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Studies of the polysaccharides of white mustard (sinapis alba)

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STUDIES OF THE POLYSACCHARIDES OF WHITE MUSTARD (SINAPIS ALBA).

by

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To my Parents

PREFACE

I would like to take this opportunity to express my sincere appreciation of the constant guidance of Dr. D.A. Rees throughout the course of this work, and to thank him for his invaluable assistance at all times. I am also grateful to Professor Sir Edmund Hirst, C.B.E., F.R.S., for the provision of library, technical and laboratory facilities, and to many members of the research staff for their co-operation. Thanks are also due to the Institute of Paper Chemistry, Appleton, Wis., U.S.A., for the provision of a grant under their Pioneering Research Program.

Part of the work in Section 1 has been published in collaboration with Sir Edmund and Dr. Rees, and a reprint has been inserted at the end of the Thesis.

Neil G. Richardson.

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GENERAL INTRODUCTION

White mustard (Sinapis alba) is a member of the cruciferous family of flowering plants and is closely related to such commercially important vegetables as cabbage, turnip and cauliflower. It is cultivated in temperate regions throughout the world where, as a young plant, it is harvested for use as a salad constituent, and, as a mature plant of some two to four feet in height, it provides the seeds from which the familiar mustard powder is prepared.

The polysaccharides of many seeds have now been investigated(1). With the exception of the cereals, attention has been directed mainly towards those mucilagenous constituents which are readily obtained by extraction of the whole seed. The structures of these polysaccharides range from the relatively simple galactomannans and glucomannans(2,3) to complex mixtures of polysaccharides composed of several different sugar units.

An example of the latter type is found in the cold water extract of white mustard seed examined by Bailey and Norris(4) which was shown to contain a mixture of acidic polysaccharides and about 43 per cent of cellulose. An aqueous solution of the ethanol precipitated mucilage was fractionated by the addition of an equal volume of saturated barium hydroxide solution which precipitated a gel. The polysaccharide material remaining in solution was shown to comprise 7 per cent pentosan, 10 per cent 6-deoxyhexosan, 24 per cent uronic anhydride, 45 per cent hexosan and 5 per cent methoxyl. Hydrolysis of this material gave L-arabinose, D-galactose and an aldobiouronic acid composed of L-rhamnose and D-galacturonic acid.

The gel contained cellulose and a second acidic fraction which was extracted from the mixture with 4 per cent sodium hydroxide solution. This extract was purified by precipitation with Fehling's solution, and shown to contain 9 per cent pentosen, 15 per cent uronic anhydride, 72 per cent hexosan and 3.4 per cent methoxyl. Hydrolysis of the extract gave L-arabinose, D-galactose, D-galacturonic acid and D-glucuronic acid.

The mucilage was also fractionated by heating for a short time with dilute sulphuric acid, which caused the precipitation of the cellulose as a fibrous gel. After removal of the gel and further hydrolysis, arabinose, galactose and two aldobiouronic acids were detected in the hydrolysate. Analysis of the mixture of disaccharides indicated that it contained a uronosyl hexose and a uronosyl 6-deoxyhexose in the proportion of 2:1. Other examples of seed mucilages of complex structure are those from linseed(5) and from the seeds of the plantain family(6).

The object of the present work was to investigate the structures of cell wall polysaccharides, especially in relation to the changes that they undergo during cell growth. Germinating mustard seeds were chosen because they seemed to offer a convenient system for such a study.

The walls of growing cells are composed of carbohydrate with smaller amounts of other materials. The carbohydrate, in the form of cellulose, forms the basic skeletal structure of the wall and is arranged in strands of microfibrils. These, in turn, are interwoven to form a network which is embedded in the non-rigid matrix material consisting of hemicelluloses and pectic substances. The pectic material is formed during the early stages of growth(7) whereas lignin is formed mainly during the development of the secondary wall when both lignin and highly orientated cellulose microfibrils are deposited in large amounts.

Cell wall expansion is thought to occur by the deposition of microfibrils at the inner surface of the primary wall, accompanied by stretching and progressive orientation of the outer framework(8). This latter process must depend, to some extent, on the physical properties of the non-fibrillar matrix materials such as their ability to participate in hydrogen bonding and in the formation of salt bridges. Recent work, reviewed by Wilson(9), has failed to relate cell wall extensibility to any particular feature of these compounds and he points out that changes in the physical structure of the cell wall may be controlled by complex metabolic processes.

The main part of the work to be described in this thesis will concern one of the cell wall polysaccharides of mustard seed cotyledons, and the structural changes which it undergoes on germination. Germinating seeds were chosen for this investigation because of the ease with which germination can be brought about at any time of the year. Mustard seeds have the additional advantage of being fatty seeds which presumably do not have large amounts of reserve polysaccharides which would complicate the isolation and investigation of cell wall polysaccharides. The cotyledons appeared to be a particularly interesting subject for study. Examination of sections under the microscope(10) has shown that, when the seeds imbibe water and begin to germinate, the cell walls in the cotyledons swell (i.e. increase in thickness) and expand (i.e. increase in area). There is little increase in the amount of polysaccharide, but experiments in which ^{14}C-sucrose was fed to the seeds at the start of germination have shown that the pectic polysaccharides are not inert during this process(11). After a brief general survey of the polysaccharides present in the organs of the seeds, attention has been concentrated on the simplest component of the mixture of pectic polysaccharides extracted from the cotyledons.

SECTION 1.

A SURVEY OF THE POLYSACCHARIDES PRESENT IN MUSTARD SEEDS AND THE ISOLATION AND INVESTIGATION OF AN ARABAN FROM THE EMBRYOS.

SECTION 1

1(a) INTRODUCTION

Methods for the extraction and purification of polysaccharides must be carefully chosen in order that the minimum amount of structural modification consistent with good yield and a high degree of purity of the end product is achieved. This is greatly facilitated if the material can be obtained from its natural source without the use of chemical reagents, as is possible in the case of gum exudates and certain polysaccharides of bacterial origin such as dextrans, but the polysaccharides of the plant cell wall, whose function is partly to serve as a chemical and physical protection to the protoplasm, do not yield readily to mild extraction procedures.

The most accessible of these polysaccharides are the pectic substances which can be extracted efficiently with hot potassium hydroxide(12) or lime water(13); but, although these methods provide convenient means of obtaining araban and galactan components of pectic complexes, they cause de-esterification and degradation by β-elimination and other reactions(14). β-elimination can also occur in hot neutral solution(15) so that extraction with solutions of ammonium oxalate(16) or EDTA(17) may also involve degradation of the native polysaccharide. When hot solvents are used for extraction, there is probably risk of glycosidic hydrolysis of furanoside linkages if the pH is less than 3. It would therefore seem desirable to use solvents of pH 4-5 for the extraction of pectic polysaccharides.

After removal of the pectic substances from the cell wall, cellulose and hemicelluloses remain. In young cells, in which there is little lignification, the latter substances can be extracted with alkali, but if extensive lignification has occurred it is necessary to delignify the material prior to alkaline extraction. Delignification procedures involve oxidation and this probably results in structural modification of the polysaccharides as well as partial depolymerisation and possible contamination of the hemicellulose fraction with degraded cellulose(18). In addition to the degradation resulting from delignification, subsequent alkaline extraction of the hemicelluloses can also involve undesirable side reactions such as oxidation (if oxygen is not excluded) and alkaline degradation by a peeling reaction from the reducing end of chains of 1,3- or 1,4-linked sugar units(19). A further reaction which occurs is the saponification of the acetyl groups known to be present in some of these compounds. A mild method by which acetylated xylans have been successfully obtained from birch woods is that involving extraction with dimethyl sulphoxide(20,21).

The different extraction conditions required for these cell wall polysaccharides can be used to effect a crude fractionation(22) but before structural investigation can be carried out, purification and fractionation of the extract is necessary. After dialysis, to remove salts and low molecular weight material, the product may be contaminated with substantial amounts of protein. The extent of this contamination can be reduced by removing the insoluble protein after denaturation by heating, but this method is not generally effective in removing the contaminant completely. Other precipitation methods for removing proteins include change of pH, "salting out" by the addition of neutral salts such as ammonium sulphate, and precipitation with organic solvents, or trichloroacetic acid(23); the last of these methods being unsuitable when acid labile polysaccharides are to be examined. Purification may also be achieved by denaturation by shaking with chloroform-amyl alcohol(24), by preferential absorption on bentonite(25), by partition into aqueous phenol(26) or by destruction of the contaminating protein with proteolytic enzymes(27). On a smaller scale, chromatographic methods employing gel-filtration or chromatography on ion-exchange cellulose can be effective and are discussed later as methods of fractionation. An example of the use of the latter method is found in the separation of wheat flour pentosan and serum albumin reported by Neukom et al.(28).

Fractionation of a polysaccharide extract is an essential preliminary to structural investigation, as the homogeneity of a polysaccharide can only be inferred from failure to achieve resolution by any available method. This problem is best approached with a prior knowledge of the chemical nature of the constituents of the mixture but, if no obvious method is indicated, fractionation can only be carried out on a "trial and error" basis.

Methods involving fractional precipitation by the addition of organic solvents to aqueous solutions are effective for the separation of components differing widely in solubility properties. When separation is possible, the tendency for polysaccharides to co-precipitate often makes it necessary for such treatment to be repeated. Cross contamination can sometimes be avoided by employing the reverse of the above process, i.e. graded extraction with aqueous solutions of non-solvents from previously isolated polysaccharide mixtures. This method has been used for the preparation of araban from commercial citrus pectin(29). Other precipitation methods include the precipitation of acidic polysaccharides such as pectic acid by the addition of inorganic salts, quaternary ammonium compounds or mineral acid(16,30,31), and the precipitation of a variety of substances by the use of complexing agents. These include copper salts and borates whose use has been reviewed by Smith(1), and barium hydroxide and lead acetate solutions which have been used for the fractionation of hemicelluloses(18). The action of these reagents is to complex with hydroxyl groups in the polysaccharide molecule. If treatment with borates does not result in precipitation, the ionic nature of the complex may lead to the formation of a precipitate in the presence of quaternary ammonium cations(32).

Purified polysaccharides can be obtained from mixtures by the destruction of the other components either enzymically(1,p.62) or by the use of acid or alkali as in purifications of pectic substances(33) in which the araban component can be destroyed by acid, and the pectic acid by alkaline degradation. The pectic substances provide an example of a further method which involves purification by the formation of derivatives. Apart from the degradation of pectic acid which occurs on methylation, methylated araban can be separated from methylated galactan by extraction of the former into ether(33). Arabans can also be purified as their acetates. The advantage of derivative formation is that the products can be purified by precipitation with ether or petroleum ether without co-precipitation due to hydrogen bonding; however, purification involving methylation suffers from the disadvantage that the original polysaccharide cannot be recovered.

The above methods are most suitable for preparative purposes as large amounts of material can be conveniently handled whereas column chromatographic methods, although generally more selective, are tedious when used preparatively. Column techniques include the use of ion-exchange celluloses such as diethylaminoethyl (DEAE)-cellulose in various salt forms, which can give separation of components depending on their acidic nature or their ability to complex with borate ions(28). Fractionation on a molecular size and shape basis is possible by chromatographic methods using gels composed of cross-linked dextran molecules. The components of a mixture which are unable to penetrate the gel structure pass through the column unretarded while chromatographic separation of the other components of the mixture may be achieved(30). These two types of chromatographic processes can be superimposed by the use of ion-exchange "Sephadexes" but this does not necessarily give improved resolution as one process may obscure the effect of the other.

On a smaller scale, ultracentrifugation, free-boundary and zone electrophoresis can be used preparatively, but they are probably of more use as powerful analytical methods for following the progress of fractionation by other means.

1(b) EXPERIMENTAL

EXPERIMENT 1. COMPARISON OF THE SUGARS PRESENT IN THE ORGANS OF THE SEEDS BEFORE AND AFTER GERMINATION.

The organs of several seeds were separated manually (before and after germination for 48 hr.) and examined by hydrolysis and paper chromatography using the methods described in the Methods section. The results of visual comparisons of spot intensities are shown in Table 1.

TABLE 1

Organ	Sugars detected after hydrolysis		
	Strong	Medium	Weak
Before germination:			
seed coat	Glucose.	Arabinose, galactose, galacturonic acid,	Mannose, rhamnose, xylose.
embryo plant	Glucose.	Arabinose, galactose, galacturonic acid.	Mannose, rhamnose, xylose.
After germination:			
cotyledons	Glucose.	Arabinose, galacturonic acid.	Galactose, xylose.
seed coat	Galacturonic acid, arabinose.	Galactose, xylose, glucose.	
root	Galacturonic acid, glucose.	Arabinose, galactose.	Xylose.
material exuded* during germination	Galacturonic acid.	Galactose, glucose, rhamnose.	Arabinose, mannose.

* This material was the slime which formed round the germinating seeds.

EXPERIMENT 2. PRELIMINARY SURVEY OF THE POLYSACCHARIDES PRESENT IN THE EMBRYOS AND SEED COATS.

The seed coats (1 g.) and the embryos (3 g.), prepared by manual separation of the whole seeds, were each subjected to extraction with the following series of reagents: (a) boiling 80% ethanol (100 ml. for 5 mins.). (b) cold water (2 x 500 ml. at room temperature for 24 hr.). (c) boiling water (2 x 500 ml. for 4 hr.). (d) 0.5% aqueous ammonium oxalate (2 x 400 ml. for 3 hr. at 65-70°). (e) delignification by the chlorite method of Jermyn(34), followed by hot water extraction (2 x 400 ml. for 3 hr.). (f) 5% (w./v.) aqueous potassium hydroxide under nitrogen (400 ml. for 16 hr. at room temperature). (g) 20% (w./v.) aqueous potassium hydroxide under nitrogen (400 ml. for 16 hr. at room temperature). The insoluble material was filtered off, washed with water and dried in a desiccator.

After each extraction, the solid residue was isolated by filtration through muslin or, when substantial disintegration had occurred, by filtration through glass fibre paper. All fractions were finally clarified by filtration through glass fibre paper. The fractions extracted by reagent (a) were evaporated to dryness under reduced pressure to give brown solids. The cold water extracted material was concentrated to <u>ca</u>. 50 ml. and dialysed against distilled water (500 ml.) to give a sub-fraction which was concentrated and freeze-dried. The higher molecular weight material and the remaining fractions were exhaustively dialysed, concentrated and freeze-dried. The yields and the results of hydrolysis and paper chromatography are given in Table 2.

TABLE 2. RESULTS OF GRADED EXTRACTIONS.

Note: For each fraction, sugars are listed in approximately descending order of concentration; those in parenthesis being relatively minor components.

	Seed Coats		Embryos	
Solvent used for extraction	Yield (%)	Sugars detected after hydrolysis	Yield (%)	Sugars detected after hydrolysis
(a) 80% ethanol	6.4	Glucose (galactose)	14	Glucose (galactose)
(b) Cold water: dialysable	7.5	Glucose, xylose, mannose (arabinose, galactose)	2.4	Glucose, xylose, galactose (galacturonic acid)
non-dialysable	6.4	Galactose, arabinose, galacturonic acid (xylose, glucose, mannose)	2.4	Arabinose, glucose (xylose, galactose, galacturonic acid)
(c) Hot water	8.6	Arabinose, galactose	3.4	Arabinose (xylose, galactose, galacturonic acid)
(d) Ammonium oxalate	16.1	Galacturonic acid, xylose (arabinose, mannose, galactose)	1.7	Arabinose (xylose, galactose, galacturonic acid, glucose)
(e) Hot water (after delignification)	14.0	Arabinose, galacturonic acid (xylose, galactose)	8.5	Arabinose, galacturonic acid (xylose, galactose, rhamnose)
(f) 5% KOH	6.4	Xylose, galacturonic acid (glucose, galactose, mannose, arabinose)	9.8	Xylose, arabinose (glucose, galacturonic acid, mannose)
(g) 20% KOH	5.4	Xylose, mannose, glucose, galacturonic acid (galactose, arabinose)	1.7	Xylose, glucose (mannose, arabinose)
Residue	16.0	Glucose (galacturonic acid)	2.4	Glucose (arabinose, galacturonic acid.)

The fractions isolated by extraction with 80% ethanol and the dialysable sub-fractions of the cold water extracts were also examined directly by paper chromatography. It appeared that the main component in each case was sucrose, with an unidentified substance also being present in smaller amount. The chromatographic mobility of this component and the reactions given with spray reagents indicated that it was a ketose-containing tetrasaccharide. Traces of raffinose were also tentatively identified. Ketoses appeared to be absent from the remaining fractions as shown by the Seliwanoff test(35). These also failed to give any colouration with an acidified solution of iodine in potassium iodide and therefore did not contain any starch-like components.

EXPERIMENT 3. A MORE DETAILED STUDY OF THE POLYSACCHARIDE COMPONENTS OF THE EMBRYOS.

Whole seeds (1 kg.) were soaked in acetone (2 vol.) twice for 48 hr., and air dried. They were then crushed in a small hand mill and the seed coats and embryo plants partially separated in a draught of air. The fractions so obtained were de-fatted by extraction in the soxhlet with a mixture of chloroform and light petroleum (1:1), and further separated in an air-draught. The sequence de-fatting, crushing and separation in an air-draught was repeated until a sufficient quantity of the embryos was practically free from contamination by seed coats and other light material. They were milled to a flour, extracted with chloroform:light petroleum (1:1) for 48 hr. in the soxhlet and dried in air (369 g.). Exhaustive extraction of the flour with boiling 80% ethanol (6 x 21.) gave a mixture of oligosaccharides similar to the corresponding fraction isolated in the small-scale experiment (Experiment 2). The residue was then extracted with cold water (5 x 5 1), then boiling water (5 x 5 1.). In each case the combined extracts were concentrated under diminished pressure and freeze-dried. The residue was extracted with 0.5% ammonium oxalate solution at 60-70°C (5 x 5 1.), and the solutions were concentrated to 4 l. and dialysed against running tap water, when a precipitate began to form. After 3 days dialysis, the suspension was removed from the sac and calcium chloride (12 g.) in aqueous solution added until precipitation was complete. The precipitate was removed on the centrifuge, washed in turn with aqueous ethanol, ethanol and ether and finally dried <u>in vacuo</u>. The supernatant solution was freed from excess calcium chloride by dialysis and freeze-dried.

The results of this series of extractions are shown in Table 3 and possible reasons for the slight differences between these results and those reported in Table 2 are: (i) some of the fractions in this experiment were isolated without dialysis, (ii) the starting material in this experiment was extracted with organic solvents before use and (iii) the ammonium oxalate extract was concentrated before dialysis thus facilitating precipitate formation. The relative amounts of the various sugars obtained on hydrolysis of these fractions were very similar to those obtained in the previous experiment. Arabinose was the major hydrolysis product from all fractions except that obtained by extraction with 80% ethanol. Hydrolysis of the Ca^{++} precipitate gave arabinose with smaller amounts of galacturonic acid and glucose (<u>ca</u>. 10% each).

<u>TABLE 3. RESULTS OF GRADED EXTRACTION OF THE EMBRYOS</u>

Solvent used for extraction	Yield[+] (%)	Protein[†] content (%)	Carbohydrate[†] content (%)
80% Ethanol	27.7	not determined	
Cold Water	6.3	47	26.7
Hot Water	6.1	60	16.1
Ammonium oxalate:			
(Ca^{++} precipitate)	1.2	36	12.8
(supernatant)	2.4	10	12.3

[+] Expressed as percentage weight of de-fatted embryos.

[†] See Methods section.

EXPERIMENT 4. FRACTIONAL PRECIPITATION OF THE MATERIAL EXTRACTED FROM THE EMBRYOS WITH HOT WATER.

(1) Fractional Precipitation with Ethanol

A pilot experiment showed that the uronic acid containing polysaccharides were almost completely precipitated by the addition of 3 volumes of ethanol to an aqueous solution. Freeze-dried hot water extract (0.03 g.) was dissolved in water (5 ml.) and ethanol was added gradually to the solution. The precipitated material was removed from time to time by centrifugation and examined by hydrolysis and paper chromatography. The results are summarised in Table 4.

TABLE 4. RESULTS OF FRACTIONAL PRECIPITATION WITH ETHANOL

Fraction	Sugars detected after hydrolysis		
	Strong	Medium	Weak
Precipitated at 44% ethanol concentration	Arabinose.	Galacturonic acid, galactose and/or glucose*	Xylose.
Precipitated at 66% ethanol concentration	Galacturonic acid, arabinose, galactose and/or glucose.	Xylose.	
Precipitated at 75% ethanol concentration	Glacturonic acid.	Arabinose, galactose and/or glucose	Xylose, mannose, ribose.
Supernatant solution	Arabinose.	Galactose and/or glucose.	Xylose, ribose.

* These sugars were incompletely separated due to "streaking".

In view of the promising results obtained in the trial experiment, a large scale fractionation was carried out by adding ethanol to

75% (v./v.) concentration to a solution (0.5% w./v.) of the freeze dried extract in water. The precipitate was allowed to flocculate (1 - 2 hr.) and removed on the centrifuge. It was washed several times with ethanol, then with ether, and dried in vacuo (yield 7.4 g. from 21 g. of freeze-dried extract). The supernatant solution was concentrated to 150 ml. and used directly for the following experiment.

(ii) Fractional Precipitation with Acetone.

Gradual addition of acetone to the solution from above gave three precipitates. At each stage the solution was left at 0°C overnight, when an oil separated. This was isolated by decanting the supernatant solution, dissolving in water, concentrating to a small volume to remove most of the acetone, and freeze-drying. After the final precipitation, the supernatant solution was concentrated and freeze-dried. Analysis by the usual methods gave the results shown in Table 5.

TABLE 5. RESULTS OF FRACTIONAL PRECIPITATION WITH ACETONE

Fraction	Yield (g.)	Carbohydrate content (%)	Sugars detected after hydrolysis		
			Strong	Medium	Weak
(A) Precipitated at 65% acetone	4.9	7.7	Arabinose	-	Glucose, mannose, galactose.
(B) Precipitated at 78% acetone	2.8	9.2	Arabinose	-	Glucose, mannose, galactose, ribose.
(C) Precipitated at 90% acetone	2.2	11.5	Arabinose	-	Glucose, galactose, ribose, mannose.
Supernatant solution	3.4	20.4	Arabinose	Ribose.	Galactose, glucose.

Because of the probable low molecular weight of the carbohydrate material in these fractions, they were not dialysed, but were purified by deionisation followed by deproteinisation. Deionisation was effected by passing solutions of the three subfractions separately through columns of Amberlite IR-120 resin (H^+ form) and Amberlite IR-45 (OH^- form). The column effluents were concentrated and freeze-dried. Deproteinisation was effected by partition into phenol (Method 4) and the results of these purifications are shown in Table 6.

TABLE 6. PURIFICATION OF THE ACETONE PRECIPITATES

Fraction and initial carbohydrate content	Deionisation step			Deproteinisation step		
	Yield (g.)	Carbohydrate content (%)	Yield* (%)	Yield (g.)	Carbohydrate content (%)	Yield* (%)
A (7.7%)	1.74	22	100	0.31	81	78
B (9.2%)	1.67	16	100	0.25	82	79
C (11.5%)	1.23	16	75	0.19	74	74

* Calculated on the basis of carbohydrate content.

EXPERIMENT 5. FRACTIONATION OF THE ACETONE PRECIPITATES BY COLUMN CHROMATOGRAPHY ON DIETHYLAMINOETHYLCELLULOSE IN THE BORATE FORM.

The column was prepared by the method of Neukom et al.(28). Diethylaminoethylcellulose (DEAE-cellulose) (25 g.) was washed (by suspension, sedimentation and decantation) with 0.5 N-hydrochloric acid, distilled water, 0.5 N-sodium hydroxide and water again - the sequence being repeated three times. Prior to each wash with water, the paste obtained by decantation was de-aerated slowly in a vacuum desiccator. The material was finally brought into the borate form by suspension in 0.2 M-sodium metaborate solution. After de-aeration and a further wash with water, the column which was to be used was filled with water and the vigorously stirred slurry was siphoned into it, (the flow rate being adjusted so that the adsorbent settled slowly under gravity). The column was tested with methyl red indicator which was retained at the top of the column when water was used as the eluent, but which moved as a discrete band on elution with 0.2 M-sodium metaborate solution. This was taken as an indication that the adsorbent had been satisfactorily packed. The dimensions of the column thus prepared were 24 cm. x 1.5 cm. diam.

The general procedure for removal of sodium metaborate from column fractions was as follows. The solution was concentrated, passed through a column of cation exchange resin (Amberlite IR-120 H^+ form), concentrated further and freeze-dried. After removal of boric acid as volatile methyl borate, the product was redissolved and again freeze-dried.

(i) Chromatographic fractionation using step-wise elution.

A portion of the deionised, deproteinised acetone precipitate)A) (83 mg.) was applied to the column in water (3 ml.) and washed on with a further quantity of water (10 ml.). The flow rate was 1.0 ml./min. and 10 ml. fractions were collected automatically for analysis with the phenol-sulphuric acid reagents. The results of eluting the column with a series of solvents are shown in Table 7.

