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# INACTIVATION OF MUSHROOM TYROSINASE BY HYDROGEN PEROXIDE*

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**Key Word Index**—*Agaricus bisporus*; Agaricaceae; mushroom; tyrosinase; inactivation; hydrogen peroxide; cupric ion.

**Abstract**—Hydrogen peroxide ($H_2O_2$) inactivates mushroom tyrosinase in a biphasic manner, with the rate being faster in the first phase than in the second one. The inactivation of the enzyme is dependent on $H_2O_2$ concentration (in the range of 0.05–5.0 mM), but independent of the pH (in the range of 4.5–8.0). The rate of inactivation of mushroom tyrosinase by $H_2O_2$ is faster under anaerobic conditions (nitrogen) than under aerobic ones (air). Substrate analogues such as L-mimosine, L-phenylalanine, *p*-fluorophenylalanine and sodium benzoate protect the enzyme against inactivation by $H_2O_2$. Copper chelators such as tropolone and sodium azide also protect the enzyme. Under identical conditions, apotyrosinase is not inactivated by $H_2O_2$, unlike holotyrosinase. The inactivation of mushroom tyrosinase is not accelerated by an $OH^{\cdot}$ generating system ($Fe^{2+}$–EDTA–$H_2O_2$) nor is it protected by $OH^{\cdot}$ scavengers such as mannitol, urate, sodium formate and histidine. Exhaustive dialysis or incubation with catalase does not restore the activity of $H_2O_2$-inactivated enzyme. The data suggest that $Cu^{2+}$ at the active site of mushroom tyrosinase is essential for the inactivation by $H_2O_2$. The inactivation does not occur via the $OH^{\cdot}$ radical in the bulk phase but probably via an enzyme-bound $OH^{\cdot}$.

## INTRODUCTION

Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxido-reductase, EC 1.14.18.1) is a copper-containing enzyme which catalyses two reactions in which molecular oxygen is the hydrogen acceptor and phenol is the hydrogen donor:

A. monophenol + $O_2$ + $AH_2$ → *o*-dihydroxyphenol + $H_2O$ + A

B. *o*-dihydroxyphenol + $\frac{1}{2}$ $O_2$ → *o*-quinone + $H_2O$,

where $AH_2$ represents a hydrogen donor [1].

$H_2O_2$ is known as an inactivator of several copper-containing enzymes [2–7], such as dopamine $\beta$-monooxygenase [6], $Cu^{2+}$–SOD [2, 3, 5] and mushroom tyrosinase [4]. The inactivation of dopamine $\beta$-monooxygenase by $H_2O_2$ was shown to be dependent on the presence of enzyme-bound $Cu^{2+}$ [6], and that of $Cu^{2+}$–SOD was attributed to the production of $OH^{\cdot}$ bound to the enzyme, which oxidized an imidazole residue at the active site [2, 3, 5].

Jolley *et al.* [4] mentioned that exposure of mushroom tyrosinase to high concentrations of $H_2O_2$ resulted in loss of both monohydroxyphenolase and *o*-dihydroxyphenolase activities and to an eventual inactivation of the enzyme, but they did not describe the conditions or attempt to explain the mechanism by which this inactivation occurs. It was shown recently [8] that $H_2O_2$ has a multiple effect on avocado polyphenoloxidase: at low concentrations it shortened the lag period of tyrosine hydroxylation, and at higher concentrations it inactivated the enzyme.

The present study was undertaken in order to describe the $H_2O_2$ inactivation phenomenon in detail, using mushroom tyrosinase as the enzyme source.

## RESULTS AND DISCUSSION

Exposure of mushroom tyrosinase to $H_2O_2$ for different periods of time resulted in a gradual loss of enzyme activity. We shall refer to this as a phenomenon of enzyme inactivation.

The rate of inactivation of mushroom tyrosinase by different concentrations of $H_2O_2$ was determined and a plot of the percent remaining activity as a function of preincubation time is shown in Fig. 1. Biphasic curves were obtained (Fig. 1) with the rate of inactivation of mushroom tyrosinase being linear and fast during the first few minutes of exposure of the enzyme to $H_2O_2$ and non-linear and slow thereafter. The data in Fig. 1 illustrates that the rate of inactivation of mushroom tyrosinase was dependent on $H_2O_2$ concentration. Fifty percent inactivation occurred after 30, 8 and 1.5 min preincubation, with 0.05, 0.5 and 5.0 mM $H_2O_2$, respectively.

Attempts to find out whether the inactivation of mushroom tyrosinase by $H_2O_2$ was reversible or not showed that exhaustive dialysis of the inactivated enzyme

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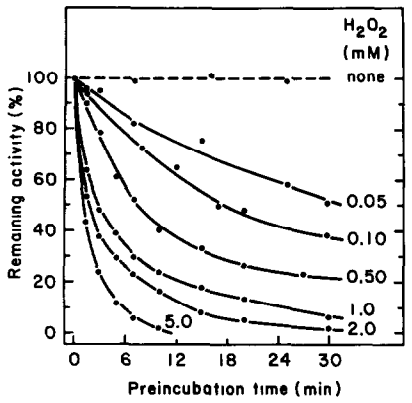



Fig. 1. Effect of different concentrations of $H_2O_2$ on the rate of mushroom tyrosinase inactivation. The preincubation mixture contained, in a total volume of 26 ml, 70 mM NaPi (pH 6.5), 650 μg mushroom tyrosinase (added last), and the indicated concentrations of $H_2O_2$. Aliquots (2 ml) were withdrawn at various times and *o*-dihydroxyphenolase activity was assayed immediately, in a total volume of 3 ml, by adding DL-DOPA at time zero so that the final concentration of DL-DOPA was 6.7 mM. *o*-Dihydroxyphenolase activity of the control (no $H_2O_2$) at time zero was 0.61 $\Delta A$ 475 nm/min, while activities at time zero in the presence of 0.05, 0.10, 0.50, 1.0, 2.0 and 5.0 mM $H_2O_2$ were 0.73, 0.76, 0.82, 0.95, 0.90 and 0.83 $\Delta A$ 475 nm/min, respectively, and were taken as 100%.

against 0.05 M NaPi buffer (pH 6.5) did not restore the activity.

*Effect of pH on the degree of inactivation of mushroom tyrosinase by* $H_2O_2$

The effect of dye-sensitized photooxidation of tyrosinase was studied by several investigators [9, 10]. Gutteridge *et al.* [9] have shown that mushroom tyrosinase and *Neurospora* tyrosinase were inactivated by dye-sensitized photooxidation and that the rate of inactivation was faster in the presence of cyanide and was pH-dependent (between pH 5.5 and 8.0). Pfiffner and Lerch [10] reported that the *Neurospora* holo-tyrosinase was not affected by dye-sensitized photooxidation but that the apotyrosinase was inactivated by this treatment and that the rate of inactivation was pH-dependent. In view of the above, the effect of pH on the rate of inactivation of mushroom tyrosinase by $H_2O_2$ was also studied. We found that the rate of inactivation of the enzyme was independent of the pH in the range of 4.5–8.0 (data not shown).

