Short Communication

Mechanism of Enhancement of IAA Oxidation by 2,4-Dichlorophenol[1, 2]

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We recently proposed a series of reactions to account for the observed formation of peroxidase compounds I and II during the aerobic oxidation of indole-3-acetic acid (IAA) by horseradish peroxidase (HRP) (3):

O_2 + HRP → HRP-O_2 (i)

HRP-O_2 + IAA → Per I (ii)

Per I + e^- → Per II + HO-IAA (iii)

Per II + IAA → HRP + IAA• (iv)

(HRP-O_2: a complex between ferriperoxidase and the biradical form of oxygen; Per I and Per II: intermediate peroxidase compounds I and II; HO-IAA: hydroxyindolenineacetic acid; IAA•: an IAA free radical.) The interaction between IAA and HRP led to enzyme inactivation or destruction, and it was postulated that this inactivation resulted from reactions between IAA free radicals and the enzyme (3). We subsequently reported a reaction time course in which 2 linear phases of IAA disappearance were observed (2). The first phase represented the enzyme catalyzed oxidation of IAA, while the second was interpreted as the oxidation of IAA by a free radical chain mechanism. Other data have been interpreted as suggesting the presence of free radicals (5, 9, 12), and the peroxidase catalyzed production of substrate free radicals is consistent with data obtained from other oxidative and peroxidative systems (6, 7, 10, 11).

2,4-Dichlorophenol (DCP) is widely employed as a promoter of the peroxidase catalyzed oxidation of IAA; however, the mechanism of promotion has not been elucidated. We now propose a mechanism of DCP action which is consistent with that shown above for the IAA-HRP interaction and with other mechanisms suggesting the formation of substrate free redicals in peroxidase catalyzed oxidations.

IAA and DCP were obtained from K and K Laboratories (New York) and analytical reagent grade HRP from Mann Research Laboratories (New York). HRP concentration was calculated by methods described previously (2, 3).

IAA oxidation was assayed in 2 ways. For the spectrophotometric assay, a Beckman DK-2A ratio recording spectrophotometer with a wavelength scan attachment was used. Scans were made from 340 to 230 nm. The Salkowski assay employed the Gordon-Weber modified reagent (4). At various times, 2 ml aliquots of the reaction mixture were added to 4 ml modified Salkowski reagent. Color was allowed to develop in the dark for 30 minutes, and the optical density at 530 nm was measured with a Beckman DB spectrophotometer.

Absorbance changes during the course of IAA oxidation are shown in figure 1. After 75 minutes the spectrum showed maxima at 254 and 248 nm. This suggested the presence of methyleneoxindole, the likely final product of peroxidase catalyzed IAA oxidation *in vitro* (5). The conversion of IAA to methyleneoxindole was not a 1-step reaction, since spectral changes continued to occur after all IAA

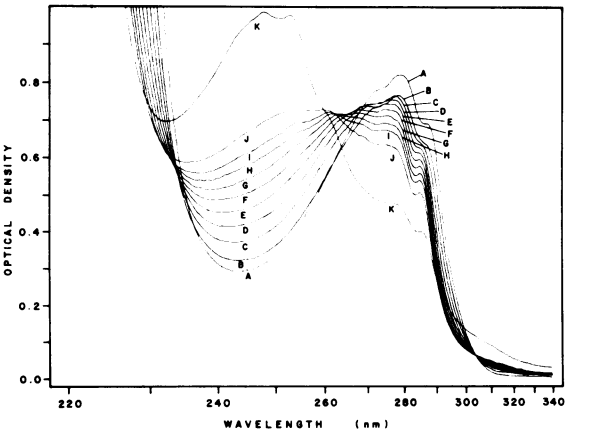

FIG. 1. Spectral changes during IAA oxidation. A through K represent times 0, 10 seconds, 2, 4, 6, 8, 10, 12, 14, 18, and 75 minutes. Reagent concentrations: IAA, 0.15 mM; HRP, 0.20 μM in 0.06 M acetate buffer (pH 4.9).

