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Review

Dehydroascorbic acid

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Abstract

Dehydroascorbic acid (DHA) is an important, interesting but somewhat enigmatic compound in biological systems. DHA has many unique properties that set it apart from ascorbic acid (AA), and DHA has functions that may be very important beyond that in the AA:DHA cycle. Future studies should help to better clarify chemical activity of DHA and related products that form from DHA, as well as to highlight the role DHA plays in normal cellular homeostasis.

Keywords: Reviews; Ascorbic acid; Dehydroascorbic acid

Contents

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1. Introduction

Dehydroascorbic acid (DHA), *threo*-2,3-hexodiulosonic acid-γ-lactone, (CAS 490-83-5) FW 174.1, is the reversibly oxidized form of ascorbic acid (vitamin C, AA). Both DHA and AA are important compounds in various dietary components. The standard representative structures of AA and DHA are shown in Fig. 1A and B. The structure labeled DHA in Fig. 1B may not be the best representation of this compound. In water, DHA is predominantly found as a hydrated hemiacetal [1] (Fig. 1C) whereas it is a dimer in the crystalline state [2] (Fig. 1D).

The formation of DHA from AA is currently felt to be the most important reaction that AA undergoes and gives AA much of its known chemical properties and physiological activity. Up until the last few decades, DHA was felt to be a product of AA oxidation rather than a species with its own important chemical and biological characteristics. Although both DHA and AA are antiscorbutic when taken orally [3,4], DHA has several properties that set it apart from AA. DHA is more reactive and unstable in solution than AA. Furthermore, DHA can be reduced to AA or readily hydrolyzed and oxidized, making it both an oxidizing and reducing agent. DHA clearly has different biological roles than AA in cell culture systems. This report reviews some of what is known about the chemistry, analysis, metabolism and biological effects of DHA.

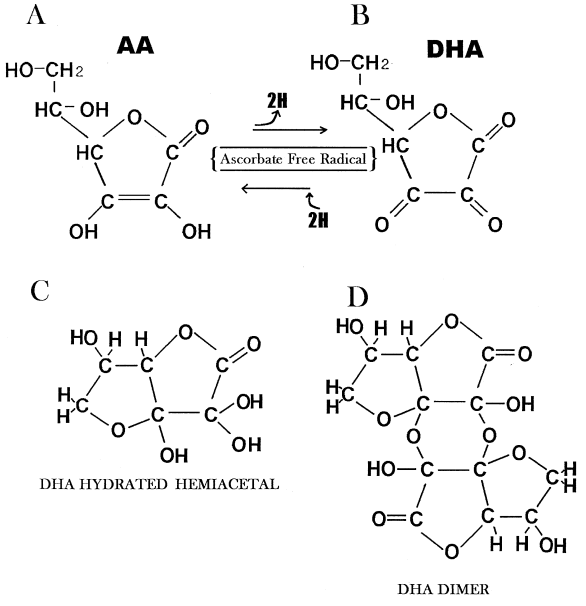

Fig. 1. The structures and the reversible reaction between AA and DHA (A, B). The hydrated hemiacetal of DHA (C) and the crystalline structure of DHA (D) are also shown.

2. Relation of ascorbic acid to dehydroascorbic acid

The relation of DHA to AA is critical to most reports concerning DHA. The oxidation of AA to DHA provides two hydrogen atoms that can be used in the reduction of biologically significant compounds (Fig. 1). Since the oxidation of AA readily occurs, it can be employed in determination of AA using electrochemical detection systems to generate a signal from current or voltage changes. The reaction in which AA is converted to DHA is a two-step process that generates free radicals, so that the conversion of AA to DHA can potentially promote both reduction and paradoxically, oxidation in a system.

The reduction of DHA by various agents also readily occurs, and this reduction leads to an increase in AA content. The measurement of AA before and after reduction is a common method to indirectly determine DHA in a sample. Due to this close interrelation of DHA to AA, the discussion of any property or measurement of DHA will frequently refer back to properties of AA in that same system.