*Is the catalytic site of the enzyme modified by* $H_2O_2$?

Mitsuda *et al.* [11] found that soybean lipoxygenase is inactivated by $H_2O_2$ or by cysteine. For 50% inactivation of the enzyme, $6 \times 10^{-6}$ M $H_2O_2$ or $7 \times 10^{-6}$ M cysteine was required. Mitsuda *et al.* [11] used the competitive inhibitor of lipoxygenase, linolelaidic acid, to prove that the catalytic site of the enzyme was the site which was modified by $H_2O_2$ or by cysteine.

The inactivation of mushroom tyrosinase by $H_2O_2$ may also be due to modification at the catalytic site of the enzyme. The effect of $H_2O_2$ on mushroom tyrosinase was therefore tested in the presence and absence of competitive inhibitors (substrate analogues), which, by occupying the catalytic site, can afford a protection against the inactivation, provided that $H_2O_2$ acts specifically on the catalytic site. Sodium benzoate, L-mimosine, L-phenylalanine and *p*-fluorophenylalanine are known as typical competitive inhibitors for tyrosinase [12–14]. Preliminary data showed, as expected, that sodium benzoate, L-mimosine, L-phenylalanine and *p*-fluorophenylalanine each inhibited *o*-dihydroxyphenolase activity of mushroom tyrosinase. The data in Table 1 demonstrate that L-mimosine was the most effective inhibitor ($I_{50} = 1.6 \times 10^{-4}$ M), while L-phenylalanine was the least effective ($I_{50} = 5.3 \times 10^{-2}$ M). The inhibition exhibited by the several substrate analogues was taken into account when studying the possibility that these substrate analogues protect the enzyme against $H_2O_2$ inactivation. The activity in the presence of the substrate analogue was taken as 100%.

It was found that L-mimosine, sodium benzoate, *p*-fluorophenylalanine and L-phenylalanine, in decreasing order, each protected the enzyme against inactivation by $H_2O_2$ in a similar pattern. The protection provided by L-mimosine against inactivation of the enzyme by $H_2O_2$ is illustrated in Fig. 2. Controls consisting of preincubation of mushroom tyrosinase with each one of the four substrate analogues (in the absence of $H_2O_2$) for a period of 30 min did not show any inactivation of the enzyme.

It is interesting to note that the extent of protection afforded by each of the substrate analogues against $H_2O_2$ inactivation seems to be directly correlated with the extent of inhibition exhibited by each substrate analogue on the enzyme as shown above in Table 1.

Better protection against $H_2O_2$ inactivation of mushroom tyrosinase was observed when preincubation of the enzyme with any of the above substrate analogues was done for 10 min prior to the addition of $H_2O_2$ than when both the substrate analogue and $H_2O_2$ were added at the same time to the enzyme. The better protection afforded by prior preincubation of the enzyme with the substrate analogue was probably due to the binding of the latter to the active site of the enzyme, thereby preventing $H_2O_2$ from getting there. The fact that substrate analogues protected mushroom tyrosinase against $H_2O_2$ inacti-

Table 1 Inhibition of *o*-dihydroxyphenolase activity of mushroom tyrosinase by substrate analogues

| Substrate analogue | $I_{50}$ (M) |
|---|---|
| L-Mimosine | $1.6 \times 10^{-4}$ |
| Sodium benzoate | $3.0 \times 10^{-3}$ |
| *p*-Fluorophenylalanine | $1.5 \times 10^{-2}$ |
| L-Phenylalanine | $5.3 \times 10^{-2}$ |

The reaction mixture contained, in a total volume of 3 ml, 6.7 mM DL-DOPA, 47 mM NaPi buffer (pH 6.5), 50 μg mushroom tyrosinase (added last), and different concentrations of each substrate analogue. $I_{50}$ = the concentration of the substrate analogue that caused 50% inhibition of *o*-dihydroxyphenolase activity of mushroom tyrosinase.

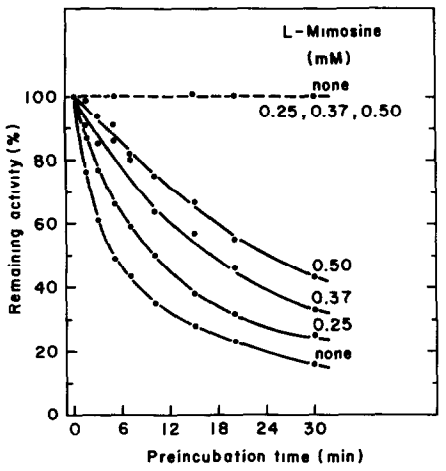



Fig. 2. L-Mimosine as a protector against inactivation of mushroom tyrosinase by $H_2O_2$. The preincubation mixture contained, in a total volume of 20 ml, 70 mM NaPi buffer (pH 6.5), 500 $\mu$g mushroom tyrosinase (added last), and L-mimosine (pH 6.5) as indicated. Control flasks (----) had no $H_2O_2$, while experimental flasks (——) contained 0.5 mM $H_2O_2$. Aliquots (2 ml) were withdrawn at various times and *o*-dihydroxyphenolase activity was assayed immediately, in a total volume of 3 ml, by adding DL-DOPA at time zero so that the final concentration of DL-DOPA was 6.7 mM. The activity of the control samples (without $H_2O_2$) was 0.49, 0.27, 0.23 and 0.20 $\Delta A$ 475 nm/min in the presence of 0, 0.25, 0.37 and 0.50 mM L-mimosine, respectively, and taken as 100%. The activity at time zero in the presence of 0.5 mM $H_2O_2$ (but without L-mimosine) was 0.72 $\Delta A$ 475 nm/min, and that in the presence of 0.5 mM $H_2O_2$ plus 0.25, 0.37 or 0.50 mM of L-mimosine was 0.48, 0.52 and 0.45 $\Delta A$ 475 nm/min, respectively, and also taken as 100%.

vation strongly suggests that $H_2O_2$ attacks the active site of the enzyme.

*Is the inactivation of mushroom tyrosinase by $H_2O_2$ via copper bound to the enzyme?*

Dopamine $\beta$-monooxygenase is a copper enzyme that is inactivated by $H_2O_2$ [6]. Skotland and Ljones [6] found that under comparable conditions, the holodopamine $\beta$-monooxygenase was inactivated by 0.25 mM $H_2O_2$, while the apoenzyme was not. The apoenzyme was inactivated only when exposed to 0.25 mM $H_2O_2$ in the presence of $CuSO_4$ (2 $\mu$M). On the basis of this and additional experiments, Skotland and Ljones [6] concluded that the inactivation of dopamine $\beta$-monooxygenase by $H_2O_2$ was dependent on the presence of enzyme-bound copper.

