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EVIDENCE AGAINST SUPEROXIDE INVOLVEMENT IN TYROSINE HYDROXYLATION BY MUSHROOM TYROSINASE*

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Abstract—The possible involvement of superoxide anions in the hydroxylation of tyrosine by mushroom tyrosinase was studied. Superoxide dismutase and scavengers of superoxide ions of smaller MW than superoxide dismutase, such as nitroblue tetrazolium and copper salicylate, had no direct effect on the monohydroxyphenolase activity of mushroom tyrosinase. The kinetics of tyrosine hydroxylation, but not of DOPA oxidation, by mushroom tyrosinase was affected by the addition of a xanthine–xanthine oxidase system. In the presence of the xanthine–xanthine oxidase system, the lag period of tyrosine hydroxylation was shortened compared to the lag period in the absence of the xanthine–xanthine oxidase system. The xanthine–xanthine oxidase system alone (without mushroom tyrosinase) had no effect on tyrosine conversion to dopachrome. Superoxide dismutase, catalase and hydroxyl radical scavengers counteracted to some extent the shortening of the lag period of tyrosine hydroxylation by mushroom tyrosinase caused by the xanthine–xanthine oxidase system. It is suggested that the shortening of the lag period is due mainly to hydroxyl radicals generated by the xanthine–xanthine oxidase system via interaction of $O_2^{\cdot-}$ and hydrogen paroxide (a Haber–Weiss type reaction). The data do not support the direct participation of superoxide anions in tyrosine hydroxylation by mushroom tyrosinase.

INTRODUCTION

The enzyme tyrosinase (also known as phenolase, catecholase, polyphenoloxidase and cresolase) can carry out two different biochemical reactions: (a) hydroxylation of monohydroxyphenols to *o*-dihydroxyphenols; monophenol + O_2 + AH_2 → *o*-dihydroxyphenol + H_2O + A;

and (b) dehydrogenation of *o*-dihydroxyphenols to *o*-quinones:

$$o\text{-dihydroxyphenol} + \tfrac{1}{2}O_2 \rightarrow o\text{-quinone} + H_2O.$$

The hydroxylation step (a) is characterized by a lag period, which can be overcome by the addition of an exogenous reductant (AH_2) [1].

The most efficient AH_2 compounds are *o*-dihydroxyphenols [2]. In the absence of an exogenous AH_2, the lag period is overcome when sufficient *o*-dihydroxyphenol is formed, so that its concentration rises to a level where it is no longer rate-limiting [3].

During the initial stage of the lag period, before the level of *o*-dihydroxyphenol is high enough to serve efficiently as AH_2, one would expect the participation of reductants other than *o*-dihydroxyphenol, or the participation of an agent that can carry out non-enzymatic hydroxylation of monohydroxyphenol. A potential candidate for both functions could be $O_2^{\cdot-}$ [4–8].

The involvement of $O_2^{\cdot-}$ in hydroxylation of monophenols was suggested in several non-enzymatic model systems [4, 9–11]. However, $O_2^{\cdot-}$ was found not to hydroxylate aromatic compounds, such as 4-hydroxybenzoic acid or salicyclic acid in the NADH–phenazine methosulfate system described by Halliwell [12].

Several investigators proposed the possible involvement of $O_2^{\cdot-}$ in some enzyme-catalysed hydroxylation reactions [4–6], as well as in reactions catalysed by oxygenases [7, 8]. Halliwell [5] reviewed the possible role of $O_2^{\cdot-}$ in non-enzymatic and enzymatic hydroxylation reactions. By analogy with other types of hydroxylases, he reasoned that $O_2^{\cdot-}$ might also be involved in the hydroxylation of monophenols carried out by tyrosinase.

Superoxide dismutase (SOD) catalyses a rapid dismutation of $O_2^{\cdot-}$ into hydrogen peroxide and oxygen and is, therefore, an efficient inhibitor of $O_2^{\cdot-}$-dependent reactions [13]. Halliwell [14, 15] compared the ability of spinach-beet chloroplasts to hydroxylate *p*-coumaric acid in the dark and in the light. In the dark, hydroxylation occurred only when an AH_2 was added and SOD did not inhibit the reaction. In the light, hydroxylation occurred in the absence of an added AH_2 and the reaction was completely inhibited by SOD. Halliwell [14] concluded that $O_2^{\cdot-}$ acts as a reductant for the tyrosinase catalysed reaction in the light-dependent reaction. However, when

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Abbreviations used: $O_2^{\cdot-}$, superoxide ion; OH, hydroxyl radical; AH_2, reductant; SOD, superoxide dismutase; NBT, Nitroblue tetrazolium; DOPA, 3,4-dihydroxyphenylalanine; X-oxidase, xanthine oxidase; EDTA, ethylenediaminetetra-acetic acid; DTPA, diethylenetriaminepenta-acetic acid.

Superoxide dismutase (superoxide:superoxide oxidoreductase) (EC 1.15.1.1).

Catalase (H_2O_2:H_2O_2 oxidoreductase) (EC 1.11.1.6).

Tyrosinase (monophenol mono-oxygenase, phenolase) (EC 1.10.3.1).

Xanthine oxidase (xanthine:O_2 oxidoreductase) (EC 1.2.3.2).

chloroplasts were incubated in the dark in the presence of an $O_2^{\cdot-}$-generating system (xanthine–xanthine oxidase), no hydroxylation of *p*-coumaric acid occurred [14].

