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Cell Wall Metabolism in Ripening Fruit[1]

V. Analysis of Cell Wall Synthesis in Ripening Tomato Pericarp Tissue Using a D-[U-^{13}C]Glucose Tracer and Gas Chromatography-Mass Spectrometry

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ABSTRACT

A gas chromatographic-mass spectrometric technique utilizing D-[U-^{13}C]glucose as a density label tracer was used to follow the synthesis of cell wall polysaccharides in pericarp discs that were excised from mature green tomato fruit (*Lycopersicon esculentum*) and allowed to ripen in culture. The biosynthetic capacity of discs from four different maturity stages was examined. Label was differentially incorporated into wall polysaccharides as the discs matured, indicating a change in the nature of wall polymers being synthesized. These differential rates of incorporation are consistent with descriptions of ripening-related cell wall compositional changes previously reported by other authors. Specific changes in wall biosynthesis noted include increased incorporation of xylosyl and mannosyl residues into hemicellulosic cell wall fractions as the discs mature and decreased incorporation of galactosyl residues into chelator-soluble pectins.

Digestion of cell wall components has been the primary focus of research for several decades aimed at understanding ripening-related tissue softening of fruits (4, 11–13, 21). Undoubtedly, the breakdown of pectic and other wall polysaccharides is an important event in ripening-related texture loss. Recent studies focusing on the role of endopolygalacturonase in tomato fruit ripening (*e.g.* 10) have questioned the idea that endopolygalacturonase is the dominant factor in tissue softening. This recognition is now driving a broadened examination of mechanisms that could explain ripening-related wall change. Included in these efforts is consideration of the idea that cell wall synthesis continuing into the ripening period could alter overall wall strength and, hence, reduce fruit firmness. This possibility was first suggested by Knee (15) in an attempt to explain softening in apple fruits, but the data he provided to support the notion were limited. Lackey *et al.* (16) provided data that suggested that the substantial loss of wall galactose seen during tomato fruit ripening was the consequence of altered rates of galactan synthesis and breakdown. This had been the only effort to demonstrate synthesis of wall components during ripening until recently.

In 1989, Mitcham *et al.* (17) showed that tomatoes are able to incorporate ^{14}C-labeled sucrose into cell-wall matrix polysaccharides well after the onset of ripening even though the total amount of cell wall material (per gram fruit fresh weight) is steadily decreasing. Synthesis continued into the red maturity stage, and they suggested that these synthetic events might be important in fruit softening. The results of this study generally indicate that cell wall polysaccharide synthesis continued well into the latter stages of tomato fruit ripening, but they provided no clear information concerning the nature of the polymers being synthesized or incorporation into individual monosaccharide residues. We have developed an analytical approach (9) to expand upon the results of Mitcham *et al.* (17) and thus get a clearer picture of the wall synthesis potential of ripening tomatoes.

Our experimental technique uses D-[U-^{13}C]glucose as a density label tracer. This can be easily applied to tomato pericarp discs whose ripening *in vitro* closely parallels the ripening of intact fruit (6). This combination of analytical approach and ripening system allows us to examine in detail incorporation at any stage of the ripening process. Detection of the label (and, thus, incorporation into the wall) is accomplished by means of a GC-MS analysis which compares the isotope ratio of selected ion pairs from individual monosaccharide residues. This method is similar to the technique of Sasaki *et al.* (18) who examined the fate of density-labeled inositol in the cell walls of germinating beans but is useful for the analysis of a greater range of monosaccharides because of the more general distribution of carbon from glucose into other sugars. The technique can also be applied to permethylated samples of cell wall polysaccharides and thus provide information regarding incorporation into specific cell wall polymer types. In this paper we demonstrate the utility of this density label technique in exploring cell wall synthesis potential and provide data that show that this potential is expressed well into advanced stages of ripening in the tomato fruit.

