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Nonenzymatic Hydroxylation of Phenylalanine by Ascorbic Acid and Cu^{++}[1]

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When phenylalanine was treated with ascorbic acid and Cu^{++} in acetate buffer (pH 6.0), the decomposition of phenylalanine was observed.

Cu^{++} accelerated remarkably the decomposition of phenylalanine as compared with other transition metal ions. In contrast, nitrogen gas, other reducing agents and radical scavengers prevented the decomposition. The results indicate that a transient free radical intermediate, formed during the autoxidation of ascorbic acid, is responsible for the decomposition.

The ascorbic acid-Cu^{++} system was found to decompose phenylalanine giving hydroxyphenylalanines such as dihydroxyphenylalanine, *m*-tyrosine, *p*-tyrosine and *o*-tyrosine.

Keywords——nonenzymatic hydroxyln.; phenylalanine; ascorbate; cupric sulfate ion-exchange chromatography; radical scavenger

The inhibitory effect of ascorbic acid and Cu^{++} on enzyme activity have been recognized by many investigators with various enzymes.[3–5] However, the mechanism of the inhibition have not yet been clear. As the first step of our study on the mechanism of the inhibitory action of ascorbic acid and Cu^{++} to enzyme activity, effects of ascorbic acid and Cu^{++} to amino acids were examined.

Edlbacher and Segesser[6] have been found that the nucleus of histidine, among other imidazoles, is easily decomposed by aeration in the presence of ascorbic acid to liberate ammonia. It has been reported that the autoxidation of ascorbic acid is affected by the presence of histidine[7] and tryptophan.[8] In addition, Lieberman and Kunishi[9] have shown that the decomposition of methionine by ascorbic acid and Cu^{++} is occurred.

The present paper deals with the hydroxylation of phenylalanine by ascorbic acid plus Cu^{++} and identification of the hydroxylated compounds and the roles played by ascorbic acid during course of the reaction.

Experimental

Materials——Special grade L-ascorbic acid and cupric sulfate were obtained from Nakarai Chemicals, Co., Ltd., Kyoto. Phenylalanine, 3,4-dihydroxyphenylalanine, *o*-tyrosine, *m*-tyrosine and *p*-tyrosine were purchased from Sigma Chemical., U.S.A. Special grade reagents were used in all other cases.

Reaction with Ascorbic Acid——The reaction mixture contained the following in 25 ml of 0.1M sodium acetate buffer (pH 6.0): phenylalanine 2×10^{-3}M, Cu^{++} 2×10^{-4}M and ascorbic acid 2×10^{-2}M. The incubation was carried out at 37° under vigorous shaking with air as the gas phase unless otherwise stated.

1) A part of this research was presented at the 96th Annual Meeting of the Pharmaceutical Society of Japan in Nagoya, April 1976.
2) Location: 5 *Nakauchicho, Misasagi, Yamashina, Kyoto 607, Japan.*
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Analysis of Products——Identification and analysis of the reaction products were carried out by the ion-exchange chromatographic techniques described previously[10] using a Hitachi Model 034 Liquid Chromatograph equipped with ultraviolet and visible detectors.

Results

Decomposition of Phenylalanine by Ascorbic Acid and Cu^{++}

When ascorbic acid and Cu^{++} were added to a vigorously shaken sodium acetate buffer (pH 6.0) containing phenylalanine, decomposition of phenylalanine was observed. As is show in Fig. 1, phenylalanine decomposed in the presence of both ascorbic acid and Cu^{++}, but no significant decomposition was occurred when ascorbic acid or Cu^{++} was omitted from the reaction mixture. It was found that the decomposition rate of phenylalanine was markedly reduced after 15 min reaction. The results may be explained on the basis of the facts that ascorbic acid and Cu^{++} react together very rapidly and approximately 90% of ascorbic acid is oxidized after 15 min incubation with Cu^{++} under the conditions described above (as judged by the decrease in λ_{max} (273 nm) of ascorbic acid).

Table I. Effect of Metal Ions on Decomposition of Phenylalanine in the Reaction with Ascorbic Acid

Metal ion	Concentration (M)	Phenylalanine remained (%)
None	0	98
Cu^{++}	2×10^{-4}	58
Fe^{++}	2×10^{-4}	96
Zn^{++}	2×10^{-4}	98
Co^{++}	2×10^{-4}	98
Ni^{++}	2×10^{-4}	98
Mn^{++}	2×10^{-4}	98

Phenylalanine (2×10^{-3} M) was incubated with ascorbic acid (2×10^{-2} M) and metal ions in 0.1 M acetate buffer (pH 6.0) for 15 min at 37°.

