

## Review

# Plant L-ascorbic acid: chemistry, function, metabolism, bioavailability and effects of processing

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**Abstract:** Humans are unable to synthesise L-ascorbic acid (L-AA, ascorbate, vitamin C), and are thus entirely dependent upon dietary sources to meet needs. In both plant and animal metabolism, the biological functions of L-ascorbic acid are centred around the antioxidant properties of this molecule. Considerable evidence has been accruing in the last two decades of the importance of L-AA in protecting not only the plant from oxidative stress, but also mammals from various chronic diseases that have their origins in oxidative stress. Evidence suggests that the plasma levels of L-AA in large sections of the population are sub-optimal for the health protective effects of this vitamin.

Until quite recently, little focus has been given to improving the L-AA content of plant foods, either in terms of the amounts present in commercial crop varieties, or in minimising losses prior to ingestion. Further, while L-AA biosynthesis in animals was elucidated in the 1960s,<sup>1</sup> it is only very recently that a distinct biosynthetic route for plants has been proposed.<sup>2</sup> The characterisation of this new pathway will undoubtedly provide the necessary focus and impetus to enable fundamental questions on plant L-AA metabolism to be resolved.

This review focuses on the role of L-AA in metabolism and the latest studies regarding its biosynthesis, tissue compartmentalisation, turnover and catabolism. These inter-relationships are considered in relation to the potential to improve the L-AA content of crops. Methodology for the reliable analysis of L-AA in plant foods is briefly reviewed. The concentrations found in common food sources and the effects of processing, or storage prior to consumption are discussed. Finally the factors that determine the bioavailability of L-AA and how it may be improved are considered, as well as the most important future research needs.

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**Keywords:** bioavailability; biosynthesis; L-ascorbate; plants; vitamin C

### HISTORICAL OVERVIEW

The disease scurvy which is now known to be the result of vitamin C (L-AA) deficiency, was described by the ancient Greeks, Egyptians and Romans and has long been associated with invading armies, navies and explorers. Even at the end of the last century, scurvy was rife amongst the gold miners of California and Alaska. The first systematic study of this problem was carried out by the naval surgeon James Lind in the

1750's, who found that the disease could be prevented and cured by the daily administration of fresh citrus fruits. However, it was not until 1907, with the demonstration by Holst and Frölich<sup>3</sup> that scurvy could be produced in the guinea pig, was it fully accepted that scurvy was the result of a dietary deficiency. Svent-Györgi isolated a compound from vegetables that he named 'crystalline hexuronic acid',<sup>4,5</sup> and which was subsequently shown to be the same as the vitamin C

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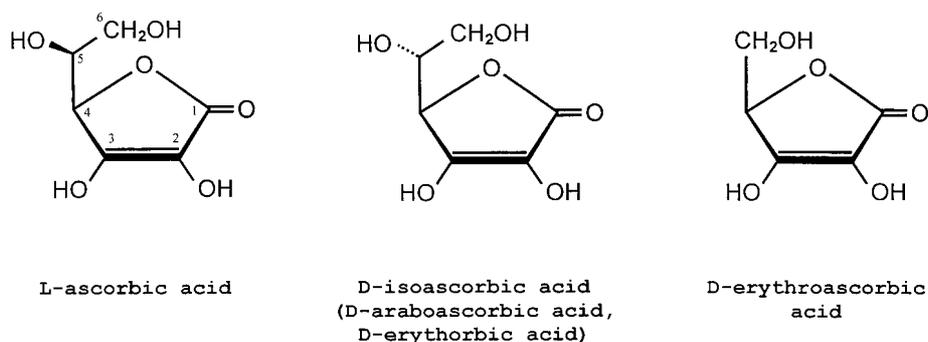


Figure 1. Chemistry of L-ascorbic acid.

isolated from lemon juice by Waugh and King.<sup>6-8</sup> The precise structure of vitamin C was determined by Haworth in 1932, and chemical synthesis achieved by Reichstein *et al* a few months later.<sup>9</sup> However, it is really only in the past 25 years that the numerous biological functions of L-AA in animals and plants have been realised. For example, the specific functions of L-AA in collagen synthesis, and consequently in the formation and maintenance of cartilage, bones, gums, skin, teeth, etc are now appreciated, and it is also now generally recognised that the activity of leucocytes and other aspects of the immune system is enhanced by L-AA. Further, as the most effective and least toxic antioxidant, there is now strong evidence to link dietary L-AA with protective effects against various oxidative stress-related diseases such as cancers, cardiovascular disease, aging and cataract formation (for review see Ref 10). Progress in the plant field has lagged behind, and it was not until the identification of the enzyme L-ascorbic acid peroxidase<sup>11,12</sup> that a plant-specific function for L-AA, as a scavenger of photosynthetically (and photorespiration) derived H<sub>2</sub>O<sub>2</sub> could be defined. Data are now also beginning to accumulate on the roles of L-AA in the apoplast and cell growth. However, much still remains to be learnt about the biochemistry and mechanisms underlying catabolism, regulation, transport and compartmentalisation of this vital nutrient in plant systems.

## CHEMICAL PROPERTIES

### Structure

Structurally, L-AA is one of the simplest vitamins. It is related to the C<sub>6</sub> sugars, being the aldono-1,4-lactone of a hexonic acid (L-galactonic or L-gulonic acid), and contains an enediol group on carbons 2 and 3. The stereoisomer of L-AA, D-isoascorbic acid (D-isoAA, D-araboscorbic acid, D-erythorbic acid), has little if any antiscorbutic activity, and should not be confused with D-erythroascorbic acid, which is the C<sub>5</sub> analogue of L-AA found in many yeasts and fungi (Fig 1), (see later).

Delocalisation of the  $\pi$ -electrons over the C<sub>2</sub>-C<sub>3</sub> conjugated enediol system stabilises the molecule and causes the hydrogen of the C<sub>3</sub> hydroxyl to become highly acidic, and to dissociate with a pK<sub>a</sub> of 4.13. Therefore at physiological pH, L-AA exists as a monovalent anion (L-ascorbate). Dissociation of the second hydroxyl takes place at pH 11.6.

Several natural derivatives of L-AA are also known.<sup>13</sup> Ascorbate-2-sulphate was initially discovered in brine shrimp cysts, and later found to be present in soft tissues and as a urinary metabolite of L-AA in animals. Ascorbate-2-sulphate is also able to relieve the signs of scurvy (at least in fish), and the enzyme ascorbate-2-sulphohydrolase has been purified and characterised.<sup>14</sup> Ascorbic acid-2-O- $\beta$ -glucur-

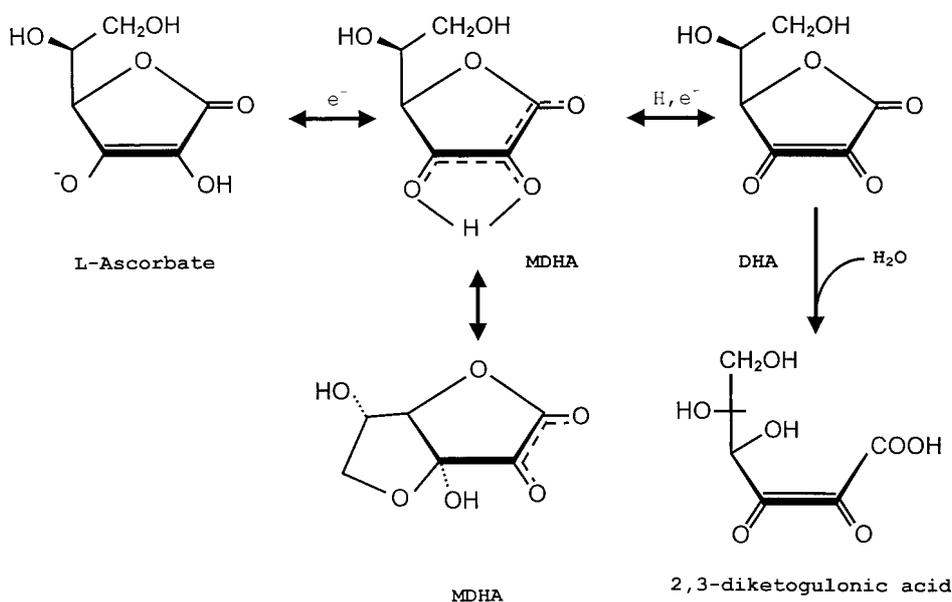


Figure 2. Oxidation of L-ascorbate.

onide and 2-*O*- $\alpha$ -glucoside have been found in humans and guinea pigs respectively,<sup>15-17</sup> and several C<sub>5</sub>-linked glycosides of 6-deoxyascorbate have been identified in mushroom.<sup>18</sup> The possible existence and function of such derivatives in plant tissues has received very little attention however. The synthetic derivatives, ascorbate-2-phosphate and ascorbate-6-palmitate have *in vitro* antioxidant properties and are used in preservation of foods, but are poorly absorbed and will not be discussed further here.

### Oxidation

L-AA is stable when dry, but solutions readily oxidise, especially in the presence of trace amounts of copper, iron and alkali. The first oxidation product of L-AA is the radical monodehydroascorbate (MDHA), also known as semidehydroascorbate, or ascorbate free radical (Fig 2), (for a discussion of reaction mechanisms, see Refs 19, 20). MDHA, with a decay constant of  $2.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  at pH 7,<sup>21</sup> is unusually stable for a radical. *In vivo* MDHA is reduced back to L-AA by the activity of the NAD(P)-dependent enzyme, monodehydroascorbate reductase (MDHAR, EC 1.6.5.4, see later), or by electron transfer reactions. If allowed to persist though, two molecules of MDHA will also spontaneously disproportionate to L-AA and DHA. DHA itself is unstable and undergoes irreversible hydrolytic ring cleavage to 2,3-diketogulonic acid in aqueous solution.<sup>22,23</sup> The half-life for this breakdown was found to be 6 min at 37 °C,<sup>24</sup> although the rates of L-AA oxidation and DHA hydrolysis will be influenced by factors such as concentration, temperature, light, pH, etc.

### Physical properties of L-AA

**Table 1.** Physical properties of L-ascorbate (adapted from Ref 354)

Property	Comments
Appearance	White, odourless, crystalline solid with sharp acidic taste
formula/mol mass	C <sub>6</sub> H <sub>8</sub> O <sub>6</sub> /176.13
m.p. (°C)	190–192
density, gml <sup>-1</sup>	1.65
pH	3 (5mgml <sup>-1</sup> ), 2 (50mgml <sup>-1</sup> )
pK <sub>1</sub>	4.17
pK <sub>2</sub>	11.57
Redox potential	first stage: E <sub>1</sub> O + 0.166V (pH 4)
Solubility (gml <sup>-1</sup> )	
water	0.33
95% ethanol	0.033
glycerol	0.01
fats and oils	insoluble
Spectral properties	
UV pH 2:	E <sub>max</sub> (1%, 1 cm), 695 at 245 nm (undissociated)
pH 6.4:	E <sub>max</sub> (1%, 1 cm), 940 at 265 nm (monodissociated form)

### BIOLOGICAL FUNCTIONS IN PLANTS AND OTHER ORGANISMS

In general three main types of biological activity can be defined for L-AA: its function as an enzyme cofactor, as a radical scavenger, and as a donor/acceptor in electron transport either at the plasma membrane or in the chloroplasts. Additionally in plants, L-AA appears to be the substrate for oxalate and tartrate biosynthesis, at least in certain species (for reviews, see Refs 10, 21, 25–31).

#### Enzymatic cofactor

One of the more clearly defined functions of L-AA in both plant and animal metabolism is to modulate a number of important enzymatic reactions. A list of the enzymes that are effected by L-AA is shown in Table 2.

Characteristically, these enzymes are mono- or dioxygenases, which contain iron or copper at the active site and which require L-AA for maximal activity.<sup>25,32-35</sup> The function of L-AA is to maintain the transition metal ion centres of these enzymes in a reduced form, and L-AA is an important enhancer of activity.<sup>25</sup> In some cases other reducing agents (at higher concentrations), can at least partially replace L-AA.

In animals, the Fe-dioxygenases involved in collagen biosynthesis require L-AA for maximal activity. L-AA is also necessary for the normal functioning of fibroblasts and osteoblasts, for carnitine and adrenal hormone biosynthesis, wound healing, folate and adrenal hormone synthesis. L-AA has also been reported to inhibit catechol-*O*-methyl transferase, thereby increasing the bioavailability of adrenaline (epinephrine).<sup>36</sup> This could have important implications in neuroendocrine control and help explain the high concentrations of L-AA found in areas of adrenaline synthesis and/or action, such as the adrenal medulla and central nervous system.

In plants, high levels of hydroxyproline-rich proteins such as the extensins are found in the cell wall.<sup>37</sup> The extensins are developmentally regulated hydroxyproline-containing glycoproteins that appear to be involved in the cross-linking of the cell wall in response to injury, and extensin genes are induced in response to wounding and pathogen attack.<sup>38,39</sup> The direct involvement of L-AA in the synthesis of plant hydroxyproline-rich proteins therefore,<sup>40-42</sup> has implications for cell expansion and cell division (see later). L-AA also specifically activates myrosinase (thioglucoside glucohydrolase), an enzyme which catalyses the hydrolysis of glucosinolates to D-glucose and an aglycone fragment (the latter subsequently rearranges to sulphate and isothiocyanate). Similarly, both 1-aminocyclopropane-1-carboxylate oxidase (ACC-oxidase) and gibberellin 3-dioxygenase, require L-AA as a cosubstrate in the biosynthesis of the important plant hormones ethylene and gibberellic acid, respectively. L-AA is additionally monovalently oxidised by violaxanthin de-epoxidase during the biosynthesis of the photoprotective pigments of the xanthophyll cycle.

**Table 2.** Enzymes requiring L-ascorbate as a cofactor or as a modulator of activity

Enzyme	Metal ion centre	Change in activity	Physiological role	Enzymatic activity
4-Hydroxyphenylpyruvate dioxygenase EC 1.13.11.27	Iron	Increase	Tyrosine metabolism	Decarboxylation and hydroxylation of 4-hydroxyphenyl pyruvic acid to homogentisic acid
gamma Butyrobetaine 2-oxoglutarate 4-dioxygenase EC 1.14.11.1	Iron	Increase	Carnitine biosynthesis	Hydroxylation of butyrobetaine to carnitine
Proline hydroxylase EC 1.14.11.2	Iron	Increase	Procollagen synthesis (animals)	Hydroxylation of proline (4-hydroxylating)
Lysine hydroxylase EC 1.14.11.4	Iron	Increase	Collagen biosynthesis (animals) Extensin biosynthesis (plants)	Hydroxylation of lysine
Procollagen proline 2-oxoglutarate 3 dioxygenase EC 1.14.11.7	Iron	Increase	Procollagen biosynthesis (animals) Extensin biosynthesis (plants)	Hydroxylation of proline (3-hydroxylating)
Trimethyllysine 2-oxoglutarate dioxygenase EC 1.14.11.8	Iron	Increase	Carnitine biosynthesis	Hydroxylation of trimethyl lysine
Cholesterol 7-alpha monooxygenase EC 1.14.13.17		Increase	Cholesterol catabolism; bile acid synthesis (animals)	Hydroxylation of cholesterol
Dopamine beta monooxygenase EC 1.14.17.1	Copper	Increase	Noradrenaline (norepinephrine) synthesis	$\beta$ -hydroxylation of dopamine
Peptidyl glycine alpha amidating monooxygenase EC 1.14.17.3	Copper	Increase	Peptide amidation in peptide hormone metabolism	C-terminal glycine amidation
Mitochondrial glycerol-3-phosphate dehydrogenase EC 1.1.99.5	Iron	Increase	NAD (P) H and ATP production; aid in insulin release	Dehydrogenation of triose phosphate
Catechol-O-methyl transferase EC 2.1.1.6		Decrease	Adrenaline (epinephrine) inactivation (animals)	Increased bioavailability of adrenaline (epinephrine)
Thymine dioxygenase EC 1.14.11.6	Iron	Increase	Pyrimidine metabolism (fungi)	7-Hydroxylation of thymine
Pyrimidine deoxynucleoside 2' dioxygenase EC 1.14.11.3	Iron	Increase	Pyrimidine metabolism (fungi)	Deoxyuridine to uridine
Deacetoxycephalosporin C synthetase	Iron	Increase	Antibiotic metabolism (fungi)	Penicillin N to deacetylcephalosporin
1-Aminocyclopropane-1-carboxylate oxidase	Iron	Increase	Ethylene (plant hormone) biosynthesis	Oxidation of 1-aminocyclopropane to ethylene and cyanofornic acid
Violaxanthin de-epoxidase		Increase	Zeaxanthin biosynthesis and the xanthophyll cycle (plants)	De-epoxidation of violaxanthin and antheroxanthin
Gibberellin 3- $\beta$ -dioxygenase EC 1.14.11.15	Iron	Increase	Gibberin (plant hormone) biosynthesis	C <sub>20</sub> oxidative decarboxylation and activation of gibberellins
Thioglucoside glucohydrolase EC 3.2.3.1		Increase	Catabolism of glucosinolates (plants)	Hydrolysis of S-glucosides

### Antioxidant functions

In both plant and animal systems L-AA interacts enzymatically and non-enzymatically with damaging oxygen radicals and their derivatives, so-called reactive oxygen species (ROS). These detoxification reactions can be considered to be an integral part of the housekeeping duties required of an aerobic existence in eukaryotic cells and the high intracellular concentrations of L-AA are an indication of the importance of these functions in eukaryotic organisms. In plants, the ability of L-AA to interact with physiologically generated ROS implicates L-AA in the modulation

of processes such as lignification, cell division and the hypersensitive response. The biological importance of the antioxidant behaviour of L-AA is that unlike other low-molecular-weight antioxidants ( $\alpha$ -tocopherol, uric acid, carotenoids, flavonoids, etc), L-AA is able to terminate radical chain reactions by disproportionation to non-toxic, non-radical products, i.e DHA and 2,3-diketogulonic acid (Fig 2). Further, since L-AA is only mildly electronegative, it can donate electrons to a wide range of substrates. Indeed, one of the most important features of the non-enzymatic antioxidant activity of L-AA, is its involvement in the regeneration

of the lipophilic, membrane-associated  $\alpha$ -tocopherol (vitamin E,  $\alpha$ -chromanoxyl), radical.

