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Biosynthesis and metabolism of ascorbic acid in plants and of analogs of ascorbic acid in fungi

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Abstract

Despite the plethora of reviews on L-ascorbic acid, few stray beyond its established roles as an agent in redox-associated reactions or its biomedical importance. Here, an attempt is made to draw attention to L-ascorbic acid biosynthesis and metabolism in plants and to structurally similar compounds in fungi. During the decade since this subject was last addressed, a rational view of ascorbic acid biosynthesis has emerged, fresh evidence of a biosynthetic role for L-ascorbic acid in oxalic acid biosynthesis has been gained, and the biosynthetic and metabolic processes associated with ascorbic acid analogs in fungi have been explored.

Keywords: Analogs of ascorbic acid; Ascorbic acid; Biosynthesis; Erythroascorbic acid; L-Galactono-1,4-lactone, L-Gulono-1,4-lactone; Metabolism; Oxalic acid; Tartaric acid; Threonic acid; Vitamin C

Contents

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1. Introduction

Ascorbic acid gains its name from its inherent property as vitamin C, the nutritional requirement of scurvy-prone animals incapable of synthesizing this compound (Moser & Bendich, 1991). Only L-ascorbic acid has this quality. Although much progress was made as regards vitamin C following its discovery in the earlier years of this century, biochemical details remained obscure until the active principal was isolated (Hughes, 1983) and its structure was elucidated in 1933 by analysis (Herbert, Hirst, Percival, Reynolds, & Smith, 1933) and synthesis (Reichstein, Grussner, & Openauer, 1933). A comprehensive review of biomedical roles for ascorbic acid appeared in 1996 (Harris, 1996).

The structure of L-ascorbic acid (a trivial name for L-*threo*-hex-2-enono-1,4-lactone) is tantalizingly simple, a six carbon aldono-1,4-lactone with a 2-keto group in its tautomeric enediol configuration. Comparison of L-ascorbic acid with D-erythroascorbic acid (a trivial name for D-*glycero*-pent-2-enono-1,4-lactone) reveal similarities in ring structure, features reflected in physical, chemical and biochemical properties (Wang, Seib, Paukstelis, Seib, & Takusagawa, 1995). D-Erythroascorbic acid will be discussed in the section on analogs of ascorbic acid.

Ascorbic acid is found in all higher plant species and probably in all algal classes. Animals which possess the capacity to oxidize L-gulono-1,4-lactone can synthesize ascorbic acid. Animals which lack this capacity (certain insects, fish, birds and mammals including primates) must find a nutritional source in order to survive, hence the designation 'vitamin C' for L-ascorbic acid. Advances in nutrition (Roig, Rivera, & Kennedy, 1993) as well as the commercial availability of ascorbic acid (Crawford & Crawford, 1980) have relieved humans from total dependence on natural food sources as a source of vitamin C. These same accomplishments have led to diminished interest in ascorbic acid biosynthesis and for many years subsequent to establishment of ascorbic acid structure, such efforts lagged. Meanwhile, progress regarding biochemical roles for ascorbic acid in plants (Smirnoff, 1996; Asada, 1997; Noctor & Foyer, 1998) continued to grow to a point where it is prudent to revisit aspects dealing with ascorbic acid biosynthesis and metabolism. Moreover, recent developments pertaining to the occurrence of ascorbic acid analogs in fungi prompts one to include these additional observations in this critique.

2. Biosynthesis of ascorbic acid

2.1. Inversion versus noninversion of the hexose carbon chain in ascorbic acid biosynthesis

In 1952, it was found that rats pretreated with the xenobiotic, chlorotone, and then fed D-[1-^{14}C]glucose excreted urine in which ^{14}C-labeled ascorbic acid was predominantly labeled at carbon 6 (Horowitz, Doerschuk, & King, 1952). Subsequent experiments with either drugged or normal rats fed D-[6-^{14}C]- or D-[2-^{14}C]glucose (Horowitz & King, 1953; Loewus, Kelly, & Hiatt, 1960; Burns, 1967) prompted a scheme for ascorbic acid biosynthesis (Fig. 1, upper scheme) Normal rats supplied with unlabeled D-glucurono-1,4-lactone, D-galacturonic acid methyl ester or selected hexonolactones provided peripheral evidence (Isherwood, Chen, & Mapson, 1954). In this scheme, D-glucose is oxidized at C6 to D-glucuronic acid. which undergoes reduction at C1 to L-gulonic acid (a convention in carbohydrate nomenclature *reverses* sequential numbering of the carbon chain so that C1 through C6 of D-glucuronic acid becomes C6 through C1 of L-gulonic acid, simply speaking, an *inversion* of the carbon chain). Subsequent lactonization of L-gulonic acid followed by oxidation gives ascorbic acid with a carbon chain sequence inverse to that of D-glucose. The presence or absence of an oxidase catalyzing the final step for ascorbic acid biosynthesis determines whether there is a vitamin C requirement.

An analogous pathway (Fig. 1, lower scheme) which invoked D-galacto/L-galacto configurations was proposed for biosynthesis of ascorbic acid in higher plants (Isherwood et al., 1954). Neither radiolabeled substrates nor the proposed primary precursor, D-galactose, was used in this study. When the scheme was tested with D-[1-, 2- or 6-^{14}C]glucose administered to detached ripening strawberries, ^{14}C was recovered predominately from carbon 1, 2 or 6, respectively, of ascorbic acid. Similar patterns were found in sucrose-derived glucose and pectin-derived D-galacturonate that served as internal controls, clear evidence of *direct* conversion, i.e., retention of the six carbon chain *without* inversion (Loewus, Jang, & Seegmiller, 1956). Subsequent studies in which D-[1-^{14}C] glucose was administered to other species including cress seedlings

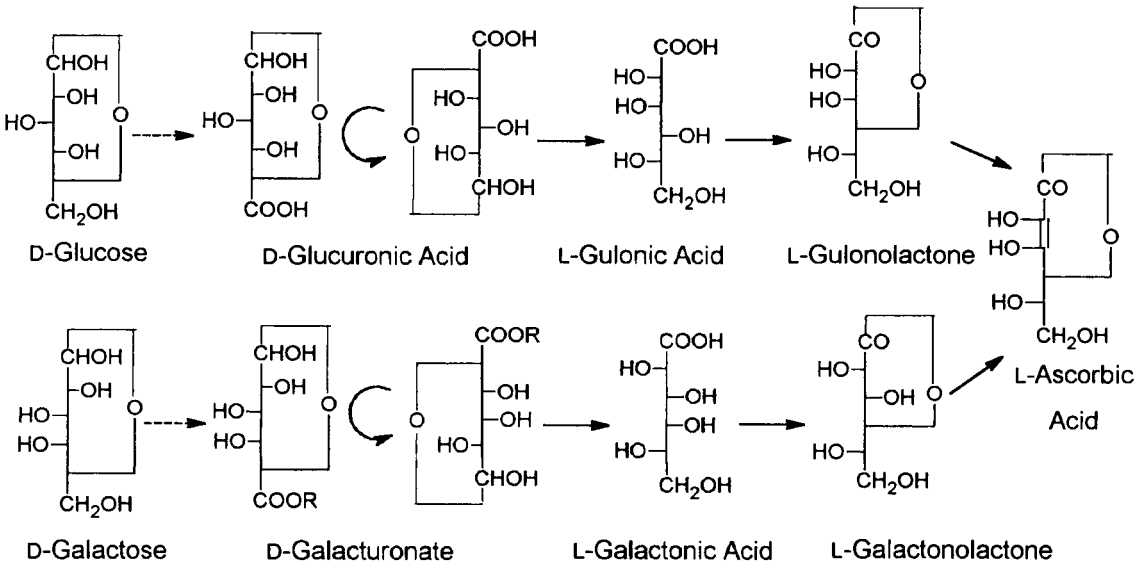

Fig. 1. Original schemes proposed for L-ascorbic acid biosynthesis: upper) rat studies, Burns (1967), lower) plant studies, Isherwood et al. (1954). In this and following figures, carbon-hydrogen bonds other than those attached to terminal carbons have been deleted for reasons of clarity.

(*Lepidium sativum* L.), parsley leaf (*Petroselinum crispum* L.), geranium apices (*Pelargonium crispum* L.), Virginia creeper leaves (*Parthenocissus quinquefolia* L.) and duckweed (*Lemna minor* L.) confirmed these observations (Loewus & Jang, 1957; Loewus, 1965; Loewus, Wagner, & Yang, 1975; Helsper, Saito, & Loewus, 1981). It should be pointed out that experiments involved in administration of D-[1-^{14}C]glucose to cress seedlings, the same plant utilized to propose the D-galacto/L-galacto scheme mentioned above, failed to show carbon chain inversion (Loewus & Jang, 1957).

