Ascorbic Acid Metabolism in Pea Seedlings. A Comparison of \textit{d}-Glucosone, \textit{l}-Sorbosone, and \textit{l}-Galactono-1,4-Lactone as Ascorbate Precursors$^{1}$

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\textit{l}-Ascorbic acid (AsA) accumulates in pea \textit{(Pisum sativum \textit{L.})} seedlings during germination, with the most rapid phase of accumulation coinciding with radicle emergence. Monodehydroascorbate reductase and dehydroascorbic acid reductase were active in the embryonic axes before AsA accumulation started, whereas AsA oxidase and AsA peroxidase activities increased in parallel with AsA. Excised embryonic axes were used to investigate the osone pathway of AsA biosynthesis, in which \textit{d}-glucosone and \textit{l}-sorbosone are the proposed intermediates. \textit{[U-\text{\textsuperscript{14}}C]}Glucosone was incorporated into AsA and inhibited the incorporation of \textit{[U-\text{\textsuperscript{14}}C]}glucose (Glc) into AsA. A higher \textit{d}-glucosone concentration (5 mM) inhibited AsA accumulation. \textit{l}-Sorbosone did not affect AsA pool size but caused a small inhibition in the incorporation of \textit{[U-\text{\textsuperscript{14}}C]}Glc into AsA. Oxidase and dehydrogenase activities capable of converting Glc or Glc-6-phosphate to glucosone were not detected in embryonic axis extracts. The osones are therefore unlikely to be physiological intermediates of AsA biosynthesis. \textit{l}-Galactono-1,4-lactone, recently proposed as the AsA precursor (G.L. Wheeler, M.A. Jones, N. Smirnoff [1998] Nature 393: 365–369), was readily converted to AsA by pea embryonic axes. Although \textit{l}-galactono-1,4-lactone did not inhibit \textit{[U-\text{\textsuperscript{14}}C]}Glc incorporation into AsA, this does not mean that it is not a precursor, because competition between endogenous and exogenous pools was minimized by its very small pool size and rapid metabolism.

AsA has an important role in the plant antioxidant system (Foyer, 1993; Conklin et al., 1996; Smirnoff, 1996), photosynthesis (Foyer, 1993), transmembrane electron transport (Horemans et al., 1994), and possibly cell expansion (Smirnoff, 1996). AsA metabolism in plants is poorly understood and, until recently (Wheeler et al., 1998), the biosynthesis pathway was not known (Fig. 1). The first plant AsA biosynthesis pathway to be suggested was via \textit{d}-GalUA and \textit{l}-GAL. In this “inversion” pathway, \textit{C1} of the hexose precursor becomes \textit{C6} of AsA (Fig. 1; Mapson and Isherwood, 1956; Mapson and Breslow, 1958; Isherwood and Mapson, 1962). This pathway is not supported by \textit{\textsuperscript{14}}C-labeling evidence (Loewus, 1963, 1988; Loewus and Mapson, 1987), even though exogenous \textit{l}-GAL is rapidly converted to AsA (Mapson and Breslow, 1958).

More recently, a pathway was proposed (Fig. 1) that involves the osones \textit{d}-glucosone and \textit{l}-sorbosone and fits the labeling data. The pathway predicts no inversion of the hexose precursor, epimerization at \textit{C5}, or conservation of the \textit{C6} hydroxymethyl group. \textit{\textsuperscript{14}}C-labeled glucosone and sorbosone are incorporated into AsA by a number of plant tissues (Saito et al., 1990). A dehydrogenase activity able to oxidize sorbosone to AsA has also been detected, but it has a very low affinity for the substrate (Loewus et al., 1990). AsA synthesis in animals follows an inversion-type pathway in which \textit{l}-GUL is the immediate precursor (Burns, 1967). Evidence that \textit{l}-GAL is the physiological precursor of AsA in plants was recently presented (Fig. 1; Wheeler et al., 1998). In this scheme \textit{l}-GAL is formed without inversion of the hexose carbon skeleton via GDP-\textit{\textit{\textbf{d}}}-Man and GDP-\textit{\textit{l}}-Gal. \textit{l}-Gal is then released and a novel enzyme, \textit{l}-Gal dehydrogenase, oxidizes it to \textit{l}-GAL. \textit{l}-GAL is then oxidized to AsA by \textit{l}-GAL dehydrogenase, a mitochondrial enzyme (Mapson and Breslow, 1958; Oba et al., 1994, 1995; Matsuda et al., 1995) that was recently cloned (\text{\textsuperscript{O}}stergaard et al., 1997).

