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A Possible Enzymatic Catabolism of L-Ascorbic Acid *via* α-Ketoaldehydes

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L- Ascorbic Acid의 생체분해

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It is shown by means of nuclear magnetic resonance spectroscopy that 3,4,5-trihydroxy-2-keto-L-valeraldehyde (L-xylosone), an α-ketoaldehyde, is formed during the oxidative catabolism of L-ascorbic acid. It is proposed that this substance serves as a substrate for the glyoxalase system by which it is transformed to L-xylonic acid. As L-xylonic acid is further oxidized to L-erythroascorbic acid, a biochemical pathway is proposed for the action of vitamin C which consists of two further γ-lactones and three different substrates of the glyoxalase system.

The enzymatic degradation of L-ascorbic acid (ASC) in biological systems has not been studied in detail yet. It is only known that ascorbate oxidase is responsible for the oxidation of ASC to dehydroascorbic acid (DHA) in plants and dopamin-β-hydroxylase or another copper-proteins may function similarly in animals (Lohmann, 1981). Furthermore, dehydroascorbatase has been described in animal tissues for the hydrolysis of dehydroascorbic acid (DHA) to 2,3-diketo-L-gulonic acid (DKG) and 2,3-diketoaldonate decarboxylase for the decarboxylation of DKG to L-xylonic and L-lyxonic acids (Kanfer *et al.*, 1960; Kagawa, 1962; Shimazono and Mano, 1961; Ashwell *et al.*, 1961) It is widely assumed that L-xylonic acid is lactonized by soluble lactonase or nonenzymatically, to L-xylonolactone which is converted to L-erythroascorbic acid (EASC) by gulonolactone oxidase (Shimazono and Mano, 1961; Ashwell *et al.*, 1961) or α-hydroxyacid dehydrogenase (Cammack, 1969). However, as we have shown recently 3,4,5-trihydroxy-2-keto-L-valeraldehyde (TKVA, L-xylosone) and EASC are produced as nonenzymatic oxidative degradation products of L-ascorbic acid (Kang *et al.*, 1982). The formation of TKVA after the enzymatic decarboxylation of DKG has also been described in bacterial systems (Kamiya, 1961).

It is obvious that the α-ketoaldehyde TKVA formed from DKG by decarboxylation may serve as a substrate of α-ketoaldehyde dehydrogenase or the glyoxalase system, the physiological substrate of which is still unknown. The glyoxalase system consists of two enzymes, glyoxalase I and glyoxalase II using reduced glutatione (GSH) as a cofactor. The substrate, *e.g.* methylglyoxal forms a hemimercaptal with reduced glutathione which is transformed to a thioester by glyoxalase I. Glyoxalase II catalyzes the separation of this thioester to GSH and D-lactic acid.

α-Ketoaldehyde dehydrogenase, on the other side, catalyzes the oxidation of α-ketoaldehyde to α-ketoacid using NAD^+ or $NADP^+$. Both enzyme systems are unspecific and can catalyze, hence, a great variety of glyoxals (Vander Jagt *et al.*, 1975; Jocelyn, 1972). It has also been described that glyoxalase system catalyzes the change of osone (α-ketoaldehyde containing aldose) to aldonic acid (Bayne and Fewster, 1956). Likewise ascorbate oxidase is a relatively unspecific enzyme which besides ascorbic acid oxidizes also other species containing a γ-lactone ring structure like araboascorbic acid or reductic acid (Katz and Wesley, 1979).

Therefore, ascorbate oxidase (or an analogous copper enzyme) in combination with the glyoxalase enzyme system, both present in living cells, will be able to catalyze the degradation of ASC *via* intermediate α-ketoaldehyde species and substances containing a γ-lactone ring like ASC. The catabolism of ASC is, thus, discussed in terms of a new understanding of the function of vitamin C in biological systems.

