"COMPLEXES OF CARBOHYDRATES WITH MAGNESIUM-IONS"

"THE ISOLATION OF AN OLIGOSACCHARIDE CONTAINING L-GALACTOSE FROM THE POLYSACCHARIDE OF *Aeodes orbitosa*"

"HORIZONTAL CELLULOSE COLUMN CHROMATOGRAPHY OF SUGARS"

by

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A THESIS

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for dad

may he see in it his own

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1 COMPLEXES OF CARBOHYDRATES WITH MAGNESIUM-IONS

1.1 ABSTRACT

Sugar complexes with anions and cations are reviewed. Evidence is presented for the formation of a complex of stoichiometry 1:1 in aqueous solution between methyl-α-D-glucopyranoside and magnesium perchlorate. The complex may be detected by the method of continuous variations. Measurements of changes in the proton chemical shifts of the glucoside in deuterium oxide, indicate that the complex is probably formed between the vicinal hydroxyl groups at C-2 and C-3 of the pyranoside ring and the hydrated cation. At elevation temperatures there is evidence for the presence of a complex of stoichiometry 2:1. Experiments conducted on cellulose indicate the possible formation of cellulose - magnesium-ion complexes. These complexes provide an explanation for the "protective action" of magnesium compounds on the cellulose portion of pulp during alkali-oxygen bleaching.

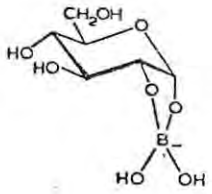
1.2 SUGAR COMPLEXES WITH ANIONS AND CATIONS

Many examples exist in different areas of carbohydrate chemistry where the interaction in solution between sugars and anions or cations has been interpreted in terms of the formation of soluble complexes. The capacity of molybdic acid or phospho-tungstic acid to complex with oligosaccharides has been used in the field of thin layer chromatography[1]. Silica gel layers impregnated with these acids have been found most effective in the separation of multi-component sugar mixtures. Paper chromatography on sheets pretreated with barium chloride and barium acetate has been effective in the efficient and rapid separation of monosaccharides[2], while a solvent system saturated with boric acid has been developed for paper chromatographic use[3]. The complexes formed between borate and the sugars are responsible for large R_f values and faster resolution times for the sugar mixtures. A comprehensive review by Böeseken[4] on the reaction between carbohydrates and boric acid has been augmented by Foster[5] and subsequently by Capon and Overend[6] and by Weigel[7].

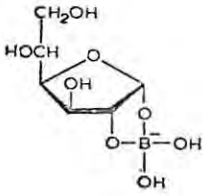
Paper electrophoresis and n.m.r. studies of complexes between polyols and diphenylborinic acid have appeared[8], while the novel application of ^{11}B n.m.r. spectroscopy[9] has indicated the ring sizes in which boron is involved during the formation of sugar complexes. Five-membered ring complexes involving O-1 and O-2 are formed with both furanoid and pyranoid forms of D-glucose, while methyl-α-D-glucopyranoside forms six-membered borate complexes in which O-4 and O-6 are bridged by boron. Methyl-α-D-galactopyranoside, however, is capable of forming a five-membered complex in which O-3 and O-4 are involved and a six-membered complex in which O-4 and O-6 are bridged by boron (Figure i).

Figure i Some borate - carbohydrate complexes

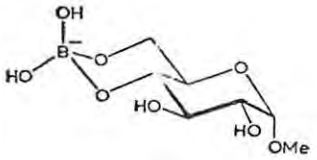
1,2- borate complex of α-D-glucopyranose



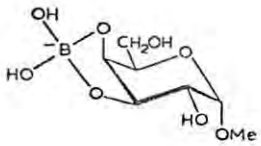
1,2- borate complex of α-D-glucofuranose



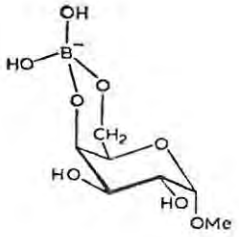
4,6- borate complex of methyl-α-D-glucopyranoside



3,4- borate complex of methyl-α-D-galactopyranoside



4,6- borate complex of methyl-α-D-galactopyranoside



Jacin[10], by the use of gas liquid chromatography, has detected and determined the formation of carbohydrate - borate complexes. The carbohydrates studied were D-glucose, methyl-α and β-D-glucopyranose, 2-deoxy-D-glucose, 3-O-methyl-α-D-glucopyranose, D-galactose, 2-deoxy-D-galactose, D-mannose, methyl-α-D-mannopyranoside and L-fucose, which were reacted with borate-ions and the products silylated after which the trimethylsilyl ethers were examined by g.l.c.

Perlin and von Rudloff[11] have reported the use of proton magnetic resonance (p.m.r.) spectroscopy in the study of tridentate complexes of periodate and some furanose derivatives, while Angyal, Greeves and Pickles[12] have indicated the structure of both periodate and borate complexes with polyols and the stereochemistry of the complexes formed (Figure ii).

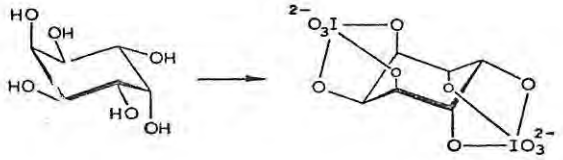


Figure ii The change in conformation necessary in neo-inositol in order to form a complex with periodate

Angyal with Klavins and Mills[13], has also investigated the tridentate complexes of some C-methyl- and C-hydroxymethyl-cyclitols with borate-ions by means of p.m.r. spectroscopy.

A procedure has been elaborated for the conversion of D-galactal into D-talose, a rare sugar[14]. The stereoselectivity of this hydroxylation by hydrogen peroxide under catalytic amounts of sodium molybdate is considered to be a consequence of the formation of an

initial transitory complex between the C-2 hydroxyl group of D-galactal and permolybdenic acid, which is subsequently decomposed to an aldose with a cis relationship of hydroxyl groups at C-2 and C-3.

Applications of carbohydrate - metal ion complexes in influencing the course of synthetic reactions have also appeared[15]. The α-furanoid and α-pyranoid forms of the methyl allosides may be predominantly formed from β-allopyranose in the presence of calcium-ions. This happens as a consequence of sugar complex formation with the cations during the glycosidation reaction with methanolic HCl.

In the cane sugar industry, the loss of sucrose as a result of molasses formation is thought to be linked with the elaboration of sucrose - salt complexes which are not amenable to crystallization[16]. In this respect, Gill[17] and co-workers indicated the possible formation of $2C_{12}H_{22}O_{11}.2NaCl$, with a known quantity of water of hydration, in a mixture of sucrose and sodium halides. Gauthier[18,19] suggested possible formulae for sucrose - salt complexes as $C_{12}H_{22}O_{11}KI.2H_2O$, and $C_{12}H_{22}O_{11}KCNS.H_2O$ while Mackenzie and Quinn[20] inferred that sucrose and potassium chloride could form compounds of the type $C_{12}H_{22}O_{11}KCl.2H_2O$. Wiklund[21] suggested the existence of complexes such as $C_{12}H_{22}O_{11}2H_2O.NaI$ and $2C_{12}H_{22}O_{11}3NaI.3H_2O$. The pioneering experiments by Reeves[22-33] on the reaction of sugar glycosides, with the cellulose solvent, cuprammonium, established beyond doubt the existence of soluble cyclic complexes and requires special mention.

Reeves was able to determine the predominant conformations of many methyl glycosides, the extent of complex formation as shown by the decrease in electrical conductivity[22] of the solutions,

and the nature of the complex[23,24], often indicated by changes in optical rotations. Reeves found that complex formation requires the presence of two hydroxyl groups in close proximity: an equatorial and an axial, or two equatorial, hydroxyl groups on adjacent carbon atoms being suitable, as are two syn-axial hydroxyl groups[22]. Reeves found the majority of the D-sugars in what he called the C-1 conformation and only methyl-α- and β-D-arabinopyranoside and methyl-α-D-idopyranoside were shown to be predominantly in the 1C conformation[29,31]. All the methyl glycosides were found to be in the chair conformation, but a few, notably the altropyranosides and lyxopyranosides appeared to contain substantial amounts of both chair conformations in equilibrium[29,31]. Reeves and Bragg[32,33] were later able to show that the diol : copper ratio in the complexes is 1:1. D-Mannosan[28] and amylose[30] complexes formed in cuprammonium solution were subsequently investigated by Reeves. The conformational assignments of the monosaccharides alluded to above have subsequently been confirmed by later work involving p.m.r. spectroscopy[34-39].

Information about sugar - metal complexes has been provided by the deft use of a wide number of physical methods. Employing Job's method[40], Ramaiah and Vishnu[41] were able to demonstrate that the decrease in optical rotation observed for sucrose - salt solutions indicated the formation of "uni-uni-molecular" sucrose - salt complexes designated Su.NaCl, Su.NaBr, Su.NaI, and Su.KCl (where Su = sucrose). At the time that this work was conducted, it was stated that "the exact composition of these complexes could be arrived at by their isolation and analysis which, however, seems to be difficult on account of their extremely high solubility"[41].

Bourne, Nery and Weigel[42] examined the chelation of metals with polyhydroxy compounds in solutions of sodium hydroxide at pH 12. An excess of metal salt solution was added to a solution of the sugar and the amount of precipitated metal ion volumetrically determined with standard E.D.T.A. With D-mannitol as the chelating agent, the metallic ions which formed soluble chelates were Cu^{++}, Ti^{+++}, Zr^{+++}, Pb^{++}, Sb^{+++}, Bi^{+++}, Fe^{++}, Fe^{+++}, Co^{++}, Ni^{++}, Th^{++++}, and UO_2^{++} while no soluble chelates were obtained with Ag^{+}, Ca^{++}, Ba^{++}, Cd^{++} and Hg^{++}. The chelating power, defined as the number of g-atoms metal/mole chelating agent, of D-glucitol, D-mannitol, dulcitol, pentaerythritol, D-glucose and D-fructose were determined for water soluble chelates with Cu^{++}, Fe^{+++}, Co^{++} and Ni^{++}.

Mills[2] investigated the association of polyhydroxy compounds with cations in solution employing the method of electrophoresis. Many of the compounds tested formed charged complexes with the metal ions in the supporting electrolytes as evidenced by the reproducible movement of the complexed sugars towards the cathode. cis-Inositol was found to be outstanding in its ability to complex with Ca^{++}, Sr^{++} and Ba^{++}. epi-Inositol and allo-inositol and cis-quercitol also showed considerable migration. Most reducing sugars showed only slight or zero movement in all the electrolytes used, with the exception of D-talose and D-ribose. Mills stated that "the degree of association will be determined by the configuration of the hydroxy compounds and the ionic radius and preferred co-ordination geometry of the metal". He concluded that the three axial hydroxyl groups in the two equivalent stable conformations of cis-inositol accounted for the strong complexing ability of this material.

Frahn and Mills[43] reported the electrophoretic rate of migration for 96 carbohydrate compounds in various support electrolytes. Sodium arsenite and basic lead acetate were found most suitable for the separation of reducing sugars including all pentoses and hexoses and the common disaccharides. Basic lead acetate was also the best electrolyte for separating the sugar alcohols (up to the heptitols) and the cyclitols, while borax was most effective for separating glycols. An attempt at correlating the configuration of stereoisomers with their mobilities was made. Frahn and Mills[43] assumed that (1) migration occurs only when at least two hydroxyl groups in a molecule of a polyhydroxy compound are united with the electrolyte ions as a cyclic complex, (2) the formation and breakdown of the complexes is reversible and very rapid, and (3) the relative mobilities of similar compounds are approximate measures of the equilibrium constants for formation of the complexes.

With borate either five-membered or six-membered cyclic complexes are possible, the equilibrium often being in favour of the complex, five-membered complexes being favoured over six-membered ones. Tridentate complexes, involving three syn-hydroxyl groups, are also possible. The dominant process in sodium arsenite and basic lead acetate is the formation of five-membered cyclic complexes with hydroxyl groups of the threo-configuration being most favoured. With sugar alcohols, in sodium arsenite and basic lead acetate five- and six-membered complexes as well as tridentate complexes are formed. The degree of complexation depending on the number of pairs of cis-hydroxyl groups present in the sugar.

The existence of molybdate complexes of saccharides in acidulated water has been confirmed by electrophoresis[44-46],

polarimetry[47,48], potentiometry[48], optical rotatory dispersion[49] and circular dichroism[50]. The electrophoretic data suggests that the complexing with molybdate-ions occurs only with polyols having an ax – eq – ax sequence of three adjacent hydroxyl groups in a six-membered ring. However, it has been shown[48-50] that this system does not cover all the possibilities of saccharide – molybdate complexing, since aldoses with free hydroxyl groups at C-1 and C-3 form complexes as well. Qualitative differences among the complexes formed the basis of a distribution of the aldoses into two groups: Group 1, for aldoses possessing a cis-cis arrangement of hydroxyl groups at C-1 and C-3, where the molybdate complexes migrate during electrophoresis and Group 2, for aldoses possessing a trans-trans arrangement of hydroxyl groups at C-1 and C-3, where the complexes do not migrate during electrophoresis.

Bilik[51,52] has reported that the C-2 hydroxyl group of aldoses undergoes inversion in acidified solutions of molybdate affording an equilibrium mixture of epimeric aldoses. The epimer predominating is that possessing a trans relationship of the hydroxyl groups at C-2 and C-3. This fact may be accounted for in terms of the steric hinderance of the C-2 hydroxyl group with the molybdate-ion during the formation of a complex which involves C-1 and C-3 hydroxyl groups. An equilibrium mixture of D-glucose and D-mannose contains these sugars in the ratio 3:1 after catalytic epimerization of either sugar with molybdate-ions. The equilibrium is attained much faster from D-mannose (3h) than from D-glucose (6h). In order to accomplish the above the conformation of either sugar must invert from the favoured C1 to 1C conformation. This is to bring the hydroxyl groups at C-1 and C-3 into the correct position

for complex formation. It would appear that the stability of the carbohydrate – molybdate complex is the driving force for the ring inversion (Figure iii).

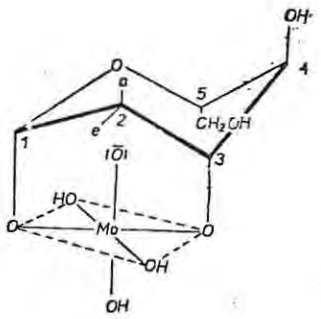


Figure iii Molybdate complex of β-D-mannose (a = H, e = OH, 1C) and β-D-glucose (a = OH, e = H, 1C) indicating the interaction between the hydrated ion and the hydroxyl group at C-2

Complexes of mannitol with metal ions in alkaline medium, previously studied by polarography and potentiometric titrations[53,54] have subsequently been studied by spectrophotometric methods[55]. The complexes formed between mannitol and cobalt (II), cobalt (III), copper (II) and nickel (II) indicated 1:1 stoichiometry of metal to carbohydrate for both cobalt species. Evidence for 1:1 and 2:1 copper – mannitol complexes was obtained and complexes with nickel were detected. The cobalt (II) complexes are tridentate, the bond forming sites being 1,2,4- 2,3,5- or 3,4,6- respectively, with the metal ion in a tridentate configuration. In the cobalt (III) complexes the mannitol acts as a bidentate ligand. The copper (II) – mannitol complexes are of two types, CuM and Cu_2M (where M = mannitol). Copper in a square planar configuration may be bound to mannitol through the 2,3- or 4,5- groups which could occur in both types of complex.

Mitzner and Behrenwald[56] have reported that the mutarotation of several sugars is affected by metal ions. In the presence of nickel nitrate (0.05M) the velocity of mutarotation of L-arabinose, D-mannose and D-fructose is considerably increased while that of D-galactose is only slightly affected. No effect on the velocity of mutarotation was observed for solutions of maltose, lactose, L-rhamnose and D-xylose. Calculations of the activation parameters as functions of the metal ion concentrations have indicated that during the mutarotation of β-D-mannose, complexes with the following metal ions are formed: nickel(II) 2:1[56], cobalt(II) 3:2, copper(II) 2:1, for copper-ion molarity $< 0.005M$, and 1:1, for copper-ion molarity $> 0.005M$ but $< 0.01M$[57], iron(II) 1:1, and zinc(II) 2:1[58]. (Ratios given as carbohydrate : metal ion).

The reaction between cadoxen[59], a solution of cadmium hydroxide in aqueous ethylene diamine, and some glycosides have been investigated by the method of polarimetry[60]. From the differences in optical rotation observed between solutions of the glycosides in cadoxen and in aqueous ethylene diamine, it was concluded that complexes are formed between cadmium and adjacent hydroxyls in the glycosides. Eleven glycosides were investigated. Methyl-β-D-glucopyranoside showed no electrophoretic mobility in cadoxen and reduced its conductivity, which proved that the complexes with this sugar are uncharged. The solubility of cellulose in cadoxen depends on complex formation involving the C-2 and C-3 hydroxyls of the anhydro-D-glucose residues.

Hygroscopic non-crystalline adducts of stoichiometry 1:1 have been prepared by Roy and Mitra[61] following the reaction of

D-glucose and maltose with the hydroxides of barium, calcium and strontium. These hydroxides have also been reacted with cellobiose, lactose, maltitol, melibiose, sucrose and α,α-trehalose[62]. The formation of 1:1 complexes have been observed in each case and have been detected by the combined use of Job's method[40] and pH measurements performed on the solutions of complexed carbohydrate. In the presence of ethylene diamine or 2-amino ethanol, the above hydroxides as well as magnesium hydroxide react with D-glucose and maltose to form sugar complexes[63]. A crystalline calcium derivative of 1,2-O-isopropylidene-D-glucose-6-phosphate has been prepared by Sahu and Mitra[64], while hygroscopic 1:2 adducts of 2-amino-2-deoxy-D-glucopyranose with calcium-, barium-, and strontium-ions have been prepared by Moulik and Mitra[65] following reaction of the carbohydrate with solutions of the metal chlorides. Treatment of glucosamine with nickel sulphate or chloride afforded a crystalline complex of stoichiometry 2:1 carbohydrate to metal. Ferrous-ions and cobalt salts were also reported by Sahu and Mitra[66] as capable of complex formation with glucosamine.

Although at least 70 crystalline complexes containing a sugar or sugar derivative and an inorganic salt in stoichiometric proportions (usually 1:1) have been isolated[67-70], their isolation does not necessarily provide evidence for the existence of stable complexes in solution. Since the review by Rendleman[71], further interesting crystalline complexes have been isolated and investigated by X-ray methods. These include β-D-mannofuranose. $CaCl_2 \cdot 4H_2O$[72], the hydrated calcium bromide salt of lactobionic acid[73], the hydrated calcium bromide complex of lactose[74,75], and calcium bromide complexes of galactose[75] and myo-inositol[75,76] and α,α-trehalose - calcium bromide monohydrate[77]. The myo-inositol

magnesium chloride - hydrate (1:1:4) complex has also been studied[78]. The structures proven for the above solids may not be assumed in solution. A general characteristic of the determinations is the indication that bond lengths and angles are appreciably altered on the formation of the complexes.

The only method of investigation which is able to indicate the structures of sugar complexes existing in solution is that of proton magnetic resonance spectroscopy. This technique, already employed most successfully in the elucidation of structural and conformational problems in carbohydrate chemistry[36, 79-85], has been shown to provide information on the structures and stability constants of soluble sugar - metal and -non metal complexes[12, 15, 86-89].

Richards and Williams[90] have reported the complex formation between aqueous zinc chloride and various cellulose-related polyhydroxy compounds. P.m.r. spectroscopy and Job's method, using polarimetry, were employed to detect complex formation between zinc-ions and methyl-β-D-glucopyranoside, methyl-α-D-glucopyranoside, and methyl 4,6-di-O-methyl-β-D-glucopyranoside. The molar ratios of salt to model compound were 1:1 for the complexes formed by the above sugars. Little or no complex formation was observed for methyl-3-O-methyl(α,β)-D-glucopyranoside, methyl-2,3,4,6-tetra-O-methyl-β-D-glucopyranoside and ethyl-3,4,6-tri-O-methyl-β-D-glucopyranoside. Richards and Williams[90] concluded that zinc chloride forms complexes with the vicinal hydroxyl groups at C-2 and C-3 in the model compounds. These workers suggested that the swelling and loss of crystallinity of cellulose in zinc chloride solutions is related to the formation of complexes between some C-2 and C-3 hydroxyl groups in the molecule and the metal ions.

McGavin, Natush and Young[91] reported that complexing with metal ions in aqueous solutions causes downfield shifts in some proton signals in the p.m.r. spectra of methyl-α-L-arabinopyranoside and methyl-α-L-arabinofuranoside. These workers concluded that two neighbouring oxygen atoms were included in the formation of a complex having small stability constants. Angyal and Davies[86] observed strong downfield shifts (0.32 p.p.m.) in the H-3 proton signal ($J_{2,3} = J_{1,4} = 3.25$ Hz) of epi-inositol in deuterated calcium chloride solution (2M). They concluded that the metal ion in the complex is located approximately in the direction of the bond from H-3 to C-3 and co-ordinated to OH-2, OH-3 and OH-4, an ax - eq - ax sequence of three hydroxyl groups. The stability constant of the complex was determined as $3M^{-1}$ (Figure iv).

