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CHANGES IN THE CHEMICAL COMPOSITION OF SUGAR CANE (*Saccharum officinarum*) DURING STORAGE

by

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Science at the University of Natal.

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I N D E X

1. PREFACE

The work described in this dissertation was carried out at the Sugar Milling Research Institute, University of Natal, Durban. The work is original, except where specifically stated in the text, and no part of the thesis has been submitted in candidature for a degree at any other University.

In accordance with regulation E 40, the candidate submits the following references, two publications supplementary to this work, in support of this candidature:

"Changes in the Chemical Composition of Sugar Cane (*Saccharum officinarum*) during storage"

The International Sugar Journal (1966) 68 331,356
Ibidem (1970) 72 195

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3. SUMMARY

An outline is given of the South African sugar industry, with particular emphasis on the unit operations which make up the industrial process for manufacturing sugar from cane.

Current knowledge of the chemistry of soluble polysaccharides is reviewed and the structures of several polysaccharides, including starch, dextran, and pullulan, are discussed.

It has been found that changes take place in the chemical composition of the juice in sugar cane (*Saccharum officinarum*) during post-harvest storage. With increasing storage time, there is a proportional decrease in the starch content of the juice, and a considerably larger proportional increase in the soluble polysaccharide content. The increased polysaccharide content was found to be due to a single glucan which, contrary to most previous publications on this subject, is definitely not a dextran. Following structural analysis, it has been established that the polysaccharide formed in stored cane had not been described before and the name "sarkaran", derived from the Sanskrit word "Sarkara", meaning "sugar" is proposed for it.

The polysaccharide was isolated from cane juice by precipitation with ethanol after the starch in the juice had been removed by centrifugation. The polysaccharide was purified by repeated dissolution in water and reprecipitation with ethanol.

Analysis by gel chromatography resulted in a single symmetrical peak, indicating that the isolated polysaccharide is homogeneous. This was confirmed by hydrolysing fractions representing a section of the ascending and a section of the descending part of the peak of the chromatogram, using the enzyme pullulanase. Chromatographic separation and quantitative analysis of the isolated oligosaccharides showed that the compositions of the two enzyme digests were identical.

Acid hydrolysis of the polysaccharide resulted in a single hexose. This was identified as glucose by paper chromatography, comparing the R_f value with that of pure glucose. Confirmation was obtained by comparing the osazone with that of glucose, using microscopic examination and determination of the melting points.

Paper electrophoresis showed the molecule to be uncharged.

Several techniques, both absolute and non absolute, were used to determine the molecular weight of the polysaccharide. A method involving viscosity determination indicated a molecular weight of 34 000 while a figure of 50 000 was obtained by gel chromatography on a Sephadex column, comparing the peak elution volume of the polysaccharide with that of dextrans of a defined molecular weight. Both these techniques are non absolute and yield rough estimates of the molecular weight. Osmometric measurement, an absolute method, showed the number average molecular weight to be 51 500. An absolute value for the weight average molecular weight of 250 000 was obtained by light scattering techniques. Data from the light scattering experiments were also used to determine a value of 200 - 250 Å for the radius of gyration R_G of the polysaccharide. End group analysis after exhaustive methylation resulted in a value of 24 000 for the number average molecular weight Mn. This indicates either that some degradation of the polysaccharide molecule occurs during the methylation procedures or that there is a certain degree of association between individual molecules.

Periodate oxidation showed that 32 percent of the glucosidic linkages are in (1 → 6) position.

The polysaccharide was exhaustively methylated by several Haworth methylations followed by a number of Kuhn methylations. The fully methylated product was methanolysed and the

methyl glucopyranosides analysed by gas liquid chromatography. The results were compared with those obtained from fully methylated starch and dextran. From the absence of disubstituted methyl derivatives in the methanolysate it was concluded that the polysaccharide is an unbranched glucan.

From the quantities of Methyl 2,3,4,6 tetramethyl-O-D-glucopyranoside, Methyl 2,3,6, trimethyl-O-D-glucopyranoside and Methyl 2,3,4, trimethyl-O-D-glucopyranoside, it was concluded that the only linkages in the glucan are (1 → 4) and (1 → 6) and that these are present in the ratio 68:32.

Enzymic hydrolysis, using pullulanase, was followed by paper chromatographic separation. Quantitative determination of the oligo-saccharides present in the enzyme digest resulted mainly in two oligosaccharides, maltotriose and maltotetraose, in nearly equal proportions. For this reason it was postulated that the polysaccharide is a maltotriose-maltotetraose polymer, and that the individual units are linked in (1 → 6) position, a linkage for which pullulanase is specific in certain configurations.

The sequence of the maltotriose and maltotetraose units in the polymer has not been investigated further, although this could be carried out by partial acid hydrolysis, followed by isolation and identification of the various oligosaccharides formed. An alternate method for the determination of the sequence of the monomers is discussed.

It was subsequently shown that the linkages in the polysaccharide are in the α configuration. The polysaccharide is highly dextra rotary and the magnitude of the rotation is comparable to that of other polysaccharides linked in α position, such as starch and dextran.

Infrared spectroscopy was used to confirm the configuration. The spectrogram of the polysaccharide contained an absorption peak at 840 cm^{-1}, which is typical of the α-anomeric absorption occurring, for example, in the IR spectrum of starch. The spectrogram exhibited no absorption peak at 891 cm^{-1}, the wavelength typical of the β-anomeric absorption in the IR spectrum of cellulose. In addition, it was found that all polysaccharides containing α (1 → 4) linkages show an absorption peak at 700 cm^{1}. This absorption peak was absent in all IR spectra obtained from various dextrans. This phenomenon has not been reported previously and it is suggested that the presence of this absorption peak in the IR spectrum of a glucan can be used to support the evidence of the presence of α(1 → 4) linkages.

It was not possible to correlate the formation of the polysaccharide with the occurrence of a specific micro organism. It is suggested that the formation of the polysaccharide is the result of enzymic reactions in the sugar cane after harvesting.

The investigation of the composition of juices from deteriorated cane has not been confined to polysaccharides. Ethanol has been isolated from the juice of some samples of stored cane which had been burnt before harvesting. The ethanol was isolated by fractional distillation and identified by measurement of the boiling point. It was confirmed, by the formation of the molybdate-xanthate complex, that the product isolated was an alcohol. The identification was further confirmed by oxidising the ethanol to acetic acid and proving the identity of the acids by paper chromatography.

It has been shown that, with the exception of two acids, the carboxylic acid composition of cane juice remains unaltered during post-harvest storage of the cane.

The two exceptions, succinic and aconitic acids, were identified from their melting points and by specific spot tests. Ion exchange was used to isolate the acids from the juice. The

eluate from the ion exchange column was concentrated and the acids separated by liquid-liquid chromatography, using a silica gel column. The levels of both aconitic and succinic acids were found to increase during the early period of storage but decreased again slowly thereafter. The percentage change was greater in the case of succinic acid, although aconitic acid was the most abundant carboxylic acid in the juice.

Lactic acid was absent from the cane juices analysed. This is surprising, as lactic acid is a common product of the metabolism of carbohydrates by micro organisms. It is suggested that the changes in acid composition during the storage of harvested cane are caused by deactivation of enzymes of the Krebs cycle.

Post-harvest deterioration of sugar cane can have serious consequences which can affect the whole Sugar Industry. Not only is crystallisable sugar lost but the products of the deterioration have adverse effects on factory processing and laboratory analysis. The problem, which will become more acute with the introduction of mechanical cane harvesting, can only be resolved through the co-operative efforts of all the parties concerned.

4. INTRODUCTION

4.1 Harvesting, transport and processing of sugar cane into sugar.

Sugar cane in Natal is an 18 month crop, but in recent years the tendency has been to shorten this time in order to obtain a bigger sugar yield per hectare per year.

Contrary to other cane growing countries in the world the cane crop in Natal is entirely hand-harvested. Before harvesting the cane is often burnt while standing in the field to remove the trash (leaves) which does not contain any sugar and reduces the yield in factory processing. This burning is carried out on approximately 50 % of the cane, the remainder being trashed by hand when harvested. Cane which has been burnt is harvested and transported to the factory as quickly as possible. The time between harvesting and processing in the factory has an important influence on the processing quality of sugar cane.

Until recently a large proportion of the cane crop has been transported by rail, either by South African Railways or by a tramline system owned and operated by the milling company. In recent years companies have moved away from rail towards road transport, which, if properly managed, is more economical and reduces transport delays. At the same time mechanical loading has been introduced and this is now practised to a large extent, usually resulting in a speeding up of deliveries. Although the introduction of road transport and better loading facilities have improved the delivery of cane to the factories, there is still in many cases a considerable delay between harvesting and processing.

Upon entering the factory the cane is weighed and the juice is extracted by a milling train or in a diffuser. In a milling train the cane is cut by two sets of cane knives and further disintegrated by a shredder before entering the mills. Normally a factory is provided with 6 sets of 3-roller mills. The juice which

is extracted from the cane is fed in counter current over the fibrous material (bagasse) of the cane. For example the juice expressed by the fourth mill is sprayed onto the bagasse discharged by the second mill. This system of counter current circulation is called imbibition. The bagasse leaving the penultimate mill is imbibed with water.

The extraction process by milling is carried out at ambient temperature (20 - 30 °C) and at the natural pH of the juice (4,8 - 5,5). The extraction efficiency of modern mills is 95 - 96 %.

The bagasse leaving the last mill has a moisture content of 50 - 52 % and contains all the unextracted sugars. It is mainly used as fuel for the factory although other uses for bagasse have been found. At present Kraft paper and particle board are manufactured from final bagasse in Natal and a small quantity is used for the production of cattle feed.

In the case of extraction by diffusion the cane is prepared in a similar manner to that used for milling. All diffusers in Natal are preceded by one mill although this is not an essential feature. The diffusion process itself is a counter current extraction carried out at 80 - 90 °C, with pH kept between 6,4 and 6,8 by the addition of lime at several points along the diffuser. The bagasse leaving the diffuser is dewatered to 50 - 52 % moisture by two 3-roller mills. The extraction efficiency in a diffuser is usually slightly higher than for a milling train, averaging 95 - 97,5 %.

The combined juice (mixed juice) obtained by milling or by diffusion, is weighed, heated to 100 °C and treated with lime to raise the pH to 7,8 - 8,0. A precipitate consisting of suspended matter, calcium phosphate, and coagulated protein is formed. A large number of other impurities is absorbed on this floc, which is allowed to settle for about 3 hours in a continuous separating vessel. Newer designs of clarifiers have made it possible to carry out this settling process in a much shorter time, but if these

vessels are used it is essential to add a high molecular flocculant in order to obtain a heavier and faster settling floc.

The overflow of the clarifiers is a clear yellow liquid, known as clear juice, which contains about 12 % sucrose. The sediment from the clarification is filtered on rotary filters and washed almost free of sugar. The filtrate and the washings are returned to the mixed juice.

The clear juice is concentrated to 60 % solids in a multiple effect evaporator; the concentrated juice is known as syrup.

For the past five years starch, originating from the cane juice has been enzymically hydrolysed during the evaporation stage. Starch is an undesirable component in raw sugar as it impedes the filtration rate in the subsequent refining process. The enzyme used is a thermostable α-amylase derived from *Bacillus subtilis*. This enzyme is commercially available in large quantities at a moderate price and is also used in the textile industry for desizing, as well as in certain detergents. The reaction for the hydrolysis is carried out in approximately 15 minutes at 73 °C and pH 6,5.

The syrup is further concentrated *in vacuo* and crystallisation of sugar takes place. This crystallisation is carried out in three consecutive stages in vessels known as vacuum pans. The operation results in a mixture of sugar crystals and mother liquor called massecuite. A massecuite is developed from a boiling of "seed grain", a mixture containing small sugar crystals which is specially prepared in a separate vacuum pan. During the boiling, syrup is added at a controlled rate to keep the massecuite at the correct supersaturation. Sugar crystallises continuously and the total quantity of massecuite in the pan increases during this process. When a pan is completely filled with massecuite a valve is opened to bring the pan to atmospheric pressure, the steam

heating is discontinued and the massecuite is discharged into a crystalliser, which is a stirred tank. Here further crystallisation takes place as a result of cooling. The massecuite is separated into A-sugar and mother liquor (A-molasses) using sieving centrifuges. The A-molasses is reboiled to produce a massecuite which yields B-sugar and B-molasses. The third boiling produces C-sugar and final molasses. Although the latter still contains a considerable quantity of sucrose it is not possible to recover this sucrose from the final molasses by further crystallisation. This is because of the large quantity of impurities present in the molasses, which form complexes with sucrose and at the same time raise the viscosity to such an extent that further crystallisation becomes impossible.

To improve the quality of the final production raw sugar, all the C-sugar and a large proportion of the B-sugar is redissolved and recrystallised, together with syrup, to form A-massecuite.

The raw sugar discharged by the centrifuges is dried in a rotary drier and weighed before leaving the factory. Sugar is transported by road and rail.

Definitions

Brix	% Total dissolved solids in a factory liquid as measured by the refractive index or specific gravity of the solution.
Pol	% sucrose as measured by a polarimeter calibrated in ° ISS (International Sugar Scale).
Purity	Pol/Brix
Impurities	All components present in factory products other than sucrose.

4.2 Object of the present investigation and lay-out of thesis

When sugar cane is stored after harvesting, several changes in its composition take place, resulting in its becoming less suitable for processing in the sugar factory.

The generally accepted disadvantages of processing deteriorated cane are lower overall recovery of sugar and slower crystallisation, especially in the third grade boilings.

Apart from deterioration due to a delay in transporting, processing difficulties have been reported in the case of burnt and frozen cane .

The object of the present investigation was to determine the nature of the various changes in chemical composition of juice in deteriorated sugar cane and to evolve an analytical method to assess the processing quality of such cane.

Cane juice is a complex mixture of soluble and insoluble organic and inorganic components and water. Because of the complexity, this investigation is not claimed to be complete, as only certain of these components could be covered. No special attention has been paid to the decrease in sucrose content of cane during storage as this generally accepted fact has been investigated by many workers in this field.

The following groups of components were investigated:

1. Polysaccharides.
2. Organic acids.
2.1 Volatile carboxylic acids.
2.2 Non volatile carboxylic acids.
3. Alcohol.

The analyses were carried out on juice obtained from Natal sugar cane varieties which had been stored for various periods after harvesting. The juice was expressed in a small-scale, 3-roller mill.

The thesis is divided into two parts:

Part I RESULTS AND DISCUSSIONS.

Part II EXPERIMENTAL DETAILS.

5. LITERATURE REVIEW OF PRODUCTS FORMED IN DETERIORATING SUGAR CANE AFTER HARVESTING.

Although a large number of deterioration products in sugar cane have been described, most of the earlier investigations were carried out in respect of the changes which take place in frozen cane. In only a few cane growing areas does frost normally occur. For this reason nearly all investigations of this problem were carried out in Louisiana.

Recently a new problem in sugar cane deterioration has resulted from the introduction of mechanical cane harvesting. Some mechanical cane harvesters chop cane into small pieces and it has been proved that these pieces are particularly subject to rapid deterioration.

The following deterioration products have been discussed: carboxylic acids, amino acids, alcohols and polysaccharides.

An increase in the amount of acids in juice from frozen cane was found by Fort and Lauritzen [2 3 4 5]. These authors reported a titratable acidity of 15 - 30 ml of 0,1 N sodium hydroxide per 100 ml of juice obtained from frozen cane. They attributed the increase in acidity almost entirely to acetic acid, which they were able to separate from the juice by steam distillation. The acidity of the steam distillate was used to judge the cane quality.

The presence of lactic acid in juice from frozen cane was mentioned by Irvine and Friloux [6 7] and in juice from mechanically harvested cane by Davis and coworkers [8 9].

Amino acids were studied in frozen cane by Irvine and Friloux but they found no apparent change as compared with the quantities found in normal sugar cane [6 7].

Diersen and coworkers [10] reported the presence of vola-

tile acids in cane molasses. They applied separation by paper chromatography and were able to find small quantities of formic, acetic, propionic, butyric, and valeric acid. These acids might not have been present in the original juice but could have been formed during the sugar processing in the factory.

Mannitol formation in juices from frozen cane was reported by Walton and Fort [11] and McCalip and Hall [12].

It is well known that a large number of changes which take place in juice composition during deterioration are caused by *Leuconostoc* or related lactic acid bacteria. *Leuconostoc* species produce dextran in sucrose containing media and form lactic and acetic acid. Fructose is reduced by *Leuconostoc* species to mannitol [11]. Gum formation in frozen cane was reported by Walton [11], Fort and Lauritzen [2 3 4 5], Friloux and Irvine [6 7]. (see foot note)*

Dextran formation in chopper harvested sugar cane has been reported by Davis and coworkers [8 9]. In this case, however, the dextran formed was not identified but was defined as a starch-free polysaccharide which precipitated in an ethanol -water mixture (1:1). This definition was based on a dextran determination published by Horsley and Nicholson [13].

Smythe studied the effect of dextran on the viscosity of massecuites and on the growth rate of sugar crystals in massecuites [14].

Leonard and Richards [15] attributed crystal elongation in boilings from chopper harvested cane to polysaccharide formation in the cane. Certain oligosaccharides e.g. raffinose, have

* The word gum in sugar technology is used for a polysaccharide or a mixture of polysaccharides, which is chemically not defined.

long been known to promote growth of the sugar crystal along the c-axis. This has recently been confirmed by Kamoda and coworkers [16]. Leonard and Richards, however, claimed that the effect of dextran in this respect was much greater than was that of oligosaccharides. The dextran formation in chopper harvested cane was found to be extremely high.

Lilienthal and Nicholson found a dextra-rotary polysaccharide in deteriorated cane, which they described as a dextran having an abnormal structure. They were not able, however, to correlate the counts of *Leuconostoc mesenteroides* in the juice with the quantity of gum found in it [17]. They established by periodate oxidation of the polysaccharide that the dextran contained an abnormally high number of $\alpha(1 \rightarrow 4)$ glucosidic linkages.

Egan and Rehbein [18] described bacterial deterioration of chopper harvested cane. The isolated organisms were predominantly *Leuconostoc mesenteroides* and *Leuconostoc dextranicum*.

Lopez Hernandez claimed that juices derived from deteriorated cane showed a redox potential which differed consederably from the redox potential of normal cane juice. The redox potential was measured between a bright platinum wire and a calomel electrode [19]. The author claimed a relationship between purity and redox potential. This was found experimentally but no theoretical explanation was given.

6. RESULTS AND DISCUSSIONS

6.1 Formation of polysaccharides in stored sugar cane

6.1.1 Review of Glucans

6.1.1.1 Starch

I Amylose

Starch is one of the various glucose polymers found in nature. It has been known for 30 years that starch is composed of different fractions. Up to about 1940 when most experiments were carried out on whole starch, there was uncertainty with regard to the heterogeneity of starch and most of the linkages had been established as being (1 → 4).

Improved structural studies became possible after a new separation technique for the fractions was applied [20] [21]. It was found that the crystalline precipitate which starch solutions deposited in the presence of ethanol or other alcohols, consisted of an alcohol-amylose complex. Many polar compounds have been shown to give complexes with amylose, n-butanol being one of the most suitable for isolation according to Muetgeert [22] [23]. Amylopectin could be recovered from the supernatant liquid.

The branched structure of amylopectin inhibits the formation of a complex with alcohols. The small amount of amylose in the amylopectin fractions could be removed by absorption on cellulose (cotton or filterpaper) [24] [25].

Analysis by ultracentrifugation confirmed the heterogeneity of starch and the average molecular weight of potato amylose was determined as being in the order of 185 000, while the molecular weight of amylopectin was found to be approximately 1 000 000 [26]. Corn amylose and amylopectin were found to have lower molecular weights.

Methylated samples of amylose gave on methanolysis Methyl-2,3,6 tri-O-methyl-D-glucopyranoside and a small amount (0,3 - 0,5 %) of Methyl-2,3,4,6 tetra-O-methyl-D-glucopyranoside, indicating that amylose contains only one non reducing terminal glucose residue for every 200 - 350 glucose residues [27] [28], thus confirming the values found for the molecular weight by ultracentrifuge determination.

The absence of disubstituted methyl derivatives in the methanolysate indicated a straight chain with the structure as shown in (I).

---O -- O -- O -- O -- O -- O -- Ø

I

O	glucose residue
Ø	reducing end group
--	(1 → 4) linkage
——	(1 → 6) linkage

In 1940 it was observed that α-D-glucosyl phosphate could be polymerised by the potato enzyme phosphorylase into a polysaccharide of about 100 glucose units per non reducing end group with a structure identical to amylose [29] [30].

The end groups in amylose have also been determined by periodate oxidation of the polysaccharide [31] [32]. The theory of, and the conditions for periodate oxidation of polysaccharides are discussed on page 68 of this thesis. The results of the periodate oxidation of amylose by Potter and Hassid [31] [32] agreed with those obtained by methylation studies.

Random depolymerisation of amylose by acid hydrolysis results in a series of oligosaccharides termed maltodextrins. The isolation of the latter, in which the glucose units are linked in α (1 → 4) mode, provides direct evidence for the existence of α (1 → 4) linkages in the original amylose. These linkages can also be identified by infrared absorption measurements as was shown by Barker, Bourne, Stacey, and Wiffen [33]. The α(1 → 4) linkage is associated with special absorption minima in the infrared as discussed on page 97 .

Initially amylose was considered as being completely unbranched. Later publications, however, revealed amylose samples having a molecular weight two or three times that calculated from the average chain length as determined by periodate oxidation, indicating slight branching [31] [34]. The same was concluded from the gel strength of amyloses [35]. It was also found that pure β-amylase converted only 70 % of amylose into maltose [36]. Earlier reports claiming the complete conversion of amylose into maltose were based on experiments using β-amylase, which was later shown to be contaminated with an enzyme called "Z-enzyme". This Z-enzyme was later found to be a weak α-amylase, cleaving the α(1 → 4) glucosidic linkages in a random manner [37].

On the other hand it was shown that oxygen was able to degrade amylose and introduce barriers to β-amylase attack [38]. The controversy was finally solved by the use of the later-discovered enzyme pullulanase, an enzyme which is able to hydrolyse α(1 → 6) linkages in special configurations. Treatment of amylose and its βlimit dextrin showed for the first time unambiguously that α-D-(1 → 6) glucosidic branch points form the natural barrier to β-amylase action and it was concluded that some amylose fractions have limited branching [39].

Amylose gives a blue complex with iodine, a property in which it differs from other α glucans. The structure of this complex is now generally accepted as being that of an amylose helix with the iodine atoms forming a linear chain within. The absorption of this iodine complex increases with increasing DP (degree of polymerisation). Dextrins having less than 5 glucose units per molecule are not coloured in iodine solution. This is attributed to the fact that 6 glucose units are required to form one turn of a helix [40].

II Amylopectin

Before starch was separated in its two components the the nature of the linkages had been established. It has long been known that starch has 20 - 30 glucose units per non reducing end group. Later more accurate investigations on amylopectin has shown this figure to be 27 [28].

The molecular weight of amylopectin is much higher than the molecular weight of 27 glucose units (4 000). Amylopectin must therefore contain many chains joined together. The isolation of Methyl-2,3 di-O-methyl-D-glucopyranoside from methylated and methanolysed amylopectin indicates that the linkages at the branch points are (1 → 6).

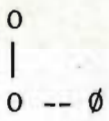
The presence of disubstituted methyl derivatives, however, can also be caused by under methylation. The presence of (1 → 6) linkages has also been proved by periodate oxidation (see page 68) and by the presence of oligosaccharides having an α (1 → 6) linkage after partial acid hydrolysis of amylopectin. One percent isomaltose (6-O-α-D-glucopyranosyl-D-glucopyranose (II)) has been isolated from an acid amylopectin hydrolysate [41].

O —— Ø

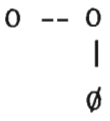
II

The amount of isomaltose exceeded by a factor of 200 times that quantity of isomaltose formed by acid reversion of glucose in control experiments [42]. This confirmed that the isomaltose originated from the α (1 → 6) linkages in the amylopectin and was not formed by side reactions.

Panose (III) and 6-O-α-maltosyl-D-glucose (IV) have also been isolated in partial starch hydrolysates [43].

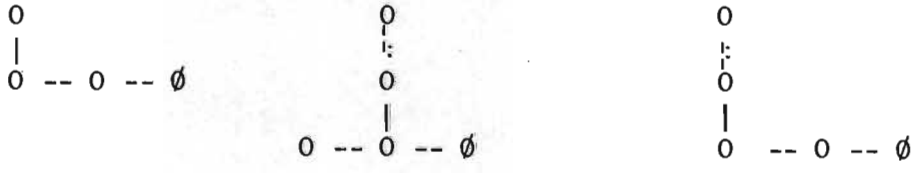


III



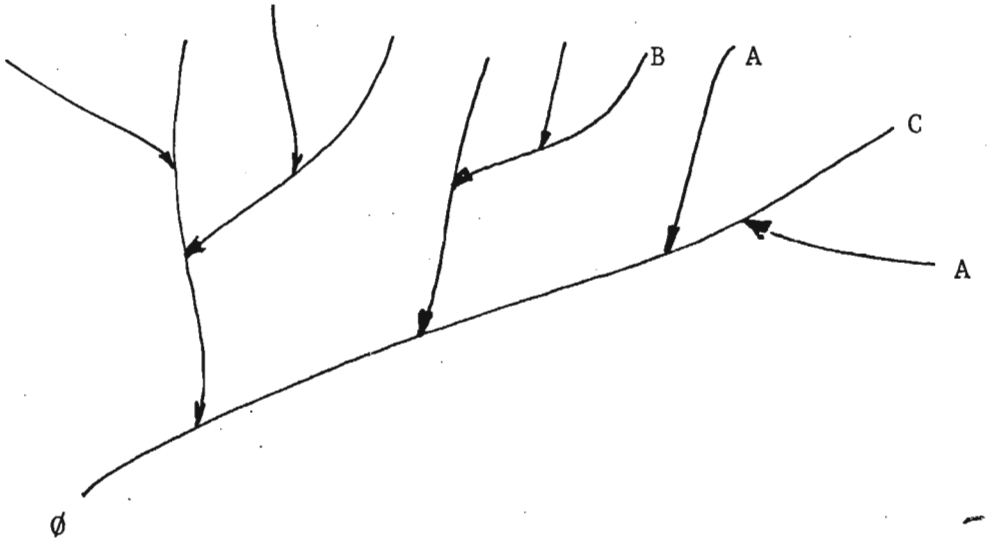
IV

Similar results have been obtained with α-amylase, an enzyme which hydrolyses randomly α (1 → 4) D-glucosidic linkages. As the α (1 → 6) linkage is not attacked, the final digest contains maltose, maltotriose and limit dextrins having a DP 4 or more (V) [44].



(V)

The presently accepted structure for amylopectin is shown in (VI).



(VI)

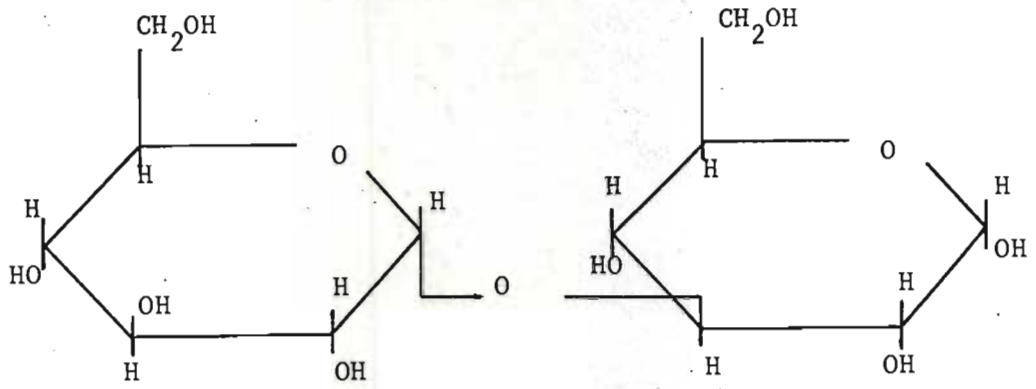
Exterior chains are those between the non reducing end group and the outermost branch points, interior chains are those betwcen two branch points. A-chains are attached by only one

α (1 → 6) linkage. B-chains, to which one or more A-chains are linked, are also attached by the reducing group to the remainder of the molecule in α (1 → 6) mode. The sole C-chain contains the only reducing end group of the molecule.

Hydrolysis by enzymes either attacking the α (1 → 4) linkage or the α (1 → 6) linkage has been used for further structural analysis. β-Amylase produces β-limit dextrin in which all the original branch points are still present. R enzyme which cleaves the outer α (1 → 6) -D-glucosidic linkages on β limit dextrin, transforms the residues of the outer chains into maltose and maltotriose. The yield of this maltotriose (12,8 %) is close to the amount which can be calculated from the structure VI (10,4 %), thus confirming a tree type structure having an equal number of A- and B-chains [45] [46] .

The average chain length in amylopectin has been determined by the concurrent action of pullulanase and β amylase [47] [48]. By this enzymic method chains having an even number of glucose units are quantitatively converted into glucose, while chains having an odd number of units are hydrolysed into maltose and glucose. The glucose can be specifically determined by the use of glucose oxidase. Assuming an equal number of chains with an even and an odd number of units, the average number of chains and the average chain length can be calculated. This method is more reproducible than periodate oxidation and can be carried out on a micro scale. Occasionally, linkages other than α (1 → 4) and α (1 → 6) have been claimed to be present in amylopectin. The identification of small amounts of glucose in the periodate oxidised and subsequently hydrolysed amylopectin has been interpreted as evidence for the presence of (1 → 3) linked glucose units [49] , or glucose units linked through the positions 2 and 4 [50] but this has also been attributed to incomplete oxidation [51].

It has also been reported that small quantities of nigerose (VII) have been found in partial acid hydrolysates of amylopectin [52] .



(VII)

Comparison with control experiments carried out under identical conditions with glucose showed that the nigerose was not formed by acid reversion of glucose [53]. It remains possible, however, that larger quantities of nigerose may be formed by the action of acid on maltose through trans-glucosidation.

More recent periodate oxidation results do not support the existence of (1 → 3) linkages [54] . For this reason it is assumed at present that amylopectin only contains α (1 → 4) and α (1 → 6) linkages.

Due to the complex structure of amylopectin, it is not possible to describe the structure of the molecule exactly. Only an average structure in terms of degree of branching, and internal and external chains length can be defined. Amylopectin is a mixture of molecules of different molecular weight and the structure may vary with the molecular weight, maturity of the plant from which starch was isolated, and with the species of the plant.

6.1.1.2 Glycogen

This polysaccharide, which can be isolated from the liver and other animal tissues, was shown from chemical studies by Karrer [55] in 1921 to have a structure closely related to starch. Acidic and enzymic hydrolysis gave similar decomposition products for both polysaccharides.

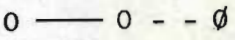
The evidence that most linkages in glycogen were (1 → 4) was supplied by Haworth and coworkers in 1929 [56] through acetylating glycogen, followed by simultaneous deacetylation and methylation. Upon hydrolysis of the fully methylated polysaccharide, 2,3,6 tri-O-methyl-D-glucopyranose was obtained with a yield of 76 percent.

Later investigations [57] showed that nine percent of methyl-tetra-O-methyl-D-glucopyranoside was present in the methanolysate, in addition to the tri-substituted derivative. From this observation it was concluded that the chain length in glycogen consisted of 12 glucose units, linked in (1 → 4) position.

Other chain lengths in glycogen have been discovered subsequently. For example, rabbit glycogen and *Mytilus edulis* glycogen contain some chains having 18 units per chain length [58] [59]. Osmotic pressure measurement by Carter and Record [60], showed that glycogens have a molecular weight in the order of 10^6, indicating a highly branched structure. The type of branching was shown to be (1 → 6) by the isolation of 2,3-di-O-methyl-D-glucose from a hydrolysate of exhaustively methylated glycogen [61].

Periodate oxidation of glycogen, followed by hydrolysis, showed glucose to be present in the hydrolysate [62]. As glucose residues which are substituted at C_2 or C_3 will not be oxidised by periodate, they will be present as glucose in subsequent acid hydrolysis [63] [64]. The amount of glucose found in these experiments was small and indicated that the glycogen would have contained not more than two to three percent of (1 → 2) or (1 → 3) linkages. This finding could also have been caused by incomplete

oxidation of the glycogen by the periodate. Partial acid hydrolysis resulted in panose (VIII) (4-O-α-isomaltosyl-D-glucose) confirming the presence of the α (1 → 6) linkage [65].



(VIII)

Small amounts of nigerose (VII) were also isolated, again indicating a small percentage of (1 → 3) linkages.

The results of other workers, however, contradicted the theory of the presence of abnormal linkages. For example, acid hydrosysates of periodate oxidised glycogens from various sources did not contain any glucose [66].

Hydrolysis of glycogen by R enzyme, followed by a determination of the number of reducing groups, was used to determine the number of (1 → 6) linkages or, in other words, the chain length. In this way the average chain length for rabbit glycogen was found to be 12,5 [67].

Enzymic analysis, involving the successive action of phosphorylase and amylo-(1 → 6) glucosidase [68] was used to confirm the multiple branch structure of glycogen. Phosphorylase in the presence of inorganic phosphate removes glucose residues from the exterior chains of glycogen:

$$G_n + m\ HOP \rightarrow G_{n-m} + m \text{ glucose 1 phosphate}$$

The phosphorylase cannot bypass the (1 → 6) inter-chain linkages. These can, however, be decomposed by the action of amylo (1 → 6) glucosidase. The successive formation of smaller and smaller limit dextrins provided evidence of the tree type structure of glycogen. If glycogen had a laminated structure, as had been suggested by Haworth [61], only one branch point would have been removed each

time by the alternate action of the two enzymes.

From a comparison of all the available information, it can be stated that the structures of glycogen and amylopectin are similar. The major difference between the two polysaccharides is the chain length and, by consequence, the iodine-binding capacity. The shorter chain length makes the interior of the glycogen molecule more compact than that of amylopectin and for this reason less susceptible to enzymic attack. Amylases degrade the interior chains (6-9 glucose units) in amylopectin more readily than those in glycogen (3-4 units). R enzyme has no appreciable effect on glycogen but hydrolyses the α-D-(1 → 6) linkages in amylopectin. The closer arrangement of the inter-chain linkages in glycogen apparently interfere with the action of R enzyme.

6.1.1.3 Fungal Starches

Starch-like polysaccharides, which give a blue colour with iodine, have been isolated from a large number of different fungi and yeasts.

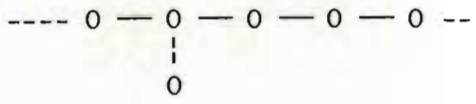
The majority of these polysaccharides has been found to be identical in structure to amylose, differing mainly in chain length [69].

6.1.1.4 Dextran

Dextran is an α-glucan produced by bacteria growing on a sucrose substrate. The backbone of the polysaccharide is formed by glucose units linked in α-D-(1 → 6) position.

Early experiments, using exhaustive methylation, followed by hydrolysis, resulted in 2,3 di-O-methyl-,2,3,4 tri-O-methyl- and 2,3,4,6 tetra-O-methyl D-glucoses in the ratio 1:3:1 [70]. Although these results were criticised on grounds of under-methylation, they have subsequently been confirmed by the investigations of Levi, Hawkins, and Hibbert [71], using improved techniques, and have also

been substantiated by other workers [72] . On the basis of the methylation studies, it was suggested that dextran contains a repetitive unit (IX).



(IX)

Other possibilities include units with longer side chains and shorter primary chains. These would give the same results in methylation studies.

Other dextrans have been isolated containing (1 → 3) glucosidic linkages in addition to the (1 → 6) linkage [73] . The strain of *Leuconostoc*, called B 512, producing these dextrans, was isolated by the Northern Utilisation Research Laboratory of the U.S. Department of Agriculture. It has been established that in magnesium deficient media, the dextran produced does not contain any (1 → 4) linkages and has an average chain length of 40-50 glucose units [74] .

The methylation results were confirmed by several workers [75] [76] using periodate oxidation techniques in which the periodate uptake and the amount of formic acid formed were measured.

The presence of (1 → 4) or (1 → 3) linked units should not, alone, be accepted as proof of branching. Periodate oxidation provides no indication about the position of the residues in a polysaccharide molecule. Linkages, additional to the predominant one, can be present in a polysaccharide molecule either as branch points or as inter-chain linkages.

Partial acid hydrolysis of dextran resulted mainly in the isolation of isomaltose (6-O-α-D-glucopyranosyl-D-glucopyranose)

(X), and isomaltotriose (XI)[77], confirming a backbone of (1 → 6) linkages in dextran. In addition, the presence of (1 → 3) linkages was confirmed by the identification of 3-O-α-D-glucopyranosyl-D-glucose (nigerose) (VII) page 30 in partially acid-hydrolysed dextran produced by a strain of *Leuconostoc* similar to B 512 [73] .

(X)

(XI)

It was found that when this strain of *Leuconostoc*, which was isolated in Birmingham, was grown on magnesium deficient media, a low molecular weight dextran having only (1 → 3) branch points and no (1 → 4) linkages was produced [74] .

Physical measurements by ultracentifugation [78] and by electron microscopy [79] , have shown that dextran exists as a branched molecule with threadlike structure, having a molecular weight up to a few million.

Senti and Hellman [80] have published data correlating the intrinsic viscosity and the molecular weight of dextran. They showed that the degree of branching increased as the molecular weight of dextran increased. For molecular weights up to 100 000 a linear relationship holds; for higher molecular weights the curvature of the plot indicates a higher degree of branching.

As is the case with most polysaccharides, the exact structure of dextran is not completely known. Moreover, molecular weight and branching varies with the strain of *Leuconostoc* and the moment of isolation of the polysaccharide from a culture. As in the case of starch, however, average molecular weight, average chain length, and type and degree of branching are known.

6.1.1.5 Mycodextran (Nigeran)

This polysaccharide was isolated from *Penicillium expansum* by Dox and Neidig [81] . A similar product is produced by *Aspergillus niger*. Complete methylation of the glucan followed by methanolysis, resulted in equal amounts of methyl- 2,3,6, and methyl- 2,3,4 tri-O-methyl-D-glucopyranoside, together with 0,3 percent of methyl- 2,3,4,6 tetra-O-methyl-D-glucopyranoside [82 83 84] . Partial acid hydrolysis resulted in maltose (XII), nigerose (XIII) and O-α-D-glucopyranosyl (1 → 4) -D-glucose (XIV).

O -- Ø O == Ø O == O -- Ø

(XII) (XIII) (XIV)

O = glucose unit
Ø = reducing end group
-- = (1 → 4) linkage
== = (1 → 3) linkage

Because no di-substituted methyl derivatives were found in the methanolysate, it was concluded that nigeran was a linear glucan, in which the units were linked alternately α-D-(1 → 3) and α-D-(1 → 4). Nigeran is therefore one of the polysaccharides of which the structure is completely defined.

6.1.1.6 Pullulan

This water soluble glucan was isolated by Bernier [85] from cultures of *Pullularia pullulans* (de Bary) Berkhout, syn. *Aureobasidium pullulans* (de Bary) Arnoud, growing on sugar-containing Czapek Dox media. This polysaccharide was shown to be extracellular and was named pullulan by Wallenfels and coworkers [86] . In addition to this polysaccharide, a jelly-like glucan adhering to the mycelium was also formed. This was identical to the α-D-(1 → 3) and α-D-(1 → 6) linked glucan isolated by Bouveng and Lindberg [87] .

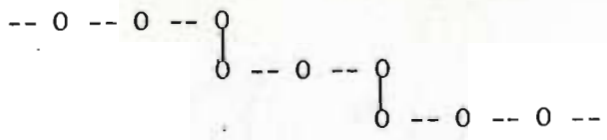
Pullulan was shown by periodate oxidation and by exhaustive methylation, to contain (1 → 4) linkages (60 %) and (1 → 6) linkages (40 %). Partial acid hydrolysis resulted in maltose and isomaltose.

In 1961 Bender and Wallenfels isolated an enzyme, produced by a strain of *Aerobacter aerogenes*, which they called pullulanase [88]. The bacteria were isolated from an air-infected solution, in which pullulan was the sole source of carbon. Later investigations showed that the enzyme was specific for the α(1 → 6) glucosidic bond [90]. More detailed investigation, however, showed that pullulanase can only act as an α-D-(1 → 6) glucanase if adjacent α(1 → 4) linkages are present in the polysaccharide acting as a substrate [88]. In this respect it is comparable to R enzyme, which was isolated from potatoes [92]. It has a debranching effect on amylopectin but does not show any activity on isomaltose or dextran. Since its discovery, pullulanase has been used frequently for structural investigations of polysaccharides containing a mixture of (1 → 4) and (1 → 6) glucosidic bonds.

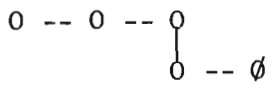
Enzymic hydrolysis of pullulan by pullulanase resulted in maltotriose as the sole decomposition product.

The combined data, coupled with the results of infra red anylsis, and the strong dextra rotation of the polysaccharide, indicated the presence of α-D-glucosidic linkages [86 89]. The molecular weight, determined by the hydrodynamic properties was found to be 235 000 [90].

From the classical chemical analysis and consideration of the products of enzymic decomposition, it was concluded that pullulan is a linear maltotriose polymer linked in α-D-(1 → 6) position (XV).

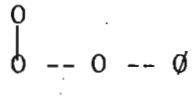


(XV)

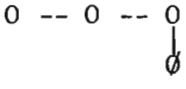


(XVI)

A very small amount of a tetrasaccharide was found in the digest by pullulanase. As it was shown that the pentasaccharide (XVI) is also a substrate of pullulanase, the limit dextrin of the pullulanase hydrolysis can only be one of the tetrasaccharides (XVII) or (XVIII), or a mixture of both [90] .



(XVII)



(XVIII)

On the other hand, Whelan and co-workers [91] found maltotetraose and a heptasaccharide in the enzymic hydrolysate of pullulan by pullulanase. They concluded on these grounds that pullulan contained 6,6 percent maltotetraose linked in α-D-(1 → 6) position to the maltotriose residues.

Because of its linear and comparatively simple structure, pullulan is one of the few polysaccharides of which the structure is completely defined. Slight differences, as published by the various workers, might be due to variation in the strain of the *Aureobasiudium pullulans* used.

6.1.2 Analysis of sugar cane for soluble polysaccharides and starch.

Sugar cane juice contains several soluble polysaccharides and one, starch which is insoluble under ambient conditions. In unheated cane juice the starch is present in the form of granules having a diameter of three to five micrometer [93]. These granules show a similar layer structure to starch granules from other plants [93].

The concentration of starch and the level and nature of soluble polysaccharides in fresh and stored sugar cane was investigated.

Many other workers in this field have reported an increase in soluble polysaccharides in juice from deteriorated sugar cane. Various analytical methods have been described, all of which are based on the insolubility of polysaccharides in aqueous ethanol.

In one of the older methods [94 95], the polysaccharides are precipitated by ethanol, followed by a filtration through a Gooch crucible precoated with a mat of asbestos fibres. The filter is dried and weighed and a second weighing is carried out after heating the crucible to 800 °C, to correct for inorganic inclusions in the polysaccharide. Instead of weighing the precipitated polysaccharide, a colorimetric determination can be carried out after resolution in water. Among suitable methods are those using sulphuric acid and anthrone [96], and sulphuric acid and phenol [97]. A third method, in which the turbidity of the precipitate formed in 50 % ethanol is measured, was described for dextran, although it is not specific for this substance [98].