Halliwell [5] found that SOD, added to reaction mixtures containing *p*-coumaric acid and tyrosinase in the presence or absence of reductants (such as ascorbate, NADH or dihydroxyfumarate), had no effect in comparison with controls (without SOD). On the other hand, hydroxylation of *p*-coumaric acid was enhanced when exposed to an $O_2^{\cdot-}$-generating system consisting of illuminated mixtures of FMN and EDTA. SOD inhibited the hydroxylation of *p*-coumaric acid in the presence of such an $O_2^{\cdot-}$-generating system [5].

Michelson and Monod [16] published a patent in which they claim that freshly cut mushrooms, apples or potatoes treated with SOD (of *Photobacterium leiognathi*, strain No. ATCC 25, 521) in dilute phosphate buffer (pH 7.8), were much less brown than control slices not treated with SOD. This finding prompted Kon [17] to test the possibility that SOD can prevent tyrosinase-induced melanin formation. However, Kon found that SOD did not inhibit the activity of mushroom tyrosinase [17].

According to Visser [18], hydroxylases require iron (II) ascorbate or are copper-containing mono-oxygenases that normally use ascorbate as co-substrate. Visser [18] has proposed a reaction sequence for various iron (II) ascorbate-dependent hydroxylases as well as for copper-containing mono-oxygenases. He suggested that the copper (I) ion replaces iron (II) and ascorbate in the copper mono-oxygenase–dopamine-β-hydroxylase and probably also in tyrosinase. Visser [18] suggested that the mechanism of action of dopamine-β-hydroxylase and of tyrosinase is similar except that, in the case of tyrosinase, *o*-diphenol replaces ascorbate as a reductant (AH_2). In this connection, it is interesting that Liu *et al.* [19] have presented evidence for the involvement of $O_2^{\cdot-}$ in the dopamine-β-hydroxylase system while Diliberto and Kaufman [20] reported lack of inhibition of dopamine-β-hydroxylase activity by SOD.

The results described above, suggesting the unlikely involvement of $O_2^{\cdot-}$ in the hydroxylation of monophenols by tyrosinase, were obtained in studies based on the use of SOD as a scavenger of $O_2^{\cdot-}$. Indeed, superoxide dismutase is a powerful inhibitor of $O_2^{\cdot-}$-dependent reactions. However, $O_2^{\cdot-}$ radicals produced at an active site of an enzyme may be inaccessible to a large molecule, such as SOD. Therefore, in this case SOD fails to inhibit the reaction and one may conclude erroneously that $O_2^{\cdot-}$ does not participate in the reaction [21].

Copper chelates (such as copper salicylate and the copper salts of lysine and tyrosine), copper sulfate, NBT and Tiron are scavengers of $O_2^{\cdot-}$ [22, 23]. Thus, for example copper salicylate, NBT and Tiron (1,2-dihydroxybenzene-3,5-disulphonic acid) were shown to inhibit several enzymatic reactions in which SOD was ineffective [19, 20].

In view of the recent suggestion of Visser [18] of a similarity between the mechanism of hydroxylation carried out by dopamine-β-hydroxylase and by tyrosinase, mentioned above, and the possibility that the use of scavengers of $O_2^{\cdot-}$ other than SOD might shed a different light on the question of $O_2^{\cdot-}$ participation in a certain reaction, we decided to examine the possibility that $O_2^{\cdot-}$ is an intermediate in the hydroxylation of monohydroxyphenols by mushroom tyrosinase with special emphasis on its possible role during the initial lag period.

RESULTS AND DISCUSSION

Effect of superoxide dismutase on the hydroxylation of monohydroxyphenols and on the oxidation of o-dihydroxyphenols by mushroom tyrosinase

In order to test whether $O_2^{\cdot-}$ is involved in tyrosinase-catalysed reactions the effect of SOD on both the monohydroxyphenolase and the *o*-dihydroxyphenolase activity of mushroom tyrosinase was studied. It was found that SOD, at levels ranging from 45 to 2000 μg/ml, had no effect on either tyrosine hydroxylation (on either the lag period or on the rate of dopachrome formation after the lag period) or on DOPA oxidation by mushroom tyrosinase.

Similar experiments were conducted using different preparations of SOD as well as of mushroom tyrosinase, different monohydroxyphenols (i.e. *p*-cresol, tyramine) or *o*-dihydroxyphenols (i.e. 4-methylcatechol, dopamine) at pH 6.5, 7.2 or 7.8, in the absence or presence of 1×10^{-4} M EDTA. In all cases, SOD had no effect on the monohydroxyphenolase or the *o*-dihydroxyphenolase activity of mushroom tyrosinase.

Control experiments conducted with the various samples of SOD showed that 6.67 μg/ml SOD per assay completely inhibited $O_2^{\cdot-}$-dependent reactions, such as epinephrine oxidation to adrenochrome, at pH 10.2, and cytochrome *c* reduction by the xanthine–xanthine oxidase system.

The preparations of SOD were checked for possible contamination by catalase and that of catalase (see below) for possible contamination by SOD. Both enzyme preparations were found to be free of cross contamination.

Effect of copper salicylate and copper sulfate on the hydroxylation of monohydroxyphenols and on the oxidation of o-dihydroxyphenols by mushroom tyrosinase

Copper chelates (i.e. copper salicylate, copper lysine, copper tyrosine) and copper sulfate exhibit superoxide dismutase-like activity, as judged by their ability to suppress the xanthine–xanthine oxidase-mediated reduction of cytochrome *c* and of NBT [21, 24–26]. In the case of propyl- and lysyl-hydroxylases, SOD does not inhibit the enzymes, while NBT or copper salicylate, copper lysine and copper tyrosine do inhibit them [22]. This was found to be the case, despite the fact that copper salicylate is *ca* 1000-fold less effective than SOD in suppressing xanthine–xanthine oxidase reduction of cytochrome *c* [24]. Similarly, the activity of catechol dioxygenase is not inhibited by SOD but is inhibited by copper salicylate [27]. Younes and Weser [21] have likewise reported that the activity of pig kidney diamine oxidase was not affected by SOD but was suppressed by copper tyrosine, copper lysine and copper sulfate.

