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Dehydroascorbic acid undergoes hydrolysis on solubilization which can be reversed with mercaptoethanol

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Abstract

Using gas chromatography–mass spectrometry, we have examined the effect of solubilization, cupric sulfate oxidation and mercaptoethanol reduction of dehydroascorbic acid. During solubilization, dehydroascorbic acid spontaneously hydrolyzed the lactone ring. The reduction of the dehydroascorbic acid hydrolysis product using mercaptoethanol resulted in the formation of ascorbic acid, suggesting that sulfhydryls are able to effect ring closure of the dehydroascorbic acid hydrolysis product. These data suggest that studies of dehydroascorbic acid should be interpreted with caution, since properties attributed to dehydroascorbic acid may actually be due to another chemical species.

Keywords: Dehydroascorbic acid; Mercaptoethanol; Ascorbic acid

1. Introduction

Ascorbic acid (AA) functions as a an antiscorbutic agent and is a cofactor in a variety of enzymatic processes [1–7]. Furthermore, AA is an excellent antioxidant in vitro and in vivo [1–7]. When exposed to oxidant stress, AA is reported to be reversibly converted to dehydroascorbic acid (DHA) [1,8]. This reversible process is responsible for the antioxidant effect attributed to AA. With continued oxidative stress, DHA is degraded to 2,3-diketogulonic acid and then to over 50 species containing five or less carbons [1,8–15]. Work done over 50 years ago suggested that DHA is quite unstable in water, and that the lactone ring spontaneously opens in aqueous solution [16,17]. Solutions in which ring opening occurred were found to have lost their antiscorbutic effect [17]. However, purified barium precipitates of 2,3-diketogulonic acid regained their antiscorbutic activity when reduced with HI [17], suggesting that the lactone ring opening may be irreversible in vivo, but could be reversed using stronger agents in vitro.

Although AA is commonly ingested for its antioxidant properties, the role of AA as an in-vivo antioxidant has been confused by data suggesting that AA also functions as a free-radical prooxidant catalyst in the presence of transition metal ions, generating hydrogen peroxide and hydroxyl radicals through the Fenton reaction [8,18]. Moreover, experimental models of oxidation injury may rely on

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reactions involving peroxidation [5,7,19,20] or transition metal-ion-induced oxidation [19,21,22]. A recent study [19] reported that DHA but not AA, prevented cupric-ion-induced low-density lipoprotein oxidation, but that AA better protected low-density lipoprotein from chemical peroxidation using azobis 2-aminopropane hydrogen chloride. These data are interesting since, AA should be readily oxidized to DHA [1,8] and solutions of DHA may consist primarily of 2,3 diketogulonic acid or other degradative products [16,17].

Much of the recent information which has been gathered in studies of DHA have relief on HPLC electrochemical analysis, in which DHA content can be inferred by measuring the AA content in a solution, gently reducing the solution with sulfhydryls such as homocysteine [19] or dimercaptopropanol [23,24] and remeasuring the AA content, based on the assumption that only DHA is reduced to AA. We have recently reported a GC–MS stable isotope dilution method to determine the content of AA and DHA in solutions [25,26], which has the advantage of measuring ions directly to derivatized DHA, but has the potential to overestimate the DHA content in solution if the DHA standard (which all measurements are made relative to) has formed other species, since the actual DHA content in the standard solution would be actually smaller than the concentration ascribed to it.

We have assessed the changes in DHA which occur during water solubilization, copper-ion-induced oxidation, and sulfhydryl reduction using GC–MS. We have found that DHA solutions exhibit complex behavior and suggest that many of the properties ascribed to DHA may actually be due to other species which form from AA and DHA.

2. Experimental

Reagents and chemicals of highest grade were obtained from Sigma (St. Louis, MO, USA), Aldrich (Milwaukee, WI, USA), Kodak (Rochester, NY, USA) and Fisher (Pittsburgh, PA, USA). Argon and nitrogen were purchased from General Air Service and Supply (Denver, CO, USA). Distilled water was purchased from Deep Rock distillers and King Soopers (Denver, CO, USA). DHA was purchased from ICN Biochemicals (Cleveland, OH, USA), and DHA dimer was purchased from Fluka (Ronkonkoma, NY, USA). N-methyl-N(*tert.*-butyldimethylsilyl) trifluoroacetamide (TBDMS) was obtained from Regis (Morton Grove, IL, USA). [$^{13}C_6$]AA (98% ^{13}C) and [6,6$^2H_2$]AA (98% ^2H) were obtained from MSD Isotopes (Montreal, Canada).