In every case insoluble material in the cane juice will interfere with the polysaccharide determination. For this reason the juice has to be filtered before the analysis. This filtration also removes the starch present in cane juice.

In the course of the present investigation, the

turbidimetric method was compared with the gravimetric method but from Fig. 1 it is apparent that insufficient correlation between the two was obtained and for this reason the former method was abandoned.

It was also established that the colorimetric methods using anthrone and sulphuric acid, and phenol and sulphuric acid gave insufficient reproducibility. The gravimetric method was found to be sufficiently reproducible, as shown in Table 1, and was employed throughout this investigation. Although the time required for the gravimetric analysis is longer than that for colorimetric methods, the amount of labour is comparable provided that a modern analytical balance is used.

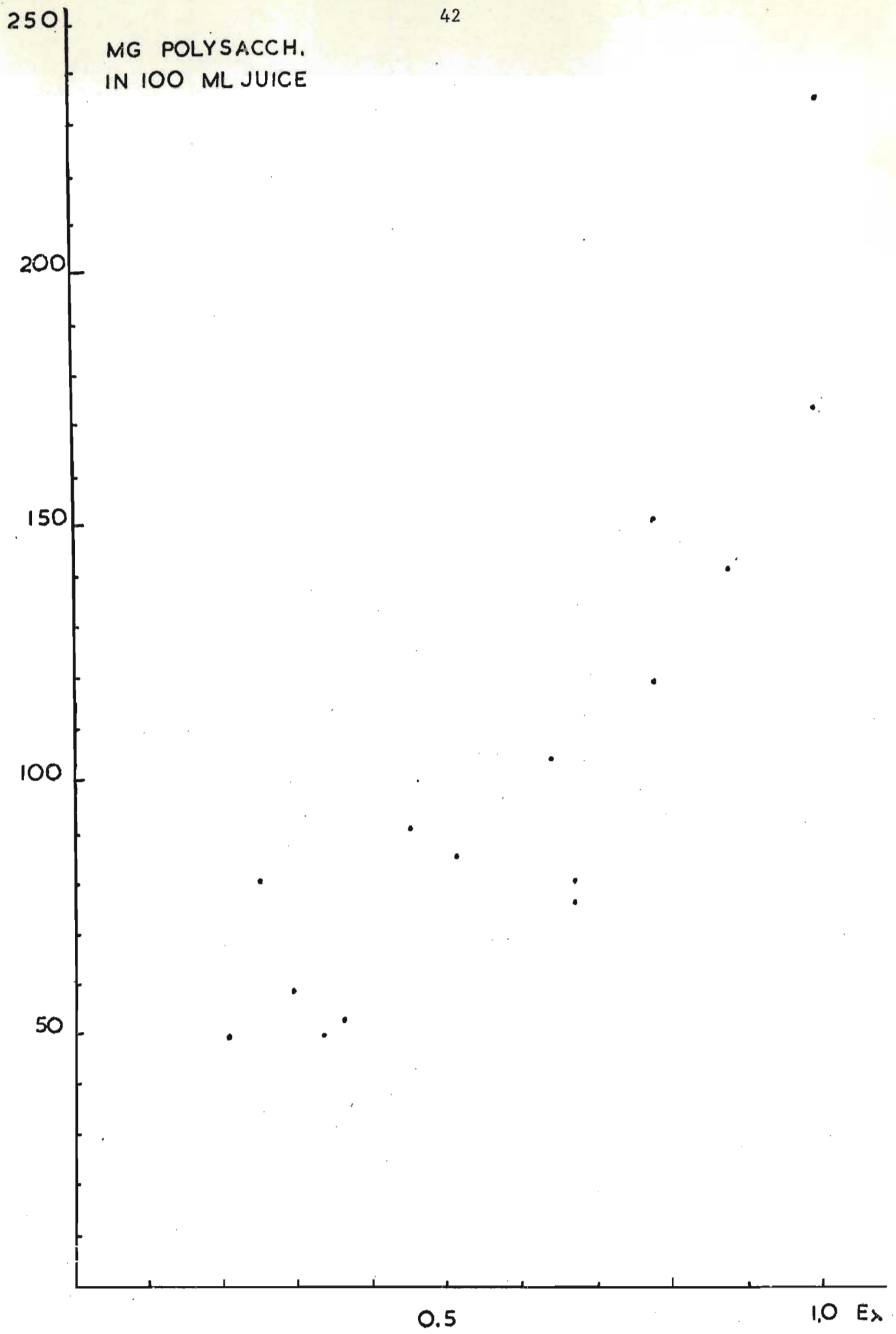


FIG. 1 CORRELATION BETWEEN TURBIDITY IN ETHANOLIC SOLUTION

Table 1

Polysaccharide content of seven subsamples of cane juice from four different consignments of sugar cane, showing the reproducibility of the analysis

		Polysaccharides expressed as percentage of total dissolved solids in juice			
		1	2	3	4
variety		N:Co 310	N:Co 310	N:Co 331	N:Co 331
days after harvest		1	6	6	14
Subsample number	1	0,92	0,47	0,41	1,23
	2	0,95	0,55	0,35	1,34
	3	0,90	0,49	0,41	1,21
	4	0,88	0,51	0,36	1,18
	5	0,90	0,58	0,44	1,20
	6	0,91	-	0,35	1,21
	7	0,88	-	-	1,23
	mean	0,91	0,53	0,39	1,23

6.1.3 Polysaccharide content of various cane juices

In Table 2 the soluble polysaccharide contents of various samples of cane juice are shown. From these data it can be concluded that the amount of polysaccharides dissolved in juice from freshly harvested cane is a reasonably constant figure. All samples which have been obtained from stored cane, however, show a higher level of soluble polysaccharides.

In Fig. 2 the soluble polysaccharide content of samples from the same pile of cane is shown after different time intervals. Whole stalks of sugar cane were stored in the open. After the first few days there was only a moderate increase in the polysaccharide level but this increased markedly after 12 days of storage.

In practice, delays of 14 days between the harvesting and processing of sugar cane do occur. The increased level of polysaccharide content is therefore of practical interest to the sugar industry and the occurrence of such long delays should be prevented.

The gradual decrease of starch in whole cane stalks during storage after harvesting is illustrated in Table 3. This decrease would be advantageous in subsequent processing by sugar factories if it were not for the fact that the decrease in starch is much smaller than the increase in soluble polysaccharides. Because the levels of change are so different the possibility that starch has been transformed into soluble polysaccharides must be excluded. In fact, the quantities determined indicate that the two processes are unrelated.

6.1.4 Isolation and purification of the polysaccharide formed in stored cane

The juice from a large quantity of sugar cane which had been stored for four weeks in the open air was obtained by crushing. The starch and other insoluble particles were removed by centri-

Table 2

Polysaccharide content of various sugar cane samples		
sample	variety	Polysaccharide expressed as percentage of total dissolved solids in juice
1 fresh cane	N:Co 310	0,35
2 fresh cane	N:Co 310	0,36
3 fresh cane	N:Co 310	0,42
4 fresh cane	N:Co 310	0,36
5 fresh cane	N:Co 310	0,41
6 fresh cane	N:Co 310	0,09
7 fresh cane	N:Co 310	0,47
8 fresh cane	N:Co 310	0,37
9 burnt cane	N:Co 310	2,86
10 cane 21 days	N:Co 331	0,88
11 cane 21 days	N:Co 331	2,07
12 cane 21 days	N:Co 331	1,66
13 cane 21 days	N:Co 331	2,42
14 burnt cane	N:Co 310	6,10

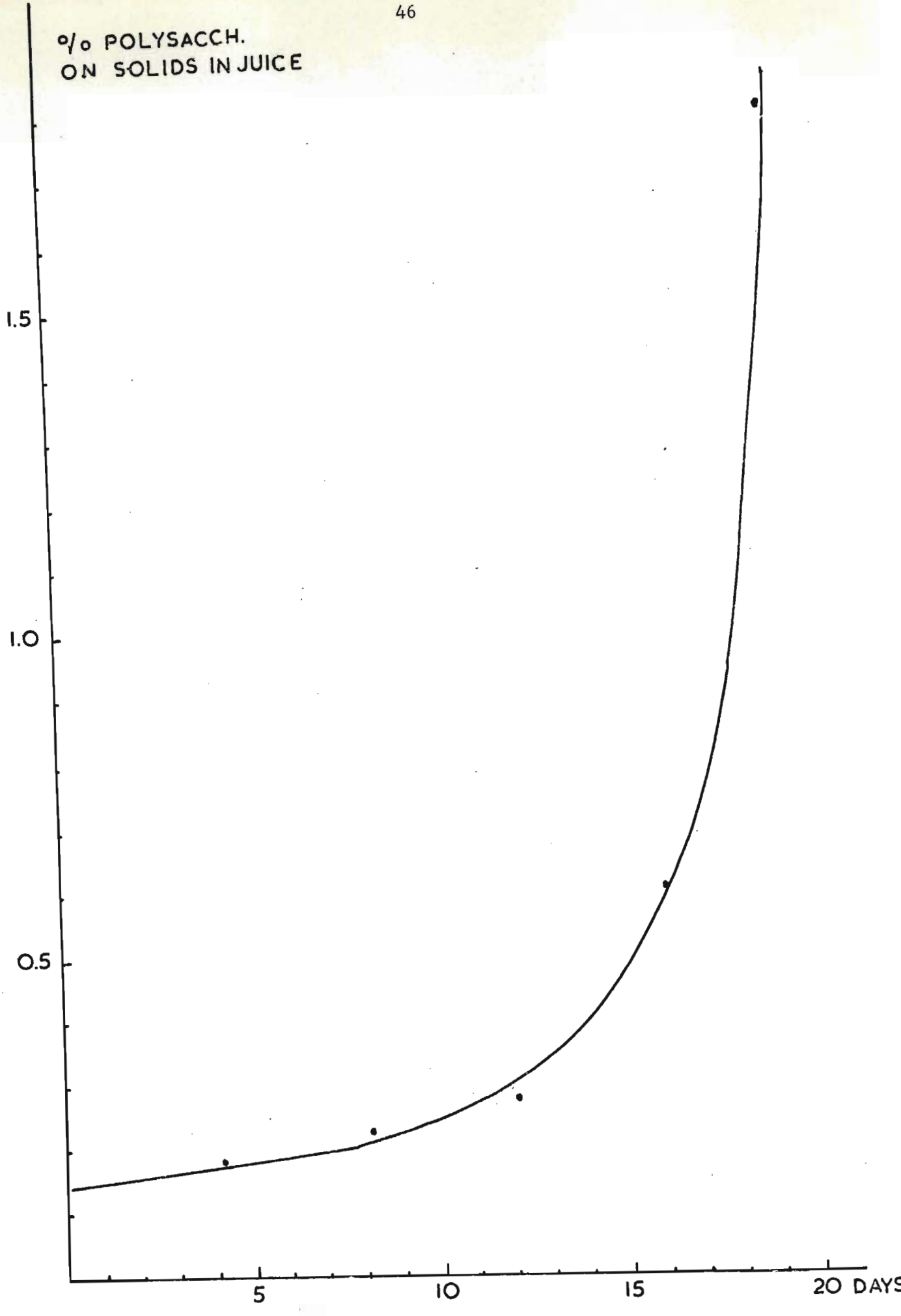


Fig. 2 SOLUBLE POLYSACCHARIDE CONTENT OF CANE VERSUS THE NUMBER OF DAYS AFTER HARVEST.

Table 3

Starch content of juice from cane with increasing interval between harvesting and crushing	
days after harvest	Starch as percentage of total dissolved solids in juice
0	0,14
2	0,12
6	0,09
8	0,06
13	0,06
15	0,05

Table 4

Composition of impurities in purified cane polysaccharide	
	percent
Protein	1,17
Starch	0,13
Ash	0,14

fuging, after which the juice was concentrated *in vacuo* to about 40 % solids and the polysaccharide precipitated with acidified ethanol. The polysaccharide was dissolved in water and re-precipitated by the addition of ethanol to a final alcohol concentration of 80 percent. Repeated re-precipitation from an aqueous solution, followed by drying, resulted in a white powder. Subsequent dialysis of an aqueous solution of the powder against cold tap water, followed by distilled water, did not result in any lowering of the ash content of the product. This step was omitted in subsequent preparations.

The analysis of the final purified product is shown in Table 4. The material was considered sufficiently pure to be used for determinations of properties and structure.

6.1.5 Homogeneity of the polysaccharide

6.1.5.1 Fractional precipitation

The homogeneity of a polysaccharide can sometimes be judged by precipitation from an aqueous solution, by an organic solvent [101]. Fractions with a lower molecular weight will be precipitated at increased concentrations of the organic solvent. The presence of a plateau in the curve drawn by plotting the percentage of polysaccharide precipitated against the solvent concentration will indicate a mixture of two different polysaccharides. The method is, however, of limited value.

Ethanol was used to test the homogeneity of a sample of the purified cane polysaccharide. The precipitation started with 50 percent ethanol and was complete at 80 percent. The results, illustrated in Fig. 3, show no indication of a plateau caused by a mixture of polysaccharides.

6.1.5.2 Gel chromatography

This method has found wide application for the separation and molecular weight determination of macro-molecules.

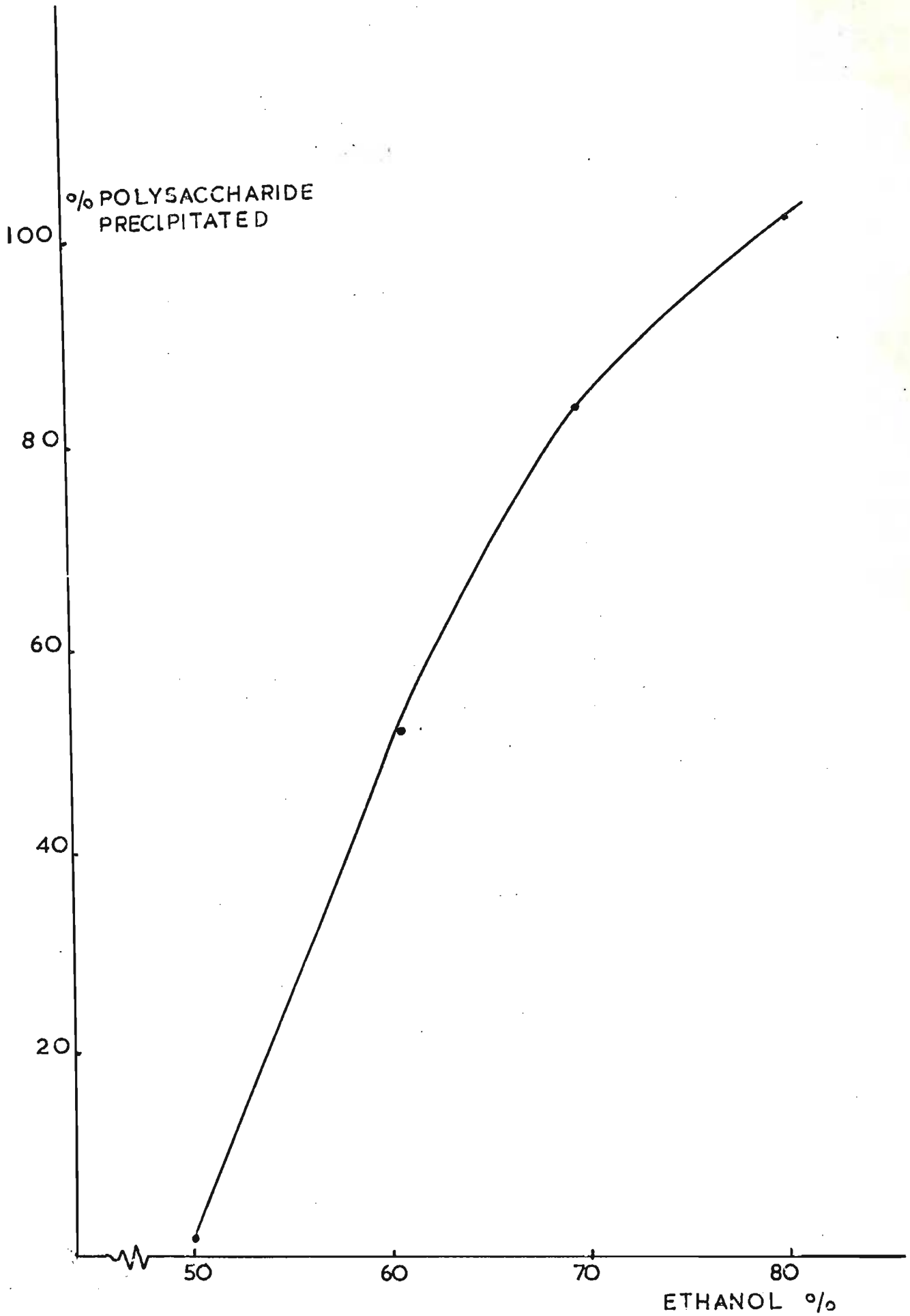


FIG. 3 FRACTIONAL PRECIPITATION OF CANE POLYSACCHARIDE

Various gels are in use, e.g. Sephadex (cross linked dextran), Polyacrylamide, Agar, Agarose and, recently, porous glass beads.

Andrews [102] determined the molecular weight of a number of proteins, using an agar column, by determining the peak elution volumes of various proteins of known molecular weight. By plotting this peak elution volume against the logarithm of the molecular weight, nearly straight lines were obtained. With the aid of these calibration graphs, the molecular weight of proteins could be determined from the measurement of their peak elution volume from the agar column. (see also page 56).

In the present investigation Sephadex and Agar were used as gel material. The latter was prefered as it resulted in higher flow rates through the column. The cane polysaccharide was analysed on a column packed with Ion Agar (5 percent, 100 mesh). The elution was carried out with distilled water. Only one symmetrical peak was eluted, indicating that the polysaccharide was homogeneous.

In a later stage of the investigation the homogeneity was again confirmed by enzymic hydrolysis of fractions eluted from the column at the beginning and end of the single peak. The composition of the enzymic hydrolysate is reported later. (Table 10, page 113).

The elution curve is shown in Fig. 4.

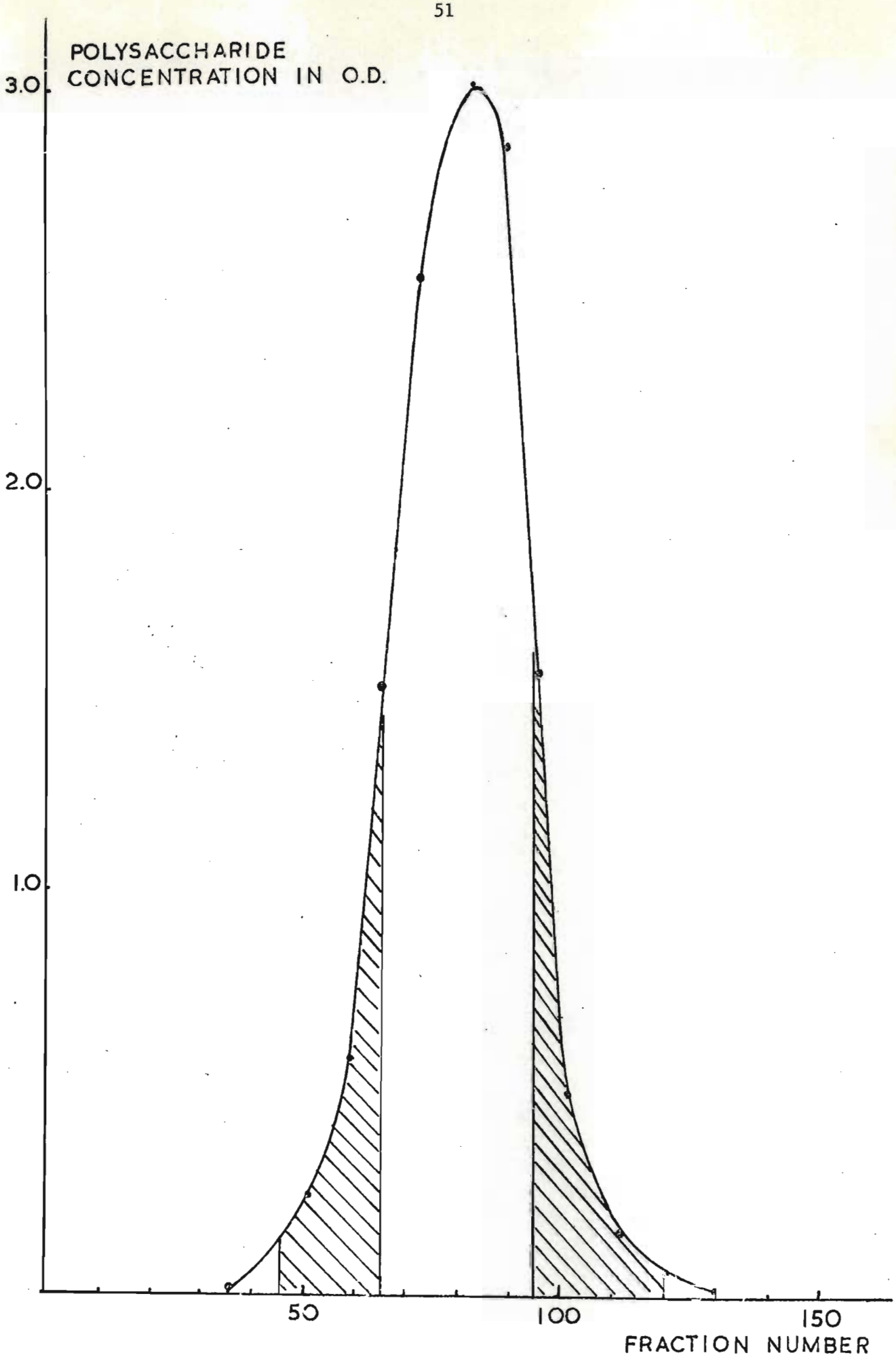


FIG. 4 GEL CHROMATOGRAPHY ON IONO AGAR OF PURIFIED CANE POLYSACCHARIDE

6.1.6 Determination of the molecular weight of the isolated polysaccharide

6.1.6.1 Available methods

Molecular weight determinations can be divided into absolute and non absolute methods. In the absolute methods the molecular weight can be calculated directly from determined physical constants, while with non absolute methods a calibration using comparable polymers of known molecular weight is required. Different methods can result in different molecular weights and in the case of a polydisperse polymer, it is customary to differentiate between the weight average molecular weight, $\bar{M}_w$ and the number average molecular weight, $\bar{M}_n$.

The following methods can be applied:

I End group analysis

An absolute method, which counts the number of molecules in a defined weight of sample and provides the number average molecular weight $\bar{M}_n$. This method tends to become inaccurate for higher molecular weights as it becomes increasingly difficult to determine the very small number of end groups present.

II Colligative methods

These methods are also absolute and include ebulliometry, osmometry, cryoscopy, and vapour pressure measurement. Essentially, these methods count the number of molecules in a defined weight of solute and provide the number average molecular weight $\bar{M}_n$. The physical constants become difficult to measure at high molecular weights and colligative methods are not considered suitable for substances with molecular weights of more than 50 000.

III Light scattering

An absolute method, which utilises the fact that intensity of the scattering of light is proportional to the square of the mass of the particles. This method determines the weight average molecular weight $\bar{M}_w$.

IV Ultracentrifugation

An absolute method in which the sedimentation velocity of the molecules is determined. The weight average molecular weight can then be calculated.

V Viscosity determination

This is an example of a non absolute method. Use has to be made of comparison with results obtained with structually similar polymers of known molecular weight. The viscosity of the solution of a polymer is determined by the size of the molecules, i.e. the radius of gyration R_G. For this reason, the correlation between size and molecular weight of the polymer under investigation must be identical to that of the polymers with defined molecular weight used for the calibration. The method does not give exactly the weight average molecular weight, but provides a value normally 10-20 % lower than the $\bar{M}_w$ for many polymers [103].

VI Gel chromatography

Also a non absolute method, gel chromatography can be used to determine the size of the polymer molecules, by comparing the peak elution volumes of polymers of defined molecular weight with the peak elution volume of the unknown, using a suitable gel column.

Just as in method V, the correlation between molecular weight and size must be identical for the polymer under investigation and those used for the calibration of the gel column.

For a polymer which is not completely monodisperse, $\bar{M}_w$ is always larger than $\bar{M}_n$. The ration $\bar{M}_w/\bar{M}_n$ can be used to judge the polydispersity of the polymer. For example, an equal weight mixture of two polymers, one with a molecular weight of 10 000 and the other of 100 000, will have a $\bar{M}_w$ of 55 000 and a $\bar{M}_n$ of 18 200.

6.1.6.2 Molecular weight determination of the isolated polysaccharide by viscosity measurement

Although calibration with comparable polymers is required, molecular weight determination by means of viscosity measurement is attractive in practice because the equipment used is far less complex than is the case with the other methods.

The chemical analysis of the isolated polysaccharide, which is described later in this thesis, showed that it could not be classified as a dextran, but that it had a structure partly related to dextran and partly related to amylose.

Because the polysaccharide had not been described previously, no formula was available which related the molecular weight to the viscosity of the polysaccharide in solution. To give an approximation, a relationship between $\bar{M}_w$ and viscosity for dextran, which was published by Marshall and co-workers, was used in this work [104]:

$$[\eta] = 10^{-1} \cdot \bar{M}_w^{0,5}$$

This relationship is valid at 25°C for dextrans with a molecular weight in the range 20 000 $< \bar{M}_w <$ 250 000.

In this formula $[\eta]$ is the limiting viscosity number, replacing the intrinsic viscosity.

$$[\eta] = \lim_{c \to 0} \cdot \eta_{sp/c}$$

The concentrations in the above formula are expressed in g/ml.

Using this relationship a value of

$$\bar{M}_w = 34\ 000$$

was obtained for one batch of the polysaccharide isolated from stored cane.

The determined value of 34 000, however, is merely an estimate, as this value was calculated using low molecular weight dextran for comparison and the polysaccharide under investigation had been shown not to be a dextran.

Viscosity determinations were also carried out on a sample of dextran which had been prepared for comparative purposes, as described in 6.1.9.1. The Marshall formula could not be applied as the average molecular weight was greater than 250 000. In its place the relationship of Senti and Hellman [80], relating log $\bar{M}_w$ and log $[\eta]$ for B 512 dextran, was used. Application of these data to the value of $[\eta]$ for the prepared dextran resulted in a molecular weight

$$\bar{M}_w = 2\ 000\ 000$$

This value is in agreement with the various published data mentioned above for the average molecular weight for dextrans and is considerably larger than the value found for the polysaccharide in deteriorated cane.

6.1.6.3 Molecular weight determination by gel chromatography

The molecular weight of the isolated polysaccharide was determined by gel chromatography using Sephadex*. The determination was carried out in a similar way to that used by Andrews for determining the molecular weights of proteins [102]. This author used ion agar (a deionised agar) as gel, and proteins of known molecular weight as markers. More recently Churms and Stephen used polyacrylamide gel (Bio-Gel) for the determination of maize starch dextrins [192]. For the determination of the molecular weight of polysaccharides, dextrans of a defined average molecular weight are commercially available*. The peak elution volumes, plotted against the logarithm of the molecular weight, fall on a straight line. Calibration of the graph was carried out by determining the peak elution volumes of three dextrans using a Sephadex G 200 column. The polysaccharide under investigation was subsequently analysed in the same way and from the peak elution volume, which was determined as 72 ml, the molecular weight was found to be

50 000 (see Fig. 4[a])

This value has to be judged with the same restrictions as that obtained by viscosity measurements. The correct value can only be obtained if the relationship between size and molecular weight of the known polymer and that of the polymer under investigation is identical.

* Pharmacia Fine Chemicals AB, Upsala, Sweden.

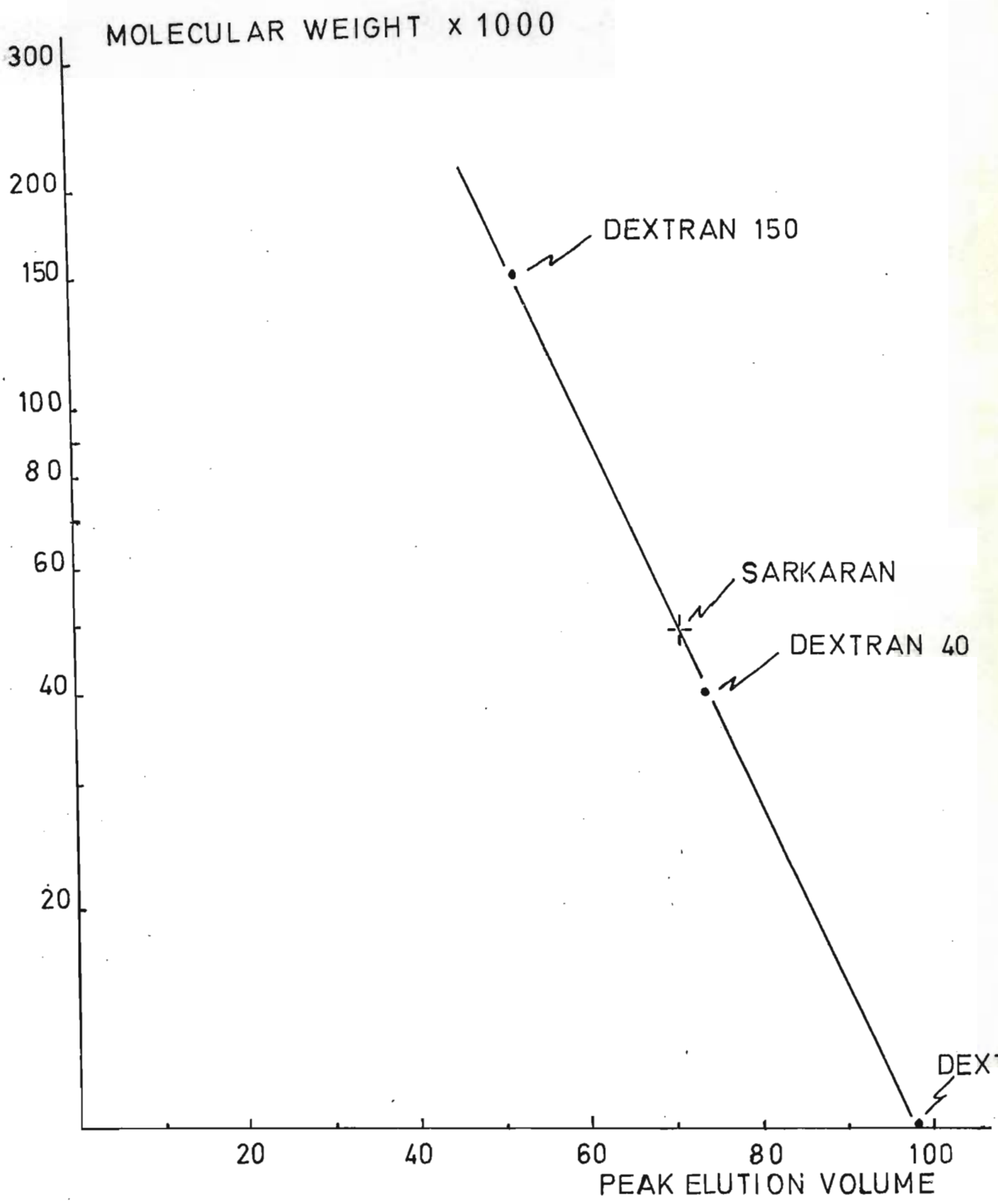


FIG. 4 [a] MOLECULAR WEIGHT DETERMINATION BY GEL CHROMATOGRAPHY.

6.1.6.4 Molecular weight determination of the isolated polysaccharide by light scattering*

Before the light scattering measurements were carried out, traces of water and suspended matter in the polysaccharide were removed by filtration, followed by freeze drying, and storage over phosphorus pentoxide.

Subsequently it was necessary to make the solutions optically clear. Clarification experiments were carried out using a 1 % (w/v) aqueous solution. This solution was filtered through a 450 nm millipore filter. The viscosity of the solution and the colour developed by phenol-sulphuric acid addition (see page 161) were determined before and after the millipore filtration. It was found that neither measurement was influenced by the filtration and it was therefore concluded that the use of 450 nm millipore filters does not affect the solution properties of the polysaccharide. Centrifugation for 4 hours at 100 000 g was tried as a pre-filtration treatment but the solutions so treated were found to give light scattering results identical to those obtained from solutions which were clarified by filtration alone.

Solutions of the polysaccharide were prepared at various concentrations (0,5 %, 1,0 %, 1,5 % and 2,0 % (w/v)) and filtered through 450 nm millipore filters.

The angular scattering intensity was determined at 5° intervals from 30 - 135°.

The refractive index difference between the solvent and the sarkaran solutions were measured on 1,0 and 2,0 %. solutions.

* This determination was carried out by Tate & Lyle Ltd., Group Research and Development.

The light scattering data were analysed by the Zimm method [193]. This method extrapolates the data to zero concentration and zero angle on a single graph. These extrapolations were necessary since light scattering theory is strictly applicable to macromolecules only at infinite dilution and zero angle [194]. For the extrapolation procedure it is necessary to calculate the Rayleigh ratio, R_θ, the ratio of the intensity of the light scattered at an angle θ to the incident direction, to the incident light intensity. This calculation must take into account the geometry of the instrument used to obtain the light scattering data, reflected light within the sample cell, and also allow for the light scattered by the solvent.

It has been shown [194] that for a polymer in dilute solution

$$\lim_{c \to o} \frac{Kc}{R_\theta} = \frac{1}{\overline{M}_w}\left[1 + \frac{16\pi^2 R_G^2}{3\lambda^2}\sin^2(\theta/2) + \ldots\ldots\right]$$

and also that

$$\lim_{\theta \to o} \frac{Kc}{R_\theta} = \frac{1}{\overline{M}_w} + 2Bc + 3Cc^2 + \ldots\ldots$$

in which

R_θ = the Rayleigh ratio
$\overline{M}_w$ = the weight average molecular weight
λ = the wavelength of the light used in measurement
c = concentration of the polymer
R_G = radius of gyration of the polymer molecules
B = their second virial coefficient
C = their third virial coefficient
K = optical constant defined by

$$K = \frac{2\pi^2 n^2 (dn/dc)^2}{\lambda^4 N}$$

where

n = refractive index of the solution

dn/dc = change in refractive index of the solution with its concentration

N = Avogadro's number

If higher powers than c and $\sin^2(\theta/2)$ are neglected; R_G, B, and $\bar{M}_w$ can be obtained from the slopes and intercepts of the graphs of $\frac{Kc}{R_\theta}$ plotted against c and $\sin^2(\theta/2)$.

The Zimm method is to plot $\frac{Kc}{R_\theta}$ versus $\sin^2(\theta/2) + K'$ where K' is an arbitary constant chosen to conveniently spread the data. Lines of constant concentration are extrapolated to zero angle, and the resulting points are joined and extrapolated to the $\frac{Kc}{R_\theta}$ axis. The intercept of the line equals $1/\bar{M}_w$ and the slope of the line is 2B. Similarly, lines of constant angle are extrapolated to the Kc/R_θ axis. The intercept is also equal to $1/\bar{M}_w$ and the slope is

$$\frac{16\pi^2 R_G^2}{3\lambda^2 \bar{M}_w}$$

Using this procedure R_G, B, and $\bar{M}_w$ were determined from the Zimm plot.

Two Zimm plots were prepared, one at 546 nm and the other at 436 nm (Fig. 4^b and 4^c). From these figures, values of $3{,}6 \times 10^{-6}$ and $3{,}8 \times 10^{-6}$ were obtained for $1/\bar{M}_w$. These result in values of 278 000 and 263 000 for the weight average molecular weight of the polysaccharide under investigation.

The Zimm plot also shows that the polysaccharide is polydisperse, because the lines joining the experimental points are curved. Curved lines of this type are characteristic of a spread of molecular weights. The experimental data for the 0,5 %

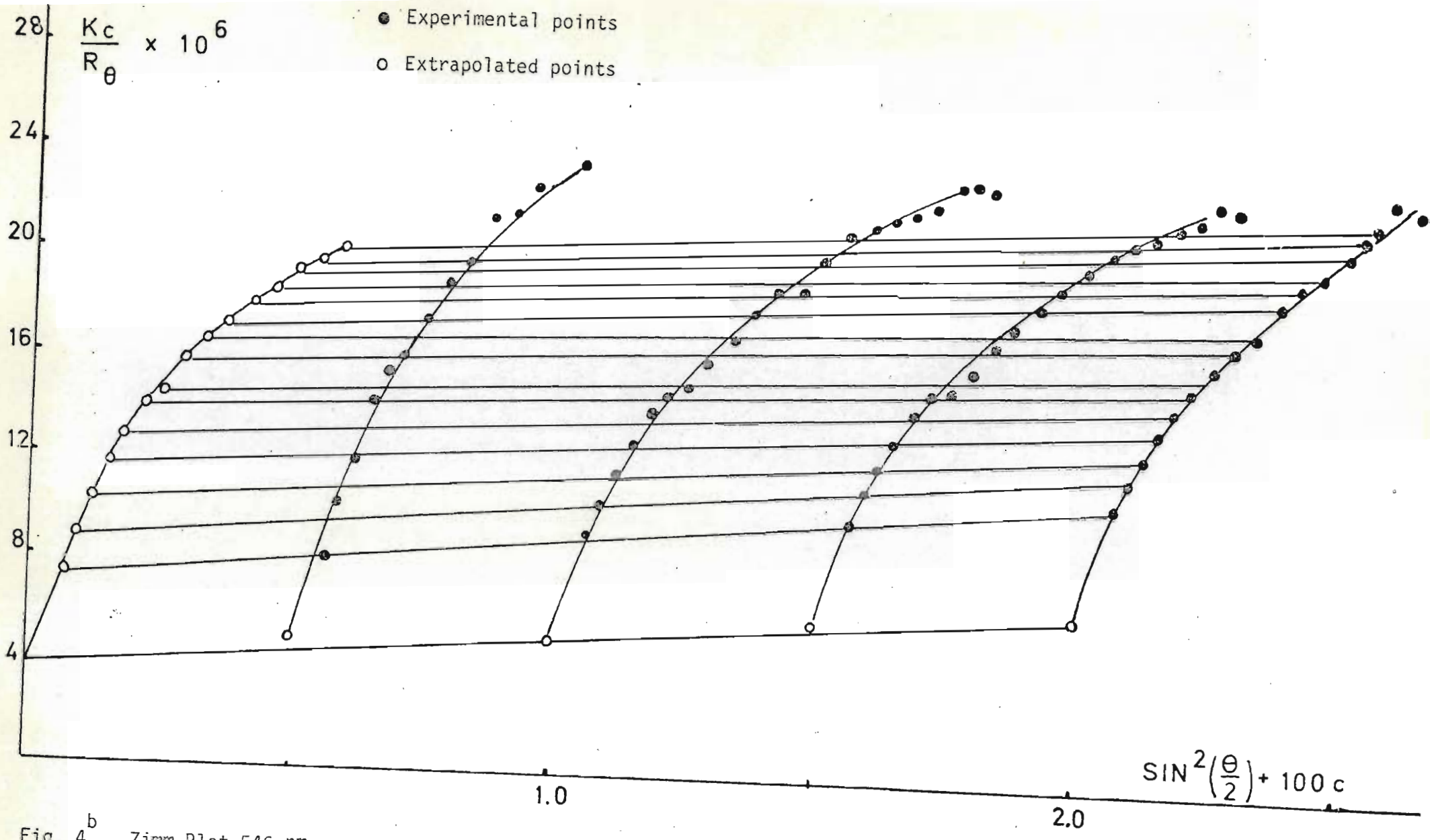


Fig. 4[b] Zimm Plot 546 nm.

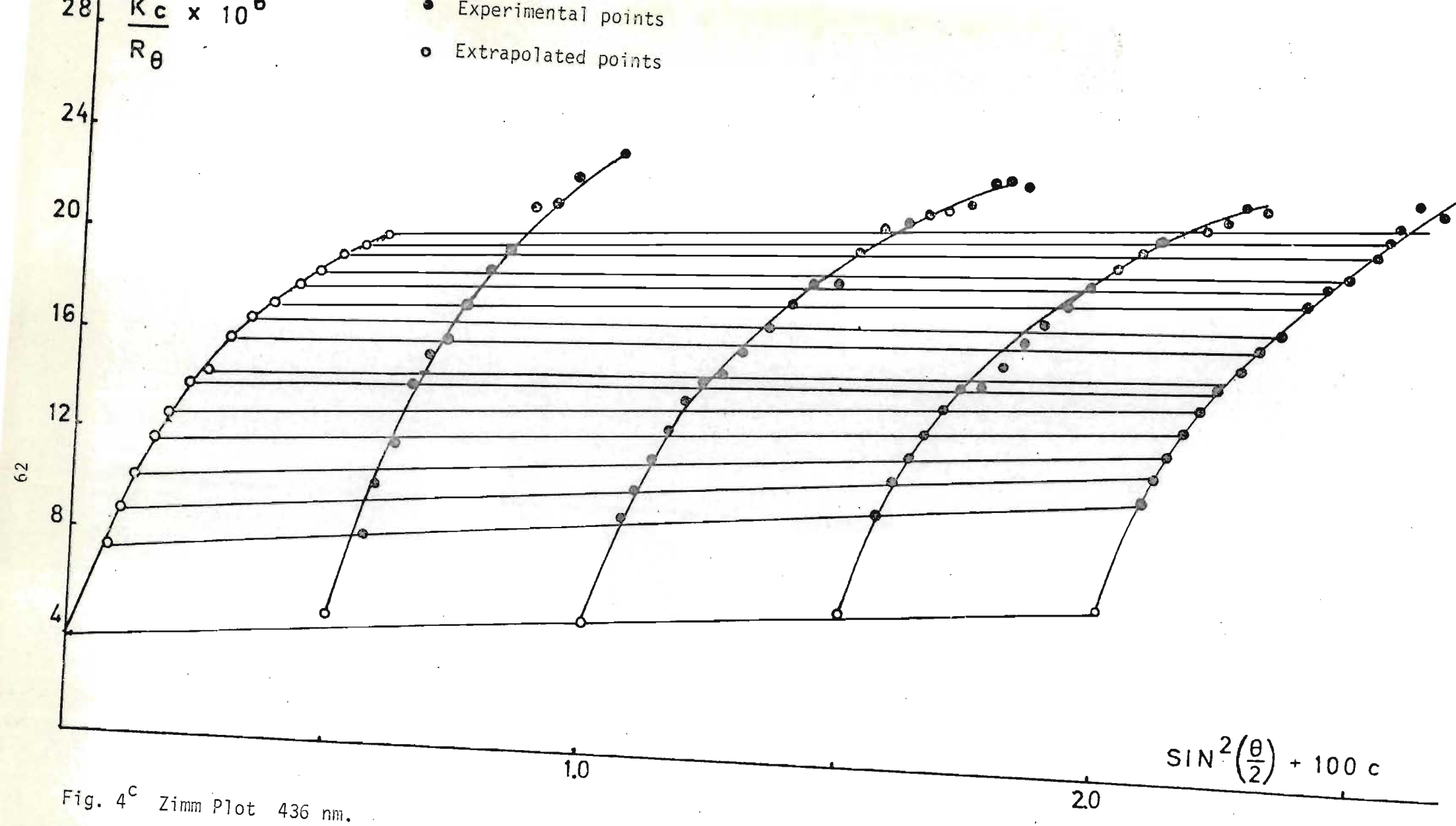


Fig. 4[c] Zimm Plot 436 nm.

solution of the polysaccharide are rather scattered due to the low scattered light intensity and consequent lower measuring accuracy.