#### ROS and oxidative stress

Reactive oxygen species (ROS) include such compounds as superoxide ( $O_2^{\cdot-}$ ), singlet oxygen ( $^1O_2$ ), hydrogen peroxide ( $H_2O_2$ ), and the highly reactive hydroxyl radical ( $OH^{\cdot}$ ) (see eg Refs 43, 44). Chloroplasts, as well as mitochondria and peroxisomes all produce ROS as byproducts of normal cellular metabolism, but this production is enhanced by a variety of environmental stresses, including drought, starvation, wounding, high salt, high light, exposure to pollutants (ozone), etc. The toxicity of ROS is considered to be due to their ability to initiate radical cascade reactions that lead to the production of the hydroxyl radical and other destructive species which can cause protein damage, lipid peroxidation, DNA damage and finally cell death. Aerobic organisms have therefore developed a range of efficient mechanisms to detoxify these species by both enzymatic and non-enzymatic means. Amongst the enzymatic mechanisms, superoxide dismutases (SOD, EC 1.15.1.1) catalyse the dismutation of superoxide to  $H_2O_2$  (for review see Refs 45, 46), and ascorbate peroxidases (APx, EC 1.11.1.11),<sup>47</sup> glutathione peroxidases (GPx, EC 1.11.1.9),<sup>48</sup> and catalases (EC 1.11.1.6), detoxify  $H_2O_2$ . Low-molecular-weight antioxidants such as glutathione ( $\gamma$ -glutamyl-L-cysteinylglycine, GSH), and L-AA, the lipophilic  $\alpha$ -tocopherol (vitamin E), as well as carotenoids and phenolics are able to non-enzymatically interact directly with ROS.<sup>44,49,50</sup> The condition of 'oxidative stress' can thus be defined as being the intracellular accumulation of toxic levels of ROS through saturation of the antioxidant defence systems.

Paradoxically however, the controlled production of ROS can also be beneficial to the organism. For example, during incompatible plant-pathogen interactions, the recognition of an invading pathogen stimulates an 'oxidative burst' and a coordinated defense response, mediated by ROS (for reviews, see Refs 30, 51-55). The mechanisms by which  $O_2^{\cdot-}$  is produced (and subsequently dismutated to  $H_2O_2$  by SOD) appear to vary between species, and include a plasma membrane NADPH oxidase (analogous to the oxidative burst of the neutrophil NADPH oxidase of mammals<sup>49,52-54,56</sup> as well as the direct production of  $H_2O_2$  from the activity of cell wall peroxidases.<sup>51,57</sup> Apart from a direct cytotoxic effect on the invading pathogen,  $H_2O_2$  also stimulates cell wall peroxidase activity and cross-linking of cell wall proteins and lignin to hinder pathogen penetration.<sup>55,58,59</sup> ROS also function as messengers, to induce an entire array of defence response genes, and finally, massive ROS accumulation can trigger a localised hypersensitive response, resulting in rapid and localised cell death, again limiting pathogen development. More recently, Alvarez *et al*<sup>55</sup> suggested that in addition to the primary oxidative burst, ROS also induce secondary

oxidative bursts in small collections of cells in distant tissues, leading to systemic acquired resistance (see also Ref 60), and Karpinski *et al*<sup>61</sup> have suggested that  $H_2O_2$  functions as a systemic signal during exposure of *Arabidopsis* leaves to high light, switching on adaptive gene expression in regions not exposed to the damaging stimulus.

Clearly, the ability of apoplastic and cytoplasmic L-AA to react non-enzymatically and enzymatically with a variety of ROS will influence these responses, and the redox status of apoplastic L-AA pools will be an additional determinant that helps define the strength and duration of these responses.

#### L-AA, photosynthesis and (photo)oxidative stress in plants

Photosynthetic organisms are particularly vulnerable to ROS since large amounts of  $O_2$  are produced in the immediate vicinity of a powerful oxidation-reduction system, capable of reducing  $O_2$  to  $O_2^{\cdot-}$ . In the light, the chloroplasts of higher plants produce ROS as a consequence of the transfer of high-energy electrons from reduced ferredoxin of the photosynthetic electron transport chain to oxygen instead of to NADP. This photoreduction of oxygen in photosystem I, is termed the Mehler reaction, and the overall transfer of electrons from water to molecular oxygen, is called pseudocyclic electron flow. Pseudocyclic electron flow provides a mechanism by which the plant is able to dissipate excess reducing power (and generate ATP), under conditions when carbon fixation is limited.<sup>27,62,63</sup> The majority of this  $O_2$  reduction is thought to proceed via reduced ferredoxin, resulting in the formation of the superoxide radical ( $O_2^{\cdot-}$ ), which again, disproportionates to  $H_2O_2$ , in a reaction catalysed by SOD (eqn (1)).



However, this simply transforms one ROS into another, and the further detoxification of  $H_2O_2$  is vital to the normal functioning of the chloroplast. Indeed, photosynthetic carbon assimilation is dependent on the stromal  $H_2O_2$ -scavenging systems for its continual operation in the light, since the thiol-modulated enzymes of the pentose phosphate pathway (Benson-Calvin cycle) are extremely sensitive to inhibition by low concentrations of  $H_2O_2$ .<sup>64</sup> Chloroplasts however lack catalase (which is located in the peroxisomes/glyoxysomes), and so L-AA is essential for chloroplast activity by serving as a substrate for APxs, to scavenge peroxide formed in the thylakoids. (eqn (2)).



APx isozymes are present in the chloroplasts as a thylakoid and a stromal form, as well as in the cytosol, mitochondria and peroxisomes.<sup>65,66</sup> Recently, a class of GSH-dependent peroxidases has also been discovered in plants<sup>48</sup> (for review, see Ref 67.), but these show homology to only one of the three classes of GPx's found in animals, namely the phospholipid

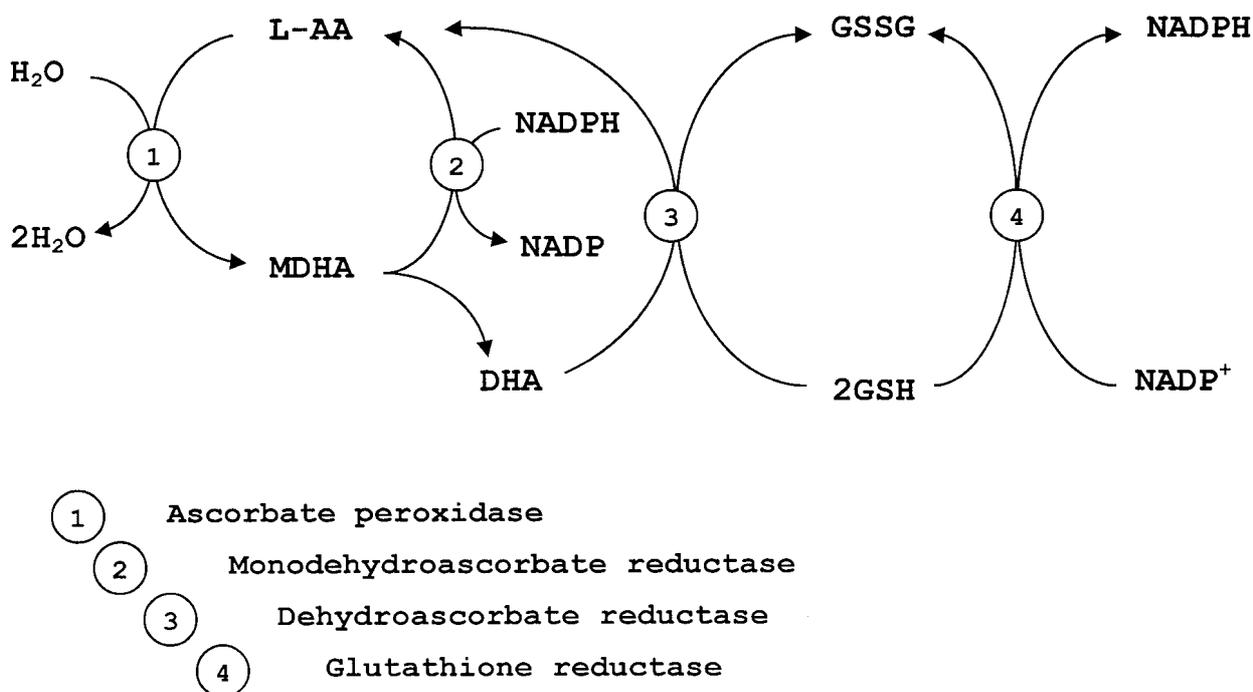


Figure 3. The ascorbate-glutathione cycle.

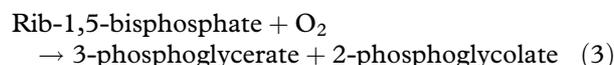
hydroperoxide glutathione peroxidases. As a consequence, chloroplastic H<sub>2</sub>O<sub>2</sub> detoxification appears to be entirely dependent on APx activity.

In the chloroplasts, MDHA generated in the reaction catalysed by APx (eqn (2)), is reduced back to L-AA either by acting as a direct electron acceptor to PSI at the reduced ferredoxin on the outside of the thylakoid membrane,<sup>68</sup> or alternatively by the ascorbate-glutathione cycle (L-AA-GSH cycle, Halliwell-Foyer-Asada pathway) (Fig 3). The L-AA-GSH cycle is a coupled series of enzymatic reactions first proposed by Foyer and Halliwell,<sup>69</sup> which links H<sub>2</sub>O<sub>2</sub> to the oxidation of light-generated NADPH. In this cycle MDHA, generated from the reduction of H<sub>2</sub>O<sub>2</sub> by APx is reduced back to L-AA by a stromal, NAD(P)H-dependent enzyme, monodehydroascorbate reductase (MDHAR, AFR reductase, EC 1.6.5.4). Any MDHA which escapes this reduction disproportionates to L-AA and DHA, and the resultant DHA is then reduced to L-AA in a reaction catalysed by the enzyme DHA reductase (DHAR, EC 1.8.5.1). The reducing power for DHAR activity is supplied by the oxidation of two GSH molecules to glutathione disulphide (GSSG). Finally, GSH in turn is regenerated from GSSG by the NADPH-dependent enzyme, glutathione reductase (EC 1.6.4.2).

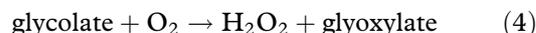
The L-AA-GSH pathway depends ultimately on reducing power derived from the light-dependent electron transport reactions of the chloroplast, or on secondary activities such as glucose-6-phosphate dehydrogenase and malate dehydrogenase for the generation of NADPH. A high proportion of the enzymes of the L-AA-GSH pathway are localised in the chloroplasts, but sufficient activity is found in other cellular compartments (cytosol, peroxisomes,

mitochondria), to drive the H<sub>2</sub>O<sub>2</sub> scavenging there as well (see Refs 65, 66, 70–75). However, as discussed by Kelly and Latzlo,<sup>76</sup> the relatively low activity of glutathione reductase, and the lack of response of this enzyme activity to stress situations (in contrast to APx and SOD), have created some controversy about the validity of the entire pathway. It may well be that in the chloroplasts, MDHA is primarily regenerated via reduced ferredoxin, and that the L-AA-GSH cycle, at least in the chloroplasts, performs a relatively minor role by scavenging DHA formed from the disproportionation of any MDHA that escapes photoreduction at the thylakoids.

The ability of ribulose-1,5-bisphosphate carboxylase, the main enzyme responsible for CO<sub>2</sub> fixation, to function as an oxygenase under CO<sub>2</sub>-limiting conditions, can also lead to photoinhibition due to the following reaction (eqn (3)):



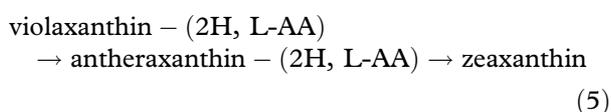
3-phosphoglycerate is a normal product of photosynthetic CO<sub>2</sub> fixation in the chloroplasts and enters the oxidative pentose phosphate pathway. However, 2-phosphoglycolate is less easily metabolised and in most higher plants is transported as glycolate to adjacent peroxisomes, where it is oxidised to glyoxylate by glycolate oxidase (eqn (4)). The glyoxylate may subsequently be used for glycine biosynthesis.



This reaction produces H<sub>2</sub>O<sub>2</sub>, which is dismutated to water and O<sub>2</sub> by the large quantities of peroxisomal catalase. Since catalase has a relatively low affinity for

$\text{H}_2\text{O}_2$  and is inactivated by light, the presence of an operational peroxisomal L-AA-GSH cycle<sup>65,66</sup> presumably serves to detoxify any  $\text{H}_2\text{O}_2$  that escapes catalase activity. The essential role of catalase in the detoxification of photorespiratory  $\text{H}_2\text{O}_2$  has recently been demonstrated using Cat1 antisense tobacco plants, which possess only 10% of the activity of wild type plants.<sup>78</sup> These antisense plants develop necrotic lesions under high light conditions, and show a large increase in the GSH pool size, due to an accumulation of oxidised glutathione.<sup>78</sup> Similar results have previously been observed in a catalase-deficient barley mutant.<sup>79</sup>

L-AA is also a cofactor for the enzyme violaxanthin de-epoxidase, which is attached to the lumen side of the thylakoid membranes and which forms the pigment zeaxanthin by the de-epoxidation of violaxanthin and antheroxanthin (eqn (5)).



Under  $\text{CO}_2$ -limiting conditions, zeaxanthin serves to dissipate excess excitation energy as heat in the light-harvesting antennae. This prevents over-reduction of ferredoxin and photodamage to the photosystem II reaction centre by the Mehler reaction. To date however, no carrier to transport L-AA across the thylakoid membrane membrane has been identified.

Therefore L-AA fulfills several functions in photosynthesis: It is a cosubstrate for enzymes involved in the biosynthesis of zeaxanthin (a photoprotectant), it is a substrate for APx in the detoxification of  $\text{H}_2\text{O}_2$  and an electron acceptor (as MDHA) for reduced ferredoxin in the photosynthetic electron transport chain, via the Mehler peroxidase reaction

#### *L-AA and the regeneration of $\alpha$ -tocopherol*

In addition to being able to interact with the superoxide and hydroxyl radicals, L-AA can also non-enzymatically reduce other radical species. Of particular importance here is the regeneration of  $\alpha$ -tocopherol (vitamin E), from the  $\alpha$ -chromanoxyl radical.<sup>27</sup>  $\alpha$ -Tocopherol is found in high concentrations in the photosynthetic membranes where it is the major lipophilic antioxidant, interacting with lipid peroxy radicals and  $\text{O}_2^-$ . Carotenoids, phenolic compounds and flavonoids are also able to scavenge  $\text{O}_2^-$ ,  $^1\text{O}_2$  and  $\text{OH}^\cdot$ .<sup>19,44</sup> Therefore chloroplastic L-AA, which may reach concentrations as high as 10–50 mM,<sup>27</sup> represents a pool of antioxidant potential that can be used to convert the highly damaging peroxy radical into non-toxic products.

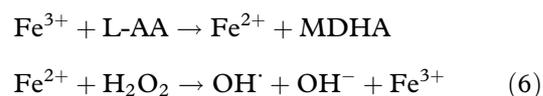
#### *Apoplasmic L-AA*

Changes in the levels and redox status of apoplasmic L-AA in response to environmental stresses have been reported by a number of groups. This has led to the realisation that apoplasmic L-AA could be involved in

the protection of cell membranes against oxidative damage, particularly as a result of exposure to ozone and other atmospheric pollutants.<sup>80–84</sup> However, while MDHAR activity has been demonstrated in the *Phaseolus* plasma membrane,<sup>85,86</sup> and ozone-responsive APX activity has been found in the extracellular matrix of several species,<sup>87</sup> no evidence for the presence in the apoplast of the other enzymes of the L-AA-GSH cycle or GSH itself, in a variety of plant species has been found.<sup>80,81,87,88</sup> Therefore, the plant cell has to possess alternative mechanisms to maintain the concentration and redox status of apoplasmic L-AA. The trans-plasma membrane transport of L-AA, DHA and the reduction of apoplasmic MDHA are discussed later. The involvement of apoplasmic L-AA in cell wall expansion and lignification is discussed below.

#### *Pro-oxidant effects of L-AA*

Under certain circumstances, L-AA can also have a pro-oxidant effect, in particular by maintaining the transition metal ions, Fe(III) and Cu(II), in their reduced forms. These metal ions react then with hydrogen peroxide to form the highly reactive hydroxyl radicals in the Fenton reaction (eqn (6)).<sup>43</sup>



There is currently no clear evidence that these reactions are of significance *in vivo* however.<sup>10,89</sup>

#### **Ascorbate, plant growth, and ascorbate oxidase**

Growth in higher plants is the result of two different processes, cell proliferation and cell elongation. Both L-AA and the enzyme ascorbate oxidase (AO) have been directly and indirectly implicated in the modulation of both.<sup>31,41,42</sup> The effects and mechanisms by which L-AA and its oxidation products may influence these processes are first discussed, followed by a discussion of the possible role of AO.

#### *L-AA and plant cell expansion*

L-AA and its oxidation products have consistently been found to influence plant cell expansion by a number of proposed mechanisms.<sup>31</sup> These include the cosubstrate requirements of the Fe-dioxygenases required for the post-translational modification of cell wall proteins,<sup>37,41,42</sup> and possibly the direct reaction of DHA with lysine and arginine side-chains to prevent cross-linking.<sup>90</sup> L-AA can directly scavenge the monolignol radicals involved in lignin biosynthesis,<sup>91,92</sup> as well as reversibly inhibit the activity of the cell wall/apoplasmic peroxidases responsible for the formation of monolignol radicals,<sup>58,92</sup> and possibly their turnover and secretion into the apoplast/cell wall.<sup>93</sup> There also appears to be a cell wall APx,<sup>80,94</sup> that could help to maintain cell wall plasticity by decreasing the availability of  $\text{H}_2\text{O}_2$  for other apoplasmic

peroxidase reactions. The addition of exogenous MDHA (but not L-AA) has been found to promote growth and rooting in *Allium*,<sup>95–97</sup> and this effect has been linked to increased cell expansion and solute uptake by stimulation of the plasma membrane H<sup>+</sup>-ATPase<sup>98</sup> as a result of transmembrane electron transport to extracellular MDHA via cytochrome *b*<sup>85,86</sup> (see later). Finally, L-AA can also serve as a substrate for oxalic acid biosynthesis.<sup>99</sup> It has been suggested that apoplastic oxalate could be responsible for the sequestering of calcium ions, which are important for the cross-linking of pectins (hemicelluloses) and cell wall strengthening. The concentration of apoplastic oxalate in turn could be regulated by germin, an abundant cell wall protein with oxalate oxidase activity (EC 1.2.3.4).<sup>31</sup> Oxalate oxidase catalyses the breakdown of oxalate to CO<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>, thus liberating calcium and H<sub>2</sub>O<sub>2</sub>, both of which would promote cell wall stiffening.