MATERIALS AND METHODS

Sodium-L-ascorbate, tetradeutroacetic acid· (CD_3COOD), deuterium chloride (DCl), deuterated sodium hydroxide (NaOD), and D_2O were purchased from Merck, Darmstadt, W. Germany, reduced glutathione from Fluka, Buchs, Switzerland, methyglyoxal, glyoxalase I (EC 4.4.1.5) and glyoxalase II (EC 3.1.2.6) from Sigma, St. Louis, U.S.A., and ascorbate oxidase (EC 1.10.3.3) from Boehringer, Mannheim, W. Germany. Dehydro-L-ascorbic acid has been prepared according to a method proposed by Holker (Hall *et al.*, 1976) and recrystallized according to a procedure proposed by Staudinger and Weis (Iio *et al.*, 1976). The ascorbate oxidase activity was tested by the decrease of the extinction of ascorbate using phosphate buffer and serum albumine for stabilization.

The UV spectra were taken with a Zeiss DMR 10 spectrophotometer at room temperature. The nuclear magnetic resonance (NMR) experiments were performed by means of a Varian XL-100-15 spectrometer operating at 100-1 MHz with 5 mm insert. The probe temperature was 27°C. Internal D_2O provided the field-frequency lock. Aquisition times of 4 second have been used. Deuterated acetate buffer (1.0 M, pD 6.6 or 7.4) has been used for maintaining constant pH conditions. The water resonance has been suppressed by an inversion-recovery pulse sequence.

RESULTS

The glyoxalase system

Methylglyoxal (MG) has been used as a substrate to test the glyoxalase enzyme system (Fig. 1). The formation of the hemimercaptal between this substance and reduced glutathione (GSH) is indicated in the ^{1}H-NMR spectrum by the broadening of the glutathione resonances (Fig. 2a). Furthermore, the H-1 of the lactoyl group can be seen at 5.93 ppm and CH_3-3 at 2.66 ppm.

After the addition of glyoxalase I to a mixture of MG and GSH a thioester is formed due to an enediol-proton transfer mechanism of the lactoyl group (Hall *et al.*, 1976). This reaction causes an upfield shift of the CH_3-3 group of MG from 2.66 to 1.68 ppm and splitts its resonance to a doublet. The H-1 of the lactoyl group disappears and a quartet of H-2 in thioester increases at 4.75 ppm, coupled with the doublet of CH_3-3 and in addition the resonances of the cysteinyl group of glutathione are shifted downfield (Fig. 2b). As can be

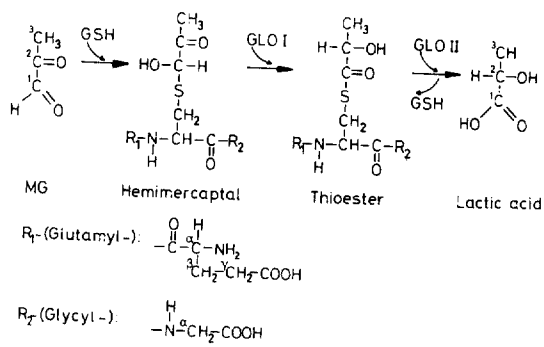

Fig. 1. *The action of the glyoxalase system, glyoxalase I (GLO I) and glyoxalase II (GLO II), with reduced glutathione (GSH) on methylglyoxal (MG), schematically.*

seen by the splitting of the cysteinyl β-CH_2 resonances due to the magnetic nonequivalency of the protons, the results of the enediol-proton transfer mechanism of MG due to the catalysis of glyoxalase I is similar to a spectrum obtained with oxidized glutathione (GSSG), when compared with spectra of GSH and GSSG (Fig. 3). Hence, the thioester consisting of MG and glutathione seems to be restricted in its internal mobility after oxidation, as it is in the case in the dimer of oxidized glutathione. However, whereas this glutathione dimer is symmetric in its structure, the thioester formed with MG is not, causing small differences in the coupling pattern.

When glyoxalase II is added to the solution, the thioester spectrum as described above disappears and the spectrum of GSH returns (Fig. 2c). In addition the upfield shift of the CH_3-3 doublet from 1.68 to 1.65 ppm and of the quartet from 4.75 to 4.46 ppm with a coupling constant of 7 Hz and the spectrum of GSH indicate the separation of thioester, resulting in the formation of free lactic acid and GSH as the final product of the glyoxalase action. Hence the activity of both glyoxalase I and II on methylglyoxal can be well monitored in detail by means of the ^{1}H-NMR technique.