TABLE 7. RESULTS OBTAINED USING STEP-WISE ELUTION

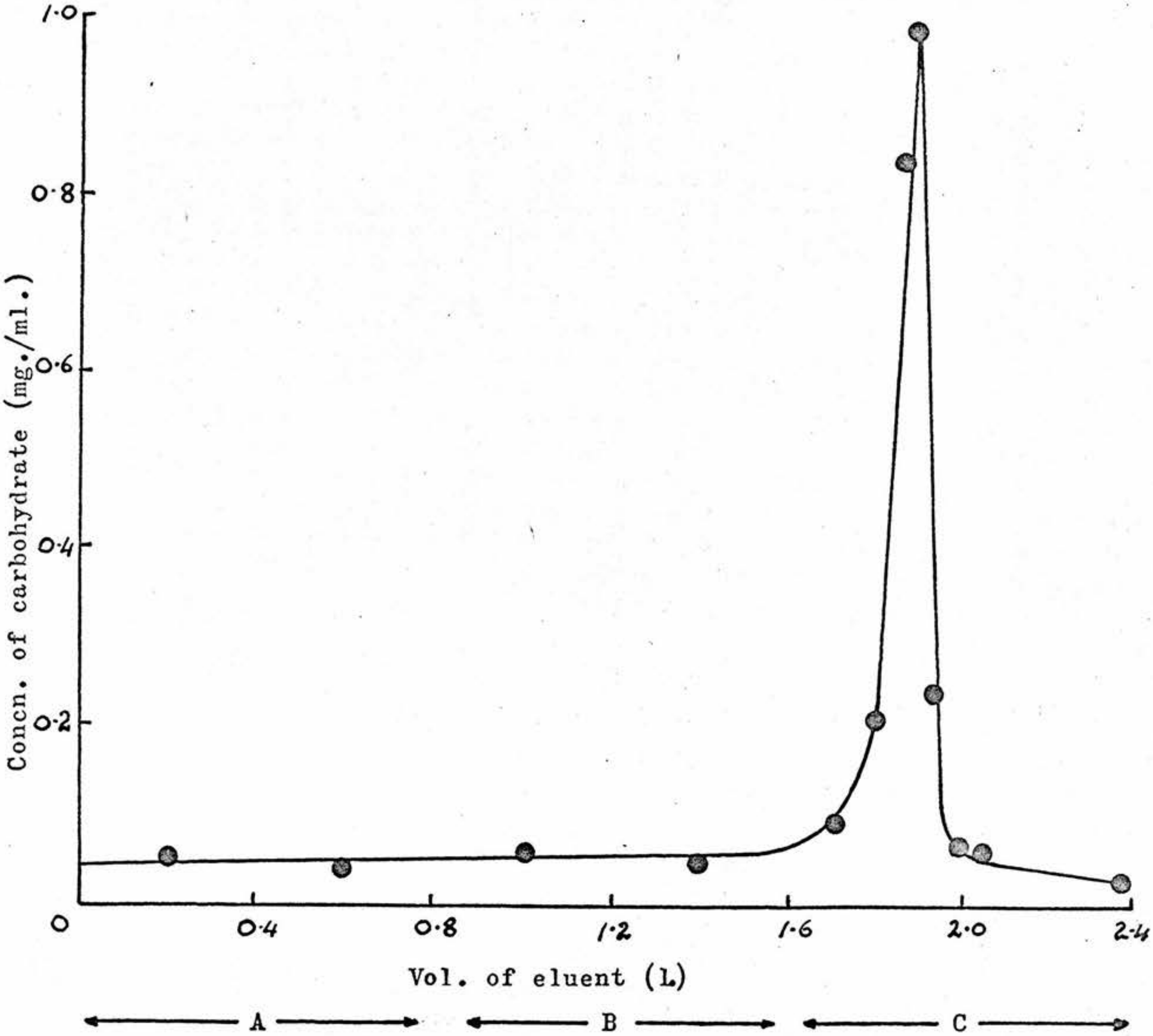
Eluent	Yield (mg.)	Carbohydrate content (%)	$[\alpha]_D$	Sugars detected after hydrolysis			
				Galatose	Glucose	Mannose	Arabinose
Water (320 ml.)	21	22	-	x	x	x	x
0.01 M-borate* (300 ml.)	7.3	-	-	x	x	x	x
0.10 M-borate (280 ml.)	20	85	-100°	trace	trace	trace	xxxx
0.50 M-borate (300 ml.)	7.6	80	-	xx	xx	x	xx

* Sodium metaborate solution.

(11) Chromatographic fractionation using gradient elution.

A further portion of the material was applied to the column in the same manner as above and the column was eluted with a linear concentration gradient from 0 - 0.5 M-sodium metaborate solution. The results indicated that a more gradual change in borate concentration was required in order to improve the separation and the following sequence of solvents was found to be suitable -
(a) water (900 ml.) (b) 0.01 M-borate (300 ml.) (c) a linear gradient from 0.01 to 0.1 M-borate (800 ml.) and (d) 0.50 M-borate (300 ml.). A flow rate of 1.0 ml. per minute was used and 10 ml. fractions were collected. These were analysed for carbohydrate by the phenol-sulphuric acid method and it was shown that most of the material eluted during the gradient elution moved as a sharp band. A typical elution diagram (obtained using the deionised, deproteinised acetone precipitate (B)) is shown in Fig. 1 and the

Fig. 1. PURIFICATION OF ARABAN ON DEAE-CELLULOSE.



Eluents: A, water; B, 0.01 M-borate; C, 0.01-0.1 M-borate (linear gradient).

corresponding results are shown in Table 8.

TABLE 8. GRADIENT ELUTION OF ACETONE PRECIPITATE (B)

Eluent	Yield (mg.)	Carbo-hydrate content (%)	$[\alpha]_D$	Sugars detected after hydrolysis			
				Galactose	Glucose	Mannose	Arabinose
Water	49.8	50	-45°	xxx	xxx	xxx	xxxx
0.01 M-borate	11.1	79	-110°	trace	trace	-	xxxx
Eluted as a "Peak" on gradient solution	98.5	92	-170°	-	-	-	xxxx
All other material	33.2	84	-140°	xx	xx	x	xxxx

Chromatographic purification of fraction (C) gave similar results but the araban was not obtained in such a high degree of purity (it was contaminated with traces of galactose, glucose, and rhamnose).

EXPERIMENT 6. LARGE SCALE PREPARATION OF CRUDE ARABAN

In the following experiment, the embryos were extracted with a hot solution of sodium diaminoethane-tetra-acetate ("EDTA") using the method described by McCready and McComb(17). Mustard seeds (4 x 2 kg.) were passed through a hand mill and immediately extracted with 90% methylated spirit at boiling point for 5 min. (5 l solvent per 2 kg. batch of seeds). After filtration and air drying, most of the seed coats were removed in a current of air, and the embryos were passed through a coarse seive which retained the unground seeds.

The material was again treated in an air-draught, and the remaining extraneous material was removed by hand. Yield of embryos: 2.9 kg. This product was extracted successively in the soxhlet for 48 hr. periods with methylated spirit (residue: 2.5 kg.) and then acetone (residue: 1.5 kg.). No further material was removed by similar extraction with chloroform or with light petroleum.

The unground embryos (1.5 Kg.) were extracted with 2 % (w./v.) EDTA solution (adjusted to pH 7.5 at 90°C; 3 x 5 l. for 3 hours each time), and then with water at 90°C (3 x 5 l. for 3 hours each time). It had been shown that, after the initial extractions with EDTA solution, the carbohydrate material could be effectively extracted with water alone. After each extraction, the suspension was filtered through muslin and the filtrate was left to stand overnight when the finer material settled out. After decanting the supernatant solution, the residue was combined with the coarser material for further extraction, and the solution was filtered through glass paper and concentrated. The residue from the extraction was washed with ethanol, then with ether, and dried in vacuo (665 g.).

The combined filtrates, after concentration to 5 l., were deproteinised by the usual method, but difficulties were encountered due to the formation of emulsions. Only one treatment with phenol was carried out at this stage and the solution, after removal of phenol by extraction into ether, was concentrated to 4 l. Ethanol (12 l.) was added and the precipitate was filtered off after 2 hours, dissolved in water (2 l.), and reprecipitated at the same ethanol concentration (yield, 75 g.) (Found: N, 1.41). The combined filtrates were concentrated to 1.5 l. and AnalaR acetone was added

(9 vols.) On leaving the solution overnight at 2°C, an oil separated out which was isolated by decantation, dissolved in water (500 ml.) and reprecipitated by the addition of nine volumes of acetone to the concentrated solution (200 ml.). The combined supernatant solutions were concentrated and freeze-dried. The oil was dissolved in water and passed through a mixed bed ion exchange column (1200 ml. IR-120, H^+ form and 1800 ml. IR-45, OH^- form). The effluent and washings, which were neutral and gave a negative flame test for sodium, were concentrated and deproteinised twice by the phenol method which presented no difficulties at this stage. On removal of residual phenol from the solution, concentration and freeze-drying, 11.5 g. of material were obtained which contained 2.0% protein and on hydrolysis gave arabinose (xxxx), galactose (x), glucose (x), and mannose (trace).

Hydrolysis of the acetone soluble fraction followed by paper chromatography showed the presence of arabinose with trace amounts of galactose glucose and ribose, while the ethanol precipitated material gave arabinose (xxxx), galacturonic acid (xx), xylose (xx), galactose (x), ribose (x), rhamnose (x) and glucose (trace).

EXPERIMENT 7. PURIFICATION OF THE CRUDE ARABAN BY PRECIPITATION WITH CETYLTRIMETHYLAMMONIUM HYDROXIDE.

The crude araban was purified by precipitation with cetyltrimethylammonium hydroxide. The method was essentially that described by Barker et al.(32). A solution (10% in water) of cetyltrimethylammonium bromide was converted into the hydroxide form by passage through a column of Amberlite IRA-400 resin

(OH^- form), and a part of this solution (2 ml.) was added to a solution of the crude araban (250 mg.) in water (30 ml.). On adding sodium hydroxide solution (1 N; 2 ml.), a flocculent white precipitate was formed. This was removed on the centrifuge after 20 minutes. (No precipitate was formed in the presence of cetyltrimethylammonium hydroxide alone or when boric acid was added). No further precipitate was obtained on addition of more detergent and alkali to the supernatant solution which was then neutralised with acetic acid, concentrated and freeze-dried. The precipitate was dissolved in water (30 ml.) containing sufficient acetic acid to give a neutral solution and then reprecipitated by the addition of alkali. After redissolution in dilute acetic acid,the detergent was precipitated by the addition of excess potassium iodide solution and removed on the centrifuge. The supernatant solution was deionised by passage through a column of mixed IR-120 resin (H^+ form) and IR-45 (OH^- form), concentrated and freeze-dried. (Yield 112 mg.) (<u>Found</u>: 98.4% anhydroarabinose) ($[\alpha]_D = -176^{\circ}$).

Deionisation of a portion of the non-precipitated material, followed by hydrolysis and paper chromatography, showed the presence of equal amounts of galactose, glucose and arabinose. The precipitated material gave only arabinose on similar treatment. By comparison with a standard mixture containing galactose, glucose, mannose, arabinose, xylose, rhamnose and galacturonic acid, it was shown by paper chromatography that if any of these sugars is present in the araban, its concentration must be less than 2%. No other components were detected on treating a paper chromatogram with the periodate-benzidine reagents (Method 8(d)).

EXPERIMENT 8. ELECTROPHORESIS OF THE ARABAN

The araban obtained by the precipitation procedure was shown, by electrophoresis (Method 9), to be indistinguishable from the araban obtained by chromatography on DEAE-cellulose. Using 0.05 M-sodium tetraborate as buffer (pH 9.2), it had $M_{\text{picric acid}}$ 0.16 and travelled as a single compact zone. This was shown to be due to borate complexing and not to any inherent acidity of the araban by the fact that it did not migrate ($M_{\text{picric acid}}$ 0.03) in 0.05 M-sodium carbonate-sodium bicarbonate buffer (pH 9.3) although it did migrate at higher pH ($M_{\text{picric acid}}$ 0.10 in 0.05 M-sodium carbonate of pH 11.4). This ionisation at higher pH was also indicated by the fact that the araban was held on strong base resin in the OH^- form but not on weak base resin.

The crude araban (obtained as the end product in Experiment 6) was shown by electrophoresis in 0.05 M-sodium tetraborate buffer to contain material of the same mobility as the araban and a similar amount of a non-migrating component. A portion of the material precipitated at 75% ethanol concentration in the same experiment was purified by dialysis and examined by electrophoresis in borate buffer. It gave a single fast moving zone with some tailing and contained no material of the same mobility as the araban.

EXPERIMENT 9. METHYLATION OF THE ARABAN

A portion of the araban prepared by the chromatographic procedure (Experiment 5) was methylated by the method of Kuhn and Trischmann(36). Araban (250 mg.) was dissolved in dimethylsulphoxide (20 ml.) and dimethylformamide (20 ml.). Barium hydroxide octahydrate (20 g.) and dimethyl sulphate (14 ml.; in four portions over 30 min.) were added to the efficiently stirred mixture at 0°C (ice bath). After stirring for 90 min. at this temperature, the ice bath was removed and the mixture was stirred at room temperature for 48 hr. Ammonia (sp. gr. 0.880; 10 ml.) was added and after being stirred for a further hour, the mixture was transferred to a separating funnel and diluted with water (200 ml.). The aqueous solution was extracted three times with 100 ml. portions of chloroform (emulsions were broken by the addition of EDTA and any remaining emulsion-like material was withdrawn with the chloroform extract). The combined chloroform extracts were washed several times with water, again retaining the residual interfacial material. After the final wash, this was retained with the aqueous phase and extracted with chloroform. The two chloroform solutions were combined, dried over anhydrous sodium sulphate and concentrated to a pale-brown glass. This was dissolved in the minimum amount of carbon tetrachloride and precipitated as an oil at 0°C by the addition of petroleum ether. The supernatant solution, which contained only trace amounts of carbohydrate, was decanted and the oil was redissolved in carbon tetrachloride and reprecipitated. Yield after drying in vacuo at 60°C, 0.225 g. (73%). (Found: OMe, 38.3%. Calc.: OMe, 38.7%).

Methanolysis followed by gas-liquid chromatography (Methods

11 and 12) gave results corresponding to a mixture of the methyl glycosides of 2,3,5-tri-O-methyl-, 2,3-di-O-methyl- and 2-O-methyl-arabinose. There was also some indication of traces of methyl 2,3,4-tri-O-methylarabinopyranosides. Formic acid hydrolysis (Method 7) followed by paper chromatography indicated the presence of the same sugars as gas-liquid chromatography, and also arabinose and a trace of a second monomethyl ether.

EXPERIMENT 10. SEPARATION AND CHARACTERISATION OF THE HYDROLYSIS PRODUCTS OF THE METHYLATED ARABAN

A stirred suspension of cellulose powder in acetone was siphoned into a column full of acetone at such a rate that the cellulose settled slowly under gravity. When the column had been packed, the composition of the solvent was gradually changed from pure acetone to that of solvent (i) below. (Final dimensions of column 50 cm. x 3.7 cm. diam.). Methylated araban (470 mg.) was hydrolysed with formic acid (Method 7). Evaporations were carried out carefully to reduce loss of the volatile 2,3,5-tri-O-methylarabinose, and the syrup was fractionated on the cellulose column using the following sequence of solvents: (i) water-saturated butan-1-ol - light petroleum (b.p. 100-120°C) (1:3, v/v ; upper layer); (ii) the same solvents mixed in the proportions 1:1 (upper layer); (iii) the same solvents mixed in the proportions 3:1 (upper layer); (iv) butan-1-ol half saturated with water. The syrup was applied to the column dissolved in a few ml. of solvent (i). About 800 ml. of each solvent were used for elution and the flow rate was 1 - 1.5 ml. per minute. Fractions (25 ml.) were collected automatically and examined by paper chromatography.

Solvent (i) eluted pure 2,3,5-tri-O-methyl-L-arabinose (180 mg.) and a mixed fraction containing 2,3,5-tri-O-methyl- and 2,3-di-O-methyl-L-arabinose (7 mg.).

Solvent (ii) eluted pure 2,3-di-O-methyl-L-arabinose (83 mg.) and a mixture containing 2,3-di-O-methyl-L-arabinose together with other sugars which, on the basis of chromatographic mobility, were thought to be 2,3,4-tri-O-methyl- and 2,4-di-O-methyl-arabinose (10 mg.).

Solvent (iii) eluted 2-O-methyl-L-arabinose (150 mg.) and a mixture containing, in addition, a small amount of a second component which might have been another mono-O-methyl ether.

Solvent (iv) eluted pure L-arabinose (25 mg.).

The relative amounts of the components present in the mixed fractions were estimated by visual comparison of spot intensities on paper chromatograms. By applying suitable corrections to the yields of the pure components, it was estimated that the methylated sugars were present in the hydrolysate in the following amounts: 2,3,5-tri-O-methyl-L-arabinose, 180 mg.; 2,3-di-O-methyl-L-arabinose, 90 mg.; 2-O-methyl-L-arabinose, 150 mg.; L-arabinose, 25 mg. and less than 5 mg. of any other methylated sugar.

The main components were characterised by the formation of either the toluene-p-sulphonylhydrazone or the amide of the derived aldonic acid. The results are given in Table 9.

TABLE 9. CHARACTERISATION OF THE HYDROLYSIS PRODUCTS OF METHYLATED ARABAN

Sugar	Derivative prepared	Melting Point			
		Observed	Literature	Authentic sample	Mixed
L-arabinose	Toluene-p-sulphonyl-hydrazone (37)	153°	154-5°	153-4°	153°
2-O-methyl-L-arabinose	Toluene-p-sulphonyl-hydrazone (37)	146°	144-6°	146°	146°
2,3-di-O-methyl-L-arabinose	Amide (38)	*158-9°	162°	*149-50°	151-2°
2,3,5-tri-O-methyl-L-arabinose	Amide (38)	136-7°	136°	137°	136°

* Although the authentic sample was obviously impure, these derivatives gave identical X-ray powder photographs.

EXPERIMENT 11. METHYLATION OF THE ARABAN BY OTHER METHODS.

(a) Barium hydroxide monohydrate was prepared by heating the octahydrate at 100°C in vacuo overnight (the system being continuously evacuated with a water pump). The equivalent weight of the product was determined by titration with hydrochloric acid and found to correspond to the monohydrate.

Araban (100 mg.) was dissolved in dimethylsulphoxide (2 ml.) and the resulting solution was diluted with an equal volume of N-methyl-2-pyrrolidone. After cooling the solution to 0°C in an ice bath, barium hydroxide monohydrate (0.4 g.) and methyl iodide (0.3 ml.) were added. The mixture was shaken at this temperature for 2 hr. then at room temperarure for a further 24 hr. The product was isolated by chloroform extraction and purified as described previously (Experiment 9) to give a similar yield of methylated araban (Found: OMe, 38.0% . Calc.: Ome 38.7%). Hydrolysis and methanolysis of this material showed it to be indistinguishable from the methylated araban previously obtained. A solution in carbon tetrachloride gave no hydroxyl absorption in the infrared

(b) The product from above was methylated further by the method of Kuhn *et al.*(39). Methylated araban (100 mg.) was dissolved in dimethylformamide (2 ml.), Methyl iodide (0.1 ml.) and silver oxide (190 mg.) were added to the solution at room temperature and the mixture was shaken for 16 hr., diluted with chloroform and filtered. The filtrate was shaken with aqueous sodium cyanide solution to dissolve silver salts, and the methylated araban was extracted with chloroform and purified as described in Experiment 9. Yield: 80 mg. The product was hydrolysed in formic acid and compared with the hydrolysate from (a) above by paper chromatography. No difference in the relative amounts of the products was observed.

EXPERIMENT 12. DETERMINATION OF THE MOLECULAR WEIGHT OF THE METHYLATED ARABAN.

This determination was carried out in benzene solution using a Vapour Pressure Osmometer (Model 301A, Mechrolab Inc., Mountainview, California), Methylated araban was prepared as described in Experiment 11(a) and the product was thoroughly dried *in vacuo* before weighing. Determinations were kindly carried out by Mr. J. Carlyle on solutions ranging from 1% to 6% concentration, and the result (7,500) was obtained by extrapolation to infinite dilution.

EXPERIMENT 13. EXTRACTION OF EMBRYOS WITH EDTA SOLUTION AT pH 4.9

The procedure used here was essentially that described in Experiment 6, but EDTA solution adjusted to pH 4.9 was used for the extraction. A batch of embryos (1.5 kg. after organic solvent extraction) gave 22 g. of crude araban. This material contained the same proportion of pure araban (determined by precipitation with detergent) and the same constituent sugars as the crude araban previously obtained (no uronic acids being present). Zone electrophoresis in borate buffer showed the two fractions to be identical.

EXPERIMENT 14. LABILITY OF THE ARABAN TO ACID HYDROLYSIS

The partial hydrolysis conditions of Andrews et al.(40) were used in this experiment. A solution of the araban in 0.01 N-sulphuric acid was heated at 80°C for 16 hours, cooled and then neutralised with solid barium carbonate. After filtration and concentration, the solution was examined by paper chromatography and shown to contain arabinose and smaller amounts of other components. This indicated that extensive hydrolysis had occurred under these conditions.

EXPERIMENT 15. EXAMINATION OF THE ARABAN BY PROTON MAGNETIC RESONANCE SPECTROSCOPY

The araban, the methylated araban and an acetylated araban (see below) were examined by proton magnetic resonance spectroscopy.

The araban was examined in D_2O solution (Method 16) and the two derivatives were examined in deuterochloroform solution. In addition, all three were examined in hexadeuterodimethylsulphoxide solution. The results are given in the Discussion.

The acetylated araban was prepared as follows. Acetic anhydride (0.3 ml.) was added to a solution of the araban (100 mg.) in pyridine (0.6 ml.). The mixture was heated at 100°C for 1 hr. in a stoppered test tube and then poured into a large volume of water. The precipitate which formed was filtered off, dried, and dissolved in the minimum amount of benzene. On diluting the solution with petroleum ether and pouring it into a large volume of this solvent, a white powder was obtained (110 mg.). A solution of this in carbon tetrachloride gave no hydroxyl absorption in the infrared. (Found: Ac, 37%. Calc.: Ac, 40%).

1(C) DISCUSSION

On graded extraction of both the embryos and seed coats of mustard seeds, polysaccharide material containing mainly arabinose, galactose and galacturonic acid was obtained first. As the extraction conditions became more severe, typical components of hemicelluloses (xylose, glucose and mannose) were increasingly more predominant as constituent sugars. Fructose was found to be present only in the oligosaccharides which were extracted almost completely with 80 % ethanol, and no evidence was found for the presence of starch in any of the fractions.

The results obtained on extraction of the seed coats with cold water differ from those reported by Bailey and Norris (summarised in the General Introduction) in that no glucuronic acid and only trace amounts of both glucose and rhamnose were found in the present survey. The early workers did not extract the seeds with ethanol prior to their investigation, and the possibility of enzymic action during cold water extraction cannot be excluded.

Two of the oligosaccharides in the 80 % ethanol extract have been identified(11). Sucrose, by the formation of the crystalline octaacetate and stachyose, by methylation and other methods. The other oligosaccharide in this fraction was probably raffinose as it was ketose-containing and gave approximately equal amounts of galactose and glucose on hydrolysis. It has been shown that these compounds are metabolised during germination and that they probably serve as energy reserves for the germinating seed(11). Nevertheless, analysis of the various parts of the seeds before and after germination revealed only minor variations in the relative amounts of the component sugars, suggesting little gross variation in the polysaccharides.

A main object of this investigation (see General Introduction) was to investigate any changes in molecular structure that occur in the cell walls of the cotyledons during the early stages of germination. An embryo preparation, which contained mainly cotyledons with a minor proportion of hypocotyls, was therefore obtained by de-fatting, winnowing etc. Graded extraction of this material showed that arabinose was the major component sugar of the more readily extracted polysaccharides, and that the other constituents of typical pectic substances were also present. Since preliminary experiments suggested that much of the arabinose was combined in a homopolysaccharide, the ultimate objective of fractionation was to obtain this araban in a pure form. The hot water extract was used to develop methods for this purpose and it was found that the uronic acid containing material could be readily removed by precipitation with ethanol. The main component sugar of the soluble fraction was arabinose, but smaller amounts of other sugars were also present at this stage. Purification of this material ("crude araban") by acetone precipitation, deionisation and deproteinisation, followed by chromatography on DEAE-cellulose, gave the araban ("mustard seed araban") in pure form.

Care had to be taken in this purification because of the possible low molecular weight and acid lability of the araban(33). It was necessary to use ion exchange resins rather than dialysis for the removal of salts, and a mild procedure for the removal of protein. The method of Westphal <u>et al</u>. involving partition of the protein into phenol, was found to be convenient and very effective for the removal of protein from mustard seed extracts. Difficulty was encountered initially due to the formation of stable emulsions, and several attempts to break these were unsuccessful. Separations were obtained on addition of n-butyric acid, but the most satisfactory method was that of simply adding more phenol to the system. In view of the low acidity of phenol (pK = 9.99), it is unlikely that any degradation occurred during deproteinisation. This was confirmed later by experiments which showed the homogeneity of the araban. In addition, random hydrolysis would probably have resulted in the detection of a certain amount of 2,5-di-O-methyl-L-arabinose in the methylation study but this was not observed.

There was a close similarity in composition between the material extracted from the embryos with cold water, hot water and ammonium oxalate, and these were therefore extracted together with EDTA solution in the preparation of a large quantity of araban for structural investigation. The araban itself was readily extracted under mild conditions (pH 4.9) but complete extraction of the other pectic materials was only possible with EDTA solution of pH 7.5. Although there was possibility of the degradation of acidic polysaccharides by β-elimination during extraction, it was necessary for other reasons (S.E.B. Gould, Ph.D. Thesis in preparation) to achieve complete extraction of all the pectic substances, and the more drastic conditions were therefore used. If the araban had been produced by β-elimination from a chain of 1,4-linked galacturonic acid units, it would have contained an α,β-unsaturated galacturonic acid residue with a characteristic absorption at 235 mμ(15). This was not observed. Furthermore, no araban would be expected on extraction at a pH at which β-elimination could not occur, but this was not found to be the case. The arabans from extraction at pH 4.9 and pH 7.5 were identical on electrophoresis (both travelling as compact zones) and both contained less than 2% of any other sugar unit. As it does not seem likely that the araban was a product of β-elimination, there was no disadvantage as far as the extraction of this polysaccharide was concerned, in using EDTA solution of the higher pH.

The results obtained during the purification of the EDTA extract were similar to those obtained on similar treatment of the hot water extract. Uronic acid-containing polysaccharides were removed by precipitation with ethanol, but the crude araban was purified by precipitation with cetyltrimethylammonium hydroxide as this was found to be more effective and more convenient than the chromatographic method. Cetylpyridinium hydroxide was also effective in precipitating the araban from alkaline solution but it is unstable and the coloured decomposition products were difficult to remove from the araban.

The fact that the araban moved as a discrete band on zone electrophoresis and when chromatographed on DEAE-cellulose, was a good indication of its homogeneity. Mr. Ian Steel has obtained additional evidence from the fact that it travelled as a single symmetrical peak on free solution electrophoresis in borate buffer, and on ultracentrifugation. The molecular weight of the araban (6,000) has been determined by Mr. Steel, using the short column ultracentrifugation method of Yphantis(41), and this value has been confirmed in the molecular weight of the methylated araban (7,500) determined by vapour pressure osmometry.

The results of the methylation analysis show the similarity between mustard seed araban and other pectic arabans. Hydrolysis of the methylated polysaccharide gave 2,3,5-tri-O-methyl-L-arabinose, 2,3-di-O-methyl-L-arabinose, 2-O-methyl-L-arabinose and L-arabinose in the molar proportions of 10:5:9:1.7. All these products were unambiguously characterised by the formation of crystalline derivatives. The above three methylated sugars have been consistently found in hydrolysates of methylated pectic arabans and the presence of arabinose is not unique as Hirst and Jones found small amounts of it in hydrolysates of methylated peanut araban(12). This had led to confusion in earlier work as it was not easily separable from 2-O-methyl-L-arabinose by the methods then available. The impure material, on conversion to the corresponding mixture of amides, gave a strong positive Weerman test for an α-hydroxy amide, and the mono-O-methyl ether was thus tentatively identified as 3-O-Methyl-L-arabinose(42). After the methylation study had been carried out (Experiments 9 and 10), it was reported that methylation with dimethyl sulphate and barium hydroxide octahydrate can give esterification of hydroxyl groups with methylsulphuric acid(43). The hydroxyl groups are then blocked to further methylation and a false impression of branching is given. The presence of arabinose in hydrolysates of the methylated araban from mustard seeds cannot be explained in this way. It was detected in about the same concentration after methylation with barium hydroxide monohydrate and methyl iodide, and after remethylation with methyl iodide and silver oxide. Nor was it a product of demethylation, as no change in its concentration was detected after hydrolysis for different lengths of time. (See Section 2).

