THE OXIDATION OF REDUCED PYRIDINE NUCLEOTIDES BY PEROXIDASE*

BY T. AKAZAWA† AND ERIC E. CONN

(From the Department of Agricultural Biochemistry, University of California, Berkeley, California)

(Received for publication, October 14, 1957)

A soluble enzyme system which catalyzes the aerobic oxidation of reduced pyridine nucleotides in wheat germ has previously been described (1). Peroxidase was shown to be a component of this system. Later, Humphreys (2) demonstrated that Mn²⁺ and an unidentified cofactor were required for aerobic oxidation of TPNH¹ in crude preparations of malic enzyme from wheat germ. Although no attempt was made to demonstrate a peroxidase requirement in this work, the protein preparations used gave strongly positive tests for peroxidase. Recently Stern and Johnston (3) have investigated a particulate enzyme system in wheat embryos which has properties in common with those described by the earlier workers.

The present study shows that reduced pyridine nucleotides are oxidized aerobically in the presence of horse-radish peroxidase, Mn²⁺, and certain phenols. The properties of this reaction are compared with those of other oxidations catalyzed by peroxidase, and a mechanism accounting for the oxidation of DPNH and TPNH is proposed.

Methods and Materials

The oxidation of DPNH and TPNH was followed by measuring the decrease in light absorption at 340 mμ. The optical density of the reaction mixture, complete except for the addition of resorcinol or other phenol, was measured initially. The reaction was then started by addition of the phenol, and readings were taken at intervals of 1 minute. The rates, expressed as the decrease in optical density units per minute, were calculated from the results observed during the interval of 1 to 6 minutes after addition of the phenol. In those instances in which the rate exceeded 0.075 optical density unit per minute, the rate was calculated from shorter in-

* This work was supported in part by grants-in-aid from the American Cancer Society and the California Division, American Cancer Society.

† Present address, Biochemistry Laboratory, Nagoya University, School of Agriculture, Anjo, Aichi, Japan.

¹ The following abbreviations are employed: DPN⁺, DPNH, TPN⁺, and TPNH, the oxidized and reduced forms of the di- and triphosphopyridine nucleotides; Tris, tris(hydroxymethyl)aminomethane; CoA, coenzyme A; Versene, ethylenediaminetetraacetic acid; IAA, indoleacetic acid.
tervals of 2 or 3 minutes. The spectrophotometric measurements were made with a Beckman model DU spectrophotometer at 23°. The blank contained all components of the reaction mixture except DPNH or TPNH.

DPNH was prepared by enzymic reduction of DPN+ and isolated as the Tris salt (4). The Tris salt was 89 per cent pure, uncorrected for water content. TPNH was prepared by reduction of TPN+ with Na₂S₂O₄ (5). Dialyzed crystalline horse-radish peroxidase (RZ = 2.40) was generously provided by Dr. A. C. Maehly. Peroxidase of about 20 per cent purity (calculated from light absorption of the protein at 403 mp) was purchased from the Worthington Biochemical Corporation. Crystalline beef liver catalase was supplied by Mr. B. Burnham. Rattlesnake venom (Crotalus adamanteus) was purchased from Ross Allen’s Reptile Institute. Crystalline alcohol dehydrogenase was prepared according to Racker (6).

Resorcinol (m.p. 109–110°) was recrystallized from benzene; 2,4-dichlorophenol (m.p. 43–45°) was recrystallized from an ethanol-water mixture. The o- and p-cresols were redistilled. Hydroxyhydroquinone was prepared by hydrolysis of the triacetate (7) with methanol-HCl (8). Gifts of the following compounds are gratefully acknowledged: Dr. H. S. Mason, 4-methylcatechol and dihydroxyfumaric acid; Dr. D. Racusen, 2-chloro-4-phenylphenol; Dr. S. Witkop, p-toluquinol; Dr. B. Volcani, 3-hydroxyanthranilic acid.

Results

Demonstration of DPNH Oxidation

When DPNH was incubated with crystalline horse-radish peroxidase and catalytic amounts of Mn⁺² and resorcinol, the reduced coenzyme was rapidly oxidized (Fig. 1). The rate of oxidation of DPNH was proportional to the amount of peroxidase employed and was independent of the concentration of DPNH until the concentration of DPNH decreased to 3 \times 10^{-5} M. If any one of the three components was omitted or if the peroxidase was denatured by heating at 100°, there was no reaction.

