Mechanism of Horseradish Peroxidase Catalyzed Epinephrine Oxidation: Obligatory Role of Endogenous O_2^- and H_2O_2[†]

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ABSTRACT: Horseradish peroxidase (HRP) catalyzes cyanide sensitive oxidation of epinephrine to adrenochrome at physiological pH in the absence of added H_2O_2 with concurrent consumption of O_2. Both adrenochrome formation and O_2 consumption are significantly inhibited by catalase, indicating a peroxidative mechanism as a major part of oxidation due to intermediate formation of H_2O_2. Sensitivity to superoxide dismutase (SOD) also indicates involvement of O_2^- in the oxidation. Although SOD-mediated H_2O_2 formation should continue epinephrine oxidation through a peroxidative mechanism, low catalytic turnover, on the contrary, indicates that O_2^- takes part in a vital reaction to form an intermediate for adrenochrome formation and O_2 consumption. Generation of O_2^- is evidenced by ferricytochrome *c* reduction sensitive to SOD. On addition of H_2O_2, both adrenochrome formation and O_2 consumption are further increased due to reaction of molecular oxygen with some intermediate oxidation product. Peroxidative oxidation proceeds by one-electron transfer generating *o*-semiquinone and similar free radicals which when stabilized with Zn^{2+} or spin-trap, α-phenyl-*tert*-butylnitrone (PBN), inhibit adrenochrome formation and O_2 consumption. The free radicals thus favor reduction of O_2 rather than the disproportionation reaction. Spectral studies indicate that, during epinephrine oxidation in the presence of catalase, HRP remains in the ferric state absorbing at 403 nm. This suggests that HRP catalyzes epinephrine oxidation by its oxidase activity through Fe^{3+}/Fe^{2+} shuttle consuming O_2, where the rate of reduction of ferric HRP with epinephrine is slower than subsequent oxidation of ferrous HRP by O_2 to form compound III. Compound III was not detected spectrally because of its quick reduction to the ferric state by epinephrine or its subsequent oxidation product. In the absence of catalase, peroxidative cycles predominate when HRP still remains in the ferric state through the transient formation of compounds I and II not detectable spectrally. Among various mono- and dihydroxyl aromatic donors tested, only epinephrine shows the oxidase reaction. Binding studies indicate that epinephrine interferes with the binding of CN^-, SCN^-, and guaiacol indicating that HRP preferentially binds epinephrine near the heme iron close to the anion or aromatic donor binding site to catalyze electron transfer for oxidation. HRP thus initiates epinephrine oxidation by its oxidase activity generating O_2^- and H_2O_2. Once H_2O_2 is generated, the peroxidative cycle continues with the consumption of O_2, through the intermediate formation of O_2^- and H_2O_2 which play an obligatory role in subsequent cycles of peroxidation.

Catecholamines such as epinephrine or norepinephrine are widely distributed in plant and animal systems. Although the physiological functions and mechanism of action of epinephrine have been extensively studied, all of the details of its catabolism are still not clear today. Epinephrine or norepinephrine is metabolized in vivo by O-methylation and oxidative deamination (*1*). However, as they contain dihydric phenolic moiety, they can be easily oxidized to adrenochrome. This oxidative pathway, in fact, occurs at physiological pH and catalytic oxidation has been demonstrated by various tissue and fluid preparations, by some oxidative enzymes and metalloproteins and also by some nonenzymatic reactions (*2*−*6*). Although the possible involvement of free radicals during ceruloplasmin and Cu^{2+}-catalyzed oxidation of epinephrine was proposed (*3*, *4*), radicals were directly shown by the use of rapid flow methods coupled with ESR[1] technique during epinephrine oxidation by ceric, permanganate, or ferricyanide and during aerial oxidation of adrenochrome at alkaline pH (*6*). The use of fast sweep electrochemical techniques has allowed the positive identification of the transient intermediates such as open-chain *o*-quinones and the precise determination of the rate of intramolecular cyclization to the substituted indole and its subsequent oxidation to the aminochrome (*7*). It is now more or less established that oxidation of epinephrine takes place through four sequential one-electron transfers to form adrenochrome through the formation of some radical intermediates (*8*) as shown in Scheme 1. Although ESR techniques have been employed to detect and identify the semiquinone species as

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[1] Abbreviations: HRP, horseradish peroxidase; LPO, lactoperoxidase; SOD, superoxide dismutase; PBN, α-phenyl-*tert*-butylnitrone; ESR, electron spin resonance; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; EDTA, ethylenediamine tetraacetate; IAA, indoleacetic acid.

