The Role of Horseradish Peroxidase in Indole-3-acetic Acid Oxidation*

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ABSTRACT: When indole-3-acetic acid (IAA) was added to horseradish peroxidase (HRP) under aerobic conditions, intermediate HRP compounds were formed which were spectrally and kinetically similar to compounds I and II formed from HRP and HOOH. Enzyme inactivation occurred as a result of the HRP–IAA interaction in the absence of a reducing agent. Reducing agent added after regeneration of the free enzyme had no effect on the amount of HRP inactivated. KCN and $Na_2S_2O_5$ inhibited the oxidation of IAA when added before the start of the reaction. When these inhibitors were added after the reaction was started, only partial inhibition was observed with KCN at a concentration sufficient to convert all of the enzyme to an inactive HRP–CN^- form, while $Na_2S_2O_5$ completely inhibited IAA oxidation. This suggests that IAA oxidation occurs mainly through a free radical chain mechanism and that the function of the enzyme is to generate the free radicals. No catalase inhibition of IAA oxidation was observed. A new mechanism for the IAA oxidase reaction catalyzed by HRP is proposed. This mechanism involves a rapid equilibrium exchange of biradical molecular oxygen with water through a coordination position of the ferric ion to yield an oxygenated form of HRP. It is suggested that the oxygenated form of HRP attacks IAA to form an intermediate compound spectrally similar to and electronically identical with the proposed compound I formed from HRP and HOOH.

The oxidative inactivation of the plant growth hormone indole-3-acetic acid (IAA)[1] by peroxidase has been intensively studied since the original discovery of IAA inactivation by plant extracts (Larsen, 1936, 1940). The literature has been lucidly reviewed by Ray (1958) and Galston and Hillman (1961). Hinman and Lang (1965) have shown that horseradish peroxidase (HRP), in the absence of added cofactors, can catalyze the oxidative conversion of IAA to either methylene oxindole or indolealdehyde. They proposed a mechanism involving the initial extraction of an electron from IAA to form a free radical and a rapid reaction of the radical with molecular oxygen to form a hydroperoxyl derivative of IAA.

These earliest steps of the reaction may, perhaps, best be examined in the light of earlier work on the reactions of HRP with simple peroxides. Soret region spectral changes in HRP were demonstrated upon the addition of hydrogen peroxide or either of two alkyl hydrogen peroxides (Chance, 1949a). Following the addition of a slight excess of HOOH to the free HRP (with an absorption maximum at 403 mμ), there was a rapid formation of a green compound (compound I) with an absorption maximum at 410 mμ and isosbestic with free HRP at 427 mμ. This compound was rapidly converted to a pale red compound (compound II) which had an absorption maximum at 417 mμ and was isosbestic with free HRP at 411 mμ. Compound II decayed spontaneously, regenerating free peroxidase.

We shall present data showing that IAA, or its derivative, can interact with HRP to form compounds spectrally similar to compounds I and II formed from HRP and simple peroxides. On the basis of the properties of these compounds, we shall develop a model for the mechanism of the "oxidase" reactions of peroxidase (Saunders *et al.*, 1964) and attempt to show a strong similarity between the "oxidase" and "peroxidase" functions of the enzyme.

Materials and Methods

Reagent grade indole-3-acetic acid (IAA) obtained from Eastman Chemical Co. was freshly dissolved (in equimolar $NaHCO_3$) each day, and the solutions were kept in a brown storage bottle at 8° to minimize decomposition.

Horseradish peroxidase (HRP) with an RZ value of approximately 2.0 was obtained from Mann Research Laboratories. The protein concentration was calculated by multiplying the optical density at 280 mμ by 1.55 and by a dilution factor. This gave protein concentrations which agreed well with the manufacturer's advertised concentration. The RZ value was calculated as the ratio $OD_{403}:OD_{275}$, and the purity was estimated on the basis of an RZ value of 3.04 for pure HRP. The peroxidase concentration was calculated from the protein

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[1] Abbreviations used in this work: DCP, 2,4-dichlorophenol; DHM, dihydroxymaleic acid; HRP, horseradish peroxidase; IAA, indole-3-acetic acid.

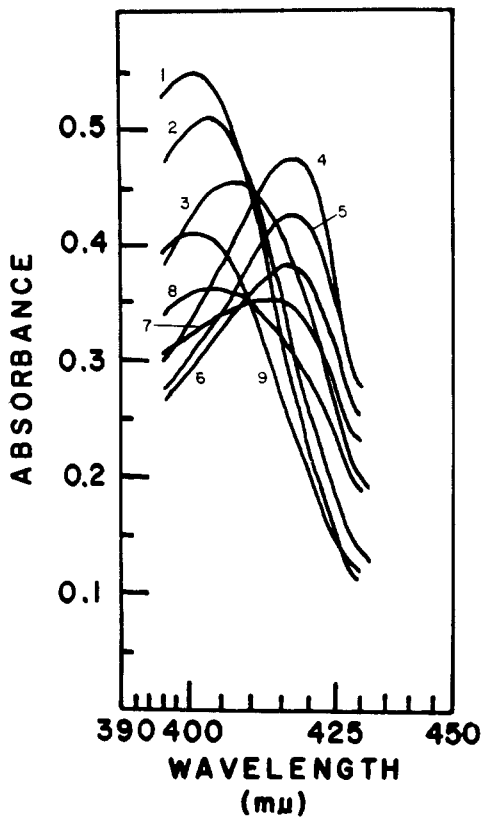


FIGURE 1: HRP spectral changes upon the aerobic addition of IAA at 20°. Curves 1–9 represent scans made at time 0, 15, 35, 60, 145, 245, 330, 360, and 480 sec, respectively. Total volume = 1.0 ml in 0.02 M citrate–phosphate buffer, pH 7.2. Reagent concentrations in cuvet: HRP = 6.4 μM, IAA = 0.12 mM.

concentration and purity, on the basis of a molecular weight of 40,000 for HRP.

