CHANGES IN CELL WALL OF MANGO FRUIT DURING RIPENING.

A Thesis submitted by

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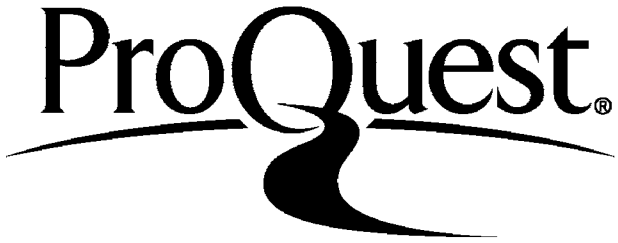

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In memory of Pam, who taught me what it meant to be a human being.

ABSTRACT

Changes in cell walls and in the levels of polysaccharide-degrading enzymes, in mesocarp of mango fruit during ripening were investigated.

Total, insoluble cell wall content declined with ripening. Galactose, arabinose, xylose and mannose were lost from the wall during ripening and autolysis of ripe mango mesocarp released arabinose and xylose into the soluble fraction. Galacturonic acid was also removed from the wall but polygalacturonase (which degrades pectic galacturonan in fruits of many other species) could not be detected. Several glycosidase activities were present in the mesocarp and, of these, β-D-galactosidase and α-D-mannosidase increased markedly during ripening. The increase in the former may be significant in the removal of wall-bound galactose accompanying ripening.

Enzyme extracts prepared from ripe mangoes released galactose from cell walls isolated from unripe fruits but soluble polysaccharides released from the wall by enzyme action in vitro could not be detected by electrophoresis. Polysaccharides, composed mainly of xylose and glucose, were co-extracted with enzymic protein from mango mesocarp and there was evidence that these polymers underwent degradation during ripening. They may be derived, partly at least, from wall hemicelluloses.

It is proposed that one process contributing

to cell wall breakdown and resultant tissue softening in the mango may be the enzymic hydrolysis of polysaccharides containing arabinose and galactose (possibly neutral pectic polymers) and that polygalacturonase activity probably does not contribute significantly to tissue softening. The mechanism for removal of galacturonan from the wall was not established but this may result from prior removal of polysaccharides containing arabinose and galactose. Data obtained also suggest that hemicelluloses in the wall may be degraded during ripening.

Critical appraisal was made of the limitations of both preparatory and analytical methods employed and proposals made for employing better methods in further work.

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I. INTRODUCTION.

I. 1. Introductory Remarks.

The purpose of the present work was to investigate changes in the composition of the cell walls of the soft, edible mesocarp tissue of mango during ripening of the fruit and to investigate, also, changes in the levels of activity of carbohydrate-degrading enzymes present in this tissue which might be related to cell wall breakdown. Such changes are central to the process of tissue softening which, alongside changes in size, shape, colour and flavour, is one of the main physical manifestations of ripening related to the eating qualities of fruits.

Fruits and their products constitute a commercially significant food commodity. The mango, in particular, is regarded as the choicest of indigenous fruits in many parts of the Orient, occupying relatively the same position in the tropics as the apple in Europe and North America (Singh, 1960; Hulme, 1971). India is, by far, the world's most important producer, her annual crop of fresh mangoes being estimated at 3-4 million tons (International Trade Centre Report, 1971) and her exports of mango products at 11,000 tons per annum (Jones, 1973). Other important mango-growing countries include Kenya, various Caribbean countries, South Africa, Egypt and Israel.

One of the limiting factors to the economic value of fruit crops is the relatively short duration of ripening of many fruits, with resultant limited storage and shelf life. Fruit rot accompanying over-ripeness

before sale is a major source of commercial loss to the fruit growing industry. This is especially true of many tropical fruits, the mango included, cultivated in climatic conditions which facilitate rapid ripening, senescence and rot and frequently in countries where the technology and infrastructure for fruit handling and storage is minimal.

Transport of fruits from the countries of cultivation to the countries of marketing poses a related problem which impinges upon the viable shelf life of the mature fruits. Some tropical and sub-tropical fruits have proved unsuitable for existing bulk methods of delaying fruit ripening during storage. The mango, for example, is particularly susceptible to "chill injury", a marked discolouration with accompanying textural and flavour changes, which renders the fruit commercially valueless upon removal from low temperature (0-5^{o}) storage (Campbell, 1959; Mattoo and Modi, 1969), a method of delaying ripening which is much applied to such temperate fruits as apples (Wills *et al.*,1976) and pears (Knee, 1973).

The useful life of most mango cultivars after harvesting when the fruits are hard and green (i.e. mature but unripe) is approximately 14 days. Ten days after harvesting the fruits are fully ripened and become susceptible to infection with microorganisms (Krishnamurthy and Subramanyam, 1973). All methods of extending this 14-day period, which are acceptable to the consumer and economically viable, by delaying or slowing ripening are

potentially of commercial value to the mango growers.

Cell wall breakdown leading to tissue softening is central to fruit ripening. Elucidation of the mechanisms of this wall breakdown in fruits is of potential value to food technologists seeking chemical or physical means of delaying or retarding ripening.

Besides dealing with what is known of mango cell wall changes occuring during ripening it is necessary, as background, to outline the chemistry of the polymers which are proposed to be present as components of the primary cell walls of dicotyledenous plant tissues, and those cell wall and related enzymic changes which have been noted in other fruit species in which ripening has been more extensively studied.

In this regard it must be borne in mind that elucidation of the chemical structure of the primary walls of higher plant tissues is in its infancy. A number of the polymers whose proposed structures are outlined below have not been isolated from sources known to contain only primary cell walls and no higher plant cell wall has been fully characterised chemically. There has been little characterisation of the constituent polymers of primary walls present in the soft tissues of fleshy fruits in particular. The relationship between structural data obtained from the primary walls of suspension-cultured cells, much-used as models for cell wall studies, and the structure of primary walls in intact plant tissues such as fruit tissue, in which each cell is cemented to the

next one by a pectin-rich middle lamella, is indefinite.

Hence, our present "knowledge" of the structures of primary cell walls in higher plants must be regarded as tentative and it is not possible to validly present a "universal model" for the primary walls of all dicotyledenous plant tissues. The best that can be achieved at the current level of knowledge is to propose somewhat speculative, generalised characteristics of dicot primary walls in general and of primary walls of soft fruit tissues in particular.

I. 2. Outline Biochemistry of the Plant Cell Wall.

I. 2. (a) Introduction

Cell walls are involved in many aspects of plant biology including morphology, growth and development of plant cells. Labavitch (1981) has recently reviewed cell wall turnover in plant development. Primary walls are laid down by and control the continuing growth of undifferentiated cells. Differentiating cells which have ceased growing develop a lignified secondary wall from the primary wall. During growth of the cell, polymers of the wall interact and change and the resulting change in wall properties can be correlated with a variation in its function. A knowledge of the detailed chemical structures of and interconnections between the constituent wall polymers should provide a basis for the understanding of both biological function and metabolic inter-relationships.

There have been several recent reviews of the chemistry of cell wall polysaccharides (Aspinall,1980,1981; Kato, 1981). Aspinall (1980) makes the point that whilst

plant polysaccharides fall into a number of well-defined structural families, some of which have long been known as cell wall components, others have only been recognised recently as arising from the wall and yet others are of undefined location and function.

Kato (1981) has described as "rather crude" the description of the primary cell wall as consisting of a network of cellulose microfibrils embedded in an amorphous matrix of pectin and hemicellulose (Northcote, 1977; Albersheim, 1978; Lamport, 1978; Preston, 1979; McNeil et al., 1979). Although it is true that these models do not completely account for the structural positions and roles of cell wall components, they do provide a framework by which some data on wall turnover during plant development can be assessed (Labavitch, 1981). As more data are generated to extend the models to cell walls as they occur in plant tissues, the models become more relevant. In spite of the present shortcomings, one thing is clear - a number of cell wall monosaccharide types can be found in a variety of polysaccharide species and in different glycosidic linkages. If we are to fully elucidate the mechanism by which a given monosaccharide is removed from the cell walls of a developing plant tissue, we must ultimately know if the missing monosaccharide was in a homo- or heteropolysaccharide and in what way the monosaccharide residues were linked to one another. Structural information of this type greatly enhances the value of wall turnover research.

I. 2. (b) Microfibrillar Component.

Crystalline cellulose fibres make up an important part of the framework of the cell walls of all higher plants. Cellulose is composed of extended chains of (1→4)-linked β-D-glucosyl residues, the glucan chains being arranged in an ordered manner and hydrogen-bonded together. The binding between approximately 40 glucan molecules results in 3.5nm diameter threadlike bodies termed elementary fibrils, these latter being further aggregated into 10 to 30nm diameter ropelike structures called microfibrils (Frey-Wyssling, 1969; Northcote, 1972; Preston, 1979).

Polymeric material containing sugars other than glucose is always found to be associated with cellulose isolated from cell walls. It is not presently clear whether these non-cellulosic polysaccharides are adsorbed on the microfibril surface, form part of individual heteroglucan chains or exist as separate chains intermingled with the outer glucan chains. Whatever the case, the location close to the microfibril surface and their similarity in structure to some of the hemicelluloses of the wall matrix material suggests a role as a key material for the entanglement of the microfibrils with the matrix (Northcote, 1972; Preston, 1979).

Northcote (1977) has suggested that the sequence of wall formation is that of synthesis and transport of matrix polysaccharides (pectins and hemicelluloses) first followed by cellulose synthesis, laid

down as microfibrils at the cell surface and woven into the matrix. Shafizadeh and McGinnis (1971) have noted that during the initial stages of cell growth the matrix is not rigid and that in the primary wall the microfibrils are laid down as a loosely-interwoven network, microfibrils being orientated at a fairly large angle to the long axis of the cell and arranged in loose bands in some regions of the cell surface. Primary cell wall cellulose glucan chains have been estimated to have degrees of polymerisation ranging from 2000 - 6000 (Marx-Figini, 1966; Marx-Figini and Schulz, 1966).

I. 2. (c) Matrix Noncellulosic Polysaccharides.

These polymers have been defined by their presence in fractions obtained by consecutive chemical extraction of isolated cell walls. The pectic polysaccharides are obtained by extracting cell walls with boiling water, EDTA or dilute acid and the hemicelluloses solubilised by the subsequent extraction of the same wall with alkali. Recently McNeil et al. (1979), have classified the pectic polysaccharides as those polymers found in covalent association with galacturonosyl-containing polysaccharides and the hemicelluloses as those polysaccharides non-covalently associated with cellulose.

I. 2. (c) 1. Hemicelluloses.

I. 2. (c) 1.1. Xylans.

Higher plant xylans contain a main chain of (1→4)-linked β-D-xylopyranosyl residues (Aspinall, 1980, 1981) with a low degree of

1→3 branching in samples from some sources (Aspinall and Stephen, 1973). These polymers are associated with hemicellulose fractions extracted from plant cell walls. Xylans from most sources contain short side-chains of other sugar residues, these side-chains being of 3 main types:

(a) single (4-O-methyl-)α-D-glucopyranosyluronic acid residues, most frequently attached to C2 of backbone xylose units.

(b) single α-L-arabinofuranosyl residues, most frequently attached to C3 of xylose units but with double branching (1→2 and 1→3) on xylose residues in more highly substituted arabinoxylans

(c) more complex side-chains in which (1→3)-linked α-L-arabinofuranosyl residues carry additional glycosyl substituents (Wilkie and Woo, 1977).

Xylose residues are frequently acetylated at C2 and/or C3 (Timell, 1964a). The acetyl groups are numerous enough to prevent alignment and molecular aggregation of the chains and are believed to influence association of the xylans with other polysaccharide complexes within the wall structure (Northcote, 1972; Preston, 1979). McNeil et al. (1979) have recently established that a glucuronoarabinoxylan constitutes 5% of the primary walls of suspension-cultured sycamore cells.

A general structure for higher plant xylans is shown in Fig.1.

I.2. (c) 1.2. Xyloglucans

Xyloglucans have been isolated from primary walls of cultured sycamore (Albersheim, 1978) and etiolated bean (Kato et al., 1977) cells. These polysaccharides possess a backbone of (1→4)-linked β-D-glucosyl residues with single α-D-xylosyl residues linked 1→6 to the glucan backbone. Some of the xylosyl residues are terminal whilst others are substituted with (1→2)-linked β-D-galactosyl residues. The xyloglucan binds tightly to purified cellulose by hydrogen bonds (Aspinall et al. 1969; Albersheim, 1978) and it is believed that the O-β-D-galactosyl-(1→2)-α-D-xylosyl side chains reduce lateral associations between xyloglucan chains giving a monolayer of xyloglucan on the surface of each cellulose microfibril (Albersheim, 1978; McNeil et al., 1979).

Fig.2 shows the proposed structure for a portion of the sycamore polymer (after Albersheim, 1978).

I.2. (c) 1.3. Mixed β-D-glucans

The status of these polymers as components of primary cell walls in dicotyledenous plants is subject to some dispute but they have been reported as minor components of primary walls

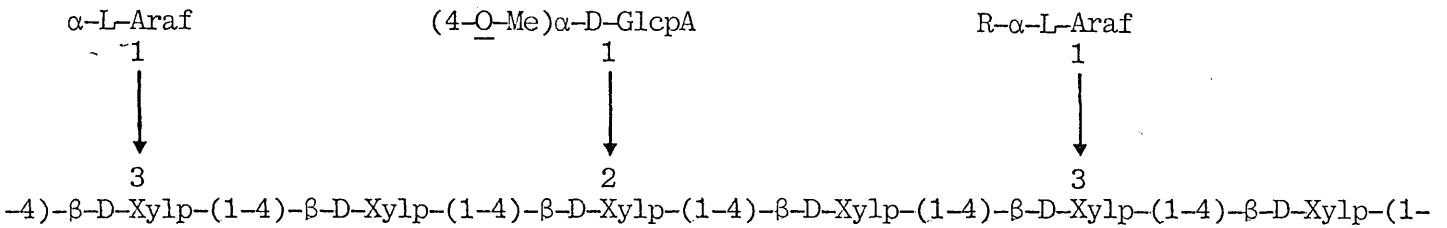


Fig.1. General structure of higher plant xylans showing commonly-encountered side chains.

Araf = arabinofuranose

Xylp = xylopyranose

(4-O-Me)α-D-GlcpA = 4-O-methyl-α-D-glucopyranosiduronic acid

R = variously-linked galactopyranose and/or xylopyranose residues in those higher plant xylans which have been characterised.

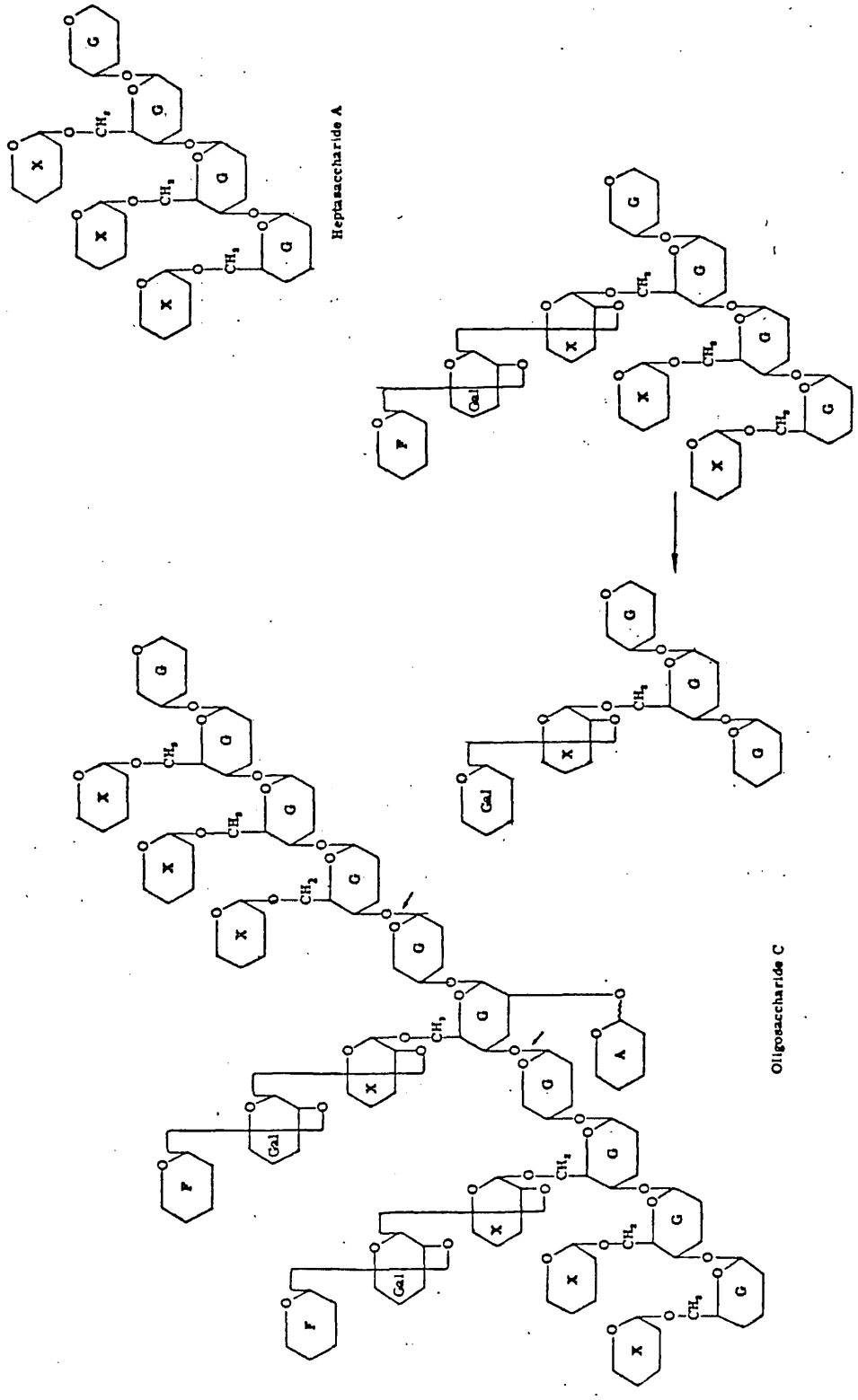


Fig.2 Proposed structure of a portion of the hemicellulosic xyloglucan of sycamore primary cell wall (after Albersheim, 1978). Endo-1,4-β-D-glucanase acting on oligosaccharide C at the bonds indicated by arrows gave rise to heptasaccharide A and nonosaccharide B. Pentasaccharide D was further derived from nonosaccharide B by the combined actions of α-L-fucosidase, α-D-xylosidase and β-D-glucosidase.

in Phaseolus hypocotyls (Buchala and Franz, 1974) and cultured Vinca rosea cells (Takeuchi and Komamine, 1978).

In monocots this glucan is a prominent structural wall component and it is from such sources that it has been structurally characterised. The polymer from oat coleoptile possesses an apparent molecular weight of 2.0×10^5 (Wada and Ray, 1978) and (1→3) and (1→4) linkages in the ratio 1:2 with a repeating structure composed of a single (1→3)-linked β-D-glucosyl alternating with two (1→4)-linked β-D-glucosyl residues (Labavitch and Ray, 1978).

I.2. (c) 1.4. Glucomannans

Glucomannans are common in higher plants being predominant in hemicelluloses of the secondary walls of gymnosperms (Timell, 1965). Pine glucomannan is a linear polysaccharide containing both (1→4)-linked β-D-mannosyl and β-D-glucosyl residues in the ratio 3:1, the residues being randomly distributed in the chain. The polysaccharide contains some terminal α-D-galactosyl residues linked to C6 of main chain hexosyl units and about half of the mannosyl residues are acetylated at C2 or C3 (Lindberg et al., 1973). These polymers are much less prominent in the hemicelluloses of angiosperms, accounting for approximately

5% of the total cell wall material (Timell, 1964a).

Methylation analyses indicate the presence of 4-linked mannosyl residues in primary walls isolated from bean hypocotyl and cultured cells of red kidney bean, soybean and tomato (Albersheim, 1976), and pea epicotyl (Gilkes and Hall, 1977). This may suggest that these walls contain mannan(s) or perhaps a glucomannan.

I.2. (c) 2. Pectic Polysaccharides

I.2. (c) 2.1. Rhamnogalacturonan

The acidic polysaccharides have a basic structure consisting of a chain of (1→4)-linked α-D-galactopyranosyluronic residues in which 2-linked L-rhamnosyl residues are interspersed. A variable number of carboxyl groups are esterified as methyl esters. The rhamnosyl residues are not randomly distributed in the chain but probably occur as rhamnosyl-(1→4)-galactopyranosyluronic acid-(1→2)-rhamnosyl units separated by short chains of 4-linked galacturonosyl residues (Aspinall, 1973, McNeil et al.,1979). Methylation analyses indicate that 50% of the rhamnosyl residues have substituents at both C2 and C4 and these residues are believed to provide the major, if not the only, branching points of the rhamno-galacturonan chain. Since no aldobiouronic acid with a galactopyranosyluronic acid

residue attached to C4 of a rhamnosyl residue has been isolated, the 2,4-linked rhamnosyl residues are probably the points of attachment of at least some of the neutral pectic side-chains (McNeil et al., 1979).

The rhamnogalacturonan obtained from the primary walls of cultured sycamore cells contains rhamnose, galacturonic acid, arabinose and galactose in the proportions 1:2:1.5:1.5 and gel filtration indicates a degree of polymerisation of about 10,000. The anomeric configuration of the rhamnosidic bonds is unknown (McNeil et al., 1979; Darvill et al., 1980). A proposed structure for the sycamore rhamnogalacturonan (after Albersheim, 1978) is shown in Fig.3.

(1→4)-linked α-D-galacturonans lacking rhamnosyl residues have been isolated from sunflower seeds (Zitko and Bishop, 1966), apple pectin (Barrett and Northcote, 1965) and the primary walls of cultured sycamore cells (McNeil et al., 1979; Darvill et al., 1980). Covalent attachment of such homogalacturonans to rhamnogalacturonan has been suggested (Darvill et al., 1980) and Rees (1972, 1975) has proposed that calcium confers rigidity to plant cell walls by inclusion of calcium ions between parallel, unesterified homogalacturonan chains. In this "egg-box model" it is proposed that each calcium

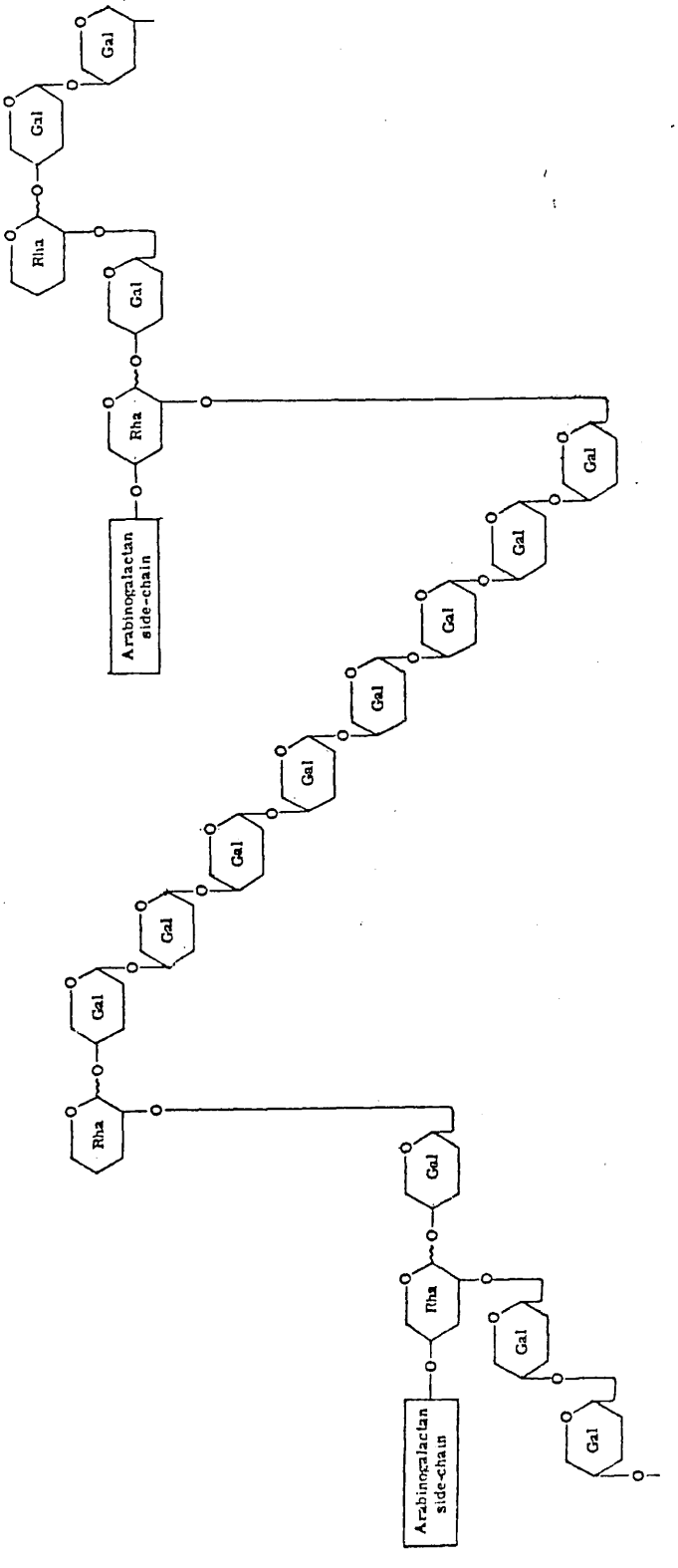


Fig.3. Proposed structure for the pectic rhamnogalacturonan of sycamore primary cell walls (after Albersheim, 1978).

ion chelates to the oxygen atoms of four galacturonosyl residues distributed between two parallel galacturonan chains, so that the ions are packed like eggs within an egg-box composed of galacturonans.

A highly complex, branched rhamnogalacturonan containing apiose, which upon hydrolysis yields ten different monosaccharides, has been identified as a minor component (3-4%) of cultured sycamore cell walls (Darvill et al., 1978). Apiogalacturonan, in which apiose and galacturonic acid are the only glycosyl constituents, has been isolated and identified as a component of the cell wall of duckweed (Duff, 1965; Beck, 1967; Hart and Kindel, 1970).

I.2. (c) 2.2. Pectic Arabinans and Galactans.

Covalent attachment of these neutral polymers to rhamnogalacturonan is suggested by their co-chromatography after liberation by endopolygalacturonase digestion of cultured sycamore cell walls (Darvill et al., 1980) and by migration of the neutral and acidic polymers as a single fraction upon electrophoresis of apple pectic polysaccharides (Barrett and Northcote, 1965). Knee et al.'s (1975) enzymic analysis of apple fruit cell walls also provided evidence of covalent association between these polymers. 2,4-linked L-rhamnosyl

residues interspersed in the rhamnogalacturonan backbone are proposed to be the sites of attachment of the neutral side-chains (McNeil et al., 1979); Darvill et al., 1980; Kato, 1981).

(1→4)-linked β-D-galactans have been isolated from citrus pectin (Labavitch et al., 1976), white willow (Toman et al., 1972) and beech (Meier, 1962) and pectic galactans containing 6-linked as well as 4-linked galactosyl residues have been isolated from various plant sources (Meier et al., 1962; Toman et al., 1972; McNeil and Albersheim, 1978). The existence of such homogalactans has been subjected to question on the basis that the isolation procedures employed would cause acid hydrolysis of arabinofuranosidic linkages in arabinogalactans (Aspinall, 1980). (1→4)-linked β-D-galactans with up to 25% of L-arabinofuranose residues in side-chains form a family of structurally-related polymers. In some plant tissues, e.g. Centrosema plumari seeds and soybean cotyledons (Aspinall, 1970) they are relatively abundant. The soybean polymer has been partly characterised and has the structure shown in Fig.4; it occurs in association with a complex rhamnogalacturonan to which the 4-linked galactan constitutes a side-chain. The mild isolation procedures employed for this neutral arabinogalactan render it unlikely to be an experimental artefact

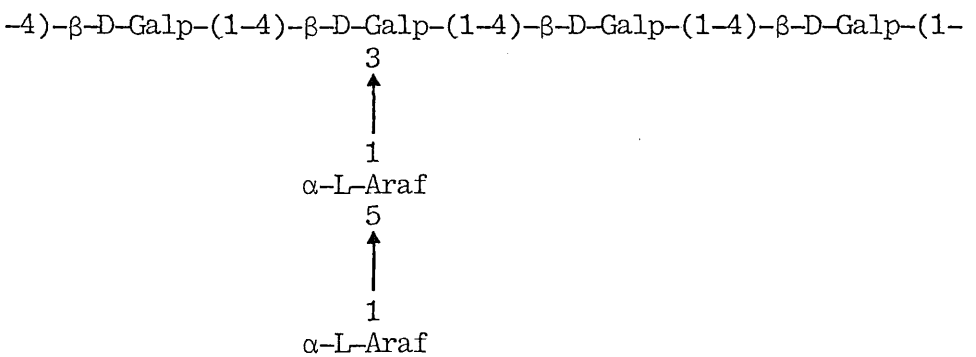


Fig.4. Partial structure of soybean cotyledon arabinogalactan (after Aspinall,1970).

Galp = galactopyranose

Araf = arabinofuranose

(Aspinall and Cottrell, 1971). No 4-linked galactan or arabinogalactan has been isolated from a purified primary cell wall but their presence is inferred by the detection of large amounts of (1→4)-linked β-D-galactosyl residues upon methylation analysis of cell walls or pectic fractions (Albersheim, 1976; Gilkes and Hall, 1977; Ring and Selvendran 1978). The galactans which have been studied have degrees of polymerisation ranging from 33 (Toman et al., 1972) to 50 (McNeil and Albersheim, 1978).

Pure arabinans have been isolated from mustard seeds (Rees and Richardson, 1966), Rosa glauca bark (Joseleau et al., 1977) and the primary walls of cultured sycamore cells (Darvill et al., 1980) and arabinans containing minor amounts of other glycosyl residues from the cell walls of many dicot plants (Hirst and Jones, 1947): Rees and Richardson, 1966; Aspinall and Cottrell, 1971; Siddiqui and Wood, 1974; Karacsonyi et al., 1975; Joseleau et al., 1977).

All of the arabinans that have been investigated are highly-branched polymers containing (1→5)-linked and (1→3)-linked α-L-arabinofuranosyl residues, generally represented by the partial structure shown in Fig.5. Some contain additional branching points provided by 2-linked

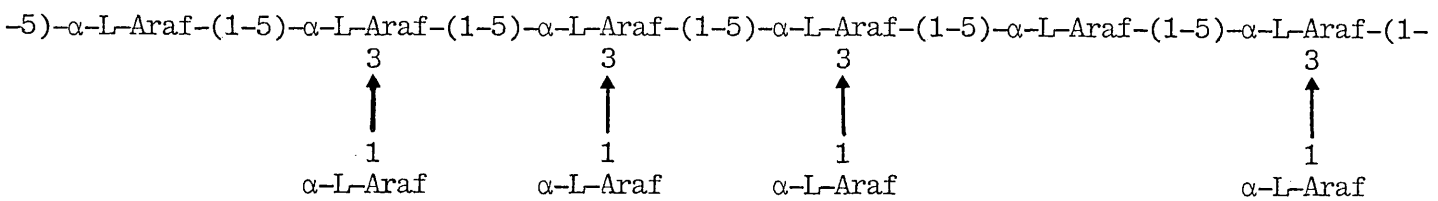


Fig.5. General partial structure of higher plant arabinans.

Araf = arabinofuranose

and perhaps 2,3-linked α-L-arabinofuranosyl residues, Methylation analysis of primary walls of cultured pea cells indicates the presence of a similar polymer (Darvill et al., 1980) and the much-studied sugar beet arabinan has been extracted in association with galacturonosyl residues which are probably derived from an acidic pectic backbone (Hough and Powell, 1960; Hullar, 1965). Estimation of the degree of polymerisation of pectic arabinans ranges from 34 (Joseleau et al., 1977) to 90 (Karacsonyi et al.,1975).

I.2. (c) 2.3. Arabino-3,6-galactans.

These so-called type II arabinogalactans are widely distributed in dicot tissues (Timell, 1965; Clarke et al., 1979) and are believed to be all similar in possessing a galactan backbone containing both (1→3)- and (1→6)-linked β-D-galactose, some of the galactosyl residues being 3,6-linked, with L-arabinofuranosyl residues present as side-chains linked (1→3) or (1→6) to galactosyl units. Beyond this general similarity there appears to be wide variation in both structure and monosaccharide composition between arabinogalactans from various sources (Clarke et al., 1979; Darvill et al., 1980); in some arabinosyl residues are present as disaccharide side-chains composed of O-β-L-arabinopyranose-(1→3)- and/or (1→5)-L-arabinofuranosyl

units (Darvill et al.,1980; Kato, 1981).

No arabinogalactan has been isolated from a source known to contain only primary cell walls. However, methylation analysis of a pectic fraction obtained from sycamore primary cell walls (Talmadge et al., 1973), suggested the presence of a pectic arabinogalactan similar to the one isolated from larch (Haq and Adams, 1961; Aspinall et al., 1968) which has been partly characterised and whose proposed partial structure is shown in Fig.6 (after Haq and Adams, 1961).

The most marked physical properties of these polymers are water-solubility, adhesiveness and ability to associate with other macromolecules (Clarke et al., 1979). Arabinogalactans in the middle lamella may function as adhesives to cement cell-cell contact. Arabinogalactans also associate with protein, e.g. suspension-cultured dicot cells secrete soluble arabinogalactan-glycoproteins into the suspension medium (Pope and Lamport, 1977; Hori and Sato, 1977, Clarke et.al., 1979). Arabinogalactans may be involved in bonding wall protein to other wall polymers or alternatively arabinogalactan-containing glycoproteins may be involved as adhesives within the wall matrix, associating with other wall polymers by non-covalent forces.

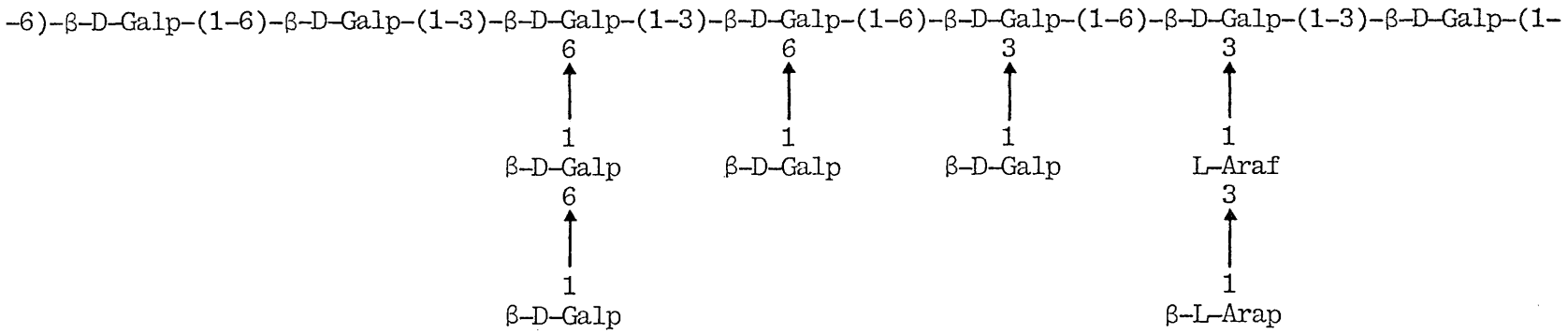


Fig.6. Proposed partial structure of larch (Larix laricina) arabinogalactan (after Haq and Adams, 1961).

Galp = galactopyranose
Arap = arabinopyranose
Araf = arabinofuranose

I.2. (d) Cell Wall Glycoproteins.

The primary walls of a wide variety of dicot plants contain a unique hydroxyproline-rich glycoprotein accounting for 2-10% of the cell wall (Lamport, 1965). All tryptic peptides derived from the glycoprotein contain at least one unit of the pentapeptide Ser-Hyp-Hyp-Hyp-Hyp (Lamport, 1973); in the intact glycoprotein most of the hydroxyproline residues are glycosylated by a tri- or tetrasaccharide of arbinofuranose and most of the serine residues are galactosylated (Lamport et al., 1973).

The available evidence suggests that the glycoprotein is not covalently attached to any of the other cell wall polymers but the evidence does not rule out the possible existence of strong, non-covalent bonding between the glycoprotein and other wall polymers (Lamport, 1978).

The arabinogalactan-containing glycoproteins secreted by suspended dicot cells (Pope and Lamport, 1977; Hori and Sato, 1977; Clarke et al., 1979) have been previously mentioned (I.2 (c) 2.3.). Unlike the hydroxyproline-rich wall glycoprotein only 4% of the arabinose residues occur as tetra-arabinosides, the majority of arabinose residues being present in an arabinogalactan (Pope and Lamport, 1977). This arabinogalactan-glycoprotein is believed to originate from the cell wall but its role (if any) is presently only a subject for speculation.

Two lectin-like protein fractions have been extracted from the cell walls of kidney bean seedlings (Kauss and Glaser, 1974; Kauss and Bowles, 1976). These

lectin-like proteins bind specifically to galactosyl residues but it has not yet been ascertained whether they contain hydroxyproline. It has been suggested that lectins may be involved in establishing a non-covalent protein-glycan network within the primary wall (Kauss and Bowles, 1976). The specificity of these cell wall lectins for galactosyl residues may indicate that pectic galactans/arabinogalactans are of especial significance in the formation of such a protein-glycan network. Recently, Frey (1982a) has suggested a role for a new amino acid (which he named isodityrosine), isolated from hydrolysates of suspended cells of several dicot species, in the formation of aromatic cross-links (similar to those of lignin) between polypeptide molecules within the cell wall. It is conceivable that glycoproteins are held within the wall structure both by non-covalent polysaccharide-protein interactions and by such cross-linking between polypeptide chains.

I.2. (e) Phenolic Components.

Lignification (i.e. impregnation of the wall structure with crystalline, hydrophobic polymers derived by enzymic dehydrogenation and subsequent polymerisation of cinnamic alcohols such as coumaryl alcohol (Freudenberg and Neish, 1968 ; Sakakibara, 1977), replacing and excluding water from the wall), is associated with formation of secondary cell walls (Northcote, 1972). Lignin does not occur in primary walls (Whitmore, 1974). However, very recently Fry (1982b, 1983) has shown ferulic and

p-coumaric acids esterified to non-reducing termini of galactan and arabinan chains forming part of the neutral pectic polymers of the primary walls of cultured spinach cells.

Amongst the possible roles suggested by Fry (1982b, 1983) for these phenolic esters is cross-linking of pectin molecules via diferulate bridges, such cross-linking possibly regulating wall extension in cell expansion growth, and as sites for initiation of lignification during the first stages of secondary wall formation after cessation of cell growth.

I.3. Primary Cell Walls.

I.3. (a) Polysaccharide Association Within the Dicot Primary Cell Wall.

In the previous section (I.2.) the chemical structures of the individual polymers thought to make up the primary cell wall were outlined. The relative dispositions of these wall constituent molecules and the nature and distribution of the covalent and non-covalent bonds between them remain subjects for controversy and speculation.

One structural model of the primary wall of suspension-cultured sycamore cells is presented by Albersheim (1978) according to the results of methylation analyses of both the isolated cell wall material and the defined fragments released by the action of specific polysaccharide-degrading enzymes. This preliminary model involves all but one of the wall polysaccharides in a covalently linked network, the exception being xyloglucan

hydrogen bonded to the cellulose microfibrils. Amongst the covalent linkages proposed in this model are:

(1) the attachment of xyloglucan molecules coating cellulose microfibrils to pectic galactan side chains by glycosidic linkage between the reducing ends of the xyloglucan chains and non-reducing ends of the galactans. The galactans are linked at their reducing ends to rhamnose residues forming part of the pectic rhamnogalacturonan.

(2) the linkage of rhamnogalacturonan to the cell wall glycoprotein through arabino-3,6-galactan attached to serine residues of the polypeptide

(3) the covalent attachment of homogalacturonan to rhamnogalacturonan

One depiction of this model is shown in Fig.7 (redrawn by Robinson, 1977 after Keegstra et al., 1973 and Albersheim, 1975).

The model structures for pectic rhamnogalacturonan with its side branches of chains of arabinosyl and galactosyl residues and for xyloglucan hydrogen-bonded to cellulose microfibrils, worked out in Albersheim's laboratory, have gained general acceptance. McNeil et al. (1979) have given the composition of the sycamore primary wall as 34% pectic polysaccharides (comprising 7% rhamnogalactouronan, 6% homogalacturonan, 9% arabinan, 9% galactan and possibly arabinogalactan and 3% apiose-containing rhamnogalacturonan), 24% hemicelluloses (comprising 19% xyloglucan and 5% glucuronoarabinoxylan), 23% cellulose and 19% hydroxyproline-rich glycoprotein.

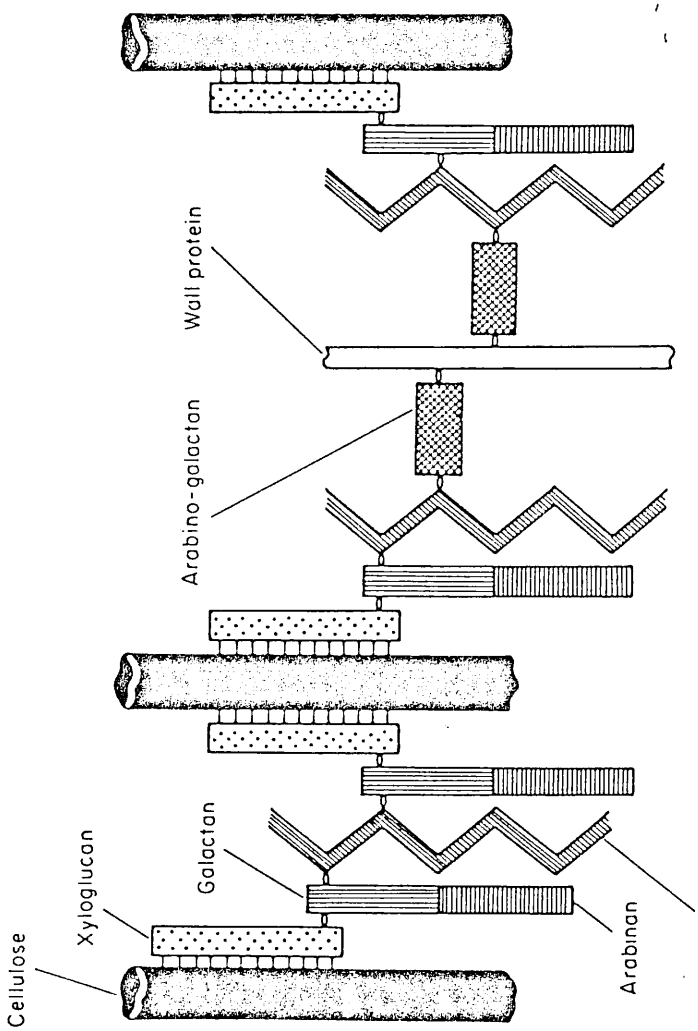


FIG. 7. Suggested scheme for the structure of the primary wall of sycamore callus cells (redrawn by Robinson (1977) from Keegstra *et al.* (1973) and Albersheim (1975) and copied with permission).

More controversial have been the proposed covalent linkages between rhamnogalacturonan, xyloglucan and other polymers in the cell wall, especially when presented in the context of a general model for the primary walls of all dicot tissues. The existence of these linkages has not been substantiated and other authors (e.g. Monro et al., 1976a) have preferred model wall structures based on a protein-glycan network(s) stabilised largely by non-covalent forces.

The "egg-box model" of Rees (1972, 1975) (see also I.2 (c) 2.1.) is an attractive model for the manner in which calcium strengthens cell walls. This model proposes that the calcium chelate results in increased rigidity of the galacturonans and in cross-linking between pectic galacturonan chains, the degree of cross-linking being sensitive to the degree of methyl esterification of the galacturonans. The possibility of other types of non-covalent interactions between the pectic polysaccharides and other cell wall polymers must certainly be considered; indeed, Preston (1979) suggests that non-covalent interactions may provide the most important interconnections between the pectic polysaccharides and other ~~other~~ cell wall polymers.

The bonding of hemicelluloses (xyloglucans and perhaps glucuronoarabinoxylans) to cellulose through multiple hydrogen bonds is clearly one of the major interconnections of the wall polymers, probably functioning to prevent cellulose microfibrils adhering to each other to form enormous aggregates (Albersheim, 1978; Kato, 1981).

Plant arabinoxylans form aggregates in solution (Blake and Richards, 1971; McNeil et al., 1979). This ability to form aggregates suggests that hemicelluloses may adhere not only to cellulose microfibrils but also to other hemicellulose molecules (and perhaps also to other wall polymers). In this way hemicelluloses may form a gel structure stabilising cellulose microfibrils within the wall matrix.

The position of wall protein within the wall matrix is the least characterised link. There is no direct evidence which demonstrates a covalent linkage between the hydroxyproline-rich glycoprotein and polysaccharides in the cell wall. Lamport and his colleagues (Mort and Lamport, 1977; Lamport, 1978) have demonstrated that complete deglycosylation of dicot primary walls by treatment with anhydrous hydrogen fluoride does not lead to protein solubilisation from the wall. They conclude that protein is cross-linked through non-covalent interactions to the polysaccharides of the cell wall. Alkaline extraction of cell walls (Selvendran, 1975; Monro et al. 1976b) yields results which support this conclusion. It may be that the hydroxyproline-rich glycoprotein (or other proteins within the wall), acting as lectins, participate in the cross-linking of wall polysaccharides (Kauss and Bowles, 1976).

I. 3. (b) Special Features of Fruit Primary Cell Walls.

Only one group of workers, Knee and his colleagues, have attempted systematic characterisation of

the constituent polymers in the primary walls of the soft tissues of fruits and elucidation of their association within the wall. Recently, Knee and Bartley (1981) reviewed the special features of fruit primary cell walls based upon their analyses of the walls of the soft tissue of apple and, to a lesser extent, of strawberry.

Histological studies of ripening fruit reveal extensive cell separation and it is natural to suppose that changes in the middle lamella are responsible for this. The Albersheim model was developed from the analysis of the walls of suspension-cultured cells and has little to say about middle lamellar structure, except by implication that it would be co-extensive with and similar to the matrix of the primary wall. Ferric hydroxamate staining followed by electron microscopy has confirmed that the middle lamella is pectin-rich (Albersheim and Killias, 1963). Chelating agents cause cell separation in non-woody plant tissues, presumably by removal of calcium from its supposed chelate with homogalacturonan chains in the middle lamella allowing separation of the chains (Ginsburg, 1961; Letham, 1962; Linhan and Hughes, 1969). Both the chelating agent sodium polyphosphate and diazomethane (which methylates free carboxyl groups of galacturonans) cause loss of cell to cell cohesion in apple tissue (Knee, 1978a) and diazomethane solubilises homogalacturonan from apple cell walls (Knee and Bartley, 1981). A fungal isoenzyme of polygalacturonase which caused separation of cells when applied to apple discs, with only limited degradation of cell walls, was concluded

by Knee et al. (1975) to selectively attack a calcium cross-linked homogalacturonan which is important in middle lamellar structure. In normally-ripening tomatoes cell wall-bound calcium decreases three-fold during the later stages of ripening in which extensive cell separation (Poovaiah, 1979) and galacturonan solubilisation (Gross and Wallner, 1979) occur.

This evidence supports the general conclusion that the cells of fleshy fruit tissues are cemented by a middle lamella in which the predominant polysaccharide is homogalacturonan and that cell to cell cohesion is dependent upon extensive cross-linking between unesterified regions of homogalacturonans by calcium ions. The proposed structure (after Knee and Bartley, 1981) for apple homogalacturonan is shown in Fig.8 (a).

The major portion of fleshy fruits consists of succulent parenchyma (Southgate, 1976); apart from the vascular tissue and specialised cells, e.g. the stone cells containing sclereids in pears, the cell walls of the fleshy parts of most fruits are unlignified (Knee and Bartley, 1981). Knee and Bartley (1981) showed that the primary walls of unripe apple and strawberry tissue contain a low proportion of hydroxyproline-rich protein and small amounts of xylose and mannose residues which are characteristic of hemicelluloses. On the other hand they contain a high proportion of galacturonic acid, galactose and arabinose which are typical of pectic polymers. The cell walls of unripe tomato fruit possess a similar gross composition with low protein content and components of the pectic

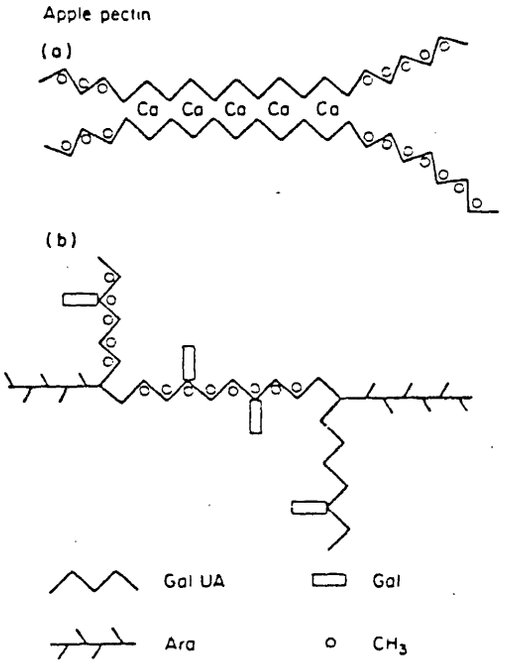


FIG. 8. Proposed structures for polygalacturonate fractions in apple fruit. (a) Sections of two "homogalacturonan" chains with linkage region. (b) Portion of branched rhamnogalacturonan. The kinks in the backbone represent rhamnose residues which are possibly the site of attachment of arabinan branches.

polysaccharides comprising 60% of the wall, galacturonic acid and galactose being particularly prominent (Gross and Wallner, 1979). There are however considerable variations in individual monosaccharides between these fruits and, in the walls of unripe pear tissue, whilst pectic components most notably galacturonic acid and arabinose are also predominant, xylose levels are considerably higher than in the walls of the other fruits (Ahmed and Labavitch, 1980a).

The tentative conclusion that may be drawn is that in those fruits which have been studied pectic polymers constitute the major proportion of the primary wall matrix. The proportion of hemicelluloses is clearly much smaller but indefinite and the limited evidence available suggests that it varies between fruit species. It must be borne in mind that, since insufficient fractionation and characterisation of the constituent polysaccharides of fruit cell walls has been carried out, the possible contribution of hemicelluloses towards the yields in wall hydrolysates of sugars like galactose and arabinose is unknown. In both tomatoes (Gross and Wallner, 1979) and pears (Ahmed and Labavitch, 1980a) at the unripe stage the proportion of non-cellulosic glucan in the wall is low and cellulose appears to make up 25% of the wall. The available evidence suggests that wall glycoprotein is quantitatively a minor wall component in fruits.

Whilst it is possible that these tentative and highly generalised conclusions about gross wall composition outlined above apply to the fleshy tissues of fruits in

general, even the limited evidence available indicates that the cell walls of different fruit species at any given ripening stage are not identical. Indeed, it seems probable that the constituent polymers and architecture of the cell walls of fleshy fruits are species-specific, a conclusion that may well apply to the tissues of all dictoyledenous plant species. The similarities between the cell walls of the soft tissues of different fruit species probably reflect (in evolutionary terms) the similar role of tissue softening in all fleshy fruits in converting the fruit into an attractive seed container.

There has been limited fractionation and characterisation of some of the constituent polysaccharides of the cell walls of some fruits. Apple cell walls contain a branched rhamnogalacturonan in which rhamnosyl residues are attached to separate side-chains of arabinose and galactose units. Enzymic analysis showed that arabinose was present in a branched (1→5)- and (1→3)-linked α-L-arabinan of molecular weight approximately 5000 whilst galactose was present in a (1→4)-linked β-D-galactan of molecular weight approximately 1000 (Knee et al., 1975; Knee and Bartley, 1981). These workers considered that this polymer shared most of the features of the pectic rhamnogalacturonan of suspension-cultured sycamore cells and concluded that it formed the main constituent of the matrix of the apple primary wall encasing the cellulose microfibrils. There is no direct evidence of a covalent linkage between this pectic polymer and hemicellulose and it seems unlikely that

the arabinan is involved in covalent links to polymers other than the rhamnogalacturonan although it may have a role in non-covalent links stabilising a network of wall glycans (Knee, 1978a; Knee and Bartley, 1981). It is not clear whether rhamnogalacturonan is covalently linked to the homogalacturonan associated with the middle lamella. The structure proposed for the branched rhamnogalacturonan (after Knee and Bartley, 1981) is shown in Fig.8 (b).

Recently Pressey (1983) isolated, from a pectic fraction liberated from tomato cell walls by the combined action of purified pectin methyl esterase and endopolygalacturonase iso-enzymes, a polysaccharide which was predominantly a (1→4)-linked β-D-galactan. During ripening of pears a high molecular weight (1.5×10^6) polymer is released from the cell walls; structural analysis of this polymer indicates that it is a galacturonan bearing arabinan side-chains consisting of a backbone of (1→5)-linked arabinosyl residues some of which bear arabinosyl side-groups at C2 and/or C3 (Ahmed and Labavitch, 1980a). This evidence is consistent with the presence in the intact, unripe pear cell wall of a pectic polymer network comparable to the branched rhamnogalacturonan described in apple cell walls (Knee and Bartley, 1981).

Chromatographic purification of an alkaline extract from apple cell walls of the small amount of hemicellulose present yields a polysaccharide of similar gross composition to the cultured sycamore cell wall xyloglucan characterised by Bauer et al.(1973) (Knee, 1973b).

The hemicellulosic fraction of pear cell walls contains both a xyloglucan of this type (Ahmed, 1978) and a (1→4)-linked β-D-xylan (Chanda et al., 1951; Ahmed, 1978).

In summary, the limited data available on the constituent polymers of the primary cell walls of soft tissues of fruits indicates a general wall architecture based on cellulose microfibrils embedded in an amorphous matrix composed of pectic polymers, hemicelluloses and a small amount of hydroxyproline-rich protein, in which the pectic polymers are the major matrix component. Some of the constituent polymers of the wall matrix appear to share the general structural characteristic of matrix polysaccharides of suspension-cultured sycamore cell walls which have been projected as a model for the primary walls of dicotyledenous plants (Albersheim, 1978). Plant cell wall structures are probably species-specific but broad similarities in wall architecture between comparable tissues of various dicot species are not ruled out. Where the walls of adjacent fruit cells meet they appear to be cemented together by a middle lamella (or intercellular matrix), in which the predominant constituent polysaccharide is homogalacturonan probably cross-linked by calcium ions between unesterified regions of adjacent chains, and which is probably co-extensive with the primary wall matrix.

I. 4. Changes in Mango Mesocarp During Ripening.

No characterisation of the structure of the cell walls of mango mesocarp (fleshy, edible tissue) or of their constituent polymers has been carried out by workers

in the field of fruit physiology. Additionally, very little sophisticated investigation of changes occurring in cell wall constituents during ripening or of enzyme activities regulating such changes has been made. Much of the work carried out has consisted of examination of gross changes in cell wall fractions, largely the pectin component, with some published data for levels of pectin methyl esterase (which de-esterifies methylated pectin) in mango mesocarp during ripening. This limited previous work, which forms the background to attempts, in the work described in this thesis, to gain further information about the changes occurring in the mescarp during ripening, is reviewed below alongside other relevant background information concerning the process of mango ripening:

The mango is a climacteric fruit, i.e. the onset of ripening is marked by a dramatic increase in the respiratory activity of the fleshy tissue (Biale, 1960; Krishnamurthy and Subramanyam, 1973; Lakshminarayana, 1973) which marks the transition from the mature but hard, green fruit at harvest to a softened, yellowing fruit in which the mesocarp changes from white to a deep yellow in colour. There is substantial evidence that this "climacteric" rise in respiration (which occurs in the majority of fleshy fruits, especially those which soften rapidly) provides the energy requirement for an intense burst of protein synthesis, including synthesis and/or activation of enzymes directly involved in the ripening process (see reviews by Hulme 1972 and Hobson, 1979). Following initiation of ripening the

level of respiratory activity returns to a low level sustained into over-ripeness. Initiation of the climacteric rise in respiration is believed to be (in part at least) mediated by the plant hormone ethylene and ripening mangoes have been shown to give off ethylene during the climacteric period which stimulates the ripening of unripe mangoes placed in their proximity (Mukerjee, 1960, Burg and Burg, 1964). The role of respiratory change, including the proposed mediatory role of ethylene, in fruit ripening has been recently and extensively reviewed by Biale and Young (1981).

Consistent with this concept of ripening as a senescent process catalysed by a range of enzyme activities many of which arise by de novo enzyme biosynthesis and/or enzyme activation, processes for which the energy requirement is provided by a hormone-mediated, transient burst of respiratory activity, in the mango a number of hydrolases and oxidases and other enzymes involved in intermediary metabolism increase significantly in activity during the period coincident with the climacteric respiratory rise (Hulme, 1971, 1972). It is significant and relevant to the purposes of this thesis that amongst these enzymes are amylase and invertase (Mattoo and Modi, 1969); starch is mobilised very early in ripening with sucrose appearing to be hydrolysed later in the ripening process (Leley et al., 1943; Biale, 1960; Hulme, 1971). It is reasonable to suppose that, in part at least, the monosaccharides liberated by these activities are utilised as respiratory substrates.

However, the only mango enzyme which has been shown to increase during the ripening period and which is

known to contribute to the degradation of a cell wall constituent in fruit tissues is pectin methyl esterase (Mattoo and Modi, 1969). This enzyme possesses the ability to demethoxylate methyl-esterified, wall-bound pectin (protopectin) and is widely distributed in fruits and several authors have proposed that, in fruits which rapidly soften, its activity precedes that of polygalacturonase which shortens the chain-length of the partly de-esterified protopectin releasing a water-soluble pectin fraction which is subject to further attack by polygalacturonase (reviews by Pressey, 1977; Hobson, 1981). There are no published data relating to activities in mango tissue of any enzyme, including polygalacturonase, involved (or potentially involved) in cell wall degradation and tissue softening other than pectin methyl esterase.

Lewis (1978) writing about the avocado, another climacteric fruit, considered that:

> "softening of the mesocarp is probably due to changes in the pectic substances. The rise in respiration is accompanied by softening, a decrease in protopectins, a rise in water-soluble pectins and a decrease in the degree of esterification".

Whilst this statement is probably oversimplified, given the probable complexity of the matrix of the cell walls of dicot tissues, including fruits, which has been reviewed earlier in this thesis (see Section I.3), there is much evidence to suggest that these processes apply generally to climacteric fruit and are under enzymic control (Hobson,1981).

The limited information available for the ripening mango, which relates mainly to changes in pectin, is largely consistent with Lewis's (1978) concept of softening in climacteric fruits described above:

The alcohol-insoluble residue of mango tissue decreases alongside tissue softening, suggesting loss of total cell wall material during ripening (Rolz et al.,1971) although in an earlier study Leley et al. (1943) were not able to show this. Total pectin also falls (Jain, 1961; Saeed, El Tinay and Khattab, 1975), suggesting conversion of part of the pectic fraction into low molecular weight compounds, although the data of Mizuta and Subramanyam (1973) show a contrary increase. Several authors (Rolz et al., 1971; Mizuta and Subramanyam, 1973; Saeed, El Tinay and Khattab, 1975) have shown that the water-soluble fraction of mango pectin increases with the onset of the ripening process and softening of texture, and two of these authors (Mizuta and Subramanyam,1973; Saeed, El Tinay and Khattab, 1975) have shown corresponding decreases in insoluble wall-bound pectin (protopectin) although no direct information on ripening-related changes in the degree of methyl-esterification of these pectin fractions is available.

In the most searching of these admittedly rather rudimentary studies (Mizuta and Subramanyam, 1973) three pectin fractions were located and examined - water-soluble (high-methoxyl); wall-bound, calgon-soluble (low methoxyl) and wall-bound, HCl-soluble (protopectin) fractions. The water-soluble fraction increased, the calgon-soluble

fraction increased during the first six days and declined later, and the HCl-soluble fraction decreased during ripening. The viscosity of the water-soluble and calgon-soluble fractions decreased during ripening. They interpreted these findings as suggesting that the strength of wall-binding of protopectin diminishes during ripening owing to de-esterification and depolymerisation.

Although Mizuta and Subramanyam (1973) concluded that there was no appreciable change in the cellulose and hemicellulose content of mango tissue during ripening and that, therefore, mobilisation of these wall components made no significant contribution to tissue softening, free xylose (Sarkar, 1963) and arabinose (Wali and Hassan, 1965) have been noted in the flesh of ripening mangoes and these sugars could originate from degradation of hemicelluloses or (in the case of arabinose) from a neutral pectic arabinan or arabinogalactan. However, given the absense of specific information on the constituent polymers of mango cell walls this is mere conjecture. A water-soluble α-D-glucan containing both (1→3) and (1→4) linkages arranged in blocks, with which both galacturonosyl and arabinosyl residues are associated, has also been located in mango mesocarp (Das and Rao, 1964, 1965). However, glucans described from the cell walls of higher plants are β-linked polymers (see I.2.(c) 1.3.) so that it is difficult to conceive that this mango polymer could be wall-derived. Its function in the fruit is presently obscure.

In summary, all that is presently established about changes in the cell walls of mango mesocarp tissue during ripening is that wall-bound pectin is apparently solubilised, probably after undergoing some degree of depolymerisation. Whilst this solubilisation of pectin could undoubtedly contribute considerably to tissue softening, particularly in dissolution of the middle lamella leading to cell separation, it seems unlikely to be the only degradative process involved. In addition, virtually nothing is known about enzyme activities in the tissue which could contribute to wall breakdown and tissue softening. Accordingly, it is made the central purpose of this thesis to seek further information about the composition of mango cell walls and its changes during ripening and also to seek evidence of carbohydrate-degrading enzyme activities which could contribute to such processes.

Because so little information concerning tissue softening during ripening in the mango specifically is available from previously-published work, it will probably be necessary to discuss experimental data obtained for the mango in the light of what is established about the ripening process, most particularly cell wall breakdown and the enzyme activities contributing to it, in other fruits in which these processes have been more extensively studied. Accordingly a review of this topic now follows as the concluding section of this introduction.

I. 5. Cell Wall and Texture Changes in Ripening Fruit.

I. 5. (a) Histological Changes.

In fleshy fruit tissues plasmadesmata, often or always associated with vesicles (Ben-Arie et al.,1979), interconnect the cytoplam of adjacent cells and in the ripe fruit are thought to give the tissue a finite amount of cohesion. In addition, turgor pressure keeps the protoplast pressed against the cell wall and this helps to maintain the tissue firmness at early stages in ripening (Hobson, 1981).

In a typical climacteric fruit such as the tomato, increase in size is by cell division for the first seven to fourteen days following petal fall (Smith, 1935; Davies and Cocking, 1965) and then by cell expansion over several weeks. During this period of cell expansion partial separation of the cell walls at the middle lamella occurs, contact being lost with adjacent cells to form intercellular spaces (Davies and Cocking, 1965; Mohr and Stein, 1969). This incipient separation is thought to be mechanical (arising from rapid cell expansion) in origin rather than enzymic. At the end of the period of cell expansion growth the fruit is mature but unripe, the ensuing onset of ripening being triggered by the respiratory climacteric rise. Near ripeness the pericarp cells become very large and cell separation much more complete, but within the cell the plasmalemma and tonoplast remain intact, both physically and physiologically (Mohr and Stein, 1969; Vickery and Bruinsma, 1973).

The linear glucan molecules in cellulose are

combined into bundles to form very elongated elementary fibrils in the primary cell walls (see I.2.(b)). In unripe avocadoes the middle lamella is quite obvious and microfibrils are packed tightly in orderly array on both sides of the middle lamella (Pesis et al., 1978). In post-climacteric fruit, no middle lamella is observed and many of the micro-fibrils are missing. Observations on ripening apple and pear tissue by Ben-Arie and associates (1979) have confirmed a progressive dissolution of the cell wall and middle lamella, but a persistence of certain organs and organelles such as the cell wall-plasmadesmata complex and the mitochondria.

From this brief summary of some of the anatomical changes that can be observed as fruit ripen the alterations in gross structure can be correlated with the marked softening of the tissue. Moreover, these modifications appear, in the main, to be brought about by a series of co-ordinated enzymic transformations and to a much smaller extent by mechanical strains generated by the extensive swelling of th tissue during fruit development (Hobson, 1981).