Although it is difficult to assess the relative contributions of these various mechanisms to cell wall expansion, it is clear that L-AA is able to modulate fundamental aspects of cell wall metabolism. In this respect the redox status of apoplastic L-AA, and the balance between L-AA and H<sub>2</sub>O<sub>2</sub> levels will directly or indirectly influence the degree of lignification and cross-linking of cell wall components. Apoplastic L-AA levels and oxidation state may in part be regulated by the cell wall protein AO, whose expression has been recently correlated to specific phases of the cell cycle and to cell growth (see below).<sup>100</sup>

#### *L-AA and plant cell division*

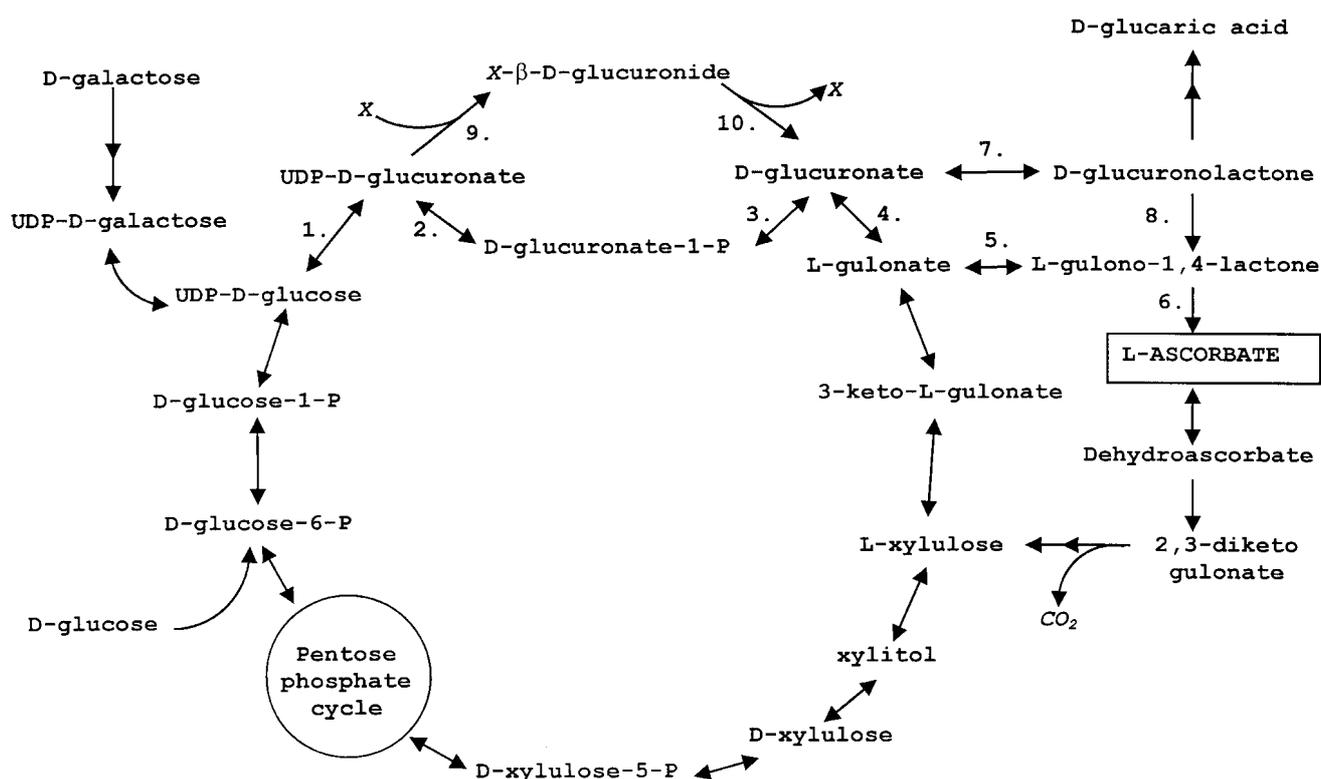
Distinct from its effects on cell expansion, are reports on the influence of L-AA on cell division and differentiation.<sup>41,101–103</sup> Exogenous L-AA has been found to accelerate the onset of cell proliferation in root primordia of *Allium*, *Pisum* and *Lupinus*,<sup>42,97,104</sup> due to an increased proportion of cells progressing through the G1/S transition.<sup>42,104–106</sup> Studies on the maize root quiescent centre, which comprises non-dividing cells arrested in G1 phase, have shown that it contains high levels of AO mRNA, protein and activity,<sup>107</sup> and that this is correlated with low or undetectable levels of L-AA. The addition of exogenous L-AA stimulates the cells of the quiescent centre to re-enter the cell cycle in both maize<sup>107</sup> and in *Allium*.<sup>105</sup> Since the product of AO activity is actually MDHA, these results may be in agreement with observations that MDHA stimulates root growth in *Allium*.<sup>97</sup> Recently, in synchronised tobacco BY-2 suspension culture, L-AA content was found to increase during cell division, and it was concluded that the concentration of DHA during G1 phase may contribute to a shortening of the cell cycle and to cell elongation.<sup>100</sup> Further, AO levels were positively correlated with the increase in DHA concentrations.<sup>100</sup> This suggests a role for AO and the L-AA/DHA ratio in determining exit from the cell division cycle.<sup>108</sup> In *Arabidopsis* root however, L-AA, GSH and

non-specific reducing agents such as dithiothreitol were all found to stimulate cell division in the root primary meristem and to extend the range of meristematic divisions.<sup>109</sup> Further, since both exogenous GSH and L-AA reversed the cessation of cell division caused by the inhibition of GSH biosynthesis, the redox status of the L-AA/DHA and the GSH/GSSG couples could be responsible for modulating internal thiols of key regulatory proteins, preventing the replication of damaged DNA under conditions of oxidative stress.

#### *3.3.3 Ascorbate oxidase*

Ascorbate oxidase (AO, EC 1.10.3.3), is a member of the 'blue' copper oxidase family of enzymes, which also includes the laccases (polyphenol oxidases), ceruloplasmin and plastocyanin.<sup>110,111</sup> AO catalyses the oxidation of L-AA to MDHA, with the concomitant reduction of molecular oxygen to water,<sup>112</sup> (for review see Ref 113). The most abundant natural sources of AO are members of the *Cucurbitaceae* (cucumber, zucchini, pumpkin, squash, melon, etc), in which AO biochemistry and expression have been extensively studied.<sup>110,114–120</sup> In the *Cucurbitaceae*, active AO is a glycosylated homodimer of 136–140 kDa, containing eight copper atoms per subunit.<sup>121</sup> AO has been shown to be a cell wall enzyme,<sup>90,118,122,123</sup> and genes encoding AO have been sequenced from cucumber,<sup>124</sup> pumpkin<sup>118</sup> tobacco,<sup>125</sup> and melon.<sup>126</sup> Genomic DNA has been isolated from cucumber,<sup>127</sup> pumpkin<sup>128</sup> and melon.<sup>123</sup> The corresponding amino acid sequences show that AO from these plants is highly conserved (75–82% identical amino acid residues), with similarities (28–30% identical amino acid residues) to laccases and human ceruloplasmin.<sup>118,124,129–131</sup> In melon, AO is coded for by a small family of at least four genes, three of which have been characterised.<sup>123</sup> This is consistent with the presence of the 12 to 14 AO isoforms that have been identified by isoelectric focusing in this species.<sup>122</sup> In tobacco a single gene coding for AO is present.<sup>125</sup>

Despite many studies, the biological function of AO is still poorly understood. However, as discussed above, increasing evidence links this enzyme to the modulation of cell expansion and/or cell division, possibly via control of the oxidation status of the L-AA/DHA redox pair.<sup>41,98,100,107</sup> In support of this, AO is induced by auxin,<sup>116</sup> and is highly expressed in fast-growing tissues<sup>90,116,123</sup> and germinating pea seeds. AO is also found localised mainly in the cell wall,<sup>123,132</sup> and AO mRNA and protein are highly expressed in flowers, ovaries and very young fruits as well as in the outer portions of the melon fruit mesocarp.<sup>122,123</sup> A similar expression pattern has also been found in non-cucurbitaceous plants.<sup>125</sup> Thus AO-mediated oxidation of apoplastic L-AA appears to be closely linked to cell elongation processes, although the mechanism by which these effects are achieved is still unclear. The availability of transgenic melon



**Figure 4.** The glucuronate pathway, L-ascorbate biosynthesis in animals and recycling of L-ascorbate via the pentose phosphate pathway. Enzymes catalysing the individual numbered reactions are given below; 1. UDP-D-glucose dehydrogenase (EC 1.1.1.22); 2. D-glucuronate-1-phosphate uridylyl transferase (EC 2.7.7.44); 3. Hydrolase; 4. D-glucuronate (hexuronate) reductase (EC 1.1.1.19); 5. Aldonolactonase (EC 3.1.1.17); 6. L-gulonolactone oxidase (EC 1.1.1.3.8); 7. Uronolactonase (EC 3.1.1.19); 8. D-Glucuronolactone reductase (EC 1.1.1.20); 9. UDP-D-glucuronosyl transferase (EC 2.4.1.17); 10.  $\beta$ -D-glucuronidase (EC 3.2.1.31).

plants carrying a sense and an antisense AO will help resolve these issues.<sup>126</sup>

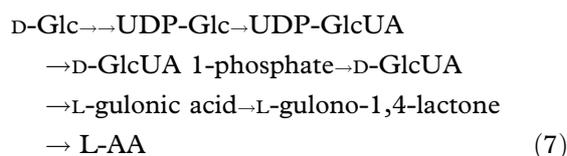
## BIOSYNTHESIS

### L-AA biosynthesis in animals

The majority of vertebrates (amphibians, reptiles, birds, and mammals) are able to synthesise L-AA, while many invertebrates and teleost fish (apart from some primitive species) are incapable of this. A few mammalian species including primates, humans and guinea pigs have lost this capability and therefore require L-AA as an essential component of their diet.<sup>133–136</sup> This deficiency has been localised to a lack of the terminal, flavo-enzyme, L-gulonolactone oxidase (GuLO, EC 1.1.1.3.8).<sup>137</sup> The gene encoding GuLO was found to be present in the human genome, but not expressed due to the accumulation of a number of mutations.<sup>136,138,139</sup> Even in vertebrates capable of synthesising L-AA, biosynthesis only takes place in a few cell types. For mammals, these are the hepatocytes, while in reptiles, amphibians and egg-laying mammals, biosynthesis takes place in the kidneys. In birds (except passeriforms which are incapable of L-AA biosynthesis), biosynthesis may take place in the kidney, liver, or both.<sup>134</sup>

The *de novo* biosynthesis of L-AA in animals was established over three decades ago,<sup>1</sup> and utilises intermediates of the D-glucuronic acid (hexuronic

acid) pathway. *In vivo*, the hexose skeleton is derived from D-glucose, mainly coming from the breakdown of glycogen,<sup>28,140</sup> and biosynthesis takes place either in the liver or kidney, which are both glycogen-storing organs (eqn (7); Fig 4) (for reviews, see Refs 28, 136).

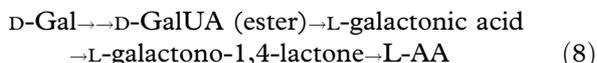


Interestingly, the terminal enzyme GuLO is not 100% specific for L-gulonolactone as substrate, but also catalyses the oxidation of the related aldono-lactones, ie L-galactonolactone (L-GL) (70–90%), D-mannonolactone (64%), and D-altronolactone (16%). The first product of the GuLO reaction is actually 2-oxo-L-gulonolactone, but this spontaneously enolises to form L-AA. GuLO utilises molecular oxygen as an electron acceptor, resulting in the concomitant formation of H<sub>2</sub>O<sub>2</sub> during biosynthesis. An important characteristic of this pathway is that it involves an apparent 'inversion' of configuration, ie early radiotracer studies using D-[6-<sup>14</sup>C]-glucose, D-[2-<sup>14</sup>C]-glucose and D-[1-<sup>14</sup>C]-glucose showed that the C<sub>1</sub> carbonyl group of L-AA is mainly formed by oxidation of the C<sub>6</sub> carbon of D-glucose (see Ref 112). Thus L-AA biosynthesis in animals follows an 'inversion'-type conversion of derivatives of D-glucose.

## L-AA biosynthesis in plants

### *Inversion pathway*

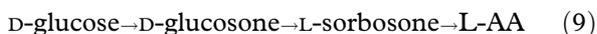
More than 45 years ago Isherwood *et al*<sup>141</sup> proposed a route for the biosynthesis of L-AA in plants based on the conversion of derivatives of D-galactose (eqn (8)). This route is analogous to the D-glucose-based pathway of animal systems discussed above, involves the conversion of derivatives of D-galactose by an 'inversion' route.



Support for this route is largely centred around the last step of the pathway where L-galactono-1,4-lactone is oxidised to L-AA by the enzyme L-galactono-1,4-lactone dehydrogenase (GLDH, EC 1.3.2.3). GLDH has now been purified and characterised from a number of different plant species,<sup>142-146</sup> and has recently been cloned from cauliflower<sup>145</sup> and sweet potato.<sup>147</sup> The substrate for GLDH, L-galactono-1,4-lactone, has also been reported to be a natural component of plant extracts.<sup>2,145</sup> Radiolabelling studies have demonstrated that D-galacturonic acid methyl ester is directly converted to L-AA without entering central carbohydrate metabolism, and a soluble enzyme activity catalysing the NADP-dependent reduction of D-galacturonic acid methyl ester to L-galactonic acid was also identified, although the affinity for its substrate was low.<sup>148</sup> However, there is currently no direct evidence for the participation of D-galactose, or sugar (nucleotide) derivatives in this route. More importantly, extensive and detailed radiotracer studies by the group of Loewus and co-workers<sup>26,112,149,150</sup> have consistently demonstrated that the majority (80%) of D-glucose is incorporated into L-AA by a pathway that does not involve inversion of the carbon skeleton. ie the C<sub>1</sub> carbonyl function of L-AA is formed by oxidation of the C<sub>1</sub> of D-glucose. Other features of the plant L-AA biosynthetic pathway are that the hydroxymethyl group at C<sub>6</sub> is conserved during synthesis and that there is a loss of label from [5-<sup>3</sup>H]-D-glucose, suggesting that this is the site of epimerisation which causes the conversion from D to L configuration. Thus in plants, L-AA biosynthesis from D-glucose proceeds via a 'non-inversion' type pathway, the opposite of the situation in animals, and the opposite of the labelling pattern predicted by the conversions of eqn (8).

### *Non-inversion pathway*

To accommodate these radiotracer data, Loewus *et al* in 1990,<sup>151</sup> proposed a new biosynthetic route from D-glucose in which no inversion of configuration occurs (eqn (9)).<sup>150-152</sup>

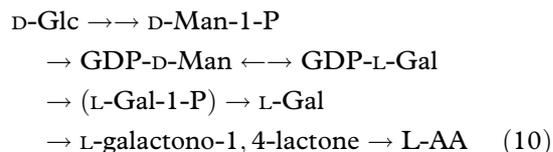


In this pathway D-glucose is first oxidised at C<sub>2</sub> to D-glucosone (D-*arabino*-hexos-2-ulose), by a pyranose-2-oxidase activity. D-glucosone is then epimerised at

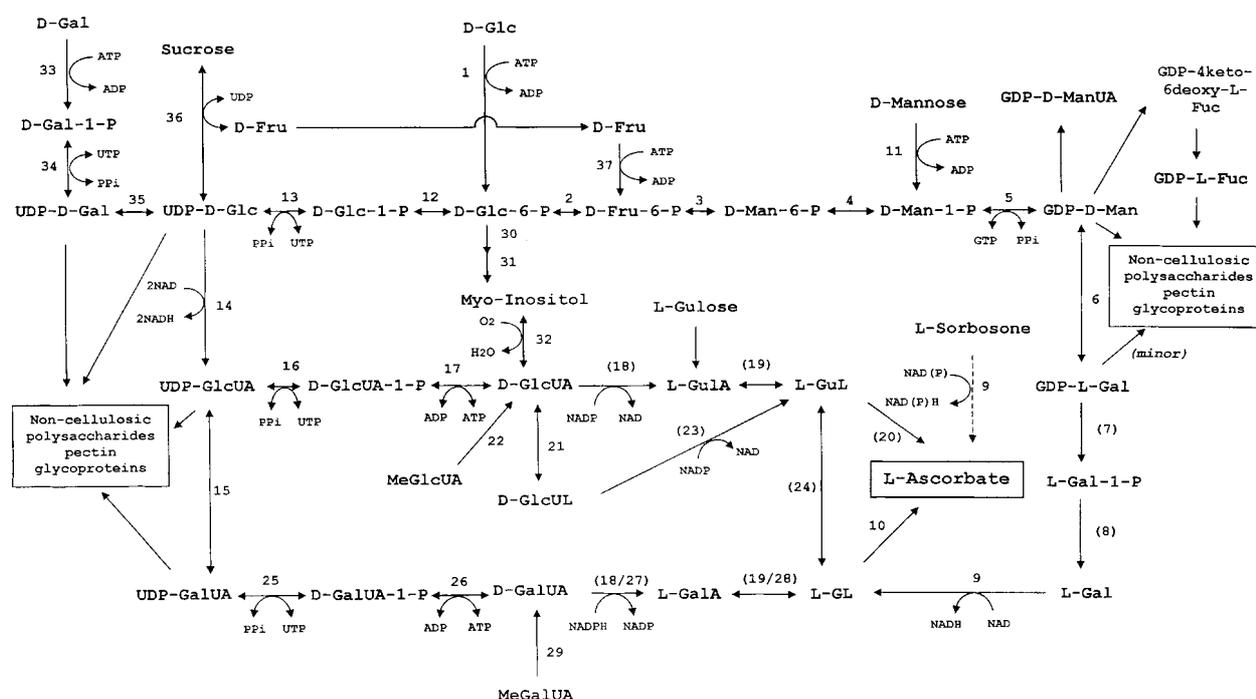
C<sub>5</sub> to give L-sorbosone (L-*xylo*-hexo-2-ulose), and C<sub>1</sub> oxidation of L-sorbosone then yields L-AA. Supporting evidence is based on the 'non-inversion' pattern of incorporation of radiolabel from D-glucose and D-glucosone into L-AA, and the partial purification of an enzyme catalysing the NADP-dependent oxidation of L-sorbosone to L-AA. In addition, nonlabelled D-glucosone and L-sorbosone both competed with radiolabelled D-glucose for L-AA biosynthesis. However, the high *K<sub>m</sub>* of sorbosone dehydrogenase for both substrates argues against a physiological role for this activity and while pyranose-2-oxidase has been identified in some Basidiomycete fungi, there has been no demonstration to date that plants possess the enzymes capable of converting D-glucose to D-glucosone or of D-glucosone to L-sorbosone.<sup>153</sup> Conklin *et al*<sup>154</sup> reported that neither D-glucosone nor L-sorbosone had any effect on L-AA accumulation in their *Arabidopsis* L-AA-deficient mutant, *vtc1*, and in *Arabidopsis* cell suspension, L-sorbosone was clearly unable to increase endogenous L-AA levels *in vivo*.<sup>155</sup> Wheeler *et al*,<sup>2</sup> reported that a newly identified enzyme, L-galactose dehydrogenase also slowly catalysed the oxidation of L-sorbosone, possibly accounting for earlier results. Recently, the conversion of D-glucosone and L-sorbosone to L-AA has been re-examined, also supporting the conclusion that this pathway is not physiologically relevant.<sup>153</sup>

### *L-galactose pathway*

Many of the contradictory data of the past decades have now been resolved by the proposal of a new pathway, which proceeds via GDP-D-mannose and GDP-L-galactose (eqn (10)).<sup>2</sup> The first part of this pathway is also utilised for the synthesis of cell wall polysaccharide precursors, while the steps after GDP-L-galactose are dedicated to L-AA biosynthesis.