D-Galactose was untested as a substrate for ascorbic acid biosynthesis by Isherwood et al. (1954). When D-[1-^{14}C]galactose was supplied to ripening strawberries, some redistribution of ^{14}C into carbon 6 of ascorbic acid was observed but as in the case of berries fed D-[1-^{14}C]glucose most of the ^{14}C was present in carbon 1 of ascorbic acid as in other metabolic products such as sucrose-derived D-glucose and pectin-derived D-galacturonic acid (Loewus & Jang, 1958). Some redistribution of ^{14}C from C1 into C6, due to hexose and triose phosphate metabolism, was encountered in all of these studies.

2.2. Question of a uronic acid intermediate in ascorbic acid biosynthesis by higher plants

Inclusion of D-galacturonic acid in the plant scheme and its reduction to L-galactono-1,4-lactone (Fig. 1, lower scheme) left unanswered such questions as the biosynthetic origin of D-galacturonic acid and the absence of naturally-occurring L-galactono-1,4-lactone. Overwhelming experimental evidence indicates that epimerization of UDP-D-glucuronic acid at C4 to form UDP-D-galacturonic acid is the major biosynthetic route to this uronic acid in plants (Feingold, 1982). Metabolic breakdown of galacturonic acid-containing oligo- or polysaccharides is the most likely source of free D-galacturonic acid. Direct oxidation of D-galactose is unlikely. As regards the proposed role of D-galacturonic acid or its putative derivative, methyl D-galacturonate, in L-galactono-1,4-lactone formation, an enzymic conversion was described (Mapson & Isherwood, 1956). When D-[1-^{14}C]galacturonic acid or methyl D-[uniformly-labeled ^{14}C]galacturonate was supplied to ripening strawberries, ^{14}C was recovered in both ascorbic acid and L-galactonic acid. With D-[1-^{14}C]galacturonic acid as the source of ^{14}C, label in both products was exclusively in C6, essentially a reduction of C1 of D-[1-^{14}C]galacturonic acid to form L-[6-^{14}C]galactonic acid/lactone with its implicit inversion of carbon chain sequence. Whether oxidation of D-galacturonic acid produced L-galactono-1,4-lactone or the free acid was undetermined (Loewus & Kelly, 1961a).

To examine the broader question of uronic acid metabolism and its putative role in ascorbic acid biosynthesis in plants, experiments were run with D-[1-^{14}C]- and D-[6-^{14}C]glucurono-1,4-lactone in ripening strawberries. ^{14}C was incorporated into ascorbic acid inverse to its site in glucuronolactone, i.e. C6 and C1, respectively, but pectin-derived D-galacturonic acid from the labeled berry had all of its ^{14}C in the same position as the form of D-glucuronolactone supplied. Interestingly, a portion of the labeled glucuronolactone

was cleaved during metabolism to yield $^{14}CO_2$ when D-[6-^{14}C]glucurono-1,4-lactone was supplied whereas D-[1-^{14}C]xylose was the labeled product of cleavage when D-[1-^{14}C]glucurono-1,4-lactone was supplied (Loewus & Kelly, 1959; Finkle, Kelly, & Loewus, 1960). Neither free D-glucuronic nor D-galacturonic acid was detected but labeled gulonic acid was found.

Detached strawberry fruit or parsley leaf supplied with *myo*-inositol cleaved this cyclitol between C1 and C6 to form D-glucuronic acid (Loewus, Kelly, & Neufeld, 1962). When *myo*-[2-^{3}H]inositol or *myo*-[2-^{14}C]inositol was used, labeled cell wall polysaccharides, L-gulonic acid, a hexaric acid (probably D-glucaric acid), and D-xylose were recovered. Neither ^{3}H nor ^{14}C was found in recrystallized ascorbic acid. Hydrolysis of the cell wall with pectinase released labeled D-galacturonate, D-glucuronic acid, D-xylose and L-arabinose. Discovery of 1L-*myo*-inositol-1-P synthase (E.C. 5.5.1.4), revealed an alternate pathway for conversion of D-glucose-6-P to UDP-D-glucuronic acid and its metabolic products, namely UDP-D-galacturonic acid, UDP-D-xylose, UDP-D-apiose, and UDP-L-arabinose, all precursors of cell wall polysaccharides; a pathway independent of oxidation of UDP-D-glucose to UDP-D-glucuronic acid by UDP-D-glucose dehydrogenase (Loewus & Loewus, 1983; Loewus, Everard, & Young, 1990b). Implicit in these findings is the conclusion that free D-glucuronic acid, generated in situ, is not reduced and is not a natural precursor of ascorbic acid in plants.

2.3. The final biosynthetic step, oxidation of L-galactono-1,4-lactone

The most significant observation to stem from Isherwood et al.'s 1954 paper, facile conversion of exogenously-supplied L-galactono-1,4-lactone to ascorbic acid in plants, has been confirmed repeatedly (Jackson, Wood, & Prosser,1961; Baig, Kelly, & Loewus, 1970; Leung & Loewus, 1985a) and the enzyme involved, L-galactono-1,4-lactone:ferricytochrome c oxidoreductase (EC 1.3.2.3), has been purified from spinach leaf (Matsuda, Ishikawa, Takada, & Shigeoka, 1995), sweet potato roots (Ôba, Ishikawa, Nishikawa, Mizuno, & Yamamoto, 1995) and cauliflower florets (Østergaardt. Persiau, Davey, Bauw, & Van Montagu, 1997). It is also involved in synthesis of ascorbic acid in wounded tissues of white potato tuber (Ôba, Fukui, Imai, Iriyama, & Nogami, 1994). Any attempt to link this activity to ascorbic acid biosynthesis in normal plant tissues must also account for overall lack of carbon chain inversion between the hexose precursor (presumably D-glucose) and ascorbic acid, cellular access to D-galacturonic acid, and its ultimate reduction to L-galactono-1,4-lactone, the essential substrate. Wound-induced synthesis has been discussed in an earlier review where it was pointed out that release of uronic acid residues stemming from degradation of cell wall polysaccharides, notably pectic and hemicellulosic components, might provide substrates for an 'abnormal' path of ascorbic acid formation (Loewus, 1980). Additional support for the view that D-glucose-to-L-ascorbic acid conversion in plants proceeds without inversion of the carbon chain is found in studies of L-ascorbic acid metabolism (Loewus, 1988). This aspect will be considered later on in this review.

While most evidence for a D-glucose-to-L-ascorbic acid conversion has been gathered in experiments involving radioisotopes in higher plants, similar studies have also been made on ascorbic acid biosynthesis in algae. Here one encounters a great diversity of chloroplast-containing organisms, some with distinct animal-like qualities, others quite plant-like (Bhattacharya & Medlin, 1998). If ascorbic acid is an essential constituent of algae, then this compound should be encountered in all algal classes. A test for noninversion or inversion of the carbon chain during conversion of D-glucose to L-ascorbic acid might reveal interesting differences in ascorbic acid biosynthesis among algal classes. The test was run on four algal classes. Only *Chlorella pyrenoidosa* (Chlorophyta) (Renstrøm, Grün, & Loewus, 1982/1983) and *Prototheca*, a related non-green species (unpublished studies), showed noninversion. Inversion was found in *Ochromonas danica* (Chrysophyta) (Helsper, Kagan, Hilby, Maynard, & Loewus, 1982), *Cyclotella cryptica* (Bacillariophyta) (Grün & Loewus, 1984), and *Euglena gracilis* (Euglenophyta) (Shigeoka, Nakano, & Kitaoka, 1979a). Algae which exhibited inversion bore cellular properties more closely allied to animals than to plants. Clearly, a comprehensive in-depth study of ascorbic acid biosynthesis that encompasses all algal classes is in order.

Although studies involving radioisotopic tracers failed to reveal an enzymic sequence for ascorbic acid biosynthesis, it did set certain constrictions on future efforts in this direction. In higher plants, D-glucose-to-L-ascorbic acid conversion proceeds without inversion of the carbon chain. A minimum of four chemical events are required:

- Oxidation of C1
- Oxidation at C2 or C3
- Epimerization or an equivalent process at C5
- Lactonization between C1 and C4

A further consideration is conservation of the hydroxymethyl group at C6 of D-glucose. A functional sequential order for these processes is crucial to any proposed biosynthetic scheme. An experimental attempt in this regard is shown in Fig. 2. Evidence for epimerization at C5 during conversion was established early-on in radioisotopic studies (Loewus & Kelly,

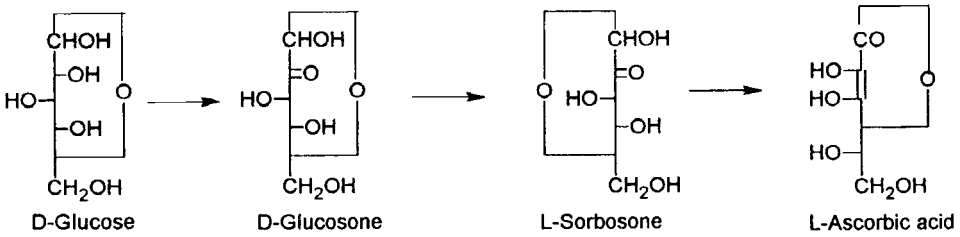

Fig. 2. Proposed 'noninversion' scheme for L-ascorbic acid biosynthesis involving oxidation of D-glucose at C1 and C2, and epimerization at C5 (Loewus et al., 1990).