MATERIALS AND METHODS

Disc Preparation and Treatment

Tomato pericarp discs were prepared from-field grown tomato fruit (*Lycopersicon esculentum* Mill. cv "Castlemart"), as described in detail by Campbell *et al.* (6). Briefly, discs (1 cm in diameter × 5 mm thick) were cut from surface-sterilized (1% commercial bleach [0.05% sodium hypochlorite], 10 min) whole fruit. This and all subsequent manipulations were performed in a sterile laminar flow hood. The excised discs

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were then placed in sterile multiwell tissue culture plates. The plates containing the discs were then stored in a sealed plastic box which was continuously flushed with filtered air at 100% RH. Discs were allowed to ripen at 20°C (the extent of ripening was based primarily on measurement of change in skin color "a*" value [6]) and were treated at various maturities. Disc color was measured using a Minolta CR-200 reflectance colorimeter and was recorded using the $L^*a^*b^*$ uniform color scale (CIELAB) with which the a^* value is a measure of hue on the green-red axis. Density label was added at the maturities listed below. The label consisted of 200 μg of D-[U-^{13}C]glucose in 50 μL H_2O. Controls consisted of 50 μL H_2O containing no label. All solutions were added to the cuticle-free surface of the discs and were sterilized before addition by filtration through a 0.45-μm Millipore filter. Discs were harvested at four maturities: green, turning, pink, and red (color stages −11, −3, +5, and +15, respectively). Labeled discs and corresponding controls were frozen 24 h after labeling.

Cell Wall Preparation

Cell walls were prepared from the frozen discs by first boiling them in 90% ethanol (to inactivate potential autolytic enzymes) and then homogenizing (Polytron) the boiled tissue in the same ethanol. Following homogenization, insoluble materials were collected by centrifugation (2000*g*, 15 min). The pellet material was resuspended in 90% ethanol and centrifuged as above. This wash procedure was repeated once more, and the pellet was isolated for further extractions. The pooled supernatants from these centrifugations were evaporated under air for later analysis. The ethanol-washed pellet (crude cell wall) was extracted overnight with 90% DMSO to remove starch (19). The starch-free cell wall material was collected by centrifugation (10,000*g*, 15 min), and the pellet was washed free of residual DMSO by several washings with 95% ethanol. Polymeric carbohydrates solubilized in the DMSO were precipitated by the addition of ethanol to 80% (v/v). Samples were then refrigerated (4°C) overnight, and precipitates were collected by centrifugation (10,000*g*, 10 min). The air-dried, starch-free pellet was next extracted with 50 mM CDTA[2], 50 mM Na acetate, pH 6.5 (10 mL/approximately 200 mg of wall), overnight. The resultant slurry was centrifuged (10,000*g*, 15 min). The supernatant was dialyzed for 36 h against deionized H_2O (three changes, 4°C) and then lyophilized. The CDTA-extracted cell wall material was resuspended in distilled H_2O, washed by centrifugation twice, and lyophilized.

Cell Wall Analysis

Samples (4–8 mg) of the dried, CDTA-extracted wall material were hydrolyzed (1 h, 121°C) in 2 N TFA. Insoluble material was collected by centrifugation (2000*g*, 5 min) and analyzed as the cellulose fraction. This was initially stirred in 67% H_2SO_4 to dissolve the cellulose, diluted with water to 2 N H_2SO_4, and heated at 121°C for 2 h. The H_2SO_4 was removed by addition of BaOH and the supernatant (containing monosaccharides generated by hydrolysis) was collected. The monosaccharides generated during TFA hydrolysis of the cell wall (TFA-soluble; noncellulosic fraction) and H_2SO_4 hydrolysis of the cellulose fraction were converted to alditol acetates (2) and examined by GC and combined GC-MS. Analysis of sugars in TFA-hydrolyzed DMSO- and CDTA-soluble materials was performed similarly.

GC and GC-MS Analysis of Cell Wall Components

GC analysis was performed using a Perkin-Elmer 8320 gas chromatograph with a flame ionization detector. Chromatography was performed using a DB-23 capillary column (30 m × 0.25 mm, i.d., J & W Scientific) using H_2 as the carrier gas and the oven held at 210°C. Quantitation was performed with a Perkin-Elmer (Sigma 10) data system.