Interestingly, this pathway utilises the same terminal enzyme GLDH, as the route originally proposed by Isherwood *et al*<sup>141</sup> (eqn (8)), so that previous observations on the conversion of L-galactono-1,4-lactone to L-AA apply. Here however, the conversion of D-glucose to L-AA occurs without inversion of the hexose carbon skeleton, thus reconciling the crucial radiolabelling data from the group of Loewus.<sup>26,99,149,150</sup> Additional evidence for this biosynthetic route is also accumulating. The authors found that L-galactose was an effective precursor of L-AA *in vivo*, and partially purified a new enzyme, L-galactose dehydrogenase from pea and *Arabidopsis thaliana*. This enzyme catalyses the NAD-dependent oxidation of the C<sub>1</sub> of L-galactose to L-galactono-1,4-lactone, with a *K<sub>m</sub>* for L-galactose of 0.3 mM. The same enzyme extract was also able to slowly oxidise L-sorbosone to



**Figure 5.** Interrelationships between possible pathways of plant L-AA biosynthesis and central plant hexose phosphate metabolism. Abbreviations: D-Fru, D-fructose; L-Fuc, L-fucose; D/L-Gal, D/L-galactose; L-GalA, L-galactonic acid; D-GalUL, D-galacturonolactone; D-Glc, D-glucose; L-GulA, L-gulonic acid; D-GlcUL, D-glucuronolactone; D-Man, D-mannose. Reactions involving dashed lines probably have no physiological relevance. Enzymatic conversions with numbers in brackets have yet to be conclusively demonstrated in the same tissue. Enzymes catalysing the individual numbered reactions are given below. 1. Hexokinase (EC 2.7.1.1) also catalyses reaction 11; 2. Glucose-6-phosphate isomerase (EC 5.3.1.9); 3. Mannose-6-phosphate isomerase, (EC 5.3.1.8); 4. Phosphomannomutase, (EC 5.4.2.8); 5. Mannose-1-phosphate guanylyltransferase (EC 2.7.7.22); 6. GDP-mannose 3,5-epimerase (E.C. 5.1.3.18); 7. Hydrolase; 8. Sugar phosphatase (EC 3.1.3.23) 9. L-galactose 1-dehydrogenase; 10. L-galactono-1,4-lactone dehydrogenase (EC 1.3.2.3); 11. D-mannose kinase/hexokinase (EC 2.7.1.1); 12. Phosphoglucomutase (EC 5.4.2.2); 13. UTP-glucose-1-phosphate uridylyl transferase (EC 2.7.7.9); 14. UDP-D-glucose dehydrogenase (EC 1.1.1.22); 15. UDP-glucuronate 4-epimerase (EC 5.1.3.6); 16. Glucuronate-1-phosphate uridylyltransferase, (EC 2.7.7.44); 17. D-glucuronokinase (EC 2.7.1.43); 18. D-glucuronate (hexuronate) reductase (EC 1.1.1.19) may also catalyse reaction 27; 19/28, Aldono-lactonase (3.1.1.17)/spontaneous; 20. L-gulonono-1,4-lactone oxidase/dehydrogenase; 21. spontaneous lactonisation, or Uronolactonase activity (EC 3.1.1.19); 22. (spontaneous) Methyltransferase activity; 23. Glucuronolactone reductase activity (EC 2.7.7.22) possibly catalyses reaction 18, and 22; 24. L-galactono-1,4-lactone 3-epimerase; 25. Galacturonate-1-phosphate uridylyltransferase; 26. Galacturonokinase (EC 2.7.1.44); 27. Hexuronate (D-galacturonate) reductase; 28/19. Aldonolactonase, or spontaneous; 29. (spontaneous) Methyltransferase activity; 30. *myo*-inositol 1-phosphate synthase (EC 5.5.1.4); 31. *myo*-inositol 1-phosphate monophosphatase (EC 3.1.3.25); 32. *myo*-inositol oxygenase (EC 1.13.99.1); 33. D-glucose 4-epimerase; 34. D-galactokinase (EC 2.7.1.6); 35. UTP-hexose 1-phosphate uridylyltransferase, (EC 2.7.7.10); 36. Sucrose synthase (EC 2.4.1.13) 37. Fructokinase (EC 2.7.1.4).

L-AA at low affinity, thus possibly accounting for earlier results<sup>150</sup> (eqn (9)). GDP-L-galactose (which is incorporated as a minor component of certain cell wall polysaccharides<sup>156,157</sup>) is synthesised from the double epimerisation of GDP-D-mannose. This reaction is catalysed by a known, but poorly characterised enzyme, GDP-D-mannose-3,5-epimerase, that was originally identified in *Chlorella pyrenoidosa* and flax.<sup>158–160</sup> The enzymes converting GDP-L-galactose to L-galactose still have to be identified in plants. However, Wheeler *et al*<sup>2</sup> further reported that incubations with radiolabelled GDP-D-mannose *in vitro* resulted in the incorporation of radiolabel into L-GL. Additional genetic evidence in support of this pathway is beginning to emerge from the characterisation of the L-AA-deficient *Arabidopsis* mutants of Conklin *et al*.<sup>154,161,162</sup> The locus of one of these mutants has recently been shown to be D-mannose-1-phosphate guanylyltransferase (GDP-mannose pyrophosphorylase). In independent work, antisense inhibition of this enzyme in potato, produced plants with foliar L-AA levels of 44–72% of wild type, and with a

30–50% reduction in the mannose content of the cell walls of leaves.<sup>163</sup> Upon transfer to soil these plants showed developmental changes leading to early senescence.<sup>163</sup>

The significance of this new biosynthetic route therefore, is that it integrates L-AA biosynthesis into the pathways of central carbohydrate metabolism, and provides connections to polysaccharide biosynthesis and protein glycosylation (Fig 5). Nonetheless, some intriguing questions remain unanswered, in particular the reports on the conversion of uronic acid derivatives to L-AA.

#### *Conversion of uronic acids to L-AA, an 'alternative' pathway?*

In addition to the substrates of eqn 10, it has long been known that plants are able to synthesise L-AA from derivatives of D-glucuronic and D-galacturonic acid.<sup>141,148,149,164–167</sup> Radiolabelling studies have shown that D-glucuronic acid, its (spontaneous) lactone D-glucuronolactone, as well as D-galacturonic acid methyl ester are converted directly to L-AA *in*

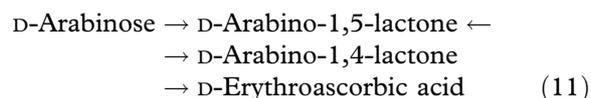
*in vivo*, by routes involving an 'inversion' of configuration.<sup>141,148,149,166,168,169</sup> These conversions occur without disruption of the carbon skeleton and with little redistribution of radiolabel.<sup>166,167,170</sup> A quantitative comparison of the rates of L-AA biosynthesis in an *Arabidopsis thaliana* cell suspension culture has shown that in addition to L-galactose and L-galactono-1,4-lactone, four other compounds are able to increase intracellular L-AA levels *in vivo*. In order of effectiveness these are; D-glucuronolactone < D-glucuronic acid methyl ester=L-gulonolactone < D-galacturonic acid methyl ester=L-galactono-1,4-lactone < L-galactose. The average rates of L-AA biosynthesis for these substrates relative to control incubations at a concentration of 15 mM, were 3:9:10:35:32 and 64, respectively.<sup>155</sup> D-galacturonic acid methyl ester could conceivably be reduced by a non-specific aldo-keto reductase activity, and this would generate L-galactono-1,4-lactone, the substrate for GLDH. However, the conversion of D-glucuronolactone, D-glucuronic acid methyl ester and L-gulonolactone to L-AA, which are not part of the L-galactose pathway, suggests the possible co-existence of a separate 'animal' type pathway. This is because purified GLDH from cauliflower meristem, spinach (*Spinacea oleracea*) leaf and *Arabidopsis* has been shown to be absolutely specific for L-galactono-1,4-lactone as substrate, with no activity towards L-gulonolactone<sup>144,145</sup> (Davey *et al* – unpublished results). Further support for this idea comes from the identification of L-gulonolactone-dependent L-AA formation in crude cellular extracts of *Arabidopsis* cell suspension culture, and which has a different sub-cellular localisation to the GLDH activity.<sup>155,171</sup> The relationship between all these possible plant biosynthetic routes is summarised in Fig 5 (adapted from Ref 155).

The physiological relevance of these uronic acid conversions must be interpreted with caution however until more data on the enzymes catalysing these reactions become available. It may be that L-AA biosynthesis from these compounds is only significant under certain circumstances or in specific tissue types. For example D-glucuronic acid and D-galacturonic acid are major components of plant non-cellulosic cell wall polysaccharides, and conversion to L-AA might represent part of a mechanism to salvage carbon arising from the breakdown of cell walls, such as occurs during abscission, fruit ripening and softening, pollen grain maturation and cell expansion during growth.

### L-AA biosynthesis in yeasts

Although early reports suggested that yeast and other micro-organisms were capable of synthesising L-AA,<sup>172,173</sup> the spectrophotometric procedures utilised were unable to distinguish between L-AA and its 5-carbon analogue D-erythroascorbic acid (Fig 1). More selective HPLC procedures later identified D-erythroascorbic acid to be a natural component of yeasts, with L-AA usually being absent unless exogenously

supplied with appropriate precursors.<sup>169,174,175</sup> Two enzymes involved in the biosynthesis of D-erythroascorbate have now been purified and cloned from *Saccharomyces cerevisiae*. D-arabinose dehydrogenase is an NADP-dependent heterodimer, which catalyses the oxidation of D-arabinose to D-arabino-1,4-lactone<sup>175,176</sup> and D-arabino-1,4-lactone is then oxidised to D-erythroascorbic acid by the flavin enzyme, D-arabino-1,4-lactone oxidase.<sup>177,178</sup> In addition to D-arabino-1,4-lactone, D-arabino-1,4-lactone oxidase also catalyses the oxidation of L-galactono-1,4-lactone and L-xylono-1,4-lactone at 85% efficiency, and L-gulonolactone at 25% efficiency. Therefore the previous identification and purification of L-galactono-1,4-lactone oxidase activity from yeast,<sup>172,173,179–181</sup> is almost certainly wrong, as demonstrated by the 100% sequence identity between the yeast 'L-galactonolactone dehydrogenase' recently published by Nishikimi's group,<sup>182</sup> and the D-arabino-1,4-lactone dehydrogenase of Huh *et al*.<sup>178</sup> Thus in yeasts, D-erythroascorbate is synthesised by a non-inversion pathway utilising C<sub>5</sub> intermediates<sup>175</sup> (eqn (11)):



### Microbial production of L-AA

Unlike eukaryotes, prokaryotes do not appear to synthesise L-AA. The interest in L-AA biosynthesis in micro-organisms therefore is focused on methods to improve the classical Reichstein–Grussner method of L-AA production. Specifically, methods to synthesise 2-keto-L-gulononic acid, which is readily converted to L-AA by acid- or base-catalysed cyclisation.<sup>183,184</sup> These methods include the L-idonic acid pathway, the 2,5-diketogluconic acid pathway, and the L-sorbose pathway (for reviews, see Refs 183, 185).

## Regulation of plant L-AA biosynthesis and control points

### Enzymatic regulation

Very little is known about the regulation of L-AA biosynthesis in plants. Nonetheless, it seems unlikely that either the terminal enzyme GLDH or the penultimate enzyme L-galactose dehydrogenase is subject to feedback control, since in the presence of exogenous L-galactono-1,4-lactone, L-AA biosynthesis occurs unabated and can lead to wilting and death of the plant.<sup>168</sup> Interestingly, studies have indicated that the L-AA-deficient *vtc* mutant appears to have a higher GLDH capacity than wild-type plants, suggesting that GLDH is upregulated in response to the lowered endogenous L-AA levels.<sup>154</sup> Wounding, which will increase the antioxidative demand of tissues, has also been reported to induce GLDH activity in sweet potato tubers,<sup>142</sup> and sweet pepper fruits.<sup>186</sup> The time scale of these responses suggests

that GLDH may be subject to broad, end-product regulation.

#### Variations in flux

The assumption that L-AA was synthesised from hexose precursors, and the strong influence of both light and photosynthesis on the L-AA content of leaves and fruit,<sup>187</sup> have long suggested a possible relationship between photosynthesis and L-AA biosynthesis.<sup>165</sup> Indeed in leaves, the supply of hexose phosphate may be critical for L-AA biosynthesis. In photosynthetic tissue Smirnoff and Pallanca,<sup>188</sup> found a linear relationship between the L-AA pool size and the soluble carbohydrate content of barley leaves, and in the dark, there is a rapid turnover and decrease of the foliar L-AA content in barley,<sup>188</sup> and *Arabidopsis*.<sup>154</sup> The fact that these losses could be prevented by feeding exogenous sugars shows that L-AA biosynthesis is not strictly light dependent and suggests that the supply of hexose phosphate could be limiting in the dark. It was found that approximately 50% of the L-AA pool turns over in about 24h.<sup>154</sup> However, L-AA biosynthesis is in general much less sensitive to carbohydrate supply in non-photosynthetic tissue.<sup>153</sup> More recently it has also been shown in pea seedlings that the rate of biosynthesis decreases when the L-AA pool is elevated, suggesting that the pathway could be controlled by feedback inhibition. Since high concentrations of D-mannose do not affect the size of the endogenous L-AA pool, it has been suggested that a possible control point lies between the conversion of GDP-D-mannose to L-galactose.<sup>2,155</sup>

The sugar phosphates which supply the hexose skeleton for L-AA biosynthesis are in rapid equilibrium with each other and act as a reservoir from which precursors are withdrawn for carbohydrate biosynthesis and glycolysis (Fig 5). The distribution of carbon flux between these various processes is thus likely to influence the overall L-AA pool size. In addition to L-AA biosynthesis, GDP-L-galactose and GDP-D-mannose are used for cell wall polysaccharide (rhamnogalacturonan I, II, and xyloglucan), and glycoprotein biosynthesis. Therefore, competition for GDP-mannose will divert carbon into glycoprotein and polysaccharide biosynthesis from L-galactose and L-AA biosynthesis (L-galactose is only a minor component of cell wall polysaccharides.<sup>156</sup> This raises the intriguing possibility that GDP-D-mannose and/or GDP-D-L-galactose may be derived not only *de novo* from D-glucose, but also from the breakdown of cell wall storage polysaccharides. Indeed, Roberts<sup>156</sup> concluded that the pattern of utilisation of radiolabelled D-mannose by corn roots was indicative of a role for D-mannose-1-phosphate guanylyltransferase in a salvage pathway for the reincorporation of free D-mannose.

#### Metabolic channelling, conjugation

The terminal enzyme of plant L-AA biosynthesis GLDH, is mitochondrial and the enzyme has consistently been purified from mitochondrial fractions of

various species.<sup>142-147</sup> In bean hypocotyls, GLDH activity was found exclusively associated with the mitochondria and no activity was detected in membrane fractions derived from microsomes, peroxisomes or the tonoplast.<sup>189</sup> GLDH activity has also been localised to the mitochondrial membranes of spinach leaf,<sup>144</sup> and the cauliflower cDNA contains a putative mitochondrial targeting signal.<sup>145</sup> No information however is available on the orientation of the active sites of GLDH. It is thus not clear whether L-AA is synthesised and released into the mitochondrial lumen, subsequently requiring transport to the cytosol as is the case in rat liver microsomes,<sup>190</sup> or whether it is released into the intramembrane space where it can freely diffuse into the cytosol.

#### Compartmentation

At the subcellular level, the presence of L-AA has been demonstrated in different cell compartments including the chloroplast, cytoplasm, mitochondria and apoplast. Routinely concentrations as high as 25–50mM have been measured in the chloroplast.<sup>27,191</sup> Vacuolar concentrations are relatively low at around 0.6mM–3mM and cytoplasmic concentrations of 20–60mM, depending on the species and the incident light intensity.<sup>192,193</sup> Approximately 25% of the cellular L-AA was recovered from the apoplast (*Picea* needles),<sup>21</sup> with concentrations in the range of 0.15–2mM.<sup>81,92</sup> Interestingly it has been reported that certain species with very high intracellular L-AA concentrations (eg horseradish root), accumulate L-AA in the large central vacuole, where presumably the low pH stabilises the molecule.

#### L-AA and lycorine

Lycorine (2,4,5,7,12b,12c-hexahydro-1H-[1,3]dioxolo[4,5-j]pyrolo[3,2,1-de]phenanthridine-1,2-diol), is a pyrolophenanthridine alkaloid present in various *Amaryllidaceae*,<sup>194</sup> which has been shown to be a powerful inhibitor of growth and cell division in higher plants, algae and yeasts.<sup>102,195,196</sup> It is also the only known inhibitor of L-AA biosynthesis, and is active at levels as low as 1µM. In certain plant species however, L-AA biosynthesis is essentially insensitive to lycorine.<sup>151,169,197</sup> These species-specific differences have been attributed to differences in the ability to regenerate L-AA via DHA reductase activity.<sup>198</sup> Lycorine has been reported to exert its activity through inhibition of GLDH *in vivo*<sup>198</sup> and *in vitro*.<sup>199</sup> For example 90% of the GLDH activity in *Vicia faba* and *Zea mays* seedlings was inhibited by 5µM lycorine<sup>199</sup> and by 10µM lycorine in cauliflower meristem.<sup>200</sup> More recently, the activity of purified GLDH from sweet potato was found to be inhibited 86% by 170µM lycorine.<sup>147</sup> However, others have found that GLDH from cauliflower is insensitive to concentrations of up to 250µM lycorine.<sup>145</sup> Assuming that the lycorine preparations are equally active, these differences are conceivably related to differences in the GLDH protein structure, the most striking of which is the

presence of a non-covalently bound FAD in the GLDH of sweet potato<sup>147</sup> and its absence in the cauliflower enzyme.<sup>145</sup> Conflicting results could therefore be due to differences in GLDH purification protocols. However, in yeasts, lycorine interacts with mitochondrial DNA,<sup>201–205</sup> and in wheat it has been reported to inhibit peptidyl transferase activity.<sup>206</sup> Consequently some caution should be exercised in the interpretation of data on L-AA metabolism obtained through the use of lycorine.

