The Oxidative Degradation of L-Ascorbic Acid via an α-Ketoaldehyde *

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Dehydro-L-ascorbic acid, the oxidation product of L-ascorbic acid, is unstable in aqueous solution and decomposed by a hydrolytic disruption of its ring structure to 2,3-diketo-L-gulonic acid. It is shown by means of UV, ^{1}H-, and ^{13}C-NMR spectroscopy that after decarboxylation 3,4,5-trihydroxy-2-keto-L-valeraldehyde, an α-ketoaldehyde, is formed. This substance is oxidized further to L-erythroascorbic acid, coupled with a reduction of 2,3-diketo-L-gulonic acid to L-ascorbic acid.

Introduction

L-Ascorbic acid (vitamin C, ASC) is known as a substance regulating the redox potential in cellular systems. Since its crystallization by Szent-Györgyi in 1928 many investigations have been performed, its complex function, however, including its detailed redox mechanism and metabolism, is still unknown. It is generally accepted that ASC is oxidized to dehydro-L-ascorbic acid (DHA) by copper proteins, *e.g.* by ascorbate oxidase in plants or domapin-β-hydroxylase or another enzyme acting similarly in animals [1 – 5]. It has also been shown that DHA can be reduced to ASC *e.g.* by reduced glutathione (6). Hence, the enzyme glutathione reductase in combination with an oxidizing protein (*e.g.* ascorbate oxidase or dopamin-β-hydroxylase) may be responsible for maintaining the redox equilibrium of the redox pair ASC-DHA.

Although DHA is known for a long time to be highly unstable in aqueous solution, its degradation processes have not been clearly explained yet. It has been suggested that DHA is delactonized to 2,3-diketo-L-gulonic acid (DKG) and metabolized further by a hitherto unidentified decarboxylating enzyme to L-xylonic and L-lyxonic acids. It has been assumed that these species are oxidizable to L-erythroascorbic acid (EASC) which contains, like vitamin C, a γ-lactone ring [7 – 9].

On the other hand, the formation of pentosone [10] or L-xylosone (*i.e.* 3,4,5-trihydroxy-2-keto-L-valeraldehyde (TKVA) after the decarboxylation of 2,3-diketo-L-gulonic acid has been described in chemical [11, 12] and bacterial systems [13]. Furthermore, in addition to an unidentified substance, ASC could be extracted with acetone and separated by paper-chromatography after decarboxylation of DHA in sodium acetate [14].

Thus, the chemical decomposition of ASC seems to be still unknown. Since the vitamin C redox system [6] as well as some of its metabolites are considered to be very important factors in living cells, this decomposition has been reinvestigated by means of NMR (Nuclear Magnetic Resonance) technique. A new model of the degradation mechanism will be proposed.

Materials and Methods

Sodium-L-ascorbate, tetradeuteroacetic acid (CD_3COOD), deuterium chloride (DCl), deuterated sodium hydroxide (NaOD), acetone, sodium acetate, potassium dihydrogen phosphate (KH_2PO_4), and D_2O were purchased from Merck, Darmstadt. Dehydro-L-ascorbic acid has been prepared according to a method proposed by Holker [15] and recrystallized according to a procedure proposed by Staudinger and Weis [16]. 3,4,5-Trihydroxy-2-keto-L-valeraldehyde (TKVA) has been prepared according to a method proposed by Salomon *et al.* [17].

The UV spectra were taken with a Zeiss DMR 10 spectrophotometer at room temperature.

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The nuclear magnetic resonance (NMR) experiments were performed by means of a Varian XL-100-15 FT spectrometer operating at 100.1 MHz with a 5 mm insert for ^{1}H- and at 25.1 MHz with a 12 mm insert for ^{13}C-measurements. The probe temperature was 27 °C. Internal D_2O provided the field-frequency lock. Aquisition times of 4 s for ^{1}H and 0.8 s for ^{13}C have been used. The ^{13}C-spectra, accumulated with delay times of 4 or 5 s, were noise decoupled or they were gated decoupled for observing the hetero coupling. Deuterated acetate or phosphate buffers were used for maintaining constant pH conditions. In some cases (Figs. 1 a and 6 a) the water resonance has been suppressed by an inversion-recovery pulse sequence.