The slope of the = 0 line is equal to 2B and that of the c = 0 line is

$$\frac{16\pi^2 R_G^2}{3\,\lambda^2\,\overline{M}_w}$$

The c = 0 line is curved and the limiting slope was used to obtain R_G.

R_G, B, and $^{dn}/dc$ are shown in Table 4[a].

Table 4[a]

Radius of gyration and second virial coefficient from Zimm plots 4[b] and 4[c]

λ	546 nm	436 nm
$^{dn}/dc$	0,149 ml.g^{-1}	0,152 ml.g^{-1}
R_G	200 Å	240 Å
B	1,3 x 10^{-6}	1,4 x 10^{-6}

As $^{dn}/dc$ appears in the equation for K, the value was determined accurately and checked using a second refractometer. This precaution was taken because a 5 % error in $^{dn}/dc$ would result in a 10 % error in $\overline{M}_w$. The agreement between the two refractometers used was within 1 %.

A small value for B is consistent with a neutral polymer and the fact that B is positive indicates the absence of particulate matter. It can be assumed therefore that the filtration prior to the light scattering measurement was satisfactory.

A value for R_G of 200-250 Å indicates that the polysaccharide molecules are present in solution as random coils rather

than rod-like structures [199], the latter show much higher values for R_G at a $\overline{M}_w$ of 250 000. This finding is consistent with the fact that the polysaccharide is a neutral polymer and also explains the comparatively low viscosities of the polymer solution and the low molecular weight value obtained by gel chromatography.

6.1.6.5 Determination of the molecular weight of the polysaccharide by osmotic pressure measurement *

This absolute determination gives a value for the number average molecular weight.

The results of the osmotic pressure measurements are shown in Fig. 4[d] as a plot of osmotic pressure per unit concentration against concentration.

The intercept with the ordinate gives a value for

$$\lim_{c \to o} \pi/c = 0,51$$

From $\overline{M}_n = \frac{RT}{\lim_{c \to o} \pi/c}$ the number average molecular weight was calculated as

$$\overline{M}_n = \frac{84,7 \times 310}{0,51} = 51\ 500.$$

The ratio

$$\frac{\overline{M}_w}{\overline{M}_n} = \frac{260\ 000}{51\ 000} = 5$$ provides additional evidence that the polysaccharide is polydisperse.

A similar wide distribution is found in other natural glucans, e.g. for starch amylopectin $\overline{M}_w/\overline{M}_n = 267$ [198].

* This determination was carried out by the Department of Chemistry, Sheffield Polytechnic, England.

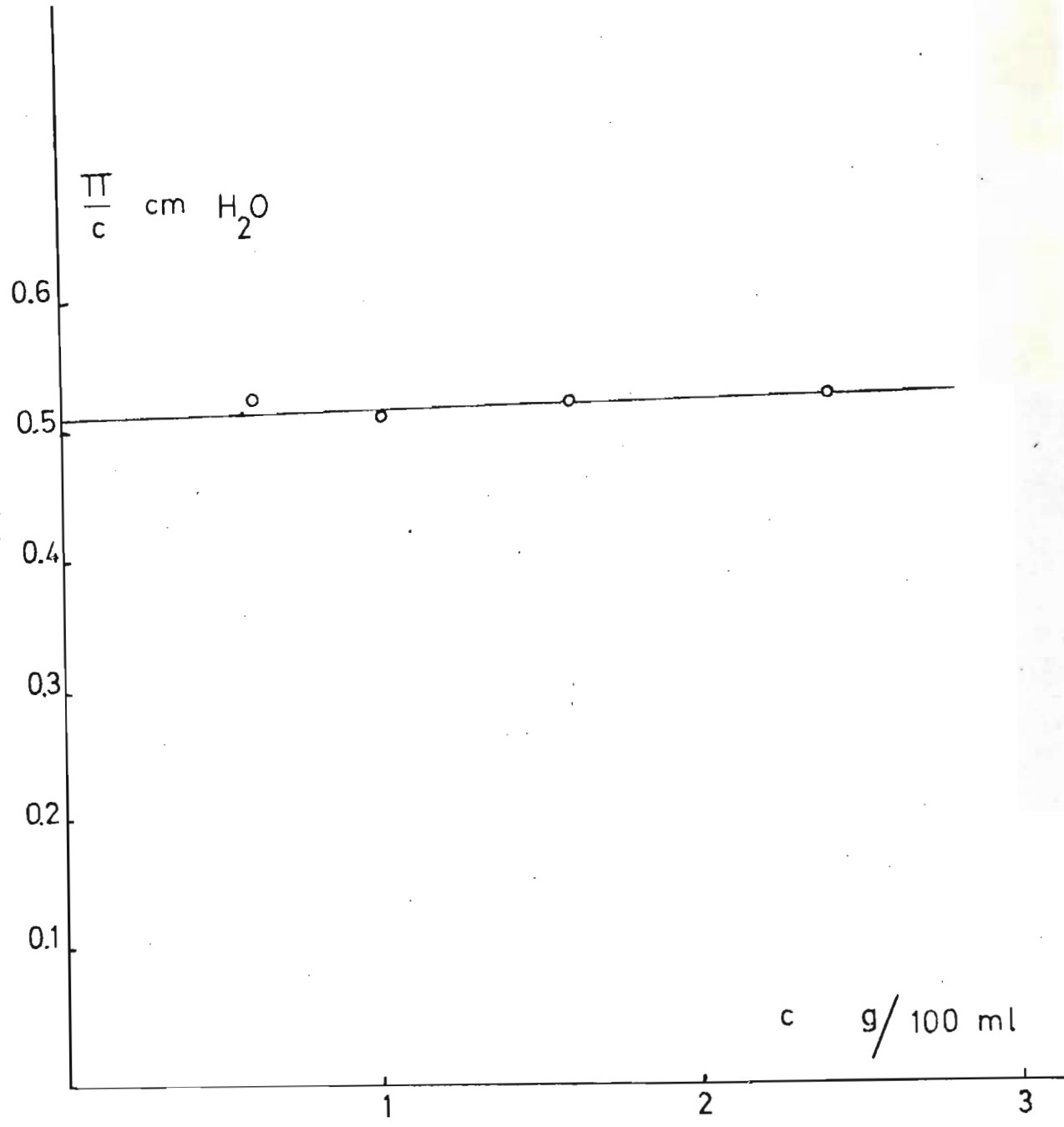


Fig 4[d] Osmotic pressure of sarkaran solutions versus concentration.

The value found for $\overline{M}_w$ = 260 000 is of the same order of magnitude as that published for other glucans (amylose, some dextrans, cellulose, pullulan) as shown in Table 4[b].

Table 4[b]

$\overline{M}_w$ of some glucans	
dextran	88 000 [105]
	50 - 525 x 10^6 [110]
glycogen	81 x 10^6 [80 106]
amylose	0,5 x 10^6 [107]
amylopectin	250 x 10^6 [108]
cellulose	0,5 x 10^6 [109]
pullulan	235 000 [90]

6.1.7 Specific rotation

The specific rotation of the polysaccharide was determined on a two percent solution in distilled water, at 20°C, using a sodium light. The value obtained was + 160°. The high dextrarotation value obtained indicates the presence of α-linkages in the glucose polymer and compares favourably with values published for dextran [111] and starch [112], which exhibit specific rotations in the order of + 200°. In the case of starch, however, the specific rotation value obtained depends very much on the method used [113]. Because of the insolubility of ungraded starch, measurements are made in alkaline solutions. Slight opacity of the solution and possible degration of the starch accounts for the variation in the values reported for $(\alpha)_D$, between + 180° and + 220°.

6.1.8 Paper electrophoresis

A simple electrophoretic method was used to investigate whether the polysaccharide was a charged or an uncharged molecule [115].

The polysaccharide was applied to a paper strip and a comparison made between its movement in an electric field and that of dextran, a neutral polysaccharide. This comparison corrects for the electro-osmotic movement of the buffer solution, in relation to the paper, caused by the charge of the filter paper [114].

After making the spots visible, the distance travelled was measured. In 8 hours, using a buffer solution of pH 5,8, dextran moved 33 mm in the direction of the cathode and the polysaccharide under investigation 25 mm. In a buffer solution of a pH 8,3, the values obtained were 34 mm for the dextran and 12 mm for the cane polysaccharide. These results showed that the polysaccharide under investigation was neutral and similar in electrophoretic behaviour to dextran. The small lateral movement in the electric field may be attributed to a slight adsorption of positive ions.

6.1.9 Determination of the structure of the polysaccharide by chemical analysis

6.1.9.1 Preparation of dextran for comparative purposes

Both data from the literature and the physical nature of the polysaccharide suggested a relation to dextran. For comparative purposes, and to test the various procedures in structural determination, dextran was produced by fermentation of a medium, containing sucrose, with *Leuconostoc mesenteroides*. This micro organism was isolated from sugar cane juice and identified by physiological tests. The dextran formed in the sucrose containing medium was isolated and purified by the same methods used for the polysaccharide in deteriorated cane. The purified dextran was subsequently analysed for protein and ash. As no trace of either

could be detected, the dextran was considered to be pure.

6.1.9.2 Identification of the hexose obtained by acid hydrolysis

The cane polysaccharide was hydrolysed by sulphuric acid, the hydrolysate neutralised, and the inorganic salts removed. The resulting aqueous solution was analysed by paper chromatography, using various eluents. A silver reagent was used to develop the spots [121] . In every case, only one spot was obtained, with the same R_f value as glucose.

To another part of the hydrolysate, phenylhydrazine and sodium acetate were added. The phenyl osazone obtained was examined microscopically and found to be identical to the phenyl osazone of D (+) - glucose. The melting point of the osazone was determined as 204°C; this was not depressed by the admixture of an authentic sample of glucosazone. The reported melting point of glucosazone is 205°C [117] . From these results it was concluded that the cane polysaccharide was a glucan.

6.1.9.3 Determination of the type of linkages, by periodate oxidation

I Reaction mechanism and products formed

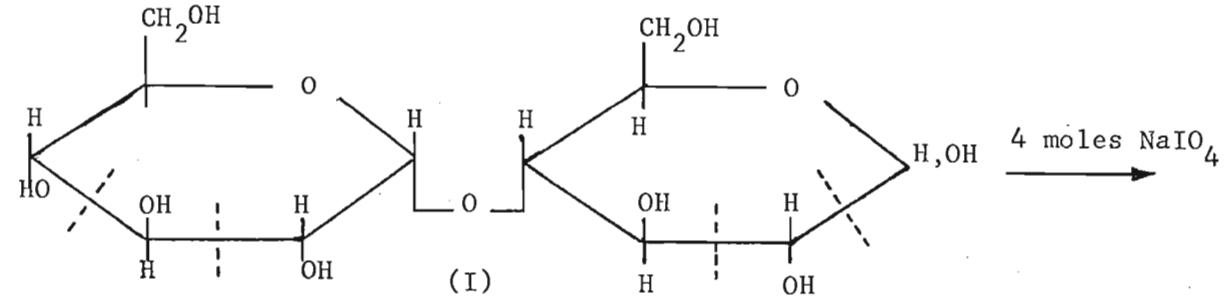
Under correct conditions of concentration, pH, and temperature, carbohydrates are oxidised by periodate. The oxidation occurs between two vicinal hydroxyl groups only, as shown for maltose (I) on page 69. Light, elevated temperature, and high concentration of the oxidant, result in side reactions and "over oxidation".

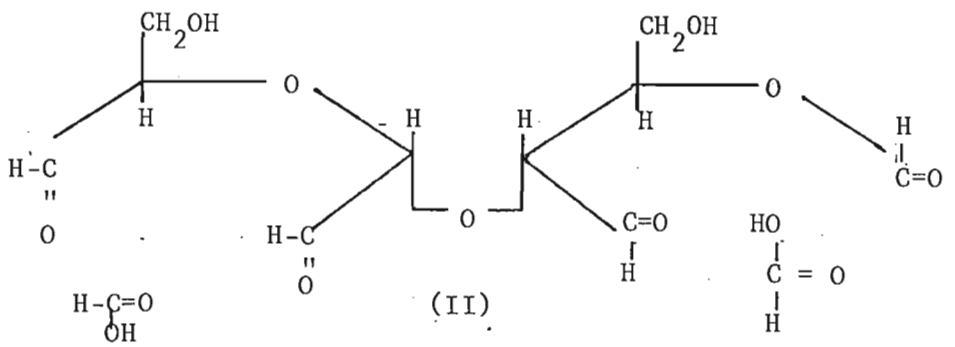
Since its discovery in 1928 by Malaprade, the oxidation method has been extensively used for the determination of the structure of carbohydrates and more particular of polysaccharides.

If three vicinal hydroxyl groups are present in the carbohydrate molecule the central group is oxidised to formic acid (see II). From the amount of periodate which is reduced and the quantity of formic acid formed, certain conclusions regarding the structure of the polysaccharide can be drawn. Provided the end groups in a chain of glucopyranose units are neglected, the results of periodate oxidation can be tabulated as follows [90 123 124].

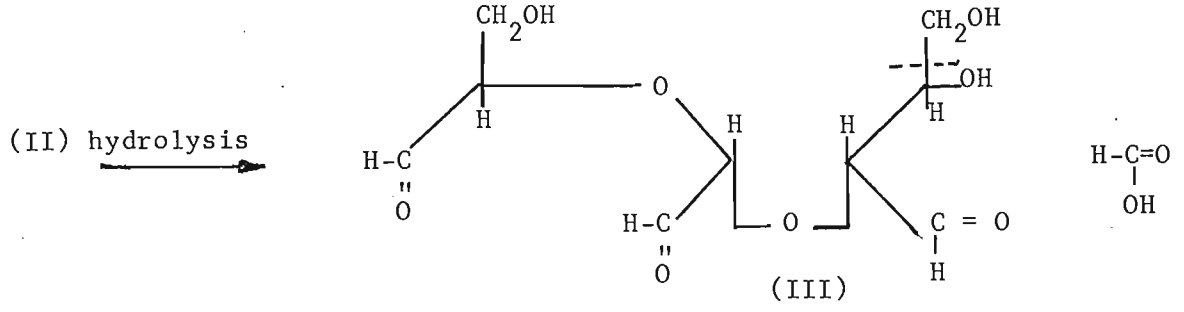
Type of linkage	$NaIO_4$ consumed. Mole/Mole glucose	Formic acid produced. Mole/Mole glucose	Residual glucose
(1 → 2)	1	-	-
(1 → 3)	-	-	+
(1 → 4)	1	-	-
(1 → 6)	2	1	-

Many investigators have used sodium meta periodate in aqueous solution. The pH of this solution is 4. During the periodate oxidation the reducing end group of a carbohydrate forms a formate ester, which will be hydrolysed very slowly at pH 4 and low temperature. The reaction for maltose is shown below:

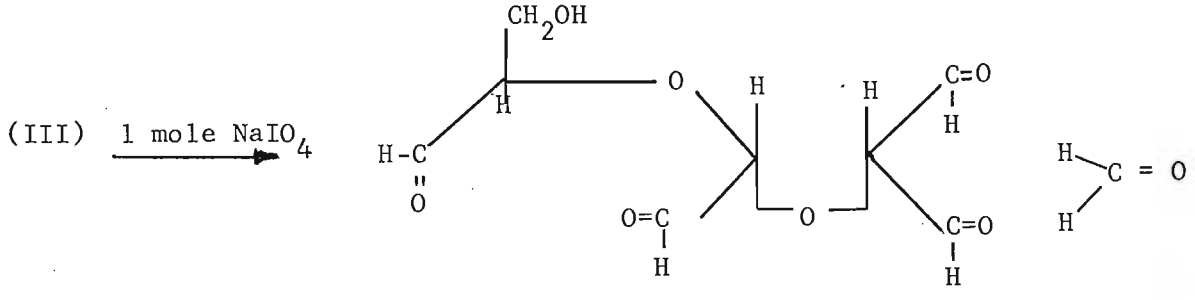




One mole of maltose reduces 4 moles of periodate. As a result 2 moles of formic acid are formed and 1 mole of formic acid is produced in the form of formate ester (II). If this formate ester hydrolyses during the oxidation reaction, a further mole of periodate is reduced (IV) and formaldehyde (V) is formed.



(III)



(IV)

Therefore, if the formate ester (III) hydrolyses during the oxidation reaction, 1 mole of maltose reduces 5 moles of sodium meta periodate.

It is obvious that this reaction mechanism is only important in the case of carbohydrate polymers with a short chain length, in which the quantity of end groups is relatively large. In long chain carbohydrate polymers, without much branching, the contribution of the end groups to the result of the periodate oxidation can be ignored. If the polysaccharide is branched, a certain amount of formic acid will be produced by oxidation of the non reducing end groups. The error produced by this oxidation will become larger with increasing branching of the polysaccharide.

Both low temperatures (0° - 6° C) and moderate temperatures (20° - 25°C) during the reaction have been used by various investigators. Although the reaction rate decreases at low temperatures, a better selective oxidation is obtained. "Over oxidation" is much smaller at 0° - 6° C than at 25°C [125].

The concentration of periodate used is normally between 0,01 and 0,1 M. The periodate consumption of the reaction mixture is usually determined by the method of Fleury and Lange [127]. The method involves the reduction of periodate to iodate, using an arsenite solution at pH 8. Iodide ions are added as catalyst.

$$IO_4^- + AsO_2^- \rightarrow IO_3^- + AsO_3^-$$

The excess of the added arsenite is titrated against a standardised iodine solution

$$AsO_2^- + I_2 + 2\,OH^- \rightarrow AsO_3^- + 2\,I^- + H_2O$$

(for experimental details see page 174)

Formic acid in the reaction mixture can be titrated with any hydroxide, after removing the excess of sodium meta periodate by the addition of ethylene glycol

$$IO_4^- + CH_2OH.CH_2OH \rightarrow IO_3^- + 2\,CH_2O + H_2O$$

The formaldehyde formed does not interfere in the titration of the formic acid. Most investigators have used barium hydroxide solution. The choice of indicator influences the final result and many have been used [126]. At pH 6 only 80 percent of the formic acid being formed will have been titrated. Using phenolhptalein the results are claimed to be only one percent too high [126].

In this investigation, sodium metaperiodate at pH 4 was used as oxidant. All oxidation reactions were carried out at 4°C in the dark to minimise side reactions. The method and conditions were first tested using pure maltose.

One mole of maltose reduces 4 moles of periodate and 3 moles of formic acid are produced (see above). During the titration of the formic acid, the formate ester has to be hydrolysed, otherwise too little formic acid will be titrated. The correct hydrolysis of the formate ester depends on the pH of the end point taken for the titration. As there appears to be little uniformity in the choice of the pH of this end point from literature data, a titration curve of pH versus volume of titrant was plotted. This titration curve was determined for the reaction mixture of maltose and sodium metaperiodate after 7 days. The pH was measured with a glass electrode and the results are shown in Fig. 7.

The curve shows that at pH 5,5 (first end point), 75 percent of the expected theoretical amount of formic acid produced has been titrated. At pH 7,4 a second end point is obtained. All the formate ester is hydrolysed at this point and the theoretical amount of formic acid is found. In this investigation 3,05 moles of formic acid per mole maltose were found, the theoretical value being 3,00 moles. All subsequent titrations for the reaction mixtures of dextran or cane polysaccharide with periodate were carried out to an end point pH 7,4, determined with a glass electrode.

The periodate consumption in the reaction mixture of maltose and sodium metaperiodate was followed, over a period of seven days, by determining the decrease in periodate after various time intervals using the method of Fleury and Lange. The results were plotted in a curve shown in Fig. 8. The curve reaches a plateau after three to four days while the theoretical amount of periodate (four moles of periodate reduced per mole maltose) is reached after seven days. The theoretical amount of formic acid produced (three moles per mole maltose) was also found after seven days.

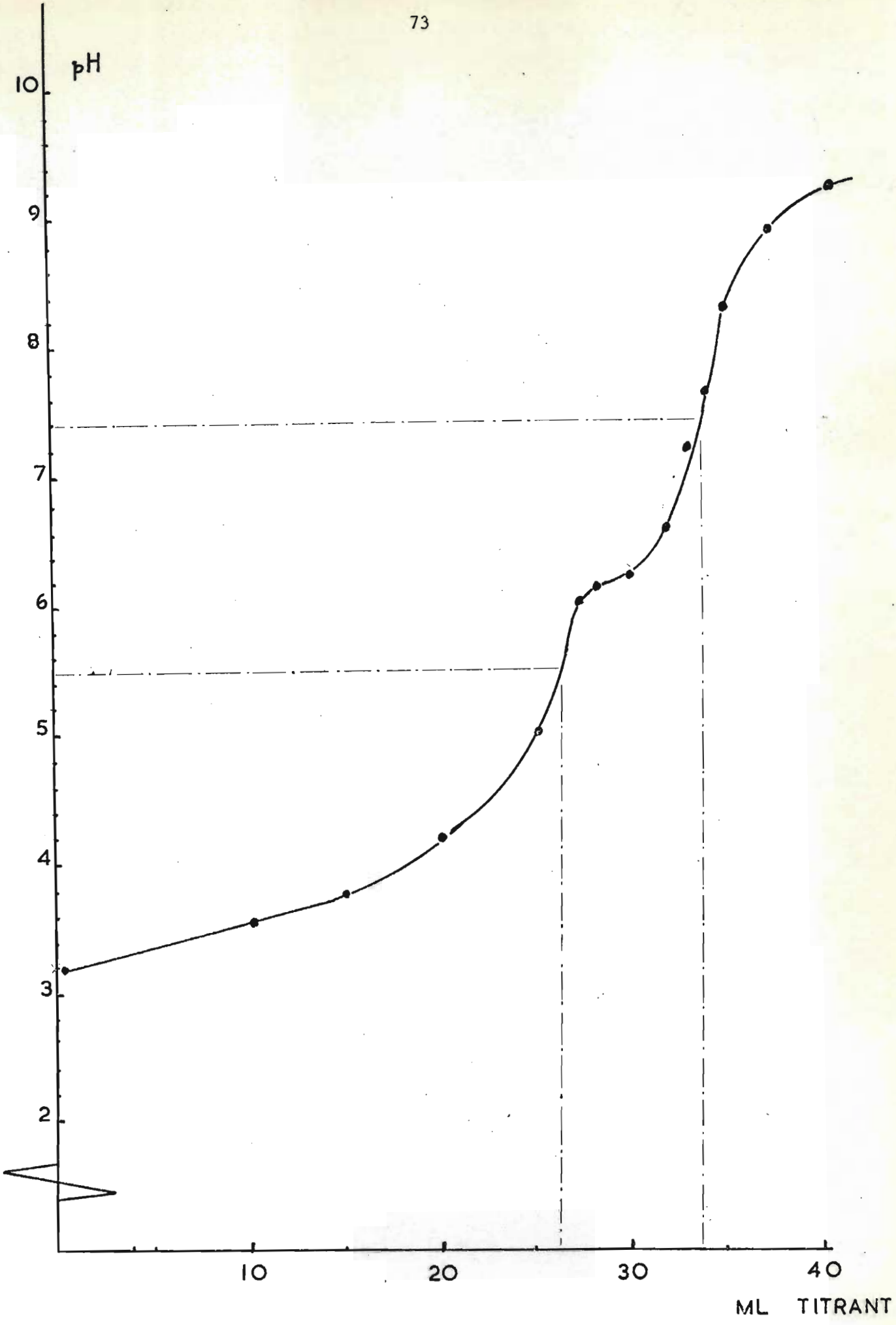


FIG. 7- TITRATION CURVE OF FORMIC ACID FORMED DURING PERIODATE OXIDATION OF MALTOSE.

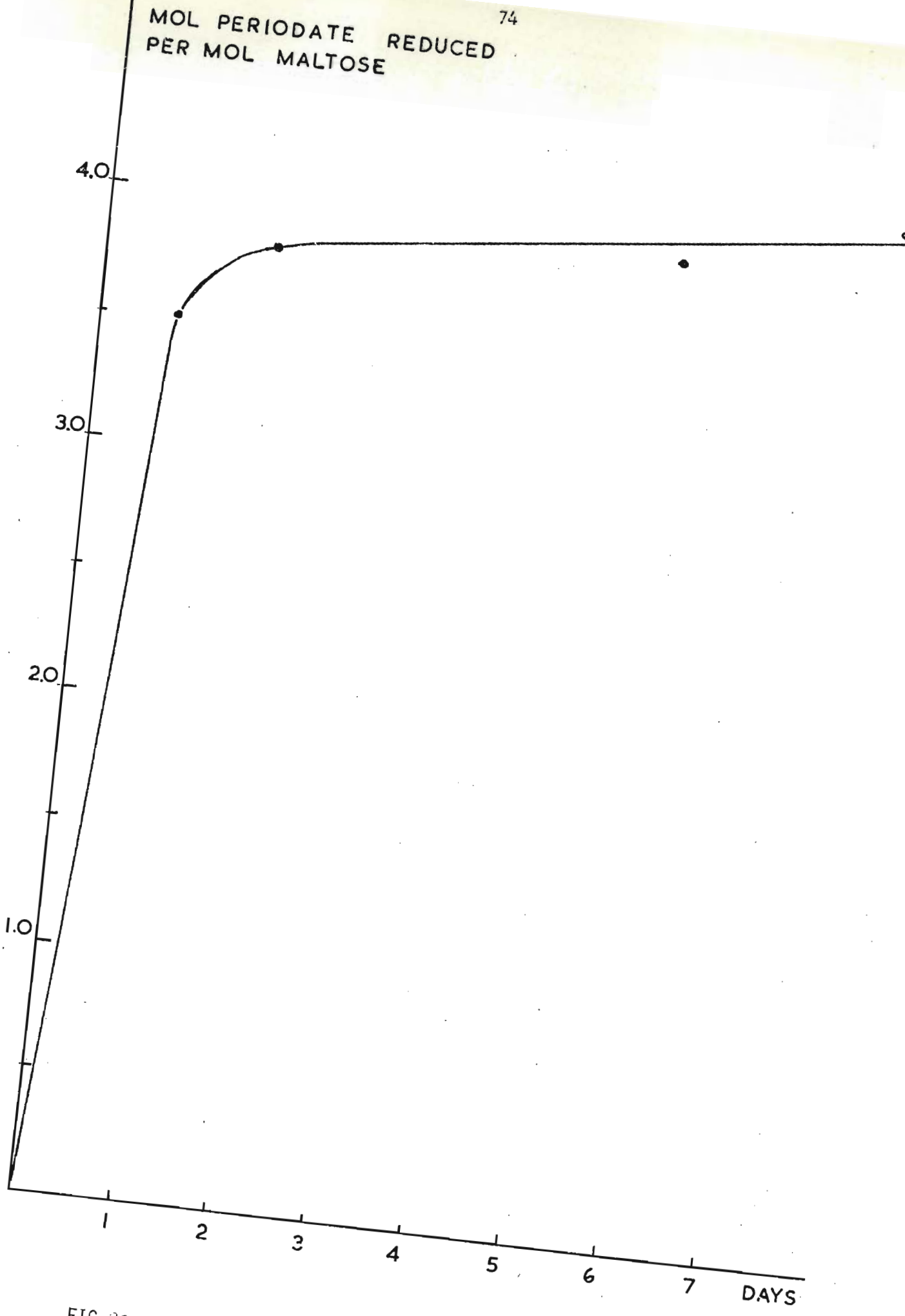


FIG. 8 PERIODATE OXIDATION OF MALTOSE VERSUS TIME

II Periodate oxidation of the dextran isolated from a culture of *Leuconostoc mesenteroides* in a sucrose containing medium.

Periodate oxidation, under the conditions previously described, was applied to dextran prepared as described under 6.1.9.1. The periodate concentration used was 0,015 M and a slight excess above the expected theoretical amount was added. This excess was established by preliminary experiments.

After seven days 1,50 moles of periodate were reduced per glucose unit (Fig. 9) and 0,77 mole formic acid was formed.

The ratio of periodate reduced to formic acid produced (2:1), indicates that only (1 → 6) linkages in the dextran and no (1 → 4) or (1 → 2) types are oxidised. If only (1 → 6) linkages in the dextran were present, however, 2 moles of periodate per glucose unit should have been consumed. The dextran is either not completely oxidised in seven days or it contains 25 percent of (1 → 3) linkages, which do not reduce periodate. (1 → 3) linkages are common in dextran as branch points and up to 40 percent (1 → 3) linkages have been found as was discussed in 6.1.1.4. [75]

III Periodate oxidation of the polysaccharide isolated from stored cane.

In the case of periodate oxidation of the polysaccharide isolated from stored cane, after 7 days, 1,08 moles of periodate were reduced and 0,26 mole of formic acid was formed per glucose unit. The formic acid must have been formed by oxidation of (1 → 6) linkages and the corresponding amount of periodate reduced by these linkages will have been 0,52 mole. Linkages of the (1 → 4) or (1 → 2) types, either separately or as a mixture will have reduced the residual 0,56 mole of periodate (see Fig. 10).

The ratio of (1 → 4) or (1 → 2) to (1 → 6) linkages is consequently 0,56:0,52/2, and it follows that the polysaccharide

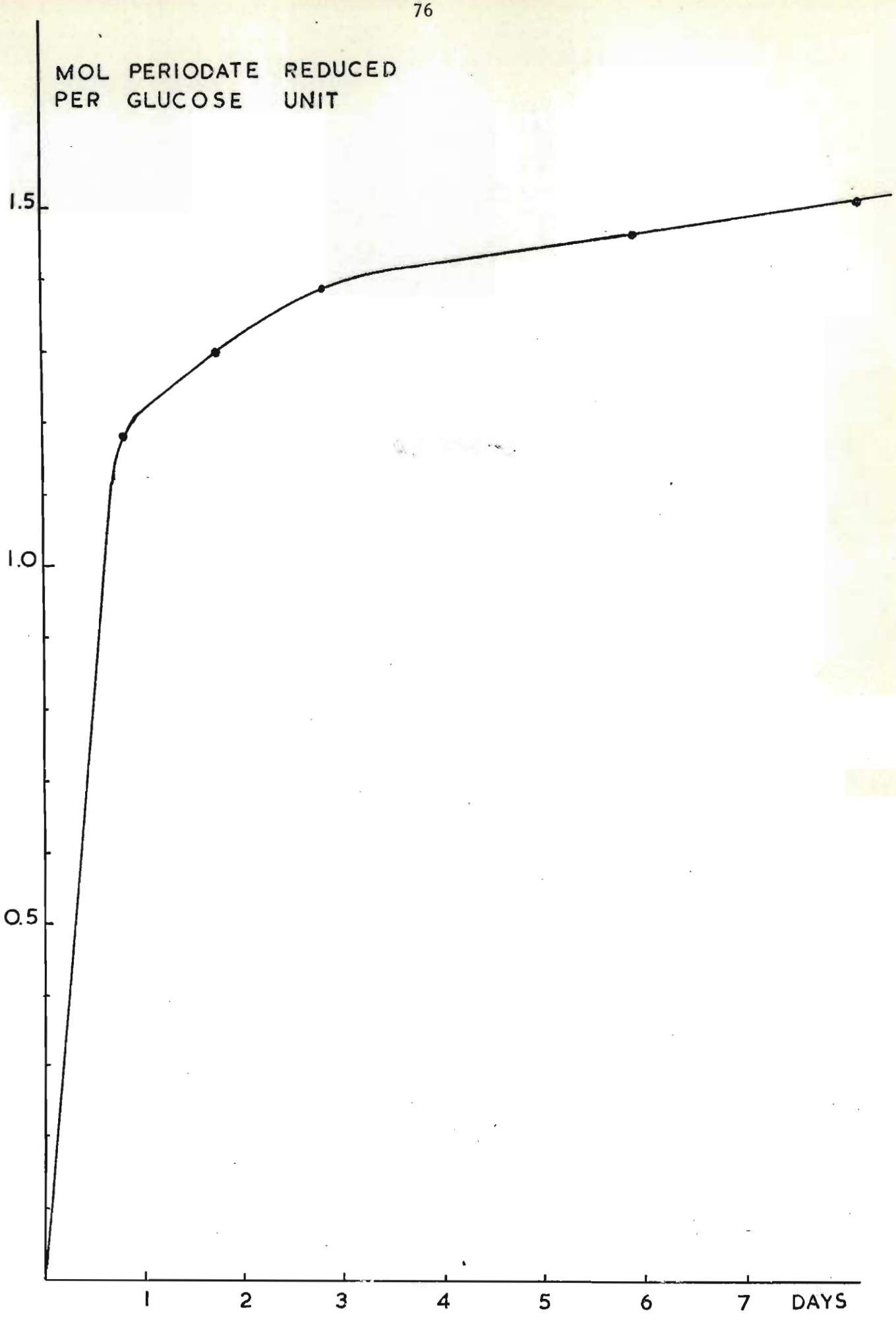


FIG. 9 PERIODATE OXIDATION OF DEXTRAN VERSUS TIME.

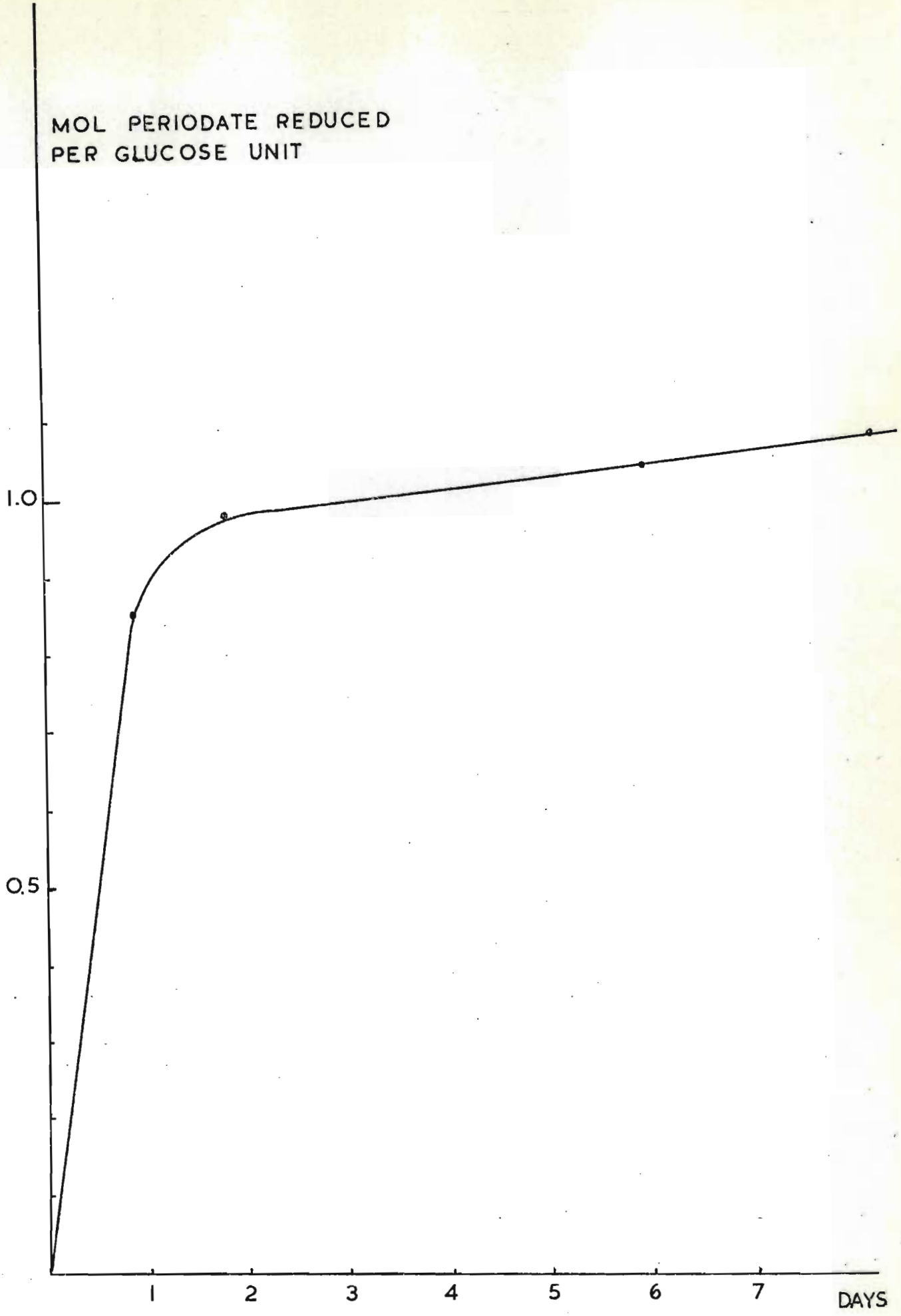


FIG. 10 PERIODATE OXIDATION OF CANE POLYSACCHARIDE VERSUS TIME.

is made up of 68 % of (1 → 4) or (1 → 2) linkages and 32 % of (1 → 6) linkages. The high ratio of (1 → 4) or (1 → 2) linkages cannot be present in the form of branch points only. Some, at least, must occur as inter chain linkages. For this reason the structure of this polysaccharide is different from dextran which, by definition, is a predominantly (1 → 6) linked α glucan .

6.1.9.4 Methylation of starch, dextran, and the polysaccharide isolated from stored cane, followed by determination of the methyl glucosides after methanolysis.

Periodate oxidation showed that the polysaccharide in stale cane did not belong to the dextran type. Methylation gives more detailed information on the structure of a polysaccharide and has the advantage over periodate oxidation in that it differentiates between (1 → 4) and (1 → 2) links.

Methylation was carried out on the cane polysaccharide, on dextran and on starch. The latter two were methylated to obtain authentic samples of methyl glucosides for comparison. In the case of starch no effort was made to separate it into amylose and amylopectin. Dimethyl glucosides were not required and the trimethyl glucosides are identical for amylose and amylopectin.

Methylation of starch followed by methanolysis results in the two methyl 2,3,6 tri-O-methyl-D-glucosides.

As dextran is a (1 → 6) linked glucan the predominant methyl glucosides are methyl 2,3,4 tri-O-methyl-α-D-glucoside and methyl 2,3,4 tri-O-methyl-β-D-glucoside.

The first methylations were carried out according to Haworth, initially at 0°C to prevent alkaline degradation of the polysaccharide.

The Haworth methylation involves treatment of the polysaccharide with an alkaline aqueous solution of dimethyl sulphate. The alkali neutralises the sulphuric acid formed during the reaction

$$ROH + Na\,OH \rightarrow RO^{-}Na^{+}$$

$$RO^{-}Na^{+} + (CH_3)_2\,SO_4^{-} \rightarrow ROCH_3 + NaCH_3\,SO_4$$

After two Haworth methylations the subsequent methylation was carried out according to Kuhn [131], by dissolving the partially methylated product in dimethyl formamide and using silver oxide and methyl iodide as the methylation mixture

$$n\,ROH + n\,CH_3I + \frac{n}{2}\,Ag_2O \rightarrow$$

$$n\,ROCH_3 + n\,AgI + \frac{n}{2}\,H_2O$$

Methylation was considered to be complete when the methoxyl content did not increase after a subsequent methylation procedure.

The methoxyl content was determined by the method of Vieböck and Brecker [129], converting the methoxyl groups into methyl iodide by heating the methylated carbohydrate with hydrogen iodide in a Zeisel apparatus. The methyl iodide formed was trapped in a bromine-containing solution of sodium acetate. Before trapping the methyl iodide, the traces of hydrogen iodide were removed by passing the gas stream through an aqueous suspension of red phosphorous. After the reaction was finished 80 % formic acid was added to the receiver solution until the bromine colour disappeared. The solution was subsequently titrated against standardised sodium thiosulphate after the addition of potassium iodide and sulphuric acid.

The reaction mechanism can be described as follows:

$$ROCH_3 + HI \rightarrow ROH + CH_3I$$

$$CH_3I + Br_2 \rightarrow CH_3Br + IBr$$

$$IBr + Br_2 + 3H_2O \rightarrow HIO_3 + 5\,HBr$$

$$HIO_3 + 5I + 5\,H^{\cdot} \rightarrow 3I_2 + 3H_2O$$

$$I_2 + 2\,S_2O_3'' \rightarrow S_4O_6'' + 2I'$$

The polysaccharide from stale cane was methylated by two Haworth methylations and five Kuhn methylations. The final methoxyl content obtained was 41,6 %.

Dextran was subjected to two Haworth methylations and three Kuhn methylations after which the methoxyl content reached 41,0 %. Difficulties arose in the methylation of starch. A Haworth methylation on soluble potato starch failed. In order to overcome this, the starch was first acetylated [147], using glacial acetic acid and acetic anhydride. Chlorine was added as catalyst. The acetyl content of the starch after this treatment was 45,3 % and the acetylated starch was dissolved in acetone and methylated according to Haworth. Two further Haworth methylations and one Kuhn methylation were carried out. After purification of the final product with chloroform and petroleum ether, the methoxyl content was found to be 44,3 %.

Completely methylated, unbranched glucan has a methoxyl content of 46,1 %. The final methoxyl contents determined for the cane polysaccharide and the dextran were low. This was partly because the initial polysaccharides were not 100 percent pure but, in addition, there are other reasons why methylated polysaccharides show a too-low methoxyl content.

It has been pointed out by Bouveng and Lindberg [137] that methylated polysaccharides are amorphous and difficult to purify. Particularly when N,N-dimethyl formamide is used in the preparation, the last traces of organic solvent are difficult to remove and the product is hygroscopic.

The absence of absorption in the region of 3 400-3 600 cm^{-1} of the infrared spectrum, the absorption characteristic of the hydroxyl group, is often used as proof of complete methylation. In the case of a hygroscopic material like the methylated polysaccharide, however, residual moisture will usually result in some absorption in the region in question [136 137].

Under-methylation of an unbranched polysaccharide would result in the presence of mono- and di-substituted methyl glucosides among the methanolysis products. Subsequent analysis proved, however, that the methylation of the cane polysaccharide had been complete and that no di-substituted methyl glucosides were present in the methanolysate.

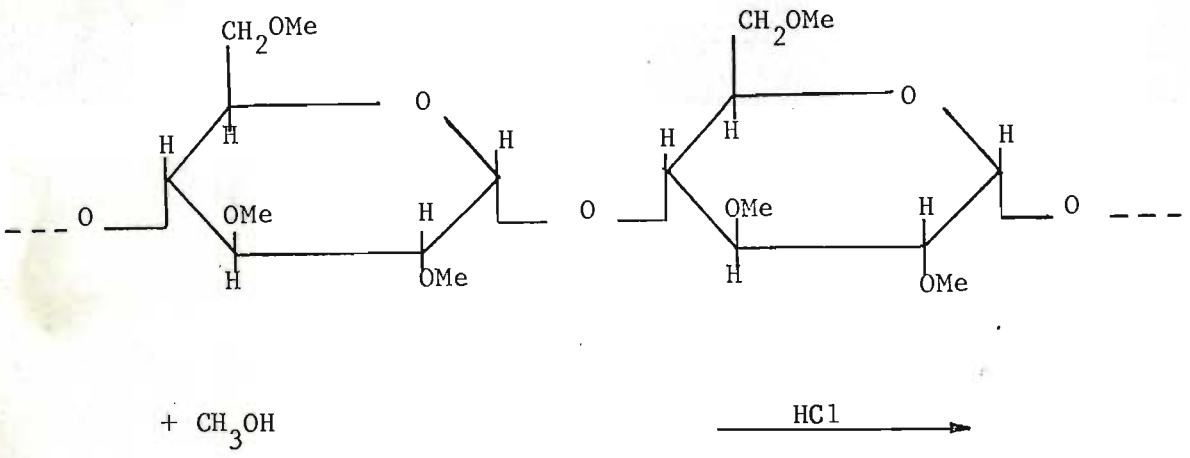
The mixture of methyl glucosides present in the methanolysate of the methylated polysaccharides can be separated and quantitatively determined by various chromatographic methods. Paper chromatography, column chromatography, and paper electrophoresis have all been used [137]. More recently, gas liquid chromatography (GLC) has been shown to have advantages over the other methods. Methyl glucosides are sufficiently volatile to be analysed at suitable temperature, by GLC, without further modification. In addition, the separation and quantitation can be carried out in one analysis.