1961b). This epimerization is accompanied by exchange of C4-bound hydrogen with the medium (Grün, Renstrøm, & Loewus, 1982). As regards oxidation at C3, experiments with D-[6-^{14}C]glucosone (D-*arabino*-[6-^{14}C]hexos-2-ulose) revealed that this putative intermediate was much more effective than D-[6-^{14}C]glucose for labelling L-ascorbic acid in bean and spinach leaves and that 90 to 98% of ^{14}C in L-ascorbic acid remained in C6 as compared to much greater redistribution of ^{14}C within glucosyl and galactosyl residues from the same experiments (Loewus & Kelly, 1961b). D-Glucosone occurs in red algae and several species of macrofungi but whether this osone occurs in higher plants has yet to be determined. If one assumes that D-to-L epimerization at C5 follows C2 oxidation, the product is L-sorbosone (L-*xylo*-hexos-2-ulose) which is oxidized at C1 by a NADP-dependent dehydrogenase found in bean and spinach leaf (Saito, Nick, & Loewus, 1990). L-Glucosone also represses conversion of D-glucose to L-ascorbic acid. L-[uniformly-labeled ^{14}C]Sorbosone is less effective in these radiolabeling studies (Loewus, Bedgar, Saito, & Loewus, 1990a). Kinetic parameters for cell-free partially purified L-sorbosone dehydrogenase, however, reveal very low affinity for the purported final intermediate, L-sorbosone.

Saito and Loewus (1992) used D-[5-^{3}H, 1-^{14}C]- and -[5-^{3}H, 6-^{14}C]glucosone to study oxidative cleavage of L-ascorbic acid to oxalic acid and L-tartaric acid in detached leaves of *Pelargonium crispum*. Both forms of labeled glucosone produced ^{14}C-labeled ascorbic acid (0.2%) but ^{3}H was lost in the conversion, an indication that epimerization at C5 involved labilization of that hydrogen-carbon bond. In another study, Saito (1996) traced conversion of D-glucosone to L-ascorbic acid and oxalate in a duckweed (*Lemna minor*). This water plant utilizes L-ascorbic acid for oxalic acid biosynthesis and accumulates this final product in discrete crystalline bundles within specialized cells called idioblasts (Franceschi, 1987). D-[1-^{14}C]- or -[6-^{14}C]-Glucosone was added to the support medium of floating plants. Over 24 h, 4% of the ^{14}C was converted to L-ascorbic acid in each experiment. A time course study of plants given D-[1-^{14}C]glucosone for 24 h followed by replacement with fresh unlabeled inorganic medium and continued growth in light over 6 days revealed a three-fold decrease of labeled L-ascorbic acid accompanied by increases, first in soluble and, subsequently, insoluble [^{14}C]oxalate. At each daily interval, disappearance of ascorbate was equivalent to labeled oxalic acid/oxalate formed, clearly a precursor–product relationship which could be extended to include D-glucosone.

Even though the scheme set forth in Fig. 2 poses serious questions concerning its presence in higher plants, it does provide clues as to the structure of intermediates involved in a direct conversion of D-glucose to L-ascorbic acid. D-Glucose is a more highly selected carbon source than D-glucose for L-ascorbic acid biosynthesis and undergoes the essential C5 epimerization. L-Sorbosone is converted to L-ascorbic acid, albeit a poorly bound substrate for the NADP-linked oxidoreductase involved.

Lycorine, a pyrrolophenanthridine alkaloid found in *Crinum* species, has been reported to be an effective inhibitor of L-ascorbic acid biosynthesis, specifically L-galactono-1,4-lactone dehydrogenase (Arrigoni et al., 1997 and earlier references therein). In the course of L-sorbosone dehydrogenase experiments described above, assays were run in the presence of 0.1 to 100 mM lycorine to test for potential inhibition of L-ascorbic acid formation. No inhibition was observed. In earlier studies on conversion of L-galactono-1,4-lactone to L-ascorbic acid in germinating lily pollen, addition of 0.4 to 140 mM lycorine inhibited germination but it had no effect on conversion of L-galactono-1,4-lactone to L-ascorbic acid (Leung & Loewus, 1985a). A similar observation was made during studies on accumulation of L-ascorbic acid in tuber-forming stolon tips of potato where the presence of lycorine failed to prevent ascorbic acid accumulation (Viola, Vreugdenhil, Daview, & Sommerville, 1998). Recently, L-galactono-1,4-lactone dehydrogenase was isolated from a mitochondrial extract of cauliflower (the same plant source used by Mapson and Breslow (1958) in their pioneering studies), purified to apparent homogeneity, and used to prepare cDNA encoding the enzyme (Østergaardt et al., 1997). A carefully purified prep-

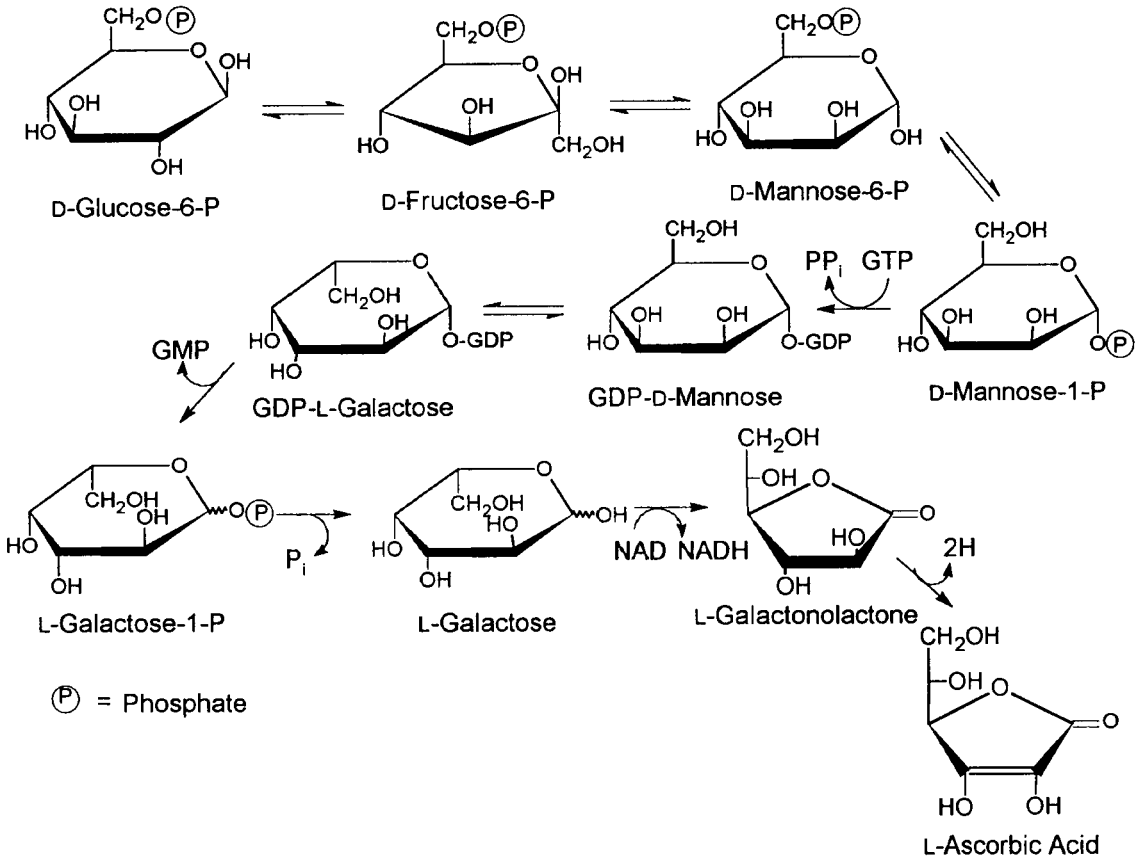

Fig. 3. New scheme proposed for L-ascorbic acid biosynthesis in higher plants by Wheeler et al. (1998). See text for details. Some anomeric bonds are undefined.

aration of lycorine (Davey et al., 1998) had no influence on this dehydrogenase activity at concentrations up to 200 mM. Moreover, reports on biological effects of lycorine (Østergaardt et al., 1997) could not be reproduced. In view of conflicting observations concerning effects of lycorine on ascorbic acid biosynthesis, use of this inhibitor as a specific tool for such studies must be regarded with caution.