### Plant L-AA transport

As discussed above, the terminal step in the plant biosynthesis of L-AA is catalysed by GLDH (eqn (10)), which is a mitochondrial enzyme.<sup>142–146</sup> There is currently no evidence to suggest that GLDH activity occurs elsewhere in the cell.<sup>189</sup> Since L-AA is a monovalent anion at physiological pHs and is unable to permeate membranes, it must be transported across the membranes of all other subcellular compartments where it is found, including chloroplasts, apoplast and vacuole.

#### *Chloroplastic transport*

Concentrations of 20–50 mM L-AA have regularly been reported in the chloroplasts.<sup>191,192</sup> The physiological role of the chloroplastic L-AA translocator appears to be the maintenance of stromal L-AA concentrations, by replenishing oxidised/degraded L-AA that escapes regeneration by the endogenous mechanisms. This chloroplastic L-AA is required for the detoxification of photosynthetically generated ROS.<sup>207</sup> In purified chloroplasts of spinach and pea, L-AA uptake was found to be a carrier-mediated process, with transport across the membrane occurring by facilitated diffusion.<sup>207,208</sup> This transport is distinct from D-glucose uptake,<sup>192</sup> but shows a surprisingly low affinity for its substrate with a  $K_m$  of around 20 mM. This suggests that the highly efficient mechanisms for L-AA regeneration within the chloroplast do not require a rapid influx of extra-chloroplastic L-AA to support ROS scavenging. Approximately 10–20% of the chloroplastic L-AA is found bound to the inner thylakoid membranes,<sup>21</sup> but transport across the thylakoid membrane was found to occur only by diffusion.<sup>192</sup> Since intralumenal L-AA is monovalently reduced to MDHA by the activity of the enzyme violaxanthin de-epoxidase,<sup>209</sup> the availability and transport of L-AA across the thylakoid membrane could act as a modulator of this enzyme activity and of the violaxanthin cycle.

#### *Plasma membrane transport*

Apoplastic L-AA is involved in the defence of cells against atmospheric-induced oxidative stress,<sup>81,82</sup> wounding,<sup>92</sup> and in the modulation of cell expansion.<sup>3</sup> Again, since GLDH is a mitochondrial enzyme, a plant plasma membrane transport system is required to maintain apoplastic L-AA concentrations and the apoplastic L-AA/DHA redox balance. In soybean, the

uptake of exogenous L-AA by roots and leaves has been shown to be an energy-requiring process.<sup>210</sup> In barley<sup>193</sup> and pea<sup>192</sup> leaf protoplasts, the uptake of L-AA and DHA displayed saturation kinetics, indicative of a carrier-mediated process possibly driven by the proton electrochemical gradient.<sup>193</sup> Using purified bean plasma membrane vesicles, it was subsequently clearly demonstrated that DHA is the preferred uptake form of L-AA and that DHA is taken up in a facilitated diffusion mechanism.<sup>211,212</sup> Further, *in vitro*, the uptake of external DHA can result in an exchange with internal (cytosolic) L-AA, and preloading vesicles with L-AA stimulates the uptake of DHA, a so-called *trans*-stimulation. The pH optimum for this high-affinity transport was 6–7.5, with a  $K_m$  for DHA of 24  $\mu\text{M}$ .<sup>213,214</sup> The identification of this carrier and its relationship to other transport proteins will depend on its purification and cloning. It is presently unclear whether additional L-AA/DHA transporters are also present in the plant plasma membrane, but there is some preliminary evidence that the plasma membrane is also able to transport L-AA via the glucose transporters. This is similar to the transport of L-AA and DHA across the plasma membrane of human neutrophils,<sup>215</sup> and rat liver microsomes,<sup>216</sup> where DHA is preferentially accumulated via the facilitative glucose transporters, and then reduced to L-AA inside the cell.

#### *Trans-plasma membrane electron transport*

None of the enzymes of the L-AA-GSH cycle appear to exist outside of the plasma membrane. Therefore alternative methods to maintain the L-AA/DHA redox status must exist. Apart from the exchange DHA carrier discussed above, there is also evidence for *trans*-plasma membrane electron transport to apoplastic MDHA, possibly from cytosolic L-AA, via the plasma membrane-associated cytochrome *b*.<sup>85,86,211</sup> This is a relatively abundant protein, at 0.1–0.5% of the total membrane protein and appears to be present in all tissue types and species examined to date (H Asard, pers communication). The cytosolic MDHA formed by this electron transport is then either reduced to L-AA by the activity of an NADH-dependent MDHA-reductase localised on the inside of the plasma membrane,<sup>217</sup> or enters the cytosolic L-AA-GSH cycle together with DHA originating from the apoplast. The *trans*-plasma membrane electron transport to MDHA has been implicated in the stimulation of the plasma membrane ATPase and growth.

#### *Intercellular transport*

In addition to intracellular transport, L-AA can also presumably be transported throughout the plant since growth on L-AA stimulates increases in foliar L-AA levels, and radiolabelled L-AA fed to the roots is recovered in other parts of the plant.<sup>210</sup> However, there is currently no information on the long-distance transport of L-AA in the xylem or phloem, and the

high pH of these fluids would presumably destabilise both L-AA and DHA, unless L-AA were transported in a stabilised form, for example as a glycosylated or phosphorylated derivative.

### Turnover and catabolism

#### *Catabolism to oxalate, tartrate and threonate*

Little is known about the processes of L-AA catabolism in plants, except that cleavage of the L-AA carbon skeleton gives rise to oxalate and tartrate. Oxalate is a normal component of higher plant tissues, possibly involved in osmoregulation and the control of calcium concentrations.<sup>218</sup> Smirnov<sup>31</sup> has suggested that the formation of oxalate from apoplastic L-AA together with oxalate oxidase activity could also have a role in cell wall expansion. Tartrate is important in the organoleptic characteristics of grape berries and it has been shown that the conversion of L-AA to tartaric acid is developmentally regulated, ie utilisation of L-AA is limited to a short period which coincides with anthesis and is determined by the location of the leaf relative to the fruit.<sup>219</sup>

Certain plant species accumulate such high levels of oxalate and tartrate that they may be referred to as either oxalate or tartrate accumulators. In tartrate accumulators such as *Pelargonium crispum* (a geranium species), early radiotracer studies with 1-[<sup>14</sup>C]-ascorbic acid showed that cleavage of L-AA at C<sub>2</sub>/C<sub>3</sub> occurs to produce oxalate and L-threonate. Oxalate is derived from the C<sub>1</sub>/C<sub>2</sub> fragment, while L-threonate is further oxidised to L-tartrate.<sup>112</sup> Surprisingly oxalate-accumulating species do not always show a concomitant accumulation of L-tartrate. This was eventually shown to be due to the decarboxylation of L-threonate to a C<sub>3</sub> product that is recycled into central hexose metabolism<sup>220</sup> (Fig 5).

In vitaceous species however (grape, virginia creeper), the formation of tartrate arises from cleavage at the C<sub>4</sub>/C<sub>5</sub> bond of L-AA (or DHA).<sup>112</sup> This generates L-tartrate and a C<sub>2</sub> fragment that is recycled into carbohydrate metabolism, possibly as glycoaldehyde. This conversion of L-AA to tartrate in grape was shown to proceed via 5-keto-L-idonic acid.<sup>221</sup> In species showing C<sub>4</sub>/C<sub>5</sub> cleavage and tartrate accumulation, L-AA conversion probably represents the major biosynthetic pathway. However, in other species the biosynthesis of oxalate from L-AA is probably not the major route, and oxalate is also formed from the oxidation of glycolic acid, the cleavage of isocitric acid by isocitrate lyase and the cleavage of oxalacetic acid by oxalacetase.<sup>152,221,222</sup>

Although the pathway for plant oxalate formation has been established by radiotracer studies, none of the enzymes involved has been identified, and it is unclear what role this biosynthetic route might play in L-AA catabolism. The conversion to oxalate would seem to represent an energetic drain on the organism since only 50% of the L-AA-derived carbon can be recycled into central metabolism (via L-threonate). Furthermore, oxidation of oxalate by oxalate oxidase gener-

ates H<sub>2</sub>O<sub>2</sub>, which only serves to increase the cellular oxidative stress and enhance L-AA turnover. Possibly, L-AA cleavage in oxalate accumulators serves another specific, unrelated function. An energetically more favourable, but hypothetical scenario is discussed in the following section.

#### *Recycling of DHA*

DHA is unstable at physiological pHs and spontaneously and enzymatically delactonises to 2,3-diketo-gulonic acid (2,3-DKG). It has been shown in animal cells that DHA can be recycled via 2,3-DKG, using the non-oxidative pentose phosphate pathway. This cycle takes place on both an intracellular and an interorganellar level, and generates triose phosphates, D-xylulose-5-phosphate and fructose-6-phosphate<sup>28,223,224</sup> The idea that L-AA can be recycled in this manner has not been specifically tested in plant tissues, but all the enzymatic conversions necessary are known to be present. Further, under conditions of oxidative stress when DHA accumulates, this cycle would provide a means of regenerating L-AA from gluconeogenic precursors that can again be used for L-AA biosynthesis, with the overall loss of only one carbon (as CO<sub>2</sub>). If this recycling follows the same route as in animal cells, 2,3-DKG is first decarboxylated to L-lyxonate and L-xylonate, before entering the pentose phosphate pathway as D-xylulose 5-phosphate. Together with erythrose 4-phosphate, D-xylulose 5-phosphate can be converted to glyceraldehyde 3-phosphate and the hexose fructose 6-phosphate, by transketolase activity. Fructose 6-phosphate then is part of the hexose phosphate pool that can be used for L-AA biosynthesis. The enzymes of the non-oxidative pentose phosphate pathway are located both in the stroma of the chloroplasts and the cytosol, although it has been questioned whether the cytosol contains the full complement of enzymes necessary for cyclic operation.<sup>225</sup> The operation of such a cycle in plant cells may explain why specific enzymes associated with the breakdown or catabolism of L-AA have not been detected to date.

### Impact of environment and culture conditions

As might be expected from its antioxidant-based functions in plant metabolism, the levels of L-AA as well as of L-AA-regenerating enzymes, are responsive to a wide variety of environmental (stress) factors. These include light, temperature, salt and drought stress, the presence of atmospheric pollutants, metals and herbicides. Furthermore these changes are often superimposed upon cell-specific and developmental changes in L-AA, and even diurnal and seasonal fluctuations.

Several groups have reported that plants increase their L-AA levels in response to high light, in a process requiring several days.<sup>21,27,188,226-228</sup> The relationship between light, hexose levels and turnover has been discussed earlier. In addition to this, however, the age and the leaf surface light intensity clearly influence

endogenous L-AA levels,<sup>27</sup> with higher concentrations being found at the top of the plant in young actively growing tissue, although there is much variation between species in the absolute values.<sup>227</sup> Interestingly, while transfer to high light conditions has little influence on L-AA levels, transfer back to dark or low light, causes a transient, rapid increase in L-AA.<sup>21</sup> This suggests that on return, there is a mismatch between turnover and biosynthesis, leading to the temporary accumulation of L-AA.

Seasonal variations in foliar L-AA and GSH levels, which could be correlated with ambient ozone concentrations, have been observed in beech (*Fagus sylvatica* L).<sup>88</sup> Schmieden *et al*<sup>229</sup> also reported on seasonal and diurnal fluctuations in L-AA levels of pine needles, with highest levels being associated with oxidative damage, due to ozone exposure, high light intensity and Mg<sup>2+</sup> deficiency. Apoplastic L-AA has been shown to be involved in ozone detoxification in spinach<sup>81</sup> and pumpkin,<sup>87</sup> although it was reported that in velvet beans (*Mucuna pruriens*), the effects of ozone, propene and isoprene on L-AA were only observed in young leaves.<sup>230</sup> The effects of ozone have been reviewed.<sup>231,232</sup> In addition to ozone, other treatments such as exposure to herbicides,<sup>233,234</sup> low temperatures,<sup>235</sup> also influence the size of the L-AA pool suggesting a clear relationship with the anti-oxidative demands of the plants.

## GENETIC CONTROLS AND PATHWAY ENGINEERING

### Plant genes characterised along the biosynthetic pathway

The first gene specifically associated with the plant L-AA biosynthetic pathway to be cloned was GLDH, cloned from cauliflower meristem,<sup>145</sup> and more recently from sweet potato.<sup>147</sup> The GLDH cDNAs from cauliflower and sweet potato show 77% identity at the amino acid level,<sup>147</sup> and significant homology to the analogous enzymes of rat (22% identity) and yeast (17% identity). This homology is situated primarily in the NH<sub>2</sub>-terminal region of the protein, suggesting that this domain has similar functional roles in these organisms.<sup>145</sup> Southern blot analysis indicates that the GLDH gene in sweet potato exists as a single copy.<sup>147</sup> Recently, the locus of the ozone-sensitive and L-AA-deficient mutant, *vtc-1* has been identified as coding for the enzyme mannose-1-phosphate guanylyltransferase (EC 2.7.7.13).<sup>154,162</sup>

### Similar genes from other organisms

The terminal enzyme of L-AA biosynthesis in animals, L-gulonolactone oxidase (GuLO), is a flavoprotein that has been characterised and purified from a number of animal species.<sup>236-240</sup> In guinea pigs and man, the non-functional gene for GuLO has been isolated and cloned and the (multiple) nucleotide changes responsible for the loss of function identified.<sup>138,139</sup> The cDNA for GuLO has also been

identified in rats,<sup>241</sup> *in vitro* synthesis of the enzyme demonstrated in rabbit reticulocyte lysates,<sup>242</sup> and the missing enzyme has been expressed in monkey cells, resulting in a restoration of the L-AA biosynthetic abilities.<sup>243,244</sup>

Yeasts preferentially synthesise D-erythroascorbic acid, the C<sub>5</sub> analogue of L-AA. The enzymes D-arabinose dehydrogenase and D-arabino-1,4-lactone oxidase involved in the biosynthesis of D-erythroascorbic acid have recently been cloned in *Saccharomyces cerevisiae*.<sup>175,176,178</sup> The 'L-galactono-1,4-lactone oxidase' independently cloned from *Saccharomyces cerevisiae*,<sup>180</sup> is 100% identical, misclassified because of the relatively broad substrate specificity of D-arabino-1,4-lactone oxidase.

### Mutants

Currently, only two L-AA-deficient mutants have been described, *vtc1-1* and *vtc1-2*.<sup>154,161,162</sup> These *Arabidopsis thaliana* EMS mutants were originally isolated on the basis of their increased sensitivity to ozone, and contain only 30% of the wild-type foliar L-AA. The *VTC-1* locus has recently been identified as coding for mannose-1-phosphate guanylyltransferase (GDP-mannose pyrophosphorylase), the enzyme responsible for the 'activation' of D-mannose-1-phosphate and the formation of GDP-mannose.<sup>62</sup> Not only are endogenous L-AA levels lower than wild type in the *vtc-1* mutants, but the rate of L-AA biosynthesis from D-mannose is also lower. Both *vtc1-1* and *vtc1-2* contain the same single point mutation in the mannose-1-phosphate guanylyltransferase gene, resulting in approximately 35% decrease in enzyme activity, although steady state GDP-mannose pyrophosphorylase mRNA levels were unaltered. GDP-mannose, in addition to being used for L-AA biosynthesis is also required for synthesis of several non-cellulosic cell wall polysaccharides, and for protein N- and O-linked glycosylation and the *vtc1* mutants also contained aberrant protein glycosylation profiles.<sup>162</sup> It cannot be excluded therefore that defective glycosylation of proteins directly or indirectly involved in L-AA metabolism due to decreased GDP-mannose levels, could be partially responsible for the decreased L-AA concentrations observed in the *vtc1* mutants. However, the mannose-1-phosphate guanylyltransferase antisense potato transformants were reported to have an unchanged protein glycosylation profile.<sup>163</sup>

## PLANT FOOD SOURCES

### Important food sources

L-AA is a universal constituent of all green plants, with the exception of dormant seeds,<sup>26,245</sup> and it is not difficult to obtain an adequate supply in the daily diet. Indeed in developed countries deficiencies are usually only associated with alcoholism and poverty. For adults, dietary needs are met by a minimum intake of 60 mg per day.<sup>29</sup> However, the preventative functions

**Table 3.** L-ascorbic acid content of selected fruits and vegetables (adapted from Refs 28, 330, 355)

Source	mg (100g) <sup>-1</sup>	μmolg <sup>-1</sup> fresh weight
Acerola (west indian cherry)	1300	73.00
Apple	2–10	0.11–0.56
Apricot	7–10	0.39–0.56
Avocado	15–20	0.84–1.12
Banana	10–30	0.56–1.68
Blackberry	15	0.84
Broccoli	113	6.35
Broccoli (cooked)	90	5.05
Brussels sprouts	87–109	4.94–6.12
Cabbage (raw)	46–47	2.64
Cauliflower	64–78	3.63–4.38
Cauliflower (cooked)	55	3.09
Carrot	6	0.34
Cranberry	12	0.67
Cherry	5–8	0.28–0.45
Blackcurrant	200–210	11.2–11.8
Redcurrant	40	2.25
Damson	3	0.17
Gooseberry	40	2.25
Gourd	8	0.45
Passion fruit	25	1.40
Grapefruit	40	1.18
Guava	230–300	13.1–16.8
Horseradish	120	6.74
Kale	186	1.01
Kale (cooked)	62	3.48
Kiwi	60	3.41
Lemon	50	2.84
Lettuce	15	0.85
Lime	25	1.40
Loganberry	30	1.68
Lychee	45	2.55
Melon	10–35	0.57–1.97
Orange	50	2.84
Orange (juice)	50	2.84
Tangerine	30	1.68
Peach	7–31	0.39–1.76
Peach (canned)	6	0.34
Pepper (green)	128	0.72
Plum	3	0.17
Pea	25	1.40
Pear	3–4	0.17–0.23
Pineapple	12–25	0.68–1.40
Pineapple (canned)	12	0.68
Pomegranate	6	0.34
Potato (new)	30	1.68
Potato (Oct, Nov)	20	1.14
Potato (Dec)	15	0.85
Potato (Jan, Feb)	10	0.57
Potato (Mar, May)	8	0.45
Potato (boiled)	16	0.90
Quince	15	0.84
Raspberry	25	1.40
Rosehip	1000	5.62
Spinach	51	2.86
Spinach (cooked)	28	1.57
Strawberry	59–60	3.37
Tomato	20–25	1.14–1.40
Tomato (juice)	16	0.90
Watercress	68–79	3.82–4.44

of L-AA in cardiovascular disease, certain cancers etc,<sup>10</sup> provide compelling arguments for an increase in dietary intakes and RDAs.<sup>246</sup>

As shown in Table 3, relatively high amounts of L-AA are found in strawberries, citrus fruits, and various vegetables, although the availability of L-AA within these food sources will be influenced by numerous factors. Virtually all of the L-AA in western diets is derived from fruit and vegetables. In the most recent UK consumption statistics,<sup>247</sup> over 85% of the daily dietary intake of L-AA was derived from fruit and fruit juices, vegetables and their products; 27 mg from fruit, 20 mg from vegetables (>85% of all). Until recently, the seasonal availability of fruit and vegetables and the signification losses of the vitamin which occurred during storage, preparation and cooking, meant that L-AA remained one of the few nutrients in which the diet can be deficient.<sup>248</sup> A summary of the average L-AA content of the major dietary sources in mg (100g)<sup>-1</sup> fresh weight is given in Table 3.

