IIIa and IVa. IIIb: mp 247—247.5°C (dec.). IVb: mp 197—200°C (recrystallized from water). *Anal.* Calcd. for $C_9H_{11}NO_6S$ (IIIb and IVb): C, 41.38; H, 4.24; N, 5.36. Found for IIIb: C, 41.39; H, 4.15; N, 5.31. Found for IVb: C, 41.30; H, 4.17; N, 5.65.

***dl*-Norepinephrine-4-O-sulfate (NE-4-S)**——A suspension of 32 mg of IVa in 24 ml of dehydrated methanol–isopropanol (3: 5, v/v) was treated with 20 mg of $NaBH_4$ in several portions over 4 h with occasional stirring and then left for a further 3 h. The solution was evaporated to dryness. The residue was dissolved in 2 ml of water and 2 N HCl was added until the pH became about 2 (pH test paper). This solution was applied to a Dowex 50W column (H^+ form, 1.6 cm i.d. × 30 cm), which was eluted with water. The ninhydrin-positive fractions eluted after the acidic fractions were collected and concentrated *in vacuo*. The white crystals that precipitated were filtered off and washed with a small portion of ethanol, 22 mg, decomp. above 152°C. *Anal.* Calcd. for $C_8H_{11}NO_6S$: C, 38.55; H, 4.45; N, 5.62. Found: C, 38.66; H, 4.47; N, 5.74.

***dl*-Norepinephrine-3-O-sulfate(NE-3-S), *dl*-Epinephrine-4-O-sulfate(E-4-S) and *dl*-Epinephrine-3-O-sulfate (E-3-S)**——These compounds were synthesized by a procedure similar to that described for NE-4-S.

From 360 mg of IIIa, 220 mg of NE-3-S was obtained after recrystallization from water, decomp. 150°C. *Anal.* Calcd. for $C_8H_{11}NO_6S$: C, 38.55; H, 4.45; N, 5.62. Found: C, 38.74; H, 4.37; N, 5.76.

From 57 mg of IVb, 40 mg of E-4-S was obtained, mp 160—161°C (methanol). *Anal.* Calcd. for $C_9H_{13}NO_6S$: C, 41.06; H, 4.98; N, 5.32. Found: C, 41.08; H, 4.97; N, 5.32.

From 400 mg of IIIb, 220 mg of E-3-S was obtained after recrystallization from water–ethanol (1: 2, v/v), mp 158—160°C (dec.). *Anal.* Calcd for $C_9H_{13}NO_6S$: C, 41.06; H, 4.98; N, 5.32. Found: C, 41.16; H, 5.03; N, 5.63.

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[Chem. Pharm. Bull. 29(7)2089—2092(1981)]

Studies on the Hydroxylation of Phenylalanine by the Ascorbic Acid–Hydrogen Peroxide System

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(Received January 26, 1981)

When phenylalanine was treated with the ascorbic acid–hydrogen peroxide system in acetate buffer (pH 4.0), *o*-tyrosine, *m*-tyrosine and *p*-tyrosine were identified as hydroxylated products.

The hydroxylation of phenylalanine in the ascorbic acid–hydrogen peroxide system was prevented by radical scavengers, *e.g.*, potassium iodide, potassium bromide, sodium thiocyanate, sodium formate, mannose, ethyl alcohol, superoxide dismutase and Tiron.

The possibility is discussed that the active species responsible for the hydroxylation of phenylalanine in the ascorbic acid–hydrogen peroxide system is the hydroxyl radical.

Keywords——hydroxylation of phenylalanine; phenylalanine; *p*-tyrosine; *m*-tyrosine; *o*-tyrosine; ascorbic acid–hydrogen peroxide system; high performance liquid chromatography

Phenylalanine is enzymically converted into tyrosine by molecular oxygen acting with phenylalanine hydroxylase and a pteridine co-factor.[1] We have recently reported that when phenylalanine is incubated *in vitro* with rat brain homogenate in the presence of a pteridine co-factor, besides *p*-tyrosine, *m*- and *o*-tyrosine are also formed.[2] The tertahydropterins are reported to undergo oxidation by molecular oxygen, possibly with the formation of hydrogen peroxide[1]; hydroxyl radicals may be formed.[3] To elucidate the mechanism of the biological hydroxylation of phenylalanine, nonenzymatic hydroxylation must be considered.