I. 5. (b) Changes in the Cell Walls.

I. 5. (b) 1. Removal of Uronic Acids.

The clearest change in wall composition that accompanies fruit softening is a decrease in wall-bound galacturonic acid (pectin) that is closely matched by an increase in soluble galacturonan (Pilnik and Voragen, 1970; Pressey, 1977; Hobson, 1981; Labavitch, 1981). In the unripe fruit a complex of calcium with pecic polymers, given the name "protopectin" (Joslyn, 1962), is believed to be

chemically and physically enmeshed with other wall polymers to form a pectin-cellulose-hemicellulose (PCH) complex, a type of giant molecule. It has been proposed that an enzyme referred to as "protopectinase" is involved in the initial stages of dissolution of the PCH complex but this enzyme is poorly characterised and its distinction from polygalacturonase is unclear (Hobson, 1981). The proposed role of calcium ions in cross-linking homogalacturonan chains, most notably in the middle lamella, has been described earlier (see section I. 3. and Fig. 8a).

The action of protopectinase in breaking up the PCH complex could actually be due to endopolygalacturonase causing shortening of galacturonan chains thereby weakening the non-covalent forces assisting cohesion of the polymers by causing release of calcium from the wall (Grant et al., 1973; Hobson, 1981). Endopolygalacturonase evidently plays a crucial role in converting wall-bound pectin to soluble form in many fruits. Because the enzyme is unable to act adjacent to an esterified galacturonosyl residue it has been thought that endopolygalacturonase could act in fruit wall metabolism only after galacturonan chains had been de-esterified by pectin methyl esterase (Pilnik and Voragen, 1970; Pressey, 1977; Hobson, 1981). Both these enzymes are widely distributed in fruit species (Hobson, 1962, 1981; Pilnik and Voragen, 1970; Pressey, 1977), the degree of esterification of pectin in many fruits decreases during ripening (Woodmansee et al., 1959; Pressey, 1977) and in a number of climacteric fruits maximum loss of firmness is coincident with rapid synthesis of endopolygalacturonase

(Pressey, 1977; Hobson, 1981; Tucker and Grierson, 1982). Pectin methyl esterase activity does not always change as fruits ripen (Pressey, 1977) so that endopolygacturonase is presumably the limiting enzyme of the two.

Although pectin methyl esterase acts both at the reducing end and at random points along the pectin backbone (Lee and MacMillan, 1970) demethoxylation of wall-bound pectin does not seem to go to completion and early in the ripening process it seems likely that accessibility of pectin methyl esterase to methoxy groups of the pectin substrate is limited. This enzyme is also strongly wall-bound (Nakagawa et al., 1971) and its release/transport to its site of action constitutes a presently unknown process which, if elucidated, could shed much light on the mechanism of pectin dissolution. Fruits also contain a number of isoenzymic forms (Delincee, 1976; Rombouts et al., 1979). A possible explanation may be that each isoenzyme is specific for the demethoxylation of pectic methylgalacturonan chains of specific length as a preliminary step for the action of endopolygalacturonase which (in tomato) also occurs as two distinct iso-enzymic forms (Grierson et al.,1980,1981). Completely de-esterified galacturonan (pectic acid) is less soluble than methylated pectin but more labile to continued polygalacturonase activity so that conversion of pectin to oligogalacturonans is probably complex in terms of solubility, with competing reactions. The degree of residual esterification of pectin solubilised in many ripening fruits is poorly characterised and further data on this question could

do much to elucidate the mechanism of pectin solubilisation.

Although this hypothesis of the sequential action of pectin methyl esterase and endopolygalacturonase is an attractive explanation for a major contribution by pectin solubilisation to cell separation and tissue softening in many fruits, it does not provide a complete answer. No form of polygalacturonase activity could be detected in some fruits, e.g. melon, cranberry and persimmon (Hobson, 1962), and other fruits, e.g. apple (Bartley, 1978) and "clingstone" varieties of peaches (Pressey and Avants, 1978) contain only exopolygalacturonase which depolymerises galacturonan more slowly by attack from the non-reducing end (McMillan and Sheiman, 1974). Alternative mechanisms of pectin solubilisation and tissue softening must be sought in such fruits.

Knee and Bartley (Knee, 1978b; Knee and Bartley, 1981) have shown that, in the apple, methyl groups donated from radiolabelled methionine continue to be incorporated into wall-bound pectin during ripening. Both the apple and "clingstone" varieties of peach soften more slowly and to a lesser extent overall and show less pectin solubilisation than fruits which contain endopolygalacturonase such as "freestone" peach varieties (Pressey et al.,1971; Pressey and Avants, 1978), strawberry (Woodward, 1972) and pear (Knee, 1973a). The pectin solubilised from the apple wall during ripening is highly methyl-esterified and of high molecular weight and does not show the progressive depolymerisation with ripening which characterises the soluble pectin fractions from those fruits containing endopolygalacturonase mentioned above (Knee and Bartley, 1981). Knee and

Bartley (1981) propose that, in fruits like the apple, the insertion into the wall and middle lamella of esterified pectin might induce solubilisation and cell separation by promoting loss of cross-linking calcium ions from the wall, lowering the cohesion of pectic galacturonan chains and inducing their disentanglement from other wall polymers with which they are non-covalently associated. It is not presently clear whether the uptake of methyl groups into the wall reflects de novo pectin synthesis or methylation of existing wall-bound galacturonan. The limitation on softening, after initiation of cell separation by this enhanced turnover of the junction zones formed by calcium ions between free carboxyl groups of non-esterified regions, may be due to pectin methyl esterase activity forming new carboxyl groups elsewhere on the pectin chains, which would be available for formation of new cross-links (Knee and Bartley, 1981). The exopolygalacturonase would presumably cause only limited degradation of pectin.

I. 5. (b) 2. Removal of Neutral Sugars.

Important as the solubilisation of pectin appears to be, the available data suggests that it is not the only process contributing to the dissolution of the cell walls of ripening fruits. Turnover and removal from the wall of neutral sugars is equally evident in a number of fruit species. These changes most often involve galactose and arabinose. There is evidence that, in some fruits at least, this loss of neutral sugars is independent of pectin solubilisation. In the apple galactose loss occurs in the absence of pectin solubilisation (Knee, 1973c, 1975) and in

tomato there is considerable turnover of wall galactose in non-softening rin mutant fruit which contain no polygalacturonase activity (Gross and Wallner, 1979). The soluble pectin of apples is deficient in neutral sugars (Knee, 1978a) whereas there is evidence that the pectic polymers of the intact, unripe apple cell wall incorporate a rhamnogalacturonan carrying galactan and arabinan side-chains (Knee et al.,1975 ; Knee and Bartley, 1981), and Bartley (1974), who demonstrated that galactose loss preceded galacturonan solubilisation from the apple cell wall, has proposed that the removal of neutral pectic side-chains may actually facilitate solubilisation of the branched rhamnogalacturonan fraction of apple pectin.

In the cell walls of fruits in general, entanglement, or even covalent attachment (although there is currently no evidence to support the latter), of pectic galacturonans with other wall polymers could depend upon neutral side chains, removal of which by enzyme action might facilitate solubilisation of galacturonan. Alternatively, removal of side-chains might facilitate access of endopolygalacturonase, which is present in many fruits, to the galacturonan backbone. Certainly,in the apple, enzymic analysis of the wall structure at various stages in ripening indicated that the loss of galactose occurred in the branched rhamnogalacturonan fraction (Knee et al., 1975).

Galactose and arabinose, in varying proportions, are lost from the cell wall during ripening of apple (Knee, 1973c; Bartley, 1976), tomato (Gross and Wallner, 1979), strawberry (Knee et al., 1977) and pear (Jermyn and Isherwood,

1956; Ahmed and Labavitch, 1980a). The enzymes responsible for removal of these monosaccharides from the wall have not been characterised. Endo-acting galactanases/arabinanases would be expected to liberate polymeric material whereas exo-acting glycosidases would be expected to liberate free monosaccharides. There is uncertainty whether simple glycosidases (i.e. those that *in vitro* normally hydrolyse p-nitrophenyl glycosides) are capable of degrading glycans by exo-activity. However, Bartley (1974) has claimed that apple β-D-galactosidase degrades (1→4)-linked β-D-galactan and liberates free galactose from apple cell walls, and Pressey (1983) has reported that one of three iso-enzymes of the same enzyme present in tomato (and which increased in activity during ripening) degraded a pectic (1→4)-linked β-D-galactan isolated from tomato cell walls, also liberating free galactose. In contrast to these findings which suggest a possible role for exo-glycosidases in the removal of neutral monosaccharides (as free sugars) from fruit cell walls, galactose and arabinose lost from the strawberry cell wall during ripening appeared in soluble polysaccharide fractions (Knee *et al.*, 1977) and the arabinan (linked to galacturonan) solubilised from the wall in ripening 'Bartlett' pear was similarly of high molecular weight (Ahmed and Labavitch,1980a). In this latter case, these authors attributed the arabinan solubilisation to polygalacturonase activity and found no arabinanase (only α-L-arabinofuranosidase) activity in this pear cultivar (Ahmed and Labavitch, 1980b). However, in the Japanese pear, in which arabinose is also lost from the

cell wall during ripening, arabinanase activity has been reported (Yamaki and Kakiuchi, 1979).

At the present level of knowledge it must be conceded that the mechanism for removal of galactose and arabinose from the cell walls of several fruit species during ripening remains a subject for speculation. It may well be that a combination of endoglycanase and exoglycosidase activities is involved or that the mechanism is different in different fruit species. Whatever the case, fruit tissues contain an impressive array of carbohydrate-degrading enzymes, a number of which increase in activity during ripening (Pressey, 1977; Labavitch, 1981) but the specific roles(if any)in ripening of which have not been characterised, with the exception of endopolygalacturonase. β-D-galactosidase in particular, which Bartley (1974) has suggested may degrade galactan in the cell wall of ripening apples, increases in activity during ripening of tomato (Wallner and Walker, 1975), apple (Bartley, 1974) and pear (Ahmed and Labavitch, 1980b).

The small quantities of monomers characteristic of hemicelluloses - xylose, glucose and mannose - do not decline in the ripening of strawberry (Neal, 1965), tomato (Gross and Wallner, 1979) and pear (Jermyn and Isherwood, 1956; Ahmed and Labavitch, 1980a) although Knee (1973c) reported mobilisation of hemicellulosic glucan in ripening apple. A number of fruits contain β-1,4-D-glucanase (Hobson, 1968); Barnes and Patchett, 1976; Pressey, 1977) and tomato also contains β-1,3-D-glucanase (Wallner and Walker, 1975). Doubt that β-1,4-D-glucanase has a primary

role in fruit softening arises from the observation that the activity of this enzyme is normal in the non-softening rin mutant of tomatoes (Poovaiah and Nukaya, 1979).

Most authors have concluded that degradation of cellulose does not contribute significantly to fruit softening, there being very little evidence of cellulose turnover during ripening (Pressey, 1977; Hobson, 1981; Labavitch, 1981; Knee and Bartley, 1981). The dissolution of the microfibrils in ripened avocadoes (Pesis et al., 1978), apples and pears (Ben-Arie et al., 1979) could as well be due to disintegration of the cell wall matrix as to direct enzymic attack upon the cellulose. Crystalline α-cellulose is very resistant to enzymic attack and can only be degraded by the combined activities of several glycanases and other enzymes such as glucose oxidase and a peroxide-utilising system (Eriksson, 1977). The necessary enzymes have not been found in fruit tissues and, in view of this, it is unfortunate that the β-1,4-D-glucanase activity which is present in some fruits should so often be called "cellulase" (Knee and Bartley, 1981).

I. 5. (c) Synthesis Versus Degradation in Cell Walls of Ripening Fruit.

Most studies of cell wall changes in ripening fruit have concentrated on degradative aspects and the possible role of biosynthetic processes has rarely been considered. A study of polysaccharide metabolism in developing strawberry fruits (Knee et al., 1977) revealed cessation of ^{14}C-glucose incorporation into wall polymers

at the onset of ripening and, in fruits labelled with $^{14}CO_2$ early in development, loss of label from wall polymers and increase in label appearing in soluble polymers during ripening. This was consistent with the predominance of degradative changes in ripening. However, biosynthesis of polysaccharides does, apparently, occur to some extent even in ripe fruits. Jermyn and Isherwood (1956) reported evidence of biosynthesis of some wall polysaccharides in ripe pears and Hough and Pridham (1956) found comparable evidence of polysaccharide biosynthesis in the mesocarp of ripe plums.

The proposed role of uptake of methyl groups into unesterified regions of galacturonan chains in the cell walls and middle lamella of apple tissue (Knee, 197b; Knee and Bartley, 1981) in possibly contributing to tissue softening during ripening has been previously discussed (section I. 5. (b) 1.). This uptake of methyl-labelled ^{14}C-methionine into pectin methyl esters continued into over-ripeness but, by contrast, the same authors found that incorporation of activity from ^{3}H-inositol into galacturonic acid residues of pectin declined sharply on ripening. Lackey et al., (1980), suggested that, in the developing tomato, a galactan component of the cell wall undergoes constant turnover and that a decrease in its rate of biosynthesis (whilst degradation continued) during ripening would account for the noted net loss of galactose residues from the wall.

In summary, it could well be that many of the constituent polymers of fruit cell walls undergo turnover on the basis of balance between biosynthetic and degradative

pathways and that the cell wall exists in dynamic equilibrium with the cytoplasm. For some of these polymers biosynthesis may continue even into over-ripeness, and intussusception of newly-synthesised polymers may even have a role in the initiation of tissue softening. However, the presently available evidence suggests that, overall, biosynthetic processes decline sharply at the onset of ripening resulting in a predominance of wall degradation over biosynthesis during ripening.

I. 5. (d) Concluding Remarks.

The involvement of other hydrolytic enzymes in addition to polygalacturonase in texture changes during the ripening of fruits seems very likely. In many types of climacteric fruit, there is substantial evidence to show that without protein synthesis, normal ripening does not occur (Hobson, 1979, 1981). Increases in the activities of enzymes such as glycosidases and other hydrolases during the ripening of fruits are common and evidence is accumulating to show that several enzymes, e.g. pectin esterase and endopolygalacturonase, have a direct or an indirect effect on tissue firmness. Although, at the present level of established knowledge, it must be considered somewhat speculative, Hobson (1981) has presented a scheme (shown in Fig.9) proposing the possible interrelations between the various factors that transform a hard, unripe fruit cell into one much more attractive for man to eat.

Available data (Strand et al., 1976; Tigchelaar et al, 1978; Ishii, 1978) suggests that endo-polygalacturonase, as well as degrading pectins of the cell

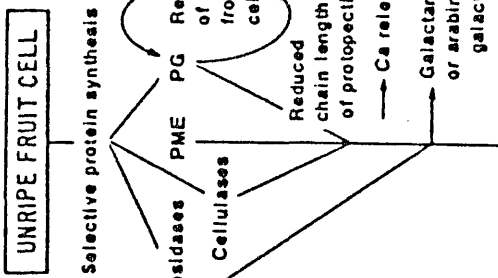


FIG. 9. Tentative scheme outlining the enzymes responsible for texture changes during the ripening of climacteric fruit.

wall and middle lamella, is also able to release from the wall considerable amounts of protein, some of which could be inactive in the bound state (Jansen et al., 1960) and on desorption could be involved in the furtherance of ripening.

It is clear that a certain amount of loss of firmness by tissue prior to incipient ripeness takes place (Besford and Hobson, 1972; Awad and Young; 1979). Glycosidases may contribute towards softening and tissue disintegration even before endopolygalacturonase is active, as softening in the locular cavities of tomatoes appears to suggest (Wallner and Walker, 1975). The more thoroughgoing changes in texture are thought to result from sequential attacks on the pectin-cellulose-hemicellulose complex, initially by pectinesterase, glycosidases and, perhaps, glucanases (often misleadingly referred to as "cellulases"), (Hobson, 1981). At the climacteric rise in respiration, a rapid synthesis of polygalacturonase overtakes the fruit cell and calcium is released at this time during which galactose and arabinose are also lost from the wall (Knee, 1973c).

We must suppose that this opens the way for the liberation and degradation of the water-soluble pectic substances, erosion of the middle lamella, disintegration of the primary cell wall and much more extensive loss of tissue firmness. The central role of endopolygalacturonase in many fruits which soften rapidly is emphasised by the behaviour of non-ripening rin tomato mutants, where in the virtual absence of this enzyme ripening is extremely slow,

tissue softening much more drawn out and resistance to fungal attack maintained for many months (Hobson, 1981).

In the light of this comparative data from other fruits cited above, in the present study especial emphasis will be placed on detecting loss from the mango cell wall during ripening of galacturonic acid, galactose and arabinose and on detecting and assaying the levels of activity at various ripening stages of enzymes which could contribute to degradation of polysaccharides containing these monomers. The ability of enzyme extracts from ripe mangoes to release fractions containing these monomers from cell walls prepared from unripe mangoes will also be tested.

II. MATERIALS AND METHODS.

II. 1. Materials.

II. 1. (a) Chemicals.

Polygalacturonic acid, pectin (citrus), polyvinylpyrrolidone, Tween-60, Tween-20, cellulase (technical) type 1, polygalacturonase (purified), fungal protease (purified) type V, and pepsin (purified) 1:60,000 were supplied by Sigma Chemicals, London.

Somogy arsenomolybdate reagent, Folin phenol reagent, N-ethyl maleimide, chloral hydrate, cysteine, 4-amino benzoic acid, naphthoresorcinol, carbazole, α-naphthol, thymol, Zerolit DM-F resin, soluble starch, D-galactose, D-galacturonic acid, D-glucuronic acid, D-glucose, D-mannose, D=xylose, L-fucose, L-rhamnose and L-arabinose were supplied by B.D.H.Ltd.

Fungal hemicellulase, myo-inositol and D-mannono-1,4-lactone were supplied by Koch-Light Ltd.

p-Nitrophenyl glycosides were supplied by Boehringer Corporation, Mannheim,with the exception of p-nitrophenyl-α-L-arabinofuranoside which was kindly donated by Dr.B.Weissmann, Dept. of Biological Chemistry, University of Illinois College of Medicine, Chicago.

Dowex-1 (200-400 mesh) and Dowex-50W (200-400 mesh) resins were supplied by Bio-Rad Ltd.

Chromosorb-W (100-120 mesh) and OV-225 were supplied by Phase Separations Ltd.

Sephadex G-100 was supplied by Pharmacia Ltd.

Silica gel thin-layer chromatography plates, 20 x 20 cm were supplied by Schleicher and Schüll Ltd.

Glass fibre paper, GF/C was supplied by Whatman Ltd.

'Repelcote' (2% solution of dimethyldichlorosilane in carbon tetrachloride) was supplied by Hopkins and Williams Ltd.

II. 1. (b) Mangoes

Mangoes of the cultivar 'Ngowe' were obtained from Geest Ltd. These fruits had been air-freighted after harvesting when the fruits were hard and green (mature but unripe). An arrangement was made with the importers so that freshly-imported fruits could be personally selected at the warehouse. Fruits selected were all externally dark green, with no trace of yellowing of the skin, and of comparable size (12-15 cm in length). The underlying mesocarp of fruits at this stage of development was hard and white in all of those fruits in which the skin was removed. By this means efforts were made to ensure genetic and developmental homogeneity of the fruits selected.

A proportion of each batch of fruits selected were immediately placed into a deep-freeze at -20^{o}, the remainder being allowed to ripen to the desired stage at room-temperature on a shaded, draught-free bench. When ripened to the desired stage (as observed by external appearance) groups of fruits were removed from each batch into the -20^{o} deep-freeze. The characteristics of fruits at the four different stages of ripening thus obtained are outlined below:

Stage 1. Unripe. Skin dark green, fleshy mesocarp hard and white. Flavour astringent, no aroma.

Stage 2. Turning. Skin tinged or 'blotched' with yellow, mesocarp pale yellow and softening. Flavour tart, slight aroma.

Stage 3. Ripe. Skin bright yellow, mesocarp dark yellow and soft. Flavour and smell aromatic.

Stage 4.Over-ripe. Yellow skin blushed with orange/pink, mesocarp orange and very soft. Flavour and smell very aromatic.

When required for use fruits were removed from the deep-freeze and allowed to thaw (in a cold-room at 4^{o}) just sufficiently so that the skin and underlying fibre could be peeled away and the mesocarp sliced with a sharp knife.

II. 2. General Methods.

II.2. (a) Physical Methods.

II.2. (a) 1. Thin layer chromatography of sugars.

The method of Menzies and Mount (1975) was followed. The experimental sample (5 ml) which contained 100 µg rhamnose (12.5µl) as an internal standard was added to Zerolit DM-F anion/cation exchange resin (2.8 g), acetate form, in a sealed tube. The resin had been pre-activated in bulk as described by Menzies and Mount (1975) and stored in a sealed, air-tight container to maintain the degree of hydration. Standard solutions containing respectively, 20, 10 and 5 µg/ml of each of fructose, galactose, glucose

sucrose and arabinose were similarly treated.

Tubes containing the sample and the resin were inverted continuously for 3 min and resin removed by centrifugation for 10 min in a bench centrifuge. The supernatant (2 ml) was freeze dried in a small siliconised glass tube. This was re-dissolved in 100-200 µl (usually 100 µl for samples and always 100 µl for standards) water; 10 µl of this solution was applied to a plastic-backed, silica gel thin-layer plate (20 x 20 cm). Samples were carefully applied at the origin as even, 1½ cm-long streaks which did not exceed 2-3 mm in width; 2 µl was applied at a time, the streak being dried with a cool hair dryer held at a distance between applications. All applications were made from a micro-syringe. On each plate 4 test samples and 3 standard sugar solutions were applied.

The plates were developed by two ascending runs (dried between runs) using ethyl acetate (60): pyridine (30): acetic acid (10): water (10), v/v, as the solvent. The plates were then quickly dried with a hair dryer and left overnight in a fume-cupboard before they were stained using a dipping reagent. A specially designed dipping tank was used which allowed the plate to be quickly and evenly dipped and the excess staining reagent uniformly skimmed from the plate surface against a bevelled, ground-glass edge (Menzies and Mount, 1975). The staining reagent used was 2% 4-amino benzoic acid in methanol containing orthophosphoric acid (3%). Hexoses and uronic acids gave a yellow/brown colour and pentoses gave a red/brown colour when

plates were heated at 130° for 5 min after removing excess methanol in a fume-cupboard.

In 2-stage reactions, plates were dipped first in 3% orthophosphoric acid in methanol and heated at 130° for 5 min. They were then dipped in 2% 4-amino benzoic acid in methanol; ketose sugars reacted rapidly at room temperature to give bright yellow colours and aldopentoses gave faint pink colours. Other aldoses only gave significant colour after further heating at 130° for 5 min, ketoses darkened to yellow/brown and aldo-pentoses to red/brown on further heating.

Plates dipped in 0.2% naphthoresorcinol in methanol containing orthophosphoric acid (3%) and heated at 130° for 5 min gave yellow/brown colours for ketose sugars and blue-grey colours for aldose sugars.

The spots in chromatograms of various standard mixtures of sugars stained by the one-stage method with 4-aminobenzoic acid are shown in Fig.10. The mobilities of standard sugars (relative to rhamnose) are given in Table 1. Fructose and mannose were not adequately resolved by this chromatographic method but could be distinguished by the 2-stage staining procedure since fructose is a ketose and mannose an aldohexose. All other sugar standards utilised were adequately resolved.

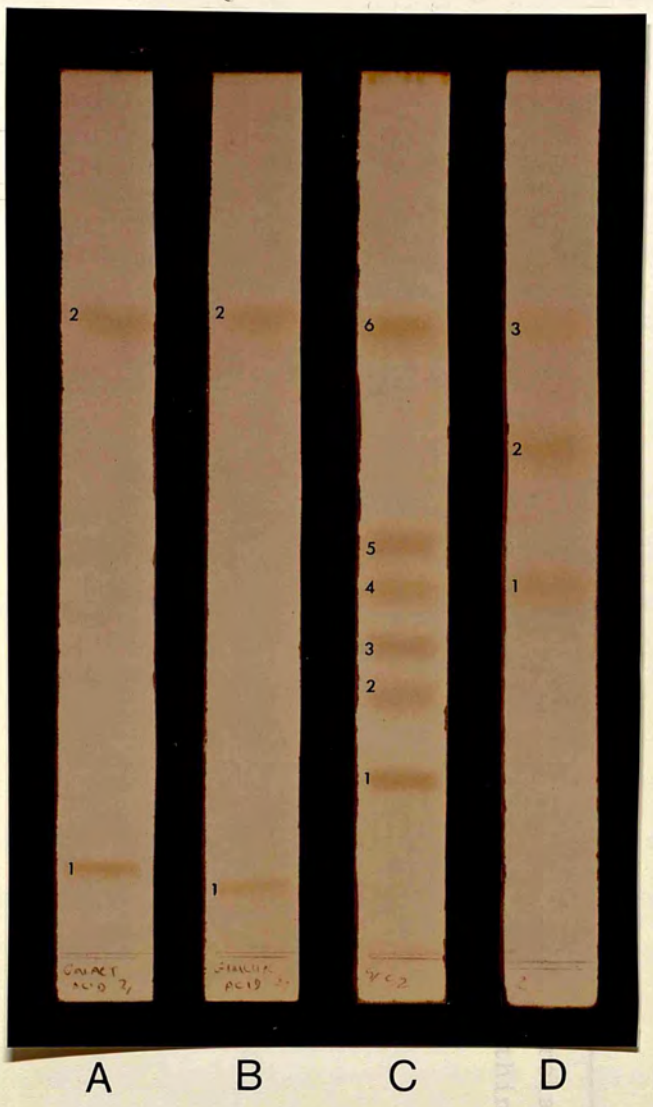


Fig.10. Thin-layer chromatograms of various mixtures of standard sugars developed by the method of Menzies and Mount (1975) and stained by the one-stage method using 4-aminobenzoic acid.

Chromatogram A:
1 - galacturonic acid
2 - rhamnose

Chromatogram B:
1 - glucuronic acid
2 - rhamnose

Chromatogram C:
1 - sucrose
2 - galactose
3 - glucose
4 - fructose
5 - arabinose
6 - rhamnose

Chromatogram D:
1 - mannose
2 - xylose
3 - rhamnose

TABLE 1. Relative mobilities of some sugars on TLC (method of Menzies and Mount, 1975)- solvent system: ethyl acetate (60): pyridine (30): acetic acid (10): water(10); v/v.

SUGAR	Mobility relative to Rhamnose on silica gel thin-layer chromatograms.
Glucuronic acid	0.10
Galacturonic acid	0.14
Sucrose	0.28
Galactose	0.42
Glucose	0.49
Mannose	0.54
Fructose	0.58
Arabinose	0.66
Fucose	0.80
Xylose	0.81

The values shown are mean values obtained from duplicate chromatograms of each sugar (5µg) separately mixed with rhamnose (5µg), applied in aqueous solution (10µl). Values obtained from chromatograms of mixtures of several sugars (eg. sucrose, galactose, glucose,fructose, arabinose; each 5µg) with rhamnose (5µg) were very close to values obtained for the separate sugars.

II.2. (a) 2. Gas-liquid chromatography of alditol acetates.

Alditol acetates were dissolved in chloroform for analysis by gas-liquid chromatography. Aliquots (1-2µl) were applied, from a micro-syringe fitted with a 16 cm needle, to a 9 ft glass column packed with 3% OV-225 on Chromosorb-W, 100-120 mesh. Oven temperature used was 180°; the injection port temperature was 300°. Nitrogen carrier-gas was used at a flow rate of 40 ml per minute. The column was mounted in a Pye Series 104 gas-liquid chromatography apparatus fitted with a flame-ionisation detector linked to a Hewlett-Packard 3370B electronic integrator for automatic print-out of peak areas. The integrator was set at a high slope sensitivity (0.01 mV/min) and operated manually. A trace of peaks eluted from the column was obtained on a 'Servoscribe' linear potentiometric chart recorder linked to the integrator. Amounts of alditol acetate present in each peak were taken as proportional to peak area and amounts of neutral sugars present in chromatographed samples were calculated by comparing them to peak area for the inositol acetate internal standard. Mannitol acetate (derived from D-mannonic acid) was similarly used as an internal standard for uronic acids. A typical chromatogram derived from neutral sugars (in this case liberated by combined acid and enzymic hydrolysis of unripe mango cell wall material), is shown in Fig.11. A typical chromatogram derived from uronic acids (in this case liberated by combined acid and enzymic hydrolysis of over-ripe mango cell wall material), is shown in Fig.12. Relative retention times (RRT) for various

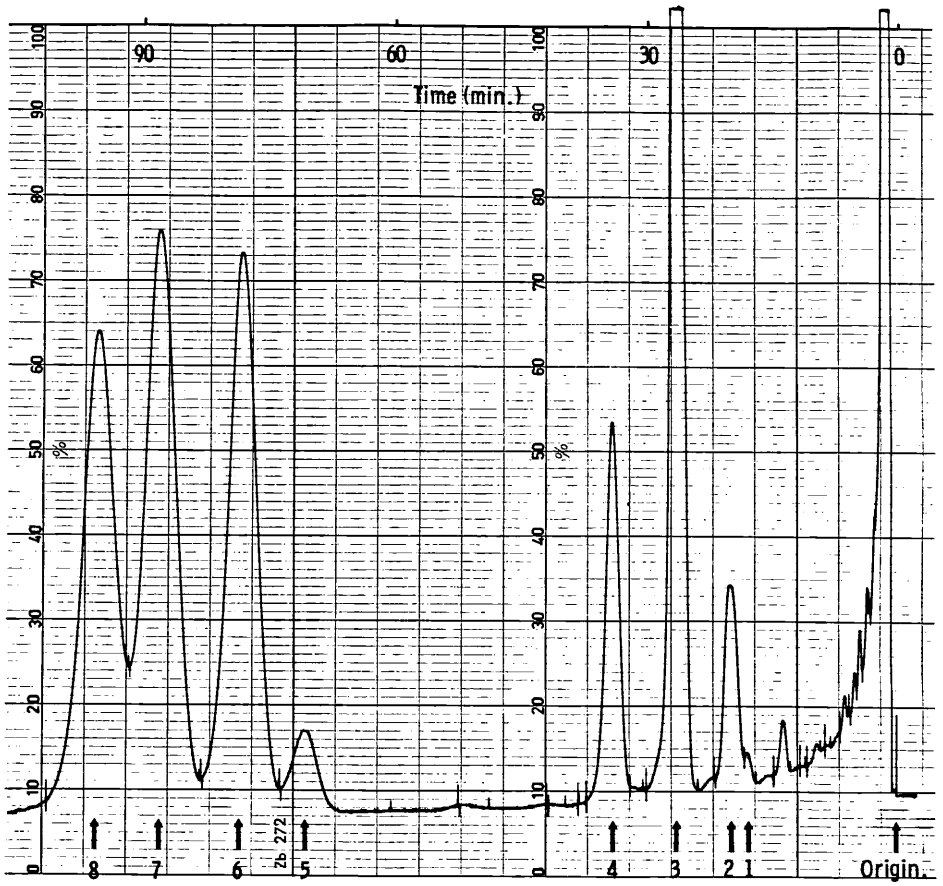


Fig.11. Typical gas-liquid chromatogram on OV-225/Chromasorb column of alditol acetates derived from neutral sugars.

1. - Rhamnose 2 - Fucose 3 - Arabinose 4 - Xylose
5. - Mannose 6 - Galactose 7 - Glucose 8 - Inositol

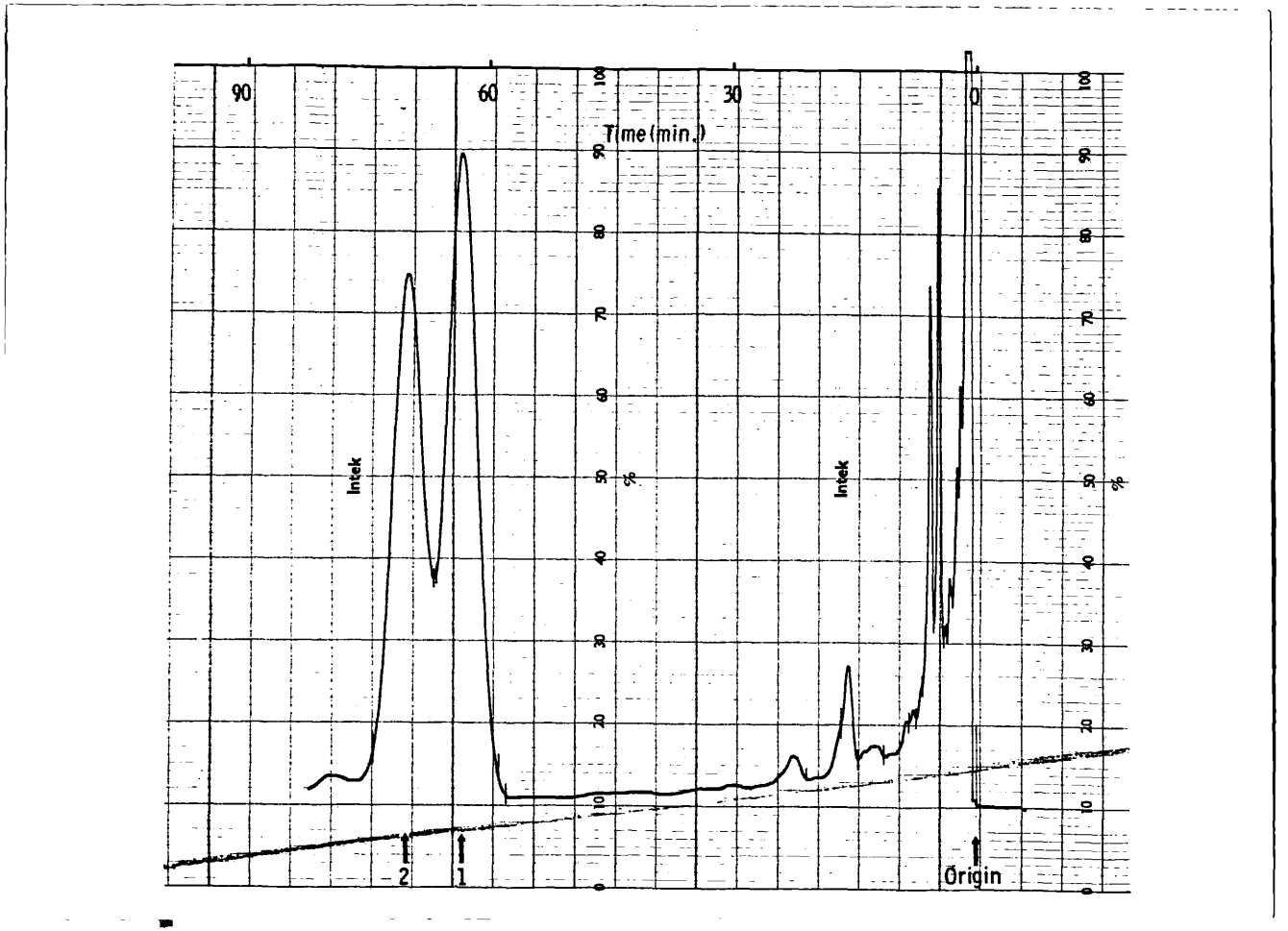


Fig.12. Typical gas-liquid chromatogram on OV-225/Chromosorb column of alditol acetates derived from uronic acid.

1. Mannuronic acid 2. Galacturonic acid.

alditol acetates, relative to retention time for inositol acetate, on the column under the conditions used are given in Table 2.

II.2. (a) 3. Glass fibre paper high voltage electrophoresis.

The deionized, concentrated samples which had been prepared for TLC (see section II,2,(a) 1) were diluted 1:2.5 with water before being also subjected to electrophoresis. Electrophoresis was carried out using the method described by Jarvis et al.(1977). Glass fibre paper (Whatman GF/C) strips (57 x 20 cm) were heated at 400^{o} for 2 hours to removed organic materials. The strips were then totally immersed in a sealed glass tank containing 2% dimethyldichlorosilane solution ('Repelcote') for 48 hours. After removal from the tank excess silane was removed by washing strips three times with toluene (400 ml) and drying in a fume cupboard. At all times strips were handled with rubber gloves. The silanised strips were re-wetted by floating for 48 hours in 0.05M sodium tetraborate (buffered with HCl to pH 9.0) containing Tween-20 (0.2%, v/v).

Electrophoresis was carried out using a Shandon water-cooled, high-voltage apparatus. The strips were lightly blotted before use with the aid of a wooden roller. Samples (1-5 µg in 2 µl) were applied at the mid-point of the strip as 1 cm-wide streaks. Four samples were run parallel on each strip. The borate buffer in the electrophoresis wells contained no Tween-20. A voltage of 1000 V (current 70-80 mA) was applied for 1 hour, (strips were subjected to pre-electrophoresis at the running voltage for

TABLE 2. Relative retention times on OV-225/Chromosorb column for various alditol acetates.

Alditol acetate	Relative retention time on OV-225 column (relative to retention time for inositol acetate)
Rhamnitol acetate	0.19
Fucitol acetate	0.21
Arabinitol acetate	0.28
Xylitol acetate	0.36
Mannitol acetate	0.74
Galactitol acetate	0.82
Glucitol acetate	0.92

The values given in this table were obtained by preparing alditol acetates from standard mixtures of each monosaccharide (1 mg) separately and inositol (1 mg) in aqueous solution (1 ml). Prepared alditol acetates were dissolved in chloroform (1 ml) and each mixture (1 µl) subjected to GLC in duplicate. Duplicate values for RRT were very close and mean values are shown in the Table. RRT values obtained by preparing alditol acetates from a mixture of all 7 monosaccharides and inositol were identified to the values obtained for each separate monosaccharide.

20 minutes before application of the samples). Immediately after electrophoresis the strips were totally immersed in methanol to fix the polysaccharide spots on the surface of the glass-fibre paper and allowed to stand for 30 minutes; the strips were then dried with hot air.

Polysaccharide spots were located by dipping the strips in α-naphthol (0.4%,w/v), in methanol containing H_2SO_4 (2% v/v) and heating for 5 min at 110°. The strips were cut out and scanned on a Joyce-Loebl 'Chromoscan' densitometer, duplicate scans being performed.

Electrophoretograms for starch (5 µg), a mixture of galactan and arabinan (5 µg) and polygalacturonic acid (5 µg) are shown in Figs 13,14,15 respectively.

II.2. (b) Assays.

II.2.(b) 1. Protein.

II.2.(b).1.1. Lowry et al's method (1951).

Extracted protein was estimated in supernatant extracts. In whole macerates, total protein was extracted for 2 hours with occasional shaking in 1M sodium hydroxide. After extraction, the solid residue was removed by centrifugation and the supernatant neutralised with 5M HCl. In all protein estimations protein was precipitated with CCl_3CO_2H (0.5M final concentration) before estimation. Precipitated protein was re-dissolved directly in 5 ml alkaline Na_2CO_3/Cu-reagent (solution C), left to stand for 10 min before addition of 0.5 ml Folin phenol reagent (diluted 1:1 with distilled water). The absorbance at 750 nm was measured after 30 minutes

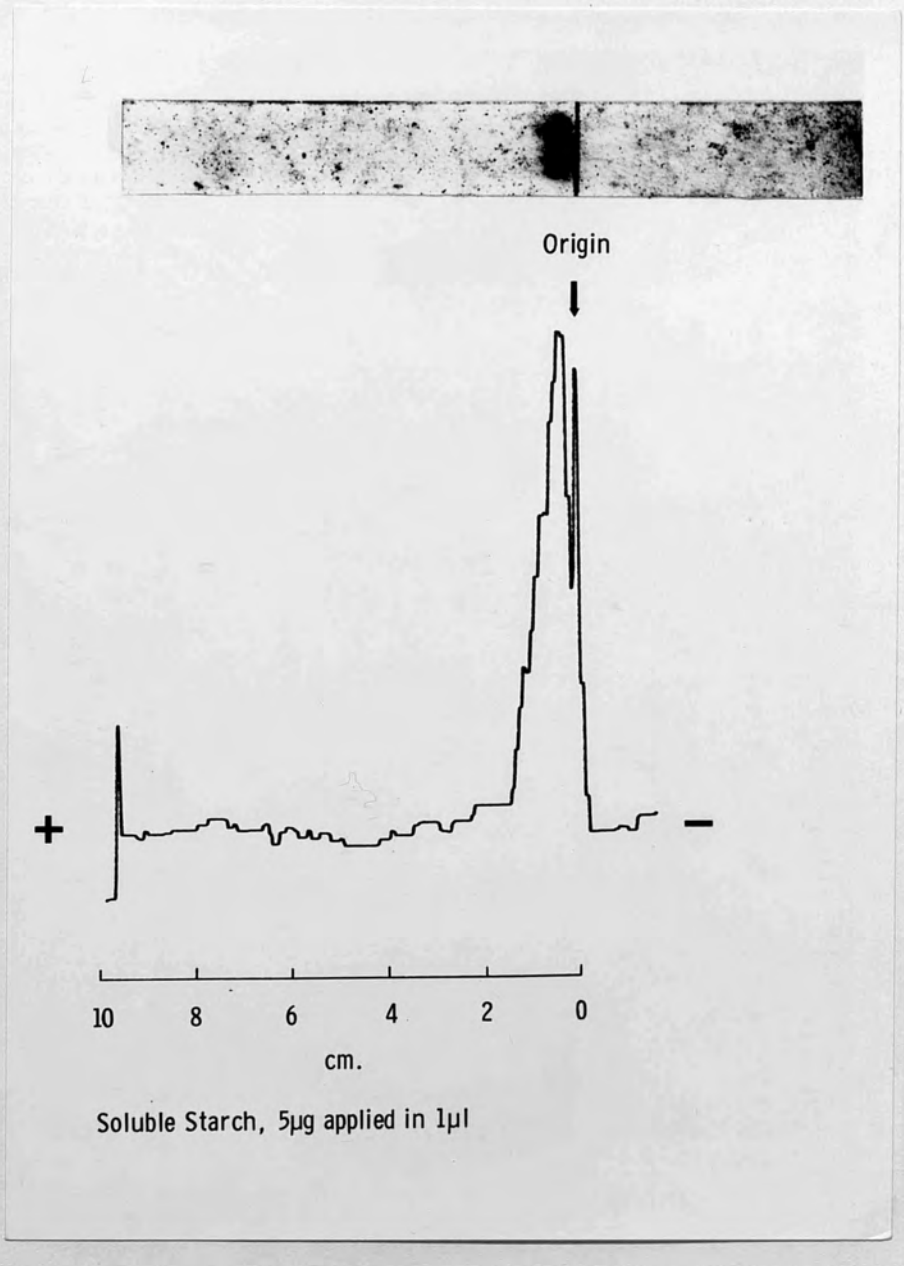


Fig.13. High-voltage electrophoresis on treated glass-fibre paper of soluble starch (method of Jarvis et al., 1977).

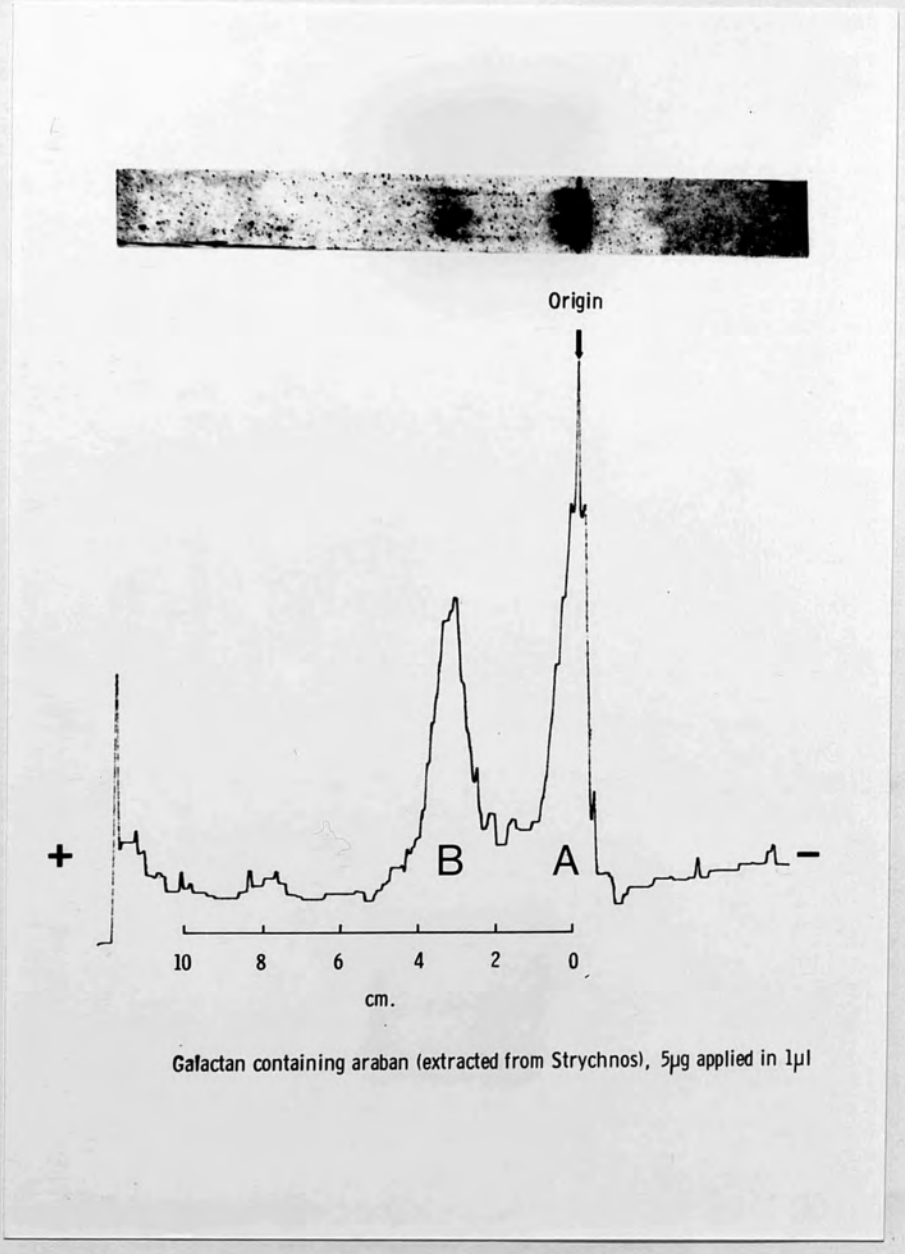


Fig.14. High-voltage electrophoresis on treated glass-fibre paper of galactan/arabinan mixture (extracted from Strychnos), (method of Jarvis et al., 1977).

A - galactan

B - arabinan.

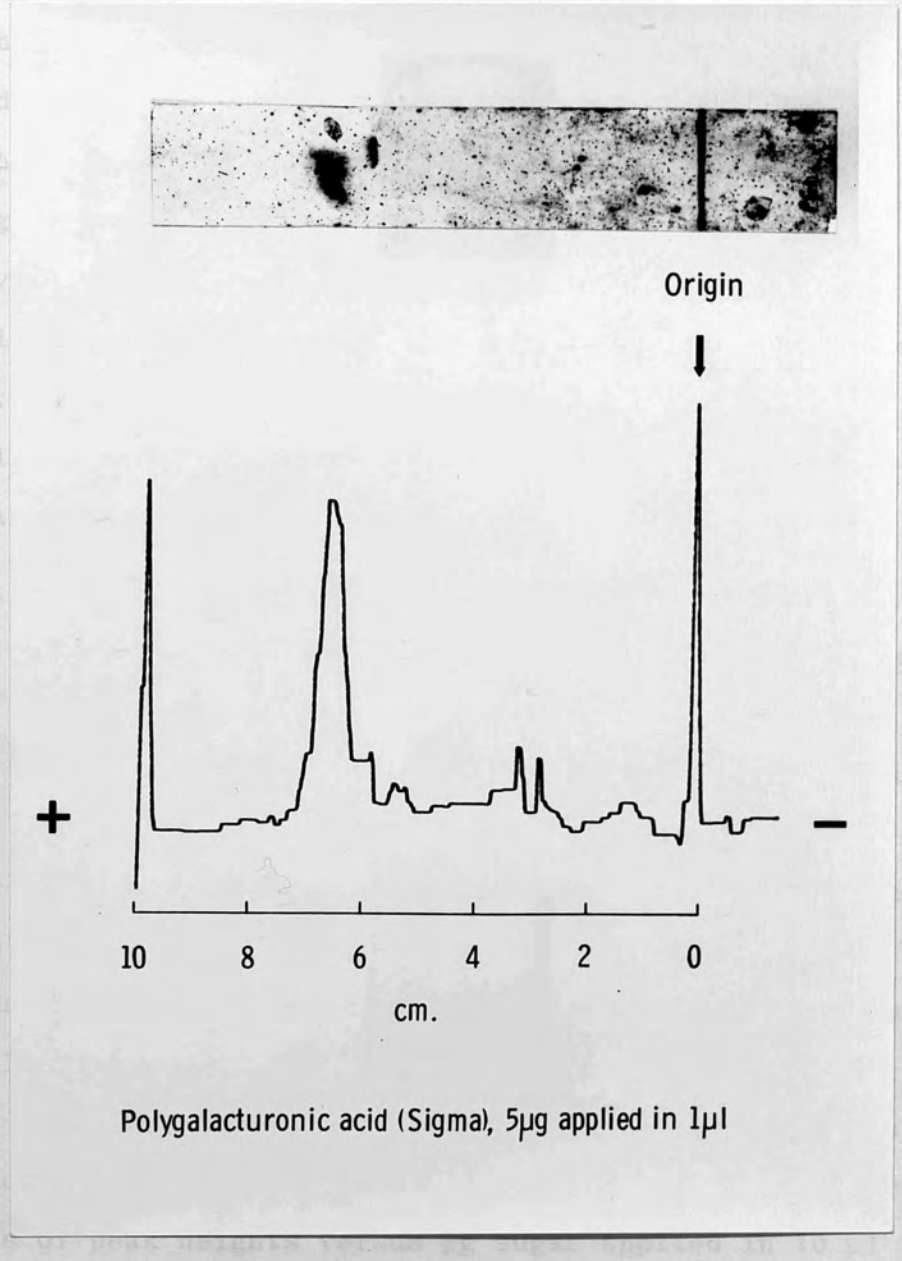


Fig.15. High-voltage electrophoresis on treated glass-fibre paper of polygalacturonic acid (Sigma), (method of Jarvis et al., 1977).

against a reagent blank containing no protein. All standard and test samples were assayed in duplicate. A linear increase in absorbance at 750 nm was obtained using bovine serum albumin (100-500 µg) - (see Fig.16). For very small amounts of precipitated protein, it was re-dissolved directly in 1 ml solution C before addition of 0.1 ml Folin phenol reagent (diluted 1:1 with distilled water). In this case the standard curve was linear in the range 20-100 µg protein (Fig.17).

II.2.(b) 2. Carbohydrates.

II.2.(b) 2.1. Densitometric method for sugars on thin-layer chromatograms.

The stained chromatographic strips (see Section II,2, (a), 1.) were carefully cut out and scanned on a Joyce-Loebl 'Chromoscan' densitometer zeroed against the white background of the chromatogram. Typical scans of standard mixtures containing various sugars are shown in Figs.18-21.

Peak height was taken as a quantitative measure of each sugar present (Menzies and Mount 1975). Standard curves of peak heights versus µg sugar applied in 10 µl samples, were prepared for a number of sugars (Figs.22-24). It can be seen that these standard curves are not linear. Because the relationship was not linear, sugars in test samples applied were not calculated from these standard curves. Instead standard sugar solutions at three different concentration (chosen to give an appropriate range of peak heights) were run on all plates alongside

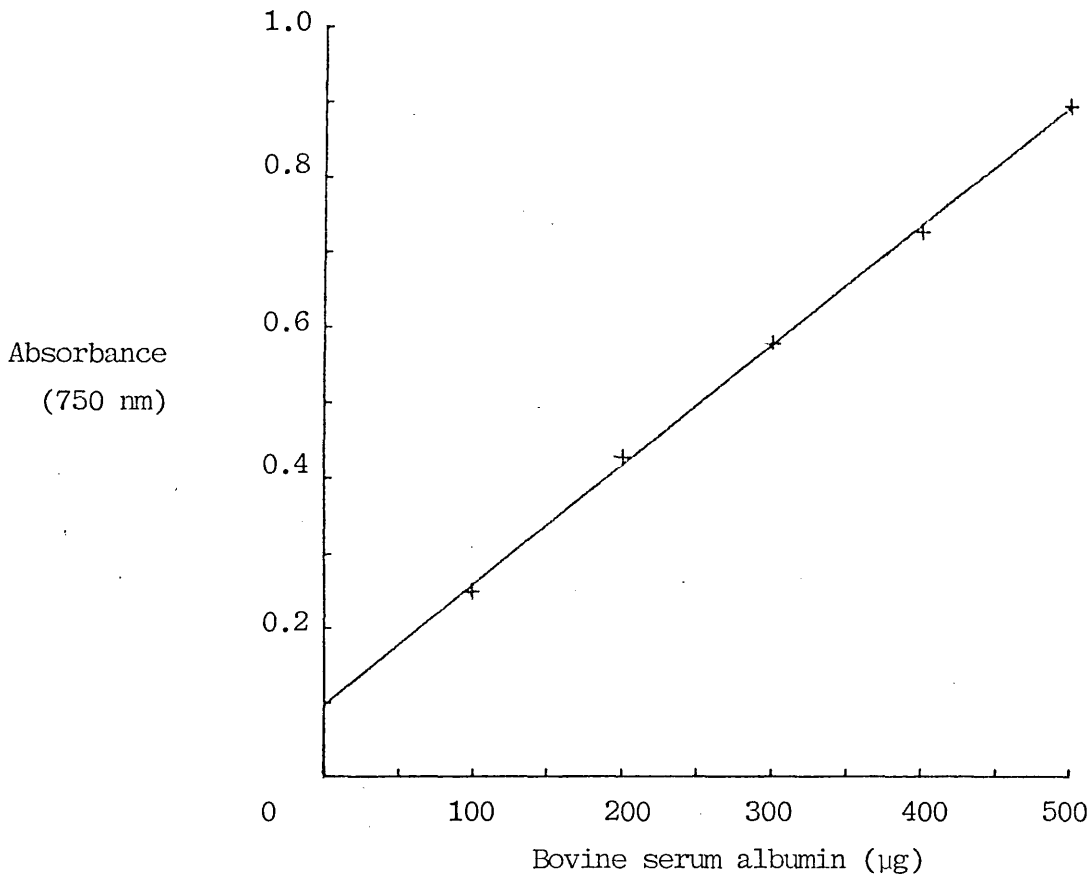


Fig.16. Standard curve for assay of protein by the method of Lowry et al., (1951).

BSA was precipitated with trichloroacetic acid and re-dissolved directly in 5 ml.Lowry solution C for assay.

Each point shown is the mean of duplicate assays and the "line of best fit" is drawn with the aid of a microcomputer linked to a printer-plotter.

Gradient = 0.0016 with 95% confidence limits 0.0015 to 0.0017

Intercept = 0.098 with 95% confidence limits 0.069 to 0.127

Correlation coefficient = 0.999.

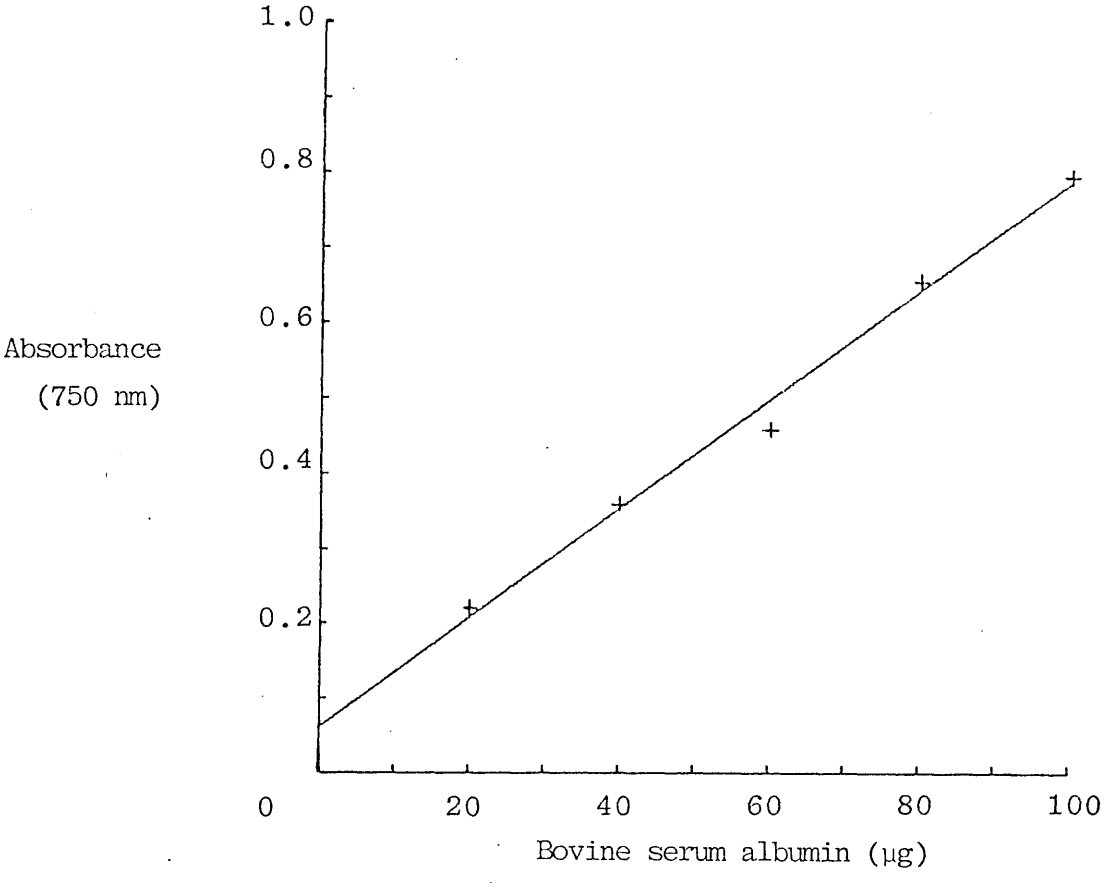


Fig.17. Standard curve for assay of protein by the method of Lowry et al., (1951).
BSA was precipitated with trichloroacetic acid and re-dissolved directly in 1 ml Lowry solution C for assay. Each point shown is the mean of duplicate assays and the "line of best fit" is drawn with the aid of a microcomputer linked to a printer-plotter.
Gradient = 0.0073 with 95% confidence limits 0.0060 to 0.0086
Intercept = 0.062 with 95% confidence limits -0.017 to 0.141
Correlation coefficient = 0.995.

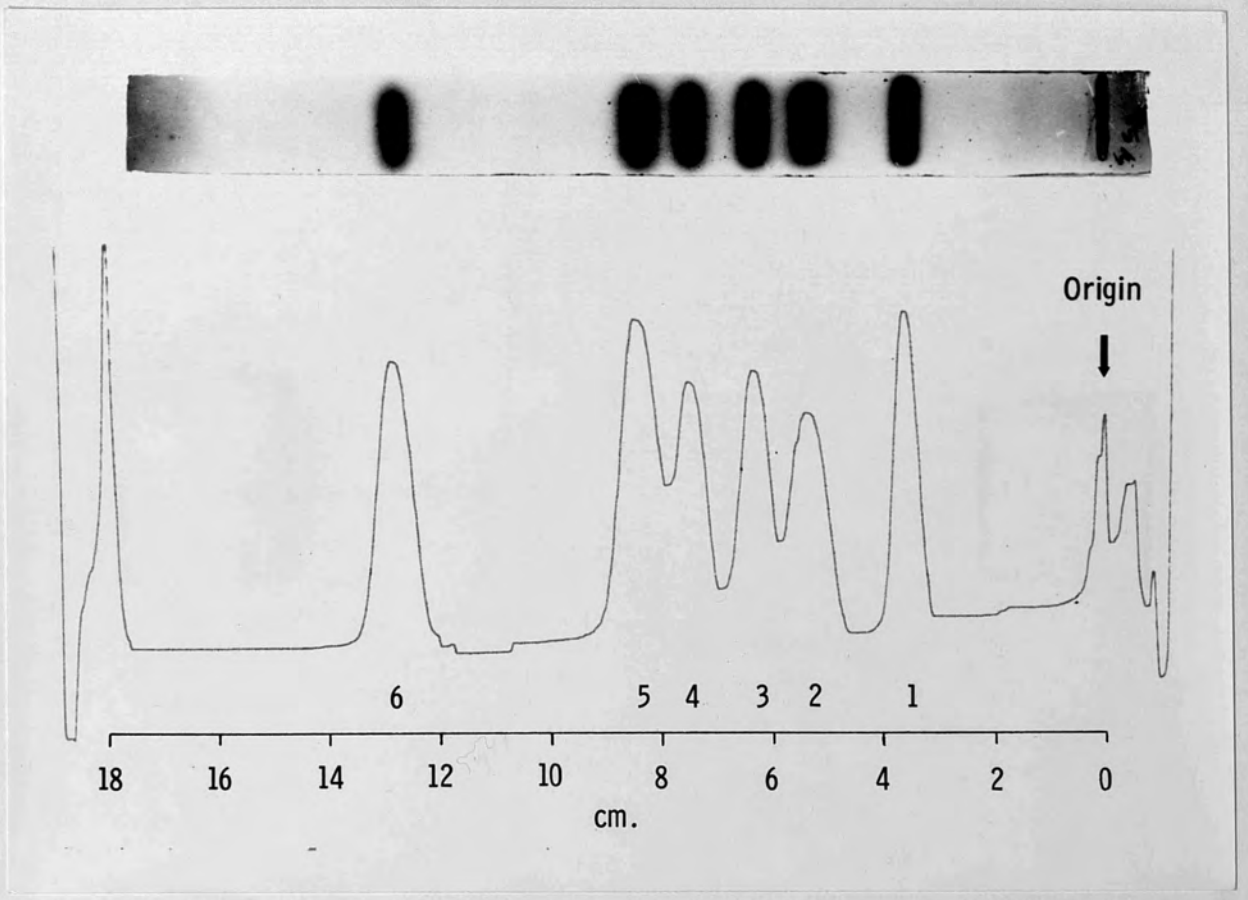


Fig. 18. Thin-layer chromatography on silica gel of sugar standards (2µg each sugar). by the method of Menzies and Mount (1975).

1. - Sucrose 2 - Galactose 3 - Glucose

4. - Fructose 5 - Arabinose 6 - Rhamnose

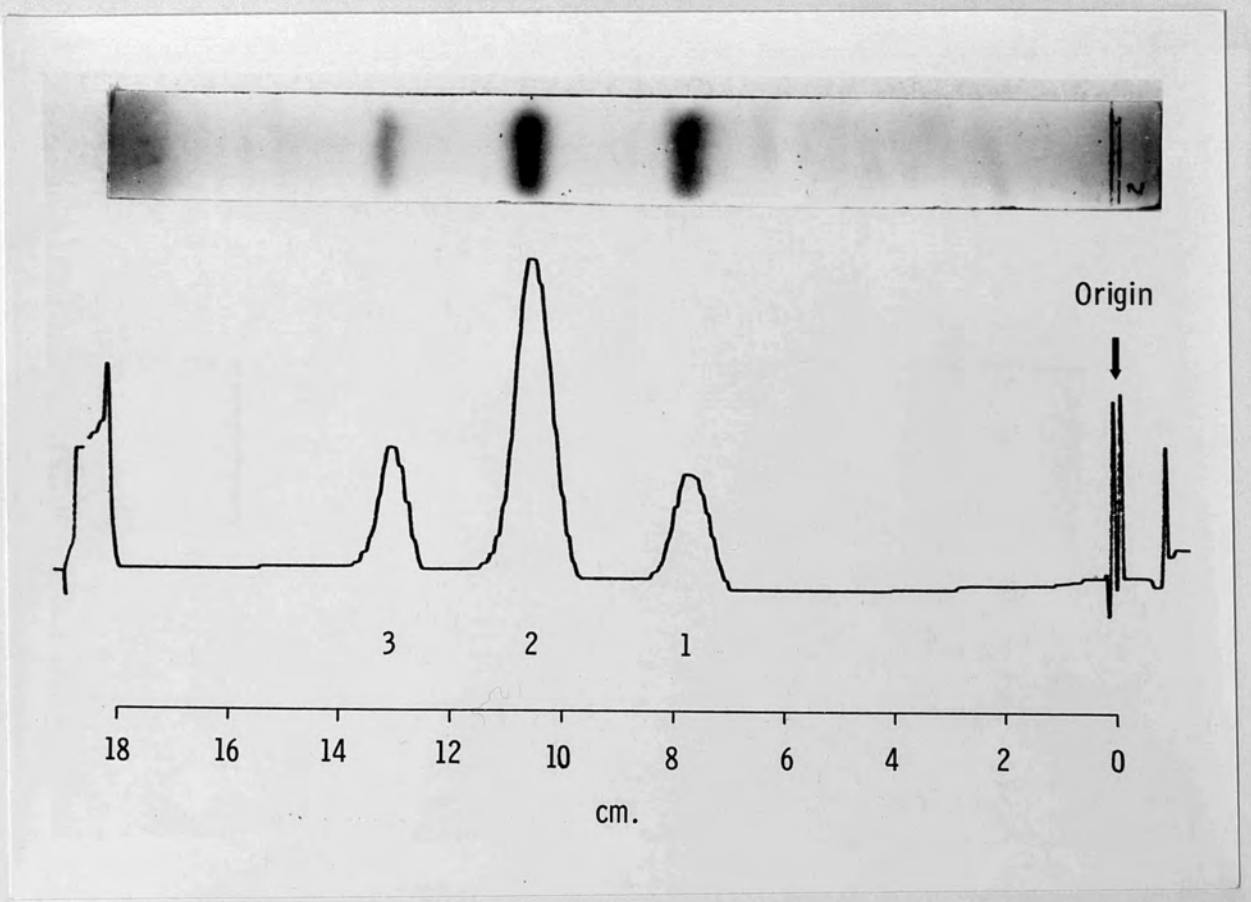


Fig.19. Thin-layer chromatography on silica gel of sugar standards (2µg each sugar) by the method of Menzies and Mount (1975).