In general, fruits tend to be the best food sources of the vitamin, with blackcurrant being especially rich at 200mg (100g)<sup>-1</sup>, strawberry (60mg (100g)<sup>-1</sup>), and the citrus fruits at 30–50mg (100g)<sup>-1</sup>. Not all fruits contain such levels, and apples, pears, and plum represent only a very modest source of L-AA (3–5mg (100g)<sup>-1</sup>). However, much fruit is eaten raw and the low pH of fruits stabilises the vitamin during storage. Variable losses occur for processed (mainly canned) fruits. Vegetables collectively show a wide range of L-AA contents with the *Brassic*s (Brussels sprouts, cauliflower and cabbage) generally containing the highest L-AA levels at 50 to >100mg (100g)<sup>-1</sup>. However, other significant sources include peas, green beans and leafy vegetables such as spinach (50–100mg (100g)<sup>-1</sup>). Other rich sources are peppers (120mg (100g)<sup>-1</sup> and gourds (185mg (100g)<sup>-1</sup>, but onions, carrots and root crops have generally much lower levels.

In terms of the relative dietary contributions of a food, it is not only the nutrient levels, but also the level of consumption of the food that is important. Detailed consumption data for fruit and vegetables in 13 European countries<sup>249</sup> show that potatoes are overwhelmingly the most highly consumed vegetable (except in Italy), with tomatoes being second (first in Italy). For fruit, apples are the highest followed by oranges, but their level of consumption is still relatively low compared to potatoes. Thus potatoes, despite having only a modest L-AA content (values range from 30mg (100g)<sup>-1</sup> in new-season potatoes down to 10mg (100g)<sup>-1</sup> in long-term (6–9 months) stored potatoes), are the major source of L-AA in the European diet (Table 4).

#### Ripening and post-harvest losses

The nutrient content of foods is subject to variation within a species but there are also differences due to environmental and cultural practices. In a study on tomatoes, Davies and Hobson<sup>187</sup> noted a seven-fold

**Table 4.** Contribution of foods to L-ascorbic acid intake in the UK diet

Contribution to Vitamin C		% in UK Diet (1997 Statistics)
Fruit of which:		27.3
	fresh fruit	13.1
	fruit juice	13.4
Vegetables		19.7
of which:	fresh potatoes	5.8
	potato products	1.1
	fresh green vegetables	2.6
	other fresh vegetables	5.0
	vegetable products	5.2

variation in L-AA content dependent on factors such as cultivar, ripeness, size, position on plant, light, soil type, and indoor or outdoor culture. Variations of this size are by no means atypical with apples reportedly showing a 10-fold variation, oranges, peas and lemon, three-fold, kiwifruit seven-fold, but more typically, potato a two-fold variation in L-AA content. Within, or additional to these variations, are maturity effects which can have a considerable but variable effect on the L-AA content. Thus Nagy<sup>250</sup> reported that the L-AA content of citrus fruits is two-fold higher in immature fruits, while Watt<sup>251</sup> showed a small decrease in potatoes as they matured. Selman and Rolfe<sup>252</sup> found that the L-AA content of peas decreased two-fold over the maturation period, and a two-fold increase was observed by Rahman *et al*<sup>253</sup> in green and yellow peppers. Salunkhe *et al*<sup>254</sup> reported a 1.6-fold increase in maturing tomato, and Kaur<sup>255</sup> reported 3.1-fold difference between mature green and ripe red tomatoes. Thus there appears to be no clear pattern as to the effect of maturation on L-AA content, as concluded by Breen in his review.<sup>256</sup> Similar-sized variations will also be caused by soil type, fertility, location and season, and also possibly by irrigation.

### Effects of processing

The processing techniques most relevant to fruits and vegetables are canning, freezing and dehydration. These techniques have been important during the past 50 to 100 years, and will continue to be important in helping to provide all-year-round availability of these foods. All fruits and vegetables are 'seasonal', and all, with the exception of certain root crops (eg potatoes), undergo progressive and in some cases quite rapid changes if stored untreated at ambient temperature. These changes are brought about by the various enzymes in the fruit/vegetable in the presence of air, and can affect colour, taste and hence overall palatability, in addition to having profound effects on nutritive values. Most prominent amongst the nutrients affected is L-AA, so much so that it is often used as a 'marker' for post-harvest deterioration. Consequently, it is essential to eliminate enzyme activity to ensure the long-term storage stability (months rather than days) of fruits and vegetables. During the canning process, this involves high-temperature treatment

(sterilisation) and sealing to exclude air; the freezing process involves a blanching stage, prior to freezing to below  $-20^{\circ}\text{C}$ ; and dehydration involves hot air treatment to drive off the water. In all cases the temperature and time of treatment reduce unwanted enzyme activity to an acceptable minimal level.

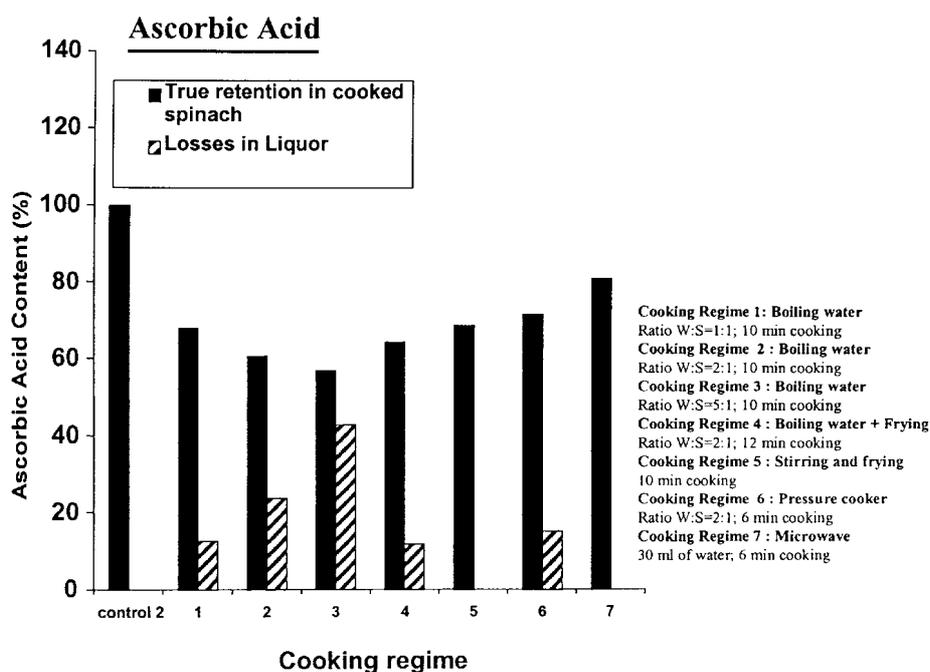
Unsurprisingly, this heat treatment also has consequences for labile nutrients and in particular for L-AA. Indeed the use of L-AA as a 'marker' vitamin in studies on the effects of processing on the nutritional value of foods means that there is a wealth of information available upon which to draw (for reviews see Refs 256, 257). It is important to note that fresh produce also undergoes post-harvest change during distribution, marketing and in-home storage, as well as during end-cooking. These changes are such that the nutritional value of the fresh produce as eaten is often quite different to that of the garden fresh.<sup>258</sup> A quote from Breene—'When properly processed a given fruit or vegetable can be as healthful, or more so, than a fresh counterpart'.

### Industrial processing

Care must be taken to harvest the crop at optimum maturity, to minimise damage during harvesting and to process as soon as possible post-harvest. This maxim applies for whichever processing method is adopted. Green leafy vegetables, peas (ex-pods), and green beans are particularly vulnerable in the immediate post-harvest period and losses of over 20% of L-AA can occur.<sup>259</sup> The subsequent thermal treatments (sterilisation in the canning process, blanching in the freezing process, fluidised-bed hot air treatment for the dehydration process) result in further losses which can be particularly significant during canning and dehydration. However, the extent of these losses is highly variable, and during canning is reported to be dependent on such factors as container construction (glass, metal etc.), pH of the food, type of steriliser (batch, continuous, ultra-high temperature) and conduction versus convection heating.<sup>260</sup> Losses of well over 50% are typical for vegetables, but are much less for most fruits and in particular acid fruits because of the stabilising effects of low pHs. It is worth noting that 'canning' by dielectric or ohmic heating can minimise nutrient loss without inflicting mechanical damage.<sup>261</sup>

The dehydration process, which is generally more widely applied to potatoes than to fruit and vegetables, can be very destructive to L-AA with losses of 75% being reported.<sup>262</sup> Such losses are inevitable because of the thermal treatment necessary to remove water. Due to the importance of potatoes as a dietary source of L-AA, it is a common, longstanding practice to restore losses by fortification.<sup>263</sup>

The losses that occur during the freezing process occur mainly at the blanching stage, again due to thermal degradation, but also through leaching of nutrients into the blanching medium. In a detailed laboratory study,<sup>264</sup> L-AA losses during blanching



**Figure 6.** Losses of L-AA arising from in home processing and cooking. Cooking regimes followed; Cooking regime 1–boiling; ratio W:S=1:1, 10 min cooking Cooking regime 2–boiling; ratio W:S=2:1, 10 min cooking Cooking regime 3–boiling; ratio W:S=5:1, 10 min. Cooking regime 4–boiling water + frying; ratio W:S=2:1, 12 min cooking Cooking regime 5–stirring and frying; 10 min cooking Cooking regime 6–pressure cooker; ratio W:S=2:1, 6 min cooking Cooking regime 7–microwave; 30 ml of water, 6 min cooking.

were found to be 10% for sweetcorn, 20% for green beans and 30% for broccoli. In earlier work by the same author,<sup>265</sup> blanching losses of 20% and 40% were found for green beans and broccoli, respectively. In a more recent study on commercially frozen produce,<sup>258</sup> average L-AA losses during the freezing process were reported to be 30% for peas, 10% for green beans, and 20% for broccoli and in three individual studies on peas, blanching losses were between 26% and 37%, illustrating the extent of variation in a well-controlled commercial system. Such losses will additionally depend on such factors as harvest damage, cutting/slicing, particle size, and type of blancher (steam/water, rotary/cabin). Thus for vegetables, losses through the freezing process are typically in the range of 10 to 40%.

In general, losses of L-AA are greatest during dehydration, are substantial but slightly less during canning, and are lowest during freezing. These conclusions need however to be viewed in the context of subsequent storage and end-cooking.

#### *In-home processing including cooking*

In-home freezing of vegetables and fruit is often used to extend the storage life of seasonally available fruit and vegetable produce. There is little reported literature on the nutritional value of such products, but where the methods employed mimic the corresponding commercial processes, then losses during processing can be expected to be much the same. Differences arise when the vegetable produce is not blanched, and in this case, while losses during the freezing process would be expected to be small, the storage capabilities of the product will be much reduced and the rate of loss of L-AA much higher.

In-home cooking can have quite a significant effect on the ultimate nutrient delivery to the consumer,

particularly that of the labile, water-soluble L-AA. The losses of L-AA that occur during the cooking of fresh fruits and vegetables are as high, if not higher, than those that occur during the cooking of processed produce. In a paper on fresh vegetable produce,<sup>266</sup> average losses from spinach were reported to be 60% through boiling, 46% through steaming, and 58% through pressure cooking. The same authors confirmed that in the cooking of broccoli and green beans, the largest losses occurred during boiling and the smallest during steaming. Surprisingly the paper also reported that large losses occur during microwave cooking, but the inference from the paper was that the microwave cooking was done in the same ratio of water to vegetable as boiling. This contrasts markedly with a cooking study on six different frozen vegetables<sup>267</sup> which reported that significantly higher levels of L-AA were retained in microwave cooking versus conventional boiling; in this case minimal water was added to vegetables during the microwave cooking.

In a recent study,<sup>268</sup> the retention of L-AA in cooked frozen spinach and the losses of the nutrients in the cooking water were examined. The study showed the importance of the ratio of water to vegetable employed, with most of the cooking loss from the vegetable being due to leaching into the water rather than thermal degradation (see Fig 6). Again the losses were lowest with microwave cooking in minimal water.

In a study on the nutrient quality of vegetables prepared by conventional and cook-freeze methods, four selected vegetables prepared by boiling were frozen and stored for six months at  $-18^{\circ}\text{C}$ . L-AA was determined on the raw vegetables, again immediately after cooking (boiling), and then after microwave heating of the stored produce. The greatest loss of L-AA occurred during boiling, with only small further losses during storage and microwave re-heating.

Losses during re-heating of canned produce are generally small, as are the losses during the reconstitution of dehydrated vegetables. However, as with all vegetables, L-AA contents will decrease if the produce is maintained at an elevated temperature, for instance if food is placed in a 'bain-marie' for a long time prior to serving and L-AA losses of 40% have been reported for cooked mashed potatoes held on a 'steam table' for a period of 1 h.<sup>269</sup>

### Stability during storage

All raw fruits and vegetables undergo a series of post-harvest changes, and the key to the stability of L-AA is the enzyme-catalysed oxidation reactions. As such the composition of the fruit or vegetable, its pH, as well as its integrity (eg whole or chopped, the extent of damage etc) all have a bearing on L-AA retention.

As might be expected, the vulnerability of different vegetables and fruits to oxidative loss of L-AA varies greatly, as indeed do general quality changes (ie spoilage). Low pH fruits (citrus fruits) are relatively stable, whereas soft fruits (strawberry, raspberry) undergo more rapid changes. Leafy vegetables (eg spinach) are very vulnerable to spoilage and L-AA loss,<sup>258</sup> whereas root vegetables (eg potatoes) retain quality (and L-AA) for many months. However, with all these products, there is a progressive loss of L-AA with time and the extent of loss is profoundly affected by temperature. Reducing the temperature from ambient (20°C) to chill (4°C) significantly reduces losses in peas, broccoli and spinach,<sup>258</sup> and further temperature reduction to freezer temperatures (ie -18°C) will additionally reduce the rate of loss. Even at these temperatures however, losses of L-AA and overall quality continue to occur. The enzyme-catalysed oxidation reactions responsible for this spoilage can be eliminated by thermal treatment and as a result of processing, frozen and canned vegetables and fruits do survive subsequent long-term storage with little or no further loss of L-AA.

### Potential for maintaining levels found in the fresh produce

All foods are complex mixtures of components that have the potential to react and interact with each other. Thus fruits and vegetables undergo changes from the moment of harvest and since L-AA is one of the more reactive compounds it is particularly vulnerable to treatment and storage conditions. In broad terms the milder the treatment and the lower the temperature the better the retention of the vitamin, but there are a host of interacting factors which affect L-AA retention.

Firstly, the rates of loss from raw (unprocessed) fruits and vegetables differ widely,<sup>270</sup> and are affected by such factors as surface area (spinach more vulnerable than sprouts), pH (stability in citrus fruits), exposure (peas in pod), protection by other oxygen scavengers (broccoli), and also enzyme (AO, ascorbic acid oxidase) activity. The mechanisms of such losses

have not been established for specific fruit and vegetables, and are likely to involve some or all of these processes to varying extents. Storage temperature however is a factor common to all the above mechanisms. Thus, storage at ambient temperature (20°C) results in greater losses than at chilling temperatures (4°C), which in turn has higher losses than deep frozen (-20°C) produce. Cut spinach deteriorates very rapidly at ambient temperature, with most of L-AA lost in two days, whilst broccoli and podded peas retain their quality for a week at ambient temperature and for several weeks at chill temperature. Not all fruit and vegetable produce is acceptable after freezing but for those that can tolerate it, both L-AA and overall quality can be maintained for a few months. However, losses cannot be eliminated, and for long-term storage, some form of processing is necessary.

Processing, such as blanching and canning, involves heat and water, and with L-AA being heat-sensitive and water-soluble, losses are inevitable. The crucial factor is the time/temperature integral needed to inactivate the key enzymes. Thus, ideally, the fruit or vegetable produce should attain the inactivation temperature rapidly, in the minimum of water, and be held at this temperature for the minimum amount of time possible. It should then be rapidly cooled with the minimum of contact with water. Inevitably compromises have to be made and it is virtually impossible to avoid some loss. The trade-off is that there is minimal further loss from the processed product during long-term storage.

### BIOAVAILABILITY

The L-AA status of humans influences many metabolic systems, including lipid, steroid and peptide metabolism, collagen synthesis, the immune system, endocrine function, control of blood pressure, iron and copper balance, haemostasis, endothelial function and fatty acid transport.<sup>25,31,271-278</sup> While affecting different systems, the biochemical mechanism of L-AA action in each, however, appears to be related to its antioxidant properties<sup>25,272,279</sup> (Fig 2).

