Plant Physiol. (1991) 97, 551–561
0032-0889/91/97/0551/11/$01.00/0

Received for publication February 19, 1991
Accepted June 4, 1991

Tracing Cell Wall Biogenesis in Intact Cells and Plants[1]

Selective Turnover and Alteration of Soluble and Cell Wall Polysaccharides in Grasses

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ABSTRACT

Cells of proso millet (*Panicum miliaceum* L. cv Abarr) in liquid culture and leaves of maize seedlings (*Zea mays* L. cv LH51 × LH1131) readily incorporated D-[U-^{14}C]glucose and L-[U-^{14}C]arabinose into soluble and cell wall polymers. Radioactivity from arabinose accumulated selectively in polymers containing arabinose or xylose because a salvage pathway and C-4 epimerase yield both nucleotide-pentoses. On the other hand, radioactivity from glucose was found in all sugars and polymers. Pulse-chase experiments with proso millet cells in liquid culture demonstrated turnover of buffer soluble polymers within minutes and accumulation of radioactive polymers in the cell wall. In leaves of maize seedlings, radioactive polymers accumulated quickly and peaked 30 hours after the pulse then decreased slowly for the remaining time course. During further growth of the seedlings, radioactive polymers became more tenaciously bound in the cell wall. Sugars were constantly recycled from turnover of polysaccharides of the cell wall. Arabinose, hydrolyzed from glucuronoarabinoxylans, and glucose, hydrolyzed from mixed-linkage (1→3,1→4)β-D-glucans, constituted most of the sugar participating in turnover. Arabinogalactans were a large portion of the buffer soluble (cytoplasmic) polymers of both proso millet cells and maize seedlings, and these polymers also exhibited turnover. Our results indicate that the primary cell wall is not simply a sink for various polysaccharide components, but rather a dynamic compartment exhibiting long-term reorganization by turnover and alteration of specific polymers during development.

A concept of plant growth which addresses development as a continuous process must integrate both cell wall "loosening" and cell wall synthesis in the mechanics of cell expansion. Hormone-induced increases in wall extensibility are unequivocal and considered by many as the rate limiting factor in expansion (for review, see ref. 39), but cell wall synthesis is indeed necessary for continuous plant growth (3, 19, 29). Most studies concerning the biochemistry of cell expansion have not addressed growth as a continuous process, and there is a resulting lack of information about how the chemistry of the cell wall may change during normal plant development. Vanderhoef and Dute (41) observed several years ago that the growth response to auxin was sometimes biphasic, exhibiting an early burst of growth as a result of a rapid induction of wall loosening and a later steady rate of growth. The second phase of sustained growth ensued after the hormone induced tissues reorganized their entire growth machinery. The continuous growth of intact plants is more similar to this second phase. Most of the evidence indicates that auxin-induced increases in cell wall synthesis are important for this sustained growth (3, 19, 29). However, other important biochemical processes not well characterized heretofore are the changing quantity and type of polysaccharide and their selective alteration *in muro*.

Polysaccharide synthesis and metabolism can be followed by the flow and accumulation of radioactivity from sugar substrates into cellular compartments and specific polysaccharides. Although incorporation and distribution of sugars can be monitored conveniently in excised tissues, excision severely limits the time course of study and interrupts normal carbon assimilation. Alternatively, cells in liquid culture offer a convenient model to study synthesis and distribution of polymers without excision or interruption of carbon supply. However, proso millet cells (*Panicum miliaceum* L. cv Abarr), like cells of many grasses (4), exhibit little cell expansion beyond that associated with cell division, and examination of the changes in wall metabolism related to further development is not possible (15). We found that maize seedlings (*Zea mays* L. cv LH51 × LH1131) readily absorbed radioactive sugar from a solution containing a surfactant, and incorporation of the radioactive sugars into cell wall polymers peaked within 20 to 30 h. The fate of the polymers synthesized in these first few hours could be observed during growth over the next 3 d. The seedlings provided a means to assess the long-term processes of wall biogenesis and the turnover of specific polysaccharides related to development.

A consequence of the hydrolytic processes of wall loosening and *in muro* alteration of polysaccharides is the recycling of sugars. In some instances recycling of sugars results in the complete turnover of some polysaccharides or specific alteration of others. For many reasons, recycling of sugars has been difficult to assess. Several studies have employed sugars such as L-arabinose and D-galactose, because salvage pathways beginning with C-1 kinases result in formation of a few specific nucleotide-sugars (20, 34), hence specific polymers (11, 36). However, assessing the recycling of sugars is a problem because C-1 kinases salvage the very sugars that recycle, and label never completely leaves these sugars. Turnover of polysaccharides can only be determined indirectly by enrichment

[1] This work was supported by Contract DE-FG02–88ER13903 from the Department of Energy. Journal paper No. 12,881 from the Agricultural Experiment Station, Purdue University.

of radioactivity in sugars that do not recycle relative to those that do (Fig. 1). We have used arabinose as a radioactive tracer because only polymers containing arabinose and xylose become radioactive. Both arabinose and xylose are formed by pathways of nucleotide-sugar interconversion whereby UDP-Glc is oxidized to UDP-GlcA and subsequently decarboxylated to UDP-Xyl (20). A C-4 epimerase interconverts UDP-Xyl to UDP-Ara (20). Arabinose can also be incorporated into polymers through a salvage pathway catalyzed by arabinokinase to form Ara-1-phosphate and a pyrophosphorylase to form UDP-Ara (20). There is no C-1 kinase for xylose, but a C-4 epimerase converts a portion of the UDP-Ara to UDP-Xyl, so only polymers containing arabinose or xylose become radioactive with this marker sugar (11, 36).

The influence of hormones on the growth physics of dicots and grasses are relatively similar; however, the chemistry of the primary cell walls of grasses is markedly different from most other plants. The primary wall of dividing grass cells is composed of cellulose microfibrils interlaced predominantly with GAXs,[2] which are linear chains of (1→4)β-D-xylan substituted variously with *t*-Ara units at *O*-3, and *t*-GlcA units at *O*-2 of the xylosyl backbone units (5, 17). GAXs can be fractionated into polymers representing various degrees of substitution and tenacity of binding, *i.e.*, alkali extractability (5, 7). Dividing cells contain HS-GAX that disappears after cell elongation ceases, concomitant with an increase of less substituted GAX (6, 7). We proposed that a water-soluble HS-GAX was the polymer synthesized, esterified to the cell wall, and made incalcitrant through cleavage of the *t*-Ara units (5, 6, 9, 12). The present study offers additional evidence for the turnover of arabinose specifically from GAX.

Most remarkable in grasses is the synthesis of different polysaccharides during specific developmental stages. Some arabinans, found in the walls of dividing cells, are no longer made during cell expansion (6, 9). Instead, new polymers, called mixed-linkage β-D-glucans (β-D-glucans), are synthesized along with GAX. β-D-Glucans are unbranched homopolymers of glucose containing linear sections of (1→4)β-D-glucose oligomers separated by single (1→3)β-D-glucose linkages which kink the molecule. In most of the polymer, the (1→3)-kinks occur at specific intervals to link approximately 10 cellotriose units to every 5 cellotetraose units (42). These sections are linked via special regions that contain longer stretches of linear (1→4)-linked units (25). Some contiguous (1→3)-linked units may be spliced into special regions to give added flexibility to the macromolecule (27). The longer stretches of (1→4)-linked units are also significant in metabolism of β-D-glucan. An endo-β-D-glucanase, discovered in the cell walls of developing maize seedlings, can hydrolyze the β-D-glucan, but only at the longer stretches of (1→4)-linked glucose (25). Hence, hydrolysis at discrete sites might disengage the β-D-glucan from microfibrils to permit expansion. β-D-Glucan may be the "molecular thread" (9) or cell wall "glue" of the grasses analogous to xyloglucan of dicots (9, 25, 27). In this report we offer the first evidence *in vivo* that β-D-glucan not only accumulates in the walls during leaf expansion, but much of it turns over during this net accumulation phase.