As can be seen in Fig. 1 (A_1 and A_3), copper salicylate and copper sulfate prolonged the lag period of tyrosine hydroxylation by mushroom tyrosinase, with the lag period increasing proportionately with increasing copper salicylate or copper sulfate concentration (in the range tested). However, the inhibitory effect of copper salicylate and of copper sulfate was not confined to the monohydoxyphenolase activity but was seen also with the *o*-dihydroxyphenolase activity [Fig. 1 (B_1 and B_3)] of the enzyme. At a concentration of 0.6 mM of copper salicylate or copper sulfate, the lag period of tyrosine hydroxylation

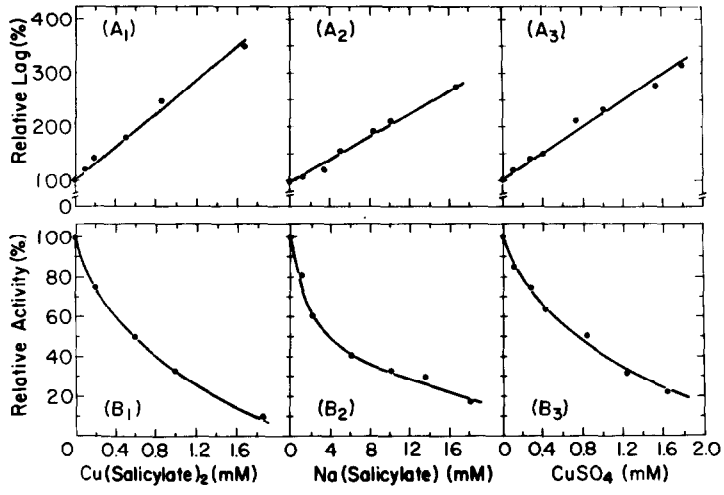

Fig. 1. Effect of copper (II) salicylate, sodium salicylate and copper sulfate on the lag period of tyrosine hydroxylation (A) and on the rate of DOPA oxidation (B) by mushroom tyrosinase. (A) The reaction mixture included 2 mM L-tyrosine, 47 mM NaPi buffer (pH 6.5), the indicated amounts of copper (II) salicylate, sodium salicylate or copper sulfate and 6.3 μg/ml mushroom tyrosinase (added last). Lag periods were computed from the curves obtained. The lag period of the controls was 420 sec and was taken as 100%. (B) The reaction mixture included 6.6 mM DL-DOPA, 47 mM NaPi buffer (pH 6.5), the indicated amounts of copper (II) salicylate, sodium salicylate or copper sulfate and 6.3 μg/ml mushroom tyrosinase (added last). Activity (ΔA_{475nm}/min) was computed from the curves obtained. The *o*-dihydroxyphenolase activity of the controls was 0.32 and was taken as 100%.

was doubled. At a similar concentration of copper salicylate and of copper sulfate, the rate of DOPA oxidation was halved [Fig. 1 (B_1 and B_3)].

The capacity of copper salicylate as a scavenger of O_2^- was also tested. It was found (data not shown) that 1.7 mM copper salicylate completely inhibited the reduction of cytochrome *c* (assayed at 550 nm) by the xanthine–xanthine oxidase system (in a reaction mixture that included 0.067 mM xanthine, 20 μg/ml xanthine oxidase, 47 mM NaPi buffer (pH 6.5) and 167 μg/ml cytochrome *c*).

The fact that copper salicylate and copper sulfate prolonged the lag period of tyrosine hydroxylation [Fig. 1 (A_1 and A_3)] suggested initially that O_2^- anions are, as proposed, required to initiate the hydroxylation reaction. However, the finding that copper salicylate and copper sulfate also inhibited *o*-dihydroxyphenolase activity to an equal extent [Fig. 1 (A and A_3)] and the fact that a 200–300-fold higher concentration of copper salicylate is needed to inhibit the mono- or dihydroxyphenolase activity of mushroom tyrosinase compared to that needed to inhibit O_2^- generation in a xanthine–xanthine oxidase system (see above), argues against this and implies rather that these compounds inhibited mushroom tyrosinase by another mechanism.

Additional experiments showed that sodium salicylate (at *ca* 20-fold higher concentration) had an inhibitory effect similar to that of copper salicylate and copper sulfate on both the monohydroxyphenolase and *o*-dihydroxyphenolase activities of mushroom tyrosinase [Fig. 1 (A_2 and B_2)]. Copper salicylate and copper sulfate had about an equal effect on the hydroxylation of tyrosine by mushroom tyrosinase; however, it took *ca* 20-fold higher concentrations of sodium salicylate to be equally effective. Therefore, Cu^{2+} appears to be the major factor in decreasing the rate of hydroxylation. The inhibition exerted by sodium salicylate can be attributed to the fact that salicylate (*o*-hydroxybenzoate), being a monohydroxyphenol, competes with substrates like tyrosine or DOPA used in the above experiments. Salicylate, being a strong chelator of Cu^{2+}, might bind also to the copper of the tyrosinase, thus inhibiting its activity. Cu^{2+}, *per se*, inhibits the activity of a copper enzyme, dopamine-β-hydroxylase; the enzyme is inhibited 60–80% with 2×10^{-4} M Cu^{2+} [28].

Effect of nitroblue tetrazolium on the hydroxylation of tyrosine by mushroom tyrosinase

O_2^- effectively reduces NBT to the blue formazan, measured at 560 nm [29] and, therefore, NBT is considered an effective scavenger of O_2^- [30, 31].

