Inositol Metabolism in Plants. V. Conversion of Myo-inositol to Uronic Acid and Pentose Units of Acidic Polysaccharides in Root-tips of Zea mays[1]

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Abstract. The metabolism of *myo*-inositol-2-^{14}C, D-glucuronate-1-^{14}C, D-glucuronate-6-^{14}C, and L-methionine-methyl-^{14}C to cell wall polysaccharides was investigated in excised root-tips of 3 day old *Zea mays* seedlings. From *myo*-inositol, about one-half of incorporated label was recovered in ethanol insoluble residues. Of this label, about 90 % was solubilized by treatment, first with a preparation of pectinase-EDTA, then with dilute hydrochloric acid. The only labeled constituents in these hydrolyzates were D-galacturonic acid, D-glucuronic acid, 4-O-methyl-D-glucuronic acid, D-xylose, and L-arabinose, or larger oligosaccharide fragments containing these units. Medium external to excised root-tips grown under sterile conditions in *myo*-inositol-2-^{14}C contained labeled polysaccharide.

When label was supplied in the form of D-glucuronate, the pattern of labeled uronic acid and pentose units in cell wall polysaccharides resembled that obtained from labeled *myo*-inositol, indicating that both substances were metabolized along a common path during polysaccharide formation, and that methylation occurred at a step subsequent to uronic acid formation. When label was supplied in the form of L-methionine-methyl-^{14}C, 4-O-methyl-D-glucuronic acid was the only labeled monosaccharide component that survived enzymatic or acid hydrolysis.

Zea mays endosperm, a known source of phytin, developed maximal phytase activity after the third day of germination. Results obtained here suggest that *myo*-inositol released by hydrolysis of phytin represents the initial precursor of a normal, possibly predominant pathway for the formation of uronic acids in plants.

Galacturonic[3], glucuronic[3], and 4-O-methyl-glucuronic acid[3] constitute the 3 major acidic glycosyl components of polysaccharides in cell walls of higher plants. Galacturonic acid, usually in the form of its methyl ester is found primarily in pectic substances, while glucuronic acid and 4-O-methyl-glucuronic acid, their carboxyl groups unsubstituted, occur as non-reducing branches attached to xylan or arabinoxylan chains. Earlier studies on the biosynthesis of pectic substances in which *myo*-inositol was utilized to generate uronic acid precursor indicated that possibly all uronsyl units of cell wall polysaccharide are derived from this cyclitol. Evidence supporting this view was obtained in some preliminary experiments on the biosynthesis of glucuronic acid and related acidic polysaccharides in which *myo*-inositol-2-^{14}C was applied as a dilute aqueous solution to root hairs of intact 2 day old *Hordeum* (barley) seedlings. A rapid incorporation of label into acidic polysaccharide, especially so-called hemicellulose B (16), an alkali-extracted, ethanol insoluble mixture of polysaccharides rich in acidic xylans was observed (3). After 32 hours as much as 80 % of applied ^{14}C was recovered in ethanol insoluble residues, much of this as pentose units, xylose[3] and arabinose[3], which were found to be labeled exclusively in carbon 5. This specific pattern of labeling is similar to patterns previously obtained from *Fragaria* (strawberry) and *Petroselinum* (parsley) tissues labeled with *myo*-inositol-2-^{14}C and indicates that *myo*-inositol was oxidatively cleaved in a stereospecific reaction to form glucuronic acid-5-[14]C. Subsequent metabolism of this product resulted in a decarboxylated intermediate that was utilized for biosynthesis of cell wall polysaccharides (10).

The present investigation has been undertaken to study processes responsible for formation of uronic acid and pentose units in acidic polysaccharides of root tips from 3 day old *Zea mays* (corn) seedlings. A preliminary report on 1 portion of this work dealing with the conversion of *myo*-inositol-2-^{14}C to 4-O-methyl-glucuronic acid units of cell wall polysaccharides in *Zea mays* root tips has appeared (30)[4]. Another paper, in which the isolation and identification of labeled constituents

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[3] Regarding these compounds: glucuronic acid refers to D-glucuronic acid, 4-O-methyl-glucuronic acid to 4-O-methyl-D-glucuronic acid, xylose to D-xylose, arabinose to L-arabinose, galacturonic acid to D-galacturonic acid, glucose to D-glucose, galactose to D-galactose, rhamnose to L-rhamnose, and methionine to L-methionine.

in the soluble portion of *Zea* root tips labeled with *myo*-inositol-2-¹⁴C is reported, is in preparation (J. Deshusses and F. Loewus, unpublished).

Materials and Methods

Germination. The *Zea mays* grain used in this study, Cornell M-4 hybrid, lot N-3971, was purchased from Agway, Inc., Syracuse, New York. Seeds grown in moist sphagnum moss were presoaked in distilled water (8 hr) and then germinated (64 hr, 28°, dark). Seedlings grown under sterile conditions were germinated (72 hr, 28°, subdued light) from seeds which had been rinsed briefly in 95 % ethanol, soaked in 2.5 % (w/v) sodium hypochlorite for 15 minutes, rinsed with sterile distilled water, and then sown, embryo-side up, in sterile petri dishes containing a layer of 0.5 % (w/v) Ionager (Consolidated Laboratories, Inc., Chicago Heights, Illinois). Root tips (1 cm) were excised from primary roots with a surgical blade while suspended in a thin film of sterile water on a glass plate.

Introduction of Labeled Compound. A selected number of root tips (usually 60) was added to a solution of labeled compound in distilled water in the outer well of an Erlenmeyer flask (50 ml) equipped with a glass center well. After adding 1 N potassium hydroxide (0.5 ml) to the center well, the flask was sealed with a rubber stopper and shaken (100 oscillations per min, 28°, subdued light) on a rotary shaker. At intervals, aliquots were removed from both wells to measure ¹⁴C uptake by the root tips and ¹⁴C content of respired carbon dioxide.

Fractionation of Labeled Ethanol Insoluble Residue. Root tips were recovered from the incubation medium by filtration, carefully rinsed to eliminate all traces of labeled medium, and then ground up in 80 % (v/v) ethanol with the aid of a motor-driven glass homogenizer. Ethanol insoluble residue was washed repeatedly with fresh portions of 80 % ethanol to remove all traces of soluble ¹⁴C, with 100 % ethanol, with ether, and then dried. Air-dried residue, referred to as ethanol insoluble residue, was used in subsequent fractionations.