In previous papers, we reported that an ascorbic acid–cupric ions system[4] and hydrogen peroxide–cupric ions system[5] were capable of hydroxylating phenylalanine to give the isomers of hydroxyphenylalanine. We suggested that hydroxyl and superoxide radicals were present in the hydrogen peroxide–cupric ions system and that the active species for hydroxylation was the hydroxyl radical.

It has long been postulated that the hydroxyl radical and superoxide radical are produced by the ascorbic acid–hydrogen peroxide system.[6,7] Recently, Shikata *et al.*[8] reported the hydroxylation of toluene by the ascorbic acid–hydrogen peroxide system.

The present paper deals with the hydroxylation of phenylalanine by the ascorbic acid–hydrogen peroxide system and the results are compared with those obtained with the ascorbic acid–cupric ions and hydrogen peroxide–cupric ions systems.

Experimental

Materials——Special grade ascorbic acid and hydrogen peroxide were obtained from Nakarai Chemicals, Co., Ltd., Kyoto. Phenylalanine, 3,4-dihydroxyphenylalanine (DOPA), *o*-tyrosine, *m*-tyrosine and *p*-tyrosine were purchased from Sigma Chemical Co., U.S.A. Superoxide dismutase from bovine blood was purchased from Miles Laboratories Ltd., U.S.A. Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid) was obtained from Nakarai Chemicals, Co., Ltd., Kyoto. All other reagents used were of analytical grade.

Method——Phenylalanine and its hydroxylated products were determined by fluorescence high performance liquid chromatography. The chromatographic conditions were as described in our previous paper.[9]

Hydroxylation——The reaction mixture contained the following components in 2 ml of 0.1 M acetate buffer: phenylalanine 4×10^{-3} M, ascorbic acid 5×10^{-3} M and hydrogen peroxide 5×10^{-3} M. The incubation was carried out at 37°C. A 50 μl-aliquot of the reaction mixture was periodically withdrawn and injected with a microsyringe into the chromatograph.

Results and Discussion

Decomposition and Hydroxylation of Phenylalanine by Ascorbic Acid and Hydrogen Peroxide

When ascorbic acid and hydrogen peroxide were added to acetate buffer (pH 4.0) containing phenylalanine, decomposition and hydroxylation of phenylalanine were observed. Figure 1 shows the time-courses of the decomposition and the hydroxylation of phenylalanine. The decomposition of phenylalanine and the formation of hydroxyphenylalanine increased with increasing concentration of ascorbic acid.

Effectors of the Decomposition and the Hydroxylation of Phenylalanine by Ascorbic Acid and Hydrogen Peroxide

To investigate the mechanism of the decomposition and hydroxylation of phenylalanine by ascorbic acid and hydrogen peroxide, the following experiments were carried out.

1) **Effect of pH**——Phenylalanine was incubated with ascorbic acid and hydrogen peroxide in acetate buffer solutions having various pH values (3.0—7.0), at 37 °C. The decomposition and hydroxylation of phenylalanine were dependent on pH (not shown in

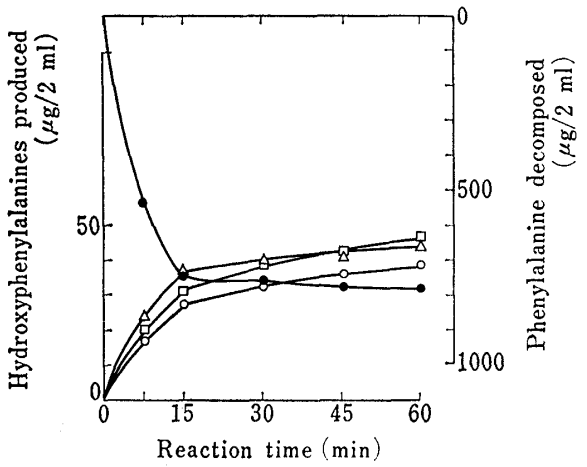

Fig. 1. Hydroxylation and Decomposition of Phenylalanine in the Ascorbic Acid–Hydrogen Peroxide System

—○—: *o*-tyrosine, —△—: *m*-tyrosine, —□—: *p*-tyrosine, —●—: phenylalanine.
Phenylalanine (1320 μg) was incubated with ascorbic acid (5×10^{-3} M) and hydrogen peroxide (5×10^{-3} M) in 2 ml of 0.1 M acetate buffer (pH 4.0) at 37°C.

the figure). The maximum rates of the decomposition and the hydroxylation of phenylalanine were found at around pH 4.0.

