Actual state of research concerning vitamin C as reflected in the literature

(Review article)

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Summary: Vitamin C (L-ascorbic acid) being essential for many living organisms, including man, became once more into the focus of interests because of its numerous physiological effects. Its anti-scurvy and anti-oxidant properties have already been recognised since long in the human body, but it turned out gradually that it has many other functions. In plants, its primary importance is defense against the photo-oxidative stress.

The present review is intended to reveal some details of the artificial synthesis of vitamin C. Emphasis is put on the metabolism of L-ascorbic acid in higher plants. Biosynthetic processes, translocation and accumulation are discussed in detail on the basis of recent results published in the scientific literature.

Key words: L-ascorbic acid, vitamin C, anti-oxidant, scurvy, L-galactonic-1,4-lacton, galacturonic acid-reductase, dehydro-ascorbic acid-reductase, phloem

Introduction

It is generally accepted that the human body cannot synthesise vitamin C, consequently, is should be acquired from our diet. Ascorbic acid is water soluble, easily emptied, thus needs to be continuously supplied. The most important sources of vitamin C are the higher plants. However, the metabolic pathways of ascorbic acid in plants have been unexplored for a long time.

The biosynthesis of ascorbic acid in the animal body was revealed on the pathway of glycuron acid (*Burns,* 1960). Burns pointed out that some mammal species, including man, are unable to synthesise ascorbic acid because the enzyme L-gulonolacton-oxidase is inactive and the process stops at the reaction that it would catalyze.

It was known that the synthesis is different in plants and animals, but some similarities have been presumed. *Isherwood* et al. (1954) supposed that in plants the pathway goes through D-galacturonic acid to the analogy of the animal pathway through D-glycuron acid. Then, *Loewus* (1963, 1987, 1988) published new information about the biosynthetic process obtained by radio-active tracing techniques.

Smirnoff et al. (2001) succeeded to prove the synthetic pathway in higher plants through D-mannose and L-galactose to ascorbic acid. However, alternatives of that pathway have been also supposed to exist by (*Agius* et al., 2003).

Another essential question besides biosynthesis is the translocation of ascorbic acid within the plant. The transport of the compound within the plant cell has been partially revealed by (*Beck* et al., 1983), but information about its further transport between the different tissues and organs is still scarce.

The least information is available about the accumulation of vitamin C in the plant. It is an interesting question why some types of fruits or other organs contain large quantities of ascorbic acid, whereas others do not.

The history of vitamin C

Ancient documents of the Old Testament, papyrus rolls from Egypt called Ebers, as well as notes of Pliny have already reported on symptoms similar to those of scurvy. Hyppocrates described the symptoms of scurvy in the ancient Greek Empire. During the Middle Ages, sailors serving on the vessels of the discoverers suffered from the unidentified disease. It was James Lind, a Scottish physician, who gave a detailed description of it and coined the name of "scurvy" in 1753. He also discovered that the consumption of citrus fruits is a valid prevention of the disease.

In 1883, Thomas Barlow distinguished the deadly scurvy from rickets. This variant of scurvy has been designated as "Barlow's disease". The deficiency symptoms of vitamin C have been known for a long time, but the compound itself was isolated only in 1928 by Albert Szent-Györgyi from different sources (pepper fruit, adrenal cortex of oxen) and it was named hexuronic acid (*Svirbely & Szent-Györgyi* 1932, 1933). In 1932, Szent-Györgyi and parallelly, Waugh and King found that vitamin C or hexuronic acid is an efficient means of curing scurvy (*Waugh & King,* 1932).

The chemical structure of vitamin C was described by two independent teams in 1933, the Harwoth team in England and the Karre team in Sweden. In 1937, Szent-Györgyi received the Nobel prize for his achievements related to vitamin C. The synthetic process of L-ascorbic acid in animals was revealed in 1965 however, the same could not be approached in plants but a couple of decennia later.

The biochemistry of vitamin C

In terms of chemistry, L-ascorbic acid is a cyclic compound of six carbon atoms, a water-soluble derivative of glucose, aldono-1,4-lacton hexon acid. It has several stereo-isomers in nature (D-iso-ascorbic acid, D-arabose-ascorbic acid, D-erythro-ascorbic acid), but those are far less efficient against scurvy. In the molecule, ene-diol groups are attached to the 2nd and 3rd carbon atoms of the cycle, which makes the molecule unstable, as its electron is easily lost in oxido-reductive environment, where it may serve either as a donor or as an acceptor. The OH-group attached to the 3rd carbon atom gives a marked acidic character to the vitamin. Several derivatives of ascorbic acid, natural and artificial, are known: among the synthetic products the ascorbic-2-phosphate and ascorbic-6-palmitate are used as preservatives in foods because of their anti-oxidant activity under in vitro conditions.

