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PHYSICAL AND STRUCTURAL STUDIES ON POLYSACCHARIDES

IAN W. STEELE, B. Sc.

Thesis presented for the Degree of Doctor of Philosophy

University of Edinburgh December 1967

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GENERAL INTRODUCTION

I Importance of Molecular Interactions

Polysaccharides function as energy reserves, antigens and structural materials. The structural role is the least understood at present and includes many of the more complex polysaccharides; it is the purpose of this thesis to consider their structural role in plants. Ideas about the structural role of polysaccharides in bacterial cell walls (Martin 1966) and animal connective tissue (Schubert 1966) are beginning to develop and will be referred to later. In plant cell walls, however, where the chemical structure of the polysaccharides is different, ideas are less advanced especially relating to non-cellulose polysaccharides. In this thesis, an attempt has been made to develop methods for the physical, and to some extent chemical, characterisation of the polysaccharides from mustard cotyledon cell walls as some work had been done previously on this system in this laboratory. A preliminary attempt has also been made to assess some of the intermolecular forces between matrix polysaccharides which might be biologically important. In this introduction, interactions between polysaccharides are discussed in general because this background will be useful for the more specific considerations which follow.

Cellulose microfibrils give a visible framework to most cell walls (Roelofsen 1959) but other polysaccharides which form a structureless matrix around the microfibrils probably also contribute to the visco-elastic (i.e. flexible, shock-absorbing) protective and water retentive properties. Cellulose chains are packed in crystalline microfibrils which are woven together to give the skeleton of the cell wall. It is not known how

the polysaccharides are held to this cellulose framework or to each other, but in seeking to explain the overall visco-elastic properties of the wall, the properties of the total assembly of chains must be considered. Although the primary structures of many cell wall polysaccharides are now known, at least in outline, we can only speculate about how they are held together. The best *in vitro* analogy, at least for young walls which have a high water content, would seem to be polysaccharide gels. The most important class of matrix polysaccharides in young cell walls are the pectic substances which are noted (and commercially useful) for their gel forming abilities; it is possible that the chains are held together in the cell wall by forces which are similar to those which give structures to these aqueous gels. A particularly striking *in vitro* polysaccharide gel is the agar gel which can contain up to 99% water, and still retain its shape. It can be melted by heating and will reset on cooling, indicating that the interactions are temporary. If these interactions hold polysaccharides in the plant cell wall together, the situation is quite different from that of the bacterial cell wall which has a very strong covalently bonded structure (Martin 1966) giving the cell good protection against osmotic damage, the bonds presumably being broken and reformed metabolically as the cell grows. In the young plant cell however, temporary interactions in the wall could conceivably be an advantage in a plastic structure which could be stretched smoothly by pressure from within the cell to give a controlled rate of growth without complicated metabolic alteration of the wall. The most widely held theory of plant cell enlargement at the present time (Wilson 1964) is indeed that the driving force is provided by this pressure, the so called

turgor pressure.

As would be expected, there is a parallel between the age of a plant cell and the flexibility of the wall. In a young cell the walls have a loose arrangement of microfibrils and a high water content, making them soft and pliable, whereas an old cell has thick walls containing more cellulose and less water and is much more rigid.

In animals, the structure giving function is not attributed to cell walls but to specialised tissue such as connective tissue in the form of skin, cartilage and bone. Each tissue has a capacity for stretching and compression which matches its specialised function. The main components are often the fibrous protein collagen and the so called protein-polysaccharides which consist of polypeptide backbones to which are attached covalently many polysaccharide chains (Rees 1965, 1967). There is variation in the chemical structure of the polysaccharide chain from one tissue to another but the dependence of the role of these protein-polysaccharides on detailed chemical structure is supported by the process of ageing. At birth, the polysaccharide consists mainly of chondroitin 4-sulphate whereas in the adult it is present as the 6-sulphate. The amount of chondroitin sulphate decreases with age and is replaced by units similar to keratan sulphate. The chondroitin sulphates are found in the rubbery type of connective tissue (e.g. cartilage) while the keratan sulphates are normally responsible for more inflexible tissue (e.g. cornea).

In order to understand how polysaccharides produce these effects in the body, it is necessary to discover the kind and number of 'cross linkages' between them. (Cross linkage in this context means any form of molecular cohesion). The theory of gelation (Flory 1953) proposes that

cross linkage extends throughout a polymer containing system if each molecule is involved in two or more cross links and this is assumed to hold for all the examples of interaction quoted.

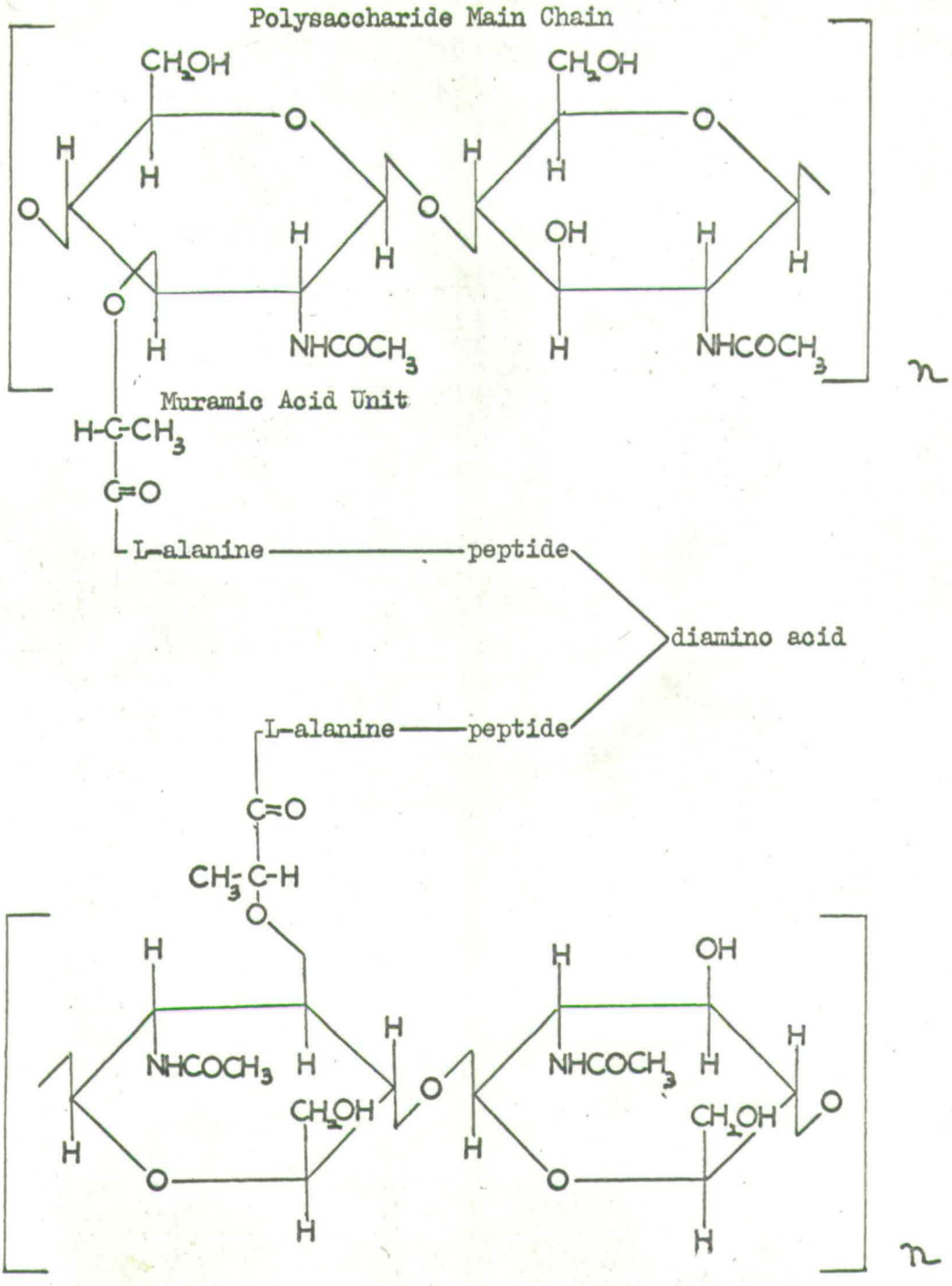
II Physical Evidence for Interaction between Polysaccharide Chains

This thesis is concerned with the properties of young plant cell walls and it would be useful to examine initially some of the possible cross linkages which might occur between polysaccharides.

1. Covalent

Most bacteria provide examples of cell walls with properties regulated by the covalent cross linkage of the structural chains (Martin 1966). The cell wall is rigid and strong and this is due to the component murein. The main chain of this polysaccharide consists of β-1,4 linked 2-acetamido-2-deoxy-D-glucose units with each alternate one modified by ether linkage to lactic acid to give muramic acid. This is the unit at which cross linkage between two murein chains, or parts of the same murein chain, occurs. The cross links are provided by peptide chains linked to the muramic acid through an L-alanine residue, any two of these chains being in turn joined together by a diamino acid (see fig. 1) e.g. diaminopimelic acid in the case of Escherichia coli. The wall is rigid because the polysaccharides are tied together covalently which allows very little possibility for stretching or rearrangement as would be possible if the bonds were of a weaker nature.

Fig. 1 Covalent Cross Linkage



2. Entanglements

Entanglement is a phenomenon seen in many types of synthetic and natural polymers of sufficiently high molecular weight. There is no precise molecular picture of an 'entanglement coupling'; the concept is used to explain non-Newtonian viscosity and the relaxation properties of extended polymeric fibres. It does not however, apply to short range molecular entwining which must exist in all polymeric systems regardless of length. Molecular entanglements are probably the result of long range interlooping (Bueche 1957) of two polymers rather than short range 'kinking' (see fig. ii) (Ferry 1967). This seems likely as the presence of bulky side groups does not markedly inhibit entanglement (Saunders et al 1959).

It is known that protein-polysaccharides have very extended chain conformations in solutions and the gel formation which occurs in concentrated solutions is most easily explained (Laurent 1966) in terms of each chain being forced to pass through the domains of others, with the result that many entanglement couplings are formed. The contribution of the polysaccharide chains to the overall visco-elastic properties of the tissue is also explained convincingly in terms of interactions between molecules through entanglement couplings. For example, the chains in nasal cartilage are polypeptides with polysaccharide side chains (chondroitin 4-sulphate) (Schubert 1966, Rees 1965) which must have considerable rigidity as there is electrostatic repulsion between the negatively charged side chains. In synovial fluid however, the chains are hyaluronic acid, a long, relatively unbranched polysaccharide with a very expanded random coil structure. The visco-elastic properties of synovial fluid are much weaker than those of cartilage and the difference between

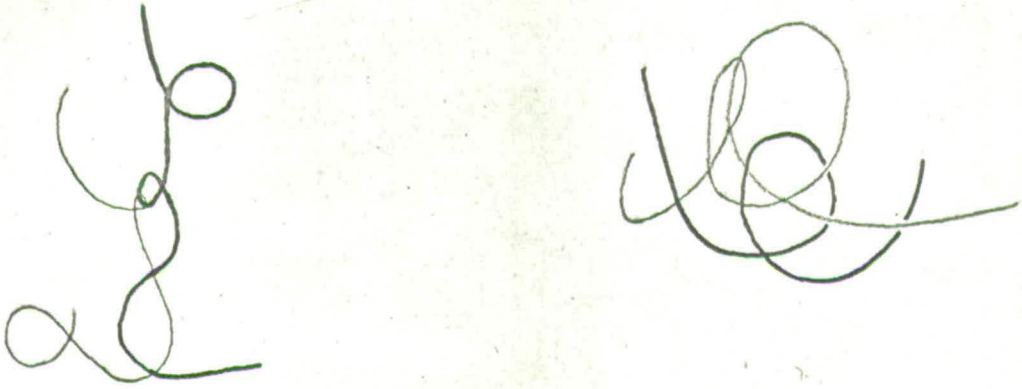
them is probably caused by a difference in the rigidity of the polymer chain.

3. Microcrystallites (Colloid Science 1949)

Cross linkage of polysaccharide chains can also occur through crystallites, in which parts of different chains come together in regions of local crystallinity. These regions may be short compared to the chain length. The classic example of a crystallite gel is regenerated cellulose (Beebe et al 1966) in which the glucose chains bind together so successfully that fibres are formed. However the substitution of carboxymethyl groups at hydroxyl groups in the D-glucose rings gives cellulose entirely different properties. When carboxymethylation is carried out, it occurs at the most accessible point; there are tightly crystalline regions which are completely inaccessible and do not react and it is these which survive to act as cross linkage points. The unbound carboxymethylated cellulose chains in separate fibres are available for entanglement and it is probable that the visco-elastic properties of carboxymethyl cellulose are caused by a combination of entanglement and crystallite cross linking (see fig.iii).

As has been shown for cellulose, minor modification to the chain units can result in different properties of the crystallite gel. Pectin, in which the main unit is methyl-D-galacturonate, forms a gel which melts on heating and resets on cooling. These gels can be weakened, or prevented from setting altogether, by the introduction of methyl or acetyl groups into the galactose ring. Side chains or the presence of L-rhamnose in the main chain, also prevent gel formation. Gel formation of this sort which is so sensitive to substitution is not explicable in terms of entanglement;

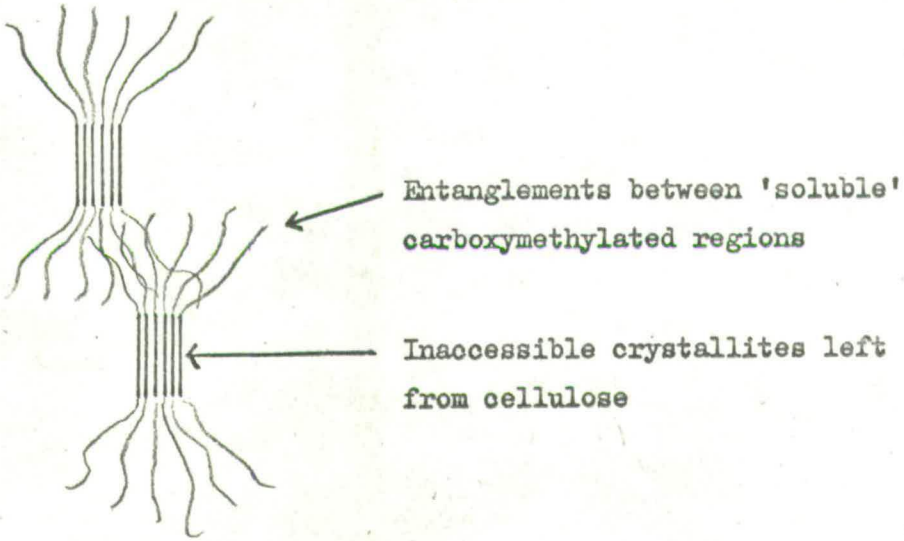
Fig. ii Entanglement



Short range local kinking

Long range interlooping

Fig. iii Interactions in carboxymethyl cellulose



instead it is the introduction of these irregularities into the crystalline regions which prevents the close fit between the chains that is required for 'crystallisation' (gel formation).

4. Hydrophobic Interactions

The effects of hydrophobic interactions are normally considered in relation to proteins where they account for some of the tertiary structural cross links (~~Bresler 1958~~, Tanford 1962, Nemethy and Scheraga 1962). The three dimensional structures of the proteins myoglobin, lysozyme and chymotrypsin (obtained from X-ray data) show them to be folded in such a way that contact between non-polar groups and water is minimised when they are in solution.

Hydrophobic interaction is a result of non-polar groups attempting to reduce their contact with water. The formation of hydrophobic bonds is favoured only because of an entropy effect; the water molecules become more ordered around exposed non-polar groups on the polymer chain (Nemethy 1967). When the hydrophobic bond is formed, the total amount of order decreases, resulting in a favourable entropy and free energy of formation.

Addition of compounds like urea and guanidine hydrochloride is suggested to reduce the ability of water molecules to hydrogen bond together and therefore the amount of 'ordered water' around a non-polar group (Frank and Franks in Nemethy 1967). This consequently reduces the strength of the hydrophobic bond and in practice results in protein denaturation as that part of its tertiary structure is lost. The strength of a hydrophobic bond increases with increasing temperature as it is an entropy dominated process, i.e. $T\Delta S$ increases in the equation for the free

energy of the system $\Delta G = \Delta H - T\Delta S$ (Nemethy and Scheraga 1962).

The gelation of methyl cellulose (Neely 1962) as temperature increases can be reinterpreted as hydrophobic bonding. Methyl cellulose is methylated heterogeneously, i.e. the distribution of methyl groups is at least partly determined by accessibility, so there are regions of fully methylated glucose units and regions of partially methylated ones. The fully methylated regions tend to be insoluble in water and gelation results from the aggregation of these regions as temperature is increased. This confirms that the process is entropy dominated and therefore that it is caused by hydrophobic bonding.

5. Electrostatic Interactions

Cross links between chains of polymers may be formed by the electrostatic attraction of oppositely charged groups. This usually does not lead to gel formation but to a general increase in molecular weight by aggregation. It may however, assist in gelation primarily caused by some other form of interaction. Association between ion-pairs has been reported (Erdi and Morawetz 1964) for the univalent cation salts of carboxyl groups on polystyrene containing small amounts (about 4 mole %) of methyl methacrylate, in benzene/methanol (90/10) solution. Complex formation, possibly chelation, with barium ions produced notably more association than did sodium ions. Similar observations have been made with polysaccharides (Greenwood and Matheson 1956); examination by ultracentrifugation of a solution of the plant gum Khaya grandifolia (47% uronic acid) containing sodium chloride and calcium ions showed a considerable increase in molecular weight over that of a salt free solution.

Plant Cell Walls

The matrix material surrounding the cellulose microfibrils in plant cell walls is composed of three main types :-

1. Pectic substances which are assumed to be the main constituents in young walls and may be either neutral polysaccharides, such as arabans (Hirst et al 1965) or, more commonly, galacturonans with various neutral side chains (Aspinall and Fanshawe 1961).
2. Hemicellulose material laid down as the cell ages and contributing to the thickening of the wall. It consists of essentially neutral polysaccharides with main chains of xylose or mannose (Aspinall 1959) but can contain glucose, arabinose and glucuronic acid as well.
3. Lignin not found in growing cells but appears in mature tissue as cementing material (Roelofsen 1959).

The pectic substances are of most importance in many young plant cell walls, as they predominate. They are being examined at present to try to find the types of molecular interaction which give the young cell wall its flexibility and other properties. The possible nature of the association between pectic molecules in the cell wall is suggested by the gels that these substances form in vitro. Such gels contain a network of macromolecules, that is, cross linked at various points. The number of cross linkages need only be small, provided they are strong enough to withstand disruptive forces such as osmosis and coulombic repulsions. The presence of repulsive forces is necessary if the gel is to exist in a swollen state.

Before discussing possible reactions of the pectic substances, it is

presumably because it breaks at least one type of cross link between the polysaccharides, thus rendering them soluble. Polysaccharides extracted by this method are usually water soluble and can be precipitated by conversion to the calcium salt. Jansen et al (1960) have shown that the calcium binding capacity of the walls of Avena coleoptile cells is that to be expected from their carboxyl content. This suggests that calcium may be naturally bound to polysaccharides by the electrostatic attraction of the carboxyl groups, perhaps with some coordination by sugar hydroxyl groups. If cations were shared by two or more chains, this would lead to cross linkage, however in the natural state only about 20% of the carboxyl groups are typically available in the form of the salt, showing that binding by purely electrostatic forces is insufficient.

Complex formation between salts and various neutral monosaccharides has been examined (Rendleman 1966) and it was concluded that the complexes were produced by chelation of the mono- or di-valent cations to the hydroxyl groups of the sugar. It seems likely that some mechanism of this sort occurs in polysaccharides as changes to the sugar residue, e.g. O-acetylation, other than to the carboxyl group, profoundly alter the gelling properties of calcium pectate (Schweiger 1964).

As mentioned earlier, temporary interactions in the young plant cell wall could be an advantage during cell expansion as the wall structure could be stretched smoothly and without the need for complicated metabolic processes. The nature of these temporary interactions is not clear but the classical theory is that purely electrostatic binding of calcium is responsible. This is unlikely because little of the pectin is in the free acid form amd also because electrostatic forces do not occupy 'fixed'

positions; however, some form of association with calcium is probable as EDTA is so successful as an extracting agent. The biological evidence (Wilson 1964) does not support the view that calcium concentration alone is responsible for controlling the flexibility of the wall and one or more other types of interaction are therefore likely to act in conjunction with it.

CHAPTER 1

APPLICATIONS OF ULTRACENTRIFUGATION AND FREE BOUNDARY ELECTROPHORESIS TO CHARACTERISATION OF COMPLEX ACIDIC POLYSACCHARIDES

Introduction

Examination of polysaccharides has been traditionally carried out using organic chemical techniques but information about polydispersity* obtained in this way is often inconclusive. There are two main problems in obtaining 'homogeneous' polysaccharide samples; firstly, a complete mild extraction procedure is needed and secondly, it is necessary to separate the mixture of polysaccharides usually obtained by extraction. The polysaccharides present in a plant tissue are usually examined for polydispersity by a fractionation procedure, e.g. :-

1. Graded extraction. The use of progressively harsher methods of extraction will yield fractions of differing composition.
2. Fractional precipitation. The gradual addition of a non-solvent to aqueous solutions of the extract may also cause fractionation although co-precipitation and the occlusion of other polysaccharides is very likely. More satisfactory results are sometimes obtained by precipitation of the extract in a cellulose column and elution of the polysaccharides by changing the composition of the solvent (Gardell 1965).

Fractional precipitation can also be achieved by the addition of

*The term 'polydispersity' denotes a polymer system containing more than one component while 'polymolecular' is used to describe a homogeneous polymer having a variation in molecular weight.

be discussed here. These can be used for separation purposes but normally the yields obtained are small, however they are well suited to the examination of already separated material for polydispersity. Gross differences in physical properties, i.e. molecular weight for ultracentrifugation and mass/charge ratio for electrophoresis, are required for separation of two components but might not be encountered as a particular extraction procedure is likely to yield polysaccharides with similar properties.

Ultracentrifugation

If the molecular weight of a polysaccharide in solution is sufficiently large, sedimentation occurs in a force-field. The number of peaks produced in the schlieren pattern ideally corresponds to the number of components in the mixture, however, if two components only differ slightly in molecular weight only one peak will be produced (Adams 1961). In a polymolecular system, the area under the schlieren peak is proportional to the concentration of the polysaccharide solution present. However, in multicomponent systems, the slower peak gains in area at the expense of the faster (Johnstone and Ogstonϕ 1946). The relative amounts of components cannot therefore be measured from the ratio of the areas under the peaks. The sedimentation coefficients (a measure of the velocity of sedimentation) of polymolecular samples are also changed by the addition of other components (Harrington and Schachman 1953) but this does not invalidate comparisons of similar mixtures of polysaccharides, e.g. the products of different extraction procedures.

A further complication is the possibility of aggregation of acidic

polysaccharides; this has been known to give completely erroneous values for sedimentation coefficients (Greenwood and Matheson 1956, Tomimatsu et al 1959).

Free Boundary Electrophoresis

This is an extremely useful technique for the detection of polydispersity (Colvin et al 1952, Northcote 1954, Foster 1953, Preece and Hobkirk 1955, Lewis and Smith 1957). It is a more sensitive tool than ultracentrifugation as even slight differences in mass/charge ratio for polysaccharides in a multicomponent system will produce separate peaks in the schlieren pattern. Some neutral polysaccharides can be examined as they can be complexed with borate buffer (Foster et al 1956) to give them a charge which will induce migration of the molecules in an electric field.

The presence of only one peak in the schlieren pattern does not prove that the polysaccharide is homogeneous and it is necessary to check the result by electrophoresis in other buffers and by ultracentrifugation. (Even this is not a final proof).

As for ultracentrifugation, the areas under the separate schlieren peaks do not give the amounts of the components in a mixture as the refractive indices of the components may differ; however it does provide a rough guide.

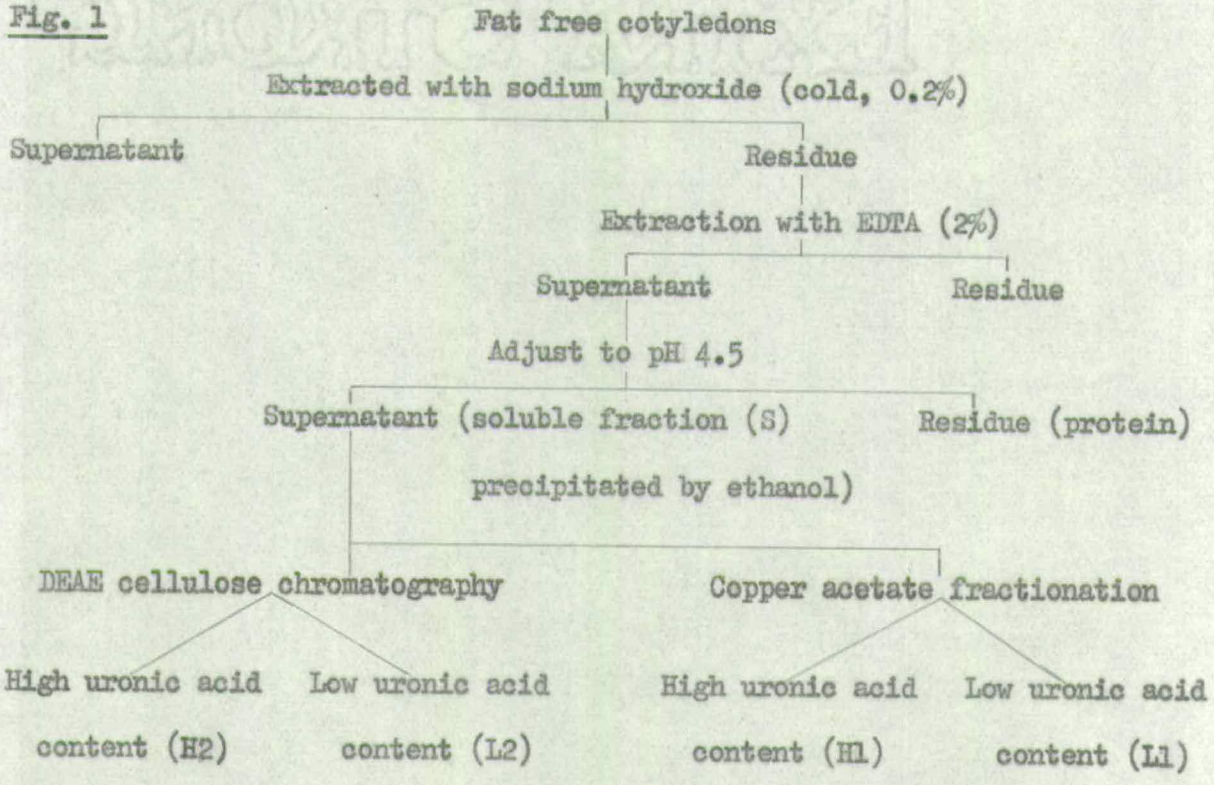
Experimental

1.1 Examination of fractions extracted from soybean cotyledons by two different methods

1.1.1 Examination of polysaccharides obtained by deproteinisation with sodium hydroxide

This was carried out by Dr. J.N.C. Whyte (1964) and Mr. A. Hamilton (1964) who provided the samples for examination. The extraction was carried out as in fig. 1.

Fig. 1



As shown in the chart, two fractionation procedures were attempted for separation of the major components of the soluble fraction (S). The products were examined by free boundary electrophoresis and ultracentrifugation.

1.1.2 Fractionation by cupric acetate

Method Free boundary electrophoresis

Fraction	Buffer	No. of Peaks	Mobility (x 10^{-5} $cm^2/s/v$)
1. S (Plate 1)	Sodium tetraborate	2	5.8, 2.2
2. L1 (Plate 2)	" "	2	7.8, 2.3
3. H1	" "	2	7.6, 2.4
4. S (Plate 3)	Sodium phosphate	1	5.1
5. L1 (Plate 4)	" "	1	9.5
6. H1	" "	1	9.0

Method Ultracentrifugation

Fraction	Buffer	Conditions	No. of Peaks
1. L1 (Plate 5)	EDTA (2%)	110,000g	2
2. H1 (Plate 5)	" "	"	2

The reason for the low value for the mobility of unfractionated S is not clear but is probably due to a higher degree of esterification in the unfractionated material. The results show clearly that fractionation was incomplete as the fraction L1 contained a considerable amount of acidic material. The proportions of the two peaks in the ultracentrifugal analysis of H1 and L1 were different indicating that each fraction was enriched in one component.

Preparative free boundary electrophoresis of a solution (0.5%) of

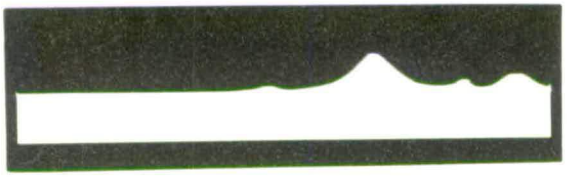


PLATE 1

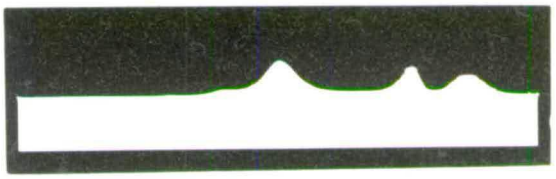
Sample : S

Time : 100 min.

PLATE 2

Sample : L1

Time : 100 min.



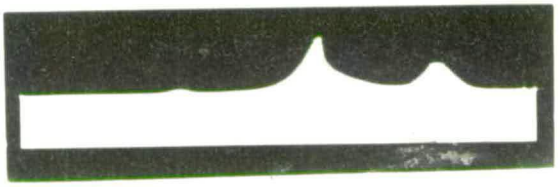


PLATE 3

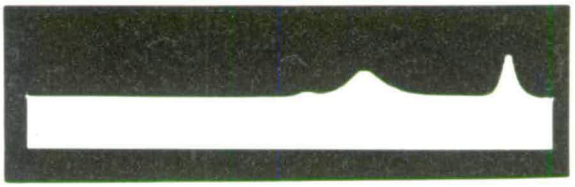
Sample : S

Time : 70 min.

