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# METABOLISM OF POLYMETHYLGALACTURONATE IN APPLE FRUIT CORTICAL TISSUE DURING RIPENING

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**Key Word Index**—*Malus domestica*; Rosaceae; apple fruit; cell wall metabolism; methylation; polygalacturonate.

**Abstract**—Changes in the composition and metabolism of polymethylgalacturonate were followed in ripening apples. After the onset of ethylene production and fruit softening total polygalacturonate decreased and the water soluble fraction increased. No change was detected in the overall degree of esterification but the esterification of the water soluble fraction increased. Incorporation of radioactivity from methionine-[$^{14}$C] into Me groups on polygalacturonate continued during ripening but incorporation from inositol-[$^{3}$H] decreased sharply. Cell separation probably depends upon the removal of low ester polygalacturonate from the middle lamella by exopolygalacturonase; the continued incorporation from methionine-[$^{14}$C] is probably due to synthesis of new polymethylgalacturonate.

## INTRODUCTION

During the ripening of apples there is limited degradation of wall-bound polymethylgalacturonate (PMG) and an increase in soluble PMG which is apparently undegraded [1]. Free carboxyl groups on the polygalacturonate are important in maintaining cell cohesion probably through cooperative binding of $Ca^{2+}$ ions [2]. It has been suggested that strawberry ripening involves methylation of free carboxyls disrupting a calcium stabilized gel structure [3]; also cell separation in cotton petiole abscission zones has been shown to be associated with incorporation from methionine-[$^{14}$C] into alkali-labile $^{14}CH_3$ groups [4]—probably in PMG. However the precursor of these Me ester groups is thought to be *S*-adenosylmethionine [5] and it seems unlikely that this would be exported to the cell wall for methylation of polygalacturonate *in situ*. Thus any increase of Me esters in the wall probably involves cytoplasmic esterification of freshly synthesized polygalacturonate. The present work attempts to resolve this problem by studying incorporation of precursors of Me groups and galacturonosyl residues. Because of the large endogenous pools of hexose sugars in fruit tissue inositol [6] was thought to be a potentially more efficient precursor of galacturonic acid than glucose.

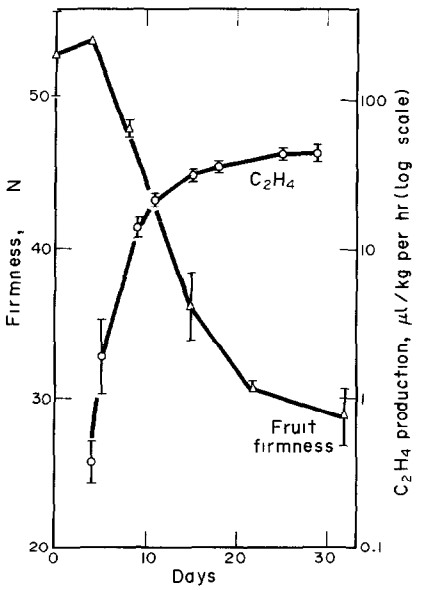


Fig. 1. Ripening of Cox's Orange Pippin apples at 12°. Vertical bars represent standard errors in this and subsequent figures.

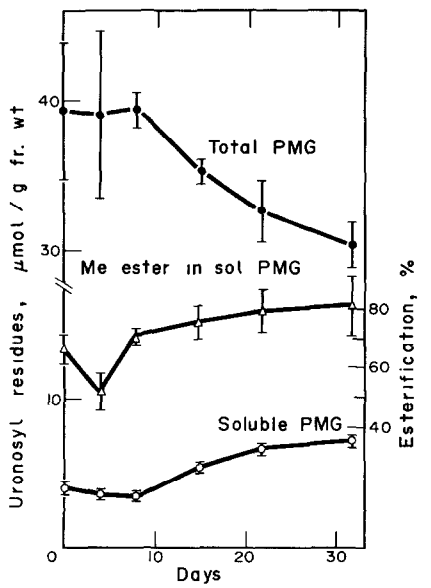


Fig. 2. Changes in polymethylgalacturonate (PMG) during ripening of apples.

## RESULTS

Careful selection of fruit resulted in uniform ripening behaviour, judged by softening and ethylene production (Fig. 1).

*Compositional changes*

There was a progressive fall in the total PMG content of the apples during ripening from 8 to 32 days after harvest (Fig. 2). The results in the figure are uncorrected for neutral sugar interference in the colorimetric assay for uronic acid. However, applying the maximum correction, on the assumption that all of the neutral carbohydrate present was composed of glucose residues, the fall in PMG was still significant ($p < 0.05$). Estimation of methanol release on saponification gave a mean percentage esterification of 78.2 for all apples with no apparent trend in time. However, the standard error of the estimations was such that a change of less than 12% could not have been detected.