2) **Effect of Radical Scavengers**—— Table I shows the effect of hydroxyl[10] and superoxide[11] radical scavengers on the decomposition and the hydroxylation of phenylalanine by the ascorbic acid–hydrogen peroxide system. The hydroxyl radical scavengers, such as potassium iodide, potassium bromide, sodium thiocyanate, sodium formate, mannose and ethyl alcohol, prevented the decomposition of phenylalanine, and reduced the total yield of tyrosines. At a certain concentration, potassium iodide completely prevented the hydroxylation. In addition, the hydroxylation of phenylalanine was prevented by superoxide radical scavengers, such as superoxide dismutase and Tiron. The mechanism of the reaction of ascorbic acid (AH_2) in the presence of hydrogen peroxide may be described by the following steps at around pH 4.0;[6,7]

$$AH_2 \rightleftarrows AH^- + H^+ \quad (1)$$

$$AH^- + O_2 \longrightarrow \cdot A + \cdot O_2H \quad (2)$$

$$\cdot A + O_2 \longrightarrow A + O_2^- \quad (3)$$

$$H_2O_2 + O_2^- \longrightarrow \cdot OH + OH^- + O_2 \quad (4)$$

TABLE I. Effect of Hydroxyl and Superoxide Radical Scavengers

Scavenger	Concentration	Phenylalanine remaining (%)	Yield of tyrosines (%)
None	0	46	100
Potassium iodide	1×10^{-2} M	97	0
	1×10^{-3} M	66	39
Potassium bromide	5×10^{-2} M	97	11
	1×10^{-2} M	79	34
Sodium thiocyanate	5×10^{-2} M	98	5
	1×10^{-2} M	84	34
Sodium formate	5×10^{-2} M	87	42
	1×10^{-2} M	61	84
Mannose	5×10^{-2} M	91	44
	1×10^{-2} M	72	88
Ethyl alcohol	5×10^{-2} M	92	24
	1×10^{-2} M	72	72
Tiron	1×10^{-3} M	76	66
	1×10^{-4} M	67	86
Superoxide dismutase	10 μg	49	96
	50 μg	51	93

Phenylalanine (4×10^{-3} M) was incubated with ascorbic acid (5×10^{-3} M) and hydrogen peroxide (5×10^{-3} M) in the presence and absence of scavengers in 0.1 M acetate buffer (pH 4.0) for 30 min at 37°C.

where ·A is the monodehydroascorbic acid radical, $\cdot O_2H$ the hydroperoxy radical, O_2^- the superoxide radical and ·OH the hydroxyl radical. In addition, superoxide radical formed from the hypoxanthine–xanthine oxidase system was found to decompose phenylalanine, but not to hydroxylate phenylalanine.[5] The above results suggest that hydroxyl and superoxide radicals formed in the ascorbic acid–hydrogen peroxide system may be responsible for the decomposition and only the hydroxyl radical may be responsible for the hydroxylation of phenylalanine.

Table II. The Hydroxylation of Phenylalanine with Various Reaction Systems

Reaction systems	Isomer distribution (%)			
	DOPA	*o*-	*m*-	*p*-
phe–AH_2–H_2O_2	—	29	35	36
phe–AH_2–Cu^{2+}	18	32	27	23
phe–H_2O_2–Cu^{2+}	—	40	34	26

The reaction mixtures [phenylalanine (phe), 4×10^{-3} M; ascorbic acid (AH_2), 5×10^{-3} M; hydrogen peroxide (H_2O_2), 5×10^{-3} M; Cu^{2+}, 5×10^{-3} M] were incubated in 0.1 M acetate buffer (pH 4.0) for 60 min at 37°C.

Hydroxylation of Phenylalanine with Various Reaction Systems

Table II shows the hydroxylation of phenylalanine with various reaction systems. As is evident from the isomer distribution in Table II, the ascorbic acid–cupric ions system differs from the ascorbic acid–hydrogen peroxide and hydrogen peroxide–cupric ions systems in terms of the formation of DOPA. To elucidate the difference, the decomposition rates of DOPA by the ascorbic acid–hydrogen peroxide and the hydrogen peroxide–cupric ions systems were compared with that by the ascorbic acid–cupric ions system. No remarkable difference in the rate of decomposition of DOPA was observed among these reaction systems. On the basis of this finding and those described in the former section, it may be concluded that the active species responsible for the hydroxylation in the ascorbic acid–hydrogen peroxide and hydrogen peroxide–cupric ions systems is the hydroxyl radical, but in the ascorbic acid–cupric ions system, some other radical(s) besides the hydroxyl radical is also responsible for the hydroxylation of phenylalanine.

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