Results and Discussion

The ^{1}H- and ^{13}C-NMR spectra of DHA, as shown in Figs. 1 a and 2 b, have been interpreted by a special bicyclic structure of this oxidized form of ASC in aqueous solution [6, 18]. DHA in its hydrated form is gradually transformed to hydrated DKG by a rupture of both rings, *i.e.* the furanose and the γ-lactone rings, due to hydrolysis. DKG exhibits a complex ^{1}H-NMR spectrum near 4.2 ppm (Figs. 1 b and c) similar to those of various sugar alcohols (*e.g.* xylitol) [19]. The corresponding ^{13}C-NMR spectrum (Fig. 2 c) has been assigned to the second form of DHA after the opening of the furanose ring system [18] and, indeed, the chemical shifts of C-4, C-5, and C-6 are very similar to those of ASC (Fig. 2 a) indicating a free side chain. However, the pH-dependence of the chemical shifts (Table I a) implies that not only the furanose ring but also the lactone ring of DHA is hydrolyzed resulting in an acid carboxyl group after delactonization. Therefore, the first degradation product of DHA, in aqueous solution, is the dihydrated form of DKG as shown in Fig. 5 a.

It has been suggested that DKG is decarboxylated after a few hours in a weakly alkaline aqueous solution [7 – 12], which should primarily result in a hydrated form of TKVA. In the ^{1}H-NMR spectrum, the H-3 resonance of this substance could be identified at 5.0 ppm, and the H-4 and CH_2-5 resonances near 4.2 ppm (Fig. 1 d). Some more information can be obtained by the ^{13}C-NMR spectrum (Fig. 2 d). As can be seen, the C-3 resonance shift is similar to

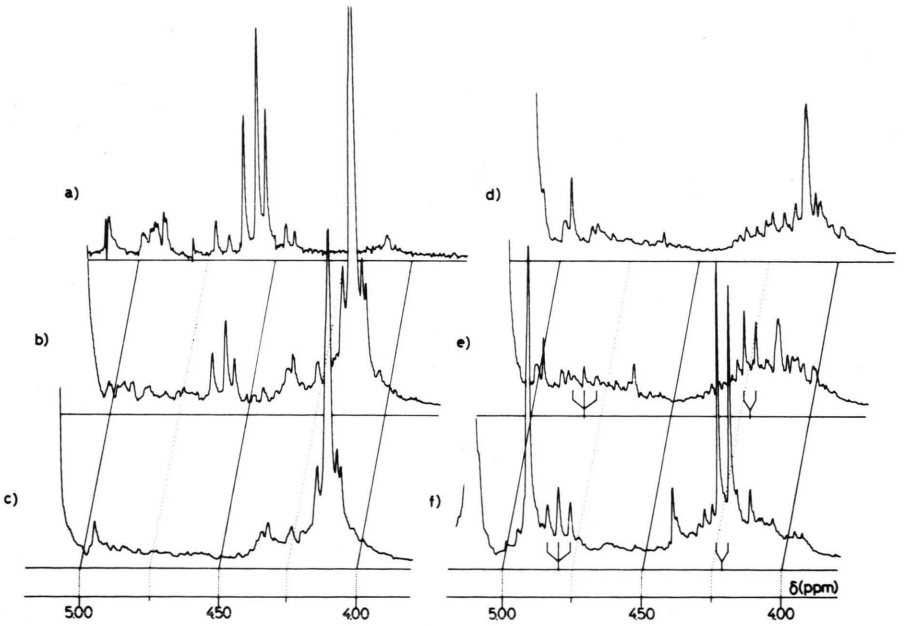

Fig. 1. ^{1}H-NMR spectrum of dehydro-L-ascorbic acid (DHA, 50 mM), taken 1 h after dissolution (a); a pD-value of 5.6 (acetate buffer) has been used in this case for stabilization. DHA spectra in 500 mM acetate buffer (pD 7.4), taken 6 h (b), 24 h (c), 96 h (d), and 168 h (e) after dissolution, and spectrum of L-erythroascorbic acid (EASC) (f), taken after extraction from the solution (e); in this case the acetate buffer was adjusted to pD 6.0 for stabilization of EASC.