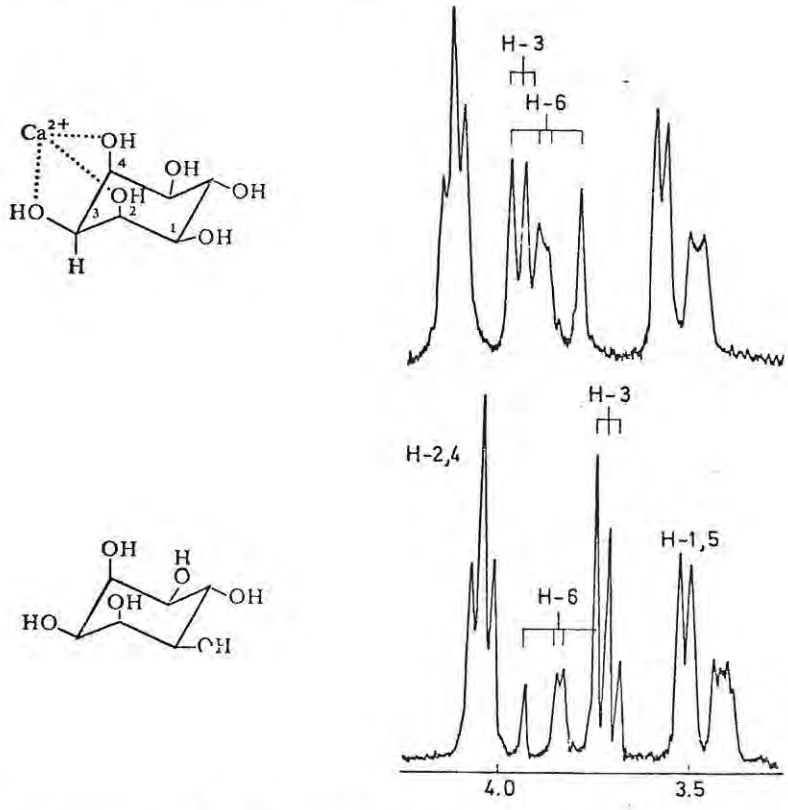


Figure iv Structure of the epi-inositol - calcium-ion complex deduced from the changes observed in the 100 MHz p.m.r. spectrum of pure cyclitol (lower spectrum) in deuterium oxide after the addition of metal ion (upper spectrum)

The changes in equilibrium composition of the tautomeric forms of D-allose in the presence of selected salts have been calculated by Angyal[87]. The stability constants of the complexes formed with the cations decrease in the following order of the salts complexed. $LaCl_3 > CaCl_2 > Ca(NO_3)_2 > Sr(NO_3)_2 > BaCl_2 > Pb(OAc)_2 > Y(NO_3)_2 > SnCl_2 > SnCl_4 > MgCl_2 > NaCl$

Weak complexes are also formed with cadmium-, tin(II)-and tin(IV)-ions while the effects with potassium and zinc are barely noticeable. No complexation occurs on addition of silver nitrate, thallium(I) nitrate, lithium chloride, caesium chloride, rubidium chloride and aluminium sulphate. Angyal[87] has reported that the optimum ionic radius of cations involved in complex formation is about 1.0 Å. In Group IA sodium with an ionic radius of 0.97 Å forms complexes more easily than the other members of the group, while in Group 2A calcium (0.99 Å) is favoured and in Group 3A lanthanum (1.02 Å) is favoured. The higher the charge on the cation, the greater the chance of complex formation.

Although nearly all the signals of the protons of the four tautomeric forms of D-allose overlap heavily, Angyal[87] has reported that the signal of the anomeric proton of each tautomer is clearly resolved, and addition of salts to solutions of D-allose in deuterium oxide have two effects on the p.m.r. spectrum: (1) the signals attributed to the α-pyranose form increase in intensity, and (2) the signal due to the α-pyranose anomeric proton shifts downfield. Since equilibrium between a sugar and its metal complex is fast on the n.m.r. time scale, only average spectra are recorded, spectra of complexed and uncomplexed sugar do not appear. The downfield shift mentioned continues until nearly all the sugar

is complexed before secondary effects, such as complexation at other less favourable sites, become apparent.

Lanthanum (III) gives the largest shifts and affects the H-2 signal the most, indicating that the ax - eq - ax arrangement of hydroxyl groups mentioned above at C-1, C-2 and C-3 favour the formation of a complex. Similar effects on the spectrum were noted with calcium chloride. A cis-cis arrangement of three hydroxyl groups at C-1, C-2 and C-3 in a five-membered ring also resulted in the formation of complexes with cations. This was indicated by the increase in the intensity of the anomeric proton signal of β-D-allofuranose (Figure v).

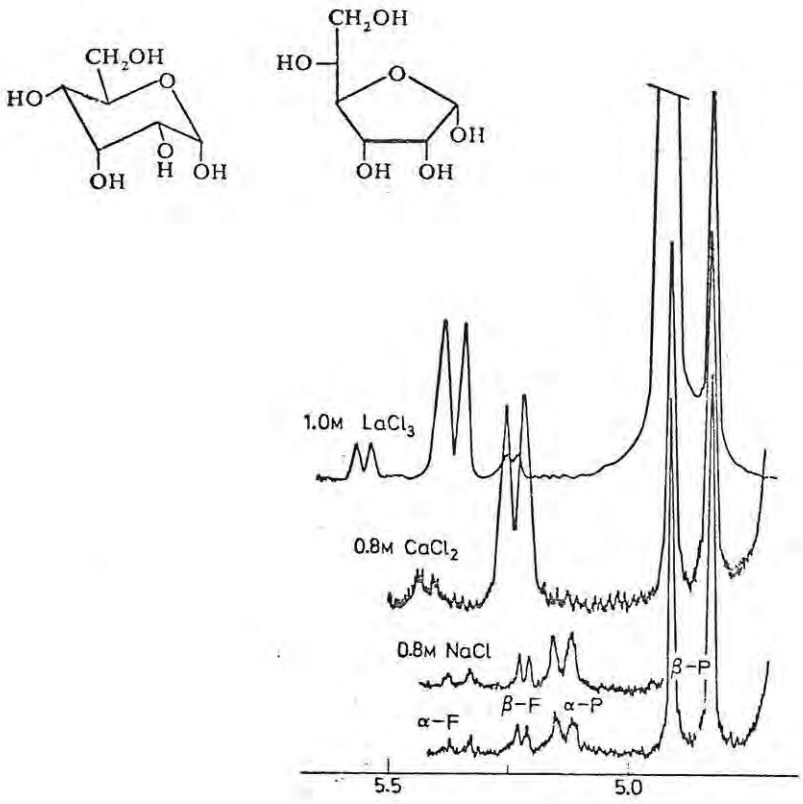


Figure v The 100 MHz p.m.r. signals of the anomeric protons of β-D-allopyranose and β-D-allofuranose increase in size and shift downfield, on addition of suitable metal ions to deuterated solutions of the sugar with the formation of sugar - metal ion complexes

D-gulose and D-glycero-D-gulo-heptose also have an ax – eq – ax sequence of hydroxyl groups at C-1, C-2 and C-3 in both the α-pyranose and α-furanose forms (actually quasi ax – quasi eq – quasi ax in the latter form), and exhibit the same effects as mentioned above, on the addition of complexing cations.

The complexing of D-ribose with metal ions presents an interesting case[87]. The α-pyranose and α-furanose forms have the ax – eq – ax sequence of hydroxyl groups and on the addition of calcium chloride, the anomeric resonance signals of these two tautomers are affected indicating complexation involving the OH-1 group. Apart from this effect the anomeric signal of the β-pyranose tautomer is also shifted and its splitting decreases from 6.4 Hz to 4.2 Hz. β-D-ribopyranose is known to exist in solution as a mixture of C1 (equatorial hydroxyls at C-1 and C-4) and 1C (axial hydroxyls at C-1 and C-4) conformers in the approximate ratio of 3:1. The 1C conformer has the desired ax – eq – ax sequence of hydroxyl groups at C-2, C-3 and C-4 and is thus able to complex with suitable cations, whereas the C1 conformer is unable to do so. The shift in the anomeric hydrogen signal and decrease in its coupling constant observed in the spectrum of D-ribose in the region of the β-D-ribopyranose anomeric proton on addition of calcium chloride, may be taken as a direct measure of the change in H-1 from an axial position (C1 conformer) to an equatorial position (1C conformer) during the formation of a complex. A similar effect is noted with methyl-β-D-ribopyranoside on the addition of calcium-ions to a deuterated solution of the carbohydrate (Figure vi).

Another sugar which changes its conformation to form a complex is D-lyxose. The α-anomer is unable to complex with cations while the β-anomer is.

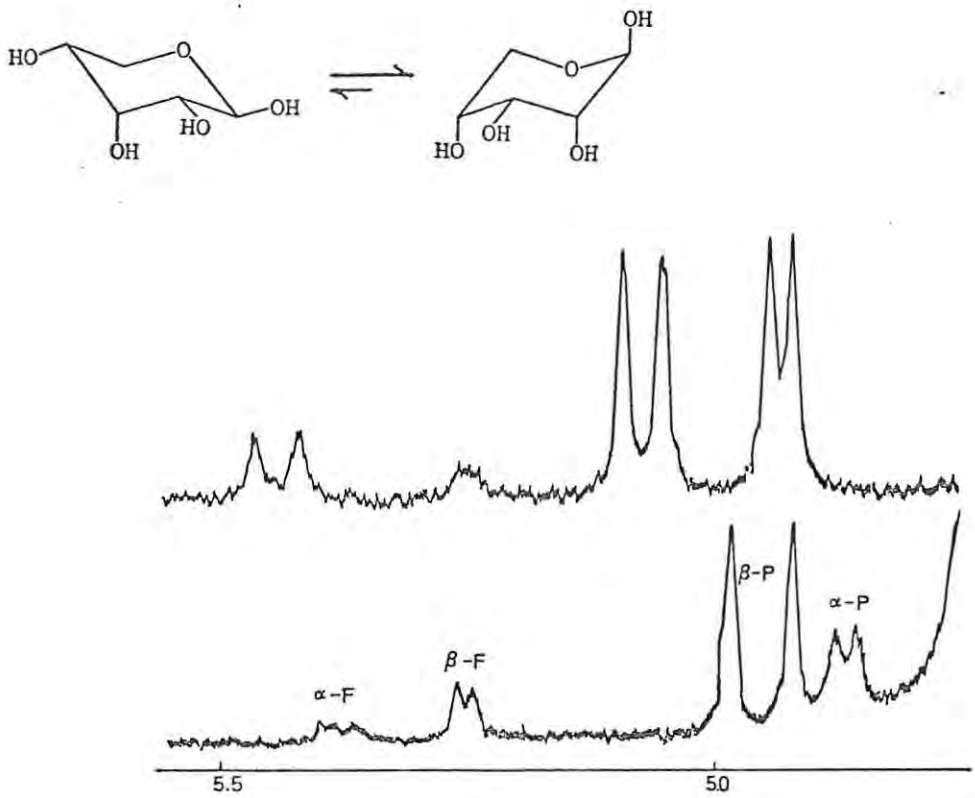


Figure vi The 100 MHz p.m.r. signals of the anomeric protons of α-D-ribopyranose and α-D-ribofuranose shift downfield and increase in size on addition of calcium chloride to solutions of the sugar in deuterium oxide, and the coupling constant of the anomeric protons of the β-D-pyranose tautomer decreases indicating a conformational change in this tautomer from C1 to 1C with the formation of complexes

Angyal, Greeves and Mills[92] have reported the complex formation of several alditols with metal ions in aqueous solution. Electrophoresis and p.m.r. spectroscopy was used to study the complexes formed with europium-ions. Complexing occurs at those sites where three consecutive oxygen atoms are in a gauche-gauche arrangement. Where this condition is not met, rotation around carbon-carbon bonds occurs, the energy required for this determining the extent of complex formation

Angyal[12] has found that the addition of magnesium and zinc to

deuterated solutions of cis-inositol shift the proton resonances downfield. Larger shifts in the resonances of the equatorial protons are noticed with these cations. This is different from shifts induced by lanthanum-ions. The latter affect axial protons more than equatorial ones. Magnesium-ions have been found to cause ring inversion of sugars by complexing for example with DL-2-deoxy-2-methyl-epi-inositol. The complexing is thought by Angyal, Greeves and Pickles[12] to occur between the oxygen atoms of the hydroxyl groups at C-1, C-3 and C-5, a tridentate site, where the distances between these oxygen atoms are close enough to form magnesium complexes.

The recorded complexes formed between magnesium-ions and carbohydrates are few, nevertheless the possibility of complexes of magnesium-ions with the anhydro-D-glucose units in cellulose could account for the "protective" action of magnesium compounds on the cellulose portion of pulp during the alkali-oxygen bleaching process. Studies by Chang[93] on the crystallite structure of cellulose have provided evidence for chain folding in the molecule. It is suggested that the concentration of these folds at some localized areas of the fibre are easily and selectively cleaved. The folds are thought not only to possess additional chemical properties, but also to control the integrity of the crystallite portion of the fibre. It is possible that during the bleaching process the severe degradation of the cellulose is prevented through the formation of cellulose - magnesium complexes at these specific sites in the molecule (Figure vii).

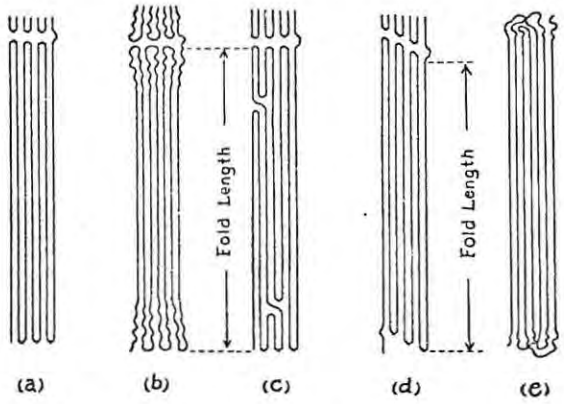


Figure vii Different folding patterns thought to exist in the crystallite structure of cellulose: (a) basic platellite, (b) generally disordered ends, (c) irregular folds, (d) staggered folds, and (e) switchboard type of folds

1.3 INTRODUCTION

Although it is known that magnesium carbonate[94-102] and other magnesium salts[103-114] "protect" the cellulose portion of pulp during alkali-oxygen bleaching, the "protective mechanism" is unclear.

Theories on the role of magnesium-ions in stabilizing organic peroxides[115-120], or deactivating heavy metal ion catalysts[121,122], or forming complexes with primary oxidized cellulose derivatives[123], have been suggested.

If the magnesium-ions were to complex directly with the hydroxyl groups on C-2 and C-3 of the anhydro-D-glucose unit in cellulose, this would constitute a further hypothesis.

In an attempt to test the validity of this last hypothesis, experiments were conducted to determine whether any reaction occurs in aqueous solution between methyl-α-D-glucopyranoside and magnesium perchlorate; and cellulose samples and metal ions.

The above monosaccharide was used as a cellulose model compound in preference to the β-anomer because of its ready availability in reasonably large amounts.

Changes in concentration of metal ion solutions after treatment of cellulose samples were used as probes to determine whether complex formation between the polymer and metal ions occurs. Were insoluble complexes to form during treatment, decreases in metal ion concentration would be observed.

1.4 RESULTS AND DISCUSSION

1.4.1 Magnesium-ion complexes with methyl-α-D-glucopyranoside

Employing the method of continuous variations[40], polarimetry measurements were made on a number of solutions of constant total molarity having differing molar ratios of methyl-α-D-glucopyranoside and magnesium perchlorate. The perchlorate salt was chosen because the anion is known to be inert and non-co-ordinating[124,125]. The difference in optical rotation between each solution and its corresponding blank, containing only the glucoside, was plotted graphically as a function of the molar ratio of the reacting species (Table I, Figures 1-4). A minimum was observed for the solution with a molar ratio of 1:1 glucoside to magnesium perchlorate, probably corresponding to the composition of a stable carbohydrate-magnesium perchlorate complex of this stoichiometry in solution[126]. At a temperature of 20° only one minimum was observed but as the temperature was increased (20 → 50°) another minimum appeared at a molar ratio of 1.33M glucoside to 0.67M magnesium perchlorate, probably indicating the formation of another soluble complex of stoichiometry 2:1 glucoside to magnesium perchlorate.

The method of continuous variations has already been successfully applied to the study of the complexes formed between sucrose and the alkaline metal salts of magnesium sulphate, calcium sulphate and barium chloride[127]. At a combined total concentration of 1M, curves exhibiting two maxima were obtained; one at a molar ratio of 0.5M sucrose to 0.5M salt, and the other at a molar ratio of 0.66M sucrose to 0.34M salt. On this evidence Ramaiah and

Vishnu[127] suggested that both 1:1 and 2:1 sucrose - alkaline earth metal salt adducts exist in solution.

Glucoside Molarity (mol ℓ^{-1})		0	0.25	0.50	0.75	1.00	1.25	1.50	1.75	2.00
Magnesium-ion Molarity (mol ℓ^{-1})		2.0	1.75	1.50	1.25	1.00	0.75	0.50	0.25	0.00
Difference in optical rotation (Deg)	TEMP. 20°	0	-0.030	-0.051	-0.069	-0.094	-0.085	-0.076	-0.032	-0.008
	TEMP. 30°	0	-0.033	-0.054	-0.073	-0.097	-0.080	-0.079	-0.029	-0.003
	TEMP. 40°	0	-0.035	-0.052	-0.073	-0.095	-0.079	-0.080	-0.027	-0.004
	TEMP. 50°	0	-0.030	-0.048	-0.066	-0.095	-0.075	-0.075	-0.025	-0.006

Table I Change in the difference in optical rotation of glucoside solutions with respect to corresponding blanks as the magnesium-ion concentration decreases

The above procedure gives no information about the location of complexing points between the glucoside ring and the magnesium perchlorate. Methyl-α-D-glucopyranoside is unable to mutarotate and it is well established that, of all the conformations possible for the molecule, the most probable and thus the most predominant conformation in solution is C1(D)[29]. In this conformation all the hydroxyl protons are in equatorial positions while the protons of the methoxyl group and those attached directly to the ring carbon atoms, except H-1, H-6 and H-6', are in axial positions.

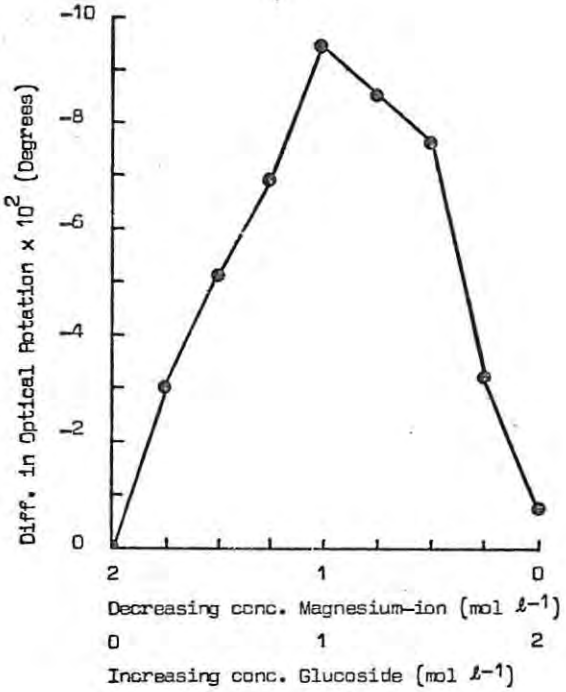


Figure 1 Graph of differences in optical rotation of glucoside with respect to blanks vs magnesium-ion concentration at a temperature of 20° (λ = 365 nm)

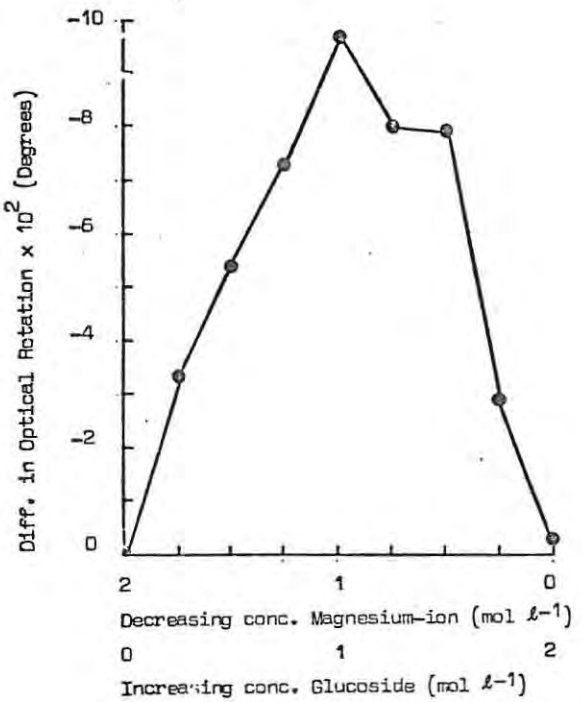


Figure 2 Graph of differences in optical rotation of glucoside with respect to blanks vs magnesium-ion concentration at a temperature of 30° (λ = 365 nm)

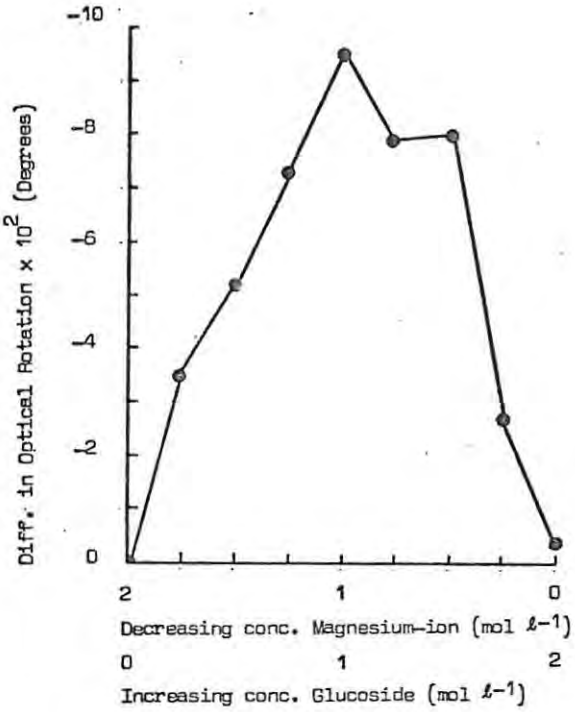


Figure 3 Graph of differences in optical rotation of glucoside with respect to blanks vs magnesium-ion concentration at a temperature of 40° (λ = 365 nm)

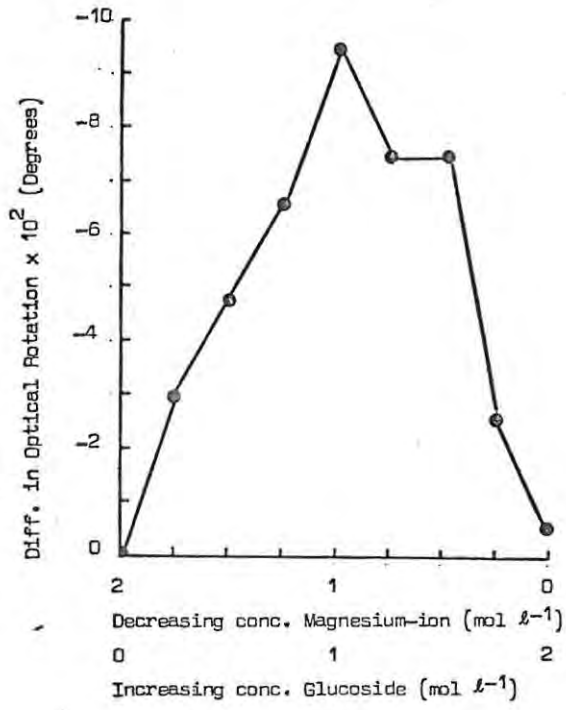


Figure 4 Graph of differences in optical rotation of glucoside with respect to blanks vs magnesium-ion concentration at a temperature of 50° (λ = 365 nm)

Although p.m.r. spectroscopy has been successfully applied to the study of carbohydrate conformations in solution[34-39,79-85], an exact analysis of a carbohydrate p.m.r. spectrum is often complicated as it involves the consideration of all the ring protons as a single interacting multiple spin system[80]. In the experiments performed spectra of pure (Figure 5) and complexed (Figure 6) methyl-α-D-glucopyranoside were incompletely assigned because of overlap in the chemical shifts of the ring protons (Δν 105.0 - 88.0 Hz).

