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STRUCTURAL STUDIES ON KLEBSIELLA
CAPSULAR POLYSACCHARIDES

by

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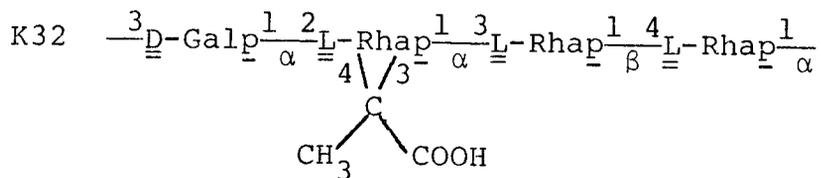
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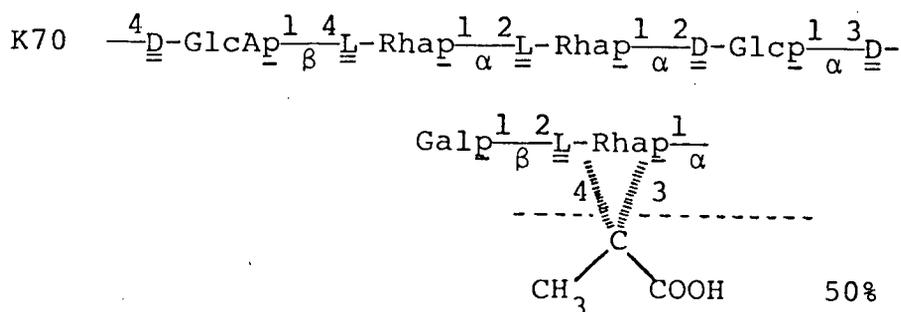
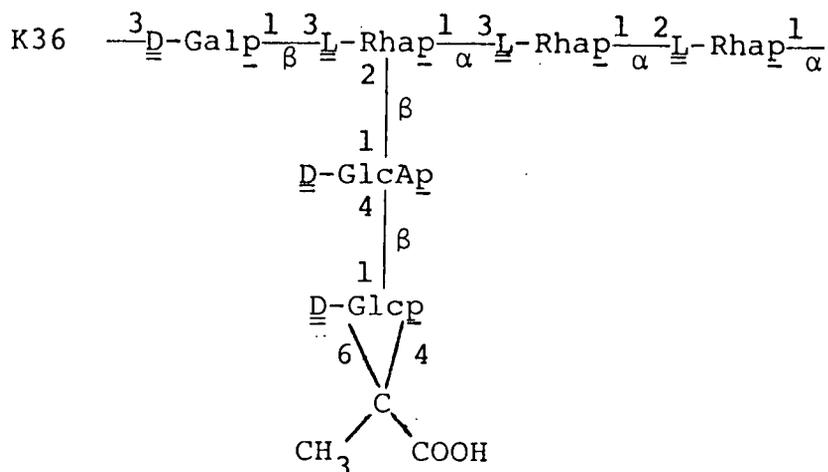
ABSTRACT

Eighty-one serologically distinct strains of Klebsiella bacteria are known. The capsular polysaccharides from these bacteria are their antigenic determinants and in order to help understand the chemical basis of serological differentiation, the detailed chemical structures of these polysaccharides are being determined.

The capsular polysaccharides isolated from Klebsiella serotypes K32, K36 and K70 are presented here and have been established using many different chemical techniques. Methylation, partial hydrolysis, periodate oxidation and β -elimination procedures have yielded analysable subunits of the polysaccharides. Extensive use has been made of n.m.r. spectroscopy (^1H and ^{13}C), mass spectrometry, gas-liquid chromatography and gel filtration in the isolation and identification of the products obtained from the various degradative techniques.

The repeating unit structures of K32, K36 and K70 are shown to be as follows:





Some features of special interest in these structures include: the extreme acid lability of the pyruvate acetal when linking hydroxyls on C₃ and C₄ of a 2-linked L-rhamnose residue (K32, K70); the existence of a β-L-rhamnose unit in the structure of K32; and the presence of the pyruvate acetal on only 50% of the linear, six sugar, repeating units of K70. It is also interesting to note that while K70 and K36 have almost the same quantitative composition the chemical structures are markedly different.

An efficient means of isolating large quantities of single repeating units of the Klebsiella polysaccharides using glycanase enzymes borne and utilised by specific

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I. INTRODUCTION

II. TECHNIQUES AND METHODS USED IN THE
STRUCTURAL ANALYSES OF POLYSACCHARIDES

I. Introduction: Bacterial Exopolysaccharides.

The cells of bacteria belonging to the family Enterobacteriaceae, which includes the genus Klebsiella, have lipopolysaccharide (L.P.S.) bound and external to the cell wall. The fact that reagents such as phenol or ethylenediaminetetraacetic acid (E.D.T.A.) can extract L.P.S. suggests the linkage between L.P.S. and the cell wall may be ionic rather than covalent. The lipopolysaccharide layer may be enveloped by a further polysaccharide layer called the extracellular capsule. Most of the Klebsiella strains possess heavy capsules (K^+) but some non-encapsulated variants (K^-) can be found. In cultures of capsulate cells 'slime' is often obtained due to the gradual release of polysaccharide from the capsule, and since Dudman and Wilkinson¹ have shown that capsular and slime polysaccharides are identical in chemical composition, it may be assumed that these polymers are physically identical. The name exopolysaccharide provides a general term for all these forms of bacterial polysaccharides found outside the cell wall. Figure I.1 shows a diagrammatic representation of a bacterial cell with capsule and slime.

Various hypothetical functions have been suggested for bacterial exopolysaccharides. Most of these have implied a protective function, such as against desiccation, against phagocytosis or against bacteriophage (virus organisms which attack bacteria). While there may certainly be some possibility of the first two roles being correct, the occur-

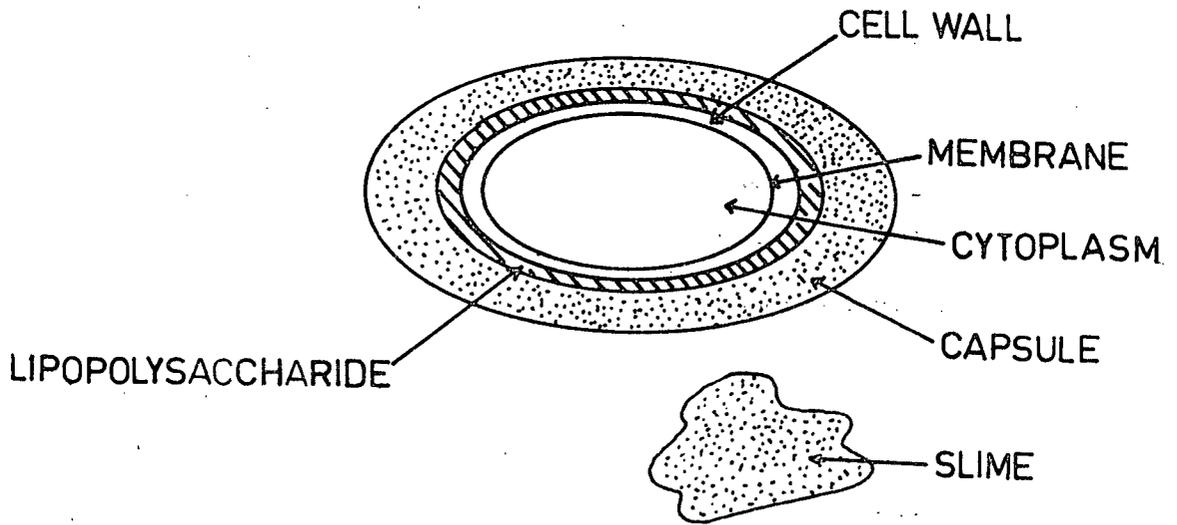


Figure I.1 Diagrammatic representation of a bacterial cell with lipopolysaccharide, capsule and slime.

rence of phages capable of inducing capsule destroying enzymes (see Appendix I) suggests that capsules frequently present no real barrier to phage infection.

Exopolysaccharides may act as determinants of specificity. This is consistent with other evidence which shows that the exposed components of a structure act as the antigenic determinants of the entire unit. Good examples of this are: the blood group antigens², whose end group monosaccharides are the antigenic determinants; and the allergenic constituents of pollen, which are located in the outer cell wall of the pollen grain³. On the basis of immunochemical tests the genus Klebsiella has been divided into approximately 80

serologically different strains^{4,5}. These strains have been designated K-types as their serological specificity is derived from the capsular (German; Kapsel) polysaccharide surrounding the bacterial cell.

Polysaccharides are generally considered to be weakly or non-antigenic, i.e. they induce only a weak response by a host's immune system--the system that produces antibodies to combat the invading antigen. This is probably because polysaccharides, even though they have high molecular weights similar to those of strongly antigenic proteins, do not have a definite three dimensional (tertiary) structure. Nevertheless, the repeating nature of the capsular polysaccharide is somehow "impressive" and does impart some antigenicity. With an isolated repeating unit from a Klebsiella polysaccharide attached to a peptide "carrier", it has been possible to isolate antibodies produced by rabbits against this carbohydrate unit⁶. When these antibodies were then tested against the intact native polysaccharide, although the antibodies did show some interaction, not all activity associated with the native polysaccharide-native antibodies were present. It might therefore be assumed that there is some contribution from the size of the polysaccharide and perhaps from the three dimensional character of the polymer.

Antigenic polysaccharides contain specific oligosaccharide units which combine with the antibody in the immunological reaction and are thus called immunodominant sites, antigenic

determinants, determinants of immunological specificity, or haptens. The structural composition of the determinant of immunological specificity can be determined by hapten inhibition^{7,8} or complement fixation inhibition studies⁹ using oligosaccharides of known structure. For the latter method oligosaccharides of known structure are very important and the need for the further development of synthetic techniques to produce these oligosaccharides remains today.

Knowledge of the molecular basis of antigen-antibody interaction and of the frequent cross-reactions which occur between microorganisms belonging to widely disparate families is not only of practical interest, but also has a broader theoretical significance; it is capable of revealing strict relations between the chemical constitution of certain microbial antigens and their immunological specificities. With this objective in mind the determination of the detailed structures of 80 Klebsiella K-types is presently being undertaken. The determination of the structures of Klebsiella K-types 32, 36 and 70 is the principle contribution of this thesis.

Nimmich^{4,5} has reported the qualitative compositions of the K-types 1 to 80, and his work has shown that most of the polysaccharides contain D-glucuronic acid or D-galacturonic acid in combination with D-mannose, D-glucose, D-galactose, and to a lesser extent, with L-rhamnose and L-fucose. Pyruvic acid, present as l-carboxyethylidene acetals, is known^{4,5} to

be present in approximately half of the polysaccharides. All Klebsiella polysaccharides contain some acidic groups, whether they be uronic acids, pyruvate acetals or keto acids, and it is the overall negative charge of these groups which is thought to impart virulence to the encapsulated bacteria.

The structures of approximately 35 Klebsiella K-types have been determined to date (see Appendix II) and a wide variety of structural features are now known to exist in these polysaccharides. The number of sugars in the repeating units ranges between three (K5) and six (K18, K28, K36, K52, K70, K81). Uronic acid residues, when present, may exist in the main chain itself (K5, K70, etc.), as branch points (K11, K21, etc.), as terminal units in a side chain (K2, K9, etc.) or as non-terminal units in a side chain. In one case (K38) two single unit side chains are linked to the same 'main chain' residue, while in others two and three unit side chains exist, and in still others, the structures are linear. To date no overall pattern is emerging from the polysaccharide structures. The diversity of structures presented by K-types which have the same qualitative composition justifies their serological differentiation, e.g. compare K-types 18, 36, 55, and 70 which each contain D-glucuronic acid, D-galactose, D-glucose and L-rhamnose. Furthermore, Klebsiella K36 and K70 polysaccharides are shown in this thesis to have completely different repeating unit structures even though they have almost the identical quantitative composition.

The existence of a defined structural unit that repeats throughout the polysaccharide from any one Klebsiella serotype makes these polymers particularly suitable as models for investigation by many different techniques. At present studies are being carried out to examine the crystalline conformations¹⁰ and solution conformations¹¹ of these polymers, the behaviour of the polysaccharides as polyelectrolytes¹², and to examine their nuclear magnetic resonance characteristics¹³.

A recent development in the structural elucidation of the Klebsiella polysaccharides has been the isolation¹⁴ and utilization¹⁵ of bacteriophage which have the ability to depolymerise the capsular polysaccharide with tremendous precision. Glycanase enzymes (enzymes which cleave glycosidic bonds) are produced by the bacteriophage and it is these enzymes which degrade the polymer into repeating units and multiples thereof. Preliminary work using the bacteriophage degradation technique is currently being pursued in this laboratory (see Appendix I, page 179). The isolation of large quantities of specific "repeat" units made possible by this method will make available excellent model oligosaccharides for ¹³C and ¹H nuclear magnetic resonance, mass spectrometric, and other studies.

II. Techniques and Methods Used in the Structural Analyses of Polysaccharides.

In order to fully characterise a polysaccharide consisting of repeating units, a structural study should determine:

- (a) The nature and number of sugar residues and their relative proportions.
- (b) The positions of linkage of the sugar residues.
- (c) The sequence of the component sugars.
- (d) The anomeric configurations of the sugars present.
- (e) If possible, the molecular weight of the polysaccharide.

Most of the techniques used to achieve these requirements have been available to the carbohydrate chemist for quite some time and many can be considered as standard. It is not my objective to go into these procedures in detail as this information is readily available in general texts, but to discuss features of special interest observed during the course of this work and to update the applications of those techniques for which significant improvements have recently been made.

While the following discussion will deal with polysaccharides with repeating units, much of the methodology described can be applied equally well to the investigation of polysaccharides with irregular structures.

II.1 Isolation and purification.

Strains of Klebsiella bacteria of known K type were obtained as agar stab cultures from Dr. I. Ørskov in Copenhagen. The bacteria were plated out on agar discs several times before use, with only strong growing, slime producing colonies being picked. Inoculation of a sucrose-yeast extract medium for 8h and subsequent incubation for three or four days on

trays of sucrose-yeast extract-agar produced a lawn of bacteria which was harvested by simply scraping the cells and slime from the agar surface.

The bacterial cells were separated from the slime solution by centrifugation and the cell free supernatant was then precipitated into ethanol. This precipitate was dissolved in water and then reprecipitated using 10% hexadecyltrimethyl ammonium bromide (CETAVLON). The precipitate, formed by the reaction of acidic groups in the polysaccharide with the CETAVLON, was then dissolved in a minimum amount of 4 M sodium chloride and then precipitated into ethanol or acetone. After this precipitate had been dissolved in water the solution was dialysed for two days against running tap water and then lyophilised.

It should be noted that following the addition of the 4 M sodium chloride to dissolve the CETAVLON-polysaccharide precipitate it is not always possible to dialyse directly this solution against tap water as might initially be thought. In some cases the precipitate will reform during the dialysis as the sodium chloride is removed. The precipitation into ethanol or acetone before dialysis prevents this reprecipitation.

The Klebsiella polysaccharides grown and purified in this manner have been found to be sufficiently pure for structural studies to be carried out. Any major impurities, such as contaminating neutral polysaccharides, would become noticeable when techniques such as nuclear magnetic resonance spectroscopy are performed as these methods rely heavily on the homogeneity of the polysaccharide structure.

II.2 Use of proton magnetic resonance (^1H n.m.r.) and ^{13}C carbon magnetic resonance (^{13}C n.m.r.) spectroscopy in structural studies.

Strong evidence for the existence of regular repeating units in Klebsiella polysaccharides comes from the fact that nuclear magnetic resonance spectra with good resolution can be obtained on solutions of the intact native polysaccharide. If a polysaccharide, with a molecular weight of approx. 1×10^6 , did not have a regular repeating unit, meaningful results from n.m.r. would be impossible to obtain. A repeating unit will give the polysaccharide an effective molecular weight between 600-1500 (depending on the number of sugar residues in the repeating unit) as far as n.m.r. studies are concerned.

II.2.1 Proton magnetic resonance.

^1H n.m.r. has been used extensively during this work and has proven to be an extremely valuable non-destructive technique^{16,17}. Several problems are encountered in performing a ^1H n.m.r. experiment on a polysaccharide. Although dimethyl sulphoxide- d_6 may be used as a solvent, deuterium oxide (D_2O) is more commonly used. The numerous hydroxyl groups present in a polysaccharide make it necessary to exchange the sample with deuterium oxide several times to convert most of the OH groups to OD groups and hence minimise interference from the temperature sensitive HOD signal. The exchange with deuterium oxide is usually performed by dissolving the polysaccharide

(approximately 15 mg) in a small volume of 99.9% deuterium oxide and then freeze drying. Following this the sample is warmed under high vacuum for several hours before the entire process is repeated. After three or four such treatments the sample is then dissolved in 100% deuterium oxide, but even after this preparation the OH, OD exchange is not entirely complete and some residual HOD signal is observed in the ^1H n.m.r. spectrum. As the HOD peak occurs in a shift region of major importance, it is necessary to move this peak. It may be shifted upfield by heating or downfield by cooling or the addition of trifluoroacetic acid. The HOD peak may also be eliminated by means of Fourier transform (F.T.) ^1H n.m.r. spectroscopy¹⁸. Use is made of the longer spin lattice relaxation time (T_1) of the HOD signal as compared to other sugar protons but this technique requires several machine hours to perform.

A second problem that is often encountered in preparing a polysaccharide sample for ^1H n.m.r. is that due to the sample viscosity. Normally a 1-2% solution of the polysaccharide can be obtained but these solutions are often so viscous that there is a loss of homogeneity, and hence resolution, when the ^1H n.m.r. experiment is performed. This problem is somewhat alleviated when the sample is heated to approximately 95° to move the HOD resonance upfield but is not altogether eliminated. It is possible to use a less concentrated sample but a 1-2% solution is at the lower limit for continuous wave (c.w.).

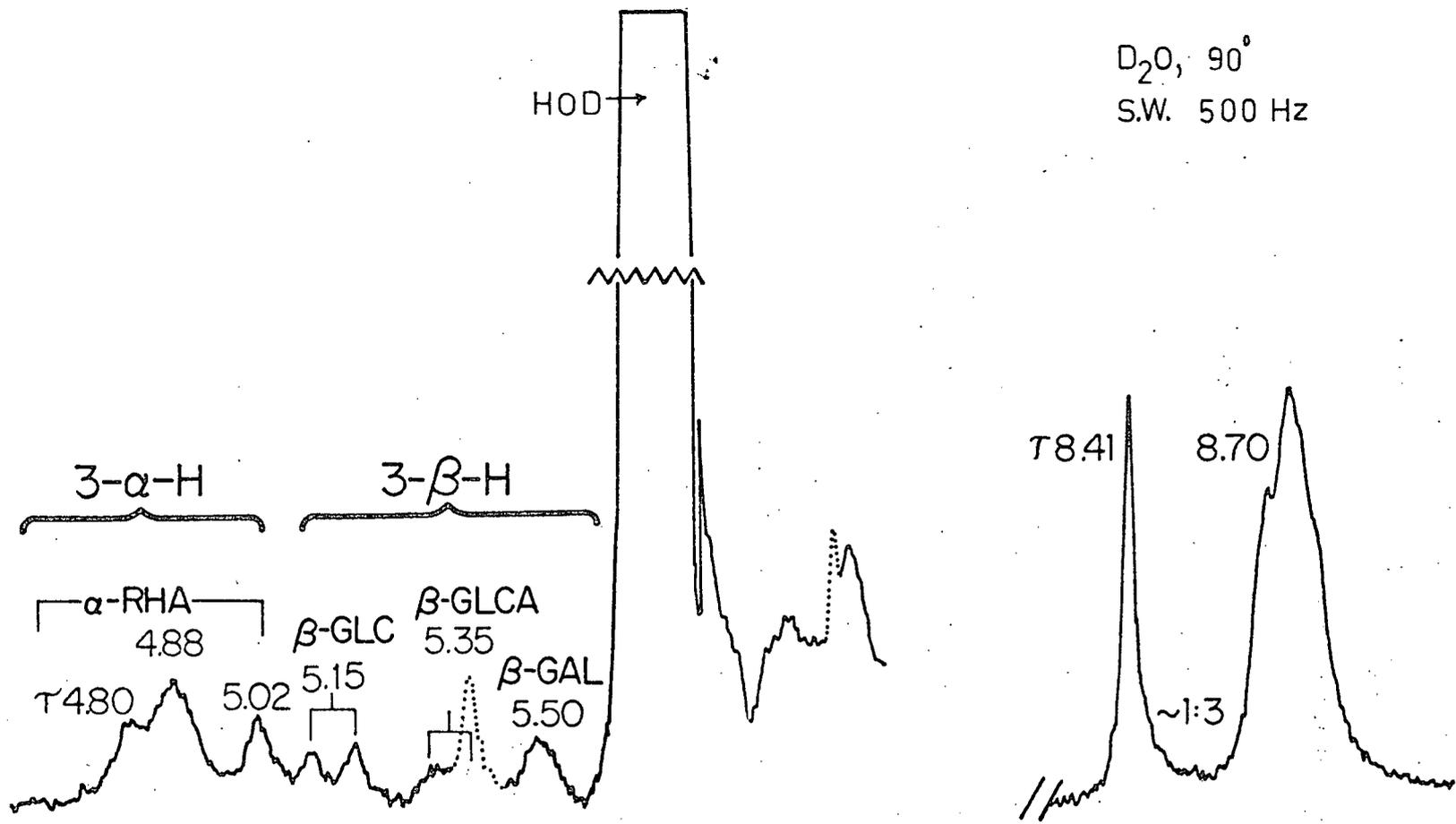


Figure II. 1 1H n.m.r. spectrum of Klebsiella K36 capsular polysaccharide.

^1H n.m.r. and F.T. ^1H n.m.r. is often necessary with more dilute solutions. A technique that is finding more widespread use is to perform an extremely mild acid hydrolysis on the native polysaccharide and to then do the ^1H n.m.r. experiment on this partially depolymerised, and far less viscous, material. Often, hydrolysis conditions can be selected so as that labile groups, such as pyruvate acetals or acetate groups, are not removed but this may not always be the case.

Information obtainable by ^1H n.m.r. includes data on: the presence or absence of pyruvate acetals, 6-deoxy sugars and acetates; the number of sugars per repeating unit; the presence of α - $\underline{\text{D}}$ or β - $\underline{\text{D}}$ anomeric signals for hexoses and α - $\underline{\text{L}}$ or β - $\underline{\text{L}}$ anomeric signals for 6-deoxy hexoses, e.g. $\underline{\text{L}}$ -rhamnose.

The ^1H n.m.r. spectrum of Klebsiella K36 (see Figure II.1, page 12) is typical of those usually obtained. The spectrum shows the presence of a pyruvate acetal (singlet at $\tau 8.41$) and moreover, shows it to be in a 1:3 ratio with respect to the broad peak at $\tau 8.70$ due to the methyl groups of three different $\underline{\text{L}}$ -rhamnose units (three doublets each with $J_{5,6}$ 6Hz). In the anomeric region ($\tau 4.5 - \tau 6.0$) three anomeric signals appear downfield of $\tau 5.0$ and can therefore be assigned tentatively to α -linkages, while three more signals appear upfield of $\tau 5.0$ and can be assigned to β -linkages. The division at $\tau 5.0$ is arbitrary but has been found to be valid in most cases. The presence of six anomeric proton signals overall indicates K36 consists of a hexasaccharide repeat unit. Spin-spin coupling

between the protons at C_1 and C_2 of sugars provides valuable information. For β -D hexoses (e.g. β -D-glucose, β -D-galactose) $J_{1,2}$ is 7-9 Hz while for α -D hexoses $J_{1,2}$ is 2-3 Hz. For the 6-deoxy-hexose L-rhamnose (6-deoxy-L-mannose) the α -L-anomeric proton has $J_{1,2} \approx 2$ Hz while the β -L anomeric proton has $J_{1,2} \approx 1$ Hz.

While tetramethylsilane is a good internal standard for ^1H n.m.r. in organic solvents, it is insoluble in D_2O and therefore cannot be used for polysaccharide samples. Sodium 2,2-dimethyl-2-silapentane-5-sulphonate (D.S.S.) or sodium 3-trimethylsilyl propionate-2,2,3,3- d_4 (T.S.P.) are possible alternatives but are difficult to remove from the sample if the polysaccharide is to be recovered. Acetone (methyl groups giving a sharp singlet at $\tau 7.77$ relative to internal D.S.S. in D_2O) has proven to be a good internal standard and the shift of the singlet is unaffected by variations in temperature.

II.2.2 ^{13}C Carbon magnetic resonance (^{13}C n.m.r.).

The first n.m.r. observations of ^{13}C nuclei were reported¹⁹ as early as 1957 but the low natural abundance of the ^{13}C nuclei (1.1%) made it necessary to work with highly soluble, low molecular weight materials. The first great breakthrough in experimental ^{13}C n.m.r. was the discovery of wide band proton decoupling in 1966²⁰. With the further development of instrumental techniques, especially Fourier transform n.m.r., ^{13}C n.m.r. has now become not only practical but also nearly comparable with ^1H n.m.r. in terms of experimental ease and

quality of results for most organic compounds. The first ^{13}C n.m.r. experiments with carbohydrates²¹ appeared in 1969 and since that time the technique has been applied extensively to monosaccharides²²⁻²⁴ and oligosaccharides²⁵⁻³¹.

The potential of this technique in determining features in polysaccharide structures has only recently been utilised. An investigation of amylose, a simple homopolymer, has been reported²⁴ and also a study on heparin, a heteropolymer with two different component sugars, has been completed^{32,33}. The ^{13}C n.m.r. spectrum of a linear glucan with (1 \rightarrow 4)- α -D and (1 \rightarrow 6)- α -D linkages showed that both the sequence and composition of the single monosaccharides could be determined^{28,34}.

Similar investigations on dextrans of known structure showed diagnostic features in the ^{13}C n.m.r. spectra for α -D-(1 \rightarrow 2)-, α -D-(1 \rightarrow 3)-, or α -D-(1 \rightarrow 4)-linkages³⁵, and the assignment of the ^{13}C n.m.r. spectrum of a mannan containing alternate β -(1 \rightarrow 3)- and β -(1 \rightarrow 4)-linked D-mannose residues was achieved with the aid of specific deuteration³⁶. Most polysaccharides give well-defined ^{13}C n.m.r. spectra but it has proven difficult to completely assign the ^{13}C resonances in all but the simplest of polymers. The objective of the technique is to postulate chemical structure on the basis of chemical shift values but until the existing methods for the assignment of individual resonances are improved, or new solutions to this problem developed, then this objective will be difficult to achieve for complex polysaccharides.

During the course of this work oligosaccharides obtained by degradative procedures on the Klebsiella polysaccharides have been examined by ^{13}C n.m.r. wherever possible, but while these experiments have provided valuable information on the isolated oligosaccharides it has proven a difficult task to correlate these spectra and the spectra of the intact polysaccharides.

The Klebsiella polysaccharides have been shown to be well suited to investigation by ^1H n.m.r. (see section II.2.1) and during the course of this work it has been shown they are equally well suited to study by ^{13}C n.m.r. Although the Klebsiella polysaccharides may contain as many as four or five different sugars the precise repeating unit structure of these materials makes it possible to examine these relatively complex heteropolymers by ^{13}C n.m.r.

The major difficulty in preparing a polysaccharide sample for ^{13}C n.m.r. is the low solubility of the material and the viscosity of the solution. At best approximately 0.2 M solutions may be obtained and often this figure is closer to 0.1 M. This low concentration and usually a repeating unit of high molecular weight therefore necessitate a large number of transients (>100,000) being completed on a sample to obtain a reasonable signal to noise ratio. As this ratio in a ^{13}C n.m.r. experiment improves as the square root of the number of total transients, there is a practical lower limit to the concentration of solution that can be used.

A normal ^{13}C n.m.r. experiment involves pulsing the sample with a short radiofrequency pulse which simultaneously excites all the ^{13}C nuclei. The subsequent precession of the ^{13}C nuclei is called a free induction decay (F.I.D.) and corresponds to the simultaneous "re-radiation" of all the energy absorbed by the ^{13}C nuclei. It takes a finite time for any one nucleus to return ("relax") to its' normal energy state. Nuclei that do not fully relax before being pulsed again may become saturated and hence will not appear in the ^{13}C spectrum when a Fourier transformation is performed on the accumulated F.I.D. signals. For most small organic molecules a pulse delay (time delay between successive pulses) of 2-5 sec is sufficient to allow most ^{13}C nuclei to relax but some nuclei, especially those in a carbonyl functionality, may require much longer delays.

Experiments have shown that the spin-lattice relaxation rates for the Klebsiella polysaccharides are very fast, e.g. approximately $20,000 \text{ msec}^{-1}$ for the ring carbons of K36 in a 1% solution at 25°C . This means that no delay is required between successive pulses and moreover, the acquisition time (time for which each F.I.D. is recorded) for these samples can be as short as 0.2 sec. The normal acquisition time for most organic molecules is about 1 sec. It should be realised however, that while these experimental conditions allow greater than five times as many transients to be completed in the same time as when "usual" conditions are employed, if some carbons

do have long relaxation rates then they will only give a partial signal or no signal in the final spectrum. This in fact occurs and a spectrum of a polysaccharide using an acquisition time of 0.2 sec and no pulse delay will not show any resonances due to carbons present as carbonyls, e.g. as in uronic acids and pyruvate acetals.

One further characteristic feature of ^{13}C n.m.r. spectra is worth mentioning. The nuclear Overhauser effect (N.O.E.) is always present in proton decoupled ^{13}C n.m.r. experiments. N.O.E. is a by-product of the proton decoupling and is derived from the proton induced relaxation of ^{13}C nuclei. In brief, the decoupled protons intensify the ^{13}C signal of the carbon to which they are attached. If the dipole-dipole relaxation mechanism were dominant for all ^{13}C nuclei in a sample, all signals would have the same integrated intensity if experimental conditions were chosen such that all nuclei were fully relaxing between pulses. In actual fact, the theoretical N.O.E. enhancement of 2.988 is not usually observed for all ^{13}C nuclei due to appreciable contributions to the ^{13}C spin-lattice relaxation from mechanisms other than the dipole-dipole mechanism. Hence the integrated intensities for ^{13}C nuclei are usually not equal and therefore widely different peak heights in a ^{13}C n.m.r. spectrum are normally observed.

The ^{13}C n.m.r. spectrum of Klebsiella K36, obtained at 90.5 MHz, is shown in Figure II.2, page 19 and is a spectrum which illustrates well the information ^{13}C n.m.r. can provide

Field; 90.5 MHz
S.W.; 20 KHz
N.T.; 50,000
A.T.; 0.2 sec
P.W.; 15 μ sec
P.D.; 0 sec

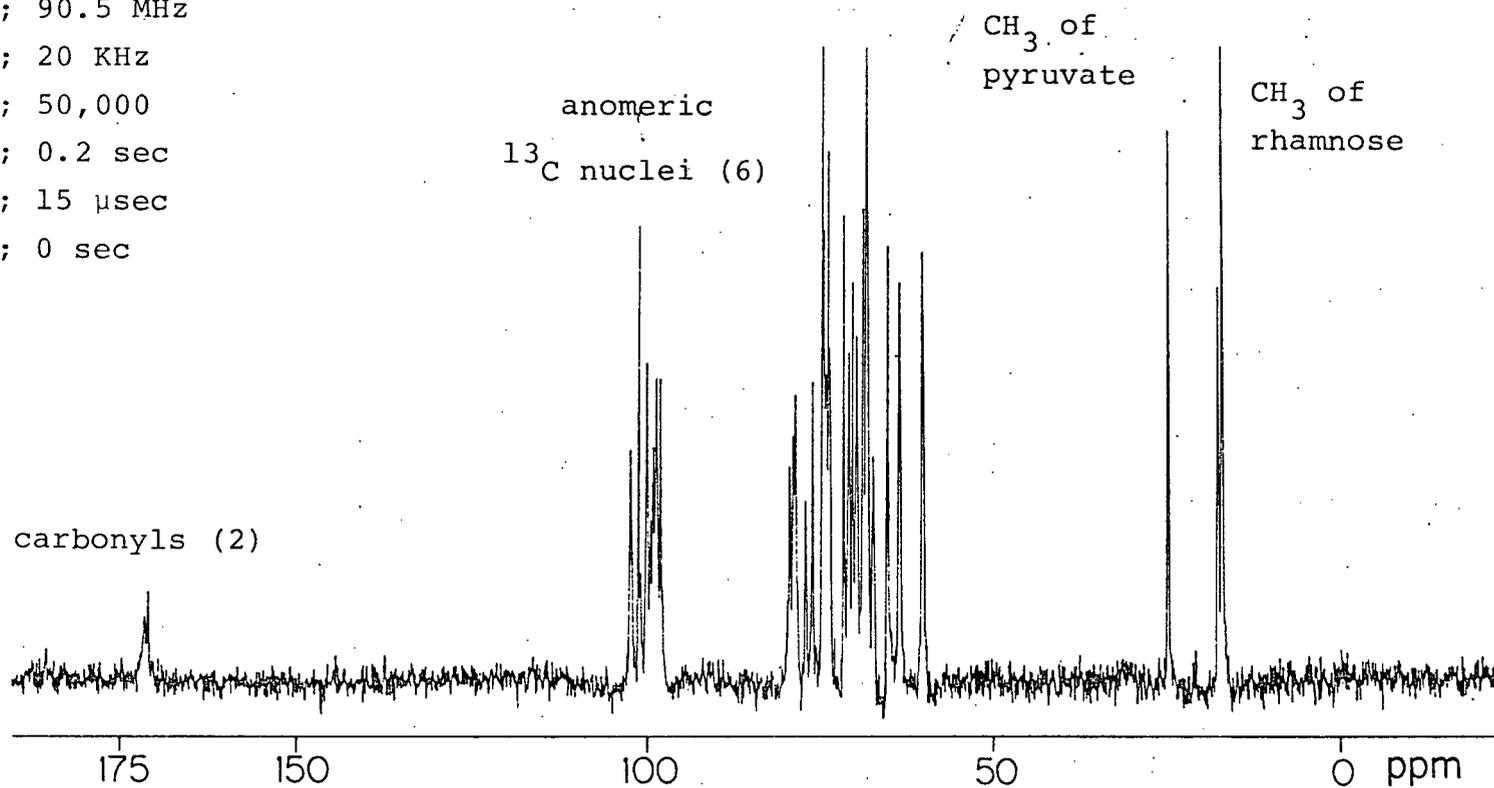


Figure II.2 ^{13}C n.m.r. spectrum of Klebsiella K36 capsular polysaccharide.

in a structural investigation. Overall 29 different carbon signals can be distinguished (theoretical is 39). At approximately 175 ppm downfield from T.M.S. two signals attributable to carbonyl ^{13}C nuclei corresponding to these nuclei in the uronic acid and pyruvate acetal are observed. At approximately 100 ppm six signals arising from anomeric (C_1) carbons are apparent and this is consistent with the structure of K36 consisting of a hexasaccharide repeating unit. In the region usually associated with ^{13}C nuclei bearing a primary alcohol (60-62 ppm) only one signal is observed. This signal probably arises from the C_6 of the D-galactose moiety in K36. In the highfield region (17-25 ppm) three signals attributable to methyl ^{13}C nuclei appear. The signal at 25 ppm is from the pyruvate acetal while the two signals at 17 ppm arise from L-rhamnose residues (6-deoxy sugars). It is probably valid to assume that the N.O.E. on these signals from the L-rhamnose moieties is equivalent and hence it could be assumed that three L-rhamnose units exist in the repeating unit. This is consistent with the structure of K36 derived by chemical methods.

It should be realised that when proton decoupled spectra are run there is a loss of all coupling information. This type of information ($J_{1,2}$ coupling constants, etc.) has proven to be very useful in ^1H n.m.r. studies. Partially coupled spectra can be obtained³⁷ in ^{13}C n.m.r., but the following two factors make this a difficult task with polysaccharides.

(i) A single ^{13}C peak will be split into two peaks of half the integrated intensity of the single peak. Hence the smaller signals are less distinguishable from baseline noise.

(ii) With single bond length C-H coupling N.O.E. effects are not observed and hence the approximate three fold signal enhancement is also lost.

Partially coupled spectra on oligosaccharides, which are more soluble and usually contain fewer "repeating unit" carbons than a polysaccharide, have been more successful and have been carried out wherever possible in this work.

A "state of the art" summary of ^{13}C n.m.r. of polysaccharides would have to conclude that while the technique does provide some very useful information that is somewhat complementary to the data obtained by ^1H n.m.r., insufficient knowledge of the influence of conformational and steric effects on ^{13}C chemical shifts does not allow unambiguous assignment of many spectral signals. More model studies using oligosaccharides of known structure are necessary to understand the finer features of the technique.

II.3 Total hydrolysis and methanolysis.

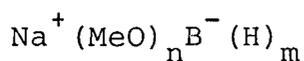
The hydrolysis of a polysaccharide and the subsequent analysis of the hydrolysis products is often performed qualitatively. However, the total hydrolysis of polysaccharides, particularly those with resistant glycosidic linkages, is a more difficult operation.

Hydrochloric, sulphuric and trifluoroacetic acids are commonly used in hydrolysis but the last named has the advantage of being easily removed under diminished pressure following the hydrolysis. 2 M Trifluoroacetic acid is usually sufficient to hydrolyse completely a neutral polysaccharide into its monomeric sugar units after 8 hours at 95° and under these conditions the degradation of these monomer units is normally considered insignificant.

Polysaccharides containing uronic acid moieties are very difficult to hydrolyse completely due to the resistance of the uronosyl bond. Normally, total hydrolysis of these polysaccharides is performed on the uronic acid reduced polymer (see Section II.5), but the reduction of uronic acids in aqueous solution is not a simple task.

A method developed in this laboratory³⁸ and used during this study, involves the use of methanolysis. The acidic polysaccharide is first treated with 3% methanolic hydrogen chloride under reflux for 16 hours. These conditions are sufficient to cleave most glycosidic bonds but not all uronosyl linkages. During this treatment the methyl ester of the uronic acid is formed and this can be reduced using sodium borohydride in anhydrous methanol. It should be noted that sodium borohydride will reduce esters in aqueous solution^{39,40} but does not proceed in 100% yield as saponification of the ester is a competing reaction. Sodium borohydride in anhydrous methanol will reduce methyl esters quantitatively and is a clean, simple reaction. When sodium borohydride is

dissolved in methanol there is a reasonably rapid reaction between the two. As a result a series of reducing species is produced which are stronger reducing agents than NaBH_4 itself in aqueous solutions. These species may be given the general formula shown below



where $n + m = 4$, and it is most probable that reduction of the ester to the alcohol is achieved by these sodium borohydride derivatives. Following the reduction a hydrolysis step is necessary to cleave those bonds that were uronosyl linkages and also to cleave the methyl glycosides which would have been formed during the treatment with methanolic hydrogen chloride. The polysaccharide can then be considered fully hydrolysed and quantitative analysis of the sugar components by g.l.c.-m.s. as their alditol acetates may be carried out. A uronic acid such as D-glucuronic acid will appear as a unit of D-glucose in the final hydrolysate and will be indistinguishable from other D-glucose units. However, if the reduction in anhydrous methanol is done using sodium borodeuteride, then only the D-glucose from D-glucuronic acid will be labelled at C_6 and can be determined by mass spectroscopy.

II.4 Methylation and methylation analysis.

Methylation analysis of polysaccharides is probably still the most widely used "tool" in the field today. The data obtained from such an analysis include information on the number of sugar residues, the type of sugar residues, the linkages between sugars and whether branching occurs in the polysaccharide. A methylation analysis is relatively straightforward and can be done on milligram quantities of material.

The analysis relies upon the etherification of every free hydroxyl group in the polysaccharide and this can be achieved by using several methods.

(1) Hakomori methylation^{41,42} - uses methyl sulphanyl anion in dimethyl sulphoxide as the base followed by treatment with methyl iodide. The technique is by far the best and one treatment is often sufficient for complete methylation.

(2) Kuhn methylation⁴³ - uses silver oxide in N,N-dimethylformamide and methyl iodide.

(3) Purdie methylation⁴⁴ - uses silver oxide in methyl iodide.

The Hakomori methylation, unlike the Kuhn and Purdie procedures, cannot be repeated on a polysaccharide containing a uronic acid because the methyl ester of the uronic acid can react with the methylsulphanyl anion to give a β -elimination (see Section II.8).

During this work all methylations were performed using the Hakomori procedure and if the etherification was not deemed to be complete (as determined by examining the hydroxyl

absorbance at 3600 cm^{-1} in the infra red spectrum) then a Purdie methylation was carried out. In all cases only one Purdie treatment was necessary to achieve total etherification.

Following the complete methylation of a polysaccharide or oligosaccharide the product is fully hydrolysed and the partially methylated monosaccharides produced are identified. For methylated material containing no uronic acid moieties this is relatively straightforward and treatment with 2 M trifluoroacetic acid at 95° for 16 hours, followed by transformation into alditol acetates and gas liquid chromatographic (g.l.c.) analysis, will give good results. Those methylated samples that do contain uronic acids are far more resistant to acid hydrolysis and treatment with 2 M trifluoroacetic acid at 95° for 16 hours will not usually cleave all the uronosyl bonds. Prior reduction of the uronic acid, which is present as its methyl ester, and then subsequent hydrolysis will alleviate this problem.

While lithium aluminum hydride in refluxing tetrahydrofuran will achieve complete reduction of the ester to the alcohol there are often losses associated with the subsequent work up. The aluminum hydroxide produced after the lithium aluminum hydride has been destroyed tends to adsorb materials, especially those with free hydroxyls. On the other hand, reductions using sodium borohydride are relatively clean and the conditions preferred during this work involve using a

Column: HIEFF 1B (6'x1/8"). 3% on Gas Chrom Q (100-120 mesh).
Programme: 165° 8 min, 2° per min to 200°.
Carrier gas: N₂, 20 ml/min.

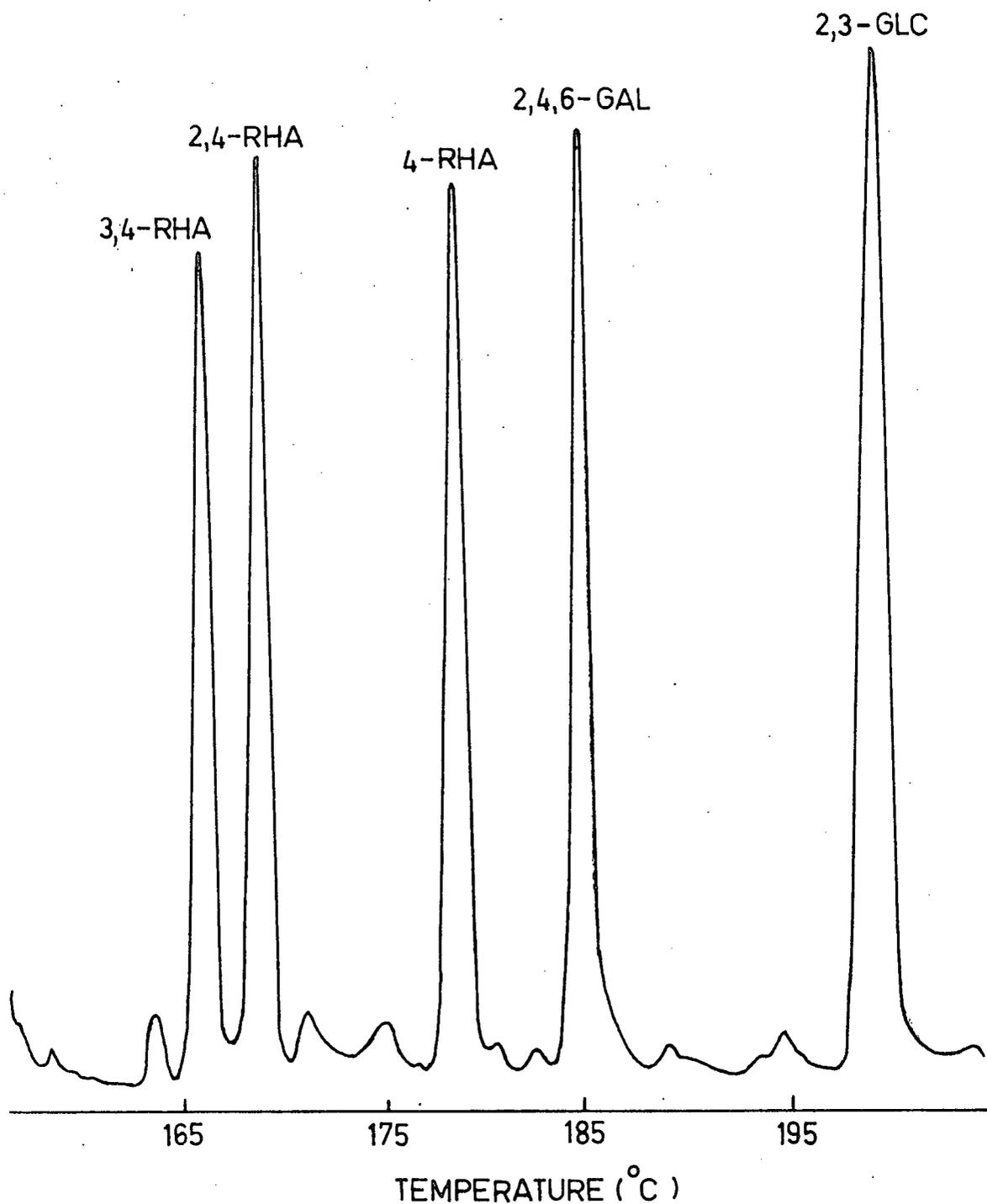


Figure II.3 G.l.c. trace obtained via methylation analysis of Klebsiella K36.

large excess of sodium borohydride in a mixture of tetrahydrofuran and ethanol (1:1). The tetrahydrofuran is necessary to dissolve completely the methylated polysaccharide. Permethylated acidic polysaccharides that have been reduced in this manner are fully hydrolysed using 2 M trifluoroacetic acid at 95° for 16 hours.

Analysis of the partially methylated monosaccharides released after hydrolysis is achieved by g.l.c. and g.l.c.-m.s. of these components as alditol acetates (see Sections II.10.1 and II.10.2.1). An example of such a g.l.c. separation is shown in Figure II.3, page 26. The components obtained are derived from the methylation analysis of Klebsiella K36.

II.5 Carboxyl reduction of acidic polysaccharides.

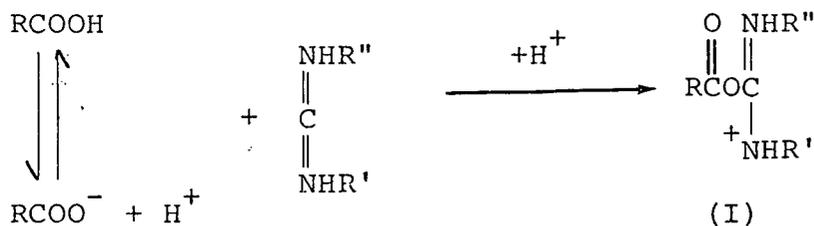
As has been pointed out already (Sections II.3 and II.4), acidic polysaccharides may present problems during structural investigations. (On the other hand, the resistance of uronosyl bonds to hydrolysis is somewhat beneficial during partial hydrolysis studies. See Section II.7.) The reduction of the acidic groups can be achieved with ease following methylation of most polysaccharides, but often reduction of the native material is desirable, e.g. to alter partial hydrolysis cleavage patterns, or to easier facilitate periodate oxidation.

The direct reduction of native polysaccharides with acidic functional groups has been achieved^{45,46} by first propionating (esterifying) the free hydroxyl groups in the poly-

saccharide with propionic anhydride in pyridine and then esterifying the carboxylic acid groups with diazomethane in tetrahydrofuran. The fully esterified material is then reduced with lithium borohydride in refluxing tetrahydrofuran. Complete reduction can be achieved by this method but several treatments may have to be carried out. The method relies upon the lithium borohydride being able to reduce the methyl ester of the carboxylic acid more quickly than the propionate esters of the hydroxyl groups in the polysaccharide.

A more recent technique developed by Conrad and Taylor⁴⁷ is quick and proceeds in good yield. The method involves the use of water soluble carbodiimides and the reaction mechanism is outlined in Figure II.4, page 29.

The initial reaction to form the intermediate (I) proceeds rapidly and consumes acid. In this way the reaction can be followed by titration to pH 4.75 with acid. Formation of (I) is usually complete within 2 hours. The reduction of the intermediate (I) must be performed under acidic conditions as (I) is unstable in basic solutions. The reduction is achieved using sodium borohydride but is an extremely inefficient process as the sodium borohydride is decomposed



Acidic Poly-
saccharide

E.D.C.*

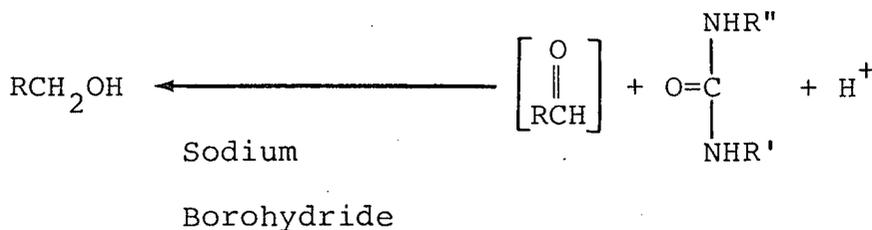
or

C.M.C.**

Carbodiimide Intermediate

Sodium Borohydride

pH 5→7



* E.D.C. ≡ 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

** C.M.C. ≡ 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide
metho-p-toluene sulphonate.

Figure II.4 Reduction of carboxylic acids in aqueous solution using carbodiimide reagents. (From Taylor and Conrad, *Biochemistry*, 11, 1383 (1972)).

rapidly in acidic conditions. Vast excesses of sodium borohydride are used and the reaction conditions are kept acidic by simultaneous titration with 4 M hydrogen chloride.

Although sodium cyanoborohydride (NaBH_3CN) does not usually reduce lactones, an attempt to use this reagent to reduce the intermediate (I) was made as sodium cyanoborohydride is stable to acid conditions. No reduction was observed however, even at pH 1.0.

II.6 Periodate Oxidation.

Since the discovery in 1928 by Malaprade⁴⁸ that sodium metaperiodate could cleave the carbon-carbon bond between vicinal diols in aqueous solution, periodate oxidation has been used extensively by the carbohydrate chemist and it remains a powerful tool for structural investigations today.

The product of periodate oxidation of a polymer is termed a 'polyaldehyde' and is reduced with NaBH_4 to give a 'polyol'. This derived polyol can provide considerable information on the structure of the polymer. Total hydrolysis (2 M T.F.A., 95°, 16 hours) of the polyol will cleave all glycosidic linkages (except where intact uronic acids still exist) and acetal linkages. The hydrolysis products can be examined qualitatively by paper chromatography or quantitatively by g.l.c.⁴⁹ The relative proportions of the surviving sugar residues and the fragments obtained from degraded sugar residues, e.g. glycerol, erythritol, etc., will give information regard-

ing some of the glycosidic linkages present in the original polysaccharide.

Much more useful information can be obtained when partial hydrolysis of the derived polyol is carried out. Smith and co-workers⁵⁰ utilised the greater susceptibility of the true acetal linkages in the derived polyol as compared to remaining intact glycosidic linkages (see Figure II.5, page 32). The acetal linkages are in general approximately 10^3 to 10^4 times more labile than the glycosidic linkages. A wide variety of hydrolysis conditions has been used for this partial hydrolysis. The range extends from 0.005 M acid at 100° for 1 hour⁵¹ to 0.5 M acid at room temperature for 8 hours⁵², but during this work 0.5 M trifluoroacetic acid at room temperature for 16 hours was found to give satisfactory results. The Smith degradation of the polyol yields glycosides of mono, di and oligosaccharides which can be analysed to give very useful structural information, especially data on sugar sequences. An example of the use of periodate oxidation and subsequent Smith degradation is shown (Figure II.5, page 32) using Klebsiella K36 as a model.

