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Formation Mechanism of the Free Radical Product and Its Precursor by the Reaction of Dehydro-L-ascorbic Acid with Amino Acid

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During the formation of radical A (**2**) and its precursor (tris(2-deoxy-2-L-ascorbyl)amine, **1**) by the reaction of dehydroascorbic acid (DHA) with amino acid, ascorbic acid (AsA) and the reduced red pigment (**3**) were newly identified, in addition to scorbamic acid (SCA) and the red pigment (**4**), as intermediate products. The addition of AsA to the DHA-amino acid reaction, as well as to the DHA–SCA reaction, greatly increased the formation of **3** and **1**. The reaction of AsA with **4** gave rapidly **3**, followed by the gradual production of **1**. From these results, a reaction pathway is proposed that **3** formed by the reduction of **4** with AsA is a key intermediate and its condensation with DHA followed by reduction with AsA might produce **2** and **1**.

The formation of free radical species during the amino-carbonyl reaction of common food constituents has been extensively studied in a series of works.[1～4] The reaction of dehydro-L-ascorbic acid (DHA) with an amino acid has been found to generate three kinds of free radical species (radicals A, B and C).[4] Among these, the radical A is particularly interesting because of its unusual stability that renders it obtainable as a blue substance on a TLC plate. Moreover we have found that the reaction mixture involves a colorless product which readily produces the radical A by air-oxidation. This precursor of radical A was identified as tris(2-deoxy-2-L-ascorbyl) amine (**1**),[5] and the radical A was also indentified as a one-electron oxidized form (**2**) of **1** by chemical and electrochemical studies.[6,7]

This paper elucidates the formation mechanism of **1** and **2** from DHA and amino acid, based on the results of a study on the roles of the individual intermediate products, ascorbic acid (AsA) and the reduced red pigment (**3**), in the reaction.

MATERIALS AND METHODS

Reagents. DHA, scorbamic acid (SCA) and the red pigment (**4**) were prepared by the methods previously described.[8] Other reagents used were of guaranteed grade.

Reaction method. A mixture of the starting materials with 95% ethanol in a test tube was refluxed in a boiling water bath.

Analyses. TLC for identification of the reaction products was performed by developing the reaction mixture with *n*-butanol–ethanol–water–acetic acid (50 : 20 : 30 : 1) using a cellulose plate (Merck).

Quantitative determination of the main intermediates was done by measurement of the visible absorbancy of the reaction mixture. **4** was at 520 nm after dilution with ethanol. **3** and **1** were at 520 nm at 10 min after diluting the reaction mixture with phosphate buffer (1/15 M, pH 7.0), and at 635 nm at 10 min after diluting with pyridine–water (1 : 1), respectively. Data from the ethanol solution were used as blanks in these measurements.

The amount of AsA was determined by GLC. The reaction mixture (0.01～0.1 ml) mixed with sorbitol so-

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Abbreviations: **1**, tris(2-deoxy-2-L-ascorbyl)amine (precursor compound to radical A); **2**, one-electron oxidized form of **1** (radical A); **3**, bis(2-deoxy-2-L-ascorbyl)amine (the reduced red pigment); **4**, two-electron oxidized form of **3** (the red pigment).

lution (1 mg/ml, 0.25 ml) was dried *in vacuo* and treated with a mixture of pyridine (0.25 ml), hexamethyldisilazane (0.1 ml) and trimethylchlorosilane (0.05 ml), then heated for 30 min in a boiling water bath. The silylated sample was analyzed by a Shimadzu GC-4BMPF equipped with a glass column, containing 1.5% silicone OV-1 on Shimalite W. The column condition was a 2-min initial hold, followed by a 5°C/min temperature rise starting from 180°C. Retention times of the silylated AsA and that of sorbitol were 9.5 and 10.0 min, respectively. Determination of the AsA content was performed from the peak intensity ratio of AsA to the standard sorbitol. The amount of DHA in the reaction mixture was also determined by GLC as above, after reduction with hydrogen sulfide.

