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Turnover of Cell Wall Polysaccharides in Elongating Pea Stem Segments[1]

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ABSTRACT

Turnover of cell wall polysaccharides and effects of auxin thereon were examined after prelabeling polysaccharides by feeding pea (*Pisum sativum* var. Alaska) stem segments ^{14}C-glucose, then keeping the tissue 7 hours in unlabeled glucose with or without indoleacetic acid. There followed an extraction, hydrolysis, and chromatography procedure by which labeled monosaccharides and uronic acids were released and separated with consistently high recovery. Most wall polymers, including galacturonan and cellulose, did not undergo appreciable turnover. About 20% turnover of starch, which normally contaminates cell wall preparations but which was removed by a preliminary step in this procedure, occurred in 7 hours. Quantitatively, the principal wall polymer turnover process observed was a 50% decrease in galactose in the pectinase-extractable fraction, including galactose attached to a pectinase-resistant rhamnogalacturonan. Other pectinase-resistant galactan(s) did not undergo turnover. No turnover was observed in arabinans, but a doubling of radioactivity in arabinose of the pectinase-resistant, hot-acid-degradable fraction occurred in 7 hours, possibly indicating conversion of galactan into arabinan. None of the above changes was affected by indoleacetic acid, but a quantitatively minor turnover of a pectinase-degradable xyloglucan was found to be consistently promoted by indoleacetic acid. This was accompanied by a reciprocal increase in water-soluble xyloglucan, suggesting that indoleacetic acid induces conversion of wall xyloglucan from insoluble to water-soluble form. The results indicate a highly selective pattern of wall turnover processes with an even more specific influence of auxin.

The question of how the plant hormone IAA induces extension growth has occupied plant physiologists ever since the discovery of auxin (27). Because it was recognized early that cell enlargement requires a weakening of the cell wall (7, 8), the search for the mode of action of auxin has focused largely on the hormone's effects on cell wall polymer metabolism.

Auxin has been shown to cause increases in the activity of polysaccharidases that have the potential to degrade cell wall polymers (3, 17, 24). Such a cleavage might weaken the cell wall and lead to elongation. Radioisotopic and quantitative analytical data have been interpreted as indicating that turnover of wall polysaccharides occurs as plant tissues grow (2, 5, 6, 14, 16, 20). Certain reports indicate that such turnover is promoted by auxin (9, 18, 26).

The published work on cell wall turnover is fraught with serious questions as to the reality and, if real, the chemical and biological significance of apparent turnover effects that have been reported, due to weaknesses in the extent and reproducibility of recovery of wall constituents, to reliance on solvent extractibility as a criterion of identity, and to the use of arbitrary and unusual experimental conditions to obtain the described effects. The present study of changes in radiocomposition of ^{14}C-labeled elongating pea stem segments was undertaken in an effort to obtain an accurate picture of what happens to the monosaccharide components of already deposited wall polysaccharides, over time, when auxin promotes wall extension.

MATERIALS AND METHODS

Seeds of *Pisum sativum*, var. Alaska, were grown in vermiculite for 7 days in the dark except for brief exposures to dim red light during watering. Under red light and using a double-bladed cutter, 8-mm segments were cut from the third internode beginning 3 mm below the apical hook.

Segments were first kept for 1 hr in water at 25 C in order to deplete the tissue's internal sugar pools (1), then incubated in uniformly ^{14}C-labeled Glc (10 μc/ml, 0.1 mc/μmole, 3 ml per sample of 30 segments) on a reciprocating shaker (120 oscillations/min) for 1 hr at 25 C. The segments were then washed in water, pooled, and mixed in a large Petri dish. Samples of 30 sections each were transferred to 5-cm Petri dishes containing 3 ml of 0.05 M Glc (unlabeled) and placed on the shaker for 3 hr.

The end of this preliminary chase period was regarded as zero time for the turnover measurement, and three samples were taken for determination of initial radioactivity. The remaining tissue samples were incubated in 50 mM Glc with or without 17 μM IAA, for an additional 7 hr (the treatment period).

After incubation, segments were chilled, rinsed with cold water, ground for 2.5 min in 5 ml of cold water in iced mortars, and centrifuged at 1000g for 2 min. The supernatant and two 5-ml water washings of the pellet were pooled. The washed pellet (crude cell wall) was frozen and dried over P_2O_5 under vacuum.

