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PECTIC SUBSTANCES FROM WHITE MUSTARD

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To my Parents and Catriona

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GENERAL METHODS

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1. Paper chromatography

Qualitative work was carried out by descending chromatography using Whatman No. 1 paper. Chromatograms were developed using the following solvent systems (v/v):

A. ethyl acetate : pyridine : water (10 : 4 : 3),

B. butan-1-ol : ethanol : water (4 : 1 : 5, upper phase),

C. butan-1-ol : ethanol : water (3 : 1 : 1, upper phase),

D. butan-2-one : water : ammonia (conc. S.G.·880) (200 : 17 : 1),

E. benzene : ethanol : water (169 : 47 : 15, upper phase).

The symbols used in measuring relative sugar mobilities are,

$$R_F = \frac{\text{distance moved by sugar}}{\text{distance moved by solvent front}}$$

$$R_G = \frac{\text{distance moved by sugar}}{\text{distance moved by 2,3,4,6-tetra-O-methyl-D-glucose}}$$

The chromatograms, having been run for a predetermined time, were air dried and the sugars located by spraying with one of the following reagents.

a) p-anisidine hydrochloride (1), as a solution (3%) in butan-1-ol. The chromatograms were heated at 120° for 5 minutes to develop the colours which are highly characteristic, especially for methylated sugars.

b) <u>alkaline silver nitrate</u> (2). The presence of polyhydroxy compounds in general may be detected by this highly sensitive reagent. Excess background was removed with thiosulphate (3).

c) <u>triphenyltetrazolium hydroxide</u> (4). The chromatogram was sprayed with a mixture (1 : 1) of aqueous triphenyltetrazolium chloride (2%) and sodium hydroxide (<u>N</u>). The colour was developed by holding the chromatogram over a boiling water bath for 5 minutes and after washing with water the chromatogram was air dried. Sugars unsubstituted at C_1 and C_2 give deep red colours.

Preparative separations of sugars were carried out on Whatman 3MM filter sheets, prewashed with methanol. The positions of the sugars were located by cutting off and developing narrow side strips. The appropriate bands of the chromatogram were then cut out and the sugars eluted with aqueous methanol.

2. <u>Gas-liquid chromatography</u> (g.l.c.) was carried out on a Pye 'Argon Chromatograph' with $^{90}Sr.$ detector, or on a Pye 'series 104 chromatograph' with dual hydrogen flame ionisation detectors and temperature programming. Sample size varied but was of the order of 0.1μl (chloroform solution). Retention times (<u>T</u>) were calculated relative to the methyl 2,3,4,6-tetra-<u>O</u>-methyl-β-glucoside peak with the following

notation for peak heights; s = strong, m = medium, w = weak, sh = shoulder. The following stationary liquid phases were used, as coatings on 80-100 mesh 'Gas Chrom P' unless otherwise stated.

1. 15% by weight polyethyleneglycol adipate,

2. 3% by weight neopentylglycol adipate,

3. 3% by weight SE-30 on 80-100 mesh 'Gas Chrom Z',

4. 10% by weight ECNSS (M).

The flow rate of argon with the 'Argon Chromatograph' was 80 ml per minute. Temperatures of operation were measured on a glass thermometer and are quoted in the text.

3. Thin layer chromatography (T.L.C.) (5) Most qualitative T.L.C. was carried out on micro-plates. Preparative work, and where better separations were required, was carried out on larger (20 x 20 cm) plates. The plates were coated with a thin uniform layer of silica gel (Kieselgel G, nach Stahl, Merck). The solvent systems used are quoted in the text. Spots or bands were detected by one of the following reagents,

i) anisaldehyde-sulphuric acid reagent (6) The plate was lightly sprayed with a solution made by dissolving anisaldehyde (1 ml) in ethanol (20 ml) and adding sulphuric

acid (conc. 1 ml, AnalaR). On heating at 120° for 5 minutes sugars give dark colours on a light background.

ii) iodine vapour. The plate was placed in a closed container with iodine crystals for several minutes. Sugars (and other organic material) give brown colours which fade rapidly on standing.

iii) diphenylamine (7) The plate was lightly sprayed with a solution of diphenylamine (1%) in ethanol. After exposure to ultra-violet radiation for 5 minutes p-toluenesulphonyl (tosyl) esters gave fluorescent green spots.

4. Cellulose columns were packed as a thick slurry in acetone. The packed column was then washed with acetone containing an increasing amount of the solvent to be used and finally equilibrated with pure solvent. The solvents used were similar to those in paper chromatography but generally contained half the water content.

5. DEAE - Sephadex columns. Diethylaminoethyl-Sephadex (A25) was allowed to swell in water overnight and all fine material removed by decantation. The material was then washed with hydrochloric acid (0.5$\underline{N}$) and then sodium hydroxide (0.5$\underline{N}$) twice, before generation in the formate form by

stirring with formic acid (15%). The resin was packed in a column and washed free with distilled water.

6. Hydrolysis. Cellular material was shaken in sulphuric acid (72% w/v) for 16 hours. The solution was then diluted to 2N and heated at 100° for 6 hours. After neutralisation with barium carbonate the solution was chilled, filtered and concentrated to a syrup. More readily soluble samples were hydrolysed directly in 2N sulphuric acid as above.

Samples, particularly methylated polysaccharides, were also hydrolysed in formic acid (45%). The solutions were heated in stoppered tubes at 100° for 16 hours. Formic acid was removed by repeated distillation of water from the hydrolysate under reduced pressure. After standing for 2-3 hours (to hydrolyse formate esters) the aqueous solution was concentrated to a syrup.

Hydrolysis was carried out at 1% w/v.

7. Methanolysis was carried out at 1% w/v by dissolving the material in methanolic hydrogen chloride (3%) in a sealed tube at 100° for 6 hours. After neutralisation with silver carbonate, filtration and evaporation to dryness, the residue was dissolved in chloroform for g.l.c. Methanolic hydrogen

chloride (3% w/v) was prepared by the careful addition of acetyl chloride (6 ml) to dry methanol (100 ml).

8. Concentration of solutions was carried out by evaporation on a rotary film evaporator under reduced pressure at or below 40°.

9. Dialysis. Polysaccharide solutions were dialysed in cellophane tubes suspended in running tap water. Chloroform was added to prevent bacterial action.

10. Nitrogen determination was carried out by a semi-micro modification of the Kjeldahl method (8). Protein content of carbohydrate material was obtained by multiplying the value above by 6.25.

11. Determination of carbohydrate content (9). The sugar solution (1 ml, containing 20 - 100 μg sugar) was placed in a test-tube and aqueous phenol (5% w/v, 1 ml) added. Sulphuric acid (conc. 5 ml, AnalaR) was added from a fast delivery pipette. When cool the optical density of the solution was measured with the EEL colorimeter using filter no. 623 (maximum transmission at 495 nm), and sugar content calculated by reference to a calibration curve.

12. Determination of methoxyl content. Analyses were performed by a commercial firm (A.H. Baird, Edinburgh or

Weiler and Strauss, Oxford).

13. Optical rotations were measured at room temperature, using the Perkin-Elmer model 141 polarimeter, in 1 dm tube.

14. Infra-red spectra were recorded using the Unicam S.P. 200 spectrophotometer. Methylated polysaccharides were examined as films made by evaporation in a dessicator of a dry chloroform solution of the sample. Other methylated samples were examined in dry carbon tetrachloride solution.

15. U.V. spectra were recorded using either the Perkin Elmer 137 or Unicam SP 800 spectrophotometer. Periodate oxidations were followed spectrophotometrically (15a) using the Unicam SP 500 instrument.

16. Removal of protein (10). Protein contaminating polysaccharide extracts was partitioned into phenol by making an aqueous solution (2%) of the extract 45% w/v with respect to phenol. The mixture was heated until it became homogeneous (approx. 75°) and then left for 16 hours at 2° in a separating funnel. The phenol layer was discarded and the procedure repeated thrice more. The excess phenol was finally removed from the aqueous layer by dialysis.

17. Methylation was carried out by one or more of the

following methods,

(a) The method of Haworth et. al. Dimethyl sulphate and sodium hydroxide were added in portions to an aqueous solution of the polysaccharide with stirring under an inert atmosphere.

(b) The carbohydrate was dissolved in N-methyl-2-pyrrolidone and methylated by addition of methyl iodide and barium hydroxide octahydrate with shaking (12).

(c) The method of Kuhn and Trischmann (13), involving the addition of dimethyl sulphate and barium hydroxide octahydrate to a solution of the carbohydrate in a mixture of dimethylformamide and dimethylsulphoxide. This method was superseded by (d) below.

(d) The method of Kuhn, Trischmann and Low(14). Methyl iodide and silver oxide were added to a solution of the carbohydrate in dimethylformamide and shaken in the dark at room temperature.

(e) As in (d) but the reaction carried out with stirring at the boiling point of methyl iodide.

Further details of the procedure and method of isolating the product are given in the text, where appropriate.

18. Demethylation (11). The methylated sugar (5 - 10 mg) was dissolved in dry dichloromethane (1 - 2 ml) and cooled to -80°. Boron trichloride (1 - 2 g), cooled to -80°, was added and the temperature kept at -80° for 30 minutes then allowed to warm to room temperature. After 16 hours the

residue was taken up in a drop of water and methanol evaporated from the solution several times under reduced pressure. The product was examined by paper chromatography in solvent A.

19. Formation of derivatives

N-phenylglycosylamine (anilide) derivative. The methylated sugar (75 mg) and aniline (1 mole, redistilled) in ethanol (5 ml) was refluxed in the dark for 1 hour. The hot solution was filtered through charcoal and evaporated to dryness. The crystalline product was recrystallised from the given solvent.

Lactones of the aldonic acids of methylated sugars were prepared as follows, The methylated sugar (75 mg) was dissolved in bromine water (3 ml) and the solution was kept in a sealed flask in the dark for 3 days. Bromine was removed by aeration and the solution neutralised with silver carbonate. Insoluble silver salts were removed by filtration and the filtrate evaporated to dryness. The lactone was extracted with acetone which on evaporation to dryness gave a syrup, from which the product crystallised. Recrystallisation was from the given solvent.

Aldonamides were prepared from the corresponding lactone, dried in a vacuum oven over phosphorus pentoxide. The lactone (50 mg) was dissolved in dry methanolic ammonia (8%, 5 ml) and kept at 0° for 2 days in a sealed flask. On evaporation of the solvent the amide crystallised and was recrystallised from the given solvent.

Toluene-p-sulphonylhydrazone derivative (15). The sugar

(100 mg) was dissolved in methanol (10 ml) and toluene-p-sulphonylhydrazide (1 mole) was added. The solution was heated under reflux for 1 hour. The product crystallised on standing at 0° for 3 days and was recrystallised from methanol and washed with ice cold methanol.

20. Melting points were obtained on a Kofler hot-stage apparatus and are uncorrected.

21. Liquid scintillation counting. All samples were counted in the Beckman Liquid Scintillation System with automatic programming. The strips of cellulose acetate or chromatography paper were immersed in liquid scintillator (10 ml) in glass vials (10 ml) with screw-tops in a constant fashion so as to minimise any orientation effect.

The scintillator was prepared by the addition of 2,5-diphenyloxazole ('PPO', 5.0g) and 1,4-Bis[2-(4-methyl-5--phenyloxazozolyl)]-benzene ('Dimethyl POPOP', 0.3g) to toluene (1 l). All chemicals were Scintillation Grade, supplied by Nuclear Enterprises (G.B.) Ltd. Conditions of counting are given in the text.

GENERAL INTRODUCTION

General Introduction

In both plant, animal and bacterial cells the active protoplasm is bounded by a lipid membrane known as the plasmalemma (16a). This performs a large variety of functions but is basically concerned with transport of materials in and out of the protoplast. In plants and bacteria however the membrane is itself surrounded by another structure known as the cell wall which imparts protection and firmness to the cell.

The osmotic pressure exerted by the protoplast, by uptake of water from a hypotonic environment, on the cell wall can be considerable and thus a fundamental property of the wall must be a high tensile strength to withstand this pressure from within. Polysaccharides account for by far the majority of wall material in young plant tissue and these polymers must be arranged so as to surround the protoplast with a mesh of the necessary properties.

In the polarising light microscope the cell wall shows birefringence whereas the layer between adjacent cells (the middle lamella) appears to be amorphous. In the electron microscope the cell wall has been shown to be composed of microfibrils of indefinite length embedded in an apparently structureless matrix (17). In higher plants the microfibrils are made up of bundles of highly orientated cellulose molecules. The staining properties of the wall and middle lamella with the basic dyes methylene blue and

ruthenium red (18) indicate that the matrix is composed, in part, of the acidic pectic substances, although hemicellulosic polysaccharides and in older cells the non-carbohydrate polymer, lignin, are also present.

Earlier work on the pectic substances has been reviewed by Hirst and Jones (19) and the constitution of pectic substances from many different sources have been recently investigated, e.g. lemon peel (20), apple (21), soybean cotyledons (22), mustard cotyledons (23) and amibilis fir bark (24).

The parent molecule of pectic substances, known as pectic acid, is a linear α-1,4 linked polymer of D-galacturonic acid. The polymer itself seems to be of limited occurrence but examples do exist (24). Usually the molecule is considerably modified in some of the following ways.

(i) by methyl esterification of the uronic acid units, the highly esterified molecule, which is known as pectin, is readily extracted with water from the tissues of many fruits and is important as the gelling agent in jam-making.

(ii) by O-acetylation of a small proportion of the uronic acid units. It is not clear whether this occurs at $C_{(2)}$ or $C_{(3)}$ or both.

(iii) by the co-occurrence of rhamnose units in the main chain. The disaccharide 2-O-α-D-galacturonosyl-L-rhamnose has been isolated from many sources, for example lucerne (25).

(iv) by the addition of single unit side chains of xylose units to the main chain through $C_{(3)}$ of uronic acid residues.

(v) by the addition of linear β-1,4 linked galactan side chains. The mode of attachment to the main chain is not clear.

(vi) by the addition of side chains which are highly branched and contain L-arabinofuranose units linked through the $C_{(3)}$ and $C_{(5)}$ positions. Again whether the attachment is direct to the backbone or through other side chains is not known.

Other homopolysaccharides, known as pectic galactan and pectic araban are also found in the pectic substances of some plants. These are closely related to the side chains mentioned above although authenticated samples are comparatively rare; the galactan of white lupin seeds (26) and the araban of white mustard cotyledons (27,28) are good examples.

The pectic substances are abundant in the walls of actively growing cells (29). In mature cells however, the more typical components are hemicelluloses and lignin. For example cambial cells may contain up to 25% of the dry weight as pectic substances as opposed to about 1% in wood cells (30a). Hemicelluloses are typified by the xylans, in which the β-1,4 linked xylose main chain carries side chains of arabinose and / or 4-O-methylglucuronic acid.

The most important of the non-carbohydrate constituents of the cell wall is lignin. This cross-linked macromolecule is formed by the polymerisation of coniferylic alcohol to give a poly-(substituted phenylpropane). In

wood a typical analysis of wall material would yield 50% cellulose, 20% (soft wood) to 30% (hard wood) hemicellulose and 20 to 30% lignin. Other important polymers are the highly insoluble cutins and suberins.

The cell wall is first formed at telophase of mitosis. In the electron microscope (16b) the initial stage is seen as an accumulation of semi-liquid droplets, each with its own membrane, known as vesicles in the central region of the mitotic spindle. These vesicles appear to originate from cytoplasmic organelles known as the Golgi bodies and the organisation of the vesicles into a lateral arrangement appears to be effected by a system of membranes joining the daughter nuclei, the endoplasmic reticulum (ER). The number of vesicles increases until the droplets coalesce to a semi-solid layer known as the cell plate. The plate at this stage has the staining characteristics of the cell wall proper and Northcote and Pickett-Heaps have shown that a pectic polymer in wheat root-cap cells is secreted in vesicles formed by the Golgi bodies (31). The cell plate now grows laterally to fuse with the longitudinal wall of the mother cell by the addition of more vesicles. The membranes of the vesicles become part of the plasmalemma and thus it appears that this important membrane also originates from the Golgi bodies. Fine protoplasmic connections known as plasmadesmata penetrate the growing wall and appear to carry profiles of ER. Even before the cell plate reaches the longitudinal wall it shows some

birefringence indicating deposition of cellulose fibrils. In the subsequent development the cell plate becomes the middle lamella bounded on either side by the primary cell walls of the daughter cells. During the rapid growth which follows mitosis, cellulose microfibrils and matrix polysaccharides are added to both the cell plate and the original wall. The latter does not become thicker because growth is coupled with considerable extension of the existing longitudinal wall. The wall at this stage, when the cell is increasing in area, is known as the primary cell wall and is thin and pliable with a high water content. The microfibrils are rather disordered and the matrix is supposed to consist of pectic substances (29).

Expansion of the young cell is frequently more pronounced in the direction of tissue growth; this is the so called 'extension growth' and must involve more stretching of the side walls than of the end walls. When viewed in the electron microscope, a side wall of a cell in extension growth shows the microfibrils of the outermost layer of the wall to be oriented in the direction of extension while the inner layers become progressively less orientated in this direction. The innermost layer (where deposition of new wall material is thought to occur), although not very highly orientated, forms a network in which the microfibrils appear to run more or less transversely to the direction of growth. These observations form the basis of the theory of 'multinet growth' (30b) which is the accepted mechanism of

extension growth. Cellulose microfibrils are deposited transversely on the inner surface of the wall, and as each layer becomes buried by new layers of wall material the microfibrils in it become orientated, by cell extension, into the direction of extension growth. Thus although turgor pressure is responsible for cell expansion the form that this will take is determined by the structure of the cell wall.

As the cell reaches maturity the wall grows in thickness by the deposition of closely packed layers of highly orientated microfibrils and hemicellulosic polysaccharides. This secondary cell wall is a rigid structure in comparison with the primary wall and effectively limits further cell expansion. Lignin is laid down only in an advanced stage of growth (30c).

The process by which cellulose chains are assembled into partly crystalline microfibrils and deposited with a particular orientation in the wall is still obscure. The matrix substances (or their precursors) are transported to the wall in the Golgi vesicles and in contrast to the microfibrils these polysaccharides are deposited at all levels in the wall (16c).

Wall extensibility, which must play a major role in growth and possibly have a controlling influence on it, might be controlled by the pectic substances of the matrix. Some of the possible interactions by which this could occur are discussed later.

The object of the work described here was to investigate the structures of pectic substances, especially in relation to the changes that they undergo during cell growth. The system chosen for the investigation was the embryo of germinating white mustard (Sinapis alba) seed, as marked biochemical and morphological changes occur here during germination.

Mustard is a representative of a family (the Cruciferae) of important vegetables (e.g. cabbage, turnip) whose polysaccharides have not been closely investigated. The seed is easily germinated in the laboratory and as it is a fatty seed the extraction and fractionation of cell-wall polysaccharides is not complicated by the presence of reserve polysaccharides. Examination under the microscope (32) has shown that as the seeds imbibe water and begin to germinate the cell walls in the cotyledons both expand and swell. Cell counts of the cotyledons during germination indicate that no cell division is taking place at this stage (33). Another investigation (34) has shown that whereas the amount of polysaccharide in the cotlyedons varies little during germination, experiments in which ^{14}C-sucrose was fed to the seeds at the start of germination indicate that synthesis of pectic polysaccharides occurs during this period. This is interpreted as turnover of pectic substances in existing cell walls.

Much of the work on seed polysaccharides has been focused on the mucilages obtained by extraction of the whole

seed e.g. mustard (35,36), linseed (37) and plantain (38). The cold water extract of white mustard seed was shown to contain a mixture of complex acidic polysaccharides composed of arabinose, galactose and galacturonic acid and a considerable proportion of cellulose (35,36). Examples of mucilages of simpler structure are the galactomannans from seeds of the Leguminosae family (39) and guaran, the galactomannan from guar seeds (39,40). More detailed investigation has, with few exceptions e.g. white mustard (27,28,34) and lupin (26), been limited to the cereals, e.g. the glucans of oats and barley grains (41).

The main part of the work described in this thesis concerns the cell-wall polysaccharides of white mustard seed cotyledons and the changes which they undergo on germination. The last chapter is concerned with the development of a method of polysaccharide structure determination applicable to polysaccharides in which unsubstituted primary hydroxyls occur only at end groups, e.g. dextran and pectic araban.

CHAPTER 1

Structural Studies of the Pectic Substances from the Cotyledons of White Mustard Seed

Introduction

As described in the General Introduction, extension growth of the young cell wall involves progressive stretching and re-orientation of the cellulose microfibrils. The physical properties of the embedding matrix must play an important role in governing wall extensibility and the biological control of this may function through the matrix polysaccharides. Growth by the 'multinet' mechanism requires the microfibrils to change their orientation and hence move relative to each other. If the matric polysaccharides were present as a weak gel this would not hinder the movement of microfibrils and might even assist this by a lubricating effect. However if a rigid gel were present severe restrictions would be imposed on the movement of microfibrils. It is a safe assumption that the matrix at this stage of growth is largely composed (in most plants) of pectic substances and hence the molecular interactions of pectic gels *in vitro* might lead to an understanding of the control of extensibility *in vivo*.

Polysaccharide gels *in vitro*, e.g. agar, can be close to a liquid in composition but still have some of the properties of a solid. The gel can be melted on heating and reset on cooling suggesting that the interactions between molecules in the gel are temporary. If this is the case *in vivo* then the plant cell wall presents a quite different situation to that in the bacterial cell wall. In the latter

the cell is in effect surrounded by a covalently bonded macromolecule (42), the bonds presumably being broken and reformed as growth occurs. Temporary interactions as opposed to covalent linkages might be more appropriate to the plant as growth could occur as a physical response to turgor pressure.

Gels *in vitro* are composed of a network of macromolecules that are cross linked at various points with the liquid occupying the spaces between polymer chains. Cross linkage in this context applies to any form of molecular association or cohesion. The number of such linkages need not be large provided they are strong enough to withstand repulsive forces (e.g. coulombic repulsion) which must be present if the gel is to exist in a swollen state. The most likely theories of interaction between molecules in polysaccharide gels are described below.

(a) Entanglements

Entanglement coupling is a rather loosely defined term: it is not applied to the short range molecular entwining which must exist in all polymeric systems but rather to long range interlooping of polymers of sufficiently high molecular weight (43). The presence of bulky side groups does not markedly inhibit entanglements (44). It is widely accepted that the protein-polysaccharides form networks in concentrated solution by entanglements. This is also thought to occur in the natural state, for example in nasal cartilage the main chains are polypeptides and the

side chains the polysaccharide chondroitin 4-sulphate (45). The rigidity is due to electrostatic repulsion between negatively charged side chains.

(b) Microcrystallites (43)

The association of polysaccharide chains can also occur by different chains coming together in regions of local crystallinity, the regions usually being short in relation to chain length. However in regenerated cellulose the chains bind together so successfully that fibres form (46). By substitution of the accessible (non-crystalline) regions with carboxymethyl groups in carboxymethyl cellulose a system with the visco-elastic properties attributable to a combination of entanglement and crystallite cross linking is formed.

(c) Hydrophobic Interations

Hydrophobic bonding is thought to account for some of the tertiary structures of proteins (47); the three dimensional structure of lysozyme (from X-ray data) shows that the molecule in solution (by extrapolation of the crystal structure) is folded such that contact between non-polar groups and water is minimised. The formation of hydrophobic bonds is an entropically favoured effect as the water molecules around exposed non-polar groups on the polymer chain become more ordered (48). Hence on formation of the hydrophobic bond the degree of order of the system as

a whole decreases, resulting in a favourable free energy of formation. By addition of compounds which reduce the ability of water molecules to hydrogen bond with each other e.g. urea and guanidine hydrochloride, the favourability of hydrophobic bonding is lost, with consequent loss of tertiary structure of the polymer. As hydrophobic bonding is an entropy controlled process the strength of the bond increases with temperature. For example methylcellulose forms gels which set on heating and melt on cooling. The cellulose derivative is prepared heterogeneously by autoclaving cellulosepretreated with sodium hydroxide (alkali cellulose) with methyl chloride. The degree of substitution is about 1.6 to 2.0 methoxyl groups per glucose residue. The distribution of methoxyl groups within the molecule is almost certainly governed by accessibility so that blocks of fully methylated glucose residues occur. The gelation is seen as the formation of stable cross linkages of the fully methylated regions of different chains by hydrophobic bonding, and as such will be favoured by increasing temperature.

(d) Polyelectrolyte effects

Cross linkages formed in this way usually do not lead to gel formation but by an aggregation effect assist in the gelation primarily caused by some other form of interaction. The uronic acid containing plant gum Khaya grandifolia in salt solution shows a considerable

molecular weight increase (as measured by ultracentrifugation) over that in salt free solution (49). Previous theories involved electrostatic attraction between ionised carboxyl (or other) groups and a polyvalent cation ('ionic bridging'). If the carboxyl groups were in different chains then a cross linkage was established. Some chelation of mono- or divalent cations to the sugar hydroxyl groups is also possible in the polymer system, although the complexes formed by low molecular weight analogues are weak (50,51).

The simple ionic bridging of negative charges by divalent cations such as Ca^{++} is not compatible with the nature of electrolyte solutions (52). The behaviour of such systems is most accurately described in terms of the polyelectrolyte character of the polymer in solution (53). The covalent structure of the molecule holds together anionic groups that would otherwise separate by repulsion. The sum total of these repulsions is such that from the free energy point of view of the system as a whole it becomes favourable to localise the cations present in the domain of the polymer, a process in itself entropically unfavourable. It has been shown that a large proportion of the counterions may be bound in this way amd that di- or trivalent cations are more tightly bound than univalent cations (54). It follows that chains which pass through each others domains will share a common atmosphere of counterions giving junction zones in which any movement of the chains away from each other will be resisted. The

effect of counterion binding will be enhanced by multivalent cations. It seems likely that cations bound in the domain of the polymer may also take part in chelation with sugar hydroxyl groups giving some further cross linking of chains. This is seen as the cation exchanging some of its water of hydration for sugar hydroxyl groups, although the extent to which this occurs is not established.

It is stressed that the bound counterions are in dynamic equilibrium with those in the bulk solution, thus no individual cation holds the chain together permanently and hence this theory is fundamentally different from the ionic bridging and chelation theories put forward previously.

Pectin gels *in vitro*

Pectins (the highly methyl-esterified molecule) are readily extracted from the tissues of fruits with boiling water and form gels on cooling. A substance of low molecular weight, usually sucrose, must be added in large quantities before the gel will set. The effect of the added solute is to make the solvent thermodynamically poorer by replacing some of the water molecules around the polymer chains with molecules which react less strongly with the polymer. The result is that the chains come out of solution to form points of cross-linkage. The loss of gel strength which occurs if the methyl ester is replaced by the ethyl or 2-hydroxyethyl ester establishes that the backbone of methyl galacturonate units is responsible for

the gelling properties rather than the neutral side chains. An O-acetyl group on (on average) every eighth methyl galacturonate unit prevents gelation and thus the mechanism would appear to be due to an intimate association of chain segments rather than to association between single sugar units. In this respect the structural requirements are similar to those involved in crystallites suggesting that a local limited crystallisation is responsible for pectin gels. Structural irregularities in the main chain, e.g. O-acetylation, insertion of rhamnose units, would thus have a weakening effect on the gel but the presence of bulky neutral side chains would have an even more drastic effect.

Pectic acid (the fully de-esterified molecule) can be converted to a gel by addition of a low concentration of Ca^{++} ions to the solution. However this property is lost on partial acetylation so that the polyelectrolyte effects discussed above cannot wholly explain the gelation. Crystallites might again be involved but as it is unnecessary to add sugar to set the gel their formation might be more favourable.

The optimum gelling conditions of low methoxyl gels (i.e. where only a portion of the uronic acid units are methyl esterified) has been shown to be a function of the methoxyl content, the concentration of Ca^{++} ions and the concentration of sucrose (55).

Pectin gels in vivo

In the natural state only about 20% of the carboxyl groups in the pectin molecule are not methyl esterified. It would seem likely that attraction through Ca^{++} binding is a contributory, rather than the major, factor in gelation. In the microscope Ca^{++} ions have been shown to occur in regions which are rich in pectic substances and complete extraction of pectic substances from plant tissue with hot water is extremely difficult unless reagents are added which remove Ca^{++} ions, confirming that Ca^{++} binding is an important interaction.

The most likely explanation of gelation in the cell wall is that the unbranched sections of the methyl galacturonate main chains are involved in local crystallisation with each other. The stability of the gel so formed is probably greatly enhanced by the polyelectrolyte effects involving Ca^{++} binding by ionised non-esterified carboxyl groups and possibly some chelation of Ca^{++} with sugar hydroxyl groups.

The gel structures in mature cell walls would be expected to be much stronger than those in cell walls having a high potential growth rate (the reasons for this are given at the beginning of the Introduction). Consequently it should be possible to rationalise the chemical structure of the pectic substances in terms of their function in the wall. The pectic substances of pollen (56) and seeds tend to be rather highly branched, consistent with an easily

deformed, fairly weak gel which would enable rapid growth to occur. In contrast the strands which are found in mature celery petioles and seem to have a purely support function have been shown by X-ray diffraction and electron microscopy to be mainly composed of short crystalline bundles of pectic substances (57). The presence of crystallites suggest that relatively unbranched sections of galacturonan chains account for the rigidity of the strands.

It is assumed that the effect of side chains and other structural irregularities on the gel strength would be similar to that observed for *in vitro* gels.

Structural studies of pectic substances

Structural analysis, while giving interesting insight into the constitution of the pectic substances, is more important in that it may enable the biological role of these polysaccharides in the cell wall to be more clearly understood.

The first stage of a structural investigation is the extraction of the material in a relatively pure state. The method of extraction adopted in this laboratory for the extraction of pectic substances involves the use of hot aqueous ethylenediamine tetra-acetic acid (EDTA) (58) solution (2% w/v). The pH must be chosen to maximise yield but minimise degradation (see following Chapter); EDTA complexes calcium ions most efficiently at a slightly alkaline pH and thus a pH of 7.5 was chosen for complete

extraction of the pectic substances. However degradation reactions are more serious under alkaline conditions and in another experiment extraction was carried out at pH 4.9. Although extraction was shown not to be as efficient at this pH, it is most unlikely that any degradation had occurred. The crude pectic mixture was obtained by precipitation from the EDTA extracts with aqueous ethanol (75%), redissolving in water, dialysis, concentration and freeze-drying. The material thus obtained is usually contaminated with appreciable amounts of protein which must be removed before proceeding with a structural analysis of the pectic substances.

