CONFORMATION AND INTERACTION
OF
POLYSACCHARIDES
OF
PLANT CELL WALLS

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Recently, progress has been made in relating the covalent structure of polysaccharides to their bulk physical properties and to their biological function by study of their conformations both in the solid state and in solution. This thesis reports the results of such investigations of tertiary structure on some of the polysaccharide constituents of the plant cell wall.

Examination by the chiroptical techniques of optical rotatory dispersion and circular dichroism of the conformational changes accompanying the sol to gel transition of pectic substances provides further evidence for a model involving association of regions of the galacturonan backbone into junction zones which establish a three dimensional gel network. Changes in the circular dichroism spectra of the carbonyl n → π* transition of low methoxy and de-esterified pectin when gelled by calcium ions have been interpreted in terms of an "egg box model" where the divalent cations are involved in specific complexing in the regular interstices which occur when polygalacturonate regions of the pectin molecules associate in an ordered conformation.

Studies on the nature of the association involved in another gelling polysaccharide system containing two unlike polysaccharide components, agar and galactomannan, have also been carried out. Here too, gelation would appear to be brought about by interaction between extensive regions of the polymers in regular ordered conformations.

Finally, investigations of some cellulose-containing plant mucilages are described. These mucilages can be considered to represent units of cell wall structure containing
crystalline cellulose in intimate association with other neutral and complex acidic polysaccharides. The mucilage particles have been shown to possess properties analogous to those of globular proteins, exhibiting internal crystallinity yet remaining soluble and also showing denaturation and dissociation behaviour.
GENERAL INTRODUCTION

Cells of the higher plants are enclosed by a structure known as the plant cell wall. This structure, apart from allowing the cell to exist in its external medium and to survive against the stress exerted on it by the force of gravity, determines the shape and texture of the cell and hence controls the form of the whole plant.

The mature cell wall can be divided into two separate structures, the primary cell wall and the secondary cell wall, which differ in function and physiological behaviour. One of the main features of the primary cell wall is its ability for rapid growth in surface area without any change in thickness of the wall whereas the opposite is true of the secondary wall. Here increase in thickness occurs without any increase in area.

Chemical Composition of the Cell Wall.

The chemical composition of primary and secondary walls also differs. It is difficult to be precise about the actual composition of each in the individual cell wall and comparisons are more easily made by studying the differences between the young growing cell wall and the full grown mature wall. The polysaccharide components of plant cell walls have traditionally been classified on the basis of solubility and variations in these and other polymer molecules present in growing cell walls are shown in Table 1.
Table 1.

Principal constituents of the growing cell wall, expressed as a percentage of its dry weight.

<table>
<thead>
<tr>
<th></th>
<th>Cellulose</th>
<th>Hemicellulose</th>
<th>Pectin</th>
<th>Lipid</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zea mays,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>coleoptiles</td>
<td>37</td>
<td>45</td>
<td>10</td>
<td>7?</td>
<td></td>
</tr>
<tr>
<td>mesocotyls</td>
<td>33</td>
<td>34</td>
<td>10</td>
<td>23?</td>
<td></td>
</tr>
<tr>
<td>ko</td>
<td>40</td>
<td>32</td>
<td>?</td>
<td>10?</td>
<td></td>
</tr>
<tr>
<td>ena</td>
<td>30</td>
<td>50</td>
<td></td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Avena,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>coleoptiles</td>
<td>42</td>
<td>38</td>
<td>8</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>25</td>
<td>51</td>
<td>1</td>
<td>4.2</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>Cotton hairs</td>
<td>32</td>
<td>19</td>
<td>21</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>Pinus,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cambium</td>
<td>25</td>
<td>26</td>
<td>24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Reference 1, page 127.

Generally, these walls contain about 30% of their dry weight as cellulose, slightly more as hemicellulose polysaccharides, around 10% as pectin and usually a smaller amount of lipid and protein.

The composition of the mature fully grown cell wall is much more variable. The cellulose content can vary between 0-97%, the lignin content between 0-30%, the hemicelluloses between 1-50% and the pectin content between 0.3-45%. Table 11 shows the comparison between growing and mature walls and also variation in the mature cell wall composition.
Table 11.

Chemical constitution of cell walls of various origin.

<table>
<thead>
<tr>
<th>Cell wall of origin</th>
<th>Cellulose</th>
<th>Hemicellulose</th>
<th>Pectin</th>
<th>Chitin</th>
<th>Lignin</th>
<th>Lipids</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arena, coleoptiles</td>
<td>25</td>
<td>51</td>
<td>1</td>
<td>-</td>
<td>+</td>
<td>42</td>
<td>95</td>
</tr>
<tr>
<td>Growing cotton hair</td>
<td>32</td>
<td>19</td>
<td>21</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>Full grown cotton hair</td>
<td>96</td>
<td>1</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
<td>0.8</td>
<td>1</td>
</tr>
<tr>
<td>Ceiba hair</td>
<td>37</td>
<td>40</td>
<td>1</td>
<td>-</td>
<td>12</td>
<td>2.5</td>
<td>+?</td>
</tr>
<tr>
<td>Linum fibres</td>
<td>85</td>
<td>9</td>
<td>4</td>
<td>-</td>
<td>0.6</td>
<td>2-5</td>
<td>+?</td>
</tr>
<tr>
<td>Corchorus fibres</td>
<td>60</td>
<td>26</td>
<td>+</td>
<td>-</td>
<td>11</td>
<td>1</td>
<td>+?</td>
</tr>
<tr>
<td>Cambium</td>
<td>25</td>
<td>26</td>
<td>24</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+?</td>
</tr>
<tr>
<td>Wood</td>
<td>41-48</td>
<td>25-40</td>
<td>0.5-1.5</td>
<td>-</td>
<td>25-30</td>
<td>+</td>
<td>+?</td>
</tr>
<tr>
<td>Petasites collenchyma</td>
<td>20</td>
<td>35</td>
<td>45</td>
<td>-</td>
<td>-</td>
<td>?</td>
<td>+?</td>
</tr>
<tr>
<td>Phycocystis sporangio phore</td>
<td>-</td>
<td>40</td>
<td>-</td>
<td>27</td>
<td>-</td>
<td>25</td>
<td>+?</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>-</td>
<td>70</td>
<td>-</td>
<td>1-3</td>
<td>-</td>
<td>8.5</td>
<td>7?</td>
</tr>
</tbody>
</table>

Adapted from Reference 1, page 2.

- not present; + present, but quantity undetermined; +? presence probable, but not strictly proved.

The structure and conformation of these polysaccharides are discussed later but we now know that cellulose is a β-1,4-glucan, hemicelluloses are an ill-defined family of molecules usually containing mainly...
xylose, mannose or galactose while pectins are made up of galacturonic acid and its methyl ester with some neutral sugars also.

Lignins are essentially high polymer materials built up of phenylpropane units or derivatives but the detailed structure of the native polymer is not well established. Fats and waxes are usually classified as substances of lipid character and these, in general, are long chain aliphatic alcohols and acids.

**Formation of the Cell Wall and Development of Ultrastructure.**

The architecture of the cell wall can be outlined in greater detail than simply the division into primary and secondary walls. The detailed morphology of the mature wall can be related to the series of events which take place during cell division or anaphase and to the subsequent life of the cell.

The first indication of a new equatorial cell wall in a dividing cell (Fig.1.) is the observation of stainable vesicles arising from Golgi bodies which coalesce to form a semi-solid layer known as the cell plate. This cell plate which contains acidic cell wall materials such as pectins, now continues to grow towards the longitudinal wall of the mother cell by further addition of Golgi vesicles. By the time it fuses with this it can be seen to be divided into three sheets, the middle lamella and two sheets one on either side of this which already contain small amounts of
Fig. 1. Schematic representation of the developmental stages of new cell walls.

(a) Formation of cell plate by coalescence of Golgi vesicles.

(b) Growth in thickness by lateral incorporation of additional vesicles.

(c) Differentiated primary wall with plasmodesms.
elementary cellulose. The membranes which surround the Golgi vesicles become part of the plasmalemma which now surround the two separate protoplasts. Further growth of the primary wall continues to take place by deposition of more vesicles containing new wall material, not only at the cell plate but around the whole cell wall. This occurs without thickening of the old wall and involves considerable extension of the existing longitudinal wall of the original mother cell.

The secondary wall makes up most of the bulk of the fully grown cell wall and forms the basis of the plant's mechanical supporting tissue. It may appear divided into several different layers, e.g., in the tracheid cells of conifers three separate layers can be distinguished (Fig. 2).

![Schematic representation of Tracheid Cell Walls according to Dadswell and Wardrop](image)

**Fig. 2.** Schematic representation of Tracheid Cell Walls according to Dadswell and Wardrop.
A further process, incrustation, can now take place to stabilise the structure of full grown cell walls. This occurs with several substances, lignins, siliceous material, suberins and cutins. These substances function in the manner of a cement which can fill up any voids and gives added tensile and compressive strength.

The ultrastructure of all plant cell walls appears to consist of a network or layering of a crystalline skeletal polysaccharide embedded in an apparently structureless, amorphous matrix polysaccharide. In all the higher plants studied so far, the skeletal polysaccharide has been shown to be cellulose. This exists as long microfibrils which have various and frequently regular orientations in an amorphous matrix of hemicellulose and pectin material.

In lower plants, such as seaweeds and algae, the crystalline skeletal polysaccharide is not always cellulose. Certain species of algae have been found to contain a β-1,3 linked xylan as a replacement for cellulose in the wall architecture. Still another substitute for cellulose, a β-1,4 linked mannan occurs as the crystalline structural component in some other species. In fact in the cell walls of Porphyra both the xylan and mannan occur as crystalline structural material in different parts of the plant. A variety of different matrix polysaccharides also occur in the lower plants replacing the hemicelluloses and pectins found in green land plants. Carrageenan, agar and
alginic acid\textsuperscript{7} all appear as matrix type polysaccharides in seaweeds.

Other analogous structural systems occur outside the plant kingdom. In the fungal cell wall, chitin\textsuperscript{8} performs a similar role to that of cellulose in higher plants and in the yeast cell wall 1,3 glucans\textsuperscript{9} have a structural role. The proteoglycan-glycosaminoglycan system\textsuperscript{10} provides a similar structural situation in mammalian tissue.

The cellulose microfibrils vary in absolute dimensions but are in the range 100-250\textsuperscript{8} wide and about half this in thickness. They are arranged in several different orientations\textsuperscript{2} in the cell wall which are revealed by electron microscopy.

In the primary cell wall of the marine alga, Valonia, which later grows in all directions, the fibrils are arranged in a dispersed random-like texture, either interwoven or superposed approximately parallel to the cell surface. This is known as a foliate texture. In walls of cells which grow in a cylindrical manner the fibrils are arranged slightly differently in a tubular texture. Here the fibrils are deposited in a direction more or less perpendicular to the direction of cell extension. The primary wall is not exclusively characterised by disperse textures and added strengthening may be due to some parallel texturing of fibrils.

In the secondary wall the fibrils are arranged of necessity, due to the high cellulose content, in a
parallel fashion. In certain types of cells such as those of ramie, linen and hemp, the fibrils are arranged parallel to the cell axis. These are known as fibrous textures. Generally the fibres in parallel textures are arranged at an angle to the longitudinal cell axis and thus are termed helical textures. The three layers in the secondary wall of the tracheids of conifers (Fig.2.) are arranged in this manner, each layer having a different helical pitch.

Yet a further arrangement is found in the mature wall of Valonia. Here the wall is built up of a succession of lamellae, having parallel orientation of their fibrils, and at an angle of approximately 60° to each other. These are termed crossed textures. In the growth of this type of cell the fibrils are thought to slide over one another due to layers of matrix material between each lamella. This type of extension occurs also between the lamellae of collenchyma and certain sclerenchyma cells of thick walled lactifers.

Conformation and Interaction of Cell Wall Polysaccharides.

In recent years studies on the behaviour of polysaccharides in the solid state and in solution has established the existence of ordered conformations in tertiary structures analogous to those ordered conformations found in the study of proteins and nucleic acids. The determination from X-ray diffraction studies of a triple helical structure for \( \beta-1,3 \) xyylan and of a
double helical structure for carrageenan both in the solid state from X-ray diffraction evidence and in solution by optical methods has led to consideration of these polysaccharides and of others as having an active role in their natural state.

Polysaccharide components of the cell wall, especially the matrix polysaccharides can thus exist in two different states, namely as a random coil or in an ordered conformation. The preference of the macromolecule to exist in one of these states is determined by the relative stability gained from the minimisation of its potential energy through cooperative interactions in the case of the ordered conformation, or the increased conformational entropy or degree of internal freedom of the random coil. The cooperative interactions can occur between molecules as well as within each molecule.

The possible ordered conformations for different polysaccharides can be classified into sub-types on the basis of conformational analysis by computer model building. From considerations of the relative energies and the absence of restricting steric interactions for the various conformations brought about by systematic rotation around the glycosidic and aglycone bonds between residues, models of the preferred ordered conformation can be obtained for different pyranoglycans.
These can be allocated to one of three different sub-types of helically ordered conformations, (Fig. 3.), an extended ribbon-like shape (type A), a wire spring-like helix (type B) or a folded or crumpled ribbon (type C). A fourth type (type D) exists for the 1,6 linked polysaccharides which have an extra bond in the linkage and are thus much more flexible. Heteropolymers containing a repeating disaccharide unit can also be analysed and allocated to a specific type but with more complicated polymers computing complexity becomes a restricting factor.

An understanding of the non-covalent associations involved in these ordered structures would appear to be
Fig. 4. Schematic representation of the types of regular conformation predicted for polysaccharides by conformational analysis in the computer.
Type A, extended and ribbon like, eg. 1,4-glucan; Type B, flexible and helical, eg. 1,3-glucan; Type C, crumpled and contorted, eg. 1,2-glucans; Type D, loosely jointed and extended, ie. 1,6 linked polysaccharides.
Cellulose has long been known to exist in a crystalline state in the cell wall and since the chemical determination of the molecular structure as a straight chain $\beta-1,4$ glucan by Hirst et al, many attempts have been made to establish the crystalline structure. Two different crystalline forms are well known, that of native cellulose or Cellulose I and regenerated cellulose or Cellulose II.

The generally accepted data for the unit cell of native cellulose is that of Meyer and Misch. They interpreted this data as a monoclinic crystal lattice having a two fold screw axis in the direction of the chain and containing four glucose residues in two antiparallel chains. The unit cell dimensions are $a:b:c$, 8.35: 10.3: 7.9 with the monoclinic angle $\beta = 84^\circ$. In this model for crystalline cellulose the linkage between the glucose residues is taken as the "straight" conformation whereas it is now accepted as being that of the bent conformation or "Hermans" conformation where the $C_6-O_2$ distance (363A) is greater and the $O_3-O_5$ distance (268A) reduced, thus allowing intramolecular H-bonding (Fig. 5.).

Experimental evidence for the "Hermans" conformation comes from polarized infra-red data for native and regenerated cellulose and from the X-ray crystal structure of celllobiose.
Fig. 5. Spatial arrangement of $\beta$-D- (1→4) - linked D-Glucopyranose rings for a $10.3$ repeat. (a) is the "straight" chain conformation and (b) the "bent" conformation.
Conformational analysis of cellobiose and cellulose\textsuperscript{21} by means of a computer enabled classification of allowed conformations for these molecules on the basis of acceptable distances between relevant atoms (no infringement of van der Waals radii) and also comparison of relative energies of the different possible conformations. The relatively small number of allowed conformations for cellulose, only about 4\%, indicates the extended inflexible nature of the molecule. These studies support the Hermans type conformation compared to that of Meyer and Misch although it was not at, but near to an energy minimum. This difference in energy could be compensated for by efficient packing of the chains which is made easier by maintaining the two fold axis rather than seeking an energy minimum for the isolated chain.

Warwicker and Wright\textsuperscript{22} envisaged the extended ribbon-like chains of cellulose laid on top of each other to form stacks which are held together by mostly non-polar contacts such as van der Waals attractions, and the stacks are then joined to each other by hydrogen bonding between equatorial hydroxyl groups (Fig.6.).

In considering the organization of the crystalline cellulose into the microfibril observed in the cell wall, two questions are especially important. Is there a biosynthetically important
Fig. 6. Diagrammatic representation of "Stacks" of cellulose chains and their possible aggregation. The approximately oval cross sections represent the view down a ribbon like cellulose chain. The fibre axis, b, is perpendicular to the page, while a and c are the other edges of the Meyer-Misch cell.
unit of aggregated cellulose chains which form these fibrils or is the distribution of the diameters merely random under the control of probability? Secondly, do the cellulose molecules all line up in a parallel fashion in the crystalline microfibril or do they, as in the Meyer-Misch interpretation, align in an antiparallel arrangement?

The original structure, a fibril built of crystalline micellar bricks of cellulose was proposed by Nageli in the nineteenth century to explain the birefringence observed with a polarised microscope. With the establishment of the crystallinity of cellulose by X-ray diffraction studies and the description of the unit cell by Meyer and Misch, the micellar theory was revived. Results obtained for the d p of native cellulose\(^23\) (ca. 10,000) tended to dismiss this idea of micelles containing straight chains of cellulose 60nm in length and arguments were then put forward for a continuous structure containing crystalline and amorphous regions. This was termed the "fringed micellar theory"\(^24\) and was supported by density measurements which showed that the cellulose was less dense than would be expected simply from the crystallographic data for completely crystalline cellulose.

Heterogeneous acid hydrolysis, which might be expected to attack amorphous regions, especially if they were to contain non-cellulosic material, yielded crystalline fibrils or rodlets 60nm in
length and 10nm wide\textsuperscript{25} as seen in the electron microscope. Electron microscopy of sections from the walls themselves indicated microfibrils of diameter 150 - 250\textmu m which appear to contain smaller strands which have been termed elementary fibrils by Muhlethaler\textsuperscript{26}.

Recently, a variety of models for the microfibril have been proposed\textsuperscript{27, 28, 29, 30}, which involve folded chain conformations for the cellulose molecules. Support for the existence of such folded chain conformations is inferred from evidence for similar conformations in cellulose triacetate and in the chitin molecule\textsuperscript{8} which closely resembles cellulose both in structure, (D-glucose is replaced by 2-acetamido-2-deoxy-D-glucose) and in function as a structural substance in anthropods.

However, there is substantial evidence against folded conformations for native cellulose\textsuperscript{34, 35} and crystallographic studies on cellotetraose\textsuperscript{36} suggests a parallel arrangement as proposed for cellulose II. Conformational analysis\textsuperscript{21} also precludes the possibility of sharp folds. Sectioning of ramie fibres and subsequent analysis of the d.p. of the cellulose has been interpreted by Muggli and Muhlethaler\textsuperscript{37} as strong evidence for extended chains.

Observations of the microfibril and related elementary fibrils by electron microscopy\textsuperscript{38} are sometimes disputed as artefacts of the technique.
However, work by Jeffries on the accessibility of hydroxyl groups in the fibrils to chemical reaction, has also been interpreted in terms of elementary fibrils.

It would appear from the weight and the nature of the evidence available at present that extended chains are present in native cellulose although no unequivocal model has yet been established for the exact nature of the microfibril as it exists in the cell wall of the living plant.

The term "hemicellulose" was first used to describe the carbohydrate substances extracted by alkali from the residue of the plant cell wall left after extraction with hot and cold water. More recently it has been taken to be the fraction extracted after the removal of lignin and pectic materials.

These hemicelluloses or polyoses as they are sometimes described contain a wide variety of polysaccharides. They are generally of much lower molecular weight than the cellulose and contain as their constituent sugars mainly, D-xylose, D-mannose, D-glucose, D-galactose, L-arabinose, 4-O-methyl-D-glucuronic acid, D-glucuronic acid and to a lesser extent various C-methylated neutral sugars.

Although these sugars are linked together to give a diverse group of polymers containing varying degrees of branching, two important types of backbone polymer appear to be commonly found. Firstly, a poly (1 → 4) β-D-xylan which is usually associated
with L-arabinose or 4-O-methyl-D-glucuronic acid branch chains and secondly, a poly (1 → 4)β-D-mannan which is accompanied by D-galactose and D-glucose residues.