The pathways and enzymes involved in oxidizing AsA and reducing the unstable oxidation products MDHA and DHA back to AsA are better characterized than the biosynthetic pathway. AsA is readily oxidized to the MDHA free radical as part of its antioxidant function. Oxidation is catalyzed by APX and AOX. MDHA disproportionates to DHA and AsA if it is not immediately reduced. DHA is unstable above pH 7.0 and irreversibly delactonizes to 2,3-diketogulonate (Loewus, 1988; Smirnoff, 1995). Under normal circumstances the AsA pool is maintained with at least 90% of the AsA in reduced form by the action of NAD(P)-dependent MDHAR and GSH-dependent DHAR. Along with glutathione reductase, which regenerates GSH, these enzymes constitute the AsA-glutathione cycle (Foyer, 1993).

We used germinating pea \textit{(Pisum sativum \textit{L.})} seedlings in the present study, which are a good system with which to

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\textbf{Abbreviations:} AOX, ascorbate oxidase; APX, ascorbate peroxidase; AsA, \textit{\textit{l}-}ascorbic acid; DHA, dehydroascorbic acid; DHAR, dehydroascorbic acid reductase; \textit{l}-GAL, \textit{l}-galactono-1,4-lactone; \textit{\textit{l}}-GUL, \textit{l}-glulono-1,4-lactone; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase.

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study AsA synthesis and turnover, because the embryonic axes accumulate AsA rapidly and exhibit large increases in APX and AOX activity and lesser increases in MDHAR and DHAR activity. Therefore, we used these axes to investigate the incorporation of putative precursors into AsA. Investigation of the metabolism of glucosone and sorbosone provided evidence against a physiological role for the osone pathway. This system has also provided evidence supporting the newly proposed AsA biosynthetic pathway via GDP-Man, GDP-L-Gal, L-Gal, and L-GAL (Wheeler et al., 1998).

**MATERIALS AND METHODS**

**Plant Culture**

Pea (*Pisum sativum* L. cv Meteor) seeds were surface-sterilized for 10 min in sodium hypochlorite (1.2% available chlorine), placed in plastic boxes between paper towels moistened with a 20% strength, nitrate-type Long Ashton nutrient medium (Hewitt and Smith, 1975), and incubated in the dark at 21°C. After the seedlings were incubated for the appropriate time the testae were removed and the seedlings were separated into cotyledons and embryonic axes.

**Enzyme Assays**

Embryonic axes were homogenized in the extraction medium described by Smirnoff and Colombé (1988) to extract MDHAR and DHAR. APX and AOX were extracted as described by Conklin et al. (1997). The assays were performed according to the methods of the following investigators: MDHAR and APX, Smirnoff and Colombé (1988); DHAR, Nakano and Asada (1980); and soluble and cell wall-bound AOX, Conklin et al. (1997). Protein was measured by the method of Bradford (1976).  

**AsA Assay**

Tissue (0.1 g) was frozen in liquid nitrogen, ground to a powder, and extracted in 1 mL of 5% perchloric acid. The homogenate was centrifuged at 12,000 g for 2 min, and the supernatant was neutralized with 5 m potassium carbonate using methyl orange indicator. The neutralized supernatant was centrifuged again and used for the AsA assay with AOX (Hewitt and Dickes, 1961). Total AsA was assayed after first reducing the sample with homocysteine (Okamura, 1980). The DHA concentration was calculated from the difference between total and reduced AsA.

**Feeding of Potential AsA Precursors to Embryonic Axes**

After dissection from seedlings, embryonic axes were incubated in glass vials on two layers of 1-cm-diameter filter-paper discs moistened with 0.4 mL of sterile nutrient medium plus the desired additions. To aid in gas exchange, the vials were enclosed in boxes with loosely fitting lids (with wet paper towels to minimize evaporation) and incubated in the dark at 20°C. When radioactive substrates were applied, the boxes were sealed to prevent the loss of 14CO2.