Although some of the first experiments with labeled *myo*-inositol involved fractionation procedures used previously in a study of cell wall polysaccharide from *Acer* cell cultures (27), later experiments, including all that were performed with sterile root tips, followed a simple 2 step procedure involving an enzymatic hydrolysis followed by a dilute acid hydrolysis. The enzyme preparation used for the first step was a crude pectinase of commercial origin (Pectinol R-10 concentrate, Rohm and Haas Company); a preparation that contained a broad spectrum of hydrolytic activities including the ability to de-esterify pectin, cleave the glycosidic bond of some aldobiouronic acids, and hydrolyze pentosans and certain hexosans as well as pectin (21).

In a typical experiment, ethanol insoluble residue from 60 root tips (25 mg) was suspended in a solution of 0.2 % (w/v) pectinase and 0.1 % (w/v) disodium EDTA (10 ml), pH 4.5, incubated for 6 hours at 37°, and then centrifuged at 1000 × *g* to sediment pectinase-resistant residue. The residue was resuspended in water (5 ml) and recentrifuged to remove further traces of soluble ¹⁴C. The washed pectinase-resistant residue was then resuspended in 1 N hydrochloric acid (5 ml) and hydrolyzed (0.5 hr, 121°, sealed tube). Residue resistant to acid hydrolysis was removed by centrifugation. Based on incorporation of *myo*-inositol-2-¹⁴C into uronic acid and pentose units, the combined enzyme-acid hydrolysis procedure solubilized about 90 % or more of the acidic polysaccharide present in ethanol insoluble residue.

Separation and Identification of Hydrolysis Products. Acid hydrolyzates were first evaporated to dryness to remove hydrochloric acid. Then each hydrolyzate, acidic or enzymatic, was passed through a column of Dowex 50 H⁺ exchange resin. Effluents from this column were transferred to a column of Dowex 1 formate (0.9 × 15 cm). Effluent of the latter contained neutral hydrolysis products, primarily glucose, galactose, rhamnose, arabinose, and xylose. Acidic constituents were eluted subsequently with a dilute formic acid gradient as described previously (27). The uronic acid content of each fraction was measured by a modified carbazole procedure (6). Further separation of constituents in the neutral effluent and in pooled fractions of the acidic eluate was accomplished by paper chromatography (30) with solvent A, ethyl acetate-pyridine-acetic acid-water (5:5:1:3, v/v); solvent B, ethyl acetate-water-acetic acid-formic acid (18:4:3:1, v/v); solvent D, benzene-*n*-butanol-pyridine-water (1:5:5:3, v/v); and solvent F, ethyl acetate-pyridine-water (8:2:1, v/v) and by paper electrophoresis in 0.1 M ammonium formate (pH 3.8, 2 hr, 20 volts/cm) with picric acid as a marker. Paper electrophoresis separated glucuronic acid and 4-O-methyl-glucuronic acid from galacturonic acid but did not resolve glucuronic acid and 4-O-methyl-glucuronic acid. The latter pair were readily separated by solvents A and B (30). Glucuronic acid was identified chemically by reduction with sodium borohydride to L-gulonate, lactonization to L-gulono-γ-lactone, and conversion to L-gulonamide. Galacturonic acid was identified chemically by reduction with sodium borohydride to L-galactonate, lactonization to L-galactono-γ-lactone, and conversion to L-galactonamide (18). Chemical identification of 4-O-methyl-glucuronic acid was reported earlier (30).

Labeled Compounds. *myo*-Inositol-2-¹⁴C was a commercial sample prepared by chemical synthesis (9). A radioactive impurity amounting to about 10 % of the ¹⁴C present was removed by passing an aqueous solution of the sample through Dowex 50 H⁺ resin. After purification, an aliquot of *myo*-inositol-2-¹⁴C was converted to its trimethylsilyl

derivative and injected on a gas chromatographic column. A single peak with the same retention volume as authentic hexakis-trimethylsilyl myo-inositol (15) was observed. Effluent gases from the chromatograph, monitored for radioactivity with a flow counter, contained a single radioactive peak coincident with the appearance of the myo-inositol derivative.

D-Glucuronic acid-1-^{14}C, a commercial sample, contained several major acidic impurities which were separated from glucuronic acid by gradient elution of the sample from a column of Dowex 1 formate resin. The purified product gave a single radioactive spot on paper chromatography in solvent A and in methyl ethyl ketone-n-butanol-acetic acid-water (3:2:2:2, v/v).

D-Glucuronic acid-6-^{14}C was prepared from D-glucurono-γ-lactone-6-^{14}C and purified as described above.

L-Methionine-methyl-^{14}C, a commercial sample, could be used without additional purification.

Samples were counted in a liquid scintillation spectrometer (Packard Model 3000) as previously described (17, 27). Insoluble residue was suspended in the liquid scintillation fluid with the aid of Cab-O-Sil. A strip scanner (Packard Model 7200) was used to locate ^{14}C labeled areas on paper.

Preparation and Assay of Phytase. Zea mays seedlings grown aseptically for various time intervals were dissected to remove embryonic tissue exclusive of the scutellum. Dissected plants were ground in 0.1 M acetate buffer, pH 5.2, in a chilled mortar, then homogenized in a Sorvall Omnimixer at top speed for 2 minutes. The milky suspension was filtered through glass wool to remove coarse particles and centrifuged (18,000 × g, 30 min). After dialysis (2 changes of 0.1 M acetate buffer, pH 5.2, 24 hr), the solution was adjusted to a volume equivalent to 1.7 ml per grain. All steps were made at 2 to 4°.

To estimate phytase activity, 0.1 ml of an incubation mixture consisting of enzyme (1.7 ml), 0.04 M magnesium sulfate (0.1 ml), and 0.01 M sodium phytate (0.2 ml) was removed, mixed with an equal volume of 10 % trichloroacetic acid, and assayed for total and inorganic phosphate (4). During incubations, assays were layered with toluene to avoid contamination by microorganisms.

Experimental Results

4-O-Methyl Glucuronic Acid from Betula *(Birch)* *Xylan.* Timell (38, 39) has reported that prolonged hydrolysis of *Betula* (birch) xylan with crude fungal pectinase releases free xylose and 4-O-methyl-glucuronic acid. In order to prepare 4-O-methyl-glucuronic acid for carrier and reference purposes, his observation was re-examined, using as an enzyme source, Pectinol R-10 concentrate. *Betula* xylan (1 g) was treated with a solution of 0.2 % pectinase and 0.1 % EDTA (40 ml, 24 hr, 40°). Soluble

acidic products were separated by ion exchange chromatography as shown in figure 1 (upper plot). Acids in the first 2 emergent peaks, 0 to 150 ml, were not characterized. The third peak, 200 to 300 ml, contained a single component, an aldobiouronic acid, tentatively identified as 2-O-(4-O-methyl-glucopyranosyluronic acid)-xylose. The last peak to emerge from the column, 300 to 450 ml, also contained a single component, 4-O-methyl-glucuronic acid.

