MAMMALIAN TYROSINASE: THE RELATIONSHIP OF COPPER TO ENZYMATIC ACTIVITY

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Copper has been reported to be an essential part of the enzyme tyrosinase prepared from various plant and insect sources (1-8). In the case of potato tyrosinase, Kubowitz (1, 2) found that the enzyme could be inhibited by reagents which combine with copper, e.g. diethyl dithiocarbamate, salicylaldoxime, and carbon monoxide. He demonstrated that treatment of the enzyme with cyanide followed by dialysis resulted in a decrease in the copper content of the enzyme preparation and a loss of enzymatic activity. Addition of sufficient cupric ions resulted in practically complete restoration of activity. Other metals (iron, cobalt, nickel, manganese, and zinc) were ineffective in restoring enzymatic activity. Allen and Bodine (3) carried out experiments on protyrosinase from grasshopper eggs similar to those of Kubowitz and obtained essentially the same results.

Several investigators have shown that copper is associated with the activity of phenoloxidases from mushrooms and tea leaf (4-8).

Hogeboom and Adams (9) demonstrated an inhibition by cyanide of the enzymatic oxidation of L-tyrosine and dihydroxyphenyl-L-alanine (dopa), using extracts from the Harding-Passey mouse melanoma; they concluded that the enzymes concerned in the oxidation of tyrosine and dopa by melanotic tissue are iron- or copper-containing enzymes. Cunningham (10) and Greenstein et al. (11) found that copper is present in human melanomas.

The experiments reported here present further evidence that mammalian tyrosinase prepared from the Harding-Passey mouse melanoma is an enzyme which requires copper for activity. This evidence is based on the demonstration that copper can be removed from the enzyme preparation with cyanide to produce a relatively inactive enzyme and that the addition of copper to such a preparation restores activity. Furthermore, sub-

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stances that combine with copper inactivate the enzyme and this inactivation is, in some instances, reversed by the addition of excess copper.

**EXPERIMENTAL**

Tyrosinase was prepared from the Harding-Passey mouse melanoma by the following procedure: Fresh mouse melanoma tissue was ground with sand and cold distilled water for 10 minutes. The mixture was treated in a Waring blender at 5° for 10 minutes and then centrifuged at 3000 r.p.m. for 30 minutes. The supernatant fluid was dialyzed twice against 100 times its volume of cold distilled water for 48 hours. The final solution had approximately 4 times the volume of the original tumor tissue. Enzymatic activity was determined manometrically by measurements of the oxygen uptake in the Warburg apparatus at 38°, in 0.1 m potassium phosphate buffer at pH 6.8. The substrate, 0.5 mg. of dopa in 0.1 m potassium phosphate buffer, was added from the side arms of the Warburg vessels to the enzyme preparation after 10 minutes equilibration of the solution at 38°. Autoxidation of dopa under these conditions is negligible for the time intervals of the experiments. In general, 3 units of enzyme were used per vessel which contained 3 ml. of reaction mixture. 1 enzyme unit is defined (12) as the amount of enzyme required to catalyze the absorption of 1 μl. of oxygen per minute by 1 mg. of substrate (dopa) when oxidation is proceeding at a maximal rate.

**Removal of Copper with Cyanide**—A method of removing copper from tyrosinase similar to that used by Kubowitz (1, 2) and Allen and Bodine (3) was followed. The enzyme preparation used in this experiment was made according to the method given above, except that the preparation was not dialyzed until after treatment with potassium cyanide. All of the procedures which follow were carried out at 5°. To 15 ml. of enzyme preparation were added 10 ml. of 1 m KCN solution and the mixture was allowed to stand overnight. The enzyme was precipitated by adding 50 ml. of saturated ammonium sulfate solution, which was likewise saturated with potassium cyanide. After 2 hours, the mixture was centrifuged at 3000 r.p.m. for 1 hour. The supernatant fluid was discarded, 10 ml. of distilled water were added to the precipitate, and the mixture was dialyzed in a cellophane bag against 2000 ml. of cold distilled water. After 4 hours, a change of water was made and dialysis was further carried out for 15 hours. The water was changed again and the dialysis was continued for another

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1 Dopa was ordinarily used as the substrate in the present study because changes in enzymatic activity could be readily observed. The enzymatic oxidation of tyrosine is complicated by the induction period in the conversion of tyrosine to dopa (12). As indicated in the text, however, tyrosine was used as the substrate in a few of the experiments.
24 hours. The dialyzed preparation was then diluted to a volume of 26 ml. with distilled water. A similar volume of tyrosinase preparation used as a control received identical treatment, except that distilled water was used in place of potassium cyanide solutions, and saturated ammonium sulfate without potassium cyanide was used to precipitate the control tyrosinase.

