EFFECT OF CATECHOL-THIOL CONJUGATES ON 
TYROSINASE-DEPENDENT TYROSINE 
HYDROXYLATION

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Summary 1. The tyrosinase reaction in the presence of a thiol 
compound was studied using mushroom tyrosinase (EC 1.10.3.1) with 
regard to catechol-thiol conjugates. 
2. Although tyrosine hydroxylation of tyrosinase was extremely de-
creased in the presence of a thiol compound, the inhibitory effect was 
removed by the addition of a pyrocatechol-cysteine conjugate, S-(2, 
3-dihydroxyphenyl) cysteine, which was not oxidized by the enzyme. 
3. The pyrocatechol-cysteine conjugate was also able to shorten the lag 
period of tyrosinase-dependent tyrosine hydroxylation. 
4. The sigmoidal reaction curve of tyrosine hydroxylation observed 
in the presence of sulfhydryl compounds was found to be caused by 
the catechol-thiol conjugates, the final products of the enzyme reaction, 
which counteract the inhibitory effect of sulfhydryl compounds. 
5. The pyrocatechol-cysteine conjugate, on the other hand, was shown 
to cause the decrease of the reaction rate of the enzyme during incubation.

Melanin formation by tyrosinase was shown to be inhibited by sulfhydryl 
compounds in vitro (1-3). This inhibitory effect of thiol compounds has been 
speculated to be involved in the regulation of the melanin biosynthesis in vivo 
(4, 5). However, because of the complication of the tyrosinase action, the effect 
of thiol compounds on tyrosinase reaction has not been clarified sufficiently. 
Our previous experiments (6) revealed that one of the inhibitory effects of sulf-
ydryl compounds on melanin formation was due to the conjugation of the thiol 
compounds with o-quinones which were the oxidation products of o-dihydroxy-
phenyl compounds.

On the other hand, tyrosinase-dependent tyrosine hydroxylation was also 
known to be inhibited by thiol compounds and this phenomenon was believed to 
be attributable to its chelation with cuprous ions in the enzyme protein (7). Recent
reports concerning melanoma indicated the presence of dopa-cysteine conjugate in the tissue and urine of patients suffering from melanoma (8, 9). In this report, we describe the effect of the catechol-thiol conjugates produced by tyrosinase action on the tyrosine hydroxylation catalyzed by mushroom tyrosinase (EC 1.10.3.1) with respect to the effect of thiol compounds. The results indicated that the catechol-thiol conjugates removed the inhibitory effect of sulfhydryl compounds and shortened the lag period of tyrosine hydroxylation. Though the conjugates were practically not oxidized by tyrosinase, the effects of the conjugates were found to be similar to the effect of dopa, which POMERANTZ and WARNER (10) reported to be a cofactor of melanoma tyrosinase.

MATERIALS AND METHODS

Materials. Mushroom tyrosinase (3,690 units/mg, grade III) was purchased from Sigma Chemicals. Radioactive L-(3,5-3H) tyrosine was obtained from the Radiochemical Centre, Amersham, England. The pyrocatechol-cysteine conjugate, S-(2,3-dihydroxyphenyl) cysteine, was chemically synthesized and purified as described in the previous paper (6). All other reagents were of the highest purity commercially available.

Radioisotopic enzyme assay. The tyrosinase-dependent tyrosine hydroxylation was performed in 0.02 M potassium phosphate buffer (pH 6.8) at 37°C in air for an appropriate time. In most cases, the tyrosinase-dependent tyrosine hydroxylation was estimated by determining the radioactivity of 3H released from L-(3, 5-3H) tyrosine. The principle of the enzyme assay was the same as that for tyrosine hydroxylase described by NAGATSU et al. (11). After an appropriate time of incubation with L-(3,5-3H) tyrosine, 0.1 ml of acetic acid and 0.1 ml of 4 mM ascorbic acid solution were added to 0.4 ml of the reaction mixture to stop the reaction. Following its dilution to 2 ml with distilled water, 10 mg of charcoal (Norit A) were added to adsorb polymerized radioactive tyrosine. After centrifugation, 1.0 ml of the supernatant was passed through the Dowex 50×8 (H+ form) column (0.5×3 cm) to separate tritiated water. The column was washed with 3 ml of distilled water. The effluent and washings were combined. The radioactivity of 1 ml of this solution was measured in BRAY’s scintillator (12). As a result of this assay, each count corresponded to the radioactivity of tritiated water in the 0.05 ml of the reaction mixture.

Assay for catechol-thiol conjugation. Formation of catechol-thiol conjugate was observed photometrically at 295 nm (6). This method was available only for the reaction system containing more sulfhydryl compound than the substrate.