Ascorbate oxidase and glyoxalase system affecting ascorbic acid

As can be seen from the time dependence of the DHA spectrum, 3,4,5-trihydroxy-2-keto-L-valeraldehyde (TKVA) is the catabolic product resulting from decarboxylation of DKG (Kang *et al.*, 1982) (Fig. 3a). This transformation may be accelerated by an enzyme 2,3-diketoaldonate decarboxylase (Kagawa, 1962). After this step, the complexation with GSH, forming a hemimercaptal, is favored if any GSH is present. Consequently, the eight lines seen in the NMR-spectrum (Fig. 3b) after the addition of GSH and glyoxalase I, are assigned to the β-CH_2 of the cysteinyl group in the thioester as has been observed in the complex between glutathione and MG (Fig. 2b).

The H-3 doublet of TKVA as well as the resonances of H-4 and CH_2-5 can also be seen; the

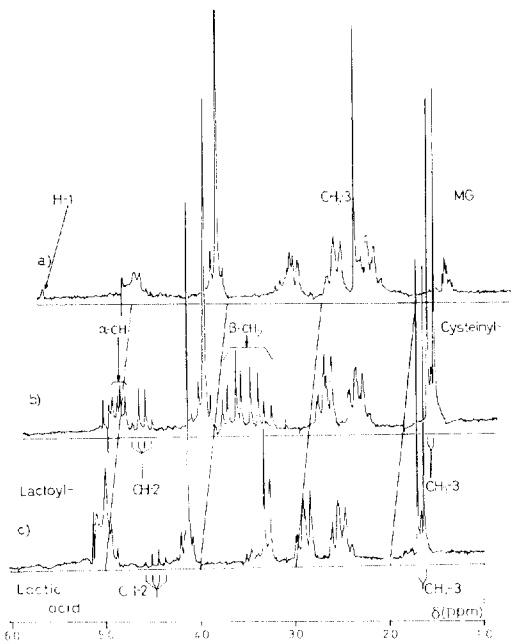

Fig. 2. *^{1}H-NMR spectra of methylglyoxal (MG, about 0.3 M in acetate buffer) treated with reduced glutathione (GSH, 0.5 M) (a), glyoxalase I (b), and glyoxalase II (c), respectively.*

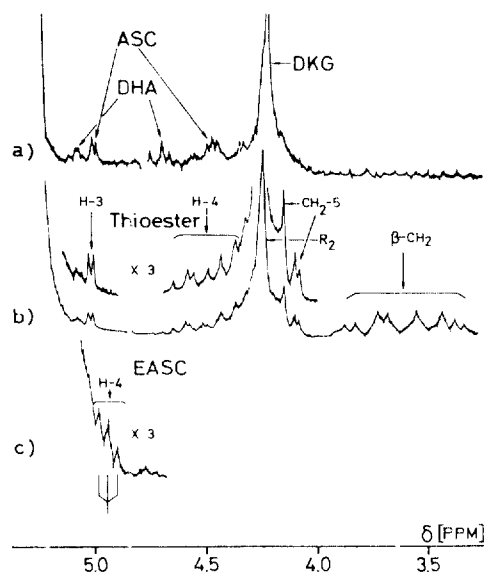

Fig. 3. *^{1}H-NMR spectra of L-ascorbic acid (ASC, 0.5 M in acetate buffer, pD 6.0) treated with ascorbate oxidase and oxygen for about 2 h (a) followed by the addition of reduced glutathione and glyoxalase I (b) and glyoxalase II (c), respectively. The spectrum (c) and the insets in spectrum (b) are enhanced by a factor of three in their intensities.*

ketoaldehyde signal at 5.02 ppm has already disappeared. Thus it may be suggested that a thioester fromation between TKVA and glutathione might play a similar role as the MG-glutathione complex. The concentration of the thioester as indicated by the NMR intensities, however, is less than would be anticipated from the glyoxalase I activity on MG with GSH as a substrate (Fig. 2b). This may be explained by an inhibition of the glyoxalase I activity due to ASC (Iio *et al.*, 1976). If a thioester is formed between TKVA and glutathione, the application of glyoxalase II should separate the thioester into GSH and L-xylonic acid and, after the addition of oxygen, to erythroascorbic acid. (EASC).