1 - Mannose 2 - Xylose 3 - Rhamnose

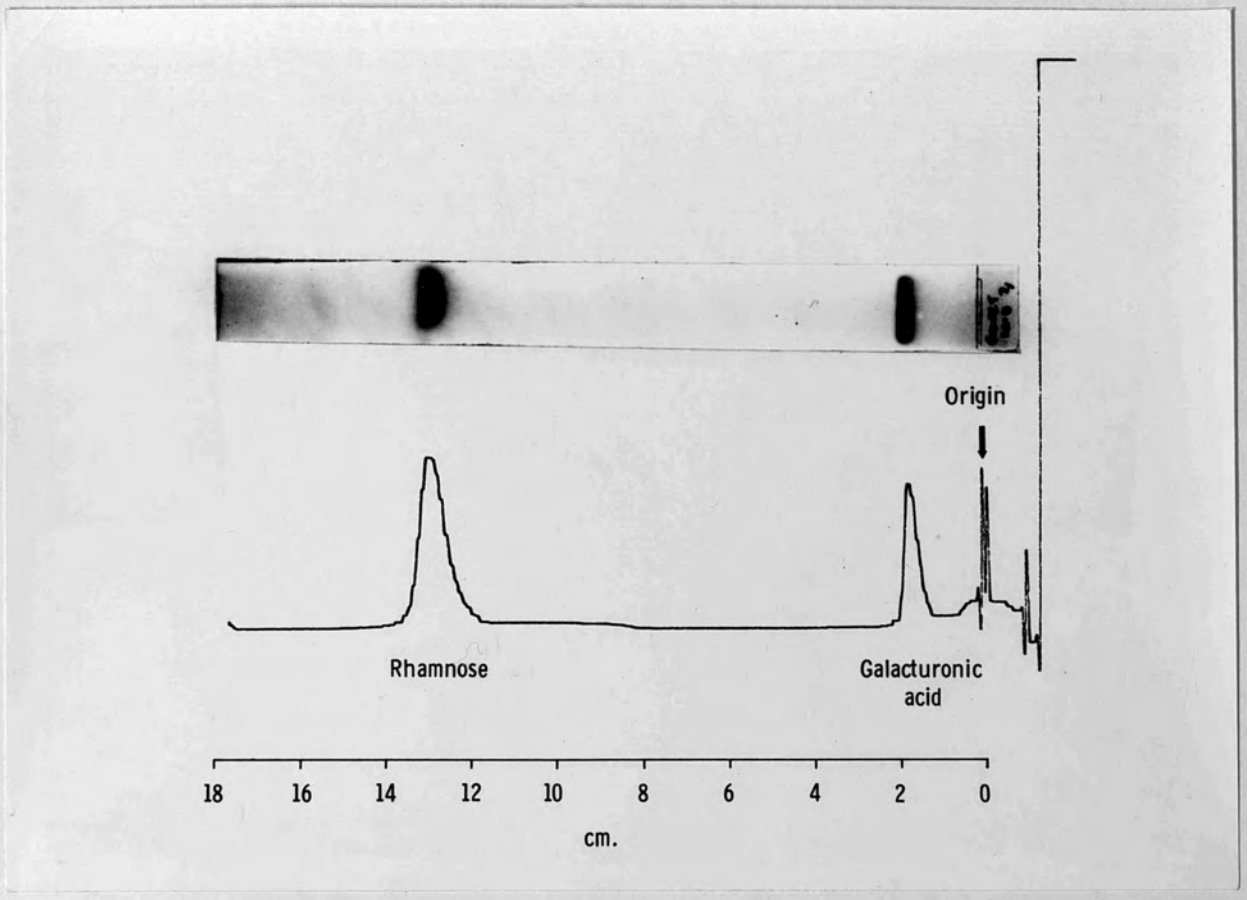


Fig.20. Thin-layer chromatography on silica gel of sugar standards (2µg each sugar) by the method of Menzies and Mount (1975).

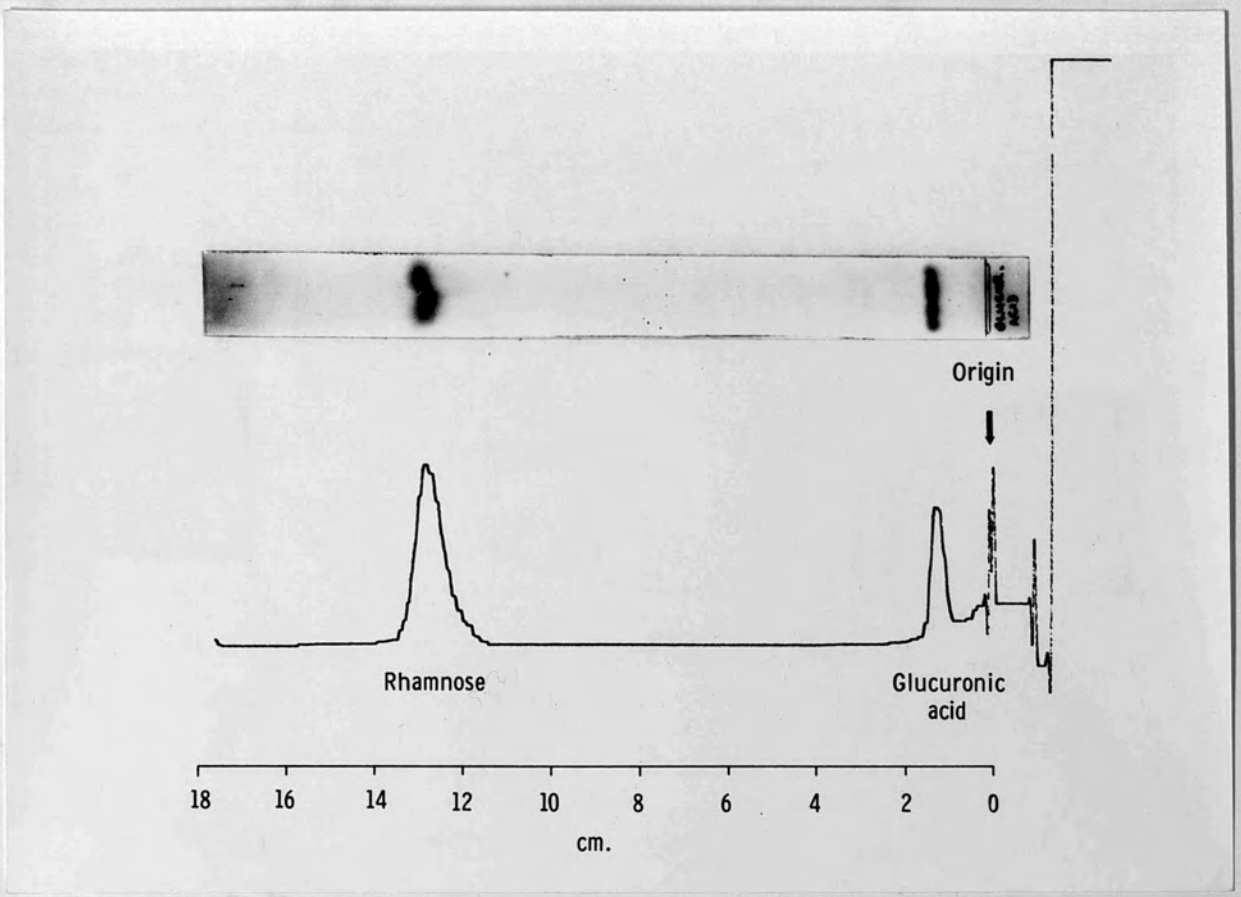


Fig.21. Thin-layer chromatography on silica gel of sugar standards (2µg each sugar) by the method of Menzies and Mount (1975).

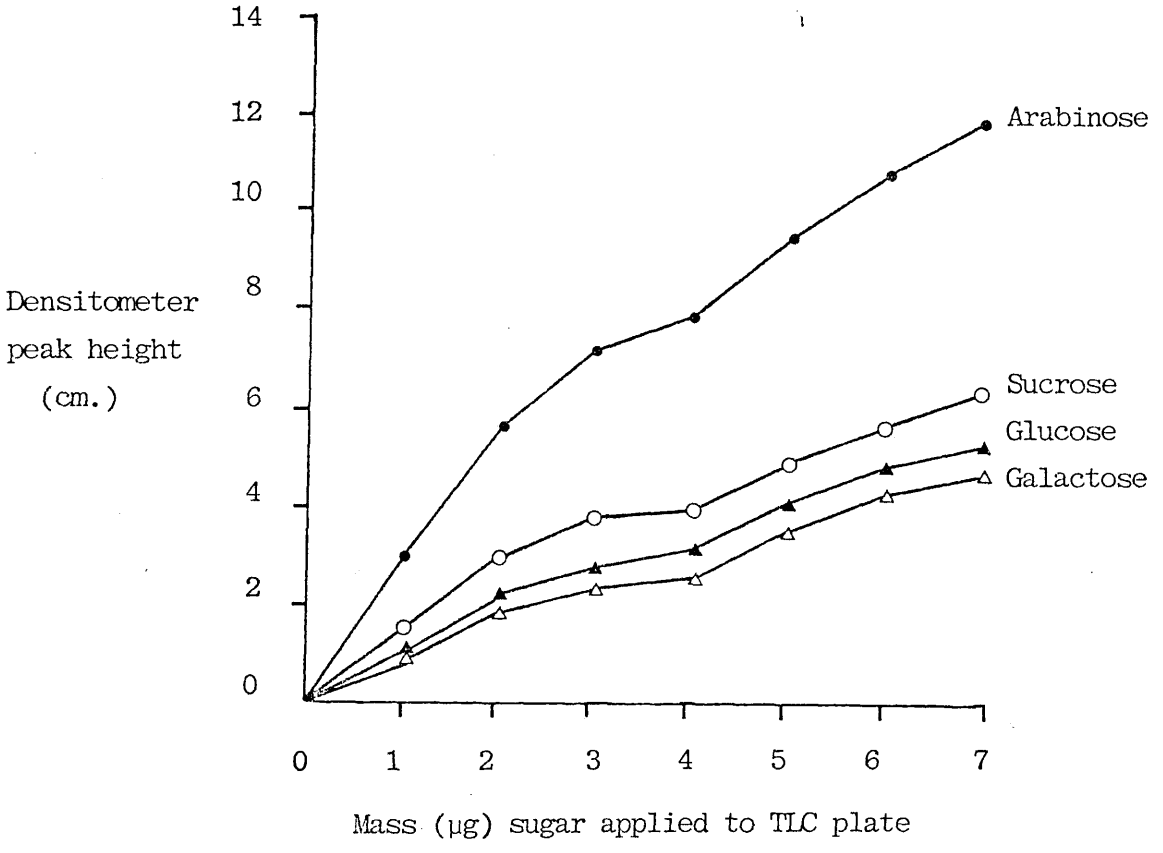


Fig.22. Standard curves for densitometric assay of sugars on silica gel thin-layer chromatograms prepared by the method of Menzies and Mount (1975).

Each point shown is the mean of duplicate densitometric assays of single thin-layer chromatograms.

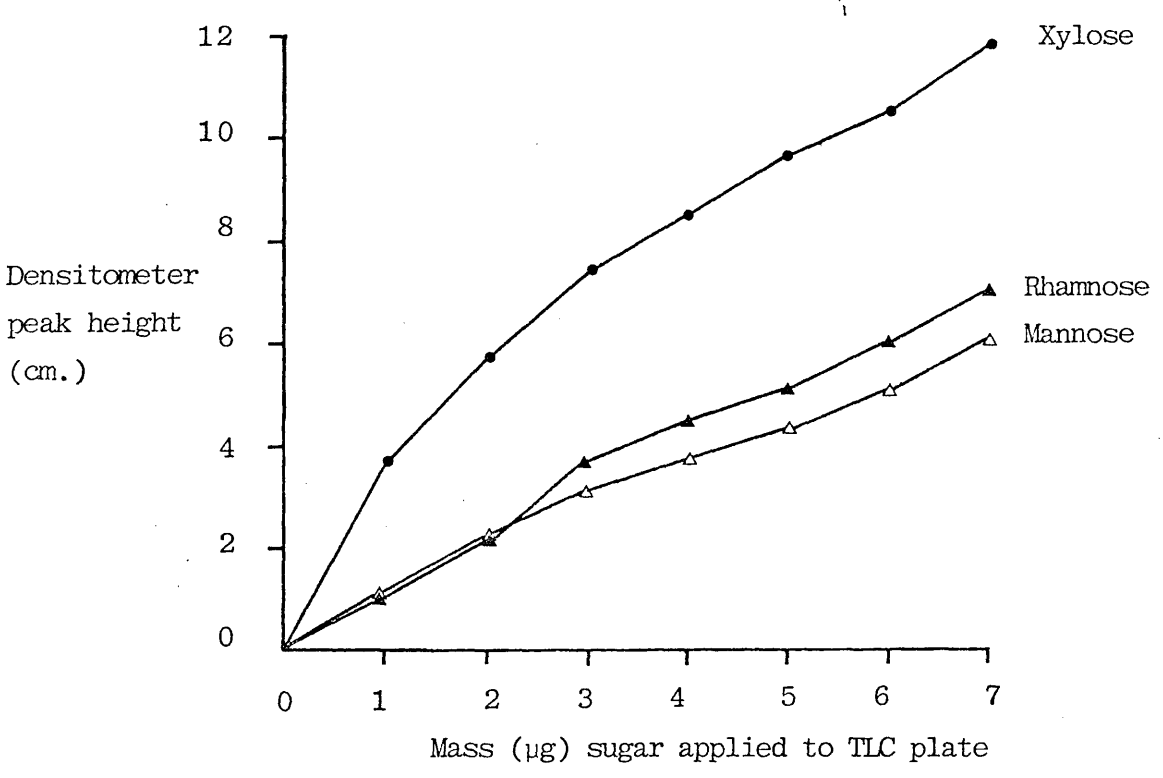


Fig.23. Standard curves for densitometric assay of sugars on silica gel thin-layer chromatograms prepared by the method of Menzies and Mount (1975).

Each point shown is the mean of duplicate densitometric assays of single thin-layer chromatograms.

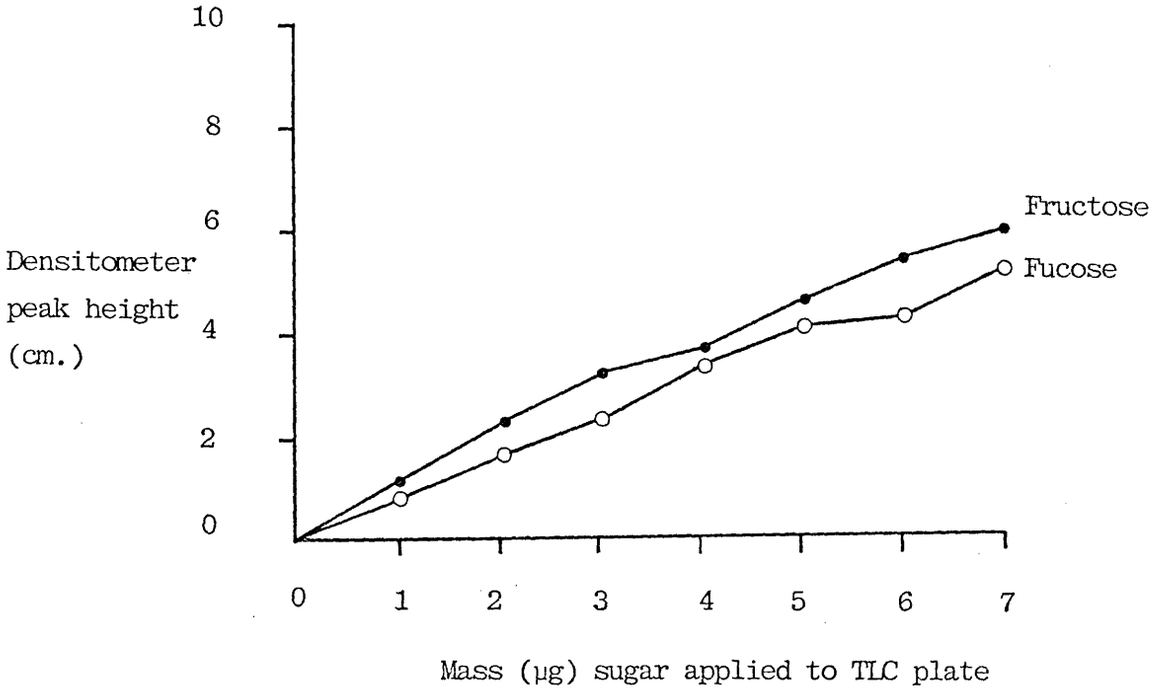


Fig.24. Standard curves for densitometric assay of sugars on silica gel thin-layer chromatograms prepared by the method of Menzies and Mount (1975). Each point shown is the mean of duplicate densitometric assays of single thin-layer chromatograms.

experimental test samples, the sample volume (10 µl) applied being kept constant for test samples and standards. Because rhamnose was added in equal amount as internal standard to both standard sugar solutions and experimental samples and all solutions were subjected to the same procedures (see section II.2. (a) 1.), rhamnose peak heights on the seven chromatograms (standard solutions at 3 different sugar concentrations and 4 different experimental samples) which could be accommodated on each thin-layer plate were comparable.

The method of calculation of sugar concentrations in experimental samples is best explained as outlined below:

Consider that on a given thin-layer plate the galactose peak appearing on the chromatogram of an experimental sample has a peak height (w cm) comparable to the galactose peak height (x cm) appearing on the chromatogram of the standard solution containing sugars at 10 µg/ml concentration, and that the corresponding rhamnose peak heights of the two chromatograms are y cm and z cm respectively. Then:

concentration (µg/ml) of galactose in experimental sample = $10. \frac{w}{x} . \frac{z}{y}$.

An advantage of this method of quantitating sugars in test samples was that it compensated for variation between plates of background reflectance and the procedure has been recommended by Menzies and Mount (1975). However, it is critically conceded that, because the relationship between peak height and mass of sugar applied to the thin layer was

not linear for the various sugars, this method of evaluating the concentrations of sugars in experimental samples is an approximation only. Because this technique was being utilised to look for significant, marked changes in sugar concentration between experimental samples this approximation was regarded as acceptable.

Each experimental sample was chromatographed only once and duplicate densitometric assays were performed on each chromatogram.

II.2. (b) 2.2. Phenol/H_2SO_4 method for assay of total carbohydrate.

The method used was based on that described by Dubois et al. (1956). The carbohydrate solution to be assayed (2 ml) containing 0-70 µg of carbohydrate was pipetted into a wide boiling tube. Phenol (80% w/v in water, 0.05 ml) was added from a micro-pipette and mixed. Concentrated H_2SO_4 (5 ml) was plunged into the carbohydrate solution from an 'Oxford' dispenser (this method of acid addition ensured rapid mixing and reproducible results). The tube was allowed to stand for 10 minutes then shaken and incubated at 30° for 20 minutes. Absorbance was measured at 490 nm against a blank containing water in place of the carbohydrate solution. All standard and test samples were assayed in duplicate. A series of standard solutions (2 ml) containing 0-70 µg galactose gave a linear increase in absorbance at 490 nm (Fig.25). Total carbohydrate in experimental samples was estimated as galactose equivalents from this calibration curve. However, it is

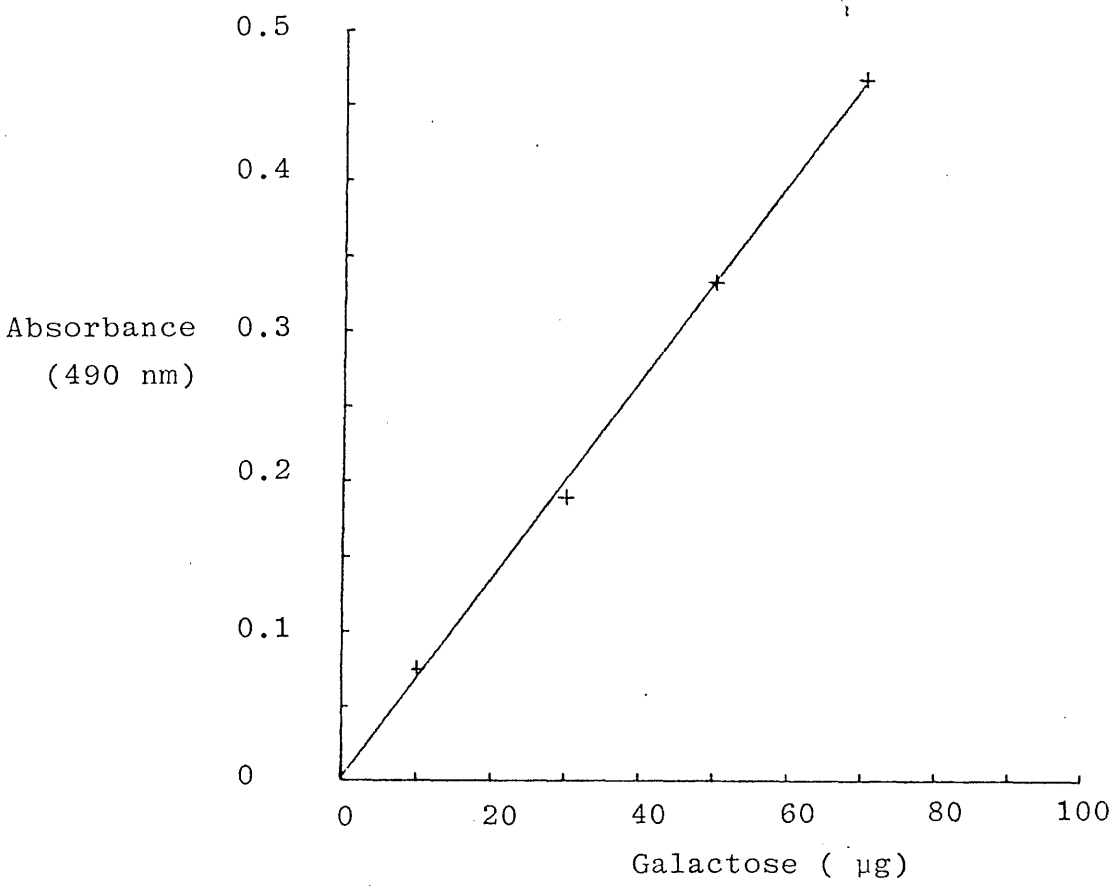


Fig.25. Standard curve for assay of total carbohydrate by the method of Dubois et al. (1956).

Each point shown is the mean of duplicate assays and the "line of best fit" is drawn with the aid of a microcomputer linked to a printer-plotter.

Gradient = 0.00665 with 95% confidence limits 0.00574 to 0.00756

Intercept = 0.0015 with 95% confidence limits -0.035 to 0.038

Correlation coefficient = 0.999

critically conceded that by this method variation is obtained between the absorbances obtained for different carbohydrates at the same concentrations (Dubois et al., 1956). Because evidence of marked changes in the level of total carbohydrate in experimental samples was being sought in the present work, this limitation was accepted.

II.2. (b).2.3. Assay of reducing sugars by the micro-cuprimetric method.

Reducing sugars were estimated microcuprimetrically (Nelson, 1944) with arsenomolybdate (Hestrin et al., 1955) as the colour-developing reagent.

To 1 ml of the sample, 0.5 ml Nelson's Cu-reagent was added and heated in a boiling water bath for 20 minutes, the test tube was then cooled and 0.5 ml arsenomolybdate reagent added and mixed. This was diluted by adding 5 ml distilled water and absorbance at 650 nm measured against a blank containing distilled water in place of the sugar solution. All standard and test samples were assayed in duplicate. The linear increase in absorbance at 650 nm obtained with galacturonic acid standard solutions (0-1000 µmol/l is shown in Fig.26. In all determinations the absorbance changes were kept within the range 0-2.0 absorbance units.

II.2.(b) 2.4. Assay of reducing sugars by the method of Foda (1957).

To 1 ml of the sugar solution, 2 ml saturated picric acid and 1 ml 20% Na_2CO_3 were added and heated in a water bath at 100^{o} for 1 hour. This was then cooled and

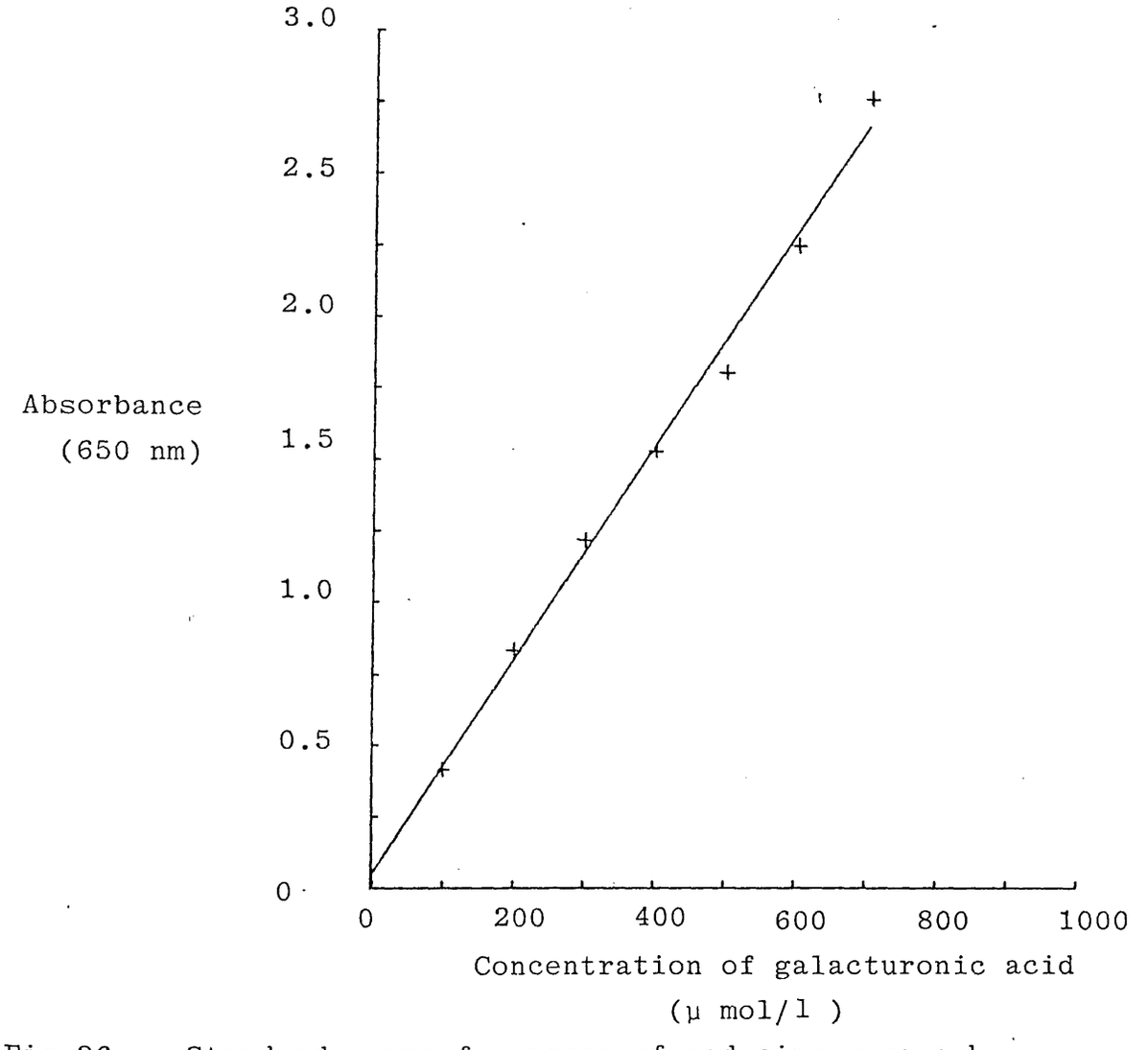


Fig.26. Standard curve for assay of reducing sugars by the microcuprimetric method (Nelson, 1944).

Each point shown is the mean of duplicate assays and the "line of best fit" is drawn with the aid of a microcomputer linked to a printer-plotter.

Gradient = 0.00376 with 95% confidence limits 0.00339 to 0.00412

Intercept = 0.049 with 95% confidence limits -0.097 to 0.194

Correlation coefficient = 0.996.

6 ml ethanol added. Absorbance at 549 nm was measured against a blank containing distilled water in place of the sugar solution. All standard and test samples were assayed in duplicate. The linear increase in absorbance at 549 nm obtained with galacturonic acid standard solutions (0-5 mmol/l) is shown in Fig. 27.

II.2. (b) 2.5. Assay of uronide content.

The total uronide was assayed in the deionised supernatant samples of enzymic digestion of cell wall preparations in some experiments and also in acid eluates of the deionising resin (Zerolit DM-F) used. For this latter process, the resin pellet was washed with water (10 ml) three times followed by centrifugation, the water washings being carefully aspirated down to the resin surface each time and discarded. The uronide bound to the resin was then eluted by addition of 5 ml 0.5M H_2SO_4 followed by shaking the tube by inversion, for 30 minutes before centrifugation and aspiration of the eluate for uronide assay. The total elution volume was 8.8 ml, 3.8 ml being the volume of water held in the packed resin (2.8 g) after water washing (this was established gravimetrically by drying the packed, wet resin by freeze-drying and storage over P_2O_5 under reduced pressure). In a control experiment, galacturonic acid (2.5 mg) and polygalacturonic acid (2.5 mg), both in solution in 0.1M acetate buffer, pH 5.0, were separately applied to a series of resin aliquots (2.8 g) and uronide assayed in both the supernatant after deionisation and the acid eluate from the resin as previously described.

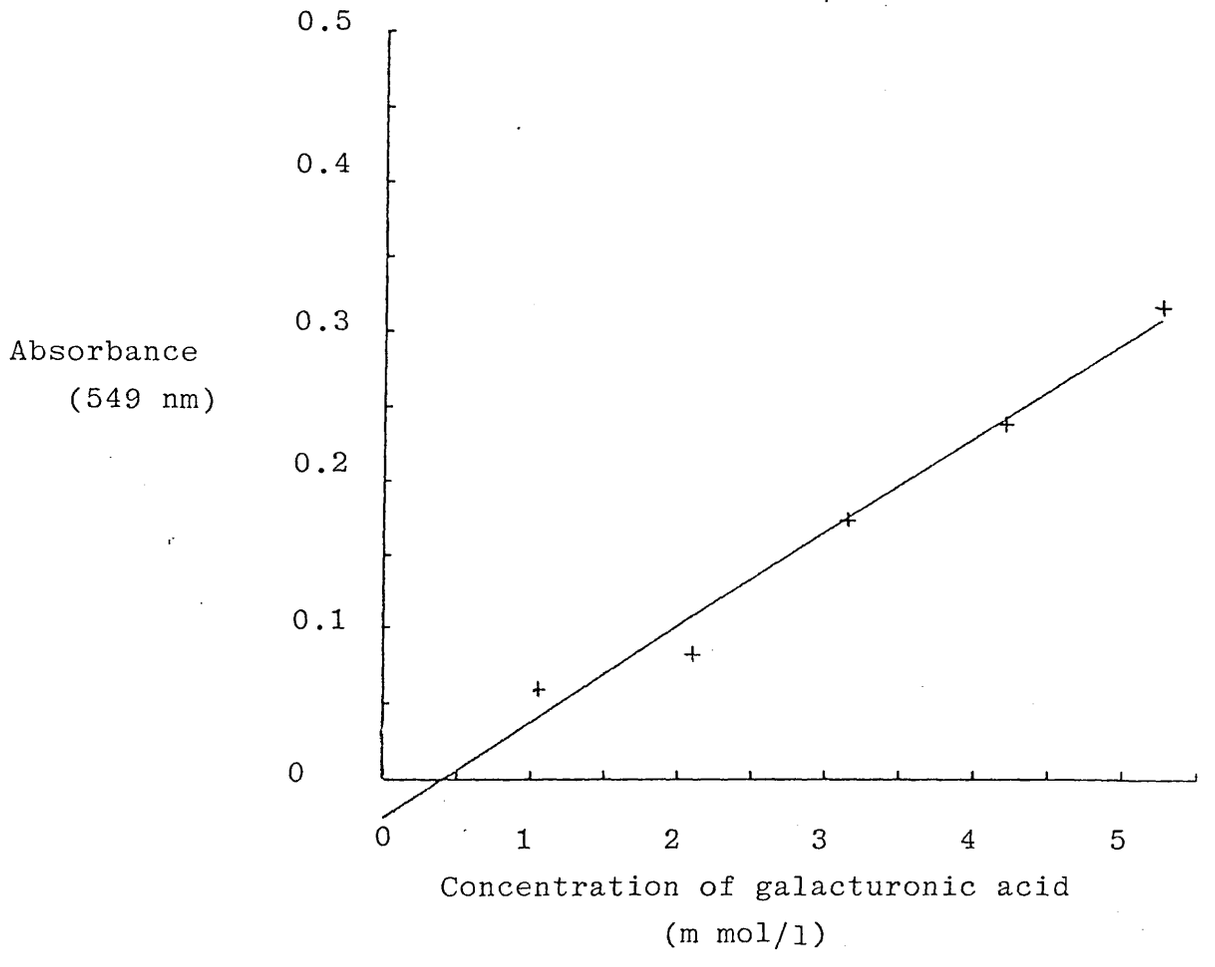


Fig.27. Standard curve for assay of reducing sugars by the method of Foda (1957).

Each point shown is the mean of duplicate assays and the "line of best fit" is drawn with the aid of a microcomputer linked to a printer-plotter.

Gradient = 0.0644 with 95% confidence limits 0.0461 to 0.0829

Intercept = -0.0275 with 95% confidence limits -0.0854 to 0.0304

Correlation coefficient = 0.988

Galacturonic acid was bound to the resin and was 80% recovered in the acid eluate; polygalacturonic acid remained essentially unbound to the resin (see Table 3).

Uronide was assayed by a method based on that described by Bitter and Muir (1962). Borax (5 ml, 0.25M) in concentrated H_2SO_4 was added from an 'Oxford' dispenser to 1 ml uronide solution (containing 0-50µg total uronide) in a wide boiling tube, and heated for 15 minutes at 100°. After cooling 0.125% (w/v) carbazole in methanol (0.2 ml) was added and heated at 100° for 10 minutes. Absorbance at 530 nm was measured against a blank containing water in place of the uronide solution. All standard and test samples were assayed in duplicate. A series of standard solutions (1 ml) containing 0-50µg galacturonic acid gave a linear increase in absorbance at 530 nm (Fig.28). Total uronide in experimental samples was estimated as galacturonic acid equivalents from this calibration curve. However, it is critically conceded that by this method contaminating neutral carbohydrate present in uronide solutions would constitute a source of interference, since neutral carbohydrate gives a diminished, partial colour reaction with carbazole (Bitter and Muir, 1962). However, since, in the present work, evidence of marked changes in uronide content of experimental samples was being sought, this limitation was accepted.

TABLE 3. Binding of galacturonic acid and polygalacturonic acid to Zerolit DM-F.

Exp.	GA* (mg) applied to Zerolit DM-F	GA(mg)recovered in supernatant after de-ionisation	% recovery in supernatant	GA(mg)recovered in eluate from Zerolit DM-F	% recovery in eluate
1	2.5	0.15	6.0	2.11	84.5
2	2.5	0.21	8.4	1.98	79.2
3	2.5	0.09	3.6	1.96	78.3
4	2.5	0.15	6.0	1.98	79.2
5	2.5	0.15	6.0	2.02	81.0
6	2.5	0.21	8.4	1.96	78.3
Mean (S.E.)	2.5 (0)	0.16 (0.02)	6.4 (0.7)	2.00 (0.02)	80.1 (1.0)
	PGA** (mg) applied to Zerolit DM-F	PGA (mg) recovered in supernatant after de-ionisation	% recovery in supernatant	PGA (mg)recovered in eluate from Zerolit DM-F	% recovery in eluate
1	2.5	1.56	62.4	0.24	9.7
2	2.5	1.41	56.4	0.26	10.6
3	2.5	1.53	61.2	0.31	12.3
4	2.5	1.56	62.4	0.33	13.2
5	2.5	1.56	62.4	0.37	15.0
6	2.5	1.44	57.6	0.31	12.3
Mean (S.E.)	2.5 (0)	1.51 (0.03)	60.4 (1.1)	0.30 (0.02)	12.2 (0.8)

*GA = galacturonic acid **PGA = polygalacturonic acid

In each experiment galacturonic acid (2.5 mg) and polygalacturonic acid (2.5 mg), both in solution in 0.1M sodium acetate buffer (5 ml) pH 5.0, were separately applied to Zerolit DM-F (2.8 g) and uronide assayed by the method of Bitter and Muir (1962) (II.2.(b) 2.5.) in both the supernatant and the acid eluate from the resin. Assay of uronide in all samples was performed in duplicate. Standard errors of means of the 6 experiments are shown in parenthesis.

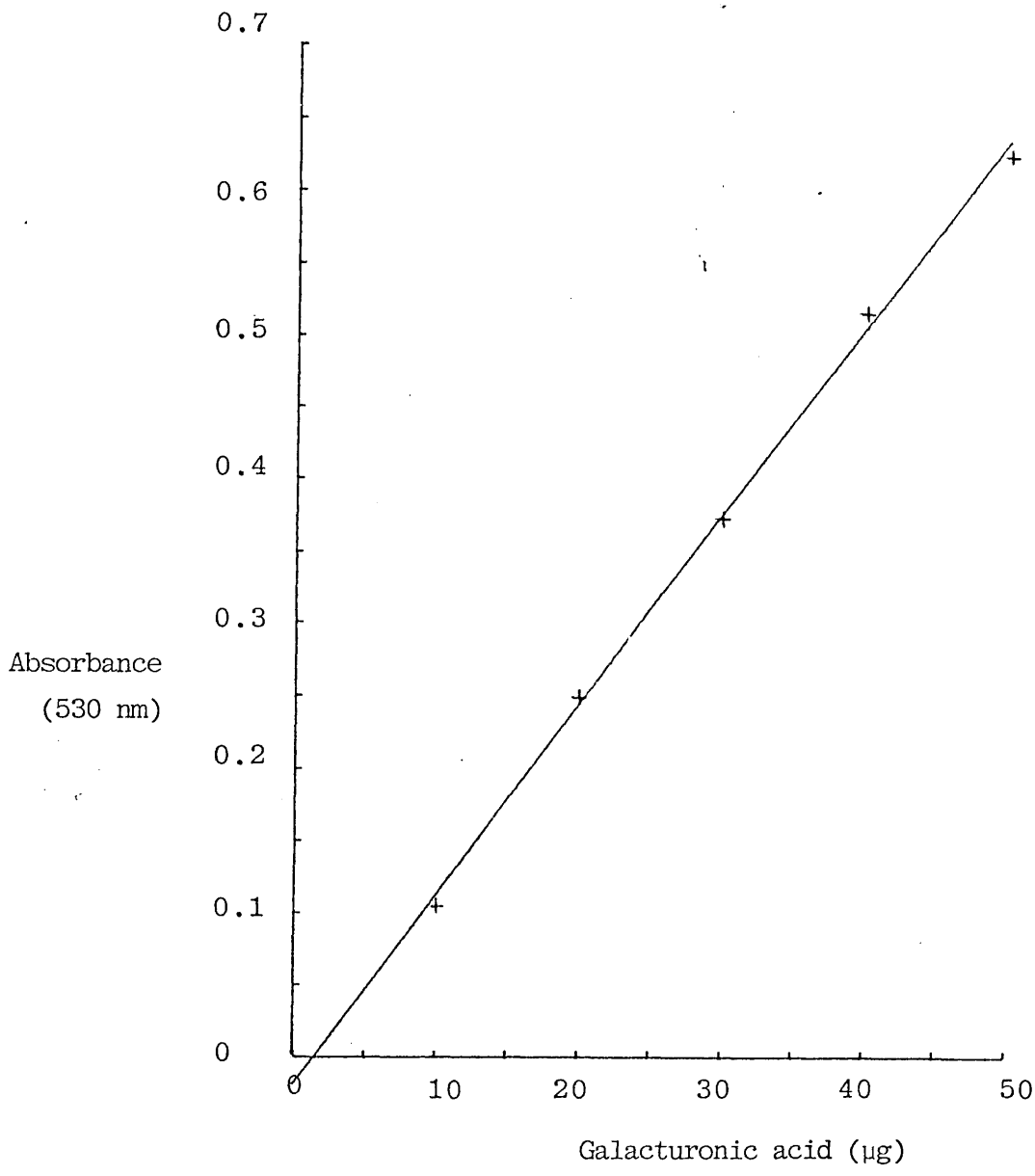


Fig.28. Standard curve for assay of uronide by the method of Bitter and Muir (1962).

Each point shown is the mean of duplicate assays and the "line of best fit" is drawn with the aid of a microcomputer linked to a printer-plotter.

Gradient = 0.01320 with 95% confidence limits 0.01214 to 0.01426.

Intercept = -0.0200 with 95% confidence limits-0.0517 to 0.0117

Correlation coefficient = 0.999.

II.2.(b) 3. Enzymes

II.2.(b) 3.1. Polygalacturonase

Enzyme solutions were incubated with 0.2% polygalacuronic acid or citrus pectin in either 0.05M potassium hydrogen phthalate buffer, pH 4.5 or 0.1M acetate buffer, pH 4.5, at 30° for measured times. Control incubation mixtures contained buffer only in place of substrate. Aliquots (1 ml) of incubation mixtures were assayed (in duplicate) for reducing sugars at timed intervals. In 0.05M potassium hydrogen phthalate buffer, pH 4.5, reducing sugars were measured by the method of Foda (1957) (see II.2.(b).2.4.). In 0.1M acetate buffer, pH 4.5, reducing sugars were measured by the microcuprimetric method (Nelson, 1944) (see II.2 (b).2.3.).

II.2.(b) 3.2. p-Nitrophenyl glycosidases

Assay mixtures, total volume 1 ml, contained 0.25-0.4 ml enzyme solution, 5 mM p-nitrophenyl glycoside (0.1 ml) and 0.1M acetate buffer, pH 5.0 (0.5-0.65 ml). Assay mixtures were incubated at 30° for measured times, reactions being started by addition of the substrate and terminated by addition of 0.1M sodium carbonate (5 ml). Assay mixtures were prepared in duplicate for each substrate. Corrections for the colour present in the enzyme solutions were made by using control reaction mixtures where enzyme solution and buffer were incubated for the appropriate time and then the substrate added, followed immediately by the sodium carbonate solution.

The p-nitrophenol liberated by hydrolase action

was determined by measurement of absorbance at 405 nm against a blank containing distilled water in place of the enzyme solution. A series of standard solutions (1 ml) containing 0-0.24 µmol p-nitrophenol, (assayed in duplicate), to which was added 0.1M sodium carbonate (5 ml), gave a linear increase in absorbance at 405 nm (Fig.29).

II.3. Extraction and Assay of Polygalacturonase.

II.3. (a). General method for extraction and assay (cf. Hobson 1962).

Thin longitudinally sliced mango mesocarp (of ripening stages 1,2,3,4) was macerated for 60 seconds at 4^{o} using a Kenwood blender in 10 sec bursts with 5 sec delay between bursts and then re-macerated with a mixture of NaCl and EDTA (10:1 w/w), using 7.5 g salt mixture/100 g tissue, for 2 min in 10 sec bursts. In some samples of fruit at ripening stage 1, 50 g of tissue was macerated with 20 ml 7.5% (w/v) solution of the salt mixture before addition of the solid salt mixture.

After standing at 4^{o} for 20 min the macerate was centrifuged at 40,000 g for 1 h and the clear supernatant collected. In cases where extracted protein was to be precipitated with $(NH_4)_2SO_4$(0-80%) the pellet was re-extracted twice with 7.5% (w/v) solution of the salt mixture (100 ml) and the supernatants pooled. Protein was precipitated by slow addition of solid $(NH_4)_2SO_4$ with stirring at 4^{o} to 80% saturation. Protein was removed by centrifugation at 40,000 g for 1 h at 4^{o} and re-dissolved in

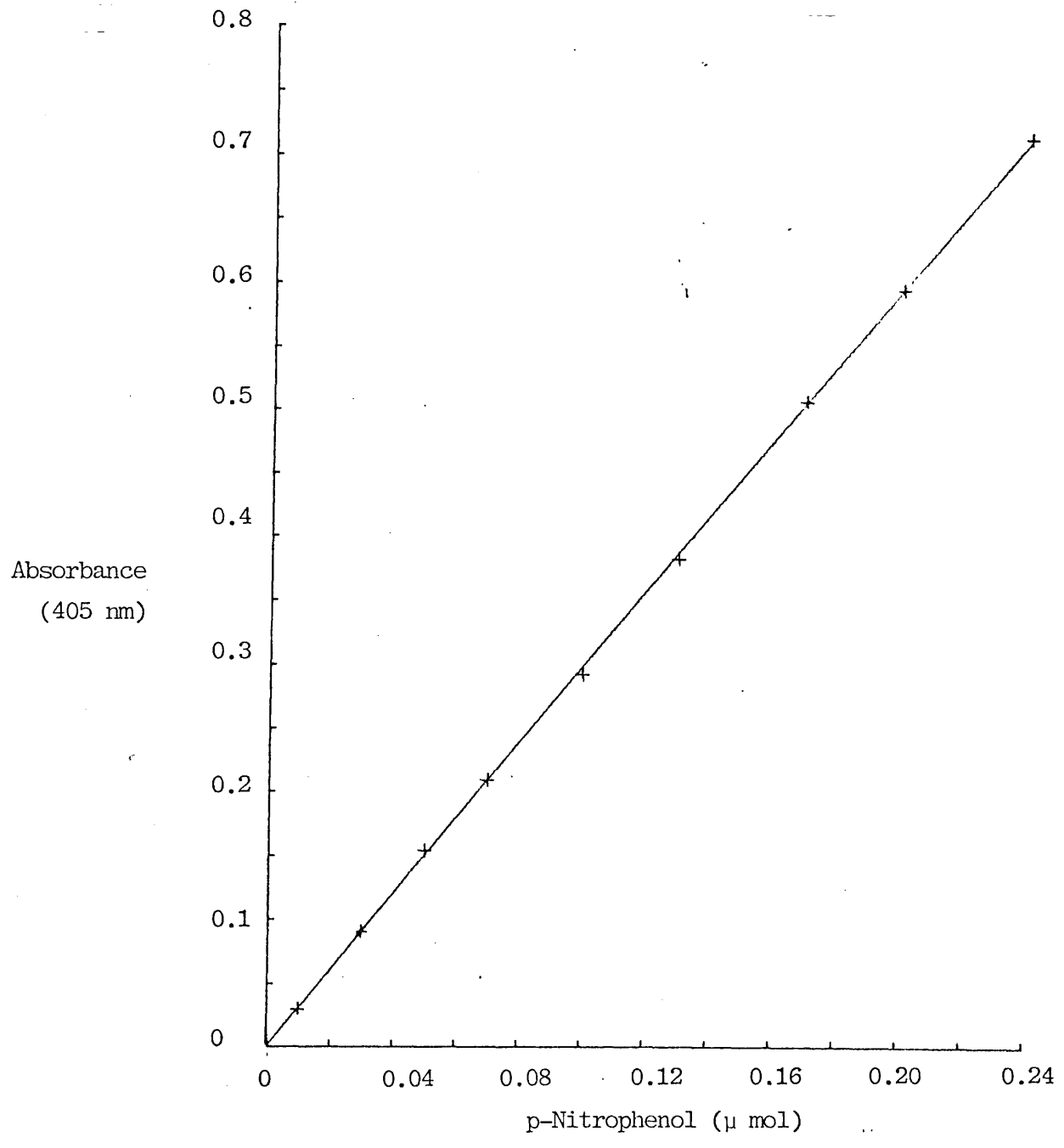


Fig.29. Standard curve for assay of p-nitrophenol in alkaline solution by measurement of absorbance at 405 nm. p-Nitrophenol solution (1 ml) was added to 0.1M sodium carbonate (5 ml) before absorbance measurement.

Each point shown is the mean of duplicate assays and the "line of best fit" is drawn with the aid of a microcomputer linked to a printer-plotter.

Gradient = 2.993 with 95% confidence limits 2.960 to 3.026

Intercept = 0.0002 with 95% confidence limits -0.0035 to 0.0039

Correlation coefficient = 1.000

0.05M potassium hydrogen phthalate buffer, pH 4.5, containing 0.1M NaCl, or 0.1M acetate buffer, pH 4.5 and dialysed against 10l of the same buffer for 24 h at 4°. In one case precipitated protein was dialysed against 2 x 10l distilled water and then against 1l 0.1M acetate buffer, pH 4.5, each dialysis for 24 h.

Crude enzyme extracts were also dialysed against 10l 0.05M phthalate buffer, pH 4.5, containing 0.1M NaCl, or 10l 0.1M acetate buffer, pH 4.5 for 24 h at 4°.

Enzyme solutions were assayed as described in II.2 (b) 3.1. The generation, by crude polygalacturonase extracted by this method from tomato pericarp tissue, of reducing power from polygalacturonic acid was linear with time when measured by the method of Foda (1957) (II.2.(b) 2.4.), (see Fig.30), or by the microcuprimetric method (Nelson, 1944) (II.2.(b) 2.3), (see Fig.31).

II.3. (b). Modifications of general method for extraction and assay.

A series of exploratory experiments for detection of polygalacturonase activity were carried out, utilising the basic Hobson (1962) technique and modifications of this method. Experiments with tomato extracts were also carried out for comparison (see II.3.(b) 9.)

In these experiments, "enzyme" preparations in 0.1M acetate buffer, pH 4.5 were assayed for polygalacturonase activity using the microcuprimetric method (Nelson, 1944) (II.2.(b) 2.3.) for assay of reducing sugars and

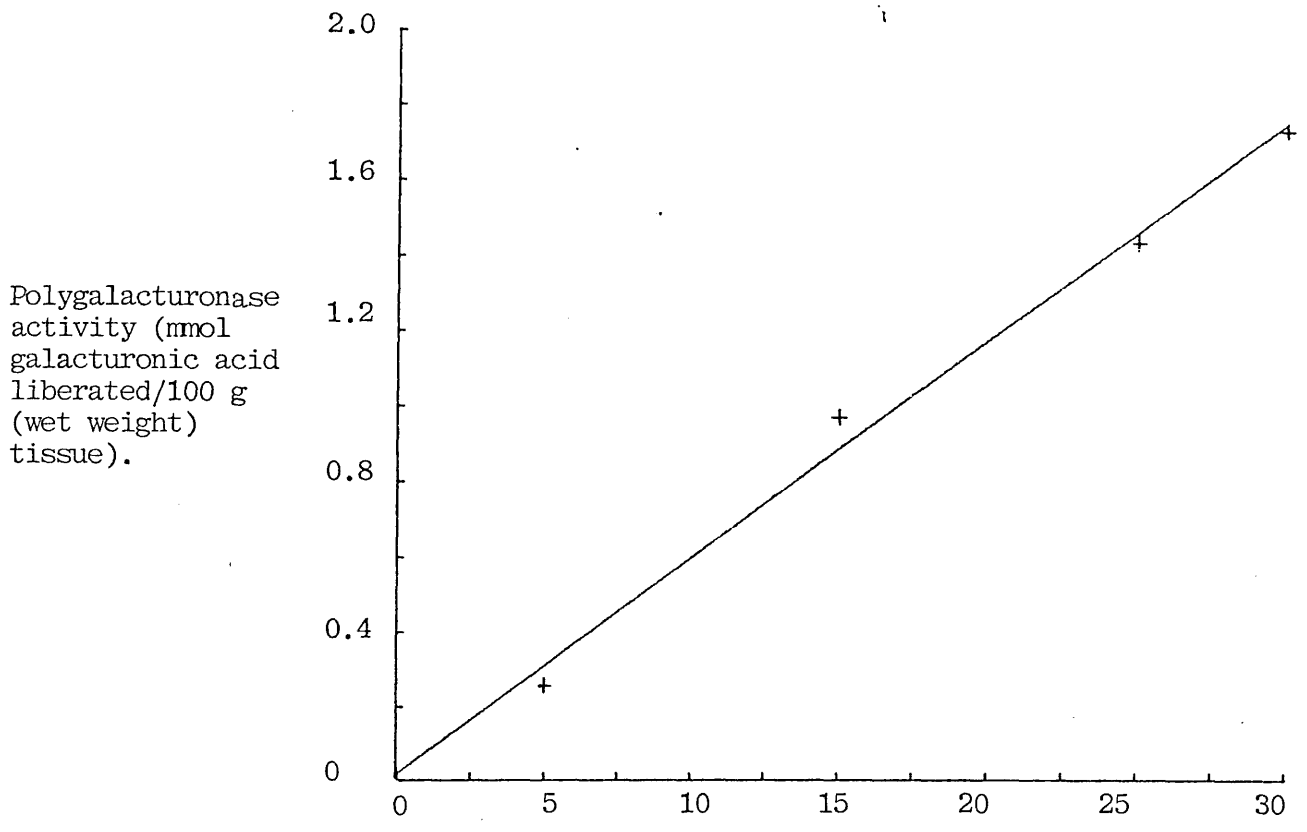


Fig.30.

Activity of polygalacturonase in tomato pericarp tissue extracted by the method of Hobson (1962).

The crude enzyme extract dialysed against 0.05M phthalate buffer, pH 4.5 containing 0.1M NaCl was assayed for hydrolytic activity against polygalacturonic acid at 30°. A control incubation mixture contained no polygalacturonic acid. Aliquots (1 ml) of incubation mixtures were assayed (in duplicate) by Foda's (1957) method for reducing sugars at timed intervals.
Each point shown is the mean of the duplicate assays and the "line of best fit" is drawn with the aid of a microcomputer linked to a printer-plotter.

Gradient = 0.0583 with 95% confidence limits 0.0417 to 0.0750
Intercept = 0.0168 with 95% confidence limits - 0.2953 to 0.3289
Correlation coefficient = 0.996.

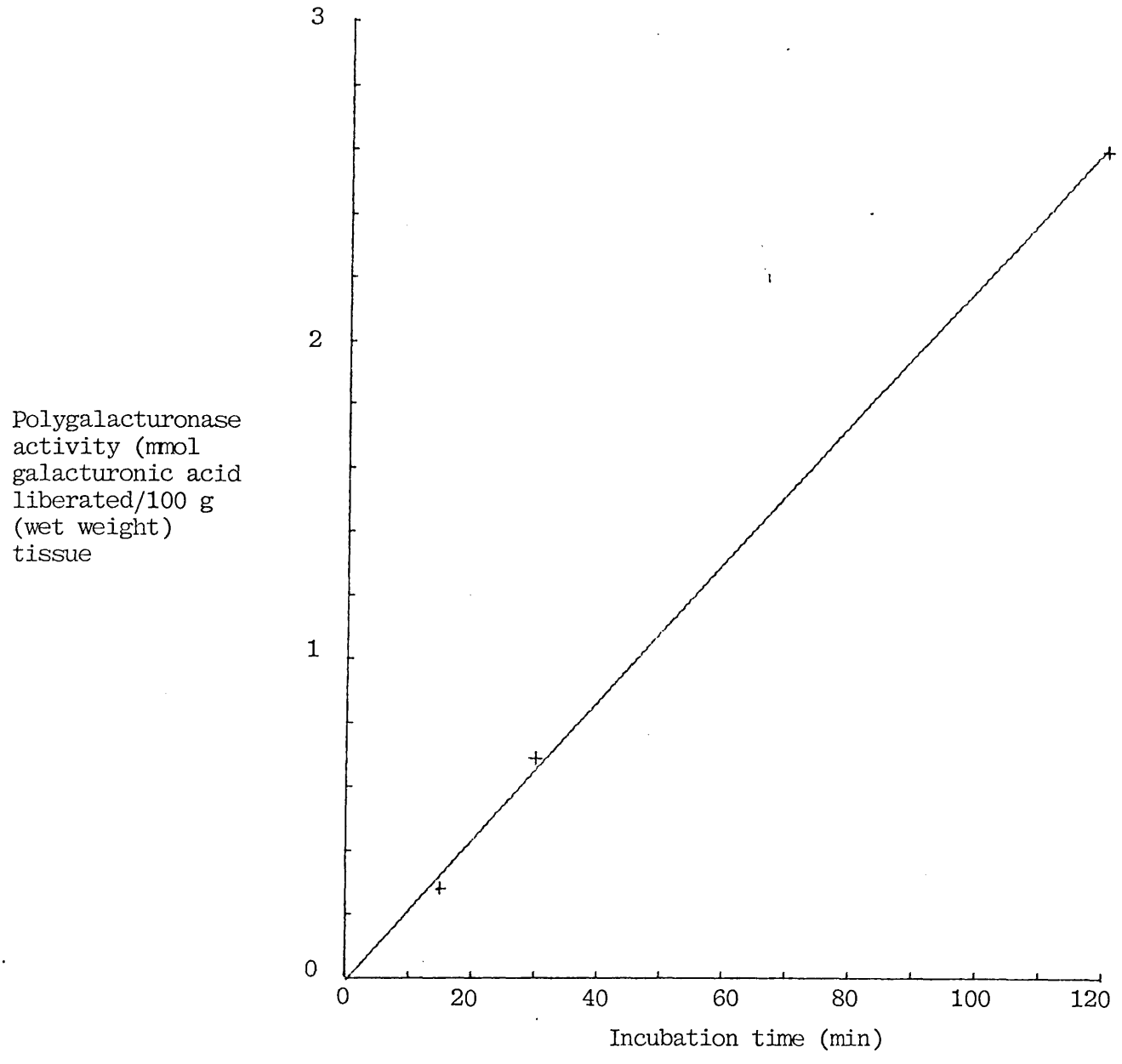


Fig.31. Activity of polygalacturonase in tomato pericarp tissue extracted by the method of Hobson (1962).

The crude enzyme extract dialysed against 0.1M acetate buffer, pH 4.5 was assayed for hydrolytic activity against polygalacturonic acid at 30°. A control incubation mixture contained no polygalacturonic acid. Aliquots (1 ml) of incubation mixtures were assayed (in duplicate) by the microcuprimetric method (Nelson, 1944) for reducing sugars at timed intervals.

Each point shown is the mean of the duplicate assays and the "line of best fit" is drawn with the aid of a microcomputer linked to a printer-plotter.

Gradient = 0.0220 with 95% confidence limits 0.0130 to 0.0310

Intercept = -0.0129 with 95% confidence limits -0.5103 to 0.4844

Correlation coefficient = 0.999.

enzyme preparations in 0.05M phthalate buffer, pH 4.5, containing 0.1M NaCl were assayed for polygalacturonase using the method of Foda (1957) (II.2.(b) 2.4.) for assay of reducing sugars.

II.3.(b) 1. Unripe, ripe and over-ripe mango slices (100 g) were separately macerated, extracted and dialysed against 0.05M phthalate buffer, containing 0.1M NaCl, as described (II.3.(a)). These crude mango extracts were assayed for hydrolytic activity against polygalacturonic acid.

II.3. (b) 2. A crude extract of over-ripe mango and an aliquot (4.6g) of the packed debris left after extraction (resuspended in 0.1M acetate buffer, pH 4.5 (20 ml)) were dialysed against the same buffer (10l) and assayed for hydrolytic activity against polygalacturonic acid.

II.3. (b) 3. Similar to II.3.(b) 2. above except that citrus pectin was used as substrate.

II.3.(b) 4. $(NH_4)_2SO_4$ (0-90%)-precipitated "enzyme" preparations from unripe, ripe and over-ripe mango in 0.1M acetate buffer, pH 4.5 were assayed for hydrolytic activity against polygalacturonic acid.

II.3.(b) 5. Aliquots of a crude extract of ripe mango were incubated with polygalacturonic acid in 0.05M phthalate buffer, containing 0.1M NaCl adjusted to a range of pH values.

II.3. (b) 6. Ripe mango (50g) was macerated with polyvinyl-pyrrolidone (10g) before extraction, dialysis against 0.05M phthalate buffer (10l) pH 4.5 containing 0.1M NaCl and assay for polygalacturonase with polygalacturonic acid as substrate.

II.3. (b) 7. An aliquot (5ml) of a $(NH_4)_2SO_4$ (0-80%)-precipitated "enzyme" preparation from over-ripe mango in 0.1M acetate buffer, pH 4.5 was applied to a Sephadex G-100 column (93 x 2.5 cm) which was eluted with the same buffer. After passage of the void volume (142ml) through the column 5ml fractions were collected and each fraction assayed for hydrolytic acitivity against poly-galacturonic acid.

Fractions were also monitored for protein content by absorbance measurement at 280nm and fractions containing protein (fractions 5 - 20, see Fig.32) were pooled and concentrated 10-fold by ultrafiltration using an Amicon PM-10 membrane at 30 p.s.i. pressure. The concentrate and the Amicon filtrate were assayed for hydrolytic activity against polygalacturonic acid.

II.3. (b) 8. Another aliquot (5 ml) of the "enzyme" preparation from II.3.(b) 7. above was dialysed against 0.1M acetate buffer (1 l), pH 4.5 containing a few crystals of thymol for 72 h. After dialysis polygalacturonase activity in the "enzyme" solution was assayed using polygalacturonic acid as substrate.