Use of specifically-labeled D-glucose in radiolabeled experiments on L-ascorbic acid biosynthesis in plants is always accompanied by redistribution patterns similar to those routinely encountered in studies of other hexose products. This is caused by partial equilibration with triose phosphate during passage of labeled hexose through the hexose phosphate pool. Generally speaking, the final pattern resulting from this process is a 20 to 40% exchange between triose moieties, resulting in appearance of 10 to 20% of the label in the diametrical position. It provides indirect evidence of the role of hexose phosphate during biosynthesis and has led to a speculative suggestion that D-glucose 6-phosphate is the ultimate source of L-ascorbic acid through a pathway distinct from the one leading to UDP-D-glucose and its products (Loewus, 1963).

2.4. A rational scheme for L-ascorbic acid biosynthesis in higher plants

Progress toward a rational interpretation of L-ascorbic acid biosynthesis in higher plants was stalled for many years by these seemingly incompatible findings which required retention of the carbon chain sequence of D-glucose, a role for hexose phosphate, an epimerization, and the curious fact that L-galactono-1,4-lactone was the most effective substrate for the final oxidation step. Then, in a single brilliant contribution, Wheeler, Jones, and Smirnoff (1998) provided a plausible pathway (Fig. 3) which accommodated all unsettled issues.

Key issues – epimerization at C5 of GDP-D-mannose to produce GDP-L-galactose, oxidation of L-galactose to L-galactonolactone by a newly discovered dehydrogenase (L-galactose:NADP$^+$ oxidoreductase), and evidence that L-galactose was readily converted to L-ascorbic acid – were all resolved in this study. When L-galactose was supplied to barley leaf slices, there was a significant increase in ascorbic acid comparable to that produced by L-galactono-1,4-lactone. Similarly, L-galactose increased the ascorbic acid level in *Arabidopsis thaliana* leaves and embryonic axes of ger-

minating pea seedlings. An enzyme capable of oxidizing L-galactose was detected in both tissues and was partially purified from pea axes. Its product showed the characteristic lactone response with hydroxylamine. It was assumed that the initial product was L-galactono-1,5-lactone, an unstable structure that rearranges spontaneously to the more stable L-galactono-1,4-lactone. The authors also detected GDP-mannose-3,5-epimerase activity in pea axes extracts and in ammonium sulfate precipitates from *A. thaliana* leaves. When GDP-D-[uniformly-labeled ^{14}C]mannose was supplied to *A. thaliana* leaves, they found free L-galactose as well as one or more acidic products in the reaction mixture, possibly L-galactose-1-P. If NAD^+ was added in the latter stages of the reaction period, ascorbic acid was produced. When the experiment was repeated with pea axes extract containing cyctochrome c as an electron acceptor for L-galactono-1,4-lactone dehydrogenase and L-ascorbic acid as carrier, radioactive ascorbic acid was detected.

Unpublished studies (S. E. Keates, F. A. Loewus and V. R. Franceschi) using the L-galactose-to-L-ascorbic acid conversion nicely demonstrates oxalic acid biosynthesis in *Pistia stratiotes*, a water-borne plant in which L-[1-^{14}C]ascorbic acid is metabolized to yield [^{14}C]oxalic acid. This experiment is described in the following section on metabolic products. Clearly, much remains to be accomplished but Wheeler et al.'s general scheme provides direction for future studies. Application to systems such as the ascorbate-deficient mutant *vtc1* of *A. thaliana* (Conklin, Pallanca, Last, & Smirnoff, 1997) accumulation of ascorbic acid in tuberizing stolon tips (Viola et al., 1998) and oxalate-accumulating plants (Franceschi & Loewus, 1995) is anticipated.

Purified L-galactono-1,4-lactone dehydrogenase (EC 1.3.2.3) from three plant sources (Matsuda et al., 1995; Ôba et al., 1995; Østergaardt et al., 1997), all highly specific for L-galactono-1,4-lactone, provide strong support for the view that this intermediate is, indeed, the immediate precursor of ascorbic acid in higher plants. Yet intact plant tissues and at least one alga do utilize L-gulono-1,4-lactone, the C3 diasteriomer of L-galactono-1,4-lactone (Isherwood et al., 1954; Baig et al., 1970; Shigeoka, Nakano, & Kitaoka, 1979b). One explanation might be the existence of a discrete L-gulono-1,4-lactone dehydrogenase in plants. An example is *Euglena gracilis* in which both activities have been detected, L-galactono-1,4-lactone dehydrogenase in mitochondrial membranes (Shigeoka et al., 1979a) and L-gulono-1,4-lactone dehydrogenase in microsomes (Shigeoka et al., 1979b). Euglenoids are free-swimming unicellular algae with animal-like features and photosynthetic capacity, possibly due to endosymbiosis (Bhattacharya & Medlin, 1998). Another possibility is epimerization of the hydroxyl function on C3 in L-gulono-1,4-lactone to produce its diastereomer, L-galactono-1,4-lactone, and vice versa. When bean leaves or immature strawberry fruits were supplied with either ^{14}C-labeled L-gulono-1,4-lactone or L-galactono-1,4-lactone, both produced labeled L-ascorbic acid as well as the corresponding aldonolactone, diastereomeric at C3 (Baig, 1969). The need for a broad survey of ascorbic acid-synthesizing activities in plants is evident.

3. Products of ascorbic acid metabolism

Plant-related functional roles involving intact L-ascorbic acid are largely related to redox properties associated with this molecule. This aspect of ascorbic acid metabolism has received considerable attention (Foyer, 1993; Arrigoni, 1994; Allen, 1995; Halliwell, 1996; Smirnoff, 1996; Smirnoff & Pallanca, 1996; Asada, 1997; Foyer, 1998; Noctor & Foyer, 1998). Of special note is Smirnoff's review (Smirnoff, 1996) in which one finds incisive evaluations of current progress on occurrence and localization of ascorbic acid, its biochemical functions and role(s) in photosynthesis and plant stress, its correlation to cell wall growth and development, and possible involvement of ascorbic acid in cell division. Less attention has been given to those processes involved in breakdown of L-ascorbic acid yet we know from studies initially used to determine its structure (Herbert et al., 1933) that treatment of ascorbate with alkaline sodium hypoiodite yields oxalate and L-threonate. Oxidation with H_2O_2 under alkaline conditions achieves the same result (Isbell & Frush, 1979). The nature of this chain-cleaving process is still only partially understood (Kwon, Foote, & Khan, 1989; Kurata, Miyake, & Otsuka, 1996; Deutsch, 1998a,b) and older views invoking dehydroascorbic and diketogulonic acids as key intermediates preceding cleavage of ascorbic acid by biological systems need closer scrutiny (Fleming, Miyashita, Quay, & Bensch, 1983; Frimer & Gilinsky-Sharon, 1995; Miyake & Shibamoto, 1995; Welch et al., 1995; Deutsch & Santhosh-Kumar, 1996; Deutsch, 1997; Jung & Wells, 1998; Miyake & Kurata, 1998). From a biological viewpoint, as will be discussed shortly, chain cleavage of L-ascorbic acid by H_2O_2 may have a major role in plant metabolism.

Ascorbic acid is rapidly synthesized during seed germination and continues to be produced in regions of active growth throughout the life of the plant yet seldom does it accumulate beyond 100 mg% of fresh weight. One might argue that certain ascorbic acid-rich tissues (i.e. rose hips, fruit of the West Indian cherry, and green walnut hulls) probably represent those plant organs where metabolic breakdown of ascorbic acid fails to keep pace with biosynthesis or where key enzy-

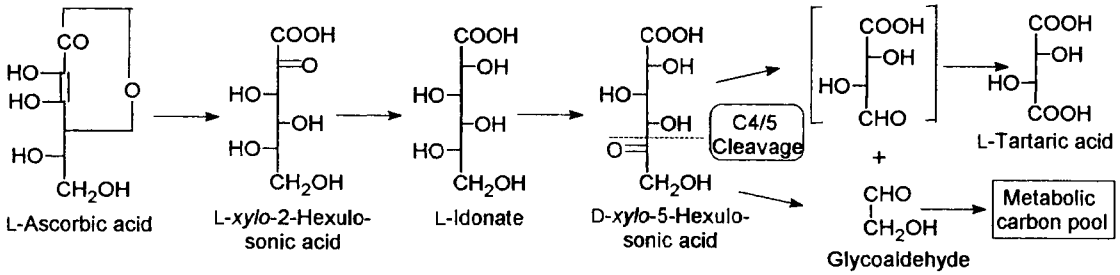

Fig. 4. C4/C5 cleavage of L-ascorbic acid to form L-tartaric acid in vitaceous plants.

mic requirements for such processes are lost or inhibited. Apart from homeostatic mechanisms needed to maintain functional roles such as control of active oxygen, the bulk of carbon products resulting from ascorbic acid metabolism accumulates either in specific end products such as L-threonic acid, L-tartaric acid, glyceric acid, oxalic acid and CO_2 or is recycled through triose and hexose phosphates. It is this aspect that is now considered.