Some important properties of L-ascorbic acid

Vitamin C is water soluble, therefore only a low quantity can be stored in the human body (compared to fat-soluble vitamins): its mean concentration in the tissues is 20 mg/kg. The pituitary gland is an exception with its 400 mg/kg concentration. Relatively high amounts of vitamin C have been found in the tissues of the liver, the brain and the leucocytes.

The amount of vitamin C can easily be determined in the blood by chemical analysis, the normal concentration is around 0.8 mg/100 ml.

Vitamin C is sensitive to heat, light and oxygen. Long-term storing or cooking may destroy it partially or completely. Because of the danger of degradation, ascorbic acid is synthesised in combination with other substances as derivatives in the industry. The food industry uses large quantities of vitamin C as a natural anti-oxidant throughout the manufacturing processes up to the packaging in order to protect the colour, flavour and nutritive qualities of the foods. The added content of reductive nitrite in meat during processing is reduced by vitamin C.

Industrial manufacturing of vitamin C

Vitamin C that is in commercial distribution is produced in different ways. It is extracted from plants by chemical synthesis according to Reichstein, by fermentation or by combining synthesis with fermentation.

The chemical synthesis of vitamin C was developed by the Polish chemist, Tadeus Reichsten, Nobel prize winner of 1933. The synthesis starts from glucose and involves several steps. The firms Roche, BASF and Takeda use this procedure also nowadays. The second step of the synthesis was the most problematic since this oxidative fermentation needed bacteria extracted from fruit flies.

The Reichstein procedure involves the following steps: first, the D-glucose is reduced to sorbitol at high temperature then the oxidation of D-sorbitol to L-sorbose is performed by means of *Gluconobacter oxidans* in a submerged fermenter with intense aeration (because the bacterium requires much oxygen). Subsequently, L-sorbose will be condensed with acetone to sorbose diacetone. In the next step 2-keto-L-gulon acid is made by oxidation using chlorine and sodium-hydroxide. Finally, 2-keto-L-gulon acid is dissolved in a mixture of organic solvents then an acid catalyser will restructure the compound to ascorbic acid. The resulting raw vitamin C is cleaned by crystallisation *(Anonym. 3., 2001)*

Several technical and chemical tricks have been developed lately in order to optimise and shorten the reactions. Presently, the efficiency of the technology of making ascorbic acid out of glucose is 60%.

Large quantities of organic solvents and reagents are used in the process. Though a portion of them is recycled, the waste material is still too much and it should be handled with special care according to severe regulations.

Fermentation in two steps

The procedure has been developed in China, where the whole industry uses that method. The licence has been purchased by several West-European factories. It needs less investment and costs of production are also lower. The steps of the procedure are the following:

Sorbitol is fermented (similarly to the Reichstein method) and oxidised to sorbose. Then, the next chemical reaction of Reichstein is substituted by another fermentation process. Thus 2-keto-gulon acid (KGA) is formed. A further chemical reaction produces raw vitamin C, which is finally cleaned by crystallisation *(Anonym. 1., 2003)*.

The two-step fermentation needs less toxic solvents and reagents, consequently, the costs of handling the waste are lower.

The development of the technology has not been finished yet. There are a number of patents submitted on the synthesis

of vitamin C. One of them aims to transform glucose by fermentation into KGA.

Another main field of research has been the direct transformation of glucose to vitamin C, by mutant micro-algae. Many scientists are convinced that the manufacturing of vitamin C will be a biotechnological process in the future.

The steps of fermentation:

In the first step, glucose will be oxidised to 2,5-diketo-o-gluconate by a selected strain of *Erwinia*, the intermediate products are o-gluconate and 2-keto-gluconate.

The second step involves a NADPH-dependent *Corynebacterium* strain, which transforms 2,5-diketo-o-gluconate to 2-keto-o-gluconate by means of the enzyme 2,5-diketo-o-gluconate reductase through a stereo-specific reaction. A "one step bio-conversion procedure" is being developed. The gene of the enzyme 2,5-diketo-o-reductase of *Corynebacterium* will be cloned and transferred to *Erwinia*, where it will be expressed. Thus the recombinant *Erwinia* strain alone will be able to perform both steps of the chemical procedure, and produce vitamin C *(Anonym. 2., 2003)*.