Most observations were made with a Beckman DK-2A recording spectrophotometer, employing either a wavelength scan or a time drive attachment. Reactions were started by adding IAA to the reaction mixture in the spectrophotometer and mixing with a Dixon stirrer (Dixon, 1954) and modified for 1-ml cuvets. A temperature-controlled, water-jacketed cuvet holder was used for reactions run at temperatures below ambient. The reagents were kept at 8°, and the reaction mixtures were allowed to equilibrate for 2 min in the cuvets before the reactions were started.

In anaerobic experiments, all reagents were gassed for at least 45 min with N_2 which had bubbled through two 1500-ml baths of 5% alkaline pyrogallol. All reagents except IAA were added to 3-ml cuvets and gassed for an additional minute. The cuvets were then capped and allowed to equilibrate to reaction temperature for an additional minute. The cuvets were opened and gassed for another minute, and IAA was added. The reaction mixture was stirred by bubbling nitrogen through the cuvet for a short time, and the cuvets were capped.

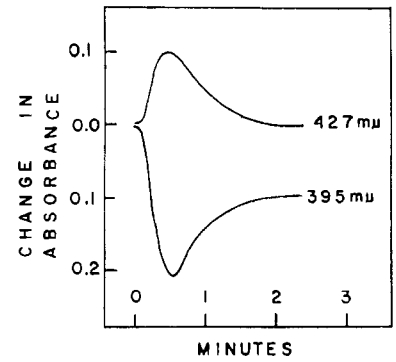


FIGURE 2: The effect of IAA on free HRP disappearance (395 mμ) and "compound II" formation (427 mμ) at 10°. Total volume = 1.0 ml in 0.02 M citrate–phosphate buffer, pH 4.8. Reagent concentration in cuvet: HRP = 5.1 μM, IAA = 30 μM.

For certain experiments, a stopped-flow chamber (French *et al.*, 1965) was used in conjunction with a Zeiss spectrophotometer. Traces in per cent transmittance were obtained on a high-speed Brush recorder and converted to absorbance. The cell was rinsed four times with each reagent; first with the enzyme dissolved in 0.02 M citrate–phosphate buffer, pH 5.0, and then with IAA dissolved in the same buffer. Slit width was maintained between 0.08 and 0.10 mm. The reactions were observed at 30°, and at least three repetitions were made at each wavelength.

Certain terms will be used throughout the paper in connection with specific processes and must necessarily be defined to avoid ambiguity. The term "disappearance of free enzyme" refers to the transition from the free enzyme to an intermediate compound form of the enzyme. The term "regeneration of free enzyme" refers to the transition from the compound II form of the enzyme back to the free enzyme. The inactivation or destruction of enzyme is referred to as the "loss of free enzyme," and is reflected in a net loss of absorbance following the regeneration of free enzyme.

Results

The spectral shift observed upon the addition of IAA to a purified HRP preparation is shown in Figure 1. The spectrum of the free enzyme, with a maximum absorption at 403 mμ, was replaced by one with a maximum of lower intensity at approximately 416–418 mμ. During the change in spectrum, an isosbestic point appeared at 410–412 mμ. Once the shift occurred there was a decreased absorbance, without a spectral shift, at the newly established maximum. This was followed by another shift back to the spectrum of the free enzyme, again with an isosbestic point in the 410–412 mμ region. The final absorbance of the regenerated free enzyme was less than the original absorbance, suggesting a destruction or inactivation of some of the en-

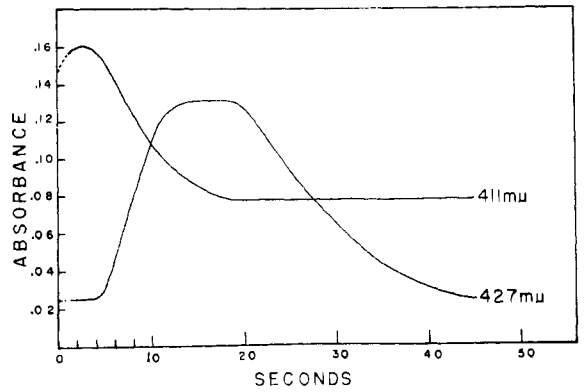


FIGURE 3: The effect of IAA on formation of intermediate HRP compounds using stopped-flow techniques at 30°. Tracings represent "compound I" (411 mμ) and "compound II" (427 mμ). Reagent concentration in cell: HRP = 3.4 μM, IAA = 50 μM, 0.02 M citrate–phosphate buffer, pH 5.0.

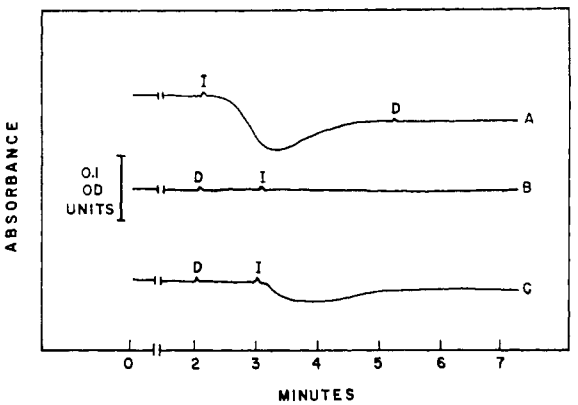


FIGURE 4: The effect of IAA and 2,4-dichlorophenol on free HRP disappearance and inactivation at 10°. I = addition of IAA, D = addition of 2,4-dichlorophenol. Total volume = 1.0 ml in 0.02 M citrate–phosphate buffer, pH 4.8. Reagent concentration in cuvet for all traces: HRP = 5.1 μM, IAA = 10 μM. Trace A, DCP = 10 μM added after enzyme regeneration; trace B, DCP = 10 μM added before IAA; trace C, DCP = 2 μM added before IAA.

zyme. During the spectral shifts another isosbestic point was observed in the 450–455 mμ region.