In view of the above it was interesting to test whether the presence of copper bound to the enzyme is also essential for the inactivation of mushroom tyrosinase by $H_2O_2$. To test this, apotyrosinase was prepared as described in the Experimental section. Apotyrosinase and holotyrosinase were each preincubated with $H_2O_2$ for different times and were then exhaustively dialysed to remove excess $H_2O_2$. The $H_2O_2$-treated apotyrosinase was then incubated with $Cu^{2+}$ (added as $CuSO_4$) in order to test if the enzyme will regain its activity. $Cu^{2+}$ was added also to the $H_2O_2$-treated holotyrosinase to serve as control. The data in Table 2 show that the activity of the $H_2O_2$-treated apotyrosinase was regained by incubation with $Cu^{2+}$, while this was not the case with the $H_2O_2$-treated holotyrosinase. The fact that the activity of the $H_2O_2$-treated apotyrosinase was regained by $Cu^{2+}$ while the $H_2O_2$-treated holotyrosinase was not, strongly indicates that $Cu^{2+}$ must be present on the enzyme in order for $H_2O_2$ to inactivate the enzyme.

Additional experiments designed to show that $Cu^{2+}$ at the active site of the enzyme was necessary for the inactivation by $H_2O_2$ to occur, were performed with the use of $Cu^{2+}$ chelators as follows: Sodium azide, sodium fluoride, tropolone and sodium thiosulphate are effective $Cu^{2+}$ chelators [15–17]. The data in Table 3 show that each of these $Cu^{2+}$ chelators inhibited *o*-dihydroxyphenolase activity of mushroom tyrosinase Mushroom tyrosinase activity was inhibited by 50% with $8.0 \times 10^{-8}$ M tropolone and with $8.0 \times 10^{-4}$ M sodium azide. The ability of these $Cu^{2+}$ chelators to protect the enzyme against $H_2O_2$ inactivation is depicted in Figs 3 and 4. The data show that sodium azide and tropolone each protected mushroom tyrosinase against $H_2O_2$ inactivation. Sodium fluoride gave a little protection while sodium thiosulphate did not give any (data not shown). If we compare the data in Figs 3 and 4 with that in Table 3, it is seen that the ability of each $Cu^{2+}$ chelator to protect the enzyme against $H_2O_2$ inactivation was related to its ability to inhibit *o*-dihydroxyphenolase activity of the enzyme. Sodium azide is a poor inhibitor (compared with tropolone; Table 3) and a poor protector of the enzyme against $H_2O_2$ inactivation, while the reverse is true for tropolone.

It must be pointed out again that the values of 100% activity of mushroom tyrosinase in the presence or absence of each $Cu^{2+}$ chelator were not the same, due to the inhibitory action of each one. The fact that the holotyrosinase was inactivated by $H_2O_2$, while the apotyrosinase was not, and the fact that $Cu^{2+}$ chelators protected the enzyme against $H_2O_2$ inactivation, indicate that the inactivation of mushroom tyrosinase by $H_2O_2$ is dependent on the presence of $Cu^{2+}$ on the enzyme.

*Is the inactivation of mushroom tyrosinase by $H_2O_2$ due to generation of $OH^{\cdot}$ radicals?*

During the inactivation of *Neurospora* tyrosinase by dye-sensitized photo-oxidation [10] and during the suicide-type inactivation of the same enzyme [18], it was shown that histidine at the active site of the enzyme was destroyed and therefore the $Cu^{2+}$ was released. $OH^{\cdot}$ radicals can attack amino acids such as tyrosine, phenylalanine, tryptophan, histidine, cysteine and methionine, and oxidize them [19–21]. Free $OH^{\cdot}$ radicals have been shown to inactivate several enzymes such as papain [22, 23], trypsin [24] and lactate dehydrogenase [25]. $OH^{\cdot}$ radicals can be generated non-enzymatically from $H_2O_2$ by two main pathways [26] as shown below:

(1A) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$ (the Fenton reaction) [27]

(1B) $Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{\cdot} + OH^-$ [28]

(2) $O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + OH^{\cdot} + OH^-$ (the Haber–Weiss reaction) [29].

Table 2. Comparison between the ability of $H_2O_2$ to inactivate apotyrosinase vs. holotyrosinase

| Part | Preincubation $H_2O_2$ (mM) | Preincubation time (min) | Reconstitution $CuSO_4$ (mM) | Activity |
|---|---|---|---|---|
| (A-1) apotyrosinase | 5 | 0 | 0 | 0.030 |
| | 5 | 0 | 0 33 | 0.090 |
| | 5 | 30 | 0 | 0.033 |
| | 5 | 30 | 0.33 | 0.091 |
| (A-2) apotyrosinase | none | 0 | 0 | 0.040 |
| | none | 0 | 0.33 | 0 125 |
| | none | 30 | 0 | 0.016 |
| | none | 30 | 0.33 | 0 110 |
| (B-1) holotyrosinase | 1 | 0 | 0 | 0.080 |
| | 1 | 0 | 0.33 | 0 096 |
| | 1 | 30 | 0 | 0.030 |
| | 1 | 30 | 0 33 | 0.040 |
| (B-2) holotyrosinase | none | 0 | 0 | 0.530 |
| | none | 0 | 0.33 | 0 570 |

Apotyrosinase (5000 $\mu$g) was preincubated with 60 mM NaPi buffer (pH 6.5) in the presence (A-1) or the absence (A-2) of 5 mM $H_2O_2$ in a total volume of 10 ml. At the indicated time, 3 ml aliquots were withdrawn and thoroughly dialysed against 5 mM NaPi buffer (pH 6.5). Holotyrosinase (2000 $\mu$g) was preincubated with 60 mM NaPi buffer (pH 6.5) in the presence (B-1) or the absence (B-2) of 1 mM $H_2O_2$ in a total volume of 4 ml. Aliquots of 2 ml were withdrawn at the indicated time and thoroughly dialysed against 5 mM NaPi buffer (pH 6.5) The reconstitution conditions were as follows: 200 $\mu$g of each sample (0.4 ml) was incubated in 0.33 mM $CuSO_4$ for 30 min in a total volume of 0.6 ml. *o*-Dihydroxyphenolase activity of 0.3 ml (100 $\mu$g of each sample) of the reconstituted mixture was then assayed in the presence of 6.7 mM DL-DOPA and 47 mM NaPi buffer (pH 6.5) in a total volume of 3 ml

Table 3. Inhibition of *o*-dihydroxyphenolase activity of mushroom tyrosinase by $Cu^{2+}$ chelators

| $Cu^{2+}$ chelator added | $I_{50}$ (M) |
|---|---|
| Tropolone | $8.0 \times 10^{-8}$ |
| Sodium azide | $8.0 \times 10^{-4}$ |
| Sodium thiosulfate | $1.2 \times 10^{-3}$ |
| Sodium fluoride | $12.0 \times 10^{-2}$ |

The reaction mixture included, in a total volume of 3 ml, 47 mM NaPi buffer (pH 6.5), 6.7 mM DL-DOPA, 50 $\mu$g mushroom tyrosinase (added last), and different concentrations of each $Cu^{2+}$ chelator. $I_{50}$ = the concentration of the $Cu^{2+}$ chelator that caused 50% inhibition of *o*-dihydroxyphenolase activity of mushroom tyrosinase.