The methods available for polysaccharide structure determination are very powerful. The first consideration is whether the extracted material is homogeneous. The heterogeneity, if present, can be of two types, (a) the extract may contain one or more well known types of polysaccharide for which efficient and specific fractionation methods are established, and, (b) within a polysaccharide type molecules have a common structural pattern but vary in size and possibly in some details of structure e.g. degree and/or distribution of branching. If the differences are fairly small no discontinuity is observed, i.e. one spectrum of molecules ~~are~~ is present, this is known as polymolecularity. However if the differences are such that discontinuity is observed, e.g. ultracentrifugation may show some separation into two fractions, then two (or more) spectra of molecules

are present, this is known as polydispersity. Electrophoresis and ultracentrifugation give clear evidence for heterogeneity as do some chemical methods e.g. fractionation on ion-exchange columns. Before further analysis it is desirable to fractionate the extract so that each fraction is as nearly as possibly homogeneous in the respect that it contains only one type of polysaccharide. Methylation analysis and Smith Degradation are examples of elegant, established methods of determining the fine structure of polysaccharides. Methylation analysis involves complete methylation of the polysaccharide followed by hydrolysis and separation and identification of the hydrolysis products. This is most satisfactorily and unambiguously achieved by the formation of crystalline derivatives but as relatively large amounts of material are required in a pure state for this, identification is frequently limited to g.l.c. and paper chromatography evidence (particularly for minor components). Although giving the nature and relative proportions of the linkages present in the polymer methylation analysis gives no information as to sequence or configuration. These are usually achieved by controlled partial acid hydrolysis followed by separation and identification of the di- and oligosaccharide products.

The pectic substances from both germinated and non-germinated mustard cotyledons have been the subject of a considerable amount of work in this laboratory. The

extractions of the pectic substances, the purification and methylation of which is described in this chapter, were carried out by Dr. J. Samuel. The 'non-germinated pectic mixture' was extracted from commercial 'White Mustard Seed Germ', (J. and J. Colman, Ltd.), and the 'germinated pectic mixture' from the cotyledons separated from commercial mustard seeds which had been germinated for four days in the dark. Obviously the former is available in much larger quantities. The sugar composition of the non-germinated pectic mixture was determined by Dr. S. Gould (59); the main components were galacturonic acid, arabinose and xylose in the molar ratio of 2 : 3 : 1 respectively, with significant amounts of galactose and rhamnose and a lesser amount of glucose. Dr. Gould also fractionated the mixture, on a column of DEAE cellulose by gradient elution with sodium phosphate buffer, into a neutral and two partially separated acidic components. The neutral fraction was composed of mainly galactose and arabinose with small amounts of glucose and xylose. The acidic components both contained similar proportions of galacturonic acid, arabinose, xylose, galactose and rhamnose, and are thus closely related. Graded hydrolysis of the mixture in 0.01 $\underline{\underline{N}}$ sulphuric acid at 100° removed nearly all the arabinose units from the polymer but liberated only traces of xylose and galactose. The arabinose units are probably present in the furanose form and occur as blocks attached indirectly to the main poly(galacturonic acid) chain. A

preliminary analysis of the hydrolysis products of the methylated pectic mixture from non-germinated cotyledons, using g.l.c. and paper chromatography to identify the products, indicated the main structural features present. The average molecule has an extremely high degree of branching and has been described in the General Introduction, but the galactose units were suggested as being present as bridge units by which other side-chains are attached to the main chain. This contrasts with the β1-4 linked galactan side-chains of other pectic substances. In an examination in the ultracentrifuge Dr. I. Steele (60) confirmed that the pectic mixture was heterogeneous, two rather broad peaks were observed. No significant differences were apparent between the germinated and non-germinated pectic mixtures in this study.

Recent investigations of pectic substances from a variety of sources have shown the presence of a number of minor structural variations other than those already mentioned. These features, listed below, probably occur as side-chains.

Feature	Source	Reference
β-D-Galp-(1→2)-D-Xylp	Soybean cotyledons	(22)
	Soybean hulls	(61)
α-L-Fucp-(1→2)-D-Xylp	Soybean cotyledons	(22)
	Soybean hulls	(61)
β-D-GpA-(1→4)-D-Galp	Soybean cotyledons	(22)
	Soybean hulls	(61)

Feature	Source	Reference
β-D-GpA-(1→6)-D-Galp	Lemon peel	(20)
	Soybean cotyledons	(22)
	Soybean hulls	(61)
β-D-GpA-(1→4)-L-Fucp	Lemon peel	(20)
	Lucerne	(25)
	Soybean cotyledons	(22)
	Soybean hulls	(61)

The object of the work described in this chapter was to correlate any changes in molecular structure (as shown by methylation analysis) of the pectic substances extracted from germinated and non-germinated mustard cotyledons with their different function in the cell wall. The most dramatic effect one might expect would be a decrease in the degree of branching during germination, enabling more rigid gels to be formed. This would also result if a redistribution of branch points occurred during germination such that blocks of unbranched galacturonic acid units became available for the formation of junction zones. Of the two mechanisms only the former would be detected by methylation analysis.

The structure of the pectic substances from mustard cotyledons requires to be put on a firmer basis and to this end the major hydrolysis products of methylated non-germinated pectic mixture (pH 7.5) were characterised unambiguously by the formation of crystalline derivatives. Also as the investigation was carried out on a relatively large scale the presence of minor features, such as those

listed above, should be detectable (if present). The comparison of the germinated and non-germinated pectic mixtures was by g.l.c. and paper chromatography of the hydrolysis products of the methylated polysaccharides. The pectic mixture extracted at pH 4.9 from non-germinated cotyledons was also compared with the above by the same methods.

Another type of polysaccharide was isolated in a fairly pure state during purification by deproteinisation of the non-germinated pectic mixture. This belongs to a family of polysaccharides known as the 'amyloids', a more detailed description of which are given in the discussion at the end of this chapter.

Experimental

Experiment 1.1. Trial purification of the pectic mixture from non-germinated cotyledons.

The crude pectic mixture was obtained (by Dr. J. Samuel) by extraction of defatted non-germinated cotyledons with aqueous EDTA (2% w/v) at 90° and subsequently with water at 90°. The EDTA and water extracts were combined and the pectic mixture precipitated with ethanol. The precipitate was redissolved, dialysed and freeze-dried.

The crude pectic mixture (140g) was deproteinised by four cycles of the treatment described in General Methods, 16. A white flocculent interfacial precipitate formed as removal of protein proceeded; both this and the clear aqueous layer gave positive reactions to the phenol-sulphuric acid reagents. Trouble was encountered from interfacial emulsions. The emulsion was retained with the carbohydrate layer and was found to diminish in amount as removal of protein reached an advanced state. After the final treatment the aqueous suspension was allowed to stand for 2 hr. and the clear supernatant removed by decantation from the precipitate. The supernatant was dialysed, concentrated and freeze-dried, (1.80g; Found, % N = 1.13). The precipitate was suspended in water, dialysed, concentrated and freeze-dried, (50.5g; Found, % N = 1.00).

The procedure was repeated on a small scale (10g)

in the presence of EDTA (2% w/v). No precipitation occurred in this experiment and removal of protein was very much easier because emulsions were absent.

Experiment 1.2. Comparison of the 'Soluble' and 'Precipitated' fractions of Experiment 1.

a) The fractions (20mg) were hydrolysed in formic acid and examined by paper chromatography in solvent A. The results are summarised below (p-anisidine hydrochloride spray).

Fraction	Sugars detected in hydrolysate				
	Strong	Medium	Weak	Very Weak	Trace
'Soluble'	Glucose	Xylose, Galactose	-	Fucose, Arabinose	Galacturonic acid
'Precipitated'	Arabinose	Xylose, Galacturonic acid, Galactose	-	Rhamnose	Glucose

b) Comparison of the fractions in the ultracentrifuge (110,000 x g at 1% concentration in 0.15M sodium chloride) was kindly carried out by Dr. I. Steele. This confirmed that the fractions were completely different. The 'Soluble' fraction gave a single sharp peak that was similar in shape and rate of sedimentation to an amyloid which had been isolated from the residue remaining after EDTA extraction of non-germinated cotyledons (59). In contrast the 'Precipitated' fraction gave two broad peaks similar in

pattern and rate of sedimentation to pectic mixtures studied previously (by Dr. Steele).

c) The 'Soluble' fraction was found to give a strong blue-green colour (λ_{max} = 650nm) with potassium triiodide in saturated sodium sulphate (62) very similar to that given by a sample of Tamarindus amyloid kindly provided by Dr. P. Kooiman. The 'Precipitated' fraction gave only a weak colour. Quantitative measurements on the colour reaction are described below.

Experiment 1.3. Estimation of the amyloid content of some fractions and extracts of non-germinated and germinated cotyledons.

The analytical method of Kooiman (62) was followed. To the aqueous polysaccharide solution (1.00 ml, containing between 0.05 and 0.30 mg of amyloid) was added an aqueous solution (0.50 ml) containing iodine (0.5 % w/v) and potassium iodide (1% w/v, AnalaR). Sodium sulphate solution (20% w/v, AnalaR, 5.0 ml) was added and the mixture shaken. After standing for 1 hr. at room temperature to allow the colour to develop, the extinction at 650nm was read in 1 cm cells against a reagent blank, (Perkin Elmer model 137 spectrophotometer).

A calibration curve of extinction against amyloid concentration (mg per ml) was made using various concentrations of Tamarindus amyloid up to 0.30 mg per ml. The graph was linear, and 1.0mg of amyloid gave an extinction of 3.78.

Kooiman's value of 1.74 for the specific extinction was obtained using 0.5cm cells.

Various extracts and fractions from both non-germinated and germinated cotyledons were analysed for amyloid content by the above method. Fractions (e) to (i) inclusive were obtained by Dr. S. Gould (59).

(a) Crude pectic mixture (see Experiment 1), (2.5 mg per ml), from non-germinated cotyledons before deproteinisation gave no colouration and had a specific extinction of zero. The reason for the apparent absence of amyloid is discussed later.

(b) The 'Soluble' fraction (see Experiment 1), (0.50 mg per ml), gave an intense colour. The specific extinction of 1.25 indicated that the fraction contained 71% amyloid.

(c) The 'Precipitated' fraction (see Experiment 1), (5.0 mg per ml), gave a weak colour. The specific extinction of 0.13 indicated an amyloid content of 3.8%.

(d) The pectic mixture (2.5 mg per ml) extracted, by a similar procedure to that outlined in Experiment 1 except with EDTA adjusted to pH 4.9, from non-germinated cotyledons was analysed. The material was purified by deproteinisation (Experiment 1.4b). A weak colour was produced, the specific extinction of 0.138 indicated an amyloid content of 4.0%.

(e) Another deproteinised pectic mixture (3.0 mg per ml) extracted with EDTA at pH 7.5 from non-germinated cotyledons gave a specific extinction of 0.19, 5.5% amyloid.

(f) A similarly extracted deproteinised pectic mixture

(3.0 mg per ml) from germinated cotyledons gave no colour. The specific extinction of zero showed the absence of amyloid.

(g) The pectic material used in (e) was fractionated on a DEAE-cellulose column by elution with a sodium dihydrogen phosphate-urea system into a neutral and two acidic fractions. The neutral fraction (0.4 mg per ml) gave a strong colour, the specific extinction of 1.64 indicating an amyloid content of 29.5%.

(h) Two neutral fractions (3.0 mg per ml and 0.4 mg per ml) obtained by a similar fractionation of the material used in (f) gave no colour. The specific extinction of zero indicated amyloid to be absent.

(i) The amyloid (0.35 mg per ml) extracted with lithium thiocyanate from the residue of non-germinated cotyledons after extraction with EDTA, and purified by complexing with cupric acetate, gave a strong colour. The specific extinction of 3.17 indicating an amyloid content of 90.5%.

Experiment 1.4. Purification of pectic material from non-germinated and germinated cotyledons.

The material purified in Experiment 1.1 was found to have been degraded by bacteria. The organism was identified as a spore-forming bacillus, probably Bacillus megaterium, which grew rapidly on pectic material. When an infected solution was left at room temperature for 24 hr. the sedimentation diagram in the ultracentrifuge showed complete disappearance of all peaks.

(a) A further batch of material (120g) was deproteinised as described in Experiment 1.1. The precipitation was again observed but complete separation of the two fractions was not successful (as shown by hydrolysis and paper chromatography); (Yield of combined fractions 59.0g; Found, % N = 0.55).

(b) The crude pectic material (23.0g) extracted with EDTA at pH 4.9 from non-germinated cotyledons was deproteinised as above. Very little precipitation or emulsion formation occurred and fractionation was not effected; (Yield 5.5g; Found, % N = 0.53).

(c) The crude pectic material extracted with EDTA at pH 7.5 from germinated cotyledons was deproteinised by Dr. Gould; Found, % N = 1.94. This was used without further purification.

Experiment 1.5. Methylation of the pectic mixtures extracted from non-germinated and germinated cotyledons.

(a) The purified pectic mixture extracted at pH 7.5 from non-germinated cotyledons (Experiment 1.4a) was methylated as follows.

The polysaccharide (39.1g) was dissolved in water (1.0 l) and methylated by the Haworth procedure. Successive daily slow additions of sodium hydroxide solution (30% w/v, 5 x 320 ml) and dimethyl sulphate (5 x 110 ml) were made, with efficient stirring under nitrogen. The temperature was kept at 0° for the first day. After the final

addition the solution was dialysed, concentrated, and remethylated by the above procedure. In all, three such cycles were carried out. The methylated polysaccharide was isolated by freeze-drying, (Yield 37.5g; Found % OCH_3 = 27.5, Calc. 40.5% from sugar ratios).

The partially-methylated pectic mixture (17.5g) was dissolved in dimethylformamide (200 ml) and methyl iodide (150 ml). Silver oxide (65g) was added and the reaction was magnetically stirred in a conical flask fitted with a double surface condenser, in a water-bath at 40° for 48 hr. Chloroform was added and the mixture was filtered to remove insoluble silver salts, which were thoroughly washed with chloroform. The chloroform solution was washed with aqueous sodium cyanide (0.5% w/v) to remove soluble silver salts. The first cyanide wash was back-extracted five times with chloroform and the combined chloroform solutions were washed with water until neutral. After drying over sodium sulphate the chloroform layer was concentrated and the product isolated by precipitation with light petroleum (b.p. $40-60^{\circ}$). (Yield 13.6g; Found, % OCH_3 = 37.9). The infra-red spectrum of the product showed only weak hydroxyl adsorption.

The product (13.6g), remethylated by the same method, gave diminished hydroxyl adsorption (Yield 12.8g; Found, % OCH_3 = 38.2).

The product (2.0g) was fractionated by dissolving in chloroform (50 ml) in a large centrifuge bottle and

slowly adding light petroleum (b.p. 40-60°). Fractions were collected by centrifugation after the addition of approximately 3 volumes, 4 volumes, and a large excess of petroleum. Each fraction was washed with petroleum and dried in vacuo.

Fraction	Yield (g)	Found % OCH_3	Calc. % OCH_3
1	0.26	37.0	40.5
2	0.17	38.4	40.5
3	1.28	37.8	40.5

Infra-red spectra confirmed that Fraction 1 was the least highly methylated. The hydroxyl adsorptions for Fractions 2 and 3 were extremely small despite the rather low methoxyl analyses, which were probably due to non-carbohydrate material.

(b) The purified 'pH 4.9' pectic mixture from non-germinated cotyldeons [Experiment 1.4 (b)] was methylated by the same procedure.

The polysaccharide (4.0g) was methylated by 3 cycles of the Haworth procedure (with amounts adjusted pro rata). A sample was isolated for analysis after each cycle. Yield 3.24g; Found, % OCH_3 = (i) 27.2, (ii) 27.9, (iii) 29.9.

The partially methylated product (3.2g) was methylated by the Kuhn procedure (Experiment 1.5a); Yield 2.46g; Found, % OCH_3 = 40.3. The infra-red spectrum showed some hydroxyl adsorption. The methylation was

repeated and gave a product with negligible hydroxyl absorption; Yield 2.08g; Found, % OCH_3 = 38.2.

The product (2.0g) was fractionally precipitated with light petroleum from chloroform solution as above. Fraction 1 (3 volumes of petroleum) was refractionated to give Fraction 1A and 1B.

Fraction	Yield (g)	Found, % OCH_3	Calc. % OCH_3
1A	0.008	34.2	40.5
1B	0.413	35.9	40.5
2	0.085	39.0	40.5
3	1.345	41.2	40.5

The infra-red spectra showed very slight hydroxyl absorption for 1A and 1B, and no hydroxyl absorption for 2 and 3. The low analysis of the unfractionated polysaccharide was presumably due to contamination with non-carbohydrate material.

(c) The pectic mixture extracted at pH 7.5 from germinated cotyledons (Experiment 4(c)) was methylated by the same procedure.

The polysaccharide (2.0g) was methylated by 3 cycles of the Haworth procedure; Yield 1.49g; Found, % OCH_3 = 25.6.

The partially-methylated product (1.4g) was remethylated by the Kuhn procedure, Yield 0.80g; Found,

% OCH_3 = 38.6. The product had only very slight hydroxyl absorption in the infra-red spectrum.

The methylated polysaccharide (0.40g) was fractionally precipitated with light petroleum from chloroform solution as above:

Fraction	Yield (g)	Found, % OCH_3	Calc. % OCH_3
1	0.115	37.08	40.5
2	0.063	40.34	40.5
3	0.203	37.20	40.5

The infra-red spectra showed very slight hydroxyl absorption in Fractions 1 and 3, but no hydroxyl absorption in Fraction 2.

Experiment 1.6. Methylation analysis of pectic mixture extracted at pH 7.5 from non-germinated mustard cotyledons.

Experiment 1.6a. Hydrolysis of methylated pectic mixture.

The methylated pectic mixture (5.0g, % OCH_3 = 38.2) was hydrolysed in formic acid. The residue after removal of excess acid was heated in 0.1$\underline{N}$ sulphuric acid (150 ml) for 6 hr. at 100° to hydrolyse formate esters, neutralised with barium carbonate, filtered and an excess of 1R 120 (H^+ form) resin added. After standing for 16 hr. the hydrolysate was applied to a column of DEAE Sephadex A25 (30g,

formate form) resin and the neutral sugars eluted with water (3 l). The neutral fraction was evaporated to dryness and dried *in vacuo* over phosphorus pentoxide (3.75g sugar). The acidic fraction was eluted with formic acid (4%, 5 l) and on concentration and drying as above weighed 1.35g. Paper chromatography in solvent B confirmed that the fraction eluted with water contained only neutral sugars and that the fraction eluted with formic acid contained only acidic material.

Experiment 1.6b. Reduction of acidic fraction.

The acidic material (1.25g) was refluxed in methanolic hydrogen chloride (3%, 100 ml) for 6 hr. The solution was neutralised with silver carbonate, filtered and evaporated to dryness. The residue was dissolved in methanol (75 ml) to which 2,2'dimethoxypropane (5 ml) was added. After standing for 16 hr. a mixture of sodium borohydride (1.0g) and sodium borotritiide (2.8 mg, specific activity 3.5 mc per mg) was added. After 48 hr. excess reagent was destroyed with dilute formic acid and the solution passed through a column of 1R 120 (H^+ form) resin. The solution was evaporated to dryness and boric acid removed by repeated distillation of methanol from the residue under reduced pressure. The residue was hydrolysed in formic acid followed by 0.1$\underline{N}$ sulphuric acid as above (Experiment 1.6a) and dried *in vacuo* over phosphorus

pentoxide (0.805g). Paper chromatography in solvent B showed incomplete reduction. Fractionation as above (Experiment 1.6a) on DEAE Sephadex (formate form) gave a neutral 'reduced' fraction (0.620g) and an acidic fraction (0.180g). A small amount (0.020g) of the acidic fraction was completely reduced as above but with a large excess of sodium borohydride and on examination by paper chromatography proved to be identical to the original neutral 'reduced' fraction.

Experiment 1.6c. Separation and identification of the components of the 'reduced' fraction.

The reduced fraction (0.615g) was dissolved in methanol (5.0 ml) and loaded on to a column (55 x 5.5 cm) of cellulose powder (Whatman, CC31) previously equilibrated with a solvent consisting of butan-1-ol : ethanol : water (6 : 2 : 1), and eluted with the same solvent (flow rate 0.6 ml per minute). Fractions (25 ml) were collected automatically and screened by paper chromatography in solvent C. On the basis of the paper chromatography results, the fractions were combined to give larger fractions which were filtered, evaporated to dryness, dried *in vacuo* and weighed.

Components of the fractions were identified by paper chromatography in solvent B and by g.l.c. on column 1 (Pye Argon Chromatograph) at 175° (see Table 1). Major

components were characterised by the formation of crystalline derivatives. Qualitative assay of the activity of the methylated sugar or parent sugar given by demethylation was by scintillation counting of relevant chromatogram strips .

Fraction 1 (0.073g). The major component of this fraction was 3,4-di-O-methylrhamnose (R_G = 0.86; T = 1.00). Trace amounts of 2,3,4-tri-O-methylgalactose (R_G = 0.86; T = 6.01) and 3-O-methylrhamnose (R_G = 0.61; T = 3.12s, 4.57w) were also present. Demethylation gave rhamnose as the only detectable product, a spot of medium intensity (p-anisidine hydrochloride spray) had a count 130 c.p.m. The sugar was thus inactive.

Fraction 2 (0.035g). This fraction was composed mainly of the sugars present in Fraction 1 together with trace amounts of two unidentified components detectable only by g.l.c. The first, (T = 1.51s, 1.74w), on the basis of its mobility in solvent C and retention times was tentatively identified as a di-O-methyl pentose. The second, (T = 8.82m, 10.94s), was shown not to be a di-O-methyl galactose or glucose obtained by reduction of the corresponding di-O-methyl uronic acid, but nonetheless on the basis of retention times is probably a di-O-methyl hexose.

Demethylation gave rhamnose and galactose. A spot of medium intensity of the former had a count of 130

c.p.m., the sugar thus was inactive. A galactose spot of medium intensity had a count of 890 c.p.m., the intermediate activity indicating that 2,3,4-tri-O-methylgalactose was only partly derived from galacturonic acid end-group.

Fraction 3 (0.010g). The fraction was composed of 2,3,4-tri-O-methylgalactose and 3-O-methylrhamnose.

Fraction 4 (0.117g). The fraction was composed of 3-O-methylrhamnose and 2,3-di-O-methylgalactose (R_G = 0.50; T = 8.60s, 11.15m, 13.90w).

Fraction 5 (0.098g). The fraction contained almost pure 2,3-di-O-methylgalactose. The sugar was characterised as the N-phenylglycosylamine derivative, which after recrystallisation from ethanol had m.p. = 153-155° undepressed on admixture with authentic 2,3-di-O-methyl-N-phenyl-D-galactosylamine of m.p. 153-155°. A spot of medium intensity of the sugar had a count of 4750 c.p.m., the sugar was thus highly active.

Fraction 6 (0.020g). The fraction was composed mainly of 2,3-di-O-methyl-D-galactose with a lesser amount of rhamnose (R_G = 0.43). A spot of weak intensity of the latter had a count of 105 c.p.m., the sugar was thus inactive.

Table 1

g.l.c. of standard compounds used in identification of components of the 'reduced fraction'

Column 1 (Pye Argon Chromatograph) at 175°

Methyl glycoside of -	T		
2,3,4-tri-O-methylgalactose	5.98		
2,3-di-O-methylgalactose	8.60s	11.24m	13.90w
2,4-di-O-methylgalactose	13.40m	15.37s	
3,4-di-O-methylgalactose	14.05s	22.25w	
3,4-di-O-methylrhamnose	0.98		
3-O-methylrhamnose	3.10s	4.59w	
4-O-methylrhamnose	3.70		
2,3-di-O-methylglucose	9.03m	11.53s	
3,4-di-O-methylglucose	7.40s	8.78m	
2,4-di-O-methylglucose	7.34m	10.42s	
2,3-di-O-methylfucose	1.04s	1.20sh	1.44m

Fraction 7 (0.195g). This fraction, which was chromatographically pure, crystallised on standing and after recrystallisation from methanol had m.p. = 148-150° and mixed m.p. = 146-148° (with authentic 2-O-methyl-D-galactose of m.p. = 146-148°). A spot of medium intensity had a count of 4375 c.p.m., the sugar was thus highly active.

Fraction 8 (0.030g). The fraction was composed of 2-O-methyl-D-galactose and 3-O-methylgalactose (R_G = 0.29).

Fraction 9 (0.10g). The major component of this fraction was 3-O-methylgalactose. Demethylation gave galactose as the only product, a spot of medium intensity of which had a count of 4310 c.p.m. The sugar was thus highly active.

Fraction 10 (< 0.005g). Continued elution yielded a small fraction, the major component of which was galactose (R_G = 0.17). A spot of weak intensity had a count of 2570 c.p.m., the sugar was thus highly active.

Experiment 1.6d. Separation and identification of the components of the neutral fraction.

The neutral material (3.6g) was dissolved in a mixture (25 ml) of methanol : butan-1-ol half saturated with water (1 : 1) and loaded on to a column (76 cm x 6 cm) of cellulose powder (Whatman, cc31) previously equilibrated with solvent (a) below. The sugars were eluted with the solvents

given below, (4 l of each), in order.

Solvent Systems:

(a) butan-1-ol half saturated with water : light petroleum (B.P. 100-120°) (1 : 3, upper phase) and butan-1-ol : light petroleum (1 : 3) mixed in ratio 1 : 1.

(b) as above but using a ratio of butan-1-ol : light petroleum of 1 : 1 in both cases.

(c) as above but using a ratio of butan-1-ol : light petroleum of 3 : 1 in both cases.

(d) butan-1-ol half saturated with water.

Fractions (250 x 25 ml, thereafter 50 ml) were collected automatically and screened by paper chromatography in solvents B, C, D and E, where appropriate. On the basis of the paper chromatography results the fractions were combined to give larger fractions which were filtered, evaporated to dryness, dried *in vacuo* over phosphorus pentoxide and weighed. Evaporation of the earlier fractions was carried out below 30° to minimise loss of the volatile tri-*O*-methyl pentose derivatives.

Components of the fractions were identified by paper chromatography on column 1 (Pye Argon Chromatograph) at 125° for Fractions 1 and 2, at 150° for Fraction 3 and thereafter at 175° (see Table 2). Major components were characterised by the formation of crystalline derivatives. Demethylation was also used in identification of sugars.

Table 2

Gas liquid chromatography of standard compounds-neutral fraction

Column 1 (Pye Argon Chromatograph)

Temperature	methyl glycoside of -	T		
125°	2,3,4-tri-O-methylrhamnose	0.42		
	2,3,4-tri-O-methylxylose	0.40m	0.53s	
	2,3,5-tri-O-methylarabinose	0.53s	0.71w	
150°	2,3,4,6-tetra-O-methylgalactose	1.76sh	1.87s	
175°	2,3,4-tri-O-methylarabinose	1.04		
	2,3-di-O-methylarabinose	1.46s	1.61w	1.78s
	2,5-di-O-methylarabinose	1.62		
	3,5-di-O-methylarabinose	1.02	2.20	
	2-O-methylarabinose	5.07s	9.10w	
	3,4-di-O-methylrhamnose	0.98		
	3-O-methylrhamnose	3.10s	4.59w	
	4-O-methylrhamnose	3.70		
	2,3,6-tri-O-methylgalactose	2.83s	3.54w	3.74w 4.10m
	2,4,6-tri-O-methylgalactose	3.55m	4.07s	
	2,3,4-tri-O-methylgalactose	5.95		
	2,4-di-O-methylgalactose	13.40m	15.37s	
	2,3,6-tri-O-methylglucose	3.01m	4.05s	
	2,3-di-O-methylglucose	9.07m	11.56s	

Fraction 1 (0.005g). The fraction contained almost pure 2,3,4-tri-O-methylrhamnose (R_G = 1.00; T = 0.42) with a trace of 2,3,5-tri-O-methylarabinose. Demethylation gave rhamnose as the only product.

Fraction 2 (1.66g). The fraction contained a mixture of 2,3,5-tri-O-methylarabinose (R_G = 0.96; T = 0.53s, 0.71w) and 2,3,4-tri-O-methylxylose (R_G = 0.94; T = 0.40m, 0.53s) with a trace of 2,3,4,6-tetra-O-methylgalactose. The major components were separated by preparative paper chromatography in solvent E to give chromatographically pure syrups, the yields indicating that the fraction contained 60% of the arabinose derivative. 2,3,5-tri-O-methylarabinose was characterised as the aldonamide, which after recrystallisation from ethanol had m.p. = 137° and mixed m.p. = 137° (with an authentic sample of 2,3,5-tri-O-methyl--L-arabonamide of m.p. = 137°).

2,3,4-tri-O-methylxylose crystallised on standing and was characterised as the aldonolactone, which after recrystallisation from diethyl ether : light petroleum (B.P. 40-60°), (1 : 1) had m.p. = 53° and mixed m.p. = 53° (with an authentic sample of 2,3,4-tri-O-methyl--D-xylonolactone of m.p. = 55°.

Fraction 3 (0.105g). The fraction contained almost pure 2,3,4,6-tetra-O-methylgalactose (R_G = 0.88; T = 1.73sh, 1.85s) with traces of the major components of Fraction 2.

The sugar was purified by preparative paper chromatography in solvent D and characterised as the N-phenylglycosylamine derivative, which after recrystallisation from ethanol had m.p. = 198° and mixed m.p. = 195° (with an authentic sample of 2,3,4,6-tetra-O-methyl-N-phenyl-D-galactosylamine of m.p. = 193°).

Fraction 4 (0.050g). The major component of this fraction was 3,4-di-O-methylrhamnose (R_G = 0.88; T = 0.9) with a smaller amount of 2,3,6-tri-O-methylglucose (R_G = 0.86; T = 3.02m, 4.03s) and a trace of tetra-O-methylgalactose.

Fraction 5 (0.055g). The major component of this fraction was 2,3,6-tri-O-methylglucose with a small amount of 3,4-di-O-methylrhamnose. 2,5-di-O-methylarabinose (T = 1.61) and 3,5-di-O-methylarabinose (T = 1.02, 2.20) were detected as minor components by g.l.c. only. Demethylation of the fraction gave glucose and rhamnose.

Fraction 6 (0.290g). The fraction contained mainly 2,3-di-O-methylarabinose (R_G = 0.74; T = 1.48s, 1.62w, 1.80m) with approximately 5% 2,3,6-tri-O-methylgalactose (R_G = 0.81; T = 2.82s, other peaks obscured) and 10% 2,4,6-tri-O--methylgalactose) (R_G = 0.76; T = 3.58m, 4.09s).

Fraction 7 (0.565g). The fraction contained almost pure 2,3-di-O-methylarabinose with a trace of 2,4,6-tri-O-

-methylgalactose. The sugar was characterised as the aldonamide, which after recrystallisation from ethanol had m.p. = 158° and mixed m.p. = 158° (with an authentic sample of 2,3-di-O-methyl-L-arabonamide of m.p. = 160°).

Fraction 8 (0.053g). The fraction contained 4-O-methylrhamnose (R_G = 0.64; T = 3.76) and 2,3,4-tri-O-methylgalactose (R_G = 0.68; T = 5.95) as the major components with a lesser amount of 2,3-di-O-methyl-L-arabinose. Demethylation gave rhamnose, galactose and arabinose.