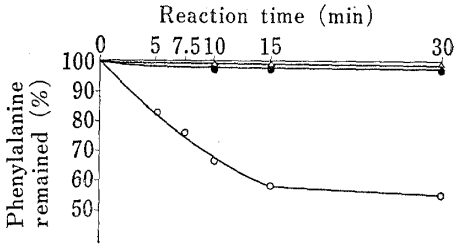

Fig. 1. Decomposition of Phenylalanine in the Reaction with Ascorbic Acid and Cu^{++}

—△—: phenylalanine (2×10^{-3}M)+ascorbic acid (2×10^{-2}M)
—●—: phenylalanine (2×10^{-3}M)+Cu^{++} (2×10^{-4}M)
—○—: phenylalanine (2×10^{-3}M)+ascorbic acid (2×10^{-2}M)+Cu^{++}(2×10^{-4}M)

A remarkable difference in the rate of decomposition was observed between the reaction mixture with Cu^{++} and that without Cu^{++}, as shown in Fig. 1. To examine the effect of the other metal ions, transition metal ions were added to the reaction mixture in stead of Cu^{++} and the decomposition of phenylalanine by ascorbic acid was determined. The results obtained are summarized in Table I. Although the decomposition of phenylalanine by ascorbic acid was markedly accelerated by the addition of Cu^{++}, all of the other cations tested had no effect. Therefore, the requirement for Cu^{++} for such decomposition is highly specific.

Effectors on the Decomposition of Phenylalanine by Ascorbic Acid and Cu^{++}

To obtain some information about the mechanism of decomposition of phenylalanine by ascorbic acid and Cu^{++}, effect of oxygen, reducing agents and radical scavengers was examined.

1) **Effect of Oxygen**——Nitrogen gas was bubbled through the reaction mixture. Under anaerobic condition, decomposition of phenylalanine by ascorbic acid and Cu^{++} was not obserbed.

2) **Effect of Reducing Agents**——Reducing agents other than ascorbic acid were added to the reaction mixture. As shown in Table II, four thiol reducing agents prevented the decomposition of phenylalanine by ascorbic acid and Cu^{++}.

3) **Effect of Radical Scavengers**——Radical scavengers were added to the reaction mixture. As shown in Table III, hydroquinone, dimethyl sulfoxide and potassium iodide prevent-

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ed the decomposition of phenylalanine by ascorbic acid and Cu^{++}. At certain concentration of potassium iodide prevented completely the decomposition by ascorbic acid and Cu^{++}.

Products Analysis

To identify the products formed by the reaction of phenylalanine with ascorbic acid and Cu^{++}, the reaction mixture was subjected to the ion-exchange chromatography, as described in experimental part. The results are shown in Fig. 2. The chromatography of reaction prod-

Table II. Effect of Reducing Agents on Decomposition of Phenylalanine in the Reaction with Ascorbic Acid and Cu^{++}

Agent	Concentration (M)	Phenylalanine remained (%)
None	0	58
Aminoethylmercaptane	2×10^{-4}	85
L-Cysteine	2×10^{-4}	90
Glutathione	2×10^{-4}	78
Dithiothreitol	2×10^{-4}	81

Phenylalanine (2×10^{-3} M) was incubated with ascorbic acid (2×10^{-2} M), Cu^{++} (2×10^{-4} M) and reducing agents in the buffer for 15 min at 37°.

Table III. Effect of Radical Scavengers on Decomposition of Phenylalanine in the Reaction with Ascorbic Acid and Cu^{++}

Scavenger	Concentration (M)	Phenylalanine remained (%)
None	0	58
Dimethyl sulfoxide	4×10^{-3}	79
Hydroquinone	4×10^{-3}	79
Potassium iodide	4×10^{-3}	100
Potassium iodide	2×10^{-3}	95
Potassium iodide	2×10^{-4}	72

Phenylalanine (2×10^{-3} M) was incubated with ascorbic acid (2×10^{-2} M), Cu^{++} (2×10^{-4} M) and scavengers in the buffer for 15 min at 37°.