Where stated, water for solubilization was degassed by running either nitrogen or argon through the water at 1–2 l/min for 30 min, and then sealing the water under an atmosphere of either nitrogen or argon. Incubations and solubilization were performed at 22°C in plastic conical tubes. Reactions were otherwise carried out on 5–20 μl aliquots in glass autosampler vials containing solutions of AA and DHA (final concentration 3–5 m*M*). 2-Mercaptoethanol (BME) was added to solutions at a final concentration of 700 to 3000 m*M* for reduction reactions with incubation at 22°C for 0.1 to 1 h, while oxidation reactions were carried out by adding cupric sulfate at a final concentration of 20–100 μM for 0.1 to 72 h following which the samples were dried at 22°C for 30 min to 2 h using a Savant (Farmingdale, NY, USA) vacuum centrifuge system. Controls without BME were run in parallel with each BME reaction.

The dried aliquots were derivatized by adding 15 μl of TBDMS and 30 μl of acetonitrile then incubating the capped samples for 2 h at 60°C. Two-μl aliquots were applied to a Hewlett-Packard (Avondale, PA, USA) 5890 gas chromatograph. Gas chromatography was carried out through a Supelco (Bellfonte, PA, USA) 10-m fused-silica capillary column (0.25 mm I.D.) using a temperature ramp of 30°C/min from 80 to 300°C with helium as a carrier, and mass spectrometry was performed on a Hewlett-Packard 5971A mass spectrometer. The scan mode was used to obtain full spectra (including the $[M-57]^+$ ion) [25–27] and appropriate retention times. Analysis was carried out by scanning and by selected-ion monitoring at the respective $[M-57]^+$ ions. The electron multiplier was at 1650–2100 V. Table 1 shows the predicted ions for AA, DHA and 2,3-diketogulonic acid.

Experiments were performed in duplicate or triplicate. Samples of unreacted starting material were analyzed in triplicate with each experiment. Chromatograms and spectra were compared to chromato-

Table 1
Predicted mass characteristics (mass or mass/charge) of ascorbic acid and related products

Compound	Mass	$[M]^+$ (in TBDMS)	$[M-57]^+$
Ascorbic acid	176	632	575
Dehydroascorbic acid	174	402	343
2,3-Diketogulonic acid	192	648	591

grams and spectra obtained from similar experiments using AA, [6,6$^2H_2$]AA, and [$^{13}C_6$]AA [25,26]. The mean values and standard deviations were determined on relative ion abundances where indicated.

3. Results

Fig. 1A shows the total-ion chromatogram (TIC) obtained when powdered DHA (ICN Pharm.) is derivatized and applied to GC–MS before solubilization in water, while Fig. 1B, C shows the TIC obtained when DHA is dissolved by agitation on a rotating wheel at 22°C for 30 min and 120 min prior to being dried and derivatized. Derivatized DHA powder (prior to solubilization) contains two major peaks, the first of which (eluting at 4.8 min) is consistent with DHA monomer [26]. The second peak, eluting at 8.1 min appeared to have an $[M-57]^+$ ion which was of a greater mass than the upper limits of the mass detector (*m/z* 650). However, major abundance ions of *m/z* 329 and 345 were found. In addition, an ion was also present at *m/z* 615. This peak was also found when AA, [6,6$^2H_2$]AA and [$^{13}C_6$]AA were incubated and oxidized with copper sulfate. However, ions of *m/z* 331, 347 and 619 were found when [6,6$^2H_2$]AA was used as the starting material, and ions of *m/z* 335, 351 and 627 when [$^{13}C_6$]AA was used as the starting material (Fig. 2). These data demonstrate that this product is a dimer (containing 12 carbon atoms) and are consistent with the compound eluting at 8.1 min being a DHA dimer [1], which would be expected to have an $[M]^+$ of *m/z* 804, and an $[M-189]^+$ of *m/z* 615.