Fraction 9 (0.030g). The fraction contained 3-O-methylrhamnose (R_G = 0.62; T = 3.10s, 4.61w) as the major component with a small amount of 2,3-di-O-methylglucose and a trace of 4-O-methylrhamnose. Demethylation gave rhamnose and a trace of glucose.

Fraction 10 (0.120g). The fraction contained 2,3-di-O-methylglucose (R_G = 0.69; T = 8.88m, 11.55s) as the major component with a small amount of 3-O-methylrhamnose. Demethylation gave glucose and a trace of rhamnose.

Fraction 11 (0.025g). The fraction contained an unidentified sugar (R_G = 0.61; T = 9.23, 12.60) as the major component. Demethylation gave glucose as the only product. The sugar was tentatively identified as a di-O-methylglucose

on the above evidence, but was neither 2,4-di-O-methylglucose (T = 7.34m, 10.42s) nor 3,4-di-O-methylglucose (T = 7.40s, 8.78m).

Fraction 12 (0.665g). This fraction contained almost pure 2-O-methyl-L-arabinose (R_G = 0.50; T = 5.05s, 9.10w) with a trace of 2,4-di-O-methylgalactose (R_G = 0.52; T = 13.30m, 15.28s). The sugar was characterised as the toluene-p--sulphonylhydrazone, which after recrystallisation from methanol had m.p. = 142-144° and mixed m.p. = 142-144° (with authentic 2-O-methyl-L-arabinose toluene-p-sulphonylhydrazone of m.p. = 145°).

Fraction 13 (0.110g). The fraction was chromatographically pure and was identified as 3-O-methylarabinose (R_G = 0.45; T = 2.93m, 3.81s, 5.86w, 7.88m) by methylation and demethylation. Methylation followed by g.l.c. gave both 2,3,5- and 2,3,4-tri-O-methylarabinoses proving the sugar to be neither 4- nor 5-O-methylarabinose. Demethylation gave only arabinose.

Fraction 14 (0.040g). The fraction contained pure arabinose (R_G = 0.23) which was characterised as the toluene-p--sulphonylhydrazone. After recrystallisation from methanol the derivative had m.p. = 155° and mixed m.p. = 153-154° (with authentic L-arabinose toluene-p-sulphonylhydrazone of m.p. = 153°).

Continued elution with solvent (d) yielded no further carbohydrate material.

Experiment 1.7. Comparison of the methylated pectic mixtures from germinated and non-germinated cotyledons.

The methylated pectic mixtures (75 mg) (Experiment 1.5a, b and c) were dissolved in dry tetrahydrofuran (5.0 ml, freshly distilled from lithium aluminium hydride) and an excess of lithium aluminium hydride added. The flasks were tightly stoppered and shaken for 16 hr., followed by heating under reflux for 2 hr. Excess reagent was destroyed by careful addition of ethyl acetate, and lithium aluminate was precipitated with the minimum amount of water. The contents of the flask were transferred to a centrifuge bottle (250 ml) with a large volume of acetone and the precipitate removed by centrifugation. The supernatant solution was decanted off and the precipitate washed once with acetone, centrifuged and the supernatant solutions combined. The precipitate gave a negative reaction to the phenol sulphuric acid reagents. The supernatant solution was evaporated to dryness. The products were examined by paper chromatography in solvent B after hydrolysis, and by g.l.c. on column 1 (Pye Argon Chromatograph) at 175° after methanolysis. The gas chromatograms are shown in Diagram 1 and retention times of component sugars are given in Table 3B. Paper chromatography showed that reduction

was complete; the component sugars were identified using standards and the following results were obtained (by visual examination; p-anisidine hydrochloride spray).

Table 3A

Sugar	Relative proportion in hydrolysate of -		
	a	b	c
2,3,5-tri-O-methylarabinose	s	s	m
2,5-di-O-methylarabinose	vw	vw	vw
2,3-di-O-methylarabinose	s	s	w
2-O-methylarabinose	m	m	tr
arabinose	w	w	-
2,3,4-tri-O-methylxylose	s	s	s
3,4-di-O-methylrhamnose	w	w	w
3-O-methylrhamnose	vw	vw	vw
rhamnose	vw	vw	vw
2,3,4,6-tetra-O-methylgalactose	w	w	w
2,4,6-tri-O-methylgalactose	w	w	w
2,3-di-O-methylgalactose	w	m	m
2,4-di-O-methylgalactose	vw	vw	vw
2-O-methylgalactose	w	w	w

s = strong, m = medium, w = weak, vw = very weak, tr = trace.

a is methylated reduced non-germinated pH 7.5 extracted pectin

b is methylated reduced germinated pH 7.5 extracted pectin

c is methylated reduced non-germinated pH 4.9 extracted pectin.

Table 3B

Gas liquid chromatography of methylated, reduced, methanolysed pectins. (see Diagram 1)

Column 1 (Pye Argon Chromatograph) at 175°

Peak	methyl glycoside of -	T			
		Standard	a	b	c
1	2,3,4-tri-O-methylxylose	0.40	0.42	0.43	0.42
2	2,3,5-tri-O-methylarabinose/ 2,3,4-tri-O-methylxylose	0.53	0.56	0.56	0.55
3	2,3,5-tri-O-methylarabinose	0.71	0.74	0.75	0.74
4	3,4-di-O-methylrhamnose	0.98	1.02	1.02	1.02
5	2,3-di-O-methylarabinose	1.46	1.49	1.49	1.49
6	2,3,4,6-tetra-O-methylgalactose/ 2,3-di-O-methylarabinose	1.61	1.69	1.68	1.69
7	2,3-di-O-methylarabinose	1.78	1.83	1.83	1.83
7a	2,3,6-tri-O-methylgalactose	3.01	2.98	2.98	2.97
8	3-O-methylrhamnose	3.10	3.21	3.20	3.21
9	2,4,6-tri-O-methylgalactose	3.55	3.65	3.67	3.67
10	2,3,6-tri-O-methylgalactose/ 2,4,6-tri-O-methylgalactose	4.10	4.13	4.13	4.13
11	2-O-methylarabinose	5.07	5.18	5.20	5.20
12	2,3,4-tri-O-methylgalactose	5.98	6.02	6.05	6.03
13	2,3-di-O-methylgalactose	8.48	8.34	8.34	8.33
14	unidentified	-	9.45	9.47	9.47
15	2,3-di-O-methylgalactose	11.20	11.16	11.16	11.15
sh	unidentified	-	12.84	12.84	12.84
16	2,4-di-O-methylgalactose	13.40	13.47	13.49	13.43
17	2,4-di-O-methylgalactose	15.37	15.40	15.42	15.43

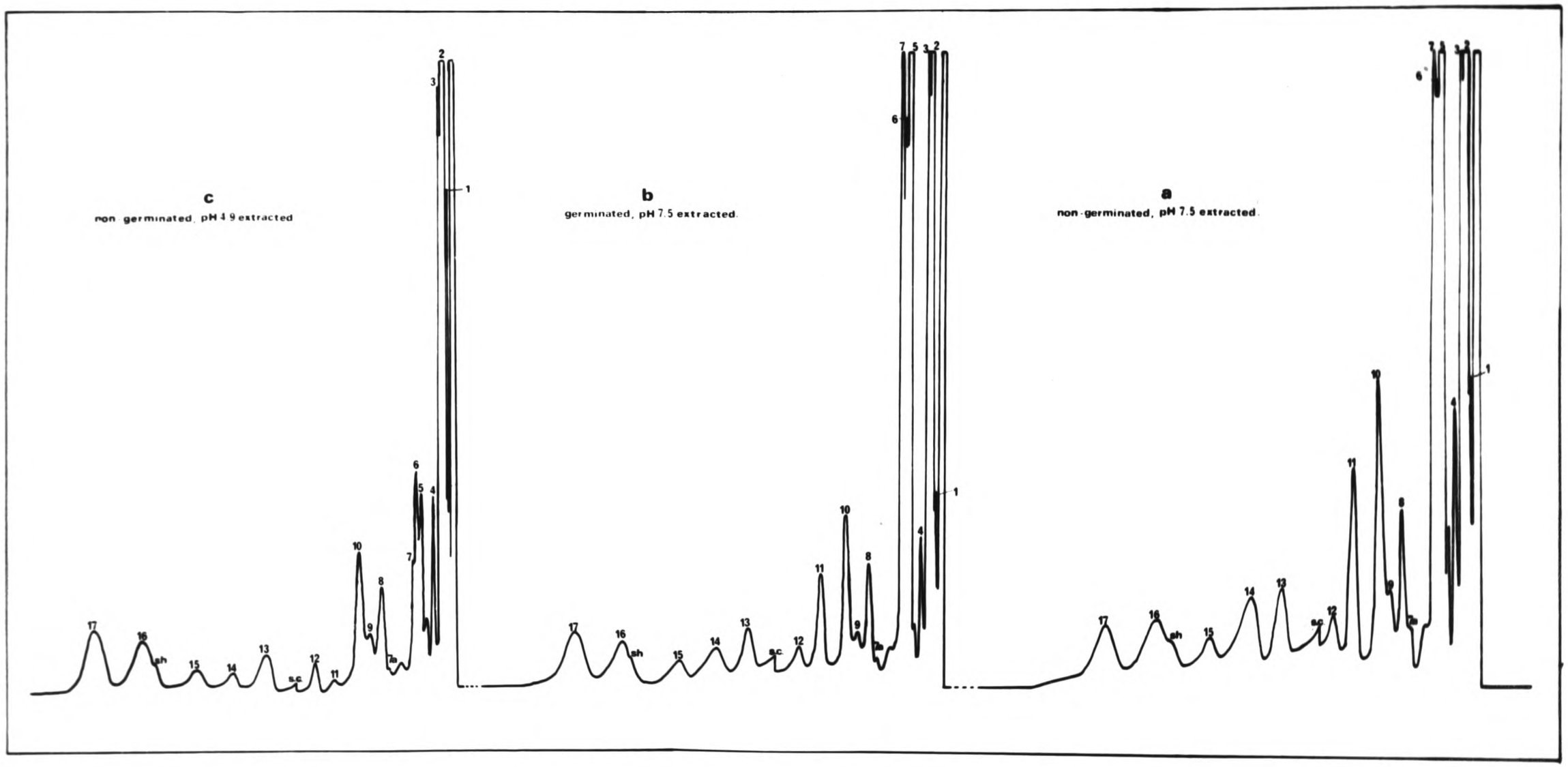


DIAGRAM 1.

Gas chromatograms of methylated reduced methanolysed pectins. (s.c. : sensitivity increased)

Discussion

(A) Previous theories as to the constitution of the pectic substances have tended to oversimplify the situation. Until fairly recently it was thought that a mixture of three homopolysaccharides was present, namely the neutral galactan and araban and the acidic galacturonan. Although authenticated samples of each of these polysaccharides have been isolated, they would seem to occur rarely. Samples of galacturonan almost invariably contain a substantial proportion of covalently linked neutral sugars (63). The arabans from guava (64) and apple (65) also contain an appreciable amount of sugars other than arabinose, particularly galactose and galacturonic acid. Barrett and Northcote (21) have shown that in alkaline solution apple pectin undergoes cleavage by an elimination mechanism (β elimination) at methyl esterified galacturonic acid units to give a fraction composed mainly of galacturonic acid, and a fraction composed mainly of neutral sugars. In the light of these results it seems likely that some arabans are elimination artefacts produced during extraction at alkaline pH, e.g. with lime water.

The results of fractionation by column chromatography and ultracentrifugation of mustard cotyledon pectin mentioned in the Introduction have shown that it is not homogeneous but that related acidic polysaccharides are present. The structural features elucidated by experiment on the whole pectin, although probably common to the

components, may thus be present to a greater or lesser degree in each component.

The pectic mixtures extracted from non-germinated cotyledons at pH 7.5 and pH 4.9, and from germinated cotyledons at pH 7.5 have been satisfactorily purified (protein content 3 - 7%) and methylated fully.

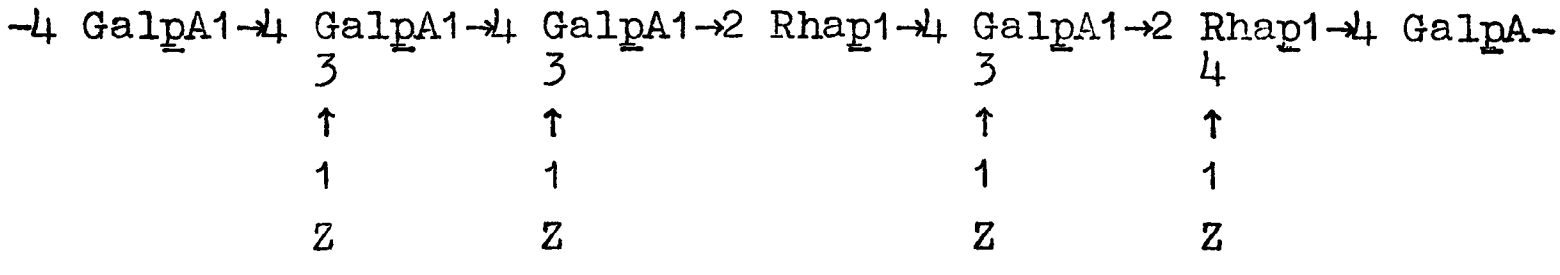
The main features of the pectin have been established by the formation of crystalline derivatives of the sugars obtained by hydrolysis of the methylated polysaccharide. The acidic sugars were identified after reduction with sodium borotritiide as the corresponding galactose derivatives. The high specific activity of the products confirmed that they had originated from galacturonic acid derivatives. The other sugars in the reduced acidic fraction (exclusively rhamnose derivatives) were unlabelled. The presence of the 1,4 linked galacturonan main chain common to pectic substances was thus confirmed (by the presence of 2,3-di-O-methyl-D-galactose), the chain being interrupted by the insertion of rhamnose units linked through $C_{(2)}$ of rhamnose (3,4-di-O-methylrhamnose) as has been established for several other pectins. As rhamnose derivatives were present in the neutral fraction in different proportions to those of the acidic reduced fraction (e.g. 4-O-methylrhamnose was found only in the neutral fraction), contiguous rhamnose units are probably also present. Recent work has shown that the pectic substances of soybean (22,61) also have contiguous rhamnose units. Approximately 60% of the

galacturonic acid and rhamnose residues carry side chains. The principal mode of branching of galacturonic acid is through $C_{(3)}$ (as shown by presence of 2-O-methyl-D-galactose), although some branching through $C_{(2)}$ (3-O-methylgalactose) and through both $C_{(2)}$ and $C_{(3)}$ (galactose) also occurs. Rhamnose is mainly branched through $C_{(4)}$ (3-O-methylrhamnose), occasionally through $C_{(3)}$ (4-O-methylrhamnose) and rarely through both $C_{(3)}$ and $C_{(4)}$ (rhamnose). Derivatives of glucose (formed by the reduction of glucuronic acid derivatives) were not observed and hence most of the minor features, e.g. glucuronosylgalactose and glucuronosylfucose, listed in the Introduction were not detected in this study. A recent study of the native pectic mixture by Dr. D. Rees (74) has, however, shown that traces of these disaccharides are present in partial hydrolysates.

The pattern of neutral sugars confirmed that branched araban side chains occur. Derivatives of arabinose account for more than 50% of the pectic mixture, and thus the side chains are of high molecular weight. The methylated sugars are typical of arabans (see Chapter 3), although 3-O-methylarabinose is comparatively rare. D-xylose was present in large amounts and exclusively as end group (2,3,4-tri-O-methyl-D-xylose) confirming the view (56) that single unit side chains of xylose residues are attached to the main chain, although other interpretations are of course possible. Galactose occurred mainly as end group (2,3,4,6-tetra-O-methyl-D-galactose) and as 2,4,6-tri-

-O-methylgalactose, indicating that 1,4 galactan, as side chain or otherwise, is not a significant structural feature of the pectic mixture. Hullar and Smith (67) in an investigation of sugar beet pectin claim that the galactose units connect the araban side chains to the galacturonan main chain, and this might well be the case with the mustard cotyledon pectin. Glucose is not a typical component of the pectic substances and the results of Experiment 1.2 show that all glucose is contained in one discrete fraction, which showed similarities with a family of polysaccharides known as the amyloids. The glucose derivatives found in the methylation study of the whole pectin are thus unlikely to be structural units of the pectic mixture.

These conclusions are in agreement with the known chemistry of the pectic substances and in particular with the general structure of mustard cotyledon pectic mixture which has been tentatively proposed (23). The average structure was represented as :-



Where Z = Xylp or araban side chain with or without galp link unit (s).

The molar ratios of component sugars were found

from the weights of column fractions. For mixed fractions the proportion of each component was estimated from the peak areas of gas chromatograms. The results are thus more valuable as a guide to the relative amounts of methylated sugars present than as an accurate analysis. The yields of 2,3,5-tri-O-methylarabinose, 2,3,4-tri-O-methylxylose and 2,3,4,6-tetra-O-methylgalactose are probably overestimated, as, due to the volatility of these sugars, the syrups were dried only briefly. The ratios (corrected for reduction in the case of galacturonic acid derivatives and for initial weight of the neutral and reduced fractions) are given below :-

2,3-di-O-methylgalacturonic acid	1.00
2-O-methylgalacturonic acid	1.14
3-O-methylgalacturonic acid	0.16
galacturonic acid	0.03
2,3,5-tri-O-methylarabinose	2.86
2,3-di-O-methylarabinose	2.50
2,5-di-O-methylarabinose	0.04
2-O-methylarabinose	2.24
3-O-methylarabinose	0.42
arabinose	0.15
2,3,4-tri-O-methylxylose	1.88
2,3,4-tri-O-methylrhamnose	0.03
3,4-di-O-methylrhamnose	0.54
3-O-methylrhamnose	0.74
4-O-methylrhamnose	0.11

rhamnose	0.03
2,3,4,6-tetra-O-methylgalactose	0.32
2,4,6-tri-O-methylgalactose	0.15
2,3,4-tri-O-methylgalactose	0.09
2,3,6-tri-O-methylgalactose	0.05
2,4-di-O-methylgalactose	0.04
2,3,6-tri-O-methylglucose	0.15
2,3-di-O-methylglucose	0.29
unidentified components (molecular weight taken as 208)	0.08

These figures may be used to derive an anhydrosugar content which can be compared with that obtained in a previous study (59) in which galacturonic acid was determined by decarboxylation and the neutral sugars colorimetrically after separation by paper chromatography.

Sugar	% anhydrosugar	
	By methylation analysis	Directly
Galacturonic acid	16.2	23.8
Arabinose	54.2	28.8
Xylose	12.4	10.9
Rhamnose	9.7	2.6
Galactose	4.5	6.1
Glucose	3.0	3.9

The comparison of the non-germinated and germinated pectic mixtures did not show the dramatic

differences expected if a decrease in the degree of branching was responsible for the role of these polysaccharides in the developing cell wall. The gas chromatograms (Diagram 1, a and b) are almost identical and do not show any major difference in the proportion of neutral sugar derivatives to 2,3-di-O-methylgalactose (formed by the reduction of 2,3-di-O-methylgalacturonic acid), although comparison of spot intensities of paper chromatograms (Table 3A) does indicate a very slight decrease in the degree of branching of the germinated pectic mixture as shown by the ratio of 2-O-methylgalactose to 2,3-di-O--methylgalactose. It would thus appear that if the pectic substances control wall extensibility through their ability to form gels via the interactions proposed for pectin gels *in vitro* then this must predominantly involve a redistribution of side-chains. This would most probably be intramolecular, so that relatively unbranched and highly branched regions of the galacturonan main chain alternate in the molecule. A structure of this type has been proposed for apple fruit pectin (21). In a pulse labelling experiment with ^{14}C arabinose, Stoddart and Northcote (68) have shown that the isolated pectin fractions of actively growing sycamore cells are metabolically related, the label passing from the neutral arabinan-galactan fraction to the acidic fractions as growth occurred. A similar function is proposed for the pectic araban from mustard cotyledons (28). Intermolecular redistribution of side-chains would thus

also seem to be a possibility, although less likely as a comparison in the ultracentrifuge (60) has not shown a molecular weight increase of any pectin fraction on germination.

Alternatively wall extensibility may be controlled by some other type of interaction involving the pectic substances.

The non-germinated pectic mixture extracted at pH 4.9 showed significant differences to those extracted at pH 7.5 (both non-germinated and germinated). Derivatives of arabinose are present in greatly diminished amounts and are, with the exception of 2,3,5-tri-O-methylarabinose, relatively minor features of the pectic mixture (Diagram 1c and Table 3A). The degree of branching is thus considerably lower, the large araban side-chains being of rather limited occurrence. This result is the opposite to that expected on the basis of the gel theory. As EDTA is less efficient at pH 4.9 the less strongly bound pectin molecules in the cell wall will be extracted preferentially. These would be expected to have a high proportion of side-chains, particularly the high molecular weight araban type. It appears that the converse is true and that the species with a high proportion of araban side-chains are more strongly bound in the wall. Whether this property is attributable to the araban side-chains or to some other property of the molecule is difficult to assess but it seems clear that the interactions between pectic substances in the wall are more

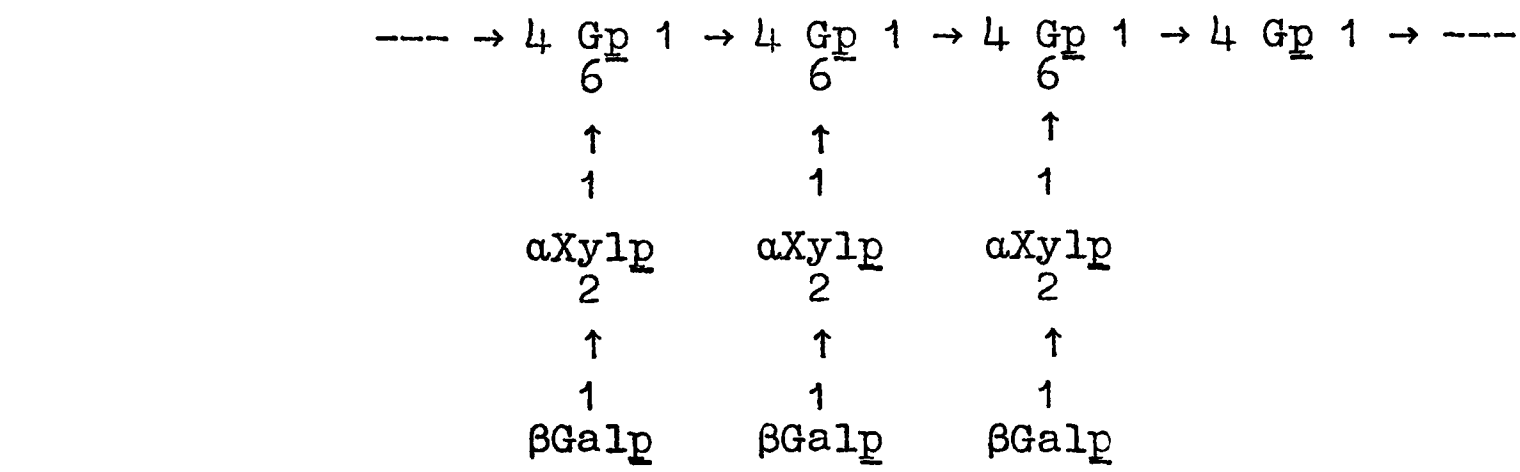
complicated than those postulated for *in vitro* gels.

The precipitation of the non-germinated pectin extracted at pH 7.5, which occurred during deproteinisation with phenol (Experiment 1.1) would confirm this view. The stages leading up to deproteinisation are; extraction with EDTA (pH 7.5), precipitation of the pectic mixture with aqueous ethanol (75%), dialysis against tap water and freeze-drying. That no precipitation occurred when deproteinisation was carried out in the presence of EDTA suggests that Ca^{++} ions are directly involved. A possible explanation is that a polysaccharide complex involving bound Ca^{++} ions is present in the cell wall, and is stabilised by protein (probably denatured) in the EDTA extract. The protein apparently confers water solubility on the system, and on deproteinisation the pectin aggregates, presumably as calcium pectate. The pectic mixture extracted at pH 4.9 showed little tendency to precipitate on deproteinisation (Experiment 1.4b), confirming the view that it is the more highly branched pectin molecules which interact more strongly, but nevertheless the uronic acid residues must be involved because of the role of the Ca^{++} ions.

(B) The fraction of the pectic mixture (non-germinated, pH 7.5 extracted) which did not precipitate on deproteinisation (Experiments 1.1 and 1.2) was shown to be homogeneous in the ultracentrifuge. The sugars given on hydrolysis, namely glucose, xylose and galactose are characteristic of a group

of polysaccharides known as xyloglucans or 'amyloids', the term amyloid denoting that, like starch, these substances form blue complexes with iodine. Amyloids have been found in the seeds of many plants and are thought to have a reserve function; in mature nasturtium seeds for example starch is absent and the amyloid forms a thick layer inside the cell wall (69). The structure of amyloids from seeds of nasturtium (70), tamarind (71), Annona muricata (72) and an amyloid isolated from the hemicellulosic fraction of mustard cotyledons (59) have been investigated.

The Tamarindus amyloid extracted from the cotyledons of tamarind (Tamarindus indica) seeds with hot water and purified by complexing with copper salts, gave on hydrolysis glucose : xylose : galactose in the ratio of 3 : 2 : 1 (71,73). The structure (71), proposed on the basis of results of methylation, partial acid hydrolysis and hydrolysis by cellulase is a β1,4-linked glucan chain branched through the $C_{(6)}$ position in three glucose units out of every four (on average), the side-chains being the disaccharide 2-O-β-D-galactopyranosyl-D-xylose.



Tamarindus amyloid, structure proposed by Kooiman (71).

The Tamarindus amyloid gives the characteristic blue-green colour reaction with potassium triiodide in saturated sodium sulphate (Experiment 1.2) with an extinction maximum at 640 nm. Amyloids with a less branched structure, i.e. a more cellulosic character, give colour reactions in which the absorption maxima are shifted, the amyloids of the seeds of the Annonaceae for example, for which a structure identical with Tamarindus amyloid except that only one in four glucose residues carry side-chains is proposed, give violet colours (72).

The amyloid from mustard cotyledons which was insoluble in EDTA (59) (the 'insoluble' amyloid) had an extinction maximum at 650 nm. Hydrolysis of this polysaccharide gave mainly glucose and xylose with some fucose and only traces of galactose. A structure related to that for Tamarindus amyloid but with side-chains in which xylose units are linked through $C_{(4)}$, and $C_{(3)}$ (instead of only $C_{(2)}$) and terminated in xylose, fucose, glucose and, rarely, galactose was proposed on the evidence of methylation, which also showed the molecule to be less branched than Tamarindus amyloid.

The 'soluble' amyloid gave the characteristic colour with potassium triiodide in saturated sodium sulphate, with an extinction maximum at 650 nm. In a methylation study carried out by Miss Ann Peattie the 'soluble' and 'insoluble' amyloids from mustard cotyledons

were compared by g.l.c. Several interesting differences were observed. The 'soluble' amyloid was shown to have a somewhat less branched (as judged from the ratio of 2,3-di-O-methylglucose to 2,3,6-tri-O-methylglucose) β1,4 linked glucan main chain and to have xylose side-chains linked only through $C_{(4)}$. The side-chains are terminated in xylose, glucose, fucose and galactose, the high proportion of the latter being a major difference between the two polysaccharides.

The 'soluble' amyloid is thus more closely related to Tamarindus amyloid than is the 'insoluble' amyloid in having less complicated side-chains, a higher proportion of which are terminated in galactose.

It would appear that a parallel situation exists with the amyloids and the pectins from mustard cotyledons in that solubility in EDTA is inversely proportional to the degree of branching.

Quantitative experiments on the iodine-staining reaction (Experiment 1.3) showed that, though less branched, the mustard cotyledon amyloids have a lower specific extinction than the Tamarindus amyloid. In starch-type polysaccharides the interaction with iodine increases as the degree of branching decreases and hence the above observation probably reflects an increased proportion of side-chain sugars. The 'soluble' amyloid was extracted equally efficiently with EDTA at pH 4.9 and pH 7.5 and occurs to the extent of about 5% by weight in the deproteinised pectic mixtures from

non-germinated cotyledons. Protein effectively prevents the colour reaction and deproteinisation is thus a necessary preliminary to amyloid determination (Experiment 1.3a). 'Soluble' amyloid was found to account for a large proportion (30%) of the neutral fraction of non-germinated pectic mixture, but was completely absent in both the neutral fraction, and the whole deproteinised pectic mixture from germinated cotyledons.

The results are consistent with the commonly held view that amyloids have a reserve function in germinating seeds. It is interesting to speculate that the amyloids might serve as a precursor of cellulose and therefore represent a store of 'soluble cellulose' in the dormant seed. The structural studies indicate that the amyloids as a class of polysaccharides are as variable in structure as, for example, the pectic substances, the differences between members of both classes arising chiefly from the proportion and structure of the side-chains.

CHAPTER 2

Incorporation of myo-inositol-2-^{3}H by germinating mustard seeds

Introduction

The use of radioactive isotopes as tracers in biosynthetic experiments is widespread even though their availability on a commercial scale is relatively recent. In most respects the isotopes are chemically identical with the stable isotope of the element but differ physically in that they emit radiation. The great value of such isotopes in biological systems is that even in enormous dilution with non-radioactive material the emitted radiation can be detected and measured, with the result that only minute quantities are required. The most commonly used tracers are compounds containing the isotopes ^{3}H, ^{14}C, ^{32}P and ^{35}S although many others are also available. Elucidation of the role of many biologically active substances and the biosynthetic pathways involved in their metabolism has been made possible by use of the radioactively labelled compounds in incorporation experiments. ^{3}H and ^{14}C both decay to a stable isotope by emission of β particles (the electron). The energy of the emitted radiation is relatively weak in ^{3}H (0.018 MeV) as compared with ^{14}C (0.15 MeV) and the radiation is classed as 'soft' and 'medium' β emission respectively. On the laboratory time scale these isotopes are fairly stable, ^{3}H having a half-life of 12.26 years and ^{14}C 5,770 years, so that negligible loss of radioactivity by decay occurs during experiment.

High energy particles (γ- and 'hard' β-radiation) are traditionally counted with the Geiger-Müller counter

(GM tube) in which the radiation produces ion-pairs in a gas held between two oppositely charged electrodes. The Ionisation Chamber and Gas-flow Counter are also examples of this approach. Disadvantages of the GM tube are that low energy particles cannot be counted, high count rates (e.g. over 5,000 per second) cannot be measured, and efficiency is low.

The scintillation counter enables particles of all energies to be counted efficiently even at extremely high count rates. In this device the emitted particles are counted via the secondary radiation which they produce in some systems. Certain fluorescent compounds can be excited to metastable states by irradiation and on returning to the ground state emit flashes of light (scintillations). In the scintillation counter these are detected by photomultipler tubes whose outputs are amplified, analysed and recorded.