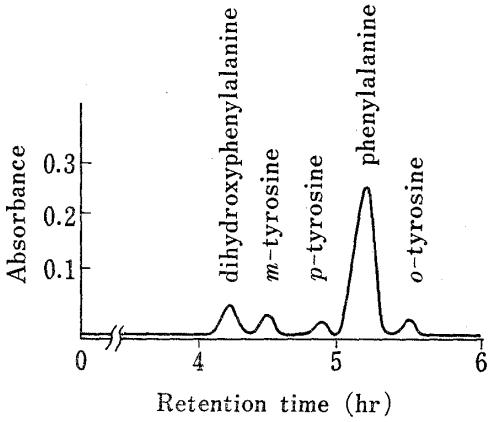

Fig. 2. Ion-exchange Chromatogram of Reaction Mixture

After 30 min reaction of phenylalanine with ascorbic acid and Cu^{++} under the conditions described in experimental part, 0.5 ml of the reaction mixture was subjected to the ion-exchange chromatograph according to the conditions reported before.[10] Color developed by ninhydrin reaction was determined at 570 nm.

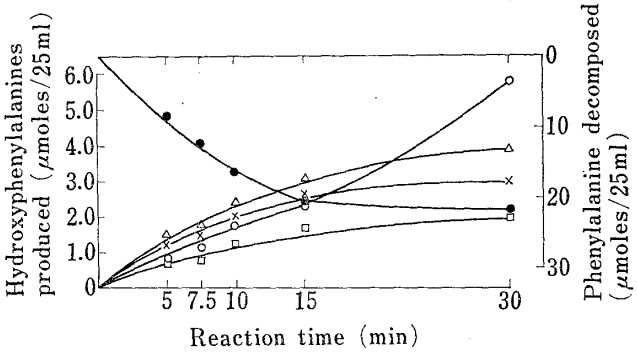

Fig. 3. The Hydroxylation of Phenylalanine in the Reaction with Ascorbic Acid-Cu^{++} System

—○—: dihydroxyphenylalanine
—△—: *m*-tyrosine
—□—: *p*-tyrosine
—×—: *o*-tyrosine
—●—: phenylalanine

The system used for the production of the hydroxylated products consisted of 5 μmoles Cu^{++}, 500 μmoles ascorbic acid and 50 μmoles phenylalanine in a volume of 25 ml of 0.1M acetate buffer (pH 6.0).

ucts revealed that dihydroxyphenylalanine, *o*-tyrosine, *m*-tyrosine and *p*-tyrosine were formed by the reaction of phenylalanine with ascorbic acid and Cu^{++}.

The relationship between the decomposition of phenylalanine and the formation of hydroxyphenylalanines was determined during the course of reaction. The results obtained are shown in Fig. 3. The amount of phenylalanine decomposed and the total amount of hydroxyphenylalanine formed at each point in Fig. 3 were estimated. From the results, it was found that 50—60% of phenylalanine was converted into the hydroxyphenylalanines.

Discussion

The following is an analysis of the results on the reaction mechanism involved in the decomposition of phenylalanine by ascorbic acid and Cu^{++}.

The addition of Cu^{++}, which is known to exert a catalytic effect upon the autoxidation of ascorbic acid, enhanced the rate of decomposition. In contrast, removal of oxygen by bubbling nitrogen gas through the reaction mixture prevented completely the decomposition reaction. The addition of reducing agents such as cystein and glutathione to the reaction mixture also prevented the decomposition. These results indicate that decomposition of phenylalanine by ascorbic acid and Cu^{++} requires the presence of molecular oxygen, and that this decomposition is accompanied by autoxidation of ascorbic acid.

Ascorbic acid is known to form free radical intermediates during its oxidation.[11–15] Radical scavengers such as hydroquinone, dimethyl sulfoxide and potassium iodide prevented the decomposition of phenylalanine. Particularly, above a certain concentration potassium iodide prevented the decomposition completely. Sulfhydryl compounds which are known to act as radical scavenger prevented the decomposition. These results indicate that the free radicals from ascorbic acid are responsible for the decomposition effect on phenylalanine. Possible radicals may be hydroxyradical (·OH), hydroperoxyradical (·OOH) and monodehydroascorbic acid radicals.[16] Further discussion, however, is not appropriate until more evidence has been accumlated.

Thus, it may be concluded that phenylalanine is oxidized by ascorbic acid and Cu^{++} in sodium acetate buffer (pH 6.0) and the isomers of hydroxyphenylalanine are produced, and that the hydroxylated compounds are the main degradation products of phenylalanine.

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