The level of water soluble PMG increased after 8 days, associated with an upward trend in the esterification of this fraction (Fig. 2). There was a significant correlation ($p < 0.05$) between the level of soluble PMG and its degree of esterification the coefficient being 0.578.

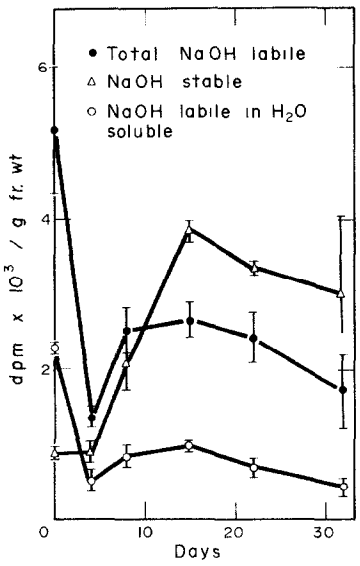


Fig. 4. Fate of radioactivity incorporated from methionine-[$^{14}$C] by discs cut from ripening apples.

*Incorporation from methionine-*[$^{14}$C]

The uptake of methionine-[$^{14}$C] by apple tissue increased in the course of ripening but the proportion of radioactivity incorporated after uptake decreased from 7 to 2% (Fig. 3a). Initially *ca* 80% of incorporation was found in the alkali-labile fraction but this decreased during ripening to *ca* 30%. Over 70% of this fraction was volatile but incubation of EtOH insoluble residues with pectinesterase failed to release radioactivity. This may have been due to the low rate of attack of the enzyme on PMG in the cell wall.

PMG was removed from cold water extracts of EtOH

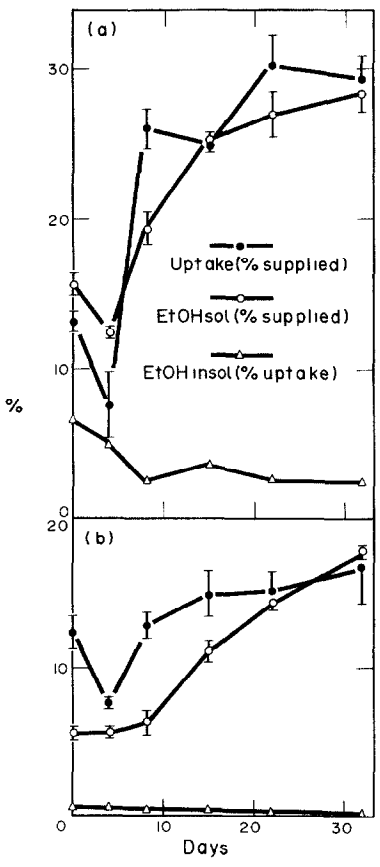


Fig. 3. Uptake and incorporation of radioactive precursors by cortical discs cut from ripening apples (a) $^{14}$C from methionine: (b) $^{3}$H from inositol

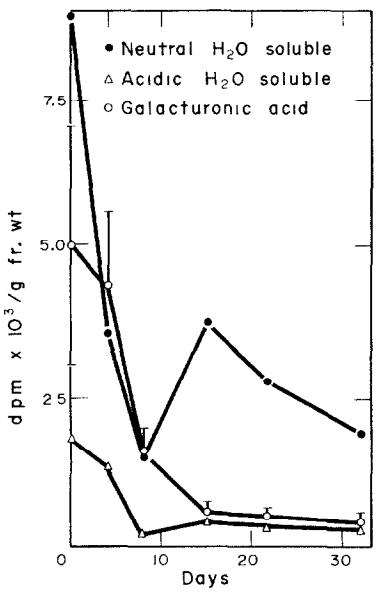


Fig. 5. Fate of radioactivity incorporated from inositol-[$^{3}$H] by discs cut from ripening apples.

insoluble residues by passage through DEAE cellulose and recovered by displacement with 0.5 M phosphate. Although *ca* 20% of total PMG was present in these preparations their radioactivity was close to background and was not more than 5% of the total alkali-labile activity. However, traces of the pectinesterase activity of the tissue survived heating with ethanol and this may account for the absence of $^{14}C$ label from soluble polymers.