In order to compare enzymatic with acidic hydrolysis, a portion of *Betula* xylan (0.73 g) was hydrolyzed in 3.6 % sulfuric acid (40 ml, 30 min, 121°, sealed tube). Ion exchange chromatography of the neutralized hydrolyzate gave an elution pattern shown in figure 1 (lower plot). Most of the uronic acid appeared in a single peak eluted between 200 to 280 ml. Paper chromatography in solvents A and B revealed 3 components in this peak of which the major component had a mobility corresponding to 2-O-(4-O-methyl-glucopyranosyluronic acid)-xylose. The small peak between 340 to 420 ml contained only 4-O-methyl-glucuronic acid.

These results emphasize the value of enzymatic hydrolysis over acid hydrolysis as a means of cleaving the glycosidic bond between 4-O-methyl-glucuronic acid and xylose.
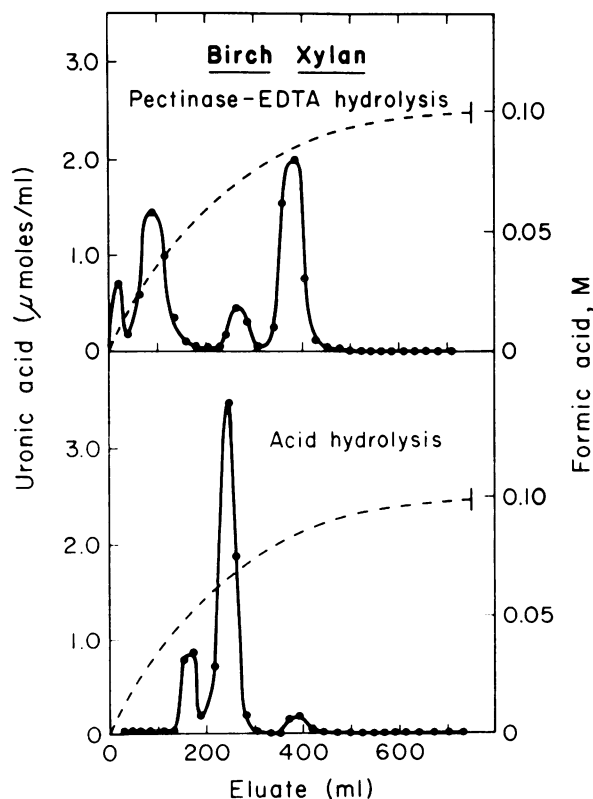

FIG. 1. Gradient elution from Dowex 1 (formate) resin of uronic acids present in hydrolyzates of *Betula* xylan.

Uronic Acids in Ethanol Insoluble Residue from Zea mays *Root Tips.* To determine if the enzymatic aldobiouronic acid cleavage observed in *Betula* xylan applied to glucuronic acid-type aldobiouronic acid as well as 4-*O*-methyl-glucuronic acid-type aldobiouronic acid, an experiment was run in which ethanol insoluble reside from 800 *Zea mays* root tips was hydrolyzed in a solution of 0.2 % pectinase and 0.1 % EDTA (40 ml, 6 hr, 37°). Pectinase-resistant residue was further hydrolyzed with N hydrochloric acid (10 ml, 30 min, 121°, sealed tube). Soluble products were separated by ion exchange chroma-
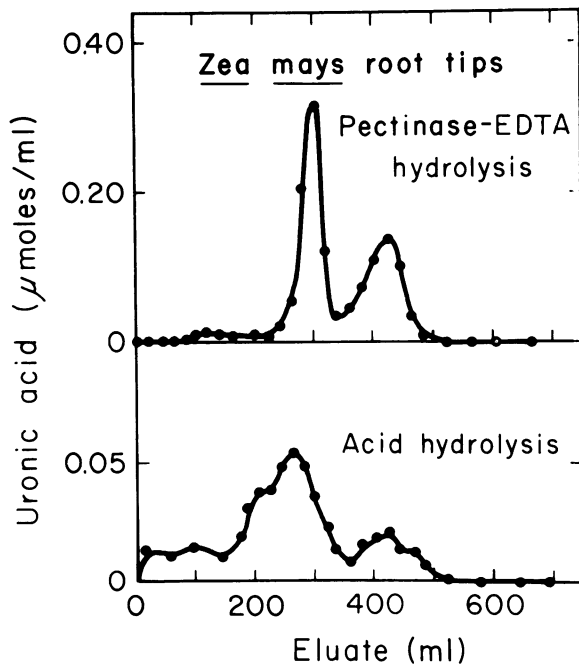

FIG. 2. Gradient elution from Dowex 1 (formate) resin of uronic acids present in successive Pectinol-EDTA and acid hydrolyzates of ethanol-insoluble residue from 800 root-tips of *Zea mays.*

tography and eluted fractions were analyzed for uronic acid (fig 2). Except for a trace of acidic oligosaccharide in early fractions, all of the pectinase-hydrolyzed acids were recovered in 2 peaks, 1 between 250 to 330 ml that contained only galacturonic acid and 1 between 350 to 480 ml that contained a mixture of glucuronic acid and 4-*O*-methyl-glucuronic acid.

The elution pattern of soluble acidic components resulting from acid hydrolysis of pectinase-resistant residue revealed a broad irregular peak between 150 to 340 ml which was found to be a mixture of 2 aldobiouronic acids, 2-*O*-(glucopyranosyluronic acid)-xylose and 2-*O*-(4-*O*-methyl-glucopyranosyluronic acid)-xylose. Some free glucuronic acid and 4-*O*-methyl-glucuronic acid, 350 to 500 ml, was also recovered. Free uronic acids were readily separated and identified by paper chromatography in solvents A and B.

Here, as in the case of *Betula* xylan, enzymatic hydrolysis proved to be a useful method for releasing free uronic acids, glucuronic acid as well as galacturonic acid and 4-*O*-methyl-glucuronic acid, from acidic polysaccharides.

