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Free Radical Intermediate in the N-Demethylation of Aminopyrine by Catalase-Cumene Hydroperoxide System

Cumene hydroperoxide-supported N-demethylation of aminopyrine catalyzed by catalase has been investigated. The transient free radical of aminopyrine was detected by electron spin resonance at room temperature. When cumene hydroperoxide was previously added to the catalase solution, the rate of the reaction and the concentration of the radical were very low. On the other hand, cumene hydroperoxide did not inhibit the catalase-catalyzed decomposition of hydrogen peroxide. On the contrary, sodium azide significantly inhibited the latter reaction and only slightly inhibited the former reaction. Methanol was not oxidized in the catalase-cumene hydroperoxide system. These results suggest that the active site of catalase for the cumene hydroperoxide-supported N-demethylation of aminopyrine is different from that for the decomposition of hydrogen peroxide.

Keywords——catalase; cumene hydroperoxide; aminopyrine; aminopyrine free radical; N-demethylation of aminopyrine; electron spin resonance; sodium azide; inhibition of catalase; cumene hydroperoxide-supported oxidation

Catalase was shown by Kadlubar *et al.*[1] to exhibit a significant aminopyrine N-demethylase activity in the presence of organic hydroperoxides. However, this activity was not well characterized. Recently, in the horseradish peroxidase-hydrogen peroxide system, the transient free radical of aminopyrine was detected by electron spin resonance (ESR) at room temperature.[2] On the other hand, the aminopyrine free radical was not detected in the metmyoglobin-cumene hydroperoxide system, although methyl radical was detected in the reaction mixture by means of a spin trapping technique.[3] In order to elucidate the mechanism of oxidative N-demethylation of substrates catalyzed by various hemeproteins, we have now studied cumene hydroperoxide-supported N-demethylation of aminopyrine catalyzed by catalase.

When cumene hydroperoxide was added to the mixture of catalase (from bovine liver, Sigma C-40) and aminopyrine, the solution turned pale blue-violet and a transient free radical was detected by ESR (JES-FE1X) at room temperature (25±1°). Fenton's reagent (ferrous ion/hydrogen peroxide in aqueous acid solution) generated identically the same free radical species from aminopyrine.[2] Fig. 1 shows time dependence of the generation of the aminopyrine free radical and production of formaldehyde and 2-phenyl-2-propanol (cumenol). The radical concentration was determined by double integration of the overmodulated ESR signal using a JEOL EC-100 computer system. The concentration of cumenol was quantitated by the use of JEOL JGC-20KFP gas chromatograph, equipped with OV-17 columns at 100°. Formaldehyde was assayed by the Nash procedure,[4] after the reaction had been quenched with 10% trichloroacetic acid and centrifuged to remove precipitated protein. The concentrations of cumenol and formaldehyde determined 2 hours after initiating the reaction, at that time the reaction is considered to be completed, were 1.3 and 0.67 mM, respectively. These results indicate that cumene hydroperoxide was almost quantitatively reduced to cumenol and the concentration of formaldehyde formed was nearly half that of cumenol. Since the decomposition of the aminopyrine free radical is complex and dose not give formaldehyde quantitatively,[5] the stoichiometry of the N-demethylation of aminopyrine can not

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5) H. Sayo and M. Masui, *J. Chem. Soc. Perkin II*, **1973**, 1640.

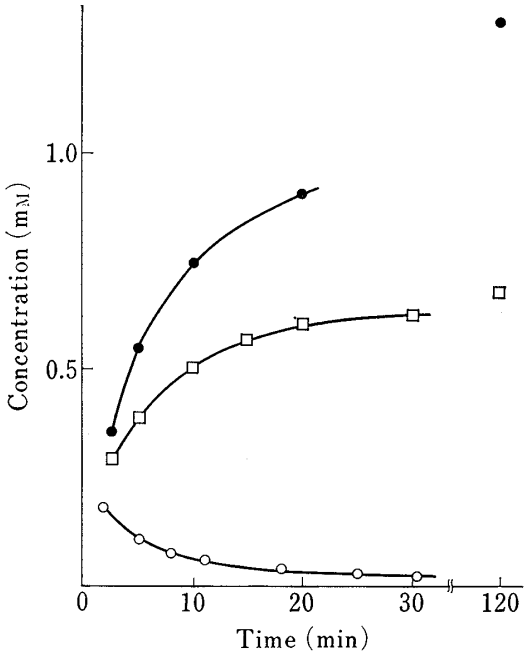

Fig. 1. Time Dependence of the Aminopyrine Free Radical and Productions of Formaldehyde and 2-Phenyl-2-propanol

The reaction mixture contained 1.4 μM catalase, 6 mM aminopyrine, and 1.5 mM cumene hydroperoxide in 0.1 M sodium phosphate buffer (pH 7.4), 25°. After the reaction was initiated by adding cumene hydroperoxide, the concentrations of the three substances were monitored as a function of time as described in the text.
○, aminopyrine free radical.
□, formaldehyde.
●, 2-phenyl-2-propanol (cumenol).

be derived from the concentration of formaldehyde alone.