The effect of IAA on the simultaneous disappearance of free enzyme and appearance of "compound II" followed by the regeneration of free enzyme as "compound II" decomposed is shown in Figure 2. Free enzyme concentration was measured at 395 mμ, the isosbestic point between HRP intermediate compounds I and II (Chance, 1949a). The formation and decomposition of "compound II" was followed at 427 mμ, which is the isosbestic point between free HRP and compound I (Chance, 1949a). Upon the addition of IAA there was a rapid decrease in the free enzyme concentration and a rapid formation of a compound spectrally similar to compound II. Again, note the loss of enzyme as reflected in a net decrease of approximately 0.1 optical density unit at 395 mμ. The time of maximum formation of "compound II" corresponded to the time of minimum concentration of free enzyme at this IAA concentration. At higher IAA concentrations, where the rate of change at 427 mμ was greater, the maximum absorbance at 427 mμ preceded the minimum absorbance at 395 mμ.

The formation and decomposition of complexes resembling compounds I and II, detected by stopped-flow techniques, are shown in Figure 3. The formation of "compound I," followed at 411 mμ, was almost complete by the end of the mixing time (0.5–0.8 sec); decomposition of "compound I" began approximately 4 sec after the addition of the reagents. It was at this time that the formation of "compound II" was observed, as measured at 427 mμ. Note that no formation of "compound II" was observed before "compound I" began to decompose. Using extinction coefficients reported by Chance (1952b), the rate of decomposition of "compound I" was calculated to be 0.2 μmole/l. per sec. This value was identical with the calculated rate of formation of "compound II" during the linear phase.

The broad maximum of "compound II" concentration observed from 14 to 18 sec after reagent addition represents that period during which the rate of formation of "compound II" equaled the rate of decomposition of "compound II." The concentration of "compound II" began to decline approximately 19 sec after reagent addition, the time that corresponded with the complete decomposition of "compound I."

2,4-Dichlorophenol (DCP), when added to the reaction mixture after regeneration of free enzyme, had no effect on the amount of enzyme lost (compare Figure 2 and Figure 4, trace A). When DCP, in a concentration equal to that of IAA, was added before IAA, no disappearance of free enzyme was observed (Figure 4, trace B). When DCP was added in amounts less than stoichio-

TABLE I: The Effect of DCP on Intermediate Compound Formation Using Stopped-Flow Techniques.[a]

	Rate (× 10^{-6} M/sec)		
	Compd I Disappearance	Compd II Appearance	Compd II Disappearance
+IAA −DCP	0.17	0.23	0.03
+IAA +DCP	ND[b]	ND	0.82

[a] Reagent concentrations in cell: HRP = 2.6 μM; IAA = 50 μM; DCP = 20 μM; 0.02 M citrate–phosphate buffer, pH 5.0, at 30°. [b] ND = not detected.

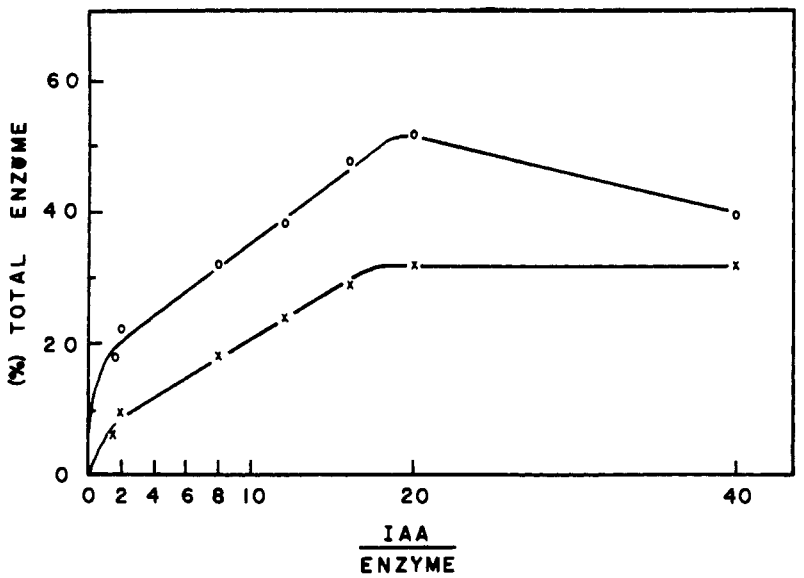


FIGURE 5: The effect of IAA:HRP ratio on the fraction of HRP complexed (○) and inactivated (×) at 10°. This figure is a composite of several experiments in 0.02 M citrate–phosphate buffer, pH 4.8, with an HRP concentration of 2–8 μM.

TABLE II: The Effect of Sequential IAA Additions on the Amount of HRP Complexed and Inactivated.[a]

IAA Addn No.	mmoles HRP ($\times 10^{-6}$)					% of HRP Complexed by this IAA Addn	Amt HRP Regenerated as % Active HRP Remaining
	Before IAA Addn	Complexed	Inactivated	Regenerated	Remaining		
I	4.98	2.59	1.65	0.94	3.33	52	29
	4.94	2.43	1.64	0.79	3.30	49	24
II	3.33	0.97	0.52	0.45	2.81	29	16
	3.30	0.88	0.41	0.47	2.89	27	16
III	2.81	0.42	0.42	0.00	2.39	16	0
	2.89	0.45	0.26	0.19	2.63	16	7
IV	2.39	0.00	0.00	0.00	2.39	0	0
	2.63	0.07	0.07	0.00	2.56	3	0

[a] IAA:HRP = 20 with first addition of IAA in 0.02 M citrate–phosphate buffer, pH 4.8, at 20°.

metric with the enzyme, some disappearance and loss of free enzyme was observed on adding IAA. As the amount of DCP was decreased, a greater amount of free enzyme disappearance and loss was observed (Figure 4, trace C). Table I presents data on the effect of DCP on intermediate compound formation using stopped-flow techniques. The rates were calculated from previously reported extinction coefficients (Chance, 1952b). In the absence of DCP, "compounds I and II" were observed, and the rate of disappearance of "compound I" was equal to the rate of formation of "compound II." When DCP was included in the IAA solution in a concentration exceeding that of the enzyme, "compound II" was fully formed by the end of the mixing time. Since we could not detect either "compound I" or the formation of "compound II," we were only able to calculate the rate of disappearance of "compound II" in the presence of DCP. This rate was approximately 300 times greater than that of the unsupplemented system, and "compound II" disappearance was completed approximately 1 sec after mixing was completed.