A complex biological and chemical process

2-keto-L-gulon acid may originate from chemical or biological processes, so the intermediary steps may differ from each other. Esterification / lactonisation: its description is partially protected by a patent. Vitamin C synthesised that way is more advantageous: the yield is higher, the reaction time is shorter therefore the volume of the reactor is smaller resulting in less degradation of the product. The alkalic type of lactonisation is less aggressive and less threatened by decay. Pollution deriving from the raw material is easy to be discarded.

Isolation/cleaning: a preliminary cleaning by ion-exchange facilitates the production of ascorbic acid of high purity, which is heat-resistant and homogenous by continuous crystallisation.

The occurrence of vitamin C in living organisms

Ascorbic acid is proved to be present in micro-organisms, which is exploited by modern fermentation procedures in producing vitamin C.

The majority of the invertebrate animals does not synthesise it, but many of the vertebrates produce their own needs. According to our present knowledge, fishes, amphibians and reptiles synthesise ascorbic acid in their kidneys. Among birds, the taxonomically older doves, ducks and hawks are also producing ascorbic acid in the kidneys. In some species of the order *Passeriformes* (Sparrow-like birds) both the kidneys and the liver are involved, in other species the liver is the only site of vitamin C-synthesis, whereas some species lack the ability to synthesise it *(Stone,* 1972).

In *Mammales*, ascorbic acid synthesis is just restricted to the liver.

The reason of relocation

The reason of the above phenomenon can be the different haematothermal bolism. The homeostatic blood temperature intensifies all of the biochemical processes, so the synthesis of glycogen has been transferred from the kidney to the liver. As ascorbic acid is mainly derived from glycogen, it has also been transferred to the liver *(Braun,* 1997)

Among *Mammales* there are but a few species, which are unable to synthesise ascorbic acid, so it is essential for them to acquire it from their diet. Such species are the guinea pig, the Indian fruit eating bat as well as some hominid apes and finally the man *(Sato & Udenfriend,* 1978).

Within the order of *Primates*, the sub-order *Prosimii* are able to synthesise ascorbic acid, whereas the *Antropoideae* have lost this ability. The first three steps of the synthesis are also performed in *Antropoideae* but the last (fourth) step is suppressed because the enzyme L-gulono-lactone-oxidase (LGO) is inactive. It is assumed that on the verge of the cretaceous and the lower eocene (cca. 58–63 million years ago) multiple mutations occurred in the gene responsible for the LGO enzyme *(Stone,* 1972).

Those mutations were not deleterious because in the last step of ascorbic acid biosynthesis toxic hydrogen-peroxide is also produced (consuming molecular oxygen), which should be neutralised by reduced glutathion. Consequently, the suppression of LGO activity abolished the danger of toxic H_2O_2.

The role of the ascorbic acid

In the animal and the human body

Ascorbic acid is mainly an anti-oxidant in the body: UV-radiation, air pollution, inflammations, tumours, etc. induce the accumulation of reactive oxygen (free radicals). Ascorbic acid is able to reduce those radicals by being oxidised. The oxidised dehydro-ascorbic acid is reduced by another anti-oxidant, the reduced glutathion *(Braun,* 1997). The key role of the ascorbic acid is also recognised in the synthesis of collagen: lysine and praline, the amino acids of pro a chains synthesised on the surface of ER (endoplasmic reticulum), are hidroxilised by specific enzymes, the hidroxilases. To the proper functioning of hidroxilases the presence of vitamin C is indispensable. Its absence causes a weakness of the collagen structure, consequently, capillary bleeding ensues. Prolonged deficiency of vitamin C ends in the development of scurvy. The loss of teeth and bleeding of gums (ulemorrhagia) are generally the first symptoms of scurvy. Vitamin C is also involved in the production of bile acids and is active in helping the absorption of iron from foods.

Vitamin C contributes to the synthesis of adrenaline and noradrenaline as well as to that of fatty acids of long chain.

It is L-ascorbic acid, which reports the inhibition of catechol-o-transferase enzyme, thus increases the availability of adrenaline.