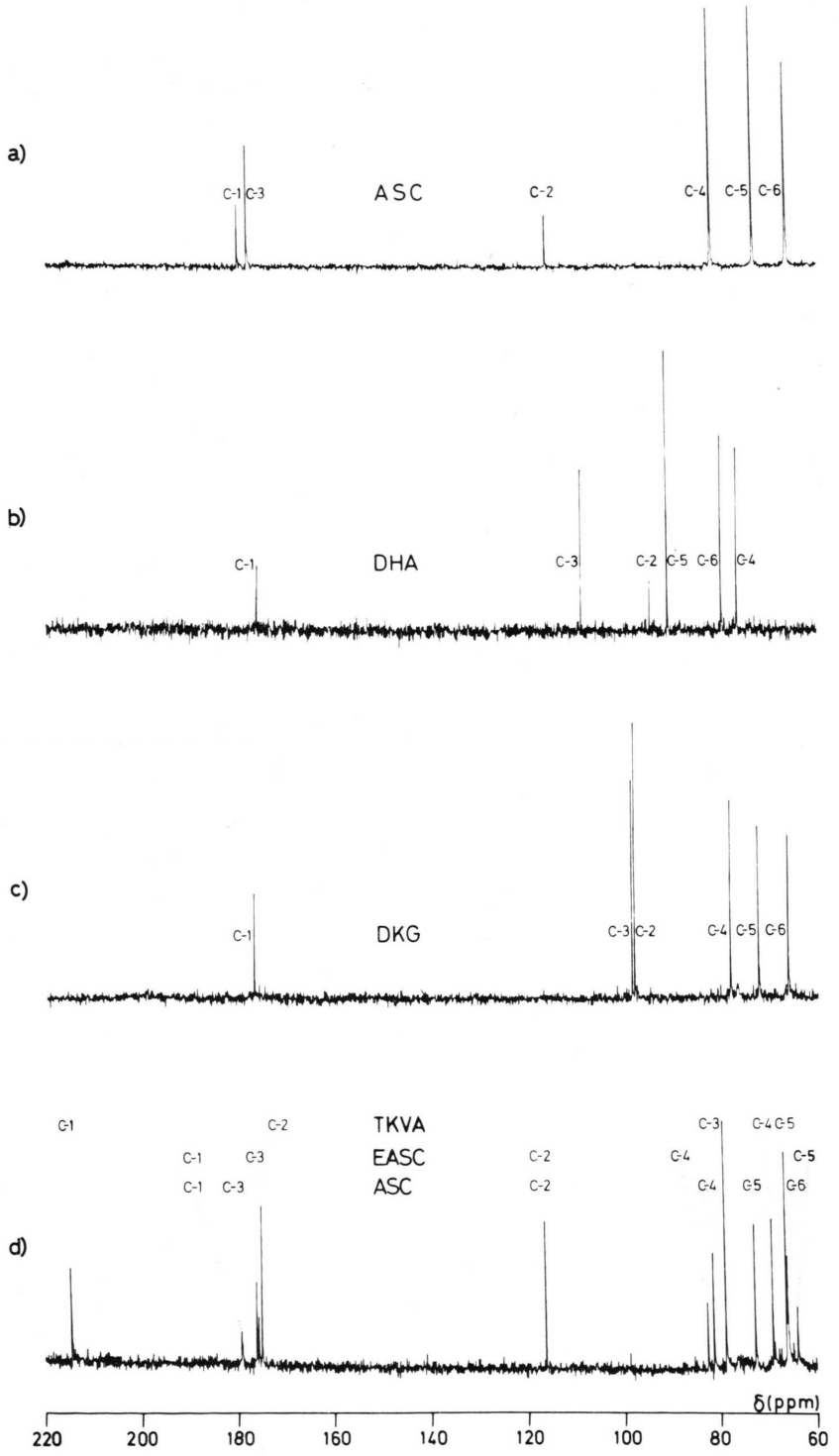

Fig. 2. Proton decoupled ^{13}C-NMR spectrum of 1 M sodium-L-ascorbate (ASC) in water (pD 7.4) (a) and of 0.5 M dehydro-L-ascorbic acid (DHA) in 2 M phosphate buffer after 1 h (b) (a pD-value of 5.6 has been used in this case for stabilization), after 2 h (pD 7.4) (c), and after 14 h (pD 7.4) (d). DKG represents 2,3-diketo-L-gulonic acid, TKVA 3,4,5-trihydroxy-2-keto-L-valeraldehyde, and EASC L-erythroascorbic acid.