From X-ray data the crystalline conformation of β-1,4 linked xylan has been interpreted as a left handed helix having a three fold screw axis and a fibre repeat of 14.8 Å. This model involves intramolecular H-bonding, as suggested by infra-red evidence, between O₃ and O₅ as in native cellulose.

Crystalline β-1,4 mannans have been isolated from ivory nuts and on the evidence of X-ray crystallography its structure is very similar to that of cellulose. The fibre repeat is found to be the same although details of a unit cell have not been completed. More commonly plant mannans contain D-glucose residues inserted into the backbone and these polymers are found to be amorphous in character.

Present studies on the role of the hemicellulose in the cell wall indicate rather intimate association with the cellulose microfibrils. X-ray and polarised infra-red data suggest that xylan in wood hemicellulose is oriented approximately parallel to fibre axis of the cellulose.

Recent work on some arabinoxylan preparations shows a conformation shift in solution from random coil to an ordered conformation. This is interpreted as a change in the β-1,4 xylan backbone to a conformation
similar to that found in the solid state which is then stabilised by intermolecular aggregation\textsuperscript{47}.

Models postulated by Fengel\textsuperscript{48} based on the concept of the elementary fibril, incorporate the idea of orientated hemicellulose between these fibrils which are then built up into the larger cellulose microfibril. The hemicelluloses could thus explain the less well bound accessible regions in the microfibril.

Another hemicellulose-like polysaccharide, a galactomannan, has been shown to interact with the tertiary structures of some polysaccharides which form multiple helices\textsuperscript{49}. This interaction with the galactomannan can cause gelation of kappa-carrageenan after the latter has been fragmented, by selective chain cleavage by Smith degradation into a non gelling polysaccharide. Considering the stereochemical similarity between the galactomannan backbone and cellulose, this interaction may be analogous to the in vivo cohesion between the skeletal polysaccharides and the gel matrix in the cell wall.

The pectic substances occur extensively in the young growing primary cell wall and the middle lamella. These polysaccharides have extremely diverse compositions containing several different sugar residues\textsuperscript{50}. They appear to exist as an amorphous rather plastic matrix in which the cellulose microfibrils are embedded. The nature and occurrence of these polysaccharides have led to their implication in theories of growth which
relate plasticity of the matrix to cell wall extension\textsuperscript{5}.

Pectin substances contain $\alpha-1,4$ linked D-galacturonic acid residues in a backbone structure which may also contain $1,2$ linked rhamnose units. This backbone is substituted by a variety of different side chains including arabinan, galactan and some acidic groups\textsuperscript{7}. A further feature of the galacturonan backbone is the extent of methyl esterification of the uronic acid residues.

The possible nature of the interaction of pectic molecules in the cell wall is suggested by their behaviour in vitro. Molecules containing a large proportion of their galacturonic acid residues as methyl esters, form into a firm gel in the presence of a high concentration of a low molecular weight solute such as sucrose, ethylene glycol or ammonium sulphate, at acid pH\textsuperscript{51}. Similar pectic gels can be formed on the addition of divalent metal ions to pectin containing mostly acidic groups. Minor structural alterations of the galacturonan backbone such as partial acetylation\textsuperscript{52} of hydroxyl groups affect the gelling ability of these pectins. Replacement of methyl groups by ethyl or 2-hydroxy ethyl esters\textsuperscript{53} also prevents gelation of the esterified pectins. This suggests that the strength of association of pectin molecules is dependent on intermolecular and possibly intramolecular distribution of ionised and esterified uronic acid residues on the
galacturonan backbone.

X-ray fibre diagrams of pectin and sodium pectate$^4$ have been interpreted as a three fold, left handed helix for the galacturonan backbone with a fibre repeat of 13.0Å. Model building computations present a slightly different model having the same three fold repeat but a right handed screw sense$^5$.

However the extended nature of the chain as a type A ribbon conformation precludes the possibility of chain associations by means of multiple helices.

Extension and Growth of the Wall.

It has been suggested that cell wall extension occurs by a procedure termed "multinet growth". Cellulose microfibrils are laid down in the plastic matrix material more or less perpendicular to the extending cell axis. As the cell grows and further fibrils are laid down, the original perpendicular fibrils in the outer regions experience a passive shift to a more diagonal orientation and eventually as the process continues they become parallel to the cell axis of the extended cell. These growth patterns could be brought about by a change in the physical, gel-like properties of the matrix materials embedding the fibrils.

To achieve this change in the properties of the matrix several means of modification of the cell wall constituents by metabolic mechanisms have been suggested. Turnover, autolysis, degradation and loosening are the most commonly used terms implying a change in wall
components which usually involves cleavage of covalent bonds.

Autolysis involves degradation of the wall by specific enzymes. Ruesink\textsuperscript{56} studied the effect of cellulase on cell wall elongation and showed that simple weakening of the cell wall was not sufficient to promote growth by pressure from within the cell. He therefore concluded that endogeneous polysaccharides are not the means by which the growth rate of coleoptiles is regulated. Other reports however indicate that isolated cell walls from oat and maize coleoptiles do contain firmly bound polysaccharide degrading activity\textsuperscript{57}.

Extensive cell wall loosening which promotes growth is brought about by auxin and other similar growth hormones, although their mode of action still remains obscure.

Turnover involves the continuous removal and synthesis of one or more components which can be anything from a complete macromolecule to a single covalent linkage. Stoddart and Northcote\textsuperscript{58} suggest that two pectic fractions are metabolically unstable in actively growing sycamore cells. By pulse labelling techniques they cite evidence for rapid turnover of a partially methylated acidic pectin fraction and a neutral arabinan-galactan fraction which appears to be transferred to the weakly acidic fraction.

Rees et al\textsuperscript{59} have studied the changes occurring in pectic arabinan of mustard cotyledons on germination.
The highly branched arabinan was found to decrease on germination and also the degree of branching lessened. These structural changes were interpreted as causing a decrease in the plasticity of the structure in keeping with the need for a strengthening of the cell wall as germination proceeded.

Examination of the pectic polysaccharides from the same mustard cotyledon showed that the galacturonan backbone was highly branched and contained a substantial number of rhamnose residues which decreased the gelling ability of the pectin. The pectin in the mustard cotyledons may thus function more as a lubricant for structural elements when enlargement occurs on germination than as a cementing matrix material as in more mature walls.

It is thus possible that metabolic turnover of a number of matrix components during growth allows the necessary changes in molecular structure and hence changes in the tertiary and quaternary structures to allow controlled extension of the cell wall.

Lamport suggests that the hydroxyproline rich protein, sometimes termed extensin, found in most cell walls has a fundamental role in the structure of the cell wall. The hydroxyproline of the protein has been found to be O-glycosidically linked to arabinose oligomers in some cases, which led Lamport to postulate a polysaccharide-protein complex giving structural form in the cell wall. Breaking of labile linkages in the
glycoprotein could then be a mechanism of extension during growth.

The present evidence on the existence of polysaccharide tertiary structures in general and particularly of those specifically involved in the cell wall suggest that these structures and the interactions involved have an important role in the biological functioning of the organism in which they occur.

The purpose of this thesis is to study the ordered conformations and interactions of some of these systems.
Chapter I

Investigation of the Gelation of Pectin: Studies by Optical Rotation.
Introduction

The pectic substances (previously discussed in the General Introduction) occur extensively in the young primary plant cell wall and are implicated in the mechanisms proposed for its extension and growth. They also occur elsewhere in plants especially in the mature thickened primary wall of the collenchyma of citrus fruits and others such as the celery petioles. It therefore seems pertinent to undertake a study of the sol to gel transition of these substances in vitro, in the hope that detailed knowledge of this system may lead to an understanding of the processes involved in the biological state.

Pectic substances occur in greatest abundance in soft tissues such as the peel of citrus fruit (ca 30%) in sugar beet (25%) and apple pomace (15%). These pectins contain mainly a linear polymer backbone of $\alpha - 1,4$ linked D-galactopyranuronic acid units (Fig. 1.1) which is now known to be covalently associated with neutral polysaccharide side chains (Table 1.1). Although an almost pure galacturonan has been isolated from pectic acid of sunflower heads it is usual to find L-rhamnose units inserted in this backbone. Present evidence suggests a chemically homogeneous polydisperse system for pectin consisting of structurally related molecular species with continuously variable proportions of neutral sugars (Fig. 1.2).
Table 1.1.

**Structural Variations in Pectic Substances**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Examples of source material</th>
</tr>
</thead>
<tbody>
<tr>
<td>...2)-L-Rhap-(1... insertion in galacturonan chain.</td>
<td>lucerne, soybean hulls, lemon peel, soybean cotyledons, mustard embryo, apple, Amabilis fir bark.</td>
</tr>
<tr>
<td>O-Acetylation of galacturonic acid residues</td>
<td>beet</td>
</tr>
<tr>
<td>&quot;Galactan&quot; side-chains, ...4)-β-D-Galp-(... n</td>
<td>soybean cotyledons, soybean hulls</td>
</tr>
<tr>
<td>Branched &quot;arabinan&quot; side-chains</td>
<td>lucerne, mustard embryo, apple</td>
</tr>
<tr>
<td>Xylp-(1... side-chains</td>
<td>mountain pine pollen, mustard embryo, lemon peel, soybean cotyledons</td>
</tr>
<tr>
<td>β-D-Galp-(1→2)-D-Xylp-(1... probably as side chains</td>
<td>soybean cotyledons, soybean hulls</td>
</tr>
<tr>
<td>α-L-Fucp-(1→2)-D-Xylp-(1... probably as side chains</td>
<td>soybean cotyledons, soybean hulls</td>
</tr>
<tr>
<td>β-D-GnA-(1→4)-D-Galp-(1... probably as side chains</td>
<td>soybean cotyledons, lemon peel, soybean hulls</td>
</tr>
<tr>
<td>β-D-GnA-(1→4)-L-Fucp-(1... probably as side chains</td>
<td>soybean cotyledons, soybean hulls, lemon peel, lucerne</td>
</tr>
<tr>
<td>D-Apiose</td>
<td>Zosteraceae</td>
</tr>
<tr>
<td>2-O-Methyl-D-xylose</td>
<td>lucerne</td>
</tr>
<tr>
<td>2-O-Methyl-L-fucose</td>
<td>plum leaves</td>
</tr>
<tr>
<td></td>
<td>sisal</td>
</tr>
</tbody>
</table>
Mild degradation of pectin by a $\beta$-elimination reaction (Fig. 1.3) leads to two polysaccharide fractions, a strongly acidic fraction containing mostly galacturonan and a neutral polysaccharide containing most of the neutral sugars present in the original.

Two types of pectin are commercially extracted from natural sources. A pectin, in which the uronic acid units are present predominately as the methyl ester, can be extracted from the intercellular layer with water or dilute acid. This is usually referred to as high methoxy pectin. A second type is usually commercially extracted with acid of a strength which simultaneously causes de-esterification thus yielding a low methoxy pectin. Similar pectin may also be extracted by the
Fig. 1.2. General structure for pectic acids.

Fig. 1.3. β-Elimination of methyl esterified pectin.
action of sodium hexametaphosphate or ethylene
diaminetetra-acetic acid. This pectin has most of the
uronic acid residues present as salts of divalent ions
such as calcium.

The phenomenon of gelation occurs widely as a
property of high polymer systems and physical and
mathematical models based on a three dimensional cross-
linked network have been successful in explaining the
solid stress resistant bulk properties shown by these
systems. Recently in polysaccharide gels this model
has been taken a step further in order to explain
gelation properties in terms of the detailed primary
and secondary structural chemistry of the components.
Gelation occurs by the association of "regions" of the
polymer molecule into "junction zones" which may be
regarded as tertiary structures analogous to the
characteristic ordered structures of globular proteins
and polypeptides.

Several different types of ordered conformation
have now been proposed for the junction zones of gelling
polysaccharides. Covalent linkages however also form
networks in synthetic polysaccharide gels such as
Sephadex and also in the peptidoglycan component of the
bacterial cell wall. Multiple helical junction zones
and aggregates of these helices occur in the gelation
of carrageenan and probably in agar also. A micellar junction zone appears to permit the gelation
of C-methylcellulose. In gelation of glycuronans, such
as pectin or alginate, junction zones involving ordered
microcrystalline regions are the most satisfactory explanation of the properties. Strength and texture, the presence of X-ray crystallinity\textsuperscript{68}, the tendency to exhibit syneresis, birefringence, and a fine structure of pores from diffusion experiments\textsuperscript{69} for calcium pectate and alginate gels are rationalised on this model and dismiss the previous unsatisfactory ideas of simple ionic bridging, or single chelation sites. Similar junction zones involving ordered regions must be envisaged for the high methoxy pectin gelation as substitution of methyl ester by the 2-hydroxy ethyl ester\textsuperscript{53} or a degree of acetylation\textsuperscript{52} of one per eight residues prevents gelation.

The ability of pectins to form gels and the respective bulk properties of these gels has been reviewed recently by Pilnik\textsuperscript{70}. Pectins are capable of forming gels at very low concentrations (even below 0.5\%) in the presence of sucrose and acid. Several additives such as glycerol, alcohols and even ammonium sulphate have been used successfully to replace the sucrose. The requirement of an H\textsuperscript{+} concentration is assumed to be necessary to suppress the ionisation of the polyelectrolyte thus minimising electrostatic repulsion between the polymer chains and allowing formation of junction zones. As discussed later in this chapter the non-esterified uronate residues also appear to be involved in the junction zones themselves. A fully esterified pectin can be gelled without lowering of the pH\textsuperscript{71}. 

In commercial applications of pectin with a low degree of esterification gelation is accomplished by addition of divalent cations, usually Ca\(^{2+}\), in the presence of a very much reduced quantity of sucrose (20-30%) as opposed to 60-70% which was used for high methoxy pectin gels. The addition of the calcium must be achieved in a controlled fashion, e.g. by gradual dissolution of insoluble salts, if a coagulated precipitate is to be avoided. A pronounced heat reversibility has been observed for certain calcium pectate gels\(^70\).

In most investigations of pectin gels, the quality of the gel is assessed by some arbitrary physical method, such as its ability to be compressed and results from different methods do not always classify gels in the same order. For the high methoxy pectin gels, increase in pectin or sucrose concentration, or a decrease in pH cause increased gel strength. Increase in the degree of esterification of pectin causes a decrease in gel strength but an increase in the rate of setting.

Similar changes in gel strength with respect to these variables are found for low methoxy pectin but here Ca\(^{2+}\) has a dominant role. Increase in Ca\(^{2+}\) concentration increases gel strength and also tends to make the gel more brittle and more liable to syneresis. Low methoxy pectin gels have been characterised by determination of gelling or setting temperatures which appear to be independent of the rate of cooling\(^70\). Increased setting
temperatures occur with increase of sucrose or calcium content.

In order to understand the properties of these pectin gels, an experimental technique must be sought which can investigate the nature and extent of association present in the junction zones. Such a technique, optical rotation, has been used successfully already to follow changes in the tertiary structure of gelatin\textsuperscript{72} and other proteins, polypeptides and more recently polysaccharides\textsuperscript{13}.

Optical rotation is a measure of the rotation of plane polarised light as it passes through a dissymmetric environment. Plane polarised light can be regarded as the sum of left and right circularly polarised light of the same amplitude and phase and the rotation arises from the different rates of propagation of the two components through the dissymmetric medium.

The speed of the light through the material medium is related to the index of refraction and the rotation can be expressed as

\[ \alpha = \frac{1800}{\lambda} \frac{1}{nL-nR} \]

where \( \alpha \) is the rotation in degrees per decimeter, \( \lambda \) is the vacuum wavelength of the light beam, and \( nL \) and \( nR \) are the different indices of refraction for left and right circularly polarised light respectively. The rotation is sometimes expresses as specific rotation \([\alpha]\) or molecular rotation \([\bar{\alpha}]\)

\[ [\alpha] = \frac{\alpha}{c} \]

\[ [\bar{\alpha}] = \frac{[\alpha]}{M/\text{1000}} = \alpha \frac{M}{\text{1000}} \]
where $C$ is the concentration of the solution in g./c.c. and $M$ is the molecular weight.

Measurements of optical rotation at specific wavelengths, especially at the sodium D-line have been widely used in the field of carbohydrates, and Hudson formulated rules for sugars and sugar glycosides based on the van't Hoff rule of optical superposition which states that, "the rotation of a molecule is made up of the algebraic sum of the rotational contributions of the various asymmetric centres".

The fundamental unsoundness of the principle is now recognised and Kauzmann suggests that, from consideration of quantum theories, the origin of optical rotation is a result of modifications of electronic motions in an asymmetric molecule by interactions between groups. The optical rotation should thus be seen as the sum of the interaction effects between groups rather than as contributions from isolated centres. These interactions may be pairwise interactions, three way interactions and so on, and may arise from interactions due to coulombic forces, dipole effects, quadrupole perturbations of electronic transitions, strong dispersion forces, charge transfer effects or interactions through covalent bonds.

On this basis Rees has attempted to interpret optical rotations of disaccharides and higher oligomers including polysaccharides in terms of a linkage rotation,

$$\Lambda = [M_{NR}] - \left\{ [M_{MeN}] + [M_R] \right\}$$
where \([M_{NR}]\) is the molecular rotation of the disaccharide, 
\([M_{MeN}]\) is the molecular rotation of the methyl glycoside of the non-reducing residue in the disaccharide and \([M_R]\) is the molecular rotation of the reducing residue.

This linkage rotation represents the optical rotation due to interactions across the glycosidic linkage minus any contribution due to the glycosidic methyl group and the hydrogen of the linkage on the reducing sugar. Using the empirical data of Brewster\(^7\) and Whiffen\(^8\) which is based on consideration of

1. interaction of non-bonded atoms which differ in polarizability, and
2. interaction of bonded atoms, taken four at a time, the linkage rotation can be derived as

\[
\left[ \frac{\Lambda}{\text{calo. D}} \right] = -105 - 120 \left( \sin \Delta \phi + \sin \Delta \psi \right)
\]

for \(\alpha\)-linked disaccharides in which the non-reducing residue is D and has the Reeves C1 conformation and where \(\Delta \phi\) and \(\Delta \psi\) are related to the angles at the linkage as defined in the general introduction (Fig.3.). A similar expression applies for \(\beta\)-linkages.

The equation was found to give very good agreement between observed and calculated rotations for \(\beta\)-cellobiose, \(\alpha\)-lactose and cyclohexaamylose in DM 80 using known standard conformations from X-ray diffraction data. Good reasons in terms of conformational analysis can be given to account for discrepancies found for some other compounds.
Using average conformations derived from computer studies, optical rotation measurements for equatorial—equatorial glycosidic linkages can be classified in terms of the conformational effect of the substituents influencing rotation around the aglycone bond\textsuperscript{15}. The rotation around the glycosidic bond did not vary much but this does not hold for the axial linkages. It would therefore appear that the optical rotation in aqueous solution is very sensitive to the linkage conformation and can be predicted roughly by considering the van der Waals repulsions between surrounding substituents due to their steric bulk.

The optical rotation of the helical and coil forms of the iota-carrageenan segments\textsuperscript{77} have been calculated\textsuperscript{78} from the linkage rotation using the X-ray diffraction data\textsuperscript{12} and the molecular rotations of the relevant methyl glycosides and disaccharides. The agreement between calculated and observed values gives impressive support for the coil to helix model for this system and also encourages the use of optical rotation in determining the exact nature of the conformational changes involved in transitions to ordered tertiary structures in other polysaccharide models.

In the case of pectin itself, although optical rotation is still expected to be sensitive to changes in conformation at the linkage, the feasibility of the application of a quantitative relationship between the linkage conformation and the rotation is hampered by the
contribution to the rotation of the carboxylate chromophore.

In this chapter some preliminary experiments have been carried out with a view to finding the most suitable pectin systems in which the conformational changes on gelation may be studied. These systems have been examined by observing the changes in optical rotation for the sol to gel transition.
Experimental

Section 1.1. Gelation of High Methoxy Pectin

Experiment 1.1.1. Gelation with Ethylene Glycol

(a) Gels were prepared by dissolving high methoxy pectin (HM-1) in a citric acid - sodium citrate buffer (0.1M) by shaking overnight (see general methods section). The buffered solution was heated on a water bath at 100°C and ethylene glycol, similarly heated, was added. The mixture was shaken and left to set for 24 hours.