Myllyla *et al.* [22] have shown that SOD does not inhibit the activity of lysyl- or propyl-hydroxylases, while NBT does. They suggested that SOD does not inhibit propyl-hydroxylase activity due to the inability of SOD to come in contact with the active site of the hydroxylase. A similar phenomenon was reported in the case of diamine oxidase [21]. Tuderman *et al.* [30] have shown that NBT was a competitive inhibitor of propyl-hydroxylase with respect to oxygen and suggested that NBT consumed an activated form of oxygen.

In view of the above, the effect of NBT on tyrosine hydroxylation by mushroom tyrosinase was tested. The data in Table 1 show that NBT, at 0.02–0.2 mM had no effect on the lag period of tyrosine hydroxylation or on the rate of dopachrome formation after the lag period.

Effect of the xanthine–xanthine oxidase system on the hydroxylation of tyrosine by mushroom tyrosinase

If O_2^- were involved in the initiation of the hydroxy-

Table 1. Effect of nitroblue tetrazolium on tyrosine hydroxylation by mushroom tyrosinase*

NBT (mM)	Lag period (sec)	Activity after lag period (ΔA_{475nm}/min)
—	342	0.23
0.02	336	0.22
0.06	366	0.21
0.10	336	0.21
0.20	324	0.22

*The reaction mixture included 3.33 mM L-tyrosine, 47 mM NaPi buffer (pH 6.5), 16.7 μg/ml mushroom tyrosinase (added last), and the indicated concentrations of NBT.

lation of tyrosine by mushroom tyrosinase then, theoretically, the addition of a source of O_2^- should stimulate the reaction. A system consisting of xanthine plus xanthine oxidase generates O_2^- effectively [29]. It was of interest to test the effect of such a superoxide-generating system on tyrosine hydroxylation by mushroom tyrosinase.

The data in Table 2 demonstrate that the addition of either xanthine or xanthine oxidase alone had no effect on tyrosine hydroxylation, but that the addition of xanthine and xanthine oxidase together shortened the lag period of tyrosine hydroxylation by mushroom tyrosinase.

The data in Fig. 2 (A) demonstrate, in some detail, the kinetics of tyrosine hydroxylation by a constant level of mushroom tyrosinase in the presence of increasing concentrations of xanthine oxidase and a constant concentration of xanthine. As can be seen from a summary of the data in Fig. 2 (B), increasing the concentration of xanthine oxidase from 0 to 280 μg/ml in the assay shortened the lag period of tyrosine hydroxylation in a proportional manner to 13% of the original value.

The shortening of the lag period was also dependent upon the mushroom tyrosinase concentration. Keeping the xanthine concentration constant, the extent to which xanthine oxidase shortened the lag period of tyrosine hydroxylation was dependent on the concentration of mushroom tyrosinase. For example, in the presence of 4.2 and 8.4 μg/ml mushroom tyrosinase, it required 25 and 18 μg/ml xanthine oxidase, respectively, to shorten the lag period by 50%.

Table 2. Effect of the xanthine–xanthine oxidase system on the hydroxylation of tyrosine by mushroom tyrosinase*

Xanthine (mM)	Xanthine oxidase (μg/ml)	Lag period (sec)	Lag period (% change)
0	0	504	100
0.07	0	504	100
0	8.3	500	100
0.07	8.3	330	65

*Each reaction mixture included 3.4 mM L-tyrosine, 34 mM NaPi buffer (pH 6.5), and the indicated concentrations of xanthine and xanthine oxidase. Mushroom tyrosinase (7 μg/ml) was added last.

Control showed that in the absence of mushroom tyrosinase, but in the presence of either 0.07 mM xanthine, or 8.3 μg/ml xanthine oxidase or both, no increase in A_{475nm} was detected even after several hr.

The effect of the xanthine–xanthine oxidase system was also tested on the *o*-dihydroxyphenolase activity of mushroom tyrosinase but it was found to have no effect on the rate of DOPA oxidation by the enzyme.

It can, thus, be concluded that the effect of the xanthine–xanthine oxidase system is limited to the monohydroxyphenolase activity and that it has no effect on the *o*-dihydroxyphenolase activity of mushroom tyrosinase.

Is the shortening of the lag period of tyrosine hydroxylation by mushroom tyrosinase caused by the xanthine–xanthine oxidase system, due to O_2^-, hydrogen peroxide or OH?

The xanthine–xanthine oxidase system can generate O_2^- and hydrogen peroxide as the products of oxygen reduction. Interaction of O_2^- and hydrogen peroxide gives rise to free OH [6, 32]. It was important to establish how these

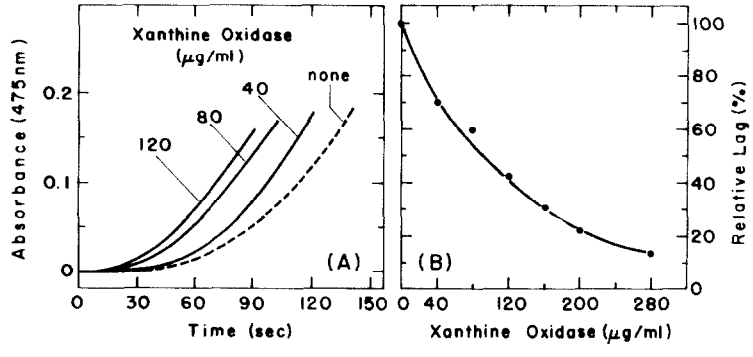

Fig. 2. Effect of the xanthine–xanthine oxidase system on the kinetics of tyrosine hydroxylation by mushroom tyrosinase. The reaction mixture included 3.3 mM L-tyrosine, 47 mM NaPi buffer (pH 6.5), 0.15 mM xanthine, the indicated amounts of xanthine oxidase and 16.6 μg/ml mushroom tyrosinase (added last). Lag periods were computed from the data in (A) and are summarized in (B).

products, each separately or in combination with each other, contribute to shortening of the lag period of tyrosine hydroxylation caused by the xanthine–xanthine oxidase system.