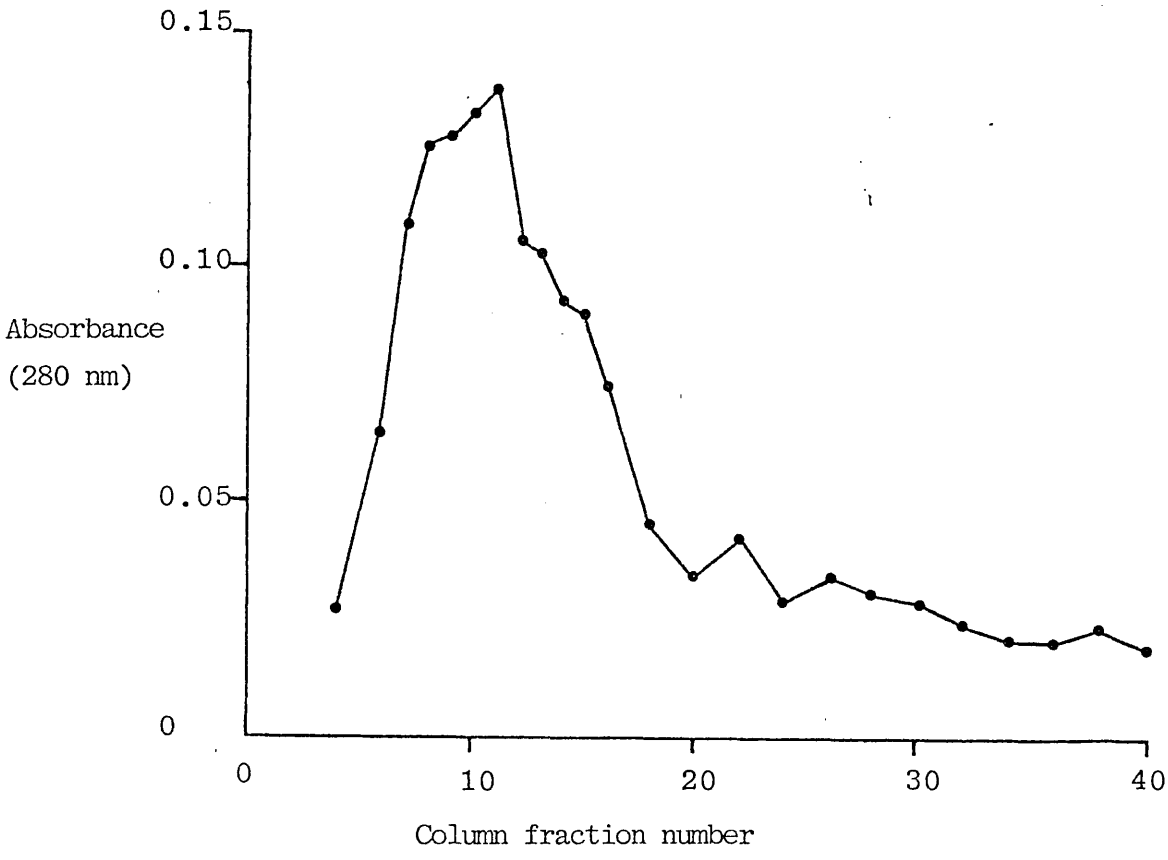


Fig.32. Chromatography of ammonium sulphate (0-80%)- precipitated fraction from extract of over-ripe mango tissue on Sephadex G-100. The mango tissue was extracted with NaCl/EDTA solution by the method of Hobson (1962), and protein in the extract precipitated with ammonium sulphate (0-80% saturation). Precipitated protein was re-dissolved in 0.1M acetate buffer, pH 4.5 and dialysed against the same buffer (2 x 1l).
5 cm^3 of the dialysed preparation was applied to a Sephadex G-100 column (93 x 2.5 cm) and, after passage of the void volume (142 cm^3), 5 cm^3 fractions eluted in 0.1M acetate buffer, pH 4.5 were collected. Protein present in column fractions was monitored by absorbance measurement at 280 nm. Fractions 5-20 were pooled, concentrated 10-fold by Amicon ultra-filtration and assayed for hydrolytic activity against polygalacturonic acid as described in II.2.(b) 3.1.

II.3. (b) 9. A crude "enzyme" preparation (extracted by the Hobson (1962) method) in 0.1M acetate buffer, pH 4.5, was made from ripe tomato tissue. This tomato extract and a $(NH_4)_2SO_4$ (0-80%)-precipitated extract from over-ripe mango in the same buffer were used to set up four separate incubation mixtures at 30^{o} for polygalacturonase assay:

1. tomato extract (1 ml)
   0.5% polygalacturonic acid in 0.1M acetate buffer (4 ml), pH 4.5
   0.1M acetate buffer (5ml), pH 4.5.
2. as 1. above with buffer only in place of polygalacturonic acid
3. tomato extract (1 ml)
   mango protein preparation (1 ml)
   0.5% polygalacturonic acid in 0.1M acetate buffer (4 ml), pH 4.5.
   0.1M acetate buffer (4 ml), pH 4.5
4. as 3. above with buffer only in place of polygalacturonic acid.

Aliquots (1 ml) were removed and assayed (in duplicate) for reducing sugars.

II.3. (b) 10. Aliquots (2 ml) of a crude extract of ripe mango, dialysed against 0.1M acetate buffer, were incubated with each of the enzymes (2mg) and at the pH values and temperatures indicated in the table below, for 2h, the reactions being stopped by cooling to 4^{o}:

Enzyme	pH of reaction mixture	Temperature
cellulase (technical,type 1)	5.0	30°
hemicellulase (fungal,crude)	5.0	30°
exo-1,3-β-D-glucanase(purified)	5.0	30°
cellulase + hemicellulase + exo-1,3-β-D-glucanase (2mg each)	5.0	30°
protease (fungal, purified)	7.0	37°
pepsin (purified)	2.3	37°
control (no enzyme added)	5.0	30°

Each digest was then separately dialysed against 0.1M acetate buffer (1l), pH 4.5 for 24 h at 4° and assayed for hydrolytic activity against polygalacturonic acid and pectin. The hydrolytic activity of the crude cellulase preparation alone (1mg/ml in 0.1M acetate buffer, pH 4.5) against polygalacturonic acid was also estimated.

II.3. (b) 11. Ripe mango tissue was extracted by modifying the Hobson (1962) extraction medium as outlined below:

1. Unmodified Hobson (1962) extraction as control.
2. Omitting EDTA.
3. Omitting EDTA and incorporating 1 mM $CaCl_2$.
4. Omitting EDTA and incorporating 1 mM $MgCl_2$.
5. Omitting EDTA and incorporating both 1 mM $CaCl_2$ and 1mM $MgCl_2$.
6. Incorporating 0.1% (v/v) Tween-60.
7. Incorporating 1mM N-ethyl maleimide.
8. Incorporating 1mM cysteine.

The various extracts were separately dialysed against 0.1M acetate buffer (1l), pH 4.5 and assayed for hydrolytic activity against polygalacturonic acid and pectin.

II.3.(b) 12. A crude extract of ripe mango dialysed against 0.1M acetate buffer, pH 4.5 was subjected to viscometric assay, Crude extract (3ml) was added to 0.25% polygalacturonic acid (12ml) in the same buffer at 30° and the mixture immediately transferred to a glass viscometer immersed in a water bath at 30°. Viscometric flow time was measured immediately and at time intervals over a 24 h period. A similar viscometric assay was set up with pectin as substrate.

II. 3. (c) Method for extraction and assay (cf.Pansolli and Belli-Donini, 1973).

Pooled, ripe mango slices were macerated (without salt addition) and the macerate centrifuged by the means described in II.3 (a). The supernatant was removed and the residue mixed with solid NaCl (50g) and 5% NaCl solution (350ml) per 1000g original wet weight tissue. The mixture was stirred slowly for 30h at 4°, centrifuged and the supernatant brought to pH 7.0 by addition of 1M phosphate buffer, pH 7.0 at 4°.

Protein was precipitated with $(NH_4)_2SO_4$ (0-90%), removed by centrifugation, re-dissolved in a small volume of 5% NaCl and dialysed twice against 5% NaCl (10 l) for 24 h at 4°.

Protein preparations were assayed for hydrolytic activity against polygalacturonic acid or pectin in

0.1M acetate buffer, pH 4.5 as described (II.2 (b) 3.1.).

II.3. (d) Method for extraction and assay (cf.Pressey and Avants, 1973).

Frozen, ripe mangoes were allowed to thaw in 0.1% sodium bisulphite pH 6.0 at 4° and pooled slices (1000g)macerated in 1l of this solution for 2 min at 4°. The macerate was centrifuged as previously described and the supernatant discarded. The residue was treated twice more to this procedure and the final residue suspended in 0.2M NaCl (2 l) and stirred slowly for 3 h at 4° and then centrifuged. The supernatant was concentrated 30-fold by ultrafiltration at 30 p.s.i. using an Amicon PM-10 membrane.

The concentrate was dialysed against 0.15M NaCl (10 l) for 24 h at 4° and traces of insoluble protein removed by centrifugation. Protein preparations were assayed for hydrolytic activity against polygalacturonic acid in 0.1M acetate buffer, pH 4.5 by generation of reducing sugars (II.2.(b) 3.1), incorporating 1 mM sodium dithionite in the incubation mixtures, or by the viscometric method (see II.3. (b) 12.).

II.4. Interaction between enzyme extracts of mango and cell wall material from unripe mangoes.

II.4. (a) Preparation of cell wall materials from mango mesocarp.

The method used was based on that described by Wallner and Walker (1975). Mango mesocarp was thinly sliced longitudinally, macerated in 20 mM phosphate buffer, pH 6.9 at 4° and centrifuged at 40,000 g for 1 h. The

pellet was washed with 20 mM phosphate buffer, pH 6.9 and re-centrifuged. The solid residue was vigorously stirred at room temperature with acetone (500 ml) for 1 h, filtered on sintered glass and washed on the filter with acetone (500 ml). This acetone extraction/washing was immediately followed by similar extraction/washing with chloroform/methanol (1:1 v/v). The wall material was dried on the filter paper under suction and stored over P_2O_5 in a desiccator under reduced pressure.

The wall material obtained was a white, finely-divided product. Walls used in these experiments were not extracted with chloral hydrate. This material was ground to a fine powder in a pestle and mortar. It was re-hydrated immediately before use by suspending in 0.1M acetate buffer, pH 5.0, and stirring at 4^{o} for 24 h.

II.4. (b) Preparation of enzyme extracts of mango.

Longitudinal slices of mango mesocarp were macerated in 0.5M acetate buffer, pH 5.0 at 4^{o} (25 g slices/100 ml buffer), using a blender at high speed in 10 sec bursts with 5 sec intervals between bursts, 12 bursts being used in all. This ensured efficient maceration and a limited temperature rise.

The macerate was stirred at 4^{o} for 2 h before centrifugation at 40,000 g for 1 h at 4^{o}. Protein was precipitated from the clear supernatant either by saturation to 80% with $(NH_4)_2SO_4$ at 4^{o} or addition of acetone cooled to -20^{o} to the supernatant at 4^{o} (final acetone concentration 50%). The acetone was quickly mixed with the supernatant and the mixture immediately centrifuged at -15^{o}. The

acetone-containing supernatant was immediately aspirated and the protein pellet quickly re-dissolved in 0.1M acetate buffer, pH 5.0 at 4°. Protein precipitated with $(NH_4)_2SO_4$ was similarly centrifuged and re-dissolved in a minimum volume of buffer.

Protein was dialysed against 0.1M acetate buffer,(10 l) pH 5.0 for 24 h at 4°. Undissolved protein was removed by centrifugation for 30 min at 100,000 g and 4° (the high centrifugal speed was necessitated by the marked viscosity of the protein solution). Protein content of all mango extracts was assayed by Lowry et al's (1951) method (II.2.(b) 1.1.).

β-D-Galactosidase was used as a marker enzyme for recovery of enzyme activity after ammonium sulphate and acetone precipitation. β-D-Galactosidase activity in the initial extract and in the protein concentrate after dialysis and removal of undissolved protein was assayed as described in II.2.(b) 3.2.

II.4. (c) Incubation of cell wall suspensions with enzyme preparations of mango.

Enzyme preparation (4 ml) was added to cell wall suspension (16 ml) containing 20 mg cell wall material (dry weight) at 4°. A zero-time aliquot was taken out immediately and kept at 4°. Three control incubations were set up and similarly treated:

1. Boiled enzyme extract (4 ml) + cell wall suspension (16 ml)
2. Enzyme extract (4 ml) + 0.1M acetate buffer, pH 5.0 (16 ml)
3. Buffer (4 ml) + cell wall suspension (16 ml).

Mixtures were incubated at 30° for 24 h with shaking, with a few crystals of thymol added as an anti-microbial agent. Reactions were stopped by cooling in ice and cell wall material removed by centrifugation at 100,000 g for 1 h at 4° followed by millipore filtration of the supernatant at 4°. Zero-time aliquots were centrifuged and millipore-filtered immediately after mixing.

The clear supernatants obtained were subjected to the following experimental procedures:

(i) Assay for total carbohydrate content by Phenol/H_2SO_4 method of Dubois et al. (1956) (II.2.(b) 2.2.).

(ii) De-ionisation for thin-layer chromatography of free sugars by the method described by Menzies and Mount (1975) (II.2.(a) 1.) and quantitation of sugars on thin-layer plates by the densitometric method (II.2.(b) 2.1.).

(iii) Assay of uronide content of supernatant after de-ionisation; elution and assay of uronide bound to Zerolit DM-F de-ionising resin. Uronide was assayed by the carbazole/H_2SO_4 method first described by Bitter and Muir (1962) (11.2.(b)2.5).

(iv) Qualitative study of polysaccharides in supernatant after de-ionisation by high voltage electrophoresis on treated glass-fibre paper (TGFP) by the method described by Jarvis et al. (1977) (11.2.(a) 3.).

(v) In one experiment (see II.4 (d) below), hydrolysis of the polysaccharide spots obtained as in II.4.(c) (iv), thin-layer chromatography of the resulting

monosaccharide products by the method described by Menzies and Mount (1975) (II.2.(a) 1.) and quantitation of these monosaccharides by the densitometric method (II.2.(b) 2.1.).

II.4 (d) Analysis of polysaccharides in enzyme/cell wall incubation mixtures.

In a single experiment, polysaccharides obtained in the soluble fraction of enzyme/cell wall mixtures, before and after incubation, were subjected to electrophoresis on glass-fibre paper (II.2. (a) 3.) and hydrolysed on the paper and the monosaccharides thus produced were analysed by thin-layer chromatography (II.2 (a) 1). Enzyme solutions, prepared by acetone precipitation as described in II.4 (b) from both unripe and over-ripe fruit were used.

II.4 (d) 1. Preparation and treatment of samples for electrophoresis.

The incubation of cell wall suspensions with enzyme preparations of mango was carried out as described in II.4 (c) and the soluble fractions of the incubation mixtures de-ionised by the procedure described by Menzies and Mount (1975) (II.2. (a) 1.). After de-ionisation each sample was freeze-dried and the dry residue re-dissolved in water (100 µl).

II.4.(d) 2. Electrophoretic method (mixed electrophoresis and TLC).

Eight separate 2 µl applications of each sample prepared as in II.4.(d) 1. were made, four

adjacent applications on each of two treated glass-fibre strips, and electrophoresis carried out as described in II.2.(a) 3. One electrophoretogram on each of the two glass-fibre strips was stained with α-naphthol to act as a marker for the polysaccharide spots obtained and the remaining six electrophoretograms were cut up into zones corresponding to stained spots appearing on the marker electrophoretograms. The six cut-out zones for each electrophoretic spot were combined and the glass-fibre cut into fine pieces and placed into a wide tube with a quickfit neck. Polysaccharide on the glass-fibre paper was hydrolysed by addition of 4M trifluoroacetic acid (10 ml) and heating at 120° for 1 h with the tubes tightly sealed. After hydrolysis the trifluoroacetic acid containing the monosaccharide products of hydrolysis was transferred into a clean, identical tube and evaporated to dryness on a rotary evaporator under reduced pressure at 50°. The fragments of paper in each hydrolysis-tube were washed three times with methanol (4 ml), the washings transferred to the tube containing the appropriate dried acid-hydrolysate and concentrated to dryness at 50° under nitrogen. Residual trifluoroacetic acid was removed by storing overnight over KOH under reduced pressure.

The contents of each tube were re-dissolved in methanol (0.5 ml) and filtered through a 2 cm diam. Buchner filter under suction to remove small shreds of paper. The tube and filter were carefully washed twice with methanol (0.5 ml) and washings and filtrate combined

in 4 ml siliconised ('Repelcote') glass tubes. The filtered shreds of paper were further washed twice on the filter with methanol (1 ml) and these washings also combined with the filtrate. Combined solutions were concentrated to dryness at 50° under nitrogen and stored overnight over P_2O_5 and KOH under reduced pressure.

The dried residue in each tube was carefully re-dissolved by washing the entire siliconised surface of the tube with water (30 µl). Each sample (10 µl) was subjected to thin-layer chromatography by the method of Menzies and Mount (1975) (II.2. (a) 1.). The remaining 20 µl was freeze-dried.

Because the thin-layer chromatograms, obtained by the immediate chromatography of each sample (10 µl), were badly streaked, the residue left by the freeze-drying of the remaining 20 µl was subjected to a further de-ionisation. The freeze-dried residue left in each tube was dissolved in water (1 ml) and applied to a small column (7 x 0.6 cm) packed with Zerolit DM-F. The inside of the tube was washed twice with water (1 ml) and the washings also applied to the column. After a second washing the column was blown through to fully expel the washing. Eluate and washings were combined and subjected to a second de-ionisation by continuous inversion with Zerolit DM-F (1 ml packed volume) for 40 min in a sealed tube. The resin was removed by centrifugation, washed with water (2 x 1 ml) and the supernatants combined and freeze-dried in a siliconised glass tube.

The entire inside surface of each tube was

washed with a 10 µl bead of water and the 10 µl applied to a thin-layer chromatogram which was developed by the method previously described (II.2.(a) 1.) and quantitated by the densitometric method (II.2. (b) 2.1.).

II.4 (e) Study of uronide in enzyme/cell wall incubation mixtures which binds to Zerolit DM-F.

In this single experiment the enzyme preparation incubated with cell wall was prepared from over-ripe fruit by ammonium sulphate precipitation as described in II.4.(b). After incubation the cell wall was removed by centrifugation and millipore filtration and the supernatant (5 ml) was de-ionised with Zerolit DM-F (2.8 g) by the procedure of Menzies and Mount (1975) (II.2.(a) 1.). The resin was recovered by centrifugation, washed with water and bound uronide eluted with 0.5M H_2SO_4 (5 ml) as described in II.2.(b) 2.5.

An aliquot (2 ml) of the acid eluate (total volume 8.8 ml) was freeze-dried in a small glass tube. The dried residue was dissolved in water (1 ml) and applied to a small column (7 x 0.6 cm) packed with Dowex 50W (200-400 mesh), H^+ form. The tube was washed three times with water (1 ml) and the washings also applied to the column, the eluates being combined and freeze-dried in a small glass tube.

The dried residue was dissolved in water (100 µl) containing 20 µg rhamnose and 10 µl of this solution was applied to a thin-layer chromatogram and developed as described in II.2. (a) 1.

Galacturonic acid (2 µg in 10 µl) was applied separately as a standard. The remainder (90 µl) of each sample was incubated at 30° with 10 mM acetate buffer (1 ml), pH 4.0 containing purified polygalacturonase (100 µg), (Sigma), for 3 h. As controls, the polygalacturonase solution was incubated with buffer (90 µl) only and with 100 µg polygalacturonic acid in the same reaction volume. The reactions were stopped by immersion in an ice bath. Reaction mixtures were de-ionised with Dowex 50W (200-400 mesh), H^+ form, packed in small colums (7 x 0.6 cm) and freeze-dried in small glass tubes. Each dried residue was dissolved in water (105 µl) except for the two controls which were dissolved in water (105 µl) containing rhamnose (21 µg); 10 µl of each sample was then applied to a thin-layer plate and developed as described in II.2 (a) 1. Standards containing 1,2 and 5 µg of both galacturonic acid and rhamnose in 10 µl were separately applied. Chromatograms were quantitated as described in II.2 (b) 2.1.

II.4. (f) Experiment with enzyme extract made in the presence of Triton X-100.

Pooled, longitudinal slices of unripe and over-ripe mangoes were macerated, by the method described in II.4.(b), in 0.5M acetate buffer, pH 5.0 containing 0.5% Triton X-100. The macerate was sonicated, using six 30-second bursts of sonicator, with 30-second intervals between bursts, at 4°. After sonication, macerates were stirred for 2 h at 4°, and the pellet removed from the extract by centrifugation as described in II.4 (b). Using

β-D-galactosidase as a marker enzyme, extractions omitting Triton X-100 and sonication yielded 77% and 86%, for unripe and over-ripe fruit respectively, of the activities extracted when Triton X-100 and sonication were utilised.

The enzyme extracts in buffer containing Triton X-100 were dialysed against 0.1M acetate buffer, pH 5.0, as described in II.4. (b). Dialaysed extracts (8 ml) were incubated at 30° with 0.1M acetate buffer, pH 5.0 (12 ml) containing finely-divided whole cell wall material (40 mg). The rest of the experimental procedures were as described in II.4 (c).

II.5. Autolysis of Mango Macerates.

II.5 (a) Preparation of samples.

Mango mesocarp (5 g) at various ripening stages was macerated in 0.1M acetate buffer, pH 4.5 (25 ml) containing 0.04% sodium azide, using a glass homogeniser with a rotary teflon pestle. The macerate was dialysed against 0.1M acetate buffer, pH 4.5 (250 ml) containing thymol + 0.04% azide at room temperature for 24 h to remove free sugars. The dialysis bag and contents were then transferred to a beaker containing 250 ml fresh buffer (with thymol + 0.04% azide) and dialysed at room temperature for a further 5 days. The dialysate was then concentrated to dryness by rotary evaporation at 40° under reduced pressure.

Monosaccharides in the dried residue were converted to alditol acetates by the method described below (II.5.(b)). Alditol acetates were analysed by

gas-liquid chromatography as previously described (II.2. (a) 2). The single alditol acetate preparation from each sample was subjected to duplicate GLC assays.

II.5 (b) Preparation of alditol acetates from monosaccharides.

The procedure employed was based on that described by Jones and Albersheim (1972). To the dried residue containing monosaccharides (5-20 mg) was added 1M NH_4OH (1 ml) containing sodium borohydride (5 mg), inositol (1 mg) as internal standard for neutral sugars, and D-mannonic acid (1 mg) as internal standard for uronic acids and reduction allowed to proceed at room temperature for 1 h. D-Mannonic acid was prepared by evaporating to dryness at 50° by rotary evaporation, 1M NH_4OH (10 ml) containing D-mannono-1,4-lactone (18.2 mg) and taking up the dried residue in 1M NH_4OH (20 ml) containing sodium borohydride (100 mg) and inositol (20 mg). One millilitre of the resulting solution contained sodium borohydride (5 mg), inositol (1 mg) and D-mannonic acid (1 mg). The borohydride reduction converted neutral sugars to their corresponding alditols and uronic acids to their corresponding aldonic acids. At the end of the reduction, glacial acetic acid was added dropwise until effervescence ceased and the sample concentrated to dryness by rotary evaporation at 40°. Borate was removed by five consecutive additions of 10% acetic acid in methanol (1 ml) followed each time by rotary evaporation to dryness.

The dried material was extracted three times

with water (1 ml), the water washings combined, Dowex-1 resin (200-400 mesh), acetate form (0.5g) added and the mixture shaken vigorously for 1 h. The resin was removed by centrifugation and the supernatant aspirated into a small tube. The resin was washed twice with water (1 ml) followed by centrifugation, and the washings were combined with the first supernatant. This combined solution containing alditols was freeze-dried and stored over P_2O_5 under reduced pressure.

Aldonic acids bound to the resin were eluted by shaking vigorously with 1M HCl (2 ml) for 1 h. The resin was removed by centrifugation and the supernatant aspirated. The resin was washed twice with 1 M HCl (2 ml) and the washings aspirated and combined with the first supernatant. The combined solution containing aldonic acids was concentrated to dryness at 40° under a stream of nitrogen and stored over solid KOH under reduced pressure to remove residual HCl. Evaporation to dryness in the presence of HCl converted the aldonic acids to aldonolactones. These were converted to alditols by adding 10 mM sodium borate, pH 7.5 (1 ml) containing sodium borohydride(10 mg) and allowing reduction to proceed for 1 h at room temperature. Reduction was stopped by addition of glacial acetic acid, and borate removed as previously described. Dried samples were stored over P_2O_5 under reduced pressure.

Alditols were acetylated by addition of acetic anhydride (0.5 ml) and pyridine (0.5 ml) in a sealed tube and heating at 100° for 1 h. Samples were

concentrated to dryness and stored over P_2O_5 under reduced pressure to remove residual acetic anhydride and pyridine.

II.6. Extraction of Glycosidases from Mango Mesocarp.

II.6 (a) Method 1.

Pooled mango slices were macerated in 0.1M acetate buffer, pH 5.0 at 4° as described earlier II.4. (b)). A measured portion of the whole macerate was taken for estimation of enzyme activity. The remainder was centrifuged as described (II.4.(b)) and the clear supernatant removed for estimation of enzyme activity. These enzyme preparations were assayed on the day of preparation without dialysis.

II.6 (b) Method 2.

Pooled, longitudinal thin slices of mango mesocarp (100g) were macerated in acetone (500 ml) cooled to -20°, in a high speed blender using six 10 sec bursts. The macerate was quickly filtered through a cooled Buchner funnel and immediately washed with 1.5l acetone (-20°) and the solvent removed under reduced pressure. The dry acetone powder thus obtained was immediately macerated in 0.5M acetate buffer, pH 5.0 (100 ml) and stirred at 4° for 24 h. A measured volume of the re-suspended acetone powder was taken for estimation of enzyme activity after dialysis for 24 h against 0.1M acetate buffer (10 l),pH 5.0 at 4°. The remainder of the macerate was centrifuged as described and the supernatant similarly dialysed before being concentrated using an Amicon ultrafiltration cell with a

PM-10 membrane.

Glycosidase activities were assayed by the method described (II.2.(b) 3.2.). All glycosidase activities were shown to be linear under the assay conditions described (see Figs. 33-38).

II.6. (c) pH - Activity relationship for mango glycosidases.

Pooled, over-ripe mangoes were used in this study, utilising Method 1 (II.6. (a)) for extraction and further concentrating the enzyme extract by $(NH_4)_2SO_4$ (80%) precipitation at 4^{o}, collecting precipitated protein by centrifugation as previously described, re-dissolving the protein in 0.1M acetate buffer, pH 5.0 and dialysing against 10 l of the same buffer for 24 h at 4^{o}. The dialysed enzyme preparation (2 ml) was added to 8 ml of a series of buffers of varying pH to give final pH values across a range 2.7-7.5. The pH values of 3.6-6.4 were achieved using 0.1M acetate buffer; pH values below 3.6 and above 6.4 were achieved using McIlvaine buffer.

The appropriate p-nitrophenyl glycoside, 5 mM, (0.1ml) was incubated in duplicate with 0.9 ml of each buffered enzyme preparation at varying pH. Incubation time for β-D-galactosidase activity was 10 min, for α-D-mannosidase activity 30 min and for α-L-arabinopyranosidase activity 90 min. Enzyme activities were assayed by the method previously described (II.2.(b) 3.2.) pH/activity relationships for the 3 glycosidases assayed are shown in Figs. 39-41.

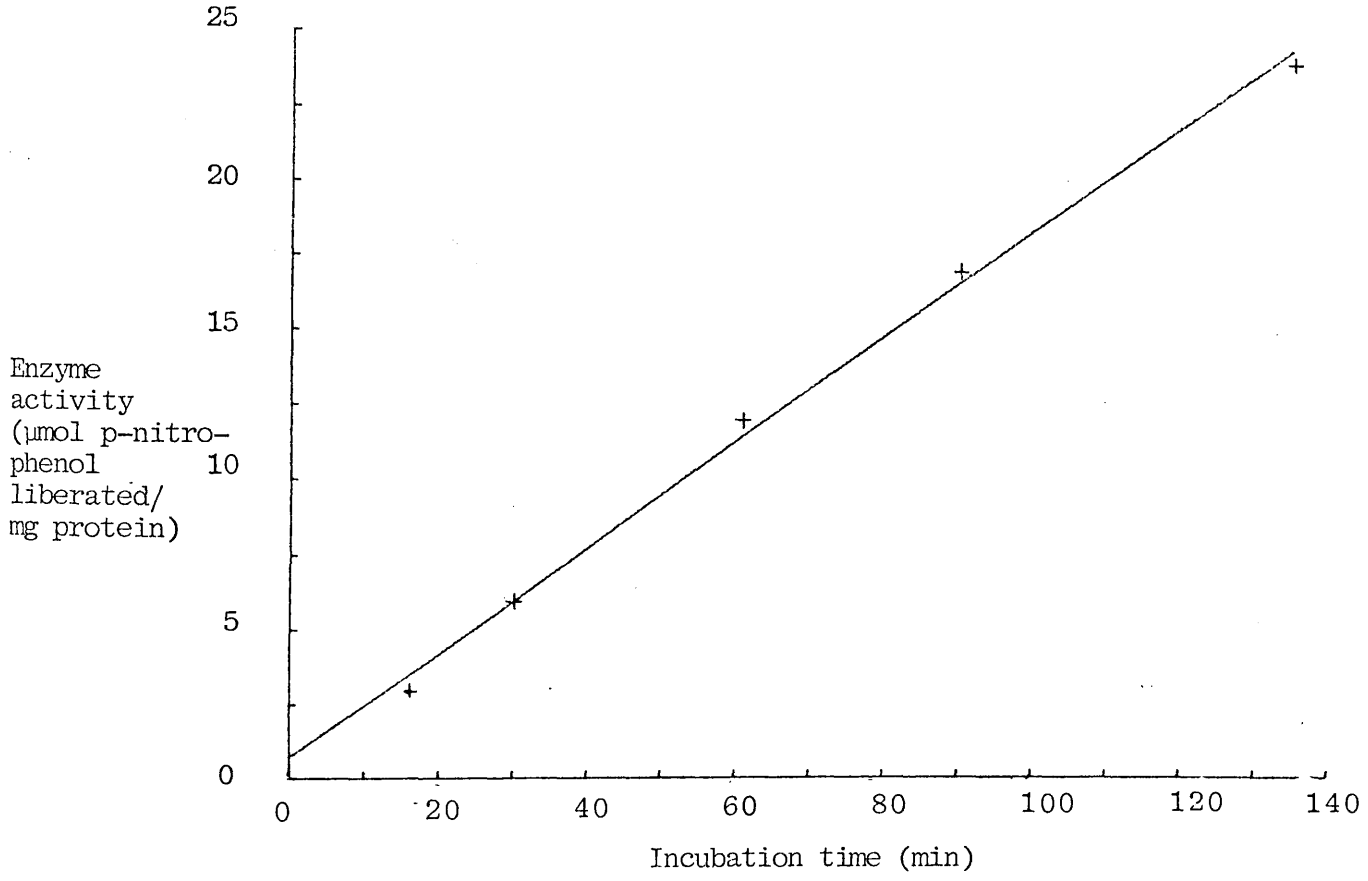


Fig. 33 Activity of β-D-glucosidase in extract of over-ripe mango mesocarp with 0.1M acetate buffer, pH 5.0.

Pooled tissue slices (25 g) from 3 mangoes were extracted with buffer (100 ml) as described (II.6.(a)) and β-D-glucosidase activity in the aqueous extract assayed by liberation of p-nitrophenol from p-nitrophenyl-β-D-glucopyranoside as described in II.2. (b) 3.2.

Each point shown is the mean of duplicate assays and the "line of best fit" is drawn with the aid of a microcomputer linked to a printer-plotter.
Gradient = 0.1762 with 95% confidence limits 0.1569 to 0.1954
Intercept = 0.7025 with 95% confidence limits -0.5766 to 1.9815
Correlation coefficient = 0.998

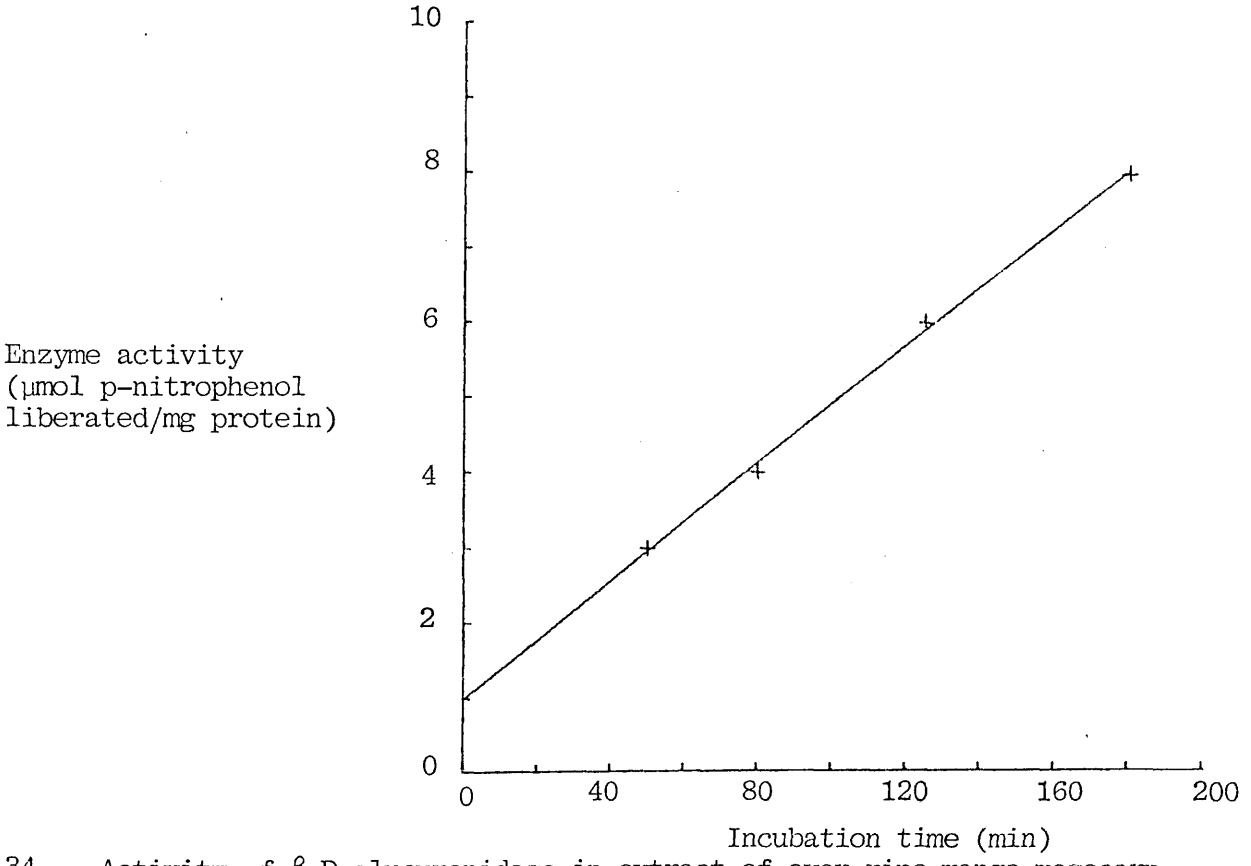


Fig.34 Activity of β-D-glucuronidase in extract of over-ripe mango mesocarp with 0.1M acetate buffer, pH 5.0

Pooled tissue slices (25 g) from 3 mangoes were extracted with buffer (100 ml) as described (II.6.(a)) and β-D-glucuronidase activity in the aqueous extract assayed by liberation of p-nitrophenol from p-nitrophenyl-β-D-glucopyranosiduronic acid as described in II.2.(b) 3.2. Each point shown is the mean of duplicate assays and the "line of best fit" is drawn with the aid of a microcomputer linked to a printer-plotter.

Gradient = 0.0391 with 95% confidence limits 0.0335 to 0.0447

Intercept = 0.9961 with 95% confidence limits 0.3880 to 1.6042

Correlation coefficient = 0.999.

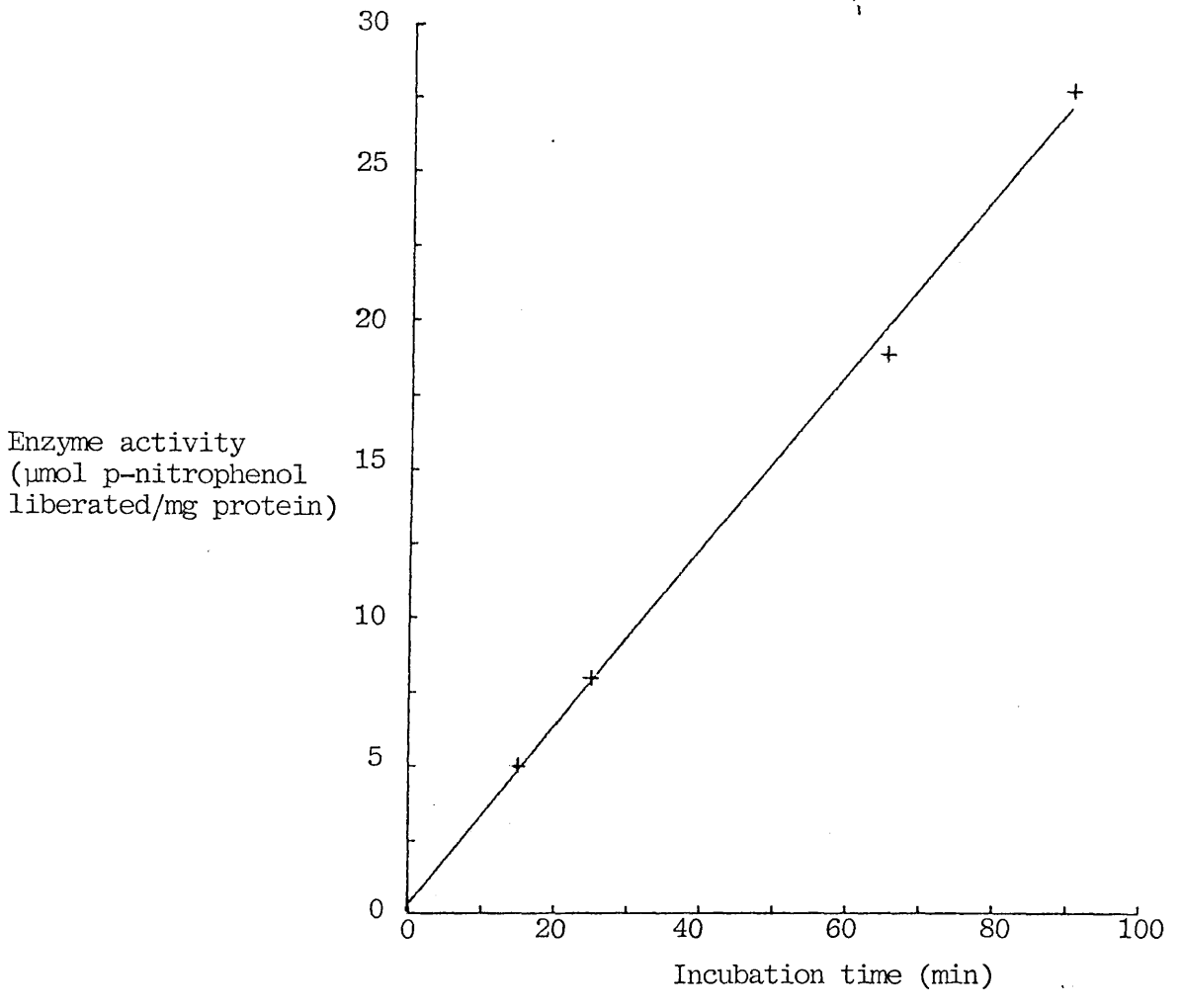


Fig.35 Activity of α-L-arabinopyranosidase extract of over-ripe mango mesocarp with 0.1M acetate buffer, pH 5.0.

Pooled tissue slices (25 g) from 3 mangoes were extracted with buffer (100 ml) as described (II.6.(a)) and α-L-arabinopyranosidase activity in the aqueous extract assayed by liberation of p-nitrophenol from p-nitrophenyl-α-L-arabinopyranoside as described in II.2.(b) 3.2.

Each point shown is the mean of duplicate assays and the "line of best fit" is drawn with the aid of a microcomputer linked to a printer-plotter.

Gradient = 0.3012 with 95% confidence limits 0.2466 to 0.3558

Intercept = 0.3169 with 95% confidence limits -2.3459 to 2.9796

Correlation coefficient = 0.998.

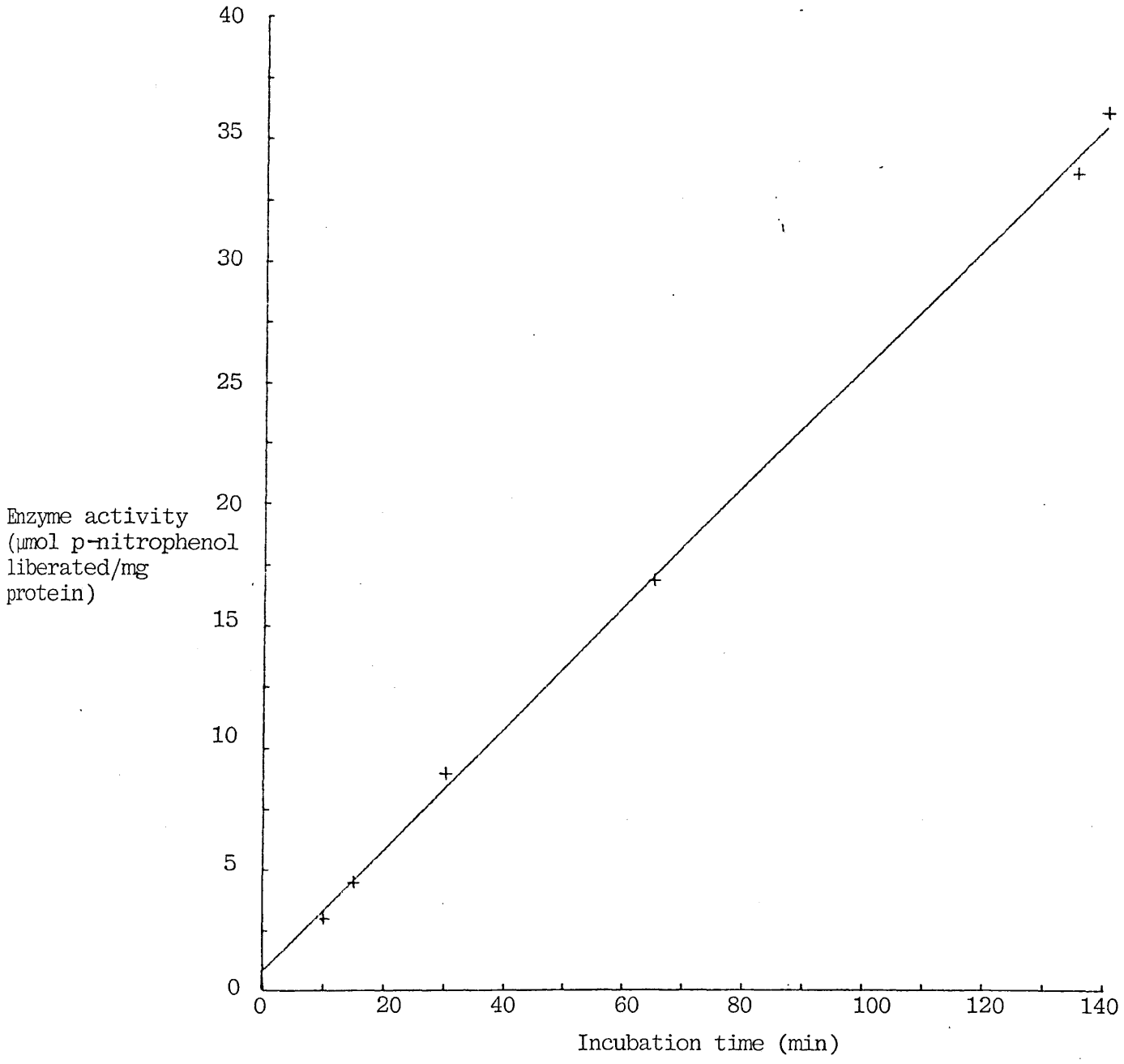


Fig. 36 Activity of α -D-galactosidase in extract of over-ripe mango mesocarp with 0.1M acetate buffer, pH 5.0

Pooled tissue slices (25g) from 3 mangoes were extracted with buffer (100 ml) as described (II.6.(a) and α-D-galactosidase activity in the aqueous extract assayed by liberation of p-nitrophenol from p-nitrophenyl-α-D-galactopyranoside as described in II.2.(b) 3.2.

Each point shown is the mean of duplicate assays and the "line of best fit" is drawn with the aid of a microcomputer linked to a printer-plotter.

Gradient = 0.2505 with 95% confidence limits 0.2382 to 0.2627.

Intercept = 0.8440 with 95% confidence limits 0.0392 to 1.6489.

Correlation coefficient = 0.999.

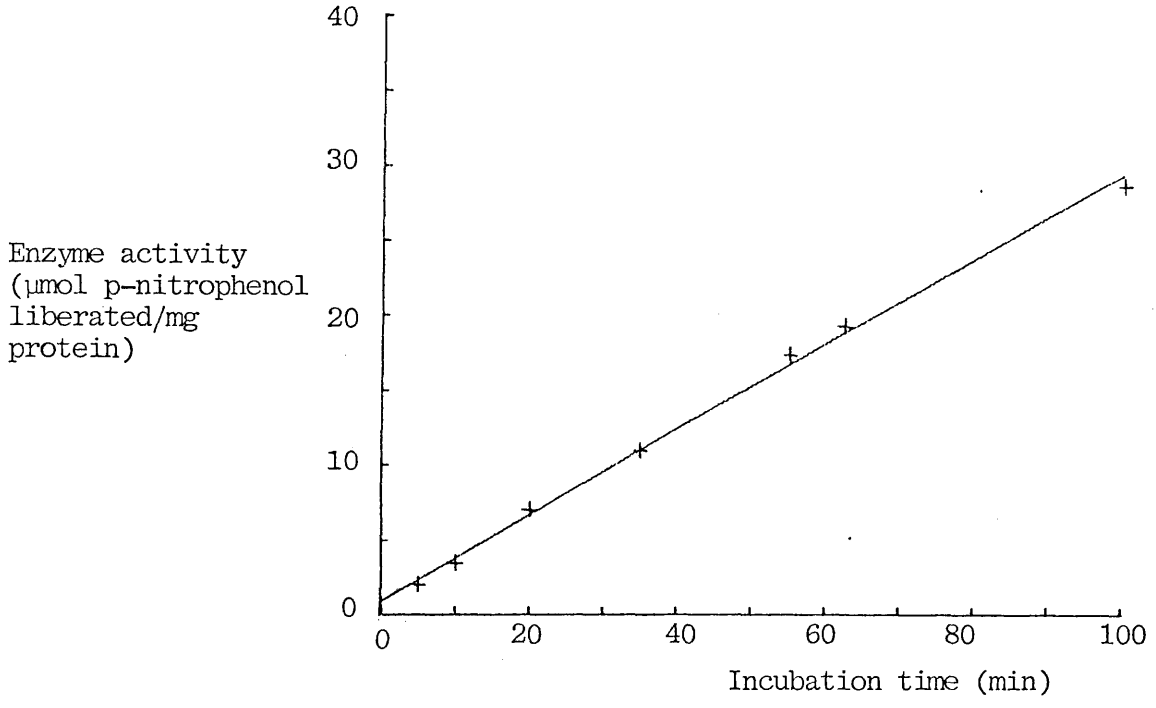


Fig. 37 Activity of α-D-mannosidase in extract of over-ripe mango mesocarp with 0.1M acetate buffer, pH 5.0.

Pooled tissue slices (25g) from 3 mangoes were extracted with buffer (100 ml) as described (II.6.(a)) and α-D-mannosidase activity in the aqueous extract assayed by liberation of p-nitrophenol from p-nitrophenyl-α-D-mannopyranoside as described in II.2.(b) 3.2.
Each point shown is the mean of duplicate assays and the "line of best fit" is drawn with the aid of a microcomputer linked to a printer-plotter.

Gradient = 0.2874 with 95% confidence limits 0.2693 to 0.3055.

Intercept = 0.9818 with 95% confidence limits 0.2365 to 1.7272.

Correlation coefficient = 0.998.

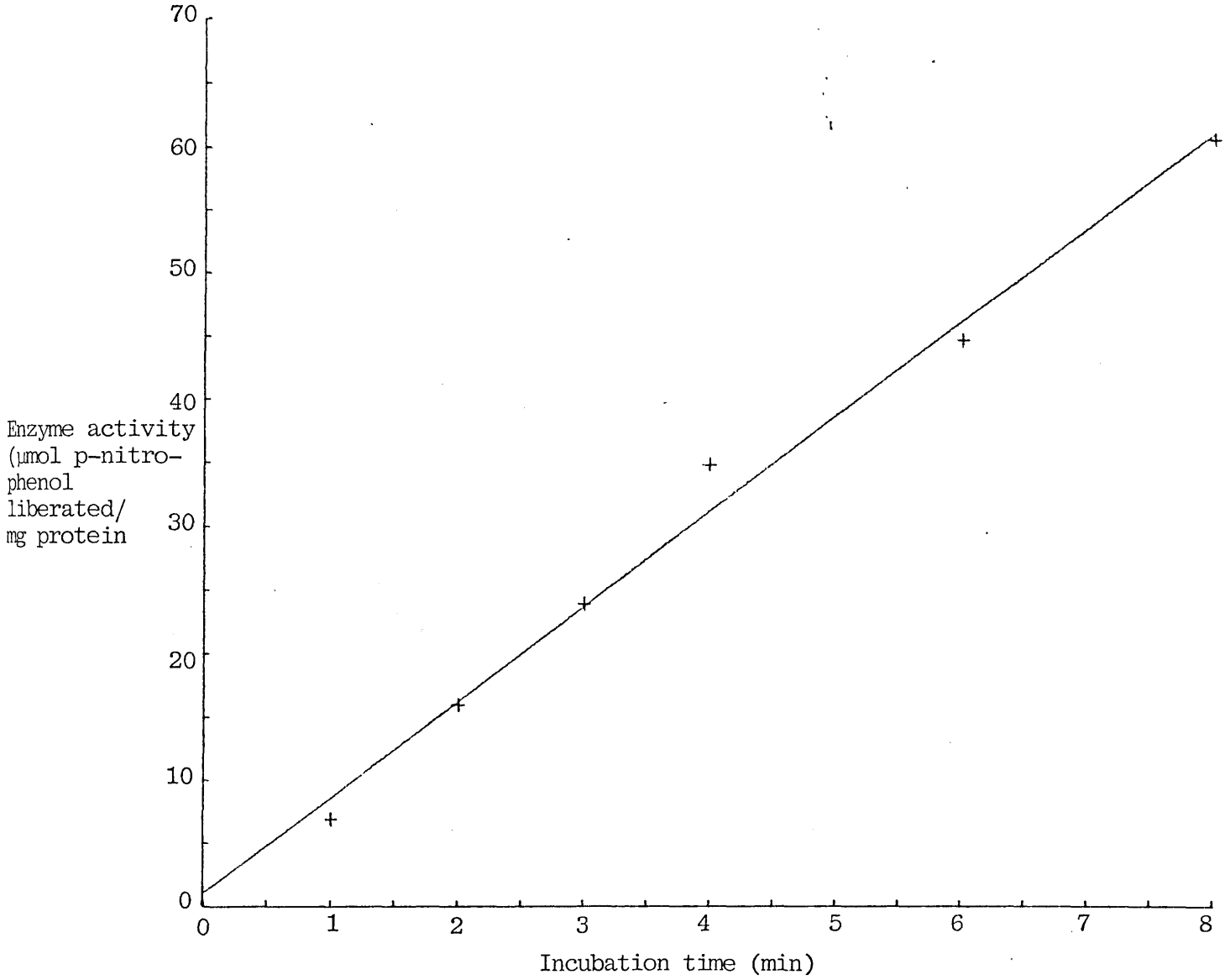


Fig. 38 Activity of β-D-galactosidase in extract of over-ripe mango mesocarp with 0.1M acetate buffer, pH 5.0.

Pooled tissue slices (25g) from 3 mangoes were extracted with buffer (100 ml) as described (II.6.(a)) and β-D-galactosidase activity in the aqueous extract assayed by liberation of p-nitrophenol from p-nitrophenyl-β-D-galactopyranoside as described in II.2.(b) 3.2.
Each point shown is the mean of duplicate assays and the "line of best fit" is drawn with the aid of a microcomputer linked to a printer-plotter.

Gradient = 7.5588 with 95% confidence limits 6.5290 to 8.5886

Intercept = 1.0980 with 95% confidence limits -3.0212 to 5.2172

Correlation coefficient = 0.995.

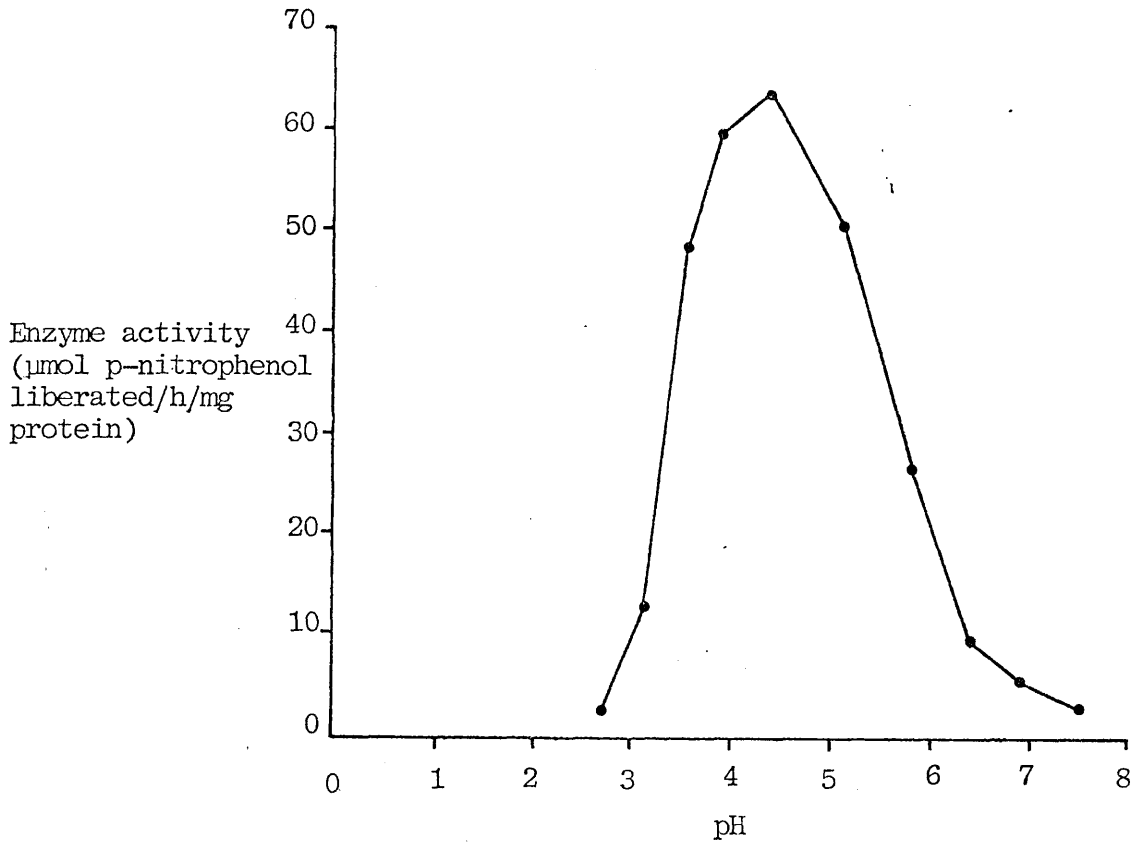


Fig. 39 Relationship between pH and activity of β-D-galactosidase in extract of over-ripe mango mesocarp with 0.1M acetate buffer, pH 5.0.

Pooled tissue slices (50g) from 4 mangoes were extracted with buffer (200 ml) as described (II.6.(a)) and protein in the extract was concentrated by $(NH_4)_2SO_4$ (80%) precipitation at 4^{o}. Dialysed protein was incubated with p-nitrophenyl-β-D-galactopyranoside adjusted to appropriate pH values and liberated p-nitrophenol assayed as described in II.2.(b) 3.2.

Each point shown is the mean of duplicate assays.

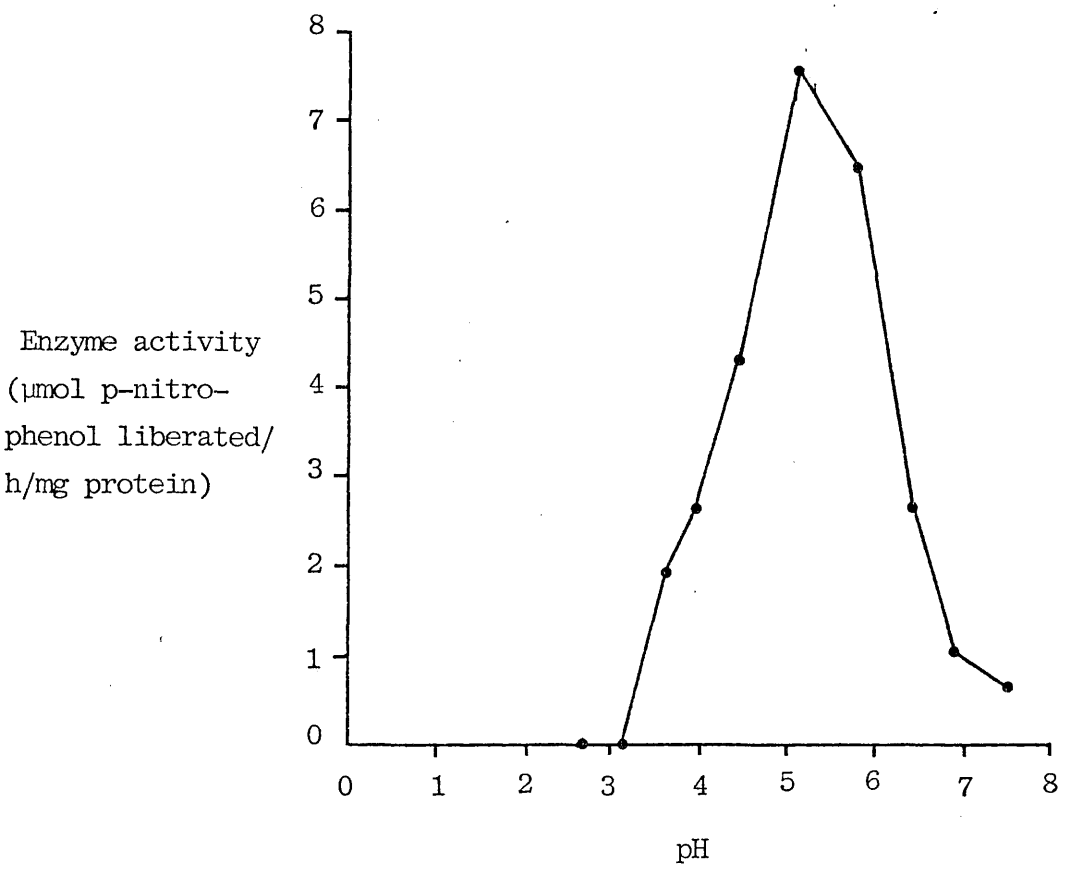


Fig. 40 Relationship between pH and activity of α-D-mannosidase in extract of over-ripe mango mesocarp with 0.1M acetate buffer, pH 5.0.

Experimental methods employed are described below Fig. 39.

Dialysed protein was incubated with p-nitrophenyl-α-D-mannopyranoside adjusted to appropriate pH values and liberated p-nitrophenol assayed as described in II.2.(b) 3.2.

Each point shown is the mean of duplicate assays.

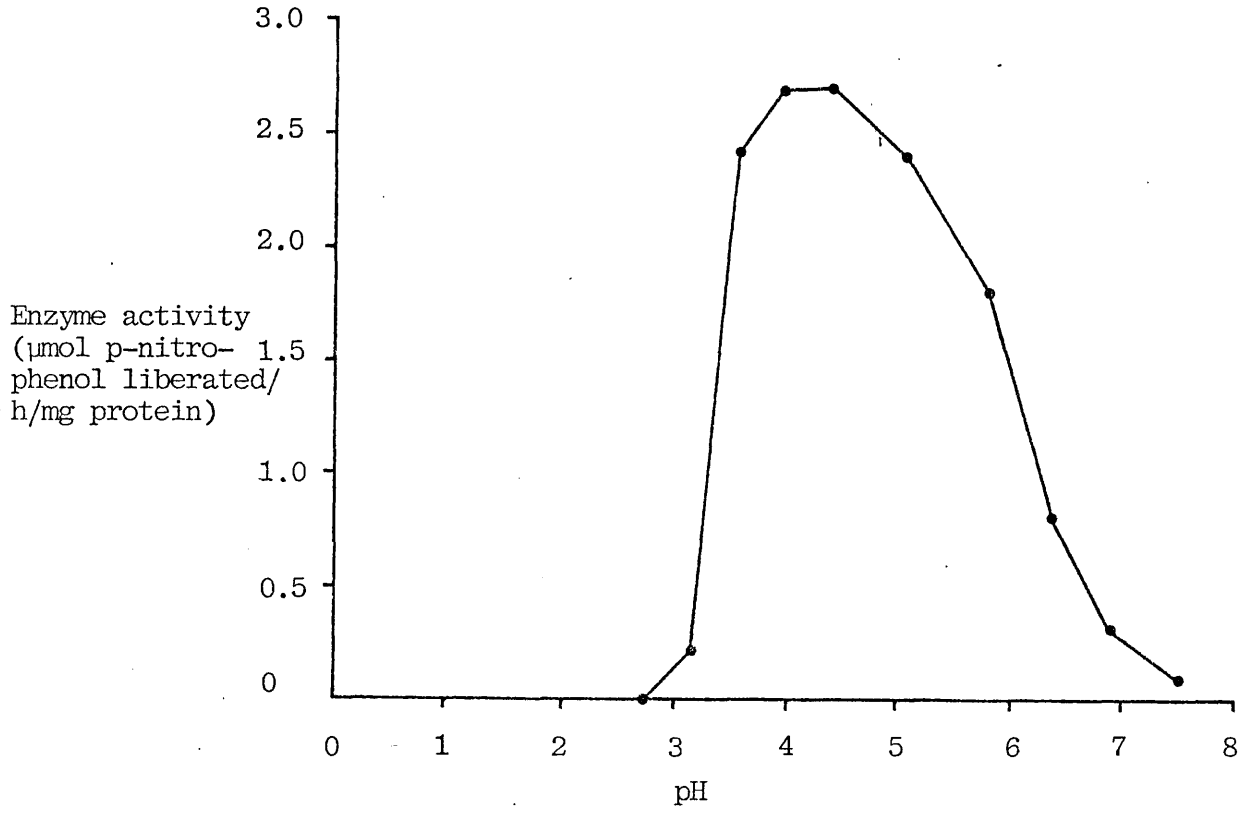


Fig .41 Relationship between pH and activity of α-L-arabinopyranosidase in extract of over-ripe mango mesocarp with 0.1M acetate buffer, pH 5.0.

Experimental methods employed are described below Fig. 39.

Dialysed protein was incubated with p-nitrophenyl-α-L-arabinopyranoside adjusted to appropriate pH values and liberated p-nitrophenol assayed as described in II.2. (b) 3.2.

Each point shown is the mean of duplicate values.

II.6. (d) Preparation of enzyme extract in the presence of polyvinylpyrrolidone.

Both unripe and over-ripe mangoes were macerated in the presence of polyvinylpyrrolidone (20 g) per 100 g tissue in 0.1M acetate buffer, pH 5.0 as previously described (II.6. (a)). Similar macerates were prepared in the absence of PVP. Glycosidase activities in the whole macerates were assayed by the method described (II.2. (b) 3.2.).

II.6. (e) Enzyme activities in various parts of stage 2 mangoes.

Stage 2(turning)mangoes often exhibit more advanced ripening on one side of the fruit than on the other. In single, stage 2 fruits, slices were cut from the ripe, yellowed side and slices cut from two separate portions of the unripe, green side. Separate macerates were made from these three sliced portions in 0.1M acetate buffer, pH 5.0 as previously described (II.6 (a)). Glycosidase activities in the whole macerates were assayed by the method previously described (II.2.(b). 3.2.).

II.7. ANALYSIS OF MANGO CELL WALLS.

II.7. (a) Preparation of materials.

II.7. (a) 1. Cell Walls.

Cell wall material from mangoes at ripening stages 1-4 prepared by the method described earlier (II.4. (a)).

II.7. (a) 2. Removal of starch from cell wall material.

In some experiments removal of starch from dried cell wall material was attempted utilising a method described by Knee (1973c). Cell wall material (2 g) was suspended in a solution of chloral hydrate (350 g) in water (98ml) and left to stand at room temperature for 48 hours. Water (250 ml) was then added and the mixture filtered on a sintered glass funnel under suction and further washed on the filter with water (500 ml), ethanol (500 ml), acetone (500 ml) and ether (500 ml). Cell wall material was allowed to dry on the filter and stored over P_2O_5 under reduced pressure before weighing.

After chloral hydrate treatment cell wall material was tested for the presence of starch by the iodine/potassium iodide method. Cell wall material (10 mg) was suspended in water (5 ml) and heated for 1 h at 100^{o}. Solid cell wall was removed by centrifugation and the supernatant aspirated; supernatant (2 ml) was added to 0.005M iodine in 0.1M potassium iodide (1 ml). The iodine/potassium iodide solution (1 ml) was also added to the solid cell wall residue.

II.7. (a) 3. Preparation of various polysaccharide fractions from cell wall material.

Dry cell wall material (2 g) was extracted with water (150 ml) for 6 h at 100^{o}. The extract was filtered while hot and the residue washed with hot water (175 ml) on a sintered glass funnel under suction. Filtrate

and washings were combined. The residue was washed with acetone (500 ml) at room temperature and allowed to dry overnight on the filter at 4^{o}. Materials in the filtrate were recovered by freeze-drying, dried over P_2O_5 under reduced pressure and weighed.

