharides. Numerous applications of the technique in studies of *Klebsiella* and *E. coli* K-antigens have been reported in the literature (see Section 3.2.3), the majority of which have involved bacteriophage enzymes with endoglycanase activity. Bacteriophage enzymes with lyase activity have been used in the studies on *Klebsiella* K5,[183] K14,[18] and K64 (ref. 185) capsular polysaccharides however, similar enzyme activity has not been reported for bacteriophages active against the *E. coli* species. Of the bacteriophage degradations reported for *E. coli* capsular polysaccharides, those on K27 (ref. 181) and K30 (ref. 25) have made use of bacteriophages initially isolated using *Klebsiella* K54 and K20 bacteria, respectively. A bacteriophage degradation has now been carried out on *E. coli* K55 polysaccharide using the lyase enzyme borne by *Klebsiella* Ø5.

5.3.2 Results and Discussion

Composition of K55 polysaccharide. - The acidic polysaccharide from *E. coli* K55 was isolated by precipitation with CTAB from five batches of bacteria grown on Mueller-Hinton agar. The isolates formed viscous aqueous solutions and were shown to be polydisperse by g.p.c. (Fig. 6A), with a range of molecular masses from M_r = 3.7 x 10^4 to M_r = 1.4 x 10^7. Treatment of the isolates with Amberlite IR-120(H^+) resin did not significantly alter the molecular mass range (Fig. 6B) as has been shown to occur for *E. coli* K37 (Section 5.2) and *Klebsiella* K39 (Section 5.4) polysaccharides. Sugar analysis of the isolates from each batch (Table V), after carboxyl reduction, showed that the amount of galactose present decreased as the incubation time for the growth was decreased although the mannose-glucose ratio remained constant. These results suggest that the K55 polysaccharide does not have galactose residues as part of its primary structure and that a galactose-rich polymer is produced by *E. coli* K55 in addition to the K55 capsular polysaccharide. This assumption is borne out by the results of

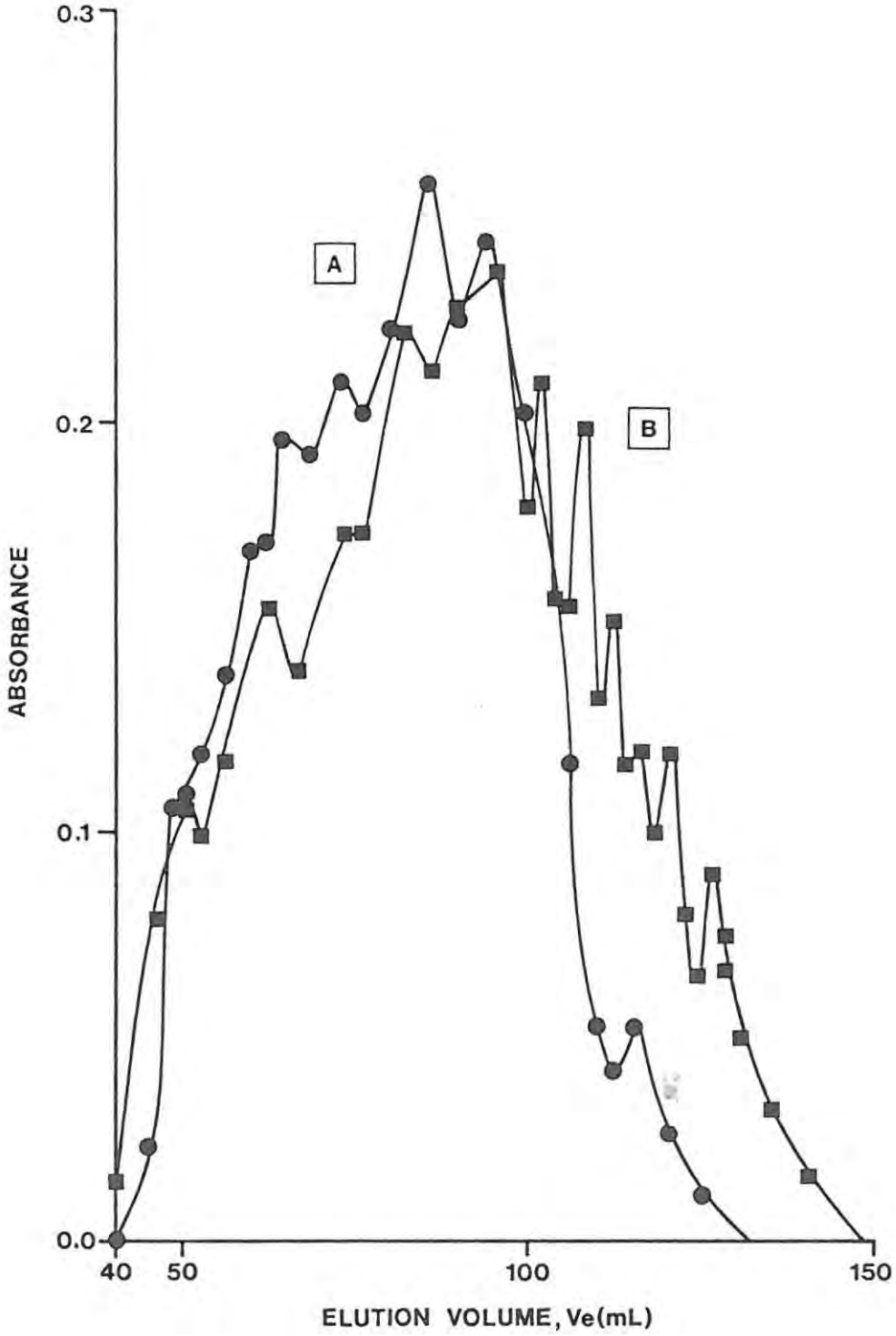


Fig. 6. Gel-permeation chromatography on Sephacryl S500: A, K55 polysaccharide (●); and B, K55 polysaccharide after treatment with Amberlite IR-120 (H^+) resin (■).

the bacteriophage degradation study performed on the K55 polysaccharide (see below). The production of the galactose-rich polysaccharide can be reduced by controlled subculturing and shorter growth times. Repeated subculturing may, however, result in the loss of K-antigen production. The presence of galactose-rich polysaccharides will be discussed further in Section 5.4.

TABLE V

SUGAR ANALYSES OF *E. coli* K55 POLYSACCHARIDE BATCHES 1-5

Sugar (as peracetylated aldononitrile)	Analysis (%)[a,b]				
	I(84)[c]	II(48)	III(36)	IV(36)	V(36)
Mannose	29	31	32	32	32
Glucose	38	52	64	55	57
Galactose	33	16	3	2	0

[a]Determined on a capillary DB-225 column at 225°. [b]I-V, Carboxyl-reduced polysaccharide from batches 1-5, respectively. [c]Parenthesized data represent the incubation time in hours for each batch.

The isolates from batches 3-5 were designated as the K55 polysaccharide. Sugar analyses of the K55 polysaccharide (Table VI, columns I and II), with and without prior carboxyl reduction, showed the polysaccharide to contain equimolar amounts of mannose, glucose, and glucuronic acid. The ^{1}H-n.m.r. spectrum of an ultrasonicated sample of K55 polysaccharide (batch 3, 7) showed three signals in the anomeric region at δ4.55 ($J_{1,2}$ 7.0 Hz), δ4.67 ($J_{1,2}$ 8.0 Hz), and δ4.72 (Table VII), corresponding to ß-linkages for glucuronic acid, glucose, and mannose residues, respectively (see below for confirmation of these assignments), and a signal for the methyl group of a pyruvate ketal (δ1.47, 3H). The ^{13}C-n.m.r. data for 7 were in agreement with the ^{1}H-n.m.r. anomeric results and showed signals at 25.48, 100.94, and 175.62 p.p.m.

which allowed the *S* configuration to be assigned to the pyruvate ketal[332,333] (Table VII). A sample of K55 polysaccharide (batch 5) after ultrasonication (8) was shown by ^{1}H-n.m.r. spectroscopy to be about 40% *O*-acetylated at the 2 position of the mannose residue (Table VII). The *O*-acetyl group, shown by a signal at δ2.18 for the methyl group, caused the signal for H2 of the mannose residue to be shifted downfield to δ5.60, a phenomenon that has recently been reported by Annison *et al.*[414] for the H2 signal (δ5.52) for a 2-*O*-acetyl-α-rhamnose residue glycosidically linked at positions 3 and 4. Acetylation of the K55 polysaccharide also caused twinning of the ß-anomeric signals and resulted in a downfield shift for the H1 signal of the ß-mannose residue (δ4.88) and an upfield shift for the H1 signals of the ß-glucuronic acid (δ4.51) and ß-glucose (δ4.59) residues. These signal assignments for **8** were confirmed by comparison with the n.m.r. data recorded for oligosaccharides derived from the K55 polysaccharide (see below).

TABLE VI

SUGAR ANALYSES OF *E. coli* K55 POLYSACCHARIDE AND DERIVED PRODUCTS

Sugar (as peracetylated aldononitrile)	Molar ratio [a,b]						
	I[c]	II[c]	III	IV	V	VI	VII
Mannose	1.00	1.00	1.00	-	1.00	1.00	1.00
Glucose	0.49	1.78	1.00	1.00	1.06	0.81	2.93
Mannitol[d]	-	-	-	0.92	-	-	1.35

[a]Determined on a capillary DB-225 column at 225°. [b]I, Native K55 polysaccharide; II, carboxyl-reduced K55 polysaccharide; III, **10**; IV, **10**-alditol; V, carboxyl-reduced **10**; VI, **11**; and VII, carboxyl-reduced **11**-alditol. [c]Batch 5 isolate used. [d]Mannitol hexa-acetate.

TABLE VII

N.M.R. DATA (500 MHz) FOR *E. coli* K55 POLYSACCHARIDE AND DERIVED PRODUCTS

^{1}H-N.m.r. data				^{13}C-N.m.r. data	
δ^a (p.p.m.)	$J_{1,2}{}^b$ (Hz)	Integral (no. of H)	Assignment^c	δ^d (p.p.m.)	Assignment^e
-4GlcAβ1-4Glcβ1-3(4,6-pyr)Manβ1- K55 polysaccharide, batch 3 (**7**)^f					
4.72	n.o.	1.0	-3(4,6-pyr)Manβ1-	176.11	COOH of GlcA
4.67	8.0	1.0	-4Glcβ1-	175.62	COOH of pyr^g
4.55	7.0	1.0	-4GlcAβ1-	103.12	-4GlcAβ1-
1.47		3.0	CH_3 of pyr	102.74	-4Glcβ1-
				100.94	C2 of pyr
				99.99	-3(4,6-pyr)Manβ1-
				60.94	C6 of Glc
				25.48	CH_3 of pyr
-4GlcAβ1-4Glcβ1-3(2-OAc 40%)(4,6-pyr)Manβ1- K55 polysaccharide, batch 5 (**8**)					
5.60	n.o.	0.4	H2 of 2-OAcMan		
4.88	n.o.	0.4	-3(2-OAc)(4,6-pyr) Manβ1-		
4.72	n.o.	0.6	-3(4,6-pyr)Manβ1-		
4.66	7.0	0.6	-4Glcβ1-		
4.59	n.o.	0.4	-4Glcβ1-(2-OAcMan)		
4.55	6.0	0.6	-4GlcAβ1-		
4.51	n.o.	0.4	-4GlcAβ1-(2-OAcMan)		
2.18		1.2	CH_3 of acetate		
1.48		3.0	CH_3 of pyr		
Δ4,5-ene-GlcAβ1-4Glcβ1-3(2-OAc 70%)(4,6-pyr)Man (**10**)					
6.21	(d, 3.5)	1.0	H4 of Δ4,5-ene-GlcA	174.02	CO of acetate
				173.63	COOH of pyr
5.64	(d, 3.2)	0.3	H2 of 2-OAcMan(β)	173.59	(Manα/β)
5.36	(m)	0.4	H2 of 2-OAcMan(α)	165.92	COOH of Δ4,5-ene-GlcA
5.23 to 5.22	n.o.	1.6	Δ4,5-ene-GlcAβ1-3(2-OAc)(4,6-pyr)Manα	141.19	C5 of Δ4,5-ene-GlcA
			-3(4,6-pyr)Manα	112.92	C4 of Δ4,5-ene-GlcA
5.11	n.o.	0.3	-3(2-OAc)(4,6-pyr) Manβ	101.29	Δ4,5-ene-GlcAβ1-

TABLE VII (*continued*)

^{1}H-N.m.r. data				^{13}C-N.m.r. data	
δ^a (p.p.m.)	$J_{1,2}{}^b$ (Hz)	Integral (no. of H)	Assignment^c	δ^d (p.p.m.)	Assignment^e
4.96	n.o.	0.1	-3(4,6-pyr)Manβ	100.91	C2 of pyr
4.65	8.0	0.1	-4Glcβ1-(Manβ)	100.11	-4Glcβ1-(Manα/β)
4.64	8.0	0.2	-4Glcβ1-(Manα)	99.87	
4.57	8.0	0.4	-4Glcβ1-(2-OAc-Manα)	99.49 99.00	-4Glcβ1-(2-OAcManα/β)
4.56	8.0	0.3	-4Glcβ1-(2-OAc-Manβ)	95.60	-3(4,6-pyr)Manα
4.47	(3.3,9.9)	0.4	H3 of 2-OAcMan(α)	95.05	-3(4,6-pyr)Manβ
4.38	(3.3,9.9)	0.3	H3 of 2-OAcMan(β)		
4.25	(m)	1.0	H3 of Δ4,5-ene-GlcA	93.95	-3(2-OAc)(4,6-pyr)Manα
3.75	(d)	1.0	H2 of Δ4,5-ene-GlcA	93.37	-3(2-OAc)(4,6-pyr)Manβ
2.18		0.8	CH_3 of acetate (Manβ)	60.84	C6 of Glc(2-OAcMan)
2.15		1.3	CH_3 of acetate (Manα)	60.79	C6 of Glc
1.59		1.4	CH_3 of pyr(Manβ)	25.35	CH_3 of pyr
1.58		1.6	CH_3 of pyr(Manα)	21.10	CH_3 of acetate
Δ4,5-ene-GlcAβ1-4Glcβ1-3(4,6-pyr)Man De-*O*-acetylated **10**					
6.20	(d, 3.9)	1.0	H4 of Δ4,5-ene-GlcA	173.86 173.79	COOH of pyr (Manα/β)
5.23	(d, 5.2)	1.0	Δ4,5-ene-GlcAβ1-	166.06	COOH of Δ4,5-ene-GlcA
5.22	n.o.	0.7	-3(4,6-pyr)Manα		
4.96	n.o.	0.3	-3(4,6-pyr)Manβ	141.31	C5 of Δ4,5-ene-GlcA
4.65	8.0	0.3	-4Glcβ1-(Manβ)		
4.64	8.0	0.7	-4Glcβ1-(Manα)	112.79	C4 of Δ4,5-ene-GlcA
4.25	(m)	1.0	H3 of Δ4,5-ene-GlcA	101.29	Δ4,5-ene-GlcAβ1-
1.57		0.7	CH_3 of pyr(Manα)	101.08	C2 of pyr
1.54		0.3	CH_3 of pyr(Manβ)	100.14 99.89	-4Glcβ1-(Manα/β)
				96.67	unassigned
				95.60	-3(4,6-pyr)Manα
				95.15	unassigned
				95.05	-3(4,6-pyr)Manβ
				60.87	C6 of Glc
				25.36	CH_3 of pyr

TABLE VII (*continued*)

^{1}H-N.m.r. data				^{13}C-N.m.r. data	
δ^a (p.p.m.)	$J_{1,2}{}^b$ (Hz)	Integral (no. of H)	Assignment^c	δ^d (p.p.m.)	Assignment^e
Δ4,5-ene-GlcAβ1-4Glcβ1-3(4,6-pyr)Mannitol (**10**-alditol)					
See TABLE IX					
11 (partially acetylated)					
6.21	(d, 3.9)	1.0	H4 of Δ4,5-ene-GlcA	173.68	COOH of pyr
				173.59	COOH of pyr
5.64	(d)		H2 of 2-OAcMan(β)	172.58	COOH of GlcA
5.37	(m)		H2 of 2-OAcMan(α)	165.95	COOH of Δ4,5-ene-GlcA
5.23	(d, 5.3)	1.0	Δ4,5-ene-GlcAβ1-		
5.21	1.7	0.7	-3(4,6-pyr)Manα +/- 2-OAc	141.21	C5 of Δ4,5-ene-GlcA
5.11	n.o.	0.1	-3(2-OAc)(4,6-pyr)Man β	112.90	C4 of Δ4,5-ene-GlcA
4.96	n.o.	0.2	-3(4,6-pyr)Manβ	103.07	-4GlcAβ1-
4.73	n.o.	1.0	-3(4,6-pyr)Manβ1-	101.26	Δ4,5-ene-GlcAβ1-
4.64	7.9	1.8	-4Glcβ1-	101.23	-3(4,6-pyr)Manβ1-
4.60	7.9	1.0	-4GlcAβ1-	100.94	C2 of pyr
4.46	(m)		H3 of 2-OAcManα	100.87	C2 of pyr
4.37	(m)		H3 of 2-OAcManβ	99.99	-4Glcβ1-
4.26	(m)	1.0	H3 of Δ4,5-ene-GlcA	99.75	-4Glcβ1-
				95.60	-3(4,6-pyr)Manα
2.18		0.3	CH_3 of acetate	95.05	-3(4,6-pyr)Manβ
2.15		0.5	CH_3 of acetate	93.94	-3(2-OAc)(4,6-pyr)Manα
1.57		3.0	CH_3 of pyr	93.37	-3(2-OAc)(4,6-pyr)Manβ
				25.33	CH_3 of pyr
				21.01	CH_3 of acetate

TABLE VII (*continued*)

^{1}H-N.m.r. data				^{13}C-N.m.r. data	
δ^a (p.p.m.)	$J_{1,2}{}^b$ (Hz)	Integral (no. of H)	Assignment^c	δ^d (p.p.m.)	Assignment^e
11-alditol					
6.15	(d, 3.7)	1.0	H4 of Δ4,5-ene-GlcA	175.69	COOH of pyr (Mannitol)
5.21	(d, 5.2)	1.0	Δ4,5-ene-GlcAβ1-	174.73	COOH of pyr (Man)
4.69	n.o.	1.0	-3(4,6-pyr)Manβ1-		
4.65	8.0	1.0	-4Glcβ1-(Man)	173.79	COOH of GlcA
4.53 / 4.52	8.0	2.0	-4GlcAβ1- -4Glcβ1-(Mannitol)	166.70	COOH of Δ4,5-ene-GlcA
4.25	(m)	1.0	H3 of Δ4,5-ene-GlcA	142.04	C5 of Δ4,5-ene GlcA
1.53		3.0	CH_3 of pyr(Man)	112.14	C4 of Δ4,5-ene GlcA
1.50		3.0	CH_3 of pyr (Mannitol)	103.82	-4Glcβ1- (Mannitol)
				103.08	-4GlcAβ1-
				101.75	-3(4,6-pyr)Manβ1-
				101.33	Δ4,5-ene-GlcAβ1-
				101.15	C2 of pyr
				100.06	-4Glcβ1-(Man)
				25.38	CH_3 of pyr(Man)
				25.13	CH_3 of pyr (Mannitol)

$^{a-g}$For legends, see TABLE III.

TABLE VIII

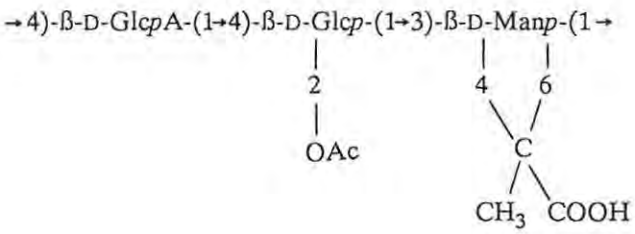
METHYLATION ANALYSES OF *E. coli* K55 POLYSACCHARIDE AND DERIVED PRODUCTS

Methylated sugar[a] (as alditol acetate)	T_R[b]	Molar ratio[c,d]					
		I	II	III	IV	V	VI
1,2,5-Man	1.45	-	-	-	-	0.67	0.73
2,4,6-Man	1.57	-	-	0.65	-	-	0.12
2,3,6-Glc	1.82	1.00	1.00	1.00	1.00	1.00	1.00
2,3-Glc	3.08	-	1.03	-	-	-	0.90
2-Man	3.81	2.65	1.27	0.30	0.51	0.16	0.71
Man	4.35	-	-	-	0.88	-	-

[a]1,2,5-Man = 3,4,6-tri-*O*-acetyl-1,2,5-tri-*O*-methylmannitol, *etc.*; all substitution patterns were confirmed by g.l.c.-m.s. [b]Retention time (T_R) relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol run under the same chromatographic conditions. [c]Determined on a capillary DB-225 column at 205°. [d]I, Native K55 polysaccharide; II, carboxyl-reduced K55 polysaccharide; III, depyruvalated K55 polysaccharide (**9**); IV, **10**; V, **10**-alditol; and VI, carboxyl-reduced **11**-alditol.

Methylation analysis. - Data for the methylation analyses of K55 polysaccharide are given in Table VIII, columns I and II. The data show the presence of a 4-linked glucose residue, a trisubstituted mannose residue and, after carboxyl reduction, a 4-linked glucuronic acid residue. Methylation analysis of a depyruvalated sample of K55 polysaccharide (**9**) gave 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol, indicating that the pyruvate group was attached to positions 4 and 6 of the mannose residue (column III). Terminal, non-reducing residues and branch point residues were not revealed during the methylation analyses. The above results, in conjunction with the n.m.r. data, suggested that the K55 polysaccharide was linear and was composed of pyruvalated trisaccharide repeating units containing varying amounts of *O*-acetylation. From the low molar ratio of the 2,3,6-tri-*O*-methylglucitol derivative in the

methylation analysis of the K55 polysaccharide (column I), it was tentatively assumed that the uronic acid was linked to the glucose residue. This assumption, although not always valid, suggested that the K55 polysaccharide was similar in structure to the *Klebsiella* K5 polysaccharide[16] (shown below). To confirm this, the bacteriophage degradation of the K55 polysaccharide using *Klebsiella* Ø5 was effected.



Klebsiella K5 structure[16]

Bacteriophage degradation. - *Klebsiella* Ø5 was isolated from sewage, purified on its host strain, and shown to carry a lyase enzyme by degradation of *Klebsiella* K5 polysaccharide.[183] Preliminary tests indicated that this bacteriophage was able to lyze *E. coli* K55 bacteria and that it could be effectively propagated in nutrient broth using this bacterium. The degradation of the isolate from batch 5 (494.7 mg) was therefore performed as described (Section 5.1.2) using a titre of 1.5 x 10^{13} PFU and yielded two pure oligosaccharides (**10** (104.8 mg) and **11** (119.5 mg)) after g.p.c. (Fig. 7). From the g.p.c. elution volumes, **10** was thought to be a trisaccharide and **11** a hexasaccharide. The thiobarbituric acid test[415] indicated the presence of a terminal hex-4-enuronic acid (4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid) moiety in both **10** and **11** but not in the native polysaccharide.

Sugar analysis of **10**, **10**-alditol, and carboxyl-reduced **10** (Table VI, columns III-V) showed that the oligosaccharide contained a glucose residue and a mannose reducing end sugar. Glucuronic acid was absent. Analysis of **10**-alditol ($NaBD_4$ reduced; Table VIII, column V), after Hakomori methylation,[113] showed an in-chain 4-linked glucose sugar and a derivative (3,4,6-tri-*O*-acetyl-1,2,5-tri-*O*-methylmannitol) resulting from a 3-linked 4,6-pyruvalated

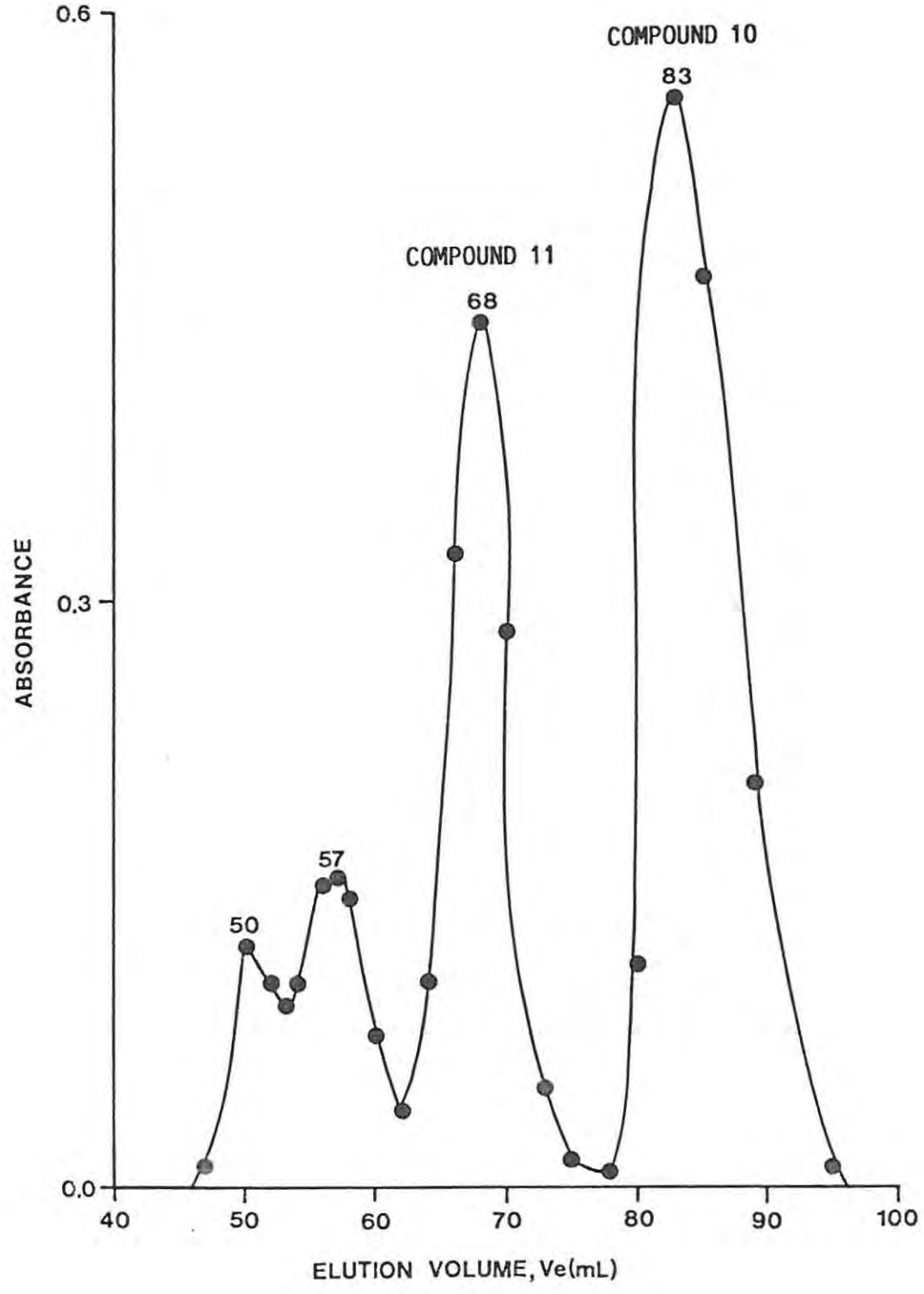
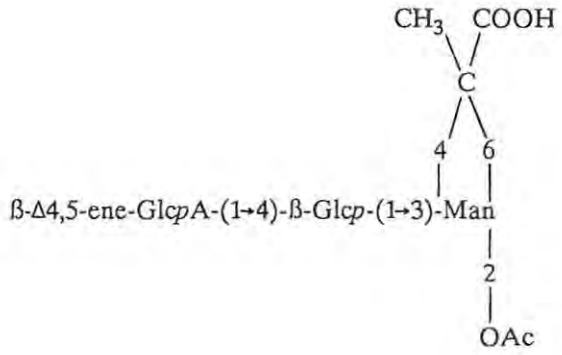


Fig. 7. Gel-permeation chromatography on Bio-Gel P4 for the bacteriophage degradation of K55 polysaccharide.

mannose reducing end group. Incomplete reduction of **10** resulted in the presence of a small amount of the 2-*O*-methylmannitol derivative in the methylation analysis, arising from the reducing end sugar. Methylation of **10** by the Prehm method[129] and subsequent analysis (see Table VIII, column IV) located an acetyl group on the 2 position of 65% of the reducing mannose residues (from the identification of mannitol hexa-acetate). N.m.r. data for **10** were in agreement with the above results (see below). The following structure of **10** can therefore be proposed.



10 (65-70% acetylated)

From sugar analyses (Table VI, columns VI and VII), methylation analyses (Table VIII, column VI), and n.m.r. data (Table VII), **11** was found to be the hexasaccharide corresponding to the double repeating unit produced by the action of *Klebsiella* Ø5 on K55 polysaccharide.

N.m.r. spectra of some derived oligosaccharides. - A heteronuclear (HETCORR) 2D shift correlated experiment (see Section 7.3.1) was performed on **10**-alditol (acid form) and the data obtained collected in Table IX. From this data the assignment of all the n.m.r. signals for this oligosaccharide alditol was possible. The spin system for the hex-4-enuronic acid (Table IX, sugar C) gives correlated peaks (p.p.m.) in the HETCORR spectrum at 5.17, 101.35 (H/C 1); 3.87, 70.65 (H/C 2); 4.25, 66.82 (H/C 3); and 6.13, 112.25 (H/C 4). Signals at 141.92 p.p.m. and

TABLE IX

DATA FROM 2D-N.M.R. ANALYSIS (500 MHz) OF **10**-ALDITOL DERIVED FROM *E. coli* K55 POLYSACCHARIDE

$\delta(^1H)^a$ (p.p.m.)	J (Hz)	$\delta(^{13}C)^b$ (p.p.m.)	Assignment
Δ4,5-enc-GlcAß1-4 Glcß1-3(4,6-pyr)Mannitol **10**-alditol[c]			
(C) (B) (A)			
Sugar A			
3.77	12.0	62.81	H/C 1′
3.94	2.7	62.81	H/C 1
4.01	m	71.26	H/C 2
4.06	1.6	76.80	H/C 3
3.76	8.3	77.34	H/C 4
4.08	m	60.77	H/C 5
3.52	12.5	67.39	H/C 6′
4.07	2.0	67.39	H/C 6
Sugar B			
4.52	8.0	103.89	H/C 1
3.44	9.2	73.87	H/C 2
3.65	9.0	74.79	H/C 3
3.70	9.1	79.72	H/C 4
3.61	m	75.31	H/C 5
3.76	12.2	61.46	H/C 6′
3.97	2.7	61.46	H/C 6
Sugar C			
5.17	5.3	101.35	H/C 1
3.87	5.1	70.65	H/C 2
4.25	4.4	66.82	H/C 3
6.13	3.8	112.25	H/C 4
-	-	141.92	C5
-	-	166.59	COOH
Pyruvate ketal			
-	-	175.51	COOH
-	-	101.00	C2
1.51	-	25.12	H/C CH_3

[a]Chemical shift measured from internal acetone, 62.23. [b]Chemical shift measured from internal acetone, 31.07 p.p.m. [c]For origin of compound, see text. For n.m.r. spectra, see Sections 7.1-7.3.

166.59 p.p.m. in the 1D ^{13}C-n.m.r. spectrum can be assigned to C5 and C6 of the hex-4-enuronic acid, respectively. These data agree well with those found by Parolis[416] for the same sugar residue produced by lyase activity on *Klebsiella* K14 polysaccharide. The ^{13}C-n.m.r. signals for the in-chain glucose unit (Table IX, sugar B) were assigned after consideration of the data published by Bradbury and Jenkins.[352] Comparison of these assignments with published results for methyl ß-glucopyranoside[349] clearly shows a downfield shift (α-effect) for the C4 resonance signal (70.6 to 79.72 p.p.m.) and upfield shifts (ß-effects) of the signals for C3 (76.8 to 74.79 p.p.m.) and C5 (76.8 to 75.31 p.p.m.). These shifts confirm that the glucose residue is linked in the 4 position. The assignment of the ^{1}H-n.m.r. signals for the glucose unit followed from the HETCORR spectrum. The remaining ^{13}C-n.m.r. signals were assigned to the mannitol residue (Table IX, sugar A) which was glycosidically linked at C3 and linked at C4 and C6 by a pyruvate ketal. The C1 (62.81 p.p.m.) and C6 (67.39 p.p.m.) resonances are readily assigned from the two correlated ^{1}H-n.m.r. peaks observed in the HETCORR spectrum, the latter signal being further downfield due to the pyruvate linkage. The ^{13}C signal at 71.26 p.p.m. is assigned to C2 of the mannitol residue (72.2 p.p.m. for mannitol)[349] and has been shifted upfield due to the glycosidic bond at C3 (ß-effect). The resonance at 60.77 p.p.m. is assigned to the C5 signal since this signal will experience a dual upfield shift due to the ketalic linkages at C4 and C6. The remaining ^{13}C-n.m.r. signals for this residue (76.80 and 77.34 p.p.m.) were assigned on the basis of the coupling constants observed in the correlated ^{1}H-n.m.r. signals from the HETCORR spectrum. The diaxial orientation of H4 and H5 is fixed due to the dioxane ring of the pyruvate ketal and the coupling constant between these two protons ($J_{4,5}$) would be expected to be large (∿8Hz), while the orientation of the H3 and H4 protons is not fixed, due to free rotation around the C3-C4 bond, and thus a smaller coupling constant would be expected. The ^{13}C-n.m.r. signal at 77.34 p.p.m. correlated to a double doublet (J 8.3 Hz) in the ^{1}H-n.m.r. spectrum at δ3.76 therefore these signals were assigned to C4 and H4 of the mannitol residue. The double doublet at δ4.06 in the ^{1}H-n.m.r. spectrum, correlated to the ^{13}C-n.m.r. signal at 76.80 p.p.m., showed coupling constants of 8.3 Hz and 1.6 Hz therefore these signals were

assigned to H3 and C3 of the mannitol residue. The ^{1}H-n.m.r. signals not discussed above were assigned from the HETCORR spectrum. The signals for the pyruvate ketal (Table IX) were assigned from the HETCORR spectrum (for the methyl group) and the 1D ^{13}C-n.m.r. spectrum (for the quarternary carbons), and confirm the assignment of the *S* configuration to this functional group.

N.m.r. data for **10** and de-*O*-acetylated **10** are presented in Table VII. The assignment of the n.m.r. data for de-*O*-acetylated **10** follows from the assignments for **10**-alditol. Due to the reducing end mannose residue the signals for the pyruvate ketal and H-1 of the ß-linked glucose residue are twinned which, in the latter case, provides further proof of the sequence for this oligosaccharide. The ^{1}H-n.m.r. spectrum of **10** is complicated by the fact that 70% of the molecules are *O*-acetylated at the 2 position of the mannose reducing end group, causing an increase in the complexity of the signals in the region from δ4.3 to δ5.7 (see Fig. 8). Signals for H1 of the reducing end mannose can be seen for the ß-anomer at δ4.96 and δ5.11 (*O*-acetylated) whilst the signals for the α-anomer resonate at the same position (δ5.22 - 5.23) as that of H1 from the ß-hex-4-enuronic acid moiety. As mentioned earlier, acetylation at position 2 of a rhamnose residue causes a significant downfield shift into the anomeric region of the spectrum of the H2 signal.[414] In the case of **10**, the ^{1}H-n.m.r. signal for H2 of the mannose residue in acetylated molecules is seen at δ5.64 (0.3H) and δ5.36 (0.4H), being split by the anomerization of the sugar. H3 of this residue will be deshielded by acetylation at position 2, the glycosidic linkage at position 3, and the pyruvate ketal linked at position 4 therefore the signal for this proton will appear appreciably downfield from its usual resonance position (δ3.83 (α) and δ3.64 (ß) for H3 of mannose).[417] The double doublets at δ4.38 ($J_{2,3}$ 3.3 Hz, $J_{3,4}$ 9.9 Hz, O.3H) and δ4.47 (0.4H) have been assigned to the signals for H3 in acetylated molecules; the resonances are split due to the ß- and α-anomers of the reducing end sugar, respectively. The assignment of the above signals for H2 and H3 of acetylated **10** is supported by the absence of these signals in the ^{1}H-n.m.r. spectrum of de-*O*-acetylated **10** (see Section 7.1.10). Finally the signals at δ4.56 and 4.57, and δ4.64 and 4.65 can be assigned to the anomeric proton of the ß-glucose residue. This glucose residue is interior to the reducing end

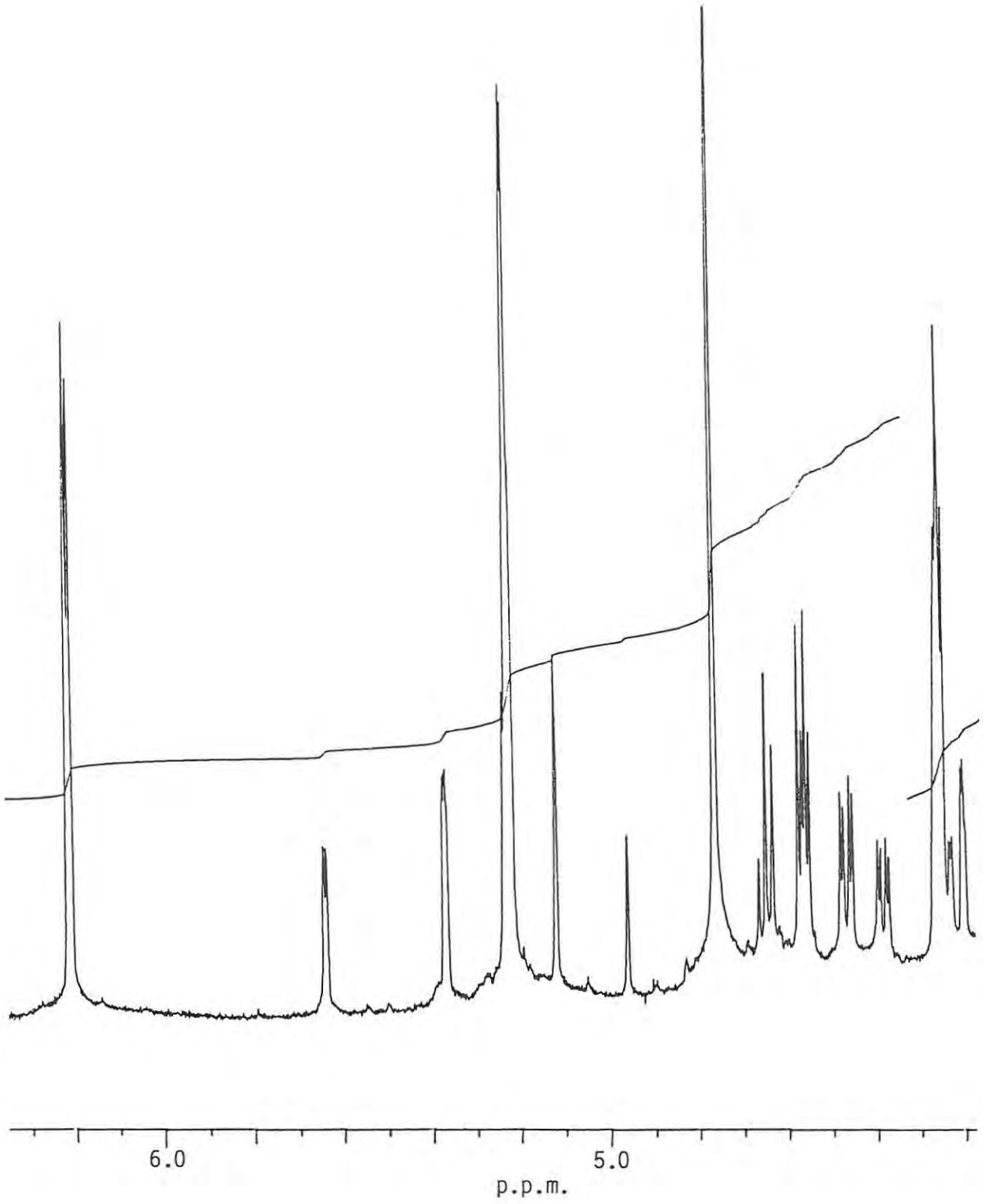


Fig. 8. ^{1}H-N.m.r. spectrum (anomeric region) of **10**.

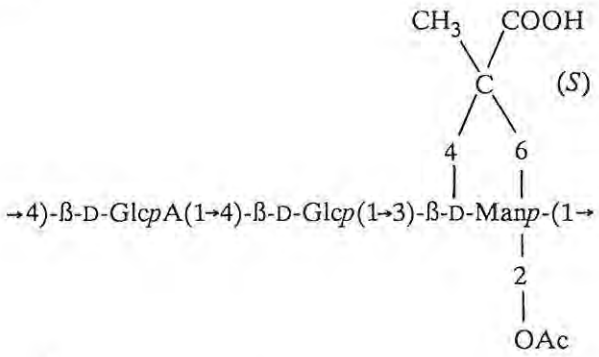
sugar and thus the signals will be twinned due to anomerization and separate signals for acetylated and non-acetylated molecules will be observed (see Table VII).

The trisaccharide obtained from the bacteriophage degradation of *Klebsiella* K5 polysaccharide[183] is identical to **10** except that the *O*-acetyl group (~20% present) has been reported to be on the 2 position of the glucose residue,[16] the site of *O*-acylation having been established for the *Klebsiella* K5 polysaccharide by Dutton and Yang[16] using the methyl vinyl ether method of de Belder and Norrman.[130] The ^{1}H-n.m.r. spectrum (500 MHz) of the trisaccharide was reported by van Dam *et al.*[183] to show complex peak patterns in the structural reporter group region and thus these workers published the ^{1}H-n.m.r. spectrum of the de-*O*-acetylated product. It is possible that the site of *O*-acylation in the *Klebsiella* K5 polysaccharide is in fact on the 2 position of the mannose residue thereby causing the above reported complexity of the ^{1}H-n.m.r. spectrum. The resonance position of the signals reported by van Dam *et al.*[183] differ from those recorded in Table VII for de-*O*-acetylated **10** since the former analysis was done on the neutral form of the trisaccharide (pD 7) and the latter on the acid form. The signal of unknown origin in the ^{1}H-n.m.r. spectrum published by van Dam *et al.*[183] appears in the spectrum of **10** and has been assigned to H1 of the ß-linked glucose residue interior to the reducing end sugar. The signal for this proton will be twinned due to the anomerization of the reducing end sugar and thus appears as a pair of overlapping doublets (δ4.64 and δ4.65, 8.0 Hz). Reduction of **10** (**10**-alditol) causes the signal to collapse to a doublet (δ4.52, 8.0 Hz) (see Tables VII and IX).