Although the methylation analysis gave no indication as to the ring size of the arabinose units in the polysaccharide (other than at the non-reducing end groups), the extensive hydrolysis under mild acid conditions (Experiment 14) indicates that most of the units are of the furanose type. (Additional evidence of this is reported in Section 2). These are joined by 1,5-linkages with a considerable proportion of 1,3-branch linkages. The presence of arabinose in the hydrolysate of the methylated polysaccharide indicates that certain of the branched units are also linked through position 2.

Proton magnetic resonance spectroscopy provided information as to the configuration of the glycosidic linkages in the polysaccharide. The spectrum of the araban in D_2O solution (Fig. 2) shows a small peak at low field (peak 1) which has not been satisfactorily assigned. Peak 4 corresponds to the protons in HOD, present in the solvent. The anomeric protons in monosaccharides generally resonate at lower fields than the other protons of the ring(44), and this is also true of polysaccharides(45). Peaks 2 and 3 were therefore assigned to the anomeric protons of the araban, and the peaks at 5 were assigned to the remaining protons. The area of the peaks at 5 was approximately five times the sum of the areas of peaks 2 and 3, in confirmation of this assignment. Although peaks 2 and 3 were not separated in this case (see below), it is likely that each of them corresponds to one of the main types of linkage in the araban (i.e. a 1,3- or a 1,5-linkage), as this slight separation was observed on several spectra and was therefore significant. If it is assumed that most of the linkages are furanose, the fact that the coupling constant for peak 3 was less than 3 c/s indicates

PROTON MAGNETIC RESONANCE SPECTRA OF ARABANS.

Fig. 2. The araban in D_2O solution.

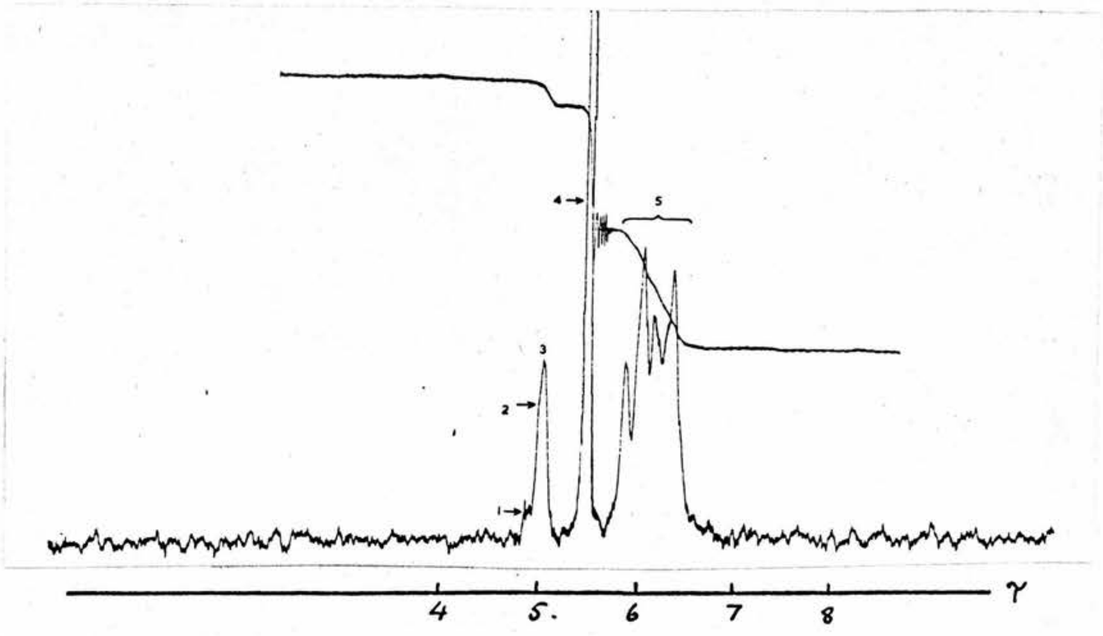
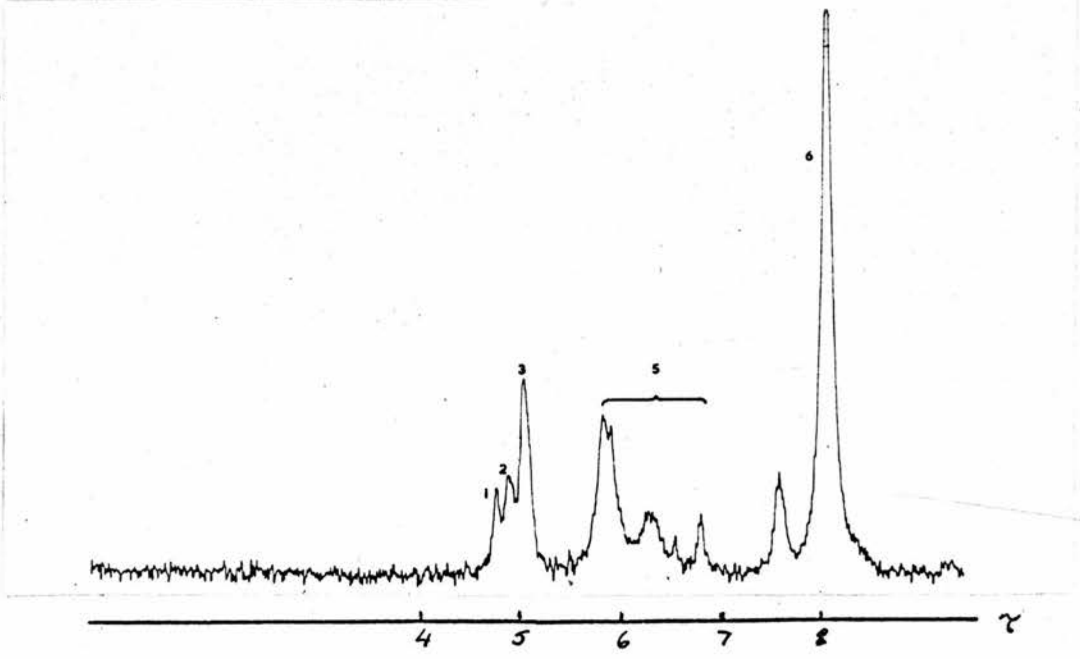


Fig. 3. The acetylated araban in hexadeuterodimethylsulphoxide solution.



Peak assignments are given in the text.

that these are α-linkages. (Methylα-L-arabinofuranoside has $J_{1,2}$ = 1 c/s, methylβ-L-arabinofuranoside has $J_{1,2}$ = 4 c/s while the values for the corresponding pyranosides are 8 c/s and 2.5 c/s respectively(46)). Of the other spectra which were examined, only that of the acetylated araban in hexadeuterodimethylsulphoxide solution (Fig. 3) gave any additional information. In this case, peaks 1,2,3 and 5 were assigned as above, and peak 6 was assigned to the protons of the acetyl groups. Peaks 2 and 3 were well separated, and the absence of any appreciable splitting (less than 3 c/s) was indicative of the α-configuration for both the 1,3- and the 1,5-linkages. This was in agreement with the negative optical rotation of the araban. However, more definite proof is required (especially in the case of the 1,3-linkage) as Andrews et al.(40) reported the isolation of 3-O-L-arabinofuranosyl-L-arabinose from sugar beet araban. This had a higher rotation than that reported for another 3-O-L-arabinofuranosyl-L-arabinose(47), and was presumably β-linked.

The araban was electrophoretically mobile in borate buffer at pH 9.2. As it did not migrate in non-borate buffer of similar pH, it was evident that this was due to borate complexing, but the mode of formation of the complex is not apparent from the methylation study. It is possible that the borate ion complexes with hydroxyl groups which are distorted due to the highly branched nature of the polysaccharide. Another possibility is that the araban, in solution, contains regions with high concentrations of hydroxyl groups, some of which will satisfy the spatial relationships necessary for complex formation. In such a situation complexes of very large ring size may be formed, and complex formation of this type has been observed where the smallest possible ring size involved 20 atoms(48).

Recent investigations of pectic substances have indicated their structural complexity. A pectic polysaccharide from lucerne has been shown to contain galacturonic acid, galactose, arabinose, rhamnose and xylose with smaller amounts of fucose, 2-O-methylfucose and 2-O-methylxylose(16). Similar complexity has been observed in the pectic substances from apple(30) and sisal(49). The fact that some of the neutral components are located within chains of galacturonic acid units is indicated by the isolation of the corresponding acidic oligosaccharides on partial acid hydrolysis. Most of the arabinose units, however, appear to be present as "blocks" on the periphery of the molecule. They can be removed by mild acid hydrolysis, and this has been found to be true of the acidic fraction of mustard seed pectin(50). A further indication of the presence of these "blocks" of arabinose units was obtained from degradative studies on a pectic polysaccharide from apple(30). The polysaccharide was extracted under slightly acid conditions and found to contain 87% galacturonic acid, 9.3% arabinose and smaller amounts of the other sugars mentioned above. It was degraded by transelimination at pH 6.8 and the eliminated fragment was found to contain 31% galacturonic acid and 51% arabinose. As this arabinose-rich polysaccharide was produced by β-elimination, it is suggested that some of the arabans investigated previously may have been produced as a result of the alkaline conditions used for extraction or methylation. Although it is possible that this has been the case, it does not appear to be true of mustard seed araban.

SECTION 2.

THE QUANTITATIVE GAS-LIQUID CHROMATOGRAPHY OF CARBOHYDRATES AND ITS APPLICATION TO THE ELUCIDATION AND COMPARISON OF POLYSACCHARIDE STRUCTURES.

SECTION 2

2(a) INTRODUCTION

The application of gas-liquid chromatography (GLC) to problems in carbohydrate chemistry has developed rapidly in recent years. It is used for both qualitative and quantitative analysis, and instruments for the separation of compounds on a preparative scale are now commercially available. The process involves repeated partition of the components of a mixture between a stationary liquid and a mobile gas. The carrier gas is passed through a heated column containing the liquid phase coated on a uniformly packed solid support of large surface area. When a sample is injected into this system, it is vapourised and resolved into its components on passage through the column.

Various instruments can be used for the detection of components in the effluent gas stream, and the most sensitive of those in common use are the ionisation detectors. An example of this type was used for most of the work to be described in this section, and involves the use of argon as the carrier gas. In this detector, a high voltage is maintained across two electrodes, and the argon passing between them is excited to a metastable energy level by a source of beta-radiation. Components emerging from the column are ionised by transfer of energy from the excited argon atoms, and the resulting change in current across the electrodes is amplified, and recorded on a moving chart. In another instrument of this type, components are detected in a similar way after thermal ionisation in a hydrogen flame. Apart from their high sensitivity, these detectors have the additional advantage of stability to minor fluctuations in temperature and flow rate.

The efficiency of a column is determined by the number of theoretical plates which it represents and this should be as high as possible in order to achieve maximum resolution. The solid support should be of small uniform particle size, and columns must be packed with care to avoid irregularities. Optimum conditions of temperature and flow rate must be chosen, and the nature and thickness of the liquid phase are equally important. The great selectivity of GLC generally makes it unnecessary for all these factors to be considered, but many of the theoretical requirements are met as a matter of routine. In order to keep running time to a minimum, temperatures are usually as high as is consistent with satisfactory resolution, the stability of the liquid phase often being a limiting factor in this respect.

The most important part of the apparatus, as far as the resolution of a mixture is concerned, is the liquid phase. These are either polar or non-polar and, generally, the polar phases are more selective. Non-polar phases, however, have the advantage of thermal stability and are useful for the separation of compounds which have a relatively large number of unsubstituted hydroxyl groups, or a high molecular weight. Thus, disaccharide acetates have been separated on a non-polar column at about 240°C.(51) Under these conditions, the mixtures of acetates from reducing disaccharides gave broad peaks and separation of anomers was poor. This is often the case with compounds of low volatility which require high operating temperatures.

Although polar liquid phases are more selective, many of them are relatively unstable at high temperatures. Under these conditions, the liquid phase slowly bleeds off the column, contaminating the detector and reducing column efficiency. However, this is less true of the polyglycols, one of which (Carbowax 20M) has been used for the separation of the acetates of various polyhydric alcohols at temperatures up to 225°C.(52) The polar nitrilesilicone polymer (XE-60) also has the advantage of stability at relatively high temperatures. The high resolving power of the less stable polar liquid phases can be made available for the separation of compounds of low volatility by reducing the amount of the liquid phase. The compounds are then less strongly held, and lower temperatures can be used with a corresponding increase in resolution. Gee and Walker(53) found that, by using thinner coatings of polyester and columns of smaller internal diameter, they could separate fully methylated di- and trisaccharides at a temperature 20°C lower than would otherwise have been possible.

An advantage of the non-polar phases (low retention volumes) can be combined with the high selectivity of the polar phases by the use of columns containing intimate mixtures of both. (The liquid phases having been coated on separate batches of solid support). By using this technique, separation of the anomers of various unsubstituted methyl glycosides was possible at about 200°C(51).

An important technical development in the field of GLC is that of temperature programming. If a sample contains a range of components which differ widely in volatility, the low temperature required for the separation of the slower moving components may give slow elution and correspondingly poor resolution of the others. In such a case, the sample can either be analysed in two operations or by the use of programmed temperature GLC. This involves the speeding up of the slower moving components by gradually raising the temperature of the column during the analysis. Two columns and two ionisation detectors are used. One of the columns effects the separation of the components and the other compensates for the change in signal resulting from the increase in temperature and additional bleeding of liquid phase from the column. By using an instrument of this type, a mixture containing the trimethylsilyl ethers of carbohydrates ranging from erythrose to trisaccharides was analysed completely in 75 minutes(54).

The application of GLC to carbohydrate chemistry is limited to the separation of those derivatives which are stable and volatile at the temperatures used. Unexpected reactions have been observed during the chromatography of certain types of compounds(55) but acetates and ethers have generally proved to be suitable. Methylation has been widely used to obtain volatile carbohydrate derivatives - in fact the first application of GLC to carbohydrate chemistry was in the separation of some fully methylated methyl glycosides(56). GLC has since been applied to the separation of complex mixtures of the isomers and anomers of methylated methyl glycosides such as are obtained on methanolysis of methylated polysaccharides. Many examples of this, and other applications of GLC to the separation of carbohydrate derivatives, have been included by Bishop in his excellent review of the subject(57).

Trimethylsilyl (TMS) ethers of carbohydrates are particularly suitable for GLC as they are easily prepared and the parent sugar can be recovered by mild hydrolysis in 50% aqueous methanol(58). Preparation involves shaking the material for a few minutes with hexamethyldisilazane and chlorotrimethylsilane in pyridine, and the reaction mixture can then be examined directly by GLC(54). Good resolution of anomers is possible on both polar and non-polar columns and a wide range of carbohydrates, including oligosaccharides(54), have been chromatographed as their TMS derivatives.

The acetates of carbohydrates are also readily prepared, and these have an advantage over TMS derivatives in that they do not decompose in the presence of moisture. The degradative rearrangement of acetylated amino sugars has been reported(55) but this does not prohibit the chromatography of acetates of carbohydrates in general. The volatility conferred by the acetyl group made possible the separation of some mono-O-methylglucoses as the pentaacetates of the corresponding glycitols(59) and even disaccharide acetates are sufficiently volatile to be amenable to GLC(51).

As previously stated, the high selectivity of GLC for the separation of complex mixtures of carbohydrates has been widely used for the rapid analysis of the methanolysis products of methylated polysaccharides. Although identifications can only be tentative, valuable information can be obtained. Under a given set of methanolysis conditions, the relative amounts of the anomers and possible ring forms of a methylated sugar are characteristic(60). The fact that one particular sugar unit in a methylated polysaccharide may give as many as four resolvable glycosides on methanolysis, is a distinct advantage for qualitative analysis. This is not true however, when quantitative information is required as the areas of incompletely resolved peaks cannot easily be measured. By converting a mixture of sugars to the corresponding glycitol derivatives, only one peak will be obtained from each of the components, but the loss of their unique ring forms may result in poor resolution on GLC. If the compounds differ sufficiently in polarity, this effect will be relatively unimportant and this was the basis of the work now to be described.

Because of the difficulty of obtaining large amounts of mustard seed araban, a method was required which would enable quantitative structural analysis to be carried out on small amounts of material. This would enable minor structural modifications, such as might occur during germination, to be detected. In addition, it would be possible to assess the effect of degradative experiments on the polysaccharide, without sacrificing large amounts of precious material. As hydrolysis of the methylated araban gave a series of products which differed in the degree of substitution of their hydroxyl groups, it was thought that the hydrolysis products would be amenable to quantitative analysis (by GLC) as the corresponding mixture of acetylated arabitols.

Several previous quantitative analyses of carbohydrate mixtures have been based on the assumption that ionisation detectors give a response which is related to the mass or concentration of the components. It is also assumed that the constant of proportionality is the same for structurally similar compounds and that the linearity is relatively unaffected by changes in operating conditions(57). On this basis, the relative amounts of the components in a mixture can be determined by comparing the areas under the various peaks. However, in several instances, these assumptions have been examined and found to be untrue, particularly for the Argon detector (61, 62, 63, 64). It therefore seemed advisable to calibrate the instrument for the particular components to be separated, and in this way to eliminate any possible errors in the preparation or chromatography of samples. For this reason, a suitable series of pure crystalline standards was prepared.

2(b) EXPERIMENTAL

EXPERIMENT 16. EXTRACTION OF THE ARABAN AFTER GERMINATION

Mustard seeds were germinated for 4 days (Method 1) and the freeze-dried products were separated into their component organs by hand. (This work was done by Mrs. S.E.B. Gould). The cotyledons (200 g. after extraction with organic solvents) were extracted with EDTA solution in the manner described in Experiment 6 (residue after drying, 132 g.). The concentrated EDTA extract was adjusted to 75% ethanol concentration, and the precipitate was removed by filtration (110 g.). Concentration of the supernatant solution followed by acetone precipitation, deionisation and deproteinisation gave crude araban (1.2 g.). Hydrolysis of this material gave mainly arabinose with smaller amounts of galactose and glucose. On purification with cetyltrimethylammonium hydroxide, a pure araban (0.50 g.) was obtained. (Found: 98% anhydroarabinose, $[\alpha]_D = -176^\circ$). This material gave only arabinose on hydrolysis and was indistinguishable from the original araban on zone electrophoresis in borate buffer, and on ultracentrifugation.

EXPERIMENT 17. METHYLATION OF THE ARABAN EXTRACTED AFTER GERMINATION.

Part of the product from above was methylated by the procedure used in Experiment 11(a) and a similar yield of methylated araban was obtained. (Found: OMe, 38.0%. Calc.: OMe, 38.7%). Neither hydrolysis followed by paper chromatography nor methanolysis followed by qualitative GLC revealed any difference between this methylated araban and those obtained from the embryos before germination.

However, a pronounced difference was detected by quantitative GLC (see Experiment 23).

EXPERIMENT 18. PREPARATION OF METHYLATED SUGARS TO SERVE AS REFERENCE COMPOUNDS FOR QUANTITATIVE GLC

(i) 2,3,5-tri-O-methyl-L-arabinose.

L-arabinose (20 g.) was shaken for 6 hr. with methanolic hydrogen chloride (0.9% ; 400 ml.). The solution was neutralised with silver carbonate, filtered and concentrated to a syrup. Part of this mixture of methyl α- and β-L-arabinofuranosides (4.0 g.) was methylated by the method described in Experiment 11(a). The product (4.2 g.) was shown by GLC (Method 12) to contain methyl 2,3,5-tri-O-methylarabinofuranosides together with small amounts of methyl 2,3,4-tri-O-methylarabinopyranosides. Formic acid hydrolysis, followed by preparative paper chromatography on Whatman 3 MM paper (Method 8) gave pure 2,3,5-tri-O-methyl-L-arabinose (2.5 g.). The separation was effected by double development with butan-2-one - water - ammonia (200:17:1).

(ii) 2,3-di-O-methyl-L-arabinose.

L-arabinose (20 g.) was converted to the mixture of methyl arabinosides by the method described above, but instead of neutralising the acid with silver carbonate, an adaptation of the method of Ness and Fletcher for the preparation of glycoside benzoates was used(56). The acid was neutralised by adding excess of pyridine, and the solvents were removed by distillation under reduced pressure at 70°C. Residual pyridine was removed by co-distillation with methanol. An attempt to block position 5 by selective O-toluene-p-sulphonylation of part of the resulting syrup by the method of Cifonelli *et al.*(66), was followed by methylation, reductive de-tosylation and hydrolysis, but this gave an unsatisfactory mixture of products. Tritylation was found to be more specific. One fifth of the syrupy mixture of glycosides was dissolved in anhydrous pyridine (200 ml.) containing triphenylchloromethane (1.2 moles), and the solution was left at room temperature for 48 hr. The reaction mixture was poured into water (2 l.) and the aqueous solution was extracted three times with chloroform. The chloroform extracts were washed three times with water, dried with anhydrous sodium sulphate, filtered and concentrated to a syrup. The last traces of pyridine were removed by co-distillation with methanol. The product was methylated by the method described in Experiment 11(b) using methyl iodide and silver oxide as methylating agents, and was then hydrolysed with formic acid. Because of the dark colouration produced during hydrolysis, it was necessary to pass an aqueous solution of the hydrolysate through a pad of decolourising charcoal four times. The resulting clear solution was evaporated to dryness and the product was shown by paper chromatography to be pure 2,3-di-O-methyl-L-arabinose. (Yield: 0.7 g.)

(iii) 2-O-methyl-L-arabinose.

L-arabinose (20 g.) was dissolved in 2% methanolic hydrogen chloride (200 ml.) and the solution was boiled under reflux for 8 hr. After neutralisation with silver carbonate followed by filtration, colloidal silver salts were precipitated by the passage of H_2S gas and filtered off. The solution was concentrated to a syrup and dissolved in methanol. Concentration of the methanol solution gave crystalline methyl β-L-arabinopyranoside (6 g. m.p., 166°C. Lit., 168°C(67)). This was dissolved in acetone (100 ml.) containing concentrated sulphuric acid (1 ml.). After 48 hr. at room temperature, the solution was neutralised with sodium bicarbonate, filtered and concentrated to give syrupy methyl 3,4-O-isopropylidene-β-L-arapinopyranoside (1.8 g.). Part of this material (1.3 g.) was methylated by the procedure used in (ii) above. Formic acid hydrolysis then gave 2-O-methyl-L-arabinose which was almost pure, as shown by paper chromatography.

EXPERIMENT 19. PREPARATION OF CRYSTALLINE DERIVATIVES FOR CALIBRATION OF GLC.

Preparations of the following derivatives of 2,3,5-tri-O-methyl-L-arabinose were attempted unsuccessfully: the p-nitrobenzoate and the 3,5-dinitrobenzoate by the method described by Rebers and Smith(68) for the preparation of similar derivatives of methylated mannoses, and the α-naphthyl carbamate and the phenyl carbamate by the method described by Bouveng(69) for the general preparation of phenyl carbamates. All the reactions appeared to go to completion as shown by thin layer chromatography (Method 10) but none of the products could be induced to crystallise. Attempts were made to purify the p-nitrobenzoate and the phenyl carbamate by chromatography on alumina and on silica-gel columns but still no crystalline product could be obtained.

Crystalline derivatives were eventually prepared after converting the methylated sugars to the corresponding arabitol derivatives. The sugar (<u>ca</u>. 100 mg.) was dissolved in water (20 ml.), excess potassium borohydride was added and the solution was left at room temperature overnight. The completeness of the reaction was checked by testing a small sample with the phenol-sulphuric acid reagents, and the excess borohydride was destroyed by the addition of dilute hydrochloric acid. The solution was evaporated to dryness on a rotary film evaporator and boric acid and the last traces of water were removed by repeated evaporation of a 1:1 mixture of benzene and methanol. The residue was dissolved in anhydrous pyridine (10 ml.) and p-nitrobenzoyl chloride was added (2.5 moles per mole of OH group). The solution was heated at 65-75°C for 30 min. and the completeness of the reaction was checked by thin layer chromatography. (Spots were located by spraying the plates with 4 N-sulphuric acid and heating over a bunsen flame for a few seconds). The reaction mixture was neutralised by the gradual addition of a saturated solution of sodium bicarbonate (with warming to prevent solidification) then diluted with water (20 ml.) and extracted three times with 10 ml. portions of chloroform. The combined chloroform extracts were washed three times with water, dried over anhydrous sodium sulphate and concentrated to a small volume. Residual pyridine was removed by co-distillation with methanol. The product was crystallised from a suitable solvent and the following derivatives were thus obtained (melting points have been corrected):

(i) 2,3,5-tri-O-methyl-1,4-di-O-p-nitrobenzoyl-L-arabitol.

The yield after crystallisation from ethanol was 60% . Repeated recrystallisation gave a product of m.p. 127-128°C.

$[\alpha]_D$ -16° (c. 1.8 in chloroform). (Found: C, 53.3; H, 4.9; N, 5.8. Calc. for $C_{22}H_{24}N_2O_{11}$: C, 53.6; H, 4.9; N, 5.7%).

(ii) 2,3-di-O-methyl-1,4,5-tri-O-p-nitrobenzoyl-L-arabitol.

The yield after crystallisation from a 1:1 mixture of acetone and ethanol was 60%. Recrystallisation gave a product of m.p. 178-179°C but on further recrystallisation, a second form was obtained which had m.p. 130°C $[\alpha]_D$ -15° (c. 1.0 in chloroform). (Found: C, 53.1; H, 4.0; N, 7.1. Calc. for $C_{28}H_{25}N_3O_{14}$: C, 53.5; H, 4.0; N, 6.7%).

(iii) 2-O-methyl-1,3,4,5-tetra-O-p-nitrobenzoyl-L-arabitol.

This compound could not initially be obtained in a crystalline form even after an attempted purification on a silica column. The reaction product was therefore dissolved in chloroform and divided into six fractions (of approximately equal weight) by fractional precipitation with light petroleum. The fractions were dissolved in the minimum amount of pyridine and methanol was added to the point of incipient turbidity at room temperature. On standing, the fourth fraction crystallised and these crystals were then used to seed all the other fractions. Recrystallisation was effected by dissolving the material in acetone and then adding an equal volume of ethanol. The material first obtained (yield 50%) had m.p. 132-133°C but on recrystallisation, a product was obtained which changed crystalline form at this temperature and melted sharply at 152-153°C. This had $[\alpha]_D$ -50° (c. 2.0 in chloroform). (Found: C, 53.6; H, 3.4; N, 7.7. Calc. for $C_{34}H_{26}N_4O_{17}$: C, 53.5; H, 3.41; N, 7.35%).