Hydrolyzed Fractions from Labeled Ethanol Insoluble Residue. To examine the distribution of ^{14}C in ethanol insoluble residue from labeled root tips, batches of 60 root tips were incubated 24 hours in distilled water containing trace levels of the ^{14}C-containing substrate. Table I lists the amount of ^{14}C given initially and the amounts recovered in final medium, respired carbon dioxide, and labeled root tips. When this study was first initiated, root tips excised from seedlings grown in moist sphagnum moss were used. It was assumed that seedlings, grown for short periods under such conditions, would be relatively free of microbiological contamination. However, the observation that appreciable ^{14}C was sometimes recovered in respired carbon dioxide (that recovered in Experiment 1M represents the highest amount encountered in 5 experiments) plus

Table I. *Recovery of ^{14}C from Root-Tips of* Zea mays *Seedlings*

Labeled compound and expt no.[1]	Conc	Total ^{14}C added	^{14}C Recovered from medium	from CO_2	Total	^{14}C Incorporated Ethanol-solubles	Ethanol-insolubles
	μmole/3 ml	μc	μc	μc	μc	%	%
myo-Inositol-2-^{14}C							
1M	0.25	2.96	0.27	0.28	2.41	29	71
1S	0.29	3.23	0.17	0.02	3.04	54	46
Glucuronate-1-^{14}C							
2M	5.86	4.51	0.61	0.51	3.39	71	29
2S	2.46	1.89	0.38	0.08	1.43	75	25
Glucuronate-6-^{14}C							
3M	3.52	2.32	1.00	0.99	0.33	68	32
3S	4.15	2.73	0.44	1.68	0.61	80	20
Methionine-methyl-^{14}C							
4M	0.09	1.21	0.04	0.03	1.14	78	22
4S	0.40	5.37	1.31	0.05	4.01	36	64

[1] Experiments lettered M refer to those performed with sphagnum moss-grown seedlings, those lettered S to sterile grown seedlings.

Table II. *Recovery of ¹⁴C from Ethanol-insoluble Fraction of Root-tips of* Zea mays *Seedlings*

Labeled compound and expt no.[1]	¹⁴C in pectinase hydrolyzate				¹⁴C in acid hydrolyzate				¹⁴C in residue Total
	Total	Cationic	Anionic	Neutral	Total	Cationic	Anionic	Neutral	
	μc	%	%	%	μc	%	%	%	μc
myo-Inositol-2-¹⁴C									
1M	0.77	0	23	77	0.74	0	8	92	0.15
1S	0.89	0	22	78	0.50	0	6	94	0.01
Glucuronate-1-¹⁴C									
2M	0.51	0	11	89	0.45	0	10	90	0.02
2S	0.09	0	29	71	0.22	0	16	84	0.03
Glucuronate-6-¹⁴C									
3M	0.09	0	82	18	0.02	0	80	20	<0.01
3S	0.07	0	86	14	0.05	0	91	9	<0.01
Methionine-methyl-¹⁴C									
4M	0.09	52	22	26	0.12	57	17	26	0.05
4S	1.89	48	23	29	0.37	50	19	31	0.31

[1] See footnote, table I.

the fact that this labeled carbon dioxide appeared as a burst about 6 to 8 hours after incubation was initiated led us to suspect a metabolic contribution by processes other than one due solely to root tips. Therefore, each experiment was repeated using sterile procedure during both germination and incubation. In Experiment 1S, a sterile run, ¹⁴C respired
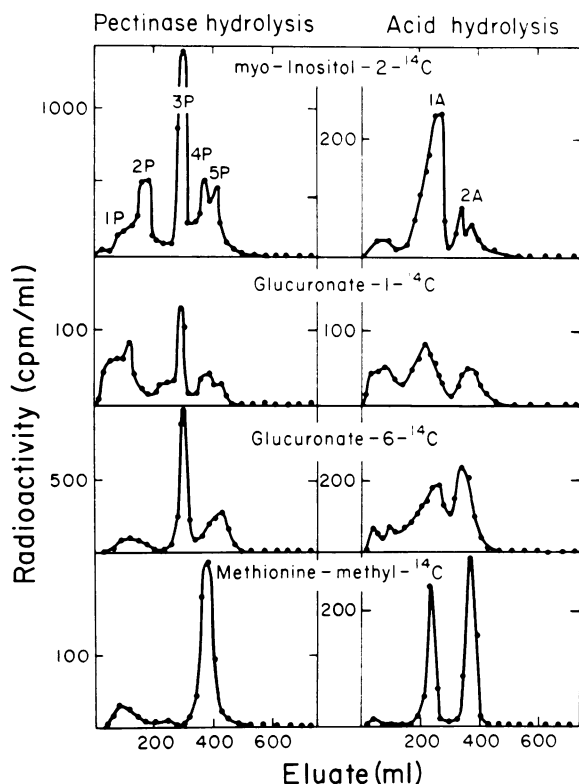

FIG. 3. Gradient elution of labeled acidic components in Pectinol-EDTA or acidic hydrolyzates from ethanol-insoluble residues of root-tips labeled with myo-inositol-2-¹⁴C, glucuronate-1-¹⁴, glucuronate-6-¹⁴C and methionine-methyl-¹⁴C

as carbon dioxide during the 24 hour incubation was less than 1 % of the administered dose. This low level of ¹⁴C in carbon dioxide respired by myo-inositol-2-¹⁴C labeled root tips has been confirmed repeatedly in recent experiments.

Ethanol insoluble residue from labeled root tips was hydrolyzed, first with pectinase-EDTA, then with acid, as has been described above. Distribution of ¹⁴C into soluble extracts following these treatments and distribution of ¹⁴C among cationic, anionic, and neutral constituents of these extracts are given in table II. Profiles of gradient elutions of labeled constituents from columns of anionic exchange resin are given in figure 3. These profiles were obtained from results involving sterile root tip experiments.

myo-*Inositol-2-¹⁴C Labeled Root Tips.* As much as 71 % of the ¹⁴C incorporated into root tips was recovered as ethanol insoluble residue. Pectinase released 50 to 70 % of this into solution, distributed approximately 1:4 between acidic and neutral constituents. The elution profile of acidic constituents (fig 3) resembles an earlier one obtained from root tips that had been grown in sphagnum moss without attention to possible contamination (30). Separation and identification by paper chromatography of constituents from pooled fractions corresponding to 1P+2P, 3P, 4P, and 5P confirmed this resemblance. Figure 4 diagrams results obtained when aliquots of each pooled fraction were chromatographed in solvent B and then scanned for radioactivity. Only acidic oligoxyloses were present in 1P+2P. Prolonged pectinase hydrolysis of 1P+2P yielded 4 labeled monosaccharides, namely, glucuronic acid, 4-O-methyl-glucuronic acid, xylose and a small amount of arabinose indicating that this fraction contained fragments of acidic xylan which were 2 or more xylose units long with a single uronic acid unit attached. Peak 3P contained only labeled galacturonic acid. Peak 4P contained only labeled 4-O-methyl-glucuronic acid and 5P only labeled glucuronic acid. The formic acid gradient used did
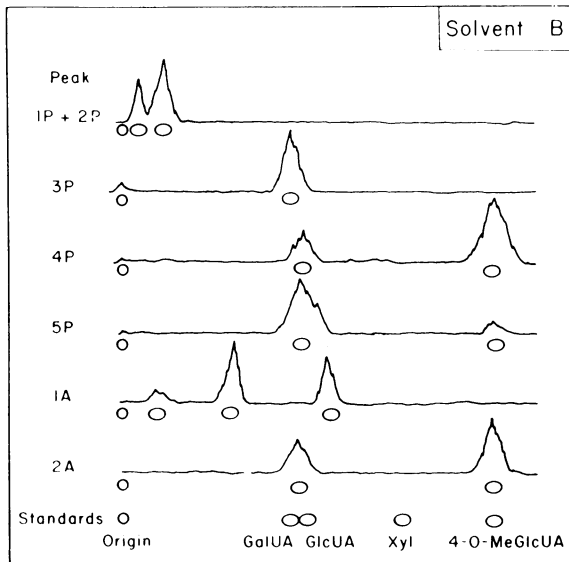
FIG. 4. Radiochromatogram scans of pectinase-hydrolyzed components 1P+2P, 3P, 4P, and 5P which had been eluted from the Dowex 1 (formate) column.