It should be noted that when during a Smith degradation the terminating glycoside originates from a 2-linked sugar residue, i.e. from a residue that has been cleaved between carbons three and four, it is necessary to include a further reduction step to reduce the aldehyde group released at C₁ during the hydrolysis (see K36 example given in Figure II.5,

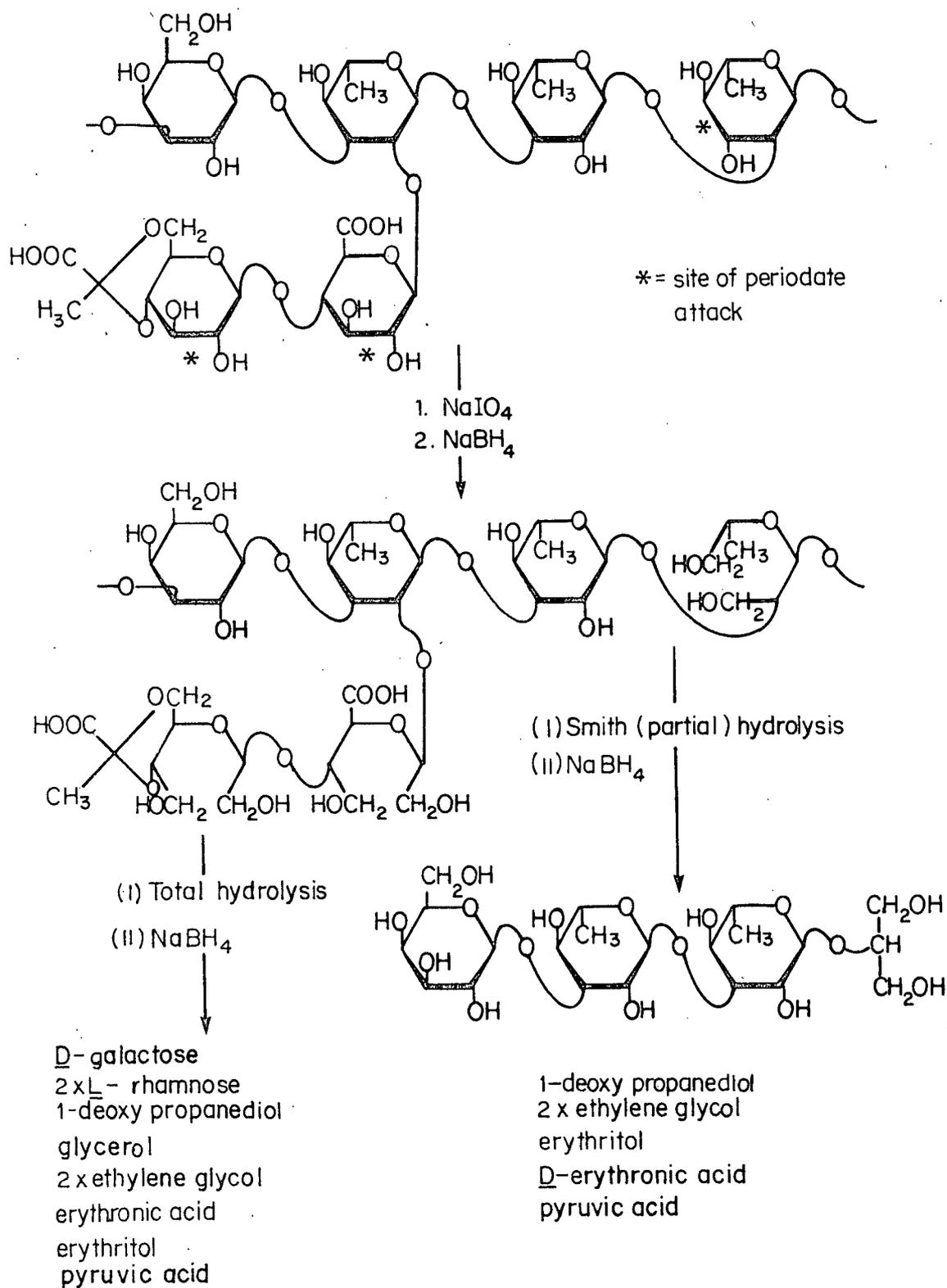


Figure II.5 Periodate degradation of Klebsiella K36.

page 32). This is needed because of the relative difficulty in working with compounds containing aldehyde functionalities.

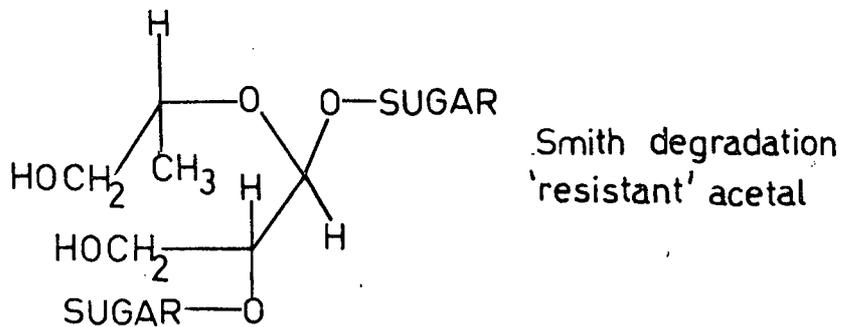
Periodate oxidation is complicated by both over and under oxidation. Over oxidation can be minimised by using dilute solutions of periodate buffered at acidic pH (e.g. pH 4.0) and by keeping the reaction mixture in the dark at 4°. Incomplete oxidation may arise due to:

1. The formation of intramolecular hemiacetals via the aldehydes generated in the initial periodate oxidation^{53,54};
2. steric hindrance that limits the accessibility of a pair of vicinal diols to periodate ions⁵⁵;
3. ionic repulsion between charged groups, e.g. carboxylate anions, and the attacking periodate molecules⁵⁶.

During periodate oxidation studies on K70 and K32, and to a lesser extent on K36, it was observed that when the oxidation was carried out in an unbuffered medium (hence the pH of the solution was approximately 4.0) the relatively labile pyruvate acetal was slowly hydrolysed and this resulted in further periodate consumption. This 'over oxidation' could be prevented in the cases of K70 and K36 but not entirely for K32.

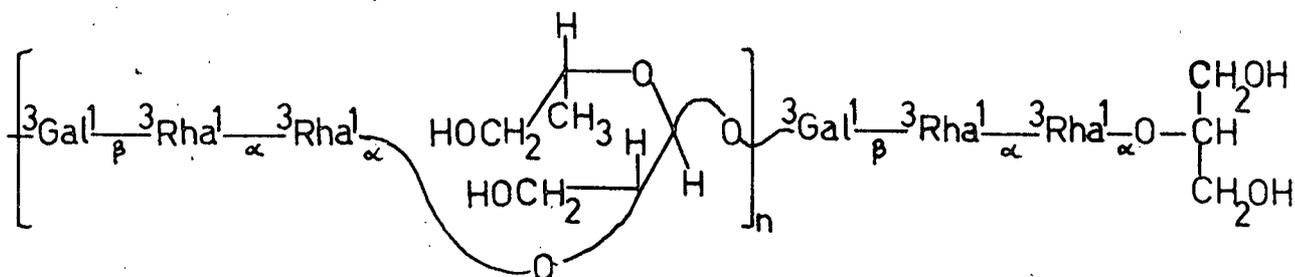
During periodate oxidation studies on K70 and K36 polysaccharides an acetal arising from the oxidation and subsequent reduction of a 2-linked L-rhamnose sugar (see page 34), was found to be unusually resistant to acid hydrolysis as compared with other similar acetals. This is even more unusual when

it is realised that the glycosidic linkages from 6-deoxy sugars are considered to be more labile than the same for a normal hexose⁵⁷, and in the ring opened state the hydrolysis of the acetal might be considered to be more easily achieved for L-rhamnose than for other 'normal' hexoses, e.g. D-glucose, D-galactose.

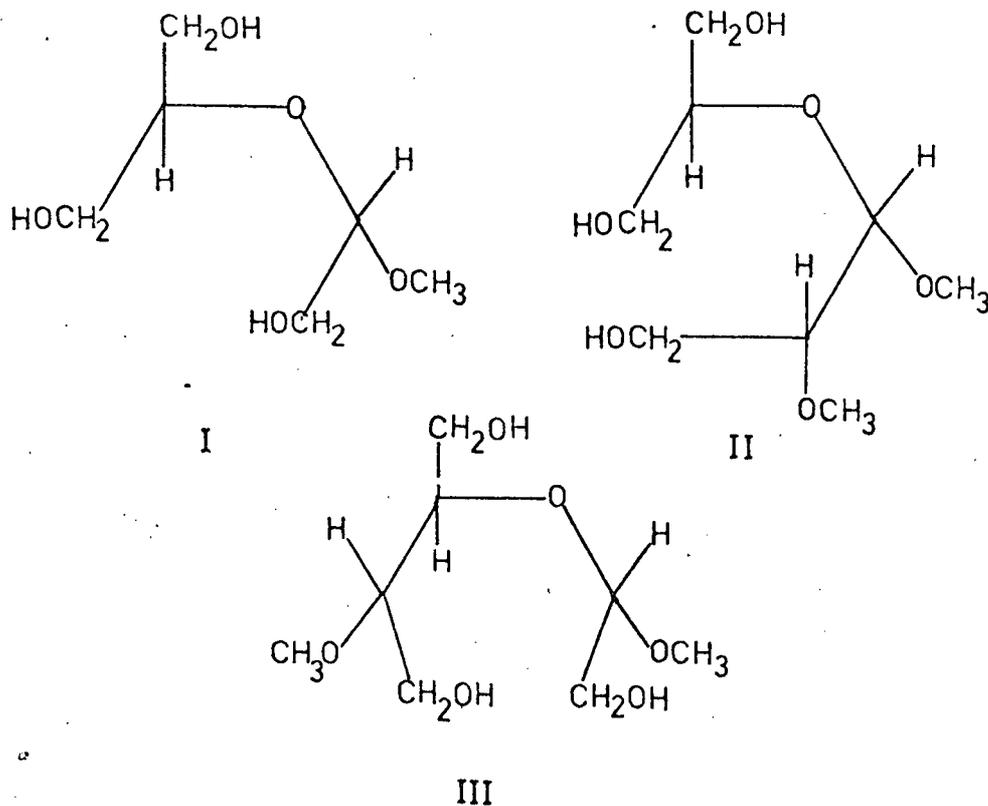


Klebsiella K36 and K70 structures both include periodate susceptible 2-linked L-rhamnose sugars. Following the Smith degradation (0.5 M trifluoroacetic acid, room temperature, 16 hours), although the expected oligomers were obtained via gel filtration, the yields of these oligomers were poor. During the gel filtration purification polymeric material eluting just after the void volume was always apparent and this would indicate incomplete hydrolysis during the Smith degradation. In the case of K36 n.m.r. spectra of this material indicated that it was very similar to the trisaccharide glyceride expected (see Figure II.5, page 32), i.e. the material

contained no sidechain D-glucuronic acid or D-glucose, and is assumed to be a mixture of polymeric material with the in-chain 2-linked L-rhamnose acetal still intact (see below).



Some further support for this phenomenon is shown in a study by Lindberg et al.⁵⁸. They compared the rate of hydrolysis of the compounds shown below.



While I and III were found to hydrolyse quite readily, II was found to be approximately 10 times more resistant to the hydrolysis conditions used. Compound II corresponds to an 'in chain' 2-linked hexose (acetylated) following periodate oxidation and NaBH_4 reduction. The reason postulated for this resistance in II is the presence of electron attracting groups in both the α and β positions to the acetal (C_1) carbon. However, all three compounds (I, II, III) were shown to hydrolyse much more readily than the parent methyl α -D-glucopyranoside.

II.7 Partial hydrolysis.

Capon⁵⁷ has comprehensively reviewed the first order rate constants for the acid catalysed hydrolysis of the glycosides of monosaccharides, and several generalisations may be made:

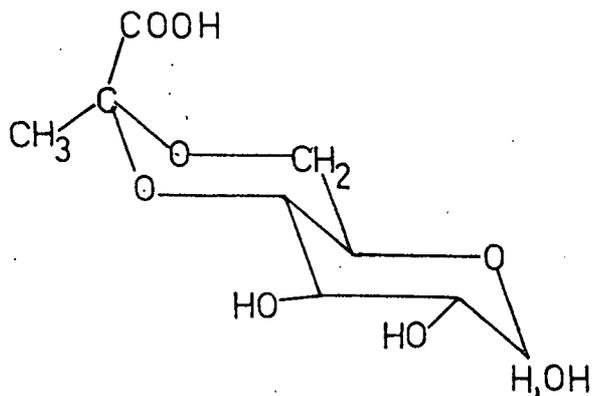
- (1) furanosides are more labile than pyranosides,
- (2) deoxy sugars are more easily hydrolysed than hexoses,
- (3) uronic acids are very resistant to hydrolysis (see Section II.2.),
- (4) amino sugars are more resistant to hydrolysis than common hexoses,
- (5) pentopyranosides are more labile than hexopyranosides,
- (6) α -glycosidic bonds are usually more labile than β -glycosidic bonds,
- (7) residues present as a sidechain are often more easily hydrolysed than when present as 'in-chain' residues.

It is therefore logical that given a heteropolysaccharide there will be some glycosidic bonds that are relatively resistant to acid hydrolysis and others that are comparatively susceptible. The result of this will be that under certain conditions of hydrolysis (acid concentration, temperature and length of hydrolysis) defined oligomeric subunits of the polysaccharide may be produced.

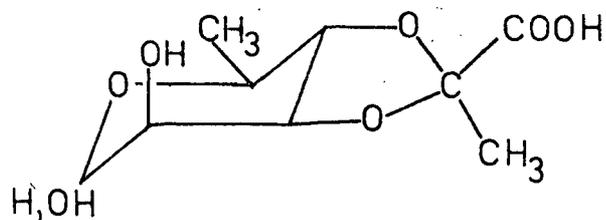
For acidic polysaccharides containing a uronic acid moiety, the resistance of the uronosyl bond usually dictates that the aldobiouronic acid, and to a lesser extent the aldotriouronic acid, will be produced in relatively large proportions. In most cases oligomers obtained via partial hydrolysis are obtained in very poor yield as those oligomers produced early on in the hydrolysis are subjected to the acid and may be hydrolysed further. Controlled, continuous removal of the oligosaccharides as they are produced is possible using the apparatus of Galanos *et al.*⁵⁹ but the problems associated with this apparatus and the large amounts of starting material available during this work (5-10 g) made it more practical to accept the poor yields during 'one-pot' partial hydrolysis studies and this technique was used throughout.

Acetals such as pyruvate acetal have been found to survive during partial hydrolysis in some cases⁶⁰, while in other instances they have been shown to be very labile⁶¹. It would appear that if the pyruvate acetal spans carbons four and six of a D-hexose sugar it is moderately stable as very little

steric strain is involved. When the acetal spans vicinal-trans positions of a hexose or 6-deoxy hexose, considerable strain is encountered. The vicinal-trans acetals are very acid sensitive. The two types of acetals mentioned are illustrated below.



4,6-O-(1-carboxyethylidene)-
D-glucose



3,4-O-(1-carboxyethylidene)-
L-rhamnose

During partial hydrolysis studies on K70, K36 and K32 no oligomers retaining the pyruvate acetal were isolated. The lability of the pyruvate acetal present as 4,6-O-(1-carboxyethylidene)-D-glucose in K36 is probably because it is terminal, and hence very accessible, on a sidechain. In K70, and especially K32, the pyruvate acetal was found to be very labile and treatment with 0.01 M trifluoroacetic acid at 100° for 30 min. cleaved it completely. During periodate oxidation studies, which are usually carried out in acidic conditions, it was necessary to buffer the reaction medium at a pH as

high as 6.5 to prevent hydrolysis of the pyruvate acetals in K70 and K32.

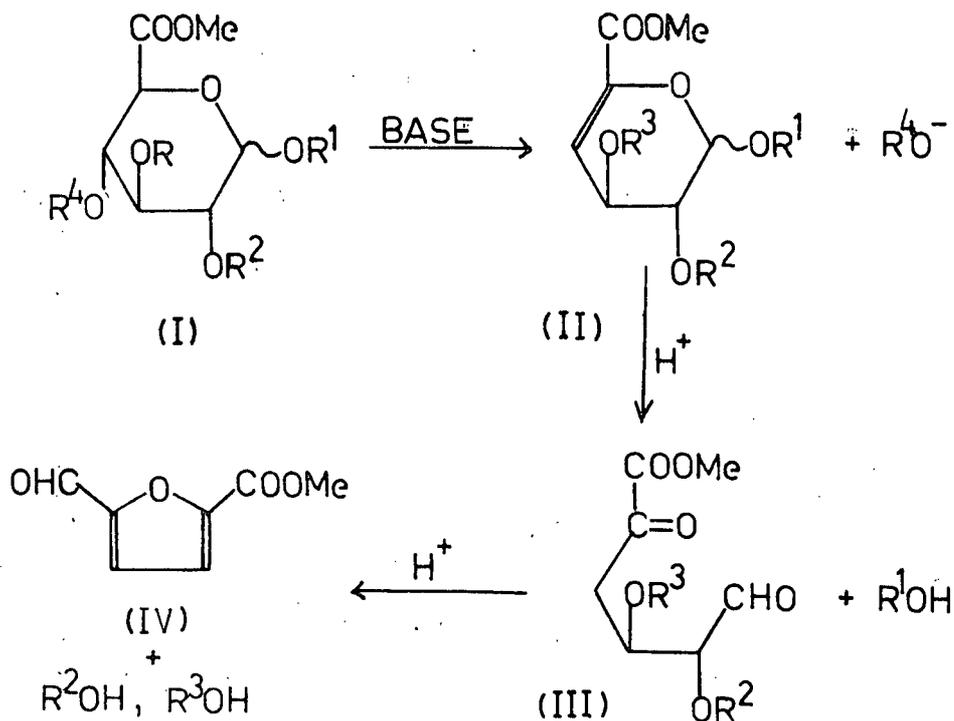
The partially hydrolysed polysaccharide material was normally separated by ion exchange chromatography into acidic and neutral fractions using Dowex 1-X2 in the formate form. The two fractions were then subjected to gel filtration and fractions, after lyophilisation, were monitored by paper chromatography (see Sections II.9.1 and II.9.5.).

II.8 β -Elimination.

The alkaline degradation of methylated polysaccharides containing uronic acid residues has recently^{62,63} been utilised to achieve specific cleavages in such polysaccharides. Kiss⁶⁴ and Aspinall⁶⁵ have investigated this reaction for compounds of low molecular weight and an extension of this work now allows valuable sequence data to be obtained for polysaccharides.

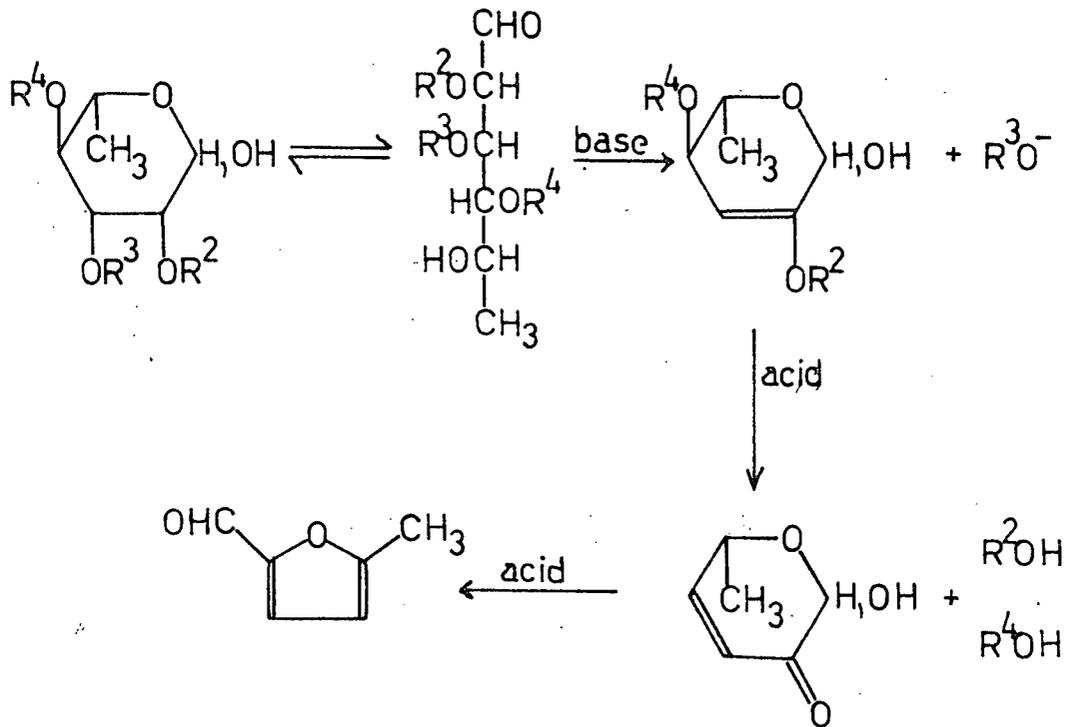
The basis for the cleavage is outlined on page 40.

The uronic acid residues (eg. I) in the permethylated polymer carry a good leaving group at position four; either a methoxyl group if the uronic acid is terminal and non-reducing, or another sugar residue. When treated with base the substituent at C₄ is eliminated as a result of the relative acidity of the ring proton at C₅. The stereochemistry of this elimination makes it such that D-galacturonic acid residues should eliminate more readily than the D-glucuronic equivalents⁶⁶.



The D-galacturonic acid has the proton at C₅ and the leaving group at C₄ in a trans diaxial arrangement. The unsaturated uronic acid derivative produced by the elimination (II) is acid sensitive as it contains an enol ether. Treatment under mild acidic conditions that are insufficient to cleave intact glycosidic bonds will degrade this unsaturated residue (II) to give, ultimately, the furan derivative (IV). During this acid treatment the substituent at C₁ of the uronic acid is released and hence the furan derivative (IV) is completely eliminated from the polysaccharide.

A further β -elimination reaction can take place if the substituent R⁴ in the uronic acid (I) is another sugar, e.g. rhamnose, as when this sugar is eliminated the resulting reducing terminus that is released is sensitive to base. Subsequently this sugar residue is also degraded during the base-acid treatment. (See page 41.)



An example of this type of degradation is shown in Figure II.6, page 42, using K70 as a substrate.

The base used to effect the elimination is either sodium methoxide in methanol or methylsulphonyl sodium in dimethylsulphoxide. The latter was used in this study. It is important to note that the methylated polysaccharide must be scrupulously dry as traces of water will result in preferential de-esterification of the ester groups when the base is added and the β -elimination will not proceed where free carboxylic acids exist. For this reason the methylated polymers

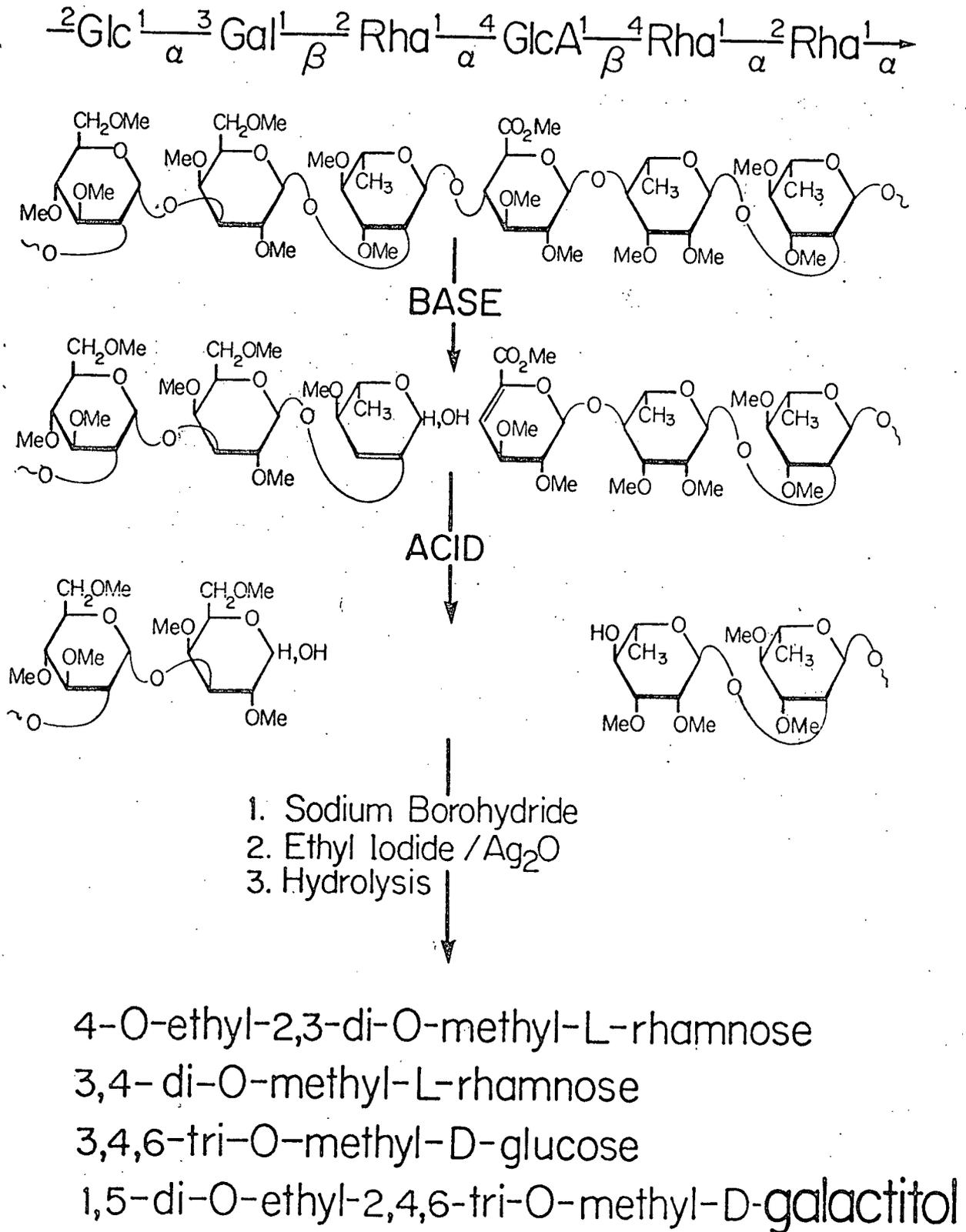
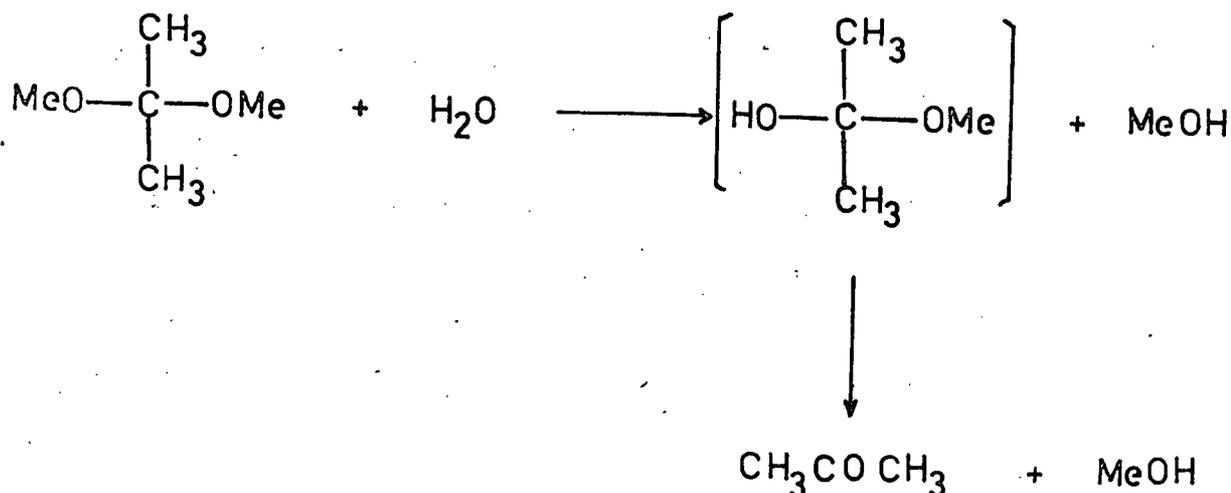


Figure II. 6 Uronic acid degradation of *Klebsiella* K70.

were stirred in the appropriate solvent with small quantities of 2,2-dimethoxypropane and toluene-p-sulphonic acid before the base was added.



The sensitivity of enol ethers to acid conditions makes the cleavage of these bonds relatively easy compared to the cleavage of glycosidic bonds in methylated polysaccharides. The usual hydrolysis conditions involve the use of either 90% formic acid at 40° for 1 hour or 10% aqueous acetic acid at 100° for 1 hour, but stronger conditions, e.g. 50% aqueous acetic at 100°, overnight, have also been used⁶⁷.

A further use for this alkaline degradation arises when the uronic acid moiety is present in the sidechain of a polysaccharide structure. Following the degradation, i.e. base and then mild acid treatments, a polymer with only a limited number of free hydroxyls will be produced. Reagents such as ruthenium tetroxide (RuO₄) are able to oxidise secondary

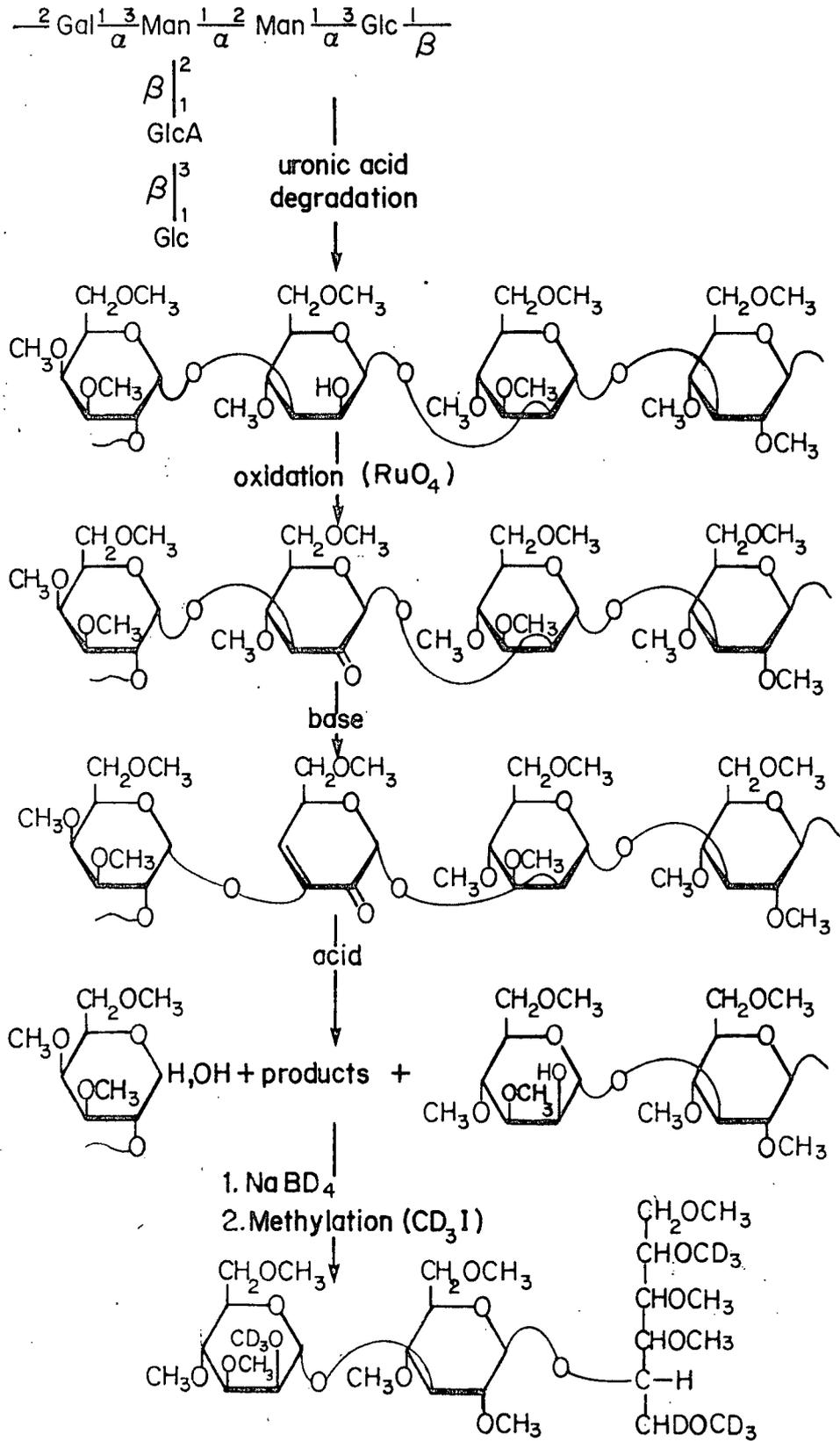


Figure II. 7 β -Elimination degradation via oxidation of a secondary hydroxyl.

alcohols to ketones and these functionalities may then be utilised in further β -elimination studies. This idea is exemplified by the work of Lindberg et al. on Klebsiella K28 (see Figure II.7, page 44).

A number of advantages associated with these eliminations based on uronic acid or keto functionalities are:

- (1) the reactions are quick and easy to perform;
- (2) they give specific cleavages and hence results are usually easily interpreted;
- (3) the specific cleavage destroys a uronic acid moiety-- a moiety which is normally resistant to degradation;
- (4) valuable sequence information may be obtained.

The fact that not all the reactions proceed in 100% yield is a disadvantage, especially where a number of sugar residues are susceptible to the basic conditions.

II.9 Separation of oligosaccharides obtained from degradations.

Degradation techniques in polysaccharide structural analyses do not give a 100% yield of a single oligomer. Often it is necessary to separate the desired product from unreacted (non-degraded) polymeric material or from contaminating compounds present as a result of side reactions. Depending upon whether methylated or non-methylated oligomers require separation or purification, preparative procedures such as paper chromatography, paper electrophoresis, liquid chromatography, gel chromatography or silica gel chromatography may be used.

II.9.1 Gel chromatography.

This technique has been used extensively in this study to separate mixtures of products obtained from partial hydrolysis, periodate oxidation and bacteriophage degradations. While gel chromatography of carbohydrates is well established, a few points are worth noting.

When selecting a gel to perform a particular separation, e.g. Sephadex G-15 might be used to separate a mixture of di, tri and tetra-saccharides, it is important to realise certain ionic interactions between the gel matrix and the carbohydrate molecules are operative. This is especially so when distilled water is the eluant and uronic acids are present. For example; if a mixture of D-glucose and L-rhamnose is separated on a column of Sephadex G-10, the D-glucose will be eluted a little before the L-rhamnose, but when the same separation is performed using Bio-Gel P-2 this order is reversed. This effect can be put to good use and, for example, to separate an aldotriouronic acid composed of a D-glucuronic acid unit and two L-rhamnose sugars from the related aldobiouronic acid Sephadex G-15 would be a better gel to select than Bio-Gel P-4. When volatile buffer solutions, e.g. water, pyridine, glacial acetic acid (1000:10:4) are used as eluants the ionic interactions experienced with distilled water alone are negated. Separations are then based solely on molecular size.

The monitoring of fractions collected from a gel column is very difficult when carbohydrates are being separated as

differential refractometry is the only continuous detection system that may be used. As the quantities of oligomers that may be separated are often small, this means of detection has only limited value. Quantitative colorimetric techniques such as the phenol-sulphuric assay⁶⁸ may be used to monitor elution profiles but these techniques are tedious to perform. During this work it was found to be much easier to first lyophilise the individual collected fractions and to then analyse them by paper chromatography--a technique that uses very little material and that can achieve very delicate separations where overlapping components occur in an individual fraction.

It is possible to separate carbohydrate mixtures that are soluble in organic solvents by gel chromatography using Sephadex LH-20. However, this technique has found only limited application and has generally been used to purify large polymeric material, e.g. permethylated or acetalated polysaccharides, from small, non-volatile reagents.

II.9.2 Silica gel chromatography.

This type of procedure may be effective in separating relatively small (hexasaccharide and smaller) derivatised oligomeric mixtures. An example of this involves the separation of two permethylated tetrasaccharides obtained from partial hydrolysis studies on K36 (see Section III, page 103).

II.9.3 Liquid chromatography.

The rapidly developing system of high pressure liquid chromatography has only recently been applied to the separation of carbohydrates⁶⁹. The separation of monosaccharides and oligosaccharides appears promising as the time required to perform these separations is short, viz., approximately 15 minutes. It would seem that this procedure is well suited to analytical separations as as little as 20 µg of an individual sugar can be detected, but preparative separations would be exceedingly tedious using small columns and exceedingly costly if larger columns were to be used. One group of workers⁷⁰ uses liquid chromatography routinely for the separation and purification of permethylated oligosaccharides.

II.9.4 Gas liquid chromatography (g.l.c.)

An extensive review of the applications of g.l.c. to carbohydrates has been published⁷¹. The technique has been widely used for the separation of derivatised monosaccharides but has not developed extensively as a tool for the separation of oligosaccharides. Of main concern is the rapid decrease in volatility as one progresses from monosaccharides to di-, tri-, oligosaccharides, etc. Oligosaccharides must be transformed into volatile derivatives and those derivatives which may be used are the:

- (1) trimethylsilyl;
- (2) trifluoroacetyl;
- (3) permethyl.

The trifluoroacetyl derivatives^o are the most volatile of these derivatives but have been used by only a few workers⁷², whereas trimethylsilyl derivatives have found more widespread application. The ease with which the original starting material can be recovered following derivatisation makes trimethylsilyl derivatives advantageous where only small amounts of material are available.

The separation or purification of permethylated oligosaccharides by g.l.c. is becoming more widely used. This has been due to the development of specific degradations of methylated polysaccharides⁶³, the realisation that mass spectroscopy of permethylated oligosaccharides can give valuable sequence information and evidence for 'volatile' terminal glycosides (see Section II.10.2.2., page 63), and the increasing availability of liquid phases for g.l.c. that are stable at quite high temperatures, viz., 250-350°. Methylation analysis of polysaccharides is used extensively and g.l.c. techniques for the analysis of partially methylated sugars (most often as alditol acetates) are now very sophisticated. Hence it is logical that these methyl ether derivatives of oligosaccharides should be subjected to g.l.c.-m.s. analysis.

Permethylated oligosaccharides are not particularly volatile and hence stable columns, such as OV-1, have normally been used. For a permethylated trisaccharide a typical retention time might be 20 min. at 275° (carrier gas; 20 ml/min.).

For reducing oligomers, α and β anomers are not usually separable as the permethylated derivatives.

II.9.5 Paper chromatography and paper electrophoresis⁷³.

Since the initial use of cellulose as an 'inert' support in adsorption chromatography⁷⁴ and the subsequent use of filter paper in partition chromatography⁷⁵ it could be said that paper chromatography has revolutionalised the study of the structure of carbohydrate polymers. The great value of the method lies in its ability to separate the components of complex mixtures of carbohydrates quickly, simply and accurately, and with the expenditure of less than a milligram of material. Often it is possible to deduce many characteristics of a component by paper chromatography alone.

The qualitative examination of a mixture of oligo and monosaccharides is still best achieved by paper chromatography. Many different solvent systems may be used and the nature of these eluting solvents often gives useful information regarding the nature of the carbohydrate material, e.g., in a basic solvent acidic components will not move from the base line.

Although preparative paper chromatography has to a large extent been superceded by gel filtration techniques, there have often been times when preparative paper chromatography would have been a simpler and faster technique. In a recent structural analysis of Klebsiella K62⁷⁶, preparative

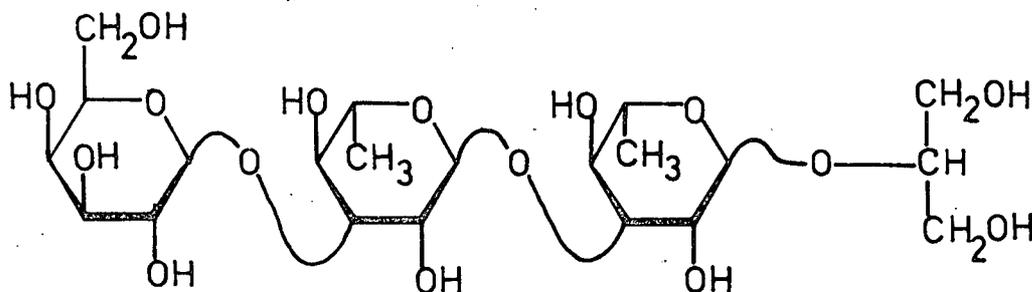
paper chromatography was used as a tool to isolate relatively large quantities of aldobio-, tri- and tetra-uronic acids.

Paper electrophoresis^{77,78} provides a convenient means for examining certain carbohydrate oligosaccharides and monosaccharides and an advantage over conventional paper chromatography is that good separations can be achieved in a relatively short period of time (2-4 hours). Buffer pH conditions are chosen so that the materials to be separated exist in a charged state, e.g. at pH 4.5 D-glucuronic acid will exist in its ionic form and will migrate under an electric potential to the anode.

II.10 Structure analysis of purified oligosaccharides.

To fully characterise an oligosaccharide it is necessary to determine several features. These are listed on page 52 using the oligosaccharide (I), shown below, as an example.

β -D-Galp-(1+3)- α -L-Rhap-(1+3)- α -L-Rhap-(1+2)-glycerol.



(I)

<u>Feature</u>	<u>Data using (I) as an example</u>	<u>Determination technique</u>
1) Quantitative composition	Gal:Rha:glycerol = 1:2:1	a) g.l.c. of alditol acetates following total hydrolysis b) ^1H and ^{13}C n.m.r.
2) Configuration of sugar components	Gal, <u>D</u> configuration 2xRha, <u>L</u> configuration	circular dichroism
3) Anomeric configuration	Gal, β - <u>D</u> - 2xRha, α - <u>L</u> -	a) ^1H and ^{13}C n.m.r. b) optical rotation
4) Positions of linkage between sugars	Gal, terminal non reducing Rha, in-chain linked at C_3 (two)	a) methylation analysis b) mass spectroscopy
5) Sequence of sugars	As shown in (I)	a) mass spectroscopy b) periodate oxidation

No single technique can give all this information and depending on the nature of the oligosaccharide different methods are chosen to demonstrate certain characteristics.

II.10.1 Gas liquid chromatography.

The application of g.l.c. has two aspects:

- (a) the quantitative determination of sugars and aglycones
- (b) the analysis of partially methylated sugars.

The total hydrolysis of an oligosaccharide in its underivatised state (see Section II.3., page 21) and subsequent reduction and acetylation will give alditol acetates of the component sugars. If an oligosaccharide has been obtained via periodate oxidation the aglycone, e.g. glycerol, erythritol, l-deoxy-erythritol, will also be present and can be identified by g.l.c., but often these small fragments are partially lost under reduced pressure during derivatisation. The alditol acetates of the common hexoses viz. L-rhamnose, D-glucose, D-galactose and D-mannose are separable by g.l.c. Two column systems used in this work are 3% SP-2340 on Supelcoport 100-120[#] and the 'hybrid' column devised by Albersheim et al.⁷⁹ The quantitation as determined by peak integration must be corrected using molar response factors (M.R.F.), but the M.R.F. for the hexoses are all the same ($\pm 2\%$).

The analysis of partially methylated sugars, obtained from the hydrolysis of methylated oligosaccharides or polysaccharides, has been best achieved by g.l.c. The field has been extensively reviewed^{71,80,81}. Several types of sugar

derivatives may be used, e.g. methyl glycosides⁸², acetates⁸³, alditol acetates⁸⁴⁻⁸⁶ and aldonitrile acetates⁸⁷. A serious disadvantage of derivatives preserving the anomeric centre is that a single methylated sugar may give rise to at least two, and possibly four, peaks on g.l.c., *viz.*, α - and β -pyranosides and α - and β -furanosides. During this study alditol acetate derivatives were used exclusively as each methylated sugar gives rise to only a single peak on g.l.c. and quantitation of peak areas can be made using response factors determined by Albersheim *et al.*⁸⁸

Analytical separations were carried out using various liquid phases. For different mixtures of partially methylated alditol acetates different columns gave optimum separation. Good separation of the three di-0-methyl-L-rhamnose isomers can be achieved using a column of HIEFF-1B but 2,3,4,6-tetra-0-methyl-D-glucose can not be separated from the 2,4-di-0-methyl-L-rhamnose when they co-exist in a mixture. To separate the latter two compounds columns of OS-138 or OV-17 can be used. Medium-polar columns such as B.D.S., OV-225, Silar 10C, and SP-1000 give good separations of trimethyl hexoses and monomethyl 6-deoxy hexoses, but no one column is guaranteed of separating all components in this region. Publications by Albersheim *et al.*⁸⁹ and Lindberg *et al.*⁸¹ list the relative retention times for a large variety of partially methylated alditol acetates. Retention times are usually quoted relative to an internal standard such as 1,5-di-0-acetyl-2,3,4,6-tetra-0-methyl-D-glucitol.

Column: HIEFF 1B (as in Figure II.3, page 26).

Programme: 160° to 180°
at 1° per
min.

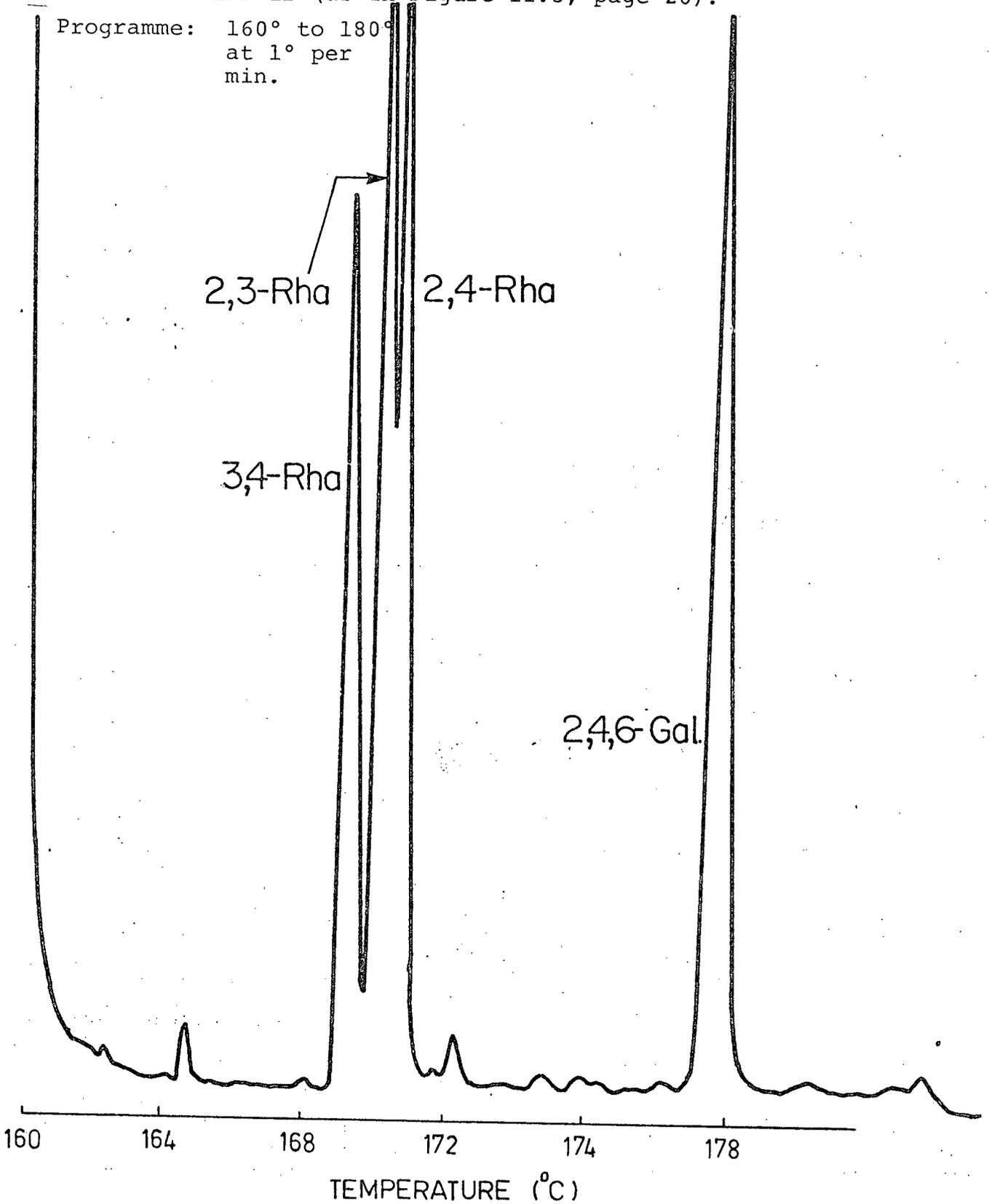


Figure II.8

G.l.c. separation of mixture of partially methylated alditol acetates obtained from Klebsiella K32 (pyruvate removed).

The identification of an individual partially methylated alditol acetate can be achieved in almost all cases by co-chromatography with authentic standards and g.l.c.-m.s. (For mass spectroscopy of partially methylated alditol acetates see Section II.10.2.1.) Where some doubt still exists after these two techniques have been fully exhausted, it is possible to preparatively collect the compound and to do physical measurements on the sample, i.e. melting point (if crystalline), ¹H n.m.r., derivatisation and then melting point. It is usually a simple matter to determine the methylation pattern of a component by mass spectroscopy and any ambiguity then lies in whether the parent sugar is galactose or glucose, etc. Demethylation⁹⁰ and reacetylation give crystalline hexaacetate derivatives of D-glucitol and D-galactitol.

An example illustrating the use of g.l.c. for separating a mixture of partially methylated alditol acetates is shown in Figure II.8, page 55. (Also see Figure II.3, page 26). Permethylated Klebsiella K32 (from which the pyruvate acetal had been previously removed) was hydrolysed, reduced and acetylated. The chromatogram reproduced in Figure II.8, shows good separation of the three subtly different dimethyl-L-rhamnose derivatives. From the chromatogram it can be concluded that K32 is composed of a tetrasaccharide repeating unit with the units being as shown, but no data on the sequence of these units are inferred by this analysis.

As a result of various degradation procedures, e.g. uronic acid degradation, it is possible to isolate oligosaccharides which contain only a limited number of free hydroxyl groups (the rest are usually present as methyl ethers). These free hydroxyl groups often represent what were linkage positions and the 'labelling' of these hydroxyls prior to subsequent hydrolysis, reduction and acetylation can yield valuable information. Use has been made of $\text{CD}_3\text{I-Ag}_2\text{O}$ to label these positions and in the final instance the once free hydroxyl can be detected in the m.s. of the partially methylated alditol acetate derived from the sugar. However, CD_3I is expensive and a labelling technique used on several occasions during this work involved etherification with $\text{EtI-Ag}_2\text{O}$. The ethyl group then present on the once free hydroxyl group is observable in the derived partially etherified alditol acetates in two ways:-

(1) The ethyl group (as compared with a methyl group) is relatively less polar and hence with polar liquid g.l.c. phases components containing ethyl groups tend to travel more quickly than their methyl analogues. Conversely, using less polar liquid phases those components with ethyl groups are characterised by having longer retention times in comparison with their methyl analogues.

(2) The ethyl group can be detected by m.s. of the derived alditol acetate of the sugar carrying the once free hydroxyl group. A characteristic shift of 14 mass units is easily detected.

The reader is referred to the analysis of the tetra-saccharide isolated from the uronic acid degradation of Klebsiella K70 and its subsequent analysis following ethylation (see Section IV, page 131) for an example of this 'labelling' technique.

II.10.2 Mass spectroscopy.

The mass spectrometry (m.s.) of organic compounds is based on fragmentation of organic molecules under electron impact, and differentiation of the resulting particles by use of the mass-to-charge ratio. In order to produce analysable ions of the compound under investigation several different ion sources can be used and these give rise to the different modes of m.s., viz. electron impact (e.i.), chemical ionisation (c.i.), field ionisation (f.i.), and field desorption (f.d.). During this work e.i., c.i. and f.d. mass spectra were recorded and more detailed data on these modes are given below.

(1) Electron impact⁹¹. This is the most widely used mode and involves subjecting the compound under investigation to a beam of electrons (normally with an energy of 70 eV) and the interaction between the electron beam and organic compound results in an energy exchange of around 10 to 20 eV. This is sufficient to cause ionisation of the molecule as the ionisation potential of the majority of organic substances ranges between 7 and 12 eV, and in many cases, causes decomposition to smaller fragment ions. Electron impact on the molecule

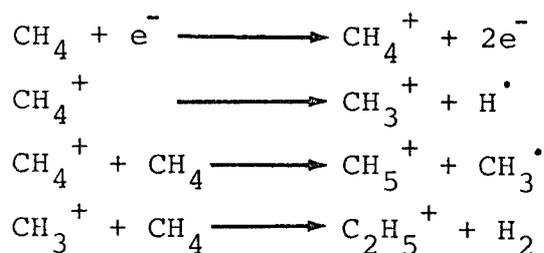
usually results in elimination of one electron, and, thus, in the formation of a positively charged ion--the so-called "molecular" or "parent" ion--designated as M^+ . The molecular ion is subsequently involved in fragmentation and rearrangement reactions to produce "daughter" ions. The abundance of any one ion is largely dependent on its stability as determined by the usual structural features in organic chemistry: e.g., tertiary ions and radicals are thermodynamically favoured over secondary and primary ones.

(2) Chemical ionisation^{92,93}. A serious shortcoming of e.i. m.s. is that many types of compounds do not give strong (or any) signal for the molecular ion. This is because during the initial electron-molecule interaction many molecules receive considerable energy above the ionisation voltage, and the molecular ion is consequently quickly destroyed by undergoing one or more bond breaking fragmentations.

The chemical ionisation process occurs with a much lower transfer of energy, and as a natural consequence, the fragmentation process is modified and greatly reduced. A quasi-molecular ion, formed by loss or gain of one hydrogen, is often the most prominent ion in the spectrum. (See page 71 for a comparison of a c.i. spectrum and e.i. spectrum.) The c.i. m.s. results from the ion-molecule reaction that occurs between the primary ions of a high pressure reactant gas and the low pressure sample gas. Both gases are introduced into the ion chamber where they are bombarded by an electron beam but virtually all primary ionisation due to the bombardment

occurs with the reactant gas. The ionised reactant gas undergoes ion-molecule reactions with itself to form a steady-state plasma which in turn reacts chemically with the dilute sample vapour.

A variety of reactant gases are used for c.i., the most common being methane, isobutane and ammonia. If methane is the reactant gas the most important ions in the reaction plasma are CH_5^+ and C_2H_5^+ .