The chemical shift of the methoxyl proton (s, 3, Δν 80.0 Hz; $-OC\underline{H}_3$) was determined following integration of the sharp singlet occurring at high field. The chemical shift of the anomeric proton (d, 1, Δν 162.7 Hz; H-1) is uniquely determined because C-1, to which it is bonded, bears two electron withdrawing oxygen atoms and hence H-1 resonates at a low field due to de-shielding effects[128]. The magnitude of the coupling constant ($J_{1,2}$ = 3.2 Hz) measured from the doublet is characteristic of H-1, H-2 protons in a gauche-equatorial-axial conformation[129].

Double resonance and spin-tickling of the anomeric proton established the chemical shift of the H-2 proton at a position upfield (72.0 Hz) from the H-1 resonance. The quartet (q, 1, Δν 90.7 Hz, $J_{1,2}$ = 3.2 Hz, $J_{2,3}$ = 7.4 Hz; H-2) collapsed to a doublet (d, 1, Δν 90.7 Hz; $J_{2,3}$ = 7.4 Hz; H-2) during double resonance experiments. Doubt exists as to the exact chemical shift of the H-2 proton as in most of the recorded spectra it was obscured by superposition of unassigned resonances and was not clearly resolved (Figure 5, Region B; Δν 96.0 - 82.0 Hz).

The chemical shift of the hydroxyl protons (s, Δν 154.0 Hz; -O<u>H</u>, <u>H</u>OD) which form an exchanging pool[128] with residual water in the deuterium oxide, was readily determined from the position of the intense downfield singlet assigned to them.

It was not possible to establish the chemical shifts of the remaining protons by double resonance techniques because of heavy overlap in the signals of H-3, H-4, H-5, H-6 and H-6'. It is suggested that they are distributed between Region A (Figure 5; Δν 108.0 - 96.0 Hz; H-3, H-4) and Region B (Figure 5; Δν 96.0 - 82.0 Hz; H-5, H-6, H-6') for the following reasons. An idealised first-order splitting pattern for H-3 and H-4 would be two similar one proton triplets of intensity 1:2:1 with $J_{2,3} = J_{3,4} = 7 - 10$ Hz. Were these protons (H-3 and H-4) magnetically equivalent, they would have almost identical chemical shifts. At 60 MHz the triplets are not clearly resolved. The resonance pattern obtained is an overlay of the two signals probably slightly displaced with respect to each other, which is observed experimentally in Region A (Figure 5) of the spectrum. Similarly first-order resonance patterns for H-5, H-6 and H-6' would be three low intensity multiplets, and at 60 MHz overlap in chemical shifts produces the pattern observed in Region B (Figure 5) of the spectrum.

The p.m.r. spectra of methyl-α-D-glucopyranoside in the presence of magnesium perchlorate were all of the same basic pattern compared with that discussed above. It may be inferred that the conformation of the glucoside in the proposed complex remains in the most stable conformation, <u>viz</u>. C1(D). Were there inversion of conformation, at least two signals for H-1 would have been detected.

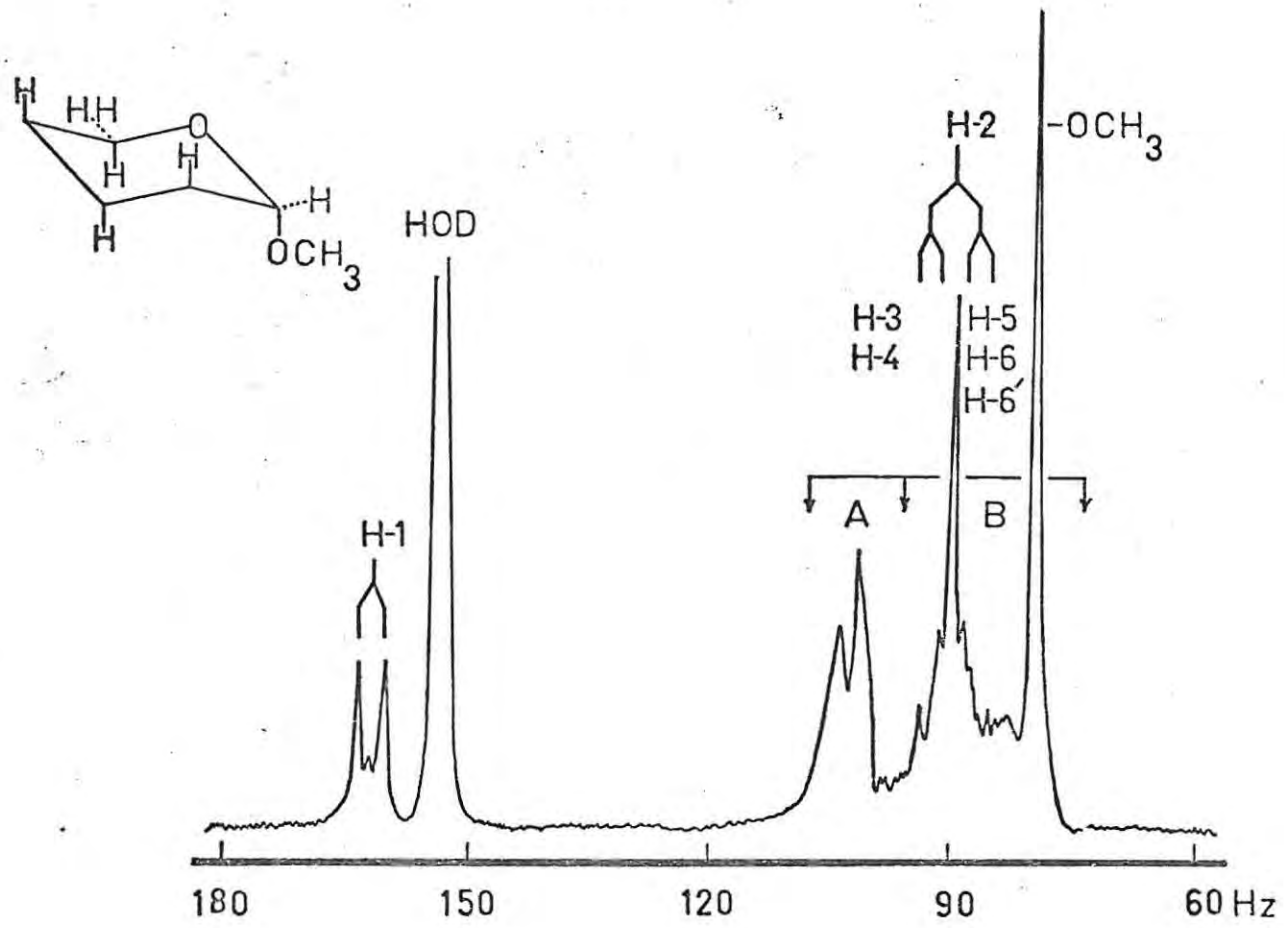


Figure 5 60 MHz P.m.r. spectrum of pure methyl-α-D-glucopyranoside

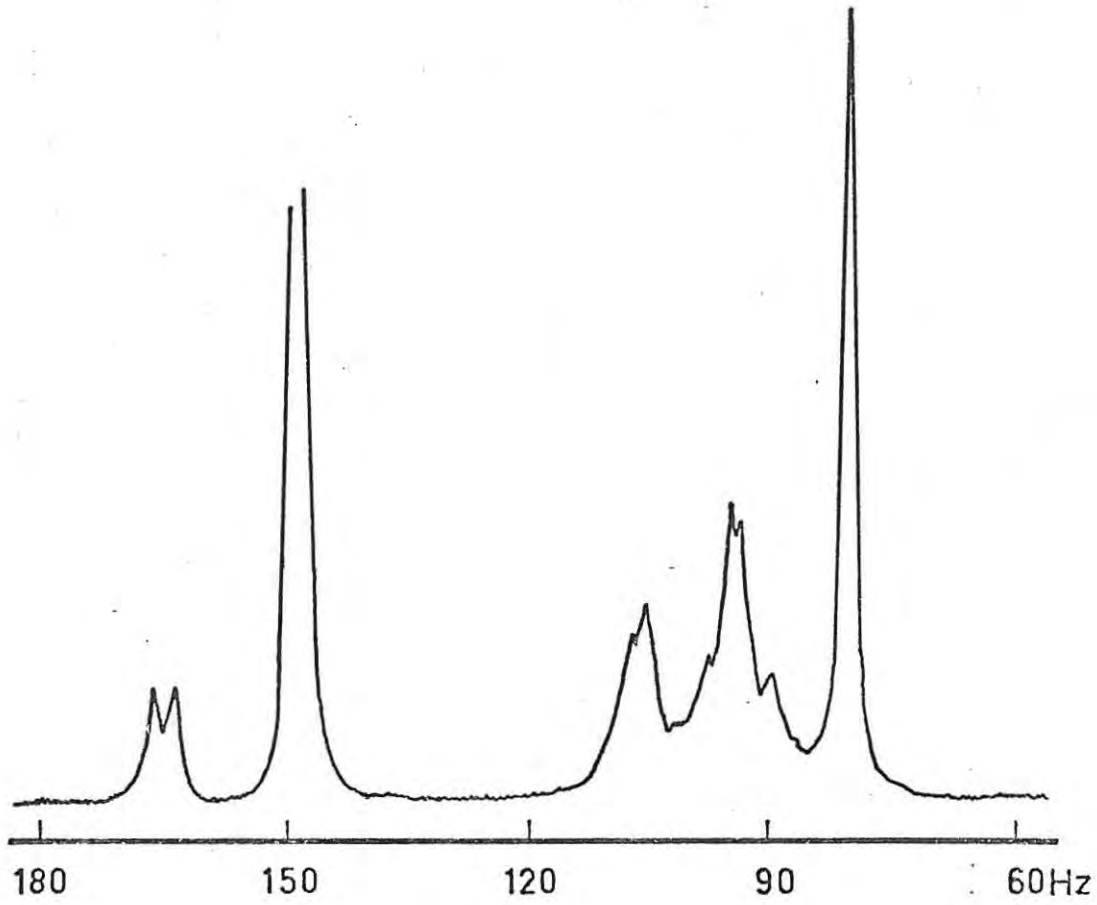


Figure 6 60 MHz P.m.r. spectrum of complexed methyl-α-D-glucopyranoside (ratio of glucoside:magnesium-ion:: 1:6)

Table II and Figure 7 indicate the small changes observed in the chemical shift of the methoxyl protons as the concentration of magnesium perchlorate is increased. The chemical shift of the singlet remains relatively unaffected which is in agreement with published findings that the possibility of an oxygen atom of an ether linkage or of the glucoside hemi-acetal linkage participating as an electron donor to bind a cation may be regarded as being slight[71]. Were there appreciable metal ion binding at this site much larger effects would have been observed.

Ratio Glucoside / Magnesium-ion	10:1	8:1	6:1	4:1	2:1	1:1	1:2	1:4	1:6
Change in Chemical Shift (Hz)	-0.7	-0.5	-0.5	-0.6	-1.0	-0.3	-0.3	-1.7	-2.0

Table II Change in chemical shift of the methoxyl protons from 80.0 Hz as the magnesium-ion concentration increases

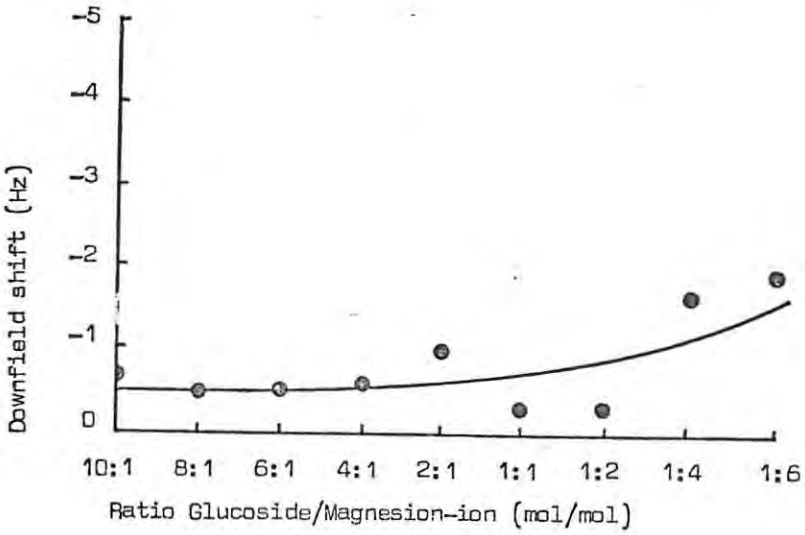


Figure 7 Graph of changes in chemical shift of the methoxyl protons as the magnesium-ion concentration increases

Increasing the concentration of magnesium perchlorate effects changes in the chemical shift and coupling constant of the anomeric proton. These are indicated in Table III and Figure 8. Apart from an initial downfield shift (0.7 Hz), a steady increase in chemical shift ($\Delta\nu$ 163.7 → 166.7 Hz) and decrease in coupling constant ($J_{1,2}$ = 3.2 → 2.9 Hz) is observed as the ratio of glucoside to magnesium-ion changes (2:1 → 1:6). This paramagnetic shift in the H-1 resonance signal may be interpreted as a deshielding of the equatorial proton as a result of interaction of OH-2 with magnesium-ions. By consideration of the Karplus equation[130] the observed decrease in coupling constant ($J_{1,2}$) may be due to an increase in the dihedral angle Φ between H-1e and H-2a.

Ratio Glucoside / Magnesium-ion	10:1	8:1	6:1	4:1	2:1	1:1	1:2	1:4	1:6
Change in Chemical Shift (Hz)	-0.7	-0.7	-0.7	-0.7	-1.0	-1.2	-1.3	-2.5	-4.0
Change in Coupling Constant (J_{12}) (Hz)	0	0	0	0	0	0.1	0.2	0.2	0.3

Table III Change in chemical shift of the anomeric proton and $J_{1,2}$ coupling constant from 162.7 Hz and 3.2 Hz as the magnesium-ion concentration increases

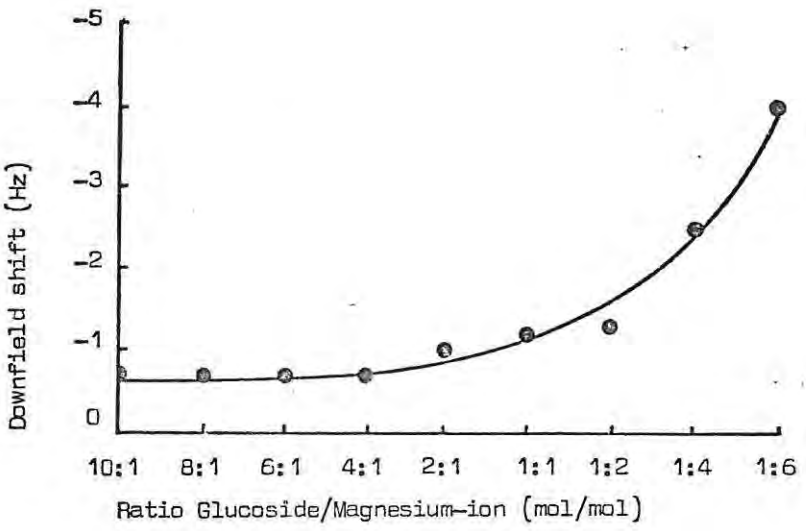


Figure 8 Graph of changes in chemical shift of the anomeric proton as the magnesium-ion concentration increases

Table IV and Figure 9 indicate the changes in chemical shift in the H-2 signal. It should be noted that the values indicated are subject to error as the centre of the quartet was only determined in double resonance experiments performed on pure glucoside. Apart from an initial downfield shift (0.7 Hz) in the resonance pattern, a steady increase in paramagnetic shift ($\Delta\nu$ 90.8 → 96.6 Hz) was observed as the ratio of glucoside to magnesium perchlorate changes (2:1 → 1:6)

Ratio Glucoside / Magnesium-ion	10:1	8:1	6:1	4:1	2:1	1:1	1:2	1:4	1:6
Change in Chemical Shift (Hz)	-0.7	-0.7	-0.7	-0.7	-0.8	-1.0	-1.4	-3.4	-5.8

Table IV Change in chemical shift of the H-2 proton from 90.7 Hz as the magnesium-ion concentration increases

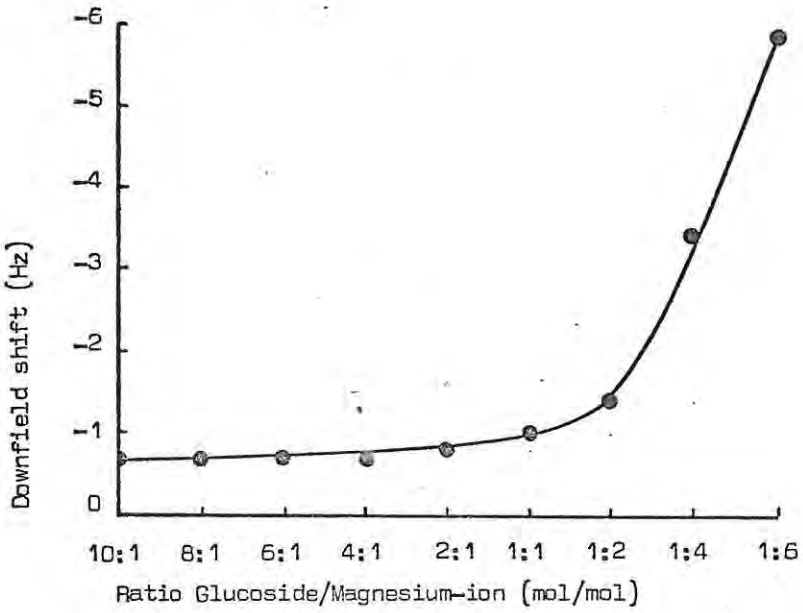


Figure 9 Graph of changes in chemical shift of the H-2 proton as the magnesium-ion concentration increases

At high concentrations of magnesium perchlorate large shifts are induced in Region B (Figure 5) of the spectrum, as shown by the downfield shift (6.4 Hz) in an unassigned peak ($\Delta\nu$ 90.3 Hz). Resonances in Region A (Figure 5) of the spectrum (unassigned multiplets, $\Delta\nu$ 104.3; 102.0 Hz) also showed relatively large downfield shifts ($\Delta\nu$ 104.3 → 108.9; 102.0 → 106.7 Hz).

In order to account for the above phenomena the appropriate molecular model of the glucoside was examined. Slight upward displacement of OH-2 (and OH-4) and concomitant downward displacement of OH-3, resulting in a small amount of torsional strain in the C-2 to C-3 (and C-3 to C-4) bonds, brings the three oxygen atoms O-2, O-3 and O-4 nearly into the same plane, the pyranose ring still remaining in the C1(D) conformation. The effect of this displacement on the normal steric arrangements of the groups mentioned is to increase the dihedral angle between

H-1e and H-2a and bring the hydroxyl groups OH-2 and OH-3 into a position favourable for the formation of a carbohydrate - metal ion complex. The driving force for this distortion in the molecule is the competition between glucoside hydroxyl groups and water molecules for the hydration of the magnesium-ions. The latter have empty d-orbitals in their valence M-shells and hence an octahedral structure is possible for the solvated magnesium-ion ($Mg\ (H_2O)_6^{++}$) [131,132].

Based on the data obtained from polarimetry measurements and that obtained from p.m.r. spectra, a structure for the glucoside - magnesium-ion complex (1:1) is proposed in which the carbohydrate acts as a bidentate ligand which displaces two water molecules from the hydration sphere of the solvated magnesium-ion to form a five-membered co-planar ring in which the oxygen atoms of the hydroxyl groups bonded to C-2 and C-3 respectively are involved without the loss of the hydroxyl protons. The complex is stabilized at high concentrations of magnesium perchlorate as a result of a shift in the equilibrium between free glucoside and complexed glucoside in favour of the latter.

1.4.2 Magnesium-ion complexes with cellulose

Experiments conducted on cellulose powder were initially performed at room temperature with unbuffered and buffered metal ion solutions (Table V). Increases in metal ion concentrations after treatment, which may be accounted for in terms of hydration and swelling of the cellulose samples, were observed. This conclusion is substantiated when one considers the changes occurring

in concentration differences, after buffering the metal ion solutions. The swelling of cellulose is known to depend on pH[133], thus the fourfold increase in concentration of magnesium-ions, and sixfold increase for calcium -ions, in the buffered solutions after 12 h, compared with the concentration increases in the unbuffered solutions after a similar time, is accounted for.

Metal ion Solution	Cellulose Treatment Time (h)	Metal ion Molarity $\times 10^3$ (mol ℓ^{-1})	pH	Cellulose Sample Weight (g)	Percentage Change
Unbuffered Magnesium Chloride	0	9.99	5.82	0	0
	12	10.01	5.32	1.0000	+0.20
	36	10.03	5.30	1.0001	+0.40
Buffered Magnesium Chloride	0	9.93	10.22	0	0
	12	10.01	10.22	1.0001	+0.80
Unbuffered Calcium Chloride	0	5.02(9)	6.16	0	0
	12	5.02(9)	6.31	1.0000	0
	36	5.05(9)	6.33	1.0000	+0.60
Buffered Calcium Chloride	0	10.37	10.20	0	0
	12	10.43	10.20	0.9999	+0.58

Table V Cellulose samples treated at room temperature

The effect of cellulose swelling, on the changes in metal ion concentrations, is in opposition to the effect of cellulose – metal ion complex formation. The swelling phenomenon "concentrates"

the metal ion solutions whereas complex formation "dilutes" them. It may be concluded that if, at room temperature, in buffered magnesium- or calcium-ion solutions, complex formation between cellulose and metal ions occurs, it does so to an extent of less than 0.8%.

An attempt was made to approximate some conditions which exist during the commercial bleaching process. The cellulose samples were heated in limited amounts of oxygen, in contact with either unbuffered or buffered metal ion solutions (Table VI). After 12 h the unbuffered solutions had remained colourless and the treated cellulose samples had retained their whiteness. No changes in metal ion concentrations and no U.V. absorbances were observed for these solutions. The diffuse U.V. reflectances of unbuffered metal ion treated cellulose samples were 60% at 275 nm, identical with that of the cellulose blanks (Figure 10; curve f). It may be concluded that neither degradation of the cellulose samples nor complex formation had occurred.

However, the buffered metal ion solutions, in contact with similar cellulose samples, had turned dark brown, the samples were noticeably coloured and precipitates had formed on the interior walls of the bottles. Concentration decreases of 37.47% in the magnesium-ion solution and 49.77% in the calcium-ion solution were observed. Both metal ion solutions after treatment of the cellulose samples had similar U.V. spectra with λmax at 272.5 nm and a broad shoulder at 300 nm (Figure 11). The diffuse U.V. reflectances at 275 nm of the dried cellulose samples, were

22% (magnesium-ion treated) and 15% (calcium-ion treated), indicating that greater degradation of the calcium-ion treated cellulose had occurred (Figure 10; curves d, a). It would appear that magnesium-ions "protect" the cellulose better than calcium-ions. Cellulose samples treated with buffer solution only, had diffuse U.V. reflectances of 17% indicating that slightly greater degradation of these samples had occurred in the absence of "protective" metal ions (Figure 10; curve b).