The ^{1}H-NMR spectrum of the latter product consists of the CH_2-5 and H-4 groups as described above. Whereas the CH_2-5 resonance is hidden by the glycyl signal of glutathione, the H-4 triplet with a coupling constant of about 4 Hz can be seen clearly (Fig. 3c). Of course, not all of the glutathione added is recovered in its reduced form because GSH is bound to TKVA only to that extent as the latter one is produced in the catabolic pathway of ASC. Nevertheless, it is shown that ASC is transformed to an α-ketoaldehyde as an intermediate step of its oxidative catabolism and transformed by the glyoxalase system and oxidized finally to EASC by molecular oxygen. This confirms similar results found by enzymatic oxidation, *e.g.* by gulonolactone oxidase (Shimazono and Mano, 1961; Ashwell *et al.*, 1961).

DISCUSSION

From the results obtained a catabolic pathway of vitamin C can be constructed which consists of subsequent reactions catalyzed alternatively by an enzyme similar to ascorbate oxidase and the glyoxalase system (Fig. 4). As it is widely assumed, ASC is oxidized to DHA, delactonized by dehydroascorbatase to DKG and then decarboxylated to L-xylonic and L-lyxonic acid (Kagawa, 1962; Shimazono and Mano, 1961: Ashwell *et al.* 1961). However, as demonstrated by the experiments described previously (Kang *et al.*, 1982), the α-ketoaldehyde TKVA might be decarboxylated from DKG by dehydroascorbatase. Since that the α-ketoaldehyde (osone) is ultimately a dehydrated form of the aldonic acid and is hydrated in higher pH ranges to its aldonic acid (Vander Jagt *et al.*, 1975; Vander Jagt *et al.*, 1972), it is difficult to identify α-ketoaldehyde (osone) from the aqueous solution chemically. This α-ketoaldehyde can be transformed by the glyoxalase system to L-xylonic acid. L-xylonic acid is lactonized by soluble lactonase or nonenzymatically to L-xylonolactone, which is oxidized by gulonolactone oxidase (Shimazono and Mano, 1961; Ashwell *et al.*, 1961) or α-hydroxy acid dehydrogenase (Cammack, 1969) to EASC, a substance which is similar to ASC in its structure and, thus, like ASC oxidizable by *e.g.* ascorbate oxidase.

It is obvious that in analogy to the results obtained for ASC the catabolism of EASC (*i.e.* oxidation and delactonization might also result in an α-ketoaldehyde, namely 2,3-diketo-L-butyraldehyde (Threosone, DKBA) (Fig. 4). As a consequence of the glyoxalase catalysis this substance might be transformed to L-threonic acid and, further, oxidized to α-hydroxytetronic acid (HT). Because this substance is again similar to ASC in its structure, it might also serve as a substrate for ascorbate oxidase. Hence, it will be oxidized to the α-ketoaldehyde, hydroxypyruvaldehyde (HPA) which, in turn, will be transformed by the glyoxalase system to glyceric acid. The production of this final product is in good concordance with former results (Reeves and Ajl, 1965). According to the biochemical pathway proposed the oxidative catabolism of ASC consists, thus, of EASC and HT, both substances containing a γ-lactone ring like ASC, and the three α-ketoaldehydes TKVA, DKBA, and HPA. On the other hand, when α-ketoaldehyde dehydrogenase may function on these α-ketoaldehydes, they may be oxidized directly to EASC, HT and hydroxypyruvic acid, respectively (Monder, 1967).