In the presence of a good proton acceptor, the ions CH_5^+ and C_2H_5^+ act as Brönsted acids and protonate the sample molecule:



These reactions are typical of those observed for alcohols, aldehydes and esters. If the sample material is not a good proton acceptor the chemical ionisation process will occur as a hydride ion abstraction:

e.g. For decane



(3) Field desorption^{94,95}. Field desorption is another technique that provides sample ionisation at relatively low energy with resultant reduced fragmentation and much increased abundance of the parent ion. In fact, in many f.d. m.s. the molecular ion is often the only major peak. In the f.d. source a very high positive electric field (in the range of 10^7 - 10^8 volts/cm) is produced between a thin emitter wire, that is coated with needle-like carbon 'whiskers', and the first slit in the spectrometer. The high electric field strength induces electron tunnelling through a potential energy barrier in the molecule, and the resulting positive ion is accelerated out of the chamber and into the mass spectrometer analyser. The sample to be investigated is put directly onto the emitter wire and unlike e.i., c.i., and f.i. sources where the sample has to be vapourised before it enters the probe, the sample coated emitter wire is put directly into the probe. For very non volatile compounds it is sometimes necessary to gently heat the emitter wire in order to produce ions.

The energy available for f.d. and subsequent excitation of a molecule is about 10-13 eV and hence many organic molecules will have very little excess energy in the parent ion to cause fragmentation. Frequently quasi-molecular ions such as $(M+H)^+$ are observed due to a surface reaction of the sample with adsorbed water on the emitter.

Carbohydrates in the underivatised state are thermally unstable and practically non volatile, and hence mass spectral

studies in the past have been performed on the more volatile derivatives, such as, methyl ethers, acetates and trimethylsilyl derivatives. The recent advances in f.i. and f.d. mass spectroscopy do however, now allow mass spectral studies on the underivatized carbohydrates.

During the course of this work mass spectroscopy was employed for two distinct purposes: the analysis of partially methylated alditol acetates in order to assign methylation patterns and in some cases parent sugar identity; and the analysis of oligosaccharides.

II.10.2.1 Analysis of partially methylated alditol acetates^{86,96,97}.

The components obtained from methylation analyses of polysaccharides, i.e., partially methylated alditol acetates, may be readily examined by m.s. and in particular, g.l.c.-m.s. The mass spectral analysis of these compounds is a routine technique performed in the e.i. mode, and considerable data are available on their fragmentation.

The partially methylated alditol acetates fragment upon ionisation and no molecular ions are seen. The fragmentation pathways for a specific compound are dictated by the methylation pattern in the molecule as scission of the carbon-carbon bonds occur with certain preferences which are outlined below.

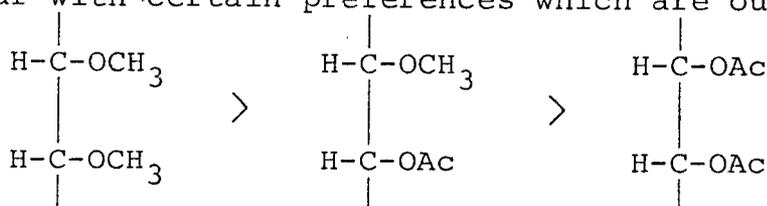


Table II.1. Primary Fragments in the Mass Spectra of Partially Methylated Sugars in the Form of Their Alditol Acetates.

Position of CH ₃	m/e														
	45	59	89	117	131	161	175	189	203	205	217	233	261	305	333
Pentoses															
2(4)				x											x
3								x							
2,3(3,4)				x				x							
2,4				x								x			
2,5	x			x								x			
3,5	x					x		x							
2,3,4				x		x									
2,3,5	x			x		x									
Hexoses															
2(5)				x											
3(4)								x					x		x
6	x														
2,3				x									x		
2,4(3,5)				x				x							
2,5				x											
2,6	x			x											
3,4								x							
3,6	x							x				x			
4,6	x					x								x	
5,6	x		x												
2,3,4				x		x		x				x			
2,3,5				x								x			
2,3,6	x			x								x			
2,4,6	x			x		x						x			
2,5,6	x		x	x											
3,4,6	x					x		x							x
2,3,4,6	x			x		x				x					
2,3,5,6	x		x	x						x					
6-Deoxyhexoses															
2				x											
3								x	x						
4					x									x	
2,3				x					x						
2,4				x	x							x			
3,4					x			x							
2,3,4				x	x	x	x								
2,3,5	x			x				x							

and permethylated derivatives¹⁰⁴⁻¹⁰⁶. While the spectra of acetate derivatives are somewhat complex due to the fact that an acetoxy group can be eliminated in four different modes, and trimethylsilyl derivatives suffer from the disadvantage of incurring a large increase in mass over the parent sugar, the only inconvenience of permethylated derivatives is that they are more difficult to make. Since it is common practice to permethylate oligosaccharides during structural studies it is natural that they be analysed by mass spectrometry.

Since fragmentation of permethylated oligosaccharides proceeds in an analogous manner to that of permethylated monosaccharide glycosides, it is convenient to look closely at the degradation pathways in the latter before considering the more complex oligosaccharides. Using permethylated D-glucose as an example, Figure II.9, page 66, outlines the most important degradation pathways for the monosaccharide derivative. The nomenclature employed by Kochetkov and Chizhov¹⁰⁴ is used throughout. The M^+ -ion is also degraded into the H- and K-series of fragments having low masses. The series A-K represent fundamental modes of fragmentation for all glycosides. Substituents, or such modifications as the introduction of deoxy groupings, can alter the relative importance of particular series of fragments. Uronic acid derivatives¹⁰⁷ follow the same major breakdown pathways.

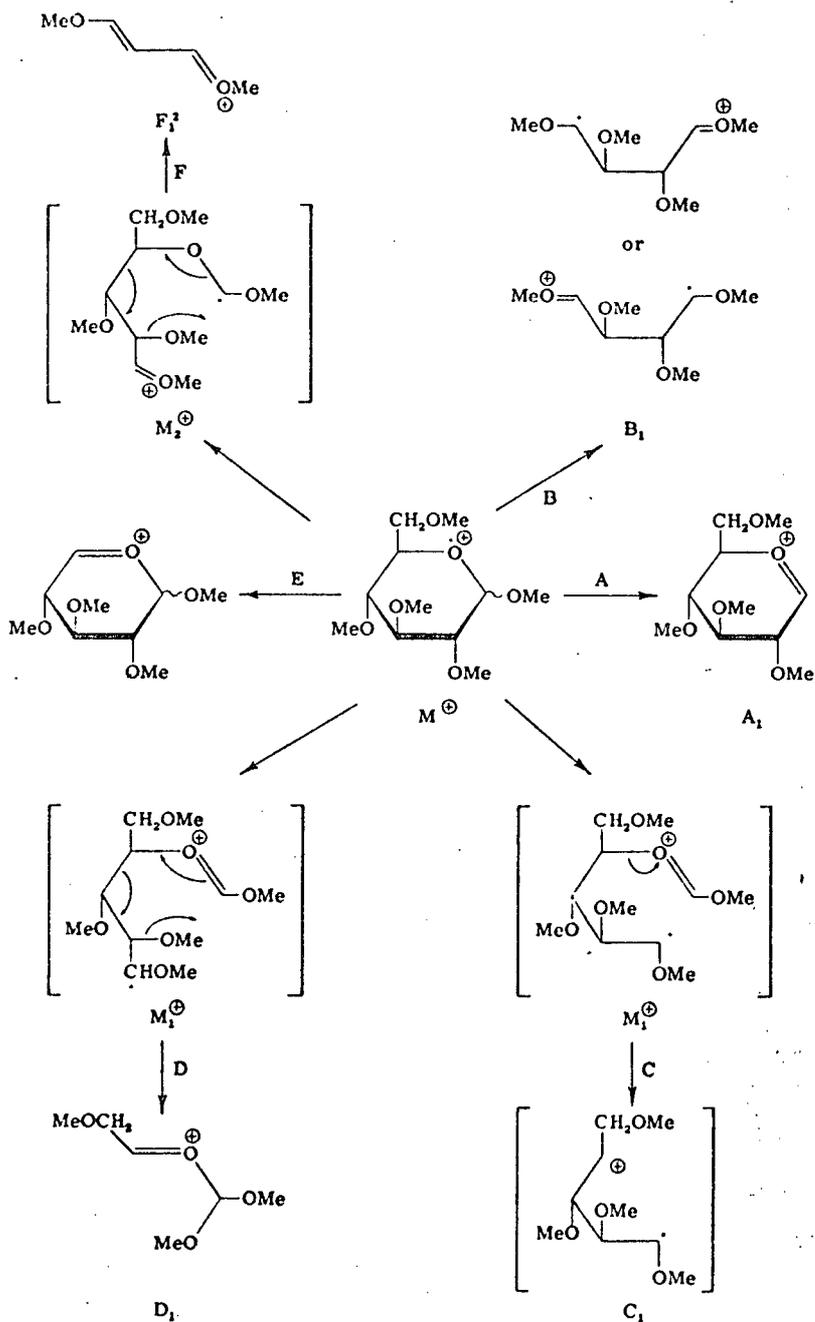
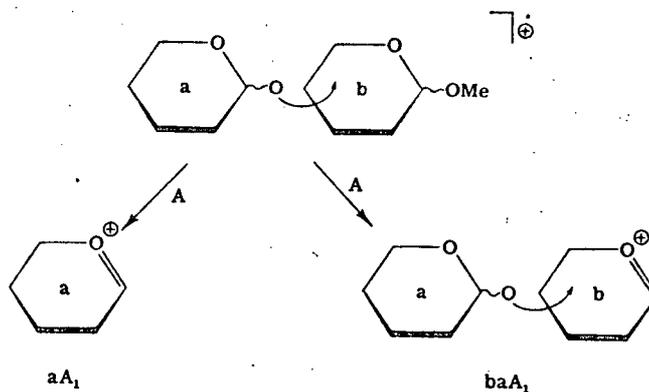


Figure II. 9

Degradation pathways of permethylated D-glucose during electron impact (e.i.) mass spectrometry. (From J. Lönngrén and S. Svensson. Adv. Carbohydr. Chem. Biochem., 29, 42 (1974)).

For permethylated oligosaccharides the nomenclature for degradation pathways is a little more complex and is illustrated below for the degradation of a disaccharide methyl glycoside.



In this scheme baA_1 denotes that the ion has been formed by cleavage of ring b following pathway A, and is substituted with ring a. The A series of fragments serves to establish the molecular weight of the oligosaccharide and its component sugar residues. The B series of fragments obtained by degradation of ring b can be used to establish the nature of the linkage between the two sugar residues (see Figure II.10, page 68).

For oligosaccharide alditols, which often arise from periodate oxidation degradations, the mass spectral fragmentations are somewhat simpler. The non-reducing sugar is identified by the A series of fragments, and the alditol part by a fragment assumed to be produced by the D sequence of fragmentation (see Figure II.11, page 69).

To illustrate the information obtainable by e.i. m.s. of an oligosaccharide alditol the spectrum of a component obtained

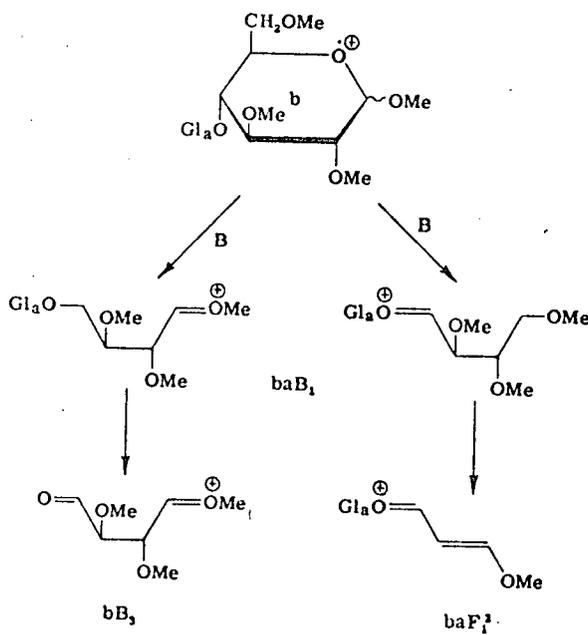
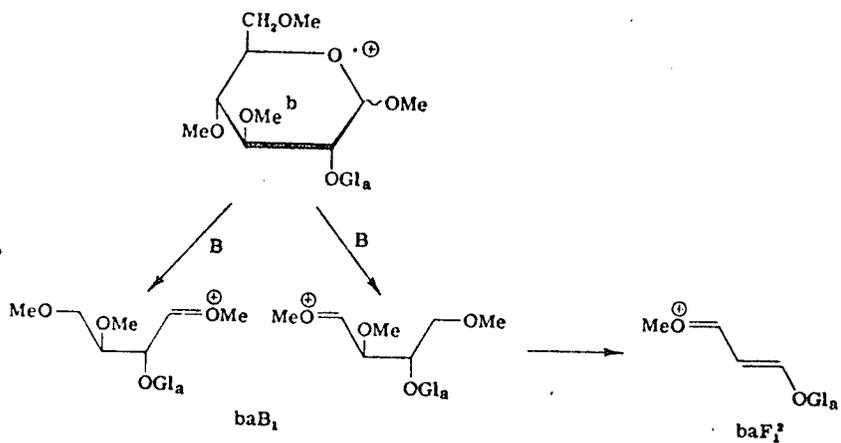


Figure II.10

Fragmentations during e.i.m.s. of permethylated disaccharides via the B pathway -- showing the nature of the linkage between two sugar residues.

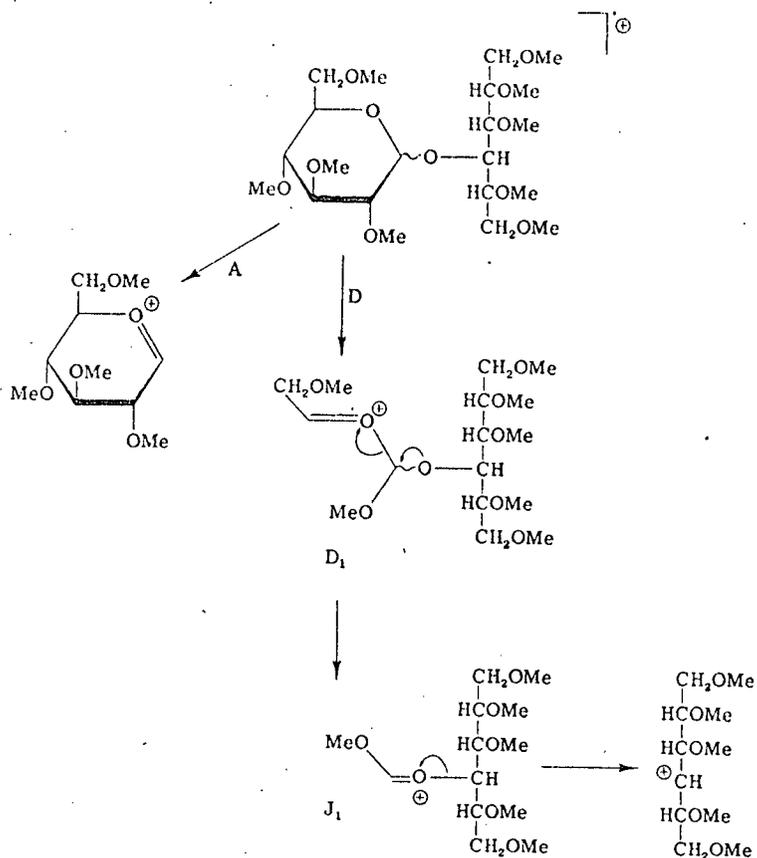


Figure II.11 Characteristic fragmentations of permethylated oligosaccharide alditols in e.i.m.s.

from Klebsiella K70 by a uronic acid degradation (see Section IV, page 130) is shown in Figure II.12, page 70. Some of the major fragments are assigned. For more detailed information regarding e.i. m.s. of permethylated oligosaccharides the reader is referred to publications by Moore and Waight¹⁰⁸, and Kärkkäinen¹⁰⁹⁻¹¹¹.

E.i. m.s. can be considered a routine technique in most laboratories but c.i. m.s. and f.d. m.s. are modes less widely

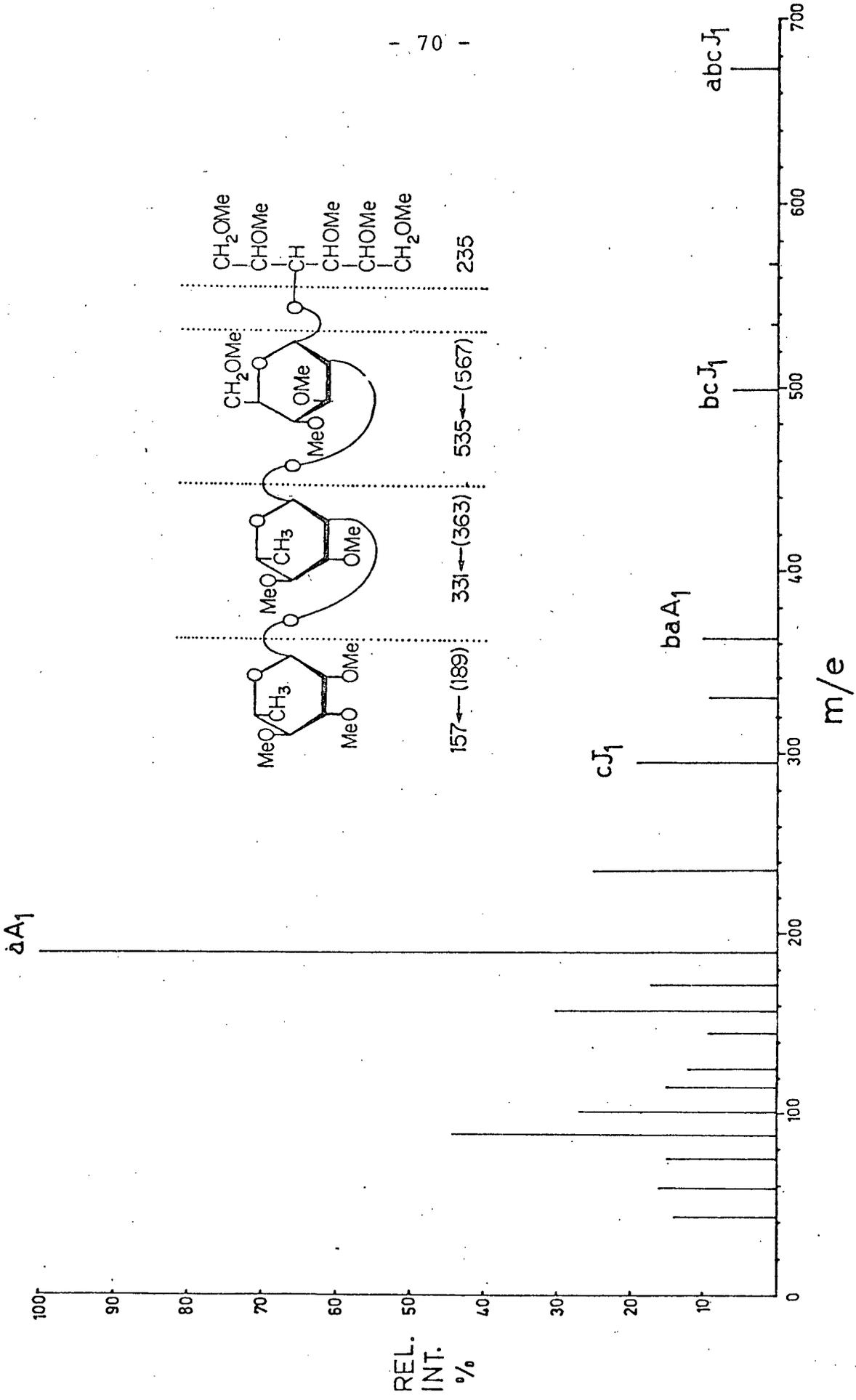


Figure II.12 The electron impact mass spectrum of a permethylated trisaccharide alditol.

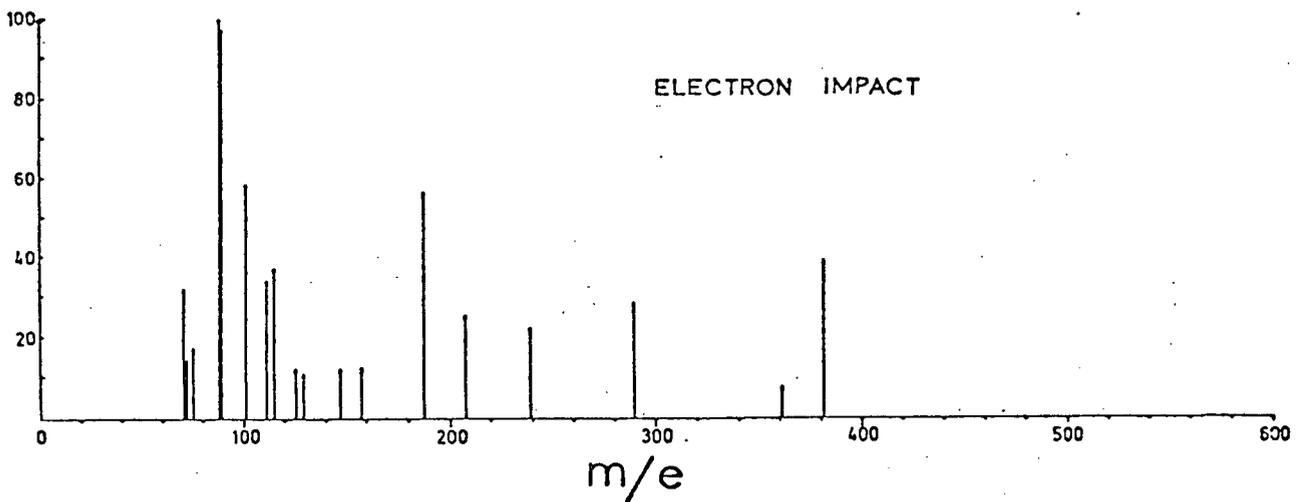
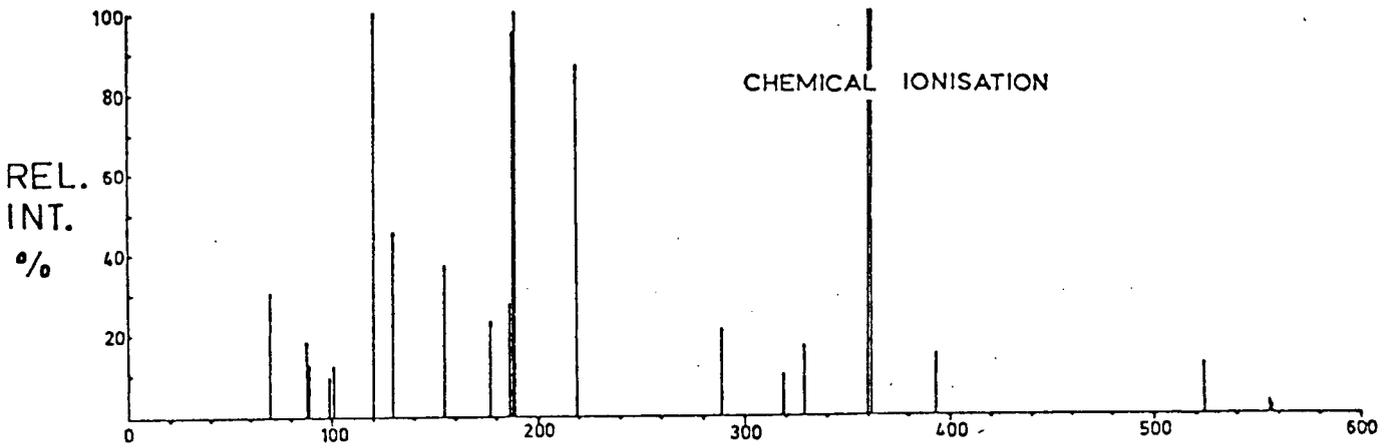
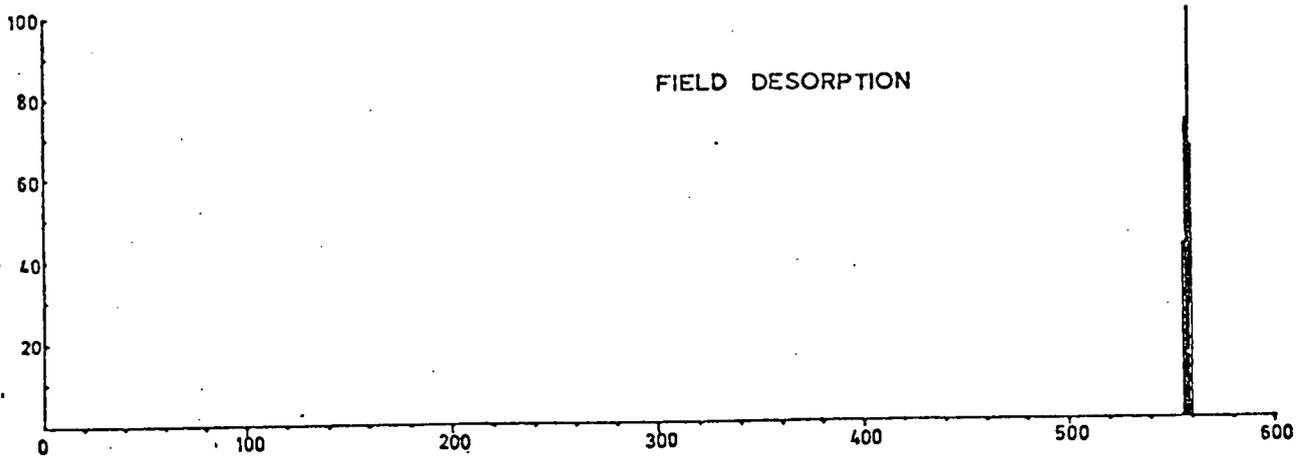
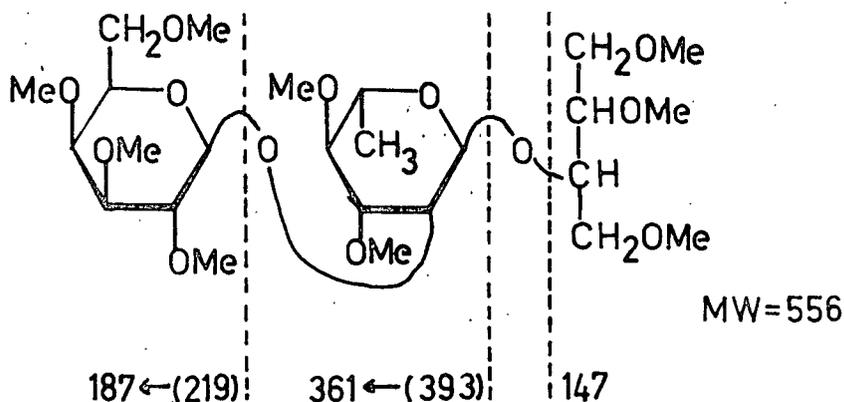


Figure II. 13 Mass spectra of a disaccharide alditol obtained using electron impact, chemical ionisation and field desorption modes.

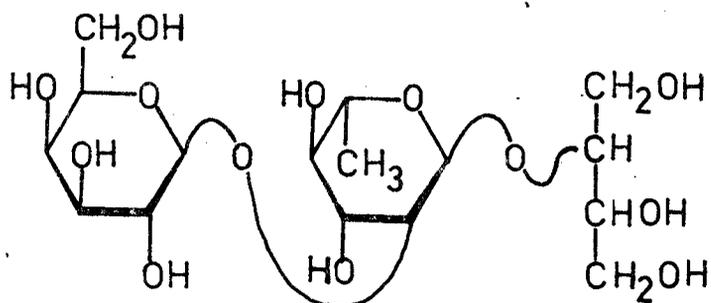
available to most chemists. During the later stages of the preparation of this thesis it was possible to have some spectra run using these 'more gentle' methods on a non-routine basis. Shown in Figure II.13, page 71, are the e.i., c.i. and f.d. spectra of a permethylated oligosaccharide with the structure shown below:



II.10.3 Nuclear magnetic resonance.

The use of n.m.r. as an investigative tool has been discussed in terms of polysaccharides in Section II.2. The technique has also been used extensively during this work to characterise oligosaccharides. Often only small quantities of pure oligomers are obtained from degradative studies and this non-destructive technique gives valuable information. For ^1H n.m.r. as little as 2 mg may be sufficient to give a good spectrum but for ^{13}C n.m.r. as much as 15 mg may be necessary to give a reasonable signal to noise ratio. There is no problem with viscosity when working with small oligosaccharides and D_2O exchange can be performed much more readily.

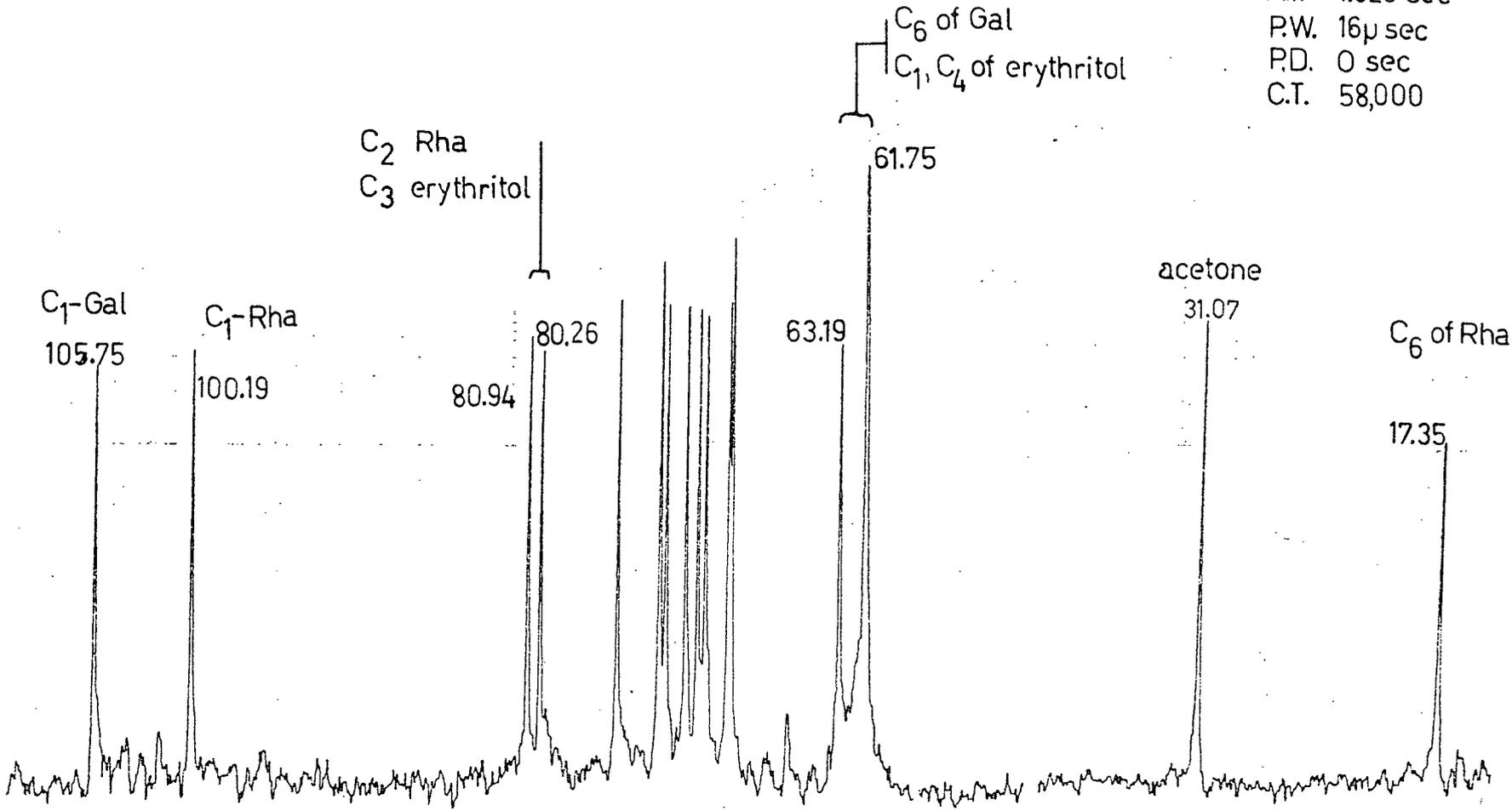
The data that can be obtained from n.m.r. studies are quite extensive and this is exemplified using the disaccharide glycoside shown on page 73.



β -D-Galp-(1-2)- α -L-Rhap-(1-2)-D-erythritol.

Shown in Figures II.14 and II.15, pages 74, 75, are both the ^1H n.m.r. and ^{13}C n.m.r. spectra of this underivatized oligomer. The ^1H n.m.r. spectrum shows the presence of one 6-deoxy sugar (L-rhamnose), and two non-reducing anomeric signals. Moreover, the chemical shift and coupling constants of these anomeric protons indicate the presence of an α -L-rhamnose moiety and a β -D-hexose (galactose). The ^{13}C n.m.r. spectrum, although not integrated, shows signals from 16 ^{13}C nuclei overall. This immediately is a good indication for a four-carbon fragment as the aglycone on the 'reducing' terminus. Also in the ^{13}C n.m.r. spectrum, signals from a 6-deoxy methyl group (δ 17.35 p.p.m.), three primary hydroxyls (61.75, and 63.19 p.p.m.), two 'linkage' carbons (80.26, 80.94 p.p.m.) and two non-reducing anomeric signals (100.19, 105.75 p.p.m.) can be

SW. 2000 Hz
A.T. 1.023 sec
P.W. 16 μ sec
P.D. 0 sec
C.T. 58,000



- 74 -

Figure II.14 ¹³C n.m.r. spectrum of β -D-Galp-(1 \rightarrow 2)- α -L-Rhap-(1 \rightarrow 2)-D-erythritol.
(See page 73.)

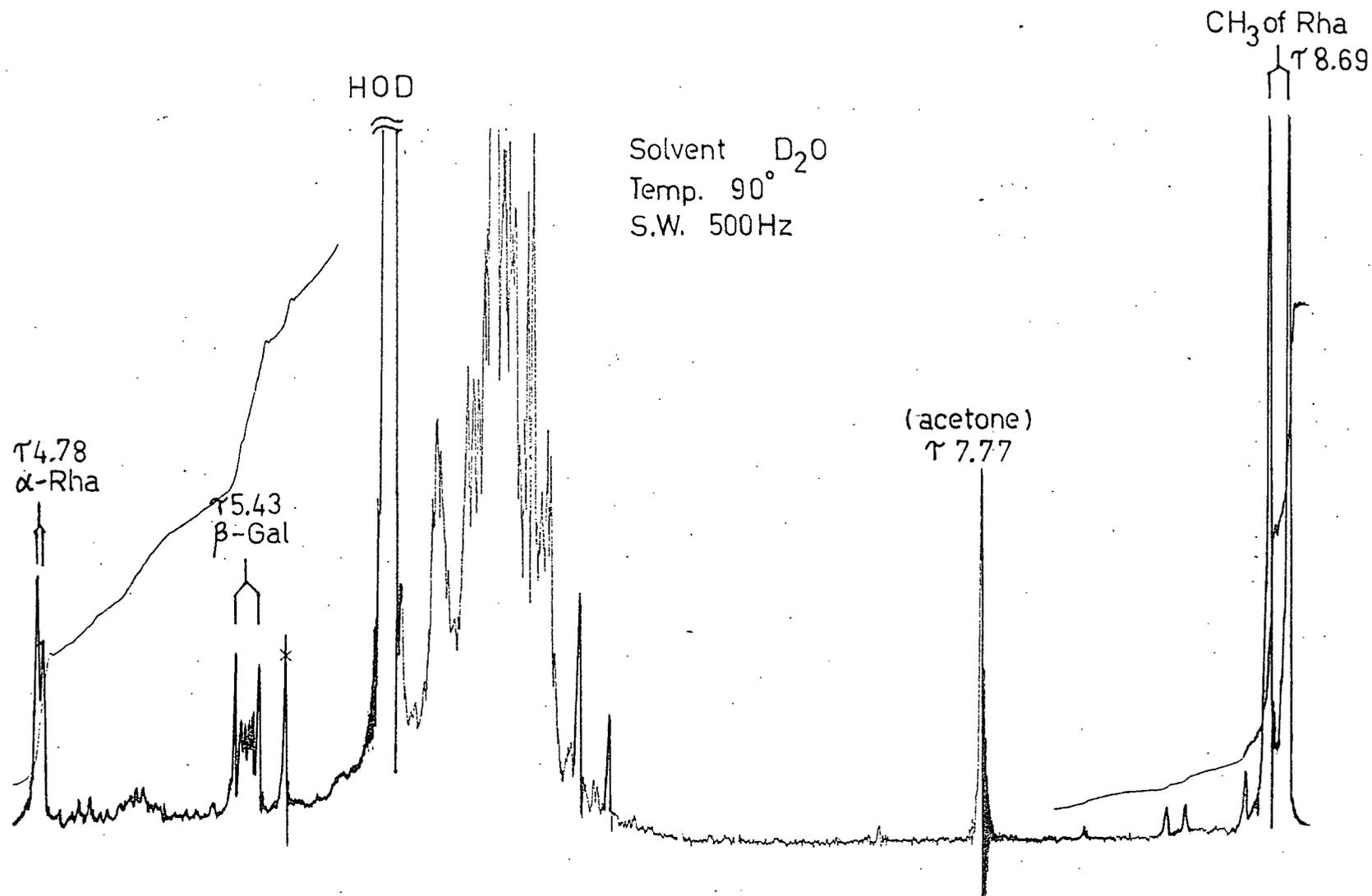


Figure II.15 1H n.m.r. spectrum of β -D-Galp-(1+2)- α -L-Rhap-(1+2)-D-erythritol.
 (See page 73.)

observed. (From the chemical shifts of the anomeric carbon nuclei signals it is usually safe to assume that signals to higher field of 102 p.p.m. are from α -linkages while those to lower field are from β -linkages. For this disaccharide glycoside a partially coupled ^{13}C spectrum³⁷ confirmed this assignment: β -D-Gal, $J_{13\text{C-H}}$ 162 Hz; α -L-Rha, $J_{13\text{C-H}}$ 172 Hz.)

The evidence for the oligomer being a non-reducing disaccharide, comprising a hexose sugar and a 6-deoxy hexose sugar, with a four carbon fragment on the reducing terminus is quite substantial. The evidence for the glycoside portion is of particular interest as these small fragments can only be 'observed' by hydrolysis studies on the underivatized oligomer and subsequent paper chromatography or g.l.c., or by mass spectroscopy. For the latter technique c.i. m.s. on the permethylated derivative or f.d. m.s. on the underivatized sample would yield molecular ions, but these analyses can not be considered 'routine' at this time.

II.10.4 Determination of D- or L-configuration of a sugar residue.

The D- or L-configuration of individual sugars can be determined by their specific oxidases (e.g., D-glucosidase and D-galactosidase) or by the sign of their circular dichroism curves as measured on suitable derivatives. For the latter, measurements at 213 m μ on alditol acetates or partially methylated alditol acetates in acetonitrile gave spectra which were

compared with those obtained from authentic samples¹¹². These components were routinely collected by preparative g.l.c.

II.11 Immunochemical methods.

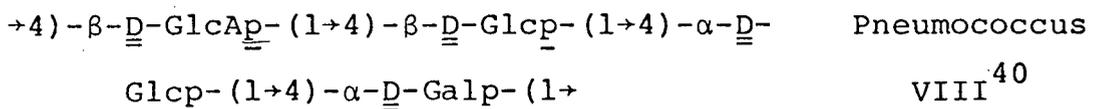
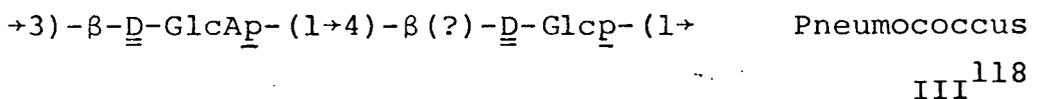
When an antigen is injected into an animal, e.g. a horse, the immune system of that organism is stimulated into producing antibodies to counteract the 'invading' antigen. The bacterial polysaccharides provide a rich source of antigenic material, and since they are often the principal antigenic determinants of the parent micro-organisms, the corresponding antisera are also frequently available. Hence the cross-reaction of a polysaccharide of unknown or uncertain chemical structure with antibodies to a polysaccharide of known structure may yield information as to one or more sugars contained in the unknown and even as to the positions at which the sugars are linked. Conversely, cross-reactivity of a polysaccharide of known structure with antibodies to a polysaccharide of unknown composition and linkage may be equally informative.

Heidelberger¹¹³ has developed methods utilising the precipitation reactions of antibody (precipitin) and antigen. This immunochemical reaction is called the precipitin test. Heidelberger et al.¹¹⁴⁻¹¹⁶ have examined the cross reactions between approximately 60 of the serologically distinct Klebsiella K-types 1-80 and antisera to some selected Pneumococcus polysaccharides. The degree of cross-reaction, as measured by the amount of precipitation, is an indication of

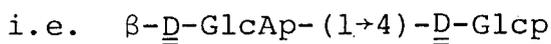
the degree of similarity of the structure of the unknown to the structure of the polysaccharide used to elicit the immune serum for the test. A series of precipitin tests has to be performed to deduce the fine partial structure of the unknown.

Cross reactions, when clear cut interpretation is possible, can yield structural information that could take months to obtain by purely organic chemical means. However, the complete structures of many of the Pneumococcus polysaccharides are not known and some strongly positive cross reactions between Pneumococcus antisera and Klebsiella polysaccharides cannot be interpreted fully. Many examples exist where this 'serological' technique has given useful information in determining a Klebsiella structure but unfortunately few positive reactions were observed for the K-types studied in this work, viz. K-types 32, 36 and 70. For clarity, however, an example using Klebsiella K5 is outlined below:

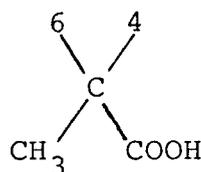
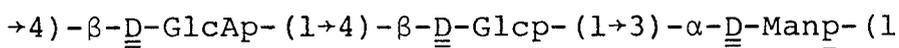
K5 polysaccharide¹¹⁷ precipitated antisera to Pneumococcus type III and Pneumococcus type VIII polysaccharides¹¹⁴. The structures of these Pneumococcus polysaccharides are not completely known but the partial structures are



A common feature of these two *Pneumococcus* polysaccharides is the presence of the cellobiouronosyl residues



It was suspected¹¹⁴ that the repeating unit for Klebsiella K5 would encompass this structural feature and this was in fact found to be the case when the structural determination of K5 was completed. The structure of Klebsiella K5 is shown below.



As more information on the Klebsiella polysaccharides becomes available, and bearing in mind the diversity of these structures determined to date, it will be most likely possible to deduce the finer details of many of the complex, and to date incomplete, *Pneumococcus* polysaccharide structures.

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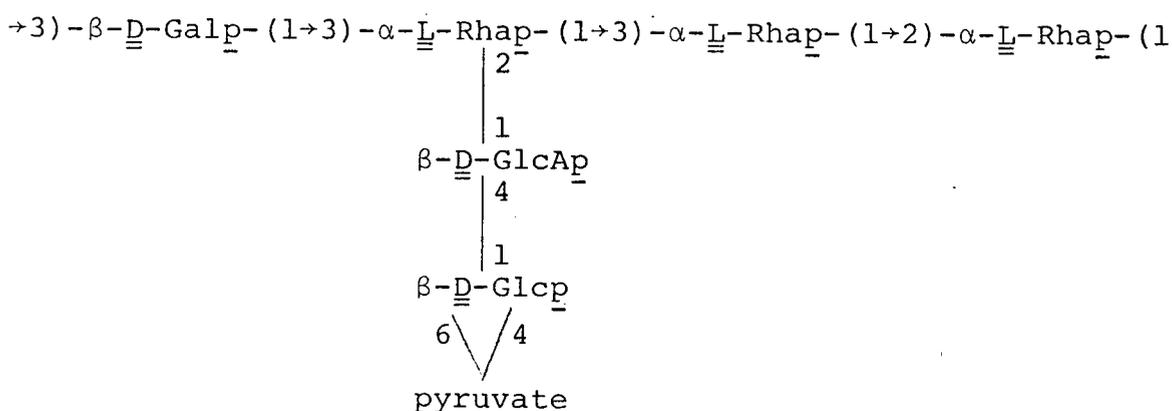
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III. THE STRUCTURE OF Klebsiella SEROTYPE K36^o
CAPSULAR POLYSACCHARIDE

III.1 Abstract

Klebsiella K36 capsular polysaccharide has been investigated using methylation, Smith-periodate oxidation and partial hydrolysis techniques. The structure was shown to consist of a hexasaccharide repeat unit as shown below. The anomeric configurations of the sugar units were determined by performing ^1H and ^{13}C n.m.r. spectroscopy on isolated oligomers obtained during the degradative studies on the intact polysaccharide.



III.2 Introduction

The bacterial genus Klebsiella is divisible into approximately 80 different serotypes which are distinguished by the structure of their capsular polysaccharides. Nimmich^{1,2} has analysed qualitatively the polysaccharide from each strain while Heidelberger and Nimmich³ have summarized the structures of the capsular polysaccharides which have been determined to date.

Many of the strains so far examined possess capsules of different qualitative compositions. There are, however, several large groups of Klebsiella bacteria which have capsules with the same qualitative analysis but with different serological reactions. One such group includes the strains of K-types K12, K36, K45, K55 and K70 whose capsules are composed of the sugars D-glucuronic acid, D-galactose, D-glucose and L-rhamnose. In an attempt to explain these serological differences on a structural basis, this group is now being examined in detail and the structure for K-type K36 here presented represents the first strain examined in this series.

III.3 Results and Discussion.

Composition and n.m.r. spectra.

Klebsiella K36 polysaccharide was prepared on an agar medium and purified by one precipitation with Cetavlon. The product had $[\alpha]_D -56^\circ$. Electrophoresis showed the material to be homogeneous.

The ¹H n.m.r. spectrum of the polysaccharide in D₂O at 90° (see Appendix III, spectrum No. 1) showed a sharp singlet at τ 8.41 indicative of a pyruvate acetal⁴. This signal was present in a ratio of 1:3 with the 6-deoxy signal due to rhamnose at τ 8.67. In the anomeric region five discernable signals were observed at τ 4.76, 2H, $J_{1,2}$ broad; τ 4.98, 1H, $J_{1,2}$ 2 Hz; τ 5.13, 1H, $J_{1,2}$ 7 Hz; τ 5.30, 1H,

$J_{1,2}$ 7.5 Hz; τ 5.50, 1H, $J_{1,2}$ broad. These chemical shifts indicate^{5,6} that the repeating unit contains six monosaccharide units, three of which are α -linked and three β -linked. More precise assignment of these signals was achieved after studying the ^1H n.m.r. spectra of the oligosaccharides obtained by partial hydrolysis; see below and Table III.1, page 92.

The ^1H n.m.r. analysis was confirmed by ^{13}C n.m.r. spectroscopy of the polysaccharide (160 mg/2 ml) which showed high field peaks at 17.6 p.p.m. (rhamnose CH_3) and 25.5 p.p.m. (pyruvate CH_3); in the anomeric region only five signals could be distinguished, at 101.4, 101.8, 102.7, 103.9 and 105.1 p.p.m. Two other signals were attributable to the C-6 carbons of hexoses and a further two signals at 173.2 and 174.2 p.p.m. were due to the carboxyl groups of the pyruvate acetal and the glucuronic acid (see Appendix III, spectrum No. 2). It is thus clear that ^1H and ^{13}C spectra provide complementary information⁷. The two sets of data show the K36 polysaccharide to be composed of three units of rhamnose, two of hexose, one uronic acid, one pyruvate acetal and having three α - and three β -glycosidic linkages.

Acid hydrolysis of the polysaccharide showed by paper chromatography the presence of glucose, galactose, glucuronic acid and rhamnose. Carboxyl reduced⁸ K36 was hydrolysed and the presence of D-glucose, D-galactose and L-rhamnose in the ratio of 2:1:3 was confirmed by gas liquid chromatography (g.l.c.) of their alditol acetates. The configurations of

Table III.1 Data on Klebsiella K36 Capsular Polysaccharide and Derived Oligosaccharides

Compound	Repeating unit of compound	τ^a (Hz)	Ratio integrals	Proton assignment
3	GlcA $\frac{1}{\beta}$ $\frac{2}{\beta}$ Rha-OH	5.37 (7) 4.63 (1.8) 5.17 (S) 8.70 (J $_{5,6}$ 6Hz)	1 0.6 0.4 3	β -GlcA α -Rha-OH β -Rha-OH CH ₃ of Rha
4	GlcA $\frac{1}{\beta}$ $\frac{2}{\beta}$ Rha $\frac{1}{\alpha}$ $\frac{3}{\alpha}$ Rha-OH	5.34 (7) 4.65 (2) 4.89 (2) 5.13 (S) 8.70 (J $_{5,6}$ 6Hz)	1 1 0.6 0.4 6	β -GlcA α -Rha α -Rha-OH β -Rha-OH CH ₃ of Rha
5	Glc $\frac{1}{\beta}$ $\frac{4}{\beta}$ GlcA $\frac{1}{\beta}$ $\frac{2}{\beta}$ Rha $\frac{1}{\alpha}$ $\frac{3}{\alpha}$ Rha-OH	5.19 (7.5) 5.35 (7) 4.63 (2b) 4.89 (1.8) 5.13 (S) 8.70 (J $_{5,6}$ 6Hz)	1 1 1 0.6 0.4 6	β -Glc β -GlcA α -Rha α -Rha-OH β -Rha-OH CH ₃ of Rha
6b	Gal $\frac{1}{\beta}$ $\frac{3}{\beta}$ Rha $\frac{1}{\alpha}$ $\frac{3}{\alpha}$ Rha-OH 2 β 1 GlcA	5.21 (7.5) 5.32 (7) 4.71 (2b) 4.91 (2) 5.13 (S) 8.70 (J $_{5,6}$ 6Hz)	1 1 1 0.6 0.4 6	β -Gal β -GlcA α -Rha α -Rha-OH β -Rha-OH CH ₃ of Rha
1	Gal $\frac{1}{\beta}$ $\frac{3}{\beta}$ Rha $\frac{1}{\alpha}$ $\frac{3}{\alpha}$ Rha $\frac{1}{\alpha}$ $\frac{2}{\alpha}$ glycerol	5.34 (7.5) 5.01 (1.8) 4.92 (1.8) 8.70 (J $_{5,6}$ 6Hz)	1 1 1 6	β -Gal α -Rha α -Rha CH ₃ of Rha
2	Rha $\frac{1}{\alpha}$ $\frac{3}{\alpha}$ Rha $\frac{1}{\alpha}$ $\frac{2}{\alpha}$ glycerol	4.94 (2) 5.03 (2) 8.70 (J $_{5,6}$ 6Hz)	1 1 6	α -Rha α -Rha CH ₃ of Rha

the glucitol hexaacetate and rhamnitol pentaacetate, as determined by circular dichroism (c.d.)⁹, were shown to be D- and L- respectively, while that of the galactose was also D- based on the c.d. of the partially methylated 2,4,6-tri-O-methyl-D-galactitol triacetate obtained during methylation studies (see later).

Methylation of original and autohydrolysed polysaccharides.

Methylation¹⁰ of K36 polysaccharide and subsequent reduction with lithium aluminum hydride, hydrolysis, derivatisation as alditol acetates and g.l.c.-m.s. analysis^{11,12} indicated that K36 is composed of a hexasaccharide repeat unit (Table III.2, column I, page 96). The presence of a monomethyl rhamnose residue is attributable to a branch point, but the absence of any terminal residue (either tetramethyl hexose or trimethyl rhamnose) indicates the pyruvate acetal must be present on a terminal sidechain sugar (see Figure III.1, page 95, for g.l.c. trace).