The distributions of ^{12}C and ^{13}C in cell wall components were determined by coupling a Hewlett-Packard model 5890 GC fitted with a DB-23 capillary column to a Hewlett-Packard model 5970 mass-selective detector. The detector could be operated in either single ion- or total ion-monitoring modes. Carrier gas for this analysis was He and the oven was programmed from 100 to 160°C at 10°C/min and then to 210°C at 2°C/min. The oven was then held at 210°C for 5 min.

Determination of the Degree of Incorporation of D-[U-^{13}C]Glucose into Cell Wall Polysaccharides

The mass spectra of the alditol acetates of the major neutral sugar (aldose) components of cell wall polysaccharides are very similar. Individual residues were identified by retention time relative to the internal standard (inositol) which has a unique mass spectrum. The extent of incorporation of [^{13}C]-D-glucose into a given residue can be determined by analysis of homologous ion fragments from alditol acetates of sugars containing primarily ^{12}C (produced during fruit growth or from endogenous sugar pools) or ^{13}C (produced after introduction of [^{13}C]glucose). These fragments will show differences in mass when analyzed by the mass-selective detector. This method is very similar to that of Sasaki *et al.* (18) in which per-C-deuterated inositol was used as a density label. Sasaki *et al.* chose to monitor the ratio of ions with masses of 187 and 191. These two fragments are secondary ions being derived from a primary ion pair (289–293) that contains four of the original carbon atoms of the labeled sugar molecules. Figure 1 shows our mass spectra for [^{12}C]glucose and [^{13}C]glucose and the structures of glucitol hexaacetates as well as the origins of the most prominent primary ion fragments from [^{12}C]- and [^{13}C]glucose. The four-carbon fragment of 289 AMU (which decomposes to form the 187-AMU daughter ion) is an excellent choice for our analysis of incorporation because the probability of a 293-AMU ion or its daughter ion (AMU 191) occurring because of the natural abundance of ^{13}C is negligible. Based on the natural abundance of ^{13}C, the predicted concentration of the 191-AMU fragment, as compared with that of its 187 counterpart, should be 0.03%. However, in several trials in which we made on-column injections of 50 μg glucose standard, we saw no 191 peak. However, if a 50-μg glucose sample is spiked with 15 ng

[2] Abbreviations: CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N'N'*-tetraacetic acid; AMU, atomic mass unit.

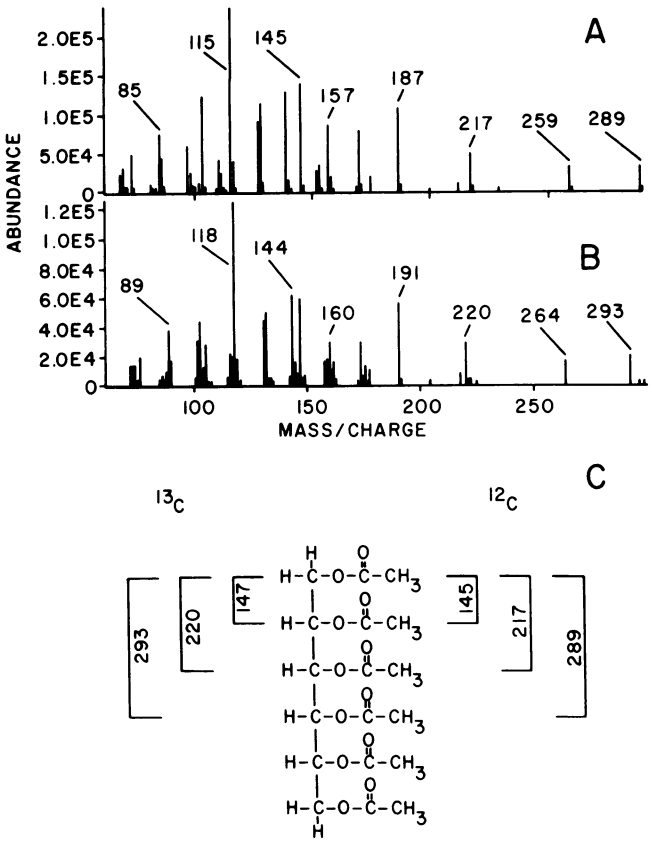

Figure 1. Mass spectra of glucitol hexacetate derived from naturally occurring glucose (A), [^{13}C]-(U)-glucitol hexaacetate (B), and the structure of the same molecule showing the origins of the most prominent ion fragments (C).