2.1. Methods for oxidizing ascorbic acid to dehydroascorbic acid

DHA can be prepared from AA through a variety of oxidation reactions employing halogens, hydrogen peroxide, ferric chloride and other agents. A common method employs the use of bromine water [5,6]. Alternatively, one can use ascorbate oxidase [7,8]. There are advantages and disadvantages to different systems, but the simplicity of bromine–water appears to make this method preferable when it can be employed.

2.2. Methods for reducing dehydroascorbic acid to ascorbic acid

Thiol-containing species readily convert DHA to AA. For example, in vitro studies have employed a variety of sulfhydryl compounds including dimercaptopropanol [9–11], mercaptoethanol [12,13], homocysteine [14,15], glutathione [16,17], and dithiothreitol [18,19].

In vivo, DHA is transported into cells, and instantly reduced to AA through a variety of enzymatic processes that involve glutathione (GSH), NADH or NADPH as a reductant. The enzymes involved include thioredoxin reductase, glutathione: dehydroascorbate oxoreductase, and 3-α-hydroxystreroid dehydrogenase [20–22].

2.3. Ascorbate free radical

As mentioned above, oxidation of AA or reduction of DHA yields free radical species referred to as the ascorbate free radical (AFR) (Fig. 1) or the semidehydroascorbate free radical. Alternatively, AFR can form in a disproportionation reaction between AA and DHA to yield 2-AFR and has an equilibrium constant of 10^{-16} to 10^{-17} at pH 7.4 [23]. The AFR has a long half-life and can be easily detected by Electron Spin Resonance/Electron Paramagnetic Resonance (ESR/EPR) (a doublet at $g=2.0054$, $aH=0.188$) in studies in which there is oxidation or reduction of the AA/DHA pair [24–27]. This free radical species is felt to be one of the major causes of the pro-oxidant effect ascribed to AA/DHA containing systems.

2.4. Hydrolysis of dehydroascorbic acid

At physiological pH, DHA is rapidly hydrolyzed to diketogulonate (DKG). There are some data to suggest that this hydrolysis is irreversible in vivo. For example, although both AA and DHA are antiscorbutic, DKG is not [28]. Some of these studies were carried out over 50 years ago, and the analytical methods employed to determine it was in fact DKG which was administered, were not as sophisticated as what is currently employed [28].

There are data to suggest that the hydrolysis of DHA may actually be partially reversible. For example, Hydro Iodic acid (HI) has been shown to reduce DKG [28]. Our laboratory has observed the formation of AA from solutions where DHA was not apparent following the incubation of the solutions with thiol containing compounds [13]. We have also observed the exchange of ^{18}O on C_1 of DHA but not AA, suggesting spontaneous hydrolysis and dehydration with lactone ring opening and closing [29]. It may be that some of the reported ''irreversibility'' of the hydrolysis may be due to experimental conditions. If the hydrolysis is thermodynamically driven at physiologic pH, it may appear to be ''irreversible'' based on feeding experiments at that same pH. Furthermore, mammalian plasma appears to possess a system that rapidly promotes the hydrolysis of DHA to DKG and blood bicarbonate levels seem important in this regard [30]. This tendency to hydrolyze in physiologic pH does not mean that hydrolysis cannot be prevented or reversed under the appropriate conditions. There are mechanisms that may attempt to prevent hydrolysis. For instance, GSH prevents the delactonization of DHA [31].

What may be of more interest is that the potentially rapid hydrolysis of DHA to DKG raises questions as to what species are present in experiments where DHA has been added. It may be that much of what is attributed to DHA is actually due to DKG or even other species that arise from DKG (see below).

2.5. Further degradation of hydrolyzed dehydroascorbic acid

Under conditions of oxidant stress, DHA, after being hydrolyzed to DKG, rapidly degrades to 5- and 4-carbon species. In vitro, the primary reactions appear to be a sequence of (irreversible) α-decarboxylations to threonate [32]. Threonate, on the other hand, is quite stable in an oxidative environment [33]. The successive decarboxylation reactions starting with DKG may have important antioxidant effects in that these reactions consume oxygen as discussed below. In biological systems, DKG may undergo decarboxylation and reduction to other 5-carbon species including lyxonic acid, xylonic acid, lyxose and xylose [34]. The compounds formed during biological degradation appear to depend on a

variety of factors including the species studied, the amount of ascorbate administered, and the state of ascorbate sufficiency at the time of study.