The quantitative copper determinations on the enzyme preparations were carried out by drying the samples, ashing at 450°, and subsequent colorimetric estimation of copper in the ash solutions with diethylthiocarbamate. The copper content of the enzyme preparation was 0.19 per cent. After treatment with cyanide, the enzyme preparation contained 0.09 per cent copper.

The activities of the tyrosinase preparations before and after treatment with cyanide, with dopa as the substrate, are demonstrated in Fig. 1. It can be seen that approximately 85 per cent of the enzymatic activity was lost after most of the copper had been removed.

The addition of cupric ions (as copper sulfate) to the cyanide-treated enzyme preparation resulted in restoration of enzymatic activity, the extent of restoration being a function of the concentration of added cupric ions; with sufficient added copper, complete enzymatic activity was restored (Fig. 2). Relative per cent restoration of activity was determined from the ratios of the maximal amount of oxygen absorbed during a 10 minute interval by the cyanide-treated tyrosinase (with varying amounts of added cupric ions) to the oxygen absorbed by the control tyrosinase.

Addition of the amount of copper that was removed by cyanide treat-
ment resulted in a restoration of approximately 90 per cent of the activity of the control tyrosinase. Kubowitz (1, 2) found it necessary to add about 10 times the amount of copper originally present to his copper-free potato tyrosinase to obtain complete restoration of activity. Allen and Bodine (3) found that twice as much copper as was originally present in grasshopper egg protyrosinase was necessary to restore complete activity.

![Graph](http://www.jbc.org/)

**Fig. 2.** The effect of added Cu++, at a constant enzyme concentration, on the restoration of enzymatic activity to a cyanide-treated dialyzed tyrosinase preparation from mouse melanoma. Substrate, dopa (8.45 X 10^-4 molar); pH 6.8; 38°.

When 0.5 mg. of tyrosine was used as the substrate in place of 0.5 mg. of dopa, there was no oxygen uptake with the cyanide-treated tyrosinase, although the control preparation catalyzed the oxidation of tyrosine after an induction period of approximately 60 minutes. Furthermore, in the presence of sufficient cupric ions to restore 100 per cent activity against dopa as the substrate, there was likewise no oxidation of tyrosine even after 4 hours. However, when small amounts of dopa (0.05 mg.) were added to this copper-activated tyrosine-tyrosinase mixture, there was complete restoration of tyrosinase activity and the rate of tyrosine oxidation was equal to that obtained with the control tyrosinase under similar conditions.
In addition to copper sulfate, the following solutions of metallic salts were added to the cyanide-treated tyrosinase: ferric nitrate, zinc sulfate, cobaltous sulfate, magnesium chloride, manganese chloride, and nickelous sulfate. These substances were added in amounts equivalent to 10 times the amount of copper sulfate required to restore 100 per cent activity. Some enzymatic activity was restored with nickelous sulfate, but none of the other metals had any effect.

In addition to potassium cyanide as an inactivator, several sulfur-containing organic compounds which form complexes with heavy metals were studied. The compounds studied were thiourea, thiouracil, glutathione, cysteine, 2,3-dithiopropanol (BAL), diethylthiocarbamate, and phenylthiourea. Compounds of these types inhibit various plant tyrosinases (1, 13-16).

Varying amounts of each of these compounds were allowed to incubate with constant amounts of enzyme (approximately 3 units) in the main reaction compartment of the Warburg vessels at 38° for 30 minutes. After this time 0.5 mg. of dopa was added from the side arms, and the oxygen consumption was recorded at 10 minute intervals for 120 minutes. The per cent activity of the tyrosinase treated with a given compound relative to that of the control tyrosinase was calculated from the ratios of the maxi-

**Fig. 3.** The effect of varying concentrations of inhibitors on the enzymatic activity, at constant enzyme concentration, of mouse melanoma extracts in the oxidation of dopa (8.45 X 10^-4 molar) at pH 6.8; 38°. Curve A, thiourea; Curve B, 2,3-dithiopropanol (BAL); Curve C, diethylthiocarbamate, Curve D, phenylthiourea.
mamalian tyrosinase and copper

values of the oxygen uptake during a 10 minute interval for the respective preparations. The results for four of these compounds are shown in Fig. 3. The inhibition curves (not shown) for glutathione, cysteine, and thiouracil had the same general S-shaped form as those shown in Fig. 3, falling between Curves A and B. The negative logarithm of the inhibitor concentration producing 50 per cent inhibition by the various inhibitors studied is recorded in Table I.