Fractionation. Figure 1 summarizes the protocol employed. Treatments with 4 × crystalline *Bacillus subtilis* α-amylase (Sigma No. A 6380, 1 mg/ml in 0.05 M phosphate buffer, pH 6.9, 12 hr) and pronase (*Streptomyces griseus* protease, Calbiochem No. 53702, 1 mg/ml in 0.05 M phosphate, pH 7.9, 48 hr) were carried out at about 25 C in the presence of a

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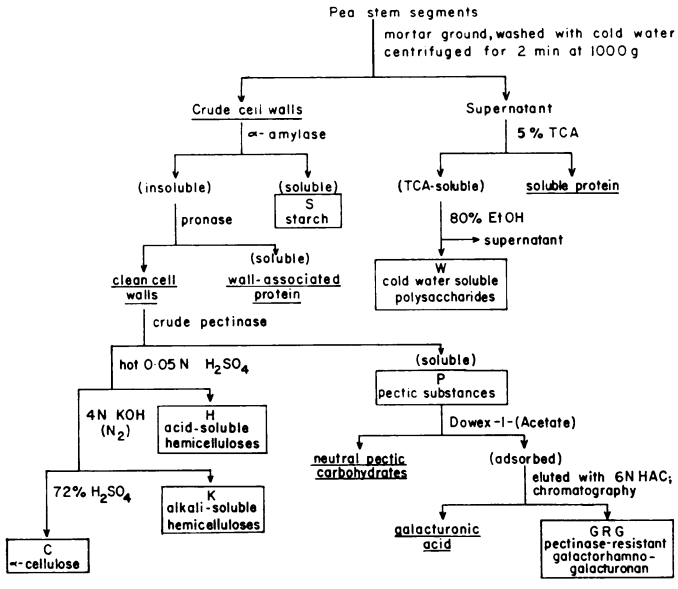

FIG. 1. Summary of preparation procedure for cell wall material and wall fractions.

drop of toluene. Pectinase treatment was carried out as described previously (22). Extraction with 0.05 N H_2SO_4 was for 5 hr at 100 C. Extraction with KOH was carried out under N_2 for 12 hr at about 25 C; the extract was neutralized by passage through Amberlite IR-120 (H^+ form). Extraction with 72% (w/w) H_2SO_4 was at 2 C for 48 hr.

All fractions, except the pectinase extract (P), were hydrolyzed in 1 N H_2SO_4 for 6 hr at 100 C, and were passed through analytical grade (Bio-Rad) Dowex-1 (acetate form) to remove SO_4^{2-}. The resin was washed with water to remove sugars and then with 6 N acetic acid to elute uronic acids; these fractions were lyophilized. Sugars were separated by descending chromatography on Whatman No. 1 paper using ethyl acetate-pyridine-water (8:2:1 by volume). Acidic components were separated using the organic phase from a mixture of 100 ml of water, 1 ml of formic acid, and 100 g of phenol. Each chromatogram was scanned with a Packard Model 7200 radiochromatogram scanner to verify the separation of components, and the peaks were eluted into vials for determination of radioactivity at 90% counting efficiency with a Beckman LS-100 liquid scintillation spectrometer.

The pectinase extract (P, Fig. 1) was passed through Dowex-1 to separate neutral and acidic fractions. The neutral material was hydrolyzed with H_2SO_4 and analyzed as described above. The acidic components were chromatographed without further hydrolysis. Scans of these chromatograms revealed two peaks. The smaller consisted of chromatographically immobile material, referred to as GRG (see "Discussion," and Fig. 1). The main peak represented over 95% of the ^{14}C-GalU[3] in all the cell wall extracts. Only small amounts of ^{14}C-GalU were obtained from fractions H and K. Because the amount of ^{14}C-GalU in these fractions was very small and did not change with time, these data are not presented.

Hydrolysis of GRG by H_2SO_4 yielded Gal, GalU, Rha, galacturonosylrhamnose, Ara, and an unknown acidic component which had a chromatographic mobility similar to 4-O-methylglucuronic acid, and which is omitted from the data presented because of lack of identification and because of its small amount (about 30% of the ^{14}C in GalU of GRG). The total amount of ^{14}C in GalU and Rha from GRG (Fig. 3) were calculated on the basis that half of the ^{14}C in galacturonosylrhamnose represents ^{14}C-GalU and the other half represents ^{14}C-Rha. Rhamnose was not obtained from any other wall fraction.

A portion of the material solubilized with α-amylase was chromatographed in *n*-propanol-acetic acid-water (8:1:2). The only chromatographically mobile radioactivity cochromatographed with maltose indicating that fraction S represents starch.

