Inhibition of Peroxidase-catalyzed Oxidations*

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Chance (1) has proposed that the peroxidase-catalyzed oxidation of dihydroxyfumaric acid proceeds in two steps. The first step is the oxidation of the hydrogen donor by oxygen, the reaction being catalyzed by a manganese-activated peroxidase and accompanied by the production of hydrogen peroxide. The second step is the oxidation of additional hydrogen donor by hydrogen peroxide, this reaction again being catalyzed by peroxidase. Akazawa and Conn (2) have proposed an analogous mechanism for the peroxidase-catalyzed oxidation of reduced diphenoylpiperidine nucleotide. An alternative mechanism for peroxidase-catalyzed oxidations was suggested by Kenten and Mann (3) who observed that manganese was oxidized during the peroxidase-catalyzed oxidations of organic acids. They considered that the manganese was oxidized enzymically, and that the manganese ions formed were necessary for the oxidation of the hydrogen donors. This function of manganese has been incorporated into the mechanism of oxidation of indoleacetic acid presented by Maciachian and Waygood (4).

Inhibitors have been employed in the present study to obtain more information on the mechanism of peroxidase-catalyzed oxidations. The data show the similarity of DPNH and indoleacetic acid oxidation and suggest that peroxidase-catalyzed oxidations may proceed by different mechanisms at different concentrations of manganese.

EXPERIMENTAL

Horseradish peroxidase was obtained from Nutritional Biochemicals Corporation (activity stated by the supplier was 60 units per mg by the assay of Polis and Shmukler (5)). Solutions were prepared by dissolving 10 mg of the powder in 100 ml of water. The solutions were stored at 5°C under toluene.

Gas exchange was measured manometrically at 30°C (6) for the oxidation of manganese (7), the nonenzymic oxidation of indoleacetic acid and the enzymic oxidation of indoleacetic acid.

Oxidation of DPNH was measured by following the decrease in optical density at 340 mµ by a Beckman DU spectrophotometer.

RESULTS

Inhibition by Chelators—Citrate and pyrophosphate inhibited the peroxidase-catalyzed oxidation of indoleacetic acid when cerium or manganese was used as the metallic cofactor (8). The inhibition in the presence of cerium is shown in Fig. 1; the results obtained when manganese replaced cerium were similar. A greater concentration of pyrophosphate than of citrate always was required for a given inhibition. Citrate and pyrophosphate inhibited by producing a lag period before oxygen uptake started. After the lag period, the rate of oxygen uptake approached the rate obtained in the absence of inhibitor. The inhibition caused by a given concentration of citrate decreased as the pH was lowered (Fig. 2), because the proportion of undissociated acid and monoanion, which do not chelate, increased as the pH was lowered.

Any alteration in the reaction mixture which accelerated the oxidation of indoleacetic acid also was effective in reducing the inhibition by a given concentration of chelator. Table I shows that an increased concentration of manganese decreased inhibition by citrate. A comparable effect of indoleacetic acid concentration was observed; as its concentration was increased from 1.5 µmoles, to 3, to 6 µmoles per vessel the lag period caused by a constant concentration of pyrophosphate (10 µmoles) was reduced from 23 minutes, to 16, to 9 minutes, respectively (the reaction mixture also contained 50 µmoles of pH 6.0 phosphate buffer, 3 µmoles of MnCl₂, 3 µmoles of resorcinol, and 0.2 ml of horseradish peroxidase solution).

Any change in the composition of the reaction mixture which slowed the rate of reaction increased the inhibition by a constant concentration of chelator. Kenten (9) and others have reported that the enzymic oxidation of indoleacetic acid is stimulated by low concentrations of resorcinol, but inhibited by higher concentrations. Supraoptimal concentrations of resorcinol increased the inhibition by pyrophosphate. When 3 µmoles of resorcinol were present per vessel, the lag periods were 12 and 5 minutes in the presence of 3 and 6 µmoles of MnCl₂ per vessel, respectively. However, when the resorcinol concentration was increased to 0 µmoles per vessel, the lag periods were 30 and 10 minutes under the same respective conditions. In this experiment there was no lag in the absence of pyrophosphate (the vessels contained 50 µmoles of pH 6.0 phosphate buffer, 3 µmoles of indoleacetic acid, 10 µmoles of pyrophosphate, except in the controls, and 0.5 ml of the horseradish peroxidase solution). Akazawa and Conn (2) also reported that the peroxidase-catalyzed oxidation of DPNH is not inhibited by pyrophosphate and citrate. However, at higher concentrations of manganese than those used by Akazawa and Conn, the reaction becomes susceptible to pyrophosphate and citrate; Fig. 3 shows that an increase in manganese concentration increases inhibition by citrate.

Akazawa and Conn (2) also reported that the peroxidase-catalyzed oxidation of DPNH was inhibited by EDTA,¹ and they contrasted this result with the report (10) that the peroxidase-catalyzed oxidation of indoleacetic acid is not affected by EDTA. In experiments with higher concentrations of manganese and re-

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¹ The abbreviation used is: EDTA, ethylenediaminetetraacetic acid.
the oxidation of DPNH gave the following changes per minute in extinction at 340 nm: 0.75, 0.80, 0.73, and 0.72, respectively (the reaction mixture contained 50 μmoles of pH 7.5 Tris buffer, 5 μmoles of resorcinol, 1 μmole of MnCl₂, 0.4 ml of DPNH solution containing 1 mg of DPNH·Na₂ per ml, and 0.5 ml of horseradish peroxidase solution in a total volume of 3.5 ml).