2.6. Antioxidant properties of dehydroascorbic acid

Although AA is the more highly reduced species in the AA:DHA pair, DHA appears to have antioxidant properties of its own, beyond that of AA. It has been shown for instance, that DHA is better than AA at protecting low-density lipoprotein from oxidation by cupric ion [35]. Investigators have shown that $^{14}CO_2$ liberation from $^{14}C_1$-DHA correlates with antioxidant activity [36]. Liquid chromatographic–mass spectrometric studies have shown evidence of rapid, successive oxidative decarboxylation reactions that take place following hydrolysis of DHA, with one oxygen atom consumed for each decarboxylation that occur [32]. It is tempting to hypothesize that the reversible oxidation of AA to DHA occurs under milder conditions, but in a situation of marked oxidative stress, DKG is formed from DHA, and irreversibly utilized (during oxidative decarboxylation) in a final attempt to remove toxic oxygen species. These studies suggest that there are reactions that take place both in vivo and in vitro which are currently not well defined, but which may have importance in the overall redox state of living organisms.

3. Analysis and measurement of dehydroascorbic acid

A variety of tools have been used to study the structure and chemical properties of DHA, and many of these tools are applied in various analytical methods which are in current use.

3.1. Crystallographic studies

In contrast to AA [37], the molecular structure of DHA was shown to be dimeric by crystallographic studies when the crystals were grown out of non-aqueous solution [2,38]. The structure appears to have a central six-membered dioxane ring surrounded by four rings of five members each. The structure inferred by the crystallographic studies is shown in Fig. 1D.

3.2. Infrared studies

The similarities and differences between DHA and AA when they are not in solution can be further appreciated by observing the infrared spectra of the two compounds [39]. The infrared spectrum of DHA (nujol mull) shows strong carbonyl absorbance at 1784 cm^{-1} (C=O), and at 3297 and 3618 cm^{-1} (O-H), and several other absorption bands (for example, C-H at 2925 cm^{-1}, 1462 cm^{-1}). In contrast, ascorbic acid shows some C=O stretch at 1754 and stronger absorbance at 1673 cm^{-1}, as well as O-H stretch at 3626, 3410, 3316 and 3217 nm, but the same broad peaks at 2924 cm^{-1} and 1460 cm^{-1}.

3.3. Ultraviolet studies

DHA in solution absorbs UV light well at 185 nm, but has little absorbance above 220 nm. This is in contrast to AA that strongly absorbs at 265 nm [19,40,41]. The difference in absorption spectra is the basis for some assays for DHA [19] and will be discussed in more detail below. In addition to direct ultraviolet absorption spectra, DHA can be derivatized with reagents such as benzamidine or dimethyl-*o*-phenylenediamine to form fluorescent compounds [42,43] in which samples containing derivatized DHA can be excited at one wavelength and the fluorescence determined at another.

3.4. ^{13}C nuclear magnetic resonance

There is a shift difference of approximately 5 ppm for [1-^{13}C]AA compared to [1-^{13}C]DHA, and a shift difference of 19 ppm between [2-^{13}C]AA and [2-^{13}C]DHA [44]. The chemical shifts for AA and DHA using ^{13}C nuclear magnetic resonance (NMR) have been shown to contain splitting which is a function of the osmolarity of the medium and the concentration of the substrate. Therefore, ^{13}C-NMR can be used to assess real time transport phenomena, although the sensitivity for DHA does not allow direct observation without the addition of supplemental DHA [44].