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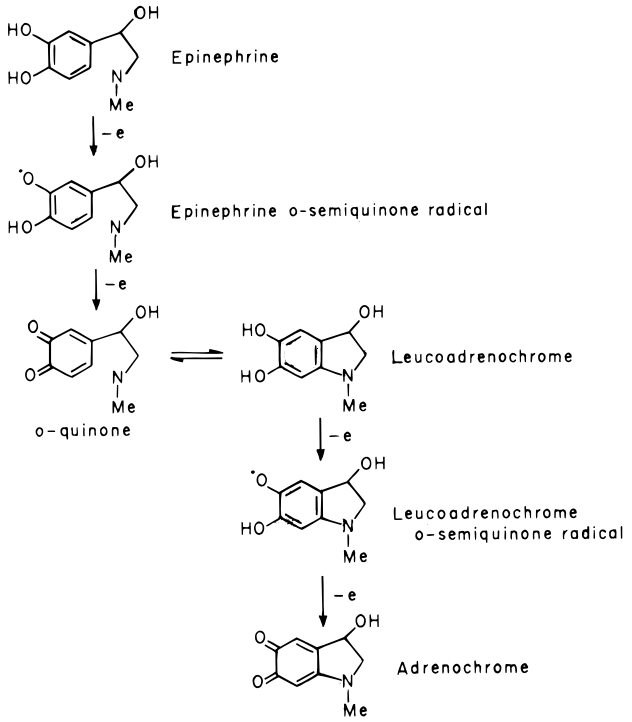
Scheme 1. Sequential One-Electron Oxidation of Epinephrine to Adrenochrome (*8*)



free radical intermediates of epinephrine oxidation (*6, 9, 10*), poor radical concentration, poor spectral resolution, and the presence of more than one spectral species due to secondary radical reaction were the associated problems of these studies. These problems were overcome by the use of the more elegant ESR spin-stabilization technique by complexing the semiquinone radicals with Zn^{2+} ions (*11*). The spin stabilization has helped unambiguous identification of primary open-chain semiquinones formed by one-electron oxidation of epinephrine and secondary semiquinones formed after the cyclization reaction (*12*). As these radicals are well-characterized (*12*), it is possible to study quantitatively the kinetics of peroxidative oxidation of catecholamines using this spin-stabilization technique with Zn^{2+} (*13*).

The mechanism of nonenzymatic aerobic oxidation of epinephrine to adrenochrome by O_2^- has also been studied (*14*−*17*). At alkaline pH, O_2^- generated as an intermediate catalyzes the autoxidation of epinephrine according to the following chain reactions (*17*):

$$RH_3^- + Me^n \rightarrow RH_3^\bullet + Me^{n-1} \quad (1)$$

$$RH_3^\bullet + O_2 \rightarrow RH_2 + O_2^- + H^+ \quad (2)$$

$$RH_2 + O_2^- + H^+ \rightarrow RH^\bullet + H_2O_2 \quad (3)$$

$$RH^\bullet + O_2 \rightarrow R + O_2^- + H^+ \quad (4)$$

$$RH_3^- + O_2^- + 2H^+ \rightarrow RH_3^\bullet + H_2O_2 \quad (5)$$

$$Me^{n-1} + O_2 \rightarrow Me^n + O_2^- \quad (6)$$

where RH_3^- represents epinephrine, R represents adrenochrome, and Me^n represents the transition metal ion. Due to the involvement of O_2^-, SOD strongly inhibits adrenochrome formation, which forms the basis of a convenient and sensitive assay of the enzyme (*17*). However, toward neutral pH, the organic radical generated in reaction 1 may also form adrenochrome through a series of dismutation reactions (*17*):

$$RH_3^\bullet + RH_3^\bullet \rightarrow RH_2 + RH_4 \quad (7)$$

$$RH_3^\bullet + RH_2 \rightarrow RH^\bullet + RH_4 \quad (8)$$

$$RH^\bullet + RH^\bullet \rightarrow R + RH_2 \quad (9)$$

Since O_2^- is not generated in these reactions, the formation of adrenochrome by this pathway is insensitive to SOD.