**Metabolism of 14C-Labeled Compounds by Embryonic Axes**

Radiolabeled compounds were fed to embryonic axes as described above. The compounds used were D-[U-14C]Glc (3 μCi; specific activity, 295 mCi mmol⁻¹; Amersham) and D-[U-14C]glucosone (3 μCi; specific activity, 295 mCi mmol⁻¹; prepared as described below). After incubation the samples were extracted in perchloric acid, and the supernatant was separated into neutral, acidic, and basic fractions by ion exchange. AsA was isolated in a fraction...
eluted from a strong anion-exchange resin by 60 mM formic acid, purified by HPLC, and its 14C content was determined with a liquid scintillation counter. Full details were given by Conklin et al. (1997).

**Synthesis of d-Glucosone**

d-Glucosone (2-keto-Glc) was prepared from d-Glc by specific oxidation at C2 using pyranose-2-oxidase purified from the basidiomycete fungus *Phanerochaete chrysosporium*. The fungus was inoculated into a medium containing 2.4% (w/v) Glc, 1.8% (w/v) cornsteep powder (Merck, Rahway, NJ), and 0.18% (w/v) MgSO4·7H2O, pH 5.5, and grown in a shaking culture at 30°C for 11 d until reaching the stationary phase. The mycelium was harvested and homogenized in 50 mM potassium phosphate buffer, pH 7.0. Pyranose-2-oxidase activity was purified by ammonium sulfate precipitation (25% saturation), hydrophobic interaction chromatography (Phenyl-Sepharose, Pharmacia), and anion-exchange chromatography (DEAE-Sepharose, Pharmacia). The enzyme preparation was dia lyzed against 20 mM Tris-HCl, pH 6.5, before use and assayed as described by Liu et al. (1983). The purified enzyme (5 units) was incubated at 28°C with 100 mg of Glc in 4 mL of sterile, distilled water with 12,500 units of catalase and 10 μL of an antibiotic/antimycotic mixture (Sigma). After 24 h of incubation, the reaction mixture was freeze dried.

Measurement of glucosone (Liu et al., 1983) showed that the conversion of Glc ranged from 90% to 100% in the different preparations. The identity and purity of the glucosone was confirmed by TLC and GC-MS. After TLC on cellulose plates with an isopropanol:pyridine:acetic acid: water (4:4:1:2, v/v) mobile phase, the product rapidly reduced tetratozium at 20°C and stained intensely blue with diphenylamine-aniline-phosphoric acid reagent (Dawson et al., 1969; Daniel et al., 1994). GC-MS (Ultra 1 capillary column interfaced with a model 5970 mass-selective detector, Hewlett-Packard) of the trimethylsilyl oxime derivative (Andrews, 1989) of the reaction product produced a peak with the same mass spectrum as the predicted fragmentation pattern for glucosone, which matched the glucosone mass spectrum in the Wiley mass-spectral database (Rev A.00.00. 1986. John Wiley & SMS Inc., New York). Blank incubations showed that the residual antibiotic mixture had no effect on the growth of the embryonic axes; therefore, the glucosone preparation was used without further purification. 14C-labeled glucosone was synthesized from d-[U-14C]Glc (specific activity, 295 mCi mmol⁻¹; Amersham) by the procedure described above.

**Assay of Glucosone Formation by Embryonic Axis Extracts**

Embryonic axes were isolated 22 h after imbibition and homogenized in ice-cold 50 mM Hepes-KOH buffer, pH 7.0, containing 5 mM MgCl₂, 0.15% Triton X-100, and 1 mM DTT. The homogenate was centrifuged at 12,000g for 2 min in a microcentrifuge. The supernatant was desalted on Sephadex G-25 (Pharmacia PD-10 columns) equilibrated with the homogenizing medium without Triton X-100. The eluent was used for assays. The reaction mixture contained, in a total volume of 100 μL, 50 μL of 50 mM Hepes-KOH, pH 6.0 or 7.0, 40 μL of desalted extract, 0.2 μCi of d-[U-14C]Glc, and 5 μL of an antibiotic/antimycotic mixture (Sigma). NAD(P) (0.1 mM) was added to determine the cofactor requirement. d-[U-14C]Glc-6-P was generated by adding 10 mM ATP and 6 units of hexokinase (Sigma) to the reaction mixture. After incubation for 2 to 4 h at 25°C, the reactions were stopped by the addition of 0.1 mL of isopropanol. After the sample was centrifuged, 10-μL aliquots were spotted onto a silica-gel TLC plate. The plates were developed with acetonitrile:0.1 mM NH₄Cl (7:3, v/v). The plate was scanned to detect 14C with a linear analyzer (Berthold Analytical, Gaithersburg, MD). Glc, Glc-6-P, and glucosone were detected on the plates with aniline/diphenylamine stain (Dawson et al., 1969). The reaction mixtures containing ATP and hexokinase were treated with 0.16 unit of alkaline phosphatase before precipitation to dephosphorylate the reaction products.