Reversal of Tyrosinase Inhibition by Addition of Copper—To study the possible reversal of tyrosinase inhibition by the addition of copper, concentrations of inhibitors giving approximately 90 per cent loss of activity were incubated with the enzyme in the Warburg apparatus as described above. After adding the substrate, dopa, the reaction was allowed to run for 40 minutes. At this time, cupric sulfate in 25 per cent excess of the inhibitor on a mole for mole basis was added from a second side arm to the reaction mixture. The inhibition of the reaction in the cases of diethyldithiocarbamate and BAL was reversed by the added cupric ions. An example of such reversal of inhibition by copper is given in Fig. 4. The inhibition produced by phenylthiourea could not be reversed by as much as 100 per cent excess of copper. Reversal by copper was not studied for thiourea, thiouracil, glutathione, and cysteine because addition to the reaction mixture of the amount of copper sulfate necessary to give an excess over the inhibitor resulted in a precipitate with the phosphate buffer.

When tyrosine was used as the substrate instead of dopa, an effect similar to that obtained in the case of cyanide inhibition was observed; viz., the addition of an excess of cupric ions to tyrosinase inhibited by diethyldithiocarbamate or BAL did not restore activity toward tyrosine unless a small amount of dopa was also present. That is, an excess of cupric ions restored activity of a diethyldithiocarbamate or BAL-inhibited tyrosinase

<table>
<thead>
<tr>
<th>Inhibitor (I)</th>
<th>- Log I (molar) for 50 per cent inhibition</th>
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<tbody>
<tr>
<td>Thiourea</td>
<td>1.75</td>
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<tr>
<td>Thiouracil</td>
<td>2.05</td>
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<tr>
<td>Glutathione</td>
<td>2.35</td>
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<tr>
<td>Cysteine</td>
<td>2.90</td>
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<tr>
<td>2,3-Dithiopropanol (BAL)</td>
<td>3.85</td>
</tr>
<tr>
<td>Diethyldithiocarbamate</td>
<td>4.00</td>
</tr>
<tr>
<td>Phenylthiourea</td>
<td>5.60</td>
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toward dopa but the preparation then had an indefinitely prolonged induction period in the oxidation of tyrosine.

The metal salts, ferric nitrate, zinc sulfate, cobaltous sulfate, magnesium chloride, and manganese chloride, had no effect on reversing the inhibition produced by diethyldithiocarbamate and BAL. Nickelous sulfate did not reverse diethyldithiocarbamate inhibition but did restore slight activity to the BAL-inhibited tyrosinase.

![Graph showing the inhibitory effect of diethyldithiocarbamate on the enzymatic oxidation of dopa by mouse melanoma preparation and the reversal of inhibition by Cu++]

**DISCUSSION**

The experimental evidence reported above indicates that tyrosinase from the Harding-Passey mouse melanoma is an enzyme which requires copper for its activity. When the enzyme preparation is treated with potassium cyanide and dialyzed, much of the copper originally present in the preparation is removed. The "copper-free" tyrosinase has little enzymatic activity. The addition to this preparation of an amount of cupric ions equal to the amount of copper removed by the cyanide-dialysis treatment restored approximately 90 per cent of the original activity. Ferric, zinc, cobaltous, magnesium, and manganese ions did not produce any effect. Nickelous ions restored some activity but were not as effective as cupric ions.

The enzyme is inhibited by certain organic thio compounds. It is likely that these compounds exert most of their action by combining with the copper required for enzymatic activity. The inhibition of tyrosinase by
the thio compounds can in some instances be reversed by the addition of an excess of cupric ions. Other metal ions do not affect the inhibition.