3.5. Mass spectrometry

3.5.1. Electron impact mass spectrometry

Electron impact mass spectrometry following deriviatization in *tert.*-butyldimethylsilyltrifluoroacetamide provides relatively specific spectra and information about the structure that can be exploited using isotopically labeled DHA. DHA has two derivatizable sites in contrast to AA that has four. Therefore the [M+] of DHA is m/z 402 compared to 632 for AA, and the predominant major ion [M−57]+ for DHA is m/z 345. AA also shows the [M−57]+ ion at m/z 575, but shows several other ions of similar abundance [8]. The electron impact spectra of both *tert.*-butyldimethylsilyl (TBDMS)-derivatized DHA and AA show decarboxylated species (m/z 301 and m/z 531). However, the electron impact generated spectrum of derivatized DHA does not as readily show structural detail as it does for derivatized AA [8]. As mentioned, crystallographic studies have determined that DHA shows a tendency to dimerize [2,38]. Electron impact mass spectrometry coupled to gas chromatography appears to identify these dimers readily (Fig. 2), whereas AA dimers are not found [13].

3.5.2. Electrospray mass spectrometry

The mass spectra obtained from DHA in solutions are somewhat complicated, since DHA forms a hydrated hemiacetal (Fig. 1, bottom left) and depending on the pH DHA is prone to hydrolyze [1]. Negative ion electrospray spectra do not show a peak at m/z 173 for DHA containing solutions [32] (Fig. 3). There is also a marked difference in negative ion-electrospray spectra of DHA at pH 4 compared to pH 7, suggesting rapid and spontaneous hydrolysis at the higher pH (Fig. 3) [32].

3.6. Electrochemical detection

AA is relatively reactive and easy to detect in coulometric and amperometric systems. As mentioned above, coulometric electrochemical detection of AA before and after reduction is commonly used to determine reduced and total AA levels, and the difference being ascribed to DHA [9,18,45–57].

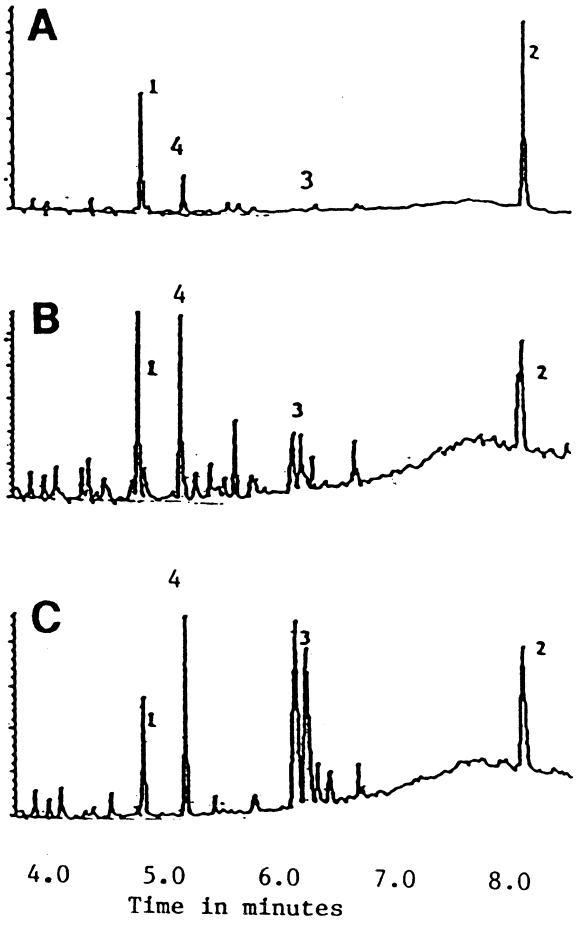

Fig. 2. Gas chromatograms of derivatized sample of DHA during incubation with water. (A) Derivatized dry powder; (B) incubation in water for 15 min followed by drying and derivatization; and (C) incubation in water for 1 h followed by drying and derivatization. The monomer is labeled (1) and the dimer is labeled (2). The peak labeled (3) is consistent with the hydrolyzed species, DKG. The peak labeled (4) is a recurrant product. (From Ref. [13] with permission).