(0.03% of 50 μg) of D-[U-^{13}C]glucose the 191 peak is easily quantitated. Running the GC-MS in the single ion monitoring mode and using larger samples enables us to quantify incorporation at even lower values.

Analysis of Ethanol-Soluble Carbohydrate

^{13}C in glucose and sucrose of the ethanol-soluble fraction of disc homogenates was measured by combined GC-MS. Paired aliquots were taken from samples. One of these was hydrolyzed in 2 N TFA, and the TFA was subsequently removed by evaporation. Both aliquots were then derivatized for GC-MS (2). The TFA treatment will both hydrolyze the glycosidic bond of sucrose and destroy the fructose generated. The ^{13}C in glucose was measured in the unhydrolyzed aliquot. ^{13}C in sucrose was calculated from the difference in the glucose identified ± hydrolysis multiplied by 2 (to account for the fructose lost).

Impact of Disc Excision (Wounding) on Cell Wall Composition and Synthesis

Immediately after excision, tomato pericarp discs show a 48-h-long period of "wound" ethylene production (6). Wounding has been shown to promote the synthesis of callose-(5) and hydroxyproline-rich glycoproteins (7), and such effects could confuse our efforts to identify ripening-related aspects of wall synthesis. The impact of disc preparation on cell wall content of 1,3-linked glucosyl residues (from callose) and hydroxyproline was determined as follows.

Discs (1 cm × 5 mm) were cut from mature-green 3 tomato fruit (locules filled with jelly, but skin and inner flesh still green) and were provided with [^{13}C]glucose (as described before) either immediately or after 48 h. Following incubation, discs were subdivided into portions containing the original cut surfaces (a 1-mm slice cut from the disc inner surface plus a 2-mm ring cut from the disc circumference) and internal tissues. Starch-free cell walls were then prepared from each of these tissue samples. Wall preparations were then methylated (3), hydrolyzed, reduced (sodium borodeuteride), and acetylated for GC-MS analysis.

Another set of discs was cut and allowed to ripen in culture. Samples (10 discs each) were taken immediately and on days 1, 2, 3, and 5 and frozen after color stage was determined (6). Samples of outer pericarp were also taken from intact tomatoes that were allowed to ripen in parallel with the discs. Starch-free cell walls were prepared from all samples. Aliquots were then hydrolyzed in 6 N HCl (105°C, 18 h). Insoluble material remaining after the hydrolysis was removed by centrifugation (700*g*, 10 min), and the HCl-containing supernatant was evaporated to dryness. This material was redissolved in H_2O and assayed for hydroxyproline (14).

Glucose labeled with ^{13}C (99%) in all six carbons was purchased from Cambridge Isotope Laboratories (Woburn, MA).

RESULTS AND DISCUSSION

The distribution of ^{13}C-labeled sugars in various tissue fractions following a 12-h incubation of discs of color stage −11 with [^{13}C]glucose is shown in Table I. The data show a better than 90% recovery of ^{13}C and indicate that the majority of the label is found in the low mol wt pool (ethanol-soluble glucose). Apparently, the flux of the glucose-sucrose pools at these stages of development is very small; we were unable to detect any increase in the [^{13}C]glucose content of the 80% ethanol-soluble fraction after hydrolysis, indicating no significant synthesis of fructose and/or sucrose from the added label. The starch content of wall material was quite low, representing only 0.04% on a dry weight basis (not shown), but there is some accumulation of [^{13}C]glucose into this fraction. However, a comparison of starch contents of [^{13}C]glucose-treated discs and controls (H_2O control or no treatment) indicates that the addition of glucose caused no net increase in the amount of starch (data not shown). The total glucose-sucrose (^{12}C plus ^{13}C) available in this sample was 4.82 mg. This means that the 200 μg of density label we added increased the endogenous pool by about 4%. We presume that this had no appreciable effect on normal wall metabolism because the addition of [^{13}C]glucose had no apparent effect on individual monosaccharide content of any cell wall fraction (data not shown).