PLATE 4

Sample : L1

Time : 45 min.



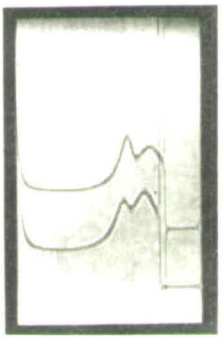


PLATE 5

Top : H1

Bottom : L1

Time : 92 min.

fraction L1 (Plate 6) was carried out in a sodium phosphate buffer. After 91 minutes, a good separation had been achieved and a sample of the acidic peak was withdrawn from the right arm of the U-tube; a similar sample of the neutral peak was withdrawn from the left arm. These solutions were dialysed and freeze dried, about 5mg of each sample being obtained. The uronic acid and total carbohydrate content of solutions of these samples were then measured using the carbazole and phenol sulphuric acid methods and these results were compared with those obtained for the original solutions (see Table 1).

Table 1

Sample	Uronic Acid Content
L1	55.2%
H1	58.6%
Stationary peak	23.1%
Mobile Peak	95.8%

1.1.3 Fractionation by DEAE cellulose column (sodium phosphate buffer pH 6.1)

This work was carried out by Dr. J.N.C. Whyte, 1964.

Method Free boundary electrophoresis

Fraction	Buffer	No. of Peaks	Mobility (x 10^{-5} $cm^2/s/v$)
1. L2 (Plate 7)	Sodium tetraborate	1	2.1
2. H2 (Plate 8)	" "	1	6.3

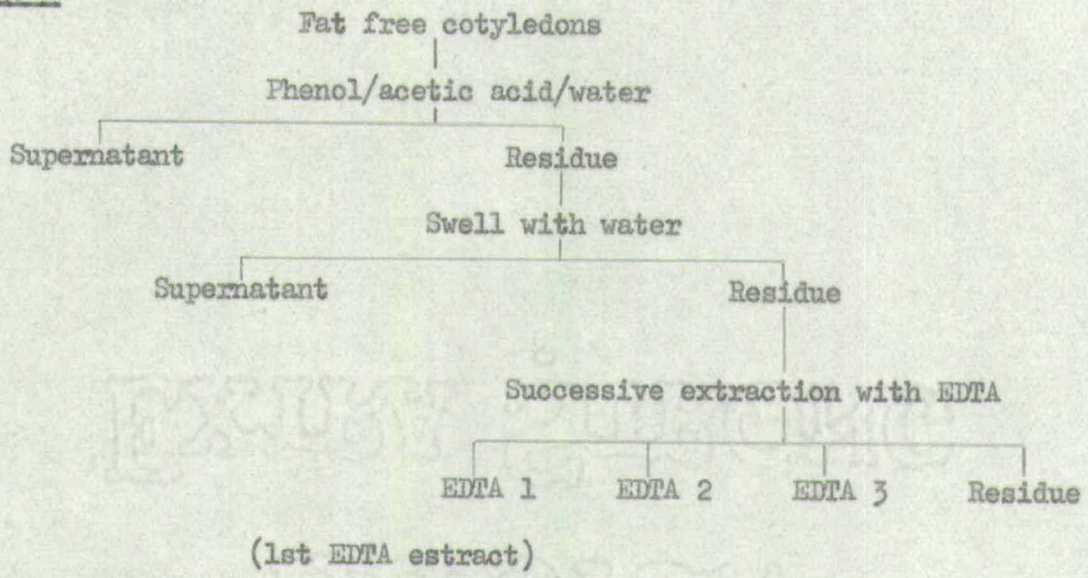
Fraction H2 above was one of two major acidic components (the other acidic fraction, not mentioned in the table, had a lower mobility). The

samples appeared to be homogeneous and the calcium separation is therefore more satisfactory than the cupric acetate fractionation.

1.1.4 Examination of polysaccharides obtained by deproteinisation with phenol/acetic acid/water (Bagdasarian et al 1964)

A small scale pilot extraction (SS) was carried out by Dr. I.M. Morrison (personal communication) and the subsequent large scale procedure by Dr. R. Begbie (personal communication) (see fig. 2).

Fig. 2



A simplified extraction scheme was carried out by Dr. R. Begbie by stirring the fat free cotyledons with water and precipitating polysaccharide material by graded addition of ethanol from one to six volumes (fractions E1 to E6).

Method Free boundary electrophoresis		No. of	Mobility
Fraction	Buffer	Peaks	($\times 10^{-5}$ cm^2/s/v)
1. SS (Plate 9)	Sodium tetraborate	2	4.9, 3.0
2. EDTA 1 (and EDTA 3) (Plate 10)	" "	3	6.0, 4.6, 2.6
3. E1 (Plate 11)	" "	3	6.6, 5.7, 2.3
4. E6 (Plate 12)	" "	2	5.5, 2.9

One can see from the above table and plates 9 and 10 that the extraction of acidic material was most successful using EDTA after deproteinisation with phenol/acetic acid/water; the product was contaminated with only a small amount of neutral material. The neutral material was obtained as a major component after extraction with cold water (without deproteinisation) followed by fractional precipitation with ethanol. The first ethanol precipitate, E1, contained least neutral polysaccharide and plate 11 shows clearly the presence of two acidic components, whereas the last ethanol precipitate, E6, contained mainly neutral and little acidic material.

1.2 Examination of polysaccharides extracted from mustard cotyledons

1.2.1 Deproteinisation and extraction of mustard cotyledons (Hirst et al 1965)

Fat free mustard cotyledons were extracted three times with EDTA (2%) and the pectic material obtained, deproteinised using phenol (Westphal et al 1952). This procedure was applied to the nongerminated cotyledons by Dr. N.G. Richardson and to the germinated cotyledons by

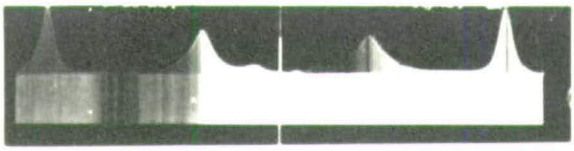


PLATE 6

Sample : L1

Time : 91 min.

PLATE 7

Sample : L2

Time : 91 min.

PLATE 8

Sample : H2

Time : 65 min.

PLATE 9

Sample : SS

Time : 118 min.

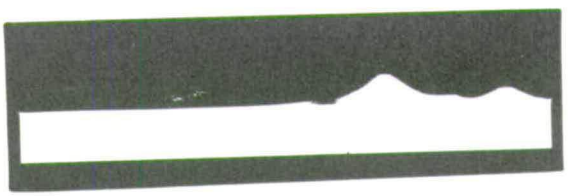


PLATE 10

Sample : EDTA 1

Time : 100 min.

PLATE 11

Sample : E1

Time : 100 min.

Dr. S.E.B. Gould.

Solutions (0.5%) of these extracts in sodium dihydrogen phosphate buffer (pH 6.0, 0.033M) were examined by ultracentrifugation at 110,000 g. Both samples produced similar schlieren patterns showing two peaks; however, the faster peak in the extract (G) from the germinated cotyledons was slightly larger than in the extract (N) from the nongerminated cotyledons. Sedimentation coefficients for this concentration were measured :-

		$S \times 10^{13}$
N -	slow peak	2.9
	fast peak	8.9
G -	slow peak	2.7
	fast peak	9.1

The corresponding peaks in N and G have very similar S values and the slight differences might be accounted for by the concentration dependence of one of the components.

The samples N and G were then examined by free boundary electrophoresis.

Fraction	Buffer	No. of Peaks	Mobility ($\times 10^{-5} cm^2/s/v$)
N (Plate 13)	Sodium phosphate	1	6.1
G (Plate 14)	" "	1	5.8
N (Plate 15)	Sodium tetraborate	3	6.9, 5.3, 4.2
G (Plate 16)	" "	3	6.3, 5.8, 4.4

1.2.2 Examination of aggregating material discovered in G

It was noticed that G showed a free boundary electrophoresis pattern with a highly mobile and very sharp peak which was opaque beyond the leading

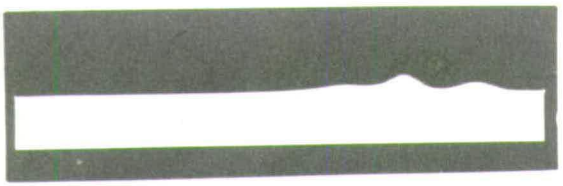


PLATE 12

Sample : E6

Time : 106 min.

PLATE 13

Sample : N

Time : 35 min.

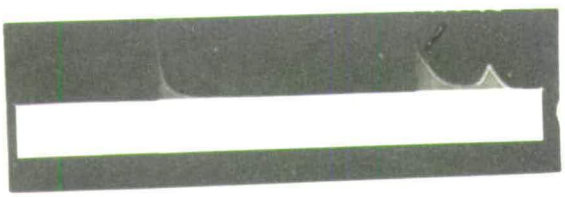


PLATE 14

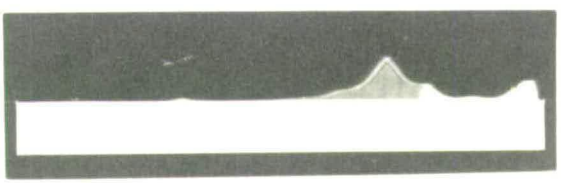
Sample : G

Time : 35 min.

PLATE 15

Sample : N

Time : 102 min.



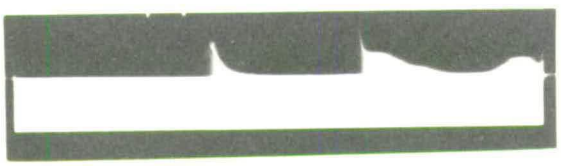


PLATE 16

Sample : G

Time : 104 min.

PLATE 17

Sample : N

Time : 102 min.

edge, suggesting the presence of a colloidal aggregate. The sample was centrifuged at 20,000 g to clarify it; this was only partially successful but on repeating the electrophoresis in sodium phosphate buffer, the size and sharpness of the aggregate peak were considerably reduced (mobility unchanged). To check the possibility that calcium and/or magnesium ions had caused the formation of aggregates, electrophoresis was carried out in sodium tetraborate buffer containing 2% EDTA (N, Plate 17; G, Plate 18).

Solutions (0.5%) of N and G in sodium chloride (0.15M) containing EDTA (2%) were examined by ultracentrifugation at 110,000 g (Plate 19). In both N and G the slower peak is enlarged on addition of EDTA.

Solutions (25ml; 0.5%) of N and G were prepared and centrifuged at 20,000 g for 1½ hours. The supernatant and aggregate were separated and the aggregate samples washed with distilled water and recentrifuged. Ultracentrifugation at 250,000 g revealed the typical pattern of N and G for the supernatant (Plate 20) while the aggregate produced no peak at all. This means that either the concentration was too low or that the dissolved aggregate had too low a molecular weight to sediment at this force.

A solution (100ml; 0.5%) of G was centrifuged at 20,000 g for 1½ hours and the supernatant was removed and freeze dried (yield = 200mg). This shows that there must have been about 60% by weight of aggregate in the original G sample. Hydrolysis (45% formic acid) of aliquots of both supernatant and aggregate fractions gave identical patterns on paper chromatography (solvent A).

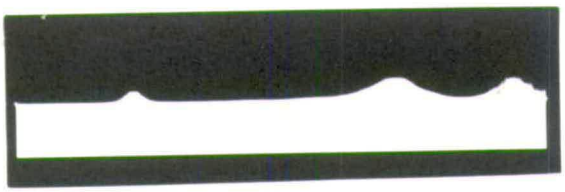


PLATE 18

Sample : G (+ EDTA)

Time : 102 min.

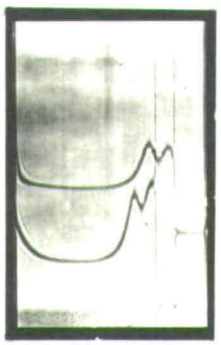


PLATE 19a

Top : G

Bottom : G
(+ EDTA)

Time : 26 min.

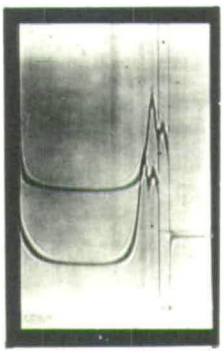


PLATE 19b

Top : N

Bottom : N
(+ EDTA)

Time : 26 min.

PLATE 20

Top : N
(supernatant)

Bottom : N
(aggregate)

Time : 15 min.

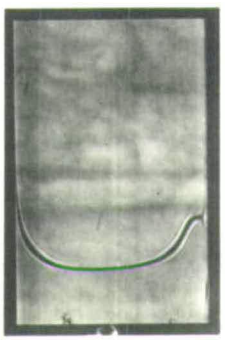


PLATE 21

Sample : G
(aggregate)

Time : 62 min.

i.e.	galacturonic acid	glucose	galactose	arabinose	xylose
	++	trace	+	+++	++

The strength of each sugar is indicated by :

- not present; + weak; ++ medium; +++ strong; ++++ very strong

Ultracentrifugation of the aggregate dissolved in EDTA (2%)/sodium chloride (0.15M) at 187,000 g gave one slow moving peak (Plate 21 c.f. Plate 20).

A long series of dialysis experiments were carried out on N and G dissolved in EDTA (2%). These clear solutions were subjected to prolonged dialysis against running tap water (the condition thought to cause the initial aggregation) and against distilled water containing Ca^{++} (35-350 p.p.m.), Mg^{++} (17-170 p.p.m.) or K^{+} (3900 p.p.m.) or combinations of all three. It proved impossible to reconstitute the gel in any significant amount.

A typical experiment was as follows :-

Samples (1g) of N and G were each suspended in water (50ml) and stirred for ½ hour; a solution (50ml) of EDTA (4%, pH 7.5) was added and the sample immediately clarified. Phenol (1g) was added to sterilise the two solutions (a strain of Bacillus megaterium was particularly troublesome throughout these dialysis experiments) and they were dialysed against running tap water for 17 days. They were then centrifuged at 20,000 g and the supernatant and the aggregate separately freeze dried and weighed. The proportion of material which was recovered in the precipitate (5.0% for N and 5.9% for G) was negligible.

An experiment was carried out to determine the protein content of these

samples :-

Freeze dried G (90mg) was shaken with water (9ml) for 3 hours and centrifuged (20,000 g) for ½ hour. The supernatant solution and precipitate were freeze dried and analysed for nitrogen by the Kjeldahl method (Belcher and Godbert 1954).

	Yield	%N
Aggregate	34.4mg	2.9
Supernatant	24.0mg	1.1

There was therefore significantly more protein in the aggregate fraction.

1.3 Ultracentrifugal examination of fractions obtained on attempted separation of the polysaccharides present in mustard cotyledons before and after germination

All column fractionations were carried out by Dr. S.E.B. Gould, 1965.

1.3.1 Elution of N from a DEAE cellulose column by an increasing concentration of sodium dihydrogen phosphate buffer (pH 6.2)

(see fig. 3)

Aqueous solutions (1%) of the various fractions in sodium phosphate buffer (0.05M, pH 6.0) were examined in the ultracentrifuge at 250,000 g. Two fractions were examined at the same time so that direct comparisons could be made (see Plate 22). (This procedure was applied to all subsequent work on DEAE cellulose column fractions). As can be seen from fig. 3, there were apparently three components in the mixture. This was borne out to a certain extent by the ultracentrifugal results, the three

components being X - a very slowly sedimenting neutral component, Y - a faster sedimenting acidic component and Z - a still faster component.

Fraction	B	C	D	E	F	H	K	M	N
Peak X	++++	+	++	-	-	-	-	-	-
Y	-	+	++	++	+	+	+	trace	-
Z	-	-	-	++	+++	+++	+++	++++	++++

Both X and Z were separated fairly well by the column but the major peak E to M is a mixture of Y and Z. As a result of this unsatisfactory separation of the acidic component Y, the fractionation was repeated using a different buffer.

The electrophoresis pattern of N (Plate 15) seemed to indicate that there was too little neutral component X present in the solution to produce a schlieren peak.

1.3.2 Repeat of 1.3.1 using sodium dihydrogen phosphate (pH 6.2) containing urea (7M) as an eluant (see fig. 4)

Fraction	A	D	E	F	G	H
Peak X	+++	+	-	-	-	-
Y	-	+++	++++	+	+	+
Z	-	-	-	+++	++++	++

Once again the neutral fraction X was obtained pure and also there was no clear separation of the components in the major peak. Fraction E corresponded to a small percentage (about 10%) of the total amount of material eluted from the column and so was of little value.

Fig. 3 Elution Diagram of N. Sodium phosphate buffer (see 1.3.1)

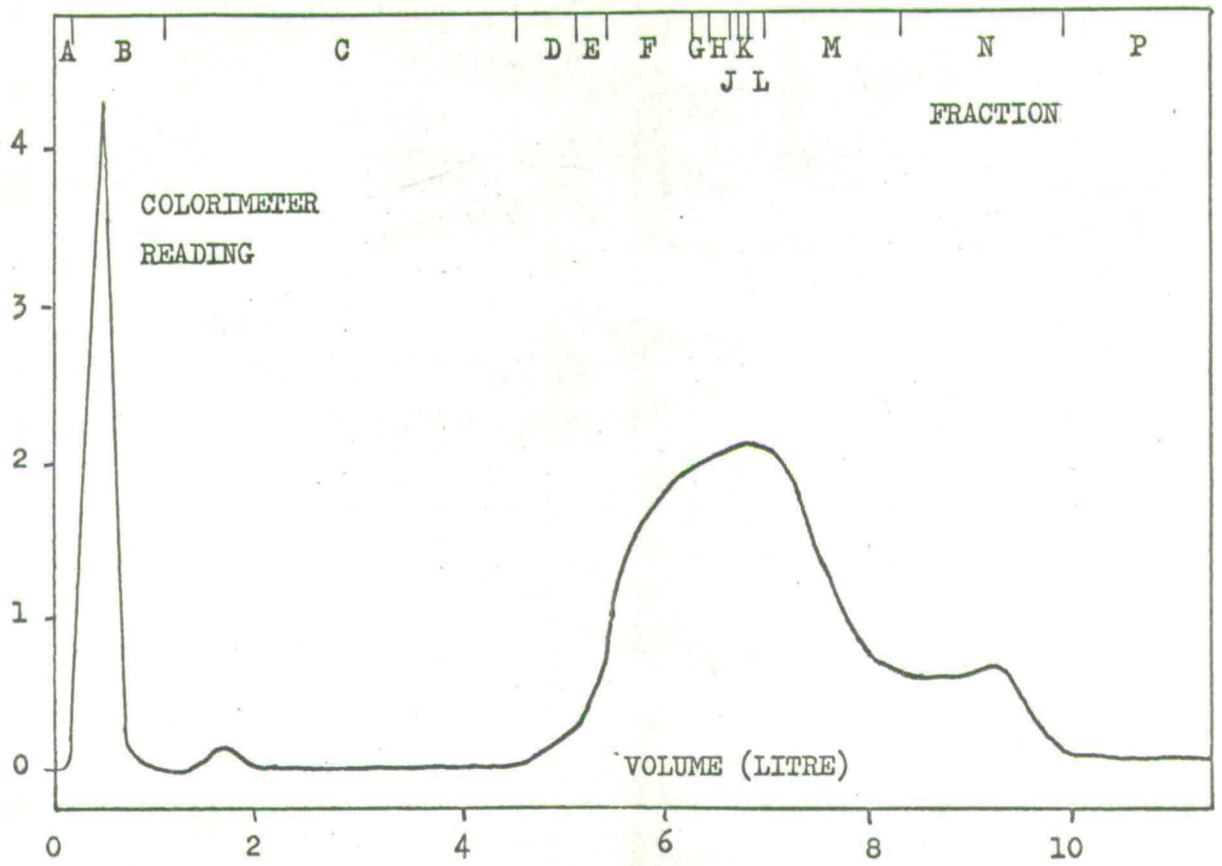
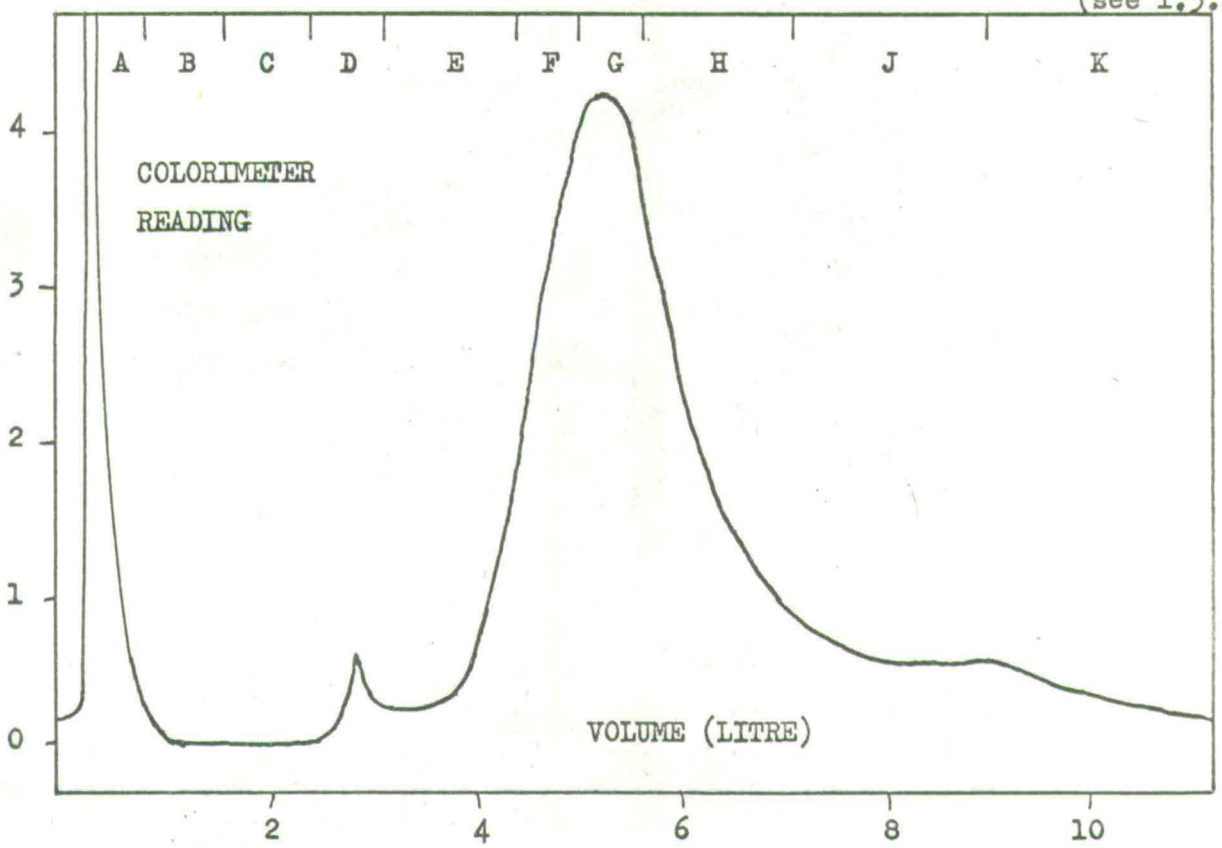


Fig. 4 Elution Diagram of N. Sodium phosphate buffer containing urea (see 1.3.2)



1.3.3 Further attempted fractionation of N using a DEAE cellulose column and sodium tetraborate (pH 9.2) as an eluant (see fig. 5)

Fraction	A	C	E	G	J	L
Peak X	++++	++++	-	-	-	-
Y	-	-	++	+++	+++	++++
Z	-	-	+++	+	+	-

The unusal feature of the fractions from this column was that the two acidic components were eluted in reverse order to that predicted from the previous results. The column achieved no better separation than the previous ones.

1.3.4 Fractionation of G on a DEAE cellulose column using sodium hydrogen phosphate and urea (7M) as an eluant (see fig. 6)

Fraction	B	E	F	G	H	J
Peak X	++++	+	-	-	-	-
Y	-	+++	++	++	++	-
Z	-	-	-	-	+	-

The pattern followed that of N in 1.3.2. The definition of the peaks in the schlieren pattern was very poor, and fraction Z did not appear in any quantity. In mobility and appearance the peak resembled the β-eliminated product from mustard pectin (see Chapter 4). It therefore seems likely that breakdown by this path had occurred under the alkaline conditions of elution.

Fig. 5 Elution Diagram of N. Sdoium tetraborate buffer (see 1.3.3)

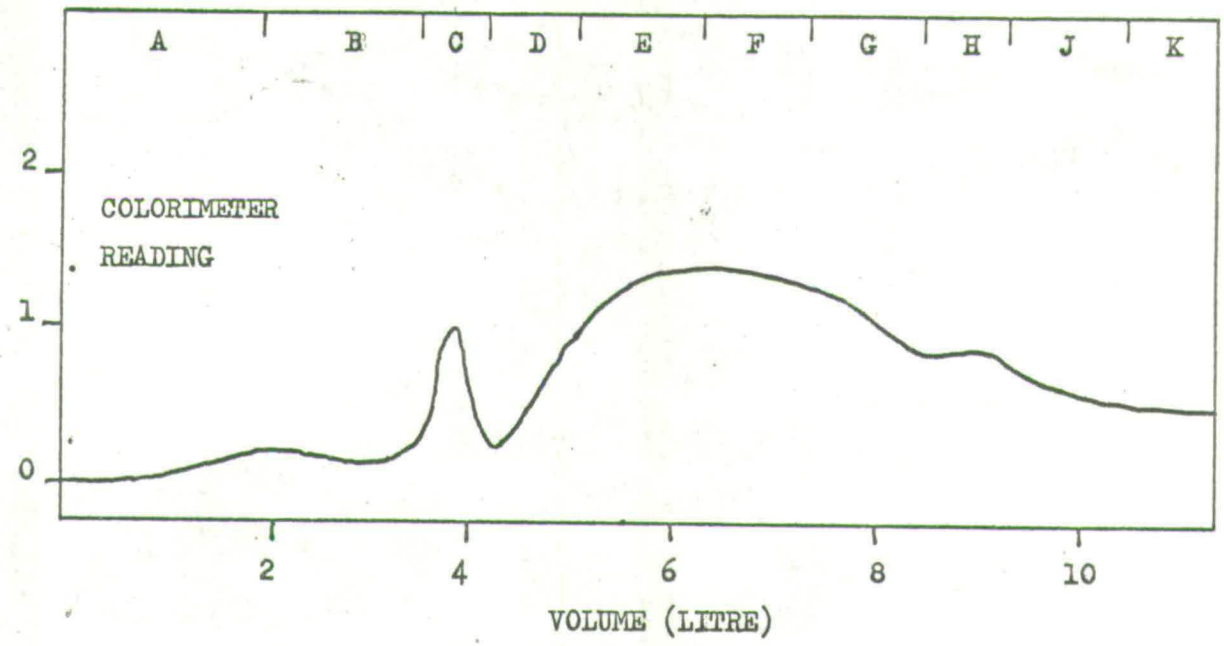
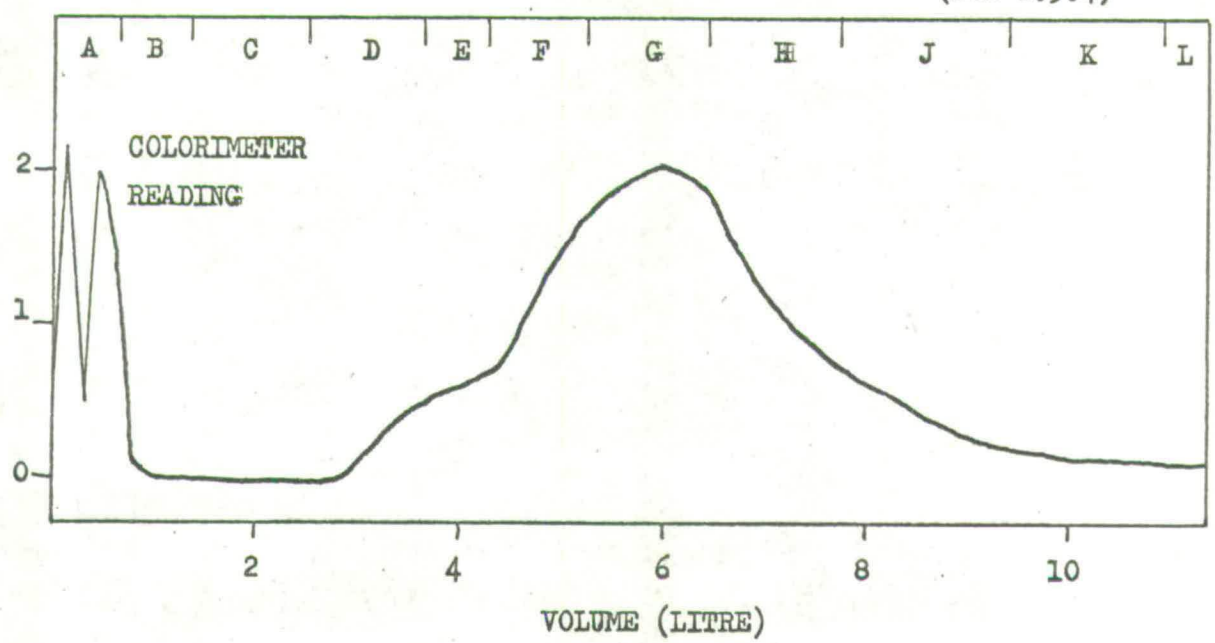


Fig. 6 Elution Diagram of G. Sodium phosphate buffer containing urea (see 1.3.4)



1.4 De-esterification of N and G

It seemed possible that the difference (in electrophoretic mobility and in retention to DEAE cellulose columns) between the two acidic peaks was due to a difference in degree of esterification.