The most commonly used technique is that of 'liquid scintillation counting'. The scintillator in this case is a liquid which has two main components; the solvent, usually toluene, xylene or dioxane of high purity, and the primary solute, usually a molecule containing several conjugated aromatic rings, for example anthracene or 2,5-diphenyloxazole. The solvent transfers the energy to the solute which emits the light. Frequently a secondary solute is added in small amounts whose function is to shift the spectrum of emitted light to longer wavelengths at which

the liquid is more transparent and photomultiplier response improved. The radioactive material may be dissolved directly (e.g. aqueous solutions such as tritiated water in dioxane based scintillator) or indirectly, by the use of a blending agent, (e.g. plasma with a quaternary amine salt) in the scintillator. Alternatively chromatogram or electrophoretogram strips, or solutions spotted onto cellulose acetate or glass fibre paper may be immersed in the scintillator and counted with fairly high efficiency. Severe loss of efficiency can occur through 'quenching', the term given to energy loss of the emitted radiation by absorption. This most commonly occurs when coloured substances, water and other compounds are present in significant amounts. A method of assessing this is given in the experimental section.

Gel, plastic and solid scintillation counting are also widely used. In the latter, which is particularly useful for γ-radiation due to its high stopping power, the sample is surrounded by a large inorganic crystal, such as sodium iodide activated with a trace of thallium, enclosed in a metal container with perspex windows.

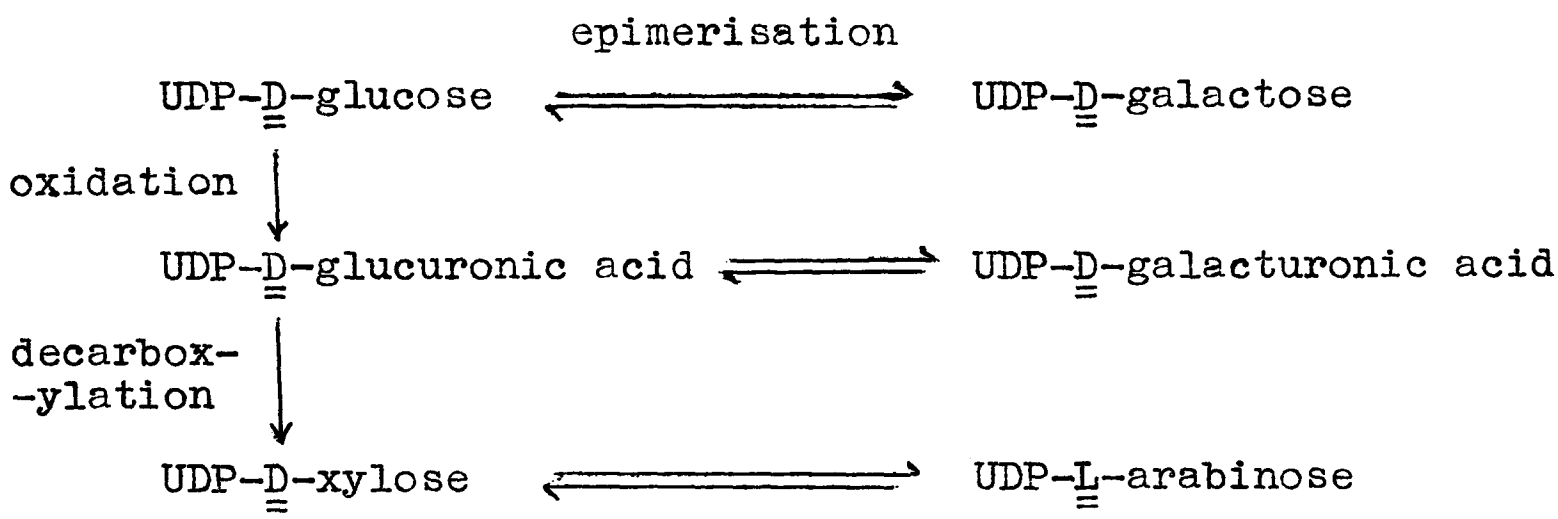
The pulse counting methods described above measure the sum of the radiation entering the counter from a sample but give no detail as to the variation in activity within a heterogeneous sample. The location of radioactivity in such a sample, e.g. a thin section of a tissue or organ, or a chromatogram or electrophoretogram, may be determined by

autoradiography (75). In this technique the property of radioactive particles to produce a latent image in a nuclear emulsion held in intimate contact with the source is utilised. A cumulative record of the radiation incident on the emulsion is thus produced and is made permanent by development and fixation. By responding in a strictly localised fashion the autoradiograph is ideally suited to studies of the distribution of activity within a sample, but as compared to scintillation counting the process is slow, complicated and more amenable to qualitative than quantitative measurements. Low energy β particles are counted with extreme efficiency, and coupled with low background effects, levels of activity as low as one disintegration per day can be detected. The results of scintillation counting and autoradiography are frequently complementary.

The biosynthesis of polysaccharides has been the object of intensive interest and radiochemical techniques applied in this field have yielded some dramatic results. In biological systems, the interconversion of sugar units and their polymerisation into oligo- and polysaccharides is effected through a group of labile compounds known as the sugar nucleotides, in conjunction with specific enzymes. The high free energy of these compounds relative to sugar glycosides (which is associated with the phosphodiester linkage) and enzyme catalysis account for the ease with which biologically useful degrees of conversion are obtained

in reactions (e.g. the direct polymerisation of glucose) which are thermodynamically unfavourable in that the products have a higher free energy than the reactants.

A series of reactions whereby the commonly occuring units of plant polysaccharides are interconverted is shown below :



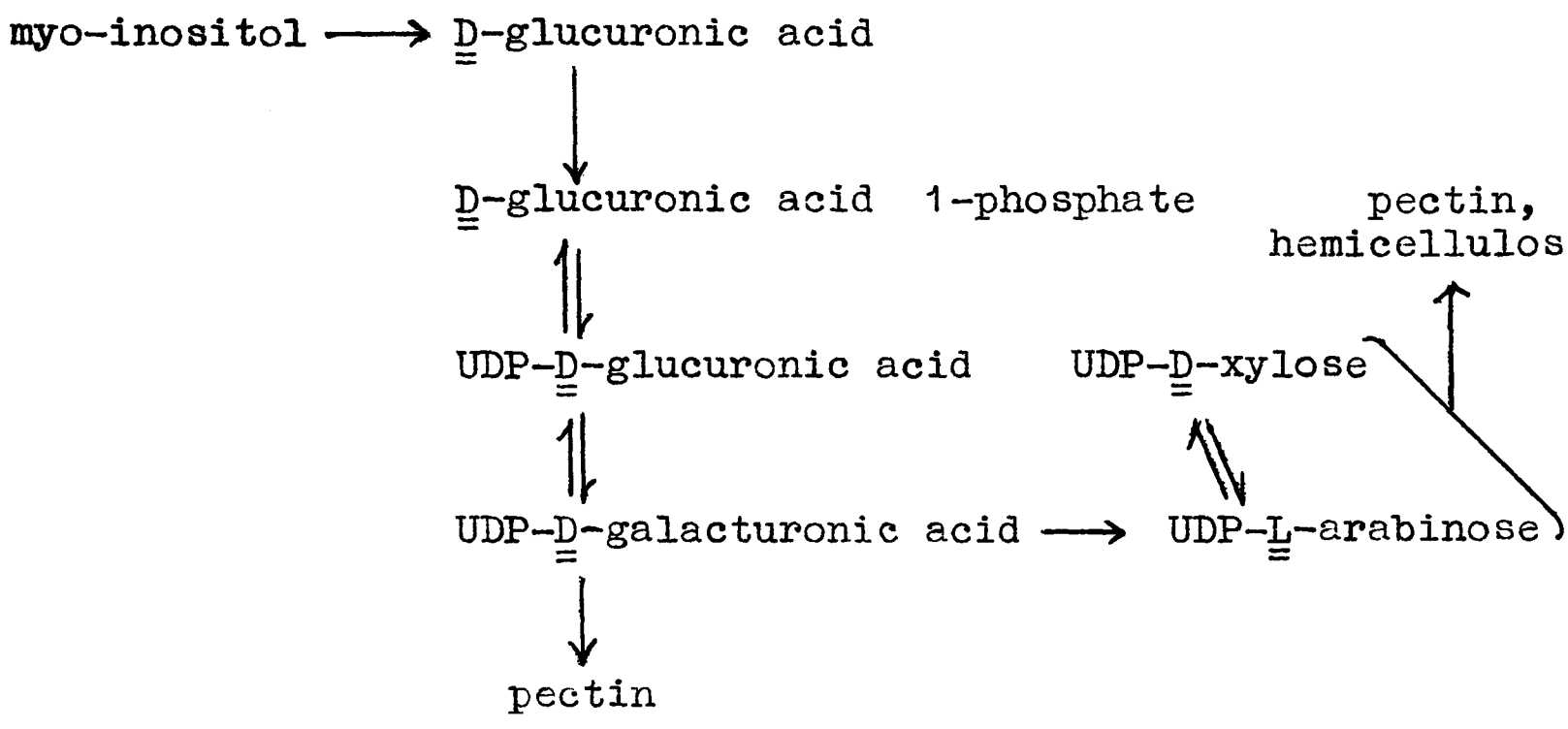
UDP is an abbreviation for uridine diphosphate. Other nucleosides, namely adenosine (A), guanosine (G), cytosine (C), and thymidine (T), are also involved in other interconversions and biosyntheses.

The discovery that phosphorylase catalysed the polymerisation of glucose from glucose 1-phosphate prompted the theory that it occupied a key role in starch and glycogen anabolism (and catabolism). The role of glucose 1-phosphate as the immediate precursor of starch and glycogen was disproved by Leloir and his colleagues who showed that both compounds are synthesised from nucleotide sugars in plants, animals and bacteria (76). The *in vitro* syntheses of many carbohydrates from ^{14}C-labelled nucleotide sugars by particulate enzyme systems from mung bean seedlings have

been reported, e.g. a cellulose-like polymer from GDP-glucose (77), glucomannan from GDP-glucose and GDP-mannose (78) and galactan from UDP-galactose (79). It has also been demonstrated that UDP-xylose is the precursor of β-1,4 xylan in corn-cobs (80). Much attention has been focussed on the biosynthesis of the pectic substances; Albersheim and Bonner (81,82) have shown that pectin in oat coleoptiles is synthesised as a methyl esterified galacturonan and that glucose and inositol will serve as uronide precursors. Methionine and formaldehyde serve as precursors of the methyl group (81,83). On the basis of these results it was postulated that the immediate precursor of pectin is the methyl esterified galacturonic acid derivative of a nucleoside diphosphate. The work of Hassid and his co-workers (84,85) however has shown that UDP-methyl-D-galacturonate was not a precursor of galacturonan in an enzyme system from mung bean seedlings, and that of the galacturonic acid nucleotides only UDP-galacturonic acid is an efficient precursor of pectin. S-adenosylmethionine has been postulated as a methyl donor to pectin in this system. Stoddart and Northcote (68) in a study of the continuous incorporation of ^{14}C-glucose into the pectin fraction of actively growing cells of sycamore callus tissue have shown that the first labelled product is a partly methyl esterified galacturonan from which the strongly acidic and weakly acidic fractions are formed by demethylation and addition of neutral side chains respectively. The authors claim that esterification could take place on

the growing chain and that the reaction may be a function of chain extension. In an autoradiographic study (in the electron-microscope) of the root cap cells of wheat seedlings fed with a pulse of ^{3}H-glucose, Northcote and Pickett-Heaps (31) have shown that labelled substances, identified as pectic polymers, are formed within the Golgi bodies and transported through the cytoplasm, and across the plasmalemma in vesicles to be deposited in the cell wall.

Inositol is a constituent of most higher plants, both in the free state and as the hexaphosphate (phytic acid). Loewus and co-workers (86) have shown that, in labelling experiments with myo-inositol-2-^{3}H with strawberry fruit and parsley leaves, inositol is converted primarily to the galacturonosyl residues of pectin. Incorporation into arabinose and xylose residues of pectin and xylose residues of hemicellulose occurs to a much lesser extent. Hexose constituents are unlabelled. It was suggested that inositol is metabolised by an oxidative pathway to glucuronic acid, (such oxidases have been observed in yeast and barley), which is then converted, via phosphorylation, to UDP-glucuronic acid. The original carbon sequence of inositol is conserved (87), $C_{(2)}$ becoming $C_{(5)}$ of the uronosyl residues.



Main features of metabolism of myo-inositol after Loewus et al. (86).

In an autoradiographic study on the germinating pollen grains of pear in a sucrose medium, Loewus and co-workers (88) have shown that incorporation of the label from myo-inositol-2-^{3}H into 70%-ethanol-insoluble material is localised in the cell wall of the pore region and pollen tube. Analysis of pectinase hydrolysates showed that virtually all of the label was in galacturonic acid and arabinose. These results were confirmed in later experiments with myo-inositol-2-^{14}C (89), in which it was also observed that with increasing period of germination the ratio of labelled pentose sugars (in Lilium longiflorum pollen grains) relative to labelled galacturonic acid increases.

Conditions for the extraction and purification of polysaccharides must be carefully controlled in order that the minimum amount of structural modification consistent with

a high yield and degree of purity of the product is attained. The graded extraction of mustard cotyledons has been described (27). Oligosaccharides and sugars are extracted with ethanol and fats with acetone. Cell wall polysaccharides are not removed by such procedures. Most of the pectic substances are extracted with hot aqueous EDTA and the remainder are then extractable with hot water. Hemicelluloses are extracted from the residue with alkali, leaving cellulose.

The pectic substances are particularly susceptible to side-reactions during extraction. Alkaline conditions provide efficient methods of extracting these polysaccharides but cause de-esterification and degradation by β-elimination and other reactions (66). β-Elimination can also occur in hot neutral solution (90). Glycosidic hydrolysis of furanoside linkages can occur in hot solutions if the pH is less than 3. Solvents of pH approximately 5 would therefore seem to involve least risk of modification of the native pectic substances but it is likely that extraction would be incomplete at this pH with EDTA. It has recently been shown (60) that a method involving the use of a mixture of EDTA and sodium lauryl sulphate at pH 5.0 is extremely effective, but there are some indications that the material extracted is modified in some way which is not attributable to the pH.

Alkaline extraction of hemicelluloses can result in degradation by a peeling reaction from the reducing end

of chains of 1,3 or 1,4 linked sugar units (91). Saponification of acetyl groups known to be present in some polysaccharides is also involved, although a mild procedure involving extraction of acetylated xylans from wood with dimethyl sulphoxide is reported (92). Although not relevant to such young tissue as mustard cotyledons, the side-reactions involved in delignification of wood prior to the extraction of hemicellulosic polysaccharides may result in oxidation, depolymerisation and contamination with degraded cellulose.

The work described in this chapter concerns the incorporation of myo-inositol-2-^{3}H into germinating mustard cotyledons. A graded extraction of germinated cotyledons established the distribution of label between the various fractions and the composition of the newly synthesised pectic substances and EDTA-insoluble material was determined after hydrolysis and separation of the sugars by paper chromatography. The location of the 70% ethanol-insoluble labelled material within the cell was determined by autoradiography of tissue sections.

Experimental

Experiment 2.1. Germination of mustard seeds supplied with myo-inositol-2-^{3}H and separation of the cotyledons.

Mustard seeds (300) were added to a solution of myo-inositol-2-^{3}H (0.10 mc, approx. 2.2 x 10^{18} cpm, in 0.80 ml water; specific activity 0.115 mc per mg) and allowed to stand with occasional shaking to ensure even soaking of the seeds. After complete absorption (2 hr), more water (0.80 ml) was added and the seeds were soaked for a further 4 hr. The apparatus used in the germination of mustard seeds was identical to that described elsewhere (34). After germination in the dark for 4 days the cotyledons and part of the hypocotyls were removed, frozen in liquid nitrogen and freeze-dried. The cotyledons were separated from the hypocotyls by hand sorting. Yield, 1.4g.

Experiment 2.2. Distribution of radioactivity in the fractions obtained by graded extraction of the cotyledons.

The cotyledons (1.4g) were extracted in the Soxhlet with aqueous ethanol (80%) (Count A) and subsequently with acetone (Count B).

The residue (0.9g) was extracted exhaustively with EDTA (disodium salt, 2% w/v, 10 x 50 ml for 3 hr each time) at 100° and pH 7.5. The extracts were combined and concentrated to 500 ml, and a sample (25 ml) removed

(Count C). The remainder was dialysed, readjusted to 475 ml and two samples (25 ml each) were removed. To one was added EDTA (6% w/v; Count D); no addition was made to the other (Count E). The remainder was deproteinised with hot 45% phenol as described in General Methods (3 cycles), dialysed, and concentrated to 425 ml. A precipitate appeared at this stage and was redissolved by the addition of EDTA (2% w/v) followed by heating at 100° for 15 minutes. The hot solution was filtered and the small amount of insoluble material dried (Count F).

Table 1. Incorporation of radioactivity into fractions of mustard cotyledons.

Solvent	Components	Count No.	Q.F.	Activity cpm x 10^{-5}	Activity %	Yield % by weight
80% ethanol	Sugars, etc.	A	1.19	26.20	57.4	23
Acetone	Fats	B	1.06	0.01	0.1	11
2% EDTA	Pectic substances, dialysable substances, protein	C	2.62	17.80	38.9	47
	Pectic substances, protein; + EDTA	D	1.02	5.65	-	-
	Pectic substances, protein; no EDTA	E	1.10	7.27	-	-
	?	F	1.00	0.00		2.5
	Pectic Substances	G	1.00	5.70	12.5	24
Residue	Cellulose, hemicelluloses, protein	H	1.00	1.66	3.6	16.5

Background count = 33 cpm. Counting time = 2 x 100 minutes per sample

The filtrate was redialysed, made to 425 ml, and a sample (25 ml) removed (Count G). The remainder was concentrated and freeze-dried. Yield, 0.27g; corrected for removal of 4 x 25 ml samples, 0.34g.

The residue after extraction of the cotyledons with EDTA was freeze-dried. Yield, 0.23g (Count H).

Preparation of samples for counting:

Accurately measured volumes (10 or 25 μl) of solutions (Count A in ethanol, Count B in acetone, Counts C, D, E, G in water or aqueous EDTA) or homogenates (Counts F and H, in water), of duplicate samples of known concentration were evaporated to dryness on strips of cellulose acetate (1.5 cm x 5.0 cm).

Counting of samples:

The strips were immersed in liquid scintillator for counting as described in General Methods. The background count was determined with a cellulose acetate blank and automatically subtracted from subsequent counts. The true activity of the sample was obtained after correction for quenching. The extent to which quenching was effecting the measured activity (the quenching factor) was obtained by 'spiking' the sample with myo-inositol-2-^{3}H, 720 cpm, and is given by

$$Q.F. = \frac{\text{Spike count (720 cpm) + Sample count (cpm)}}{\text{[Spike + sample] count (cpm)}}$$

The true activity was calculated by multiplication of the observed activity by the quenching factor. The results are shown in Table 1.

Experiment 2.3. Distribution of radioactivity in the component sugars of the EDTA-soluble and EDTA-insoluble fractions of the cotyledons.

The deproteinised freeze-dried EDTA-soluble material (Experiment 2.2.) (50 mg) was hydrolysed in sulphuric acid (1 N, 10 ml) at 100° for 16 hr. The residue after EDTA extraction (50 mg) was dissolved by shaking overnight in sulphuric acid (72% w/v, 1 ml) at room temperature; after dilution with water (9 ml), the solution was heated at 100° for 6 hr. Each hydrolysate was neutralised with barium carbonate, chilled, filtered and evaporated to dryness. The residue was taken up in water (1 ml) for paper chromatography. Each solution was spotted in duplicate on the chromatogram which was developed in solvent A, marked off in 1 cm strips on the longitudinal axis and cut in half between the two spots. One half was sprayed (reagent a) to locate the sugars; the other was cut into 1 cm strips numbered from the origin, which were assayed for radioactivity in the liquid scintillation counter (General Methods). The background count was determined with a blank strip of chromatography paper (1 cm x 6 cm) and subtracted automatically from subsequent counts. The results are given

in Table 2 (EDTA-soluble fraction) and Table 3 (EDTA-insoluble fraction).

Table 2. Radioactivity in the component sugars of the EDTA-soluble fraction of mustard cotyledons.

Component	Activity		Relative concentration by spray reagent (a)
	cpm	%	
Oligosaccharides	436	65.4	inestimable
Galacturonic acid	92	13.8	+++
Galactose	11	1.6	+++
Glucose	8	1.2	+
Arabinose	97	14.5	+++++
Xylose	18	2.7	+++
Rhamnose	5	0.8	+

Background = 28.5 cpm (automatically subtracted)
Counting time = 3 x 10 minutes each sample

Table 3. Radioactivity in the component sugars of the EDTA-insoluble fraction of mustard cotyledons.

Component	Activity		Relative concentration by spray reagent (a)
	cpm	%	
Oligosaccharides	29	19	-
Galacturonic acid	29	19	++
Galactose	12	8	+
Glucose	31	21	++++
Arabinose	19	12.5	trace
Xylose	31	21	+

Background = 28.5 cpm (automatically subtracted)
Counting time = 1 x 100 minutes each sample

Experiment 2.4. Autoradiography of the cotyledons

Mustard seeds (10) were supplied with a solution of myo-inositol-2-^{3}H and germinated as described in Experiment 2.1.

The cotyledons were removed and washed with a solution of myo-inositol (2% w/v, 50 ml) at 0° for 1 hr, followed by a solution of formaldehyde (4%, 3 x 30 ml for 30 minutes each time) and then in myo-inositol (2%, 50 ml) overnight. The cotyledons were thoroughly washed with water and then taken through a series of aqueous ethanol solutions (ethanol concentration increasing from 0 to 70% v/v in 10% intervals, 50 ml for 30 minutes each time) and finally stored in aqueous ethanol (70%, 10 ml).

Authoradiographs of sections of the cotyledons were kindly prepared by Dr. M.M. Yeoman. The method employed is described below.

The cotyledons were embedded in wax and thin sections (a few cells thick) cut from them. The wax was gradually dissolved from the sections which were finally washed with water. Slides were prepared to take the sections as follows: the slide was degreased in chromic acid before dipping in an aqueous solution of gelatin (5g per 1) and chrome alum (0.5g per 1). The slide was drained and dried such that an even layer was produced and the section mounted on the prepared slide. Subsequent stages were carried out in the dark-room under a Wratten series 1 (red)

filter. The nuclear emulsion (Kodak K2) was melted and an equal volume of water was added. The solution was thoroughly mixed and kept at 55°. The slide was dipped in the emulsion for 30 sec, removed and drained in an upright position, and allowed to dry very slowly. The slide was then stored, in a sealed box containing silica gel, at 4° for 14 days. After development (Kodak D19) and fixing, the slide was washed and dried.

The autoradiographs were examined microscopically (Leitz 'Orthoplan' system); some of the microphotographs obtained are shown in the Discussion to this chapter.

Discussion

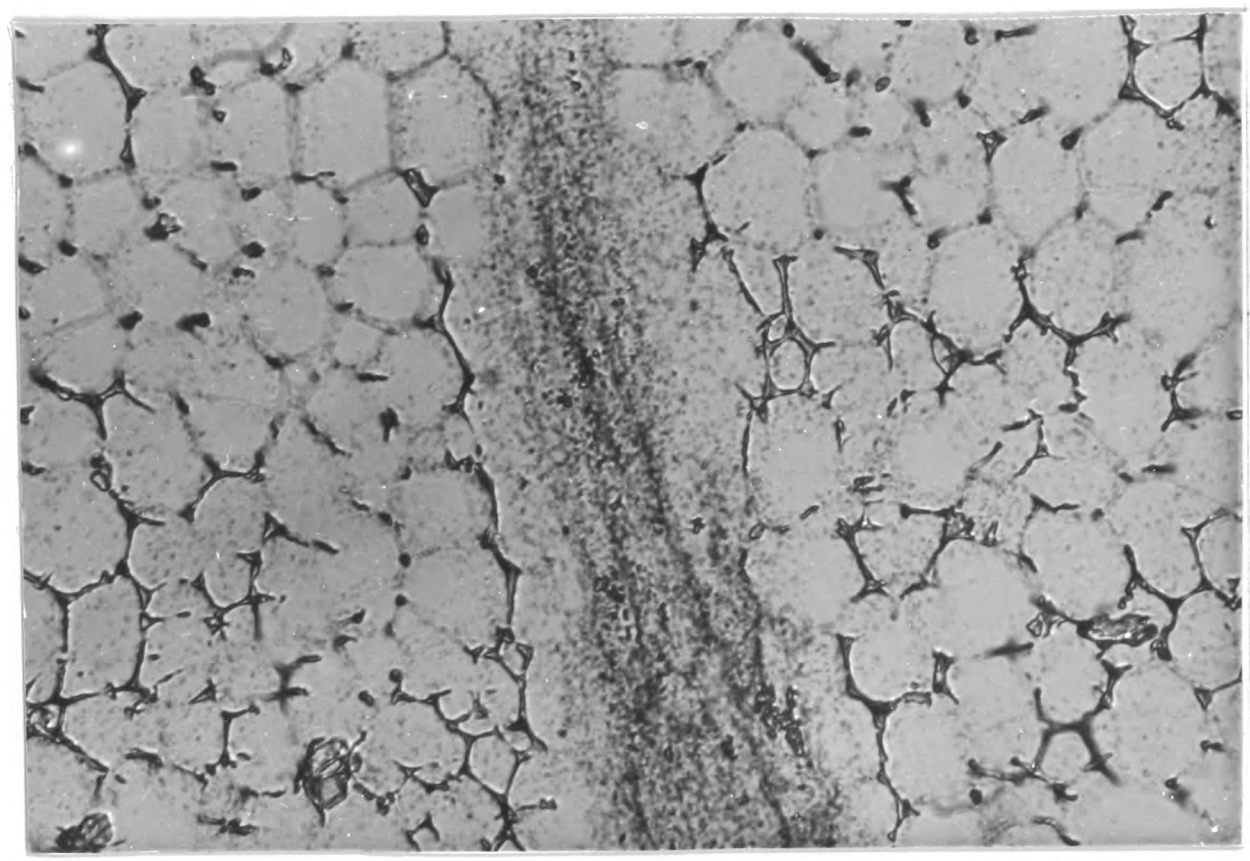
Examination of the results of Experiment 2.2 (Table 1) shows that a large proportion (57%) of the total activity was contained in the 80% ethanol-soluble fraction, presumably mainly in unutilised myo-inositol. Fats were unlabelled. Of the activity recovered in the 80% ethanol-insoluble residue, 91.5% was contained in the EDTA-soluble fraction and 8.5% in the EDTA-insoluble residue. Dialysable substances accounted for a considerable proportion (nearly 60%) of the activity in the EDTA-soluble fraction. Low molecular weight carbohydrates which were not extracted by ethanol for accessibility reasons probably account for most of this (no break-up of the cotyledons occurred in ethanol), although polysaccharides of relatively low molecular weight

such as the araban, and degradation artefacts of the pectic substances will also contribute to the activity. Protein was unlabelled as shown by the small change in counts caused by deproteinisation. The pectic substances accounted for just over 40% of the activity in the EDTA-soluble fraction. In contrast hemicelluloses and cellulose were weakly labelled.

On hydrolysis and paper chromatography the dialysed, deproteinised EDTA-soluble fraction was shown (Table 2) to have the typical component sugars of pectic substances. A large proportion (nearly 80%) of the label was present in galacturonic acid and galacturonic acid-containing oligosaccharides which are resistant to hydrolysis; most of the remaining activity was contained in arabinose. Xylose was relatively weakly labelled and only traces of activity were found in the hexoses.

Hydrolysis and paper chromatography of the EDTA-insoluble reside (Table 3) showed glucose to be the major component, although other sugars derived from hemicelluloses and residual pectin were also present. A large proportion (over 50%) of the label in this fraction was contained in the component sugars of the residual pectin (oligosaccharides, galacturonic acid and arabinose); the remainder was present in xylose, glucose and galactose - typical sugars of the hemicellulose and cellulose fraction.

The autoradiographs of the cotyledons (Experiment 2.4. and Figures 1a and 1b) show that the 70% ethanol-



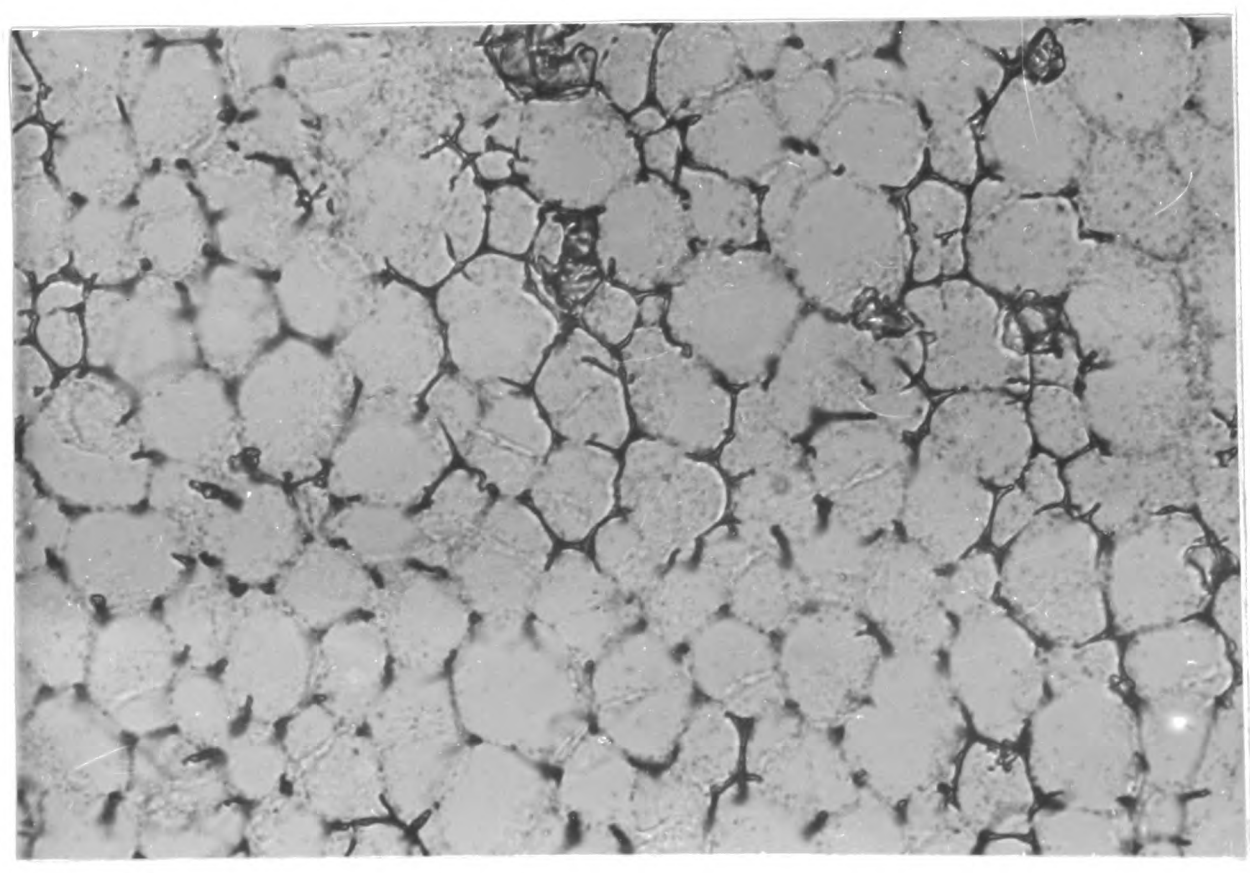


Figure 1. Autoradiographs of white mustard cotyledons (x 40)

Below : Figure 1a, parenchyma

Above : Figure 1b, as in 1a but showing vascular tissue

insoluble label is contained in the region of the cell wall and middle lamella (as shown by developed silver grains in the nuclear emulsion), but resolution is not high enough to show whether the middle lamella is labelled, although there are indications that it is not. The cytoplasm is virtually unlabelled but it is interesting to note (Figure 1b) that the vascular tissue is labelled.