Determination of absolute configuration. - The absolute configuration for the neutral sugar residues in the K55 polysaccharide was determined by g.l.c. analysis of the acetylated (-)-2-octyl glycosides prepared from an acid hydrolyzate of the polysaccharide, with and without prior carboxyl reduction. These residues were found to have the D-configuration.

5.3.3 Conclusion

The investigation of the *E. coli* K55 polysaccharide allows the following structure for the repeating unit to be proposed.



The primary structure differs from that of the *Klebsiella* K5 polysaccharide[16] only in the reported site of *O*-acetylation, the ester being on the 2 position of the glucose residue in the K5 polysaccharide. The similarity in structures suggests a possible immunological cross-reactivity between the two capsular antigens.

The usefulness of the bacteriophage degradation technique is illustrated in this study of the *E. coli* K55 polysaccharide. The use of an enzyme isolated from another source (*Klebsiella* Ø5) suggests that these degradations may be extended to include polysaccharides from sources other than bacteria. The slight variation in the structures of the *E. coli* K55 and *Klebsiella* K5 polysaccharides indicates that minor structural variations can be tolerated by the lyase enzyme, thus extending the number of potential substrates. Oligosaccharides containing acid labile groups (*e.g.* acetates) can be isolated intact and thereby provide more structural information about the original polysaccharide.

Lyase activity has been reported for bacteriophages acting on *Klebsiella* K5,[183] K14,[18] and K64 (ref. 185) capsular polysaccharides. A structural feature common to these polysaccharides is a sterically hindered mannopyranosyl unit attached to the 4 position of a glucopyranosyluronic acid unit. This arrangement allows a facile axial-equatorial ß-elimination reaction (lyase activity) to occur in preference to endoglycanase activity and produces a terminal hex-4-enuronic acid moiety.

5.3.4 Experimental

General methods. - See Section 5.1. G.p.c. of the isolates from batches 1-5 (∿3.0 mg) was performed on a column of Sephacryl S500 as described in Section 5.1.1 (Fig. 6). The procedure proposed by Hascall *et al.*[415] for the thiobarbituric acid test was followed using 1 mg samples of **10**, **11**, and K55 polysaccharide and the resultant solutions were analyzed by spectrophotometry.

Preparation and properties of K55 polysaccharide. - An authentic culture of *E. coli* O9:K55 (N 24c) was obtained from Dr I. Ørskov (Copenhagen). Five batches of the bacteria were grown on Mueller-Hinton agar as described (Section 5.1.2) and incubated for the periods specified in Table V. Isolation of the acidic polysaccharide was achieved by precipitation with CTAB to give yields of 513, 674, 461, 496, and 980 mg for batches 1-5, respectively. The total sugar ratio of the isolates (∿2 mg), after carboxyl reduction and hydrolysis (8 h), was measured by g.l.c. analysis (225°) of the PAAN derivatives (Table V). The sugar analysis of K55 polysaccharide (batch 5), with and without prior carboxyl reduction, was carried out as described in Section 5.1.2 (Table VI, columns I and II). Samples of K55 polysaccharide from batches 3 (17.2 mg, *7*) and 5 (19.2 mg, 8) were dissolved in water (2 mL) and ultrasonicated for 0.5 h to reduce the viscosity prior to n.m.r. analyses (Table VII).

Methylation analysis. - K55 polysaccharide (33.8 mg, acid form) was methylated once by the Hakomori method[113] (potassium dimsyl[115]) and then by the Kuhn method.[399] The permethylated product (14.0 mg) was methanolyzed (19 h) and the methanolyzate split into two portions; the first portion was hydrolyzed (8 h) and derivatized (alditol acetates) whilst the second was carboxyl reduced ($NaBH_4$) prior to hydrolysis (8 h) and derivatization (alditol acetates). G.l.c. (205°) and g.l.c.-m.s. analyses of these derivatives gave the results in Table VIII, columns I and II. K55 polysaccharide (6.1 mg) in the acid form was dissolved in water (5 mL) and heated at 100° for 40 min. The solution was then filtered, dialyzed against running tap water (3500 Mw cut-off, 1 d), and freeze-dried to give depyruvalated K55 polysaccharide **9** (4.0 mg). Methylation of **9** (Hakomori,[113] potassium dimsyl[115]) followed by

hydrolysis (16 h) and derivatization (alditol acetates) gave the results in Table VIII, column III.

Bacteriophage degradation. - A sample of *Klebsiella* Ø5 was obtained from Prof. S. Stirm (Freiburg-Zahringen). The bacteriophage was propagated on *E. coli* K55 in nutrient broth as described in Section 5.1.2 until a crude solution (600 mL) containing 2.2 x 10^{13} PFU was obtained. The solution was purified to give a bacteriophage solution (1.5 x 10^{13} PFU, 230 mL) to which was added K55 polysaccharide (batch 5, 494.7 mg) and the degradation allowed to proceed for 5 d at 30°. The bacteriophage degradation products, **10** (104.8 mg) and **11** (119.5 mg), were isolated from the degraded K55 polysaccharide solution by g.p.c. as described in Section 5.1.2 (Fig. 7). The results of the g.l.c. analyses of **10** (∿1.8 mg), with and without prior carboxyl reduction, as the PAAN derivatives are given in Table VI, columns III and V. **10** (5.5 mg) was reduced with sodium borodeuteride and a sample of the product (**10**-alditol, 1.0 mg) was analyzed by the Morrison method[76] (Table VI, column IV). The remainder of **10**-alditol was methylated (Hakomori,[113] potassium dimsyl[115]) and derivatized (alditol acetates), after hydrolysis (18 h), for analysis by g.l.c. (205°) and g.l.c.-m.s. (Table VIII, column V). A sample of **10** (4.7 mg) was methylated by the Prehm method[129] (see Section 5.1.2) and similarly analyzed (Table VIII, column IV). **11** (1.3 mg) was hydrolyzed (19 h) and derivatized (PAAN's) whilst a second sample of **11** (2.4 mg) was reduced ($NaBH_4$), methanolyzed (10 h), carboxyl reduced, hydrolyzed (10 h) and derivatized (PAAN's). The derivatives produced were analyzed by g.l.c. (225°) and the results presented in Table VI, columns VI and VII. A sample of **11** after reduction ($NaBD_4$) was methylated (Hakomori,[113] potassium dimsyl[115]), methanolyzed (19 h), carboxyl reduced, and derivatized to give the partially methylated alditol acetates which were analyzed by g.l.c. (205°) and g.l.c.-m.s. (Table VIII, column VI). Samples of **10** (40.1 mg), **10**-alditol ($NaBH_4$, 17.0 mg), de-*O*-acetylated **10** (Section 5.1.2, 11.3 mg), **11** (41.8 mg), and **11**-alditol ($NaBH_4$, 19.2 mg) were analyzed by n.m.r. spectroscopy (Tables VII and IX).

Determination of absolute configuration. - K55 polysaccharide (5.2 mg) was hydrolyzed (18 h) and converted to the (-)-2-octyl glycosides[99] whilst a second sample (6.4 mg) was methanolyzed, carboxyl reduced and similarly derivatized (Section 5.1.2). These derivatives were analyzed by g.l.c. at 230°.

5.4 ELUCIDATION OF THE *KLEBSIELLA* K39 CAPSULAR POLYSACCHARIDE STRUCTURE USING BACTERIOPHAGE DEGRADATION.

5.4.1 Introduction

The capsular polysaccharide from *Klebsiella* K39 was originally thought to be composed of D-glucuronic acid, D-glucose, D-mannose, and D-galactose.[9] D-Galactose has however, now been shown not to be part of the K39 polysaccharide structure, its presence being due to a galactose-rich polysaccharide contaminant. The structure of the K39 polysaccharide was first investigated by Leek[418] who isolated two oligosaccharides, **A1** and **A2**, from a partial hydrolysis study of the polysaccharide. Sugar analyses and ^{1}H-n.m.r. spectra of **A1** showed that the compound was a pentasaccharide composed of glucose, mannose, and glucuronic acid in a molar ratio of 1:2:2. ^{1}H-N.m.r. spectroscopy (**A1** and **A1**-alditol) indicated that the two mannose residues were α-linked and that the uronic acid residues were ß-linked (data (400 MHz) presented in Table XII). Methylation analysis of **A1**, carboxyl-reduced **A1**, and **A1**-alditol showed a 3-linked glucose reducing end group, a non-reducing glucopyranosyluronic acid terminus, and three in-chain sugar residues (one 4-linked glucopyranosyluronic acid residue and two 2-linked mannose residues). Sequencing of **A1** was achieved by e.i.-m.s. of the borodeuteride-reduced, permethylated oligosaccharide. From these analytical results, the following structure for **A1** was proposed.

ß-Glc*p*A-(1→2)-α-Man*p*-(1→4)-ß-Glc*p*A-(1→2)-α-Man*p*-(1→3)-Glc

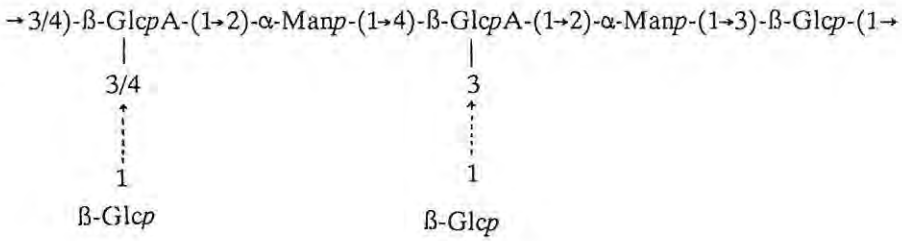
A1 (12)

Similar analyses, with the exception of e.i.-m.s., were performed on **A2** which was shown to be the tetrasaccharide corresponding to **A1** minus the glucose reducing sugar.

The methylation analyses of K39 polysaccharide indicated that the repeating unit for the polysaccharide had a branch-point at one of the glucopyranosyluronic acid residues (identified from 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methylglucitol found after carboxyl reduction) and that glucose was the terminal non-reducing residue in the side-chain (identified from 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol). Methylation analysis of the product after mild acid hydrolysis of K39 polysaccharide showed the appearance of a di-*O*-methylglucitol acetate

derivative and a concomitant decrease in the amount of the above monomethyl derivative in a carboxyl-reduced sample, thus indicating that the side-chain had been cleaved. Since all of the in-chain residues were still accounted for in the methylation analysis, the side-chain was considered to be composed of a single glucose unit.

From the above results, a partial structure for the K39 polysaccharide was proposed as shown below.



Leek[418] was unable to unambiguously locate the site of the pendant ß-glucopyranosyl residue and could not explain the presence of a 3-linked galactose derivative in the methylation analyses. The side-chain was readily cleaved from the polysaccharide during the partial hydrolysis study and thus the oligosaccharide representing the intact repeating unit was not isolated. The milder degradative process of bacteriophage-mediated depolymerization has now been applied to the K39 polysaccharide, resulting in the isolation of the intact repeating unit for the polysaccharide and thereby the elucidation of the complete structure for this antigen (see ref. 178).

5.4.2 Results and Discussion

Composition of K39 polysaccharide. - The polysaccharide was isolated by precipitation with CTAB from several batches of the *Klebsiella* K39 bacteria grown on three different agar media, *viz.* sucrose-rich agar, nutrient agar, and Mueller-Hinton agar. Sugar analysis of the individual isolates, without carboxyl reduction (Table X), showed that they contained varying amounts of mannose, glucose, and galactose however, the mannose-glucose ratio appeared to be

constant whereas the amount of galactose varied. The ^{1}H-n.m.r. spectrum of the polysaccharide from batch 2 (predominantly galactose-containing isolate) showed poor signal resolution in the anomeric region (Section 7.1.14), suggesting that this polymer did not have a structural repeating unit. A similar analysis of the isolate from batch 6 (minimal galactose present) gave well defined signals for six anomeric protons corresponding to the signals reported for the material isolated originally[418] (Section 7.1.15). Immunoelectrophoresis tests (performed by Drs I. and F. Ørskov (Copenhagen)) showed that the polysaccharide from batch 6 corresponded to the standard test antigen for *Klebsiella* K39 but not that from batch 2.[419] The production of a galactose-rich polymer has been noted in related studies on *E. coli* (see Section 5.3) but this is the first time it has been reported for the *Klebsiella* genus. While no systematic study has been done, it appears that the proportion of galactose may be reduced by growing *Klebsiella* bacteria on sucrose-rich agar at 30° for a short period of time. The polysaccharide from batch 6 (designated as the K39 polysaccharide) was used for further investigation.

TABLE X

SUGAR ANALYSES OF *Klebsiella* K39 GROWTH ISOLATES

Sugar (as peracetylated aldononitrile)	Analysis (%)[a,b]					
	I	II	III	IV	V	VI
Mannose	2	0	14	5	13	39
Glucose	3	0	16	6	15	48
Galactose	75	80	54	67	57	3

[a]Determined on a capillary DB-225 column at 225°. [b]I-VI represent batches 1-6, respectively, without carboxyl reduction (see text).

TABLE XI

SUGAR ANALYSES OF *Klebsiella* K39 POLYSACCHARIDE AND DERIVED PRODUCTS

Sugar (as peracetylated aldononitrile)	Molar ratio[a,b]			
	I	II	III	IV
Mannose	1.64	2.10	1.57	2.11
Glucose	2.00	4.00	1.00	3.00
Glucitol[c]	-	-	0.70	0.70

[a]Determined on a capillary DB-225 column at 225°. [b]I, Native K39 polysaccharide; II, carboxyl-reduced K39 polysaccharide; III, **14**-alditol; and carboxyl-reduced **14**-alditol. [c]Glucitol hexa-acetate.

The K39 polysaccharide was shown to be polydisperse by g.p.c. (Sephacryl S500) with two major peaks corresponding to M_r = 1.0 x 10^7 and 6.2 x 10^6 (Fig. 9A). After treatment with Amberlite IR-120(H^+) resin, a product giving a single broad peak (M_r = 3.4 x 10^4) was obtained (Fig. 9B). Although no phosphate analyses were performed on this polysaccharide, these g.p.c. results may be due to a similar type of micellar deaggregation discussed for the *E. coli* K37 polysaccharide (Section 5.2). G.l.c. analysis of the native polysaccharide and a carboxyl-reduced sample suggested that the polysaccharide contained equimolar amounts of mannose, glucose, and glucuronic acid (Table XI, columns I and II). ^{1}H-N.m.r. spectroscopy (see Table XII) of a partially autohydrolyzed sample of the polysaccharide (**13**) indicated the presence of six anomeric protons corresponding to two α-linkages (δ5.20 and δ5.45) and four β-linkages (δ4.93, $J_{1,2}$ 8.0 Hz (1H) and δ4.53-4.71 (3H)). These signals were assigned on the basis of previously reported data[418] and by studying the oligosaccharides derived during this investigation. ^{13}C-N.m.r. data for **13** (Table XII) substantiated these results.[349] No signals for non-carbohydrate substituents were observed in the n.m.r. spectra.

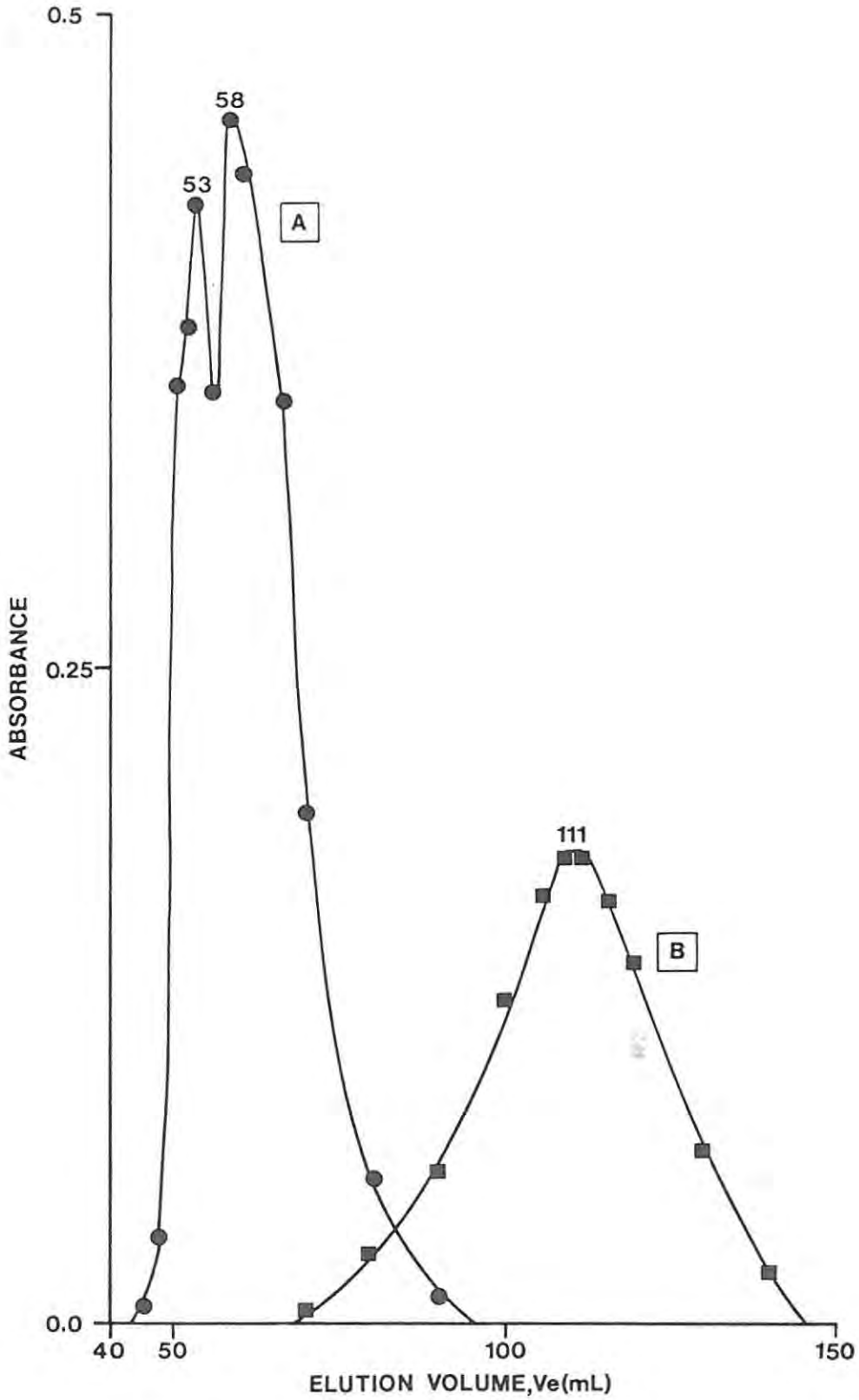


Fig. 9. Gel-permeation chromatography on Sephacryl S500: A, K39 polysaccharide (●); and B, K39 polysaccharide after treatment with Amberlite IR-120(H^+) resin (■).

TABLE XII

N.M.R. DATA (500 MHz) FOR *Klebsiella* K39 POLYSACCHARIDE AND DERIVED PRODUCTS

^{1}H-N.m.r. data				^{13}C-N.m.r. data	
δ[a] (p.p.m.)	$J_{1,2}$[b] (Hz)	Integral (no. of H)	Assignment[c]	δ[d] (p.p.m.)	Assignment[e]
-3(Glcβ1-4)GlcAβ1-2Manα1-4GlcAβ1-2Manα1-3Glcβ1- (F) (E) (D) (C) (B) (A)				Autohydrolyzed K39 polysaccharide (**13**)[f]	
5.45	n.o.	1.0	-2Manα1- (B)	102.75	-4GlcAβ1- (C)
5.20	n.o.	1.0	-2Manα1- (D)	102.60	-3,4GlcAβ1- (E)
4.93	8.0	1.0	Glcβ1- (F)	102.42	Glcβ1- (F)
4.71 to 4.53		3.0	-3Glcβ1- (A) -4GlcAβ1- (C) -3,4GlcAβ1- (E)	101.30	-3Glcβ1- (A)
				100.91	-1Manα1- (D)
				100.15	-2Manα1- (B)
GlcAβ1-2Manα1-4GlcAβ1-2Manα1-3Glc (**12**)[g] (E) (D) (C) (B) (A)					
5.47	n.o.	1.0	-2Manα1- (B)	102.98	-4GlcAβ1- (C)
5.23	4.0	0.4	-3Glcα (A)	102.31	GlcAβ1- (E)
5.15	n.o.	1.0	-2Manα1- (D)	100.92	-2Manα1- (D)
4.66	8.0	0.6	-3Glcβ (A)	100.19	-2Manα1- (B)
4.62	8.0	1.0	-4GlcAβ1- (C)	96.74	-3Glcβ (A)
4.57	8.0	1.0	GlcAβ1- (E)	92.98	-3Glcα (A)
GlcAβ1-2Manα1-4GlcAβ1-2Manα1-3Glucitol (**12**-alditol)[g] (E) (D) (C) (B) (A)					
5.37	n.o.	1.0	-2Manα1- (B)		
5.27	n.o.	1.0	-2Manα1- (D)		
4.58	8.0	1.0	-4GlcAβ1- (C)		
4.51	8.0	1.0	GlcAβ1- (E)		

TABLE XII (*continued*)

^{1}H-N.m.r. data				^{13}C-N.m.r. data	
δ^a (p.p.m.)	$J_{1,2}{}^b$ (Hz)	Integral (no. of H)	Assignment^c	δ^d (p.p.m.)	Assignment^e
Glcβ1-4GlcAβ1-2Manα1-4GlcAβ1-2Manα1-3Glc (14)^h					
(F)	(E)	(D)	(C) (B) (A)		
5.46	n.o.	0.4	-2Manα1-(3Glcα)(B)	100.14	-2Manα1-(3Glcα)(B)
5.45	n.o.	0.6	-2Manα1-(3Glcβ)(B)	100.04	-2Manα1-(3Glcβ)(B)
5.23	4.0	0.4	-3Glcα (A)	92.95	-3Glcα (A)
5.15	n.o.	1.0	-2Manα1- (D)	100.86	-2Manα1- (D)
4.66	8.0	0.6	-3Glcβ (A)	96.66	-3Glcβ (A)
4.62	8.0	1.0	-4GlcAβ1- (C)	102.28	-4GlcAβ1- (C)
4.60	8.0	1.0	-4GlcAβ1- (E)	102.78	-4GlcAβ1- (E)
4.51	8.0	1.0	Glcβ1- (F)	102.93	Glcβ1- (F)
Glcβ1-4GlcAβ1-2Manα1-4GlcAβ1-2Manα1-3Glucitol (14-alditol)					
(F)	(E)	(D)	(C) (B) (A)		
5.27	n.o.	1.0	-2Manα1- (B)	103.04	-4GlcAβ1- (C)
5.20	n.o.	1.0	-2Manα1- (D)	102.93	-4GlcAβ1- (E)
4.63	8.0	1.0	-4GlcAβ1- (C)	102.26	Glcβ1- (F)
4.62	8.0	1.0	-4GlcAβ1- (E)	101.48	-2Manα1- (B)
4.54	8.0	1.0	Glcβ1- (F)	100.81	-2Manα1- (D)

$^{a-f}$For legends, see TABLE III. ^{g}400 MHz data from D.M.Leek (ref. 418). ^{h}^{1}H- and ^{13}C-N.m.r. data are correlated from the HETCORR spectrum (see Section 7.3.2).

Methylation analysis. - The methylation analyses of K39 polysaccharide (Table XIII, columns I and II) gave results that were in accordance with the published partial structure (see Introduction). The polysaccharide was shown to contain a glucopyranosyluronic acid branch point and a glucose residue at the non-reducing terminus of the side-chain. From the partial hydrolysis studies reported by Leek,[418] the side-chain was composed solely of the glucose residue however, its specific site of attachment to the polymeric chain was unknown. To resolve this problem, a bacteriophage degradation was performed on K39 polysaccharide.

TABLE XIII

METHYLATION ANALYSES OF *Klebsiella* K39 POLYSACCHARIDE AND DERIVED PRODUCTS

Methylated sugar[a] (as alditol acetate)	T_R[b]	Molar ratio[c,d]			
		I	II	III	IV
1,2,4,5,6-Glc	0.48	-	-	1.15	0.20
2,3,4,6-Glc	1.00	0.81	0.60	1.00	0.24
3,4,6-Man	1.65(1.42)	1.75	1.95	2.39	2.00
2,4,6-Glc	1.65(1.52)	1.00	0.93	-	-
2,3-Glc	3.54	-	1.00	-	1.76
2-Glc	4.95	-	0.65	-	-

[a]1,2,4,5,6-Glc = 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylglucitol, *etc.*; all substitution patterns were confirmed by g.l.c.-m.s. [b]Retention time (T_R) relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyglucitol run under the same chromatographic conditions. [c]Determined on a capillary DB-225 column at 205°; where derivatives were unresolved, separation and quantification was achieved on a capillary DB-17 column at 195°, the relative retention times being given in parentheses. [d]I, Native K39 polysaccharide; II, carboxyl-reduced K39 polysaccharide; III, **14**-alditol; and IV, carboxyl-reduced **14**-alditol.

Bacteriophage degradation.[172] - *Klebsiella* Ø39 was isolated from local sewage water, purified, and built up on its host strain to a titre of 1.3 x 10^{13} PFU (145 mL). The bacteriophage solution was used to degrade K39 polysaccharide (504.5 mg) and subsequent dialysis of the reaction mixture gave oligomeric material from which **14** (178.2 mg), **15** (171.5 mg), and **16** (87.7 mg) were isolated by g.p.c. (Fig. 10). From the elution volumes of these fractions, **14** was thought to be the intact repeating unit of the polysaccharide, with **15** and **16** being the dimer and trimer thereof, respectively. **15** and **16** were not examined further. It is interesting to note the high yield of oligomeric material (~87%) recovered from this specific degradation technique.

Sugar analysis of **14** by the Morrison method,[76] with and without carboxyl reduction (Table XI, columns III and IV), showed that the oligosaccharide contained six sugar residues in an equimolar ratio of mannose, glucose, and glucuronic acid, with a glucose reducing end group. ^{1}H- and ^{13}C-n.m.r. spectral analyses (Table XII) confirmed that **14** was a hexasaccharide with a glucose sugar at the reducing end. The ^{1}H-n.m.r. signal for the α-mannose residue interior to the terminal reducing group could be readily identified due to twinning observed in the expanded ^{1}H-n.m.r. spectrum (δ5.45, -2Manα1-(3Glcß); δ5.46, -2Manα1-(3Glcα)), these assignments being made after consideration of the α/ß ratio of the reducing end signal. The identification of this mannose signal allowed the signal at δ5.15 to be assigned to the second α-mannose residue in **14**. A 2D heteronuclear correlation (HETCORR) spectrum of **14** (see Section 7.3.2), in conjunction with the reported spectral assignments for the oligosaccharides derived from K39 polysaccharide,[418] allowed only a limited assignment of the remaining anomeric signals of **14**, although the anomeric linkages could still be elucidated. By comparing the n.m.r. spectra of **12** (Table XII), which constitutes the repeating unit of the backbone of the K39 polysaccharide (see partial structure), with those of **14**, an additional β-signal is observed in the spectra of **14**, indicating that the side-chain was ß-linked.

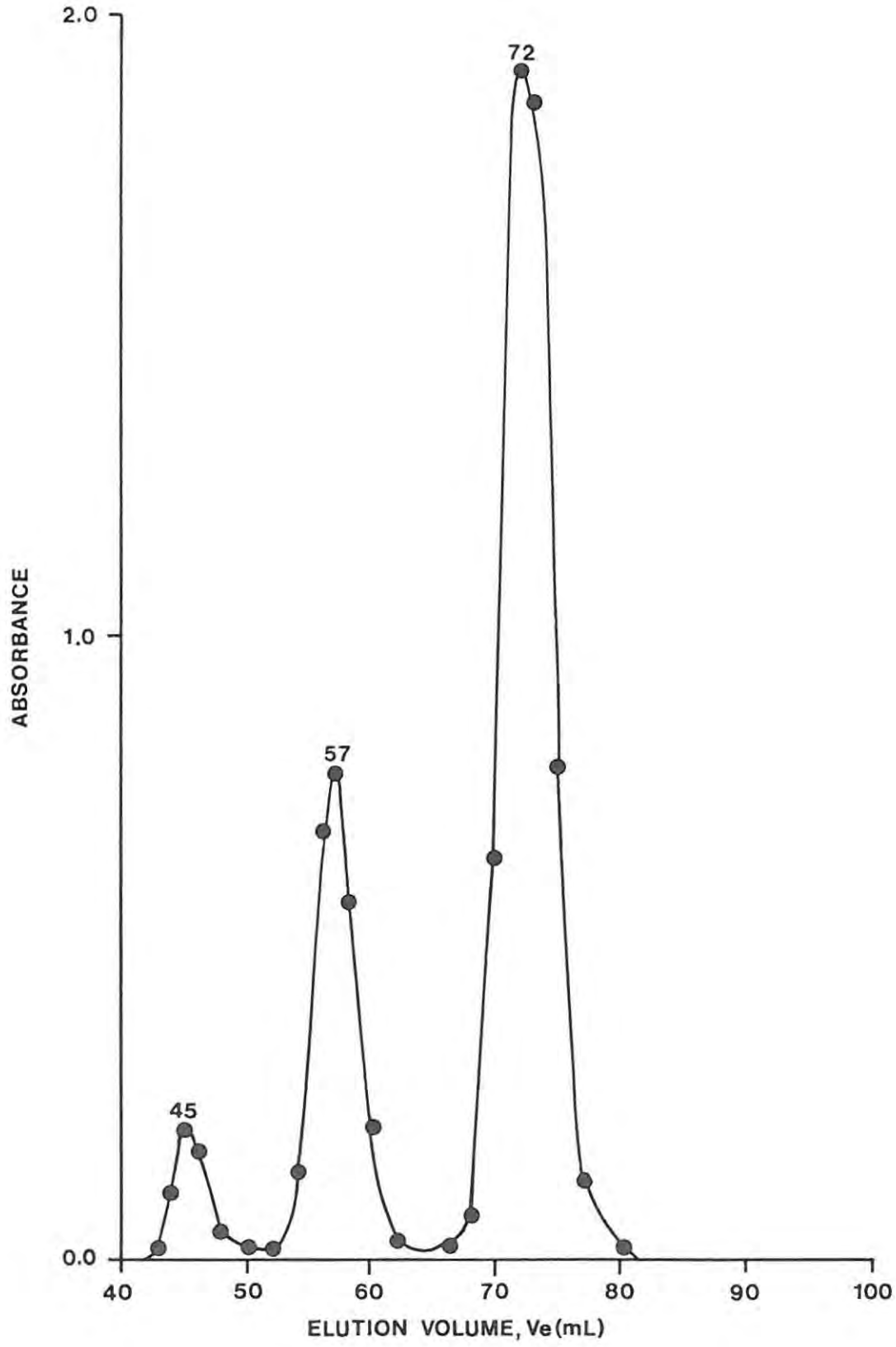


Fig. 10. Gel-permeation chromatography of K39 bacteriophage degradation products on Bio-Gel P4.

Methylation analyses of borodeuteride-reduced **14**, with and without carboxyl reduction (Table XIII, columns III and IV), showed it to be a straight-chain hexasaccharide with (1) a 3-linked glucose reducing end group, (2) two 2-linked in-chain mannose residues, (3) both in-chain glucopyranosyluronic acid residues 4-linked, and (4) a terminal non-reducing glucose residue. The low molar ratio of the 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol derivative from this terminal unit was due to losses during the evaporation steps in the analysis. By comparison with the structure of **12**, the following structure for **14** can be proposed.

ß-Glc*p*-(1→4)-ß-Glc*p*A-(1→2)-α-Man*p*-(1→4)-ßGlc*p*A-(1→2)-α-Man*p*-(1→3)-Glc

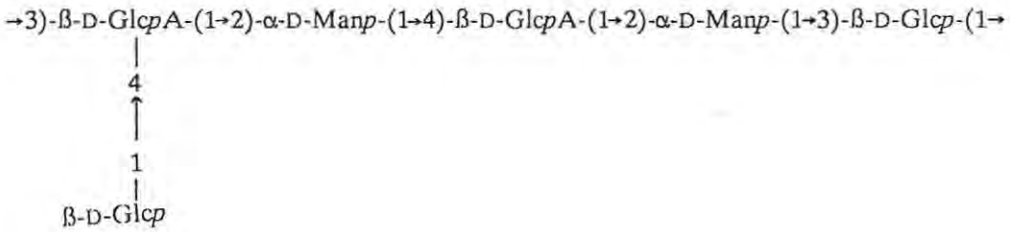
14

The results of the methylation analysis of **14**, when compared to those for the native K39 polysaccharide, enable the exact linkage site of the side-chain to be determined and thus allow a complete structure for the repeating unit of the *Klebsiella* K39 polysaccharide to be proposed.

Determination of absolute configuration.[99] - The peracetylated (-)-2-octyl glycosides prepared from an acid hydrolyzate for a carboxyl-reduced sample of the K39 polysaccharide were analyzed by g.l.c. (230°) and found to have the D-configuration.

5.4.3 Conclusion

From the above results, the partial structure for the *Klebsiella* K39 polysaccharide reported by Leek[418] can be amended and the following structure for this antigen may be proposed.



This is the first structure in the *Klebsiella* K-antigen series shown to have two uronic acid residues per repeating unit. Immunochemical studies[13] indicate that the *Klebsiella* K39 antigen has structural features in common with the *Klebsiella* K1 (ref. 15) and K31 (ref. 420) antigens. Comparison of the above structures shows that all three contain a 3-linked ß-glucopyranosyl unit linked to a substituted glucopyranosyluronic acid residue. This may constitute the immunodominant region for these antigens. The glucopyranosyluronic acid residues, however, vary in their degree and site of substitution, and their anomeric configuration. The results of the bacteriophage degradation of the K39 polysaccharide show that *Klebsiella* Ø39 has an endoglucosidase activity and that a high yield of oligomeric material is possible using this technique. These results are also consistent with the generalizations that bacteriophages tend to cleave ß-linkages at sterically hindered sites with the resultant reducing sugar residue being substituted at position 3 (see Section 3.2.3).

5.4.4 Experimental

General methods. - See Section 5.1. The sucrose-rich agar used for the growth of *Klebsiella* K39 bacteria was composed of the following materials: sucrose, 45.0 g; sodium chloride, 3.0 g; yeast extract, 3.0 g; dipotassium hydrogen phosphate, 1.5 g; magnesium sulphate heptahydrate, 0.4 g; calcium carbonate, 0.3 g; agar, 22.5 g; and distilled water to 1.5 L. The suspension was autoclaved as previously described.

Preparation and properties of K39 polysaccharide. - An authentic culture of *Klebsiella* K39 (7749) was obtained from Dr I. Ørskov (Copenhagen) and grown in six batches of six trays as described in Section 5.1.2 but with the following changes to the method: batches 1-3 were grown on sucrose-rich agar at 37° for 4 d; batch 4 was grown on Mueller-Hinton agar at 37° for 5 d; batch 5 on nutrient agar with 2 $g.L^{-1}$ sodium chloride at 37° for 4 d; and batch 6 on sucrose-rich agar at 30° for 3 d. The acidic polysaccharide was isolated as described, with yields of 288, 535, 402, 472, 150, and 1916 mg for batches 1-6, respectively. The isolate from batch 6 was found to be the K39 polysaccharide (see Section 5.4.2). G.p.c. (Sephacryl S500) was performed on K39 polysaccharide (3.1 mg) and on a sample of K39 polysaccharide

after passage through a column of Amberlite IR-120(H^+) resin (3.0 mg), as described in Section 5.1.1 (see Fig. 9). The K39 polysaccharide had $[\alpha]_D$ + 7° (*c* 5.9, water). A sample of K39 polysaccharide (55.2 mg, acid form) was heated in aqueous solution for 0.5 h at 80°, the solution was then dialyzed (3500 Mw cut-off) and freeze-dried to give **13** (35.9 mg) which was used for ^1H- and ^{13}C-n.m.r. studies (Table XII).

Sugar composition. - Samples (3-4 mg) of the isolates from batches 1-6 were hydrolyzed overnight and the hydrolyzates analyzed as the PAAN derivatives by g.l.c. (225°) as described (Table X; and Table XI, column I for batch 6 isolate). Dried K39 polysaccharide (batch 6, 9.8 mg) was methanolyzed, carboxyl reduced, and similarly analyzed after hydrolysis (Table XI, column II).

Methylation analysis. - K39 polysaccharide (47.9 mg, acid form) was methylated by the Hakomori method (sodium dimsyl)[113,398] and the etherification of all free hydroxyl groups was shown to be complete by i.r. spectroscopy. A sample of the permethylated product (10 mg) was hydrolyzed (19 h), derivatized (alditol acetates), and analyzed by g.l.c. (205°) and g.l.c.-m.s. (Table XIII, column I). A second sample (10 mg) was carboxyl reduced after methanolysis and analyzed as above (Table XIII, column II).

Bacteriophage degradation.[172] - The bacteriophage degradation was conducted as described in Section 5.1.2. *Klebsiella* Ø39 was isolated from local sewage, purified by repicking single plaques, and propagated on its host strain, *Klebsiella* K39, in nutrient broth until a titre of 2.5 x 10^{13} PFU was reached. This solution was purified to give a bacteriophage solution (145 mL) containing 1.3 x 10^{13} PFU to which K39 polysaccharide (504.5 mg) was added. After 72 h at 30° the degradation was terminated (freeze-dried) and the oligosaccharide fractions were isolated by g.p.c. as described (Fig. 10) to give **14** (178.2 mg), **15** (171.5 mg), and **16** (87.7 mg).

A sample of **14** and **14**-alditol (produced by sodium borodeuteride reduction of **14** in deuterium oxide solution) were deuterium exchanged for n.m.r. studies (Table XII). **14** (4.9 mg) was reduced ($NaBH_4$) and methanolyzed as described earlier. A portion of the methanolyzate was hydrolyzed (18 h) while the remainder was carboxyl reduced then hydrolyzed (16 h). The

hydrolyzates thus produced were analyzed as the PAAN derivatives by g.l.c. (225°) (Table XI, columns III and IV). **14**-Alditol (10 mg), after n.m.r. analyses, was permethylated (Hakomori, sodium dimsyl)[113,398] then methanolyzed. The methanolyzate was split into two portions; the first was hydrolyzed (17 h) and the second was carboxyl reduced then hydrolyzed (16 h). The derived alditol acetates from each hydrolyzate were analyzed by g.l.c. (205°) and g.l.c.-m.s. (Table XIII, columns III and IV).

Determination of absolute configuration.[99] - K39 polysaccharide (13.7 mg) was methanolyzed (18 h), carboxyl reduced, hydrolyzed (8 h), and then derivatized to the (-)-2-octyl glycosides as described. These derivatives were analyzed by g.l.c. (230°).

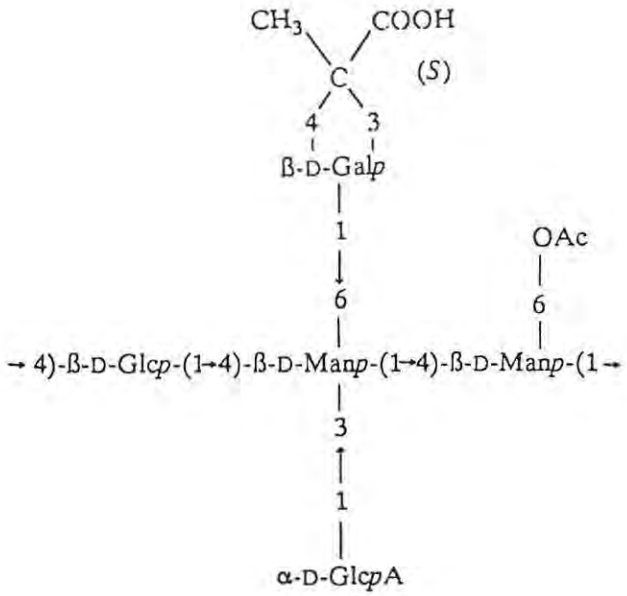
5.5 BACTERIOPHAGE DEGRADATION OF *KLEBSIELLA* K30 CAPSULAR POLYSACCHARIDE

5.5.1 Introduction

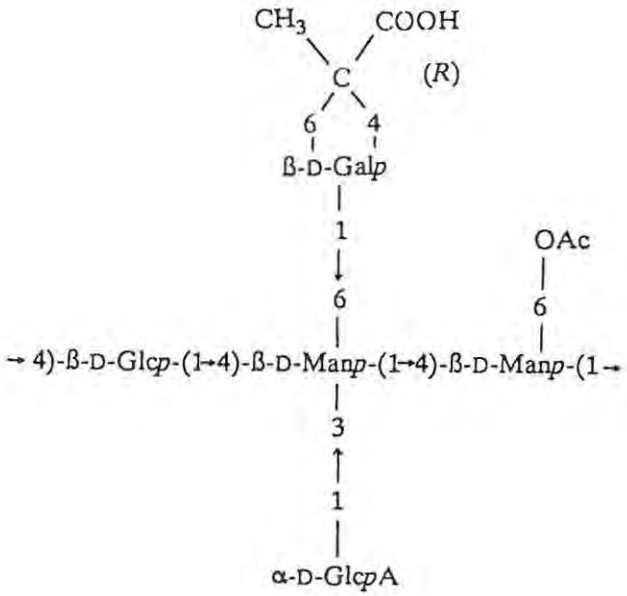
The structure of *Klebsiella* K30 (ref. 19) and K33 (ref. 20) capsular polysaccharides vary only in the degree of *O*-acyl substitution of the repeating units. Approximately one in every three repeating units are acetylated in the former polysaccharide while each repeating unit in the latter polysaccharide is substituted (Fig. 11). The structural study of the *Klebsiella* K69 polysaccharide using *Klebsiella* Ø69 (ref. 21) has revealed that the structure of the antigen is very similar to the K30 polysaccharide structure (Fig. 11), the only difference being the position of substitution by the pyruvate ketal; the ketal is 3,4-linked (*S*-configuration) in K30 and 4,6-linked (*R*-configuration) in K69. These structural similarities suggest that *Klebsiella* Ø69 may be able to cleave the K30 and K33 polysaccharides. As part of an investigation to study these possibilities, the bacteriophage degradation of K30 polysaccharide using *Klebsiella* Ø69 was effected.