This dimer-product was present in both DHA preparations prior to exposure to water, and was formed from AA in the presence of cupric ions. To determine if dimerization occurred in the aqueous

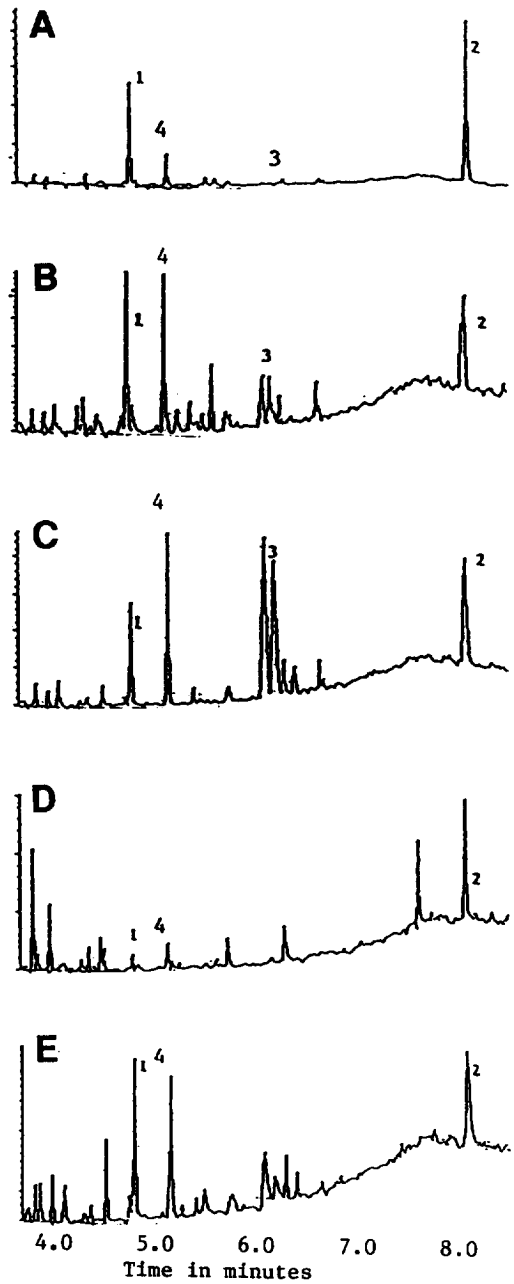

Fig. 1. Total-ion chromatograms (*m/z* 150–650) from (A–C) DHA (ICN Pharm.) and (D,E) DHA dimer (Fluka). (A) dry powder; (B) 30 min in water; (C) 120 min in water; (D) dry powder; (E) 120 min in water. The peak marked 1 is DHA monomer. The peak marked 2 is the dimer. The peaks marked 3 are consistent with isomers of 2,3-diketogulonic acid. The peak marked 4 is a DHA rearrangement product.

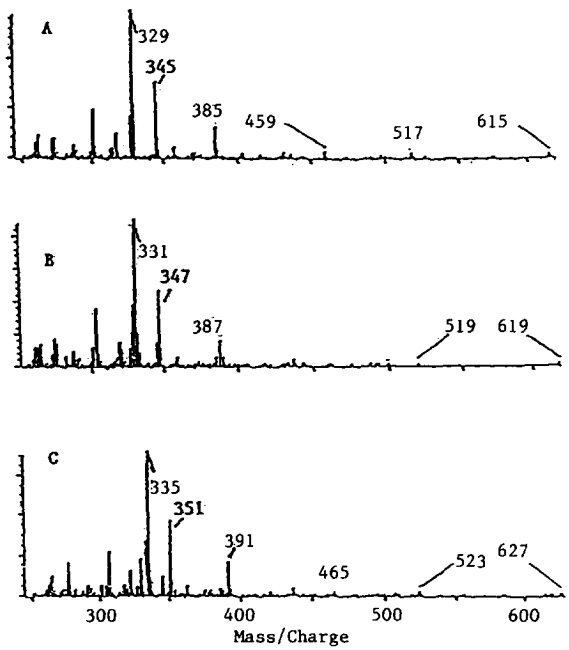

Fig. 2. Spectra of the DHA dimer eluting at 8.1 min when derived from: (A) AA or DHA; (B) [6,6$^2H_2$]AA; (C) [$^{13}C_6$]AA.

state, or if it occurred when samples were dried (i.e., drying samples prior to TBDMS-derivatization), either AA or [6,6$^2H_2$]AA was incubated with copper sulfate for 2 h following which the opposite isotopic form (either [6,6$^2H_2$]AA or AA) was added and the incubation was continued for an additional 2 h. If a stable dimer formed in the aqueous state, then two major peaks for the $[M-189]^+$ ions, *m/z* 615 (formed from DHA–DHA) and *m/z* 619 (formed from [6,6$^2H_2$]DHA–[6,6$^2H_2$]DHA) would be present, with very little product at *m/z* 617. If DHA dimerization was unstable in solution, but stable after water was removed during sample drying, prior to derivatization with TBDMS, then we would expect a normal distribution with the abundance being greatest at *m/z* 617, and similar but lesser abundances of *m/z* 615 and 619. Table 2 shows the abundances of the proposed $[M-189]^+$ ions when different isotopic mixtures of AA were incubated with cupric ions. These data are most consistent with the stable dimer forming at the end of the experiment, during sample drying.