Solutions (10ml, 1.2%) of N and G in sodium hydroxide were stirred for 14 hours and allowed to stand for a further 10 hours. Then an aliquot (10ml) of a solution of sodium hydroxide (100ml, 0.1N) containing boric acid (2.475g) was added to each solution. The solution was dialysed against sodium tetraborate buffer (0.05M) for 2 days before free boundary electrophoresis.

Fraction	Buffer	No. of Peaks	Mobility (x 10^{-5} cm^2/s/v)
N (Plate 23)	Sodium tetraborate	2	6.0, 4.5
G (Plate 24)	" "	3	7.5, 6.9, 4.6

If these results are compared with those for the original N and G samples, it will be seen that the mobilities of all components have increased. The very fast peak (μ = 6.9) in N (Plate 15) appears to have been reduced but the schlieren pattern in G is virtually unchanged. The presence of at least two acidic peaks in both samples after de-esterification indicates a more fundamental difference between the polysaccharides.

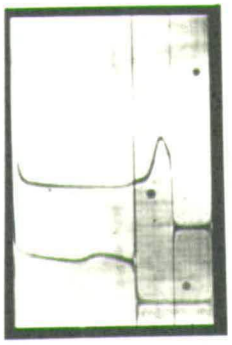


PLATE 22a

Top : Fraction B

Bottom : Fraction C

Time : 30 min.

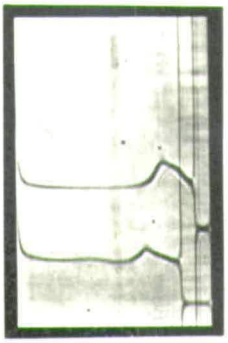


PLATE 22b

Top : Fraction D

Bottom : Fraction E

Time : 49 min.

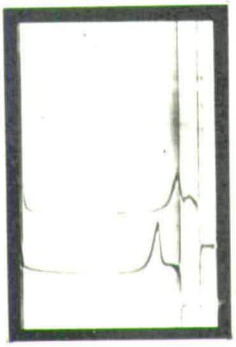


PLATE 22c

Top : Fraction H

Bottom : Fraction M

Time : 15 min.

PLATE 23

Sample : N

Time : 102 min.

PLATE 24

Sample : G

Time : 102 min.

Discussion

Although ultracentrifugation and free boundary electrophoresis have not been widely used in the study of pectic substances up to the present time, they have been used in this work to look for differences not detectable by conventional analysis. For example, it proved impossible to distinguish between the various acidic fractions obtained from DEAE cellulose chromatography of mustard seed pectin by hydrolysis and paper chromatography but ultracentrifugation showed at least two recognisably different acidic polysaccharides which were partly separated on the column. This was confirmed by free boundary electrophoresis.

Because these polysaccharides are so similar chemically, it is possible that they are degradation products, produced by alkaline β-elimination (Barrett and Northcote 1964) from the same parent molecule which was broken up during extraction. β-elimination during the alkaline deproteinisation of soybean cotyledons (see Chapter 4) could account for the difference in the mobilities of the acidic components from those obtained for the material deproteinised using phenol/acetic acid/water although it is probable that de-esterification of carboxyl groups occurred before β-elimination could proceed to any great extent (Zitko and Bishop 1965). These problems are discussed fully in relation to mustard polysaccharides in Chapter 4.

The ultracentrifugation and free boundary electrophoresis results in this chapter confirm the value of DEAE cellulose chromatography for the separation of pectic substances from mustard and soybean cotyledons and show also that the fractions obtained are genuinely different components. Fractional precipitation, as illustrated by the copper acetate and ethyl

alcohol methods, seems to be less powerful.

Ultracentrifugation and free boundary electrophoresis are complementary and should be used in conjunction to check the polymolecularity or polydispersity of a sample. Free boundary electrophoresis requires a considerable amount of material (about 80mg) which is often not available, e.g. a small fraction from a column, and so it would in some cases be preferable to use ultracentrifugation (requiring only about 10mg). The choice of method is usually governed by its success in each particular case; ultracentrifugation was therefore used for the examination of mustard polysaccharide fractions, while electrophoresis was found to be more useful for soybean polysaccharides.

The results indicate that soybean pectic material extracted by the methods described, contained two distinct acidic species and one neutral component, the latter being a small proportion (1.4%) of the total. The mustard extract similarly contained two acidic and one neutral component, the two acidic fractions being partially separable by chromatography. Physical differences (i.e. in molecular weight or mass/charge ratio) were not found between polysaccharides obtained before and after germination. An interesting property of the acidic polysaccharides isolated both before and after germination was their tendency to form aggregates (gels) when the crude extracts were dialysed against hard tap water and then deproteinised. Both N and G aggregates were much more freely soluble in EDTA than in water suggesting that divalent cations such as Ca^{++} or Mg^{++} might have a role in aggregation. However, once the gel had been dissolved it proved impossible to reconstitute it by straight forward dialysis against hard tap water. It seems that either the presence of protein was

necessary during the dialysis stage or treatment with phenol after dialysis was required. The former seems the most likely possibility as a significant amount of protein remained in the G gel after deproteinisation with phenol.

Aggregate formation of this type is a new property of pectin and might reflect the way that calcium and perhaps other cations participate in the natural cross linking of pectic substances in the cell wall (for background see Bonner 1961). From a practical point of view, it is to be recommended that pectic substances should be dissolved in EDTA (2%) before examination by ultracentrifugation or free boundary electrophoresis so that aggregates do not complicate the analysis.

CHAPTER 2

PHYSICAL CHARACTERISATION OF MUSTARD ARABANS

Introduction

The pectic araban from mustard seeds is unusual in being a homopolysaccharide containing no units other than L-arabinose. Most other pectic arabans reported in the literature contain at least a small proportion of other sugars, notably galacturonic acid, galactose and rhamnose. For example, the araban from sugar beet has been extensively studied and contains these sugars (Hullar 1963, Andrews et al 1959); reported molecular weights (Ingelman 1945, Tomimatsu and Palmer 1963) vary from 10,000 to 70,000. These molecular weights are probably those of fragments formed by β-elimination (see Chapter 4) of a much larger molecule consisting of a galacturonic acid-containing main chain with large arabinose-containing side chains in addition to other sugar units. Production of fragments similar to those above has been found for apple pectin on heating at neutral pH (Barrett and Northcote 1965) and so it can be concluded that this holds for other arabans heated at neutral or alkaline pH (Sengupta et al 1965).

The possibility that mustard araban is a similar artefact produced as a result of β-elimination during extraction has been rejected (Hirst et al 1965) as this would leave a considerable amount of galacturonic acid main chain attached to the araban. Cleavage occurs at 10%, or less, of the methyl galacturonate units during β-elimination (Albersheim et al 1960) under the extraction conditions; continued treatment of the sugar beet araban at alkaline pH results in a loss of some, but not all, of the

acidic units (Hullar 1963). It is impossible to detect the presence of galacturonic acid or other foreign units in the mustard arabans by hydrolysis and paper chromatography, and examination of the ultraviolet spectrum for the characteristic absorption caused by C4 - C5 unsaturation in a uronic acid unit was negative. Further evidence is that similar amounts of araban are produced when extraction is carried out with EDTA at pH 7.5 or pH 4.9, the latter being much less favourable for the β-elimination reaction.

The role of the araban in the cell wall is not clear. Its molecular weight is small (7,500 for the methylated araban, Rees and Steele 1966) and thus interactions of the type described in the General Introduction are likely to be weak and occur to a very limited extent. It has been shown to possess a high degree of branching which diminishes significantly upon germination (Rees and Richardson 1966). Perhaps it has a plasticizing rather than a stiffening effect by keeping the more strongly interacting molecules apart from each other. The yield of araban drops after germination which together with the decrease in branching would cause stiffening of the wall as the organ matures by allowing the other pectic polysaccharides to interact more closely.

Experimental

2.1 Extraction of Araban (4) from nongerminated mustard seeds by EDTA at pH 4.9

Crude araban, which had previously been extracted (by Dr. N.G. Richardson) from unground, solvent extracted embryos using EDTA at pH 4.9 and deproteinised twice with phenol (45% by weight) was purified using detergent (Hirst et al 1965). This method was much less laborious than the DEAE cellulose column method and appeared to give just as pure a product.

Cetyl trimethyl ammonium bromide (Cetavlon) was converted into the hydroxide by passing an aqueous solution (10% w/v) through a column of Amberlite IRA 400(OH^-) resin. A part of this solution (4ml) was added to an aqueous solution of the crude araban (500mg in 60ml). Sodium hydroxide (1N, 4ml) was added and the flocculent white precipitate was collected by centrifugation. The precipitate was redissolved in water (60ml) containing a very small amount of acetic acid (approximately neutral pH) and reprecipitated with sodium hydroxide. The precipitate, collected by prolonged centrifugation, was dissolved in very dilute acetic acid (2ml). The araban was obtained by precipitation with methyl ethyl ketone (18ml) and the white sticky material obtained dissolved in water (10ml) and passed through a small Sephadex (G 15) column to remove salts. The pure araban solution was concentrated and freeze dried (yield 85mg).

The anhydro-arabinose content was 100% (phenol-sulphuric acid method, see General Methods) and the optical rotation $[\alpha\ ^{20}_{D}]$ was -173°. Hydrolysis and paper chromatography showed only arabinose was present (the maximum amount of any other sugar present was less than 2% of the whole).

The other arabans used for the physical studies were extracted by a similar technique using EDTA at pH 7.5 which is thought to encourage β-elimination. Extraction of some nongerminated (Araban 1) and germinated seeds (Araban 3) was carried out by Dr. N.G. Richardson and an extraction of some 'commercial white mustard seed germ' (J. and J. Colmans, Araban 2) was carried out by Mr. J.W.B. Samuel.

2.2 Examination of the arabans by free boundary electrophoresis

Electrophoresis is only possible if the molecule under examination is charged or is complexed with a charged species. The complexing agent used was sodium tetraborate (0.05M) but the reason for complex formation is not clear since model compounds, e.g. methyl α- and methyl β-arabinofuranoside, that contain the main structural features of the araban, do not form complexes (Foster 1957). Three explanations for complexing by the araban are possible :-

1. The single reducing unit in each polysaccharide molecule may be able to form a complex with borate.
2. Strain may be present in the polysaccharide molecule so altering the distance between the hydroxyl groups and permitting complex formation.
3. Complex formation may be caused by borate ions bridging different sugar units.

2.2.1 Free boundary electrophoresis of arabans

Electrophoresis (E = 6.5v/cm) of each araban in AnalaR sodium tetraborate buffer (0.05M, pH 9.2) for 178 minutes produced one diffuse symmetrical peak and no other peaks were detected. The boundary remained stationary if a non-complexing buffer (sodium phosphate, pH 6.0) was used.

Araban	Mobility (10^{-5} $cm^2/s/v$)
(1)	2.2
(2)	2.3
(3)	2.2
(4) See Plate 25	1.9

2.3 Sedimentation coefficients (S_{20}) of arabans

Ultracentrifugation (334,000 g) of each araban (1%) in sodium chloride (0.15M) solution for 98 minutes produced a diffuse symmetrical peak. Care was taken to ensure that the concentration of all four arabans was the same as the sedimentation coefficients may be concentration dependent.

Araban (see Plates 26 and 27)	S_{20} (10^{-13})
(1)	1.32
(2)	1.23
(3)	1.41
(4)	1.24

2.4 Examination of the molecular weight distribution by ultracentrifugation of the arabans in a synthetic boundary cell

The solutions used in this experiment were part of those made up for the previous experiment. The special (12mm) aluminium centrepiece (for use at high speeds) required to produce the synthetic boundary was supplied by Dr. C.T. Greenwood.

The cell was half filled with a solution of the araban and two pockets in the side of the centrepiece were filled with solvent (sodium chloride, 0.15M, 0.25ml). As the rotor accelerated slowly, the cell distorted slightly

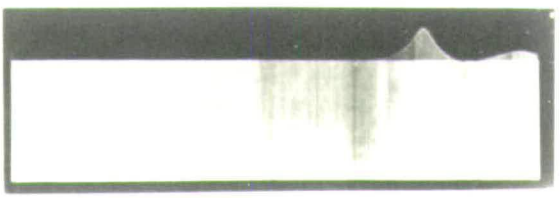


PLATE 25

Sample : Araban (4)

Time : 178 min.

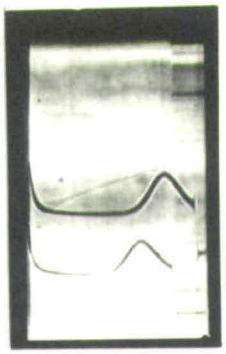


PLATE 26

Top : Araban (1)

Bottom :
Araban (3)

Time : 98 min.

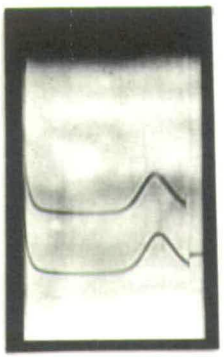


PLATE 27

Top : Araban (2)

Bottom :
Araban (4)

Time : 98 min.

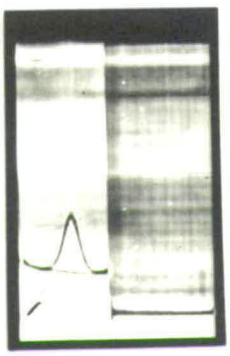


PLATE 28

Sample :
Araban (4)

Time : 15 min.

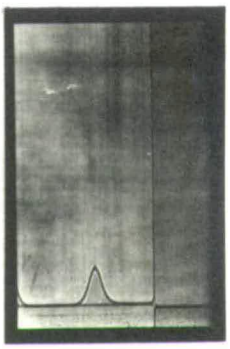


PLATE 29

Sample :
Araban (1)

Time : 16 min.

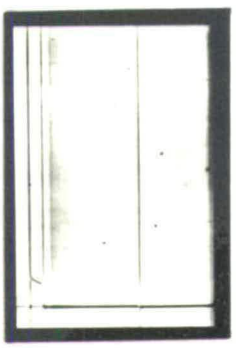


PLATE 30

Sample : Araban (1)

Time : 120 min.

(at about 8,000 r.p.m.) and allowed the solvent to run slowly from the two pockets and layer itself on top of the araban solution. As soon as the synthetic boundary was formed, the rotor was accelerated hard to 67,770 r.p.m. (334,000 g) and the schlieren pattern photographed after 15 minutes (see Plate 28).

2.5 Determination of the molecular weight (weight average) of the Araban (1) by the 'short column method' (Yphantis 1960)

The molecular weight (M_w) is determined using the equation :-

$$M_w = \frac{RT\, dc/dr}{\bar{r} C_o \omega^2 (1 - \bar{V}\rho)} \qquad (1)$$

where ω is the angular velocity, $\bar{V}$ is the partial specific volume of the solution, i.e. araban, ρ is the density of the solution, dc/dr is the concentration gradient at the point at which the concentration is C_o and $\bar{r}$ is the distance of that point from the centre of rotation.

The use of this equation was made easier (Van Holde and Baldwin 1958) by the fact that in a short column (under 5mm) C_o is within 1% of the initial concentration at the mid-point of the solution (where $\bar{r} = (a + b)/2$ a and b being the distances from the centre of rotation to the meniscus and base respectively).

2.5.1 Determination of $(1 - \bar{V}\rho)$

A pyknometer similar to that used by Lipkin et al 1944 (see fig. 7) was used for determining $(1 - \bar{V}\rho)$. The pyknometer was calibrated using AnalaR benzene (density at 25°C = 0.87340g/cc).

Calibration was carried out by weighing the carefully cleaned and

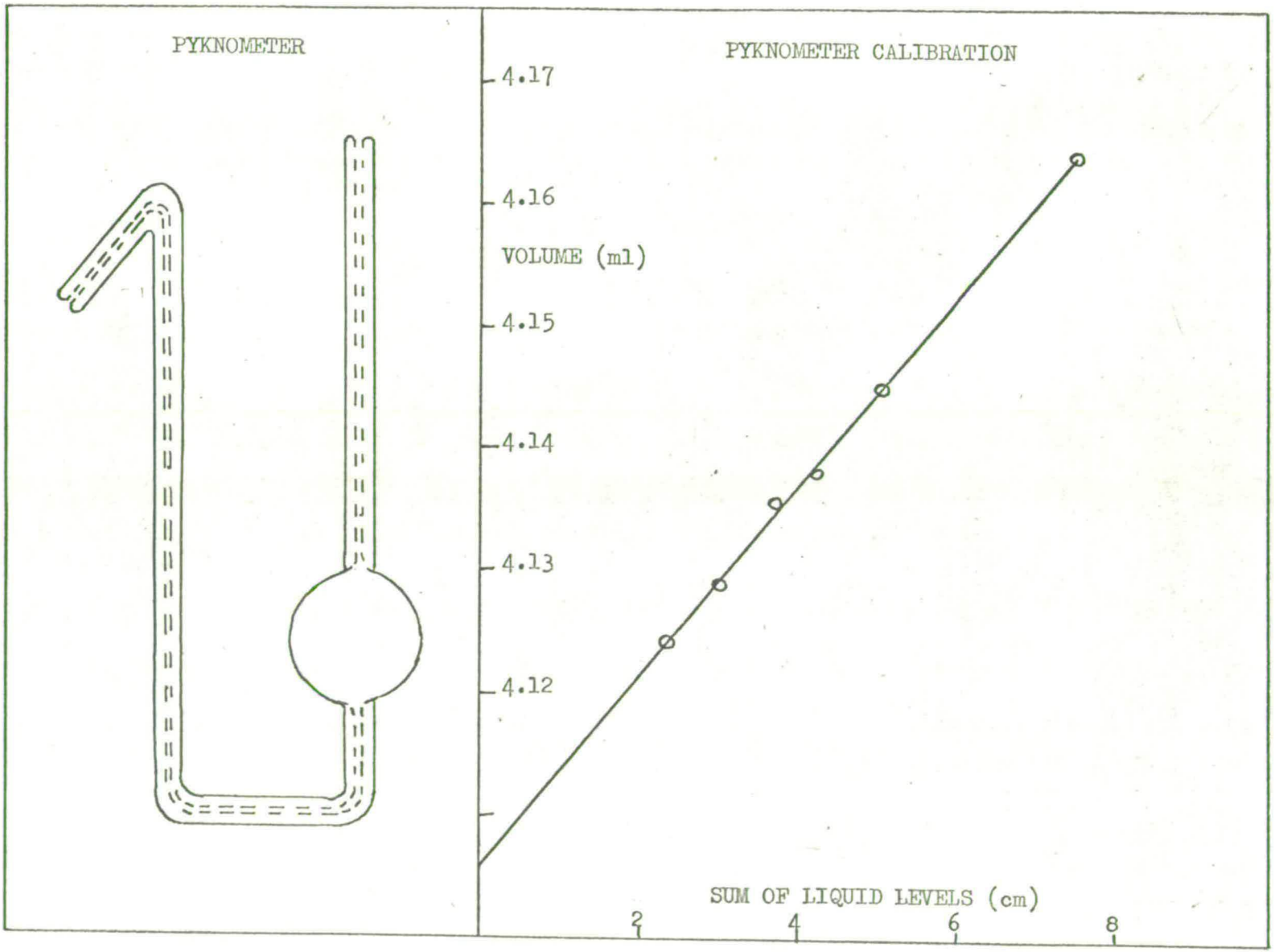

PYKNOMETER
PYKNOMETER CALIBRATION
4.17
4.16
VOLUME (ml)
4.15
4.14
4.13
4.12
SUM OF LIQUID LEVELS (cm)
2
4
6
8

Fig. 7

dried pyknometer empty and filled with different amounts of benzene. After filling, the pyknometer was placed in a thermostatted water bath ($25^{\circ}C \pm 0.002^{\circ}C$) for ½ hour and the heights of the liquid levels above the reference marks were recorded. As the density of the benzene was known it was possible to plot a graph of volume of the pyknometer (V) against sum of the heights of the liquid levels (S) (see fig. 7).

The equation of the graph was worked out by applying the method of least squares to the values for V and S (calculated using a KDF9 computer with a program written in Atlas Autocode by Dr. R.M. Taylor). It was necessary to do this as insufficient accuracy was available by simply plotting the graph.

$$V = 0.00787S + 4.10611 \qquad (2)$$

The term $\bar{V}$ appearing in equation (1) is the reciprocal of the density, the partial specific volume. This can be defined as the increase in volume of a solution when 1g of solute, i.e. araban, is added to a large volume of solvent.

For a two component system, the partial specific volume of component 1, $\bar{V}_1$ is given by :-

$$\bar{V}_1 = (\delta v/\delta m_1)m_2$$

where V is the volume of solution and m_1, m_2 are the weights of the two components.

The specific volume of solution is :-

$$V = v/(m_1 + m_2)$$

$$\therefore v = V(m_1 + m_2) \qquad (i)$$

$$\text{Hence } \bar{V}_1 = (\delta v/\delta m_1)m_2 = V + (m_1 + m_2)dV/dm_1$$

Now the weight fraction of component 2 is :-

$$W_2 = m_2/(m_1 + m_2)$$

$$dW_2 = - m_2 dm_1/(m_1 + m_2)^2 \quad \text{when } m_2 \text{ is constant}$$

$$= - W_2 dm_1/(m_1 + m_2) \qquad \text{(ii)}$$

Combining (i) and (ii)

$$\bar{V}_1 = V - W_2 dV/dW_2$$

Now expressing this in terms of the density of the solution (ρ)

$$dV/dW_2 = - (1/\rho^2).(d\rho/dW_2)$$

and $W_2 = (1 - W_1)$

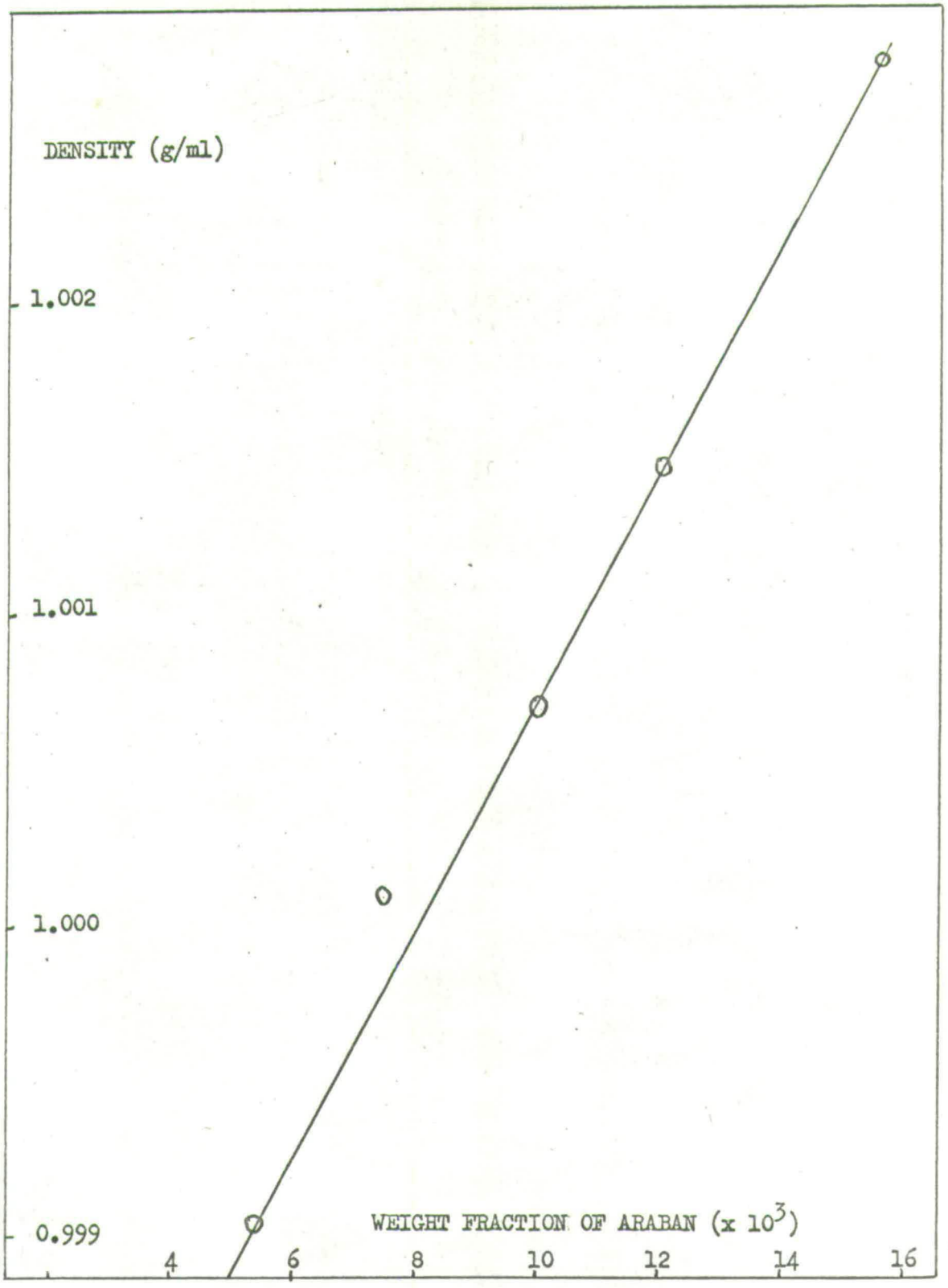
$$\therefore \quad \bar{V}_1 = V + \frac{(1 - W_1)}{\rho^2}.\frac{d\rho}{dW_2}$$

$$= \frac{1}{\rho}\left(1 - \frac{1 - W_1}{\rho}.\frac{d\rho}{dW_1}\right)$$

$$\therefore \quad (1 - \bar{V}_1\rho) = \frac{(1 - W_1)}{\rho}.\frac{d\rho}{dW_1} \qquad (3)$$

Using equation (3), $(1 - \bar{V}_1\rho)$ can be calculated from the densities of a series of polymer solutions of known concentrations. The previously calibrated pyknometer was used (see equation 2) to determine the densities of five solutions of araban (0.5%, 0.8%, 1.0%, 1.2% and 1.5%) in distilled water (10ml); the weight fraction of araban (W_1) present in each solution was calculated. The gradient ($d\rho/dW_1$) of the graph of density plotted against weight fraction (see fig. 8) was calculated using the method of least squares (by KDF9 computer).

Fig. 8 Density versus Weight Fraction of Araban



$$d\rho/dW_1 = 0.38 \pm 0.02$$

Now $\frac{1 - W_1}{\rho} = 0.99$ (for 0.8%, 1.0% and 1.2% solutions)

$$\therefore \underline{1 - \bar{V}\rho = 0.38} \quad (\text{" " " " " " " "})$$

2.5.2 Determination of concentration, C_o, by means of synthetic boundary runs (Klainer and Kegeles 1955; Ginsburg et al 1956)

Synthetic boundary runs were carried out at 30,200 g on the 0.8%, 1.0% and 1.2% araban solutions to determine the concentration, C_o, in terms of arbitrary units; the area between the schlieren peak and the base line being proportional to the concentration. A standard 12mm epoxy resin synthetic-boundary, double-sector cell was used for this, a small amount of araban solution (0.2ml) being placed in one sector and a larger amount of water (0.5ml) in the other. As the rotor was accelerated, water was forced slowly through a channel between the sectors and layered itself on top of the araban solution, so forming the synthetic boundary; the water remaining in the other sector of the cell provided a base line. Photographs were taken as soon as the schlieren peak had become fairly broad (2-10 minutes, see Plate 29). Runs were not continued for more than 15 minutes as, after this time, the peak began to sediment. A phase plate angle of 80° was used for all runs involving the calculation of this molecular weight.

The area under the peak was obtained by measuring the height (ΔH) of the peak above the base line at set intervals (0.03cm) across it.

$$\text{Area} = (\text{interval}).\Sigma \Delta H$$

However, as various lenses are incorporated into the ultracentrifuge

to make the schlieren peak easily seen, the area must be corrected for these magnification factors. Only the horizontal magnification need be taken into account as the vertical magnification 'cancels out' in a later stage of the calculation. A further correction is necessary, as in the short column runs which follow, a 30mm epoxy resin cell was used.

Araban Concentration	Area of Peak	Corrected Area (C_o = Area x $\frac{2.5}{2.224}$)
0.8%	0.104cm^2	0.116cm^2
1.0%	0.135cm^2	0.151cm^2
1.2%	0.153cm^2	0.171cm^2

2.5.3 Sedimentation equilibrium in a short column of araban solution (Yphantis 1960)

The correct speed of rotation for these runs, 15,220 r.p.m. (16,800 g) was determined by a trial and error method based on values quoted by Yphantis. If the speed of rotation is too high, the concentration at the centre point of the column of liquid will differ significantly from C_o, the original concentration. If the speed is too low, it will take a long time before equilibrium is reached so removing the advantage this method has over the Archibald (1947) technique.

The equilibrium runs were carried out in standard 30mm epoxy resin double-sector cells, with an artificial base in one sector provided by 0.1ml of FC 43 (perfluorotributylamine, a dense, volatile liquid purchased from B.D.H.). This gave the short column an easily distinguishable base when it was being centrifuged. Araban solution (0.15ml) was added to the same sector as the FC 43 and a larger volume of solvent (water, 0.5ml) was

added to the other sector to provide a base line for the curve. Samples were run in the ultracentrifuge for 2½ hours (at 16,800 g) photographs being taken at half-hourly intervals. Equilibrium was judged to have been reached when the height of the mid-point of the curve above the base line (dc/dr in equation 1) remained constant for 30 minutes (see Plate 30). The only other unknown variable, $\bar{r}$, the distance of the mid-point of the araban solution from the centre of rotation, was also obtained from the photograph of this equilibrium position (this value was corrected for horizontal magnification). Equilibrium was reached by the araban solutions (0.8%, 1.0% and 1.2%) after 2 hours and dc/dr and $\bar{r}$ were measured from photographs taken at that time (phase plate angle of 80°).