The gels were characterised by recording their melting points under a standard set of conditions. This was accomplished by injecting each gel with mercury beads (1 μm), placing the tube containing the gel on a water bath and then heating. The melting point was taken as the temperature at which the mercury bead began to move rapidly through the gel. Setting points were determined by dropping mercury beads (1 μm) through the solution as it cooled. The setting point temperature was taken as the temperature at which the beads first failed to drop. Results are shown in Table 1.2. The heating and cooling rates of the water bath were not constant but varied between 0.2-2.0°C min.⁻¹

(b) A second series of gels were made up to determine the effect of pH. These were made up by a different method. The pectin (HM-1) was dissolved in citrate buffer (50ml, 0.1M) and distilled water (50ml) by boiling in an evaporating dish. Ethylene glycol was added and the solution was then evaporated until the contents weighed 100g.
Table 1.2.
Gelation of High Methoxy Pectin (HM-1) with Ethylene Glycol.

<table>
<thead>
<tr>
<th>Pectin (g/100ml of gel)</th>
<th>Ethylene glycol (ml/100ml of gel)</th>
<th>pH of Buffer</th>
<th>Melting Point (°C)</th>
<th>Setting Point (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>50</td>
<td>3.3</td>
<td>41</td>
<td>33</td>
</tr>
<tr>
<td>2.0</td>
<td>55</td>
<td>3.3</td>
<td>44</td>
<td>37</td>
</tr>
<tr>
<td>2.0</td>
<td>60</td>
<td>3.3</td>
<td>49</td>
<td>39</td>
</tr>
<tr>
<td>2.1</td>
<td>65</td>
<td>3.3</td>
<td>57</td>
<td>41</td>
</tr>
<tr>
<td>2.0</td>
<td>70</td>
<td>3.3</td>
<td>59</td>
<td>43</td>
</tr>
<tr>
<td>2.1</td>
<td>75</td>
<td>3.3</td>
<td>62</td>
<td>50</td>
</tr>
<tr>
<td>0.5</td>
<td>50</td>
<td>3.3</td>
<td>no gel</td>
<td>-</td>
</tr>
<tr>
<td>0.5</td>
<td>55</td>
<td>3.3</td>
<td>38</td>
<td>28</td>
</tr>
<tr>
<td>0.5</td>
<td>60</td>
<td>3.3</td>
<td>39</td>
<td>31</td>
</tr>
<tr>
<td>0.5</td>
<td>65</td>
<td>3.3</td>
<td>41</td>
<td>34</td>
</tr>
<tr>
<td>0.5</td>
<td>70</td>
<td>3.3</td>
<td>51</td>
<td>37</td>
</tr>
<tr>
<td>0.5</td>
<td>75</td>
<td>3.3</td>
<td>56</td>
<td>41</td>
</tr>
<tr>
<td>0.5</td>
<td>50</td>
<td>3.0</td>
<td>no gel</td>
<td>-</td>
</tr>
<tr>
<td>0.5</td>
<td>55</td>
<td>3.0</td>
<td>55</td>
<td>27</td>
</tr>
<tr>
<td>0.5</td>
<td>60</td>
<td>3.0</td>
<td>60</td>
<td>32</td>
</tr>
<tr>
<td>0.5</td>
<td>65</td>
<td>3.0</td>
<td>69</td>
<td>35</td>
</tr>
<tr>
<td>0.5</td>
<td>70</td>
<td>3.0</td>
<td>76</td>
<td>40</td>
</tr>
<tr>
<td>0.5</td>
<td>75</td>
<td>3.0</td>
<td>80</td>
<td>39</td>
</tr>
</tbody>
</table>
The mixture was then transferred to sample tubes and allowed to set at room temperature. Results are shown in Table 1.3.

**Table 1.3.**

Effect of pH on high methoxy pectin (HM-1) - ethylene glycol gels.

<table>
<thead>
<tr>
<th>Pectin (g/100g of gel)</th>
<th>Ethylene glycol (ml/100g of gel)</th>
<th>pH of Buffer</th>
<th>Melting Point (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>70</td>
<td>2.1</td>
<td>90</td>
</tr>
<tr>
<td>0.5</td>
<td>70</td>
<td>2.8</td>
<td>73</td>
</tr>
<tr>
<td>0.5</td>
<td>70</td>
<td>3.4</td>
<td>33</td>
</tr>
<tr>
<td>0.5</td>
<td>70</td>
<td>3.6</td>
<td>no gel</td>
</tr>
</tbody>
</table>

**Experiment 1.1.2. - Gelation with Sucrose and Sorbitol.**

Since sucrose is commonly used to gel high methoxy pectin in commercial applications, gels were prepared containing pure sucrose by the method used in Experiment 1.1.1. (b). Under the conditions used, gels were made which did not melt on heating to 100°C on a water bath. When transferred to an oil bath these gels could be melted at higher temperatures and on subsequent cooling they reset. Sucrose - pectin gels have a very different appearance to those made with ethylene glycol. Whereas the ethylene glycol gels are clear, wobbly and easily cut (ie maintaining the cut edge), the sucrose gels, in contrast, are stodgy, glue-like and not easily cut. Use of sorbitol as additive forms similar gels to those involving sucrose. Results are shown in Table 1.4.
Table 1.4.

High Methoxy Pectin (HM-1) Gels containing Sucrose or Sorbitol.

<table>
<thead>
<tr>
<th>Pectin (g/100g of gel)</th>
<th>Additive (g/100g of gel)</th>
<th>pH of Buffer</th>
<th>Melting Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>75 Sucrose</td>
<td>3.0</td>
<td>ca 120</td>
</tr>
<tr>
<td>0.5</td>
<td>69 &quot;</td>
<td>3.0</td>
<td>ca 120</td>
</tr>
<tr>
<td>0.6</td>
<td>66 &quot;</td>
<td>3.0</td>
<td>ca 110</td>
</tr>
<tr>
<td>0.5</td>
<td>45 &quot;</td>
<td>3.0</td>
<td>no gel</td>
</tr>
<tr>
<td>0.5</td>
<td>77 Sorbitol</td>
<td>3.0</td>
<td>ca 100</td>
</tr>
<tr>
<td>0.5</td>
<td>66 &quot;</td>
<td>3.0</td>
<td>100</td>
</tr>
<tr>
<td>0.6</td>
<td>65 &quot;</td>
<td>3.0</td>
<td>100</td>
</tr>
<tr>
<td>0.6</td>
<td>51 &quot;</td>
<td>3.0</td>
<td>95</td>
</tr>
</tbody>
</table>

On being left to cool to room temperature the solutions reset.

Experiment 1.1.3. - Investigation of the Role of the Additive.

Since it is known that several quite different additives can be used to cause high methoxy pectin to gel an attempt was made to rationalise their role in the gelation process. Gels were formed by the procedure in Experiment 1.1.1. (b) with the concentration of the additives varying while the pectin concentration (HM-1, 0.5g/100g of gel) and the pH of 3, were kept the same. The minimum amount of each additive required to cause gelation is tabulated below, Table 1.5.

Experiment 1.1.4. - Preparation and Gelation of a Fully Esterified Pectin.

The previous citrus high methoxy pectin (HM-1) was
used to prepare a fully methyl esterified pectin by the method of Deuel.  

The pectin (20g) in water (2L) was passed through a column of Amberlite resin IR-120 (analytical grade) in the H⁺ form. The eluted pectin was precipitated with 96% ethanol and washed with absolute ethanol and ether. This material was then dispersed in ether at -30°C and a pre-cooled ethereal solution of diazomethane was added. The temperature was achieved by adding solid carbon dioxide to an acetone bath. The reaction was allowed to proceed for 25 minutes with continuous stirring after which the excess diazomethane was filtered off. The precipitate was then washed successively with cold alcohol, alcohol, acetone and ether.

Table 1.5.
The approximate minimum concentration of additive required to cause gelation of high methoxyl pectin (HM-1, 0.5g/100g of gel) in citrate buffer (0.1M, 50ml/100g of gel) at pH3.0.

<table>
<thead>
<tr>
<th>Additive</th>
<th>Concentration (g/100g of gel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>60</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>55</td>
</tr>
<tr>
<td>Ethylene Glycol</td>
<td>53</td>
</tr>
<tr>
<td>Propan - 1,2 - diol</td>
<td>47</td>
</tr>
<tr>
<td>Propan - 1,3 - diol</td>
<td>47</td>
</tr>
<tr>
<td>Butan - 1,4 - diol</td>
<td>40</td>
</tr>
<tr>
<td>Butan - 2,3 - diol</td>
<td>40</td>
</tr>
<tr>
<td>Glycerol</td>
<td>60</td>
</tr>
</tbody>
</table>
The dried pectin (0.66g/100g) was gelled with ethylene glycol (80g/100g) at pH 6 (0.1M, citrate buffer). This was taken to indicate almost complete esterification. Infra-red studies indicated a strong absorption due to the carbonyl group of the ester (1740cm$^{-1}$) and negligible absorption for the carboxyl of the acid (1607cm$^{-1}$).

Further gels were made up by dissolving the pectin in distilled water and then mixing in ethylene glycol. The fully esterified pectin could be gelled at concentrations as low as 0.3g/100ml of gel in the presence of ethylene glycol (70ml/100ml of gel).

These gels could be melted by heating on a water bath but they appeared to melt irregularly over a wide range of temperature. The more concentrated gels had to be heated to 100°C before they melted.
Section 1.2. Gelation of Low Methoxy Pectin.

Experiment 1.2.1. - Comparison of Different Methods of Gelation with Divalent Cations.

Usually, in the commercial applications of gelation of low methoxy pectin, sucrose is used as an additive. Since it was essential to leave out the sucrose if optical rotation studies were to be carried out, different methods of forming low methoxy gels with divalent cations were investigated in order to achieve a suitable system for these optical rotation studies.

Methods

(a) Low methoxy pectin, (2.0% w/v) was dissolved in citrate buffer (75ml/100ml of gel, 0.05M, pH 3.0) by shaking. The solution was then heated on a water bath at 100°C and a solution of hot CaCl₂ was added (50-400mg Ca²⁺/100ml of gel). The pectin gelled on cooling. This gel, after ageing overnight, could be melted easily by heating on a water bath. It reset on cooling.

(b) Low methoxy pectin, (1.4% w/v) was dissolved in distilled water by heating. After cooling, sodium citrate (1.25% w/v) and calcium hydrogen orthophosphate (0.4% w/v) were added to the solution and finally citric acid (1.25% w/v) which caused the pectin to gel on standing.

These gels did not melt easily but appeared to break up and become more liquid in character with heating. They did not reset to the original gel on cooling.

Using this procedure, but adding calcium chloride in the place of calcium hydrogen orthophosphate also produced pectin gels.
Low methoxy pectin, (0.5% w/v) was dissolved in distilled water and placed in dialysis tubing. This was then immersed in a solution of calcium chloride (12mM) for 16 hours. A firm gel was formed.

**Experiment 1.2.2. - Gelation of Pectic Acids and Pectin Amides.**

Several pectin samples were saponified with alkali to obtain pectins without any ester groups (see general methods section). These pectic acids formed firm clear gels on dialysis against Ca$^{2+}$ ions.

Pectin which is saponified by treatment with ammonia produces a pectin which contains amide groups as well as ester and carboxyl groups. These pectins could be gelled by dialysis against calcium but they could also be gelled by dialysis against acid, especially after further removal of the methyl ester with alkali.

**Experiment 1.2.3. - Effect of Calcium on the Gelation of Low Methoxy Pectin (LM-1).**

Gels containing varying amounts of calcium ions were prepared by the method described in Experiment 1.2.1. (a). The quality of the gels was assessed by testing the gel (100g) with a Bloom Gelometer. The results are shown in Fig.1.4.
Experiment 1.2.4. - Effect of Different Divalent Cations on Gelation.

The ability of different cations to cause gelation of low methoxy pectin (LM-2) was studied by preparing gels by the method used in Experiment 1.2.1. (b) replacing the calcium hydrogen orthophosphate with different quantities of metal chlorides. The gels were assessed on the Boucher Jelly Tester. Table 1.6. indicates the approximate minimum quantity of cation required for gelation or precipitation of the pectin and also the maximum gel strength obtained for the particular metal pectate gel as determined from graphs similar to that for the Ca$^{2+}$ in Fig.1.4.
<table>
<thead>
<tr>
<th>Cation</th>
<th>Ionic Radius for $M^{2+}$</th>
<th>Minimum Concentration required to cause Gelation or Precipitation (Millimoles/100ml of gel)</th>
<th>Maximum Gel Strength</th>
<th>Concentration at Maximum (Millimoles/100ml of gel)</th>
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<tr>
<td>Pb</td>
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<td>0.25</td>
<td>87</td>
<td>0.45</td>
</tr>
<tr>
<td>Ba</td>
<td>1.34</td>
<td>0.30</td>
<td>90</td>
<td>0.75</td>
</tr>
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<td>Sr</td>
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<td>62</td>
<td>1.50</td>
</tr>
<tr>
<td>Ca</td>
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<td>1.10</td>
<td>76</td>
<td>1.60</td>
</tr>
<tr>
<td>Cd</td>
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<td>1.25</td>
<td>95</td>
<td>3.00</td>
</tr>
<tr>
<td>Sm</td>
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<td>1.50</td>
<td>94</td>
<td>2.50</td>
</tr>
<tr>
<td>Mn</td>
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<td>2.00</td>
<td>Precipitate*</td>
<td>-</td>
</tr>
<tr>
<td>Zn</td>
<td>0.74</td>
<td>2.20</td>
<td>&quot;</td>
<td>-</td>
</tr>
<tr>
<td>Fe</td>
<td>0.74</td>
<td>2.50</td>
<td>&quot;</td>
<td>-</td>
</tr>
<tr>
<td>Co</td>
<td>0.72</td>
<td>3.20</td>
<td>&quot;</td>
<td>-</td>
</tr>
<tr>
<td>Ni</td>
<td>0.69</td>
<td>3.00</td>
<td>116</td>
<td></td>
</tr>
</tbody>
</table>

* at very much higher concentrations (ca. 5.0 millimoles/100ml of gel) a weak gel was formed.
Section 1.3. Optical Rotation Studies on Pectin Gels.  

Experiment 1.3.1. - Optical Rotation of High Methoxy Pectin - Ethylene Glycol Gels.  

The sol to gel transitions for high methoxy pectin-ethylene glycol gels were studied by following the changes in the optical rotation as the temperature was varied.  

The rotations were measured on the Perkin-Elmer 141 Polarimeter in thermostated cells as described in the general methods section.  

Fig. 1.5. shows the changes in rotation which occur for a high methoxy pectin, (HM-1, 20% w/v), ethylene glycol (60% v/v) gel in citrate buffer (0.1M, pH 3.3) when the temperature is altered at a rate of 1°C/min. The temperature was held at 45°C for 20 minutes on both the heating and cooling runs. The gels were made up by the method in Experiment 1.1.1. (a). Fig. 1.6. shows the changes which occur for a series of gels in which the pectin (HM-1, 0.5% w/w) and the pH 3.0 (50% v/w, 0.1M, citrate buffer) were kept the same but the amount of ethylene glycol was varied. In Fig. 1.7. the effect of pH of the buffer on optical rotation is shown. The gels in Fig. 1.6. and 1.7. were made by the method described in Experiment 1.1.1. (b).  

Fig. 1.8. shows the effect of changing the concentration of pectin present in a glycol gel. The rotations of the solutions were multiplied by the appropriate concentration factor to facilitate comparison.
Fig. 1.5. Optical rotation changes during the setting and liquifaction of high methoxy pectin (2.0% w/v, HM-1) -ethylene glycol (60% v/v) gel in citrate buffer (40ml/100ml of gel, 0.1M, pH 3.3). Rate of change of temperature was 1°C/minute. The temperature was held at 45°C for 20 minutes on both heating and cooling.
Fig. 1.6. Changes in optical rotation on cooling solutions of high methoxy pectin (0.5% w/w, HM-1) in citrate buffer (50ml/100g of solution, 0.1M, pH 3.0) containing various amounts of ethylene glycol, A- none, B- 32% w/w, C- 54% w/w, D- 65% w/w and E- 75% w/w. C, D and E formed gels at low temperature.
Fig. 1.7. Changes in optical rotation on cooling solutions of high methoxy pectin (0.5% w/w, HM-1) and ethylene glycol (70% v/w) in citrate buffer (50ml/100g of solution, 0.1M) at various pH values. A- 3.8, B- 3.5 and C- 2.8. B and C formed gels at low temperature.
Fig. 1.8. Changes in optical rotation on cooling solutions of high methoxy pectin (HM-1) and ethylene glycol (70% v/v) in citrate buffer (50mL/100mL of solution, 0.1M, pH 3.0) with different pectin concentrations; 0.5% w/v (continuous line), 0.05% w/v (broken line). To facilitate comparison, the rotation of 0.05% pectin solution has been multiplied by 10. Only the 0.5% solution gelled at low temperature.
Experiment 1.3.2. Optical Rotation of High Methoxy Pectin Gels containing different Additives.

Similar graphs of optical rotation against temperature were obtained for high methoxy pectin (HM-1)-glycerol and high methoxy pectin (HM-1)-sorbitol gels. In the case of the sorbitol the contribution due to the sorbitol was eliminated by subtracting the readings which were obtained for the same sorbitol solution without the pectin present. Gels were prepared by the method in Experiment 1.1.1. (b) and the results are shown in Fig. 1.9. and 1.10.

Experiment 1.3.3. Optical Rotation of Fully Esterified Pectin.

The fully esterified pectin obtained in Experiment 1.1.4. was gelled with ethylene glycol and the gel to sol transition on heating was followed by optical rotation. The results are shown in Fig. 1.11. together with the optical rotation readings at different temperatures for the solution of pectin alone.

Experiment 1.3.4. Optical Rotation of Pectic Acid.

A sample of high methoxy pectin which had been de-esterified with alkali, (PA-1, 0.5% w/v) (see general methods) was dissolved in citrate buffer (0.1M) and heated to 100°C on a water bath. The sample was transferred to the optical rotation cell and readings taken as the temperature was lowered. In the sample at pH 2.0 there was definite evidence of aggregation at low temperature
Fig. 1.9. Changes in optical rotation on cooling solutions of high methoxy pectin (0.5% w/w, HM-1) in citrate buffer (50ml/100g of solution, 0.1M, pH 3.0) containing glycerol, A- 53% w/w and B- 80% w/w. Only B formed a gel at low temperature.
Fig. 1.10. Changes in optical rotation on cooling solutions of high methoxy pectin (0.5% w/w, HM-1) in citrate buffer (50ml/100g of solution, 0.1M pH 3.0) containing various amounts of sorbitol, A- 45% w/w, B- 60% w/w and C- 75% w/w. The rotations shown are those due to the contribution from the pectin alone, i.e., after subtraction of rotation due to sorbitol solutions in the absence of pectin. Only C formed a gel at low temperature.
Fig. 1.11. Changes in optical rotation on cooling solutions of fully esterified pectin (0.5% w/v), A- alone and B- containing ethylene glycol (70% v/v). B formed a gel at low temperature.
as the solution became opaque when viewed along the cell path. The results are shown in Fig. 1.12.
Fig. 1.12. Changes in optical rotation on cooling a solution of de-esterified pectin (0.5% w/v, PA-1) in citrate buffer (0.1M, pH 3.0).
Discussion

The experimental results given above can be explained in terms of the concept of junction zones in the gelation of pectin. For reasons indicated in the introduction to this chapter, these zones involve parts of the galacturonan backbone in "tie points" between macromolecules to cause gelation of pectins.

The galacturonan backbone can contain different proportions of methyl ester groups, undissociated acid groups, ionised carboxylate groups and in some cases, amide groups. The results now reported support the view that by providing suitable conditions, each of these variations can be encouraged to become part of an ordered zone. For example, pectin containing a high proportion of uronic acid groups as opposed to ester groups can be caused to gel with addition of ethylene glycol, provided the ionisation of the pectin is kept low. In the extreme case, a pectin containing no ester groups can be made to gel in this way by using a buffer solution at a pH of around 2. The importance of the rhamnose units present in the backbone as natural breaks in the regular ordered conformation can thus be seen. These "kinks" in the conformation provide a means of regulating the association of the pectin chains.
The gelation of pectins can be divided into three classes,

(a) those gels which require addition of large quantities of co-solute,

(b) those gels to which a divalent metal ion has to be added to induce gelation, and,

(c) pectin containing amide groups which gel under acidic conditions.