Procedural details, and representative scans illustrating the character and completeness of the chromatographic separations, were presented previously (13).

RESULTS AND DISCUSSION

Turnover of pea cell wall polysaccharides was sought in a pulse-chase experiment in which cell wall material was first labeled extensively during a 1-hr incubation of pea stem segments in ^{14}C-glucose (the pulse period). The tissue was then incubated (the chase period) in a concentration of unlabeled glucose sufficient to maintain a high rate of wall synthesis, and thus to dilute endogenous sources of radioactive sugar taken up during the pulse period.

Preliminary experiments showed that radioactivity increased at least slightly in all wall fractions for up to 3 hr after transfer to chase medium, presumably due to continued incorporation of previously formed labeled wall precursors or to incomplete isotope dilution of soluble pools or to both. Because such incorporation would obscure the occurrence of turnover processes, in the final experimental design a 3-hr chase period in unlabeled glucose was given before harvesting replicate initial samples. At this time IAA was added to tissue samples which were to receive auxin, and incubation of replicate plus- and minus-auxin samples in a chase medium containing unlabeled glucose was continued for 7 additional hours before they were harvested and worked up by the same procedure as used for the initial samples. This final 7-hr portion of the chase period is referred to hereafter as the treatment period.

Data given in Figure 2 show that IAA added to pea segments by this procedure caused a strong and typical promotion of elongation. This promotion continued for at least the 7-hr duration of the treatment period during which the test for turnover was made.

The data given in Figure 3, on changes in wall radiocomposition during the treatment period, are taken from a single experiment of the type described above. This type of experiment was actually performed four times. Figure 4 summarizes the results of all these experiments in terms of the relative change in each constituent during treatment with IAA.

The four complete experiments gave satisfyingly consistent results, with respect both to amounts of radioactivity in individual sugars among replicate tissue samples and to changes in radioactivity with time, seen in the various sugars of different extracts. We attribute this to achievement of a consistently high recovery of labeled wall components by the procedure described in "Materials and Methods." Over 99% of the radiolabel in the wall fraction was extracted by the series of steps described in "Materials and Methods," and over 80% of the label was recovered in chromatographically indentifiable components after a series of operations, each of which inevitably involved some loss. We conclude from these facts that the principal changes in radioactivity observed during the chase period are real and not due to variations in extraction or recovery.

The data show, contrary to previous reports (9, 14, 18), that turnover of wall polysaccharides is not general, but that turn-

[3] Abbreviations: GalU: galacturonic acid; GRG: galactorhamnogalacturonan.

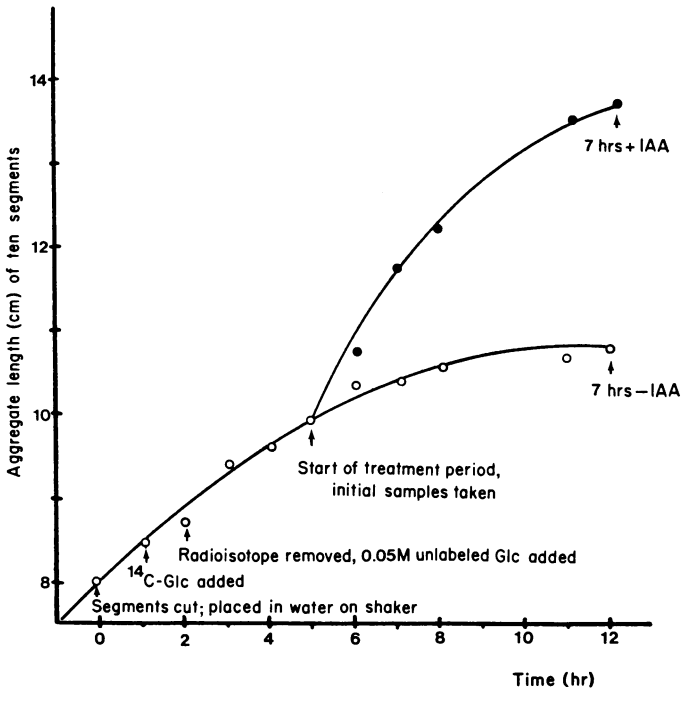

FIG. 2. Changes in tissue segment length over the course of a typical turnover experiment.

over does occur and is specific to certain monomeric components of particular wall polymers.