3.7. Separation methods

There are several chromatographic methods employed to separate AA from DHA, and to characterize DHA. Reversed phase high-performance liquid chromatography (HPLC) using octadecyl columns (C_{18}) are commonly employed [18,19,58–62] (Fig. 4). Aminopropylsilane columns (NH_2) also appear to provide good separation [1,63]. Fused-silica (capillary electrophoresis) has been reported [14,15,64].

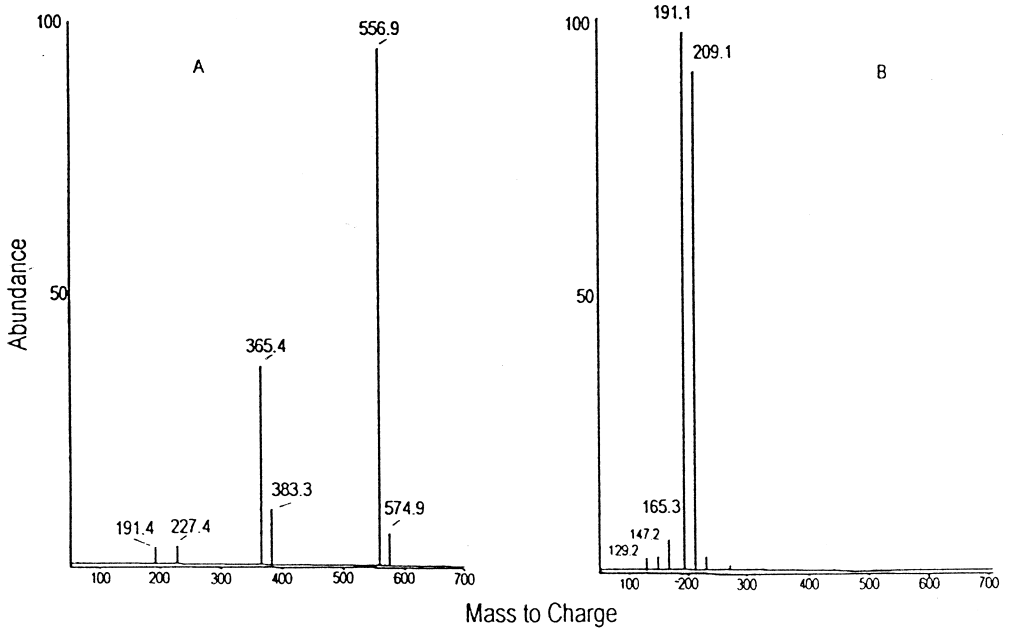

Fig. 3. The electrospray-negative ion mass spectra of DHA in water at pH 3 3 (A) and pH 7 4 (B). (From Ref. [32] with permission.) The ion m/z 365 in (A) is the negative ion of 2DHA:H_2O clusters (174+174+18−1) while the ion m/z 556 9 is 3DHA:2H_2O. This ion is gone at pH 7.4 and is replaced by m/z 191 and m/z 209 in (B) which is likely represent DKG and DKG:H_2O.

Excellent separation between AA and DHA can be obtained by gas chromatography on derivatized samples using dimethylsiloxane columns [8,13].

4. Biology of dehydroascorbic acid

4.1. Dehydroascorbic acid in plants

Although DHA is detectable in plant tissues, it is controversial whether or not DHA serves a physiologic role. Some authors feel that DHA will not accumulate in plant cells, and therefore, much of the DHA measured in plants is artifactual due to the creation of DHA during sample processing [65]. The role played by DHA in plant metabolism is somewhat controversial as evidenced by recent commentaries [66,67]. Data has recently been presented which suggests that uptake of DHA is preferred over AA in plant membranes, and that a DHA:AA exchange mechanism exists in plants [68]. This is particularly interesting in light of the DHA:AA transport studies in animal cells discussed below.