In class (a) gelation occurs on addition of various solutes (Table 1.5.) to solutions of pectin buffered to acidic pH. The requirement for an acidic environment has been explained in terms of the minimisation of the electrostatic repulsion between the polyelectrolyte chains which then allows association of these chains to occur.

It would now appear from the optical rotation of ethylene glycol - high methoxy pectin gels (discussed later), the optical rotation of pectic acids (Fig. 1.12) and the ability of pectic acid to gel in the presence of ethylene glycol at low pH, that the undissoilated acidic residues are actually involved in the junction zones themselves. A contribution from the undissoilated acid groups to the strength of association of the pectin in the junction zones would be in keeping with the resulting gel strengths of high methoxy pectins of different degrees of esterification. As stated in the introduction, increasing the degree of esterification decreases the gel strength.

Present evidence suggests that biopolymer molecules in solution are surrounded by a hydration shell
which have a mediating effect on the conformation which the biopolymer adopts in solution. The function of the additives in pectic gels can be understood in terms of the degree to which they effect this hydration and the nature of this influence.

Alteration of the solvent to include high concentrations of solute will necessarily markedly reduce the mole fraction of water available for hydration of polymer species. Also the solute molecules themselves may well be bound to water molecules to give hydrated species and thus also diminish the available water. These factors are reflected in measurements of the activity of the water present in the system. Calculation of water activities for sorbitol-water and glycerol-water systems of the same composition as in Table 1.5. did not however produce similar values, but in both cases the activities were very much reduced by the solute.

If ordered water structures are involved in the solvation of the random coil conformation or in the solvation of the junction zones then the addition of solute would cause breakdown or modification of this structure to an extent that varies with the nature of the particular solute. Contributions to these interactions may occur by different types of hydration, such as hydrophobic hydration in which alkyl residues or similar non-polar groups are known to have a structuring effect on water. The polar hydrophobic groups or atoms, eg. oxygen or nitrogen atoms would also affect the structured water by acting as proton acceptors for H-bonding of the water.
Apart from the role the additive plays in modification of water structure in the system, in the case of pectin containing uronic acid residues, change in the solvent composition would influence the degree of dissociation of these acidic groups. Since addition of these compounds to aqueous solutions of pectin would reduce the dielectric constant of the medium, a consequent reduction in the degree of dissociation of the acidic species in solution would be expected which would encourage gelation.

In experiment 1.1.1. pectin gels made with different concentrations of ethylene glycol appear to show a hysteresis on melting and setting. The different temperatures recorded for liquefaction and solidification may be explained by the differing and substantial rates of cooling and heating employed in the determinations. Extremely slow rates or extrapolation to an infinitely slow rate may eliminate the apparent hysteresis. The optical rotation against temperature for these gels also shows reversibility, Fig. 1.5.

The gels in class (b) depend on the interaction of divalent cation (Ca$^{2+}$ is usually employed) with the polyelectrolyte macromolecule. The gels can be prepared by different methods (Experiment 1.2.1.) which give rise to gels varying in their physical properties. Preparation hot by addition of calcium chloride solution to the buffered pectin solution produces gels on cooling which show distinct heat reversibility and have an opaque character in the gel state. Gels prepared by slower
diffusion of cation, eg. using dicalcium phosphate from which the calcium ions are slowly released after addition of acid, appear to be stronger, show more irregular and higher melting points and are less opaque.

These observations are consistent with the idea of microcrystalline junction zones already put forward to explain the existence of these gels. The increased strength of the gel with increase in calcium concentration (Fig. 1.4.) is thus explained by increased junction zone formation in the gel. A decrease in strength at very large concentrations of cation may be due to partial collapse of the gel towards a precipitate (ie. less network more junction zones).

The different cations show a definite tendency to cause ordered binding of pectin which is dependent on their ionic radius (Table 1.6.). An ion with ionic radius of around $12\text{R}$ is most efficient in causing gelation of pectin and ions with smaller (or larger in the case of $\text{Ba}^{2+}$) radii appear to be less efficient in binding into ordered regions. This suggests a requirement for a geometrical fit in the region of binding.

The third class of gel (c) would be expected to involve ordered junction zones also, in which the amide group possibly has an additional dominant role in the stabilization and strength of the association.

The optical rotation variations with temperature for high methoxy pectin gels containing a co-solute show a gradual and continuous increase in the rotation on cooling.
from a hot solution to a cold gel. These changes may be considered to be brought about by three factors,

1. Changes due to alteration of the linkage geometry on gelation and thus alteration of the linkage contribution \( \Lambda \) to the rotation,

2. Changes due to alteration of the solvent, e.g., in an ethylene glycol-water solvent system, replacing water may affect the partial rotatory contribution of some asymmetric groups in the molecule, and,

3. Changes due to changes in the contribution from the carbonyl chromophore of the uronic acid or ester brought about by gelation.

The observed linkage rotation has been calculated for pectin \(^7\) from consideration of the monomer, oligomers and the methyl glycosides and an exceedingly large anomalous contribution \( \Lambda_{\text{obs}} \) \( D \) 215 - 284° is obtained compared with a value of \( \Lambda_{\text{calc}} \) \( D \) -19° calculated from the dihedral angles from x-ray data. The empirical equation determines that the value for the linkage rotation cannot possibly exceed a value of 135° and is unlikely on steric grounds to exceed 100°. Thus it would appear that, in the case of the uronic acid in pectin, a very large partial rotation contribution from the carbonyl chromophore swamps the optical rotation contribution from interactions across the linkage. It is therefore not possible to observe by optical rotation studies alone these changes in linkage geometry if they do in fact occur.
Alteration of the solvent by addition of ethylene glycol or a similar less polar solvent might be expected to increase the influence of the polar interactions present in the pectin. Enhancement of these interactions could change the conformation and hence the optical rotation of the solution.

The most likely explanation of the gross changes observed in these systems is that they are due to changes in the contribution from the chromophore as these regions of the backbone form junction zones.

For the fully esterified pectin gel with ethylene glycol (Fig. 1.11.) the optical rotation increases continuously with temperature without showing any evidence of a cooperative change on the onset of gelation. Melting and setting behaviour, however, suggests that gelation is a cooperative process, so we therefore have to conclude that either,

1. junction formation is not cooperative and cooperativity is to be explained in terms of a Stockmayer mechanism\(^5\), in which junction zones are formed and broken over the whole temperature range or,
2. the junction zones are formed in a cooperative manner but we are unable to observe this by optical rotation.

Gelation of high methoxy pectin, which contains acid groups in buffer at a pH of 3.0, with ethylene glycol shows an increasing sigmoidal character in the optical rotation versus temperature curve as the concentration of glycol increases.
Since addition of ethylene glycol would decrease the degree of dissociation of these acidic groups in the pectin and as pectic acid alone in buffer solution at pH 3.0 shows a similar although less emphatic sigmoidal change in optical rotation (Fig. 1.12), it would appear that the enhancement of cooperativity might involve the carboxylate groups in the chain. These high methoxy gels also show sharper (ie more cooperative) behaviour in melting and setting compared to fully esterified pectin. The optical rotation for gels including buffers of different pH also show this tendency to more cooperative behaviour as the pH is lowered. The optical rotation studies would therefore seem to support the view that non-esterified uronic acid residues are involved in the junction zones and enhance the cooperativity.

In general, increased cooperativity is to be explained by

(a) longer lengths of polymer involved in junction zones,
(b) larger aggregates forming, or,
(c) greater enthalpy of formation.

(a) is hardly likely as the pectin used was the same in both cases, apart from the difference in degree of esterification. It is probable that increased enthalpy is the reason as these groups may enhance the exothermic association of chains by H-bonding in the junction zones.
It now appears that optical rotation studies are not as informative as was first hoped due to the presence of the very large contribution of the chromophore. This large contribution is in keeping with the relatively stiff type of chain which would be expected to occur in solution. Association of these chains could thus be envisaged with a minimum alteration of conformation and therefore little effect on the optical rotation.

If more detailed information about the packing and geometry of the junction zones is to be obtained then studies of the circular dichroism of these gels should be carried out which can yield information on the environment of the chromophore itself without background contributions from other groups present. Such studies are described in Chapter II.
Chapter II

Introduction

In recent years circular dichroism has developed into an important spectroscopic technique for studying the configuration and conformation of certain organic molecules.

Circular dichroism is similar to optical rotation being a measure of the interaction of circularly polarised light with a dissymmetric molecule, but for circular dichroism, the difference in absorption of left and right circularly polarised light by specific chromophores is measured. Thus for circular dichroism to be applicable, the compound must either contain a chromophore which absorbs in the ultraviolet or visible region of the electromagnetic spectrum or such a chromophore must be introduced into the molecule.

The dichroism arises from the different absorption coefficients of the medium for left and right polarised light which results in the light wave becoming elliptically polarised (Fig. 2.1.). The ellipticity per unit length is given by

$$\theta = \frac{\Delta}{\lambda} (k_l - k_r)$$

where $\theta$ is in radians and $k_l$ and $k_r$ are the absorption coefficients for left and right polarised light respectively. For comparison purposes the molecular ellipticity $[\theta]$ is usually quoted

$$[\theta] = \frac{\theta \times 18 M}{C}$$

where $C$ is the concentration in g/cc, and $M$ is the molecular weight.
Fig. 2.1. Effect of an optically active absorbing sample on plane polarised light.

$\alpha$ - optical rotation.
$\theta$ - circular dichroism.
The optical rotatory dispersion curve arising from a specific transition can be calculated from the circular dichroism spectrum by the Kronig-Kramers transformation equations 87. The position of the circular dichroism maximum may also be calculated by applying the Drude equation 88 to the optical rotatory dispersion curve. The two techniques can thus be used in conjunction. The forms of the individual contributions of a chromophore to the spectra recorded by these techniques are shown in Fig. 2.2.

Since their development, these techniques have been used chiefly to investigate the carbonyl chromophore in rigid asymmetric structures such as steroids and polycyclic terpenes. The keto group has a weak absorption band at about 300nm which has been assigned to the $n \rightarrow \pi^*$ transition. This transition is fairly sensitive to induced asymmetry and therefore is very suitable for circular dichroism studies. The effect on the circular dichroism of the carbonyl chromophore by the other substituents in the molecule close by it, has been formulated into empirical rules such as the Carbonyl Octant Rule 89. This rule allocates the sign of the contribution of neighbouring groups depending on their spatial distribution relative to the carbonyl. Several other similar empirical rules have also been developed for other chromophores.

Apart from these rules which deal mainly with interpretation in terms of configuration in rigid molecules, circular
Fig. 2.2. Idealized O.R.D., C.D. and U.V. spectra for an isolated chromophore. Left, dextrorotatory molecule; right, laevorotatory molecule.
Dichroism is also extremely sensitive to the distribution of different conformational forms in solution. Changes in the population of different states, induced by changes in temperature, are reflected in the circular dichroism spectrum. Circular dichroism studies have been used extremely successfully to study conformation of proteins and polypeptides where, in some cases, spectra can be interpreted in terms of quantitative contributions from the three basic ordered structures present, α-helix, pleated sheet and random coil.

In the field of carbohydrate chemistry application of circular dichroism has been more restricted due to the absence of suitable chromophores which may be studied. In simple unsubstituted monosaccharides, such as glucose, a spectral band begins around the lower limit of present instrumentation although recently the extrema of these bands have been recorded on a vacuum ultraviolet circular dichroism spectrometer. In many cases chromophores must therefore be introduced into the compound under study by preparation of derivatives, dye binding or complexing with metal ions. However, a few accessible chromophores do exist in some monosaccharides and related natural polysaccharides, such as the N-acetyl groups of certain acetamidodeoxy sugars, and the circular dichroism of these molecules and the application of the technique to polysaccharides in general has been reviewed by Morris and Sanderson.
For example, N-acetyl - D - glucosamine and N-acetyl - D - galactosamine both show similar negative circular dichroism bands centred around 210nm while N-acetyl - D - mannosamine gives a band of opposite sign. N-acetyl muramic acid which is a major component of peptidoglycans of bacterial cell walls shows a circular dichroism spectrum which is a combination of the contributions due to the carbonyl of the muramic acid and the N-acetyl chromophore.

Studies of the circular dichroism and optical rotatory dispersion of oligosaccharides and mucopolysaccharides containing these groups can be useful in furnishing additional evidence in determination of structure and also in determining the nature of linkages and type of substitution present in sugar units.

Uronic acids, the type of monomer unit involved in the pectic polysaccharides, have also been studied by circular dichroism and the sign of the dominant band has been shown to depend mainly on the configuration at C5. Methyl-α-D-glucuronoside and galacturonoside show a band around 210nm which is typical of the carbonyl n → π* transition. However, for both methyl-α-D-glucuronoside and methyl-α-D-mannuronoside which have an equatorial hydroxyl group at C4, a negative band occurs at higher wavelength which is not observed in the corresponding glycosides with this group axial. It has been postulated that this negative band is also due to the n → π*
carbonyl absorption but that it represents a particular rotational conformation of the carboxyl group about the C_5 - C_6 bond. If the ring oxygen lies in the plane of the chromophore, stabilisation of the excited state of the carbonyl by conjugation with the non-bonded orbitals of the ring oxygen has been suggested with consequent lowering of the energy of the excited \( \pi^* \) state and thus of the transition itself. This conformation would be favoured by hydrogen bonding between the carboxyl and the hydroxyl group at C_4 when this is equatorial. This explanation is in accord with the increase in the negative band in changing to a non-polar solvent which would encourage H-bonding. At lower wavelength (190nm) the onset of a large band occurs which, in the case of aldohexoses, has been attributed to excitation of the unshared electrons of the pyranoid oxygen and appears to be influenced by the configuration at C_4 but, in the case of the uronic acids, the carbonyl \( \pi - \pi^* \) transition is likely to make a stronger contribution in this spectral region.

Circular dichroism is therefore sensitive to the conformations adopted by the chromophore in the uronic acids, which encouraged the investigation of polysaccharides containing these monomer units. A study by circular dichroism of alginate, a seaweed polysaccharide containing D-mannuronic acid and L-guluronic acid, has been undertaken by Thom, in parallel with the present study of pectins to be described in this chapter.
The polysaccharide structure is based on $\beta-1,4$ linked D-mannuronopyranose in the C1 ring form and $\alpha-1,4$ linked L-guluronopyranose in the C2 ring form. These residues have been further shown to exist in a blockwise arrangement containing regions of guluronic acid, regions of mannuronic acid and regions where guluronic and mannuronic acids alternate.

The circular dichroism spectra of the two monomers shows bands of opposite sign and this difference is also maintained in the spectra of the isolated blocks of guluronic acid and of mannuronic acid. Consequently the composition of alginates can be characterised by mixing of the "canonical spectra" of the three possible block structures to give the observed spectrum of the polysaccharide.

Gelation of alginate with calcium ions has been shown to result in a change in the circular dichroism spectrum which has implicated the guluronic acid blocks as the part of the molecule involved in the junction zones on gelation. This conclusion would also be expected from the different binding affinities of Ca$^{2+}$ for alginates of different compositions.

In this chapter, experimental studies using circular dichroism have been carried out on the gelation of several pectin samples, and results, together with the results of conformational and X-ray crystallographic studies available and ion binding information, have allowed a model of the nature of the junction zones present in pectin gels to be postulated.
**Experimental**

**Experiment 2.1 Circular Dichroism of Pectin Solutions**

(a) **Comparison of Different Pectins**

Solutions of various pectins (0.05% - 0.5% w/v) as their sodium salts were made up in distilled water, and their circular dichroism spectrum recorded on the Cary 61 spectropolarimeter as described in the general methods section.

The spectra of all pectins recorded contained a positive peak starting at a wavelength around 250nm and showing a maximum in the region 200 - 210nm similar to that recorded for the methyl-α-D-galacturonoside. It was not possible to record the low wavelength end of this band as this was outside the scope of the instrument.

The differences in the spectra observed for different pectin samples were not very marked. In Fig. 2.3 the influence of increasing sodium uronate content is shown. The fully esterified sample was prepared from the high methoxy pectin (HM-1) shown and the completely de-esterified sample was also prepared from this pectin by saponification with sodium hydroxide (see general methods).

The spectra of several other samples of low methoxy pectin as their sodium salts were also recorded, and these were essentially very similar to high methoxy pectin (HM-1) having the peak maxima at a slightly lower wavelength, 203 - 205nm compared to 207nm. Pectin samples prepared by de-esterification of these low methoxy pectin samples had spectra identical to the de-esterified sample shown.
in Fig. 2.3, having a peak maxima at 202nm.

The spectrum of amide pectin (Fig. 2.14) which contains amide groups at C6 as well as ester and acid residues, is also similar to the above spectra, but differs in showing the onset of a large negative peak at low wavelength. The maximum for the positive peak occurs at 208nm.

(b) Influence of pH.

The circular dichroism of a sample of de-esterified pectin from high methoxy pectin (PA-1) was recorded at various pH’s by addition of dilute acid (1N-HCl) or alkali (1N-NaOH) to the solution. The maximum of the positive peak was seen to change from 202nm for the neutral solution to 211-212nm for the acidic solution at a pH of less than 3 (Fig. 2.4). Similar changes in circular dichroism with change in pH were observed for other pectin samples containing non-esterified residues. The circular dichroism of the fully esterified pectin, however, was found to be independent of pH in the range 2-7.

(c) Influence of Temperature.

The effect of increase in temperature on the circular dichroism of several pectins was investigated. In all cases a decrease in the amplitude of the spectrum was observed as the temperature increased. For fully esterified pectin (Fig. 2.5), high methoxy pectin at acidic pH (Fig. 2.6) and high methoxy pectin at neutral pH the magnitude of the change appears to be very similar.
Fig. 2.3. Circular dichroism spectra of solutions of pectin (0.05% w/v) with various methyl ester content. A, fully esterified pectin from HM-1; B, high methoxy pectin, HM-1; C, pectic acid from HM-1. Solutions were run in a 10mm path length cell at 25°C.
Fig. 2.4. Changes in circular dichroism spectra with change in pH for a solution of pectic acid (0.05% w/v, PA-1). A - pH 6.5, B - pH 4.2 and C - pH 2.8. Solutions were run in 10mm path length cell at 25°C.
A solution containing fully esterified pectin (0.5% w/v) and ethylene glycol (70% v/v) was transferred hot to a circular dichroism cell (1mm path length) and then allowed to set to a gel. The circular dichroism was recorded at 25°C and then at 90°C after heating to this temperature to melt the gel. A similar procedure was followed in recording the spectra of the pectin solution in the absence of ethylene glycol. The results are shown in Fig. 2.5.

An identical experiment was carried out with a gel containing high methoxy pectin (HM-1, 0.5% w/v) in citrate buffer (40% v/v, 0.1M, pH 3.0) and ethylene glycol (60% v/v). The results of the circular dichroism studies are shown in Fig. 2.6.

The circular dichroism of this gel was recorded at several temperatures, and a graph of ellipticity maximum against temperature produces a curve (Fig. 2.7) similar to that observed in the optical rotation studies on this gel (Fig. 1.6).

A second high methoxy pectin (HM-2) was prepared from the fully esterified sample by partial de-esterification with acid (see general methods) to 80% esterification. The circular dichroism of the gel at 25°C and the sol at 90°C formed by this pectin (HM-2, 0.5% w/v) in citrate buffer (30% v/v, 0.1M, pH 2.5) with ethylene glycol (70% v/v), were recorded. The increase in amplitude on changing from the sol to the gel was very similar to that occurring for the
Fig. 2.5. Changes in circular dichroism spectra with change in temperature for an aqueous solution of fully esterified pectin, (0.5% w/v) [broken line] and for a gel containing ethylene glycol, (70% v/v), [continuous line]. Cell path length, 1mm.
Fig. 2.6. Changes in circular dichroism spectra with change in temperature for a solution of high methoxy pectin (0.5% w/v, HM-1) in citrate buffer (40ml/100ml of solution, 0.1M, pH 3.0) [broken line], and for a gel containing ethylene glycol (60% v/v) [continuous line]. Cell path length, 1mm.
Fig. 2.7. Graph of ellipticity maximum against temperature for the high methoxy pectin - ethylene glycol gel in Fig. 2.6.
fully esterified sample (Fig. 2.5). However, the change in optical rotation with change in temperature showed more cooperative behaviour (Fig. 2.8) similar to that observed for the original high methoxy pectin, HM-1 (Fig. 1.6), possibly confirming that this results from the presence of the acidic groups.