2.5.4 Calculation of the molecular weight of the araban in solution (0.8%, 1.0% and 1.2%)

Values for the molecular weight were calculated using equation (1) :-

$$M = \frac{RT\ dc/dr}{\bar{r}C_o\omega^2(1 - \bar{V}\rho)}$$

In this equation the following are constants for these experiments :-

$R = 8.32 \times 10^7$ ergs/mole/°C

$T = 298°A$

$\omega^2 = 3.95 \times 10^6$ radians/second

$1 - \bar{V}\rho = 0.38 \pm 0.02$

The molecular weights calculated were corrected to the nearest hundred and are quoted in the following table :-

Concentration of araban solution	0.8%	1.0%	1.2%
dc/dr (cm)	0.304	0.366	0.419
$\bar{r}$ (cm)	7.052	7.051	7.018
C_o (cm^2).	0.116	0.151	0.171
Molecular Weight	6100	5700	5800

Discussion

The molecular weight of the araban obtained by ultracentrifugation is small, corresponding to 40-45 L-arabinose units, and is similar to the value found for the methylated polysaccharide using vapour pressure osmometry. The similarity of these two results, one a weight average value (ultracentrifugation) and the other a number average result, indicates that the molecular weight distribution is small. The weight average molecular weight, M_w, can only be the same as the number average value, M_n, in a system containing polymers of identical molecular weight as :-

$$M_w = \frac{\Sigma N_i M_i^2}{\Sigma N_i M_i} \qquad \text{and} \qquad M_n = \frac{\Sigma N_i M_i}{\Sigma N_i}$$

where N_i is the number of molecules of any kind, i, and M_i is their molecular weight.

There is little variation in sedimentation coefficient between arabans from nongerminated seeds, but this is within the experimental error. The error here (about 7%) is mainly due to inaccuracy in measuring the photographic plate as the peaks were diffuse. However, in the case of the molecular weight determination, the major error arises from the pyknometry (determination of $1 - \bar{V}\rho$); for example, an error in the gradient, $d\rho/dW_1$, of 0.02 has a consequent error in the molecular weight of about 300 and all other errors are insignificant in comparison.

There is little change in the physical properties of the araban after germination. That there is negligible difference in the electrophoretic mobility is surprising as study of the methylated polysaccharide (Rees and Richardson 1966) has shown that there is considerable reduction in the

degree of branching during germination. The extent of complex formation might be expected to be related to the degree of branching if it is dependent either on strained conformation at individual sugar units, or on bridging of different sugar units by the borate ions. However, this was not borne out by the results. This shows that the sedimentation and electrophoretic diagrams may not always be sensitive enough to detect significant differences in structure.

The low value for the molecular weight of the arabans confirms that they are not degradation artefacts split from a larger pectin molecule. Even only one foreign unit in a molecule of this size would represent 2% of the weight and therefore be readily identified by hydrolysis and paper chromatography. The consistent nature of the sedimentation coefficients is further proof, as even slight variations in the extraction procedure of the arabans would yield molecules with noticeable differences in physical properties due to different degrees of degradation.

CHAPTER 3

A METHOD FOR THE EXTRACTION OF PECTIC SUBSTANCES AND ITS MODE OF ACTION

Introduction

The ultimate objective of structural analysis of pectic polysaccharides is to determine their biological role in plant cell walls. It is therefore very important that these polysaccharides extracted from plant tissue should be unmodified. Different cells with the same function in a particular plant organ are likely to have reached different stages of development and it is probable that the pectic polysaccharides in the cell wall will also have a different stage of development. This means that pectic polysaccharides from the organ are likely to have a distribution of molecules. It is therefore necessary that the polysaccharide should be completely extracted otherwise an unrepresentative sample is obtained.

Unfortunately, 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 pectic substances have been traditionally extracted using potassium hydroxide (Hirst and Jones 1947) or lime water (Hirst and Jones 1948) 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 (see Chapter 4) and other reactions (Whistler and BeMiller 1958). Indeed, it seems likely that the so-called 'neutral components' have often been degradation artefacts (Barrett and Northcote 1965). β-elimination can also occur in hot neutral solutions so that extraction with solutions of ammonium oxalate (Aspinall and Fanshawe 1961) or EDTA

(McCready and McComb 1952) may also involve degradation of the native polysaccharide. When hot solvents are used for extraction, there is the 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.

The problem of a complete, mild extraction scheme is difficult without a knowledge of the way in which pectic polysaccharides are bound into the cell walls. Some of the various modes of polysaccharide interaction have been discussed in the General Introduction and it is probable that the pectic polysaccharides are bound to each other or to protein by one or more of these interactions. Suppression of these interactions should enable easy extraction of the polysaccharides.

A very successful procedure involves the use of EDTA which can sequester divalent cations which might be binding the polysaccharides to each other and/or to proteins by electrostatic forces and possible chelation. Unfortunately, EDTA is most efficient above about pH 7 and this could lead to unwanted degradation. An alternative efficient divalent cation chelation agent is Calgon (sodium hexametaphosphate) which is effective at or below pH 4.0 (Letham 1962).

It was suggested in the General Introduction that pectin forms crystallite gels by packing main galacturonosyl chains together, these being held at a distance from one another by electrostatic repulsion of the carboxyl groups and so prevents the formation of fibres as is the case for cellulose. Work in this laboratory on gels of similar types, has suggested that hydrophobic bonding may be responsible for their existence (Anderson 1966). It is possible that pectin gels could be partly the result of the hydrophobic

binding together of non-polar regions of sugar units formed by axial C—H groups; the presence of methyl esters might enhance this effect. For these reasons, compounds like urea and guanidine hydrochloride should prove successful extractants as they have the ability to break down the water structure around hydrophobic sites by reducing the strength of the hydrogen bonds between water molecules. This, along with their ability to break hydrogen bonds by competition for the active sites, should make them efficient extraction agents.

The use of compounds which are able to form hydrophobic bonds with themselves, e.g. micelles, and with other molecules containing hydrophobic sites, might also reduce the strength of any such interactions between polysaccharide molecules. Such an agent would also destroy protein-polysaccharide and protein-protein interactions caused by hydrophobic bonding as well as being able to denature proteins. Examples of compounds which form hydrophobic bonds are straight-chain alkyl sulphonate detergents. For example, sodium lauryl sulphate is known to be an efficient protein denaturant by virtue of its hydrophobic hydrocarbon chain and so may prove useful in the extraction of polysaccharides.

Hydrophobic binding has been put forward as an explanation for the formation of gels by kappa-carrageenan (Anderson 1966). It was found that sodium lauryl sulphate, guanidine hydrochloride and urea all inhibit gelation of agarose, an uncharged polysaccharide similar to kappa-carrageenan. This could be due to disruption of hydrophobic bonds and/or electrostatic interactions, but the former is most likely as agarose is uncharged. A series of experiments is described at the end of this chapter to devise methods which could be used to test the possibility that pectins also form

gels by hydrophobic binding. This is relevant to the biological problem because of the possibility that polysaccharide interactions in the cell walls resemble those in reconstituted gels (Rees 1967).

Experimental

3.1 Urea extraction of the residue remaining after EDTA extraction of nongerminated mustard embryos

A mixture of residue (25g) and urea (240g) was made up to 500ml with water and stirred for 24 hours. After centrifugation, the carbohydrate content of the supernatant solution was measured by the phenol/sulphuric acid method (see general methods). The extraction was repeated a further seven times and a graph plotted of the extraction number versus the optical density (at 490m μ) (fig. 9). The supernatant solutions were bulked and dialysed against running tap water for 7 days, then concentrated and freeze dried to give 3.9g of material. The phenol/sulphuric acid test was calibrated using D-glucose and subsequently used to determine the 'carbohydrate content' of the extracted material (10%). The weight of the final residue (after drying) was 11g and the sugars observed after hydrolysis and paper chromatography are given in Table 2.

3.2 Guanidine hydrochloride extraction of the residue remaining after EDTA extraction of nongerminated mustard embryos

This extraction was very similar to the previous one. Residue (25g) and guanidine hydrochloride (48g) in a total volume of 500ml were stirred for 24 hours and the extraction repeated a further nine times. The supernatant solutions were bulked, dialysed for 6 days against running tap water and freeze dried (0.2g; carbohydrate content 7.0%). The dried residue from the extraction weighed 20.5g and contained the sugars noted in Table 2. The course of the extraction is shown in fig. 9.

3.3 Extraction of defatted mustard embryos at 20°C with urea (8M) containing EDTA (2%)

This extraction was very similar to those described above. Ground embryos (25g) urea (240g) and EDTA (10g) in a total volume of 500ml were stirred for 24 hours. After ten treatments, very little further carbohydrate was extracted (fig. 10). After freeze drying the dialysed, concentrated supernatant solution, the weight of material (carbohydrate content 2.5%) obtained was 2.0g. The dried residue weighed 11g (see Table 2).

The extraction was very poor as the embryos did not disintegrate. In an attempt to overcome this, 5g of the residue from the above extraction was homogenised (Potter glass homogeniser) and the extraction repeated three further times. It was impossible to follow the extraction as the material was colloidal. A clear solution was obtained with difficulty by filtration through colite, and dialysis and freeze drying produced 0.3g material.

3.4 Extraction of defatted embryos at 100°C with urea (7M) containing EDTA (2%)

The extraction procedure was the same as used in 3.3 except that the temperature was 100°C. After four extractions, the supernatant solutions were bulked, dialysed for 6 days, concentrated and freeze dried (12g; carbohydrate content 8.5%). The residue weighed 2.0g after freeze drying and contained the sugars shown in Table 2. The progress of the extraction is shown in fig. 10.

3.5 Extraction of defatted embryos at 40°C with urea (12M) containing EDTA (2%)

A mixture of ground embryos (25g) urea (350g) and EDTA (10g) were

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Fig. 9 Extraction of the residue after EDTA extraction of mustard embryos, with urea (8M) (continuous line) and guanidine hydrochloride (broken line)

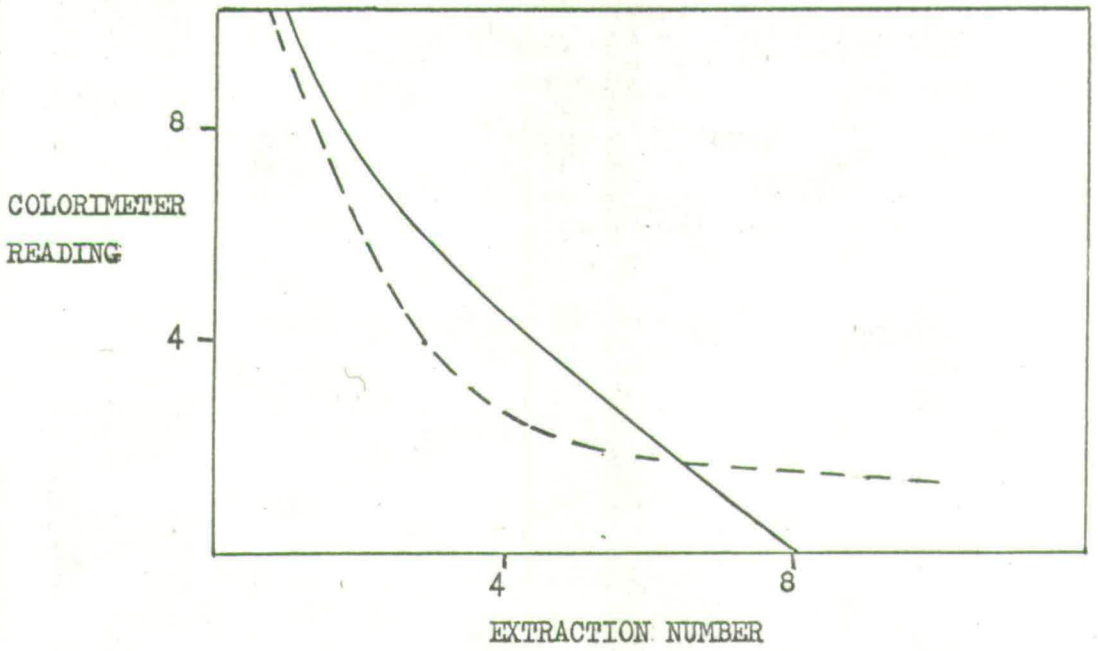
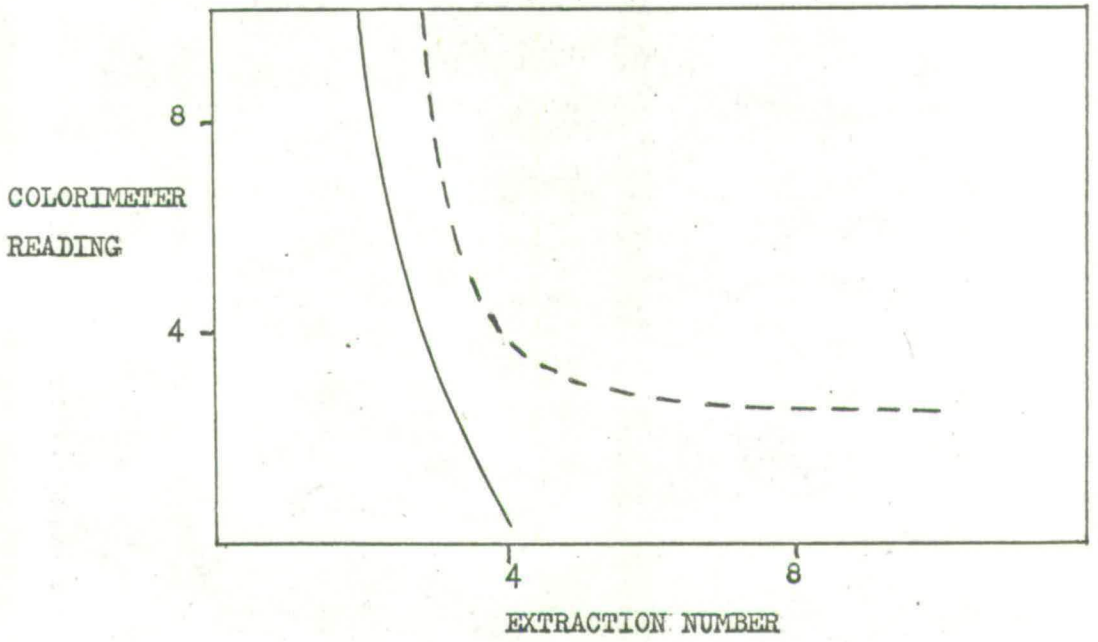


Fig. 10 Extraction of mustard embryos with urea (7M) containing EDTA (2%) at 100°C (continuous line) and urea (8M) containing EDTA (2%) at 20°C (broken line)



mixed with water (500ml) and stirred at 40°C for 24 hours. The mixture was filtered through muslin and the residue subjected to the same treatment a further five times. Fig. 11 shows the progress of extraction and the sugars observed after hydrolysis and paper chromatography of the residue are given in Table 2. The extracts were worked up in the usual manner (yield 11.4g; carbohydrate content 7.5%). The weight of the dried residue was 0.9g.

3.6 Extraction of defatted embryos with urea (12M) in a rotary ball mill

A mixture of ground embryos (25g) and urea (350g) in water (500ml) was milled for 24 hours. The mixture was centrifuged, the supernatant solution analysed for carbohydrate content, and the residue extracted a further six times. Fig. 11 shows the progress of extraction and Table 2 the sugars present in the residue. The extracts were worked up in the usual manner (yield 6.1g; carbohydrate content 12.0%). The weight of the dried residue was 3.1g.

3.7 Extraction after deproteinisation

Deproteinisation was attempted with phenol/acetic acid/water (Bagdasarian et al 1964) as follows. Ground embryos (50g) were ball milled with phenol(1kg) acetic acid (1 litre) and water (1 litre) for 24 hours and the precipitate was collected by centrifugation. The extraction was repeated twice on this precipitate which was finally washed with ethyl alcohol (90%) and ether. The yield of dried material was 9.1g (nitrogen content 4.9%, see general methods). This carbohydrate enriched material was subjected to the following series of small scale extractions to determine whether polysaccharides could be extracted from it more easily

than from the non-deproteinised embryos.

3.7.1 Extraction of deproteinised residue with EDTA at 20°C

The residue (1.0g) was shaken with EDTA (0.4g) and water (20ml) for 24 hours. The extraction was repeated a further nine times, the supernatant solution being analysed for carbohydrate at each stage. The progress of the extraction is shown in fig. 12. The weights of the final residue and of the dialysed freeze dried extract were 0.9g and 0.06g respectively. The sugars given on hydrolysis of the final residue are listed in Table 3.

3.7.2 Extraction of deproteinised residue with EDTA (2%) at 100°C

The method was similar to that used in 3.7.1, the only difference being that EDTA (2%, pH 4.9) was used at 100°C. The yield of extracted material after dialysis and freeze drying was 0.23g (carbohydrate content 30%). The residue (0.23g) showed galacturonic acid after hydrolysis. The detailed results are shown in Table 3 and fig. 12.

3.7.3 Extraction of deproteinised residue with sodium hexametaphosphate

Sodium hexametaphosphate (2%, pH 4.9) was used in place of EDTA in an experiment similar to 3.7.1. Care was taken to adjust the pH of the solution to 4.0 using a pH meter. The deproteinised meal (1g) was extracted for eight 24 hour periods at room temperature using sodium hexametaphosphate (0.4g) in water (20ml) for each extraction. The yield of extracted material after dialysis and freeze drying was 0.5g (carbohydrate content 44%). The residue weighed 0.3g and hydrolysis, followed by paper chromatography showed only a trace of galacturonic acid. The other sugars are listed in Table 3. The progress of the extraction is

Fig. 11 Extraction of mustard embryos with urea (12M) (continuous line) and with urea (12M) containing EDTA (2%) at 40°C (broken line) (see 3.5 and 3.6)

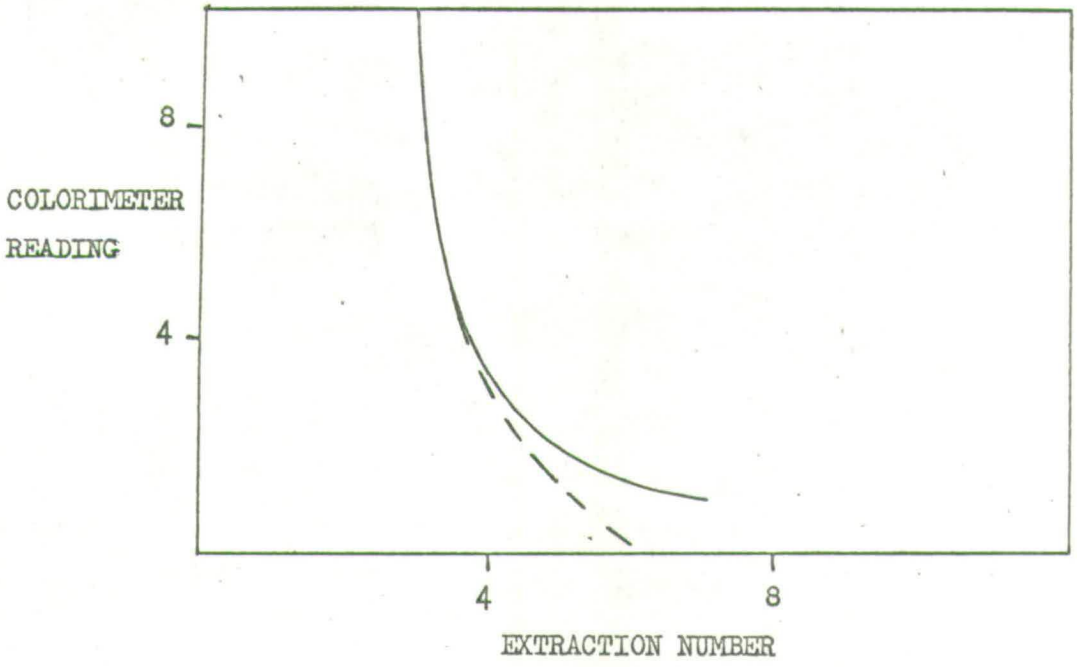
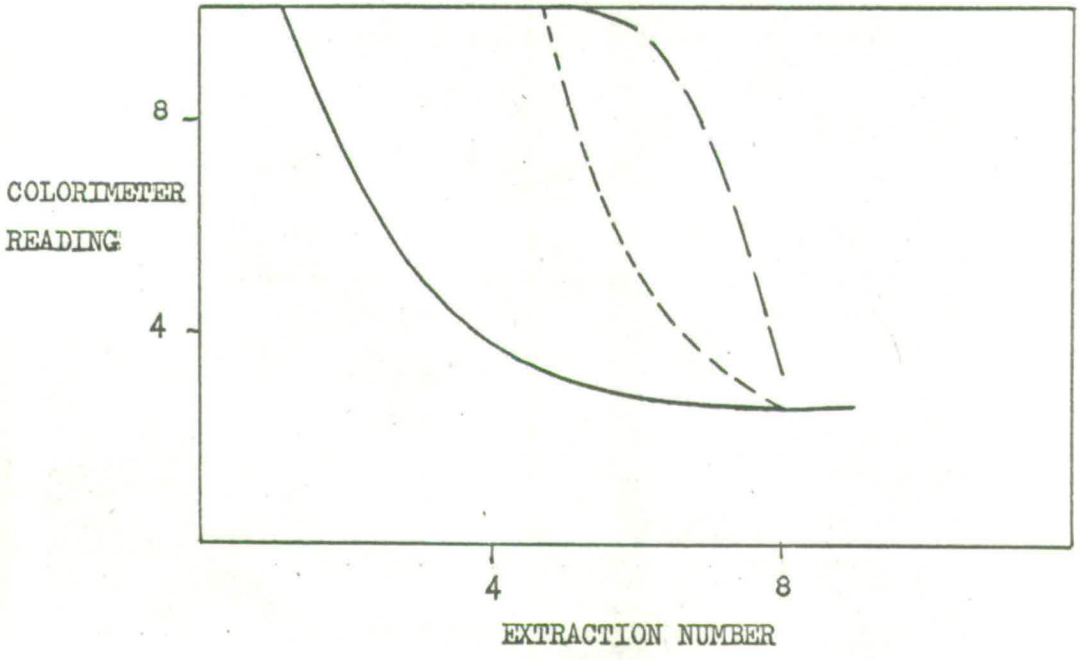


Fig. 12 Extraction of deproteinised residue with sodium hexametaphosphate (2%) at 20°C (long dashed line) and with EDTA (2%) at 100°C (short dashed line) and at 20°C (continuous line) (see 3.7.1, 3.7.2 and 3.7.3)



given in fig. 12.

3.7.4 Extraction of deproteinised residue with urea (12M) containing EDTA (2%)

Urea (14.1g) was used as well as EDTA in a method similar to that in 3.7.2. The progress of the extraction is shown in fig. 13. The weights of the final residue and of the dialysed, freeze dried extract was 0.3g and 0.09g respectively. The sugars given on hydrolysis of the final residue are listed in Table 3.

3.7.5. Extraction of deproteinised residue with sodium thiocyanate (50% w/v) containing EDTA (2%)

Sodium thiocyanate (20g) (Gould 1965) was used as well as EDTA in a method similar to 3.7.1. The progress of the extraction is shown in fig. 13. The weights of the final residue and of the dialysed, freeze dried extract were 0.4g and 0.09g respectively. The sugars given on hydrolysis of the final residue are listed in Table 3.

3.7.6. Extraction of deproteinised residue with guanidine hydrochloride (7M) and with guanidine hydrochloride (7M) containing EDTA (2%)

The residue (1.0g) was shaken with guanidine hydrochloride (12.3g) and water (20ml) for 24 hours. The extraction was repeated a further five times, the supernatant solution being analysed for carbohydrate at each stage. The residue was then extracted several times in the same way but with the incorporation of EDTA (0.4g) into the mixture. The progress of the extractions is shown in fig. 14. The weights of the final residue and of the dialysed, freeze dried extract were 0.3g and 0.25g respectively.

Fig. 13 Extraction of deproteinised residue with urea (7M) containing EDTA (2%) (continuous line) and with sodium thiocyanate (50%) containing EDTA (2%) (broken line) (see 3.7.4 and 3.7.5.)

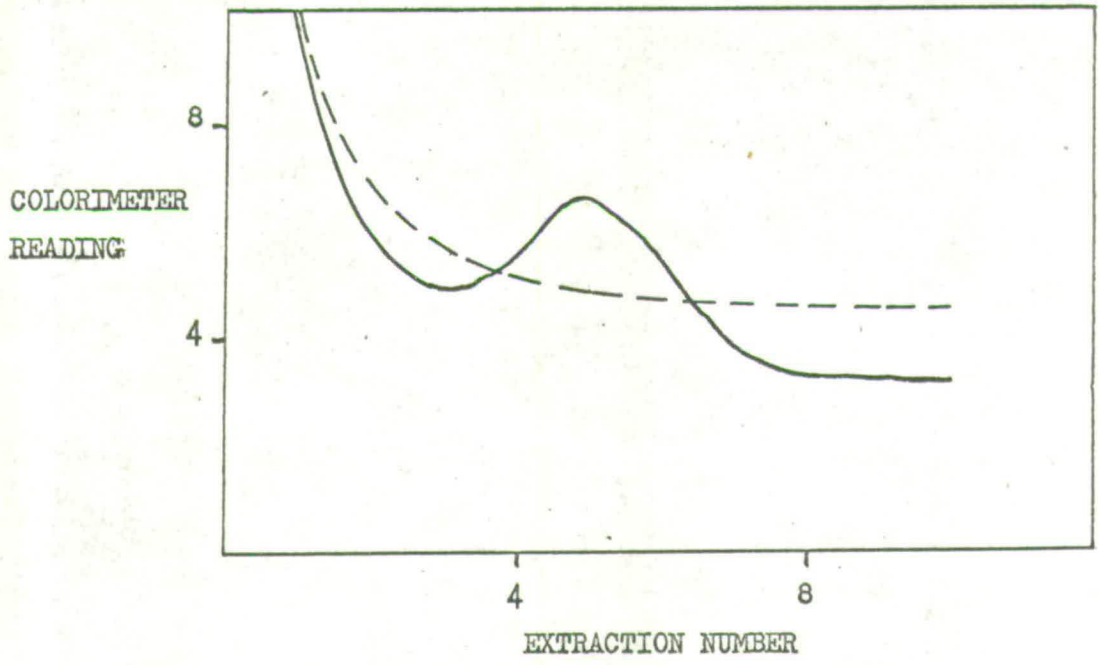
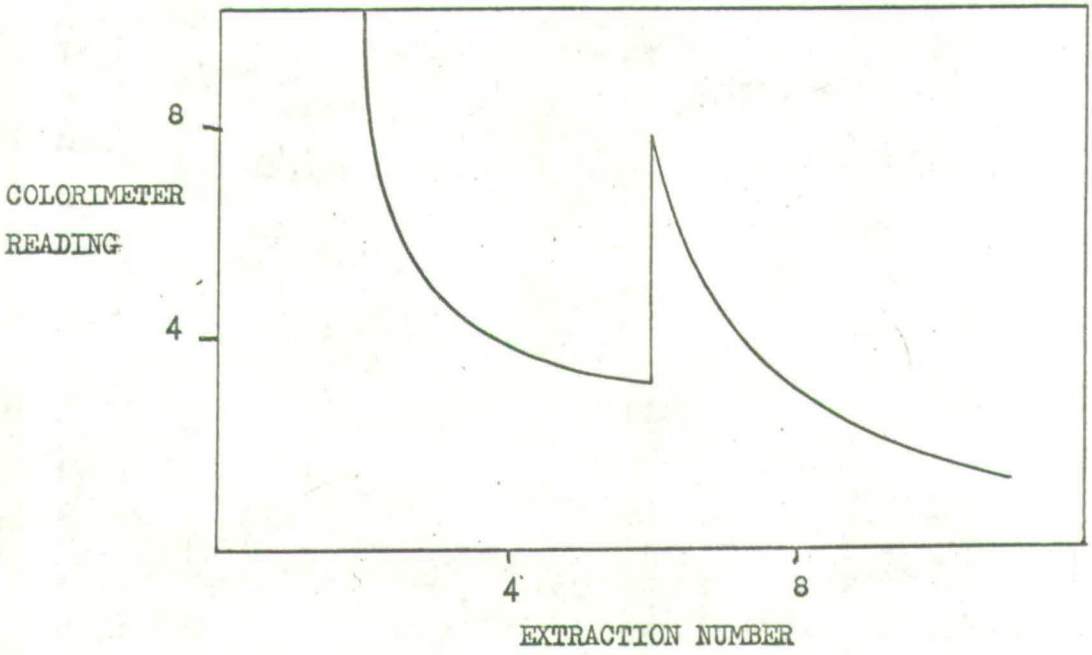


Fig. 14 Extraction of deproteinised residue with guanidine hydrochloride (7M) then guanidine hydrochloride (7M) containing EDTA (2%) (see 3.7.6)



The sugars given on hydrolysis of the final residue are listed in Table 3.

3.7.7 Extraction of deproteinised residue with formamide-water mixtures

Phenol/sulphuric acid tests show that very little polysaccharide was extracted when the residue was shaken with formamide-water mixtures in the proportions 2:1 or 1:2.

3.8 Extraction of mustard embryos with guanidine hydrochloride (7M) containing EDTA (2%) at 40°C

Defatted embryos (12.5g) were dispersed in water (250ml) containing guanidine hydrochloride (166g) and EDTA (5g) and stirred at 40°C for 3 hours. The residue was extracted three times in the same way and then a further thirteen times for 24 hour periods. The progress of the extraction is shown in fig. 15. The weights of the final residue and of the dialysed, freeze dried extract were 1.1g and 4g (carbohydrate content 8.5%) respectively. The sugars contained in the residue are shown in Table 2.

3.9 Extraction of defatted embryos with sodium hexametaphosphate at 80°C

Embryos (5g) were extracted at 80°C for 24 hours with a solution of sodium hexametaphosphate (2%, pH 4.0) in water (125ml). This was repeated six times and the yield of extracted material after dialysis was 2.7g (carbohydrate content 4.5%). The residue (1g) gave galacturonic acid as well as other sugars (Table 2) on hydrolysis. The detailed results are shown in Table 2 and fig. 16.