In view of the above, it was important to establish whether $OH^{\cdot}$ radicals are the active species responsible for the inactivation of mushroom tyrosinase by $H_2O_2$. This was tested on the basis of the following rationale: (a) addition of a factor known to accelerate $OH^{\cdot}$ generation from $H_2O_2$ should accelerate the rate of inactivation of the enzyme by $H_2O_2$; (b) addition of $OH^{\cdot}$ scavengers should slow down the rate of inactivation of the enzyme by $H_2O_2$.

(a) *Effect of* $OH^{\cdot}$ *generating system.* $OH^{\cdot}$ radicals can be generated in a system consisting of $H_2O_2$, $Fe^{2+}$ and EDTA (the Fenton-type system described by Walling *et al.* [27], or by Cohen and Cederbaum [30]). To produce $OH^{\cdot}$ radicals from $H_2O_2$, a complex of $Fe^{2+}$ with an excess of EDTA was prepared in order to prevent the oxidation of $Fe^{2+}$ by air. The $Fe^{2+}$–EDTA complex can react with $H_2O_2$ and produce $OH^{\cdot}$ in the same way that $Fe^{2+}$ alone reacts with $H_2O_2$ in a Fenton reaction.

It is theoretically possible that in our experiments $H_2O_2$ reacts with contaminant $Fe^{2+}$ that may be present in the reaction mixtures (traces in NaPi buffer), or with $O_2^{\cdot-}$ (generated by oxidation of $H_2O_2$ [31]) to yield $OH^{\cdot}$ (reactions 1 and 2). Such $OH^{\cdot}$ can attack amino acid(s) at the active site of the enzyme and oxidize it, causing irreversible inactivation of the enzyme.

It was reasoned that if the inactivation of mushroom tyrosinase by $H_2O_2$ was by $OH^{\cdot}$, then exposure of the enzyme to $H_2O_2$ plus $Fe^{2+}$–EDTA should accelerate the inactivation relative to that obtained in the presence of $H_2O_2$ alone. DTPA (diethylene triaminepentaacetic acid) can also complex with $Fe^{2+}$ but, compared with $Fe^{2+}$–EDTA, $Fe^{2+}$–DTPA does not generate $OH^{\cdot}$ radicals from $H_2O_2$ [32]. It was therefore important to compare the effect of $Fe^{2+}$–EDTA–$H_2O_2$ ($OH^{\cdot}$ generating system) with that of $Fe^{2+}$–DTPA–$H_2O_2$ ($OH^{\cdot}$ non-generating system) as to the rate of inactivation of

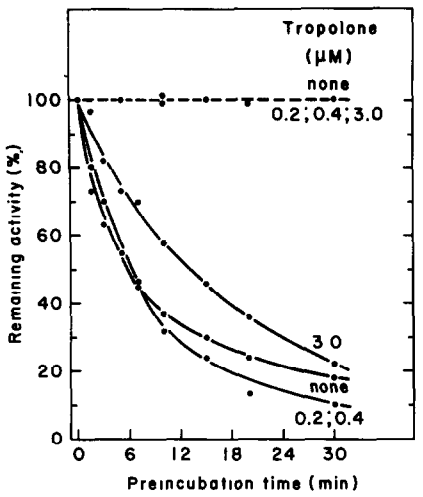



Fig. 3. Tropolone as a protector against inactivation of mushroom tyrosinase by $H_2O_2$. The preincubation mixture contained, in a total volume of 20 ml, 70 mM NaPi buffer (pH 6.5), 500 μg mushroom tyrosinase (added last), and tropolone (pH 6.5), as indicated. Control flasks (----) had no $H_2O_2$, while experimental flasks (——) contained 0.5 mM $H_2O_2$. Aliquots (2 ml) were withdrawn at various times and *o*-dihydroxyphenolase activity was assayed immediately, in a total volume of 3 ml, by adding DL-DOPA at time zero so that the final concentration of DL-DOPA was 6.7 mM. The activity of the control samples (without addition of $H_2O_2$) was 0.48, 0.30, 0.25 and 0.05 $\Delta A$ 475 nm/min in the presence of 0, 0.2, 0.4 and 3.0 μM tropolone, respectively, and taken as 100%. The activity at time zero in the presence of 0.5 mM $H_2O_2$ (but without tropolone) was 0.72 $\Delta A$ 475 nm/min and that in the presence of 0.5 mM $H_2O_2$ plus 0.2, 0.4 and 3.0 μM of tropolone was 0.46, 0.38 and 0.17 $\Delta A$ 475 nm/min, respectively, and also taken as 100%.

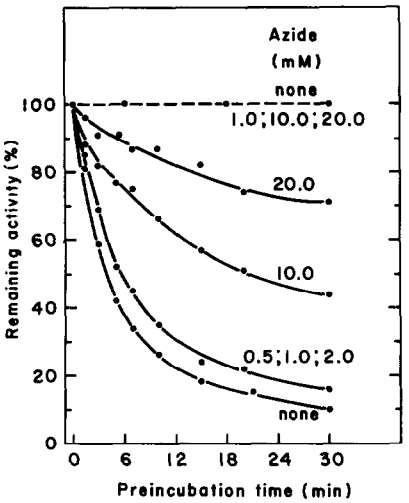



Fig. 4. Sodium azide as a protector against inactivation of mushroom tyrosinase by $H_2O_2$. The preincubation mixture contained, in a total volume of 20 ml, 70 mM NaPi buffer (pH 6.5), 500 μg mushroom tyrosinase (added last) and sodium azide (pH 6.5) as indicated. Control flasks (----) had no $H_2O_2$, while experimental flasks (——) contained 0.5 mM $H_2O_2$. Aliquots (2 ml) were withdrawn at various times and *o*-dihydroxyphenolase activity was assayed immediately, in a total volume of 3 ml, by adding DL-DOPA at time zero so that the final concentration of DL-DOPA was 6.7 mM. The activity of the control samples (without addition of $H_2O_2$) was 0.53, 0.45 and 0.09 $\Delta A$ 475 nm/min in the presence of 0, 0.5 and 10 mM sodium azide, respectively, and taken as 100%. The activity at time zero in the presence of 0.5 mM $H_2O_2$ (but without sodium azide) was 0.77 $\Delta A$ 475 nm/min and that in the presence of 0.5 mM $H_2O_2$ and 0.5 mM or 1.0 mM of sodium azide was 0.51 and 0.16 $\Delta A$ 475 nm/min, respectively, and also taken as 100%.

mushroom tyrosinase. While $Fe^{2+}$–EDTA was expected to accelerate the inactivation of the enzyme by $H_2O_2$, $Fe^{2+}$–DTPA was expected to have no effect at all.