PMG was isolated similarly from hot water extracts and this showed appreciable activity (Fig. 4). The PMG in these preparations accounted for 53% (s.e. = 2.2%) of the total but the volatile alkali-labile activity accounted for 40% of total at first and fell to 10% at the end of the experiment. After pectinesterase treatment *ca* 60% of the radioactivity was extracted into ether and about 10% was recovered as methyl dinitrobenzoate after TLC.

Pronase treatment rendered 90% of the alkali-stable radioactivity methanol soluble.

*Incorporation from inositol-*[$^3$H]

The percentage uptake of inositol-[$^3$H] was generally lower than that of methionine-[$^{14}C$] and the amount of activity incorporated into the EtOH insoluble fraction was always less than 1% of uptake (Fig. 3b).

Incorporation into galacturonic acid and into $H_2O$ soluble material decreased sharply but continued after the onset of ripening (Fig. 5).

## DISCUSSION

The above results show that there is no gross change of esterification of the whole PMG in ripening apple. This contrasts with some earlier findings when, using less direct analytical methods, increases [7] or decreases [8] of esterification were recorded. If the soluble PMG represents material derived from the middle lamella region, the increase of esterification of this fraction would be in keeping with the hypothesis that intercellular cohesion and PMG solubility are determined by the proportion of free carboxyl groups. The fall in total galacturonic acid residues conflicts with some earlier observations of their constancy during ripening of apples [7, 9, 10] but a fall has frequently been observed, if not always remarked upon [11–13]. The decrease can be readily accounted for now that a cell wall degrading exopolygalacturonase has been discovered in apple fruit [14].

Incorporation from methionine-[$^{14}C$] into material which was volatile after alkali treatment of the high MW fraction of plant tissues has generally been taken to be equivalent to Me esters of polygalacturonate. Exhaustive investigation has failed to show incorporation into other cell-wall ester groups [15]. The alkali-labile, radioactive material from apple tissue has only been partly characterised. The partial recovery of activity in methyldinitrobenzoate is thought to be due to manipulative losses and there are no grounds for thinking that the volatile product of alkali treatment represented anything other than the methyl ester of polygalacturonate.

The incorporation from methionine-[$^{14}C$] and inositol-[$^3$H] into PMG in unripe apple discs was as expected for fruit tissue which presumably still had the capacity for cell expansion and hence wall synthesis. The continuance of this incorporation after the onset of ripening was more surprising, particularly since the overall quantity of PMG was declining. The sharper decline in incorporation from inositol than in that from methionine could be due to increased dilution of some intermediate in an enlarged endogenous pool or to a switch from the inositol pathway to PMG synthesis from glucose.

The turnover of PMG observed leads to a more dynamic interpretation of cell wall changes in ripening apples. Cell separation could be the result of degradation of existing, largely insoluble PMG and its replacement by a freshly synthesized, highly esterified polymer. This is consistent with the observation that the wall-bound PMG of ripe apples was of lower MW than that of the corresponding fraction from unripe fruit or the soluble PMG from ripe fruit. Much of the wall-bound PMG is highly branched and this feature would presumably limit attack by exopolygalacturonase. However the removal of galactose side chains [13] would perhaps make the molecule more labile to the enzyme. Whether the removal of these chains has any other effect awaits elucidation of the role, if any, of neutral side chains in wall structure.

## EXPERIMENTAL

Fruits were harvested from the outer branches of a single apple tree (*Malus domestica* Borkh., cv Cox's Orange Pippin on M 26 rootstock) on 16 September 1976. Selected fruits between 110 and 125 g were placed individually in containers each supplied with a flow of air (0.5 l./hr) at 12°. Samples (0.5 ml) of air passing over each apple were regularly injected for GLC on an alumina column with FID to measure $C_2H_4$ production. On each sampling date 3 apples were taken and the firmness of their flesh measured in 3 positions using a penetrometer with an 8 mm plunger [16].

*Analytical methods.* Slices of cortical tissue (10 g) from each apple were disintegrated in 40 ml EtOH and refluxed 40 min. After filtration through sintered glass (porosity 3) the residue was washed with EtOH–$H_2O$ (4:1), EtOH and $Et_2O$ and allowed to equilibrate with air at room temp. Subsamples of EtOH insoluble residue representing 1 g fr. wt were analysed for $H_2O$ soluble and total polygalacturonate by a colorimetric procedure [17]. The total carbohydrate content of extracts was estimated colorimetrically with sulphonated α-naphthol reagent [18] and simultaneous equations based on reaction of glucose and galacturonic acid standards in this and the uronic acid assay were solved to enable the latter to be corrected for interference from neutral carbohydrate. MeOH derived from alkaline hydrolysis of soluble polygalacturonate and EtOH insoluble residues was estimated by GLC [1].