3.10 Extraction of defatted embryos with sodium hexametaphosphate at 100°C

The procedure was the same as in 3.9 except the temperature was 100°C. The yield of extracted material after dialysis and freeze drying was 1.2g

Fig. 15 Extraction of mustard embryos with guanidine hydrochloride (7M) containing EDTA (2%) at 40°C (see 3.8)

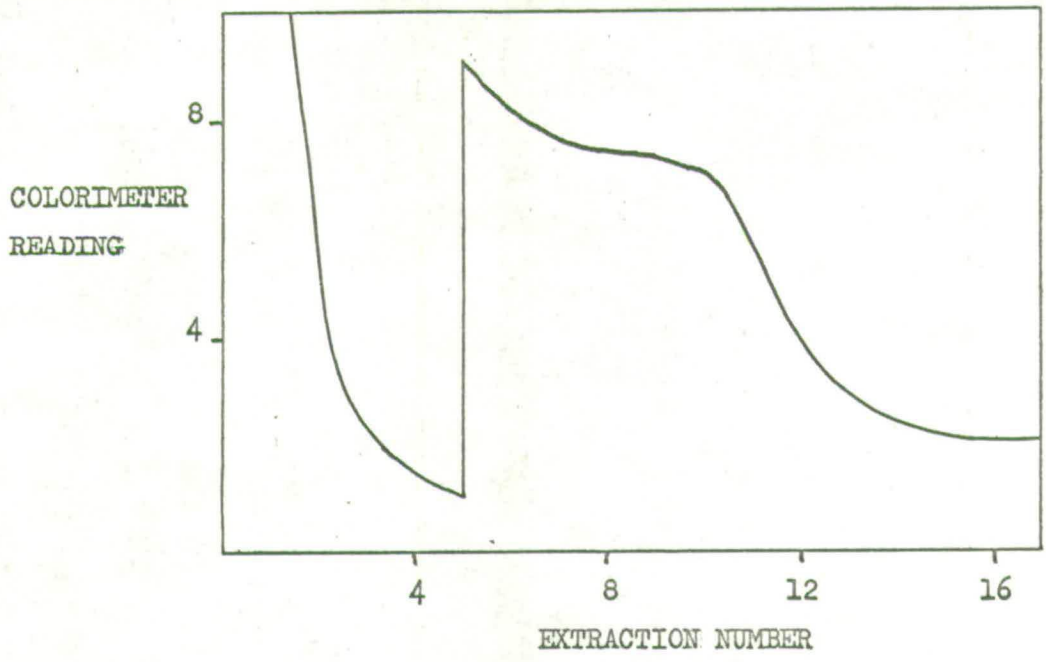
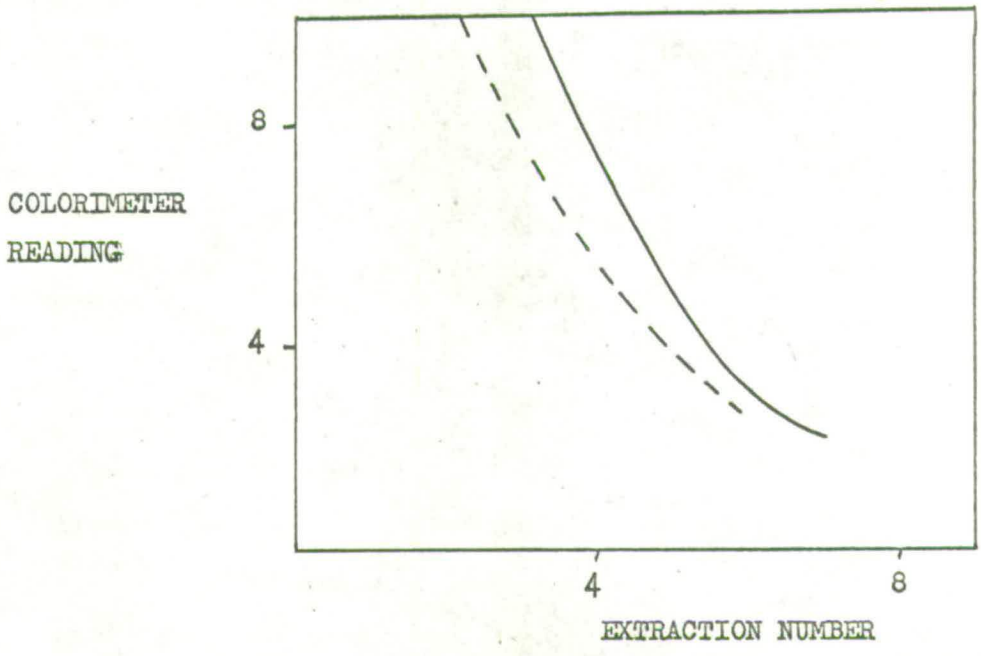


Fig. 16 Extraction of mustard embryos with sodium hexametaphosphate at 80°C (continuous line) and at 100°C (broken line) (see 3.9 and 3.10)



(carbohydrate content 10%) and the yield of the residue was 0.6g. Other results are given in Table 2 and fig. 16.

3.11 Extraction of defatted embryos with EDTA (2%) containing sodium lauryl sulphate (0.2M)

Sodium lauryl sulphate (7.2g) with EDTA (2.5g) and defatted mustard seed embryos (5g) were heated in a total volume of 125ml for 3 hours at 100°C. A control experiment showed that the pH of the extracting solution was 5.0. The mixture was filtered and the residue extracted in the same way a further three times and finally extracted twice for 24 hours. The six extracts were combined and dialysed against running tap water to remove EDTA. Methyl ethyl ketone (10 volumes) was added to precipitate the polysaccharide free from sodium lauryl sulphate (yield 5g; carbohydrate content 2.5%). The residue weighed 0.17g after drying. Other results are given in Table 2 and fig. 17. It would appear that extraction of pectic substances was complete because the residue gave no galacturonic acid on hydrolysis and little arabinose.

3.12 Extraction of defatted embryos with EDTA (2%) containing sodium lauryl sulphate (0.2M) at 80°C and 60°C

This series of extractions was similar to that in 3.12 except that extraction was for seven periods of 24 hours each. The yield of extracted material (at 80°C) was 3.2g (carbohydrate content 3.0%) and that of the residue was 0.26g. In a similar series of extractions at 60°C, the corresponding yields were 2.8g (carbohydrate content 6.0%) and 0.76g. The progress of both extractions is shown in fig. 17. In neither case was the extraction of galacturonic acid-containing polysaccharide complete (Table 2).

3.13 Extraction of defatted embryos with sodium lauryl sulphate at 100°C in the absence of EDTA

Defatted embryos (5g) were extracted five times for 24 hour periods with sodium lauryl sulphate (7.2g) in 125ml water at 100°C. The yield of extracted material (carbohydrate content 7.5%) was 6.0g (contaminated with sodium lauryl sulphate) and that of the residue (0.35g). Although the results (Table 2) indicate that the extraction was efficient, control experiments carried out showed that the pH of sodium lauryl sulphate solution was 9.0 and it is therefore likely that degradation of polysaccharides occurred and facilitated extraction.

3.14 Extraction of defatted embryos with sodium hexametaphosphate (2%) containing sodium lauryl sulphate (0.2M) at 80°C

Sodium lauryl sulphate (7.2g) was heated with sodium hexametaphosphate (2g) and defatted embryos (5g) in a total volume of solution of 125ml at 80°C for 24 hours. This was repeated a further five times. At the beginning of each extraction, the solution was carefully buffered to pH 4.0. The yield of extracted material was 0.9g (carbohydrate content 10%) and that of the residue was 0.58g. The progress of the extraction is shown in fig. 18. The hydrolysis products of the residue (Table 2) showed that extraction of pectic substances was incomplete.

3.15 Extraction of defatted embryos with sodium hexametaphosphate (2%) containing EDTA (2%) and sodium lauryl sulphate (0.2M) at 80°C

The extraction was similar to that in 3.14 except that the solution included EDTA (2g). The yield extracted was 1.5g (carbohydrate content 9%) and that of the residue was 0.56g. The progress of the extraction is shown

Fig. 17 Extraction of mustard embryos with EDTA/sodium lauryl sulphate at 100°C (continuous line) 80°C (short dashed line) and 60°C (long dashed line) (see 3.11 and 3.12)

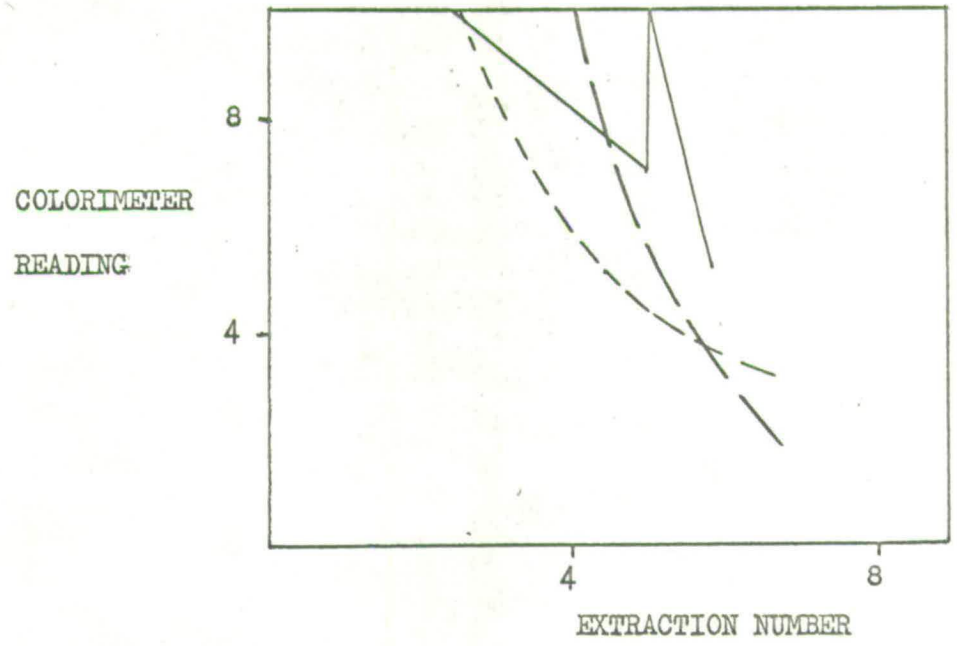
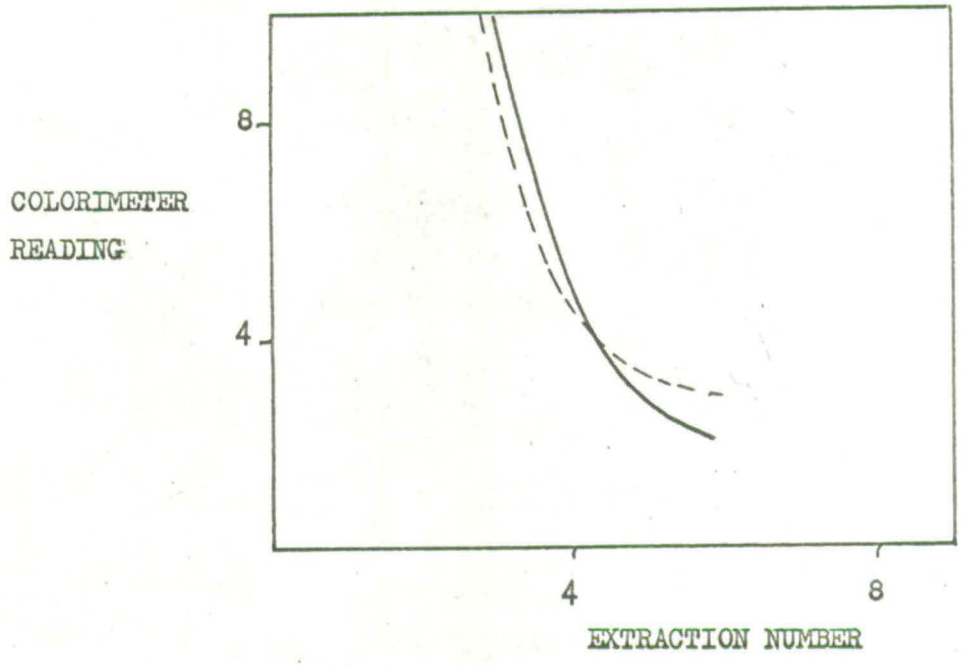


Fig. 18 Extraction of mustard embryos with sodium hexametaphosphate/sodium lauryl sulphate at 80°C (continuous line) and sodium hexametaphosphate/ sodium lauryl sulphate/EDTA at 80°C (broken line) (see 3.14 and 3.15)



in fig. 18. The hydrolysis products of the residue (Table 2) show that extraction was incomplete.

Table 2 Sugars detected after hydrolysis of residues from the extraction of mustard embryos

Extraction Conditions*	Sugars detected by paper chromatography after hydrolysis**					
	Galacturonic Acid	Glucose	Galactose	Arabinose	Xylose	Rhamnose
3.1	++	++	-	++	+++	+
3.2	++	+	++	+++	++	-
3.3	++	+	++	+++	+++	+
3.4	++	+++	++	++	++	-
3.5	++	++	++	+++	++	+
3.6	++	+	++	+++	++	+
3.8	++	+	++	+++	++	+
3.9	+	++	++	trace	+++	-
3.10	+	+	++	-	+++	-
3.11	-	+	+	+	++	-
3.12 (80°C)	+	++	++	++	+++	trace
(60°C)	+	+	++	+++	+++	trace
3.13	trace	++	++	++	+++	-
3.14	+	++	++	-	+++	-
3.15	+	++	++	-	+++	-

Notes :- See page 59

Table 3 Sugars detected after hydrolysis of residues from the extraction of deproteinised material

Sugars detected by paper chromatography after hydrolysis**

Extraction Conditions*	Galacturonic Acid	Glucose	Galactose	Arabinose	Xylose	Rhamnose
3.7.1	++	trace	+	+++	++	trace
3.7.2	+	+	++	-	++	-
3.7.3	trace	+	++	+++	++	-
3.7.4	++	trace	+	+++	++	trace
3.7.5	+++	trace	+	+++	++	trace
3.7.6	+	+	++	+++	++	-

Notes :-

* The letters in this column refer to the corresponding headings in the text.

** For hydrolysis, approximately 20mg of each residue was shaken for 24 hours with 72% w/v sulphuric acid (2ml). After dilution to 20ml, the mixture was heated at 100°C for 6 hours then neutralised by shaking three times with octyldimethylamine (5% in chloroform) and examined by paper chromatography in solvent A (see general methods). Chromatograms were sprayed with p-anisidine hydrochloride.

3.16 Large scale extraction of the pectic polysaccharides from the cotyledons of nongerminated and germinated seeds

3.16.1 The separation of cotyledons from nongerminated seeds (Richardson 1965)

A large quantity of whole seeds was crushed using a hand mill and the enzymes inactivated by heating with ethanol (80% v/v) at 80°C for 10 minutes. After a thorough sifting and winnowing procedure to obtain pure cotyledons (1140g) oligosaccharides were removed by solvent extraction (soxhlet) with ethanol (48 hours). Fats were removed in a similar manner using acetone as a solvent. The yield of air dried cotyledons was 580g.

3.16.2 The separation of cotyledons from germinated seeds (Gould 1965)

Mustard seeds were germinated on moist filter paper (Whatman 3MM) in the dark. After four days they were harvested, frozen immediately in liquid nitrogen and freeze dried. The seedlings were crushed in a hand mill and subjected to the same sifting and winnowing procedure as described in 3.16.1. As this was complicated by the presence of roots, the final pure sample of cotyledons (330g) was obtained by hand sorting. The cotyledons were solvent-extracted with ethanol and acetone and gave 137g of fat free cotyledons.

3.16.3 Large scale extraction using sodium lauryl sulphate (0.2M) and EDTA (2%) at 100°C

Fat free cotyledons (580g) from nongerminated seeds were stirred with water (2 litres) containing sodium lauryl sulphate (115g) and EDTA (40g) for 3 hours at 100°C. The supernatant was filtered off through muslin and the process was repeated twice on the residue. Five further extractions

were then carried out, heating the mixture at 100°C for 24 hours. The bulked extracts (16 litres) were then centrifuged to remove cell debris and methylated spirit (4 volumes, 64 litres) was added to precipitate the pectic material. The mixture was allowed to stand for 24 hours and the supernatant siphoned off. The precipitate was centrifuged, stirred with water (5 litres) containing a small amount of mercuric iodide for sterilisation and dialysed for 10 days against running tap water. The mixture (13 litres) was centrifuged and the precipitate (probably denatured protein) was discarded as it contained only a trace of carbohydrate (by phenol/sulphuric acid test). The supernatant was concentrated to 3 litres and deproteinised (three times) by heating with phenol (45% w/w, 2.3kg) at 75°C (Westphal et al 1952). It was shaken six times with 1½ litres of ether to remove residual phenol, concentrated and freeze dried. The yield of pectic polysaccharides (N') was 40.7g (N = 0.64%) and gave the usual pattern of sugars (see Table 4) on hydrolysis (formic acid) and paper chromatography (solvent A).

Physical Properties

1. Free boundary electrophoresis in sodium tetraborate buffer (containing 2% EDTA) produced one slow moving, diffuse peak (Plate 31) and a similar pattern (with $\mu = 6.3 \times 10^{-5} cm^2/s/v$) was obtained in sodium phosphate buffer.
2. Ultracentrifugation at 110,000 g of a 1% solution containing sodium chloride (0.15M) and EDTA (2%) gave one slow sedimenting peak ($S_{20} = 2.05 \times 10^{-13}$; see Plate 32).

The extraction was repeated on cotyledons (137g) from germinated seeds

PLATE 31

Sample : Pectin from non-germinated seeds

Time : 111 min.

PLATE 32

Sample : Pectin from germinated seeds

Time : 102 min.

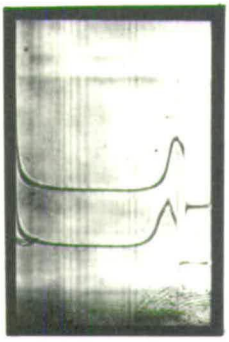


PLATE 33

Top : Pectin from germinated seeds

Bottom : Pectin from non-germinated seeds

Time : 27 min.

obtained in 3.16.2 above; the quantities of reagents used were scaled down in proportion. The yield of pectic polysaccharides (G') was 17.4g (N = 0.85%) and gave a similar pattern on hydrolysis and paper chromatography to that obtained for the material from non-germinated seeds (Table 4).

Physical Properties

1. Free boundary electrophoresis in sodium tetraborate buffer (containing 2% EDTA) produced a slow moving, very diffuse peak (Plate 32) and a similar less diffuse peak (with mobility 8.0 x 10^{-5} $cm^2/s/v$) in sodium phosphate buffer.
2. Ultracentrifugation at 110,000 g of a 1% solution containing sodium chloride (0.15M) and EDTA (2%) gave an almost identical pattern (Plate 33) to that obtained for the material for non-germinated seeds (S_{20} = 2.10 x 10^{-13}).

Table 4 Sugars obtained after hydrolysis and paper chromatography

	Galacturonic Acid	Galactose	Glucose	Arabinose	Xylose
N'	++	+	-	++++	+++
G'	++	+	-	++++	+++

3.17 Examination of the setting properties of some polysaccharide gels

3.17.1 Changes in optical rotation during setting

All polarimetry was carried out in a thermostatted cell (1dm) using a Perkin-Elmer Model 141 automatic polarimeter with variable slit width (a similar instrument without this control gave unsatisfactory results).

Aqueous solutions of agarose (1% and 0.5%) and the potassium salt of two carrageenans (kappa 3% and iota 5%) were examined. Each was placed in the cell at about 80°C, equilibrated at this temperature for 20 minutes and the optical rotation at up to three wavelengths (365, 546 and 578mμ) measured. The temperature was lowered by 5-10°C and the optical rotation measured after 20 minutes (see figs. 19, 20, 21 and 22). The process was repeated until room temperature had been reached. In the case of agarose, once room temperature had been reached, it was heated to about 80°C again, readings being taken at approximately 10°C intervals.

A distinct change in optical rotation was observed when the gels set; the ones with negative rotation (agarose) immediately became more negative and the ones with positive rotation (carrageenan) became more positive.

Possible Systematic Errors

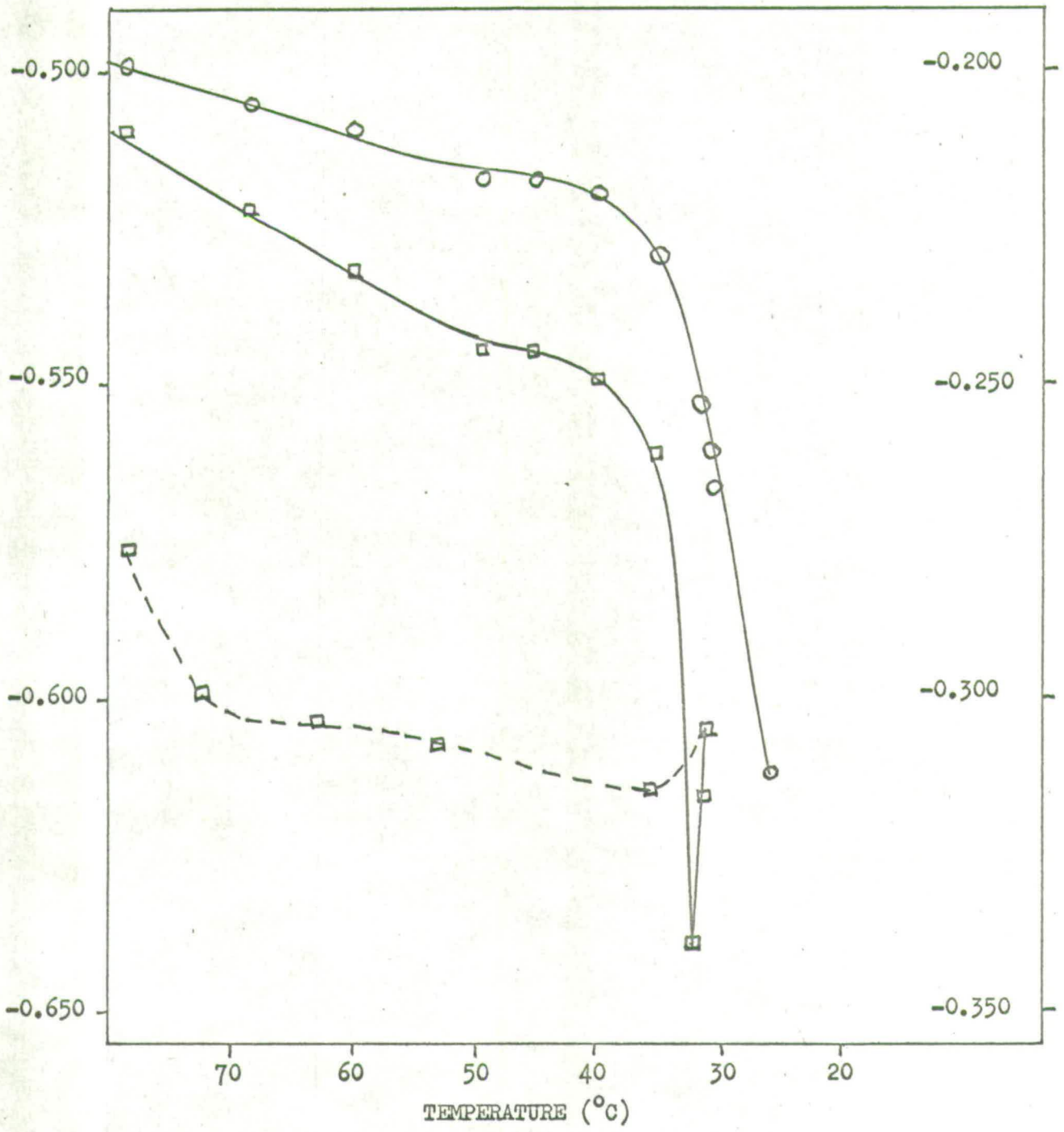
1. Variation of optical rotation with temperature.

   An aqueous solution (3%) of α-methyl D-glucoside was examined in a manner similar to that described for the polysaccharide gels (see fig. 23). There were only slight deviations from a straight line as the temperature was lowered.

2. Change in refractive index upon setting.

   The refractive index of the polysaccharide solutions was measured (Abbé refractometer) at intervals on cooling from 67.5°C to 25°C (see Table 5).

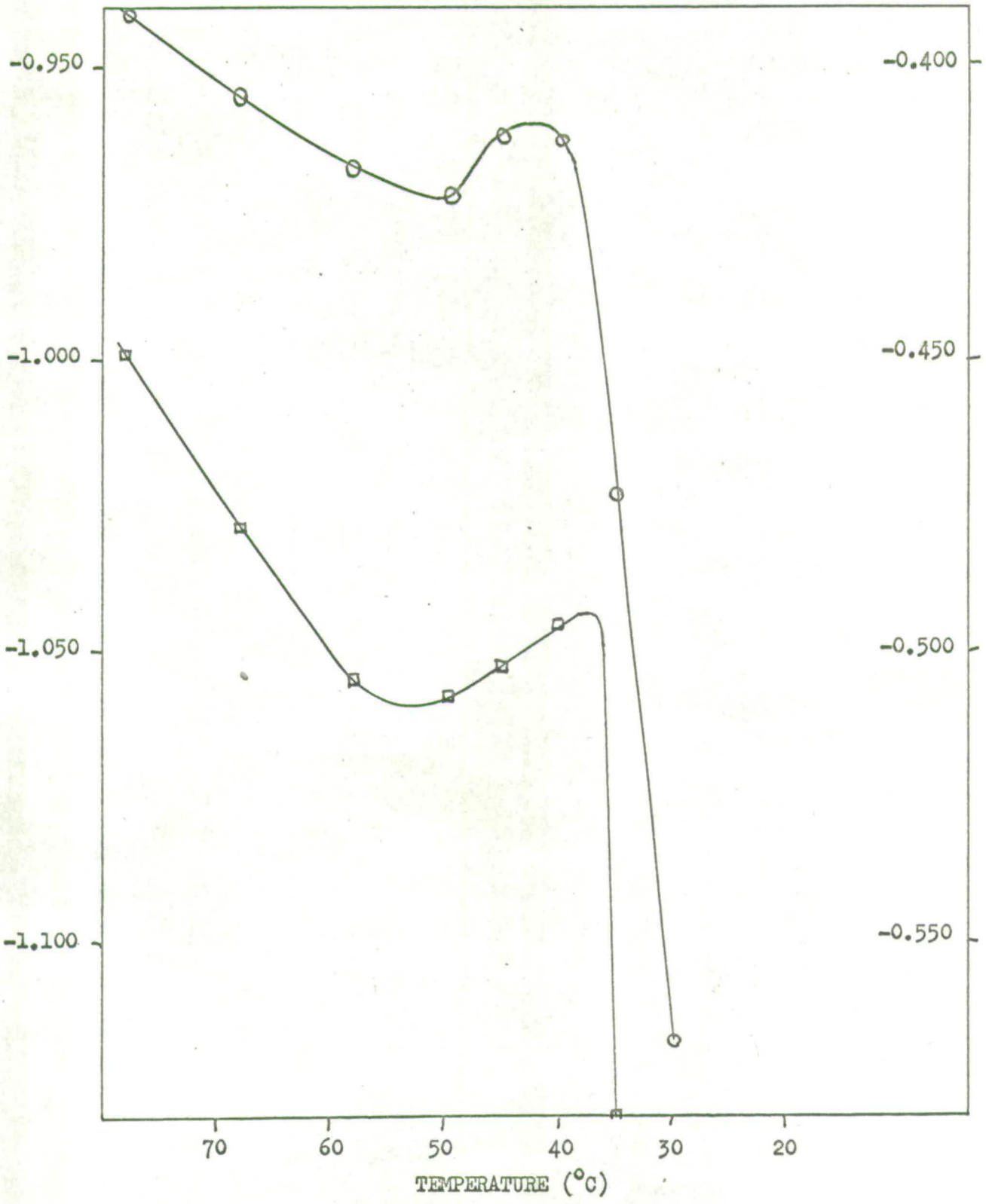
Fig. 19 Optical rotation of agarose (0.5%) solution on reducing the temperature (continuous line) and on reheating (broken line)



Optical rotation at 365mμ (denoted by □)

Optical rotation at 546mμ (denoted by ○)