Hence, in speculating about the activity of vitamin C in living cells the ASC-DHA equilibrium may be an essential precondition, maintained by

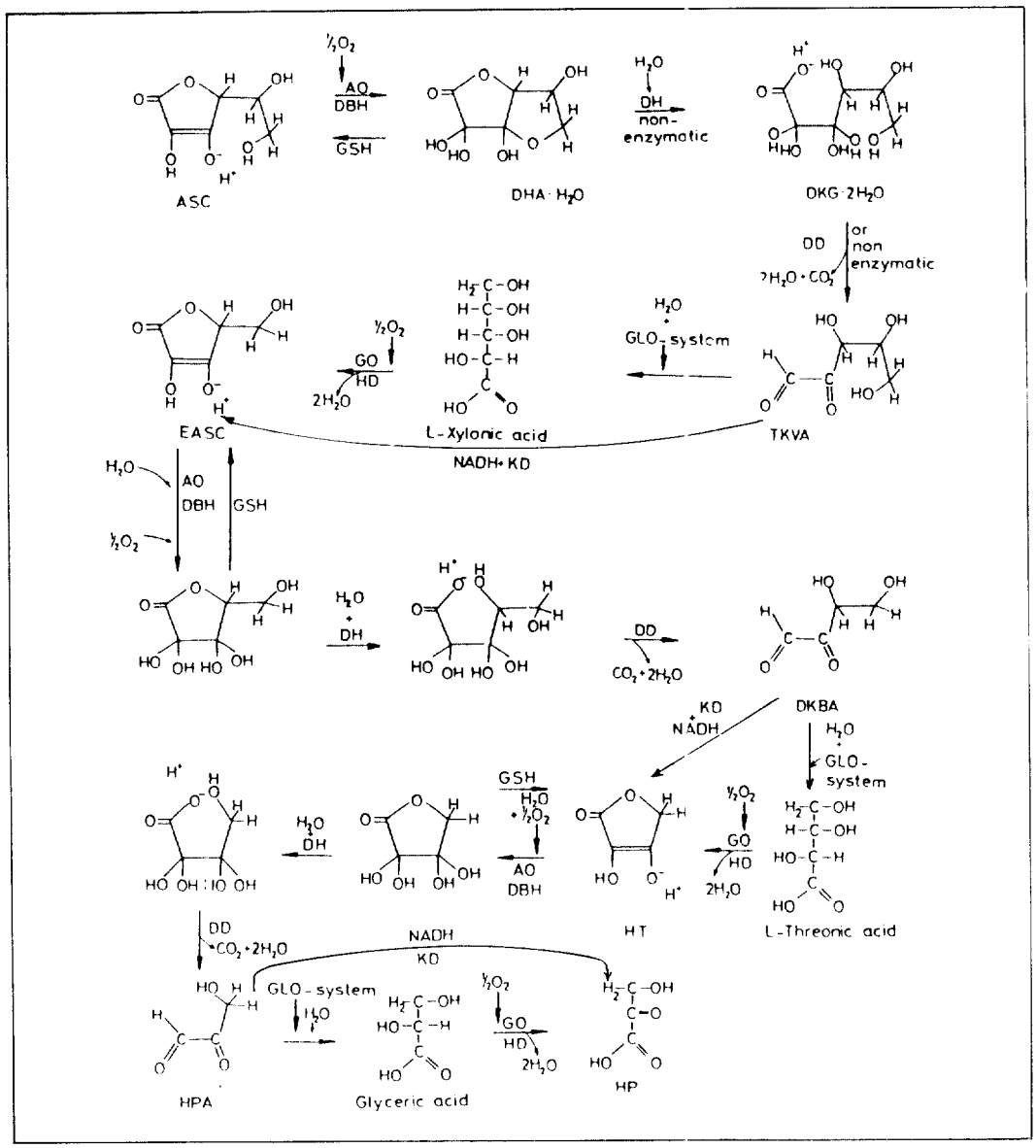

Fig. 4. *A model for the catabolism of L-ascorbic acid via the glyoxals 3,4,5-trihydroxy-2-keto-L-valeraldehyde (TKVA), 2,3-diketo-L-butyraldehyde (DKBA), and hydroxypyruvaldehyde (HPA) by the enzymic action of ascorbate oxidase (AO) and glyoxalase (GLO) system using reduced glutathione (GSH) as a cofactor. Abbreviation: DBH, dopamine-β-hydroxylase; DH, dehydroascorbatase; DD, 2,3-diketoaldonate decarboxylase; KD, α-ketoaldehyde dehydrogenase.*