Galactans and galacturonans. The most extensive metabolism of a cell wall component that was indicated by the data is the decrease in ^{14}C-Gal in the material extractable by pectinase (Figs. 3 and 4). Since no compensating increases in ^{14}C-Gal occurred in other polymer fractions, it is clear that a true turnover of cell wall galactan is occurring in pea tissue. This agrees with indications of wall galactan turnover in developing tissues, that have been obtained previously both by chemical analysis (11, 21, 25) and by radioisotopic methods (9, 18).

A change similar to the above mentioned decrease in ^{14}C-Gal occurred in the acidic fraction of the material solubilized by pectinase. In addition to liberating a large amount of ^{14}C as free GalU (Fig. 3), the pectinase treatment released an acidic, chromatographically-immobile polymer that yielded neutral sugars (chiefly Gal and Rha), GalU, and galacturonosylrhamnose upon acid hydrolysis. Because of its monomeric components, this polymer was designated the galactorhamnogalacturonan (GRG) fraction. The composition of GRG is much like that of the pectic polymer complexes recently described for sycamore cell walls (10, 23), and suggests a similarity in the pectic polymer structure of pea and sycamore cell walls. The radioactivity found in GRG consistently decreased by about 25% during the treatment period (Fig. 4). This decrease was due mainly to the decline in ^{14}C-Gal in GRG to about one-half the initial level (Fig. 3); a similar decrease in ^{14}C-Ara, and some decrease in ^{14}C-Rha, in GRG also occurred.

The results just described indicate occurrence of a special kind of turnover in which neutral sugar residues are removed from an acidic heteropolysaccharide without its complete degradation (*i.e.*, of its polygalacturonic acid chains). The similarity between the percent decreases in the Gal of GRG and in the free Gal released from the cell wall by pectinase extraction suggests that both of these decreases may represent the same turnover phenomenon, or at least may share a common enzymatic basis.

In contrast to Gal, the data consistently indicated that the GalU of pea cell walls does not undergo turnover (Figs. 3 and 4). This differs from data on certain other tissues (11, 19) and contradicts earlier reports on pea cell walls (14, 18, 26).

None of the turnover processes described above gave any indication of being affected by IAA, the changes during the treatment period being very similar with or without IAA.

Arabinans. Surprisingly, a large and statistically highly significant increase of ^{14}C-Ara in the hot, dilute acid-soluble fraction (H, Figs. 3 and 4) occurred during the treatment period in all the experiments. No compensating decreases in ^{14}C-Ara occurred in any other wall fraction (except for the relatively very small decrease in ^{14}C-Ara in GRG noted above) or in water-soluble material. The nearly steady ^{14}C levels in other wall monomers discussed above, and in Ara of other fractions of the wall, is evidence that ^{14}C in endogenous metabolite pools had been thoroughly diluted during the 3-hr chase in unlabeled Glc that preceded the test for turnover, during which the increase in ^{14}C-Ara was detected. This excludes the possibility that ^{14}C-Ara is being formed from prelabeled soluble metabolites, or from the turnover of starch, because there is no apparent way in which the ^{14}C-Glc from starch could be selectively converted only into Ara units of fraction H.

One explanation for the increase in labeled arabinan is that

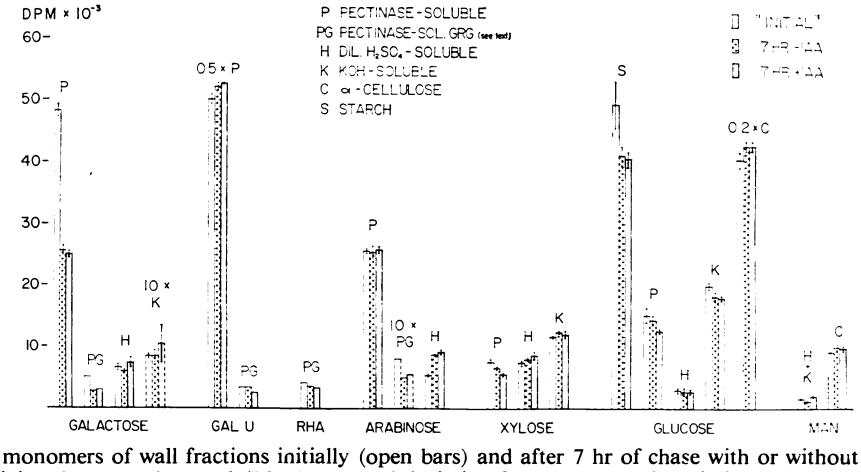

FIG. 3. Radioactivity in monomers of wall fractions initially (open bars) and after 7 hr of chase with or without IAA. Data is from a single experiment, with each bar giving the mean dpm and (I bar) standard deviation from the mean for triplicate samples (for data without deviations the triplicate samples were pooled prior to chromatographic separation of components). Note that data for very abundant and very minor components have been multiplied by a scale factor, as indicated, to show them on a common graph.