Fig. 2 shows the dependence of the DPNH oxidation system on the presence of oxygen. When the complete reaction mixture was incubated in a Thunberg tube in nitrogen (Curve I), little oxidation of DPNH occurred. Only slow oxidation resulted when, after 14 minutes, the reaction mixture was carefully transferred to the spectrophotometer cuvette without aeration. Then, at 20 minutes, air was bubbled through the reaction mixture for 45 seconds, and rapid oxidation of DPNH occurred. Since the amount of DPNH oxidized far exceeded the amount of resorcinol or Mn⁺² added, oxygen must have served as the terminal oxidizing agent. Curve II shows that DPN⁺ is formed when DPNH is oxidized. This was estab-
lished when the addition of ethanol and yeast alcohol dehydrogenase at 38 minutes resulted in the reduction of DPN+.

The oxidation of DPNH was also followed manometrically. The data in Fig. 3 were obtained when 7.6 μmoles of DPNH were incubated in the Warburg respirometer in the presence of peroxidase and catalytic amounts of resorcinol and Mn²⁺. The final oxygen uptake corresponded to 0.5 mole of oxygen per mole of DPNH. Only slight oxygen uptake occurred when any component of the reaction mixture was omitted.

**Properties of DPNH Oxidation System**

The data presented in Figs. 1, 2, and 3 were obtained with dialyzed, crystalline horse-radish peroxidase (RZ = 2.40). However, identical results were obtained with horse-radish peroxidase of lower purity, and the properties of the DPNH-oxidizing reaction were examined in more detail with this preparation. Under the conditions described for Fig. 1, TPNH was oxidized approximately twice as rapidly as DPNH. The optimal pH for the oxidation of DPNH was 6.0. Most of the experiments described
here, however, were carried out at pH 7.4 in Tris or phosphate buffer because of the instability of DPNH at pH values below neutrality.

An examination of the metal requirement of the DPNH-oxidizing system showed that Mn\(^{+2}\) was highly specific. The following cations, when tested at 10\(^{-6}\) M, were inactive: Ni\(^{+2}\), Cd\(^{+2}\), Cu\(^{+2}\), Ca\(^{+2}\), Al\(^{+3}\), Fe\(^{+3}\), Mg\(^{+2}\), and Zn\(^{+2}\). Only in the case of Co\(^{+2}\) was there any indication that another metal could substitute for Mn\(^{+2}\), and in this case anomalous results were obtained. In the presence of 10\(^{-5}\) M Mn\(^{+2}\), 0.33 \(\mu\)mole of DPNH was oxidized in a period of 20 minutes. In the presence of 10\(^{-5}\) M Co\(^{+2}\), the initial oxidation rate was the same, but the oxidation of DPNH ceased after about 50 per cent of the DPNH was oxidized. When the concentration of resorcinol or Co\(^{+2}\) was increased 10-fold, no significant change in the extent of DPNH oxidation occurred. It was concluded that Co\(^{+2}\) was not equivalent to Mn\(^{+2}\), and no further studies were made. Experiments showed that there was an optimal concentration of 10\(^{-5}\) M for Mn\(^{+2}\) in the DPNH-oxidizing system. At 10\(^{-4}\) M Mn\(^{+2}\), the rate of DPNH oxidation decreased.
about 50 per cent; at $10^{-2} \text{M} \text{Mn}^{+2}$, the oxidation of DPNH was inhibited 100 per cent.

In order to obtain some information concerning the mechanism of the oxidation of DPNH, we investigated the ability of different phenols to substitute for resorcinol. The compounds examined were classified according to their action as one of three types: active, inactive, or inactive and inhibitory. Table I, which lists the active phenols, shows that the active compounds are phenols which are not readily oxidized to quinones. The rates of DPNH oxidation observed when the active phenols were tested at a concentration of $1.7 \times 10^{-5} \text{M}$ are given in Table I. Although the majority of the studies reported in this paper were carried out with resorcinol, three other phenols were more active. In the case of resorcinol the rate of DPNH oxidation reached a maximum when the resorcinol concentration was increased. From data obtained, a Michaelis constant of $3.8 \times 10^{-5} \text{M}$ was calculated for resorcinol.