5.5.2 Results and Discussion

Bacteriophage degradation of K30 polysaccharide. - *Klebsiella* K30 bacteria were grown on sucrose-rich agar as described earlier (see Section 5.4) and the acidic capsular polysaccharide (**17**) was isolated in the usual way. *Klebsiella* Ø69, isolated from local sewage water, was found to lyze cultures of *Klebsiella* K30 and therefore the bacteriophage was propagated on this host (see Section 5.1.2). A purified bacteriophage solution containing 7.6 x 10^{12} PFU (450 mL) was used to degrade *Klebsiella* K30 polysaccharide (0.5 g). Subsequent dialysis gave a mixture of oligosaccharides from which **18** (72.3 mg) and **19** (105.7 mg) were isolated by g.p.c. (Fig. 12). The relatively low yield of oligosaccharides from this degradation is probably due to the low concentrations of bacteriophage and K30 polysaccharide used.[172] From the g.p.c. elution volumes for **18** and **19**, these fractions were thought to consist of the repeating unit and the double repeating unit of the K30 polysaccharide, respectively. **19** was not further examined.



Klebsiella K30 (30% *O*-acetylated)[19]
Klebsiella K33 (100% *O*-acetylated)[20]



Klebsiella K69 (30% *O*-acetylated)[21]

Fig. 11. The structures of the *Klebsiella* K30, K33, and K69 capsular polysaccharides.

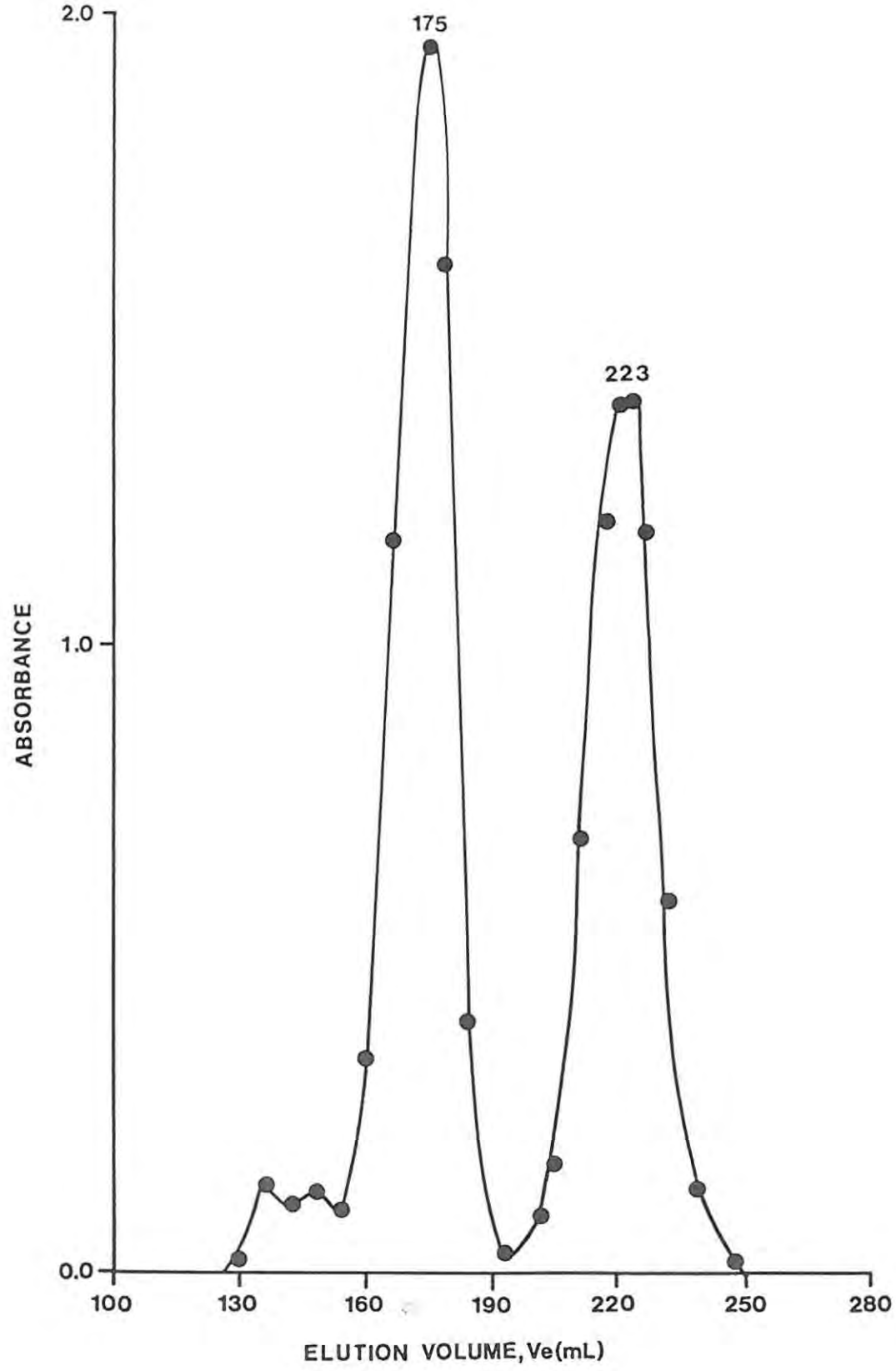


Fig. 12. Gel-permeation chromatography on Bio-Gel P4 for the bacteriophage degradation of *Klebsiella* K30 polysaccharide.

Chemical analysis of 18. - Sugar analyses of **18**, with and without prior carboxyl reduction (Table XIV, columns I and II), showed that this oligosaccharide contained mannose, glucose, galactose, and glucuronic acid in the molar ratio of 2:1:1:1. Reduction of **18** prior to sugar analysis (column III) indicated that the reducing end sugar was mannose and that **18** was a pentasaccharide. The concept of a pentasaccharide was supported by n.m.r. data (see below) which also revealed the presence of an *O*-acetyl group in **18** (80% present). **18** was methylated using the Prehm method[129] to enable the location of the acetyl group. The results of the methylation analyses (Table XV) are consistant with the known structure of the K30 repeating unit, where enzymatic cleavage of the glycosidic bond of the non-branched mannosyl residue by the bacteriophage has occurred (see Fig. 11). The presence of 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylmannitol indicates that the site of *O*-acetylation is on position 6 of the reducing end mannose sugar. Interestingly, the trimethylated alditol acetate expected from the non-acetylated reducing end residue (20% present from the n.m.r. data) was not observed in these methylation analyses. This indicates that **18** is fully acetylated and that the *O*-acetyl group was cleaved during the preparation of the oligosaccharide sample for n.m.r. analysis. If this is the case, then the K30 polysaccharide isolated contains one *O*-acyl substituent per repeating unit, thereby differing from the structure of the K30 polysaccharide reported by Lindberg *et al.*[19]

Mass spectrometric analysis of 18. - Direct probe e.i.-m.s. (20 eV) was performed on the permethylated (Prehm method[129]) sample of **18** (Fig. 13A). The peak for the molecular ion (*m/z* 1164, acetylated **18**; *m/z* 1136, non-acetylated **18**) was not observed in the mass spectrum although peaks for A_1-type fragments resulting from the molecular ion were recorded (see Fig. 13B). The presence of the *m/z* 901 peak ($M^+ - 263$) and the absence of a peak at *m/z* 929 indicate that the *O*-acetyl substituent was situated on the reducing end sugar of **18**, thereby supporting the results of the methylation analyses. The peak at *m/z* 177 (Fig. 13C) is indicative of a trisubstituted glycosyl residue from which non-reducing units have been cleaved by A_1-type fragmentation. The presence of this peak in the mass spectrum supports the

TABLE XIV
SUGAR ANALYSES OF 18 DERIVED FROM *Klebsiella* K30 POLYSACCHARIDE.

Sugar (as peracetylated aldononitrile)	Molar ratio[a,b]		
	I	II	III
Mannose	1.47	2.10	0.50
Glucose	1.12	2.09	1.13
Galactose	1.00	1.00	1.00
Mannitol[c]	-	-	1.54

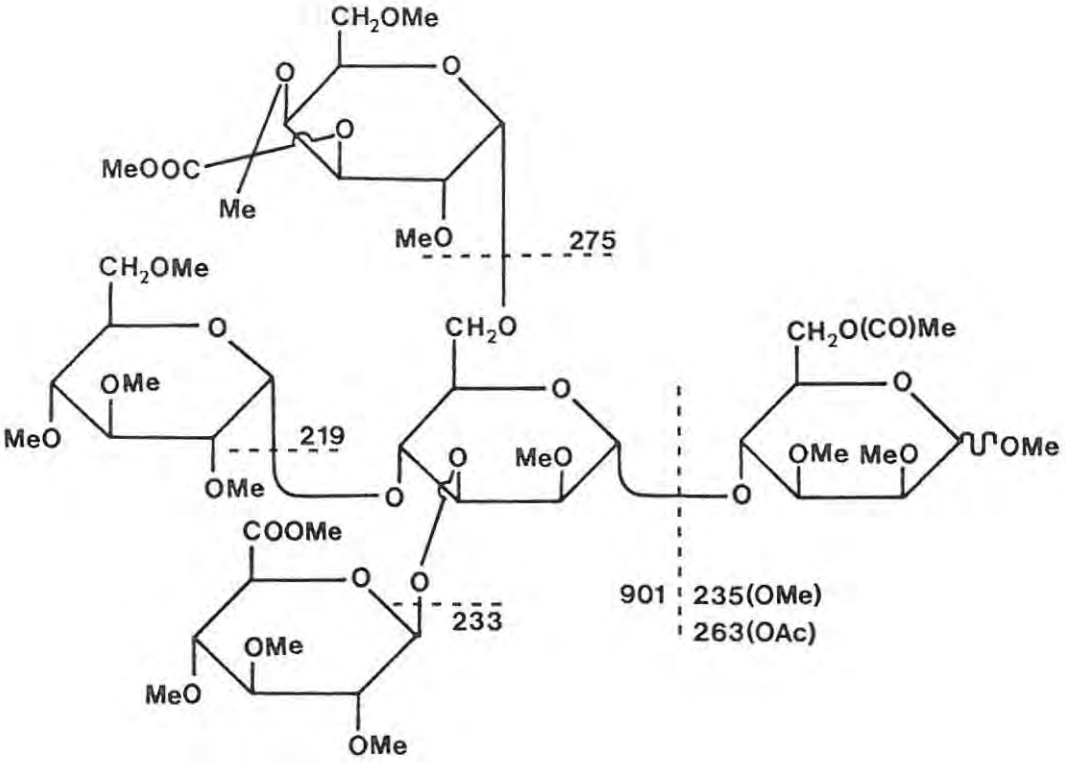
[a]Determined on a capillary DB-225 column at 225°. [b]I, **18**; II, carboxyl-reduced **18**; and III, **18**-alditol. [c]Mannitol hexa-acetate.

TABLE XV

METHYLATION ANALYSES OF 18 DERIVED FROM *Klebsiella* K30 POLYSACCHARIDE

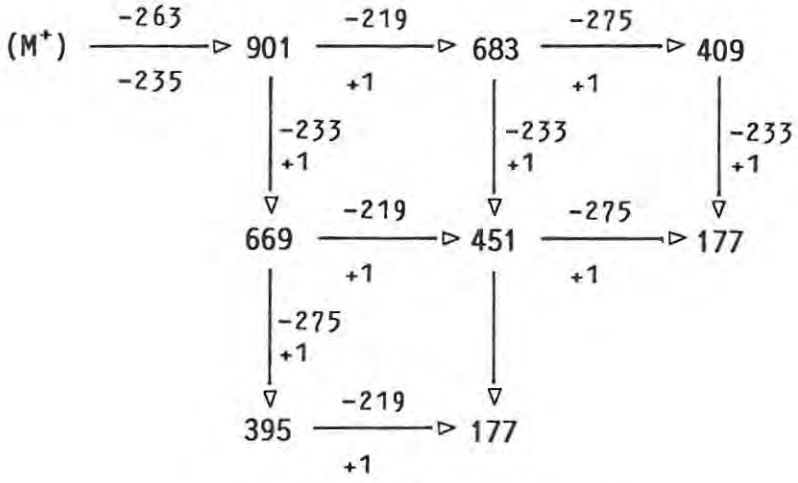
Methylated sugar[a] (as alditol acetate)	T_R[b]	Molar ratio[c,d]	
		I	II
2,3,4,6-Glc	1.00	1.00	1.00
2,3,4-Glc	1.80	-	0.57
2,6-Gal	2.77	0.63	0.80
2,3-Man	3.06	0.71	0.90
2-Man	3.82	0.38	0.85

[a]2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, *etc.*; all substitution patterns were confirmed by g.l.c.-m.s. [b]Retention time (T_R) relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol run under the same chromatographic conditions. [c]Determined on a capillary DB-225 column at 205°. [d]I, **18**; and II, carboxyl-reduced **18**.

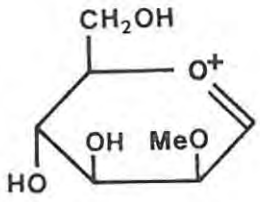


A. Permethylated **18**

(M^+ = *m/z* 1164 - acetylated)
(M^+ = *m/z* 1136 - non-acetylated)



B. A_1-Type fragments (*m/z*)

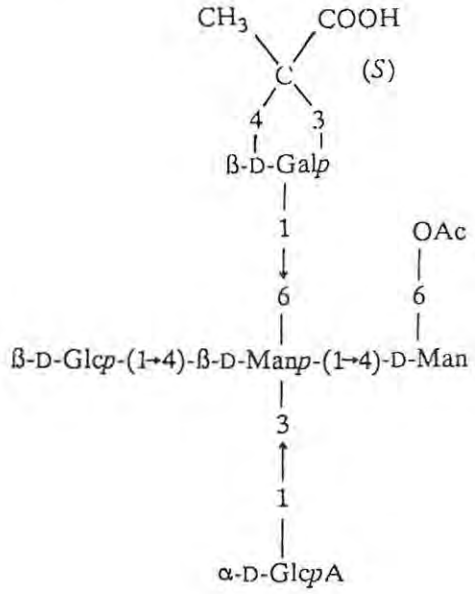


C. Mass fragment *m/z* 177

Fig. 13. Mass spectrometric analysis of permethylated **18**.

concept of the ion at *m/z* 901 being a branched tetrasaccharide species. These mass spectral results are in agreement with the results from the chemical analyses of 18.

The following structure for 18 can therefore be proposed.



18

The n.m.r. data discussed below support this proposal.

N.m.r. analyses of derived oligosaccharides. - The ^{1}H-n.m.r. spectrum of 18 showed the presence of a pyruvate ketal (δ1.69) and indicated that 80% of the molecules were *O*-acetylated (from signals at δ2.15 and δ2.17). The partial acetylation of 18 complicated the ^{1}H-n.m.r. spectrum and thus the data were not further analyzed. The ^{13}C-n.m.r. spectrum of 18 was also complicated however, DEPT spectroscopy (see Section 7.3.3) showed, *inter alia*, the signals at 61.34 and 63.96 p.p.m. in the spectrum to be due to CH_2 (C6) carbon nuclei (see below for discussion). To simplify the n.m.r. analysis, a de-*O*-acetylated sample (de-*O*-acetylated 18) and a reduced ($NaBD_4$) sample (18-alditol) of 18 were prepared and analyzed using 1- and 2-D n.m.r. experiments (see Sections 7.3.4 - 7.3.7).

18-alditol was analyzed using heteronuclear (HETCORR) and homonuclear (COSY) 2D shift correlated experiments in conjunction with 1D ^{1}H- and ^{13}C-n.m.r. experiments, and the data are collected in Table XVI. The 1D experiments showed the presence of a pyruvate ketal

TABLE XVI

N.M.R. DATA FOR SOME OLIGOSACCHARIDES DERIVED FROM *Klebsiella* K30 POLYSACCHARIDE

3,4-pyr-Galβ1-6(Glcβ1-4)(GlcAα1-3)Manβ1-4(6-OAc 80%)Man (**18**)[a]
(A) (B) (C) (D) (E)

	^{1}H data[b]		^{13}C data[c]	^{1}H data		^{13}C data
	δ (p.p.m.)	J (Hz)	δ (p.p.m.)	δ (p.p.m.)	J (Hz)	δ (p.p.m.)
	18-alditol			De-*O*-acetylated **18**		
Sugar A						
1	4.51	8.4	102.89	4.55	8.4	102.59
2	3.52	7.5	73.74[d]	3.53	7.5	73.81
3	4.30	5.6	79.80	4.34	5.9	80.01
4	4.24	2.0	76.13[e]	4.27	1.9	76.38
5	4.07		73.84[d]	4.08		73.81
6	≈3.94		61.53	≈3.89		61.60
6′	≈3.94			≈3.89		
COOH pyr			175.58			174.78
C2 pyr			107.35			107.00
CH_3 pyr	1.67		23.79	1.69		23.70
Sugar B						
1	4.53	7.9	103.25	4.57	7.9	103.16
2	3.28	9.3	74.16	3.28	9.4	74.15
3	3.50	9.2	76.23[e]	3.50	9.2	76.24
4	3.30	10.3	70.80	3.30	9.4	70.82
5	3.51	2.0/7.5	77.48	3.51	2.6/7.5	77.51
6	3.99	12.6	61.88	4.01	12.4	61.91
6′	3.74			3.74		
Sugar C						
1	5.21	4.0	102.27	5.23	4.0	102.35
2	3.57	9.8	72.15	3.57	9.8	72.35
3	3.83	9.5	74.16	3.83	9.8	74.07
4	3.58	10.1	72.47	3.60	10.0	72.07
5	4.37		72.47	4.37		72.43
6			174.20			173.80

TABLE XVI (*continued*)

3,4-pyr-Galβ1-6(Glcβ1-4)(GlcAα1-3)Manβ1-4(6-OAc 80%)Man (18)[a]
(A) (B) (C) (D) (E)

	^{1}H data[b]		^{13}C data[c]	^{1}H data		^{13}C data
	δ (p.p.m.)	J (Hz)	δ (p.p.m.)	δ (p.p.m.)	J (Hz)	δ (p.p.m.)
Sugar D						
1	4.84	n.o.[f]	100.66	4.78	n.o.	100.90
2	4.24	4.0	71.70	4.24	2.7	71.56
3	3.88	10.0	81.94	~3.89	~9.0	81.89
4	4.08	10.0	78.13	4.15	9.9	78.25
5	3.76	1.9/5.5	74.84	~3.77		74.75
6	4.34	11.1	68.77	4.32		68.08
6′	3.97			~4.00		
Sugar E						
1			63.21	5.18(α)	1.4	94.62
				4.91(β)	0.8	94.49
2	~3.93		71.43	~4.00(α)		71.38
				~4.01(β)		71.64
3	3.78		70.22	~4.00(α)		69.85
				~3.78(β)		72.99
4	4.08		73.50	~3.87(α)		77.95
				~3.87(β)		77.95
5	3.93	5.8/3.3	71.53	~4.17(α)		72.57
				~3.43(β)		75.50
6	3.66	11.8	63.21	~3.83(α)		61.35
				~3.83(β)		61.35
6′	3.82					

[a]For origin of compounds, see text. For n.m.r. spectra, see Sections 7.1-7.3. [b]Chemical shift measured from internal acetone, δ2.23. [c]As in *b* with acetone 31.07 p.p.m. [d,e]Assignments may be interchanged. [f]n.o. = not observed.

(δ1.67; 23.79, 107.35, and 175.58 p.p.m.) and the *S* configuration was assigned to this functional group on the basis of these chemical shifts.[332,333] The low-field position of resonance for the non-protonated carbon of the ketal (107.35 p.p.m.) has been reported by Gorin *et al.*[356] and is indicative of a 3,4-pyruvalated galactose residue. The COSY spectrum of **18**-alditol (Fig. 14) was used to map out the spin systems for the various sugar residues (labelled A - E in Table XVI). The ^{1}H-n.m.r. signals for sugar B were readily assigned from the COSY spectrum and, by comparison with data reported for methyl glycosides,[417] indicated that the sugar was a ß-glucopyranosyl residue. The ^{13}C-n.m.r. signals correlating to the ^{1}H-n.m.r. signals for sugar B in the HETCORR spectrum were compared with the data published by Boch and Pedersen[349] for methyl ß-glucopyranoside and the results support the above conclusion about sugar B. The ^{1}H- and ^{13}C-n.m.r. signals for sugar C were similarly analyzed. The analysis of this spin system was facilitated by the presence of virtual long-range couplings in the COSY spectrum (Fig. 14) between the H1-H5, H1-H3, and H3-H5 nuclei and by the large coupling constants present in this residue. After comparison with data published by Cavagna *et al.*[378] and those recorded by Ravenscroft[421] for glucopyranosyluronic acid residues, the signals for sugar C were assigned to an α-glucopyranosyluronic acid residue. The $J_{1,2}$ coupling constant for sugar C (4.0 Hz) supported this assignment.

The ^{1}H-n.m.r. signals for sugar A were mapped out from the COSY spectrum. The signals for H1 (δ4.51, $J_{1,2}$ 8.4 Hz) and H2 (δ3.52) compare favourably with the data reported for methyl ß-galactopyranoside.[417] Comparison of the remaining signals for sugar A with those of the methyl galactoside showed that the signals for H3 (δ4.30) and H4 (δ4.24) of sugar A appear appreciably further downfield than the corresponding signals in the galactoside (δ3.64 and 3.92, respectively) while the signal for H6 (∿δ3.94) is similar to that of the galactoside (δ3.80). ^{13}C-N.m.r. signals for sugar A, obtained from the HETCORR spectrum using the ^{1}H-n.m.r. signal assignments, show downfield shifts for the C3 (79.80 p.p.m.) and C4 (76.13 p.p.m.) resonances when compared with the reported signal resonances for methyl ß-galactopyranoside (73.8 and 69.7 p.p.m., respectively),[349] indicating that these positions in the sugar residue were substituted. These results suggested that sugar A was a ß-galactopyranosyl residue substituted

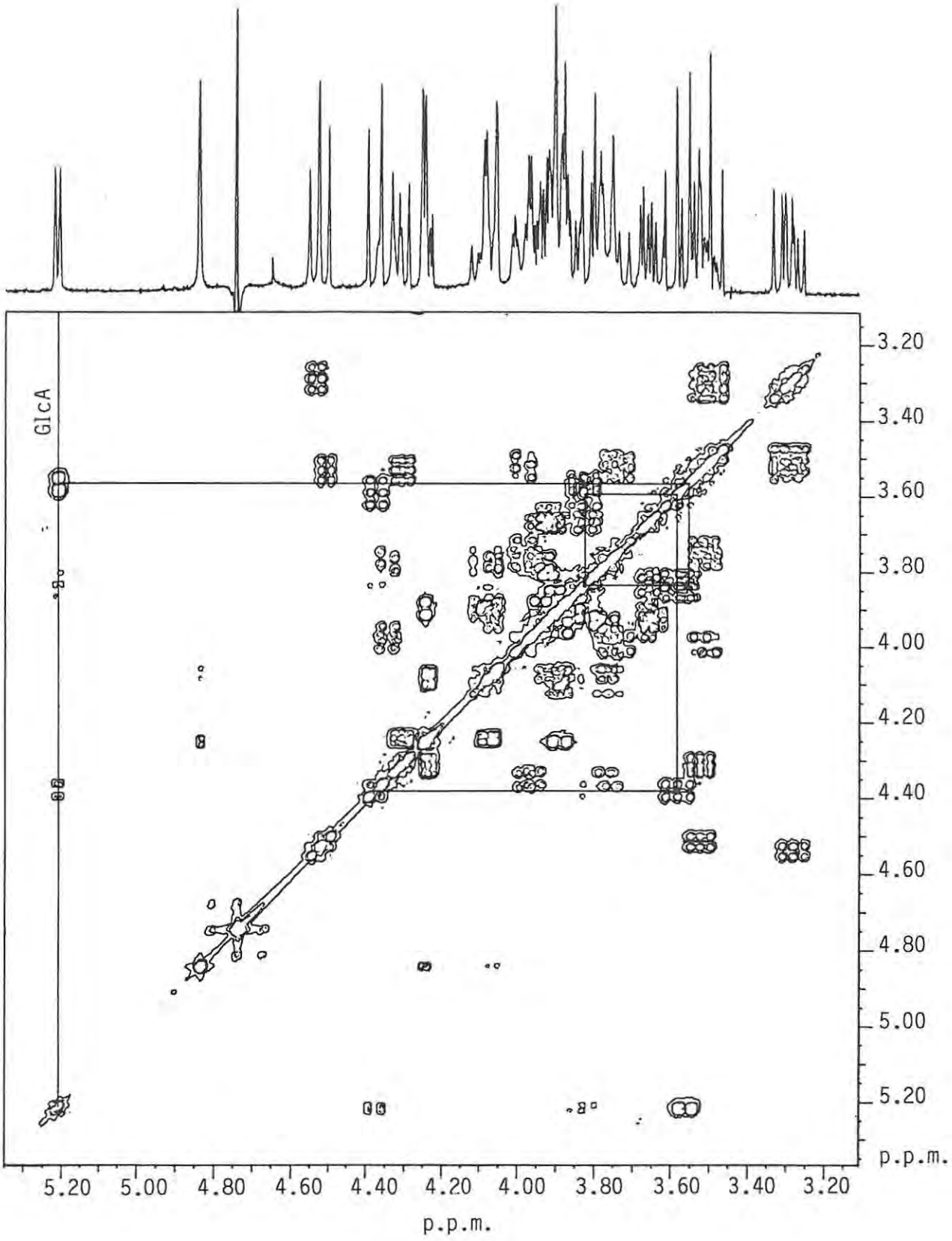


Fig. 14. COSY spectrum of 18-alditol.

at positions 3 and 4. The ^{1}H- and ^{13}C-n.m.r. signals for sugar D were assigned following the same procedure as for sugar A. Virtual long-range coupling between the H1-H4 nuclei in the COSY spectrum (Fig. 14) helped analyze the spin-system for sugar D. The $J_{1,2}$ coupling constant for sugar D could not be measured from the H1 signal (δ4.84) in the ^{1}H-n.m.r. spectrum, these results indicating that the sugar was a ß-mannopyranosyl residue. Comparing the assignments made for sugar D with the ^{1}H- and ^{13}C-n.m.r. data published for methyl ß-mannopyranoside[349,417] showed downfield shifts for the signals of positions 3, 4, and 6. Sugar D was considered to be a 3,4,6-trisubstituted ß-mannopyranosyl residue from the above results.

The spin system for sugar E, the alditol from the reducing end sugar of **18**, could not be mapped out from the COSY spectrum of **18**-alditol because the cross-peaks were too close to the diagonal-peaks. The assignments for this residue (Table XVI) were made by considering the ^{13}C-n.m.r. signals that were unassigned in the 1D ^{13}C-n.m.r. spectrum of **18**-alditol and comparing these with ^{13}C-n.m.r. data reported for alditols.[349] Prior knowledge of the type of residue (mannitol) and the site of substitution (position 4) were necessary for these assignments to be made. The ^{1}H-n.m.r. signals were assigned from the HETCORR spectrum of **18**-alditol using the ^{13}C-n.m.r. signal assignments. The ^{1}H- and ^{13}C-n.m.r. signal assignments for sugar E require verification using further 2D-n.m.r. experiments.

HETCORR, COSY, and 1D ^{1}H- and ^{13}C-n.m.r. experiments were performed on de-*O*-acetylated **18** and the results are presented in Table XVI. The signal assignments for sugars A-D follow directly from the assignments made for **18**-alditol. The ^{1}H- and ^{13}C-n.m.r. signal assignments for sugar E were again made by considering the signals remaining in the 1D ^{13}C-n.m.r. and HETCORR spectra after the signals for sugars A-D had been assigned and thus need further verification. It was possible, however, to unambiguously assign the C6 signal (61.35 p.p.m.) for sugar E. As mentioned earlier, the ^{13}C-n.m.r. spectrum of **18** showed signals at 61.34 and 63.96 p.p.m. due to C6 carbon nuclei. The 63.96 p.p.m. signal was absent from the ^{13}C-n.m.r. spectrum of de-*O*-acetylated **18** and there was a concomitant significant increase in the size of the signal recorded at 61.35 p.p.m. These results indicate that the *O*-acetyl group is located on the 6 position of the reducing end sugar in **18**

While the above n.m.r. analyses provide much useful information concerning **18**, the complete structure for this oligosaccharide cannot be unambiguously elucidated from the n.m.r. experiments performed. The structure of **18** can, however, be determined if the n.m.r. results are used in conjunction with the data from the chemical and mass spectrometric analyses presented earlier.

5.5.3 Conclusion

The bacteriophage degradation of *Klebsiella* K30 polysaccharide using *Klebsiella* Ø69 has allowed the isolation of the intact repeating unit for this antigen, showing the usefulness of this technique where acid labile substituents are present. The fact that *Klebsiella* Ø69 degraded the K30 polysaccharide is interesting since it shows that some structural variations in the enzyme substrate for this bacteriophage can be tolerated. This variation in substrate specificity increases the possible usefulness of the enzyme carried by *Klebsiella* Ø69 for the degradation of polysaccharides from sources other than *Klebsiella* K69 and K30.

5.5.4 Experimental

Bacteriophage degradation of K30 polysaccharide. - An authentic culture of *Klebsiella* K30 (7824) was obtained from Dr I. Ørskov (Copenhagen) and grown on sucrose-rich agar as described (Section 5.4.4). The acidic capsular polysaccharide was isolated by precipitation with CTAB (Section 5.1.2). *Klebsiella* Ø69, isolated from local sewage water by Mr P.L. Hackland, was propagated in nutrient broth using *Klebsiella* K30 until a titre of 1.6 x 10^{13} PFU was reached. The crude solution was purified (Section 5.1.2) to give a final bacteriophage solution (450 mL) containing 7.6 x 10^{12} PFU. The K30 polysaccharide and the bacteriophage solution were prepared by Dr L.A.S. Parolis and Miss S.D. Mee. K30 polysaccharide (0.5 g) was dissolved in the bacteriophage solution and the mixture was left for 5 d at 37°. Oligosaccharide fractions, **18** (72.3 mg) and **19** (105.7 mg), were isolated from the degraded material by g.p.c. as described (Section 5.1.1, Fig. 12).

Chemical analysis of 18. - A sample of **18** (0.5 mg) was hydrolyzed (18 h) and the hydrolyzate converted into the PAAN derivatives. A second sample (1.6 mg) was methanolyzed (18 h) and carboxyl reduced prior to hydrolysis (8 h) and derivatization (PAAN's). Both sets of derivatives produced above were analyzed by g.l.c. (225°) (Table XIV, columns I and II). A sample of **18** (1.2 mg) was reduced ($NaBH_4$), hydrolyzed (20 h), and derivatized (PAAN's). These derivatives were similarly analyzed by g.l.c. (Table XIV, column III). **18** (5.6 mg) was methylated using the Prehm method[129] (Section 5.1.2) and a portion of the permethylated product was subject to direct probe e.i.-m.s. (20 eV) on a Hewlett-Packard 5988A mass spectrometer (Fig. 13). A sample of the remaining permethylated **18** was hydrolyzed (20 h) and derivatized (alditol acetates) while the rest was methanolyzed (20 h), carboxyl reduced, hydrolyzed (10 h), and derivatized (alditol acetates). The partially methylated alditol acetate derivatives were analyzed by g.l.c. (205°) and g.l.c.-m.s. (Table XV).

N.m.r. analyses of derived oligosaccharides. - **18** (27.0 mg) was prepared for 1D n.m.r. analyses as described in Section 5.1.1. A sample of **18** (26.9 mg) was reduced with sodium borodeuteride (40 mg) in deuterium oxide (1 mL) to give **18**-alditol while a second sample of **18** (19.4 mg) was de-*O*-acetylated by the method of Amemura *et al.*[400] (see Section 5.1.2). 1D (^{1}H- and ^{13}C-n.m.r.) and 2D (HETCORR and COSY) experiments were performed on **18**-alditol and de-*O*-acetylated **18**, and the results are presented in Table XVI.

6. CONCLUSION

6. CONCLUSION

A general trend in the structural elucidation of polysaccharides towards the analysis of higher oligosaccharides can be seen from the literature reviewed in this thesis. The trend has been facilitated by advances in technology which have made instrumental techniques such as f.a.b.-m.s. and 2D-n.m.r. spectroscopy available for general research use. The feasibility of analyzing small amounts of carbohydrate material has become realistic due to improved instrumental capabilities and thus new areas of research have been opened up. At the same time, more detailed information concerning the fine structure of known carbohydrate materials can be elucidated. However, the use of the older, established chemical and instrumental techniques is still necessary for the analysis of many carbohydrates.

The determination of the structure of three bacterial capsular antigens, *viz.* *E. coli* K37 and K55, and *Klebsiella* K39, is presented in this thesis. The *E. coli* K37 capsular antigen has been shown to be the first in the *E. coli* species thus far examined to owe its acidity solely to the presence of a 1-carboxyethylidene ketal. The structure of the *E. coli* K55 antigen varies from that of the *Klebsiella* K5 antigen only in the site of *O*-acetylation. The similarity in structures tends to suggest that immunological cross-reactions may occur between these two antigens. The *Klebsiella* K39 capsular antigen is unique within the *Klebsiella* genus in that it contains two uronic acid residues per repeating unit in the polysaccharide. The elucidation of the *Klebsiella* K39 antigen structure may help rationalize the immunological cross-reactions observed between the antigen and the *Klebsiella* K1 and K31 antigens by defining chemically an immunodominant region within the structures.

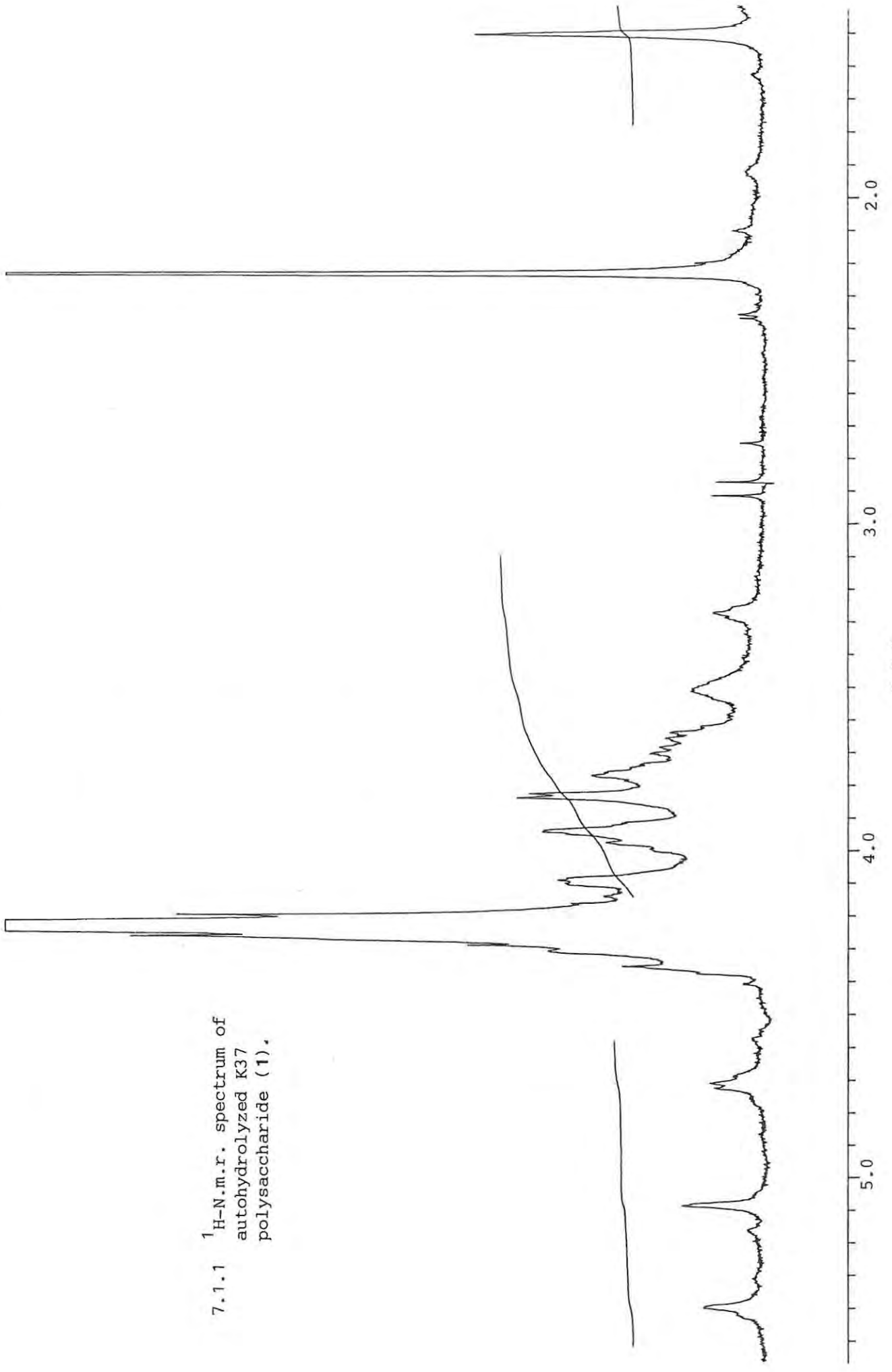
This thesis also emphasizes the usefulness of bacteriophage degradation as a technique for the structural elucidation of polysaccharides containing repeating unit structures. Bacteriophages provide a readily available source of enzymes which may be used to degrade polysaccharide material. The bacteriophage degradation of *E. coli* K55 polysaccharide illustrates that bacteriophage-borne enzymes may be used to degrade chemically related carbohydrate materials. Minor structural variations, such as the site of *O*-acetylation, may not be important for bacteriophage activity. The use of this technique for the isolation of

oligosaccharides containing labile substituents is also shown from this degradation and from the degradation of *Klebsiella* K30 polysaccharide. This latter degradation further emphasizes that structural variations in the substrate may be tolerated by the bacteriophage-borne enzymes. These tolerances may make the technique of bacteriophage degradation applicable to fields other than the structural elucidation of bacterial capsular antigens. The ability of the bacteriophage-borne enzymes to degrade substrates which differ chemically is maybe a factor which provides survival advantages to the bacteriophage. The use of the bacteriophage degradation technique for the structural elucidation of the *Klebsiella* K39 polysaccharide illustrates the advantages of the technique where the polysaccharide contains labile glycosidic bonds which are readily cleaved during standard chemical analyses. The enzymatic degradation of polysaccharides will also allow high yields of specific oligosaccharides to be recovered.

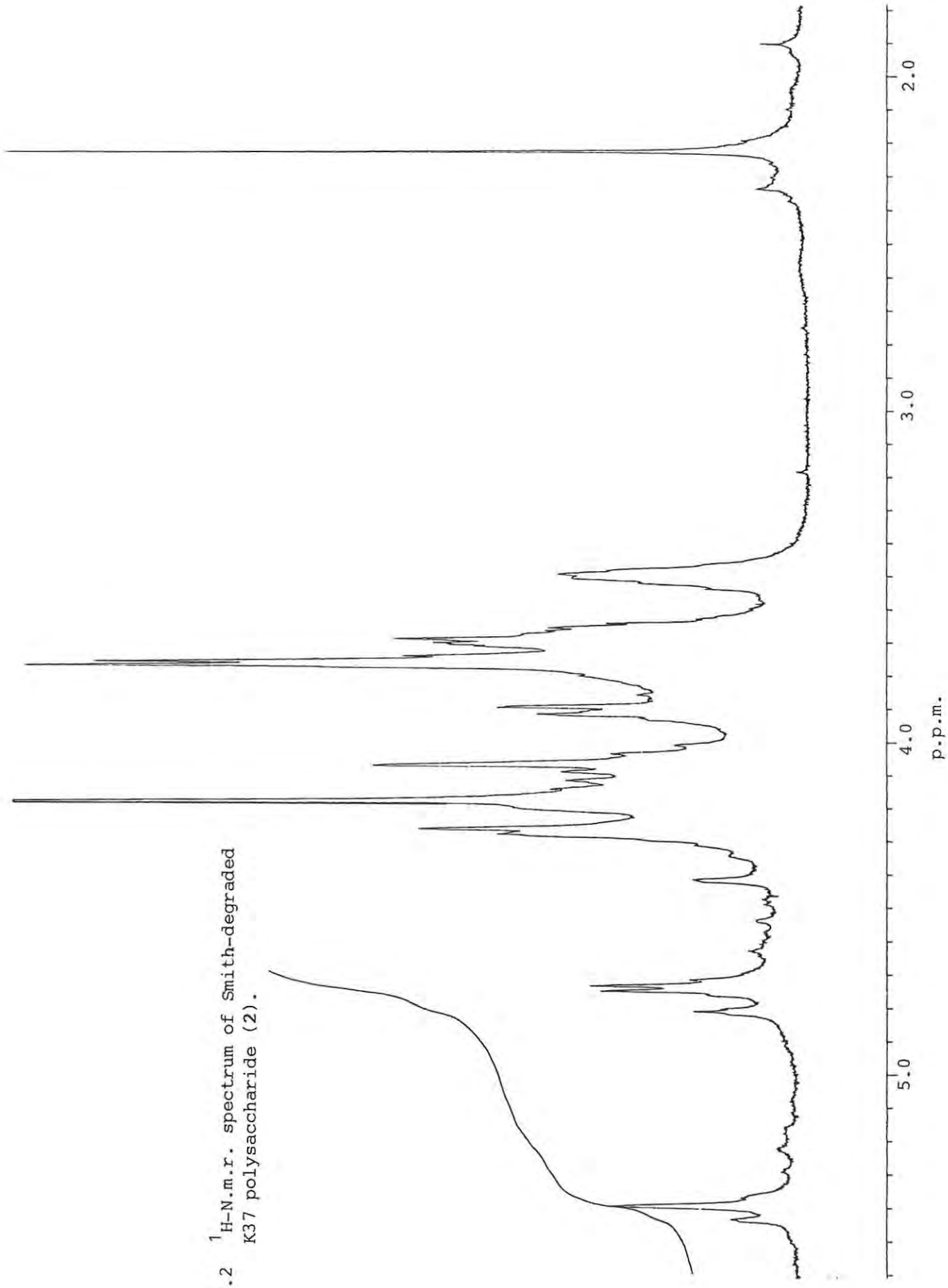
In the future, the structural analysis of carbohydrate materials will tend to become based upon the use of instrumental techniques whereby oligosaccharides derived by specific degradation techniques are analyzed. This format will provide more structural information about the saccharide and allow the rapid, accurate analysis of small samples.

