SOFTENING AND THE CELL WALL ENZYMES OF STRAWBERRY FRUIT

A thesis
submitted in partial fulfilment
of the requirements for the Degree
of
Master of Horticultural Science
in the
University of Canterbury

by
B.J. PATCHETT

Lincoln College
1974

CONTENTS

LIST OF TABLES

LIST OF TABLES (Cont'd)

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The softening of fruit during ripening is one of the tangible aspects of the increase in metabolic activity that occurs with the onset of ripening.

The collapse of the cell walls of fruit during the after-ripening period is probably the most important factor which leads to the decline in quality of harvested fruit. Strawberries can only be held for a short time, under normal conditions, before a decline occurs in quality and therefore consumer appeal. The collapse of cell walls is generally attributed to the solubilization and hydrolysis of the pectin fraction of the cell wall under the influence of the pectic enzymes.

The work described in this dissertation is an attempt to measure the relative importance of the enzymes responsible for softening during the ripening and after ripening phase of strawberry fruit.

CHAPTER II

SOFTENING OF FRUIT

I. BIOCHEMISTRY OF SOFTENING

(1) Softening of fruit

During the physiological life of a fruit, changes in cell wall structure occur. Pectin content and structure in particular are known to be modified over this period. The degree of esterification, molecular weight, neutral sugar components and acetylation may all change during this time (Pilnik and Voragen 1970), but the variety of methods used for fractional extraction of pectin is so great that comparison between different authors becomes tenuous.

In general, it appears that pectin levels decrease during ripening and storage but confusion remains as to the real cause of softening. Neal (1965), for example, suggested that the separation of cells he observed during ripening could be due to complete esterification of pectin along certain layers of the middle lamella which would sever the cationic crosslinks and allow the cells to separate. Others, however, have reported that esterification of total pectin decreases during ripening (Dolando et al 1966, Sterling and Kalb 1959).

Jermyn and Isherwood (1956) have made an intensive study of the polysaccharides from pear cell walls.

Changes in the amounts of xylan, araban, polygalacturonic acid, galactan and cellulose were followed during storage. A fall in total polysaccharides occurred with a major loss of araban and significant losses occurring in galactan and cellulose suggesting that the cell wall can act as a reserve for respiration. There is a sharp rise in the total polysaccharides and some fractions at the over-ripeness stage indicating that the relationship between cell wall and cytoplasm involves synthesis of polysaccharides as well as breakdown.

Shewfelt et al (1971) worked on the pectic constituents of ripening peaches and showed with four varieties that pectinic acids increased to 80% from 20% of total pectin during ripening. Pectic acids remained less than 10%. Three fractions were isolated and assayed for molecular weight and methoxyl content. Molecular weight decreased during ripening for all 3 fractions consistent with Pressey et al (1971), but only protopectin showed a consistent decrease in esterification.

Gee et al (1959) working with pears showed that the percentage esterification of pectin is maximal in mature fruit and decreases in ripening fruit. The methoxyl content of polysaccharides was shown to fall during the storage of pears (Jermyn and Isherwood 1956) which could be accounted for by the fall in methoxyl content of the pectin fraction. Wade (1964) could show no change in the ester groupings of the insoluble cell wall polysaccharides of strawberries.

The hydroxyls of Carbon-2 and Carbon-3 of the galacturonic acid units in the pectin chain were considered to be free from ester by Beavan and Jones (1947). Schweiger (1966) pointed out that the binding of divalent ions to pectin involves both the carboxyl and hydroxyl groups.

Sterling (1961) looked at the physical state of cellulose in ripening peaches by X-ray crystallography. This work showed an increase in the diameter of cellulose micelles during the ripening period. It was concluded that only limited degradation of cellulose occurred. Jermyn and Isherwood (1956) showed a small decrease in cellulose during ripening and storage of pears.

Knee (1973) examined the polysaccharide changes in cell walls of apples ripening both on and off the tree. This work showed that soluble polyuronides increased during ripening especially in detached fruit. DEAE - cellulose chromatography showed that this polyuronide was free from neutral sugar residues. Soluble neutral polysaccharides and glycoproteins did not change during ripening. Decreases in hemi-cellulose were correlated with losses of wall glucan.

(2) Softening of Strawberries

Only a limited amount of work has been done on softening of strawberry fruit. Neal (1965) and Wade (1964) have extracted chemical constituents of different stages during ripening.

Neal (1965) considered 3 stages of ripening with two varieties of strawberries. On ripening the middle lamella of the cortical parenchyma cells separated into two, one part going to each cell. This action could be simulated by the action of EDTA and reversed by Ca^{++} ions. Complete methylation of pectin was shown to decrease firmness with no influence shown by Ca^{++}ions. Decreasing firmness was therefore attributed to the methylation of pectin which inhibits the firming effect of Ca^{++} in spite of the presence of pectin methylesterase (PE). A change in the ionic stabilization of the middle lamella rather than degradation of the pectin molecule was assumed to occur. Neal measured the polysaccharide content of the extracts obtained. These results showed that total anhydro-uronic acids remained almost constant over the ripening period and that no major change occurred in the overall composition of the cell walls. This was used to support his contention that textural changes are subtle rather than a reorganization of total cell wall composition.

Wade (1964) extracted the polysaccharides from 2 varieties of strawberries over 3 stages of ripeness from firm white berries to uniformly red firm berries. Wade showed by microscopy an increase in size of the parenchyma cells, as did Neal (1965), but a decrease in contact between adjacent cells. Wade could show no major changes over the period studied of component anhydro sugars or ester groupings of the insoluble cell wall polysaccharides.

Woodward (1962) showed limited softening of strawberries between 20 and 40 days after petal fall during which gross synthesis of polyuronides occurred. Very little polyuronide was liberated from the cell wall during this time. He proposed a general theory, consistent with the results of Wade (1964), and Neal (1965), that cell wall polysaccharides are rearranged during ripening to allow for greater plasticity.

Strawberry pectin was studied by Beaven and Jones (1947), and, McComb and McCready (1957), measured the acetyl percentage of strawberry pectin. Neither of these studies, however, were related to the ripening period.

Pectin esterase activity was shown in ripe Cambridge Favourite strawberries (Neal 1965). Gizis (1964) also showed PE activity in strawberries. Leuprecht and Schaller (1968) studied the activity of PE in strawberry puree and made a thorough investigation of the titrimetric method for following the saponification of pectin. Their study was concentrated on the optimum conditions for PE activity of frozen strawberry puree including assessment of optimum NaCl concentration, pectin concentration, pH level and temperature.

Neal (1965) could find no depolymerization activity suggestive of polygalacturonase activity in strawberries and Gizis (1964) and Staden and Doesburg (1962) suggest that micro organisms may be responsible for the depolymerisation of pectin they observed

II THE ENZYMES RESPONSIBLE FOR SOFTENING

Pilnik and Voragen (1970) have reviewed the recent material on pectin esterase (PE) and polygalacturonase. Consequently only papers more recent and more relevant to this study will be mentioned here.

(1) Pectin esterase

Hultin and Levine (1963) found 3 forms of banana pectin esterase by differential extraction which were further studied by Hultin et al (1966). These enzymes had different pH optima and reacted differently to inactivation by detergent and temperature. Following purification by ammonium sulphate fractionation, each fraction showed unique behaviour on ion exchange columns of DEAE- and CM-cellulose.

Four forms of tomato pectin esterase have been obtained by Pressey and Avants (1972) by separation on DEAE- Sephadex. These forms varied in their behaviour towards various treatments such as SDS, pH and NaCl concentration, stability to heat and molecular weight. The number of components and relative concentration of each component varied according to variety and ripeness of fruit. Fractions collected from column chromatography of green Marion tomatoes had two peaks of PE activity but ripe Marion had three peaks; one peak between the two corresponding to green fruit. In addition the first peak was lower and the third peak higher in activity than the corresponding peaks of green fruit.

Lee and Macmillan (1970) describing the mode of action of tomato PE found that 50% of the enzyme initiated activity near the reducing end of highly esterified pectin molecules. The balance of the enzyme activity occurred at secondary loci.

Wood and Siddiqui (1971) have recently described a new method for measurement of pectin ester content and PE activity as have Bartolome and Hoff (1972).

(2) Polygalacturonase

Plant polygalacturonase (P.G.) has not been greatly studied and then usually in situ without previous extraction or purification. Hobson (1962), however, has investigated the sodium EDTA extracts from various fruits and vegetables and shown PG activity in tomatoes, avocado, pears and bananas.

Neal (1965), could find no depolymerisation activity in strawberry fruit and the activity shown by Gizis (1964) could be due to microbial activity.

A detailed study of tomato fruit PG was made by Hobson (1964) which confirmed earlier work (Demain and Phaff 1957, Patel and Phaff 1958) that at least two PG enzymes were present. It has been suggested (Foda 1957, McColloch and Kertesz 1948) that one of the enzymes made a decreasing contribution to overall activity towards full ripeness of the tomato. Hobson (1964) could show no PG activity in green fruit but a two hundred fold rise from the green-orange to red stage.

Pressey et al (1971) have shown PG activity in peaches by a different assay which revealed enzymes of low activity previously undetected. PG activity rose rapidly during ripening and was accompanied by a decrease in firmness of the fruit and an increase in water soluble pectin. Total pectin remained virtually constant.

Macmillan et al (1964) showed that an exopolygalacturonate lyase from Clostridium multi-fermentans removes unsaturated digalacturonic acid. This enzyme will not degrade glycosidic linkages in pectins that are highly esterified with methoxyl groups. Plant proteins that inhibit polygalacturonases secreted by plant pathogens have been described by Albersheim and Anderson (1971). These inhibitors differentiate between the polygalacturonases of different pathogens.

Hatanaka et al (1969) have shown the presence of an exopolygalacturonase which produces digalacturonic acid by removing disaccharide units from the non-reducing end of the substrate. This enzyme would cleave trigalacturonic acids only very slowly and digalacturonic acid units not at all.

Besford and Hobson (1972), from studies on tomato fruit transeliminases concluded that pectin transelimin-ase and pectic acid transeliminase were not significant in contributing to softening of tomatoes.

(3) Cellulase

Cellulase has been little studied in fruit. The role of cellulase in cell wall softening is not clear. Several lines of evidence militate against cellulose hydrolysis being a primary cause of flesh softening.

Cellulose micelles undergo little degradation in pears (Jermyn & Isherwood, 1956) or peaches (Sterling 1961) during ripening. The cellulose content of apples decreases only slightly as they ripen in storage (Kertesz et al, 1959). Wade (1964) from his studies on the cell wall polysaccharides of strawberries concluded that cellulose levels remained the same during the ripening period. Hasegawa and Smolensky (1971) showed a dramatic rise in cellulase activity of dates during ripening. They suggested that this implied a greater involvement of cellulase activity in dates due to more extensive breakdown of cell walls. Cellulase activity of tomato has been observed to increase during ripening. Hall (1964), Hobson, (1968), Dickinson & McCollum (1964).