The data in Fig. 5 show that addition of the $Fe^{2+}$–EDTA complex to a reaction mixture consisting of DOPA, NaPi buffer (pH 6.5), mushroom tyrosinase and $H_2O_2$ did not increase the rate of inactivation of the enzyme compared with the rate of inactivation of the enzyme in the presence of $H_2O_2$ alone. Instead, the addition of $Fe^{2+}$–EDTA to the reaction mixture offered protection against $H_2O_2$ inactivation. The protection by $Fe^{2+}$–EDTA is probably due to the decomposition of $H_2O_2$ by the $Fe^{2+}$–EDTA complex (via the Fenton reaction) resulting in a lower concentration of $H_2O_2$ in the system.

Similar results were obtained by the use of $Fe^{2+}$–DTPA complex instead of the $Fe^{2+}$–EDTA (data not shown); addition of $Fe^{2+}$–DTPA to the preincubation mixture (containing mushroom tyrosinase, $H_2O_2$ and buffer) offered protection against $H_2O_2$ inactivation probably also due to the decomposition of $H_2O_2$ by $Fe^{2+}$–DTPA.

(b) *Addition of* $OH^{\cdot}$ *scavengers.* Of the various known $OH^{\cdot}$ scavengers [19], mannitol, formate, urate and histidine are particularly suitable ones for our study, since they do not interact with $H_2O_2$ and they have low $M_r$s which make them easily accessible to the active site of the enzyme. Preincubation of mushroom tyrosinase with $H_2O_2$ in the presence of the above $OH^{\cdot}$ scavengers did not offer any protection against $H_2O_2$ inactivation compared with preincubation of the enzyme with $H_2O_2$ alone (Table 4). A total preincubation time of 45 min resulted in no difference from the control. The data shown in Table 4 are the remaining activity after 10 min of preincubation.

The fact that addition of $Fe^{2+}$–EDTA to $H_2O_2$ as a means of accelerating $OH^{\cdot}$ production did not enhance the inactivation of the enzyme, and that addition of $OH^{\cdot}$ scavengers did not protect the enzyme against inactivation by $H_2O_2$, indicate the unlikelihood that the inactivation of mushroom tyrosinase by $H_2O_2$ is due to an attack of the enzyme by free $OH^{\cdot}$ produced in the bulk phase.

*Is the inactivation of mushroom tyrosinase by* $H_2O_2$ *due to the formation of a dead-end product (oxy-oxytyrosinase)?*

According to Jolley *et al.* [4], addition of a very low concentration of $H_2O_2$ (15 μM) to mushroom tyrosinase (4 μM) produces oxytyrosinase which can be destroyed by the addition of catalase.

According to Eickman *et al.* [33], Lerch [18], Solomon [34] and Winkler *et al.* [35], the mechanism of action of tyrosinase is as follows: mettyrosinase ([Cu(II)Cu(II)]; also referred to as T resting) is reduced by an endogenous

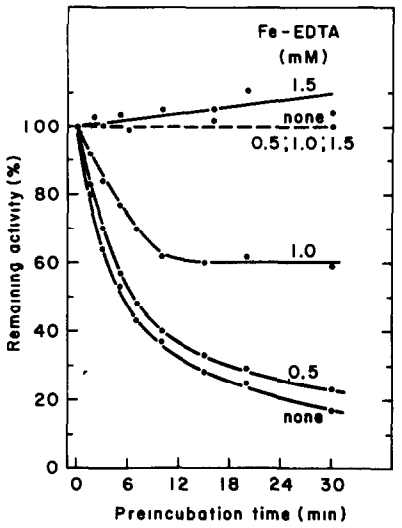
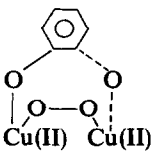



Fig. 5. Effect of the $Fe^{2+}$–EDTA complex on the inactivation of mushroom tyrosinase by $H_2O_2$. The preincubation mixture contained, in a total volume of 20 ml, 70 mM NaPi buffer (pH 6.5), 500 $\mu$g mushroom tyrosinase (added last), and $FeSO_4$ (pH 6.5), as indicated. $FeSO_4$ was added as the Fe–EDTA complex, which was prepared fresh by mixing 0.01 mM $FeSO_4$ and 0.03 mM EDTA (pH 6.5). Control flasks (----) had no $H_2O_2$, while experimental flasks (——) contained 0.5 mM $H_2O_2$. Aliquots (2 ml) were withdrawn at various times and *o*-dihydroxyphenolase activity was assayed immediately, in a total volume of 3.0 ml, by adding DL-DOPA at time zero so that the final concentration of DL-DOPA was 6.7 mM. The activity of the control samples (without $H_2O_2$) was 0.44, 0.45 and 0.44 $\Delta A$ 475 nm/min in the presence of 0, 0.5, 1.0 and 1.5 mM $Fe^{2+}$–EDTA, respectively, and taken as 100%. The activity at time zero in the presence of 0.5 mM $H_2O_2$ (but without $Fe^{2+}$–EDTA) was 0.67 $\Delta A$ 475 nm/min and that in the presence of 0.5 mM $H_2O_2$ plus 0.5, 1.0 and 1.5 mM $Fe^{2+}$–EDTA was 0.62, 0.47 and 0.38 $\Delta A$ 475 nm/min, respectively, and also taken as 100%.