7. ANNEXURES

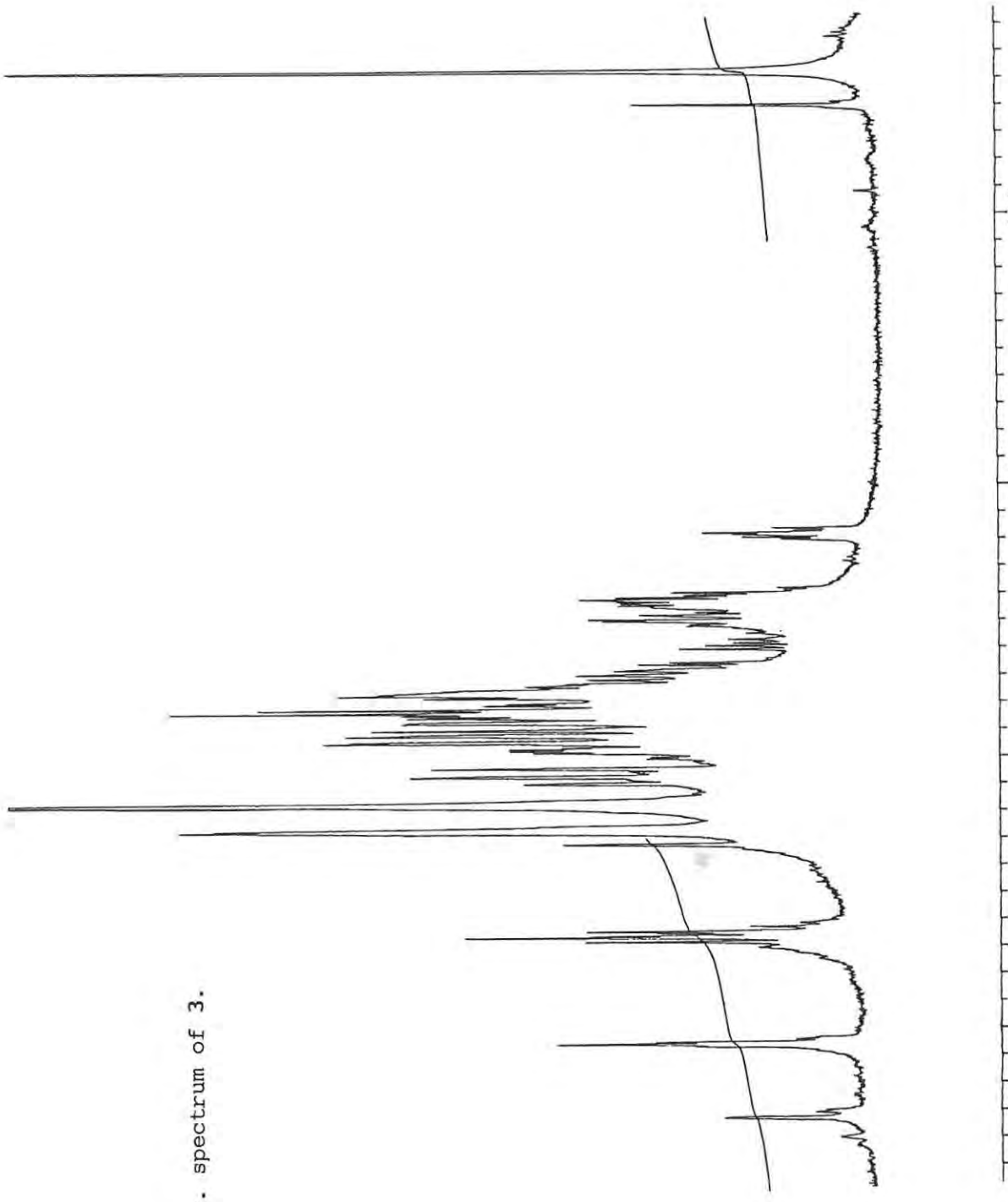
7.1 PROTON NUCLEAR MAGNETIC RESONANCE SPECTRA



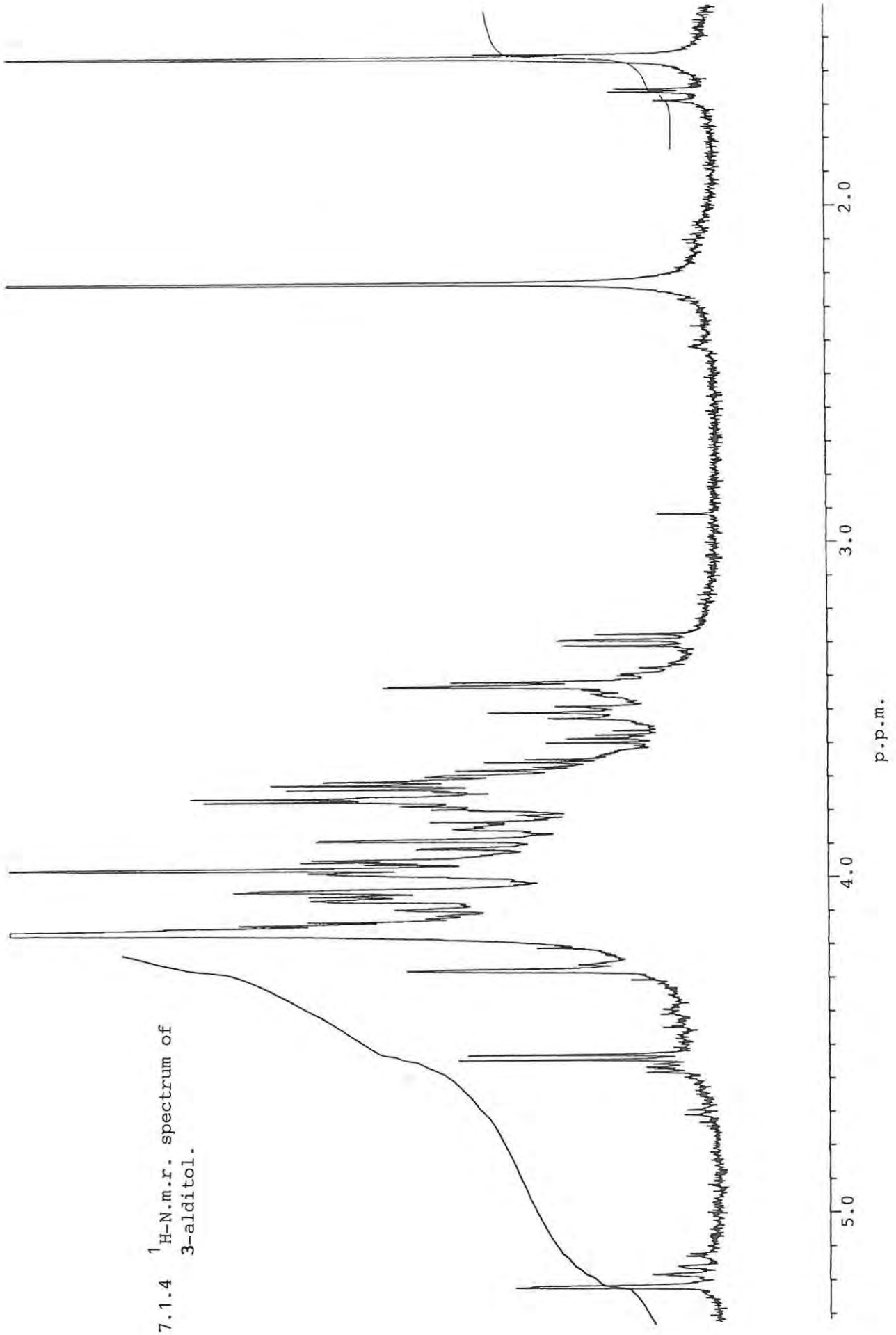
7.1.1 ^{1}H-N.m.r. spectrum of autohydrolyzed K37 polysaccharide (1).



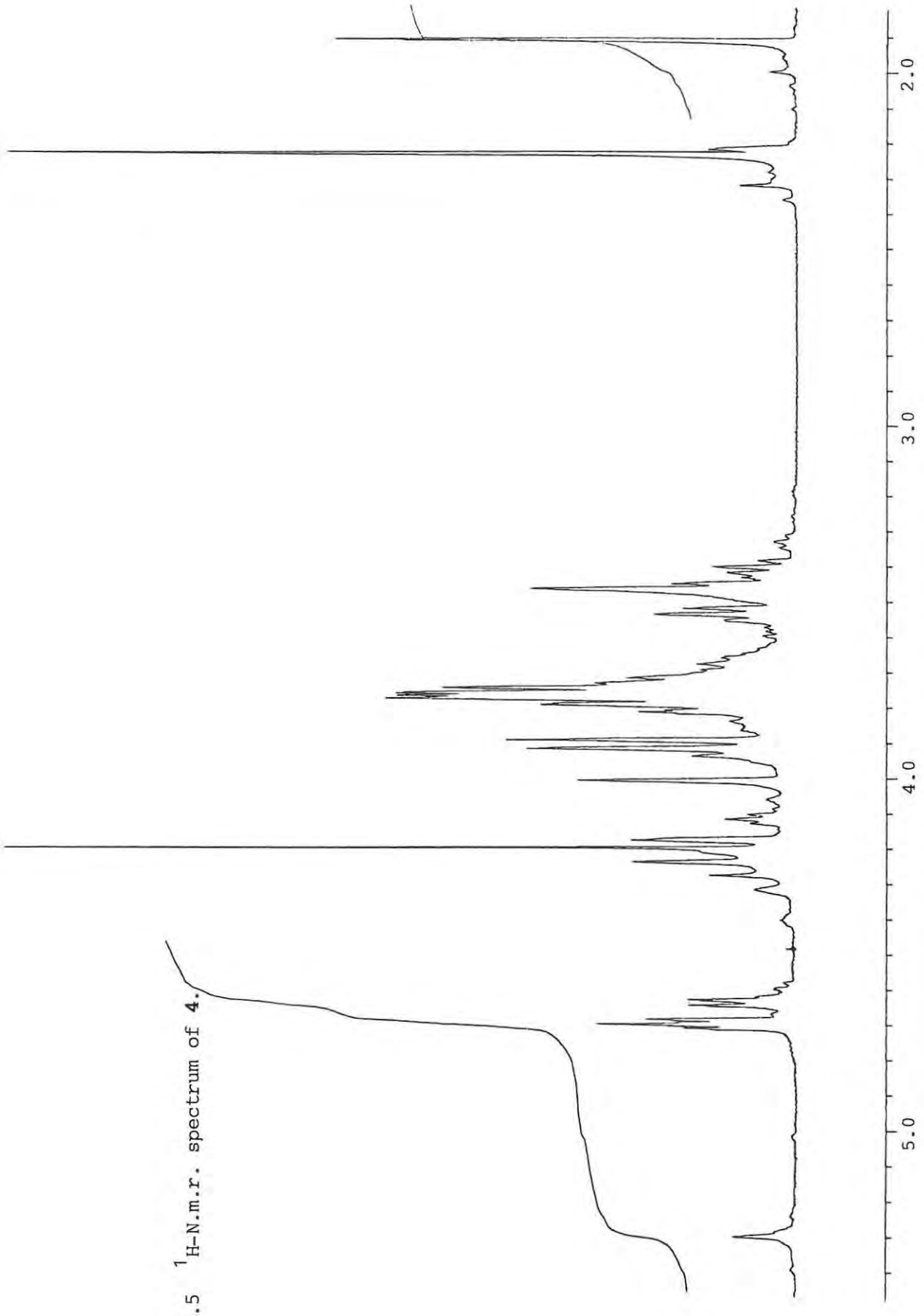
7.1.2 ^{1}H-N.m.r. spectrum of Smith-degraded K37 polysaccharide (2).



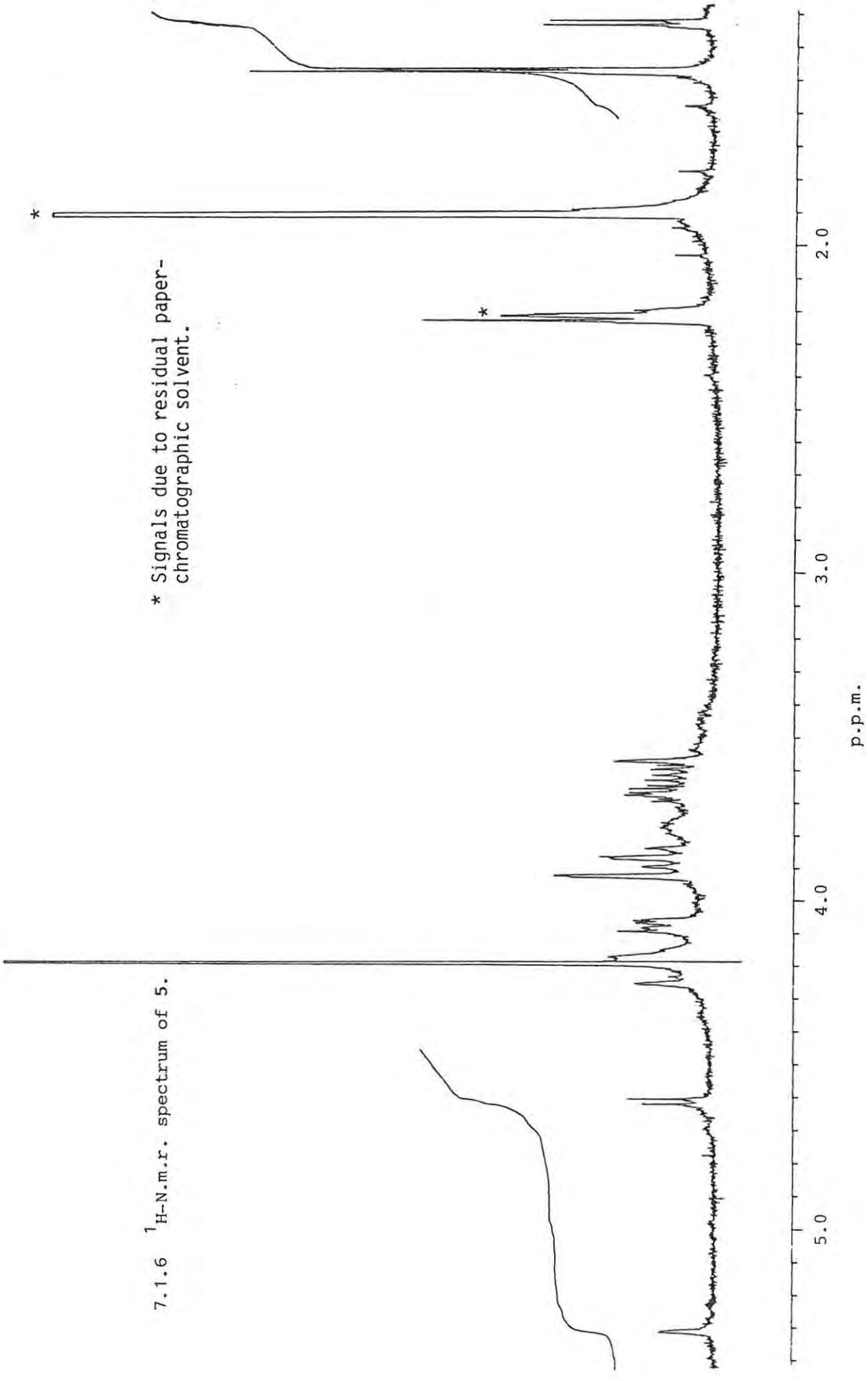
7.1.3 ^{1}H-N.m.r. spectrum of 3.



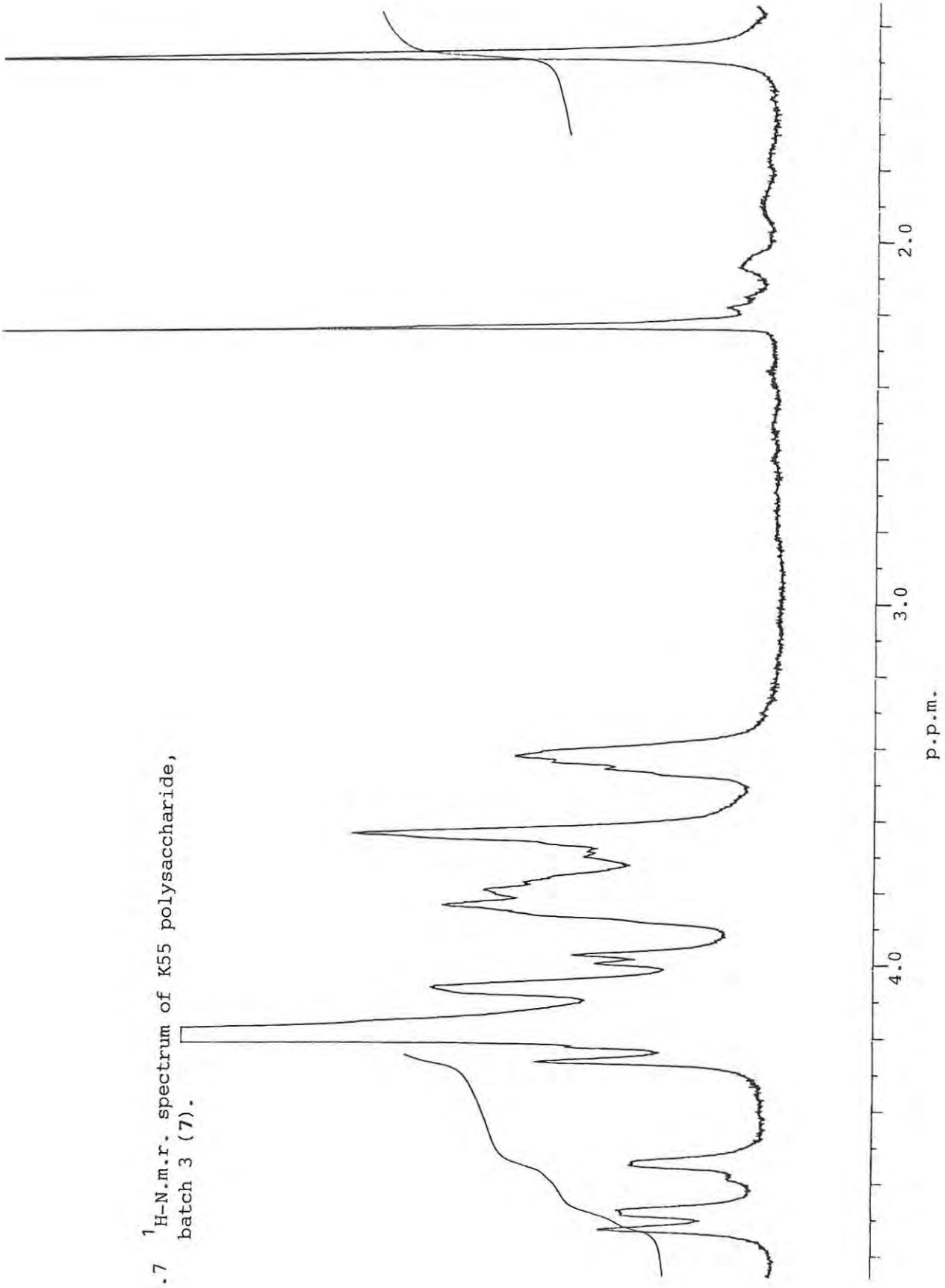
7.1.4 ^{1}H-N.m.r. spectrum of 3-alditol.



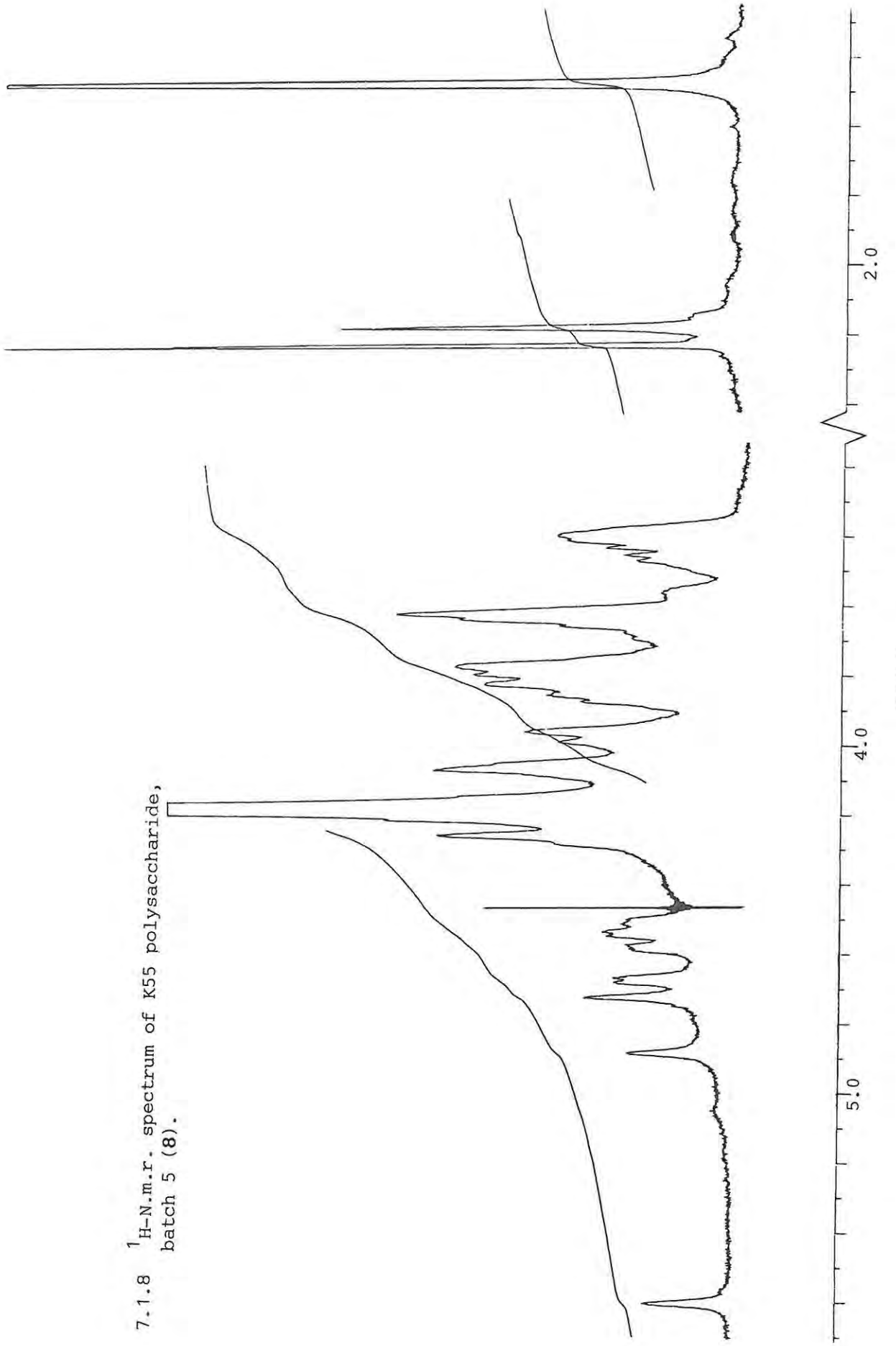
7.1.5 ^{1}H-N.m.r. spectrum of **4**.



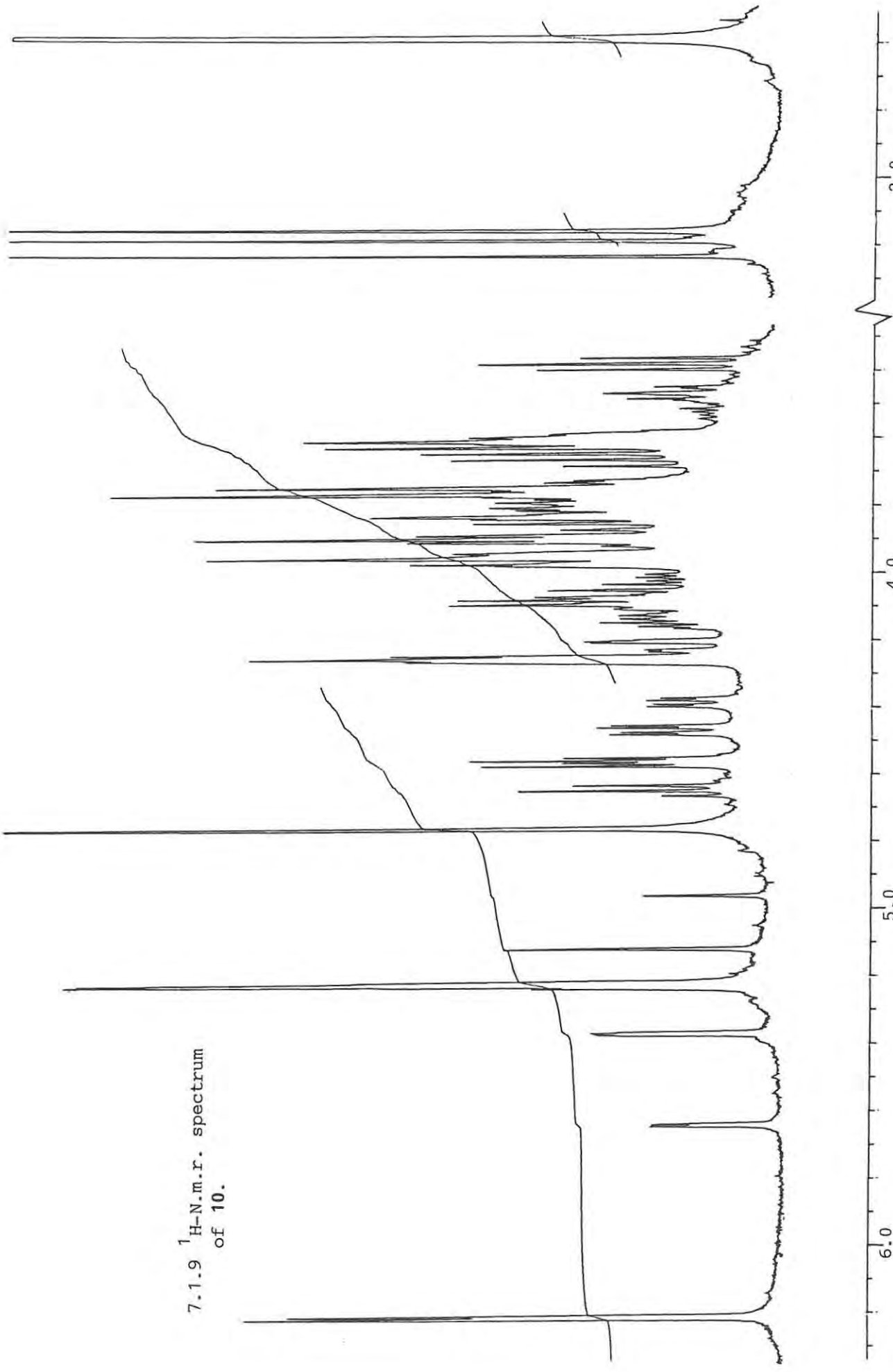
7.1.6 ^{1}H-N.m.r. spectrum of 5.



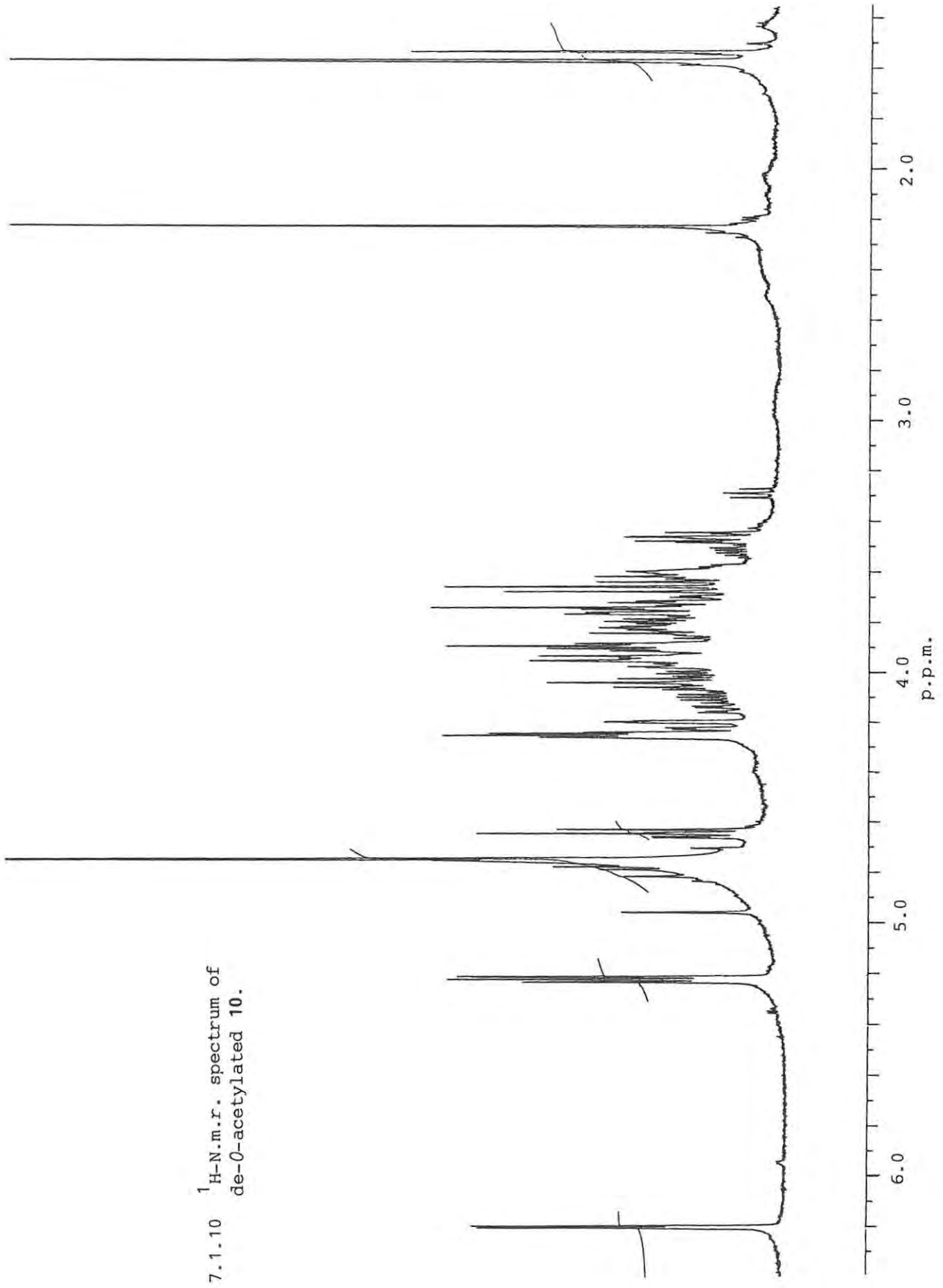
7.1.7 ^{1}H-N.m.r. spectrum of K55 polysaccharide, batch 3 (7).



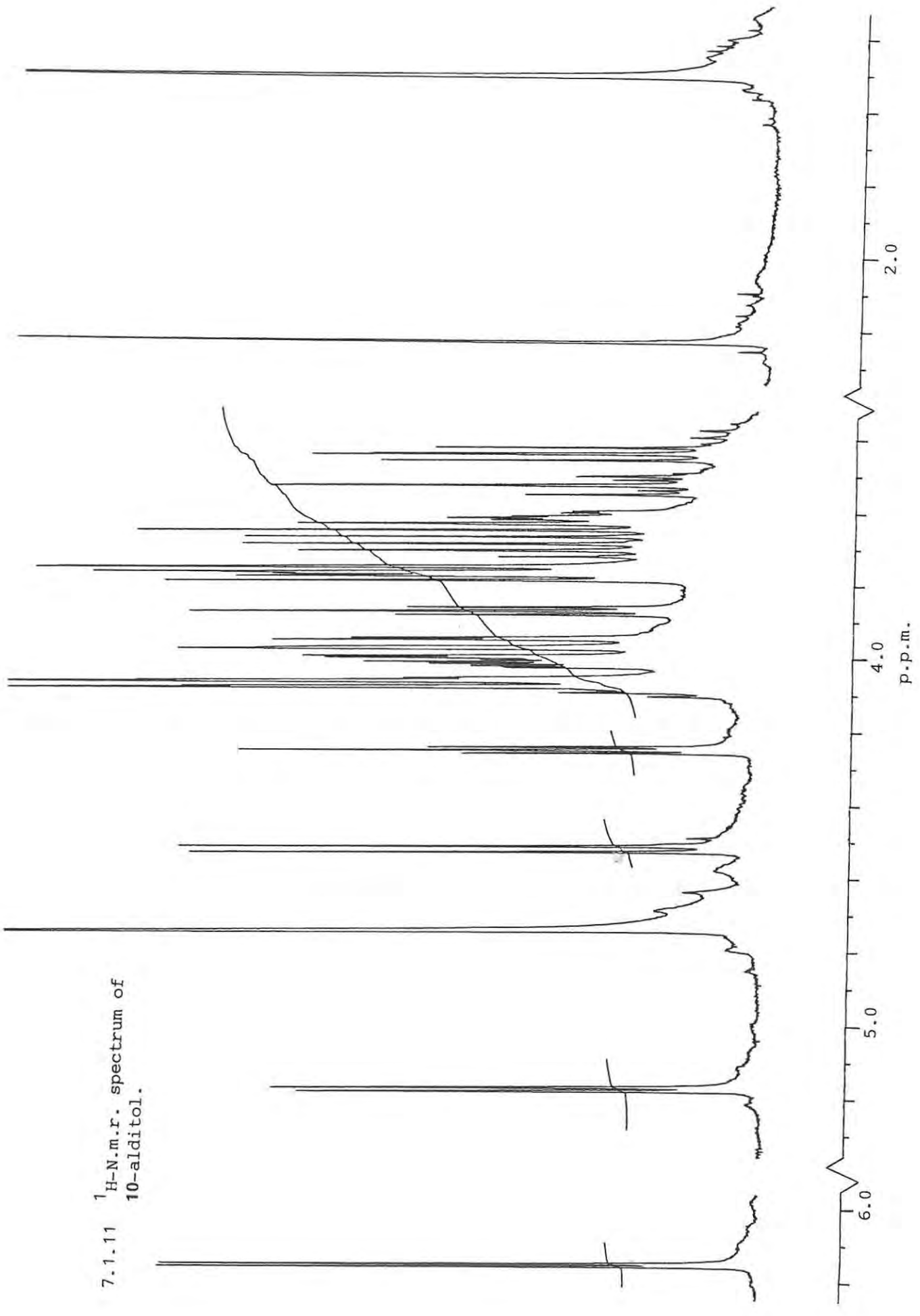
7.1.8 ^{1}H-N.m.r. spectrum of K55 polysaccharide, batch 5 (8).



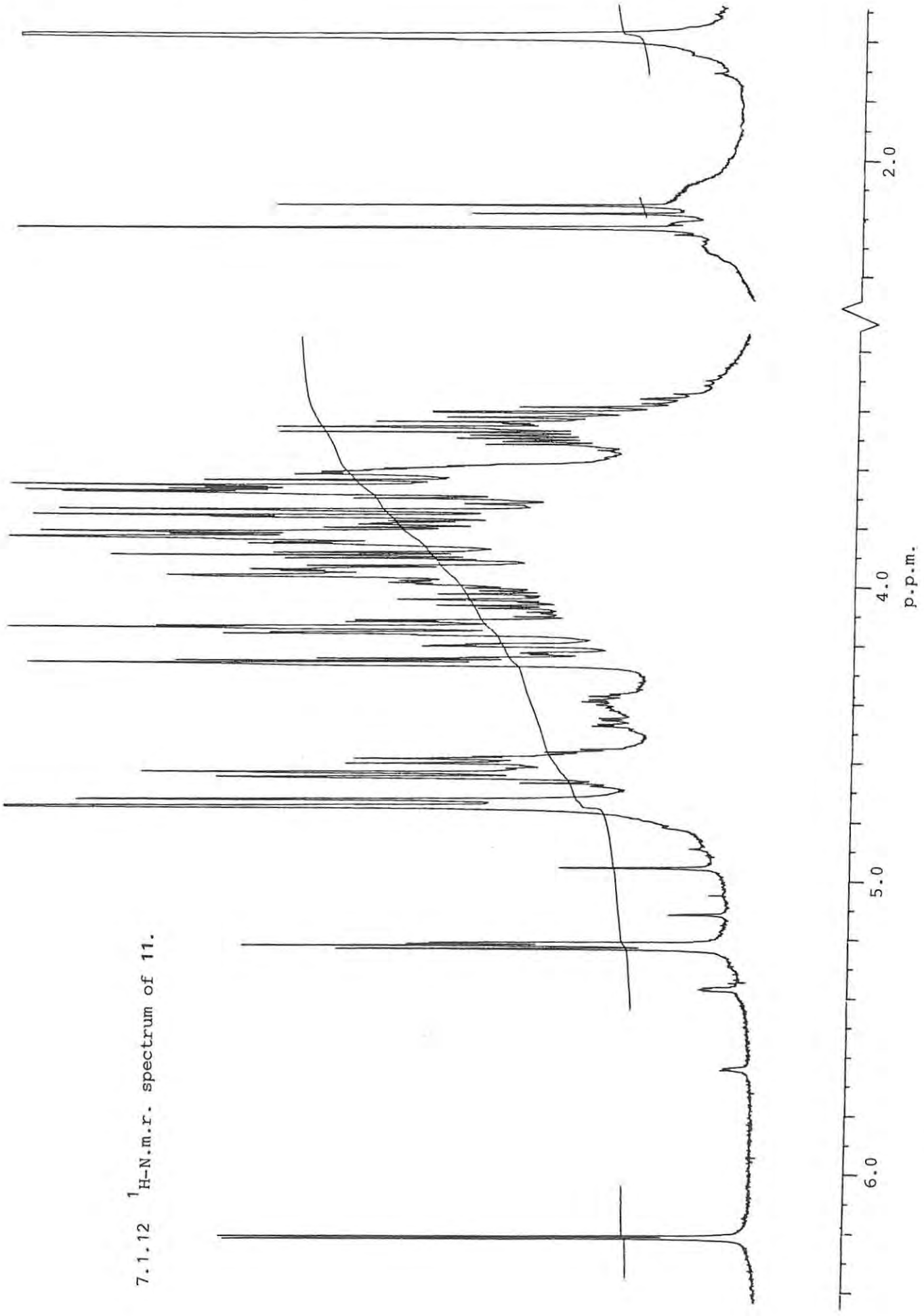
7.1.9 ^{1}H-N.m.r. spectrum of **10**.



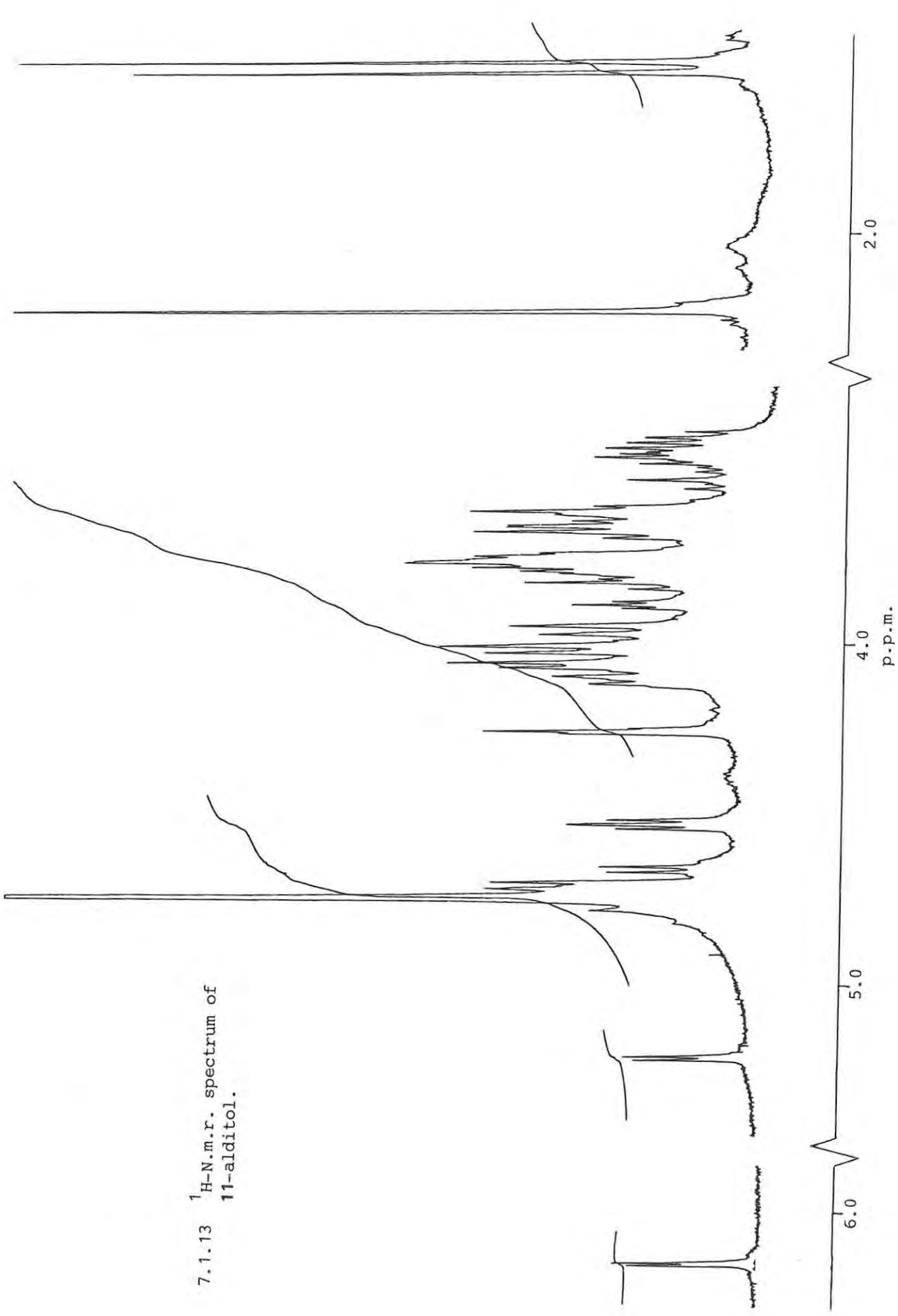
7.1.10 ^{1}H-N.m.r. spectrum of de-*O*-acetylated **10**.