ascorbate oxidase or a similar copper oxidase and GSH in combination with glutathione reductase. In addition to that equilibrium, however, and as a consequence of the chemical instability of DHA, the α-ketoaldehydes formed by degradation may also be part of the complex molecular mechanism of vitamin C. They may *e.g.* be responsible for blocking protein and DNA synthesis, inhibiting, thus, the cell proliferation. All three α-ketoaldehyde species formed during the catabolism of DHA are possibly substrates of the glyoxalase system. The α-ketoaldehydes $C_3H_4O_2$ (*e.g.* MG) to $C_{13}H_{24}O_2$ are active cell inhibitors with the highest efficacy for compounds with six C-atoms (Együd

and Szent-Györgyi, 1966). α-Ketoaldehydes described as being part of the DHA catabolism, TKVA (5), DKBA (4), and HPA (3) are near this optimum.

The oxidation of ASC produces two substances with a γ-lactone ring. Both, EASC and HT, are reductants like ASC (Thompson, 1980; Szent-Györgyi, 1977) and their oxidized forms may be reduced by GSH as shown in the case of ASC (Sapper *et al.*, 1982). Therefore, each ASC molecule may act three times as a so-called "retine" (ASC, EASC, and HT). The balance between the γ-lactone rings and α-ketoaldehydes is influenced by GSH acting as a reductant and a cofactor in the glyoxalase system. This agrees well with observations according to which the GSH level is enhanced *e.g.* during the growth of plants, *i.e.* during a phase of frequent cell proliferation (Jocelyn, 1972). GSH reduces the oxidized forms of the γ-lactone species as well as transforms the α-ketoaldehydes. Finally, the α-ketoaldehyde concentration decreases and with the onset of DNA and protein synthesis the cells are able to proliferate again.

The relatively high ASC activity found at the edge of tumor tissue (Lohmann *et al.*, 1981) may result from the oxidation of the α-ketoaldehydes to EASC and HT, respectively. Hence, the enhanced ASC activity corresponds to a decreased over all levels of α-ketoaldehydes and, therefore, facilitates the cell proliferation. Indeed, an enhanced cell proliferation is characteristic for most of the tumor cells. On the contrary, α-ketoaldehyes inhibit tumor growth. Since α-ketoaldehydes are produced during the ASC metabolism, vitamin C may be active against tumors also by these species depending on the balance of the participating enzyme systems.

It has been reported that the catabolic forms of ASC. DHA and DKG are diabetogenic in rats (Meglasson and Hazelwood, 1982) and in human patients (Chatterjee and Banerjee, 1979; Lohmann *et al.*, 1982) and that osones (α-ketoaldehydes) are related to diabetes mellitus (Bayne and Fewster, 1956). This, again, confirms that the molecular action of vitamin C is not exhausted by the well known reducing ability of ascorbic acid, but has to be considered as a consequence of a variety of degradation products, especially α-ketoaldehydes.

적 요

L-Ascorbic acid의 생체 산화분해과정중 α-ketoaldehyde의 한 종류인 3, 4, 5-trihydroxy-2-keto-L-valeraldehyde (L-xylosone)가 형성된다는 사실을 핵자기공명스펙트럼분석법으로 확인하였다. 이 물질은 glyoxalase system에 의해 L-xylonic acid로 변환되고 계속해서 L-erythroascorbic acid로 산화된다. 이러한 근거 위에서 vitamin C의 분해과정이 vitamin C이외의 두종류의 γ-lactones과 3종류의 α-ketoaldehydes로 구성된 분해경로를 갖는다는 사실을 제안하였다.

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