*Incorporation of precursors.* Discs of cortical tissue (2.5 g) from each apple 15 × 2 mm were incubated 1 hr at 20° in 10 ml 0.4 M sucrose containing 1.25 μCi L-methionine methyl-[$^{14}C$] (52 μCi/μmol) and 6.25 μCi myo-inositol 2-[$^3$H] (5 mCi/μmol). (The methionine was dissolved in $H_2O$ and aliquots were freeze dried and stored under $N_2$ at −20°.) After incubation the liquid was decanted and the discs were washed ×3 with $H_2O$ before they were disintegrated in 10 ml EtOH. After refluxing 30 min the suspension was filtered and the residue was washed with EtOH–$H_2O$ (4:1) and $Et_2O$ as above. The aq. and EtOH washings were assayed for their radioactivity by mixing aliquots with 10 ml PPO (4 g/l.) in toluene-2-ethoxyethanol (6:4) before scintillation counting as described below.

*Radioassay on* EtOH *insoluble residue.* Portions of residue corresponding to 0.25 g tissue were taken for each determination. The unfractionated residue and material remaining after various extractions were assayed for radioactivity after suspension in 5 ml PPO-toluene (4 g/l.) containing 250 mg fused silica. To assay alkali-labile material, to each was added 0.3 ml 0.5 M NaOH and after 30 min at 20° 0.1 ml M HOAc and 2 ml MeOH. After centrifugation the supernatant was withdrawn and the residue washed ×2 with 2 ml MeOH by resuspension and

centrifugation; to the combined MeOH supernatants was added 5 ml PPO-toluene (8 g/l.). After radioassay the non-volatile activity was measured by evaporating to dryness at 35° and redissolving in toluene–MeOH (1·1). Portions of residue were incubated in 2 ml $H_2O$ 17 hr at 20° or 4 hr at 98° and filtered through glass fibre paper (Whatman GF/A). The filtrate was applied to a column of 0.5 ml DEAE cellulose (Whatman DE 52) equilibrated with 5 mM NaPi 1 mM EDTA (pH 6.5) and eluted with 2 ml of this buffer to give the neutral fraction. The acidic fraction was eluted with 2 ml 0.5 M NaPi 1 mM EDTA (pH 6.5) [19]. To avoid precipitation, aliquots (0.4 ml) of soluble polysaccharide fractions were incubated 16 hr at 20° with 10 µl pectinase soln (10 mg/ml, Koch Light) before addition of 10 ml PPO–toluene–ethoxyethanol. Aliquots of selected acidic fractions were also assayed after incubation with 50 units tomato pectinesterase (Sigma) 16 hr at 20° and extraction with $Et_2O$. The dried ($Na_2SO_4$) $Et_2O$ extract was refluxed 60 min with 50 mg 2,4 dinitrobenzoyl chloride and the methyl dinitrobenzoate recovered after TLC on Si gel [20]. EtOH insoluble residue was incubated with 2.5 mg pronase (Sigma Type VI) in 1 ml 0.05 M NaPi (pH 6.5) 24 hr at 20°. After addition of 9 ml MeOH and centrifugation 4 ml supernatant was mixed with 5 ml PPO–toluene (8 g/l.). Alkali-labile $^{14}C$ remained in the residue after pronase treatment.

To estimate activity in galacturonic acid further portions of residue were incubated with 1 ml 0.1 M NaOH 10 mM EDTA 60 min at 20°. After addition of 10 µl HOAc 50 µl (0.1 mg) pectinase soln (Koch Light) and 2 drops toluene were added and incubation continued 24 hr at 20°. EtOH (8 ml) was added to each and after centrifugation the supernatant was evaporated at 40° and redissolved in 80 µl $H_2O$. Aliquots (20 µl) were applied to Whatman No. 3 paper and developed with EtOAc–$C_5H_5N$–HOAc–$H_2O$ (5:5:1:3) in a tank equilibrated with EtOAc–$C_5H_5N$–$H_2O$ (40:11:6) [21]. Galacturonic acid in samples was located by reference to marker strips visualized with $AgNO_3$–NaOH and eluted with $H_2O$. (No oligomers were detected in similarly visualized sample strips.) The eluates were freeze dried and redissolved in 0.25 ml $H_2O$ before addition of PPO–toluene–ethoxyethanol. All samples were counted in an 'Intertechnique' SL 30 scintillation counter at efficiencies ranging from 12 to 25 % for $^3H$ and 30–55 % for $^{14}C$.

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