RESULTS AND DISCUSSION

1. Formation of AsA

It has been previously shown[9)] that the formation of **1**, the precursor compound to **2**, was increased markedly by the prior addition of AsA to the reaction system of DHA and amino acid. This seems to suggest that, in a system without added AsA, **1** and **2** are produced by a reaction involving AsA produced by the DHA-amino acid reaction. In order to verify this supposition, the reaction mixture of DHA with amino acid was examined by TLC to confirm the presence of AsA. Among the spots observed in the TLC of the reaction mixture of DHA with Phe in ethanol (Fig. 1A), a considerable amount of AsA was detected as ninhydrin-positive spot, at an *Rf* of about 0.6, and which was also detectable with Tillman's reagent or a UV lamp. Further confirmation and quantification were accomplished by use of GLC. As shown in Fig. 1B, a peak at t_R 9.5 min on GLC was in good agreement with that of the authentic AsA. The amount of AsA produced by the reaction of various amino acids with DHA, along with the yields of **4** and **3**, and the browning are shown in Table I. AsA was found commonly in most cases with various amino acids, although the yield based on DHA ranged widely from 0 to 18%, and this was roughly proportional to the intensity of browning.

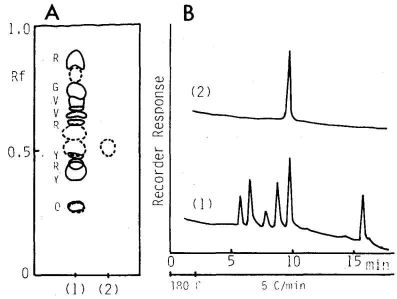

FIG. 1. TLC (A) and GLC (B) of the Reaction Mixture of DHA (0.5 M) with Phe (0.25 M) in Ethanol.

(1), reaction mixture; (2), authentic AsA.

TLC: Y, yellow; R, red; V, violet; G, green. ◌ , ninhydrin positive.

GLC: Silylated derivatives.

TABLE I. REACTION BETWEEN DHA AND AMINO ACID

Estimation of products and the degree of browning.[a]

	AsA (mM)	**4**[b]	**3**[b]	Browning[b]
α-Ala	27.5	65	82	110
β-Ala	20.0	97	65	102
Val	32.5	30	50	122
Phe	45.0	25	67	110
Pro	28.7	5	5	27
Ser	22.5	15	17	20
Trp	72.4	70	55	248
Tyr	0.0	5	0	5
Glu	8.7	10	3	15
Met	16.2	20	25	127
Glu	0.0	5	3	10
Arg	24.4	32	47	25

[a] DHA (0.5 M) and amino acid (0.25 M) was refluxed for 20 min in ethanol.

[b] O.D. × dilution factor.

2. Formation of the reduced red pigment (**3**)

The reaction of AsA with 4 has been known to enhance the formation of radical C[4)], as well as **1** (see also § 5). These suggest that the reduction of **4** caused by AsA or other reducing agents results in the formation of **1**. Therefore, products detectable by the reaction of **4** with AsA were investigated. The systems DHA–Phe–AsA and DHA–SCA–AsA were also examined because of their superiority to the systems without AsA in the formation of **1** as shown in the latter sections. Figure 2 shows the TLC of the reaction mixtures of the three systems **4**–AsA, DHA–Phe–AsA and DHA–

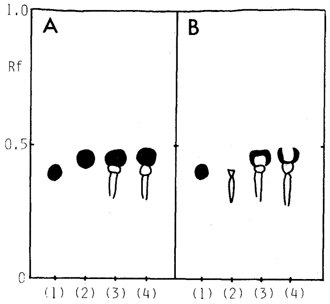

FIG. 2. Effect of Atmosphere on the TLC of the Reaction Mixture in Ethanol.

A: Development under air. B: Development under nitrogen. (1), authentic **4**; (2), **4** (0.05 M)+AsA (0.1 M), at 70°C for 10 min; (3), DHA (0.4 M)+Phe (0.2 M)+AsA (0.2 M), refluxed for 6 min; (4), DHA (0.2 M)+SCA (0.1 M)+AsA (0.1 M), at 80°C for 10 min.

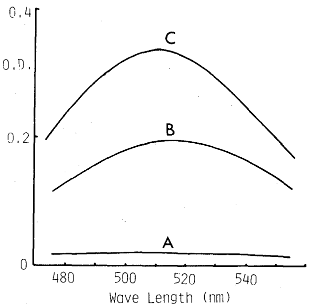

FIG. 3. Visible Spectra of the Ethanol Extract from *Rf* 0.45 Fraction on TLC that Corresponds to Fig. 2.