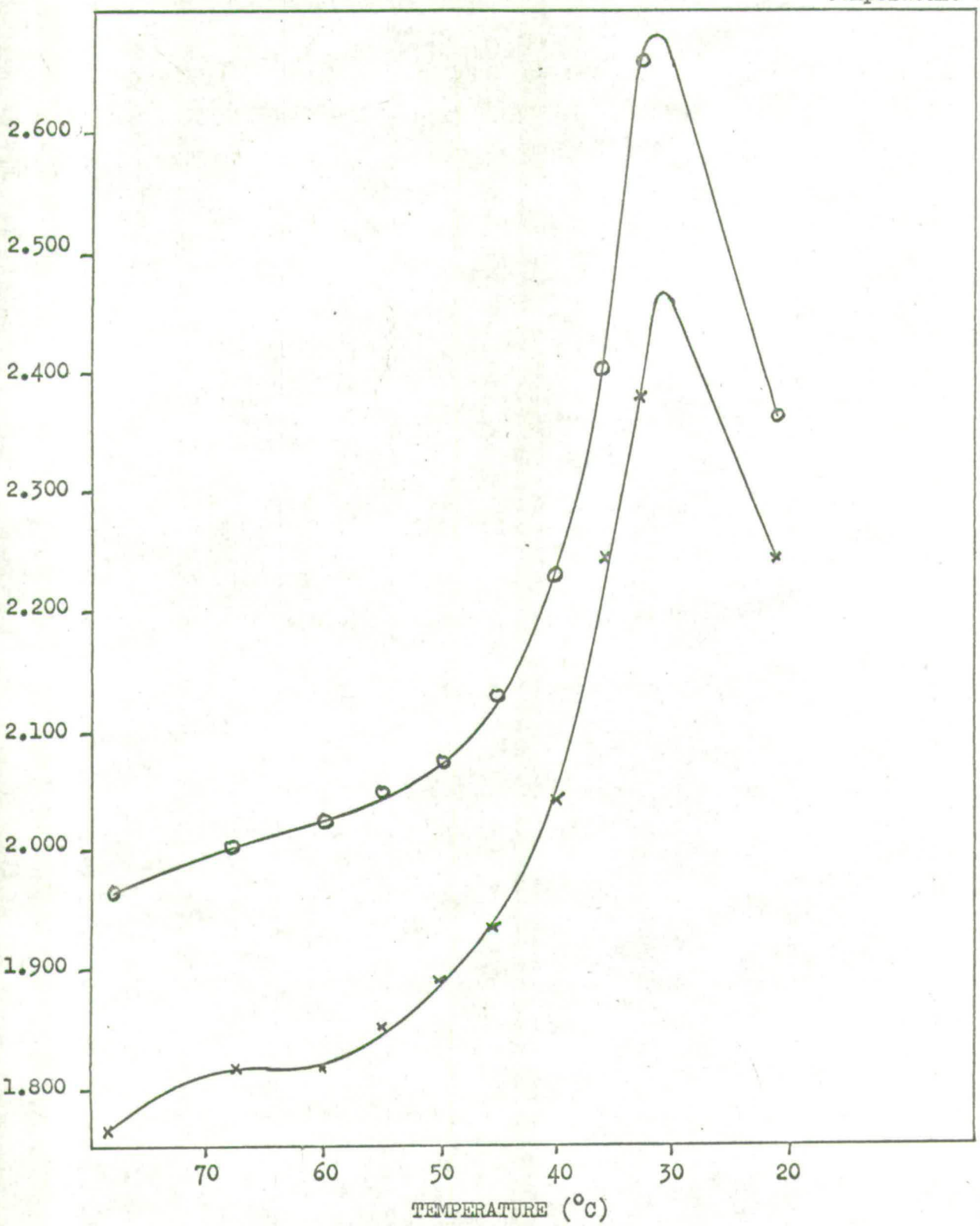
Fig. 20 Optical rotation of agarose (1.0%) solution on reducing the temperature



Optical rotation at 365mμ
(denoted by □)

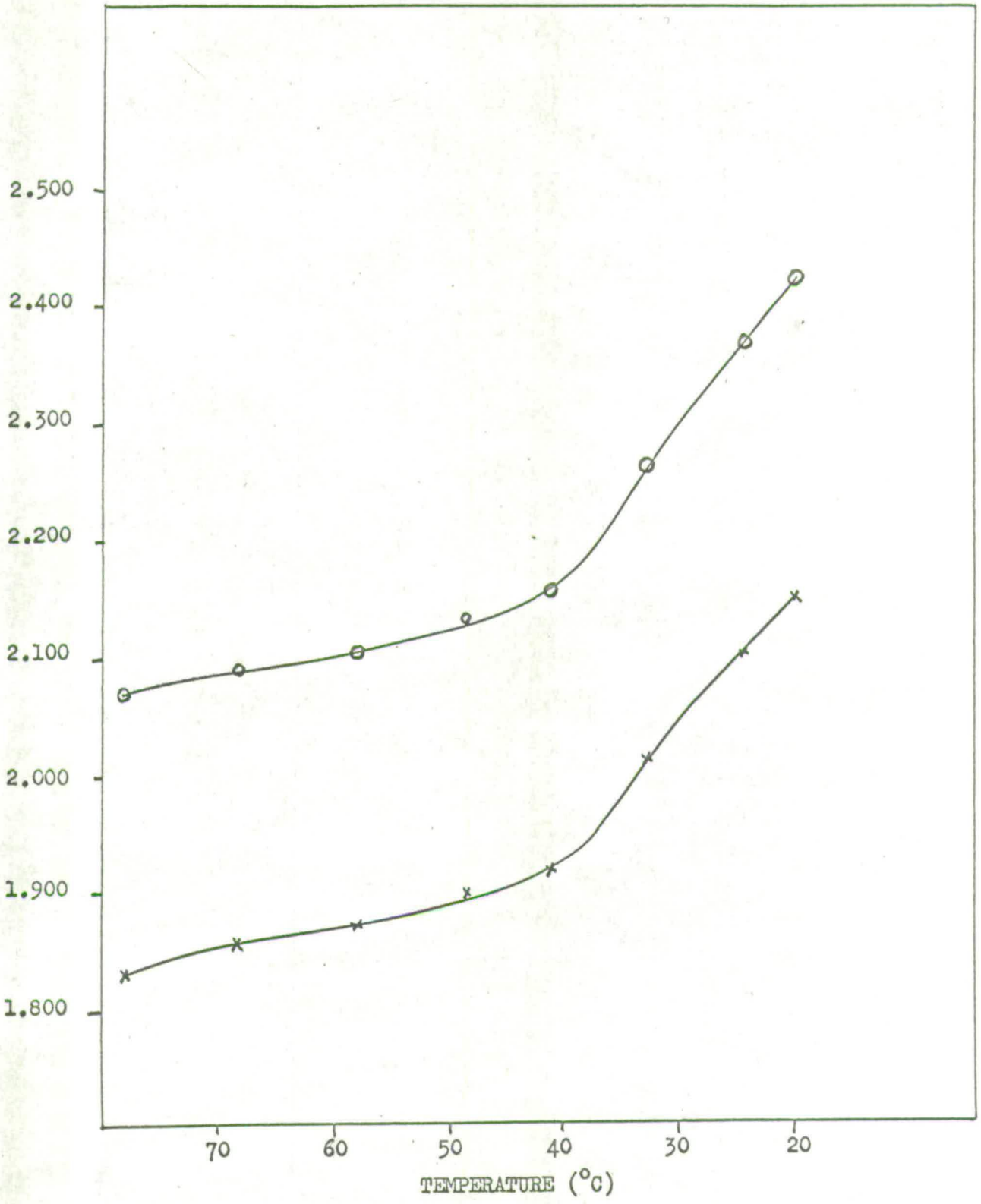
Optical rotation at 546mμ
(denoted by ○)

Fig. 21 Optical rotation of kappa-carrageenan solution (3%) on reducing the temperature



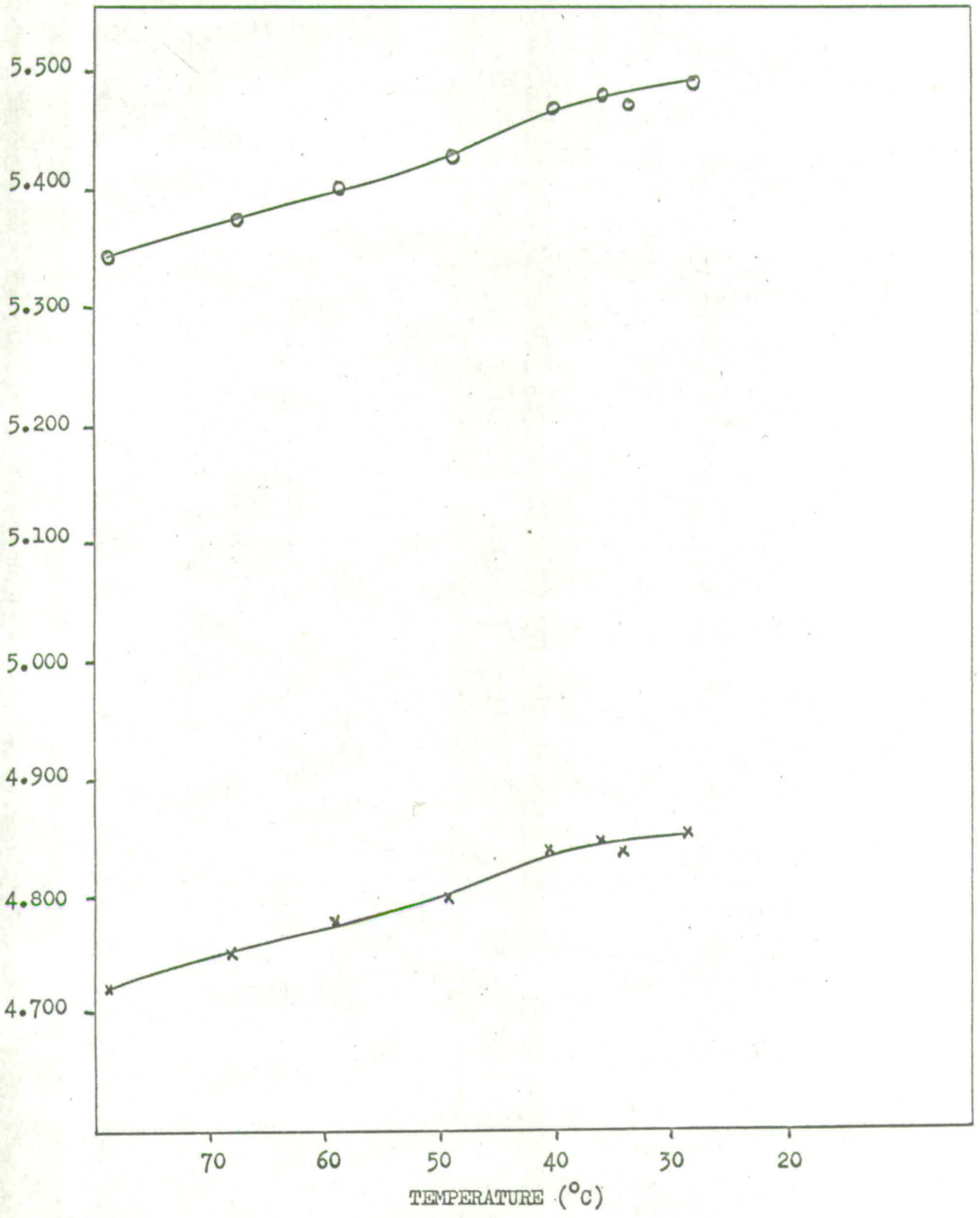
Optical rotation at 546mμ (denoted by o) and 578mμ (denoted by x)

Fig. 22 Optical rotation of iota-carrageenan (5%) on reducing the temperature



Optical rotation at 578mμ (denoted by x) and at 546mμ (denoted by o)

Fig. 23 Optical rotation of α-methyl glucoside (3%) on reducing the temperature



Optical rotation at 578mμ (denoted by ×) and at 546mμ (denoted by o)

Table 5

Refractive Indices

Temperature Water	α-methyl D-glucoside (3%)	Agar (½%)	Carrageenan Kappa- (3%)	Carrageenan Iota- (5%)
67.5°C	1.330	1.326	1.329	1.331
60°C	1.332	1.328	1.330	1.332
50°C	1.334	1.329	1.332	1.333
45°C	1.334	1.330	1.333	1.335
40°C	1.335	1.331	1.334	1.336
35.5°C	1.336	1.333	1.335	1.337
30°C	1.337	1.334	1.335	1.337
25°C	1.337	1.334	1.336	1.338

3. Light scattering and reduction in transmission

Light scattering caused by setting of a gel should tend to depolarise light. An attempt was made to examine the effect of light scattering by adding calcium carbonate (5mg) to a solution of α-methyl D-glucoside (3%, 10ml). A similar experiment was carried out using carbon instead of calcium carbonate as it was possible that a reduction in the intensity of the light passing through the solution might be responsible for a change in the rotation. The optical rotation at 578mμ, 546mμ and 378mμ was measured before and after the addition of calcium carbonate or carbon.

Optical rotation of :-	Wavelength 578mμ	546mμ	365mμ
α-methyl D-glucoside	4.929	5.571	13.903
" " and carbon	4.915	5.561	13.870
" " and calcium carbonate	4.918	5.561	13.870

3.17.2 The effect of electrolytes on the setting temperature of gels

The melting points of gels containing anions at different concentrations were recorded (Tables 6 and 7). The gels used were commercial agar (0.4%, Oxoid Ionagar No. 2) agarose (0.5%, a very pure sample, RENJ 5116, supplied by Marine Colloids) kappa-carrageenan (3% and 2%, REX 3148 isolated from Chondrus crispus by Marine Colloids) and iota-carrageenan (2% and 1%, REX 5201 isolated from Eucheumia spinosum by Marine Colloids). The effect of addition of varying amounts of sodium lauryl sulphate to agar (0.4%) and methyl cellulose (1.5%) (B.D.H.) gels was also examined.

All the gel samples in a particular series were heated in sealed tubes in a gently heated, stirred water bath (rate of increase of temperature = 0.75°C/minute) until they melted. The melting point was taken as the temperature at which the gel flopped on shaking the tube gently.

3.17.3 Gels from fully methylated citrus pectin

An attempt was made to prepare a fully methylated citrus pectin gel using the method of Deuel et al (1950). This was successful only on the addition of sucrose (50% w/w) and the sol obtained on melting was too viscous for reliable melting point determination. Attempts to reduce the concentration of pectin or sucrose content prevented the gel from setting.

Table 6 Melting point of a gel at 0.25M salt concentration (°C)

Salt	Agar 0.4%	Agarose 0.5%	Carrageenan Kappa- 3%	Kappa- 2%	Iota- 2%	Iota- 1%
-	80	74	47	28	Not	set
Sodium trichloroacetate	75	65	56	47	60	32
Sodium thiocyanate	72	68	62	74	38	62
Lithium thiocyanate	70	73	59	59	60	53
Potassium thiocyanate	72	74	61	72	47	63
Sodium perchlorate	71	73	67	81	48	59
Sodium iodide	75	67	65	83	57	59
Sodium acetate	77	81	61	49	65	31
Sodium bromide	78	81	62	83	55	64
Sodium chloride	80	82	59	50	62	31
Urea	78	76	47	Not set	Not	set
Guanidine hydrochloride	78	76	62	40	65	21

Table 7 Melting point of agarose (0.4%) at 1.0M salt concentration

Salt	Melting Point (°C)
-	75
Sodium trichloroacetate	Not set
Sodium thiocyanate	" "
Lithium thiocyanate	" "
Potassium thiocyanate	" "
Sodium perchlorate	" "
Sodium iodide	" "
Sodium acetate	83
Sodium bromide	70
Sodium chloride	73
Urea	70
Guanidine hydrochloride	74

Table 8

Gel	Amount of added sodium lauryl sulphate (g/25ml of gel)	Melting Point (°C)
Agar (0.4%)	0	85
	0.2	83
	0.4	83
	0.7	83
	1.0	83
	3.0	Not set
	5.0	Not set
Methyl cellulose (1.5%)	0	80
	0.6	Not set
	1.0	Not set

Discussion

One of the important aims in obtaining a polysaccharide from its natural source, is that extraction should be complete. In the case of mustard pectin, it was shown (Gould 1965, Richardson 1965) that pectic substances were completely extracted by EDTA (pH 7.5, 100°C) by the fact that the residue gave no galacturonic acid or arabinose on hydrolysis. The success of this method (Hirst et al 1965) suggested that divalent cations, possibly calcium, are involved in the binding of pectic polysaccharides into the cell walls (c.f. General Introduction). Unfortunately, β-elimination and de-esterification (see Chapter 4) are likely to occur to some extent, and a new method is therefore required without these drawbacks. The search for new methods for complete extraction was based on attempted suppression of certain types of possible molecular interaction in the plant tissue.

The most obvious alternative is a divalent cation sequestering agent which would be effective at low pH. Sodium hexametaphosphate works at low pH but did not achieve complete extraction from fat free mustard embryos. EDTA at pH 4.9 (extraction carried out by Dr. N.G. Richardson) is also not completely effective and this suggests that the removal of cations is not the whole answer to complete extraction; there must be another form of interaction holding the polysaccharide into the cell wall.

Deproteinisation, followed by extraction using sodium hexametaphosphate, gave good results even in the cold although some combined arabinose remained in the residue. That the removal of protein was necessary before complete extraction could be achieved suggests, as did the work on pectin gels described in Chapter 1, that protein may play a part in the binding of

pectic substances into the cell wall.

Interaction between molecules in the plant cell wall might be related to that in synthetic gels (General Introduction). The recent suggestion that hydrophobic bonding might be important in certain polysaccharide gels (Anderson 1966) in turn means that this type of interaction may exist *in vivo*. Urea, guanidine hydrochloride, sodium thiocyanate and sodium lauryl sulphate are all thought to be compounds with the ability to break or compete for hydrophobic bonds. Of the reagents of this type that were tried, extraction of pectic material from defatted mustard seeds was only complete with the sodium lauryl sulphate/EDTA mixture used at 100°C.

The choice of an extraction method was therefore between this system and sodium hexametaphosphate after deproteinisation. Neither is as mild as could be wished, but neither suffers from the drawback of using EDTA (pH 7.5) at 100°C which is that pectic substances are likely to be degraded severely during extraction. Sodium hexametaphosphate was considered less satisfactory as it was necessary to deproteinise the seeds first with a mixture containing acetic acid and this might have caused degradation. The large scale extractions of germinated and non-germinated seeds were therefore carried out using sodium lauryl sulphate/EDTA at pH 5.0.

These large scale experiments extracted significantly more polysaccharide material (25% more) than the original EDTA method (Hirst *et al* 1965, Gould 1965). The extracts also contained much less protein after treatment with phenol. This may have been due to the ability of sodium lauryl sulphate to denature protein during extraction and make it easier to separate from the polysaccharide.

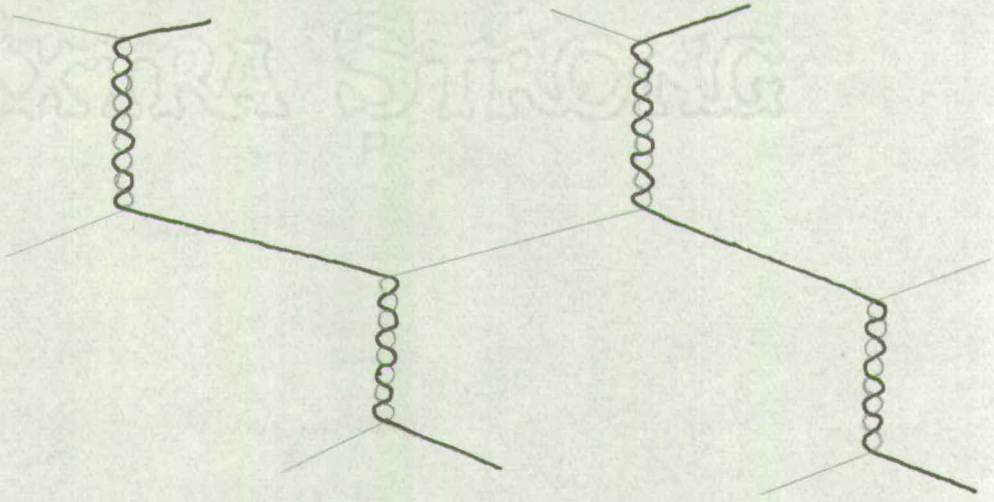
The patterns produced on ultracentrifugation of these new materials

were rather different from those obtained for the pectin extracted at pH 7.5 (Chapter 1, Plate 20). Only one slow peak was obtained in both samples and this corresponded approximately to the slow-sedimenting material in the original pectic extract. It is possible that the faster sedimenting material was not completely absent but was obscured by physical effects in the ultracentrifuge cell. This hypothesis is supported by the distortion of the leading edge of the slow peak after prolonged ultracentrifugation at high speed. This could only account for part of the loss of the fast component; another explanation is required for the remainder. One possibility is that extensive degradation of this material had taken place during extraction, though this is difficult to understand as the conditions were much milder than those in the original extraction. An extraction carried out on fat free mustard embryos using EDTA at pH 4.9 (by Dr. N.G. Richardson) gave pectic material which contained both fast and slow peaks indicating that the loss of the fast peak is not a function of the low pH. Another explanation is that the fast peak is due to a physical aggregate produced by some special condition of the original extraction which survives from the natural state, but is dissociated by extraction with sodium lauryl sulphate. Insufficient information is available to select the most probable answer.

Free boundary electrophoresis also produced a single peak in both samples. Although the new pectin from non-germinated seeds had a similar mobility to that of the original N, the mobility of the pectin from germinated seeds was significantly higher than that of the original G and both N samples. This was the only difference noticed between the pectins from non-germinated and germinated seeds.

The action of sodium lauryl sulphate is probably due either to the removal of protein surrounding the polysaccharides in the cell wall (supported by the fact that sodium hexametaphosphate is more successful after deproteinisation) or to the solubilisation of polysaccharides perhaps by hydrophobic bonding to it. To test whether the latter hypothesis is reasonable, work was done on some gels as model systems. One of these gels (kappa-carrageenan) has been examined by X-ray diffraction (Anderson 1966) and appears to possess a hydrophobic face consisting of axial -H units. Other methods were developed using gels in the hope that they could then be applied to pectins to show the nature of the binding in gel formation.

The current theory of gel formation by polysaccharides of the carrageenan type is that the chains couple with the formation of helices :-



The helices may gain stability by electrostatic or possibly also hydrophobic interaction. If this mechanism is correct, a change in optical rotation should occur when the gel sets because dissymmetry of the sugar units will favour either a left or right handed helix (unless the axis is twofold) and hence there will be a change in the dissymmetry of the system

as a whole. This prediction has now been confirmed experimentally for agar, agarose, kappa- and iota-carrageenan. The change in rotation appears to be genuine as there was no refractive index change as the gel set [optical rotation is proportional to $n^2 + 2$, where n is the refractive index (Steiner 1965)]. Similarly, any anomaly due to light scattering is expected to decrease the optical rotation (by depolarisation) rather than increase it.

The positive rotation of kappa- and iota-carrageenan became more positive and the negative rotation of agarose became more negative when the gels set. It is possible that this is due to formation of helices with opposite senses (see above).

It should be possible to apply this method to pectin gels provided that glycerol is used in place of sucrose to make the pectin set. The sucrose would provide a very high background rotation which might obscure any change.

The experiments on the effects of salts and other hydrophobic bond breaking compounds upon the melting points of gels was prompted by the work of Hamaguchi and Geiduschek (1961) on the denaturation of DNA using salts. They have produced a table of anions with increasing power to denature DNA and have ascribed this power to the weakening of hydrophobic bonds. The effects of these salts on agar and agarose gels paralleled their effect on DNA as shown in the following table, in which results given in the experimental section are presented in a different form.

Sodium salt used	Melting Point of DNA in 4M salt (°C)	Melting Point of 0.5% agarose gel in 0.25M salt (°C)	Melting Point of 0.4% agarose gel in 1M salt (°C)
CCl_3COO^-	25	65	Not set
CNS^-	68	68	Not set
ClO_4^-	74	73	Not set
I^-	76.4	67	Not set
Br^-	89.1	81	70
Cl^-	90	82	73
CH_3COO^-	83.5	81	83

Further similarities are that, like DNA, agarose gels are 'denatured' by urea but less effectively than by trichloroacetate, thiocyanate, perchlorate and iodide. Hydrophobic bonding could indeed be important and this method should be applicable to pectin gels if the setting problem can be overcome (see Experiment 3.17.3). It might be feasible with a fully esterified pectin of high molecular weight because this might be expected to show a sharper melting point. There was little correlation for kappa- and iota-carrageenan, very likely because of complications caused by electrostatic effects; these were not serious for DNA presumably because of the high salt concentration. Because agar and agarose were extremely sensitive to anion concentration, low salt concentrations were used with polysaccharide gels so that they could be compared under the same conditions. However, it should be possible to repeat the experiments with kappa- and iota-carrageenan, using salt concentrations which are high enough to eliminate

electrostatic complication.

If sodium lauryl sulphate can bind hydrophobically to gel molecules, it will compete for the active sites with other chains so preventing cross linking at this point. In practice, the melting points of agar and methyl cellulose gels were not depressed by the addition of sodium lauryl sulphate; until a certain concentration of detergent was reached, the gels would not set. This all or nothing effect is not surprising as it has been shown that the melting point of a gel is not dependent to any great extent on the number of cross linkages by the fact that agar gels of different concentrations have very similar melting points. Large depression of the melting point would only be expected when most of the linkage points had been broken.

In summary, the existence of helices in plant cell walls or synthetic gels as linkage points between pectic molecules, has not been proved or disproved by the work in this chapter. However, two methods new to polysaccharide chemistry have been investigated and appear to show promise. It might be possible to detect helix formation during gelation by optical rotation changes and a study of the use of denaturants might give information on the cohesive forces whether or not helices are present.

It is still not known whether extraction with sodium lauryl sulphate/EDTA is effective because of its action on protein or polysaccharide, but it does seem to offer a new selective, mild method for the complete extraction of pectic substances.

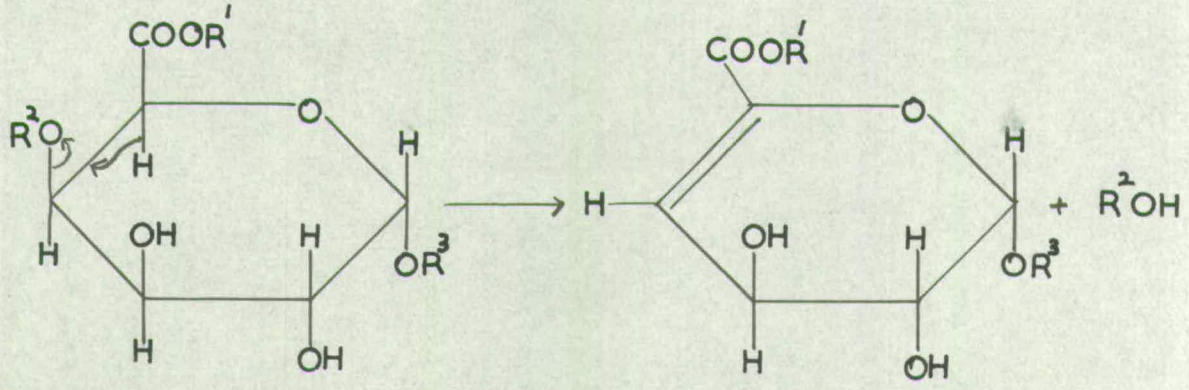
CHAPTER 4

BASE CATALYSED FRAGMENTATION OF PECTINS AS A STRUCTURAL TOOL

Introduction

Esterified uronic acid units in polysaccharides are very susceptible to fragmentation when aqueous solutions are heated at neutral or alkaline pH. This is probably not connected with the 'peeling' reaction (Whistler and BeMiller 1958) which involves removal of successive single sugar units from the reducing end of a polysaccharide chain under alkaline conditions. Almost certainly, the main path is a β-elimination reaction which is distinguished by the production of an unsaturated sugar unit, see fig. 24.

Fig. 24



This unsaturation gives rise to a maximum in the ultraviolet spectrum at 235mμ and provides a ready means of checking if β-elimination has taken place. Prolonged heating of the neutral pectin solutions produces a further peak in the ultraviolet spectrum at 265mμ indicating that unsaturated compounds of increased conjugation are produced. The extent

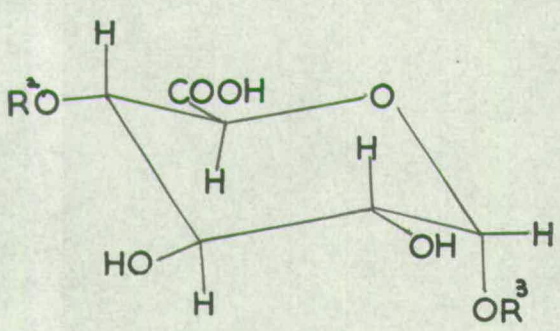
of reaction under the conditions normally used, i.e. heating an aqueous polysaccharide solution buffered at pH 6.8 for about 2 hours, is small with cleavage occurring at only 10% of the available uronic ester units (Albersheim et al 1960); this is partly due to competing de-esterification. The reaction has been successfully applied to the study of the structure of apple and other pectins (Northcote et al 1965, 1967).

Enzyme catalysed eliminations are also known, for example, for hyaluronic acid (Linker et al 1956) chondroitin sulphates (Linker et al 1960) alginic acid (Preiss and Ashwell 1962) and pectic substances (Macmillan and Vaughn 1964). Although the specificity of enzyme reactions is often helpful in structure investigations, it may be inconvenient and an efficient chemical method would be useful. An alternative method is needed and it seemed that if alkoxide was used as a base, trans-esterification rather than de-esterification would take place. Model compounds of low molecular weight have been shown to undergo β-elimination when treated with methanolic sodium methoxide (Heim and Neukom 1962). However, with compounds of higher molecular weight, for example, 2-hydroxyethyl alginate which seems to consist entirely of 1,4 linked uronic acid derivatives (Hirst et al 1964, 1965; Rees and Samuel 1967) there is no visible reaction. The addition of a water-scavenger, methanolic 2,2-dimethoxypropane (5% w/v) to the polysaccharide before the addition of methoxide causes the reaction to occur to a considerable extent (Samuel 1967). This is measured by the absorption at 235mμ and by positive reaction to thiobarbituric acid. Separation of the products of this reaction shows the presence of a series of oligosaccharides with the ultraviolet and infrared bands characteristic of C4 - C5 unsaturated esters. The absorption at 235mμ disappears on

borohydride reduction or on ozonolysis (after which oxalic acid dihydride is isolated). These observations indicate that β-elimination occurs as with the smaller molecules. Paper electrophoresis indicates that partial de-esterification occurs during the reaction.