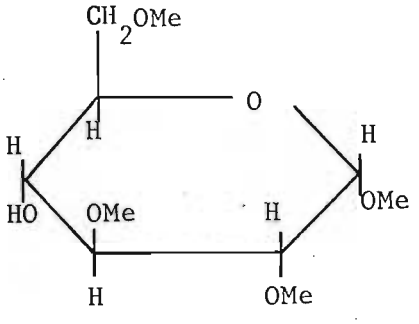
In the present investigation, the separation and quantitative determination of the methanolysis products of the methylated polysaccharides was carried out by GLC.

The procedure used for methanolysis involved heating the methylated polysaccharides in absolute methanol containing 5 % hydrochloric acid. After the reaction was complete, the acid was removed by treatment with an excess of silver carbonate. The remaining silver carbonate and the silver chloride formed were removed by filtration and the solution of methyl glucosides in methanol was analysed without further concentration or separation

of the methanol.

The methanolysis can be illustrated as follows:





Methyl-2,3,6 tri-O-methyl-D-glucopyranoside

The methyi glucosides resulting from the methanolysis of the methylated polysaccharides were analysed in two stages. First, the more volatile tetra- and trimethyl glucosides were analysed by gas chromatography. The dimethyl glucosides were analysed in a second GLC analysis at a higher temperature. Because the di-substituted glucosides were expected to be present in a much lower concentration than the more fully substituted methyl glucosides, the second analysis was carried out at a more sensitive setting of the attenuator of the gas chromatograph.

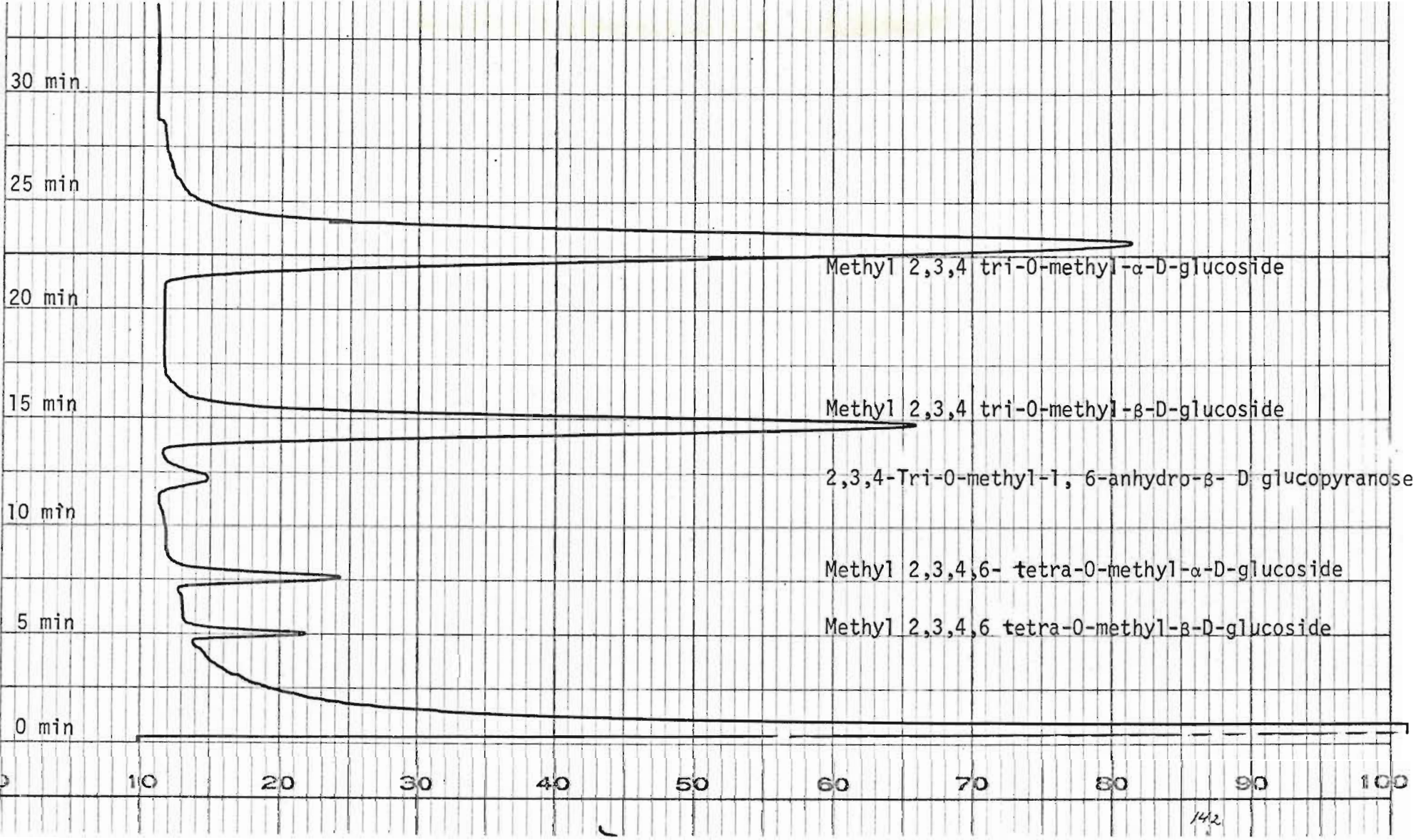
Eight chromatograms of the various methyl glucosides of the cane polysaccharide, dextran, and starch are shown in Figs. 11 - 16.

The T-values obtained for the methyl glucosides of the methylated starch and dextran, which are listed in Table 5, are in agreement with those reported in the literature [133]. In the chromatogram of the methylated cane polysaccharide, the peaks of methyl-2,3,6 tri-O-methyl-β-D-glucoside and methyl-2,3,4 tri-O-methyl-α-D-glucoside partly overlap. The areas of the peaks were determined by triangulation and in the case of these two overlapping peaks, the top parts were extrapolated.

The response per mole of partially substituted methyl glucosides to the flame ionisation detector is less than that of the more fully substituted derivatives. For this reason the peak areas of the methyl glucosides of 2,3,4-tri-O-methyl-D-glucopyranose and 2,3,6-tri-O-methyl-D-glucopyranose were divided by 0,85 and 0,75 respectively, thereby obtaining molar proportions [134]. The final results, calculated in relation to one mole of methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside, are shown in Table 6.

Comparison of the T-values obtained for the methyl glucosides of the methylated cane polysaccharide and those obtained for methylated starch and dextran (Fig. 11, 12, 13), provides proof of the presence in the cane polysaccharide of inter-chain linkages in (1 → 4) and (1 → 6) position, while other linkages are absent. These results were confirmed by enzymic hydrolysis of the cane polysaccharide, as discussed later.

Because it is almost impossible to maintain absolutely constant column conditions in respect to temperature and carrier gas flow, it is not very satisfactory to determine coincidence of T-values in two separate analyses [151]. This is clearly illustrated by comparing Figs. 12 and 13. Although the instrument settings were not changed and the two analyses were carried out one directly after the other, the temperature of the column dropped by 3°C. For this

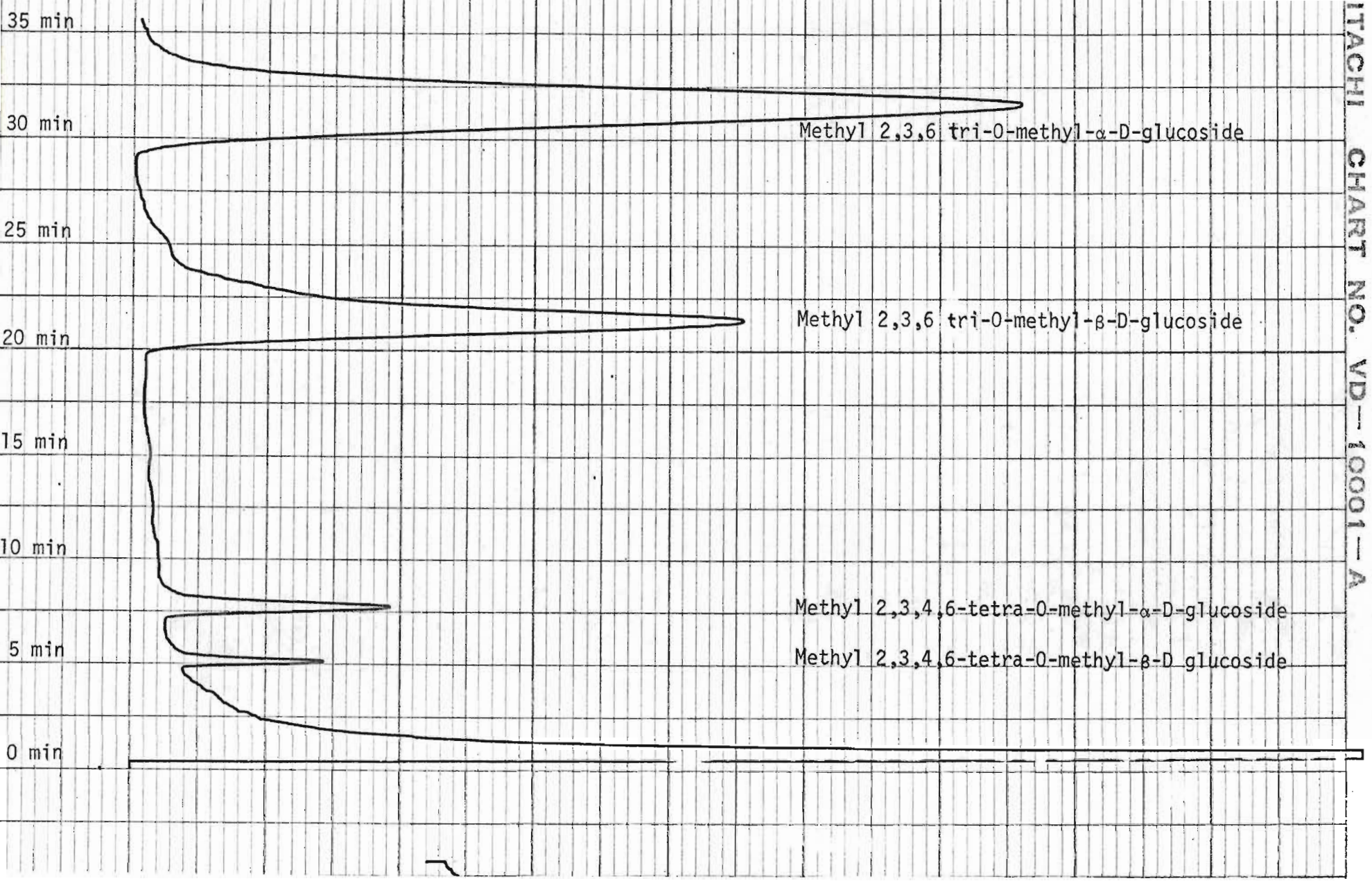


. 11 DEXTRAN.

Temperature: 142°C

Detector sensitivity:
5×10^{-10}A F.S.D.

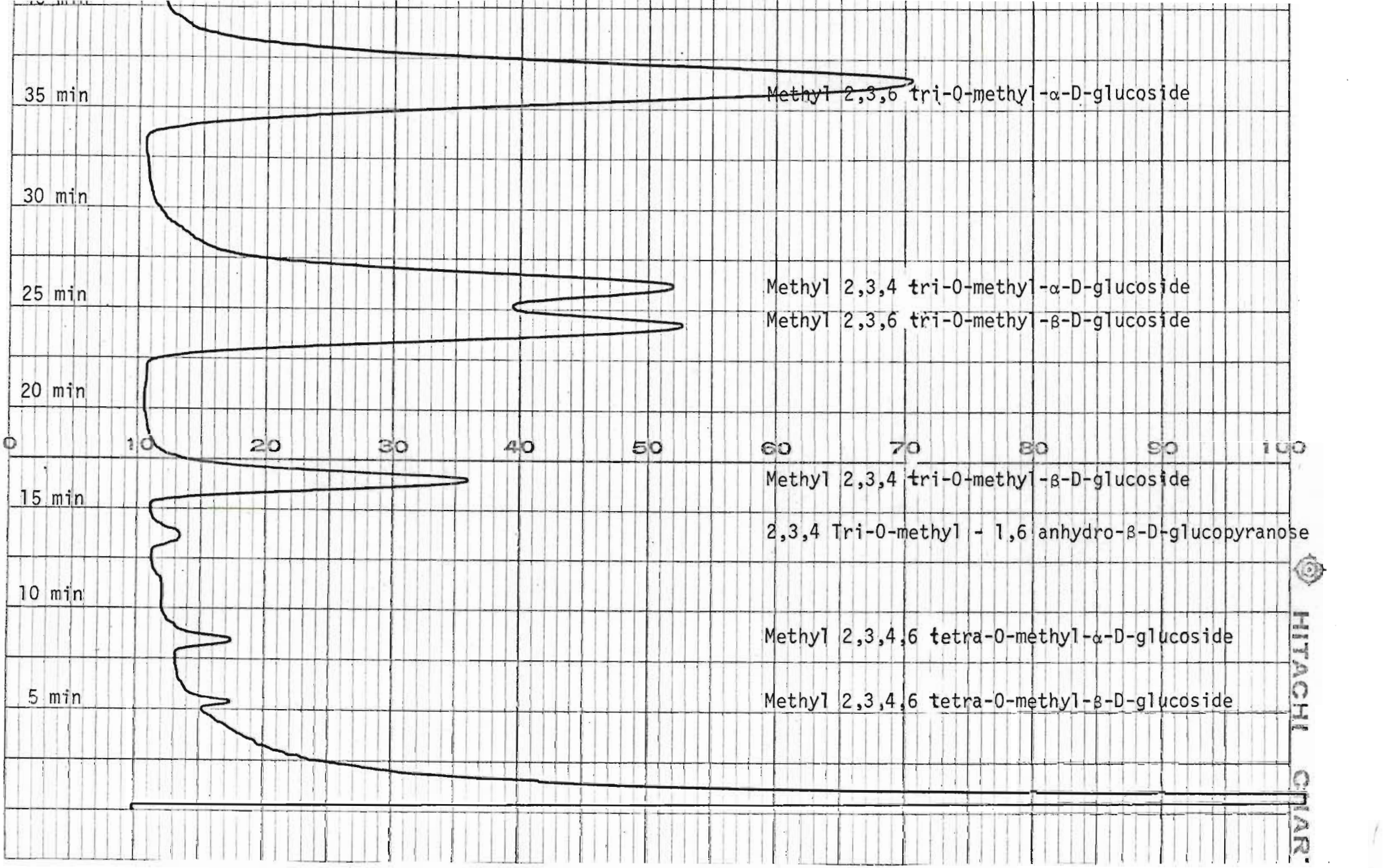
Column 1/8 inch 6 feet
Packing Di ethylene glycol succinate poly ester 5% on Chromosorb
Carrier Gas N_2 55 ml per min



12 STARCH

Temperature: 142° C
Detector sensitivity
5×10^{-10}A F.S.D.

Column 1/8 inch. 6 feet
Packing Di ethylene glycol succinate poly ester 5% on Chromosorb W
Carrier gas N_2 55 ml/min.



IG. 13 SARKARAN

Temperature: 139°C

Detector sensitivity:
5×10^{-10} A F.S.D.

Column 1/8 inch 6 feet
Packing Di ethylene glycol succinate polyester 5% on Chromosorb W Carrier Gas N_2
55 ml per min.

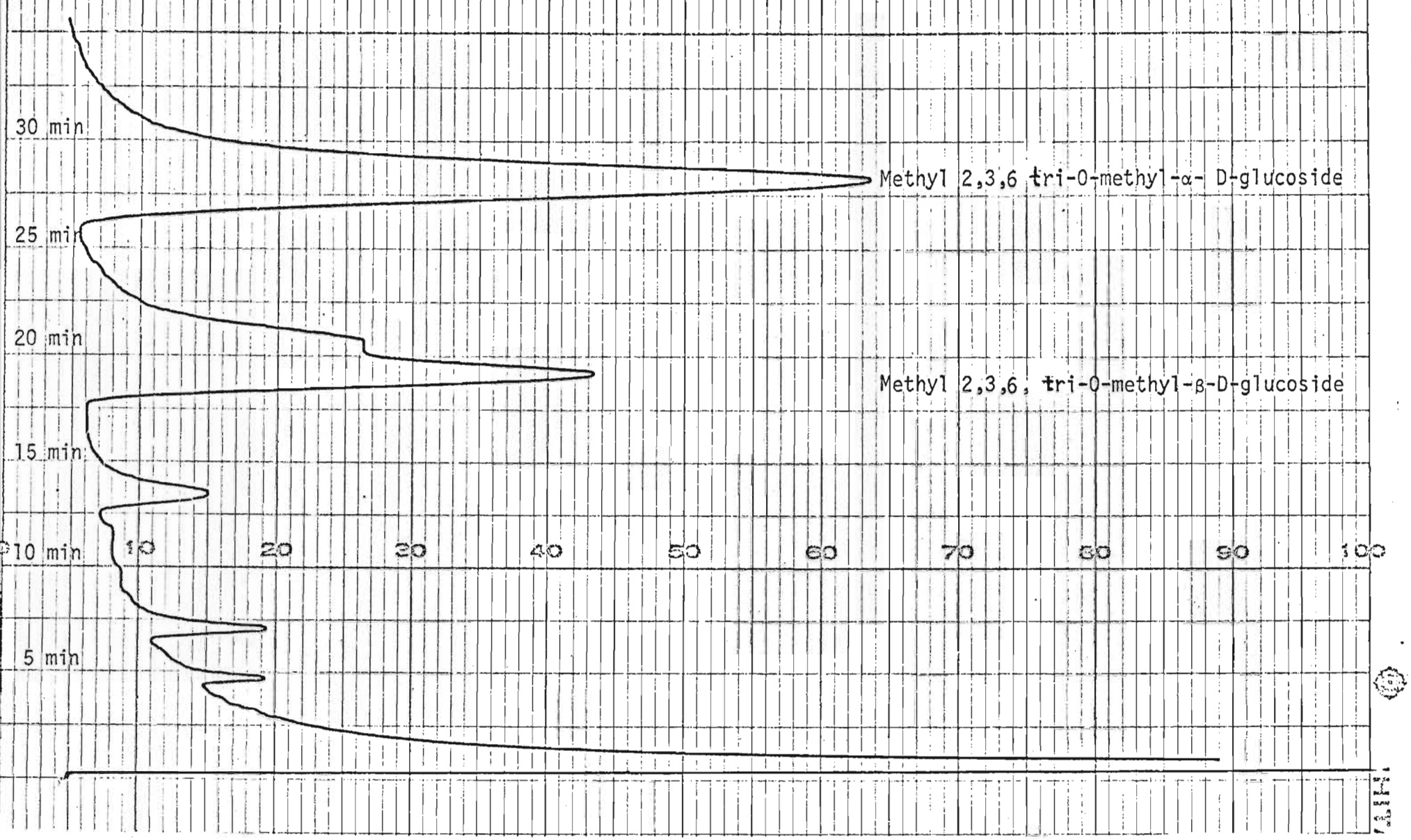


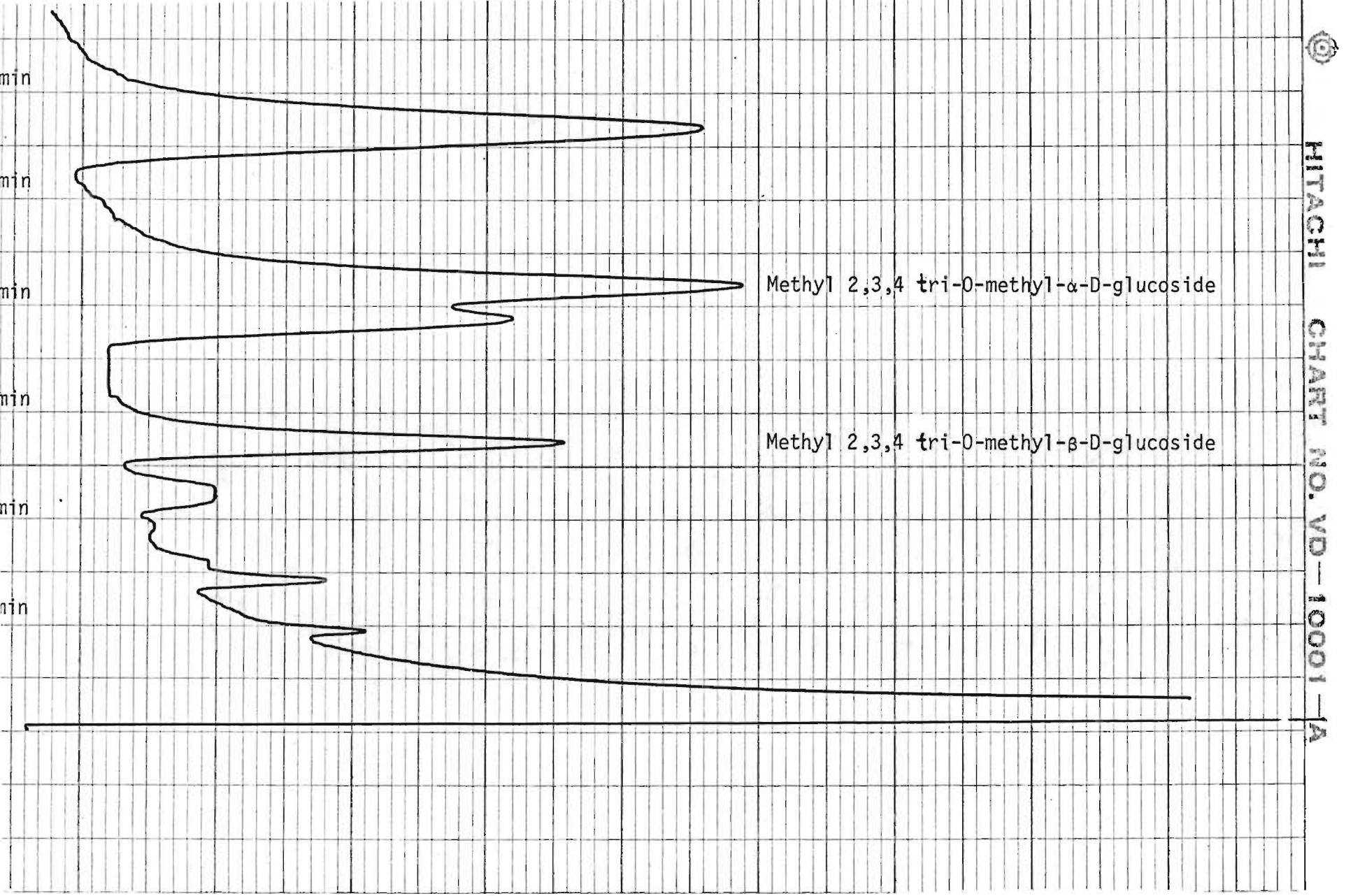
FIG. 13 [a]

SARKARAN
Mixed with
Starch

Temperature: 137°C

Detector sensitivity:
5×10^{-10} A F.S.D.

Column 1/8 inch 6 feet
Packing Di ethylene glycol succinate poly-ester 5% on Chromosorb W Carrier Gas N_2 55 ml per min.



13 b

SARKARAN
Mixed with
dextran

Temperature: 137°C
Detector sensitivity:
5×10^{-10} A F.S.D.

Column 1/8 inch 6 feet
Packing Di ethylene glycol succinate poly ester
5% on Chromosorb W Carrier Gas N_2 55 ml per min.

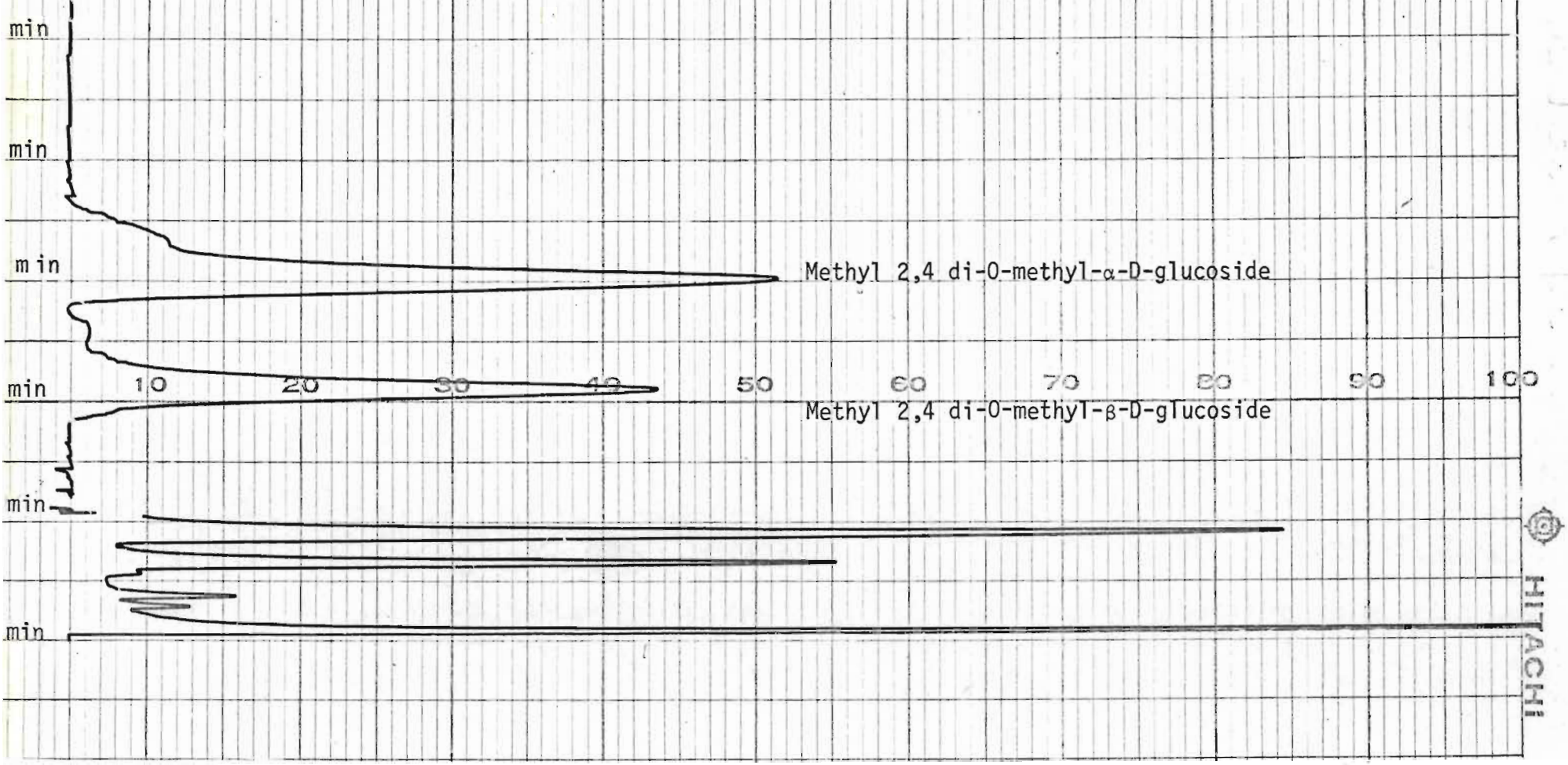


FIG. 14 DEXTRAN Temperature : 180°C

Detector sensitivity :

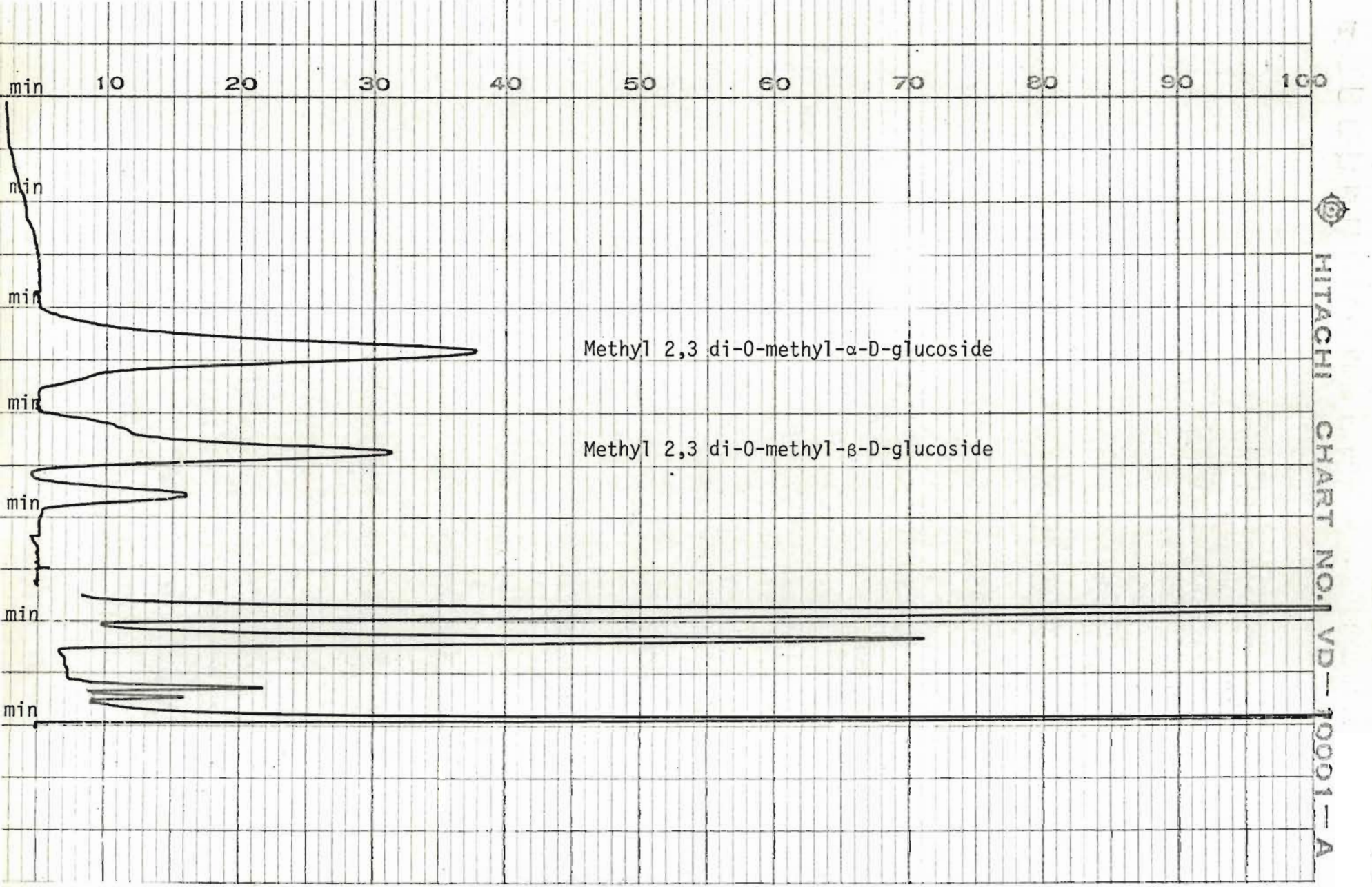
0-6 min 5×10^{-9} A F.S.D.

6-30 min 5×10^{-10} A F.S.D.

Column $\frac{1}{8}$ inch 6 feet

Packing Di-ethylene glycol succinate poly ester 5% on Chromosorb W

Carrier Gas N_2 55 ml per min



90

i. 15 STARCH

Temperature: 180°C

Detector sensitivity:

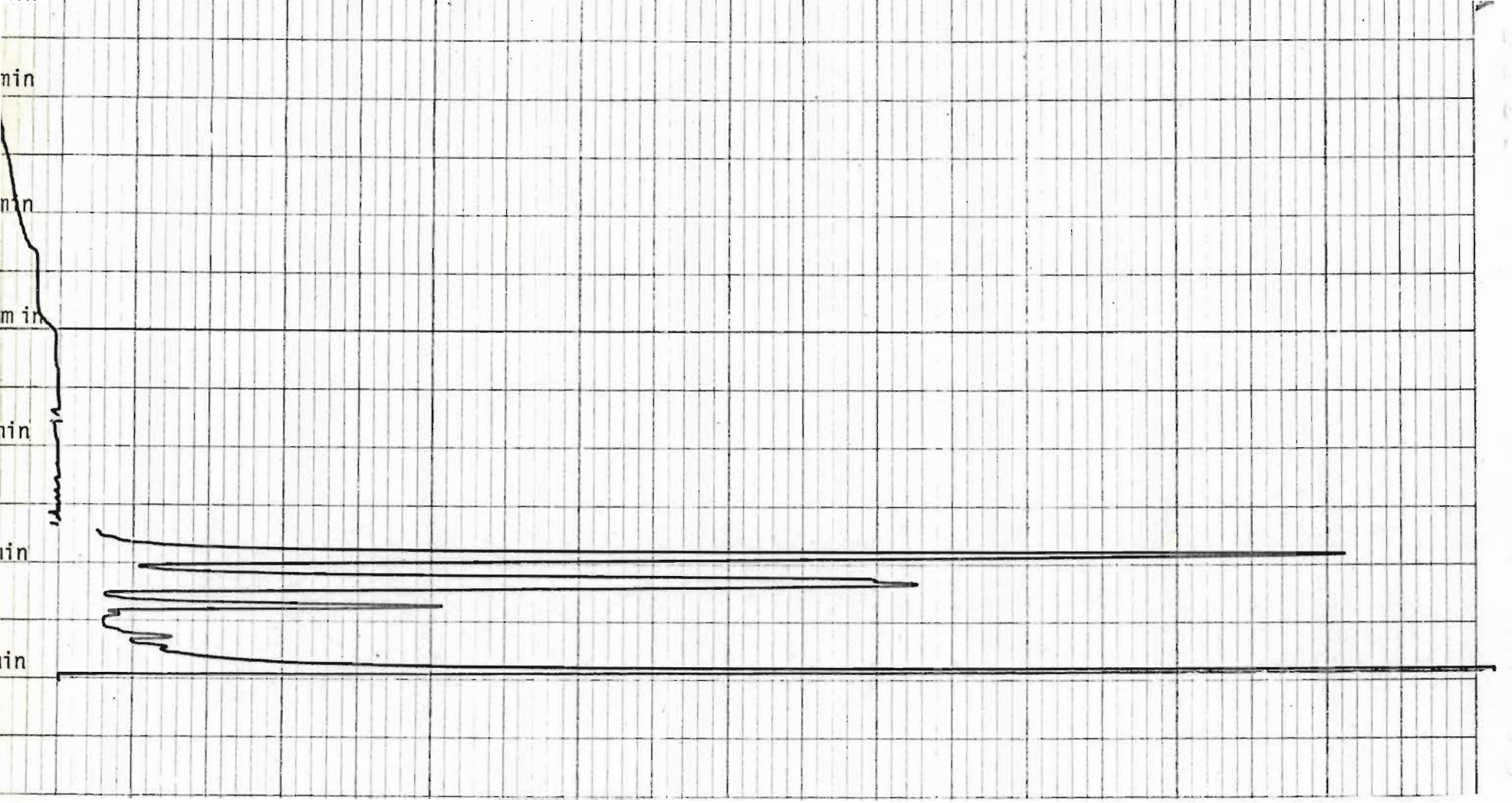
0-6 min 5×10^{-9} A F.S.D.

6 - 30 min 5×10^{-10} A F.S.D.

Column 1/8 inch 6 feet

Packing Di ethylene glycol succinate poly ester 5% on Chromosorb

Carrier gas N_2 55 ml per min



16 SARKARAN

Temperature: 180°C

Detector sensitivity:

0-6 min 5×10^{-9} A F.S.D.
6-30 min 5×10^{-10} A F.S.D.

Column 1/8 inch 6 feet

Packing Di ethylene glycol succinate poly ester 5% on Chromosorb

Carrier Gas N_2 55 ml per min

reason the methyl 2,3,6 tri-O-methyl-α-D-glucoside in Fig. 12 emerged after 32 minutes, while the same compound in Fig. 13 emerged 37 minutes after the injection into the gas chromatograph.

To overcome this difficulty, the T-values of the methyl glucosides in the methanolysate of the cane polysaccharide were determined by peak enhancement, by mixing the methanolysate with a standard. The methanolysates of starch and dextran, in which the methyl glucosides are known, were used as standards. A chromatogram of a mixture of the cane polysaccharide and starch methanolysates is shown in Fig. 13[a]. Peak enhancement of the two methyl-2,3,6 tri-O-methyl-D-glucosides is evident. In Fig. 13[b], a mixture of cane polysaccharide and dextran methanolysates is shown. In this case there has been peak enhancement of the two methyl-2,3,4, tri-O-methyl-D-glucosides. These two figures show that there is complete coincidence in retention time between the standards and the analysis mixture.

Coincidence in retention time of an unknown component and a standard is not usually sufficient for the complete elimination of uncertainty and ambiguity. Further confirmation is required by infrared analysis or other physical measurement, following isolation of the fraction under investigation.

In this case, however, this confirmation is unnecessary, as only methyl glucosides can be present in the mixture. According to data from the literature [133], all possible methyl glucosides show different retention times on a column packing similar to that used for the present analysis. Literature data were available for Butane 1-4 diol succinate polyester as liquid phase in the column.

In addition, the identity of the linkages was further confirmed by enzymic analysis as discussed later.

In the second analysis at higher temperature, the di-O-methyl-D-glucosides were determined. To accommodate the alteration in the response to the ionisation detector, the peak areas were divided by 0,5 to obtain molar proportions. The quantities were again related to one mole of methyl-2,3,4,6 tetra-O-methyl-

D-glucoside [134] .

The results are shown in Figs. 14, 15 and 16. The quantity of di-O-methyl-D-glucosides in dextran methanolysate is given in Table 7. For the present investigation, however, the amounts of the di-O-methyl-D-glucosides in the methanolysate of methylated starch and dextran were not of importance. These methyl glucosides were only used to determine the T-values in the gas chromatogram, in order to be able to compare these values with those obtained for methylated cane polysaccharide.

In Figs. 14 and 15, it is shown that the dimethyl glucosides emerge between 10 and 20 minutes after the start of the analysis. The major branch points in the amylopectin fraction of starch are α (1 → 6) linkages and the dimethyl glucosides are the methyl 2,3 di-O-methyl-D-glucosides. In dextran the major branch points are α (1 → 4) linkages or in some dextrans α (1 → 3) linkages, which may constitute as much as 40 % [135] , and the dimethyl glucosides are either methyl 2,3 di-O-methyl-D-glucosides or methyl 2,4 di-O-methyl-D-glucosides, depending on the type of dextran.

The presence of disubstituted derivatives in a methanolysate of a fully methylated polysaccharide is a measure of the amount and type of branching. As discussed earlier, however, there is the possibility of under-methylation, which can also result in the formation of di-substituted methyl derivatives. This possibility is normally ruled out before undertaking the GLC analysis, by infrared analysis of the exhaustively methylated product.

In Fig. 16 the GLC analysis of the methanolysate of the exhaustively methylated cane polysaccharide is shown. It can be seen that no products emerge from the column between 10 and 20 minutes after the injection, proving the absence of dimethyl glucosides. In fact no product emerged from the column between five minutes after injection and 30 minutes after injection, when the analysis was discontinued.

Two conclusions were drawn from this result:

I The polysaccharide was completely methylated. As a result it was considered superfluous to confirm complete methylation by infrared analysis.

II The polysaccharide under investigation has an unbranched structure.

From the quantitative interpretation of the gas chromatogram obtained, the percentage of (1 → 4) linkages in the molecule of cane polysaccharide can be calculated. In Table 6, the molar quantities of methyl 2,3,6 tri-O-methyl-D-glucoside and methyl 2,3,4 tri-O-methyl-D-glucoside are listed as 100,5 ± 0,5 and 49,0 ± 0,5 respectively. The percentage (1 → 4) linkages can therefore be calculated as

$$\frac{100,5}{149,5} \times 100 = 67,5\ \%$$

ignoring the end group. This value agrees well with the figure of 68 %, previously determined by periodate oxidation (page 78).

Because the polysaccharide is unbranched, the molecular weight can be calculated from the data in Table 6, assuming that no degradation has taken place during the methylation procedure. As there is one end group per 150 glucose units, the molecular weight is

$$150 \times 160 = 24\ 000$$

This end group analysis determines the number average molecular weight $\bar{M}_n$. This molecular weight was determined by osmotic pressure measurement 6.1.6.5 (page64) as being 51 500. The lower value obtained by end group analysis indicates that some degradation of the polysaccharide has taken place during the various methylation steps. Another possibility that there is some

Table 5

T values of methyl glucosides obtained by analysing methanolysates of methylated starch and dextran		
Values are relative to Methyl-2,3,4,6-tetra-O-methyl-β-D-glucopyranoside		
	β	α
Methyl-2,3,4,6-tetra-O-methyl-D-glucoside	1,00	1,53
Methyl-2,3,4-tri-O-methyl-D-glucoside	2,95	4,65
Methyl 2,3,6-tri-O-methyl-D-glucoside	4,17	6,10

Table 6

Molar quantities of tetra- and tri-methyl glucosides in a methanolysate of exhaustively methylated cane polysaccharide.	
Values are relative to Methyl-2,3,4,6-tetra-O-methyl- D-glucopyranoside	
	Moles
Methyl-2,3,4,6 tetra-O-methyl-D-glucosides	1,0
Methyl-2,3,4-tri-O-methyl-D-glucosides	49,0
Methyl-2,3,6-tri-O-methyl-D-glucosides	100,5

Table 7

Molar quantities of dimethyl glucosides in a methanolysate of exhaustively methylated dextran and cane polysaccharide		
Values are relative to Methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside		
	2,3,4,6 G	Total Me_2 G
dextran	1,0	5,9
cane polysaccharide	1,0	nil

Table 8

Infrared frequencies of amorphous cyclohexamylose, cycloheptamylose, amylodextrin and amylose in the range 700 - 1 000 cm^{-1} [140].

cyclo hexamylose	cyclo heptamylose	amylose	amylo-dextrin	Tentative assignment
948	943	928	928	Ring vibration
934	936	-	-	Ring vibration
856	856	852	852	C_1 group vibration
842	845	-	-	C_1 group vibration
750	753	759	759	Ring breath vibration
703	703	705	706	Ring vibration

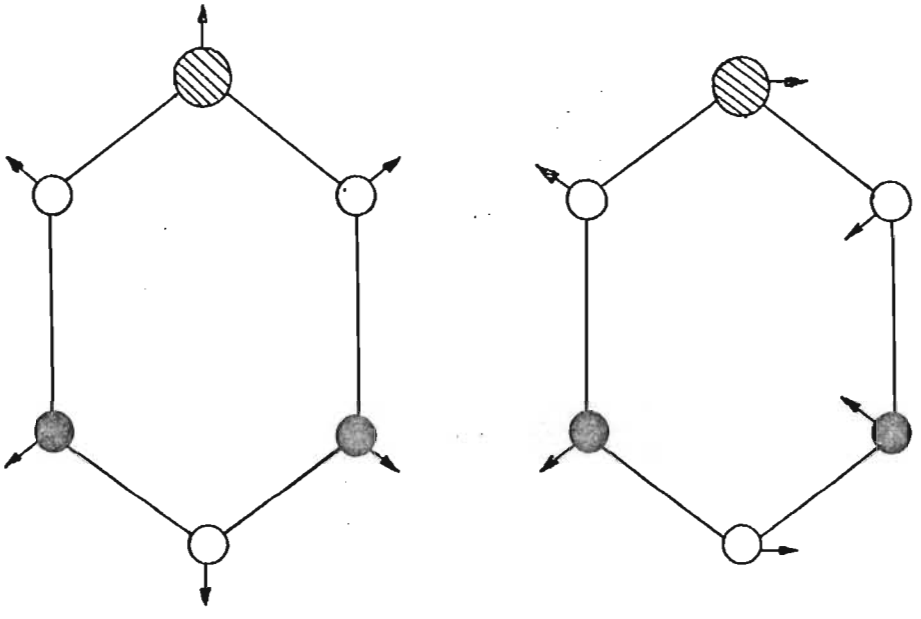
association of polysaccharide molecules [195] .