Ten min (A) and 18 hr (B) after extraction with ethanol. One min and 18 hr after extraction with pH 7 phosphate buffer, superimposed (C).

SCA–AsA. When these reaction mixtures were developed on TLC in the presence of air, the red spots detected in each system appeared at the same *Rf* value of 0.45, which was somewhat higher than that of the authentic **4** (Fig. 2A). On the other hand, on the TLC under a nitrogen atmosphere, the red spot was absent or hardly detectable (Fig. 2B). When this plate was exposed to air for half an hour or more, however, the red spots gradually appeared at the same *Rf* as those observed in Fig. 2A. When the newly-appeared red spots were extracted with water and developed again on the TLC, the red spots gave an identical *Rf* value to that of authentic **4**. This finding revealed that the reaction of **4** with AsA, as well as those of the DHA–SCA–AsA and DHA–Phe–AsA systems, produce a colorless product which is easily converted to **4** by air-oxidation.

To isolate this colorless product, preparative TLC of the reaction mixture of DHA–SCA–AsA (the same as in Fig. 2) was done under a nitrogen atmosphere, and the fraction corresponding to *Rf* 0.45 was extracted with ethanol. As shown in Fig. 3, the ethanol extract was colorless and initially showed no absorption around 500 nm but, after standing overnight in air it turned red and showed a spectrum having a maximum at 515 nm. When the extract from TLC was diluted with phosphate buffer (pH 7), the solution turned red instantaneously and gave a spectrum having a maximum at 510 nm. These correspond to the absorption maxima of the authentic **4** in water and in ethanol, which are at 510 and 515 nm, respectively. Moreover it was confirmed that the red color of **4** in ethanol readily disappeared by catalytic reduction with hydrogen and palladium black, but the dilution of the reduced solution with neutral phosphate buffer in air restored the red color.

These results indicate that the product isolated from the TLC fraction around *Rf* 0.45 is the reduced product of **4**, bis(2-deoxy-2-L-ascorbyl)amine (**3**). Quantification of this product in the reaction mixture was performed by measurement of the red color that appeared after dissolution with phosphate buffer (pH 7). The amount of **3** in the reaction mixtures of DHA with different amino acids are shown in Table I. The amount of 3 produced was approximately proportional to that of **4** in all cases.

3. Quantification of the intermediate products during the reaction of DHA with Phe

When a mixture of DHA and Phe in ethanol was refluxed, the mixture immediately colored red and then brown. As shown in Fig. 4, **4** increased during the first several minutes of the reaction, and then decreased gradually to a constant level. **3** initially increased almost in

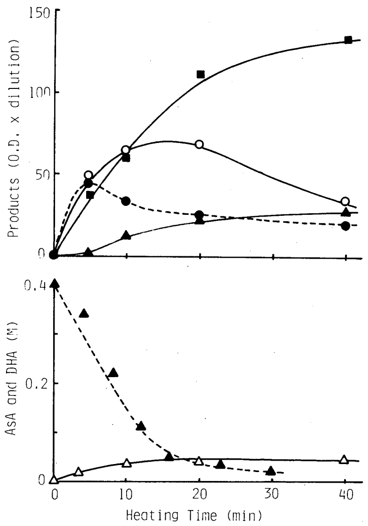

FIG. 4. Formation of Products by the Reaction of DHA (0.5 M) with Phe (0.25 M) in Ethanol.

--●--, **4**; —○—, **3**; —▲—, **1**; ---▲---, DHA; —△—, AsA; —■—, degree of browning.

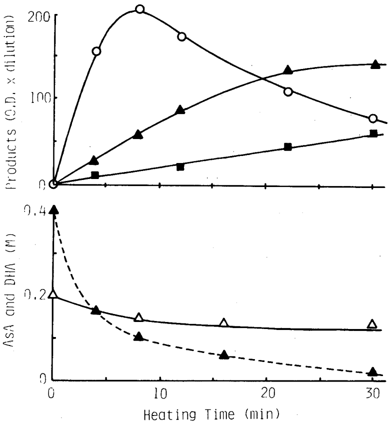

FIG. 5. Formation of Products by the Reaction of DHA (0.5 M) with Phe (0.25 M) in Ethanol in the Presence of AsA (0.25 M).