Neuro-endocrinous peptides (vasopressine, oxitocine) also require the presence of vitamin C, and amongst the amino acids, the decomposition of tyrosine as well as the metabolism of folic acid are dependent on it.

The transport across cell membranes and elements of the immune system are also controlled by it. An important function of it is preventing the building up of carcinogenic nitroso-amines (which are threatening from foods containing nitrites)

The binding between haemoglobin and oxygen is also a part of its beneficial action.

The role ascorbic acid in plants

In plants, the main role of ascorbic acid is the same as in the human body, i.e. the anti-oxidant function: detoxification of reactive oxygen radicals (ROS) and regeneration of the damage caused by them. ROS are always produced but accumulate especially in stress situations. Superoxid-dismutase enzymatically catalyses the dismutation of superoxid to hidrogen-peroxid (*Smirnoff*, 2000). The latter is then decomposed further by ascorbic acid peroxidase, glutation-peroxidase and catalase. Anti-oxidants of smaller molecules (ascorbic acid, glutation, and tokoferol) deactivate ROS in non-enzymatic ways (*Inze & Van Montagu*, 1995).

Another function of ascorbic acid in photosynthesising plants is performed in the chloroplasts, where the photo-oxidation causes stress (*Foyer* et al., 1991). Many reactive radicals are induced by the incident light, and some electrons of high energy are transferred from the electron chain of ferredoxin to oxygen instead of the NADP. This process taking place in the *photochemical system 1* (PSI) is called Mehler-reaction. The electron transport from water to oxygen is known as pseudo-cyclic electron-flow. That is a mechanism, which serves to dissipate the superfluous reductive force if there is a lack of available carbon. Since the chloroplast lacks catalase, hidrogen-peroxid should be neutralised by ascorbic acid-peroxidase using ascorbic acid. Ascorbic acid-peroxidase catalyses the transformation to monodehydro-ascorbic acid (MDHA), by oxidation. The MDHA can be reduced in two ways, either by the reduced ferredoxin of the outer tylacoid membrane reduces the electron-acceptor of PSI, or the MDHA is reduced through the ascorbate-glutathion cycle (*Foyer* et al., 1994; *Alscher* et al., 1997).

Ascorbic acid and ascorbic acid-oxidase participate, indirectly or directly, in the proliferation and wall elongation of plant cells. Where the ascorbic acid oxidase activity is higher in the cell walls, growth is faster (*Smirnoff*, 1996).

LAA is also a substrate of the biosythesis of the oxalic acid or the tartaric acid at least in some species (*Loewus*, 1988).

Ascorbic acid helps the regeneration of a tokoferol associated to the membrane by reducing the a tokoferil radicals. Those lipotropic anti-oxidants are also involved in the elimination of noxious radicals during photosynthesis (*Foyer*, 1993).

L-ascorbic acid alsohas an effect on mono- and di-oxigenases, in the active center of which iron or copper is found. Ascorbic acid (LAA) maintains the redox state of the central metallic ion and so it is responsible for the maximal activity of the enzyme. Such oxigenase enzymes are:

Proline-hidrolase enzyme is involved in cell growth, it hidrolises the proline of the glycoproteins in the cell wall (Glucoproteins are necessary for cell division and growth). Hidroxilysed glucoproteins controll the extensine in the cross-ties of the cell wall, which is important in mending scars and meeting pathogen intrusions. (*Arrigoni* et al., 1994, 1997). Thioglucosid-glucohidrolase (mirosinase) catalyses the hidrolysis of glucosinolates into glucose and aglucon fragment.

Gibberelin-3-dioxigenase and amino-cyclo-propane-1-carboxylate-oxidase is also dependent on LAA (*Smirnoff*, 2000b)

Violaxanthin-de-epoxidase is attached to the lumen of tylacoid membranes in the xanthophyll cycle and catalyses the de-epoxydation of violaxanthin or anthaeroxanthin to zeaxanthin (*Smirnoff & Wheeler*, 2000; *Smirnoff*, 2000a).

Steps of biosynthesis leading to vitamin C

In yeasts

Previously, it was assumed that yeasts and other micro-organisms are able to synthesise ascorbic acid (LAA) (*Heick* et al., 1972). However, the distinction between LAA and the analogous D-erythro-ascorbic acid was not possible by the formerly used spectrophotometric methods. Later, HPLC facilitated the conclusion that D-erythro-ascorbic acid is a regular component of yeasts and the appearance of LAA is only possible if special external precursors are applied (*Nick et al.*, 1986; *Kim* et al., 1996).