6.1.10 Infrared Spectroscopic Analysis

Infrared absorption of the glucose pyranoid ring vibrations are in the region of 700 - 950 cm^{-1}.

An absorption at 917 ± 13 cm^{-1} has been said to be due to anti-symmetrical C-O-C stretching [138], although some later investigators have assigned this absorption to symmetrical C-O-C stretching.

Most workers in this field, however, assign the absorption at 770 ± 14 cm^{-} to symmetrical ring vibration. These two ring vibrations are illustrated below:



Symmetrical ring breathing

Anti-symmetrical ring bending

carbon above xy plane

carbon below xy plane

oxygen

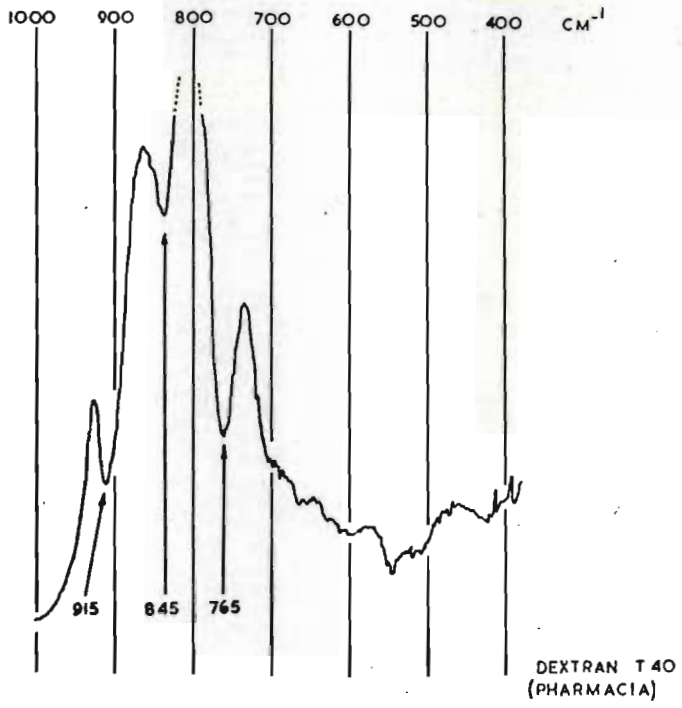
Arrows represent displacements of the nuclei from their equilibrium position.

The α anomeric absorption at 844 ± 8 cm^{-1} and the β-anomeric absorption at 891 ± 7 cm^{-1}, originally assigned to C-H deformations at C_1 [138], have more recently been ascribed to vibration of the whole grouping at C_1 [139].

According to the literature, a difference exists between the absorptions for the α (1 → 4) glucopyranose ring and the α (1 → 6) glucopyranose ring. For starch, which is mainly linked in the α (1 → 4) mode, the antisymmetrical ring vibration absorption is at 930 cm^{-1}, and the symmetrical ring vibration absorption at 758 cm^{-1}. For dextran, these absorptions are 917 cm^{-1} and 768 cm^{-1} respectively [138]. These shifts are small, however, and it is questionable whether these differences should be used to base a conclusion on the structure of a polysaccharide.

In addition to the absorptions already mentioned, the pictures of the infrared spectra determined for pullulan, cane polysaccharide, and starch (Figs. 17, 18 and 19) show an absorption at 700 cm^{-1}. This absorption has been reported previously for starch by Casu and Reggiani [140], who published the band assignments from available information as listed in Table 8. The same absorption peak is present in infrared spectra of starch published more recently by Dawoud and Majawar [197]. The absorption at 700 cm^{-1} for starch was tentatively assigned as a ring vibration. The spectra, which are shown in Fig. 19 exhibit this ring vibration to be present in all α glucans containing (1 → 4) linkages in large quantity. It was found, however, that the absorption peak at 700 cm^{-1} was absent from all the infrared spectra of dextrans determined in this investigation.

According to Spedding [152], this difference between the infrared spectra of polysaccharides containing α (1 → 4) glucosidic linkages and those of polysaccharides containing mainly α (1 → 6) linkages has not been published elsewhere. It is suggested that this difference in infrared spectra might be used as additional confirmation in the structural analysis of these polysaccharides.



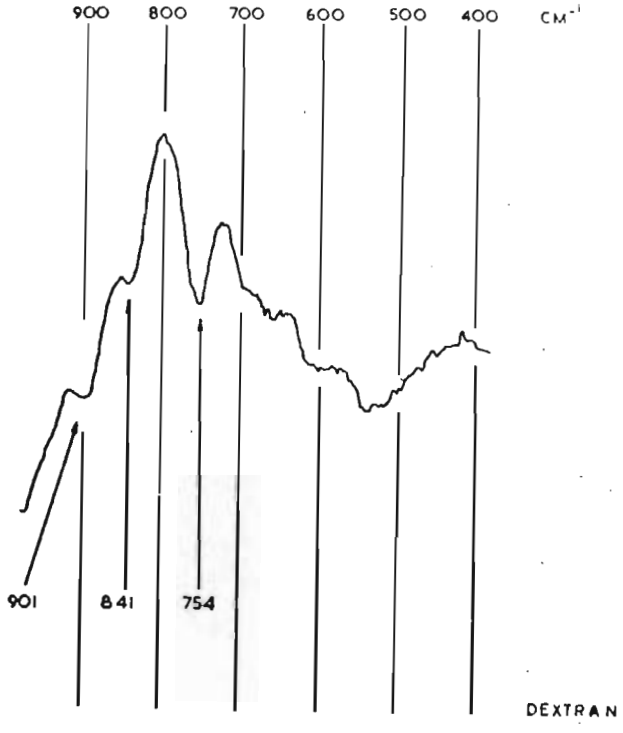


FIG. 17 INFRARED SPECTRA OF DEXTRAN.

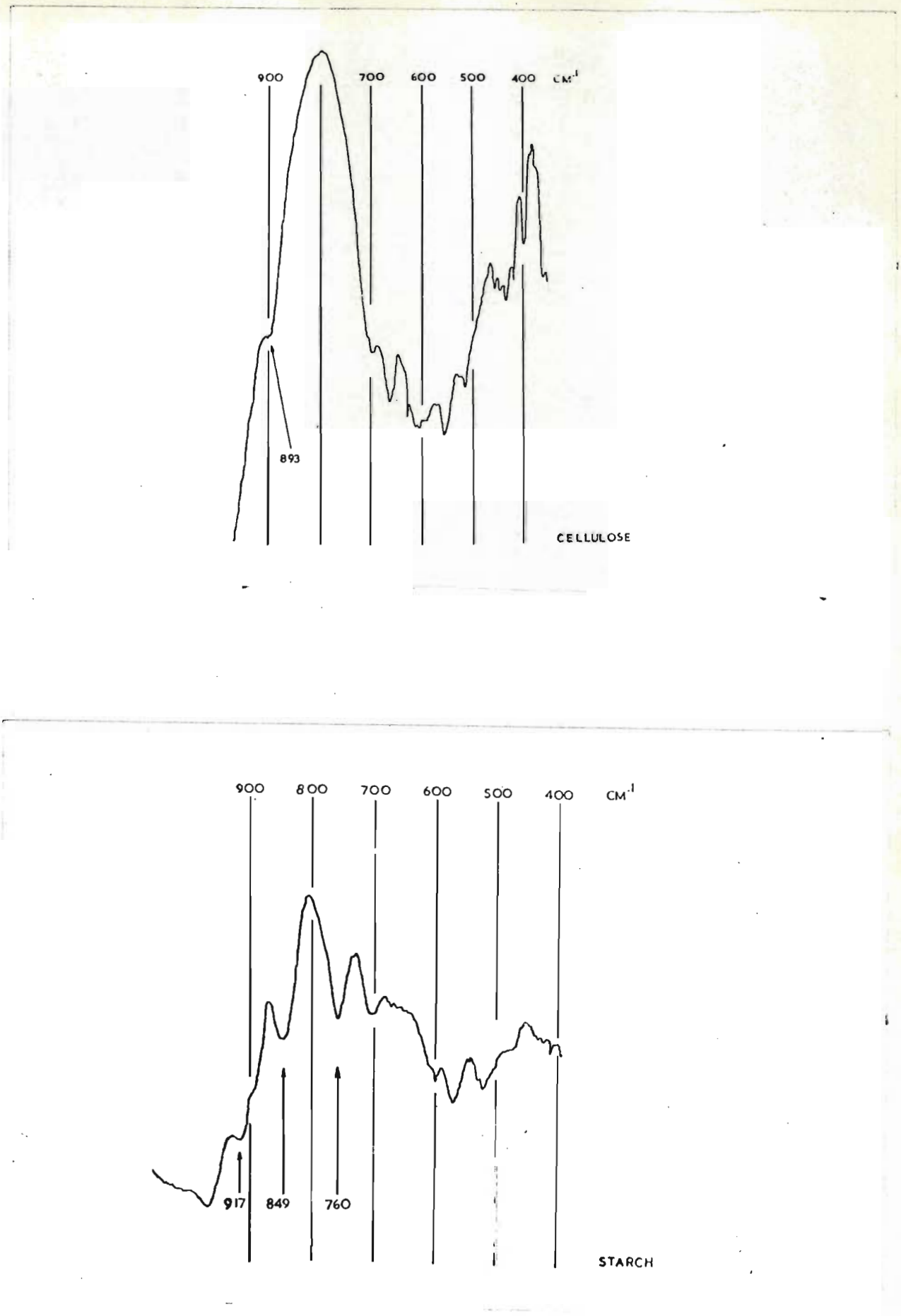
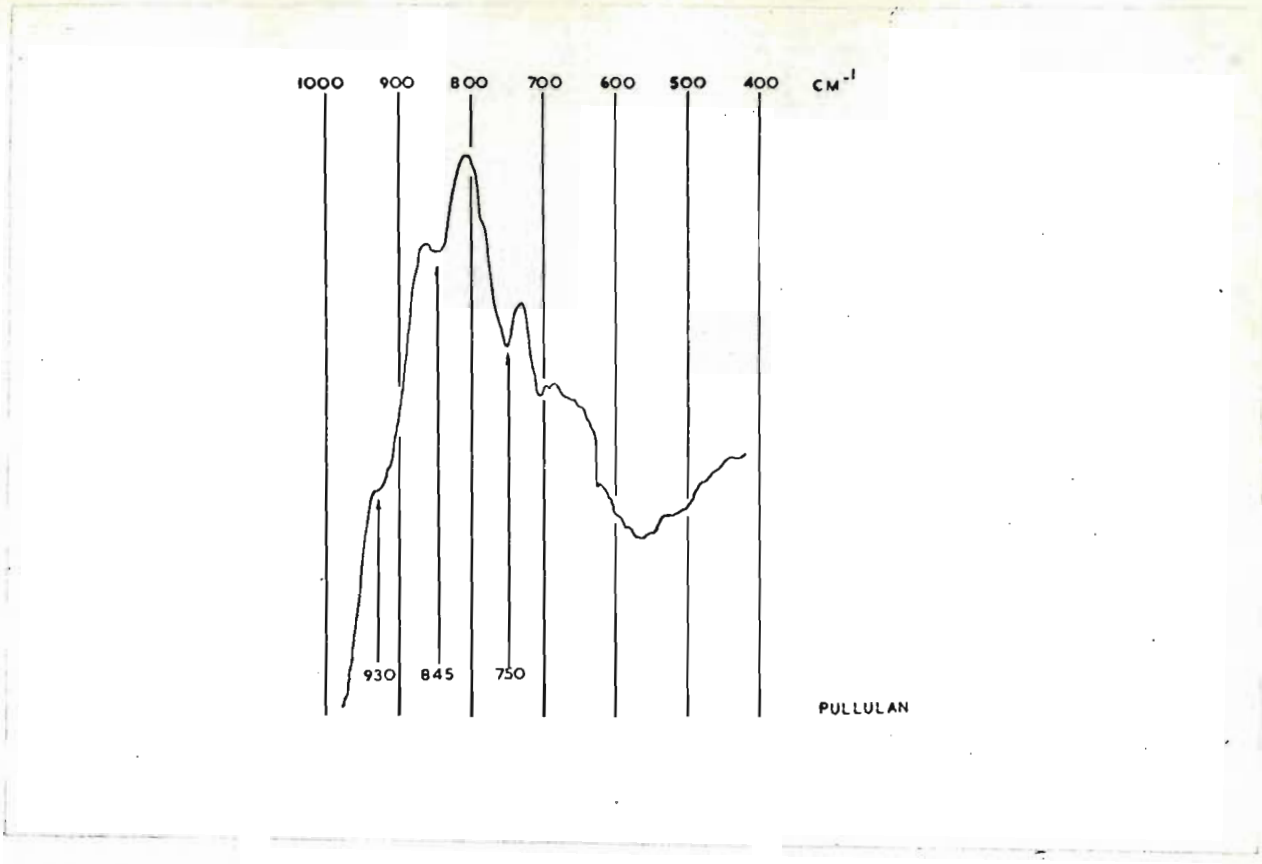


FIG. 18 INFRARED SPECTRA OF CELLULOSE AND STARCH.



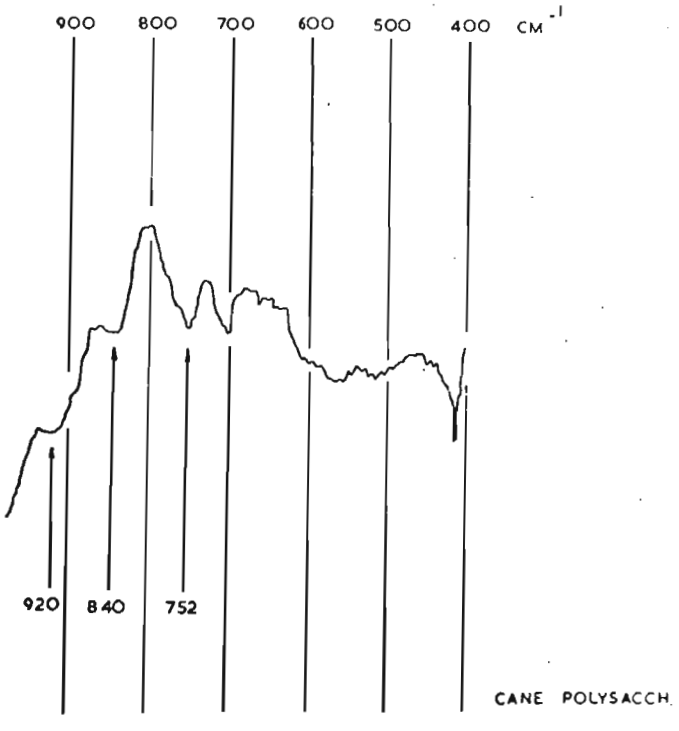


FIG. 19 INFRARED SPECTRA OF PULLULAN AND SARKARAN.

The difference in wave number of the absorption peaks of the α-(844 cm^{-1}) and the β-mode (891 cm^{-1}) of the linkage is sufficiently large to be conclusive. The infrared spectrum of cane polysaccharide shows an absorption peak at 840 cm^{-1} (Fig. 19). For comparison, a spectrum of cellulose was determined (Fig. 18), in which a shoulder appears at 893 cm^{-1}.

It is concluded that the linkages in cane polysaccharide are in the α-mode because:

(a) The infrared spectrum shows a peak at 840 cm^{-1}.
(b) The polysaccharide has a high dextrarotation (page 66).

6.1.11 Investigation of the structure of cane polysaccharide by means of enzymic hydrolysis

6.1.11.1 Application of enzymes for structural determinations

In recent years, methods involving enzymic hydrolysis and synthesis have become powerful tools for determining the structure of polysaccharides. These methods are used in conjunction with chemical techniques which, by themselves, have limitations.

It is, for example, difficult to obtain complete methylation of a highly branched polysaccharide, and equally difficult to avoid "over oxidation" of the reducing end group of a polysaccharide during reaction with periodate [148]. As enzymic methods are based on a different mechanism, they are not subject to the same disadvantages. In addition, more detailed information can be obtained with hydrolytic enzymes, because of their high specificity for the decomposition of a particular bond. Various enzymic methods have been mentioned in the earlier section: "Review of glucans" (see page 24).

The enzymic hydrolysis of a specific linkage can be followed by monitoring the enzyme digest through tracing the decrease in viscosity, change in optical rotation, or increase in reducing power, or by chromatography. Both chromatography and

measurement of reducing power were used during this investigation.

In studies of this nature it is essential that the enzyme preparation used be homogeneous and not a mixture of two or more different enzymes. If this is not the case, no valid conclusions can be drawn from the results of the hydrolysis.

It was decided to use enzymic analysis to confirm the structure of the polysaccharide isolated from stored cane. Chemical determination had shown that the polysaccharide is an unbranched glucan having α (1 → 4) and α (1 → 6) linkages in the ratio 67,5:32,5. This agrees very closely with the structure of pullulan (see page 37), as determined in 1961 by Bender and Wallenfels, using the enzyme pullulanase [88] . Because of the similarities between the structures of the two polysaccharides, it appeared likely that the cane polysaccharide would be attacked by pullulanase and it was decided to use this enzyme in an attempt to obtain confirmation of the structure.

For this purpose, pullulanase had to be prepared and isolated *). In order to be able to control the production and to test the strength of the isolated enzyme, it was also necessary to produce and isolate the substrate, the polysaccharide pullulan.

6.1.11.2 Production and isolation of pullulan

Two strains of *Aureobasidium pullulans* were tried for the production of pullulan. The culture received from the "Centraal Bureau voor Gist en Schimmel cultures" at Baarn, Holland, produced a small quantity of a mannose polymer and this was not further investigated. A second strain obtained from the "Royal Free Hospital School of Medicine", London, England, formed pullulan on a sucrose containing, Czapek Dox medium. The pullulan was produced by this organism in shake cultures and was precipitated from these shake cultures by ethanol. The crude pullulan which was purified by repeated solution in distilled water and precipitation by

* The enzyme is now commercially available.

ethanol, was finally obtained as a voluminous white powder. The purity of the pullulan was checked by acid hydrolysis and the only sugar present in the hydrolysate was glucose. This was proved by comparing the R_f value with that of pure glucose, using paper chromatography.

Enzymic hydrolysis with pullulanase (see page 107) resulted in maltotriose only, as proved by paper chromatography. The R_f value of the hexose polymer in the hydrolysate was compared with that of a series of malto-oligosaccharides prepared by partial acid hydrolysis of a sample of pure amylose. Such a hydrolysate contains only malto-oligosaccharides having an α (1 → 4) linkage. As amylose contains no branch points, isomalto-oligosaccharides with the α (1 → 6) linkage are absent. The amylose was prepared from potato starch, using the method of Schoch [141], by precipitation of the amylose-butanol complex from a hot solution of potato starch, followed by centrifugation.

For enzymic hydrolysis with pullulanase, it is essential that both the polysaccharide under investigation and the pullulanase preparation used should be completely free from amylase. If amylase is present, the maltotriose or any other malto-oligosaccharide containing α (1 → 4) linkages will be further hydrolysed into maltose, in which case no valid conclusions on the presence of α (1 → 6) linkages can be drawn.

The chromatograms published by Wallenfels [88] show hydrolysates obtained with pullulanase which contain not only maltotriose but also glucose and maltose. Later it became evident that the pullulanase used by Wallenfels contained small quantities of glucamylase. This glucamylase could be destroyed by heating the enzyme. This fortunately did not impair the pullulanase activity, as pullulanase is a fairly thermostable enzyme [149].

A picture of the chromatogram obtained in this work is shown in Fig. 20. The absence of maltose or glucose in the chromatogram of pullulan hydrolysate shows that, in this investigation, neither the pullulan nor the pullulanase contained any amylase. The results were compared with those obtained by the action of a sample of pure pullulanase, received from Wallenfels, on pullulan. In all cases the only hydrolysis product in the enzyme digest was maltotriose.

6.1.11.3 Production and isolation of pullulanase

Wallenfels identified the organism which was used in the preparation of pullulanase as *Aerobacter aerogenes* [88]. A type culture received from the "Laboratory for Technical Microbiology" at Delft, Holland failed to produce pullulanase. A strain obtained from the "Royal Free Hospital School of Medicine" at London did, however, produce the enzyme. This strain proved to be atypical in its acid formation in a dextrose-peptone medium.

Organisms of the coli-aerogenes group are normally classified according to the so-called Imvic test. This test determines indole formation, acid formation in a methyl red containing medium, the Voges-Proskauer reaction*, and the possibility of the organism using citrate as the only carbon source.

The result of the test for the "Delft" strain and the "London" strain are shown below:

	Indole	Methyl red	Voges-Proskauer	Citrate
A.aerogenes (Delft)	-	-	+	+
A.aerogenes (London)	-	+	+	+

* This reaction determines the organisms ability to form 1 acetyl 1 hydroxy ethane (acetyl methyl carbinol $CH_3CO.CHOH.CH_3$).

The "Delft" organism exhibited the reactions of a type culture of *A. aerogenes*. The "London" strain formed acid in a glucose containing medium. This identifies the strain as an intermediate between *Eschericia coli* and *Aerobacter aerogenes*. These intermediate forms have been isolated regularly by other investigators, although they are less common than the typical forms of the two micro organisms mentioned.

The enzyme was produced in extra-cellular form in a maltose-containing medium in a shake culture. The production was followed by testing the culture liquid for enzymic activity in a pullulan solution. This test was based on the formation of reducing sugar (maltotriose) from pullulan by the pullulanase. The reducing sugar was determined by the ferricyanide micro method of Hagedorn and Jensen [143]. Although this is a very old method (1923) used for the quantitative determination of reducing sugar in blood, it was found to be accurate and reproducible for all determinations on small amounts of reducing sugars. The most critical requirement is the pH of the reaction mixture. The method has an advantage over the more popular and more modern Somogyi method, or its modifications, in that it is not influenced by air oxidation [144]. The major disadvantage is a lower specificity, which precludes its use for complex mixtures.

According to the present system, the unit of pullulanase activity (U) is defined as the amount which will catalyse the transformation of 1 μ equivalent of the substrate or group attacked per minute under optimum conditions of pH and temperature [142].

Wallenfels defined the unit of pullulanase activity as the quantity of the enzyme which increases the reducing power of a pullulan solution by 1 mg maltose equivalent in 1 hour at 40°C [88].

Using these conditions for the enzymic hydrolysis, the liquid showed an activity of 2,5 U per 100 ml after 3 days of cultivation.

The enzyme was precipitated with acetone from the filtered culture liquid and further purified by repeated solution and precipitation. The final product obtained was a slightly coloured dry powder. It was subsequently found that the enzyme had better keeping qualities in impure form. For this reason the impure product was kept under refrigeration and further purified just before use. The pure preparation received from Wallenfels also showed a gradual decrease in activity during storage.

6.1.11.4 Action of various enzymes on cane polysaccharide and pullulan

The rate of decomposition of solutions of cane polysaccharide (2 %) and pullulan (2 %) by the two enzymes amylase and pullulanase, was investigated at the optimum pH and temperature for the enzyme used.

The reaction was followed by determining the increase in reducing power of the enzyme-polysaccharide mixture at various time intervals. This reducing power was expressed as mg maltose. As the reducing power of maltotriose is approximately three quarters that of maltose, the maltotriose content in the enzyme digest was about 33 percent higher than the maltose equivalent values.

Figs. 21 and 22 show that neither pullulan nor the cane polysaccharide are attacked by malt amylase or bacterial α-amylase, while both polysaccharides are attacked in the same way by pullulanase. The final maltose equivalent value obtained was 12, which corresponds to about 16 mg of maltotriose. As the total amount of polysaccharide added in each experiment was 20 mg (1 ml 2 % solution), it can be seen that, in both cases, 80 % of the polysaccharide had been hydrolysed at the end of the experiment.

In a similar experiment, using dextran, no increase in reducing power was found after 25 hours of incubation with pullulanase. This confirmed the reports in the literature that pullulanase has no activity on this polysaccharide.

After removal on the enzyme by precipitation with ethanol*, the hydrolysate obtained by the action of pullulanase on the cane polysaccharide was deionised, using a mixed bed ion exchanger, and concentrated. Using the paper chromatographic method previously used for the pullulan hydrolysate, the concentrate was compared with a series of α (1 → 4) malto-oligo-saccharides obtained from partially hydrolysed amylose.

The combined paper chromatogram is shown in Fig. 20. It is evident from the results that, while pullulan is a maltotriose polymer with the units linked in α (1 → 6) position, the cane polysaccharide is predominantly a maltotriose-maltotetraose polymer, with the individual units linked in α (1 → 6) position.

In addition to maltotriose and maltotetraose in the hydrolysate, other oligosaccharides are present in small quantities. The ratio of these oligosaccharides was determined by quantitative paper chromatography. This quantitative determination was carried out by a modification of the method published by Whistler and Hickson [120] . These authors found that the phenol-sulphuric acid and anthrone-sulphuric acid methods were not reproducible, although they are frequently recommended for this type of analysis [144] .

* Ethanol was preferred to acetone in this case as dilute ethanol is a better solvent for oligosaccharides.

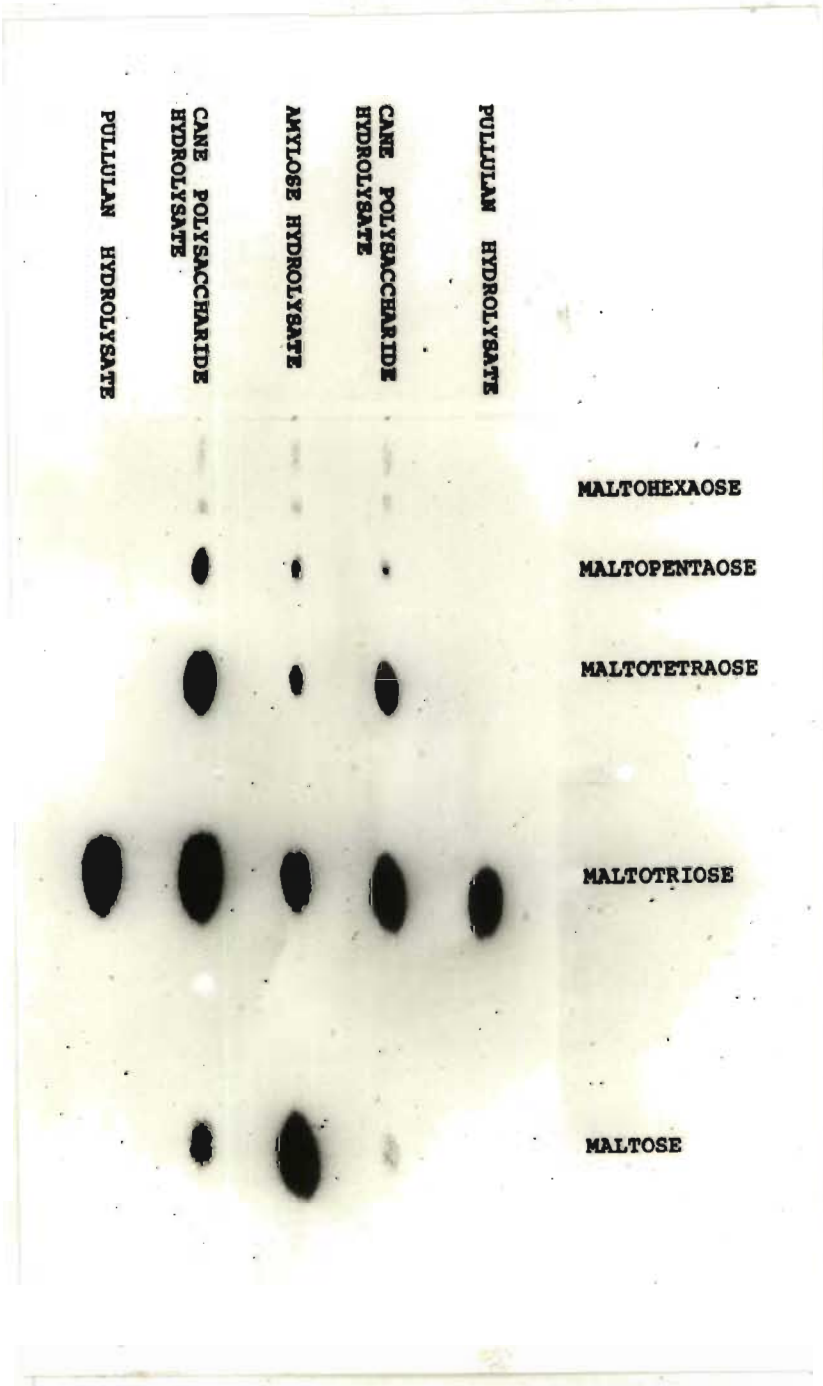


FIG. 20 PAPER CHROMATOGRAM OF AN ENZYMIC HYDROLYSATE OF SARKARAN PULLULAN AND A PARTIAL ACID HYDROLYSATE OF AMYLOSE

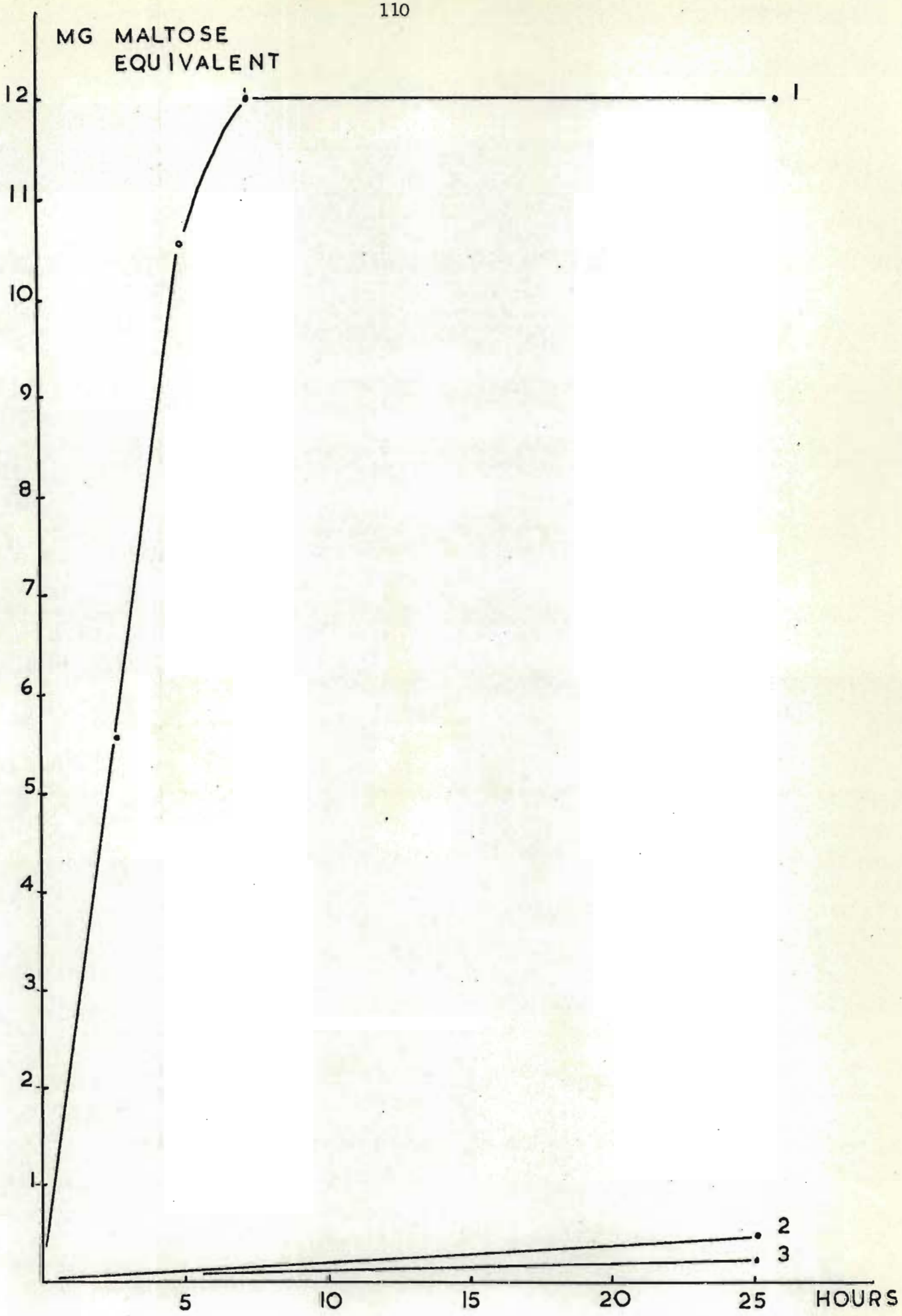


FIG. 21 ACTION OF ENZYMES ON PULLULAN 1 Pullulanase
2 Malt amylase

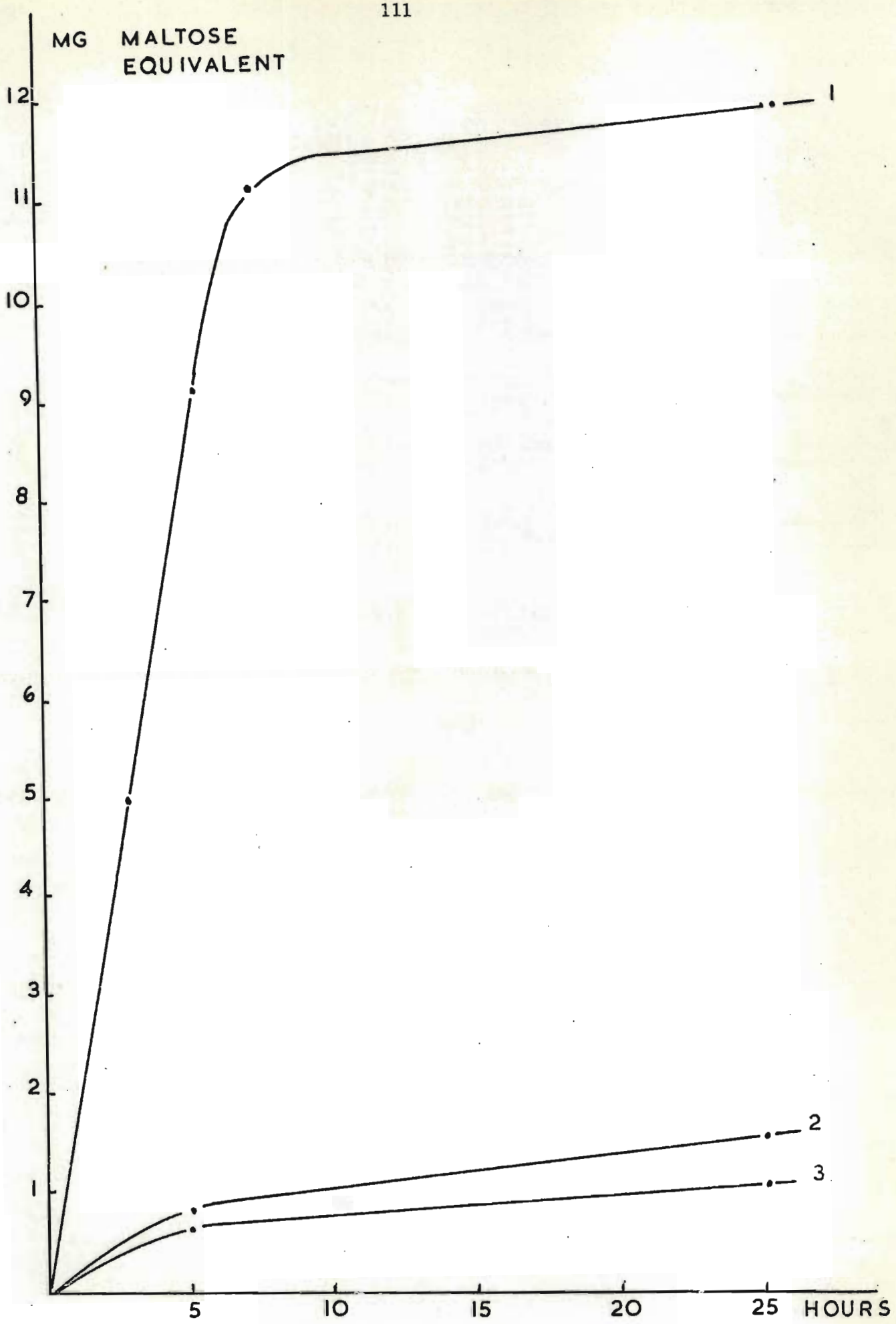


FIG. 22 ACTION OF ENZYMES ON CANE POLYSACCHARIDE (SARKARAN)

1 Pullulanase
2 Malt amylase
3

As an alternative, Whistler and Hickson used the ferricyanide method, after eluting the oligosaccharides from the paper and hydrolysing them in 0,01 N hydrochloric acid.

This method was tried, but it was found that with defined quantities of maltose, incomplete hydrolysis took place and only 75 % of the added maltose was determined in the subsequent titration as glucose. Increasing the acid concentration to 2 N resulted in complete hydrolysis. At this acid concentration, however, paper fibres, which were eluted with the oligosaccharide, were found to interfere. As the fibres are hydrolysed by 2 N acid to reducing sugars, the recovery of a defined amount of maltose was found to be too high. The problem was overcome by removing the fibres by filtration through a membrane filter, prior to acid hydrolysis. With this modification no interference occurred and recoveries of 98 % were obtained.

The quantities of oligosaccharides present in the cane polysaccharide hydrolysate are shown in Table 9. From these quantities, the ratio of α (1 → 4) linkages to the total number of linkages can again be calculated. The results can be summarised as follows:

Method of determination	α (1 → 4) links as percentage of total links.
Periodate oxidation	68
Methylation followed by G.L.C. of methanolysate	67,5
Oligosaccharides in a pullulanase hydrolysate	73

Table 9

Percentage of various oligosaccharides in a pullulanase hydrolysate of cane polysaccharide	
Maltose	5,1
Maltotriose	49,8
Maltotetraose	36,7
Maltopentaose	6,9
Maltohexaose	1,5

Table 10

Comparison of oligosaccharide content of pullulanase hydrolysates from chromatographically fractionated cane polysaccharide. Fraction 45 - 63 corresponds to the ascending portion, and Fraction 90 - 120 to the descending portion of the peak obtained by separating the polysaccharide by gel chromatography, using ion agar (see Fig. 4).

	fraction 45 - 63	fraction 90 - 120
Maltose	4,3	3,2
Maltotriose	50,1	49,3
Maltotetraose	38,4	37,1
Maltopentaose	5,6	7,2
Maltohexaose	1,6	3,0

Considering the limits of accuracy of the analytical methods used, there is reasonable agreement between the results obtained by the three determinations. The close agreement between the first two results is probably coincidental, as the limits of accuracy of both these methods are no better than five percent.

It is only in the case of a polysaccharide like pullulan, where only one oligosaccharide is obtained in the enzymic hydrolysate, that it is possible to calculate the ratio of the two linkages exactly. This is because the value is not dependent on an analytical determination of the quantity of oligosaccharide. In the cane polysaccharide hydrolysate, however, two different oligosaccharides are present as major constituents, together with small amounts of other oligosaccharides. The accuracy of the final result will therefore be dependent on the accuracy of each separate determination.

6.1.11.5 Homogeneity of the cane polysaccharide

The possibility that the material obtained from stored cane could be a mixture of two or more polysaccharides, has been mentioned previously in the discussions on gel chromatography and the fractional precipitation of the polysaccharide with ethanol.* Although only one symmetrical peak was obtained during these experiments, a mixture of polysaccharides might have escaped detection because of insufficient resolution of the gel column. Assuming that this was the case, it would be unlikely that the polysaccharides corresponding to the first half and the second half of the peak, would show identical hydrolysis products after treatment with pullulanase.

The fractions 45 - 63 from the chromatographic separation shown in Fig. 4, were collected and combined. After concentration, they were hydrolysed by pullulanase. The hydrolysis products were determined by quantitative paper chromatography.

* Section 6.1.5.1 and 6.1.5.2.

The same procedure was followed for the fractions corresponding to the descending part of the peak (90 - 120). The results are shown in Table 10. From these results it is evident that the structure of the polysaccharide is uniform over the whole peak area. It is concluded that the polysaccharide isolated from stale cane is homogeneous, and not a mixture of two or more different polysaccharides.

6.1.12 Conditions for the formation of the polysaccharide

6.1.12.1 Influence of moisture

Pieces of sugar cane stalk (N:Co 310) were kept for 3 weeks at 100 % relative humidity. After this period the juice was expressed and the total polysaccharide content was determined. The results were compared with the amount of polysaccharide obtained from pieces stored under normal atmospheric conditions (70-80 % R.H.). In a third experiment pieces of sugar cane were kept in an air stream, saturated with moisture.

The combined results, together with the polysaccharide content of juice from the fresh cane, are listed in Table 11.

These results show that little increase in polysaccharide content occurs in a moist atmosphere. Drying out of the pieces of cane appears to be essential for the formation of the polysaccharide. Aeration makes no difference to the final result.

It is difficult to provide a satisfactory explanation for this phenomenon. In general, provided sufficient air is available in the case of aerobic organisms, bacterial action will be promoted rather than restricted by the presence of moisture. On the other hand, drying out of sugar cane can result in the formation of lengthwise cracks in the rind, which would make the cane more susceptible to bacterial infection from the outside.

Table 11

The influence of moisture on the polysaccharide formation in stored sugar cane. (N:Co 310)	
	Total soluble polysaccharide expressed as percentage of the dry solids in cane juice
original cane	0,20
after 21 days at 100 % R.H.	0,50
after 21 days at 100 % R.H. with aeration	0,50
after 21 days in normal atmosphere	2,63

Table 12

Polysaccharide formation in sugar cane impregnated with penicillin G	
	Total soluble polysaccharide expressed as percentage of the dry solids in cane juice
original cane	0,32
cane impregnated with penicillin (20 Units/ml) after 18 days storage	2,94
control test impregnated with water; after 18 days storage	2,68

6.1.12.2 Formation of the polysaccharide under aseptic conditions

An attempt was made to investigate the formation of polysaccharide in sterilised, stored sugar cane. The only sure way to sterilise pieces of sugar cane would involve heating with steam under pressure. This treatment would, however, denaturate all proteins, including enzymes, present in the sugar cane and for this reason another approach was tried.