Table II lists those phenols which were inactive and those which were both inactive and inhibitory. The latter compounds were judged to be inhibitory if DPNH oxidation did not occur after resorcinol was added to
a mixture containing DPNH, Mn$^{2+}$, peroxidase, and the phenol being examined. In addition, when ascorbic acid, dihydroxyfumaric acid, and $p$-

### Table I

Phenolic Compounds Active in DPNH-Oxidizing System

<table>
<thead>
<tr>
<th>Phenolic compound</th>
<th>$-\Delta$ optical density at 340 mp per min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resorcinol</td>
<td>0.069</td>
</tr>
<tr>
<td>2,4-Dichlorophenol</td>
<td>0.156</td>
</tr>
<tr>
<td>2-Chloro-4-phenylphenol</td>
<td>0.108</td>
</tr>
<tr>
<td>$p$-Cresol</td>
<td>0.080</td>
</tr>
<tr>
<td>$p$-Chlorothiophenol</td>
<td>0.060</td>
</tr>
<tr>
<td>1-Naphthol</td>
<td>0.050</td>
</tr>
<tr>
<td>3,4-Dimethylphenol</td>
<td>0.042</td>
</tr>
<tr>
<td>$m$-Cresol</td>
<td>0.027</td>
</tr>
<tr>
<td>$o$-Cresol</td>
<td>0.024</td>
</tr>
<tr>
<td>Phenol</td>
<td>0.013</td>
</tr>
<tr>
<td>$m$-Hydroxybenzoic acid</td>
<td>0.011</td>
</tr>
<tr>
<td>Orcinol</td>
<td>0.005</td>
</tr>
</tbody>
</table>

The conditions were those described for Fig. 1 except that 40 $\mu$ of peroxidase (Worthington) were employed.

### Table II

Inactive and Inhibitory Phenolic Compounds

<table>
<thead>
<tr>
<th>Inactive</th>
<th>Inactive and inhibitory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrogallol</td>
<td>Hydroquinone</td>
</tr>
<tr>
<td>Phloroglucinol</td>
<td>Catechol</td>
</tr>
<tr>
<td>Naphthoresorcinol</td>
<td>o-Aminophenol</td>
</tr>
<tr>
<td>Hydroxyhydroquinone</td>
<td>2,4-Dinitrophenol</td>
</tr>
<tr>
<td>Thymol</td>
<td>2,4-Dichloro-4-nitrophenol</td>
</tr>
<tr>
<td>$p$-Toluquinol</td>
<td>4-Methylcatechol</td>
</tr>
<tr>
<td>2,6-Dichlorobenzene-</td>
<td>2,6-Dichlorobenzene-</td>
</tr>
<tr>
<td>-indo-3'-chlorophenol</td>
<td>indophenol</td>
</tr>
<tr>
<td>Picro acid</td>
<td>3-Hydroxyanthranilic acid</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>2-Naphthol*</td>
</tr>
</tbody>
</table>

The compounds were tested at a concentration of $1.7 \times 10^{-6}$ M in the complete reaction mixture described for Fig. 1. 40 $\mu$ peroxidase (Worthington) were employed.

* These compounds were slightly active (0.005 to 0.010 optical density units per minute) but were inhibitory to the oxidation occurring in the presence of resorcinol.

aminobenzoic acid were tested at $1.7 \times 10^{-6}$ M, they were inactive. Anthranilic acid at the same concentration was inactive and inhibitory.
Earlier work (1, 2, 9, 10) has shown the occurrence in higher plants of soluble enzyme systems which catalyzed the oxidation of DPNH and TPNH by O₂. Extracts from several different plants and from two yeast preparations were tested for their ability to substitute for the phenol requirement of the DPNH-oxidizing system. The extracts were prepared by grinding one weight of the plant tissue or yeast sample with 2 volumes of H₂O and heating at 100° for 10 minutes. 0.05 ml. of the centrifuged, clarified extract was tested in the spectrophotometric test system. Active extracts were obtained from the cotyledons and the hypocotyls of 5 day-old etiolated lupine seedlings, the hypocotyls of etiolated peanut seedlings, wheat germ, and yeast extract. Preparations obtained from carrot roots, spinach leaves, white potato tubers, mushrooms, sweet potato roots, and dried brewers' yeast were inactive. It is interesting that mushrooms and sweet potatoes which have active polyphenolases and the natural substrates for the phenolase complex (11) did not yield an extract which could substitute for the phenol in the DPNH-oxidizing reaction.