The cell wall residue, after hot water extraction, was treated with 4M KOH (25 ml) under a flow of nitrogen and with occasional shaking for 2 h at room temperature. The alkali extract was filtered and washed with 4M KOH (20 ml) and then with water (2 x 20 ml) on a sintered glass funnel directly into glacial acetic acid to give pH 7.0 for the combined filtrate and washings. Absolute ethanol was added to this (80% v/v) and the mixture allowed to stand overnight at 4^{o} to ensure full precipitation of the polysaccharides. The precipitate was isolated, washed consecutively with ethanol (400 ml), acetone (400 ml), and ether (400 ml), dried over P_2O_5 under reduced pressure and weighed.

The residue remaining on the sintered glass funnel was immediately washed with water (500 ml), ethanol (300 ml), acetone (300 ml) and ether (300 ml), dried over P_2O_5 under reduced pressure and weighed.

II. 7. (b) Hydrolysis of polysaccharides.

Cell wall material or extracted fractions of cell wall material were subjected to the procedure described below:

The sample (9-12 mg) with added inositol (1 mg) as an internal standard for neutral sugars was heated in a sealed

tube with 4M trifluoroacetic acid (2 ml) for 2 hours at 120°. The contents of the tube were concentrated to dryness by rotary evaporation at 40° under reduced pressure and allowed to stand overnight over solid KOH under reduced pressure in a desiccator to remove residual trifluoroacetic acid.

In some experiments polysaccharides were hydrolysed by a 2-stage procedure - first stage hydrolysis with trifluoroacetic acid - second stage hydrolysis with a mixture of polysaccharide-degrading enzymes as described below:

The sample, after hydrolysis with and removal of trifluoroacetic acid, was suspended in 10 mM acetate buffer, pH 4.5 (2 ml) containing 100 µg of each of three enzymes; cellulase (technical type 1), hemicellulase (crude, fungal) and polygalacturonase (purified,fungal). All enzyme mixtures were dialysed overnight at 4° against the same buffer before use. The sample was incubated with the enzyme mixture at 30° for 6 h.

In order to rule out contribution of monosaccharides from carbohydrate impurities in the enzyme mixtures used, "enzyme only" blanks were treated under identical experimental conditions. These blanks contained only the polysaccharide-degrading enzyme mixture, without cell wall, and were incubated alongside samples containing cell wall material. No alditol acetate peaks appeared on gas-liquid chromatograms (see II.2.(a) 2.) derived from these blanks subjected to the derivatisation

procedure (see II.5. (b)) confirming that the enzyme mixtures contributed little or no extraneous monosaccharides to the products of hydrolysis.

II.7. (c) Analysis of monosaccharides produced by hydrolysis of cell wall materials.

Monosaccharides were converted to alditol acetates as described earlier (II.5. (b)). Alditol acetates were analysed by gas-liquid chromatography (see II.2.(a) 2). The single alditol acetate preparation from the hydrolysate of each cell wall or polysaccharide sample was subjected to duplicate GLC assays.

II.8. STATISTICAL METHODS (see Moroney (1965); Snedecor and Cochran (1967)).

II.8. (a) Calculation of sample standard deviation and standard error of mean.

In calculating these estimators, methods appropriate for small samples when sampling from a normally distributed population were used.

The sample standard deviation (denoted by s) of the mean ($\bar{X}$) of a sample containing n items is given by the formula:

$$s = \sqrt{\frac{\Sigma(X-\bar{X})^2}{n-1}}$$

First, each deviation $(X-\bar{X})$ is squared. Next the sum of squares, $\Sigma(X-\bar{X})^2$, is divided by (n-1), one less than the sample size. The result is the mean square or sample variance (s^2). Finally, the extraction of the

square root yields the sample standard deviation (s).

The standard error of the mean is given by $\frac{s}{\sqrt{n}}$

II.8. (b) Calculation of values of Student's t.

In dealing with small samples it can not be assumed that the sample variance is an unbiased estimate of the population variance. Account is taken of the bias in small samples, applying Bessel correction, and referring the resultant ratio to Student's t distribution.

In calculating values of t for testing significance of difference between the means of two samples:

$$t = \frac{\text{Difference of Means}}{\text{Standard Error of Difference}}$$

Assume that sample 1 (containing n_1 items) has mean $\bar{X}_1$ and sample standard deviation s_1 and that sample 2 (containing n_2 items) has mean $\bar{X}_2$ and sample standard deviation s_2.

The first step, in applying Bessel correction, is to make a pooled estimate of the variance ($\hat{\sigma}^2$) - on the Null Hypothesis that the two samples are drawn from populations identical both as to mean and variance:

$$\hat{\sigma}^2 = \frac{n_1 s_1^2 + n_2 s_2^2}{n_1 + n_2 - 2}$$

from which $\hat{\sigma}$ is found by extracting the square root.

The best estimate of the standard error ($\hat{\sigma}_w$) for the difference of the means of the two samples is given

by

$$\hat{\sigma}_w = \hat{\sigma}\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

so that:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\hat{\sigma}_w}$$

The calculated value of Student's t is referred to tables of Student's t with number of degrees of freedom equal to $n_1 + n_2 - 2$ to establish the significance of difference (if any) between the two means.

II.8. (c) Calculation of product moment correlation coefficient.

The degree of correlation between sets of x,y co-ordinates is measured by the so-called product moment correlation coefficient (r) which is given by:

$$r = \frac{1}{N}\sum\left(\frac{x-\bar{x}}{\sigma_x}\right)\left(\frac{y-\bar{y}}{\sigma_y}\right)$$

where N = number of x,y co-ordinates, $\bar{x}$ and $\bar{y}$ are respectively the mean of all the x values and the mean of all the y values and σ_x and σ_y are respectively the standard deviations of all the x values and all the y values.

It may be shown that the correlation coefficient (r) cannot exceed +1 or be less than -1 in value. A value of +1 denotes perfect functional relationship between y and x, an increasing x being associated with an increasing y. When r is equal to -1, we again have a perfect functional relationship, but this time an increasing x is associated with a decreasing y. When $r = 0$, there is no relation at all between x and y, they are not correlated. Other

intermediate values of r indicate that, while there is not a strictly functional relationship between the variables, there is a trend. If the coefficient is positive, increasing x tends to be associated with increasing y, while, if the coefficient is negative, increasing x tends to be associated with decreasing y.

It may also be shown that it does not matter what value of x or y we choose as our origins of measurement, nor what units the quantities x and y are measured in. The value of r remains constant for a given distribution throughout all changes of this type.

In the present work a BBC microcomputer with disc software was programmed to calculate correlation coefficient from input x,y co-ordinates.

II.8. (d) Obtaining straight lines of "best fit" for x,y co-ordinates.

If two quantities, y and x, are related by a straight line law, the equation expressing the relationship will be of the form

$$y = mx + c$$

where m is the parameter expressing the slope of the line (that is, by how much y increases for an increase of unity in the value of x) and c is the parameter (intercept) which tells at what value the straight line cuts the axis of y. Wherever there is a straight line trend between two quantities y and x, it is possible to find values for the two parameters m and c, which give the equation of the straight line which is the best fit to the points in the

graph. There is always some straight line which is a better fit than all other straight lines.

The generally-accepted criterion for "best fit" is the "least squares" one. The equation for the line of best fit is that which gives a minimal sum of squares of the discrepancies between values of y predicted by this equation and observed values of y, for various values of x.

The mathematically most straightforward procedure for finding the values of m and c which make the sum of the squares of these discrepancies as small as possible, utilising the observed values of x and y is:

$$m = \frac{\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{N}}{\Sigma x^2 - \frac{(\Sigma x)^2}{N}}$$

and

$$c = \frac{(\Sigma x)(\Sigma xy) - (\Sigma y)(\Sigma x^2)}{(\Sigma x)^2 - N(\Sigma x^2)}$$

where

N = number of x,y co-ordinates

Σx = summation of x values

Σy = summation of y values

Σxy = summation of xy values for various values of x

Σx^2 = summation of x^2 values

The equation relating y to x may also be found from:

$$y - \bar{y} = r\left(\frac{\sigma_y}{\sigma_x}\right)(x-\bar{x})$$

where $\bar{x}$ and $\bar{y}$ are respectively the mean of all the x values and the mean of all the y values, σ_x and σ_y are respectively the standard deviations of all the x values and all the y

values and r is the calculated correlation coefficient.

In the present work a BBC microcomputer with disc software was programmed to calculate values of m and c in the equation giving the straight line of best fit for input x,y co-ordinates. A Radio Shack TRS-90 printer/plotter peripheral linked to the microcomputer plotted points and drew the line of best fit for each set of co-ordinates.

II.8. (e) Calculation of confidence limits on gradient and intercept of straight lines of best fit.

II.8. (e) 1. Gradient.

First each value of X is substituted in the equation of the line to give a corresponding value $\hat{Y}$. At each value of X the deviation from regression $(d_{y \cdot x}) = Y - \hat{Y}$ (i.e. the observed value of Y minus the value of Y predicted from the equation of the line) is found. Each deviation is squared and the squares summed to give $\Sigma d_{y \cdot x}^2$. The mean square deviation from regression $s_{y \cdot x}^2$ is given by:

$$s_{y \cdot x}^2 = \frac{\Sigma d_{y \cdot x}^2}{(n-2)}$$ where n = number of paired co-ordinates

and the sample standard deviation from regression $(s_{y \cdot x})$ is the square root.

Σx^2 is found by finding the mean $(\overline{X})$ of the X values, calculating the deviation from the mean $(x = X - \overline{X})$ for each value, squaring each and summing the squares. The sample standard deviation of the regression coefficient (s_b) is then given by:

$$s_b = \frac{s_{y \cdot x}}{\Sigma x^2}$$

The numerical values of the confidence limits on the gradient (M) of the line are given by $M \pm t.s_b$, the appropriate value of t at (n-2) degrees of freedom being found from a table showing values of t for calculating confidence limits at the 95% level.

II.8. (e) 2. Intercept

Once the confidence limits on the gradient are known the confidence limits on the intercept on the Y axis are easily calculated.

$\overline{X}$ = mean of the X values

$\overline{Y}$ = mean of the Y values

$M_u = M + t.s_b$; upper confidence limit on gradient

$M_l = M - t.s_b$; lower confidence limit on gradient

If C_u is upper confidence limit on intercept and C_l is lower confidence limit, then:

$$C_u = \overline{Y} - M_l\overline{X} \quad \text{and}$$

$$C_l = \overline{Y} - M_u\overline{X}$$

In the present work a Commodore Series 4000 microcomputer with disc software was programmed to calculate 95% confidence limits on both gradient and intercept of the "best fit" straight line obtained for input x,y co-ordinates.

III. RESULTS.

III.1. The analysis of mango cell walls at various stages of ripeness.

III.1. (a) Yield of cell wall materials from tissue at various stages of ripeness.

The recovery of cell wall material from pooled mango tissues at 4 different stages of ripeness is shown in Table 4. The attempt was made to remove starch by treatment of the dry wall material with chloral hydrate solution (a method applied to apple cell walls by Knee (1973c)). Although the data in Table 4 indicate that material (varying from 23% of the dry weight of the initial wall preparation in unripe fruit to 42% in turning fruit) was extracted in chloral hydrate from walls derived from tissue at all four stages of ripeness, only over-ripe walls were found to be starch-free after this treatment, as revealed by testing of the walls with iodine/KI solution. Hence chloral hydrate treatment was apparently unsuccessful in the case of the mango.

The variation between figures obtained in the two separate experiments (eg. 2.40g and 1.84g respectively, for dry weight of chloral hydrate-extracted wall from unripe fruit) was probably the result of difficulty in controlling gravimetric losses of material during the prolonged and tedious preparative procedures. However, despite this variation, overall Table 4 indicates a decline in total "cell wall" (chloral hydrate-extracted) material during ripening . Mangoes do not change in size nor does the water content of the mesocarp change

TABLE 4. Recovery of cell wall material and cell wall polysaccharide fractions from mango mesocarp at various stages of ripening.

Stage of Ripening	Experiment	Wet weight of mango tissue (g)	Dry weight of wall material before CHE* (g)	Dry weight of wall material after CHE* (g)	Dry weight of water-soluble fraction (g)	Dry weight of KOH-soluble fraction (g)	Dry weight of residue (g)
Unripe	1	100	2.79	2.40			
	2	100	2.71	1.84	0.40	0.27	0.40
Turning	1	100	2.78	2.04			
	2	100	2.45	0.97	0.38	0.17	0.17
Ripe	1	100	1.41	1.01			
	2	100	1.45	1.03	0.19	0.28	0.29
Over-ripe	1	100	1.05	0.62			
	2	100	1.34	0.98	0.09	0.20	0.36

The results shown above are from 2 separate experiments in both of which pooled mango tissue from 3 separate fruits at each ripening stage was used. The chloral hydrate-extracted wall material was fractionated in experiment 2 only. Ripening stages are defined in Section II.1.(b).

The procedure for preparation of wall materials from mango tissue is described in II.4.(b); the procedures for extraction with chloral hydrate and fractionation of wall materials are described in II.7.(a) 2. and II.7.(a) 3. respectively.

* Chloral hydrate extraction.

during ripening. Stability of the water content was established by taking pooled tissue slices from 3 separate fruits at each of the 4 ripening stages and dehydrating in an oven at 110°. The water contents (% by weight) of the tissues were: unripe, 89.8 (± 0.9); turning, 89.4 (± 0.1); ripe, 89.4 (± 0.9) and over-ripe, 91.4 (±0.4). Each of these figures represents the mean of 4 separate determinations and standard errors of means are given in parenthesis. Since mangoes do not change in size/water content during ripening, wet weight of mesocarp tissue (100g) is a reasonable measure of sample volume of tissue taken for "cell wall" gravimetric determination, so that data in Table 4 indicate a true decline in total "cell wall" content of the mesocarp tissue during ripening. The yield of chloral hydrate-extracted wall material from over-ripe tissue was only approximately 40% of that from unripe tissue, taking the average of the figures obtained from the 2 experiments carried out.

The product from unripe and turning fruit was white and finely divided whereas wall material from ripe and over-ripe fruit retained a faint yellow/brown coloration which may have been due to oxidised phenolic compounds or adsorbed carotenoid pigments.

In one of the two experiments performed (exp.2 in Table 4) the chloral hydrate-extracted cell wall material at each stage of ripeness was fractionated into hot water-soluble, alkali-soluble and residual fractions. Only about 70% by weight of the chloral hydrate-extracted

wall was recovered in the combined fractions; some losses presumably occurred during the preparative procedures. There was a marked decrease in the yield of the hot water-soluble fraction with increasing ripening suggesting that loss of hot water-soluble polymers makes a major contribution to the overall decline in cell wall content of the tissue which accompanies ripening. The results also suggest a more modest decline in the amount of the insoluble residue during ripening.

III.1. (b) Monosaccharide composition of hydrolysates of cell wall material obtained from mangoes at various stages of ripeness.

In order to test the reproducibility of the procedures employed for hydrolysis of cell wall preparations and subsequent conversion of liberated monosaccharides to alditol acetates which were quantitated by gas-liquid chromatography, separate cell wall preparations obtained from single fruits at 2 different ripening stages were each subjected to 3 separate hydrolyses/monosaccharide analyses using the combined acid and enzymic hydrolysis procedure. The results obtained are shown in Table 5. It can be seen that monosaccharide data obtained were generally reproducible, although there were some variations, especially of the two minor constituents, fucose and rhamnose. Presumably, the multiple stages in both the cell wall hydrolysis and derivatisation procedures account for these variations.

The effectiveness of two different hydrolytic

TABLE 5. Recoveries of monosaccharides from mango cell wall preparations subjected to repeated analyses. (The combined acidic and enzymic hydrolysis procedure was used.

Monosaccharide	Mass (mg) of monosaccharide released by hydrolysis from 10 mg (dry weight) cell wall preparation. Unripe cell wall (Stage 1)				Turning cell wall (Stage 2)			
	Analysis 1	Analysis 2	Analysis 3	Mean (S.E.)	Analysis 1	Analysis 2	Analysis 3	Mean (S.E.)
Rhamnose	0.05	0.06	0.06	0.06 (0.004)	0.01	0.05	0.04	0.03 (0.01)
Fucose	0.11	0.15	0.15	0.13 (0.01)	0.04	0.09	0.09	0.07 (0.02)
Arabinose	0.61	0.68	0.69	0.66 (0.02)	0.64	0.93	0.75	0.77 (0.08)
Xylose	0.27	0.33	0.33	0.31 (0.02)	0.14	0.17	0.15	0.15 (0.01)
Galactose	0.36	0.40	0.39	0.38 (0.01)	0.24	0.36	0.30	0.30 (0.03)
Glucose	3.58	3.49	3.34	3.47 (0.07)	2.90	3.85	2.63	3.13 (0.37)
Galacturonic acid	0.93	1.03	1.16	1.04 (0.07)	0.94	0.72	0.79	0.82 (0.07)
Total monosaccharides	5.91	6.12	6.11	6.04 (0.07)	4.92	6.16	4.77	5.28 (0.44)
% (by weight) of cell wall obtained in total monosaccharides after hydrolysis.	59%	61%	61%	60% (1%)	49%	62%	48%	53% (4%)

The cell wall samples were prepared from single mano fruits at each ripening stage and extracted with chloral hydrate. In the 3 separate analyses a separate portion (10 mg) of the cell wall preparation was hydrolysed first with trifluoroacetic acid ($2cm^3$). Each of the 3 acid hydrolysates (after drying) was then further hydrolysed with a separately prepared mixture of polygalacturonase, hemicellulase and cellulase in 10mM acetate buffer, pH 4.5 ($2cm^3$). The dried hydrolysates were converted to alditol acetates as described in II.5.(b) and analysed by glc. as described in II.2.(a)2.Each data item shown is the mean of duplicate glc assays of a single alditol acetate sample. The mean of the 3 separate analyses (n = 3) for each cell wall preparation is shown in the table with standard error of the mean (S.E.) in parenthesis.

procedures for cell walls, trifluoroacetic acid alone and similar acid hydrolysis followed by a mixed enzymic digestion, was compared. The cell walls used in this experiment were not extracted with chloral hydrate. The results obtained are shown in Table 6. In general the two-step hydrolytic method released more monosaccharide than acid by itself and, in particular, arabinose and galacturonic acid. The two-step hydrolytic procedure was used for all subsequent hydrolyses.

In all hydrolysis studies with intact cell wall preparations, both those not extracted with chloral hydrate (Table 6) and those subjected to chloral hydrate extraction (Table 7), the yields of monosaccharides from unripe walls were significantly higher than those from the more mature walls, suggesting that unripe walls contain higher levels of readily-hydrolysable polysaccharides than walls from ripening fruit.

There is some variation between the data from individual monosaccharides in Tables 6 and 7 where the 2-stage hydrolysis has been used. This is probably due to some physiological variation in the fruits selected at each ripening stage. The quantitatively significant monosaccharides present in the wall preparations analysed were glucose, galactose, arabinose, xylose and galacturonic acid.

In both walls extracted with chloral hydrate (Table 7) and those not chloral hydrate-extracted (Table 6), the levels of arabinose, galactose and glucose in cell wall preparations declined during ripening. Statistically

TABLE 6. Determination of monosaccharides in hydrolysates of cell wall preparations from mango mesocarp at various stages in ripening using different hydrolysis procedures.

Monosaccharide	Mass (mg) of monosaccharide released by hydrolysis from 10 mg (dry weight) cell wall preparation.					
	ACID HYDROLYSIS ONLY			ACID HYDROLYSIS FOLLOWED BY ENZYMIC HYDROLYSIS		
	Unripe	Ripe	Over-ripe	Unripe	Ripe	Over-ripe
Rhamnose	0.01	0.03	0	0.01	0.02	0
Fucose	0.20	0.09	0.16	0.18	0.11	0.13
Arabinose	0.87	0.28	0.31	1.32	0.29	0.29
Xylose	0.43	0.29	0.52	0.33	0.31	0.45
Galactose	1.14	0.18	0.40	1.02	0.26	0.35
Glucose	1.12	0.94	0.25	1.13	1.40	0.21
Galacturonic acid	0.38	0.16	0.40	1.34	0.95	1.12
Total monosaccharides	4.15	1.96	2.04	5.33	3.34	2.55
% (by weight) of cell wall obtained in total monosaccharides after hydrolysis	42%	20%	20%	53%	33%	26%

The cell wall samples were prepared from single mango fruits at each ripening stage (not extracted with chloral hydrate). The hydrolysis procedures are described in II.7.(b). The dried hydrolysates were converted to alditol acetates as described in II.5 (b) and analysed by glc as described in II.2.(a) 2.

Each data item shown is the mean of duplicate glc assays of a single alditol acetate sample.

TABLE 7. Determination of monosaccharides in hydrolysates of cell wall preparations from mango mesocarp at various stages in ripening. (The combined acidic and enzymic hydrolysis procedure was used).

Monosaccharide	Mass (mg) of monosaccharide released by hydrolysis from 10 mg (dry weight) cell wall preparation							
	Unripe		Turning		Ripe		Over-ripe	
Rhamnose	0.02	(0.02)	0.02	(0.005)	trace		0	
Fucose	0.15	(0.07)	0.09	(0.01)	0.05	(0.002)	0.10	(0.01)
Arabinose	0.95	(0.19)	0.92	(0.13)	0.30	(0.09)	0.20	(0.02)
Xylose	0.28	(0.05)	0.19	(0.02)	0.25	(0.05)	0.31	(0.01)
Galactose	0.67	(0.16)	0.53	(0.16)	0.17	(0.05)	0.22	(0.01)
Glucose	2.24	(0.75)	2.37	(0.67)	0.64	(0.32)	0.13	(0.04)
Galacturonic acid	0.93	(0.07)	0.86	(0.04)	1.43	(0.20)	1.06	(0.07)
Total monosaccharides	5.23	(0.31)	4.98	(0.12)	2.84	(0.24)	2.01	(0.11)
% by weight of cell wall obtained in total monosaccharides after hydrolysis.	52%	(3%)	50%	(1%)	28%	(2%)	20%	(1%)

The results shown above represent the mean of 3 experiments in which cell wall material was prepared from different single fruits at each ripening stage in each experiment. Wall preparations were extracted with chloral hydrate. The hydrolysis procedures are described in II.7.(b). The dried hydrolysates were converted to alditol acetates as described in II.5.(b) and analysed by glc as described in II.2.(a) 2. Single alditol acetate samples prepared from each wall hydrolysate were assayed by glc in duplicate .
Each data item shown is the mean of the 3 values (each value itself the mean of duplicate glc assays) obtained from the 3 separate experiments (i.e. $n = 3$), with standard error of the mean shown in parenthesis.

analysing the data in Table 7 by means of Student's t-test, applying the Bessel correction for small samples, the difference between the mean arabinose level in unripe walls (0.95mg/10mg dry wall) and that in over-ripe walls (0.20mg/10mg dry wall) is significant at the 5% level ($t = 3.18$ with 4 degrees of freedom), applying a 2-tail test. The mean galactose level in unripe walls (0.67mg/10mg dry wall) is significantly higher than the level in over-ripe walls (0.22mg/10mg dry wall) at the 5% level ($t = 2.3$ with 4 degrees of freedom), applying a 1-tail test. Similarly, the mean glucose level in unripe walls (2.24mg/10mg dry wall) is significantly higher than the level in over-ripe walls (0.13mg/10mg dry wall) at the 5% level ($t = 2.3$ with 4 degrees of freedom), applying a 1-tail test. Interpreting the data for these three monosaccharide consituents in the light of the overall loss of cell wall from the fruit tissue during ripening noted in Table 4, it is clear that ripening of the mango is accompanied by a marked loss of these three monosaccharides from the mango tissue. However, in the case of glucose, because wall preparations were contaminated with starch it is not possible to reach meaningful conclusions regarding loss of wall glucan during ripening.

Tables 6 and 7 both reveal that, for xylose and galacturonic acid, although there is some variation between ripening stages for the recoveries in wall hydrolysates of these monosaccharides, overall there is no significant change in their levels in the wall preparations from the unripe

fruit to over-ripeness. However, interpreting the data in the light of the noted loss of cell wall material from the fruit tissue during ripening (Table 4), it appears that xylose and galacturonic acid are lost from mango tissue with ripening of the fruit. Loss of these constituents which constitute an unchanging proportion of the wall clearly contributes to the overall loss of wall material from the tissue, although this loss is less marked than losses of arabinose and galactose which simultaneously decline in the proportion of the wall which they constitute.

Rhamnose and fucose appear as only minor constituents of the wall material but, applying to these monosaccharides the same argument applied to the more abundant constituents, Tables 6 and 7 also suggest loss of these monosaccharides from mango tissue during ripening.

III.1. (c) Fractionation of the cell wall and determination of the monosaccharide composition of the fractions from unripe, ripe and over-ripe fruit.

The probability that the relatively crude fractionation procedures (utilising hot water and alkali extraction) resulted in some overlap between the fractions of pectic, hemicellulosic and residual "cellulosic" polysaccharides was accepted in this work. However, the hot water-soluble fraction was taken as a crude extract of pectic polymers, the alkali-soluble fraction as a crude hemicellulose extract and the insoluble residue as a crude cellulosic fraction. Recoveries of monosaccharides obtained by combined acid and enzymic hydrolysis of these crude

fractions obtained from chloral hydrate-extracted walls of fruits at various stages of ripeness are shown in Table 8.

Only small amounts of rhamnose were present in all fractions and fucose was present largely in the alkali-soluble fraction in which it appeared to decline during ripening. The gravimetric yield of this wall fraction from 100g (wet weight) tissue did not vary greatly with ripening (see Table 4), although there is a loss of intact wall material during ripening. Like fucose, xylose and mannose were present largely in the alkaline extract. Mannose, which could not be detected by hydrolysis of intact walls, is presumably a minor wall constituent present in hemicellulosic polysaccharides which is concentrated by alkaline extraction. Mannose declined whilst xylose remained at a stable level in this fraction with ripening. Xylose in the insoluble, residual fraction, however, declined during ripening. Interpreting these findings in the light of data provided by Table 4, it seems likely that there is a modest loss of these three monosaccharides as a consequence of wall dissolution during ripening.

Glucose declined in all fractions with ripening but the known contamination of wall preparations with starch renders this finding impossible to clearly interpret. Arabinose and galactose similarly declined in all fractions with ripening. Arabinose (and its decline during ripening) was particularly prominent in the hot water-soluble fraction, the yield of which fraction from 100g (wet weight) tissue fell with ripening. (See Table 4). This suggests a marked

TABLE 8 Determination of monosaccharides in hydrolysates of polysaccharide fractions extracted from cell wall preparations from mango mesocarp at various stages in ripening. (The combined acidic and enzymic hydrolysis procedure was used).

Monosaccharide	Mass (mg) of monosaccharide released by hydrolysis from 10 mg (dry weight) cell wall fraction								
	H_2O-soluble fraction			KOH-soluble fraction			Insoluble residue		
	Unripe	Ripe	Over-ripe	Unripe	Ripe	Over-ripe	Unripe	Ripe	Over-ripe
Rhamnose	0.04	0.01	0	0.03	0.01	0.01	0.02	trace	0
Fucose	0.04	0.06	0.04	0.41	0.34	0.30	0.06	0.03	0.03
Arabinose	1.02	0.80	0.32	0.18	0.22	0.14	0.30	0.10	0.13
Xylose	0.02	0.01	0.01	2.21	2.10	2.08	0.32	0.11	0.12
Mannose	0.09	0.02	0.01	0.28	0.19	0.17	0.03	0.03	0.02
Galactose	0.30	0.17	0.11	1.18	0.77	0.71	0.26	0.19	0.16
Glucose	4.52	0.65	0.04	3.56	1.78	2.13	2.20	0.50	0.22
Galacturonic acid	0.70	1.65	2.28	0.13	0.05	0.12	0.18	0.14	0.11
Total monosaccharides	6.72	3.38	2.81	7.99	5.42	5.66	3.36	1.09	0.79
% (by weight) of wall fraction obtained in total monosaccharides after hydrolysis.	67%	34%	28%	80%	54%	57%	34%	11%	8%

The cell wall preparations were obtained from single mango fruits at each ripening stage and extracted with chloral hydrate. The procedures for fractionation of the cell wall preparations are described in II.7.(a) 3. and the procedures for hydrolysis of wall fractions are given in II.7.(b). The dried hydrolysates were converted to alditol acetates as described in II.5.(b) and analysed by glc as described in II.2.(a) 2.
Each data item shown is the mean of duplicate glc assays of a single alditol acetate sample.

loss of arabinose (most particularly from hot water-soluble polysaccharides) from the cell wall accompanying ripening. Somewhat surprisingly, galactose was most prominent in the alkali-soluble fraction, but its decline in all three wall fractions suggests that, like arabinose, there is marked loss of galactose from the cell wall during ripening. These data for arabinose and galactose are consistent with the data obtained from hydrolysis of intact cell walls (see Tables 6 and 7).

The data obtained for galacturonic acid are somewhat difficult to interpret. Predictably, galacturonic acid was most prominent in the hot water-soluble fraction, a fraction in which gravimetric yield (per 100g fresh tissue) declined with ripening. (See Table 4). However, the recovery of galacturonic acid after hydolysis of 10mg of this water-soluble fraction increased with ripening (see Table 8). Hence it is difficult to make from this experiment the overall conclusion that galacturonic acid is lost from mango cell walls during ripening, a conclusion drawn from data provided by hydrolysis of intact cell walls (see Tables 6 and 7) interpreted in the light of Table 4.

In all of these experiments involving combined acid and enzymic hydrolysis, of both intact cell wall preparations and fractions obtained from such walls, it was noted that the % (by weight) of the starting material obtained in total monosaccharides after hydrolysis declined steadily with ripening of the tissue from which the wall materials were obtained, indicating that wall materials prepared from

ripe fruit were less labile to the hydrolytic procedures employed than wall materials prepared from unripe fruit. To consider just one example, in Table 7, 52% (by weight) of the unripe cell wall is recovered in total monosaccharides after hydrolysis whereas only 20% of the over-ripe wall is similarly recovered. It is critically conceded that failure to achieve 100% hydrolysis of all wall materials constitutes a defect in the work carried out and (to some extent) a complicating factor in interpreting data, since it introduces uncertainty about the monosaccharide composition of that portion of the wall materials which resists the hydrolytic procedures. This failure to completely hydrolyse cell wall materials will be further discussed in the Discussion (section IV).

III.2. Autolysis of homogenised mango mesocarp.

In this experiment homogenates of mango tissue at different stages of ripeness in sodium acetate buffer, pH 4.5 (after an initial dialysis to remove free sugars) were allowed to autolyse in dialysis bags at room temperature for 5 days. Sodium azide and thymol were present to prevent the growth of microorganisms. The final dialysate (which was not de-ionised) was evaporated to dryness and free monosaccharides in the dried residue converted to alditol acetates for analysis by glc.

The results obtained are shown in Table 9. Recoveries of total monosaccharides represent only a small proportion of the 100 mg of each dried residue analysed since, clearly, much of this residue was composed of sodium acetate

TABLE 9 Determination of monosaccharides in dialysates of autolysing mango mesocarp.

Monosaccharide	Monosaccharide (mg) present in dried residue (100 mg) of dialysate		
	Unripe	Ripe	Over-ripe
Rhamnose	0.05	0.02	0.05
Arabinose	0.02	0.01	0.16
Xylose	0.01	0.01	0.03
Fructose	0.40	0.62	2.37
Glucose	0.88	1.38	5.90
Total monosaccharides	1.36	2.04	8.50

Complete experimental details are given in section II.5. The alditol acetates obtained from monosaccharides present in the dried residues were analysed by glc as described in II.2.(a) 2. Each data item shown is the mean of duplicate glc assays of a single alditol acetate sample.

from the buffer. However, the total monosaccharides recovered increased significantly with ripening. Much of this increase can be attributed to increased recovery of glucose and fructose. The relatively high levels of these two monosaccharides, even at the unripe stage, suggest that they were present as free sugars in the mango tissue before homogenisation and were not completely removed by the initial dialysis. It is thus not possible to reach meaningful conclusions about the release of glucose into the soluble fraction as a result of enzymic action against wall polysaccharides.

Rhamnose appeared in the soluble fraction but there was no indication that it increased in amount during ripening. Xylose was also present in small amounts in the soluble fraction and increased in the over-ripe stage. The most striking change occurring during ripening was the 8-fold increase in arabinose in the soluble fraction from the unripe to the over-ripe stage. These results suggest autolytic degradation of xylose and arabinose-containing polysaccharides during the later stage of ripening of mango tissue.

III.3. A search for polygalacturonase activity in mango mesocarp.

III.3. (a) Introduction.

Because of the established importance of both endo- and exopolygalacturonase activities in ripening of a number of different fruits (see I.5. (b) 1.), considerable experimental efforts were made to see if polygalacturonase activity was present in mango mesocarp.

III.3. (b) Initial experiments.

In these initial studies (see Tables 10-13) efforts were made to extract polygalacturonase from mesocarp tissues at different stages of ripening with various salt solutions bearing in mind that the enzyme might be bound in some way or associated with an inhibitor and, hence, also including a number of potential activating factors and varying the pH. The assay for enzyme activity was based upon the generation of reducing groups from polygalacturonic acid (and, in some cases, polymethylgalacturonic acid), a method which detects both endo- and exo-polygalacturonase activities. Both the conversion of picrate to picramate (Foda, 1957) and the more sensitive Nelson (1944) micro-cuprimetric method utilising arsenomolybdate as the colour-developing reagent (Hestrin et al., 1955) were used for quantitative measurement of the reducing power produced. Control incubations were employed in which enzyme extracts were incubated in the absence of the polygalacturonic acid substrate. These controls were used to compensate for possible autolysis of carbohydrate present in the enzyme preparations and which could not be removed (see p. 215).

Higher levels of protein were extracted from ripe than from unripe fruit although there was a wide variation in the amount of protein extracted between individual tissue samples at any given ripening stage (see Tables 10-13). This variation in the main probably reflects differences in the level of physiological development of the fruits which were not apparent from the external appearance, i.e. the method

TABLE 10. Assay of NaCl/EDTA extracts of mango mesocarp tissue for polygalacturonase activity.

Incubation time at pH 4.5 (min)	Concentration of reducing sugars in incubation mixture (mmol galacturonic acid equivalents/l.)					
	Unripe enzyme preparation		Ripe enzyme preparation		Over-ripe enzyme preparation	
	+PA*	-PA	+PA	-PA	+PA	-PA
0	1.85	1.35	1.70	1.15	2.05	1.15
30	1.70	1.20	1.70	1.20	1.70	0.85
120	1.60	1.10	2.00	1.10	1.90	0.75

Protein concentrations (µg/ml) in the incubation mixtures were: Unripe enzyme preparation - 7
Ripe enzyme preparation - 46
Over-ripe enzyme preparation - 79

Total extracted protein (mg/100 g fresh weight tissue) in the enzyme preparations was:
Unripe enzyme preparation - 3.7
Ripe enzyme preparation - 25.1
Over-ripe enzyme preparation - 43.4

*PA = citrus polygalacturonic acid

Experimental details are given in Section II.3. (b) 1. Reducing sugar concentrations in duplicate aliquots (1 ml) of each incubation mixture were assayed by the method of Foda (1957) as described in II.2.(b) 2.4. Protein concentrations were also assayed in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1. Each data item shown represents the mean of the duplicate assays.

TABLE 11. Assay of extracts of ripe mango mesocarp tissue with NaCl solutions containing various additives for polygalacturonase and polymethylgalacturonase activities.

	Concentration of reducing sugars in incubation mixture (mmol galacturonic acid equivalents/l)						Protein concentration in incubation mixture (µg/ml)	Total extracted protein (mg/100g fresh weight tissue)
	+PA*		+MPA**		+ buffer alone			
Incubation time at pH 4.5 (min)	0	180	0	180	0	180		
Solution								
7.5% NaCl/EDTA (10:1)	0.18	0.27	1.04	0.83	0.17	0.18	18	15.5
7.5% NaCl	0.28	0.26	0.81	0.78	0.17	0.17	6	5.0
7.5% NaCl containing Ca^{2+} (10^{-3}M)	0.29	0.30	0.69	0.72	0.19	0.19	16	14.0
7.5% NaCl containing Mg^{2+} (10^{-3}M)	0.27	0.28	0.95	0.65	0.18	0.18	10	8.8
7.5% NaCl containing Ca^{2+} (10^{-3}M) and Mg^{++}(10^{-3}M)	0.26	0.25	1.00	0.72	0.16	0.15	9	7.7
7.5% NaCl/EDTA (10:1) containing Tween-60 (0.1%)	0.25	0.24	0.97	0.85	0.16	0.15	11	9.5
7.5% NaCl/EDTA (10:1)containing N-ethyl maleimide (10^{-3}M)	0.29	0.26	1.04	0.70	0.18	0.17	17	14.2
7.5% NaCl/EDTA (10:1)containing cysteine (10^{-3}M)	0.34	0.28	0.90	0.73	0.21	0.20	21	18.0

*PA = citrus polygalacturonic acid

**MPA = citrus polymethylgalacturonic acid

Experimental details are given in Section II.3.(b) 11.

Reducing sugar concentrations in duplicate aliquots (1 ml) of each incubation mixture were assayed by the micro-cuprimetric method of Nelson (1944) as described in II.2.(b) 2.3. Protein concentrations were also assayed in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1. Each data item shown represents the mean of the duplicate assays.

TABLE 12. Assay of NaCl/EDTA extract of ripe mango mesocarp tissue and of residual tissue pellet for polygalacturonase and polymethylgalacturonase activities.

Incubation time at pH 4.5 (min)	Concentration of reducing sugars in incubation mixture (mmol galacturonic acid equivalents/l.)					
	Soluble supernatant			Tissue pellet		
	+PA*	+MPA+	+buffer alone	+PA	+MPA	+buffer alone
0	0.45	1.25	0.22	0.45	1.20	0.18
180	0.42	1.12	0.21	0.40	1.12	0.18

Protein concentration in the soluble supernatant incubation mixture was 0.18 mg/ml; total extracted protein in the soluble supernatant was 102.4 mg/100 g fresh weight tissue.

*PA = citrus polygalacturonic acid

+MPA = citrus polymethylgalacturonic acid

Experimental details are given in Section II.3. (b) 2.

Reducing sugar concentrations in duplicate aliquots (1 ml) of each incubation mixture were assayed by the microcuprimetric method of Nelson (1944) as described in II.2.(b) 2.3. Protein concentrations were also assayed in duplicate by the method of Lowry et al., (1951),as described in II.2.(b) 1.1. Each data item shown represents the mean of the duplicate assays.

TABLE 13 . Assay of NaCl/EDTA extracts of ripe mango mesocarp tissue for polygalacturonase tissue at varying pH values.

	Concentration of reducing sugars in incubation mixture (mmol galacturonic acid equivalents/l.)		
Incubation time (h)	0	4	20
Incubation pH			
3.0	2.27	2.37	2.27
3.5	2.27	2.47	2.47
4.0	2.37	2.42	2.68
4.5	2.37	2.47	2.42
5.0	2.47	3.04	2.50
5.5	2.63	2.68	2.73
6.0	2.73	2.83	2.94

Protein concentration in the incubation mixtures was 79 µg/ml; total extracted protein in the enzyme preparation was 43.4 mg/100 g fresh weight tissue.

Experimental details are given in Section II.3.(b) 5. Reducing sugar concentrations in duplicate aliquots (1 ml) of each incubation mixture were assayed by the method of Foda (1957) as described in II.2. (b) 2.4. Protein concentrations were also assayed in duplicate by the method of Lowry et al. (1951) as described in II.2. (b) 1.1.

Each data item shown represents the mean of the duplicate assays.

used for selection. In none of these initial experiments (Tables 10-13) was significant activity detected when using either polygalacturonic or polymethylgalacturonic acids as substrates and examining soluble extracts and tissue pellets. In one experiment (Table 13) using NaCl/EDTA extracts of ripe mango mesocarp and varyihg the pH, incubations were extended up to 20 h with negative results.

When polymethylgalacturonic acid (MPA in Tables 11 and 12) was used as substrate the level of reducing sugars in incubation mixtures was considerably higher than when polygalacturonic acid (PA) was used as substrate, suggesting contamination of the polymethylgalacturonic acid with reducing sugars. Small, apparent changes in measured reducing power of some incubation mixtures (not significant in terms of polygalacturonase activity) during the course of incubation were not consistent (in some cases reducing power appeared to increase slightly after incubation and in other cases it appeared to decrease slightly) and such changes were attributed to experimental error in reducing sugar assays.

III.3. (c) Experiments designed to remove potential inhibitors of polygalacturonase activity.

Further experiments were carried out in an attempt to remove possible inhibitors associated with the enzymic protein. In this connection it should be noted that Mattoo and Modi (1970) have isolated proteinaceous inhibitors of amylase, peroxidase and catalase from the soft tissue of both unripe mangoes and unripe bananas. Pansolli and Belli-donini (1973) also claimed that a polygalacturonase

inhibitor was present in the soft tissue of grape and Weurman (1953) demonstrated the presence of a soluble, thermostable inhibitor of polygalacturonase in pear fruit. The masking of enzyme activity by binding to macromolecules other than protein (polysaccharides) was also considered and to investigate these possibilities a NaCl/EDTA extract of mango mesocarp was incubated with various hydrolytic enzymes. The digests were dialysed against sodium acetate buffer, pH 4.5 at 4^{o} before assaying for enzyme activity using both polygalacturonic acid and polymethylgalacturonic acid as substrates. Here again, however, no degradation of either substrate occurred (Table 14). The crude cellulase preparation used for some digestions did, itself, however possess polygalacturonase activity (Table 15).

Treatment of NaCl/EDTA extracts of mesocarp at various stages of ripening with 80 or 90%-saturated $(NH_4)_2SO_4$ solution in an attempt to precipitate the enzyme and at the same time to remove possible inhibitors of polygalacturonase yielded a preparation with no activity (Table 16). In order to investigate whether a bound inhibitor (possibly polysaccharide) might be removed by autolysis by endogenous enzyme(s), the ammonium sulphate-precipitated protein fraction from over-ripe fruit was dialysed against sodium acetate buffer, pH 4.5 for an extended period (72 h) at 22^{o} in the presence of thymol, before assaying for polygalacturonase activity. No enzyme activity was detected after this treatment (Table 17). In an attempt to remove a possible, non-covalently bonded

TABLE 14. Assay of NaCl/EDTA extract of ripe mango mesocarp tissue for polygalacturonase and polymethylgalacturonase activities after digestion of the extract with various hydrolytic enzymes.

	Concentration of reducing sugars in incubation mixture (mmol galacturonic acid equivalents/l.)					
	+PA*		+MPA+		+ buffer alone	
Incubation time at pH 4.5 (min)	0	180	0	180	0	180
Hydrolytic enzyme(s) added						
Cellulase	0.18	1.60†	0.80	0.93	0.10	0.11
Hemicellulase	0.27	0.32	0.90	0.85	0.21	0.20
Cellulase + hemicellulase	0.14	1.70†	0.55	0.60	0.08	0.10
Exo-β-(1→3)-D-glucanase	0.23	0.21	0.85	0.65	0.17	0.16
Protease	0.12	0.12	0.50	0.53	0.06	0.05
Pepsin	0.20	0.18	0.68	0.85	0.12	0.12
No enzyme	0.31	0.28	1.00	1.08	0.23	0.24

Protein concentration in the mango enzyme extract was 0.2 mg/ml and all hydrolytic enzymes were added to the extract at concentrations of 1 mg/ml. After digestion (2 h) and dialysis against sodium acetate buffer, pH 4.5, extract (0.2 ml) was added to substrate solution or buffer (0.8 ml).
Total extracted protein in the mango enzyme preparation was 20.2 mg/100g fresh weight tissue.
*PA = citrus polygalacturonic acid. +MPA = citrus polymethylgalacturonic acid. † See Table 15.

Reducing sugar concentrations in duplicate aliquots (1 ml) of each incubation mixture were assayed by the micro-cuprimetric method of Nelson (1944) as described in II.2. (b) 2.3. Protein concentrations were also assayed in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1. Each data item shown represents the mean of the duplicate assays.

TABLE 15. Assay of cellulase preparation used for digestion of mango enzyme extract for polygalacturonase activity.

Incubation time at pH 4.5 (min)	Concentration of reducing sugars in incubation mixture (mmol galacturonic acid equivalents/l).	
	+PA*	-PA
0	0.13	0.03
180	1.15	0.04

Cellulase (1 mg/ml in 0.1M acetate buffer, pH 4.5) solution (0.2 ml) was added to 0.25% polygalacturonic acid (0.8 ml) or buffer (0.8 ml) at pH 4.5.

*PA = citrus polygalacturonic acid.

Reducing sugar concentrations in duplicate aliquots (1 ml) of each incubation mixture were assayed by the microcuprimetric method of Nelson (1944) as described in II.2. (b) 2.3.

Each data item shown represents the mean of the duplicate assays.

TABLE 16 Assay of fractions, obtained by treating NaCl/EDTA extracts of mango mesocarp tissue with 90%-saturated $(NH_4)_2SO_4$, for polygalacturonase activity.

Incubation time at pH 4.5 (min)	Concentration of reducing sugars in incubation mixture (mmol galacturonic acid equivalents/l)					
	Unripe enzyme preparation		Ripe enzyme preparation		Over-ripe enzyme preparation	
	+PA*	-PA	+PA	-PA	+PA	-PA
0	1.70	1.25	3.05	2.45	3.15	2.00
30	1.45	1.25	2.55	2.20	2.55	1.85
120	1.35	1.00	2.90	2.35	3.15	2.10

Protein was precipitated from extracts with 90%-saturated ammonium sulphate, dissolved in 0.05M phthalate buffer, pH 4.5, and dialysed against buffer at 4° for 24 h to remove ammonium sulphate.

Dialysed protein (2 ml) was added to 0.2% polygalacturonic acid solution (8 ml), pH 4.5.

Protein concentrations (µg/ml) in the incubation mixtures were:

Unripe enzyme preparation	- 4
Ripe enzyme preparation	- 204
Over-ripe enzyme preparation	- 399

Total extracted protein (mg/100 g fresh weight tissue) in the enzyme preparations was:

Unripe enzyme preparation	- 1.1
Ripe enzyme preparation	- 57.1
Over-ripe preparation	- 109.7

*PA = citrus polygalacturonic acid

Experimental details are given in Section II.3. (b) 4.

Reducing sugar concentrations in duplicate aliquots (1 ml) of each incubation mixture were assayed by the microcuprimetric method of Nelson (1944) as described in II.2. (b) 2.3. Protein concentrations were also assayed in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1. Each data item shown represents the mean of the duplicate assays.

TABLE 17. Assay of fraction, obtained by treating NaCl/EDTA extract of over-ripe mango mesocarp tissue with 80%-saturated $(NH_4)_2SO_4$ and dialysing precipitated protein for 72 h at 22°, for polygalacturonase activity.

Incubation time at pH 4.5 (min)	Concentration of reducing sugars in incubation mixtures (mmol galacturonic acid equivalents/l.)	
	+PA*	-PA
0	0.44	0.30
30	0.43	0.31
65	0.46	0.33
135	0.46	0.31
180	0.44	0.31
1505	0.54	0.34

Protein was precipitated from extracts with 80%-saturated ammonium sulphate, dissolved in 0.1M acetate buffer, pH 4.5 and dialysed against buffer at 4° for 24 h to remove ammonium sulphate.
The protein solution was further dialysed against buffer at 22° for 72 h in the presence of thymol.
Dialysed protein (1 ml) was added to 0.2% polygalacturonic acid solution (9 ml), pH 4.5.
The protein concentration in the incubation mixture was 50 µg/ml; total extracted protein in the enzyme preparation was 28.1 mg/100 g fresh weight tissue.

*PA = citrus polygalacturonic acid
Experimental details are given in Section II.3. (b) 8.

Reducing sugar concentrations in duplicate aliquots (1 ml) of each incubation mixture were assayed by the microcuprimetric method of Nelson (1944) as described in II.2.(b) 2.3. Protein concentrations were also assayed in duplicate by the method of Lowry et al. (1951) as described in II.2. (b) 1.1.
Each data item shown represents the mean of the duplicate assays.

inhibitor of low molecular weight the ammonium sulphate-precipitated protein fraction from over-ripe fruit was applied to a Sephadex G-100 column and eluted with 0.1M acetate buffer, pH 5.5. Fractions containing protein, which eluted as a single band (see Fig.32, p. 119) were pooled and concentrated by ultrafiltration using an Amicon PM-10 membrane. No polygalacturonase activity was present in the concentrated protein fraction or the Amicon filtrate (Table 18).

Enzyme activities extracted from plant tissues can be inhibited by polyphenols which are simultaneously extracted (Hulme and Jones, 1963; Loomis and Battaile, 1966; Richmond and Biale, 1967; Van Sumere et al., 1975). It is generally agreed that high molecular weight polyphenols of the tannin type interact with proteins by multiple hydrogen bonding of phenolic groups to 'peptide' ('amide') bonds (Loomis and Battaile, 1966; Van Sumere et al.1975). This problem may be overcome by addition of polyvinylpyrrolidone (PVP), (which rapidly hydrogen bonds to the phenolics) (Gustafson, 1954), to the extracting medium. For soluble enzyme protein the most effective procedure is to use high molecular weight, insoluble PVP which is then centrifuged off (together with bound polyphenols) along with cell debris (Loomis and Battaile, 1966). Extraction of mango mesocarp with NaCl/EDTA solution in the presence of insoluble PVP gave soluble extracts with no detectable polygalacturonase activity (Table 19). It was noteworthy,however, that in incubation mixtures containing enzyme extracted in the presence of PVP (+PVP in Table 19) the measured concentration

TABLE 18. Assay for polygalacturonase of fractions obtained from an NaCl/EDTA extract of over-ripe mango mesocarp tissue by treatment with 80%-saturated $(NH_4)_2SO_4$ followed by Sephadex G-100 chromatography and concentration of the eluted protein by Amicon filtration.

Incubation time at pH 4.5 (min)	Concentration of reducing sugars in incubation mixtures (mmol galacturonic acid equivalents/l.)			
	Amicon PM-10 concentrate		Amicon PM-10 filtrate	
	+PA*	-PA	+PA	-PA
0	0.56	0.51	0.10	0.03
180	0.56	0.51	0.11	0.03

Protein was precipitated from the extract with 80%-saturated ammonium sulphate, dissolved in 0.1M acetate buffer, pH 4.5 and dialysed against distilled water (twice) and buffer (once) at 4° to remove ammonium sulphate. All dialyses were for 24 hours. Dialysed protein was applied to a Sephadex G-100 column and eluted with 0.1M acetate buffer, pH 5.5. Fractions containing protein (which eluted as a single band, see Fig.32, p.119), were pooled and concentrated (x10) by Amicon ultrafiltration. Amicon concentrate (0.5 ml) or filtrate (0.5 ml) was added to 0.2% polygalacturonic acid solution (0.5 ml), pH 4.5.

Final protein concentration in the incubation mixture containing Amicon concentrate was 0.25 mg/ml; total extracted protein in the enzyme preparation was 28.1 mg/100 g fresh weight tissue.

*PA = citrus polygalacturonic acid

Experimental details are given in Section II.3.(b) 7. See also Fig.32, p.

Reducing sugar concentrations in duplicate aliquots (1 ml) of each incubation mixture were assayed by the microcuprimetric method of Nelson (1944) as described in II.2. (b) 2.3. Protein concentrations were also assayed in duplicate by the method of Lowry et al. (1951) as described in II.2. (b) 1.1.
Each data item shown represents the mean of the duplicate assays.

TABLE 19. Assay of NaCl/EDTA extract, containing polyvinylpyrrolidone (PVP), of ripe mango mesocarp tissue for polygalacturonase activity.

Incubation time at pH 4.5 (min)	Concentration of reducing sugars in incubation mixtures (mmol galacturonic acid equivalents/l.)			
	Extract + PVP		Extract -PVP	
	+PA*	-PA	+PA	-PA
0	3.20	1.25	5.00	3.30
10	2.90	1.05	4.40	2.80
30	2.55	1.20	4.10	2.65
60	2.50	1.10	4.25	2.70
120	2.20	1.20	4.50	3.15

Protein concentration in the incubation mixture (-PVP) was 70 µg/ml; total extracted protein in the enzyme preparation (-PVP) was 10.6 mg/100 g fresh weight tissue. Protein was assayed only in mixtures not containing PVP as a portion of the PVP preparation used proved to be soluble and inhibited the Lowry (1951) colour reaction for protein.

*PA= citrus polygalacturonic acid

Experimental details are given in Section II.3. (b). 6.

Reducing sugar concentrations in duplicate aliquots (1 ml) of each incubation mixture were assayed by the method of Foda (1957) as described in II.2.(b) 2.4. Protein concentrations were also assayed in duplicate by the method of Lowry et al. (1951) as described in II.2. (b) 1.1.

Each data item shown represents the mean of the duplicate assays.

of reducing sugars was consistently lower than in incubation mixtures containing enzyme extract in which PVP was not incorporated in the extraction medium. The simplest explanation for this puzzling finding is that (for obscure reasons) the extract prepared in the presence of PVP contained less extracted reducing sugars (the problems encountered in the removal of carbohydrate from mango enzyme extracts are discussed later in this thesis, see p.215). However, it should be noted that, although the PVP employed was described by the manufacturers (Sigma Corporation) as "High molecular weight, Insoluble", in practice some of the PVP (presumably a lower molecular weight PVP contaminant) was found to dissolve forming a cloudy solution. It was found that the Lowry (1951) reaction for protein was inhibited in mixtures containing enzyme extracted in the presence of PVP. In place of the expected reaction, a thick white precipitate of re-precipitated PVP formed immediately, along with precipitated protein, on addition of trichloroacetic acid to the aqueous sample (see II.1.(b) 1.1.). This precipitated PVP re-dissolved, along with precipitated protein presumed also to be present, in Lowry solution C but no colour reaction developed on subsequent addition of Folin phenol reagent. The chemical nature of this interference is obscure except, perhaps, that PVP is known to hydrogen bond strongly to phenolic compounds. In retrospect, it is conceded that the possibility that this dissolved PVP might in some way have interfered also with the Foda (1957) picrate reduction assay for reducing sugars (see II.1. (b) 2.4), giving a reduced colour reaction, cannot be entirely discounted, even though this may seem an

unlikely speculation. The author could find nothing in the literature describing interference of dissolved PVP in such reactions.

The extraction technique described by Pansolli and Belli-donini (1973) based on $(NH_4)_2SO_4$ precipitation at pH 7.0 (see Section II.3. (c), p. 122) was originally designed for the removal of an unidentified inhibitor from polygalacturonase in fruits from which the enzyme had previously been reported to be absent (Hobson, 1962; Mannheim and Siv, 1969).. Ripe and over-ripe mango mesocarp tissues were extracted by this technique but the resulting extracts contained no detectable polygalacturonase or polymethylgalacturonase activity (Table 20).

In a further experiment designed to demonstrate whether or not a polygalacturonase inhibitor was present in mango, a ripe mesocarp extract was added to an active polygalacturonase preparation from tomato. There was, however, no inhibition of the tomato enzyme indicating the absence of mango inhibitor or at least an inhibitor capable of combining with tomato polygalacturonase (Table 21).

TABLE 20. Assay of extracts of mango mesocarp tissue, prepared by method of Pansolli and Belli-donini (1973), for polygalacturonase and polymethylgalacturonase activity.

Incubation time at pH 4.5 (min)	Concentration of reducing sugars in incubation mixtures (mmol galacturonic acid equivalents/l.)					
	Ripe enzyme preparation			Over-ripe enzyme preparation		
	+PA*	+MPA+	+ buffer alone	+PA	+MPA	+buffer alone
0 -	0.36	1.25	0.31	0.27	0.71	0.18
60	0.44	1.28	0.25	0.31	0.90	0.19
180	0.43	1.28	0.28	0.30	0.95	0.21

Tissue was macerated and centrifuged at 4^{o}. The tissue pellet was extracted with 5% sodium chloride solution for 30 h at 4^{o} and the extract brought to pH 7.0. Protein was precipitated from the extract with 90%-saturated ammonium sulphate, dissolved in 5% NaCl and dialysed against 5% NaCl (twice) at 4^{o} to remove ammonium sulphate. Dialysed protein (2-4 ml) was added to 0.2% polygalacturonic acid or polymethylgalacturonic acid solution (6-8 ml) in 0.1M acetate buffer, pH 4.5.

Protein concentrations (µg/ml) in the incubation mixtures were: Ripe enzyme preparation - 19
Over-ripe enzyme preparation - 333

Total extracted protein (mg/100 g fresh weight tissue) in the enzyme preparations was:

Ripe enzyme preparation - 1.4
Over-ripe enzyme preparation - 50.0

*PA = citrus polygalacturonic acid; +MPA = citrus polymethylgalacturonic acid.
Experimental details are given in Section II.3. (c).

Reducing sugar concentrations in duplicate aliquots (1 ml) of each incubation mixture were assayed by the microcuprimetric method of Nelson (1944) as described in II.2. (b) 2.3. Protein concentrations were also assayed in duplicate by the method of Lowry et al. (1951) as described in II.2. (b) 1.1.
Each data item shown represents the mean of the duplicate assays.

TABLE 21. Effect of NaCl/EDTA extract of ripe mango mesocarp tissue on tomato polygalacturonase activity.

Incubation time at pH 4.5 (min)	Galacturonic acid (μmol) liberated/100 g fresh weight tomato tissue			
	Tomato enzyme only		Tomato enzyme + mango preparation	
	+PA*	-PA	+PA	-PA
15	137	0	133	0
30	347	0	375	0
120	1313	0	1047	0

Protein concentration in the incubation mixtures containing tomato enzyme only was 20 μg/ml; total extracted protein in the tomato preparation was 24.5 mg/100 g fresh weight tissue.

Protein concentration in the incubation mixtures containing tomato enzyme and mango preparation was 70μg/ml; total extracted protein in the mango preparation was 28.1 mg/100 g fresh weight tissue.

*PA = citrus polygalacturonic acid

Experimental details are given in Section II.3. (b) 9.

Reducing sugar concentrations in duplicate aliquots (1 ml) of each incubation mixture were assayed by the microcuprimetric method of Nelson (1944) as described in II.2.(b) 2.3. Protein concentrations were also assayed in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1.

Each data item shown is based on the mean of the duplicate assays.

III.3. (d) Experiments designed to detect low levels of polygalacturonase activity in mango extracts.

Pressey and Avants (1973) have developed a method of polygalacturonase extraction and assay (see Section II.3 (d), p.123) which they used for fruits, such as peach, which possess very low levels of enzyme activity. This procedure was therefore tried with ripe mango mesocarp tissue. Ripe mango tissue was chosen since it was considered that activity was likely to be maximal at this developmental stage. The basis of the assay method is that sodium dithionite is incorporated into the incubation mixture of enzyme and substrate and this inhibits galacturonic acid oxidase which, if present, oxidises the galacturonic acid liberated by polygalacturonase and therefore interferes with the assay of reducing sugar. No polygalacturonase activity was found in soluble fractions from the mango using this technique (Table 22).

Viscometry is a highly sensitive technique for following degradation of viscous polysaccharide solutions as the breaking of only a single internal bond in a polysaccharide chain results in a marked fall in viscosity. Viscometry is, therefore, an ideal method for the detection of endo-polygalacturonase activity which catalyses a rapid reduction in the molecular weight of pectic acid. The fall in viscosity accompanying exo-polygalacturonase activity is much slower since the molecular weight of the substrate only decreases gradually as terminal glycosyl residues are sequentially removed. Viscometric assay of extracts of ripe mango mesocarp in NaCl/EDTA solution and of extracts

TABLE 22. Assay of extract of ripe mango mesocarp tissue, prepared by method of Pressey and Avants (1973), for polygalacturonase activity.

Incubation time at pH 4.5 (min)	Concentration of reducing sugars in incubation mixtures (mmol galacturonic acid equivalents/l.)	
	+PA*	-PA
0	0.57	0.62
75	0.62	0.72
150	0.57	0.72

Tissue was macerated in 0.1% sodium bisulphite at 4^{o}, centrifuged and the supernatant discarded. The pellet was washed twice with 0.1% sodium bisulphite and extracted at 4^{o} for 3 h with 0.2M NaCl. The extract was concentrated (x30) by Amicon (PM-10) ultrafiltration and dialysed against 0.15M NaCl at 4^{o}. Dialysed protein (3 ml) was added to 0.2% polygalacturonic acid solution (7 ml) in 0.1M acetate buffer, pH 4.5 containing 1mM sodium dithionite.

Protein concentration in the incubation mixtures was 66 µg/ml; total extracted protein in the enzyme preparation was 1.4 mg/100 g fresh weight tissue.

*PA = citrus polygalacturonic acid

Experimental details are given in Section II.3.(d).

Concentration of reducing sugars in duplicate aliquots (1 ml) of each incubation mixture was assayed by the micro-cuprimetric method of Nelson (1944) as described in II.2.(b) 2.3. Protein concentrations were also assayed in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1.

Each data item shown represents the mean of the duplicate assays.

of the same tissue prepared by the method of Pressey and Avants (1973) utilising both pectic (polygalacturonic) acid and polymethylgalacturonic acid as substrates, revealed no enzyme activity even after prolonged incubations (Tables 23 and 24). The small decreases in flow time noted in both viscometric experiments could indicate very low levels of exo-polygalacturonase/exo-polymethylgalacturonase activity but such small changes after long term incubation is not considered to be reliable evidence.

Given the wide range of experimental techniques utilised in the attempt to detect polygalacturonase activity in the mango and the negative results obtained in all cases, it seems unlikely that the enzyme is present in the fruit.

III.4. Glycosidases in mango mesocarp.

III.4. (a) Introduction.

Mango mesocarp at various stages of ripeness was assayed for a number of glycosidases in anticipation that the activities detected might provide information on the ripening process.

Activities were assayed by the generation of p-nitrophenol from the appropriate p-nitrophenyl glycosides and the development of a yellow colour in alkaline solution for colorimetric estimation at 405 nm. With the assay procedure employed it should be borne in mind that low levels of activity (0-20 units/100 g fresh weight tissue) were just detectable; for example, an enzyme extract containing of the order of 10 units of activity/100 g tissue would only increase the optical density by approximately

TABLE 23. Viscometric assay of NaCl/EDTA extract of ripe mango mesocarp tissue for polygalacturonase and polymethylgalacturonase activities.

Incubation time at pH 4.5 (min)	Viscometer flow time (min)	
	+PA*	+MPA+
0	4.4	8.7
30	4.4	9.2
60	4.4	8.5
120	4.4	8.5
180	4.4	8.4
1380	4.4	8.2

Protein concentration in the incubation mixtures was 0.18 mg/ml; total extracted protein in the enzyme preparation was 102.4 mg/100 g fresh weight tissue.

*PA = citrus polygalacturonic acid; +MPA = citrus polymethylgalacturonic acid

Experimental details are given in Section II.3.(b) 12.

In this experiment single viscometers containing each incubation mixture were set up and single determinations of flow time made at timed intervals.

Protein concentration was assayed in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1.

Protein concentration given represents the mean of the duplicate assays.

TABLE 24. Viscometric assay of extract of ripe mango mesocarp tissue, prepared by method of Pressey and Avants (1973), for polygalacturonase activity.

Incubation time at pH 4.5 (min)	Viscometer flow time (min)
0	7.0
60	7.0
240	6.9
1260	6.5

Protein concentration in the incubation mixture was 66 µg/ml; total extracted protein in the enzyme preparation was 1.4 mg/100 g fresh weight tissue.

Experimental details of the method of enzyme extraction are given in Section II .3. (d). Experimental details of the viscometric assay are given in II.3.(b) 12.

In this experiment a single viscometer containing mango enzyme extract and citrus polygalacturonic acid as substrate in 0.1M acetate buffer, pH 4.5, was set up and single determinations of flow time made at timed intervals.

Protein concentration was assayed in duplicate by the method of Lowry et al (1951) as described in II.2.(b) 1.1. Protein concentration given represents the mean of the duplicate assays.

0.05 units, even after 3 h incubation with the substrate. Hence, such low levels of activity are clearly just within the limits of sensitivity of the assay and for this reason are probably subject to greater error and less reproducibility.

All enzyme activities were expressed as units of activity (1 unit of activity = 1 μmol p-nitrophenol liberated per h) present in 100 g fresh weight tissue; it was unnecessary to calculate activity in terms of unit/100 g dry weight tissue since the water content of the mango mesocarp, surprisingly, underwent no significant change during ripening (see III.1. (a) p. 157).