The extent to which [^{13}C]glucose is incorporated into components of the CDTA-soluble fraction of disc cell walls during incubations at four ripeness stages is shown in Table II.

Table I. *Distribution of [^{13}C]-(U)-Glucose and Its Metabolites following a 24-h Incubation with Mature-Green 3 Discs*

Pericarp discs cut from mature-green 3 tomato fruit (color stage −11) were labeled with 200 μg D-[U-^{13}C]glucose. The discs were treated as described in "Materials and Methods." The resulting fractions were analyzed by GC and GC-MS to determine the extent to which label was incorporated into individual residues.

Fraction	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Total
	μg neutral sugar residue per disc derived from ^{13}C-glc							*μg ^{13}C per fraction*
80% ethanol supernatant					0.015		163.100	163.115
DMSO extract			0.069		0.010	0.095	0.267	0.441
CDTA extract			0.080			0.650	0.143	0.873
2 N TFA-soluble fraction			1.866	0.124	0.024	7.689	1.292	10.990
Cellulose					0.120		18.100	18.220
Total ^{13}C recovered								193.639

Galactose and arabinose are the predominant neutral sugars in this fraction early in ripening; rhamnose content increases as ripening proceeds. Incorporation of ^{13}C into galactose is relatively high at the green stage, but relative ^{13}C content decreases substantially as disc ripening continues. In contrast, the relative ^{13}C content of the arabinose of this fraction increases slowly throughout disc ripening, and the ^{13}C content of the increasing rhamnose component increases dramatically at the pink and red stages. The presence of glucose in this fraction is somewhat unexpected and probably represents starch remaining after DMSO treatment. Accumulation of ^{13}C in the glucose-containing component of the CDTA extract is low initially and decreases to zero as ripening continues.

Similar information for the 2 N TFA-soluble (noncellulosic) wall fraction is provided in Table III. Again, at the green stage, there is a significant incorporation of density label into galactosyl residues. This decreases with disc ripening in a manner similar to that for the CDTA-soluble material. The patterns of labeling of the arabinosyl and rhamnosyl residues in the wall also parallel those for the CDTA fraction. Different, however, are the data for xylose, mannose, and glucose. Incorporation into the [^{13}C]glucose component increases from the green to the turning stages, remains constant into the pink stage, and then increases slightly as ripening advances. The incorporation of label into xylose peaks at the turning stage, and ^{13}C incorporation into the mannose component increases substantially as the discs become fully red.

Analysis of the 2 N TFA-insoluble (cellulosic) cell wall fraction indicates that there is continued synthesis of cellulosic polymers during ripening. The amounts of [^{13}C]glucose in cellulosic glucose at stages −11, −3, +5, and +15 are 0.28, 0.35, 0.40, and 0.30%, respectively. Incorporation into the mannose produced during hydrolysis of this "cellulose" fraction increased from 0.10% at color stage −11 to 0.40% at color stage +15.

Although there appears to be some impact of excision on disc synthesis of polysaccharide, this wound effect seems to run its course within 48 h of disc preparation. Callose-type polymers (measured as 1,3-linked glucosyl residues) were present in very low abundance in cell walls of freshly cut discs (0.014% of the 2 N TFA-soluble fraction). The content of these residues increased by 30% in cell walls prepared from the directly wounded surfaces of discs by 12 h after excision (incubated with [^{13}C]glucose for 12 h, starting immediately after cutting) and remained steady at the higher level at 60 h postexcision. No change in 1,3-glucosyl residue content was seen in walls from interior (*i.e.* unwounded) portions of discs. Although there was incorporation of ^{13}C into callose during the 0- to 12-h incubation, no incorporation was noted in the discs incubated with [^{13}C]glucose beginning at 48 h postexcision.