Together, the proso millet cells in liquid culture and intact maize seedlings allowed the observation of long-term synthesis and distribution of polymers typical of cell division, cell elongation, and differentiation in grasses. In addition, we used

[2] Abbreviations: GAX, glucuronoarabinoxylan; HS, highly substituted; β-D-glucan, mixed-linkage (1→3,1→4)β-D-glucan; AGP, arabinogalactan-protein.

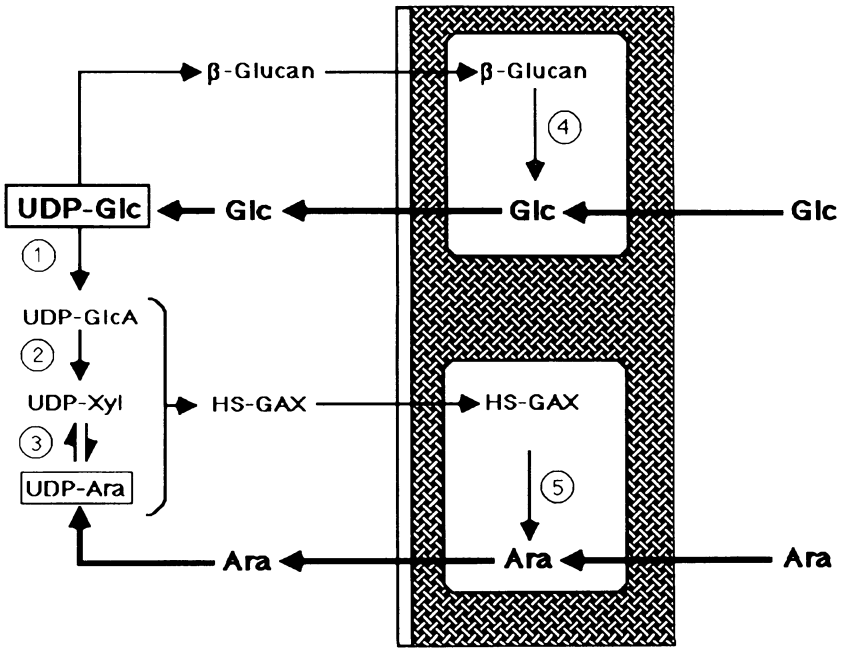

Figure 1. Representation of the pathways of uptake and recycling of arabinose and glucose from the cell wall during maize leaf development. Radioactive glucose is incorporated into UDP-Glc and used directly for synthesis of β-D-glucan. A substantial portion of the UDP-Glc pool is diverted via enzymes of the sugar-nucleotide interconversion pathways; a UDPG dehydrogenase (*1*), a decarboxylase (*2*) and a C-4 epimerase (*3*). UDP-GlcA, UDP-Xyl, and UDP-Ara are substrates used in the synthesis of HS-GAX. After the initial incorporation of glucose into β-D-glucan, continual turnover of the glucan by endo- and exo-β-D-glucanases *in muro* (*4*) results in the recycling of glucose and eventual accumulation of radioactivity in the xylosyl units of GAX which do not turnover. Radioactive arabinose is incorporated directly into UDP-Ara via a salvage pathway catalyzed by a specific C-1 kinase and pyrophosphorylase, and UDP-Xyl is synthesized by action of a C-4 epimerase (*3*). Arabinose cleaved selectively from either AGP or GAX by arabinosidases (*5*) would continue to cycle through the salvage pathway, and the turnover can be observed indirectly by slow conversion of the radioactivity into the xylosyl units of GAX which do not turnover.

gas chromatography-radiogas proportional counting to achieve capillary column separation and quantitation of mass and radioactivity in partially methylated alditol acetate derivatives. Such measurements permitted determination not only of the fate of individual sugars, but also of specific polysaccharides based on the diagnostic linkages they formed. We report on three experiments designed to assess the dynamics of polymers in the cell walls of dividing, expanding and differentiating cells of grasses.

MATERIALS AND METHODS

Plant Material

Cells of proso millet (*Panicum miliaceum* L. cv Abarr) were grown in liquid culture and have been maintained for over 5 years with subculture every 14 d (15). Cells at mid-logarithmic phase were subcultured into fresh medium containing 20 mM D-glucose and incubated an additional 11 h to ensure recovery from transfer shock. Cells had absorbed enough glucose that the medium was adjusted slightly upward to 10 mM glucose at the start of the experiments. Seeds of maize (*Zea mays* L. cv LH51 × LH1131) were soaked in running tap water overnight and sown in plastic trays filled with moist vermiculite. The plants were grown at 26°C under red fluorescent lamps (Sylvania, F40PK) for 8 days until the first leaf had expanded and the second leaf was just beginning to unfurl. Seedlings were watered every three days with a dilute nutrient solution (Miracle-Gro). Sixteen to 20 seedlings were size-selected for each experiment, and the remainder were excised at the base of the coleoptile and discarded.

Pulse-Labeling with Radioactive Sugars

For millet cells, 250 μL of 250 μM (10 μCi) L-[U-^{14}C] arabinose in water was filter-sterilized and added to 0.3 g fresh weight of cells in 10 mL of millet medium (15) in 50-mL Erlenmeyer flasks. Entire flasks of cells were harvested intermittently by filtration on a single disk of Whatman No. 4 filter paper, rinsed briefly in fresh nonradioactive medium without sugar, weighed, and frozen in liquid nitrogen. Several experiments tested various amounts of arabinose alone, combinations of glucose and arabinose, and the efficacy of different modes of chase, including filtration and resuspension in nonradioactive medium. The transfer shock was significant in these latter cases and was rejected in favor of a 100-fold dilution of label by addition of L-arabinose to 25 mM. We found that unlabeled cells treated similarly remained healthy for at least 1 d after the 3-h experiments. Results reported here were the mean of two samples for each time point in two independent pulse-chase experiments. To investigate long-term changes in sugar distribution, 1 g fresh weight of mid-logarithmic phase cells was suspended in 25 mL of medium containing 100 mM D-glucose and incubated for 12 h in 125-mL Erlenmeyer flasks. One microcurie of L-[U-^{14}C]arabinose (250 mCi/mmol) in 100 μL of water was filtered-sterilized and added to the shaking flasks. Samples in duplicate were harvested as described every day for up to 4 d.

For experiments with seedlings, 100 μCi of L-[U-^{14}C]arabinose or 250 μCi of D-[U-^{14}C]glucose were dissolved in 1 mL of 0.3% Tween 20 in water, and 50 μL of the solution was pipeted into the cup-like whorl formed by the leaves of seedlings. An ink mark was made on the outside of the first leaf to note the portion directly exposed to the solution. Two seedlings per time point were harvested by excision at the base of the coleoptile. Negligible radioactivity was found in the roots and they were discarded. The leaves were excised into pieces representing the furled portions 0.5 cm below the ligule of the first leaf, the expanding portion up to the mark denoting the original level of the pulse solution, and the expanded leaves above the mark. The sections were rinsed with water for a few seconds to remove any unabsorbed label from the surface, and the sections were frozen in liquid nitrogen. Most of the radioactivity remained in the mid-section in direct contact with the radioactive solution. Radioactivity absorbed by the basal section slowly accumulated in upper sections during the course of the experiment (data not shown). Although the three sections per seedling were analyzed separately, all results presented here were the sum of the total radioactivity absorbed and incorporated per seedling, *i.e.*, the sum of data from the three sections.