A sample of Klebsiella K36 polysaccharide was autohydrolysed at pH 2.2 overnight. A ¹H n.m.r. spectrum of the recovered non-dialyzable polymer showed the absence of pyruvate acetal and of reducing protons, consistent with a product having still a high degree of polymerisation (see Appendix III, spectrum No. 3). In the anomeric region signals observed had the same chemical shift as for the ungraded K36 polysaccharide but the signal at τ 5.13, $J_{1,2}$ 7 Hz

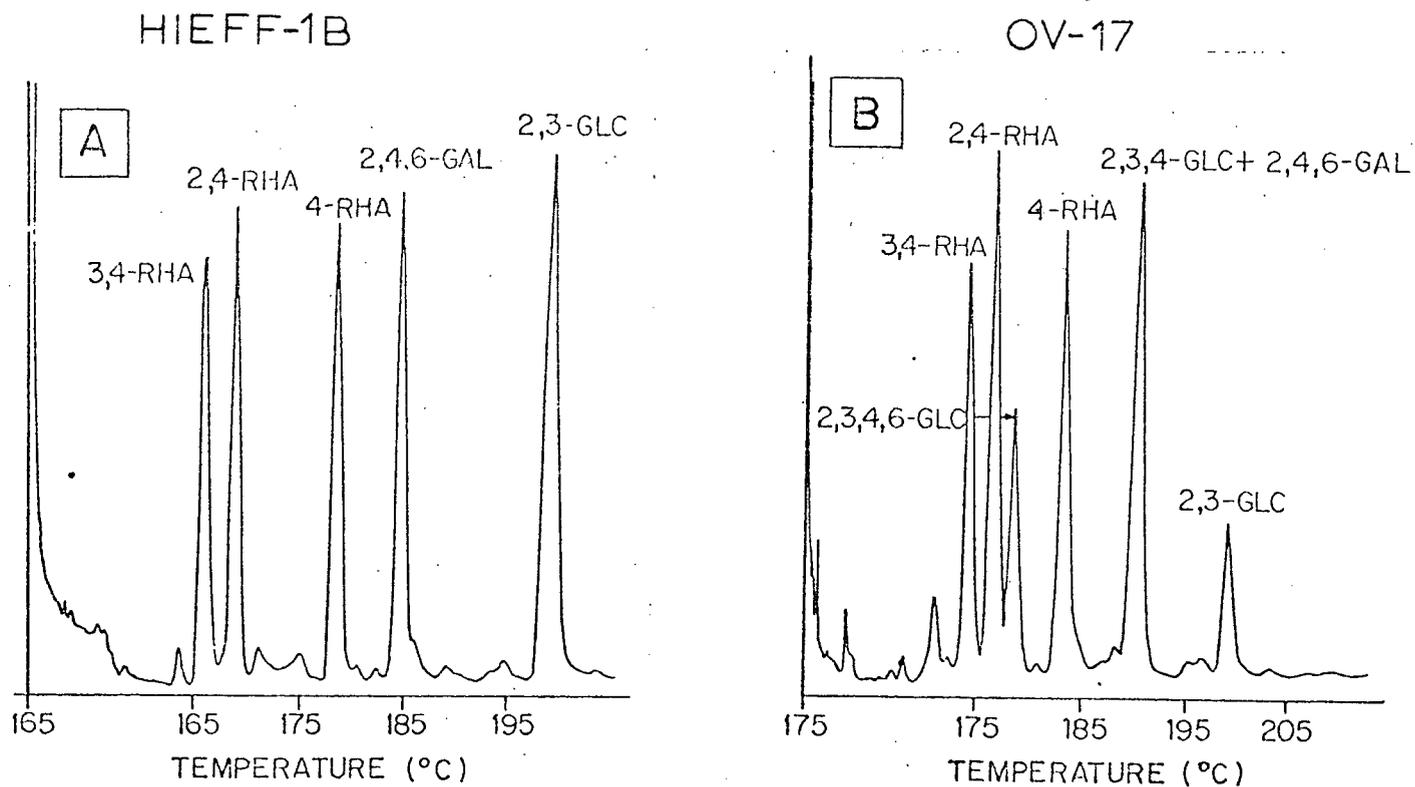


Figure III.1 G.l.c. separation of partially methylated alditol acetates obtained from: A, native K36 polysaccharide; B, degraded K36 polysaccharide. For column details see Table III.2, page 96.

Table III.2 Methylation Analyses of Original and Degraded K36 Capsular Polysaccharide

Methylated Sugars ^a (as Alditol Acetates)	T ^b			Mole % ^{c,d}		
	Column A ^e (HIEFF 1B)	Column B ^f (OV 17)	Column C ^g (OV 225)	I	II	III
	3,4-Rha	0.87	0.79	0.90	16.6	17.0
2,4-Rha	1.00	0.91	0.96	16.3	19.7	20.6
2,3,4,6-Glc	1.00	1.00	1.00		10.6	8.4
4-Rha	1.45	1.24	1.40	16.9	20.0	19.0
2,4,6-Gal	1.77	1.59	1.68	20.3	} 25.0	17.5
2,3,4-Glc	1.77	1.59	1.77			6.2
2,3-Glc	2.32	2.00	2.56	30.0	7.5	7.4

^a3,4-Rha = 1,2,5-tri-0-acetyl-3,4,-di-0-methyl-L-rhamnitol, etc.

^bRetention time relative to the alditol acetate derivative of 2,3,4,6-tetra-0-methyl-D-glucose. ^cI, original polysaccharide, column A; II, degraded polysaccharide (see text for details), column B; III, degraded polysaccharide, column C.

^dValues corrected using 'Effective carbon response' molar-response factors¹³. ^eProgramme; 165° for 8 minutes and then 2° per minute to 200°.

^fProgramme; 175° for 8 minutes and then 2° per minute to 210°.

^gProgramme; 180° for 8 minutes and then 2° per minute to 200°.

did not integrate as a complete proton; the reason for this is explained below.

Methylation of this degraded material and reduction with lithium aluminum hydride followed by derivatisation and g.l.c.-m.s. analysis (Table III.2, columns II, III, page 96) gave a complex mixture of components which could not be separated completely on any one g.l.c. column. Analysis of the mixture on columns of OV 17, OV 225 and HIEFF 1B (see Figure III.1, page 95) enabled all peaks to be resolved. The presence of a terminal glucose unit demonstrates that the pyruvate acetal is attached to this residue in the original polysaccharide. The relative molar ratios of 2,3,4-tri-O-methyl-D-glucose and 2,3,4,6-tetra-O-methyl-D-glucose indicate that the D-glucuronic acid residue is also present in the side chain and that the terminal glucosidic bond is being partially cleaved (~40%) during the autohydrolysis; this is consistent with the ¹H n.m.r. spectrum.

Periodate Oxidation.

Periodate oxidation¹⁴ of K36 polysaccharide proceeded rapidly with 3 moles of periodate being consumed per mole of repeat unit after 25 hours (theoretical = 3 moles). The consumption gradually increased to a value of 4.2 moles per mole of repeat unit over a further 48 hours. By ¹H n.m.r. of the sodium borohydride reduced periodate product it was evident that part of this slow increase was due to the hydrolysis of the pyruvate acetal at the reaction pH of 3.0.

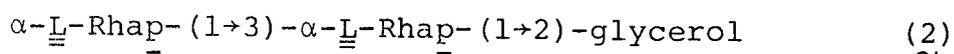
Smith hydrolysis¹⁵ of the sodium borohydride reduced periodate product (3 mole uptake) using 0.5 M trifluoroacetic acid at room temperature for 16 hours and subsequent reduction again with sodium borohydride yielded a mixture which was resolved by gel chromatography (Bio-Gel P-4). A significantly large amount of polymeric material was eluted shortly after the void volume followed by an oligosaccharide derivative, oligomer 1. This incomplete hydrolysis, using trifluoroacetic acid to effect the Smith degradation, of a polyol derived from these capsular polysaccharides was observed first¹⁶ during studies on K18 and has been noted subsequently¹⁷ in the case of K55. There are earlier reports¹⁸ on incomplete hydrolysis attributed to uronic^{19,20} acid or ester sulfate²¹ groups, but it is particularly surprising in the three bacterial examples cited that in each case it is an oxidised rhamnose unit which is resistant to the acid conditions used.

Oligomer 1 ($[\alpha]_D -60^\circ$) was obtained pure and, after hydrolysis and paper chromatography, was shown to contain rhamnose, galactose and glycerol. ¹H n.m.r. of 1 (see Appendix III, spectrum No. 4) indicated (Table III.1, page 92) the presence of three non-reducing anomeric signals; one β hexose signal at τ 5.34 ($J_{1,2}$ 7.5 Hz) attributable to the D-galactose and two further signals at τ 5.01 ($J_{1,2}$ 1.9 Hz) and τ 4.92 ($J_{1,2}$ 1.8 Hz) due to two α -L-rhamnose signals. ¹³C n.m.r. of 1 (see Appendix III, spectrum No. 5) gave a spectrum with 21 carbon signals which indicated that in addition to the

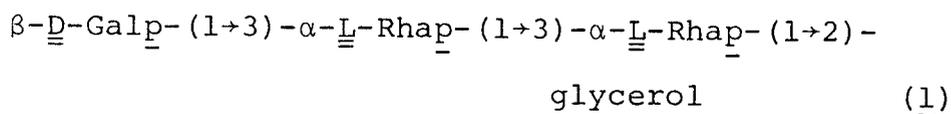
three hexose sugars a three carbon fragment (glycerol) was present on the 'reducing' terminus. Attempted acetolysis of 1 in an effort to remove the small aglycone (glycerol) preferentially²² was unsuccessful.

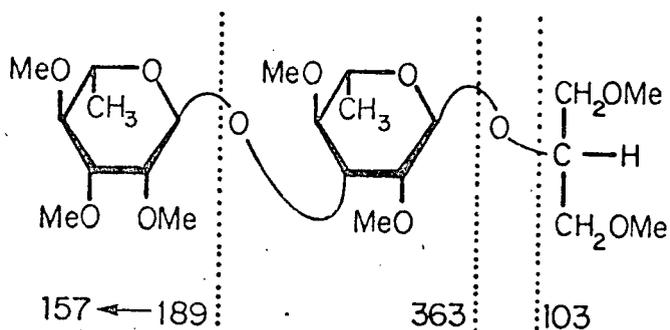
Smith periodate degradation of 1 and gel chromatography of the resulting material yielded oligomer 2 ($[\alpha]_D -67^\circ$) which gave only rhamnose and glycerol on hydrolysis. In the ¹H n.m.r. spectrum (see Appendix III, spectrum No. 6) the only anomeric signals corresponded to two α -L-rhamnose protons (non-reducing) at τ 4.94 ($J_{1,2}$ 2 Hz) and τ 5.03 ($J_{1,2}$ 2 Hz). Methylation analysis of 2 gave 2,3,4-tri-O-methyl-L-rhamnose and 2,4-di-O-methyl-L-rhamnose (the volatile 1,3-di-O-methylglycerol derivative was lost under reduced pressure during work up). Mass spectra of fully methylated 2 using electron impact (e.i.), chemical ionisation (c.i.) and field desorption (f.d.) modes²³ also confirmed the presence of two 6-deoxy hexoses and a glycerol moiety. A parent peak at m/e 482 was obtained in the f.d. spectrum. The c.i. spectrum also gave a parent peak (M-1) at m/e 481 and the origins of other major peaks in the spectrum are shown on page 100.

The structure of 2 is thus established as

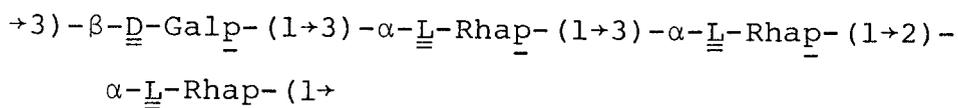


and that of 1 as





The methylation analysis of the original and autohydrolyzed polymers indicates that the side chain is the disaccharide unit $\underline{\underline{D}}\text{-Glc} \frac{1}{\beta} \frac{4}{\underline{\underline{D}}}\text{-GlcA}$ — and it is the glucose unit that carries the 4,6- $\underline{\underline{O}}$ -(1-carboxyethylidene) acetal. It is thus now possible to deduce that the glycerol fragment in $\underline{\underline{1}}$ above originated from a 2-substituted $\underline{\underline{L}}$ -rhamnose residue. Hence, the backbone of K36 is established as



Attachment of side chain

Having determined the sequence of the tetrasaccharide backbone, the nature of the side chain and that the sugar on

which branching occurs yields 4-0-methyl-L-rhamnose in the methylation analysis, the only remaining problem is to identify which of the three rhamnose units constitutes the branch point. This was achieved by examination of the products of partial hydrolysis.

Characterization of oligosaccharides from partial hydrolysis

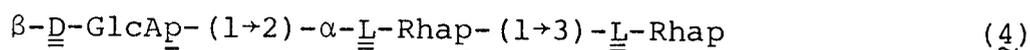
Partial hydrolysis of K36 polysaccharide (2 M trifluoroacetic acid, 3 h, 95°) and separation of acidic and neutral components by ion exchange yielded a mixture of acidic oligomers which were separated by gel permeation chromatography (Bio-Gel P-4). Three pure oligomers (3, 4, 5) were obtained in decreasing quantities.

Compound 3 [α]_D -12°, by ¹H and ¹³C n.m.r. (see Table III.1, page 92) was shown to contain one non-reducing β anomeric signal and two reducing anomeric signals attributable to a 6-deoxy hexose (see Appendix III, spectra No.'s. 7, 8). Methylation, lithium aluminum hydride reduction, hydrolysis, and g.l.c. of the alditol acetates gave 2,3,4-tri-0-methyl-D-glucose and 3,4-di-0-methyl-L-rhamnose. The structure of 3 is thus established as

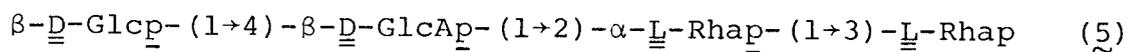


Compound 4, [α]_D -31.3°; ¹H n.m.r. (see Table III.1, page 92) indicated in the anomeric region the presence of one β non-

reducing signal, one α non-reducing signal, and two signals due to a reducing 6-deoxy hexose (see Appendix III, spectra No.'s 9, 10). Methylation of 4 and subsequent lithium aluminum hydride reduction, hydrolysis and derivatisation as alditol acetates gave, in addition to the two components obtained from 3, 2,4-di-0-methyl-L-rhamnose. Compound 4 is therefore established as



Compound 5 was obtained in only very small quantity. ^1H n.m.r. (see Table III.1, page 92) indicated the presence of two non-reducing β anomeric signals, one α non-reducing anomeric signal, and two signals attributable to a reducing 6-deoxy hexose (see Appendix III, spectrum No. 11). Methylation analysis (as described for compounds 3 and 4) yielded 2,3,4,6-tetra-0-methyl-D-glucose, 2,3-di-0-methyl-D-glucose, 2,3-di-0-methyl-L-rhamnose and 3,4-di-0-methyl-L-rhamnose in equal proportions. Having established the structure of 4, it is possible to assign the structure of 5 as



A partial hydrolysis of K36 using 0.3 M trifluoroacetic acid for 3 hours at 95° was also performed. After separation of acidic and neutral components the acidic compounds were

III.4 Experimental

General Methods

Descending paper chromatography was carried out using Whatman No. 1 paper and the following solvent systems (v/v) were used: (A) Freshly prepared 1-butanol-acetic acid - water (2:1:1); (B) ethyl acetate-pyridine - water (8:2:2). Chromatograms were developed using silver nitrate²⁵. Concentrations were carried out under reduced pressure at bath temperatures which did not exceed 40°. Analytical g.l.c. separations were performed using a Hewlett Packard 5700 instrument fitted with dual flame ionisation detectors. An Infotronics CRS-100 electronic integrator was used to measure peak areas. Stainless steel columns 1/8 inch outside diameter were used with a carrier gas flow rate of 20 ml/min. Columns used were (A) 3% of HIEFF 1B on Gas Chrom Q (100/120 mesh); (B) 3% of OV 17 on the same support; (C) 3% of OV 225 on the same support; (D) 0.2% of polyethylene glycol succinate, 0.2% of polyethylene glycol adipate, 0.4% of XF 1150 on the same support. All columns were 6 feet in length. Preparative g.l.c. was performed using an F and M model 720 instrument with dual thermal conductivity detectors. Columns (6 feet by 1/4 inch) analogous to those used for analytical separations were used. G.l.c.-m.s. was carried out using a Micromass 12 instrument fitted with a Watson-Biemann separator. Spectra were recorded at 70 eV with an ionisation current of 100 µA and an ion source temperature of 200°. Other electron

impact m.s. were run on a MS 902 instrument while chemical ionisation spectra (of oligosaccharide derivatives) were recorded on a Finnigan 3200 quadrapole mass spectrometer. ^1H n.m.r. spectra were run on a Varian XL-100 instrument. Samples run in D_2O were exchanged and freeze dried three or four times in 99.9% D_2O and then finally dissolved in 100% D_2O . Acetone (τ 7.77, measured against aqueous sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS)) was used as an internal standard. Spectra were recorded at approximately 90° . Spectra of methylated derivatives were run using CDCl_3 as solvent with an internal Me_4Si standard. ^{13}C n.m.r. spectra were obtained using a Varian CFT-20 instrument and were run at ambient temperature in 50% D_2O using acetone (31.07 p.p.m. from DSS) as internal standard. Circular dichroism spectra were recorded on a Jasco J20 automatic recording spectropolarimeter. Optical rotations were measured at $23 \pm 2^\circ$ on a Perkin-Elmer model 141 polarimeter using a 10 cm cell. IR spectra were recorded using a P.E. 457 spectrophotometer. Gel permeation chromatography was carried out using a column (2.5 x 120 cm) of Bio-Gel P-4 (minus 400 mesh). The column was irrigated with water at a flow rate of approximately 7 ml/h. Fractions (1-2 ml.) were collected, freeze dried and chromatographed on paper.

Preparation and properties of K36 capsular polysaccharide.

A culture of Klebsiella K36 (8306) was obtained from Dr. I. Ørskov, Copenhagen, and was grown on a medium of 8 g

NaCl, 4 g K_2HPO_4 , 1 g $MgSO_4 \cdot 7H_2O$, 2 g $CaCO_3$, 120 g sucrose and 8 g Bacto yeast extract in 4 l of water for 4 days. The cells were harvested and diluted to 1600 ml with water containing 1% phenol. This solution was then centrifuged for 6 hours at 30,000 r.p.m. in a Beckman T4 zonal rotor. After this time the clear supernatant was separated and concentrated to approximately 400 ml. Crude polysaccharide, obtained by precipitation into ethanol (2 l), was redissolved in 400 ml. of water, precipitated with 10% Cetavlon, redissolved in 4 M NaCl (500 ml.) and then dialysed against tap water overnight. Lyophilization of this solution yielded 10 g of the polysaccharide, $[\alpha]_D -56^\circ$ (c 3.4, water).

Carboxyl-reduction of the native polysaccharide.

A sample of the polysaccharide was reduced using the procedure described by Taylor and Conrad⁸. Two treatments were required to achieve complete reduction as estimated by titration. One 'treatment' is described as follows. The polysaccharide (approximately 1 g) in the Na salt form was dissolved in approximately 100 ml of distilled water and the pH adjusted to 4.75 by the addition of 0.1 M HCl. A ten fold molar excess (over the total moles of carboxyl groups available for reduction in the polysaccharide) of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluene sulphonate (8 g) was added as the solid. As the reaction proceeded the pH began to rise and this was compensated for by titration to

pH 4.75 with the 0.1 M HCl. After approximately 5 hours the pH remained almost constant indicating the coupling reaction was complete. A total of 14.8 ml of 0.1 M HCl had been consumed. NaBH₄ (120 ml of a 2 M solution) was then added while the pH was kept between 5 and 7 by the simultaneous addition of 4 M HCl. During this part of the procedure octanol was used as an antifoaming agent and a gentle stream of air was directed at the reaction surface to also reduce foaming. The 'reduced' solution was dialysed against running tap water for two days and subsequently lyophilised.

Sugar and methylation analysis of native polysaccharide.

Hydrolysis of a sample (20 mg) of carboxyl-reduced K36 with 2 M trifluoroacetic acid at 95° overnight and subsequent derivatisation of the liberated monosaccharides as alditol acetates gave L-rhamnitol pentaacetate, D-galactitol hexaacetate (m.p. 168°) and D-glucitol hexaacetate (m.p. 99°) in the ratio of 2.98:1.00:2.01. (Column D; programmed at 120° for 8 minutes and then 1°/min to 200°.) Circular dichroism of the rhamnitol pentaacetate showed $\epsilon_{213}^{\text{MeCN}} - 1.12$ and the glucitol hexaacetate $\epsilon_{213}^{\text{MeCN}} + 1.83$. Circular dichroism of 2,4,6-tri-O-methyl-D-galactitol triacetate obtained from the total hydrolysis of methylated K36, was shown to be positive. Methanolysis of K36 polysaccharide using refluxing 3% methanolic hydrogen chloride overnight yielded methyl α -L-rhamnopyranoside; (m.p. 108 - 110°).

Methylation of K36 using Hakomori conditions followed by a subsequent Purdie²⁶ treatment yielded a product which showed no hydroxyl absorption in the i.r. spectrum. The Hakomori methylation was performed as follows. The polysaccharide (~1 g), in the 'free acid' form, was carefully dried under a heat lamp and high vacuum overnight. In a 250 ml round bottom flask fitted with a rubber serum cap and containing a stirrer bar, the polysaccharide was dissolved in 50 ml of dried and distilled dimethyl sulphoxide. The flask was flushed with dry nitrogen. Methyl sulphanyl anion (20 ml of a 2 M solution) was then added and the reaction left to proceed with stirring for approximately 6-8 hours. After this time a small portion of the reaction mixture was removed and tested with triphenylmethane to ensure excess methylsulphanyl anion was present. Methyl iodide (10 ml) was then added while the reaction was kept at 4° in an ice bath. After 0.5 hours the solution was dialysed against running tap water for approximately 20 hours and lyophilised. Yield; 900 mg. The Purdie methylation was performed as follows. The material obtained from the Hakomori treatment (above) was dissolved in 15 ml of methyl iodide and the solution was refluxed for 48 hours, during which time 4 x 0.5 g of Ag₂O was added. The Ag₂O was then separated by centrifugation and refluxed in 150 ml of CHCl₃ for 3 hours and removed by centrifugation again. The combined supernatants (MeI and CHCl₃) were evaporated and yielded 550 mg of the fully methylated polysaccharide. The methylated polysaccharide, as shown by i.r. and ¹H n.m.r.

(CDCl_3), contained impurities and was purified by precipitation into petroleum ether (30-60°).

The purified methylated material was reduced with lithium aluminum hydride in refluxing tetrahydrofuran overnight and, following hydrolysis with 2 M trifluoroacetic acid at 95° for 8 hours, the mixture was reduced with sodium borohydride and then acetylated. G.l.c. (column A; programmed at 160° for 4 minute and then 1° per minutes to 190°) and m.s. of the collected components allowed their assignment as in column I, Table III.2, page 96.

Methylation analysis of degraded polysaccharide.

Autohydrolysis at pH 2.2 for 16 hours at 95° gave a very soluble polymer of K36 which, after dialysis against running tap water overnight and lyophilization, was examined by ^1H n.m.r. (D_2O , 90°, see Appendix III, spectrum No. 3). The degraded material was methylated (as above) and subsequently reduced with lithium aluminum hydride in refluxing tetrahydrofuran overnight. Hydrolysis, reduction and acetylation, as performed on the native polysaccharide, followed by g.l.c. analysis showed the presence of seven components. (See columns II and III, Table III.1, page 92). A column of OV 17 (column B; programmed at 170° for 4 minutes and then 1°/min to 190°) achieved complete separation of 2,3,4,6-tetra-0-methyl-D-glucose and 2,4-di-0-methyl-L-rhamnose, while the separation of 2,3,4-tri-0-methyl-D-glucose and 2,4,6-tri-0-methyl-D-galactose was achieved on OV 225 (column C; programmed at 170°

for 8 minutes and then 2° per minute to 200°).

Periodate Oxidation.

Capsular polysaccharide (1 g) was dissolved in 250 ml of a solution of NaIO₄ (0.05 M) and NaClO₄ (0.2 M)²⁷. The pH of this solution was 2.7. The solution was kept in the dark at 4°. After 20 hours 3 moles of periodate per repeat unit had been consumed (theoretical = 3), rising to 4.5 moles after 172 hours.

Following the addition of ethylene glycol (2 ml) after 25 hours, reduction with sodium borohydride, dialysis, deionisation, lyophilization and removal of borate, the product was hydrolysed (0.5 M trifluoroacetic acid) at room temperature overnight. After removal of the acid and subsequent reduction with sodium borohydride the material (2 x 175 mg) was applied to the top of a Bio-Gel P-4 column (2.5 x 120 cm). Elution with water gave a range of polymeric products appearing soon after the void volume (blue dextran) followed by pure oligomer 1 which had R_{Glc} 1.0 (solvent A).

Oligomer 1, 35 mg, had $[\alpha]_D -60.7^\circ$ (c 2.5, water) and was examined both by ¹H n.m.r. (D₂O, 90°) and ¹³C n.m.r. spectroscopy (see Appendix III, Spectra No.'s 4,5). Hydrolysis of 1 (0.5 M trifluoroacetic acid, 4 hours, 95°) showed by paper chromatography the presence of galactose, rhamnose and glycerol.

Periodate oxidation of 1 (12 mg) in 0.05 M NaIO₄ at 4° overnight and subsequent addition of ethylene glycol, reduction

with sodium borohydride, Smith hydrolysis (0.5 M trifluoroacetic acid at room temperature overnight) and reduction with sodium borohydride yielded material which was applied to the top of a Bio-Gel P-4 column (2.5 x 120 cm). Component 2 was isolated (4 mg) having R_{Glc} 0.73 (solvent A) and $[\alpha]_D -67^\circ$ (c 0.35, water). 1H n.m.r. of 2 showed anomeric signals at τ 4.94, 1H, $J_{1,2}$ 2 Hz and τ 5.03, 1H, $J_{1,2}$ 2 Hz (see Appendix III, spectrum No. 6). Hydrolysis of a small portion of oligomer 2 gave only L-rhamnose and glycerol by paper chromatography. Methylation of 2 yielded 2.5 mg of the permethylated derivative having R_F 0.38 on t.l.c. (ethyl acetate). M.s. of this derivative in the f.d. mode gave major peaks at m/e 481, 482, 483 and 484 corresponding to M-1, M, M+1 and M+2 respectively. Chemical ionisation (methane) m.s. of this same derivative gave a spectrum which showed, inter alia, the following fragments (relative intensities in brackets): 88(11), 99(12), 125(27), 127(29), 129(10), 149(10), 159(11), 189(100), 190(12), 205(49), 233(9), 363(49), 364(10), 391(18), 481(2). The peak at 391 is attributed to a dioctyl phthalate impurity. Hydrolysis of the permethylated derivative of 2 (2 M trifluoroacetic acid, 6 hours, 90°) and subsequent derivatisation yielded 2,3,4-tri-O-methyl-L-rhamnose and 2,4-di-O-methyl-L-rhamnose in equal amounts. The dimethyl glycerol fragment was too volatile to be isolated during work up.

Partial hydrolysis of polysaccharide.

K36 (1 g) was hydrolysed for 3 hours at 95° in 2 M trifluoroacetic acid. After removal of the acid by evaporation with several portions of water, the mixture was neutralised (NaOH) and then applied to the top of a column (2 x 20 cm) of Dowex 1-X2 (formate form). The column was then washed with 1 ℓ of distilled water. The acidic components were eluted with 10% HCOOH and after evaporation to dryness this material weighed 350 mg. Paper chromatography (solvent B) showed no neutral monosaccharides were present in the mixture.

Gel chromatography (Bio-Gel P-4; 2 x 175 mg) of the neutralised (NaOH) oligomers did not give good separation of the mixture of components. Selected fractions containing small oligomers were rerun (Bio-Gel P-4) yielding pure aldobiouronic acid 3 (60 mg), aldotriouronic acid 4 (35 mg), and an acidic tetrasaccharide 5 (4 mg).

The aldobiouronic acid 3 (R_{Glc} 1.0, solvent A) showed $[\alpha]_D -12^\circ$ (c 1.2, water)²⁸. ¹H n.m.r. (D₂O, 90°) showed anomeric signals at τ 5.37, 1H, $J_{1,2}$ 7 Hz; τ 4.63, 0.6 H, $J_{1,2}$ 1.8 Hz; and τ 5.17, 0.4 H, singlet (see Appendix III, spectra No.'s 7,8). The ¹³C n.m.r. spectrum showed two anomeric signals; one 104.9 p.p.m. downfield from T.M.S. attributable to the C₁ of the D-glucuronic acid and another at 93.7 p.p.m. corresponding to the C₁ of the reducing α -L-rhamnose. No resonances for either the reducing β -L-rhamnose or the C₆ of a hexose²⁹ were observed. Hakomori methylation of 3 yielded permethylated aldobiouronic acid which on t.l.c. had R_F 0.80

(ethyl acetate). Reduction of this compound with lithium aluminum hydride in tetrahydrofuran yielded a compound with R_F 0.32 on t.l.c. (ethyl acetate:ethanol, 9:1). Subsequent hydrolysis (2 M trifluoroacetic acid at 95° for 6 hours), reduction and acetylation yielded two components as their alditol acetates in a 1:1 ratio corresponding to 2,3,4-tri-0-methyl-D-glucose and 3,4-di-0-methyl-L-rhamnose (g.l.c. column A). G.l.c.-m.s. confirmed the assignment of the above components.

The aldotriouronic acid 4 (R_{Glc} 0.88, solvent A) showed $[\alpha]_D -31^\circ$ (c 1.1, water)³⁰. 1H n.m.r. (D_2O , 90°) showed anomeric signals at τ 5.34, 1H, $J_{1,2}$ 7 Hz; τ 4.65, 1H, $J_{1,2}$ 2 Hz; τ 4.89, 0.6 H, $J_{1,2}$ 1.8 Hz; and τ 5.13, 0.4 H, singlet. The ^{13}C n.m.r. spectrum displayed signals in the anomeric region at 105.2 p.p.m., 101.6 p.p.m. and two signals (94.7 p.p.m., 94.1 p.p.m.) attributable to the α and β reducing anomeric carbons of rhamnose (see Appendix III, spectrum No.'s 9,10).

Hakomori methylation of 4 yielded permethylated aldotriouronic acid having R_F 0.70 on t.l.c. (ethyl acetate). Reduction with lithium aluminum hydride in tetrahydrofuran yielded the corresponding product having R_F 0.27 on t.l.c. (ethyl acetate:ethanol, 9:1). Hydrolysis (2 M trifluoroacetic acid at 95° for 6 hours), reduction and acetylation yielded the alditol acetates corresponding to 2,3,4-tri-0-methyl-D-glucose, 3,4-di-0-methyl-L-rhamnose and 2,4-di-0-methyl-L-rhamnose, (g.l.c. column A), in the ratio 1:1:1. G.l.c.-m.s. confirmed the methylation pattern of the above components.

Compound 5 ($R_{\underline{Glc}}$ 0.64, solvent A) gave a ^1H n.m.r. spectrum (see Appendix III, spectrum No. 11) having anomeric resonances at τ 5.35, 1H, $J_{1,2}$ 7 Hz; τ 5.19, 1H, $J_{1,2}$ 7.5 Hz; τ 4.63, 1H, $J_{1,2}$ 2 Hz; τ 4.89, 0.6 H, $J_{1,2}$ 1.8 Hz; and τ 5.13, 0.4 H, singlet. Methylation of 5 yielded the permethylated derivative having $R_{\underline{F}}$ 0.75 on t.l.c. (ethyl acetate:ethanol, 92:8). Reduction of this material with lithium aluminum hydride in tetrahydrofuran gave a compound with $R_{\underline{F}}$ 0.37 on t.l.c. (chloroform:acetone, 2:1). Hydrolysis and conversion to alditol acetates (same as for compounds 1 and 2) yielded four components in a 1:1:1:1 ratio. G.l.c. (column B) and g.l.c.-m.s. identified these as being the alditol acetates of 2,3,4,6-tetra-0-methyl-D-glucose, 2,3-di-0-methyl-D-glucose, 3,4-di-0-methyl-L-rhamnose and 2,4-di-0-methyl-L-rhamnose. Compound 5 is therefore β -D-Glc \underline{p} -(1 \rightarrow 4)- β -D-GlcAp-(1 \rightarrow 2)- α -L-Rhap-(1 \rightarrow 3)- α -L-Rhap.

A partial hydrolysis of K36 (200 mg) using 0.3 M trifluoroacetic acid at 95° for 3 hours was carried out. Treatment of the hydrolyzate as described for the preceding partial hydrolysis yielded, in addition to components 3, 4 and 5 (above), a component 6 (8 mg; $R_{\underline{Glc}}$ 0.64, solvent A) which was initially thought to be a hexasaccharide from its ^1H n.m.r. spectrum (see Appendix III, spectrum No. 12).

Compound 6 was reduced with lithium borodeuteride and then methylated. Two components, with $R_{\underline{F}}$ 0.73 (compound 6a) and $R_{\underline{F}}$ 0.57 (compound 6b) on t.l.c. (ethyl acetate:ethanol,

92:8) were obtained. Column separation on silica gel yielded 6a (4.5 mg) and 6b (2.5 mg).

Reduction of 6a with lithium aluminum hydride in tetrahydrofuran gave a compound with R_F 0.37 on t.l.c. (chloroform: acetone, 2:1). Hydrolysis reduction, acetylation and g.l.c. separation (column B) showed 6a to be identical to permethylated compound 5. G.l.c. and g.l.c.-m.s. gave the alditol acetates corresponding to 2,3-di-0-methyl-D-glucose, 2,4-di-0-methyl-L-rhamnose and 2,3,4,6-tetra-0-methyl-D-glucose, together with 3-0-acetyl-1,2,4,5-tetra-0-methyl-L-rhamnitol. (Some of the latter component, monodeuterated at C_1 , was lost during derivatisation.)

Reduction of 6b with lithium aluminum hydride in tetrahydrofuran gave a component with R_F 0.30 on t.l.c. (chloroform: acetone, 2:1). Hydrolysis reduction and acetylation followed by g.l.c./g.l.c.-m.s. (column A) gave peaks corresponding to the alditol acetates of 2,3,4-tri-0-methyl-D-glucose, 4-0-methyl-L-rhamnose and 2,3,4,6-tetra-0-methyl-D-galactose, together with 3-0-acetyl-1,2,4,5-tetra-0-methyl-L-rhamnitol. The ratio of the four components was 1:1:1:0.5 with 50% of the volatile tetra-0-methyl-L-rhamnose derivative, monodeuterated at C_1 , being lost under vacuum during work up. Compound 6b is thus the permethylated derivative of the tetrasaccharide as shown on the following page.

III.5 Bibliography for Section III.

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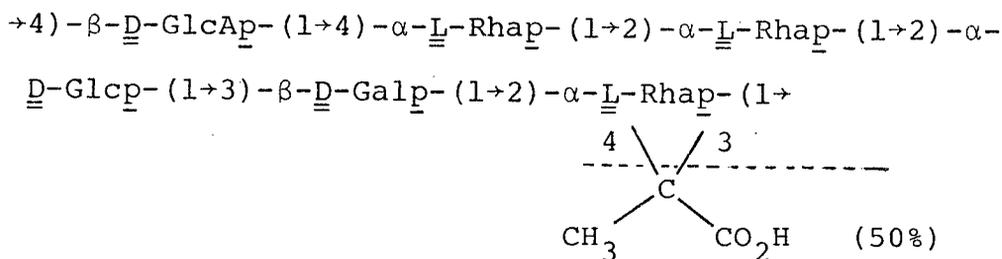
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IV. THE STRUCTURE OF Klebsiella SEROTYPE
K70 CAPSULAR POLYSACCHARIDE

IV.1 Abstract

Using the techniques of methylation analysis, uronic acid degradation, partial hydrolysis and periodate oxidation the structure of the capsular polysaccharide from Klebsiella serotype K70 has been investigated. Nuclear magnetic resonance was used extensively to characterise fragments obtained as a result of the various degradation procedures. The existence of a linear hexasaccharide repeat unit as shown below, with a pyruvate acetal attached to a 2-linked L-rhamnose residue on every second repeating unit, has been demonstrated.



IV.2 Introduction

Of the 81 different Klebsiella serotypes the structural analyses of approximately 30 of the capsular polysaccharides produced by these bacteria have been reported. Klebsiella K70 has been shown to contain glucuronic acid, galactose, glucose and rhamnose and is one of 11 serologically different K-types having this same qualitative composition^{1,2}. We report here the results of our structural investigation of this polysaccharide.

IV.3 Results and Discussion

The polysaccharide, isolated as previously described³ (see Section III.4, page 106), had $[\alpha]_D -43^\circ$ (c 2.8, water). Proton magnetic resonance (^1H n.m.r.) indicated the presence of one pyruvic acid acetal per 12 sugar residues^{4,5} (see Appendix III, spectrum No. 13). In the anomeric region (τ 4.5 - τ 5.5) six proton signals were observed while a nine proton doublet at τ 8.7, due to the CH_3 groups of 6-deoxy sugars, was also apparent (see Table IV.1, page 124). Carbon magnetic resonance (^{13}C n.m.r.) information⁶ was in agreement with the ^1H n.m.r. data (see Appendix III, spectrum No. 14), and in addition, indicated the presence of two hexose sugars; (two signals were observed between 60-62 p.p.m. indicative of the signals from C_6 of hexose sugars). The ^1H and ^{13}C n.m.r. spectra therefore indicate the presence of three rhamnose residues and two hexose residues. Knowing the qualitative composition of K70 capsular polysaccharide it can be deduced that the remaining sugar residue in the repeating unit must be a glucuronic acid unit.

Hydrolysis of K70 and paper chromatography of the hydrolysate indicated the presence of glucose, glucuronic acid, galactose and rhamnose. Progressive hydrolysis of the polysaccharide, monitored by paper chromatography, did not show the release of any one sugar preferentially and indicated K70 was probably a linear polysaccharide. Methanolysis of Klebsiella K70, reduction with sodium borohydride in dry

TABLE IV.1 N.M.R. Data for *Klebsiella* K70 Polysaccharide and Isolated Oligosaccharides^a

Compound	¹ H τ ^b (J _{1,2}) ^c , integral, assignment ^d	p.p.m. ^e	¹³ C assignment ^f
GlcA $\frac{1}{\beta}$ $\frac{4}{\beta}$ Rha-OH 1	4.89 (1.8 Hz), 0.6H, α-Rha-OH	103.70	β-GlcA
	5.14 (S), 0.4H, β-Rha-OH	94.55	α-Rha-OH
	5.28 (8 Hz), 1H, β-GlcA	93.23	β-Rha-OH
	8.70, (J _{5,6} 6 Hz), 3H, CH ₃ of Rha	17.82	CH ₃ of Rha
Glc $\frac{1}{\alpha}$ $\frac{3}{\alpha}$ Gal $\frac{1}{\beta}$ $\frac{2}{\beta}$ Rha-OH 2	4.59 (1.8 Hz), 0.6H, α-Rha-OH	105.51	β-Gal ^g
	4.85 (3.5 Hz), 1H, α-Glc	104.77	
	5.13 (S), 0.4H, β-Rha-OH	96.33	α-Glc
	5.34 (7*), 1H, β-Gal	93.92	α-Rha-OH
	8.70, (J _{5,6} 6 Hz), 3H, CH ₃ of Rha	93.59	β-Rha-OH
		17.56	CH ₃ of Rha

TABLE IV.1 Continued

Gal $\frac{1}{\beta}$ -Rha $\frac{1}{\alpha}$ -erythritol	4.78 (1.8 Hz), 1H, α -Rha	(16 signals overall)
	5.43 (7.5 Hz), 1H, β -Gal	105.75 β -Gal
	8.70, ($J_{5,6}$ 6 Hz), 3H, CH ₃ of Rha	100.19 α -Rha
		63.19 } { C ₆ of Gal
		61.77 } { C ₁ of erythritol
		61.74 } { C ₄ of erythritol
		17.35 CH ₃ of Rha
Gal $\frac{1}{\beta}$ -glycerol	5.45 (7.5 Hz), β -Gal	(9 signals overall)
		103.33 β -Gal
		62.44 } { C ₆ of Gal
		61.90 } {
		61.78 } { C ₁ , C ₃ of glycerol

TABLE VI.1 Continued

Native K70 Polysaccharide	4.78 (S*), 1H,	α -Rha	105.7	
	4.90 (S*), 2H	} α -Rha	103.8	six unassigned
	5.03 (S*), 1H		} α -Glc	
	5.23 (7 Hz), 1H,	β -GlcA	101.7	signals
	5.45 (7 Hz*), 1H,	β -Gal	100.9	
	8.41 (S), 1.5H,	CH_3 of pyruvate	95.7	
	8.70, ($J_{5,6}$ 6 Hz), 9H,	CH_3 of Rha	62.20	} C_6 of Gal
			61.30	
			17.50	CH_3 's of Rha

Footnotes:

^aFor origin of oligosaccharides 1, 2, 5, and 6 see text. ^bChemical shift taken relative to internal acetone; τ 7.77 downfield from D.S.S. ^cS=singlet. Those values marked with an asterisk were broad signals. ^de.g., α -Rha=proton on C_1 of L-Rha residue which is α -linked (Gal=D-Gal). ^eChemical shift quoted as p.p.m. downfield from T.M.S. relative to internal acetone; 31.07 p.p.m. from D.S.S. ^fAs for ^d but for anomeric ^{13}C nuclei. ^gTwo values are given for $^{13}\text{C}_1$ of β -Gal as the chemical shift of this carbon atom is affected by α - and β -equilibrium of the reducing Rha residue. 105.51 = β -Gal(α -Rha), 104.77 = β -Gal(β -Rha).

methanol and then hydrolysis, yielded a mixture of sugars which was shown by paper chromatography to contain only glucose, galactose and rhamnose. Reduction and acetylation to convert this mixture to alditol acetates and gas chromatographic (g.l.c.) analysis confirmed the presence of the above three sugars in the proportions 31:17:52. This result indicated that K70 contained glucose, glucuronic acid, galactose and rhamnose residues in the ratio of 1:1:1:3 respectively, and that the polysaccharide consisted of a hexasaccharide repeat unit. The glucose and rhamnose were shown to be of D- and L- configuration respectively by the circular dichroism⁷ curves of the alditol acetates. The configuration of the galactose was shown to be D- by the circular dichroism curve of the 2,4,6-tri-O-methyl derivative obtained from methylation analysis.

Methylation analysis^{8,9} of the native polysaccharide and of a sample of K70 which had been autohydrolysed at pH 2.2 for 16 hours, confirmed the existence of a hexasaccharide repeat unit (see Table IV.2, page 128). The concomitant increase of 3,4-di-O-methyl-L-rhamnose and loss of L-rhamnose after the pyruvate acetal had been removed, located the acetal on a 2-linked L-rhamnose and confirmed the existence of this substituent on every second repeat unit of Klebsiella K70.

Partial acidic hydrolysis of K70 was performed using 0.5 M trifluoroacetic acid at 95° for 45 minutes. Following separation of acidic and neutral material by ion exchange

TABLE IV.2 Methylation Analyses of Native and Depyruvalated^a
Klebsiella K70 Capsular Polysaccharide

<u>Methylated sugars^b</u> (as alditol acetates)	T ^c	<u>Mole %^d</u>	
		I	II
3,4-Rha	0.89	22.6	33.1
2,3-Rha	1.00	17.0	16.9
3,4,6-Glc	1.50	17.2	17.3
Rha	1.58	10.2	
2,4,6-Gal	1.67	16.6	17.3
2,3-Glc (from <u>D</u> -GlcA)	2.40	16.3	15.3

^aDepyruvalated \equiv K70 polysaccharide autohydrolysed at pH 2.2 for 16 hours at 95°.

^b3,4-Rha \equiv 1,2,5-tri-O-acetyl-3,4-di-O-methyl-L-rhamnitol, etc.

^cRetention time of partially methylated alditol acetates, relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol, on a column of 3% of HIEFF 1B on Gas-Chrom Q (100-120 mesh) programme at 160° for 8 min. and then 2° per min. to 190°.

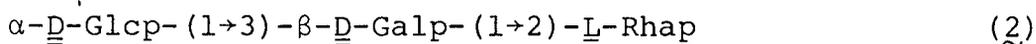
^dColumn I; native polysaccharide. Column II; depyruvalated polysaccharide.

chromatography each fraction was analysed using a Bio-Gel P-4 gel permeation column. From the acidic fraction a pure oligomer 1 with $[\alpha]_D -30^\circ$ was isolated¹⁰. The 1H and ^{13}C n.m.r. spectra were in agreement with 1 being an aldobio-uronic acid (see Table IV.1, page 124, and also Appendix III, spectrum No.'s 15, 16). Compound 1 was reduced with lithium borodeuteride and then methylated. Subsequent reduction with lithium aluminum hydride, hydrolysis and g.l.c.-m.s.^{9,11} analysis of the liberated partially methylated monosaccharides as alditol acetates, gave components confirming the structure of 1 as being



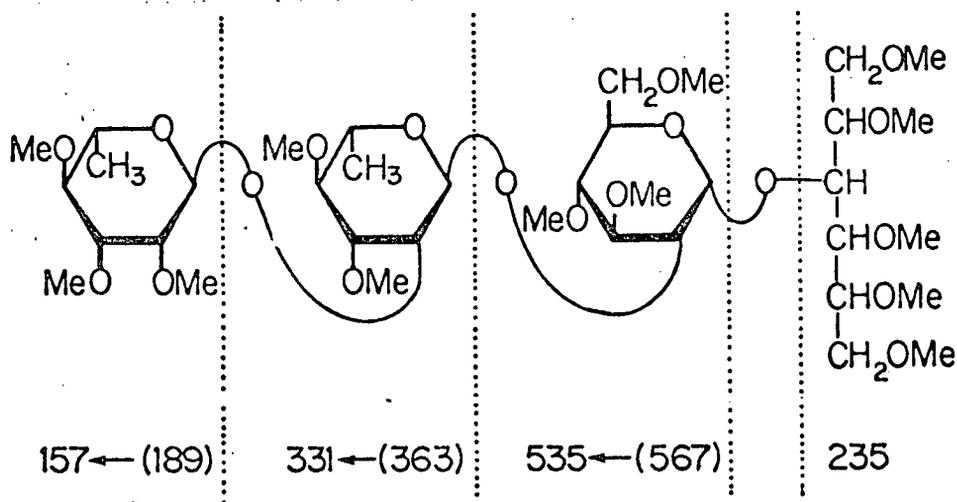
The neutral material obtained from the partial hydrolysis was also separated on a Bio-Gel P-4 column. Oligomer 2 with $[\alpha]_D +10^\circ$ was isolated and from the 1H and ^{13}C n.m.r. spectra (see Table IV.1, page 124, and also Appendix III, spectra No.'s 17, 18) was thought to be a trisaccharide. The ^{13}C n.m.r. spectrum of 2 was of particular interest in that the terminal reducing sugar (easily recognisable in 1H and ^{13}C n.m.r. as a rhamnose residue) was affecting the chemical shift of the non-terminal (middle) anomeric carbon. Hence, the C_1 signals from the reducing rhamnose at 93.92 p.p.m. (α) and 93.59 p.p.m. (β) influenced the anomeric carbon on the middle sugar so that it gave two signals at 105.51 and 104.77 p.p.m. The chemical shift (approximately 105 p.p.m.)

of this double signal allowed it to be assigned to a β -hexose. Reduction of 2 with lithium borodeuteride and subsequent methylation yielded a product which was hydrolysed, reduced, acetylated and analysed by g.l.c.-m.s.^{9,11}. Identification of the three components observed (see Experimental) established 2 as being



A sample of Klebsiella K70 from which the pyruvate acetal had been selectively removed by autohydrolysis, was permethylated and then subjected to a uronic acid degradation¹². This technique can provide valuable information in sequencing a linear polysaccharide, even though the β -elimination reactions may not go to completion. After treatment with methylsulphinyl anion in dimethyl sulphoxide and subsequent mild acid treatment to cleave the enol ethers formed during the elimination reactions, the mixture of products was reduced with sodium borohydride in dry methanol. Methylation of this reduced mixture with Purdie's reagents and chromatography on silica gel facilitated the isolation of a component 3a which, by ¹H n.m.r., was shown to have signals at τ 4.88 (2H, $J_{1,2}$ broad) and τ 5.08 (1H, $J_{1,2}$ 3 Hz) in the anomeric region (see Appendix III, spectrum No. 19). The mass spectrum of 3a (e.i.) was in agreement with that expected for a trisaccharide alditol¹¹. The origins of some pertinent fragments are outlined in the accompanying diagram. It is perhaps

worthy of note that besides the fragments aA_1 , baA_1 , $cbaA_1$, by which the sequence of $3a$ can be determined, the main fragments arose from the cJ_1 , bcJ_1 and $abcJ_1$ ions. These latter fragments are in agreement with the inter glycoside linkages being as shown

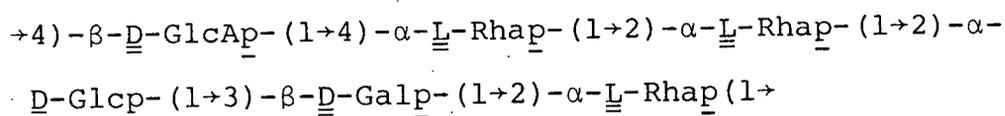


Hydrolysis of $3a$ and analysis by g.l.c.-m.s.^{9,11} of the alditol acetates gave peaks corresponding to 1,2,4,5,6-penta-0-methyl-D-galactitol, 2,3,4-tri-0-methyl-L-rhamnose, 3,4-di-0-methyl-L-rhamnose and 3,4,6-tri-0-methyl-D-glucose.

Ethylation of the sodium borohydride reduced uronic acid degradation product gave a component $3b$ and analysis of this material as described for the permethylated product ($3a$) gave 1,5-di-0-ethyl-2,3,6-tri-0-methyl-D-galactitol, 4-0-ethyl-2,3-di-0-methyl-L-rhamnose, 3,4-di-0-methyl-L-rhamnose and 3,4,6-tri-0-methyl-D-glucose. Bearing in mind that the sequence of compound 2 (obtained from partial

hydrolysis) is already known it is possible to write the structure of 3a as the permethylated derivative of α -L-Rhap-(1 \rightarrow 2)- α -L-Rhap-(1 \rightarrow 2)- α -D-Glc_p-(1 \rightarrow 3)-D-galactitol. The 4-O-ethyl-2,3-di-O-methyl-L-rhamnose obtained from 3b indicates the terminal rhamnose was linked through the hydroxyl on C-4 in the polysaccharide. The sequence of reactions in the β -elimination of K70 is outlined in Figure II.6, page 42.

The results obtained from partial hydrolysis and uronic acid degradation studies are sufficient to establish the repeating unit of K70 as being that shown below

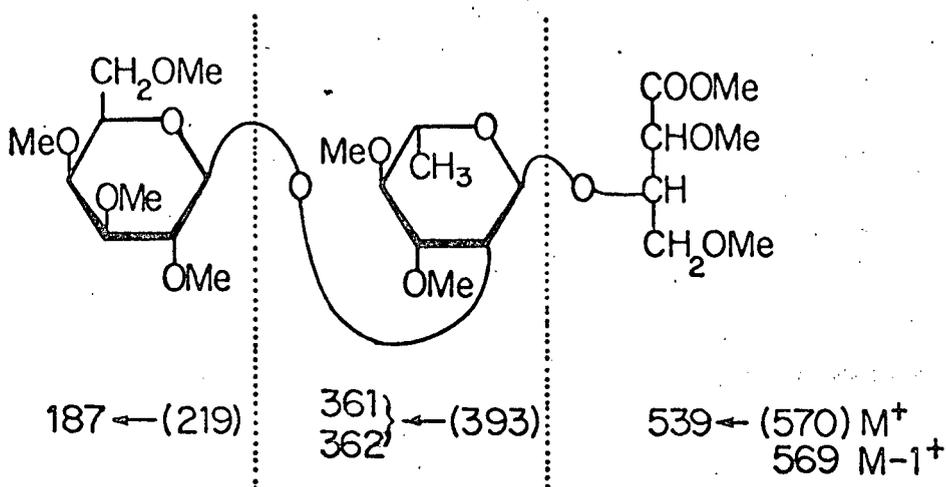


The pyruvate moiety, shown to occur on only 50% of the repeating units by methylation analysis and ^1H n.m.r. of K70, is present as an acetal spanning the hydroxyls on C_3 and C_4 of a 2-linked rhamnose. The presence of two 2-linked rhamnoses in the repeating unit made it necessary to locate the pyruvate more precisely. This was done using periodate oxidation¹³.