**RESULTS**

**AsA Accumulation during Pea Seed Germination**

The AsA concentration in the embryonic axes and cotyledons of pea seeds was measured during the first week of germination in the dark. In all cases DHA constituted 10% to 20% of the total AsA pool; therefore, all results refer to the total. The dry seeds contained very little AsA. The AsA pool of the embryonic axes started to increase 10 h after imbibition, and between 24 and 50 h the concentration increased rapidly (Fig. 2B). The rapid increase corresponded to the emergence of the radicle (Fig. 2A). AsA concentration decreased 50 h after imbibition (Fig. 2B), corresponding to the emergence of the plumule and a phase of rapid growth in fresh and dry weight of the embryonic axis (Fig. 2A), suggesting that the rate of AsA synthesis does not increase to keep pace with growth at this point. The AsA concentration in the cotyledons also increased between 24 and 50 h after imbibition (Fig. 2B); however, the cotyledons always contained considerably less AsA than the embryonic axes (Fig. 2B).

**Activity of AsA-Metabolizing Enzymes in Embryonic Axes during Germination**

APX and AOX activities were both undetectable 10 h after imbibition and then increased rapidly until 40 h after imbibition (Fig. 3). APX and AOX activity followed a time course similar to that of the increase in AsA concentration during germination (Figs. 2B and 3, A and B). The pattern of MDHAR and DHAR activity during germination differed from that of APX and AOX in that appreciable activity was detected early in germination (Fig. 3, C and D). The contrasting patterns of APX/OX and MDHAR/DHAR activity suggest that the embryonic axis has a relatively high capacity to regenerate oxidized AsA from a very early stage of germination but that the AsA-oxidizing enzymes have very low activity until AsA accumulation begins.
Characteristics of AsA Accumulation by Isolated Embryonic Axes

Embryonic axes retained their ability to synthesize and accumulate AsA after the removal of their cotyledons. The total AsA content doubled during the 6 h after embryonic axes were excised from seedlings 24 h after imbibition (Table I), even though 50% of the soluble carbohydrate pool was lost during this time (data not shown). The axes also grew by water uptake during this period (Table I). A range of sugars was supplied to isolated embryonic axes to assess the role of carbohydrate supply in AsA accumulation. Suc, d-Glc, d-Fru, d-Man, l-Man, d-Gal, and l-rhamnose (5–25 mM for periods of 6–24 h) had no effect on the AsA pool size or on the expansion growth of the axes (data not shown). Isolated embryonic axes, therefore, are a suitable system with which to investigate AsA metabolism, because they can accumulate AsA at a rate (0.1–0.2 μmol h⁻¹ g⁻¹ fresh weight) comparable to that of intact seedlings in the absence of an exogenous carbon source.

The Effect of Putative AsA Precursors on AsA Pool Size

In the absence of an influence of sugars, a range of more specific AsA precursors was fed to the embryonic axes (Table I). l-GAL increased the AsA pool. l-GUL, the AsA precursor in animals (Burns, 1967), caused a small and statistically insignificant increase in AsA pool size; this effect was consistently noted in several experiments. d-Glucosone and l-sorbosone have been suggested as AsA precursors in plants (Saito et al., 1990). When fed to embryonic axes, up to 5 mM l-sorbosone had no effect on AsA pool size or expansion growth (Table I). In contrast, 5 mM l-glucosone prevented AsA accumulation (Table I). At 25 mM, glucosone also inhibited embryonic axis expansion growth, suggesting that it is toxic at high concentrations (Table I). The effect of l-GAL and glucosone together was also investigated (Table I). The conversion of l-GAL was apparently inhibited but not prevented by glucosone. Because the increase after the addition of both compounds (1.17 μmol g⁻¹ compared with no addition) was similar to the difference between the calculated net change caused by either compound alone (1.08 μmol g⁻¹), it appears that glucosone does not interfere directly with the oxidation of l-GAL to AsA.