not fully resolve 4P and 5P; therefore, these peaks usually overlapped or at worst resulted in a broad single peak from which the relative amounts of each acid was estimated after further separation by paper chromatography in solvent A and/or B. Presence of ^{14}C in galacturonic acid, 3P, and glucuronic acid, 5P, was confirmed by diluting aliquots of 3P and 5P with appropriate carrier uronic acid, reduction to the corresponding L-aldonic acid, and conversion of that acid to its amide (table III).

Additional ^{14}C-labeled material was recovered from the anionic exchange column by elution with 3 N formic acid, undoubtedly fragments containing 2 or more uronic acid units attached to pectinase-resistant xylose chains.

Xylose and arabinose (^{14}C ratio, 4:3) accounted

Table III. *Identification of Galacturonic Acid and Glucuronic Acid Recovered by Pectinase-Hydrolysis of Ethanol-insoluble Residues from Root Tips Labeled with myo-Inositol-2-^{14}C*

	Specific radioactivity
	dpm/mmole
From 3P	
D-Galacturonate, NaCa salt[1]	
1st crystallization	70,000
2nd crystallization	68,000
L-Galactonamide	65,000
From 5P	
D-Glucuronate, Ca salt[2]	
1st crystallization	6070
2nd crystallization	5930
L-Gulono-γ-lactone	5920
L-Gulonamide	5890

[1] Diluted with 100 mg of unlabeled NaCa galacturonate.
[2] Diluted with 166 mg of unlabeled Ca glucuronate.

for nearly all label in the fraction rendered soluble by pectinase and not bound by ion exchange resins. Paper chromatography in solvents D and F showed the presence of galactose, glucose, and rhamnose, but these hexoses were unlabeled. About 30 % of labeled material remained at the origin of chromatograms. Further acid hydrolysis of these oligomers gave only labeled pentose.

Acid hydrolysis of pectinase-resistant residue released nearly all label still bound. Less than 10 % of this was uronic acid. The remaining 90 % or more was xylose and arabinose (^{14}C ratio, 2:1). Gradient elution of acids is shown in figure 3. The major peak, 1A, preceeded by a smaller (unlettered) peak of acidic oligosaccharides, contained 2 labeled components, 2-O-(glucopyranosyluronic acid)-xylose and 2-O-(4-O-methyl-glucopyranosyluronic acid)-xylose. Peak 2A was a mixture of glucuronic acid and 4-O-methyl-glucuronic acid, here partially resolved.

Residue resistant to both pectinase and acid still contained a small but significant amount of ^{14}C. Hydrolysis with H_2SO_4 (25), neutralization, and paper chromatography revealed a large amount of unlabeled glucose, a small amount of labeled arabinose and xylose, and a trace of labeled uronic acid. Apparently the residue was mainly cellulose with traces of acidic xylan still present.
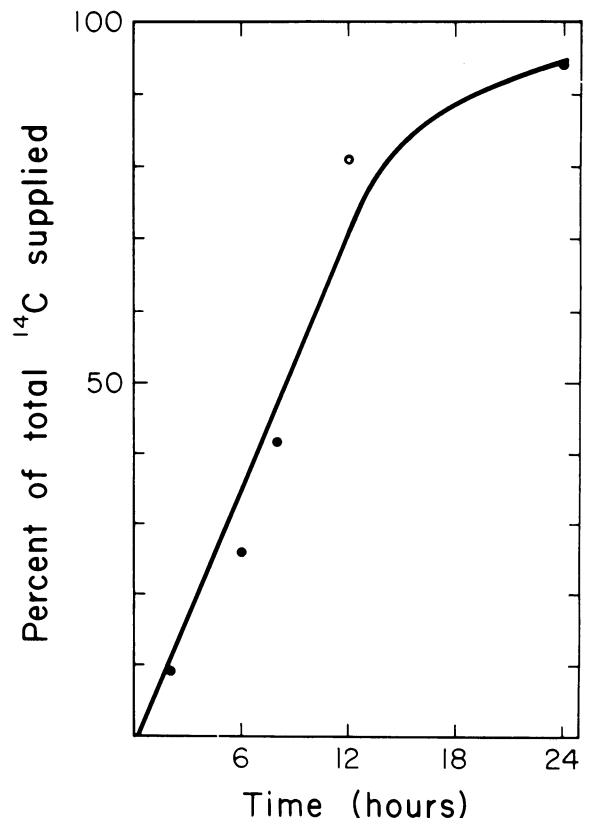

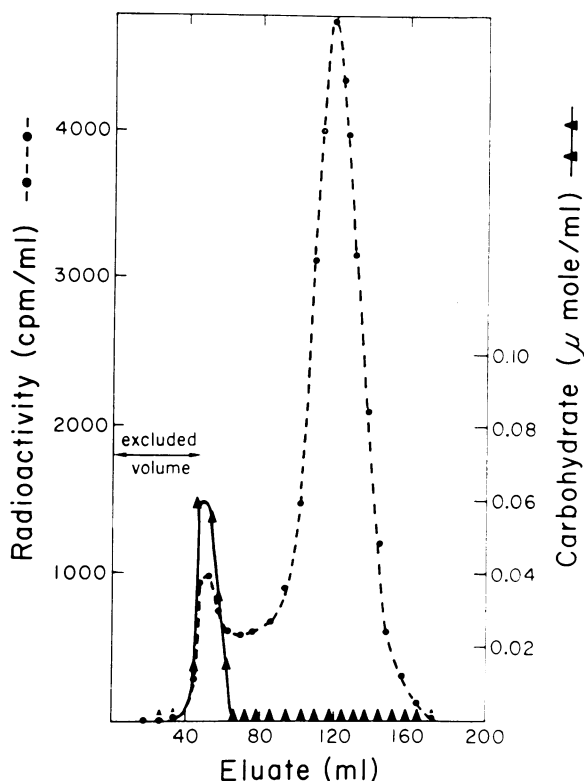FIG. 5. Uptake of myo-inositol-2-^{14}C, by root-tips of Zea mays.