D-erythro-ascorbic acid is formed in two steps. In the first, D-arabinose dehydrogenase (a NADP-dependent heterodimer) catalyses the oxidation of D-arabinose to D-arabino-1,4-lacton. Subsequently, D arabino-1,4-lakton-oxidase completes the synthesis to D-erythro-ascorbic acid (*Spickett* et al., 2000).

In animals and in the human body

The synthesis of vitamin C in animals was revealed already in 1960. Even in those animals which are able to synthesise LAA the activity is restricted to special cell types only.

The substrate of the process is glucose derived from stored glycogen (*Bánhegyi* et al., 1997). The UDP-glucose is oxidised to D-glucuronic acid, then the discharge of one molecule of water results in D-glucuronic-acid-lactone. It is reduced to L-gulono-1,4-lacton (meanwhile, an inversion happens on the first carbon-atom), then L-gulono-lactone-oxidase enzyme transforms it to 2-keto-L-gulono-1,4-lactone. As the last step, 2-leto-L-gulono-1,4-lactone is spontaneously enolised to L-ascorbic acid. This

is the last step of the synthesis, which is suppressed in man because of the mutation of gulono-lactone-oxidase (*Nikishimi*, 1994), thus ascorbic acid as a final product cannot appear.

In plants

The follow-up of the biosynthetic pathway of vitamin C in plants was started during the 1950s, but the results were dubious. Isherwood et al. proposed (more than 45 years ago) a possible explanation of the LAA biosynthesis according to the analogy of the animal pathway, starting from D galactose (*Isherwood et. al.*, 1954): D-galactose → D-galacturonic acid-ester → L-galacturonic acid → L-galactono-1,4-lactone → L-ascorbic acid. The whole process of synthesis has been detected in successive phases, which revealed the interactions.

The last step is catalysed by L-galactono-1,4-lactono-dehydrogenase (GLDH), which has been isolated and purified from several plant species, moreover, it has been cloned taken from cauliflower and sweet potato (*Østergaard* et al., 1997; *Imai* et al., 1998)

Loewus et al. (1961, 1987, 1988) applied radioactive tracing and proved that no inversion happens in the carbon chain sceleton during the synthesis of LAA.

In the 1990s, *Nick Smirnoff* et al. (2001); *Wheeler* et al. (1998) used ascorbic acid deficient *Arabidopsis thaliana* mutants for the identification of genes active in the synthesis of ascorbic acid. GC-MS and NMR tests have been performed in order to check the metabolic consequences of the mutations (*Smirnoff* et al., 2003b).

The locus of the *vtcl* has been spotted successfully as responsible for the GDP-mannose-pyro-phosphorylase enzyme, which served as a proof of the supposed metabolic pathway.

The L-galactose way (*Conklin* et al., 1997, 1999, *Conklin* et al., 1997) starts by D-glucose and reaches L-ascorbic acid through nine or ten steps. The synthesis takes place in the cytosol except for the last step, which ensues in the mitochondria (*Smirnoff*, 2000b).

The D-glucose is transformed to D-glucose-6P by hexokinase, then it becomes D-fructose-6P via the effect of glucose-phosphate-isomerase. In the next step, D-mannose-6P is formed by mannose-phosphate-isomerase (mannose is an important carbohydrate in the cell wall of plants), phospho-mannose-mutase transforms it into D-mannose-IP. Subsequently, GDP-D-mannose is formed in a reaction catalysed by GDP-mannose-pyrophosphorylase. GDP-mannose-3,5-epimerase transforms it into GDP-L-galactose. The next step is L-galactose, a rather rare sugar. Oxidation results in L-galactono-1,4-lactone and another oxidation produces L-ascorbic acid. The liberated (e-) reduces the cytochrom C, terminal oxidase. The same enzyme is responsible for the last step of oxidation, which has been recognised by Isherwood earlier. It was also justified that Loewus was right when he stated that the synthesis of ascorbic acid is completed without inversion (*Smirnoff*, 2000).

This new biosynthetic pathway is significant because it integrates ascorbic acid into the central carbohidrate metabolism and indicates that there is a relationship between the biosynthesis of polysacharides and the glucolysation of proteins.

An alternative way of vitamin C synthesis in plants

(*Loewus* et al., 1961) observed that the content of L-ascorbic acid in strawberry fruits increases during the ripening process. He also discovered the transformation of galacturonic acid into ascorbic acid during ripening.