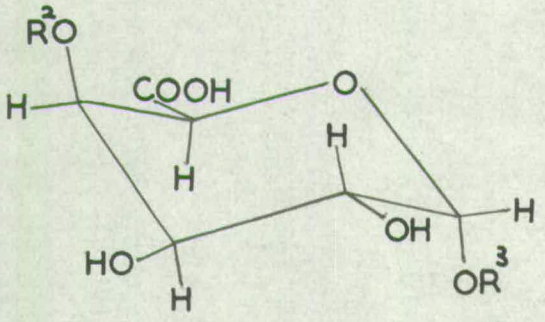
The required stereochemistry for stereospecific elimination is present in a gulopyranosyluronate but not in a mannopyranosyluronate unit. However, comparison of alginates containing the two types of unit in different proportions suggests that the reaction is not stereospecific. This has been confirmed because the methyl ester methyl ether of Leiocarpan A (Aspinall and McNab 1965) in which all the uronic acid units have the 'wrong' (c.f. D-glucuronic acid) configuration (see fig. 25) undergoes rapid reaction.

Fig. 25



α-D-glucuronic acid

'Wrong' relationship between eliminated groups



α-D-galacturonic acid

Correct relationship between eliminated groups

The lack of stereospecificity is probably due to some E1 c.b. character in the mechanism. The ionisation of the proton at C5 is probably easy and will occur more readily than removal of the glycosyl substituent so that formation of the double bond at C4 - C5 precedes the ejection of the

substituent at C4.

If the elimination reaction is prolonged, the peak at 235mμ progressively disappears and new absorptions at longer wavelengths take its place; this shows that unsaturated units analagous to those in fig. 24 are not the end products. Further elimination ought to cause the release of these units which would ketonise, but no trace of α-keto acids have been found using paper chromatography or colour reactions.

The average chain length of the product from this reaction is about four units. This value is obtained from the extinction coefficient at 235mμ. The conclusions from these experiments are that :-

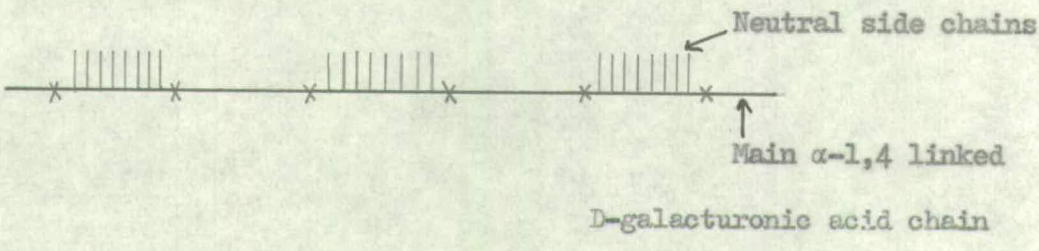
1. Extensive breakdown occurs on heterogeneous treatment with methanolic sodium methoxide, provided that a chemical method is used for the removal of bound water from the polysaccharide.
2. The rate of reaction is not usefully sensitive to the stereochemistry of the glycopyranosyl unit.
3. The unsaturated product which is first formed does not accumulate but undergoes further reaction.
4. Fragmentation of the chain is incomplete, perhaps partly because of accessibility effects similar to those which limit heterogeneous hydrolysis (Haug et al 1966).

The nature of the products from this reaction have not led to any new structural information about alginic acid. It has however, been of considerable use in the isolation of a new sugar (B) (McCleary 1967) which may be a branched chain uronic acid from the slime polysaccharide of Escherichia coli K 12. Treatment of the 2-hydroxyethyl ester with 2,2-dimethoxypropane/methanol/methoxide followed by chromatographic

purification gives a fair yield of B. About 50% of the B units can be isolated as monomer if ethyl acetate is included in the reaction mixture, presumably because water, which is produced in side reactions and would normally cause de-esterification, is removed by the formation of the acetate ion from ethyl acetate which is present in large excess. Methylation analysis of the polymeric residue confirms that there is little change in polysaccharide structure apart from the removal of B and destruction of glucuronic acid units. This reaction provides the only method at present available for the isolation of unsubstituted B for investigation of its structure. Together with the fact (shown also by Roden and Markowitz 1966) that most of the uronic acid may be recovered as 3-O-D-glucopyranosyluronic acid-D-galactose, it establishes the sequence at most chain ends as B-glucuronic acid-galactose.

The potential value of this method of polysaccharide fragmentation is well illustrated by Barrett and Northcote's (1965) work on apple pectin. The products of breakdown in sodium phosphate buffer were separated into two distinct components, one of which is acidic and has a low molecular weight, while the other has a much lower uronic acid content and a high molecular weight. Assuming that random cleavage occurs, these results are interpreted in terms of a block type distribution of side chains (see fig. 26)

Fig. 26



A random distribution of side chains would not account for these results unless there were only a few very large ones.

As there are few methods available for assessing the distribution of branch points, this procedure promises to find many applications. If degradation were complete, the side chains could be isolated individually to provide further evidence for polysaccharide structure. Most of the side chains which occur in pectic substances are more acid labile than the uronic acid containing backbone and it is therefore difficult to establish their size and detailed structure by other methods. Mustard pectin, for example, contains arabinofuranose-rich side chains which are readily removed by autohydrolysis or treatment with dilute acid.

Experimental

4.1 Citrus pectin

The citrus pectin (supplied by Dr. J.W.T. Craig) used in the following experiments was isolated from freshly cut citrus peel by cold water extraction followed by precipitation with ethanol. Examination of this pectin using free boundary electrophoresis showed that it was homogeneous ($\mu = 10.8 \times 10^{-5} cm^2/s/v$) in sodium tetraborate buffer. The high mobility indicates a low degree of esterification.

4.1.1 β-elimination of citrus pectin in sodium phosphate buffer (0.2M, pH 6.8)

Two samples of citrus pectin (300mg each) were dissolved in hot water (60ml) and a hot aqueous solution (60ml) of sodium phosphate (0.2M, pH 6.8) was added; one sample was heated on a boiling water bath for 2 hours and the other for 20 hours. At the end of these times, the samples were cooled, dialysed against distilled water (five changes) for six days and freeze dried. The ultraviolet spectra of the undialysed samples were also recorded.

Sample	Yield	Ultraviolet Maxima (mμ)	
2 hours	250mg	235 (w)	275 (w)
20 hours	120mg	235 (m)	270 (m)

In this and the tables that follow, the strength of absorption is indicated by :-

vw = very weak; w = weak; m = medium; s = strong; vs = very strong

Free boundary electrophoresis

Sample	Buffer	No. of Peaks	Mobility (x 10^{-5} $cm^2/s/v$)
2 hours	Sodium tetraborate	2	10.5, 9.9
20 hours	" "	1	13.5

4.1.2 Heterogeneous β-elimination of citrus pectin in methanolic sodium methoxide

A methanolic solution of sodium methoxide was prepared by dissolving sodium (500mg) in methanol (100ml) previously dried by shaking with 2,2-dimethoxypropane (5ml) for 24 hours. All methanolic sodium methoxide mentioned in this Chapter was used at this concentration unless otherwise stated.

Two samples of citrus pectin (300mg each) were shaken with methanol (60ml) containing 2,2-dimethoxypropane (4.5ml) for 1 hour. Methanolic sodium methoxide (60ml) was then added to each and one sample was shaken for ½ hour and the other for 20 hours. At the end of these periods, the reactions were stopped by the addition of excess solid carbon dioxide; water was added and the solutions concentrated to remove methanol. The ultraviolet spectra of the samples were examined and the samples dialysed against distilled water for six days.

Sample	Yield	Ultraviolet maxima (mμ)			
½ hour	110mg	230 (s)	280 (s)		
20 hours	70mg	230 (m)	280 (vs)	335 (w)	355 (w)

Free boundary electrophoresis

Sample	Buffer	No. of Peaks	Mobility ($\times 10^{-5} cm^2/s/v$)
½ hour	Sodium tetraborate	4	12.2, 10.4, 7.5, 4.1
20 hours	" "	4	13.0, 10.9, 7.0, 4.7

4.1.3 Examination of the molecular state of citrus pectin degraded by sodium phosphate using Sephadex (G-75) gel filtration

A Sephadex (G-75) column ($4.9 cm^2$ x 85cm) was prepared by the addition of a continuously stirred slurry of the previously swollen (in 0.1M sodium chloride) gel.

A large sample of citrus pectin (750mg) was degraded using a method identical with that in 4.1.1 and the product dialysed for six days against a large volume of distilled water. The freeze dried material (320mg) was dissolved in sodium chloride (0.1M, 10ml) and applied to the Sephadex column as a layer on top of the gel, care being taken not to disturb the surface of the gel. The material was eluted with sodium chloride (0.1M) solution and fractions (2ml) were collected; an aliquot (1ml) of every second fraction was removed for analysis by the phenol/sulphuric acid method (see fig. 27).

Samples A, B and C were obtained by bulking tubes 0-55, 56-135 and 136-200 respectively. These samples were dialysed against distilled water, freeze dried and their ultraviolet spectra examined. On hydrolysis and paper chromatogrpahy, all three samples were shown to contain the same sugars; galacturonic acid, galactose and arabinose, there being less galacturonic acid in sample A than in B or C.

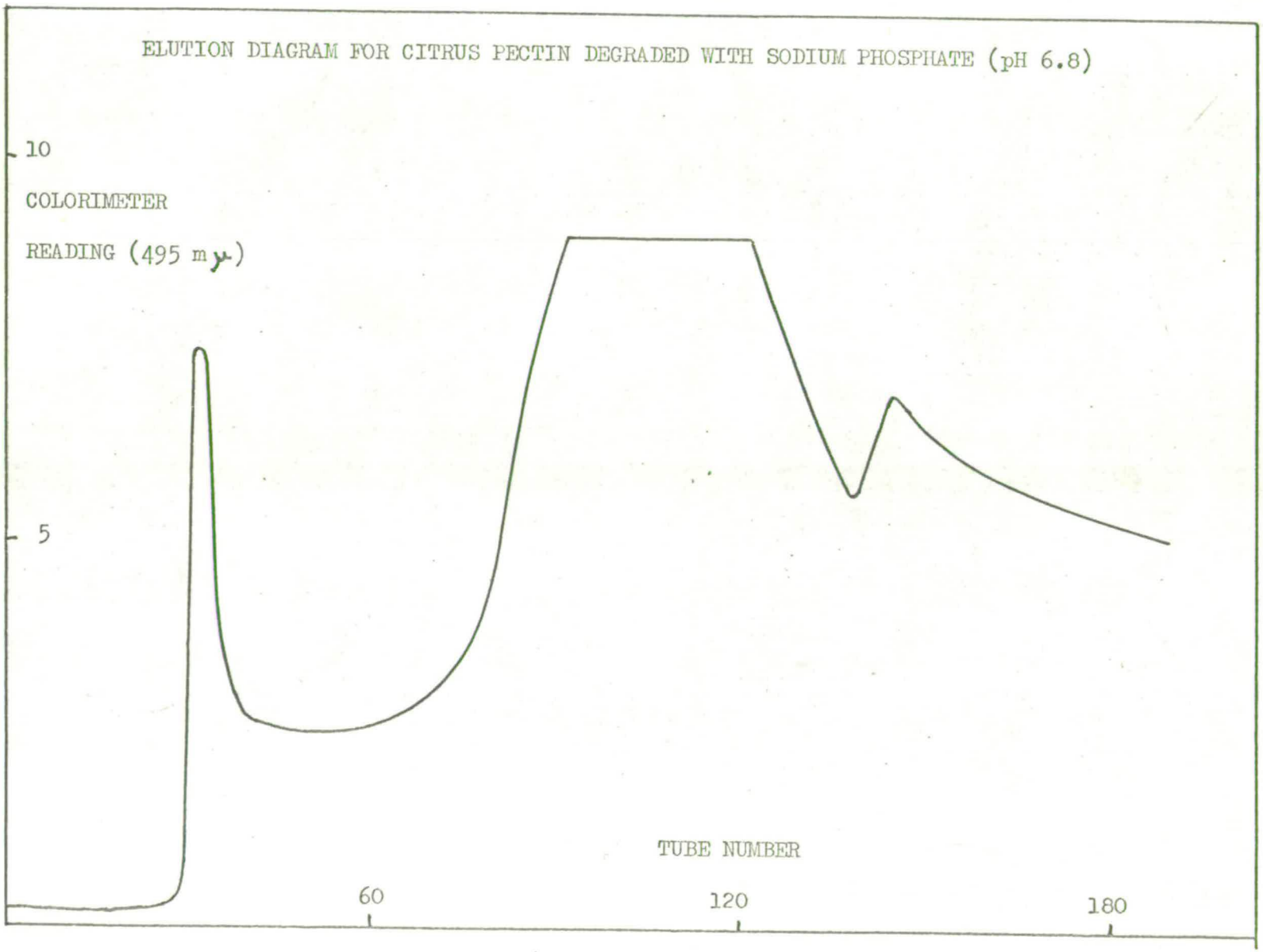

ELUTION DIAGRAM FOR CITRUS PECTIN DEGRADED WITH SODIUM PHOSPHATE (pH 6.8)
10
COLORIMETER
READING (495 mμ)
5
TUBE NUMBER
60
120
180

Fig. 27

Sample	Yield	Ultraviolet Maxima (mμ)
A	30mg	230 (w)
B	130mg	230 (m)
C	20mg	230 (w) 275 (w)

4.1.4 Preparation of the 2-hydroxyethyl ester of citrus pectin (Deuel 1947)

An aqueous solution of citrus pectin (1%, 200ml) was passed through a column of IR 120 (H^+) resin and the resulting free-acid form stirred for five days with ethylene oxide (25ml). More ethylene oxide (25ml) was added and the solution stirred for a further seven days. The neutral solution was dialysed against distilled water (five days) and freeze dried. The mobility of esterified citrus pectin in sodium tetraborate solution was $7.2 \times 10^{-5} cm^2/s/v$ indicating that by no means all the available carboxyl groups were esterified. (The mobility was at least twice that of most polysaccharides known to be neutral).

4.1.5 Fragmentation of esterified citrus pectin in sodium phosphate buffer

A hot aqueous solution of esterified citrus pectin (0.5%, 300ml) was added to an equal volume of a hot sodium phosphate buffer solution (0.2M, pH 6.8) and the resulting mixture heated on a boiling water bath for five hours. Samples (100ml) were withdrawn ½, 1, 2, 3, 4 and 5 hours after the start and immediately cooled. All the samples showed strong absorption only at 235mμ. They were dialysed against distilled water (five days) and freeze dried (Yields :- 100, 93, 91, 90, 87, 80mg respectively).

Free boundary electrophoresis

Sample	Buffer	No. of Peaks	Mobility (x 10^{-5} $cm^2/s/v$)
½ hour	Sodium tetraborate	2	10.9, 2.6
1 hour	" "	3	11.4, 6.1, 2.6
2 hours	" "	3	12.2, 6.2, 2.8
5 hours	" "	3	13.4, 6.6, 3.5

4.2 Tragacanthic acid

Gum Tragacanth consists of a soluble and an insoluble fraction. The soluble fraction contains the tragacanthic acid which is extracted for examination as it possesses many of the typical features of plant pectins, e.g. a main chain consisting of α-1,4 linked D-galacturonic acid units interrupted by L-rhamnose, side chains containing D-xylose and other sugars (Aspinall and Baillie 1963).

4.2.1 Extraction of tragacanthic acid from Gum Tragacanth (Baillie 1961)

A sample of commercial Gum Tragacanth (20g) was stirred with water (2 litres) for 12 hours, stood at 0°C for a further 12 hours and finally stirred again (12 hours). The resultant viscous mixture was passed through a 'Sharples Super Centrifuge' (eight times) and the supernatant eluted through a column of mixed resins (IR 120 (H^+) and IR 4B (OH^-)). The solution was concentrated (1 litre) and then stirred for 12 days in the presence of ethylene oxide (125ml). As the solution was neutral after this time it was dialysed against running tap water (seven days) and freeze dried (7g).

Examination of the esterified tragacanthic acid ($\mu = 5.2 \times 10^{-5}$ $cm^2/s/v$)

by free boundary electrophoresis in sodium tetraborate buffer showed the presence of a small amount of contaminating neutral material ($\mu = 3.5 \times 10^{-5} cm^2/s/v$). The addition of acetone (600ml) to an aqueous solution of this mixture, precipitated the esterified tragacanthic acid (5.5g). The neutral material (0.2g) was precipitated after the addition of more acetone (1200ml).

4.2.2 β-elimination of esterified tragacanthic acid in sodium phosphate buffer

A sample of esterified tragacanthic acid (1g) was dissolved in water (200ml) by heating to 100°C. Sodium phosphate buffer (0.2M, pH 6.8; 200ml) was added and the solution heated for 20 hours on a boiling water bath. Aliquots (60ml) were withdrawn after ½, 1 1/4, 2, 3, 4 and 20 hours and were immediately cooled. They were dialysed against a large volume of distilled water for 6 days and freeze dried.

Sample	Yield	Ultraviolet Maxima (mμ)	
1 1/4 hours	140mg	235 (vw)	275 (vw)
20 hours	120mg	235 (m)	275 (m)

Free boundary electrophoresis

Sample	Buffer	No. of Peaks	Mobility (x $10^{-5} cm^2/s/v$)
1 1/4 hours	Sodium tetraborate	2	5.9, 4.0
20 hours	" "	2	8.0, 5.0

4.2.3 Heterogeneous β-elimination of esterified tragacanthic acid in methanolic sodium methoxide

The method employed was similar to that described for citrus pectin in 4.1.2, the reactions being stopped after 1 hour and 20 hours.

Sample	Yield	Ultraviolet Maxima (mμ)
1 hour	235mg	-
20 hours	230mg	280 (m)

Free boundary electrophoresis

Sample	Buffer	No. of Peaks	Mobility (x 10^{-5} $cm^2/s/v$)
1 hour	Sodium tetraborate	1	5.9
20 hours	" "	2	6.1, 4.1

4.3 Mustard pectin

The mustard pectins used in the following experiments were those extracted from non-germinated and germinated seeds by Dr. N.G. Richardson and Dr. S.E.B. Gould respectively, using the method described in 1.2.1. As was shown by the results in Chapter 1, they contain a mixture of at least two acidic and one neutral polysaccharide.

4.3.1 β-elimination of N in sodium phosphate buffer

β-elimination was attempted on an aqueous solution of N (0.5%, 100ml) by heating it with sodium phosphate buffer (0.2M, pH 6.8) at 100°C for 20 hours. The ultraviolet spectrum of the resulting solution showed no absorption at 235 or 275mμ.

4.3.2 Heterogeneous β-elimination of N in methanolic sodium methoxide

A sample of N (0.5g) was shaken with methanol (100ml) containing 2,2-dimethoxypropane (7.5ml) for 20 hours. Methanolic sodium methoxide (100ml) was added and the mixture shaken for a further 20 hours. The reaction was stopped by the addition of excess solid carbon dioxide, water was added and the methanol removed by evaporation at reduced pressure. The sample was treated with IR 120 (H^+) resin to remove sodium ions and the ultraviolet spectrum recorded (very weak peaks at 235mμ and 278mμ).

4.3.3 Preparation of the 2-hydroxyethyl ester of N and G (i.e. NE and GE) using ethylene oxide

Samples of N (4g) and G (1g) were stirred with water (200ml and 50ml respectively) for four hours and EDTA (4%) added (200ml and 50ml respectively). Both solutions immediately became clear and were passed through a mixed resin column (IR 120(H^+) and IR 45 (OH^-)) and were concentrated to their original volumes. Ethylene oxide (48ml and 12ml respectively) was added to the two solutions (pH 3.0) with shaking and they were left to stand at room temperature for six weeks with occasional stirring and further additions of ethylene oxide. Both solutions (pH 3.8) were concentrated to half their original volume and further ethylene oxide (24ml and 6ml respectively) added. After 24 hours the pH of both solutions had risen to about 5.0 and they were then dialysed for four days against distilled tap water (yield :- NE 3.33g, GE 0.50g). Ultracentrifugation of solutions (1%) of the two samples at 185,000 g produced only one slow moving peak in each, the fast sedimenting components of N and G being absent (see Plates 34 and 35).

Ultracentrifugal examination of N during esterification

An aqueous solution of N (1%, 2ml) was passed through IR 120 (H^+) and IR 45 (OH^-) resins to remove ions. The solution was concentrated to its original volume and divided into three equal parts. One portion (P) was sterilised by the addition of phenol (0.07g). Ethylene oxide (0.9ml) was added to another portion (E) and the third sample was left unadulterated. The samples were left to stand in sealed tubes, aliquots (1ml) being withdrawn after 1, 2, 4, 7, 14, 28 and 44 days. As both phenol and ethylene oxide (Meynell and Meynell 1965) are excellent bacteriocidal agents, the possibility of bacterial degradation in those samples was discounted; however, the unsterilised sample rapidly became infected and was discarded.

Before examination of samples P and E by ultracentrifugation (185,000 g) they were neutralised by the addition of saturated sodium bicarbonate solution (2 drops). Initially, both P and E gave very similar sedimentation diagrams (see Plate 36) but after 44 days both peaks present in E were very much slower than the peaks in P. Both of these solutions were then dialysed against running tap water for two days whereupon E produced one slow-sedimenting peak which corresponded to the slow moving component in P. There was no trace of the fast moving peak in E (Plate 37).

4.3.4 Small scale β-elimination studies on NE using methanolic sodium methoxide

All the β-elimination experiments in this section were carried out on NE (50mg) using the procedure described in 4.3.2 with correspondingly scaled down quantities of reagents. The various modifications of this

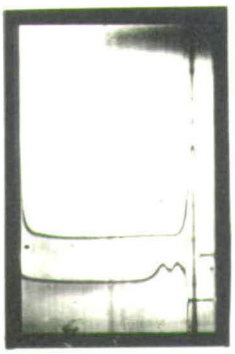


PLATE 34

Top : NE

Bottom : N

Time : 18 min.

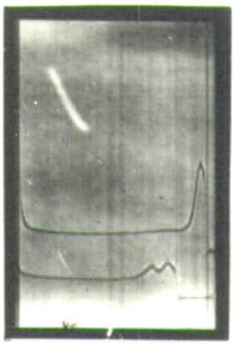


PLATE 35

Top : GE

Bottom : N

Time : 18 min.

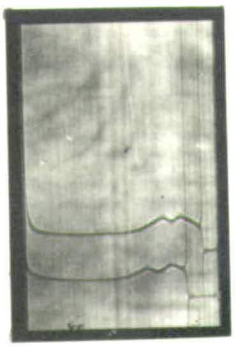


PLATE 36

Top : P

Bottom : E

Time : 50 min.

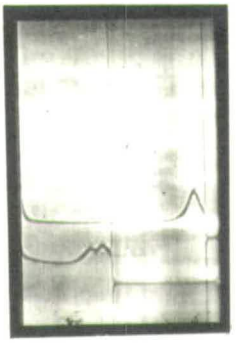


PLATE 37

Top : E

Bottom : P

Time : 22 min.

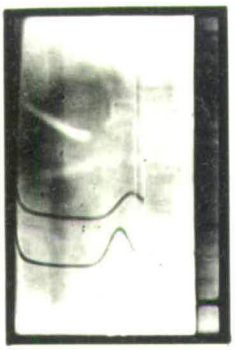


PLATE 38

Top : NE 9

Bottom : Araban (1)

Time : 49 min.

procedure which were attempted are as follows :-

1. Standard methanolic sodium methoxide

This was carried out using the procedure described in 4.3.2 and the results were used for comparison purposes. The product (yield 33mg) from this experiment had a small absorption maximum at 230mμ; comparison of the extinction coefficient of this peak with values obtained by Macmillan and Vaughn (1961) shows that the average degree of polymerisation of the material is about 90.

2. Methanolic sodium methoxide and ethyl acetate

In this experiment, NE was suspended in methanol/ethyl acetate (4:1 v/v) containing 2,2-dimethoxypropane (1.5ml). The methanolic sodium methoxide was added in the usual manner. The freeze dried product (36mg) had a similar degree of polymerisation to that in 1.

An experiment carried out using the same reagents at 40°C gave similar results.

3. Methanolic sodium methoxide (high concentration) and ethyl acetate

The same solvent system was used as for the previous experiment, but the methanolic sodium methoxide used for the β-elimination had a much higher concentration (sodium (0.50g) dissolved in methanol (20ml) containing 2,2-dimethoxypropane (1.5ml)) than was normally used. The freeze dried product (41mg) again showed (from the ultraviolet spectrum) that little β-elimination had taken place.

4. Prolonged β-elimination under very anhydrous conditions

A sample of NE (50mg) was treated with methanol (40ml) containing 2,2-dimethoxypropane (5ml) for 24 hours. Methanolic sodium methoxide

(10ml; high concentration, as prepared in 3.) was added and the mixture shaken for 48 hours. The sample was worked up in the usual manner (yield 28mg). The extinction coefficient of the peak at 225mμ indicated a degree of polymerisation of about 40.

A few drops of an aqueous solution of NE were placed on a transparent disc of silver chloride and the water evaporated under vacuum. The infrared spectrum of this sample was recorded after equilibration in a continuous stream of nitrogen saturated with deuterium oxide to shift the water peak at 1720cm^{-1} which would otherwise have obscured the carboxylate absorption. This showed that the sample was still highly esterified.

4.3.5 Homogeneous β-elimination of NE and GE using methanolic sodium methoxide

Samples (200mg) of NE and GE were dispersed in dimethylsulphoxide (10ml) by shaking for 24 hours, 2,2-dimethoxypropane (1ml) was added and the solutions shaken for a further 24 hours. Methanolic sodium methoxide (5ml) was added and the samples were shaken overnight. The solutions were neutralised by the addition of excess solid carbon dioxide and water (50ml) added. They were then passed through IR 120 (H^+) resin and concentrated to their original volume (10ml). After addition of more dimethylsulphoxide (10ml) fractional precipitation was carried out by the addition of small aliquots of methyl ethyl ketone; after excess (120ml) of this had been added and no further material could be precipitated, the solutions were evaporated to dampness under high vacuum to give fraction (11).

Fraction	Volume (ml) of methyl ethyl ketone to precipitate	Weight (mg) NE	GE
1	14	1.0	trace
2	20	-	1.0
3	24	-	2.0
4	28	-	-
5	32	20.0	2.0
6	36	1.0	2.0
7	40	0.5	2.0
8	48	3.0	3.5
9	80	90.0	101.0
10	120	13.0	-
11	-	about 10	
		138	123

Ultracentrifugation (330,000 g) of fraction NE 9 produces one peak which sediments at a rate slightly slower than that of mustard araban showing that it may have a low molecular weight (Plate 38).

Some of the larger fractions were examined by hydrolysis (formic acid, 45% v/v) and paper chromatography (Table 9) with solvent A, the spots being developed by spraying with p-anisidine hydrochloride dissolved in water-saturated butan-1-ol.

Table 9

Fraction		Galacturonic Acid	Galactose	Glucose	Arabinose	Xylose	Rhamnose
NE	5	-	-	-	-	++	-
	7	trace	++	-	trace	++	trace
	8	++	++	-	++	+++	+
	9	++	+	-	+++	+	+
	10	++	trace	-	+++	trace	trace
	11	trace	+	-	++	trace	-
GE	5	-	++	-	+	++	+
	7	trace	++	-	+	++	trace
	8	++	++	-	++	++	trace
	9	++	+	-	+++	trace	trace
	11	trace	+	-	++	trace	-

The infrared spectra of deuterated films of fractions NE 9 and GE 9 showed that there was little de-esterification. As a precautionary measure, the material was re-esterified before repeating the β-elimination procedure. Fractions NE 9 and GE 9 were methyl esterified by shaking them with a solution of diazomethane in ether (+ 10% methanol) for 2 hours. Diazomethane was prepared by the gradual addition of a slurry of p-tolylsulphonylmethylnitrosamide (100g) in ether (500ml) to a heated mixture of potassium hydroxide (30g), water (50ml), 2-ethoxyethanol (175ml) and ether (50ml); the diazomethane being obtained as a solution in ether by distillation.

The methanol, ether and excess diazomethane were removed by evaporation

and both samples were subjected to a further β-elimination reaction using the same conditions as described above. Fractional precipitation was carried out on the product dissolved in dimethylsulphoxide (5ml).

Volume (ml) of methyl ethyl ketone to precipitate	Weight (mg) NE	GE
5	1.0	4.5
15	36.0	35.0
25	-	-

The entire esterification and β-elimination procedure was repeated again and gave the major fraction (NE 9.0mg; GE 15mg) on the addition of three volumes of methyl ethyl ketone. The above series of experiments was repeated to check the results and comparable yields were obtained at all stages. Test experiments in which NE and GE were precipitated from dimethylsulphoxide solution by methyl ethyl ketone (under 1 volume) have confirmed that the degraded material has a much lower molecular weight than either NE or GE. The pectins required a similar volume of methyl ethyl ketone to precipitate them from solutions in dimethylsulphoxide.

4.3.6 Effect of the β-elimination conditions on mustard araban

A sample (100mg) of araban from non-germinated seeds was subjected to the procedure described in 4.3.5. The product was precipitated from dimethylsulphoxide solution (10ml) using methyl ethyl ketone. These results were checked by precipitating undegraded araban from dimethylsulphoxide solution (10ml) using methyl ethyl ketone.

Volume (ml) of methyl ethyl ketone to precipitate	Weight of product precipitated	Weight (mg) of undegraded araban precipitated
10	-	-
15	25	14
20	50	35
25	14	33
30	5	11
45	-	-

The similarity between the two fractionation patterns indicates that no degradation of the araban has occurred. Most of the araban in either sample was precipitated by the addition of 2.5 volumes of methyl ethyl ketone, whereas the degraded NE and GE samples required between 2.5 and 4.0 volumes to precipitate them. This suggests that the degraded material has a lower molecular weight than the araban and this is confirmed by ultracentrifugation (see Plate 38).

Discussion

Fragmentation in neutral buffer using the methods of Albersheim et al (1960) and Barrett and Northcote (1965) has been applied to three representative pectic polysaccharides. This procedure has been compared with a new method involving heterogeneous β-elimination with sodium methoxide.

The degradation of citrus pectin using sodium phosphate produced absorption at 235mμ and 270mμ representing increasingly conjugated degradation products (Albersheim et al 1960). Prolonged treatment with sodium methoxide gave further conjugated products and a much reduced yield of non-dialysable material. There was a 77% reduction in weight using this method while the corresponding loss using sodium phosphate was 60%. It would appear that sodium methoxide produces more extensive degradation.