Table I. pH-dependence of the ^{13}C chemical shifts δ (in ppm from TMS) of 2,3-diketo-L-gulonic acid (a) and 3,4,5-trihydroxy-2-keto-L-valeraldehyde (b).

a) 2,3-diketo-L-gulonic acid			
	pD 7.4	pD 2.9	$\Delta\delta$
C-1	177.53	176.70	− 0.83
C-2	97.96	98.31	+ 0.35
C-3	97.41	97.22	− 0.19
C-4	77.14	76.94	− 0.20
C-5	71.15	70.84	− 0.31
C-6	64.89	64.92	+ 0.03

b) 3,4,5-trihydroxy-2-keto-L-valeraldehyde			
	pD 7.4	pD 2.9	$\Delta\delta$
C-1	216.28	218.82	+ 2.54
C-2	176.25	167.09	− 9.16

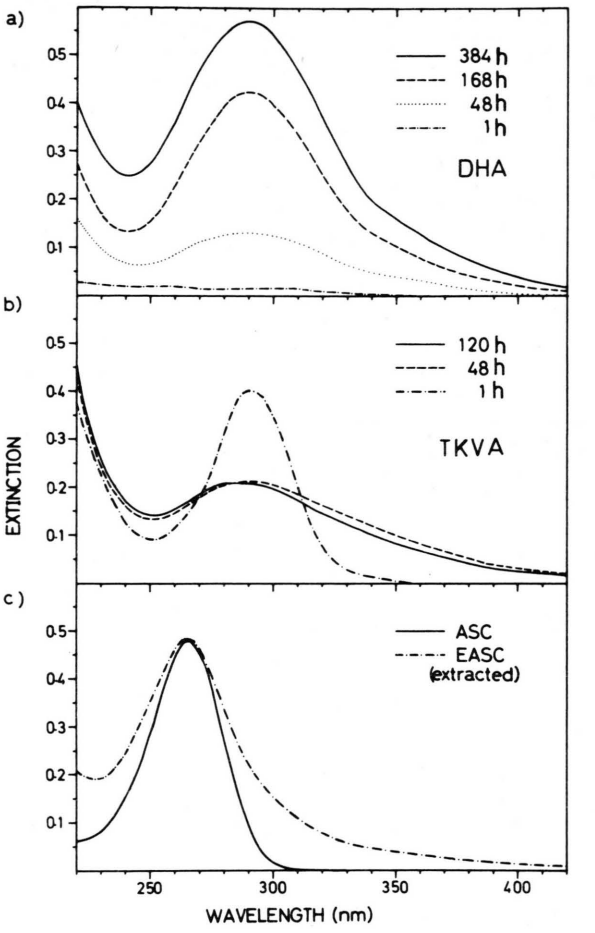

Fig. 3. Optical spectra in acetate buffer (pH 6.0) of dehydro-L-ascorbic acid (DHA) (0.33 mM) taken after 1, 48, 168, and 384 h (a), of synthesized 3,4,5-trihydroxy-2-keto-L-valeraldehyde (TKVA) (0.33 mM) taken after 1, 48, and 120 h (b), and of sodium-L-ascorbate (ASC) (0.037 mM) and of acetone-extracted L-erythroascorbic acid (EASC) (the same solution as used for Fig. 1 f) (c).