**Experiment 2.3. Studies of the Gelation of Pectin with Calcium Ions by Circular Dichroism.**

From experiments described in Chapter I it was found that the most suitable method of forming clear calcium pectate gels was by dialysis in calcium chloride solution. For circular dichroism studies, solutions of pectin (0.2 – 2.0% w/v) were introduced into a glass cell (1mm - path length) and the aperture sealed by covering with dialysis membrane. The cell was then immersed in a calcium chloride solution. The circular dichroism of the pectin was recorded before dialysis and at appropriate intervals during dialysis until the spectrum was found to remain unaltered.

The changes in the circular dichroism spectrum for de-esterified pectin from LM-2 as Ca$^{2+}$ ions are dialysed into the cell are shown in Fig. 2.9. The positive peak appears to be reduced in amplitude and its maximum is shifted to a lower wavelength, close to the limit of the spectrometer's resolution. When the spectrum of the pectin after dialysis is subtracted from the original solution spectrum, a gaussian curve results which has a maximum at 208 – 209nm. The same difference curve resulted when other de-esterified samples were used.
Fig. 2.8. Changes in optical rotation on cooling a solution of high methoxy pectin (0.5% w/v, HM-2), [ex. fully esterified pectin], in citrate buffer (30ml/100ml of solution, 0.1M, pH 2.5) containing ethylene glycol (70% v/v). The solution gelled at low temperature.
Fig. 2.9. Circular dichroism spectra of a solution of pectic acid (0.5% w/v, PA-2) and of the gel after diffusion of Ca^{2+} to a concentration of 60mM. Cell path length, 1mm. The broken line shows the difference spectrum.
In the case of low methoxy pectins (Fig. 2.10) a similar shift of the circular dichroism spectrum occurs on dialysis against calcium, but the resulting difference spectra, although very similar to the curves obtained for the de-esterified pectins, having a similar gaussian shape and peak maximum at around 208 - 209nm, are smaller in amplitude.

Amide pectins also gel on dialysis against calcium, but the changes occurring in the circular dichroism are more complex (Fig. 2.11). The difference spectrum is initially, at high wavelength, very similar to that for non-amidated pectin. But at low wavelengths the spectrum for the gelled pectin has changed from strongly negative in the solution to positive and thus exhibits the onset of a negative band in the difference spectrum. This region of the spectrum can be seen to be characteristic of the amide group by comparison of amidated and non-amidated pectin samples and thus the changes observed on calcium gelation seem to suggest the involvement of the amide group in the gelation mechanism.

Experiment 2.4. Gelation of Pectin with other Divalent Cations.

Gelation of de-esterified pectin from LM-2 (0.5% w/v) with other divalent cations was monitored by circular dichroism as described above for calcium.

Several cations, Ba$^{2+}$, Sr$^{2+}$ and Ni$^{2+}$ (60mM solutions) were found to have a very similar effect to that of Ca$^{2+}$ on the
Fig. 2.10. Circular dichroism spectra of a solution of low methoxy pectin (0.5% w/v, LM-2) and of the gel after diffusion of Ca$^{2+}$ to a concentration of 60mM. Cell path length, 1mm.
The broken line shows the difference spectrum.
Fig. 2.11. Circular dichroism spectra of a solution of low methoxy amide pectin (0.5% w/v) and of the gel after diffusion of Ca$^{2+}$ to a concentration of 12mM. Cell path length, 1 mm. The broken line shows the difference spectrum.
circular dichroism of the pectin. The amplitude of the positive peak reduced in size which gave rise to difference spectra, similar to that found for calcium, having a peak maximum between 206 - 209nm.

Two other divalent cations, Mg$^{2+}$ and Cu$^{2+}$ did not however comply with this general picture. The dialysis of Mg$^{2+}$ (12mM) into low methoxy pectin (LM-2, 0.5% w/v) resulted in a very small change in the circular dichroism spectrum which can be correlated with the observation that magnesium does not readily cause gelation or precipitation of pectin even at substantial concentrations of cation.

Cu$^{2+}$ cations cause extremely rapid gelation of pectin but in contrast to the above cases the circular dichroism spectrum of low methoxy pectin (LM-2, 0.2% w/v, 12mM Cu$^{2+}$) was found to increase in amplitude and the transition originates at a much higher wavelength, 300nm (Fig. 2.12) as opposed to 245nm for calcium gelation. Under different conditions, however, with de-esterified pectin from LM-2 (0.5% w/v, 60mM Cu$^{2+}$) a more specific change in the spectrum occurred. Initially the amplitude decreased as for the other divalent cations but as gelation proceeded a broadening of the transition also occurred.

**Experiment 2.5. Circular Dichroism Studies on the Gelation of Amide Pectin by Acid.**

Gelation of amide pectin in the circular dichroism cell was also achieved by dialysis in hydrochloric acid (12mM).
Fig. 2.12. Circular dichroism spectra of a solution of low methoxy pectin (0.2% w/v, LM-2) and of the gel after limited diffusion of Cu$^{2+}$. 
The gelation was followed as in the previous experiments.

The amplitude of the circular dichroism peak for amide pectin increased in size on dialysis against the acid and the peak maximum shifted from 208nm to 214nm. A similar change was observed with the de-esterified sample of amide pectin (Fig. 2.15.).
Fig. 2.13. Circular dichroism spectra of a solution of de-esterified amide pectin (0.5% w/v) and of the gel after diffusion of hydrochloric acid to a concentration of 12mM. Cell path length, 1mm.
Discussion

The circular dichroism studies on pectins reported above, together with other supporting evidence now suggest a more detailed explanation of the gelation of these substances in terms of a network of crystalline junction zones. By consideration of the possible nature of association of these crystalline zones, a more exact model can be proposed.

In experiment 2.1 the circular dichroism spectra for solutions of several different pectin samples were recorded, and these show very similar spectra to those recorded for the methyl-\(\alpha\)-D-galacturonopyranoside which represents the monomeric unit present in pectin. This suggests that the absorption is due to the same proposed \(n \rightarrow \pi^*\) transition of the carboxyl chromophore present at \(C_5\). The slight differences in spectra with change in composition of the pectin sample (Fig. 2.3) can be explained by considering the separate contributions to the circular dichroism of the constituent carbonyl-containing chromophores, i.e. the methyl ester, the sodium salt of the acid and the undissociated acid. The \(n \rightarrow \pi^*\) transition in each of these groups would be expected to require different energies and thus would show bands in the circular dichroism with maxima at different wavelengths. In the fully esterified pectin this maximum occurs at 210nm (Fig. 2.3) while the maximum for the sodium salt of the completely de-esterified sample occurs at 202nm. When the pH of this solution is lowered the maximum is found
to shift from 202nm to 212nm as the degree of
dissociation of the carboxyl groups in the pectin
decreases.

Pectins containing different degrees of esterification
show circular dichroism bands which fall between the two
extreme cases shown in Fig. 2.3. The circular dichroism
is therefore sensitive to the ester content of the
individual pectin but the nature and magnitude of the
observed differences drastically limit the accuracy of
any attempt to resolve spectra on this basis. Even less
accessible is information regarding the intramolecular
distribution of the chromophores.

On gelation of fully esterified pectin with ethylene
glycol the circular dichroism shows an increase in
amplitude while remaining a broad positive band in
essentially the same position (Fig. 2.5). This increase
in the optical activity can be interpreted in terms of
the incorporation of polymer chains into junction zones
with a consequent "locking" of the conformation of the
ester groups by restriction of rotation around the C5-C6
bond and thus contributing to an overall narrowing of the
distribution of these rotational states. This alteration
from the statistical distribution of rotational conformers
in solution to the distribution heavily biased in favour
of the specific conformer in the junction zones results
in the increased amplitude without affecting the position
or width of the n → n* transition. In contrast, the
change occurring when the non-gelling pectin solution in
the absence of glycol is heated through the same temperature range, is much less emphatic. Here the spectrum (Fig. 2.5) was only slightly increased in amplitude on cooling which would be expected for a general decrease in the rotational distribution of the chromophore with lowering of temperature rather than the specific ordering of the gel state.

In the gelation of high methoxy pectin with ethylene glycol the increase in the amplitude of the circular dichroism band for the sol to gel transition is significantly greater than that for the fully esterified sample (Fig. 2.6). The gross changes can be explained in the same manner as above, but this difference in the circular dichroism behaviour may be correlated with the presence of acidic groups in the polymer molecule and specifically with the incorporation of these groups into the junction zones as already suggested in Chapter I. The carboxyl group would be incorporated into the association in a rigid conformation which may possibly contribute to the stability by involvement in hydrogen bonding. Fig. 2.7 shows the variation in the amplitude of the circular dichroism for the high methoxy pectin as the temperature varies. The resultant curve has a sigmoidal shape similar to the optical rotation - temperature curves for these gels (Fig. 1.6) which again implies the more cooperative nature of this gelation process.
An alternative explanation for the smaller increase in amplitude on gelation of fully esterified pectin may concern possible differences in molecular weight of this sample and the high methoxy pectin from which it was prepared. Reduction of molecular weight could lead to fewer residues being involved in the junction zones on gelation which would thus cause a decrease in the circular dichroism change when gelation takes place. Such a reduction in molecular weight on treatment of pectin with diazomethane, using similar conditions to those employed here, has been suggested by both Neukom and Smit. Analytical centrifugation of the present fully esterified sample indicated that slight depolymerisation may have occurred but this could not have been so extensive as to inhibit gelation even at very low concentrations of pectin (Experiment 1.14).

The results from circular dichroism and optical rotation of the acid de-esterified sample showed that although the increase in the amplitude of the circular dichroism on gelation was similar to that for the fully esterified sample, the introduction of acidic groups into the pectin made the gelation process more cooperative (Fig. 2.8) confirming this role for the acidic residues in gelation.

Similar spectral changes arise in the gelation of pectins containing amide groups where gelation is brought about by dialysis against acid. Here also the amplitude of the circular dichroism is found to increase markedly (Fig. 2.13).
Although the transition shifts from a maximum at 207-208 nm to 213-214 nm as the pectin is converted from the salt form to the undissociated acid, the large increase in amplitude can be accounted for in terms of the explanation already put forward for the highly esterified pectin gel. The role of the amide group in the gelation could not be further established by circular dichroism, as the most easily recognised part of the contribution from this chromophore appears to originate at the less accessible lower wavelength region.

When de-esterified pectin is gelled by dialysis against Ca$^{2+}$ solution, a very different change is observed in the circular dichroism spectrum (Fig. 2.9.). Here the band, in contrast to the highly esterified samples, decreases in amplitude and the maximum shifts to a wavelength below 200 nm. When the change occurring during gelation is calculated by subtracting the spectrum for the gel from that for the initial solution, a difference band results which is essentially gaussian and centred around 208 nm (Fig. 2.9.). We can now interpret this difference spectrum in terms of the cooperative involvement of the chains of uronate residues in coordination with calcium cations to form specific ordered regions providing junction zones between the pectin chains.

The change in the spectrum on gelation can be considered as the appearance of a much less positive, zero or negative n $\rightarrow$ $\pi^*$ band for the carbonyl chromophores as they associate into the junction zones with a corresponding disappearance of part of the sodium pectate spectrum and thus a consequent
decrease in the overall observed n → ν* band. This change can be explained by reference to the "octant type" effect of substituents around the chromophore which involves the division of the space surrounding the chromophore by its plane of symmetry and the allocation of a definite contribution to the sign of the circular dichroism by each of the substituent parts of the molecule depending on the area of space into which they fall. Work by Klyne has indicated that the sign of the contribution from a substituent is also dependent on its electronegativity, which gives rise to the term "anti-octant" behaviour. Recent X-ray studies of the crystal structure of sodium calcium and sodium strontium galacturonates have shown that the divalent ion is located (Fig. 2.14) at the apex of a pyramid involving the O⁵, O⁶ and the oxygen of a water molecule. If a similar situation occurs in the polymer when calcium ions complex with the uronic acid residues, then the strongly positively charged cation will be in proximity to the partially negatively charged pyranoid oxygen which has the dominant influence on the sign of the observed spectrum of the pectin solution. A strongly positively charged entity in this location therefore causes complex perturbation of its environment resulting in a large contribution to the circular dichroism, of opposite sign to that of the pyranoid oxygen, and thus a consequent decrease or reversal of the overall spectrum.

In gelation of low methoxy pectins with calcium, changes
Fig. 2.14. Stereochemical relationships in the crystal structure of calcium galacturonate.

Fig. 2.15. Proposed "Egg-box" model for gelation of pectins with divalent calcium ions.
in the circular dichroism occur which can also be explained by complexing of the cation with acidic residues into ordered regions. The changes on gelation in this case (Fig. 2.10) are not so emphatic and a significant positive contribution still remains at high wavelengths compared to the fully de-esterified sample. The difference spectrum is therefore smaller but essentially very similar. These facts suggest that the ester groups in the molecule are not involved in the junction zones when this pectin is gelled with calcium.

When pectin was gelled with several other divalent cations similar changes were observed in the circular dichroism and for the Sr, Ba and Ni, these changes originate from similar ordered junctions where these cations replace calcium ions. In the case of Cu²⁺ cation, this is not always so and much less specific ordering can result in an increase in the amplitude of the spectrum and a significant broadening of the transition to include much higher wavelengths (300nm). This would seem to indicate strong complexing of a much less specific nature than for the above cations. Similar strong complexing is frequently observed for Cu where the interaction in one case has been found strong enough to induce distortion of the xylose ring in a xylose-serine-Cu complex Which gives rise to non-equivalent sugar residues and a consequent deviation from symmetry.

Dialysis against calcium also causes gelation of amide
pectin and the changes observed by circular dichroism (Fig. 2.11) indicate that here too the uronic acid residues are complexing with cations to produce ordered intermolecular association. There also appears a simultaneous change in the low wavelength part of the spectrum which changes from strongly negative to positive. On comparing this with non-amidated pectins, this area of the dichroism can be correlated with the amide group and so the changes on gelation indicate that complexing with calcium or ordering of these groups also occurs on gelation.

The specific interactions between pectin and divalent cations can now be explained in terms of an "egg-box" model (Fig. 2.15). The polygalacturonate chains in the pectin align in a regular ordered conformation and cooperative intermolecular association is brought about by the divalent cations which occupy regular interstices between the chains.

The exact site of the cation is suggested by the X-ray crystallographic structures already mentioned in the explanation of the circular dichroism results, where favourable coordination of divalent cations with sugar residues occurs at O6, O5 and the oxygen of a water molecule. This type of triangular coordination has also been shown to occur between Ca2+ and oxygen functions in other compounds. The calcium cation may therefore be expected to complex with a suitably arranged set of oxygen atoms in each pectin chain. Computer model
building studies\textsuperscript{113} suggest that coordination with triangles of oxygen atoms is indeed possible, involving the $O_6$ and $O_5$ and the $O_2$ or $O_3$ of either of the adjacent residues while maintaining twofold chain symmetry. The ability of the individual chain to associate into junction zones would then be influenced by the size of the cation which complexes with an oxygen triangle in each of the two chains associating.

This model for binding of the polyelectrolyte chain to different cations has also been successfully applied to the gelation of alginate polysaccharide where the selectivity of the polyguluronate blocks for specific cations in preference to polymannuronate is explained by the radius ratio effect\textsuperscript{114}, implied in this type of model.

The cooperative nature of the interaction between galacturonate chains and calcium cations is also suggested from ion binding studies on oligogalacturonates where calcium ion activity in solution shows a stepwise decrease beyond a certain threshold degree of polymerisation\textsuperscript{115}. For calcium pectate, the calcium activity coefficient\textsuperscript{116} was found to be very low compared to that of the monomer and of the same order as for polymethacrylate which has a significantly greater linear charge density. The stability constants for calcium pectates also show a similar stepwise dependence on the degree and distribution of ester groups present in the pectin molecule\textsuperscript{117}.

In pectin gelation, therefore, the polygalaoturonic acid
backbones associate by adopting a regular geometry in which favourable interactions are encouraged by complexing with the divalent metal cation. The presence of an amide group or an undissociated acidic group can be easily accommodated in this structure but for the much bulkier ester group, interactions would appear to be unfavourable and this group therefore inhibits the gelation. Although gelation of highly esterified pectins, in the absence of cations, can be thought of as occurring from a similar association of chains, the detailed geometry may be different. Here, the methyl ester groups play a part in the association which can also involve amide and undissociated acid functions but electrostatic repulsion prevents the incorporation of carboxylate groups.
Chapter III

Studies on a Synergistic Polysaccharide System.
Introduction
In this chapter some investigations of a polysaccharide system involving interaction between the ordered conformations of two different macromolecules are described.

This system, in which reversible changes in conformations and association occur between agarose or carrageenan and galactomannans, has been postulated as a model system for association of similar polysaccharide tertiary conformations in the natural cell wall and thus as a model for the biological cohesion involved between the skeletal phase and the gel phase of the matrix.

Kappa carrageenan in an ordered double helical tertiary structure and agarose which, on present evidence, also appears to adopt a similar type of ordered structure, interact with a galactomannan polysaccharide, locust bean gum, to form firm gels at concentrations of carrageenan and agar at which no gel would normally exist in the absence of the galactomannan.

This synergistic interaction between two unlike polysaccharides which possess ordered tertiary structures is the first example of a quaternary structure for polysaccharides.

Carrageenan and agar are matrix polysaccharides which are extracted from red seaweeds of the class, Rhodophyceae. The idealized structures of kappa carrageenan and agarose are shown in Fig. 3.1. The carrageenan consists of a
AGAROSE (idealized)

KAPPA CARRAGEENAN (idealized)

Fig. 3.1.
repeating disaccharide unit, a 1,4 linked – 3,6 – anhydro – α – D – galactopyranose and a 1,3 linked – β – D – galactopyranose – 4 – sulphate. The 3,6 – anhydro unit can also exist as the 2-sulphate or be replaced by α – D – galactopyranose – 6 – sulphate. In agar the idealized structure is represented by a repeating disaccharide unit containing 1,4 linked – 3,6 – anhydro – α – L – galactopyranose and 1,3 linked β – D – galactopyranose 118. The idealized structure is usually modified by the presence of L-galactose sulphate in the place of the 3,6-anhydro residues 119 or there may also be partial replacement of D-galactose residues with the pyruvic acid acetal, 4,6 – 0 – (1-carboxy-ethylidene) – D – galactose 120. Agar therefore exists as a complex spectrum of polysaccharides, ranging from the virtually neutral molecule, agarose, to a highly changed galactan121.

The galactomannans may be regarded as a family of polysaccharides which are obtained from various leguminous seeds such as the locust bean or carob bean (Ceratonia siliqua). The covalent structure of these polymers is based on a linear β – 1,4-linked mannopyranose backbone to which α – 1,6 linked galactopyranosyl residues are attached 122. Evidence from enzymic hydrolysis indicates that these galactose units are not distributed along the backbone in a random fashion but occur in blocks as represented in Fig. 3.2. Courtois and Le Dizet 123 studied the action of α- galactosidases and β- mannosidases on various galactomannans and concluded that their
Fig. 3.2. Schematic representation of the binding of the unsubstituted mannan regions (B) of galactomannans with helical polysaccharides (A). The regions of the galactomannan carrying galactose substituents (C) provide the connections between helices.
galactosidase hydrolysed off the 1,6 linked galactose residues which were next to an unsubstituted mannose residue. The mannosidase yielded low molecular weight oligomers of mannose identical to those which would occur from treatment of a pure $\beta$-1,4 linked mannann and also a non-dialysable fraction which when analysed had a mannose to galactose, M/G, ratio of 1. When fenugreek galactomannan, which had an M/G of 1.08, was treated with the $\alpha$-mannosidase, no reaction was detected. However, initial treatment with the $\alpha$-galactosidase increased the M/G to 1.2 indicating that the enzyme must have hydrolysed off some of the galactose substituents to leave unsubstituted mannose residues in the molecule. Subsequent treatment with the $\beta$-mannosidase was then possible which gave a non-dialysable fraction in the product which also had an M/G ratio of 1.