According to (*Smirnoff*, 2001) there are also alternative biosynthetic ways besides the one through galactose. *Fernanda Agius* et al. (2003) proved the earlier assumptions according to which ascorbic acid was formed through galacturonic acid under *in vivo* conditions. This synthetic path facilitates the use of carbonic compounds arising from pectin after the injury of cell walls in certain organs (e.g. in ripening fruits). This is supported by the increasing ascorbic acid content of fruits during the ripening process *(Davey* et al., 2000).

Agius et al. (2003) isolated and characterised the gene of the enzyme D-galacturonic acid-reductase (GalUR) from strawberry. They claimed that the D-galacturonic acid-reductase participated in the synthesis of ascorbic acid. It has been observed that ascorbic acid content correlates with the expression of galacturonic acid-reductase during fruit ripening.

In several *Fragaria* species, galacturonic acid-reductase level and vitamin C content have been measured. The parallel variation in the two compounds has been verified. The gene of GalUR being overexpressed in *Arabidopsis thaliana* increased the ascorbic acid content to two to three times the original and it was further increased in transgenic lines due to adding galacturonic acid. In transgenic strawberry plants, antisense lines have also been formed, which lowered the expression of pectin-liase. As a result, fruits became more firm (which is attributed to the reduced solubility of pectin in cell walls), but ascorbic acid content was substantially lower.

Smirnoff et al. (2003a) claim that synthesis of ascorbic acid through galacturonic acid exists in strawberry, but radioactive tracing technique proved that this way plays only a minor role in the synthesis of vitamin C under *in vivo* conditions. According their opinion, NDP-D-galacturonic acid is perhaps formed from NDP-D-galactose and part of it is built into pectin within the Golgi apparatus then is transferred to the cell wall, where it will be methylised. When pectin is injured, D-galacturonic acid is released. Other parts of NDP-D-galacturonic acid are hidrolysed, directly, into D-galacturonic acid.

According to *Smirnoff* (2003a), the role of pectin-lyase is not clear in the synthesis of ascorbic acid. The enzyme in question does not enable the direct release of D-galacturonic acid from pectin, it is rather bound to the β type of elimination.

The identification of the gene of galacturonic acid-reductase enzyme will help to explore the biosynthesis of ascorbic acid also in unicellular organisms. Though there is little evidence, ti has been proved that in some taxonomic groups *(Chrysophyta, Bacillarophyta, Euglenophyta)* the path is manifested through galacturonic acid (*Smirnoff*, 2003a).

Regulation of ascorbic acid synthesis in plants

Conklin et al. (1997) observed that in mutants deficient in ascorbic acid (*vtc*), the capacity of L-galactono-1,4-lactone-dehydrogenase (GLDH) increased compared to the wild types. They concluded that the low ascorbic acid content "upregulated" the GLDH. Proofs exist that in photosynthetic tissues, there is a linear relation between the LAA pool and the quantity of soluble carbohydrates in barley leaves. In darkness, quick turnover and a drop in LAA has been experienced. The loss of LAA could be prevented by adding sugars. These results indicate that the synthesis of LAA does not depend on light and that the hexose-phosphate supply becomes a limiting factor in darkness (*Smirnoff & Pallanca*, 1995)

Tissues, which do not photosynthesise, are less susceptible to carbohydrate supply. Experiments with peas revealed a decline in the rate of biosynthesis due to an increased level of LAA, which is attributed to the inhibiting feedback mechanism.

An increased D-mannose did not cause any change in the LAA pool, as if there was a critical rate between the quantities of GDP-mannose and L-galactose.

Sugar-phosphates participating in acorbic acid synthesis become easily saturated and act as a reservoir, which transfers precursors to the carbohydrate synthesis and to the glucolysis. It is probable that the synthesis of different carbohydrates also influence the whole process of LAA synthesis. In additio, GDP-mannose and GDP-galactose are involved in the synthesis of the cell wall and of glucoproteins, too.

Decomposition and regeneration of L-ascorbic acid

Ascorbic acid is oxidised to dehydro-ascorbic acid through mono-dehydro-ascorbic acid. Dehydro-ascorbic acid is partially regenerated by means of dehydro-ascorbic acid-reductase (DHAR) as a catalyser. Thus DHAR has a decisive role in regulating the redox status of ascorbic acid.