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[2] Much of this material was included in a doctoral thesis submitted by L. R. Fox to the Graduate Division of the University of California, Santa Barbara.

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Table I. *Spectral Changes and IAA Consumption During the IAA Oxidase Reaction*

Aliquots of reaction mixture simultaneously assayed spectrally and by Salkowski assay. Reagent concentration: IAA, 0.20 mM; HRP, 0.75 μM in 0.06 M citrate-phosphate buffer, pH 5.8.

Time	IAA consumed	Optical density 300 nm	255 nm
min	*μmoles*		
0	0	0.170	0.510
0.25	1.26	0.170	0.735
3	3.42	0.170	0.860
6	3.70	0.170	0.950
9	3.72	0.185	1.000
14	3.72	0.205	1.070

had disappeared (table I). During the course of the reaction an isosbestic point appeared at 300 to 304 nm, and its existence was correlated with the presence of IAA. Once the IAA had disappeared, absorption at 300 to 304 nm increased. These data indicated that peroxidase catalyzed the oxidation of IAA to some first product, and that this was ultimately converted to methyleneoxindole. These findings are in agreement with those of Ray (8) and of Hinman and Lang (5).

DCP at low concentrations greatly increased the rate of IAA oxidation (table II). Fox *et al.* proposed a mechanism for the oxidation of IAA which suggested that IAA free radicals were produced by a reaction of IAA with the secondary intermediate compound (compound II) of HRP (3), and Chance showed that the transition from compound I to compound II was accelerated in the presence of hydrogen donors (1). Using stopped flow techniques, such an accelerated transition between compound I and compound II was observed when DCP was included in the reaction mixture (3). Thus, DCP may have promoted IAA oxidation by accelerating the formation of compound II, resulting in a more rapid production of IAA free radicals.

This mechanism is not, however, the only way in which DCP promoted the oxidation of IAA. When DCP was added to a reaction mixture 75 seconds after the start of the reaction [during the nonenzymic phase (2)], the rate of IAA oxidation

Table II. *The Effect of Dichlorophenol on the Rate of IAA Oxidation*

IAA determined by Salkowski assay. Reagent concentration: IAA, 0.15 mM; HRP, 0.13 μM in 0.06 M acetate buffer (pH 5.0).

DCP concentration	IAA oxidized	Promotion
μM	*μmoles/min*	%
0	1.44	...
1.0	2.28	58
5.0	4.48	211
10.0	5.64	292

was nearly double that of the control, the rates being 68 and 36 μmoles/minute, respectively. The rate of IAA oxidation during the enzymic phase (2) in the absence of DCP nearly equaled the rate during the nonenzymic phase in the presence of DCP. Thus, one might reason that DCP acted to restore the enzymatic oxidation of IAA. However, this explanation is unsatisfactory since, when DCP was added to an HRP reaction mixture following inactivation of the enzyme, no reactivation of the destroyed HRP was observed (3).

Ray postulated a 2-step reaction to account for the spectral changes observed during IAA oxidation catalyzed by *Omphalia* peroxidase (8). The spectra of the first and second products (A and B) were isosbestic at 261 nm. In the present study, when DCP was added to the reaction mixture before all of the IAA was consumed (determined on the basis of the isosbestic point at 300–304 nm), there was an immediate change in the spectrum of the reaction mixture. Subsequent changes in the spectrum revealed the loss of the isosbestic point at 300 to 304 nm and the establishment of a new isosbestic point at 262 nm (fig 2). This indicated that DCP, added before or after the reaction was started, accelerated the conversion of IAA to a first product (A in Ray's terminology).