FIG. 6. Separation on a column of Sephadex G-50 of labeled components present in the final incubation medium from experiment 1S.

Root tips incubated in a solution containing a micromolar concentration of *myo*-inositol-2-^{14}C, as represented by these experiments, removed about 80 % of the ^{14}C present in the medium within the first 12 hours (fig 5). Thereafter, uptake was very slow and there was still about 6 % of the label in the medium at the end of each 24 hour incubation. A portion of the final medium from Experiment 1S was reduced to a small volume, placed on a column of Sephadex G-50 (32×2 cm), and eluted with distilled water (fig 6). Fractions were assayed for ^{14}C and for total carbohydrate. A labeled carbohydrate-rich peak was eluted in the region of the void volume of the column. This was followed by a large radioactive peak in the region of small molecules. There was no detectable carbohydrate in the latter fractions.

The carbohydrate-rich peak fractions were pooled and hydrolyzed with N hydrochloric acid (30 min, 121°, sealed tube). Hydrolyzed fragments were separated by ion-exchange and paper chromatography. About 30 % of the ^{14}C was bound to anionic resin. The only labeled acidic components that could be eluted by dilute formic acid (about one-third of the bound ^{14}C) were glucuronic acid and an aldobiouronic acid with the mobility of 2-O-(4-O-methyl-glucopyranosyluronic acid)-xylose. All of the label not bound to the resin was found in xylose and

arabinose (^{14}C ratio 2:3). Over 95 % of the ^{14}C eluted in the small molecule peak (110–135 ml) was recovered as *myo*-inositol. This was demonstrated after addition of carrier *myo*-inositol (160 mg) to a portion of this solution (9000 cpm). The *myo*-inositol was recrystallized several times from ethanol, converted to its hexacetate by treatment with acetic anhydride (1.5 ml) and H_2SO_4 (50 μl), and the hexacetate recrystallized twice without loss of specific activity.

Glucuronic Acid-1-^{14}C and -6-[14]C Labeled Root Tips. Glucuronic acid is readily decarboxylated (loss of carbon 6) in plants but its metabolic path is such that carbon 1 is oxidized to carbon dioxide very slowly (10, 24, 32). In the experiments with sterile root tips reported here (2S and 3S, table I), glucuronic acid-1-^{14}C labeled root tips lost less than 4 % of applied ^{14}C as carbon dioxide in 24 hours whereas 61 % appeared as labeled carbon dioxide when glucuronic acid-6-^{14}C was given. No significance can be attached to results relating to labeled carbon dioxide obtained in Experiments 2M or 3M since these were run without precautions concerning sterility.

Less ^{14}C was incorporated into ethanol insoluble residue with glucuronic acid-1-^{14}C than with *myo*-inositol-2-^{14}C but distribution of label in the residue was similar in both cases, a result to be expected if glucuronic acid is an intermediate in conversion of *myo*-inositol to cell wall polysaccharide since both precursors contained label in carbon atoms which were retained during conversion to uronic acid and pentose. Carbon-14 was present in each of the uronic acid products, galacturonic, glucuronic, and 4-O-methyl glucuronic acid and in both pentoses, arabinose and xylose.

With glucuronic acid-6-^{14}C, only uronic acids in ethanol insoluble residue of maize root tips became labeled. As seen in table II, over 80 % of labeled fragments released by pectinase and acid hydrolysis was recovered as acidic constituents. The small amount of label not retained by the anionic column, reported here as "neutral" constituents, was not identified. In the absence of labeled pentoses, peaks 1P+2P and the irregular peak preceeding 1A (fig 3), fractions in which the proportion of xylose to uronic acid is probably much greater than 1:1, were less pronounced relative to subsequent aldobiouronic and uronic acid peaks, 3P and 4P+5P, than those obtained in Experiments 1 and 2. The absence of label in pentose or hexose fractions is taken as evidence that glucuronic acid did not recycle label into the hexose phosphate pool during its 24 hour incubation. The presence of labeled 4-O-methyl-glucuronic acid in both pectinase and acid hydrolyzates is taken as evidence that a methylation of glucuronic acid occurred subsequent to its incorporation.

Methionine-methyl-[14]C Labeled Root Tips. Higher plants utilize methionine, supplied exogenously, as a source of methyl groups for biosynthesis

of *O*-methyl ethers in phenolic substance (7, 12) or methyl ester in pectic substance (28, 31). Our observation that glucuronic acid is converted, in part, to 4-*O*-methyl-glucuronic acid during its incorporation into cell wall polysaccharide suggested that methionine might act as a methyl donor and that one might use methionine-methyl-^{14}C as a specific marker for 4-*O*-methyl-glucuronic acid units. This was tested in Experiment 4 (table I). Very little ^{14}C appeared in respired carbon dioxide regardless of whether or not precautions were taken to maintain sterile conditions. However, the distribution of ^{14}C between ethanol soluble and ethanol insoluble fractions in sterile root tips differed considerably from that found under non-sterile conditions. Although the subsequent distribution of label into various fractions after pectinase and acid hydrolysis is given for both Experiments 4M and 4S for purposes of comparison, only data from 4S is included in results discussed here.

Pectinase and acid hydrolysis released 88 % of the label incorporated in ethanol insoluble residue. Of this, about 50 % was retained by cationic resin while another 30 % bound neither by cationic nor anionic resins, was recovered in the final effluent as unidentified "neutral" constituents. Attention was focused, in this study, to that portion bound to anionic resin. Gradient elution with 0 to 0.1 N formic acid gave the elution profile shown in figure 3. Except for minor amounts of label in acidic oligoxyloses and a trace of 2-*O*-(4-*O*-methyl-glucopyranosyluronic acid)-xylose, all label eluted was present as 4-*O*-methyl-glucuronic acid. More ^{14}C-containing material, presumably fragments of acidic xylan containing 2 or more labeled 4-O-methyl-glucuronic acid residues per molecule, was eluted by the addition of 3 N formic acid to the column.

Acid hydrolysis of pectinase-resistant residue confirmed the specific nature of label derived from methionine as it appeared in acidic xylan. The elution profile of acidic constituents from this hydrolysis (fig 3) contained 2 sharp peaks of radioactivity, one corresponding to labeled 2-*O*-(4-*O*-methyl-glucopyranosyluronic acid)-xylose between 200 and 260 ml and the other, labeled 4-*O*-methyl-glucuronic acid, between 320 and 400 ml. The identity of these acids was confirmed by paper chromatography in solvents A and B.