The conclusions to be reached from the above observations are :

(i) Myo-inositol-2-^{3}H is incorporated mainly into the pectic substances of mustard cotyledons rather than other cell-wall polysaccharides.

(ii) The incorporation of label within the pectic substances is mainly into galacturonic acid and arabinose (and to a lesser extent xylose); very little label is incorporated into hexose components. The newly-synthesised pectin has a ratio of galacturonic acid : arabinose : xylose of 1.00 : 1.05 : 0.20 compared with that of 1.00 : 3.25 : 0.77 present in the pectin of non-germinated cotyledons (derived from the results of methylation analysis). The reduction in neutral sugars signifies a dramatic decrease in the degree of branching of the pectin synthesised during germination, despite the fact that galacturonic acid is underestimated due to incomplete liberation during hydrolysis.

(iii) The newly synthesised pectin is located in the cell wall. The vascular tissue is also the site of 70% ethanol-insoluble labelled material. This could be due to

incomplete extraction of 70% ethanol-soluble substances from this tissue, but it is more likely that it reflects the synthesis of considerable amounts of new cell wall material as a result of differentiation.

CHAPTER 3

Smith Degradation of an araban component of the pectic substances from mustard cotyledons

Introduction

Many of the existing methods of polysaccharide structural analysis involve controlled fragmentation of the polymer. These procedures entail selective cleavage of one linkage rather than another, followed by characterisation of the products. The elucidation of the branching pattern of amylopectin by Peat et al. (93) using specific enzymes is a classical example of the highly controlled breakdown of a polysaccharide. Unfortunately direct chemical methods are much less specific but, as the selection of pure specific enzymes is somewhat limited, these are frequently the only methods available. β-Elimination of uronic acid-containing polysaccharides has been employed (21,94) but elimination reactions are not yet in such general use as solvolysis procedures. The reverse specificities of aqueous acid hydrolysis and acetolysis has been employed in determination of the structure of some dextrans (95). Chemical modification of sugar units can be used to alter the cleavage pattern of the polysaccharide either by stabilising a particular linkage (some such methods, and one in particular, are described in the following Chapter) or by rendering it extremely labile.

A powerful approach to the characterisation of the branching pattern in many polysaccharides involves the use of periodate degradation methods; lability at the oxidised unit enabling controlled breakdown under mild conditions. Other structural features may also be detected by this

procedure. In the Barry Degradation the periodate oxidised polysaccharide is treated with phenylhydrazine. The main drawback is that the work-up is complicated and the osazone products are difficult to handle. Treatment of the oxidised polysaccharide with sodium borohydride followed by mild acid hydrolysis (the Smith Degradation) is more powerful because the reaction is exceptionally clean and the products relatively easy to characterise. The nature and relative yields of the Smith Degradation products, coupled with the results of methylation and other studies often enables a considerable narrowing down of the possible structures of a polysaccharide. In favourable cases where experiment has determined the nature of the branching pattern, the distribution of the side-chains (i.e. regular, block or random) on the main chain may be elucidated. Aspinall and Ross (96) showed (by methylation, enzymic hydrolysis and other experiments) that rye flour arabinoxylan has a β-1,4 xylan main chain to which single arabinose side-chains are attached through the $C_{(3)}$ position. The isolation of 2-O-β-D-xylopyranosylglycerol; O-β-D-xylopyranosyl-(1→4)--O-β-D-xylopyranosyl-(1→2)-glycerol, and O-β-D-xylopyranosyl--(1→4)-O-β-D-xylopyranosyl- (1-4)-O-β-D-xylopyranosyl-(1→2)--glycerol in the ratio of 7.5 : 2.2 : 1 but no products of higher molecular weight, showed that the distribution of side-chains is rather random and that no more than 3 adjacent xylose units are ever branch points. The estimation of Smith Degradation products in the above study was by elution

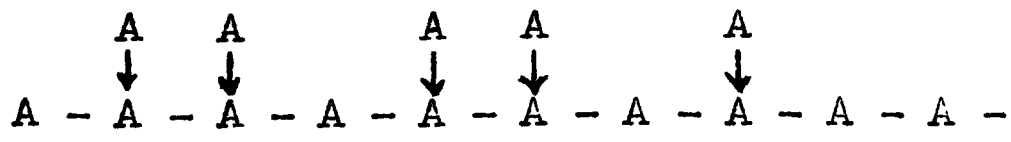
from paper chromatograms followed by determination of carbohydrate content with the phenol-sulphuric acid reagents. The accuracy of this procedure is probably low, due to incomplete elution and possible contamination with cellulose from the paper. Glycerol cannot be estimated by the above method. Estimation by weighing involves a large amount of starting material with a subsequent lengthy separation of the products. This chapter describes a method whereby accurate ratios of Smith Degradation products may conveniently be obtained on a micro scale, with particular reference to an araban component of the pectic substances of mustard cotyledons (from both germinated and non-germinated seeds).

The araban is readily isolated because it is not precipitated with 70% ethanol from the EDTA extract as are the other pectic substances. Addition of a large excess of acetone or butan-2-one to the ethanol filtrate precipitates the araban in a crude state, which is then purified by precipitation from alkaline solution with cationic detergents (27,97). The product has an anhydroarabinose content of 98% or better. Substantial evidence has been put forward (27,28,98) in support of the view that the polysaccharide is a genuine homoaraban rather than a β-elimination artifact of pectin as may have been the case with several arabans isolated from other sources. The araban may well be metabolically related to pectin in some way.

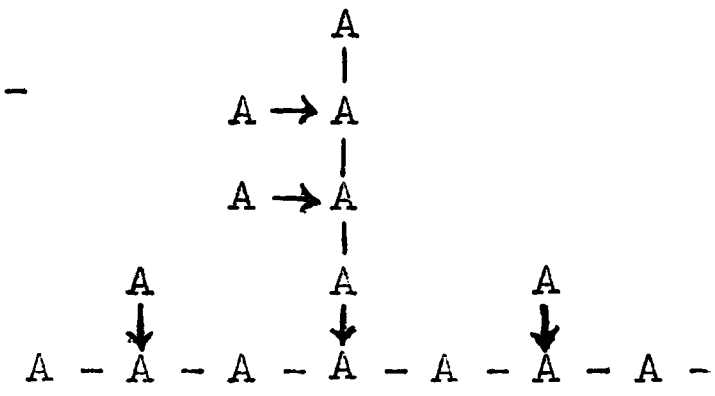
The araban has been shown to be homogeneous in the ultracentrifuge and on free solution electrophoresis in

borate, and arabans extracted at different pH values, extracted from different batches of non-germinated seeds and extracted from cotyledons after germination are identical in these respects (99). The molecular weight given by ultracentrifugation and vapour pressure osmometry (on the methylated polysaccharide) is about 6,000 corresponding to a molecule containing approximately 45 anhydroarabinose units. Other studies by Rees and Richardson (28,98) have established the principal structural features of the araban. The negative rotation (-180°) and the ease of hydrolysis indicate that the polymer is composed of α-linked arabinofuranose units. The configuration of the linkages is confirmed by enzymic hydrolysis with an α-arabinosidase (100). Sugars other than arabinose were not detectable by various methods and the UV spectrum showed no peak at 235 nm due to unsaturated 4,5-uronic acid (as would be present in a β-elimination artifact). Methylation analysis has established the main structural features of the araban; the pattern of methylated sugars is closely related to that obtained from mustard cotyledon pectin (see Chapter 1), namely, 2,3,5-tri-O-methyl-L-arabinose, 2,3-di-O-methyl-L--arabinose, 2-O-methyl-L-arabinose and L-arabinose. Trace amounts of 2,5-di-O-methylarabinose and a second monomethyl ether (probably 3-O-methylarabinose by analogy with pectin) were also detected. The furanose ring form is thus confirmed for end-group at least. Arabinose was only present in small amounts; the molecule is thus joined by

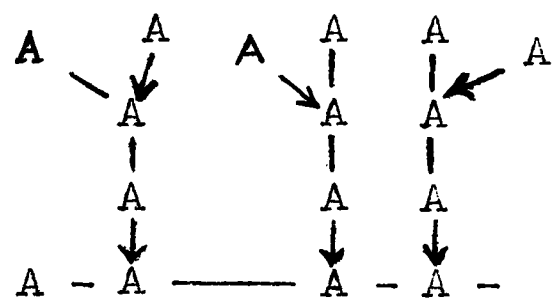
1,5 linkages with a considerable proportion of 1,3 branch linkages and a further small proportion of units branched through both the $C_{(2)}$ and $C_{(3)}$ positions. Further discrimination between the possible structures which may be proposed on the basis of methylation analysis was made from the results of quantitative g.l.c. of the methylated araban and the methylated Smith Degraded araban. The gas chromatograph detector response was calibrated with crystalline compounds and the hydrolysis products of methylated araban and the methylated degraded araban chromatographed as the arabitol acetate derivatives. The absolute proportion of the structural units was thus determined. The results from the methylated araban showed that, in whole numbers, the araban molecule contains 16 end units, 11 chain units and 15 branch units, when the small proportion of 1,2 linkages and the end units associated with them are ignored. No 2-O-methylarabinose or 2,5-di-O-methylarabinose was detected (as the alditol acetate derivatives) among the methylation products of the degraded araban and thus the araban, unlike beet araban (67), contains no contiguous 1,3 linkages. It was also shown that most of the oxidisable units (i.e. end units or chain units) occur in nonadjacent positions so that a structure having a substantial proportion of a linear component may be excluded. Even this considerable evidence does not lead to a unique structure for the araban. Remaining possible structures are given below (after Rees and Richardson, 28), although hybrids of these are also possible:



'comb' or
'herring bone' I



'tree' II



'bush' III

where —— denotes 1,5 linkage

⟶ denotes 1,3 linkage

A denotes arabinofuranosyl residue

The distribution of branch points within the molecule may be determined by a Smith Degradation in which the ratio of the products is accurately determined. Diagram 1 describes the products which would be obtained from the araban and the structural units from which they derive. The substituted glycerol derivatives obtained would thus be expected to be : 1-O-α-L-arabinofuranosylglycerol; O-α-L-arabinofuranosyl-(1→5)-O-α-L-arabinofuranosyl-(1→1)--glycerol and higher homologues (if present). If arabinosylglycerol is the major product obtained then the branch units tend to occur in isolated positions, and similarly, if only higher homologues are observed, then the

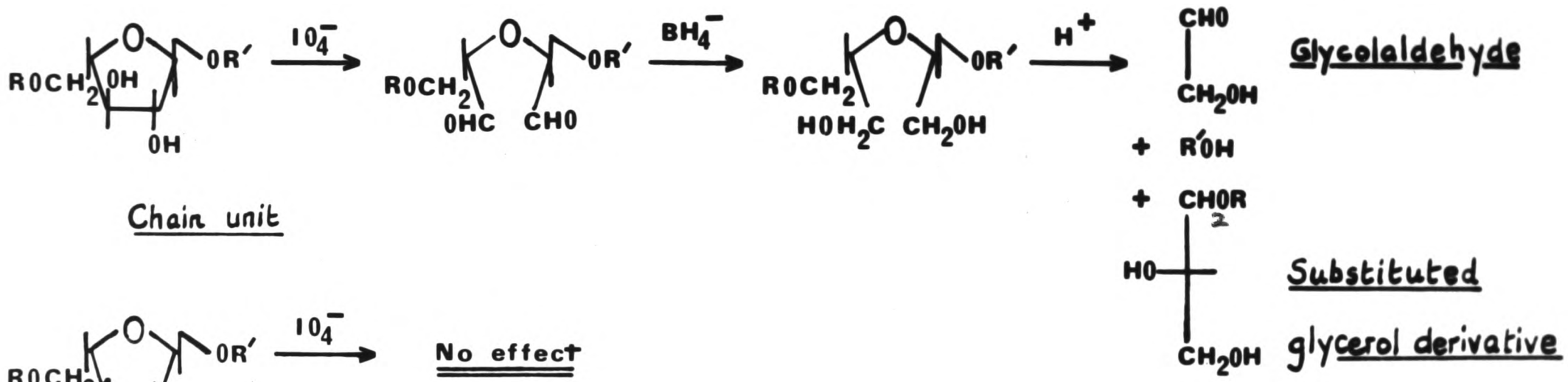


Diagram 1. Smith Degradation of Araban.

branch units occur in contiguous positions. Patterns of products between these extremes would represent a more randomly branched structure. For a structure such as I ('Herring Bone') the yields of Smith Degradation products from a randomly branched molecule can be predicted statistically. The 'randomness' of the branching pattern of the araban may thus be assessed if it is assumed that the structure approximates to I.

Periodate oxidation of the arabans from germinated and non-germinated mustard cotyledons was followed by reduction with sodium borotritiide and mild hydrolysis. The yields of the labelled products, separated by paper chromatography, was calculated from the activities obtained by liquid scintillation counting.

Experimental

Experiment 3.1. Extraction and purification of araban from germinated cotyledons.

The pectic substances were extracted from the defatted cotyledons (137g) of germinated seeds with an aqueous solution of EDTA (2%) and sodium lauryl sulphate (0.2M) at 100°, and precipitated at 70% ethanol concentration. The crude araban was precipitated as an oil by the addition of butan-2-one (15 volumes) to the supernatant and was isolated by decantation. The product was reprecipitated twice by dissolving in water and adding butan-2-one. The final precipitate was a solid which was removed on the centrifuge, dissolved in water and freeze-dried. Yield, 2.65g.

Hydrolysis and paper chromatography (solvent A) showed that, in addition to arabinose, traces of glucose and galactose were present.

The crude material was purified by precipitation with cetyltrimethylammonium hydroxide. An aqueous solution (10%) of cetyltrimethylammonium bromide was converted to the hydroxide by passing through a column of Amberlite 1RA-400 (OH^-) resin and part of this solution (20 ml) was added to the crude araban (2.5g) in water (300 ml). On slow addition of sodium hydroxide (1 $\underline{N}$, 20 ml) a flocculent white precipitate was formed which was removed after 1 hr by

centrifugation. The precipitate was dissolved in water (300 ml) containing enough acetic acid to give a neutral solution, thenreprecipitated with alkali. This procedure was repeated once. After redissolution in dilute acetic acid, the detergent was removed by precipitation with excess potassium iodide solution followed by centrifugation at 0°. The supernatant solution was filtered and passed through a mixed bed ion exchange column (IR 120 H^{+} and IR 45 OH^{-} resins). Tests showed that the effluent was free from iodide (silver nitrate test) and potassium ions (flame test). The solution was concentrated and freeze-dried. Yield, 0.120g. Found, 100% anhydroarabinose (hydrolysis and paper chromatography, and quantitative phenol-sulphuric acid determination), $[\alpha]_D$ - 177° (c 0.66 in water). In the ultracentrifuge (240,000g) the araban was identical with samples isolated before (60).

Experiment 3.2. Periodate oxidation of the arabans extracted from germinated and non-germinated cotyledons.

The araban from non-germinated cotyledons was prepared by Dr. N. Richardson (98). The polysaccharides were dried exhaustively in vacuo at 60°.

Equal volumes (10.0 ml) of sodium periodate solution (0.080 M) and each polysaccharide (0.050 M with respect to anhydroarabinose units) were mixed and left in the dark at room temperature. The reduction of periodate was followed spectrophotometrically (15a) until the reaction was

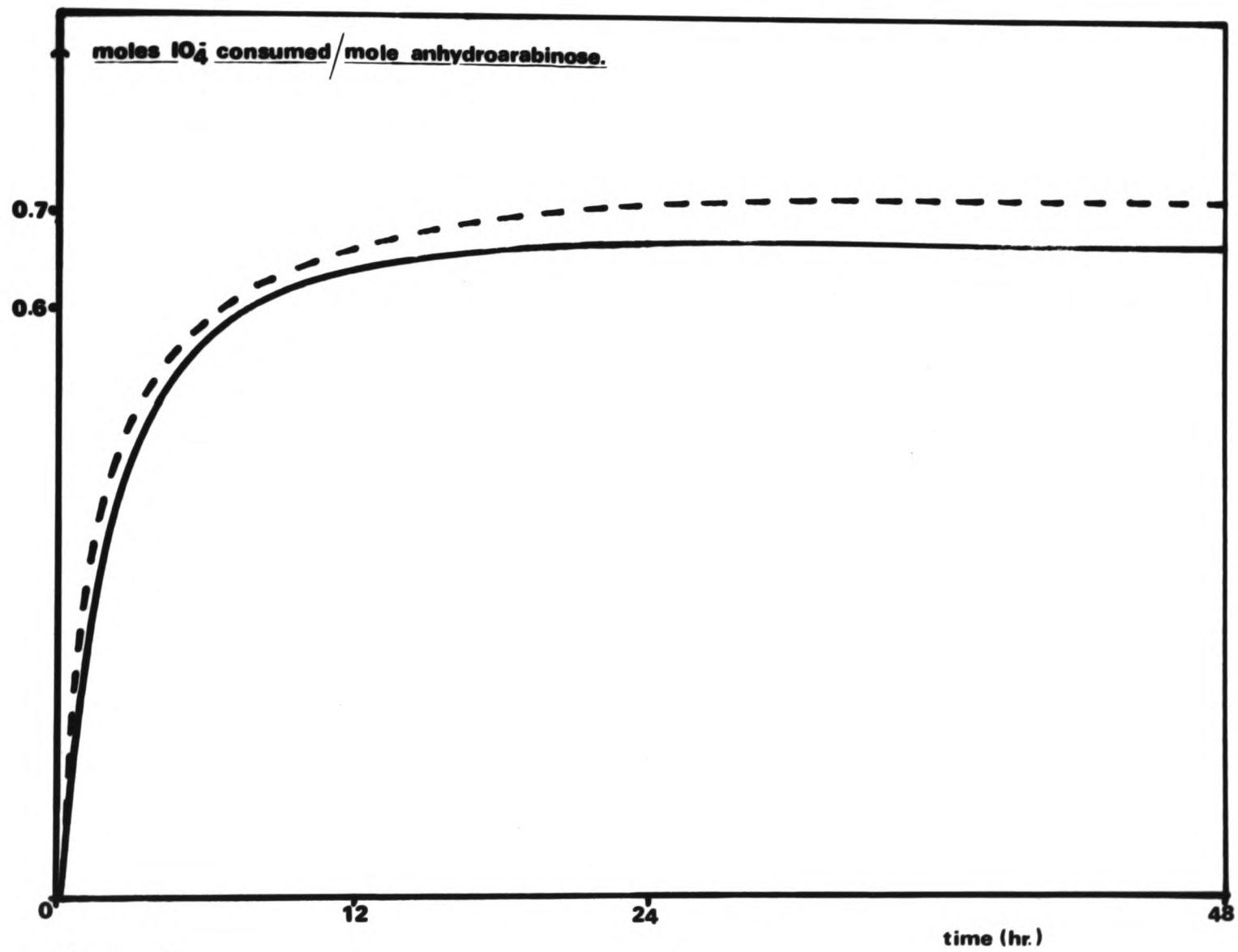


diagram 1.

Periodate uptake of arabans.

nongerminated.

germinated.

complete. The final values correspond to the consumption of 0.67 and 0.71 moles of periodate per mole anhydroarabinose for the 'non-germinated' and 'germinated' arabans respectively (see Diagram 1). The residual periodate was destroyed with a small excess of ethylene glycol.

Experiment 3.3. Smith Degradation of araban from non-germinated cotyledons.

Sodium borohydride (0.2g) was dissolved in water (20 ml) and left to stand overnight, then added to sodium borotritiide (0.2 - 0.5mg, specific activity 19 mc per mg) and the solution was added to the periodate oxidised araban. After 60 hr at room temperature the excess reagent was destroyed with the minimum volume of hydrochloric acid (1 $\underline{\underline{N}}$). The solution was passed through a mixed bed ion exchange column (IR 120 H^+ and IR 45 OH^- resins), evaporated to dryness under reduced pressure and boric acid removed as the volatile methyl borate by repeated distillation of methanol from the residue. The residue was dissolved in sulphuric acid (1.0 $\underline{\underline{N}}$, 8 ml) and left at room temperature. Samples (approx. 2 ml) were removed after 5 mins, 1 hr, 16 hr, and 24 hr; neutralised with barium carbonate, chilled and filtered. The filtrates were evaporated to dryness and redissolved in water (0.2 ml).

The products were examined by paper chromatography

in solvent C with glycerol and arabinose as controls. With reagent b (alkaline silver nitrate reagents) a series of spots was obtained from the 16 hr and 24 hr hydrolysates, but glycerol was the only detectable component in the 5 minute and 1 hr hydrolysates. The mobilities of the components and tentative assignments are given below.

Tentative identity	Rf
Glycolaldehyde	0.8 to 1.0
Glycerol	0.64
Arabinosylglycerol	0.52
Arabinobiosylglycerol together with arabinose	0.39
Arabinotriosylglycerol	0.28
Arabinotetraosylglycerol	0.20

With reagent c (triphenyltetrazolium hydroxide spray) free arabinose was detected in the 16 hr and 24 hr hydrolysates; it therefore follows that some non-specific hydrolysis had occurred under these conditions.

The hydrolysates were double spotted on chromatograms and developed in solvent C (Whatman no. 1 paper; 20 hr). The dried chromatograms were ruled off in numbered 1 cm strips perpendicular to the longtitudinal axis and cut in half between the two spots. One half was cut into strips and counted in the liquid scintillation counter as described in General Methods; the other was sprayed (reagent b) so as to correlate components with activities. A typical result

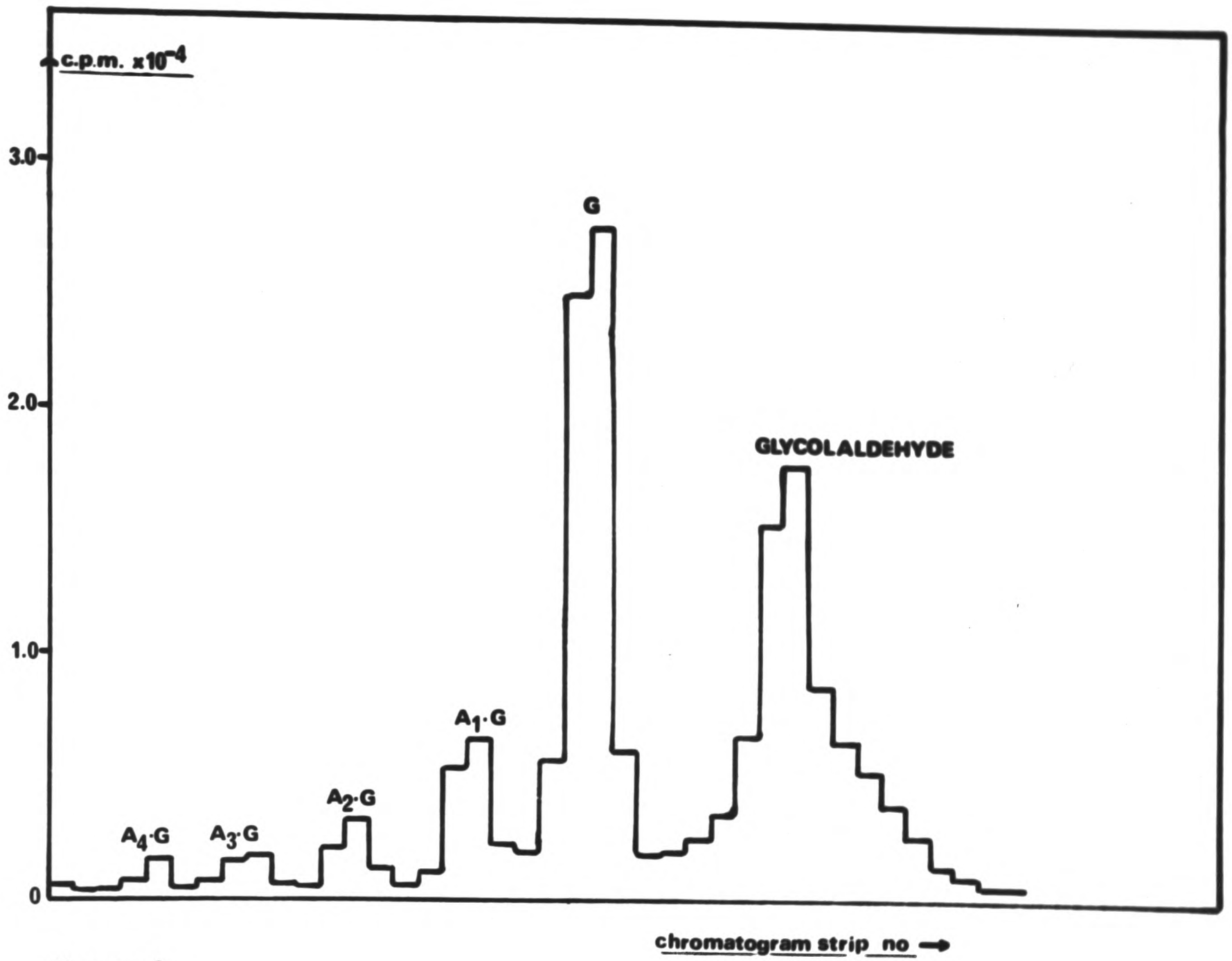


diagram 2.

Smith Degradation products of germinated araban (24 hr.)

is shown in Diagram 2. Nearly all of the activity in the 5 minute and 1 hour hydrolysates remained at the origin showing that hydrolysis was incomplete; negligible activity was present at the origin in the 16 hr and 24 hr hydrolysates. The activities of the components were obtained by summation of the relevant counts, the results for the 16 hr and 24 hr hydrolysates are given below.

Component	Activity of Component cpm x 10^{-3}	
	16 hr	24 hr
Glycolaldehyde	not measured	not measured
Glycerol (G)	117.39	83.66
Arabinosylglycerol (A_1G)	25.95	16.45
Arabinobiosylglycerol (A_2G)	12.71	8.77
Arabinotriosylglycerol (A_3G)	6.63	4.27
Arabinotetraosylglycerol (A_4G)	2.41	1.66
Total glycerol count (tG)	165.09	114.81

Counting time 1 x 50 min per sample.

These results are discussed fully later. As significant non-specific hydrolysis was occurring, the Smith Degradation of the 'germinated' araban was carried out at a wide range of hydrolysis times so that the extent to which this side-reaction was affecting the yields of the substituted glycerol products might be determined.

Experiment 3.4. Smith Degradation of araban from germinated cotyledons.

The periodate oxidised 'germinated' araban was reduced and dissolved in dilute sulphuric acid exactly as described above. Samples (approx. 1 ml) were removed from the hydrolysis after 3 hr, 6 hr, 12 hr, 24 hr, 48 hr, 72 hr, 96 hr and 192 hr and neutralised and prepared for paper chromatography (solvent C) as described above. With the alkaline silver nitrate reagents a similar pattern of spots to that observed before was obtained from all the hydrolysates except the 3 hr sample. Glycerol was virtually the only detectable product in this hydrolysate showing hydrolysis was incomplete after this time. The triphenyltetrazolium hydroxide reagent showed liberation of free arabinose after 12 hr, the amount of which increased with hydrolysis time. The activities of the components were obtained as before (Experiment 3.3). A large proportion of the activity of the 3 hr sample remained at the origin and the results for this are thus not significant.

Component	Activity of Component cpm x 10^{-3}						
	6 hr	12 hr	24 hr	48 hr	72 hr	96 hr	192 hr
Glycolaldehyde	not measured						
G	62.45	49.10	62.75	86.63	72.75	190.15	267.75
A_1G	10.05	9.60	13.15	18.75	15.35	33.45	40.67
A_2G	2.75	4.58	4.83	7.05	5.48	11.79	11.05

	6 hr	12 hr	24 hr	48 hr	72 hr	96 hr	192 hr
A_3G	1.65	2.71	2.20	2.35	1.83	5.70	8.61
A_4G	1.70	1.60	1.98	1.57	1.30	2.63	3.81
tG	78.60	67.59	84.91	116.35	96.71	243.72	331.89

Counting time 1 x 60 min per sample.

Theory

In the experiments described above the label is contained solely in glycerol and the glycerol moiety of the other degradation products (if glycolaldehyde is ignored). Hence the activities of these products accurately reflect their concentrations on a molar basis. It is stressed that the identities assigned to the components are at this stage tentative.

The following abbreviations are used : G = glycerol, A_1G = arabinosylglycerol, A_2G = arabinobiosylglycerol, A_3G = arabinotriosylglycerol, A_4G = arabinotetraosylglycerol, tG = total glycerol (i.e. free and combined) = $A_1G + A_2G + A_3G + A_4G + G$.

Correction for non-specific hydrolysis obviously requires values for the concentrations of the products at as many hydrolysis times as possible. The rate of this reaction was thus calculated for the 'germinated' araban only (as several hydrolysis times were employed) and used for both arabans on the assumption that the rate of non-specific hydrolysis would be similar.

(A) 'Germinated' araban

The values of the concentrations of the products (excluding glycolaldehyde), as measured by their activities (Experiment 3.4.), at different hydrolysis times are shown below :

Table 1.