*o*-dihydroxyphenol (or by exogenously added reductants such as ascorbate or $NH_2OH$) to deoxytyrosinase [Cu(I)Cu(I)]. Deoxytyrosinase binds to molecular oxygen, forming oxytyrosinase which exists as a peroxide form [Cu(II)Cu(II)$O^2$] [33]. Oxytyrosinase (thought to be the active species) interacts with the substrate (either a monohydroxy- or *o*-dihydroxyphenol) to form a ternary complex [34]. The ternary complex formed between oxytyrosinase and *o*-dihydroxyphenol is pictured as follows [34]:



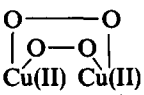

In our experiments (Fig. 1), the concentration of $H_2O_2$ is very high relative to the concentration of the enzyme (0.5 mM $H_2O_2$ to 0.2 $\mu$M mushroom tyrosinase). It is conceivable that in the presence of relatively high concentrations of $H_2O_2$, tyrosinase is converted to an

Table 4. Effect of addition of $OH^{\cdot}$ radical scavengers on the inactivation of mushroom tyrosinase by $H_2O_2$

| Radical scavenger added | Concentration (mM) | Remaining activity (%) after 10 min preincubation |
|---|---|---|
| Potassium urate | 0 | 41 |
| | 3.75 | 37 |
| Mannitol | 0 | 34 |
| | 75.0 | 31 |
| | 150.0 | 25 |
| Sodium formate | 0 | 35 |
| | 25.0 | 27 |
| | 50.0 | 27 |
| L-Histidine | 0 | 38 |
| | 5.0 | 35 |
| | 15.0 | 28 |

The preincubation mixture included, in a total volume of 26 ml, 70 mM NaPi buffer (pH 6.5), 650 $\mu$g mushroom tyrosinase (added last), 0.5 mM $H_2O_2$, and various $OH^{\cdot}$ radical scavengers, as indicated. Aliquots (2 ml) were withdrawn at various times and *o*-dihydroxyphenolase activity was assayed immediately, in a total volume of 3 ml, by adding DL-DOPA at time zero so that the final concentration of DL-DOPA was 6.7 mM.

oxy-oxytyrosinase. The oxy-oxytyrosinase




being a hypothetical intermediate analogous to the ternary complex formed between oxytyrosinase and *o*-dihydroxyphenol. Oxy-oxytyrosinase is probably a "dead-end" form of tyrosinase that cannot bind monohydroxy- or *o*-dihydroxyphenols and can therefore be considered as an inactivated form of the enzyme. If this were the case, then catalase might be expected to destroy oxy-oxytyrosinase (if it has access to the complex), and consequently the enzyme might regain its activity.

The possibility that an oxy-oxytyrosinase is formed by an interaction of the enzyme with $H_2O_2$ was tested as follows:

(a) Mushroom tyrosinase was preincubated with $H_2O_2$, aliquots were withdrawn at different time intervals, and *o*-dihydroxyphenolase activity was then assayed with DL-DOPA as the substrate, in the presence or absence of catalase. The data in Fig. 6 show that the first phase of $H_2O_2$-inactivation of mushroom tyrosinase was identical in the presence or absence of catalase, while the second phase was not. In the second phase, catalase offered some protection against $H_2O_2$ inactivation, probably due to the destruction of $H_2O_2$ in the reaction mixture by catalase.

(b) Mushroom tyrosinase was preincubated with 0.5 mM $H_2O_2$ for 0, 15 and 30 min. The resultant $H_2O_2$-inactivated enzyme was dialysed exhaustively overnight to remove $H_2O_2$. The dialysed $H_2O_2$-inactivated enzyme was then incubated with catalase for 10 min and its *o*-dihydroxyphenolase activity was assayed with DL-DOPA as the substrate. The data in Table 5 illustrate that the $H_2O_2$-inactivated enzyme did not regain activity as a result of 10 min incubation with catalase.

These results indicate one of the following possibilities: (i) that the inactivation of mushroom tyrosinase by $H_2O_2$ is not likely to be due to oxy-oxytyrosinase formation; (ii) that catalase does not have access to the active site of

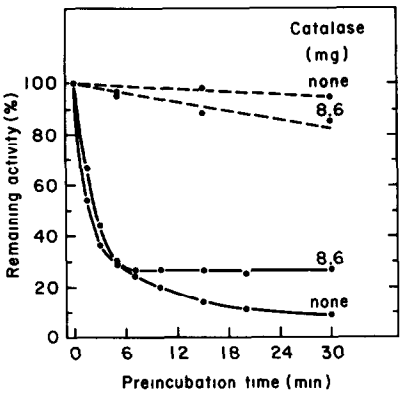



Fig. 6. Effect of catalase on $H_2O_2$-inactivated mushroom tyrosinase. The preincubation mixture contained, in a total volume of 20 ml, 70 mM NaPi buffer (pH 6.5) and 500 $\mu$g mushroom tyrosinase (added last). Control flasks (----) had no $H_2O_2$, while experimental flasks (——) contained 0.5 mM $H_2O_2$. Aliquots (2 ml) were withdrawn at various times and *o*-dihydroxyphenolase activity was assayed, in a total volume of 3 ml, by adding DL-DOPA at time zero, so that the final concentration of DL-DOPA was 6.7 mM, in the presence or absence of 8.6 mg catalase. The activity of the control samples (without $H_2O_2$) was 0.65 and 0.69 $\Delta A$ 475 nm/min in the presence of 0 or 8.6 mg catalase, respectively, and taken as 100%. The activity at time zero in the presence of 0.5 mM $H_2O_2$ (but without catalase) was 1.09 $\Delta A$ 475 nm/min, and that in the presence of 0.5 mM $H_2O_2$ and 8.6 mg of catalase was 0.66 $\Delta A$ 475 nm/min, respectively, and also taken as 100%.

Table 5. Effect of catalase on the inactivation of mushroom tyrosinase by $H_2O_2$

| Preincubation $H_2O_2$ (mM) | time (min) | Activity after dialysis before treatment with catalase ($\Delta A$ 475 nm/min) | after treatment with catalase ($\Delta A$ 475 nm/min) |
|---|---|---|---|
| None | 0 | 0.47 | 0.50 |
| None | 15 | 0.47 | 0.50 |
| None | 30 | 0.47 | 0.50 |
| 0.5 | 0 | 0.080 | 0.085 |
| 0.5 | 15 | 0.035 | 0.035 |
| 0.5 | 30 | 0.015 | 0.020 |

The preincubation mixture contained, in a total volume of 20 ml, 47 mM NaPi buffer (pH 6.5) and 500 $\mu$g mushroom tyrosinase (added last), in the presence or absence of 0.5 mM $H_2O_2$. At the indicated time, 4 ml aliquots were withdrawn and dialysed overnight against 0.05 M NaPi buffer (pH 6.5) to remove $H_2O_2$. Samples of the dialysed mixtures were then incubated with 8 mg catalase for 10 min and assayed by adding DL-DOPA at time zero so that the final concentration of DL-DOPA was 6.7 mM. Other samples were assayed in the absence of catalase in the same way.

mushroom tyrosinase; and (iii) that oxy-oxytyrosinase cannot be acted upon by catalase.

*Comparison between the rate of inactivation of mushroom tyrosinase under aerobic vs. anaerobic conditions*

As mentioned above, oxytyrosinase is thought to be the active species of mushroom tyrosinase to which either a monohydroxy- or dihydroxyphenol binds [18]. Interaction of oxytyrosinase with *o*-dihydroxyphenols results in the formation of *o*-quinones and the concomitant reduction of oxytyrosinase to mettyrosinase [18]. The conversion of oxytyrosinase to mettyrosinase occurs either directly or via a hypothetical intermediate [Cu(I)Cu(I)$O^2$] [18].