RESULTS

Effect of sulfhydryl compound on tyrosinase reaction

The effect of thiol compounds on the tyrosinase reaction was studied using
Fig. 1. Inhibition of tyrosine hydroxylation by glutathione. The reaction mixture contained 50 μg of mushroom tyrosinase and 5.0 μCi of L-(3,5-3H)tyrosine (0.1 mM) in 2 ml of 0.02 M phosphate buffer (pH 6.8). After the incubation was carried out at 37°C in the presence (○) or absence (•) of glutathione, the radioactivity of released 3H in 0.2 ml of the reaction mixture was measured at each point as described in MATERIALS AND METHODS. Dotted line indicates the half count of that added by glutathione.

Glutathione as a representative of thiol compounds. Glutathione significantly depressed the release of 3H from L-(3,5-3H) tyrosine as shown in Fig. 1. In our previous experiments glutathione was shown to be conjugated with dopa at position 5 of the benzene ring quantitatively (6). Therefore, in the presence of thiol compounds tyrosinase-dependent release of 3H was caused not only by the hydroxylation of L-(3,5-3H) tyrosine but also by the conjugation of thiol compounds with 3H-dopaquinone which was produced from 3H-dopa by dopa oxidation. For this reason, when glutathione was present in the reaction mixture, half of the total 3H released from L-(3,5-3H) tyrosine had to be compared with the other glutathione-free group. When long term incubation was carried out, dopa-glutathione conjugation rate which had been depressed initially by glutathione had become gradually high and resulted in the sigmoidal reaction curve (Fig. 2). Such an increase of the conjugation rate was evoked even in the presence of 10 times as large amount of glutathione as that of tyrosine. Thus, this phenomenon was not thought to be caused by the decrease of glutathione, an inhibitor of tyrosine hydroxylation, according to its conjugation with the substrate. Other conceivable mechanisms of the increase of the reaction rate were as follows: 1) the character of the enzyme was changed to be insensitive to glutathione during incubation, 2) the enzyme activity was strengthened to overcome the inhibitory action of glutathione, 3) small amount of catechol-thiol conjugate produced by the enzyme reaction affected the enzyme to recover its activity.
Fig. 2. Time course of the tyrosinase-dependent conjugate formation in the presence of glutathione. The reaction mixture contained 50 µg of tyrosinase, L-tyrosine (0.1 mM) and glutathione (1 mM, ○; 0.2 mM, ×; 0.1 mM, △) in 2 ml of 0.02 M phosphate buffer (pH 6.8). Other conditions for incubation were the same as that described for Fig. 1.

Our preliminary experiments suggested that the last mechanism was the most probable because the preincubation of tyrosinase with glutathione in the presence or absence of the substrate did not activate the enzyme itself after its separation from low molecular material by gel filtration. Thus the relationship between reaction product and the enzyme activity was studied in the next experiments.

Effect of pyrocatechol-cysteine conjugate on tyrosinase activity in the presence of glutathione

Pyrocatechol-cysteine conjugate was used for the experiments in place of the conjugate of tyrosine with glutathione because of the difficulty in obtaining pure tyrosine-glutathione conjugate. These conjugates were not practically oxidized by the tyrosinase as reported previously (6). The effect of pyrocatechol-cysteine conjugate on the tyrosinase reaction in the presence of glutathione is shown in Fig. 3a, in which the tyrosinase activity was measured photometrically by dopa-glutathione conjugate formation at 295 nm. The enzyme activity was clearly restored by the addition of pyrocatechol-cysteine conjugate. Moreover, the same result was obtained by measuring the release of ³H from L-(3,5-³H) tyrosine (Fig. 3b). These results indicate that the pyrocatechol-cysteine conjugate dependent stimulation of tyrosine-glutathione conjugation was caused by the increase of tyrosine hydroxylation. No other conjugate except catechol-thiol conjugates was detected in these incubation mixture.
Removal of inhibitory effect of glutathione by pyrocatechol-cysteine conjugate

To elucidate the mechanism of the enzyme stimulation by pyrocatechol-cysteine conjugate, we examined whether the conjugate activated the enzyme directly or only removed the inhibitory effect of glutathione. As the concentration of

| Glutathione (M x 10^-4) | Pyrocatechol-cysteine conjugate |
|-------------------------|--------------------------------|
|                         | 0 M                      | 10^-5 M                  | 10^-4 M                  |
| 1                       | 0.0323                   | 0.0805                   | 0.167                    |
| 2                       | 0.0146                   | 0.0710                   | 0.161                    |
| 4                       | 0.0109                   | 0.0645                   | 0.161                    |
| 6                       | 0.0097                   | 0.0605                   | 0.170                    |
glutathione was increased in the incubation mixture, tyrosinase-dependent tyrosine hydroxylation was shown to become slower (Table 1). While, in the presence of pyrocatechol-cysteine conjugate, inhibitory action of glutathione was reduced and was entirely eliminated by $10^{-4}$ M of pyrocatechol-cysteine conjugate. In the next experiment, glutathione was added to the medium containing tyrosinase and pyrocatechol-cysteine conjugate. As shown in Fig. 4, the release of $^3$H from L-(3,5-$^3$H) tyrosine was doubled when glutathione was added. This increase, however, was clearly caused by the conjugation of $^3$H-dopaquinone with glutathione as described above. For this reason, the count of $^3$H in the presence of glutathione had to be halved to compare with the other count for the enzymic hydroxylation activity. Thereby, it was clear that glutathione did not inhibit tyrosine hydroxylation when pyrocatechol-cysteine conjugate was present in the medium and this fact indicates that the conjugate prevented the inhibitory effect of glutathione.