Hydrolysis time (hr)	Ratio of activities					
	$\frac{G}{tG}$	$1 - \frac{G}{tG}$	$\frac{A_1G}{tG}$	$\frac{A_2G}{tG}$	$\frac{A_3G}{tG}$	$\frac{A_4G}{tG}$
6	0.795	0.205	0.128	0.0350	0.0210	0.0216
12	0.727	0.273	0.142	0.0676	0.0401	0.0237
24	0.739	0.261	0.155	0.0568	0.0259	0.0232
48	0.745	0.255	0.161	0.0605	0.0202	0.0135
72	0.753	0.247	0.159	0.0567	0.0189	0.0135
96	0.780	0.220	0.138	0.0485	0.0234	0.0104
192	0.819	0.181	0.125	0.0340	0.0168	0.0066

The variations of the concentrations of glycerol, arabinosylglycerol, arabinobiosylglycerol etc. with hydrolysis time are shown in Diagrams 3, 5, 6, 7 and 8 (solid lines). The general decrease in the concentrations of the substituted glycerol products and increase in free glycerol after extended periods of hydrolysis shows the significance of the non-specific effect. The best smooth curve through these points was extrapolated to zero time to give the following concentrations of the Smith Degradation products of the germinated araban as intercepts (expressed as fractions of the total glycerol):

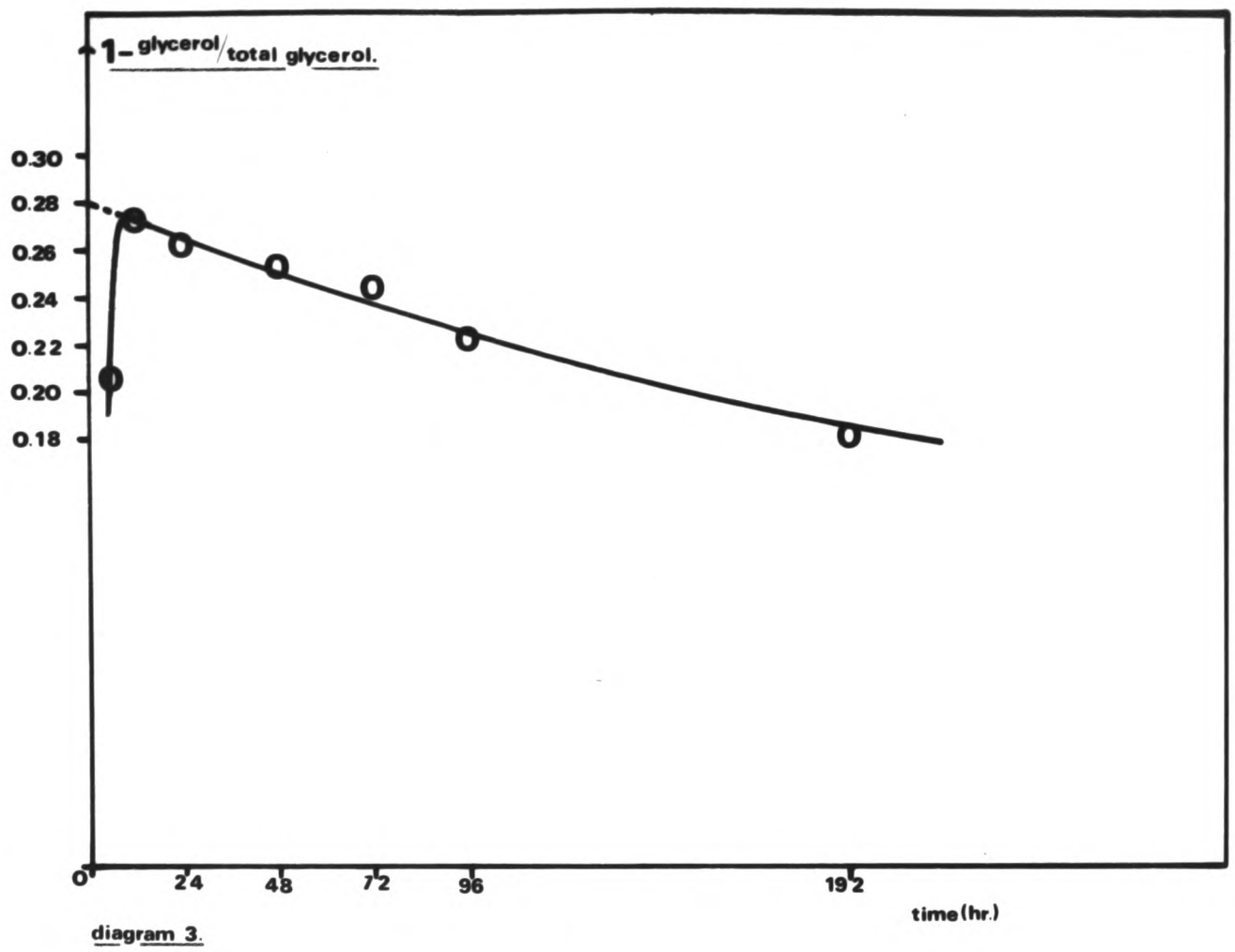


diagram 3.

1- glycerol / total glycerol v hydrolysis time.

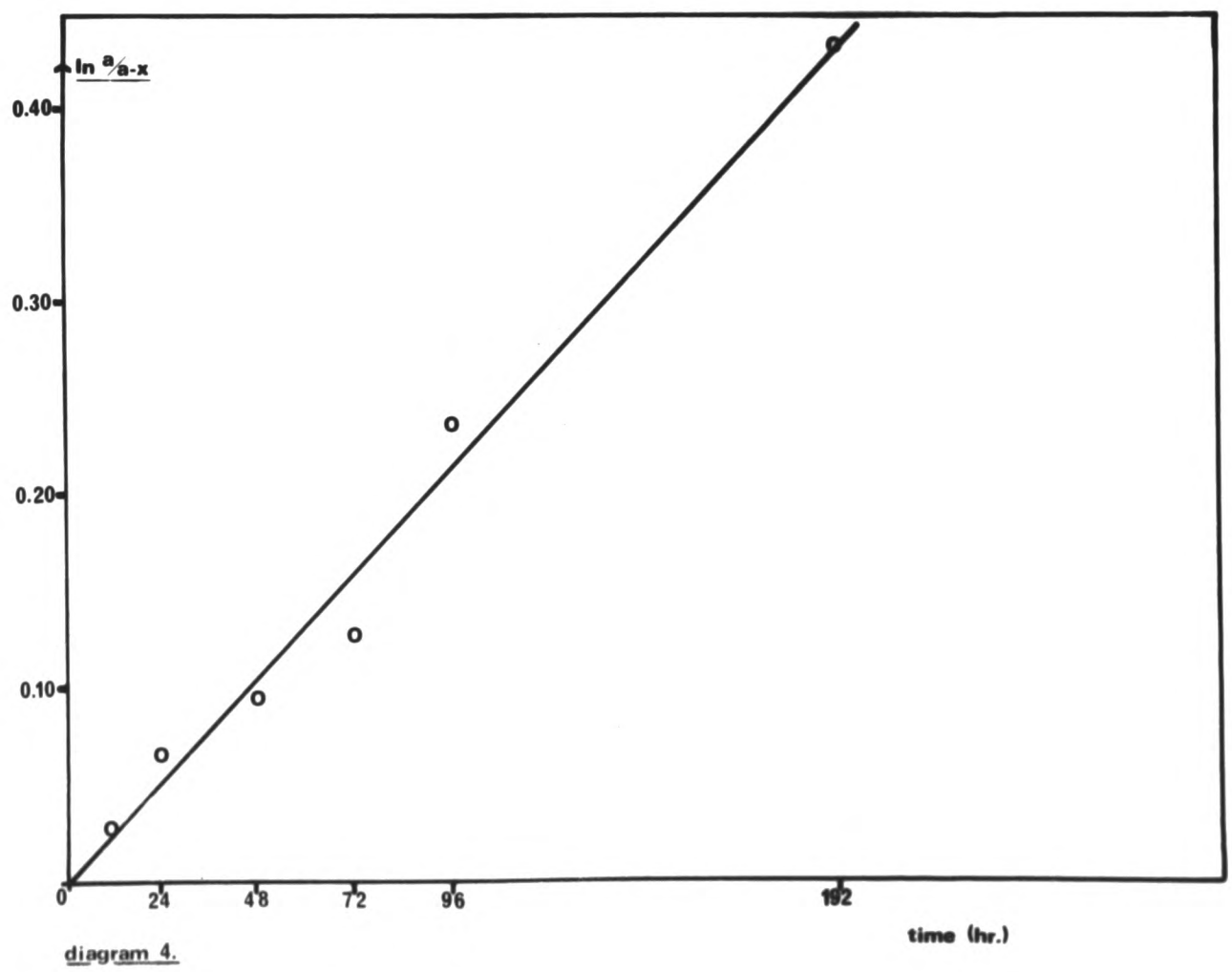


diagram 4.

First order plot of hydrolysis of arabinosylglycerol linkages.

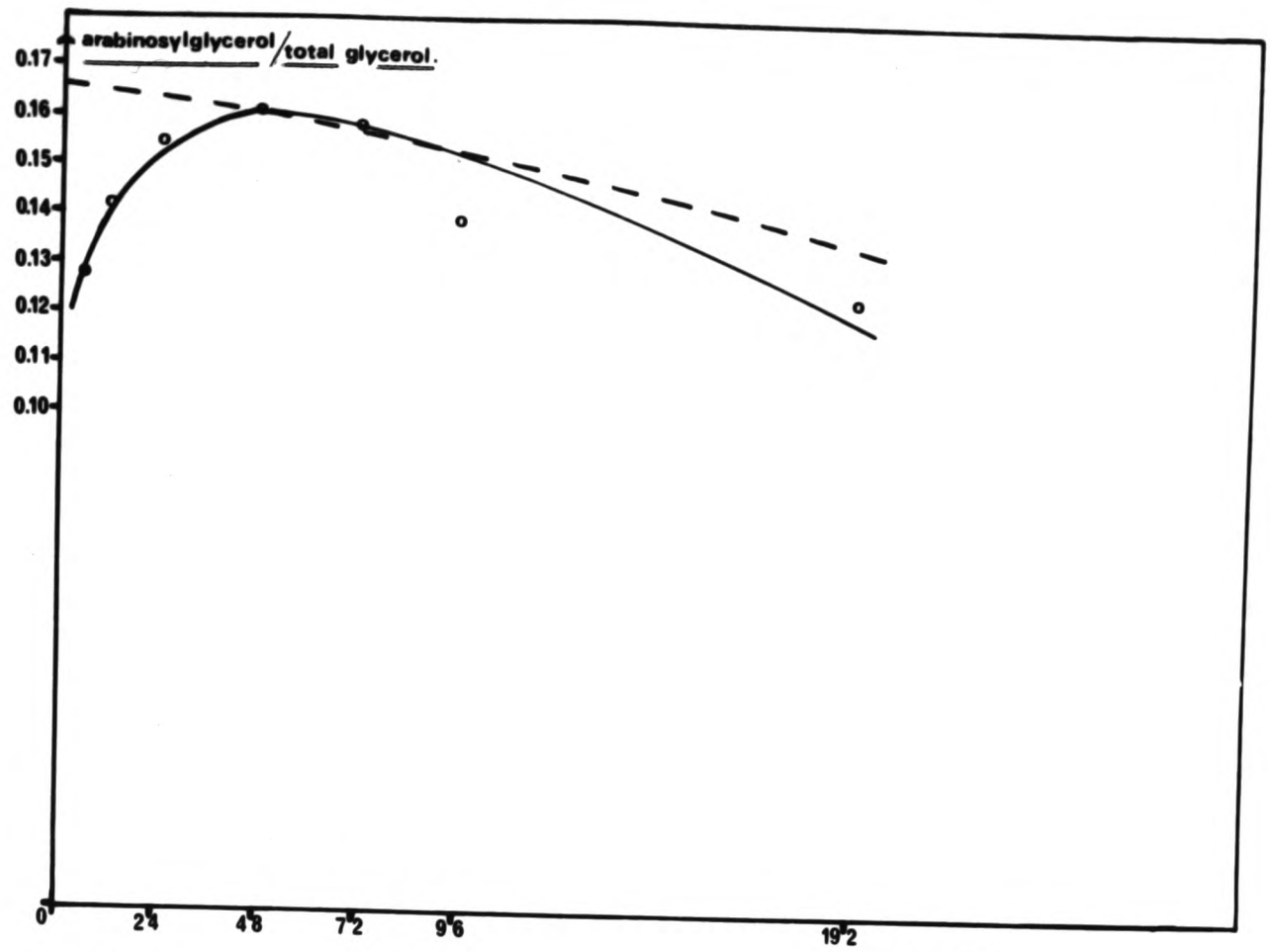


diagram 5.

Arabinosylglycerol concentration vs. time.

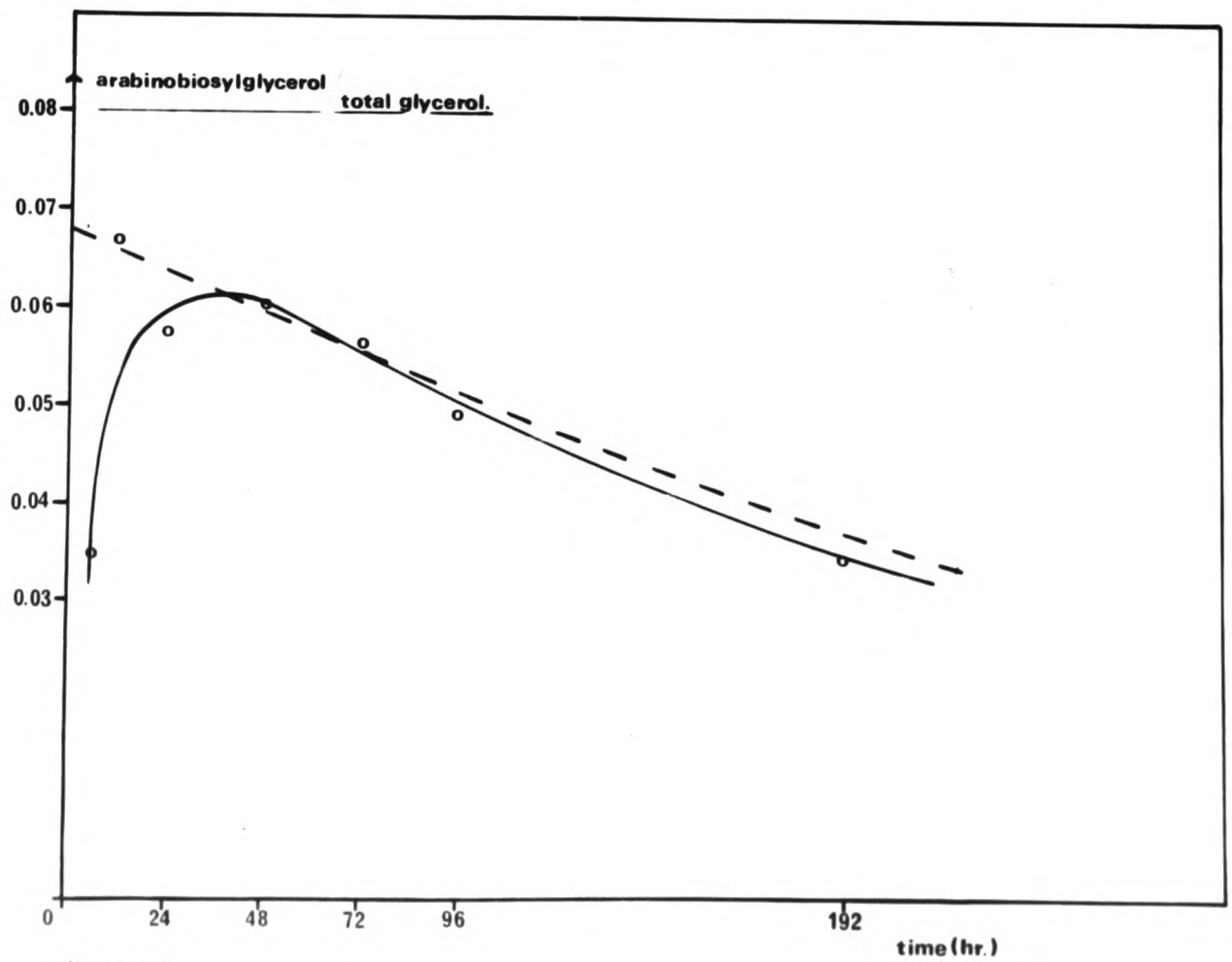


diagram 6

Arabinobiosylglycerol concentration vs. time.

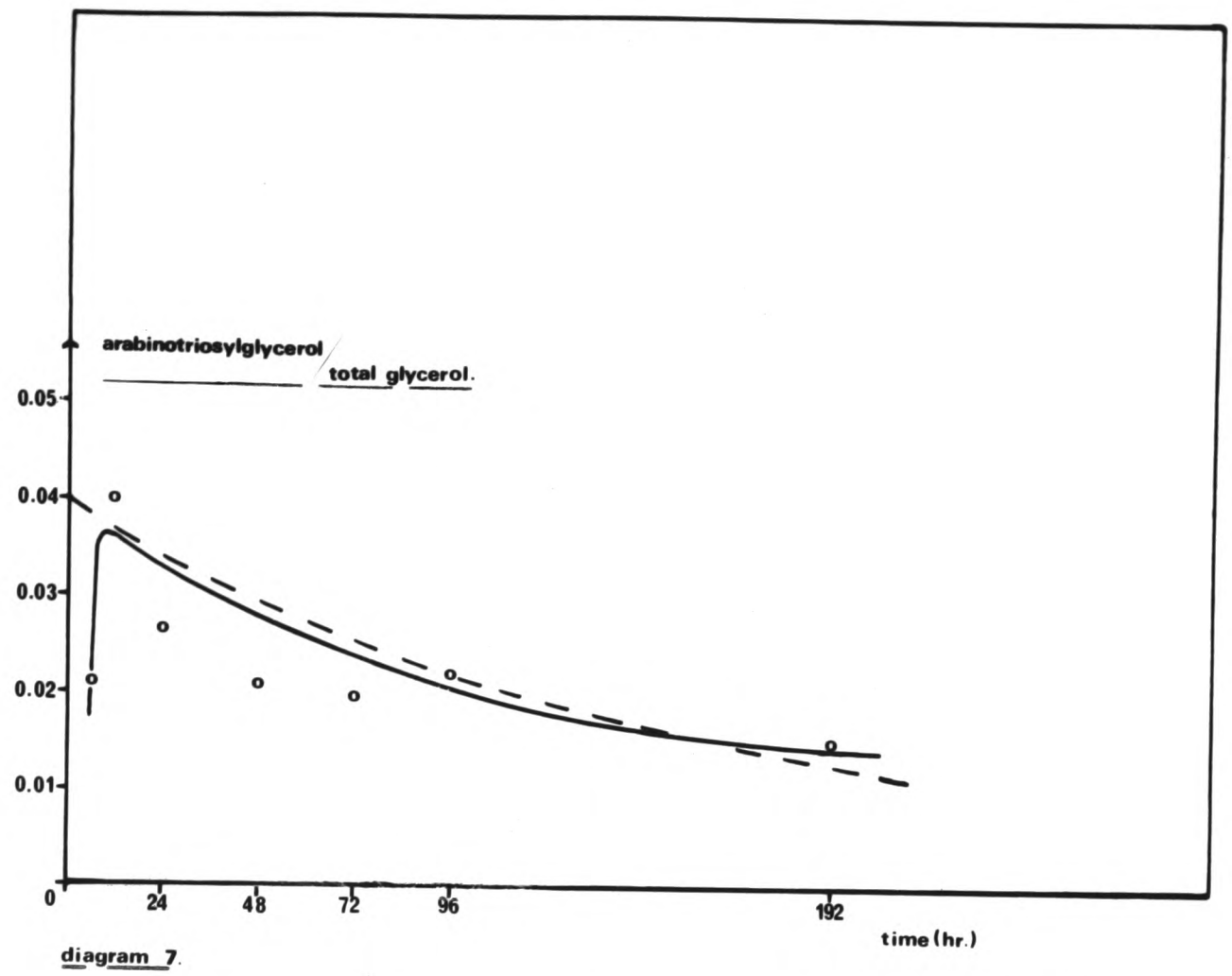


diagram 7.

Arabinotriosylglycerol concentration vs. time.

o—— experimental.
— — — calculated.

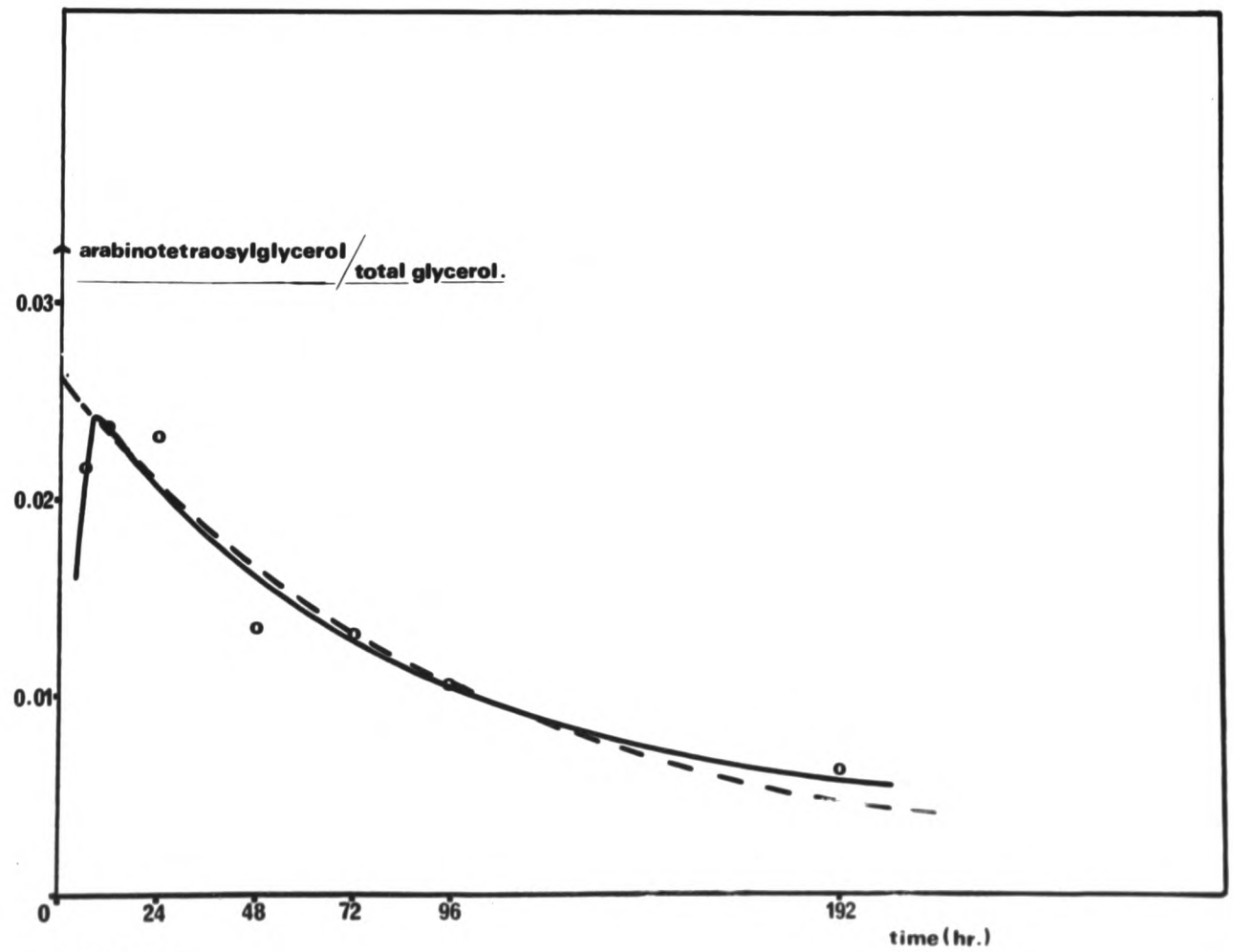


diagram 8.

Arabinotetraosylglycerol concentration vs. time.

o—— experimental
— — — - calculated

arabinosylglycerol	0.166
arabinobiosylglycerol	0.068
arabinotriosylglycerol	0.040
arabinotetraosylglycerol	0.026

Determination of the rate constant of non-specific hydrolysis would enable theoretical curves to be constructed.

(B) Rate constant of non-specific hydrolysis.

It is assumed that the hydrolysis is first order and that all arabinosyl linkages are hydrolysed at a similar rate.

The most accurate concentration is likely to be that of glycerol, i.e. $\frac{G}{tG}$, as glycerol is the major product. The rate of hydrolysis of arabinosyl linkages was thus calculated from the variation of $1 - \frac{G}{tG}$ with hydrolysis time as shown in Diagram 3. Extrapolation to zero time gave the value 0.28. According to first order kinetics $\ln \frac{a}{a-x} = kt$, where a is $(1 - \frac{G}{tG})_{t_o} = 0.28$, x is $(1 - \frac{G}{tG})_t$ and k is the first order rate constant. The plot of $\ln \frac{a}{a-x}$ against hydrolysis time is shown in Diagram 4. The value of k as given by the slope is 6.4×10^{-7} sec^{-1}.

(C) Kinetics of the non-specific hydrolysis of the Smith Degradation products.

The first order equations are : (where subscript

t refers to concentration at time t and subscript o to initial concentration)

arabinotetraosylglycerol : $\frac{A_4G}{tG}t = \frac{A_4G}{tG}o \; e^{-4kt}$

(this equation is valid as no higher products were observed)

arabinotriosylglycerol $\frac{A_3G}{tG}t = \left[\frac{A_3G}{tG}o + \frac{A_4G}{tG}o \; (1-e^{-kt})\right] . e^{-3kt}$

arabinobiosylglycerol $\frac{A_2G}{tG}t = \left[\frac{A_2G}{tG}o + \left(\frac{A_4G}{tG}o + \frac{A_3G}{tG}o\right).\left(1-e^{-kt}\right)\right].e^{-2kt}$

arabinosylglycerol $\frac{A_1G}{tG}t = \left[\frac{A_1G}{tG}o + \left(\frac{A_4G}{tG}o + \frac{A_3G}{tG}o + \frac{A_2G}{tG}o\right).\left(1-e^{kt}\right)\right].e^{-kt}$

Theoretical curves were computed by insertion of the 'to' values of section (A) and the rate constant (6.4×10^{-7} sec^{-1}) in the above equations. The programme was written by Dr. D. Rees in Edinburgh University Atlas Autocode (Iso version) and run on the English Electric KDF9 computer. These curves, shown in Diagrams 5,6, 7 and 8 (broken lines), have a similar shape to the experimental curves.

(D) Non-germinated araban

The values of the concentrations of the degradation products, as measured by their activities (Experiment 3.3), for the 16 hr and 24 hr hydrolysates are shown below:

Table 2.

Hydrolysis time (hr)	Ratio of Activities					
	$\frac{G}{\overline{tG}}$	$1 - \frac{G}{\overline{tG}}$	$\frac{A_1G}{\overline{tG}}$	$\frac{A_2G}{\overline{tG}}$	$\frac{A_3G}{\overline{tG}}$	$\frac{A_4G}{\overline{tG}}$
16	0.711	0.289	0.157	0.0770	0.0402	0.0146
24	0.729	0.271	0.144	0.0765	0.0372	0.0145

These values and the value of the rate constant determined for the germinated araban on insertion into the above equations give, on averaging, the following concentrations of the Smith Degradation products of the 'non-germinated' araban corrected for non-specific hydrolysis (expressed as fraction of the total glycerol) :

		16 hr	24 hr
arabinosylglycerol	0.152	0.158	0.145
arabinobiosylglycerol	0.0814	0.0807	0.0821
arabinotriosylglycerol	0.0437	0.0443	0.0430
arabinotetraosylglycerol	0.0175	0.0169	0.0181

(E) Statistical yield of Smith Degradation products from a randomly branched araban.

It is assumed, for simplicity, that the araban has only single unit side-chains, i.e. the structure is of type I illustrated in the Introduction.

Let the number of 1,3 linked end-groups per

molecule be E and the number of 1,5 linked residues in the main chain be L. The degree of polymerisation (DP) is thus E + L. The probability that any arabinose residue is a branch point is $\frac{E}{L}$, and the probability that it is not branched is $1 - \frac{E}{L}$.

(a) Yield of Glycerol :

(i) General case excluding glycerol from unsubstituted non-reducing end-groups. The feature required for this is two or more successive unsubstituted arabinose units, i.e. - A - A -. The probability that such a pair of units exists is $(1 - \frac{E}{L})^2$ and as the molecule contains (L - 1) possible pairs the yield of glycerol will be $(L - 1)(1 - \frac{E}{L})^2$ per molecule. (It is obvious that overlapping eligible pairs, i.e. where more than two successive unsubstituted arabinose residues occur, do not interfere with the release of glycerol from each other).

(ii) Glycerol released from an unsubstituted end-group of the 1,5 linked main chain. The probability that this exists is $(1 - \frac{E}{L})$ and hence the yield of glycerol is $(1 - \frac{E}{L})$ per molecule.

(iii) Glycerol released from the 1,3 linked end-groups. The yield of glycerol from this is E per molecule.

$$\underline{\text{Total yield of glycerol}} = (L - 1)(1 - \frac{E}{L})^2 + (1 - \frac{E}{L}) + E.$$

(b) Yield of arabinosylglycerol :

(i) General case excluding arabinosylglycerol from a substituted 1,5 linked end-group. The feature required for this is

$$-A-\overset{\overset{A}{\downarrow}}{A}-A-.$$

The probability that such a fragment exists is $(1 - \frac{E}{L})^2 \frac{E}{L}$ and as the main chain contains (L - 2) trisaccharide segments the yield of arabinosylglycerol is $(L - 2)(1 - \frac{E}{L})^2(\frac{E}{L})$ per molecule. (As before the yield is not affected by overlapping of eligible fragments.)

(ii) Arabinosylglycerol released from substituted 1,5 linked end-groups. The feature required for this is $\overset{\overset{A}{\downarrow}}{A}-A$. The probability that this exists is $(1 - \frac{E}{L})(\frac{E}{L})$, and hence the yield of arabinosylglycerol is $(1 - \frac{E}{L})(\frac{E}{L})$ per molecule.

Total yield of arabinosylglycerol =

$$(L - 2)(1 - \tfrac{E}{L})^2(\tfrac{E}{L}) + (1 - \tfrac{E}{L})(\tfrac{E}{L}).$$

(c) General case: similarly it may be shown that the yield of any fragment containing N arabinose residues is

$$(L - 1 - N)(1 - \tfrac{E}{L})^2(\tfrac{E}{L})^N + (1 - \tfrac{E}{L})(\tfrac{E}{L})^N \text{ per molecule.}$$

Molecular weight determinations have shown a DP of approximately 45 for both the 'germinated' and 'non-germinated' arabans. The statistical yields of the Smith Degradation products which would be obtained from a

randomly branched molecule having a DP of 45 was computed for various values of E (programme written by Dr. D. Rees, as above).