Hall (1963) using an assay involving the reduction in viscosity of CM cellulose over a 20 hour incubation period, showed sodium chloride extracts of tomatoes to possess cellulase activity. Extracts from locular tissue showed higher activity than extracts from pericarp tissue.

Hobson (1968) showed acetone precipitates from tomatoes to possess cellulase activity. He found cellulase activity to be high in immature fruit

subsequently declining, but increasing two fold in passing from mature green to the red ripe stage. Cellulase activity stayed at this level while P.G. activity continued to rise as fruits went from ripe to over-ripe. There was no correlation between cellulase activity and firmness of several varieties of tomato. Potentate, in fact, although a firm variety, contained twice as much cellulase activity as Harbinger, a soft variety. Hobson concluded that cellulase only complemented the activity of P.G. and P.E.

CHAPTER III

MATERIALS AND METHODS

I PREPARATION OF THE FRUIT

Red Gauntlet strawberries were picked and placed in categories according to the stage of ripeness and then stored at 3°C for two hours before extraction.

For the pectin esterase experiments strawberries were placed in the following categories:

I	Small green
II	Large green
III	Reddish
IV	Red ripe
V	Over ripe (ripe strawberries were stored at room temperature for three days)

After cold storage 25gm samples were taken from each category for extraction.

For polygalacturonase and cellulase experiments only three categories of strawberries were taken:

G	Green mature
R	Red ripe
OR	Over ripe (held 2-4 days after picking at red ripe stage)

The strawberries were stored at 3°C for two hours prior to extraction as for the PE experiments

II EXTRACTION PROCEDURES

All extraction procedures were carried out at 3°C

(1) Method 1 Pectinesterase

Crude preparations of P.E. were obtained after the method of Hultin and Levine (1963).

25gm of strawberries were homogenised with 50ml of 0.15M NaCl and 0.25gm of Polyvinyl pyrrolidone (P.V.P.) used to complex inhibitors (polyphenols) as recommended by Nagel and Patterson (1967). This homogenate was allowed to stand for 10-15 minutes then centrifuged at 500g. The supernatant was retained and the residue re-extracted twice with 20ml of H_2O.

50ml of the collected supernatants were taken to 15% saturation by addition of solid ammonium sulphate and centrifuged to obtain a clear solution which was dialysed against distilled water before chromatography on a DEAE- cellulose column.

(2) Method 2 Polygalacturonase and Cellulase

This method is essentially that of Pressey and Avants (1971).

25gm of strawberries were homogenised with 25ml of 1M NaCl and 0.25gm of PVP for 5 minutes at 3°C. This homogenate was centrifuged at 8000g for 20 minutes. The residue and supernatant were dialysed separately against distilled water overnight and 1ml aliquots taken for enzyme assays.

(3) Method 3 Polygalacturonase and Cellulase

Extraction after Hobson (1968). 20gm of strawberries and 1gm of salt mixture (4:1/NaCl : EDTA) were homogenised with 25mls of H_2O and 0.2gm of PVP. This slurry was taken to pH8.0 by 1N NaOH and kept at 3°C for one hour, then centrifuged at 800g for 3 minutes. The supernatant was filtered through muslin and retained; the residue was re-extracted twice, for 15 minutes each time, with 20mls of distilled water. Cold acetone was added to the pooled supernatants until 70-72% with respect to the ketone. This mixture was left to stand overnight at -20°C, centrifuged, and the supernatant discarded; the container with the residue being left at -20°C for at least a further 12 hours.

The enzyme was subsequently redissolved in 10% ammonium sulphate and aliquots taken for assay.

When dialysis was introduced into the procedure immediately preceding the acetone treatment complete loss of enzyme activity occurred.

III ENZYME ASSAYS

(1) Pectin Esterase

The samples to be tested for PE activity were assayed by the method of Abou Akkada and Howard (1961).

The pH in all cases was maintained at pH 6.8, the temperature at 35°C. 0.2ml of 10% H_2SO_4 was used to stop enzyme activity.

Every fifth fraction tube off the DEAE- cellulose column was assayed for PE activity.

For the overall assay of PE over the ripening period ten fraction tubes immediately adjacent to the first peak of PE activity for each category were pooled and held at 3°C.

(2) Polygalacturonase

1ml of 1% polygalacturonic acid was placed in a test tube with 3mls of buffer, either phosphate buffer pH 6.8, or citrate-phosphate buffer pH 5.0. 1ml of enzyme solution was added to this and incubated for 16 hours at 30°C. Standards were run with 1ml galacturonic acid (100ug/ml), 3mls of buffer and 1ml of distilled water. Blanks contained all ingredients except the enzyme which was replaced by 1ml H_2O.

After 16 hours the samples were heated in boiling water for 15 minutes, cooled, centrifuged, and supernatants analysed for reducing sugars by the arsenomolybdate method (Nelson, 1944 and Somogyi, 1952).

The method of Dische (1947) for analysis of hexuronic acids was unsuccessful under these conditions in spite of attempts to precipitate and remove protein by $Zn(OH)_2$ or Keiselguhr treatment.

The increase in end groups during incubation was followed by periodate oxidation (Sterling 1966).

(3) Cellulase

The assay system was the same as that described for polygalacturonase. 1% CM - cellulose was used as substrate, glucose (300ug/ml) was used as standard. Citrate-phosphate buffer at pH 5.0 was used throughout the assays, temperature was maintained at 30°C.

The increase in reducing sugars was measured by the arsenomolybdate method (Nelson 1944, Somogyi 1952).

IV PERIODATE OXIDATION

The method used was basically the method for assaying the non reducing end groups of glycogen as described by Sterling, (1966).

To 1ml of polygalacturonic acid or, 1ml of polygalacturonic acid incubated with polygalacturonase extract, was added 1ml of 0.2M sodium periodate, mixed and placed in the dark at 0°C. The uptake of periodate was measured every 12 hours up to 48 hours by spectrophotometry at 223 nm. After 48 hours 0.1 ml of ethylene glycol was added to each reaction vial, mixed, and stood at room temperature for 1 hour. Controls were set up with ethylene glycol added to destroy the periodate before the addition of carbohydrate.

To measure the formic acid produced by periodate treatment 1ml of 1% KI was added to produce a quantitative yield of iodine (I_2). This was titrated against 0.002N $Na_2 S_2 O_3$ (sodium thiosulphate).

V ANALYSIS OF REDUCING SUGARS

Sugars were measured by the Nelson-Somogyi method (Nelson 1944, Somogyi 1952).

Incubation mixes were boiled 20 minutes and centrifuged. 1 ml aliquots of the supernatant were placed with 1 ml of Nelsons reagent and analysed for reducing sugars.

A standard curve was constructed with standard solutions containing from 0 - 300 ug glucose/ml. From the standard curve the sugar content of test solutions was obtained. Optical densities (O.D.) were measured at 520nm.

VI METHANOL DETERMINATION

The methanol content of PE incubation mixes was determined by the method of Abou Akkada and Howard (1961).

1 ml of the acidified mixture from the incubation was placed in the outer well of a Conway unit, the inner well containing 1.2 ml of 10% v/v H_2SO_4.

Standard tubes containing water (1.5 ml) and methanol (0.5 ml of 0.01% v/v solution, i.e. 40ug of methanol) were incubated at the same time as the test solutions. The reading obtained with the methanol standard was used to calculate the quantity of methanol in the other tubes.

VII CHROMATOGRAPHY

(1) Paper Chromatography

Paper chromatography for galacturonic acid and oligouronides followed the method of Abou Akkada and Howard (1961).

(2) Ion Exchange Chromatography of P.E.

A column of DEAE- cellulose was used for ion exchange chromatography.

An eluting solution of 0.02M PO_4 buffer pH 6.8 was used, with a linear concentration gradient of NaCl and a limiting concentration of 1M as used by Hultin et al (1966). 3 ml fractions were collected automatically and the O.D. of these fractions at 280nm determined.

Between samples the column was washed with 200 ml of 2N HCl and twice with 200 ml of distilled water. The column was re-equilibrated with 150 ml of 0.02M PO4 buffer pH 6.8 and held in the buffer until the next run.

In preliminary experiments changes in the eluting solution, of pH from 6.8 to 6.0 and the limiting concentration of NaCl from 1M to 2M were tested for the effect on elution of P.E. A change in pH, or in the limiting concentration of NaCl, had the effect of flushing total protein and P.E. from the column.

CHAPTER IV

RESULTS AND DISCUSSION

I Pectinesterase

Extracts were made from ripe strawberries by method I and assays were run for PE activity. Activity was shown in the crude extract which was then subjected to separation on a DEAE- cellulose column. The protein elution pattern (OD 280nm) and enzyme activity of the eluted fractions exhibited the characteristics shown in Figure III.

Subsequently extracts were taken from fruit of the five stages of ripeness.

I	Small green
II	Large green
III	Reddish
IV	Red ripe
V	Over ripe

These extracts were subjected to column chromatography and assays run for PE activity on the fractions obtained. The results of the enzyme assays and the elution patterns of protein in the solutions are shown in Figures I - V. Two assays were run on the same eluate samples and these are labelled (1) and (2) on the graphs. The figures from the enzyme assays are shown in Appendix (i). The figures I - V indicate two peaks of activity of PE.

The first peak (Peak I) appeared in fractions which were eluted at the same point as the peak of the protein in the extract (as measured by OD at 280nm) at fraction 10. The second peak (Peak II) eluted later in the region of fraction 25. This behaviour suggests a different ionic character between the enzyme eluted at the two peaks. Peak II was retained by DEAE- cellulose and was therefore behaving as an anion. The enzyme at Peak I was not retained. It is therefore suggested that Peaks I and II represent distinct pectinesterases (PE I, PE II).

For each stage of ripeness two incubations were run on the same effluent samples and these are plotted separately on the graphs. The second incubation was of lower activity (in ug of methanol) except for PE I in Stage II (Fig II) and Stage IV (Fig IV).

The second incubation of stage IV shows considerably higher activity than the first incubation in virtually all fractions. Storage unstable inhibitors of PE could be postulated to account for this behaviour but it is probably more reasonable to assign it to the variability between runs which can occur with chromogenic methods of analysis and this method in particular (Howard Pers. Comm. 1973).

There is a fall in activity for the second assay in the other extractions which is due to a decline in the activity of the enzyme solutions which had been stored. Four days elapsed between the first and second

TABLE I

Pectinesterase activity in strawberry extract during ripening.

Stage of Ripeness	Activity (ug methanol)	%inc. over I
I	3.17	
II	3.74	18%
III	4.64	46%
IV	5.43	71%
V	4.14	31%

TABLE II

Level of PE I, PE II and PE I and PE II during ripening.