III.4. (b) Glycosidase activities in extracts of fresh mango tissue (at various stages of ripeness) with 0.1M acetate buffer, pH 5.0.

Whole homogenates of unripe mango mesocarp in 0.1M sodium acetate buffer, pH 5.0 contained 6 different glycosidase activities (Table 25): β-D-glucosidase, α- and β-D-galactosidases α-D-mannosidase, α-L-arabinopyranosidase and β-D-glucuronidase, the last two of these enzymes being present at very low levels. Only 3 activities changed with ripening: β-D-galactosidase increased 13-fold overall from unripe (stage 1) to over-ripe (stage 4) with a marked increase between the turning (2) and over-ripe (4) stages. The optimum pH for this enzyme is 4.5 (see Fig.39, p. 143) A similar pattern was noted for α-D-mannosidase (12-fold overall change from unripe to over-ripe), but with a marked increase between the unripe (1) and turning (2) stages.

This enzyme has a different pH optimum - 5.2 (see Fig.40, p. 144). α-L-arabinopyranosidase activity was very low in unripe fruit but increased sharply with ripening. This activity has the same pH optimum - 4.5,(see Fig. 41, p.145) as β-D-galactosidase and it is possible that one enzymic protein is responsible for both activities as α-L-arabinopyranosides have similar configurations to β-D-galactosides (Wallenfels and Malhotra, 1961). However, the ratio of β-D-galactosidase activity to α-L-arabinopyranosidase activity in over-ripe mesocarp was 18:1, this ratio was not the same at other stages of ripening; for example, in turning mesocarp the ratio was 30:1. These different values for the ratio of the two enzyme activities suggest that two different enzymic proteins are involved. It should also be noted, however, that the levels of α-L-arabinopyranosidase in both unripe and turning tissue were very low and, perhaps, not reliable for the reasons discussed previously (p.196), so that the ratios of the two enzyme activities in unripe and turning tissue may not be reliable. It is probably safest to draw no firm conclusion as to whether α-L-arabinopyranosidase and β-D-galactosidase activities reside on the same or different proteins in the mango.

β-D-Galactosidase, α-D-mannosidase and α-L-arabinosidase underwent significant changes in specific as well as total activities during ripening (Table 25). Glycosidase activities which were not detected at any stage of mango mesocarp development included α-D-glucosidase, β-D-mannosidase, α- and β-D-xylosidases, β-L-arabinopyranosidase, α- and β-L-fucosidases and α-D-galacturonidase.

TABLE 25 Enzyme activities in whole homogenates of mango mesocarp in 0.1M sodium acetate buffer (pH 5.0).

Enzyme	Unripe		Turning		Over-ripe	
	Total Activity (units*/100 g wet tissue)	Specific activity (units/mg protein)	Total Activity (units/100g wet tissue)	Specific activity (units/mg protein)	Total Activity (units/100g wet tissue)	Specific activity (units/mg protein)
β-D-Glucosidase	17.0	0.08	18.1	0.07	23.1	0.11
α-D-Galactosidase	40.5	0.19	46.6	0.18	52.5	0.25
β-D-Galactosidase	142.7	0.67	391.1	1.51	1904.7	9.07
α-D-Mannosidase	29.8	0.14	269.4	1.04	363.3	1.73
α-L-Arabinopyranosidase	2.1	0.01	13.0	0.05	105.0	0.50
β-D-Glucuronidase	6.4	0.03	10.4	0.04	4.2	0.02
Total protein (mg/100 g wet tissue)	213		259		210	

*1 unit of enzyme activity = 1 μmol p-nitrophenol liberated/h.

Whole homogenates were prepared as described in Section II.6.(a) from pooled mesocarp slices obtained from 3 mangoes at each stage of ripeness.

Glycosidase enzyme assays were carried out in duplicate as described in II.2.(b).3.2. and protein assays were also carried out in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1.

Each data item shown represents the mean of the duplicate assays.

'Turning' (stage 2) fruit are frequently yellow-skinned on one side and green on the other although in some fruits 'yellowing' imparts a more 'blotchy' appearance with yellow patches distributed over both sides of the fruit. So when sampling 'turning' fruit in an attempt to even out these variations, the whole mesocarp from both sides was removed and cut into slices which were evenly mixed. In many experiments such slices from several 'turning' fruits were pooled. The actual variation between the levels of glycosidase activities present in the mesocarp on the clearly distinguishable green and yellow sides of 'turning' fruit was investigated by sampling, separately, the tissues on the two sides of 3 separate fruits and measuring β-D-galactosidase and α-D-mannosidase activities. The level of α-D-mannosidase (total and specific activities) was generally higher in the yellow portions of the fruit than in the green but β-D-galactosidase activities in yellow tissue were only marginally higher (see Table 26). Statistically analysing data for α-D-mannosidase activities in Table 26 by means of Student's t (one-tail) test, applying the Bessel correction for small samples, the mean level of <u>total</u> activity in tissue underlying yellow skin (60 units of activity/100g (wet weight) tissue) is significantly <u>higher</u> than the mean level in the "2nd sample" of tissue underlying green skin (23 units of activity/100g (wet weight) tissue), at the 5% level ($t = 2.45$ with 4 degrees of freedom). The mean level of <u>specific</u> activity in tissue underlying yellow skin (0.26 units of activity/mg protein) is significantly <u>higher</u> than the mean level in the "2nd

TABLE 26 Enzyme activities in whole homogenates of mango mesocarp in 0.1M sodium acetate buffer (pH 5.0) prepared from different parts of single 'turning' (stage 2) fruits.

Enzyme	Fruit No.	Yellow-skinned tissue: Total activity (units*/100g wet tissue)	Yellow-skinned tissue: Specific activity (units/mg protein)	Green-skinned tissue (1st sample): Total activity (units/100g wet tissue)	Green-skinned tissue (1st sample): Specific activity (units/mg protein)	Green-skinned tissue (2nd sample): Total activity (units/100g wet tissue)	Green-skinned tissue (2nd sample): Specific activitiy (units/mg protein)
β-D-Galactosidase	1	193	0.84	135	0.53	139	0.42
	2	186	0.79	184	0.79	157	0.63
	3	151	0.68	136	0.65	131	0.59
	Mean (S.E.)	177(13.0)	0.77(0.05)	152(16.2)	0.66(0.08)	142(7.7)	0.55(0.06)
α-D-Mannosidase	1	63	0.28	9	0.04	8	0.02
	2	69	0.29	48	0.21	43	0.17
	3	47	0.21	18	0.09	18	0.08
	Mean (S.E.)	60(6.6)	0.26(0.03)	25(11.8)	0.11(0.05)	23(10.4)	0.09(0.04)
Total Protein (mg/100g wet tissue)	1	229		254		330	
	2	236		232		251	
	3	221		208		223	
	Mean (S.E.)	229(6.1)		231(18.8)		268(32.0)	

*1 unit of enzyme activity = 1 μmol p-nitrophenol liberated/h.

Separate fruits (1,2 and 3) were used in 3 experiments and in each case two green-skinned tissue samples and one yellow-skinned sample were examined. Whole homogenates were prepared from these tissue samples as described in II.6.(a), (see also II.6.(e)).

Glycosidase enzyme assays were carried out in duplicate (see II.2.(b) 3.2.) and protein assays were also carried out in duplicate by the method of Lowry et. al. (1951) as described in II.2.(b) 1.1.
Each data item shown represents the mean of the duplicate assays.

The means shown in the table are the means of the 3 separate values obtained in the 3 experiments (i.e. n = 3), each of these 3 values being itself the mean of duplicate assays, with standard errors (S.E.) of the means given in parenthesis.

sample" of tissue underlying green skin (0.09 units of activity/mg protein) at the 5% level (t = 2.76 with 4 degrees of freedom).

Utilising a Student's t (two-tail) test, also with Bessel correction, the mean level of total α-D-mannosidase activity in tissue underlying yellow skin (60 units of activity/100g (wet weight) tissue) is significantly different from the mean level in the combined "1st sample" and "2nd sample" of tissues underlying green skin (24 units of activity/100 g (wet weight) tissue) at the 2.5% level (t = 2.89 with 7 degrees of freedom). The corresponding mean specific activities in tissue underlying yellow skin (0.26 units of activity/mg protein) and green skin (0.10 units of activity/mg protein) are significantly different at the 2.5% level (t = 3.04 with 7 degrees of freedom.

It seems clear that the increase in glycosidase activities accompanying yellowing of the fruit occurs locally in the mesocarp underlying the yellow skin whilst activities in the slower-ripening part of the fruit remain at lower levels. This tends to emphasize that the changes in activities are, in some way, associated with ripening.

The inhibition of plant enzymes by polyphenolics (Hulme and Jones, 1963; Loomis and Battaile, 1966; Richmond and Biale, 1967; Van Sumere et al., 1975) and the use of polyvinylpyrrolidone in the extraction medium to surmount this probelm (Gustafson, 1954; Loomis and Battaile, 1966) have been previously discussed (p. 184). In order to establish whether phenolic substances affected glycosidase activities in mango mesocarp, homogenates of unripe and over-ripe tissue prepared in the presence and absence of polyvinylpyrrolidone (PVP) were compared. The results obtained are shown in Table 27; incorporation of PVP appeared to slightly enhance both the total and specific

TABLE 27. Enzyme activities in whole homogenates of mango mesocarp in 0.1M sodium acetate buffer (pH 5.0) in presence and absence of polyvinylpyrrolidone.

Enzyme	Experiment	Unripe				Over-ripe			
		-PVP		+PVP		-PVP		+PVP	
		Total activity (units */ 100g wet tissue)	Specific activity (units/mg protein)	Total activity (units/ 100g wet tissue)	Specific activity (units/mg protein)	Total activity (units/ 100g wet tissue)	Specific activity (units/mg protein)	Total activity (units/ 100g wet tissue)	Specific activity (units/mg protein)
β-D-Galactosidase	1	180	0.87	234	1.13	2787	11.56	2641	10.96
	2	141	0.60	165	0.70	1112	4.50	1089	4.41
α-D-Mannosidase	1	47	0.23	73	0.35	249	1.03	360	1.49
	2	30	0.13	34	0.14	237	0.96	226	0.91
α-L-Arabinopyranosidase	1	16	0.08	7	0.03	171	0.71	160	0.66
	2	nil	nil	nil	nil	94	0.38	84	0.34
Total protein (mg/100 g wet tissue)	1			208				241	
	2			235				247	

*1 unit of enzyme activity = 1 μmol p-nitrophenol liberated/h.

In experiments 1 and 2 samples of pooled tissue from 3 fruits at each stage of ripeness were used (different fruits were used in the two experiments). Whole homogenates of these tissue samples were separately prepared in the presence and absence of PVP as described in II.6.(d).

Glycosidase enzyme assays were carried out in duplicate as described in II.2.(b) 3.2. and protein assays were also carried out in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1. Protein was assayed only in homogenates not containing PVP as a portion of the PVP preparation used proved to be soluble and inhibited the Lowry (1951) colour reaction for protein; protein figures presented in the table are for these homogenates.

Each data item shown represents the mean of the duplicate assays.

activities from unripe fruit preparations but made no difference to the levels with those from over-ripe fruit. In general, the data obtained for the 3 glycosidase activities measured were comparable with the data obtained for these glycosidases in unripe and over-ripe mesocarp shown in Table 25, although Experiment 2 in Table 27 showed rather lower activities at the over-ripe stage. It appears, therefore, that inhibition by polyphenols is not a significant phenomenon affecting the assay of mango preparations.

The soluble glycosidase activities were investigated after centrifuging the homogenates, from mesocarp at ripening stages 1,2 and 4 at 40,000 g for 1 h. The % recovery figures for the supernatants shown in Table 28 are based upon activities in Table 25. Generally speaking, there is an increase in the % recovery of activity in the soluble extracts for all the glycosidases present as the tissue goes from the unripe to the 'turning' stage. In addition, with β-D-glucosidase, α-L-arabinopyranosidase and, perhaps, β-D-glucuronidase there appears to be a further increase in extractability as the tissue goes from 'turning' to over-ripe. The high protein levels in the homogenates (Table 25) compared with soluble extracts (Table 28) result in higher specific activities for all the glycosidases in the latter at all stages of ripening. With ripening, extractable protein increases but the specific activities of β-D-galactosidase, α-D-mannosidase and α-L-arabinopyranosidase all increase.

TABLE 28 Enzyme activities in 0.1M sodium acetate buffer, pH 5.0 cell free extracts of mango mesocarp.

Enzyme	Unripe			Turning			Over-ripe		
	Total activity (units*/ 100 g wet tissue)	Specific activity (units/ mg protein)	% Recovery+	Total activity (units/ 100 g wet tissue)	Specific activity (units/ mg protein)	% Recovery+	Total activity (units/ 100 g wet tissue)	Specific activity (units/ mg protein)	%Recovery+
β-D-Glucosidase	2.0	0.9	11.8	4.8	1.0	26.5	17.3	2.3	74.9
α-D-Galactosidase	9.9	4.5	24.4	25.9	5.4	55.6	27.0	3.6	51.4
β-D-Galactosidase	42.0	19.1	29.4	318.2	66.3	81.4	1368.0	182.4	71.8
α-D-Mannosidase	5.9	2.7	19.8	212.2	44.2	78.8	305.3	40.7	84.0
α-L-Arabinopyranosidase	nil	nil	0	4.8	1.0	36.9	77.3	10.3	73.6
β-D-Glucuronidase	nil	nil	0	6.2	1.3	59.6	3.7	0.5	88.1
Total protein (mg/100 g wet tissue)	2.2			4.8			7.5		

*1 unit of enzyme activity = 1 μmol p-nitrophenol liberated/h.

+ Based on total enzyme activities of whole homogenates (see Table 25).

Enzyme extracts were prepared as described in II.6.(a) from pooled mesocarp slices obtained from 3 mangoes at each stage of ripeness.
Glycosidase enzyme assays were carried out in duplicate as described in II.2.(b) 3.2. and protein assays were also carried out in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1.

Each data item shown represents the mean of the duplicate assays.

The next experiment performed was a comparison of the 3 major glycosidase activities in both whole homogenates and 0.1M acetate buffer, pH 5.0 extracts of mesocarp from single mangoes with the previously-obtained activities from pooled mango mesocarp at the unripe and over-ripe stages. The data obtained for the single fruits are shown in Table 29 and comparing these activities with those in whole homogenates, (see Table 25) the same general pattern of an increase in β-D-galactosidase, α-D-mannosidase and α-L-arabinopyranosidase activities with ripening were noted. However, there was some notable differences between the two sets of results. Firstly, total extracted protein from the single fruits was higher, especially at the over-ripe stage (424 compared to 210 mg/100 g fresh weight tissue), contributing to lower specific activities for all enzymes at both ripening stages in the single fruits. Secondly, the levels of activity of α-D-mannosidase were lower in the single fruits at both ripening stages and α-L-arabinopyranosidase activity in the homgenates from single over-ripe fruit (152.6 units/100 g tissue) was higher than the level (105.0 units/100 g tissue) from the pooled over-ripe fruits.

Comparing the extracted activities (i.e. comparing Table 29 with Table 28) the most notable differences were at the over-ripe stage. The extracted and specific activities of β-D-galactosidase and α-L-arabinopyranosidase from the single fruit at this ripening stage were higher than these activities from pooled, over-ripe fruits. In contrast, the extracted activity (305.3 units/100 g tissue, 84% recovery) of α-D-mannosidase from pooled, over-ripe fruits was consid-

TABLE 29. Enzyme activities in whole homogenates and buffer (0.1M sodium acetate, pH 5.0) cell free extracts of mango mesocarp.

Whole Homogenates	Unripe		Over-ripe	
	Total activity (units*/ 100 g wet tissue)	Specific activity (units/mg protein)	Total activity (units/100 g wet tissue)	Specific activity (units/mg protein)
Enzyme:				
β-D-Galactosidase	107.7	0.37	1908.0	4.50
α-D-Mannosidase	5.8	0.02	284.1	0.67
α-L-Arabinopyranosidase	nil	nil	152.6	0.36
Total Protein (mg/100 g wet tissue)	291		424	

Buffer Extract	Total activity units/100 g wet tissue)	% recovery†	Specific activity (units/mg protein)	Total activity (units/100 g wet tissue)	% recovery†	Specific activity (units/mg protein)
β-D-Galactosidase	41.1	38.2	15.8	1757.0	92.1	319.5
α-D-Mannosidase	6.0	103.4	2.3	134.7	47.4	24.5
α-L-Arabinopyranosidase	6.0	**	2.3	130.9	85.8	23.8
Total Protein (mg/100 g wet tissue)	2.6			5.5		

*1 unit of enzyme activity = 1 μmol p-nitrophenol liberated/h.

† Based on total activities in the whole homogenates. (**Measured total activity for α-L-arabinopyranosidase in the buffer extract of 6.0 units/100 g unripe tissue compared to nil activity in the corresponding homogenate would make the % recovery in the extract of this enzyme infinitely high! This inconsistent finding is believed to be the result of experimental artefact).

Enzyme preparations were made as described in II.6.(a) from single fruits at both stages of ripeness and assayed within 24 h of preparation.

Glycosidase enzyme assays were carried out in duplicate as described in II.2.(b) 3.2. and protein assays were also carried out in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1.

Each data item shown represents the mean of the duplicate assays.

erably higher than this activity (134.7 units/100 g tissue, 47.4% recovery) from the single, over-ripe fruit.

The most likely source of these differences is undetected variation between fruits since they were selected on the basis of appearance only. The extent of the variation suggests that for true, reliable mean values for levels of glycosidase activities it would probably be necessary to pool tissues from a much larger number of fruits but such experiments would be limited by cost and availability of the mangoes. The pooling of 3 fruits in the present study was clearly adequate for revealing the general pattern of changes in glycosidase activities with ripening but insufficient for satisfactorily eliminating variation between individual fruits at any given ripening stage.

One strange finding noted in Table 29 was that total activity for α-L-arabinopyranosidase of 6.0 units/100g (wet weight) tissue was measured in the buffer extract of unripe mango compared to nil activity detected in the corresponding whole homogenate from which this extract was obtained by centrifugation. If treated as a true finding, this would make the % recovery of α-L-arabinopyranosidase in the buffer extract (based on total activity in the whole homogenate) infinitely high! It is highly unlikely that a homogenate containing no measurable activity would reveal activity in the soluble fraction after removal of the homogenised tissue and this single finding was inconsistent with all other findings for glycosidase activities in the mango. In addition, the apparent activity in the extract was very low and the probable unreliability of such low measured activities has been

discussed previously (see p. 196). Hence, it was considered that this inconsistent finding for α-L-arabinopyranosidase activity in unripe fruit probably arose from experimental artefact.

A single experiment was conducted to look for α-L-arabinofuranosidase activity in mango mesocarp at the unripe, 'turning' and over-ripe stages of ripening. The substrate (p-nitrophenyl-α-L-arabinofuranoside) for this assay is not commercially available but a small quantity, sufficient for one experiment, was obtained from a private source (see Section II.1.(a), p. 75). It was considered important to look for this activity since it was shown that arabinose was lost from mango mesocarp cell walls during ripening (see Section III.1. (b), p. 162) and, as stated earlier, it is known that arabinose in primary cell wall polysaccharides occurs largely in the furanose form (see arabinan, p. 35 and arabinogalactan, p. 37). The levels of β-D-galactosidase activity were also measured as a 'control' to enable comparison between the levels of α-L-arabinofuranosidase and the previously-determined levels of other glycosidases present in mango mesocarp.

The results for this experiment are shown in Table 30 and a comparison of these with the results obtained for β-D-galactosidase in whole homogenates (Table 25), shows a similar pattern of increase of β-D-galactosidase activity with ripening. The unripe mango mesocarp contained a low level of α-L-arabinofuranosidase, the total and specific activities of which increased somewhat with ripening. However,

TABLE 30. A comparison of β-D-galactosidase and α-L-arabinofuranosidase activities in whole homogenates of mango mesocarp in 0.1M sodium acetate buffer (pH 5.0) with cell free extracts (0.1M acetate buffer, pH 5.0 from the homogenates.

ENZYME	Unripe				Turning				Over-ripe			
	Homogenate		Cell-free extract		Homogenate		Cell-free extract		Homogenate		Cell-free extract	
	Total activity (units*/ 100g wet tissue)	Specific activity (units/ mg protein)	Total activity (units/ 100g wet tissue)	Specific activity (units/ mg protein)	Total activity (units/ 100g wet tissue)	Specific activity (units/ mg protein)	Total activity (units/ 100g wet tissue)	Specific activity (units/ mg protein)	Total activity (units/ 100g wet tissue)	Specific activity (units/ mg protein)	Total activity (units/ 100g wet tissue)	Specific activity (units/ mg protein)
β-D-Galactosidase	108.1	0.38	25.1	10.95	103.3	0.21	19.1	8.10	1904.8	4.47	1346.1	183.64
α-L-Arabinofuranosidase	8.7	0.03	18.6(?)	8.10	20.0	0.04	16.8	7.14	37.2	0.09	38.9	5.30
Total protein (mg/100g wet tissue)	284.5		2.3		491.9		2.4		426.1		7.3	

*1 unit of enzyme activity = 1 μmol p-nitrophenol liberated/h.

Enzyme preparations were made as described in II.6.(a) from pooled mesocarp slices obtained from 3 fruits at each stage of ripeness.

Glycosidase enzyme assays were carried out in duplicate as described in II.2.(b) 3.2. and protein assays were also carried out in duplicate by the method of Lowry et al (1951) as described in II.2.(b) 1.1.

Each data item shown represents the mean of the duplicate assays.

even in the over-ripe fruit, the level of this enzyme activity was very low compared to those of β-D-galactosidase and α-D-mannosidase and no firm conclusion may be safely drawn about the possible contribution of α-L-arabinofuranosidase to the degradation of arabinose-containing polymers in mango cell walls during ripening.

III.4. (c) Glycosidase activities in extracts of acetone powders, prepared from mango mesocarp (at various stages of ripeness), with 0.5M acetate buffer, pH 5.0.

The final experiment conducted was to utilise a different extraction technique for glycosidases, namely to prepare acetone-powders from pooled mango mesocarp tissue at various stages of ripening and to extract these preparations with acetate buffer of higher molarity (0.5M) in an attempt to enhance the levels of activity extracted by concentrating the protein and perhaps removing inhibitors. Total (soluble and bound) activity was estimated by re-suspending the acetone powders in 0.5M sodium acetate buffer, pH 5.0, prior to incubation with the substrate (see Section II.6. (b), p.135).

However, by comparing the results obtained with the re-suspended acetone powders from mango mesocarp (Table 31) to the corresponding activities present in whole homogenates of mesocarp tissues (Table 25), the main difference noted was the somewhat lower values for total tissue protein, at all ripening stages, obtained in the case of the re-suspended acetone powders, which contributed to the apparently higher specific activities for the glycosidases in these preparations. It is possible that some protein was denatured by acetone rendering it less readily extractable with 1M alkali for protein assay. A second difference was the lower level of α-D-mannosidase activity in re-suspended acetone powder prepared from unripe mango mesocarp than in the corresponding

TABLE 31. Enzyme activities in acetone powder homogenates of mango mesocarp in 0.5M sodium acetate buffer (pH 5.0).

Enzyme		Unripe		Turning		Over-ripe	
		Total activity units*/ 100 g wet tissue)	Specific activity (units/ mg protein)	Total activity (units/ 100 g wet tissue)	Specific activity (units/ mg protein	Total activity (units/ 100 g wet tissue)	Specific activity (units/ mg protein)
β-D-Glucosidase	(a)	26.7	0.17	29.0	0.16	40.9	0.23
	(b)	16.5	0.10	34.4	0.20	37.7	0.25
α-D-Galactosidase	(a)	40.8	0.26	41.6	0.23	58.7	0.33
	(b)	36.3	0.22	48.2	0.28	64.9	0.43
β-D-Galactosidase	(a)	141.3	0.90	391.0	2.16	1470.3	8.26
	(b)	117.2	0.71	424.8	2.47	1541.7	10.21
α-D-Mannosidase	(a)	1.6	0.01	208.1	1.15	331.1	1.86
	(b)	3.3	0.02	246.0	1.43	309.5	2.05
α-L-Arabinopyranosidase	(a)	7.8	0.05	7.2	0.04	94.3	0.53
	(b)	1.6	0.01	12.0	0.07	105.7	0.70
β-D-Glucuronidase	(a)	12.6	0.08	12.7	0.07	12.5	0.07
	(b)	8.2	0.05	17.2	0.10	13.6	0.09
Total protein (mg/100 g wet tissue)	(a)	157		181		178	
	(b)	165		172		151	

* 1 unit of enzyme activity = 1 μmol p-nitrophenol liberated/h.

For each developmental stage 3 fruits were selected, the mesocarp tissues combined and duplicate (a and b) acetone powders prepared, as described in II.6.(b).

Glycosidase activities in the re-suspended acetone powders were assayed in duplicate as described in II.2.(b) 3.2. and protein assays were also carried out in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1.

Each data item shown represents the mean of the duplicate assays.

buffer homogenate of this tissue whereas the levels in 'turning' and over-ripe tissue were comparable. In both types of enzyme preparation (Tables 25 and 31) it is noteworthy that α-D-mannosidase appears to be tightly bound to the particulate fraction in unripe mango. The enzyme cannot be unstable to the acetone treatment since its activity does not suffer from 'exposure' to acetone at the 'turning' and over-ripe stages but it may be less stable to acetone when tightly bound. It is possible that some specific characteristic of the location and bound state of α-D-mannosidase in the unripe mango wall renders it particularly susceptible to exposure to acetone (even at -20^{o}) although such a hypothesis is, admittedly, speculative.

Apart from difference in α-D-mannosidase activities, the results obtained with the two types of homogenates were essentially similar for all the other glycosidases and the pattern of changes in activities during ripening (Tables 25 and 31) was generally comparable. Summarising the two sets of results, low activities of β-D-glucosidase and α-D-galactosidase were present and very low levels of β-D-glucuronidase; all three of these activities underwent little change during ripening. The total and specific activities of α-D-mannosidase increased greatly between the unripe and 'turning' stages of ripening, with some increase continuing to the over-ripe stage. β-D-Galactosidase activity similarly increased during ripening with a particularly marked change between the 'turning' and over-ripe stages. α-L-Arabinopyranosidase activity was

very low at the initial ripening stages and displayed a marked change only between the 'turning' and over-ripe stages.

Inspection of the glycosidase activities in the soluble supernatants obtained from the acetone powders (Table 32) confirms the results obtained with the supernatants from buffered extracts of tissue (Table 28), and indicates that all glycosidases detected in the mango are more tightly bound to the particulate cell wall components in unripe fruit than in fruit at later stages of ripening, the % recoveries of total activities in the soluble supernatants (compared to total activities in the homogenates of acetone powders) being lower at the unripe than at later stage of ripeness.

III.5 Interactions between soluble mango extracts and unripe mango cell wall preparations.

III.5. (a) Introduction

To further investigate changes in the mango cell wall associated with ripening the ability of soluble 'enzyme' extracts from mangoes at different ripening stages to liberate carbohydrate from insoluble cell wall prepared from unripe mangoes was examined. Unripe cell wall was chosen as the most suitable substrate on the assumption that the wall at this ripening stage would have undergone minimal enzymic modification in the intact fruit. Carbohydrate solubilised from the wall was analysed for the presence of free monosaccharides and for glycans which, it was anticipated, might be liberated from wall polysaccharides by endo-glycanase activity.

TABLE 32. Enzyme activities in 0.5M sodium acetate buffer (pH 5.0) cell free extracts of acetone powders of mango mesocarp.

Enzyme		Unripe			Turning			Over-ripe		
		Total activity (units*/ 100 g wet tissue)	Specific activity (units/ mg protein)	% Recovery+	Total activity (units/ 100 g wet tissue)	Specific activity (units/ mg protein)	% Recovery+	Total activity (units/ 100 g wet tissue)	Specific activity (units/ mg protein)	% Recovery+
β-D-Glucosidase	(a)	nil	nil	0	21.0	10.5	72.4	40.0	15.4	97.8
	(b)	1.1	0.7	6.7	21.1	8.8	61.3	25.0	13.9	66.3
α-D-Galactosidase	(a)	16.0	10.7	39.2	23.0	11.5	55.3	46.0	17.7	78.4
	(b)	16.0	10.0	44.1	27.1	11.3	56.2	38.0	21.1	58.6
β-D-Galactosidase	(a)	10.1	6.7	7.1	336.0	168.0	85.9	1286.0	494.6	87.5
	(b)	18.1	11.3	15.4	301.9	125.8	71.1	1064.0	591.1	69.0
α-D-Mannosidase	(a)	nil	nil	0	149.0	74.5	71.6	276.1	106.2	83.4
	(b)	nil	nil	0	163.0	70.0	68.3	223.9	124.4	72.3
α-L-Arabinopyranosidase	(a)	1.0	0.7	12.8	4.0	2.0	55.6	59.0	22.7	62.6
	(b)	1.0	0.6	60.0(?)	6.0	2.5	50.0	60.0	33.3	56.8
β-D-Glucuronidase	(a)	nil	nil	0	7.0	3.5	55.1	13.0	5.0	104.0
	(b)	nil	nil	0	9.1	3.8	52.9	10.1	5.6	74.3
Total protein (mg/100 g wet tissue)	(a)		1.5			2.0			2.6	
	(b)		1.6			2.4			1.8	

* 1 unit of enzyme activity = 1 μmol p-nitropehnol liberated/h.

+ Based on total enzyme activities of whole homogenates of acetone powders (Table 31).

For each developmental stage 3 fruits were selected, the mesocarp tissues combined and duplicate (a + b) acetone powders prepared which were subsequently extracted with buffer, as described in II.6.(b).

Glycosidase activities in the buffer extracts were assayed in duplicate as described in II.2.(b) 3.2. and protein assays were also carried out in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1.

Each data item shown represents the mean of the duplicate assays.

III.5. (b) Properties of the 'enzyme' preparations.

A complicating factor was the presence in the 'enzyme' preparations of significant amounts of soluble carbohydrate. Crude buffer extracts of mango tissue possessed considerable viscosity presumably due to the presence of polysaccharides and the solutions also contained free sucrose, glucose and fructose which were identified by thin-layer chromatography on silica gel by the method of Menzies et.al. (1973) (see II.2. (a) 1.). Fructose and sucrose gave reactions for ketose sugars when thin-layer plates were stained by the 2-stage method with 4-aminobenzoic acid or with naphthoresorcinol.

Attempts were made to remove this carbohydrate by precipitation of the enzymic protein from the crude extracts either by saturation to 80% with ammonium sulphate or addition of acetone (-20^{o}) to the extract at 4^{o} (final acetone concentration 50%, v/v). In both cases the precipitates were recovered by centrifugation and then subjected to dialysis (see II.4. (b)). However, the total carbohydrate content of the resulting solutions, as measured by the phenol/H_2SO_4 method of Dubois et al. (1956), could not be reduced below 0.2%, w/v. In this respect it is noteworthy that in earlier attempts to extract the enzyme polygalacturonase from mango tissue, sequential extraction with NaCl/EDTA solution by the method of Hobson (1962), ammonium sulphate precipitation, dialysis, chromatography on Sephadex G-100 and Amicon ultrafiltration of the protein-containing fractions in the chromatographic eluate (see II.3. (b) 7., p. 118 and Fig.32, p. 119) failed to

remove reducing sugars, as measured by the microcuprimetric method of Nelson (1944), from the extract (see Table 18, p. 185). It is clear that removal of carbohydrates (including carbohydrates of low molecular weight) from association with protein in extracts of mango tissue is not a simple or straightforward task.

The final enzyme preparations after precipitation with ammonium sulphate (80%-saturated) or acetone (50%, v/v) still contained free sucrose, glucose and fructose and, after removal of these free sugars by electrophoresis on glass fibre paper by the method of Jarvis et al. (1977), polysaccharide fractions were obtained which on acid hydrolysis yielded glucose, xylose, galactose, arabinose and another sugar which was tentatively identified as mannose (see also pp. 79 and 241 and Tables 43 and 44). Polysaccharides were therefore presumably co-precipitating with the protein and glycoproteins may also have been present in the extracts. It is difficult to explain, however, why free sucrose, glucose and fructose were not removed from the mango 'enzyme' extracts by the procedures used but it is worth noting that a similar problem has been encountered in the preparation of enzyme extracts from banana fruits (Caygill, personal communication). These low molecular weight carbohydrates must presumably be physically bound in the colloidal polysaccharide/protein mixture.

In retrospect, it is conceded that this contamination of mango 'enzyme' extracts with carbohydrates placed serious limitations on the value and possibilities of these

enzyme extract/cell wall incubation experiments and that more rigorous and far reaching attempts, utilising a wide range of gel permeation and ion-exchange media, to remove the carbohydrate contaminants should have been made.

β-D-Galactosidase activities in the final enzyme preparations and in the initial crude extracts were compared to give some indication of the likely losses of other carbohydrate-degrading activities which occurred during the preparative procedures. The recoveries of β-D-galactosidase and protein during preparation of the 'enzymes' are shown in Table 33. The results indicate that the precipitation procedures removed only moderate amounts (22-34%) of β-D-galactosidase activity and it was hoped that other relevant hydrolytic activities would also survive these procedures.

III.5. (c). Release of soluble carbohydrate from re-suspended cell walls by 'enzyme' preparations.

Acetone-precipitated 'enzyme' preparations from unripe fruit released no soluble carbohydrate from cell wall. Similar preparations from over-ripe fruit catalysed an apparent 14% increase in the total carbohydrate in the soluble fraction, although the high level of background carbohydrate in the incubation mixtures (referred to in III.5.(b)) prevented the attributing of statistical significance (by the Student's t-test) to this change. The change did not occur in the boiled enzyme 'control' incubation. These results are shown in Table 34.

With the ammonium sulphate-precipitated 'enzyme'

TABLE 33. Protein and β-D-galactosidase activity in 'enzyme' preparations from mango.

		Total extractable protein (mg/100 g fresh tissue)	β-D-Galactosidase (*units/100 g fresh tissue)	β-D-Galactosidase % recovery
Crude extracts	(Unripe fruit	10.8 (3.0)	112 (18)	100
	(Over-ripe fruit	39.0 (10.3)	994 (91)	100
Acetone-precipitated preparations	(Unripe fruit	8.2 (2.6)	75 (18)	67
	(Over-ripe fruit	3.0 (1.2)	776 (91)	78
$(NH_4)_2SO_4$-precipitated preparations	Over-ripe fruit	25.2 (10.8)	658 (70)	66

*1 unit of enzyme activity = 1 μmol p-nitrophenol liberated/h.

'Enzyme' preparations were made from single mango fruits. Separate crude extracts were prepared from 4 unripe fruits and from 8 over-ripe fruits. Separate acetone-precipitated preparations were made from 4 unripe fruits and from 3 over-ripe fruits. Separate ammonium sulphate-precipitated preparations were made from 5 over-ripe fruits.
In each preparation β-D-galactosidase activity was assayed in duplicate as described in II.2.(b) 3.2 and protein was assayed in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1.

Each data item shown is the mean of the several values obtained with the separate preparations (each value being itself the mean of duplicate assays) with standard error of the mean shown in parenthesis.

Details of the methods used for 'enzyme'preparation are given in II.4.(b).

TABLE 34. Total soluble carbohydrate in incubation mixtures of acetone-precipitated mango enzyme preparations with cell walls from unripe mangoes.

Incubation mixture	Total soluble carbohydrate (mg/ml) in incubation mixture			
	Zero time		24 h incubation	
	Unripe preparation*	Over-ripe preparation+	Unripe preparation*	Over-ripe preparation+
Enzyme + cell wall suspension	0.45 (0.08)	0.43(0.10)	0.46(0.09)	0.49(0.10)
Boiled enzyme + cell wall suspension	0.43 (0.07)	0.40(0.08)	0.44(0.08)	0.39(0.09)
Enzyme + buffer	0.38 (0.08)	0.35(0.07)	0.38(0.07)	0.37(0.09)
Buffer + cell wall suspension	0.06 (0.01)	0.06(0.01)	0.06(0.01)	0.06(0.01)

* Protein concentrations in incubation mixtures were 18.0 (6.7) µg/ml

\+ Protein concentrations in incubation mixtures were 6.4 (2.3) µg/ml

Resuspended cell walls were separately incubated with 'enzyme' preparations made from 4 separate unripe fruits and 3 separate over-ripe fruits.

In each incubation mixture total soluble carbohydrate was assayed in duplicate by the phenol/H_2SO_4 method of Dubois et al (1956) as described in II.2.(b) 2.2. and protein was assayed in duplicate by the method of Lowry et al (1951) as described in II.2.(b) 1.1.

Each data item is the mean of the several values obtained with the separate preparations (each value being itself the mean of duplicate assays) with standard error of the mean shown in parenthesis.

Full experimental details are given in II.4.(c).

preparations from over-ripe fruit there was an even greater (28%) apparent release of soluble carbohydrate from the wall although, again, statistical significance could not be attributed to the change for the reason stated. The release of carbohydrate was again prevented by boiling the 'enzyme' fraction (see Table 35). It is important to note that the protein concentration in the incubation mixture (65.2µg/ml) was here ten times greater than in the case of the incubation mixture with acetone-precipitated preparation from over-ripe fruit (6.4µg/ml - see Table 34).

An attempt to locate more carbohydrate-releasing activity was made by extracting mango tissues with acetate buffer containing Triton-X-100 to release potentially-bound enzymes. The results obtained by incubating these preparations with cell wall are given in Table 36. It should be noted that the incubation mixtures in this experiment contained significantly higher levels of total carbohydrate than the incubation mixtures containing precipitated enzyme fractions (cf. 0.36 mg/ml in enzyme + buffer incubation mixture in Table 35 with 1.20 mg/ml in the corresponding incubation mixture in Table 36). In this regard it should be noted that the crude initial extract of mango tissue (without additional precipitation procedures) containing Triton X-100 was used, suggesting that the precipitation procedures with acetone and ammonium sulphate (whilst failing to reduce carbohydrate in 'enzyme' preparations to acceptably low levels, as discussed in III.5(b)) did remove some carbohydrate from the crude extracts. Protein levels in

TABLE 35. Total soluble carbohydrate in incubation mixtures of ammonium sulphate-precipitated enzyme preparations with cell walls from unripe mangoes.

Incubation mixture	Total soluble carbohydrate (mg/ml) in incubation mixture	
	Zero time	24 h incubation
Enzyme + cell wall suspension	0.39 (0.06)	0.50 (0.06)
Boiled enzyme + cell wall suspension	0.40 (0.05)	0.39 (0.04)
Enzyme + buffer	0.36 (0.04)	0.36 (0.05)
Buffer + cell wall suspension	0.06 (0.02)	0.06 (0.02)

Protein concentrations in incubation mixtures were 65.2 (20.3) µg/ml

Resuspended cell walls were separately incubated with 'enzyme' preparations made from 5 separate over-ripe fruits.

In each incubation mixture total soluble carbohydrate was assayed in duplicate by the phenol/H_2SO_4 method of Dubois et al (1956) as described in II.2.(b) 2.2. and protein was assayed in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1.

Each data item shown is the mean of the 5 values obtained with the 5 separate preparations (each value being itself the mean of duplicate assays) with standard error of the mean shown in parenthesis.

Full experimental details are given in II.4.(c).

TABLE 36. Total soluble carbohydrate in incubation mixtures of Triton-extracted mango enzyme preparations with cell walls from unripe fruit.

Incubation mixture	Total soluble carbohydrate (mg/ml) in incubation mixture			
	Zero time		24 h incubation	
	Unripe preparation	Over-ripe preparation	Unripe preparation	Over-ripe preparation
Enzyme + cell wall suspension	1.35	1.70	1.35	1.85
Boiled enzyme + cell wall suspension	1.35	1.75	1.35	1.70
Enzyme + buffer	1.20	1.55	1.25	1.55
Buffer + cell wall suspension	0.18	0.25	0.25	0.25

In single experiments resuspended cell walls were incubated with 'enzyme' preparations made from pooled tissues from 3 mangoes at each ripening stage, extracted in acetate buffer - Triton X-100.

In each incubation mixture total soluble carbohydrate was assayed in duplicate by the phenol/H_2SO_4 method of Dubois et al. (1956) as described in II.2·(b) 2.2.

Protein concentrations were not determined owing to interference by the Triton in the Lowry et al. (1951) assay.

Full experimental details are given in II.4·(f).

incubation mixtures containing Triton X-100-extracted 'enzyme' preparations were not measured because of the interference of Triton X-100 with the Lowry et al. (1951) protein assay. The data in Table 36 indicate that 'enzyme' extracts of over-ripe fruit made in the presence of Triton X-100 exhibited no apparent marked increase in carbohydrate-releasing activity against cell walls, compared to the activity of ammonium sulphate-precipitated 'enzyme' preparations from over-ripe fruit (Table 35).

TLC analysis of all the incubation mixtures containing acetone-precipitated 'enzyme' preparations (Table 37) showed the presence of sucrose together with smaller amounts of galactose, glucose, fructose and arabinose: in the case of over-ripe fruit 'enzyme' the levels, particularly in the case of sucrose, glucose and fructose, were higher than in the incubation mixtures containing 'enzyme' from unripe fruit.

The acetone-precipitated unripe mango preparations when incubated alone showed little change, either qualitatively or quantitatively, in carbohydrate constituents as judged by thin-layer chromatography and these preparations also exhibited no marked activity towards the cell walls as judged by galactose release.

When the acetone-precipitated over-ripe preparations were incubated with cell walls the most significant observation was the increase in the galactose content of the incubation mixtures and inspection of the control digests indicated that this must have been wall-derived.

TABLE 37. TLC-detectable soluble sugars in incubation mixtures of acetone-precipitated mango enzyme preparations with cell walls from unripe mangoes.

Incubation mixture		Soluble sugars (µg/ml) in incubation mixture			
		Zero time		24 h incubation	
		Unripe preparation*	Over-ripe preparation+	Unripe preparation*	Over-ripe preparation+
Enzyme + cell wall suspension	Sucrose	30.0(5.7)	37.5(8.5)	27.9(7.4)	29.5(6.9)
	Galactose	1.6(1.2)	5.9(1.1)	5.3(2.5)	30.0(5.8)
	Glucose	6.1(1.7)	9.4(0.8)	10.9(1.8)	26.6(2.4)
	Fructose	5.9(1.1)	8.7(0.2)	7.7(0.8)	18.9(3.6)
	Arabinose	0.1(0.1)	0.8(0.1)	0.2(0.2)	1.6(0.6)
Boiled enzyme + cell wall suspension	Sucrose	30.1(5.2)	39.8(8.4)	25.1(5.0)	37.3(8.5)
	Galactose	1.0(1.0)	0.5(0.5)	1.1(1.1)	0.5(0.5)
	Glucose	4.8(1.5)	7.3(0.7)	6.1(1.3)	7.7(0.6)
	Fructose	5.6(0.9)	7.7(0.8)	6.4(1.6)	8.0(0.8)
	Arabinose	0.1(0.1)	0.1(0.1)	0 (0)	0.2(0.2)
Enzyme + buffer	Sucrose	24.2(5.6)	42.2(11.1)	23.1(5.7)	29.2(7.3)
	Galactose	0.9(0.9)	0.8(0.4)	2.3(1.6)	3.0(0.7)
	Glucose	5.3(1.5)	7.9(0.3)	8.5(0.9)	17.8(2.1)
	Fructose	6.3(1.0)	8.1(0.4)	8.1(1.0)	17.0(2.7)
	Arabinose	0.1(0.1)	0.3(0.3)	0 (0)	1.0(0.5)
Buffer + cell wall suspension	Sucrose	1.3(0.5)	4.7(2.6)	1.2(0.3)	5.0(2.5)
	Galactose	0 (0)	0 (0)	0 (0)	0 (0)
	Glucose	0 (0)	0 (0)	0 (0)	0 (0)
	Fructose	0 (0)	0 (0)	0 (0)	0 (0)
	Arabinose	0 (0)	0 (0)	0 (0)	0 (0)

*Protein concentrations in incubation mixtures were 18.0 (6.7) µg/ml
+Protein concentrations in incubation mixtures were 6.4 (2.3) µg/ml
Resuspended cell walls were separately incubated with enzyme preparations made from 4 separate unripe fruits and 3 separate over-ripe fruits.
In each incubation mixture soluble sugars were separated by TLC as described by Menzies and Mount (1975) (II.2. (a) 1.) and assayed on the single chromatograms by duplicate densitometric assays as described in II.2. (b) 2.1.; and protein was assayed in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1. Each data item shown is the mean of the several values obtained with the separate preparations (each value being itself the mean of duplicate assays) with standard error of the mean shown in parenthesis. Full experimental details are given in II.4. (c).

The mean level of galactose in the soluble fraction of enzyme-cell wall incubation mixtures after 24 hours of incubation (30.0µg/ml) was significantly different from the mean level at zero time, (5.9µg/ml) at the 2.5% level, applying the 2-tail Student's t-test with the Bessel correction for small samples (t = 4.01 with 4 degrees of freedom) (see Table 37).

No firm conclusions could be drawn concerning levels of sucrose, fructose and glucose in incubation mixtures because of contamination of 'enzyme' preparations with these sugars. Contamination of cell wall preparations with starch (see p. 157) also ruled out firm conclusions regarding release of glucose from wall glucans.

The results obtained with ammonium sulphate-precipitated 'enzyme' fractions from over-ripe fruit (see Table 38 and Fig.42 (a), (b)) were essentially similar and again free galactose was released from the cell wall. The mean level of galactose in the soluble fraction of enzyme-cell wall incubation mixtures after 24 hours of incubation (29.1µg/ml) was significantly different from the mean level at zero time (5.3µg/ml) at the 0.1% level, applying the same test of significance as above (t = 5.54 with 8 degrees of freedom).

In Table 39, where mango tissues from pooled fruits were extracted with acetate buffer containing Triton X-100, the results obtained with the over-ripe fruit extract again indicated that this extract released soluble galactose when incubated with cell wall. It is notable

TABLE 38. TLC-detectable soluble sugars in incubation mixtures of ammonium sulphate-precipitated enzyme preparations from over-ripe fruit with cell walls from unripe mangoes.

Incubation mixture	Sugar	Concentration of sugars (µg/ml) in incubation mixture	
		Zero time	24 h incubation
Enzyme + cell wall suspension	Sucrose	18.7 (6.2)	13.0 (5.7)
	Galactose	5.3 (0.9)	29.1 (4.2)
	Glucose	9.0 (1.3)	20.9 (2.1)
	Fructose	8.1 (0.8)	11.8 (2.1)
	Arabinose	2.2 (0.5)	3.4 (0.4)
Boiled enzyme + cell wall suspension	Sucrose	23.6 (7.2)	21.4 (6.2)
	Galactose	0 (0)	0 (0)
	Glucose	7.4 (1.1)	7.9 (1.0)
	Fructose	6.4 (0.8)	7.3 (0.7)
	Arabinose	1.4 (0.4)	1.8 (0.5)
Enzyme + buffer only	Sucrose	18.2 (6.1)	13.0 (4.7)
	Galactose	0 (0)	0 (0)
	Glucose	9.3 (1.4)	13.4 (2.2)
	Fructose	8.3 (1.2)	11.6 (2.1)
	Arabinose	1.9 (0.4)	2.7 (0.7)
Buffer + cell wall suspension	Sucrose	0.5 (0.3)	0.7 (0.3)
	Galactose	0 (0)	0 (0)
	Glucose	0 (0)	0 (0)
	Fructose	0 (0)	0 (0)
	Arabinose	0 (0)	0 (0)

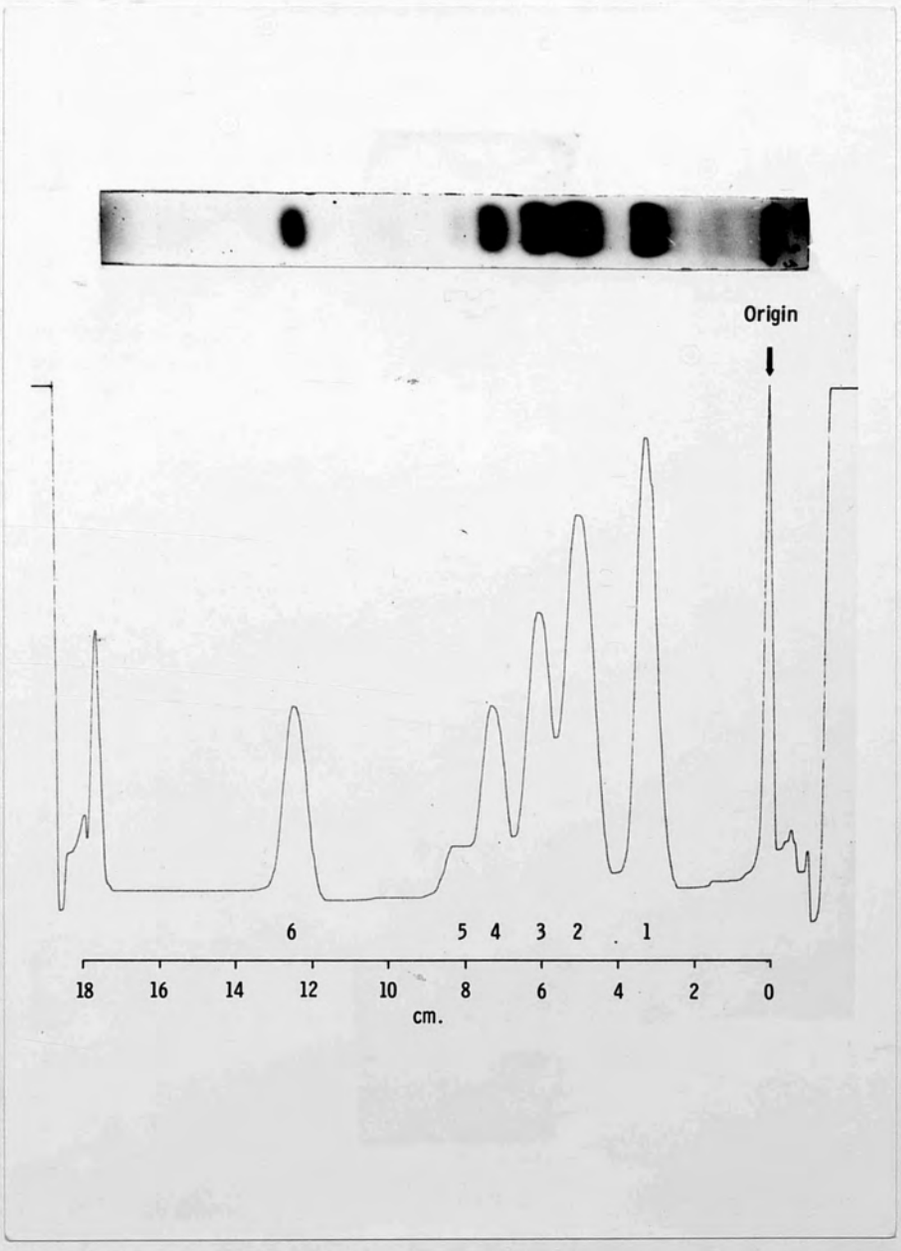
Protein concentrations in the incubation mixtures were 65.2 (20.3) µg/ml.

Resuspended cell walls were separately incubated with 'enzyme' preparations made from 5 separate over-ripe fruits.

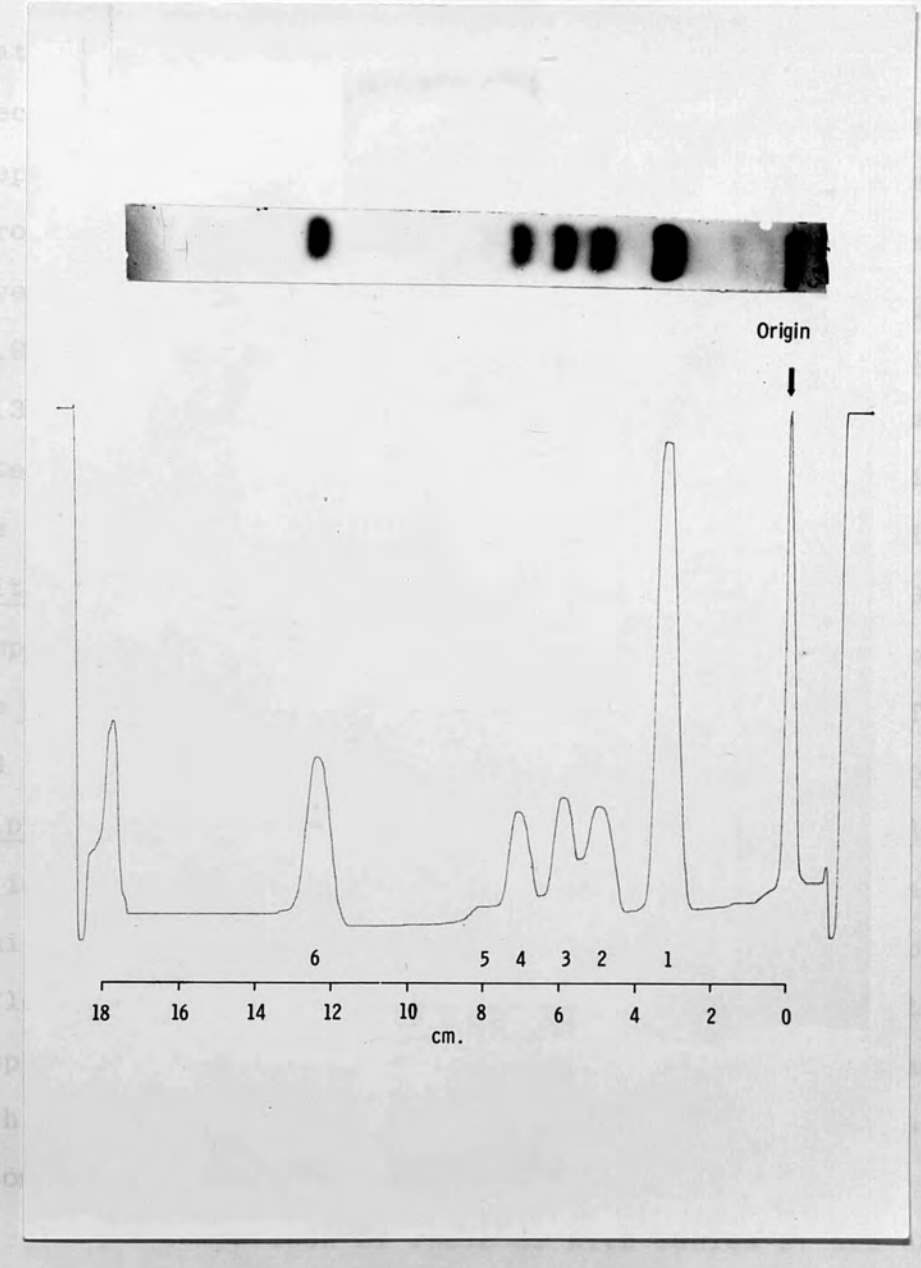
In each incubation mixture soluble sugars were separated by TLC as described by Menzies and Mount (1975) (II.2. (a) 1.) and assayed on the single chromatograms by duplicate densitometric assays as described in II.2.(b) 2.1.; and protein was assayed in duplicate by the method of Lowry et al. (1951) as described in II.2. (b) 1.1.

Each data item shown is the mean of the 5 values obtained with the 5 separate preparations (each value being itself the mean of duplicate assays) with standard error of the mean shown in parenthesis.

Full experimental details are given in II.4.(c).



(b)



(a)

Fig. 42. Thin-layer chromatograms by the method of Menzies and and Mount (1975) of free monosaccharides in an incubation mixture of an ammonium sulphate-precipitated 'enzyme' preparation from over-ripe mangoes with cell walls from unripe mangoes.

(a) zero time; (b) 24 h incubation.

1 - Sucrose; 2 - galactose; 3 - glucose;

4 - fructose; 5 - arabinose; 6 - rhamnose. (I.S.)

For experimental details see Section II.4. (c).

that utilising this extract, which was subjected to no further precipitation procedures and which was used immediately after preparation, the level of galactose in the soluble fraction at zero time (5µg/ml) was closely comparable to the corresponding levels in incubation mixtures containing acetone-precipitated (5.9µg/ml - see Table 37) and ammonium sulphate-precipitated (5.3µg/ml) - see Table 38) 'enzyme' preparations, whereas after 24 hours of incubation, galactose concentration in the soluble fraction of the incubation mixture containing Triton-extracted 'enzyme' had reached 82µg/ml (Table 39) compared to only 29.5µg/ml in the corresponding fraction of the acetone-precipitated 'enzyme' incubation mixture (Table 37) and 29.1µg/ml in the corresponding fraction of the ammonium sulphate-precipitated 'enzyme' incubation mixture (Table 38). It is clear that the extract incorporating Triton contained a higher level of galactose-liberating activity, possibly reflecting enhancement by Triton of the extraction of the responsible enzyme or the freshness of the Triton extract, with no activity losses due to precipitation procedures, or a combination of these factors.

Comparison of Table 39 with Tables 37 and 38 also reveals that the Triton extract contained much higher levels of free sucrose, fructose and glucose than the 'enzyme' preparations resulting from precipitation with acetone or ammonium sulphate (e.g. cf. fructose concentrations at zero time in the over-ripe 'enzyme + buffer' incubation mixtures - 158µg/ml in Table 39 compared with 8.1µg/ml and 8.3µg/ml in Tables 37 and 38 respectively. This is consistent with the higher level of total carbohydrate detected by the

TABLE 39. TLC-detectable soluble sugars in incubation mixtures of Triton-extracted mango enzyme preparations with cell walls from unripe fruit.

Incubation mixture	Sugar	Concentration of sugars (µg/ml) in incubation mixture			
		Unripe preparation	Over-ripe preparation	Unripe preparation	Over-ripe preparation
Enzyme + cell wall suspension	Sucrose	> 700	> 700	> 700	> 700
	Galactose	0	5	0	82
	Glucose	128	116	98	287
	Fructose	147	149	122	303
	Arabinose	0	0	0	0
Boiled enzyme + cell wall suspension	Sucrose	> 700	> 700	> 700	> 700
	Galactose	0	0	0	0
	Glucose	79	107	95	91
	Fructose	98	146	120	126
	Arabinose	0	0	0	0
Enzyme + buffer	Sucrose	> 700	> 700	> 700	> 700
	Galactose	0	0	0	0
	Glucose	122	131	110	199
	Fructose	138	158	134	212
	Arabinose	0	0	0	0

In single experiments resuspended cell walls were incubated with 'enzyme' preparations made from pooled tissues from 3 mangoes at each ripening stage, extracted in acetate buffer - Triton X-100.

In each incubation mixture soluble sugars were separated by TLC as described by Menzies and Mount (1975) (II.2.(a) 1.) and assayed on the single chromatograms by duplicate densitometric assays as described in II.2.(b) 2.1. Protein concentrations were not determined owing to interference by the Triton in the Lowry et al. (1951) assay.

Full experimental details are given in II.4.(f).

phenol/H_2SO_4 method of Dubois et al. (1956) in the Triton-extracted 'enzyme' preparations (Table 36), which has been previously discussed (p. 220), and suggests that the precipitation procedures with acetone and ammonium sulphate are responsible for removing a considerable proportion of the free sugars from the crude mango extracts.

Although (as discussed above in comparing Table 39 with Tables 37 and 38) Triton X-100 extracts of over-ripe fruit released more galactose detected by TLC from cell walls than 'enzyme' preparations from over-ripe fruit prepared by precipitation with acetone or ammonium sulphate, this was not reflected in any apparent marked increase in the total carbohydrate solubilised from the wall, detectable by the phenol/H_2SO_4 method of Dubois et al. (1956), induced by the Triton extract as compared with the ammonium sulphate-prcipitated 'enzyme' preparation (see comparison between Tables 35 and 36, pp.221 & 222. However, it must be borne in mind that free galactose in the incubation mixtures constituted only a small part of the total soluble carbohydrate. For example, in the 'enzyme + cell wall' incubation mixtures containing ammonium sulphate-precipitated 'enzyme' preparations after 24 hours of incubation the mean soluble galactose concentration was 29.1µg/ml (Table 38) compared with a mean total soluble sugar concentration of 78.2µg/ml (Table 38) and a mean soluble carbohydrate concentration of 500µg/ml (Table 35). Thus a major part of the carbohydrate in this soluble fraction evidently consists of soluble polysaccharide, and the differences in the level of galactose released are

not likely to significantly affect the levels of total soluble carbohydrate detected by the phenol/H_2SO_4 method of Dubois et al. (1956) (galactose in the soluble fraction of the corresponding incubation mixture containing Triton extract constituted only 82µg/ml (Table 38) out of a total soluble carbohydrate concentration of 1.85mg/ml (Table 36)).

The 'enzyme(s)' in the ammonium sulphate-precipitated over-ripe fruit preparations responsible for galactose release from cell walls exhibited optimal activity at about pH 5.1 (Fig.43). The time course for galactose liberation from cell wall by this enzyme preparation is shown in Fig.44.

III.5. (d) Soluble acidic carbohydrate of low molecular weight in incubation mixtures of mango 'enzyme' preparations with unripe mango cell walls.

The occurrence of low molecular weight acidic carbohydrates in the soluble fractions from the experiments in which the acetone- and ammonium sulphate-precipitated 'enzyme' preparations were incubated with cell walls (see Tables 40 and 41) was examined by elution of the Zerolit DM-F resins, which were used to deionise these fractions, with dilute sulphuric acid. No free galacturonic or glucuronic acids could be detected in the acidic eluates by thin-layer chromatography (Menzies and Mount (1975)) but all the eluates (Tables 40 and 41) gave positive uronic acid reactions with the carbazole reagent; the highest values for uronic acid content were obtained from samples containing the ammonium sulphate-precipitated 'enzyme' fraction from

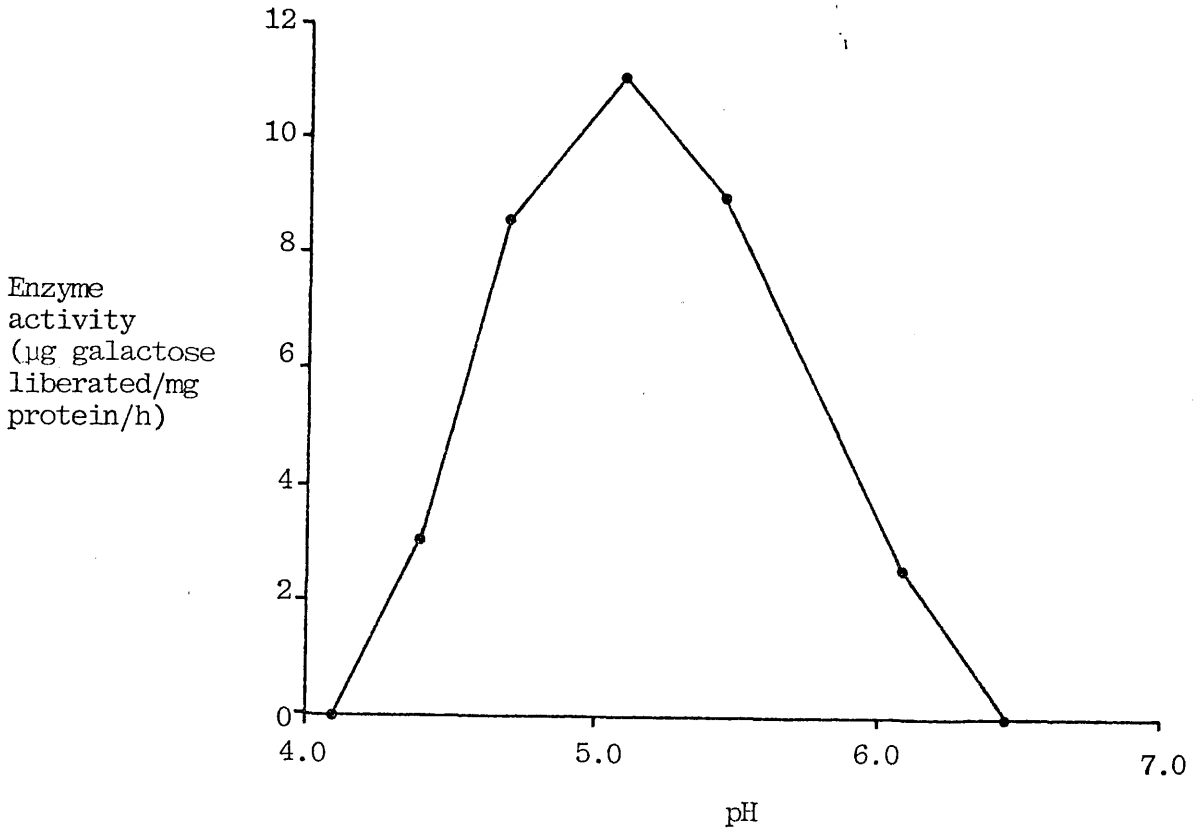


Fig.4 3 Effect of pH on the liberation of galactose from unripe cell walls by an ammonium sulphate-precipitated 'enzyme' fraction from pooled mesocarp of 3 over-ripe mangoes.