The 'germinated' araban has been shown (Experiment 3.1.) to consume 0.71 moles of periodate per mole of anhydroarabinose. This corresponds to a molecule having, on average, 13 1,3 linked end-groups, 13 1,5 linked branch units in the main chain and 19 unsubstituted 1,5 linked chain units. Similarly the periodate consumption of the non-germinated araban (0.67 moles) corresponds to a molecule containing, on average, 15, 1,3 linked end-groups, 15 branch units and 15 unsubstituted chain units.

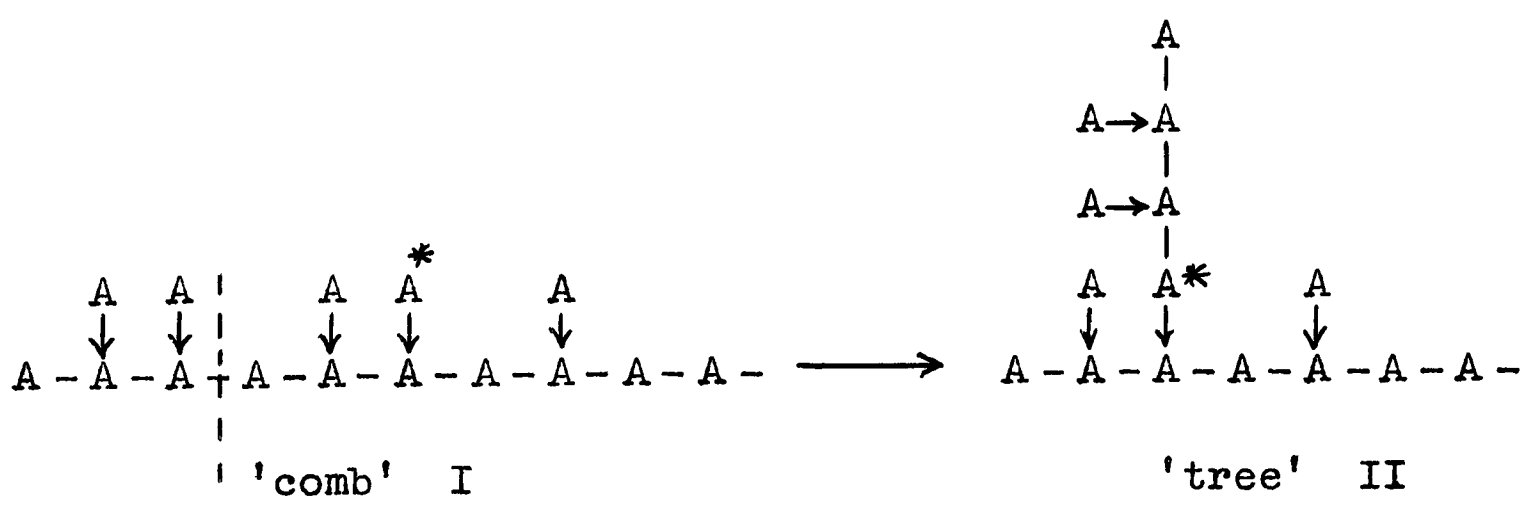
The following yields of Smith Degradation products were predicted for a randomly branched molecule having a DP of 45 and 13 and 15 1,3 linked end-groups (i.e. E = 13 and 15) respectively (expressed as a fraction of the total glycerol) :

Component	E = 13	E = 15
arabinosylglycerol	0.142	0.126
arabinobiosylglycerol	0.056	0.061
arabinotriosylglycerol	0.022	0.029
arabinotetraosylglycerol	0.009	0.014
higher homologues	0.006	0.013

The above considerations apply only to a 'comb' or 'herring-bone' structure. The yields which would be obtained from the other allowed structures, II ('tree') and III ('bush') are somewhat different.

Tree-type structures :

A 'tree-type' branch can be generated from a comb-type molecule by splitting a bond and transferring the reducing end of the chain as shown below.



Factors affecting the yields of the degradation products :

(i) The number of end-groups remains the same.

(ii) Splitting the chain means that there is one less eligible disaccharide fragment, two less trisaccharide fragments and N + 1 fewer ways of choosing a A - $(A)_N$ - A segment (this is the segment required for a product containing N arabinose residues, as shown above).

(iii) Despite (i) there is now 1 extra eligible end segment

(from the end of the new branch).

(iv) The 1,3 linked arabinose unit A^* may now be part of an oligosaccharide (if the adjacent unit in the branch is itself branched). A^* is always oxidisable and is thus always cleaved (the absence of contiguous 1,3 linkages showed this unit is never branched); this means that the branches behave as separate comb-type molecules and no new products will be formed. These considerations may be embodied into a correction factor which when added to the general expression for the yield of products from a 'comb-type' molecule gives the expression for a 'tree-type' molecule. If Q 'tree-type' branches are introduced in the manner described above, then (using the notations above) :

Correction for (ii) Add $- Q(N + 1)(1 - \frac{E}{L})^2(\frac{E}{L})^N$

Correction for (iii) Add $Q(1 - \frac{E}{L})(\frac{E}{L})^N$

Correction for (iv) Add $Q(1 - \frac{E}{L})(\frac{E}{L})^N$

Correction for glycerol only : Add $- Q$

$\therefore$ <u>Total correction</u> $= Q(\frac{E}{L})^N(1 - \frac{E}{L})\left[2 - (N + 1)(1 - \frac{E}{L})\right]$,
plus a further $- Q$ for glycerol only.

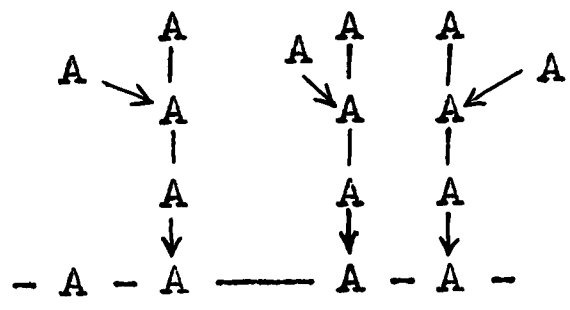
As an example when DP = 45 and E = 15 (i.e. L = 30) the correction factor simplifies to $\frac{Q}{2^{N+2}}(3-N)$ plus a further $-Q$ when N = 0.

If 4 and 8 tree-type branches (i.e. Q = 4,8) for example are introduced, then the following variations in the yields (expressed as fractions of the total glycerol) would be observed.

	Yield of component			
Component	Q = 0	Q = 4	Q = 8	Observed trend
Glycerol	0.767	0.729	0.695	decrease
Arabinosylglycerol	0.127	0.159	0.192	increase
Arabinobiosylglycerol	0.061	0.069	0.077	increase
Arabinotriosylglycerol	0.030	0.030	0.030	little change
Arabinotetraosylglycerol	0.014	0.12	0.010	decrease

The decrease in glycerol is easily rationalised : the number of 1,3 linked end-groups, which always give rise to glycerol in I, is decreased as branches are added on.

Bush-type structures :



'bush' III

In this case no distinction is made between 1,5 and 1,3 linked end-groups. For a molecule with DP = 45 and 15 end-groups, 7 such branches would be present. These

side-chains and the branch points plus the end-group of the main chain would account for 36 residues. This leaves 9 oxidisable units in the main chain. The yield of glycerol (free) would be maximised if the branches were present in a block and in this case the yield of glycerol from the main chain would be 8 units with a total yield of free glycerol of 23 units. Thus the minimum ratio of arabinosylglycerol/glycerol yields would be $^{7}/_{23} = 0.304$. It is intuitively obvious that a structure of type III would give a very much higher yield of arabinosylglycerol relative to higher products than the more random type I and II structures.

Discussion

The observed Smith Degradation products indicate that both the 'germinated' and 'non-germinated' arabans have irregularly branched structures in which no more than four adjacent arabinose units are ever branch points.

The accuracy of the values obtained for the yields of the products may be checked against the periodate consumption as follows :

$$\text{Periodate consumption} = \frac{\text{oxidised units}}{\text{oxidised units} + \text{unoxidised units}}$$

$$= \frac{tG}{tG + tA}$$

where tG = total glycerol as defined above (p.106) and tA = total arabinose = $\frac{A_1G}{tG} + 2\frac{A_2G}{tG} + 3\frac{A_3G}{tG} + 4\frac{A_4G}{tG}$.

As tG = 1,

Periodate consumption = $\frac{1}{1 + tA}$

By inserting the values of the total arabinose, the measured and calculated periodate consumption may be compared.

Araban	Periodate consumption		
	Calculated	Measured	Error %
'Germinated'	0.655	0.71	7.8
'Non-germinated'	0.66	0.67	1.5

The values of the total arabinose are thus rather high, especially for the 'germinated' araban. The major source of error is likely to be in measurement of the activity of A_4G because the activity relative to background was not high (see Diagram 2) and any error is magnified in the calculation of the total arabinose.

The yields of the products may be compared with the yields which would be obtained from a randomly branched 'comb-type' structure having the same periodate consumption. Yields are expressed as fractions of the total glycerol as before :

Component	Yield from 'germinated'			Yield from 'non-germinated'		
	Observed	Predicted	Obs/Pre	Observed	Predicted	Obs/Pre
A_1G	0.166	0.142	1.17	0.152	0.126	1.20
A_2G	0.068	0.056	1.22	0.081	0.061	1.33
A_3G	0.040	0.022	1.82	0.044	0.029	1.52
A_4G	0.026	0.009	2.99	0.018	0.014	1.29
Higher homologues	-	0.006		-	0.013	

The yields of the substituted glycerol products are generally rather higher than predicted for the randomly branched 'comb' structure which would have a similar periodate consumption. The absence of fragments higher than A_4G might account for this in part and some over-estimation of the products (or under-estimation of glycerol) might also contribute. In ascending the series A_1G to A_4G, the ratio of the observed yield to the predicted yield increases showing that A_4G, A_3G and, to a lesser extent, A_2G, are more favoured than A_1G.

'Bush-type' structures (III) are unlikely for the araban as the theory shows that in the degradation products there would be a much higher proportion of A_1G and a much lower proportion of A_2G, A_3G and A_4G. Similarly, increasing 'tree-type' character has been shown to increase the yields of A_1G and A_2G and decrease the yield of A_4G, as compared with a 'comb' structure, and thus substantial

'tree-type' (II) character is not likely. It is most likely that the structure of mustard araban is an irregular 'comb-type' with a bias in favour of contiguous branch points. This bias is more pronounced in the 'germinated' araban and may reflect some redistribution of branches during germination.

Generally, although the labelling method of determining the yields of Smith Degradation products was rather less accurate than was desired it is more accurate and convenient than other existing methods and may be used on a micro scale. Correction for non-specific hydrolysis would seem to be desirable in quantitative work especially where furanoside linkages are involved.

CHAPTER 4

Characterisation of polysaccharide structures by glycoside stabilisation with toluene-p-sulphonates (tosylates): model experiments with dextran

Introduction

Characterisation of polysaccharide structures by stabilising a particular linkage to acid conditions is an attractive method of obtaining additional evidence to that given by the periodate degradation methods discussed in the previous chapter. The aim of the work described here was to develop such a method which would be applicable to polysaccharides in which unsubstituted primary hydroxyls occur only at the end-groups, such as dextran and araban. The significance of the evidence which would be obtained by characterisation of the terminal linkages in the above polysaccharides will be discussed later. The catalytic oxidation procedure of Aspinall (101) could be applied to such polysaccharides as this also depends on selective reaction of primary hydroxyl groups. These groups are oxidised to carboxylic acid groups which then confer the desired stability towards acid hydrolysis. Analysis of the aldobiuronic acid products characterises the terminal linkages. Unfortunately the oxidation requires extended periods and is far from quantitative, probably due to difficulties in obtaining all the primary hydroxyl groups of the polymer in the correct orientation on the catalyst surface, and these are the major drawbacks of the method.

The acid hydrolysis of glycosides, though the subject of intensive investigation, is not yet fully understood. Most of the work has been limited to the

pyranosides, the kinetics of the hydrolysis being followed polarimetrically. In the usual mechanism of hydrolysis of the pyranosides (102) the initial step is the rapid equilibrium-controlled protonation of the glycosidic oxygen to give the conjugate acid. This is followed by the slow rate-determining heterolysis of the conjugate acid with the formation of a carbonium-oxonium ion and an alcohol. The former reacts rapidly with water to give the parent sugar. If the rate-determining step were bimolecular involving a molecule of water the transition state, assumed to approximate to the carbonium-oxonium ion, would be more highly ordered than the ground state leading to negative entropies of activation. Positive values have been obtained for a wide variety of pyranoside glycosides and for glycosides of pyranosiduronic acids (103) indicating the less ordered transition state is involved. In contrast negative entropies of activation have been recorded for some furanoside glycosides (104) indicating that a different mechanism is likely, interpreted as either a bimolecular displacement involving a molecule of water or a mechanism involving ring opening.

Formation of the carbonium-oxonium ion intermediate (i.e. an ion in which the positive charge is stabilised by sharing between $C_{(1)}$ and the ring oxygen) requires that $C_{(2)}$, $C_{(3)}$, the ring oxygen and $C_{(5)}$ become co-planar, and thus involves a conformational change from the chair to the half-chair form. The formation is sterically impeded by

partial eclipsing of the substituents on $C_{(2)}$ and $C_{(3)}$ and of those on $C_{(4)}$ and $C_{(5)}$. When an electron-withdrawing substituent is present in the glycone the inductive effect will destabilise the carbonium-oxonium ion and hence raise the activation energy of the hydrolysis reaction. It follows that both steric and electronic factors affect the formation and stability of the carbonium-oxonium ion and hence the rate of hydrolysis.

The nature of the aglycon may also have a marked effect on the rate of hydrolysis. Feather and Harris (105) have observed that axially orientated substituents at $C_{(1)}$ are hydrolysed more slowly than their equatorial counterparts, presumably because the former are stabilised by the anomeric effect (106). The methyl glucuronosides are, surprisingly, hydrolysed at a similar rate to the methyl glucosides (107), whereas other glycosides of glucuronic acid (and particularly where the aglycon is another sugar unit, as in a polymer) are extremely stable (108). This is a dramatic example of the size of the aglycon affecting the rate of hydrolysis, although it has been shown that the entropies of activation of the methyl glycosides of glucuronic acid are considerably lower than for most pyranoside glycosides (107) and this probably reflects that a somewhat different mechanism is operating. The electron affinity of the aglycon does not appear to be as important as the size factor in the glucuronosides and it is thought that the effect of the carboxyl group on $C_{(5)}$ plus the size

of the $C_{(1)}$ substituent combine to give the overall result.

The considerable literature on the acid hydrolysis of glycosides (both pyranoside and furanoside) has recently been reviewed (109). By analogy with the carboxyl stabilising effect it might be expected that a large electron-withdrawing substituent on the primary hydroxyl of the non-reducing unit of a disaccharide would stabilise the glycosidic linkage to hydrolysis. Such a substituent in a 1,6 linked hexosan, or possibly a 1,5 linked pentosan, would enable the terminal linkage to be characterised. The use of toluene-p-sulphonates (tosylates) to this end is the subject of this chapter.

These esters are themselves acid stable and glycosides of 2-O-toluene-p-sulphonyl pyranoses are known to be stable to acid hydrolysis (110). Measurements have shown (111) that tosylates at $C_{(6)}$ should be sufficiently electron-withdrawing to stabilise the glycoside to a useful extent, the inductive substituent constant (σ_1) for $-CH_2O.SO_2.C_6H_4.CH_3$ of about 0.37 compares favourably with 0.39 for -COOH and 0.07 for $-CH_2OH$. In preliminary experiments in this laboratory by Dr. N. Richardson (98), the hydrolysis of methyl 6-O-toluene-p-sulphonyl-α-D--glucopyranoside was stabilised by a factor of between 3 and 5 as compared with methyl α-D-glucopyranoside. Although this is not sufficiently large to be useful as such, it is greater than for the corresponding uronoside (107) and by analogy with the uronic acid series (108) would be expected

to be very much greater at the disaccharide level (i.e. where the aglycon is another sugar unit). Experiments with dextran ensued, with the ultimate aim of applying the method (if successful) to the mustard seed araban.

Dextrans are glucans produced by strains of Leuconostoc mesenteroides and other bacteria on a sucrose substrate. The polysaccharides have very high molecular weights (up to several million) and fractions obtained by controlled partial hydrolysis are used as substitutes for blood plasma. Determination of the fine structure of dextrans is thus important in view of the immunological factors involved.

Early work has been reviewed by Neely (112). Dextrans are essentially α-1,6 linked but differ in some structural details which vary according to origin. The most common variation is a degree of branching through the $C_{(3)}$ and/or $C_{(4)}$ positions of the glucose residues, although branching through $C_{(2)}$ is also reported (113). Some dextrans also have α-1,3 and/or α-1,4 linked inter-chain residues (114). The principal problem as regards fine structure concerns the length and structure of side-chains. The dextrans produced by L. mesenteroides NRRL B-1415 (α-1,4 branched) and NRRL B-1416 (α-1,3 and α-1,4 branched) have been investigated by enzymatic methods (with glucamylase) and the dextrans produced by NRRL-1415 and NRRL-1375 (α-1,3 branched) by the catalytic oxidation procedure (115). The results showed that most, if not all

of the side-chains consist of single glucose units and it was suggested that this structural feature might be common to other branched dextrans. Similar conclusions have been reached on the basis of physical measurements (112).

The particular dextran used in this work was that from L. mesenteroides NCIB 2706 and has been shown by periodate oxidation studies (116) to contain 18% 1,3-type linkages and about 3% 1,4-type linkages. The molecule is thus sufficiently highly branched to be a suitable model for the development of the technique. P.m.r. spectroscopy of the deuterated dextran (98) confirmed the degree of branching was of the order of 20%, the interpretation of the spectrum being based on the assignments of Pasika and Cragg (117). The tosylated derivative of the dextran was prepared by Dr. Richardson (98). To obtain the most selective substitution possible the tosyl groups were introduced after triphenylmethylation (tritylation), methylation and detritylation. Conditions were chosen to obtain complete primary substitution in the first stage and the product was found to be over-tritylated to the extent of 20%. Examination after the second stage showed tetra-O-methylglucose to be completely absent proving the location of the majority of trityl groups. In preliminary hydrolyses of the O-methyl-O-tosyldextran Dr. Richardson showed that glycoside stabilisation was apparent under methanolysis conditions and in 50% aqueous monochloroacetic acid solution but that detosylation and other reactions occurred in formic acid

solution. This was attributed to the reducing power of formic acid. The usefulness of the specific tosyl ester spray of Jackson and Hayward (7 and General Methods) was confirmed for locating these esters on thin layer chromatograms.

The aim of the work described in the experimental section of this chapter was to establish optimum hydrolysis conditions and to characterise the stabilised linkage(s). The structural features of the native dextran were established by a methylation analysis, indentification of the methylated sugars being based on g.l.c. and paper chromatography. A survey of the specificities of some acids towards the co-occurring linkages in dextran and O-methyldextran gave a background for the hydrolysis of the tosylated derivative. Various attempts to isolate the tosylated products of hydrolysis are described.

Experimental

Experiment 4.1. Preparation of dextran.

The dextran was prepared from a culture of Leuconostoc mesenteroides NCIB 2706 obtained from the Torry Research Station, Aberdeen. The culture medium contained sucrose (10%), yeast extract (1%), potassium hydrogen phosphate (0.5%), and peptone (0.25%), in tap water, and was adjusted to pH 7.0 with dilute sodium hydroxide solution. Two 50 ml and two 2 l conical flasks containing 25 ml and 1 l of medium respectively were plugged with paper-wrapped cotton wool and sterilised in an autoclave. The small flasks were innoculated with the organism and incubated for 2 days at 26°. The resulting viscous solutions were transferred to the large flasks which were similarly incubated. The dextran was isolated from solution by the slow addition of 1.5 volumes ethanol with stirring. The gummy product was purified by redissolving in water (2 l), boiling the resultant solution for 45 minutes to coagulate proteins and centrifuging. The supernatant solution was filtered and the dextran reprecipitated with ethanol. On redissolving the gum in water and freeze-drying, 100g. of dextran was obtained. (Found, %N = 0.75)

Experiment 4.2. Methylation of dextran.

Dextran (11.0g) was dissolved in N-methyl-2-

-pyrrolidone (220 ml). The solution was cooled to 0°, and methyl iodide (33 ml), dissolved in N-methyl-2-pyrrolidone (220 ml), and barium hydroxide octahydrate (77g) were added. The mixture was shaken at 0° for 2 hr. and then at room temperature overnight. Methyl iodide (110 ml) and barium hydroxide octahydrate (264g) were added and shaking was continued for 24 hr. The mixture was transferred to a separating funnel with water. The product was extracted with chloroform (3x) and the chloroform layer washed with sodium thiosulphate solution (1%), water (3x), and dried over sodium sulphate. The syrup was diluted with a small volume of benzene and precipitated with light petroleum (b.p. 40-60°). The white fibrous product was purified by reprecipitation (8.98g, 65%). Anal. Calc. for tri-O-methylhexose polymer : %OCH_3 = 45.6. Found, % OCH_3 = 37.4.

The incompletely methylated material (5.9g) was dissolved in dimethylformamide (180 ml) and methyl iodide (60 ml) and silver oxide (30g) were added. The mixture was magnetically stirred in a conical flask fitted with a double surface condenser, in a water-bath at 40°. After 6 hr methyl iodide (60 ml) and silver oxide (30g) were added and the reaction refluxed for 48 hr. The product was obtained as described in Experiment 1.5. (a) and was purified by the precipitation procedure above (4.15g, 46% overall). Anal. Found, % OCH_3 = 43.5%.

The product (4.0g) was remethylated by the same method (3.05g, 35% overall). Anal. Found, % OCH_3 = 43.6.

The infra-red spectrum showed negligible hydroxyl absorption. The product was fractionated, by the addition of light petroleum (b.p. 40-60°) to a chloroform solution of the methylated polysaccharide, into four subfractions of approximately equal weight. Anal. Found, % OCH_3 = 45.3, 44.0, 42.9, 38.5.

The fully methylated unfractionated material (20 mg) was methanolysed and examined by g.l.c. (Pye Argon Chromatograph) on column 2 at 160° and 175°. The results are given below.

Temperature	methyl glycosides of -	T (standard)	T (dextran
160°	2,3,4,6-tetra-O-methylglucose	1.00, 1.46	1.00, 1.46
	2,3,4-tri-O-methylglucose	2.32, 3.32	2.33, 3.29
	2,4,6-tri-O-methylglucose	2.88, 4.48	2.85, 4.38 (traces)
175°	2,4-di-O-methylglucose	6.18, 8.54	6.10, 8.54
	3,4-di-O-methylglucose	7.54	-
	2,3-di-O-methylglucose	12.01	-

Hydrolysis and paper chromatography in solvents B and D respectively confirmed the presence of 2,3,4,6-tetra--O-methylglucose (R_G = 0.98, 1.00), 2,3,4-tri-O-methylglucose (R_G = 0.89, 0.80), 2,4,6-tri-O-methylglucose (traces only, R_G = 0.82, 0.54) and 2,4-di-O-methylglucose (R_G = 0.65, 0.35). A small amount of another, unidentified, component having R_G = 0.80 and 0.90 in solvent B and D respectively and T =

1.92 (160°) may have been 1,6-anhydro-2,3,4-tri-O-methylglucose.

Separation of the hydrolysis products of fully methylated dextran (2g) by preparative paper chromatography in solvent D yielded 2,4-di-O-methylglucose (0.168g) and a mixture of 2,3,4-tri-O-methylglucose and 2,3,4,6-tetra-O-methylglucose (1.020g). Both products were exhaustively dried *in vacuo* over phosphorus pentoxide at 40°. This corresponds to approximately 15% branching.

Experiment 4.3. Comparison of the stabilities of the 1,3- and 1,6-glycosidic linkages in dextran and methylated dextran under various solvolysis conditions.

The general method used is as follows. Samples (10 - 20 mg) of dextran or methylated dextran were hydrolysed for varying times under the conditions given and, after neutralisation or removal of acid, each hydrolysate was methylated, methanolysed and examined by g.l.c. on column 1 at 175° (Pye Argon Chromatograph). The relative rate of hydrolysis of the 1,3- and 1,6-glycosidic linkages is given by the variation of the relative concentration of 2,3,4-tri-O-methylglucose and 2,4,6-tri-O-methylglucose with hydrolysis time. This was conveniently measured from the peak heights of the slower peak of each tri-O-methylglucoside peaks.

(a) Sulphuric acid (1N) hydrolysis of dextran.

Samples (20 mg) of dextran were dissolved in sulphuric acid (4N, 1.0 ml) and the solutions diluted with water (3.0 ml), and heated for varying times at 100°. After neutralisation, filtration and evaporation to dryness each residue was methylated by Kuhn and Trischmann's method (13) which involves the use of barium hydroxide octahydrate and dimethyl sulphate in a mixture of dimethylformamide and dimethylsulphoxide. The absence of di-O-methylglucose derivatives in the hydrolysis products after 6 hr and 24 hr indicate that undermethylation was not significant.

The results indicate that preferential hydrolysis of 1,6-linkages occurs in this system.

Hydrolysis time (hr)	Methyl 2,3,4-tri-O-methylglucoside peak height / Methyl 2,4,6-tri-O-methylglucoside peak height
0	22.0
0.66	7.6
1.0	12.0
2.0	8.4
6.0	1.1
24.0	1.7

(b) Formic acid hydrolysis of dextran and methylated dextran.

Samples (10 mg) of dextran and methylated dextran were heated with formic acid (45% w/v, 2 ml) for varying

periods at 100°. After removal of acid (General Methods) and drying, the hydrolysates were methylated, methanolysed and examined by g.l.c. as described above.

Hydrolysis time (hr)	Methyl 2,3,4-tri-O-methylglucoside peak height / Methyl 2,4,6-tri-O-methylglucoside peak height	
	dextran	methylated dextran
0	22.0	22.0
0.75	12.1	36.3
1.0	15.3	26.6
2.0	13.5	38.6
6.0	14.0	22.2
24.0	5.0	40.7

This result was suspect due to slight undermethylation.

The results indicate that both linkages are hydrolysed at about the same rate in this system with the methyl ether groups appearing to confer some stability on the 1,6-linkage.

(c) Chloroacetic acid hydrolysis of dextran and methylated dextran.

In order to assess the difficulties involved in the removal of the acid and possible side-reactions (such as reversion), D-glucose (20 mg) was dissolved in aqueous

chloroacetic acid (50% w/v, 2 ml) and heated at 100° for 24 hr. The acid was removed by evaporation on the rotary evaporator using an oil-pump and a bath temperature of 60° but required repeated distillation of water from the residue to effect efficient removal. On examination by paper chromatography in solvent A no traces of disaccharides or products other than glucose were observed and it was concluded that reversion was not significant.

(i) Dextran (20 mg) was heated in chloroacetic acid (50%, 2 ml) at 100° for 12 hr. After removal of the acid and drying, the product was methylated (as in (a) above), methanolysed and examined by g.l.c. Although slight undermethylation was observed the results clearly showed that 1,6-linkages were being hydrolysed at a faster rate than 1,3-linkages in this system.

(ii) Samples (12 mg) of methylated dextran were heated in chloroacetic acid (50%, 2 ml) for varying periods at 100°. After removal of acid and methylation as above the infra-red spectra showed slight hydroxyl absorptions. The samples were remethylated in dimethylformamide with methyl iodide and silver oxide under reflux as in Experiment 4.2. After methanolysis the following results were obtained.

Hydrolysis time (hr)	Methyl 2,3,4-tri-O-methylglucoside peak height / Methyl 2,4,6-tri-O-methylglucoside peak height
0	22.0
1.5	13.6
20.0	1.6
72.0	2.1

The results again indicate preferential hydrolysis of the 1,6-linkage.

Experiment 4.4. Comparison of the rate of hydrolysis of O-methyldextran and O-methyl-O-tosyldextran in chloroacetic acid.

The hydrolysis of O-methyldextran and O-methyl-O--tosyldextran was conveniently followed by the colorimetric estimation of the degree of polymerisation as the reaction proceeded (118). Aliquots of the reaction at varying times were reduced with sodium borohydride and the optical density of the reaction with the phenol-sulphuric acid reagents (General Methods) determined. Preliminary experiments showed that equimolar amounts of methyl α-D-glucoside and methyl 6-O-toluene-p-sulphonyl-α-D-glucoside gave the same optical density in the phenol-sulphuric acid reaction. The presence of the tosyl group in dextran was thus unlikely to

affect the validity of the results. It was found however that large amounts of sodium borohydride supressed the colour reaction and that addition must be rigidly controlled. (Excess borohydride was used to both neutralise the acid and reduce the sugars).

Equimolar samples of O-methyldextran (20 mg) and O-methyl-O-tosyldextran (22.8 mg) were heated with chloroacetic acid (50%, 2.0 ml) in glass stoppered tubes with mechanical shaking at 100°, 84.5° and 69° in thermostat baths. O-methyldextran dissolved readily but the tosyl derivative was insoluble for some time (2.5 hr. at 84.5°), and this explains the anomalous results for the shorter hydrolysis times. Samples (0.010 ml) were removed at intervals, diluted with water (1.00 ml) and a weighed excess of sodium borohydride (0.100g) was added in portions. After standing for at least 24 hr with occasional shaking, excess borohydride was destroyed by the slow addition of concentrated sulphuric acid (AnalaR, 1.00 ml). Water (1.00 ml) was added followed by the phenol-sulphuric acid reagents and the optical density measured. The optical density corresponding to 90% hydrolysis was determined by tenfold dilution of the hydrolysates after reaction and addition of 0.010 ml of this, diluted with water (1.00 ml), to premixed sodium borohydride and concentrated sulphuric acid in the normal proportions, followed by the phenol-sulphuric acid reagents.