Symbols are same as those in Fig. 4.

the same manner as **4** for about 15 min and then decreased gradually with further heating. Comparing with the formation of these products, **1**, a precursor compound to **2**, appeared in the solution a little later than the above two products and increased gradually. It was also seen that DHA decreased rapidly with the reaction time, along with a gradual formation of AsA.

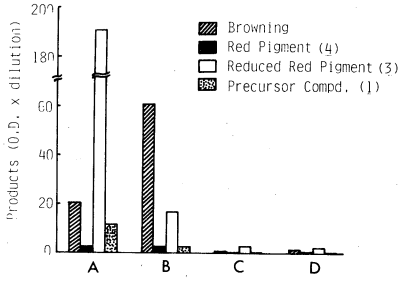

FIG. 6. Relative Amount of the Products Formed by the Reaction System Containing SCA.

A, DHA (0.2 M)+SCA (0.1 M)+AsA (0.1 M); B, DHA (0.2 M)+SCA (0.1 M); C, SCA (0.1 M)+AsA (0.1 M); D, SCA (0.1 M).

Mixtures in ethanol was heated at 80°C for 15 min.

Figure 5 shows the formation of these products in the case of the DHA–Phe–AsA system. Here, the amounts of **3** and **1** were significantly increased as compared to the reaction system without AsA shown in Fig. 4.

These facts suggest that AsA contributes greatly to the formation of **3**, and that **1** is produced successively from **3**.

The browning of the reaction mixture was suppressed by the addition of AsA. The effect could reasonably be understood by the assumption that the reduction of **4** by AsA caused the suppression of the main browning reaction process, which proceeds through the formation of a yellow pigment by the reaction of **4** with SCA, as has been mentioned in one of the preceding papers.[10)]

4. *Formation of the precursor compound* (**1**) *in the systems involving SCA*

SCA has been considered to be the first product during the reaction of DHA and amino acid, and a direct precursor that produces **4**.[8)] The formation of **1** by the reaction of SCA with DHA and/or AsA was therefore examined, and the results are shown in Fig. 6. No appreciable amount of the products was obtained in the systems of SCA alone or with AsA (C and D). The reaction of SCA with DHA (B) gave a high degree of browning along with the formation of **3**. Moreover, the addition of AsA to the DHA–SCA system resulted in a marked increase in the formation of **3** and **1** accompanied by an apparent decrease in the browning reaction, as was observed for the above-mentioned DHA–Phe system (Figs. 4 and 5).

5. *Formation of the precursor compound* (**1**) *in the systems involving the red pigment* (**4**)

The formation of **1** in the reaction systems involving **4** was examined, because **4** has been considered to be the condensation product of SCA with DHA.[8] It was shown that the heating of **4** alone as well as **4** with DHA or SCA scarcely produced **1**, while the reaction of **4** with AsA gave a high yield of **1**.

Figure 7 shows a time course for the formation of **1** and **3** by the reaction of **4** with AsA. Although the reduction of **4** to **3** proceeded very quickly, the increase in **1** was gradual. This is in accord with the assumption that **3** produced from **4** by the reduction with AsA is necessary for the formation of **1**.

Figure 8 shows the effect of AsA concentration on the yield of **1** by the reaction of **4** with AsA. The formation was enhanced by increasing the concentration of AsA up to the molar ratio of 1 : 2 between **4** and AsA, which suggests that the formation of **1** requires two mol of AsA for one mol of **4**. The solution of **3** (10 mg/0.5 ml) in ethanol, prepared by catalytic reduction as described in § 2, was heated with AsA (20 mg) or with DHA (20 mg) at 70°C for 1 hr. A TLC of these reaction mixtures indicated no formation of **1**. In contrast to this, when **3** was heated with a mixture of DHA and AsA (10 mg each), a large amount of **1** was detected by both TLC and visible absorption after the pyridine-treatment. This showed that the presence of one mol of DHA, which is the product of the reaction of AsA with **4**, is necessary for the formation of **1**.