Stage of Ripeness	PE I	PE II	PE I & PE II
I	4.05	4.97	9.02
II	5.05	6.37	11.42
III	8.66	8.27	16.93
IV	5.83	6.5	12.33
V	3.00	6.16	9.16

assay for each stage of ripeness except for Stage II (Fig II) when 12 days elapsed.

An increase in activity of both PE's is observed over ripening but PE II shows a greater proportion of the total activity. PE I, however, shows a distinct increase in contribution to total PE activity at stage III.

The behaviour of PE from strawberries obtained here is very similar to fraction II of Rultin et al (1966) for banana PE. Similar to the results shown here, their fraction retained on DEAE- cellulose was the major fraction. In addition their unretained fraction was shown to be very similar to a PE obtained by a different extraction procedure. This supports the suggestion made here that two PE enzymes are present in strawberries.

By comparing Stage I and Stage V a distinct difference in the pattern of activity can be shown. Stage V shows a distinct and sustained level of activity over Stage I particularly in PE II. Over all the stages of ripeness, however, a definite change in the pattern of activity, as was shown by Pressey and Avants (1972) for tomato pectinesterase, cannot be supported by these results.

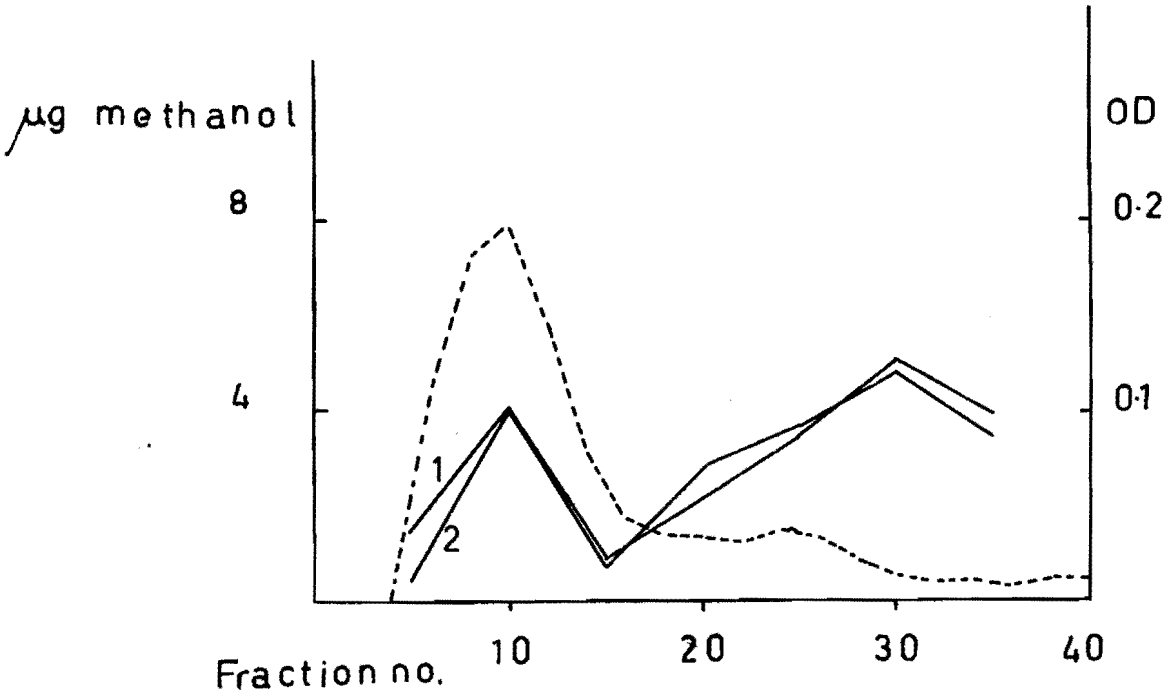
30ml of enzyme solution immediately adjacent to the first peak of enzyme activity were taken for each stage of ripeness and subjected to assays to assess the pattern of enzyme activity over the ripening and after-ripening period. Results are shown in Appendix (ii) and graphed in Figure VI. Two

incubations were run and the means plotted on the graph. This graph shows the level of PE I activity at each stage of ripeness, reflecting what is assumed to be the in situ behaviour of PE over the ripening period. These results show quite clearly a distinct pattern of PE activity during and after ripening in strawberries. There is a low level of activity even in small green fruit which increases during ripening and declines again during the after-ripening period with a peak of activity between Stage III and Stage IV.

The means of PE activity for Peak I and the means for Peak II for each stage of ripeness are plotted separately in Figure VII to show the relative activity of PE I and PE II during ripening. The sum of the means of PE I and PE II for each stage of ripeness are plotted in Figure VIII to show the pattern of total PE activity over the ripening and after-ripening period.

PE I shows lower activity in Stages IV and V and the suggestion is made that PE I is less stable during the after-ripening period than PE II. It is noticed (Figs. IV and V) that PE II is making a much greater contribution to the overall pectinesterase activity during the after-ripening period, indeed PE II maybe responsible for softening during the after-ripening period in strawberries. PE I could well be active and of most consequence to the fruit during the green and mature stages of fruit development when it contributes to the "sliding growth" phenomenon. The

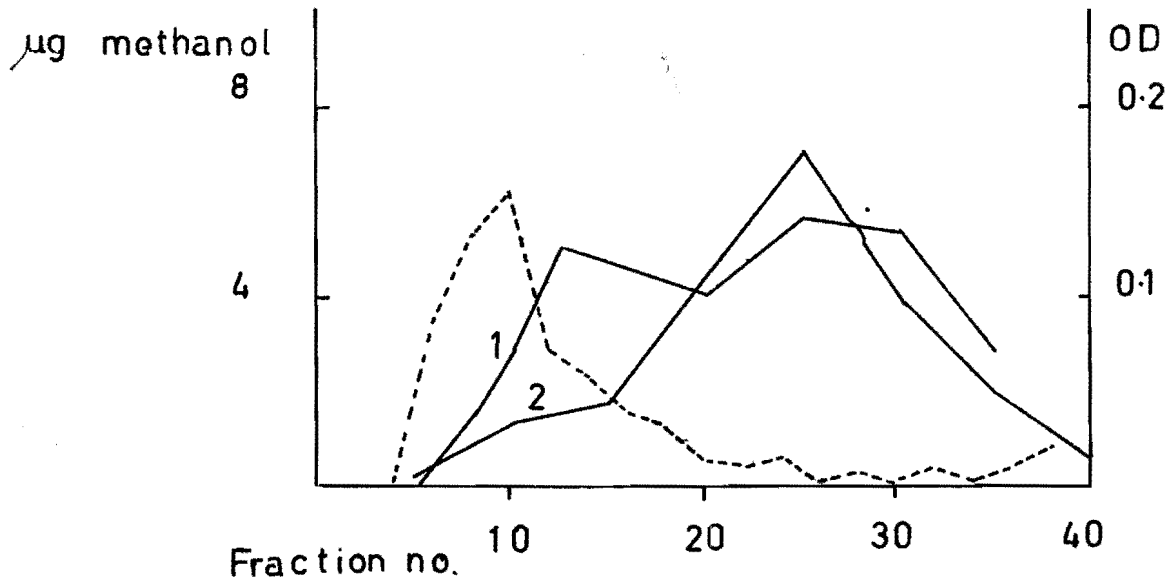
FIGURE I PE activity in fractions from STAGE I extracts



--- Protein level in eluate (OD 280nm)

—— PE activity in duplicated incubations (yield in µg methanol)

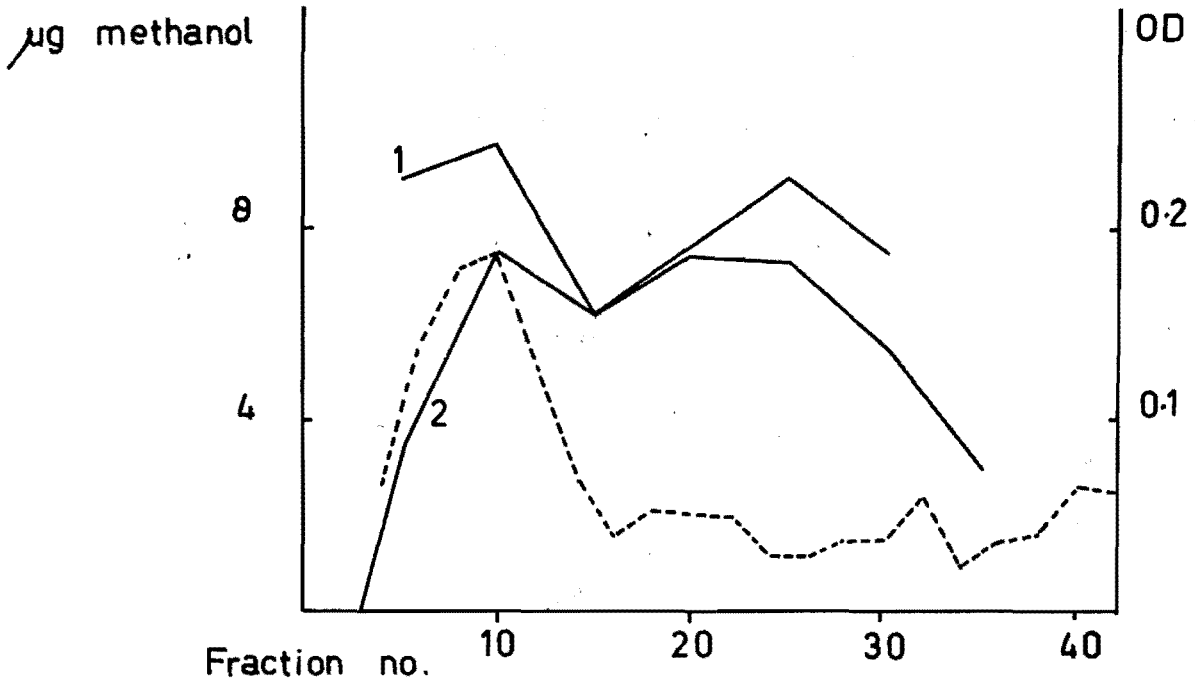
FIGURE II PE activity in fractions from STAGE II extracts



--- Protein level in eluate (OD 280nm)

—— PE activity in duplicated incubations (yield in µg methanol)