Blank determinations performed on the buffered metal ion solutions, in the absence of cellulose samples, indicated concentration decreases of 90.99% for magnesium-ions or 4.63% for calcium-ions. Although both of these solutions (pH 10) after preparation were clear and remained so even after being stored, when heated, insoluble metal hydroxides were precipitated. Magnesium and calcium hydroxides are less soluble at high temperatures[134], hence the observed percentage decreases in metal ion concentrations in the blank determinations are explained. Comparison of these decreases with those occurring in the solutions in contact with the cellulose samples, indicates differences of 53.48% for the magnesium-ion solution and 45.14% for the calcium-ion solution which cannot be accounted for in terms of the precipitation of insoluble metal hydroxides. An attempt to explain these differences will be made later.

Metal ion Solution	Cellulose Treatment Time(h)	Metal ion Molarity x 10^3 (mol ℓ^{-1})	pH	Cellulose Sample Weight (g)	Percentage Change
Unbuffered Magnesium Chloride	0	9.99	5.81	0	0
	12	9.99	5.82	2.0003	0
	12	9.98	5.80	0	-0.10
Buffered Magnesium Chloride	0	9.93	10.20	0	0
	12	5.20(9)	10.19	2.0005	-37.47
	12	0.89(9)	10.21	0	-90.95
Unbuffered Calcium Chloride	0	5.02(9)	6.14	0	0
	12	5.02(9)	6.15	2.0001	0
	12	5.02(9)	6.14	0	0
Buffered Calcium Chloride	0	10.37	10.20	0	0
	12	5.20(9)	10.20	2.0008	-49.77
	12	9.89	10.21	0	- 4.63

Table VI Cellulose samples treated at 120° (air-saturated)

The same effects as discussed above, though to a lesser extent were observed when cellulose samples were treated with buffered nitrogen saturated metal ion solutions (Table VII). No concentration differences were observed for these solutions, which remained uncoloured, as did the respective cellulose samples in contact with them during the heating period. The solutions showed no U.V. absorbances and the diffuse U.V. reflectance of each of the samples was 60%, as before (Figure 10; curve f)

For the buffered nitrogen saturated solutions, however, the blank corrected, percentage decreases in concentration of magnesium- and calcium-ions were 55.07% and 28.59% respectively. This will be discussed later. Both solutions were brown in colour and exhibited U.V. absorbtion spectra similar to those already discussed for the air-saturated buffered metal ion solutions. The cream coloured dried cellulose samples had diffuse U.V. reflectances of 27% and 22% for magnesium-ion and calcium-ion treated celluloses respectively, while the cellulose samples treated with nitrogen-saturated buffer solution only, had diffuse U.V. reflectances of 20% (Figure 10; curves e, d, c). These values are improvements on those mentioned earlier indicating that exclusion of oxygen from the system decreases the amount of cellulose degradation which occurs at 120°.

After treatment, all buffered metal ion solutions in contact with cellulose samples, were subjected to descending paper chromatography. D-glucose was applied as standard and the chromatograms were developed for 18 h in both ethyl acetate - acetic acid - formic acid - water (18:3:1:4) and ethyl acetate - pyridine - water (8:2:1) solvent systems. Visualization was achieved after spraying the dry papergrams with either p-anisidine hydrochloride[135] or alkaline permanganate-periodate spray reagents[136]. No reducing or non-reducing sugars were detected. Molisch tests on the same metal ion solutions were negative.

To determine whether cellulose samples could cause concentration differences when treated with unbuffered metal ion solutions,

Metal ion Solution	Cellulose Treatment Time(h)	Metal ion Molarity x 10^3 (mol ℓ^{-1})	pH	Cellulose Sample Weight (g)	Percentage Change
Unbuffered Magnesium Chloride	0	10.23	5.82	0	0
	12	10.23	5.81	1.0006	0
	12	10.22	5.80	0	- 0.10
Buffered Magnesium Chloride	0	10.17	10.15	0	0
	12	5.86(1)	10.00	1.0005	-42.37
	12	0.26(0)	10.00	0	-97.44
Unbuffered Calcium Chloride	0	10.18	6.15	0	0
	12	10.18	6.15	1.0005	0
	12	10.18	6.14	0	0
Buffered Calcium Chloride	0	10.14	10.15	0	0
	12	7.18(1)	10.00	1.0004	-29.18
	12	10.08	10.00	0	- 0.59

Table VII Cellulose samples treated at 120° (nitrogen-saturated)

experiments with nitrogen-saturated alkaline magnesium sulphate solutions (pH 10) were conducted (Table VIII). A characteristic of the experiments was the relatively small differences in metal ion concentrations, but large pH changes in the metal ion solutions. None of the solutions in contact with cellulose samples showed U.V. absorbances and the diffuse U.V. reflectance spectra of the samples, indicated no cellulose degradation.

The pH of the sodium hydroxide solution, used in the preparation of the alkaline magnesium sulphate, changed by 4.34 units during a 12 h heating period in contact with a cellulose sample, an observation which may be explained in terms of the swelling phenomenon mentioned earlier. In the heated metal ion solutions, small amounts of insoluble magnesium hydroxide were formed. The decreases in the magnesium-ion concentration with respect to the original metal ion solutions, would suggest that complex formation had occurred. However, the accompanying decreases in pH, and metal ion concentration increases with respect to the blanks, indicate rather that complex formation was probably negligible while the amount of swelling large.

Metal ion Solution	Cellulose Treatment Time(h)	Metal ion Molarity x 10^3 (mol ℓ^{-1})	pH	Cellulose Sample Weight (g)	Percentage Change
Alkaline Magnesium Sulphate	0	10.05	9.67	0	0
	1	10.05	5.95	1.0002	0
	1	10.04	5.95	1.0002	-0.10
	1	10.00	8.47	0	-0.50
	12	10.00	4.69	1.0005	-0.50
	12	10.01	4.51	1.0010	-0.40
	12	9.91	6.60	0	-1.39
Sodium Hydroxide	0	100.0	9.94	0	.
	1	Mg^{++} Absent	7.92	1.0001	.
	12	Mg^{++} Absent	5.60	1.0009	.

Table VIII Cellulose samples treated with alkaline metal ion solutions at 120° (nitrogen-saturated)

In order to verify the last conclusion, pretreated cellulose samples were used in a further experiment (Table IX). The samples were pretreated by heating with either alkaline magnesium sulphate or sodium hydroxide. The magnesium-ion treated cellulose samples and the solutions in contact with them remained uncoloured while the sample heated with sodium hydroxide turned beige and the solution light brown. The samples were then washed with water (pH 1) to free the fibres from residual metal ion solutions and insoluble adsorbed magnesium hydroxide precipitate. After a second treatment with alkaline magnesium sulphate in the cold, the magnesium-ion pretreated cellulose samples, caused very slight increases in metal ion concentrations, while the alkali pretreated sample, caused a decrease in metal ion concentration.

It may be concluded that the formation of cellulose - metal ion complexes to an extent of at least 0.30% occurs concomitantly with the swelling phenomenon. A possible reason for this small value may be explained in terms of the swelling of the cellulose. The observed change in metal ion concentration is the nett result of two opposing effects: swelling and complex formation. In this experiment the latter effect was the more dominant. To obtain a more precise measure of the concentration changes produced by complex formation, the swelling effect would have to be quantitatively defined. This was not possible using the method already outlined.

Metal ion Solution	Cellulose Treatment Time(h)	Temperature (°C)	Metal ion Molarity x 10^3 (mol ℓ^{-1})	pH	Cellulose Sample Weight (g)	Percentage Change
Alkaline Magnesium Sulphate	0	20	0	11.46	0	0
	1	120	0.86(0)	9.08	1.0000	+
	1	120	0.85(0)	8.94	1.0000	+
	1	120	0	11.21	0	0
	0	20	10.03	9.05	0	0
	5	20	10.04	7.05	1.0000	+0.10
	5	20	10.04	7.10	1.0000	+0.10
	5	20	10.00	6.75	1.0004	−0.30
Sodium Hydroxide	0	20	25	12.05	0	.
	1	120	Mg^{++} Absent	9.71	1.0004	0

Table IX Pretreated cellulose samples treated again at room temperature

However, it may be concluded that, in unbuffered but alkaline magnesium sulphate solutions, cellulose is "protected" by the metal ions when heated to a moderately high temperature.

The experiments discussed above were performed on samples of commercially pure cellulose. Pilot experiments were then performed on crude pine pulp and cotton linters (Table X). Relatively large quantities of magnesium sulphate were absorbed by the samples, at room temperature. This could be explained in terms of an ion-exchange process. The pulp, being unbleached,

would contain ligin which is able to form weak salts with the magnesium-ions present during treatment. Similarly the cotton linters could contain free carboxyl groups responsible for the uptake of metal ions.

Metal ion Solution	Sample	Treatment Time(h)	Temperature (°C)	* Metal ion Concentration	pH	Sample Weight (g)	Percentage Change
Alkaline Magnesium Sulphate	Pulp	0	20	2.46(6)	10.05	0	0
		0.5	20	2.34(0)	8.95	1.0023	-5.11
		0	20	1.23(4)	9.45	0	0
		0.5	20	1.15(8)	8.45	1.0012	-6.16
		0.75	120	1.18(0)	6.98		-4.38
		0.75	120	1.23(1)	8.75	0	-0.24
	Cotton Linters	0	20	1.23(9)	9.25	0	0
		0.5	20	1.20(3)	8.95	1.0344	-2.91
		0.75	120	1.19(5)	7.45		-3.55
		0.75	120	1.22(8)	8.98	0	-0.89

* (mg Mg^{++} g sol^{-1} g $sample^{-1}$)

Table X Pulp and cotton linters treated with metal ion solutions

The method used to detect possible cellulose - metal ion complexes is unable to differentiate between an ion-exchange process and complex formation, since both have the same nett result on the concentration changes in metal ion solutions. It is for this reason that discussion of some of the earlier results was delayed until this point.

Reports have been published suggesting that degradation of

cellulose molecules during alkali-oxygen treatment at high temperatures starts by random cleavage of the D-glucosidic bonds along the molecular chain[137] whereupon terminal D-glucose end groups are formed which are attacked[138], resulting in a stepwise "peeling" of the same type as that occurring during low temperature alkali treatment of hydrocellulose[139]. Formation of carboxylic end groups render the terminal groups stable to further attack[140]. Manley[141] has reported that this bond cleavage is not random and that evidence exists for "weak links" at every eight glucose unit in the cellulose molecule, which might be the points of oxygen attack leading to the gross depolymerization of the cellulose, known to occur during oxygen-alkali bleaching[121].

The above suggestions could explain the take up of magnesium-ions by the cellulose powder samples during treatment with air-saturated buffered metal ion solutions, but fail to explain the substantial metal ion uptake in nitrogen-saturated buffered metal ion solutions, In the absence of oxygen the degradation of the cellulose is attenuated, hence the results obtained for nitrogen-saturated buffered metal ion solutions would suggest that complex formation between the cellulose and metal ions occurs.

The experiments discussed above have established that the cellulose molecule is able to bind, in an insoluble way, magnesium- or calcium-ions.

The infrared spectra, (Figure 12) of treated and untreated cellulose powder samples were identical and showed no absorbances which could be unequivocally assigned to carboxyl groups, indicating that if present, the concentration was very low. It may be concluded that gross changes in the cellulose after treatment are absent, since were this the case, differences in the infrared spectra would be evident. The appearance of carboxyl peaks in the spectra would suggest ionic binding of the metal ions and since this is not the case, the hypothesis of complex formation is strengthened.

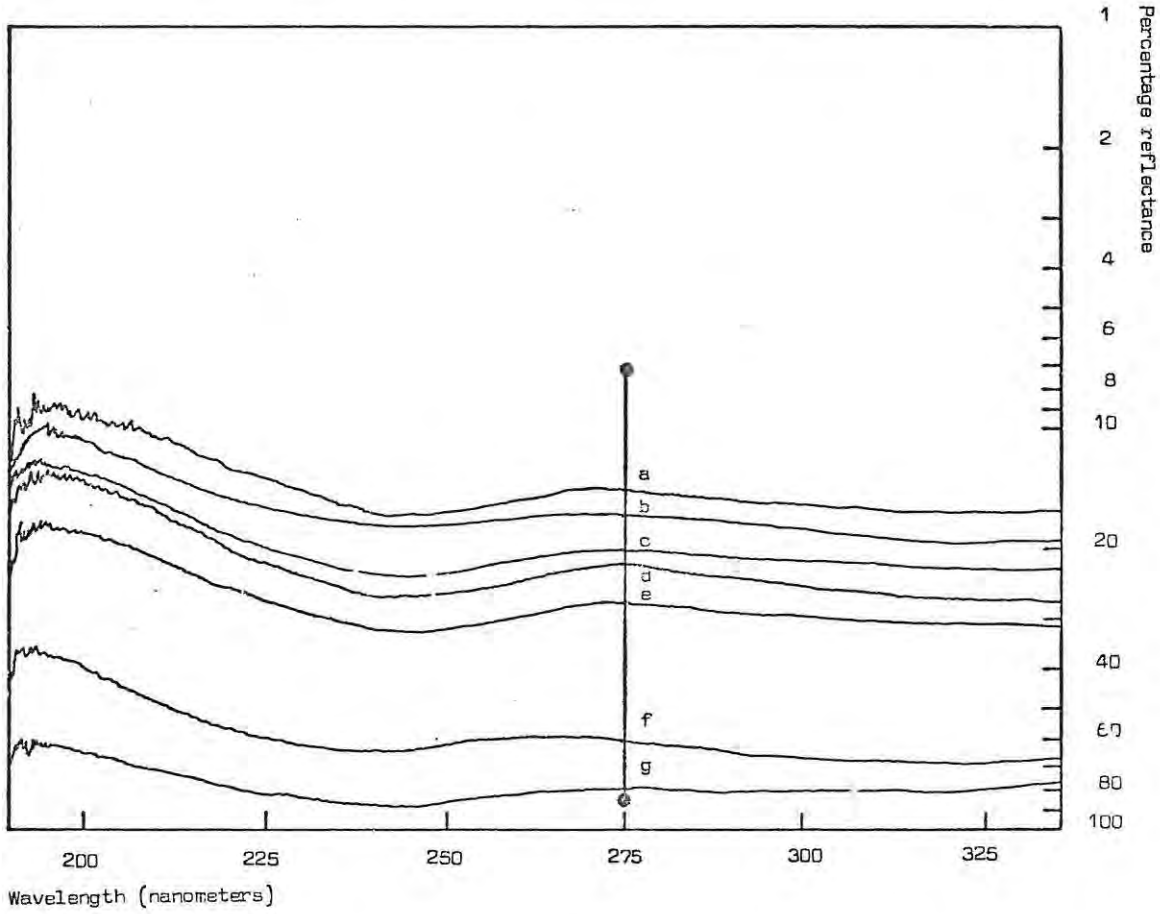


Figure 10 Diffuse ultra-violet reflectance spectra of selected cellulose samples Curves: a = 15%; b = 17%; c = 20%; d = 22%; e = 27%; f = 60% (cellulose blank) g = 80% (magnesium oxide standard)

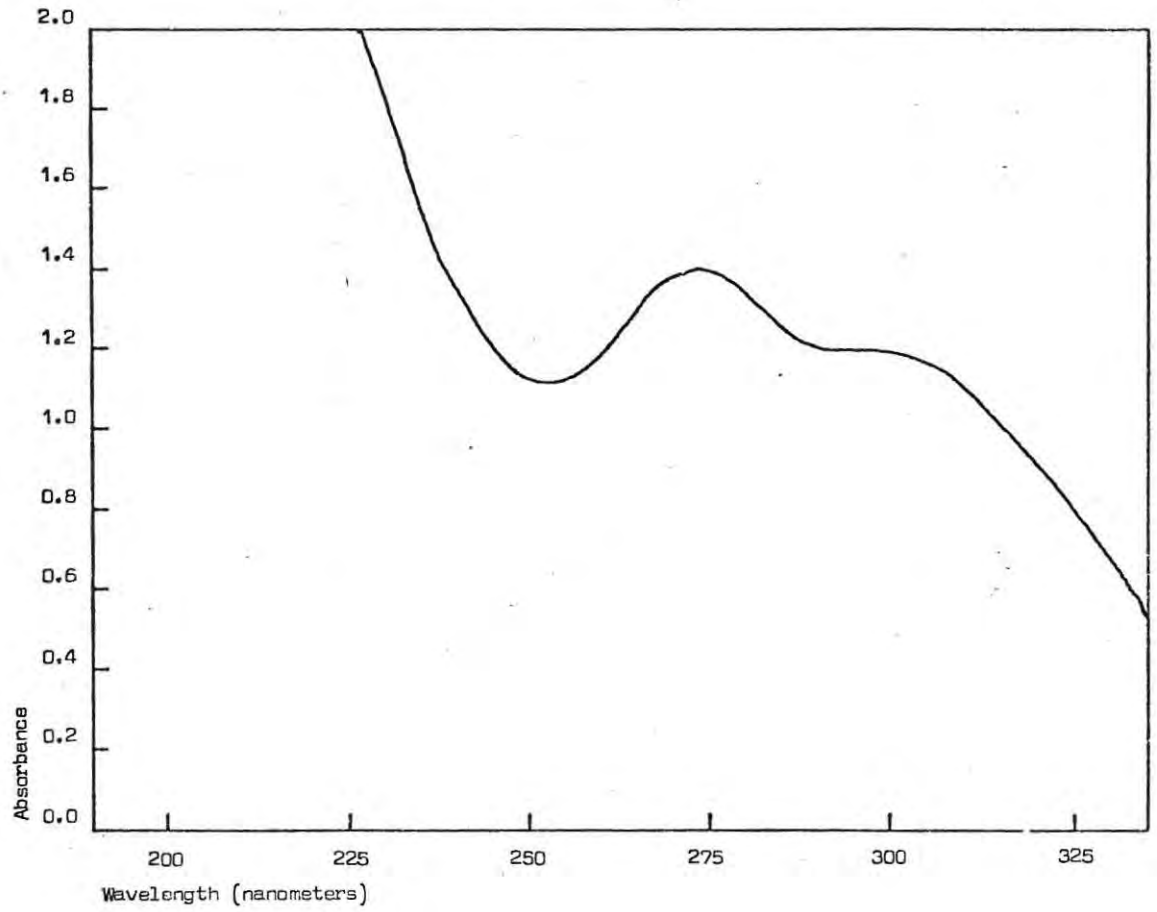


Figure 11 Ultra-violet absorption spectrum of typical metal ion solution after heating with a cellulose sample

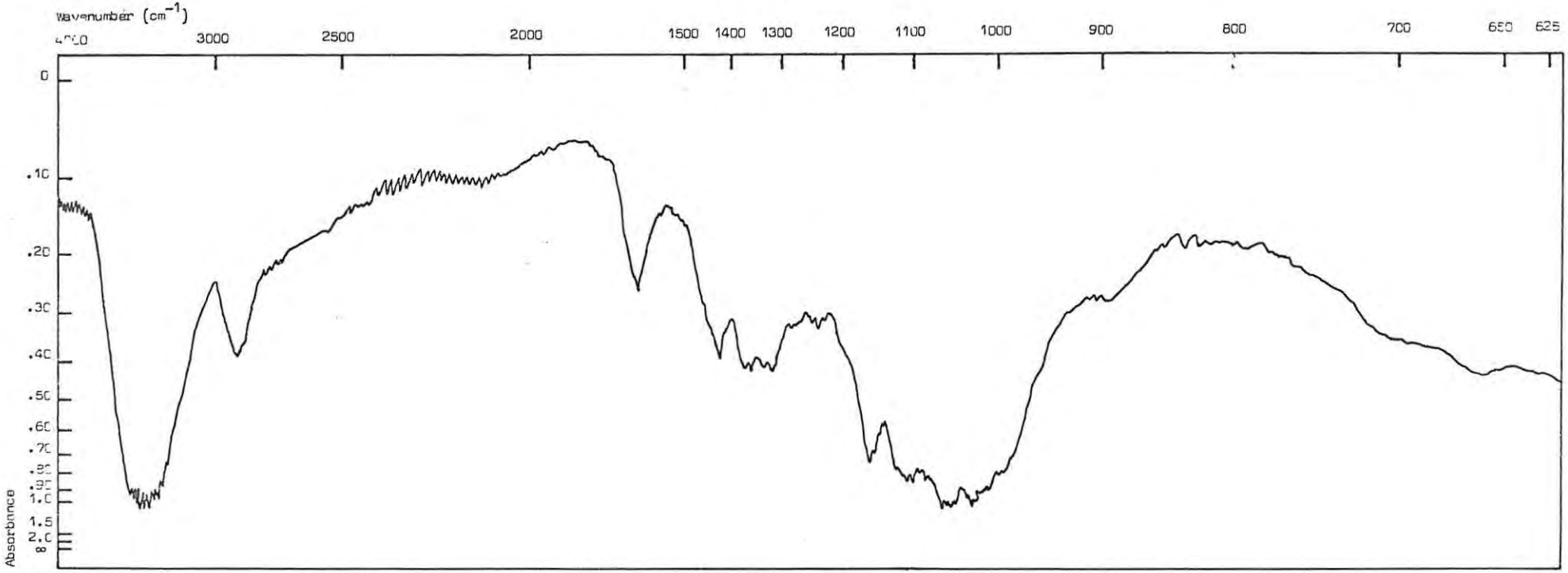


Figure 12 Infrared spectrum of a typical cellulose sample

1.5 EXPERIMENTAL

1.5.1 Polarimetry

Optical rotations (± 0.001°) were recorded on a Perkin-Elmer 141 polarimeter at 589, 578, 546, 436 and 365 nm in matched 0.1 dm water jacketed quartz cells at temperatures (± 0.05°) of 20, 30, 40 and 50°, in distilled water.

1.5.2 Solutions for polarimetry

Methyl-α-D-glucopyranoside, dried over P_2O_5 at 60°/0.1 mm for 24 h, had m.p. 166 - 167°; $[\alpha]_D^{20}$ + 158.95° (Lit. m.p. 167°; $[\alpha]_D^{20}$ + 158 - 159°)[90]. A stock solution (100 ml) of approximately 2.5M $Mg(ClO_4)_2.6H_2O$ was standardized volumetrically with E.D.T.A.[142]. Each separate solution was prepared as follows. The calculated amount (± 0.1 mg) of methyl-α-D-glucopyranoside, corresponding to the desired molarity was dissolved in the minimum of water in a volumetric flask (10.0 ml). The required volume of standard stock magnesium-ion solution, calculated to give the desired molarity, was then run in from a semimicro burette. The solution was made up to volume with water, the flask and its contents being maintained at constant temperature (20°) in a water bath. Blank solutions of glucoside and magnesium perchlorate were prepared similarly. The same volumetric flask was used for all solutions so ensuring that no variations arose from flask differences. All solutions remained clear and transparent, even after standing for several weeks at 20°.