The effects of the IAA:HRP ratio on the fraction of the enzyme entering into complex and on the fraction inactivated by the interaction with IAA are shown in Figure 5. This figure is a composite of several experiments with different enzyme concentrations and is,

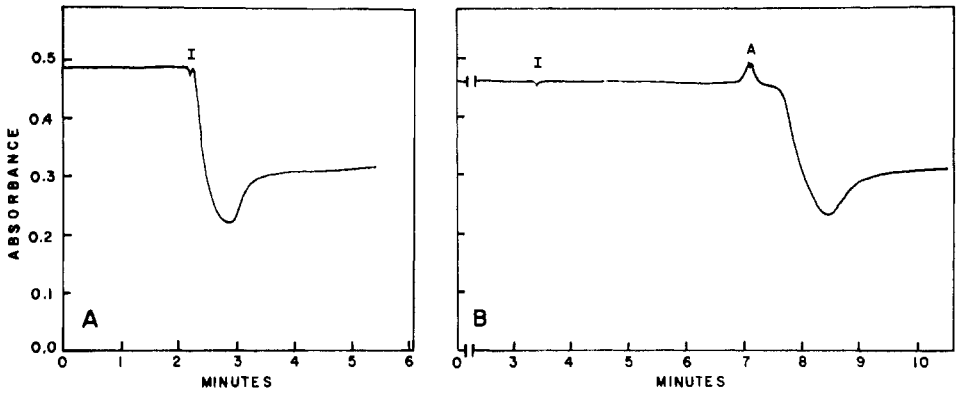


FIGURE 6: The effect of IAA and anaerobiosis on free HRP disappearance at 10°. (A) The aerobic adddition of IAA (I); (B) the anaerobic addition of IAA followed by the addition of air (A). Total volume = 1.0 ml in 0.02 M citrate–phosphate buffer, pH 4.8. Reagent concentration in cuvet: HRP = 5.1 μM, IAA = 0.10 mM.

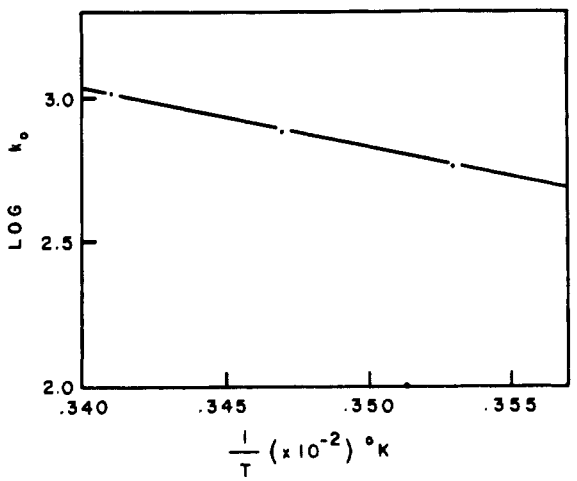


FIGURE 7: An Arrhenius plot of the IAA–HRP interaction at 10, 15, and 20°. Total volume = 1.0 ml in 0.02 M citrate–phosphate buffer, pH 5.8. Reagent concentration in cuvet: HRP = 5.2 μM, IAA = 20 μM.

therefore, expressed in relative terms. An extinction coefficient of 85.0 mM^{-1} cm^{-1} was calculated for HRP at 395 mμ. The change from initial to minimum absorbance at 395 mμ was used to calculate the amount of enzyme complexed, and the difference between the initial and final absorbances was used to calculate the amount of enzyme lost. As the amount of IAA relative to HRP concentration was increased, there was an increased amount of enzyme participating in complex formation and an increased amount of enzyme inactivated. The optimum IAA:HRP ratio was about 20, at which approximately 50% of the enzyme participated in complex formation and about 25% was inactivated or destroyed.

The effects of sequential additions of IAA to the enzyme are shown in Table II. IAA was added to the reaction mixture, complexing occurred, and regeneration of free HRP was completed before the subsequent addition of IAA. The IAA concentration was such that the maximum amount of complex would form upon the first addition of IAA. With the first IAA addition (I) approximately 50% of the enzyme molecules participated in complex formation, and some were inactivated. That fraction which was not inactivated, but regenerated, is expressed as a percentage of the free enzyme which remained following enzyme inactivation. After the first IAA addition approximately 27% of the free enzyme remaining was that which had regenerated from the first complexing. With the second IAA addition (II) approximately 28% of the enzyme complexed with IAA, and the amount regenerated represented about 16% of the enzyme remaining following inactivation due to the second complexing. Upon the third addition of IAA (III), 16% of the enzyme was complexed and all of this enzyme was inactivated. In a later experiment, an enzyme preparation was exposed to successive treatments with IAA at a dosage such that the first addition did not yield maximum complex formation. After nine additions of IAA the enzyme lost its ability to complex with IAA. Fifty per cent of the enzyme had been inactivated by the IAA–HRP interaction, the same percentage of enzyme lost during the interaction at the optimal IAA:HRP ratio.