When one molecule of tyrosinase acts on its phenolic substrates, the enzyme undergoes rapid inactivation every $5 \times 10^3$ turnovers, a phenomenon referred to as suicide-type inactivation [18]. Lerch [18] suggested that the suicide-type inactivation of tyrosinase is caused by OH$^\cdot$ bound to the enzyme (enzyme–$Cu^{2+}$–OH$^\cdot$). The latter is thought to be generated from an incomplete reduction of the hypothetical intermediate [Cu(I)Cu(I)$O^2$]. In the case of *Neurospora* tyrosinase, the enzyme-bound OH$^\cdot$ then attacks histidine-306 at the active site of the enzyme, leading to loss of activity [18].

According to Jolley *et al.* [4], mettyrosinase can interact directly with $H_2O_2$ in the presence of oxygen to form oxytyrosinase. Oxytyrosinase [Cu(II)Cu(II)$O^2$] is in equilibrium with deoxytyrosinase [Cu(I)Cu(I)] and oxygen [4]. Deoxytyrosinase is also obtained by an interaction of mettyrosinase with $H_2O_2$ under anaerobic conditions [4].

Hodgson and Fridovich [3] reported that the rate of $H_2O_2$ inactivation of CuZn–SOD was twice as fast under nitrogen as under 100% oxygen and explained this phenomenon by suggesting that oxygen protects the enzyme against $H_2O_2$ inactivation by competing with $H_2O_2$.

A comparison was made between the rate of inactivation of mushroom tyrosinase by $H_2O_2$ under aerobic vs. anaerobic conditions. The data in Fig. 7 show that the rate of inactivation of mushroom tyrosinase by $H_2O_2$ is faster under anaerobic conditions (nitrogen) than under aerobic ones (air). Controls without $H_2O_2$ were not affected by nitrogen (Fig. 7). Fifty percent inactivation of the enzyme by $H_2O_2$ occurred after 9 min in the presence of air, but after only one min under nitrogen (Fig. 7). The rapid rate of inactivation of mushroom tyrosinase by $H_2O_2$ by nitrogen was irreversible, judged by the observation that bubbling air through a mixture containing the enzyme, $H_2O_2$ and NaPi buffer, which had been preincubated previously under nitrogen, did not restore any of the activity.

We would like to suggest that the inactivation of mushroom tyrosinase by $H_2O_2$ may be due to the formation of tyrosinase Cu(II)–OH$^\cdot$. The latter can be produced by interaction of $H_2O_2$ with $Cu^+$ present in deoxytyrosinase, yielding OH$^\cdot$ bound to the enzyme in a Fenton-type reaction [28] as follows:

(3) Cu(I)Cu(I) + $H_2O_2$ → Cu(II)Cu(II)–OH$^\cdot$
deoxytyrosinase tyrosinase Cu(II)–OH$^\cdot$

Tyrosinase Cu(II)–OH$^\cdot$ could oxidize histidine at the active site of the enzyme, causing the enzyme's inactivation, since histidines are known to be the $Cu^{2+}$ ligands of the enzyme [18]. It is likely that as a result of the

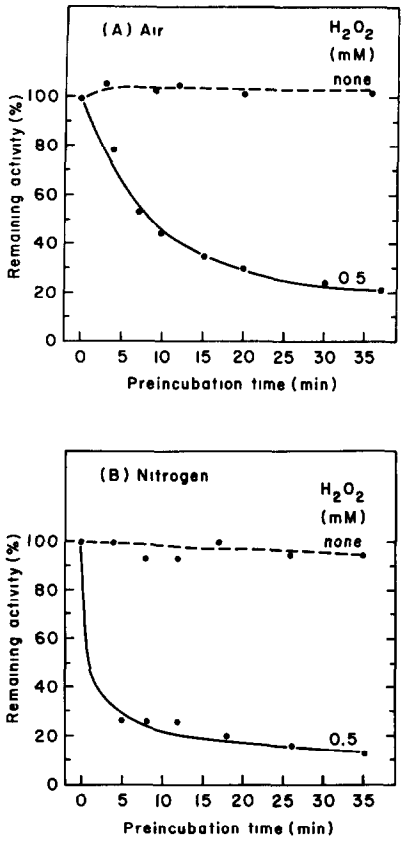



Fig. 7. Comparison between the rate of mushroom tyrosinase inactivation by $H_2O_2$ in air and under nitrogen. (A) The reaction mixture included, in a total volume of 26 ml, 70 mM NaPi buffer (pH 6.5), in the presence or absence of 0.5 mM $H_2O_2$ as indicated. (B) The reaction mixture included, in a total volume of 26 ml, 70 mM NaPi buffer (pH 6.5), in the presence or absence of 0.5 mM $H_2O_2$ as indicated. Air was bubbled through the reaction mixture (A) for 60 min and purified nitrogen (99.99%), passed through an oxygen trap, was bubbled through the reaction mixture (B) for 60 min, after which 260 $\mu$g mushroom tyrosinase was added to each. The reactions were kept under nitrogen (*without bubbling*) for an additional 40 min incubation period. In both (A) and (B), 2 ml aliquots were withdrawn at various times and *o*-dihydroxyphenolase activity was assayed immediately in a total volume of 3 ml by adding DL-DOPA at time zero, so that the final concentration of DL-DOPA was 6.7 mM.

destruction of histidine, $Cu^{2+}$ would be released from the active site of the enzyme.

The differences in the rate of inactivation of mushroom tyrosinase by $H_2O_2$ under aerobic vs. anaerobic conditions (Fig. 7) can be explained as follows: under aerobic conditions, $H_2O_2$ interacts with mettyrosinase to form oxytyrosinase, and the latter is in equilibrium with deoxytyrosinase [4] which can then interact with excess $H_2O_2$ to produce tyrosinase Cu(II)–OH$^-$. Oxygen can compete with $H_2O_2$ for deoxytyrosinase, converting it to oxytyrosinase. Under such conditions, the relative concentration of deoxytyrosinase would be low. This is shown in the following scheme:

Aerobic

(4) Cu(II)Cu(II) $\xrightarrow{H_2O_2}$ Cu(II)Cu(II)$O^2$

mettyrosinase oxytyrosinase

$O_2$ ↑ ↓

Cu(II)Cu(II)–OH$^-$ $\xleftarrow{H_2O_2}$ Cu(I)Cu(I)

tyrosinase Cu(II)–OH$^-$ deoxytyrosinase

Under anaerobic conditions mettyrosinase can interact with $H_2O_2$ to produce deoxytyrosinase [4] and the latter can then interact with excess $H_2O_2$ to produce tyrosinase Cu(II)–OH$^-$. Under such conditions, the relative concentration of deoxytyrosinase would be high. This is shown in the following scheme:

Anaerobic

(5) Cu(II)Cu(II) $\xrightarrow{H_2O_2}$ Cu(I)Cu(I)

mettyrosinase deoxytyrosinase

↓$H_2O_2$

Cu(II)Cu(II)–OH$^-$

tyrosinase Cu(II)–OH$^-$

The presence of a relatively high concentration of deoxytyrosinase under anaerobic compared with aerobic conditions would lead to a high production of tyrosinase Cu(II)–OH$^-$, as shown in scheme (3). This would explain our finding that the rate of $H_2O_2$-inactivation of mushroom tyrosinase is faster under anaerobic conditions than under aerobic ones (Fig. 7).