No impact of disc preparation on cell wall content of

Table II. *Neutral Sugar Composition of the CDTA-Soluble Tomato Fruit Cell Wall Fraction and the Percentage of each Residue Derived from the Density Label*

Pericarp discs were prepared from mature-green 3 tomato fruit. Density label was applied to sets of 20 discs each at four color stages (−11, −3, +5, +15). Following a 24-h incubation, the discs were frozen and subsequently extracted, and the CDTA-soluble fraction was isolated as described in "Materials and Methods." Quantification and analysis of label distribution was accomplished by GC and GC-MS. Data presented for each sugar residue are its mole percentage of total neutral sugars and, in parentheses, the percentage of that sugar that is ^{13}C labeled.

Sugar	Color Stage			
	−11	−3	+5	+15
Rha	10.1 (0)	14.2 (0.08)	18.2 (0.21)	28.0 (0.74)
Fuc	0.8 (0)	1.0 (0)	1.3 (0.04)	1.7 (0.06)
Ara	25.1 (0.04)	25.8 (0.05)	26.4 (0.06)	21.6 (0.09)
Xyl	2.6 (0)	2.9 (0)	3.1 (0)	4.9 (0)
Man	2.5 (0)	2.9 (0)	2.9 (0)	3.1 (0)
Gal	48.7 (0.09)	44.2 (0.06)	40.1 (0.01)	37.2 (0)
Glc	8.9 (0.02)	8.0 (0.02)	7.6 (0)	4.2 (0)
Total CDTA-soluble neutral sugar as a % of total cell wall dry weight	0.47	0.55	0.59	0.64

hydroxyproline was noted. The initial wall content of hydroxyproline in discs was low (0.01% of wall weight) and increased with ripeness stage (to 0.02% of wall weight), but a similar pattern was seen in cell walls prepared from ripening fruits that had reached comparable ripening stages (data not shown).

The data in Tables II and III indicate that the tomato pericarp's capacity for cell wall synthesis utilizing exogenously supplied precursor is maintained well into ripening. The amount of incorporation in ripening pericarp is low compared with that shown in actively growing tissue. For example, Sasaki *et al.* (18) provided labeled myoinositol to growing bean hypocotyls and showed as much as 20% labeling of xylosyl and galacturonsyl residues after 72 h. However, the ripening tomato fruit is a senescing organ. Furthermore, it has been developing and producing cell walls from ^{12}C-labeled precursors for 50 to 60 days. It is not surprising that the proportion of ^{13}C in its cell walls should be relatively low after a short exposure to D-[U-^{13}C]glucose. It is possible that what we are measuring is a synthesis that is artifactually promoted by the addition of suitable substrate. We do not think this to be the case. The amount of precursor added amounted to only a few percent of the total glucose/sucrose content of discs, and the feeding had no measurable impact on the relative sugar content of cell wall fractions. Furthermore, the patterns of change in the extent to which the [^{13}C]glucose was incorporated into wall material show interesting parallels to data describing overall sugar changes in walls prepared from ripening tomato pericarp. Several studies (12, 20), including our own with pericarp discs (ref. 6 and data presented in Table III), have shown a pronounced decrease in wall content of galactose. Lackey *et al.* (16) suggested that some of this decrease could be due to a ripening-associated slowing of synthesis of galactan in combination with a continuing loss of galactans from the wall (*i.e.* a change in turnover rate). Our data (Table III) indicate a substantial decline in relative incorporation into wall galactose as ripening continues. Cell wall xylose content increases as ripening progresses (ref. 6 and Table 3). Although this could represent a relative increase in xylose (as other sugars decline in relative importance), the fact that incorporation of ^{13}C into wall xylosyl residues increases until the turning stage suggests that at least some of this increase is a consequence of changes in patterns of wall synthesis. Tong and Gross (21) reported an increase in the amount of 4- and 4,6-linked mannosyl residues in hemicelluloses extracted from ripe tomato pericarp. Our data (Table III) also show a late increase in wall mannose content and indicate that this is accompanied by a substantial increase in relative ^{13}C incorporation.