Zhong Chen et al. (2003) isolated the gene of DHAR from wheat and expressed it in tobacco and maize. They observed that the augmented expression of the DHAR enzyme was 32–100 times higher, and as a consequence, ascorbic acid content increased to 2–4 times higher levels in transformed plants compared to the oxidised form and also the level of glutathion increased. Results prove that not only the stimulation of biosynthesis but also the overexpression of DHAR having a role in the redox cycle lead to an increased level of vitamin C in the plant.

The other part of the oxidised dehydro-ascorbic acid is irreversibly hidrolysed into 2,3-diketo-gulonic acid and is integrated into the oxidative pentose-phosphate cycle.

Translocation and accumulation of vitamin C in plants

Information on the genes coding the enzymes involved in the biosynthesis of ascorbic acid is rudimentary, as well as data concerning the distribution and locations of vitamin C synthesis within the organs of the plant are scarce. Neither it is known, whether ascorbic acid is synthesised directly in the storing tissues or should be transported there from elsewhere. Moreover, what is the fate of ascorbic acid, how does it change in the storing organs?

In the generally high ascorbic acid content of the leaves there is but little difference between herbaceous and woody plants.

In edible tissues which do not contain chlorophyll, there is a large and unexplained variation in vitamin C content. In fruits, e.g. in camu camu the concentration is 2700 mg/l00g, whereas in medlar it is only 3 µg/l00g. Differences are most likely not due to the taxonomic belonging. In rose hips the content is around 4000 times higher than in the medlar fruit belonging to the same family, *Rosaceae* (*Viola* et al., 2001). Ascorbic acid content of fruits is influenced by the growing site, weather conditions, mineral supplies, growing type, post harvest conditions, storing, etc. According to some researchers, the regulation of ascorbic content is less severe in the storing organs than in leaves, where its presence is essential for photosynthesis.

On the subcellular level, it has been observed that LAA is concentrated in the chloroplasts, apoplasts and in the mitochondria. It is interesting that in the root of horse radish LAA is accumulated in the central vacuole, where the pH is probably low and it stabilises the molecule.

The short distance translocation of ascorbic acid is much better known than the long distance transport within the organism. According to monovalent anions, LAA alone cannot penetrate the membranes under physiological conditions, so it is transported by carrier molecules. *Foyer* et al. (1991) found that in purified chloroplasts of pea and spinach the uptake of ascorbic acid is a carrier-mediated process, though it crosses the tilacoid membranes by facilitated diffusion. At the same time, 10–20% of LAA is bound within the tilacoid membranes.

Rautenkratz et al. (1994) claimed that the uptake of LAA/DHA shows a kinetic saturation in leaves of barley and pea, which indicates the presence of a carrier-mediated transport, maintained by a proton-gradient.

In purified vesicles of bean membrane, it was proved that DHA crosses the membrane more easily by carrier mediated diffusion than *LAA* (*Horemans* et al., 1997).

Franceschi & Tarlyn (2002) made very interesting new observations related on the long-distance transport of ascorbic acid. Accumulation of radio-active (C^{14}) marked L-ascorbic acid was measured in three different plant species (*Arabidopsis thaliana, Medicago sativa, Impatiens walreiana*). Using autoradiography and HPLC techniques it was determined that LAA is accumulated in the phloem of the intact plant, or is transported to the root tips, shoots, reproductive organs, but not into the mature leaves. From the phloem of *Arabidopsis thaliana* juice was extracted by the "aphid stylectomy" technique, which facilitated the direct detection of L-ascorbic acid. In experiment based on autoradiography, accumulation has been traced in the smaller and larger veins of thin leaves of *I. walreiana* after 12 and 24 hours of incubation, whereas in *A. thaliana* and *M. sativa*, it was difficult to decide whether the patterns are representing ascorbic acid or some of the derivatives (oxalic acid, tartaric acid).

Micro-autoradiographic trials prove that traces are found in the phloem and not in the xylem. Ascorbic acid is moving from the source, the leaves, through conductive elements of the phloem to the sink. This statement is essential because though studies reported that ascorbic acid and its oxidised form, dehydro-ascorbic acid can permeate the membranes of the cytoplasm, it has not been proven that its long distance transport takes place via the phloem.

Kollist et al. (2001) proved that ascorbic acid and dehydro-ascorbic acid are transported together, from the apoplast into the symplast, i.e. in tissues of the intact leaf.