## EXPERIMENTAL

*Mushroom tyrosinase.* Unless otherwise indicated, *o*-dihydroxyphenolase activity was assayed in a reaction mixture that included, in a total vol. of 3 ml, 47 mM NaPi buffer (pH 6.5), 6.7 mM freshly prepared DL-DOPA and mushroom tyrosinase (added last). The formation of dopachrome was followed spectrophotometrically at 475 nm (using a Varian 635 spectrophotometer equipped with a recorder) as a function of time. The reaction was linear for about 90 sec and activity ($\Delta A$ 475 nm/min) was estimated from the initial linear portions of the curves.

*Preparation of apotyrosinase.* Apotyrosinase was prepared according to the method of ref. [36], by exhaustive dialysis of mushroom tyrosinase against 10 mM KCN (pH 7) for 24 hr, followed by five exchanges against 50 mM NaPi buffer (pH 6.5) for another 24 hr. Partial reconstitution of apotyrosinase was achieved by incubating apotyrosinase with 100-fold excess of $CuSO_4$ for 30 min.

$H_2O_2$ concn was determined spectrophotometrically at 240 nm using Em (240 nm) of 43.6 $M^{-1}$ $cm^{-1}$.

*Materials* Mushroom tyrosinase (grade III), catalase (C-100) from bovine liver, DL-DOPA, DTPA, K urate, tropolone, $NaN_3$, $Na_2S_2O_3$, L-mimosine, L-phenylalanine and *p*-fluorophenylalanine were obtained from the Sigma; EDTA, mannitol, HCOONa, L-histidine, NaF and $C_6H_5COONa$ were obtained from BDH; $H_2O_2$ was purchased from Merck.

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## REFERENCES

1. Mason, H. S. (1965) *Annu. Rev. Biochem.* **34**, 595.
2. Bray, R. C., Cockle, S. A., Fielden, E. M., Roberts, P. B.,

Rotilio, G. and Calabrese, L. (1974) *Biochem. J.* **139**, 43.
3. Hodgson, E. K. and Fridovich, I. (1975) *Biochemistry* **14**, 5294.
4. Jolley, R. L., Evans, L. H., Makino, N. and Mason, H. S. (1974) *J Biol. Chem.* **249**, 335.
5. Sinet, P. M. and Garber, P. (1981) *Arch. Biochem. Biophys.* **212**, 411.
6. Skotland, T. and Ljones, T. (1980) *Arch. Biochem. Biophys.* **201**, 81.
7. Weinryb, I. (1966) *Biochemistry* **5**, 2003.
8. Kahn, V. (1983) *Phytochemistry* **22**, 2155.
9. Gutteridge, S, Dickson, G. and Robb, D. (1977) *Phytochemistry* **16**, 517.
10. Pfiffner, E. and Lerch, K. (1981) *Biochemistry* **20**, 6029.
11. Mitsuda, H., Yasumoto, K. and Yamamoto, A. (1967) *Agric. Biol. Chem.* **31**, 853.
12. Gutteridge, S. and Robb, D. (1975) *Eur. J. Biochem.* **54**, 107.
13 Hashiguchi, H. and Takahashi, H. (1977) *Molec. Pharmacol.* **13**, 362.
14. Pomerantz, S. H. (1963) *J. Biol. Chem.* **238**, 2351.
15. Goldstein, M., Lauber, E. and McKeregham, M. R. (1964) *Biochem. Pharmacol.* **13**, 1103.
16. Kidani, Y., Ohkuma, K., Hirose, J. and Noji, M. (1980) *Arch. Biochem. Biophys.* **200**, 452
17. Vamos-Vigyazo, L. (1981) *CRC Critical Reviews in Food Science and Nutrition* **15**, 49.
18. Lerch, K. (1981) in *Metal Ions in Biological Systems* (Sigel, H., ed.) Vol. 13, p. 143. Marcel Dekker, New York.
19. Dorfman, L. M. and Adams, G. E. (1973) *National Standard Reference Data System—National Bureau of Standards* **46**, 1.
20. Hiller, K. O., Masloch, B., Gobl, M. and Asmus, K. D. (1981) *J Am. Chem. Soc.* **103**, 2734.
21. Lal, M., Lin, W. S., Gaucher, G. M and Armstrong, D. A. (1975) *Int. J. Radiat. Biol.* **28**, 549.
22. Clement, J. R., Lin, W. S. and Armstrong, D. A. (1977) *Radiat. Res* **72**, 427.
23. Lin, W. S, Armstrong, D. A. and Lal, M. (1978) *Int. J. Radiat. Biol.* **33**, 231.
24. Masuda, T., Ovadia, J. and Grossweiner, L. I. (1971) *Int. J. Radiat. Biol.* **20**, 447.
25. Buchanan, J. D. and Armstrong, D. A. (1976) *Int. J. Radiat Biol.* **30**, 115.
26. Korycha-Dahl, M. B. and Richardson, T. (1978) *CRC Critical Reviews in Food Science and Nutrition* **10**, 209.
27. Walling, C., Partch, R. E. and Weil, T. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 140.
28. Gutteridge, J M. C. (1982) *Biochem. Soc. Trans.* **10**, 72.
29. Haber, F. and Weiss, J (1934) *Proc. R. Soc. London, Ser. A.* **147**, 332.
30. Cohen, G. and Cederbaum, A. I. (1980) *Arch. Biochem. Biophys.* **199**, 438.
31. Bernofsky, C. and Wanda, S.-Y. C. (1983) *Biochem. Biophys. Res. Commun.* **111**, 231.
32. Halliwell, B. (1978) *FEBS Letters* **92**, 321.
33. Eickman, N. C., Solomon, E. I., Larrabee, J. A., Spiro, J. G. and Lerch, K. (1978) *J. Am. Chem. Soc.* **106**, 6529.
34. Solomon, E. I. (1981) *Copper Proteins* (Spiro, T. G., ed.) p. 41. John Wiley, New York.
35. Winkler, M. E, Lerch, K and Solomon, E. I. (1981) *J. Am. Chem. Soc.* **103**, 7001.
36. Kertesz, D., Rotilio, G., Brunori, M., Zito, R. and Antonini, E. (1972) *Biochem. Biophys. Res. Commun.* **49**, 1208.