The effect of SOD or of catalase on shortening of the lag period caused by the xanthine–xanthine oxidase system on tyrosine hydroxylation by mushroom tyrosinase was tested in order to establish whether O_2^- alone, hydrogen peroxide alone, or both, are needed. As pointed out earlier, the preparation of SOD was checked for possible contamination by catalase and that of catalase for possible contamination by SOD. The enzymes were found to be free of such contamination. The data in Table 3 demonstrate that either catalase or SOD partially reversed the shortening of the lag period of tyrosine hydroxylation caused by the xanthine–xanthine oxidase system.

Control experiments showed that catalase or SOD alone had no effect on tyrosine hydroxylation by mushroom tyrosinase and that catalase alone had no effect on xanthine oxidase activity (assayed by uric acid production). As already mentioned above, SOD alone had no effect on tyrosine hydroxylation by mushroom tyrosinase.

Additional control experiments showed that SOD or catalase, added separately or in combination with either xanthine alone or xanthine oxidase alone, had no effect on the lag period. SOD or catalase were effective in counteracting the shortening of the lag period of tyrosine hydroxylation by mushroom tyrosinase only when both xanthine and xanthine oxidase were present (Table 3).

Does hydrogen peroxide, formed in the xanthine–xanthine oxidase system, shorten the lag period of tyrosine hydroxylation by mushroom tyrosinase?

Hydrogen peroxide is a product in the xanthine–xanthine oxidase system when oxygen is the electron acceptor. Hydrogen peroxide, at relatively low levels, can shorten the lag period of hydroxylation of monohydroxyphenols by mushroom tyrosinase [3, 33; Kahn, V., unpublished results]. Thus, the shortening of the lag period of tyrosine hydroxylation by mushroom tyrosinase in the presence of the xanthine–xanthine oxidase system seen above (Fig. 2) could possibly be due to the hydrogen peroxide generated in the system. The data in Fig. 3 represent a comparison of the capacity of exogenously added hydrogen peroxide and the ability of the xanthine–xanthine oxidase system to shorten the lag period of tyrosine hydroxylation by mushroom tyrosinase.

The data in Fig. 3(A) show that when exogenous hydrogen peroxide is added, concentrations of 2 and 8 mM shorten the lag period of tyrosine hydroxylation by 50% and 80%, respectively. The data in Fig. 3(B) also show that, in the presence of 0.3 mM xanthine, the lag period was shortened by 50%, with 66 μg/ml xanthine oxidase.

The final products of the reaction of xanthine oxidase with xanthine are uric acid and hydrogen peroxide in a 1:1 ratio. Thus, the maximum concentration of hydrogen peroxide that can be produced by the action of excess

Table 3. Effect of SOD or of catalase on the shortening of the lag period of tyrosine hydroxylation (by mushroom tyrosinase) caused by the xanthine oxidase system*

Part	Mushroom tyrosinase (μg/ml)	Xanthine (mM)	Xanthine oxidase (μg/ml)	SOD (μg/ml)	Catalase (μg/ml)	Lag period (sec)	Lag period (% control)
A	8.3	—	—	—	—	620	100
	8.3	0.125	40	—	—	460	74
	8.3	0.125	40	333	—	546	88
	8.3	0.125	40	—	200	570	92
	8.3	0.250	80	—	—	397	64
	8.3	0.250	80	333	—	564	91
	8.3	0.250	80	—	200	620	106
	8.3	—	—	333	—	620	100
	8.3	—	—	—	200	632	102
B	16.7	—	—	—	—	450	100
	16.7	0.033	60	—	—	288	64
	16.7	0.033	60	250	—	405	90
	16.7	0.033	60	—	220	423	94
	16.7	—	—	250	—	450	100
	16.7	—	—	—	220	454	101
C	33.3	—	—	—	—	330	100
	33.3	0.033	60	—	—	175	53
	33.3	0.033	60	250	—	251	76
	33.3	0.033	60	—	220	297	90
	33.3	—	—	250	—	330	100

*The reaction mixture included 3.3 mM L-tyrosine, 47 mM NaPi buffer (pH 6.5), and the indicated concentrations of xanthine, xanthine oxidase, SOD, catalase and mushroom tyrosinase (added last).

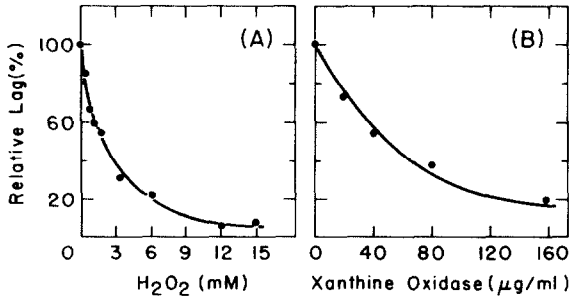

Fig. 3. Comparison between the effect of exogenously added hydrogen peroxide (A) and of the xanthine–xanthine oxidase system (B) on tyrosine hydroxylation by mushroom tyrosinase. The reaction mixture included 3.3 mM L-tyrosine, 47 mM NaPi buffer (pH 6.5), 16.7 μg/ml mushroom tyrosinase (added last) and the following additions: (A) hydrogen peroxide as indicated; (B) 0.3 mM xanthine and the indicated amounts of xanthine oxidase. Lag periods were computed from the curves obtained. The lag period in the control was 320 sec and was taken as 100%.