Because of the possible presence of solvent of crystallisation, compounds (ii) and (iii) were recrystallised (unchanged m.p. and mixed m.p.) by the addition of ethanol to their chloroform solutions. The absence of ethanol was apparent from the infrared spectra of the products, and the absence of chloroform was indicated by the Beilstein halogen test, Compound (i) was also shown to give no hydroxyl absorption in the infrared. The nuclear magnetic resonance spectra of the compounds in deuterochloroform solution gave the expected ratios of methyl protons to aromatic protons to other protons. As additional confirmation, all the peaks were satisfactorily assigned to individual protons or groups of protons for each compound.

To complete the series of standard compounds for calibration a sample of L-arabitol pentabenzoate was kindly provided by Mr. W.D. Annan.

EXPERIMENT 20. PREPARATION OF STANDARD COMPOUNDS FOR GLC

Samples of each of the arabitol derivatives were prepared for GLC in the following way. The derivative (ca. 1 mg.) in a B10 Quickfit test tube was dissolved in dry benzene (0.2 ml.) and the solution was diluted with dry methanol (0.3 ml.) containing 0.1 mg. of sodium per ml. After standing overnight at room temperature, de-esterification was shown to be complete by thin layer chromatography using 4 N-sulphuric acid as spray reagent (see Experiment 19). The solution was acidified by the addition of a drop of acetic acid, and evaporated to dryness (Method 17). The residue was dissolved in a 1:1 mixture of acetic anhydride and pyridine (0.3 ml.) and heated in the stoppered test tube for 1 hr. at 100°C. The reaction was again shown to have gone to completion by thin layer chromatography. Methanol (0.3 ml.) was added and the solution was concentrated to a small volume (Method 17). This treatment removed part of the acetylation reagents. Complete removal was achieved by five such treatments, and the sample was then dissolved in a drop of chloroform and examined by GLC.

The instrument used was a Pye Argon Gas Chromatograph and the column was packed with a 3 % coating of XE-60 on Gas-Chrom P. An operating temperature of 152°C (registered on a mercury thermometer) and a flow rate of 80 ml. per min. were found to be suitable. Samples were introduced using the "closed injection system". All the samples gave only two peaks on GLC, one corresponding to the acetylated arabitol derivative and one corresponding to the methyl ester of the carboxylic acid moiety of the original standard compound. A mixture, containing the acetylated arabitol derivatives and erythritol tetraacetate, was examined by GLC and all the components were separated. The retention volumes, relative to that of erythritol tetraacetate, are given in Table 10 together with those of some other compounds. In view of the good separation achieved, erythritol tetraacetate was used as an internal standard for calibration.

TABLE 10. RETENTION VOLUMES RELATIVE TO THAT OF ERYTHRITOL TETRAACETATE

Compound	Relative retention volume
Methyl p-nitrobenzoate	0.40
Acetate of:-	
2,3,5-tri-O-methyl-L-arabitol	0.57
2,3,4-tri-O-methyl-L-arabitol	0.74
erythritol	1.00
2,5-di-O-methyl-L-arabitol	1.18
2,4-di-O-methyl-L-arabitol	1.49
2,3-di-O-methyl-L-arabitol	1.51
2,3,4,6-tetra-O-methyl-D-galactitol	1.57
2-O-methyl-L-arabitol	2.56
L-arabitol	3.62

EXPERIMENT 21. CALIBRATION OF GAS LIQUID CHROMATOGRAPH.

A standard solution was prepared in which the arabitol derivatives were present in approximately the same molar proportions as was found (Experiment 10) to exist between the corresponding constituents of the methylated araban. The following amounts of the derivatives were accurately weighed out and dissolved in acetone (10 ml.) in a standard flask: tri-O-methyl-L-arabitol derivative, 25.1 mg.; di-O-methyl-L-arabitol derivative 14.4 mg.; mono-O-methyl-L-arabitol derivative, 34.7 mg. and L-arabitol derivative, 5.81 mg. (solution A).

Using this solution and a standard solution of erythritol (245 mg.) in methanol (50 ml.), mixtures were prepared as shown in Table 11.

TABLE 11. PREPARATION OF STANDARD MIXTURES FOR QUANTITATIVE GLC

Mixture No.	Volume of solution A (ml.)	Volume of solution B (ml.)
1.	1.00	0.050
2.	1.00	0.100
3.	0.50	0.100
4.	1.00	0.250

The standard mixtures were prepared for GLC by the method described in Experiment 20. The same amounts of solvents and reagents were used, and again all reactions went to completion. For quantitative analysis, a detector voltage of 1500 volts was used. A detector sensitivity of "X 10" was used for all peaks except that corresponding to L-arabitol pentaacetate, for which this was changed to "X 1". Various amounts of the mixture of acetates derived from standard mixture 3 (Table 11) were injected onto the column, and graphs relating peak height (arabitol derivative) with peak height (erythritol derivative) were constructed (Figs. 4 & 5). These showed that this peak height ratio was independent of sample size. Similar linear relationships were obtained using the other standard mixtures. When all the mixtures had been chromatographed, calibration graphs were constructed, relating the ratio of peak height (arabitol derivative) : peak height (erythritol derivative) with the corresponding molar ratio (Figs. 6 & 7). A typical chromatogram is shown later (Fig. 8).

Variation of peak height (arabitol dve.) with peak height (erythritol dve.).

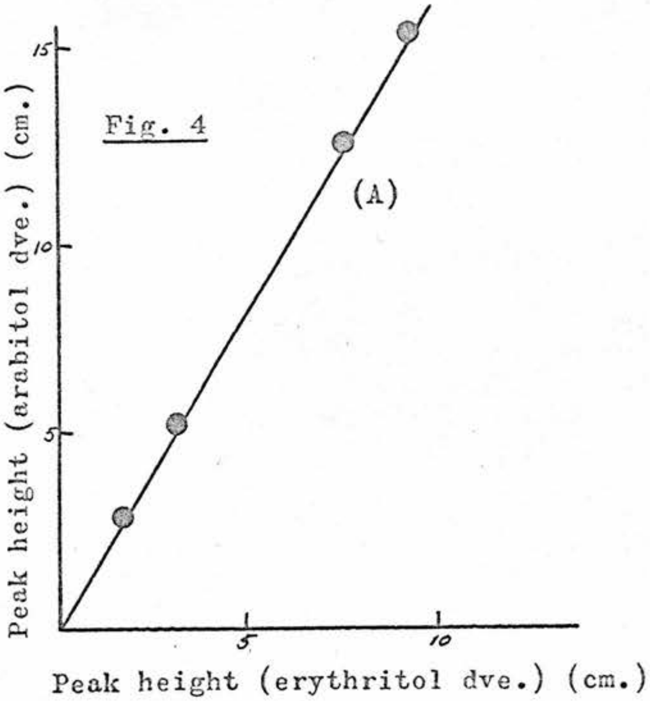


Fig. 4

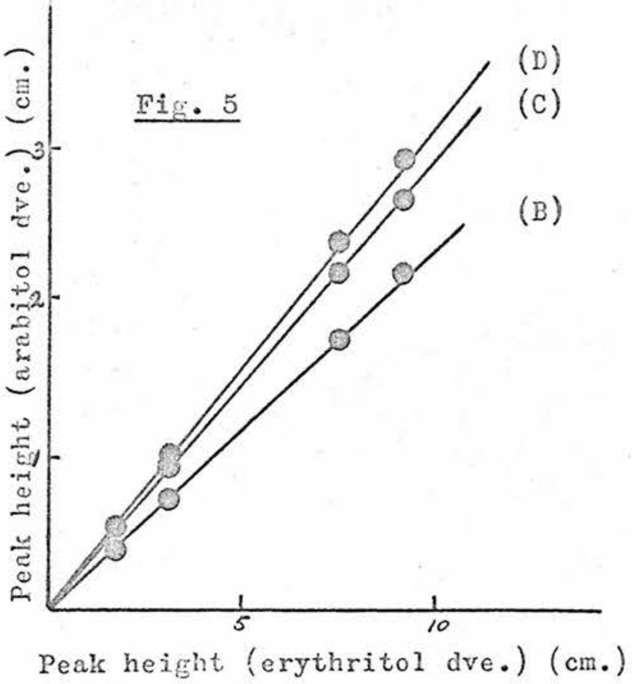


Fig. 5

Variation of peak height ratio with molar ratio.

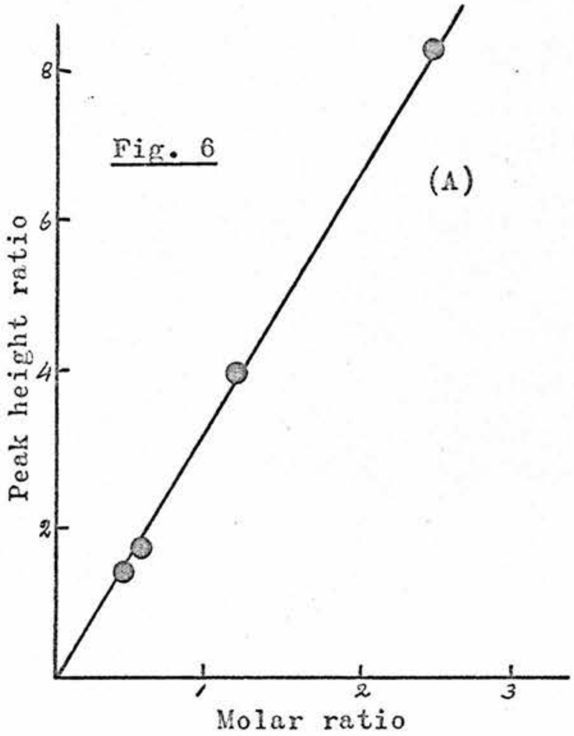


Fig. 6

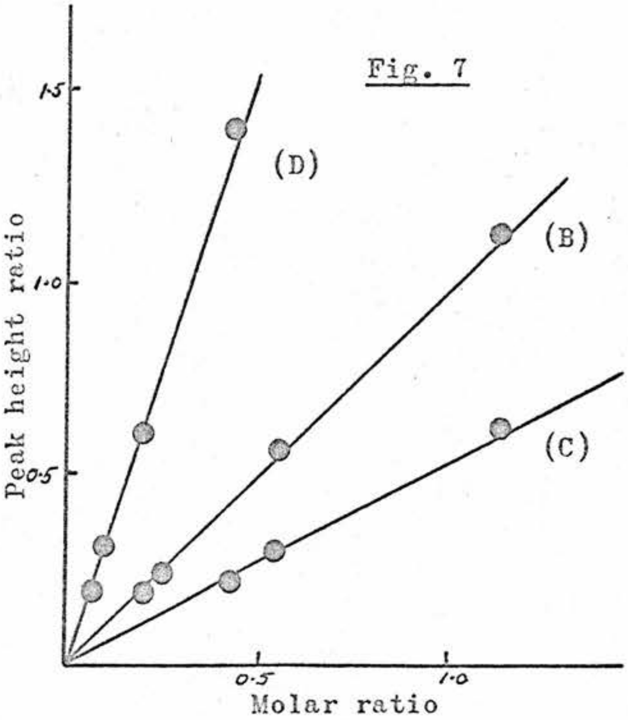


Fig. 7

(A), tri-O-methyl derivative; (B), di-O-methyl derivative; (C), mono-O-methyl derivative; (D), arabitol derivative.

EXPERIMENT 22. PREPARATION OF METHYLATED ARABANS FOR QUANTITATIVE GLC.

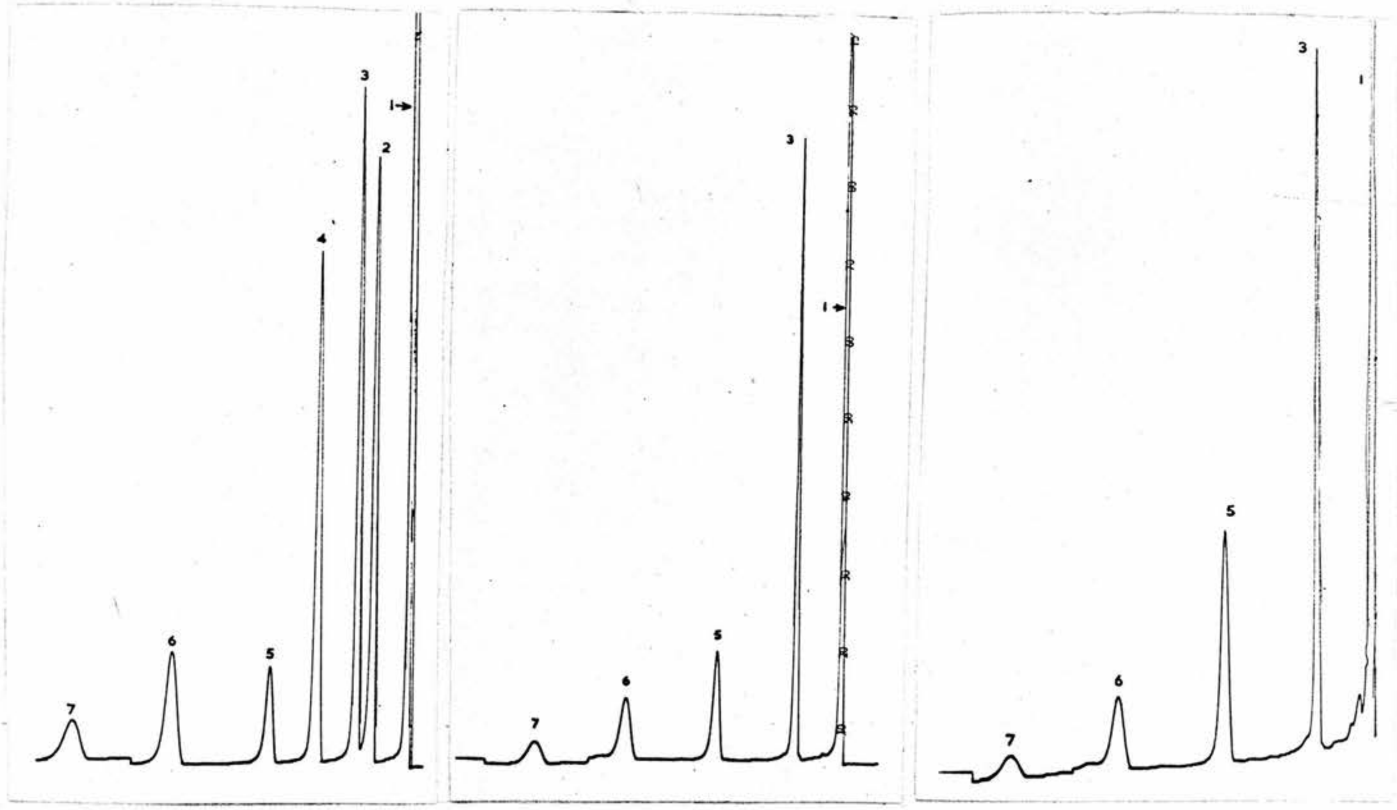
For quantitative GLC, arabans were methylated with barium hydroxide monohydrate and methyl iodide as described in Experiment 11(a). The methylated araban (ca. 2 mg.) was hydrolysed at 1% concentration in 45% formic acid at 100°C for 16 hr. The hydrolysate was adjusted to about pH 8 by the careful addition of ammonia. Potassium borohydride (ca. 15 mg.) was added and the solution was left at room temperature for 4 hr., when a second addition of borohydride was made (ca 15 mg.). (A large excess was required as the ammonium formate appeared to buffer the solution at a pH at which hydrolysis of the borohydride was fairly rapid. After a further 16 hr., the completeness of the reduction was checked using the phenol - sulphuric acid reagents, and the solution was deionised by passage through a column of IR-120 resin (H^+ form) (5 ml.). The eluate and washings were concentrated to a small volume on a rotary film evaporator (bath temperature, 30°C), transferred to a B 10 Quickfit test tube and evaporated to dryness (Method 17). Boric acid was removed as methyl borate by repeated evaporation of methanol, and the residue was acetylated and prepared for GLC by the method described in Experiment 20.

EXPERIMENT 23. ANALYSIS OF METHYLATED ARABANS BY QUANTITATIVE GLC.

Samples derived from methylated arabans were analysed without the addition of erythritol, as absolute quantitative analysis was not normally required. For a chromatogram from such a sample, a hypothetical value for peak height (erythritol derivative) was chosen so that the resulting ratios of peak height (arabitol derivative) : peak height (erythritol derivative) were within the ranges calibrated. By reading off the corresponding molar ratios from the calibration graphs, the molar proportions of the arabitol derivatives in the sample were determined.

The following araban samples were methylated and examined by quantitative GLC: (a) the araban prepared in Experiment 7, (b) an araban extracted by Mr. J.W.B. Samuel from a commercial preparation (White Mustard Seed Germ) which consisted mainly of embryos, (c) the araban prepared in Experiment 16 by extraction of mustard seed cotyledons after germination. Typical chromatograms are shown in Fig. 8. The results (Table 12) are expressed for an "average molecule" containing 45 arabinose units, this being the average degree of polymerisation indicated by molecular weight determinations on sample (a).

Fig. 8. TYPICAL GAS-LIQUID CHROMATOGRAMS.



Standard compounds.

Araban.

Araban after germination.

Peaks: 1, chloroform; 2, methyl nitrobenzoate; 3, tri-O-methyl dve.; 4, erythritol dve.; 5, di-O-methyl dve.; 6, mono-O-methyl dve.; 7, arabitol dve. (with sensitivity control at "X3")

TABLE 12. RESULTS OF ANALYSIS OF METHYLATED ARABANS

Sample	% Ome	Molar proportions found			
		tri-O-methyl derivative	di-O-methyl derivative	mono-O-methyl derivative	arabitol derivative
(a)	38.0	19.2	11.5	12.3	2.1
(b)	-	18.5	11.2	13.1	2.1
(c)	38.0	16.2	17.2	9.8	1.9

All these methylated arabans gave no hydroxyl absorption in the infrared.

An "absolute" determination was carried out in the following way. A sample of methylated araban (a) was accurately weighed and hydrolysed as described in Experiment 22. A known amount of erythritol was added to the hydrolysate, and the sample was prepared for GLC in the usual way. By using the "found" peak height (erythritol derivative) instead of a hypothetical value, it was shown that the analytical figures, which were identical to those shown in Table 12, represented about 90% of the structural units of the methylated araban.

EXPERIMENT 24. CONSIDERATION OF SOME POSSIBLE SYSTEMATIC ERRORS IN THE ANALYSES

a) Preferential decomposition of some of the sugars during hydrolysis.

Samples from methylated arabans were analysed by quantitative GLC after hydrolysis periods of 6, 16 and 36 hr. No differences in the relative amounts of the products were detected.

b) Errors incurred in the work-up of the hydrolysates.

Part of the standard mixture of arabitol derivatives (solution A, Experiment 21) was de-esterified in the usual way, and the reaction mixture was evaporated to dryness. The residue was dissolved in 45% formic acid, neutralised with ammonia, and potassium borohydride (30 mg.) was added. When hydrolysis of the borohydride was complete (16 hr.), the solution was deionised and prepared for analysis by quantitative GLC. The composition of the mixture was found to be unchanged. This showed that there was no anhydride formation(70) due to the acid conditions caused by resin treatment, and that no error was being introduced by preferential adsorption by the resin.

c) Analysis of mixtures of the standard compounds.

As the composition of the standard mixture (Experiment 21) differed slightly from those of samples derived from methylated arabans, synthetic mixtures were prepared which were of similar composition to those analysed. Analysis of these confirmed that the calibration was valid for the analysis of methylated araban samples. The synthetic mixtures were prepared by accurate weighing of the standard compounds on a Cahn electrobalance. They were then de-esterified and acetylated for analysis. The results are shown in Table 13.

TABLE 13. ANALYSIS OF MIXTURES OF THE STANDARD COMPOUNDS

Component	Molar proportions present in mixture	
Derivative of:		
2,3,5-tri-O-methyl-L-arabitol	10.0 (found: 10.0)	10.0 (found: 10.0)
2,3-di-O-methyl-L-arabitol	5.4 (found: 5.5)	7.9 (found: 7.7)
2-O-methyl-L-arabitol	5.2 (found: 5.1)	4.6 (found: 4.4)
L-arabitol	1.4 (found: 1.3)	1.0 (found: 1.1)

d) A further verification of the purity of the standard compounds.

In view of the slight discrepancy between the number (Table 12) of end groups and branch points in the araban as shown by quantitative GLC, the relative purities of the corresponding standard compounds were confirmed in the following way. A solution of chloroform in deuterochloroform was prepared (*ca*. 20% v/v). Using this as solvent, solutions of the derivative of 2,3,5-tri-O-methyl-L-arabitol (0.300 M) and the derivative of 2-O-methyl-L-arabitol (0.150 M) were accurately prepared and examined by proton magnetic resonance spectroscopy. In both spectra, the integral of the signal due to the aromatic protons was compared with that due to the protons of chloroform, and the standard compounds were shown to be of similar purity. The results are given in Table 14.

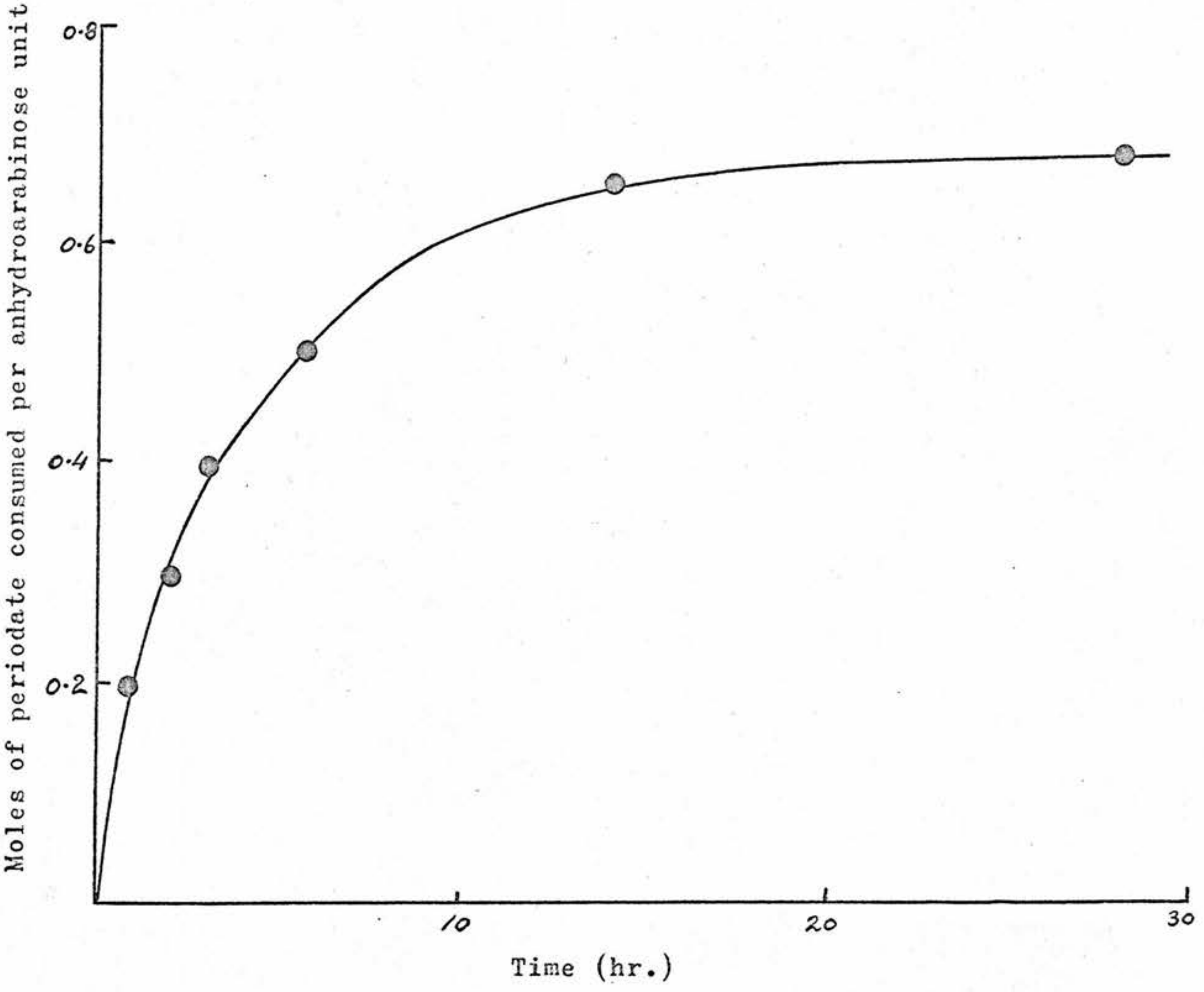
TABLE 14. COMPARISON OF THE PURITY OF THE STANDARD COMPOUNDS BY PROTON MAGNETIC RESONANCE SPECTROSCOPY.

Compound	Integral of signal from:		Ratio
	aromatic protons (a)	chloroform protons (b)	(a)/(b)
Derivative of tri-O-methyl-L-arabitol	4.45	2.85	1.56
Derivative of mono-O-methyl-L-arabitol	4.45	2.90	1.53

EXPERIMENT 25. SMITH DEGRADATION(71) OF THE ARABAN.

Equal volumes (30 ml.) of a stock solution of sodium metaperiodate (0.0800 M) and a solution of mustard seed araban (0.0440 M with respect to monomer units) were mixed and left in the dark at room temperature. The reduction of periodate was followed spectrophotometrically by the method of Aspinall and Ferrier(72) and the rate of consumption is shown in Fig. 9. The final value corresponded to 0.69 moles of periodate per anhydroarabinose unit. When the oxidation was complete, the residual periodate was destroyed by the addition of an excess of ethylene glycol. Excess potassium borohydride was added and the solution was left at room temperature for 2 days. It was then deionised using a mixture of IR-120 (H^+ form) and IR-45 (OH^- form) resins, and concentrated to dryness under reduced pressure at 30°C. Boric acid was removed as volatile methyl borate and, after withdrawal of a small sample, the residue was dissolved in 1N-sulphuric acid (20 ml.) and left at room temperature. At intervals, samples (2 ml.) were withdrawn. These were

Fig.9 . Periodate oxidation of the araban.



neutralised with solid barium carbonate, evaporated to dryness and methylated by the method described in Experiment 11(a). The methylated products were quantitatively analysed by the gas-liquid chromatographic method and the results are shown in Table 15.

To check that hydrolysis only took place at the oxidised units, a solution of araban in 1 N-sulphuric acid was left at room temperature for 144 hr. It was then isolated, methylated and analysed in the same way as the Smith-degraded samples. The results of this control experiment are also shown in Table 15.

TABLE 15. ANALYSIS OF THE PRODUCTS OF SMITH DEGRADATION AND OF ACID TREATMENT OF THE ARABAN.