that of C-4 of ASC, as observed in the corresponding proton NMR spectrum; the C-4 and C-5 resonances are located close together. The resonances at 216.3 and 176.3 ppm represent a part of the molecule which cannot be detected by the ^1H-NMR method. These resonances have to be assigned to C-1 and C-2, representing a carbonyl group associated to a COH group by a double bond. This assignment is confirmed by the pH-dependence of the C-2 resonance (Table I b), which is similar to that of C-3 of ASC [20]. Hence, it may be concluded that an α-ketoaldehyde is formed by the decarboxylation of dihydrated DKG, *i.e.* that the hydrated TKVA, formed primarily, is dehydrated. It might also be possible that DKG is dehydrated first followed by a decarboxylation resulting in TKVA [13]. The formation of a similar pentosone has also been suggested [10]. However, this form of DKG could not be identified in the spectrum (Fig. 2 c). The downfield shifts of the H-3 and C-3 resonances are due to the electronic resonance structure between C-1 and C-2. However, in a coupled spectrum (not shown in Fig. 2 c) no coupling of C-1 with any directly bound proton could be observed. Thus, a tautomeric structure of TKVA has to be assumed with a ketone group at C-1 and a dissociable hydroxyl group at C-2, a conformation which has also been described for methylglyoxal [21].

As shown in Figs. 1 e and 2 d, in addition to TKVA two other substances seem to be present. If the samples were extracted by acetone [13], only the resonances of TKVA disappeared (s. ^1H-spectrum, Fig. 1 f). The other two substances were identified as ASC and EASC, using paper-chromatography. Since their structures and physico-chemical properties are very similar, they are very difficult to separate chemically. Consequently, the corresponding chemical shifts of C-5 and C-6, and of C-3 and C-4 are very similar (Figs. 2 a and 2 d). The chemical shift of C-5 of EASC which corresponds to that of C-6 of ASC shows a triplet in the proton coupled spectrum (Fig. 4). From these results, it seems to be evident that DKG is unstable like DHA. It is decarboxylated to TKVA and oxidized further to EASC.

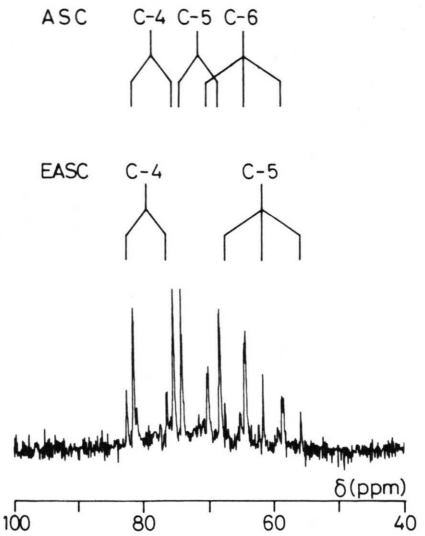

Fig. 4. Proton coupled ^{13}C-NMR spectrum of an extract from the solution of Fig. 2 d.

The degradation described can also be followed by optical spectra (Figs. 3 a and b). The absorption band at 290 nm, presumably similar to that of a pentosone [10], may be related to TKVA, and that near 260 nm, seen only as a shoulder in Figs. 3 a and b, may correspond to both EASC and ASC, as shown with higher intensity in the extracted solution (Fig. 3 c).

The regeneration of ASC in the degradation pathway of DKG can be explained by the assumption that it oxidizes its own degradation product TKVA to EASC and is reduced, thereby, to ASC (Fig. 5 c).

Naturally, DKG can also be reduced to ASC by other reductants, *e.g.* OH^- ions, under highly basic conditions. This can be seen in the ^{1}H- and ^{13}C-spectra of Fig. 6. However, in the neutral pH-range this reaction may be neglected.

Therefore, it might be concluded, that DKG is not decarboxylated to L-xylonic and L-lyxonic acid, as it has been assumed formerly [7 – 9], but rather to a tautomeric structure of TKVA which, then, is oxidized to EASC. The tautomeric equilibrium of TKVA may depend on the medium, and some other structures may also be possible under conditions different from those used in our experiments [14]. Furthermore, the concentrations of DKG and TKVA as well as the production of ASC and EASC should depend on the reaction rates of the decarboxylation and dehydration processes relative to those of the redox reactions. Therefore, the decomposition of DHA in aqueous solution, as summarized in Fig. 7, is, of course, dependent on changes of pH, temperature, or other factors as well.