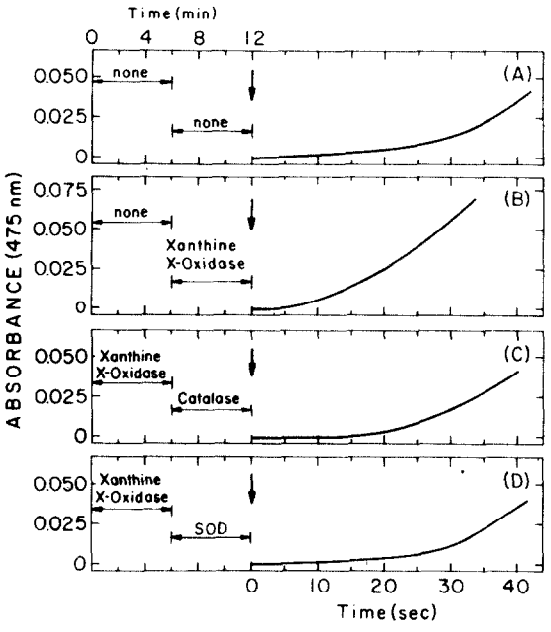

Fig. 4. Effect of reaction products generated by the xanthine–xanthine oxidase system on tyrosine hydroxylation by mushroom tyrosinase. Preincubation conditions were as follows. (A) 47 mM sodium phosphate buffer (pH 6.5) preincubated for 10 min. (B) 47 mM sodium phosphate buffer (pH 6.5) preincubated for 5 min. Then, 0.25 mM xanthine and 80 μg/ml xanthine oxidase were added and the mixture was preincubated for another 5 min. (C) 0.25 mM xanthine, 80 μg/ml xanthine oxidase and 47 mM sodium phosphate buffer (pH 6.5) were preincubated for 5 min. Then, 400 μg/ml catalase was added and the mixture was preincubated for another 5 min. (D) 0.25 mM xanthine, 80 μg/ml xanthine oxidase and 47 mM NaPi buffer (pH 6.5) were preincubated for 5 min. Then, 333 μg/ml SOD was added and the mixture was preincubated for another 5 min. Each reaction mixture was gently swirled throughout the 10 min preincubation period. At the end of the total 10 min preincubation period, L-tyrosine (3.3 mM final concentration) and mushroom tyrosinase (8.3 μg/ml final concentration) were added to each reaction mixture (indicated by the arrow); this represented time 0 for tyrosine hydroxylation. All concentrations given are for the final assay conditions of tyrosine hydroxylation (conducted in a volume of 3 ml).

xanthine oxidase on 0.3 mM xanthine is 0.3 mM. This concentration of hydrogen peroxide is much lower than that required to be added exogenously (Fig. 3A) to obtain a comparable shortening effect on the lag period of tyrosine hydroxylation by mushroom tyrosinase. It can, therefore, be concluded that the maximum concentration of hydrogen peroxide that can be produced by the xanthine–xanthine oxidase system is too low to account for all of the shortening of the lag period caused by the xanthine–xanthine oxidase system.

Does the xanthine–xanthine oxidase system increase the level of DOPA (AH_2)?

As noted above, reactions of mushroom tyrosinase with L-tyrosine in the presence of the xanthine–xanthine oxidase system had longer lag periods in the presence of SOD than in its absence. However, it must be emphasized that SOD, at any of the concentrations tested, counteracted the effect of the xanthine–xanthine oxidase system on the lag period to a limited extent only.

The data presented in Fig. 4, obtained under somewhat different experimental conditions than those in Table 3, demonstrate that the removal of either hydrogen peroxide or O_2^- by pretreatment with catalase (Fig. 4C) or SOD (Fig. 4D) counteracts the stimulating effect otherwise caused by the xanthine–xanthine oxidase system (Fig. 4B) on tyrosine hydroxylation. Thus, the data in Table 3 and Fig. 4 indicate that both O_2^- and hydrogen peroxide, formed by the xanthine–xanthine oxidase system, are needed to shorten the lag period of tyrosine hydroxylation by mushroom tyrosinase.

The superoxide anion can act as either a reducing agent or an oxidizing agent [34]. For example, the superoxide anion can reduce cytochrome *c* or oxidize ascorbate:

$$\text{cytochrome } c\ (Fe^{3+}) + O_2^- \rightarrow \text{cytochrome } c\ (Fe^{2+}) + O_2,$$

$$\text{ascorbate} + 2O_2^- + 2H^+ \rightarrow \text{dehydroascorbate} + 2H_2O_2.$$

Superoxide anions have been shown to serve as a reducing agent for tyramine hydroxylation by dopamine-β-hydroxylase [35]. In view of the capacity of O_2^- to act as a reducing agent [34], it is possible that O_2^- could serve as an AH_2 for tyrosine hydroxylation by mushroom tyrosinase. Moreover, perhaps O_2^- could reduce dopaquinone, thus increasing the initially low level of endogenous DOPA to enable DOPA to act effectively as AH_2. If O_2^- could indeed reduce dopaquinone, then one would expect that a system generating O_2^- would shorten the lag period of tyrosine hydroxylation. It would then be expected that only SOD, but not catalase, should counteract the shortening of the lag period of tyrosine hydroxylation by mushroom tyrosinase caused by the xanthine–xanthine oxidase system.