Figure 2. Ascorbate concentration in relation to development during pea seedling germination. Dry weight changes (A) and total AsA pool size (B) in cotyledons (○) and embryonic axes (●) during germination are shown. First arrow in A indicates radicle emergence; second arrow indicates plumule emergence. Values are means ± SD (n = 3).

Figure 3. Specific activity of AsA-metabolizing enzymes in pea embryonic axes during germination. A, APX; B, AOX (AO); C, MDHAR (MDAR); and D, DHAR. Values are means ± SD (n = 3).
Comparison of D-[U-14C]Glc and D-[U-14C]Glucosone Incorporation into AsA

Glucosone metabolism was further investigated by following the metabolism of D-[U-14C]Glc and D-[U-14C]glucosone for 2, 4, and 6 h. The labeled glucosone contained no carrier, and its concentration was 25 μM. At this level the glucosone did not inhibit AsA accumulation in the embryonic axes (Table II) compared with unlabeled glucosone at 5 μM (Table I). The uptake of labeled glucosone was 20% to 35% slower than that of Glc (Table II). The tissue was extracted and fractionated by ion-exchange chromatography into major classes of compounds. The distribution of label between these fractions is compared in Table III. Compared with [14C]Glc, more label from glucosone remained in the soluble fraction and less label was incorporated into insoluble material (cell walls and protein) and respired as CO2. Within the soluble fraction the same proportion was incorporated into acidic compounds after metabolism of both compounds; however, a much smaller proportion of label from glucosone was found in the basic fraction (amino acids), whereas a much higher proportion was found in neutral compounds. A detailed study of the products of glucosone metabolism is not presented here, but HPLC analysis of the acidic fraction showed label in compounds with the same retention time as malic and citric acids.

Label in AsA was determined after separation of the 60 mM formic acid eluate by HPLC (Table II). In agreement with previous studies, only 1% to 2% of Glc was incorporated into AsA (Saito et al., 1990; Conklin et al., 1997). However, about 4% of the glucosone label was found in

<table>
<thead>
<tr>
<th>Table I. The effect of putative ascorbate precursors on ascorbate concentration and fresh weight of pea embryonic axes</th>
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</thead>
<tbody>
<tr>
<td>Values are means ± SD (n = 3). Values within each experiment followed by different letters are significantly different (P = 0.05) according to one-way analysis of variance and the least-significant range test.</td>
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<tr>
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<td></td>
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<tr>
<td>25 mM for 8 h</td>
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<tr>
<td>No addition</td>
</tr>
<tr>
<td>D-Glucose</td>
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<tr>
<td>L-GAL</td>
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<tr>
<td>L-GUL</td>
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<tr>
<td>D-Glucosone</td>
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<tr>
<td>5 mM for 6 and 24 h</td>
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<tr>
<td>Initial (0 h)</td>
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<tr>
<td>No addition 6 h</td>
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<tr>
<td>D-Glucosone 6 h</td>
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<tr>
<td>L-GAL 6 h</td>
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<tr>
<td>Control 24 h</td>
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<tr>
<td>D-Glucosone 24 h</td>
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<tr>
<td>L-GAL 24 h</td>
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<tr>
<td>Combined L-GAL and D-glucosone for 6 h</td>
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<tr>
<td>Initial (0 h)</td>
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<tr>
<td>No addition 6 h</td>
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<tr>
<td>D-Glucosone (5 mM)</td>
</tr>
<tr>
<td>L-GAL (5 mM)</td>
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<tr>
<td>D-Glucosone (5 mM) plus L-GAL (5 mM)</td>
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<thead>
<tr>
<th>Table II. Comparison of the incorporation of [U-14C]Glc and [U-14C]glucosone into AsA by pea embryonic axes</th>
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</thead>
<tbody>
<tr>
<td>AsA was separated by HPLC from the 60 mM formic acid eluate shown in Table III. Values are means ± SD (n = 2). Values within columns followed by different letters are significantly different (P = 0.05) according to one-way analysis of variance and the least-significant range test.</td>
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<td></td>
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<tr>
<td>[U-¹⁴C]Glc</td>
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<td>2 h</td>
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<td>4 h</td>
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<td>6 h</td>
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<td>[U-¹⁴C]Glucosone</td>
</tr>
<tr>
<td>2 h</td>
</tr>
<tr>
<td>4 h</td>
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<tr>
<td>6 h</td>
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</table>
AsA. Glucosone, therefore, is an effective precursor for AsA compared with Glc when applied at low concentrations, but it inhibits AsA accumulation at 25 mM.