The electrophoretic mobilities of the material from both procedures increased to a limiting value ($13.5 \times 10^{-5} cm^2/s/v$) after 20 hours' reaction, confirming that the degree of esterification drops during fragmentation and it is probable that this causes the reaction to slow down. Gel filtration produced an elution pattern similar to that of Barrett and Northcote, with two main fractions, a minor and relatively neutral component and an acid-rich fraction. The production of material poor in uronic acid is confirmed by the free boundary electrophoresis results. It seems that citrus pectin, like apple pectin (Barrett and Northcote 1965) might contain the neutral side chains grouped in blocks and complementary regions of main chain which are free from branch units.

Esterification of citrus pectin (80% of the uronic acid groups already existing as the methyl ester) using ethylene oxide, was carried out in an attempt to increase the amount of fragmentation before

de-esterification causes the reaction to stop. This did not appear to be successful as even after short treatment with sodium phosphate, the material had regained its original mobility thereafter increasing more slowly to the limiting value. The 2-hydroxyethyl group would appear to be more easily removed during the reaction than the methyl group which exists in the natural pectin.

The success of either method was not nearly so marked when applied to acidic polysaccharides which are highly branched. In the case of tragacanthic acid (40% uronic acid) which does not possess the blocks of L-arabinose units present in citrus pectin, these being replaced by numerous short side chains which contain D-xylose and sometimes L-fucose units, neither method achieves extensive breakdown as judged by the yield of non-dialysable product. The peak (very minor) with low mobility ($3.5 \times 10^{-5} cm^2/s/v$) observed during free boundary electrophoresis of the esterified tragacanthic acid, is a contaminating neutral component not completely removed by fractional precipitation. However, during prolonged treatment with sodium methoxide or sodium phosphate, it increases in size and mobility, suggesting that there might be a degradation component formed in a similar manner to the low mobility components in citrus pectin.

Mustard pectin is similar to tragacanthic acid in that it has a low uronic acid content (25%) and a high degree of branching. The postulated structure (Gould 1965) is :-

galpA 1,4 galpA 1,4 galpA 1,4 galpA 1,2 rhap

```
3        3                 3        3
↑        ↑                 ↑        ↑
1        1                 1        1
Z        Z                 Z        Z
```

where Z = xylopyranose units or large blocks of arabinose units with structure similar to that of mustard araban.

The aim of the experiments with this polysaccharide was to break the main galacturonosyl chain into as many pieces as possible and so characterise the side chains. Cleavage of every galpA 1→4 galpA linkage would give the 'araban' blocks as the largest fragment to survive this process. It may be calculated from the methylation results of Dr. S.E.B. Gould (1965) that the average size of the side chains is about ten units or about a quarter of the size of the neutral araban; each araban side chain would still be attached to one unsaturated galacturonic acid residue.

None of the previous methods of β-elimination produced any significant degradation as judged by the low ultraviolet absorption maxima at 235mμ and 270mμ.

A successful fragmentation procedure was found in which the esterified pectin was dispersed in dry dimethylsulphoxide before treatment with sodium methoxide. It was confirmed that the procedure did not cause degradation of the side chains themselves, by subjecting the araban (which contains no D-galacturonic acid units) to this procedure. The starting material was recovered from the dimethylsulphoxide solution by the addition of the same volume of methyl ethyl ketone as was required by the untreated araban.

The major fraction after fragmentation of both mustard pectins had a slightly smaller molecular weight than mustard araban. On repeating

treatment with base however, further degradation occurred whereas the araban itself was stable. This suggests that the fraction consisted of a series of short arabinose-containing side chains attached to a fragment of galacturonan main chain, and therefore that the side chains themselves are considerably smaller than the araban. This qualitative confirmation of the estimate from methylation results is welcome because the latter is subject to considerable error from undermethylation and other sources. Any simple biological relationship between the neutral araban and the araban side chains, such as was considered earlier (Hirst, Rees and Richardson 1965) would therefore now seem to be unlikely.

There was no noticeable difference between the pattern of products from N and G; similar yields of precipitate were obtained at each concentration of methyl ethyl ketone and when retreated with sodium methoxide, each major fraction again degraded to a similar extent. This suggests that there can be little difference in the distribution or degree of branching between the two pectins.

Treatment of the free-acid form of mustard pectins with ethylene oxide to produce the 2-hydroxyethyl ester caused the disappearance of the fast sedimenting material (ultracentrifuge). There are two reasonable explanations for this effect :-

1. There may be degradation of the larger, fast sedimenting material by a fragmentation reaction similar to β-elimination with ethylene oxide or ethylene glycol acting as a nucleophile. This is difficult to envisage at low pH although a precedent does exist in Heim and Neukom's (1962) report that diazomethane will cause this type of degradation.
2. A second and less likely explanation is that the fast sedimenting

material may be an aggregate which dissociates upon esterification. The reluctance of mustard pectins to esterify could then be explained as the aggregates would not be readily accessible to the ethylene oxide. However, the fast sedimenting material is still present when a solution of N in EDTA (2%) is examined in the ultracentrifuge; there being sufficient EDTA present to swamp any ionic interactions that may occur and to remove divalent cations that might cause aggregation of the type noted earlier (Greenwood and Matheson 1956).

GENERAL METHODS OF INVESTIGATION

Ultracentrifugation (Schachman 1959)

A Spinco Model E analytical ultracentrifuge was used for this work. The solution was contained in a sector shaped cell clamped between two quartz discs and held in a cylindrical housing. The optical path length through the cell was either 12mm or 30mm. The force-field chosen for any particular run was the maximum available under the given conditions. The solvent systems used were sodium phosphate (0.033M, pH 6.0) sodium chloride (0.15M) and sodium chloride (0.15M) containing EDTA (2%, pH 7.5).

Sedimentation coefficients, S, were calculated using the equation :-

$$S = \frac{2.303}{\omega^2} \frac{d \log}{dt} (7.3 - \frac{x}{M})$$

where x is the distance (cm, measured from photographs using a Pye microcomparator) travelled by the schlieren peak in a given time t, ω is the velocity of angular rotation and M is a correction factor due to the magnification of the optical system.

Free Boundary Electrophoresis (reviewed by Alberty 1948)

A Spinco Model H Electrophoresis-Diffusion instrument was used here to examine polysaccharide samples for heterogeneity. The 11ml cell was used for all the quantitative work while the 80ml cell was used preparatively.

The apparatus is designed so that the field strength, E, is greatest in the cell; the cross sectional area, q, being least there. The field strength can be calculated from the equation :-

$$E = \frac{i}{qK}$$

where i is the current (amps.) and K is the specific conductivity of the buffer.

The value for the field strength for a given buffer has to be chosen (by varying the current) so that there is not enough heat generated in the cell to cause convection and hence destruction of the boundaries. Great care has to be taken that the temperature of the cell and buffer vessels are at the same temperature ($4^{o}C$) as the water bath before electrophoresis is commenced.

The two buffers used throughout this work are sodium tetraborate (0.5M, pH 9.2) with E = 6.5 v/cm and a maximum current of 16ma., and sodium phosphate (0.033M, pH 6.0) with E = 8.5 v/cm (maximum current 10ma.) the latter being prepared by the addition of 1/30M sodium dihydrogen phosphate (9 litres) and 1/30M disodium hydrogen phosphate (1.125 litres).

The electrophoretic mobility, μ, of a peak produced during electrophoresis is calculated from :-

$$\mu = \frac{\Delta x/t}{E}$$

where Δx is the distance (cm) travelled by the peak in time t (secs.).

The peak which is present in all the runs and marks the position of the boundary before electrophoresis is begun, is caused by a buffer concentration change at this point. This peak unfortunately obscures the presence of neutral material (immobile) in non-complexing buffer systems. Some plates in the text will contain two almost identical (mirror image) patterns and this is due to the fact that it is possible to photograph both arms of the U-tube simultaneously. Normally only the left arm (descending) is examined.

Acid Hydrolysis

Cellular material was suspended in sulphuric acid (72% w/v) and shaken until it dissolved. After dilution to 2.0N with respect to acid, the solution was heated at 100°C for 4 hours, neutralised with calcium carbonate, filtered and concentrated to a syrup for examination by paper chromatography.

Samples were also hydrolysed with formic acid (45% v/v). The solutions were heated in sealed tubes at 100°C for 16 hours and could then be used directly for chromatography as the formic acid evaporates.

Carbohydrate Content (Dubois et al 1956)

Sugar solution (1ml) was placed in a test tube and aqueous phenol (1ml, 5% w/v) added. Concentrated AnalaR sulphuric acid (5ml) was added from a fast delivery pipette so that the solution boiled. When cool, the optical density of the solution at 495mμ was measured with an EEL Colorimeter or Unicam SP 500 Spectrophotometer and the amount of sugar present obtained by reference to a calibration curve for a standard solution of the appropriate sugar.

Concentration of Polysaccharide Solutions

This was carried out under reduced pressure at 40°C or less using a rotary film evaporator.

Dialysis

Solutions were dialysed in a cellophane tube against distilled or running tap water. Chloroform or mercuric iodide was added to prevent bacterial action.

Electrophoresis

This was carried out in a pyridine/acetic acid buffer (0.2M) at pH 6.2 with a voltage gradient of 14 v/cm, picric acid and glucose being used as markers.

Germination of Mustard Seeds

A single layer of seeds was spread out on a double thickness of Whatman 3MM paper supported by a stainless steel tray. The ends of the paper sheets were allowed to dip into troughs of water in which the water levels were not more than 2cm below the level of the sheets. The whole apparatus was left in a dark room at room temperature for four days (Gould 1965).

Nitrogen Determination

A semi-micro modification of the Kjeldahl method was used (Belcher and Goldbert 1954).

Optical Rotations

These were measured at room temperature using a 1dm polarimeter tube.

Paper Chromatography

Qualitative chromatography was carried out using Whatman No.1 and No.4 papers. The solvent (solvent A) used was ethyl acetate/pyridine/water (10:4:3) and the chromatograms were developed using a p-anisidine hydrochloride spray (prepared by dissolving p-anisidine hydrochloride (3%) in water saturated butan-1-ol containing a trace of stannous chloride) and heating in an oven at 110°C for 5 minutes.

Uronic Acid Content (Stark 1950)

Sugar solution (1ml) was placed in a test tube, cooled in iced water and concentrated AnalaR sulphuric acid (6ml) was added. The solution was heated in a boiling water bath for 20 minutes, cooled and an alcoholic solution (0.1%, 0.2ml) of carbazole was added. The sample was allowed to stand for 2 hours and the optical density at 530mμ measured. The amount of uronic acid units present was obtained by reference to a calibration curve for a standard solution of the appropriate sugar.

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Two papers have been published in conjunction with Dr. D.A. Rees, Dr. N.G. Richardson and Dr. S.E.B. Gould and are enclosed at the end of this thesis. A further paper is due to be published in Carbohydrate Research with Dr. D.A. Rees, Dr. J.W.B. Samuel and Dr. C.W. McCleary.

PECTIC POLYSACCHARIDES IN THE GROWTH OF PLANT CELLS: MOLECULAR STRUCTURAL FACTORS AND THEIR ROLE IN THE GERMINATION OF WHITE MUSTARD

By

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PECTIC POLYSACCHARIDES IN THE GROWTH OF PLANT CELLS: MOLECULAR STRUCTURAL FACTORS AND THEIR ROLE IN THE GERMINATION OF WHITE MUSTARD

By SHEILA E. B. GOULD, DR. D. A. REES, N. G. RICHARDSON and I. W. STEELE

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THE part that the plant cell wall plays in growth has been discussed very thoroughly by plant biologists in recent years[1]. Much of the speculation and experiment has stemmed from the hope that wall extensibility, which must play a major part in cell growth and which may have a controlling influence on it, might be adjusted through one set of molecular interactions. 'Cross-linkages' between pectic components of the wall have often been suggested to have this kind of key role; a discussion of the molecular interactions which could exist between pectic polysaccharides in the cell wall is therefore given here from a chemical point of view together with a preliminary account of a chemical approach to the investigation of these interactions in one system.

The possible nature of the association between pectic molecules in the cell wall is suggested by the gels that these substances form *in vitro*. Such gels contain a network of macromolecules that is cross-linked at various points; the number of cross-linkages need only be small, provided they are strong enough to withstand disruptive forces such as osmosis and coulombic repulsion. The presence of repulsive forces is necessary if the gel is to exist in a swollen state. When the galacturonic acid units are methyl-esterified, gels can be formed in the presence of a high concentration of a low-molecular-weight solute such as sucrose, glycerol or ammonium sulphate[2]. Minor changes in molecular structure can prevent gelation[3,4], suggesting that the cross-linkages are secondary valence forces between parallel polysaccharide chains that contain large numbers of consecutive methyl galacturonate units in close contact. The strength of the association in this type of gel is probably, therefore, affected by the distribution of the methyl galacturonate units available for bonding (that is, not carrying any other substituent). Gel formation can occur whether or not esterification is complete[5], but a proportion of any non-esterified units must occur largely in the undissociated state, or in association with divalent cations (see following). The

gel must be stabilized by the presence of solutes which presumably act, in large part at least, by diminishing the osmotic pressure which would otherwise be disruptive.

A different type of gel formation occurs *in vitro* in the presence of divalent cations such as calcium when the polysaccharide is de-esterified to a sufficient degree. Although charge neutralization might be one factor in the mechanism, it seems likely that other effects such as hydrogen bonding between chains and/or co-ordination of the cation by hydroxyl groups are also involved. Otherwise it would be difficult to understand why partial acetylation should affect gel formation[6,7], why cation binding should be influenced by the stereochemistry of the uronic acid units[8], and why acidic polysaccharides such as polysaccharide sulphates should not form gels with divalent cations. The strength of the association in this type of gel is dependent on the intermolecular, and possible intramolecular, distribution of ionized uronic acid units[9].

Neutral sugars occur in two types of covalent combination in galacturonans. Units of rhamnose, and possibly of other sugars, occur (normally in small amounts) as interruptions in the galacturonan chain; without a more detailed understanding of the stereochemistry of the secondary bonding involved in cross-linking, their effect on gel properties cannot be predicted. The second type is in side-chains which are sometimes very large; it appears that they can often be isolated by degradation of the galacturonan chain with alkali[10]. These side-chains would be expected to prevent the alignment of galacturonan chains, and polysaccharides with numerous such side-chains might lose the capacity to form a stable gel structure in the cell wall[11], or to do so by different mechanisms. A number of plant slimes appear to be examples of this type. These are laid down in cell walls, often associated with cellulose microfibrils[12]. In water they swell to such an extent that the cell is burst; but this effect is not observed if the water contains solute to diminish the osmotic pressure sufficiently. The swelling might be due to an inability to form a compact gel structure, owing to the lack of strong inter-chain binding between the molecules. The slimes seem often to contain a high proportion of neutral sugar units in the main 'galacturonan' chain, numerous short side-chains also being present (for example, cress seed[13] and linseed[14] mucilages). A similar effect of increased branching in preventing alignment and aggregation of polysaccharide chains has been noted with certain arabinoxylans[15]. In the 'normal' pectic polysaccharides that have so far been thoroughly studied[10,16,17], relatively few side-chains occur (although this proportion seems rarely to be negligible), and the continuity of the galacturonan chain is less interrupted by other sugar units.

This brief discussion of the chemistry of pectic substances indicates the way in which these molecules are likely to be associated in the middle lamella and perhaps also in the cell wall proper, though here especially their association might be modified by the presence of other types of molecule. The fact that naturally occurring pectic substances are often highly esterified[18] suggests that association via methyl-esterified galacturonic acid units might sometimes be important. The polysaccharide molecules might be synthesized and laid down such that there is a higher proportion of intermolecular relative to intramolecular cross-linkages than can be achieved in synthetic gels. The structure might not then need to be stabilized to exist, as it must *in vitro*, by the control of solute concentration, *p*H and/or cations present. Even should osmotic stabilization be required, it might be provided by other wall components such as different polysaccharides[19]. Calcium, and perhaps other cations, probably participate in the natural cross-linking of pectic polysaccharides. This is suggested by the maceration and improved extraction of pectic substances that is brought about by chelating agents and agents that precipitate calcium ions[20,21] and by the co-occurrence of calcium and pectic substances in the same region[20,22]. Such 'cation bridges' might add extra stability to a 'methyl ester linked' gel, or function independently. The presence of covalent ester linkages between wall polysaccharides is another possible type of cross-linkage to be borne in mind. There are therefore many types of molecular change which would alter the association between pectic substances *in vivo*, including changes in the molecular distribution of acetyl and methyl ester groups and neutral sugar units. Although the metabolic control of extensibility could conceivably be achieved through only one of these, it is equally likely that the situation is more complicated. The discussion presented here has been simplified by considering only the association between pectic polysaccharides, and no attention has been given to the role of other wall components which might indeed be more important, especially in some species where the concentration of pectic polysaccharides is very low[23].

Against this background, we have undertaken a chemical study of the pectic polysaccharides of white mustard cotyledons, to look for molecular changes which might correlate with biological changes in the cell walls during germination[24,25]. The pectic extract was readily separated into a neutral araban and a mixture consisting chiefly of acidic polysaccharides[24]. Several types of structure are consistent with the results of structural studies on the araban, but it is only relevant here to give one of them (Fig. 1). The other possibilities contain the same structural units in the same proportions, but differ in the way in which the units are arranged. After germination, the araban was present in smaller amount and methylation

analysis coupled with quantitative gas chromatography showed that the proportion of branched units in the 'main chain' (Fig. 1) has diminished from 56 to 40 per cent.

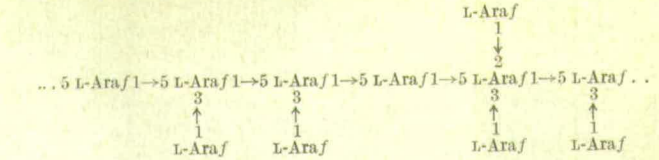
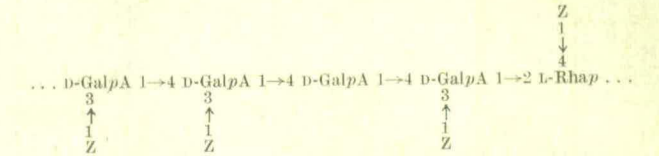


Fig. 1. Possible structure for mustard araban: note that this is only one of the types of structure consistent with present evidence

The acidic polysaccharide fraction is very complex and contains several components which appear to be related in that they give similar mixtures of sugars on hydrolysis. The main structural features indicated by experiments on the mixture are shown in the form of a composite typical molecule (Fig. 2). The average degree of branching is quite remarkable, and might be related to the observation that the cotyledon cell walls appear to swell markedly at the onset of germination (cf. the discussion of plant slimes here). One of the acidic pectic components showed a specific tendency to aggregate in the presence of low concentrations of calcium ions, with the formation of an insoluble gel. This behaviour was most conveniently examined using the ultracentrifuge. The relative concentration of this aggregating component increased very much during the germination period.



where Z is D-Xylp . . . or highly branched side-chains containing only arabinose units, perhaps resembling the neutral araban shown in Fig. 1

Fig. 2. Structural units present in the mixture of acidic polysaccharides from mustard embryos

The general conclusions to be drawn from these experiments are as follows. During the early stages of germination, the pectic polysaccharides in the cotyledons remain little changed in terms of total amount present, sugar composition on hydrolysis—and even in electrophoretic mobility which implies similarity with respect to such properties as charge density and perhaps degree of esterification. Dramatic changes are only revealed by more detailed examination of the chemistry of the wall components. The araban, a highly soluble polysaccharide which by analogy with other systems would be expected to have little capacity for intermolecular bonding, decreases in amount during germination to about one-third of the original level. At the same time, the degree of branching is diminished and therefore the capacity for cross-linking of the fraction of araban that remains is

probably increased. The acidic polysaccharides (very much the major pectic components) change in such a way that they would appear by their aggregation behaviour to have an increased capacity for intermolecular bonding by 'cation bridging' after germination. These various factors would be expected to result in a firmer, less-extensible overall association. A decrease in the deformability of the cell wall would be expected on biological grounds, because the period in which the alteration in visible form of the sall was most rapid was complete at the point at which cotyledons were taken for analysis (after 4 days, germination). In view of this correlation, it seems possible that pectic polysaccharides are involved in the metabolic control of cell enlargement; their role could either be confined to the middle lamella[1], or be more general throughout the whole wall. The detailed chemistry of the process is not yet clear, but the general factors that might apply are outlined in the first part of this article and the evidence suggests that the chemistry is more complicated than has often been imagined in previous discussions. This function of the pectic polysaccharides would not exclude the possibility that enlargement is dependent on other biochemical changes as well.

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gram (Figure 1) with that published by Hullar (1963), suggests that beet araban is more heterogeneous than mustard seed araban, as might be expected on the basis of this discussion.

Our genuine arabans differ in optical rotation from arabans that have been isolated with the aid of harsh methods. Our specific rotations are in the range −173 to −180°, compared with the range −55 to −100° for several samples that have been shown to contain other sugars (Andrews *et al.*, 1959; Hullar, 1963; Sengupta *et al.*, 1965).

When mustard seeds germinate, the araban in the cotyledons diminishes in amount and the degree of branching decreases (Rees and Richardson, 1966). The molecular weight and the molecular weight distribution would appear from the sedimentation diagrams to be little altered during this process. No partly degraded araban was detected after germination. It follows that if the change in branching is brought about by simple addition or subtraction of arabinose units, then all, or nearly all, of the araban molecules in the cotyledons must be affected equally to give a small average shift in molecular weight. The most likely possibility is perhaps that the structural changes result from turnover of some or all of the araban molecules, rather than simple modification. It is also possible that the araban in the resting seeds is heterogeneous with respect to degree of branching and that the most densely branched molecules are selectively and completely metabolized during germination.

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TABLE I: Physical Constants of Mustard Arabans.

Sample[a]	$[\alpha]_D$ (water), Deg	Electrophoretic Mobility[b]	Sedimentation Coefficient[c]
1	−176	2.2	1.32
2	−180	2.3	1.23
3	−176	2.2	1.41
4	−173	1.9	1.24

[a] Samples 1–3 correspond to the sample numbers defined earlier (Rees and Richardson, 1966, Table IV). Sample 4 is the araban whose isolation by extraction at pH 4.9 is described in this paper. [b] $\mu \times 10^5$ cm^2 v^{-1} sec^{-1} using 0.5% solution of araban in 0.05 M sodium tetraborate (pH 9.2) at 6.5 v/cm (current 16 ma). [c] Apparent s_{20} with a 1% solution of araban in 0.15 M NaCl at 334,000g.

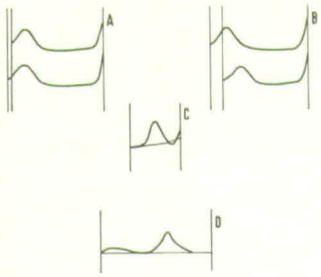

FIGURE 1: Sedimentation and electrophoresis diagrams for mustard arabans. (A) Sample 2 (upper) and sample 4 (lower); photograph after 98 min at 334,000g (1% araban solution in 0.15 M NaCl in 12-mm aluminium cell at 20°); bar angle 65°. (B) Sample 1 (upper) and sample 4 (lower); conditions as in A. (C) Ultracentrifugation of araban sample 4 under similar conditions to A and C but in a synthetic boundary cell (upper solution 0.15 M NaCl); photograph after 15 min; araban samples 1–3 behaved similarly to this. (D) Free boundary electrophoresis of 0.5% solution of araban sample 4 in 0.05 M sodium tetraborate (pH 9.2); photograph is of the ascending limb after 178 min at 6.5 v/cm (current 16 ma). The araban travels from left to right as a negatively charged borate complex; araban samples 1–3 gave peaks that were exactly alike in shape (for mobilities see Table I). Sample numbers are defined in Table I.

derived from one batch of seeds and samples 1 and 3 from another.

Discussion

The araban molecule is evidently quite small (it contains about 45 sugar units) and evidence from the sedimentation and electrophoresis diagrams, from the correspondence between the number-average and weight-average molecular weights, and from criteria used earlier (Hirst *et al.*, 1965) would suggest that it is not grossly heterogeneous. Chemical evidence has been given previously that the araban does not contain a linear component (Rees and Richardson, 1966). The apparent similarity of the arabans isolated before and after germination (Table I), however, shows that, as criteria of homogeneity, the sedimentation and electrophoresis diagrams are not sensitive to differences in degree of branching of the araban because it has been shown that these polysaccharide preparations differ quite markedly in this respect. It would therefore be unsafe to assume that individual molecules in a given araban preparation are nearly identical with respect to the degree of branching.

Arabans which occur naturally as side chains in larger molecules and which are released as elimination products during extraction at alkaline pH appear to remain attached to segments of the original main chain (Rees and Richardson, 1966; Hullar, 1963; Barrett and Northcote, 1965). The proportion of sugars other than arabinose would therefore depend on the size of the side chain. The molecular weight of mustard seed araban is low enough for such a main chain segment to have been detected, even had it consisted of only one unit. This molecular weight determination therefore provides further evidence that mustard seed araban is not a degradation artifact. Barrett and Northcote (1965) suggested that the parent pectins from which "araban artifacts" may be formed by eliminative degradation during extraction may have higher proportions of covalently bound neutral sugars associated with certain regions of the main chain. It is therefore likely that "araban side chains" closely situated to each other would be released in attachment to the same main chain segment. This would account for the wide difference between the molecular weight of mustard seed araban (6×10^3) and that of Barrett and Northcote's araban formed by degradation of apple pectin (2×10^5 or more). The conditions for release of the araban in Barrett and Northcote's work (sodium phosphate buffer, pH 6.8) would have caused little degradation subsequent to the elimination reaction. The much harsher lime water treatment which is used in the preparation of beet araban would, however, be expected to cause further degradation of the main chain segment by "peeling" (Whistler and BeMiller, 1958). Partial or complete separation of the "araban side chains" from each other would occur and a product with a lower molecular weight would be expected. Reported molecular weights for arabans isolated by this and similar methods are in the range $1–7 \times 10^4$ (Ingleman, 1945; Andrews *et al.*, 1959; Hullar, 1963; Tomimatsu and Palmer, 1963; Sengupta *et al.*, 1965). Comparison of our electrophoresis dia-

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Polysaccharides in Germination. Physical Characterization of the Pectic Araban of White Mustard Cotyledons*

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ABSTRACT: The molecular weight of mustard seed araban, determined by sedimentation equilibrium and vapor pressure osmometry, corresponds to a molecule containing about 45 sugar units. Arabans extracted at different pH values, extracted from different batches of resting seeds, and extracted from cotyledons after germination are all homogeneous and similar in the ultracentrifuge and on free solution electrophoresis in borate. These findings confirm that mustard seed araban is not a degradation artifact of a large heteropolysaccharide, and show that the change in araban structure which occurs with germination cannot take place by the simple addition or simple partial removal of arabinose units from a polysaccharide subfraction.

Mustard cotyledons contain an araban which is considered to be a natural component of the plant cell wall, quite separate and distinct from the side chains of similar structure attached to the acidic backbone of a co-occurring larger polysaccharide (Hirst *et al.*, 1965; Rees and Richardson, 1966; Gould *et al.*, 1965). The araban diminishes in amount during germination, and undergoes changes in molecular structure (Rees and Richardson, 1966). In this paper, we report physical results which throw some further light on the changes during germination and which confirm that the araban is not a degradation artifact. The results make an interesting comparison with physical data published for arabans that are thought to be artifacts.

Materials and Methods

Most of the general methods have been described already (Hirst *et al.*, 1965; Rees and Richardson, 1966). A sample of araban was extracted from commercial "White Mustard Seed Germ" (J. and J. Colman, Ltd., Carrow Works, Norwich, U. K.) using EDTA at pH 4.9, and purified by fractional precipitation with ethanol, acetone, and cetyltrimethylammonium hydroxide (Hirst *et al.*, 1965). After purification on a column of Sephadex G-15 and freeze-drying, the yield (2.5 g/kg) was only slightly less than had been obtained by extraction at pH 7.5 (3.5 g/kg). The difference probably arose because extraction of other wall polysaccharides and of protein is more efficient at pH 7.5 and there is therefore better breakdown of the tissue which results in more efficient extraction of the araban. The product contained 100% arabinose and had $[\alpha]_D$ −173° (water). Only arabinose was detectable after hydrolysis and paper chromatography.

Other araban specimens and the methylated arabans were those used earlier (Rees and Richardson, 1966). Ultracentrifugation was with the Spinco Model E. Free solution electrophoresis was in sodium tetraborate solution (0.05 M, pH 9.2) using the Beckman H apparatus. A vapor pressure osmometer (Model 301A, Mechrolab Inc., Mountainview, Calif.) was used for determination of the molecular weight of the methylated araban in benzene solution.

Results

Molecular Weights. The short-column technique in the ultracentrifuge (Yphantis, 1960) was used to determine the molecular weight in aqueous solution of the araban from resting seeds ("sample 1"). The value for $1 - \bar{V}\rho$ (where $\bar{V}$ is the partial specific volume of the araban, ρ is the density of the solution) was 0.38. The molecular weights determined at 0.8, 1.0, and 1.2% concentrations were 6100, 5700, and 5800, respectively (essentially weight-average values). A number-average determination on the di-*O*-methylaraban (also "sample 1") by vapor pressure osmometry over a concentration range of 1–6% followed by extrapolation to infinite dilution gave a value of 7500, corresponding to a value of about 6200 for the parent araban.

Ultracentrifugation and Electrophoresis. The arabans isolated previously from different batches of resting seeds and from germinated cotyledons by extraction at pH 7.5 (Rees and Richardson, 1966) and the product of extraction with EDTA at pH 4.9 were similar in the ultracentrifuge and on free solution electrophoresis. Typical diagrams are shown in Figure 1, and the results of some measurements are listed in Table I. Most of the small differences in sedimentation coefficients may be related to the fact that samples 2 and 4 were

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