The specificity of xanthine oxidase for an electron acceptor during the conversion of xanthine to uric acid is low. Oxygen, dyes, ferricyanide and quinones can each serve as an electron acceptor [36]. Therefore, theoretically another possible route by which the DOPA level could be increased due to the addition of the xanthine–xanthine

oxidase system is the following: xanthine oxidase could act on xanthine and use dopaquinone, rather than oxygen, as the hydrogen acceptor, thus producing uric acid and DOPA. In this case, neither SOD not catalase should counteract the shortening of the lag period by mushroom tyrosinase caused by the xanthine–xanthine oxidase system.

It is well-known that ascorbate can reduce dopaquinone to DOPA and becomes oxidized to dehydroascorbate [37]. This is illustrated in an experiment (Fig. 5) in which DOPA is oxidized to dopachrome (measured at 475 nm) by mushroom tyrosinase. The presence of ascorbate in such a system results in a temporary initial delay (lag period) in dopachrome formation, the duration of the delay being dependent on the level of ascorbate present. As soon as all the ascorbate is oxidized to dehydroascorbate, dopaquinone is converted to dopachrome and a net dopachrome formation is observed (Fig. 5).

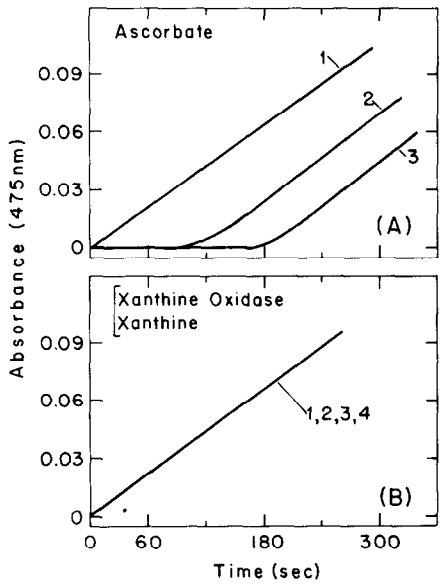

Fig. 5. Does the xanthine–xanthine oxidase system increase the level of AH_2 (DOPA)? The reaction mixture included 0.67 mM DL-DOPA, 47 mM sodium phosphate buffer (pH 6.5), 0.83 μg/ml mushroom tyrosinase (added last) and either ascorbate (A) or the xanthine–xanthine oxidase system (B). (A) Contained ascorbate (mM) as follows: (1) = none, (2) = 7, (3) = 33. Each reaction in (B) contained 0.25 mM xanthine and the following concentrations of xanthine oxidase (μg/ml): (1) = none, (2) = 80, (3) = 160, (4) = 240.

The possibility that the xanthine–xanthine oxidase system converts dopaquinone to DOPA by any of the above mechanisms was tested under the same conditions as those where ascorbate was shown to convert dopaquinone to DOPA (Fig. 5). However, no delay in dopachrome formation was detected when 0.25 mM xanthine and different levels of xanthine oxidase (80–240 μg/ml) were added to a reaction mixture containing 0.67 mM DOPA, 47 mM sodium phosphate buffer (pH 6.5) and 0.83 μg/ml mushroom tyrosinase (Fig. 5B).

The fact that either SOD or catalase counteracted the shortening of the lag period caused by the xanthine–xanthine oxidase system (Fig. 4) and the fact that there was no delay in DOPA oxidation in the presence of the xanthine–xanthine oxidase system (Fig. 5) suggest that the shortening of the lag period, caused by the xanthine–xanthine oxidase system, was not a result of an increase in the level of DOPA due to reduction of dopaquinone by any of the possible mechanisms mentioned above.

Do ·OH, produced by the xanthine–xanthine oxidase system shorten the lag period of tyrosine hydroxylation by mushroom tyrosinase?

Free ·OH can be generated from hydrogen peroxide and $O_2^{\cdot-}$ according to the (iron-catalysed) Haber–Weiss reaction [38] as well as by slightly modified reactions, as suggested by some investigators [36, 39, 40].

Goscin and Fridovich [9] have described a model system in which $O_2^{\cdot-}$, formed by the xanthine–xanthine oxidase system, in the presence of iron salts, caused a non-enzymatic hydroxylation of *p*-cresol. It is possible that, in the experiments described above, some tyrosine molecules are hydroxylated non-enzymatically by hydroxyl radicals, generated by an interaction of hydrogen peroxide and $O_2^{\cdot-}$ in the xanthine–xanthine oxidase system. This would increase the level of endogenous AH_2 molecules (DOPA) generated in the reaction mixture compared with its level in the absence of the xanthine–xanthine oxidase system. Such a possibility could explain the shortening of the lag period of tyrosine hydroxylation by mushroom tyrosinase caused by the xanthine–xanthine oxidase system.

Inhibition of a reaction by both catalase and SOD, as well as by hydroxyl radical scavengers, can be interpreted as being dependent on free hydroxyl radicals generated from hydrogen peroxide and $O_2^{\cdot-}$ by the Haber–Weiss reaction mentioned above. For example, Brawn and Fridovich [41] have recently shown that exposure of Col E1 DNA to the xanthine–xanthine oxidase system resulted in strand scission. Catalase, SOD or hydroxyl radical scavengers protected the DNA against strand scission by the xanthine–xanthine oxidase system. EDTA–iron catalysed the DNA strand scission while DTPA–iron prevented it. Based on the above data, they concluded that the actual DNA-attacking agent was probably the ·OH generated by a metal catalysed interaction between $O_2^{\cdot-}$ and hydrogen peroxide [41].

The effect of ·OH scavengers on the ability of the xanthine–xanthine oxidase system to shorten the lag period of tyrosine hydroxylation by mushroom tyrosinase was tested. Ethanol and mannitol are particularly suitable scavengers for ·OH since they do not interact with $O_2^{\cdot-}$ [42]. The data in Table 4 demonstrate that the shortening of the lag period of tyrosine hydroxylation by the xanthine–xanthine oxidase system can be counteracted by ethanol, mannitol or histidine. Mannitol was the least effective of the three compounds.