Sugar cane was cleaned by scrubbing with soap, after which it was dipped in mercuric chloride and washed with sterilised water. This cane was subsequently stored under sterile conditions. After the storage period, the treated cane showed the same increase in polysaccharide content as was shown by the untreated cane used as control. Sterilisation of pieces of sugar cane by this method will never be entirely complete, however. It is of interest to note that investigations have shown that healthy and undamaged sugar beet contains micro organisms in the intracellular spaces [145]. These would probably be unaffected by a sterilisation procedure similar to that described above.

In a second experiment, an attempt was made to inhibit the growth of micro organisms by impregnating the pieces of cane with a solution of penicillin G.

It was first established that *Leuconostoc mesenteroides* was inhibited by penicillin G. This test was carried out using Petri dishes filled with juice tryptone agar. A bacteria-free zone, 12 mm in diameter was obtained around a filter paper disc containing 1,3 Units of penicillin G.

The polysaccharide content of juice extracted from cane which had been impregnated with penicillin G before storage, was compared with a control, in which the cane had been stored after impregnation with sterile water. The results which are shown in Table 12, show that the penicillin G had no effect on the formation of polysaccharide in the stored cane.

It was therefore concluded that *L. mesenteroides* is not responsible for polysaccharide formation in stored cane under the conditions prevalent in Natal.

6.1.12.3 Attempts to isolate a micro organism responsible for the polysaccharide formation in stored cane

Juice from deteriorated cane was plated out on Petri dishes filled with juice-tryptone agar. After incubation, the colonies which formed a polysaccharide on the agar were transferred into flasks containing Czapek Dox solution with 10 % sucrose. These flasks were incubated on an orbital shaking machine and the polysaccharide which was formed in the Czapek Dox-sucrose solution was isolated by precipitation with ethanol, as described previously for pullulan. A two percent solution of the isolated polysaccharide was subsequently incubated with pullulanase and the increase in reducing power of the enzyme digest measured. As there was no evidence of enzymic hydrolysis, it was concluded that the polysaccharide formed by the randomly isolated micro organisms was not affected by pullulanase and was therefore not the same as the polysaccharide formed in stored sugar cane.

As was seen in the previous section, the fact that the polysaccharide forms in stored cane in the presence of penicillin G precludes the possibility that *Leuconostoc* species are responsible for its formation. This conclusion is supported by the differences in the structures of cane polysaccharide and dextran, and by the absence of lactic acid in deteriorated cane, a subject which is treated in greater detail in a later section.

Using plate techniques, it was not possible to isolate an organism which produced *in vitro* a polysaccharide identical to that found in stored cane. It is likely therefore that the glucan formation during storage is a reaction caused by enzymes in the cane. In addition, if the polysaccharide is produced by micro organisms, it is difficult to explain why drying out of the cane is essential for the formation. It seems more probable that the polysaccharide formation is a reaction of the cane to a change in environment.

A similar, enzymic polysaccharide formation has been described in sugar beets by Devillers and Loilier [146]. In this case, the reaction takes place if the beet is damaged and stops after about one hour. The actual increase in polysaccharide content is small. These authors concluded that the formation of this polysaccharide represents an attempt by the beet to provide protection against invasion by micro organisms.

6.1.13 Conclusion

The polysaccharide which is formed during the storage of sugar cane, was isolated from the juice by precipitation with ethanol. The crude polysaccharide was purified by repeated reprecipitation from aqueous solutions, with ethanol.

Only one peak resulted from separation by gel chromatography. The homogeneous nature of the polysaccharide was confirmed by collecting fractions corresponding to the ascending and descending sections of this peak. Enzymic hydrolysis of these fractions, using pullulanase, resulted in identical oligosaccharides, while the ratio between the oligosaccharides formed was exactly the same for both fractions. It would have been virtually impossible to obtain this result if a mixture of more than one polysaccharide had been present.

Several methods were used to determine the molecular weight of the polysaccharide.

Using viscosity determinations a molecular weight of approximately 30 000 was obtained. Gel chromatography using a Sephadex G 200 column resulted in a $\overline{M}_w$ of 50 000.

Both methods however are non absolute measurements and the accuracy of results obtained is dependent on the polymers used for calibration. Essentially, both methods depend more on the size of the polymers than on their molecular weight and correct values for $\overline{M}_w$ can be expected only if the polymer under investigation and the polymers used for calibration are of identical structure.

The only polysaccharide available for comparison was dextran. The formulae used in the calculation for $\bar{M}_w$ from viscosity data are valid for low molecular dextran. The Sephadex column used for gel chromatography was calibrated with dextrans of a defined molecular weight.

Subsequent determination of the $\bar{M}_w$ by light scattering revealed a value for $\bar{M}_w$ of 260 000. In addition it was found that the radius of gyration R_G of the molecule was 200 - 240 Å.

From this value for R_G it was concluded that the molecules of the polysaccharide are random coils and this is consistent with the comparatively low viscosity of sarkaran solutions.

Osmotic pressure measurements showed that the number average molecular weight of the polysaccharide is 51 500.

The ratio $\bar{M}w/\bar{M}n$, approximately 5, indicates that the polysaccharide is polydisperse. This in agreement with $\bar{M}w/\bar{M}n$ ratios for other polysaccharides. All natural polysaccharides which have been described have been shown to be polydisperse.

The methanolysate of the fully methylated polysaccharide contained no di-substituted methyl glucosides and it was therefore concluded that the polysaccharide is unbranched. Analysis of the methanolysate by gas chromatography showed that one end group is present for every 150 glucose units. From these data it was calculated that the number average molecular weight of the polysaccharide, $\bar{M}_n$, is 24 000.

This value for $\bar{M}_n$ is lower than that found by osmotic pressure measurements. This indicated that either some degradation of the polysaccharide molecules has taken place during methylation or that there is a slight association of the polysaccharide molecules in aqueous solution. Such an association has been reported for dextran by Daker and Stacey [195].

Electrophoretic measurement revealed that the polysaccharide is uncharged. Acid hydrolysis resulted in a hydrolysate containing only glucose and the polysaccharide was therefore classified as a glucan.

Periodate oxidation indicated that the polysaccharide contains 32 % (1 → 6) linkages and 68 % either (1 → 4) or (1 → 2) linkages. Contrary to all previous publications, the polysaccharide formed in stored cane cannot be classified as a dextran, which is a predominantly α (1 → 6) linked glucan. This conclusion was confirmed by exhaustive methylation of the glucan, followed by methanolysis. Gas chromatographic analysis of the methanolysate showed that 32,5 % of the linkages are (1 → 6) and 67,5 % (1 → 4). The possible presence of (1 → 2) linkages was ruled out by this analysis.

The polysaccharide is dextrarotatory in aqueous solution, having a specific rotation similar in order of magnitude to starch and dextran. It was concluded that the linkages between the glucose units are of the α configuration and this was confirmed by infrared analysis, using a potassium·bromide disc.

In addition, it was established that the IR-spectra of those α-glucans containing a high percentage of α (1 → 4) linkages, exhibit an absorption peak at 700 cm^{-1}. This absorption peak was absent from the IR-spectra of all the samples of dextran which were analysed. It is suggested that the existence of an absorption peak at 700 cm^{-1} in the IR-spectrum of a glucan can be used to confirm the presence of α (1 → 4) linkages.

The conclusions gained from classical chemical analyses were confirmed and augmented by enzymic hydrolysis of the glucan. The enzyme used was pullulanase, which is specific for the hydrolysis of α (1 → 6) glucosidic linkages in the presence of α (1 → 4) glucosidic linkages. From the results of this enzymic analysis, the glucan was defined more precisely as being a maltotriose-maltotetraose

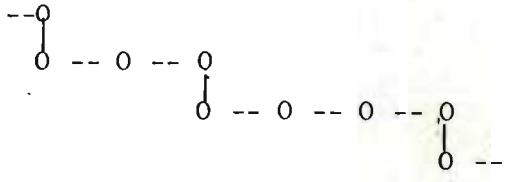
polymer in which the oligosaccharide units are linked in α (1 → 6) configuration. The ratio of maltotriose to maltotetraose was found to be 50:37.

From the results of chemical determinations by periodate oxidation and exhaustive methylation, it was difficult to differentiate between the isolated polysaccharide and pullulan. Both were found to be α-glucans while the ratios of α (1 → 4) to α (1 → 6) linkages, 32,5:67,5 in the case of the isolated polysaccharide and 33,3:66,6 in the case of pullulan, are too close to be used with confidence for purposes of identification. The difference between the two polysaccharides was revealed by enzymic analysis with pullulanase. Whereas the cane polysaccharide was found to be composed of both maltotriose and maltotetraose units, pullulan is exclusively a maltotriose polymer.

No description of an α-glucan, having the same structural characteristics as the polysaccharide isolated from stored cane, has been found in the literature. The name "sarkaran", which is derived from the Sanskrit word for sugar, has been suggested for the cane polysaccharide.

The sequence of maltotriose - maltotetraose units in sarkaran has not been determined. Several different structures are possible, one of these is illustrated in (I).

Structure A

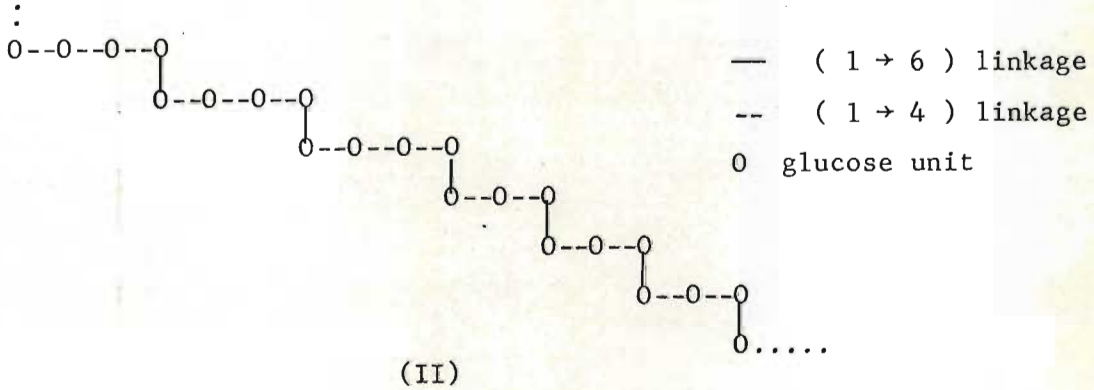


(I)

In this structure, regularly alternating maltotriose and maltotetraose units are linked in a α (1 → 6) position.

Another possible structure is illustrated in (II). In this case, a large number of maltotetraose units, linked in α (1 → 6) position, are followed by a chain of similarly linked maltotriose units.

Structure B



(II)

To complicate the problem still further, any variety of combinations of these two extremes is also possible. In addition, the end group configuration has not been completely defined.

The conventional procedure for studying the sequence of monosaccharide units in a polysaccharide molecule includes partial hydrolysis of the polysaccharide, followed by chromatographic separation of the oligosaccharides formed. In many cases the oligosaccharides can be identified, providing information from which valuable conclusions may be drawn.

From a consideration of the facts already known about the structure of sarkaran, the oligosaccharides of interest will be the various isomers of maltoheptaose and malto-octaose. These are the only oligosaccharides which are large enough to enable valid conclusions to be drawn on the sequence of maltotriose and maltotetraose in the parent polysaccharide.

Although chromatographic separation of oligosaccharides has been studied by several investigators, most of the published reports concern only the separation of oligosaccharides up to a degree of polymerisation of seven or eight. Whistler and Hickson showed that a mixture of malto-oligosaccharides can be separated using paper chromatography, and that the members of the iso-series have lower chromatographic mobility than those of the normal series [120]. This is illustrated in Fig. 23, in which the R_f values obtained by Whistler are plotted on a logarithmic scale against the degree of polymerisation (D.P.) of the oligosaccharides.

More recently, oligosaccharides have been separated by gas chromatography of the trimethyl silyl ethers. Beadle, using a gas chromatograph which was temperature programmed up to 410°C, successfully separated oligosaccharides up to D.P. 7 [153]. The advent of stationary phases for G.L.C. which are stable up to 500°C (a polycarborane-siloxane called "Dexil") [191] has provided the possibility that the D.P. limit can be extended upwards. This elevated temperature separation can only be used, however, if the silyl ethers of the higher D.P. oligosaccharides are stable up to 500°C.

Other techniques which may prove useful for separating malto-oligosaccharides include carbon-celite chromatography and gel chromatography.

After separation and isolation of the oligosaccharides under consideration, they can be identified by systematic decomposition, or by comparison with reference samples.

The decomposition can be carried out either by chemical or enzymic hydrolysis, followed by identification of the resulting mono- and oligosaccharides and, from this, the type of linkage in the originally isolated oligosaccharide.

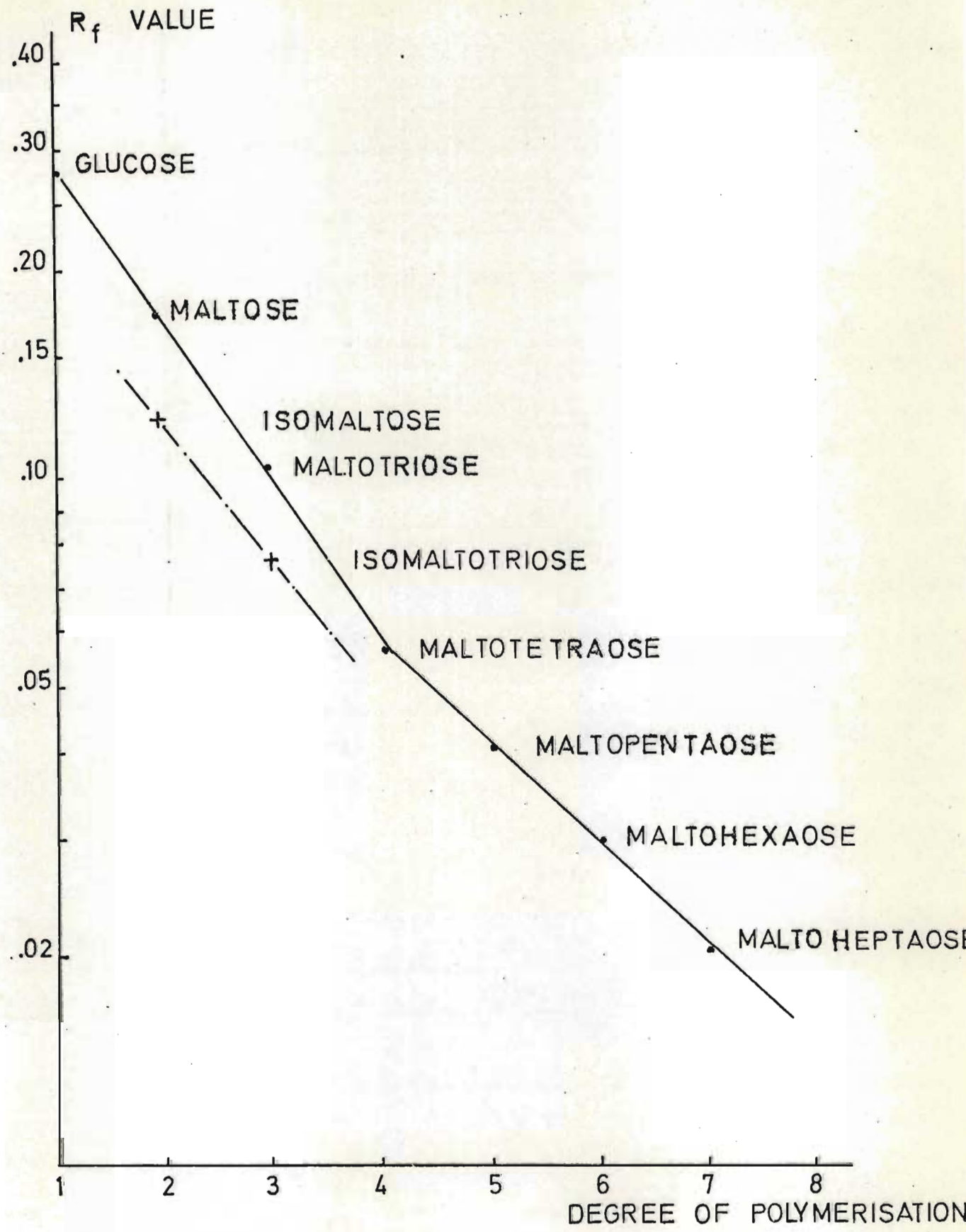


FIG. 23 PAPER CHROMATOGRAPHIC SEPARATION OF MALTO-OLIGOSACCHARIDES. R_f VALUE PLOTTED AGAINST D.P.

The need for reference samples of the various isomers of maltohepataose and malto-octaose represents a further obstacle to the use of conventional techniques. Samples of these oligosaccharides are not available commercially and are extremely difficult to synthesise. Because of this difficulty it is pertinent to consider using a different analytical technique, in which partial acid hydrolysis would be combined with enzymic hydrolysis. If pullulanase was used, the only references needed would be maltotriose and maltotetraose.

The partial acid treatment will hydrolyse sarkaran randomly and the hydrolysate will contain a mixture of oligosaccharides. It should be possible to use chromatography to isolate the group of maltononaose isomers from the hydrolysate.

A list of all the maltononaose isomers which could be present in a partial acid hydrolysate of sarkaran, is given in Tables 13 and 14. The list has been divided into two sections, containing the isomers which would result from hydrolysis of the two extreme structures, A and B, depicted in (I) and (II) (page 122,123) respectively. Because the acid hydrolysis is random, the relative amounts of the various isomers in the hydrolysate will be dictated by the original structure of the polysaccharide.*

Hydrolysis of the isolated maltononaose fraction, using pullulanase, would result in a hydrolysate containing oligosaccharides, some of which are limit dextrins. Wallenfels studied the compounds represented by (III), (IV) and (V) and showed that III is a substrate for pullulanase whereas IV and V are likely to be limit dextrins [90] .

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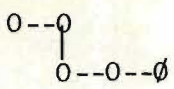
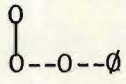
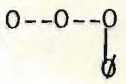
*Amylose is known to be hydrolysed faster by acids than is dextran and the $\alpha(1\rightarrow6)$ branch points in amylopectin are hydrolysed more slowly by acid than the $\alpha(1\rightarrow4)$ interchain linkages. Although little is known about rates of acid hydrolysis in polysaccharide chains having a mixture of $\alpha(1\rightarrow6)$ and $\alpha(1\rightarrow4)$ interchain linkages, it can be expected that the $\alpha(1\rightarrow6)$ linkages present in sarkaran will be hydrolysed at a slower rate than the $\alpha(1\rightarrow4)$ linkages. This will not change the predicted ratio of maltotriose to maltotetraose in the case of structure I (see bottom of table 13) but will probably result in a ratio slightly smaller than the predicted 7/4 in the case of structure II (bottom table 14)



(III)



(IV)



(V)

-- α (1 → 4) linkage

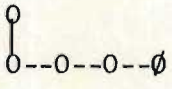
— α (1 → 6) linkage

O glucose unit

Ø reducing end group

It is reasonable to assume that compound (VI), will also be a limit dextrin, though this should be confirmed experimentally.

Analysis of the pullulanase hydrolysate for maltotriose and maltotetraose could be used to determine the composition of the maltononaose fraction. This information could be used, in turn, to interpret the structure of the parent polysaccharide.



(VI)

The major products which would be formed by pullulanase hydrolysis of each of the possible nonaose isomers are listed in Tables 13 and 14. The tables include the amounts of maltotriose and maltotetraose, together with the probable limit dextrins.

It is evident from these tables that, in the case of structure A ((I) Page 122), equal quantities of maltotriose and maltotetraose will be formed. With the nonaose isomers derived from structure B ((II) page 123), however, the ratio of maltotriose to maltotetraose will be 7:4. If the structure of sarkaran is a combination of the two extreme examples, the ratio of maltotriose to maltotetraose in the pullulanase digest of the nonaose fraction will be between 7:4 and 1:1

The oligosaccharides in the enzyme digest could be separated and isolated by paper chromatography. Quantitative determination of the eluted maltotriose and maltotetraose could then be used in an attempt to identify the structure of the original sarkaran. The attempt may be successful if the structure of sarkaran is close to one of the extreme examples. If the actual structure is a complex combination of these extremes, however, the errors inherent in the various separation stages and analytical determinations will prohibit the formation of a definite conclusion about the sequence of units in the polysaccharide.

Finally, exhaustive experimentation failed to locate a micro organism which could be responsible for the formation of sarkaran in sugar cane during storage. The fact that dry conditions promote the formation of sarkaran is a further indication that the polysaccharide is not produced by a micro organism.

It is suggested that sarkaran results from the action of one or more enzymes in the cane, and that these enzymes become active after harvesting because of a disturbance to the normal metabolic equilibria in the cane.

Table 13

Maltotriose and maltotetraose present in a pullulanase digest of isomeric nonaoses. Nonaose mixture, which would be obtained by partial acid hydrolysis of sarkaran.

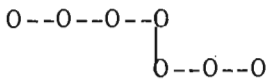
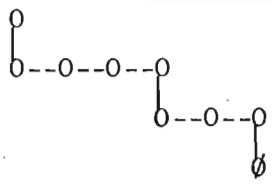
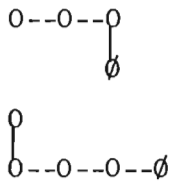
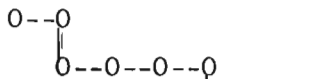
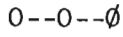
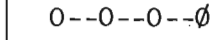
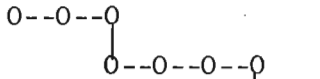
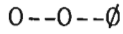
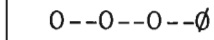
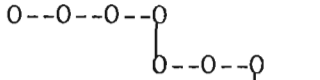
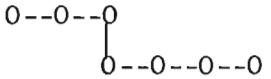
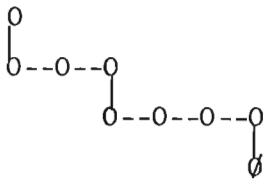
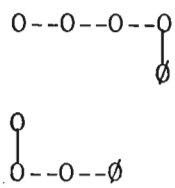
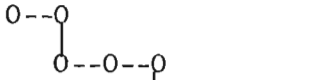
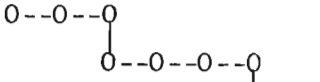
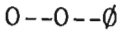
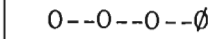
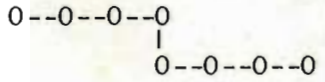
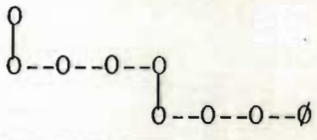
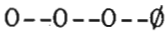
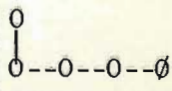
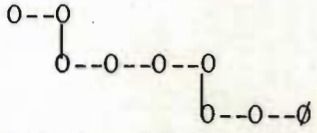
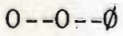
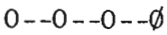
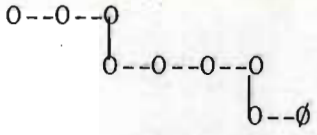
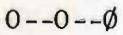
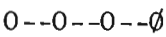
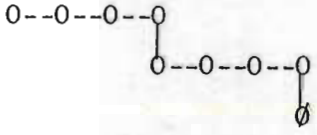
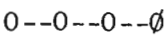
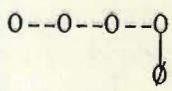
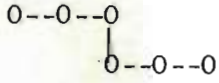
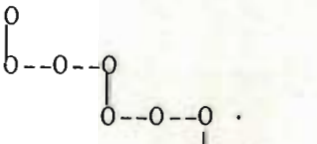
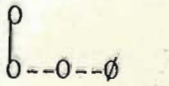
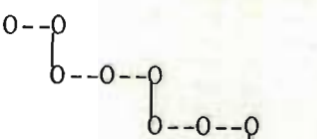
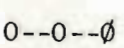
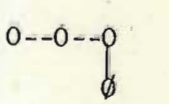
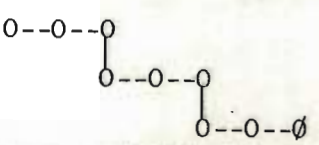
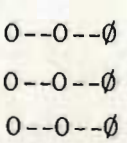
Structure I	PARTIAL ACID HYDROLYSIS	HYDROLYSIS BY PULLULANASE		
	Possible nonaoses formed	maltotriose	maltotetraose	limit dextrin
				
				
				
				
				
				
				
Molar amounts		5	5	

Table 14

Structure II	PARTIAL ACID HYDROLYSIS	HYDROLYSIS BY PULLULANASE		
	Possible octaoses formed	maltotriose	maltotetraose	limit dextri
				
				
				
				
				
				
				
Molar amounts		7	4	

6.2 FORMATION OF ALCOHOL

Samples of juice from deteriorated cane were analysed for volatile organic compounds, by distillation through an efficient fractionating column.

One fraction of distillate, representing a narrow boiling range, 78 - 80°C was collected. It was assumed from the boiling point that this was ethanol, formed by microbial activity during cane deterioration. The identity of the alcohol as ethanol was confirmed as follows:

I After dilution, the alcohol in the distillate was oxidised by chromic acid into acetic acid. This was converted to the ammonium salt and identified by paper chromatography [154]. An R_f value identical to that of ammonium acetate (0,56) was obtained, using ammonia in ethanol as eluent.

II A positive reaction was obtained to the molybdate-xanthate test for primary and secondary alcohols [155].

Subsequently, several samples of juice from stale cane were distilled in the same way and the volume of ethanol collected was measured. The collected fraction was assumed to be 96 % ethanol, as this is the concentration which can be expected from an efficient fractionating column. The results are shown in Table 15. It is evident that ethanol is not a product which normally occurs in stale cane juice but can be associated with cane which has been burnt to remove trash before cutting. With this pre-harvest treatment, alcohol formation may be both rapid and severe, as indicated by one sample, which, after six days storage, showed an alcohol content of 1 %. It is obvious that burning damages cane to such an extent that micro organisms can invade and ferment the juice.

Table 15

Percentage ethanol by volume in juices from cane (N:Co 310) after different pre-harvest treatments and storage intervals.	
Type of pre-harvest treatment and number of days after harvest	ethanol content %
not burnt 6 days	0
not burnt 11 days	0
not burnt 25 days	0
burnt 4 days	0,3
burnt 10 days	0,3
burnt 29 days	0,5
burnt 6 days	1,0

Table 16

Acidity of steam distillates from juice from stale and fresh cane. (variety N:Co 376)		
Sample	acidity, in mg equivalent per 100 ml of juice	amount of acid expressed in ppm acetic acid
immediately after harvest	0,06	38
immediately after harvest	0,03	15
after 10 days of storage	0,13	76
after 22 days of storage	0,15	93
frozen cane (from Dalton area)	0,16	98

No other volatile products were detected by this distillation method.

6.3 FORMATION OF ORGANIC ACIDS

6.3.1 Volatile acids

6.3.1.1 Previous work

Previous investigators determining the volatile acid content of cane juice, used steam distillation of an acidified juice sample followed by titration of the distillate [2 3 4 5 10].

Another approach is to use a single method for determining the total content of both volatile and non volatile carboxylic acids. In general, the acids in plant juices are determined by isolation using ion exchange, followed by elution and chromatographic separation. These techniques can be combined, by using ion exchange chromatography.

The steam distillation method is simple and can readily be applied to routine analysis. Fort and Lauritzen [2] used steam distillation to determine the amount of excess acidity in sugar juices, obtained from frozen cane in Louisiana. They took 100 ml samples of juice and collected the first 25 ml of distillate which they assumed would contain the total amount of volatile acids. They defined " excess acidity" as the amount of acid, in mg equivalent, in excess of the amount found by applying the same technique to a sample of juice from normal cane. The authors found values between 0,06 and 0,7 mg equivalent for the excess acidity in juice from frozen cane.

Friloux and Irvine [6 7] found that the increase in juice acidity in frozen cane did not correlate with the amount of deterioration.

They also found that immature cane showed a high acid content.

Using the same steam distillation method for juice from deteriorated cane in Natal, Young and Buchanan [175] found figures for the excess acidity not higher than 0,03 mg equivalent per 100 ml of cane juice.

6.3.1.2 Present Investigation

A distillation method similar to that described by previous workers, was used for determining volatile acids in cane juice. The juice was acidified with phosphoric acid and the mixture distilled. The distillate was collected and titrated against 0,01 N sodium hydroxide solution. The results are shown in Table 16.

It is evident that there is very little increase in acidity during the deterioration of Natal cane. Even a rare sample of frozen cane exhibited acidity figures very much lower than those reported from Louisiana.

The method of Barker and Kennedy [154] was used in an attempt to analyse for individual acids. The steam distillates were made alkaline, by adding ammonia, and concentrated. Analysis by paper chromatography revealed no trace of volatile organic acids in any of the concentrated distillates obtained from juice from stale cane.

The analytical method was subsequently tested using cane juice to which butyric and acetic acids had been added. When the complete procedure had been followed, chromatographic analysis resulted in two spots with R_f values at 0,50 and 0,66, consistent with the two acids added.

It is concluded that the formation of volatile organic acids during deterioration of Natal sugar cane is negligible.

6.3.2 Non volatile mono- and dicarboxylic acids

6.3.2.1 Previous Investigations

Numerous methods have been published for the analysis and isolation of organic acids in plant and fruit juices. A quick and easy paper chromatographic method, published by Stark et al [156], is applicable to most mono- and dicarboxylic acids.

Separation by silica gel column chromatography has been used by various workers [157 158 159 160]. Water-saturated silica gel is used as the stationary phase and the silica gel is acidified with sulphuric acid in order to prevent tailing of the chromatogram due to ionisation of the organic acids. Fractions are collected from a suitable sized column, in 3 to 10 ml quantities.

Ion exchangers can be used in place of silica gel as column packing for the separation of plant acids. Borodkin and Berger [171] described a method in which the ion exchange column is eluted with dilute formic acid. After the fractions have been collected, the formic acid is removed by azeotropic distillation with chloroform and the fractions are titrated using coulometry.

More recently, gas liquid chromatography has been used for the separation of carboxylic and keto-carboxylic acids. Because of insufficient volatility, it is necessary to esterify the acids and both methyl and ethyl esters are used. According to many authors, the best method for esterification is carried out by using diazo methane [161-169]. Gee, however, recommends esterification with methanol, hydrogen chloride, and thionyl chloride, [170] especially for di- and tricarboxylic acids. According to the author this method results in less side reactions.

Trimethyl silyl ethers have also been used for the separation of acids. Eposito [176] describes a method in which the formation of the ethers is carried out on the chromatographic

column. More recently, Oldfield [172] analysed the organic acids in sugar beet juice by isolating them using ion exchange methods. The acids were eluted as ammonium salts and concentrated to dryness. The dry salts were subsequently silylated with BSA [N,O - *bis* (trimethyl silyl) acetamide] and analysed by GLC.

6.3.2.2 Investigation into a suitable analytical method for the determination of non volatile carboxylic acids in cane juice.

Little has been published on the analysis of acids in cane juice. Roberts and Martin determined mono- and dicarboxylic acids in juice for Louisiana cane, by chromatography on columns packed with silica gel [173] [184]. The most important acids found were aconitic, malic, glycolic, citric, and oxalic acid, while others were present in minute quantities.

Although the actual analysis by gas liquid chromatography requires little time, sample preparation is rather complex. The acids have to be isolated from the sample of cane juice and then dried completely before esterification into the methyl- or ethyl esters for GLC analysis. Because of this complexity a paper chromatographic method was preferred for cane juice. Good separations were obtained when applying the method of Stark et al [156] to a mixture of aconitic, succinic and malic acids, each in a concentration of 1 %. Serious tailing resulted, however, when the method was applied to a juice sample concentrated to 50 % dissolved solids. Separation of acids in this way was evidently impossible.

To overcome the tailing problem, the acids were separated by ion exchange from the rest of the juice components, prior to the paper chromatographic analysis. Only aconitic acid could be distinguished in the resulting chromatogram. Other acids, if present, were in too low concentration to be detected.

Liquid chromatography by means of columns has the advantage that larger quantities of material can be separated without overloading. Even if sensitive analytical detection methods are applied, there may be difficulty in detecting trace quantities of compounds in the presence of large amounts of similar materials, using paper chromatography. In such cases, column chromatography could be advantageous. For organic acid analysis, an additional advantage of column chromatography is that the individual acids can be isolated in sufficient quantities for identification through typical reactions.

Roberts and Martin combined gradient elution and silica gel chromatography using mixtures of chloroform and n-butanol. The elution was started with pure chloroform and the n-butanol concentration was increased by five percent after every 100 ml. This increase in polarity can be carried out by using two or more specially designed mixing vessels [174] [159] . By changing the dimensions of these vessels, any gradient can be obtained.

The use of mixtures of butanol and chloroform with silica gel columns creates an additional difficulty. The silica gel in the column has a predetermined water content and, unless the eluent is saturated with water, the risk is run of depleting the column of its stationary phase. The solubility of water in the eluent alters in sympathy with the change in butanol/chloroform ratio. As a result the automatic preparation of a gradient eluent, although not impossible, is rather complex. When presaturated chloroform and butanol are mixed, water droplets are formed which interfere with the proper operation of the column. Isherwood and Wager [159] overcame this difficulty by separating the droplets in a pre-column, followed by conditioning the eluent through a layer of silica gel before it entered the analytical column.

In the present experiments, the eluents were prepared manually. The gradient was changed step-wise by feeding into the column 100 ml portions of butanol-chloroform mixtures and increasing the n-butanol concentration of successive portions by five percent.

It soon became evident that the type of silica gel used for the separation was very important. Although good separations of acids using commercial silica gel have been reported, most authors favour "Mallinckrodt Silica Gel for Chromatography". At the time of this investigation, however, this type of silica gel could not be obtained.

A mixture of aconitic, succinic, and citric acids was separated on commercially available silica gel ("May and Baker Silica Gel for Chromatography"). In the resulting chromatogram, poor separation and very diffuse peaks against an irregular background were obtained (see Fig. 24).

Later, the silica gel was prepared by grinding and classification to 100 mesh, followed by washing with hydrochloric acid but the treatment did not improve the separation of the acid mixture.

Silica gel prepared from water glass and classified to 100 mesh gave better results, although the column had to be packed and operated using air pressure. The acids were not quantitatively eluted, however, due to the absorptive properties of the gel.

Repeated washing with hydrochloric acid, followed by water, improved the quality of the gel to the extent that the acids could be completely eluted from the column. When the same gel was used three weeks later, however, the retention times of the acids, especially those with a low R_f value, had changed. In addition, the acids were no longer quantitatively eluted from the column. These results show that the type of gel used for the separation of acids is very important and that commercially available gels (BDH, and May and Baker) are unsuitable for this purpose. Treatment with hydrochloric acid is essential for removing impurities such as iron and aluminium. The structure of the gel is also important, as is shown by the increase in absorptive properties during storage. Tristram [178] reported that gels which initially did not show the undesirable property of absorbing organic acids often exhibited these properties after ageing. It is important to take account of this ageing effect when preparing

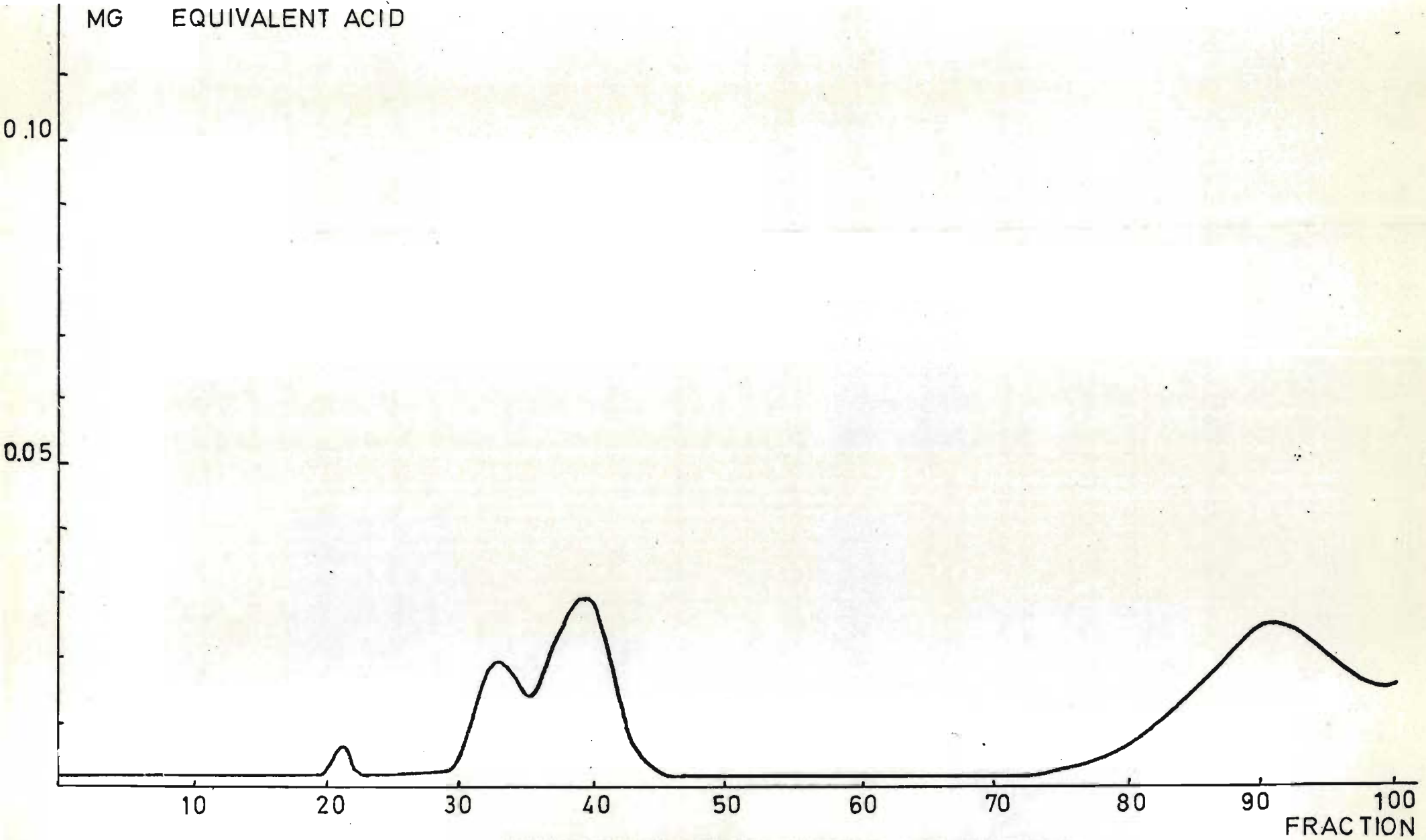


FIG. 24 CHROMATOGRAM OF ACONITIC, SUCCINIC AND CITRIC ACID USING M & B SILICAGEL.

silica gel for chromatography and it is normal to store the gel for a sufficient length of time before the last treatment with hydrochloric acid. A suitable procedure for the preparation of silica gel for chromatography is described by Isherwood [159] .

Gel prepared according to Isherwood's prescription was used for all subsequent analyses. A chromatogram of a test mixture of acids is shown in Fig. 25. The areas under each peak were integrated and the total quantity of acid eluted was calculated. The amounts of acids introduced on the column and the quantities which were eluted are shown in Table 17. Citric acid was not completely eluted while, for the other acids, the accuracy of the determination was about five percent. The fractions were titrated against 0,01 N sodium hydroxide. The titration was complicated by the fact that the eluent used was not miscible with water. To avoid this difficulty, Isherwood [159] used alcoholic sodium hydroxide as titrant and phenolphthalein in alcohol as indicator, so that the titration was carried out in one phase, instead of in two immiscible liquids.

When this method was applied, it was found that the end point tended to be undefined. A much sharper end point was obtained by titration in an aqueous medium. Bulen and Varner [158] used aqueous sodium hydroxide as titrant and phenol red as indicator. During the titration the fractions had to be shaken vigorously to obtain sufficient contact between the two phases. An improvement was obtained by the addition of teepol to the indicator solution [160] . The shaking, however, caused a considerable drift in the endpoint due to carbon dioxide absorption from the atmosphere. It is probable that part of the background irregularity in the initial chromatograms was due more to drift in the titration end point than to properties of the silica gel.

A stable endpoint was obtained by mixing the two phases in a stream of carbon dioxide-free air. At the same time, this provided a blanket during the titration.

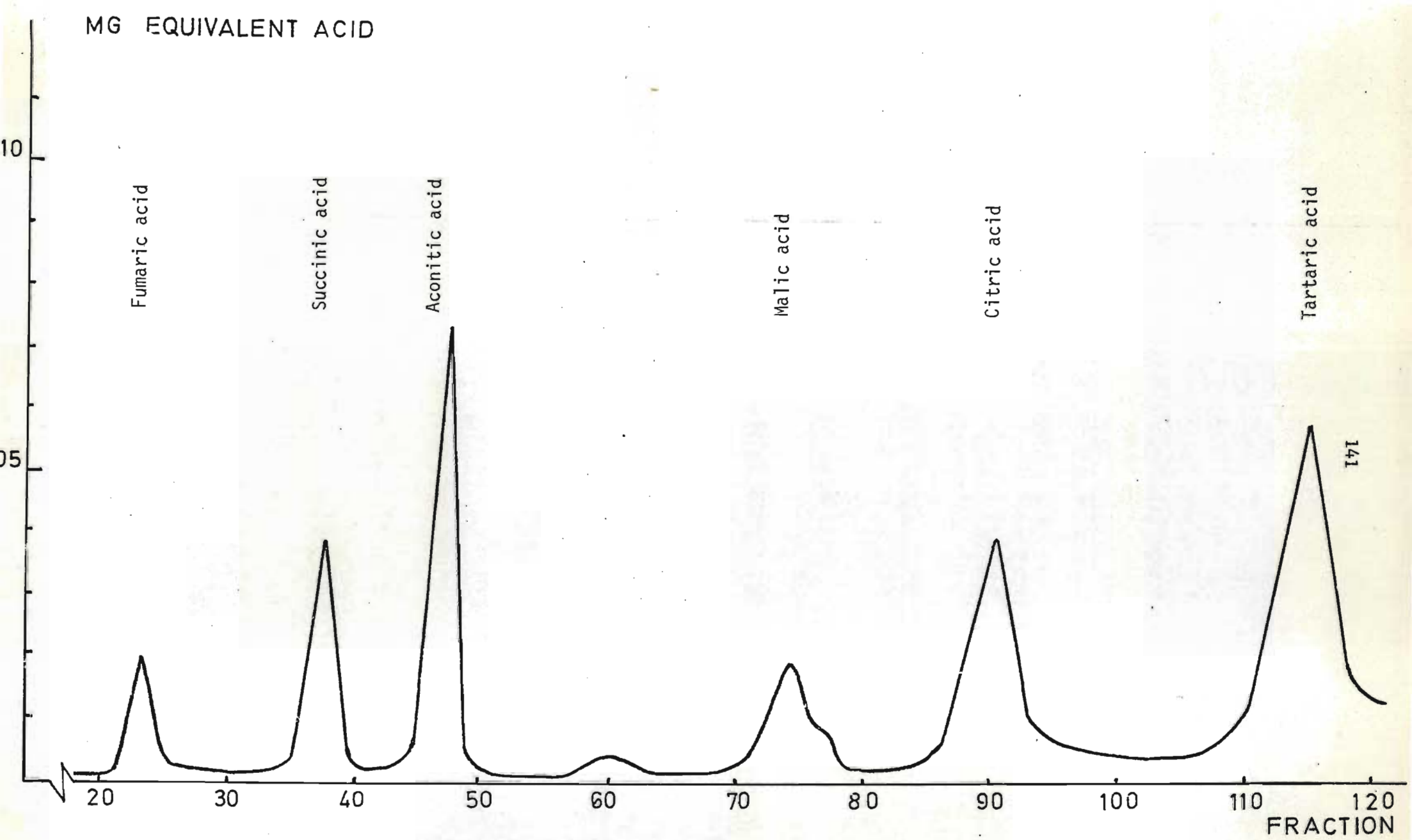


FIG. 25 CHROMATOGRAM OF A TEST MIXTURE OF ORGANIC ACIDS.