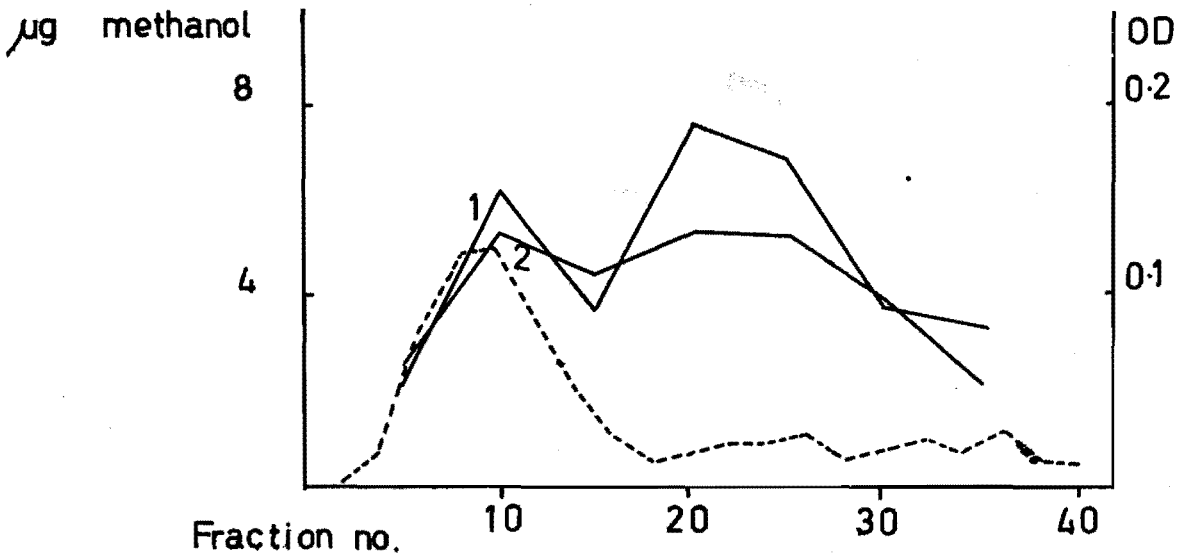
FIGURE III PE activity in fractions from STAGE III extracts



---- Protein level in eluate (OD 280nm)

—— PE activity in duplicated incubations (yield in µg methanol)

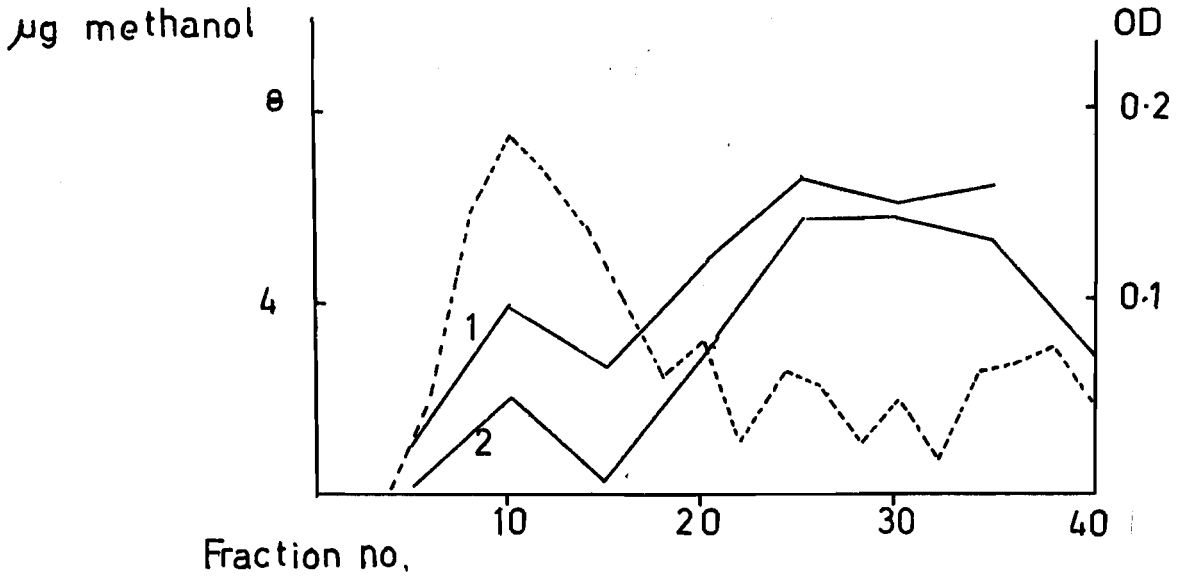
FIGURE IV PE activity in fractions from STAGE IV extracts



--- Protein level in eluate (OD 280nm)

—— PE activity in duplicated incubations (yield in µg methanol)

FIGURE V PE activity in fractions from STAGE V extracts



--- Protein level in eluate (OD 280nm)

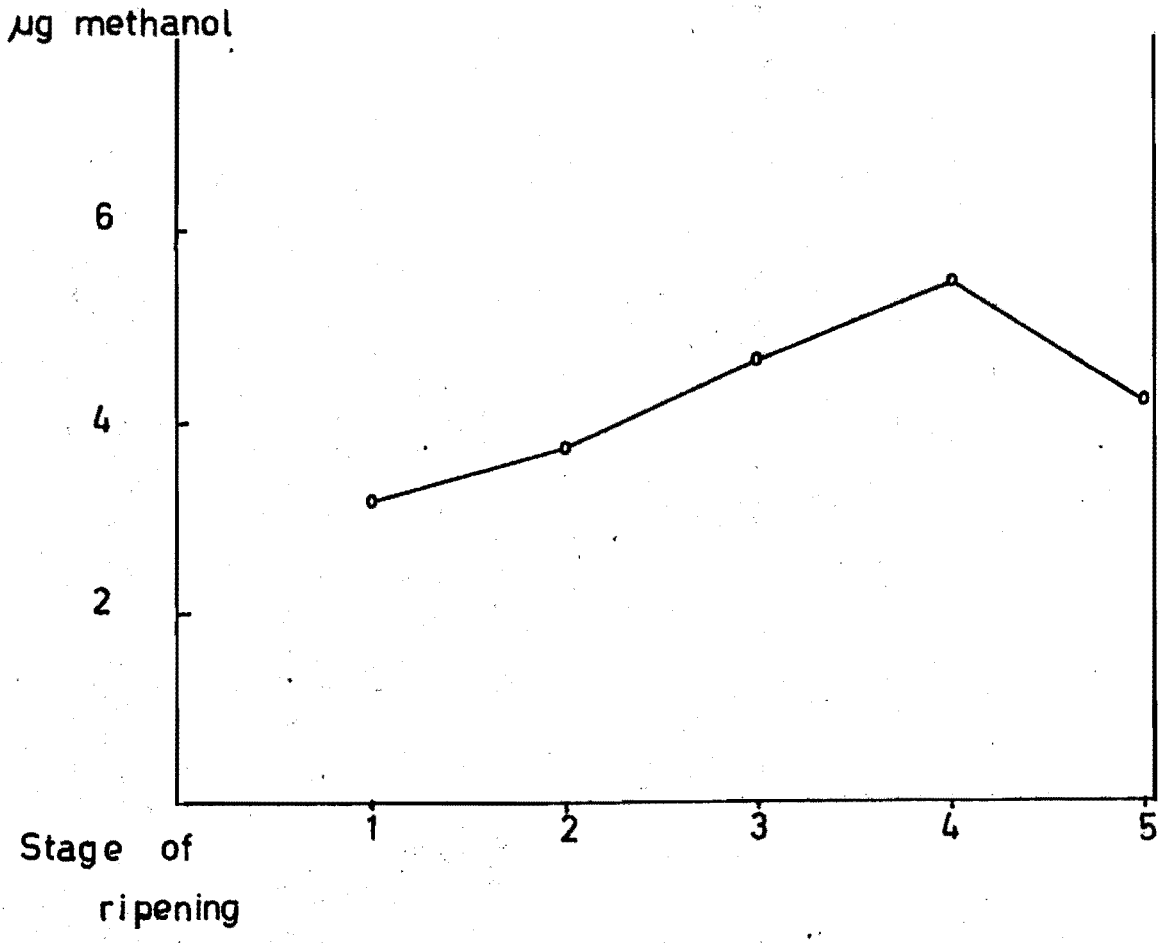
—— PE activity in duplicated incubations (yield in µg methanol)

suggestion has been made for tomatoes that the function of one of the four isoenzymes located maybe to create initiation points for other enzymes (Pressey and Avants 1972).

It appears therefore that during ripening PE II gradually increases in activity to the ripe stage (Stage III) when it tends to level off and gradually decline. PE I on the other hand increases more rapidly commencing at a later stage, reaches a peak about the same time as PE II and then decreases more rapidly also. The level of activity in both enzymes during the most active period (Stage III - IV) is quite similar.

The PE activity described in this work follows a typical pattern of enzyme activity for cell wall enzymes during ripening (Dilley 1970). Neal (1965) showed PE activity in strawberries and contends that changes in the cationic content of the pectinous middle lamella are related to changes in the methylation of that material. Doesburg (1957) has suggested that softening in apples is due to a transfer of calcium and other divalent metal cations from the wall into the cell, and Rees (1969), has pointed out that calcium ions are unlikely to form a strong linkage and has suggested that Ca^{++} may aid the packing of polygalacturonate chains by fitting neatly into a microcrystalline structure and neutralizing mutually repulsive charges. It is suggested here that PE may effect the cell wall by upsetting the balance of these charges consequently causing a deterioration in