When powdered "DHA dimer" (Fluka) was derivatized without prior solubilization, several peaks were found, including the product eluting at 8.1 min (Fig. 1D). Although poorly soluble, the supernatant of a 1 mg/ml solution obtained after 2 h of agitation of this same material also contained the peak at 8.1 min. In addition, the water solubilization appeared to lead to formation of DHA monomer (Fig. 1E).

Table 2
Formation of dehydroascorbic acid dimer based on relative abundances of $[M-189]^+$ ions from ascorbic acid and [6,6$^2H_2$] ascorbic acid during 4-h incubations with cupric sulfate

Reaction	*m/z*		
	615	617	619
A ×2 h then add B ×2 h	1.02±0.04	2.13±0.40	1.20±0.41
B ×2 h then add A ×2 h	1.39±0.10	2.36±0.42	0.84±0.13
A+B ×4 h	0.91±0.18	1.66±0.26	0.74±0.16

A=ascorbic acid; B=[6,6$^2H_2$] ascorbic acid.

As shown in Fig. 1, the solubilization of DHA monomer (ICN Pharm.) also appeared to lead to the formation of new peaks eluting at 5.2 and 6.1–6.2 min. There were no obvious differences observed in the total-ion chromatograms of solubilized DHA whether the water had been degassed and agitated in air, nitrogen or argon, suggesting that dissolved oxygen was not responsible for this change. Spectra for these new peaks are shown in Fig. 3f.

Based on the spectrum (Fig. 3A), and the formation of a similar substance from [$^{13}C_6$]AA (not shown), the product eluting at 5.2 min (Fig. 1) contains six carbons, and has an $[M-57]^+$ ion *m/z* 459. This product therefore contains three derivatizable sites and has an identical mass as DHA. These data are consistent with this substance being a conjugated rearrangement product, either 2,3,6-trihydroxy 2,4-dienoic acid lactone, or the isomer R-345 described by Kimoto et al. [15].

The products eluting at 6.1–6.2 min (Fig. 1) contained apparent $[M-57]^+$ ions of *m/z* 591, consistent with the ring opening to form 2,3-diketogulonic acid. However, the retention times (relative to A) and spectra differed from products we have previously identified with $[M-57]^+$ ions of *m/z* 591, which arise when AA solutions are incubated in hyperoxic water [25]. The peaks at 6.1–6.2 min were also found in 5 m*M* solutions of AA,

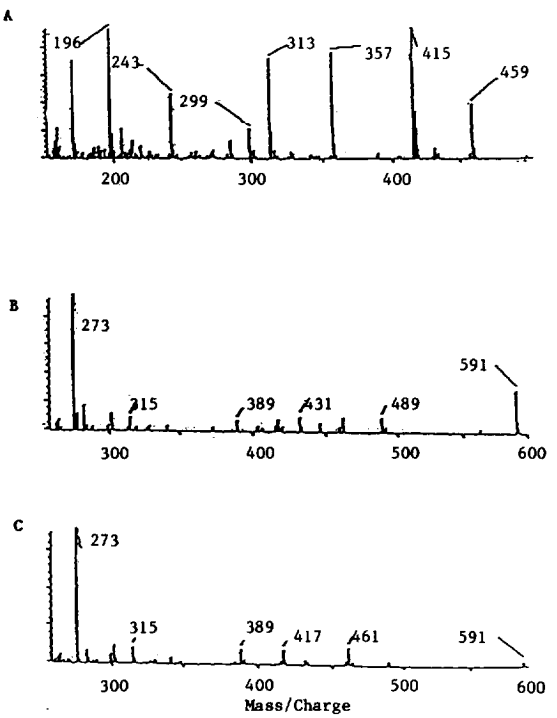

Fig. 3. Spectra of the peaks eluting at (A) 5.1 min (shown as peak 4 in Fig. 1), (B) 6.1 min, and (C) 6.2 min in solubilized DHA (shown as peaks 3 in Fig. 1).

[6,6^{2}H$_2$]AA and [^{13}C$_6$]AA when incubated in 50 μM cupric sulfate solutions for 2–72 h. These data suggested that different species could arise from AA solutions that have an underivatized M_r of 192. Since AA has a M_r of 176, and DHA has a M_r of 174, the products with a M_r of 192 could arise from either water addition to DHA or singlet oxygen addition to AA.