A series of absorbance traces at 395 mμ are shown in Figure 6. Figure 6A shows the disappearance, regeneration, and loss of free enzyme upon the addition of IAA. When IAA was added to the same reaction mixture under anaerobic conditions, there was no disappearance of free enzyme (Figure 6B). When air was subsequently bubbled through this reaction mixture, there was a typical, although somewhat slower, disappearance of free enzyme. Approximately the same amount of enzyme disappeared and was lost in both cases (traces A and B).

An Arrhenius plot of the IAA–HRP interaction is shown in Figure 7. The rate of free HRP disappearance was measured at 395 mμ, pH 5.8, at three different temperatures. The pH value was chosen so that the rate

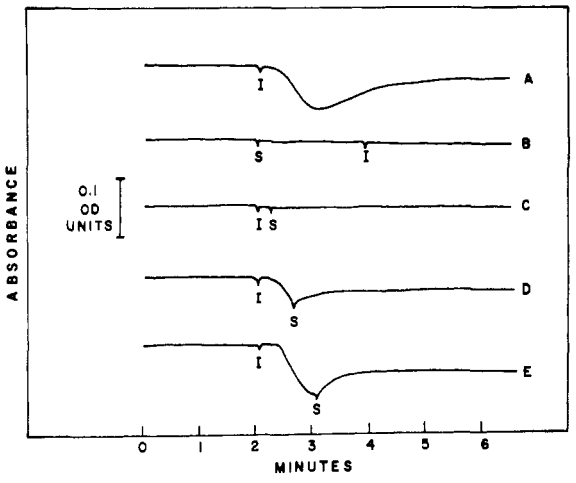


FIGURE 8: The effect of sodium metabisulfite and IAA on free HRP disappearance at 10°. I = addition of IAA, S = addition of $Na_2S_2O_5$. Trace A = addition of IAA to HRP alone; trace B = addition of $Na_2S_2O_5$ before IAA; trace C = addition of $Na_2S_2O_5$ during the short lag period following IAA addition; trace D = addition of $Na_2S_2O_5$ when approximately one-half of the potential HRP disappearance had occurred; trace E = addition of $Na_2S_2O_5$ at time of maximum free HRP disappearance. Total volume = 1.0 ml in 0.02 M citrate–phosphate buffer, pH 4.8. Reagent concentration in cuvet: HRP = 5.1 μM, IAA = 8.0 μM, $Na_2S_2O_5$ = 10 μM.

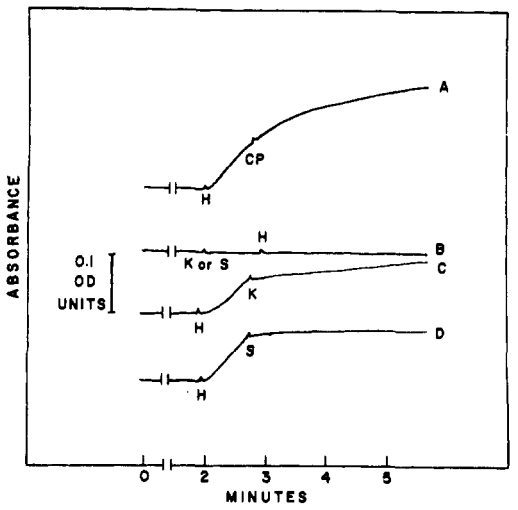


FIGURE 9: The effect of KCN and $Na_2S_2O_5$ on IAA oxidation (261 mμ) at 10°. CP = addition of buffer, H = addition of HRP, K = addition of KCN, S = addition of $Na_2S_2O_5$. Trace A = addition of HRP followed by addition of buffer approximately 45 sec later; trace B = addition of KCN or $Na_2S_2O_5$ before HRP; trace C = addition of KCN approximately 45 sec after HRP; trace D = addition of $Na_2S_2O_5$ approximately 45 sec after HRP. Total volume = 1.0 ml in 0.02 M citrate–phosphate buffer, pH 5.0. Reagent concentration in cuvet: HRP = 0.53 μM, IAA, KCN, and $Na_2S_2O_5$ = 0.15 mM.

would be sufficiently slow for accurate measurement at the highest temperature. The measured rates were used to calculate the observed second-order rate constant (k_0). The Arrhenius activation energy (E_a) was obtained from the equation

$$E_a = -2.303R \frac{d(\log k_0)}{d(1/T)}$$

The Arrhenius energy of activation was found to be −9.33 kcal/mole. The Gibbs free energy of activation (ΔF^*), the entropy of activation (ΔS^*), and the enthalpy of activation (ΔH^*) were calculated from the following formulas, the values obtained being given in Table III.

$$\Delta F^* = -2.303RT \log \left[\frac{k_0 h}{k_b T}\right]$$

$$\Delta H^* = -2.303R \left[\frac{d(\log k_0)}{d(1/T)} + T\right]$$

$$\Delta S^* = \frac{\Delta H^* - \Delta F^*}{T}$$

where h = Planck's constant and k_b = Boltzmann's constant.

Potassium cyanide is known to form an HRP–CN^- complex, producing a spectral shift in the Soret region so that a new maximum appears at approximately 423 mμ (Keilin and Hartree, 1951). When we added KCN to the enzyme preparation there was a decrease in absorbance at 395 mμ, and a new maximum appeared at approximately 421 mμ. The enzyme treated with KCN was unable to interact with IAA.