The 'enzyme' preparation was incubated with cell walls resuspended in 0.1M acetate buffer adjusted to appropriate pH values.

Galactose in the soluble fractions was separated by TLC as described by Menzies and Mount (1975) (II.2.(a) 1.) and assayed on the single chromatograms by duplicate densitometric assays as described in II.2.(b) 2.1. Protein was assayed in duplicate by the method of Lowry et al. (1951) as described in II.2.(b)1.1 Each point shown is the mean of the duplicate assays.

Full experimental details are given in II.4·(c).

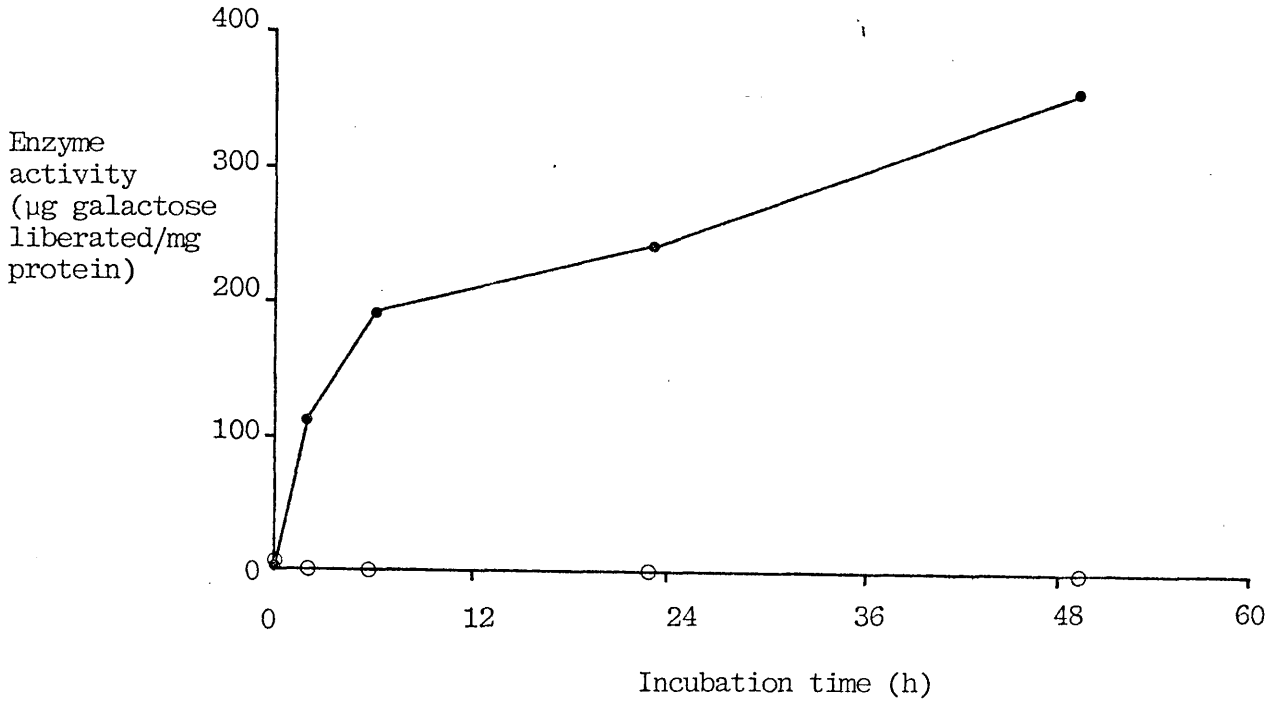


Fig.44. Galactose release from unripe cell walls by an ammonium-sulphate precipitated 'enzyme' fraction from pooled mesocarp of 3 over-ripe mangoes.

— • — , Active enzyme; — o — , boiled enzyme control.

The 'enzyme' preparations were incubated with cell walls resuspended in 0.1M acetate buffer, pH 4.5. At timed intervals aliquots were removed from incubation mixtures, cooled in melting ice and undegraded cell walls removed by centrifugation and 'Millipore' filtration.
Galactose in the soluble fractions was separated by TLC as described by Menzies and Mount (1975) (II.2.(a) 1.) and assayed on the single chromatograms by duplicate densitometric assays as described in II.2.(b) 2.1. Protein was assayed in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1.

Each point shown is the mean of the duplicate assays.
Full experimental details are given in II.4.(c).

TABLE 40. Soluble acidic carbohydrate bound by Zerolit-DM/F ion-exchange resin in incubation mixtures of acetone-precipitated enzyme preparations with cell walls from unripe mangoes.

Incubation mixture	Acidic carbohydrate (expressed as µg galacturonic acid/ml) in incubation mixtures			
	Unripe preparation*	Over-ripe preparation+	Unripe preparation*	Over-ripe preparation+
Enzyme + cell wall suspension	11 (3.5)	38 (5.0)	12 (1.5)	36 (7.0)
Boiled enzyme + cell wall suspension	16 (3.5)	43 (7.0)	15 (3.0)	41 (10.0)
Enzyme + buffer	10 (3.0)	39 (6.0)	10 (3.5)	36 (5.0)
Buffer + cell wall suspension	8 (4.0)	12 (3.0)	8 (3.0)	12 (4.0)

*Protein concentrations in incubation mixtures were 18.0 (6.7)µg/ml

+Protein concentrations in incubation mixtures were 6.4 (2.3)µg/ml

Resuspended cell walls were separately incubated with enzyme preparations made from 4 separate unripe fruits and 3 separate over-ripe fruits. Incubation mixtures were cooled in melting ice and, after removal of undegraded cell walls by centrifugation and 'Millipore' filtration, were treated with Zerolit DM-F ion-exchange resin as described in II.2.(b) 2.5. After water-washing of the resin, acidic carbohydrate remaining bound to the resin was eluted with dilute H_2SO_4 (see II.2. (b) 2.5.). In each acidic eluate total uronic acid content was assayed in duplicate by the method of Bitter and Muir (1962) as described in II.2.(b) 2.5. Protein in the incubation mixtures was assayed in duplicate by the method of Lowry et al. (1951) as described in II.2.(b) 1.1.

Each data item shown is the mean of the several values obtained with the separate preparations (each value being itself the mean of duplicate assays) with standard error of the mean shown in parenthesis.

Full experimental details of the incubation procedures are given in II.4.(c).

TABLE 41 Soluble acidic carbohydrate (total uronic acids) bound by Zerolit DM/F ion-exchange resin in incubation mixtures of ammonium sulphate-precipitated enzyme preparations with cell walls from unripe mangoes.

Incubation mixture	Experiment	Acidic carbohydrate (expressed as mg galacturonic acid equivalents/ml) in incubation mixtures	
		Zero time	24 h incubation
Enzyme + cell wall suspension	1*	0.14 (0.31)++	0.12 (0.37)++
	2+	0.04 (0.58)	0.05 (0.71)
Boiled enzyme + cell wall suspension	1	0.15 (0.33)	0.15 (0.30)
	2	0.04 (0.56)	0.05 (0.53)
Enzyme + buffer	1	0.15 (0.25)	0.14 (0.25)
	2	0.04 (0.51)	0.04 (0.52)
Buffer + cell wall suspension	1	0.02 (0.02)	0.02 (0.02)
	2	0 (0.08)	0 (0.08)

* Protein concentration in incubation mixture was 30.5 µg/ml

\+ Protein concentration in incubation mixture was 132.0 µg/ml

The results shown above represent 2 separate experiments using enzyme preparations from separate over-ripe fruits.

Enzyme preparations were incubated with cell walls resuspended in 0.1M acetate buffer, pH 4.5. Incubation mixtures were cooled in melting ice and, after removal of undegraded cell walls by centrifugation and 'Millipore' filtration, were treated with Zerolit DM-F ion-exchange resin as described in II.2.(b) 2.5. After water-washing of the resin, acidic carbohydrate remaining bound to the resin was eluted with dilute H_2SO_4 (see II.2.(b) 2.5).

In each acidic eluate total uronic acid content was assayed in duplicate by the method of Bitter and Muir (1962) as described in II.2. (b) 2.5. Protein in the incubation mixtures was assayed in duplicate by the method of Lowry et al. (1951) as described in II.2. (b) 1.1.

++ N.B. In this Table all figures in parenthesis show total soluble carbohydrate (mg/ml), as measured in duplicate by the phenol/H_2SO_4 method of Dubois et al. (1956), in incubation mixtures before treatment with Zerolit DM-F ion exchange resin.

Each data item shown is the mean of the duplicate assays.
Full experimental details of the incubation procedures are given in II.4. (c).

over-ripe fruit.(see Table 41). In the latter case, hydrolysis of the acidic eluates from the Zerolit resin with polygalacturonase (see II.4. (e)) yielded galacturonic acid (detected by thin-layer chromatography - Menzies and Mount (1975)) from incubation mixtures containing the 'enzyme' preparation and from incubation mixtures containing only cell wall material and buffer.

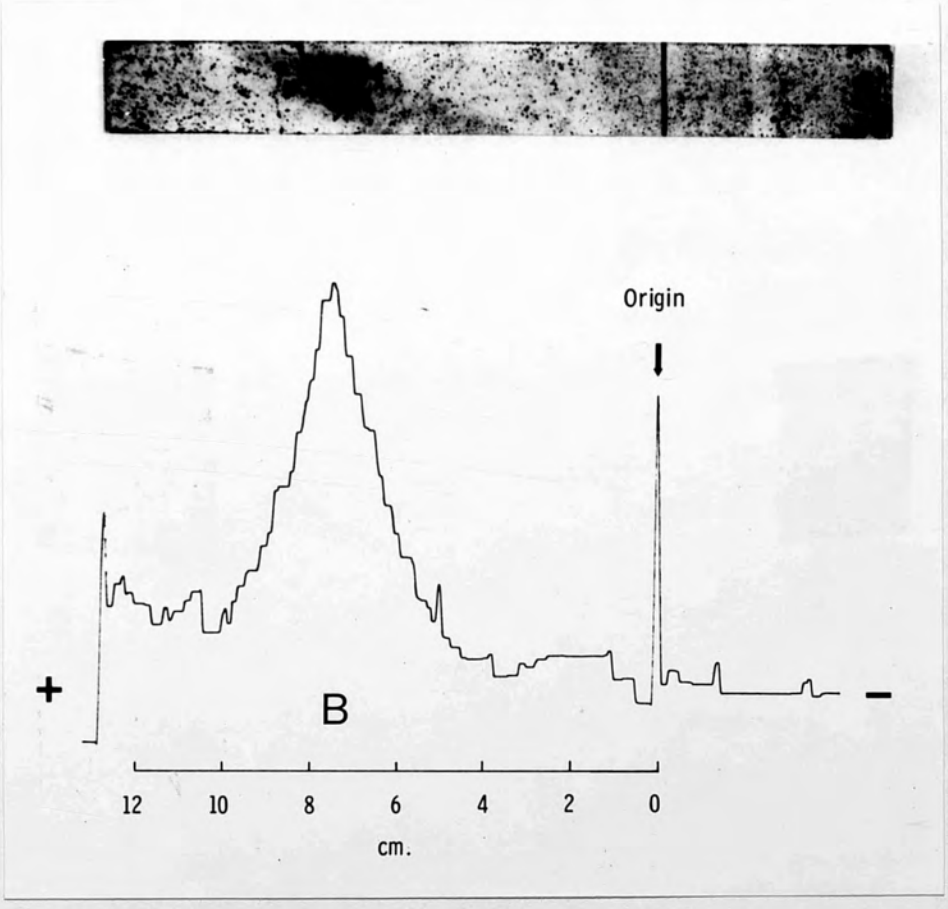
No uronic acid-containing material appeared to be released by enzyme activity when cell walls were incubated with either the acetone-precipitated (Table 40) or ammonium-sulphate precipitated (Table 41) 'enzyme' fractions. It was apparent that most of the soluble acidic material originated from the 'enzyme' preparations and, to a lesser degree, from the cell wall preparations where there appeared to be leaching of a small amount of bound uronic acid into the buffer solution. It was assumed that the acidic material derived from both these fractions was polyuronide of relatively low molecular weight as Zerolit DM-F does not bind high molecular weight polygalacturonic acid (see Table 3, Section II.2. (b) 2.5.).

The higher carbazole 'readings' given by the acidic Zerolit eluates derived from the over-ripe acetone-precipitated 'enzyme' preparations compared with the unripe (Table 40) presumably result from the presence in the former of higher levels of pectic materials which are derived from the cell wall in fruits at this stage of development.

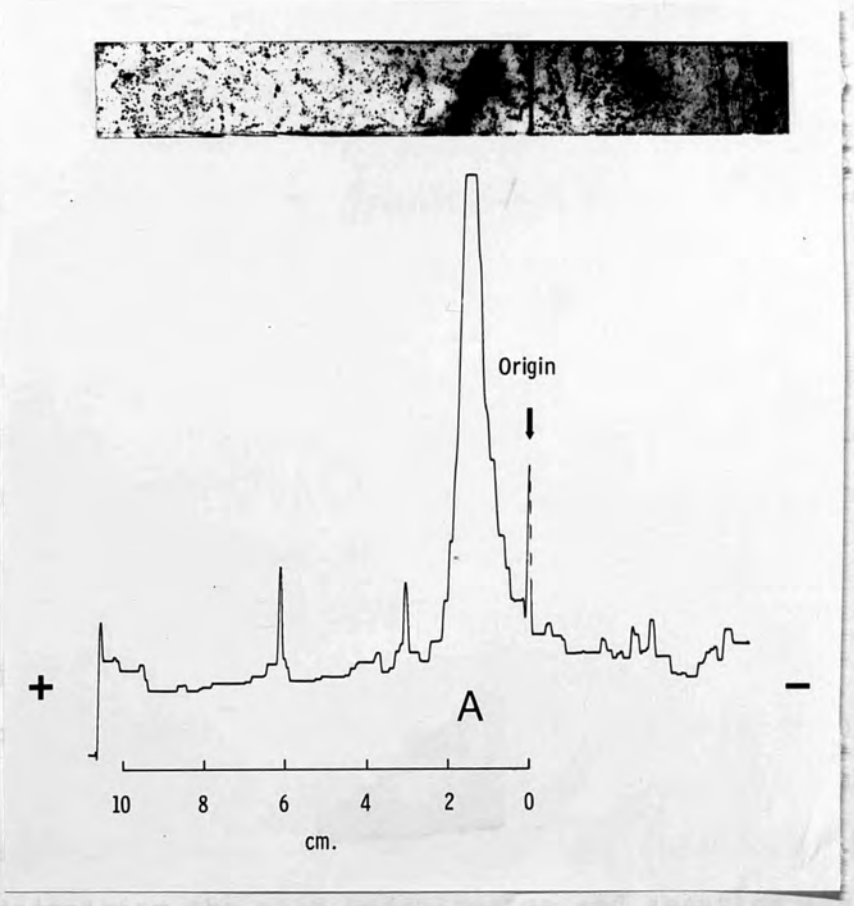
III.5. (e). Investigation of polysaccharides in soluble fractions of incubation mixtures of mango 'enzyme' preparations with unripe mango cell walls.

In the final stages of this study attempts were made to investigate the possible release of polysaccharides from resuspended cell walls as might occur, for example, if the mango enzyme preparations possessed endo-galactanase or other endoglycanase activities. To achieve this the deionised, soluble fractions of incubation mixtures of cell walls with acetone-precipitated 'enzyme' fractions from over-ripe and unripe mangoes were examined by glass-fibre paper high voltage electrophoresis (Jarvis et al. (1977)) in 0.05M sodium tetraborate, pH 9.0. The polysaccharides were visualised by heating with an acidified solution of 1-naphthol in methanol and the resulting bands were scanned with a recording densitometer (see II.2. (a) 3.).

In the case of the acetone-precipitated enzyme preparation from over-ripe fruit, the incubation mixture at zero time contained a single major polysaccharide fraction (A) with low electrophoretic mobility (Fig.45 (a)). After 24 h incubation this had disappeared (Fig.45 (b)) and was replaced by a single major fraction (B) with a significantly higher mobility. When these electrophoretic patterns were compared with those obtained from control incubations containing (1), enzyme alone and (2), boiled enzyme with cell wall they were observed to be identical



(b)



(a)

Fig.45 Glass-fibre paper high-voltage electrophoresis of the 'neutral' polysaccharides in the soluble fraction of an incubation mixture of an acetone-precipitated 'enzyme' preparation from over-ripe fruit with cell walls from unripe fruit.

(a) zero time; (b) 24 h incubation.

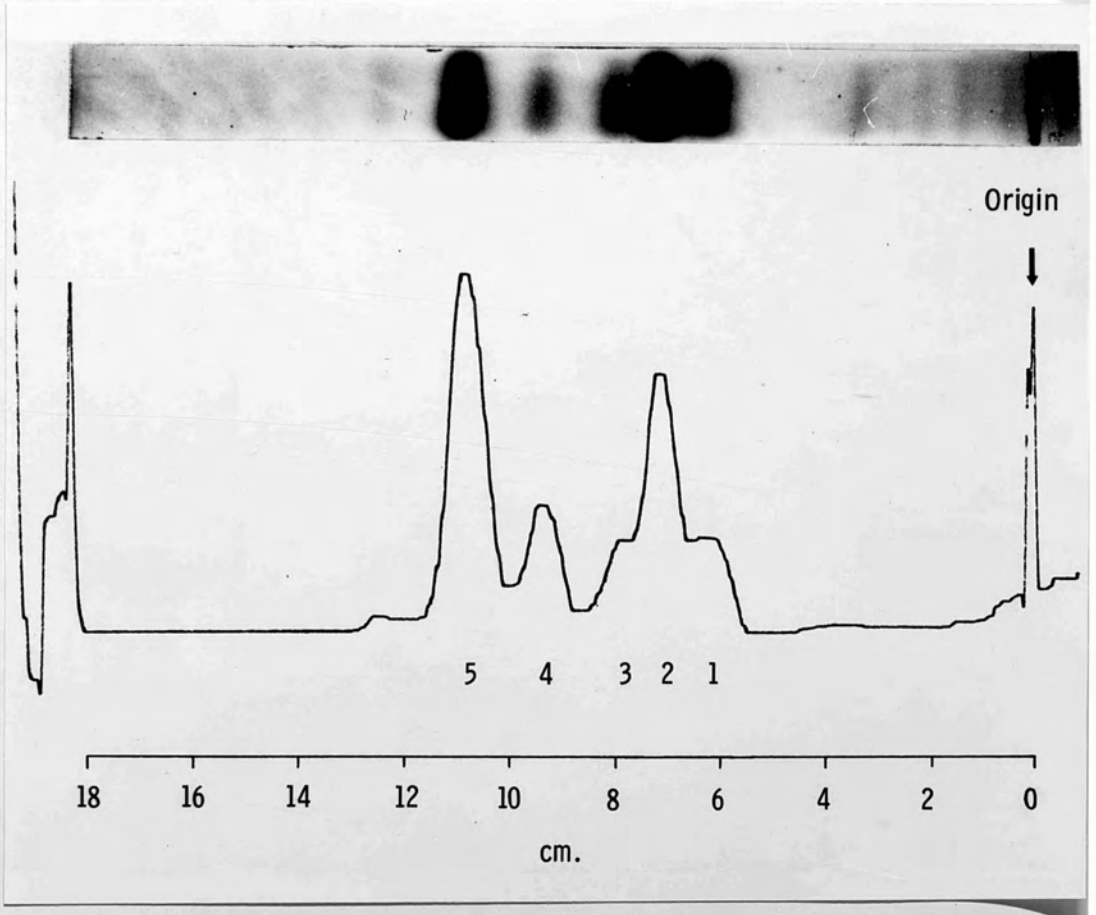
After incubation undegraded cell walls were removed by centrifugatio and 'Millipore' filtration and the soluble fraction was de-ionised with Zerolit DM-F before electrophoresis. Carbohydrate on the electrophoretograms was detected by heating with acidified 1-naphtho in methanol and scanned with a recording densitometer.

Full experimental details are given in II.4.(c) and II.2.(a) 3.

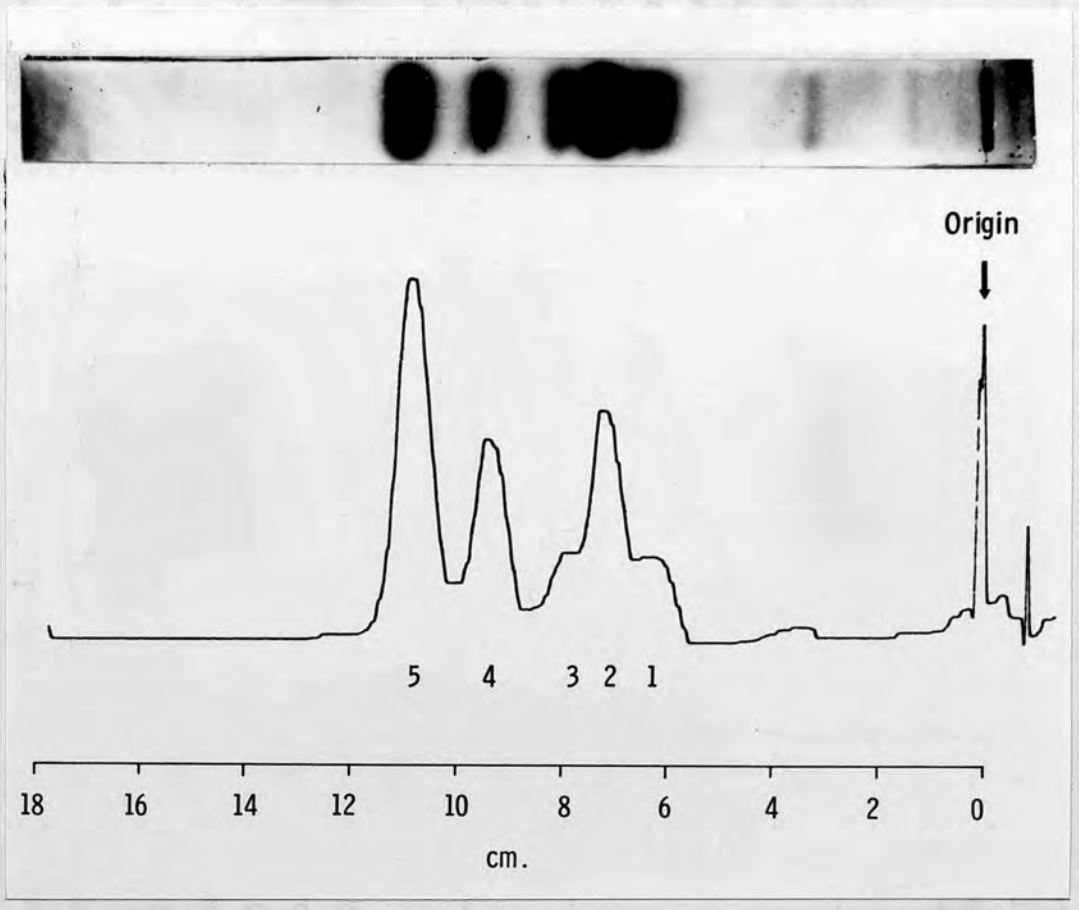
at zero time. After 24 h incubation the 'enzyme alone' control showed no significant difference from the pattern in Fig.45 (b) and there was no change during incubation in the case of the 'boiled enzyme' control. Incubation of cell walls alone yielded no detectable carbohydrate bands on the electrophoretogram.

The overall results therefore suggested that soluble polysaccharide present in the 'enzyme' preparation was being enzymically degraded during the incubation and that there was little or no release into the soluble fraction of cell wall polysaccharides.

The covalently bound monosaccharides present in the major polysaccharide bands, A and B, from the cell wall-enzyme incubation mixtures at zero time (Fig.45(a)) and 24 h (Fig.45 (b)) respectively were examined by hydrolysing the polysaccharides on the paper with trifluoroacetic acid, deionising the acid hydrolysates and assaying the monosaccharide products by quantitative thin-layer chromatography (Menzies and Mount, 1975) - see II.4.(d) 2. The main monosaccharides present (Table 42 and Fig.46) appeared to be glucose and xylose together with significant amounts of galactose, mannose and arabinose but the proportions of the monosaccharides were similar in both polysaccharide fractions with the possible exception of arabinose which was somewhat lower in the fraction B obtained after 24 h incubation. The sugar which was tentatively identified as mannose did not give the ketose sugar reaction but, instead, reacted as an aldohexose in the 2-stage staining



(b)



(a)

Fig.46. Thin layer chromatograms of monosaccharides obtained by acid hydrolysis of polysaccharides isolated by glass-fibre paper electrophoresis from the deionised soluble fraction of an incubation mixture of an acetone-precipitated 'enzyme' preparation from over-ripe mangoes with unripe mango cell walls.

(a) Zero time (fraction A); (b) 24 h incubation (fraction B).

1 - Galactose; 2 - glucose 3 - mannose 4 - arabinose

5 - xylose.

Undegraded cell wall was removed from incubation mixtures by centrifugation and 'Millipore' filtration and the soluble fractions deionised with Zerolit DM-F before electrophoresis by the method of Jarvis et al.(1977) (see II.2.(a)3. Polysaccharide bands on the electrophoretograms were hydrolysed on the paper with trifluoroacetic acid and the acid hydrolysates de-ionised with Zerolit DM-F. Monosaccharides in the acid hydrolysates were separated by TLC as described by Menzies and Mount (1975) and scanned on a recording densitometer as described in II.2·(b) 2.1.

TABLE 42. Monosaccharides released by acid hydolysis of polysaccharide fractions obtained by glass-fibre paper electrophoresis of polysaccharides present in the deionised soluble fraction of an incubation mixture of an acetone-precipitated enzyme preparation from over-ripe mangoes with unripe mango cell walls (see Fig. 49 (a) and (b).

Monosaccharide	Bound monosaccharide (µg)/ml incubation mixture in major electrophoretic fractions.	
	Zero time Fraction A	24 h incubation Fraction B
Galactose	23.8	26.7
Glucose	67.3	74.2
Mannose	25.9	26.6
Arabinose	21.4	13.1
Xylose	43.4	42.7
Total soluble carbohydrate (µg/ml) in incubation mixture	425	505

Protein concentration in the incubation mixture was 1.2 µg/ml.

The results shown above represent a single experiment.

Undegraded cell wall was removed from incubation mixtures by centrifugation and 'Millipore' filtration and the soluble fractions de-ionised with Zerolit DM-F before electrophoresis by the method of Jarvis et al (1977). Polysaccharide bands on the electrophoretograms were hydrolysed on the paper with trifluoroacetic acid and the acid hydrolysates de-ionised with Zerolit DM-F. Monosaccharides in the acid hydrolysates were separated by TLC as described by Menzies and Mount (1975) and estimated on the single chromatograms by duplicate densitometric assays as described in II.2. (b). 2.1.

In the incubation mixtures total soluble carbohydrate was assayed in duplicate by the phenol/H_2SO_4 method of Dubois et al. (1956). and protein was assayed in duplicate by the method of Lowry et al. (1951).

Each data item shown is the mean of the duplicate assays.

Full experimental details are given in sections II.4.(c) and II.4.(d).

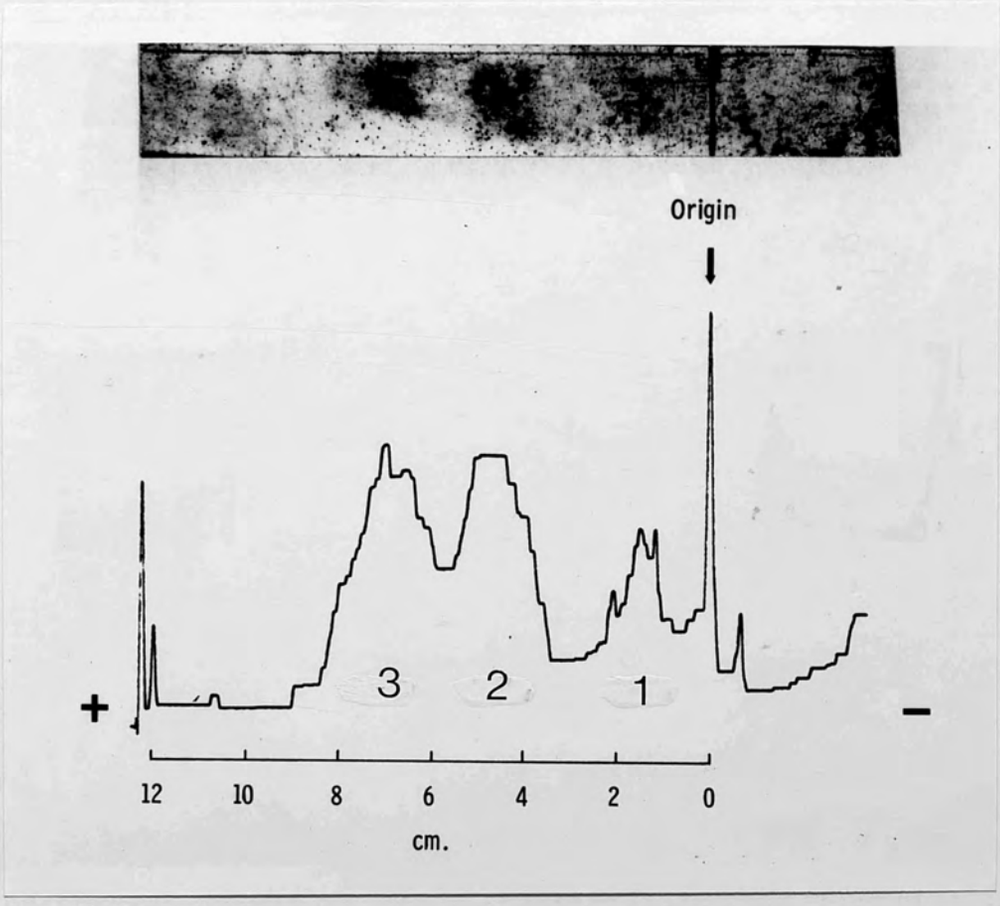
procedure with orthophosphoric acid and 4-aminobenzoic acid (see II.2. (a) 1., p. 79) which was used for these chromatograms. On this basis the likelihood of fructose contamination(fructose and mannose are not adequately resolved on TLC by the Menzies and Mount (1975) method - see p. 79) was discounted. It was also unlikely that free fructose or sucrose contaminating the 'enzyme' preparation would have similar electrophoretic mobility to the polysaccharide fractions in the 'enzyme' preparation.

The recoveries of monosaccharides from these electrophoretic fractions (based on the assay of the total soluble carbohydrate in the incubation mixtures by the phenol/H_2SO_4 method of Dubois et al., 1956) present in the incubation mixtures were of the order of 40% (see Table 42). This low value cannot seemingly be accounted for by the presence of acid-stable aldobiouronic acid linkages in the electrophoretic fractions since only minute traces of uronic acid could be found in the acid hydrolysates of these fractions (see Fig. 46 (a), (b)) and none was detected on the Zerolit resin which was used to deionise the acid hydrolysates before thin-layer chromatography. The trifluoroacetic acid hydrolysis procedure which was employed has been shown to liberate galacturonic acid from cell wall polysaccharides (see Section III.1 (b)). Presumably, the low recovery of monosaccharides in hydrolysates of the polysaccharide fractions from the electrophoretograms results from the removal of relatively low molecular weight acidic carbohydrate (see III.5. (d), p. 232) by the initial

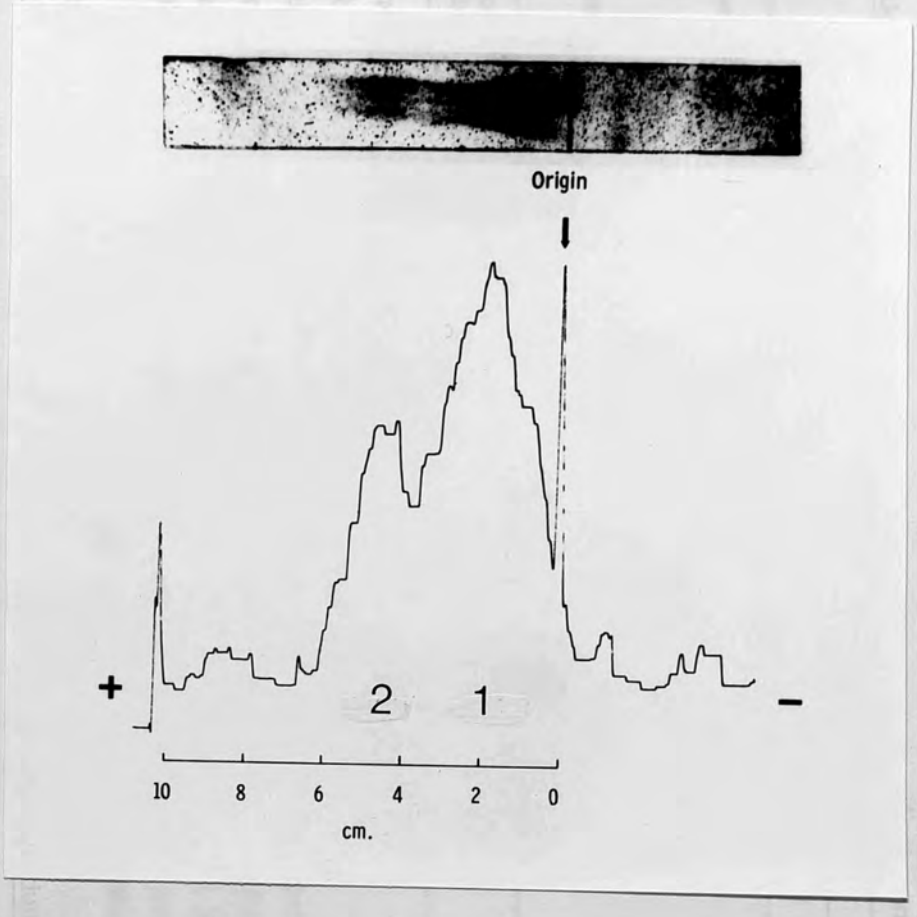
de-ionisation preceding electrophoresis, the separation of neutral sugars in the incubation mixtures from the polysaccharides during electrophoresis and the error introduced by a comparison of total carbohydrate and combined monosaccharides measured by two different assay methods.

A similar investigation was carried out using an acetone-precipitated enzyme fraction from unripe mangoes and as with the over-ripe preparation there were no electrophoretic indications of cell wall degradation leading to release of polymers. The electrophoretic patterns shown in Fig. 47 (a), (b) could, by examination of the controls, again be accounted for by changes in the enzyme preparation alone. They show a more complex mixture of carbohydrate polymers in both zero time and 24 h incubation mixtures in comparison with the studies involving the over-ripe 'enzyme' (Fig. 45 (a), (b)). At zero time the unripe mango preparation contained two major polysaccharide fractions, 1 and 2, and on incubation for 24 h the less mobile component 1 decreased and a third, rapidly-moving polymer (3) appeared.

Quantitative analysis by trifluoroacetic acid hydrolysis and thin-layer chromatography (Menzies and Mount, 1975) of the combined monosaccharides present in the polysaccharide fractions isolated from these glass-fibre electrophoretograms is given in Table 43. Recoveries were again low, presumably for the reasons given previously (see p. 245). Fraction 1 obtained from both zero time



(b)



(a)

Fig.47. Glass-fibre paper high-voltage electrophoresis of the 'neutral' polysaccharides in the soluble fraction of an incubation mixture of an acetone-precipitated enzyme preparation from unripe fruit with cell walls from unripe fruit.

(a) zero time; (b) 24 h incubation.

After incubation undegraded cell walls were removed by centrifugation and 'Millipore' filtration and the soluble fraction was de-ionised with Zerolit DM-F before electrophoresis. Carbohydrate on the electrophoretograms was detected by heating with acidified 1-naphthol in methanol and scanned with a recording densitometer.

Full experimental details are given in II.4.(c) and II.2.(a) 3.

TABLE 43. Monosaccharides released by acid hydrolysis of polysaccharide fractions obtained by glass-fibre paper electrophoresis of polysaccharides present in the de-ionised soluble fraction of an incubation mixture of an acetone-precipitated enzyme preparation from unripe mangoes with unripe mango cell walls (see Fig. 50 (a) and (b)).

Monosaccharide.	Bound monosaccharide (µg)/ml incubation mixture in major electrophoretic fractions				
	Zero time		24 h incubation time		
	Fraction 1	Fraction 2	Fraction 1	Fraction 2	Fraction 3
Galactose	33.4	77.3	17.2	52.3	16.4
Glucose	97.3	99.1	64.2	49.5	90.5
Mannose	30.5	26.7	16.3	14.2	61.7
Arabinose	25.2	13.9	13.9	11.4	7.0
Xylose	66.3	60.9	30.2	19.2	30.9
Total soluble carbohydrate (µg/ml) in incubation mixture	460		490		

Protein concentration in the incubation mixture was 2.0 µg/ml.

The results shown above represent a single experiment.

Undegraded cell wall was removed from incubation mixtures by centrifugation and 'Millipore' filtration and the soluble fractions de-ionised with Zerolit DM-F before electrophoresis by the method of Jarvis et al. (1977). Polysaccharide bands on the electrophoretograms were hydrolysed on the paper with trifluoroacetic acid and the acid hydrolysates de-ionised with Zerolit DM-F. Monosaccharides in the acid hydrolysates were separated by TLC as described by Menzies and Mount (1975) and estimated on the single chromatograms by duplicate densitometric assays as described in II.2. (b) 2.1.

In the incubation mixtures total soluble carbohydrate was assayed in duplicate by the phenol/H_2SO_4 method of Dubois et al. (1956) and protein was assayed in duplicate by the method of Lowry et al. (1951).

Each data item shown is the mean of the duplicate assays.

Full experimental details are given in Sections II.4. (c) and II.4.(d).

and 24 h incubations of the unripe enzyme preparation was particularly rich in glucose and xylose and both had similar overall compositions which, in turn, resembled the compositions of polysaccharide fractions A and B obtained from the over-ripe enzyme preparation (Table 42). Fraction 2 isolated after 24 h incubation (Fig. 47 (b)) possessed a higher proportion of galactose and a lower xylose content than the fraction with similar electrophoretic mobility at zero time (Fig. 47 (a)). Both of these fractions possessed a significantly higher galactose content than either fraction (A and B) isolated from over-ripe 'enzyme' preparations (Fig. 45 (a), (b)). Fraction 3, the apparently low molecular weight (fast-moving) product which appeared after incubation of the unripe 'enzyme' had a very low galactose content and a high proportion of mannose in comparison with fractions 1 and 2.

As previously mentioned, uronic acid 'spots' appearing on TLC of acid hydrolysates of the soluble polysaccharides separated by glass-fibre paper electrophoresis from mango 'enzyme' preparations (see Fig.46 (a), (b)) were too faint for quantitation with the reflectance densitometer and no galacturonic acid could be detected on the Zerolit DM-F resins used to de-ionise these acid hydrolysates before TLC (see p. 232). The acid hydrolysates also contained no trace of rhamnose, a sugar often associated with galacturonic acid in the pectic polymers of higher plants (see I.2. (c) 2.1., p. 29). These findings suggest that the soluble

polysaccharides present in the mango 'enzyme' preparations, which were revealed by electrophoretic separation, did not include pectic polymers of the rhamnogalacturonan or homogalacturonan types. However, it is critically conceded that the polysaccharide fractions on the glass-fibre electrophoretograms were not examined for galacturonic acid residues by digestion with polygalacturonase, although trifluoroacetic acid hydrolysis alone would be expected to release galacturonic acid in detectable quantity if the polysaccharides contained a significant proportion of galacturonan (see p. 261).

The soluble fractions which were applied to glass-fibre paper for electrophoresis did yield a reaction with the carbazole reagent (a reaction more marked in preparations from over-ripe than in unripe fruit) - see Table 44, but this is not conclusive evidence of the presence of significant amounts of uronic acid residues since neutral carbohydrate has been shown to interfere with the carbazole reaction (Bitter and Muir, 1962).

TABLE 44. Carbazole reaction for acidic carbohydrate (total uronic acids) in soluble fractions after Zerolit DM-F treatment from incubation mixtures of acetone-precipitated mango enzyme preparations with cell walls from unripe mangoes.

Incubation mixture	Exp.	Reaction to carbazole (expressed as µg galacturonic acid equivalents/ml in soluble fractions			
		Zero time		24 h incubation	
		Unripe preparation	Over-ripe preparation	Unripe preparation	Over-ripe preparation
Enzyme + cell wall suspension	1	0.08 (0.43)*	0.25 (0.43)+	0.07 (0.39)*	0.21 (0.51)+
	2	0.19 (0.64)**	0.26 (0.61)++	0.17 (0.68)**	0.27 (0.66)++
Boiled enzyme + cell wall suspension	1	0.08 (0.37)	0.20 (0.42)	0.07 (0.37)	0.25 (0.42)
	2	0.15 (0.61)	0.22 (0.54)	0.14 (0.65)	0.25 (0.53)
Enzyme + buffer	1	0.06 (0.31)	0.20 (0.36)	0.07 (0.29)	0.18 (0.37)
	2	0.15 (0.58)	0.24 (0.46)	0.14 (0.56)	0.25 (0.52)
Buffer + cell wall suspension	1	0 (0.08)	0 (0.06)	0 (0.07)	0 (0.06)
	2	0 (0.05)	0 (0.08)	0 (0.07)	0 (0.09)

*Protein concentration in incubation mixture was 15.3 µg/ml.
**Protein concentration in incubation mixture was 34.6 µg/ml.
+Protein concentration in incubation mixture was 1.2 µg/ml.
++Protein concentration in incubation mixture was 10.0 µg/ml.

The results shown above represent 2 separate experiments using enzyme preparations from separate unripe fruits and 2 separate experiments using enzyme preparations from separate over-ripe fruits.
After incubations undegraded cell walls were removed by centrifugation and 'Millipore' filtration and the soluble fractions treated with Zerolit DM-F ion-exchange resin. After Zerolit DM-F treatment total uronic acid content of soluble fractions was assayed in duplicate by the method of Bitter and Muir (1962). Protein in the incubation mixtures was assayed in duplicate by the method of Lowry et al. (1951)
N.B. In this table all figures in parenthesis show total soluble carbohydrate (mg/ml), as measured in duplicate by the phenol/H_2SO_4 method of Dubois et al. (1956), in incubation mixtures before treatment with Zerolit DM-F ion-exchange resin

Each data item shown is the mean of the duplicate assays.

Full experimental details of the incubation procedures are given in II.4.(c).

IV. DISCUSSION.

IV.1. Changes in mango cell walls during ripening.

IV.1. (a) Preparation of the cell wall material.

Homogeneity of the mango tissue as far as appearance was concerned was ensured by careful removal of the skin and underlying fibre before taking slices of the mesocarp for maceration. The attempts to ensure both genetic and developmental homogeneity of the mangoes selected at various ripening stages have already been described in Section II.1 (b).

Water-soluble materials were removed from the tissue by maceration with and several subsequent washes with phosphate buffer pH 6.9 at 20°. After each washing the insoluble material was recovered by centrifugation and the pellet then resuspended in fresh buffer by maceration. It was considered that this repeated procedure would be likely to remove both free sugars and readily water-soluble polymers such as the "soluble pectin" fraction. The single, brief buffer-washing procedure employed by Gross and Wallner (1979) during the isolation of walls from tomato pericarp did not remove all of the "soluble pectin" fraction; further material could be extracted with water at 30°. It is claimed by Knee (1973 b, c; 1978 a,b) that a similar fraction extracted at 20° from apple tissue consists of high molecular weight galacturonan which has been solubilised from the wall by enzyme action *in vivo* but not substantially degraded.

After repeated phosphate buffer washes the insoluble mango residue was recovered by centrifugation and

dehydrated with organic solvents (Section II.4. (a)). This is a necessary step in producing cell walls suitable for monosaccharide-composition studies but heating, as a means of dehydration was avoided as it produces artefacts formed by condensations between proteins and other wall components (Van Soest and McQueen, 1973). Solubilisation of polysaccharides can also sometimes occur with hot solvents (Gaillard, 1958). The product from unripe and turning fruit was white whereas wall material from ripe and over-ripe fruit retained a faint yellow/brown coloration which may have been due to oxidised phenolic compounds or adsorbed carotenoid pigments.

Delignification of these isolated walls (a procedure, for example, employed by Jermyn and Isherwood (1956) (cf Wise et al., 1946) to remove lignin from pear preparations containing secondary walls) was considered unnecessary since the primary walls of the parenchymatous mango mesocarp were thought to contain little of this phenolic polymer.

Attempts were made to remove starch from these cell wall preparations by extraction with a solution of chloral hydrate (see Section II. 7.(a) 2.) as described by Knee (1973c) for apple cell walls. However, the technique was not successful with mango since an iodine test showed that wall preparations from all stages of fruit development, except over-ripe, still contained traces of starch.

Table 4 (see Section III.1. (a), p. 158) suggests that chloral hydrate did remove some material from mango wall preparations and it is critically conceded that its

effect on mango walls was indeterminate and that the composition of the fraction extracted with chloral hydrate ought to have been fully established. The possibility that polysaccharides other than starch are extracted by this procedure should not be overlooked.

IV.1. (b) Fractionation of the mango cell walls.

Cell wall preparations were resolved into crude pectic, hemicellulosic and 'cellulosic' fractions by extraction with hot water followed by alkali (see Section II.7. (a) 3.).

Some degree of overlap between the wall fractions was accepted as a probable consequence of the relatively unsophisticated fractionation procedure employed. Extraction of pectin from the cell walls with water at 100^{o} would not have removed the insoluble calcium salt of pectic acid: this would have required the use of EDTA or ammonium oxalate solution. Pectin was recovered from the aqueous extract by freeze-drying. No attempt was made to separate other water-soluble polysaccharides dissolving at this stage from the pectic polysaccharides.

Extraction of the crude hemicellulosic fraction, after removal of the pectic polymers, was carried out using alkali at room temperature and under nitrogen, since a low temperature and the exclusion of oxygen is necessary to prevent extensive changes in these polysaccharides under high pH conditions (Southgate, 1976). The alkaline extract was neutralised with acetic acid and the hemicellulosic fraction precipitated with ethanol and collected by centrifugation. It was dried by careful washing

with organic solvents at -10^{o} and finally placed over P_2O_5.

The cell wall residue which is insoluble in strong alkali is often designated α-cellulose and in most tissues this β-1,4-linked glucan is the major component. However, some xylose is almost invariably present in such α-cellulose fractions, and frequently other sugars are also present in traces (Northcote, 1972; Southgate, 1976; Preston, 1979). Whether these represent contaminants or a measure of the heterogeneity of the cellulose microfibrils is uncertain. However, it is reasonable to suppose that they are derived from polysaccharides that are intimately associated with cellulose and that, from the point of view of cell wall structure, they form part of the microfibrillar network (see also page 22).

IV. 1. (c) Changes in the total cell wall content of mango tissue during ripening.

Reference to Table 4 (see Section III.1. (a), p. 158) indicates that there is a marked decline in the "total cell wall" (after chloral hydrate treatment) content of mango mesocarp tissue during ripening of the fruit. This finding is consistent with the findings of Rolz et al. (1971) for the mango and with the histological observations of Ben-Arie and associates (1979) which confirmed a progressive dissolution of the cell wall and middle lamella in the soft tissues of apple and pear as ripening proceeded. Gradual loss of cell wall material as the tissue softens may well be a general feature of the ripening of fleshy

fruits; the cell separation resulting from such dissolution is likely to make a major contribution to textural change. Fractionation of the cell wall material (see exp. 2 in Table 4) suggested that the major losses of this cell wall material in the mango came from losses of the hot water-soluble ('pectic') and insoluble residue (after sequential extraction with hot water and alkali) fractions. A criticism of the present work is that, although the losses of the insoluble residue were more modest than those of the hot water-soluble fraction, the changes in the former were not substantially further investigated when they might potentially have proved to be of considerable interest, possibly indicating degradation of cellulose in ripening mangoes.

Earlier authors (Rolz et al., 1971; Mizuta and Subramanyam, 1973; Saeed et al., 1975) have shown that, as ripening proceeds in the mango, wall-bound (insoluble in cold aqueous solutions) pectin decreases and this is accompanied by a corresponding increase in "soluble pectin" (extractable with aqueous solutions at 18-20^{o}). The major decline, reported in the present study, during ripening of the wall fraction extracted with hot water is consistent with these findings, since this fraction would correspond to "wall-bound pectin". However, it is critically conceded that, in the present study, only changes in the wall material prepared by subjecting homogenised mango tissue to repeated buffer washes followed by extraction with organic solvents were investigated. The buffer washes would be likely to remove the "soluble pectin" fraction and no attempt was made

to investigate this latter fraction. Examination of changes in "soluble pectin" would probably have enhanced the value of the findings reported in the present study for changes in the hot water-soluble (wall-bound pectin) fraction of the wall. It is also conceded that contamination of wall preparations with starch (see p. 157) diminished,to some extent, the reliability of gravimetric measurement of wall fractions since starch would be likely to be extracted during wall fractionation procedures, especially into the hot water-soluble fraction.

IV.I. (d) Hydrolysis of cell walls and wall fractions.

Although the hydrolysis procedure (trifluoroacetic acid hydrolysis followed by digestion with a mixture of polysaccharide-degrading enzymes - see Section II.7. (b)) employed in the present study was reasonably reproducible (see Table 5, Section III.I. (b), p. 161), total hydrolysis of polysaccharides in the cell wall preparations was not achieved by these methods. In hydrolyses of unripe cell walls the yield (by weight) of the total monosaccharides did not exceed 60% of the weight of cell wall material hydrolysed (see Tables 5 - 7). Further, the gravimetric yield of total monosaccharides after hydrolysis decreased with increasing ripeness of the tissue from which wall material was prepared, presumably reflecting removal from the wall, by enzymic action in the tissue during the ripening process, of polysaccharides more labile to the hydrolytic procedures employed. Comparable problems were encountered in the hydrolysis of fractions obtained from cell wall preparations, which were particularly

marked in the case of the insoluble wall residue left after extraction of wall preparations with hot water and alkali (see Table 8, p. 168). These combined problems, of inability to totally hydrolyse wall preparations and different labilities to the hydrolytic procedures employed of cell wall materials prepared from tissue at different stages of ripeness, placed serious limitations on the interpretation of the monosaccharide composition data obtained from these hydrolysis studies.

Such technical problems of incomplete polysaccharide hydrolysis are compounded by the apprehension that the employment of severe hydrolytic conditions to ensure full hydrolysis of the most resistant cell wall polysaccharides would be likely to cause degradation of the monosaccharide products of other, more labile, polysaccharides. Complete hydrolysis of glycosidic linkages in many non-cellulosic polysaccharides can be achieved with dilute mineral acids; however, degradation of the component monosaccharides can readily occur in the presence of oxygen or other constituents, notably protein. Monosaccharides in the furanoid form seem to be more susceptible to degradation than those that are pyranoid (Southgate, 1976). With regard to the hydrolysis of polysaccharides containing uronic acid residues it should be noted that glycosidic linkages involving these residues are more resistant to acid hydrolysis than the corresponding linkages between neutral sugars. Because aldobiouronic acids are more stable towards acid than neutral disaccharides,

the former are often obtained in high yields on partial, acid hydrolysis of acidic polysaccharides (Whistler and Richards, 1958; Marchessault and Ranby, 1959; Hamilton and Thompson, 1960; Nakano and Ranby, 1962; Semke et al., 1964; Timell, 1964b; Timell et al., 1965; Capon and Ghosh, 1965; De and Timell, 1967).

Thermodynamic data (Timell et al., 1965; De and Timell, 1967) suggest that acidic disaccharides undergo acid hydrolysis by a different mechanism from neutral disaccharides but the nature of this difference in mechanism is not presently understood. The uronic acid group exerts a stabilising effect on the uronosidic bond (Capon and Ghosh, 1965; De and Timell, 1967). This resistance of aldobiouronic acids to acid hydrolysis is believed to result from steric factors which render difficult the formation of the partially planar carbonium ion intermediate in the hydrolysis reaction. It has been found that polar groups in the aglycon of glycosiduronic acids, but not of glycosides, have a profound influence on their stability towards acids (Timell et al., 1965; Saunders and Timell, 1967). The results of Capon and Ghosh (1965) suggest that it may be possible to hydrolyse polysaccharides containing uronic acids preferentially at the uronosidic linkages by carrying out the reactions in solutions of low acidity.

When hydrolytic conditions are sufficiently severe, extensive destruction of both uronic acids and other glycosyl constituents occurs: in the case of uronic acids decarboxylation (Tracey, 1948) and lactonisation

(Blake and Richards, 1968) are known to result. These technical difficulties should be particularly borne in mind when interpreting data for galacturonic acid in the hydrolyses that were carried out in the present study. Trifluoroacetic acid, which is volatile and easily removed by evaporation was used for the hydrolysis of mango cell wall and polysaccharide fractions. Albersheim and his associates (1967) found this procedure to be as efficient as the usage of mineral acids. The conditions chosen were the optimum described by these workers for the most efficient hydrolysis with the minimum of degradation. For the hydrolysis of cellulose, which is highly resistant to both dilute mineral and trifluoroacetic acids, it is normally necessary to first bring it into solution in 72% (w/w) H_2SO_4, followed by dilution to an acid strength of about 1M before boiling under reflux (Saxena et al., 1961; Updegraff, 1969). It is critically conceded that, in the present study, this procedure should have been employed for hydrolysis of the insoluble wall residue remaining after extraction of wall preparations with hot water and alkali since monosaccharide yields from hydrolysis of this fraction were particularly low. Reliable monosaccharide composition data for this fraction would probably have added considerable insight to its noted decrease in the wall during ripening of the fruit (see Table 4, p. 158).

In an attempt to overcome the several problems of incomplete hydrolysis outlined above, trifluoroacetic acid hydrolysis of cell wall and polysaccharide fractions was

followed, in most experiments, by digestion with a mixture of polysaccharide-degrading enzymes. The mixture used contained polygalacturonase, hemicellulase and cellulase. This 2-stage hydrolytic procedure enhanced the yield of monosaccharides obtained by acid hydrolysis alone (see Table 6, p. 163) but as mentioned previously (p.258) total hydrolysis of cell wall preparations was not achieved. The cellulase preparation employed seems, in particular, to have been ineffective since the insoluble wall residue after hot water and alkali extraction was particularly resistant to hydrolysis (see Table 8, p. 168).

Gas-liquid chromatography was used for the qualitative and quantitative analysis of monosaccharides.

The procedure for conversion of the monosaccharide products of hydrolysis to alditol acetates was as described by Jones and Albersheim (1972) (see Section II.5. (b)). To facilitate accurate quantitation, myoinositol was incorporated, before the hydrolysis step, as an internal standard for neutral sugars and D-mannonic acid was incorporated, after hydrolysis, as an internal standard for uronic acids. It was recognised that the incorporation of these standards would not accurately compensate for losses of monosaccharides caused by degradation during hydrolysis.

IV. 1. (e). Changes in monosaccharide composition of mango cell walls during ripening.

Despite the limitations of hydrolysis previously mentioned (see p. 258) the results obtained provided evidence of marked changes in the monosaccharide composition

of mango cell walls during the ripening process.

In all hydrolysis studies with intact cell wall preparations (see Tables 6 and 7, Section III.1. (b)) the yields of monosaccharides from unripe preparations were significantly higher than those from the more mature walls. This presumably relates to the presence of higher levels of readily-hydrolysable wall polysaccharides and contaminating starch in the unripe walls compared to those from ripening fruits.

A major defect of the present study was that calculation of gravimetric data for individual monosaccharides obtained on hydrolysis of cell wall material should have been referred to a stated initial wet weight (e.g. 100g) of mango mesocarp tissue, which would have been facilitated if the gravimetric yield of cell wall material/100g wet tissue had been estimated for all wall preparations hydrolysed. This was not done and thus it will be necessary to interpret the gravimetric yields for monosaccharides/10mg dry wall preparation displayed in Tables 6 - 8 by taking into account the marked decrease during ripening of the total cell wall material/100g wet mango tissue revealed by the data in Table 4 and which has been previously discussed (see p. 256).

Tables 6 and 7 reveal that quantitatively the most significant monosaccharides present in intact cell walls preparations were galactose, arabinose, glucose, xylose and galacturonic acid. During ripening the proportions of both galactose and arabinose in the wall fell markedly and it is clear that these monosaccharides were being removed

from the cell walls in the tissue. Because of known contamination of wall preparations with starch it is not possible to reach meaningful conclusions about the possible removal of glucose from wall glucan(s) during ripening. The proportions of galacturonic acid and of xylose in the wall remained relatively stable during ripening but, since it is known that there is a marked decrease in the total wall material present in the tissue with ripening (Table 4), then it must be concluded that loss of these monosaccharides from the wall must also have contributed to the overall wall loss. Rhamnose and fucose were present only as minor constituents of the wall preparations but the data also indicate some loss of these monosaccharides during ripening.

Examination of the composition of fractions extracted from cell walls at different stages of ripeness (see Table 8) indicated that galactose and arabinose were present in all three wall fractions obtained,in all of which both of these monosaccharides declined during ripening. The decline of arabinose was particularly marked in the hot water-soluble fraction which itself declined during ripening (Table 4). Galactose was most prominent in the alkali-soluble fraction but declined with ripening in all wall fractions. The results obtained for galacturonic acid were somewhat puzzling. This monosaccharide was concentrated in the hot water-soluble fraction, consistent with its usual presence in pectic galacturonans in plant cell walls (see Section I.2. (c) 2.1.). The proportion

contributed by galacturonic acid to this fraction increased with ripening and (despite the indication from data in Table 4 that the water-soluble fraction of the wall declined with ripening) it is difficult to correlate the data obtained for galacturonic acid in Table 8 with the clear indication from data in Tables 6 and 7 that overall loss of galacturonic acid from intact cell walls in mango tissue occurs during ripening. Rhamnose was present in small amounts in all three wall fractions in all of which it declined with ripening, the most marked decline being manifested in the hot water-soluble fraction.

Xylose, mannose and fucose were concentrated in the alkali-soluble fraction consistent with the usual presence of these monosaccharides in plant hemicellulosic polysaccharides (see Section I.2.(c) 1.), with xylose being quantitatively the most significant monosaccharide in this fraction. Mannose and fucose declined to some extent in this fraction with ripening. The apparent stability during ripening of xylose in the alkaline extract (Table 8), a wall fraction which itself appears relatively stable during ripening (Table 4), is somewhat inconsistent with the indication from data obtained by hydrolysis of intact cell walls at different stages of ripeness (Tables 6 and 7) that xylose was lost from the wall during ripening. Xylose, however, declined with ripening in the alkali-insoluble wall residue.

Despite the several inconsistencies between the data obtained for hydrolysis of intact cell walls (Tables 6 and 7) and the data obtained for hydrolysis of wall

fractions (Table 8) mentioned above it is possible to reach general conclusions regarding changes in monosaccharide composition of mango cell walls during ripening. It should be borne in mind that data for intact walls were obtained from several experiments whereas data for wall fractions represent only a single experiment.

No mango cell wall polysaccharide has been characterised and the structures of cell walls in plant tissues may well be species-specific. However, by analogy with the acidic polysaccharides isolated from other plant sources (see Section I.2. (c) 2.1.) it is reasonable to propose that the removal from mango cell walls during ripening of galacturonic acid and rhamnose may represent removal from the wall of polymers forming part of the pectic backbone. Several earlier authors (Jain, 1961; Rolz et al., 1971; Mizuta and Subramanyam, 1973; Saeed et al., 1975) have published findings suggesting that solubilisation of pectin from the cell wall occurs during the tissue softening which accompanies mango ripening. Removal of wall-bound galacturonic acid ('protopectin') with a corresponding increase in soluble, high molecular weight galacturonan is a process which accompanies tissue softening during ripening of a wide range of fruits (Pilnik and Voragen, 1970; Pressey, 1977; Hobson, 1981; Labavitch, 1981), (see Section I.5. (b) 1.). The results reported for changes in mango wall monosaccharide composition in the present study suggest that mango ripening may resemble the ripening of other fruits in this respect.

The marked loss of arabinose and galactose from mango cell walls during ripening suggests that arabinans and galactans may also be removed from the wall as ripening proceeds. The loss of arabinose is especially marked from the hot water-soluble fraction of the wall and, by analogy with the pectic polymers of other plant tissues including some containing only primary cell walls (see Section I.2. (c) 2.2.), this could indicate removal of a pectic arabinan forming part of the neutral pectic polymers of mango walls, although this must be regarded as speculative since no such polymer has been characterised from mango cell walls. Some workers, e.g. Keegstra et al., 1973; Albersheim, 1975 for sycamore cells and Knee et al., 1975; Knee and Bartley, 1981 for apple cortical tissue, have suggested that in the primary walls of these tissues arabinans and galactans are attached as side-chains to a pectic rhamnogalacturonan backbone and that the former neutral polymers may form links between the pectic backbone and other cell wall polysaccharides such as hemicelluloses, stabilising the wall matrix. However, the existence of such links has not been firmly established, either in these tissues or in the primary walls of other dicot species, and it would be dangerous to extend this highly tentative 'model' to the mango cell wall in which no polysaccharide has been structurally characterised. The Albersheim (1975) model may apply only to the sycamore primary cell wall and there may, indeed, be no general model for the structure of the primary cell wall which is applicable to all dicot species.

However, loss of arabinose and galactose from the cell wall during ripening, reported here for the mango, has been noted during the ripening of other fruits. Removal of these sugars could result from the action of endo-arabinanase(s)/endo-galactanase(s) releasing soluble polymers from the wall, possibly combined with the further activity of exo-acting glycosidases further degrading solubilised fragments, but this is purely speculative since no such mechanism has been established in any fruit species, although arabinanase activity has been detected in the Japanese pear (Yamaki and Kakiuchi, 1979) and α-L-arabinofuranosidase activity has been detected in 'Bartlett' pear (Ahmed and Labavitch, 1980b) and in both of these pear cultivars arabinose loss from the wall is a prominent feature of ripening. As previously mentioned in Section I.5.(b) 2. loss of arabinose and galactose from the wall is associated with ripening of several fruits; in the strawberry (Knee et al., 1977) arabinose and galactose residues lost from the wall appear in soluble fractions together with solubilised polygalacturonate and in pear (Ahmed and Labavitch, 1980a) the substantial amounts of arabinose lost from the wall appear as a soluble arabinan of high molecular weight, to which galacturonan is attached. In the apple (Knee, 1973c) neutral carbohydrate in the pectic fraction extracted from the wall decreases during ripening and, correspondingly galactose and arabinose are lost from the wall; as with the strawberry (Knee et al., 1977) the solubilised polygalacturonate in apple (Knee 1973c) appears to be free of

neutral residues. In the tomato, Gross and Wallner,1979, also, the ripening-related decline in galactose and arabinose content of the wall appears to be separate from galacturonan solubilisation. Loss of these monosaccharides from the mango cell wall suggests a further similarity between the ripening processes occurring in mango and in some other fruits.

The indications of overall loss of xylose, mannose and fucose from the intact mango cell wall together with decline of arabinose and galactose in the alkali-soluble wall fraction during ripening suggest that hemicellulosic polysaccharides in the mango wall are being solubilised from the wall. It is difficult to relate this finding to the data in Table 4 which suggest that the alkali-soluble fraction of the wall does not decline in amount during ripening. It is possible that hemicelluloses in the wall undergo turnover, the monosaccharides which are removed from the wall being replaced by polysaccharides containing other monosaccharides, e.g. glucans, but this must be regarded as purely speculative, especially since starch contamination of wall preparations prevented meaningful conclusions about turnover of wall glucans during ripening. It must also be stressed that loss of these monosaccharides from the wall is only suggestive of the removal of hemicelluloses on the basis of analogy with the monosaccharide composition of hemicellulosic polysaccharides in other plant species (see Section I.2. (c) 1.) since the constituent polysaccharides of the mango primary cell wall have not been characterised.