Fractionation of Material

Frozen cells and leaves were homogenized in fivefold excess ice-cold 50 mM Tes(NaOH), 50 mM NaCl, 30 mM ascorbate, pH 5.5, in a glass-glass motorized homogenizer. The walls were pelleted by centrifugation at 1200*g* for 5 min, and the supernatant was brought to 80% ethanol and incubated overnight at −20°C to precipitate soluble polymers. The cell walls and debris were washed sequentially with ice-cold 0.5 M NaCl (5×), water (2×; ambient temp), $CHCl_3$:MeOH (1:1, v/v) at 45°C for 30 min (2×), methanol (2×; ambient temperature), and water (2×; ambient temperature). Ten milliliters of DMSO were added to the pellet, and the suspension was stirred vigorously for 24 h to remove starch (13). The insoluble material was washed twice with water, suspended in water, and lyophilized.

Two to 25 mg of cell walls were extracted twice in 10 mL of each concentration of 0.1 M, 1.0 M, and 4.0 M KOH (supplemented with 3 mg/mL $NaBH_4$) under nitrogen with vigorous stirring for 1 h each. The insoluble material remaining after each extraction was pelleted by centrifugation at 2500*g*, and the supernatants of appropriate KOH concentration were combined, chilled, neutralized with glacial acetic acid, and dialyzed extensively against deionized water. Samples were lyophilized and weighed.

Enzymic Analysis of the Mixed-Linkage β-D-Glucan

Samples of the 4 M KOH fraction containing the bulk of the β-D-glucan from the cell walls were suspended in 0.8 mL of 20 mM Na-acetate, 20 mM NaCl, pH 5.5, and divided into two 0.4-mL fractions in Eppendorf centrifuge tubes. Fifty microliters of a preparation of *Bacillus subtilis* endo-β-D-glucanase (14) or a buffer blank were added to the suspensions, and the samples were incubated overnight at 37°C under a drop of toluene to inhibit bacterial growth. The endoglucanase cleaves (1→4)β-D-glucosyl linkages but only if preceded by a (1→3)-linkage (14, 24). The products released from digestion of purified β-D-glucan are mostly cellobiosyl- and cellotriosyl-

(1→3)β-D-glucose and small amounts of longer oligomers. One milliliter of ethanol was added to each of the samples, and after chilling to ice temperature, the samples were microfuged for 10 min. One milliliter of the ethanol soluble material was assayed for radioactivity, and radioactivity in β-D-glucan was estimated as the difference between digested and undigested samples.

Preparation of Polysaccharides from Sonicated Membranes

Elongated, etiolated coleoptiles were gently crushed in ice-cold 100 mM potassium phosphate buffer, pH 7.6, containing 0.25 M sucrose in a chilled mortar and pestle. The homogenate was filtered through cheesecloth, and the remaining cell wall debris was removed by centrifugation at 800*g* for 10 min. The supernatant was centrifuged at 280,000*g* for 1 h to pellet all membranes and vesicles. The pellet was resuspended in buffer and sonicated with a Branson model W185 Sonifier at 80 W for 2 min. The resuspension was centrifuged again at 280,000*g* for 1 h. The first and second supernatant liquids were brought to 80% ethanol and incubated at −20°C. The ethanol-precipitated materials and the final membrane pellet were washed three times with 80% ethanol and once with chloroform:methanol (1:1 v/v) at 45°C for 30 min. The insoluble materials were digested sequentially in 0.5 mL water with 1 unit of RNAase then 1 unit of proteinase K (Boehringer). The digests were brought to 80% ethanol, then heat killed, and washed three times with 80% ethanol. Purified polysaccharides were derivatized for sugar composition analysis as described below.

Sugar Composition and Linkage Analysis

Samples of each fraction (1–2 mg) were hydrolyzed with 2 M TFA containing 1 μmol of *myo*-inositol (internal standard) for 90 min at 120°C in a heating block. Samples were withdrawn for determination of radioactivity by liquid scintillation spectroscopy. The TFA was evaporated under a stream of nitrogen and the sugars were derivatized to alditol acetates (2) as modified by Carpita and Shea (16). Other samples were per-*O*-methylated with Li^+ methylsulfinylmethanide and methyl iodide according to Kvernheim (30) as modified by Carpita and Shea (16). Lyophilized samples (1–2 mg) were stored in a vacuum desiccator over P_2O_5 overnight. The tubes were sealed with serum sleeve stoppers, and 1 mL of anhydrous DMSO (Pierce Chemical) was added by syringe. The tubes were evacuated and sonicated for 2 h in a water bath warmed to 50°C. Nitrogen was introduced by a syringe needle with a second needle inserted as an escape valve, and 0.5 mL of 2.5 M *n*-butyllithium in hexanes (Aldrich Chemical) was added very slowly to the vigorously stirred DMSO suspension. After evaporation of much of the hexane in about 10 min, the homogeneous solution was gently stirred for 2 h until the yellowish solution began to develop a bluish tinge. Iodomethane was added drop-wise until the solution turned clear and then the remainder of 0.5 mL was added. Nitrogen flow was stopped, and samples were stirred for 1 h.

The methylated polymers were recovered after addition of water to the mixture and partitioning into chloroform. The chloroform extracts were washed five times with 10-fold excess of water, the chloroform was evaporated, and the methylated polymers were hydrolyzed in 2 M TFA for 90 min at 120°C. The sugars were then reduced and acetylated as described, and gas-liquid chromatography-electron impact mass spectrometry of nonradioactive samples was used to verify all derivative structures (16).

Methodology for gas-liquid chromatography-radiogas proportional counting has been described (12, 38). Briefly, the alditol acetates were separated by gas-liquid chromatography on a 0.75-mm × 30-m wide-bore glass capillary column of SP-2330 (Supelco, Bellefonte, PA) temperature was programmed from 170°C to 240°C at 5°C/min with a 6 min hold at the upper temperature. The partially methylated alditol acetates were separated on the same column. Temperature was programmed from 160°C to 210°C at 2°C/min and then to 240°C at 5°C/min with a 4 min hold at the upper temperature. Injector and transfer line were at 250°C with helium as the carrier gas at 5 mL/min. Separations were carried out with a gas chromatograph coupled to a Packard model 894 gas proportional counter (United Technologies, Downer's Grove, IL). Samples were injected in ethyl acetate. Helium was added directly to the column effluent at 118 mL/min for make-up gas, and N_2 sweep gas was added to the flame ionization detector to total 30 mL/min; the effluent was split 2% to the flame-ionization detector and 98% to the proportional counter. The signals were calibrated with known amounts of radioactive alditol acetate standards.