3.1. Cleavage of L-ascorbic acid – tartrate biosynthesis

In 1956, Hough and Jones (1956) suggested that L-ascorbic acid might be a precursor of (+)-tartaric acid through loss of C1 + C2 of the former followed by subsequent oxidation of terminal carbons of the C_4 fragment. Their suggestion was tested in detached grape leaf, a tartrate-accumulator, infiltrated with L-[6-^{14}C]ascorbic acid (Loewus & Stafford, 1958). Negligible ^{14}C was recovered in tartaric acid but over 85% of the ^{14}C was present in glucose, fructose, sucrose and unidentified higher oligo- or polysaccharides. Ninety percent of the ^{14}C in glucose was equally distributed among C1, C3, C4 and C6, a pattern characteristic of ^{14}C-labeled carbon recycled through triose phosphate metabolism. In 1969, Saito and Kasai administered L-[1-^{14}C]ascorbic acid to immature grape berries and discovered that after 24 h of metabolism, 72% of the ^{14}C in the soluble fraction was recovered as tartaric acid with most of that ^{14}C in carboxyl carbon. Together, these experiments involving L-[1-^{14}C]- and -[6-^{14}C]ascorbic acid indicate that ascorbic acid was cleaved between C4 and C5, with the C_4 fragment converted to L-(+)-tartaric acid (Wagner, Yang, & Loewus, 1975) and the C_2 fragment recycled back into hexose phosphate. These results support direct D-glucose-to-L-ascorbic acid conversion inasmuch as grape tissue produced only carboxyl-labeled tartaric acid from L-[1-^{14}C]ascorbic acid.

Pelargonium, a geraniaceous plant, also accumulates L-tartaric acid (Stafford, 1961). When L-[1-^{14}C]- or -[6-^{14}C]ascorbic acid were supplied to *P. crispum* leaves, only the latter labeled tartaric acid, exclusively in carboxyl carbon. L-[1-^{14}C]Ascorbic acid, on the other hand, produced labeled oxalic acid (Wagner & Loewus, 1973). Here, in contrast to the grape experiments, L-ascorbic acid was cleaved between C2 and C3. When L-[6-^{14}C]galactono-1,4-lactone was used as source of label to *P. crispum* leaves, 90% of the ^{14}C present in ascorbic acid was in C6 and 96% of the ^{14}C in tartaric acid was in carboxyl groups (the method of degradation did not distinguish C1 from C4 of tartaric acid). When uniformly labeled L-[^{14}C]galactono-1,4-lactone was supplied, specific radioactivities in both products, tartaric acid and oxalic acid, were identical. Subsequent studies using L-[4-^{14}C]-, -[4-^{3}H]-, and -[uniformly-labeled ^{14}C]ascorbic acid confirmed the distinctive nature of C4/C5 cleavage in grape as compared to C2/C3 cleavage in *P. crispum* (Williams & Loewus, 1978a; Williams, Saito, & Loewus, 1979). Tartaric acid labeling from ascorbic acid in grape was highest in days preceding or at anthesis but declined with berry maturity (Williams & Loewus, 1978a; Saito & Loewus, 1979).

The biosynthetic origin of tartaric acid in plants is only partially understood. Stafford (1959) identified genera in three families (Geraniaceae, Vitaceae and Leguminosae) where high levels of tartaric acid are found. *Bauhinia reticulata*, a leguminous plant, is reported to contain D-(−)-tartaric acid rather than the commonly occurring L-(+) form, but its biosynthesis is unknown. Of the three known pathways to L-(+)-tartrate, two arise from L-ascorbic acid and one from D-gluconate [(Wagner & Loewus, 1973; Williams & Loewus, 1978a; Williams et al., 1979; Saito & Kasai, 1982; 1984; Malipiero, Ruffner, & Rast, 1987; Saito & Loewus, 1989a, b, c). The first pathway (Fig. 4), occurs in grape and other vitaceous plants (Saito & Kasai, 1969; 1982; 1984; Malipiero et al., 1987).

The second pathway (Fig. 5), probably more significant due to its generation of oxalic acid, may exist in many plant families even though its role as a source of tartaric acid is more restricted (Wagner & Loewus, 1973; Yang & Loewus, 1975; Nuss & Loewus, 1978;

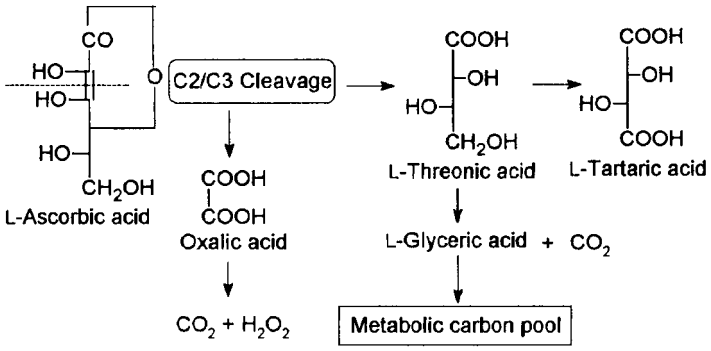

Fig. 5. C2/C3 cleavage of L-ascorbic acid to form oxalic acid, L-threonic acid and/or L-tartaric acid in geraniaceous and oxalate-accumulating plants.

Williams & Loewus, 1978a; Williams et al., 1979; Helsper & Loewus, 1982; Saito & Loewus, 1992; Saito, Ohmoto, & Kuriha, 1997).

The third pathway (Fig. 6) to tartaric acid does not involve ascorbic acid. Instead, D-gluconic acid is converted to D-*xylo*-5-hexulsonic acid, the third intermediate in Fig. 4. Carbon chain cleavage at C4/C5 follows (Saito & Loewus, 1989a, b; Saito, 1994).

This third pathway is present in leguminous plants and, possibly, as a very minor pathway in vitaceous plants (Saito & Loewus, 1989b). In a survey of 32 cultivars of *Phaseolus vulgaris* L., only seven cultivars accumulated significant levels of tartrate (Saito & Loewus, 1989a). These cultivars also showed increases in tartrate when administered D-gluconate. When D-*xylo*-5-hexulsonate was supplied to these seven tartrate accumulators and to seven nonaccumulators, all fourteen cultivars exhibited increases in tartrate. Cleavage of L-ascorbate or D-*xylo*-5-hexulsonate between C4 and C5 may be more common among plant species than noted here and a survey for conversion of D-*xylo*-5-hexulsonate to L-tartrate in higher plants is indicated. This process always leads to recycling of C5+C6 into triose and hexose phosphate metabolism. Conceivably, this C_2 fragment is glycoaldehyde or an equivalent enzyme-bound intermediate. Attempts to link it to glycolate metabolism through use of such glycolate inhibitors as semicarbazide·HCl, isonicotinic acid hydrazide, or α-hydroxy-2-pyridine-methane sulfonate were inconclusive (Saito & Loewus, 1979).

3.2. Cleavage of L-ascorbic acid between C2 and C3 – oxalate biosynthesis

Oxalic acid biosynthesis in plants has been an object of research and conjecture for many years (Hodgkinson, 1977). In the course of studies on the biosynthetic connection between ascorbic acid and tartaric acid in *P. crispum* (Wagner & Loewus, 1973), oxalic acid was identified as the C_2 fragment corresponding to C1+C2 of ascorbic acid. The possibility that ascorbic acid might be a significant carbon source of oxalic acid in plants generally, prompted further studies using oxalate-accumulating plants (*Amaranthus*, *Begonia*, *Beta*, *Chenopodium*, *Halogeton*, *Lemna*, *Nicotiana*, *Oxalis*, *Rheum*, *Rumex*, *Spinacia* and *Triticum*). When L-[1-^{14}C]ascorbic acid was administered to seedlings or detached leaves (or in the case of *Lemna* to the intact plant), [^{14}C]oxalic acid was formed (Yang & Loewus, 1975; Nuss & Loewus, 1978). Dehydro-L-ascorbic acid was equally effective; possibly due to its facile reduction to L-ascorbic acid by dehydroascorbic acid reductase (Foyer, 1998). Diketo-L-gulonic acid was ineffective as a carbon source for oxalic acid due to irreversibility following hydrolysis of dehydroascorbic acid. Little, if any, tartaric acid was

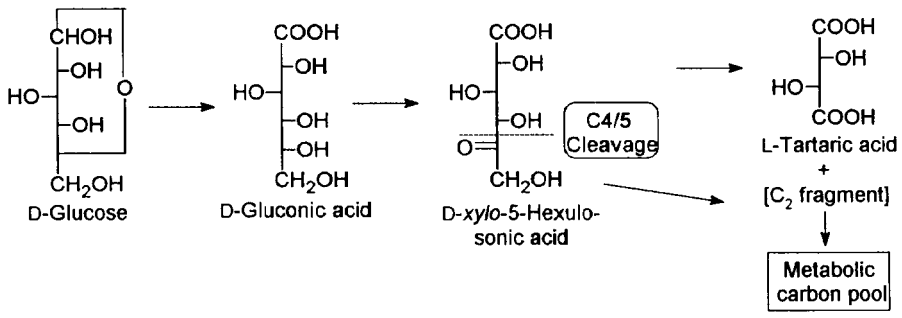

Fig. 6. C4/C5 cleavage of D-gluconic acid to form L-tartaric acid in leguminous plants.