The molar proportions are expressed in terms of the original molecule which contained 45 arabinose units.

Component	Molar proportions found					
	Acid treatment of the reduced periodate-oxidised araban for:				Acid treatment of the araban for:	
	0 hr.	8.5 hr.	24 hr.	69 hr.	0 hr.	144 hr.
Derivative of:						
Tri-O-methylarabitol	0	5.7	8.7	7.7	19.2	19.1
Di-O-methylarabitol	0	7.0	5.5	6.4	11.5	12.7
Mono-O-methylarabitol	12.0	1.5	0	0	12.3	11.0
Arabitol	2.1	0.05	0	0	2.1	1.9

EXPERIMENT 26. INVESTIGATION OF THE RELATION BETWEEN PEAK AREA AND CONCENTRATION.

A standard mixture of the acetylated arabitol derivatives prepared from solution A (Experiment 21) was analysed by measurement of peak areas on GLC. The column packing, temperature and flow rate were as described previously, except in the case of the flame ionisation detector when temperature programming was used. The following detectors were investigated.

(i) An argon detector. This was the instrument which was used in the previous experiments, and peak areas were measured with an electronic integrator. Results were reproducible to ± 5% .

(ii) A flame ionisation detector (Pye 104). Temperature programming (120 - 180°C at 3° c/min.) gave symmetrical peaks which were measured by "triangulation". Results were reproducible to ± 5%.

(iii) A gas density balance (Griffin D6 Chromatograph). Because of the detector's low sensitivity, large samples had to be applied. This resulted in considerable tailing of peaks and the peak corresponding to arabitol pentaacetate was so broad that it was not measured. Although the electronic integrator was used, results were only reproducible to ± 10% . The results of these experiments are shown in Table 14 and the figures quoted are the average values of four determinations.

TABLE 16. RESULTS OF PEAK AREA MEASUREMENTS

Component	Known composition of mixture		Relative peak areas		
	Weight Proportions	Molar Proportions	(i)	(ii)	(iii)
Derivative of:					
tri-O-methylarabitol	100	100	100	100	100
di-O-methylarabitol	50	45	35	47	45
mono-O-methylarabitol	107	89	62	95	95
arabitol	22	17	8	13	-

2(c) DISCUSSION

Quantitative GLC was found to be a suitable method for the characterization of arabans and degraded arabans in terms of the proportions of the different types of structural units present; indeed, the application of this method has led to some important conclusions regarding the structure of mustard seed araban and its changes on germination. The hydrolysis products of the methylated polysaccharides were reduced and acetylated to give mixtures of arabitol derivatives that were separated and analysed by GLC. For calibration purposes it was necessary to prepare a series of pure crystalline reference compounds. The p-nitrobenzoates of 2,3,5-tri-O-methyl-L-arabitol, 2,3,-di-O-methyl-L-arabitol and 2-O-methyl-L-arabitol and the pentabenzoate of L-arabitol were found to be suitable. The p-nitropenzoates were all new compounds; they were readily prepared from the parent alditols, and shown to be pure by their sharp melting points, proton magnetic resonance spectra, and by analysis. Their only drawback as analytical standards was that they exhibited polymorphism. For this reason, purification by recrystallisation was sometimes complicated. In the calibration procedure, weighed amounts of the reference compounds were mixed with the internal standard (erythritol), deacylated with sodium methoxide, and the mixture of products was acetylated with acetic anhydride in pyridine. The deacylation procedure was shown to be quantitative by thin layer chromatography. Although the results of the analyses given here are normally expressed as ratios, the use of the internal standard enabled the absolute amounts to be determined. The absolute values showed that the results accounted for 90% of the weight of the araban. The remainder is presumed to have been lost by acid catalysed decomposition of the methylated sugars during hydrolysis; control experiments (discussed below) showed that this did not lead to a disproportionate loss of any of the components.

Comparison of three types of detector suggested that calibration was necessary for accurate analysis, especially for the "argon" detector because this was not equally sensitive to all the compounds being determined. The flame ionisation detector showed reasonable uniformity of response on a molar basis for three of the four components, but this might have been fortuitous since there is no obvious theoretical basis for this correlation. Further work will be necessary to clarify this point. Accurate quantitative analysis (without calibration) should be possible with the gas density balance, because there is a well established theoretical relationship between the detector signal and the concentration of material in the carrier gas emerging from the column. Unfortunately, the low sensitivity of this detector resulted in complications in the analysis and consequent loss of accuracy and reproducibility.

Having calibrated for quantitative analysis, various methylated arabans were examined. One of these was obtained from a commercial preparation of "mustard seed germ" and was found to be almost identical to the araban originally prepared. Analysis of the latter polysaccharide gave the molar proportions of 2,3,5-tri-O-methyl-L-arabinose: 2,3-di-O-methyl-L-arabinose: 2-O-methyl-L-arabinose: L-arabinose as 19.2: 11.5 : 12.3 : 2.1. The corresponding results obtained in the methylation study of the same araban

(Experiment 10) were 18:9:16:3. This discrepancy is presumably due to the experimental errors involved in separation of the hydrolysis products on a cellulose column, and indicates the unsuitability of such methods for the detection of minor structural differences.

The araban extracted (in diminished yield) from the cotyledons after germination was indistinguishable from the original polysaccharide by both zone electrophoresis and ultracentifugation Hydrolysis of the methylated polysaccharide gave the same products as were previously obtained, but quantitative GLC revealed a distinct difference in the relative proportions of these. It appears that the metabolic changes that occur during the first four days of germination result in a pronounced decrease in the degree of branching in the araban, and this can be seen on visual comparison of the chromatograms (Fig. 8).

The reproducibility of the results of these analyses was excellent. Independent determinations of the tri-O-methyl, di-O-methyl and mono-O-methyl derivatives, involving rehydrolysis of the methylated polysaccharide and all subsequent operations, almost invariably gave results within ± 2% of the mean. Similar agreement was also obtained when independent determinations actually involved independent methylation and all subsequent operations. As the acetate of 2,3,5-tri-O-methyl-L-arabitol was the major component of all the samples analysed and had the lowest retention volume, the sizes of the peaks corresponding to the other components were, of necessity, rather small. This limited the accuracy of determinations of arabitol pentaacetate, as this had the largest retention volume and was no more than a minor component in any of the samples. The necessary electronic amplification of this peak magnified the effect of base-line drift and "noise", and determinations of this component were only reproducible to ± 7%.

Although the reproducibility of the method was satisfactory, there is a possibility that there might have been systematic errors in the determinations. This is indicated because the results (Table 12) should show the number of end-units in the molecule to be equal to the number of branch-units plus one. This requirement was satisfied by sample "b" (18.5 end-units to 17.3 branch units) but not by sample "a" (19.2 end-units to 16.5 branch-units) or sample "c" (16.2 end-units to 13.6 branch-units). It is unlikely that the lack of agreement in the case of sample "c" was due to a molecular weight effect, as all three arabans were indistinguishable in the untracentrifuge. Various tests showed that these discrepancies were not due to incomplete hydrolysis, preferential decomposition during hydrolysis, errors incurred in the work-up of the reduced hydrolysis products, or errors due to impurities in the standard compounds used for calibration. The accuracy of the calibration was also verified by analysis of synthetic mixtures of similar composition to those obtained from methylated arabans. None of these experiments indicated any source of error. If there is an undetected source of error, it would not invalidate the method for the structural comparison of different arabans, as it would be common to all determinations. However, the slight discrepancies do indicate that it might be unwise to place as much reliance on the accuracy of the method as would seem justified by the excellent reproducibility.

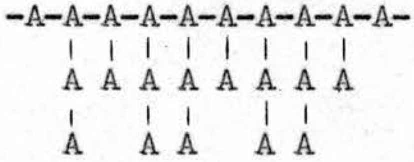
On periodate oxidation, the araban consumed 0.69 moles of periodate per anhydroarabinose unit. This was in good agreement with the value (0.68) calculated from the results of quantitative GLC (Table 12). The oxidised polysaccharide was then subjected to the Smith degradation procedure and the progress of the mild acid hydrolysis of the modified araban was followed by methylation and quantitative GLC. In order to determine the effect of non-specific hydrolysis during acid treatment, a control experiment was carried out on the original araban. The results of this (Table 15) show that even on extended treatment the extent of non-specific hydrolysis is relatively small. A further indication to this effect was that analysis of the sample obtained from the degraded araban, after 69 hr. of acid treatment, showed the presence of only trace amounts of the acetate of 2,3,4-tri-O-methylarabitol. This derivative, which is completely separable from all the other components (Table 10), would have been expected if any free arabinose had been present in the acid solution as a result of non-specific hydrolysis.

These considerations, together with the results of gas-chromatographic analysis before mild acid hydrolysis of the modified araban (Table 15), indicate that the degradation proceeded according to expectation, and that the results can safely be used to deduce fine structural details of the araban.

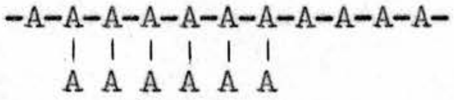
The acid lability of the araban (Experiment 14) indicated that there were furanose linkages in the molecule. This was confirmed, in the case of the end-units, by the results of the methylation study (Experiment 10). The detection of the acetate of 2,3,5-tri-O-methyl-L-arabitol on analysis of the degraded araban proves that some of the branch units are also present in the furanose ring form, and these two results, combined, account for more than half the arabinose units in the polysaccharide.

The results of analysis of the native araban (Table 12) show that some of the branch-units in the molecule must be contiguous, as there are more branch-units than chain-units. This conclusion is qualified by the results of Smith degradation:

(i) All the branch-units in the araban cannot be contiguous - otherwise no 2,3,5-tri-O-methylarabitol derivative would have been detected on analysis of the degraded polysaccharide. This eliminates such possibilities as -



or



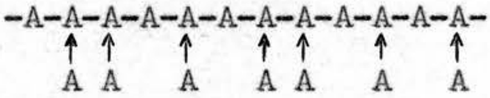
(ii) No branch units can be attached to adjacent branch units by 1,3-linkages. This follows from the fact that no 2,5-di-O-methylarabitol or 2-O-methylarabitol derivatives were detected on analysis of the degraded araban.

In further interpreting the results of the degradation experiment, the following structural features will be ignored:
a) the 1,2-linkages and the end-units associated with them and
b) the possible presence of a branch-point on the reducing unit of the molecule. The original araban molecule contains approximately 16 end-units, 11 chain-units and 15 branch-units. Only those units which are originally present as branch-units survive the degradation. Taking the mean of the determinations after 24 and 69 hr. hydrolysis (Table 15) it is clear that in whole numbers

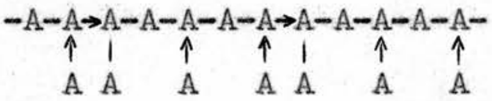
9 of these become end-units while 6 remain substituted at C-5 by another sugar unit. The 9 which become end-units must originally have been substituted at C-3 and C-5 by oxidisable units, while the remaining 6 must have been substituted at C-3 with an oxidisable unit and at C-5 with another branch unit. A total of 24 oxidisable units must therefore be attached through 1,3- or 1,5-linkages to branch units. As there are only 28 such oxidisable units in the araban molecule, it follows that most of the oxidisable units are in isolated (i.e. non-contiguous) positions, and consequently that most of the end-units must be directly attached to branch-units.

These conclusions do not indicate a unique structure for the araban, but they do diminish the number of possibilities. Some of the remaining possible structures are shown below. The symbol - denotes a 1,5-linkage and the symbol → denotes a 1,3-linkage.

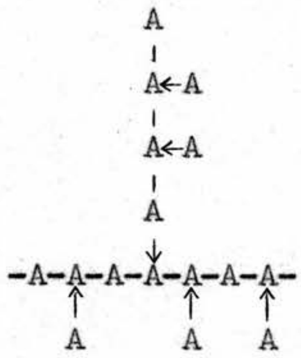
A. <u>Comb-type structures</u>, with a distribution of side-chains such that most of the chain-units are isolated:



However, by criterion (ii) above, certain structures of this type are not possible, e.g.

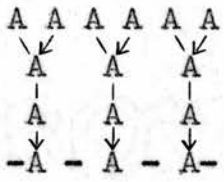


B. <u>Tree-type structures</u>



By criterion (ii), no branch-units in the side chains can be attached directly to the main chain by 1,3-linkages.

C. <u>Bush-type structures</u>:



Characteristics of any of these possible types of structure may be present to a greater or lesser degree in the araban molecule. Similar but much less detailed results have been obtained by application of the Barry degradation procedure to the arabans from sugar beet(73) and Guava(74). Further investigation is obviously required before more definite conclusions can be drawn. This may be achieved by a study of the molecular weight distribution of the products which were obtained on degradation of mustard seed araban or by application of the method described in Section 3 of this Thesis.

SECTION 3

3(a) INTRODUCTION

The main part of the Introduction to this Section is an outline of the present knowledge of the factors which determine the rates of hydrolysis of glycosides in aqueous acid. This will be necessary background for the description and discussion of an attempt to devise a method by which further information could be obtained about the fine structure of mustard seed araban. The basic aim was to characterise the terminal linkages of the polysaccharide by stabilising them towards hydrolysis so that the corresponding disaccharide or branched trisaccharide would remain after hydrolysis of the other linkages in the polymer. The structural significance of the information that would be so obtained, is discussed below. Aspinall's method(75), which involves catalytic oxidation of a proportion of the primary hydroxyl groups to carboxylic acid groups (which then confer the desired stabilisation) could conceivably be applied to this problem, because oxidizable units are only present at the non-reducing chain ends of the polysaccharide. However, this method is limited because it is not usually possible to achieve oxidation of more than a small proportion of the hydroxyl groups - presumably because the macromolecule is not readily adsorbed on the catalyst in the correct orientation. In these circumstances the quantitative significance of the results would not be clear. Quantitative considerations are important in the present argument (see below) and the possibility of devising a new method for stabilising the terminal linkages was therefore investigated.

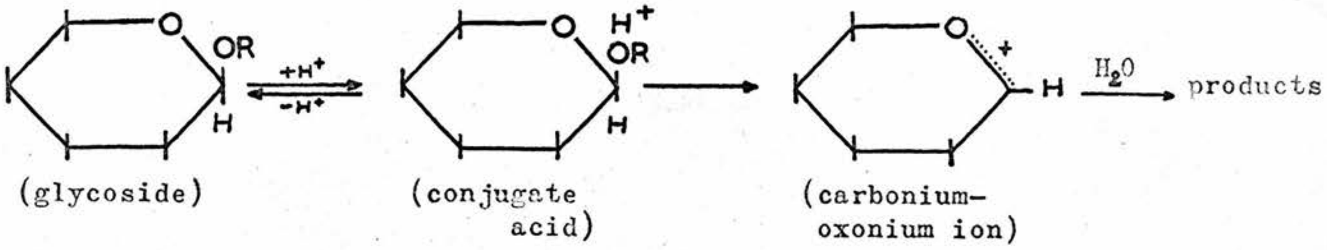
Although a considerable amount of interest has been shown in the relative stabilities of various glycosides towards acid catalyzed hydrolysis in aqueous systems, the factors that determine the reaction rates are not fully understood. Kinetic studies have been confined almost exclusively to pyranosides; predictions of the rates of hydrolysis of furanosides must therefore be made cautiously and by analogy with pyranosides. In using such analogies, the possibility must be borne in mind that pyranosides and furanosides might be hydrolysed by somewhat different mechanisms(76).

The first step in the hydrolysis reaction (Fig. 10) is considered to be the rapid, equilibrium-controlled protonation of either the glycosidic or the ring oxygen. Depending on the position of protonation, two mechanisms have been proposed for the remaining stages of the reaction. One of these is that protonation at the glycosidic oxygen is followed by slow, unimolecular heterolysis of the conjugate acid, with the formation of an alcohol and a cyclic carbonium-oxonium ion which reacts rapidly with water to give the parent sugar. The other is that protonation occurs at the ring oxygen and is followed by slow heterolysis to give an acyclic carbonium-oxonium ion which combines with water to give the alcohol and reducing sugar. Banks _et al._(77) have shown that the hydrolysis of methyl α-D-glucoside and the methanolysis of phenyl α- and β-D-glucosides do not proceed via ring opening, and the other mechanism is generally favoured.

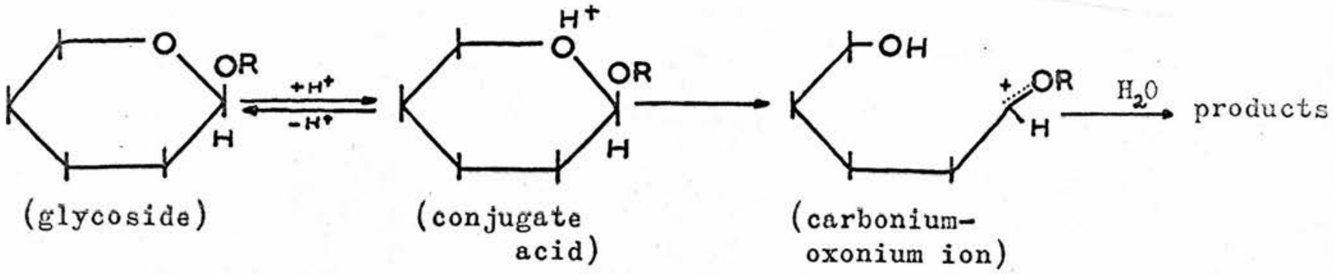
The unimolecularity of the rate-determining heterolysis step is indicated by the fact that, for glycosidic hydrolyses in general, the plot of the logarithm of the rate constant against the Hammett

FIG. 10. PROPOSED MECHANISMS OF HYDROLYSIS OF GLYCOSIDES.

A. Cyclic pathway.



B. Acyclic pathway.



acidity function(78) is a straight line of unit slope(79, 80). Also, if the reaction were bimolecular involving a molecule of water, the transition state would be more highly ordered than the ground state. This would be reflected in a negative entropy of activation but positive values have been obtained for a wide variety of compounds in agreement with the formation of a less ordered transition state(81, 76).

Although the rate-determining step in the reaction is the heterolysis of the conjugate acid, the equilibrium concentration of this acid must be an important factor in determining glycoside stability. Feather and Harris(82) compared the rates of hydrolysis of anomeric methyl glycosides and found that those in which the aglycone was axially orientated were more stable. They attributed this to the fact that an axially orientated glycosidic oxygen is relatively inaccessible to protonation, and that formation of the conjugate acid is therefore hindered. Steric factors play an important part in determining glycoside stability, particularly in relation to the ease of formation of the carbonium-oxonium ion. This ion will tend to assume a half-chair conformation and its formation will be impeded by non-bonded interactions between substituents on C-2 and C-3 and between those on C-3 and C-5. Timell(83) interpreted the stability of the methyl glucuronosides in terms of the interaction between the carboxyl group on C-5 and the hydroxyl group on C-4 but Edward(84) suggests that the carbonium ion would actually be sterically favoured by hydrogen bonding between the carboxyl group and the C-4 hydroxyl. The relative stabilities of a wide range of methyl glycosides have been interpreted in stereochemical terms by Feather and Harris(82). They also point out that the stable conformation of a glycoside may be influenced by the size of the aglycone, and that such considerations are important in interpreting the relative hydrolysis rates of oligosaccharides.

It is difficult to draw conclusions about the steric effects of various aglycones, as increase in size is usually paralleled by a change in electronic interaction with the glycosidic oxygen. Electronic factors must play a part in glycoside hydrolysis, influencing both the equilibrium concentration of the conjugate acid, and subsequent heterolysis. It has been suggested that the presence of an electron withdrawing group in the aglycone has relatively little effect on the stability of the glycoside(81) because, although protonation is impeded by the smaller electron density at the glycosidic oxygen, this is offset by the enhanced rate of heterolysis that results. Opposing effects would also be expected if an electron-releasing substituent were present in the aglycone, but Armour et al.(79) found that the rate of hydrolysis of tertiary butylβ-D-glucopyranoside is much faster than that of the corresponding methyl glycoside. This is because the two reactions proceed by different mechanisms, the former involving O-alkyl fission to give the more stable alkyl carbonium ion.

When an electron-withdrawing substituent is present in the glycone, the inductive effect impedes both the formation of the conjugate acid and its subsequent heterolysis. Thus the rapid hydrolysis rates of 2-deoxy-D-glucopyranosides, as compared with the parent glycosides, can be attributed to inductive stabilisation by the hydroxyl group on C-2 in the latter case(79). This might also be the reason why 2-O-toluene-p-sulphonyl-glycosides are so stable to acid hydrolysis(85). It is possible that a similar effect operates when an electron-withdrawing substituent is present on C-5 of the glycone and the stability of glycuronosides is well known. However, the carboxyl stabilising effect has so far defied rational explanation. If it were the result of purely electron effects, at least the same degree of stabilisation would be expected as in the case of a 6-chloro-6-deoxy-glycoside by consideration of polar substituent constants(86); in fact, methyl 6-chloro-6-deoxyα-D-glucopyranoside is hydrolysed more than six times slower than the corresponding methyl glucuronoside(83). It is also significant in this context that methyl 6-O-methyl-D-glucopyranosides are hydrolysed more slowly than the corresponding glucuronosides although no inductive stabilisation would be expected(83).

By comparing the stabilities of phenyl glycosides in which the electron affinity of the aglycone differed, it was found that the hydrolysis rates of phenyl β-D-glucosides were lowered by reducing the glycosyl group electron density(87). (This indicates that the effect on the ease of protonation is predominant in this instance). Such an effect was not observed in the case of glucuronosides and this was explained by a consideration of the polarisability of the reaction centre. If the inductive effect of the carboxyl group reduces the electron density in this region, the polarisability will also be reduced, and changes in the electron affinity of the aglycone will be less significant. Although this indicates that the inductive effect of the carboxyl group is felt at the reaction centre, it does not imply that this is the predominant factor in the stabilisation nor that protonation necessarily occurs at the glycosidic oxygen. The large difference in entropies of activation between methyl glucosides and methyl glucuronosides(83) indicates that different mechanisms might be involved in the hydrolyses of these compounds. A further complication is that the nature of the glycone appears to have a profound effect on the magnitude of the carboxyl stabilising effect. Thus, when the aglycone is a methyl group, the stabilisation is relatively insignificant but this is not so when the methyl substituent is replaced by a phenyl group or another sugar unit. As stated above, the electron affinity of the aglycone does not effect the stability of glucuronosides and it would appear that the size of the C-1 substituent, plus some undefined effect of the carboxyl group, combine to give the overall result.

Although the nature of the carboxyl stabilising effect is not understood, 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 in a similar way. Such a substituent in a 1,6-linked hexosan or possibly a 1,5-linked pentosan would enable the terminal glycosidic linkages to be characterised. An investigation of the possibility of using the toluene-p-sulphonyl (tosyl) ester group for the stabilisation of these linkages in mustard seed araban has been carried out. The tosyl group was chosen because it is stable to acid and is similar to the carboxyl group in being relatively large and also electron-withdrawing(88). Preliminary experiments carried out by Mr. E. Farr showed that primary tosylation stabilises methyl arabinofuranosides by a factor of about four and, by analogy with the carboxyl stabilising effect, it might be expected that this would be even more pronounced at the disaccharide level.

As direct tosylation of carbohdrates does not occur specifically at the primary hydroxyl groups, the intention was to block these by triphenylmethylation, methylate the remaining hydroxyl groups, remove the blocking group and then stabilise the terminal linkages by tosylation. Hydrolysis of this araban derivative would then give a mixture of methylated arabinoses and stabilised disaccharides which, on reductive de-tosylation, methylation and hydrolysis, would give a mixture of products which would reflect the fine structure of the polysaccharide. In this way, it would be possible to distinguish between some of the possible structures described in Section 2 of this Thesis. If the proposed scheme went according to expectation, the comb-type structure (page 72) would give mainly tri-O-methyl-L-arabinoses, with di-O-methyl-L-arabinoses as the only other products; the bush-type structure (page 73) would give mainly tri-O-methyl-L-arabinoses, with 2-O-methyl-L-arabinose as the only other product while the tree-type structure would give all three types of product. The two extreme structures could therefore be distinguished while the tree-type would give the same result as a combination of the other two.

It was anticipated that such a scheme might present practical difficulties, and only limited amounts of the araban were available. A dextran was therefore used to establish the correct conditions for the various stages and to determine whether any appreciable stabilisation did occur at the disaccharide level. The reason for choosing a dextran for this purpose was its close structural similarity to the araban. The dextrans are predominantly α-1,6-linked glucans with varying degrees of branching which may be through any of the remaining hydroxyl groups depending on the source of the polysaccharide. Almost all the primary hydroxyl groups must therefore be on the terminal glucose units (as in mustard seed araban). The particular dextran which was used in this work has been shown by periodate oxidation studies(89) to contain 18% 1,3-type linkages and 3% 1,4-type linkages and was therefore sufficiently highly branched to be a suitable model compound.

After a preliminary verification that the stabilising effect of the tosyl group operates in the glucose series, conditions for the homogeneous triphenylmethylation of the dextran were established, and the remaining synthetic stages of the scheme were carried out. The methylated, tosylated dextran was then subjected to acid treatment and the stabilising effect of the substituent was assessed.

3(b) EXPERIMENTAL

EXPERIMENT 27. COMPARISON OF THE STABILITIES OF GLYCOSIDES

Various glycosides were hydrolysed at 1% concentration in either 45% formic acid, 1 N-sulphuric acid of 1 N-hydrochloric acid by heating at 100°C. Samples were withdrawn at intervals and examined by thin layer chromatography. Formic acid hydrolysates were prepared for examination by Method 7 and mineral acid hydrolysates were neutralised with silver carbonate and examined directly by thin layer chromatography. The reaction products from unmethylated glycosides were separated by double development using a 25% solution of methanol in benzene but for those from methylated glycosides, single development with a 10% solution of methanol in benzene was used. Spots were normally located with the anisaldehyde-sulphuric acid spray reagent, but toluene-p-sulphonate esters were located with the specific spray reagent of Jackson and Hayward(90) (Method 15). By visual comparison of spot intensities at different times during the reactions, the half-lives of the various glycosides were estimated. The results are shown in Table 17. Hydrolysis of the tosylated compounds gave more than one product in each case and the significance of this is considered in the Discussion.

TABLE 17. HALF LIVES OF GLYCOSIDES UNDER HYDROLYSIS CONDITIONS

Compound	Half life (hr.) in:-		
	HCl	H_2SO_4	HCOOH
Methyl α-D-glucopyranoside	0.7	1.5	-
Methyl 6-O-toluene-p-sulphonyl-α-D-pyranoside	3.5	4.5	-
Methyl 2,3,4,6-tetra-O-methyl-α-D-glucopyranoside	-	-	8
Methyl 2,3,4-tri-O-methyl-6-O-toluene-p-sulphonyl-α-D-glucopyranoside	-	-	30

SECTION 3

THE DEVELOPMENT OF A METHOD FOR THE STRUCTURAL ANALYSIS OF POLYSACCHARIDES BY STABILISATION OF SELECTED GLYCOSIDIC LINKAGES TO ACID HYDROLYSIS.