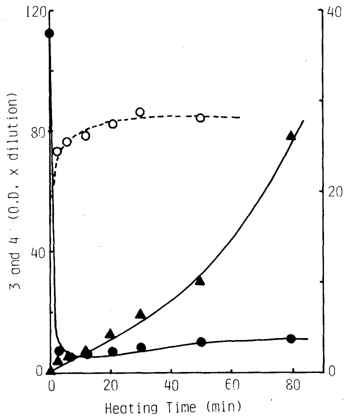

FIG. 7. Formation of Products by the Reaction of **4** (0.05 M) with AsA (0.1 M) in Ethanol, at 70°C.
—●—, **4**; --○--, **3**; —▲—, **1**.

6. *Formation mechanism of radical A* (**2**) *and its precursor* (**1**)

Based on the results described above, a pathway for the formation of **2** and **1** by the reaction of DHA with amino acid is proposed as shown in Fig. 9. Here, the initial step is the formation of **4**, as has already been studied.[8] In the next stage, the reaction of **4** with other substances proceeds in two separate ways; one of these is its reaction with SCA to produce radical B and the yellow product followed by the reactions to give the brown product,[10] and the other is that with AsA to produce **2** and **1** through **3**. Radical C has been known to be produced from **4** and AsA,[4] and therefore it should be the intermediate in the reduction of **4** to **3**.

Two pathways are probable for the formation of **1** from **3**: (1) direct formation by its condensation with AsA to give the radical A precursor, and (2) formation by reduction after condensation with DHA. The first, how-

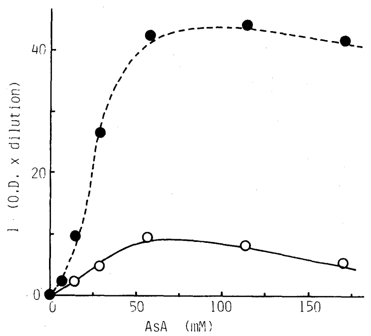

FIG. 8. Effect of AsA Concentration on the Formation of **1** by the Reaction with **4** (27 mM), at 70°C.
—○—, heating for 30 min; ---●---, heating for 2 hr.

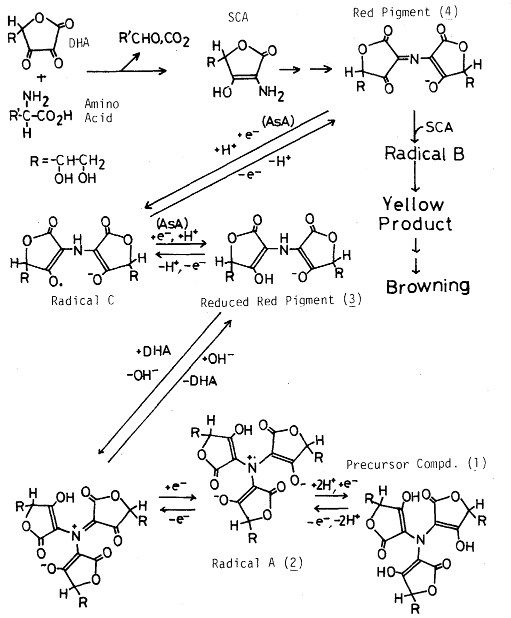

FIG. 9. Probable Pathway for the Formation of **2** and **1**.

ever, was refected by the fact that the reaction of **3** with AsA gave no precursor compound. The second seems more reasonable and is proposed as the probable pathway. The reaction that follows, that is, the reaction between **1** and **2** has already been determined.[6,7)]

In a previous paper,[6)] the conversion of **2** to **4** by air-oxidation has been interpreted as the hydrolysis of the oxidized **1** directly producing **4** and AsA. However, the present mechanism seems to suggest that the process of hydrolysis of the oxidized **1** initially gives DHA and **3**, and the latter subsequently is oxidized to **4**. Moreover, we have designated **1** as the precursor compound to give **2**, but in the present mechanism it was shown that **1** is instead the finally reduced product through **2**.

The formation of radical B and the yellow product constitutes the main pathway of the normal browning reaction,[10)] while the formation of **1** and **2** occurs under reducing conditions, where the normal browning reaction is relatively suppressed.

In this report, a large part of the processes of this very interesting reaction between DHA and amino acid, which produces the red pigments and its reduced form, the yellow product and the three kinds of free radical products and their precursors, has been clarified by identification and quantification of these intermediate products. The pathway of this reaction has been shown to involve complicated mutual redox-reactions between the starting materials, intermediates and the products.

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