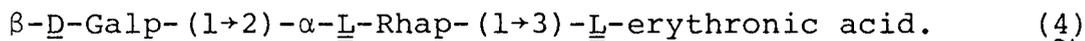
When Klebsiella K70 polysaccharide was reacted with sodium periodate¹⁴ a plateau value was obtained corresponding to the consumption of six moles of oxidant per true repeat unit (taken as twelve sugar units). This value is

three moles below the theoretical value. However, following reduction of the polyaldehyde product, dialysis, and treatment again with the periodate solution, a further 4.2 moles of periodate was consumed. (This value rose to 5 moles after 156 hours.) These results indicate that hemiacetal formation¹⁵ is occurring during the initial periodate treatment. By ¹H n.m.r. it was shown that the twice periodate-oxidised polyalcohol contained only a trace of the pyruvate acetal and it was assumed this had been cleaved by hydrolysis at the reaction pH of 4.2. Another sample of Klebsiella K70 was subjected to oxidation in a buffered solution (pH 5.0) of periodate and after 24 hours the product was reduced with sodium borohydride and the polyalcohol was oxidised again using the same conditions. The polyalcohol derived from this sequence of reactions was then methylated and following reduction with lithium aluminum hydride and hydrolysis, the partially methylated sugars were analysed by g.l.c.-m.s.^{9,11} as their alditol acetates. Only 1,2,3,4,5-penta-0-acetyl-L-rhamnitol and 1,3,5-tri-0-acetyl-2,4,6-tri-0-methyl-D-galactitol were found to be present in a 1:2 ratio. This result indicated the pyruvate acetal was still present on the doubly oxidised polymer and that complete periodate oxidation had been achieved. The polyalcohol from the fully oxidised polymer was then subjected to a Smith hydrolysis¹⁶ and reduced with sodium borohydride. The mixture of products was separated by gel permeation chromatography giving three

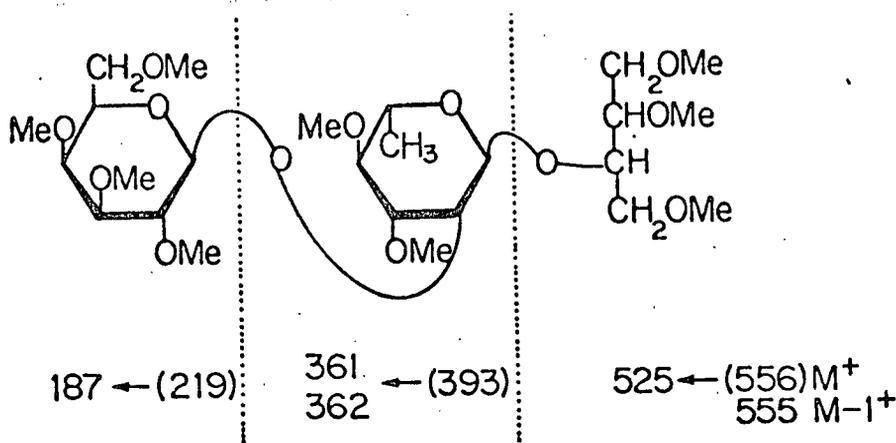
pure oligomers; 4, 5 and 6. Compound 4 showed $[\alpha]_D -16^\circ$ and ^1H n.m.r. spectroscopy (see Table IV.1, page 124, and also Appendix III, spectra No.'s 20, 21) revealed signals attributable to anomeric protons at τ 4.72 (1H, $J_{1,2}$ 1.8 Hz) and τ 5.43 (1H, $J_{1,2}$ 7.5 Hz). Hydrolysis of 4 showed by paper chromatography the presence of galactose, rhamnose and two other components which were indistinguishable from erythronic acid and erythronolactone. Reduction of 4 with sodium borohydride gave an oligomer which was chromatographically indistinguishable from oligomer 5 and some unchanged starting material. Methylation of 4 gave a product which had a strong absorbance at 1750 cm^{-1} in the i.r. spectrum. The mass spectrum of permethylated 4 using the chemical ionisation (c.i.) mode¹⁷ was consistent with the structure given. The source of some pertinent fragments is illustrated:



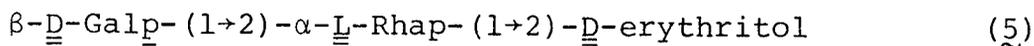
Oligomer 4 is therefore shown to be



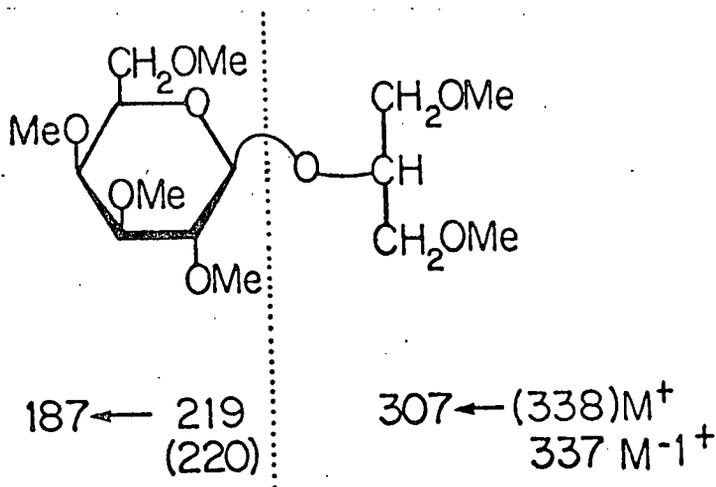
Compound 5 had $[\alpha]_D +4.5^\circ$ and the ^1H n.m.r. spectrum showed signals at τ 4.78 (1H, $J_{1,2}$ 2 Hz) and τ 5.43 (1H, $J_{1,2}$ 7.5 Hz) in the anomeric region. In the ^{13}C n.m.r. spectrum 16 signals overall were observed; two signals in the anomeric region at 105.75 and 100.19, and three signals in the region (60-63 p.p.m.) associated with the C-6 of hexose sugars (see Table IV.1, page 124, and also Appendix III, spectra No.'s 22,23). Hydrolysis of 5 showed by paper chromatography the presence of rhamnose, galactose and erythritol. Methylation of 5 yielded a product which upon hydrolysis and conversion to alditol acetates gave components corresponding to 2,3,4,6-tetra-0-methyl-D-galactose and 3,4-di-0-methyl-L-rhamnose. The mass spectrum of permethylated 5 in the c.i. mode was consistent with the structure shown below. Some pertinent fragments are indicated:



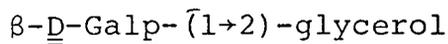
Compound 5 is therefore established as being



Compound 6, $[\alpha]_D +3.7^\circ$ was shown by ^{13}C n.m.r. to contain nine carbons overall. In the anomeric region one signal at 103.33 p.p.m. was apparent and three signals between 60-63 p.p.m. were observed. In the ^1H n.m.r. spectrum only one signal at τ 5.45 (1H, $J_{1,2}$ 7.5 Hz) was apparent in the anomeric region (see Table IV.1, page 124, and also Appendix III, spectra No.'s 24, 25). Hydrolysis of 6 showed by paper chromatography the presence of galactose and glycerol. Methylation of 6 and subsequent hydrolysis gave a component corresponding to 2,3,4,6-tetra-O-methyl-D-galactose as its alditol acetate on g.l.c.-m.s. The mass spectrum of permethylated 6 (c.i.) was consistent with the structure shown below. Some pertinent fragments are indicated:



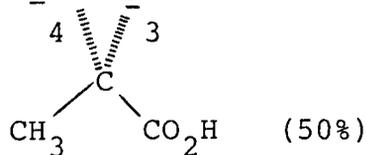
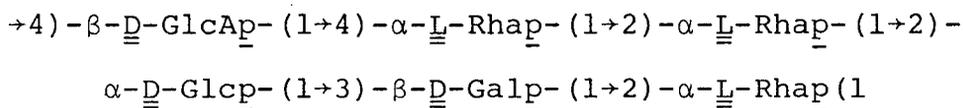
Oligomer 6 is thus established as being



(6)

The isolation of oligomers 4, 5, 6 is compatible with the linear sequence of K70 shown previously. Compound 5 results from the spontaneous lactonisation of periodate oxidised D-glucuronic acid residues during the double oxidation-reduction procedure used and the subsequent partial reduction of this lactone with sodium borohydride results in the reduction of the acid group to a primary alcohol. Effectively, some of the D-glucuronic acid residues give rise to erythronic acid and some give rise to erythritol, either of which are found as terminating glycosides in 4 and 5. The survival of the 2-linked rhamnose through the periodate oxidation indicates that the pyruvate acetal was attached to this sugar during the oxidation.

The evidence presented for Klebsiella K70 is consistent with the structure being as shown below. Because of the good resolution obtained in ^1H and ^{13}C n.m.r. studies of K70, it is probable that the pyruvate acetal is distributed evenly throughout the polysaccharide and is therefore present on every second repeat unit.



IV.4 Experimental

General methods

Instrumentation used was the same as previously described³ (see Section III.4, page 105). For descending paper chromatography the following solvent systems (v/v) were used: (A) (2:1:1) freshly prepared 1-butanol-acetic acid-water; (B) (8:2:2) ethyl acetate-pyridine-water. Analytical g.l.c. separations were performed using stainless steel columns (1/8 inch x 6 feet) with a carrier gas flow rate of 20 ml/min. Columns used were (A) 0.2% of polyethylene glycol succinate, 0.2% of polyethylene glycol adipate, 0.4% of XF 1150 on Gas Chrom Q (100/120 mesh); (B) 3% of HIEFF 1B on the same support. Analogous columns (1/4 inch x 6 feet) were used for preparative g.l.c. separation.

Preparation and properties of Klebsiella K70 capsular polysaccharide.

A culture of Klebsiella K70, obtained by courtesy of Dr. I. Ørskov, was grown and isolated as described previously for Klebsiella K36³. The polysaccharide showed $[\alpha]_D -43^\circ$ (c 2.8, water). The ¹H n.m.r. spectrum of K70 was recorded on a solution of 20 mg/ml in D₂O at approximately 90° and the ¹³C n.m.r. spectrum was recorded on a solution of 150 mg/ml in 50% D₂O at 40°.

Sugar analysis.

This was performed as described before³, viz. methanolysis, reduction with sodium borohydride in anhydrous methanol, hydrolysis, reduction and acetylation. The alditol acetates derived from rhamnose, galactose and glucose were separated by g.l.c. (column A, programmed from 120° to 190° at 1° per minute) and found to be present in the ratio of 31:17:52 respectively. Preparative g.l.c. gave galactitol hexaacetate (m.p. 166-168°) and glucitol hexaacetate (m.p. 99°). Circular dichroism of the rhamnitol pentaacetate showed $\epsilon_{213}^{\text{MeCN}} - 1.52$, and of the glucitol hexaacetate showed $\epsilon_{213}^{\text{MeCN}} + 1.83$. By comparison with authentic standards this confirmed the L- and D- configurations of the two sugars respectively. The configuration of the galactose residue was determined by examining the circular dichroism curve of 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-galactitol, obtained during methylation analysis. This component showed $\epsilon_{213}^{\text{MeCN}} + 0.26$ and by comparison with an authentic standard, established the galactose as being in the D-configuration.

Methylation analysis.^{8,9}

A sample of Klebsiella K70 which had previously been passed through a column of Amberlite IR-120(H⁺) was methylated by the Hakomori⁸ procedure. Methylation was found to be incomplete and treatment with silver oxide in methyl iodide (Purdie) was required to obtain a product

showing no absorbance at 3600 cm^{-1} in the i.r. spectrum. A ^1H n.m.r. spectrum of this material showed a broad doublet at τ 8.76 (due to the CH_3 of rhamnose) which integrated as 9 protons and a sharp singlet at τ 8.43 (due to the CH_3 of the pyruvate acetal). These two signals were in the ratio of 6:1. In the anomeric region signals were observed at τ 4.81, 2H, $J_{1,2}$ broad; τ 5.05, 2H, $J_{1,2}$ 2 Hz; τ 5.27, 1H, $J_{1,2}$ 7 Hz; and τ 5.52, 1H, $J_{1,2}$ 7 Hz.

Reduction of a sample of methylated K70 with lithium aluminum hydride in refluxing tetrahydrofuran overnight yielded a product with no carbonyl absorption (1750 cm^{-1}) in the i.r.. Hydrolysis with 2 M trifluoroacetic acid at 95° overnight and subsequent reduction with sodium borohydride followed by acetylation in pyridine-acetic anhydride yielded a mixture of partially methylated alditol acetates. This mixture was analysed by g.l.c.-m.s.^{9,11}. The alditol acetates of 3,4-di-0-methyl-L-rhamnose, 2,3-di-0-methyl-L-rhamnose, L-rhamnose, 3,4,6-tri-0-methyl-D-glucose, 2,4,6-tri-0-methyl-D-galactose and 2,3-di-0-methyl-D-glucose were identified in the ratio of 1.5:1:0.5:1:1:1. (See Table IV.2, page 128 for exact ratios and column used.)

A sample of Klebsiella K70 which had been autohydrolysed on a steam-bath for 16 hours at pH 2.2 was methylated as described for the native K70 polysaccharide. In the ^1H n.m.r. spectrum the singlet at τ 8.43 was absent. Reduction with lithium aluminum hydride, hydrolysis, reduction with

sodium borohydride and acetylation as for the non-degraded, methylated material yielded a mixture of partially methylated alditol acetates which were analysed by g.l.c.-m.s.^{9,11}.

The same components as obtained from methylated native K70 were identified with the exception that no L-rhamnose was present. The ratios of the five components were 2:1:1:1:1.

(See Table IV.2, page 128 for exact ratios and g.l.c. column used.) The concomitant loss of the 1,2,3,4,5-tetra-O-acetyl-L-rhamnitol and gain in the amount of 1,2,5-tri-O-acetyl-3,4-di-methyl-L-rhamnitol indicates that the pyruvate residue was present in the native polysaccharide as an acetal spanning positions three and four of a 2-linked rhamnose.

Partial acidic hydrolysis.

Klebsiella K70 polysaccharide (500 mg) was hydrolysed with 0.5 M trifluoroacetic acid at 95° for 45 minutes. After removal of the acid by several successive evaporations with water, the material was dialysed against tap water overnight. The nondialysable material (250 mg) was lyophilised and subjected to the same treatment as above, i.e. hydrolysis and dialysis. The dialysable material from the hydrolyses (400 mg) was then separated into neutral and acidic components using Dowex 1-X2 (formate form) resin.

The acidic components were neutralised with sodium hydroxide and then applied to the top of a Bio-Gel P-4 column (1.8 x 100 cm) which was subsequently irrigated with

water at a flow rate of 4 ml/h. Fractions (1-2 ml) were collected, lyophilised and analysed by paper chromatography. A major component (1, 50 mg) with R_{Glc} 1.0 (solvent A) and $[\alpha]_D -30^\circ$ (c 1.3, water)¹⁰, was obtained. 1H n.m.r. of 1 gave signals attributable to anomeric protons at τ 5.28, 1H , $J_{1,2}$ 8 Hz; τ 4.89, 0.6H, $J_{1,2}$ 1.8 Hz; and τ 5.14, 0.4H, singlet. A 6 Hz doublet (3 protons) at τ 8.70 was also apparent (see Appendix III, spectrum No. 15). This spectrum is consistent with 1 being an aldobionuronic acid with a reducing rhamnose unit. In the ^{13}C n.m.r. spectrum (see Appendix III, spectrum No. 16) a nonreducing signal at 103.70 p.p.m. and 2 reducing signals at 94.55 p.p.m. and 94.23 p.p.m. were observed in the anomeric region. No signal attributable to the C_6 of a hexose was observed while a signal at 17.82 p.p.m. (CH_3 of 6-deoxy sugar) was apparent.

Reduction of 1 with lithium borodeuteride and subsequent methylation, hydrolysis (2 M trifluoroacetic acid, 95° , overnight) reduction with sodium borohydride and acetylation yielded two components identified by g.l.c.-m.s. (column B) as 1,5,6-tri-0-acetyl-2,3,4-tri-0-methyl-D-galactitol and 4-0-acetyl-1,2,3,5-tetra-0-methyl-L-rhamnitol. The latter component was monodeuterated at C_1 . (Some of the tetramethyl rhamnitol derivative was lost under diminished pressure during work up.)

The neutral oligomers from the partial hydrolysis of K70 were separated by gel chromatography using Bio-Gel P-4

also. Besides obtaining large quantities of monosaccharides, an oligomer 2, (12 mg) with R_{Glc} 0.50 (solvent A) and $[\alpha]_D +10^\circ$ (c 1.2, water) was isolated. A ^1H n.m.r. spectrum of 2 (D_2O , 90°) gave signals in the anomeric region as follows: τ 5.34, 1H, $J_{1,2}$ 7 Hz (broad); τ 4.85, 1H, $J_{1,2}$ 3.5 Hz; τ 4.59, 0.6 H, $J_{1,2}$ 1.8 Hz and τ 5.13, 0.4 H, $J_{1,2}$ singlet. A doublet at τ 8.70, 6 Hz, integrating as three protons was also observed (see Appendix III, spectrum No. 17). In the ^{13}C n.m.r. spectrum (see Appendix III, spectrum No. 18), besides a signal at 17.56 p.p.m. attributable to the CH_3 of a 6-deoxy sugar, five signals assigned to anomeric carbons were observed. Two signals from the C_6 of hexoses were also apparent (refer to Discussion and Table IV.1, page 124, for the assignment of the signals in the anomeric region).

Compound 2 was reduced with lithium borodeuteride and subsequently methylated. Hydrolysis with 2 $\underline{\underline{M}}$ trifluoroacetic acid, reduction with sodium borohydride and acetylation yielded alditol acetates corresponding to 2,3,4,6-tetra-0-methyl-D-glucose and 2,4,6-tri-0-methyl-D-galactose together with 2-0-acetyl-1,3,4,5-tetra-0-methyl-L-rhamnitol. G.l.c. and g.l.c.-m.s. were performed using column B. Some ($\sim 50\%$) of the tetra-0-methyl-rhamnitol derivative, monodeuterated at C_1 , was lost under diminished pressure during work up.

Uronic Acid Degradation.¹²

A sample of Klebsiella K70 which had been autohydrolysed at pH 2.2 for 16 hours at 95° (pyruvate acetal removed) was methylated and carefully dried. This material (140 mg) was then dissolved in 20 ml of a mixture of dimethylsulphoxide and 2,2-dimethoxy-propane (19:1). To this solution 5 mg of toluene-p-sulphonic acid was added and the mixture was stirred under nitrogen for 2 hours. Methylsulphinyl anion (2 M, 15 ml) was then added and the reaction was stirred overnight. Following the addition of 50% aqueous acetic acid to adjust the pH to 6.0 the solution was extracted with chloroform (3 x 25 ml). The combined chloroform extracts were back extracted with water (25 ml). The methylated material obtained from the chloroform extraction was then treated with 10% aqueous acetic acid at 95° for 1 hour. Removal of the acetic acid was achieved by lyophilisation. The degraded material was then reduced with sodium borohydride in dry methanol overnight to give a component 3 and this reduced product was divided into two equal portions. One portion was methylated using methyl iodide and silver oxide. Purification on silica gel (ethyl acetate) yielded component 3a (7 mg) with R_F 0.15 (ethyl acetate). ¹H n.m.r. of 3a (CDCl₃) revealed signals in the anomeric region at τ 4.88 (2H, $J_{1,2}$ broad) and τ 5.08 (1H, $J_{1,2}$ 3 Hz). Two doublets (6 Hz each) at τ 8.72 and τ 8.76, each integrating as three protons were also apparent (see Appendix III,

spectrum No. 19). Electron impact m.s. of 3a showed, inter alia, the following peaks: m/e 88(45), 101(28), 157(29), 189(100), 235(26), 295(19), 331(9), 363(9), 499(4), 535(3), 567(2) and 673(4). Hydrolysis of 3a (2 M trifluoroacetic acid, 95°, 6 hours) and g.l.c.-m.s. analysis of the alditol acetate derivatives of the partially methylated sugars gave peaks corresponding to 1,2,4,5,6-penta-0-methyl-D-galactitol, 2,3,4-tri-0-methyl-L-rhamnose, 3,4-di-0-methyl-L-rhamnose and 3,4,6-tri-0-methyl-D-glucose. A second portion of 3 was ethylated using ethyl iodide and silver oxide and following purification on silica gel (ethyl acetate) yielded a component 3b (6.2 mg) with R_F 0.19 (ethyl acetate). Hydrolysis of 3b and analysis by g.l.c.-m.s. as alditol acetates gave peaks corresponding to 4-0-ethyl-2,3-di-0-methyl-L-rhamnose 1,5-di-0-ethyl-2,4,6-tri-0-methyl-D-galactitol, 3,4-di-0-methyl-L-rhamnose and 3,4,6-tri-0-methyl-D-glucose.

Periodate Oxidation of K70 polysaccharide.

A sample (400 mg) of native polysaccharide was dissolved in 100 ml of a solution of NaIO₄ (0.05 M) and NaClO₄ (0.2 M). The pH of this solution was 4.2. The reaction was allowed to proceed at 4° in the dark and the periodate consumption was followed by removing 5 ml aliquots which were analysed by the Muller-Friedberger¹⁸ method. Periodate consumption reached a plateau value after 26 hours of 6 moles per repeat unit of Klebsiella K70 (theoretical; 9

moles). Following the addition of ethylene glycol, dialysis, reduction with sodium borohydride and lyophilisation, the modified polysaccharide was subjected to a second oxidation as described above. A further 4.2 moles of periodate per repeat unit of K70 was consumed after 20 hours and this value gradually increased to 5.0 moles after 156 hours. Conversion of this material to the polyalcohol (as above) yielded 250 mg. of a non-dialysable polymer which did not contain any pyruvate acetal (as shown by ^1H n.m.r.).

Periodate oxidation of K70 polysaccharide (1 g) was also performed using a solution of NaIO_4 (0.05 M), NaClO_4 (0.2 M) and buffered at pH 5.0 with a sodium acetate buffer. After 24 hours the reaction was stopped by the addition of ethylene glycol and after dialysis overnight against running tap water, the material was reduced with sodium borohydride. Excess hydride was destroyed by the addition of acetic acid and the pH of the solution was adjusted to 6.5. Dialysis and lyophilisation yielded 950 mg of a polymeric material. Treatment again with NaIO_4 and NaClO_4 at pH 5.0 as described above and subsequent work up yielded 700 mg of a polymer which was considered to have undergone complete periodate oxidation.

A sample of the periodate oxidised product was methylated by the Hakomori procedure and then reduced using lithium aluminum hydride in refluxing tetrahydrofuran overnight. Subsequent hydrolysis (2 M trifluoroacetic acid, 95°, over-

night), reduction and acetylation yielded a mixture of alditol acetates which were analysed by g.l.c.-m.s. (column A). The alditol acetates of L-rhamnose and 2,4,6-tri-O-methyl-D-galactose were found to be present in the ratio of 1:2.

The remainder of the periodate oxidised material was then subjected to a Smith hydrolysis using 0.5 M trifluoroacetic acid at room temperature overnight and reduced with sodium borohydride. The mixture of products was then applied to the top of a Bio-Gel P-2 column (160 x 2.5 cm) which was irrigated with distilled water. Three pure oligomers (4, 5, 6) were isolated. Compound 4 (60 mg), $R_{Glc} 0.92$ (solvent A), had $[\alpha]_D -16^\circ$ (c 2.1, water). In the 1H n.m.r. spectrum signals attributable to anomeric protons were observed at τ 4.78, 1H, $J_{1,2}$ 1.8 Hz and τ 5.43, 1H, $J_{1,2}$ 7.5 Hz (see Table IV.1, page 124, and Appendix III, spectrum No. 20). The ^{13}C n.m.r. spectrum of 4 (see Appendix III, spectrum No. 21), was not completely consistent with the proposed structure for 4 due to the very slow relaxation of the terminal erythronic acid moiety. Hydrolysis of 4 (2 M trifluoroacetic acid, 95°, 6 hours) and paper chromatography (solvent A) revealed the presence of rhamnose, galactose and two further components with mobilities equivalent to erythronolactone and erythronic acid respectively. Methylation of 4 gave a component which showed a strong absorbance at 1750 cm^{-1} in the i.r. spectrum. The mass spectrum of

permethylated 4 (c.i.) showed, inter alia, the following peaks: m/e 111(64), 155(17), 186(15), 187(99), 219(55), 303(17), 361(100), 362(96), 539(13), 568(2), 569(3.0) and 570(1.5). Reduction of permethylated 4 with sodium borohydride in dry methanol and subsequent hydrolysis, reduction and acetylation gave a mixture of alditol acetates which were analysed by g.l.c.-m.s. The alditol acetates of 2,3,4,6-tetra-0-methyl-D-galactose and 3,4-di-0-methyl-L-rhamnose in equal amounts were identified. (The volatile di-0-methyl erythritol derivative was lost under reduced pressure during work up.)

Oligomer 5 (60 mg), R_{Glc} 0.96 (solvent A), had $[\alpha]_{\text{D}}$ +4.5° (c 1.5, water). In the ^1H n.m.r. spectrum (D_2O , 90°) signals were observed in the anomeric region at τ 4.78, 1H, $J_{1,2}$ 2 Hz and τ 5.43, 1H, $J_{1,2}$ 7 Hz. A doublet (6 Hz) at τ 8.70 integrating as three protons was also present (see Appendix III, spectrum No. 22). In the ^{13}C n.m.r. spectrum (see Appendix III, spectrum No. 23) 16 signals overall were observed. Two signals (105.75 p.p.m. and 100.19 p.p.m.) were assigned as arising from anomeric carbons while three signals appeared in the region associated with the C_6 of hexoses (60 to 62 p.p.m.). The ^{13}C n.m.r. spectrum is in agreement with an oligomer comprising a hexose sugar, a 6-deoxy sugar and a four-carbon aglycone. Hydrolysis of 5 (2 M trifluoroacetic acid, 95°, 4 hours) revealed galactose, rhamnose and erythritol by paper chromatography (solvent A). Methylation of 5 yielded a product with R_{F} 0.23 (ethyl

acetate). The mass spectrum (c.i.) of permethylated 5 showed, inter alia, the following peaks: m/e 71(65), 88(55), 101(49), 103(40), 112(100), 133(75), 145(39), 155(68), 163(75), 186(60), 220(99), 307(64), 337(30), and 338(6). Hydrolysis of permethylated 5 and subsequent derivatisation yielded alditol acetates corresponding to 2,3,4,6-tetra-0-methyl-D-galactose and 3,4-di-0-methyl-L-rhamnose. The volatile tri-0-methyl erythritol derivative was lost under vacuum during work up.

Compound 6 (20 mg), R_{Glc} 1.14 (solvent A), had $[\alpha]_D +3.7^\circ$ (c 0.8, water). In the 1H n.m.r. spectrum (D_2O , 90°) only one signal at τ 5.45, 1H, $J_{1,2}$ 7.5 Hz, was observable in the anomeric region. In the ^{13}C n.m.r. spectrum nine signals were observed overall. The presence of one signal (103.33 p.p.m.) in the anomeric region and three signals between 60-62 p.p.m. are in agreement with 6 being a hexose sugar linked through C_1 to a three carbon fragment (see Appendix III, spectra No.'s 24, 25). Hydrolysis (2 M trifluoroacetic acid, 95° , 4 hours) of 6 and paper chromatography (solvent A) showed the presence of galactose and glycerol. Methylation of 6 gave a compound with R_F 0.34 (ethyl acetate) and the mass spectrum (c.i.) of this component showed, inter alia, the following peaks: m/e 71(65), 75(24), 88(55), 101(49), 103(40), 112(100), 127(33), 133(75), 145(37), 155(68), 163(75), 186(60), 220(99), 307(64), and 337(30). Hydrolysis of this permethylated derivative yielded

only 1,5-di-0-acetyl-2,3,4,6-tetra-0-methyl-D-galactose after derivatisation as alditol acetates. Once again, the volatile di-0-methyl glycerol moiety was lost during work up.

IV.5 Bibliography for Section IV.

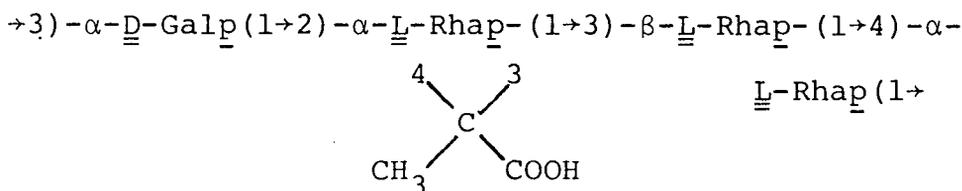
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V. THE STRUCTURE OF KLEBSIELLA SEROTYPE K32
CAPSULAR POLYSACCHARIDE

V.1 Abstract

The capsular polysaccharide from Klebsiella K32 has been studied using methylation, periodate oxidation and partial hydrolysis techniques. The polysaccharide is shown to comprise a four sugar repeating unit as shown below. Features of interest in this structure include the presence of a β -linked L-rhamnose sugar, and the extreme acid lability of the pyruvate acetal. N.m.r. has been used extensively to establish the nature of the anomeric linkages and to identify oligosaccharides obtained by the various degradative techniques used.



V.2 Introduction

Nimmich^{1,2}, in a qualitative analysis of the capsular polysaccharide from Klebsiella K32, found the presence of only galactose, rhamnose, and pyruvic acid. The absence of any uronic acid indicates that as a capsular antigen the polysaccharide relies on the pyruvic acid acetal for its overall negative charge and hence its virulence. Klebsiella K types 56³ and 72⁴ have been investigated previously and the present study completes the structural analysis of this trio of Klebsiella serotypes in which pyruvic acid is the only acid component.

V.3 Results and Discussion

Composition and n.m.r. spectra.

Isolation and purification of the polysaccharide were carried out as previously described⁵. One Cetavalon precipitation was performed. The purified material had $[\alpha]_D +113^\circ$. ¹H n.m.r. of the polysaccharide in deuterium oxide showed the presence of four anomeric protons and also indicated that three 6-deoxy sugars (rhamnose residues) and one pyruvate acetal were present per repeating unit^{6,7a,7b}. Acid hydrolysis of the polysaccharide and paper chromatography of the hydrolysate indicated the presence of rhamnose and galactose. Quantitation as alditol acetates proved these two sugars to be present in the proportions 72:28 respectively. The rhamnose was shown to belong to the L-series by circular dichroism measurements on the alditol acetate⁸ while the galactose was confirmed as being in the D-configuration by the action of D-galactostat on the free sugar.

Methylation of original and partially depyruvalated polysaccharide.

Complete methylation of K32 proved to be difficult. Due to the lability of the pyruvate acetal (see later) the polysaccharide could not be converted to the free acid form without the loss of a substantial proportion of the acetal. Methylation of the polysaccharide was hindered by the poor solubility

in dimethylsulfoxide of the native material in the sodium salt form. Two successive Hakomori⁹ treatments and a subsequent Purdie¹⁰ methylation were necessary to achieve a product which showed no hydroxyl absorbance in the infra red. Methylation analysis^{11,12} of the native polysaccharide and material that had been converted to its free acid analogue by passage through ion-exchange resin (Table V.1, page 157, columns I and II) indicated that Klebsiella K32 is composed of a linear tetrasaccharide repeat unit comprising three L-rhamnose residues (linked through positions two, three, and four respectively) and one D-galactose residue (linked through position three). The pyruvic acid unit is present as an acetal spanning position three and four of the two-linked L-rhamnose residue. Separation of the partially methylated alditol acetates corresponding to the 2,3-, 2,4-, and 3,4-di-O-methyl-L-rhamnose derivatives was achieved by gas liquid chromatography using a polar liquid phase of HIEFF-1B (see Table V.1, page 157 for details).

Periodate oxidation¹⁴.

Periodate oxidation of native K32 polysaccharide proceeded rapidly and after 40 hours analysis of the oxidised polymer (see Experimental) indicated complete oxidation. The oxidation was performed in 0.05 M sodium periodate and was buffered at pH 6.5 to minimise hydrolysis of the pyruvate acetal. However, the analysis of the polymer after 40 hours

Table V.1 Methylation Analyses of Native and Depyruvalated^a
Klebsiella K32 Capsular Polysaccharide

Methylated sugar ^b	T ^c	Mole % ^{d,e}	
		I	II
3,4-Rha	0.88	-	15.1
2,3-Rha	0.91	26.8	25.6
2,4-Rha	1.00	27.0	23.3
Rha	1.53	21.6	8.2
2,4,6-Gal	1.61	24.7	28.0

^aConversion of the native polysaccharide into the 'free acid' form by passage through IR-120(H⁺) resulted in approximately 50% of the pyruvate acetal groups being removed. ^b3,4-Rha ≡ 3,4-di-0-methyl-L-rhamnose, etc. ^cRetention time of corresponding alditol acetate relative to 1,5-di-0-acetyl-2,3,4,6-tetra-0-methyl-D-glucitol. Column used was 3% of HIEFF-1B on Gas Chrom Q (100-120 mesh) programmed from 160° to 190° at 1° per min. Column dimensions: 6' x 1/8". ^dFigures are corrected using molar response factors given by Albersheim et al.¹³ ^eI, native polysaccharide; II, depyruvalated polysaccharide.

Table V.2 N.M.R. Data for *Klebsiella* K32 Capsular Polysaccharide and Isolated Oligosaccharides.^a

Compound	¹ H n.m.r.		¹³ C n.m.r.	
	$\tau^b(J_{1,2})^c$, integral, assignment ^d		p.p.m. ^e	assignment ^f
Gal $\frac{1}{\alpha}$ 2Rha $\frac{1}{\alpha}$ 3Rha $\frac{1}{\beta}$ 3l-deoxy- <u>D</u> -erythritol 1	4.81 (1.8 Hz), 1H, α -Rha		(22 signals overall)	
	4.92 (3.5 Hz), 1H, α -Gal		100.41	} { α -Rha β -Rha
	5.21 (1 Hz), 1H, β -Rha		100.35	
	8.69 ($J_{5,6}$ 6 Hz), 6H, CH ₃ of Rha's		98.42	α -Gal
	8.79 (6 Hz), 3H, CH ₃ of l-deoxy- <u>D</u> -erythritol		62.06	} {C ₆ of Gal C ₄ of l-deoxy- <u>D</u> -erythritol
			61.76	
			17.95	} {CH ₃ of Rha's CH ₃ of l-deoxy- <u>D</u> -erythritol
			17.55	
		17.36		
Rha $\frac{1}{\beta}$ 3l-deoxy- <u>D</u> -erythritol 2 or 1b	5.24 (1 Hz), 1H, β -Rha		(10 signals overall)	
	8.69 ($J_{5,6}$ 6 Hz), 3H, CH ₃ of Rha		100.69	β -Rha
	8.79 (6 Hz), 3H, CH ₃ of l-deoxy- <u>D</u> -erythritol		62.10	C ₄ of l-deoxy- <u>D</u> -erythritol
			17.54	} {CH ₃ of Rha CH ₃ of l-deoxy- <u>D</u> -erythritol
			18.05	

Table V.2 Continued

Gal ¹ _α ² glycerol	4.83 (3 Hz), α-Gal	(9 signals overall)
3		98.89 α-Gal
~		61.28 } { C ₆ of Gal
		62.01 } {
		62.28 } { C ₁ , C ₃ of glycerol
Gal ¹ _α ² Rha ¹ _α ³ Rha ¹ _β ⁴ Rha-OH	4.80 (1.8 Hz), 1H, α-Rha	Insufficient material
	4.87 (1.8 Hz), 0.6H, α-Rha-OH	
4	4.94 (3 Hz), 1H, α-Gal	
~	5.12 (1 Hz), 0.4H, β-Rha-OH	
	5.25 (1 Hz), 1H, β-Rha	
	8.69 (J _{5,6} 6 Hz), 6H, CH ₃ of Rha's	
	8.79 (6 Hz), 3H, CH ₃ of 1-deoxy-D-erythritol	
$\frac{3}{\text{Gal}} \frac{1}{\alpha} \frac{2}{\text{Rha}} \frac{1}{\alpha} \frac{3}{\text{Rha}} \frac{1}{\alpha} \frac{4}{\text{Rha}} \frac{1}{\alpha}$	4.80 (2 Hz), 1H, α-Rha	Incomplete
$\begin{array}{c} \text{CH}_3 \\ \diagdown \\ \text{C} \\ \diagup \\ \text{COO}^- \text{Na}^+ \end{array}$	4.93 (b), 2H, α-Gal and α-Rha	
NATIVE K32 POLYSACCHARIDE	5.25 (S), 1H, β-Rha	
	8.41 (S), 3H, CH ₃ of acetal	
	8.70 (J _{5,6} 6 Hz), 9H, CH ₃ of Rha's	

^aFor origin of oligosaccharides 1, 2, 3 and 4 see text. ^bChemical shift taken relative to internal acetone; τ 7.77 downfield from D.S.S. ^cb≡broad, S≡singlet. ^de.g. α-Rha≡

(continued over page)

Table V.2 Continued

proton on C₁ of L-Rha residue which is α -linked. (Gal=D-Gal). ^eChemical shift quoted as p.p.m. downfield from T.M.S. relative to internal acetone; 31.07 p.p.m. from T.M.S. ^fAs for ^e, but for anomeric ¹³C nuclei.

oxidation at this pH indicated that some (15%) of the pyruvate acetals had in fact been removed. The derived polyol was subjected to a Smith degradation¹⁵ and the resulting mixture of oligosaccharides was separated using gel chromatography. Three chromatographically pure components, 1, 2, 3, were isolated.

Component 1 (120 mg) showed $[\alpha]_D +102^\circ$. ^1H and ^{13}C n.m.r. (see Appendix III, spectrum No. 26) studies indicated 1 to be a trisaccharide glycoside consisting of one galactose residue (α -linked), two rhamnose residues (one α -linked and the other β -linked), and 1-deoxyerythritol (see Table V.2, page 158 for data). The ^1H n.m.r. spectrum of 1 is shown (see Figure V.1, page 162) and clearly demonstrates the presence of three non-reducing anomeric proton signals. The signal at τ 5.21 with $J_{1,2}$ 1 Hz is from the anomeric proton of the β -linked L-rhamnose residue and is easily distinguishable from the anomeric proton signal (τ 4.81, $J_{1,2}$ 1.8 Hz) of the α -linked L-rhamnose residue. The full assignment of the spectrum appears in Table V.2, page 158. Field desorption mass spectrometry^{16,17} of 1 gave peaks at m/e 561, 583 and 599 corresponding to $(M+1)^+$, $(M+\text{Na})^+$ and $(M+\text{K})^+$ respectively. Periodate oxidation of 1 and subsequent reduction, Smith degradation and gel filtration yielded a component 1b, with $[\alpha]_D +104^\circ$. ^1H and ^{13}C n.m.r. indicated 1b is composed of one rhamnose residue (β -linked) and a deoxyerythritol fragment. (See Table V.2, page 158, and Appendix III, spectra No.'s 27,

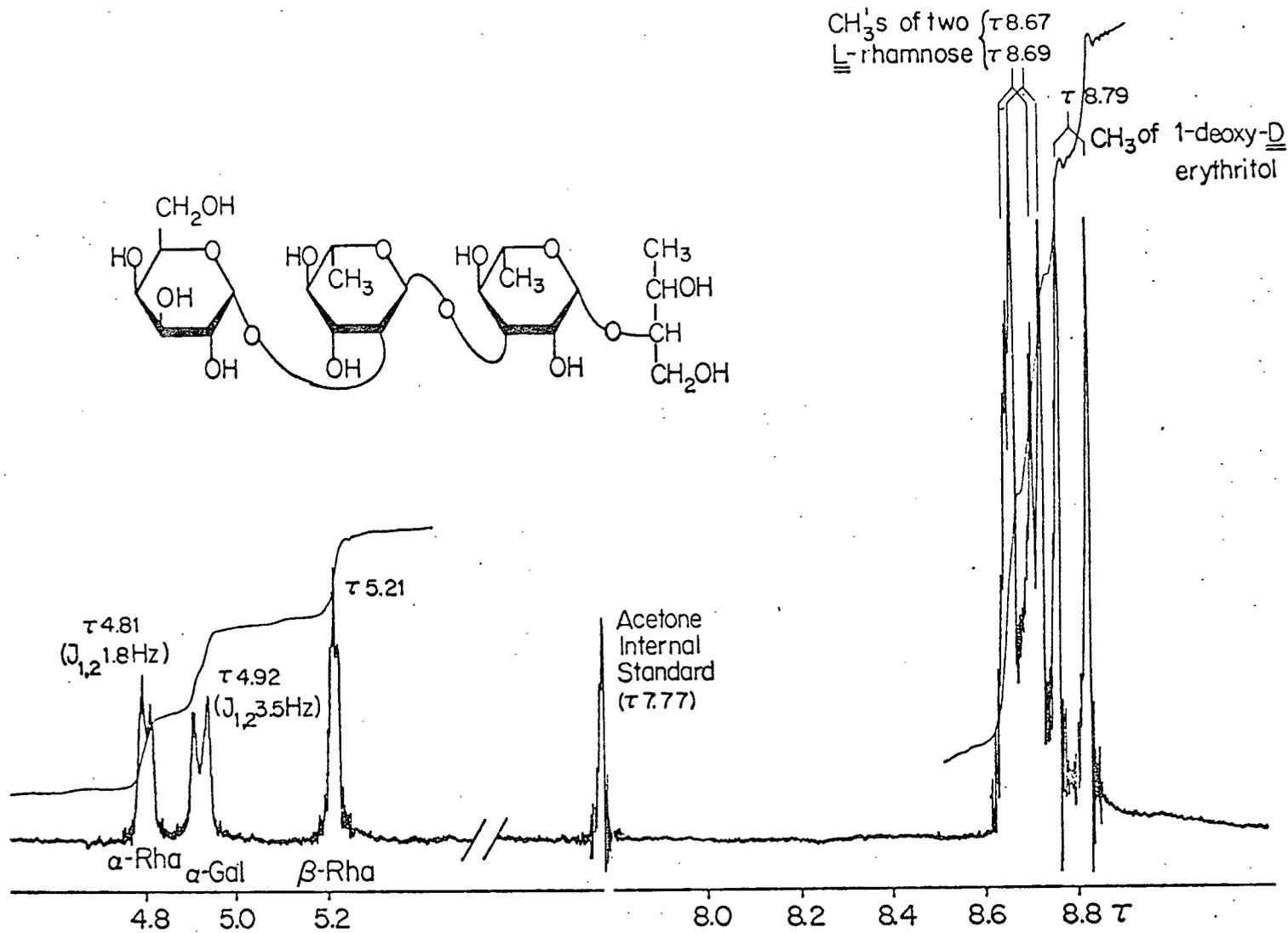
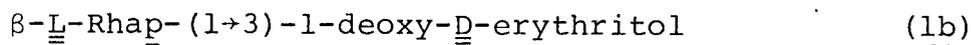
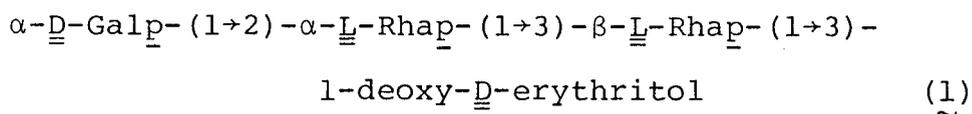


Figure V.1 ^1H n.m.r. spectrum of α -D-Galp-(1 \rightarrow 2)- α -L-Rhap-(1 \rightarrow 3)- β -L-Rhap-(1 \rightarrow 3)-1-deoxy-D-erythritol. (1)

28, for n.m.r. data). Hydrolysis of a small portion of 1b and paper chromatography of the hydrolysate gave two components indistinguishable from rhamnose and l-deoxyerythritol. Field desorption mass spectrometry of 1b gave peaks at m/e 253, 275 and 291, corresponding to $(M+1)^+$, $(M+Na)^+$ and $(M+K)^+$ respectively. The survival of the deoxyerythritol (which is obtained initially from periodate attack at a four linked L-rhamnose residue) during the periodate oxidation of oligosaccharide 1 indicates that the linkage to this terminating glycoside must be to position three. The structure of component 1b is therefore established as being



The structure of oligosaccharide 1 can therefore be written as



Oligosaccharide 2 (20 mg), obtained from the periodate oxidation of the native polysaccharide, was indistinguishable from component 1b (above) in every respect.

Oligosaccharide 3 (20 mg), also obtained from the periodate oxidation of the native polysaccharide, showed $[\alpha]_D +118^\circ$.

Hydrolysis and paper chromatography revealed the presence of only galactose and glycerol. ^1H and ^{13}C n.m.r. studies (see Table V.2, page 158, and Appendix III, spectrum No. 29 for data), were in agreement with $\underline{3}$ comprising one $\underline{\text{D}}$ -galactose moiety α -linked to a glycerol fragment. Field desorption mass spectrometry of $\underline{3}$ gave peaks at m/e 255, 277 and 293, corresponding to $(\text{M}+1)^+$, $(\text{M}+\text{Na})^+$ and $(\text{M}+\text{K})^+$ respectively. The structure of oligosaccharide $\underline{3}$ is therefore established as being

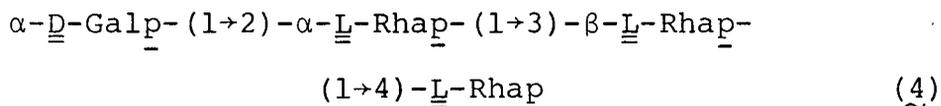


The results of the periodate oxidation studies on Klebsiella K32 are summarised in Figure V.2, page 164. Identification and characterisation of components $\underline{1}$, $\underline{1b}$, $\underline{2}$ and $\underline{3}$ establishes the tetrasaccharide repeating structure of the polysaccharide and also locates the pyruvate acetal.

Partial hydrolysis.

Although the structure of K32 capsular polysaccharide can be deduced from the periodate oxidation data (above), supporting evidence was sought using the technique of partial hydrolysis. Mild acid hydrolysis of K32 native polysaccharide resulted in very non-specific cleavage of the glycosidic linkages. As a result a large number of very similar oligosaccharides was simultaneously released. Examination by paper chromatography of a progressive acid hydrolysis of a

small amount of native polysaccharide indicated that maximum oligosaccharide production occurred after 9 hours treatment at 95° with 0.03 M trifluoroacetic acid. Alternatively, using the polysaccharide in its 'free acid' form (pH 3.0) maximum oligosaccharide production occurred after 18 hours autohydrolysis at 95°. Using the latter conditions a larger sample of K32 was hydrolysed and then dialysed against a fixed volume of water. The dialysable material was examined by gel chromatography and a pure oligosaccharide 4 (4 mg) was obtained. ¹H n.m.r. (see Appendix III, spectrum No. 30) indicated 4 to be a tetrasaccharide containing three L-rhamnose residues, one of which was reducing (see Table II for ¹H n.m.r. data and interpretation). Reduction of 4 with sodium borodeuteride and subsequent methylation analysis yielded the acetylated derivatives of 2,3,4,6-tetra-0-methyl-D-galactitol, 3,4-di-0-methyl-L-rhamnitol, 2,4-di-0-methyl-L-rhamnitol and, from the reducing terminus of 4, 1,2,3,5-tetra-0-methyl-L-rhamnitol. These components were obtained in approximately equal amounts but some of the last named component, monodeuterated at C₁, was lost under reduced pressure during derivatisation. In light of the sequence of oligosaccharide 4 obtained by periodate oxidation the structure of the reducing tetrasaccharide 4 may be written as shown below



The pyruvate acetal in K32 is perhaps the most acid sensitive acetal examined to date in the Klebsiella polysaccharides. Part of the reason for this susceptibility to acid hydrolysis is that the acetal is spanning trans-diequatorial vicinal hydroxyls. Only as a result of a large degree of distortion of the sugar ring can these two hydroxyl groups be brought into a reasonably planar orientation that is necessary for the formation of this type of acetal. The existence of a pyruvate acetal attached to the 3- and 4-positions of a L-rhamnopyranosyl residue has been noted previously^{4,21}. Pyruvate acetals spanning vicinal trans-diequatorial hydroxyls (0-2 and 0-3) of a 4-linked D-glucuronosyl residue²², and the same positions of a D-galactosyl residue²³, have been demonstrated. In all these cases the acetal has been found to be easily removed under mild acid conditions.

V.4 Experimental

General Methods.

Equipment for m.s., n.m.r., g.l.c. and g.l.c.-m.s. was the same as in the investigation of Klebsiella K36 polysaccharide⁵. Field desorption mass spectrometry was performed using an A.E.I. M.S. 902 mass spectrometer equipped with an e.i./f.i./f.d. source. The columns used for g.l.c. separations were (A) 3% of HIEFF-1B on Gas Chrom Q (100-120 mesh) and (B) 0.2% of polyethylene glycol succinate, 0.2% of polyethylene

glycol adipate, 0.4% of XF-1150 on the same support. For descending paper chromatography the following solvent systems (v/v) were used: (A) freshly prepared 2:1:1 1-butanol-acetic acid-water; (B) 8:2:2 ethyl acetate-pyridine-water.

Preparation and properties of K32 capsular polysaccharide.

This was performed as previously described⁵. The isolated polysaccharide showed $[\alpha]_D +113^\circ$ (c 3.8, water). ^1H n.m.r. spectroscopy was performed on the polysaccharide in the sodium salt form and revealed signals in the anomeric region that integrated as four protons relative to the signals at τ 8.41 (3H, singlet) and τ 8.70 (9H, $J_{5,6}$ approx. 6 Hz). For the assignment of the anomeric proton signals see Table V.2, page 158.

Sugar and methylation analysis of native and partially depyruvalated polysaccharides.

Hydrolysis of a sample of native K32 polysaccharide with 2 M trifluoroacetic acid for 6 hours at 95° and subsequent derivatisation of the liberated monosaccharides as alditol acetates gave peaks corresponding to rhamnitol pentacetate and galactitol hexaacetate in the proportions 72:28 respectively (Column B; programmed at 120° for 8 min and then at 1°/min to 200°). Preparative g.l.c. gave galactitol hexaacetate (m.p. 168°) and rhamnitol pentaacetate. Circular dichroism of the latter component showed $\epsilon_{213}^{\text{MeCN}}$ -1.20 and by comparison

with authentic standards confirmed the sugar as being in the L-configuration. The configuration of the galactose was shown to be D by the action of D-galactostat on a portion of the free sugar isolated by gel filtration of a hydrolysed sample of the polysaccharide.

Methylation of K32 capsular polysaccharide in the sodium salt form was performed using the Hakomori procedure⁹. Difficulty was encountered in dissolving the polysaccharide in dimethylsulfoxide and agitation in a sonicator for two days at room temperature was needed to achieve complete solution. Two successive Hakomori methylations were performed and these were followed by a Purdie¹⁰ treatment using silver oxide in methyl iodide. The final product showed no absorbance at 3600 cm^{-1} in the i.r. spectrum and had $[\alpha]_{\text{D}} +10^{\circ}$ (c 2.2, chloroform). Hydrolysis of this material using 2 M trifluoroacetic acid at 95° for 16 hours and subsequent derivatisation as alditol acetates gave a mixture of components which was analysed by g.l.c.-m.s. as detailed in Table V.1, column I.

A sample of K32 capsular polysaccharide that had been passed through a column of Amberlite IR 120 (H^{+}) was shown by ^1H n.m.r. to have lost approximately 70% of the pyruvate acetal groups. Methylation of this partially depyruvalated material in the free acid form proceeded without complication and the subsequent methylation analysis results are shown in Table V.1, page 157, column II.

Periodate Oxidation.

A sample (616 mg) of Klebsiella K32 capsular polysaccharide in the sodium salt form (with a full complement of pyruvate) was dissolved in 1ℓ of a phosphate buffer at pH 6.5. The buffer was 0.05 M in NaIO_4 . The solution was stirred at 4° in the dark for 40 hours after which time ethylene glycol (10 ml) was added. Dialysis of the solution against tap water overnight was followed by reduction with sodium borohydride (1 g). The solution was then neutralised with glacial acetic acid, dialysed overnight and lyophilised to yield 610 mg of the derived polyol. A small portion (10 mg) of this polyol was hydrolysed with 2 M trifluoroacetic acid and by paper chromatography (solvent B) the present of 1-deoxyerythritol, galactose and rhamnose was confirmed. Reduction and acetylation of this hydrolysate gave a mixture of alditol acetates which was shown by g.l.c.-m.s. (column B) to contain components corresponding to rhamnose and galactose in the proportions 63:37 respectively (theoretical 66:33). This result indicated oxidation was complete.

Smith hydrolysis of the polyol using 0.5 M trifluoroacetic acid at room temperature overnight was followed by removal of the acid by evaporation with successive portions of water and then reduction with sodium borohydride in water overnight. After the usual workup, lyophilisation yielded 520 mg of material which was investigated using gel filtration chromato-

graphy. The material (3 x 150 mg) was applied to a column of Bio-Gel P-4 (2.5 x 160 cm) which was irrigated with distilled water at a flow rate of approximately 7 ml/h. Fractions (2 ml) were collected, lyophilised individually, and examined by paper chromatography (solvent A). Three pure components, 1, 2 and 3, with R_{Glc} values of 1.15, 1.75 and 1.14 respectively (solvent A) were isolated.

Oligosaccharide 1, 120 mg, had $[\alpha]_D +102^\circ$ (c 3.2, water) and was examined by 1H and ^{13}C n.m.r. spectroscopy (see Table V.2, page 158, and Appendix III, spectrum No. 26 for detail). Hydrolysis of 1 (2 M trifluoroacetic acid at 95° for 6 hours) revealed the presence of rhamnose, galactose, and l-deoxyerythritol by paper chromatography (solvent A). The mass spectrum (f.d.) of 1 gave peaks, inter alia, at m/e 561(36), 562(12), 583(100), 584(34), 585(17), 599(21) and 600(22). The major peaks at m/e 561, 583 and 599 correspond to $(M+1)^+$, $(M+Na)^+$, and $(M+K)^+$ respectively. Methylation of a small portion of 1 yielded a product which upon hydrolysis with 2 M trifluoroacetic acid at 95° for 16 hours and subsequent derivatisation was shown by g.l.c.-m.s. (column A) to contain the alditol acetates of 2,3,4,6-tetra-0-methyl-D-galactose, 3,4-di-0-methyl-L-rhamnose, and 2,4-di-0-methyl-L-rhamnose in equal proportions. The volatile tri-0-methyl-l-deoxy-D-erythritol component was lost under reduced pressure during work up.

Periodate oxidation of 1 (30 mg) with 0.05 M sodium periodate for 24 hours and sodium borohydride reduction in

the usual manner gave a product which was hydrolysed with 0.5 M trifluoroacetic acid at room temperature for 16 hours (Smith hydrolysis). The hydrolysate was investigated using gel chromatography and was applied to a Bio-Gel P-4 column (2.5 x 160 cm) and the column was irrigated with distilled water. A pure component 1b (7 mg) was isolated and showed $[\alpha]_D +104^\circ$ (c 0.78, water). ^1H and ^{13}C n.m.r. data for 1b are recorded in Table V.2, page 158, and Appendix III, spectra No.'s 27, 28. Hydrolysis of 1b and paper chromatography (solvent B) of the hydrolysate gave components indistinguishable from authentic rhamnose and l-deoxy-erythritol samples. The mass spectrum of 1b (f.d.) gave peaks, inter alia, at m/e 253(100), 254(24), 275(30) and 291(11). The peaks at m/e 253, 275, and 291 correspond to $(\text{M}+1)^+$, $(\text{M}+\text{Na})^+$ and $(\text{M}+\text{K})^+$ respectively. Oligosaccharide 1b is therefore shown to be $\beta\text{-}\underline{\text{L}}\text{-Rhap-(1}\rightarrow\text{3)-1-deoxy-}\underline{\text{D}}\text{-erythritol}$, and 1 is established as being $\alpha\text{-}\underline{\text{D}}\text{-Galp-(1}\rightarrow\text{2)-}\alpha\text{-L-Rhap-(1}\rightarrow\text{3)-}\beta\text{-}\underline{\text{L}}\text{-Rhap-(1}\rightarrow\text{3)-1-deoxy-}\underline{\text{D}}\text{-erythritol}$.