Inhibition by Phenolic Compounds—Kenten (9) reported that peroxidase-catalyzed oxidation of indoleacetic acid was inhibited by catechol and several other phenolic compounds. Waygood et al. (10) showed that catechol inhibited by causing a lag period. The inhibition by catechol becomes apparent over a very narrow concentration range (Fig. 4). In this range, the lag period rapidly increased from zero to infinity (Fig. 5). The nonenzymic

sorcinol than those used by Akazawa and Conn, the rate of oxidation of DPNH was not affected significantly by EDTA. In the presence of 0, 10, 20, and 30 μmoles of EDTA per cuvette,
oxidation of indoleacetic acid was affected similarly by catechol (Fig. 4b); the increase in concentration required to proceed from insensitivity to total inhibition was very small. Since the peroxidase-catalyzed oxidation of manganese has been considered as a partial reaction in the oxidation of indoleacetic acid, the effect of catechol on this reaction was also determined (Fig. 4c). Low concentrations of catechol stimulated the oxidation of manganese and indoleacetic acid. Gortner and Kent (11) previously have observed stimulation of indoleacetic acid oxidation by low concentrations of inhibitory substances. The inhibition of manganese oxidation at higher concentrations of catechol was not as marked as the inhibition of indoleacetic acid. The effect of catechol on the oxidation of manganese therefore does not completely explain the effect of catechol on the enzymic oxidation of indoleacetic acid.

\[ \text{Fig. 3. Effect of manganese concentration on the citrate inhibition of DPNH oxidation.} \]
\[ \text{a. 2 \mu moles of MnCl}_2 \text{ per cuvette; b. 1 \mu mole of MnCl}_2 \text{ per cuvette; c. 0.7 \mu mole of MnCl}_2 \text{ per cuvette.} \]
\[ \text{O-O, no citrate; - - - , 2 \mu moles of citrate per cuvette.} \]
\[ \text{In addition to the above components, the reaction mixture contained 50 \mu moles of Tris buffer, pH 7.5; 5 \mu moles of resorcinol; 0.4 mg of disodium DPNH; 0.3 ml of horseradish peroxidase solution, and water to make a total volume of 3.5 ml.} \]

\[ \text{DISCUSSION} \]

\[ \text{The peroxidase-catalyzed oxidations of dihydroxyfumaric acid (13), phenylacetaldehyde (14), dicarboxylic acids (3), indoleacetic acid (15), phenylpyruvic acid (16), 2-nitropropane (17), and DPNH (2) have in common the property of stimulation by manganese.} \]
\[ \text{It may be hypothesized that the mechanisms of these reactions also have something in common. However, differences in the oxidations of DPNH and indoleacetic acid have} \]
The lag period caused by chelators and some phenolic compounds probably results from their trapping or inactivation of an essential intermediate and the simultaneous inactivation of the inhibitor, when it forms a complex with the intermediate. With the chelators, the effect can be explained as a trapping of manganic ions, as they are formed, until the number of manganic ions formed exceeds the capacity of the chelator. Increasing the rate of formation of manganic ions, for example by increasing the concentration of manganous ions, reduces the lag period. Reinert et al. (21) have observed that the lag period caused by diethylthiocarbamate is reduced by increasing manganese concentration. Increasing the indoleacetic acid concentration reduces the lag period, probably by competing more effectively with the chelator for manganic ions.

The lag period inhibition caused by phenolic compounds has at least three apparently logical explanations: Waygood et al. (10) concluded that the phenolic inhibitors react directly with manganic ions because they decolorize solutions of manganiversene; Ray (22) has pointed out that the phenolic inhibitors may act by virtue of their property of free radical inhibitors; finally, the phenolic inhibitors may form a nonreactive complex with the enzyme (11). The data still are insufficient to support a choice among these possibilities.

The unusual enzyme concentration-activity curve shown for pineapple "indoleacetic acid oxidase" (23), in which high enzyme concentrations were apparently inhibitory, is explicable in the light of the discovery of the exponential increase in inhibition caused by some phenolic compounds. When naturally occurring inhibitor is added with the enzyme, its exponential effect soon overcomes the stimulatory effect of increased enzyme concentration.

**SUMMARY**

1. Citrate and pyrophosphate inhibited oxidation of indoleacetic acid by horseradish peroxidase when manganese or cerium was used as the metallic cofactor.
2. This inhibition of indoleacetic acid oxidation by chelators was reduced by increasing either the concentration of manganese or the concentration of indoleacetic acid. The inhibition was increased by increasing the concentration of resorcinol to levels which were superoptimal for oxidation in the absence of chelator.
3. The inhibition by citrate of reduced diposphopyridine nucleotide oxidation was increased by increasing the concentration of manganese.
4. The effect by catechol and ferulic acid as inhibitors of indoleacetic acid oxidation increased approximately exponentially as their concentration was increased.

**BIBLIOGRAPHY**

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