1.5.3 P.m.r. spectroscopy

Spectra were recorded for deuterated solutions at 60 MHz on a Perkin-Elmer R 12 spectrometer. Dry redistilled methyl cyanide (2M%) was used as an internal reference from which chemical shifts

were measured in Hz, and provided a locking signal for field-frequency stabilization (± 0.1 Hz in 5 min). A sweep range of 5 p.p.m. was employed. Mean chemical shifts were calculated (± 0.3 Hz) from four spectra. The sweep rate was kept constant (0.8 Hz per sec.) and the H_1 level optimised (at 5) to prevent saturation. The sensitivity for all resonance signals, except those of the methoxyl protons, was kept constant (at 1) and then changed (to 0.5). All solutions were preheated to the probe temperature (35.0 ± 0.1°) prior to recording the spectra and the field homogeneity set to a resolution of 0.33 Hz half height width for the quartet of acetaldehyde, with a signal to noise ratio greater than 28:1 as measured on a 1% ethyl benzene solution in CCl_4.

Double resonance experiments at a sweep range of 10 p.p.m. were performed in the field sweep mode. The frequency offset of the decoupling field was ahead (72 Hz) of the observation field frequency. Spin-tickling expermiments were conducted at constant H_1 with variation of H_2 (0.5 units per time) per spectrum.

1.5.4 Solutions for p.m.r. spectroscopy

Powdered $Mg(ClO_4)_2.6H_2O$ was heated in a test tube under vacuum (200°; 0.1 mm) the product was analysed at intervals and the heating stopped (24 h) when no further change in the stoichiometry of the product $[Mg(ClO_4)_2.2H_2O]$ [143] took place. Each separate solution was prepared as follows. Methyl-α-D-glucopyranoside (97.1 ± 0.1 mg) was weighed into a glass stoppered test tube (3.5 ml; B 10), followed by a mass of $Mg(ClO_4).2H_2O$ calculated to yield the desired carbohydrate to magnesium ratio. D_2O (1.00 ml; 99.75%, E. Merk, Uvasol), containing the internal reference, was added by means of a calibrated semimicro syringe. The tube was then stoppered and

shaken to effect solution, which was filtered through a sintered glass disc (G4; 10 mm) prior to recording the spectrum.

1.5.5 Cellulose experiments

In the first series of experiments, samples of Whatman Chromedia fibrous cellulose powder (CF 11) were treated with buffered and unbuffered solutions of either magnesium or calcium chloride, for 12 h at either room temperature, or 120° under air or nitrogen. Blank determinations were made on buffered and unbuffered metal ion solutions in the absence of cellulose, and the cellulose was heated with water or buffer, in the absence of metal ion solutions.

The buffer in a second series of experiments, was replaced with sodium hydroxide solution (pH 10); the temperature was fixed at 120°, and contact times with alkaline magnesium chloride solutions under nitrogen of 1 h or 12 h were allowed. The necessary blank determinations were made.

A third series of experiments on cellulose samples which had been pretreated for 1 h at 120°, with either sodium hydroxide (pH 12) or alkaline magnesium sulphate solution were conducted. These samples were treated for a further 5 h at room temperature, with the alkaline metal ion solution. As before the necessary blank determinations were made.

In a similar series of experiments, samples of pine pulp (N.P. 122B, Kappa No. 25.8) and samples of cotton linters, supplied by SAPPI were treated with solutions of alkaline magnesium sulphate, either at room temperature for 0.5 h, or at 120° for 0.75 h. Blank determinations on the heated alkaline metal ion solutions were made.

All samples were magnetically stirred (Section 4.1 Plate F and G)

in sealed screw top bottles (Section 4.1 Plate H) previously cleaned with chromic acid, detergent and boiling water, after which metal ion contaminants were found to be absent. All solutions were prepared from distilled deionized water saturated either with air or nitrogen. The reagents were of analytical grade and volumetric apparatus was of grade A. Ammonia/ammonium chloride buffer was prepared as usual[142]. Solutions were centrifuged when necessary and their metal ion concentrations determined as before by titration with E.D.T.A.[142], pH Adjustments of the distilled water were effected by hydrochloric acid or sulphuric acid or sodium hydroxide.

The ultra-violet absorbances of selected solutions, before and after treatment, were recorded on a Unicam SP 800 ultra-violet spectrophotometer. Selected metal ion treated cellulose samples were dried (P_2O_5, 0.01 mm Hg, 4 days) prior to recording their diffuse ultra-violet reflectance spectra on the SP 800 fitted with an SP 890 diffuse reflectance accessory. Their infrared spectra were recorded on a Beckman IR 8 infrared spectrophotometer.

2 THE ISOLATION OF AN OLIGOSACCHARIDE CONTAINING L-GALACTOSE FROM THE POLYSACCHARIDE OF *Aedes orbitosa*

2.1 ABSTRACT

The occurrence of L-sugars in selected red seaweed polysaccharides is reviewed. The extraction of the water soluble polysaccharide elaborated by the seaweed Aeodes orbitosa is described. From a large scale partial hydrolysis of the polymer two oligosaccharides, 4-O-β-D-galactopyranosyl-D-galactose and 4-O-β-D-galactopyranosyl-L-galactose were isolated, the latter in very small amount. The presence of the L-galactose residues in the polymer is discussed.

2.2 L-SUGARS IN RED SEAWEED POLYSACCHARIDES

Studies on the polysaccharides elaborated by a number of red seaweeds have indicated that they are essentially composed of D-galactose or modified D-galactose units, linked by an alternating sequence of α 1 → 3 and β 1 → 4 glycosidic bonds[144]. The polysaccharides are complex macromolecules and contain sulphate ester groups the concentration of which varies from 0%, as in the neutral agarose fraction of agar, to as much as 36% in one form of the carrageenans[145]. Both the position and concentration of these sulphate ester groups are largely responsible for the wide variety of gels obtainable from agar-type polysaccharides[146,147]. Current belief is that all of the sulphated polysaccharides isolated from the Rhodophyceae are related by a common group of monosaccharides with a degree of sulphation, and other variables, dependent on species and environmental conditions[144]. Apart from the presence of the D-sugars alluded to above, amounts of L-galactose and modified forms of this enantiomer have been detected, successfully isolated and characterized from some red seaweed polysaccharides. In most cases the L-sugars occur to a lesser extent than the D-sugars. In a semi-qualitative discussion which follows, the presence of D-sugars will be assumed, and mentioned only where necessary as an aid to explanation, while the L-sugars and their mode of linkage in selected agar-type, carrageenan-type, Grateloupiaceae and miscellaneous polysaccharides will be highlighted.

Agar-type polysaccharides abound in a number of species of red seaweeds, among which are the Gelidium, Gracilaria, Acanthopeltis, Ahnfeltia, Ceramium, Campylaephora, Phyllophora and Pterocladia. The first L-sugar to be isolated from an agar-type

polymer was 3,6-anhydro-L-galactose[148-152]. The mode of linkage of this unit, in the macromolecule extracted from Gelidium amansii, was established by the isolation and characterization of 2-O-methyl-3,6-anhydro-L-galactose dimethyl acetal[151] from the methanolysis products of the methylated polymer, while the isolation and characterization of agarobiose[152] (4-O-β-D-galactopyranosyl-3,6-anhydro-L-galactose) from a partial hydrolysis of agar, and agarobiose dimethyl acetal[153] from a partial methanolysis, provided still further information on the arrangement of the L-sugar in the polysaccharide. Enzymic hydrolysis of agar with Pseudomonas kyotoensis afforded two crystalline oligosaccharides, neoagarobiose[154] (3-O-α-L-3,6-anhydro-galactopyranosyl-D-galactose) and neoagarotetraose[155] (A_L - α - (1 → 3) - G_D - β - (1 → 4) - A_L - α - (1 → 3) - β - G_D, where A_L = 3,6-anhydro-L-galactose and G_D = D-galactose) thus defining the gross structure of agar. A further two L-sugars, detected in agar-type polysaccharides, though to a varying extent were L-galactose, and 4-O-methyl-L-galactose, the latter occurring in such small quantities in the polymer from Gelidium amansii, that Araki et al[156] considers it to have no structural significance, while the former was considered to be a contaminant[157]. Investigation on the agar-type polysaccrarides extracted from species of the Gracilaria, viz: G. debilis, G. compressa, G. foliifora, G. domingenis, G. damaecornis and G. ferrox by Duckworth et al[158], furnished similar results to those outlined above, which agree with the postulated general formula for the neutral polysaccharide fraction of agar, made earlier by Araki[157]. It was concluded that the polymer chain was terminated on the non-reducing end with 3,6-anhydro-L-galactose and

on the reducing end with D-galactose.

The polysaccharides elaborated by the Porphyra species are examples of modified agar-type polymers. For example both P. capensis[159] and P. umbilicalis[160] have been shown to contain both D-galactose and 3,6-anhydro-L-galactose. The presence of L-galactose in the polymer has also been established, while Turvey and Rees[161] have indicated the presence of L-galactose 6-sulphate as an integral component of the polysaccharide of P. umbilicalis. Turvey and Williams[162], have suggested that the simplest structure of porphyran is a chain of alternating α-1,3-linked D-galactose and β-1,4-linked L-galactose units, with some of the D-galactose being 6-O-methylated and the L-galactose occurring either as the 6-sulphate or as the 3,6-anhydride. These suggestions have been corroborated by the isolation and characterization from porphyran, of the following four oligosaccharides[162], containing L-galactose or its modified forms: $G_D - \beta - (1 \rightarrow 4) - G_L$ 6-SO_4; 6-O-Me-$G_D - \beta - (1 \rightarrow 4) - G_L$ 6-SO_4; G_L 6-$SO_4 - \alpha - (1 \rightarrow 3) - G_D$ and G_L 6-$SO_4 - \alpha - (1 \rightarrow 3)$ -6-O-Me-G_D, where G_D = D-galactose; G_L 6-SO_4 = L-galactose 6-sulphate and 6-O-Me-G_D = 6-O-methyl-D-galactose. The similarity of the porphyran polysaccharides to the agar-type polysaccharides lies in the presence of β- (1 → 4)-linked L-galactose units, whereas the individuality of these polymers is evident in that only part of the L-galactose occurs as the 3,6-anhydride while the remainder is present as the L-galactose 6-sulphate.

Another polysaccharide which may be regarded as a modified agar-type is the galactan elaborated by Laurentia pinnatifida[163]. The presence of L-galactose, 2-O-methyl-L-galactose, 3,6-anhydro-L-galactose and 3,6-anhydro-2-O-methyl-L-galactose and L-galactose 6-sulphate have all been established as components of the

polysaccharide. Bowker and Turvey[164] have suggested that the L-galactose and 2-O-methyl-L-galactose units are linked through position 4, while the D-residues are linked through position 3. This polysaccharide is closely related to that briefly discussed above for the Porphyra species, however its individuality lies in the fact that some of the 3,6-anhydro-L-galactose and L-galactose units are 2-O-methylated.

A large group of red seaweed polysaccharides containing little or no L-sugars are those known as the Carrageenan polysaccharides, which system had been shown to be a spectrum of several related polymers referred to as ι-, κ-, λ-, μ-, ν-, and ξ-carrageenan[3, 165-175]. In early work on whole carrageenan, Johnston and E.G.V. Percival[176] reported the presence of L-galactose units in the polymer. Rees et al[168], however, have been unsuccessful in detecting this sugar in hydrolysates of either κ- or λ-carrageenan, which had been treated with D-galactose adapted yeasts. Although the κ-carrageenan-type polysaccharide extracted from Gigartina tenella[177] afforded very small amounts of L-galactose on methanolysis and hydrolysis, the presence of this sugar in the polymer is thought to be a contaminant. The κ-carrageenan-type polymer, however, extracted from Gigartina skottsbergii has been reported by Cerezo[178,179] to contain L-galactose units.

Nunn, Parolis et al[180-187], have isolated sulphated galactans, most of which are methylated, from some seaweeds of the Grateloupiaceae family. This family of seaweeds consists of nineteen genera[188], ten of which are represented in South Africa[189]. The polysaccharides so far examined are from Aeodes orbitosa[180,181], Phyllymenia cornea[182,183], Aeodes ulvoidea[184], Pachymenia carnosa[185,186], Phyllymenia hieroglyphica[190], Pachymenia hymantophora[173] and Grateloupia

elliptica[191]. The L-sugars found in the above polymers are indicated in Table a. Oligosaccharides (Table b) containing L-galactose units have also been isolated from the various polymers studied and indicate the mode of linkage of the L-sugars in these polymers. Of the polymers which contain 4-O-methyl-L-galactose, only that from Aeodes ulvoidea has been shown to contain this sugar in more than trace quantity. The mode of linkage of the 4-O-methyl-L-galactose in the polymer has recently been established and reported[187].

Saccharide unit	Aeodes orbitosa	Aeodes ulvoidea	Phyllymenia cornea	Pachymenia carnosa	Grateloupia elliptica
L-galactose	+	+	+		+
2-O-methyl-L-galactose		+		+	+
4-O-methyl-L-galactose	+	++	+	+	
2-O-methyl-3,6-anhydro-L-galactose					+

Table a L-units present in Grateloupiaceae polysaccharides

Oligosaccharide	Aeodes orbitosa	Aeodes ulvoidea	Phyllymenia cornea	Pachymenia carnosa
$G_D\,\beta(1\rightarrow4)G_L$	+	detected chromato-graphically	+	
$4G_L\,\alpha(1\rightarrow6)G_D$		+		
$G_D\,\beta(1\rightarrow4)2G_L$		+		+
$4G_L\,\alpha(1\rightarrow6)[G_D\,\beta(1\rightarrow4)]G_D$		+		
G_D = D-galactose; G_L = L-galactose; $2G_L$ = 2-O-methyl-L-galactose; $4G_L$ = 4-O-methyl-L-galactose				

Table b Oligosaccharides, containing L-units, isolated from Grateloupiaceae polysaccharides

Of the various miscellaneous polysaccharides elaborated by other members of the Rhodophyceae, the polymer syntehsised by Anatheca dentata (Solieriaceae)[192,193] requires special mention, since in this polysaccharide the ratio of D-galactose to L-galactose is 1.6:1. Most of the L-galactose has been shown to be 4-linked while unbranched D-galactose units have been shown to be 3-linked, with both enantiomers occurring as non-reducing end groups. Evidence for the existence of disulphated and trisulphated L-galactose units in the macromolecule exists. Partial hydrolysis of the polymer afforded the following L-galactose containing oligosaccharides: 4-O-β-D-galactopyranosyl-L-galactose, 3-O-α-L-galactopyranosyl-D-galactose and O-β-D-galactopyranosyl-(1 → 4)-O-α-L-galactopyranosyl-(1 → 3)-D-galactose, indicating that a substantial part of the polymer is made up of α-(1 → 3)-linked D-galactose and β-(1 → 4)-linked L-galactose residues and in this respect resembles one of the extreme agar structures postulated by Duckworth and Yaphe[194].

Another miscellaneous sulphated polysaccharide containing L-sugars is that obtained after decalcification of Corallina officinalis[195]. L-galactose 6-sulphate and L-galactose 4-sulphate were obtained on mild acid hydrolysis of the polymer. The mucilage of Gloiopeltis furcata has been shown by Hirase et al[196,197], to contain 3,6-anhydro-L-galactose and L-galactose. This macromolecule differs from the porphyran-type polysaccharides mentioned above since it contains no 6-O-methyl-D-galactose units and is distinguished from agarose by its high sulphate ester and L-galactose content. A similar polysaccharide is elaborated by Gloiopeltis cervicornis[173]. The mucilage from Polysiphonia fastigiata[198] afforded L-galactose on hydrolysis as well as 6-O-methyl-L-galactose and 3,6-anhydro-

L-galactose, while tentative evidence of L-galactose 6-sulphate in the mucilage has also been obtained.

The fact, that in most cases the amount of D-galactose and its modified forms occurring in the polysaccharides of the Rhodophyceae is greater than the amount of L-galactose and its modifications, makes the detection, isolation and characterization of the latter enantiomer far from routine. Polysaccharides still to be extracted from members of the Rhodophyceae, which as yet have not been investigated may in the future reveal polymers in which the D/L - galactose ratio is inverted and the L-enantiomers occur in higher concentrations. The versatility of Nature does not preclude this possibility.

2.3 INTRODUCTION

Previous work on aeodan, the polysaccharide(s) elaborated by the red seaweed Aeodes orbitosa (Grateloupiaceae), indicates that the basic structure is an alternating sequence of an α-(1 → 3)-and a β-(1 → 4)-linked galactopyranose unit[180,181]. The polymer is sulphated (27.5% calc. as $NaSO_3^-$) and differs from the carrageenan system of polysaccharides in the location of the sulphate ester groups. Although the major sulphated units have already been shown to be (1 → 3)-linked galactose 2-sulphate and probably (1 → 3)-linked galactose 2,6-disulphate, the presence of some sulphate-free (1 → 3)-linked units has been established. Most of the (1 → 4)-linked galactose residues are sulphate free. The monosaccharide residues found in the polymer are D-galactose (58%), monomethylgalactoses (10%), consisting of 2-O-methyl-D-galactose, 4-O-methyl-L-galactose and 6-O-methyl-D-galactose, and trace amounts each of xylose and glycerol[180]. Oligosaccharides already isolated and characterized from a partial acid hydrolysate of the polymer, are 4-O-β-D-galactopyranosyl-D-galactose and 3-O-α-D-galactopyranosyl-D-galactose[181].

Further paper chromatographic studies on partial hydrolysates of aeodan indicated the presence of an oligosaccharide with a R_g value equal to that of authentic 4-O-β-D-galactopyranosyl-L-galactose[192]. This would suggest the hitherto undetected presence of L-galactose residues in the polymer. Hence a partial hydrolysis of the polymer on a large scale was conducted in an attempt to isolate and characterize the L-galactose containing disaccharide. This would further define the structure of aeodan.

2.4 RESULTS AND DISCUSSION

Only four oligosaccharides containing L-galactose or methylated L-galactose units have been isolated from hydrolysates of the polysaccharides elaborated by seaweeds of the Grateloupiaceae species. The oligosaccharides are: (1) 4-O-β-D-galactopyranosyl-L-galactose[199]; (2) 6-O-(4-O-methyl-α-L-galactopyranosyl)-D-galactose[187]; (3) 4-O-β-D-galactopyranosyl-2-O-methyl-L-galactose[187] and (4) O-(β-D-galactopyranosyl)-(1 → 4)-O-[4-O-methyl-α-L-galactopyranosyl-(1 → 6)]-D-galactose[187]. The isolation of oligosaccharides (1) and (2) suggests that some of the (1 → 4)-linked D-galactose units in the polymers may be replaced by their L-enantiomers. This may not occur in all the polymers as L-galactose units have still to be determined in the polysaccharides of Phyllymenia hieroglyphica[190] and Pachymenia hymantophora[173]. The isolation of oligosaccharide (2) from the polymer of Aeodes ulvoidea, demonstrated for the first time the mode of linkage of 4-O-methyl-L-galactose in a red seaweed polysaccharide[187]. The sugar previously shown[184] to be present as a non-reducing end group has recently been shown to be linked as a single branch unit attached to position 6 of the 4-linked galactose residues in the (1 → 4), (1 → 3)-linked polysaccharide chain. Oligosaccharide (1), chromatographically identified as a trace component of the hydrolysate of the polysaccharide from Phyllymenia cornea[182] has now been isolated and characterized from a partial hydrolysate of of the polymer(s) of Aeodes orbitosa. The fact that the sugar occurs in such low concentration may be interpreted in either of two ways: (a) some of the D-galactose units in the polymer could occur as L-galactose units (already hinted at as a general feature

of these polymers) or (b) the sugar could be part of a contaminating polysaccharide co-occurring with the major polymer extracted from Aeodes orbitosa. Previous ultracentrifuge studies[180] on purified polymer exhibited a single sharp peak indicating that the polymer is probably homogeneous and an extended type of molecule. The present work was done on a sample of polysaccharide obtained from weed harvested at a different time from that mentioned above, and which could have contained a second polysaccharide as mentioned above. Although it is not possible, with present evidence, to exclude the possibility of the L-sugar having arisen from a contaminating polysaccharide, it seems highly improbable when comparing the polymer from Aeodes orbitosa with the other Grateloupiaceae polysaccharides.

2.5 EXPERIMENTAL

Concentration of solutions was carried out at 40°/20 mm, and specific rotations were measured in water. Corrected melting points were determined in capillaries on a Gallenkamp melting point apparatus. Infrared spectra were recorded on a Beckman IR - 8 spectrophotometer using KBr discs. Paper chromatography, using Whatman No. 1 sheets, was performed in the following solvent systems: (A) ethyl acetate - acetic acid - formic acid - water (18:3:1:4) and (B) ethyl acetate - pyridine - water (8:2:1). Visualization of the dry papergrams was achieved after spraying with p-anisidine hydrochloride reagent[135]. R_g values refer to rates of movement relative to that of galactose.

2.5.1 Isolation and purification of the polysaccharide

Fresh Aeodes orbitosa (4.4 kg) was washed and mixed with water (18 l). Acetic acid was added to pH 2 - 3 to aid in rapid disintergration of the weed. Steam was passed into the mixture (1 h) with periodic stirring during which time the pH rose to between 6 and 7. The crude extract was rapidly passed through a basket centrifuge while still hot, yielding a murky pale brown mucilage. Steam was then passed into the mixture for a second time (15 mins) and the extract centrifuged through a Sharples continuous centrifuge (20,000 rpm). This afforded a clear, pale, yellow-brown liquid (20 l) which was stored in a refrigerator overnight. A colloidal precipitate which appeared was removed by further centrifugation of the cold solution. Precipitation into alcohol (5 vols) and washing with ethanol and finally with ether afforded, after drying in a vacuum oven (45°, 0.1 mm, 12 h, silica gel), an off-white product (460 g, 10.4% on a wet wt. basis).

Further purification of the polysaccharide (100 g) was effected by dissolution in water (1.5 l), centrifugation of the solution, and precipitation into ethanol (5 vols). After three cycles a white fluffy product (60 g), $[\alpha]_D^{20}$ + 77° (c 0.5) was obtained on drying (45°, 0.1 mm, 12 h, silica gel).

2.5.2 Determination of a suitable time for the partial hydrolysis of the polysaccharide

The polysaccharide (5 g) dissolved in sulphuric acid (0.5 M) 150 ml), was heated (98°) under reflux on a water bath. Aliquots (1 ml) were removed at intervals (0.5 h), neutralized (barium carbonate), centrifuged, decanted and evaporated to dryness on individual watch glasses. Each residue, in a minimum of water (2 drops) was applied to a sheet of chromatography paper and the chromatogram developed in solvent A (12 h). After visualization, a hydrolysis time of 3.5 h appeared to give the maximum amount of the desired oligosaccharide.