As shown in Fig. 4, the spectra of the peaks eluting at 6.1 and 6.2 min were essentially identical, in contrast to the spectra of the substances with a predicted M_r of 192 which arose from AA during oxygen exposure [25]. When solutions of AA and [^{13}C$_6$]AA were incubated in 50 μM cupric sulfate for 72 h (to allow both adequate time for ring opening through solubilization, and oxidant stress), both the previously described products containing m/z 591/597 [25] and the currently described products were seen (Fig. 5). The spectra and time course of formation suggest that these products are isomers of 2,3-diketogulonic acid. These data show that the formation of the particular isomers appears to depend on the conditions of oxidation or solubilization.

To test whether sulfhydryls could reduce the ring-opening product (to form A), 6 mM solutions of DHA were incubated in water at 22°C for 96 h and

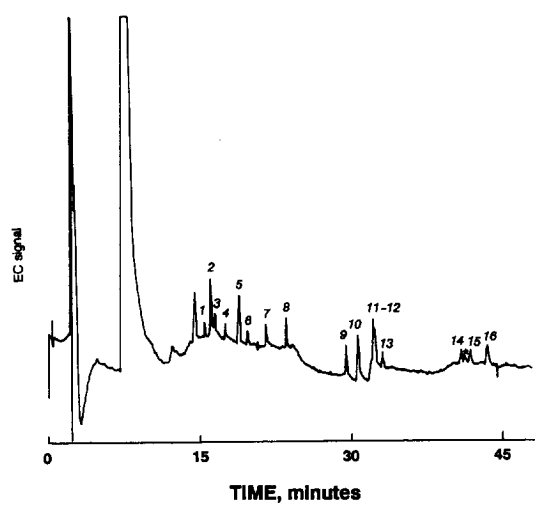

Fig. 4. Spectra of peaks eluting at 6.1, and 6.2 min after incubating (A) AA, (B) [6,6^{2}H$_2$]AA, and (C) [^{13}C$_6$]AA in 50 μM cupric sulfate for 8 h.

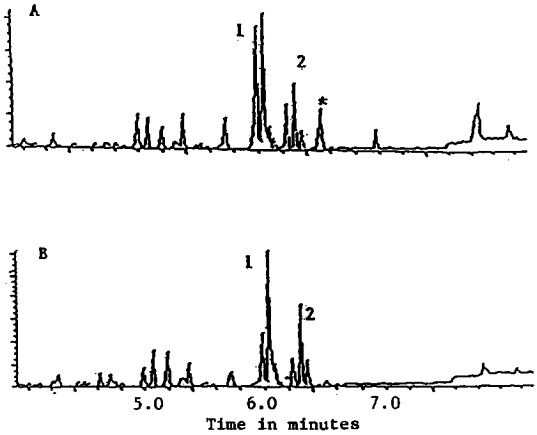

Fig. 5. Total-ion chromatograms of (A) AA, and (B) [$^{13}C_6$]AA following 72 h incubation in 50 μM cupric sulfate. The peak marked (*) in (A) is from [6,6$^2H_2$]AA added at the end of the 72-h incubation. Peaks marked 1 have an apparent $[M-57]^+$ ion of m/z 591 and spectra as shown in Fig. 4. Peaks marked 2 also have spectra with an apparent $[M-57]^+$ ion of m/z 591, but do not contain m/z 277 as a major component. Spectra for these peaks have been previously published by us [21].

an aliquot was further incubated in 700 mM BME at 22°C for 1 h. As shown in Fig. 6A, the majority of the ion current (70%) derived from solubilized DHA eluted at 6.1–6.2 min, consistent with the ring-opening product, while the rest of the ion current was attributable to DHA, DHA dimer, and the product 2,3,6-trihydroxy 2,4-dienoic acid lactone/R-345. Following incubation with BME (Fig. 6B Fig. 7), the ring-opening product, and the peaks attributable to DHA disappeared, and the majority of the non-BME ion current (65%) eluted in a peak with a retention time and spectrum identical to AA. There was no residual DHA, ring-opening product, or trihydroxydienoic acid lactone following BME reduction.