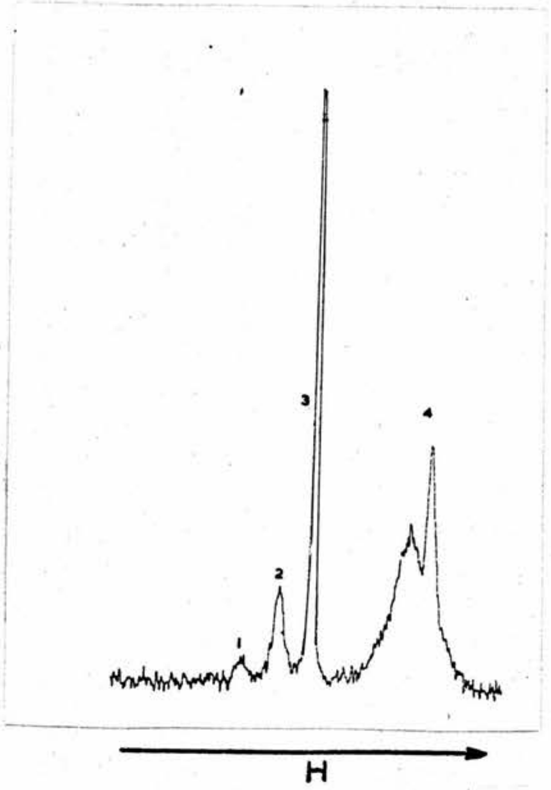
EXPERIMENT 28. PREPARATION OF DEXTRAN.

The polysaccharide 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%) and was adjusted to pH 7.0 with sodium hydroxide solution. Two 50 ml. and two 2 l. conical flasks containing 25 ml. and 1 l. of the culture medium respectively were plugged with cotton wool and sterilized in an autoclave. The two small flasks were inoculated with the organism (observing the normal bacteriological precautions) and were incubated at 24°C for two days. The resulting viscous solutions were carefully transferred to the larger flasks which were similarly stored. The polysaccharide was isolated from solution by the slow addition of 1.5 volumes of ethanol with stirring. The gummy product was further purified by dissolving in water (2 l.), boiling the resulting solution to coagulate proteins (30 mins.) and centrifuging. The supernatant solution was filtered and the polysaccharide material was reprecipitated by the addition of ethanol. On redissolving the gum in water and freeze-drying, 60 g. of dextran were obtained. (Found: N, 0.7%).

EXPERIMENT 29. EXAMINATION OF THE DEXTRAN BY PROTON MAGNETIC RESONANCE SPECTROSCOPY.

The proton magnetic resonance spectrum of deuterated dextran in D_2O solution is shown in Fig. 11. Peak 3 was assigned to the protons of HOD, present in the solvent. Following Pasika and Cragg(45), Peak 1 was assigned to the anomeric protons involved

Fig. 11. PROTON MAGNETIC RESONANCE SPECTRUM OF THE DEXTRAN.



Peaks: 1, the anomeric protons at branch points; 2, the anomeric protons in the main chain; 3, HOD; 4, the remaining protons.

in the branch-linkages and peak 2 to the anomeric protons in the main chain. The ratio of the areas of these two peaks indicated that about 20% of the linkages in the molecule were branch-linkages. The sum of these two areas was approximately one sixth of the area of the peaks at 4, in confirmation of these assignments.

EXPERIMENT 30. METHYLATION OF DEXTRAN.

Dextran (0.50 g.) was dissolved in N-methyl-2-pyrrolidone (20 ml.). The solution was cooled to 0°C, and methyl iodide (1.5 ml.; dissolved in 20 ml. of N-methyl-2-pyrrolidone) and barium hydroxide octahydrate (3.6 g.) were added. The mixture was shaken in an ice bath for 2 hr., then at room temperature overnight. Methyl iodide (5 ml.) and barium hydroxide octahydrate (12 g.) were added, and shaking was continued for a further 24 hr. at room temperature. The product was obtained by chloroform extraction as described in Experiment 9 for the preparation of methylated araban, and the chloroform solution was concentrated to a syrup. This was diluted with a small volume of benzene, then with petroleum ether, and poured into a large volume of petroleum ether. The white fibrous product was filtered off and purified by reprecipitation. Yield, 0.50 g. (79%). (Found: OMe, 38.5. Calc.: OMe, 45.6%).

This incompletely methylated material (0.50 g.) was dissolved in dimethylformamide (10 ml.), and methyl iodide (1.5 ml.) and silver oxide (2.7 g.) were added. The mixture was shaken at room temperature for 16 hr., diluted with dimethylformamide, and filtered. The filtrate was shaken with an aqueous solution of sodium cyanide. Chloroform extraction, followed by precipitation with petroleum ether as described above, gave a white fibrous product. Yield, 0.45 g. (71 % for the two operations). (Found: OMe, 40.0. Calc.: OMe, 45.6 %).

A sample of this material was methanolysed and examined by GLC (Methods 11 and 12). The pattern of peaks corresponded to that expected for a mixture of the methyl glycosides of 2,3,4,6-tetra-O-methyl-, 2,3,4-tri-O-methyl- and 2,4-di-O-methyl-glucose. A more detailed examination of the dextran, after complete methylation by successive treatments with methyl iodide and silver oxide at 40°C, revealed a small amount of 2,4,6-tri-O-methylglucose showing that there were also some 1,3-linkages in the main chain. This study, which was carried out by Mr. N.J. Wight, also confirmed that about 20% of the units in the molecule were branch-units.

EXPERIMENT 31. INVESTIGATION OF THE SUITABILITY OF VARIOUS SOLVENTS FOR THE TRITYLATION OF DEXTRAN

The dextran was insoluble in pyridine and did not go into solution on prolonged heating with pyridine containing a large excess of triphenylchloromethane (trityl chloride). As the polysaccharide was soluble in dimethylsulphoxide, and was not precipitated by the addition of an equal volume of pyridine, the possibility of using this solvent mixture for the tritylation of dextran was investigated. Methyl α-D-glucopyranoside was used as a model compound for trial experiments. As the dextran was fairly insoluble in the intended solvent and contained relatively few primary hydroxyl groups, the trial experiments were carried out on dilute solutions of the glucoside in order to obtain comparable conditions. Both the solvents were dried over type 4a molecular seive, and a mixture containing equal volumes of each was prepared. Methyl α-D-glucopyranoside (7 mg.) and trityl chloride (100 mg. - 10 moles per mole of glucoside) were dissolved in 1 ml. of the solvent mixture and the solution was heated at 100°C. Samples were withdrawn at intervals and examined by thin layer chromatography (Method 10) using a 15 % solution of methanol in benzene as developing solvent. The presence of pyridine did not effect the separations but interfered with the anisaldehyde-sulphuric acid spray reagent. For this reason, plates were strongly heated after development, sprayed, and heated again. Tritylated products gave yellow spots during the first few seconds and, on further heating, gave the characteristic colours of carbohydrate derivatives. The results showed that tritylation was complete in 1 hr. but that there were also traces of fast moving compounds (presumably the products of secondary tritylation) at this stage.

A similar experiment was carried out at 23.5°C and in this case primary tritylation was complete in 24 hr. Traces of other products were detected after 70 hr. When only three moles of trityl chloride were used, tritylation was incomplete even on prolonged heating at 100°C, and this indicated that the solvent mixture contained impurities which were reacting with the tritylating agent.

The purities of the solvents were tested by making use of the fact that, by thin layer chromatography, methyl trityl ether can be separated from trityl chloride and triphenylcarbinol which do not separate. Benzene was used as developing solvent in these experiments and spots were located by spraying the plates with 4 N-sulphuric acid and heating over a bunsen flame. Trityl chloride (100 mg.) was dissolved in pyridine (1 ml.), and the solution was heated at 100°C for 5 min. (heating for a longer time did not alter the final result). An excess of anhydrous methanol (o.25 ml.) was then added, and the solution was heated for a further 5 min. (again further heating did not alter the result). The reaction mixture was examined by thin layer chromatography and two spots were detected. The faster moving of these had the same mobility as trityl methyl ether while the slower one had the same mobility as triphenylcarbinol. By comparing the intensities of these spots, it was estimated that the ratio of the faster moving component to the slower moving component was about 10:1. In a similar experiment in which trityl chloride (10 mg.) was dissolved in pyridine (1 ml.), this ratio was about 3:1. In view of the difficulty of accurately estimating the intensity of the bright yellow colouration, this was taken as an indication that the pyridine was sufficiently pure for the purposes of this investigation.

Similar experiments were carried out in which varying amounts of trityl chloride were dissolved in 1 ml. of a 1:1 mixture of dimethyl sulphoxide and pyridine. When 10 mg. of trityl chloride were used, only the slower moving spot was detected on thin layer chromatography; 50 mg. gave both spots of equal intensity and 100 mg. gave about five times as much of the faster moving component.

This clearly showed that an impurity in the dimethylsulphoxide was reacting with the trityl chloride and, as the product of this reaction had the same chromatographic mobility as triphenyl carbinol, it was assumed that this impurity was water.

In an attempt to purify the dimethylsulphoxide further, it was shaken with barium oxide for two days and recovered by distillation under reduced pressure. Almost half the solvent distilled over before a constant boiling point was reached (80°C), and this fraction was discarded. The purified solvent was examined by the method described above and similar results were obtained. No improvement resulted from repeated passage of the "purified" solvent through a column of type 4a molecular seive. Commercial dimethylsulphoxide was found to be much less pure than the solvents used in the above experiments but fractional distillation under reduced pressure gave a product of similar purity. The low boiling fraction was discarded as described above, and distillation was stopped when about 10% of the solvent still remained. This procedure was adopted for future experiments.

Mixtures of N-methyl-2-pyrrolidone or dimethylformamide and pyridine 1:1 were also found to be suitable solvents for the tritylation of methylα-D-glucopyranoside but attempts to purify these solvents were equally unsuccessful. For solubility reasons, neither of these solvent systems is suitable for the tritylation of dextran.

EXPERIMENT 32. TRITYLATION OF DEXTRAN.

The results of the previous experiments indicated that dimethylsulphoxide could be used as a solvent for the tritylation of dextran provided it was purified beforehand and provided that a large excess of trityl chloride was used. In the following experiments, dimethylsulphoxide was purified by distillation as described in Experiment 31, and the amount of trityl chloride used was 10 times the amount required for primary tritylation of the end-units (assuming 20 % branching).

Dextran (0.5 g.) was dissolved by heating in dimethylsulphoxide (8.3 ml.), and the resulting solution was diluted with pyridine (8.3 ml.). Trityl chloride (1.73 g.) was added and the solution was stored at 23.5°C. At intervals, portions of the viscous solution were withdrawn and diluted with an excess of methanol. Each sample was heated at 100°C for a few minutes to destroy the residual trityl chloride and examined by thin layer chromatography (see Experiment 31) to verify that methyl trityl ether had been produced and that there had in fact been an excess of reagent left. The solution was then poured in a thin stream into a large volume of methanol and the white fibrous product was collected by centrifugation. This was washed with methanol (by centrifugation) until the washings no longer gave a yellow colouration with concentrated sulphuric acid, and was then dried at 60°C *in vacuo*. The products were analysed by method 14 and the derived percentage O-trityl contents were plotted against time (Fig. 12). In an attempt to increase the specificity of the reaction, a similar experiment was carried out at 0°C and the results are also shown in Fig. 13.

As no pronounced increase in specificity was achieved by

Fig. 12. Tritylation of dextran at 23.5°C

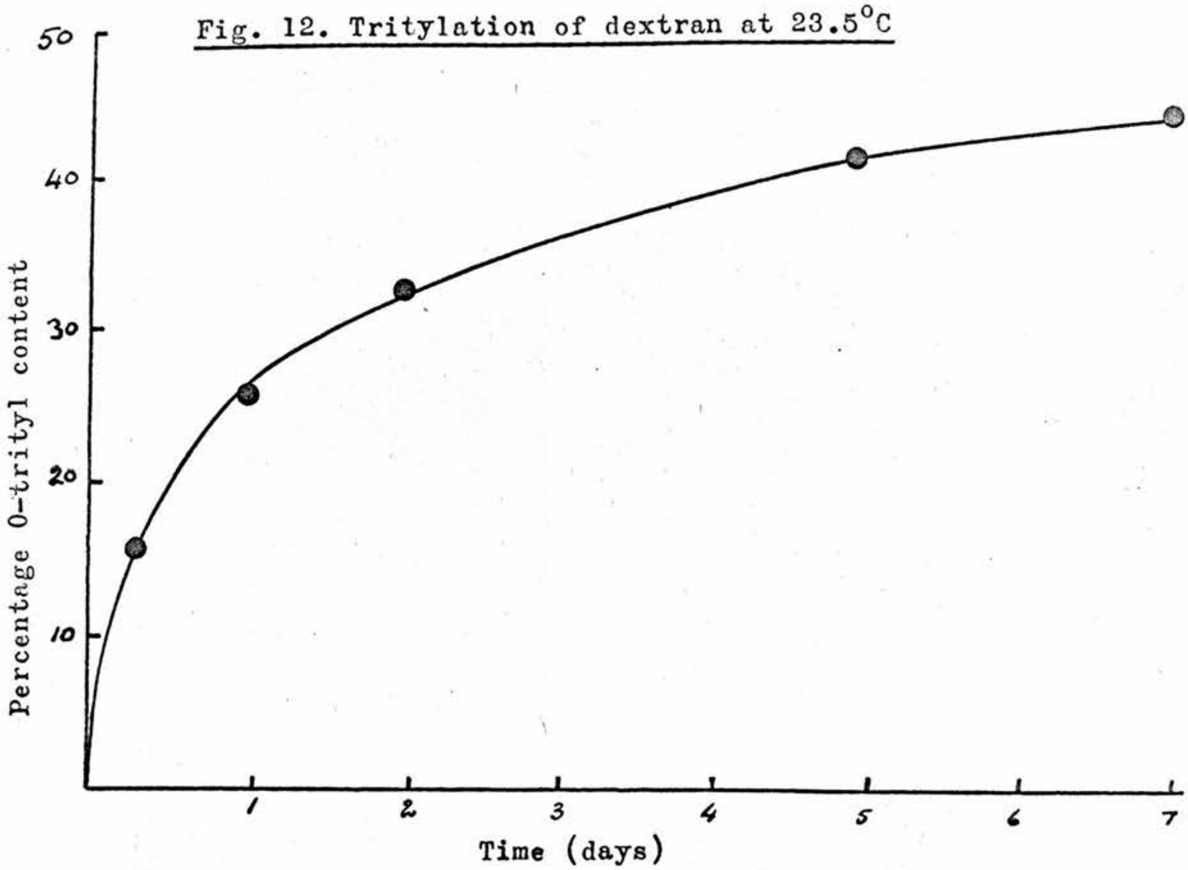
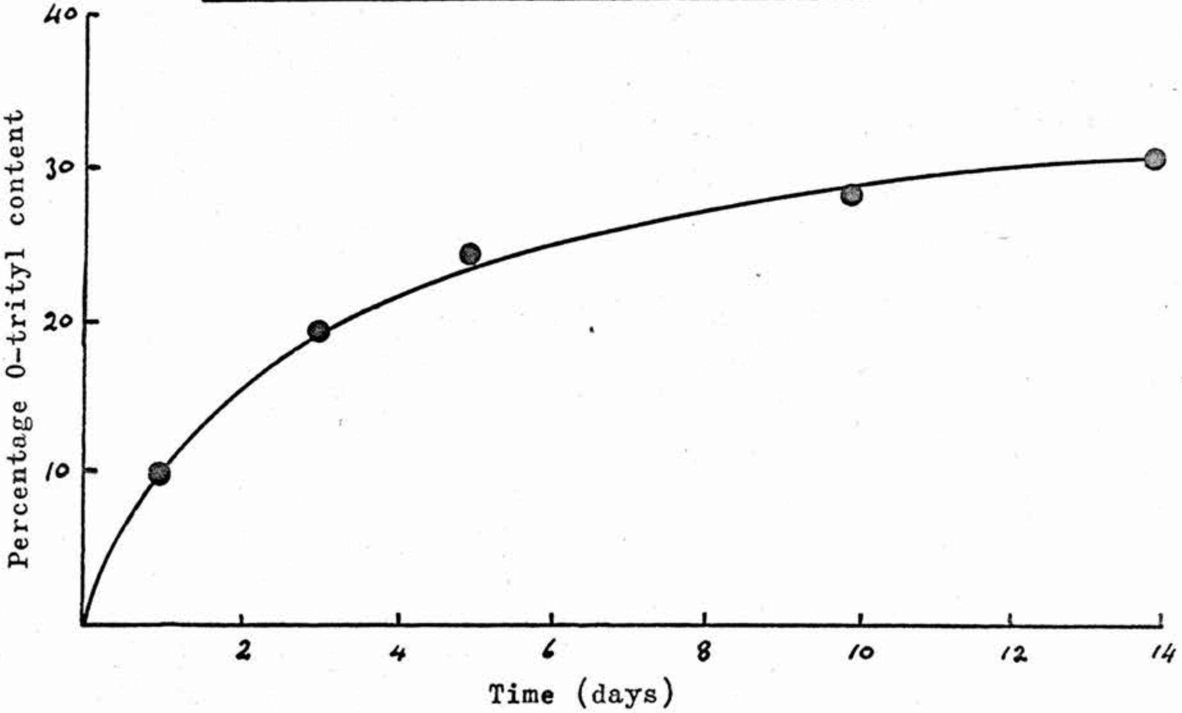


Fig. 13. Tritylation of dextran at 0°C.



this change in temperature, a large amount of dextran was tritylated at 23.5°C. Dextran (9.0 g.) was dissolved in dimethylsulphoxide (150 ml.) and the solution was diluted with pyridine (150 ml.). Trityl chloride (31 g.) was added and the solution was stored at 23.5°C for 27 hr. The product was isolated as described above and dried _in_ _vacuo_ at 60°C. Yield, 9.2 g. (79% on basis of 20% branching; Found: OTr, 29.2. Calc. for 20% branching: OTr, 24.6 %).

EXPERIMENT 33. METHYLATION OF TRITYLATED DEXTRAN.

Tritylated dextran (5.0 g.) was dissolved in dimethylformamide (100 ml.), Methyl iodide (60 ml.) and barium hydroxide octahydrate (150 g.) were added to the solution at 0°C and the mixture was shaken at this temperature for 2 hr. After shaking for a further 16 hr., the methylated product was isolated as a white powder as described in Experiment 30. Yield, 4.4 g. Part of this material (3.7 g.) was dissolved in dimethylformamide (100 ml.). Methyl iodide (10 ml.) and silver oxide (20 g.) were added, and the mixture was heated under reflux (40°C) with magnetic stirring. After 16 hr., a second addition of methylating reagents was made, and heating and stirring were continued for a similar period. The product was isolated as a white powder by the method described in Experiment 30 and found to give no hydroxyl absorption in the infrared. Yield, 3.6 g. (73 %). (Found: OMe, 32.3; OTr. 24.0. Calc; OMe, 32.4; OTr, 24.8 %). A sample of this material was methanolysed, and the products were examined by GLC (Methods 11 and 12). No peaks corresponding to the methyl glycosides of 2,3,4,6-tetra-O-methylglucose were detected.

EXPERIMENT 34. DETRITYLATION.

Heterogeneous detritylation of the product from the above experiment (using methanolic hydrogen chloride) was found to be ineffective and the reaction was therefore carried out homogeneously. The methylated, tritylated dextran (3 g.) was dissolved in chloroform (20 ml.), and methanolic hydrogen chloride, prepared from acetyl chloride (0.18 ml.) and methanol (80 ml.), was added. The resulting solution was left at room temperature overnight, neutralised with silver carbonate, filtered and concentrated to a syrup. The syrup was dissolved in chloroform and petroleum ether was added to incipient precipitation. On pouring this solution into a large volume of petroleum ether, a grey-white powder was obtained which was further purified by reprecipitation. Yield 1.5 g. (65 %). This product was shown to contain less than 0.4 % OTr. (Found: OMe, 39.1. Calc.: 42.4 %).

EXPERIMENT 35. TOLUENE-P-SULPHONYLATION.

The product of detritylation (1.4 g.) was dissolved in pyridine (20 ml.) containing toluene-p-sulphonyl chloride (1.35 g.) and the solution was left at 37°C overnight. The reaction mixture was diluted with water (500 ml.) and the aqueous solution was extracted four times with 200 ml. portions of chloroform. The combined extracts were washed four times with 500 ml. portions of water and then dried over anhydrous sodium sulphate, filtered and concentrated to a syrup. The product was isolated by precipitation with petroleum ether and was found to give a slight hydroxyl absorption in the infrared. Complete esterification was achieved on retreatment with toluene-p-sulphonyl chloride and the final product was a light yellow powder. Yield, 1.30 g. (78 %). (Found: OMe, 33.8; S, 2.9. Calc.: OMe, 35.4; S, 3.3 %).

EXPERIMENT 36. INVESTIGATION OF THE ACID STABILITY OF THE DEXTRAN DERIVATIVE.

The hydrolysis products of methylated dextran which differ in the degree of substitution of their hydroxyl groups can be separated by thin layer chromatography using 15% methanol in benzene as developing solvent. A small portion of the methylated tosylated dextran was hydrolysed in 45% formic acid (Method 7) and the hydrolysate was examined by thin layer chromatography. It was found that the main carbohydrate product had the same mobility as a tri-O-methylglucose but that there were several other unidentifiable products. Essentially the same result was obtained after hydrolysis for 12, 16 and 60 hr. The spot corresponding to the main carbohydrate product gave a negative reaction with the spray reagent specific for toluene-p-sulphonate esters (Method 15) while some of the others gave a positive reaction. A positive reaction was also given by some of the products which did not react with anisaldehyde-sulphuric acid spray reagent. None of the products had the same mobility as toluene-p-sulphonic acid. The pattern of tosyl ester-like products was similar to that obtained on formic acid hydrolysis of methyl 2,3,4-tri-O-methyl-6-O-toluene-p-sulphonyl-α-D-glucopyranoside (Experiment 27). These results were interpreted in terms of the reducing properties of formic acid (see Discussion).

Further experiments were carried out under methanolysis conditions. These involved heating samples in 3 % methanolic hydrogen chloride at 100°C in sealed tubes for 2 hr. Under these conditions, methylated dextran is completely methanolysed and the products are separable on thin layer chromatography using the same solvent as above. Methyl 2,3,4-tri-O-methyl-6-O-toluene-p-sulphonyl-α-D-glucopyranoside gave a spot of the same mobility as the starting material and no other products were detected with either of the spray reagents used above; no de-tosylation therefore occurred under these conditions. Examination of the methanolysate of the methylated, tosylated dextran showed that almost all the carbohydrate products had the same mobility as methyl tri-O-methylglucosides. Small amounts of products which were probably methyl di-O-methylglucosides were also detected. The major carbohydrate spot gave a positive reaction with the specific spray reagent, and only trace amounts of other materials gave a similar reaction. No products of the same mobility as methyl 2,3,4-tri-O-methyl-6-O-toluene-p-sulphonyl-α-D-glucopyranoside were detected, and the numerous non-carbohydrate, tosyl ester-like products that were observed on formic acid hydrolysis were absent.

3(C) DISCUSSION

A comparison of the rates of hydrolysis of various methyl glucosides (Experiment 27) revealed that the presence of the tosyloxy substituent on C-6 stabilised the glycosidic linkage to acid hydrolysis. The observed stabilisation was greater than between methylα-D-glucopyranoside and methylα-D-glucopyranosiduronic acid(83) and there was therefore reason to believe that it might be even more pronounced if another sugar unit were present as the aglycone (see Introduction to this Section). This possibility was investigated by preparing a fully methylated dextran derivative in which all the primary hydroxyl groups were tosylated. This was achieved by selective tritylation followed by complete methylation, de-tritylation and tosylation. Methanolysis of the product then gave a sound indication that the terminal glycosidic linkages were indeed stabilised.

The preliminary experiments (Experiment 27) indicated that hydrolyses of the 6-tosylated glucosides were accompanied by other reactions. The non-methylated compound on aqueous mineral acid hydrolysis gave two non-tosylated carbohydrate reducing products, while the methylated glucoside, which for solubility reasons was hydrolysed with aqueous formic acid, gave a variety of products. Some of the latter products gave a positive reaction for tosyl esters with the specific spray, and were non-carbohydrate. It was later shown (Experiment 36) that methanolysis of the methylated glucoside gave no such artefacts (it was of course not possible to assess with certainty the degree of stabilisation in this experiment).

It would seem therefore that sugar hydroxyl groups are involved in the formation of these artefacts in mineral acid, probably by intramolecular displacement of the tosyloxy substituent as the first stage. A further complication in the formic acid hydrolyses was that detosylation appeared to be taking place by a reductive pathway. This emerged from experiments (see below) on the hydrolysis of the stabilised dextran derivative in this medium, when numerous artefacts were observed; in contrast, preliminary experiments (not reported in this Thesis) have shown that artefacts are not formed when the hydrolysis is carried out in aqueous acetic acid-hydrochloric acid, or 50% chloroacetic acid.

In order to determine the stabilising effect of the tosyloxy group at the polysaccharide level, a dextran with a suitable proportion of primary hydroxyl groups was prepared (see Introduction to this Section). Examination of this polysaccharide by proton magnetic resonance spectroscopy showed that about 20% of the units in the molecule were branch-units. This was confirmed by the results of the methylation study carried out by Mr. N.J. Wight which also showed that the polysaccharide was composed of 1,6-linked glucose units with 1,3-branch linkages. The proportion of 1,3-linkages in the main chain was sufficiently small to be disregarded as a potential complication.

Because the objective of this investigation was to assess the stabilising effect of the tosyloxy substituent, one of the ultimate aims was to determine the yield of the stabilised disaccharide after acid hydrolysis. For this reason it was necessary to stabilise as many of the terminal glycosidic linkages as possible, while limiting secondary tosylation to a minimum. Homogeneous reaction conditions and a low temperature were therefore used for the tritylation of the dextran. A 1:1 mixture of dimethylsulphoxide and pyridine was found to be a suitable solvent for this reaction although impurities in the dimethylsulphoxide made careful purification necessary. In several abortive experiments, in which impure dimethylsulphoxide was used, the O-trityl content of the dextran was found to have reached a maximum at less than the theoretical value. When the thin layer chromatographic technique (Experiment 31) had been developed, it was shown that this was because there was consumption of trityl chloride by the solvent with the result that there was none left in the reaction mixture, and the tritylation of the dextran therefore ceased.

The colorimetric method for the analysis of tritylated dextrans (Method 14) was found to be more convenient than the gravimetric method(91) for the rapid analysis of small amounts of material. The use of the Cahn electrobalance reduced volumetric manipulations of the viscous solutions to a minimum and the results were reproducible to ± 5%.