The effects of adding sodium metabisulfite ($Na_2S_2O_5$) to the reaction mixture at various times are shown in Figure 8. Trace A shows the usual free enzyme disap-

TABLE III: Thermodynamic Constants for the IAA–HRP Interaction.[a]

	Temperature (°C)		
	10	15	20
ΔF^* (kcal/mole)	13.0	13.1	13.1
ΔH^* (kcal/mole)	−10.6	−10.6	−10.6
ΔS^* (kcal/deg mole)	−83.4	−82.3	−80.9

[a] Constants calculated from formulae in text using rate constants calculated at each temperature. Total volume = 1.0 ml in 0.02 M citrate–phosphate buffer, pH 5.8. Reagent concentrations in cuvet: HRP = 5.2 μM, IAA = 20 μM.

pearance upon the addition of IAA. When $Na_2S_2O_5$ was added to HRP there was no effect on the enzyme, but the subsequent addition of IAA caused no disappearance of free enzyme (trace B). When metabisulfite was added after IAA, during the short lag period, there was still no observed free enzyme disappearance (trace C). If $Na_2S_2O_5$ was added when approximately one-half of the potential complex had formed, there was an immediate cessation of complexing with about 50% of the complexed enzyme being regenerated to free enzyme (trace D). When the metabisulfite was added at the time of maximum complex formation (minimum free enzyme absorbance), there was no effect on the amount of enzyme complexed, but the amount of enzyme lost was increased by about 4% over the control value (trace E). Using stopped-flow techniques, neither "compound I" nor "compound II" was detected when sodium metabisulfite was included in the IAA solution.

The rate of IAA disappearance from HRP-containing solutions was used as a measure of over-all reaction rate. This disappearance may be followed as a change in optical density at 261 mμ (Ray, 1956). The effects of an enzyme inhibitor (KCN) and a free radical inhibitor ($Na_2S_2O_5$) on the rate of IAA disappearance are shown in Figure 9. When either inhibitor was added before the enzyme no disappearance of IAA was observed (trace B). When KCN was added approximately 45 sec after the reaction had started the oxidation of IAA was only partially inhibited (trace C). However, when $Na_2S_2O_5$ was added to the reaction 45 sec after the start the reaction was completely inhibited (trace D). At the enzyme concentration used the formation and decomposition of intermediate compounds of the enzyme is completed in a short time, while the disappearance of IAA continues long after "compound II" has regenerated to the free enzyme.

Table IV shows the effect of twice crystallized beef liver catalase (Mann Research Laboratories) on the rate of IAA disappearance at pH 5.2, followed at 261 mμ (Ray, 1956). Catalase was added to the IAA solution at the time this solution was prepared (treated IAA) in order to ensure that no free hydrogen peroxide was present. The catalase was added to the IAA in such amount that its concentration in the cuvet would equal 0.18 μM. The data of Table IV indicate that catalase had no effect on the rate of IAA oxidation catalyzed by HRP whether catalase was added to the cuvet just prior to IAA or to the IAA at the time of its preparation. Also, catalase was unable to effect by itself the oxidation of IAA in our system. These observations are in disagreement with previous reports of inhibition by catalase of IAA oxidation (Galston *et al.*, 1953; Kenten, 1955), which has been ascribed to a dialyzable factor in the catalase preparations (Waygood *et al.*, 1956).

TABLE IV: The Effect of Catalase on IAA Oxidation.

Enzyme ($\times 10^{-7}$ M)		IAA ($\times 10^{-4}$ M)		IAA Oxidation ($OD_{261m\mu}$/20 min)
HRP	Catalase	Untreated	Treated[a]	
2.0		1.0		0.12
2.0	2.0	1.0		0.12
2.0	0.2		1.0[a]	0.13
	2.0	1.0		0.00

[a] An IAA solution to which catalase was added at the time of solution preparation so that the catalase concentration in the cuvet was 1.8×10^{-7} M. All values given are concentrations in the cuvet. Total volume = 1.0 ml in 0.02 M acetate buffer, pH 5.2, at 20°.

Discussion

Nature of the Intermediate Compounds. The terminology used in this paper refers to the HRP intermediates which are analogous to those described previously (Chance, 1949a, 1952b; George, 1953), and are assigned their respective numbers on the basis of absorption maxima, isosbestic points, and spectral kinetics (see introduction). The changes in the HRP Soret spectrum upon the aerobic addition of IAA are shown in Figure 1. We propose that the observed spectral shift is due to a mixture of compounds I and II resulting from an incomplete conversion of compound I to compound II, a phenomenon observed when reducing agents are not added (Chance, 1952b; George, 1952). The data in Figure 3 support the existence of an authentic compound I and present clear evidence for its conversion to compound II. No evidence for the formation of compounds III and IV was detected by our procedures. The absence of compound III was not unexpected in that the presence of this compound appears to be associated with high peroxide concentrations, and these experiments were performed in the absence of any peroxide or known peroxide-generating systems. No evidence for compound IV was obtained, even though stopped-flow tracings were continued for a longer period of time than shown in Figure 3. Note, however, that in Figures 1 and 2 and in Table III there is an apparent loss of enzyme either during or following the decomposition of the intermediate HRP compounds.

Reducing agents have been shown to accelerate the transitions between the intermediate compounds of HRP formed from simple peroxides (Chance, 1952b; George, 1953). The effect of DCP, a reducing agent, on the formation and decomposition of compounds I and II is shown in Figure 4. The apparent lack of free enzyme disappearance appeared to be the result of accelerated transitions between the intermediate compounds of peroxidase, as indicated by the stopped-flow data in Table I. In the presence of DCP, compound II was already fully formed by the time mixing was completed, and the total decomposition of compound II occurred approximately 1 sec after mixing was completed. This suggests that the intermediate compounds of the enzyme formed during the HRP–IAA interaction are not only spectrally and kinetically similar to the

intermediate compounds formed from simple peroxides, but that they are also similar in their behavior toward reducing agents.

As shown in Figure 5, intermediate compound formation was observed with an IAA:HRP ratio as low as 2, at which the absolute concentration of IAA was 10 μM, and the amount of enzyme complexed increased with an increase in IAA concentration to an optimum IAA:HRP ratio of 20. At the optimal ratio, only 50% of the enzyme present participated in intermediate compound formation, suggesting either that only half of the enzyme absorbing in the Soret region is capable of forming these compounds during the HRP–IAA interaction or that not all of the different molecular forms of the enzyme (Jermyn and Thomas, 1954) were able to interact with IAA.