Fig. 4. Effect of glutathione of tyrosinase-dependent tyrosine hydroxylation in the presence of the pyrocatechol-cysteine conjugate. The reaction mixture contained 5 $\mu$g of tyrosinase, 2.5 $\mu$Ci of L-(3,5-$^3$H)tyrosine (0.25 mM), the pyrocatechol-cysteine conjugate (0.1 mM) and glutathione (0.25 mM, ○; 0 mM, ×) in 2 ml of 0.02 M phosphate buffer. Other incubation conditions and measurement of radioactivity were the same as those described in the legend to Fig. 1. Dotted line indicates the half count of that added by glutathione.

Removal of lag period of tyrosinase by pyrocatechol-cysteine conjugate

The next experiments were carried out in the absence of the thiol compound to make clear whether the conjugate was able to activate tyrosinase itself. The results indicated that pyrocatechol-cysteine conjugate had an ability to remove the lag time of the enzyme reaction (Fig. 5a). While, in spite of the increase of the initial velocity of the reaction, a higher concentration of the conjugate rather inactivated the enzyme during the incubation (Fig. 5b).

From these results, it was clarified that the inhibitory effect of thiol compounds
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Fig. 5. Effect of the pyrocatechol-cysteine conjugate on tyrosinase dependent tyrosine hydroxylation. (a) The reaction mixture contained 5 µg of tyrosinase, 2.5 µCi of L-(3,5-3H)tyrosine (0.25 mM) and the pyrocatechol-cysteine conjugate (0.1 mM, △; 0.01 mM, ○; 0 mM, ×) in 2 ml of 0.02 M phosphate buffer (pH 6.8). Other incubation conditions and measurement of radioactivity was the same as those described in the legend to Fig. 1. (b) The reaction mixture contained 50 µg of tyrosinase, 5.0 µCi of L-(3,5-3H)tyrosine (0.1 mM) and the pyrocatechol-cysteine conjugate. The concentration of the conjugate and other incubation conditions were the same as those of (a).

On tyrosinase was removed by catechol-thiol conjugates which were produced by the enzyme reaction in the presence of thiol compounds and that the conjugates caused the removal of the lag time of the tyrosine hydroxylation as shown in the following scheme.

**DISCUSSION**

In case of mammalian tyrosinase, dopa was found to shorten the lag time of the tyrosine hydroxylation (13). Moreover, this enzyme was reported to require dopa as a cofactor in vivo by POMERANTZ and WARNER (10). In the present experiments, the catechol-thiol conjugates were also shown to play a role as cofactors for mushroom tyrosinase, though the conjugates themselves were not practically oxidized by the enzyme. These compounds were not only able to diminish the lag time but also removed the inhibitory effect of thiol compounds. Ascorbic acid has been found to shorten the lag time (13) and to remove the inhibitory effect of thiol compounds (our unpublished data). However, our preliminary experiments showed that a comparatively small amount of ascorbic acid (10⁻⁶ M) had
lower ability to remove lag time caused by thiol compounds than the same amount of pyrocatechol-cysteine conjugate did. The role of ascorbic acid in dopamine β-hydroxylase was reported to be the reduction of the cupric ion in the enzyme to the cuprous ion (14). Then it is likely that ascorbic acid affected tyrosinase with its reducing power. DUCKWORTH and COLEMAN (15) suggested in their study of physicochemical and kinetic properties of mushroom tyrosinase that the enzyme had an activator site to be bound to o-dihydroxyphenyl derivatives. Therefore, the effect of the conjugates shown in the present experiments may be related to this activator site. In general, inhibitory effect of thiol compounds on tyrosinase was thought to be caused by its chelation with cuprous ions in the enzyme (7). Then one may consider the following probable mechanisms for the removal of the thiol-induced inhibition by the conjugates: 1) cuprous ion-thiol chelation was replaced by the conjugates to form cuprous ion-conjugates chelation, 2) attachment of the conjugates to the enzyme caused the conformational change of the enzyme to permit itself to be resistant against thiol compounds. To elucidate such mechanisms, more precise experiments have to be carried out with regard to active site, activator site and other conformation of the enzyme. Hydroxylation of p-coumaric acid by spinach beet phenolase was reported to have lag time and o-dihydroxyphenol was shown to remove or shorten the lag time (16). These phenomena were very similar to the disappearance of the lag time of tyrosinase reaction by the conjugates. Therefore, these two phenomena were likely to involve the same fundamental mechanism.

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