2.5.3 Partial hydrolysis of the polysaccharide

Polysaccharide (48 g) was hydrolysed with sulphuric acid (0,5 M 180 ml) under reflux on a water bath (98°). The solution was cooled, neutralized (barium carbonate) and after centrifugation was decanted from the precipitate (barium sulphate). The neutral hydrolysate was deionized by slow sequential passage through ion-exchange resins [IRA 120(H^+), IR 200(CH_3 COO^-)] and concentrated to afford a brown syrup (18.13 g). The syrup was taken up in a minimum of distilled water and applied to a charcoal-Celite column (1:1, 75 x 7 cm) which had been prewashed with water, aqueous formic acid (2%), aqueous ethanol (20%) and finally with water again.

2.5.4 Isolation and characterization of two oligosaccharides

The column was eluted with water (25 l) until free of monosaccharides, as indicated by the absence of these sugars in the chromatograms (solvent A and B) of concentrated fractions (30 ml) of column eluate. The monosaccharides were not separated or characterized as their removal was all that was required. Gradient elution of the column (0 - 5% aqueous ethanol over 50 l) was then initiated and every tenth fraction chromatographically screened (solvent A) for the presence of the L-galactose containing oligosaccharide. Similar fractions were bulked, concentrated under vacuum and afforded two main fractions: Fractions I and II.

Fraction I The syrup (0.715 g, eluted with 35 l, 2 - 3% aqueous ethanol) was shown by chromatography (solvent A and B) to contain 4-O-β-D-galactopyranosyl-D-galactose, R_g 0.35 (A) and 0.30 (B) with a trace of a second saccharide R_g 0.32 (A) and 0.26 (B). The syrup, taken up in methanol (8 ml) was set aside to crystallize. A crop of crystals (0.710 g) which appeared after a few days was filtered off and recrystallized from methanol (5 ml) affording a product (0.708 g); $[\alpha]_D^{20}$ + 94.8 (c 0.74) (5 min) → 62.5° (final), m.p. 208 - 209°, mixed m.p. 205 - 206° with authentic 4-O-β-D-galactopyranosyl-D-galactose[185]. The infrared spectrum (Figure 13) of the oligosaccharide was identical with that of authentic 4-O-β-D-galactopyranosyl-D-galactose and a chromatogram of a total hydrolysate revealed the presence of galactose only. The mother liquors from both crystallizations were kept pending further analysis.

Fraction II The syrup (120 mg, eluted with 15 l, 3 - 4% aqueous ethanol) was treated as above. A further amount (10 mg) of 4-O-β-D-galactopyranosyl-D-galactose was obtained [m.p. and mixed m.p. 205 - 206° with authentic 4-O-β-D-galactopyranosyl-D-galactose, $[\alpha]_D^{20}$ +60° (c 0.25)]. The mother liquor obtained after removal of this oligosaccharide was combined with those from Fraction I (see above). The syrup (118 mg), applied to sheets of Whatman No. 1 chromatography paper was eluted with solvent B. The sugar zones were determined (after 5 days) by the standard method and then carefully combined to give two fractions each of which was subjected to further preparative chromatography (solvent A). After three such cycles, Fraction IIa (8 mg), relatively rich in 4-O-β-D-galactopyranosyl-D-galactose, and Fraction IIb (28.3 mg) was obtained. Fraction IIa was not further investigated. The crude syrup from Fraction IIb (28.3 mg), taken up in ethanol (5 ml) was decolourized by treatment with activated charcoal, concentrated (1.5 ml) and set aside to crystallize. Crystals (13.0 mg) subsequently isolated were recrystallized from a minimum of ethanol affording a product (11.7 mg), R_g 0.32 (A) and 0.26 (B), $[\alpha]_D^{20}$ -60 (c 0.47) (6 min) → -47° (final), m.p. 237 - 238°, mixed m.p. 235 - 236° with authentic 4-O-β-D-galactopyranosyl-L-galactose[192]. The infrared spectrum (Figure 14) of this oligosaccharide was identical with that of authenic 4-O-β-D-galactopyranosyl-L-galactose and a chromatogram of a total hydrolysate revealed the presence of galactose only. Further elution of the column (15 l, 10% aqueous ethanol) afforded chromatographic evidence (solvent A) of further saccharides which however were present in very low concentrations (barely visible in chromatograms in solvent A and B). The elution of the column was then terminated.

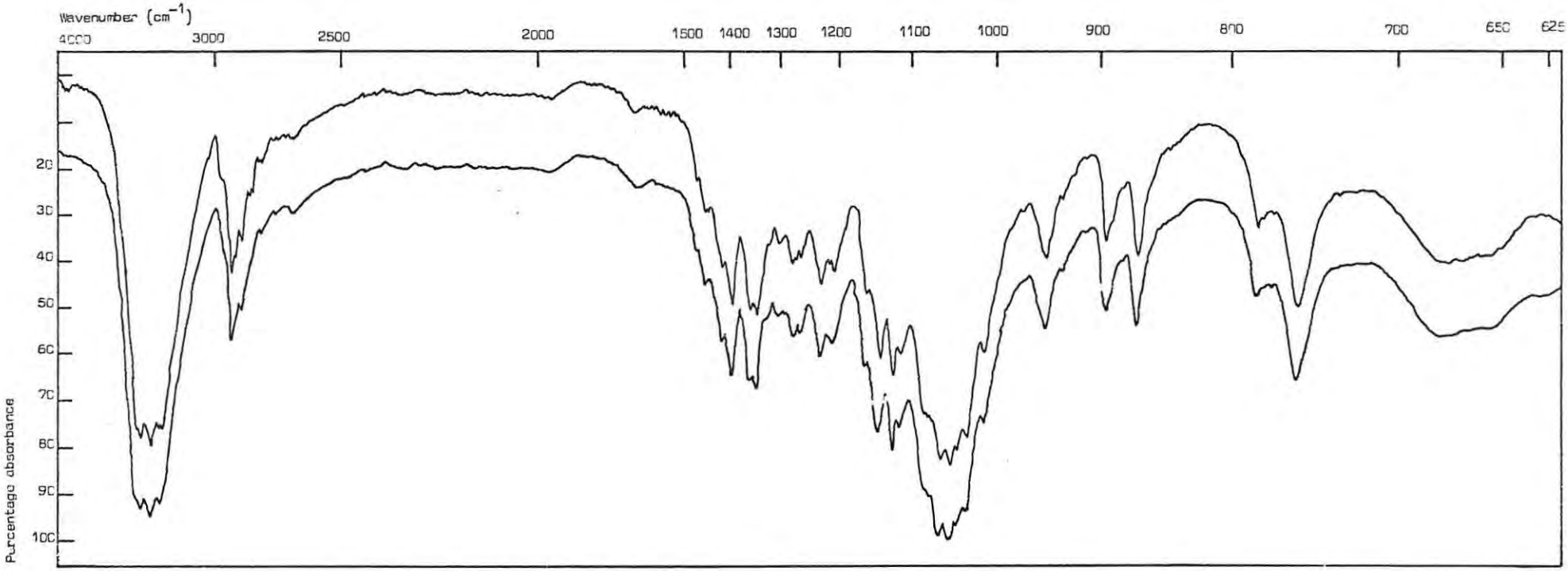


Figure 13 Infrared spectra of authentic (upper) and isolated (lower) 4-O-β-D-galactopyranosyl-D-galactose

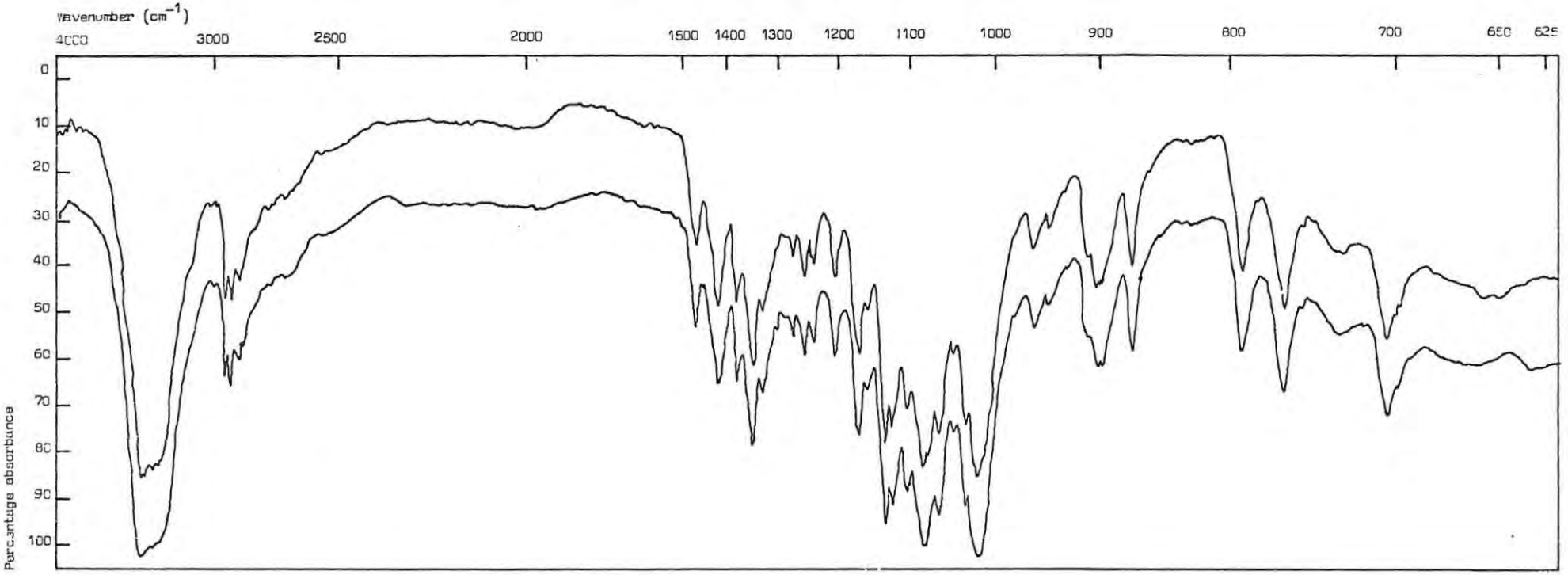


Figure 14 Infrared spectra of authentic (upper) and isolated (lower) 4-O-β-D-galactopyranosyl-L-galactose

3 HORIZONTAL CELLULOSE COLUMN CHROMATOGRAPHY OF SUGARS

3.1 ABSTRACT

Selected applications of the method of cellulose powder column chromatography for the period 1966 to 1971 are reviewed. The method of horizontal cellulose column chromatography of sugars is described with examples of its application. The advantages of the method are discussed.

3.2 APPLICATIONS OF CELLULOSE COLUMN CHROMATOGRAPHY

The technique of separating sugars on columns of cellulose powder was developed by Hough et al[200-203]. A powerful method thus became available for separating the complex mixtures often associated with a wide variety of investigations. Excellent reviews by Balston and Talbot[204] and by Hough[205] and Binkley[206] have already appeared. Following these are reviews by Whistler and Be Miller[207] and Gardell[208]. Since the period (ca 1949 - 1955) covered in these reviews, a variety of publications, well in excess of 250, has appeared, indicating the effective use of cellulose powder column chromatography. This review covers selected publications for the period 1966 - 1971; these being representative of the application of this method in the field of carbohydrate chemistry.

Cellulose column chromatography is frequently used in the field of sugar synthesis. Ishizu et al[209] successfully separated the lactones of the saccharinic acids produced when D-xylose is reacted with aqueous calcium hydroxide at 25° for 300 h. Kochetkov et al[210] prepared novel phosphorous containing sugars by reaction of levoglucosan with hypophosphorous acid, and separated them as sodium salts on cellulose columns eluted with a mixture of iso-propanol and ammonium hydroxide. Nifant'ev et al[211] prepared salts of (6-deoxy-D-glucos-6-yl) phosphorous acid and (6-deoxy-D-galactos-6-yl) phosphorous acid in a similar reaction and were able to resolve them by cellulose column chromatography. In order to elucidate the structure of selected cardiac glycosides, partially acetylated gitoxins were prepared by hydrolysis of penta-O-acetyl-gitoxin and compared with those isolated from natural sources[212].

The cellulose column used in the separation of these synthetic compounds was impregnated with formamide prior to development of the chromatogram, which was effected by a series of solvent systems, viz; n-heptane - methyl ethyl ketone (1:1); n-heptane - xylene - methyl ethyl ketone (3:3:4); xylene - methyl ethyl ketone (2:1) and then finally elution with xylene - methyl ethyl ketone (1:1), affording the four new partially acetylated gitoxins. In the synthesis of D-mannose 6-sulphate Lloyd et al[213], used cellulose column chromatography to isolate and purify the sulphated sugar. Several oligosaccharides have been synthesised and purified by chromatography of the crude reaction products on columns of powdered cellulose. Lindberg et al[214] prepared 4-O-β-D-galactopyranosyl-D-xylose and purified this on a column eluted with ethyl acetate - acetic acid - water (3:1:1), while Swiderski[215] having synthesised methyl-β-cellobioside (from acidic deacetylation of octa-acetyl (α,β)-cellobiose), purified the glycoside on a column eluted with ethyl acetate - propanol - water (5:3:2). Ekborg et al[216] compared the synthetic oligosaccharide 2-O-β-D-glucopyranosyl-β-L-arabinopyranose, purified as the benzyl derivative on a column of cellulose powder eluted with ethyl acetate - pyridine - water (8:2:1), with that obtained on graded hydrolysis of the alkaloid glycoside from a Malaxis species, while Rutherford et al[217] separated primeverulose (6-O-β-D-xylopyranosyl-D-fructose) from primeverose (6-O-β-D-xylopyranosyl-D-glucose) on a cellulose column following partial rearrangement of the primeverose on reaction with aqueous ammonia. A column of micro-crystalline cellulose eluted with benzene - methanol (10:1) was

used to resolve the products of the persulphate oxidation of D-glucose[218].

Many antibiotics, nucleosides and nucleotide components, have sugar moieties and thus may be effectively purified by cellulose column chromatography. Umezawa et al[219] described the synthesis and subsequent purification of O-(2-amino-2-deoxy-α-D-glucofuranosyl) deoxystreptamine from the reaction products obtained by acid reversion of 2-amino-2-deoxy-α-D-glucopyranose and streptamine, while the antibiotic paromamine was purified by cellulose column chromatography, using the solvent system butanol - pyridine - water - acetic acid (6:4:3:1)[220]. Trehalosamine, found to be effective against Mycobacterium tuberculosis 607, was also successfully purified by cellulose column chromatography[221]. Farkas[222], synthesised and purified several methyl 1-deoxy-D-psicofuranosides, C-1 substituted with halo-atoms and mercapto-groups; while Tokuyama et al[223] prepared, and successfully isolated L-sorbosylthymines and related compounds from the reaction products applied to a column of cellulose powder, which was eluted with the solvent system butanol - ethanol - water (4:5:1). Hyflo super-cel was effectively used to separate 5' -O-methylthymidine and its derivatives on columns of the modified cellulose, with an eluting solvent system of ethyl acetate - iso-propanol - water (12:1:6)[224]. Fujii et al[225] separated the products of the reaction between D-glucose and ammonia, obtaining pure 4(5)-D-(-)-glycero-7,3-dihydroxypropyl imidazole, while L-rhamnose was reacted similarly to produce products from which 4(5)-(L-erythro-2,3-dihydroxybutyl) imidazole was successfully resolved[226]. Ikehara et al[227] have described the purification of a novel cyclonucleoside having an 8,3'-O-anhydro-linkage. 8,3'-anhydro-8-oxy-9-β-D-(2-deoxy-D-threo-pentofuranosyl) adenine was separated

from its mixture with 2-deoxy adenosine on a cellulose column eluted with iso-propanol - ammonia - water (7:1:2). The solvent system butanol saturated with water - concentrated ammonia (100:1) was effective in the separation of 2'-deoxy- and 3'-deoxyadenosine, two purine cyclonucleosides, from adenosine[228] while 9-α-D-ribofuranosyl-6-thiopurine was separated from 6-benzylthio-9-α-D-ribofuranosyl purine on a cellulose column eluted with butanol - water (86:14)[229]. Fischer et al[230] indicated the use of the method in the successful resolution of 2-methyl-D-glucopyrano-[1', 2':4,5]-2-imidazoline from 2-acetamido-2-deoxy-D-glucose following the reaction of the latter compound with ethyl iminoacetate hydrochloride.

The application of cellulose column chromatography is not only confined to the synthetic field. Numerous examples abound in the literature indicating the application of the method to the study of carbohydrate natural products. Eppenberger et al[231], obtained pure crystals of stapelogenin from the seeds of Stapelia gigantea after cellulose chromatography of seed extracts, while Davy et al[232] employed multiple chromatographic columns with solvent systems of butanol - iso-propanol - water (1:6.5:2.5) and ethyl acetate - pyridine - water (8:2:1) to isolate lychnose, raffinose, the two isomers O-α-D-galactopyranosyl-(1 → 1)-β-D-fructofuranosyl-α-D-galactopyranoside and O-α-D-galactopyranosyl-(1 → 3)-β-D-fructofuranosyl-α-D-glucopyranoside, as well as O-β-D-fructofuranosyl-(2 → 1)-β-D-fructofuranosyl-α-D-glucopyranoside from the seeds of Silene inflata. The flowers of the Liliaciae have yielded flavonoid mixtures which were amenable to separation on columns of cellulose powder, in this respect Skrzypczakowa[233] has isolated five flavonoids and the 7-diglycoside

of apigenin from extracts of Colchicum autumnale. Flavonoids from the leaves of Cynara scolymus[234], Mentha piperta[235] and derivatives of the alkali-labile glucoside, piptoside, from "bitter vine" (Piptocalyx moorei)[236] leaves have all been purified and in some cases isolated by cellulose column chromatography of the appropriate extracts. Selected flavonoids were separated from the leaves of Folium belladonae[237] using different solvent systems, viz: ethyl acetate - formic acid - water (10:2:3), acetic acid - water (15:85) and ethyl acetate - pyridine - water (2:1:2) on several cellulose columns, while the methanolic extracts of soapwort roots (Saponaria officinalis)[238] were resolved, affording hyposide, gentiobiose and a new non-reducing pentasaccharide saponarose, [β-D-galactopyranosyl-(1 → 3)-α-D-galactopyranosyl-(1 → 3)-α-D-galactopyranosyl-(1 → 3)-α-D-galactopyranosyl-α-D-glucopyranose]. The pentasaccharide was eluted with butanol saturated with water and butanol - acetic acid - water (4:1:5) solvent systems.

In structural investigations on many polysaccharides, recourse at some time or other to the method of cellulose powder chromatography is necessary. Heterogenous treatment of 2-O-hydroxyethylalginate with sodium methoxide in methanol afforded a series of oligosaccharides which McCleary et al[239] were able to separate and characterize following cellulose column chromatography of the reaction products. Glycerol, 2-O-methyl-D-galactose and D-galactose was successfully separated by Nunn et al[180] from the hydrolysis products of the red seaweed polysaccharide elaborated by Aeodes orbitosa. Hydrolysis of the methylated polymer yielded a sugar mixture which was resolved by cellulose column chromatography with either butanol half saturated with water, or

ethyl acetate - acetic acid - formic acid - water (18:3:1:4) solvent systems. The hydrolysate of the native and methylated polysaccharide from Phyllymenia cornea[182, 183] was fractionated on a cellulose column as was the hydrolysate of the polysaccharide from Aeodes ulvoidea[184] and Anatheca dentata[192]. Graded hydrolysis of cashew nut shell polysaccharide afforded disaccharides which were characterized following their successful resolution on a cellulose column[240].

The fact that conventional cellulose column chromatography of carbohydrates has been used as a fundamental separatory method for more than twenty years is sufficient testimony to its reliability, versatility and effectiveness. Recently a new technique of horizontal cellulose column chromatography of sugars has been developed by Nunn et al[241], where rapid reproducible separations of monosaccharide mixtures are easily effected in a very much shorter time than that required for conventional cellulose column chromatography.

3.3 INTRODUCTION

The separation of sugars on a column of cellulose powder is an established technique[203]. In general such separations are time consuming, involve frequent attention, large volumes of eluting solvent and great numbers of fractions, which collected on an automatic fraction collector, have subsequently to be sorted and combined by reference to paper chromatograms.

Furthermore, it is general experience that solvent systems which separate sugars on paper do not as a rule effect similar separations on cellulose columns. Sugars tend to travel much faster on cellulose columns, with a resulting loss in resolution, when using solvent systems designed for paper chromatography. In order to achieve good separations on cellulose columns it is usually necessary to use a solvent mixture [e.g. n-butanol - water (95:1)] in which the sugars have low solubilities consequently involving very large volumes of eluate. The method of horizontal cellulose chromatography[241] overcomes some of these disadvantages.

3.4 DISCUSSION AND RESULTS

The horizontal column of cellulose powder [Plate A, (H)] packed in a nylon tube[242-244] [Plate A, (E,F)] draws solvent by capillary action from the constant level reservoir [Plate A, (C)] through the cellulose wick in the supply tube [Plate A, (D)]. The solvent elutes the sugars, placed at the head of the column (between dotted lines in Plate A), at approximately the same rate as would occur on a sheet of Whatman No. 1 chromatography paper using the same solvent system.

A "chicken feeder" cut-off system maintains the solvent level in the constant level reservoir. The correct level, empirically determined, is critical. If too high, column elution is too fast, due to syphoning effects, and a loss in resolution results. If too low, solvent passage through the column is excessively slow.

During development of chromatograms, using prototypes of the apparatus, appreciable changes in composition of the eluting solvent system occurred. This was due to volatile solvent components evaporating from the constant level reservoirs. The latter were modified, with a corresponding improvement in column performance, until the solvent area, open to the atmosphere, was as small as practically possible (Plates B and C).

It was found necessary to notch the glass base of the column supply tube allowing the escape of bubbles which sometimes collected at the head of the supply wick and impaired solvent flow.

The end of the column drain tube was constricted, facilitating eluate flow from the drain wick. Failure to accomplish this led

to solvent hold up on the column which caused large diffusion effects and marked loss of resolution of the sugar mixture.

Since there is a correlation between the R_f value of sugars on a paper chromatogram and the position of the same sugars on the column, the length of time necessary for resolution of the sugar mixture may be judged by developing conventional descending paper chromatograms of a sample of the sugar mixture, applied to the column, in the same solvent system. If the sugar mixture is a syrup, it is taken up in a small volume of water, to which the cellulose powder is added. The water is then removed in a vacuum at 50° and the resulting powder placed on the column. After termination of the column chromatogram, the sugar bands may be easily located.