found in these plants which led to renewed interest in the metabolic fate of the C_4 fragment. To explore this question, a general procedure was developed for preparation of specifically-labeled forms of L-ascorbic acid to trace the fate of any particular atom in the molecule (Williams & Loewus, 1978b). A survey of oxalate-accumulating species supplied with L-[1-^{14}C]-ascorbic acid and L-[uniformly-labelled ^{14}C]-ascorbic acid (Nuss & Loewus, 1978) revealed that the latter contained one third as much ^{14}C in the C_2 fragment, oxalic acid, as was present in tissues labeled with L-[1-^{14}C]-ascorbic acid, solid evidence of C2/C3 carbon-chain cleavage. Five-day-old seedlings of *Rumex crispus* (curlydock) labeled with [^{14}C]oxalic acid released over twice as much $^{14}CO_2$ as those labeled with L-[1-^{14}C]-ascorbic acid. With seven-day-old seedlings, the ratio of $^{14}CO_2$ release between [^{14}C]oxalic acid-labeled and L-[1-^{14}C]-ascorbic acid-labeled seedlings increased five-fold. Although the source of this $^{14}CO_2$ was unclear at the time of the study, it now known to arise from two sources, decarboxylation of L-threonic acid and oxidation of oxalic acid by oxalate oxidase.

A more detailed study of ascorbic acid metabolism in *Rumex* labeled with various forms of labeled ascorbic acid revealed that in this plant L-threonic acid and its metabolic products were significant products (Helsper & Loewus, 1982). In *Rumex* leaves labeled with L-[uniformly-labeled ^{14}C]ascorbic acid for 24 h, percent distribution of ^{14}C was threonic acid 14, tartaric acid 1, oxalic acid 11, other compounds 49, residue 14, and CO_2 11. When *Rumex* leaves were labeled with ammonium L-[uniformly-labeled ^{14}C]threonate, percent distribution of ^{14}C was threonic acid 14, tartaric acid 6, oxalic acid <1, other compounds 38, residue 21, and CO_2 21. *Rumex* leaves labeled with L-[uniformly-labeled ^{14}C]threono-1,4-lactone gave results comparable to the latter. Clearly, tartaric acid is a metabolic product of L-threonic acid.

Gas chromatography of the trimethylsilylated threonate fraction from both experiments revealed the presence of glycerate as well as threonate. These metabolic processes are included in Fig. 5. In *Rumex* leaves labeled with L-[4-^{3}H]- or -[6-^{3}H]ascorbic acid, 20 and 7%, respectively, of the ^{3}H was recovered in threonic acid whereas no ^{3}H was found in tartaric acid although tartaric acid was present to the extent of 0.1 to 0.2 mg/g fresh weight. Oxidation of threonic acid from L-[4-^{3}H]ascorbic acid-labeled leaves with nitric acid produced labeled tartaric acid with no change in specific radioactivity, evidence that ^{3}H was attached to an internal carbon atom. Oxidation of threonic acid from L-[6-^{3}H]ascorbic acid-labeled leaves by metaperiodate released C4 as formaldehyde which contained all of the ^{3}H originally present in the threonic acid. In experiments with dual-labeled substrate, L-[4-^{3}H + uniformly-labeled ^{14}C]- or -[6-^{3}H + uniformly-labeled ^{14}C]ascorbic acid, no loss or exchange of ascorbic acid-bound ^{3}H was detected throughout the metabolic period but results also revealed that one of the two hydrogens on C4 of threonic acid was exchanged with the medium during its formation.

Decarboxylation of L-threonate produced L-glycerate. In *Rumex*, [uniformly-labeled ^{14}C]ascorbate as well as [uniformly-labeled ^{14}C]threonate produced labeled sucrose by recycling the labeled C_3 fragment through triose and hexose phosphate. Details regarding this path of metabolism are lacking but a reasonable suggestion is conversion of L-glycerate to D-glycerate via hydroxypyruvate followed by phosphorylation to phosphoglycerate.

3.3. Concerning C2/C3 and C4/C5 cleavage mechanisms of L-ascorbic acid

The biochemical mechanisms involved in C2/C3 and C4/C5 cleavage of L-ascorbic acid are poorly understood. Most information comes from chemical studies on ascorbic acid in solution. An effort to probe tartaric acid and oxalic acid formation in these two types of ascorbic acid cleavage was undertaken by Saito et al. (1997) through use of plant tissues labeled with ^{14}C-ascorbic acid administered in the presence of $^{18}O_2$ or $H_2{}^{18}O$. They used young leaves of *Pelargonium*, parsley, bean and grape, plant types employed in previous studies on ascorbic acid by Saito. Without going into experimental details which can be found in their paper, their data strongly suggested that C2/C3 cleavage of L-ascorbic acid involved both oxygenase and hydrolyase activities while that of C4/C5 cleavage involved only hydrolysis.

3.4. Concerning the biosynthetic path of oxalic acid in higher plants

Historically, oxalic acid biosynthesis has focused on involvement of intermediary compounds of plant metabolism; oxaloacetate, glycolate and glyoxylate, as well as plant constituents such as citrate which give rise to these organic acids. When it became evident that ascorbic acid was cleaved between C2 and C3 in oxalate-accumulating plants, focus shifted to hexose as an ultimate source of oxalate carbon in agreement with D-glucose-to-L-ascorbic acid conversion in higher plants. Implicit in these findings was the conclusion that C1 + C2 of D-glucose provided the carbon source of oxalate. Still, attempts to ferret out biochemical details accommodating a D-to-L transition and a role for L-galactono-1,4-lactone dehydrogenase were lacking until Wheeler et al. (1998) discovered that L-galactose was a crucial intermediate (Fig. 3). To test the putative role of L-galactose as precursor of ascorbic acid and, ultimately, of oxalic acid, an axenic culture

of *Pistia stratiotes* was selected as the experimental model (Tarlyn, Kostman, Nakata, Keates, & Franceschi, 1998) (Sarah E. Keates, Frank A. Loewus, Vincent R. Franceschi, unpublished studies). Calcium oxalate is the end product of a high capacity calcium regulation system in this plant but little is known concerning the source of oxalic acid used for this important regulatory system.

The experiment compared oxalic acid and calcium oxalate labeling patterns from L-[1-^{14}C]- and L-[6-^{14}C]ascorbic acid with those from L-[1-^{14}C]galactose. [^{14}C]Oxalic acid was the control. These labeled compounds were supplied to intact *P. stratiodes* via the culture medium. After a period of uptake previously shown to furnish a reasonable amount of ^{14}C to plant tissues, the plants were analyzed for labeled soluble and insoluble (calcium oxalate) oxalic acid pools in whole plant extracts. In addition, plants were chemically fixed and embedded for microautoradiography to determine if labeled oxalic acid was incorporated into calcium oxalate crystals within the plant. Of the extractable label in ascorbic acid, oxalic acid, and calcium oxalate, 32% was in calcium oxalate when [1-^{14}C]ascorbic acid was used versus only 2% when [6-^{14}C]ascorbic acid was used. Clearly, C1 + C2 of ascorbic acid was the carbon source of oxalic acid and calcium oxalate in *P. stratiotes* as found earlier in *Pelargonium* (Wagner & Loewus, 1973) and *Rumex* (Helsper & Loewus, 1982). In plants grown with L-[1-^{14}C]galactose, 39% of the extractable label was in calcium oxalate. In plants grown with the control, [^{14}C]oxalic acid, 85% of the extractable label was present in calcium oxalate. Current data indicate that:

- Ascorbic acid is a major substrate for synthesis of oxalic acid used for calcium regulation via calcium oxalate formation.
- Oxalic acid is derived from C1 + C2 of ascorbic acid.
- L-Galactose is just as effective as L-ascorbic acid in oxalic acid synthesis (which can be interpreted as evidence that L-galactose is converted first to ascorbic acid in *P. stratiotes*, as Wheeler et al. (1998) discovered in barley leaf slices, embryonic axes of pea seedlings, and *Arabidopsis* leaves.

Microautoradiography showed that virtually all the acid-extractable insoluble label (calcium oxalate) from [L-[1-^{14}C]galactose, L-[1-^{14}C]ascorbic acid, or [^{14}C]oxalic acid was located in calcium oxalate crystals produced by a specialized cell type whose primary function is high capacity calcium sequestration. Label from [6-^{14}C]ascorbic acid was heavily incorporated into cell walls as well as a large unidentified pool of nonoxalic acid soluble materials, but not into calcium oxalate, an observation consistent with results from experiments using L-threonic acid in *Rumex* (Helsper & Loewus, 1982). Related to the above experiments, it was found that the level of soluble and insoluble oxalic acid in the plant increased with increasing calcium in the growth medium. In addition, the amount of label incorporated into both soluble and insoluble oxalic acid when [1-^{14}C]ascorbic acid was fed to the plants increased with increasing calcium availability, indicating that the biosynthetic pathway from ascorbic acid to oxalic acid is inducible by calcium. This is consistent with ascorbic acid being a major precursor for oxalic acid used in the high capacity calcium regulation system in plants. This is a new concept in terms of the function of ascorbic acid in higher plants, and further points to the importance of this organic acid in the basic physiology of plant systems.