These results were therefore interpreted in terms of the block structure shown in Fig. 3.2. The amount of galactose branching and hence the block arrangement varies depending on the source of the galactomannan, eg. the amount of galactose relative to mannose increases progressively from locust bean gum, to tara (Caesalpinia spinosa), to guar (Cyamopsis tetragonolobus) to fenugreek (Trigonella foenum-graecum).

The gelation of the galactomannan with carrageenan or agar has been postulated as being due to a structure resulting from the association of chain segments of the carrageenan or agar into "junction zones" which are then
joined together by interaction with galactomannan chains which run through two or more junction zones (Fig. 3.3.). This then allows the galactomannan to provide additional network and thus strengthen the gel.

The unsubstituted regions of the mannan backbone have been implicated in this association as the galactomannans with high proportions of galactose, e.g. fenugreek where almost every mannose residue is substituted by galactose, shows very little, if any, tendency to synergise while the most successful interaction occurs when the $M/G$ ratio is high as is the case for locust bean gum.

It has been established both from X-ray evidence and by optical rotation studies as discussed in the introduction to Chapter I that the carrageenan exists as a double helix in the gel state and that a three dimensional network of these helices is encouraged by the existence of helix terminating "kinks" in each chain which are due to replacement of the $3,6$-anhydro residues by $6$-sulphate containing units, and which appear to be involved in the biological control of the tissue texture. Similar kinks are present in the agar molecule arising from the replacement of some $3,6$-anhydro residues by $L$-galactose sulphate residues.

Selective cleavage of these kinks in iota carrageenan by periodate oxidation, borohydride reduction and subsequent hydrolysis produces segmented carrageenan which has been shown by optical rotation, osmometry and light scattering to exist as double helices in
solution. Addition of galactomannan to similar segments from kappa carrageenan causes gelation, which can be followed by optical rotation and shows a similar shift to that which occurs for the native carrageenan - galactomannan gel, thus providing evidence for the gelling interaction.

Segments formed from agar by similar cleavage were also made to gel by addition of galactomannan.

The optical rotation against temperature for agar in the presence of galactomannan (Fig. 3.4.) compared to that for an agar solution indicates the existence of a conformational change in this synergistic system which may be due to an ordering of the conformation of the galactomannan as gelation occurs. The galactomannan in solution alone did not appear to undergo any such conformational change on lowering the temperature as no change is observed in the optical rotation. The optical rotation shift which occurs on gelation of agar has been interpreted in a similar manner to that discussed for carrageenan, as representing a coil to double helix transition. A double helical structure of left handed helices containing three or four disaccharide units per turn has been computed to agree with both the available data from X-ray diffraction and the optical rotation transition, although elucidation of the exact ordered structure has not been claimed due to lack of satisfactory X-ray evidence so far.
Fig. 3.4 Optical rotation variations with temperature (above) for a gelling mixture of agarose (0.05%) and galactomannan (0.1%) and (below) for a non-gelling solution of agarose (0.05%) alone. To facilitate comparison, the upper curve has been adjusted by subtracting the contribution expected from the galactomannan alone.
The conformation of the β-1,4 mannann backbone in the crystalline state has already been elucidated from X-ray diffraction evidence for guar galactomannan 127 and for mannan 128, and from computer model building 15, indicating that the backbone exists in an extended ribbon-like form. The optical rotation shift for the agarose-galactomannnan gel has thus been interpreted in terms of the above extended helical conformation for the mannan backbone. Two periods of a three-fold extended helical conformation are envisaged in attachment to three periods of the helical agarose conformation.

Further evidence for a specific interaction between the galactomannan and agar is furnished by freeze-thaw experiments. When gels of these polysaccharides are first frozen and then thawed out, much of the liquid originally held in the gel synereses out and the gel network collapses into a gel-like precipitate. Analysis of the liquid shows that most of the locust bean gum is retained in the network but in the case of fenugreek galactomannan, over 80% of the original is found to be syneresed.

The behaviour of the optical rotation coil to helix transition for carrageenan in the presence of galactomannan has been explained by induced helix formation at a higher temperature, which in terms of the proposed model arises from stabilization of the helix by binding with the galactomannan thus providing an example of a ligand induced conformational change in a polysaccharide system 129.
In this chapter further studies which have been carried out on the agar-galactomannan system are described. Methods have been used to modify the galactomannan molecules with the intention of establishing the stoichiometry involved in the interaction. The possibility of interactions of this quaternary nature between galactomannan and pectin in its tertiary structure have also been investigated.
Experimental

Experiment 3.1. Attempted Isolation of Synergistic Regions of Galactomannan.

An experiment was devised to establish the parts of the galactomannan chain which are involved in the interaction in several synergistic gelling systems, by treatment with periodate which would not be expected to attack the units of the galactomannan participating in the association and therefore allow these to be identified.

Solutions or gels (20ml) of the following polysaccharides (see Table 3.1.) were made up and allowed to age overnight. The gels were then broken up and a solution of sodium periodate was added and the reaction shaken at room temperature for 3 days. The excess periodate was destroyed by ethylene glycol and the products were reduced by sodium borohydride (1g). After dialysis against running tap water for two days, the solution was concentrated and hydrolysed with formic acid (45% v/v) at 100°C overnight. The resultant hydrolysates were analysed by paper chromatography in solvent A using sprays A and B for the development.
## Results

<table>
<thead>
<tr>
<th>Solution or Gal</th>
<th>Concentration (mg)</th>
<th>Periodate (C2M) used</th>
<th>Sugars present in hydrolysate.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBG</td>
<td>50</td>
<td>20mL</td>
<td>GluA Gal Glu MAN</td>
</tr>
<tr>
<td>FG</td>
<td>50</td>
<td>20mL</td>
<td>+ + + +</td>
</tr>
<tr>
<td>DAK</td>
<td>150</td>
<td>10mL + 1g</td>
<td>tr + + + +</td>
</tr>
<tr>
<td>AG:LBG</td>
<td>100:50</td>
<td>20mL</td>
<td>+</td>
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<tr>
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<td>100:50</td>
<td>20mL</td>
<td>+</td>
</tr>
<tr>
<td>KC:FG</td>
<td>150:150</td>
<td>10mL + 0.5g</td>
<td>++ + + +</td>
</tr>
<tr>
<td>KC:LBG</td>
<td>150:150</td>
<td>10mL + 0.5g</td>
<td>++ + + +</td>
</tr>
<tr>
<td>LC:LBG</td>
<td>150:150</td>
<td>10mL + 0.5g</td>
<td>+++ + + +</td>
</tr>
<tr>
<td>DAK:LBG</td>
<td>150:150</td>
<td>10mL + 1g</td>
<td>tr + + + +</td>
</tr>
</tbody>
</table>

LBG - locust bean gum, FG - fenugreek gum, DAK - deacetylated keltrol, AG - agar, KC - kappa carrageenan and LC - lambda carrageenan.

GLUA - glucuronic acid, GAL - galactose, GLU - glucose and MAN - mannose.

The locust bean gum has M/G ratio of 3.8 and the fenugreek galactomannan, 1.08.

Keltrol is an acidic polysaccharide produced by the bacterium *Xanthomonas campestris* NRRL B-1459 130. It contains D-glucose, D-mannose, D-glucuronic acid and acetyl groups in the ratio 2.8:3.0:2.0:1.7 and also 3.0-3.5% pyruvic acid attached to certain glucose residues by a 4,6-O-(1-carboxyethylidene) linkage 131. Recently the polysaccharide has been shown to gel after de-acetylation
and optical rotation studies indicate a coil to ordered structure transformation occurs

Lambda carrageenan is a non gelling carrageenan which differs from kappa in containing 1,4-linked-D-galactose units as their 6-sulphate and also some of the 1,3-linked-D-galactose units as their 2-sulphate or 2.6-disulphate but contains no 4-sulphated residues.

The results indicate that not all of the galactomannan is attacked by the periodate as mannose is present in both the hydrolysate from the locust bean gum and that from fenugreek gum. The amount of mannose still remaining in the synergistic gel systems cannot therefore be attributed directly to those regions of the mannan backbone which are associated in the gel.

**Experiment 3.2. Depletion of the Synergistic Interaction by Modification of Galactomannan with Periodate as followed by Optical Rotation.**

Initially agar (0.05% w/v) and locust bean gum (0.5% w/v) gels were treated with periodate above the gelling temperature and the system was then studied by optical rotation as the gel set. This proved unsatisfactory as the agar is attacked quite substantially (also followed by optical rotation) which is unexpected in terms of the idealized structure of agarose and the usual mechanism of periodate oxidation, although other radical mechanisms have been proposed.

An experiment was therefore carried out in which the galactomannan LBG was first modified and the effect on the
synergism caused by this modification was then followed by optical rotation.

LBG (5 ml, 1.0% w/v) was cooled to 3-4°C on a Tecam water bath provided with a cooling device and a sodium periodate solution (0.25M, 1 ml) also at the same temperature was added. The reaction was allowed to proceed for the allocated time and the periodate was then destroyed with ethylene glycol (1 ml). The solution was heated to around 60°C, and agar (7 ml, 0.1% w/v) also at 60°C was added. The solution was then transferred to a heated optical rotation cell and the optical rotation followed as the solution was cooled.

The results are shown in Fig. 3.5.

As can be seen in Fig. 3.4, the optical rotation of an agar-locust bean gum gel shows a distinct rise at around 29°C with the onset of gelation, while the sol to gel transition for agar alone also occurs at around 29°C but gives a change of opposite sign. The loss of synergistic activity of the IEG could thus be represented by the difference in the optical rotation of the solution at 35°C and 25°C. When there is no impairment of synergism this value will be that of the agar-locust bean gum gel itself and when there is complete destruction of synergism the value would be expected to correspond to that of the agar gel itself. Fig. 3.6 shows the loss of synergistic ability of the galactomannan as the reaction proceeds at the above conditions. Some other reaction conditions which however did not allow complete destruction
Fig. 3.5. Comparison of optical rotation variations with temperature for agarose at a non-gelling concentration (0.05% w/v) and for synergistic mixtures with periodate modified galactomannans (0.36% w/v). To facilitate comparison, the synergistic mixtures have been adjusted by subtracting the contribution expected from the modified galactomannans alone. The figures refer to the time (mins) of the periodate reaction.
Fig. 3.6. Graph of the total change in optical rotation on cooling synergistic mixture of agar and modified galactomannans after various oxidation treatments.

Continuous line - using conditions in Expt. 3.2. (Fig. 3.5).
Broken line - as for Expt. 3.2. but with 0.1M sodium periodate at 25°C.
Dotted line - as for Expt. 3.2. but with 0.05M sodium periodate at 41°C.
Circles - modified galactomannans from Expt. 3.3.
of synergism are also plotted.

**Experiment 3.3. Preparation of Modified Galactomannans.**

Modified galactomannans were prepared by essentially the same method as used in Experiment 3.2, but on a larger scale.

Six solutions of locust bean gum (1% w/v, 300ml) were made up by autoclaving at 121°C for a few minutes. These solutions were cooled to 3-4°C and sodium periodate (60ml, 0.25M) also cooled to 3-4°C was added with shaking. The reactions were stopped by addition of ethylene glycol (15ml) after 2, 4, 10, 17, 20 and 24 minutes respectively. After dilution to 1 litre, sodium borohydride (2.5g) was added to each flask, which was then stirred at room temperature for two days. The samples were dialysed against running tap water for a further three days and finally freeze dried.

The samples were examined for their synergistic ability by optical rotation as in Experiment 3.2, and the results are shown in Fig. 3.6 and 3.7.

When solutions containing agar (0.05% w/v) and modified galactomannan (0.5% w/v) were made up hot at 121°C and then allowed to cool, the LBG-2, 4, and 10 all formed gels, but LBG-17 had only a very little gel character while LBG-20 and 24 had no gel character at all. A similar experiment was carried out with de-acetylated keltrol and with kappa carrageenan. Using non-gelling concentrations of these polysaccharides, the modified galactomannans,
Fig. 3.7. Comparison of optical rotation variations with temperature for agarose (0.05% w/v) and periodate/borohydride modified galactomannans (0.36% w/v). To facilitate comparison the synergistic mixtures have been adjusted by subtracting the contribution expected from the modified galactomannan alone. The figures refer to the time (mins) of the periodate reaction.
LBG-2, 4 and 10 formed gels while the others did not.

**Experiment 1.4.** Synergistic Action of Modified Galactomannans as Monitored by Freeze-Thaw Experiments.

A series of gels were made up containing agar (0.5% w/v) and the modified galactomannans (0.1% w/v) by autoclaving at 121°C. The solutions set on cooling and were allowed to age overnight. After freezing for a further 24 hours the gels were allowed to thaw out. The gel network contracted to form a solid and the liquid in the gel syneresed out. The syneresed liquid was then analysed for carbohydrate material by the phenol-sulphuric acid assay method of Dubois et al. 1934.

**Results**

<table>
<thead>
<tr>
<th>Galactomannan</th>
<th>Carbohydrate concentration of syneresed liquid due to galactomannan (g w/v).</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBG</td>
<td>0.008</td>
</tr>
<tr>
<td>LBG-2</td>
<td>0.018</td>
</tr>
<tr>
<td>LBG-4</td>
<td>0.026</td>
</tr>
<tr>
<td>LBG-10</td>
<td>0.037</td>
</tr>
<tr>
<td>LBG-17</td>
<td>0.066</td>
</tr>
<tr>
<td>LBG-20</td>
<td>0.069</td>
</tr>
<tr>
<td>LBG-24</td>
<td>0.075</td>
</tr>
</tbody>
</table>

LBG-2, 4 etc. refers to the locust bean gum modified for 2 mins, 4 mins. etc.
Analysis of the galactomannans were carried out by a method devised by Dr. A. Morrison of Unilever Research which was an adaptation of the method of Albersheim et al\textsuperscript{135}.

The galactomannan sample (10-20mg) together with the internal standard, inositol (1ml from a solution containing 10mg/ml) and trifluoro-acetic acid (\textsuperscript{4}N, 1ml) was heated in a sealed tube in a pressure cooker at 121°C for 1 hour. After removing the reagents in vacuo on a Buchi rotavapor at 50-60°C, the hydrolysate was reduced by addition of ammonium hydroxide solution (1N, 0.5ml) and sodium borohydride (10-20mg) for 1 hour. The excess borohydride was then destroyed by glacial acetic acid added dropwise and the reagents removed in vacuo at 50-60°C. Successive evaporations with methanol (1-2ml) removed the residual borate.

The sample was then acetylated by heating at 121°C for 3 hours with acetic anhydride (1ml). The reagent was then removed in vacuo at 50-60°C and the sample taken up in chloroform.

The analysis of the alditol acetates formed was carried out by gas liquid chromatography on a Hewlett Packard 11 gas chromatograph with a glass column (6ft x 1mm ID) of 10% ECNSS-M on JJ Celite (80-100mesh). The nitrogen carrier gas pressure was 20 p.s.i. and the hydrogen pressure was 22.5 p.s.i. The runs were temperature programmed for
5 minutes at $140^\circ$C and the temperature increased to $180^\circ$C at $4^\circ$C/min. The injection temperature was $210^\circ$C.

**Results**

### Sample Mole fraction of sugars present in hydrolysates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Galactose</th>
<th>Mannose</th>
<th>Erythritol</th>
<th>Threitol</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBG</td>
<td>0.208</td>
<td>0.792</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LBG-2</td>
<td>0.193</td>
<td>0.754</td>
<td>0.020</td>
<td>0.012</td>
<td>0.021</td>
</tr>
<tr>
<td>LBG-4</td>
<td>0.167</td>
<td>0.746</td>
<td>0.042</td>
<td>0.005</td>
<td>0.040</td>
</tr>
<tr>
<td>LBG-10</td>
<td>0.125</td>
<td>0.676</td>
<td>0.088</td>
<td>0.007</td>
<td>0.104</td>
</tr>
<tr>
<td>LBG-17</td>
<td>0.113</td>
<td>0.649</td>
<td>0.120</td>
<td>0.015</td>
<td>0.103</td>
</tr>
<tr>
<td>LBG-20</td>
<td>0.108</td>
<td>0.631</td>
<td>0.146</td>
<td>0.010</td>
<td>0.106</td>
</tr>
<tr>
<td>LBG-24</td>
<td>0.091</td>
<td>0.606</td>
<td>0.153</td>
<td>0.011</td>
<td>0.139</td>
</tr>
</tbody>
</table>

### Sample Mannose Erythritol ratio (M/E) Mannose Galactose ratio (M/G)

<table>
<thead>
<tr>
<th>Sample</th>
<th>M/E</th>
<th>M/G</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBG</td>
<td>-</td>
<td>3.8</td>
</tr>
<tr>
<td>LBG-2</td>
<td>37.7</td>
<td>3.9</td>
</tr>
<tr>
<td>LBG-4</td>
<td>17.8</td>
<td>4.5</td>
</tr>
<tr>
<td>LBG-10</td>
<td>7.7</td>
<td>5.4</td>
</tr>
<tr>
<td>LBG-17</td>
<td>5.4</td>
<td>5.7</td>
</tr>
<tr>
<td>LBG-20</td>
<td>4.4</td>
<td>5.8</td>
</tr>
<tr>
<td>LBG-24</td>
<td>4.0</td>
<td>6.7</td>
</tr>
</tbody>
</table>

**Experiment 3.6.** Preparation of Galactomannan Segments from the Modified Galactomannans by Mild Hydrolysis.

A sample of each of the modified galactomannans was hydrolysed in $H_2SO_4$ (1N) for 48 hours at RT. The acidic solution was neutralised with barium carbonate, filtered through glass paper and treated with Amberlite IR-120 ($H^+$)
resin to remove any remaining cations. The resulting solutions of oligomers of the galactomannan were finally freeze-dried.

A freeze-thaw experiment was carried out with these galactomannan segments (0.1% w/v) and agar (0.5% w/v). Segments from the modified galactomannans, LBG-2, LBG-4 and LBG-10 were retained in the gel network indicating interaction. The results are shown below.

**Results**

<table>
<thead>
<tr>
<th>Segments</th>
<th>Carbohydrate concentration of syneresed liquid due to galactomannan (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBG-2</td>
<td>0.068</td>
</tr>
<tr>
<td>LBG-4</td>
<td>0.075</td>
</tr>
<tr>
<td>LBG-10</td>
<td>0.082</td>
</tr>
<tr>
<td>LBG-17, 20, 24</td>
<td>0.100</td>
</tr>
</tbody>
</table>

When these segments from LBG-2, 4 and 10 (0.1% w/v) were added to a gelling concentration of agar (0.1% w/v) the gelation was inhibited and the solutions showed less gel character than for the agarose alone which is in contrast to the action of the modified galactomannans themselves. Using the segments from LBG-2, the solution remained free flowing and showed no visible gel character. The agar solutions containing segments from LBG-4 and LBG-10 showed increasing gel character, the LBG-10 solution being very similar to the gel for agar alone.

Similar behaviour was observed when optical rotation studies were attempted on these segments. Additions of segments
(0.5% w/v) to the agar (0.05% w/v) did not induce gelation but caused the solutions to become opaque at low temperatures (below 30°C) preventing observation of any conformational change which might be taking place.

This anti-synergistic behaviour has also been seen for agar and low molecular weight galactomannan fractions obtained by enzymatic hydrolysis, and can be explained by an increased tendency for aggregation of the agarose helices in the presence of galactomannan and thus partial precipitation when the galactomannan molecules are too short to provide additional network.

**Experiment 3.7.** Attempted Separation and Analysis of Galactomannan Segments by T.L.C.

Separation of the galactomannan oligomeric series from the hydrolysis of Experiment 3.6. was attempted by the method of Nash Huber et al 136 as used for the maltodextrin series.