The limited data available suggest that monosaccharides characteristic of hemicelluloses, most particularly xylose, may be quantitatively more significant in the mango cell wall than in the cell walls of the soft tissues of other fruits (see Section I.3. (b), p. 50 and I.5. (b) 2., p. 68). Also, mobilisation of mango hemicelluloses during ripening would indicate a difference between mango and some other fruits which have been studied in this respect. The small quantities of monosaccharides, characteristic of hemicelluloses, present in the wall - xylose, mannose and glucose - do not decline during the ripening of strawberry (Neal,1965); tomato (Gross and Wallner, 1979) and pear (Jermyn and Isherwood,1956; Ahmed and Labavitch, 1980a) although Knee (1973c) reported mobilisation of hemicellulosic glucan in the cortical tissue of ripening apple.

IV.2. Autolysis of homogenised mango mesocarp.

In this experiment in which homogenised mango mesocarp at various stages of ripeness was allowed to autolyse, after an initial dialysis for the purpose of removing free sugars in the homogenates (see Section II.5. (a) and Table 9, Section III.2), the quantitatively most prominent monosaccharides present in the soluble fractions at all stages of ripeness were glucose and fructose and these increased with ripening. It was considered that the high levels of these sugars resulted from free sugars in the homogenate, not removed entirely by the initial dialysis, and their increase is consistent with previous published findings (Leley et al., 1943; Biale, 1960; Hulme, 1971) indicating that starch and sucrose are mobilised during mango ripening with

corresponding increases in the levels of the constituent monosaccharides in the tissue. Hence no conclusions were drawn concerning the possibility that degradation of wall glucan contributed to soluble glucose. It should be borne in mind that with the procedures employed for alditol acetate formation from sugars (see Section II.5. (b)) fructose would be converted into a mixture of mannitol acetate and glucitol acetate. Since mannose was only a very minor component of mango cell walls (see Sections III.1 (c) and IV. 1. (e)) all mannitol acetate appearing on glc was attributed to fructose. Mannose in the soluble fraction could not have been distinguised from fructose by the methods employed in any case. Hence the figures presented for sucrose in Table 9 represent the figures obtained for mannitol acetate but it should be noted that fructose also contributes to the formation of glucitol acetate and this contribution is incorporated in the recovery figures for glucose in Table 9.

Rhamnose appeared in the soluble fraction at all stages of ripeness but its level did not appear to change with ripening. This sugar has been associated with pectic backbone polysaccharides in other plant cell walls (see Section I.2. (c) 2.1.) and its appearance in the soluble fraction could indicate autolytic degradation of such pectic polymer(s) in the mango cell wall but galacturonic acid, the major constituent of such polymers, was not detected in the soluble fraction.

Arabinose in the soluble fraction increased

8-fold at the stage of over-ripeness from the level at the unripe stage. This observation suggests possible autolytic degradation, through enzyme activity in the mango homogenates, of arabinan(s) in the ripening mango cell wall and is consistent with the finding that, during ripening, a marked loss of arabinose from mango cell wall preparations was noted (see Sections III.1. (b) and IV.1. (e)). Wali and Hassan (1965) previously reported the appearance of free arabinose in mango mesocarp during ripening. Galactose, another sugar which also was removed from the wall during ripening, however, was not detected in the soluble fraction of autolysing mango homogenates. This latter finding was surprising in view of the fact that in the present study the loss of galactose from mango wall preparations with ripening was comparable with the loss of arabinose.

Xylose in the soluble fraction increased in the stage of over-ripeness. This finding suggests possible autolytic degradation of xylose-containing polysaccharides in the mango cell wall and that such activity is enhanced in the late stage of ripening. This observation is consistent with the conclusion reached in the present study (see Sections III.1 (b) and IV.1 (e)) that removal of xylose from wall polysaccharides contributed to the overall loss of cell wall from mango mesocarp tissue which occurred during ripening (see Table 4, Section III.1 (a)). In a previous report Sarkar (1963) noted the appearance of free xylose in the mesocarp of ripening mangoes and this author also speculated that the free sugar originated from

the breakdown during ripening of hemicellulosic polysaccharides containing xylose present in the mango cell walls.

It should be stressed that only free monosaccharides were sought in the dialysates; no attempt was made in this experiment to look for bound monosaccharides in soluble polymers which might be released by autolytic wall degradation.

IV. 3. A search for polygalacturonase activity in mango mesocarp.

A prolonged and searching attempt was made to locate this enzyme activity in mango mesocarp tissue at various stages of ripeness. The experimental methods employed have been fully described in Section II.3. Three separate extraction techniques were utilised; one of these, extraction with NaCl/EDTA solution (the method described by Hobson (1962)), was applied with a wide range of experimental modifications. Both generation of reducing power and viscometry were tried in attempts to assay the enzyme activity against both polygalacturonic and polymethylgalacturonic acids as substrates. Considerable effort was made to remove potential inhibitors from association with the enzyme and this, together with the evidence provided by the failure of mango enzyme extract to inhibit polygalacturonase activity in a tomato preparation, militates against the presence of such an inhibitor in the extract.

Such an exhaustive and time-consuming investigation was made in view of the established role of polygalacturonase(s)

activity in converting cell wall-bound pectin to soluble form, such activity apparently making a major contribution to tissue softening during ripening, through dissolution of the cell wall matrix and middle lamella leading to cell separation, in a wide range of fruits investigated, as previously discussed extensively in Section I.5. (b) 1., p. 62 . From previous reports (Rolz et al., 1971; Mizuta and Subramanyam, 1973; Saeed et al., 1975) (see Section I.4., p.57) it is apparent that a comparable process of the solubilisation of high molecular weight, wall-bound pectin occurs during the softening of mango mesocarp accompanying ripening. This, in fact, is the only mechanism of cell wall dissolution in ripening mangoes which has, to any extent, been investigated in previous work. Further, in the present work, it was concluded that removal of galacturonic acid (presumed to originate in high molecular weight galacturonan by analogy with the acidic polysaccharides of other plant cell walls - see Section I.2. (c) 2.1.) from the wall contributed to the overall loss of cell wall material which occurred in mango mesocarp tissue during ripening (see Sections III.1. (b) and IV.1. (e)).

Very few of the fleshy fruit species investigated have been shown to lack polygalacturonase (either endo- or exo-acting) activity. Only melon, cranberry and persimmon have been claimed to lack polygalacturonase activity, in an early study by Hobson (1962) which has not been subsequently repeated or re-investigated with regard

to these particular fruit species by other workers, and several other fruit or root species also claimed in this report to lack polygalacturonase have been reported by other authors to contain the activity (e.g. grape (Pansolli and Belli-Donini, 1973), cucumber (Bell, 1951; Pressey and Avants, 1975a; McFeeters et al., 1980) and carrot root (Pansolli and Belli-Donini, 1973; Pressey and Avants, 1975b). The reported levels of polygalacturonase activity in other fruits, in terms of units of activity per 100 g fresh tissue, range from 0.06 in the pear to 3.68 in the tomato, where 1 unit of activity is defined as 1 g galacturonic acid monohydrate liberated from polygalacturonic acid per h (Hobson, 1962). At the outset it was therefore anticipated that mango mesocarp would contain polygalacturonase activity and that, in common with a number of other climacteric fruits (Pressey, 1977; Hobson, 1981; Tucker and Grierson, 1982), enhanced levels of activity would accompany ripening, providing a possible explanation for the reported solubilisation of wall-bound pectin (Rolz et al., 1971; Mizuta and Subramanyam, 1973; Saeed et al., 1975) accompanying tissue softening in the mango mesocarp.

However, despite the prolonged investigation, the activity could not be detected in the tissue at any stage of ripeness (see Section III.3.). The possibility that it is present cannot be totally discounted (although this seems unlikely). In spite of the precautions taken, the firm binding to the mango enzyme of a specific inhibitor in this particular fruit can not be completely ruled out,

but it seems improbable. The possibility that enzyme activity remained undetected perhaps because of the absence of unidentified co-factors or because the enzyme is present as an inactive proenzyme must be considered. If the latter were the case there was no evidence of active enzyme being produced at any developmental stage of the fruit, and, therefore, the hypothetically inactive form would be of little apparent physiological significance.

On the balance of the evidence provided by this investigation it would appear that the mango mesocarp must possess significantly lower polygalacturonase activity than the pear cortex and it is concluded that, as reported for the melon, tangerine and persimmon (Hobson, 1962) the mango possesses insignificant amounts of the active enzyme and, therefore, it is probably of little importance in the ripening process.

IV. 4. Glycosidases in mango mesocarp.

IV.4. (a) Introduction.

The exact physiological role of simple glycosidases, (i.e. those that *in vitro* normally hydrolyse p-nitrophenyl glycosides), in ripening fruit is presently unknown (see Section I.5. (b) 2., p. 67) and there is uncertainty about the nature of the natural substrates for these enzymes. Bartley (1974), for example, reported that the wall-bound β-D-galactosidase of apple cortex possessed the ability to hydrolyse, *in vitro*, a galactan with β-1,4 links obtained from potato pectin and Pressey (1983) found that one

of three iso-enzymes of this enzyme in tomato, which increased in activity during ripening, possessed the ability to degrade a similar polysaccharide isolated from tomato fruit cell walls. In both cases, free galactose was liberated. In contrast, Wallner (1978) found that this tomato enzyme had no activity against apple cortex cell walls, which contain a β-1,4-linked pectic galactan. Furthermore, Ahmed and Labavitch (1980b) demonstrated that the α-L-arabinofuranosidase extracted from the soft tissue of pear fruit did not generate reducing sugar, *in vitro*, from a purified arabinan. The ability of a glycosidase to hydrolyse a p-nitrophenyl glycoside is no indication that the enzyme will necessarily hydrolyse any naturally occurring molecule which possesses glycosyl residues for which the enzyme is specific (*cf.* Pharr *et al.*, 1976). Even if a glycosidase acted as an exo-glycanase it is unlikely that it could cleave beyond the branch points of cell wall polysaccharides. Several glycosidase activities, to which it is difficult to assign any role in wall breakdown and tissue softening, have been located in fruit tissues; for example the most active glycosidases in the pear cortex are α-D-galactosidase and α-D-mannosidase (Ahmed and Labavitch, 1980b), but analysis of pear fruit cell walls gave no indication of α-linked galactans or mannans and little change in wall mannose or galactose content occurred during pear ripening (Ahmed and Labavitch, 1980a).

However, although it is clear that the cellular role of glycosidases is open to speculation, nevertheless

the most likely natural substrates for their activity are phenolic glycosides and soluble glycan fragments of low molecular weight, i.e. small oligosaccharides, derived from endo-glycanase activities against cell wall polysaccharides. The possibility that these enzymes may also partly degrade branched cell wall polysaccharides by exo-activity against linear chains containing appropriate linkages as far as branching points, or even fully degrade unbranched polysaccharides, (e.g. β-1,4-linked D-galactans such as those isolated from citrus pectin (Labavitch et al., 1976) and tomato fruit cell walls (Pressey, 1983)) by similar activity, must also be considered.

IV.4.(b) Increases in activities of glycosidases in mango during ripening.

In mango mesocarp, the activities which demonstrated the most marked increases during ripening were α-D-mannosidase and β-D-galactosidase (see Section III.4., p. 196). It is difficult to designate a role for the former enzyme in mango wall breakdown since mannose appeared to be only a minor component of the hemicellulosic fraction of the mango wall (see Section III.1.,p. 167), in contrast to certain other plant cell walls in which mannose-containing polysaccharides are major constituents, e.g. mannans in the cell walls of ivory nut endosperm (Timell, 1965), galactomannans in legume seeds (Dea and Morrison, 1975) and glucomannans and galactoglucomannans in gymnosperms (Timell, 1964a, 1965) In all of these above-mentioned polysaccharides the mannosyl residues are β-D-mannosidically linked and

α-D-mannosyl residues have not been reported to occur in plant cell walls. In the present study, β-D-mannosidase could not be detected in mango mesocarp and β-D-mannanase activity was not assayed.

However, it should be borne in mind that the breaking of just a single glycosidic bond could be highly significant in the ripening process and minor wall components and low levels of enzymes which may interact with them should not be ignored when attempting to explain the process. For this reason it is critically conceded that, in the present study, it would probably have been constructive to assay mango mesocarp for mannanase activity.

The high levels of β-D-galactosidase activity in mango mesocarp and its marked increase during ripening would strongly suggest that this enzyme is involved in the observed loss of galactose from the mango cell wall with ripening (see p. 162) and the ability of 'enzyme' extracts of over-ripe mango mesocarp (which contain β-D-galactosidase activity - see Table 33, Section III.5. (b), p.218) to liberate free galactose from cell walls prepared from unripe mangoes (see Section III.5. (c), p. 223). The ability of this enzyme to hydrolyse galactans is subject to question (see p.276) but β-D-galactosidases extracted from at least two other fruits - apple (Bartley, 1974) and tomato (Pressey, 1983) - have been reported to degrade β-1,4-linked D-galactans derived from the pectic fractions of tissues containing only primary cell walls. No such polysaccharide has been characterised in mango cell walls, but pectic

galactans are widespread in plant tissues (see Section I.2. (c) 2.2., p. 32) and it can be speculated that mango β-D-galactosidase may be active during the ripening process against a galactan which forms a component of the mango cell wall, most likely, by comparison with other plant cell walls, a neutral component of the pectic polysaccharides.

It has been previously stated (see p. 197) that it is unsafe to conclude whether the other major glycosidase activity which increases during mango ripening - α-L-arabinopyranosidase - results from β-D-galactosidase activity against p-nitrophenyl-α-L-arabinopyranoside or constitutes a separate enzyme activity. It should also be noted that arabinose in cell wall polysaccharides occurs largely in the form of α-linked L-arabinofuranose units (see Sections I.2. (c) 1.1., p. 23 and I.2. (c) 2.2., p. 32), although the xyloglucan of sycamore cell primary wall contains a small proportion of terminal, α-linked L-arabinopyranose residues (Albersheim, 1975, 1976, 1978; Darvill et al., 1980), - see also Fig.2, p. 27 . It is conceivable that an α-L-arabinopyranosidase activity could play a role in the detachment of such arabinopyranose residues from mango cell wall polysaccharides. No marked α-L-arabinofuranosidase activity could be detected in mango mesocarp (see Table 30, p. 209).

A further factor for consideration is whether the observed increases in glycosidase activities result from the synthesis of enzymes during the ripening process or merely represent increases in activity as the enzymes are being

detached from the wall where in the bound form they are inactive or only partially active. In this connection it is interesting to note the suggestion made by Hankins and Shannon (1978) that some lectins in seeds are wall-bound, inactive proteins which acquire enzymic (hydrolase) activities during germination. Ripening is a process of senescence and it may be surmised that once this process has commenced further synthesis of enzymes is unlikely. However, there is abundant evidence to suggest that in climacteric fruits, which include the mango, the respiratory 'burst' which appears to initiate ripening is accompanied by considerable de novo synthesis of enzymes which catalyse the final senescence of the fruit (Hulme, 1972; Hobson,1979) (see p. 54).

This rapid enzyme synthesis during the climacteric period could readily account for the increases in mango glycosidase activities observed between the unripe (1) and 'turning' (2) stages of ripening. But, it is more difficult to attribute continued measurable increases in activity beyond the 'turning' stage into over-ripeness, (which is particularly noticeable in the case of mango β-D-galactosidase), to continued synthesis of enzymes. It seems more logical to attribute this further increase to continuing solubilisation/ activation of enzyme molecules. However, Knee et al.(1977) reported that in the strawberry (an apparently non-climacteric fruit) incorporation of {^{14}C}-proline into the wall and synthesis of wall glycoprotein continued into late ripening when hydration and dissolution of the middle lamella and wall

matrix materials were advanced. Thus it seems that, in one fruit at least, biosynthesis of protein may continue during senescence so that the possibility of comparable biosynthesis of enzymes by senescent mango can not be totally dismissed.

Finally, it is conceded that, in the present study, the information provided by assay of the levels of activity of glycosidases in mango mesocarp at different stages of ripeness would probably have been complemented and greatly extended by similar assay of the levels of glycanases in the tissue, utilising purified polysaccharides of characterised structure as substrates. In particular, the levels of galactanase and arabinanase activities in mesocarp would, potentially, have been of great interest, in view of the observation that galactose and arabinose were the monosaccharides most markedly removed from mango cell walls during ripening (see Section III.1. (b), p. 162). The indications from results obtained in the present study that hemicelluloses of the mango cell wall may be mobilised during ripening (see p. 296) suggest that β-D-xylanase and β-D-mannanase activities may also have been of potential interest.

IV. 4. (c) Solubilisation of mango glycosidases from mango cell walls during ripening.

From examination of levels of activities of glycosidases in buffer extracts (see Section III.4.(b), Table 28, p. 204 and Table 32, p. 214) compared to the levels in whole homogenates containing the particulate wall

fraction (Tables 25, p. 198 and 31, p. 211) the conclusion may be drawn that, in the mango, glycosidase enzyme are more tightly bound to the particulate fraction (cell wall) in unripe fruit than in 'turning'/over-ripe fruit. In a study of β-D-galactosidase activity in ripening apples, Bartley (1974), in contrast to the present findings with mango, reported that enzyme activitity in the soluble fraction closely followed changes in the cell wall-bound activity (which increased steadily with ripening), the soluble activity remaining at around 25% of the wall-bound activity. It should be noted that, in this study, Bartley (1974) treated the apple tissue with acetone prior to buffer extraction which was carried out by re-suspension of the acetone-powder in buffer, followed by freezing and thawing. Pharr et al. (1976) did not directly compare extracted glycosidase activities from tomato mesocarp with wall-bound activities but they did show that the specific activity and concentration (units/g dry weight walls) of β-D-glucosidase was greater in cell walls from unripe, green fruits than in walls from firm, ripe fruits. This could indicate solubilisation of this enzyme from the wall during ripening, but since total β-D-glucosidase activity (units/g fresh weight) in tomato mesocarp also declined with ripening, it could simply reflect the gradual loss of an enzyme which is mainly wall-bound at all stages (the specific activity of β-D-glucosidase in isolated tomato mesocarp walls was 4 times greater than its specific activity in extracts of intact mesocarp tissue). In contrast, the concentration and specific activity of

α-D-galactosidase in both isolated walls and whole tissue of tomato mesocarp changed little with ripening (the specific activity in the walls was only half the specific activity in extracts of both unripe and ripe tissue). This evidence suggests that α-D-galactosidase is not solubilised from the tomato mesocarp wall during ripening. Ahmed and Labavitch (1980b), in their study of glycosidases in the soft tissue of pear fruit, did not compare extracted activity with activity in the particulate fraction at various stages of ripening.

The present finding that the extractability of glycosidase enzymes from intact mango tissue apparently increases with ripening is thus not matched by any firm report obtained from another fruit. It is assumed that the present findings indicate stronger binding of the enzymes to the cell wall in unripe than in ripe fruit and if glycosidases become less tightly bound, and thus more soluble, as the fruit ripens, this may have physiological significance. It is possible that the initial stages of ripening involve solubilisation of glycans from the wall by the activity of 'endo-acting', wall-bound enzymes so that, by the stage of over-ripeness, possible substrates for exo-glycosidase activity (oligosaccharides produced by endo-glycanase-catalysed degradation of wall polysaccharides) would be located in the cytoplasm. The loosening of glycosidases from the wall and their entry into the cytoplasm to catalyse the final breakdown of these soluble oligosaccharides is consistent with this concept of the ripening

process.

Alternatively, it is possible that these glycosidases are only physiologically important when attached to the wall where they may contribute *in situ* to the degradation of polysaccharides in the wall matrix during ripening. Consideration must be given to the possibility that wall-bound exo-glycosidase activity might precede the action of endo-glycanase(s) in ripening, i.e. the removal of a terminal group or groups would then allow internal glycosidic bonds in wall polysaccharides to be cleaved. Solubilisation of bound glycosidases from the mango cell wall during ripening may be simply the result of wall degradation. Wall-bound glycosidases have been demonstrated in apple (Bartley, 1974) and tomato (Pharr *et al.*, 1976) fruits and Bartley (1974) has demonstrated that an increase in the β-D-galactosidase activity associated with the apple wall occurred simultaneously with loss of galactose residues from the wall during ripening. Glycosidases have also been found bound to cell walls in other plant tissues, e.g. cultured *Convolvulus* (Klis *et al.*, 1974; Pierrot and Van Wielink, 1977) and sycamore (Keegstra and Albersheim, 1970; Klis *et al.*, 1974) cells, carrot callus (Parr and Edelman, 1975) and pea (Murray and Bandurski, 1975) and bean (Nevins, 1970; Jaynes *et al.*, 1972) coleoptile tissue. Correlation has been demonstrated between the levels of activity of wall-bound glycosidases and cell growth in cultured *Convolvulus* and sycamore cells (Klis *et al.*,1974) and in pea coleoptiles (Murray and Bandurski, 1975) and it is possible that the

wall-bound enzymes play a role in "loosening" the primary cell walls of these tissues during growth. It may be speculated that such "wall loosening" bears comparison with dissolution of the primary cell walls of the soft tissues of fruits during ripening, in which wall-bound glycosidases may also play a role, although it should be borne in mind that fruit ripening is a senescent process rather than a process of active growth.

Finally, the possibility that cytoplasmic glycosidases were adsorbed onto cell walls during preparative procedures, and that this adsorbtion was more marked in unripe mangoes can not be overlooked as adsorbtion of cytoplasmic proteins to cell walls during comparable preparative procedures in plant coleoptiles has been reported (Kivilaan et al., 1959, 1961; Johnson et al., 1974; Huber and Nevins, 1977) and consequently Pharr et al., (1976), recommended non-aqueous procedures for the preparation of cell walls from tomato mesocarp to prevent such adsorbtion.

In summary, until more is known about the natural substrates for and mechanism of activity of glycosidases, assigning their role (if any) in fruit ripening must remain purely speculative. More knowledge is required of the nature and significance of the binding sites for these enzymes in cell walls and of the mechanism (if any) of their catalytic activities against wall components and/or wall fragments released by other enzyme activities.

IV. 5. Soluble mango extracts and their activity against cell walls prepared from unripe mangoes.

The buffer extracts of mango mesocarp employed were partly concentrated by precipitation with 80%-saturated ammonium sulphate or acetone before testing their activities against the cell wall preparations. The 'enzyme' preparations from over-ripe fruit exhibited activity against the cell walls whereas those from unripe fruit did not. The main activities identified were the ability of the enzyme extracts to increase the level of total carbohydrate (determined by the phenol/H_2SO_4 method of Dubois et al., 1956) in the soluble fraction after incubation with cell walls (see Section III.5. (c), Tables 34-36) and to release galactose (identified and quantitated by TLC by the method of Menzies and Mount, 1975) from the cell walls (see Section III.5.(c),Tables 37-39 and Fig.42). In the former case, (i.e. release from the wall of soluble carbohydrate), the high level of background carbohydrate in the 'enzyme' preparations, which has been previously discussed (see Section III.5 (b), p. 215), prevented the increase in total carbohydrate in the soluble fraction from being shown as statistically significant but the change was consistent in all the experiments conducted and, since the increase of galactose in the soluble fraction was statistically significant (see p. 225), it was concluded that the increase in total soluble carbohydrate probably represented a real change.

These results suggest clearly that over-ripe

mangoes possess enzymic activity against cell walls from unripe fruit, (which were considered to be walls likely to have undergone minimal previous enzymic degradation in the intact fruit from which they were prepared), which results in release of carbohydrate(s) from the unripe wall and, specifically, that galactose is released. The absence of such activity from 'enzyme' preparations from unripe mangoes suggests that the development of this activity is part of the ripening process. The amount of galactose released does not account in full for the total soluble carbohydrate apparently released so that the nature of other soluble carbohydrates released has not been firmly established in the present study. This liberation of galactose supports the evidence obtained from analysis of mango cell walls at different stages of ripeness (see Section III.1.(b), p. 162) that loss of galactose from the wall contributes to the overall loss of cell wall material from the softening mesocarp which accompanies mango ripening (see Table 4, p. 158). In Section IV.4. (b) (p.279) the possibility that β-D-galactosidase, an enzyme which increases markedly in activity in mango mesocarp during ripening and which was shown to be present in the mango 'enzyme' preparations obtained by precipitation (see Table 33, p. 218), may contribute to this galactose removal from the wall was discussed. The observed pH optimum for this 'enzymic' galactose release from cell walls was 5.2 (Fig. 43 , p. 233) whereas β-D-galactosidase in mango mesocarp exhibited a pH optimum of 4.5 (Fig.39, p. 143).

Loss of galactose from the wall accompanies the ripening of fruits of several other species (see p. 65) and the mango may share with these species a common mechanism of cell wall degradation in this regard.

It was considered a surprising finding that, in view of evidence obtained in the present study that arabinose was lost from the mango cell wall during ripening (see Section III.1.(b), p. 162) and that free arabinose appeared in the soluble fraction of autolysing over-ripe mango mesocarp, (see Section III. 2., p. 172) the precipitated 'enzyme' preparations from over-ripe fruit did not release free arabinose on incubation with unripe cell walls. Similarly, no free galacturonic acid or xylose, the other major monosaccharide constituents of mango cell walls which, apparently, are lost from ripening mango cell walls (see Section III.1.(b), p. 166) were released by the activity of these 'enzyme' preparations against the unripe cell wall.

Indeed, these 'enzyme'/cell wall incubation experiments provided little insight into the mechanism by which galacturonic acid is apparently removed from the mango wall during the ripening process, a process which in several other fruits (see section I.5. (b) 1.) is considered to be of major significance in disentangling acidic pectic polymers from other polysaccharide constituents of the primary wall, resulting in dissolution of the cell wall and middle lamella with resultant cell-separation and tissue softening. In the present study, no soluble carbohydrates containing galacturonic acid were shown to be released from the unripe wall

by incubation with the precipitated mango 'enzyme' preparations. The small amount of soluble carbohydrate containing galacturonic acid, which was presumed to be of relatively low molecular weight because it bound to Zerolit DM-F ion-exchange resin unlike high molecular weight galacturonan which does not (see Table 3, p. 109), detected in the incubation mixtures was derived not from enzyme activity against the cell wall during the course of incubation but appeared to originate both from the wall preparation (by leaching into the suspending buffer) and from the 'enzyme' preparations used (see Tables 40 and 41, pages 235 and 236). The only indication that galacturonan fragments may be released from the wall into the soluble fraction in intact mangoes during ripening came from the finding that acetone-precipitated 'enzyme' preparations from extracts of over-ripe mangoes gave a more marked reaction with carbazole (for total uronic acids) than similar preparations from unripe mangoes (see Table 44, p. 252), and this finding is unreliable because the 'enzyme' preparations also contained neutral carbohydrates which have been shown to interfere with the assay for total uronic acids, employing carbazole, described by Bitter and Muir (1962). However, the fraction containing galacturonic acid which bound to Zerolit Dm-F resin (used to de-ionise the soluble fractions before subjecting them to electrophoresis on glass-fibre paper) in 'enzyme' preparations from over-ripe mangoes also gave a more marked carbazole reaction than the comparable fraction obtained from unripe mango 'enzyme' preparations.

In the light of these findings, the inability in the present study to demonstrate release of fragments containing galacturonic acid from unripe mango cell walls incubated *in vitro* with 'enzyme' preparations from over-ripe mangoes is puzzling.

When the soluble fractions (after de-ionisation) of mango 'enzyme'/cell wall incubation mixtures were subjected to glass-fibre paper electrophoresis (cf. Jarvis *et al.*, 1977), no 'novel' polysaccharide fractions, whose presence in the soluble fraction could be attributed to enzymic degradation of the cell wall during the incubation, could be detected. The polysaccharides present in the soluble fractions apparently originated in the 'enzyme' preparations having (presumably) been co-precipitated with protein from the buffer extracts of mesocarp tissue (see Section III.5. (e), Figs. 45 and 47). When these polysaccharides were hydrolysed and the monosaccharide products separated by TLC only traces of galacturonic acid were located, insufficient to be quantitated by densitometric assay (see Section III.5. (e), Tables 42 and 43 and Figure 46). This is considered another puzzling finding since, as galacturonic acid was lost from the mango cell wall during ripening, 'enzyme' preparations from mangoes, with which a considerable amount of soluble carbohydrate remains associated, might be expected to contain bound galacturonic acid, especially in over-ripe fruit.

The results of these electrophoretic studies were disappointing since, at the outset, it was anticipated that particularly 'enzyme' preparations from over-ripe fruit would

release glycans from the unripe cell wall preparation into the soluble fraction, providing insight into the mechanisms by which enzymic depolymerisation of mango cell wall polysaccharides occurred during ripening. In particular, release of soluble glycan fragments incorporating arabinose, galactose and galacturonic acid was anticipated. It was appreciated that the method employed for preparation of the cell wall material, which involved repeated buffer washing of the insoluble (cell wall) fraction of the unripe mango mesocarp, would be likely to remove 'soluble pectin' from the wall preparations but, at the unripe stage of development, it was considered that 'soluble pectin' would be at a minimum and that much of the galacturonic acid in the wall would remain wall-bound and be subject to release as soluble galacturonan when incubated *in vitro* with 'enzyme' preparations from over-ripe fruit.

Polygalacturonase activity could not be detected in mango mesocarp (see Section III.3., p. 193) suggesting that marked depolymerisation of galacturonan in the mango cell wall is not likely to occur during ripening in contrast to the mechanism proposed for other fruits in this respect (Pressey, 1977; Hobson, 1981; Labavitch, 1981). The structures of the mango cell wall and of its constituent polysaccharides have not been characterised but the pectic polymers from other plant cell walls have been proposed to consist of an acidic galacturonan backbone bearing side-chains of neutral polysaccharides containing galactose and arabinose (Aspinall, 1973, 1981; McNeil *et al.*,1979;

Darvill et al., 1980; Kato, 1981), (see also Sections I. 2. (c) 2, p. 29 and I.3. (a), p. 42). The so-called "model" for the dicot primary cell wall first proposed by Keegstra et al (1973) suggested that the pectic rhamnogalacturonan backbone interacted with other wall polysaccharides such as hemicelluloses through the neutral pectic side-chains, and that such interaction contributed to the cohesion and integrity of the wall matrix. Although this model may apply only to the cultured sycamore cell primary wall for which it was proposed it has gained wide currency in cell wall research and in the apple, the fruit species in which constituent polysaccharides of the primary cell wall have been most closely studied (Barrett and Northcote, 1965; Knee et al., 1975; Knee, 1978a; Knee and Bartley, 1981) the structures of the pectic polymers appear to be similar to those proposed in the sycamore cell wall model.

In the present study (see Section III.1 (b), p. 162) it was noted that the mango cell wall, like the apple cell wall (Knee, 1973c), lost galactose and arabinose during ripening. In the apple the loss of these neutral sugars was accompanied by solubilisation of galacturonan which was free of these neutral residues (Knee 1973c) to which it appears to be attached in the intact apple wall (Barrett and Northcote, 1965; Knee et al., 1975; Knee, 1978a; Knee and Bartley, 1981). If in the mango mesocarp cell wall galacturonan is linked to neutral arabinans/ galactans which bind it into the wall structure, then it may be speculated that removal of arabinose and galactose by

enzymic degradation of these neutral polysaccharides could provide a mechanism (not involving polygalacturonase) for the solubilisation of acidic pectin from the cell walls of ripening mango. Such solubilisation has been reported by earlier authors (Rolz et al.,1971; Mizuta and Subramanyam, 1973; Saeed et al., 1975).

However, such a mechanism would be expected to cause the appearance in the soluble fraction of undegraded galacturonan. Since mango 'enzyme' preparations could not be shown to release soluble galacturonan from unripe mango cell walls in vitro, in the present study, it must be concluded that the means by which galacturonic acid is removed from the mango mesocarp cell wall during ripening has not been established and remains a subject for further investigation. The mechanism(s) by which arabinose and galactose are removed are also worthy of further enquiry and such studies should be linked with systematic chemical characterisation of the constituent polysaccharides of the mango wall at various stages of ripeness.

The compositions of the polysaccharide fractions, which appeared to be derived from the 'enzyme' preparations, appearing on glass-fibre paper electrophoretograms were of interest. However, without knowing the degree of heterogeneity of the various polysaccharide fractions obtained by electrophoresis and the detailed structures of the components it is impossible to reach other than tentative conclusions regarding the observed changes.

The over-ripe and unripe mango 'enzyme' preparations obviously possessed a catalytic activity which resulted in an

apparent decrease in the degree of polymerisation of the endogenous polysaccharides, which are presumably cell wall-derived, since in both preparations (see Section III.5.(e), p. 238) new polysaccharides of higher electrophoretic mobility appeared after incubation. In the case of the over-ripe fruit preparations (see Fig.45 (a) and (b), p.240) the polysaccharides appeared to be relatively high molecular weight materials migrating as single electrophoretic bands. In the unripe preparations polysaccharides with a wider range of molecular weights were obvious, suggesting possible ripening-related changes. Two components (1 and 2; Fig.47) in the unripe preparation possessed relatively high proportions of galactose (Table 43, p. 249) compared to fractions (A and B; Fig. 45 (a), (b)) obtained from over-ripe preparations. This can be equated with removal of galactose from wall polysaccharides during the ripening process. The conclusion is supported by other findings: cell wall analyses (Section III.1. (b), p.162) indicated wall-bound galactose release, enzyme preparations from over-ripe mangoes liberated galactose from unripe cell walls (Section III.5. (c),p.223) and β-D-galactosidase activity in mango mesocarp increased with ripening (Section III.4. (b), p. 196). All these results indicate that galactose loss from the cell wall is an important factor in mango ripening.

The polysaccharides extracted from unripe and over-ripe mangoes were similar in general composition (see Tables 42 and 43), containing largely neutral sugars (predominantly xylose and glucose with smaller amounts of

galactose and arabinose) and traces of uronic acids. Galactose and arabinose in the extracted polysaccharides may, in part, be derived from neutral pectic polymers. The possibility that starch in the mango mesocarp may contribute to the glucose recovered can not be discounted, although starch has very low electrophoretic mobility (see Fig. 13, p. 88) and would only be expected as a possible contaminant of the electrophoretic fractions of lowest mobility.

The monosaccharide composition of the extracted polysaccharides certainly suggests wall-derived fragments largely of hemicellulosic origin. The presence in the soluble fractions of such polysaccharides tends to reinforce indications, provided by the noted decline of xylose, mannose and fucose in mango cell walls during ripening (Section III.1.(b), pp. 165-167) and by the appearance of free xylose, increasing in over-ripeness, in the soluble fraction of autolysing mango mesocarp (Section III.2., p.172), that hemicelluloses may be mobilised in the mango cell wall during ripening. The apparent decrease in the degree of polymerisation of the polysaccharides upon incubation of the preparations (p.295) suggests that mango mesocarp may contain enzymic activities which degrade hemicelluloses.

V. CONCLUDING REMARKS.

1. In some respects the ripening of the mango appears similar to the ripening of other fruits studied, most notably in the observed decline of galacturonic acid, galactose and arabinose in the cell wall. However, in other respects, notably the absence of polygalacturonase activity at all stages during ripening and indications that, in the mango, hemicellulosic polysaccharides of the wall may be degraded, the data obtained indicate that the ripening mango differs significantly from many other fruits studied.

2. In future studies better characterisation of the structures of the mango cell wall and of its constituents, at various stages of ripeness, will be required. Entire, starch-free cell walls are required. Removal of starch may require sonication and/or repeated digestion with amylase followed by dialysis of cell wall preparations. Improved methods, e.g. extraction of polysaccharide fractions from walls with selective solvents, purification of polysaccharides and glycoproteins by column chromatography and refined analysis of the resultant fractions obtained by selective hydrolysis techniques, enzymic analysis and glycosyl linkage analysis, need to be employed to achieve these objectives. The structural significance in mango cell walls of proteins and of phenolic components, if present (as well as of polysaccharides) needs to be established.

3. At various stages of ripeness both the intact tissue and the wall preparations should be critically monitored using microscopy.

4. More accurate methods for analysing monosaccharides should be employed (HPLC might be fruitful in this respect) and these should be coupled with hydrolytic methods devised to ensure total hydrolysis of all starch-free, entire cell wall and constitutent polysaccharide preparations.

5. In all further studies calculation of monosaccharide recoveries in cell wall analysis should be referred to 100 g wet mango mesocarp tissue. All data should be suitably replicated and assessed for their statistical significance.

6. The development during ripening of the 'soluble pectin' (as well as of other polysaccharides extractable in aqueous buffers at room temperature, if present) should be investigated in mango mesocarp, in addition to the investigation of the insoluble cell wall.

7. Further enzyme studies must be made using enzyme preparations from which at least all simpler sugars and preferably all other non-protein-bound carbohydrates have been removed. Mango mesocarp at various stages of ripeness should be investigated for glycanase activities using, as substrates,

purified polysaccharides of known structure, preferably extracted from the cell wall of mango itself. Purified p-nitrophenyl glycosidases extracted from mango mesocarp should also be investigated for hydrolytic activity against such substrates to establish the physiological significance (if any) of these enzyme activities in mango during ripening.

8. Mango mesocarp should be investigated for protease activities and for the possible significance of such activities in structural changes of the cell wall during ripening.

REFERENCES

AHMED, A.E.R. (1978).
Ph.D. thesis, University of California, Davis.

AHMED, A.E.R. and LABAVITCH, J.M. (1980a).
Plant Physiol. 65, 1009-1013.

AHMED, A.E.R. and LABAVITCH, J.M. (1980b).
Plant Physiol. 65, 1014-1016.

ALBERSHEIM, P. (1975).
Sci.Am. 232, 80-95.

ALBERSHEIM, P. (1976).
In "Plant Biochemistry" (J.Bonner and J.E.Varner, eds.).
Academic Press, New York. pp. 225-274.

ALBERSHEIM, P. (1978).
In "Biochemistry of Carbohydrates II. Int.Rev.Biochem.
Vol. 16" (D.J.Manners, ed.). University Park Press,
Baltimore. pp. 127-150.

ALBERSHEIM, P. and KILLIAS, U. (1963).
Am. J. Bot. 50, 732-745.

ALBERSHEIM, P., NEVINS, D.J., ENGLISH, P.D. and
KARR, A. (1967). Carbohydr. Res. 5, 340-345.

ASPINALL, G.O. (1970).
In "The Carbohydrates" (W.Pigman and D.Horton, eds.),
Vol. IIB. Academic Press, New York. pp. 515-536.

ASPINALL, G.O. (1973).
In "Biogenesis of Plant Cell Wall Polysaccharides"
(F.Loewus, ed.). Academic Press, New York. pp. 95-115.

ASPINALL, G.O. (1980).
In "The Biochemistry of Plants". Vol.3. Carbohydrates:
Structure and Function". (J.Preiss, ed.). Academic
Press, London and New York. pp. 473-500.

ASPINALL, G.O. (1981).
In "Encyclopedia of Plant Physiology. New Series
Vol. 13B. Plant Carbohydrates II. Extracellular
Carbohydrates" (W.Tanner and F.A.Loewus, eds.).
Springer-Verlag, Berlin. pp. 3-8.

ASPINALL, G.O. and COTTRELL, I.W. (1971).
Can.J.Chem. 49, 1019-1022.

ASPINALL, G.O. FAIRWEATHER, R.M. and WOOD, T.M. (1968).
J.Chem.Soc. (C). pp. 2174-2179.

REFERENCES (continued)

ASPINALL, G.O., MOLLOY, J.A. and CRAIG, J.W.T. (1969).
Can.J.Biochem. 47, 1063-1070.

ASPINALL, G.O. and STEPHEN, A.M. (1973).
In "MTP International Review of Science" (G.O. Aspinall, ed.), Org.Chem.Series 1, Vol. 7. Butterworth, London. pp. 285-313.

AWAD, M. and YOUNG, R.E. (1979).
Plant Physiol. 64, 306-308.

BARNES, M.F. and PATCHETT, B.J. (1976).
J.Food Sci., 41, 1392-1395.

BARRETT, A.J. and NORTHCOTE, D.H. (1965).
Biochem. J. 94, 617-627.

BARTLEY, I.M. (1974).
Phytochem. 13, 2107-2111.

BARTLEY, I.M. (1978).
Phytochem. 17, 213-216.

BECK, E. (1967).
Z.Pflanzenphysiol 57, 444-461.

BELL, T.A. (1951).
Bot.Gaz. 113, 216-221.

BEN-ARIE, R., KISLER, N. and FRENKEL, C. (1979).
Plant Physiol. 64, 197-202.

BESFORD, R.T. and HOBSON, G.E. (1972).
Phytochem. 11, 2201-2205.

BIALE, J.B. (1960).
Advan. Food Res. 10, 293-354.

BIALE, J.B. and YOUNG, R.E. (1981).
In "Recent Advances in the Biochemistry of Fruits and Vegetables" (J.Friend and M.J.C. Rhodes, eds.). Academic Press, London and New York. pp. 1-39.

BITTER, T. and MUIR, H.M. (1962).
Analytical Biochem. 4, 330-334.

BLAKE, J.D. and RICHARDS, G.N. (1968).
Carbohydr. Res. 8, 275-281.

BLAKE, J.D. and RICHARDS, G.N. (1971).
Carbohydr. Res. 18, 11-20.

REFERENCES (continued)

BUCHALA, A.J. and FRANZ, G. (1974).
Phytochem. 12, 1373-1376.

BURG, S.P. and BURG, E. (1964).
Plant Physiol. 39, supplement - Proc.Ann.Mtng.
Plant Physiol, 1964, University of Colorado,
Boulder, page X.

CAMPBELL, W.C. (1959).
Proc. 19th Ann. Meeting Fla. Mango Forum,
Hort. Absts. 21 (1), p.11.

CAPON, B. and GHOSH, B.C. (1965).
Chem. Commun. No.22, 586-587.

CAYGILL, J.
Tropical Products Institute, London (private
communication).

CHANDA, S.K., HIRST, E.L. and PERCIVAL, E.G.V. (1951).
J.Chem.Soc., 1240-1246.

CLARKE, A.E., ANDERSON, R.L. and STONE, B.A. (1979).
Phytochem. 18, 521-540.

DARVILL, A.G.,McNEIL, M., and ALBERSHEIM, P. (1978).
Plant Physiol. 62, 418-422.

DARVILL, A.G.,McNEIL, M., ALBERSHEIM, P. and
DELMER, D.P. (1980).
In "The Plant Cell" (N.E.Tolbert, ed.).
Academic Press, New York. pp. 91-162.

DAS, A. and RAO, C.V.N. (1964).
Tappi 47, 339-343.

DAS, A. and RAO, C.V.N. (1965).
Aust. J. Chem. 18, 845-850.

DAVIES, J.W. and COCKING, E.F. (1965).
Planta 67, 242-253.

DE, K.K. and TIMELL, T.E. (1967).
Carbohydr. Res. 4, 177-181.

DEA, I.C.M. and MORRISON,A. (1975).
Advan. Carbohydr. Chem. Biochem. 31, 241-312.

DELINCEE, H. (1976).
Phytochem. 15, 903-906.

REFERENCES (continued)

DUBOIS, M., GILLES, K.A., HAMILTON, J.K., REBERS, P.A. and SMITH, F. (1956).
Analytical Chem. 28, 350-356.

DUFF, R.B. (1965).
Biochem. J. 94, 768-772.

EASTY, D.B. (1962).
J.Org.Chem. 27, 2102-2106.

ERIKSSON, K.E. (1977).
In "Cell Wall Biochemistry Related to Specificity in Host-Plant Pathogen Interactions" (J.Raa and B.Solheim, eds.).
University Tromso Press, Tromso, Norway. pp.71-83.

FODA, Y.M. (1957).
Ph.D. thesis, University of Illinois.

FREY-WYSSLING, A. (1969).
Fortschr. Chem. Org. Naturst. 26, 1-30.

FRY, S.C. (1982a).
Biochem. J. 204, 449-455.

FRY, S.C. (1982b).
Biochem. J. 203, 493-504.

FRY, S.C. (1983).
Planta 157, 111-123.

GAILLARD, B.D.E. (1958).
J.Fd. Sci. Agric. 9, 170-177.

GILKES, N.R. and HALL, M.A. (1977).
New Phytol. 78, 1-15.

GINSBURG, B.Z. (1961).
J.Exp. Bot. 12, 85-107.

GRANT, G.T., MORRIS, E.R., REES, D.A., SMITH, P.J.C. and THOM, D. (1973).
FEBS Letters 32, 195-198.

GRIERSON, D., TUCKER, G.A. and ROBERTSON, N.G. (1981a).
In "Recent Advances in the Biochemistry of Fruits and Vegetables" (J.Friend and M.J.C.Rhodes, eds.).
Academic Press, London and New York. pp. 149-160.

GRIERSON, D., TUCKER, G.A. and ROBERTSON, N.G. (1981b).
In "Quality in Stored and Processed Vegetables and Fruits" (P.W. Goodenough and R.K.Atkin, eds.).
Academic Press, London and New York. pp. 179-191.

REFERENCES (continued)

GROSS, K.C. and WALLNER, S.J. (1979).
Plant Physiol. 63, 117-120.

GUSTAFSON, K.H. (1954).
Svensk Kem. Tidskr. 66, 359-362.

HAMILTON, J.K. and THOMPSON, N.S. (1960)
Pulp Paper Mag. (Canada) 61, T263-T272.

HANKINS, C.N. and SHANNON, L.M. (1978).
J. Biol.Chem. 253, 7791-7797.

HAQ. C. and C.A.ADAMS (1961).
Can. J. Chem. 39, 1563-1573.

HART, D.A. and KINDEL, P.K. (1970).
Biochem. J. 116, 569-579.

HESTRIN, S., FEINGOLD, D.S. and SCHRAMM, M. (1955).
In "Methods in Enzymology", Vol. 1 (Colowick, S.P. and Kaplan, N.O., eds.).
Academic Press, New York. p.249.

HIRST, E.L. and JONES, J.K.N. (1947).
J.Chem. Soc., pp.1221-1225.

HOBSON, G.E. (1962).
Nature 195, 804-805.

HOBSON, G.E. (1968).
Ann.Rep.Glasshouse Crops Res.Inst. 1967, 134-136.

HOBSON, G.E. (1979).
Curr. Adv. Plant Sci. 11, 1-11.

HOBSON, G.E. (1981).
In "Recent Advances in the Biochemistry of Fruits and Vegetables" (J.Friend and M.J.C.Rhodes, eds.).
Academic Press, London and New York. pp. 123-132.

HORI, H. and SATO, S. (1977).
Phytochem. 16, 1485-1487.

HOUGH, L. and POWELL, D.B. (1960).
J. Chem. Soc. pp. 16-22.

HOUGH, L. and PRIDHAM, J.B. (1956).
Nature. 177, 1039.

HUBER, D.J. and NEVINS, D.J. (1977).
Plant Physiol. 60, 300-304.

HULLAR, T.L. (1965).
Dissertation Abstr. 26, 671.

REFERENCES (continued)

HULME, A.C. (1971).
In "The Biochemistry of Fruits and Their Products".
(A.C.Hulme, ed.). Academic Press, London.
pp. 233-254.

HULME, A.C. (1972).
J. Food Technol. 7, 343-371.

HULME, A.C. and JONES, J.D. (1963).
In "Enzyme Chemistry of Phenolic Compounds".
(J.B.Pridham, ed.). Pergamon Press, Oxford.
Chapter 10, pp. 97-120.

International Trade Centre (UNCTAD/GATT). (1971).
"The Markets for Selected Exotic Fruit Products in the U.K., the Federal Republic of Germany, Switzerland and the Netherlands". Report, Geneva.

ISHII, S. (1978).
Plant Physiol. 62, 586-589.

JAIN, N.L. (1961).
Rev. Food Technol. 3, 131-162.

JANSEN, E.F., JANG, R. and BONNER, J. (1960).
Food Res. 25, 64-72.

JARVIS, M.C., THRELFALL, D.R. and FRIEND, J. (1977).
Phytochem. 16, 849-852.

JAYNES, T.A., HASKINS, F.A., GORZ, H.J. and KLEINHOES,A. (1972).
Plant Physiol. 49, 277-279.

JERMYN, M.A. and ISHERWOOD, F.A. (1956).
Biochem. J. 64, 123-132.

JOHNSON, K.D., DANIELS, D., DOWLER, M.J. and RAYLE, D.L. (1974).
Plant Physiol. 53, 224-228.

JONES, A. (1973).
"The Market for Mango Products with Particular Reference to the U.K." Tropical Products Institute Report, London.

JONES, T.M. and ALBERSHEIM, P. (1972).
Plant Physiol. 49, 926-936.

JOSELEAU, J.P., CHAMBAT, G., VIGNON, M. and BARNOUD, F. (1977).
Carbohydr. Res. 58, 165-175.

REFERENCES (continued)

JOSLYN, M.A. (1962).
In "Advances in Food Research" (C.O.Chichester, E.M.Mrak and G.F.Stewart.,eds.). Academic Press, New York and London. pp.1-107.

KARACSONYI, S., TOMAN, R., JANECEK, F. and KUBACKOVA, M. (1975).
Carbohydr. Res. 44, 285-290.

KATO, K. (1981).
In "Encylcopedia of Plant Physiology. New Series, Vol. 13B. Plant Carbohydrates II, Extracellular Carbohydrates (W.Tanner and F.A. Loewus, eds.). Springer-Verlag, Berlin. pp. 29-46.

KATO,Y., ASANO, N. and MATSUDA, K. (1977).
Plant Cell Physiol. 18, 821-829.

KAUSS, H. and BOWLES, D.J. (1976).
Planta 130, 169-174.

KAUSS, M. and GLASER, C. (1974).
FEBS Lett. 45, 304-307.

KEEGSTRA, K. and ALBERSHEIM, P. (1970).
Plant Physiol. 45, 675-678.

KEEGSTRA, K., TALMADGE, K.W., BAUER, W.D. and ALBERSHEIM, P. (1973).
Plant Physiol. 51, 188-196.

KIVILAAN, A., BEAMAN, T.C. and BANDURSKI, R.S. (1959).
Nature 184, B.A.81 - B.A.82.

KIVILAAN, A., BEAMAN, T.C. and BANDURSKI, R.S. (1961).
Plant Physiol. 36, 605-610.

KLIS, F.M., DALHUIZEN, R. and SOL, K. (1974).
Phytochem. 13, 55-57.

KNEE, M. (1973a).
J.Food Sci. Agric. 24, 1137-1145.

KNEE, M. (1973b).
Phytochem. 12, 637-653.

KNEE, M. (1973c).
Phytochem. 12, 1543-1549.

KNEE, M. (1978a).
Phytochem. 17, 1257-1260.

REFERENCES (continued)

KNEE, M. (1978b).
Phytochem. 17, 1261-1264.

KNEE, M. and BARTLEY, I.M. (1981).
In "Recent Advances in the Biochemistry of Fruits and Vegetables" (J.Friend and M.J.C.Rhodes, eds.).
Academic Press, London and New York. pp. 133-148.

KNEE, M., FIELDING, A.M., ARCHER, S.A. and LABORDA, F. (1975).
Phytochem. 14, 2213-2222.

KNEE, M., SARGENT, J.A. and OSBORNE, D.J. (1977).
J.Exp. Bot. 28, 377-393.

KRISHNAMURTHY, S. and SUBRAMANYAM, H. (1973).
Trop.Sci. 15, 167-193.

LABAVITCH, J.M. (1981).
Ann. Rev. Plant Physiol. 32, 385-406.

LABAVITCH, J.M. and RAY, P.M. (1978).
Phytochem. 17, 933-937.

LABAVITCH, J.M., FREEMAN, L.E. and ALBERSHEIM, P. (1976).
J.Biol. Chem. 251, 5904-5910.

LACKEY, G.D., GROSS, K.C. and WALLNER, S.C. (1980).
Plant Physiol. 66, 532-533.

LAKSHMINARAYANA, S. (1973).
J.Hort.Sci. 48, 227-233.

LAMPORT, D.T.A. (1965).
Adv. Bot. Res. 2, 151-218.

LAMPORT, D.T.A. (1973).
In "Biogenesis of Plant Cell Wall Polysaccharides" (F.Loewus, ed.). Academic Press, New York. pp. 149-164.

LAMPORT, D.T.A. (1978).
In "Frontiers of Plant Tissue Culture" (T.A.Thorpe, ed.). The Bookstore, Univ. Calgary, Canada.
pp. 235-244.

LAMPORT, D.T.A., KATONA, L. and ROERIG, S. (1973).
Biochem. J. 133, 125-131.

LEE, M. and MacMILLAN, J.D. (1970).
Biochemistry 9, 1930-1934.

LELEY, V.K., NARAYANA, N. and DAJI, J.A. (1943).
Indian J. Agr. Sci. 13, 291-299.

REFERENCES (continued)

LETHAM, D.S. (1962).
Exp. Cell.Res. 27, 352-355.

LEWIS, C.E. (1978).
J.Food Sci.Agric. 29, 857-866.

LINDBERG, B., ROSELL, K.G. and SVENSSON, S. (1973).
Sven. Papperstidn. 76, 383-384.

LINEHAM, D.J. and HUGHES, J.C. (1969).
J.Sci. Fd. Agric. 20, 119-123.

LOOMIS, W.D. and BATTAILE, J. (1966).
Phytochem. 5, 423-438.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and
RANDALL, R.J. (1951).
J.Biol.Chem. 193, 265-275.

MacMILLAN, J.D. and SHEIMAN, M.I. (1974).
Advan. Chem. Ser. No.136, 101-130.

MANNHEIM, C.H. and SIV, S. (1969).
Fruchtsaft Industrie 14, 98-103.

MARCHESSAULT, R.H. and RANBY, B.G. (1959).
Svensk Papperstidn. 62, 230-240.

MARX-FIGINI, M. (1966).
Nature 210, 754-755.

MARX-FIGINI, M. and SCHULZ, G.V. (1966).
Naturwissenschaften 53, 466-474.

MATTOO, A.K. and MODI, V.V. (1969).
Proc.Int.Conf. Trop. and Sub-trop. Fruits.
London. pp.111-115.

MATTOO, A.K. and MODI, V.V. (1970).
Enzymologia 39, 237-247.

McFEETERS, R.F., BELL, T.A. and FLEMING, H.P. (1980).
J.Food Biochem. 4, 1-16.

McNEIL, M. and ALBERSHEIM, P. (1978).
Unpublished findings cited in Darvill, A.G., McNeil, M.,
Albersheim, P. and Delmer, D.P. (1980). In "The
Plant Cell" (N.E.Tolbert, ed.). Academic Press,
New York, pp. 91-162.

REFERENCES (continued)

McNEIL, M., DARVILL, A.G. and ALBERSHEIM, P. (1979).
Fortschr. Chem. Org. Naturst. 37, 191-248.

MEIER, H. (1962).
Acta Chem. Scand. 16, 2275-2283.

MENZIES, I.S. and MOUNT, J.N. (1975).
Med. Lab. Tech. 32, 269-276.

MIZUTA, T. and SUBRAMANYAM, H. (1973).
Unpublished findings cited in H.Subramanyam, S. Krishnamurthy and H.A.B. Parpia (1975), Adv. Food Res. 21, 223-305.

MOHR, W.P. and STEIN, M. (1969).
Can. J. Plant. Sci. 49, 549-553.

MONRO, J.A., PENNY, D. and BAILEY, R.W. (1976a).
Phytochem. 15, 1193-1198.

MONRO, J.A., BAILEY, R.W. and PENNY, D. (1976b).
Phytochem. 15, 175-181.

MORONEY, M.J. (1965; latest re-print 1982).
"Facts from Figures", Penguin Books, Harmondsworth, U.K.

MORT, A.J. and LAMPORT, D.T.A. (1977).
Anal. Biochem. 82, 289-309.

MUKERJEE, P.K. (1960).
Hort. Adv. 4, 138-141.

MURRAY, A.K. and BANDURSKI, R.S. (1975).
Plant Physiol. 56, 143-147.

NAKAGAWA, H., SEKIGUCHI, K., OGURA, N. and TAKEHANA, H. (1971).
Agr.Biol. Chem. 35, 301-307.

NAKANO, J. and RANBY, B.G. (1962).
Svensk Papperstidn. 65, 29-33.

NEAL, G.E. (1965).
J.Sci.Food Agric. 16, 604-611.

NELSON, N. (1944).
J. Biol. Chem. 153, 375-380.

NEVINS, D.J. (1970).
Plant Physiol. 46, 458-462.

REFERENCES (continued)

NORTHCOTE, D.H. (1972).
Ann. Rev. Plant Physical. 23, 113-132

NORTHCOTE, D.H. (1977).
In "The Synthesis, Assembly and Turnover of Cell Surface Components" (G.Poste and G.L.Nicolson, eds.). North Holland, Amsterdam. Vol. 4. pp. 717-739.

PANSOLLI, P. and BELLI-DONINI, M.L. (1973).
Agrochimica 17, 365-368.

PARR, D.R. and EDELMAN, J. (1975).
Planta 127, 111-119.

PESIS, E., FUCHS, Y. and ZAUBERMAN, G. (1978).
Plant Physiol. 61, 416-419.

PHARR, D.M., SOX, H.N. and NESBITT, W.B. (1976).
J. Am. Soc. Hort. Sci. 101, 397-403.

PIERROT, H. and VAN WIELINK, J.E. (1977).
Planta 137, 235-242.

PILNIK, W. and VORAGEN, A.J.G. (1970).
In "The Biochemistry of Fruits and Their Products" (A.C.Hulme, ed.). Vol. 1. Academic Press, London. pp. 53-87.

POOVAIAH, B.W. (1979).
Commun. Soil Sci. Plant Anal. 10, 83-88.

POOVAIAH, B.W. and NUKAYA, A. (1979).
Plant Physiol. 64, 534-537.

POPE, D.G. and LAMPORT, D.T.A. (1977).
Plant Physiol. 59, 894-900.

PRESSEY, R. (1977).
In "Enzymes in Food and Beverage Processing". (R.L.Ory and A.St.Angelo, eds.). Am.Chem.Soc., Washington. pp. 172-191.

PRESSEY, R. (1983).
Plant Physiol. 71, 132-135.

PRESSEY, R. and AVANTS, J.K. (1973).
Plant Physiol. 52, 252-256.

PRESSEY, R. and AVANTS, J.K. (1975a).
J. Food Sci. 40, 937-939.

PRESSEY, R. and AVANTS, J.K. (1975b).
Phytochem. 14, 957-961.

REFERENCES (continued)

PRESSEY, R. and AVANTS, J.K. (1978).
J.Food Sci. 43, 1415-1417.

PRESSEY, R., HINTON, D.M. and AVANTS, J.K. (1971).
J.Food Sci. 36, 1070-1073.

PRESTON, R.D. (1979).
Ann. Rev. Plant Physiol. 30, 55-78.

REES, D.A. (1972).
Biochem. J. 126, 257-273.

REES, D.A. (1975).
In "Biochemistry of Carbohydrates" (W.J.Whelan, ed.).
M.T.P. Internat. Rev. Sci. Biochem.Ser.1, Vol.5.
Butterworth, London; University Park Press, Baltimore.
pp. 1-42.

REES, D.A. and RICHARDSON (1966).
Biochemistry 5, 3099-3107.

RICHMOND, A. and BIALE, J.B. (1967).
Biochim. Biophys. Acta 138, 625-627.

RING, S.G. and SELVENDRAN, R.P. (1978).
Phytochem. 17, 745-752.

ROBINSON, D.G. (1977).
In Adv. Bot. Res., Vol. 5 (H.W.Woolhouse, ed.).
Academic Press, London and New York. pp. 89-151.

ROLZ, C., DESHPANDE, S., PAIZ, L., ORTIZ, J.,
FLORES, M.C., SANCHEZ, M. and DE ORTEGA, M. (1972).
Turrialba 22, 65-72.

ROMBOUTS, F.M., WISSENBURG, A.K. and PILNIK, W. (1979).
J.Chromatogr. 168, 151-161.

SAEED, A.R., EL TINAY, A.H. and KHATTAB, A.H. (1975).
J. Food Sci. 40, 203-204.

SANKAR, K.P. (1963).
Sci. Cult. 29, 51-59.

SAUNDERS, T. and TIMELL, T.E. (1967).
Unpublished findings cited in De, K.K. and Timell, T.E.,
(1967), Carbohydr. Res. 4, 177-181.

SAXENA, K.K., MUKHERJEE, R. and MAHADEVAN, V. (1961).
J.Sci. Ind. Res. 20C, 186-188.

SELVENDRAN, R.P. (1975).
Phytochem. 14, 2175-2180.

REFERENCES (continued)

SEMKE, L.K., THOMPSON, N.S. and WILLIAMS, D.G. (1964).
J. Org. Chem. 19, 1041-1047.

SHAFIZADEH, F. and McGINNIS, G.D. (1971).
Adv. Carbohydr. Chem. Biochem. 26, 297-349.

SIDDIQUI, I.R. and WOOD, P.J. (1974).
Carbohydr. Res. 36, 35-44.

SINGH, L.B. (1960).
"The Mango". Leonard Hill, London.

SMITH, O. (1935).
N.Y.Agric. Exp. Stn., Ithaca, Mem. No. 184, 1-16.

SNEDECOR, G.W. and COCHRAN, W.G. (1967).
"Statistical Methods", Iowa State University Press, Iowa.

SOUTHGATE, D.A.T. (1976).
In "Fiber in Human Nutrition". (G.A.Spiller and R.J.Amen, eds.). Plenum Press, New York and London. pp. 31-72. (p.61).

STRAND, L.L., RECHTORIS, C. and MUSSELL, N. (1976).
Plant Physiol. 58, 722-725.

TAKEUCHI, Y. and KOMAMINE, A. (1978).
Physiol. Plant 42, 21-28.

TALMADGE, K.W., KEEGSTRA, K., BAUER, W.D. and ALBERSHEIM, P. (1973).
Plant Physiol. 51, 158-173.

TIGCHELAAR, E.C., McGLASSON, W.B. and BUESCHER, R.W. (1978).
Hort. Science 13, 508-513.

TIMELL, T.E. (1964a).
Adv. Carbohydr. Chem. 19, 247-302.

TIMELL, T.E. (1964b).
Chem. and Ind. pp. 503-504.

TIMELL, T.E. (1965).
Adv. Carbohydr. Chem. 20, 409-483.

TIMELL, T.E. ENTERMAN, W., SPENCER, F. and SOUTES, E.J. (1965).
Canad. J. Chem. 43, 2296-2305.

TOMAN, R., KARACSONYI, S. and KUBACKOVA, M. (1972).
Carbohydr. Res. 25, 371-378.

REFERENCES (continued)

TRACEY, M.W. (1948).
Biochem. J. 43, 185-189.

TUCKER, G.A. and GRIERSON, D. (1982).
Planta 155, 64-67.

UPDEGRAFF, D.M. (1969).
Analyt. Biochem. 32, 420-424.

VAN SOEST, P.J. and McQUEEN, R.W. (1973).
Proc.Nutr. Sci. 32, 123-130.

VAN SUMERE, C.F., ALBRECHT, J., DEDONDER, A.,
De POOTER, H. and PE, I. (1975).
In "Chemistry and Biochemistry of Plant Proteins",
(J.B. Harborne and C.F. Van Sumere, eds.).
Academic Press, London. pp. 211-264.

VICKERY, R.S. and BRUINSMA, J. (1973).
J.Exp.Bot. 24, 1261-1270.

WADA, S. and RAY, P.M. (1978).
Phytochem. 17, 923-931.

WALI, Y.A. and HASSAN, Y.M. (1965).
Proc.Amer.Soc.Hort. Sci. 87, 264-269.

WALLENFELS, K. and MALHOTRA, O.P. (1961).
Advan. Carbohydr. Chem. 16, 239-298.

WALLNER, S.J. (1978).
J. Am. Soc. Hort. Sci. 103, 364-373.

WALLNER, S.J. and WALKER, J.E. (1975).
Plant Physiol. 55, 94-98.

WEURMAN, C. (1953).
Acta Botan. Neerl. 2, 107-110.

WHISTLER, R.L. and RICHARDS, G.N. (1958).
J. Amer. Chem. Soc. 80, 4888-4891.

WHITMORE, F.W. (1974).
Plant Physiol. 53, 728-731.

WILKIE, K.C.B. and WOO, S.C. (1977).
Carbohydr. Res. 57, 145-162.

WILLS, R.B.H., SCOTT, K.J. and FRANKLIN, M.J.,
(1976).
Phytochem. 15, 1817-1818.

REFERENCES (continued)

WISE, L.E., MURPHY, M. and D'ADDIECO, A.A. (1946).
Paper Tr. J. 122, 35-43.

WOODMANSEE, C.W., McCLENDON, J.H. and SOMERS, G.F. (1959).
Food Res. 24, 503-514.

WOODWARD, J.R. (1972).
J. Food Sci. Agric. 23, 465-473.

YAMAKI, S. and KAKIUCHI, N. (1979).
Plant Cell Physiol. 20, 301-309.

ZITKO, V. and BISHOP, C.T. (1966).
Can. J. Chem. 44, 1275-1282.