Phytase Activity in Zea mays *Endosperm.* Extracts of ungerminated grain or of grain which had been incubated 24 hours under conditions specified for preparation of sterile seedlings contained endogenous, non-dialyzable phosphate that was slowly released as inorganic phosphate under assay conditions whether sodium phytate was present or absent. Figure 7 shows results obtained in the presence of sodium phytate. For ungerminated seeds, the release was about 100 μg phosphorus per grain per 24 hours. Addition of sodium phytate did not increase the amount of phosphate released. With boiled extract, no P_i was released. Between the
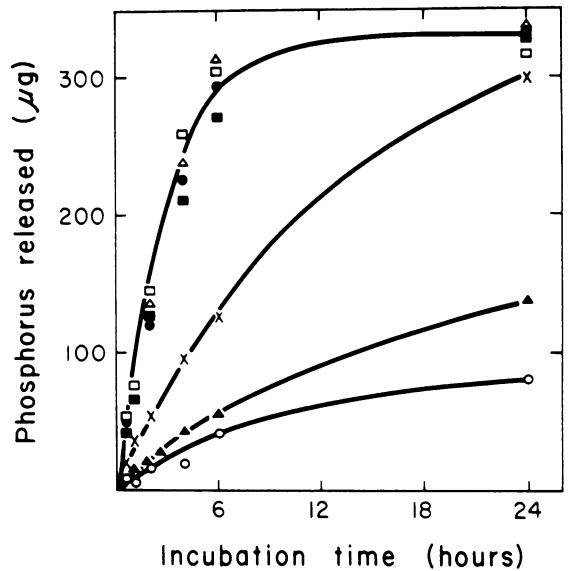

Fig. 7. Phosphorus release from sodium phytate by homogenates prepared from *Zea* grain germinated for 0 (○), 1 (▲), 2 (X), 3 (●), 4 (■), 5 (△), and 6 (□) days.

first and third day of germination, phytase activity rose to a maximum, as shown in figure 8, and remained at this high level for at least 4 days. After the third day, sufficient phytase was present to hydrolyze within 6 hours all sodium phytate added (equivalent to about 300 μg of phosphorus per grain) (fig 8). There was no detectable release of P_i from endogenous, non-dialyzable phosphate after the third day. It is significant, that once phytase activity appeared, pure phytate was hydrolyzed completely. It appears likely that this activity represents true phytase and not that of non-specific phosphatase.
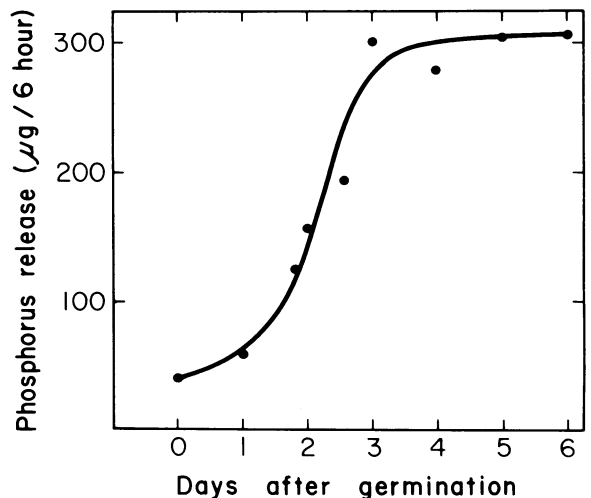

Fig. 8. Time course of appearance of phytase activity in *Zea mays* grain following germination.

Discussion

Previous studies with *Fragaria* fruit, *Petroselinum* leaves, germinating *Pyrus* pollen, and cultured *Acer* (sycamore) cells have shown that *myo*-inositol is rapidly and efficiently utilized for biosynthesis of pectic substances (16, 27). Work with *Hordeum* seedlings (16) and now with *Zea mays* seedlings emphasize the role played by *myo*-inositol as a precursor of uronosyl and pentosyl units in acidic polysaccharides normally associated with so-called hemicellulose and other polysaccharides found in secondary cell wall substance. Results with *myo*-inositol closely resemble those obtained with glucuronate, indicating that these 2 substances follow a common pathway of metabolism during cell wall formation.

Zea mays was chosen for this investigation because it is a plant rich in acidic xylans. The chemical nature of these polysaccharides has been studied by others (3, 37). In the root, especially that of newly germinated seedlings, cells rely on metabolites supplied from elsewhere in the plant body for growth and development. This heterotropic behavior provides a useful experimental condition for study of carbohydrate metabolism free of processes related to photosynthesis. It is also known that deposition of polysaccharide in root tips continues unchanged for a limited time after excision (25, 26).

Procedures usually used to fractionate cell wall carbohydrate depend upon differential solubilities of various polysaccharide constituents. At best, these procedures are arbitrary. Many experiments preceeding those reported in this study involved initial extraction of cell wall residue with cold water, then enzymatic hydrolysis of pectic substance resistant to water extraction followed by an alkaline extraction of the remaining non-cellulosic polysaccharides. The information thus gained permitted us to simplify the extraction procedure, limiting it to 2 steps, an enzymatic hydrolysis followed by hydrolysis with dilute hydrochloric acid of residue resistant to the first step. The enzyme preparation used, Pectinol R-10 concentrate, contained a wide spectrum of hydrolytic activities. Under conditions used here, it released into solution all of the polysaccharide-bound galacturonic acid together with quantities of glucose, galactose, arabinose, xylose, glucuronic acid, and 4-O-methyl-glucuronic acid and a trace of rhamnose. Essentially all pectic substance present in ethanol insoluble residue was hydrolyzed as well as part of the so-called hemicellulose (non-cellulosic cell wall polysaccharides resistant to neutral extractives but readily solubilized by alkali). This enzyme preparation also contained proteases since an appreciable portion of the material removed from the hydrolyzate by passage through a column of Dowex 50 H⁺ resin was identified as free amino acids. This was clearly evident in experiment 4S when the enzyme released appreciable quantities of label which was retained by Dowex 50 (H⁻) ion exchange resin. Pectin esterase was present (28) but we found no evidence of pectin transeliminase although the latter activity

has been reported to be present in some samples of this preparation (2). McClendon and Kreisher (21) were able to separate 16 active fractions representing no less than 8 different hydrolase activities from a crude pectinase preparation, similar to the one employed here, by column chromatography on cellulose phosphate. Perhaps the most notable enzymatic activity present in the Pectinol R-10, at least from the point of view pressed in the present study, was that which catalyzed hydrolytic cleavage of the glycosidic bond linking glucuronic acid or 4-O-methyl-glucuronic acid to xylose. Normally, this bond is characterized chemically by its unusual stability toward acid hydrolysis as was evident in all acid hydrolyses described here, in which aldobiouronic acids rather than free uronic acids were major products.