Table 17

Separation of organic acids on a silica gel column.		
Fraction	mg acid introduced	mg acid recovered
1. Fumaric acid	2,0	2,0
2. Succinic acid	4,8	4,6
3. Aconitic acid	9,5	8,9
4. l-Malic acid	4,2	4,7
5. Citric acid	14,3	12,2
6. Tartaric acid	20,0	20,8

Table 18

Acid content of various sugar cane samples, expressed as ppm on dissolved solids in juice.					
Peak number	Normal cane N:Co 310	Normal cane N:Co 310	Normal cane N:Co 376	3 weeks after harvest N:Co 310	3 weeks after harvest N:Co 376
1.	100	50	24	-	-
2.	200	110	100	-	-
4.(succinic)	300	240	180	740	506
5.(aconitic)	2 940	580	2 170	575	1 240
6.	530	160	160	330	630
8.	890	560	970	530	210
9.	290	460	540	400	103

6.3.2.3 Analysis of acids in juice from normal and deteriorated cane

Roberts and Martin [173] determined the organic acids in cane juice by column chromatography, using the dry material obtained by freeze drying the juice.

In the present investigation, juice was clarified by heating with lime followed by filtration. Calculation showed that this clarification does not interfere with the acid determination. The most insoluble calcium salt formed from organic acids present in sugar cane is calcium oxalate, with a solubility product of 6×10^{-4}. The calcium concentration in clarified juice is 0,05 g per 100 ml (700 mg CaO per litre juice), therefore the oxalate concentration has to be higher than $1,2 \times 10^{-2}$ before calcium oxalate will begin to precipitate. The concentrations of individual organic acids in cane juice, apart from aconitic acid, are below 120 mg per litre. Aconitic acid has a more soluble calcium salt.

The clarified juice was evaporated and dried *in vacuo*, and the dry residue was introduced on to the top of the silica gel column. The quantity of material taken was such that the concentration of the major acids was approximately 0,01 mg equivalent. Despite all precautions, the large quantity of sucrose in the sample caused major interference and the column blocked after the passage of 500 ml of eluent.

It is obvious that pretreatment is an essential step in the chromatographic analysis of acids in sugar cane juice. Lee and Resnik [177] described the chromatographic analysis of acids in tobacco leaves. They separated the acids in the extract from the leaves by ion exchange, prior to chromatographic analysis. These authors used an Amberlite resin in the carbonate form to exchange the anions of the acids present. The anions were subsequently eluted using an ammonium carbonate solution and the resulting eluate contained the ammonium salts of the acids in an excess of the ammonium

carbonate solution. Heating at 70°C decomposed the ammonium carbonate into ammonia and carbon dioxide. The ammonia and carbon dioxide were driven off and, after evaporation in vacuo, the dry ammonium salts of the acids present in the extract of tobacco leaves were obtained. This method was tested on a solution of succinic acid. More than 95 % of the quantity of succinic acid introduced into the ion exchange column was recovered from the eluate after the decomposition of the excess ammonium carbonate.

Special attention was paid to the analysis of lactic acid. This acid is a common product of bacterial metabolism and for this reason its presence in deteriorated cane was thought to be likely.

Bulen and Varner [158] found that lactic acid was not separated from succinic acid on a silica gel column. These acids emerge as one peak if mixtures of chloroform and butanol are used as eluent. The two acids can be separated, however, if mixtures of benzene and chloroform are used as eluent.

A test was carried out on a mixture of pure lactic and succinic acids. The chromatogram is shown in Fig. 26. Although the separation is not complete, the presence of two acids can be clearly distinguished. The chromatogram also shows a small amount of an impurity emerging before the two main peaks. Although the lactic acid used was analytically pure, it is common to find this impurity present in all commercial lactic acids [158].

The ion exchange method described above, followed by the analysis of the eluted ammonium salts of the organic acids on a silica gel column, was applied to clarified juice.

The ammonium salts of the acids were dissolved in sufficient sulphuric acid to release the free acids and the resulting solution was mixed with sufficient silica gel to obtain a free flowing powder.

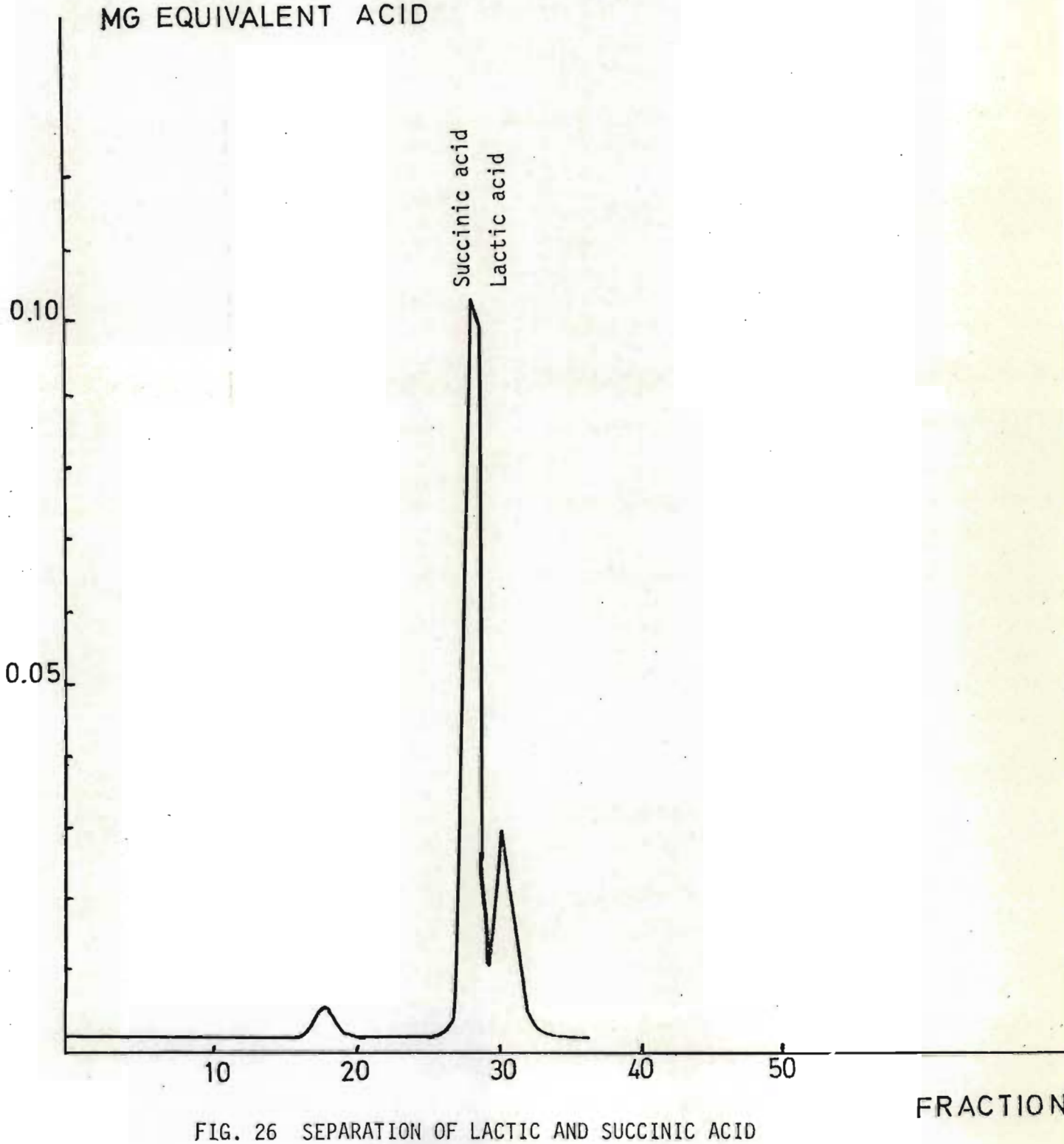


FIG. 26 SEPARATION OF LACTIC AND SUCCINIC ACID USING BENZENE-BUTANOL MIXTURES AS ELUENT.

In Fig. 27, a chromatogram of a normal cane sample is shown, while that of a deteriorated cane sample is shown in Fig. 28.

It can be seen that the quantities of acids alter during deterioration. The small changes in the peaks 1-3 can be ignored as they only represent trace amounts of acids. The amount of acid in the 4th peak (fraction 32-40) increases during deterioration. Comparison of the retention time with that of the test sample indicated that this acid is either lactic or succinic acid, or a mixture of both. The contents of tubes 32-40 in each chromatogram were combined and concentrated to near dryness. The resulting syrup was mixed with silica gel and fed into another silica gel column, which was subsequently eluted with benzene-n-butanol mixtures.

In this second analysis, only one peak was obtained from the samples investigated. After complete evaporation of the eluent, a crystalline acid, which was identified as succinic acid (see section 6.3.2.4) was recovered. As a result of this second chromatographic analysis with benzene-butanol mixtures, it was proved that lactic acid was absent from the cane samples under investigation.

The results of the organic acid analyses of juices from various samples of sugar cane, both fresh and deteriorated, are listed in Table 18.

In addition, a test batch of harvested cane was used to monitor the progress of deterioration during storage in the open air. Samples drawn from this batch were analysed by chromatography for organic acids. The results, shown in Table 19, indicate clearly that the succinic acid content increases steeply during the initial period of deterioration but decreases gradually thereafter. A similar trend is followed by aconitic acid but the increase as a percentage of the original concentration is less pronounced. In addition, the variation in the aconitic acid content in normal cane varies considerably, while the concentration of succinic acid is more constant.

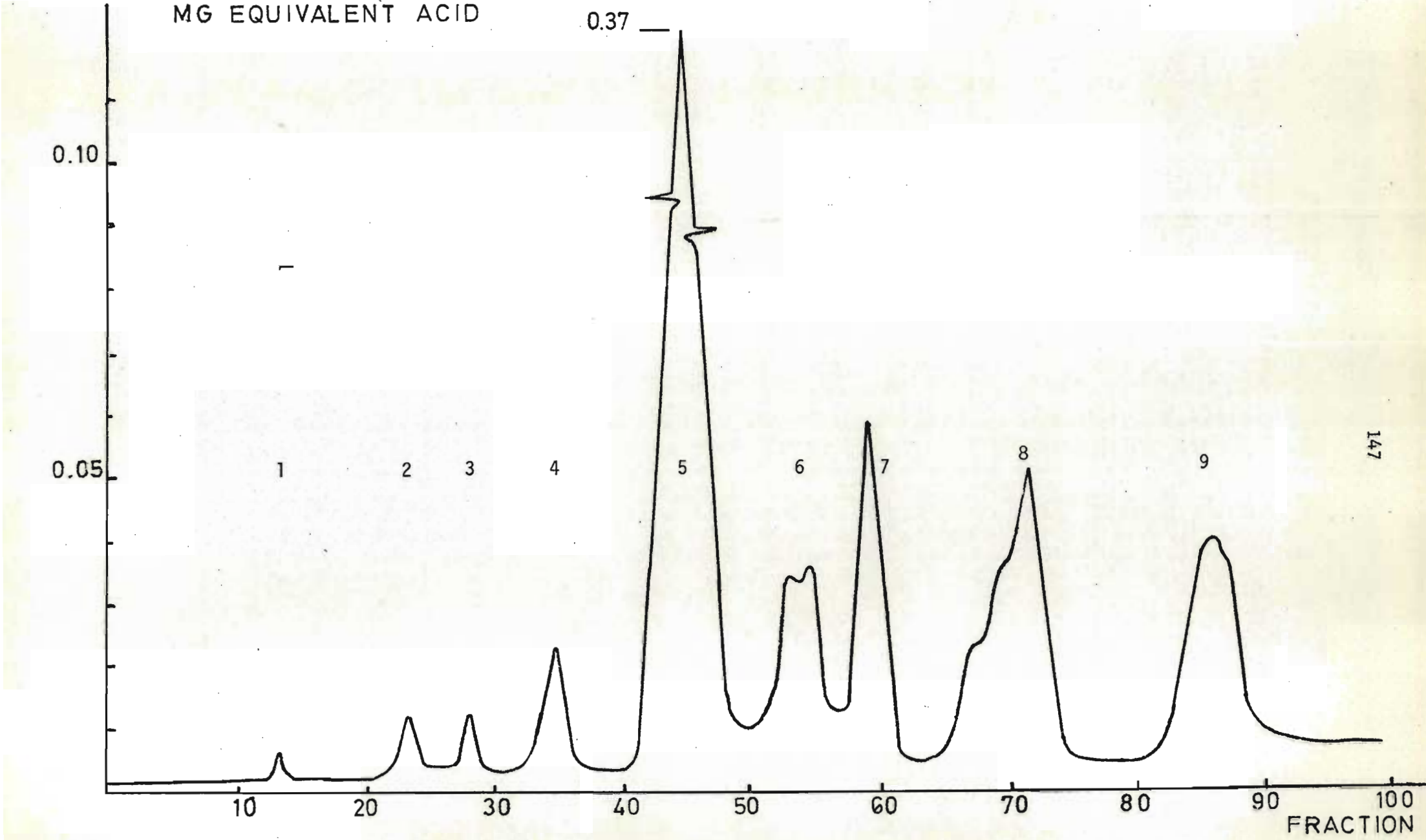


FIG. 27 CHROMATOGRAM OF ORGANIC ACIDS IN 200 ML OF JUICE FROM NORMAL SUGAR CANE.

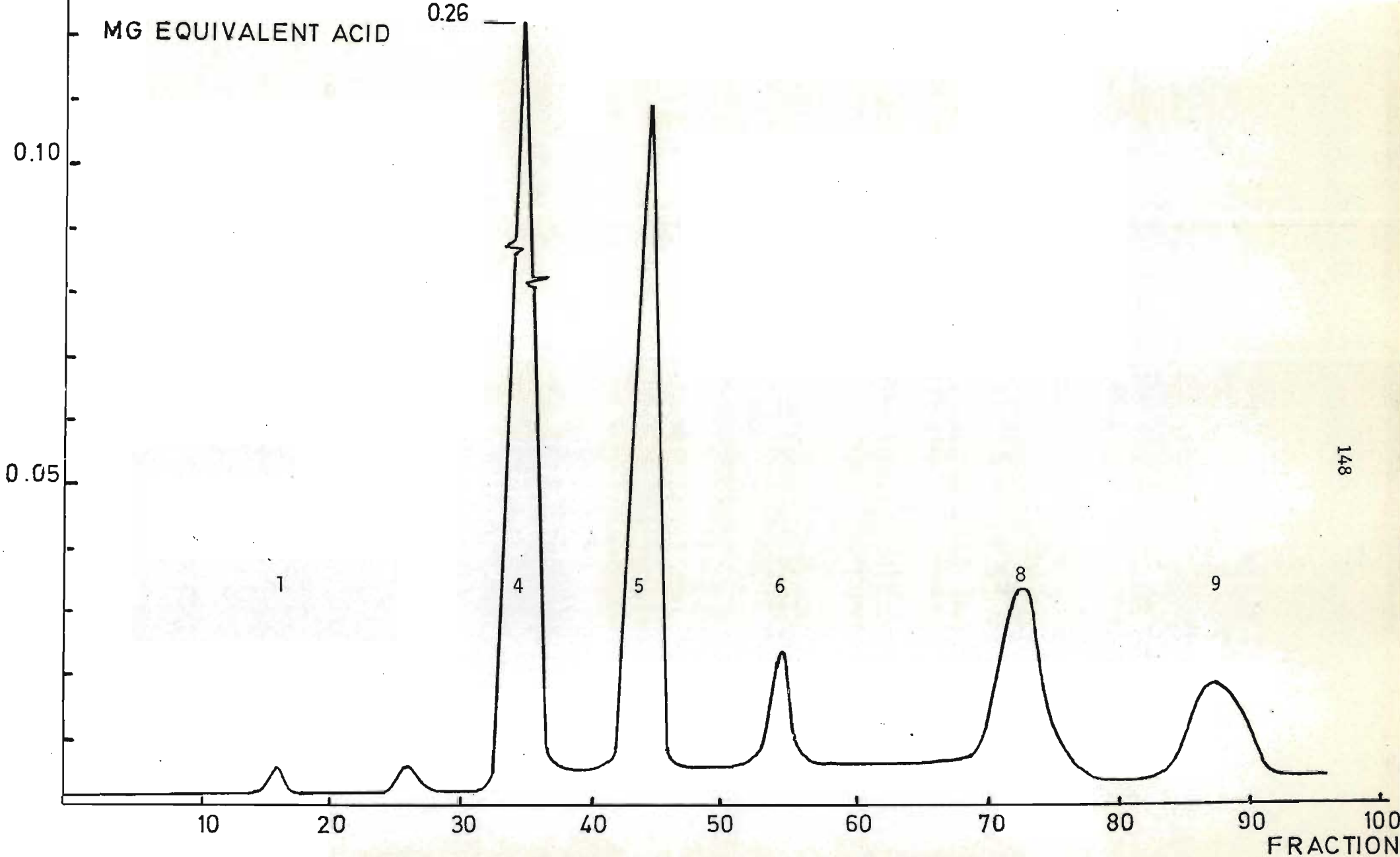


FIG. 28 CHROMATOGRAM OF ORGANIG ACIDS IN 200 ML OF JUICE FROM DETERIORATED CANE.

Table 19

Acid content of samples of cane stored for various times after harvest, expressed as ppm on dissolved solids in juice.
Cane Variety N:Co 310

Peak number	days after harvest 0	6	15	21	24
1.	90	32	40	35	38
2.	110	32	82	40	50
4.	80	350	750	350	250
5.	800	1 900	4 000	1 500	2 500

6.3.2.4 Identification of some acids in cane juice after separation by chromatography

The identification was carried out on the acids emerging in peaks 4 and 5 of the chromatogram. Peak 4 was chosen because the concentration of this acid changes during deterioration while the acid in peak 5 is present in the highest concentration in all the samples of sugar cane analysed. The other acids, which were present in only small quantities, were not investigated further.

Fraction 32-40, the equivalent of peak 4, was investigated using the following tests:

I The pyrrole test according to Feigl [179].

With p-amino-benzaldehyde a pink stain was obtained. This is specific for succinic acid, succinates, or succinimide.

II The fluorescein test [180].

With resorcinol and sulphuric acid a red solution was obtained with an intense green fluorescense, indicating the presence of phthalic or succinic acid. Although this reaction is common to all acids with carboxylic groups in 1,2 position, tests showed that only phthalic acid gave the same intense green fluorescense as succinic acid. All the other carboxylic acids tested gave a less intense and different colour using this reaction.

III The melting point of the isolated and dried crystals was determined as 181,5°C. Admixture of pure succinic acid (BDH) did not depress this value. The melting point published in literature is 183°C [181].

IV Paper chromatographic analysis of the acid using a mixture of phenol, water and formic acid as eluent, resulted in a spot with the same R_f value as succinic acid.

From the combined results of these tests it was concluded that the isolated acid was succinic.

The acid present in the fractions 42-48 was identified as aconitic acid. Aconitic acid has been shown to be the most important acid present in sugar cane from Louisiana [184] . This is evidently also the case in Natal sugar cane.

The following reactions were carried out for identification purposes:

I No melting point could be established. A decomposition point was found at 170°C. According to the literature the decomposition point is 190°C (capillary in oil bath) [183] . Decomposition points depend however on the rate of heating and on the temperature at which heating is started.

II Reaction with acetic anydride gave a violet colour. This is specific for aconitic acid. [182]

III A paper chromatogram using phenol, water and formic acid as eluent [156] , resulted in a spot with R_f value identical to that of aconitic acid.

6.3.3 Conclusion

The most abundant acid present in cane grown in various sugar producing countries is aconitic acid [173] . In common with a large number of plant species, succinic, aconitic, malic, and glycolic

acids normally occur in cane [184], but the total quantity of carboxylic acids found is usually small. The investigation into sugar cane grown in South Africa confirmed these findings.

The same acids were found in deteriorated cane as in normal cane and, ignoring trace amounts of unidentified acids, the concentrations were approximately the same. It was found, however, that succinic acid can increase to abnormal concentrations during deterioration. This increase occurs in the initial period of deterioration, after which the concentration slowly declines. It is considered unlikely that the increase in succinic acid content has any specific influence on the sugar manufacturing process but any increase in the non-sucrose content of cane will contribute to the formation of a larger quantity of molasses. Aconitic acid follows a similar pattern but the changes are less pronounced.

Succinic and aconitic acid are acids of the so called "citric acid" or "Krebs" cycle. In terms of this cycle, carbohydrate is oxidised to carbon dioxide and water via pyruvic acid. Changes in the composition of "Krebs cycle" acids in plants have been reported. Unripe cane harvested in Louisiana was found to have a higher acid content than ripe cane.

A diurnal variation in malic acid content has been found in excised leaves from some plants. The acid level rose in the dark and fell during the day, with an opposite variation in starch content [185] [186].

Changes in citric acid content during the growth of sugar beet were recently reported by Schiweck [187].

From the results of the present investigation, it appears likely that the various enzymes responsible for the individual steps in the "citric acid cycle", lose their activity gradually during the storage of the sugar cane after harvesting. It is also apparent, however, that not all the enzymes lose activity at the same rate.

Consequently, there is a disturbance in the normal acid balance in the plant.

It is remarkable that lactic acid was not detected during this investigation on deteriorating sugar cane. Lactic acid is common in bacterial fermentations and is a normal metabolic product of *Leuconostoc mesenteroides* and other lactic acid bacteria found in sugar cane. The absence of lactic acid in deteriorated sugar cane indicates that no appreciable fermentation by lactic acid bacteria occurrs during whole-stalk* cane deterioration.

* This expression is used to differentiate between this type of deterioration and that which follows mechanical harvesting.

7. INDUSTRIAL IMPORTANCE OF SUGAR CANE DETERIORATION

A most important aspect of cane deterioration from the industrial point of view is the decrease in the amount of sucrose available for crystallisation as commercial sugar. Whether this loss is suffered by the farmer or by the sugar manufacturer, the overall result is a loss in national income. A considerable amount has been written regarding this type of loss, and there is fairly general agreement concerning its magnitude and importance [188].

Another important aspect of cane deterioration is connected with polysaccharide formation. Two properties of sarkaran have an effect on sugar manufacture: its dextrarotation and its viscosity in solution.

The specific rotation of sarkaran has been found to be approximately three times that of sucrose. In an analysis by direct polarimetric measurement, any quantity of sarkaran present in cane juice will be recorded as the equivalent of a three-times larger quantity of sucrose. It has been established experimentally that the conventional clarification with dry lead subacetate powder, prior to the polarimetric measurement, does not remove any sarkaran from the juice. In the double polarimetric method for the determination of sucrose according to Jackson and Gillis [189], the concentration of hydrochloric acid (0,8 N), temperature (65°C), and time (30 min.), are sufficient to invert all the sucrose present but will not hydrolyse the sarkaran. For this reason the dextrarotary readings due to the polysaccharide, before and after inversion of the sucrose, are identical and will cancel out in the final calculation. As a result, the sucrose content as determined by this method is unaffected by sarkaran. It is known that polysaccharides are included in the sucrose crystal during crystallisation. All raw sugars contain starch, together with certain amounts of other polysaccharides. It is the convention to determine the sucrose content of raw sugar by direct polarimetric measurement, after clarification with lead subacetate solution. As sarkaran is not removed by this clarification procedure, raw sugars which contain this polysaccharide will be awarded a polarisation figure higher

than they deserve on the merits of their sucrose contents. A similar situation obtains when the sugar contains starch in a water-soluble form. These inflated figures can have considerable financial implication, as practically all raw sugar payments are based on the direct polarisation determination.

The increased viscosity of sugar house products caused by the presence of sarkaran, can have a marked influence on factory operations. When deteriorated cane is processed, crystallisation can be retarded or even stopped completely, and it is not uncommon for lower-grade boiling times to be increased two or three fold. Viscosity also has considerable effect on the exhaustion of final molasses and losses of sucrose in final molasses often increase during periods when a high proportion of the cane processed by a factory has suffered deterioration. Inclusion of impurities in the sucrose crystal can cause crystal deformation. This can result in a reduction in both sugar quality and sugar recovery. Various authors have named dextran, raffinose, and other oligosaccharides as the culprits responsible for the elongation of sucrose crystals along either the b - or the c - axis[15 16 190]. The effect of sarkaran on the habit of sucrose crystals has not been investigated extensively, but the possibility that the products formed in deteriorating cane can cause crystal deformation, should not be discounted.

A determination of the soluble polysaccharides present in cane is indicated as a means for assessing the degree of deterioration. The analysis can be carried out either by the gravimetric method described in section 6.1.2 or by the quicker but less accurate procedure of measuring the turbidity after the addition of ethyl alcohol to the expressed juice (6.1.2). It has been established, by analysis of a large number of cane samples, that the normal polysaccharide content of juice from undeteriorated cane is approximately 0,3 % of the total dissolved solids. Any appreciable increase in this value will indicate a delay between the times of harvesting and delivery of the cane to the factory.

The provision of information on the freshness of cane will be of limited value unless the tool provided by the analyst can be used as part of an organisational system for minimising the delays which follow harvesting.

A successful solution to the serious problem of sugar cane deterioration can only be found through the cooperative efforts of Agriculture, Transport, and Factory. This problem will become one of even greater urgency if cutting of cane by hand is replaced by mechanical harvesting.

PART II

8. EXPERIMENTAL DETAILS

8.1 FORMATION OF POLYSACCHARIDES

8.1.1 Analysis of sugar cane juice for soluble polysaccharides and starch

In the following experiments, juice was obtained from the sugar cane sample by passing the cane four times through a three roller laboratory mill, using a small amount of imbibition water. Under these conditions the extraction efficiency was 60 - 70 %. In those cases where there was insufficient material to use the mill, extraction was carried out by pressing shredded sugar cane in a hydraulic press.

8.1.1.1 Gravimetric method for soluble polysaccharides

Juice (25 ml) was filtered through Whatman No. 5 filter paper and the first 5 ml was discarded. Acidified alcohol (100 ml) (see note 1) was added to 15 g of the clear filtrate. The precipitate formed was allowed to coagulate overnight and was then filtered through a Gooch crucible and washed with 80 % alcohol followed by absolute alcohol. The crucible was dried for one hour at 100 - 110°C, cooled, and weighed. It was subsequently heated at 800°C for one hour, cooled, and reweighed. The difference between the two weights was recorded as soluble polysaccharide.

The results were expressed as a percentage of the total dissolved solids in the cane juice. The quantity of total dissolved solids was determined by measuring the refractive index of the filtered cane juice. The refractometer was calibrated in degrees Brix, which is the scale normally used in the sugar industry. One degree Brix is equivalent to 1 g dissolved solids per 100 g of juice. Although the relationship is completely valid only for pure sucrose solutions, it can be applied with reasonable accuracy to impure sugar solutions as well.

Note 1: Absolute ethanol (150 ml) mixed with 1:1 HCl (30 ml)

8.1.1.2 Turbidimetric method for soluble polysaccharides

One hundred ml of acidified alcohol was added to 15 g of filtered cane juice. The optical density at 400 nm was recorded 15 minutes after the addition of the ethanol, using a 2 cm cell. As the reading is influenced by the colour of the juice, a blank was prepared by adding 100 ml of hydrochloric acid solution (see note 1) to 15 g of the juice filtrate [98] .

Figure 1 (page 42) compares results by the gravimetric method and by means of turbidimetric measurement.

8.1.1.3 Colorimetric methods for soluble polysaccharides

For these methods, the polysaccharide precipitate obtained with acidified ethanol was centrifuged off, washed with 80 % ethanol followed by absolute ethanol, and dissolved in boiling water. After cooling this solution was made up to 100 ml. A suitable aliquot was then taken for reaction with either sulphuric acid and phenol [96] or sulphuric acid and anthrone [97] .

8.1.1.4 Determination of starch [99] .

One hundred ml of absolute ethanol and 2 g of Kieselguhr were added to 100 ml of juice. The mixture was stirred and left standing for one hour. The sample was then filtered through a matt of Kieselguhr (2 g) supported on Whatman No. 5 filter paper in a 5 cm Buchner funnel. The cake was washed with 80 % ethanol and absolute ethanol and transferred into a beaker. Forty ml of 35 % calcium chloride solution was added after which the mixture was boiled for 15 minutes to dissolve the starch, cooled, and transferred to a 100 ml volumetric flask. After making up to volume and additional 1,7 ml of water was added, to correct for the volume of the Kieselguhr. The suspended Kieselguhr was separated in a centrifuge, 10 ml of the supernatant liquid was pipetted into a 50 ml volumetric flask to which was added in turn, 10 ml of distilled water, 2,5 ml of 2 N acetic acid,

Note 1: 15 ml concentrated hydrochloric acid
+ 165 ml distilled water.

0,5 ml of 10 % potassium iodide solution, and 5 ml of 0,01 N potassium iodate solution. After making up to volume, the absorption was measured at 600 nm using a 1 cm cell. The reading was compared with a standard curve, prepared using soluble starch (note 1).

8.1.2 Isolation and purification of the polysaccharide in stored cane

Cane juice was expressed from sugar cane, which had been stored for four weeks in the open. Starch and other suspended matter was removed by centrifuging in a continuous Alfa Laval separator. The clear juice was concentrated at a pressure equivalent to 7,5 cm mercury, to a dissolved solids content of 40 %. To this syrup, 3,5 times its volume of acidified alcohol was added. After standing overnight, the precipitate formed was filtered, washed with aqueous ethanol (70 % V/V), and redissolved in water. The polysaccharide was reprecipitated by adding ethanol to a final concentration of 70 % V/V. This procedure was repeated once more and the final product was dried *in vacuo* over phosphorus pentoxide.

The product was redissolved in water and dialysed through a cellulosic membrane (Visking tubing), which retains compounds with a molecular weight over 8 000. The dialysis was carried out against tap water at room temperature. Analysis indicated that the ash content was not lowered by dialysis, the undialysed preparation, however, was already nearly ash-free.

Analysis for protein was carried out by a micro Kjeldahl determination [100]. One ml of the aqueous polysaccharide solution was pipetted into a test tube and 2 mg of catalyst (note 2) added.

Note 1: Pure soluble starch prepared by J.T. Baker Chemical Co. Phillipsburg N.J.

Note 2: Magnesium sulphate 7 aq. (2,9 g) selenium powder (0,28 g) and potassium sulphate (9,1 g).

After the addition of 0,1 ml of concentrated sulphuric acid the tube was placed in a specially designed sand bath which was covered with an asbestos sheet to keep the top part of the tube cool. A blank determination was carried out simultaneously. The mixture was kept at 310 - 320°C for 1 hour, after which it was cooled to room temperature and 4 ml 1N sodium hydroxide solution was added and the contents of the tube mixed. A 3 ml sub-sample was pipetted into a colorimetric tube together with 2 ml of water and 0,1 ml of Nessler reagent. The tubes were shaken and the absorption determined after 30 minutes at 490 nm. The nitrogen content so determined was multiplied by 6,28 to give the protein content.

Starch in the polysaccharide was determined by the method described for cane juice.

8.1.3 Homogeneity of the polysaccharide

8.1.3.1 Fractional precipitation

9,38 g of the polysaccharide was dissolved in 300 ml of water. Ethanol was added stepwise over a concentration range from 50 % - 80 %. The precipitate formed after each concentration increase was filtered off through a Gooch crucible, dried at 105°C, and weighed.

8.1.3.2 Gel chromatography

Sufficient Ionagar (note 1) to pack a column 2,5 x 45 cm (note 2) was prepared according to Andrews [102]. A solution of 5 % agar gel was autoclaved for 20 - 30 minutes at a pressure of 2 bar. The gel was cooled overnight and disintegrated in distilled water, using a rotating blade homogeniser (note 3). The resulting slurry

Note 1: Deionised agar Oxiod Limited London S.E.1
Note 2: Standard column Pharmacia A.B. Upsala, Sweden.
Note 3: Kitchen liquidiser Bamix.

was physically screened through a 60 mesh followed by a 100 mesh, using a flat spatula. The resulting-100 mesh granules were poured into the column as an aqueous slurry. The packing was carried out under gravity and, finally, a pressure equivalent to a 60 cm water column was applied.

A solution containing 78 mg of the isolated and purified cane polysaccharide dissolved in 2 ml of distilled water was introduced on top of the column. Elution was carried out with distilled water and 2 ml fractions were collected. The fractions were analysed for polysaccharide content by the phenol and sulphuric acid method . 1 ml of 5 % phenol solution and 5 ml of concentrated sulphuric acid were added to the 2 ml fraction and the optical density measured at 490 nm, after the tubes had been standing for 20 minutes at room temperature.

8.1.4 Determination of the molecular weight of the polysaccharide

8.1.4.1 By viscosity measurement

The viscosity measurements were carried out using a Hoeppler viscosimeter. The temperature of the viscosimeter was kept constant by circulating water from a controlled water bath. The measurement was carried out at three different concentrations of the polysaccharide.

I Polysaccharide from stored cane

Measuring temperature 30°C

Concentration of polysaccharide in water g/ml	falling time of ball in sec.	spec. gravity of solution
0,0000	76,3	0,996
0,0102	94,0	1,003
0,0204	118,5	1,005
0,0306	148,0	1,007

$$\eta \text{ spec.} = \frac{\eta \text{ solution}}{\eta \text{ solvent}} - 1$$

$$\eta = T \times (S_1 - S_2) \times B$$

in which

η = viscosity in centipoises
S_1 = specific gravity of ball (2,414)
S_2 = specific gravity of solution
B = ball constant
T = falling time of ball in sec.

$$\eta \text{ spec.} = \frac{T \times (S_1 - S \text{ solution})}{T \times (S_1 - S \text{ solvent})} - 1$$

For a concentration of 0,0102 g/ml

$$\eta \text{ spec.} = \frac{94 \times (2{,}414 - 1{,}003)}{76{,}3 \times (2{,}414 - 0{,}996)} - 1 = 0{,}228$$

$$\frac{\eta \text{ spec.}}{c} = \frac{0{,}228}{0{,}0102} = 22{,}4$$

Similar calculation for the other concentrations results in

c	=	0,0204 g/ml	spec./c	=	26,9
c	=	0,0306 g/ml	spec./c	=	30,4

These three values obtained were plotted as shown in Fig. 5. Extrapolation to c = 0 results in

$$(\eta) = 18{,}4$$

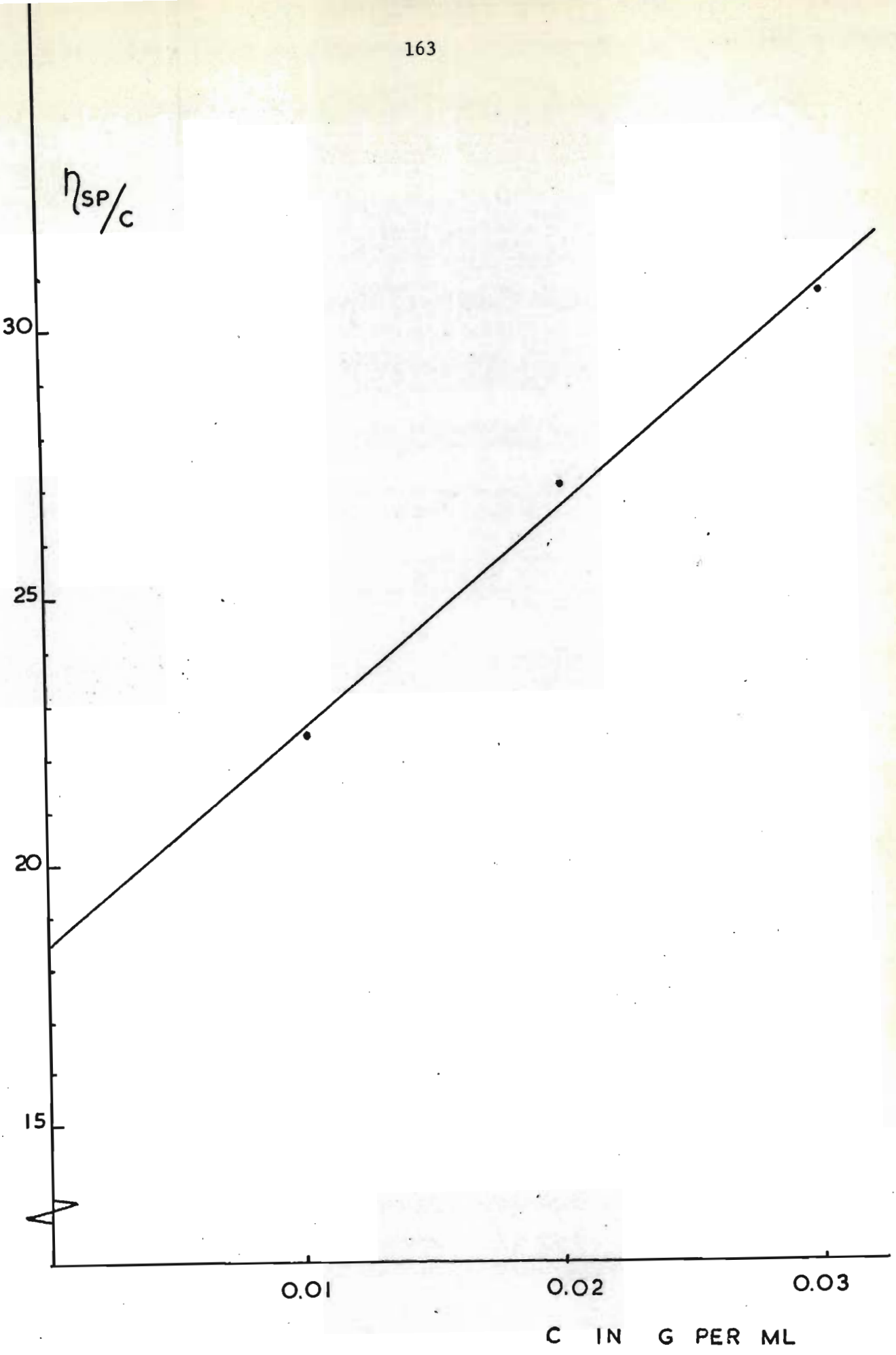


FIG. 5 η_{sp}/c VERSUS THE CONCENTRATION OF AN AQUEOUS SOLUTION OF CANE POLYSACCHARIDE

Applying $(\eta) = 10^{-1} \cdot \bar{M}_w^{0,5}$

$\sqrt{\bar{M}_w} = 10 \times (\eta)$

$\bar{M}_w = \left[10 \times (\eta)\right]$

$= (10 \times 18,4)$

$\triangleq 34,000$

II Dextran

Using the same procedure, a determination was carried out on a sample of dextran prepared by fermentation with *Leuconostoc mesenteroides* (described in 8.1.7.1).

As the viscosity of the dextran solution was higher than that of the solutions of cane polysaccharide, the measurement was carried out on more dilute solutions. Because the density of these dilute solutions differed by less than 0,1 % from the density of distilled water, no correction for density has been applied.

The following results were obtained:

dextran concentration in g per ml.	falling time of ball in sec.
0,000	83,6
0,001	90,4
0,002	97,8
0,003	105,6

η spec. = 90,4/83,6 - 1 = 0,081
η spec./c = 81
η spec. = 97,8/83,6 - 1 = 0,170
η spec./c = 85
η spec. = 105,6/83,6 - 1 = 0,236
η spec./c = 88

These values are plotted in Fig. 6, extrapolation to c = 0 results in

$$(\eta) = 77,6$$

The formula used for the polysaccharide from stored cane is not applicable in this case as the molecular weight is greater than 250 000.

Senti and co-workers [80] have published a graph for log (η) versus log $\overline{M}_w$ for dextran B 512. In this graph (η) is expressed as instrinsic viscosity, based on a concentration in g per ml.

For a value of $(\eta) = 0,78$, the corresponding molecular weight would be

$$\overline{M}_w = 2 \times 10^6$$

8.1.4.2 Molecular weight by gel chromatography

For this determination a 15 x 350 mm column was packed with Sephadex G 200. A 2 mg portion of polysaccharide in aqueous solution was applied to the top of the column and eluted with water. Two ml fractions were collected and the quantity of polysaccharide in the fractions determined using the phenol-sulphuric acid method [97]. The peak elution volume was compared with results obtained by applying the identical procedure to dextrans of known molecular weight (note 1). A typical calibration graph is shown in Fig. 4[a] page .

Note 1: Dextran 150, Dextran 40, and Dextran 10 supplied by Pharmacia Fine Chemicals A.B.,

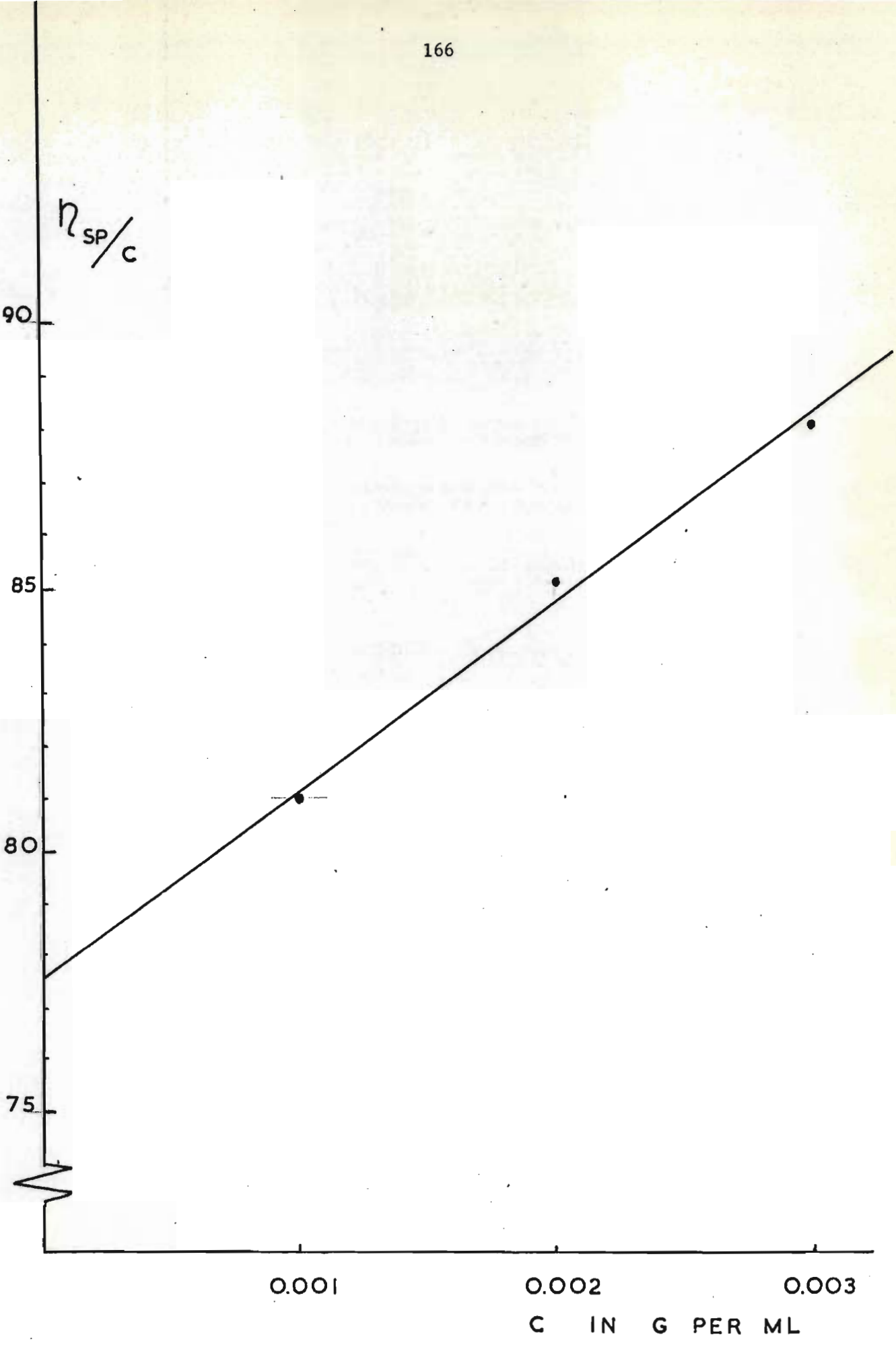


FIG. 6 nsp/c VERSUS THE CONCENTRATION OF AN AQUEOUS SOLUTION OF DEXTRAN.