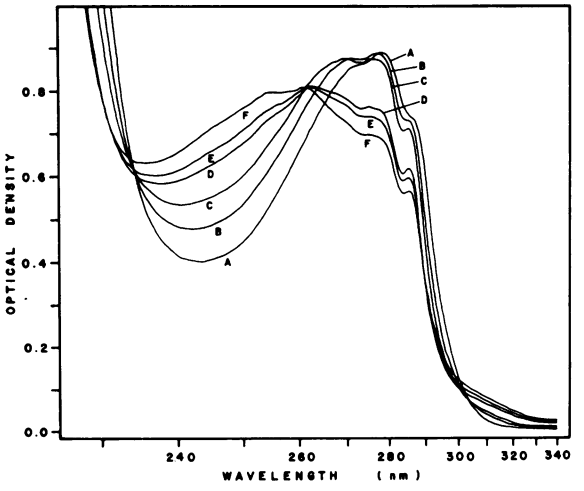

FIG. 2. Spectral changes upon addition of dichlorophenol 435 seconds after reaction started. A through F represents times 10 seconds, 2, 4, 8, 10, and 15 minutes after start of reaction. Spectra after DCP addition not corrected for dilution. Reagent concentrations: IAA, 0.15 mM; DCP, 10 μM; HRP, 0.19 μM in 0.06 M acetate buffer, pH 4.9.

Reactions v to xi are proposed to explain the promotive effects of DCP on IAA oxidation.

$$\text{Per I} + \text{DCP} \rightarrow \text{Per II} + \text{DCP}\cdot \quad \text{(v)}$$
$$\text{Per II} + \text{DCP} \rightarrow \text{HRP} + \text{DCP}\cdot \quad \text{(vi)}$$
$$\text{Per II} + \text{IAA} \rightarrow \text{HRP} + \text{IAA}\cdot \quad \text{(vii)}$$
$$\text{DCP}\cdot + \text{IAA} \rightarrow \text{DCP} + \text{IAA}\cdot \quad \text{(viii)}$$
$$\text{IAA}\cdot + O_2 \rightarrow \text{IAA-}O_2\cdot \quad \text{(ix)}$$
$$\text{IAA-}O_2\cdot \rightarrow \text{P}\cdot + CO_2 + H_2O \quad \text{(x)}$$
$$\text{DCP} + \text{P}\cdot \rightarrow \text{DCP}\cdot + \text{P} \quad \text{(xi)}$$

Compound I formation occurred in the manner described previously (3). Reaction v explains the accelerated transition between compounds I and II observed in the presence of DCP (3). That both DCP (vi) and IAA (vii) reacted with compound II was suggested by the accelerated rate of decomposition of compound II in the presence of DCP (3). When reactions v to vii are compared to reactions i to iv, it is evident that at least twice as many free radicals were produced during the IAA-HRP interaction in the presence of DCP as in its absence. It is further proposed that DCP acts to maintain the free radical chain by undergoing reactions viii and xi. A reaction of DCP with P•, rather than IAA• or IAA-O_2•, is proposed because apparently reactions ix and x occur so rapidly that these compounds are probably not significant intermediates in the free radical chain (5).

This mechanism (v to xi) can also explain the promotive effect of DCP on IAA oxidation when the phenol was added during the nonenzymic phase of IAA destruction. It is proposed that in the absence of DCP, the free radical chain was maintained by reactions between free radical intermediates of IAA oxidation (P•) and IAA, producing IAA• and P. When DCP was added during the nonenzymic phase it reacted with free radical intermediates of IAA oxidation, as in reaction xi, and the DCP• produced would subsequently react with IAA (as in reaction viii). If P• was more reactive toward DCP than toward IAA, and the DCP• produced by such a reaction was less stable than P•, then the reactivity of free radicals in the system was increased when DCP was added, even though the free radical concentration was not altered. It is likely that DCP• is more reactive than free radical intermediates of IAA oxidation, since the indole ring could stabilize the odd electron more readily than could the benzene ring system of DCP.

Thus, it is proposed that DCP promoted IAA oxidation by increasing the concentration of free radicals (when the enzyme was active), and by the formation of free radicals which were more reactive than those present during IAA oxidation in the absence of DCP. Furthermore, it is proposed that when DCP was present during the enzyme catalyzed phase of IAA oxidation, IAA free radicals (or P•) reacted with DCP rather than with HRP, thus preventing enzyme inactivation (3).

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