Relevant studies display that there are protein-type carrier molecules that transport ascorbic acid and dehydro-ascorbic acid. The distribution of those carrier molecules is, however, unknown in different cell types.

Franceschi et al. (2002) treated intact leaves of *Arabidopsis* and *Medicago* with galactono-1,4-lactone by the technique of compartment method. They observed that L-ascorbic acid content increased to 7–8 times the original level, whereas in untreated leaves it only doubled or tripled. L-ascorbic acid content in the treated leaves and accumulation in the sink tissues was proportional to the amount of galactono-1,4-lactone added. At the same time, galactono-1,4-lactone has not been detected in the sink tissues. It has been concluded that ascorbic acid is synthesised in tissues of the source and is transported through the phloem to the sink. Experiments performed with L galactose gave similar results.

When examining the ascorbic acid producing ability of different organs, it was stated that the biosynthetic capacity of mature leaves is 3–10 times higher while their rate of turnover is much lower than those of the sink tissues.

It is probable that ascorbic acid is more easily synthesised and stored in the photosynthetic tissues, where more assimilated carbon is available than in the fast growing sink organs.

DeGara et. al. (1997) pointed out in their papers that synthesis of ascorbic acid in the tissues is limited mainly by the availability of the substrate, and not so much by the biosynthetic capacity. The level of ascorbic acid in the sink tissues is determined to a certain extent by the quantity of the products of source origin.

The transport of ascorbic acid is proportional to the requirements of the sink tissues. Translocation of ascorbic acid in the phloem is a regulating factor of the development of sink tissues because ascorbic acid is of vital importance in the processes of cell division and growth.

Summary

Research on with vitamin C has been accelerated since the last years of the past century. Many researchers studied the relevant questions from different points of view. In the new millennium, intensive research is carried out by numerous laboratories of the world. What is the reason of the increased interest? The present review is intended to find an answer to this question. Information is accumulating rapidly on the numerous vital functions of ascorbic acid in maintaining the health of the human body. However, as the synthesis of that vital compound cannot be performed in the human body, people should acquire it from their diet. It is still generally accepted that the required daily dose of vitamin C for a man is around 60–70 mg. In fact, that is sufficient only for the maintenance of a subclinical level of scurvy. Famous researchers, as Albert Szent-Györgyi and Linus Pauling consumed more than one gram of vitamin C daily. According to Szent-Györgyi, it should be eaten as a common food instead of measuring the milligrams. Thus the recommended daily dose of an average man should vary between 1–10 g. Stress, smoking, environmental pollution all need to be compensated by ascorbic acid. How can we ingest the sufficient amount of vitamin C? It would be the simplest to take some tablets. However, there are some researchers who emphasise the difference between the manufactured and the natural vitamin C, stating that the latter is much more efficient. So fruits and vegetables have to be preferred as sources of vitamin C. That is the reason why so many researchers endeavour to explore the metabolism of ascorbic acid in plants, and the same motives urged us to start our research. Decisive steps have been made in that direction, the main features and all intermediates together with the enzymes responsible for the process have been revealed. It is clear that the synthesis of vitamin C takes place mainly in the cytoplasm of the cells except for the last step, which is performed in the mitochondria. The redox cycle as well as the decomposition of ascorbic acid are known. Less information is available about the transport between plant organs and even less about overproduction and/or accumulation. Up-to-date biochemical and molecular techniques help in answering the so-far unanswered questions, which will enable us to regulate the natural ascorbic acid content of our crop and foods in the future. The development of the most efficient technologies should be completed with the domestication of new species, which are more promising than the established crops and still

unexplored. Vitamin C content of fruits varies between wide boundaries. Thus we claim that the existing assortments do not give a sufficient answer to the questions raised about the numerous processes related to L-ascorbic-acid biosynthesis. The plants to be studied produce or accumulate extreme amounts of LAA (their ascorbic acid content exceeds 1% of the fresh weight). Such plants are the camu-camu (*Myricaria dubia* L.), the acerola (*Malpighia glabra*) and some rose species and varieties. Domestic and foreign institutes, which are co-operating in this field with our laboratory, provide for the availability of the research objects mentioned. A further objective of our program is the breeding of alfalfa varieties with high vitamin C content for the purpose of producing protein concentrates, vegetables with high vitamin C content (egg plant, pepper, tomato) as well as fruits with increased vitamin C content (apple, apricot, grape).

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