Oligosaccharide 2 (20 mg) was identical to component 1b in every respect.

Component 3 (20 mg) showed $[\alpha]_D +117^\circ$ (c 0.4, water). The mass spectrum (f.d.) of 3 gave peaks, inter alia, at m/e 255(29), 277(17), and 293(100). These peaks correspond to $(\text{M}+1)^+$, $(\text{M}+\text{Na})^+$ and $(\text{M}+\text{K})^+$ respectively. The ^1H and ^{13}C n.m.r. spectra of 3 are detailed in Table V.2, page 158, and Appendix III, spectrum No. 29. Hydrolysis

of a small portion of 3 and subsequent paper chromatography (solvent A) of the hydrolysate showed two components indistinguishable from authentic samples of galactose and glycerol. Component 3 was chromatographically identical to an authentic sample of α -D-Galp-(1 \rightarrow 2)-glycerol.

Partial Hydrolysis.

Klebsiella K32 (0.5 g) was autohydrolysed at pH 3.0 for 18 hours on a steam bath. This material was then dialysed against a fixed volume of water and lyophilisation yielded 175 mg of dialysable oligosaccharides containing at least six different components, as indicated by paper chromatography (solvent A). Gel chromatography using a column (2.5 x 170 cm) of Sephadex G10 with a flow rate of 10 ml/h and subsequent lyophilisation of fractions and examination by paper chromatography revealed that the separation of the mixture was poor. However, careful examination did allow the isolation of a small amount of a pure oligomer 4 (4 mg). ^1H n.m.r. of 4 (see Table V.2, page 158, and Appendix III, spectrum No. 30 for detail) indicated the presence of one D-galactose residue (α -linked) and three L-rhamnose residues (one α -linked, one β -linked and one residue reducing). Oligosaccharide 4 was reduced with NaBD_4 and subsequently methylated. The permethylated derivative was hydrolysed with 2 M trifluoroacetic acid at 95° for 8 hours, reduced, acetylated and analysed by g.l.c.-m.s. (column A). Partially methylated alditol acetates corresponding to 2,3,4,6-tetra-

O-methyl-D-galactose, 3,4-di-O-methyl-L-rhamnose, 2,4-di-O-methyl-L-rhamnose and 1,2,3,5-tetra-O-methyl-L-rhamnitol were identified. (The latter component was deuterated at C₁.)

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APPENDIX I. BACTERIOPHAGE DEPOLYMERISATION
OF KLEBSIELLA K32 CAPSULAR
POLYSACCHARIDE.

APPENDIX I.

Bacteriophage Depolymerisation of Klebsiella K32 Capsular
Polysaccharide

Introduction.

An efficient method for specifically degrading polysaccharides into more easily analysed subunits, and in particular for the Klebsiella polysaccharides into repeating units, has been a challenge to workers in the field of polysaccharide structural determination for many years.

If such specific cleavages could be achieved then many problems, viz. viscosity, accessibility, solubility, associated with handling the undegraded polymers and in determining their detailed structure could be eliminated. Low yield, non specific techniques such as partial hydrolysis have in the past been used extensively to obtain oligosaccharides which are needed to elucidate the detailed structure (sequence data in particular) of many polysaccharides, but in several instances the isolation of a definite subunit, e.g. a repeating unit in the Klebsiella polysaccharides, would provide a far easier and simpler means to a structural determination.

A method whereby high yields of oligosaccharides can be obtained by selective fragmentation of some Klebsiella capsular polysaccharides has recently been developed¹. The method relies on the specificity of enzymes, born and utilised by bacteriophage, that are capable of depolymerising Klebsiella

polysaccharides. Although it was originally thought that capsulate bacteria were generally phage resistant, bacteriophage have now been isolated for several different species, including the genus Klebsiella². In many cases a characteristic feature of phage infection when an actively growing 'lawn' of bacteria is infected by a relatively small number of phage, is the formation of plaques surrounded by large clear haloes³. The haloes comprise an area in which polysaccharide depolymerase enzymes, induced in phage infected bacteria, destroy capsular material without affecting bacterial viability.

Specific bacteriophage, i.e. bacteriophage that show depolymerase activity with a particular Klebsiella serotype, can be prepared for the majority of the 81 serotypes in considerable amounts using lysis techniques, precipitation with polyethylene glycol and isopycnic centrifugation^{1,4}. This, together with the fact that the phage will depolymerise the cell free exopolysaccharide by itself, makes available a powerful and selective cleavage tool.

The bacteriophage degradation of Klebsiella K32 is presented here. This work represents some of the initial experiments carried out in this laboratory using bacteriophage and since the completion of the depolymerisation of K32 other polysaccharides have been similarly examined with very encouraging results.

Experimental

General techniques and media.

Most of the techniques used are described in detail by Stirm et al.¹. Phosphate-buffered physiological saline (P.B.S.) was made up using 8.5 g NaCl, 1.76 g Na₂HPO₄·12H₂O, and 0.1 g KH₂PO₄ in 1 l of water. "Standard" liquid broth or medium contained 5 g Bactopeptone, 3 g Bacto beef extract, and 2 g NaCl per litre of water. "Standard" agar plates were made using a solution of "standard" liquid broth to which 15 g of agar per litre had been added; 8.5 cm disposable plastic plates were used.

Bacteriophage and bacteria.

The bacteriophage used was Klebsiella bacteriophage No. 32, originally isolated from sewage⁵ and kindly given to us by Dr. S. Stirm, Max Planck-Institut für Immunbiologie, Freiburg, Germany. The host for this bacteriophage is Klebsiella 6837 (K32) and the slime polysaccharide was isolated from this bacterium as previously described. (See procedure for Klebsiella K36, Section III.4, page 106).

Correlation of optical density and bacteria concentration.

A flask of liquid medium was inoculated with a culture of actively growing Klebsiella K32 bacteria and vigorously aerated at 37°. Aliquots were removed at 30 minute intervals, appropriately diluted (10^{-5} to 10^{-8}) with liquid medium and

a small quantity (0.1 ml) of the diluted solution was incubated on an agar plate for 12-16 hours. Individual bacterial colonies could then be counted. The optical density of the actively growing bacterial solution was recorded at each 30 minute interval. A plot of optical density versus the logarithm of the number of colonies is shown in Figure AI.1, page 183.

Preparation of bacteriophage (ψ).

(a) Tube lysis. An active bacterial culture of Klebsiella K32 was obtained by successive replatings on agar plates. 7x5 ml of sterile liquid medium was then inoculated with the bacteria by the addition of 0.5 ml of an actively growing liquid K32 bacterial culture. These seven test tubes were incubated at 37° and at 30 minute intervals the tubes were inoculated with 0.5 ml of a solution of liquid medium containing ψ 32. Continued incubation resulted in the first few tubes changing from the cloudy solution associated with actively growing K32 bacteria to a clear solution (lysis). After the last tube had cleared the incubation was continued for 30 minutes and then a few drops of CHCl_3 was added to the tube and the mixture was shaken well. A phage "titre" on the solution was performed by successively diluting a small volume (0.1 ml) of the clear liquid with liquid medium and then applying approximately 0.03 ml of these dilutions to a 'lawn' of actively growing Klebsiella K32. (The lawn of K32 was prepared by inoculating 2 ml of liquid medium with an actively growing colony of Klebsiella K32 and incubating this

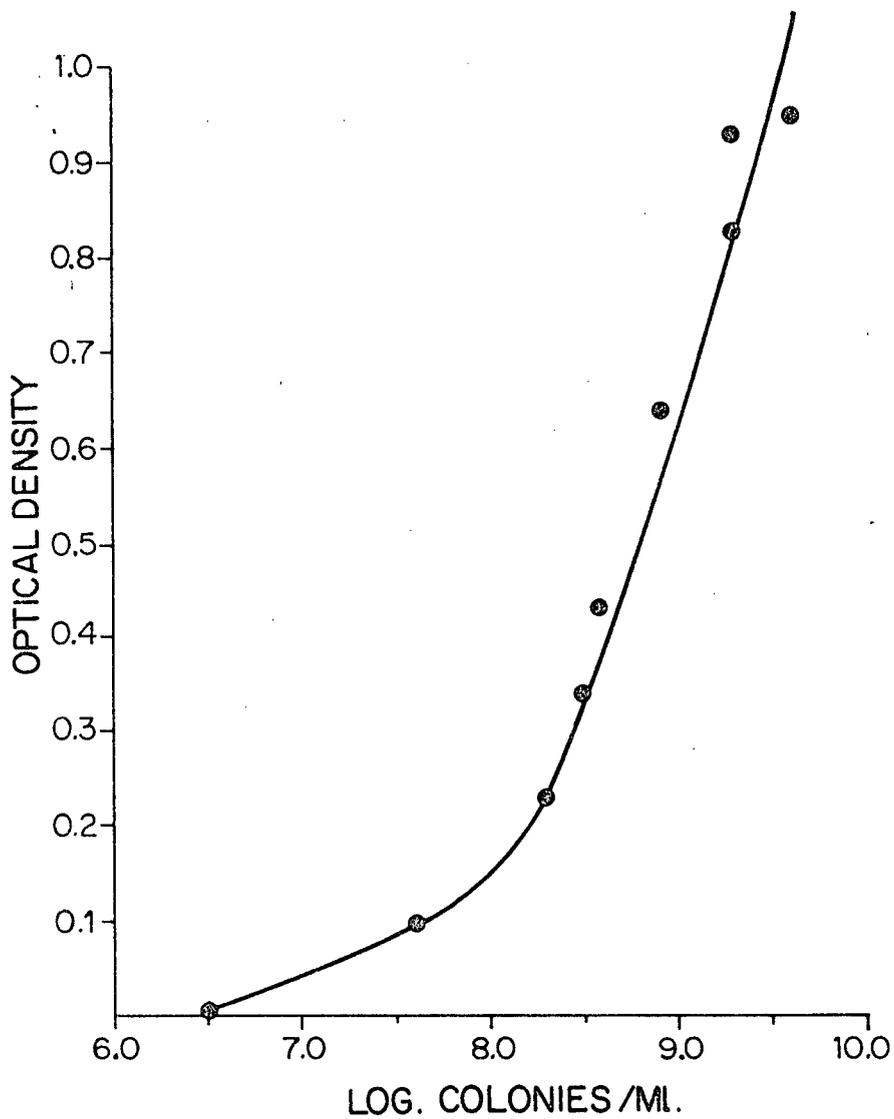


Figure AI.1 Correlation of optical density and colony concentration for Klebsiella.K32.

culture for 3 hours. An agar plate, previously dried for approximately 1 hour in the incubator at 37°, was covered with this liquid culture, left for 5 minutes and then the excess liquid was removed. Incubation for 30 minutes gave a stable 'lawn' of Klebsiella K32.) Individual bacteriophage were observed as clear spots (approximately 0.3 cm. in diameter) on the bacterial lawn after incubation for 16 hours. At high phage concentrations individual phage could not be distinguished but at more suitable dilutions, e.g. 10^{-8} to 10^{-10} , individual 'haloes' could be easily counted. As a result of a single tube lysis of this nature an assay yielded 10^9 plaque forming units (P.F.U.) per ml. of medium in the last tube to completely clear.

(b) Small flask lysis. This technique is essentially the same as that described for the tube lysis. As larger volumes of liquid medium can be used the overall total of bacteriophage can be increased even though the phage titre per ml. may not be significantly higher. In a typical small flask lysis 50 ml. solutions of K32 cultures were inoculated with 1.5 ml. of a phage solution containing 10^9 P.F.U./ml (from tube lysis). In an analogous manner to that described for the tube lysis, titration of the final flask to completely clear gave a titre of 1.2×10^{10} P.F.U./ml.

(c) Bottle lysis. Three 300 ml. bottles each containing

250 ml of an actively growing liquid culture of K32 were vigorously aerated at 37°. A small amount of a silicon antifoam agent (Dow antifoam FG-10 emulsion) was added to each. The optical density of each flask was monitored and at appropriate optical density readings (calculated such that the ratio of total bacteriophage to total bacterial colonies was approximately 3:1) aliquots of liquid phage cultures were added and the optical density monitoring continued. A subsequent drop in optical density indicated lysis had occurred. The results of a typical bottle lysis are shown in Figure AI.2, page 186. A bottle lysis might typically yield 400 ml of a solution with a titre of 3.0×10^{10} P.F.U./ml.

(d) One litre flask lysis. In an analogous manner to that described for the bottle lysis three one litre flasks, each containing 600 ml of liquid medium, were inoculated with K32 bacteria, aerated and incubated to appropriate optical densities, and then bacteriophage solutions were added. A typical result of such a lysis might yield 1400 ml of a phage solution with a titre of 3×10^{10} P.F.U./ml.

Bacteriophage concentration.

A solution (1400 ml) of liquid medium containing 3×10^{10} P.F.U./ml (4.2×10^{13} P.F.U. in total) was centrifuged at 5000 g for 15 minutes just prior to the addition of 160 g of polyethylene glycol. This solution was left at 4° for

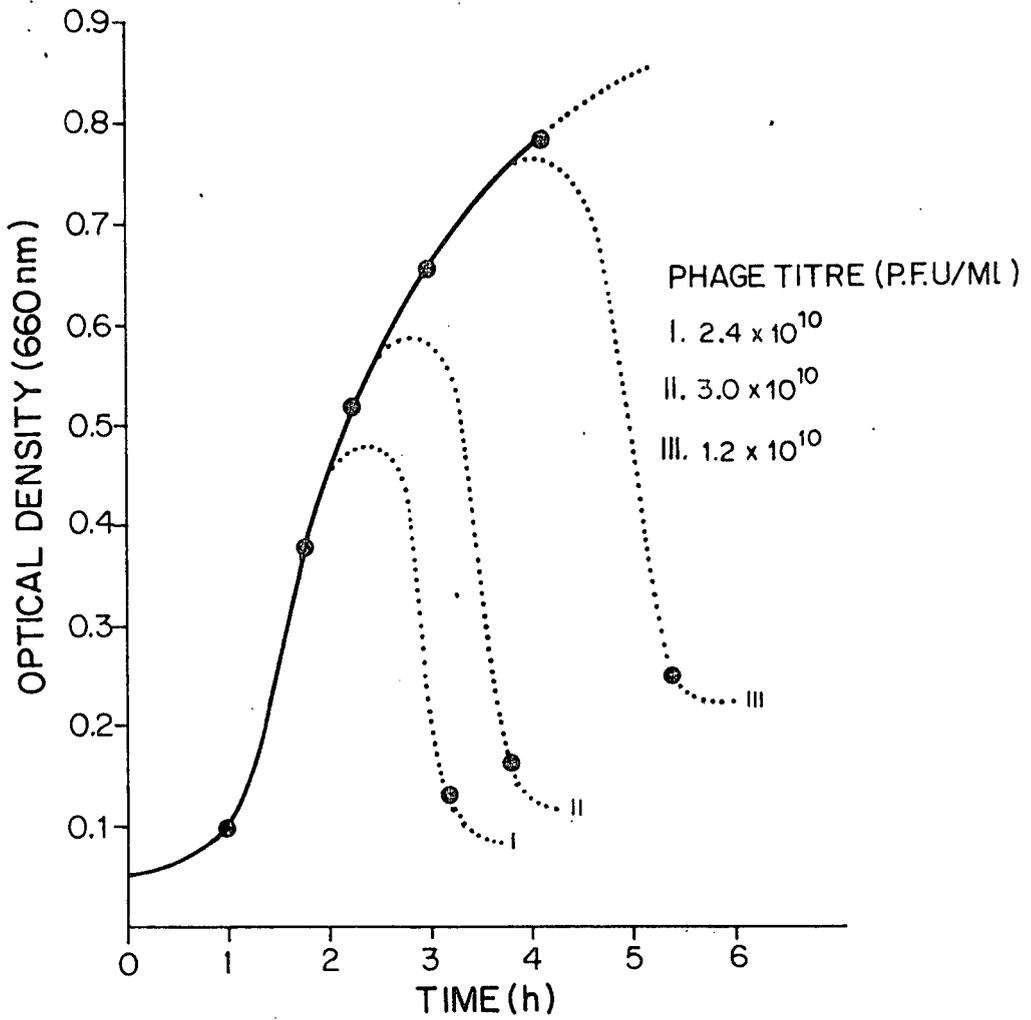


Figure AI.2

Results of a typical bottle lysis of Klebsiella K32 with bacteriophage ψ 32.

three days. The fine misty grey precipitate that formed during this period was separated by centrifugation at 20,000 g for 30 minutes. The precipitate was then suspended in physiologically buffered saline (P.B.S.) with the aid of a syringe and 20 gauge hypodermic. Centrifugation at 5000 g for 15 minutes removed a large amount of a dense precipitate which upon titration was shown to contain approximately 1% of the total plaque forming units. The milky supernatant from this slow speed centrifugation was then centrifuged at 100,000 g for 1 hour and a solid phage pellet was obtained. (The clear supernatant was subsequently shown to contain less than 1% of the total P.F.U.) The phage pellet was resuspended in P.B.S. (10 ml) but appeared inhomogeneous. A low speed centrifugation (30 minutes, 5000 g) gave a precipitate (which was subsequently shown to contain less than 1% of the total P.F.U.) and a milky white supernatant. At this point the bacteriophage were present in 12 ml of a P.B.S. solution with a titre of 1.8×10^{12} P.F.U./ml. (2.4×10^{13} P.F.U. in total).

Purification of bacteriophage.

The bacteriophage were purified by isopycnic centrifugation in a linear CsCl gradient. In two cellulose nitrate tubes for a Beckman S.W.27 rotor (tube capacity 38.5 ml each), two linear gradients were formed simultaneously using a three channel peristaltic pump. The two solutions used to form the gradient were made up as follows; 32.35 g CsCl

in 32.14 ml of Tris HCl, and 6.47 g of CsCl in 31.15 ml of Tris HCl. Sufficient space was left in the S.W.27 tubes to load approximately 7 ml of phage suspended in P.B.S. onto each gradient. The loaded gradients were spun at 86,000 g for 1.5 hours. An opalescent band (lowest band) was clearly visible after this time and this was collected by piercing the cellulose nitrate tube from the side using a syringe and new hypodermic needle. The refractive index of this material correlated with a density of 1.44 g/ml. and was dialysed against P.B.S. to remove the CsCl .

Electron microscopy.

The conditions and staining techniques used for electron microscopy were those described by Stirm and Freund-Mölbart⁵. The electron micrographs clearly showed the icosahedral bacteriophage head and the spikes attached directly to the head. This morphology has been classified by Bradley⁶ as type C.

Conditions of depolymerisation.

800 mg. of Klebsiella K32 capsular polysaccharide was dissolved in 400 ml of P.B.S. (pH 7.0) and to this solution was added a total of 1×10^{13} P.F.U. in 30 ml of P.B.S. The mixture was incubated at 37° for a total of 48 hours. At various time intervals (see Figure AI.3, page 189) the viscosity of the mixture was measured using an Ostwald viscosimeter (a portion of the depolymerising reaction mixture was left in the viscosimeter at 37°). At the same time aliquots of the reaction mixture were removed and tested

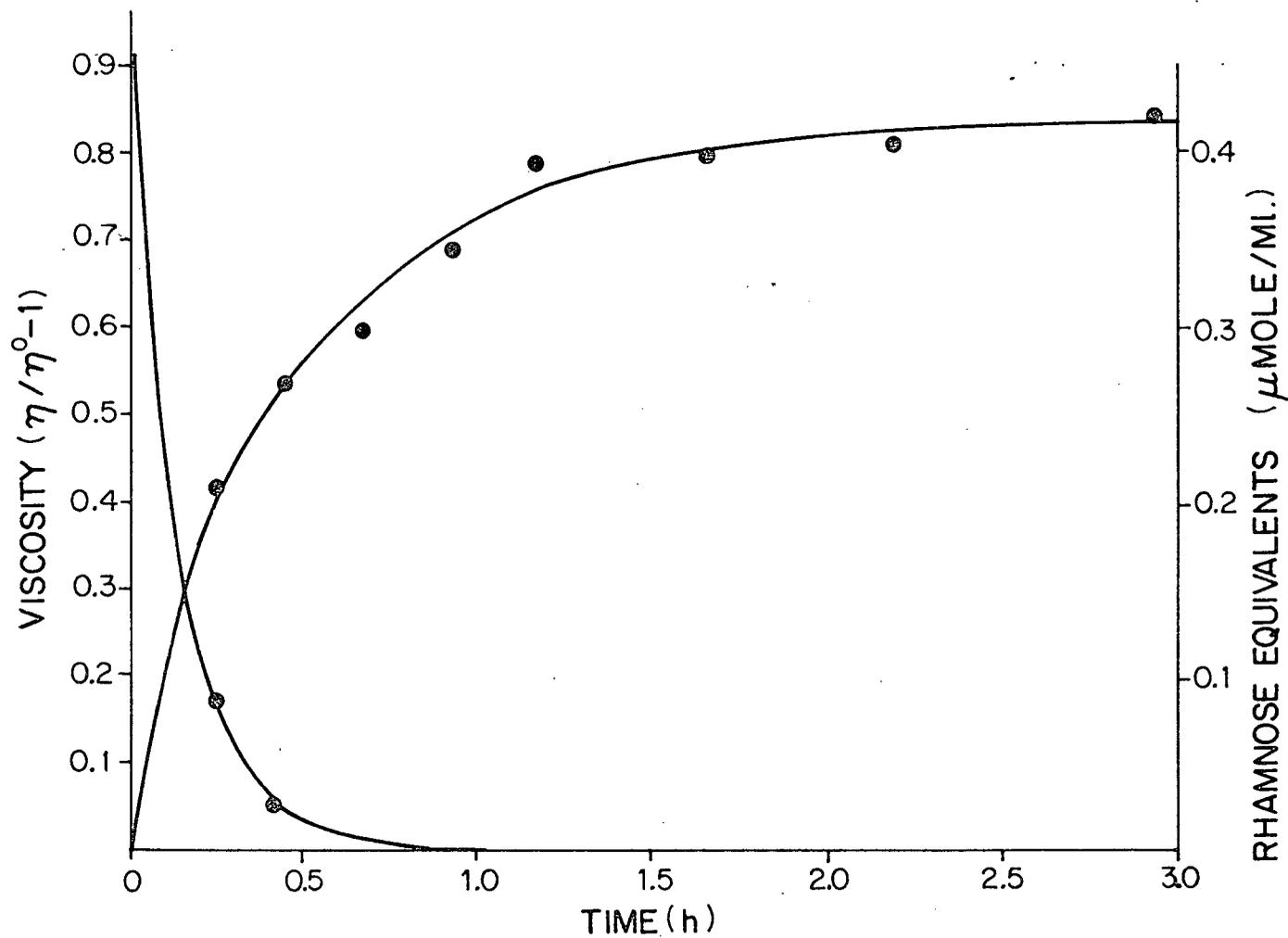


Figure AI.3 Bacteriophage depolymerisation of Klebsiella K32 capsular polysaccharide.

for 'reducing power' using $K_3[Fe(CN)_6]$ ⁷. L-Rhamnose was used to establish a standard curve. The changes in viscosity and reducing power are recorded in Figure AI.3, page 189.

Purification and separation of depolymerised material.

Portions of the crude lyophilised depolymerisation mixture (2x1.5 g) were desalted using a column of Sephadex G10 (100 cm x 19.5 cm²). The column was eluted with a water-pyridine-glacial acetic acid (1000:10:4) buffer (pH 4.5) at a flow rate of 25 ml/h and carbohydrate material was localised using the Molisch test⁸. Part of the desalted material (430 mg) was dialysed against 3 x 1ℓ of distilled water and lyophilisation yielded 64.5 mg (15%) of non-dialysable material and 350 mg of dialysable material that was considered to contain no oligomers containing more than approximately 12 sugar residues.

The dialysable material (320 mg) was then applied to the top of a D.E.A.E.-A25 Sephadex column. (The column, 90x1.5 cm, was packed in 0.5 M Tris/HCl buffer (pH 7.2) and then equilibrated with 0.025 M Tris/HCl buffer--at least 10 column volumes of the latter buffer are required to achieve equilibration as determined by performing conductivity measurements.) The material was applied as a solution in 0.025 M Tris/HCl (2 ml). The column was eluted (10 ml/h) with 0.025 M Tris/HCl (140 ml) and a linear salt gradient (from 0 to 0.35 M NaCl) was then begun. Fractions (2 ml)

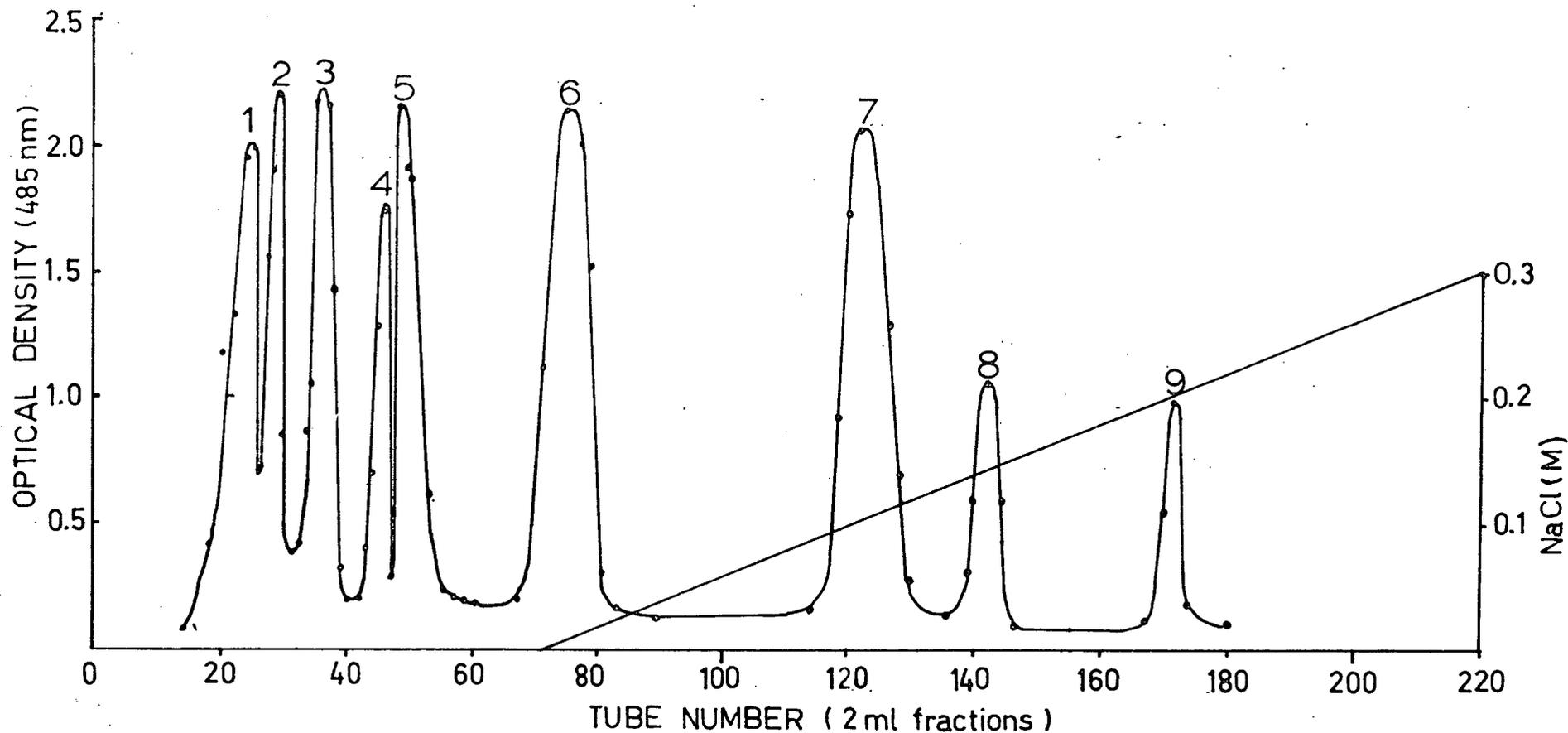


Figure AI.4 Ion exchange chromatography of Klebsiella K32 oligosaccharides obtained by bacteriophage depolymerisation. D.E.A.E. Sephadex A25 equilibrated with 0.025 M Tris/HCl buffer was used and eluted with a linear 0 to 0.3 M NaCl gradient in the same buffer. Fractions were analysed with phenol-sulphuric acid.

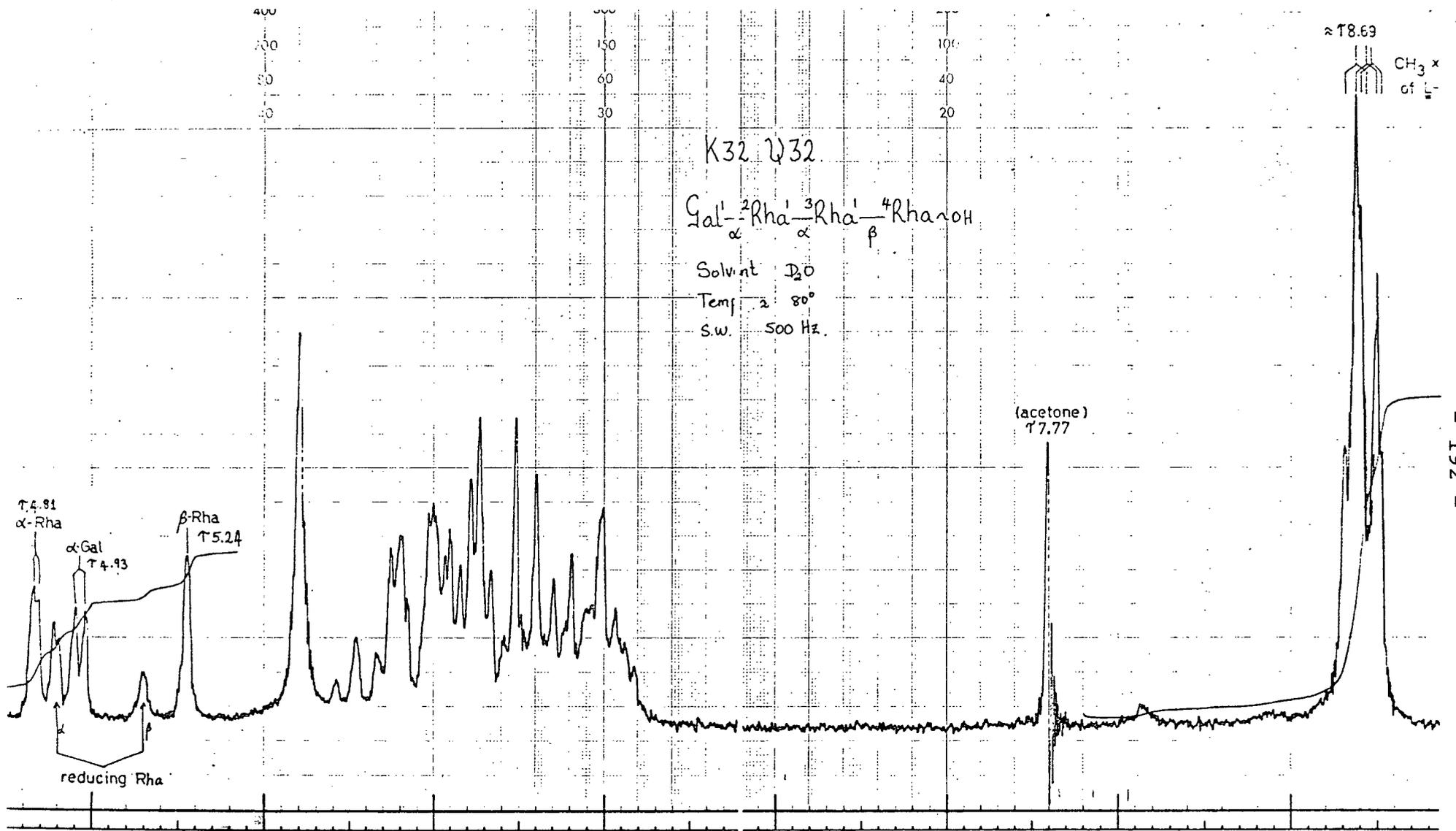
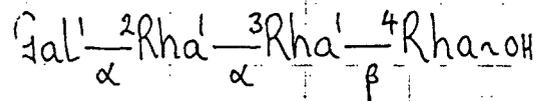


Figure AI.5 ¹H n.m.r. of neutral repeating unit from phage degraded K32.

1	34	2021.5	101.07
2	46	2008.9	100.44
3	47	1970.0	98.50
4	25	1891.1	94.55
5	16	1836.3	94.31
6	28	1672.4	83.62
7	17	1665.2	83.26
8	37	1627.0	81.34
9	42	1536.4	76.82
10	74	1457.0	72.84
11	24	1449.2	72.45
12	54	1440.2	72.01
13	60	1436.2	71.80
14	71	1428.6	71.42
15	35	1420.5	71.02
16	45	1409.3	70.46
17	58	1403.4	70.17
18	105	1400.5	70.02
19	38	1394.0	69.70
20	44	1381.6	69.08
21	28	1352.2	67.60
22	37	1235.5	61.77
23	48	621.7	31.08
24	89	351.4	17.57
25	61	349.1	17.45

K32 ψ 32



S.W. 2000 Hz

A.T. 1.023 sec.

P.W. 18 μ .sec.

P.D. 1.0 sec.

C.T. 41,000.

3 \times CH₃ of Rha

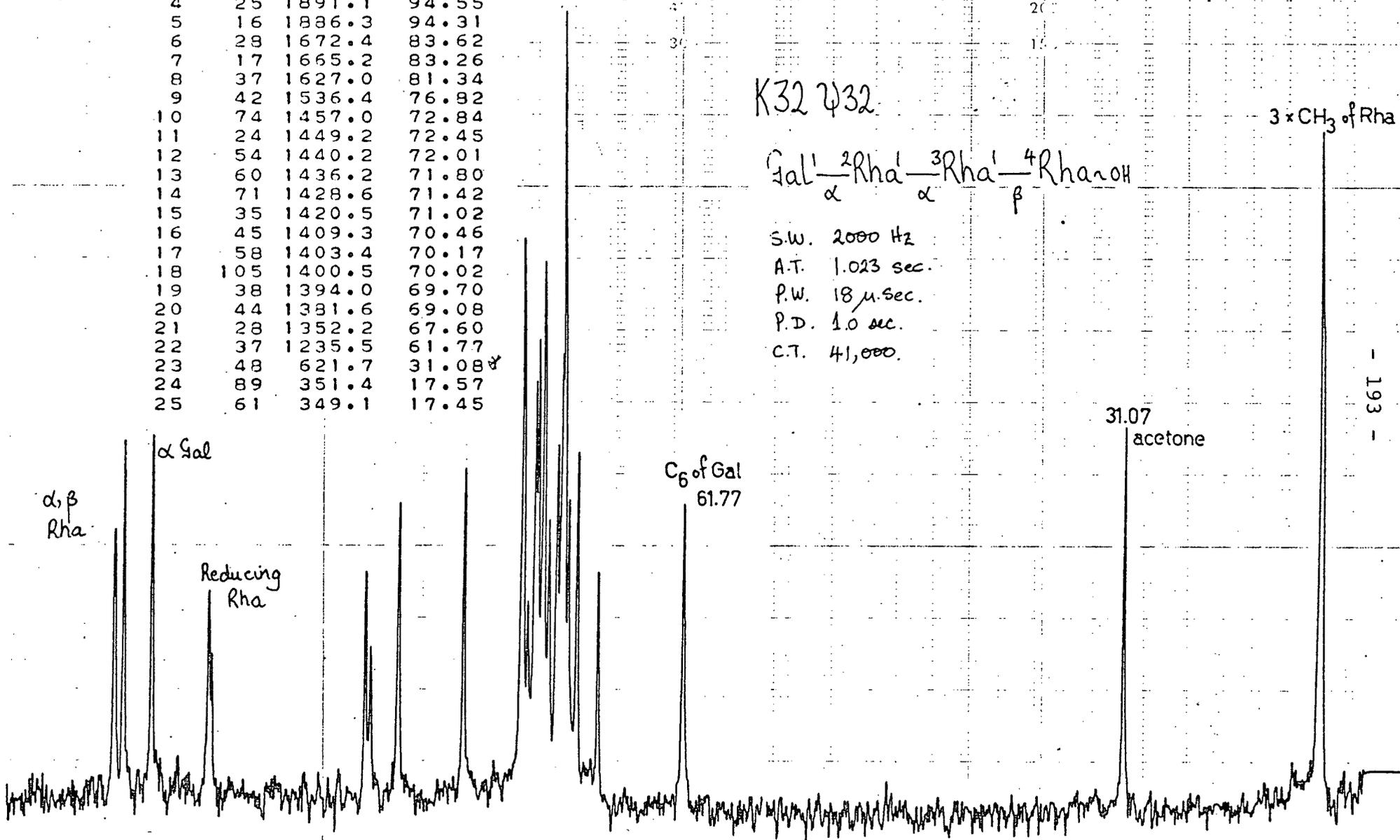


Figure AI.6 ¹³C n.m.r. of neutral repeating unit from phage degraded K32.

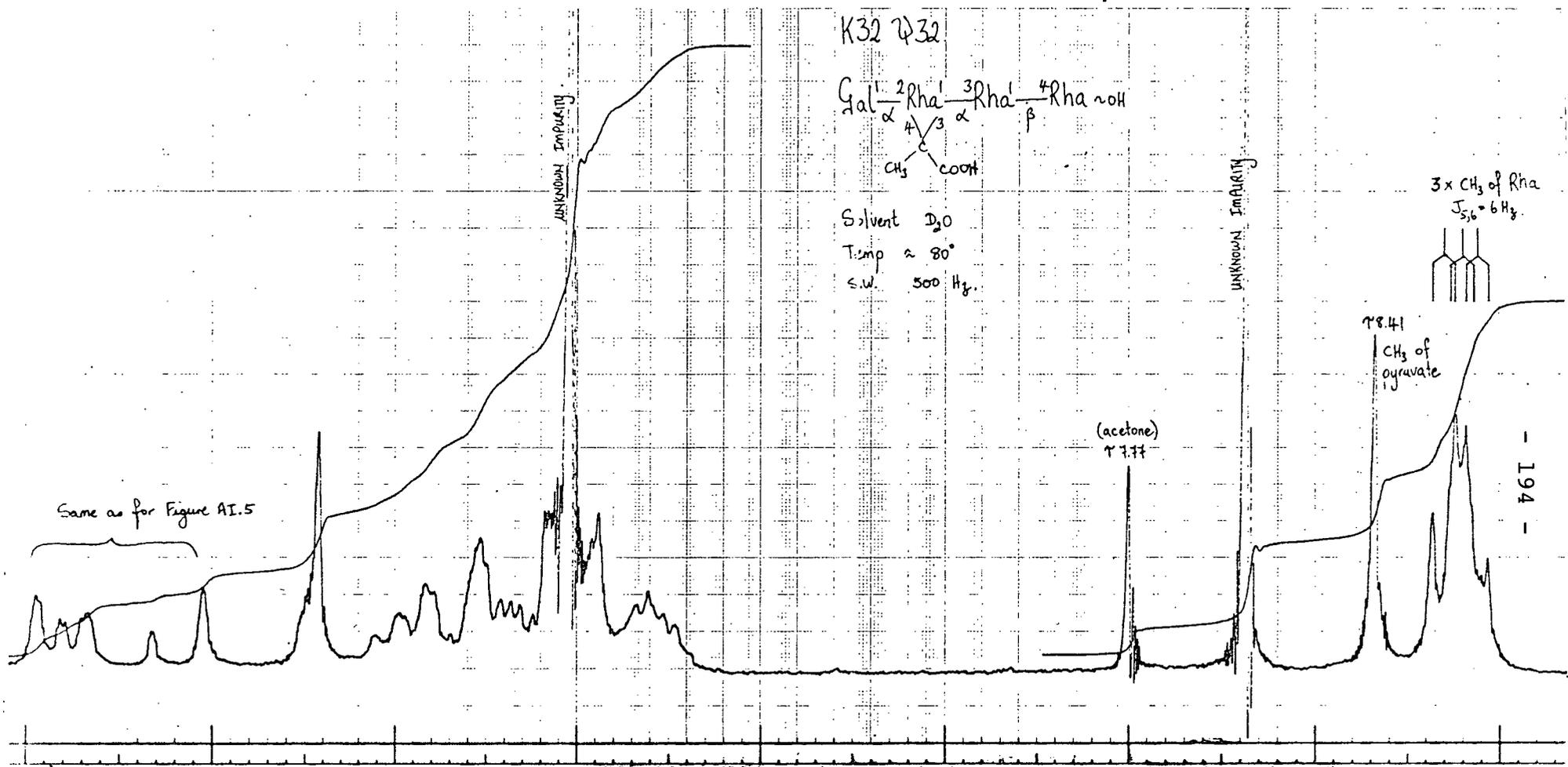


Figure AI.7 ¹H n.m.r. of acidic repeating unit from phage degraded K32.

1	23	2168.8	108.44
2	33	2037.0	101.84
3	28	2019.0	100.95
4	36	1981.5	99.07
5	21	1889.4	94.46
6	16	1884.7	94.23
7	23	1670.1	83.50
8	13	1663.0	83.14
9	31	1633.5	81.67
10	28	1537.1	76.85
11	28	1524.0	76.20
12	32	1495.1	74.75
13	35	1455.5	72.77
14	15	1446.8	72.33
15	53	1434.5	71.72
16	91	1427.9	71.39
17	26	1419.3	70.96
18	68	1400.6	70.03
19	27	1391.7	69.58
20	33	1380.2	69.01
21	23	1351.0	67.54
22	30	1243.9	62.19
23	24	1242.0	62.10
24	119	1205.6	60.28
25	15	621.6	31.08
26	30	470.1	23.50
27	27	361.4	18.07
28	60	350.4	17.52

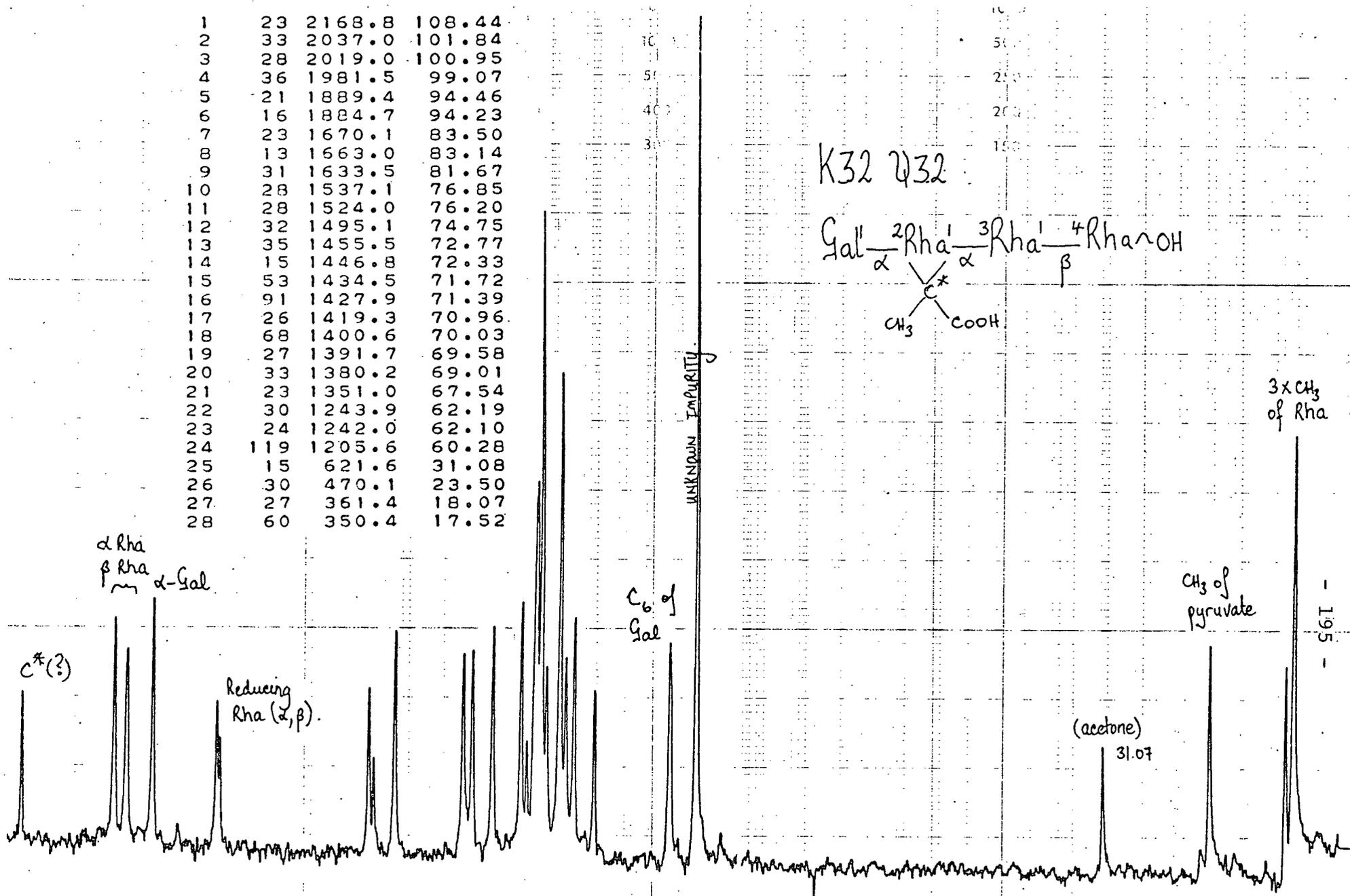


Figure AI.8

¹³C n.m.r. of acidic repeating unit from phage degraded K32.

were collected and examined using the phenol-sulphuric acid assay⁹. The elution profile for this column separation is shown in Figure AI.4, page 191. The peaks are labelled 1-9. The n.m.r. spectra of peak 3 are shown in Figure AI.5, AI.6, pages 192 and 193, and those of peak 7 in Figures AI.7, AI.8, on pages 194 and 195. Other spectra were recorded for peaks 1, 2, and 6, but are not shown here.

Discussion

The growing of $\psi 32$ in order to utilise the specific depolymerising action of the glycanase enzyme liberated by the bacteriophage proceeded as expected and after 4-6 weeks sufficient purified bacteriophage were propagated to degrade approximately 800 mg of capsular polysaccharide that had previously been isolated. The depolymerisation of the polysaccharide proceeded rapidly and was essentially complete after 3-4 hours.

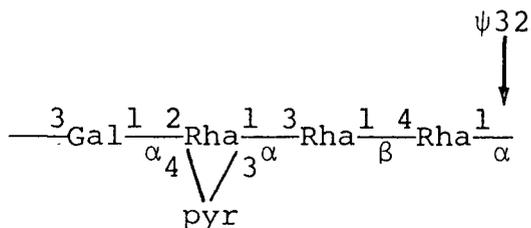
The separation of the oligosaccharides obtained as a result of the bacteriophage action was not as clear cut as hoped for. Dialysis of the carbohydrate material (after desalting on G10) resulted in only 15% of the oligosaccharides remaining in the dialysis bag. To a first approximation this was taken to mean that 15% of the original polysaccharide had a degree of polymerisation (D.P.) greater than 12; i.e., the majority (85%) of material was of lower D.P. than 12.

The profile shown in Figure AI.4 as a result of the D.E.A.E.-Sephadex separation is complex. Much of the carbo-

hydrate material did not bind to the support (peaks 1→5) and ^1H n.m.r. examination of these components confirmed the absence of any pyruvate acetal. The neutral oligosaccharides were thus separated solely according to size (rather than charge) on the column. The pyruvate acetal in Klebsiella K32 is very labile (see section V.3, page 168), and was probably lost during the desalting of the depolymerisation mixture. This desalting was performed on a Sephadex G-10 column equilibrated with a water-pyridine-glacial acetic acid (1000:10:4) buffer (pH 4.5). Those components (peaks 6→9) eluted by the linear salt gradient did contain some pyruvate acetal groups (as established by ^1H n.m.r.). However, component 6, shown by ^1H n.m.r. to be a tetrasaccharide, did not contain its full complement of pyruvate and hence this must have been lost during desalting of peak 6 after the D.E.A.E.-Sephadex separation or during preparation of the ^1H n.m.r. sample. Also, the appearance of spurious peaks in the n.m.r. spectra of some of the components suggests that a further complication in this separation may be present.

The n.m.r. spectra shown in Figures AI.5→8 indicate that the glycanase enzyme responsible for depolymerising K32 capsular polysaccharide is a α -rhamnosidase. The component 3 represents a single repeating unit of K32 capsular polysaccharide without the pyruvate acetal and component 7 the same unit with the acetal. (The latter component also has an unknown impurity.) By examination of n.m.r. spectra of

these two components it is possible to locate the point of phage cleavage more specifically as shown below.



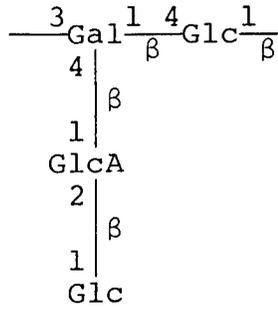
The lability of the pyruvate acetal in K32 complicates the final separation of the specifically depolymerised products obtained from the action of $\psi 32$. In another case in this laboratory using Klebsiella K21, where this complication did not arise, very good results were obtained and from 1 g of polysaccharide 200 mg of the single repeating unit of K21 and 200 mg of the double repeating unit were obtained¹⁰.

APPENDIX I. References

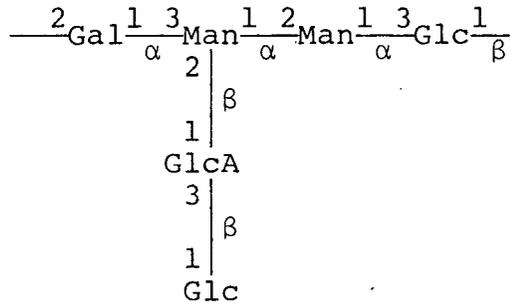
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APPENDIX II. THE KLEBSIELLA POLYSACCHARIDES
OF KNOWN STRUCTURES.