3.5. Oxidation of oxalic acid

In higher plants, oxalic acid is further oxidized to $2CO_2 + H_2O_2$ by oxalate oxidase (EC 1.2.3.4). Experiments in which L-[1-^{14}C]- or [uniformly-labeled ^{14}C]ascorbic acid was fed to plants suggest that this may be a significant internal source of CO_2. While it is still too early to speculate on the potential role of oxalate as a reservoir of CO_2 for photosynthesis in oxalate-accumulating plants in the sense prescribed by malate in C_4 photosynthesis, such a possibility should be considered. *Oxalis oregana* Nutt. (sorrel) is a likely model for such a study (Yang & Loewus, 1975; Nuss & Loewus, 1978). This plant is a ubiquitous ground cover in densely shaded habitat of Pacific Northwest rainforests in North America and exhibits a remarkable response to intense sunflecks by changing orientation of its leaflets to minimize light interception which may create changes in irradiance of 200-fold within seconds (Bjorkman & Powles, 1981). This process protects against light-induced injury to the photosynthetic apparatus (Powles & Bjorkman, 1981). In *O. oregana* where much of the oxalic acid is soluble and is not sequestered as calcium oxalate, one can construct a hypothetical 'C_2' path of carbon for photosynthesis as outlined in Fig. 7. Such a scheme includes C2/C3 cleavage of the carbon chain of L-ascorbic acid with L-threonic acid undergoing decarboxylation while oxalic acid accumulates as free acid. In subsequent steps, three carbons from ascorbic acid recycle through hexose metabolism into cell constituents. Oxalate oxidase activity may be light-regulated to release CO_2 at optimal moments of photosynthesis while H_2O_2, also released in this process, replenishes the oxalic acid pool through cleavage of ascorbic acid. Conceivably, a homeostatic mechanism controls ascorbic acid biosynthesis. Potentially, 50% of the carbon from ascorbic acid is available for photosynthesis in one turn of this cycle.

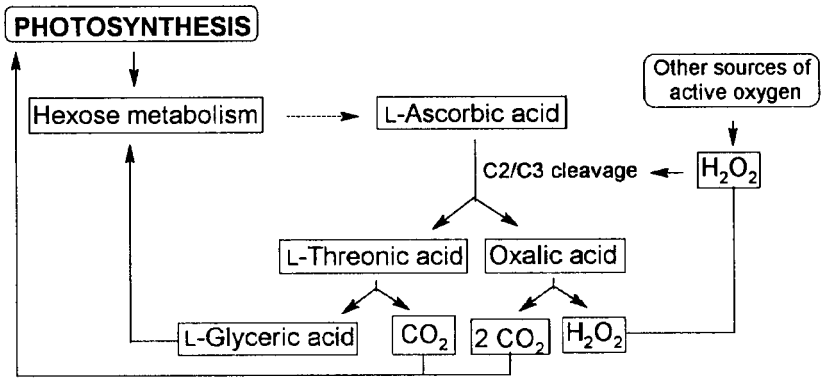

Fig. 7. Hypothetical 'C2' pathway of photosynthesis utilizing CO_2 from products of C2/C3 cleavage of L-ascorbic acid.

4. Biosynthesis and metabolism of analogs of ascorbic acid in fungi

*4.1. D-Erythroascorbic acid (*D-glycero-*pent-2-enono-1,4-lactone)*

Reports of ascorbic acid in several species of yeasts (Heick, Graff, & Humpers, 1969a; Heick, Stewart, Graff, & Humpers, 1969b) prompted Leung and Loewus (1985b) to reexamine this possibility with a more selective, sensitive method of analysis (Grün & Loewus, 1983). *Lypomyces starkeyi*, a yeast reported by Heick et al. (1969b) to contain appreciable ascorbic acid, was found to be devoid of this compound although it did contain an electrochemically-reactive constituent distinguishable from ascorbic acid and readily oxidized in the presence of oxygen and activated charcoal. Under aerobic conditions *L. starkeyi* produced an electrochemically-reactive product which could be separated on a HPLC cationic resin column from ascorbic acid (retention time relative to ascorbic acid, 0.85). When *L. starkeyi* was stored under anaerobic conditions for several days, a second electrochemically-reactive product corresponding to authentic D-erythroascorbic acid (retention time relative to ascorbic acid, 1.15) accumulated. GC/MS and NMR analysis of the latter product confirmed its identity as D-erythroascorbic acid. Acid-soluble extracts from *Saccharomyces cerevisiae* contained erythroascorbic acid but not the unidentified product (Nick, Leung, & Loewus, 1986). D-Erythroascorbic acid, a five carbon analog of L-ascorbic acid, is present in the imperfect fungus *Candida* (Murakawa, Sano, Yamashita, & Takahashi, 1977). Its enantiomeric identity was based on experiments which showed that D-arabinose and D-arabinono-1,4-lactone stimulated production of this analog of ascorbic acid (Murakawa et al., 1977). Enantiomeric L-erythroascorbic acid has been detected as a product of L-ascorbic acid catabolism in animals (Ashwell, Kanfer, Smiley, & Burns, 1961; Jung & Wells, 1998). D-Erythroascorbic acid has been found in several fungal species (Dumbrava & Pall, 1987; Kim, Seib, & Chung, 1993; Okamura, 1994; Loewus, Saito, Suto, & Maring, 1995; Okamura, 1998; Keates, Loewus, Helms, & Zink, 1998) and has been chemically synthesized from D-xylose in a simple three-step procedure (Shao et al., 1993). Apart from D-erythroascorbic acid, other fungal ascorbic acid analogs and their glycosides currently known include 6-deoxy-ascorbic acid, 6-deoxy-5-*O*-(α-D-xylopyranosyl)-ascorbic acid, 6-deoxy-5-*O*-(α-D-glucopyranosyl)-ascorbic acid and 5-*O*-(α-D-glucopyranosyl)-erythroascorbic acid (Okamura, 1994), 5-*O*-(α-D-xylopyranosyl)-D-erythroascorbic acid (Okamura, 1998) and 5-*O*-(α-D-galactopyranosyl)-D-erythroascorbic acid (Keates et al., 1998). The identity of the ascorbic acid analog detected in aerobically-grown *L. starkeyi* remains unknown.

Analogs and their glycosides identified by Okamura (1994, 1998), were isolated from eatable mushrooms (Basidiomycetes). D-Erythroascorbic acid and galactosyl-D-erythroascorbic acid, the principal analogs present in *Sclerotinia sclerotiorum* (Ascomyces) have been studied in the author's laboratory (Loewus et al., 1995; Keates et al., 1998). Both compounds are substrates for ascorbic acid oxidase and serve as precursors of oxalic acid in *S. sclerotiorum*. The galactosyl analog appears to be more stable in solution and it accumulates in sclerotia, the hard resting structures formed by aggregation of *S. sclerotiorum* hyphae, which provide a useful source of this compound for structural studies (Keates et al., 1998).

In the presence of H_2O_2 under alkaline conditions, D-erythroascorbic acid is cleaved between C2/C3 to yield oxalic acid and D-glyceric acid (Jung & Wells, 1998), a process analogous to peroxygenation of L-ascorbic acid (Isbell & Frush, 1979). Glycosidation of ascorbic acid analogs appears to be a common feature in fungi. In every case, substitution occurs at carbon 5 but the monosaccharidic residue may be D-glucose, D-

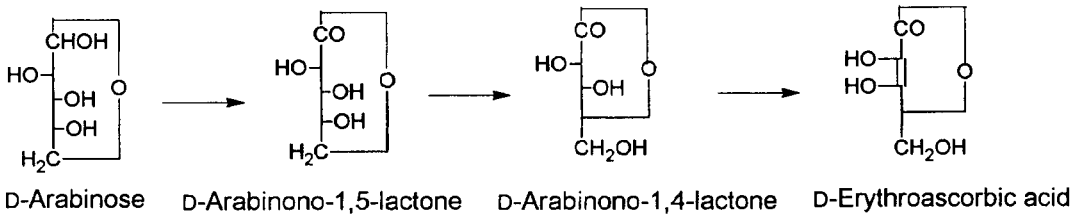

Fig. 8. Scheme proposed for biosynthesis of D-erythroascorbic acid from D-arabinose in fungi (Murakawa et al., 1977; Huh et al., 1994; Kim et al., 1996).

xylose, or D-galactose, depending upon the species involved. Presumably, glycosidation follows synthesis, possibly serving as a mechanism for sequestering the glycoside in stable form. Whether the glycoside undergoes oxidative cleavage to release oxalic acid and aldonic acid as is observed with free D-erythroascorbic acid or must first undergo hydrolysis of the glycosidic bond needs to be determined. Implicit in this relationship between ascorbyl analogs and their glycosides in fungi is the question of metabolic regulation, a mechanism whereby control of oxidative products is maintained. The enantiomeric assignment of ' D' to erythroascorbic acid recovered from fungi by Murakawa et al. (1977) has been confirmed by chemical synthesis (Shao et al., 1993 and earlier papers therein) and by NMR analysis (Shao et al., 1993; Keates et al., 1998). Structurally, D-erythroascorbic acid has a five-membered lactone ring stereochemically identical to that of L-ascorbic acid. Both compounds are substrates for ascorbate oxidase (Keates et al., 1998). Oxidation by H_2O_2 leads to oxalic acid and D-glyceric acid in the case of erythroascorbic acid and to oxalic acid and L-threonic acid in the case of ascorbic acid.