An alternative procedure for locating the sugar bands to that used in pilot chromatograms involves the division of the entire column into 40 or 50 equal parts, immediately on termination (Plate D). These are allowed to dry out, a minimum of warm methanol is added to each and the extracts subjected to paper chromatography. This method is easy to perform and slightly more sensitive in the overlap regions of the chromatogram.

Because of the slow even rate of solvent flow induced by the horizontal position (Plate B), chanelling effects were absent and good separations were achieved. This was not the case in experiments performed on columns in a vertical position, where both chanelling and rapid solvent flow produced no separation at all.

Table XI indicates the results obtained when using different

solvent systems. Although good separation was achieved on control papergrams in the n-butanol - acetic acid - water (5:1:2) system this was not the case on the horizontal column. Possible reasons could be overloading of the cellulose and the relatively large volume of water in the solvent system. A good match between sugar resolution on paper and that occurring on the cellulose powder column is related to the state of hydration of the cellulose powder. When hydrated to the same extent as the chromatography paper good resolution occurs.

Cellulose Weight (g)	Development Time (h)	Developer	Sugar Applied	Weight (g)	Column Position	Sugar Detected
164.6	51.0	n-Butanol - Acetic acid - Water (5:1:2)	Galactose Arabinose Ribose Rhamnose	0.0401 0.0410 0.0424 0.0411 0.1646	1-15 16-18 19-22 23-24 25-29 30-34 35-End	nil Galactose Gal + Arab Gal + Arab + Rib Gal+Arab+Rib+Rham Rhamnose nil
150.6	32.5	Ethyl acetate- Acetic acid - Water (15:5:3)	Galactose Arabinose Ribose Rhamnose	0.0298 0.0305 0.0308 0.0303 0.1214	1-5 6-10 11 12-17 18-20 21-24 25-29 30-End	nil Galactose Gal + Arab Arabinose Arab + Rib Rib + Rham Rhamnose nil

Table XI Resolution of a standard sugar mixture on 50 cm horizontal columns using different solvents

The solvent system ethyl acetate - acetic acid - water (15:5:3) gave better results although the ribose was not resolved. The solvent system yielding the best results was ethyl acetate - acetic acid - formic acid - water (18:3:1:4).

Using a three component sugar mixture and unwashed cellulose

powder, good resolution and sugar recovery was achieved (Table XII). The total weight of recovered syrup (0.1537 g) when compared with the weight of sugar applied (0.1225 g) indicates an excess of 31.2 mg. In order to obtain a good quantitative recovery the experiment was repeated using cellulose which had been washed and carefully dried at constant humidity. A final charcoal treatment of selected syrups followed by filtration through sintered discs removed contaminating cellulose fibres, yielding very good recoveries (galactose 88.02%, arabinose 90.44%, and ribose 93.22%). The washing of the cellulose powder also improved the resolution of the column as indicated by the relatively small syrup weights in the overlap region of the column chromatogram.

Using the technique of horizontal cellulose column chromatography, rapid reproducible results were obtained for a number of runs where separations were usually equal to, and sometimes superior to those obtained on a vertical cellulose column eluted with n-butanol - water (95:5).

Development Time(h)	Sugar Applied	Weight (g)	Column Position	Cellulose Weight(g)	Sugar Detected	Syrup Weight (g)	Recovery Excess (g)	Weight Purified syrup(g)
			1-5	24.74	nil	0.0058		
			6-14	37.10	Galactose	0.0386		
	Galactose	0.0404	15-16	10.73	Gal + Arab	0.0111	0.0312	
25	Arabinose	0.0413	17-24	32.90	Arabinose	0.0321	for unwashed Cellulose	not Recorded
	Ribose	0.0408	25-27	16.05	Arab + Rib	0.0189		
		0.1225	28-End	56.60	Ribose	0.0472		
				178.12		0.1537		
			1-6	22.40	nil	0.0068		
			7-15	37.24	Galactose	0.0439		0.0404
	Galactose	0.0459	16-17	8.70	Gal + Arab	0.0070		
24.5	Arabinose	0.0387	18-24	30.30	Arabinose	0.0385	0.0211 for washed Cellulose	0.0350
	Ribose	0.0487	25-27	11.68	Arab + Rib	0.0093		
		0.1333	28-End	49.42	Ribose	0.0489		0.0454
				159.94		0.1544		

Table XII Resolution of a standard sugar mixture on 50 cm horizontal columns using the solvent ethyl acetate - acetic acid - formic acid - water (18:3:1:4)

3.5 EXPERIMENTAL

All solvents were either distilled or fractionated before use (see Appendix, Section 4.2). Concentration of solutions was carried out at 40°/20 mm with a rotary evaporator. Paper chromatograms on Whatman No. 1 paper were developed in ethyl acetate – acetic acid – formic acid – water (18:3:1:4) and were visualized after spraying with p-anisidine hydrochloride reagent[135]. Whatman Chromedia fibrous cellulose powder (CF 11) was used as the column solid phase. Nylon tubing was supplied by Walter Coles Co. Ltd., P. O. Box 42, Plastic Works, 47–49 Tanner Street, London, S.E.1, United Kingdom.

3.5.1 Preparation of a column

The column supply tube [Plate A, (D)] stoppered with a rubber bung at its notched end, was filled with dry cellulose powder, which was compacted with a vibrator after each addition[243]. When the supply tube was nearly full an intimate mixture of cellulose powder (10 g) and the sugars to be resolved was compacted into position.

One end of a length of nylon tubing [60 x 5 cm; Plate A (E,F)] was then stretched over the free end of the column supply tube and the other end of the nylon tube stretched over the stem of a wide-necked funnel. The apparatus was supported in a vertical position by two clamps, one fixed round the supply tube and the other round the funnel stem, care being taken to ensure that the nylon tube was firmly gripped. Dry cellulose powder, poured into the funnel and down the nylon tube in small quantities, was thoroughly compacted[243] onto the sugar mixture by means of the vibrator, after each addition.

When the nylon tube was firmly packed to within 10 cm from

the end, the funnel was removed and the column drain tube [Plate A, (G,I)] previously filled with dry compacted cellulose powder was inserted in its place. The packed column [Plate A, (H)] was then supported in a horizontal position. The constant reservoir [Plate A (C)] after removal of the rubber bung from the supply tube, was connected to the latter and the solvent reservoir [Plate A, (A)] filled with the required solvent, was placed in position. Springs were stretched between hooks above and below each of the respective joints to hold them in place.

3.5.2 Development of a chromatogram and location of the separated sugars

Chromatogram development began on opening the solvent reservoir key [Plate A, (B)]. A series of decending paper chromatograms of the sugar mixture placed on the column, were concurrently developed in the normal manner in the same solvent system as used on the column. Visualization of these at intervals, gave a good indication of the progress in resolution of the sugar mixture on the column. The column was terminated after a suitable time by removal of the supply and drain tubes and location of the sugar bands was achieved by the following method.

The nylon tube was marked off at intervals (1 cm) with a felt tipped pen, along its entire length. A transverse slit was made with a razor blade at each mark and an amount of wet cellulose powder about the size of a match head removed from the column with a pair of tweezers [Plate E]. Each sample was repeatedly pressed (5 times) in turn onto its corresponding marker on the base line at the top of a sheet of Whatman No. 1 chromatography paper. The tweezers were decontaminated by washing with water and

methanol, before removal of each sample. The sampling process was repeated until samples from all marked off column positions had been applied to their corresponding markers on the paper sheet.

The nylon tube was then slit along its entire length and the column broken up into sections [Plate D], roughly equal in size, which were placed in individual beakers (30 ml) and allowed to dry. The "sampling" chromatogram was developed overnight and visualized in the usual manner.

Column sections containing the same sugar, as indicated by the "sampling" papergram, were bulked and the sugar extracted with warm methanol to afford a chromatographically pure syrup. Selected syrups, dissolved in a minimum of methanol, were treated with charcoal to remove cellulose contaminants and filtered through sintered glass discs (G6, 1.5 cm). Removal of the methanol from some of these purified syrups resulted in their crystallization on standing.

3.5.3 Determination of the purity of the cellulose powder

Cellulose powder (35.0 g) was stirred with hot methanol (150 ml), the slurry was filtered through a sintered glass funnel (G4, 5 cm) and the filtrate evaporated to dryness in a pre-weighed flask under reduced pressure (30°, 10 mm Hg). The deposit was 7.8 mg.

3.5.4 Purification of a bulk amount of cellulose powder

Cellulose powder (500 g) after washing with distilled deionized water (4 x 5 l), followed by filtration under suction through a sintered glass funnel (G4, 15 cm) was dried in an oven (silica gel, 100°, 24 h). The dry powder laid out flat on sheets

of filter paper, was allowed to rehydrate at constant temperature and humidity (20°, 50%).

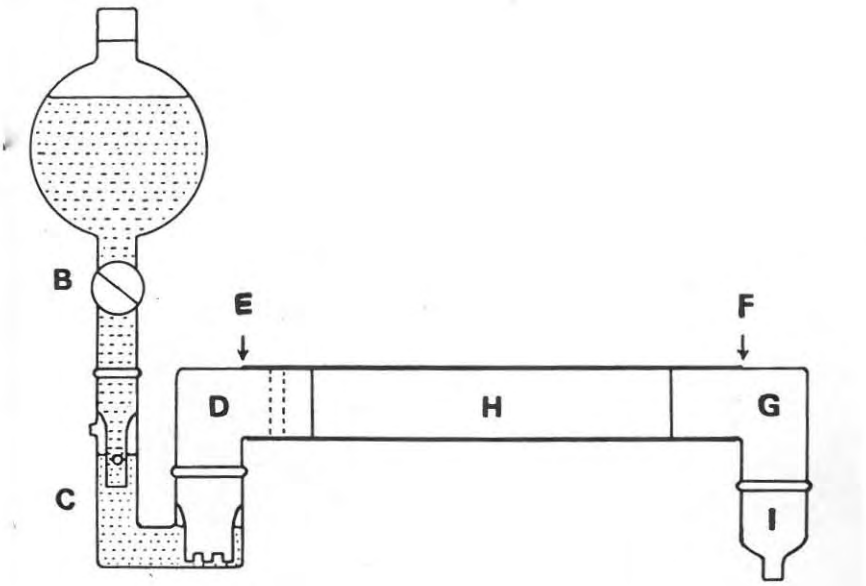


Plate A Schematic diagram of the horizontal column

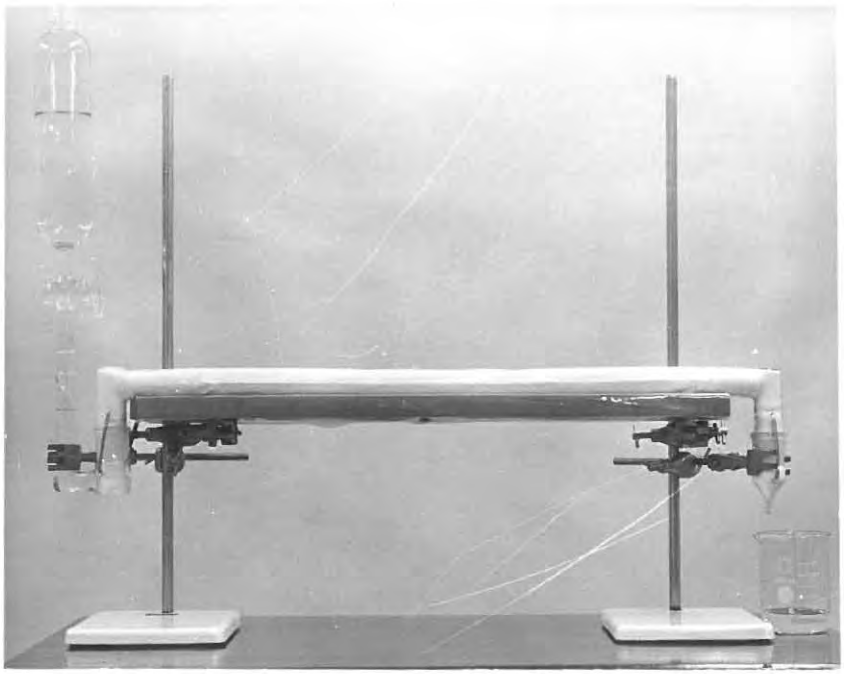


Plate B The horizontal column in operation

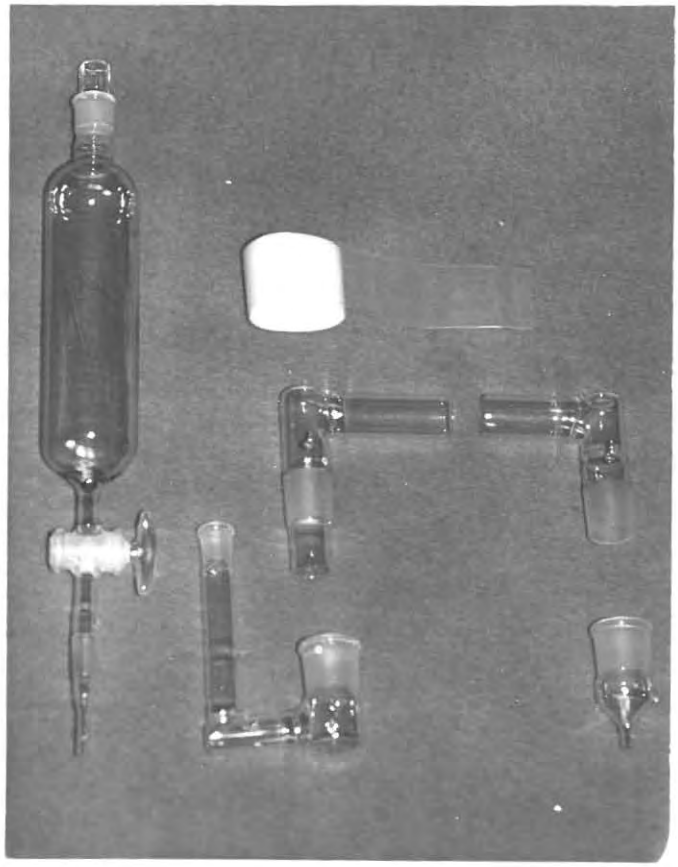


Plate C The components of the column

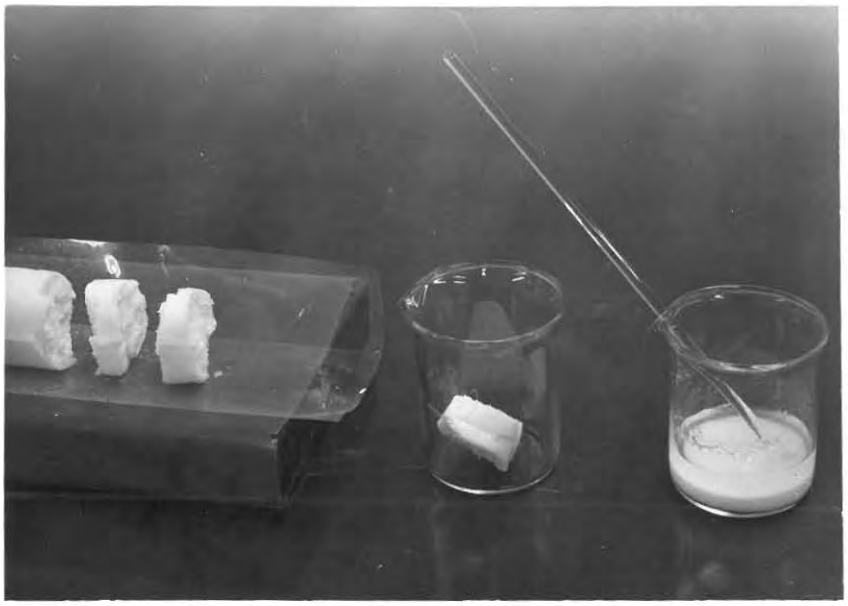


Plate D Division of the cellulose column into sections before locating the sugar bands

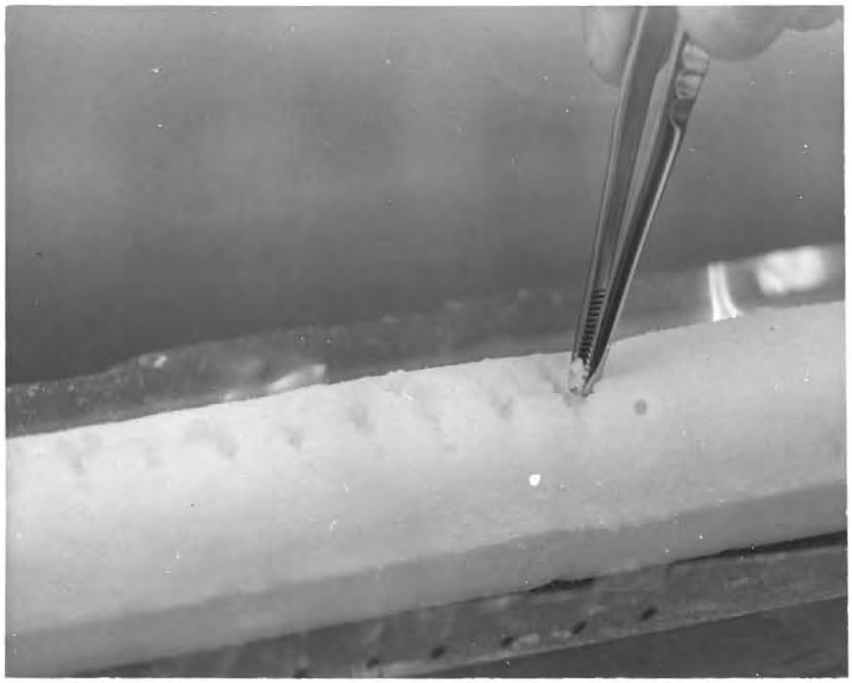


Plate E Sampling the cellulose at intervals to detect the position of the resolved sugars

4 APPENDICES

4.1 THE CONSTRUCTION OF A FOUR PLACE MAGNETIC STIRRER

4.1.1 Introduction

In experiments where several solutions have to be magnetically stirred and maintained at a chosen temperature, individual magnetic stirrer units with independent heating elements are found to be unreliable and inadequate in meeting both of the above requirements. No two units have the same heating characteristics and the use of one unit only, although obviating this difficulty, is impractical because of the time delay between experiments. Experiments in which cellulose samples were identically treated for extended periods with different metal ion solutions at moderately high temperatures, necessitated the construction of a portable inexpensive four place magnetic stirrer unit from readily available materials. The unit (Plate F and G) consists of three parts: the heating bath, the base and motors, and a control box for adjustment of the stirring rate.

4.1.2 The heating bath

The heating bath made from copper sheet, is commercially available. It is circular (28 x 15 cm) and has a heating collar (500 watt, 8 cm) which is supplied with power (250 V) through a sunvic simmerstat. It is filled to the required depth with glycerine into which the reaction bottles, containing the cellulose samples and metal ion solutions, are placed. The bottles are secured by terry clips fixed to aluminium supports. The latter clip over the side of the heating bath and are held in position in line with the stirrer motors, by fastening the respective bolt in each support.

4.1.3 The base and motors

Four Pullin millimotors (code M 94315/1, 9 V D.C.), individually bolted into screw top aluminium cans (May and Baker tablet containers), which are then packed with foam rubber, are mounted below an insulating asbestos disc (33 x 0.6 cm) by means of individual rivetted aluminium tripod supports. The balanced brass magnet holders, in which the respective magnets (Eclipse horseshoe) have been carefully aligned and secured to minimize vibrations, are bolted onto individual motor shafts. Four holes (6.5 cm) are cut through the asbestos disc to allow for vertical adjustment of the motors and magnets so that the latter may be positioned to maximize the coupling with the stirrer bars in the respective solutions. The asbestos base is supported on three aluminium legs (20 x 1.2 cm) which are bolted to the disc at evenly spaced points near its perimeter.

4.1.4 The control box

The circuit (labelled from L. to R. in Figure 15 and 16) consists of a conventional power supply unit which is connected to a custom built transistorized control section. Full-wave rectification of transformed (T1; 250 → 8 V) alternating mains voltage is achieved by the silicon bridge rectifier (BR1). The voltage is smoothed (C5) and applied to the free running astable multi-vibrator (TR6, TR7; R11 - R14; C3, C4). The square-wave voltage output of the latter is then applied (via C2) to the monostable multi-vibrator (TR4, TR5; R5 - R10; C1), the switching of which may be smoothly controlled by variation of R7. A string of D.C. pulses whose mark to space ratio may be varied is obtained at R4. The pulses are shaped in the pulse shape and buffer amplifier (TR2, TR3; R1 - R4) and applied to TR1,

the driver amplifier. A pulsed D.C. is thus delivered to the motors, which are connected in parallel, allowing smooth alteration of the turning speed of the magnets while retaining torque. The light emitting diode (L.E.D.) indicates when power is applied to the circuit and D1 protects TR1 from back e.m.f's. which may be produced by the motors. The circuit is protected by the fuse (F1; 0.25 A).

The component values are as follows:

TR1 : 2N3055; TR2 : 2N1711; TR3 – TR7 : BC107;

R1 : 100 Ω (1 watt); R2 : 2.7 kΩ; R3, R5, R9 : 10 k Ω;

R4, R10 : 4.7 kΩ; R6 : 39 kΩ; R7 : 5 kΩ (linear);

R8 : 560 Ω; R11 : 470 Ω; R12, R13 : 15 kΩ; R14 : 2.2 kΩ;

R15 : 470 Ω; C1, C2, C4 : 0.1 μF; C3 : 0.22 μF;

C5 : 3000 μF (electrolytic): D1 : BY 100; BR1 : International silicon bridge rectifier (5 A rating); F1 : 0.25 A;

T1 : 250 → 8 V Transformer (5 A rating).