While L-AA interacts with specific enzyme systems, it can also be said to have a more generic role as an antioxidant *in vivo*.<sup>279,280</sup> L-AA contributes around 10-15% of the 'total antioxidant power' of fasting plasma,<sup>281,282</sup> and up to 30% after a large oral dose. Some cells, including monocytes and adrenal cells, have L-AA concentrations up to 100 fold that of plasma, and various fluids, such as the aqueous humour, seminal fluid and gastric juice, have L-AA levels five to ten fold that of plasma.<sup>283</sup> This implies that these areas have an elevated antioxidant requirement. L-AA scavenges reactive species within the aqueous systems of the body, protecting protein and DNA from oxidative damage.<sup>280,284-286</sup> L-AA may also recycle vitamin E, the main lipid-soluble antioxidant, and may prevent initiation of lipid peroxida-

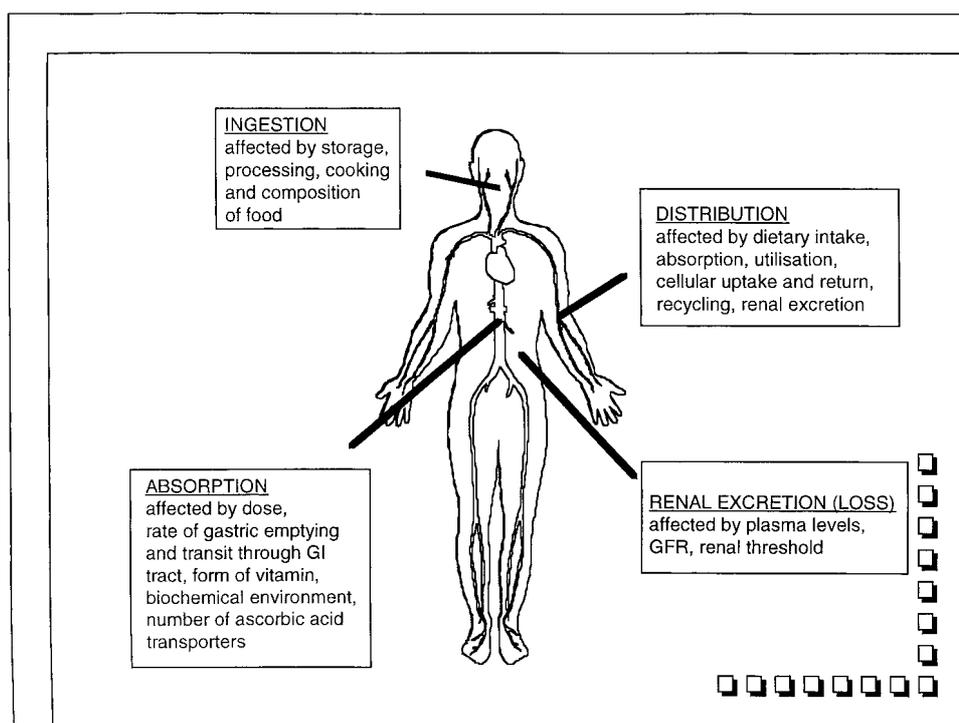


Figure 7. Factors controlling bioavailability.

tion.<sup>279,287</sup> Oxidative damage to protein, DNA and lipid is associated with chronic degenerative diseases including cataracts, cancers and coronary heart disease.<sup>281,288–291</sup>

There is strong and consistent epidemiological evidence of a role for L-AA in lowering risk of chronic degenerative disease.<sup>292–297</sup> This, together with the experimentally supported biochemical rationale of its efficacy,<sup>33,279,286,298–300</sup> and its low toxicity,<sup>301</sup> has led to an awareness that lowering of overall risk of disease by dietary means should be considered when reassessing recommended or required nutritional intakes (Food and Nutrition Board, Institute of Medicine, Washington DC, NAS 1997). Before an informed reassessment of the required nutritional intake for L-AA can be made however, it is necessary to review and extend current data on the bioavailability of L-AA, to design and evaluate strategies which can be used to modulate bioavailability, and to evaluate the overall health benefit of different levels of L-AA nutritional status.<sup>302</sup>

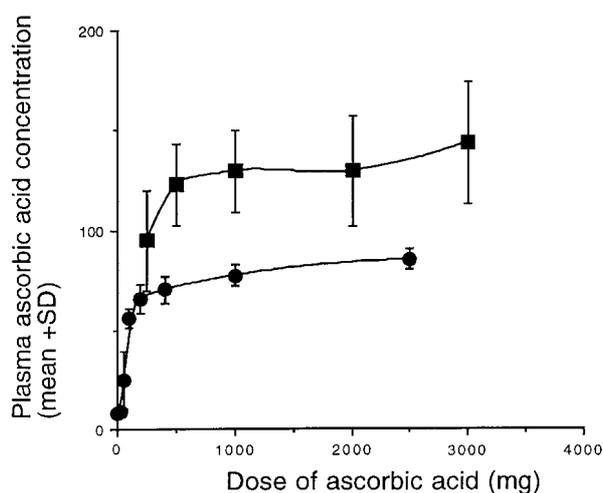
#### Overview of methods used to assess bioavailability and current data

The concept of nutrient 'bioavailability' encompasses the processes of ingestion, absorption, distribution, utilisation and loss. Bioavailability can be defined as 'the fraction of an ingested nutrient which may have the potential to meet functional demands in target tissues.' To date, bioavailability has usually been assessed by measuring the amount of an oral dose which enters the circulating plasma, for example by monitoring the post-ingestion area under the curve (AUC).<sup>276,303–305</sup> This approach has yielded useful results (for review see Ref 276), but data are in many

cases likely to be inaccurate due to nutrient utilisation within the circulation, movement from plasma into the extravascular spaces, cellular uptake and renal loss, as well as leakage or release from cells. All these processes may lead to a gross misrepresentation of bioavailability (Fig 7).

Plasma levels of L-AA are controlled, but not completely defined, by dietary intake. The concentration of L-AA in the plasma of apparently healthy fasting adults ranges between 25 and 100  $\mu\text{mol litre}^{-1}$ .<sup>77,279,306</sup> There is no clearly defined lower level which represents adequacy; nor has an upper limit to the desirable range been established, although fasting levels of  $\geq 60 \mu\text{mol litre}^{-1}$  have been reported to indicate tissue saturation.<sup>307</sup> L-AA is concentrated in various tissues and fluids, most notably in the brain, the eye, the internal organs and white blood cells. Intracellular L-AA concentrations can reach millimolar concentrations, but the fasting plasma concentrations correlate significantly with red blood cell, leucocyte and platelet levels if the intake is stable.<sup>77,279,307–311</sup>

L-AA is absorbed from the gastrointestinal tract and enters tissue cells by a sodium-dependent active transport mechanism, mainly in the jejunum, and by a passive absorptive pathway.<sup>283</sup> Ingestion causes a dose-related increase in the plasma L-AA concentration.<sup>312,313</sup> Mean peak plasma L-AA concentrations after a 0.5 g or 1.0 g dose of the vitamin are in the range 120–130  $\mu\text{mol litre}^{-1}$ , and mean increases in plasma L-AA concentration of 45–66  $\mu\text{mol litre}^{-1}$  were reported following ingestion of 0.5–1.5 g.<sup>77,309,313,314</sup> Plasma L-AA concentrations increase within 15–20 min of ingestion, and peak at 1–2 h post-ingestion.<sup>307,313</sup> This response is independent of the prevailing L-AA nutri-



**Figure 8.** Influence of dietary dose of L-ascorbate on mean plasma L-ascorbate levels.

tional status, as assessed by the fasting plasma L-AA concentration,<sup>313</sup> and is not related to age or sex, although the response may be delayed in depleted subjects.<sup>77,276</sup> Plasma L-AA concentrations drop rapidly after 6 h post-ingestion and regain basal (fasting) levels by 12 h post-dose.<sup>307–309,315</sup>

The form in which the vitamin is taken may influence absorption. For example, more L-AA may be absorbed when it is taken with food, perhaps owing to a longer gastrointestinal transit time. Sustained release preparations have however produced variable results, and the incorporation of bioflavonoids in such preparations did not produce the expected increase in L-AA bioavailability.<sup>276,315</sup> It has also been suggested that some individuals may be better absorbers of L-AA than others. While this is not supported by results of controlled repletion studies, where the inter-individual variability of response to the same L-AA dose appeared to be quite small,<sup>307</sup> there is experimental evidence that the number of intestinal brush border transporters in teleost fish varies inversely with habitual L-AA intake.<sup>316</sup> This could imply that individuals usually taking low doses of L-AA have a more efficient absorption mechanism than those whose diets are rich in L-AA.

The relative amount of L-AA absorbed from a single oral dose decreases from nearly 100% at low doses, to 80% at 180 mg, 50% at 1.5 g and 16% at 12.0 g.<sup>77,276,313,314</sup> While there is no true plateau of post-ingestion response, ingestion of  $\geq 1.0$  g, in a single dose does not cause a significantly greater response than that induced by 0.5 g (Fig 8). Fasting plasma and leucocyte levels also increase with the size of the dose, but responses level out at intakes of  $\geq 200$  mg per day<sup>307</sup> As with the acute response, no true plateau was seen in fasting plasma L-AA levels following increased intakes, but further increases at intakes of  $>200$  mg per day were relatively small<sup>307</sup> (Fig 3). Megadoses of L-AA appear to have no advantage therefore, at least in terms of increasing

systemic supply in either the immediate post-absorptive or in the fasting state.<sup>313,317</sup>

Large doses of L-AA lead to increased renal loss of the vitamin. Plasma levels after doses of 1–2 g can approach  $200 \mu\text{mol litre}^{-1}$ ,<sup>313</sup> well above the renal reabsorptive threshold of around  $85 \mu\text{mol litre}^{-1}$ .<sup>318</sup> Even a modest dose however, may cause a post-ingestion 'spike' of L-AA into the plasma, raising the concentration above the renal threshold.<sup>313</sup> Renal loss will also be increased – lowering bioavailability – if the renal threshold is decreased, due to an increased glomerular filtration rate, or an increase in other constituents of the glomerular filtrate which share the same reabsorptive mechanism. An example of this might be D-glucose. Thus, hyperglycaemia might affect the bioavailability of L-AA by interfering with several processes, including gastrointestinal L-AA absorption, cellular uptake and recycling from DHA, as well as renal reabsorption.

Mechanisms controlling tissue L-AA uptake and the relative distribution of L-AA within the body are not clear. DHA is reported to be removed rapidly from plasma by erythrocytes, thus maintaining the normally very low or undetectable levels of DHA in the circulating plasma.<sup>319</sup> Once taken up, intracellular DHA is reported to be immediately reduced within erythrocytes to L-AA by a glutathione-mediated pathway.<sup>320</sup> This intracellular 'recycling' of L-AA may serve to remove potentially harmful DHA and maintain a reservoir of L-AA which can replenish plasma levels as these are depleted during utilisation, and as L-AA moves into the extravascular space. Whether this process is restricted only to erythrocytes is not known, but erythrocytes do not accumulate L-AA. Leucocytes, and other tissues which concentrate L-AA may well have a specific *in situ* requirement for L-AA. To date, however, the physiological rationale behind the pattern of distribution is not clear; rates of passage into and out of tissues are not known, and while 3% of the body pool is reported to be degraded,<sup>318</sup> turnover at specific sites of utilisation remains to be established. Tests which largely measure absorption and renal excretion of L-AA do not assess those aspects of 'bioavailability', which are needed here. What is needed are tests which can monitor the dynamics of L-AA distribution throughout the body, and its interaction with other chemical species.

It may be possible to follow the distribution and turnover of L-AA *in vivo* using [<sup>13</sup>C]-labelled L-AA and gas chromatography/mass spectrometry.<sup>321</sup> This stable isotope of L-AA may also be imaged *in situ* using organ or whole body nuclear magnetic resonance spectroscopy (NMR).<sup>322,323</sup> However, these techniques are sophisticated, expensive and technically demanding, and are currently still in the developmental stage. Nonetheless, they are potentially very powerful, sensitive and specific tools to study L-AA bioavailability.

In summary, the bioavailability of L-AA is generally assessed by measuring plasma levels after an oral dose.

This assesses the absorption and release into the plasma, but does not determine tissue uptake, release, utilisation, recycling, loss or overall turnover. Current bioavailability data are therefore incomplete and new and complementary methods of assessing the various components effecting bioavailability are needed.

### Improving bioavailability measurements

L-AA is distributed unevenly throughout the hydrophilic systems of the body. Target sites are not clear, and supply and demand at different sites and even within the same site are likely to be highly variable, independent of each other, and largely unpredictable. To date, there are no data on the 'true' bioavailability of L-AA in humans. Bioavailability measurements in current usage are indirect and restricted to assessing only some of the individual processes which together define and control bioavailability (Fig 7). A more informative approach would be to measure functional indicators, or biological markers, of nutrient action.<sup>324,325</sup> As discussed, direct imaging of [<sup>13</sup>C]-L-AA using NMR would provide a more direct way of assessing bioavailability. It would then be possible to monitor the fate of L-AA from ingestion to degradation or excretion under different nutritional, physiological and pathological conditions. In combination with the use of functional indicators, this would provide clear insight into the processes of ingestion, absorption, distribution, utilisation and loss. Bioavailability would then be a quantifiable reality, rather than an unmeasurable concept.

#### Functional Indicators

A reliable functional indicator, or biological marker, of nutrient action in humans must meet certain criteria, as outlined below (see also Ref 325).

1. There must be a measurable dose-response relationship; ie the functional indicator must respond in a sensitive, specific and predictable manner to changes in the concentration/supply of the micronutrient.
2. The functional indicator must be present in body fluids, structures or cells which can be sampled or imaged in some way, ie it must be accessible for measurement.
3. There must be suitable and available analytical tools, ie the functional indicator must be in a form and quantity which can be measured objectively and reproducibly.
4. The functional indicator must relate to a physiological (biological) or pathological endpoint, ie it must reflect a change, in the target tissue or fluid, which has a direct impact on health.

Three classes of functional indicators – molecular, biochemical and physiological – have been described.<sup>325</sup> Each class differs in its relation to the mechanism of action of L-AA, to the ease with which samples can be taken and analysed, and to the known relevance to health.

*Molecular indicators.* These can be described as relating to effects on the function or activity of specific molecules, such as enzymes or DNA. The effects are likely to be mediated (or prevented) directly by L-AA. This type of functional indicator may be very specific and results may reflect a dynamic 'real time' influence of L-AA. Sampling however is problematical, since intracellular constituents are likely to be needed, and the relevance to health may not always be clear. Nevertheless, imaging techniques offer a potential tool to observe molecular indicators *in situ*. Until such imaging techniques become available however, the use of molecular indicators will be limited, but potential targets are:

1. the activity of enzymes which are dependent on L-AA for maximum activity (see Table 2);
2. the half-life of adrenaline at sites of synthesis and/or action;
3. oxidative changes to DNA, protein and/or lipids.

*Biochemical indicators.* These can be described as relating to effects of L-AA on the levels of biochemical constituents in body fluids. These will often be the result of molecular changes, and so may be somewhat removed from the direct action of L-AA. Although some specificity may be lost, samples are likely to be easier to obtain and their relationship to health may be less conjectural. Potentially useful biochemical markers of L-AA include:

1. deoxy pyridinoline:total collagen cross links;
2. free carnitine levels;
3. ratio of MDHA:L-AA.

*Physiological indicators.* These can be described as relating to the effects of L-AA on homeostatic processes or organ systems resulting in physiological (or pathological) change. These are likely to be the result of biochemical changes, and may be distant in both time and space from the direct action of L-AA. Physiological indicators, such as blood pressure, D-glucose homeostasis and endothelial function can be relatively simple to measure and have a clear relationship to health. They are likely to be influenced by many factors however and so the specificity for L-AA is poor. The specificity of particular physiological indicators will be increased if mapped to biochemical indicators, which, in turn, can be mapped to molecular indicators of L-AA function.

Measuring reliable functional indicators, covering the spectrum from molecular change to biological endpoint, will assess functional outcome and identify need. Plasma concentrations measure absorption and potential supply to sites of need. 'Real-time' imaging of L-AA distribution and interaction could reveal actual supply and turnover within individual sites. Together, these measurements will provide the information needed to assess 'the fraction of an ingested nutrient which may have the potential to meet

functional demands in target tissues' – ie true bioavailability. With this information there can then be an objective and scientific re-examination of current recommendations regarding L-AA intake. Furthermore, strategies to modulate bioavailability can be evaluated in terms of efficacy and desirability.

#### Factors which may modulate bioavailability

Modulating the processes of ingestion, absorption, distribution, utilisation or loss of L-AA will clearly affect its bioavailability. The amount ingested in food is difficult to estimate with any degree of accuracy as it is affected by many factors, including the type of food, and the conditions under which food is grown, stored, processed, cooked and served. Within the gastrointestinal tract, L-AA reacts with other food components, for example nitrites, iron or copper. Indeed this may well be a useful function of dietary L-AA and fits within the definition of bioavailability. Such reactions however, influence the amount of L-AA available for absorption and systemic distribution. With regard to absorption, the larger the amount of L-AA ingested at any one time, the lower the relative, but not absolute absorption. The absorbed quantity is also affected by the rate of gastric emptying, the rate of transit through the jejunum and possibly, by the amount ingested over the previous days or weeks. If intake is found to affect the number of transporters, then the amount absorbed from a large dose may be greater in individuals whose diet is generally low in L-AA. Bioavailability will also be affected by the presence of other substances that share the same transport mechanisms and so compete for absorption, such as D-glucose. High D-glucose content in the food mixture and/or extracellular fluids may inhibit absorption of L-AA and/or DHA.<sup>283,326</sup>

With regard to the release of absorbed L-AA into the systemic circulation, hepatic 'harvesting' of L-AA from the portal circulation has not been shown, and neither is L-AA 'inactivated' by being bound to proteins in plasma. It is possible that if there is an inflammatory process within the liver some of the newly absorbed L-AA might be used before it reaches the circulation – but this would reflect increased demand, rather than lower bioavailability *per se*.

The amount of L-AA present in the circulating plasma represents the sum of the basal level plus the newly absorbed dietary L-AA plus the amount 'returned' to the plasma from cells. From this are subtracted the amount of L-AA 'used' within the plasma, the amount transported into cells, interstitial spaces and tissue fluids, and the amount which has been filtered and not reabsorbed by the kidney. Increased demand in specific areas, for example at sites of inflammation, may lower the amount available at other sites. Leucocytes, which are rich in L-AA, are produced in large numbers and migrate towards and accumulate within areas of damage or infection. This draining of L-AA resources targets the vitamin towards specific sites of need, but does not represent a decrease in overall bioavailability. Such increased

demand however, may lead to a relative deficiency elsewhere. Return of L-AA to the plasma, for example from erythrocytes, may help adjust the balance, but the role and control of this mechanism is unclear. If continual reduction of DHA within erythrocytes is important in maintaining L-AA, then intracellular levels of GSH, NAD(P)H and the pathways by which these are produced will also affect bioavailability of L-AA.