The Effect of Putative Precursors on the Synthesis of AsA from [U-14C]Glc

Saito et al. (1990) carried out competition experiments in which unlabeled osones were allowed to compete with [14C]Glc for incorporation into AsA over 24 h. Both compounds competed, suggesting that they could be intermediates in AsA biosynthesis. Similar experiments were carried out with embryonic axes but with much shorter labeling times (2 h). d-Glucosone, l-sorbosone, and l-GAL were fed to embryonic axes, and their effect on the incorporation of [U-14C]Glc into AsA was determined (Table IV). None of the precursors affected [14C]Glc uptake or its incorporation into the major fractions (data not shown). D-Glucosone inhibited synthesis of AsA from Glc and decreased the pool size of AsA in the embryonic axes. l-Sorbosone decreased the proportion of [14C]Glc incorporated into AsA from 1.76% to 1.54%. Although statistically significant, this effect was very small. l-GAL increased the AsA pool size but did not decrease [14C]Glc incorporation into AsA. It was not possible to detect a peak corresponding to l-GAL after HPLC of the neutral fraction from the embryonic axes that had been fed with l-GAL.

Table IV. The effect of d-glucosone, l-sorbosone, and l-GAL on the incorporation of [U-14C]Glc into ascorbate by pea embryonic axes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Ascorbate (μmol g⁻¹ fresh wt)</th>
<th>% of total label</th>
<th>% 14C in Ascorbate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.84 ± 0.04</td>
<td>0.191 ± 0.021</td>
<td>1.95 ± 0.18</td>
</tr>
<tr>
<td>d-Glucosone</td>
<td>1.11 ± 0.07</td>
<td>0.036 ± 0.004</td>
<td>0.46 ± 0.09</td>
</tr>
<tr>
<td>l-Sorbosone</td>
<td>1.51 ± 0.13</td>
<td>0.128 ± 0.014</td>
<td>1.54 ± 0.06</td>
</tr>
<tr>
<td>l-GAL</td>
<td>1.37 ± 0.20</td>
<td>0.100 ± 0.011</td>
<td>1.16 ± 0.16</td>
</tr>
</tbody>
</table>

DISCUSSION

Development of the AsA System during Pea Seed Germination

Pea seeds contain low concentrations of AsA and DHA. Similarly, low concentrations have been found in bean seeds at maturity (Arrigoni et al., 1992) and in a wide range of other species (N. Smirnoff, unpublished results). AsA is oxidized and DHA is destroyed during the desiccation phase of seed development (Arrigoni et al., 1992), which would explain the lack of AsA in seeds. There is a lag of

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20 h after imbibition before the AsA content of the embryonic axis increases rapidly. This increase just precedes radicle emergence. The embryonic axes have very low APX and AOX activity before 20 h. This corresponds with the lack of AOX activity in developing bean seeds and the loss of APX activity during desiccation (Arrigoni et al., 1992). These enzymes have a coordinated increase in their activity in parallel with AsA content, which suggests that the expression of the AsA system during germination is highly controlled. The correlation between growth of the radicle and AOX activity supports the suggestion that wall AOX activity is related to cell wall expansion (Smirnoff, 1996).

There is also a correlation between AOX activity and the zone of cell expansion in pea and maize roots (Mertz, 1961; Suzuki and Ogiso, 1973). In developing cucurbit fruits, AOX activity is greatest in tissues undergoing rapid cell expansion and is induced by IAA (Esaka et al., 1992). Critical experiments are now needed to probe the role of AOX in cell expansion. The *vtc1* Arabidopsis mutant, which has 30% of the wild-type level of AsA, does not show marked growth reduction or altered development (Conklin et al., 1996) under normal growth conditions. Therefore, assessment of the role of AsA may require mutants with lower AsA concentrations. Low APX activity during early germination could protect the small pool from oxidation. The relatively high MDHAR and DHAR activity during early germination could also protect the small pool from loss by oxidation. The rapid AsA accumulation between 20 and 30 h after imbibition makes pea embryonic axes a useful system with which to detect enzymes and intermediates involved in AsA synthesis.