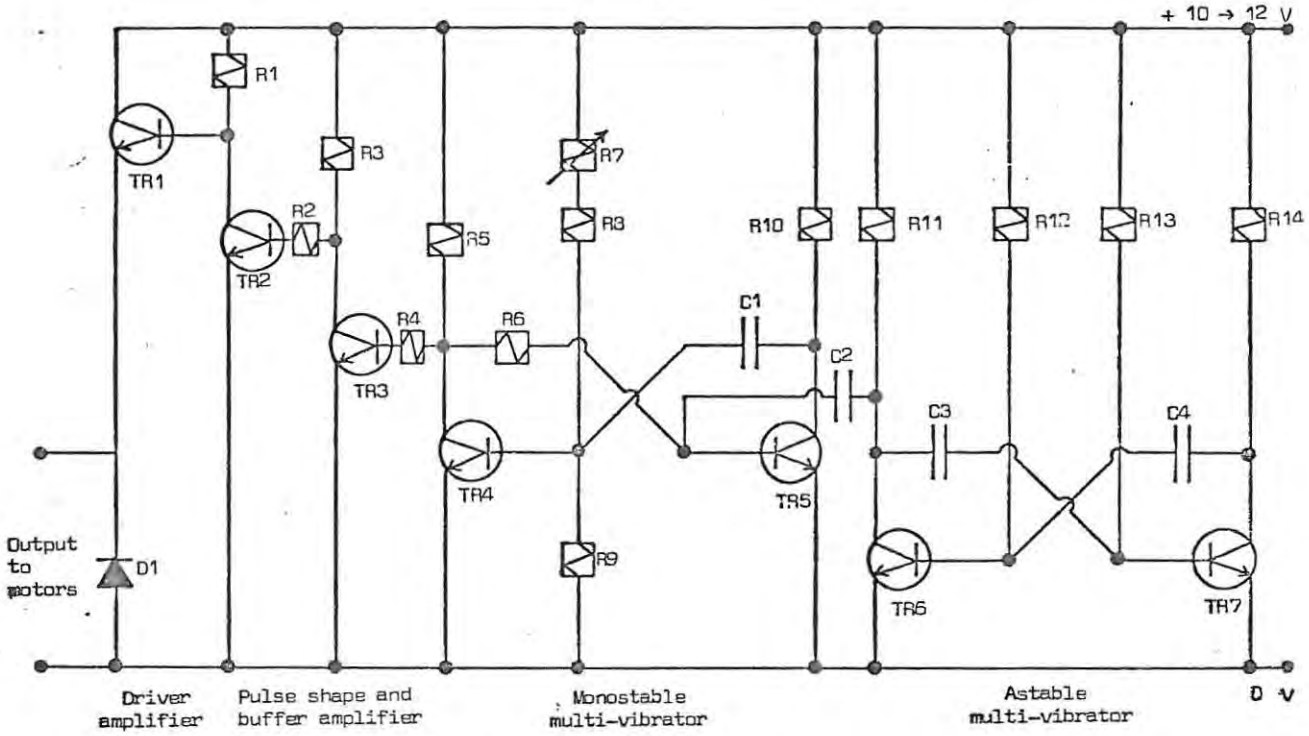


Figure 15 Transistorized control section of control box circuit

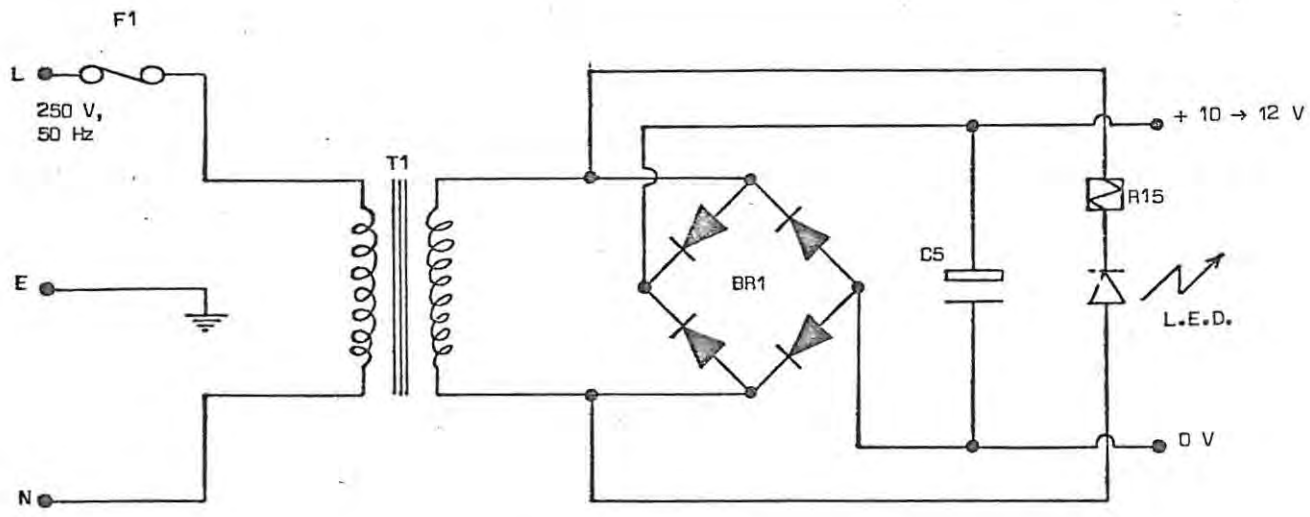


Figure 16 Power supply for transistorized control section of control box circuit

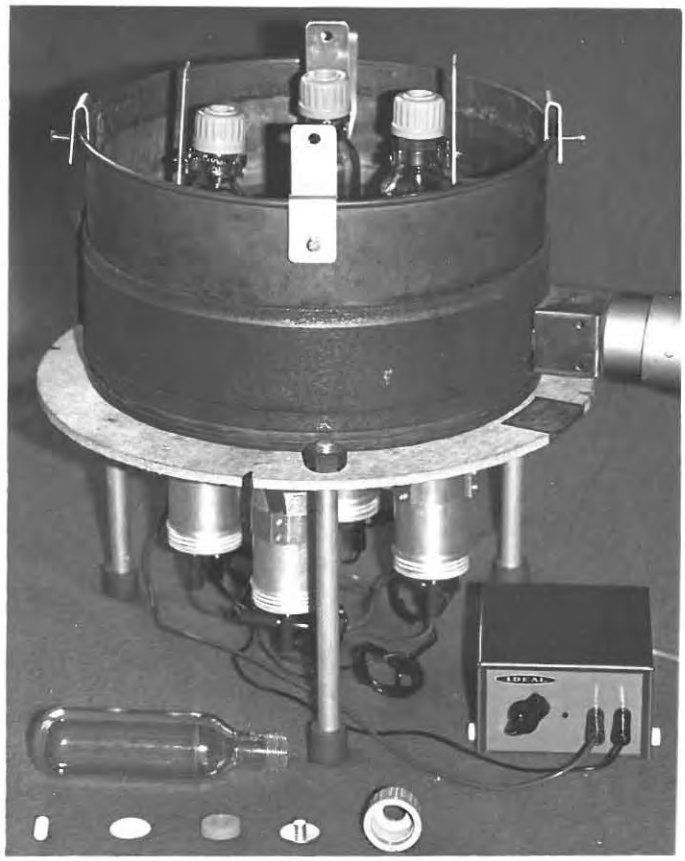


Plate F Heating bath and reaction bottles

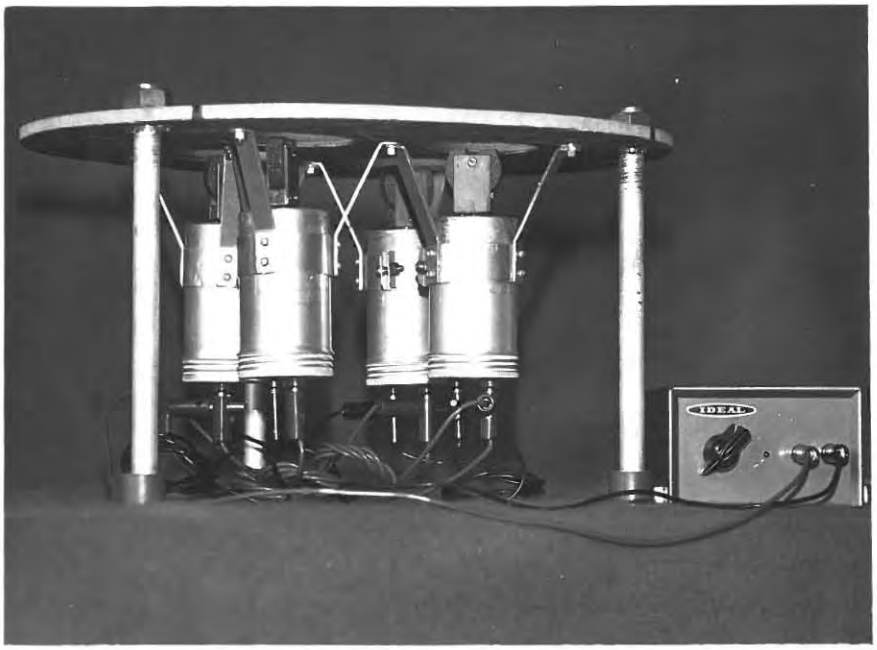


Plate G Magnetic stirrer unit

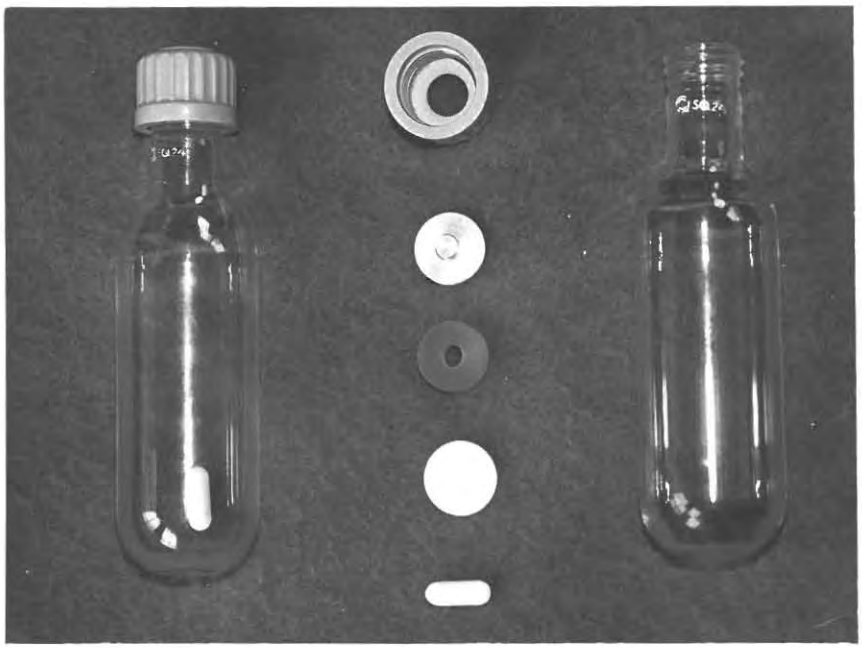


Plate H Typical screw top reaction bottles showing plastic screw top, aluminium insert, neoprene washer, teflon seal and teflon covered stirring bar

4.2 CONSTRUCTION OF A LARGE SCALE FRACTIONAL DISTILLATION APPARATUS

4.2.1 Introduction

Experience in the separation of carbohydrate mixtures by preparative chromatographic techniques involves very large amounts of solvents and it is essential that pure redistilled solvents are used. Small scale distillations, although meeting immediate demands for amounts of pure solvent, are generally a waste of time, since more often than not they have to be repeated several times in the course of a chromatographic separation on a preparative scale. A ready supply of fractionated solvents is more convenient. For this reason a large scale still, capable of straight distillation or fractionation of solvents, with a throughput of 10 l per day, is a valuable piece of fundamental laboratory equipment[245].

Theoretical and practical considerations of fractional distillation equipment have been expertly, and succinctly treated in standard texts[246], and will not be mentioned here. Recently distillation systems have been reviewed[247]. A still (Plate I) was constructed from readily available materials to meet the above demands and is in almost continuous operation, maintaining a stock of fractionated or distilled solvents, for use in our laboratories. A brief description of the apparatus and its construction follows.

4.2.2 The still head

A vertical, co-axial twin spiral, water cooled, total condenser was constructed having a low holdup, a large vapour capacity, no tendency to flood and a low pressure drop. Product rate is controlled by a flow regulating valve which allows a steady stream of condensate to flow from the reflux condenser to the product

receiver (Plate J). Excess condensate flows back to the rectifying section as reflux. This valve is a modification of that used by Willingham and Rossini[248]. A teflon rod (6 mm diameter), tapered at the tip (from 6 mm to 3.5 mm over 12.5 mm), may be raised from, or lowered into, a lapped ground glass valve seat by appropriate rotation of a knurled teflon shank. This is threaded into a teflon gland having a standard B19/26 cone which fits into a corresponding socket at the top of the still head. When the column is on total reflux, condensate delivered to the plug keeps the orifice covered without the valve leaking. The reflux ratio, visually monitored by comparison between the drop rate at the valve and that at an identical ground glass valve seat adjacent to the former, but feeding the column, may be smoothly and continuously adjusted and set from total reflux to total take off. The product rate valve has substantially the same pressure on both sides and hence there is very little hold up and no blow back of condensate. Anhydrous distillation is possible by connection (B10/19 socket and cone) of a suitable moisture trap to the still head. Vapour temperature may be monitored at the top of the still head on the thermometer provided. A standard B40/38 cone connects the still head to the column.

4.2.3 The column

In order to achieve the closest possible contact between rising vapour and descending liquid with an approach towards equilibrium conditions, a packed column (100 x 4.5 cm) was constructed. The good rectification efficiency with high throughput, relatively small hold up and pressure drop generally attributed to such a column makes

the latter favoured for enrichment purposes. Berl saddles (6 mm), supported on a glass grid at the base of the column, provided a compact shaken down dump packing in excess of 90 cm. Before removal of product, the rectification efficiency is optimized with the teflon valve closed, by flooding the column at the start of the distillation. This causes removal of entrapped air and spreads the reflux uniformly over the packing surface. The column has a standard B45/40 cone at its base.

4.2.4 Column compensation

For efficient enrichment and maximum amount of separation of components, the rectification system described above must operate under adiabatic conditions. A compensating jacket was constructed to fulfil this requirement. Seventy parallel notches were obliquely cut with a diamond tipped saw into opposite sides of a glass tube (93 x 6 cm). Resistance ribbon (10.5 Ω/m) was tightly wound through the notches and secured at the top and bottom of the tube by copper bands. The return lead from the top of the spiral heater was brought to the base through a length of 1 mm (I.D.) glass tubing. The spiral heater is protected by an outer glass jacket (93 x 8 cm). Circular end plates (11.5 x 1 cm) with appropriate concentric spacing channels and under tension from three brass rods, locate and secure the heating tube and outer protective jacket respectively. The column, supported at its upper end by the top end plate when the still pot is being charged, may be easily withdrawn from the compensating jacket for cleaning or removal of the dump packing. A thermometer is let down through a small hole in the top end plate to record the temperature of the rectification system.

4.2.5 The still pot

The still pot is a conventional 10 l round bottomed, flange flask (Quickfit FR 10 LF), which is connected to the rectification system by means of a modified flange to socket (B45/40) adaptor (Quickfit DA 7/100), which has a side arm, closed during a distillation with a B24 stopper. A circular spring clip, secured by means of a bolt and butterfly nut, holds the two flanges in intimate, and vapour tight contact. The still pot, after being charged, through the side arm mentioned above, is heated by a 10 l mantle (Electrothermal MY 6409), initially with both the upper and lower 250 V, 500 watt elements in circuit and in the final stages of a distillation, with the lower element only.

A heating mantle is used thus avoiding the gross buoyancy effects and the inherent "mess" associated with a "liquid" heating bath. In order to prevent bumping through superheating of the distilland, small amounts of clean dry porous pumice pieces are added to the still pot prior to charging. The still pot may be lowered away from or raised up to the flange to socket adaptor when necessary by means of a "lab jack" fixed permanently to the underside of the mantle. During operation the mantle and "lab jack" support the weight of the column and still head.

4.2.6 Electrical and water supply

Two 250 V variable transformers, rated respectively at 5 A and 2.5 A, supply current (50 Hz) independently to the mantle and spiral heater in the compensating jacket. Current adjustment to the mantle is smooth; the heat supply is even and vapour can thus be generated in the still pot at a steady, uniform and controllable rate.

Similarly, the current supply to the spiral heater may be easily and smoothly adjusted to prevent heat loss through the column wall, by the vapours entering the rectification section; and to minimize gross heat flow from the spiral heater to the column which may cause vaporization of a portion of the reflux.

The current supply to both transformers is controlled by three respective microswitches in an M6 type relay, sensitive to the pressure of water flowing through the condenser coils. A slight drop in water pressure is indicated by a warning light while a further pressure drop causes the mantle and then the spiral heater to be switched off. A distillation is thus protected against water failure and accidental switch on since current is only supplied after water flow is established in the condenser.

Current to the relay is via a 24 h Sangamo time switch, enabling the time of switch off to be programmed. Manual switch on is necessary after charging the still pot. A pressure reducer connected in front of the relay prevents mains water pressure from damaging the condenser coils.

4.2.7 The product receiver

The product receiver which may be lowered and raised by a "lab jack" is a 5 l erlenmeyer flask connected by a B45/40 joint to a key, with a 10 mm bore, which is in turn connected through a flexible system of S19 and S29 spherical joints to the still head.

When full, automatic cut-off of the product rate valve occurs, and condensate is then returned to the still pot as reflux. After closing the key in the receiver line, a fresh empty flask may be substituted for the full one and on opening the key, the fractionation

may be continued without recourse to adjustment of the product rate valve. At this stage the time of switch off should be set. The apparatus thus requires the minimum of attention.

4.2.8 Setting the variable transformers

Table XIII indicates recommended settings for the variable transformers when fractionating the solvents indicated. No guide to reflux ratios can be given since these depend on the nature and number of contaminants present in the distilland and thus have to be empirically determined.

Distilland	Uncorrected boiling point(°C)	Mantle variac	Column variac	Column temperature (°C)
Acetic acid	113.0	190	140	112.5
Acetone	53.8	140	80	52.0
Benzene	78.1	170	100	76.3
Chloroform	61.0	150	70	59.2
Dichloromethane	38.5	150	50	37.0
Ethyl acetate	76.6	170	80	74.8
Ethyl alcohol	76.2	180	100	75.5
Hexane	67.5	160	90	67.0
Methyl alcohol	61.5	180	80	60.2

Table XIII Recommended settings for column and mantle variacs

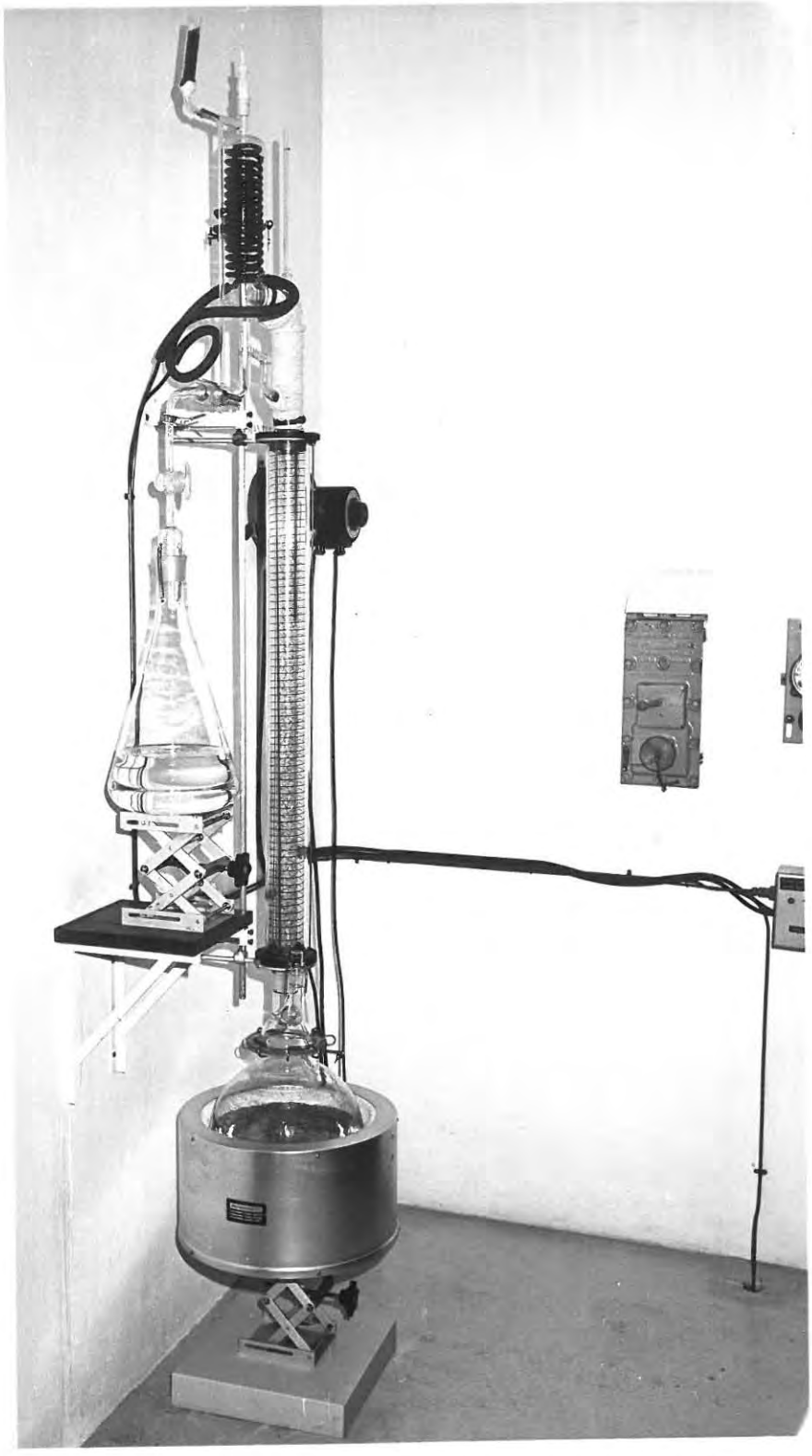


Plate I The large sacle fractional distillation apparatus

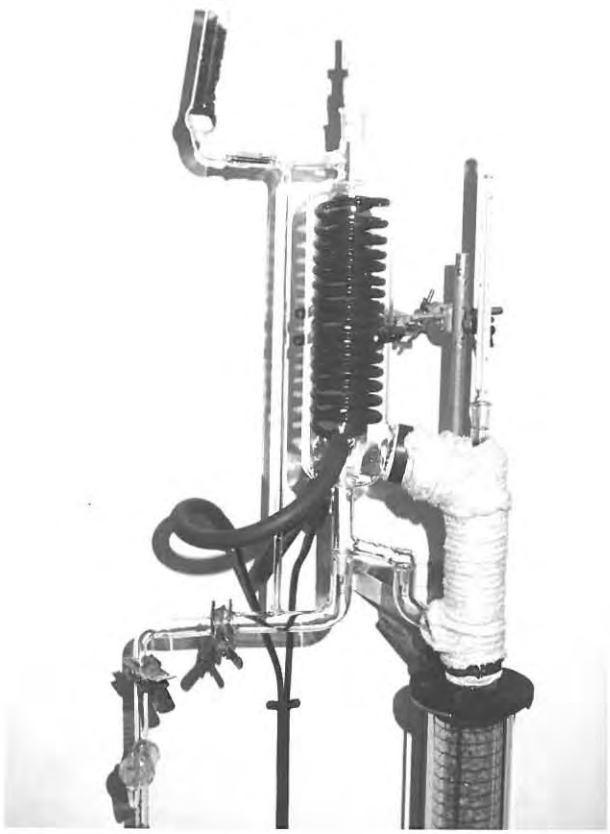


Plate J Fractionating head, showing the product rate valve

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