T.L.C. plates were made up with Silica Gel G and Kieselguhr G (3:1) to a thickness of 400 microns. The solutions of galactomannan segments were applied to the plates as 7mm streaks and the irrigations carried out in a solvent system of propan-1-ol-nitromethane-water (5:2:3). The irrigation was repeated twice, with thorough drying between each. The oligomers were detected by spraying with a 50% solution of concentrated sulphuric acid and heating at 150°C for 30 mins.

The LBG-24 segments separated into the constituent oligomers up to a d.p. of 11 but there appeared to be a small amount of carbohydrate material still remaining at the origin.
For the other segments from LBG-2, 4, 10, 17 and 20, the quantity of material, still at the origin after development, increased from LBG-20 to LBG-2 while there was a corresponding decrease in the oligomers, d.p. 2-11, and in the case of LBG-2 virtually all of the material remained at the origin.

The system therefore was not very satisfactory as a means of separation of segments of higher d.p. probably because of their insolubility in the solvent. However, from the separation achieved for LBG-24 segments it may be concluded that fairly long oligomers must be present, at least of degree of polymerisation greater than 11 and substantially greater in segments of LBG-2.

Experiment 3.8. Minimum Length of the Agarose Interaction

A freeze-thaw experiment was carried out with the agar sample and a series of oligosaccharides made up from the basic neagarobiase disaccharide unit of agarose which were obtained from Dr. W. Yaphe.

Agar (0.5% w/v), which itself was purified by freezing and thawing twice, and the oligosaccharides (0.1% w/v) were dissolved with heating. The solution was allowed to cool and the resultant gel was aged overnight. The gel was frozen for a further 24 hours and after thawing the syneresed liquid was collected. This liquid and a solution of the original oligosaccharides (0.1% w/v) were compared by T.l.c. by the method of Duckworth and Yaphe 137.

The separations were carried out on microcrystalline cellulose (Camag. D.S.O. - 250 thickness) in the solvent
system, butan-1-ol ethanol, water (3:2:2). The plate was developed with modified naphthoresorcinol reagent which is sensitive to the 3,6-anhydro-L-galactose present. The spray reagent contains two parts ethanolic sulphuric acid (375ml ethanol plus 100ml concentrated sulphuric acid) and one part naphthoresorcinol solution (0.2% in ethanol), A bright blue colour appeared for the oligosaccharides a few hours after spraying.

The two solutions did not show any difference in their composition of oligomers, both containing the oligosaccharides neoagarobiose, neoagarotetraose up to the neoagarododecaose. It is therefore concluded that regions of d.p. greater than 12 are required for formation of the helical ordered structure in agarose.

**Experiment 3.9. The Possible Synergising Ability of Galactomannan with Pectins.**

Addition of locust bean gum galactomannan (0.5 - 3.0% w/v) to high methoxy or low methoxy pectin (1.0% w/v) did not produce any evidence of gelation.

In gels formed by addition of ethylene glycol, replacement of 0.5% w/v of the pectin in a 1.0% w/v high methoxy pectin gel by 0.5% w/v galactomannan necessitates the addition of a further 5-10% w/v of glycol to cause gelation than would be required for a 1.0% w/v pectin gel itself. Since the galactomannan (1.0% w/v) alone forms a gel-like mixture with ethylene glycol (55% v/v), it was therefore concluded that it is unlikely that there is any positive interaction of the pectin and galactomannan of the kind
already described for the agar system.

When locust bean gum (1% w/v) was added to low methoxy pectin (2% w/v), this appeared to prevent proper gelation on subsequent addition of calcium ions.
Discussion

After oxidation with periodate, the interaction of galactomannan with agarose is changed in a manner that is consistent with the model already proposed for the synergistic interaction. The gross reduction in the interaction by a random statistical oxidation of a few of the units in the mannan chain, points clearly to the involvement of lengths of mannan backbone containing several mannose residues.

The initial experiment, Experiment 3.1, was based on the hope that bound galactomannan would be protected from periodate oxidation by involvement of either the hydroxyl group on C₂ or C₃ or both in the association. The experiment also attempted to compare the quantitative interaction of the galactomannan with several different polysaccharides by estimation of the remaining mannose after oxidation of the gels. This did not prove possible because later experiments showed that incomplete oxidation of the mannose results in solutions of the locust bean gum and fænugreek galactomannans themselves. This resistance to oxidation is possibly due to inter-residue hemi-acetal formation with the nearest hydroxyl of the neighbouring residue, thus protecting it from oxidation. At mannose residues which are substituted with galactose, this type of hemi-acetal formation could occur rather than the intra-residue type with ₀₆.  

In Experiment 3.2 the problem is approached from a slightly different angle. Here the galactomannan is first allowed
to react with the periodate and then this modified galactomannan is examined for synergistic activity. The periodate attacks the galactomannan molecules in a random manner oxidising the sugar residues by splitting the sugar ring between two adjacent hydroxyls and thus converting to the open chain dialdehyde (Fig. 3.8). The effect expected is to remove the possibility for intimate fit and ordered binding of the polymer molecule at this point. An increase in the time of exposure to oxidation increases the number of "breaks" in the structure and thus decreases the average length of unaltered mannose units available for interaction with the co-synergist. The galactose will also be attacked by the periodate in the same manner but this would not be expected to change its role in the system.

The optical rotation results (Fig. 3.5 and 3.7) provide a good illustration of the gradual disappearance of the synergism as the time of oxidation is increased. As seen in Fig. 3.4, agarose shows a fall in optical rotation when it converts to the proposed helical structure. When the galactomannan binds with conversion to an ordered structure it makes a positive contribution which overrides the change due to the agarose and therefore a gross positive change is observed for the synergistic mixture. Measurement of the change in optical rotation as mixtures of agar and the modified galactomannans are cooled through the gel point provides a means of estimating the degree of interaction present between the polysaccharides (Fig. 3.6).
Fig. 3.8. Products which could form during limited periodate oxidation of galactomannan followed by borohydride reduction. Also shown are possible products which might occur after subsequent mild hydrolysis.
In Experiment 3.3 large batches of modified galactomannans were prepared but this time the reaction included a reduction to convert the aldehydic groups to the corresponding alcohols. These samples, which were also oxidised for different periods of time, were examined for synergistic ability (Fig. 3.7) and were found to fit quite accurately onto the original graph for the reaction (circles on Fig. 3.6).

In the 20 and 24 minute reaction there appears to be little if any synergistic interaction in evidence. The 17 minute sample shows very slight evidence of interaction which would seem to indicate that there are still a few regions left in the oxidised molecule which are capable of attaching to the agarose. The samples oxidised for 10, 4 and 2 minutes show increasing interaction in that order.

The freeze-thaw experiment (Experiment 3.4) also indicates a decrease in the interaction as the oxidation is increased but here there is an apparent anomaly when taking into account the optical rotation result. With the locust bean gum galactomannan itself, around 90% is retained in the gel after thawing, while for the 24 minute modified sample 25% appears to be still retained even though the optical rotation does not indicate any interaction. This would possibly be due to the fact that only one site of interaction for each molecule may be required to enable it to be retained in the network in the freeze-thaw experiment while the optical rotation provides a quantitative
assessment of the interaction. This is confirmed by the freeze-thaw experiment with segments (Experiment 3.6) which were obtained by mild hydrolysis of the original modified galactomannans because only the segments from the 2, 4 and 10 minute samples are held in to measurable extent.

Analysis of the modified galactomannans was carried out by hydrolysis and subsequent determination by gas-liquid chromatography of the products. The hydrolysis yields mannose from the unaltered regions of the backbone and erythritol from every oxidised mannose unit. The products obtained from the oxidised galactose units are glycerol or threitol depending on the extent of the oxidation. The ratio of the mannose to erythritol can be calculated for each sample and thus provide an estimation of the average length of the unoxidised mannan regions or blocks in the backbone of the modified samples. The mannose to erythritol ratio for the 17 minute sample is 5.4 but only the very high extreme of the distribution of chain length must interact in the gel because virtually none of the segments are retained in the freeze-thaw and only a very slight interaction is evident from the optical rotation results. Therefore the binding length is much larger than 5.4.

Perhaps a more useful estimate for the minimum length of unoxidised mannose residues required for the synergistic interaction can be obtained from the 2 minute sample. Here the mannose to erythritol ratio is 38 and less than half (ca 35% by weight) of the hydrolysed segments are
retained in the freeze-thaw experiment so it is perhaps reasonable to assume that the minimum d.p. required for interaction of the segments is greater than 35. This figure, of course, is very dependent on the accuracy of the estimation of erythritol which is present in rather small quantity.

From the t.l.c. analysis for the 24 minute sample the minimum length for interaction can certainly be regarded as greater than a d.p. of 11 while similarly the minimum d.p for the agarose interaction to its ordered conformation would appear to be greater than 12.

In conclusion it would seem that rather long lengths of unsubstituted mannan backbone are required for what is most likely a sharp cooperative interaction with the agarose helical structure. The longer the region of attachment, the more stable or stronger the interaction is likely to be.

The interaction between galactomannan polysaccharides and other polysaccharide tertiary structures has already been mentioned as a possible model for biological cohesion for polysaccharides in the cell wall although the gel forming polysaccharides agar, carrageenan and keltrol do not themselves occur in vivo with galactomannan. In the plant cell wall polysaccharides with overall similarity to the mannan backbone, such as cellulose, xylan and perhaps mannan itself occur in the presence of pectin which is the gelling matrix polysaccharide corresponding to the agar
and carrageenan in seaweeds and therefore interaction might be expected to occur between galactomannan and pectin. However the experiments carried out showed no evidence of any such interaction and the two polysaccharides appeared to behave in an additive fashion. The galactomannan appeared to inhibit the formation of the calcium pectate gel.
Chapter IV

Denaturation and Dissociation Behaviour of Cellulose Containing Mucilages.
Introduction
In the previous three chapters, the conformation and interaction in two artificially induced polysaccharide systems have been investigated. The constituent polysaccharides of these systems were first extracted by chemical and physical methods prior to the study of their ordered conformation in solution. The results of these investigations provide important information as to the possible nature and function of these polysaccharides in the biological state.

Results are now reported of investigations on a system which parallels directly the in vivo situation in the plant cell wall, namely cellulose containing mucilages.

In most extraction procedures for polysaccharides the conditions used must disrupt any associations present in the natural state but, for these mucilages, extraction is achieved by soaking in cold water the seeds of mustard (*Brassica sinapis Alba*), cress (*Lepidium Sativum*), quince (*Cydonia*) and flax (*Linum usitatissimum*) and therefore the products obtained may be expected to contain their constituent polysaccharides in an unmodified state in which the tertiary and quaternary structure is little altered.

The mucilages have long been known to contain a polysaccharide whose properties resemble cellulose and also in close association with this, pectic substances. They therefore provide a model for the investigation of the likely nature of cohesion in the plant cell wall.

Structural studies on the seed-coat mucilages of mustard
and cress have been carried out by Bailey \textsuperscript{141} over thirty years ago. The mustard mucilage was fractionated by addition of saturated barium hydroxide solution which produced a gelatinous material containing cellulose and also L-arabinose, D-galactose, D-galacturonic acid and D-glucuronic acid as constituent sugars. The polysaccharide material left in solution contained L-arabinose and D-galactose and also an aldobiuronic acid composed of L-rhamnose and D-galacturonic acid.

A more recent study of mucilage obtained from cress seed has been carried out by Tyler \textsuperscript{142}. This also gave two components from fractional precipitation with ethanol but both proved to have similar constituents, only differing in the amount of cellulose present, the second fraction being virtually cellulose free. Both fractions were shown by hydrolysis and paper chromatography to contain galacturonic acid, 4-O-methylglucuronic acid, galactose, arabinose, xylose and rhamnose with traces of glucose.

Methylation of the cellulose-free fraction and further fractionation of this with chloroform was shown to yield a methylated xyloarabinan and a methylated acidic polysaccharide. The structure shown in Fig. \textsuperscript{4.1} was put forward for the xyloaraban although the main chain could also contain 1,4-linked arabinopyranosyl or 1,3-linked arabinofuranosyl units.
Fig. 4.1. Probable structure of cress seed xyloaraban.

Results of structural studies on the acidic polysaccharide fraction suggested that the structure shown in Fig. 4.2. most accurately represents the polysaccharide.

$$\rightarrow 5 - \text{L-Araf}_{1} \rightarrow 5 - \text{L-Araf}_{1} \rightarrow 5 - \text{L-Araf}_{1} \rightarrow$$

$$\text{3} \quad \text{3} \quad \text{3}$$

$$\uparrow \quad \uparrow \quad \uparrow$$

$$\text{1} \quad \text{1} \quad \text{1}$$

$$\text{L-Araf}_{1} \quad \text{L-Araf}_{2}$$

$$\text{3} \quad \text{3}$$

$$\uparrow \quad \uparrow$$

$$\text{1} \quad \text{1}$$

$$\text{D-Xylp} \quad \text{D-Xylp}$$

Most common side chain

$$4 - \text{D-GalpA}_{1} \rightarrow 4 - \text{D-GalpA}_{1} \rightarrow 2 - \text{L-Rhap}_{1} \rightarrow$$

$$\text{4} \quad \uparrow$$

$$\text{1}$$

Fig. 4.2. Approximate structure of the acidic polysaccharide.

About one-third of the rhamnose residues in the mucilage were branched at both the 3 and 4 positions although a few terminal rhamnose units also occurred. A less common side chain consisting of $$- \text{D-Xylp (1} \rightarrow 4) - \text{D-Galp}$$ was also shown to be present.

These mucilages therefore would appear to contain, together with cellulose, a highly branched acidic component and a neutral polysaccharide similar to polysaccharide fractions which have been shown to occur in other parts of mustard seeds.
The presence of cellulose as a component of these mucilages had not been definitely established until recent work by Skerrett\textsuperscript{144}. Previous evidence was based on the glucose content and the appearance of an insoluble fibrous precipitate under certain conditions. Chemical analysis and X-ray diffraction patterns of the precipitate obtained with dilute acid from \textit{noriasa} mucilage\textsuperscript{145} suggested that this material was very similar to cotton cellulose. 

Muhtethaler\textsuperscript{146} also provided indirect evidence by observing cellulose-like microfibrils by electron microscopy.

Infra-red spectroscopy and X-ray fibre diffraction studies\textsuperscript{144} have now shown the presence of cellulose II in mustard and cress mucilages which is the crystalline form occurring in the cellulose microfibrils of the plant cell wall (see general introduction).

Enzymatic hydrolysis of the fibrous material was also shown to yield the expected di- and oligosaccharides of the cellobiose series and showed no evidence of any anomalous branching or any sugar other than glucose in the products.

Analytical ultracentrifugation and Tiselius electrophoresis of the mustard and cress mucilages showed the marked physical heterogeneity of these extracts which may be due to different components or to different cellulose contents present in the mucilage particles.

Further studies\textsuperscript{147} by X-ray fibre diffraction crystallography of the mucilages themselves yielded diffraction patterns with d-spacings corresponding to those for cellulose I but also gave a reflection which provided a reasonable fit with a 030 strong meridional reflection found for crystalline pectin. From this evidence it was deduced that the mucilages contain crystalline pectin with the same orientation as that of the cellulose fibril.
A tentative model was thus proposed for the mucilage particle (Fig. 4.3.).

It is suggested that this model represents the situation present in the plant cell wall but here in the mucilage the galacturonan backbone of the pectin is modified to a much greater extent and so a complete coherent matrix is not formed and the seeds are thus able to swell and exude the particles as a gel-like mass.

In this chapter experiments are described concerning the effect on the mucilage of several reagents which would be expected to affect non-covalent associations and interactions in biopolymer systems and thus possibly to lead to an understanding of the cohesive forces involved.
Crystalline Cellulose Microfibrils

Hemicellulosic sheath

Neutral pectic polysaccharides

Pectin network cross-linked by crystallites

Fig. 4.3. Schematic representation of the cross section of the mucilage particle.
Experimental

Experiment 4.1. Extraction of Mustard and Cress Mucilages.

Mustard seeds (Brassica Sinapis Alba, 1000g) were soaked in distilled water (8 litres) for 24 hours with occasional stirring during which time the solution became slightly viscous and the seeds themselves were surrounded by a capsular gel. The solution and gel were separated from the seeds by squeezing through muslin. The extract, which was a dirty yellow colour, was centrifuged (2000 r.p.m. in the M.S.E. "Major") to remove most of the suspended material. The mucilage was then precipitated with methylated spirits and the isolated precipitate, after further washing with methylated spirits, was dried in a vacuum oven at 48°C over phosphoric oxide. A yield of 18.7g was obtained.

Curled cress seeds (Lepidium sativum, 1000g) were also extracted in this manner yielding 22.7g of mucilage.

The isolated mucilage precipitates could be dissolved in distilled water to form rather viscous solutions (1% w/v) which were a dirty yellowish colour and still contained some insoluble impurities.

The mustard mucilage was therefore further purified by means of a preparative ultracentrifuge as follows.

The extracted mucilage (1% w/v in 10% w/v aqueous urea, 8ml) was carefully layered onto a concentrated aqueous urea solution (40% w/v, 12ml) in a polypropylene centrifuge tube so that the former solution remained floating on the latter. The solution was then centrifuged on the M.S.E. Model 50 Preparative Ultracentrifuge using the 3 x 20ml swing out head at 20,000 r.p.m. (44,000 x g) for 3 hours.
The bottom fraction (10ml) in each tube was isolated by piercing the tube with a hollow needle and draining off the liquid. This fraction was termed the "heavy fraction". The top 10ml, the "light fraction", was also collected. The polysaccharide material in each fraction was isolated by dialysis against running tap water and then freeze-dried. The two fractions (0.3% w/v in 10% w/v urea) were examined in the Analytical Beckmann Spinco Model E Ultracentrifuge at 24,630 r.p.m. Both samples showed similar hypersharp Schlieren peaks which appear to sediment through the cell at the same rate. These peaks show that the cellulose fibril-soluble polysaccharide complex is present in both fractions but in the light fraction there also appeared a considerable amount of low molecular weight material which did not sediment away from the origin.

**Experiment 4.2. Effect of Different Reagents on the Mucilage Complex.**

The associations present in the mucilage complex were investigated by treatment of the whole mucilage with several reagents which are known to have a denaturing influence on protein and DNA. The extent to which precipitation or thickening of the mucilage solutions occurs with these various reagents was assessed in the hope that the changes brought about could be correlated with possible polysaccharide interactions involved in the complex.

Preliminary experiments attempted to assess visually the amount of fibrous material precipitated from mucilage solution (0.5% w/v) by the reagents (Table 4.1.)
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Conditions</th>
<th>Action on the mucilage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>4M</td>
<td>Boiled for 30 secs</td>
<td>Solution became very gel-like</td>
</tr>
<tr>
<td>E.D.T.A.</td>
<td>1%</td>
<td>&quot;</td>
<td>No change detected</td>
</tr>
<tr>
<td>Sodium lauryl sulphate</td>
<td>1%</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>E.D.T.A. plus sodium lauryl sulphate</td>
<td>1%</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCNS</td>
<td>1M</td>
<td>80°C for 3 hours</td>
<td>Precipitate observed</td>
</tr>
<tr>
<td>Sodium trichloroacetate</td>
<td></td>
<td>80°C for 30 mins</td>
<td></td>
</tr>
</tbody>
</table>

The $\text{CNS}^-$ and $\text{CCl}_3\text{CO}_2^-$ anions therefore appeared to cause alteration of the complex and precipitation of an insoluble polysaccharide fraction. However, the mode of action of these reagents may be complicated by the fact that the pH rose to between 8 and 9.

The mucilage concentration was found to be another variable which influences the ability of reagents to cause precipitation of the mucilage. When the concentration of the mucilage was reduced to 0.2% w/v a precipitate formed when a solution of mucilage containing E.D.T.A. (2% w/v) plus sodium lauryl sulphate (2% w/v) was boiled briefly. A similar result was obtained for mucilage (0.13% w/v) in urea (8M).

Attempts to assess quantitatively the effect of reagents by phenol-sulphuric acid assay of the polysaccharide remaining after removal of precipitated material were not successful as results were found to be extremely irreproducible.
An alternative method was therefore devised in which the whole mucilage (0.4 - 0.5% w/v) was treated with reagent by refluxing gently for 2½ hours. The solution was then cooled and the precipitate formed was collected by centrifugation (M.S.E. Major, at 5,000 r.p.m. using the angle head), washing once with reagent and then thrice with water before freeze-drying.