7.1.11 ^{1}H-N.m.r. spectrum of **10**-alditol.

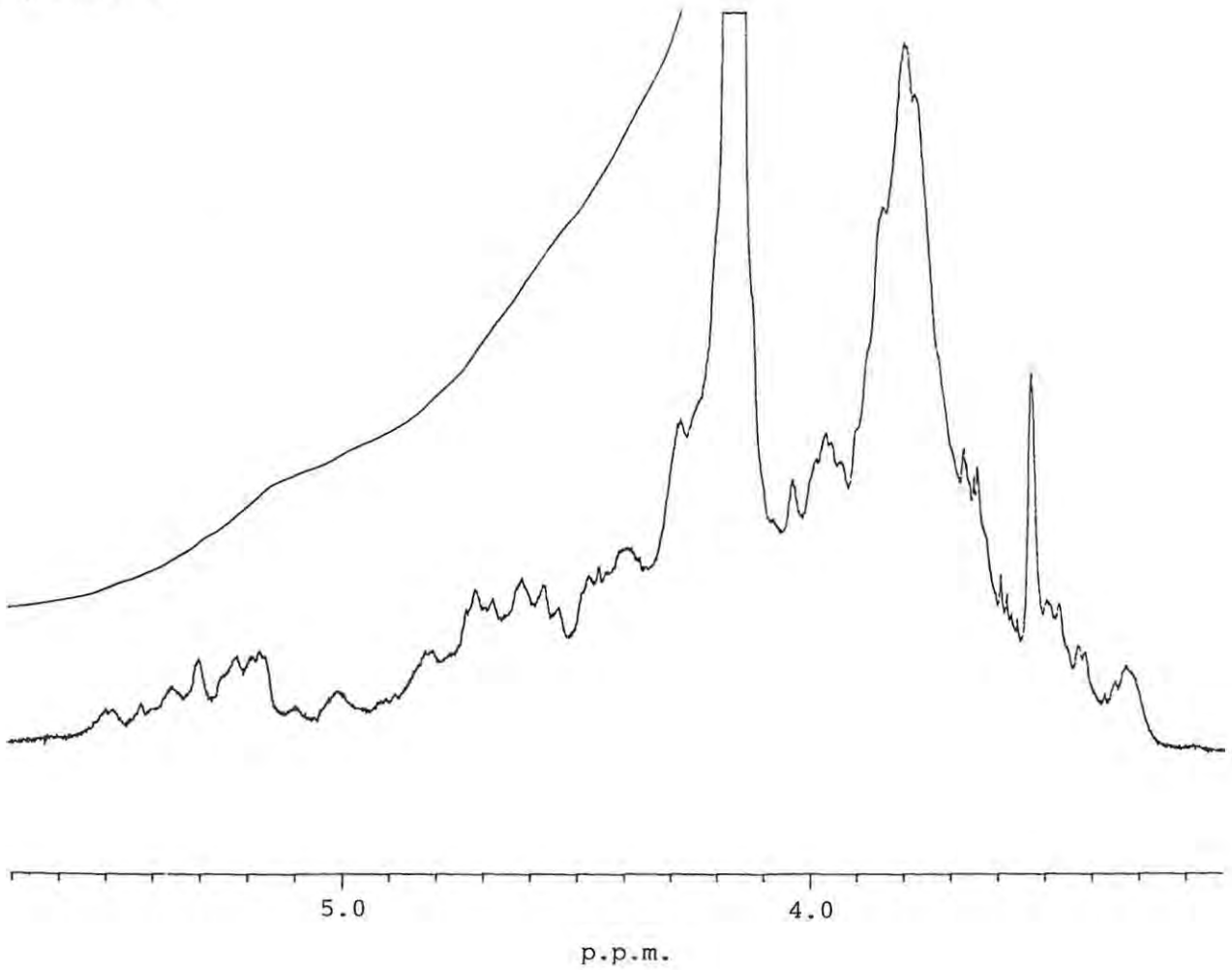


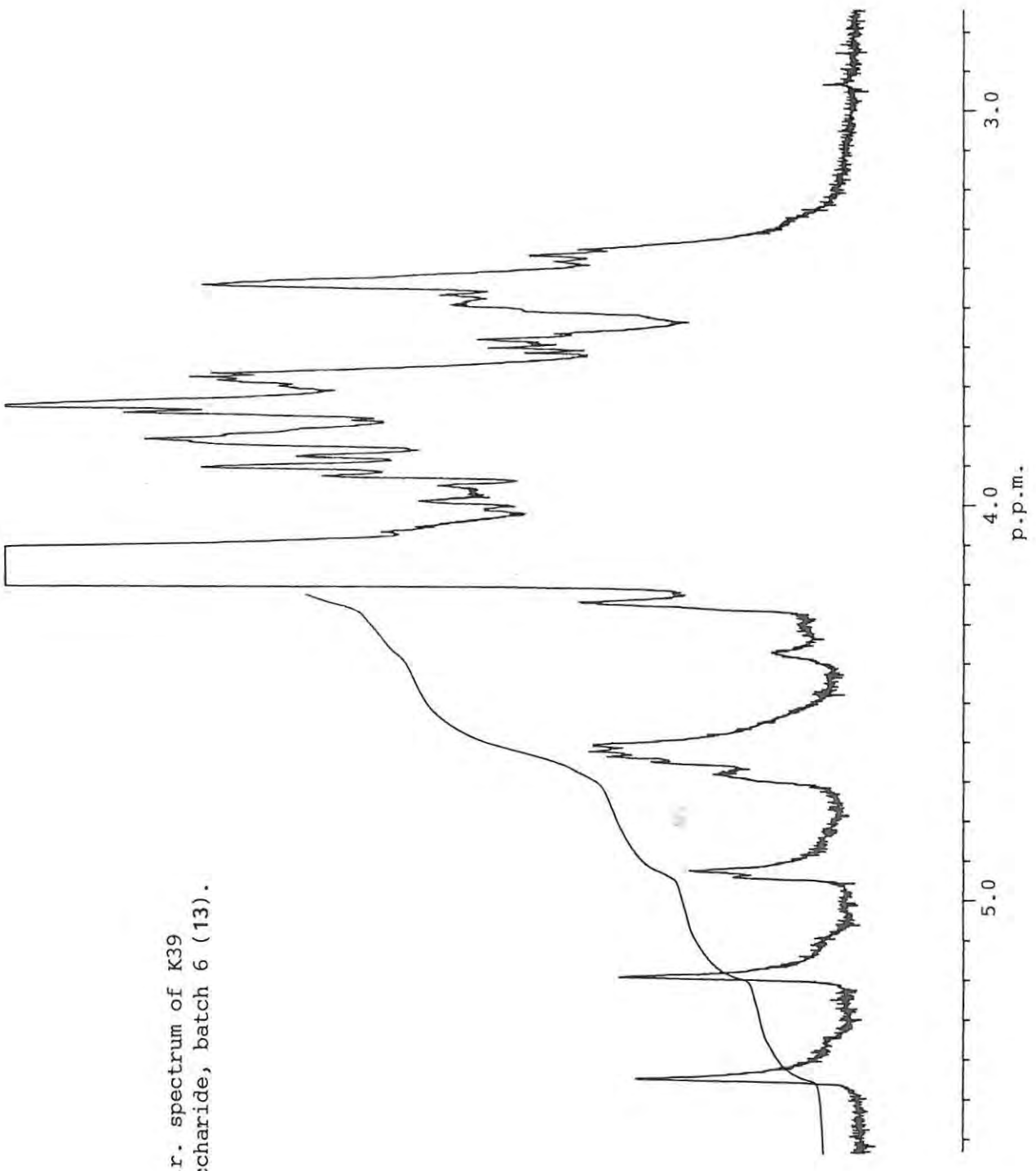
7.1.12 ^{1}H-N.m.r. spectrum of **11**.



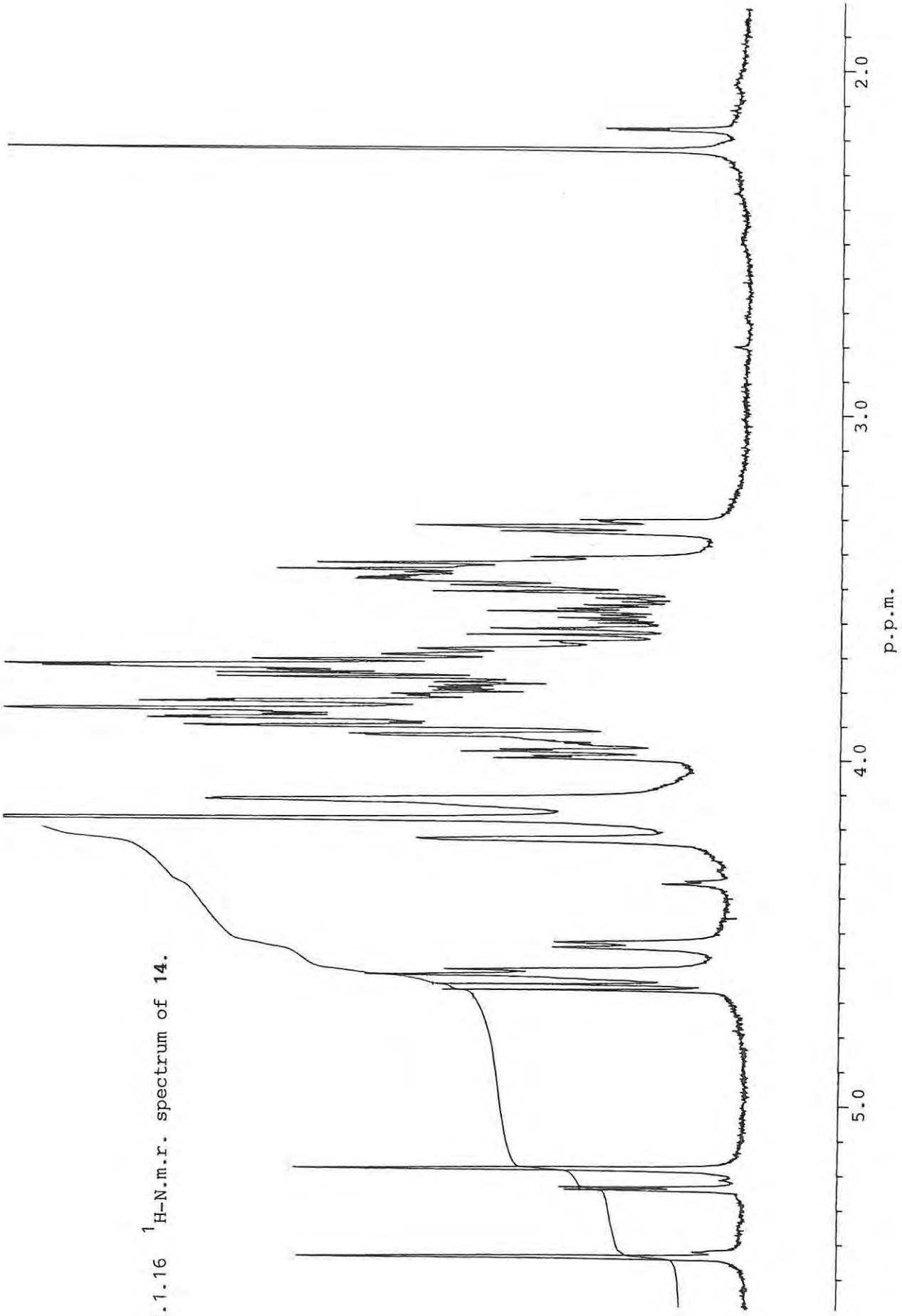
7.1.13 ^{1}H-N.m.r. spectrum of **11**-alditol.

7.1.14 ^{1}H-N.m.r. spectrum of K39 polysaccharide, batch 2.

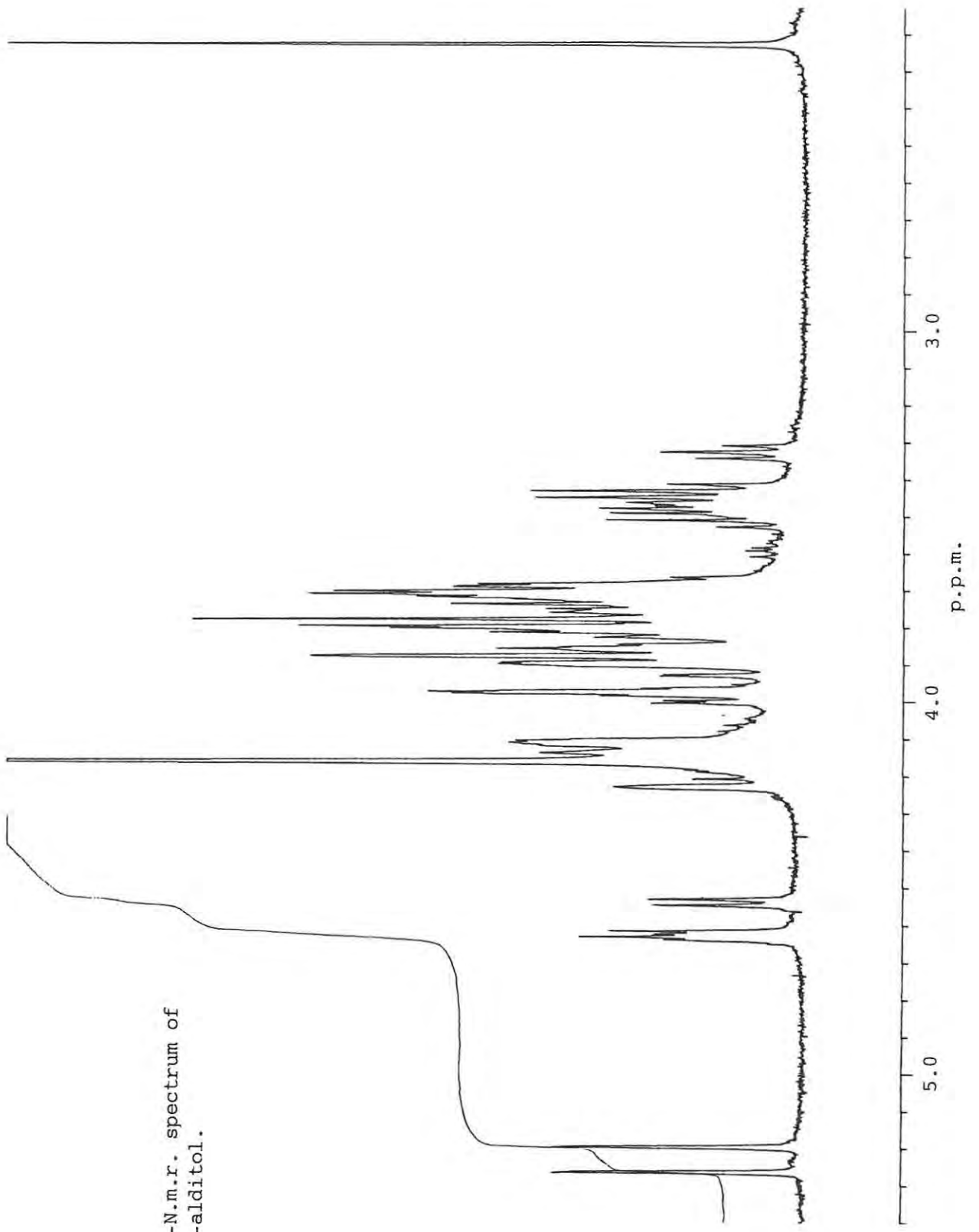




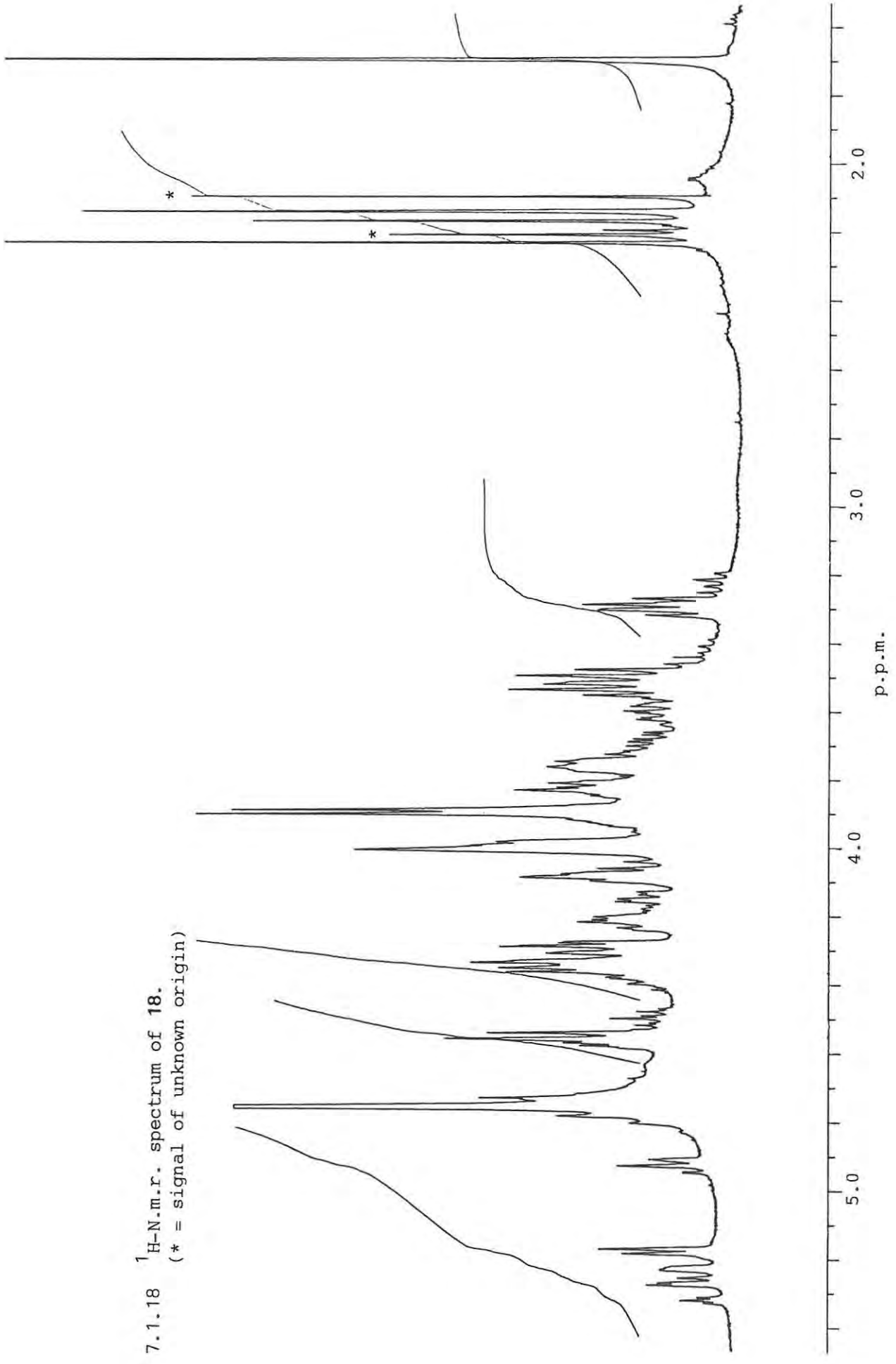
7.1.15 ^{1}H-N.m.r. spectrum of K39 polysaccharide, batch 6 (13).



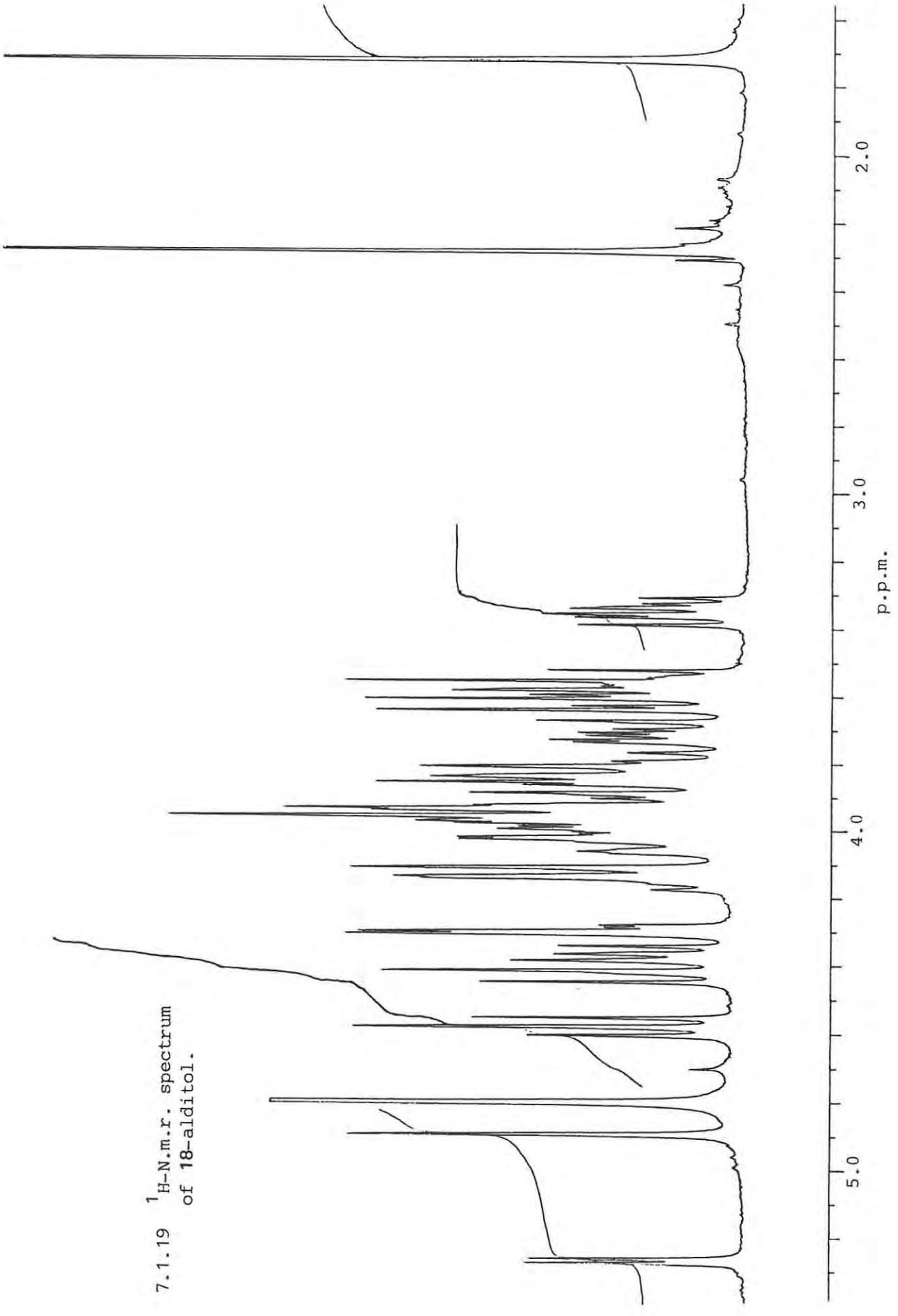
7.1.16 ^{1}H-N.m.r. spectrum of **14**.



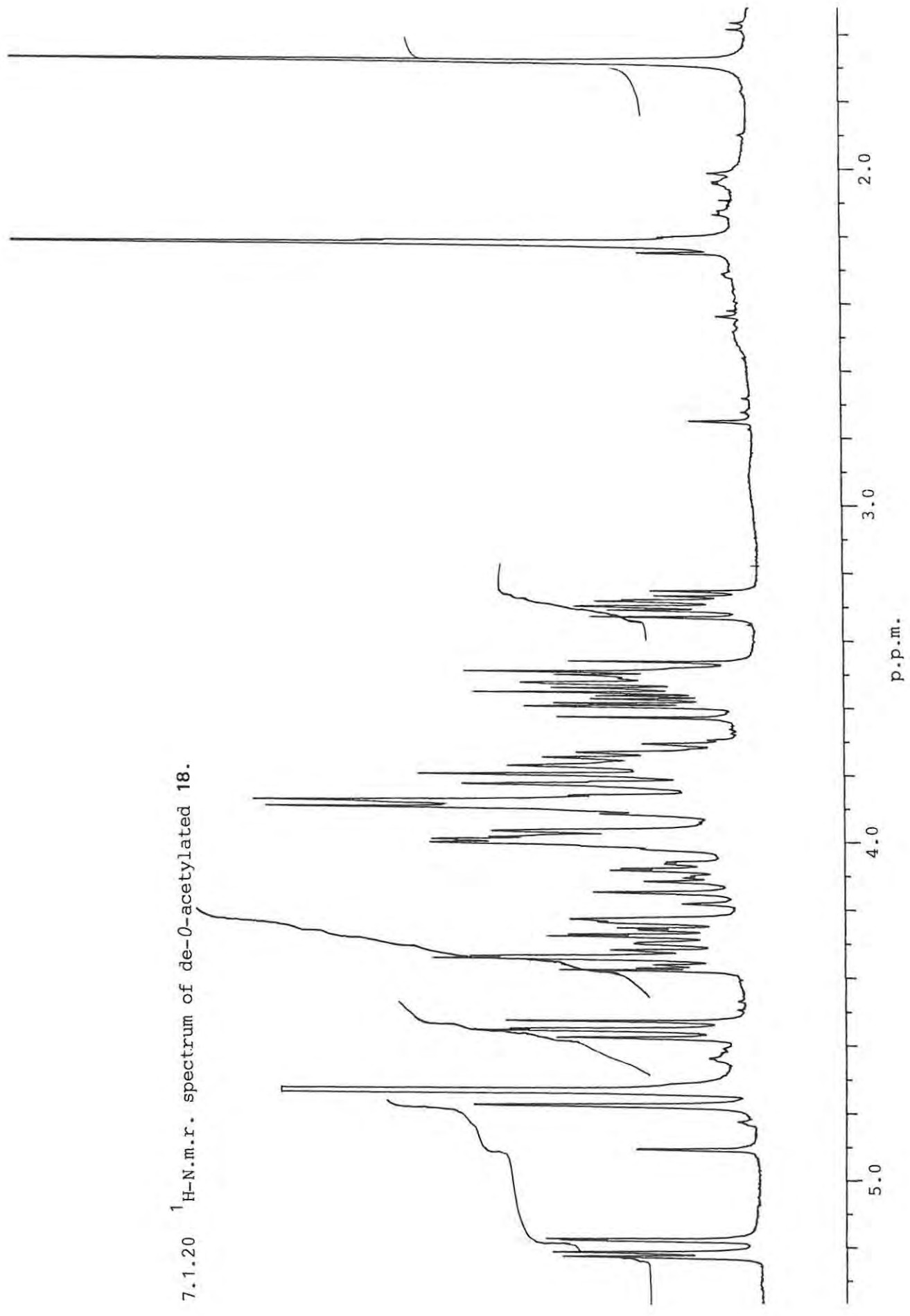
7.1.17 ^{1}H-N.m.r. spectrum of **14**-alditol.



7.1.18 ^{1}H-N.m.r. spectrum of **18**.
(* = signal of unknown origin)

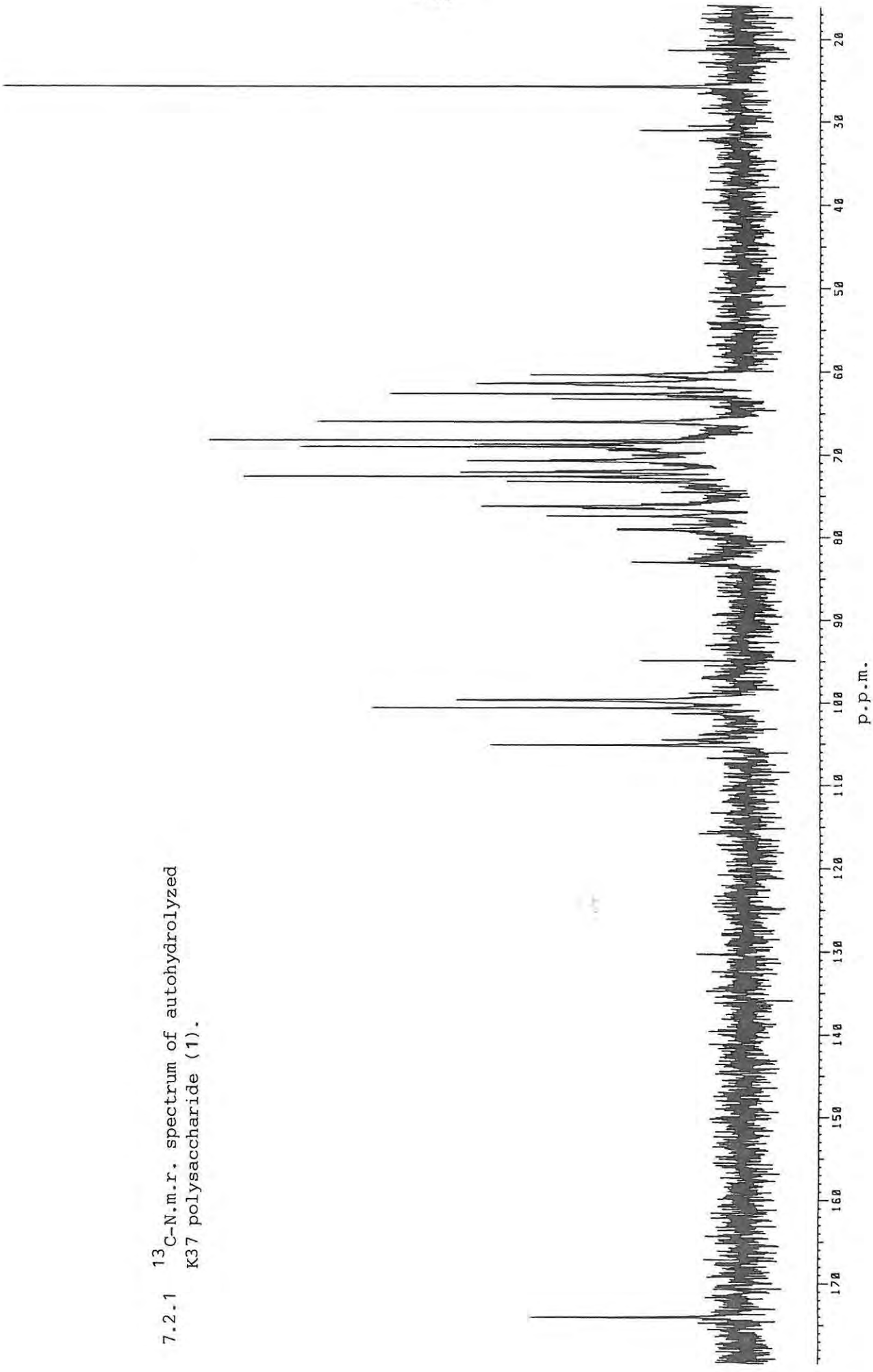


7.1.19 ^{1}H-N.m.r. spectrum of **18**-alditol.



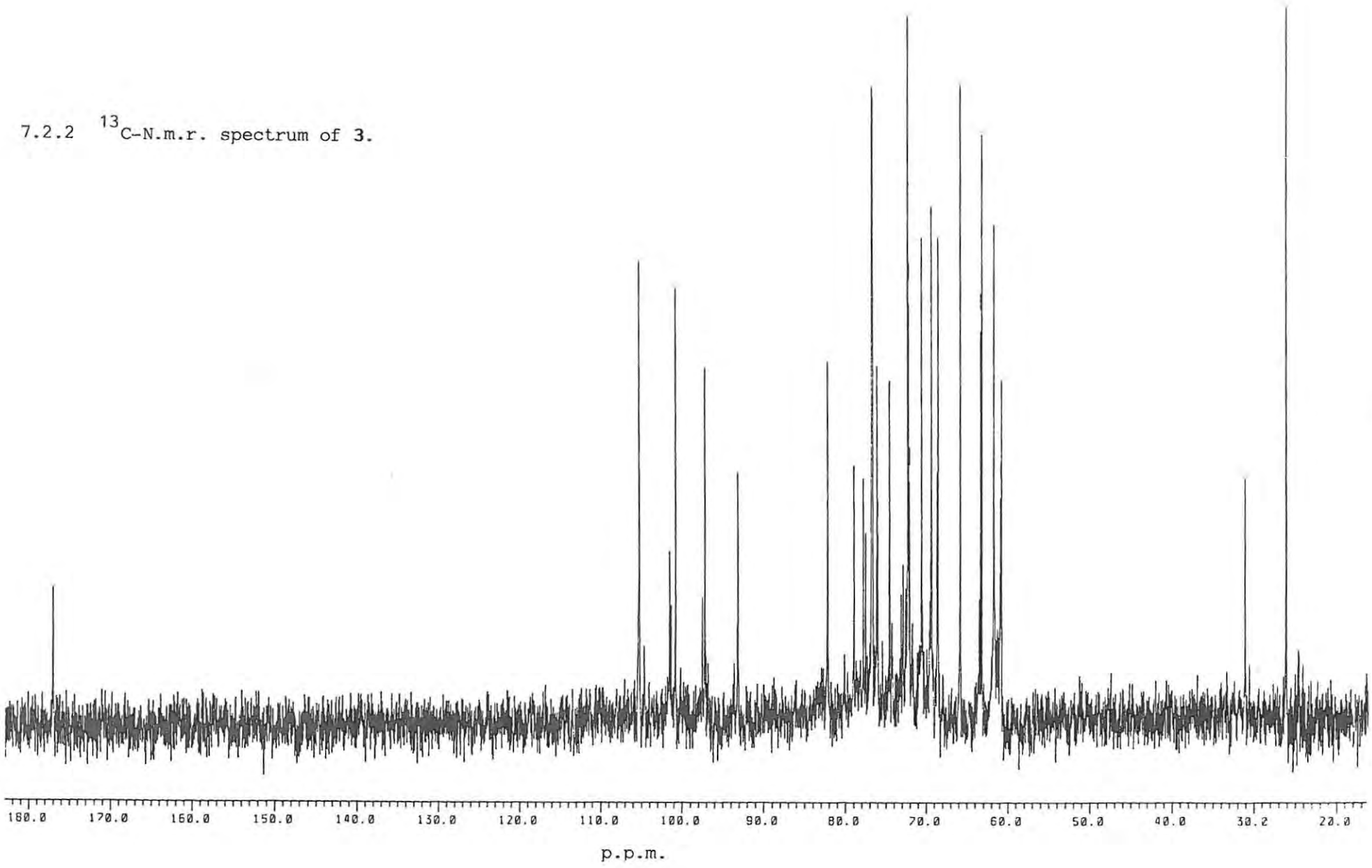
7.1.20 ^{1}H-N.m.r. spectrum of de-*O*-acetylated **18**.

7.2 CARBON-13 NUCLEAR MAGNETIC RESONANCE SPECTRA

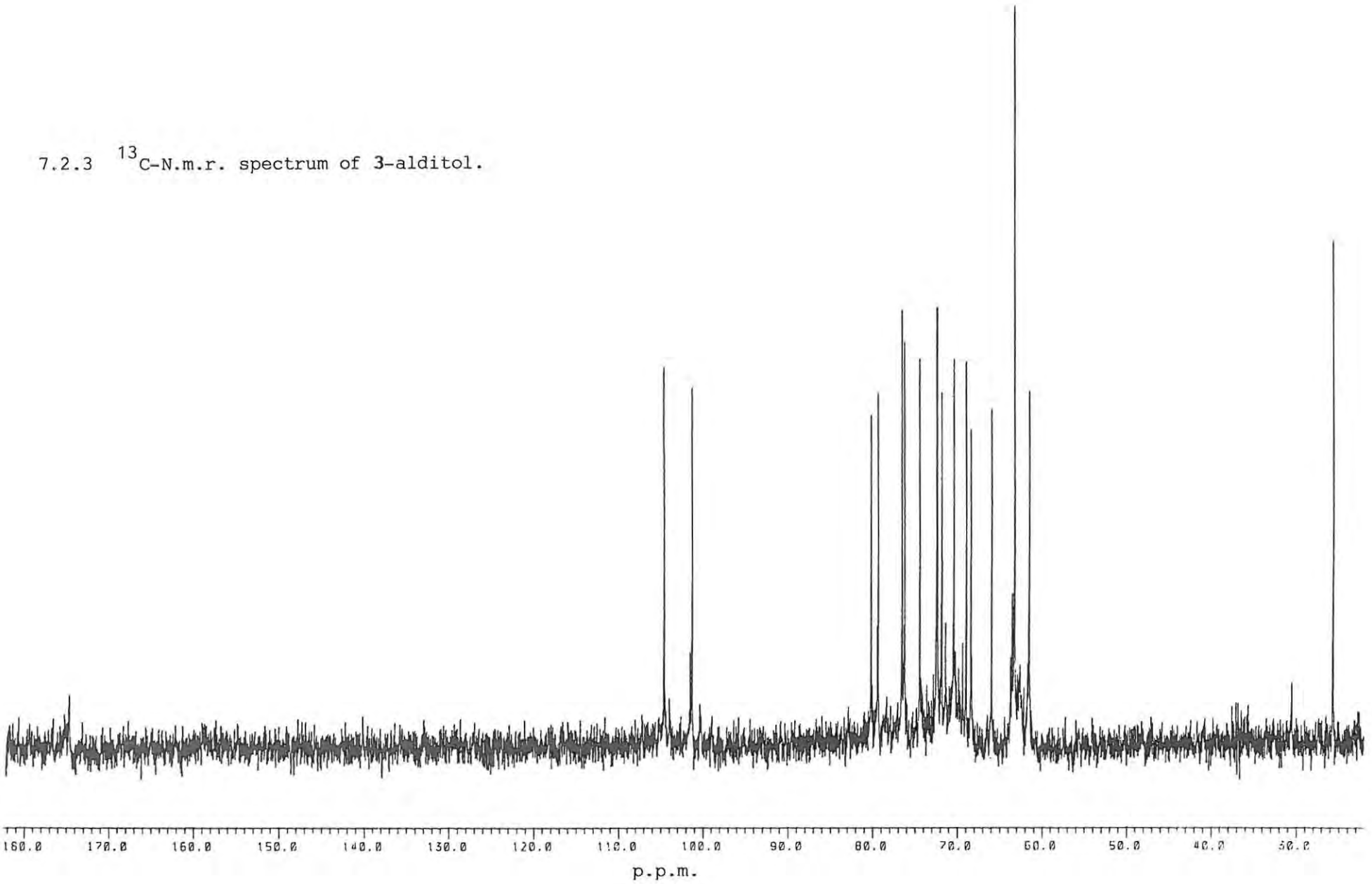


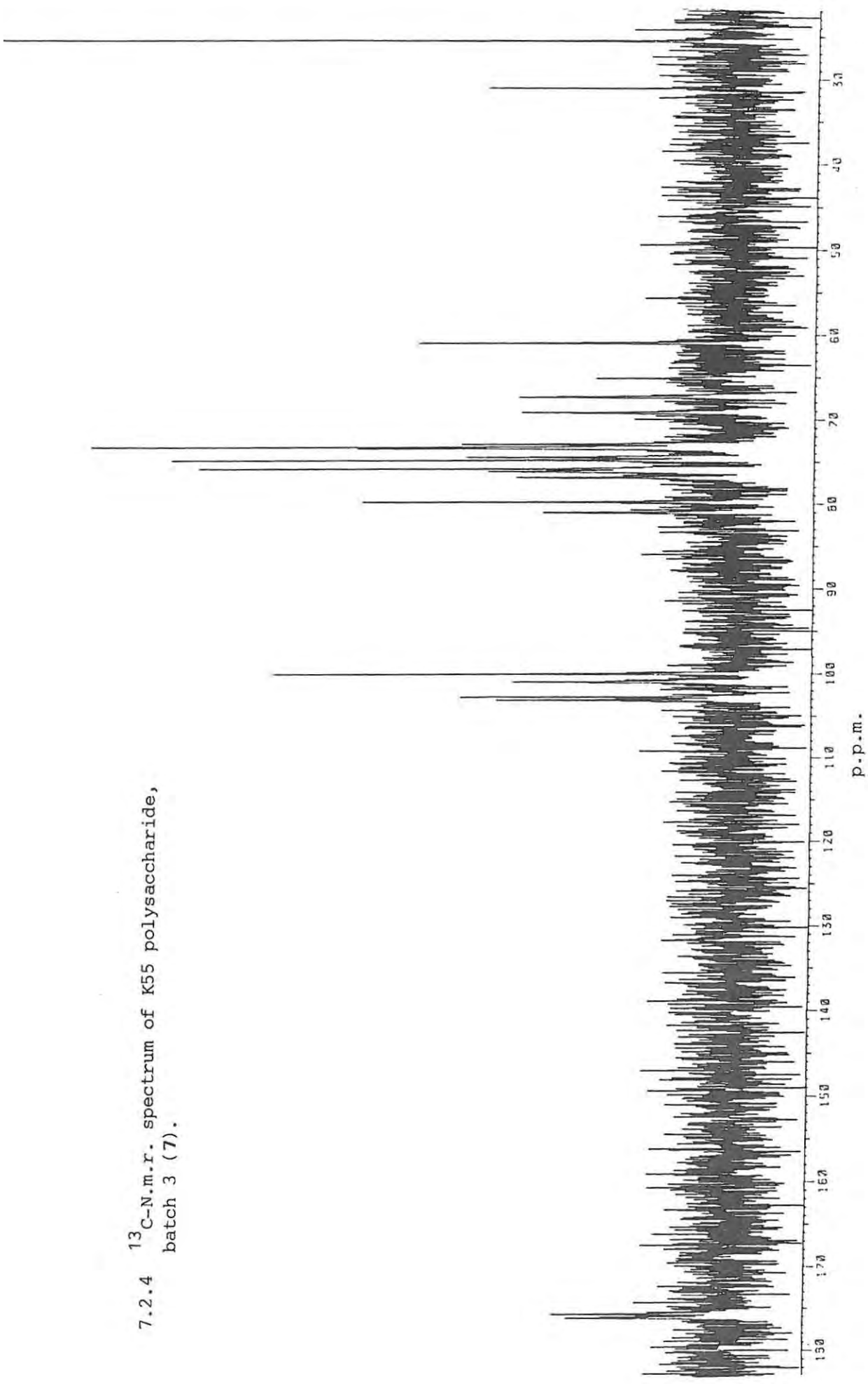
7.2.1 ^{13}C-N.m.r. spectrum of autohydrolyzed K37 polysaccharide (1).