Humphreys (2) observed that the TPNH oxidase of wheat germ was stimulated by an unidentified cofactor found in CoA preparations of low purity (0.9 per cent CoA) but not in purified samples (75 per cent CoA). In the present work, a coenzyme mixture containing 7 per cent DPN⁺, 7 per cent TPN⁺, and 3 per cent CoA also contained a compound which could substitute for the phenol requirement of the DPNH peroxidase system. However, highly purified DPN⁺, TPN⁺, CoA, oxidized glutathione, and reduced glutathione were free of the compound. No attempt was made to identify the unknown compound in the coenzyme mixture.

As previously mentioned, several phenolic compounds were inhibitory to the DPNH-oxidizing system. Table III shows that two of these phenols, hydroquinone and catechol, were strongly inhibitory at \(7 \times 10^{-6}\) M. In addition to phenols, compounds such as KCN and hydroxylamine, which are known to affect iron enzymes, were also inhibitory. Concentrations of KCN and hydroxylamine which inhibited approximately 50 per cent are listed in Table III. Sodium azide, \(\alpha,\alpha'\)-dipyridyl, diethylthiocarbamate, and \(\alpha\)-phenanthroline were not nearly as effective, however. Cysteine was inhibitory, and Cu²⁺ was also a highly efficient inhibitor at the concentration of \(3 \times 10^{-6}\) M. Perhaps the most interesting inhibitor is the enzyme catalase. Small amounts of extensively dialyzed catalase strongly inhibited DPNH oxidation. Since heat-inactivated catalase did not inhibit, the action of catalase is evidently associated with its catalytic ability.

The inhibition by catalase suggested that \(H₂O₂\) was an intermediate in the DPNH oxidation. However, the manometric data in Fig. 3 indicated that \(H₂O₂\) did not accumulate during the course of the reaction. Attempts
were made to detect H₂O₂ in the reaction mixture during the course of the reaction. Although the analytical method employed (12) would have detected as little as 0.05 μmole of H₂O₂, there was no evidence of H₂O₂ accumulation in the reaction mixtures. It should be pointed out that an amount of H₂O₂ equivalent to the resorcinol present in the reaction mixture (0.05 μmole) might be utilized by peroxidation of the phenol. This is due to the fact that resorcinol is an effective donor for the peroxide-peroxidase Complex II (13).

**TABLE III**

*Inhibitors of DPNH Oxidation Catalyzed by Peroxidase*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7 × 10⁻⁶ M hydroquinone</td>
<td>98</td>
</tr>
<tr>
<td>7.6 × 10⁻⁶ “ “</td>
<td>98</td>
</tr>
<tr>
<td>7.6 × 10⁻⁶ “ catechol</td>
<td>85</td>
</tr>
<tr>
<td>1.7 × 10⁻⁴ “ potassium cyanide</td>
<td>58</td>
</tr>
<tr>
<td>3.3 × 10⁻⁴ “ hydroxylamine hydrochloride</td>
<td>65</td>
</tr>
<tr>
<td>1.7 × 10⁻⁴ “ sodium azide</td>
<td>0</td>
</tr>
<tr>
<td>3.3 × 10⁻⁵ “ α,α’-dipyridyl</td>
<td>10</td>
</tr>
<tr>
<td>3.3 × 10⁻⁵ “ diethylthiocarbamate</td>
<td>0</td>
</tr>
<tr>
<td>1 × 10⁻⁴ M o-phenanthrolone</td>
<td>27</td>
</tr>
<tr>
<td>1.7 × 10⁻⁴ M cysteine</td>
<td>70</td>
</tr>
<tr>
<td>3.3 × 10⁻⁵ “ cupric sulfate</td>
<td>97</td>
</tr>
<tr>
<td>200 γ catalase</td>
<td>98</td>
</tr>
<tr>
<td>200 “ “ heat-inactivated</td>
<td>2</td>
</tr>
</tbody>
</table>

The conditions were those described for Fig. 1 except that 40 γ of peroxidase (Worthington) were employed. The inhibition was calculated from the rate of oxidation observed in the presence of the inhibitor compared with the rate in the absence of the inhibitor.