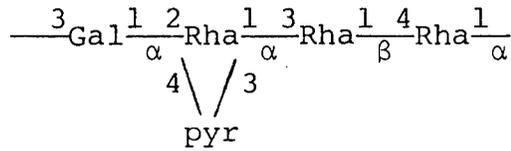
K25



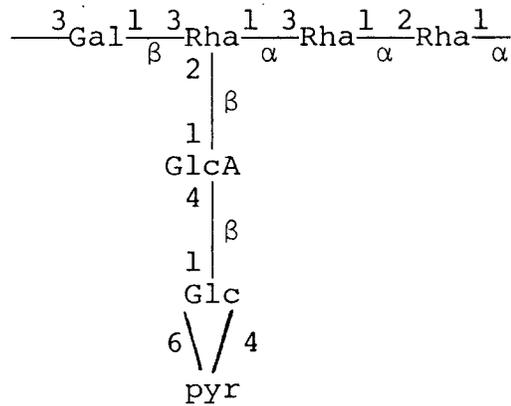
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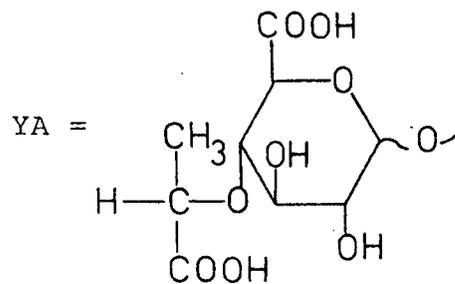
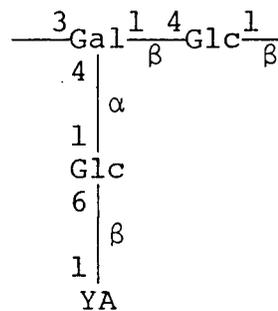
K32



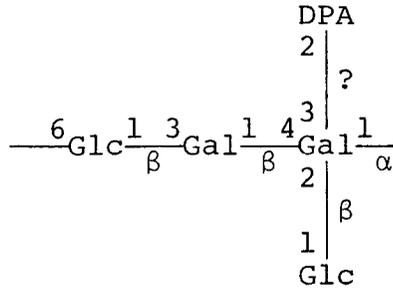
K36



K37

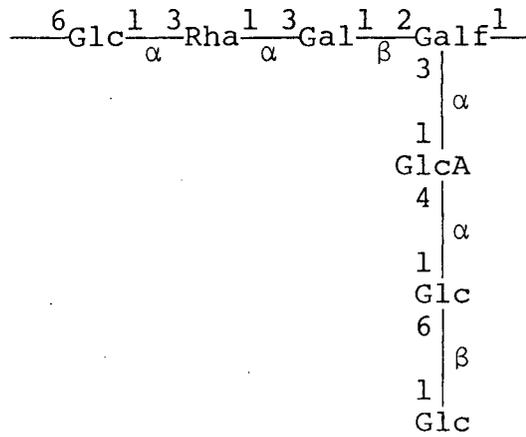


K38

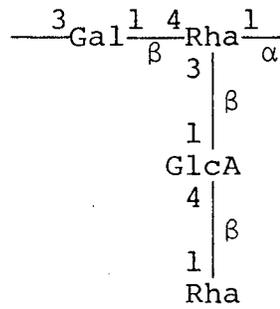


DPA = 3-deoxy-L-glycero-pentulosonic acid

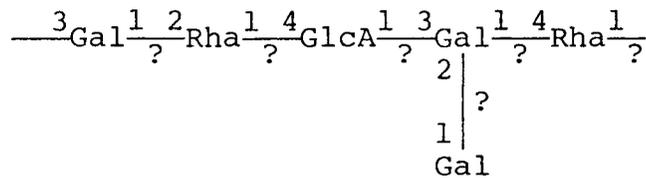
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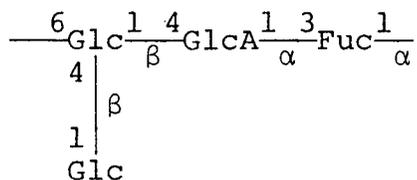
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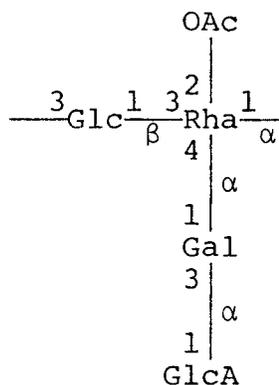
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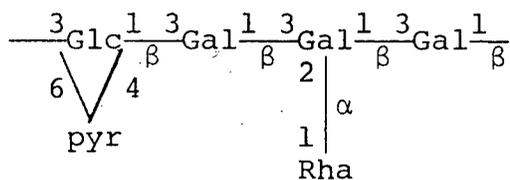
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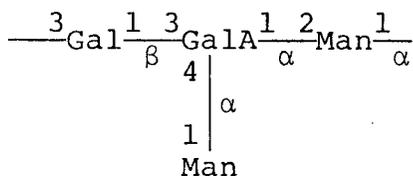
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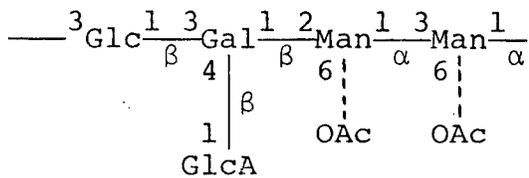
K56



K57



K59



(dotted lines indicate OAc's not
(on all residues

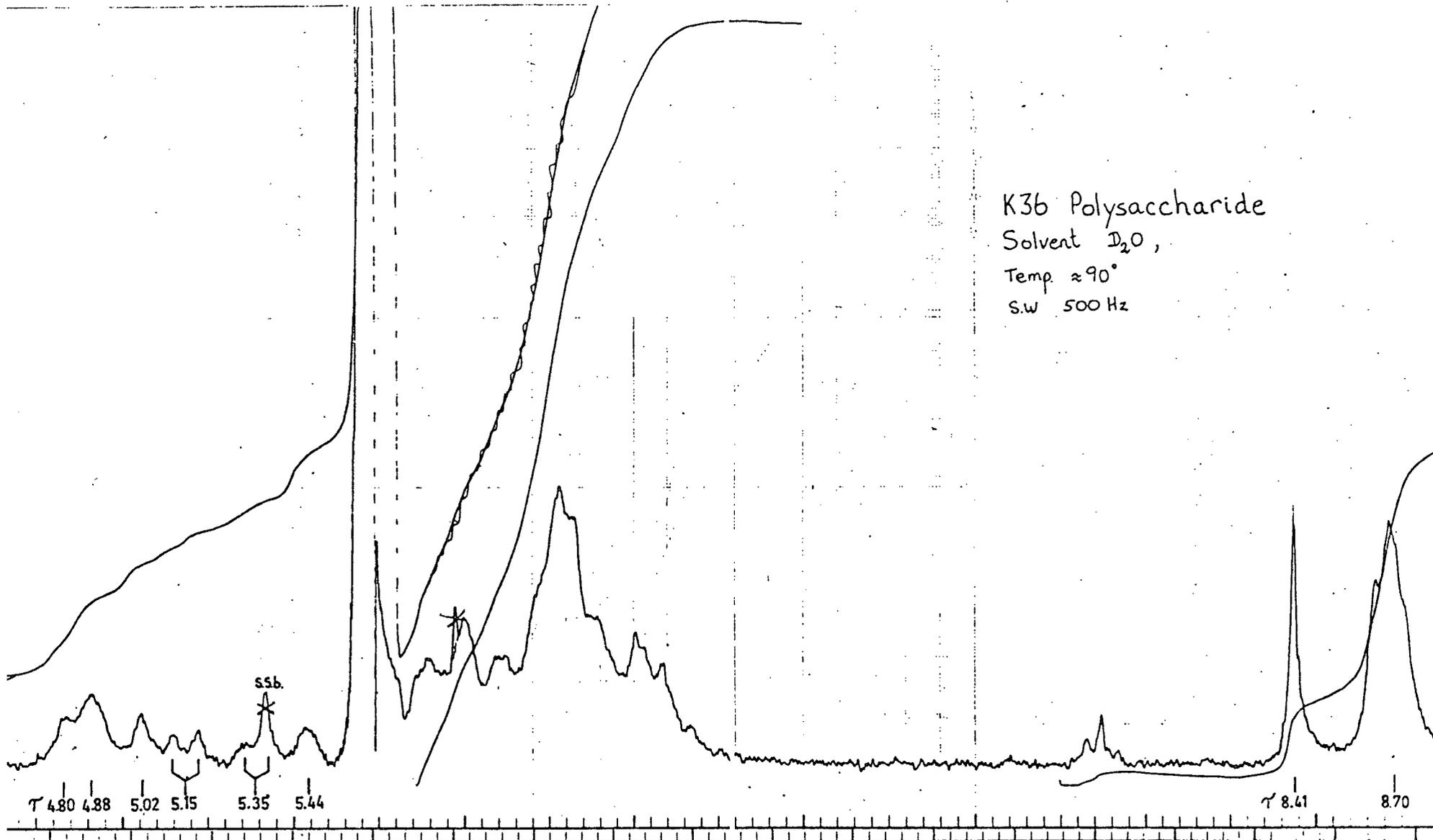
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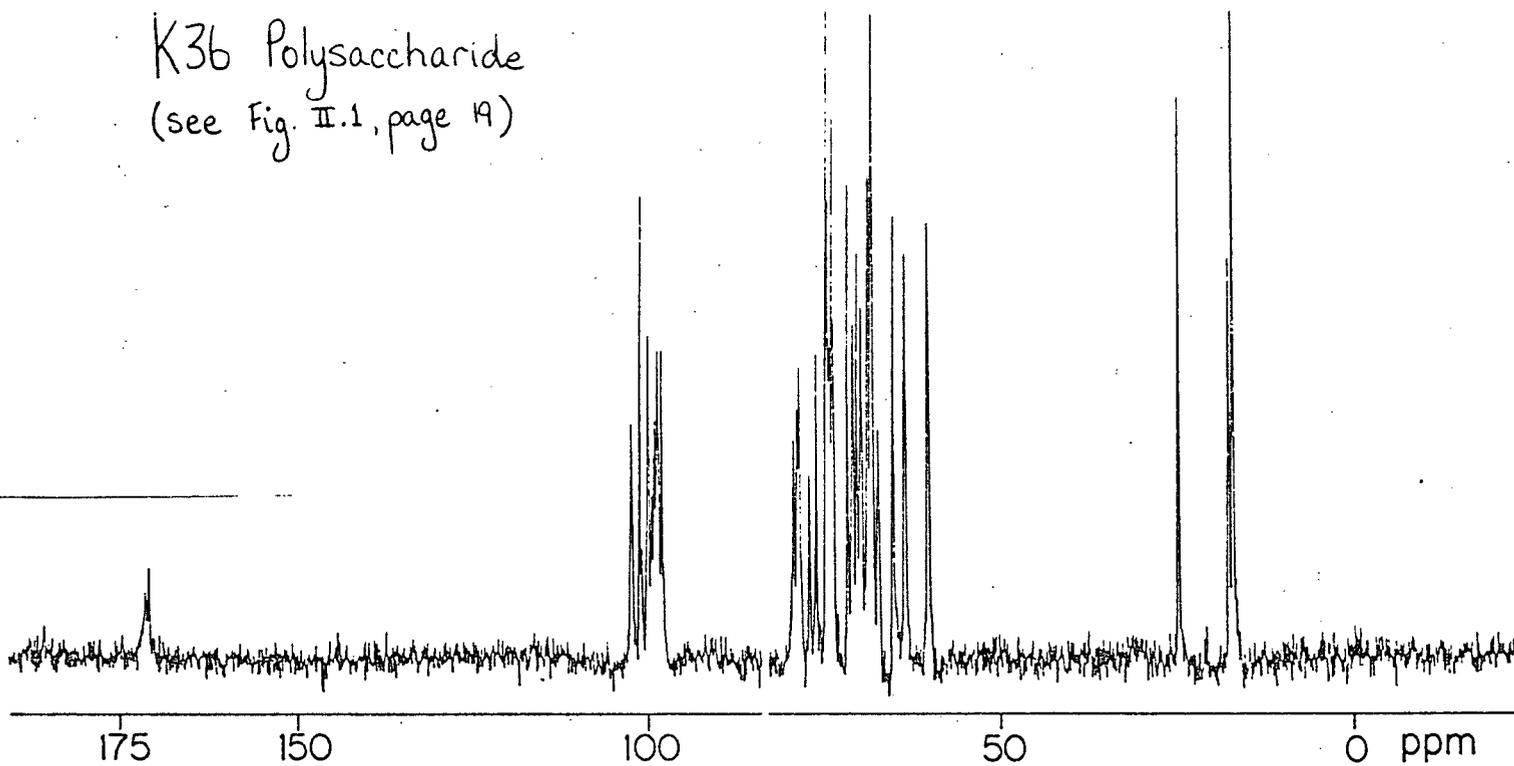
APPENDIX III. N.M.R. SPECTRA.



K36 Polysaccharide
Solvent D_2O ,
Temp $\approx 90^\circ$
S.W. 500 Hz

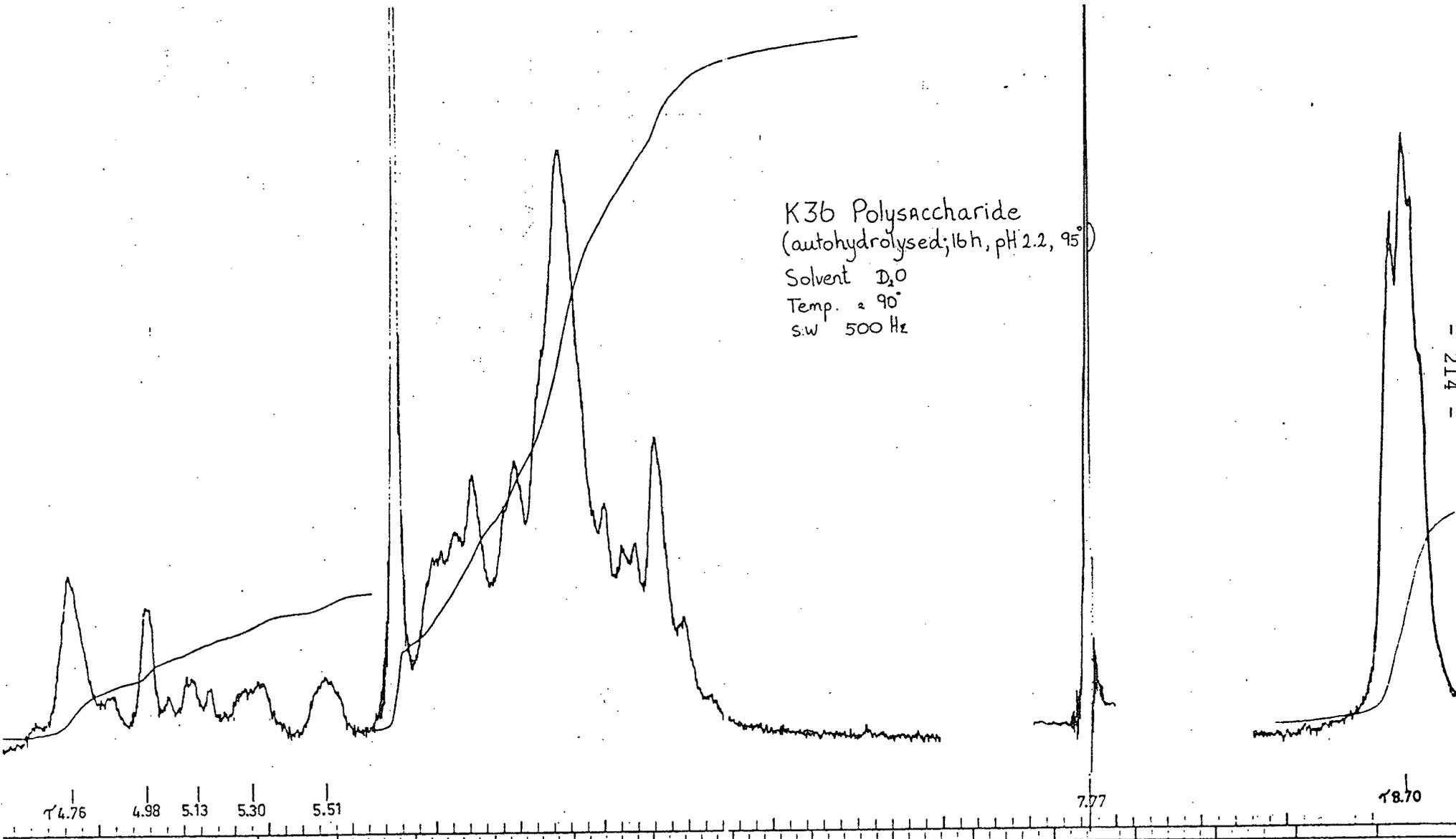
Spectrum No. 1.

K36 Polysaccharide
(see Fig. II.1, page 19)

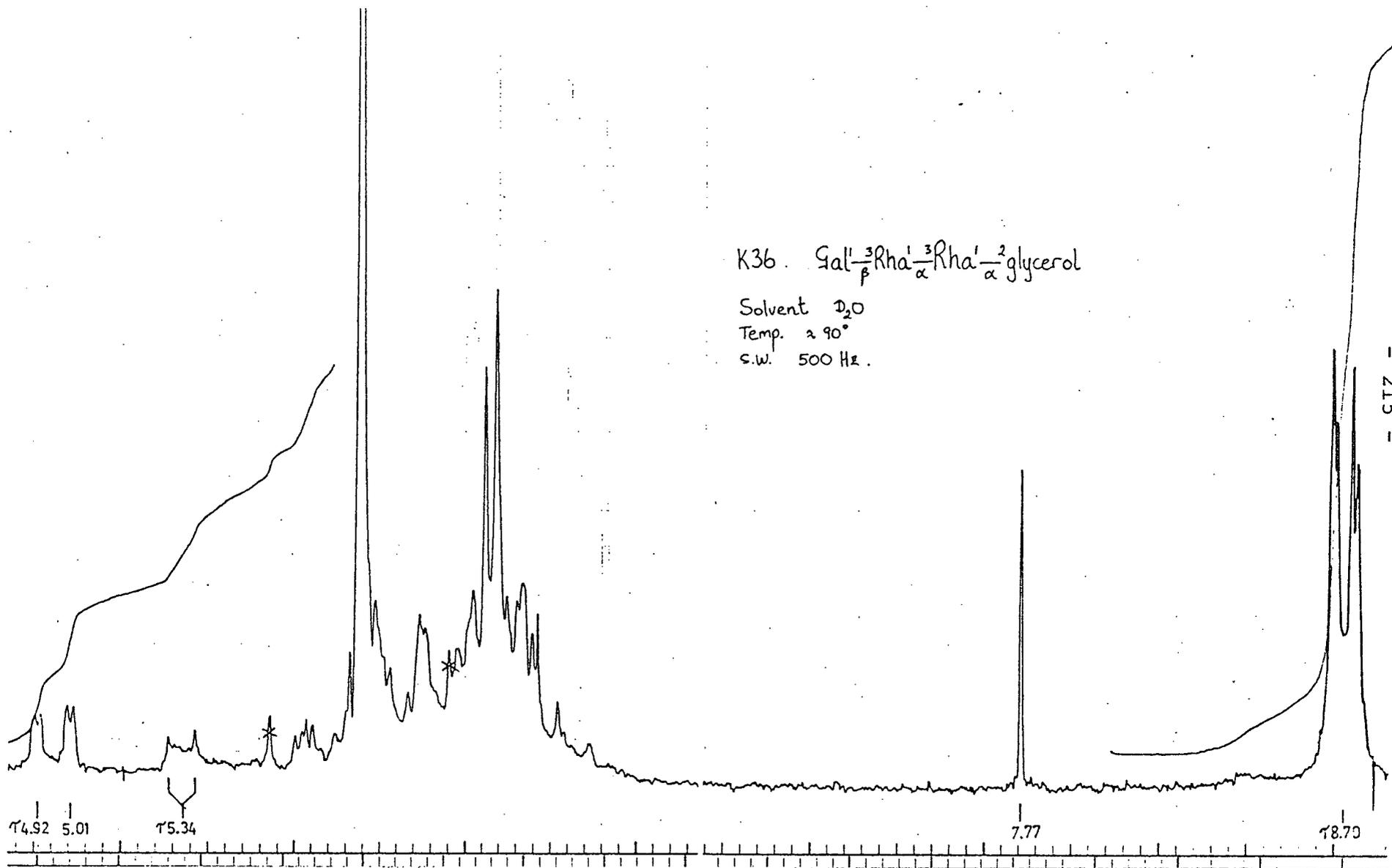


Spectrum No. 2.

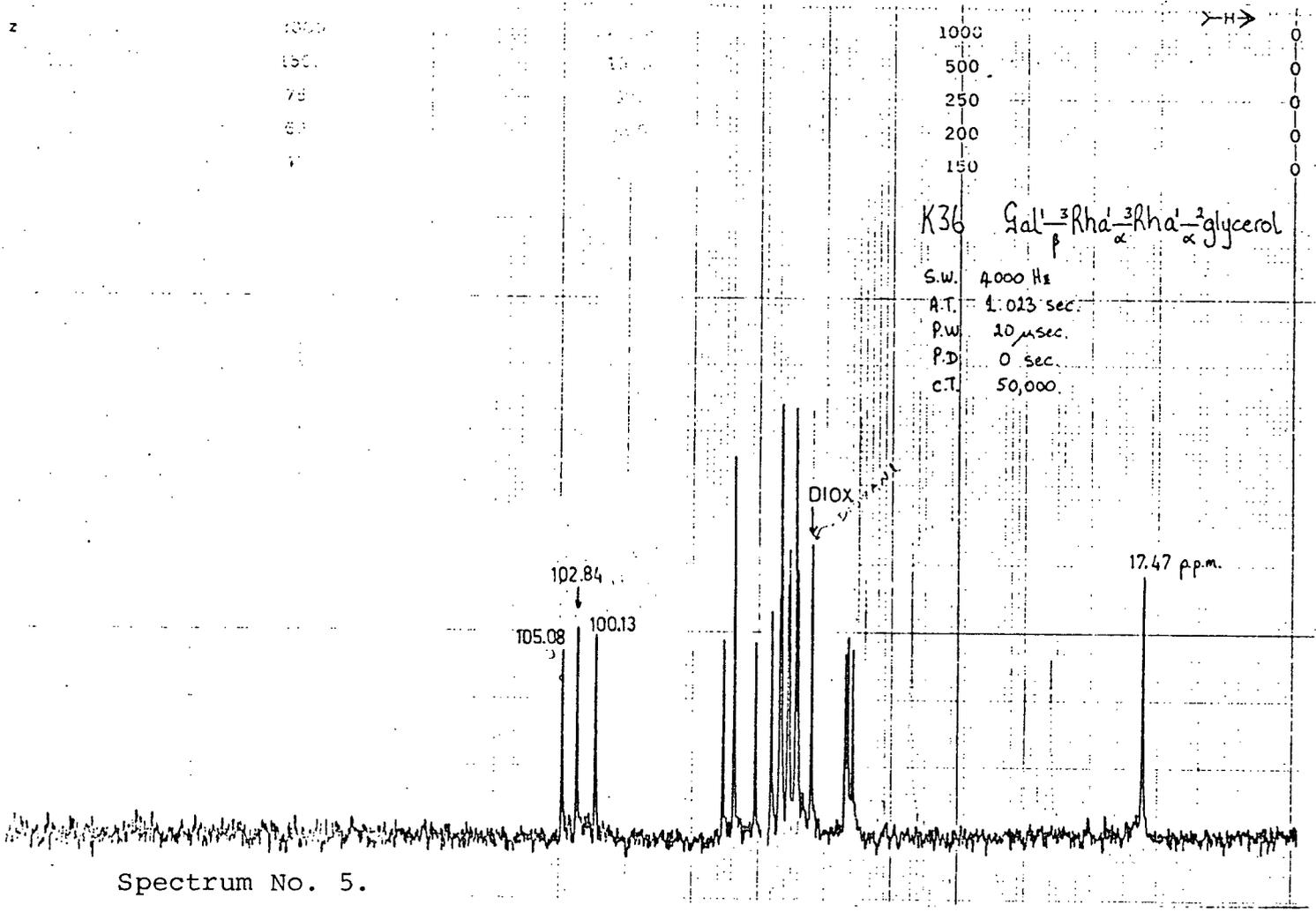
K3b Polysaccharide
(autohydrolysed; 16h, pH 2.2, 95°)
Solvent D₂O
Temp. ≈ 90°
S.W. 500 Hz



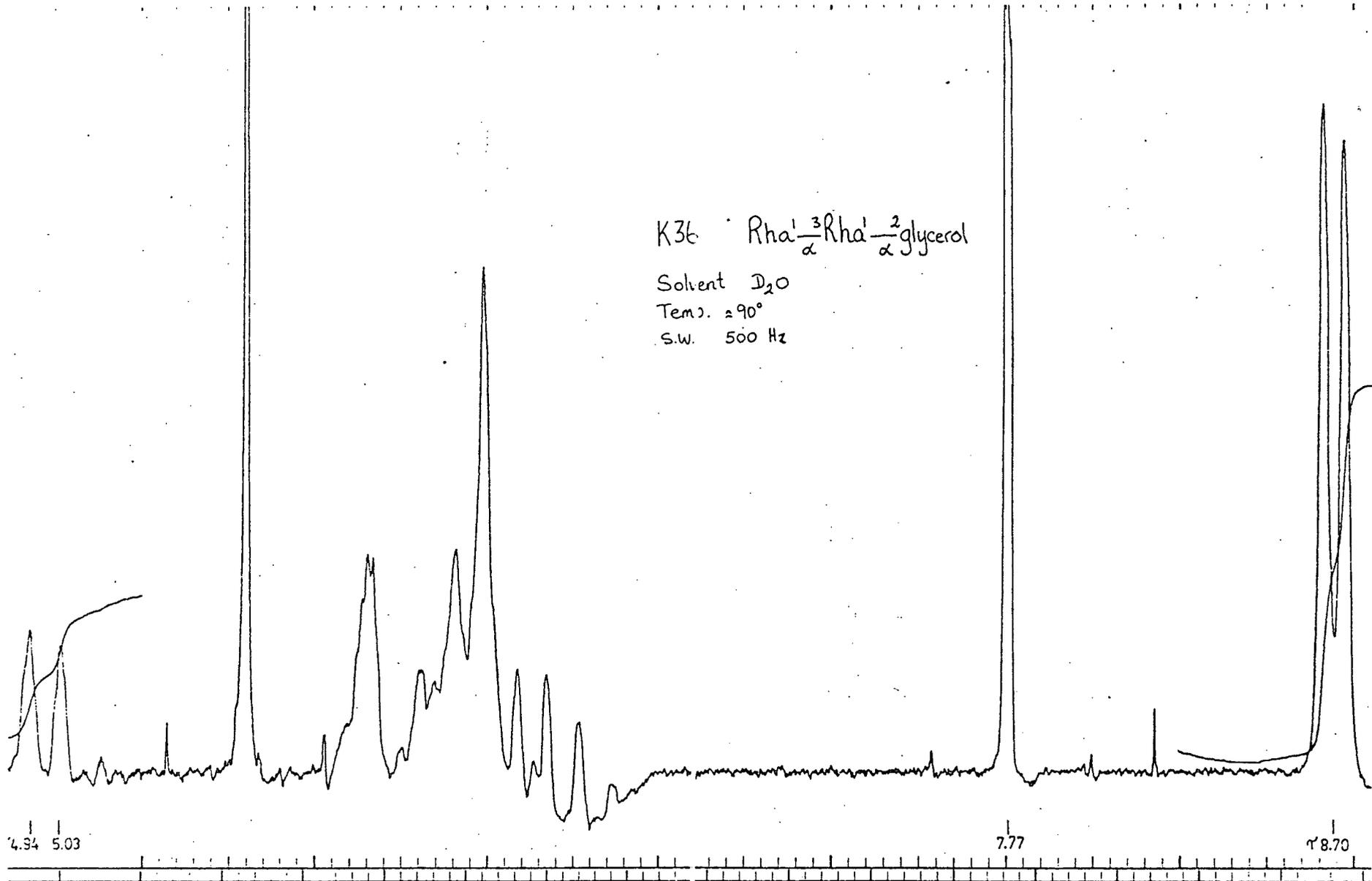
Spectrum No. 3.



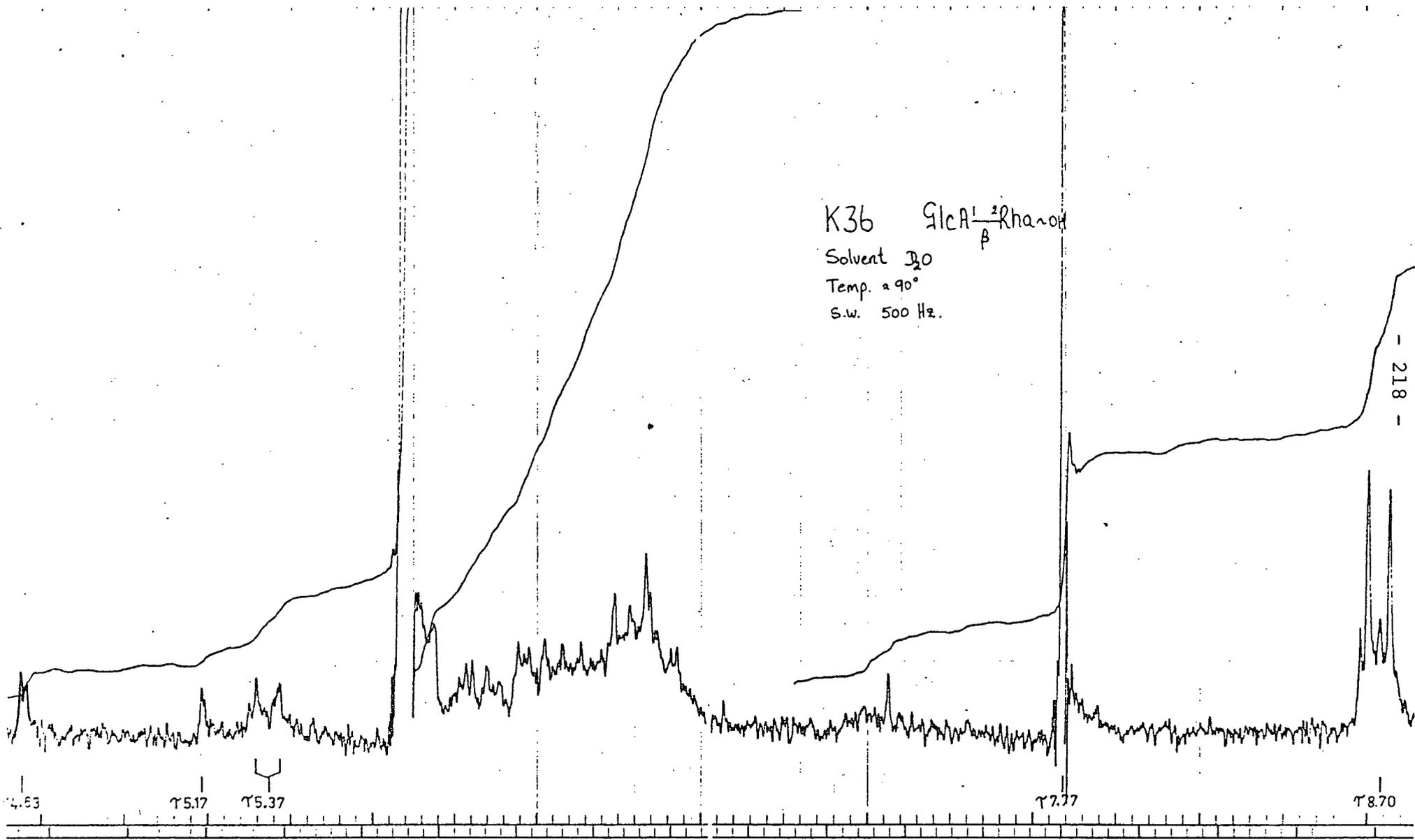
Spectrum No. 4.



Spectrum No. 5.



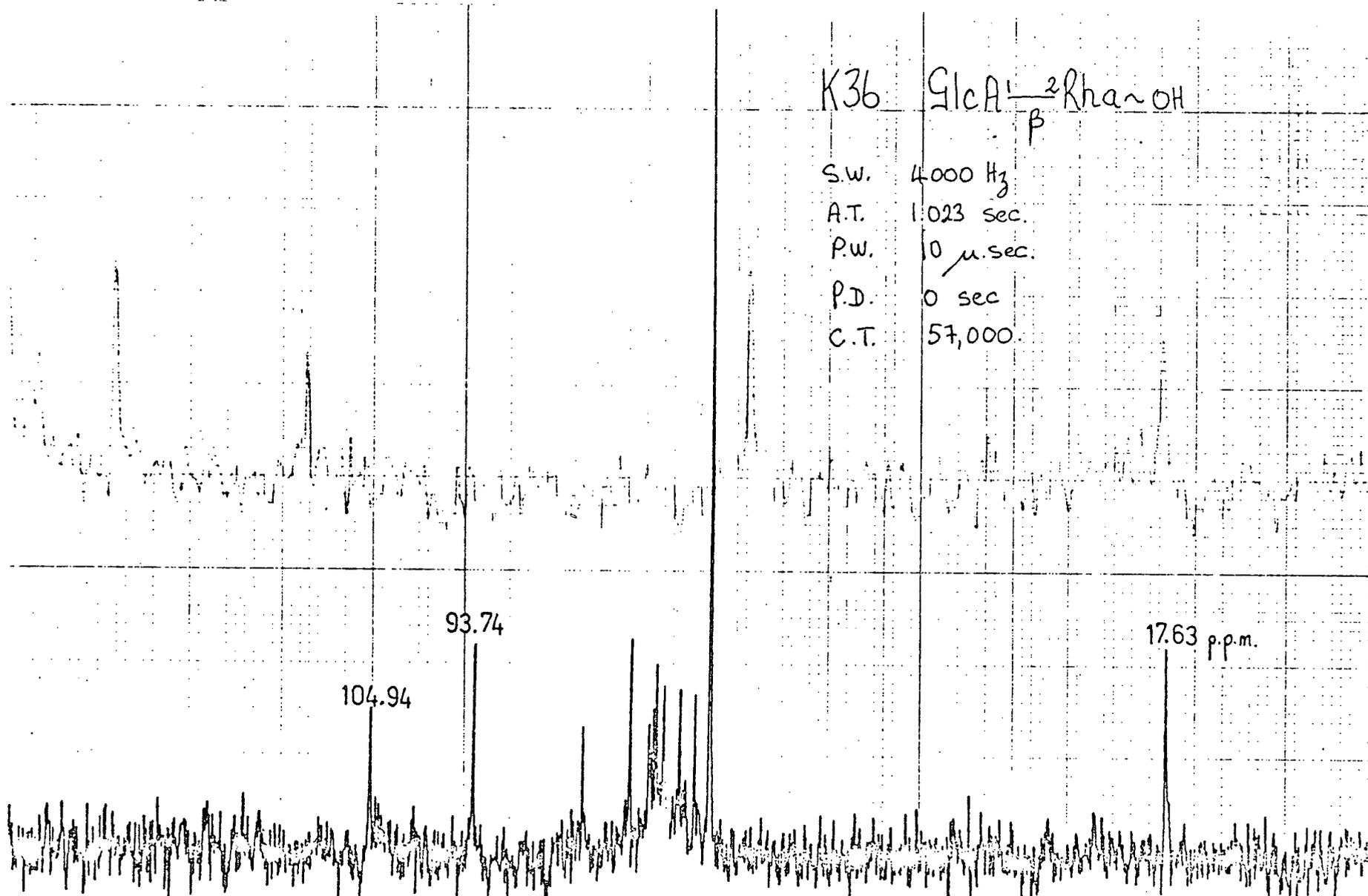
Spectrum No. 6.



Spectrum No. 7.

K36 GlcA¹-_β2Rha-OH

S.W. 4000 Hz
A.T. 1.023 sec.
P.W. 10 μ.sec.
P.D. 0 sec
C.T. 57,000



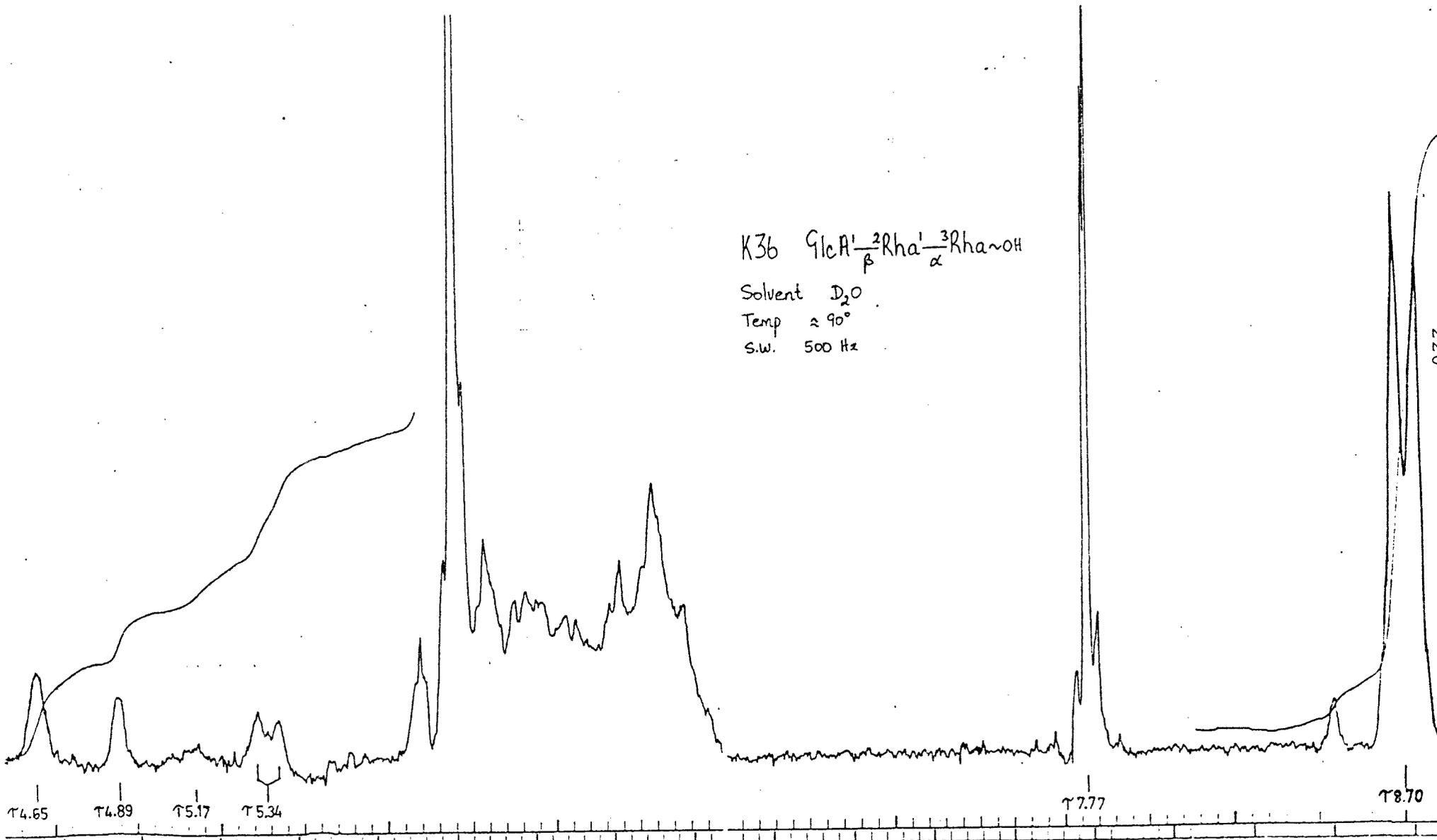
Spectrum No. 8.

K36 GlcA¹_β-2Rha¹_α-3Rha-OH

Solvent D₂O

Temp ≈ 90°

S.W. 500 Hz



Spectrum No. 9.

K36 $\text{GlcA}'_{\beta} - \text{Rha}'^2_{\alpha} - \text{Rha}\alpha^3 - \text{OH}$

S.W. 4000
A.T. 1.023 sec.
P.W. 20 μ . sec.
P.D. 0 sec.
C.T. 150,000

DIOX.

17.43

105.06
101.64

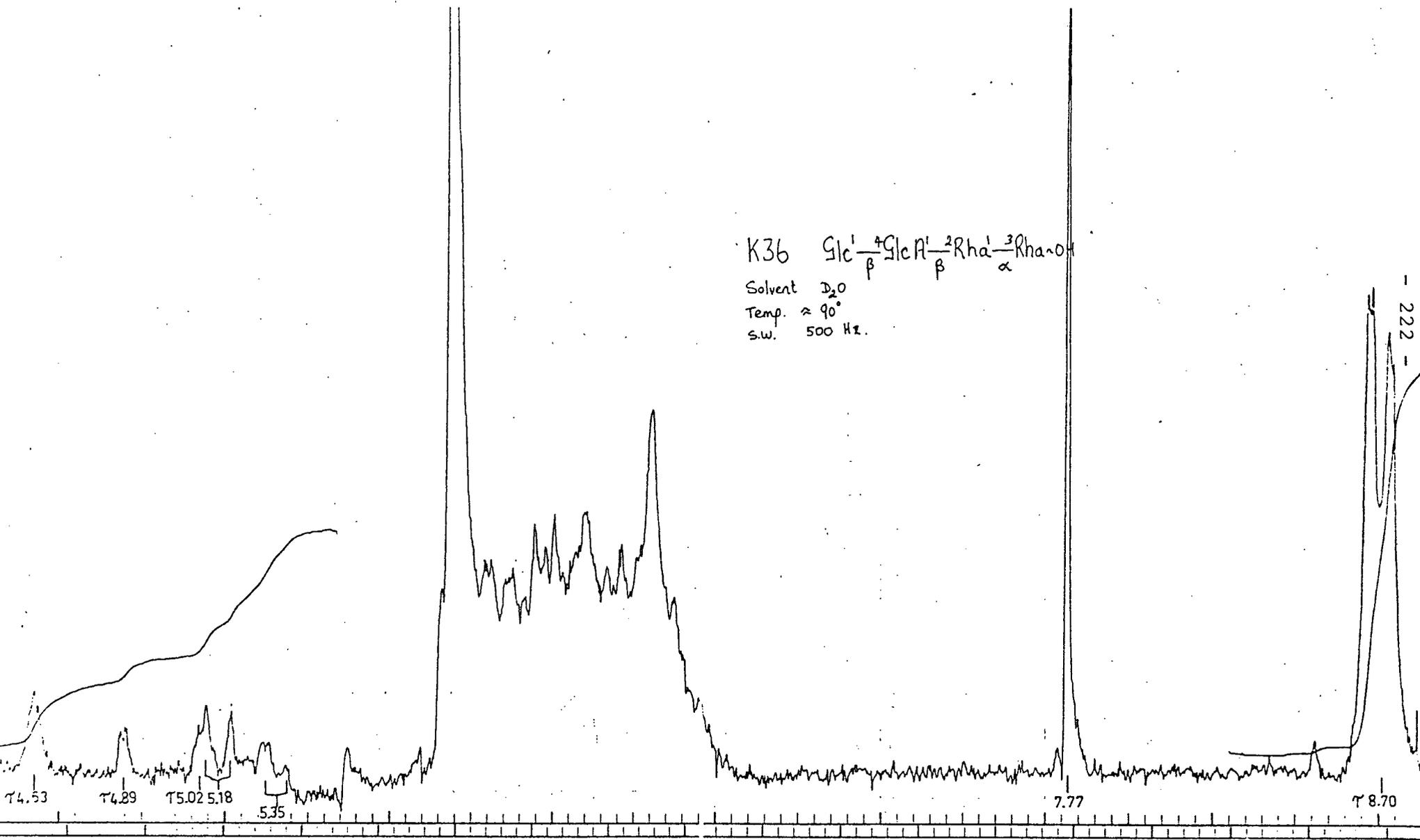
94.76

Spectrum No. 10.

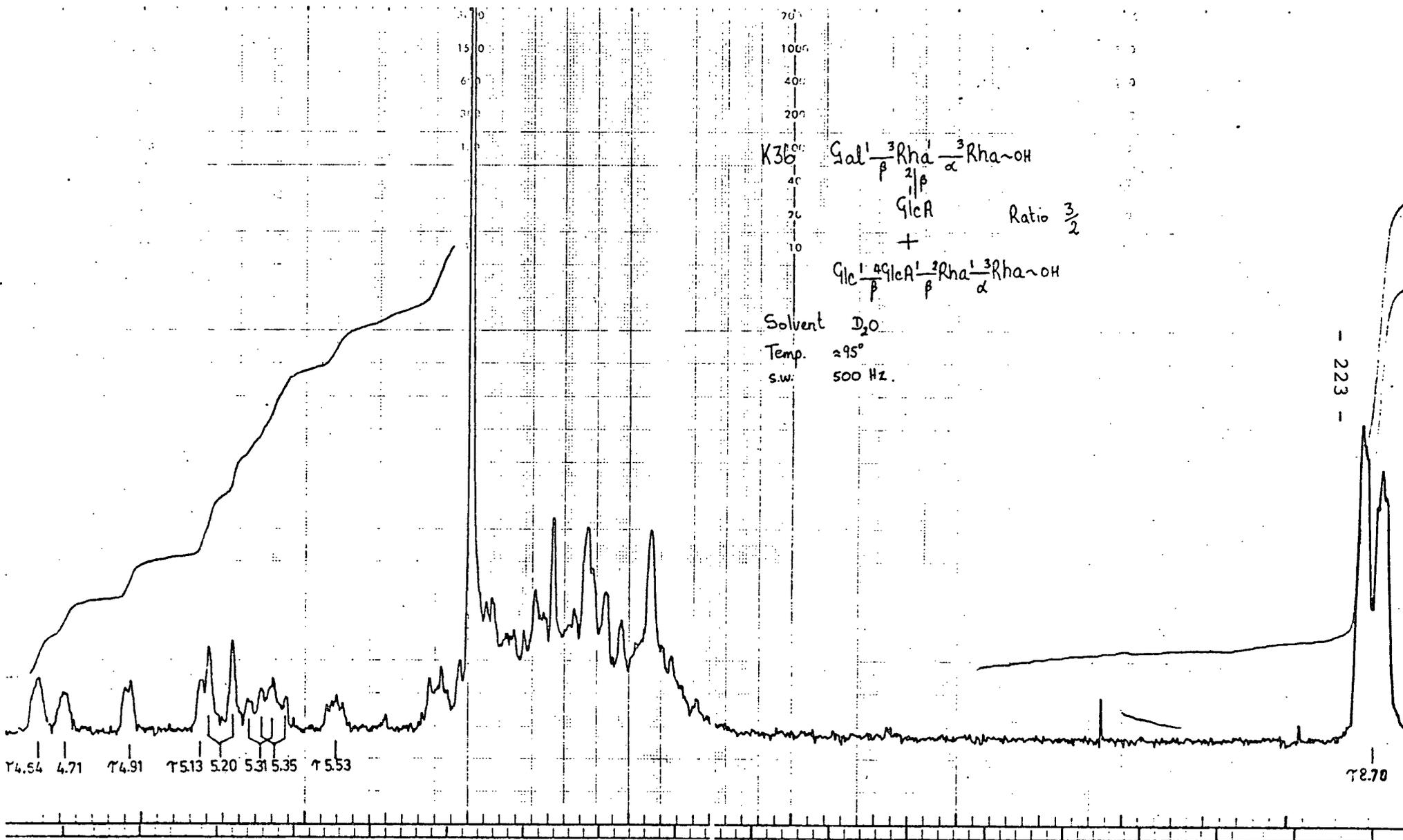
K36 $\text{Glc}'_{\beta} - \text{Glc}''_{\beta} - \text{Rha}'_{\alpha} - \text{Rha}''_{\alpha}$

Solvent D_2O
Temp. $\approx 90^\circ$
s.w. 500 Hz.

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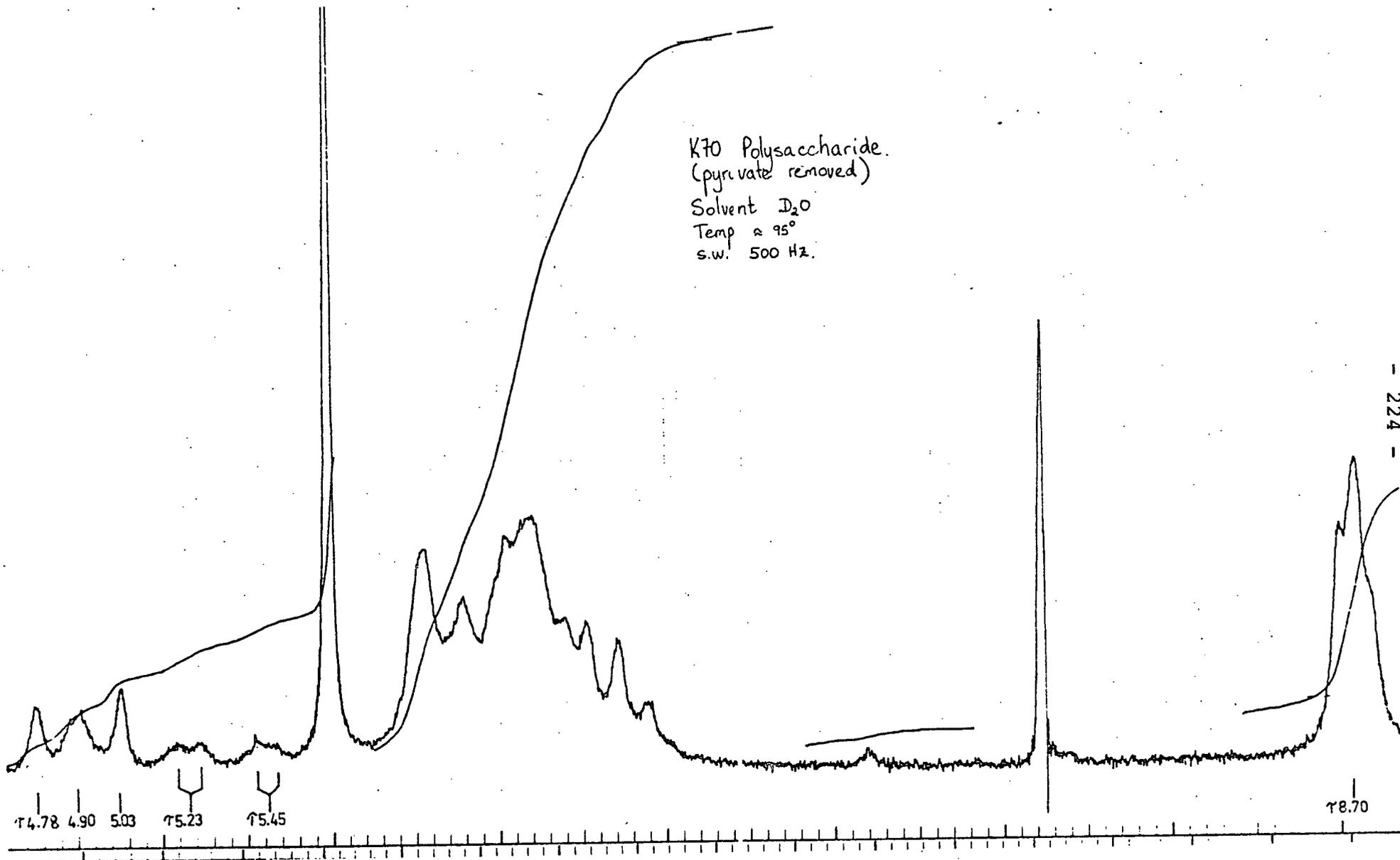


Spectrum No. 11.



Spectrum No. 12.

K70 Polysaccharide.
(pyruvate removed)
Solvent D₂O
Temp ≈ 95°
s.w. 500 Hz.

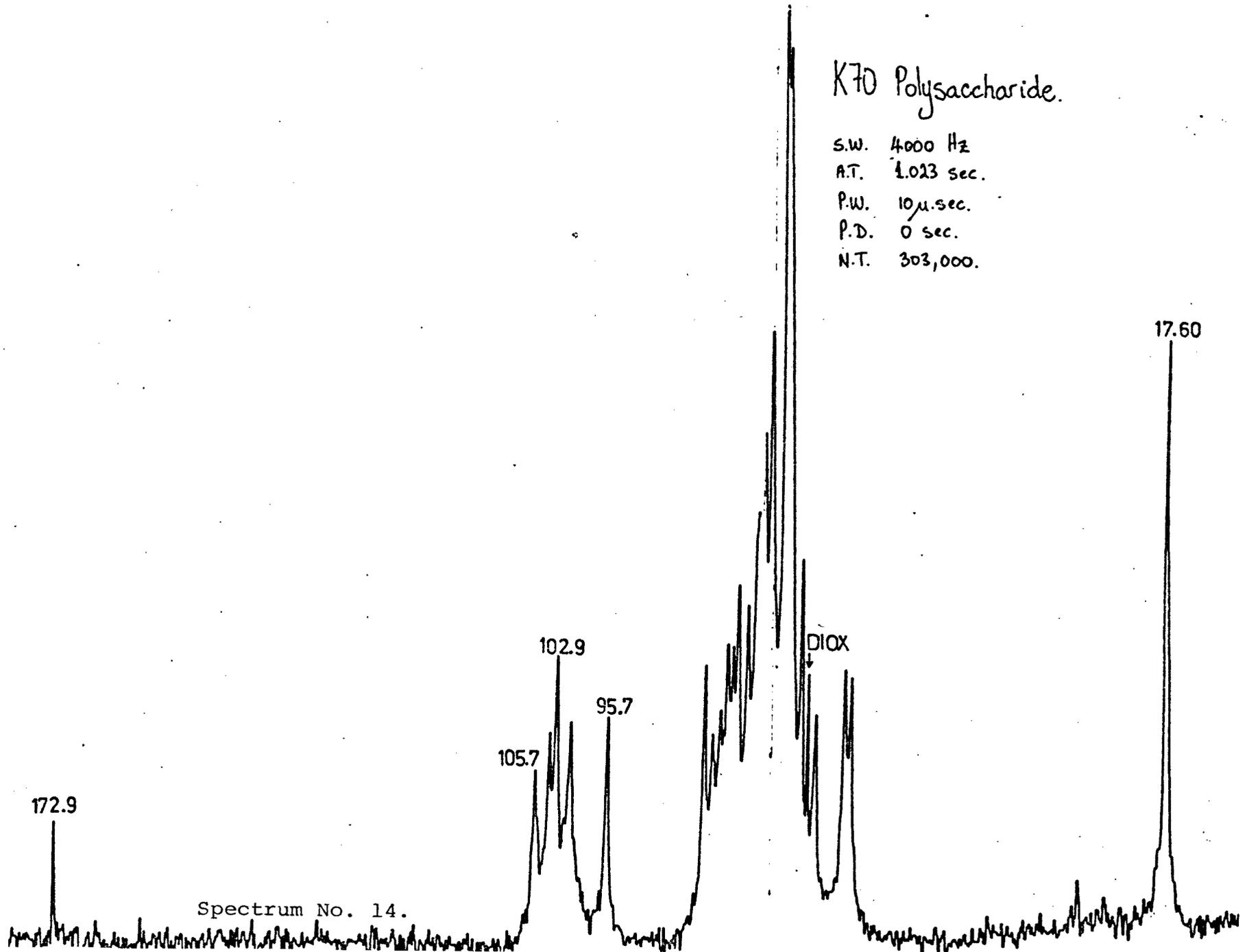


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Spectrum No. 13.

K70 Polysaccharide.

S.W. 4000 Hz
A.T. 1.023 sec.
P.W. 10 μ .sec.
P.D. 0 sec.
N.T. 303,000.



Spectrum No. 14.