### The Osones as Intermediates of AsA Biosynthesis in Pea Embryonic Axes

The osone pathway was proposed to explain the lack of inversion of the Glc carbon skeleton during AsA synthesis (Loewus et al., 1990; Saito et al., 1990). Neither glucosone nor sorbosone has been detected in plant extracts, and therefore, if they are present their concentration is very low. Although l-GAL was proposed as a precursor many years ago, it has been assumed that it could be synthesized from Glc only by inversion of the carbon skeleton. However, the recently proposed biosynthetic pathway of AsA in plants suggests that the immediate precursor is l-GAL, which can be synthesized from Glc without inversion (Fig. 1; Wheeler et al., 1998). Therefore, it remains to be explained how l-glucosone and l-sorbosone can act as AsA precursors. The alternative possibilities are that the osones provide a second minor AsA-biosynthesis pathway or that they are analogs of intermediates in the l-GAL pathway.

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**Figure 4.** TLC separations of the reaction products of Glc metabolism by pea embryonic axis extracts. Extracts were incubated with [U-14C]Glc in 50 mM Hepes-KOH buffer at pH 7.0, and the reaction mixture was separated by TLC on silica plates. Radioactive peaks were detected by scanning the plates after 2 h of incubation (A), 4 h of incubation (B), 4 h of incubation with NAD+ (C), or 4 h of incubation with *P. chrysosporium* pyranose-2-oxidase (D). The arrows indicate the mobility of D-Glc and D-glucosone standards.
**D-Glucosone and L-sorbose did not increase the AsA pool even when supplied at a high concentration. They also had no effect in Arabidopsis leaves (Conklin et al., 1997). The inability of D-glucosone and L-sorbose to elevate the AsA pool is surprising, because they are proposed as intermediate AsA precursors and label from [14C]glucosone and [14C]sorbose is incorporated into AsA (Saito et al., 1990). The evidence suggests that the osones are not physiological precursors of AsA. For one thing, the dehydrogenase proposed by Loewus et al. (1990) to oxidize sorbose to AsA has an extremely high $K_m$ for sorbose. Second, in an isotope-dilution experiment, exogenous sorbose did not compete strongly with incorporation of [14C]Glc into AsA in pea embryonic axes. Saito et al. (1990) showed competition in their similar but longer-term experiments with spinach leaves. Furthermore, a small proportion of exogenous [14C]sorbose is incorporated into AsA (Saito et al., 1990). The explanation may be related to the affinity of L-Gal dehydrogenase to oxidize L-sorbose to AsA with low affinity (Wheeler et al., 1998): exogenous sorbose, therefore, would have a very small effect because its affinity for L-Gal dehydrogenase is too low to compete with endogenous L-Gal.**

In pea, [14C]glucosone was twice as effective as Glc as an AsA precursor, which is in agreement with the results of Saito et al. (1990). However, millimolar concentrations of exogenous glucosone appeared to be moderately toxic. Glucosone at a concentration of 5 mM inhibited AsA accumulation, whereas at 25 mM water uptake by the embryonic axes was inhibited. Although toxicity could be attributed to impurities or breakdown products in the glucosone preparation, it has also been noted that bean shoots and spinach leaves wilt when supplied with glucosone via the transpiration stream (Saito et al., 1990).

Glucosone toxicity at millimolar concentrations has been reported in a number of organisms. Glucosone is phosphorylated by hexokinase, and toxicity has been attributed to interference with carbohydrate metabolism (Bayne and Fewster, 1956) and to oxidative damage in the presence of transition metal ions (Nakayama et al., 1992). Sorbose has no evident toxic effects on pea embryonic axes or spinach and bean leaves (Saito et al., 1990), which is in agreement with the observation that a variety of osones only glucosone is toxic (Bayne and Fewster, 1956). D-Glucosone inhibited [14C]Glc incorporation into AsA, as was also found in spinach leaves (Saito et al., 1990). This does not mean that glucosone lies on the AsA-biosynthetic pathway, because of the direct inhibitory effect of glucosone on AsA accumulation discussed above. Nevertheless, [14C]glucosone is incorporated into AsA; therefore, it must be converted to an intermediate of AsA biosynthesis.