With the fractionation procedure developed in this study it was possible to demonstrate clearly the precursor roles of *myo*-inositol, glucuronic acid, and methionine in the biosynthesis of 4-O-methyl-glucuronic acid units of acidic polysaccharides in cell wall of *Zea mays* root tips. *myo*-Inositol acts as a general precursor of all uronic acid and pentose units encountered in cell walls of higher plants, including D-apiosyl units in *Lemna* (29). Glucuronic acid, though less effective than *myo*-inositol, probably because of extensive metabolism to other end-products such as L-gulonic acid (20) and D-glucaric acid (14), is also converted to the same cell wall units. Evidence for methylation of glucuronic acid at a step following its formation has been obtained by isolation of labeled 4-O-methyl-glucuronic acid from root tips labeled with glucuronic acid-1-¹⁴C and -6-¹⁴C as well as from root tips labeled with the 1 carbon donor, methionine-methyl-¹⁴C. Kauss and Hassid have shown that S-adenosyl methionine-¹⁴C (methyl) also acts as a methyl donor to glucuronosyl units in a particulate cell-free preparation from *Zea mays* (13). Their work suggests that methylation proceeds after polysaccharide formation but the evidence is, by no means, conclusive.

Earlier work on distribution of ¹⁴C in fractions obtained from ethanol insoluble carbohydrate residue of *Hordeum* seedlings suggested that labeled products of *myo*-inositol-2-¹⁴C were utilized for cell wall biosynthesis in a non-competitive manner since arabinose and xylose units recovered from successive hydrolytic extractions had similar specific activities (16), an observation confirmed in fractionation studies of *Zea mays* root tips (unpublished) preceeding the present work. Moreover, evidence gathered thus far suggests that *myo*-inositol may have a primary role as an intermediate in the biosynthesis of cell wall polysaccharides. For example, in experiments in which *Petroselinum* leaves were labeled with glucose-1-¹⁴C, then transferred to either water or 1 % *myo*-inositol and allowed to metabolize the label for 42 hours (19), distribution patterns of ¹⁴C in *myo*-inositol and galacturonic acid (pectin) recovered from the leaves revealed about 80 % of the ¹⁴C of each molecule retained in carbon

1 (most of the remaining ^{14}C in carbon 6) regardless of whether the leaves had been held in water or myo-inositol solution. In contrast to this, the pattern in sucrose-derived glucose was greatly influenced by the presence of excess myo-inositol which caused redistribution of an additional 20 % of the ^{14}C from carbon 1 into other carbons (see table V, reference 16). These results may be interpreted as evidence in favor of a path of biosynthesis from hexose (glucose) to uronic acid and pentose units of the cell wall in which myo-inositol is intermediate (fig 9). In the presence of excess myo-inositol, utilization of glucose 6-phosphate would be blocked and hexose phosphates would have greater opportunity to equilibrate with the triose phosphate pool. Redistribution of label from carbon 1 into other carbons would result. The fact that this redistribution is reflected in the sucrose-derived glucose, a product of uridine diphosphate glucose, but not in galacturonosyl units of pectin, a product of uridine diphosphate glucuronic acid, is significant. Statements such as those which suggest that myo-inositol is inactive in the metabolism of plants (33) or that its role is primarily that of a growth factor of unknown function (34) should be regarded cautiously. Recent reports that galactianol has a functional role as a cofactor in galactosyl transfers (35, 36) are compatible with the idea of a functional role for myo-inositol as well as galactose moiety of this galactoside. Conceivably, galactinol is formed in the photosynthetic regions of the plant, then translocated to sites of cell wall formation where galactose units are utilized or transferred while the myo-inositol is oxidized to glucuronic acid and metabolized as shown in this and previous studies.

The wide-spread occurrence of myo-inositol and

its derivatives in the plant world is well documented (23). Particular interest, with regard to the present study, centers on phytin, the hexaphosphoric acid ester of myo-inositol, the form of myo-inositol most common to seeds of higher plants. As Posternak clearly points out in his book, the fate of myo-inositol arising from hydrolysis of phytin is largely unknown. Historically, phytin has been regarded as a phosphate reserve in seeds. It is not always clear whether or not phytases, liberated by seeds during germination, hydrolyze part or all of the phosphate ester bonds in phytin. In an attempt to clarify this point, an experiment was run in which samples of a highly purified sodium phytate were hydrolyzed by a series of crude phytase preparations prepared at successive intervals following germination of grains of Zea mays. Phytase activity was low in ungerminated grains. Twenty-four hours after germination, phytase activity was measurably greater, increasing rapidly up to the third day after germination, then remaining at a uniformly high level up to 6 days, the extent of the experiment. Other workers (1, 5, 22) have observed a similar pattern of phytase activity in germinating seeds from several plant species. Phytase activity from a single Zea mays grain, less the embryo, after 3 days of germination was sufficient to hydrolyze completely to P_i nearly 2.7 mg of sodium phytate·32 H_2O in 6 hours, equivalent to the release of nearly 50 μg of myo-inositol per hour. Bianchetti and Sartirana (5) found that P_i controls, in part, the formation of phytase in germinating Triticum embryos. Such control would also regulate release of free myo-inositol. In germinating Phaseolus vulgaris seed (8, 11), breakdown of phytin in the cotyledons was correlated with a fall in total myo-inositol content, with a translocation of myo-inositol to the young plant including the root, and with growth of the embryo. From our present results, it is evident that utilization of myo-inositol as precursor material for the biosynthesis of newly deposited cell wall substance must be given serious consideration in future studies that are directed towards the functional role of phytin during germination.
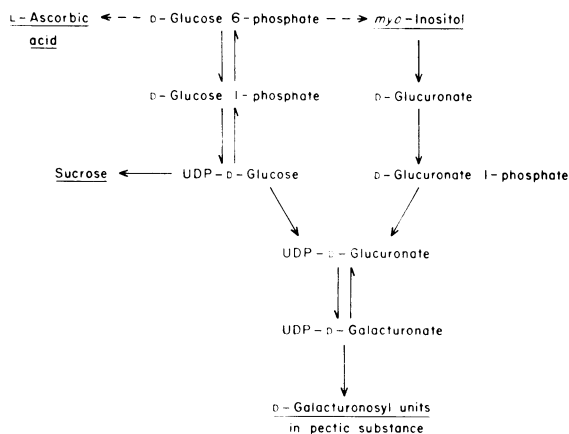

FIG. 9. A diagram depicting alternate pathways for conversion of D-glucose to D-galacturonosyl units of pectic substance. Compounds that have been underlined indicate those products which have been isolated and analyzed for ^{14}C distribution in the experiment in which D-glucose-1-^{14}C was administered to parsley leaves (19). Multistep reactions (broken arrows) have been distinguished from those generally regarded as due to a single enzyme (solid arrows).

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