The products obtained were examined by hydrolysis and paper chromatography as follows.

Mucilage material (50mg) was dissolved in sulphuric acid (70% v/v, 1ml) and agitated periodically by means of a Whirlimix for 2 hours by which time complete solution was achieved. The tube was kept in a cold room (0-2°C) for 48 hours before dilution to an acid concentration of 1N and heating on a water bath (100°C for 10 hours). The hydrolysate was neutralised with barium carbonate and filtered before treatment with Amberlite IR-120 (H⁺ form) resin and then finally evaporated to a small volume.

Paper chromatography was carried out with Whatman No.1 paper using solvent systems A or B and developed with spray A.

The results for the mustard mucilage are shown in Table 4.2. Similar results for cress mucilage are also shown in Table 4.3.
<table>
<thead>
<tr>
<th>Material</th>
<th>GalA</th>
<th>Gal</th>
<th>Glu</th>
<th>Ara</th>
<th>Man</th>
<th>Xyl</th>
<th>Rha</th>
</tr>
</thead>
<tbody>
<tr>
<td>whole mucilage</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>&quot;heavy fraction&quot;</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ppt. with water</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ppt. with E.D.T.A. (2%)</td>
<td>tr/+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ppt. with E.D.T.A. (2%) + sodium lauryl sulphate (2%)</td>
<td>tr/+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ppt. with sodium lauryl sulphate (2%)</td>
<td>tr/+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ppt. with potassium thiocyanate (40%)</td>
<td>tr/+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ppt. with potassium chloride (2%)</td>
<td>tr/+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ppt. with urea (8M)</td>
<td>tr/+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ppt. with magnesium sulphate (2%)</td>
<td>tr/+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4.3. Hydrolysis Products from Cress Mucilage after Precipitation with Reagents.

<table>
<thead>
<tr>
<th>Material</th>
<th>GalA</th>
<th>Gal</th>
<th>Glu</th>
<th>Ara</th>
<th>Man</th>
<th>Xyl</th>
<th>Rha</th>
</tr>
</thead>
<tbody>
<tr>
<td>whole mucilage</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>ppt. with water</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>ppt. with E.D.T.A. (2%)</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td>tr</td>
</tr>
<tr>
<td>ppt. with E.D.T.A. (2%) + sodium lauryl sulphate (2%)</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td>tr</td>
</tr>
<tr>
<td>ppt. with sodium lauryl sulphate (2%)</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td>tr</td>
</tr>
<tr>
<td>ppt. with potassium chloride (2%)</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td>tr</td>
</tr>
</tbody>
</table>

From the data shown in Table 4.2 and Table 4.3, it can be seen that the above reagents do not strip all the non-cellulosic polysaccharide from the mucilage particle. A further experiment was therefore carried out on the mucilage using a harsher alkaline treatment. Three samples of mucilage (0.5% w/v) were treated with sodium hydroxide (4% w/v) and sodium borohydride (1% w/v) and left for 24 hours with occasional stirring. The resulting precipitates were isolated by filtration, washed with sodium hydroxide-sodium borohydride solution and then with water. One sample (Sample 1) was further washed with acetone and dried in vacuo while the remaining two samples were again treated with the above reagent mixture but at 50°C for 3 hours. One of these samples (Sample 2) was then isolated with acetone and dried in vacuo. Sample 3 was heated for a further 30 minutes at 100°C before also being isolated in the same manner.
Hydrolysis and paper chromatography were carried out as for the previous samples. The results are shown in Table 4.4.

<table>
<thead>
<tr>
<th>Material</th>
<th>GalA</th>
<th>Gal</th>
<th>Glu</th>
<th>Ara</th>
<th>Man</th>
<th>Xyl</th>
<th>Rha</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>-</td>
</tr>
<tr>
<td>Sample 2</td>
<td>tr</td>
<td>tr</td>
<td>++</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>-</td>
</tr>
<tr>
<td>Sample 3</td>
<td>+</td>
<td>+</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Experiment 4.3. Attempt at Quantitative Analysis of Mucilage Precipitates.

Since it was difficult to identify minor differences in hydrolysate compositions by paper chromatography, a method was sought which would produce a more quantitative assessment of their composition. Although any method would be unlikely to give absolute values for the original mucilage precipitate due to the resistance of the uronosyl linkages to cleavage by hydrolysis, a quantitative comparison might however be obtained.

(a) Analysis by Gas-Liquid Chromatography

The sugars present in the mucilage hydrolysate were converted to their methyl glycosides or methyl ester methyl glycoside in the case of the galacturonic acid followed by trimethylsilyl ether formation.

Hydrolysate (ca. 10mg) and methanolic hydrogen chloride (2ml, 3%) were placed in a sealed tube in a boiling water bath for 16 hours. After neutralization with silver carbonate, the solution was filtered and evaporated to dryness.
To this, dimethylsulphoxide (2ml) and trimethylchlorosilane: hexamethyldisilazane, 5:1 (1ml) were added and the flask shaken occasionally over a period of one hour. The upper layer which had separated was pipetted off into water (5ml), shaken, transferred to a further 5ml of water, shaken and finally withdrawn and dried over anhydrous magnesium sulphate.

The resulting trimethyl ethers in hexamethyldisiloxane and those of the individual constituent sugars prepared in the same manner were chromatographed on a Pye Argon Chromatograph using a wide range of column materials. The most satisfactory separation achieved was with a SE-30 (7%) on 60/80 mesh Gas Chrom P column (5ft. x 0.5cm), at a temperature of 145°C and an argon flow rate of 85ml/min. The results are given in Table 4.5.
Table 4.5. Identification of Sugar Components of Mucilage Hydrolysate.

<table>
<thead>
<tr>
<th>Mucilage</th>
<th>Rha</th>
<th>Man</th>
<th>Xyl</th>
<th>Ara</th>
<th>Gal</th>
<th>Glu</th>
<th>GalA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.20</td>
</tr>
<tr>
<td>0.23</td>
<td>0.24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.22 (minor)</td>
</tr>
<tr>
<td>0.33</td>
<td>0.32 (minor)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.32</td>
</tr>
<tr>
<td>0.36</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.36</td>
</tr>
<tr>
<td>0.55</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.54</td>
<td></td>
<td>0.54</td>
</tr>
<tr>
<td>0.65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.64</td>
<td></td>
<td>0.64</td>
</tr>
<tr>
<td>0.79</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.74 (minor)</td>
<td></td>
<td>0.69</td>
</tr>
<tr>
<td>0.92</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.93 (minor)</td>
<td></td>
<td>0.92</td>
</tr>
</tbody>
</table>

* Retention times are given relative to the trimethylsilyl ether of methyl-β-D-glucopyranoside.

(b) Liquid Chromatography

A high speed liquid chromatography set up with automatic online monitoring of eluant was made available for analysis of the hydrolysates by Mr. J.S. Hobbs of Unilever Research, Colworth.

The method involved separation of the neutral sugars from the acidic components on an anion exchange column and then further separation of neutral sugar mixture on a cation exchange column.

The hydrolysates (10-20mg) were first evaporated to dryness before dissolving in acetic acid (2M, 50-100μl). To this a known quantity of ribose was added (ca. 5mg in 2M acetic acid) and the sample was then eluted with acetic acid (2M) through a column (30cm x 0.5cm) containing a strongly basic
anion exchange resin (Technicon Type -S chromobeads in the acetate form) at room temperature. The neutral sugar components eluted immediately after the column dead volume and were collected while the acidic species were estimated by comparison of their peak areas with those of standard solutions of galacturonic acid. The eluted sugars were detected by a differential refractometer (Water Associates). The collected neutral sugars were evaporated to dryness and redissolved in 85% aqueous ethanol (50-100 μl) before passing down a column (100 x 0.4 cm) containing a cation exchange resin (Bio Rad - Aminex A-6, 17.5 ± 2.5) in the trimethyl ammonium form. The elution was carried out with an ethanol water solution (85%) at 65°C with a flow rate of 0.4 ml/min and the eluant monitored with a Pye moving wire detector. The individual sugars present were determined relative to the ribose internal standard using the response factors determined from a standard mixture. The results of the analysis of the mucilage precipitates obtained in Experiment 4.2. are shown in Table 4.6.
### Table 4.6. Analysis by Liquid Chromatography of the Hydrolysed Precipitates obtained from the Mustard Mucilage after Treatment with Reagents.

<table>
<thead>
<tr>
<th>Material</th>
<th>Estimated acidic components</th>
<th>Rha</th>
<th>Glu</th>
<th>Man</th>
<th>Xyl</th>
<th>Ara</th>
<th>Gal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole mucilage</td>
<td>34</td>
<td>14</td>
<td>100</td>
<td>20</td>
<td>6</td>
<td>10</td>
<td>71</td>
</tr>
<tr>
<td>Water treated ppt</td>
<td>26</td>
<td>4</td>
<td>100</td>
<td>18</td>
<td>3</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>Heavy fraction from ultra centrifugation</td>
<td>7</td>
<td>4</td>
<td>100</td>
<td>17</td>
<td>7</td>
<td>6</td>
<td>38</td>
</tr>
<tr>
<td>KCl treated ppt</td>
<td>*</td>
<td>1</td>
<td>100</td>
<td>25</td>
<td>7</td>
<td>10</td>
<td>44</td>
</tr>
<tr>
<td>EDTA</td>
<td>tr</td>
<td>1</td>
<td>100</td>
<td>21</td>
<td>5</td>
<td>4</td>
<td>28</td>
</tr>
<tr>
<td>NaLS</td>
<td>3</td>
<td>1</td>
<td>100</td>
<td>10</td>
<td>4</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>KCNS</td>
<td>tr</td>
<td>1</td>
<td>100</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Alkaline boro hydride treatment at RT</td>
<td>tr</td>
<td>1</td>
<td>100</td>
<td>3</td>
<td>nd</td>
<td>nd</td>
<td>2</td>
</tr>
<tr>
<td>&quot; at 50°C</td>
<td>tr</td>
<td>nd</td>
<td>100</td>
<td>3</td>
<td>nd</td>
<td>nd</td>
<td>4</td>
</tr>
<tr>
<td>&quot; at 100°C</td>
<td>tr</td>
<td>nd</td>
<td>100</td>
<td>3</td>
<td>nd</td>
<td>nd</td>
<td>5</td>
</tr>
</tbody>
</table>

* impurity in the sample obscured the acidic peaks.
tr - trace, nd - not detected.

The hydrolysate of the whole mucilage contained four acidic components, two of which had retention times corresponding to galacturonic acid and the galacturonosyl rhamnose dimer.

Due to the rather small quantities of acidic species present in most of the samples analysed, an estimate of the total content of acidic species was made by comparison with standard solutions of galacturonic acid.
Experiment 4.4. Circular Dichroism of Mustard Mucilage.

The possibility of using circular dichroism to follow changes in polysaccharide associations in the mucilage under certain conditions was investigated.

The "heavy fraction" of the mucilage from ultracentrifugation (0.2% w/v) was dissolved in deionised water and the circular dichroism recorded in a 10mm path length cell at 25°C. The solution was then treated at 100°C for an hour before cooling to 25°C and again recording the circular dichroism spectrum. Finally the solution was boiled for two minutes and the spectrum subsequently recorded at 25°C.

The procedure was then repeated for the "heavy fraction" dissolved in potassium chloride solution (2% w/v).

Due to the nature of the mucilage solution being examined, the circular dichroism spectra showed considerable noise in the signal. However the estimated spectra are shown in Fig. 4.4.
Fig. 4.4. Circular dichroism spectra of the "heavy fraction" of mustard mucilage (0.2% w/v) before and after "denaturation".

- in deionised water.
- in deionised water after heating to 100°C for 1 hour.
- in deionised water after further boiling for 2 minutes.
- in 2% potassium chloride.
- in 2% potassium chloride after boiling for 2 minutes.

All spectra were recorded in 10mm path length cell at 25°C.
Discussion

From the experiments carried out above and the previous studies on the identification of cellulose, the characteristic properties of the mucilage particle can now be described in terms of biopolymer associations analogous to those established for globular proteins. Although containing a high proportion of crystalline cellulose, the mucilage particle retains its solubility in water due to the intimate association with pectic material and therefore parallels the situation in certain soluble proteins, such as myoglobin, where the protein has a high degree of internal ordered structure. The mucilage particle also shows behaviour which is typical of the denaturation behaviour of ordered structures in protein chemistry. When a solution of the mucilage is boiled, coagulation and the formation of a gelatinous precipitate occurs. This denaturation process was facilitated by several reagents including those used extensively to cause denaturation of proteins.

The analysis of the constituent sugars after hydrolysis of the denatured material shows that when denaturation is achieved by boiling in water the composition of the denatured particle and the original mucilage are very similar, the denatured mucilage showing only a slight decrease in the acidic pectic component. The purification of the mucilage by preparative ultracentrifugation also yields a particle (heavy fraction) which shows a decrease in this component which suggests that some pectin material
may be present as a separate entity or alternatively more loosely attached to the mucilage particle than the remainder. However, the urea used to establish the density gradient for ultracentrifugation may also have affected the original mucilage particle. 

Denaturation with several other reagents, potassium chloride, E.D.T.A., sodium lauryl sulphate and potassium thiocyanates shows more extensive loss of the acidic component from the mucilage particle suggesting that these reagents cause partial dissociation of the complex together with denaturation. It therefore appears that in the case of these reagents non-covalent associations between the acidic component and the rest of the complex are being disrupted. When a more drastic alkaline borohydride treatment which can also affect covalent linkages was applied to the mucilage, mostly all of the non-cellulosic material was stripped from the particle. Only a small quantity of mannose and galactose together with glucose appeared in the hydrolysate from the fibrous material obtained from the treatment. However trace quantities of acidic sugars also appeared.

These results comply with the proposed model for the association of the polysaccharides in the mucilage particle (Fig. 4.3.) where the cellulose fibril is envisaged as having most intimate association with a neutral polysaccharide, in the case of the mustard mucilage, most likely a mannan and then further encapsulation by neutral xyloaraban and acidic pectic material.
Circular dichroism studies showed that the "heavy fraction" of the mucilage particle has a negative spectrum which decreases in amplitude when the particle is denatured. This change in the spectrum was much more emphatic when denaturation was carried out in potassium chloride, the spectrum of the denatured particle in this case became positive.
The negative spectrum of the mucilage was unexpected when comparison is made with other pectic material but this may possible be explained by the presence of proteinaceous impurities for which evidence was also provided by ultraviolet absorption spectra. However, the recorded spectra also contained considerable signal noise and most likely exhibit similar spectral distortion to that frequently envisaged in the circular dichroism of most particulate biomembrane suspensions 151.

Interpretation of these spectra in terms of tertiary structure was therefore not possible.
The evidence presented in this chapter therefore represents further support for treating polysaccharides as biopolymers in which a molecular description of tertiary and quaternary structures is essential to a more complete understanding of their function in biological systems.
GENERAL METHODS

Materials

High Methoxy Pectin (HM-1) was a commercial sample of citrus slow set pectin (unstandardised, approx. grade 230) obtained from H. P. Bulmers Ltd., Hereford.

Firmagel, Type 135 (LM-1) was a low methoxy citrus pectin also obtained from the above source.

Low Methoxy Pectin, Type CE 320 (LM-2) was a commercial sample of pectin obtained from Chemical Exchange (UK) Ltd. Pectic Acid samples were obtained by alkali de-esterification of high and low methoxy pectins.

PA-1, de-esterified HM-1.

PA-2, de-esterified LM-2.

Low Methoxy Amide Pectin was a laboratory prepared sample obtained from Professor W. Pilnik, University of Wageningen, Holland.

Fully Esterified Pectin was prepared from HM-1 as described in Chapter I.

A highly esterified pectin (HM-2) was prepared from the above fully esterified sample by partial de-esterification with acid. All pectin samples were converted to their sodium salts before use.

An analysis of the approximate composition of the above pectin samples was carried out by Mr. D. Welti, Unilever Research by infra-red spectroscopy.
Pectin | % of uronic acid units present as methyl ester | % of uronic acid in total sample
--- | --- | ---
HM-1 | 61 | 88
HM-2 | 78 | 91
LM-1 | 30 | 91
LM-2 | 27 | 87
Amide pectin | 26 (28% amide) | -

Acarose (code REX 5468) was provided by Marine Colloids Inc.
Locust Bean Gum (code REX 5924) was also provided by Marine Colloids Inc.
Kappa-carrageenan (code REX 5401) was prepared from Chondrus crispus also in the laboratory of Marine Colloids Inc.
Lambda-carrageenan (code RENJ 5263) was prepared from Gigartina canaliculata also in the above laboratory.
Fenugreek gum was prepared by R. Moorhouse, Unilever Research from the seeds of Trigonella foenum-graecum by the method of Andrews et al.
Keltrol was obtained from Kelco Company, New Jersey, U.S.A.

De-esterification of Pectin Samples
Complete de-esterification of pectin was carried out by an alkaline treatment while partial de-esterification was accomplished in an acidic aqueous alcohol system

Pectin (20g) was dissolved in water (1000ml) containing 80ml of 1N sodium hydroxide. The solution was allowed to stand at room temperature for 2 hours and was then centrifuged at 20,000xg. Hydrochloric acid (18% v/v) was then added dropwise to the clear solution with vigorous stirring. When precipitation was complete, the pectic acid gel was squeezed through muslin and washed twice with 60% acidified
ethanol (5ml conc. HCl per litre) then successively with 60% ethanol, ethanol and ether. The air dried product was then converted to the sodium salt from the titration of a 1% solution with NaOH (0.2N). The sodium pectate was isolated by freeze drying.

Partial de-esterification was achieved by suspending pectin (3g) in a mixture of isopropyl alcohol (81ml), water (33ml) and conc. hydrochloric acid (11ml) at 38°C for 14 hours during which the suspension was periodically stirred. The pectin was then filtered off, washed with 60% v/v isopropyl alcohol until free of chloride and subsequently with isopropyl alcohol and ether before air drying.

Preparation of Citrate Buffer

Citrate buffers were made up by addition of sodium citrate (0.1M) to a solution of citric acid (0.1M) until the desired pH was achieved.

Optical Rotation Measurements

These were carried out on a Perkin-Elmer 141 polarimeter in a 1dm. path length thermostated cell. The temperature was controlled by means of a water circulating bath (Haake) fitted with a contact thermometer which controlled the temperature. Unless otherwise stated optical rotation readings were taken after allowing the system to equilibrate for 30 minutes or more at the desired temperature. The rotation measurements were taken at several wavelengths as readings at 365 and 436nm. were increased in amplitude and highest light intensities occurred at 436 and 546nm. Measurements at 589nm. are given here as these are usually quoted in the literature. Cell blanks were also measured
for the various experiments but these were found to be very small compared to the actual readings and to the changes in readings.

**Circular Dichroism Measurements**

Measurements of the circular dichroism spectra was carried out on Cary 61 spectropolarimeter. Temperature control of the cell holder was achieved by the same system as in the optical rotation methods. Cell blanks were allowed for.

**Phenol-Sulphuric Acid Assay for Carbohydrate in Freeze-Thaw Experiments.**

Aqueous phenol (1ml, 5% w/v) was added to the sample solution (1ml containing ca. 0.1mg carbohydrate material). To this concentrated sulphuric acid (5ml) was added rapidly. After cooling the solution for 30 minutes, the optical density was measured on a Pye Unicam SP 600, UV spectrophotometer at 485nm.

The carbohydrate present in the syneresed liquid was estimated by comparison of their optical density readings with that of the original solutions of synergist. Allowance was made for any syneresed material from the gelling polysaccharide by measuring blanks in the absence of the synergist.

**Paper Chromatography**

Paper chromatography was carried out on Whatman No.1. paper by descending chromatography in the following solvent systems.

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

After development for the required time, the chromatograms were air-dried and sugars located by one of the following spray reagents.

A. p-anisidine hydrochloride, as a 2% solution in n-butanol. The chromatograms were heated for 5 - 10 minutes at 120°C to
develop the colours which are characteristic of individual sugars.

B. Aniline oxalate, saturated solution in ethanol. The chromatograms were heated at 120°C for 5 - 10 minutes to develop the sugar spots.
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