Wheeler et al. (1998) have proposed that label from glucosone appears in AsA, because reduction of the carbonyl group at C2, if nonstereospecific, produces 50% Glc and 50% D-Man. d-[14C]Man is incorporated into AsA much more effectively than Glc (Wheeler et al., 1998). A ketose reductase that can reduce Fru at C2 occurs in maize endosperm (Doehlert, 1987) and an enzyme of this type could also reduce glucosone. Alternatively, reduction of glucosone at C1 would form Fru, and Fru-6-P would then be readily converted to Man-6-P by phosphomannose isomerase. Aldose reductase reduces glucosone specifically at C1, forming Fru (Kotecha et al., 1996), and this enzyme has been reported in plants (Roncarati et al., 1995). On the basis of the present evidence we suggest that glucosone is converted to Fru or Man. The products of [14C]glucosone metabolism must be examined in more detail to provide evidence for or against this hypothesis.

Further evidence against glucosone and sorbose as precursors of AsA is provided by the apparent lack of enzyme activities that could oxidize Glc and Glc-6-P to glucosone in pea embryonic axes. Embryonic axis extracts were examined for pyranose-2-oxidase activity similar to that in glucosone-forming basidiomycete fungi such as *P. chrysosporium* (Daniel et al., 1994) and also for NAD(P)-linked dehydrogenase activity. No glucosone was formed from [14C]Glc in any of these assays. The pyranose-2-oxidase from *P. chrysosporium* produced glucosone when added to the assay, showing that, if produced by embryonic axis extracts, it would have been stable under the assay conditions. Inclusion of ATP and hexokinase to investigate the possibility that Glc-6-P could be a substrate also failed to produce glucosone. Although the possibility cannot be excluded that the extraction and assay conditions were inadequate, these results suggest that the embryonic axes cannot synthesize glucosone from Glc. In summary, it is suggested that D-glucosone and L-sorbose are not likely to be physiological intermediates of AsA synthesis, but they can both act as pseudosubstrates that are converted to AsA (Fig. 1). This explains the logic of proposing these compounds as intermediates of AsA synthesis (Loewus et al., 1990; Saito et al., 1990).**

**1-GAL as an AsA Precursor**

In the present study, pea embryonic axes converted 1-GAL to AsA, as has been found in all higher plant tissues examined thus far. This reaction is catalyzed by a mitochondrial 1-GAL dehydrogenase (Mapson and Breslow, 1958; Oba et al., 1994, 1995; Matsuda et al., 1995; Østergaard et al., 1997). 1-GUL, the AsA precursor in animals, had a small effect on the AsA pool in pea. Purified cauliflower and spinach 1-GAL dehydrogenase has little or no activity toward 1-GUL (Matsuda et al., 1995; Østergaard et al., 1997). However, partially purified potato tuber 1-GAL dehydrogenase supported 1-GUL oxidation at a rate that was 20% of that of 1-GAL (Oba et al., 1994), suggesting that there is interspecific variability in enzyme specificity.

If 1-GAL is the physiological precursor for AsA, an isotope-dilution experiment should reveal competition between 1-GAL and [14C]Glc for label incorporation into AsA. Competition between Glc and 1-GAL was not detected, but this does not rule out 1-GAL as the precursor, because the conditions under which the interpretation of the isotope-dilution experiment is valid were not met. In an isotope-dilution experiment it is assumed that, if a compound lies on the biosynthetic pathway, then an unlabeled exogenous supply will dilute the specific activity of the endogenous pool being synthesized from a labeled precursor farther back in the pathway. It also assumes that the
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pool is large relative to the flux so that it is not all converted to AsA during the course of the experiment. However, the pool size of L-GAL is very small (Baig et al., 1970; Østergaard et al., 1997) and its conversion to AsA is very fast, which suggest the possibility that endogenous and exogenous L-GAL are fully converted during the course of the experiment, thus avoiding competition. Therefore, these results do not provide evidence against L-GAL being the precursor of AsA, as was proposed by Wheeler et al. (1998).