The plot of optical density against hydrolysis time

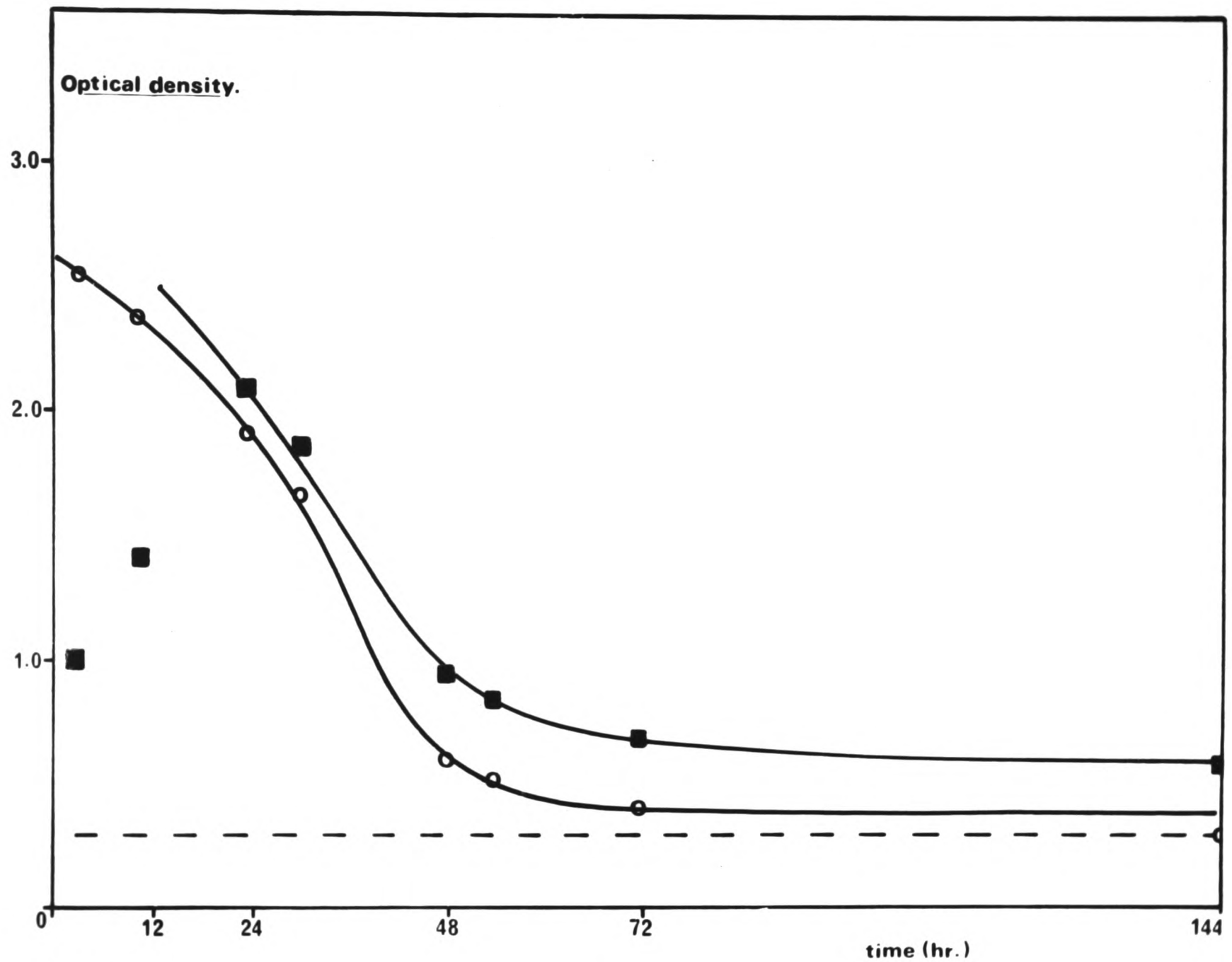


diagram 1.

Chloroacetic acid hydrolysis (84.5°) of : ■— O-methyl-O-tosyldextran
—o— O-methyldextran

is shown in diagram 1 for the 84.5° hydrolysis. (The broken line corresponds to 90% hydrolysis). The second and slower phase of the hydrolysis of O-methyl-O-tosyldextran was more pronounced at 84.5° and 69° than at 100°, there being little difference in this respect between 84.5° and 69°. However the absolute rate of hydrolysis was inconveniently slow at 69° (approximately 18 days for 80% hydrolysis) and the most suitable conditions for optimum yield of stabilised fragments from O-methyl-O-tosyldextran was judged to be 72 hr at 84.5°.

Experiment 4.5. Thin Layer Chromatography of the hydrolysis products of O-methyl-O-tosyldextran.

The optimum conditions established above were employed. In addition to a small scale (20 mg) hydrolysis of O-methyl-O-tosyldextran, similar samples of O-methyldextran and O-methyl-O-triphenylmethyldextran were hydrolysed. After removal of the acid as above, portions (2 mg) were dissolved in a small volume of acetone and examined by T.L.C. on silica gel micro-plates (General Methods). After double development in benzene-ethanol-water-ammonia (200 : 47 : 15 : 1) tosyl esters were located by use of the specific spray (iii). Subsequent spraying with the general reagent (i) located all the carbohydrate components.

Only the O-methyl-O-tosyldextran hydrolysate gave a positive reaction with the specific spray, the only

significant UV fluorescing spot had the same mobility as 2,3,4-tri-O-methylglucose and was considerably slower than 2,3,4-tri-O-methyl-6-O-tosylglucose. 2,3,4,6-tetra-O--methylglucose was only significant in the hydrolysate of O-methyldextran. 2,3,4-tri-O-methylglucose (major component) and 2,4-di-O-methylglucose were present in all the hydrolysates.

Some resolution of the tosylated material and 2,3,4-tri-O-methylglucose was obtained on larger plates (20 x 5 cm) by development in benzene-ethanol (2 : 1) or chloroform-methanol (25 : 1) but this was not sufficiently good to allow separation on a preparative scale. In these solvents the tosylated material did not appear to be entirely homogeneous. A wide variety of other systems was examined, with silica, alumina and cellulose plates, but the results were usually inferior.

Experiment 4.6. Gel filtration of the hydrolysis products of O-methyl-O-tosyldextran.

Attempted separation of the components of the hydrolysate (20 mg) on a column of Sephadex LH-20 (30 x 2.5 cm) by elution with ethanol was not successful. The column fractions were screened by UV spectrophotometry (λ_{max} 223 nm) but on examination of the combined absorbing material by T.L.C. the original mixture was obtained.

Separation was similarly unsuccessful when the mixture (30 mg) was applied to a column (40 x 2 cm) of

Dowex 50 resin (50 WX2, 200 - 400 mesh, Ba^{++} form) and eluted with aqueous ethanol (40%).

Experiment 4.7. Paper chromatography of the hydrolysis products of O-methyl-O-tosyldextran.

The hydrolysates of O-methyl-O-tosyldextran and O-methyl-O-triphenylmethyldextran (Experiment 5) were examined by paper chromatography in solvents B, E and a solvent F consisting of benzene-ethanol-water-light petroleum (b.p. 60 - 80°) (169 : 47 : 15 : 130). Tosyl esters were located by spraying the chromatogram with the specific spray and examination under UV radiation as described in General Methods under T.L.C. (iii). Subsequent overspraying with p-anisidine hydrochloride located all the sugars present. The results are summarised below.

Component	Relative concentration in hydrolysate of:		R_G in solvent system:		
	-tosyl deriv.	-triphenylmethyl deriv.	B	E	F
2,4-di-O-methylglucose	weak	weak	0.55	0.09	0.05
2,3,4-tri-O-methylglucose	strong	strong	0.87	0.46	0.24
2,3,4,6-tetra-O-methyl--glucose	-	-	1.00	1.00	1.00
unidentified product	weak	weak	1.04	1.14	1.14
tosyl ester	medium	-	1.14	1.27	1.46

Solvent F gave good separation of the tosyl ester component from the other sugars. The unidentified component was shown not to be residual chloroacetic acid.

Experiment 4.8. Separation of the hydrolysis products of O-methyl-O-tosyldextran by cellulose column chromatography.

The results of Experiment 4.7. suggested that purification of the tosyl ester fraction might be possible by cellulose column chromatography in solvent F. O-methyl-O-tosyldextran (0.320g) was hydrolysed in aqueous chloroactic acid under the standard conditions developed in Experiment 4.4. After exhaustive removal of acid the hydrolysate was dried in vacuo at 50° (0.446g). Paper chromatography showed a similar composition to the hydrolysate examined previously. The UV spectrum in ethanol solution showed a peak at 223 nm identical with that given by methyl 6-O-tosyl-α-D-glucoside. The infra-red spectrum (as a film) showed bands at 1160 cm^{-1} (medium) and 1380 cm^{-1} (weak) attributable to the tosyl group, and also a very strong carbonyl absorption at 1740 cm^{-1} suggesting residual chloroacetic acid or, less likely, that chloroacetate esters were present. This would account for the discrepancy between the weights of starting material and the hydrolysate.

The syrup was treated twice with aqueous ammonium carbonate (1% w/v, 10 ml) followed, after standing for 2 hr, by evaporation to dryness under reduced pressure

at 60°. Water was distilled from the residue several times until ammonia was no longer detectable by smell. The infra-red spectrum showed that the band at 1740 cm^{-1} had been shifted to 1600 cm^{-1} confirming that a carboxylic acid had been present but was now converted to the ammonium salt. There was a slight residual absorption at 1740 cm^{-1} which was not affected by brief (30 minute) de-esterification of the hydrolysate in methanolic 2,2-dimethoxypropane (5%) with sodium methoxide. The cleavage of chloroacetate esters may not lead to the disappearance of the residual 1740 cm^{-1} band as the product (methyl chloroacetate, b.p. 150°) might not have been removed by evaporation. After neutralisation with solid carbon dioxide and evaporation to dryness, exhaustive extraction of the salty residue with ethanol (6 x 10 ml) followed by evaporation gave a clear syrup (300 mg).

The syrup was dissolved in ethanol (5 ml) and loaded onto a cellulose column (70 x 3.5 cm) and eluted with a system essentially similar to solvent F but half-saturated with water. Fractions (10 ml) were collected automatically and screened by spotting onto paper and spraying with p-anisidine hydrochloride. Fractions were combined on the basis of the results into three larger fractions which were evaporated to dryness and dried <u>in vacuo</u>.

Fraction	Weight (mg)	Main Component(s)
1	105	tosyl ester
2	61	2,3,4-tri-O-methylglucose
3	17	2,4-di-O-methylglucose, 2,3,4-tri-O-methylglucose

The composition of Fractions 2 and 3 were confirmed by paper chromatography, T.L.C. (silica gel; benzene-ethanol, 2 : 1, solvent) and g.l.c. on column 2.

Although Fraction 1 appeared to be pure by paper chromatography, T.L.C. on silica gel plates with butan-2-one as developing solvent showed two main components detectable with iodine vapour. Subsequent application of the specific spray for tosyl esters (after allowing the iodine to evaporate) showed a positive reaction for the faster one only. The syrup (105 mg) was purified by preparative T.L.C. on silica gel plates (butan-2-one solvent). The faster moving component was located with iodine vapour, eluted from the silica gel with acetone, filtered and evaporated to dryness (60 mg). Examination of the purified material by T.L.C. (conditions as above) showed that it did not move as a discrete spot. The main components were two partly resolved spots; minor amounts of tosylated material which moved slightly faster and slower than the above were detected but may possibly have been caused by streaking of the major components.

Separation of the components of the tosyl ester fraction appeared to be beset with many difficulties, the streaking mentioned above, which occurred to some extent in all the systems examined, being one. The line of approach involving direct identification of the tosylated disaccharide(s) was thus abandoned in favour of an indirect method described in the next experiment.

Experiment 4.9. Identification, after methylation, reductive detosylation and remethylation, of the methanolysis products of O-methyl-O-tosyldextran.

Methylation followed by detosylation and remethylation of the methanolysis products of O-methyl-O-tosyldextran should yield fully-methylated isomaltose (6-O-α-D-glucopyranosyl-D-glucose) and/or fully-methylated nigerose (3-O-α-D-glucopyranosyl-D-glucose), the relative yields being diagnostic of the branching pattern of the dextran. Incomplete methanolysis at non-stabilised linkages would also yield the methylated disaccharides and correction for this was made by addition of an internal standard. Identification of the fully methylated disaccharide derivatives was by g.l.c. and relative yields were calculated from peak areas.

(a) Preparation of standard compounds for g.l.c.

Nigerose and isomaltose samples were kindly

provided by Professor D.J. Manners and Dr. J.R. Turvey. Each disaccharide (10 mg) was dissolved in dimethylformamide (1.0 ml) and methyl iodide (1.0 ml) and silver oxide (0.100g) added to the solution at 0°. The reaction was carried out in a sealed tube with shaking in the dark at room temperature for 48 hr. The product was obtained by evaporation of the chloroform solution obtained by the procedure described in Experiment 1.5. (a). Three such methylations were carried out.

Octa-O-methylsucrose, kindly provided by Dr. J.C.P. Schwarz, was used as the internal standard.

The compounds prepared were shown to be separable from each other and from octa-O-methylsucrose by g.l.c. on column 4 (General Methods) (Pye series 104 chromatograph) at 210°. The retention times (T_s) were expressed relative to acta-O-methylsucrose, and are in the range to be expected from the literature (119).

Octa-O-methylsucrose, T_s 1.00
Methyl hepta-O-methylisomaltosides, T_s 1.44 2.02
Methyl hepta-O-methylnigerosides, T_s 1.82 2.35

(b) Investigation of the methanolysis products of O-methyl-O-tosyldextran.

Samples (25 mg) of O-methyl-O-tosyl dextran were heated in sealed tubes with methanolic hydrogen chloride (3%, 2.5 ml) at 100° for 0.5, 1.5, 4.5, 13.5 and 40.5 hr.

The material dissolved instantly. After neutralisation with silver carbonate, filtration and evaporation to dryness, each residue was dissolved in dimethylformamide (2.0 ml). Methyl iodide (0.5 ml) and silver oxide (0.2g) were added and the reaction carried out as in (a), above, and worked up in the usual way. The procedure was repeated twice. The products were dried *in vacuo* and examined by infra-red spectroscopy in dry carbon tetrachloride solution. No trace of hydroxyl absorption was observed for any sample, showing methylation to be complete. Examination of the methylated methanolysates by g.l.c. on column 4 (Pye series 104 chromatograph) at 210° showed isomaltosides as the only detectable disaccharide products (T_s, 1.48, 2.00, 2.60 (minor, unidentified). To each sample was added octa-*O*-methylsucrose as the internal standard. The UV spectra of the samples were recorded in ethanol solution.

After evaporation to dryness, each methanolysate was dissolved in aqueous isopropanol (80%). Reductive detosylation (120) was carried out by addition of an excess of sodium amalgam (2%) to the solution at 60° over 24 hr., followed by addition of solid carbon dioxide, filtration and evaporation. The product was extracted from the salty residue with acetone which was filtered and evaporated to dryness. The residue was dissolved in chloroform which was washed with water and evaporated to dryness. The absorptions at 223 nm of the products in ethanol solution were virtually undiminished. The treatment with sodium

amalgam was repeated using a very large excess which was added over 48 hr to a solution of the product in aqueous ethanol (80%) at 65°. After working up as above, greatly diminished absorptions at 223 nm were observed in all the products.

After remethylation and g.l.c. by the same procedure, the increase in methylated disaccharides was measured from the peak areas of g.l.c. charts, using the internal standard. Areas were measured by triangulation. Both isomaltosides and nigerosides were identified on the basis of retention times (T_s, 1.48, 1.98, 2.34 (except in product after 0.5 hr), 2.60 (minor)). The faster nigeroside peak was not observed, probably due to only trace amounts of this derivative being present. The assignment of the peaks to disaccharide derivatives was supported by the gradual build-up of peak areas with methanolysis time. The maximum increase in the proportion of isomaltose plus nigerose derivatives relative to the internal standard, (8.22 fold), was observed after 40.5 hr methanolysis and it is likely that greater increases may have resulted from even longer times. The yield of nigerose derivatives relative to isomaltose derivatives was very small and the significance of this is discussed later.

Methanolysis time (hr)	Ratio of isomaltose + nigerose derivatives : octa-O-methyl--sucrose		Relative increase in methylated disaccharides ($^{B}/_{A}$)
	Before detosylation (A)	After detosylation (B)	
0.5	0.528	0.833	1.579
1.5	0.609	1.395	2.291
4.5	1.146	3.473	3.031
13.5	0.666	2.400	3.603
40.5	0.524	4.305	8.219

The 40.5 hr product was examined further by g.l.c. using the same apparatus and column as above but with temperature programming from 140 - 210° (increasing by 6° per min to 210°, followed by 5 min at this temperature) to estimate the relative amounts of disaccharide derivatives and tetra-O-methylglucosides (no other methylated glucoside derivatives were observed). Measurement of peak areas of the 40.5 hr methanolysate gave:

Tetra-O-methylglucosides	2.16 cm^2
Hepta-O-methylisomaltosides	2.10 cm^2
Hepta-O-methylnigerosides	0.17 cm^2

On the assumption that the flame ionisation detector gives a response on a weight basis for compounds of such related structure these values represent a molar ratio of tetra-O-methylglucosides : hepta-O-methylisomaltosides :

hepta-O-methylnigerosides of 100 : 54 : 4. The accuracy of these values is probably no more than ± 10%. The earlier results have shown that less than one eighth of the total yield of disaccharides is attributable to incomplete hydrolysis at non-stabilised linkages.

Experiment 4.10. Identification, after methylation, reductive detosylation and remethylation, of the hydrolysis products of O-methyl-O-tosyldextran.

The purified tosyl ester fraction (25 mg) isolated by cellulose column chromatography and T.L.C. from the chloroacetic acid hydrolysate of O-methyl-O-tosyldextran (Experiment 4.8.) was methylated twice by the procedure described in the previous experiment. The infra-red spectrum showed methylation to be complete. The UV spectrum in ethanol solution showed the usual strong absorption at 223 nm. Detosylation was carried out in aqueous ethanol (80%) by the addition of a large excess of amalgam to the solution at 65° over 48 hr. After working up as described in the previous experiment the UV spectrum showed considerable de-esterification had been effected. The product was remethylated and examined by g.l.c. by the methods described previously. Isomaltosides were detected on the basis of retention times, expressed relative to octa-O-methylsucrose, (T_s, 1.48, 2.00, (2.60 minor, unidentified)) but not nigerosides.

This result confirmed the conclusion of the previous experiment, i.e. that the stabilised disaccharides were predominantly derivatives of isomaltose.

Experiment 4.11. Relative yields of methyl ethers from O-methyl-O-tosyldextran and O-methyl-O-triphenylmethyldextran.

The polysaccharide (10 mg) was heated with methanolic hydrogen chloride (3% w/v, 1 ml) at 100° for 41 hr. in a sealed tube. After neutralisation with silver carbonate, filtration and evaporation, the products were analysed by g.l.c. on column 1 at 175° (Pye Argon Chromatograph). 2,3,4-tri-O-methylglucose (T 2.34, 3.24; 2.35, 3.24, standard) and 2,4-di-O-methylglucose (T 7.14, 10.14; 7.13, 10.16, standard) were identified as the only major components of both methanolysates. The ratio of 2,4-di-O-methylglucose to 2,3,4-tri-O-methylglucose was calculated from the peak heights of both 2,4-di-O-methyl--glucoside peaks and the peak height of the faster 2,3,4-tri-O-methylglucoside peak (the slower peak was off scale).

dextran derivative	Sum of peak heights of 2,4-di-O-methyl--glucoside (cm)	Peak height of 2,3,4-tri-O-methylglucoside (cm) (faster peak)	2,4-di-O--methyl--glucose / 2,3,4-tri-O--methyl--glucose
-O-tosyl-	1.15	9.53	0.121
-O-triphenylmethyl	1.08	18.70	0.058

The results indicated that the yield of 2,4-di-O--methylglucose from O-methyl-O-tosyldextran was approximately 2.1 times that obtained from O-methyl-O-triphenylmethyldextran under these conditions.

Discussion

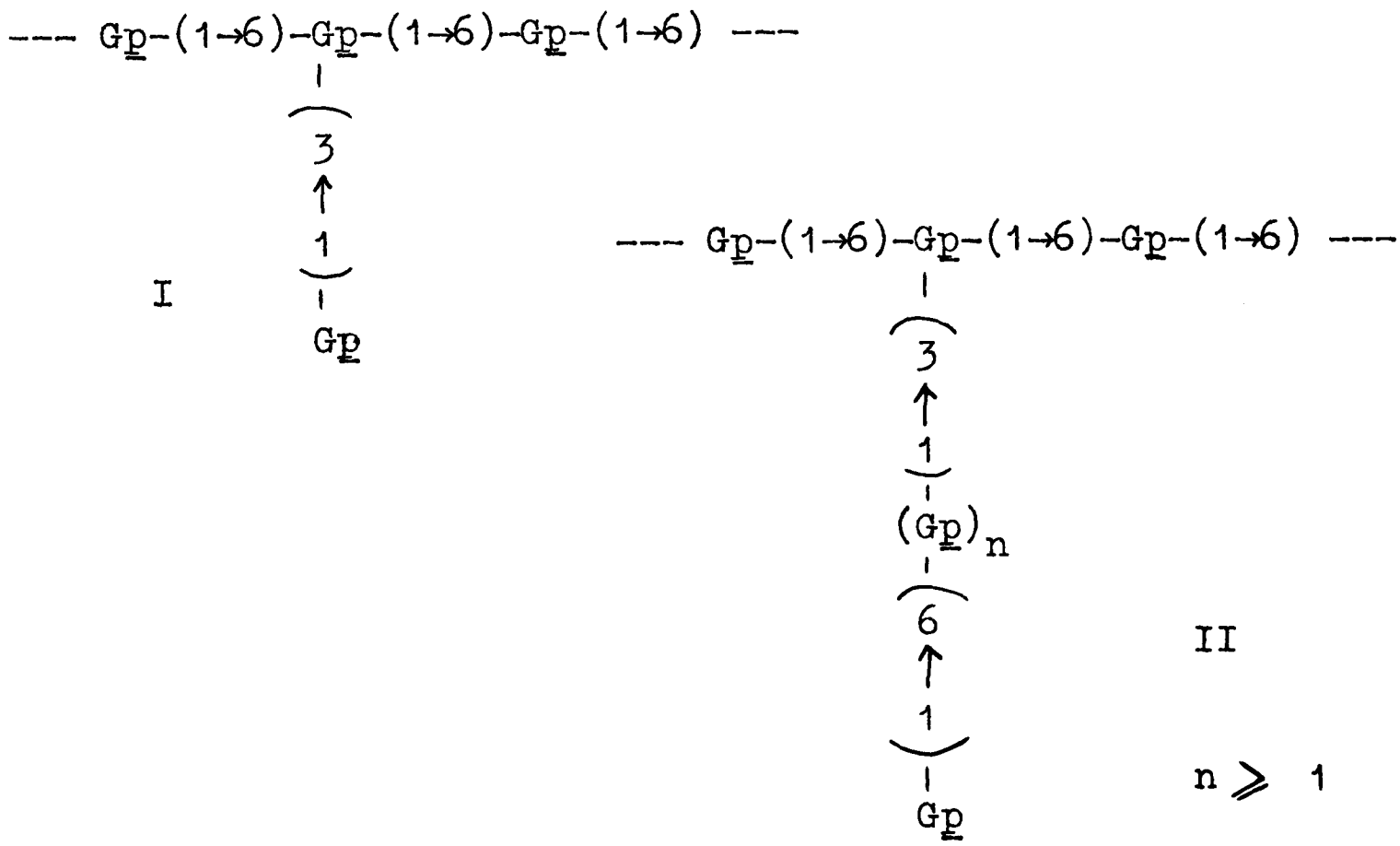
Analysis of the hydrolysis products of the methylated polysaccharide confirmed that the dextran from L. mesenteroides NCIB 2706 is of a well-known structural type in which 1,6-linked glucopyranose residues are branched through the $C_{(3)}$ position. Traces of 2,4,6-tri-O-methyl--glucose were also detected indicating that a small proportion of 1,3-linked inter-chain residues was also present, but no traces of 2,3-di-O-methylglucose, 3,4-di-O--methylglucose, 2,3,6-tri-O-methylglucose or 3,4,6-tri-O--methylglucose were found. The presence of 1,4-type linkages which were suggested on the basis of periodate oxidation (116) was thus not confirmed. The preparative-scale paper chromatographic separation of the hydrolysate (Experiment 4.2.) showed that 2,4-di-O-methylglucose accounted for 15% of the sugars present (on a molar basis). The accuracy of the procedure is low and considering this the result is in fairly good agreement with the degree of branching (approximately 20%) given by the p.m.r. and periodate oxidation studies (see Introduction).

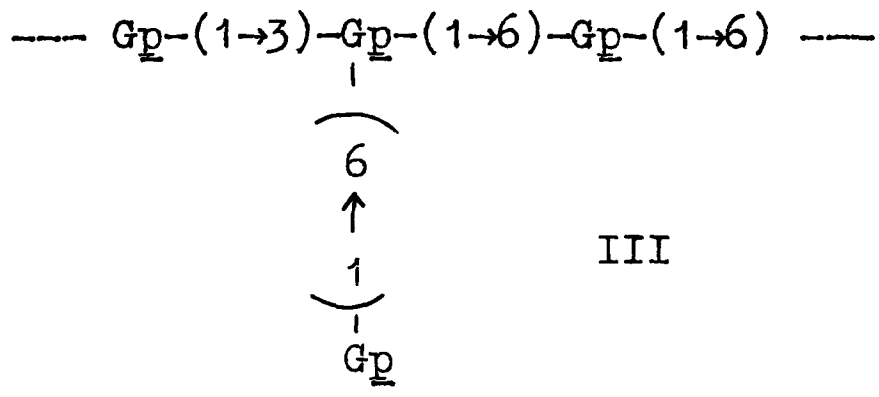
The survey of different solvolysis procedures with both dextran and O-methyldextran gave information as to the specificities of these reagents towards the 1,6- and 1,3-linkages. Goldstein and Whelan (95) have observed that whereas the 1,3-linkages of dextran are more labile than the 1,6-linkages in aqueous acid, the reverse is true under acetolysis (acetic anhydride and sulphuric acid) conditions. Mr. J. Campbell has found that the 1,6-linkages of O-methyl--dextran are preferentially cleaved under both methanolysis and acetolysis conditions, a similar procedure to Experiment 4.3. being used to determine the relative rate of hydrolysis of the two linkages. The results of Experiment 4.3. show that in dilute sulphuric acid or 50% aqueous chloroacetic acid, the 1,6-linkages of dextran are hydrolysed more rapidly than the 1,3-linkages. This was also observed in the chloroacetic acid hydrolysis of O-methyldextran (for solubility reasons hydrolysis of the methylated polysaccharide in dilute sulphuric acid was not attempted). Formic acid did not exhibit any marked specificity for either linkage in dextran or O-methyldextran. The observation that acid hydrolysis of dextran leads to preferential cleavage of the 1,6-linkage is contrary to that observed before.

The hydrolysis of O-methyl-O-tosyldextran, with the object of isolating the stabilised disaccharide fragments, was initially carried out in 50% chloroacetic acid. Methanolysis could also have been used but not dilute sulphuric acid as the tosylated derivative is even more

insoluble than O-methyldextran. Formic acid would lead to side-reactions as mentioned in the Introduction. Measurement of the average degree of polymerisation for samples withdrawn from the hydrolysate (Experiment 4.4.) showed that the reaction levelled off at about 80% hydrolysis (Diagram 1), consistent with the stabilisation expected from the degree of branching. The hydrolysis of O-methyldextran was also incomplete (about 90%) under similar conditions and hence not all of the stabilisation observed in O-methyl-O-tosyldextran is attributable to the tosyl ester. The conditions for the optimum yield of stabilised fragments was judged to be 72 hr at 84.5°.

Possible structures of the dextran are shown below. Hybrids of these structures are also possible.





III

Purification of the tosylated component(s) of the chloroacetic acid hydrolysate by T.L.C. using a variety of systems was unsuccessful as satisfactory separation from 2,3,4-tri-O-methylglucose - the other major hydrolysis product - could not be obtained. Similarly, attempts at purification by gel filtration were unsuccessful. Partition chromatography on cellulose (paper and column) was a limited success. Unfortunately the column fraction containing the tosylated components was heavily contaminated with non-carbohydrate material (presumably degradation products) due to the high mobility (R_G 1.14 in solvent B). This fraction after purification by T.L.C. showed two major components.

2,4-di-O-methylglucose was observed in the hydrolysis products by T.L.C., paper chromatography and in a fraction from the cellulose column, indicating that single unit side-chains of the type found in I are not present in substantial amounts (assuming the terminal linkage is stable).

After methylation of the 40.5 hr methanolysis product from O-methyl-O-tosyldextran the tosyl esters were removed by sodium amalgam. Remethylation and analysis by

g.l.c. then showed the presence of tetra-O-methylglucosides, hepta-O-methylisomaltosides and hepta-O-methylnigerosides in the approximate molar ratio of 100 : 54 : 4. When the treatment with amalgam was omitted the proportion os isomaltosides fell to less than one eighth of the value above; this arose from the incomplete hydrolysis of non-tosylated parts of the molecule. The proportion of the 58 disaccharide molecules that are attributable to incomplete hydrolysis of non-stabilised linkages and / or non-specific substitution may be calculated as follows. On the basis of the degree of branching (20%) and if methanolysis at non-stabilised linkages were complete, tetra-O-methylglucosides and disaccharide glycosides would be present in the (molar) ratio of 100 : 33. Thus if x molecules of disaccharide have arisen from incomplete hydrolysis and / or non-specific substitution then :

$$\frac{100 + 2x}{58 - x} = \frac{100}{33} \quad , \quad \text{i.e.} \quad x = 15.$$

Thus no more than 15 disaccharide molecules can have arisen in this way. Therefore 6-O-tosylisomaltosides must be present in at least tenfold excess over 6-O-tosylnigerosides and the structure of the dextran thus corresponds more closely to II or III than I. These conclusions were confirmed by Experiment 4.10. in which the tosyl ester fraction obtained by cellulose column chromatography of the chloroacetic acid hydrolysate of O-methyl-O-tosyldextran yielded only

isomaltosides, after methylation, detosylation and remethylation.

A further distinction between the possible structures is that only II should give derivatives of 2,4-di-O-methylglucose under cleavage conditions which retain the terminal linkage. This sugar has been identified in the hydrolysate. In quantitative terms, the relative yields of 2,4-di- and 2,3,4-tri-O-methylglucose derivatives from II should be in the ratio of 1 : 2, if the terminal linkage is retained. To avoid calibration, as would otherwise be advisable with compounds which contain different numbers of hydroxyl groups, the ratio was determined by g.l.c. relatively to O-methyl-O-triphenyl--methyldextran for which the ratio should be 1 : 4 (the trityl group confers no stability and is rapidly removed under acid conditions). A tosylated derivative of structure II should therefore yield twice as much 2,4-di-O-methyl--glucoside as this polysaccharide. The experimental figure (Experiment 4.11.) of 2.1 : 1 is within 5% of this figure and it is concluded that the dextran has a structure which approximates to II.

Shortly after this work was complete Lindberg and Svensson (121), by application of the catalytic oxidation procedure, reported side-chains of length greater than one unit in NRRL B-512 dextran.

It is probably a general drawback of the method that indirect structure proofs must be used to characterise

the tosylated disaccharide fragments. It would be more satisfactory to isolate the products in the pure state but the pronounced tendency of these compounds to streak in chromatographic systems, plus contamination with small amounts of products arising from non-specific substitution, complicate their purification.

The ultimate object of applying the method to the pectic araban from mustard seeds has not yet been achieved. The native araban, unlike dextran, is soluble in pyridine and Mrs. L. Alexander has successfully tosylated the polysaccharide directly with toluene-p-sulphonyl chloride at -15° in this solvent. Application of the methods described above would enable the terminal linkage(s) of the araban to be characterised, and hence conclusions as to the fine structure of the molecule to be drawn.

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