Therefore, it can be concluded that the shortening of the lag period of tyrosine hydroxylation by mushroom tyrosinase, caused by the addition of the xanthine–xanthine oxidase system, is mainly due to ·OH generated by an interaction between $O_2^{\cdot-}$ and hydrogen peroxide (Table 4). It is very likely that the ·OH formed by an interaction of $O_2^{\cdot-}$ and hydrogen peroxide (via a Haber–Weiss type reaction) generated by the

Table 4. Effect of ·OH scavengers on the shortening of the lag period of tyrosine hydroxylation (by mushroom tyrosinase) caused by the xanthine–xanthine oxidase system*

Xanthine (mM)	Xanthine oxidase (μg/ml)	Histidine (mM)	Ethanol (mM)	Mannitol (mM)	Lag period (% control)
—	—	—	—	—	100
0.125	80	—	—	—	62
0.125	80	6.6	—	—	80
0.125	80	20.0	—	—	85
0.125	80	33.0	—	—	97
—	—	6.6	—	—	103
—	—	20.0	—	—	102
—	—	33.0	—	—	104
—	—	—	—	—	100
0.125	80	—	—	—	60
0.125	80	—	3.2	—	75
0.125	80	—	6.4	—	81
0.125	80	—	32.0	—	95
—	—	—	3.2	—	103
—	—	—	6.4	—	104
—	—	—	32.0	—	107
—	—	—	—	—	100
0.125	80	—	—	—	58
0.125	80	—	—	32.0	82
0.125	80	—	—	160.0	86
—	—	—	—	32.0	101
—	—	—	—	160.0	103

*The reaction mixture included 1 mM L-tyrosine, 47 mM NaPi (pH 6.5), 4.2 μg/ml mushroom tyrosinase (added last) and the indicated concentrations of xanthine, xanthine oxidase and OH scavengers. The lag period of controls was 550 sec and was taken as 100%.

xanthine–xanthine oxidase system, hydroxylate non-enzymatically a few molecules of tyrosine to yield DOPA. The DOPA formed acts as an efficient AH_2, thus shortening the lag period of tyrosine hydroxylation by mushroom tyrosinase. It only takes 1×10^{-6} M DOPA to completely eliminate the lag period [3]; therefore, direct detection of DOPA produced would be difficult.

The results of the experiments described in this paper do not support the involvement of $O_2^{\cdot-}$ in either: (a) the direct formation of the first few molecules of DOPA (AH_2) necessary to initiate the hydroxylation of tyrosine by mushroom tyrosinase during the lag period; or (b) the hydroxylation of tyrosine by mushroom tyrosinase during the linear phase of the reaction (following the lag period).

EXPERIMENTAL

Materials. Mushroom tyrosinase (grade III), xanthine oxidase (grade I), catalase (C-100) from bovine liver, superoxide dismutase (type I) from bovine blood, cytochrome *c* from horse heart (type VI), L-epinephrine, uric acid, nitroblue tetrazolium (grade III), L-tyrosine, DL-DOPA and L-ascorbic acid were from Sigma; H_2O_2 was from Merck; L-histidine and mannitol were from BDH. All other chemicals were reagent grade. Copper (II) salicylate was prepared as described by the method of ref. [24].

Mushroom tyrosinase. Unless otherwise indicated, monohydroxyphenolase and *o*-dihydroxyphenolase activities were assayed in reaction mixtures that included: (a) for monohydroxyphenolase, 4.2 mM L-tyrosine, 47 mM NaPi buffer (pH 6.5), 8.3 μg/ml mushroom tyrosinase (added last); or (b) for *o*-dihydroxyphenolase 6.7–8.3 mM DL-DOPA, 47 mM NaPi buffer (pH 6.5), 4.2 μg/ml mushroom tyrosinase (added last).

The rate of formation of dopachrome as a function of time was followed at 475 nm in a Varian 635 spectrophotometer equipped with a recorder. The lag period of tyrosine hydroxylation was estimated by extrapolation of each curve to the *x*-axis as suggested in ref. [43]. When DOPA was the substrate, *o*-dihydroxyphenolase activity was computed from the initial linear portion of each curve, and expressed as $\Delta A_{475\,nm}$/min. Mushroom tyrosinase was found to be free of SOD, catalase or peroxidase activities.

Superoxide dismutase. Superoxide dismutase (SOD) activity was assayed by its ability to inhibit the following reactions. (a) Autoxidation of epinephrine to adrenochrome at alkaline pH [44]. This was followed by *A* at 480 nm in a 3 ml reaction mixture that included 1.33 mM L-epinephrine, 33.3 mM Na_2CO_3 (pH 10.2) and 0.13 mM EDTA. Activity of SOD was assayed according to its ability to inhibit adrenochrome formation. Usually, 80% inhibition was obtained with 6.7 μg/ml SOD under the above conditions. (b) Reduction of cytochrome *c* by the xanthine–xanthine oxidase system [45]. This was followed by *A* at 550 nm in a 3 ml reaction mixture that included 167 μg/ml cytochrome *c*, 47 mM NaPi buffer (pH 6.5), 0.1 mM xanthine and 40 μg/ml xanthine oxidase.

Catalase. Catalase activity was assayed by measuring the decomposition of H_2O_2 at 240 nm [46].

Xanthine oxidase. Xanthine oxidase activity was assayed by measuring uric acid production from xanthine at 290 nm or cytochrome *c* reduction at 550 nm [29]. Controls showed that tyrosine (3.33 mM) had no effect on the activity of xanthine oxidase.

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