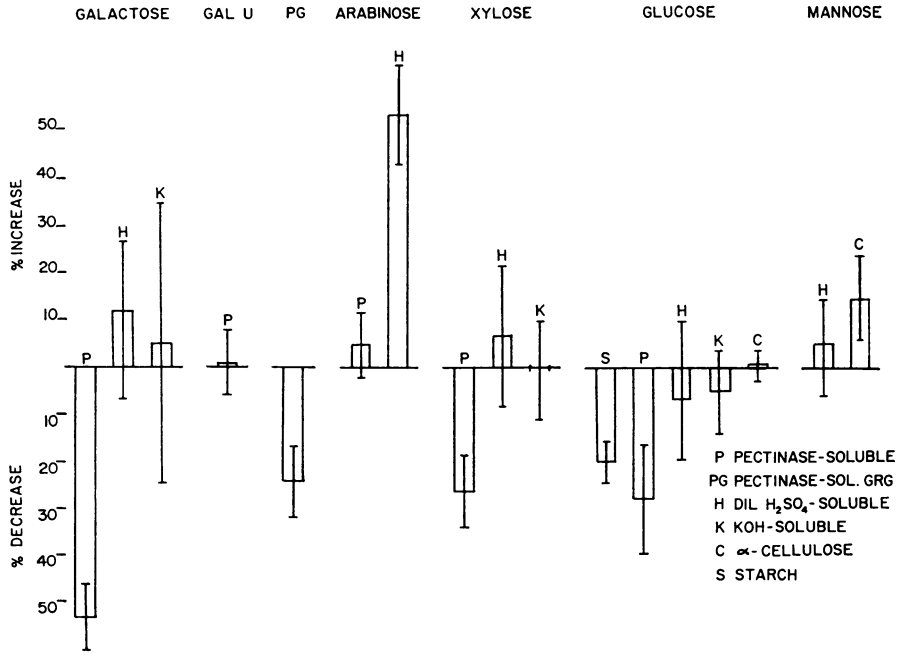

FIG. 4. Changes in radioactivity of wall constituents averaged for four complete pulse-chase experiments similar to and including Figure 3. Radioactivity after 7 hr with IAA minus initial radioactivity is expressed as a percentage of the initial radioactivity of each constituent. The standard deviation for this difference among the four experiments is given by the I symbol. Since GRG was not hydrolyzed and chromatographed in all experiments, data for change in total (unhydrolyzed) GRG are presented.

Table I. *Comparison of Changes in Neutral Sugars of Water-soluble and Pectinase-extractable Polysaccharides*

Data give the mean ^{14}C, ± standard deviation, for triplicate tissue samples at the end of the pretreatment chase period (initial) and after 7 additional hours with or without IAA.

	Gal	Glc	Ara	Xyl
	cpm			
Fraction P (pectinase extractable)				
Initial	48,250 ± 1,800	15,150 ± 1,300	25,000 ± 500	7,600 ± 400
7 hr − IAA	25,600 ± 900	14,400 ± 800	25,400 ± 1,050	6,550 ± 200
7 hr + IAA	25,100 ± 650	12,600 ± 250	26,850 ± 550	5,550 ± 250
Fraction W (water soluble)				
Initial	12,450 ± 1,400	4,000 ± 550	3,700 ± 350	2,550 ± 350
7 hr − IAA	9,900 ± 500	2,900 ± 350	2,800 ± 300	2,000 ± 150
7 hr + IAA	11,300 ± 900	5,750 ± 100	3,250 ± 400	3,850 ± 250

labeled galactan is being converted to arabinan. Such a conversion has been proposed before (12), but never demonstrated, and is consistent with our data (there is a large decrease in the ^{14}C-Gal of fraction P). However, this explanation encounters a number of difficulties. Removal of C-6 of Gal to form Ara has not been demonstrated previously and would, most likely, have to be accompanied by a contraction of the pyranose ring of Gal to the furanose ring that is common for Ara. It is difficult to envision how these changes might occur if the galactan backbone is not degraded to the monosaccharide level. On the other hand, if the galactan is degraded to Gal before its conversion, this Gal must be converted to Ara via a special route that avoids the isotope dilution occurring in the pathway(s) by which Gal and Ara are formed from Glc.