It is interesting to note that, when tyrosine is used as the substrate for tyrosinase inhibited by cyanide treatment or by the compounds listed in Table I, activity is not restored by the addition of cupric ions unless a small amount of dopa is also present. That is, "reactivated" tyrosinase has an indefinitely prolonged induction period in the oxidation of tyrosine alone, yet in the presence of small amounts of dopa the reactivated enzyme is fully as active against tyrosine as was the original untreated enzyme. This parallels our earlier experience with various fractionation procedures supposedly designed to separate "tyrosinase" from "dopa oxidase," and is further evidence that under certain circumstances the distinction between these two activities is no longer valid (12).

It is an extremely difficult task to prove categorically that an enzyme molecule itself contains copper. For such proof, one would have to obtain a pure enzyme preparation that satisfies all the criteria required of a homogeneous preparation. One would then have to demonstrate the constancy of the copper content in the active enzyme under a variety of conditions. Finally, removal of copper should lead to a loss of enzymatic activity. Activity should be restorable on the addition of sufficient copper in the proper form.

We have not been able to carry out this detailed proof to show that mammalian tyrosinase is a copper-containing enzyme because we have not as yet been able to obtain a pure tyrosinase preparation. Mammalian tyrosinase activity is associated with the particulate matter of cells (12) and no way has been found to free the enzyme from the particles or to obtain particulate preparations with purely tyrosinase activity. Even without this rigorous proof, however, we feel that the evidence presented above justifies the conclusion that copper is as firmly associated with mammalian tyrosinase activity as it is with tyrosinase from plant and insect sources.

The inhibition curves shown in Fig. 3 vary somewhat in shape. The curves for BAL and diethyldithiocarbamate inhibition have a steep slope and correspond in form to the theoretical curve resulting from plotting the enzymatic activity against the logarithm of the total inhibitor concentration in accordance with the following equation:

\[ I = \left( \frac{1 - a}{a} \right) K + 2(1 - a)E \]  

where \( I \) = the molar concentration of the inhibitor (total), \( E \) = the molar concentration of the enzyme (total), \( a \) = the fractional activity of the enzyme, and \( K \) = the dissociation constant of the enzyme-inhibitor complex.
This equation represents the following enzyme-inhibitor reaction (17):

\[ \text{E} + 2\text{I} \rightleftharpoons \text{E}2\text{I} \]

The curve for thiourea inhibition is similar to the theoretical curve obtained from the relationship:

\[ I = \left( \frac{1 - a}{a} \right) K + (1 - a)E \]

(2)

This relationship corresponds to the following enzyme-inhibitor reaction:

\[ \text{E} + \text{I} \rightleftharpoons \text{E}\text{I} \]

The shape of the activity-inhibitor curves (Fig. 3) may indicate the number of molecules of inhibitor which combine with active enzyme centers. Thus one might conclude from our data that either 1 or 2 molecules of thiourea, BAL, or diethyldithiocarbamate combine with one active enzyme center. However, the experimental difficulties associated with obtaining precise curves, together with the admitted complexity of the enzyme system concerned, make us hesitant to reach such definite conclusions concerning the stoichiometric relation between enzyme and inhibitor until more adequate data are available. In this connection, it is interesting to note that DuBois and Erway (13), using potato tyrosinase and inhibitors similar to those described here, found straight line relationships between percent activity and the logarithm of inhibitor concentrations. However, their data cover the range of activity between 20 and 85 percent only, and in this range it is difficult to establish the true shape of the activity-inhibitor curve.

The phenylthiourea inhibition curve cannot be represented by any simple enzyme-inhibitor reaction. The fact that this inhibition cannot be reversed by cupric ions indicates that phenylthiourea may inhibit tyrosinase by a way other than that of binding copper.

The curve in Fig. 2, which represents the restoration of enzymatic activity by the addition of cupric ions to a "copper-free" tyrosinase preparation, is of somewhat the same type as the activity-inhibitor curves just discussed, but is even less susceptible to quantitative interpretation. This is no doubt due in part to our inability to obtain an enzyme preparation which was completely free from copper and also devoid of enzymatic activity. However, the relation between added copper and enzyme activity shown in Fig. 2 suggests to us the strong possibility that it represents a reversible equilibrium between enzyme molecules and copper ions. Further study will likewise be necessary to clarify this point.
1. Mammalian tyrosinase requires copper for enzymatic activity.
2. Compounds that combine with copper inhibit mammalian tyrosinase; in some instances, this inhibition can be reversed by added cupric ions. Other metal ions do not have this effect.
3. The nature of the reaction between tyrosinase and inhibitors and between "copper-free" tyrosinase and copper is discussed.

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