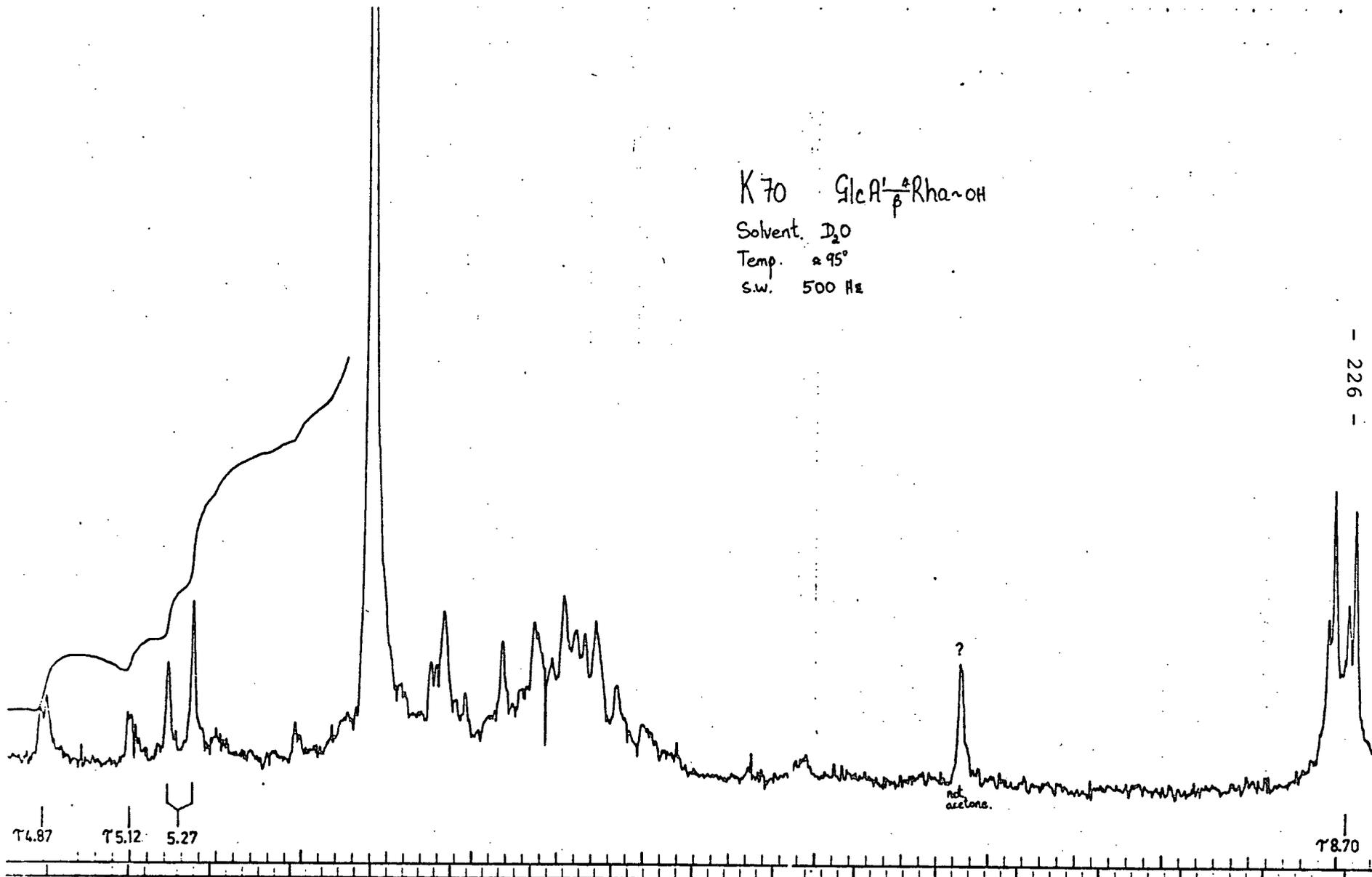
K70 $\text{GlcA}'_{\beta}\text{Rha-OH}$

Solvent. D_2O

Temp. $\approx 95^\circ$

S.W. 500 Hz

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Spectrum No. 15.

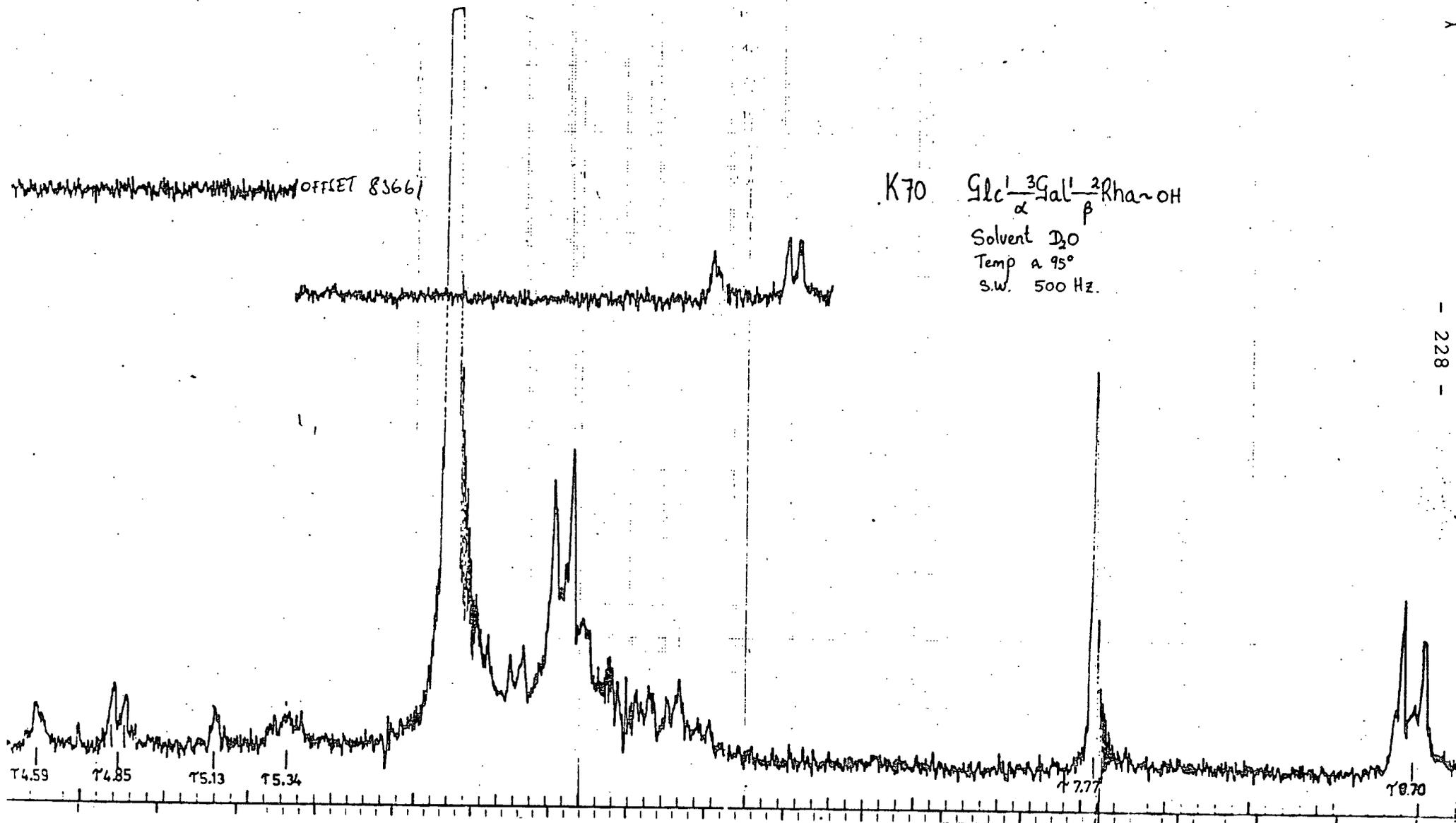
N°	Int.	pp.m.
1	79	103.89
2	51	94.63
3	28	94.26
4	51	82.14
5	27	81.70
6	79	76.19
7	29	75.56
8	84	74.30
9	33	73.69
10	99	72.22
11	57	71.77
12	31	71.28
13	53	70.89
14	50	67.54
15	100	31.07
16	53	17.78

K70 GlcA- β -RharOH

S.W. 2000
 A.T. 1.023 sec.
 P.W. 10 μ .sec.
 P.D. 1/2 sec.
 C.T. ?

31.07

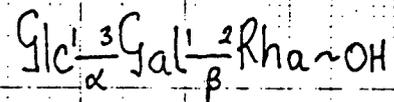
Spectrum No. 16.



K70 $\text{Glc}^1_{\alpha}\text{-Gal}^3_{\beta}\text{-Rha-OH}$
Solvent D_2O
Temp 95°
S.W. 500 Hz.

Spectrum No. 17.

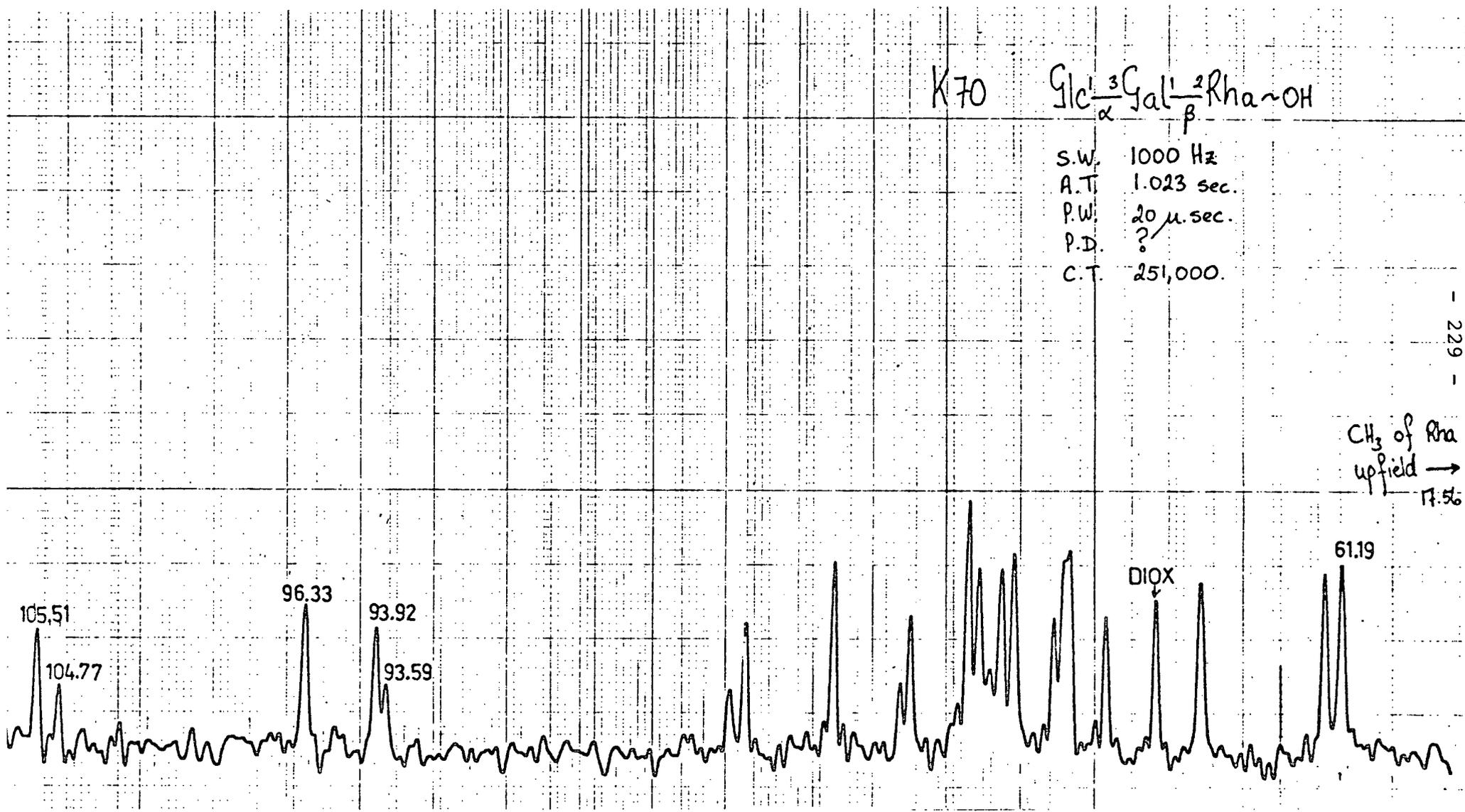
K70



S.W. 1000 Hz
A.T. 1.023 sec.
P.W. 20 μ .sec.
P.D. ?
C.T. 251,000.

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CH₃ of Rha
upfield \rightarrow
17.56

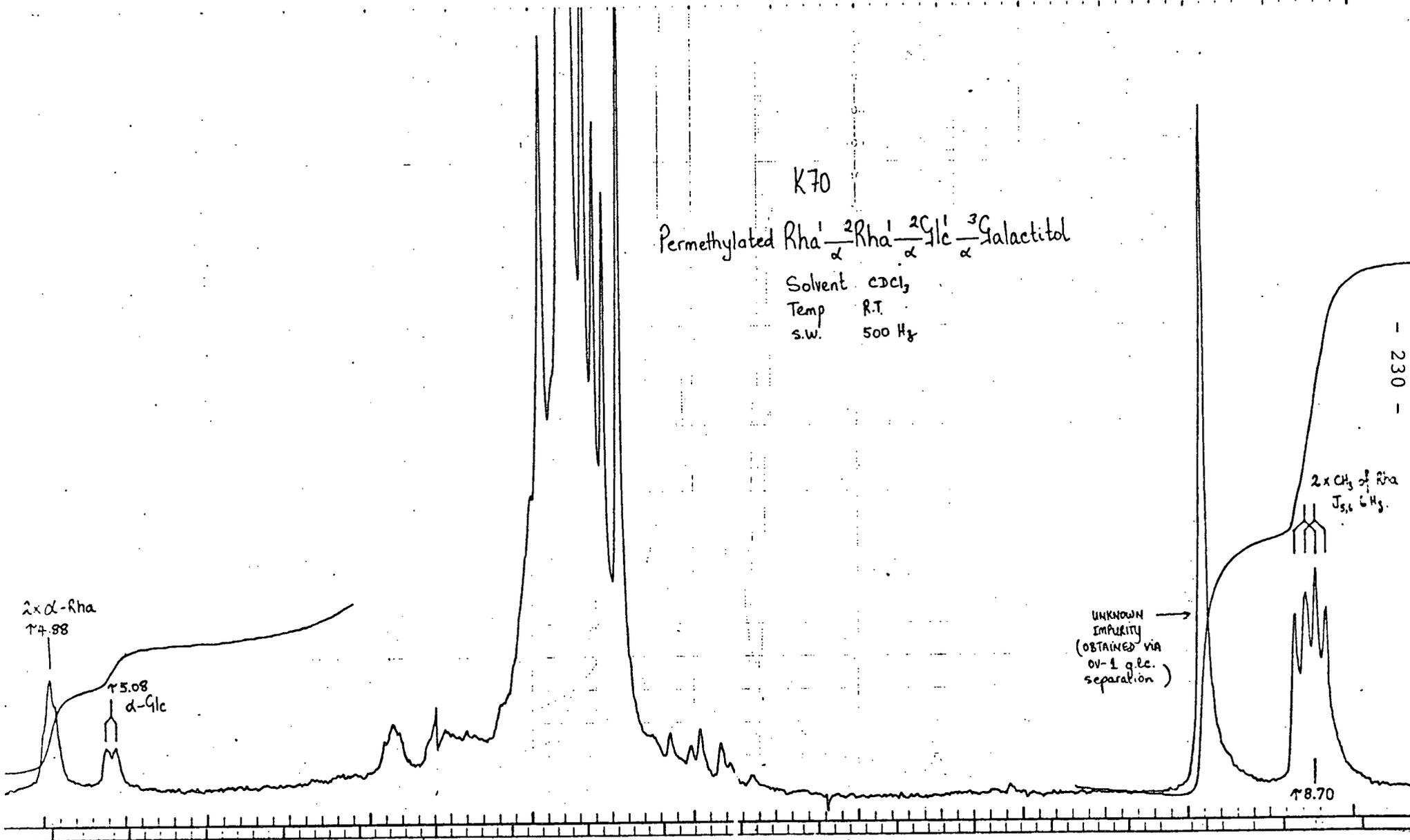


Spectrum No. 18.

K70

Permethyloated Rha¹_α-Rha²_α-Glc¹_α-Galactitol³_α

Solvent: CDCl₃
Temp: R.T.
s.w.: 500 Hz



2x α-Rha
↑ 4.88

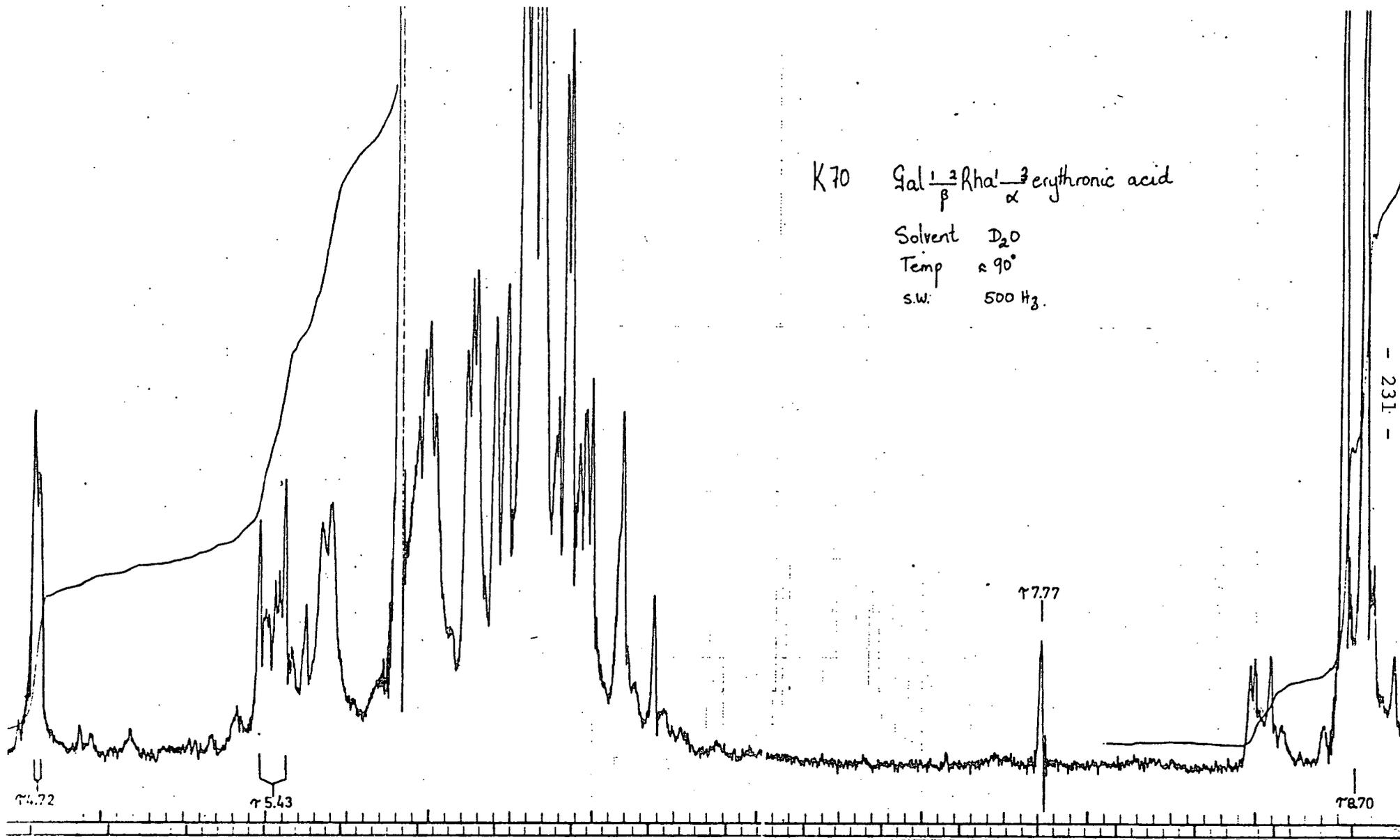
↑ 5.08
α-Glc

UNKNOWN
IMPURITY
(OBTAINED VIA
OV-1 g.l.c.
separation)

2x CH₃ of Rha
↑ 8.70

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Spectrum No. 19.



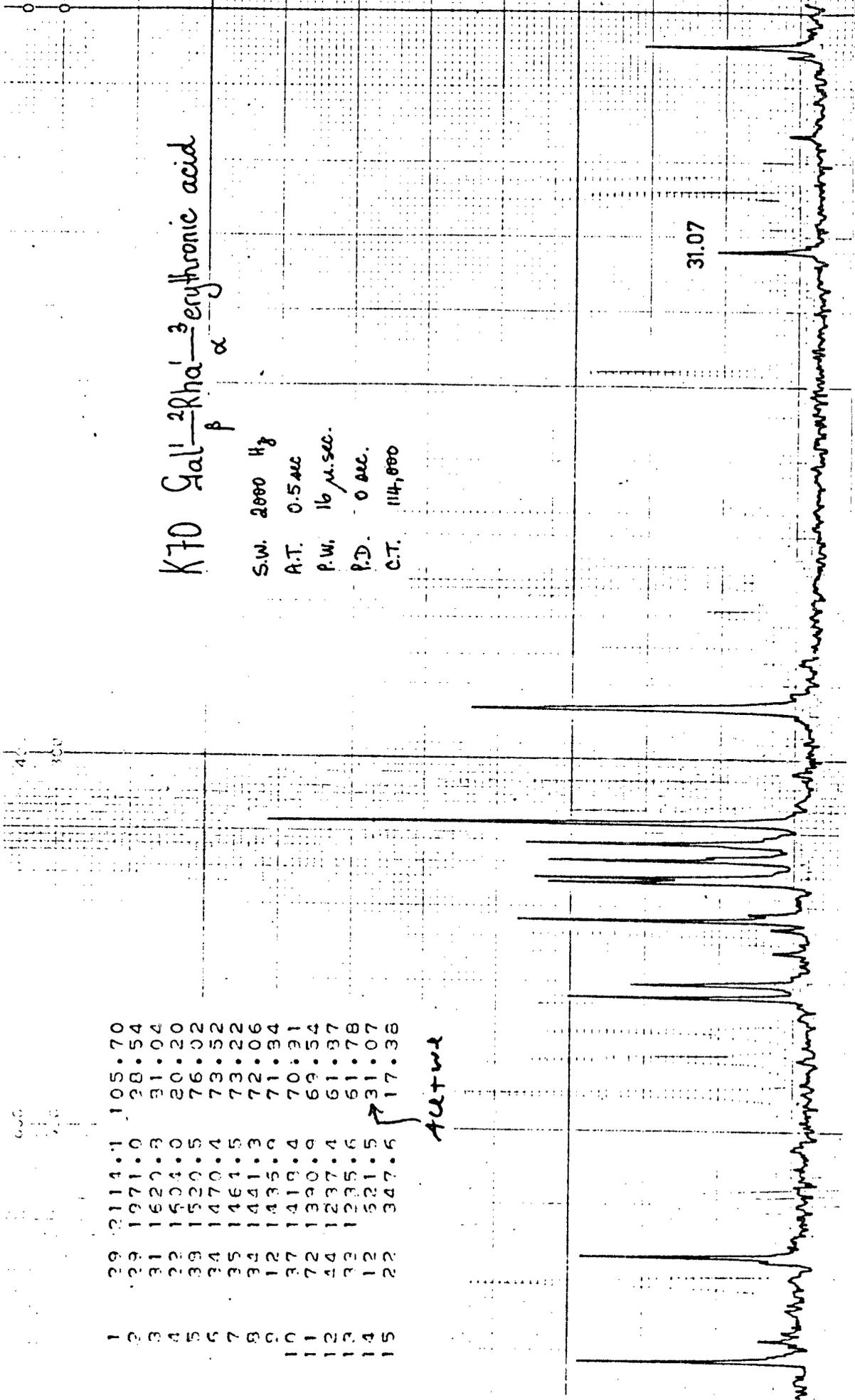
Spectrum No. 20.

K7D Gal¹-²Rha¹-³erythronic acid

S.W. 2000 Hz
 A.T. 0.5 sec
 P.W. 16 μ .sec.
 P.D. 0 sec.
 C.T. 114,000

1	29	2114.1	105.70
2	29	1971.0	38.54
3	31	1620.3	31.04
4	22	1504.0	20.20
5	33	1520.5	76.02
6	34	1470.4	73.52
7	35	1461.5	73.22
8	34	1441.3	72.06
9	12	1435.0	71.34
10	37	1419.4	70.31
11	72	1390.9	69.54
12	44	1237.4	61.37
13	33	1235.6	61.78
14	12	521.5	31.07
15	22	347.6	17.38

Acetone

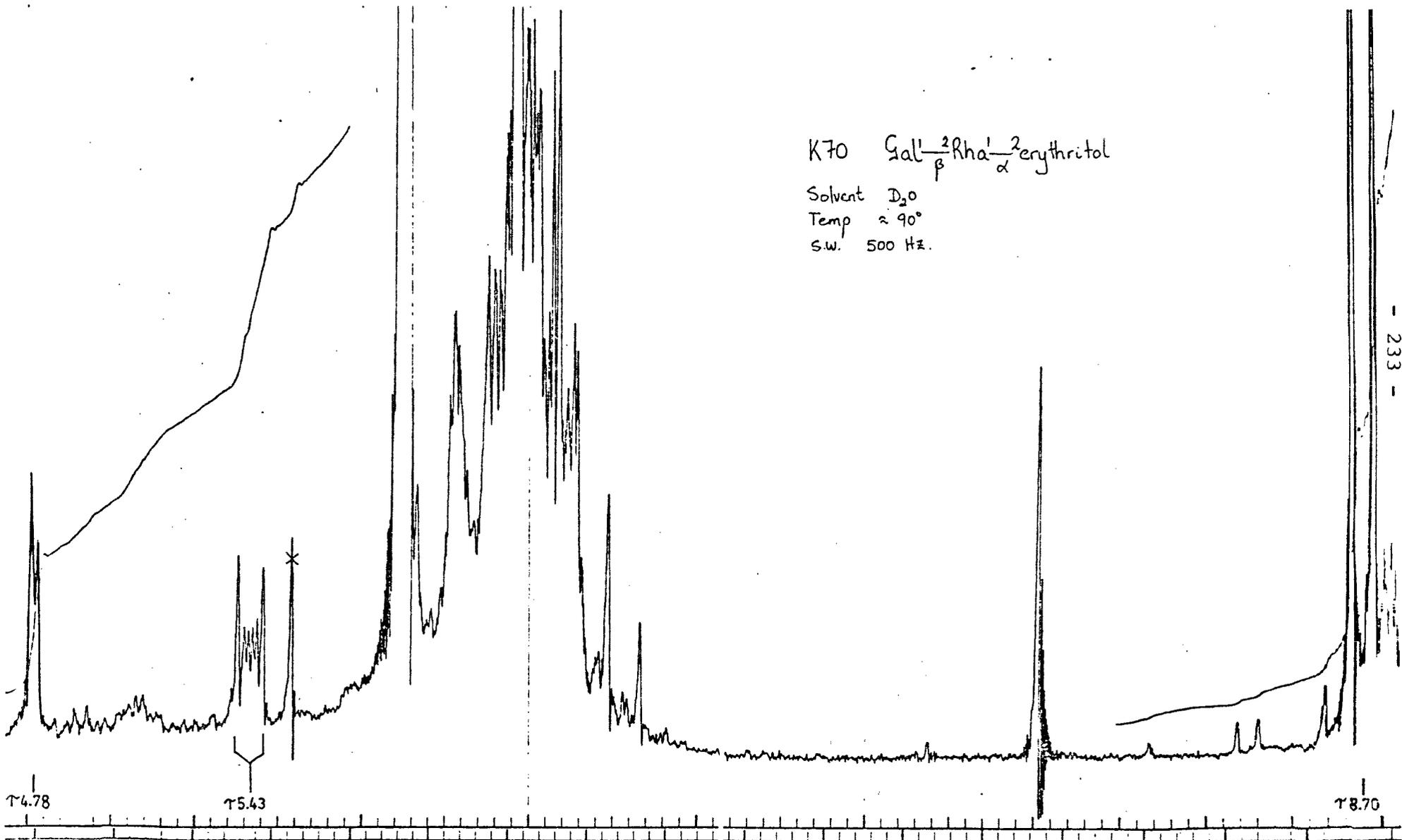


K70 Gal_β¹⁻²Rha_α¹⁻²erythritol

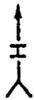
Solvent D₂O

Temp ≈ 90°

S.W. 500 Hz.



Spectrum No. 22.



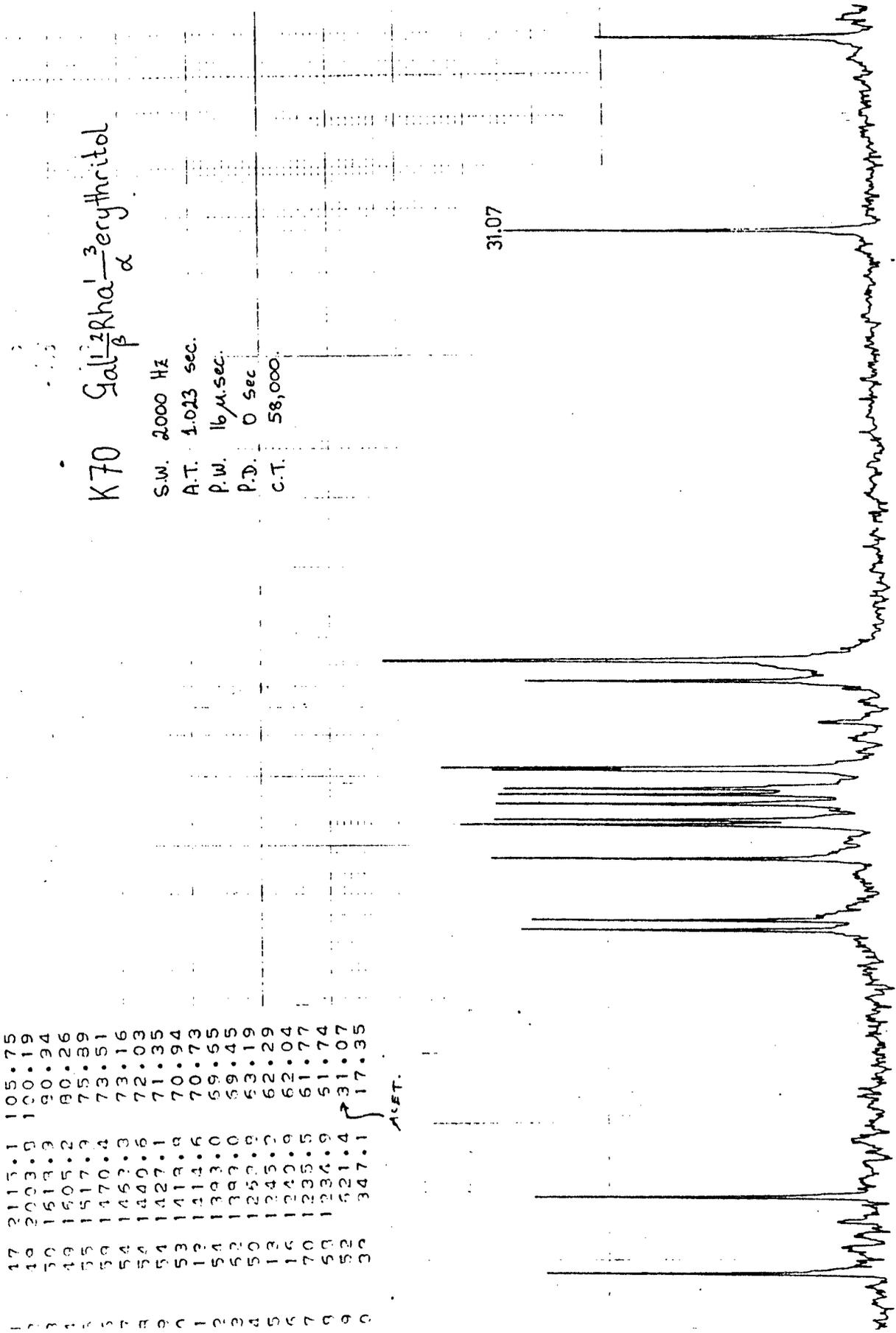
K70 Gal^{1,2}Rha¹-³erythritol

S.W. 2000 Hz
 A.T. 1.023 sec.
 P.W. 16 μ .sec.
 P.D. 0 sec.
 C.T. 58,000

17	2115.1	105.75
18	2003.9	100.19
19	1613.9	90.94
19	1505.2	80.26
25	1517.9	75.89
39	1470.4	73.51
54	1452.3	73.16
54	1440.6	72.03
54	1427.1	71.35
53	1419.8	70.94
12	1414.6	70.73
54	1393.0	69.55
52	1392.0	69.45
44	1252.9	63.19
55	1245.2	62.29
16	1240.9	62.04
70	1235.5	61.77
58	1234.9	61.74
52	521.4	31.07
30	347.1	17.35

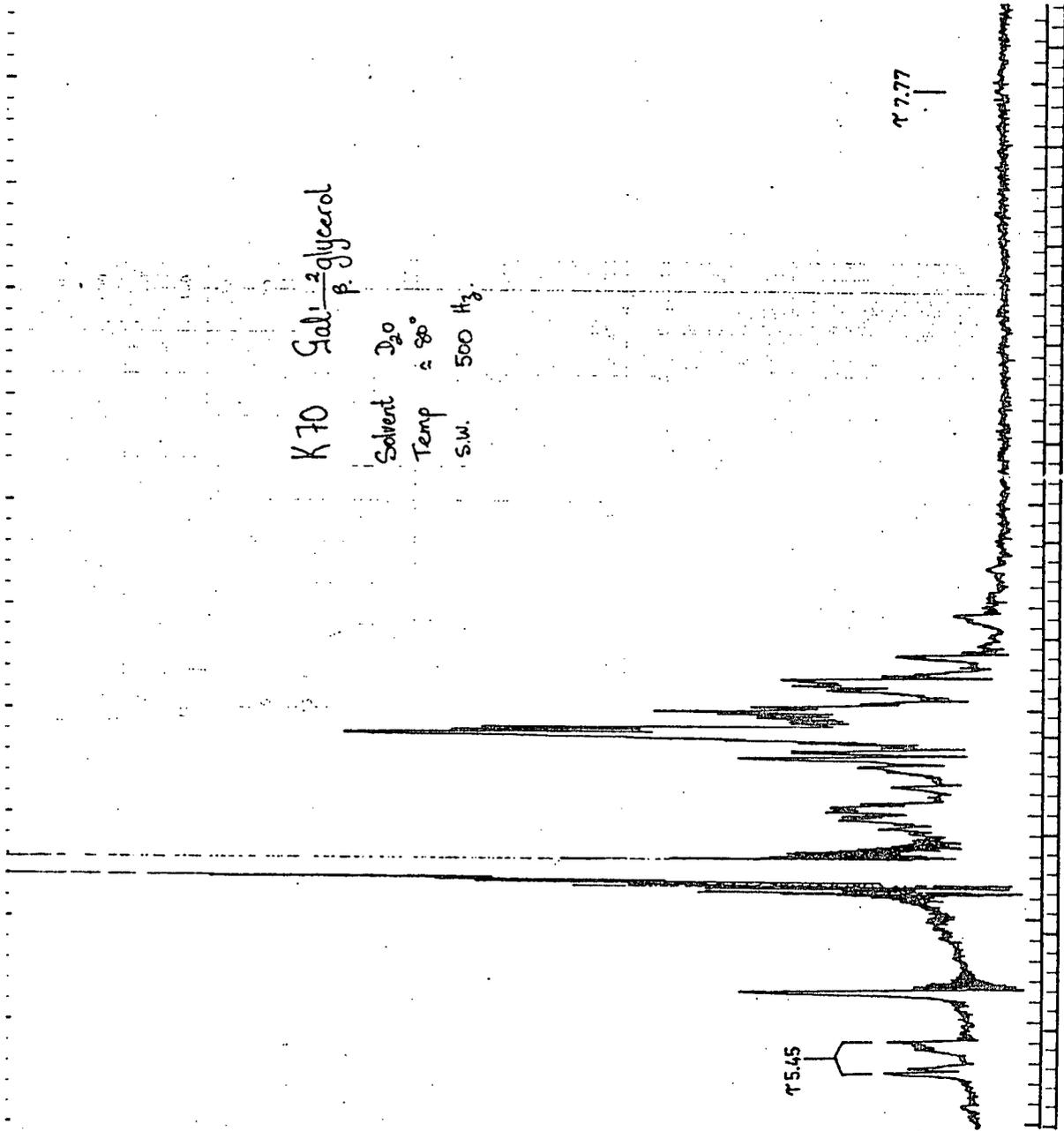
ASFT.

31.07



K70 Sal.² glycerol
P. glycerol

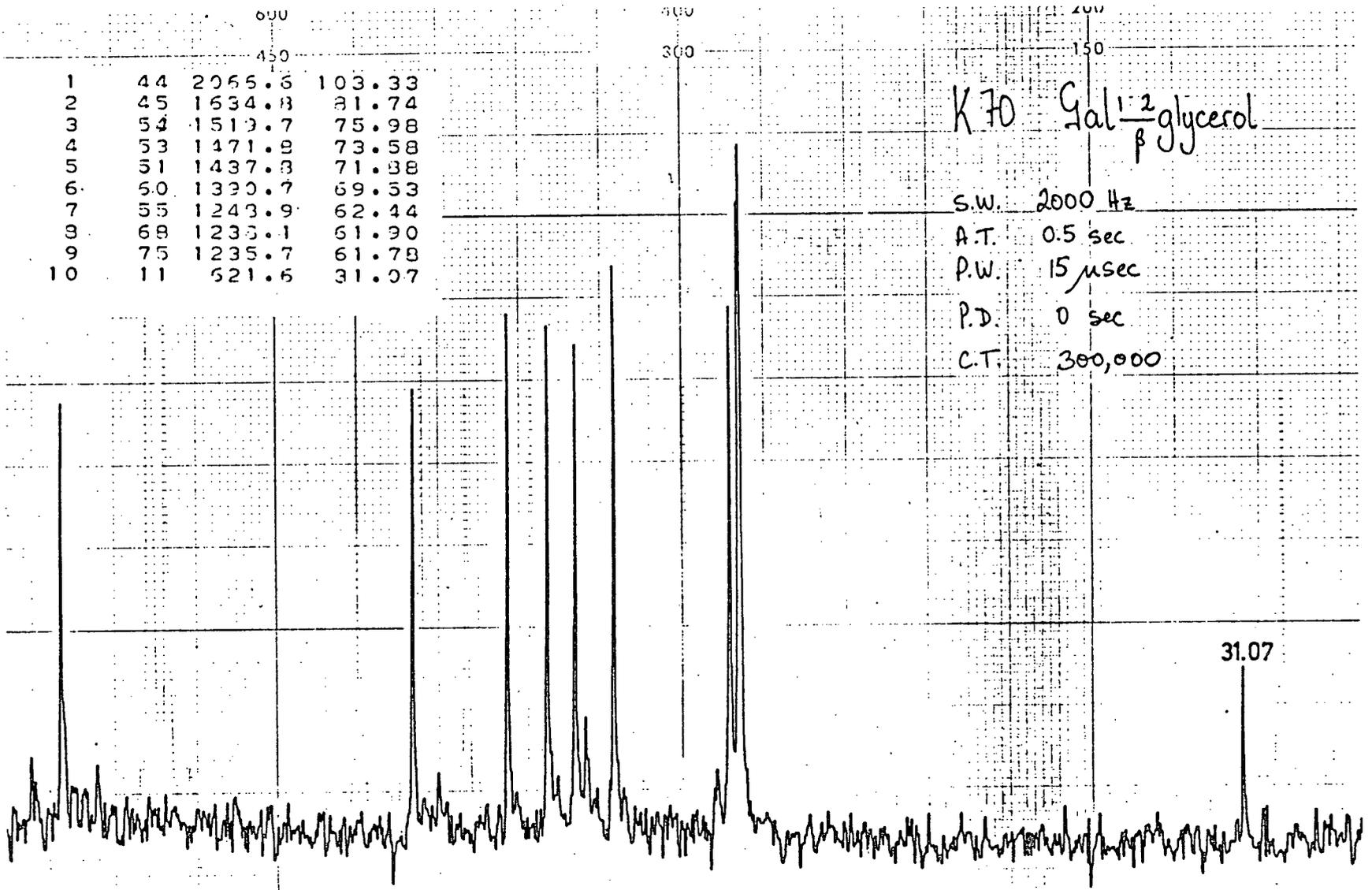
Solvent D₂O
Temp ≈ 80°
S.W. 500 Hz



		600		
		450		
1	44	2055.6	103.33	
2	45	1634.8	31.74	
3	54	1513.7	75.98	
4	53	1471.8	73.58	
5	51	1437.3	71.38	
6	50	1330.7	69.53	
7	55	1243.9	62.44	
8	68	1235.1	61.90	
9	75	1235.7	61.78	
10	11	521.6	31.07	

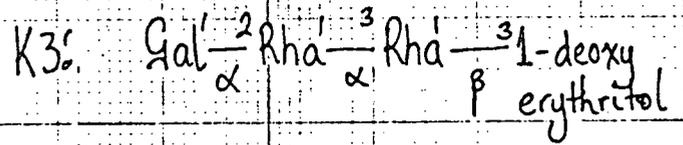
K70 Gal¹⁻²/_β glycerol

S.W. 2000 Hz
 A.T. 0.5 sec
 P.W. 15 μsec
 P.D. 0 sec
 C.T. 300,000

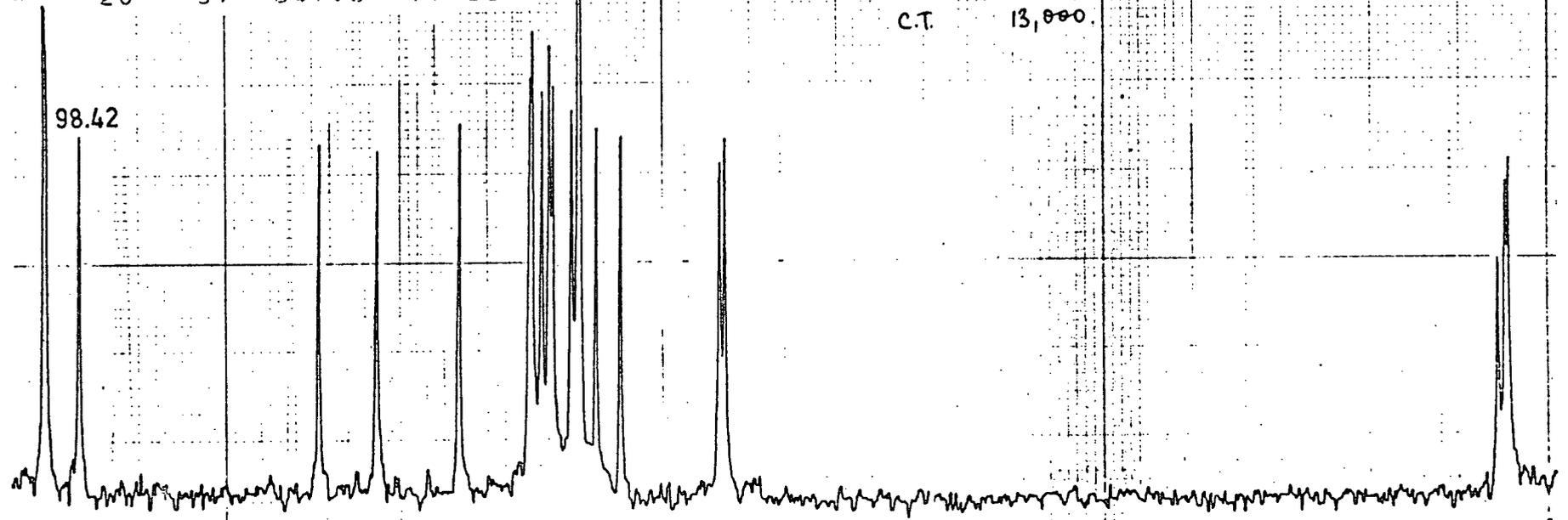


Spectrum No. 25.

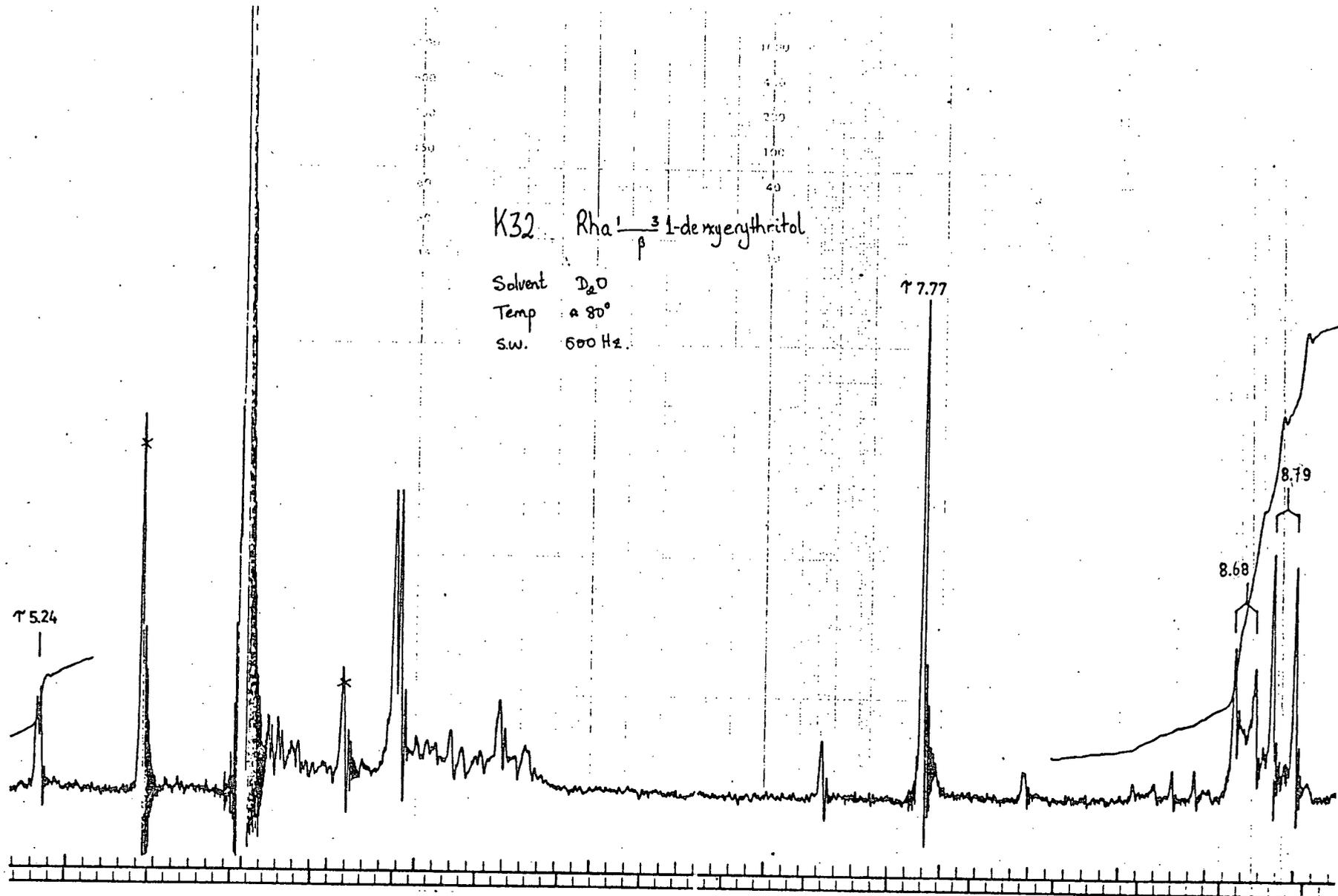
1	55	2003.3	100.41
2	54	2007.0	100.35
3	40	1353.4	98.42
4	39	1693.2	84.65
5	39	1627.5	81.39
6	41	1535.5	76.77
7	47	1455.1	72.90
8	52	1453.5	72.67
9	15	1443.0	72.14
10	50	1431.9	71.74
11	45	1430.2	71.54
12	43	1407.7	70.48
13	100	1407.7	70.03
14	41	1331.6	69.08
15	40	1251.3	67.71
16	37	1241.3	62.06
17	39	1235.4	61.76
18	26	359.2	17.95
19	35	351.1	17.55
20	37	347.3	17.36



S.W. 2000 Hz
 A.T. 0.6 sec.
 P.W. 18 μ sec.
 P.D. 0 sec
 C.T. 13,000.



Spectrum No. 26.



Spectrum No. 27.

K32 Rha¹ - β -D-3-deoxyerythritol

S.W. 2000 Hz

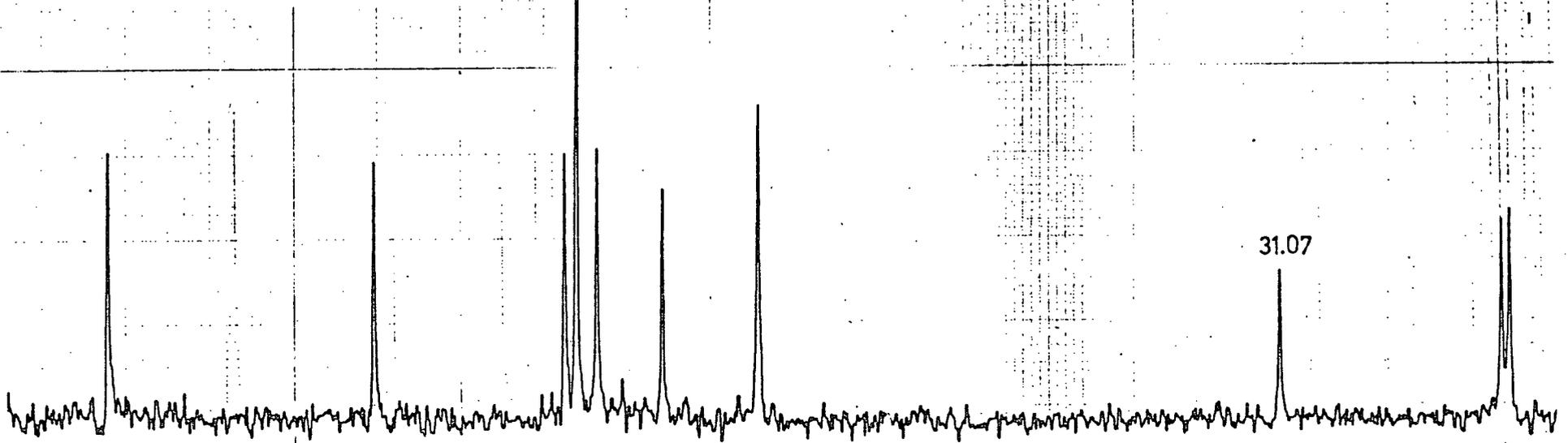
A.T. 0.5 sec.

P.W. 18 μ sec

P.D. 0 sec

C.T. 114,000

1	29	2014.0	100.69
2	27	1696.6	34.32
3	23	1471.9	73.59
4	50	1457.3	72.39
5	22	1433.4	71.67
6	23	1355.2	67.76
7	33	1242.1	62.10
8	13	621.4	31.07
9	19	351.0	18.05
10	21	351.0	17.54

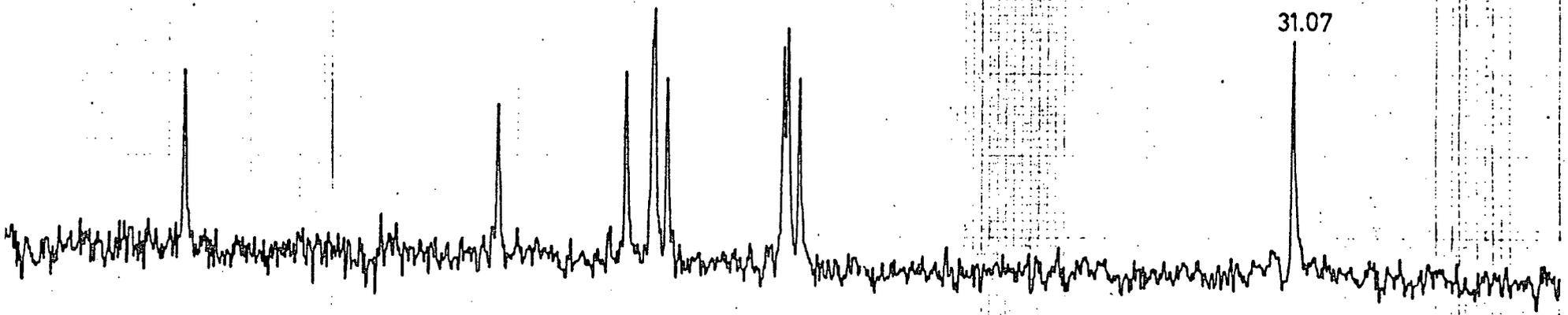


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K32 Gal¹⁻² glycerol
d

S.W. 2000 Hz
A.T. 0.6 sec.
P.W. 17 μsec.
P.D. 0 sec.
C.T. 94,000

1	12	1377.9	38.89
2	15	1532.6	79.63
3	20	1433.0	71.39
4	23	1405.1	70.30
5	27	1403.4	70.17
6	19	1387.5	59.37
7	23	1245.7	62.28
8	25	1240.2	62.01
9	19	1225.7	61.28
Act, 0	23	521.3	21.06



Spectrum No. 29.