4. Discussion

DHA solutions exhibit complex behavior, making the analysis of DHA-containing solutions and the attribution of functions to any component of these solutions very difficult. Our data are in agreement with other investigators who have shown that DHA

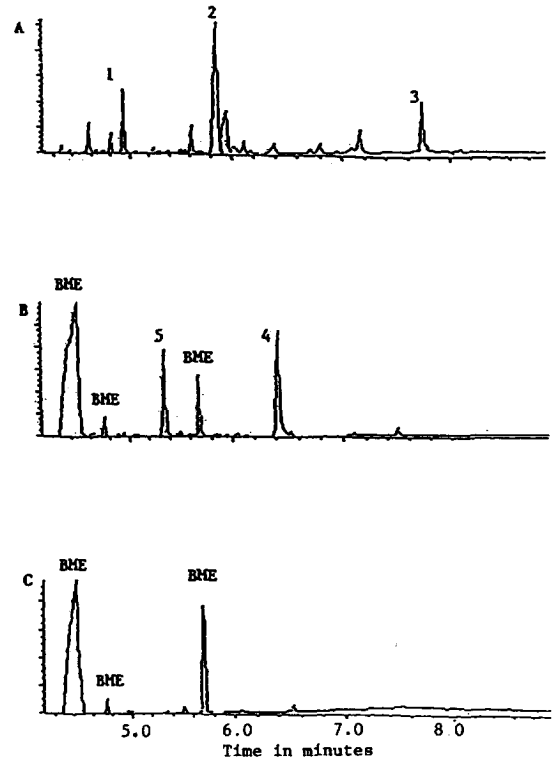

Fig. 6. Total-ion chromatograms made from: (A) solution of DHA (ICN Pharm.) incubated in water at 22°C for 97 h; (B) an aliquot of the solution of DHA removed after 96 h and then incubated for 1 h in 100 M excess of BME; (C) BME blank. The peaks in (B) derived from BME are marked. The numbers in each chromatogram give the identity of the peak: (A), 1=DHA monomer; 2=ring hydrolysis product, "2,3-diketogulonic acid"; 3=DHA dimer; (B), 4=AA; 5=unknown reduction product.

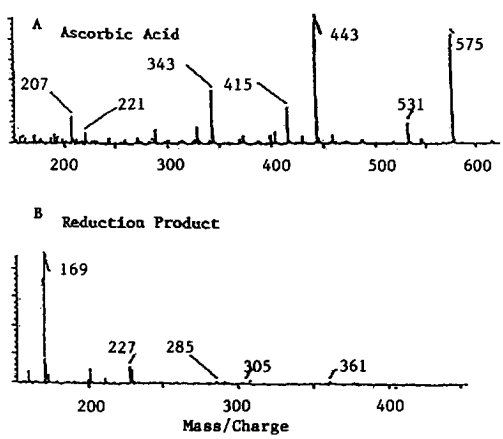

Fig. 7. Spectra of the 4, 5 formed during reduction of solubilized DHA (Fig. 6B). (A) AA; (B) unknown reduction product.

solutions rapidly change [15–17]. Furthermore, we have shown that reduction using sulfhydryls is sufficient to drive compounds other than DHA back to AA.

These data raise questions concerning the use of HPLC electrochemical measurements of DHA in which sulfhydryl reduction was used to convert DHA to AA, since the current work shows that open-ring products which are not DHA can also be reduced with a resulting rise in AA. However, since sulfhydryls are readily available in biological systems, it is interesting to note that the open-ring products apparently do not have antiscorbutic effect [17]. This suggests that biological sulfhydryls may not reduce these open-ring compounds in vivo.

Many of the problems encountered in the study of DHA can be circumvented through the use of GC–MS isotope dilution assays [25,26]. However, caution is still indicated, since DHA solutions tend to degrade quickly, even during solubilization. This makes it difficult to assign a true value to a standard in an isotope dilution assay. For example, if one makes a fresh 1 m*M* DHA solution (174 μg/ml) to standardize a stable isotope DHA solution, and the majority of the fresh solution is in the open-ring form, the stable isotope solution will be assigned a higher value than the amount of DHA monomer which is actually present.

Other methods of DHA analysis have recently been published which use different separation and detection systems [28,29]. It is unclear which method of analysis will ultimately offer an advantage in terms of sensitivity or specificity. It is likely that each analytical method adds some information, and that a combination of methods may best provide insight to the overall role DHA plays in biological systems.

It is likely that much of the literature published concerning the analysis of DHA contains values which actually differ from the true concentration of DHA monomer. Furthermore, much of the activity ascribed to DHA in chemical and biological systems may actually be due to other species derived from DHA. However, as workers continue to define the behavior of DHA, and as analytical methods improve, we expect that many of the interesting paradoxes found in AA research will be better understood.

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