FIGURE VI Total PE activity during ripening in crude strawberry extracts



Activity measured as the µg methanol formed

FIGURE VII Levels of PE I and PE II during ripening

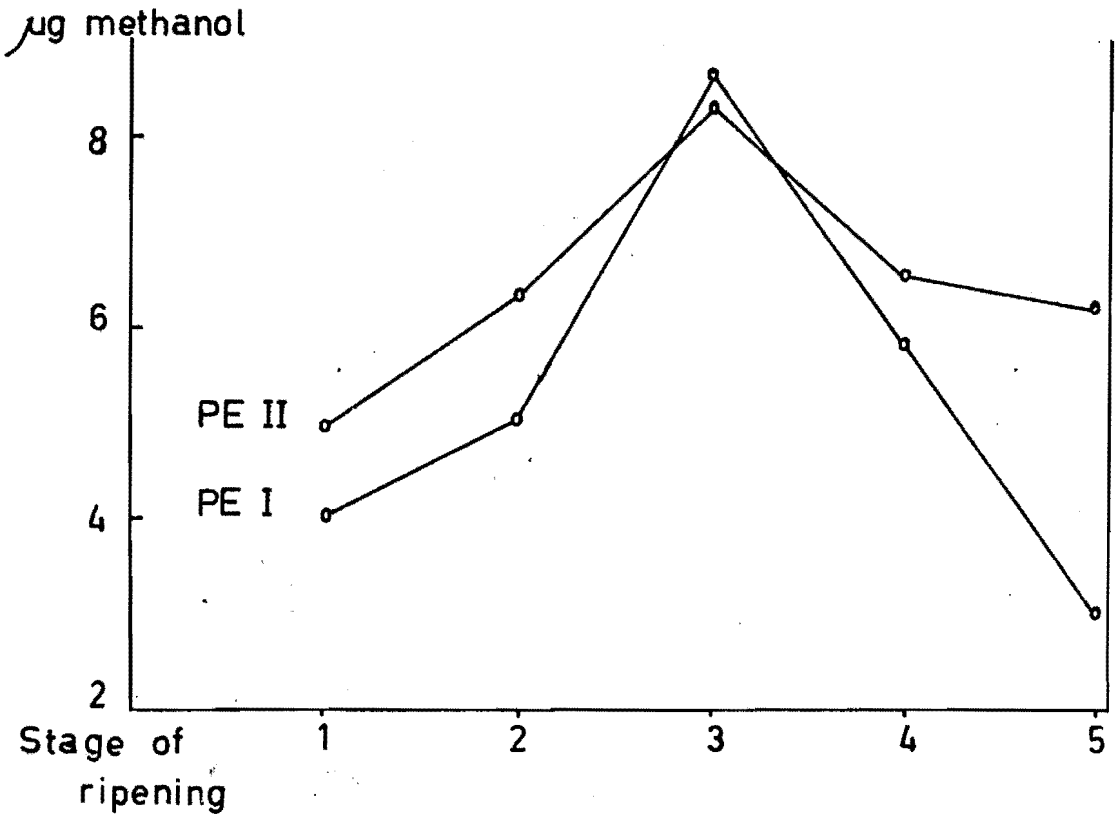
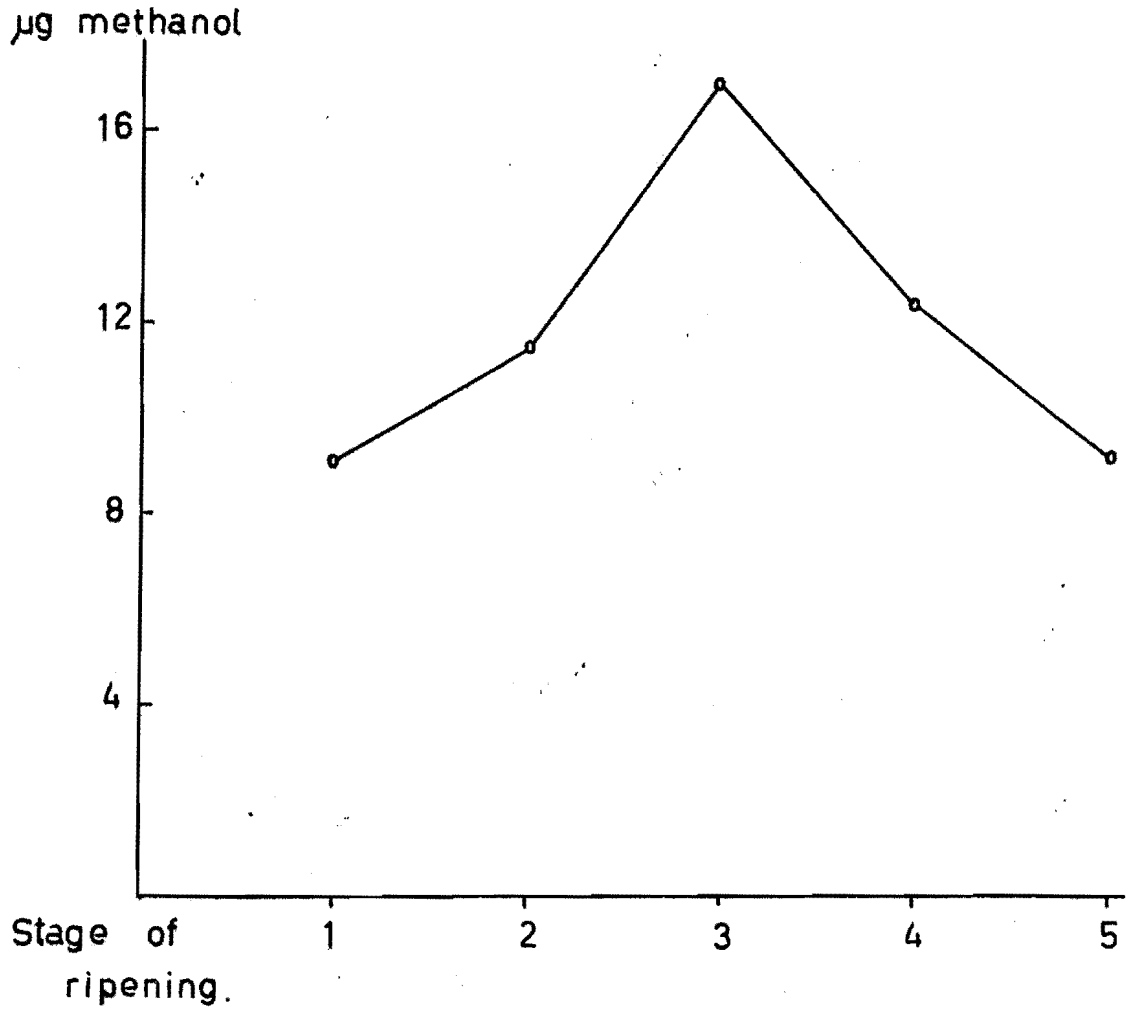


Figure VIII Total PE activity during ripening



structural relationships which macroscopically appear as softening.

II Cellulase

In preliminary experiments using Method II to extract cellulase from 'R' strawberries both the residue and supernatant fractions of the extract were tested for cellulase activity. The results are presented in Table III. These results show higher sugar levels in the controls (boiled enzyme) than in the treatment (enzyme active). This result could be attributed to hydrolysis of polysaccharides introduced with the enzyme extract during boiling to destroy the enzyme in controls prior to incubation.

Following this attempts were made to precipitate unwanted material principally by $Zn(OH)_2$ treatment and this was partially successful (Table IV). The background level of reducing sugar was decreased by this treatment but still remained between 5 - 30 ug/ml. Controls in the assay of the residue fraction of the extract continued to exhibit higher reducing sugar levels than the treatments. These difficulties suggested that a different extraction procedure should be employed.

Hobsons (1968) extraction procedure (Method III) was of considerable success in his tomato cellulase determinations and it was employed in this investigation for all subsequent cellulase determinations.

TABLE III

Cellulase activity of strawberry extracts by Method II from 'R' strawberries

	Control	Treatment
Residue	286	142
	300	142
Supernatant	70	47
	70	47

Yield ug glucose /ml

TABLE IV

Cellulase activity of extracts by Method II following $Zn(OH)_2$ precipitation

	Control	Treatment
Residue	142	94
	94	47
Supernatant	286	286
	300	300

Yield ug glucose /ml

TABLE V

Cellulase activity of strawberry extracts Method III

	Strawberry extract	Commercial cellulase preparation
Control	0.5	54
Treatment	18	108

ug glucose /ml

Preliminary experiments using an extraction from 'R' strawberries and a commercial cellulase preparation showed cellulase activity in both preparations (Table V). Strawberry enzyme released approximately 20 ug glucose over the sixteen hour incubation period while the commercial cellulase released approximately 50 ug over the same time.

It was found impossible to obtain zero levels of reducing sugar in controls in spite of dialysis, ethanol precipitation, ammonium sulphate precipitation, $Zn(OH)_2$ precipitation, or Keiselguhr treatment and retain enzyme activity. It was consequently necessary to run all the assays with controls from each extraction for each category of ripeness and assume that increases in reducing sugar above the control were due to enzyme activity.

The following treatments were carried out for 'G', 'R' and 'CR' fruit.

Treatment	I	Incubation with substrate and enzyme extract
	II	Incubation with substrate and boiled enzyme extract
	III	Incubation with no substrate and enzyme
	IV	Incubation with no substrate and boiled enzyme

The results are presented in Table VI and in Table VII results are presented for a parallel experiment in which treatments III and IV were deleted.

These results indicate the difficulties of working with crude enzyme preparations and sensitive chromogenic reagents. They also indicate most clearly the necessity for a full set of controls. If Treatments III and IV had been deleted from Table VI it would have been easy to ascribe the increases in reducing sugar to cellulase activity.

There is the suggestion of cellulase activity in these results. However, statistical analysis could show no significant difference between treatments. Table VII is strongly suggestive of cellulase activity, however, the absence of sufficient controls as in the results on Table VI throws doubt on any conclusions to that effect. There is perhaps a suggestion of activity but a more intensive study and more satisfactory extraction procedure and method of assay is required.

The standard curve for Nelson's method of estimating reducing sugars is present in Figure IX.

Hobson (1968) concluded that the cellulase enzyme he located in tomatoes was of little significance to the softening of tomato fruit, and Wade (1964) concluded that cellulose levels remained the same during ripening in strawberries. It appears that although cellulase activity has been demonstrated for some fruit (Hall 1963, Hobson 1968, Hasegawa and Smolensky 1971) that this activity is not important to the overall softening of fruits, with the notable exception of dates. Cellulose levels changed only marginally in pears (Jermyn and Isherwood 1956), peaches (Sterling 1961) and strawberries (Wade 1964).

TABLE VI

Cellulase activity in strawberry Extracts (Method III) from 'G', 'R', and 'OR' fruit (1)

ugm glucose /ml

		G		R		OR	
Treatment	I	182	156	176	195	137	128
	II	147	133	190	182	98	108
	III	167	156	220	205	137	118
	IV	156	147	205	172	137	104

The inability to demonstrate a significant change in cellulase activity here together with the previous work suggests that over the ripening and after-ripening period for strawberries cellulase activity is not of great importance to softening.

A more sophisticated technique may be necessary to show the breakdown of cell wall constituents into smaller macromolecules. Breakdown into molecules which are smaller than those *in situ* but still large enough to show up as macromolecules could have a dramatic effect on viscosity and consequently firmness (Pilnik and Voragen 1970). Pilnik and Voragen claim that for an endo polygalacturonase specific viscosity is down to half when only 2-3% of glycosidic linkage are split.

This reasoning also applies to cellulose. An endo enzyme or enzyme specific for points of attachment of side chains could easily have a dramatic effect on the overall texture of the softening fruit which would not necessarily show up as a dramatic increase in enzyme activity. In addition glucan contributes 25% of the total cell wall in Cambridge Favourite strawberries (Wade 1964) so that a small change in average molecular weight could have a dramatic effect on cell wall integrity.

Pressey *et al* (1971) prepared high MW soluble pectic substances from peaches which suggest that a considerable degree of endo enzyme splitting which although causing a textural change may reveal little if any change in pectin levels during ripening.