From the observed second-order rate constant, we have calculated a Gibbs free energy of activation of 13.1 kcal/mole for the HRP–IAA interaction (Table III). Using reported rate constants (Chance, 1949b), we have also calculated the Gibbs free energy of activation for various peroxides (kcal/mole): HOOH, 7.88; MeOOH, 8.27; EtOOH, 8.40. The free energies of activation tend to increase with increasing size of the R group in ROOH, and IAA is consistent with this trend.

Oxygen Requirement and the Absence of Hydrogen Peroxide. When catalase was added to the aerobic HRP–IAA system, no inhibition of IAA oxidation was observed (Table IV). This, together with the absence of compound III, shows that hydrogen peroxide was not present in our system and that the observed compounds I and II were not the result of an interaction between HRP and oxidatively generated HOOH. Oxygen was an absolute requirement for the formation of the intermediate compounds of the enzyme during the HRP–IAA interaction (Figure 6) and for the oxidation of IAA catalyzed by HRP (unpublished data). In Figure 6, note that upon the addition of air to the anaerobic reaction mixture there was an immediate formation of the intermediate compounds of HRP. The absence of a lag in the formation of intermediate compounds between the anaerobic and aerobic phases suggests that HOOH was not oxidatively generated from IAA and that a hydroperoxyl derivative of IAA was not formed prior to interaction with the enzyme.

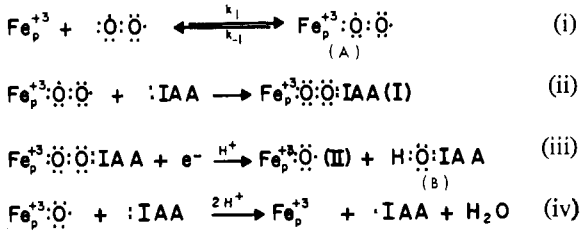
Evidence for the Existence of Free Radicals. Using electron paramagnetic resonance techniques it has been shown that substrate free radicals were formed during the peroxidative oxidations of hydroquinone, ascorbic acid, and dihydroxyfumaric acid (Yamazaki *et al.*, 1960). The existence of free radicals during the oxidation of IAA catalyzed by peroxidase has also been inferred by Yamazaki and Souzu (1960) and by Ray (1962). If the oxidation of IAA is strictly an enzyme-catalyzed reaction, then an enzyme inhibitor (such as KCN) should inhibit the oxidation of IAA completely, whether it is added before or after the start of the reaction. On the other hand, if the oxidation proceeds through a free radical mechanism, a free radical inhibitor (such as $Na_2S_2O_5$) added before or after the start of the reaction should inhibit the reaction completely, but KCN should be less effective after initiation of the reaction. The data presented in Figure 9 suggest the existence of free radicals during IAA oxidation. When KCN was added after the start of the reaction only partial inhibition of IAA oxidation was observed, while $Na_2S_2O_5$ completely inhibited the reaction. Using stopped-flow techniques we also observed that $Na_2S_2O_5$ completely inhibited the formation of compounds I and II in our system (see Results), suggesting that free radicals are required for the HRP–IAA interaction.

Enzyme Inactivation. The data presented in Figures 1 and 2 and in Table II indicate that some kind of enzyme inactivation occurred as a result of the HRP–IAA interaction. We have demonstrated the absence of HOOH during the interaction and therefore rule out enzyme inactivation due to a high peroxide concentration. Figure 3 demonstrates the formation of compound II, and this, together with the data presented in Figure 1, suggests that enzyme inactivation occurred either during or following the decomposition of compound II. It has been shown that the transitions from compound I to compound II and from compound II to the free enzyme involve one-electron reductions (Chance, 1952a; George, 1953), and that these transitions occur even with pure, crystalline samples of HRP in the absence of reducing agents (Chance, 1949a). It was suggested that the spontaneous transitions were due to reducing groups on the enzyme and that when reducing agents were present in the reaction mixture they could catalyze the transitions either directly or indirectly by reducing the oxidized "endogenous donors" (Chance, 1949b, 1952b). The data in Figure 4 show that DCP, a reducing agent, had no effect on the amount of enzyme inactivated when it was added following enzyme inactivation. Yet when DCP was present in the reaction mixture before the HRP–IAA interaction occurred, no enzyme inactivation was observed (Figure 4). Our data suggest that the observed HRP inactivation during the HRP–IAA interaction involves the removal of electrons from the enzyme, but the mode and agent of the electron withdrawal is not known.

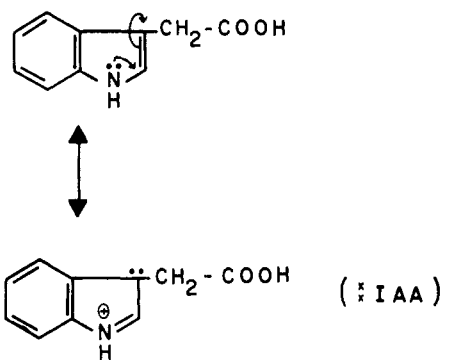
Mechanism of the HRP–IAA Interaction. To summarize, for the formation of intermediate compounds of the enzyme during the HRP–IAA interaction we have shown: (1) the absolute requirement for O_2; (2) a requirement for free radicals and the probable formation of free radicals during the oxidation of IAA; (3) the formation of enzyme intermediate compounds I and II; (4) that compound I is converted to compound II in a manner analogous to the one-electron reductions of intermediate compounds formed from simple peroxides, and that this electron may come from either an added reducing agent (DCP) or the enzyme itself, the latter possibly resulting in enzyme inactivation; (5) the lack of endogenous hydrogen peroxide; and (6) the absence of compounds III and IV.