RESULTS

Incorporation of Radioactivity in Cells in Liquid Culture

Proso millet cells in liquid culture were used for study of cell wall synthesis in dividing cells without disruption of normal metabolism. Radioactivity from arabinose was absorbed quickly and incorporated into buffer-soluble and cell wall fractions (Fig. 2). Over 70% of 250 μM arabinose was absorbed from the medium in 2 h, and chase was accomplished by addition of arabinose to 25 mM. Radioactivity decreased rapidly in both the soluble sugar fraction and the buffer-soluble fraction. These decreases were closely followed by an increase of radioactivity in the cell wall (Fig. 2). Accumulation of radioactivity in the extracellular medium was less than 5% of that accumulated in the cell wall (data not shown). Using sugar composition and linkage analyses similar to that shown in Figure 3, we demonstrated that accumulation of radioactivity in the cell wall was indeed only in arabinose and xylose and mostly into GAX, confirmed by the large amounts of label in *t*-Ara, 4-Xyl, and 3,4-Xyl. Other linked arabinosyl units (2-, 3-, 5-, 2,5-, and 3,5-linked) and *t*-Xyl, perhaps from xyloglucan, constituted the remainder of the radioactive sugars. The ratio of radioactive Ara:Xyl in the cell walls was about 2.3:1, indicative of HS-GAX. However, the buffer-soluble polymers were enriched in arabinogalactan rather than GAX, indicated by an Ara:Xyl ratio of 6:1.

In another approach, a pulse of radioactive arabinose was given to millet cells, and the radioactivity was chased into nucleotide-sugar and cell wall pools by continuous flow of glucose supplied in the culture medium. Here again, most of

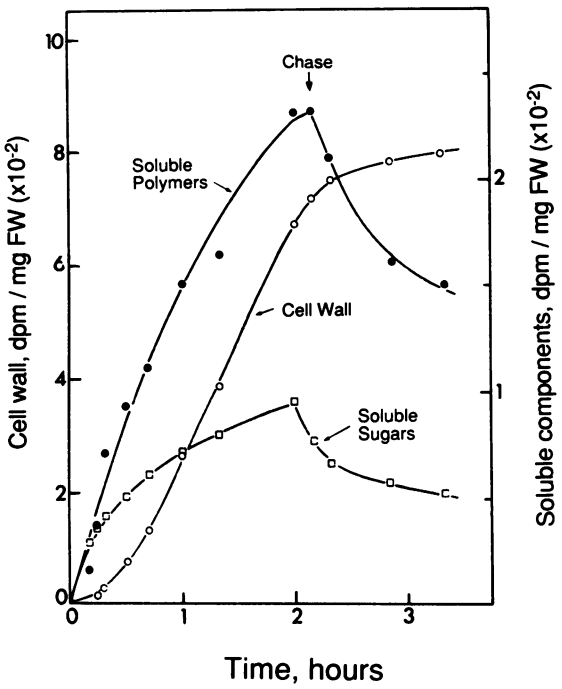

Figure 2. Incorporation of arabinose into soluble polymers of proso millet cells and transfer of radioactivity to the cell wall during chase with unlabeled arabinose. Cells were incubated in 20 mM D-glucose overnight, adjusted to 10 mM before introduction of 250 μM (10 μCi) L-[U-^{14}C]arabinose at time 0 h. After 2 h, chase was accomplished by addition of unlabeled L-arabinose to 25 mM. Cells were collected by filtration, rinsed briefly in unlabeled medium, and separated into these three major fractions.

the buffer-soluble polymers chased to the cell wall (Fig. 4) or culture medium (not shown). The soluble sugar pool decreased only slightly, indicating a metabolically inactive vacuolar pool. We were interested in assessing the relative amount of turnover from the cell wall pool, particularly from arabinose units of GAX. These estimates could be made only indirectly because of the nature of the salvage pathway (Fig. 1). Arabinose hydrolyzed from polymers and reabsorbed would simply shuttle back into UDP-Ara and UDP-Xyl. However, each cycle would result in conversion of more of the arabinose to xylose. If total radioactivity in the wall was constant and xylose did not recycle, then turnover from the cell wall would be observed by a decrease in the ratio of radioactive Ara:Xyl. In the millet cells, the ratio decreased only slightly from 2.3 to 2.2, indicating little turnover and maintenance of GAX with relatively high degrees of arabinose substitution. These data were consistent with our analysis of the proso millet GAX whose degree of substitution was essentially constant throughout the culture cycle (15). In contrast, the degree of substitution of GAX fell markedly during cell elongation in coleoptiles and leaves (6, 7). We then explored ways to follow the fate of radioactive sugars in intact seedlings during development from cell division to cell elongation.

Incorporation of Radioactivity in Intact Seedlings

We were able to follow the fate of radioactive glucose and arabinose during incubations up to 4 d after pulse-labeling. At the time of the pulse, the first leaf was about 8 cm long, and the second leaf grew to about 20 cm during the course of the experiment. During this time the mass of the cell wall increased about fourfold from 5 to 21 mg per seedling (Fig. 5A). Individual plants absorbed different amounts of label

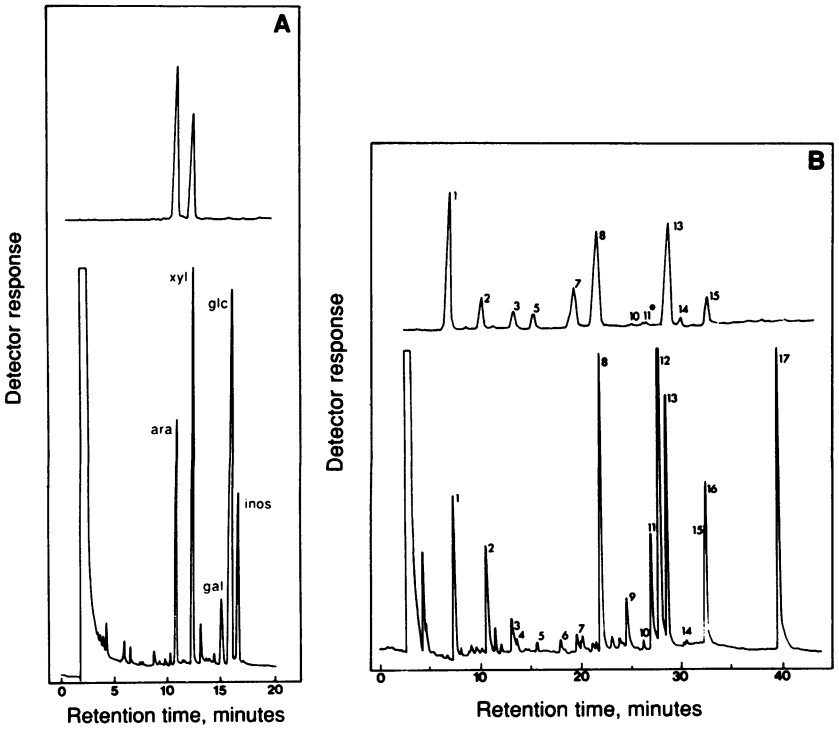

Figure 3. Incorporation of radioactivity from L-[U-^{14}C]arabinose into specific sugars and their respective linkage groups. Radiolabeled cell walls were either hydrolyzed to monosaccharide for preparation of alditol acetates or prepared for methylation analysis. Lower traces are the flame ionization detector signals for detection of mass and the upper traces are the signals from radiogas proportional counting for detection of radioactivity. A, Sugar composition of the 4 M KOH fraction from maize seedlings (mid-section) 31 h after pulse with L-[U-^{14}C]arabinose. Radioactivity from arabinose was incorporated only into arabinose and xylose. B, Separation of partially methylated alditol acetates from this same fraction. Major peaks identified in similar runs by GC-MS (16) were: 1, *t*-Ara*f*; 2, *t*-Xyl; 3, 2-Ara*f*; 4, *t*-Glc; 5, 3-Ara*f*; 6, *t*-Gal; 7, 5-Ara*f*; 8, 4-Xyl; 9, 3-Glc; 10, 2,5-Ara*f*; 11, 4-Gal (*unresolved from 3, 5-Ara*f* [labeled] and underacetylated 4-Glc [16]); 12, 4-Glc; 13, (2+)3,4-Xyl; 14, arabinitol pentaacetate; 15, xylitol pentaacetate; 16, 4,6-Glc; 17, *myo*-inositol hexaacetate (internal standard).