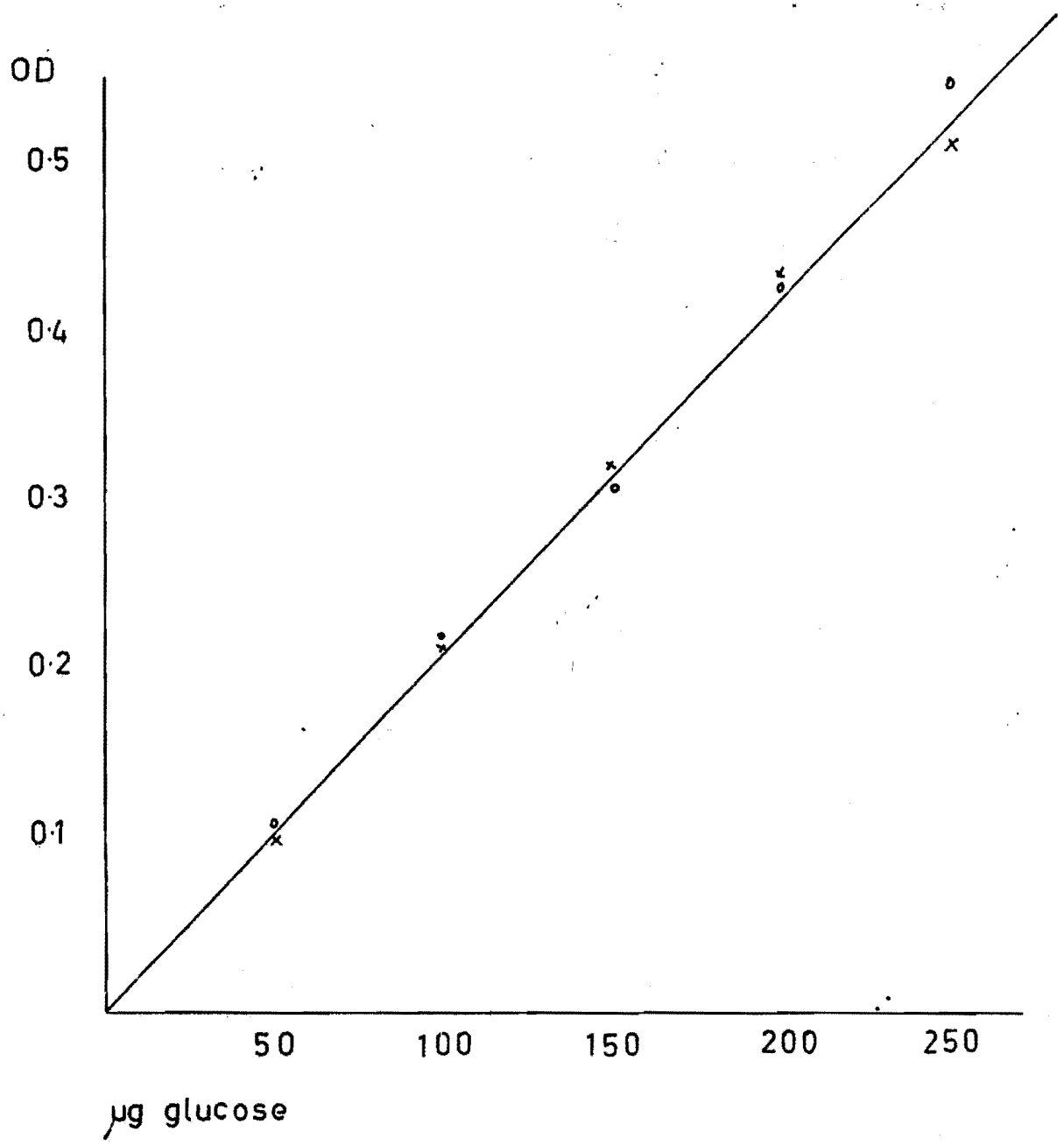
TABLE VII

Cellulase activity in extracts from 'G', 'R' and 'OR' fruit (2)

	G	R	OR
Treatment	14	28	118
Control	4	4	14

ug glucose /ml

FIGURE IX Standard curve for Nelson's method for estimation of reducing sugars using glucose



Cellulose may perhaps show a similar breakdown to high MW substances.

However, as Hobson (1968) has suggested, the role of cellulase is probably insignificant compared with the effect of the pectic enzymes on softening.

III Polygalacturonase

Initially attempts were made to extract polygalacturonase from 'R' strawberries following the extraction procedure Method II. The carbazole method of Dische (1950) for polyuronides, and the Nelson-Somogoyi (1944, 1952) method for reducing sugars failed to show any significant enzyme activity and paper chromatography of the incubation mixes failed to show the presence of galacturonic or glucuronic acids. It was concluded that this extraction procedure did not contain a polygalacturonase that has galacturonic acid as an end-product.

To investigate the possibility of an enzyme that splits polygalacturonic acid into oligouronides the periodate uptake of the incubation products were assessed.

The products of enzyme incubation (2 ml) were used for periodate oxidation. Duplicates of the periodate oxidation were run for both the active enzyme and the extracts in which the enzyme was destroyed by boiling prior to incubation with polygalacturonic acid. These results are shown in Table VIII. This extract was from the supernatant fraction. In a repeat

TABLE VIII

Periodate uptake by polygalacturonase incubation products (O.D 223nm)

	Uptake After 24 Hr	48 Hr	OD 750 formic acid formed
Control	0.96	1.13	0.045
	1.04	1.16	0.056
Treatment	1.11	1.16	0.052
	1.08	1.33	0.041

TABLE IX

Periodate uptake by polygalacturonase incubation products for residue and supernatant fractions of Method II

	Periodate uptake at 48 Hr	Titre ($Na_2S_2O_3$) ml
Residue control	1.15	26.4
	1.02	29.2
Treatment	0.98	29.1
	0.964	27.0
Supernatant control	0.34	74.4
	0.51	59.2
Treatment	0.542	60.9
	0.46	65.4

experiment both the residue and supernatant fractions of the extraction procedure were tested for an increase in end groups. These results are presented in Table IX. Back titration, with sodium thiosulphate, of iodine uptake by the formic acid which appeared during periodate oxidation is also shown in that table.

The results in Table IX show that some periodate is used in the oxidation of carbohydrate bonds resulting in the formation of formic acid. However, little difference is exhibited in the periodate uptake between treatments and controls. The titration of the iodine formed by the different samples is consistent with this result. It would appear therefore that an enzyme which cleaves polygalacturonic acid chains into oligouronides has not been isolated with the techniques employed in this investigation.

The possibility of locating a cell wall polygalacturonase by acetone precipitation was explored by following the method of Hobson (1968), Method III. Preliminary results (Table X) with incubations of extracts from 'R' strawberries suggested the possibility of polygalacturonase activity. A 30% and 50% increase in reducing sugar was measured for the enzyme over the controls. Problems similar to those experienced in the cellulase extracts were encountered in cleaning up the extract. Dialysis of the extract prior to acetone precipitation during the extraction procedure resulted in a complete loss of enzyme activity and attempts to remove the unwanted material after incubation were difficult. Ethanol precipitation (AbourAkkada and

Howard 1961) and ammonium sulphate precipitation were successful in removing polysaccharides and protein and in clarifying the solutions but the background sugar levels remained. The hint of enzyme activity in the preliminary experiments suggested that extracts should be taken from G, R and OR fruit and the polygalacturonase levels assayed. The extracts from G, R and OR fruit were assayed separately for enzyme activity and the results of the separate incubations are presented in Tables XI, XII, XIII, XIV. Paper chromatography of the incubation products from R extracts are shown in Figure X.

G and OR extracts do not show any difference in sugar levels between the treatments and controls suggesting the absence of polygalacturonase activity discernable by this method. R extracts have shown an increase in reducing sugar which is significant at the 10% level for one extract (Table XI) but non significant at the 10% level in another extract (Table XII) when subjected to an F test. When the extracts were subjected to paper chromatography to identify the end products no spots were revealed corresponding to galacturonic acid nor was any significant difference between any of the acidic spots revealed on the chromatograph.

These results suggest that while an enzyme responsible for the increase in reducing sugar maybe active in the R extracts, this enzyme does not produce galacturonic acid as an end product and is not therefore

TABLE X

Preliminary test for Polygalacturonase activity using extracts of Method III

(x 10^{-3} g galacturonic acid)

Treatment		I	II
Replication	1	0.8	0.6
	2	0.8	0.4

Treatment I Enzyme active

Treatment II Enzyme destroyed

TABLE XI

Polygalacturonase activity in 'R' strawberries (1)

(x 10^{-3} g galacturonic acid)

	Treatment I	II
Replication 1	1.2	1.8
2	1.38	1.6

F Significant at 10% level

Treatment I Enzyme destroyed

Treatment II Enzyme active

TABLE XII

Polygalacturonase activity in 'R' strawberries (2)

(x 10^{-3} g galacturonic acid)

	Treatment I	II
Replication 1	1.45	1.76
2	1.4	1.84

F Non significant at 10% level

Treatment I Enzyme destroyed

Treatment II Enzyme extract active

TABLE XIII

Polygalacturonase activity in 'G' strawberries

(x 10^{-3} g galacturonic acid)