The following proposed mechanism is our attempt to rationalize our findings with those of previous workers. It should be pointed out that the structures of compounds I and II are far from being understood and that there is evidence that the representation of the

enzyme as ferriperoxidase–H_2O may be incorrect (George and Lyster, 1958). However, the structures we shall propose for compounds I and II formed in our system are electronically identical with those proposed for these compounds formed from simple peroxides (Theorell *et al.*, 1952), and we shall represent the enzyme as ferriperoxidase–H_2O for simplicity.



Reaction i is a rapid exchange between water and the biradical form of molecular oxygen through a coordination position of the ferric ion. This is proposed as a very rapid equilibrium where k_{-1} is much larger than k_1, so that only a very small equilibrium concentration of the oxygenated enzyme (A) is formed in the absence of substrates. This oxygenated form of the enzyme would be a good electrophile, because of the biradical nature of the coordinated oxygen. On the other hand, the IAA molecule can obtain a nucleophilic nature by building up a two-electron excess on the 3 position of the indole ring through a mesomeric release of the extra pair of electrons on the nitrogen atom (reaction v). Thus, the



oxygenated form of the enzyme could attack the excess electrons on the IAA molecule to yield the proposed compound I, as shown in reaction ii. The proposed structure for compound I formed from IAA is electronically identical with the structure proposed by Theorell *et al.* (1952) for compound I derived from methyl hydrogen peroxide (C). These reactions explain

both the requirement for oxygen and free radicals for the formation of compound I in our system.

Chance (1952a) showed that the conversion of compound I to compound II was a one-electron reduction of compound I, the electron being provided either by a reducing group on the enzyme (Chance, 1949b, 1952b) or by some reducing agent in the reaction mixture. Such a conversion is represented by reaction iii in our proposed system. With the addition of an electron from a reducing group on the enzyme, from an added reducing agent, or possibly from a second IAA molecule, compound I disassociates to produce a proposed 3-hydroxyindolenine-3-acetic acid (B) and compound II. The electronic structure proposed for compound II is identical with that proposed by Theorell *et al.* (1952) for compound II formed from methyl hydrogen peroxide (D). George (1952) demonstrated that compound II was one oxidizing equivalent above the free enzyme and was converted to the free enzyme by a one-electron reduction. In our system we propose that in the absence of reducing agents a second molecule of IAA can effect this reduction, as shown in reaction iv. The products of this reaction would be the free enzyme and an IAA radical, produced by the abstraction of one of the excess electrons carried at the 3 position of the indole ring. The IAA radical produced in reaction iv could then, by an autocatalytic, free radical mechanism, undergo the reaction series proposed by Hinman and Lang (1965) leading to methyleneoxindole. Using electron paramagnetic resonance techniques, Yamazaki *et al.* (1960) have detected substrate free radicals during the peroxidation of several peroxidase substrates. These findings lend support to the postulated generation of IAA free radicals in reaction iv since the free radical generation in the peroxidative systems involves the same intermediate enzyme structures as proposed for our oxidase system.

Hinman and Lang (1965) have proposed a mechanism for the oxidation of IAA leading to methyleneoxindole in which hydroperoxyl-IAA was an intermediate. If the hydroperoxyl-IAA was formed prior to interaction with the enzyme in our system, a rapid rate of autoxidation of IAA would be expected, and a lag would have been noted in the anaerobic–aerobic phase change, but this was not substantiated by our data.

Our proposed mechanism rationalizes the oxidase function of HRP with the structural data for the intermediate HRP–HOOH compounds more consistently than do previously proposed mechanisms. The intermediate compounds formed in this reaction can be rationalized into the same electronic structures as those proposed for intermediate compounds formed from simple peroxides. In so doing, the requirement for the enzyme to produce HOOH in one reaction and use it as substrate in the next was eliminated. This type of mechanism may explain the oxidase function of peroxidase in systems other than the IAA oxidase system. In fact, because of the ready availability of O_2 as compared with HOOH, the primary *in vivo* function of HRP may be as an oxygenase rather than a peroxidase.

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The Mechanism of Bilirubin Toxicity Studied with Purified Respiratory Enzyme and Tissue Culture Systems*

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ABSTRACT: The mechanism of bilirubin toxicity was studied in purified respiratory enzyme and whole cell systems. Like Amytal, a classical electron-transport inhibitor, bilirubin inhibited reduced nicotinamide-adenine dinucleotide oxidase but had little effect on succinate oxidase. Experiments with electron-transport fragments indicated that the locus of action of bilirubin was similar to Amytal. However, unlike Amytal, bilirubin had a powerful uncoupling effect on oxidative phosphorylation in whole mammalian cells in tissue culture.

Simultaneous measurements of oxygen uptake and glycolysis in intact cells further indicated that uncoupling was the primary toxic action of bilirubin on oxidative metabolism. Bilirubin also inhibited amino acid incorporation into protein, another property that distinguishes the mode of action of bilirubin from the barbiturates. Other bile pigments either had no effect or were effective only at greatly increased concentrations over that of bilirubin on electron-transport systems from purified enzymes or on whole cells in tissue culture.

The mechanism of bilirubin toxicity is poorly understood in spite of the interest this complex problem has received. The reward for its solution is twofold. Certainly there is academic interest in understanding the mode of action of any such potent biological inhibitor. However, there is also a very practical application in clinical medicine, that is, to help understand and perhaps prevent the brain damage that results in the newborn from the excessive accumulation of unconjugated bilirubin. The newborn infant lacks a significant level of activity of the enzyme, glucuronyl transferase, essential in the detoxication of bilirubin. This factor together with other circumstances unique to the newborn causes him to be especially vulnerable to this metabolic poison.

Previous workers have linked bilirubin toxicity to an effect on terminal oxidation. Day (1954) reported that bilirubin depressed the respiration of chopped rat brain. Cytochrome *c* reversed this inhibition. The same author, utilizing several different fresh tissue systems, extended these observations to include a series of tetrapyrrole pigments (Day, 1956). Bilirubin inhibited the

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