As shown in Table I, when cumene hydroperoxide was previously added to the catalase solution and allowed to stand for 5 min and then mixed with the aminopyrine solution, the rate of the reaction and the concentration of the radical were very low (run 2). This indicates that an excess amount of cumene hydroperoxide in the absence of aminopyrine impairs the N-demethylase activity of catalase. However, as shown in Table II, cumene hydroperoxide did not inhibit the catalase-catalyzed decomposition of hydrogen peroxide. On the contrary, sodium azide significantly inhibited the latter reaction (Table II) and only slightly inhibited the former reaction (run 3 and 4). Moreover, methanol, which is an effective reductant of compound I of catalase, was not oxidized in the catalase-cumene hydroperoxide system (run 5).

These experimental results strongly suggest that compound I of catalase is not responsible for the N-demethylation of aminopyrine by cumene hydroperoxide. Although methyl radical was claimed to be an oxidant in the metmyoglobin-cumene hydroperoxide system,[3] neither methyl radical nor acetophenone was

Table I. Effects of Added Compounds and the Order of their Additions on the Rate of the Reaction and the Concentration of the Aminopyrine Free Radical in the Catalase-catalyzed System at 25°

Run	Order of additions to the catalase solution[a] 1	2	3	Rate of HCHO formation[b]	Rate of cumenol formation[b]	Aminopyrine free radical[d] mM
1	Aminopyrine	Cumene hydroperoxide		56(100)[c]	88(100)[c]	0.18(100)[c]
2	Cumene hydroperoxide	Allowed to stand for 5 min	Aminopyrine	5.4(9.6)	10(12)	0.004(2.2)
3	NaN_3 (0.4 mM)	Aminopyrine	Cumene hydroperoxide	48(86)	75(85)	0.14(78)
4	NaN_3 (2.0 mM)	Aminopyrine	Cumene hydroperoxide	40(71)	61(69)	0.10(56)
5	Methanol	Cumene hydroperoxide		0	0	

a) The final concentrations of various agents are as follows; catalase 1.4 μM, aminopyrine 6 mM, cumene hydroperoxide 1.5 mM, NaN_3 0.4 or 2.0 mM, and methanol 6 mM.
b) HCHO or cumenol mol/min/mol catalase.
c) Numbers in parentheses are the values relative to run 1.
d) The concentration was determined 2 min after initiating the reaction.
e) All experiments were carried out in 0.1 M sodium phosphate buffer, pH 7.4.

Table II. Effects of Added Compounds on the Catalase-catalyzed Decomposition of H_2O_2

Additions to the catalase solution (1.4 μM)[a]	Activity[b]	% of control
Control	11100	100
+NaN_3 (0.4 mM)[a]	5200	47
+NaN_3 (2.0 mM)[a]	800	7.2
+Cumene hydroperoxide (1.5 mM)[a]	11100	100

a) The concentration in the mixture of catalase and additives. The mixture was allowed to stand for 5 min and then diluted with 0.05 M phosphate buffer (pH 7.0) for the assay.
b) Sigma units per mg protein. One Sigma unit will decompose one μmol of H_2O_2 per minute at pH 7.0 at 25°, while the H_2O_2 concentration falls from 10.3 to 9.2 μmol per ml of reaction mixture.

detected by spin trapping technique[6] and gas chromatography in our system. Therefore, the active site of catalase for the cumene hydroperoxide-supported N-demethylation is considered to be different from that for the decomposition of hydrogen peroxide. Detailed studies are now in progress.

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6) The spin traps used were 0.1 M phenyl-*tert*-butylnitrone and 0.1 M 5,5-dimethyl-1-pyrroline-1-oxide. E.G. Janzen and B.J. Blackburn, *J. Am. Chem. Soc.*, **91**, 4481 (1969); E.G. Janzen and J. I-Ping Liu, *J. Magn. Resonance*, **9**, 510 (1973); A.N. Saprin and L.H. Piette, *Arch. Biochem. Biophys.*, **180**, 480 (1977).

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Eugeniin, a New Ellagitannin from Cloves

A new ellagitannin, eugeniin was isolated from cloves, the dried flower buds of *Eugenia caryophyllata* Thunb., and the structure was determined to be 1,2,3-trigalloyl 4,6-hexahydroxydiphenoyl β-D-glucopyranose.

Keywords——ellagitannin; eugeniin; clove; *Eugenia caryophyllata*; Myrtaceae; partial hydrolysis; tannase

As a part of chemical investigation on tannins from crude drugs, we have examined the tannins in colves, the dried flower buds of *Eugenia caryophyllata* Thunb. (*Syzygium aromatica* Merr et Perry, Myrtaceae), and isolated a new tannin named eugeniin, together with ellagic acid and gallic acid. This paper deals with the structure determination of eugeniin.

Eugeniin (I), a light tan amorphous powder, $[\alpha]_D$ +57.1° (acetone), is very soluble in water, MeOH, acetone and EtOAc, and shows a blue color with $FeCl_3$ reagent and dark green coloration (after 30 min) with AcOH–$NaNO_2$ reagent,[1] being characteristic of ellagitannin.

1) E.C. Bate-Smith, *Phytochemistry*, **11**, 1153 (1972).