Reactive oxygen species (ROS), generated as a result of post-ischaemic reperfusion, inflammation, toxins, pollutants, cigarette smoking, UV light etc interact with L-AA and cause a decrease of L-AA levels. The relative amounts of vitamin E and uric acid present may also affect the L-AA concentrations, if, as is thought, the tocopheroxyl radical ( $\alpha$ -chromoxy radical), and the urate radical are 'repaired' by L-AA.<sup>327,328</sup> Again this will decrease the amount of bioavailable L-AA unless the supply is increased.

Renal loss of L-AA is controlled by the plasma concentration and by the glomerular filtration rate. L-AA is freely filterable, and the reabsorption mechanism saturable. Plasma concentrations above the renal threshold of around  $85 \mu\text{mol litre}^{-1}$  will consequently result in increased loss. Urinary loss will increase at plasma L-AA levels lower than this, however, if the glomerular filtration rate is increased. This may be due to, for example, exercise, hypertension, pregnancy, or to changes in the pressure of the surrounding atmosphere, and will cause an increase in the total filtered load. This results in saturation of the reabsorptive capacity at relatively modest plasma L-AA concentrations. L-AA will also be lost in urine if there is competition for reabsorption between L-AA and another substance within the glomerular filtrate. Urinary excretion represents true loss, rather than utilisation or redistribution. It is possible that renal loss might be a regulatory mechanism to prevent the accumulation of excess L-AA, but this is not known.

#### Strategies for enhancing bioavailability

Enhancing bioavailability is important in economic terms – more can be achieved with less. In addition, if the putative benefits to health of an increased supply of L-AA are confirmed, strategies that enhance bioavailability will lower the potentially enormous costs of increasing the recommended nutritional intake at the population level.<sup>329</sup> Based on our knowledge of factors which may modulate bioavailability and, as outlined above, it is possible to list a number of potential or theoretical strategies to enhance the bioavailability of L-AA (Table 5). Some of these strategies are impracticable and others may have undesirable side effects. They are therefore presented not as recommendations but rather as suggestions to fuel discussion and debate on the subject and to generate ideas that will lead to realistic and practical strategies of enhancing L-AA bioavailability.

To summarise, current bioavailability data on L-AA are based largely on post-ingestion increases in the

**Table 5.** Overviews of strategies to enhance bioavailability of L-ascorbic acid

<i>Process</i>	<i>Strategy</i>	<i>Rationale</i>	<i>Effect</i>	<i>Comment</i>
<i>Ingestion</i>	Inhibit activity of ascorbate oxidase/prevent transcription of ascorbate oxidase/delete gene for ascorbate oxidase	Prevent loss of ascorbic acid in fruits/vegetables owing to oxidation to DHA	Increase ascorbic acid content of the fruit/vegetables	Requires genetic manipulation of plants; may be detrimental to plant tissues and/or interfere with plant growth/ripening/reproduction/defence
	Overexpression of enzymes for synthesis of ascorbic acid	Increase ascorbic acid production	Increase ascorbic acid content of the fruit/vegetables	Requires genetic manipulation of plants; may be detrimental to plant tissues and/or interfere with plant growth/ripening/reproduction/defence
	Improve growing/storage conditions	Minimise loss of ascorbic acid	Maximise ascorbic acid content of stored and processed foods	Revised database of ascorbic acid content of plants grown and stored under different conditions needed
	Add antioxidant preservatives to foods during processing and packaging	Minimise loss through non-enzymatic autooxidation	Maximise ascorbic acid content of stored and processed foods	Antioxidants could be toxic to humans or compete for absorption; toxicity and interaction studies needed
	Reduce time, temperature and contact with air during cooking	Minimise loss through non-enzymatic autooxidation	Maximise ascorbic acid content of stored and processed foods	Revised database of ascorbic acid content of foods prepared/cooked under different conditions needed
<i>Absorption</i>	Use small, frequent doses	Avoid saturating absorption mechanism	Increase relative and absolute amount absorbed from each dose ingested	Simple strategy
	Use 'pulse and withdraw' feeding pattern	Maximise the number of intestinal transporters	Increase relative and absolute amount absorbed from each dose ingested	Human studies comparing dose/response to different doses at different habitual intakes needed
	Choose higher bioavailability food formulation	Aid speed and/or efficiency of absorption	Increase relative and absolute amount absorbed from each dose ingested	Systematic comparison of response to different preparations and food mixtures in fasting and fed state needed
	Delay transit through GI tract	Retain at sites of absorption	Increase relative and absolute amount absorbed from each dose ingested	Impracticable
	Avoid high glucose environment	Prevent competitive inhibition of absorption	Increase relative and absolute amount absorbed from each dose ingested	Study of response to ingestion of ascorbic acid +/- glucose needed
<i>Distribution</i>	Apply topically as cream, eyedrops; give as i.v. infusion	Direct supply to eye, skin and systemic circulation	Increase concentration at specific sites	Ascorbic acid is acidic, direct application may cause irritation or have toxic effects; animal then human studies needed
<i>Utilisation</i>	Improve erythrocyte GSH levels	Increase recycling/return from erythrocytes to plasma	Increase half-life and efficiency of each ascorbate molecule	Impracticable
<i>Loss</i>	Keep plasma concentration < 85 µmol litre <sup>-1</sup>	Keep level below renal threshold	Prevent renal loss	Can be readily achieved by keeping individual doses < 200 mg
	Maintain normal blood pressure and volume	Avoid increase in glomerular filtration rate	Prevent or minimise renal loss	Increase in glomerular filtration rate unavoidable in pregnancy, during exercise and in pressurised surrounding, eg aircraft

plasma L-AA concentration. This information is useful but incomplete as it measures the potential supply to sites of need, but does not map the fate of dietary L-AA entering the systemic circulation. Nor does it reveal where L-AA is most needed, or when and why. Research is needed to extend current data and to address these issues.

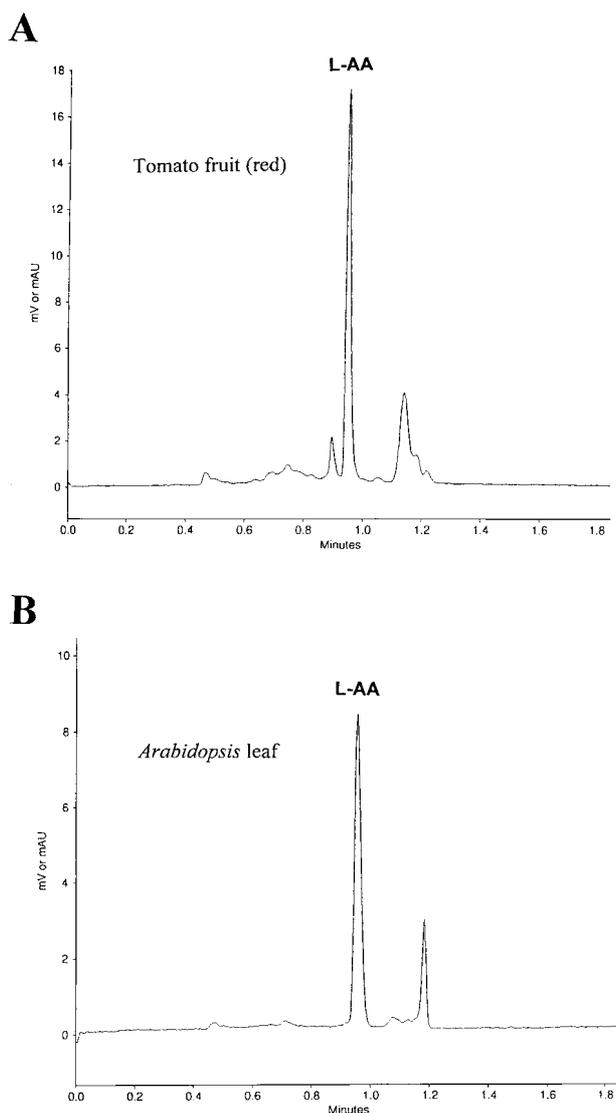
### ANALYTICAL METHODOLOGIES

The interest in L-AA metabolism over the years has given rise to a plethora of procedures for the quantification of L-AA and DHA. Unfortunately in many cases these methods have been developed for the analysis of specific tissue types, and considerable

caution should be exercised in the transfer of these methods to the analysis of different tissues. This is particularly so when working with plants, which contain large amounts of potentially interfering compounds. Reviews covering L-AA analysis can be found in Refs. 22, 112, 330

### Extraction

It is essential when analysing L-AA to inactivate degradative enzymes and to fix the L-AA/DHA redox equilibrium. L-AA is readily oxidised under alkaline conditions, so the use of a high ionic strength, acidic extraction solvent is required to suppress metabolic activity upon disruption of the cell and to precipitate proteins. A metal chelator such as EDTA is also



**Figure 9.** HPCE analysis of plant L-AA contents. Analysis of crude 3% metaphosphoric extracts of plant tissues, by 'short end' injection. UV detection at 267nm. Separations carried out on a 41.2cm (6.2cm to detector)  $\times$  50  $\mu$ m ID fused silica capillary thermostatted at 20°C. Injection of sample for 6s  $\times$  0.8psi on detector side of capillary. Separations carried out at -26kV, with a background electrolyte of 200mM borate pH 9.0, containing 10% v/v acetonitrile and 0.1mM EDTA. Capillary regenerated for use in between injections by rinsing with 0.1M HCl, and background electrolyte.

usually required. In the analysis of plant tissue, highest recoveries are obtained with 3–5% metaphosphoric acid/1mM EDTA (Davey *et al* – unpublished data). Recoveries with 10% trichloroacetic acid by comparison were particularly poor, a phenomenon that has previously been observed (see Refs. 331, 332). Oxalate at 5% has also been reported to extract only 85% of the total L-AA available and samples are less stable than in metaphosphoric acid.<sup>333</sup> Perchloric acid at 5% produced similar yields to metaphosphoric acid, but it is a less efficient protein precipitator.<sup>334</sup> We have found that plant L-AA/DHA is stable in 3% metaphosphoric acid/1mM EDTA for a minimum of 24h when stored at 4°C or less. This is in agreement with the results of

Lykkelsfeldt *et al*,<sup>335</sup> in the analysis of blood L-AA levels.

### Spectrophotometric procedures

Prior to about 1980, most assays for L-AA content were based on spectrophotometric determinations. Such methods include the reduction of 2,6-dichlorophenol-indophenol (DCIP) by L-AA, and the reduction of ferric iron to ferrous iron by L-AA, followed by the formation of a chromogenic ferrous iron complex, using chelators such as 2,2'-dipyridyl, and ferrozine. Another widely used procedure is the oxidation of L-AA to DHA and 2,3-DKG, followed by reaction with 2,4-dinitrophenylhydrazine. After treatment with sulphuric acid, a coloured product is formed that absorbs at 520nm. Fluorometric determinations are based on the condensation of DHA with *o*-phenylenediamine to form a quinoxaline derivative. In all cases these spectrophotometric procedures suffer from lack of sensitivity and lack of specificity of reaction.<sup>22</sup> The use of the enzyme AO avoids the problem of reaction specificity, and the oxidation of L-AA to DHA by AO is followed either by the decrease in L-AA absorbance at 260nm, or by complexation with ferric iron. However, this procedure cannot be used to directly measure DHA.

### HPLC

HPLC analysis with electrochemical detection,<sup>333,336–338</sup> with fluorescence detection,<sup>332</sup> refractive index detection,<sup>339</sup> or UV detection,<sup>340,341</sup> avoids the problems of non-specific interference, and analyses display high selectivity and sensitivity. There are a number of robust, accurate protocols, but in general analysis times are relatively long due to the need to recondition columns between analyses and the fact that samples need to be analysed individually. Further, DHA usually needs to be determined after reduction of DHA with a suitable reducing agent. Recently HPLC methods for the simultaneous analysis of L-AA and DHA have appeared,<sup>342,343</sup> however it remains to be seen how suitable these procedures are for the analysis of complex biological samples.

### HPCE

Increasingly in recent years, the high resolution and efficiencies of high-performance capillary electrophoresis (HPCE), have been used for L-AA analysis (see for example Refs 344–349). In comparison to HPLC separations, HPCE analyses are faster, require only nl of sample and have much higher peak capacities. In addition the open tube format allows for more rapid turnaround times between analyses. However, in general, the concentration limits of detection are higher than for HPLC. A typical example of the type of separation possible is shown in Fig 9 (M Davey, unpublished data).

### Analysis of ascorbate derivatives

Analytical methods are available for the analysis of

various L-AA derivatives by HPLC<sup>15,350-352</sup> and HPCE.<sup>353</sup>

## PRIORITY RESEARCH NEEDS

### Metabolism

At the level of plant L-AA metabolism, it is clear that despite over 60 years of active research, there remain several areas of fundamental importance that are still poorly understood. The resolution of these questions is of prime importance towards achieving future goals such as engineering crops to contain stably enhanced L-AA levels, increased nutritional values, and improved stress resistance and yields.

Nonetheless, the recent identification of the L-galactose-based pathway of plant L-AA biosynthesis represents a major advance, and opens up the possibility for the future metabolic engineering of plant L-AA metabolism. This task will be aided by the availability of cDNAs for several enzymes of the pathway (GLDH, and mannose-1-phosphate guanylyl-transferase), and L-AA-deficient mutants, but without doubt, the identification purification and cloning of the remaining enzymes of this pathway is of paramount importance. The question of 'alternative' routes of L-AA biosynthesis via uronic acids still remains open however, and again resolution of these issues will require characterisation of the enzymes involved and further study.

Despite some tantalising glimpses, little is known about the mechanisms governing the control of plant L-AA levels *in vivo*. In some species there appears to be a direct correlation between soluble carbohydrate levels, as source of hexose precursors for L-AA biosynthesis and L-AA pool size, and this reinforces the long-observed relationship between light intensities (photosynthesis) and L-AA levels. Perhaps surprisingly, the activity of the terminal enzyme, GLDH also appears to be upregulated in response to stress and lowered L-AA pool. However the point(s) at which control over flux through this pathway is exerted, and perhaps the consequences for related metabolic routes still have to be elucidated. Related to this are the mechanisms of L-AA turnover, regeneration and catabolism, as the difference between these competing processes will determine the size of the steady state L-AA pool.

Ascorbate oxidase (AO) and ascorbate peroxidase (APx) are the only two enzymes known that specifically oxidise L-AA. The essential role of APx in H<sub>2</sub>O<sub>2</sub> scavenging, and the regeneration of oxidised L-AA, either by photosynthetic electron transport or by the L-AA-GSH cycle has received much attention, and many details appear well understood. However, the function of AO in plant metabolism still needs clarification. Evidence is accumulating for a role for AO in regulating cell wall expansion, and possibly cell division, but whether AO is actually involved in the catabolism and turnover of L-AA is unclear. It is known that L-AA can serve as a substrate for oxalate,

threonate and tartrate, but again the importance of this route in governing the rate of turnover of L-AA is unknown. The possibility of the recycling of DHA and 2,3-diketogulonic acid via the pentose phosphate pathway, as proposed here, has not received any attention, but would appear energetically more favourable.

To date, the majority of studies on plant L-AA biosynthesis and metabolism have focused on biochemical approaches, such as enzyme purification, (radiolabelled) substrate incubations, etc. However with the recent cloning of GLDH,<sup>145,147</sup> and the availability of mutants with altered L-AA biosynthetic capabilities,<sup>154,161</sup> attention will increasingly turn to the tools of the molecular biologist to unravel some of the more intractable problems in this field. Widely used plant model systems, in particular *Arabidopsis thaliana* and *Nicotiana tabacum* for which transformation techniques are well established will undoubtedly be at the forefront of this (fundamental) research. However, we can already consider turning our attention to more commercially relevant species, where the particular problems associated with L-AA metabolism during fruit/storage tissue development and ripening can be addressed.

### Nutrition and bioavailability

The criteria used to define nutritional requirements and recommended intakes are a critical issue, as intake is a key controlling factor in defining the bioavailability of L-AA. The World Health Organization/Food and Agriculture Organization/International Atomic Energy Agency committee define a nutritional requirement as 'the lowest continuing level of nutrient intake that, at a specified efficiency of utilisation, will maintain the defined level of nutriture in the individual'.<sup>329</sup> There are three areas within this definition which must themselves be defined or described and this requires answers to the following questions:

- (i) How can the lowest continuing level of nutrient intake be assessed?
- (ii) how can the specified efficiency of utilisation be defined?
- (iii) How is the defined level of nutriture (nutritional status) decided upon?

A further point, which must also be considered, is the difficulty of translating data from individuals into public health policy designed to meet individual needs within each population. This definition of nutritional requirement, therefore, is a Pandora's box, full as it is of the unknown.

By focusing, in reverse order, on the three areas identified, this definition of nutritional requirement may be put in the context of current knowledge, and areas of priority research may become clear. Put simply, intake may control the outcome, but the outcome defines the requirement. The complex mechanisms controlling relationships and attainable out-

comes however, are not well understood. Research is needed to:

- define the level of nutriture required; this can be taken to mean the level at which the desirable, health-related outcome is achieved, and above which there is either no further benefit or toxicity may develop
- define the specified efficiency of utilisation: this can be taken to mean the varying response or outcome to different levels of ascorbic acid status, or a dose (intake)-response (benefit) relationship
- assess the lowest continuing level of nutrient intake necessary; this can be taken to mean the minimum amount in the diet which would provide the optimal dose response, or cost/benefit ratio, to achieve the desirable outcome.

Such research hinges upon the use of clear, agreed, measurable and objective health-related outcomes, ie reliable functional indicators for ascorbic acid, and for other micronutrients, must be identified, and optimal or threshold values established for each. The current lack of suitable functional indicators severely restricts human studies of bioavailability, and seriously hampers objective and scientific reassessment of nutritional requirements.

In conclusion, the search for reliable functional indicators of L-AA nutritional status must be regarded as a priority research area. A further area in which work is needed is imaging techniques. The ability to observe the 'real-time' distribution and utilisation of L-AA within target sites could reveal currently unknown functions of L-AA and control mechanisms, and help to identify possible functional indicators. These techniques, in addition to those currently available, will permit true bioavailability to be assessed, the need for increased nutritional status to be investigated, strategies to enhance bioavailability to be evaluated, and the potential toxicity of high L-AA intake to be explored.

The increasing recognition of the protective functions of elevated dietary L-AA intakes in a number of disease states will undoubtedly lead to increased interest in nutritional aspects of this vitamin. The exciting progress now taking place in the study of fundamental aspects of plant L-AA metabolism, raises the realistic possibility that such increases in population daily dietary intakes can be achieved by the stable engineering of important plant food sources, without the need for supplementation, or for a change in the dietary eating habits as a whole. Such gains can be complemented by improvements in techniques for food processing and storage.

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