4.2. Biosynthesis of D-erythroascorbic acid in fungi

The enzymic components of the biosynthetic pathway from D-arabinose to D-erythroascorbic acid as originally proposed by Murakawa et al. (1977) (Fig. 8) were isolated from *Candida albicans* and characterized in elegant detail by Sa-Ouk Kang and his colleagues (Huh et al., 1994; Kim, Huh, Kim, Hwang, & Kang, 1996).

The first step, oxidation of D-arabinose, is catalyzed by a $NADP^+$-specific D-arabinose dehydrogenase which was purified 2750-fold from crude cell extract of *C. albicans* (Kim et al., 1996) and oxidizes D-arabinose, L-fucose, L-xylose and L-galactose to the corresponding aldonolactone, possibly the aldono-1,5-lactone which then rearranges to the more stable aldono-1,4-lactone. D-Arabinose and L-fucose exhibit highest and nearly equal rates with purified enzyme. The final step, oxidation of D-arabinono-1,4-lactone, is catalyzed by a flavin-containing enzyme which was purified 639-fold from the mitochondrial fraction of *C. albicans* (Huh et al., 1994). Again, D-arabinono-1,4-lactone exhibited highest activity but L-galactono-1,4-lactone, L-xylono-1,4-lactone and L-gulono-1,4-lactone were also readily oxidized. The product formed with D-arabinono-1,4-lactone was D-erythroascorbic acid and H_2O_2 was released in stoichometric amount. Kang provides an interesting discussion in which the properties of his D-arabinono-1,4-lactone oxidase are compared to two L-galactono-1,4-lactone oxidases isolated from *Saccharomyces cerevisiae* by Nishikimi, Noguchi, and Yagi (1978), and by Bleeg and Christensen (1982). Kang concludes '...from a physiological viewpoint that the name L-galactono-1,4-lactone oxidase from *S. cerevisiae* should be changed to D-arabinono-1,4-lactone oxidase...'.

Quite recently, Nishikimi, Ohta, and Ishikawa (1998) found a homologue of rat L-gulono-1,4-lactone oxidase in a database of proteins encoded by the genome of the yeast *Saccharomyces cerevisiae*. When amplified and expressed as a fusion protein with glutathione *S*-transferase in *Escherichia coli*, they found L-galactono-1,4-lactone oxidase activity similar to purified yeast L-galactono-1,4-lactone oxidase. Their finding strengthens an earlier proposal of theirs that '...L-galactono-1,4-lactone oxidase and L-gulono-1,4-lactone oxidase are homologous enzymes which evolved from a common ancestral enzyme...' (Nishikimi et al., 1978). It remains to be determined whether this proposal extends to L-galactono-1,4-lactone dehydrogenase in higher plants or the two ascorbic acid-synthesizing activities in *Euglena gracilis*.

4.3. Concerning the carbon source of oxalic acid for phytopathogenic infection

The oxalate-producing quality associated with ascorbyl analogs has prompted a more detailed study in which *Sclerotinia sclerotiorum* served as model organism (Keates, 1988; Loewus et al., 1995; Keates, Franceschi, & Loewus, submitted). *S. sclerotiorum* is a fungal phytopathogen of considerable agronomic importance (Purdy, 1979). Oxalic acid production by this fungus plays a key role during initiation of infection

by plant tissue, a synergistic process of cell wall maceration involving polygalacturonase (Lumsden, 1979). Comparative studies of oxalic acid production by wild type and oxalic acid-deficient mutants have confirmed its pathogenicity determinant properties (Godoy, Steadman, Dickman, & Dam, 1990; Keates, Zhang, Loewus, & Franceschi, 1996). An investigation of potential precursors for oxalate production in culture and *in planta* (Keates et al., submitted) turned up the interesting fact that L-ascorbic acid was the best carbon source for oxalic acid production in culture (in both mycelium and sclerotia). D- and L-Arabinose were also effective sources of oxalic acid. In culture, L-ascorbic acid was the best carbon source for erythroascorbic acid production. The ubiquitous nature of ascorbic acid in higher plants prompts an interesting question – does *S. sclerotiorum* utilize L-ascorbic acid (a plant-generated carbon source) for oxalic acid production during infection of the host or does it depend upon its own cellular resources, notably D-erythroascorbic acid and comparable analogs. To examine this matter (Keates et al., submitted), two species of bean, one susceptible to infection by *S. sclerotiorum* and the other resistant, were infected with either wild type or an oxalate-deficient mutant. After 4 d, bean leaves were analyzed for ascorbic acid, erythroascorbic acid, galactosyl erythroascorbic acid, oxalic acid and oxalate. Uninfected (control) leaves had the highest concentration of ascorbic acid, mutant-infected leaves less, and wild type-infected leaves the least, one-third the amount present in uninfected tissues. Galactosyl erythroascorbic acid was detected in wild type-infected but not in mutant-infected leaves. Free erythroascorbic acid was only detected in wild type-infected leaves from the resistant species. Oxalic acid and its salts were not detectable in uninfected leaves but accumulated to high levels in wild type-infected leaves from both susceptible and resistant leaves. These preliminary results suggest that host-derived ascorbic acid is utilized for oxalic acid production in wild type-infected leaves but the experiment fails to reveal whether erythroascorbic acid or its galactosyl derivative is involved. Perhaps a better way to approach the question would be through manipulation of the ascorbic acid level in the host, for example by supplying L-galactono-1,4-lactone to bean plants both before and during infection (Baig et al., 1970)].

5. Concluding remarks

It now appears that L-ascorbic acid and ascorbic acid analogs like D-erythroascorbic acid share a common pattern of biosynthetic intermediates in steps preceding the final product:

$$\text{Aldose} \longrightarrow \text{Aldone} - 1,5 - \text{lactone} \longrightarrow \text{Aldone} - 1,4 - \text{lactone} \longrightarrow \text{Ascorbic acid (or analog)}$$

While this sequence may apply generally in the case of plants and fungi, it must be qualified as concerns animal species competent in ascorbic acid biosynthesis where reduction of D-glucuronic acid to L-gulonic acid is generally regarded as the carbon source of L-gulono-1,4-lactone but other possibilities have not been excluded.

In plants, the postulated aldose precursor of L-ascorbic acid is L-galactose (Wheeler et al., 1998), a product of D-mannose metabolism in corn roots (Roberts, 1971). In fungi, D-arabinose is the likely source of D-erythroascorbic acid (Murakawa et al., 1977; Loewus et al., 1995; Kim et al., 1996) and L-fucose a potential source of 6-deoxy-L-ascorbic acid (Okkamura, 1994). In both plants and fungi the cofactor for the aldose dehydrogenase is $NADP^+$-specific. Contraction of the aldonolactone from a 6 to a more stable 5 member ring structure would be a spontaneous process within the cellular milieu. As noted earlier, the final step, oxidation of the aldono-1,4-lactone, is catalyzed by a flavin-bound oxidase, possibly FAD. Certain algal species may have selected an aldose-like precursor with a configuration resembling L-gulonic acid as substrate for the first step. The diphosphate ester of L-*xylo*-hex-2-enonic acid (also referred to as 2-keto-L-gulonic acid) is found among photosynthetically-produced metabolites of *Chlorella pyrenoidosa* (Moses, Ferrier, & Calvin, 1962) and could conceivably furnish a carbon source for the final step in *Euglena* (Shigeoka et al., 1979b). This paucity of information may soon be remedied as fresh interest develops regarding new functional role(s) for ascorbic acid and its analogs.

Apart from its well established roles as an intermediate in redox cycles, as a scavenger of active oxygen, and its involvement in prolyl oxidation, L-ascorbic acid emerges in the plant literature as a carbon source of metabolites including L-tartaric, L-threonic, L-glyceric, oxalic acids and intercellular CO_2 as well as means for recycling carbon into products of triose and hexose phosphate metabolism. Its involvement in oxalic acid biosynthesis needs to be studied in greater detail since the possibility that it may be an internal source of CO_2 for photosynthesis has not been tested. Hopefully, this critique will stimulate renewed interest in this ubiquitous constituent of plants and its analogs in fungi.

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