A high degree of specificity was observed in the tritylation of methylα-D-glucopyranoside at 23.5°C (Experiment 31). This was not so in the tritylation of the dextran - even at 0°C (Figs. 12 and 13); this is presumably because there are five times as many secondary hydroxyl groups in the dextran, though other factors may also be involved. The conditions that were finally chosen were such as to ensure complete primary tritylation of the polysaccharide for the reasons stated above. A certain amount of secondary tritylation was unavoidable under these conditions but greater selectivity may be possible when the experiment is repeated with the more highly branched araban. Also, the araban is soluble in pyridine and could therefore be tritylated using a much smaller excess of trityl chloride as it would not be necessary to allow for large amounts of impurities in the solvent.

Methylation of the tritylated dextran showed that complete primary tritylation had been achieved as no methyl glycosides of 2,3,4,6-tetra-O-methylglucose were detected after methanolysis of part of the product. Analysis of the dextran derivatives after de-tritylation and after tosylation gave good agreement with the calculated values although the sulphur content of the final product was rather low. This was not consistent with the results of infrared spectroscopy which showed no hydroxyl absorption, and was presumably due to an analytical error in the sulphur determination or in the original determination of the O-trityl content of the tritylated dextran.

Formic acid hydrolysis of the methylated tosylated dextran gave several products - some of which were similar to those obtained on similar hydrolysis of methyl 2,3,4-tri-O-methyl-6-O-tosyl-α-D-glucopyranoside (Experiment 27). There was obviously no stabilisation under these conditions and this was attributed to the removal of the stabilising substituent by reductive de-tosylation. After subjection of methyl 2,3,4-tri-O-methyl-6-O-tosyl-α-D-glucopyranoside to severe methanolysis conditions (Experiment 36), the reaction mixture was examined by thin layer chromatography and found to contain only material of the same mobility as the original compound. It is possible that the anomeric methyl glycosides of this compound are not separable by thin layer chromatography and that methanolysis did occur under these conditions but the relevant conclusion was that the tosyloxy substituent was not removed (methyl 2,3,4-tri-O-methyl-D-glucosides are separable from methyl 2,3,4-tri-O-methyl-6-O-tosyl-α-D-glucopyranoside by thin layer chromatography). Methanolysis of the terminal glycosidic linkages in the dextran derivative would give the methyl glycosides of 2,3,4-tri-O-methyl-6-O-tosyl-D-glucopyranose (the tosylate ester being stable under these conditions). At least the α-anomer of these would have been identified by its known chromatographic mobility. No compound of this mobility was detected in the methanolysate which showed, almost conclusively, that the terminal linkage was stable to methanolysis. A possible explanation of these results is that the glycoside was stable, while the disaccharide was methanolysed to give exclusively the β-glycoside of the tosylated moiety (having the same mobility as a methyl tri-O-methylglucoside on thin layer chromatography). This seems most unlikely and it is almost certain that the terminal glycosidic linkages in the dextran were stabilised by the presence of the tosyloxy substituent on C-6 of the end-units. The other tosyl ester-like products which were detected in trace amounts were presumably the result of stabilisation of other linkages in the molecule by primary tosylation of the 1,3-linked chain units or secondary tosylation of other units.

The methanolysis conditions used in these experiments were shown to be sufficiently drastic for the complete depolymerisation of methylated dextran. As the stabilised linkages remained intact during this treatment, quantitative determination of the stabilised products was unnecessary. Useful structural information could be obtained, however, by isolating and characterising these products, and this is an obvious follow-on from the present work. Fractionation of the methanolysis products would be complicated by the presence of different anomeric forms of the same compound, and hydrolysis in an aqueous system would be more suitable. Unfortunately, some of the methanolysis products are insoluble in mineral acids (as would be expected for such tosyl esters) and acid hydrolysis in aqueous methanol gives glycoside formation. The dextran derivative is, however, soluble in 50% aqueous monochloro-acetic acid, and preliminary experiments have shown that hydrolysis is possible in this solution. Characterisation of the stabilised disaccharides would show whether the polysaccharide contained single unit side-chains and, if so, whether these were linked through position 3 or position 6 of the units in the main-chain. This information would be obtained in quantitative terms and, as such, would be a valuable contribution to the present knowledge of the fine structure of dextrans.

GENERAL METHODS

1. Germination of seeds. The seeds used in these experiments (Brassica sinapis alba) were purchased from a local seed merchant. For germination, they were soaked in water overnight and spread out on a double thickness of Whatman 3 MM paper. The paper was supported on a glass plate, and its ends were immersed in troughs of water. After germination in the dark for a suitable time, the seeds were plunged into liquid nitrogen and freeze-dried.
2. Concentration of polysaccharide solutions. Solutions were concentrated under reduced pressure using a rotary film evaporator and a bath temperature of 40°C.
3. Dialysis. Polysaccharide solutions were dialysed for 3 days in cellophane tubes suspended in running tap water. Chloroform was added to prevent bacterial action.
4. Removal of protein from mustard seed extracts. The method used was that of Westphal *et al.*[26]. A solution of the extract (2% w/v) was adjusted to 45% phenol concentration (w/v), shaken for 30 min. and left at 2°C overnight when the phenol layer was discarded (if no such layer was formed, more phenol was added to the system and it was again left at 2°C). The procedure was usually repeated twice more, and the residual phenol was removed by extraction into ether before concentration of the solution and freeze-drying. Emulsions formed during extraction with ether were broken by the addition of Cetavlon.

5. Carbohydrate contents. These were determined, using arabinose as standard, by the phenol-sulphuric acid method(92) and are quoted as percentage anhydroarabinose contents.

6. Protein contents. These were calculated by multiplying the percentage content of N (Kjeldahl) by 6.

7. Hydrolysis. Cellular material was suspended in 72% (w/v) sulphuric acid and shaken until it dissolved. After dilution to 1.5 N-sulphuric acid concentration, the solution was heated at 100°C for 4 hr., neutralised with calcium carbonate, filtered and concentrated to a syrup for examination by paper chromatography. Polysaccharide material was hydrolysed at 1% concentration in 1 N-sulphuric acid by heating at 100°C for 16 hr. The cooled hydrolysate was then neutralised and prepared for further examination as described above.

   Methylated polysaccharides were also hydrolysed at 1% concentration. A portion of the methylated material was dissolved in 90% formic acid, and the resulting solution was diluted with an equal volume of water. After heating the solution at 100°C for 16 hr., the hydrolysate was concentrated to a syrup on a rotary film evaporator (bath temperature 40°C) and the last traces of formic acid were removed by co-distillation with water. The residue was dissolved in water and left at room temperature overnight to ensure complete hydrolysis of formyl esters, and the solution was then concentrated to a syrup for further examination.

8. Paper chromatography. Whatman No. 1 or No. 4 paper was used for qualitative work and Whatman 3MM paper for preparative experiments. In the latter cases, the mixtures of sugars were applied as streaks which were not quite extended to the edges

of the papers. After development, the positions of the resulting bands were located by spraying test strips with the appropriate reagent. The solvent systems used for paper chromatography in general were: for neutral sugars, ethyl acetate-pyridine-water (8:2:1, by vol.); for uronic acids, ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by vol.); for methylated sugars, butan-1-ol-ethanol-water 4:1:5, by vol.) or butan-2-one-water-conc. ammonia (200:17:1, by vol.). The position of spots on chromatograms were located by treatment with one of the following spray reagents:

(a) p-Anisidine hydrochloride. (For reducing sugars).

The air-dried chromatogram was sprayed with a solution (3% w/v) of p-anisidine hydrochloride in water-saturated butan-1-ol containing a trace of stannous chloride, and heated at 110-120°C for about 5 min. (Stannous chloride was added so that uronic acids could be detected).

(b) Aniline oxalate. (For reducing sugars).

Chromatograms were treated as above with a saturated solution of aniline oxalate in ethanol. This spray reagent was used mainly for methylated sugars.

(c) Urea oxalate. (For ketoses).

Chromatograms were sprayed with a saturated solution of urea oxalate in 80% aqueous ethanol and heated at 100°C until blue-black spots appeared on a colourless background.

(d) Periodate-benzidine. (For compounds containing glycol groups).

A 2.28% aqueous solution of periodic acid (1 ml.) was diluted with acetone (29 ml.). The chromatogram was passed through this solution, left at room temperature for 7 min, and passed through a solution prepared by dissolving benzidine (1.84 g.) in a mixture of acetic acid (6 ml.) and acetone (1 l.). White spots were obtained on a blue background after a few minutes at room temperature.

9. Zone electrophoresis. The paper was held between two sheets of polythene which were clamped between two cooling blocks. The potential gradient was normally 40 v/cm. and the running time 1 hr. Prior to each run, the paper was immersed in the buffer and then blotted. After positioning on the apparatus, it was spotted with the solutions to be examined. When a filter paper support was used, the spots were located with the potassium periodocuprate spray, followed by rosaniline(93). With glass fibre paper, the spray was anisaldehyde and sulphuric acid in ethanol(94). In all cases, cafeine (visible under an ultraviolet lamp) was used as an endosmotic marker. The anisaldehyde sulphuric acid spray reagent was a solution of anisaldehyde (1 ml.) and AnalaR conc. sulphuric acid (1 ml.) in ethanol (20 ml.). The paper was sprayed very lightly with this reagent to minimise background colouration, and then heated at 110-120°C for about 5 min. Sugars gave dark spots on a colourless background.

10. Thin layer chromatography. Silica gel(Kieselgel G Nach, Stahl., Merck) was added to about 4 ml. of water (with shaking) until a creamy suspension was obtained. This was sufficient for the preparation of about 12 plates. Part of the suspension was poured onto a clean microscope slide and smeared over the surface. By tilting and gently agitating the plate, a thin homogeneous layer was obtained. This was left at room

temperature to set, heated at 100°C for 20 min. and stored in a desiccator. Samples were applied as spots of about 1 mm. diam. and plates were developed for 5-10 min. with solutions of methanol in benzene. Unless otherwise stated, the anisaldehyde-sulphuric acid spray reagent was used (see Method 9) and the plate was then gently heated over a bunsen flame for a few seconds.

11. <u>Methanolysis</u>. The sample was heated in a sealed tube with dry methanolic hydrogen chloride (3%) for 6 hr. The resulting solution was neutralised with silver carbonate, filtered and concentrated to a syrup at room temperature under reduced pressure.

12. <u>Gas-liquid chromatography</u>. This was carried out with a Pye Argon chromatograph fitted with a ^{90}Sr detector. For the examination of mixtures of methylated glycosides, the conditions described by Aspinall(60) were used.

13. <u>Optical rotations</u>. These were measured at room temperature using a 1 dm. polarimeter tube. Water was used as solvent unless otherwise stated.

14. <u>Determination of triphenylmethoxyl contents</u>. These were determined by a method based on that of Duffield and Nussbaum(95) in which the reagent used is a 1:1 mixture of sulphuric acid and methanol (v/v) and in which the colouration due to the triphenylmethyl carbonium ion is measured. A calibration graph was constructed using a standard solution of triphenyl carbinol (50 mg.) in methanol (50 ml.). Aliquots were micropipetted into "EEL" colorimeter tubes, diluted with the acid reagent (8 ml.) and the solutions were then stirred with a glass rod. The optical densities were

measured in the colorimeter using filter no. 621 (maximum transmission at 450 mμ) and in this way, a calibration graph covering the range 10-100 μg. was obtained. Samples of tritylated dextran (0.1 mg.) were accurately weighed using the Cahn electrobalance, and transferred to colorimeter tubes. Reagent (<u>ca</u>. 1 ml.) was then added from a burette and the sample was dissolved by warming to 80°C and swirling the tube for about 1 min. The solution was then diluted to 8 ml. with reagent from the burette and, after stirring, the optical density was measured. Control experiments using mixtures of triphenylcarbinol and native dextran showed that neither heating nor the presence of dextran in concentrations similar to those encountered in typical analyses interferes with the reading obtained. The colouration was also shown to be stable for a few hours.

15. <u>Detection of tosyl esters</u>. The spray reagent of Jackson and Hayward(90) was used. This was a 1% solution of diphenylamine in ethanol. Plates were sprayed with the reagent and exposed to ultraviolet radiation (from a Camag Universal UV Lamp) for about 5 min. Fluorescent spots were obtained on a colourless background.

16. <u>Proton magnetic resonance spectroscopy</u>. Spectra were taken at 60 mc/s. Unless otherwise stated polysaccharides were freeze-dried from D_2O solution three times and 10% solutions of the deuterated products (in the same solvent) were examined.

17. <u>Evaporation of solutions from B 10 Quickfit test tubes</u>. The test tube was attached by means of a B 10 cone and length of rubber tubing to a water pump. Evaporations were carried out under reduced pressure by swirling the test tube in a beaker of water at 30°C.

REFERENCES

(1) Smith, F., and Montgomery, R., Chemistry of Plant Gums and Mucilages (Reinhold Publishing Corp., New York, 1959) p.324.

(2) Horvei, K.F., and Wickstrom, A., Acta Chem. Scand., 18, 833 (1964).

(3) Thompson, J.L., and Jones, J.K.N., Canad. J. Chem., 42, 1088 (1964).

(4) Bailey, K., and Norris, F.W., Biochem. J., 26, 1609 (1932).

(5) Hunt, K., and Jones, J.K.N., Canad. J. Chem., 40, 1266 (1962).

(6) Laidlaw, R.A., and Percival, E.G.V., J., 528 (1950).

(7) Northcote, D.H., Symp. Soc. Exp. Biol., 17, 157 (1963).

(8) Roelofsen, P.A., The Plant Cell Wall, Encyclopedia of Plant Anatomy, vol. 3, part 4. (Gebruder Borntraeger, Berlin, 1959).

(9) Wilson, K., International Review of Cytology, 17, 1 (1964).

(10) Prof. R. Brown, F.R.S., Personal Communication.

(11) Gould, S.E.B., and Rees, D.A., J. Sci. Food Agric. (in press).

(12) Hirst, E.L., and Jones, J.K.N., J., 1221 (1947).

(13) Hirst, E.L. and Jones, J.K.N. J., 2311 (1948).

(14) Neukom, H., and Deuel, H., Chem. & Ind. (London), 638 (1958).

(15) Albersheim, P., Neukom, H., and Deuel, H., Arch. Biochem. Biophys., 90, 46 (1960).

(16) Aspinall, G.O., and Fanshaw, R.S., J., 4215 (1961).

(17) McCready, R.M., and McComb, E.A., Analyt. Chem., 24, 1986 (1952).

(18) Lindberg, B., International Union of Pure and Applied Chemistry, Proceedings of the Wood Chemistry Symposium, Montreal, Canada (1961) (Butterworths, London, 1962) p. 67.

(19) Whistler, R.L., and BeMiller, J.N., Adv. Carbohydrate Chem., 13, 289 (1958).

(20) Timell, T.E., J. Amer. Chem. Soc., 82, 5211 (1960).

(21) Bouveng, H.O., Garegg, P.J., and Lindberg, B., Acta Chem. Scand., 14, 742 (1960).

(22) Thornber, J.P., and Northcote, D.H., Biochem. J., 81, 449 (1961).

(23) Bell, D.J., and Young, F.C., Biochem. J., 28, 882 (1934).

REFERENCES (Contd.)

(24) Sevag, M.G., Lockman, D.B., and Smollens, J., J. Biol. Chem., 124, 425 (1938).

(25) Adams, M., Richtmeyer, N.K., and Hudson, C.S., J. Amer. Chem. Soc., 65, 1369, (1943).

(26) Westphal, O., Luderitz, O., and Bister, F., Z. Naturforsch., 7b, 148 (1952).

(27) Jeanloz, R.W., and Forchielli, E., J. Biol. Chem. 186, 495 (1950).

(28) Neukom, H., Deuel, H., Heri, W.G., and Kundig, W., Helv. Chim. Acta, 43, 64 (1960).

(29) Hirst, E.L., and Jones, J.K,N., J., 452 (1939).

(30) Barret, A.J., and Northcote, D.H., Biochem. J., 94, 617 (1965).

(31) Hirst, E.L., and Jones, J.K.N., J., 454 (1939).

(32) Barker, S.A., Stacey, M., and Zweifel, G., Chem. & Ind., 330 (1957).

(33) Hirst, E.L., and Jones, J.K.N., Adv. Carbohydrate Chem., 2, 235 (1946).

(34) Jermyn, M.A., Modern Methods of Plant Analysis, vol. 2, Ed. by K. Paech & M.V. Tracey. (Springer-Verlag, Berlin, 1955) p. 203.

(35) Roe, J.H., Epstein, J.H., and Goldstein, N.P., J. Biol. Chem., 178, 839 (1949).

(36) Kuhn, R., and Trischmann, H., Chem. Ber., 96, 284 (1963).

(37) Aspinall, G.O., and Baillie, J., J., 1714 (1963).

(38) Laidlaw, R.A., and Percival, E.G.V., Adv. Carbohydrate Chem., 1, 1 (1952).

(39) Kuhn, H., Trischmann, H., and Low, I., Angew. Chem., 67, 32 (1955).

(40) Andrews, P., Hough, L., and Powell, D.B., Chem. & Ind., 658 (1956).

(41) Yphantis, D.A., Ann. New York Acad. Sci., 88, 586 (1960).

(42) Beaven, G.H., Hirst, E.L., and Jones, J.K.N., J., 1865 (1939) and earlier papers.

(43) Wallenfels, K., Bechtler, G., Kuhn, R., Trischmann, H., and Egge, H., Angew. Chem. Internat. Edn., 2, 515 (1963).

(44) Ferrier, R.J., and Williams, N.R., Chem. & Ind., 1696 (1964).

(45) Pasika, W.M., and Cragg, L.H., Canad. J. Chem., 41, 293 (1963).

(46) Capon, B., and Thacker, D., Proc. Chem. Soc., 369 (1964).

REFERENCES (Contd.)

(47) Smith, F., and Stephen, A.M., J., 4892 (1961).

(48) Morawetz, H., in Rice, S.A., and Nagasawa, M., Polyelectrolyte Solutions (Academic Press, New York and London, 1961) p. 207.

(49) Aspinall, G.O., and Canas-Rodriguez, A., J., 4020 (1958).

(50) Mrs. S.E.B. Gould, unpublished result.

(51) Jones, H.G., and Perry, M.B., Canad. J. Chem., 40, 1339 (1962).

(52) Eposito, G.G., and Swann, M.H., Analyt. Chem., 33, 1851 (1961).

(53) Gee, M., and Walker, H.J.Jr., Analyt. Chem. 34, 651 (1962).

(54) Sweeley, C.C., Bentley, R., Makita, M. and Wells, W.W., J. Amer. Chem. Soc., 85, 2497 (1963).

(55) Bishop, C.T., Cooper, F.P., and Murray, R.K., Canad. J. Chem., 41, 2245 (1963).

(56) McInnes, A.G., Ball, D.H., Cooper, F.P., and Bishop, C.T., J. Chromatog., 1, 556 (1958).

(57) Bishop, C.T., Adv. Carbohydrate Chem., 19, 95 (1964).

(58) Hedgley, E.J., and Overend, W.G., Chem. & Ind. (London), 378 (1960).

(59) Bishop, C.T., and Cooper, F.P., Canad. J. Chem., 38, 388 (1960).

(60) Aspinal, G.O., J., 1676 (1963).

(61) Hause, J.A., Hubicki, J.A., and Hazen, G.G., Analyt. Chem., 34, 1567 (1962).

(62) Mr. N.S. Anderson, personal communication.

(63) Dr. J. Turvey, personal communication.

(64) Dr. C.C. Sweeley, personal communication.

(65) Ness, R.K., and Fletcher, H.G. Jr., J. Amer. Chem. Soc., 80, 2007 (1958).

(66) Cifonelli, M., Cifonelli, J.A., Montgomery, R., and Smith, F., J. Amer. Chem. Soc., 77, 121 (1955).

(67) Jones, J.K.N., Kent, P.W. and Stacey, M., J., 1341 (1947).

(68) Rebers, P.A., and Smith, F., J. Amer. Chem. Soc., 76, 6097 (1954).

(69) Bouveng, H.O., Acta Chem. Scand., 15, 87 (1961).

REFERENCES (Contd.)

(70) Baddiley, J., Buchanan, J.G., and Carss, B., J., 4138 (1957).

(71) Goldstein, I.J., Hay, G.W., Lewis, B.A., and Smith, F., Amer. Chem. Soc. Meeting, Boston, April 1959, Abs. Papers, 3D.

(72) Aspinall, G.O., and Ferrier, R.J., Chem. & Ind. 1216 (1957).

(73) Finan, P.A., and O'Colla, P.S., Chem. & Ind., 493 (1958).

(74) Sengupta, A.K., Mukherkee, A.K., and Rao, C.V.N., Austral. J. Chem., <u>18</u>, 851, (1965).

(75) Aspinall, G.O., Cairncross, I.M., and Nicolson, A., Proc. Chem. Soc., 270 (1959).

(76) Overend, W.G., Ross, C.W., and Sequeira, J.S., J., 3429 (1962).

(77) Banks, B.E., Meinwald, Y., Rhind-Tutt, A.J., Sheft, I., and Vernon, C.A., J., 3240 (1961).

(78) Hammett, L.P., and Deyrup, A.J., J. Amer. Chem. Soc., <u>54</u>, 2721 (1932)

(79) Armour, C., Bunton, C.A., Patai, S., Selman, L.H., and Vernon, C.A., J., 412 (1961).

(80) Bunton, C.A., Lewis, T.A., Llewellyn, D.R., and Vernon, C.A., J., 4419 (1955).

(81) Timell, T.E., Canad. J. Chem., <u>42</u>, 1456 (1964).

(82) Feather, M.A., Harris, J.F., J. Org. Chem., <u>30</u>, 153 (1965).

(83) Timell, T.E., Chem. & Ind., 503 (1964).

(84) Edward, J.T., Chem. & Ind. (London), 1102 (1955).

(85) Jones, J.K.N., and Nicholson, W.H., J., 3050 (1955).

(86) Taft, R.W. Jr., in Steric Effects in Organic Chemistry. Ed. by Newman, M.S., (Wiley, London, 1956), p. 556.

(87) Semke, L.K., Thompson, N.A., and Williams, D.G., J. Org. Chem., <u>29</u>, 1041 (1964).

(88) Hine, J., and Ramsay, O.B., J. Amer. Chem. Soc., <u>84</u>, 973 (1962).

(89) Dr. D.H. Hutson, personal communication of results obtained by application of the methods of Jeanes <u>et al</u>., J. Amer. Chem. Soc., <u>76</u>, 5041 (1954).

(90) Jackson, M., and Hayward, L.D., J. Chromatog., <u>5</u>, 166 (1961).

REFERENCES (Contd.)

(91) Hearon, W.M., Hiatt. G.D., and Fordyce, C.R., J. Amer. Chem. Soc., 65, 2449 (1943).

(92) Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F., Analyt. Chem., 28, 350 (1956).

(93) Bonner, T.G., Chem. & Ind., 345 (1960).

(94) Stahl, E., and Kaltenbach, U., J. Chromatog., 5, 451 (1961).

(95) Duffield, A.M., and Nussbaum, A.L., Analyt. Biochem., 7, 254 (1964).

ABSTRACT OF THESIS

Name of Candidate NEIL GEORGE RICHARDSON

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Title of Thesis STUDIES OF THE POLYSACCHARIDES OF WHITE MUSTARD (SINAPIS ALBA).

SECTION 1. Graded extraction ~~of both~~ the embryos and the seed coats of mustard seeds revealed little difference between their polysaccharide components in terms of the sugar units present. Polysaccharide material containing the major component sugars of typical pectic substances was extracted from each under relatively mild conditions, while hemicellulosic material became increasingly more predominant as the conditions became more severe. A more detailed examination of the polysaccharides of the embryos revealed the presence of an araban (mustard seed araban) which was isolated in pure form. This was homogeneous on electrophoresis, on ultracentrifugation, and when chromatographed on DEAE-cellulose in the borate form. A control extraction and other experiments indicated that the araban was not a product of β-elimination from a larger and more complex molecule. The lability of the araban to acid hydrolysis suggested that many of the units were of the furanose type. Methylation analysis showed the polysaccharide to be composed of 1,5-linked arabinose units with a considerable proportion of 1,3-branch linkages. In addition, some of the units which were linked through positions 3 and 5 were also linked through position 2. The high negative value of the optical rotation suggested that the units were α-linked and further evidence of this was obtained by proton magnetic resonance spectroscopy. The molecular weight of the araban (6,000) was determined by ultracentrifugation, and this value was confirmed by vapour pressure measurements made on solutions of its methylated derivative.

SECTION 2. A quantitative gas-liquid chromatographic method was developed for structural analysis and comparison of arabans. The hydrolysis products of the methylated polysaccharides were reduced and acetylated to give mixtures of arabitol derivatives which were separated and analysed/

Use other side if necessary.

analysed by GLC. A series of pure crystalline reference compounds was prepared and used for calibration purposes (erythritol tetra-acetatebeing used as an internal standard). The analytical method was then used for the determination of the proportions of the different types of structural units present in mustard seed araban. Further applications were in the comparison of this araban with one extracted after germination of the seeds, and in following the course of the Smith periodate degradation of the araban. The results showed that germination brought about a pronounced decrease in the degree of branching in the polysaccharide, and the degradation experiment enabled fine structural details to be deduced.

<u>SECTION 3</u>. A comparison of the rates of hydrolysis of various methyl glucosides showed that the presence of a tosyloxy substituent on C-6 stabilises the gylcosidic linkage to acid hydrolysis. The possibility of using this substituent for the stabilisation of selected glycosidic linkages in a polysaccharide was investigated. A branched dextran was prepared and characterised by methylation analysis and proton magnetic resonance spectroscopy. By selective tritylation followed by complete methylation, de-tritylation and tosylation, a derivative was prepared in which all the primary hydroxyl groups were tosylated. Examination of the methanolysis productsof this derivative gave convincing evidence that the terminal glycosidic linkages were stabilised by the presence of the tosyloxy substituent.

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Seed Polysaccharides and their Role in Germination

A SURVEY OF THE POLYSACCHARIDE COMPONENTS OF MUSTARD SEEDS WITH SPECIAL REFERENCE TO THE EMBRYOS

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1. Methods were developed for the extraction, fractionation and purification of the more soluble polysaccharides of mustard-seed embryos. 2. One of these components was a pure homopolysaccharide, an araban, which was characterized by analysis, optical rotation, chromatography on diethylaminoethylcellulose and electrophoresis; the hydrolysis products of the methylated polysaccharide were isolated and characterized by the formation of crystalline derivatives. From these studies it emerges that mustard-seed araban is very similar to the family of pectic arabans, except that it is more highly branched than usual and contains a proportion of 1→2-linkages. 3. A survey of the other polysaccharides of mustard seed, both in the embryos and in the seed coats, suggests a predominance of pectic-type polysaccharides.