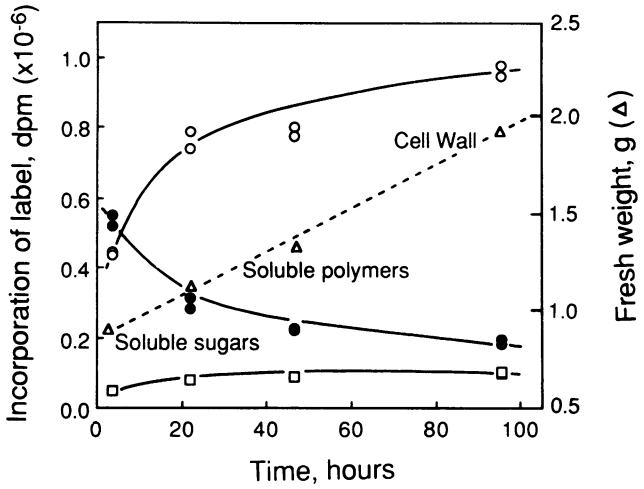

Figure 4. Incorporation of arabinose into soluble polymers of proso millet cells and transfer of radioactivity to the cell wall during chase with the normal supply of glucose. Cells in mid-logarithmic growth were transferred to fresh medium containing 100 mM D-glucose and incubated for 12 h to permit cells to overcome transfer shock. Cells were then pulsed with 250 μM (1 μCi) L-[U-^{14}C]arabinose and incubated for up to 4 d.

during the first hours, but total incorporation reached a maximum between 20 and 30 h after addition of label (Fig. 5A). An estimation of relative incorporation and subsequent metabolism was obtained by calculating the proportion rather than total amount of label in each fraction (Fig. 5B).

As in the long-term pulse of millet cells (Fig. 4), the proportion of radioactivity in soluble sugar remained fairly constant and buffer soluble pools decreased slightly. The proportion of radioactivity in the cell wall from labeling with either arabinose or glucose was greatest 20 to 30 h after the pulse then decreased slightly (Fig. 5B). Although the cell wall pool of label was essentially saturated after just 1 d, there were considerable changes in the distribution of the label in various fractions of the wall and in the individual sugars of polysaccharides. In general, there was a slow conversion of loosely bound polymers to tightly bound polymers, indicated by their relative solubility in KOH (Fig. 6). During the first few hours of labeling with glucose, the wall material extracted by dilute alkali contained relatively high proportions of radioactivity, but material extracted by 4 M KOH gained label at the expense of the 0.1 M and 1.0 M KOH extracted materials, while the proportion of radioactivity in crystalline cellulose increased relative to acetic-nitric-digestible material in α-cellulose (Fig. 6A). Modest decreases in 0.1 M and 1.0 M KOH extracted materials and apparent conversion to 4.0 M KOH extracted materials was also observed after pulse-labeling the seedlings with arabinose (Fig. 6B). These data indicate that synthesis of wall "precursor" polysaccharides is quite rapid, but their subsequent integration into the wall is a much slower process.

The distribution of radioactivity in the various sugars of wall polymers also changed in each individual fraction, and pulse-labeling with arabinose or glucose accentuated these differences. The mole% of arabinose was fairly constant during growth, but that of xylose increased slightly relative to glucose and galactose (Fig. 7A). The proportion of radioactivity from D-[U-^{14}C]glucose incorporated into glucan accounted for nearly half of the accumulated radioactivity in the first few hours but fell markedly to less than 20% by the end of the 4-d incubation (Fig. 7B). We interpret these data to mean that glucose cleaved selectively from glucan was reabsorbed by the cells and reentered the nucleotide-sugar pool for synthesis of new polysaccharides. Because the flow of carbon in the nucleotide-sugar pool is from UDP-Glc → UDP-Xyl → UDP-Ara, and because there is a greater sink for xylose than for arabinose, any glucose returning to the cytoplasmic pool from hydrolysis of glucans would enrich xylose relative to arabinose and glucose (Figs. 1 and 7). Release of radioactivity from the wall by the *B. subtilis* endoglucanase also indicated a rapid accumulation of label in β-D-glucan followed by substantial loss of radioactivity even though the mass of β-D-glucan increased about threefold (Fig. 8). Hence, the relative loss of radioactive glucose from the cell wall (Fig. 7) was traced directly to loss of radioactivity from mixed-linkage β-D-glucan (Fig. 8).

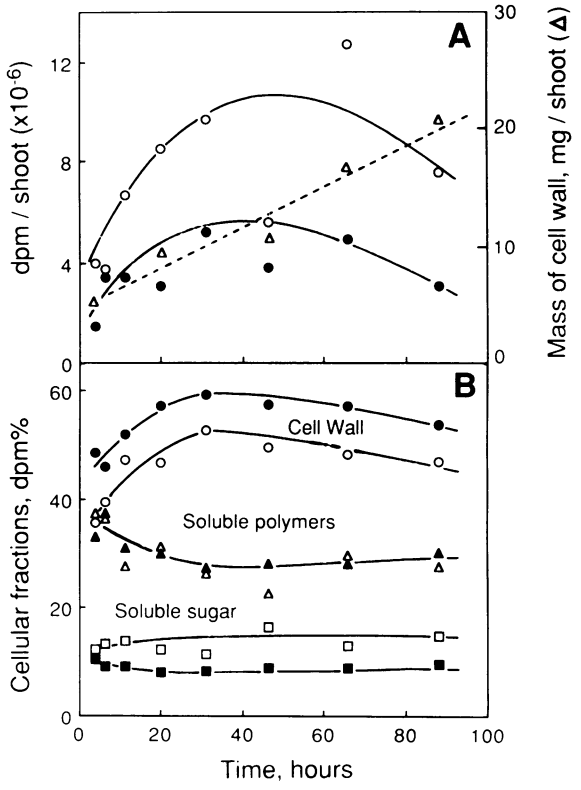

Figure 5. Incorporation of radioactivity from D-[U-^{14}C]glucose and L-[U-^{14}C]arabinose into cell wall, buffer-soluble polymers, and soluble sugar pools of maize seedlings. Seedlings with a single expanded leaf were size selected, and 5 μCi of L-[U-^{14}C]arabinose or 12.5 μCi of D-[U-^{14}C]glucose in water containing 0.3% Tween 20 were pipetted into the cup-like whorl of leaves at time 0 h. After growth, seedlings were excised at the base and rinsed free of unabsorbed label. Radioactivity in the roots and mesocotyl was negligible. A, Total radioactivity accumulated per plant. B, Proportion of total label accumulated in cell wall, soluble polymer, and soluble sugar pools. Closed symbols, L-[U-^{14}C]arabinose; open symbols, D-[U-^{14}C]glucose.