		Treatment I	II
Replication	1	0.8	0.8
	2	0.8	0.8

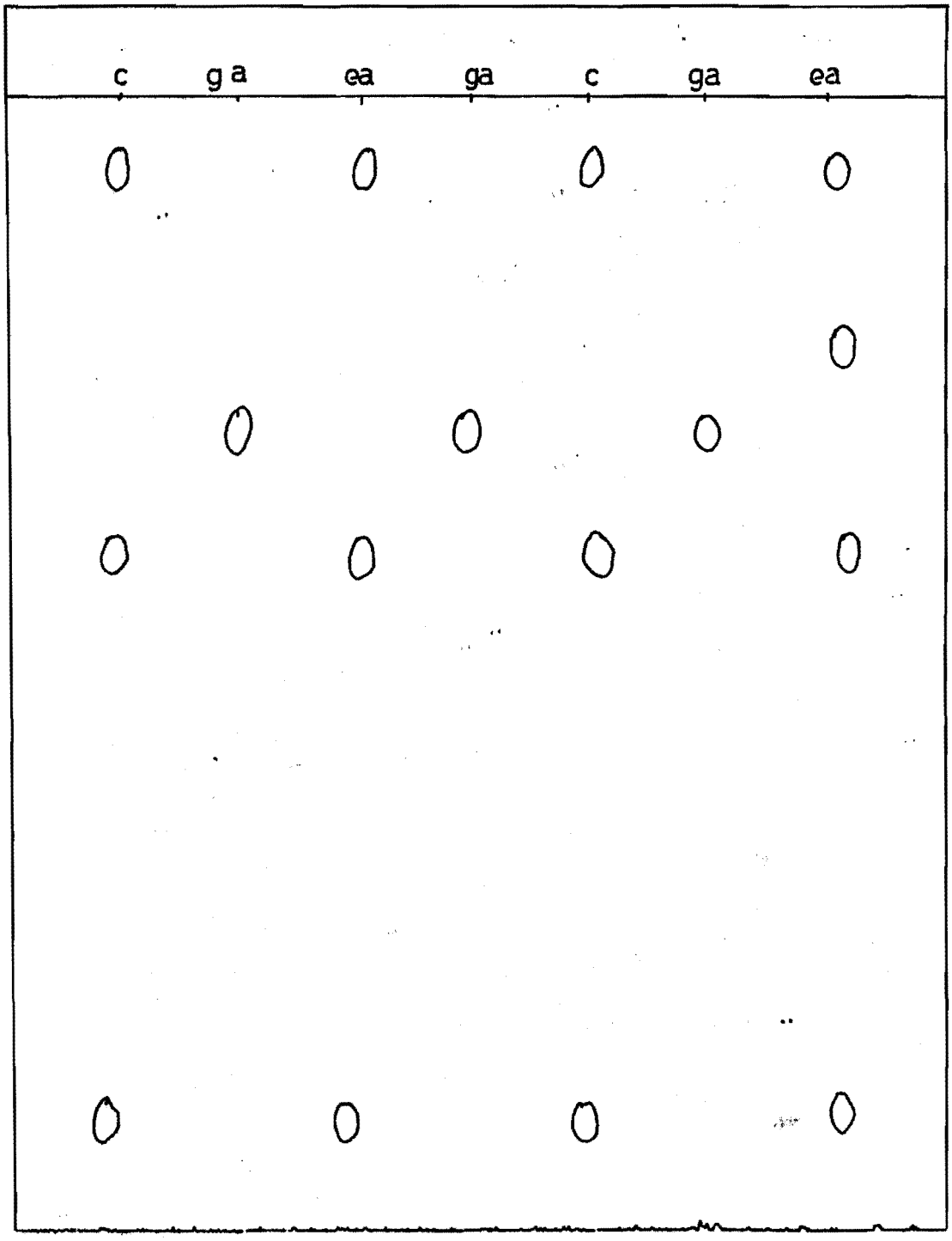
TABLE XIV

Polygalacturonase activity in 'OR' strawberries

(x 10^{-3} g galacturonic acid)

		Treatment I	II
Replication	1	1.0	1.2
	2	1.0	1.0

FIGURE X Chromatograph of the end products of the polygalacturonase assays



c Enzyme destroyed

ea Enzyme active

ga Galacturonic acid

a polygalacturonase enzyme. Presumably the low enzyme activity shown in the R extracts is due to enzyme activity on cell wall polysaccharides other than the artificial substrate.

Although polygalacturonase activity could not be demonstrated here polygalacturonic acid degrading enzymes have been demonstrated in other fruits; tomatoes, avocado (Hobson 1962). Neal (1965) and Gizis (1964) could not demonstrate any polygalacturonase activity in strawberries and the inability of Wade (1964) to show significant differences of uronic acids between ripe or unripe strawberries supports the work described here. The inability to show the presence of an enzyme splitting off oligouronides suggests, that for strawberries at least the concept of a chain splitting enzyme contributing significantly to softening may be in doubt. The exopolygalacturonate lyase from Clostridium multifermentans (Macmillan et al 1964) which removes units of unsaturated digalacturonic acid from a polygalacturonic acid chain raises the possibility of a similar enzyme in fruits, and the observations (Lee and Macmillan 1970) on the nature of tomato PE suggests that the enzymes responsible for wall softening maybe of more specific character than has been ascribed to them formerly.

IV Conclusion

The phenomenon of cell wall softening during fruit ripening is far from well understood and it has

become accepted to ascribe softening to pectin degrading enzymes. The ease with which PE activity has been shown in many fruit, and its relationship to methylation of pectin and the firming effect of calcium ions has led to its implication in softening.

The demonstration of molecular weight dependent polygalacturonase activity (Patel and Phaff 1948, Pressey and Avants 1971) which may also be dependent on the presence or absence of methylated carboxyls has resulted in the concept of PE initiation of pectin degradation followed by polygalacturonase chain splitting activity. It has been assumed that such activity could account for the degradation of cell walls and consequently softening. The presence of polygalacturonase activity has been confirmed in a number of fruits, (Hobson 1962) and the recent addition of peaches to the list (Pressey et al 1971) by a new method has supported the concept. However, the inability to conclusively show polygalacturonase in strawberries by a number of workers (Neal 1965, Gizis 1964) and the inability to show polygalacturonase or cellulase activity in this work raises some question as to the soundness of the concept.

It has all-ready been shown that only small differences in the polysaccharide components of cell walls need to be exhibited for significant effects on softening, and it is possible that a refined technique may reveal cellulase or polygalacturonase activity that has been previously undetected (Pressey et al 1971). On the other hand it could be that enzymes specific for some of the small side chains such as arabinose,

galactose or rhamnose or the concentrated areas of rhamnose residues (Aspinall 1970) within the polygalacturonic acid chain may have a significant effect on wall integrity. Side chains may be broken by enzyme activity specific for certain sugars, perhaps even for specific sequences or combinations of sugars. The rigid structure of the cell wall may be consequently weakened and allow the turgor pressure within the cell to cause wall extension or softening.

Some workers (Rees 1969, Neal 1965, Doesburg 1957) have tried to account for the breakdown of cell walls by considering the effect of actions on firming. Calcium is implicated in this activity and it is suggested that Ca^{++} may neutralize mutually repulsive charges and allow close packing of polygalacturonate chains. Neal (1965) suggests that this phenomenon occurs in spite of PE activity. An increase of PE activity during ripening as shown here, however, is difficult to correlate with a decrease in methylation of pectin which would lower the Ca^{++} level in the cell wall, if Ca^{++} level is proportional to its firming effect. Rees (1969) has suggested that Ca^{++} ions aid by neutralizing mutually repulsive charging and allowing the polysaccharide chains consequently to fit more closely together. If this is so when PE cleaves the methoxyl units off the chain it may cause an upset in the balance of charges in the wall resulting eventually in softening. In this way it would be possible for PE activity to contribute significantly to the softening phenomenon. It is possible that in strawberries at

least PE activity may be of sufficient importance to account for fruit softening and even that the two PE enzymes proposed in this work are responsible for cell elongation and wall softening by their effect on the cationic composition of the cell wall. Possibly one of the enzymes is primarily responsible for wall extension during growth and the other for wall softening during ripening.

Nagel and Patterson (1967) have shown a decrease in PE activity during maturation of pears. This is supported by Weurman (1954), and Gee et al (1959) who showed PE activity to be greatest in immature pears where the percentage esterification of pectin was lowest and minimal in mature fruit when percentage esterification is greatest. Perhaps in this case PE adds methoxyl units in this way interfering with the cationic firming of the tissue.

Probably the key to understanding the softening phenomenon during fruit ripening lies in an understanding to a much greater degree in the nature of the polysaccharides in the wall. When the three dimensional nature of polysaccharide chemistry is more understood and refined technique enables accurate measurement of low enzyme activity of greater specificity than currently expected, we will be able to explore the ramifications of cell wall biochemistry more thoroughly.

ACKNOWLEDGMENTS

1 To Dr. M.F. Barnes for the supervision of the work

2 To Professor B.H. Howard for the pectinesterase assay and encouragement to undertake the work

3 To Mrs S.J. Coleman for the typing of the thesis and forbearance under duress

4 To Robyn for patience and sympathy

5 To the many staff and students whose criticism and assistance lightened the burden

REFERENCES

1. Abou Akkada, A.R. and Howard, B.H. 1961. The Biochemistry of Rumen Protozoa. 4. Decomposition of Pectic Substances. Biochem. J. 78: 512-517

2. Albersheim, P. and Anderson, A.J. 1971 Proteins from Plant Cell Walls inhibit Polygalacturonase secreted by plant pathogens. Proc. Nat. Acad. Sci. 68: 1815-1819

3. Aspinall, G.O. 1970 Polysaccharides. Pergamon Press. Oxford. 228p.

4. Bartolome, L.G. and Hoff, J.E. 1972 Gas chromatographic technique for assay of pectin methylesterase. J. Ag. Food Chem. 20: 262-266

5. Beaven, G.H. and Jones, J.K.N. 1947. Pectic substances. Part V. The molecular structure of strawberry and apple pectic acids. J. Chem.Soc. 150: 1218-1220

6. Besford, R.T. and Hobson, G.E. 1972. Pectic enzymes associated with the softening of tomato fruit. Phytochem. 11: 2201-2205

7. Demain, A.L. and Phaff, H.J. 1957. As quoted by Pilnik, W. and Voragen, A.G.J. in Biochemistry of Fruit and their Products. Vol I 1970. ed. Hulme A.C. Academic Press, London. 788p.

8. Dickinson, D.B. and McCollum, J.P. 1964 Cellulase in Tomato Fruits. Nature 203: 525-526

9. Dilley, D.R. 1970. In Biochemistry of Fruit and their Products Vol I, 1970 ed. Hulme A.C. Academic Press London 788p.

10. Dische, Z. 1950. Carbazole reaction of polyuronides. J.Biol. Chem. 183: 489-494

11. Doesburg, J.J. 1957. Relation between the solubilization of pectin and the fate of organic acids during maturation of apples. J.Sc.Fd. Ag. 8: 206-216

12. Dolendo, A.L., Luh, B.S. and Pratt, H.K. 1966. Relation of Pectic and Fatty acid changes to Respiration Rate during ripening of Avocado Fruit. J.Fd.Sc. 31: 332-336

13. Foda Y.H. 1957. As quoted by Hobson, G.E. 1964, in Polygalacturonase in normal and abnormal Tomato Fruit. Biochem J. 42: 324-332

14. Gee, M., Reeve, R.M., and McCready, R.M. 1959. Reaction of hydroxylamine with pectinic acids - degree of esterification of pectic substances in fruit. J. Ag. Fd. Chem. 7: 34-38

15. Gizis, E.J. 1964. As quoted by Pilnik, W. and Voragen, A.G.J. in Biochemistry of Fruit and their Products. 1970. ed Hulme Academic Press London. 788p.