Incorporation of ^{13}C into the neutral sugar residues of the chelator-soluble "pectin" fraction changes during the course

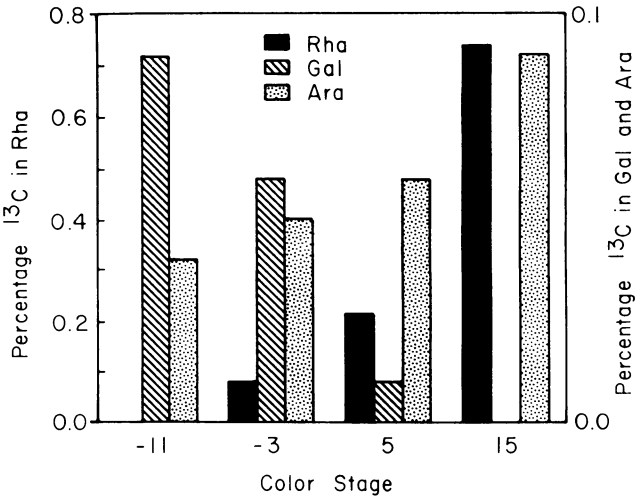

Figure 2. Comparison of the levels of incorporation of D-[U-^{13}C] glucose into Rha, Ara, and Gal in the CDTA-soluble tomato fruit cell wall fraction. Fractions were isolated and analyzed as described in the legend for Table II.

Table III. *Neutral Sugar Composition of the 2 N TFA-Soluble Tomato Fruit Cell Wall Fraction and the Percentage of Each Residue Derived from the Density Label*

Tomato fruit cell wall material, isolated after the CDTA extraction, was hydrolyzed with 2 N TFA, and the resulting monosaccharides were treated as described in "Materials and Methods." Analysis of composition and label distribution was accomplished by GC and GC-MS. Data presented for each sugar residue are its mole percentage of total neutral sugars and, in parentheses, the percentage of that sugar that is ^{13}C labeled.

Sugar	Color Stage			
	−11	−3	+5	+15
Rha	9.7 (0)	9.7 (0)	12.1 (0.005)	14.6 (0.01)
Fuc	0.8 (0)	0.8 (0)	0.9 (0)	1.1 (0.003)
Ara	19.9 (0.08)	18.2 (0.09)	17.4 (0.1)	14.2 (0.07)
Xyl	14.6 (0.005)	18.4 (0.07)	22.8 (0.12)	24.5 (0.05)
Man	6.9 (0.002)	6.9 (0.002)	7.0 (0.002)	9.8 (0.080)
Gal	37.4 (0.32)	34.2 (0.25)	25.1 (0.09)	21.2 (0.05)
Glc	10.4 (0.05)	11.9 (0.09)	14.2 (0.1)	14.8 (0.06)
Total 2 N TFA-soluble neutral sugar as a % of total cell wall dry weight	14.8	13.5	12.2	11.2

of the experiment (Table II, Fig. 2). That a change in rhamnogalacturonan synthesis accompanies ripening is suggested by the pronounced increase in ^{13}C labeling of the rhamnosyl residues of the CDTA-soluble fraction (Fig. 2). Efforts to determine to what extent pectin synthesis changes during ripening and whether this synthesis contributes to the ripening-associated increases in soluble pectins reported by several laboratories (6, 12, 19) are underway. Thus far, we have used an approach that involves methanolysis followed by sodium borodeuteride reduction of carboxylmethyl esters (1) to study uronosyl residues in our preparations and have detected no ^{13}C-incorporation into galacturonosyl residues. Whether this is an indication of a technical problem, that glucose is not the correct precursor or that no uronide synthesis is occurring, is not yet clear. An important question that must be addressed is how (if at all) synthesis of new wall polysaccharides can contribute to the weakening of cell wall connections that has been commonly associated with fruit tissue softening.

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