The systematic exploration of plant polysaccharides by methylation and other methods has been proceeding for over 30 years and many structural types are now recognized (Aspinall, 1962). In addition, some aspects of the biosynthesis and biological functions of the simpler polysaccharides are understood (Leloir, 1961). One of the next aims in the study of complex polysaccharides must be to understand in chemical terms their biological importance and their biosynthetic relations, one molecule with another. Perhaps the richest and most varied source of polysaccharides in the plant is the cell wall; in view of the synthesis and development of cell walls that occurs in germinating seeds, we have chosen this system for a study of polysaccharide degradation, synthesis and transformation in plants. As a start to this programme we are investigating the polysaccharides of white mustard (*Sinapis alba*). This seed was chosen because the plant can be conveniently grown and handled in the laboratory, because it is a representative of a family of important vegetables (e.g. cabbage, turnip) about whose polysaccharides remarkably little is known, and because it is a fatty seed in which the extraction and fractionation of cell-wall polysaccharides would presumably not be complicated by the presence of reserve polysaccharides.

As a preliminary to more detailed work, we have made a survey in broad outline of the polysaccharides that are present in the seed. This forms the subject of the present paper.

MATERIALS AND METHODS

Materials. Agricultural white mustard (*Brassica sinapis alba*) was purchased from a local seed merchant.

Protein contents of freeze-dried extracts. These were calculated by multiplying the percentage content of N (Kjeldahl) by 6.

Carbohydrate contents. These were determined, with arabinose as standard, with the phenol–sulphuric acid reagents (Dubois, Gilles, Hamilton, Rebers & Smith, 1956).

Paper chromatography. The solvents used were: for neutral sugars, ethyl acetate–pyridine–water (8:2:1, by vol.); for uronic acids, ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.); for methylated sugars, butan-1-ol–ethanol–water (4:1:5, by vol.). Spots were located by spraying the paper with *p*-anisidine hydrochloride in butanol, or aniline oxalate in ethanol, followed by heating in an oven at 110–120°.

Paper and glass-fibre electrophoresis. Several buffers were used, as mentioned in the text. The paper was held between two pieces of polythene, which were clamped between two cooled copper blocks. The potential gradient was normally 100v/cm. and the running time 1hr. When a filter-paper support was used, the spots were located with potassium periodocuprate spray, followed by rosaniline (Bonner, 1960). With glass fibre, the spray was anisaldehyde and sulphuric acid in ethanol (Stahl & Kaltenbach, 1961); it was important in this case to spray the electrophoretogram lightly, otherwise a pronounced background colour was obtained. In all cases caffeine (visible under an ultraviolet lamp) was used as the endosmotic marker.

Gas-liquid chromatography of methylated sugars as their methyl glycosides. This was carried out with a Pye Argon chromatograph fitted with a ^{90}Sr detector, essentially as described by Aspinall (1963).

Column chromatography of the araban on DEAE-cellulose. The column was prepared in the borate form by the method of Neukom, Deuel, Heri & Kündig (1960), and tested by adsorption of methyl red indicator. The dye was retained at the top of the column for as long as washing was continued with water. When the eluent was changed to 0·2M-sodium metaborate, the dye moved down as a discrete band, this being taken as an indication that the column was satisfactorily packed.

The washed column (24cm. × 1·5cm. diam.) was loaded with polysaccharide (0·2g.) and eluted in turn with (i) water (900ml.), (ii) 0·01M-sodium metaborate (300ml.), (iii) a linear gradient from 0·01M- to 0·10M-sodium metaborate (800ml.) and (iv) 0·5M-sodium metaborate. The flow rate was 0·5ml./min., and 10ml. fractions were collected automatically for analysis with the phenol–sulphuric acid reagents (Dubois *et al.* 1956).

Separation into seed coats and embryos. Whole seeds were passed through a small hand mill into boiling aq. 80% (v/v) ethanol. After 5–20min. in this solution they were drained, partly separated in a draught of air and then extracted in the Soxhlet extractor, first with ethanol and then with acetone. No further material was removed from the embryos with chloroform or with light petroleum. After further purification by sieving and in the air draught, the fractions were obtained by hand sorting.

RESULTS

Preliminary survey of the polysaccharides in the embryos and seed coats

For these experiments, the parts were separated by hand and were not extracted with organic solvents before use. The seed coats (1g.) and the embryos (3g.) were each treated with the following series of reagents: (*a*) boiling 80% (v/v) ethanol (100ml.); (*b*) cold water (2 × 500ml. at room temperature for 24hr.); (*c*) boiling water (2 × 500ml. for 4hr.); (*d*) aq. 0·5% ammonium oxalate (2 × 400ml. for 3hr. at 65–70°); (*e*) delignification by the chlorite method (Jermyn, 1955) followed by hot-water extraction (2 × 400ml. for 3hr.); (*f*) aq. 5% (w/v) potassium hydroxide under nitrogen (400ml. for 16hr. at room temperature); (*g*) aq. 20% (w/v) potassium hydroxide under nitrogen (400ml. for 16hr. at room temperature). The insoluble material was separated on a filter, washed with water and dried in a desiccator.

All fractions except those isolated with reagent (*a*) were purified by dialysis and isolated as freeze-dried solids. Part of each product was suspended in 72% (w/v) sulphuric acid and shaken until it dissolved. After dilution to 1·5N-sulphuric acid concentration, the solution was heated on a boiling-water bath for 4hr., neutralized with calcium carbonate, filtered, concentrated under diminished pressure and examined by paper chromatography. The results are given in Table 1. Some additional results, given in Table 2, were obtained with fractions that were isolated in a separate series of extractions on a larger scale. In this series, the fractions obtained by cold- and by hot-water extraction were not dialysed before being freeze-dried, but the procedure was otherwise the same as for the small-scale experiment. The fraction isolated with reagent (*a*) contained the low-molecular-weight carbohydrates

Table 1. *Sugars detected after hydrolysis of the polysaccharide fractions from mustard seed*

Experimental details are given in the text. For each fraction, sugars are listed in approximately descending order of concentration; those in parentheses are relatively minor components.

	Seed coats		Embryos	
Solvent used for extraction	Yield (%)	Sugars detected after hydrolysis	Yield (%)	Sugars detected after hydrolysis
Cold water	6·4	Galactose, arabinose, galacturonic acid (xylose, glucose, mannose)	2·4	Arabinose, glucose (xylose, galactose, galacturonic acid)
Hot water	8·6	Arabinose, galactose, galacturonic acid (xylose, rhamnose, glucose, mannose)	3·4	Arabinose (xylose, galactose, galacturonic acid)
Ammonium oxalate	16·1	Galacturonic acid, xylose (arabinose, mannose, galactose)	1·7	Arabinose (xylose, galactose, galacturonic acid, glucose)
Hot water (after delignification)	14·0	Arabinose, galacturonic acid (xylose, galactose)	8·5	Arabinose, galacturonic acid (xylose, galactose, rhamnose)
5% KOH	6·4	Xylose, galacturonic acid (glucose, galactose, mannose, arabinose)	9·8	Xylose, arabinose (glucose, galacturonic acid, mannose)
20% KOH	5·4	Xylose, mannose, glucose, galacturonic acid (galactose, arabinose)	1·7	Xylose, glucose (mannose, arabinose)
Residue	16·0	Glucose (galacturonic acid)	2·4	Glucose (arabinose, galacturonic acid)

Table 2. *Yield and analyses of the fractions obtained by large-scale extraction of mustard-seed embryos*

Experimental details are given in the text. Reasons for the differences between the yields in this experiment and corresponding ones in Table 1 are: (i) some of the fractions in this experiment were isolated without dialysis; (ii) the starting material in this experiment was more thoroughly extracted with organic solvents before use.

Solvent used for extraction	Yield (%)	Protein content (%)	Carbohydrate content (%)
Cold water	6·3	47	26
Hot water	6·1	60	16·1
Ammonium oxalate	3·6	19	12·5

of the seed. These were characterized as sucrose and stachyose, together with traces of raffinose.

Large-scale extraction and purification of the araban

The unground solvent-extracted embryos (1·5 kg.) were extracted with aq. 2% (w/v) EDTA solution (adjusted to pH 7·5 with sodium hydroxide) (3 × 5 l. at 90°) and then with water (3 × 5 l. at 90°), since it was found that after the initial treatments with EDTA the carbohydrate material could be extracted with water alone. After each extraction, the suspension was filtered through muslin, or, in the later extractions, by which time the embryos had disintegrated into a flour, through glass paper (residue 665 g.). When the combined extracts had been concentrated under diminished pressure to about 5 l., the viscous solution was passed through the Sharples super-centrifuge (twice) and then phenol added to give a concentration of 45% (w/v). After shaking for 30 min. the emulsion was set aside at 2° (Westphal, Luderitz & Bister, 1952), but the layers did not separate. It was eventually found that the emulsion could be broken by warming to 70° and adding *n*-butyric acid until the milkiness disappeared (approx. 500 ml.). The aqueous layer that separated on cooling was extracted twice with ether to remove residual phenol, whereupon similar difficulties were encountered as a result of emulsion formation (broken in this case by dilution and the addition of about 3 g. of Cetavlon). In view of these difficulties, further phenol extractions were postponed until a later stage.

After concentration *in vacuo* to 4 l., the solution was adjusted to 75% (v/v) ethanol concentration. The precipitate was removed on a filter, redissolved in water and reprecipitated at the same ethanol concentration. After redissolution, dialysis (trial experiments had shown that there was no loss of carbohydrate through the membrane), phenol extraction (twice) and removal of the residual phenol by dialysis, the polysaccharide was isolated by freeze-drying (yield, 75 g.) (Found: N, 1·41%). After complete acid hydrolysis (N-sulphuric acid at 100° for 16 hr.) and neutralization (calcium carbonate) paper chromatography showed the presence of arabinose as the major component, together with substantial amounts of galacturonic acid and xylose and smaller quantities of galactose, glucose and rhamnose.

The combined filtrates from the ethanol precipitation were concentrated *in vacuo* (1·5 l.) and acetone (9 vol.) was added with stirring. On leaving the mixture overnight at 2°, an oil separated that was dissolved in water (500 ml.), concentrated *in vacuo* (200 ml.) and reprecipitated with acetone. The oil was dissolved in water and passed through a mixed-bed ion-exchange column (1200 ml. of Amberlite IRA-120 and 1800 ml. of Amberlite IR-45). The effluent and washings (which were neutral and gave a negative flame test for sodium) were concentrated and freeze-dried (Found: N, 2·5%; carbohydrate, 85%).

The freeze-dried material was dissolved in water and deproteinized with phenol. After removal of the residual phenol with ether, the aqueous layer was concentrated and freeze-dried (yield, 11·5 g.) (Found N, 0·33%). Hydrolysis and paper chromatography showed the presence of arabinose as the major component, with small amounts of other sugars (galactose, glucose and a trace of ribose), which were readily removed by further purification (see below).

Chromatography on DEAE-cellulose. Part of the impure araban from the 75%-ethanol-soluble fraction was purified by a procedure similar to that described in the Materials and Methods section, but on a larger scale (1·5 g. of polysaccharide). Although this technique is very useful as an analytical tool (Fig. 1), it is cumbersome and inconvenient for preparative use; for these purposes, purification with Cetavlon (see below) is now preferred. Most of the arabinose was contained in a single band, which was discrete and well-defined, and separated from the other polysaccharide components. The pooled fractions containing the araban were passed through cation-exchange resin, and the boric acid was removed as volatile methyl borate. The product thus isolated gave no other sugar on hydrolysis and paper chromatography, and had $[\alpha]_D$ −157° (water).

Purification by precipitation with cationic detergents. The two reagents investigated were cetylpyridinium hydroxide and cetyltrimethylammonium hydroxide, in each case the method of Barker, Stacey & Zweifel (1957) being used. No precipitation of polysaccharide occurred in the presence

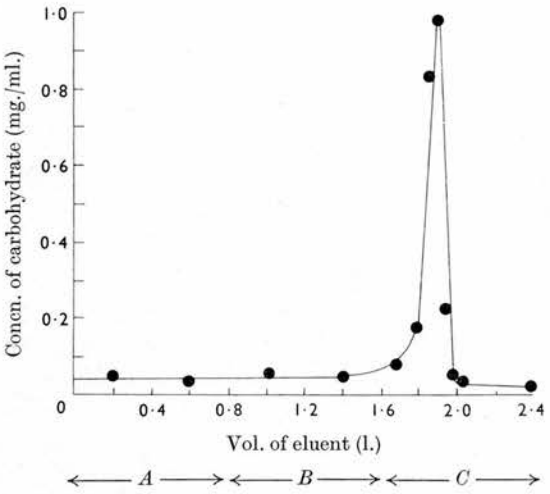

Fig. 1. Typical chromatogram of araban on DEAE-cellulose (material purified from hot-water extract of embryos). Experimental details are given in the text. Eluents: *A*, water; *B*, 0·01 M-borate; *C*, 0·01–0·1 M-borate (linear gradient).

of either of the reagents alone or when boric acid was added. Part of the polysaccharide was, however, precipitated when either of the reagents was added together with sodium hydroxide, and this formed the basis of an excellent method for fractionation. Both reagents gave the same fractionation, but cetyltrimethylammonium hydroxide is preferred because it is much the more stable. Cetylpyridinium hydroxide is very difficult to work with, because immediately it is prepared from the chloride it begins to decompose to coloured materials that are difficult to remove from the product.

The fractionation was performed as follows: an aqueous 10% (w/v) solution of cetyltrimethylammonium bromide (Cetavlon, purchased from British Drug Houses Ltd., Poole, Dorset) was passed through a column of Amberlite IRA-400 resin (OH^- form). A part of this solution (2ml.) was added to the crude araban (0·250g.) in water (30ml.). On adding N-sodium hydroxide solution (2ml.) a flocculent white precipitate was formed that was removed on the centrifuge after 20 min. No further precipitate was obtained by the addition of more detergent and alkali to the supernatant solution. The supernatant solution was neutralized with acetic acid, concentrated and freeze-dried. The precipitate was dissolved in 30ml. of water (containing sufficient acetic acid to give a neutral solution) and then reprecipitated by the addition of alkali and centrifuged. After redissolving in very dilute acetic acid, the polysaccharide was obtained by the addition of 9vol. of butan-2-one. It was dissolved in water and passed slowly through a column of mixed Amberlite IR-120 resin (H^+ form) and IRA-400 ($CO_3{}^{2-}$ form). The solution was concentrated and freeze-dried (yield, 0·113g.) (Found: anhydroarabinose, 98·4%); it had $[\alpha]_D$ −176° (water).

Deionization of a part of the supernatant solution, followed by hydrolysis and paper chromatography, showed approximately equal amounts of galactose, glucose and arabinose.

Electrophoresis of the araban. The araban, whether purified by either technique, travelled as a single compact zone on electrophoresis under all conditions. In 0·05M-sodium tetraborate, pH 9·2, it had $M_{\text{picric acid}}$ 0·16. That this movement was genuinely due to complex-formation and not to the presence of inherent acidic functions was shown by the fact that there was no movement ($M_{\text{picric acid}}$ 0·03) in 0·05M-sodium bicarbonate–sodium carbonate buffer, pH 9·3. The weakly acidic groups that are indicated by the movement at higher pH ($M_{\text{picric acid}}$ 0·10 in 0·05M-sodium carbonate, pH 11·4) are probably hydroxyl groups.

Methylation. The procedure is based on that of Kuhn & Trischmann (1963). Polysaccharide purified by the chromatographic method (0·250g.) was dissolved in dimethyl sulphoxide (20ml.), and dimethylformamide (20ml.) was added. After cooling in an ice bath, barium hydroxide octahydrate (20g.) followed by dimethyl sulphate (14ml.; in four portions over 30min.) was added to the efficiently stirred mixture at 0°, and stirring continued for a further 90min. At this point, the ice bath was removed and stirring continued for a further 48hr. Ammonia (sp.gr. 0·88; 10ml.) was added, followed by stirring for 1hr., and the mixture was transferred to a separating funnel with chloroform (250ml.). After the chloroform layer had been washed several times with water (the stable emulsion that forms may be broken by the addition of EDTA), it was dried over sodium sulphate and concentrated to a pale-yellow glass [yield, 0·225g. (73%)] (Found: OMe, 38·3. Calc.: OMe, 38·7%).

Tentative identification of the methylated sugars formed on hydrolysis. A sample of the methylated product (1–2mg.) was sealed in a Pyrex test tube with methanolic 3% (w/v) hydrogen chloride (0·5ml.) and heated on a boiling-water bath for 6hr. After neutralization with silver carbonate, filtration and evaporation to dryness at room temperature, the residue was dissolved in a drop of chloroform and examined by gas–liquid chromatography. The pattern of peaks corresponded to that expected for a mixture of the methyl glycosides of 2,3,5-tri-*O*-methyl-, 2,3-di-*O*-methyl- and 2-*O*-methyl-arabinose. There was also some indication of traces of methyl 2,3,4-tri-*O*-methylarabinoside.

A further small portion of the polysaccharide

(1–2mg.) was hydrolysed in aq. 45% (v/v) formic acid (0·05ml.) at 100° for 3hr. Paper chromatography of the hydrolysate indicated the presence of the same methylated sugars as had gas–liquid chromatography, and in addition there was evidence for a small amount of arabinose and traces of a second monomethyl ether.

Hydrolysis and separation of the methylated sugars on a cellulose column. Methylated polysaccharide (0·5g.) was dissolved in 45% formic acid (100ml.) and heated at 100° for 3hr. The solution was carefully concentrated to dryness in a rotary evaporator, a bath temperature less than 40° being used to prevent loss of the volatile 2,3,5-tri-*O*-methylarabinose. The syrup was left in an evacuated desiccator over sodium hydroxide pellets for 24hr., redissolved in water and allowed to stand at room temperature for 24hr., before once more being concentrated to a syrup (0·470g.). This syrup was separated on a cellulose column (50cm. × 3·7cm. diam.) by using the sequence of solvents: (i) water-saturated butan-1-ol–light petroleum (b.p. 100–120°) (1:3, v/v; upper layer); (ii) the same solvents but mixed in the proportions 1:1 (upper layer); (iii) the same solvents but mixed in the proportions 3:1 (upper layer); (iv) butan-1-ol half saturated with water. About 800ml. of each solvent was used. Fractions (25ml.) were collected automatically and examined by paper chromatography. The first solvent eluted pure 2,3,5-tri-*O*-methyl-L-arabinose (0·180g.), identified as the amide of the derived aldonic acid (m.p. 136–137°, not depressed on mixing with authentic material; cf. Laidlaw & Percival, 1952), followed by a mixed fraction (0·007g.) containing 2,3,5-tri-*O*-methylarabinose and 2,3-di-*O*-methylarabinose. The second solvent eluted pure 2,3-di-*O*-methyl-L-arabinose (0·083g.), identified as the amide of the derived aldonic acid, m.p. 158–159° (cf. Laidlaw & Percival, 1952). Unfortunately, the sample available of the authentic derivative was rather impure (m.p. 149–150°), but the mixed m.p. indicated the identity of the two compounds (mixed m.p. 151–152°), and the X-ray powder photographs were identical. A small mixed fraction (0·010g.) was obtained by continued elution with the second solvent; this contained 2,3-di-*O*-methylarabinose, 2,3,4-tri-*O*-methylarabinose and traces of another component that might have been 2,4- or 2,5-di-*O*-methylarabinose. The third solvent gave 2-*O*-methyl-L-arabinose (0·150g.), identified as the crystalline toluene-*p*-sulphonylhydrazone (m.p. and mixed m.p. 146°; cf. Aspinall & Baillie, 1963), and a mixture (0·020g.) containing in addition a small amount of a component that might have been a second mono-*O*-methyl ether. The fourth solvent eluted L-arabinose (0·025g.), which was identified as the toluene-*p*-sulphonylhydrazone (m.p. and mixed m.p. 153°; cf. Aspinall & Baillie, 1963).

DISCUSSION

So far as we are aware, the only other published investigations of mustard-seed polysaccharides are those of Bailey & Norris (1932) and Bailey (1935), who isolated a mucilage in 2% yield by extraction of the whole seed with cold water. This was fractionated into cellulose and two acidic polysaccharides, each of which contained galactose, galacturonic acid and arabinose. This agrees well with our own results for the composition of the cold-water extract of the seed coats (Table 1), although we have not found rhamnose and glucuronic acid (which were also reported by the early workers) in this fraction.

The most striking feature of the results of the survey (Table 1) is the preponderance of those sugars that are thought of as major constituents of pectic materials, i.e. galactose, arabinose and galacturonic acid. Indeed, with the exception of some of the less-soluble 'hemicellulosic' and cellulosic fractions giving xylose or glucose as the main sugar on hydrolysis, all fractions contained these three sugars with one of them as the main sugar.

We have chosen the polysaccharides of the embryos for further study because it is presumably in this part of the seed that the more marked biochemical changes occur on germination. A particularly simple extraction pattern is to be seen here. Polysaccharides having arabinose as the major sugar component are extracted first, but after their removal and when the extraction conditions are more severe xylose becomes predominant. No evidence was found for the presence of starch (iodine-staining) or fructans (Seliwanoff test). Considerable amounts of protein were present in all fractions (e.g. Table 2). In view of the close similarity in composition between the fractions obtained from the embryos with cold water, hot water and ammonium oxalate, these were extracted together for more detailed investigation. Preliminary experiments showed that EDTA (McCready & McComb, 1952) was a more efficient solvent than ammonium oxalate for these polysaccharides, and it was therefore used for the preparative extraction. After purification, two main fractions were obtained, both giving arabinose on hydrolysis. In one case relatively small amounts of other sugars were also present, notably galacturonic acid, but the second fraction ('mustard-seed araban') gave only arabinose. The araban moved as a discrete band on electrophoresis and when chromatographed on DEAE-cellulose (Fig. 1). The physical properties suggested a similarity with pectic arabans (cf. Hirst & Jones, 1946), and this was confirmed by the results of methylation analysis.

The four major products of hydrolysis of the methylated polysaccharides were L-arabinose, 2-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-L-arabinose and 2,3,5-tri-*O*-methyl-L-arabinose; these were isolated in pure form by column chromatography in the molar proportions 2:11:6:12 and characterized unambiguously by the formation of crystalline derivatives. From the weights of these sugars it appears that they represent at least 95% of the structural units in the araban. Although there is no direct evidence from our results about the ring size of the arabinose units in the polysaccharide other than at the non-reducing end groups, it seems likely that they are all of the same type, i.e. furanose, the units being joined by 1→5-linkages with a considerable proportion of 1→3-branch linkages. The high negative value for the optical rotation suggests that the units are α-linked. The isolation and characterization of a small amount of arabinose from the methylated polysaccharide after hydrolysis indicates that there is a second mode of branching in the polysaccharide, namely through position 2 of a relatively small proportion of units that are also linked through positions 3 and 5. It is unlikely that this arabinose arises merely from incomplete methylation of the polysaccharide, since it was repeatedly observed in about the same concentration after different methylation experiments, and its concentration was not diminished by remethylation by different techniques. The structure indicated by these results is typical of that normally associated with pectic arabans (Hirst & Jones, 1946; Hough & Powell, 1960), except that it is rather more highly branched and that it contains a proportion of 1→2-linkages.

Preliminary fractionation and methylation experiments with the other main fraction of the EDTA extract (D. A. Rees, N. G. Richardson & J. W. B. Samuel, unpublished work) suggest that the major component is a 'pectic araban' containing a significant proportion of covalently bound galacturonic acid and other sugar units. The results of both these methylation studies therefore reinforce the general impression that a high concentration of pectic-type polysaccharides is present in mustard seed. The possibility that these two polysaccharides are related chemically or biologically is under investigation. It seems unlikely that the neutral araban is an artifact that is split from a larger acidic polysaccharide by base-catalysed elimination during extraction, e.g. at uronic acid units, because extraction with EDTA at different pH values (4·9 and 7·5) gave the neutral araban in similar yields.

Purification of mustard-seed araban is readily effected by methods that make use of the ability of the molecule to form a complex with borate ions, or of the fact that the hydroxyl groups ionize in alkaline solution, or of both. A rationalization of the purification methods in these terms is consistent with the electrophoretic behaviour of the araban described in the Results section. It is surprising that the araban forms a complex with borate since model compounds (e.g. methyl α- and β-arabinofuranoside and methyl α- and β-xylopyranoside) that contain the major structural features that are consistent with the methylation results do not form complexes (Foster, 1957). Three explanations of the complex-formation may be suggested that might be separately or jointly responsible: (i) it is likely that the reducing arabinose unit in each polysaccharide molecule will be able to form a complex with borate; (ii) in the highly branched araban molecule it is possible that some strain is present arising from steric factors, and that this leads to the distortion of some sugar units, thereby altering the distance between hydroxyl groups and making complex-formation possible; (iii) borate ions might form a complex with the polysaccharide by bridging different sugar units.

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REFERENCES

Aspinall, G. O. (1962). In *The Chemistry of Carbon Compounds*, vol. 5, pp. 150–167. Ed. by Rodd, E. H. Amsterdam and New York: Elsevier Publishing Co.
Aspinall, G. O. (1963). *J. chem. Soc.* p. 1676.
Aspinall, G. O. & Baillie, J. (1963). *J. chem. Soc.* p. 1714.
Bailey, K. (1935). *Biochem. J.* **29**, 2477.
Bailey, K. & Norris, F. W. (1932). *Biochem. J.* **26**, 1609.
Barker, S. A., Stacey, M. & Zweifel, G. (1957). *Chem. & Ind.* p. 330.
Bonner, T. G. (1960). *Chem. & Ind.* p. 345.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
Foster, A. B. (1957). *Advanc. Carbohyd. Chem.* **12**, 81.
Hirst, E. L. & Jones, J. K. N. (1946). *Advanc. Carbohyd. Chem.* **2**, 235.
Hough, L. & Powell, D. B. (1960). *J. chem. Soc.* p. 16.
Jermyn, M. A. (1955). In *Modern Methods of Plant Analysis*, vol. 2, p. 203. Ed. by K. Paech & M. V. Tracey. Berlin: Springer-Verlag.
Kuhn, R. & Trischmann, H. (1963). *Chem. Ber.* **96**, 284.
Laidlaw, R. A. & Percival, E. G. V. (1952). *Advanc. Carbohyd. Chem.* **7**, 1.
Leloir, L. F. (1961). *Harvey Lect.* **36**, 23.
McCready, R. M. & McComb, E. A. (1952). *Analyt. Chem.* **24**, 1986.
Neukom, H., Deuel, H., Heri, W. J. & Kündig, W. (1960). *Helv. chim. acta*, **43**, 64.
Stahl, E. & Kaltenbach, U. (1961). *J. Chromat.* **5**, 351.
Westphal, O., Luderitz, O. & Bister, F. (1952). *Z. Naturf.* **7b**, 148.