16. Hall, C.B. 1963. Cellulase in tomatoes. Nature 220: 1010-1011

17. Hall, C.B. 1964 Cellulase activity in tomato fruits according to portion and maturity. Bot. Gaz. 125: 156-157

18. Hatanaka, C. et al. 1969. Enzymic degradation of pectic acid. XIII. New exopolygalacturonase producing digalacturonic acid from pectic acid. Nippon Nogei Kagaku Kaishi 43: 764

19. Hasegawa, S. and Smolensky, D.C. 1971 Cellulase in dates and role in fruit softening. J.Fd.Sc. 36: 966-967

20. Hobson, G.E. 1962. Determination of Polygalacturonase in fruits. Nature 195: 804-805

21. Hobson, G.E. 1964. Polygalacturonase in normal and abnormal tomato fruit. Biochem. J. 92: 324-332

22. Hobson, G.E. 1968. Cellulase activity in tomato. J.Fd. Sc. 33: 588-592

23. Hultin, H.O. and Levine, A.S. 1963. Occurence of multiple molecular forms of PE. Archs. Biochem. Biophys 101: 396-402

24. Hultin, H.O., Sun. B, and Bulger, J. 1966. Pectin methyl esterases of the Banana. Purification and properties. J.Fd.Sc. 31: 320-327

25. Jermyn, M.A. and Isherwood, F.A. 1956. Changes in the cell wall of pear during ripening. Biochem. J. 64: 123-132

26. Kertesz, Z.I., Eucare, M. and Fox, G. 1959. Apple Cellulose. Fd.Res. 24: 14-19

27. Knee, M. 1973. Polysaccharide changes in cell walls of ripening apples. Phytochem 12: 1543-1549

28. Lee, M. and Macmillan, J.D. 1970. Mode of action of pectic enzymes. III Site of initial action of tomato pectinesterase on highly esterified pectin. Biochem. 9: 1930-1934

29. Leuprecht, H. and Schaller, A. 1968 Ergebnisse methodologisher Untersuchungen hinsichtlich der Messung der Pektinmethyl esterase - Aktivitat in aufgetautem Tiefgefrier - Erdbeer puree. Fruchtsaft. Ind. 13: 50-74

30. Macmillan, J.D. Phaff, H.J. and Vaughn, R.H. 1964. The pattern of action of an exopolygalacturonic acid transeliminase from Clostridium multifermentans. Biochem 3: 572-578

31. McComb, E.A. and McCready, R.M. 1957. Determination of acetyl in pectin and in acetylated carbohydrate polymers. Anal. Chem. 29: 819-821

32. McCulloch, R.J. and Kertesz, Z.I. 1948 Tomato polygalacturonase. Arch. Biochem. 17: 197

33. Nagel, C.W. and Patterson, M.E. 1967. Pectic enzymes and development of pear. J.Fd.Sc. 32: 294-297

34. Neal, G.E. 1965. Changes occurring in the cell walls of strawberries during ripening. J.Sc.Fd.Ag. 16: 604-611

35. Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J.Bio. Chem. 153: 375-380

36. Patel, D.S. and Phaff, H.J. 1958. On the action of purified "tomato polygalacturonase". Food Res. 23: 693-694

37. Patel, D.S. and Phaff, H.J. 1959. On the mechanism of action of yeast endo-polygalacturonase on oligo-galacturonides and their reduced and oxidised derivatives J.Bio. Chem. 234: 237-241

38. Pilnik, W. and Voragen, A.G.J. 1970. Pectic substances and other uronides. In Biochemistry of Fruit and their Products Vol 1 1970. ed Hulme A.C. Academic Press London. 788p

39. Pressey, R. and Avants, J.K. 1971. The effect of substrate size on the activity of tomato polygalacturonase. J.Fd.Sc. 36: 486-489

40. Pressey, R. and Avants, J.K. 1972. Multiple forms of pectinesterase in tomatoes. Phytochem. 11: 3139-3142

41. Pressey, R., Hinton D.M. and Avants, J.K. 1971. Development of polygalacturonase activity and solubilization of pectin in peaches during ripening. J.Fd.Sc. 36: 1070-1072

42. Rees, D.A. 1969. As quoted by Knee, M. 1973. Phytochem 12: 1543-1549

43. Schweiger R.G. 1966. Quoted by Pilnik,W. and Voragen, A.G.J. in Biochemistry of Fruit and their Products, Vol I. 1970 ed. Hulme, A.C. Academic Press London 788p

44. Shewfelt, A.L., Paynter, V.A & Fen, J.J. 1971. Textural changes and molecular characteristics of pectic constituents in ripening peaches. J.Fd.Sc. 36: 573-575

45. Somogyi, M. 1952. Notes on sugar determination. J.Bio.Chem. 195: 19-23

46. Staden, O.L. and Doesburg, J.J. 1961 In Ann. Rep. 1961 pp60-62. Inst. Res. on Storage and Process. of Hort. Prod. I.B.V.T. Wageningen.

47. Sterling, Clarence. 1961. Physical state of cellulose during ripening of peach. J.Fd.Sc. 26: 95-98

48. Sterling, C. and Kalb, A.J. 1959. Pectic changes in peach during ripening. Bot.Gaz. 121:111-113

49. Sterling, Chaykin. 1966. Biochemistry Laboratory Technique.

50. Wade, F. 1964. Insoluble cell wall polysaccharides of strawberries. J.Sc.Fd. Ag. 15, 51-56

51. Weurman, C. 1954. Pectinase in pears. Acta Botan. Neerl. 3, 108-113

52. Wood, P.J. and Siddiqui, I.R. 1971 Determination of methanol and its application to measurement of pectin ester content and pectinesterase activity. Anal. Biochem. 39: 418-428

53. Woodward J.R. 1962. Physical and chemical changes in developing strawberry fruit. J.Sc.Fd.Ag. 23: 465-473

Appendix (i)

OD and PE activity (ug methanol) for fractions obtained after chromatography on DEAE- cellulose for Stage I - Stage V strawberries. Yields from Assay 1 and Assay 2 were averaged.

STAGE I

Fraction No.	OD Assay 1	OD Assay 2	ug methanol
5	0.01	0.02	0.9
10	0.07	0.06	4.0
15	0.02	0.01	0.9
20		0.04	2.8
25	0.06	0.06	3.7
30	0.09	0.07	5.0
35	0.07	0.05	3.7

STAGE II

Fraction No.	OD Assay 1	OD Assay 2	ug methanol
5	0.0	0.0	0.0
8		0.02	1.0
10	0.03	0.05	2.3
12		0.08	4.7
15	0.04	0.02	1.5
20	0.09	0.06	4.7
25	0.15	0.09	6.4
30	0.08	0.08	4.7
35	0.04	0.04	2.3
40	0.01		0.5

STAGE III

Fraction No.	OD Assay 1	OD Assay 2	ug methanol
5	0.18	0.06	6.2
10	0.19	0.13	8.7
15	0.12	0.11	6.2
20	0.15	0.13	7.6
25	0.18	0.13	8.2
30	0.15	0.10	6.7
35		0.05	3.0

STAGE IV

Fraction No.	OD Assay 1	OD Assay 2	ug methanol
5	0.04	0.04	2.3
10	0.09	0.11	5.9
15	0.08	0.6	4.0
20	0.09	0.13	6.5
25	0.09	0.12	5.9
30	0.07	0.07	4.0
35	0.04	0.06	2.7

STAVE V

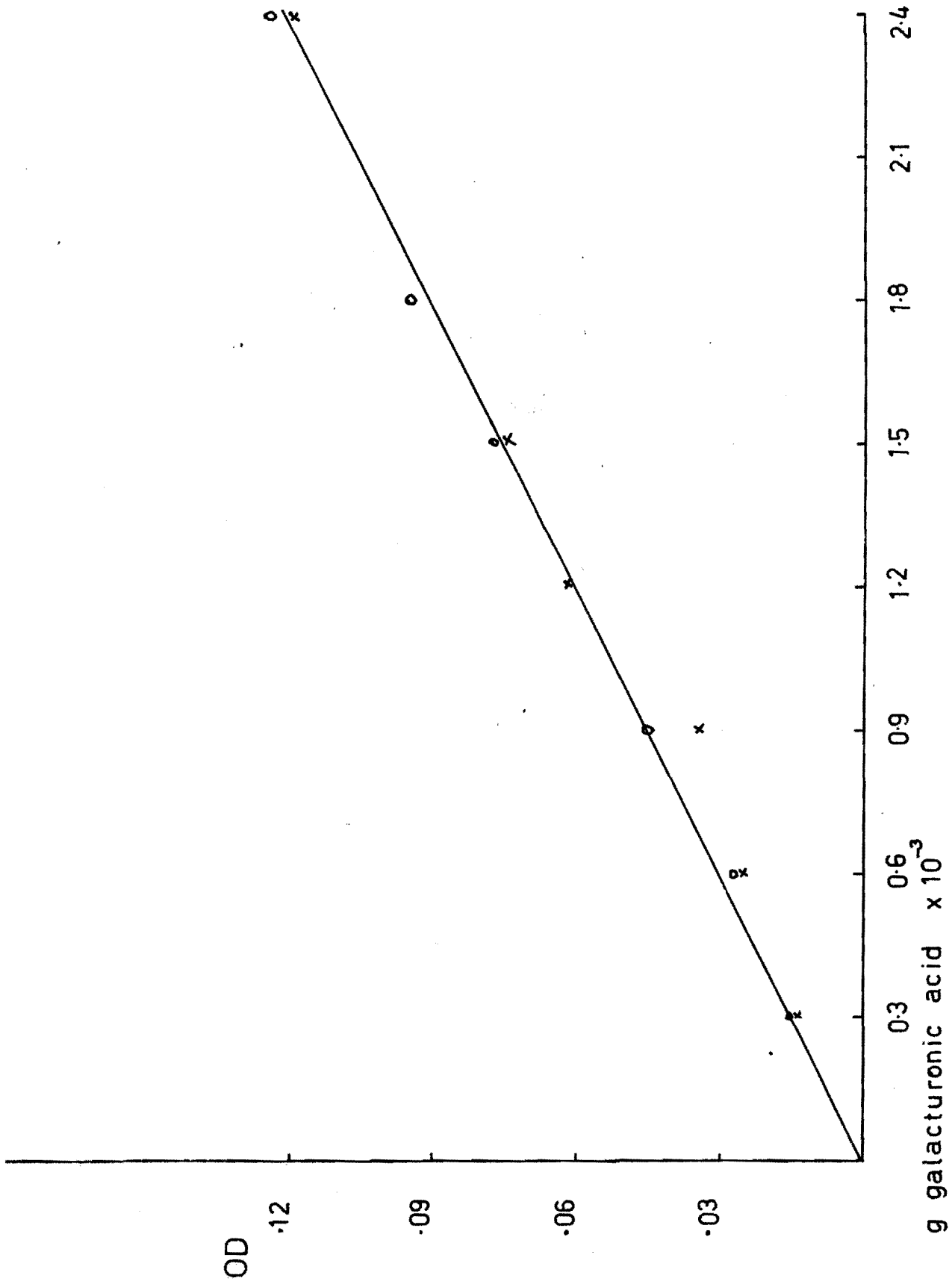
Fraction No.	OD Assay 1	OD Assay 2	ug methanol
5	0.02	0.0	0.4
10	0.08	0.04	3.0
15	0.05	0.01	1.5
20	0.09	0.06	3.9
25	0.13	0.12	6.5
30	0.12	0.12	5.9
35	0.12	0.11	5.9
40		0.06	3.0

Appendix (ii)

PE activity in duplicated samples collected from Peak I

Stage of Ripeness	Yield	ug methanol	Average
I	3.34	3.0	3.17
II	3.82	3.6	3.71
III	4.74	4.55	4.74
IV	6.41	4.45	5.43
V	5.31	3.04	4.17

APPENDIX (iii)



Standard curve for Nelson's method for estimation of reducing sugars using galacturonic acid