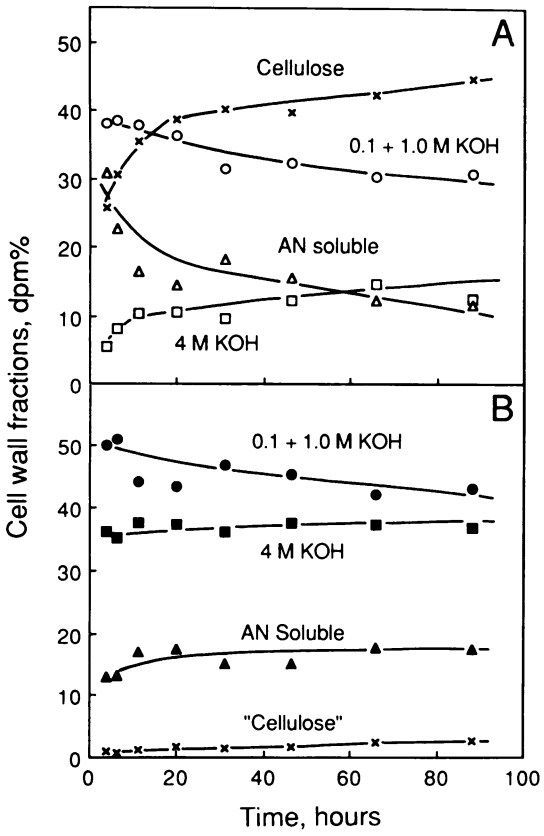

Figure 6. Distribution of radioactivity in KOH and acetic-nitric acid (AN soluble) subfractions of the cell walls of maize seedlings, pulse-labeled as in Figure 5. A, Radioactivity in fractions of seedlings pulsed with D-[U-^{14}C]glucose. B, Radioactivity in fractions of seedlings pulsed with L-[U-^{14}C]arabinose.

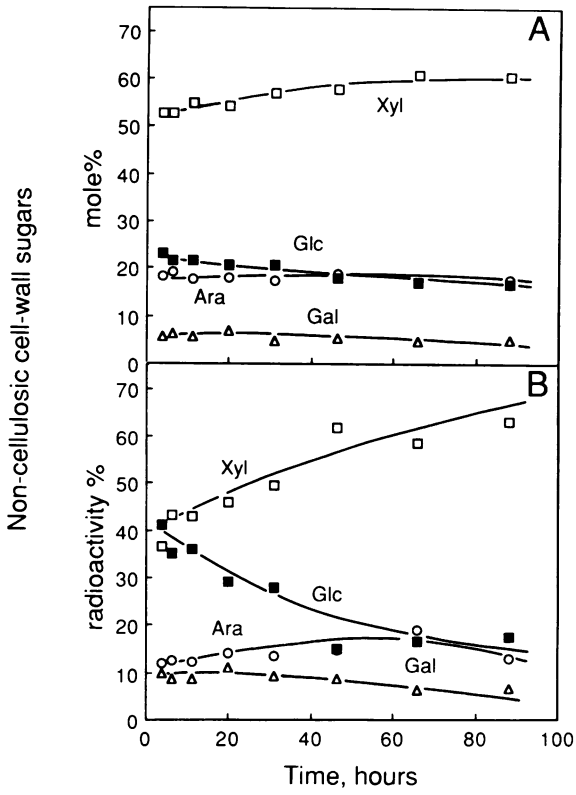

Figure 7. Distribution of mass and radioactivity of the noncellulosic sugars of the cell wall of maize seedlings, pulse-labeled with D-[U-^{14}C]glucose as in Figure 5. A, Mole percent of total noncellulosic sugar (mean of glucose- and arabinose-labeled seedlings). B, Radioactivity percent from D-[U-^{14}C]glucose.

In contrast to labeling with D-[U-^{14}C]glucose, the flow of carbon from labeling with L-[U-^{14}C]arabinose was UDP-Ara → UDP-Xyl through a salvage pathway. Initially, the interconversion of arabinose to xylose resulted in a nearly equal proportion of radioactivity in cell-wall arabinose and xylose (Fig. 9A). However, as exogenous arabinose was incorporated into the cell wall (Fig. 5B), radioactivity in arabinose decreased relative to that in xylose (Fig. 9A). The decrease in the Ara:Xyl ratio was greatest in the 4 M KOH fraction of the cell wall and less pronounced in the 1 M KOH fraction. The 0.1 M KOH fraction maintained high ratios of Ara:Xyl, indicative of HS-GAX, throughout the pulse (Fig. 9B). Methylation analysis with radiogas proportional counting as illustrated in Figure 3B verified that the ratio of the specific activities of 3,4-Xyl:4-Xyl in GAX in the 4 M KOH fraction was initially 1.4:1 and fell concomitantly to 1.2:1 by 31 h and to 1:1 by 88 h. These data indicate that loss of *t*-Ara from the xylosyl *O*-3 of GAX was indeed responsible for the change in the ratio of labeled Ara:Xyl.

Buffer-Soluble Polysaccharides

The buffer-soluble polymers in both proso millet cells and maize seedlings were likely the precursor polysaccharides of

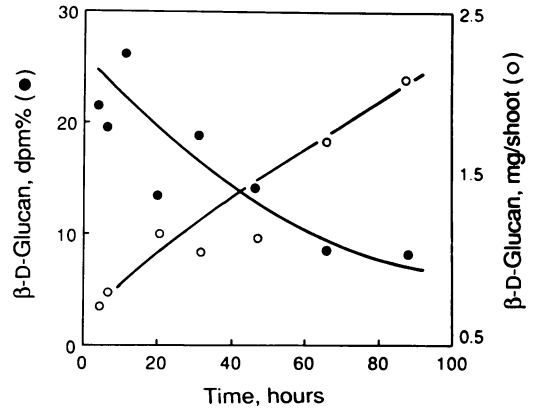

Figure 8. Radioactivity and mass of cell wall β-D-glucan in the 4 M KOH fraction of maize seedlings, pulse-labeled with D-[U-^{14}C]glucose as in Figure 5. Radioactivity was released specifically by digestion of the mixed-linkage β-D-glucan with the *B. subtilis* endo-β-D-glucanase. Mass of β-D-glucan was determined by quantitative linkage analysis as described elsewhere (16).

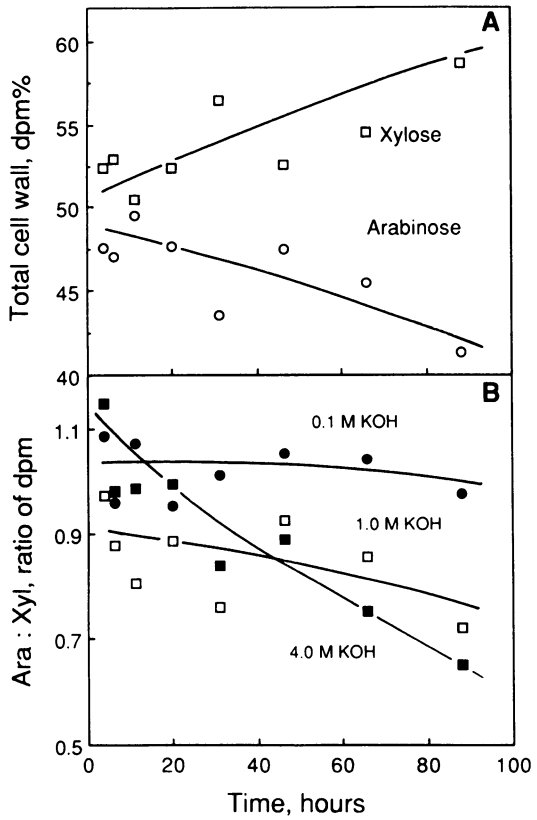

Figure 9. Changes in the ratio of radioactive arabinose to xylose in noncellulosic polymers of the cell wall, pulse-labeled with L-[U-^{14}C] arabinose as in Figure 5. A, Percent of total radioactivity in arabinose and xylose in total noncellulosic cell wall sugar. B, Ratios of radioactivity in arabinose and xylose calculated for individual KOH fractions of the cell wall. Values are from integration of the signals from radiogas proportional counting of alditol acetates as described in Figure 3A.