7.2.2 ^{13}C-N.m.r. spectrum of **3**.

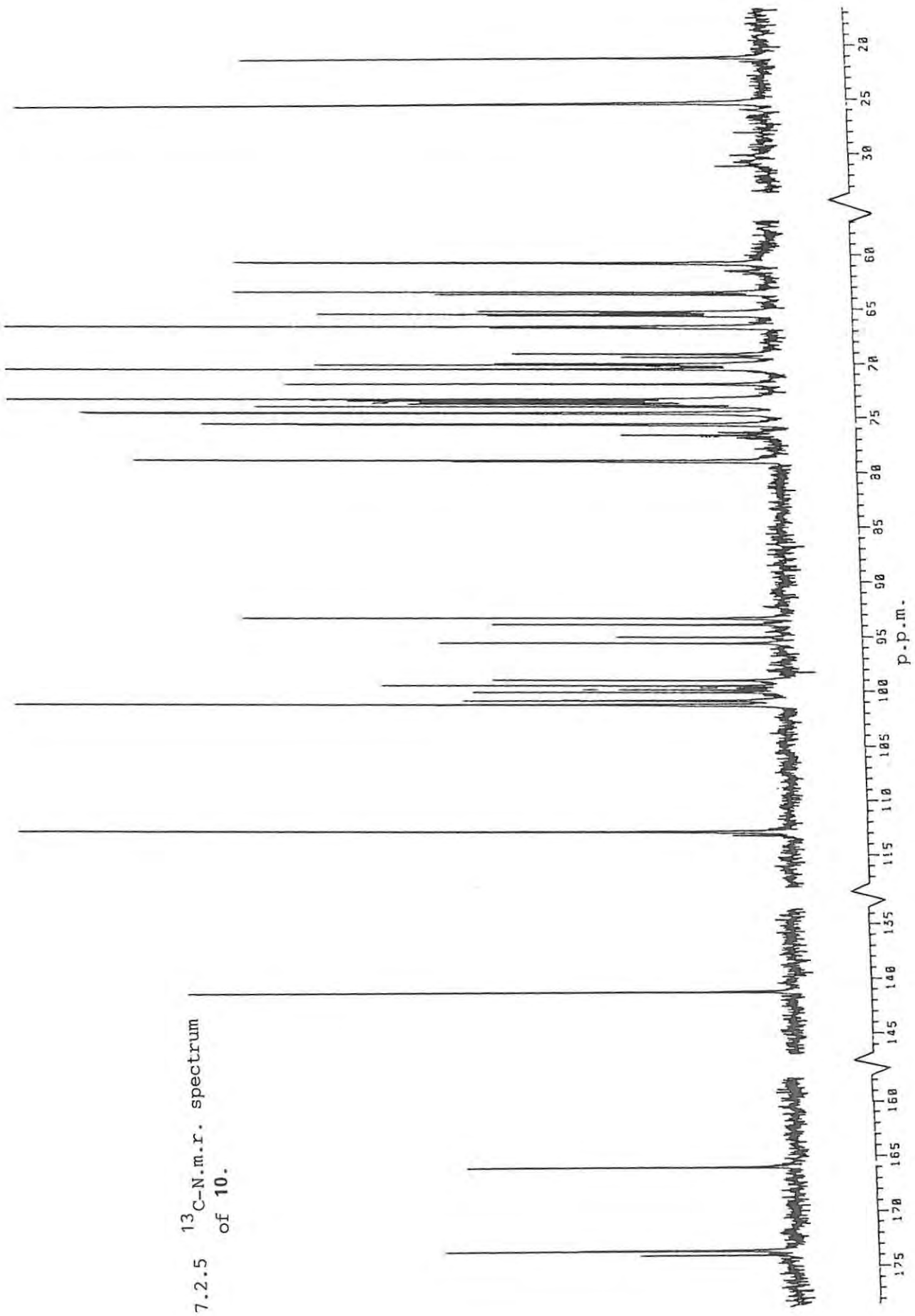


7.2.3 ^{13}C-N.m.r. spectrum of 3-alditol.

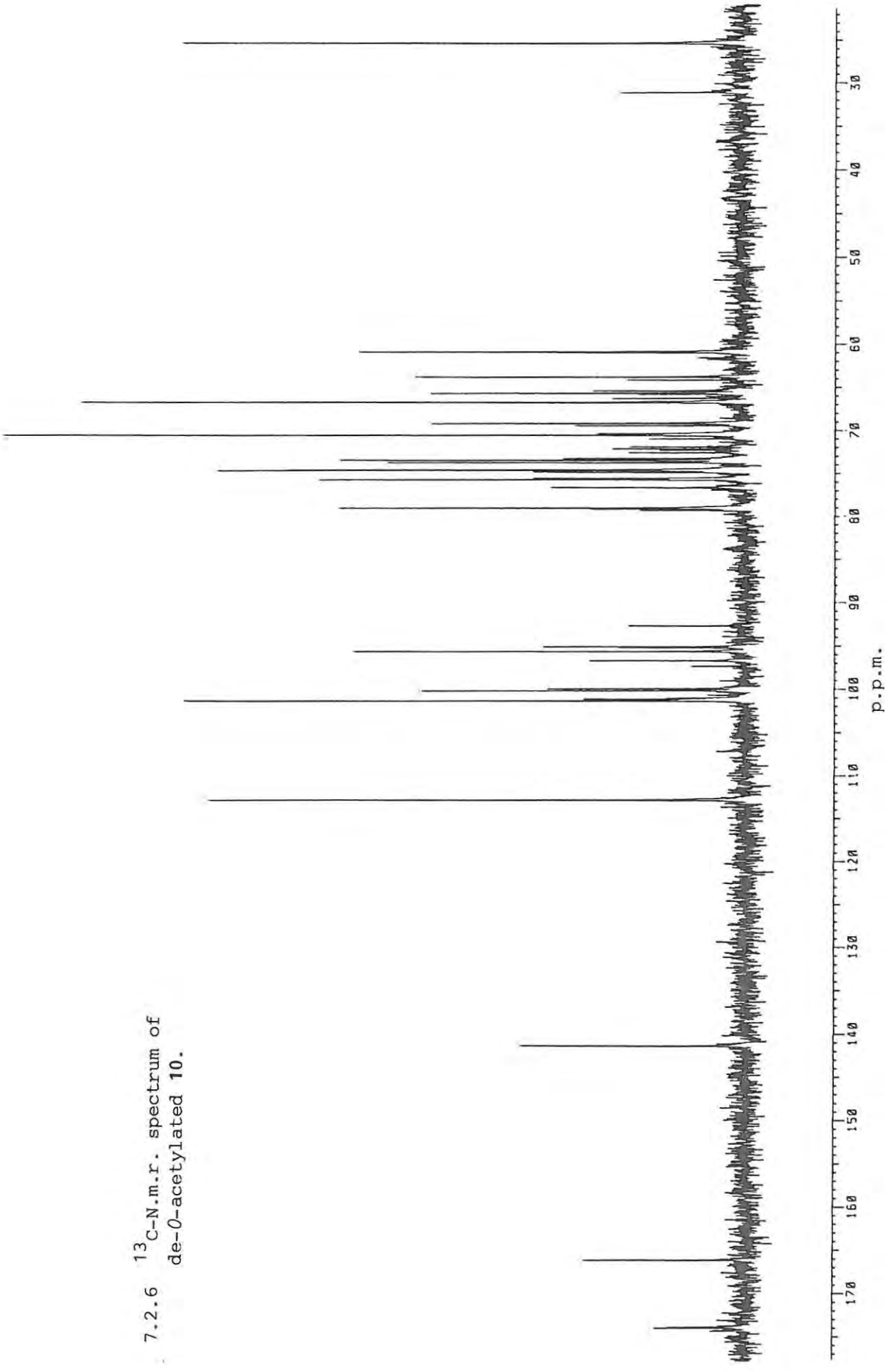




7.2.4 ^{13}C-N.m.r. spectrum of K55 polysaccharide, batch 3 (7).

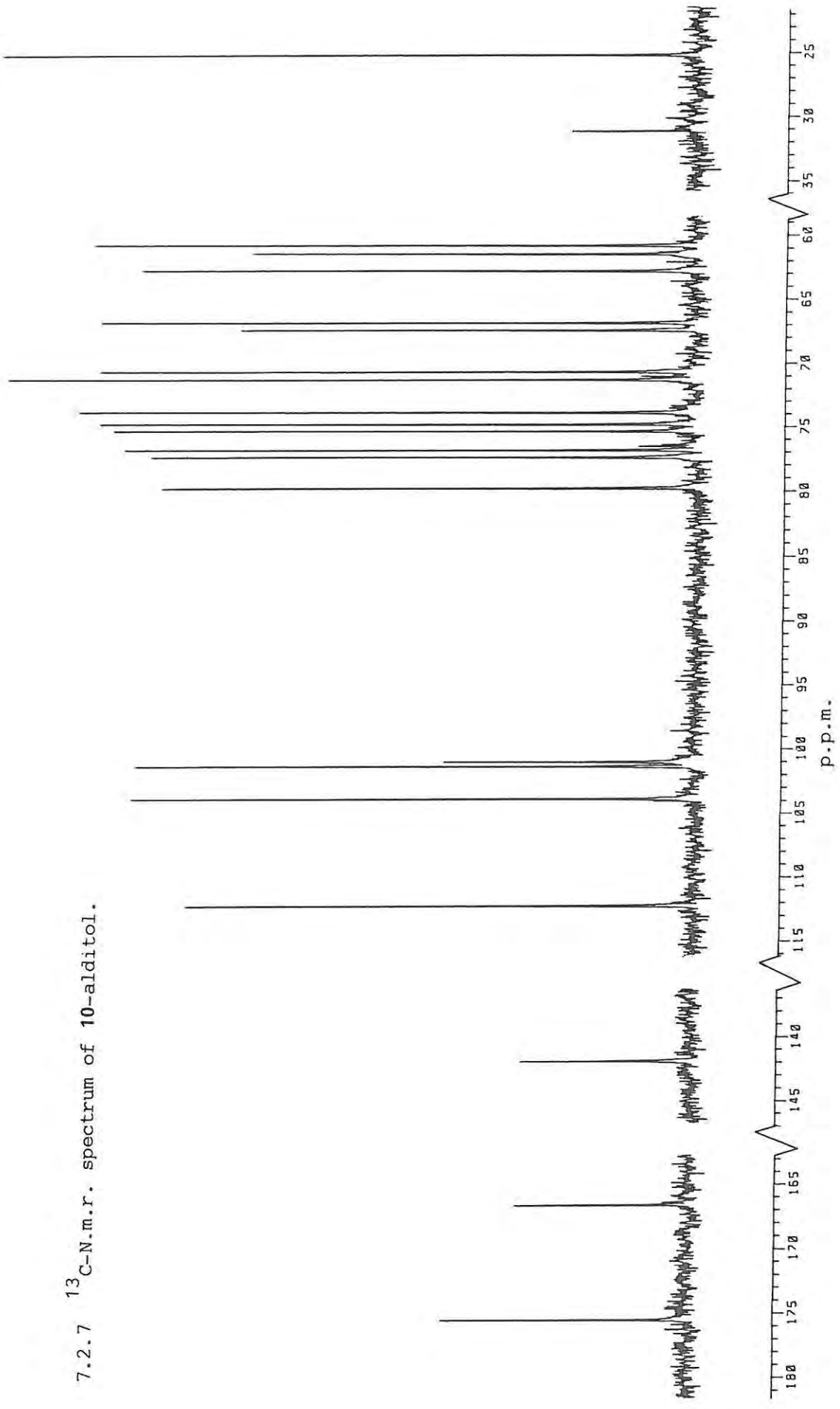


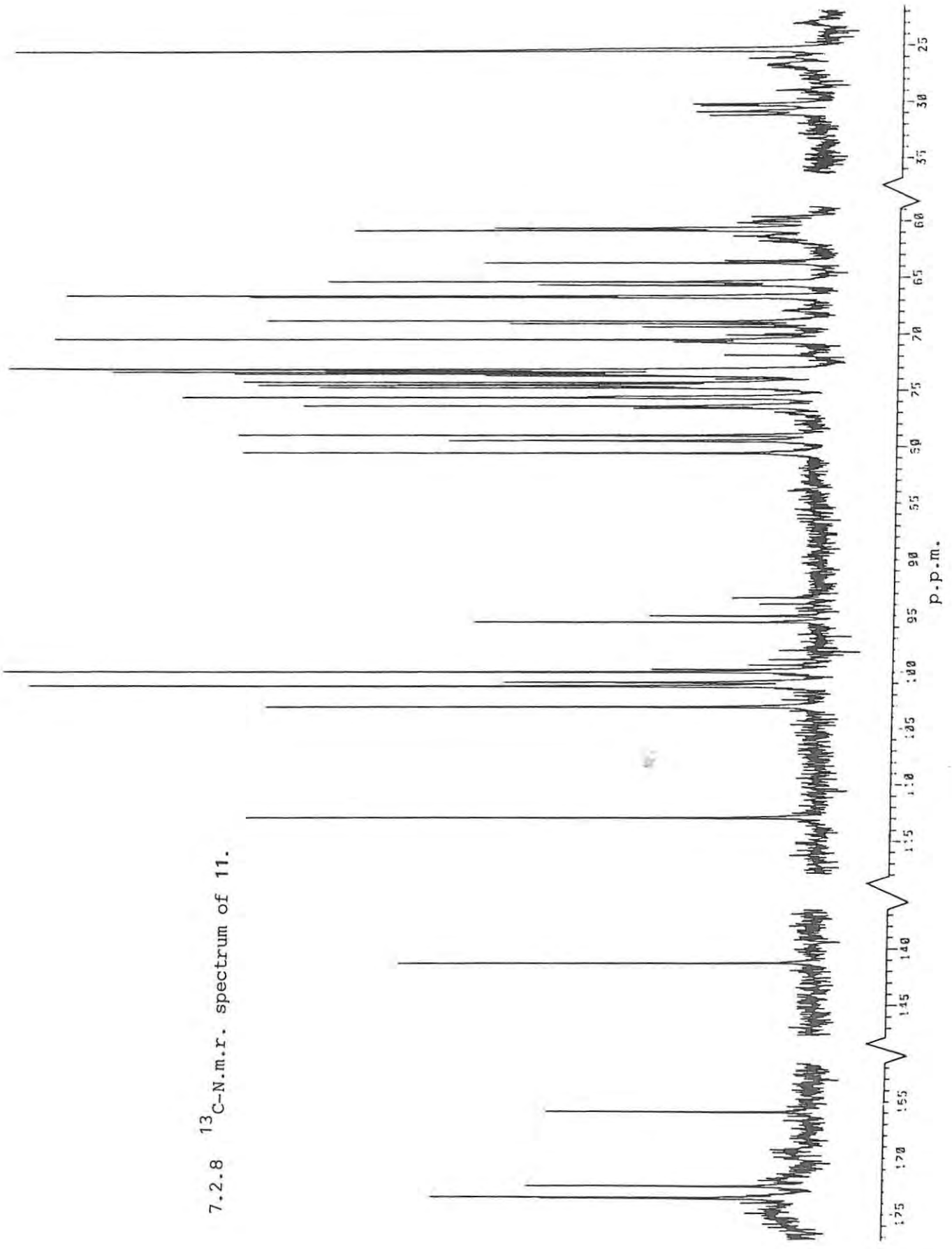
7.2.5 ^{13}C-N.m.r. spectrum of **10**.



7.2.6 ^{13}C-N.m.r. spectrum of de-*O*-acetylated **10**.

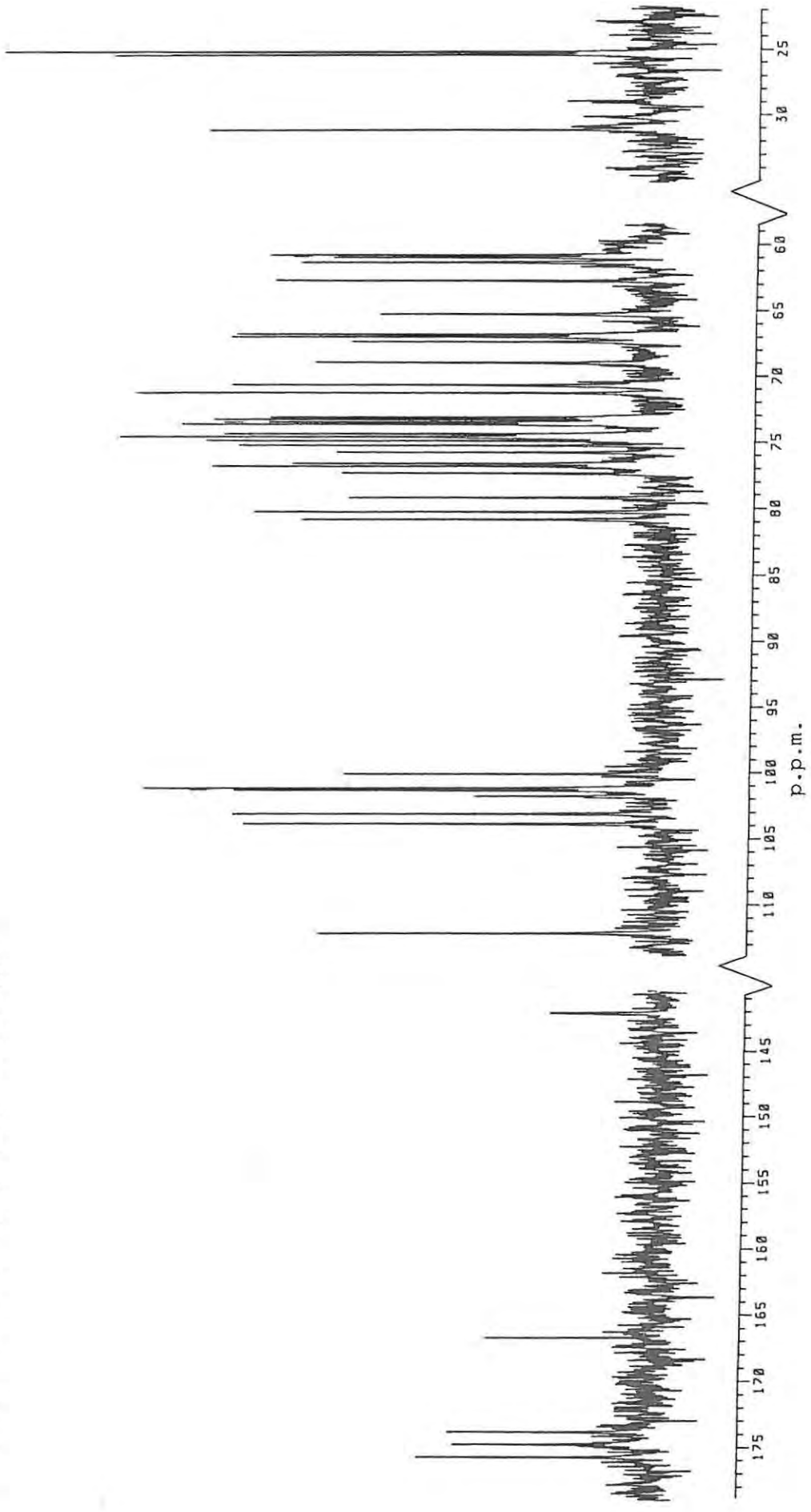
7.2.7 ^{13}C-N.m.r. spectrum of 10-alditol.



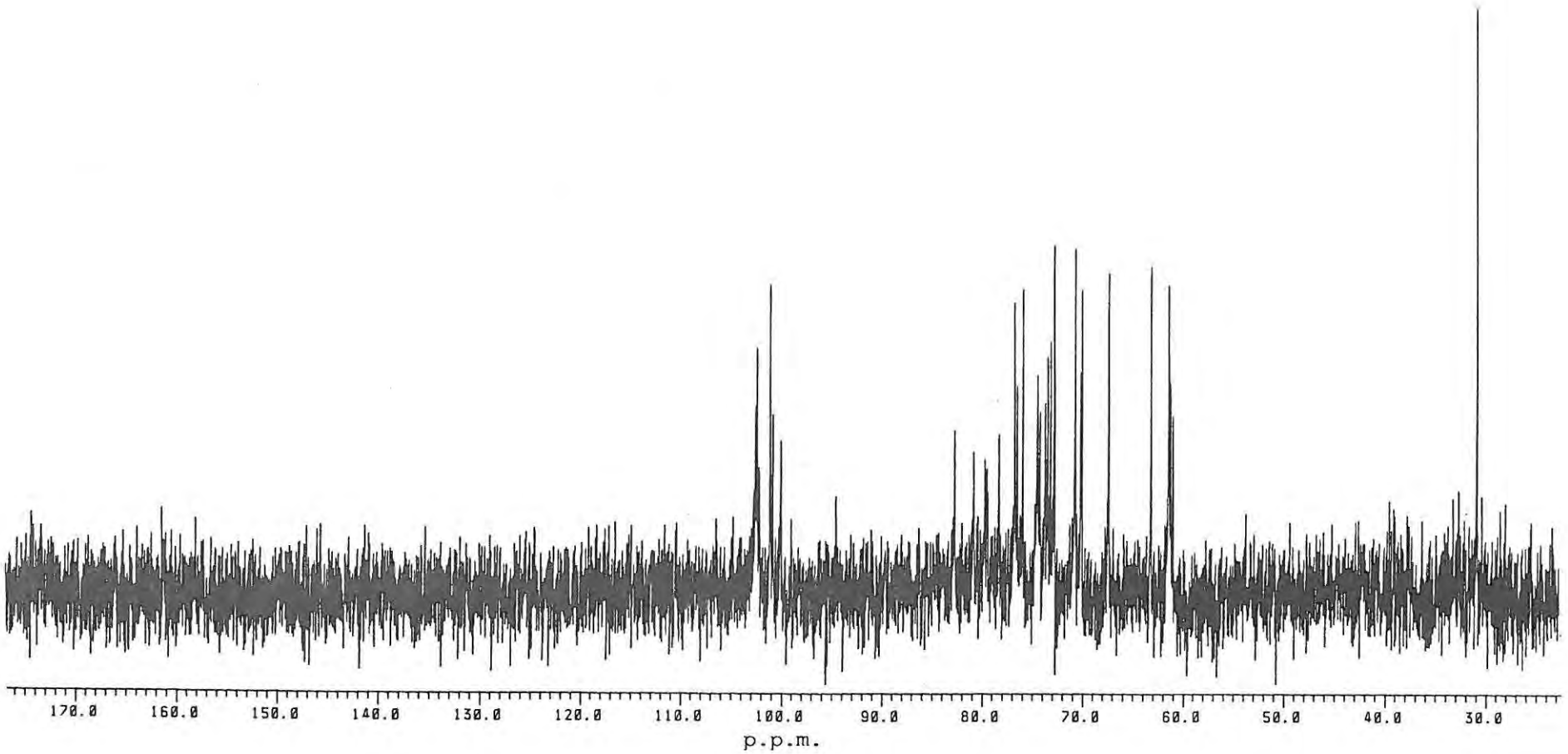


7.2.8 ^{13}C-N.m.r. spectrum of 11.

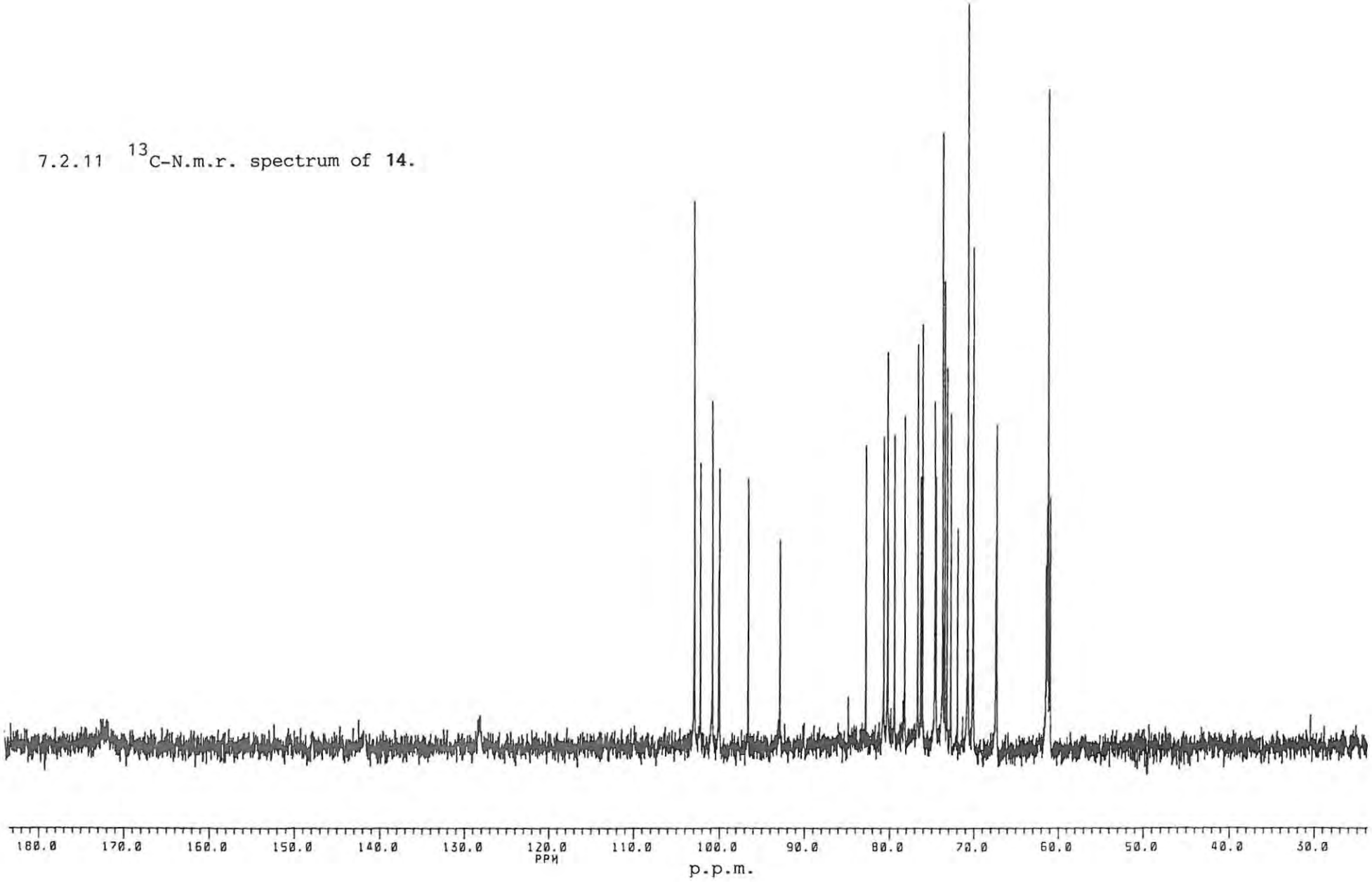
7.2.9 ^{13}C-N.m.r. spectrum of **11**-alditol.

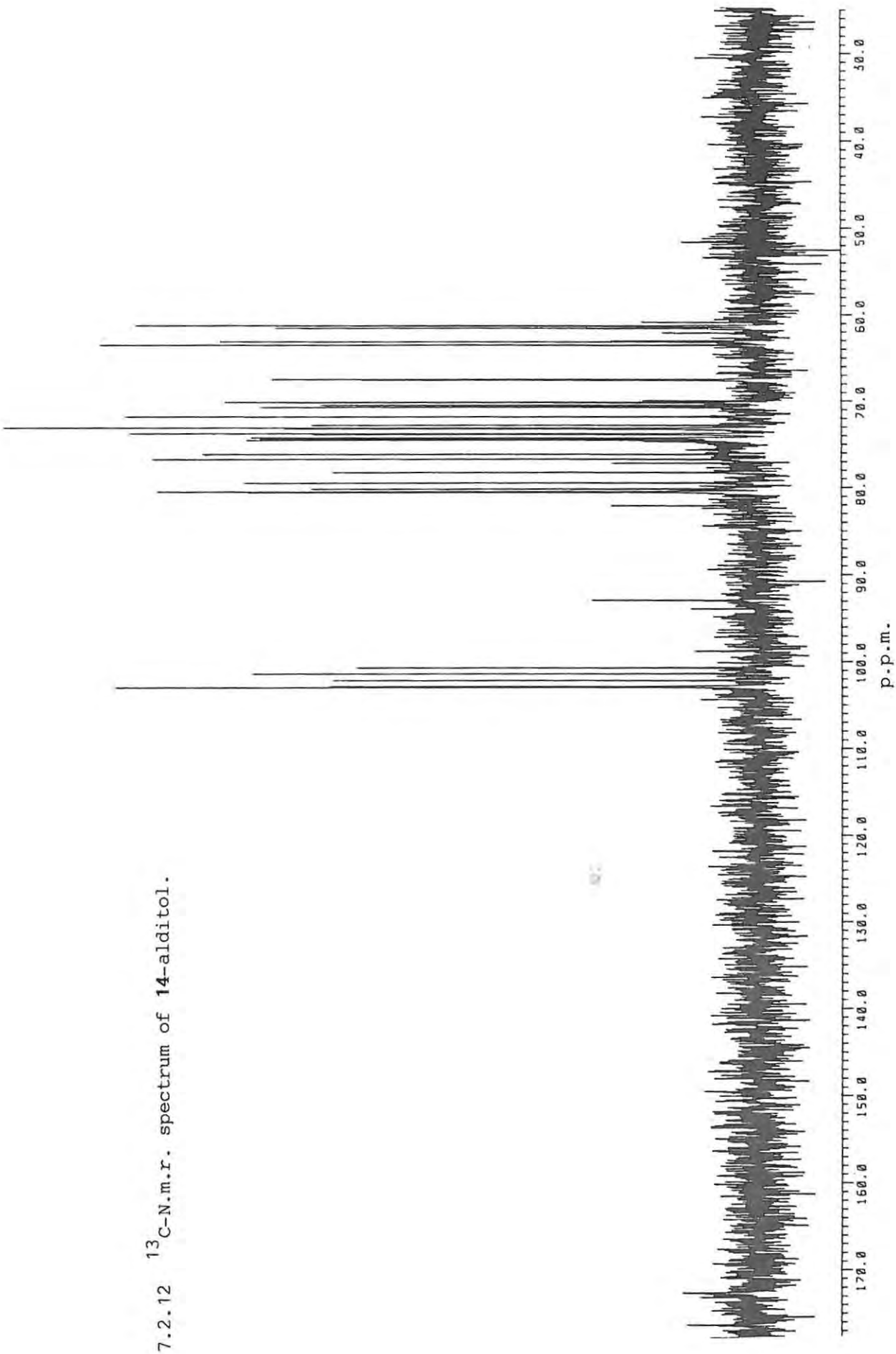


7.2.10 ^{13}C-N.m.r. spectrum of K39 polysaccharide, batch 6 (**13**).



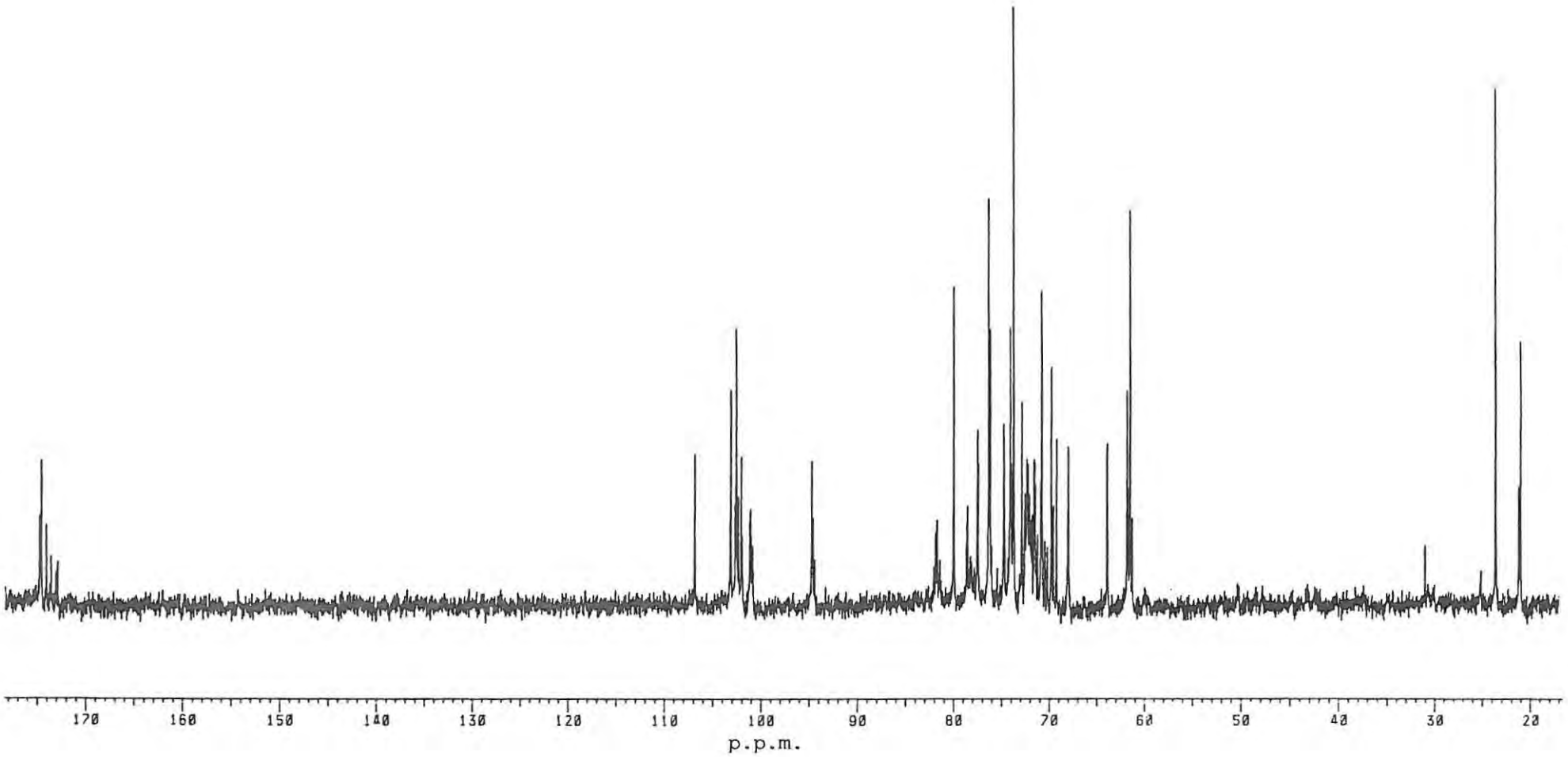
7.2.11 ^{13}C-N.m.r. spectrum of **14**.

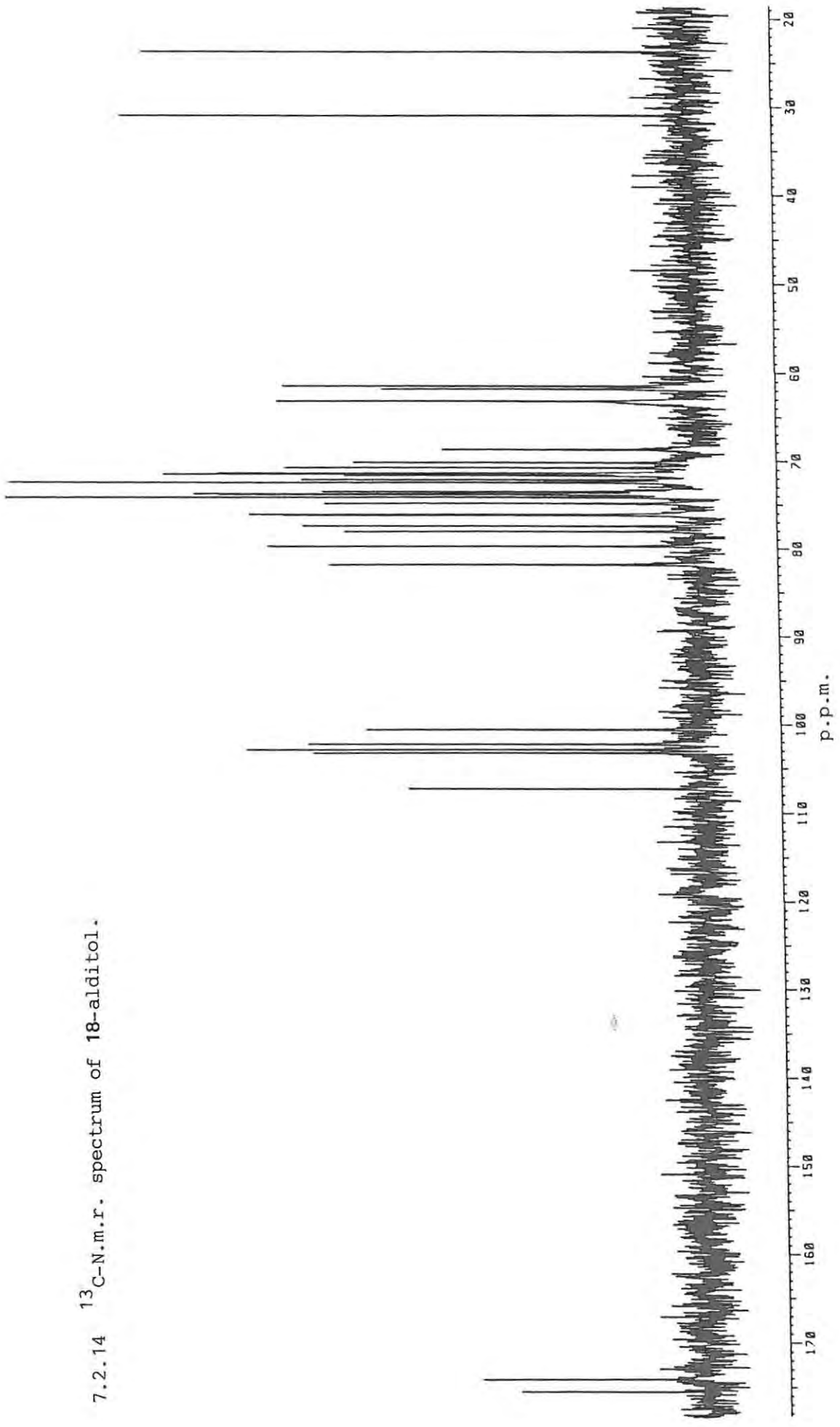




7.2.12 ^{13}C-N.m.r. spectrum of **14**-alditol.

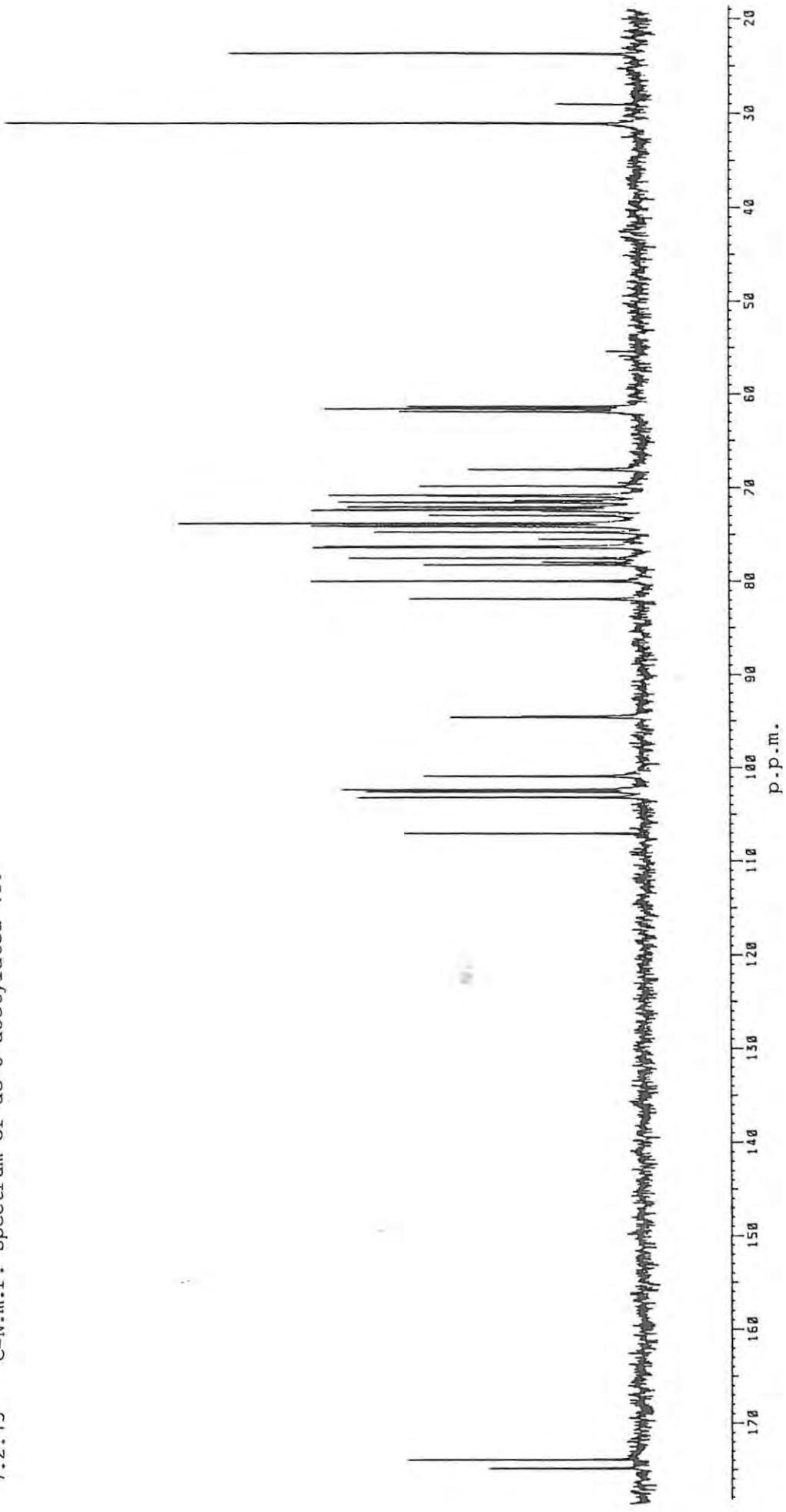
7.2.13 ^{13}C-N.m.r. spectrum of **18**.