Chance has shown that DPNH can react with horse-radish peroxidase Complex II (13). Therefore, the effect of adding H₂O₂ to reaction mixtures containing DPNII, peroxidase, Mn⁺², and resorcinol was examined (Table IV). When 0.3 μmole of H₂O₂ was added to the complete reaction mixture (Experiment 1), the rate of DPNH oxidation was increased about 70 per cent. When Mn⁺² was omitted from the reaction mixture (Experiment 3), the DPNH was still rapidly oxidized upon addition of H₂O₂. This observation indicates that resorcinol is oxidized in the presence of H₂O₂ and peroxidase alone (13) and that the phenol oxidation product in turn can oxidize DPNH. The quinones formed by oxidation of o- and p-dihydrichinols are known to oxidize DPNH non-enzymically (14, 15). The fact that DPNH oxidation occurred in Experiment 3 when Mn⁺² was omitted also suggests that Mn⁺² functions in the formation of H₂O₂ in reaction
mixtures containing Mn\(^{+2}\), resorcinol, and peroxidase. When resorcinol or when both Mn\(^{+2}\) and resorcinol were omitted, H\(_2\)O\(_2\) addition did not cause appreciable oxidation of DPNH (Table IV). Therefore, DPNH was not oxidized by H\(_2\)O\(_2\) and peroxidase under the conditions employed, even though DPNH can serve as a hydrogen donor for peroxidases (13, 16).

Similar results were obtained when H\(_2\)O\(_2\) was generated enzymically in the reaction mixture by the addition of L-leucine and snake venom containing L-amino acid oxidase (Table IV). The amounts of L-leucine and venom added were capable of producing 3 \(\mu\)moles of H\(_2\)O\(_2\) in 15 minutes, an amount far in excess of the amount of DPNH oxidized in the same interval.

### Table IV

**Effect of Adding H\(_2\)O\(_2\) to Peroxidase System Oxidizing DPNH**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Conditions</th>
<th>Rate of DPNH oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H(_2)O(_2) added</td>
</tr>
<tr>
<td>1</td>
<td>Complete</td>
<td>0.145</td>
</tr>
<tr>
<td>2</td>
<td>Omit H(_2)O(_2)</td>
<td>0.085</td>
</tr>
<tr>
<td>3</td>
<td>&quot; Mn(^{+2})&quot;</td>
<td>0.140</td>
</tr>
<tr>
<td>4</td>
<td>&quot; resorcinol&quot;</td>
<td>0.005</td>
</tr>
<tr>
<td>5</td>
<td>&quot; resorcinol and Mn(^{+2})&quot;</td>
<td>0.005</td>
</tr>
</tbody>
</table>

0.3 \(\mu\)mole of H\(_2\)O\(_2\) or 5 \(\mu\)moles of L-leucine and 1 mg. of dialyzed snake venom were added, except where indicated, to the complete reaction mixture described in Fig. 1. 40 \(\gamma\) of horse-radish peroxidase (Worthington) were employed. The rate of DPNH oxidation is expressed as the decrease in optical density units per minute. In Experiments 1 and 2, DPNH oxidation was initiated by adding resorcinol. In Experiments 3, 4, and 5, the H\(_2\)O\(_2\) or L-leucine and venom were added last.

Other iron porphyrin proteins were examined for their ability to substitute for peroxidase in the DPNH-oxidizing system. Those examined were cytochrome c, hemoglobin, and catalase; all were inactive. Hemin was also inactive.

### DISCUSSION

Horse-radish peroxidase possesses some unusual catalytic properties. In addition to catalyzing the oxidation of a wide variety of hydrogen donors by H\(_2\)O\(_2\) (13), horse-radish peroxidase has long been known to act as an aerobic oxidase for dihydroxyfumaric acid (17, 18). Recently, Mason et al. have shown that peroxidase can catalyze hydroxylation reactions in the presence of dihydroxyfumaric acid (19). In the presence of Mn\(^{+2}\), peroxidase catalyzes the aerobic oxidation of phenylacetaldehyde (20) and phenylpyruvic acid (21). It catalyzes the oxidation of Mn\(^{+2}\) to Mn\(^{+3}\) (22) in
the presence of $\text{H}_2\text{O}_2$ and certain phenols. Finally, in the presence of $\text{Mn}^{+2}$ and certain phenolic compounds, peroxidase will catalyze the aerobic oxidation of dicarboxylic acids (23) and indoleacetic acid (24–26).