8.1.4.3 Molecular weight by light scattering*

Four grams of the polysaccharide were dissolved in 200 ml water. This solution was filtered, followed by freeze-drying. A white solid with a "fluffy" texture was obtained. This solid was kept over phosphorus pentoxide and assumed to contain a negligible amount of water. From this dried polysaccharide, 0,5 %, 1 %, 1,5 % and 2 % ($^{w}/v$) aqueous solutions were prepared, stirred for 24 hours at room temperature, and kept thereafter in the refrigerator.

The four solutions prepared were clarified by several filtrations through 450 nm millipore filters, collecting the filtrate in the light scattering cell enclosed in a dust-free air stream. The cell was rigorously cleaned with chromic acid, repeatedly rinsed with dust-free distilled water, and finally flushed with freshly distilled methanol in a special enclosed still constructed in the laboratories of Tate & Lyle, Research & Development. The cell was covered before transfer to the light scattering photometer.

The angular scattering intensity of each solution was measured using a Brice Phoenix light scattering photometer, Series 2 000. Measurements were made at wavelengths 546 nm and 436 nm.

At the same wavelengths the refractive index differences between water and the sarkaran solutions were measured directly on the 1,0 % and 2,0 % solutions, using a differential refractometer constructed in the abovementioned laboratories. Calibration of the refractometer was carried out using 1,0 % and 2,0 % sucrose solutions. The measured refractive indices were further checked on a Brice Phoenix differential refractometer at Kodak Research Laboratories, England. The agreement between these measurements was within 1 %.

The angular scattering intensity of each solution was measured, that of the solvent was determined using a freshly filtered (450 nm) sample. Measurements were made at 5° intervals from 30° to 135°. The incident intensity was measured by setting the detector to 0° and attenuating the beam with neutral density filters, so that all readings were made with the same photo multiplier EHT. In this way,

* Determined by Tate & Lyle, Research & Development.

absolute scattering values are obtained without reference to standard scatterers or amplification factors. Scattering intensities were reproducible to 2 - 3 % in duplicate measurements.

The Rayleigh ratio was calculated, taking into account the geometry of the instrument used, reflected light within the sample cell, and also the light scattered by the solvent.

For the Brice Phoenix 2 000 instrument, these factors are contained in the equation.

$$R_\theta = \frac{TDan^2.}{1{,}049\ h} \quad \frac{r.}{r'} \quad \frac{\sin\theta}{1+\cos^2\theta} \left[\frac{1}{(1-R)^2\ (1-4R^2)}\right] \times$$

$$\left[\left[\frac{G_\theta.F}{G_W\ Soln} - \frac{G_\theta.F}{G_W\ Solv.}\right] - 2R\left[\frac{G_{180°-\theta}}{G_W\ Soln}\ F - \frac{G_{180°-\theta}}{G_W\ Solv}\ F\right]\right]$$

in which

a,T,D h. r/r'are instrument constants,

n = the refractive index of the solvent

R = the fraction of the light reflected at the glass liquid interface.

G_θ = The galvanometer reading for angle θ(proportional to light intensity at a fixed EHT)

G_W/F = Galvanometer reading proportional to incident light intensity

F = Attenuating factor of neutral density filter

To correct the observed intensity at angle θ it is necessary to know the intensity at (180 - θ). The photometer measured in the range 30° - 135°, and so readings of G for θ = 140°, 145°, 150° are required to correct the observed intensities at 40°, 35° and 30°. These were obtained by multiplying G_{135} by

$$\frac{1 + \cos^2 \theta}{1 + \cos^2 135°} \cdot \frac{\sin 135°}{\sin \theta}$$

which provides a slight underestimate of the true backward scattering, but represents the best available approximation.

The above equation for R_θ gives this ratio for each value of θ and concentration c. Data were collected for 25 angles of observation, 4 concentrations, and 2 wavelengths, resulting in 200 calculations of R_θ.

A computer program was written (by Dr. B. Orchard, Tate and Lyle Research and Development) to calculate R from the experimental data.

8.1.4.4 Number average molecular weight by osmotic pressure measurement

Osmotic pressure measurements were carried out using a Hewlett-Packard Series 500 membrane Osmometer.

Aqueous solutions of the polysaccharide were prepared as described under 8.7.3. Measurements were carried out at 37°C. Pressures were expressed in cm water column.

8.1.5 Determination of the Specific rotation

1,0166 g of cane polysaccharide was dissolved in 50 ml of distilled water and the optical rotation was determined using a Hilger polarimeter with a sodium light source. The 20 cm polarimeter tube was maintained at a temperature of 20°C. The rotation was + 6,5°

$$(\alpha) = \frac{100 \text{ x}}{\text{c x l}}$$

in which

α = angular rotation in degrees
c = concentration of solute in g per 100 ml
l = tube length in dm

The specific rotation was

$$(\alpha)^{20°}_{\text{sodium light}} = \frac{100 \times 6,5}{2,0332 \times 2} = + 160°$$

8.1.6 Paper Electrophoresis

A simple paper electrophoresis apparatus, described by Lederer [115], was used. A paper strip (Whatman No. 1), 47 cm long, was placed between two glass plates, to provide cooling and prevent evaporation. Pieces of paper were hung on each side and dipped into beakers containing buffer solution. Carbon electrodes, connected to a stabilized 300 V D.C. supply, were placed in each beaker. This resulted in a field of 6,5 V per cm.

The strength of the phosphate-citrate buffer solution was adjusted so that a current of 8 mA was obtained. Two buffer solutions were used, one of pH 5,8 the other of pH 8,5. After the paper was moistened, the polysaccharides were applied in the form of a spot in the centre of the paper strip using a microsyringe. After 8 hours the paper strip was removed and dried at room temperature in a horizontal position. This precaution was taken to prevent any lateral movement of the polysaccharide spot during drying.

The paper was subsequently sprayed with a 1 % aqueous solution of sodium periodate and left for 15 minutes in the dark at room temperature. It was then sprayed with an aqueous sodium carbonate solution containing 2 % potassium iodide.

The polysaccharide was oxidised faster by the periodate than was the filter paper. The excess periodate liberated iodine from the second reagent and the developed spots appeared white on a yellow background.

8.1.7 Determination of the structure of the polysaccharide by chemical analysis

8.1.7.1 Preparation of dextran

It was first necessary to isolate the micro-organism. Dilute cane juice was spread on Petri dishes containing juice tryptone agar (note 1) and incubated for 3 days at 25°C. At the end of this time, a number of slime-forming colonies had developed. One of these was dispersed in sterilised water and a drop of the resultant liquid spread on juice tryptone agar. After 3 days, only slime-forming colonies had developed. One of these was transferred to a culture tube and maintained on a medium consisting of malt extract agar and calcium carbonate. Further transfer to a new tube of nutrient was carried out once a month.

The isolated bacterium was a non motile spherical organism, which produced acid and carbon dioxide in glucose-containing media, and formed slime in sucrose-containing media. From these characteristics, it was identified as belonging to the genus *Leuconostoc*. It was also found that the bacterium produced acid in a medium containing arabinose as the sole carbon source. This is a specific property of *Leuconostoc mesenteroides* which is used to differentiate this bacterium from the other species in the genus [116].

Note 1:

Tryptone	10 g
Sodium chloride	1 g
Agar	20 g
Filtered cane juice	1 000 g
pH	7,0

A sucrose-containing medium (note 1) was prepared and sterilised for 15 minutes at a pressure of 1,6 bar in conical flasks closed with a cotton wool plug. These solutions were inoculated with the isolated organism and incubated for 3 weeks at 25°C.

The dextran formed was precipitated using the method described for cane polysaccharide (page 159). Repeated purification by precipitation with ethanol resulted in an ash and protein-free product.

8.1.7.2 Acid hydrolysis of the cane polysaccharide and identification of the hexose formed

Two hundred mg of the polysaccharide was dissolved in 5 ml of 4 N sulphuric acid and maintained at 100°C for 5 hours in a sealed tube. After cooling, the tube was opened and the solution neutralised with solid barium carbonate. The precipitate formed and the excess barium carbonate were removed by filtration and washed with distilled water. The combined filtrate and washings were concentrated *in vacuo* to a volume of 0,5 ml. A three microlitre portion of this concentrate was applied to a sheet of chromatographic paper (Whatman No. 1). The following eluants were used

propanol	: ethyl acetate	: water	7:1:2	[118]
n-butanol 1	: ethanol	: water	4:1:1	[119]
ethyl acetate	: acetic acid	: water	3:1:3	[119]
n-butanol 1	: ethanol	: water	2:1:1	[120]

Note 1:

sucrose	100 g	ferrous sulphate	0,01 g
di-K hydrogen phosphate	1 g	peptone	3 g
magnesium sulphate 7 aq	0,5g	water	1 000 g
potassium chloride	0,5g	pH	7,0

After drying, the sheet was impregnated with 0,1 N silver nitrate in acetone (note 1). To obtain a lightly coloured background the sheet was immersed once rapidly in a bath of the reagent and dried in the dark. After drying, the sheet was dipped in the following solution:

sodium hydroxide	40 %	25 ml
ethanol		475 ml

After thorough washing, the spots were fixed by immersing the paper in a solution containing:

sodium thiosulphate	50 g
sodium meta bisulphate	7,5 g
acetic acid	7,5 ml
water	500 ml

Subsequently the paper was washed and dried. Various hexoses and pentoses were applied to the same paper as references. These included arabinose, ribose, xylose, mannose, glucose, fructose, and galactose. The positions of the spots obtained from the references were compared with the spot obtained from the polysaccharide hydrolysate.

The phenylosazone was prepared by adding to 4 ml of the purified hydrolysate containing about 200 mg carbohydrate, 0,6 g of sodium acetate and 0,4 g of phenyl hydrazine hydrochloride. The mixture was kept in a boiling water bath for 5 minutes, after which crystallisation occurred. The crystals were separated by filtration using a microfilter, recrystallised from hot 60 % ethanol, and dried *in vacuo*.

Note 1: Water was added to this reagent until the solution became clear.

8.1.7.3 Periodate oxidation

8.1.7.3.1 Analytical methods

I Oxidation of maltose

100 mg of pure maltose was dissolved in 50 ml water and cooled to 4°C (equivalent to 0,2924 millimol). This solution was mixed with a cooled solution containing 400 mg of sodium meta periodate (equivalent to 1,896 millimol) in 40 ml of distilled water. The mixture was made up to 100 ml with distilled water at 4°C and kept at 4°C in the dark. At daily intervals, the periodate reduction was determined using the method of Fleury and Lange [127] . A 5 ml aliquot of the solution was pipetted into a 100 ml flask. Ten ml of saturated sodium bicarbonate solution was added, followed by 20 ml of 0,01495 N sodium arsenite solution, and 2 ml of 20 % potassium iodide solution. The resulting solution was kept in the dark at 20°C for 15 minutes and then titrated against 0,0091 N iodine solution. A solution containing 400 mg of sodium meta periodate in 100 ml of distilled water used as a blank. It was maintained at 4°C in the dark and was titrated daily. The results of the maltose oxidation were corrected for the decomposition of the periodate in the blank.

II Titration of the formic acid formed [126] .

When the periodate oxidation was complete (after 7 days), a 25 ml aliquot of the resultant solution was treated with a few drops of ethylene glycol, and left to stand for one hour in the dark. After this procedure to remove the excess periodate, the formic acid was titrated against standard barium hydroxide solution (0,00673 N). The titration was monitored with a pH meter provided with a glass-calomel electrode pair. After the initial experiment, the endpoint was determined as pH 7,4.

The blank solution was treated in the same way and titrated to a pH of 7,4. This titration value (0,2 ml) was applied as correction.

8.1.7.3.2 Oxidation of dextran

A solution containing 150 mg of dextran (equivalent to 0,926 millimol of glucose units) was treated with 321 mg of sodium meta periodate (equivalent to 1,5 millimol) in 100 ml of water as described above. The periodate reduction was determined daily (see Fig. 9, page 76). The formic acid formed was determined after seven days.

8.1.7.3.3 Oxidation of cane polysaccharide

The quantities used and the corrections applied for the oxidation of cane polysaccharide were the same as for dextran (see 8.1.7.3.2).

8.1.7.4 Methylation of starch, dextran, and cane polysaccharide and determination of the methanolysis products

8.1.7.4.1 Haworth methylation

Fifteen g of polysaccharide was dissolved in 150 ml of an ice-cooled 30 % aqueous solution of sodium hydroxide. The solution was placed in a three neck flask, provided with a reflux condensor and stirrer. The flask was cooled in an ice bath. 180 ml of dimethyl sulphate and 250 ml of 30 % sodium hydroxide solution were added over a period of 10 hours, in 30 equal portions. Foaming was prevented by the addition of some diethyl ether.

When dextran was methylated it was more difficult to keep in solution and 4 x 50 ml portions of acetone were added at intervals [130] . After the last addition of DMS, the reaction mixture was stirred for a further few hours.

The temperature during the second Haworth methylation was gradually increased from ambient at the start to a final 50°C. At the end of the reaction the mixture was cooled in ice and neutralised with 5 N sulphuric acid. In the case of dextran, the acetone was first removed by distillation. The salts were subsequently removed by dialysis against running tap water in "Visking" tubing. The residue was concentrated and the final product dried. Complete drying between

the methylation steps was avoided, as this rendered the product nearly insoluble. Only the small portion required for a methoxyl determination was dried completely.

Starch was acetylated prior to the Haworth methylation [147]. Twenty g of soluble starch was treated with 6 times its weight of glacial acetic acid through which chlorine had been bubbled for a few seconds. This mixture was stirred for 30 minutes at 20°C. After the addition of 400 g of acetic anhydride, containing a small amount of sulphur dioxide, the temperature was raised to 55°C. This mixture was stirred for 4 hours. A clear solution was obtained and poured into an excess of cold water. The precipitate formed was washed for two days with water, followed by dilute alcohol, and finally ether, after which it was dried *in vacuo*. The acetyl content was determined by boiling an aliquot of the acetylated starch for 30 minutes with 1 N sodium hydroxide and titrating the hydroxide which remained after the deacetylation.

The acetylated starch was dissolved in acetone and methylated in the same way as dextran.

8.1.7.4.2 Methoxyl determination.

This determination was carried out on 5 - 10 mg of the methylated polysaccharide, dried over phosphorus pentoxide. The sample was weighed in a short piece of 3 mm O.D. glass tube, closed at one end. The scrubber of the Zeisel apparatus was filled with 7 ml of acid-free phosphorus slurry. The receiver was filled with 5 ml of a solution prepared by dissolving 10 g of sodium acetate in 57 ml of glacial acetic acid. Three drops of bromine were added to the solution in the receiver.

The sample in the glass tube was dropped into the flask and 5 ml of hydriodic acid was added. Carbon dioxide was passed through the apparatus for about 5 minutes, after which the flask was heated on a glycerol bath at 140 - 160°C for 1,5 hour.

After cooling, the receiving solution was washed into a 150 ml conical flask containing 3 ml of a 20 % aqueous solution of sodium acetate. The mixture was diluted to 50 ml and a few drops of 80 % formic acid were added until the bromine colour disappeared. Five ml of 20 % potassium iodide solution and 5 ml of sulphuric acid were added and the mixture was titrated against 0,05 N sodium thiosulphate solution.

The hydriodic acid was prepared from commercial hydroiodic acid to which a few ml of hypophosphorous acid had been added. This mixture was distilled in a stream of carbon dioxide, and the constant boiling point fraction (125°C) was collected in 5 ml portions in small test tubes. These tubes were sealed under carbon dioxide and stored in a refrigerator in the dark until used.

8.1.7.4.3 Kuhn methylation [131].

Ten g of the partially methylated polysaccharide prepared by Haworth methylation was dissolved in 120 ml of dry dimethyl formamide. Forty five ml of methyl iodide, dried over calcium chloride, was added to this solution. The mixture was stirred at room temperature in a three neck flask provided with a reflux condenser. Forty five g of silver oxide was added in small portions over a period of 8 hours, after which the stirring was continued for two days. After centrifuging to remove the insolubles, the precipitate was washed 5 times with 50 ml portions of DMF followed by 5 x 50 ml of chloroform. The washings were combined with the supernatant liquid. This combination was washed with 500 ml of 1 % potassium cyanide solution. The aqueous layer was separated and washed 5 times with chloroform. The combined chloroform solutions were washed a further 5 times with water after which the solution was concentrated to near dryness by vacuum distillation. The final residue was dried in air, followed by drying over phosphorus pentoxide *in vacuo*.

The methylated starch was purified in a slightly different way. It was dissolved in a small quantity of chloroform, after which petroleum ether was added and the mixture heated. The

precipitate formed was separated by filtration and dried *in vacuo*.

The DMF for the methylation was dried over phthalic anhydride after which it was distilled *in vacuo*.

The silver oxide was prepared by adding a hot, filtered solution of silver nitrate (100 g per 500 ml) to a hot filtered solution of barium hydroxide (100 g per litre) [132]. The precipitated silver oxide was washed with boiling water until the excess of barium hydroxide was removed. The filtered product was dried and stored in a desiccator.

8.1.7.4.4 Methanolysis and gas chromatographic analysis of the methyl glucosides (see note 1).

The methylated starch, dextran, and cane polysaccharide were methanolysed in a sealed test tube at 105°C for 15 hours, using absolute methanol containing 5 % hydrochloric acid. After cooling, the tubes were opened and the solution was neutralised with silver carbonate and filtered. The filtrate was used for chromatographic analysis [150].

The instrument used was a Perkin Elmer F 11 gas chromatograph, fitted with a flame ionisation detector. A 1,80 m, 3 mm O.D. stainless steel column was packed with 5 % diethylene glycol succinate polyester on Chromosorb W. The carrier gas was nitrogen at a flow rate of 55 ml per minute.

Tetra- and trimethyl glucosides were analysed at an oven temperature of 140°C. The dimethyl glucosides were analysed using the same column at an oven temperature of 180°C.

Note 1: Initially, no gas chromatograph was available. A sample of dextran and cane polysaccharide was analysed by Dr. J. McD Blair of the University of Cape Town, using a Beckman GC-2A gas chromatograph.

8.1.8 Infrared spectra

Ten to twenty mg of the dried and powdered polysaccharide was mixed with 200 - 300 mg of potassium bromide, under an infrared lamp, to prevent absorption of moisture. Discs were made from the mixture by pressing at 4 500 bar and the spectra were recorded using a Perkin Elmer model 521 spectrophotometer.

Reproductions of the spectra are shown in Figs. 17, 18 and 19 (page 99-101).

8.1.9 Enzymic hydrolysis of the cane polysaccharide

8.1.9.1 Production of pullulan

The strain of *Aureobisidium pullulans* received from London was maintained in malt agar slants. These tubes were used to inoculate 500 ml round bottomed flasks containing the following medium:

ammonium sulphate	3 g
dipotassium hydrogen phosphate	1 g
magnesium sulphate 7 aq.	0,5 g
potassium chloride	0,5 g
ferrous sulphate 7 aq.	0,01 g
sucrose	100 g
tap water	1 000 ml
yeast extract	100 g
pH	7,2

The flasks were plugged with cotton wool and shaken for 5 days on an orbital shaker at 25°C. The cells were subsequently separated from the medium by centrifuging. The solution was filtered through kieselguhr and the polysaccharide was precipitated from the filtrate by addition of acetone in the ratio 1,2 volumes of acetone per one volume of filtrate. The precipitate was separated by centrifuging, redissolved in water, and reprecipitated with acetone. This procedure was repeated twice. The finally precipitated

pullulan was obtained as a white voluminous powder. No difference was observed in the final product if glucose was used instead of sucrose as a carbon source in the culture medium.

The acid hydrolysis and chromatography of the hydrolysate was carried out as described for the cane polysaccharide (page 172).

Enzymic hydrolysis with pullulanase was carried out by incubating for 4 hours at 40°C a mixture containing:

9 ml of 0,2 M	phosphate buffer, pH 6,0 (note 1)
10 mg of	pullulanase powder
1 ml of 2 %	pullulan solution

Residual amylase activity in the pullulan sample was destroyed by heating to 90°C for 15 minutes prior to use.

After the incubation, the residual enzyme was removed by adding acetone in the ratio 1,6 volumes acetone per one volume of the digest. The precipitate formed was left to stand overnight and removed by centrifugation. The clear liquid was deionised using a small quantity of Amberlite MB 1 mixed bed ion exchange resin, after which the deionised solution and the wash water from the washing of the resin were combined and concentrated under vacuum.

The chromatogram was run for 36 hours using n-butanol: ethanol:water (2:1:1) as eluent. Spots were developed as described previously (page 172).

Note 1: citric acid 0,1 gmol per 1 000 ml, sodium phosphate 0,2 gmol per 1 000 ml, in the ratio 3,68:6,32.

Amylose was prepared according to Schoch [141] . A 3 % aqueous solution of potato starch was autoclaved at 2,5 bar absolute at pH 6,0. n-Butanol was added to the hot solution to a final butanol concentration of 10 %. The mixture was stirred continuously during cooling and then kept in a refrigerator. After separation of the precipitate by centrifuging, the amylose was washed with 10 % methanol, followed by absolute methanol and dried under vacuum.

One hundred mg of amylose was partially hydrolysed by heating for 20 hours with 5 ml of 0,1 N sulphuric acid, at 105°C, in a sealed test tube. The unhydrolysed starch was subsequently removed by precipitation with 5 ml of ethanol, followed by filtration. The filtrate was deionised using 250 mg of Amberlite MB 1 resin and the deionised solution was concentrated *in vacuo*.

This concentrate was used as a reference in chromatograms showing the complete series of α (1 → 4) malto oligosaccharides.

8.1.9.2 Production and isolation of pullulanase

The two strains of *Aerobacter aerogenes* were tested, using the standard tests for the coli-aerogenes group.

(a) Indole test

The organism was grown in tryptone water (note 1) for 24 hours at 35°C. After incubation the content of the tube was shaken with 1 ml of the following reagent:

para-dimethyl aminobenzaldehyde	5 g
n- amyl alcohol	75 ml
hydrochloric acid (s.g.1,19)	25 ml

A red colour formation occurs if the reaction is positive.

Note 1: Tryptone 10 g per 1 000 ml, sodium chloride 5 g per 1 000 ml.

(b) Methyl red test

The organism was incubated for 5 days at 30°C in a medium containing:

peptone	5 g
dextrose	5 g
dipotassium hydrogen phosphate	5 g
water	1 000 ml

After incubation, 5 drops of methyl red indicator were added to 5 ml of the culture liquid.

A red colour is formed if the reaction is positive.

(c) Voges-Proskauer test

Five ml of 10 % potassium hydroxide solution was added to 5 ml of the culture liquid, prepared for the methyl red test (see (b)). A red colour is formed after 6 hours of standing if the reaction is positive.

(d) Citrate test

The organism was incubated in a medium containing:

sodium citrate 5,5 aq.	2,8 g
sodium ammonium phosphate 4 aq.	1,5 g
potassium phosphate, primary	1,0 g
magnesium sulphate 7 aq.	0,2 g
water	1 000 ml

The test registered positive if growth continued, as this indicated that the organism was able to use citrate as the only source of carbon.

The strain from London was used for pullulanase production. The bacteria were cultivated and maintained on agar slants of the following composition.

malt extract	3 g
peptone	3 g
sodium chloride	0,7 g
dipotassium hydrogen phosphate	0,7 g
glucose	6 g
agar	25 g
water	1 000 ml
pH	7,0

These slants were incubated for 3 days at room temperature, subsequently the cells were transferred into 500 ml round bottomed flasks containing 250 ml of the following medium:

malt extract	3 g
peptone	3 g
sodium chloride	0,7 g
dipotassium hydrogen phosphate	0,7 g
glucose	6 g
water	1 000 ml
pH	7,0

The flasks were shaken at room temperature for 2 days. The culture liquid from these flasks was used to inoculate the main culture.

Six ml of the preculture was transferred to similar round bottomed flasks containing 250 ml of the main culture broth:

magnesium sulphate 7 aq.	0,5 g
sodium nitrate	5 g
dipotassium hydrogen phosphate	1 g

ferrous sulphate 7aq.	0,01	g
maltose	5	g
peptone	8	g
water	1 000	ml
pH	6,6	

The main culture was shaken for 3 days at room temperature on an orbital shaker. The activity of the culture liquid was tested by mixing together the following:

phosphate buffer pH 6,0 (note 1)	7 ml
culture liquid	2 ml
pullulan solution, 2 %	1 ml

This mixture was incubated at 40°C for 4 hours. The increase in reducing sugars was measured by titrating the original mixture and the digest after 4 hours with a ferricyanide reagent, using the method described by Hagedorn and Jensen [143] .

The broth, which showed sufficient enzyme activity, was collected and centrifuged at 3 000 r.p.m. to remove most of the bacterial cells. The solution was then filtered through kieselguhr and the clear filtrate cooled to 4°C.

Cold acetone was added to the filtrate, in the ratio 3 volumes acetone to 2 volumes filtrate, and the mixture was kept overnight in the refrigerator. The precipitate was centrifuged off and purification carried out by extracting the crude product with 50 ml of 0,2 M phosphate - citrate buffer, pH 6,8. The remaining solids were further extracted with 25 ml of buffer solution for 12 hours, after which the extracts were combined and centrifuged. This enzyme extract was used for enzymic hydrolysis. Prior to its use this extract was heated for 1 min at 90°C to destroy traces of amylase.

Note 1: Citric acid 0,1 g mol per 1 000 ml, sodium phosphate 0,2 g mol per 1 000 ml, in the ratio 3,68: 6,32.

8.1.9.3 Action of various enzymes on cane polysaccharide and pullulan

20 mg of either the cane polysaccharide or pullulan was dissolved in 10 ml of phosphate - citrate buffer, adjusted to the optimum pH for the particular enzyme to be used. The enzyme was added and the mixture stirred and incubated at 40°C. The quantities of each enzyme used and the pH for each test were as listed below:

enzyme type	mg enzyme powder	pH of buffer
malt amylase	5	5,5
B. subtilis amylase	10	6,5
pullulanase	10	6,0
blank	-	5,0

The reducing power of the enzyme substrate mixture was determined at various time intervals, using the ferrycyanide method of Hagendorn and Jensen [143]. A blank was included but no increase in reducing power was found in the blank after 25 hours. The results are plotted in Figs. 21 and 22. A similar experiment was carried out using 20 mg of dextran and 10 mg of pullulanase powder in a phosphate-citric acid buffer at pH 6,0.

For the quantitative determination of the oligosaccharides formed by the hydrolysis of cane polysaccharide with pullulanase, 48 mg of cane polysaccharide was dissolved in 0,02 M phosphate - citric acid buffer, pH 6,0 and boiled for 5 minutes to destroy traces of amylase. After cooling, 92 mg of dry pullulanase powder was added and dissolved. This mixture was incubated for 4 hours at 40°C, after which the residual enzyme was precipitated by adding cold ethanol in the ratio 1,6 volumes ethanol to one volume of the digest. The solution was kept overnight and the precipitate removed by centrifugation. The supernatant liquid was deionised by shaking with 5 % $^{w}/_{v}$ of Amberlite MB 1 mixed bed ion exchanger. The resin was filtered off and washed with distilled water. The filtrate and combined washings were concentrated under vacuum.

For the chromatographic analysis, 45 µl of the concentrate, containing 2,5 mg of total sugars, was applied along a 5 cm wide section of the starting line of the Whatman 3 MM paper chromatogram. Guide spots were placed at a distance of 4 cm on either side of this section and the paper was eluted with n-butanol : ethanol : (2:1:1), for 60 hours. Subsequently, the paper was dried and the guide strips were cut off and developed with silver nitrate reagent. The positions of the various oligosaccharides derived from the concentrate were located with the aid of the guide strips. Each portion was cut into ten pieces, placed in a 100 ml beaker, covered with distilled water, and left soaking for one hour. The solution was then filtered through a 0,45 µm membrane filter, the filter washed four times with 5 ml water, and the final filtrate made up to 25 or 50 ml, depending on the oligosaccharide concentration present in the fraction. A 10 ml aliquot of this solution was pipetted into a boiling tube and 2 ml of 10 N hydrochloric acid was added. The tube was placed in a boiling water bath for 25 minutes, after which 2 drops of 0,005 % bromo thymol blue solution were added. After cooling, the solution was neutralised with 10 N sodium hydroxide. The solution was then titrated according to the method of Hagedorn and Jensen [143]. A strip of chromatographic paper taken from between the oligosaccharide fractions was treated in the same way as the paper containing the fractions themselves and used as a blank.

The final result, expressed in mg of glucose, was corrected for the degree of polymerisation of the oligosaccharide, to obtain the amount of oligosaccharide present in each fraction.

8.1.10 Conditions of formation of the polysaccharide

8.1.10.1 Influence of moisture

Sugar cane was cut into 10 cm pieces and divided into three subsamples. One was kept under atmospheric conditions in a tray; the second was placed in a large flask with some distilled water at the bottom; the third was stored in a closed flask provided with inlet and outlet tubes, through which a stream of water-

saturated air was passed continuously.

The polysaccharide content of the filtered juice from the original cane and from these three subsamples after they had been stored for 3 weeks, was determined by ethanol precipitation as described in an earlier section.

8.1.10.2 Formation of polysaccharide under aseptic conditions

Sugar cane pieces, 10 cm long, were placed in a large suction flask into which was poured a solution containing 20 000 units of penicillin G per litre. A vacuum pump was applied to the flask for about one hour until air no longer escaped from the pieces of cane. This procedure was adopted to ensure that all vascular bundles and inter cellular spaces were filled with the penicillin solution. After the treatment, the pieces were drained and stored in a tray.

The sample used for control was impregnated with distilled water, using the same technique.

The sensitivity of *Leuconostoc mesenteroides* towards penicillin G was determined on juice-tryptone agar:

cane juice (filtered)	1 000 g
sodium chloride	1 g
tryptone	10 g
agar	20 g

This medium was poured into Petri dishes and, after solidification, a suspension of *L. mesenteroides* in sterilised water was spread evenly over the surface. Penicillin G solutions of different concentrations were prepared. Discs of filter paper, 7 mm in diameter, were impregnated with 1 µl portions of these solutions and placed on the surface of the suspension in the Petri dishes. After 3 days incubation at room temperature, the bacteria-free areas were measured.

8.1.10.3 Isolation of micro organisms

Juice from deteriorated cane was diluted with sterilised water and spread evenly over Petri dishes filled with juice-tryptone agar. After 3 days of incubation at room temperature, those isolated colonies which had produced a polysaccharide were selected. The polysaccharide formation was judged by the slimy appearance of the colony.

Bacteria from these colonies were inoculated into 500 ml round bottomed flasks, containing sterilised Czapek-Dox solution to which 10 % sucrose had been added. The cultures were then shaken for five days at room temperature on an orbital shaking machine.

The bacteria cells were removed by centrifugation and the polysaccharide formed in the shake culture precipitated using ethanol. The crude polysaccharide was purified by repeated dissolution and precipitation, and the purified product was dried *in vacuo*.

The action of pullulanase on a 2 % solution of these isolated polysaccharides was investigated, as described earlier, by using the ferricyanide titration to measure the increase in reducing power during incubation.

9. FORMATION OF ALCOHOL

Five hundred ml of the cane juice sample was used for distillation. A 60 cm fractionating column was packed with stainless steel gauze rings (Dixon rings) and provided with an electrically heated jacket. The temperature of the jacket was maintained near the boiling point of the fraction to be collected. A reflux condenser, on top of the column, was fitted with a tap to control the reflux ratio. This ratio was adjusted to obtain efficient separation; the approximate ratio of reflux to product was 9 to 1.

The distillate obtained was refluxed for one hour with a mixture containing 250 ml of water, 50 ml of concentrated sulphuric acid and 100 g of potassium dichromate. This treatment was used to oxidise the alcohol to the corresponding acid. After the reaction

was completed, 80 ml of the mixture was distilled and collected. This distillate was adjusted with ammonia to pH 9 and concentrated to a few millilitres. A 1 µl spot was placed on a sheet of Whatman No. 4 chromatographic paper which previously had been washed with 1 % aqueous oxalic acid solution, followed by distilled water, and dried at room temperature. This treatment was used to prevent the occurrence of ghost spots. The spot was eluted in ascending mode with 25 % ammonia : ethanol, in the ratio 1:100.

After elution the paper was dried at room temperature and sprayed with a solution containing 50 mg of bromophenol blue and 200 mg of citric acid in 100 ml of water. The spots appeared blue on a yellow background.

For the preparation of the molybdate-xanthate complex, a drop of the distillate was mixed with a drop of carbon disulphide and some powdered sodium hydroxide. The mixture was shaken for 5 minutes, after which one drop of 1 % ammonium molybdate solution was added. This mixture was then acidified with 2 N sulphuric acid and shaken with a few drops of chloroform. A violet colour in the chloroform layer proved the presence of primary or secondary alcohols.

10. FORMATION OF ORGANIC ACIDS

10.1 Analysis of volatile acids in Natal sugar cane

Two ml of 80 % phosphoric acid was added to 400 ml of cane juice. The mixture was distilled until 250 ml of distillate had been collected. Twenty five ml of this distillate was titrated against 0,01 N sodium hydroxide, using phenolphtalein as indicator.

The residual 225 ml of distillate was treated with ammonium hydroxide to pH 9 and concentrated *in vacuo* to 1 ml. One µl of this concentrate was applied to Whatman No. 4 chromatographic paper

which had been previously washed with an oxalic acid solution. The spot was eluted in ascending mode with 25 % ammonia : ethanol, (1:100).

After elution, the paper was dried at room temperature and sprayed with a solution containing 50 mg bromophenol blue and 200 mg citric acid in 100 ml water.

The method was tested by dissolving 1 g of acetic acid and 1 g of butyric acid in 1 litre of cane juice. Clear spots were obtained using the method described above, and the respective R_f values were 0,50 and 0,66.

10.2 Non volatile organic mono- and dicarboxylic acids

10.2.1 Investigation into analytical methods for carboxylic acids

10.2.1.1 Paper chromatography according to Stark [156].

An aqueous solution of analytically pure aconitic, succinic, and malic acids was prepared, each in a concentration of 1 %.

One µl of the solution was applied to a sheet of Whatman No. 1 chromatographic paper and eluted in ascending mode. The eluent used was:

phenol	3 g
water	1 ml
formic acid 90 %	1 %

The inorganic acids present as impurities in the phenol have an R_f value of less than 0,25. It is necessary to take precautions to prevent contamination of the chromatogram by these impurities. For this reason, only three quarters of the available paper length was used and the sample spots were applied along a line one quarter of the paper length above the surface of the eluent in the reservoir.

The paper was eluted for 16 hours, dried at room temperature, and sprayed with the following reagent:

bromo-phenol blue	50 mg
ethanol 95 %	100 ml

This reagent mixture was made slightly alkaline with sodium hydroxide. Acids appeared on the paper as yellow spots on a blue background.

10.2.1.2 Ion exchange

A 250 mm long, 10 mm diameter column was filled with Amberlite IRA 400 resin in OH^- form. Fifty ml of cane juice was passed through this column and followed by distilled water, until all the sugars had been removed.

The acids were eluted with 20 ml of 0,1 N sodium hydroxide. One µl of the eluate was applied to chromatographic paper and treated as described in the previous section (10.2.1.1).

10.2.1.3 Silica gel column chromatography

(a) Purification of the silica gel (note 1)

The gel was ground in a agate mortar and sieved through a 100 mesh screen. The screened powder was treated with concentrated hydrochloric acid, washed with distilled water until acid free, and then dried.

(b) Column packing

Twenty two grams of silica gel, which had been dried overnight at 105°C, was mixed with 9 ml of 0,5 N sulphuric acid. If this amount of acid was exceeded, the silica gel became sticky and would not flow freely.

Note 1: May and Baker Silica Gel for Chromatography.

Chloroform was equilibrated by shaking in a separating funnel with 0,5 sulphuric acid. The chloroform layer was separated and filtered through dry filter paper to remove any small droplets of sulphuric acid.

The acid-treated silica gel powder was mixed with equilibrated chloroform to form a slurry which was washed into a 180 mm long, 16 mm diameter, glass column.

(c) Application of the acids

The acids were dissolved in the smallest possible quantity of 0,5 N sulphuric acid and mixed with sufficient dry silica gel powder to obtain a free flowing powder. This powder was mixed with a few ml of equilibrated chloroform and poured onto the top of the column.

A mixture of aconitic, malic, and succinic acids was used for trial purposes. A gradient elution programme was followed and 10 ml fractions were collected. These fractions were titrated against 0,01 N sodium hydroxide solution.

The elution programme used was as follows:

i	chloroform 100 %	100 ml, fractions 1-10
ii	chloroform 95 % n-butanl 5 %	100 ml, fractions 11-20
iii	chloroform 90 % n-butanol 10 %	100 ml, fractions 21-30
	progressing to:	
x	chloroform 55 % n-butanol 45 %	100 ml, fractions 91-100

All eluents were equilibrated with 0,5 N sulphuric acid and filtered before use.

(d) Titration, indicator solutions

Isherwood found that an alcoholic solution of phenolphthalein was a suitable indicator for the titration of the acid fractions with sodium hydroxide in alcohol [159].

A mixture of 100 mg of phenol red and 5,7 ml of 0,05 N sodium hydroxide diluted to 100 ml with distilled water, was found to be a better indicator. Before use this mixture was diluted with an equal quantity of 2 % Teepol solution.

The titrant used was 0,01 N aqueous sodium hydroxide solution. Depending on the amount of acids present in the fractions, the titration was carried out with either a 5 ml microburette or a 50 ml standard burette. A stream of air from which carbon dioxide had been removed by bubbling it through concentrated potassium hydroxide solution in a wash bottle, was passed through the titration vessel during the titration.

(e) Preparation of silica gel according to Isherwood [157].

A little methyl orange was added to a solution of 2,75 kg of commercial water glass, diluted with twice its volume of water. The solution was filtered and 10 N hydrochloric acid was added, in a thin stream, with vigorous stirring. When the mixture became acid, all the lumps were broken up by stirring, and a further 200 ml of 10 N hydrochloric acid was added.

The mixture was allowed to stand for several hours and then filtered through a Buchner funnel, using hardened filter paper. After the residue had been dried as dry as possible by suction, it was suspended in 5 litres of 10 N hydrochloric acid and allowed to stand overnight. The mixture was subsequently filtered and washed with 10 litres of 5 N hydrochloric acid; care was taken to ensure that the residue in the filter did not break up during washing.

The filtrate was checked to ensure that no ferric ions were present. The gel was then washed with about 20 litres of distilled water, until free of chloride ions, and finally washed with 10 litres of absolute ethanol.

The powder was dried in air and stored for two weeks. After this storage period, it was suspended in 7 litres of 10 N hydrochloric acid and allowed to stand overnight. The gel was then filtered and washed in sequence with 10 litres of 5 N hydrochloric acid, distilled water until chloride free, and ethanol.

The gel was dried in warm air and screened through a 100 mesh screen. Before use it was dried under vacuum over phosphorus pentoxide.

A column was packed with this gel, using the techniques described earlier (10.2.1.3). The water-absorbing capacity was much higher than for the other gels used. A column height of 180 mm was obtained in a 16 mm column, by mixing 8 g of the prepared gel with 8 ml of 0,5 N sulphuric acid.

10.2.2 Analysis of acids in normal and deteriorated sugar cane

10.2.2.1 Clarification of the juice

Saccharated lime was added to the juice to pH 8,0. The mixture was then boiled for 5 minutes and filtered through filter paper.

10.2.2.2 Isolation of acids by ion exchange using the method of Lee and Resnik [177].

A 10 mm diameter glass column was filled with 20 ml of Amberlite IRA 400 in chloride form. The resin was converted from the chloride form into the carbonate form by washing it with 500 ml of 2 N sodium carbonate solution. The surplus sodium carbonate was

subsequently removed by washing with 100 ml of 1,5 N ammonium carbonate solution, followed by distilled water.

A trial was carried out using a solution of 25,5 mg of succinic acid in 50 ml distilled water. After the column had been rinsed with distilled water, the acid was eluted with 1,5 N ammonium carbonate solution, which was collected in a 100 ml volumetric flask. A 25 ml aliquot was heated at 70°C until the smell of ammonia had disappeared.

The residual ammonium succinate solution was passed through a column containing Amberlite 120 cation exchanger in hydrogen form. The succinic acid which resulted was titrated with 0,01 N sodium hydroxide solution using phenol red as indicator. The recovery of acid was 95 %.

The acids in clarified cane juice were isolated by passing a mixture of 200 ml of clarified juice and 200 ml of distilled water through the Amberlite IRA 400 ion exchange column. The eluate was concentrated to dryness.

10.2.2.3 Separation of lactic and succinic acids on silica gel

A powdered mixture containing 8,8 mg of lactic acid and 10,6 mg of succinic acid was poured into the silica gel column. The acids were eluted using the following programme:

i	95 ml benzene, 5 ml n-butanol
ii	90 ml benzene, 10 ml n-butanol
iii	85 ml benzene, 15 ml n-butanol
iv	80 ml benzene, 20 ml n-butanol

This programme was sufficient to ensure complete elution of all the succinic and lactic acids. The results are shown in Fig. 26.

10.2.2.4 Identification of aconitic acid and succinic acid isolated from cane juice

(a) The pyrrole test

Some of the isolated succinic acid crystals and a few milligrams of zinc dust were heated together in a test tube. The tube was covered by a disc of filter paper which had been moistened with a 5 % solution of para-dimethyl aminobenzaldehyde in a mixture of 80 % benzene and 20 % trichloro-acetic acid. The formation of a pink colour indicated the presence of succinic acid.

(b) The fluorescein test

A few milligrams of the isolated succinic acid were mixed with some resorcinol and a few drops of concentrated sulphuric acid and heated for 5 minutes. After cooling water was added and the solution was made alkaline with sodium hydroxide. A brilliant green fluorescence indicated succinic acid.

(c) The presence of aconitic acid was confirmed by boiling a few crystals of the isolated acid in 1 ml of acetic anhydride. A violet colour developed, indicating the presence of aconitic acid.

II LITERATURE REFERENCES

PREFACE

Titles of journals in the references are abbreviated according to the list prepared by the American Standards Association, Sectional Committee Z39 on Library Work and Documentation [200] .

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