It is known that DHA and DKG are inhibitors of cell growth and this has been explained by their structural similarity to glyoxals (α-ketoaldehydes) which have been described by Szent-Györgyi *et al.* as "retines" [22]. These substances inhibit cell division without damaging the cells. These so-called retines are described as transformable to non-inhibiting substances by the glyoxalase enzyme system [23 – 27] or by α-ketoaldehyde dehydrogenase [28]. Whereas these systems are found in all

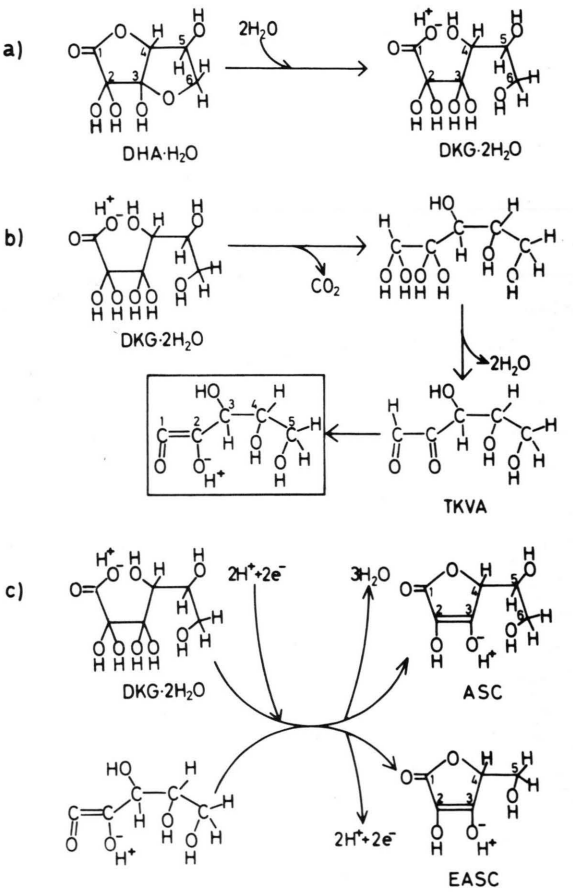

Fig. 5. The reaction mechanisms of hydrolysis of dehydro-L-ascorbic acid (DHA) to 2,3-diketo-L-gulonic acid (DKG) (a), of decarboxylation of DKG to 3,4,5-trihydroxy-2-keto-L-valeraldehyde (TKVA) (b), and of oxidation-reduction of DKG and TKVA to L-ascorbic acid (ASC) and L-erythroascorbic acid (EASC).

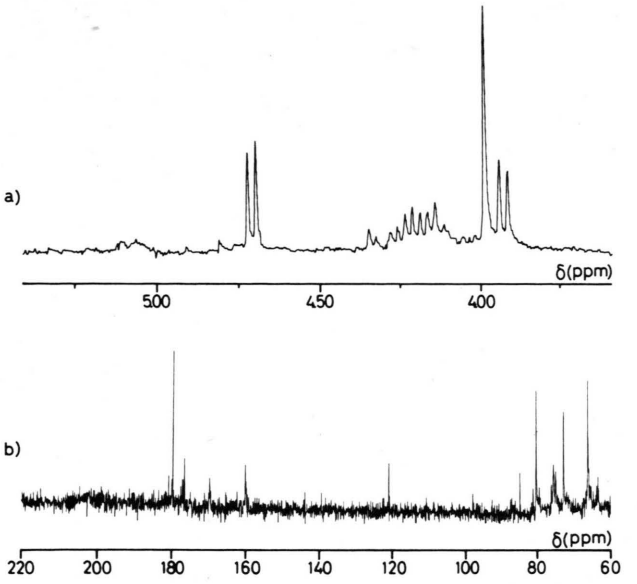

Fig. 6. ^{1}H-NMR spectrum of 50 mM dehydro-L-ascorbic acid (DHA) in pD 10 taken after 2 weeks (a) and proton decoupled ^{13}C-NMR spectrum of 500 mM dehydro-L-ascorbic acid measured in pD 3 after 6 h in pD 10 (b).

organisms and nearly all tissues, their biological substrates are not known yet [25].