Although a detailed mechanism has been proposed for the oxidation of IAA (26), there are several observations which indicate that the oxidation of DPNH does not occur in the same manner. First, recrystallized and extensively dialyzed catalase strongly inhibited the oxidation of DPNH by the peroxidase system (Table III). This is in direct contrast to the findings of Waygood et al. (25), who observed that catalase did not inhibit the oxidation of IAA but rather catalyzed IAA oxidation in the presence of $\text{Mn}^{+2}$ and resorcinol. Secondly, $10^{-2}$ m citrate and $10^{-2}$ m pyrophosphate did not inhibit the oxidation of DPNH, but $10^{-2}$ m Versene inhibited 83 per cent. These results are exactly opposite to those obtained by Waygood et al. (25) in the oxidation of IAA. The latter authors suggested that citrate and pyrophosphate were inhibitory to the oxidation of IAA because these anions formed stable complexes of relatively low oxidation-reduction potentials with $\text{Mn}^{+3}$. Finally, the concentrations of $\text{Mn}^{+2}$ ($10^{-3}$ to $10^{-2}$ m) employed in the studies by either Kenten and Mann (23) or Waygood et al. (25, 26) were 100 to 1000 times as high as the $\text{Mn}^{+2}$ concentration which is optimal for DPNH oxidation. Indeed, $10^{-2}$ m $\text{Mn}^{+2}$ completely inhibited the oxidation of DPNH.

The oxidation of DPNH by the peroxidase system more closely resembles the aerobic oxidation of dihydroxyfumaric acid catalyzed by peroxidase (17, 18) and the oxidation of tryptophan catalyzed by tryptophan peroxidase (27). All of these reactions are inhibited by low concentrations of $\text{Cu}^{+2}$ and by inhibitors of heavy metal enzymes. In these reactions oxygen is consumed, and $\text{H}_2\text{O}_2$ does not accumulate. However, $\text{H}_2\text{O}_2$ may be involved because catalase inhibits all three reactions.

The aerobic oxidation of dihydroxyfumaric acid, which is catalyzed by horse-radish peroxidase, has been investigated in detail by Chance (18). The reaction was studied at $4^\circ$, at which it was clearly separated from the peroxidatic oxidation of dihydroxyfumarate catalyzed by peroxidase. Chance proposed that a ternary complex composed of $\text{Mn}^{+2}$, peroxidase, and $\text{H}_2\text{O}_2$ is the catalyst for the aerobic oxidation of dihydroxyfumarate. In the oxidation of DPNH by peroxidase it seems reasonable to propose that a similar ternary complex catalyzes the oxidation of the phenol (ROH) by $\text{O}_2$ with the formation of $\text{H}_2\text{O}_2$ and an oxidized phenolic product (RO·) (Equation 1). Although DPNH might react with $\text{O}_2$ under similar conditions, the following observations indicate that the phenol is the reduc-

$$2\text{ROH} + \text{O}_2 \xrightarrow{\text{peroxidase}} 2\text{RO}· + \text{H}_2\text{O}_2$$  (1)
tant. First, there is no spectrophotometric evidence for oxidation or other modification of DPNH unless the complete reaction mixture composed of peroxidase, Mn\(^{2+}\), and resorcinol is present. If DPNH were the reductant, some evidence of oxidation would be expected in the absence of resorcinol. Secondly, compounds other than DPNH and TPNH (i.e. dicarboxylic acids (23), IAA (24, 26), ferrocytochrome \(c_2\) and reduced glutathione\(^2\)) are aerobically oxidized in the presence of peroxidase, Mn\(^{2+}\), and resorcinol or certain monophenols. This suggests that the initial step is the formation of the oxidized phenolic product (RO\(\cdot\)), which in turn can oxidize a variety of other compounds.

If the phenol is oxidized according to Equation 1, the oxidized phenol (RO\(\cdot\)) could then be reduced by DPNH either enzymically or non-enzymically (Equation 2). Since only a small amount of \(H_2O_2\) would be required

\[
2RO\cdot + DPNH + H^+ \rightarrow 2ROH + DPN^+ \tag{2}
\]

to form and maintain the ternary complex, it is suggested that the major amount of \(H_2O_2\) produced in Equation 1 can oxidize the phenol in a typical peroxidase reaction (Equation 3) (28). The oxidized phenol (R'O\(\cdot\)) produced in Equation 3 could then be reduced by DPNH (Equation 4). This series of four reactions would account for (1) the functioning of resorcinol and Mn\(^{2+}\) in catalytic concentrations, (2) the observed stoichiometry for \(O_2\) consumption and DPNH oxidation, and (3) the inhibition of the DPNH oxidation by catalase. The latter, by competing for \(H_2O_2\), would prevent the formation of the ternary complex.