No effect of IAA on arabinan metabolism was observed.

Glucans and Mannan. Significant turnover was detected in starch (S, Fig. 4). An erroneous impression of substantial cell wall glucan turnover would have been created had contaminating starch grains not been first removed by the preliminary α-amylase extraction. Turnover of starch was not affected by auxin.

The data for glucose of α-cellulose (C, Figs. 3 and 4) gave no indication of occurrence of turnover, in spite of the presence of cellulase and auxin effects thereon in this tissue (3, 4, 15).

The data of Figure 3 suggests a 5% increase in ^{14}C-cellulose during the treatment period. This increase was barely significant statistically, and in fact was not seen in most of the experiments (Fig. 4).

The amount of ^{14}C in mannose, about 80% of which appeared in fraction C, increased during the treatment period in all experiments (Figs. 3 and 4). The average increase of about 14% of the initial value is known with only low precision, because mannose is such a minor cell wall constituent, but could well be real in light of the much more dramatic increase in ^{14}C-Ara discussed above.

Xyloglucans. All four turnover experiments indicated the occurrence of a relatively small but significant decline of ^{14}C-Glc and ^{14}C-Xyl in the pectinase-extractable fraction P (Figs. 3 and 4; Table I), especially in the presence of IAA. On the other hand, no significant change in ^{14}C-Glc or ^{14}C-Xyl occurred in acid- or alkali-soluble fractions H and K (Figs. 3 and

4). The pectinase used for extraction of fraction P evidently contained a xyloglucanase, since all the ^{14}C-Glc and ^{14}C-Xyl in fraction P was released as glucose-xylose oligosaccharides (data not shown). The glucan and xylan of fractions H and K, which are resistant to pectinase, are on this account presumed to be chemically distinct from xyloglucan P, and it is interesting that, as in the case of the galactans, the pectinase-susceptible xyloglucan P is subject to turnover *in vivo*, whereas the pectinase-resistant glucans and xylans of fractions H and K are not.

The decrease in ^{14}C-Xyl and ^{14}C-Glc in fraction P was approximately doubled by treatment with IAA (Fig. 3; Table I), this being the only detected wall turnover process that was affected by auxin. Averaged over all four pulse-chase experiments, during the treatment period ^{14}C-Glc decreased 11 ± 3.5% (standard deviation) without IAA, and 28 ± 12% in the presence of IAA. Corresponding figures for ^{14}C-Xyl are 17 ± 4% (minus IAA) and 24 ± 8% (plus IAA).

Cold Water-soluble Polysaccharides (Fraction W). These were examined initially as a possible source of ^{14}C-Ara to explain the increase in ^{14}C-Ara in fraction H, but the small change in ^{14}C-Ara (Table I) in fraction W was not sufficient to justify the postulate (Fig. 3). A small decrease in ^{14}C-Gal also occurred in fraction W. However, in the case of Glc and Xyl, ^{14}C decreased during the treatment period in the absence of IAA, but actually rose in tissues being treated with IAA (Table I). This phenomenon has been observed in numerous subsequent experiments.

The differences in ^{14}C of Glc and Xyl in fraction W between minus and plus IAA tissue at the end of the treatment period were approximately equal and opposite to the plus-minus IAA differences in these sugars in fraction P of the cell wall (Table I). This suggests that water-soluble xyloglucan is being produced from the water-insoluble cell wall xyloglucan P by a turnover process that is promoted by auxin. The alternative explanation that glucan and xylan in fraction W are being formed *de novo* from previously labeled substrate pools seems very unlikely in view of the lack of such changes in most of the numerous other polysaccharide constituents.

The suggested IAA-promoted turnover process would constitute a modification of insoluble xyloglucan without its complete degradation, since tests showed that the (alcohol-insoluble) Glc and Xyl in fraction W were indeed polymeric. A possibly similar change in solubility of galacturonan has been observed during ripening of fruits (11). It is apparent from the minus-IAA data that glucan and xylan in fraction W are subject to degradation to alcohol-soluble products that are excluded from fraction W. The question whether Glc and Xyl in fraction W are actually derived from xyloglucan P by a turnover process requires structural comparison between the respective polymers. This, and the important question whether these xyloglucan effects of IAA reflect the biochemical action of auxin on the cell wall which makes it capable of extension during cell enlargement, will be the subject of further reports.

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