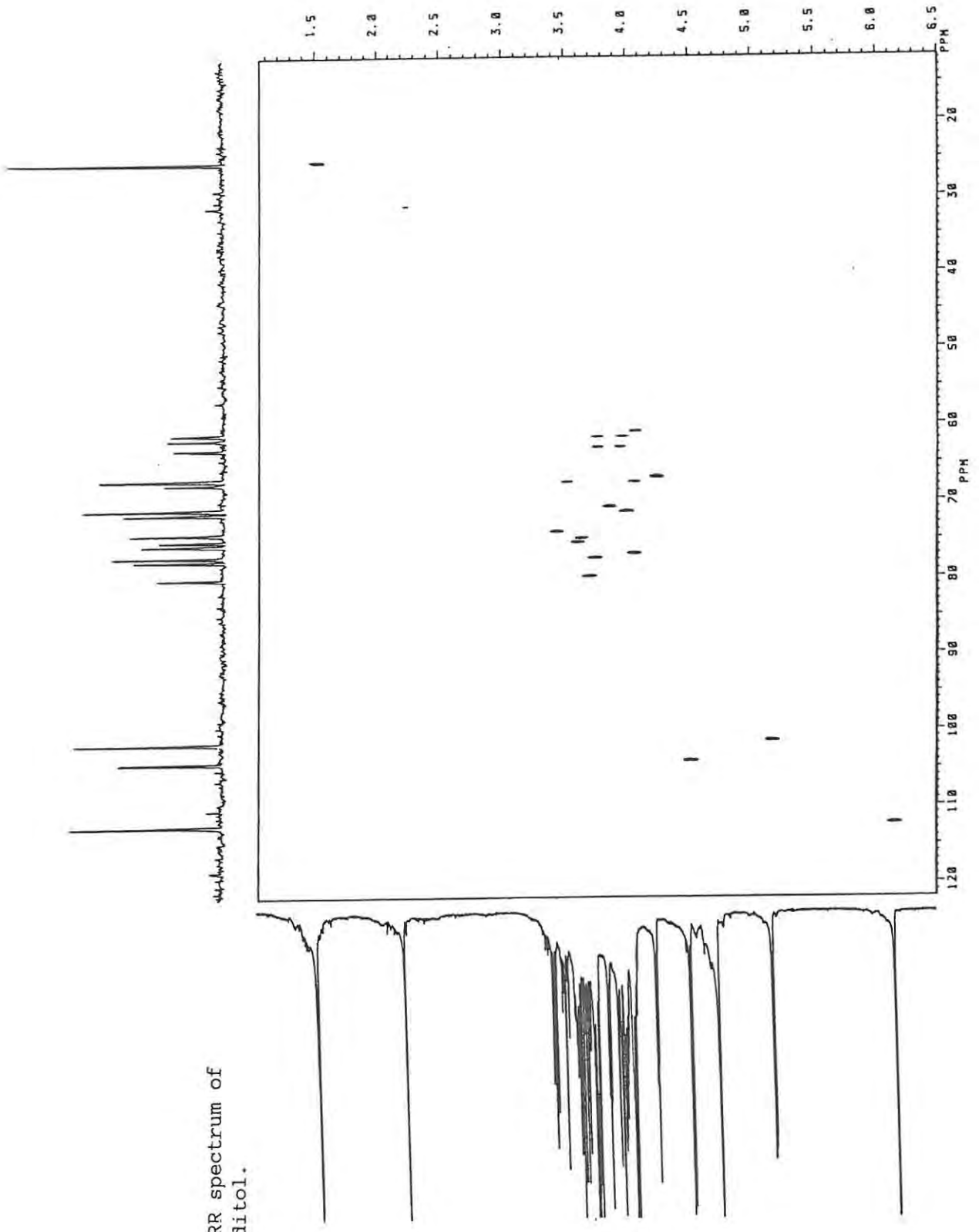


7.2.14 ^{13}C-N.m.r. spectrum of **18**-alditol.

7.2.15 ^{13}C-N.m.r. spectrum of de-*O*-acetylated **18**.

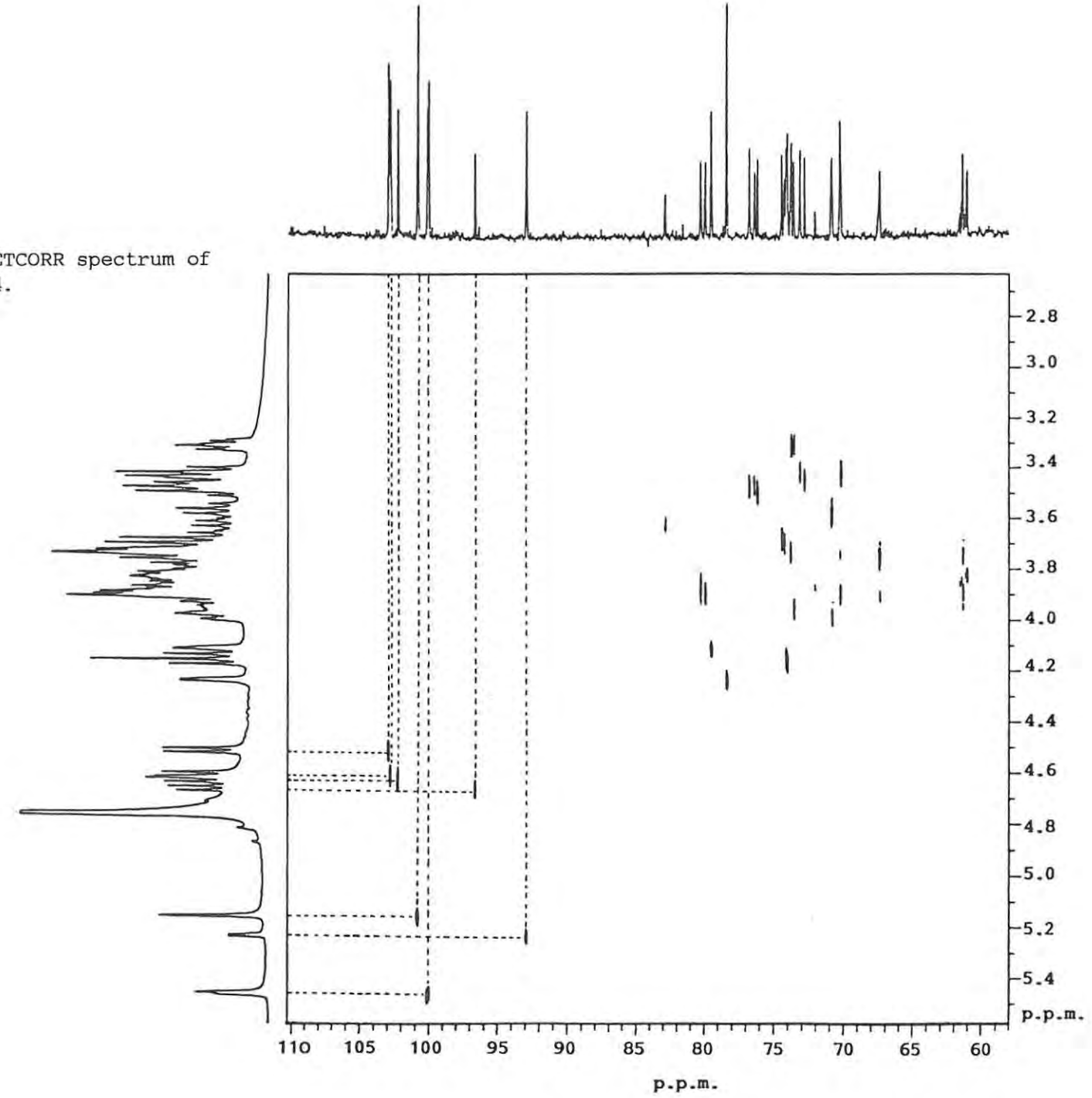


7.3 2D NUCLEAR MAGNETIC RESONANCE SPECTRA

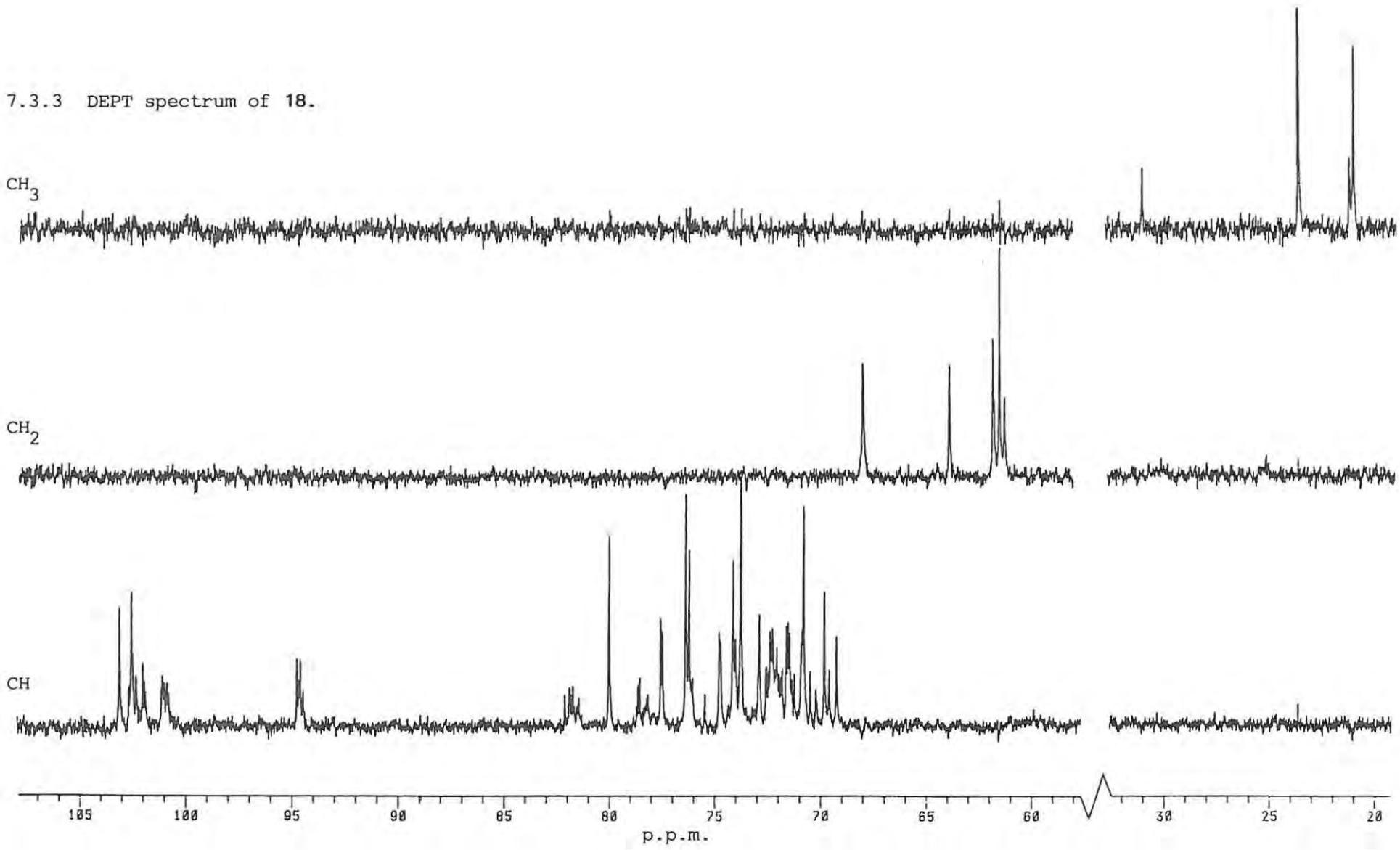


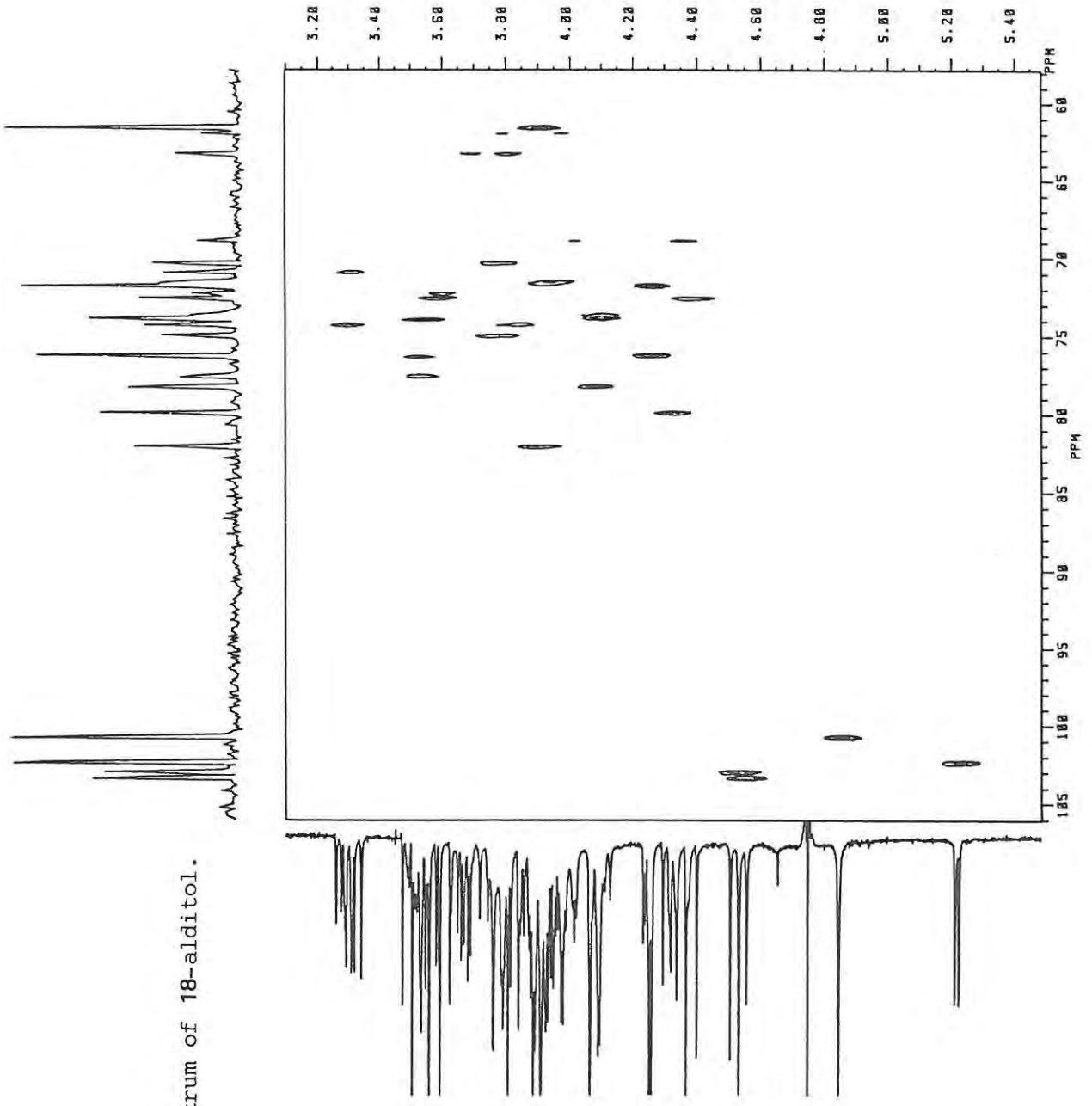
7.3.1 HETCORR spectrum of 10-alditol.

7.3.2 HETCORR spectrum of **14**.

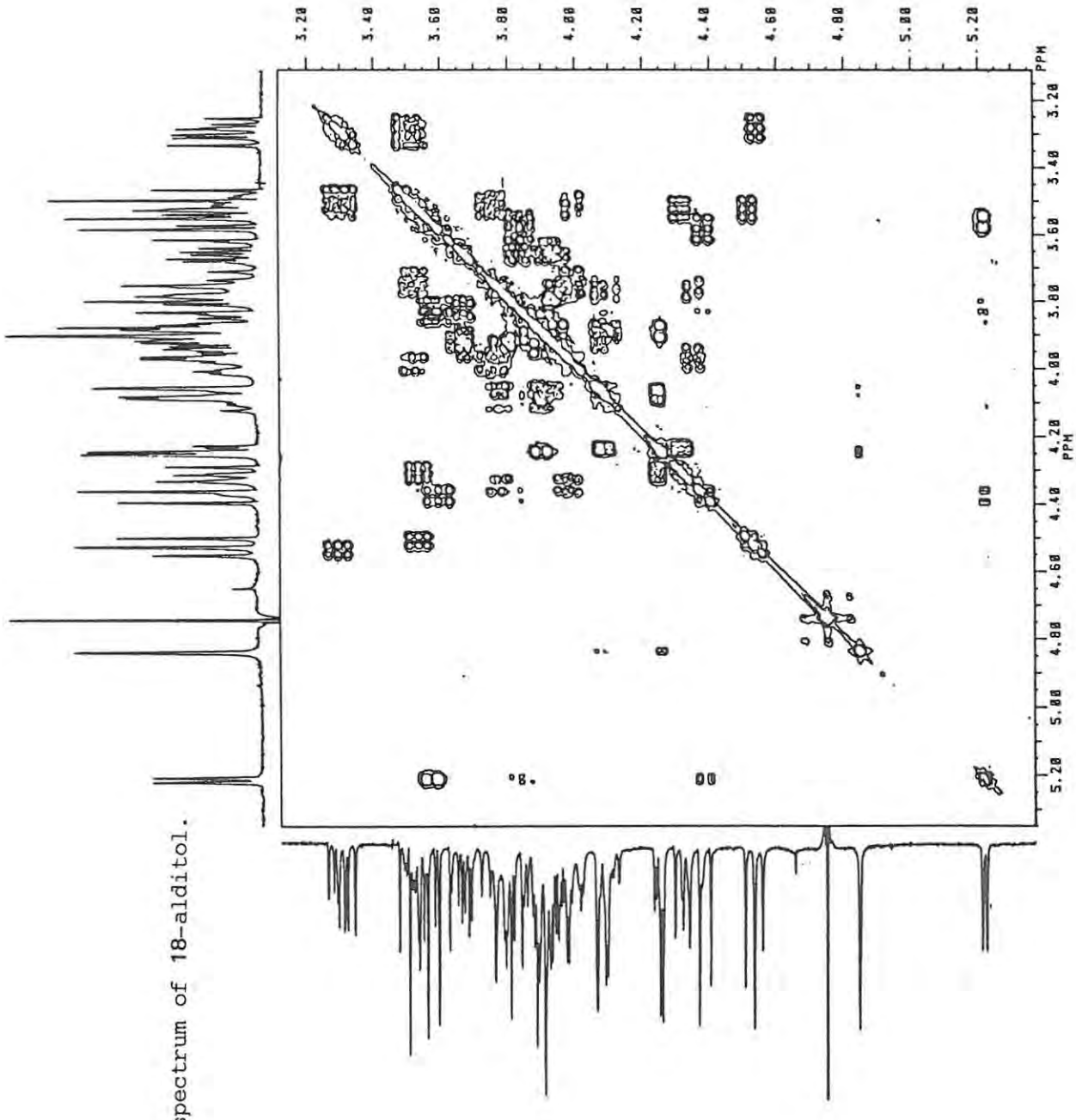


7.3.3 DEPT spectrum of **18.**



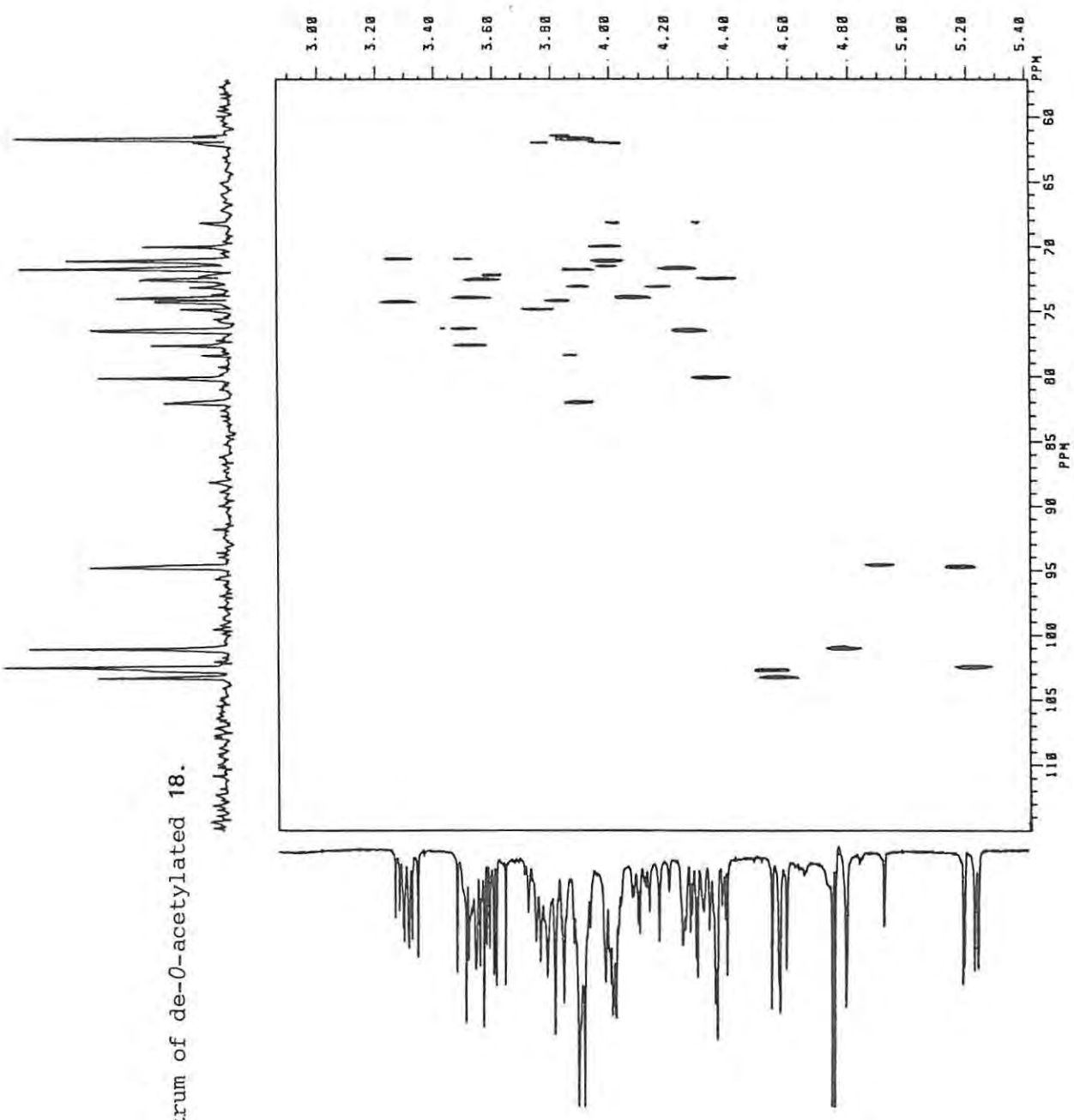


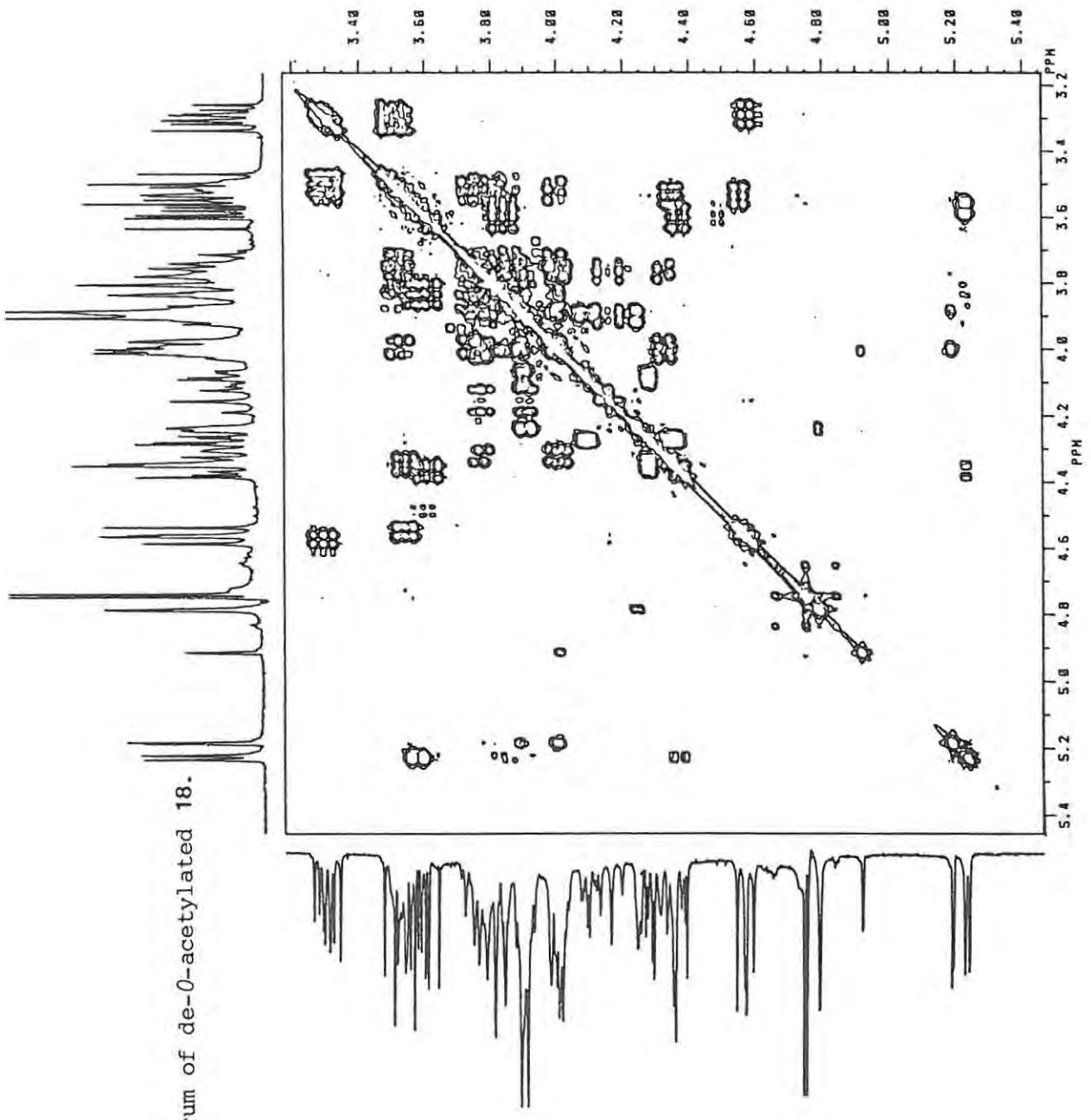
7.3.4 HETCORR spectrum of 18-alditol.



7.3.5 COSY spectrum of 18-alditol.

7.3.6 HETCORR spectrum of de-*O*-acetylated **18**.





7.3.7 COSY spectrum of de-*O*-acetylated 18.

8. REFERENCES

8. REFERENCES

1. C.T. BISHOP AND H.J. JENNINGS, in G.O. ASPINALL (Ed.), *The Polysaccharides*, Vol. 1, Academic Press, New York, 1982, pp. 291-330.
2. S.M. HAMMOND, P.A. LAMBERT, AND A.N. RYCROFT, *The Bacterial Cell Surface*, Croom Helm, London, 1984, pp. 57-118.
3. M.E. BAYER AND H. THUROW, *J. Bacteriol.*, 130(1977)911-936.
4. I. ØRSKOV, F. ØRSKOV, B. JANN, AND K. JANN, *Bacteriol. Rev.*, 41(1977)667-710.
5. I. ØRSKOV AND M.A. FIFE-ASBURY, *Int. J. Syst. Bacteriol.*, 27(1977)386-387.
6. B. LINDBERG AND W. NIMMICH, *Carbohydr. Res.*, 48(1976)81-84.
7. W. NIMMICH AND G. KORTEN, *Pathol. Microbiol.*, 36(1970)179-190.
8. C. ERBING, B. LINDBERG, J. LÖNNGREN, AND W. NIMMICH, *Carbohydr. Res.*, 56(1977)377-381.
9. W. NIMMICH, *Z. Med. Microbiol. Immunol.*, 154(1968)117-131.
10. W. NIMMICH, *Acta Biol. Med. Germ.*, 26(1971)397-403.
11. W. NIMMICH AND W. MÜNTER, *Z. Allg. Mikrobiol.*, 15(1975)127-129.
12. G.G.S. DUTTON AND A.V.S. LIM, *Carbohydr. Res.*, 123(1983)247-257.
13. M. HEIDELBERGER, W. NIMMICH, J. ERIKSEN, AND S. STIRM, *Acta Pathol. Microbiol. Scand., Sect. B*, 86(1978)313-320.
14. B. LINDBERG AND L. KENNE, in G.O. ASPINALL (Ed.), *The Polysaccharides*, Vol. 2, Academic Press, New York, 1983, pp. 287-363.
15. C. ERBING, L. KENNE, B. LINDBERG, J. LÖNNGREN, AND I.W. SUTHERLAND, *Carbohydr. Res.*, 50(1976)115-120.
16. G.G.S. DUTTON AND M.-T. YANG, *Can. J. Chem.*, 51(1973)1826-1832.
17. G.G.S. DUTTON, A.M. STEPHEN, AND S.C. CHURMS, *Carbohydr. Res.*, 38(1974)225-237.
18. L.A.S. PAROLIS, Ph.D. Thesis, Rhodes University, 1985.
19. B. LINDBERG, F. LINDH, J. LÖNNGREN, AND I.W. SUTHERLAND, *Carbohydr. Res.*, 76(1979)281-284.
20. B. LINDBERG, F. LINDH, J. LÖNNGREN, AND W. NIMMICH, *Carbohydr. Res.*, 70(1979) 135-144.
21. P.L. HACKLAND, M.Sc. Thesis, Rhodes University, 1986.
22. Y.-M. CHOY AND G.G.S. DUTTON, *Can. J. Chem.*, 51(1973)3015-3020.

23. J.P. KAMERLING, B. LINDBERG, J. LÖNNGREN, AND W. NIMMICH, *Acta Chem. Scand., Sect. B*, 29(1975)1-6.

24. J.-P. JOSELEAU AND M.-F. MARAIS, *Carbohydr. Res.*, 77(1979)183-190.

25. A.K. CHAKRABORTY, H. FRIEBOLIN, AND S. STIRM, *J. Bacteriol.*, 141(1980)971-972.

26. S.M.R. STANLEY, M.Sc. Thesis, Rhodes University, 1986.

27. H. NIEMANN, A.K. CHAKRABORTY, H. FRIEBOLIN, AND S. STIRM, *J. Bacteriol.*, 133(1978)390-391.

28. A.K. CHAKRABORTY, H. FRIEBOLIN, H. NIEMANN, AND S. STIRM, *Carbohydr. Res.*, 59(1977)525-530.

29. E. ALTMAN AND G.G.S. DUTTON, *Carbohydr. Res.*, 138(1985)293-303.

30. M. HEIDELBERGER, W.F. DUDMAN, AND W. NIMMICH, *J. Immunol.*, 104(1970) 1321-1328.

31. M. HEIDELBERGER AND G.G.S. DUTTON, *J. Immunol.*, 111(1973)857-859.

32. M. HEIDELBERGER, W. NIMMICH, J. ERIKSEN, G.G.S. DUTTON, S. STIRM, AND C.T. FANG, *Acta Pathol. Microbiol. Scand., Sect. C*, 83(1975)397-405.

33. M. HEIDELBERGER AND W. NIMMICH, *Immunochemistry*, 13(1976)67-80.

34. M.M. LEVINE, in R. GERMANIER (Ed.), *Bacterial Vaccines*, Academic Press, London, 1984, pp. 187-235.

35. G. SEMJEN, I. ØRSKOV, AND F. ØRSKOV, *Acta Pathol. Microbiol. Scand., Sect. B*, 85(1977)103-107.

36. E.C. GOTSCHLICH, B.A. FRASER, O. NISHIMURA, J.B. ROBBINS, AND T.-Y. LIU, *J. Biol. Chem.*, 256(1981)8915-8921.

37. M.A. SCHMIDT AND K. JANN, *FEMS Microbiol. Lett.*, 14(1982)69-74.

38. F. ØRSKOV, V. SHARMA, AND I. ØRSKOV, *J. Gen. Microbiol.*, 130(1984)2681-2684.

39. F. ØRSKOV AND I. ØRSKOV, Personal communication to H. Parolis.

40. L.A.S. PAROLIS, H. PAROLIS, AND G.G.S. DUTTON, Abstracts of the XIIIth International Carbohydrate Symposium, Ithaca, New York, 1986, p. 235.

41. G.G.S. DUTTON AND A. KUMA-MINTAH, Abstracts of the XIIIth International Carbohydrate Symposium, Ithaca, New York, 1986, p. 167.

42. Y.-M. CHOY AND G.G.S DUTTON, Abstracts of the XIIIth International Carbohydrate Symposium, Ithaca, New York, 1986, p. 151.

43. G.G.S. DUTTON, D.N. KARUNARATNE, AND A.V.S. LIM, Abstracts of the XIIIth International Carbohydrate Symposium, Ithaca, New York, 1986, p. 166.

44. B. JANN, T. DENGLER, AND K. JANN, *FEMS Microbiol. Lett.*, 29(1985)257-261.

45. T. DENGLER, B. JANN, AND K. JANN, *Carbohydr. Res.*, 142(1985)269-276.

46. F.-P. TSUI, R.A. BOYKINS, AND W. EGAN, *Carbohydr. Res.*, 102(1982)263-271.

47. W.F. VANN, T. SODERSTROM, W. EGAN, F.-P. TSUI, R. SCHNEERSON, I. ØRSKOV, AND F. ØRSKOV, *Infect. Immun.*, 39(1983)623-629.

48. B.A. FRASER, F.-P. TSUI, AND W. EGAN, *Carbohydr. Res.*, 73(1979) 59-65.

49. Z. DISCHE, *Methods Carbohydr. Chem.*, 1(1962)477-514.

50. M. DUBOIS, K.A. GILLES, J.K. HAMILTON, P.A. REBERS, AND F. SMITH, *Anal. Chem.*, 28(1956)350-356.

51. S.M. PARTRIDGE, *Nature*, 158(1946)270-271.

52. S.M. PARTRIDGE AND R.G. WESTALL, *Biochem. J.*, 42(1948)238-250.

53. S.C. CHURMS, in G. ZWEIG AND J. SHERMA (Eds.), *Handbook of Chromatography*, Vol. 1, CRC Press, Florida, 1982.

54. A.G. McINNES, D.H. BALL, F.P. COOPER, AND C.T. BISHOP, *J. Chromatogr.*, 1(1958)556-557.

55. J. DROZD, *J. Chromatogr.*, 113(1975)303-356.

56. C.T. BISHOP, *Adv. Carbohydr. Chem.*, 19(1964)95-147.

57. G.G.S. DUTTON, *Adv. Carbohydr. Chem. Biochem.*, 28(1973)11-160.

58. G.G.S DUTTON, *Adv. Carbohydr. Chem. Biochem.*, 30(1974)9-110.

59. M.F. LAKER, *J. Chromatogr.*, 184(1980)457-470.

60. S.W. GUNNER, J.K.N. JONES, AND M.B. PERRY, *Chem. Ind.(London)*, (1961) 255-256.

61. S.W. GUNNER, J.K.N. JONES, AND M.B. PERRY, *Can. J. Chem.*, 39(1961)1892-1899.

62. J.S. SAWARDEKER, J.H. SLONEKER, AND A. JEANES, *Anal. Chem.*, 37(1965)1602-1604.

63. A.B. BLAKENEY, P.J. HARRIS, R.J. HENRY, AND B.A. STONE, *Carbohydr. Res.*, 113(1983)291-299.

64. F. DASGUPTA, G.W. HAY, W.A. SZAREK, AND W.L. SHILLING, *Carbohydr. Res.*, 114(1983)153-157.

65. C.C. SWEELEY, R. BENTLEY, M. MAKITA, AND W.W. WELLS, *J. Am. Chem. Soc.*, 85(1963)2497-2507.

66. C.C. SWEELEY, W.W. WELLS, AND R. BENTLEY, *Methods Enzymol.*, 8(1966)95-108.

67. J. SHAPIRA, *Nature*, 222(1969)792-793.

68. D.G. LANCE AND J.K.N. JONES, *Can. J. Chem.*, 45(1967)1995-1998.

69. B.A. DMITRIEV, L.V. BACKINOWSKY, O.S. CHIZHOV, B.M. ZOLOTAREV, AND N.K. KOCHETKOV, *Carbohydr. Res.*, 19(1971)432-435.

70. R. VARMA, R.S. VARMA, AND A.H. WARDI, *J. Chromatogr.*, 77(1973)222-227.

71. R. VARMA, R.S. VARMA, W.S. ALLEN, AND A.H. WARDI, *J. Chromatogr.*, 86(1973) 205-210.

72. F.R. SEYMOUR, E.C.M. CHEN, AND S.H. BISHOP, *Carbohydr. Res.*, 73(1979)19-45.

73. C.C. CHEN AND G.D. McGINNIS, *Carbohydr. Res.*, 90(1981)127-130.

74. G.D. McGINNIS, *Carbohydr. Res.*, 108(1982)284-292.

75. R.H. FURNEAUX, *Carbohydr. Res.*, 113(1983)241-255.

76. I.M. MORRISON, *J. Chromatogr.*, 108(1975)361-364.

77. R. VARMA AND D. FRENCH, *Carbohydr. Res.*, 25(1972)71-79.

78. R.A. LAINE AND C.C. SWEELEY, *Anal. Biochem.*, 43(1971)533-538.

79. T.P. MAWHINNEY, M.S. FEATHER, J.R. MARTINEZ, AND G.J. BARBERO, *Carbohydr. Res.*, 75(1979)C21-C23.

80. H.J. CHAVES DAS NEVES, A.M.V. RISCADO, AND H. FRANK, *Carbohydr. Res.*, 152(1986)1-6.

81. S. HONDA, N. YAMAUCHI, AND K. KAKEDI, *J. Chromatogr.*, 169(1979)287-293.

82. D.T. WILLIAMS AND J.K.N. JONES, *Can. J. Chem.*, 44(1966)412-415.

83. F. EISENBERG, JR., *Carbohydr. Res.*, 19(1971)135-138.

84. F.R. SEYMOUR, E.C.M. CHEN, AND J.E. STOUFFER, *Carbohydr. Res.*, 83(1980)201-242.

85. T.P. MAWHINNEY, M.S. FEATHER, G.J. BARBERO, AND J.R. MARTINEZ, *Anal. Biochem.*, 101(1980)112-117.

86. J.-R. NEESER AND T.F. SCHWEIZER, *Anal. Biochem.*, 142(1984)58-67.

87. J.C. LINDEN AND C.L. LAWHEAD, *J. Chromatogr.*, 105(1975)125-133.

88. R. SCHWARZENBACH, *J. Chromatogr.*, 117(1976)206-210.

89. R.B. MEAGHER AND A. FURST, *J. Chromatogr.*, 117(1976)211-215.

90. K.B. HICKS, P.C. LIM, AND M.J. HAAS, *J. Chromatogr.*, 319(1985)159-171.

91. S. HONDA, T. KONISHI, S. SUZUKI, K. KAKEHI, AND S. GANNO, *J. Chromatogr.*, 281(1983)340-344.

92. C.F. SNYDER, H.L. FRUSH, H.S. ISBELL, A. THOMPSON, AND M.L. WOLFROM, *Methods Carbohydr. Chem.*, 1(1962)524-534.

93. 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.

94. G. AVIGAD, D. AMARAL, C. ASENSIO BRETONES, AND B.L. HORECKER, *J. Biol. Chem.*, 237(1962)2736-2743.

95. J.N.C. WHYTE AND J.R. ENGLAR, *Carbohydr. Res.*, 57(1977)273-280.

96. C.H. LOCHMÜLLER AND R.W. SOUTER, *J. Chromatogr.*, 113(1975)283-302.

97. G.J. GERWIG, J.P. KAMERLING, AND J.F.G. VLIEGENTHART, *Carbohydr. Res.*, 62(1978)349-357.

98. G.J. GERWIG, J.P. KAMERLING, AND J.F.G. VLIEGENTHART, *Carbohydr. Res.*, 77(1979) 1-7.

99. K. LEONTEIN, B. LINDBERG, AND J. LÖNNGREN, *Carbohydr. Res.*, 62(1978)359-362.

100. M.R. LITTLE, *Carbohydr. Res.*, 105(1982)1-8.

101. H. SCHWEER, *J. Chromatogr.*, 243(1982)149-152.

102. H. SCHWEER, *J. Chromatogr.*, 259(1983)164-168.

103. W.A. KÖNIG, I. BENECKE, AND H. BRETTING, *Angew. Chem., Int. Ed. Engl.*, 20(1981) 693-694.

104. W.A. KÖNIG, I. BENECKE, AND S. SIEVERS, *J. Chromatogr.*, 217(1981)71-79.

105. R. OSHIMA, Y. YAMAUCHI, AND J. KUMANOTANI, *Carbohydr. Res.*, 107(1982)169-176.

106. H. BJÖRNDAL, C.G. HELLERQVIST, B. LINDBERG, AND S. SVENSSON, *Angew. Chem., Int. Ed. Engl.*, 9(1970)610-619.

107. P.-E. JANSSON, L. KENNE, H. LIEDGREN, B. LINDBERG, AND J. LÖNNGREN, *Chem. Commun.*, University of Stockholm, 8(1976).