In an effort to obtain experimental evidence for Equation 1, oxygen uptake was measured in reaction mixtures containing \(10^{-2}\) M resorcinol and catalytic amounts of peroxidase and Mn\(^{2+}\). Although we observed resorcinol oxidation with high concentrations of Mn\(^{2+}\) (10\(^{-2}\) M) and thereby confirmed Maclachlan and Waygood's findings (28), no oxygen consumption occurred at the lower concentrations of Mn\(^{2+}\) used in the studies on DPNH oxidation. It is possible that the postulated oxidation product (RO\(\cdot\)) might accumulate in the absence of DPNH and be self-inhibiting. In the presence of DPNH, however, the oxidation product (RO\(\cdot\)) would not accumulate (Equation 2).

The nature of the oxidized phenolic products (RO\(\cdot\) and R'O\(\cdot\)) postulated in Equations 1 and 3 is unknown. However, if they are phenoxy

\*Akazawa, T., and Conn, E. E., unpublished observations.
radicals, it is extremely unlikely that they exist free in aqueous solution. Instead, such radicals may be associated with, and even stabilized to some extent by, the enzyme in a peroxidase-radical complex. In this event the oxidation of DPNH by the peroxidase-phenoxy radical complex would be analogous to the oxidation of various donor molecules by the peroxidase-H₂O₂ Complex II.

One puzzling property of the DPNH oxidation system, as well as the IAA and dicarboxylic acid oxidations, is the requirement for monophenols or resorcinol, *i.e.* phenolic compounds which are not readily oxidized to quinones. The recent experiments of Mason *et al.* (19) suggested that initially hydroxylation of the phenol might occur in the presence of DPNH and peroxidase and that the hydroxylated phenol might be the active agent in the DPNH oxidation. To test this possibility, hydroxyhydroquinone and 4-methylcatechol, the products resulting from hydroxylation of resorcinol and p-cresol, respectively (19), were examined in the DPNH oxidation reaction. Hydroxyhydroquinone was inactive, and 4-methylcatechol was inactive and inhibitory (Table II). Mason has examined the ability of DPNH to substitute for dihydroxyfumaric acid in the hydroxylation reaction. Under conditions in which resorcinol and p-cresol were rapidly hydroxylated in the presence of dihydroxyfumaric acid, hydroxylation did not occur with DPNH.³

The physiological significance of the oxidation of reduced pyridine nucleotides by the peroxidase system described herein is unknown, although the observations which led to these studies were encountered in numerous plant tissues (1, 2, 9). The properties of the reaction are similar in some respects to those of a particulate enzyme system recently described (3). The oxidation of DPNH and TPNH by the peroxidase system is of interest since, like the oxidations of dihydroxyfumaric acid, IAA, and certain dicarboxylic acids, it shows that peroxidase may catalyze oxidations independently of external sources of H₂O₂.

**SUMMARY**

The reduced forms of diphosphopyridine and triphosphopyridine nucleotides are rapidly oxidized in the presence of crystalline horse-radish peroxidase and catalytic amounts of Mn⁺² and certain phenols. 1 atom of oxygen is consumed per mole of nucleotide oxidized.

The requirement for Mn⁺² is specific. The phenols which are active are either monohydric phenols or resorcinol. However, not all monohydric phenols are active. The reaction is inhibited by catalase, by certain other phenols, cysteine, cupric ions, and by inhibitors of heavy metal enzymes.

³ Mason, H. S., private communication.
The mechanism proposed for the reaction is patterned after the aerobic oxidation of dihydroxyfumaric acid catalyzed by horse-radish peroxidase.

BIBLIOGRAPHY
THE OXIDATION OF REDUCED PYRIDINE NUCLEOTIDES BY PEROXIDASE

T. Akazawa and Eric E. Conn


Access the most updated version of this article at http://www.jbc.org/content/232/1/403.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/232/1/403.citation.full.html#ref-list-1