4.2. Membrane transport of dehydroascorbic acid

Uptake of dehydroascorbate across membranes appears to take place through specific transporters and is separate from membrane transport of AA. For example, uptake of DHA is inhibited by certain monosaccharides, but is not inhibited by AA. Studies using plant [68] and animal tissues [69–71] show that DHA transport is more efficient than AA transport. In animal tissues, DHA transport utilizes hexose transporters of the GLUT-family [6,72]. Microsomes and cell membranes appear to use similar types of transporters for DHA uptake. Expression cloning in Xenopus oocytes has clearly demonstrated involvement of both GLUT1 and GLUT3 in this process [6]. These studies are intriguing, in that they suggest very efficient recycling of AA following oxidation at the cell surface. The in vivo role of these transporters is not totally defined in either organisms which synthesize AA (such as the rat) compared to organisms which require AA in the diet (such as primates). However, as seen above, a large body of literature now exists to show that

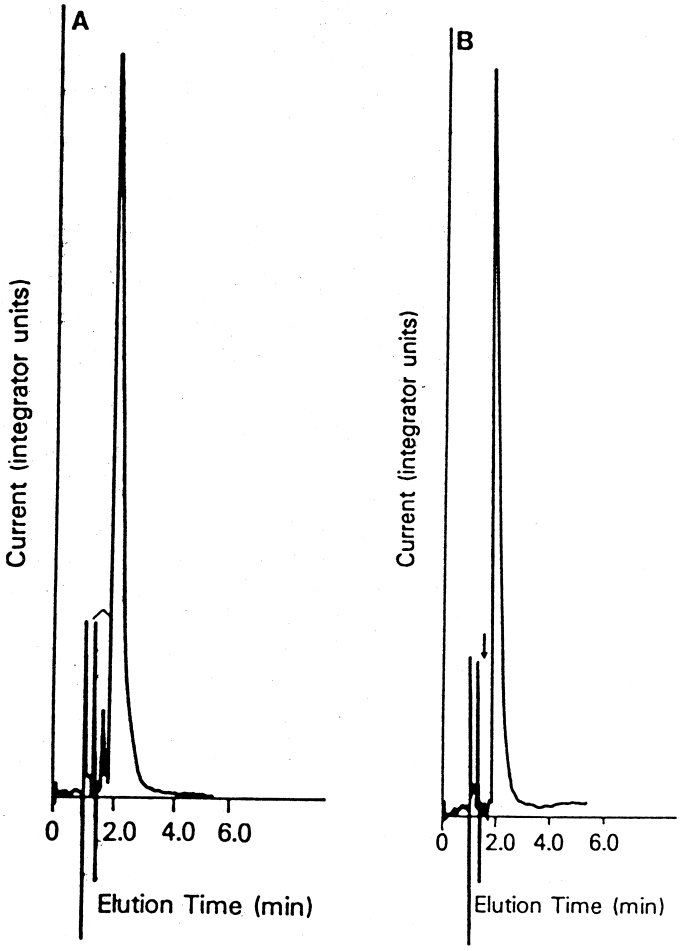

Fig. 4. HPLC on an octadecyl reversed-phase column, before and after ascorbic oxidase. (From Ref. [48], used with permission). The small arrow marks the time of elution of AA.

DHA is a preferentially transported species in AA metabolic pathways.

4.3. Dehydroascorbic acid recycling

It appears that DHA, once transported into a cell, is rapidly reduced back to AA. This reduction is important in maintaining adequate cellular levels of AA. Glutathione dependent enzymes are the most important species in this regard [20,22]. In addition, DHA reduction from rat liver cytosol has been shown to occur through 3-α-hydroxystreroid dehydrogenase [21].

The ready reduction of DHA to AA likely explains the antiscorbutic effect of DHA. The observed decreased antiscorbutic potency of DHA compared to AA [4] can be explained by the rapid hydrolysis of DHA to DKG [42].

As noted above, DHA rapidly hydrolyzes to DKG in physiologic solutions, so that the rate of uptake and the rate of DHA degradation will both influence the amount of DHA that ultimately crosses a membrane. It seems likely that AA, which is oxidized to DHA in the immediate vicinity of the cell membrane, is more likely to be recycled than DHA that is formed further away from membranes in the extracellular compartment. Measurements of DHA are likely therefore to consist of two pools – one of

which is cycled back to AA through a membrane, and another, which is hydrolyzed to DKG and further oxidized to species such as threonate.

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