108. J. LÖNNGREN AND S. SVENSSON, *Adv. Carbohydr. Chem. Biochem.*, 29(1974)41-106.

109. H. RAUVALA, *Carbohydr. Res.*, 72(1979)257-260.

110. T. PURDIE AND J.C. IRVINE, *J. Chem. Soc.*, 83(1903)1021-1037.

111. H.O. BOUVENG AND B. LINDBERG, *Adv. Carbohydr. Chem.*, 15(1960)53-89.

112. B. LINDBERG, *Methods Enzymol.*, 28(1972)178-195.

113. S. HAKOMORI, *J. Biochem. (Tokyo)*, 55(1964)205-208.

114. J. FINNE, T. KRUSIUS, AND H. RAUVALA, *Carbohydr. Res.*, 80(1980)336-339.

115. L.R. PHILLIPS AND B.A. FRASER, *Carbohydr. Res.*, 90(1981)149-152.

116. A.B. BLAKENEY AND B.A. STONE, *Carbohydr. Res.*, 140(1985)319-324.

117. J.P. PARENTE, P. CARDON, Y. LEROY, J. MONTREUIL, B. FOURNET, AND G. RICART, *Carbohydr. Res.*, 141(1985)41-47.

118. T. NARUI, K. TAKAHASHI, M. KOBAYASHI, AND S. SHIBATA, *Carbohydr. Res.*, 103(1982)293-295.

119. T.J. WAEGHE, A.G. DARVILL, M. McNEIL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 123(1983)281-304.

120. P.J. HARRIS, R.J. HENRY, A.B. BLAKENEY, AND B.A. STONE, *Carbohydr. Res.*, 127(1984)59-73.

121. D.M.W. ANDERSON AND G.M. CREE, *Carbohydr. Res.*, 2(1966)162-166.

122. U. ZÄHRINGER AND E.T. RIETSCHEL, *Carbohydr. Res.*, 152(1986)81-87.

123. I. CIUCANU AND F. KEREK, *Carbohydr. Res.*, 131(1984)209-217.

124. G. LARSON, N. KARLSSON, G.C. HANSSON, AND W. PIMLOTT, *Carbohydr. Res.*, 161(1987)281-290.

125. A. ISOGAI, A. ISHIZU, J. NAKANO, S. EDA, AND K. KATO, *Carbohydr. Res.*, 138(1985) 99-108.

126. E.G. GROS AND S.M. FLEMATTI, *Chem. Ind. (London)*, (1966)1556-1557.

127. I.O. MASTRONARDI, S.M. FLEMATTI, J.O. DEFERRARI, AND E.G. GROS, *Carbohydr. Res.*, 3(1966)177-183.

128. J. ARNARP, L. KENNE, B. LINDBERG, AND J. LÖNNGREN, *Carbohydr. Res.*, 44(1975) C5-C7.

129. P. PREHM, *Carbohydr. Res.*, 78(1980)372-374.

130. A.N. DE BELDER AND B. NORRMAN, *Carbohydr. Res.*, 8(1968)1-6.

131. D. ROLF AND G.R. GRAY, *J. Am. Chem. Soc.*, 104(1982)3539-3541.

132. V.N. REINHOLD, E. COLES, AND S.A. CARR, *J. Carbohydr. Chem.*, 2(1983)1-18.

133. D. ROLF, J.A. BENNEK, AND G.R. GRAY, *J. Carbohydr. Chem.*, 2(1983)373-383.

134. J.A. BENNEK, D. ROLF, AND G.R. GRAY, *J. Carbohydr. Chem.*, 2(1983)385-393.

135. J.-G. JUN AND G.R. GRAY, *Carbohydr. Res.*, 163(1987)247-261.

136. A. VAN LANGEHOVE AND V.N. REINHOLD, *Carbohydr. Res.*, 143(1985)1-20.

137. J.U. BOWIE, P.V. TRESCONY, AND G.R. GRAY, *Carbohydr. Res.*, 125(1984)301-307.

138. D. ROLF AND G.R. GRAY, *Carbohydr. Res.*, 131(1984)17-28.

139. D. ROLF, J.A. BENNEK, AND G.R. GRAY, *Carbohydr. Res.*, 137(1985)183-196.

140. J.A. BENNEK, M.J. RICE, AND G.R. GRAY, *Carbohydr. Res.*, 157(1986)125-137.

141. S.J. ANGYAL AND K. JAMES, *Aust. J. Chem.*, 23(1970)1209-1221.

142. J.J. MARSHALL, *Adv. Carbohydr. Chem. Biochem.*, 30(1974)257-370.

143. B. LINDBERG, J. LÖNNGREN, AND S. SVENSSON, *Adv. Carbohydr. Chem. Biochem.*, 31(1975)185-240.

144. R.D. GUTHRIE AND J.M. McCARTHY, *Adv. Carbohydr. Chem.*, 22(1967)11-23.

145. J.N. BeMILLER, *Adv. Carbohydr. Chem.*, 22(1967)25-108.

146. A.S. PERLIN, *Adv. Carbohydr. Chem.*, 14(1959)9-61.

147. I.J. GOLDSTEIN, G.W. HAY, B.A. LEWIS, AND F. SMITH, *Methods Carbohydr. Chem.*, 5(1965)361-370.

148. J. KISS, *Adv. Carbohydr. Chem. Biochem.*, 29(1974)229-303.

149. G.O. ASPINALL, *Pure Appl. Chem.*, 49(1977)1105-1134.

150. A.J. MORT AND D.T.A. LAMPORT, *Anal. Biochem.*, 82(1977)289-309.

151. A.J. MORT AND W.D. BAUER, *J. Biol. Chem.*, 257(1982)1870-1875.

152. A.J. MORT, J.-P. UTILLE, G. TORRI, AND A.S. PERLIN, *Carbohydr. Res.*, 121(1983) 221-232.

153. A.J. MORT, *Carbohydr. Res.*, 122(1983)315-321.

154. Y.A. KNIREL, E.V. VINOGRADOV, A.S. SHASHKOV, B.A. DMITRIEV, AND N.K. KOCHETKOV, *Carbohydr. Res.*, 112(1983)C4-C6.

155. C. BOSSO, T. DEFAYE, A. DOMARD, A. GADELLE, AND C. PEDERSEN, *Carbohydr. Res.*, 156(1986)57-68.

156. M.-S. KUO AND A.J. MORT, *Carbohydr. Res.*, 145(1986)247-265.

157. A.J. MORT, M.-S. KUO, AND P. KOMALAVILAS, Abstracts of the XIIIth International Carbohydrate Symposium, Ithaca, New York, 1986, p. 229.

158. J.M. LAU, M. McNEIL, A.G. DARVILL, AND P. ALBERSHEIM, *Carbohydr. Res.*, in press.

159. D.E. BRADLEY, *Bacteriol. Rev.*, 31(1967)230-314.

160. B.H. PARK, *Virology*, 2(1956)711-718.

161. M.H. ADAMS AND B.H. PARK, *Virology*, 2(1956)719-736.

162. M.E. BAYER, H. THUROW, AND M.H. BAYER, *Virology*, 94(1979)95-118.

163. J.C. HUMPHRIES, *J. Bacteriol*, 56(1948)683-693.

164. C. EKLUND AND O. WYSS, *J. Bacteriol.*, 84(1962)1209-1215.

165. P.F. BARTELL, T.E. ORR, AND G.K.H. LAM, *J. Bacteriol.*, 92(1966)56-62.

166. I.W. SUTHERLAND, *Biochem. J.*, 104(1967)278-285.

167. I.W. SUTHERLAND AND J.F. WILKINSON, *Biochem. J.*, 110(1968)749-754.

168. E.C. YUREWICZ, M.A. GHALAMBOR, D.H. DUCKWORTH, AND E.C. HEATH, *J. Biol. Chem.*, 246(1971)5607-5616.

169. S. STRIM AND E. FREUND-MÖLBERT, *J. Virol.*, 8(1971)330-342.

170. D. RIEGER-HUG AND S. STIRM, *Virology*, 113(1981)363-378.

171. K.R. YAMAMOTO, B.M. ALBERTS, R. BENZINGER, L. LAWHORNE, AND G. TRIEBER, *Virology*, 40(1970)734-744.

172. 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.

173. G.G.S. DUTTON, A.V. SAVAGE, AND M. VIGNON, *Can. J. Chem.*, 58(1980)2588-2591.

174. M. BEURRET AND J.-P. JOSELEAU, *Carbohydr. Res.*, 157(1986)27-51.

175. H. THUROW, H. NIEMANN, C. RUDOLPH, AND S. STIRM, *Virology*, 58(1974)306-309.

176. H. NIEMANN, B. KWIATKOWSKI, V. WESTPHAL, AND S. STIRM, *J. Bacteriol.*, 130(1977)366-374.

177. J.L.DI FABIO, D.N. KARUNARATNE, AND G.G.S. DUTTON, *Carbohydr. Res.*, 144(1986) 251-261.

178. A.N. ANDERSON, H. PAROLIS, G.G.S. DUTTON, AND D.M. LEEK, *Carbohydr. Res.*, 167(1987)279-290.

179. G.G.S. DUTTON AND D.N. KARUNARATNE, *Carbohydr. Res.*, 138(1985)277-291.

180. S. STIRM, F. ALTMANN, AND F.M. UNGER, Abstracts of the 3rd European Symposium on Carbohydrates, Grenoble, France, 1985, p. 51.

181. I.W. SUTHERLAND, K. JANN, AND B. JANN, *Eur. J. Biochem.*, 12(1970)285-288.

182. E. ALTMAN, G.G.S. DUTTON, AND A.M. STEPHEN, *S. Afr. J. Sci.*, 82(1986)45-46.

183. J.E.G. VAN DAM, H. VAN HALBEEK, J.P. KAMERLING, J.F.G. VLIEGENTHART, H. SNIPPE, M. JANSZE, AND J.M.N. WILLERS, *Carbohydr. Res.*, 142(1985)338-343.

184 (a) N. RAVENSCROFT, E.H. MERRIFIELD, AND A.M. STEPHEN, *S. Afr. J. Sci.*, 81(1985)380-381. (b) N. RAVENSCROFT, A.M. STEPHEN, AND E.H. MERRIFIELD, *S. Afr. J. Sci.*, 81(1985)381-382.

185. N. RAVENSCROFT, A.M. STEPHEN, AND E.H. MERRIFIELD, *Carbohydr. Res.*, 167(1987)257-267.

186. G.G.S. DUTTON AND E.H. MERRIFIELD, *Carbohydr. Res.*, 85(1980)C13-C14.

187. G.G.S. DUTTON, K.L. MACKIE, A.V. SAVAGE, D. RIEGER-HUG, AND S. STRIM, *Carbohydr. Res.*, 84(1980)161-170.

188. P.C. HALLENBECK, E.R. VIMR, F. YU, B. BASSLER, AND F.A. TROY, *J. Biol. Chem.*, 262(1987)3553-3561.

189. A.J.P. MARTIN AND R.L.M. SYNGE, *Biochem. J.*, 35(1941)1358-1368.

190. A.T. JAMES AND A.J.P. MARTIN, *Biochem. J.*, 50(1952)679-690.

191. J.J. VAN DEEMTER, F.J. ZUIDERWEG, AND A. KLINKENBERG, *Chem. Eng. Sci.*, 5(1956)271-289.

192. T.A. ROONEY, in R.R. FREEMAN (Ed.), *High Resolution Gas Chromatography*, 2nd ed., Hewlett-Packard, 1981, pp. 7-28.

193. M. NOVOTNY, *Anal. Chem.*, 50(1978)16A-32A.

194. J. EYEM, *Chromatographia*, 8(1975)456-462.

195. K. GROB AND G. GROB, *J. Chromatogr.*, 213(1981)211-221.

196. M.L. DUFFY, *Int. Lab.*, April (1986)78-87.

197. K. TESAŘÍK, *J. Chromatogr.*, 65(1972)295-302.

198. A.G.W. BRADBURY, D.J. HALLIDAY, AND D.G. METCALF, *J. Chromatogr.*, 213(1981) 146-150.

199. G. HOLZER, J. ORÓ, S.J. SMITH, AND V.M. DOCTOR, *J. Chromatogr.*, 194(1980)410-415.

200. C. GREEN, V.M. DOCTOR, G. HOLZER, AND J. ORÓ, *J. Chromatogr.*, 207(1981)268-272.

201. J. KLOK, E.H. NIEBERG-VAN VELZEN, J.W. DE LEEUW, AND P.A. SCHENCK, *J. Chromatogr.*, 207(1981)273-275.

202. R. OSHIMA, A. YOSHIKAWA, AND J. KUMANOTANI, *J. Chromatogr.*, 213(1981)142-145.

203. A.B. BLAKENEY, P.J. HARRIS, R.J. HENRY, B.A. STONE, AND T. NORRIS, *J. Chromatogr.*, 249(1982)180-182.

204. N. SHIBUYA, *J. Chromatogr.*, 208(1981)96-99.

205. E. BARRETO-BERGTER, L. HOGGE, AND P.A.J. GORIN, *Carbohydr. Res.*, 97(1981)147-150.

206. R. GEYER, H. GEYER, S. KÜHNHARDT, W. MINK, AND S. STIRM, *Anal. Biochem.*, 121(1982)263-274.

207. J.A. LOMAX AND J. CONCHIE, *J. Chromatogr.*, 236(1982)385-394.

208. G. EKLUND, B. JOSEFSSON, AND C. ROOS, *J. Chromatogr.*, 142(1977)575-585.

209. D.P. SWEET, R.H. SHAPIRO, AND P. ALBERSHEIM, *Carbohydr. Res.*, 40(1975)217-225.

210. R.F. ADDISON AND R.G. ACKMAN, *J. Gas Chromatogr.*, 6(1968)135-138.

211. G.P. BELUE AND G.D. McGINNIS, *J. Chromatogr.*, 97(1974)25-31.

212. G.P. BELUE, *J. Chromatogr.*, 100(1974)233-235.

213. G.D. McGINNIS AND P. FANG, *J. Chromatogr.*, 130(1977)181-187.

214. N.W.H. CHEETHAM AND P. SIRIMANNE, *J. Chromatogr.*, 196(1980)171-175.

215. M. D'AMBOISE, D. NOËL, AND T. HANAI, *Carbohydr. Res.*, 79(1980)1-10.

216. G.D. McGINNIS AND P. FANG, *Methods Carbohydr. Chem.*, 8(1980)33-43.

217. L.A.TH. VERHAAR AND B.F.M. KUSTER, *J. Chromatogr.*, 220(1981)313-328.

218. J. LEHRFELD, *J. Chromatogr.*, 120(1976)141-147.

219. G.R. HER, S. SANTIKARN, V.N. REINHOLD, AND J.C. WILLIAMS, *J. Carbohydr. Chem.*, 6(1987)129-139.

220. S. HONDA, Y. MATSUDA, M. TAKAHASHI, AND S. GANNO, *Anal. Chem.*, 52(1980) 1079-1082.

221. S. HONDA, T. KONISHI, AND S. SUZUKI, *J. Chromatogr.*, 299(1984)245-251.

222. R.E. REIM AND R.M. VAN EFFEN, *Anal. Chem.*, 58(1986)3203-3207.

223. L.M. SANTOS AND R.P. BALDWIN, *Anal. Chem.*, 59(1987)1766-1770.

224. D. LECACHEUX, Y. MUSTIERE, R. PANARAS, AND G. BRIGAND, *Carbohydr. Polymers*, 6(1986)477-492.

225. T.R. COVEY, E.D. LEE, A.P. BRAINS, AND J.D. HENION, *Anal. Chem.*, 58(1986) 1451A-1461A.

226. M.W. SPELLMAN, M. McNEIL, A.G. DARVILL, P. ALBERSHEIM, AND A. DELL, *Carbohydr. Res.*, 122(1983)131-153.

227. S. SANTIKARN, G.R. HER, AND V.N. REINHOLD, *J. Carbohydr. Chem.*, 6(1987)141-154.

228. N.V. ESTEBAN, D.J. LIBERATO, J.B. SIDBURY, AND A.L. YERGEY, *Anal. Chem.*, 59(1987)1674-1677.

229. G.H. LATHE AND C.R.J. RUTHVEN, *Biochem. J.*, 62(1956)665-674.

230. *Gel Filtration Theory and Practice*, Technical literature, Pharmacia Fine Chemicals, Uppsala, Sweden, 1983.

231. S.C. CHURMS, *Adv. Carbohydr. Chem. Biochem.*, 25(1970)13-51.

232. D.M.W. ANDERSON AND J.F. STODDART, *Anal. Chim. Acta*, 34(1965)401-406.

233. K.A. GRANATH, *Methods Carbohydr. Chem.*, 5(1965)20-28.

234. R.L. WHISTLER AND A.K.M. ANISUZZAMAN, *Methods Carbohydr. Chem.*, 8(1980)45-53.

235. E. GRELLERT AND C.E. BALLOU, *Carbohydr. Res.*, 30(1973)218-219.

236. J.F. KENNEDY AND J.E. FOX, *Carbohydr. Res.*, 54(1977)13-21.

237. M. JOHN, G. TRÉNEL, AND H. DELLWEG, *J. Chromatogr.*, 42(1969)476-484.

238. N.K. KOCHETKOV AND O.S. CHIZHOV, *Methods Carbohydr. Chem.*, 6(1972)540-554.

239. N.K. KOCHETKOV AND O.S. CHIZHOV, *Adv. Carbohydr. Chem.*, 21(1966)39-93.

240. J. LÖNNGREN AND S. SVENSSON, *Adv. Carbohydr. Chem. Biochem.*, 29(1974)41-106.

241. T. RADFORD AND D.C. DE JONGH, *Biochem. Appl. Mass Spectrom.*, 1st Suppl. (1980) 255-310.

242. H. KRONE AND H.D. BECKEY, *Org. Mass Spectrom.*, 2(1969)427-429.

243. H.D. BECKEY AND H.-R. SHULTEN, *Angew. Chem., Int. Ed. Engl.*, 14(1975)403-415.

244. K. LEVSEN AND H. SCHWARZ, *Angew. Chem., Int. Ed. Engl.*, 15(1976)509-519.

245. V. KOVÁČIK, E. PETRÁKOVÁ, V. MIHÁLOV, I. TVAROŠKA, AND W. HEERMA, Abstracts of the XIIth International Carbohydrate Symposium, Utrecht, The Netherlands, 1984, p.496.

246. P.A. FINAN, R.I. REED, AND W. SNEDDEN, *Chem. Ind.(London)*, (1958)1172.

247. K. BIEMANN, D.C. DE JONGH, AND H.K. SCHNOES, *J. Am. Chem. Soc.*, 85(1963) 1763-1770.

248. D.C. DE JONGH AND K. BIEMANN, *J. Am. Chem. Soc.*, 85(1963)2289-2294.

249. N.K. KOCHETKOV, N.S. WULFSON, O.S. CHIZHOV, AND B.M. ZOLOTAREV, *Tetrahedron*, 19(1963)2209-2224.

250. N.K. KOCHETKOV AND O.S. CHIZHOV, *Tetrahedron*, 21(1965)2029-2047.

251. V. KOVÁČIK, Š. BAUER, J. ROSÍK, AND P. KOVÁČ, *Carbohydr. Res.*, 8(1968)282-290.

252. V. KOVÁČIK, Š. BAUER, AND J. ROSÍK, *Carbohydr. Res.*, 8(1968)291-294.

253. V. KOVÁČIK, V. MIHÁLOV, S. KARÁCSONYI, AND R. TOMAN, *Carbohydr. Res.*, 58(1977)203-208.

254. B. FOURNET, G. STRECKER, Y. LEROY, AND J. MONTREUIL, *Anal. Biochem.*, 116(1981)489-502.

255. J.H. BANOUB, F. MICHON, R. ROY, A. GROUILLER, AND H. BAZIN, *Carbohydr. Res.*, 144(1985)127-136.

256. L.S. GOLOVKINA, O.S. CHIZHOV, AND N.F. VUL'FSON, *Izv. Akad. Nauk SSSR, Ser. Khim.*, 11(1966)1915-1926.

257. P.-E. JANSSON AND B. LINDBERG, *Carbohydr. Res.*, 86(1980)287-292.

258. H. BJÖRNDAL, B. LINDBERG, AND S. SVENSSON, *Carbohydr. Res.*, 5(1967)433-440.

259. H. BJÖRNDAL, B. LINDBERG, Å. PILOTTI, AND S. SVENSSON, *Carbohydr. Res.*, 15(1970)339-349.

260. H.B. BORÉN, P.J. GAREGG, B. LINDBERG, AND S. SVENSSON, *Acta Chem. Scand.*, 25(1971)3299-3308.

261. K. AXBERG, H. BJÖRNDAL, Å. PILOTTI, AND S. SVENSSON, *Acta Chem. Scand.*, 26(1972)1319-1325.

262. O.S. CHIZHOV, N.V. MOLODTSOV, AND N.K. KOCHETKOV, *Carbohydr. Res.*, 4(1967) 273-276.

263. D.C. DE JONGH, T. RADFORD, J.D. HRIBAR, S. HANESSIAN, B. BIEBER, G. DAWSON, AND C.C. SWEELEY, *J. Am. Chem. Soc.*, 91(1969)1728-1740.

264. H.B. BORÉN, P.J. GAREGG, L. KENNE, Å. PILOTTI, S. SVENSSON, AND C.-G. SWAHN, *Acta Chem. Scand.*, 27(1973)3557-3562.

265. G. PETERSSON, *Tetrahedron*, 25(1969)4437-4443.

266. J. SZAFRANEK, C.D. PFAFFENBERGER, AND E.C. HORNING, *Carbohydr. Res.*, 38(1974) 97-105.

267. F.R. SEYMOUR, R.D. PLATTNER, AND M.E. SLODKI, *Carbohydr. Res.*, 44(1975)181-198.

268. F.R. SEYMOUR, M.E. SLODKI, R.D. PLATTNER, AND A. JEANES, *Carbohydr. Res.*, 53(1977)153-166.

269. C.A. STORTZ, M.C. MATULEWICZ, AND A.S. CEREZO, *Carbohydr. Res.*, 111(1982)31-39.

270. D.P. SWEET, R.H. SHAPIRO, AND P. ALBERSHEIM, *Biomed. Mass Spectrom.*, 1(1974) 263-268.

271. O.S. CHIZHOV, B.A. DMITRIEV, B.M. ZOLOTAREV, A.YA. CHERUYAK, AND N.K. KOCHETKOV, *Org. Mass Spectrom.*, 2(1969)947-952.

272. D.C. DE JONGH, *J. Org. Chem.*, 30(1965)1563-1570.

273. J. KÄRKKÄINEN, *Carbohydr. Res.*, 11(1969)247-256.

274. J. KÄRKKÄINEN, *Carbohydr. Res.*, 17(1971)1-10.

275. J. KÄRKKÄINEN, *Carbohydr. Res.*, 17(1971)11-18.

276. J.K. SHARP AND P. ALBERSHEIM, *Carbohydr. Res.*, 128(1984)193-202.

277. W.F. DUDMAN AND M.J. LACEY, *Carbohydr. Res.*, 145(1986)175-191.

278. D.B. KASSEL AND J. ALLISON, *Anal. Chem.*, 58(1986)1670-1675.

279. B. MUNSON, *Anal. Chem.*, 43(1971)28A-43A.

280. A.M. HOGG AND T.L. NAGABHUSHAN, *Tetrahedron Lett.*, 47(1972)4827-4830.

281. D. HORTON, J.D. WANDER, AND R.L. FOLTZ, *Carbohydr. Res.*, 36(1974)75-96.

282. R.C. DOUGHERTY, J.D. ROBERTS, W.W. BINKLEY, O.S. CHIZHOV, V.I. KADENTSEV, AND A.A. SOLOV'YOV, *J. Org. Chem.*, 39(1974)451-455.

283. M. McNEIL AND P. ALBERSHEIM, *Carbohydr. Res.*, 56(1977)239-248.

284. B.W. LI, T.W. COCHRAN, AND J.R. VERCELLOTTI, *Carbohydr. Res.*, 59(1977)567-570.

285. C. SCHRÖDER, A.K. SHUKLA, J. RIEDEMANN, AND R. SCHAUER, Abstracts of the XIIth International Carbohydrate Symposium, Utrecht, The Netherlands, 1984, p. 497.

286. R.A. HANCOCK, K. MARSHALL, AND H. WEIGEL, *Carbohydr. Res.*, 49(1976)351-360.

287. Y. TANAKA, R.K. YU, S. ANDO, T. ARIGA, AND T. ITOH, *Carbohydr. Res.*, 126(1984)1-14.

288. D.J. SURMAN AND J.C. VICKERMAN, *J. Chem. Soc., Chem Commun.*, (1981) 324-325.

289. M. BARBER, R.S. BORDOLI, R.D. SEDGWICK, AND A.N. TYLER, *J. Chem. Soc., Chem. Commun.*, (1981)325-327.

290. J.E. BARTMESS AND L.R. PHILLIPS, *Anal. Chem.*, 59(1987)2012-2014.

291. M. BARBER, R.S. BORDOLI, R.D. SEDGWICK, AND A.N. TYLER, *Nature (London)*, 293(1981)270-275.

292. L.S. FORSBERG, A. DELL, D.J. WALTON, AND C.E. BALLOU, *J. Biol. Chem.*, 257(1981)3555-3563.

293. A. DELL, H.R. MORRIS, H. EGGE, H. VON NICOLAI, AND G. STRECKER, *Carbohydr. Res.*, 115(1983)41-52.

294. A. DELL, W.S. YORK, M. McNEIL, A.G. DARVILL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 117(1983)185-200.

295. A. DELL AND C.E. BALLOU, *Carbohydr. Res.*, 120(1983)95-111.

296. A. DELL, J. OATES, C. LUGOWSKI, E. ROMANOWSKA, L. KENNE, AND B. LINDBERG, *Carbohydr. Res.*, 133(1984)95-104.

297. C. BOSSO, J. DEFAYE, A. HEYRAUD, AND J. ULRICH, *Carbohydr. Res.*, 125(1984)309-317.

298. D. CHATTERJEE, G.O. ASPINALL, AND P.J. BRENNAN, *J. Biol. Chem.*, 262(1987)3528-3533.

299. A. DELL AND P.R. TILLER, Abstracts of the 3rd European Symposium on Carbohydrates, Grenoble, France, 1985, pp. 2-3.

300. G.G.S. DUTTON AND Z. LAM, Abstracts of the XIIIth International Carbohydrate Symposium, Ithaca, New York, 1986, p. 168.

301. P.L. HACKLAND, H. PAROLIS, A. DELL, AND P.R. TILLER, Unpublished results.

302. A. DELL AND M.E. ROGERS, Abstracts of the XIIIth International Carbohydrate Symposium, Ithaca, New York, 1986, p. 274.

303. A. DELL AND P.R. TILLER, *Biochem. Biophys. Res. Commun.*, 135(1986)1126-1134.

304. V.N. REINHOLD, S.A. CARR, AND B.N. GREEN, Abstracts of the XIIth International Carbohydrate Symposium, Utrecht, The Netherlands, 1984, p. 491.

305. V.N. REINHOLD, Abstracts of the XIIIth International Carbohydrate Symposium, Ithaca, New York, 1986, p. 273.

306. F. BLOCH, W.W. HANSEN, AND M. PACKARD, *Phys. Rev.*, 69(1946)127.

307. F. BLOCH, *Phys. Rev.*, 70(1946)460-474.

308. F. BLOCH, W.W. HANSEN, AND M. PACKARD, *Phys. Rev.*, 70(1946)474-485.

309. E.M. PURCELL, H.C. TORREY, AND R.V. POUND, *Phys. Rev.*, 69(1946)37-38.

310. R. BENN AND H. GÜNTHER, *Angew. Chem., Int. Ed. Engl.*, 22(1983)350-380.

311. D.L. TURNER, *Prog. NMR Spectros.*, 17(1985)281-358.

312. G.A. MORRIS, *Magn. Reson. Chem.*, 24(1986)371-403.

313. T.C. FARRAR, *Anal. Chem.*, 59(1987)679A-690A.

314. T.C. FARRAR, *Anal. Chem.*, 59(1987)749A-761A.

315. R.U. LEMIEUX, R.K. KULLNIG, H.J. BERNSTEIN, AND W.G. SCHNEIDER, *J. Am. Chem. Soc.*, 79(1957)1005-1006.

316. R.U. LEMIEUX, R.K. KULLNIG, H.J. BERNSTEIN, AND W.G. SCHNEIDER, *J. Am. Chem. Soc.*, 80(1958)6098-6105.

317. L.D. HALL, *Adv. Carbohydr. Chem.*, 19(1964)51-93.

318. B. COXON, *Adv. Carbohydr. Chem. Biochem.*, 27(1972)7-83.

319. L.D. HALL, *Adv. Carbohydr. Chem. Biochem.*, 29(1974)11-40.

320. B. COXON, *Methods Carbohydr. Chem.*, 6(1972)513-539.

321. D.R. BUNDLE AND R.U. LEMIEUX, *Methods Carbohydr. Chem.*, 7(1976)79-86.

322. G. KOTOWYCZ AND R.U. LEMIEUX, *Chem. Rev.*, 73(1973)669-698.

323. P.E. PFEFFER, *J. Carbohydr. Chem.*, 3(1984)613-639.

324. R.H. MARCHESSAULT, M.G. TAYLOR, C.A. FYFE, AND R.P. VEREGIN, *Carbohydr. Res.*, 144(1985)C1-C5.

325. B. MATSUHIRO, A.B. ZANLUNGO, AND G.G.S. DUTTON, *Carbohydr. Res.*, 97(1981)11-18.

326. M. KARPLUS, *J. Chem. Phys.*, 30(1959)11-15.

327. M. KARPLUS, *J. Am. Chem. Soc.*, 85(1963)2870-2871.

328. T.B. GRINDLEY AND C. WICKRAMAGE, *J. Carbohydr. Chem.*, 4(1985)171-192.

329. Y.-M. CHOY, G.G.S. DUTTON, A.M. STEPHEN, AND M.-T. YANG, *Anal. Lett.*, 5(1972) 675-681.

330. G.M. BEBAULT, Y.-M. CHOY, G.G.S. DUTTON, N. FUNNELL, A.M. STEPHEN, AND M.-T. YANG, *J. Bacteriol.*, 113(1973)1345-1347.

331. P.-E. JANSSON, L. KENNE, AND E. SCHWEDA, *J. Chem. Soc., Perkins Trans. 1*, (1987)377-383.

332. P.J. GAREGG, B. LINDBERG, AND I. KVARNSTRÖM, *Carbohydr. Res.*, 77(1979)71-78.

333. P.J. GAREGG, P.-E. JANSSON, B. LINDBERG, F. LINDH, J. LÖNNGREN, I. KVARNSTRÖM, AND W. NIMMICH, *Carbohydr. Res.*, 78(1980)127-132.

334. J.L. DI FABIO, G.G.S. DUTTON, AND H. PAROLIS, *Carbohydr. Res.*, 133(1984)125-133.

335. C.W.M. GRANT, L.D. HALL, AND C.M. PRESTON, *J. Am. Chem. Soc.*, 95(1973)7742-7747.

336. C.M. PRESTON AND L.D. HALL, *Carbohydr. Res.*, 37(1974)267-282.

337. L.D. HALL AND C.M. PRESTON, *Carbohydr. Res.*, 29(1973)522-524.

338. L.D. HALL AND C.M. PRESTON, *Carbohydr. Res.*, 49(1976)3-11.

339. L.D. HALL AND C.M. PRESTON, *Carbohydr. Res.*, 41(1975)53-61.

340. J.H. BRADBURY AND J.G. COLLINS, *Carbohydr. Res.*, 71(1979)15-24.

341. J.F.G. VLIEGENTHART, H. VAN HALBEEK, AND L. DORLAND, *Pure Appl. Chem.*, 53(1981)45-77.

342. J.F.G. VLIEGENTHART, L. DORLAND, AND H. VAN HALBEEK, *Adv. Carbohydr. Chem. Biochem.*, 41(1983)209-374.

343. G.C. LEVY, R.L. LICHTER, AND G.L. NELSON, *Carbon-13 Nuclear Magnetic Resonance Spectroscopy*, 2nd ed., John Wiley and Sons, New York, 1983.

344. R. FREEMAN, H.D.W. HILL, AND R. KAPTEIN, *J. Mag. Reson.*, 7(1972)327-329.

345. K. BOCK AND L.D. HALL, *Carbohydr. Res.*, 40(1975)C3-C5.

346. H.J. JENNINGS AND I.C.P. SMITH, *Methods Enzymol.*, 50(1978)39-50.

347. R. BARKER, H.A. NUNEZ, P. ROSEVEAR, AND A.S. SERIANNI, *Methods Enzymol.*, 83(1982)58-63.

348. P.A.J. GORIN, *Adv. Carbohydr. Chem. Biochem.*, 38(1981)13-104.

349. K. BOCK AND C. PEDERSEN, *Adv. Carbohydr. Chem. Biochem.*, 41(1983)27-66.

350. K. BOCK, C. PEDERSEN, AND H. PEDERSEN, *Adv. Carbohydr. Chem. Biochem.*, 42(1984)193-225.

351. K. DILL, E. BERMAN, AND A.A. PAVIA, *Adv. Carbohydr. Chem. Biochem.*, 43(1985)1-49.

352. J.H. BRADBURY AND G.A. JENKINS, *Carbohydr. Res.*, 126(1984)125-156.

353. K. BOCK, I. LUNDT, AND C. PEDERSEN, *Tetrahedron Lett.*, 13(1973)1037-1040.

354. K. BOCK AND C. PEDERSEN, *J. Chem. Soc., Perkins Trans. 2*, (1974)293-297.

355. K. BOCK AND C. PEDERSEN, *Carbohydr. Res.*, 145(1985)135-140.

356. P.A.J. GORIN, M. MAZUREK, H.S. DUARTE, M. IACOMINI, AND J.H. DUARTE, *Carbohydr. Res.*, 100(1982)1-15.

357. M.J. KING-MORRIS AND A.S. SERIANNI, *J. Am. Chem. Soc.*, 109(1987)3502-3508.

358. M.K. McINTYRE AND G.W. SMALL, *Anal. Chem.*, 59(1987)1805-1811.

359. J.C. CHRISTOFIDES AND D.B. DAVIES, *J. Chem. Soc., Chem. Commun.*, (1985)1533-1534.

360. J.C. CHRISTOFIDES, D.B. DAVIES, J.A. MARTIN, AND E.B. RATHBONE, *J. Am. Chem. Soc.*, 108(1986)5738-5743.

361. R.E. HOFFMAN, J.C. CHRISTOFIDES, D.B. DAVIES, AND C.J. LAWSON, *Carbohydr. Res.*, 153(1986)1-16.

362. K.G.R. PACHLER, E.B. RATHBONE, AND A.M. STEPHEN, *Carbohydr. Res.*, 47(1976) 155-157.

363. A. BAX, W. EGAN, AND P. KOVÁČ, *J. Carbohydr. Chem.*, 3(1984)593-611.

364. B. COXON, Abstracts of the XIIth International Carbohydrate Symposium, Utrecht, The Netherlands, 1984, p. 509.

365. J.-R. BRISSON AND J.P. CARVER, *J. Biol. Chem.*, 258(1983)1431-1434.

366. D.A. CUMMING, D.S. DIME, A.A. GREY, J.J. KREPINSKY, AND J.P. CARVER, *J. Biol. Chem.*, 261(1986)3208-3213.

367. D.G. DAVIS AND A. BAX, *J. Am. Chem. Soc.*, 107(1985)7197-7198.

368. S. SUBRAMANIAN AND A. BAX, *J. Mag. Reson.*, 71(1987)325-330.

369. W.P. AUE, E. BARTHOLDI, AND R.R. ERNST, *J. Chem. Phys.*, 64(1976)2229-2246.

370. H.C. TORREY, *Phys. Rev.*, 76(1949)1059-1068.

371. O.W. SØRENSEN, G.W. EICH, M.H. LEVITT, G. BODENHAUSEN, AND R.R. ERNST, *Prog. NMR Spectros.*, 16(1983)163-192.

372. L.D. HALL AND G.A. MORRIS, *Carbohydr. Res.*, 82(1980)175-184.

373. C. MORAT, F.R. TARAVEL, AND M.R. VIGNON, *Carbohydr. Res.*, 163(1987)265-268.

374. L.D. HALL, S. SUKUMAR, AND G.R. SULLIVAN, *J. Chem. Soc., Chem. Commun.*, (1979)292-294.

375. W. CURATOLO, L.J. NEURINGER, D. RUBEN, AND R. HABERKORN, *Carbohydr. Res.*, 112(1983)297-300.

376. L.D. HALL, G.A. MORRIS, AND S. SUKUMAR, *J. Am. Chem. Soc.*, 102(1980)1745-1747.

377. G.A. MORRIS AND L.D. HALL, *J. Am. Chem. Soc.*, 103(1981)4703-4711.

378. F. CAVAGNA, H. DEGER, AND J. PULS, *Carbohydr. Res.*, 129(1984)1-8.

379. W.J. GOUX AND C.J. UNKEFER, *Carbohyar. Res.*, 159(1987)191-210.

380. G. EICH, G. BODENHAUSEN, AND R.R. ERNST, *J. Am. Chem. Soc.*, 104(1982)3731-3732.

381. M.A. BERNSTEIN, L.D. HALL, AND S. SUKUMAR, *Carbohydr. Res.*, 103(1982)C1-C6.

382. M.A. BERNSTEIN AND L.D. HALL, *J. Am. Chem. Soc.*, 104(1982)5553-5555.

383. C. JONES, *Carbohydr. Res.*, 139(1985)75-83.

384. G. MASSIOT, C. LAVAUD, D. GUILLAUME, L. LE MEN-OLIVIER, AND G. VAN BINST, *J. Chem. Soc., Chem. Commun.*, (1986)1485-1487.

385. V.K. DUA, B.N.N. RAO, S.-S. WU, V.E. DUBE, AND C.A. BUSH, *J. Biol. Chem.*, 261(1986)1599-1608.

386. J. FEENEY, T.A. FRENKIEL, AND E.E. HOUNSELL, *Carbohydr. Res.*, 152(1986)63-72.

387. T. NISHIDA, C.R. ENZELL, AND G.A. MORRIS, *Mag. Reson. Chem.*, 24(1986)179-182.

388. M. IKURA AND K. HIKICHI, *Carbohydr. Res.*, 163(1987)1-8.

389. J. DABROWSKI, A. EJCHART, M. KORDOWICZ, AND P. HANFLAND, *Mag. Reson. Chem.*, 25(1987)338-346.

390. J.N. SCARSDALE, J.H. PRESTEGARD, S. ANDO, T. HORI, AND R.K. YU, *Carbohydr. Res.*, 155(1986)45-56.

391. D.H. HUANG, N.R. KRISHNA, AND D.G. PRITCHARD, *Carbohydr. Res.*, 155(1986)193-199.

392. P.-E. JANSSON, H. LENNHOLM, B. LINDBERG, U. LINDQUIST, AND S.B. SVENSON, *Carbohydr. Res.*, 161(1987)273-279.

393. P.-E. JANSSON, L. KENNE, AND T. WEHLER, *Carbohydr. Res.*, 166(1987)271-282.

394. D.G. DAVIS AND A. BAX, *J. Am. Chem. Soc.*, 107(1985)2820-2821.

395. A. BAX, A. ASZALOS, Z. DINYA, AND K. SUDO, *J. Am. Chem. Soc.*, 108(1986)8056-8063.

396. L. LERNER AND A. BAX, *Carbohydr. Res.*, 166(1987)35-46.

397. R.A. BYRD, W. EGAN, M.F. SUMMERS, AND A. BAX, *Carbohydr. Res.*, 166(1987)47-58.

398. H.E. CONRAD, *Methods Carbohydr. Chem.*, 6(1972)361-364.

399. R. KUHN, H. TRISCHMANN, AND I. LOW, *Angew. Chem.*, 67(1955)32.

400. A. AMEMURA, T. HARADA, M. ABE, AND S. HIGASHI, *Carbohydr. Res.*, 115(1983)165-174.

401. Y.-M. CHOY, F. FEHMEL, N. FRANK, AND S. STIRM, *J. Virol.*, 16(1975)581-590.

402. L.M. BEYNON AND G.G.S. DUTTON, Abstracts of the XIIIth International Carbohydrate Symposium, Ithaca, New York, 1986, p. 141.

403. A.I. VOGEL, *Textbook of Quantitative Inorganic Analysis*, 4th ed., Longmans, London, 1979, p. 756.

404. K. JANN, B. JANN, F. ØRSKOV, I. ØRSKOV, AND O. WESTPHAL, *Biochem. Z.*, 342(1965)1-22.

405. D. HUNGERER, K. JANN, B. JANN, F. ØRSKOV, AND I. ØRSKOV, *Eur. J. Biochem.*, 2(1967)115-126.

406. K. JANN, B. JANN, K.F. SCHNEIDER, F. ØRSKOV, AND I. ØRSKOV, *Eur. J. Biochem.*, 5(1968)456-465.

407. L.-B. NHAN, B. JANN, AND K. JANN, *Eur. J. Biochem.*, 21(1971)226-234.

408. C.S. HUDSON, *J. Am. Chem. Soc.*, 31(1909)66-86.

409. P.A.J. GORIN AND F.J.T. SPENCER, *Can. J. Chem.*, 42(1964)1230-1232.

410. P.A.J. GORIN AND T. ISHIKAWA, *Can. J. Chem.*, 45(1967)521-532.

411. V.L. DI NINNO, R.A. BELL, AND E.L. McCANDLESS, *Biomed. Mass Spectrom.*, 5(1978) 671-673.

412. W.E. TREVELYAN, D.P. PROCTER, AND J.S. HARRISON, *Nature*, 166(1950)444-445.

413. R.D. GUTHRIE, *Methods Carbohydr. Chem.*, 1(1962)435-441.

414. G. ANNISON, G.G.S. DUTTON, AND E. ALTMAN, *Carbohydr. Res.*, 168(1987)89-102.

415. V.C. HASCALL, R.L. RIOLO, J. HAYWARD, JR., AND C. REYNOLDS, *J. Biol. Chem.*, 247(1972)4521-4528.

416. H. PAROLIS AND L.A.S. PAROLIS, Unpublished results.

417. K. BOCH AND H. THØGERSEN, *Ann. Reports NMR Spectros.*, 13(1982)1-57.

418. D.M. LEEK, M.Sc. Thesis, University of British Columbia, 1982.

419. I. ØRSKOV AND F. ØRSKOV, Personal communication to H. Parolis.

420. C.-C. CHENG, S.-L. WONG, AND Y.-M. CHOY, *Carbohydr. Res.*, 73(1979)169-174.

421. N. RAVENSCROFT, Personal communication to H. Parolis.