We suggest that the α-ketoaldehyde TKVA as formed from DHA by degradation serves as a substrate of the glyoxalase system. As shown by Szent-Györgyi [25], the glyoxals $C_3H_4O_2$ (*e.g.* methylglyoxal) to $C_{13}H_{24}O_2$ are active cell inhibitors with the highest efficacy for compounds with six C-atoms. TKVA (5 carbons) is near that optimum. Thus, this substance may be an effective inhibitor of cell proliferation and presumably also responsible for a similar activity which has been ascribed to DHA and DKG formerly [22]. Hence, vitamin C in its reduced (ASC) and oxidized (DHA) forms may not only serve as an ubiquitous redox system in living cells, but might be involved in other fundamental biological functions due to some of its degradation products.

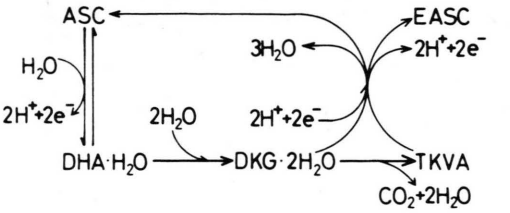

Fig. 7. A model for the degradation and regeneration of L-ascorbic acid (ASC) via dehydro-L-ascorbic acid (DHA), 2,3-diketo-L-gulonic acid (DKG), and 3,4,5-trihydroxy-2-keto-L-valeraldehyde (TKVA) to L-erythroascorbic acid (EASC) and L-ascorbic acid (ASC).

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[1] E. Cameron, L. Pauling, and B. Leibovitz, Cancer Research **39,** 663 – 681 (1979).
[2] W. Lohmann, J. Schreiber, and W. Greulich, Z. Naturforsch. **34c,** 550 – 554 (1979).
[3] A. Szent-Györgyi, The living state and cancer, Marcel Dekker, New York 1978.
[4] G. Münker, Ph.D. thesis, Freiburg 1962.
[5] W. Lohmann, Z. Naturforsch. **36c,** 804 – 808 (1981).
[6] H. Sapper, S.-O. Kang, H.-H. Paul, and W. Lohmann, Z. Naturforsch. in press.
[7] O. Touster, Ann. Rev. Biochem. **31,** 407–450 (1962).
[8] Y. Kagawa, J. Biochem. **51,** 134–144 (1962).
[9] J. Kanfer, G. Ashwell, and J. J. Burns, J. Biol. Chem. **235,** 2518–2521 (1960).
[10] E. Mögel, Ph.D. thesis, Karlsruhe 1968.
[11] M. C. Nath and E. P. M. Bhattathiry, Nature **176,** 787–788 (1955).
[12] E. S. West and R. E. Rinehart, J. Biol. Chem. **146,** 105–108 (1942).
[13] S. Kamiya, J. Vitaminol. **7,** 19–26 (1961).
[14] H. von Euler und B. Eistert, Chemie und Biochemie der Reduktone und Reduktonate, Ferdinand Enke Verlag, Stuttgart 1957.
[15] J. R. Holker, J. Chem. Soc. London I **1955,** 579–580.
[16] Hj. Staudinger and W. Weis, Hoppe-Seyler's Z. Physiol. Chem. **337,** 284–285 (1964).
[17] L. L. Salomon, J. J. Burns, and C. G. King, J. Amer. Chem. Soc. **74,** 5161–5162 (1952).
[18] J. Hvoslef and B. Pedersen, Acta Chem. Scand. **B33,** 503–511 (1979).
[19] C. J. Pouchert and J. R. Campbell, The Aldrich Library of NMR spectra, Aldrich Chemical Company, USA, 1974.
[20] R. Matusch, Z. Naturforsch. **32b,** 562–568 (1977).
[21] E. Racker, J. Biol. Chem. **190,** 685–696 (1951).
[22] J. A. Edgar, Nature **227,** 24–26 (1970).
[23] H. Otsuska and L. G. Együd, Curr. Mod. Biol. **2,** 106–110 (1968).
[24] A. Szent-Györgyi, L. G. Együd, and J. A. McLaughlin Science **155,** 539–541 (1967).
[25] L. G. Együd and A. Szent-Györgyi, Proc. Nat. Acad. Sci. USA **55,** 388–393 (1966).
[26] L. G. Együd and A. Szent-Györgyi, Proc. Nat. Acad. Sci. USA **56,** 203–207 (1966).
[27] A. Szent-Györgyi, Science **149,** 34–37 (1965).
[28] C. J. Monder, J. Biol. Chem. **242,** 4603–4609 (1967).