the cell wall and other polymers associated with internal membranes. To investigate these possibilities, we prepared soluble and membrane-associated polysaccharides from developing maize coleoptiles and determined their monosaccharide composition. Membranes and vesicles were sedimented by centrifugation and resuspended in buffer then sonicated to release only entrapped polysaccharides. After sonication, the membranes that sedimented contained much higher amounts of glucose, perhaps from glucan rendered insoluble by sonication, and mannose, a sugar expected from intrinsic *N*-linked glycoproteins (Table I). More than 90% of the polysaccharides recovered were initially in the soluble fraction, and sonication released additional material of a similar sugar composition (Table I). We interpret this to mean that buffer-soluble polymers were contained in very labile secretory vesicles and most of the vesicles were disrupted during homogenization. The high proportion of arabinose and galactose (Table I), and the detection of mostly 3-, 6-, and 3,6-Gal units and *t*-Ara units typical of type II AGP (21), indicated that AGP comprised the bulk of buffer-soluble polymers. However, AGP comprised an exceptionally small amount of the primary walls of maize seedlings and was associated primarily with a pectic fraction containing polygalacturonic acid, rhamnogalacturonan, and HS-GAX (10). Therefore, AGP in the buffer-soluble fraction was either a static pool in the cytoplasm or a pool of polymer-laden vesicles that turnover rapidly after emptying their contents at the cell wall.

When maize seedlings were labeled with D-[U-^{14}C]glucose, over 30% of the radioactivity of soluble polymers was initially in glucose (Fig. 10). The radioactivity disappeared from glucose with longer incubations, and arabinose and galactose combined for much of the remainder of the radioactive sugars. Because the proportion of radioactivity in the soluble polymers remained fairly constant (Fig. 5B), transfer of radioactive glucose in glucan to the cell wall and subsequent return of glucose would result in slow enrichment of radioactivity in arabinose and galactose as long as the AGPs remained the predominant polymer synthesized and loaded in secretory vesicles. Similarly, the buffer-soluble polymers were relatively poor in xylose, indicating that the GAX (HS-GAX) was a small portion of the total polysaccharide. Return of arabinose to the soluble pool after cleavage from HS-GAX would concentrate in the AGP rather than GAX, and high ratios of Ara:Xyl were maintained (Fig. 10). Accurate estimation of the extent of AGP turnover in seedlings was not possible because C-1 kinase salvage pathways are active for both arabinose and galactose (20).

DISCUSSION

Kinetics of Labeling and Cell Wall Turnover

There were substantial differences between cells in liquid culture and seedlings in the kinetics of pulse-labeling. When liquid cultures of proso millet cells were exposed to radioactive arabinose in the first and second experiments, the cells absorbed it within minutes and there was little delay before the pool of buffer-soluble polymers was labeled (Figs 2 and 4). A short delay was observed before radioactivity appeared in the cell wall fraction, indicating that synthesis of polysaccharides occurred within minutes, but secretion and integration of polymers into the cell wall required about 30 min (Fig. 2). When radioactivity in these cells was chased with 100-fold excess unlabeled arabinose, the buffer-soluble polymers were quickly chased to the cell wall (Fig. 2). In the second experiment using proso millet cells, a pulse of arabinose was also chased from buffer soluble polymers to the cell wall, this time by the normal supply of glucose. However, the chase time was extended compared to the first experiment and radioactivity in the cell wall continued to rise 4 d after the pulse (Fig. 4). In the third experiment with maize seedlings the time course of labeling was similar to that in the second experi-

Table I. *Distribution of Sugars in Buffer-Soluble and Membrane-Associated Polysaccharides from Maize Coleoptiles*

Fraction	Ara	Xyl	Man	Gal	Glc
Supernatant [12,600]^a	23	13	9	32	21
Sonication soluble [1000]	30	14	6	37	10
Membrane [90]	18	22	18	11	28

^{a} Total nanomoles recovered.

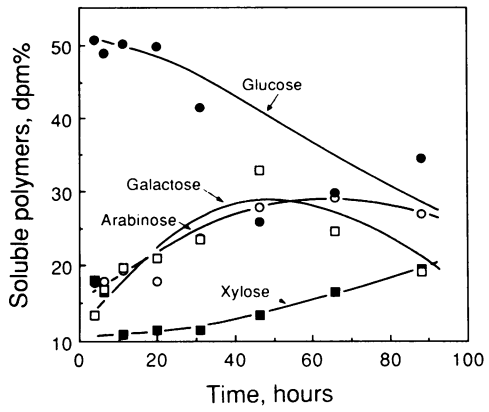

Figure 10. Changes in the distribution of radioactivity in the sugars of buffer-soluble polymers of maize seedlings, pulse-labeled with D-[U-^{14}C]glucose as in Figure 5. Values are from integration of the signals from radiogas proportional counting of alditol acetates as described in Figure 3A. Symbols: (●) glucose; (○) arabinose; (□) galactose; (■) xylose.

ment; radioactivity from glucose and arabinose was chased from buffer-soluble to cell wall polymers in about 20 to 30 h (Fig. 5B). However, radioactivity in the cell wall of maize seedlings actually decreased after 30 h. In the latter two experiments, there was a substantial pool of buffer-soluble polymers that did not appear to chase to the cell wall. However, kinetic analysis of the distribution of radioactivity in the individual sugars of these fractions showed that they were a dynamic population whose turnover was masked by reintroduction of labeled sugars from the cell wall.

Foremost in discussion of the kinetics of pulse-labeling of the maize seedlings was the observation that the mass of leaves increased fourfold during the same time that the total amount and proportion of label in the wall decreased (Fig. 5, A and B). This slight decrease in proportion was traced to a selective loss of label in arabinose and glucose relative to that in xylose (Figs. 7 and 9). These data indicate significant turnover of specific polymers that would eventually return monosaccharides to the cytoplasmic pool. Radioactive glucose from β-D-glucan (Fig. 8) and radioactive arabinose from the HS-GAXs and AGPs (Fig. 9) constituted a major portion of the polymers that turn over. The radioactive glucose hydrolyzed from wall β-D-glucan would be siphoned off continually into other sugars through the interconversion of nucleotide sugars (Fig. 1). Likewise, label from arabinose cleaved from GAX or AGP would gradually be transferred to xylose through the salvage pathway and the C-4 epimerase. Such a selective turnover of arabinose was indicated by the observation of a substantial change in the ratio of Ara:Xyl of certain wall fractions well after maximum accumulation of total radioactivity. High ratios of radioactive Ara:Xyl were maintained in the cytoplasmic pool of polymers because of the predominance of AGP in cytoplasmic polymers (Fig. 10).

Turnover of the AGPs

Turnover of the buffer-soluble polymers was striking in the cells in liquid culture (Figs. 2 and 4), whereas the proportion of label in buffer-soluble polymers of the seedlings was less pronounced (Fig. 5B). The high proportion of arabinose and galactose in polymers of the buffer soluble pool (Table I) and an enrichment of radioactivity in arabinose and galactose from D-[U-^{14}C]glucose (Fig. 10) indicated that AGPs were the predominant molecules synthesized in the buffer soluble pool. The type II AGPs are well known secretory molecules of cells in liquid culture (21) including cereals (1). One might expect that a large portion of the turnover of the buffer-soluble pool observed in the millet cells would be a result of secretion of the material to the extracellular medium, but the quantity of label lost from the buffer-soluble pool was about the same as that accumulated in the cell wall (Figs. 2 and 4) and less than 5% of the label was recovered in polymers of the extracellular medium (data not shown). Even though the secretory vesicles of cereal cells contained as much as 70% AGPs (Table I), the AGPs constituted only 10 to 20% by weight of accumulated polysaccharide at stationary phase of liquid culture (15). The AGPs were an even smaller portion of the total polysaccharides of cell walls of seedlings (10). Given that (a) AGPs were the major component of the buffer-soluble polysaccharides, (b) the amount of AGP that actually accumulated in the cell wall was very small (10), and (c) the rates of turnover of label from the AGP-rich buffer-soluble pool during pulse-chase were extremely rapid (Fig. 2), then a substantial portion must turn over at the cell wall with its sugars returned to the cytosol for synthesis of new polymers.

Various lines of circumstantial evidence also support a turnover cycle for AGPs. Activities of both β-D-galactosidase and α-L-arabinosidase are substantial and are sometimes correlated with growth (26, 31, 40). As mentioned, C-1 kinases specific for arabinose and galactose ensure rapid recycling into the nucleotide-sugar pool (20). Hydroxyproline can also be converted back to proline in grasses (18), indicating a turnover of hydroxyproline-rich proteins. Although turnover of extensin during growth has been postulated, the AGP peptides are more likely the candidates for turnover in the extensin-poor grasses. We have suggested that AGPs may serve some role in the secretion of newly synthesized cell wall polymers, perhaps as shuttle molecules that bind to polymers to render them soluble in Golgi vesicles until they arrive at their proper sites of assembly at the cell surface (12). Although this is possible, we await more than circumstantial evidence to support such a role.

Changes in Degree of Substitution of GAX

Discovery of a transient HS-GAX that was replaced by a less substituted GAX in rapidly elongating maize coleoptiles indicated that the HS-GAX may be the polymer synthesized and exported, and that other GAX of lower degrees of substitution arose by hydrolysis of the arabinosyl units (6, 7, 9, 12). Data to support this hypothesis include: the waning of synthesis of HS-GAX upon termination of elongation in coleoptiles (6, 7), the disappearance of HS-GAX in mature leaves (7), and the accumulation of GAX with low degrees of substitution in coleoptiles and mature leaves (6, 7). Also, pulse-chase labeling studies of excised maize coleoptiles showed that a small amount of label turned over from the HS-GAX fraction to other GAX (5). However, wall synthesis was im-

paired after 12 h of incubation of the excised tissues, and further chase could not be observed.

Our experiments with intact seedlings add further support to the hypothesis that arabinose was hydrolyzed from GAX to yield relatively unsubstituted xylans capable of tight hydrogen-bonding to other matrix polymers. Radioactivity was initially loosely bound and became more tightly bound upon further growth (Fig. 6). Also, the ratio of Ara:Xyl in the more tenaciously held fractions fell markedly (Fig. 9B) well after the maximum incorporation of radioactivity in the cell wall (Fig. 5, A and B). Using methylation analysis with radiogas proportional counting as illustrated in Figure 3B, we verified that loss of *t*-Ara from the xylosyl *O*-3 of GAX was indeed responsible for the change in the ratio of labeled Ara:Xyl.

Loss of substitution increases the ability of the GAX to hydrogen bond to cellulose (7), and data presented here support the hypothesis that loss of the *t*-Ara units also results in a tighter integration of GAX into the wall matrix. However, part of the increase in tenacity of binding is also a result of phenolic crosslinks (7, 8). Ferulic acid monomers are the most abundant of the hydroxycinnamic acid components (8), and most of the GAX is held by etherified units rather than diferulic acid (8, 37). It is important to know exactly how loss of arabinose units and the crosslinking of GAX by phenolic substances is coordinated during later stages of expansion. Ferulic acid is attached on arabinosyl units of GAX (28), but, ironically, the amount of ferulic acid increases markedly after cell expansion just at the time when marked decreases in arabinosyl substitution occur (6, 8, 35). How this special metabolism relates to developmental modification of GAX during cell expansion remains in question and should be examined more closely.

Turnover of Mixed-Linkage β-D-Glucan

Although mixed-linkage β-D-glucan is one of the more dynamic polymers of grass species, there is considerable argument concerning the direct involvement of this polymer in cell elongation. Analogous to the paradigm of xyloglucan in the Dicotyledonae, one hypothesis has been put forward that β-D-glucan interlaces the cellulose microfibrils and phytohormones induce hydrolysis of the β-D-glucan to facilitate cell expansion. Evidence to support this hypothesis includes the selective loss of glucose from walls of excised sections of coleoptiles (32) and the accelerated loss of glucose with auxin treatment (33). Huber and Nevins (24) have shown that the loss of glucose was specifically from hydrolysis of β-D-glucan. However, unlike the hydrolysis of xyloglucan, auxin-induced hydrolysis of β-D-glucan was not accelerated in isolated walls *in vitro* (25), and accumulation rather than hydrolysis was more closely correlated with the rate of elongation in intact tissues (6, 22). These two observations do not support the hypothesis that hydrolysis of β-D-glucan is an important component in the growth of grasses. Xyloglucan is a constitutive polymer in the walls of dicots, and its hydrolysis and subsequent reduction in proportion of the wall can sometimes be observed (23). However, β-D-glucan is synthesized only during cell expansion and may approach one-third of the entire hemicellulose fraction (6). Therefore, the net amount of glucan in the wall may inaccurately reflect its rate of hydrolysis. More recently we showed that β-D-glucan accumulated asymmetrically in the more rapidly growing lower half of gravistimulated oat pulvini, and this accumulation correlated closely with extensibility (22). In the special case of gibberellin-deficient dwarf maize, hydrolysis of a portion of the β-D-glucan was suspected. Mixed-linkage β-D-glucan accumulated in leaves of gibberellin-deficient dwarf maize, but the proportion decreased after treatment with gibberellin, concomitant with a stimulation of growth and increases in specific activity of a cell wall endo-β-D-glucanase (14). Once elongation of leaves ceased, the amount of β-D-glucan decreased markedly (14).

In the intact maize seedlings, the mass of β-D-glucan per leaf increased about threefold despite a decrease in the proportion of radioactivity during leaf expansion (Fig. 8). Because the proportion of radioactivity in the glucan decreased well after the peak of incorporation into the cell wall, and the specific activity of glucose relative to xylose decreased as well, there was an indication that the radioactive pool cycled through the nucleotide sugar interconversion pathways more than once. Our data provide the first evidence for turnover of the mixed-linkage β-D-glucan *in vivo* during development (Fig. 8). How cellulose and β-D-glucan are organized, and how the synthesis and degradation of β-D-glucan is balanced to meet the physical requirements for cell expansion remain unclear.

SUMMARY

Cells in liquid culture and intact seedlings are able to absorb radioactive sugar precursors for synthesis of cell wall polymers. Our experiments indicate that (a) there exists a system for turnover of AGPs from the buffer-soluble pool distinct from cell wall metabolism; (b) there is substantial re-organization of the GAX polymers, including hydrolysis of a substantial portion of their terminal α-L-arabinosyl units, and (c) the mixed-linkage β-D-glucan polymers exhibit marked turnover even though there is net accumulation during growth of the leaves.

ACKNOWLEDGMENT

Technical assistance by Anna Olek is greatly appreciated.

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