Horseradish peroxidase (donor H_2O_2 oxidoreductase EC 1.11.1.7) catalyzes the oxidation of various benzene metabolites, hydroquinones, and catechols by H_2O_2 to the corresponding quinones through the well-known peroxidative one-electron-transfer mechanism and the product being formed either by the disproportionation reaction between the free radicals or by the reduction of molecular O_2 by the radicals to form O_2^- (*18*−*21*). The mechanism of the peroxidative oxidation of catecholamines has been studied by ESR spin-stabilization technique using the HRP−H_2O_2 system (*11*−*13*) which generates *o*-semiquinone as a primary one-electron oxidation product which by disproportionation may give rise to *o*-quinone. With deprotonation of the amino group in the side chain, *o*-quinone undergoes 1,4 intramolecular cyclization to form leucoadrenochrome which may be rapidly oxidized to adrenochrome. It is possible that leucoadrenochrome may undergo two one-electron transfers to form adrenochrome via the intermediate formation of leucoadrenochrome semiquinone (*12*). ESR spin-stabilization technique has also identified the *o*-semiquinone intermediate as a one-electron oxidation product formed during lactoperoxidase-catalyzed norepinephrine oxidation in the presence of H_2O_2 (*22*).

Although the products and the radical intermediates in HRP-catalyzed epinephrine oxidation in the presence of H_2O_2 have been identified and characterized (*11*−*13*), the molecular mechanism of oxidation by HRP is unusually complex as it also catalyzes epinephrine oxidation in the absence of added H_2O_2 followed by peroxidation by endogenous H_2O_2 as shown in this study. HRP exhibits oxidase activity toward a number of substrates in the absence of H_2O_2 under aerobic conditions. Since Swedin and Theorell (*23*) demonstrated that HRP can catalyze aerobic oxidation of dihydroxyfumerate, the mechanism of this oxidase reaction has been extensively studied and summerized (*24*). Among various substrates, dihydroxyfumerate, indoleacetic acid, indole butyrate, oxalate, dihydroxytartarate, phenylacetaldehydes, NADH, and NADPH may be mentioned as a few which are oxidized by the HRP oxidase system (*24*). The O_2-consuming indoleacetic acid and NADH oxidations by HRP oxidase in the absence of H_2O_2 may be regarded as typical examples and extensive studies have already been made on the mechanism (*24*−*36*). However, very little is known about the mechanistic aspect of the oxidation of epinephrine by HRP in absence of added H_2O_2 at physiological pH. Evidence is presented to show that HRP can catalyze oxidation of

epinephrine to adrenochrome in the absence of added H_2O_2 with concomitant consumption of O_2. The reaction is initiated by its oxidase activity and proceeds through its peroxidase activity by endogenous H_2O_2 (sensitive to catalase) with the generation of free radicals via the one-electron-transfer mechanism. Sensitivity to SOD indicates that O_2^- plays a vital role in epinephrine oxidation and O_2 consumption, and free-radical-mediated O_2 consumption, rather than the disproportionation reaction of the free radicals, is the favorable pathway for adrenochrome formation, the findings which were not evident in earlier studies. Binding studies indicate that epinephrine preferentially interacts at a site close to the heme iron for efficient electron transfer during oxidation. We suggest that oxidation of epinephrine by HRP is initiated by its oxidase activity but proceeds through its peroxidase activity with the consumption of O_2 by a radical intermediate, where endogenous O_2^- and H_2O_2 play a vital role in the catalytic formation of adrenochrome.

MATERIALS AND METHODS

Materials. Horseradish peroxidase (HRP type VIA, RZ = 3.0), lactoperoxidase (LPO, RZ = 0.88), superoxide dismutase, α-phenyl-*tert*-butylnitrone, ferricytochrome *c*, hemin, catalase, guaiacol, *p*-hydroquinone, *p*-cresol, resorcinol, and epinephrine were purchased from Sigma. *n*-Butanone, KSCN, and $ZnSO_4$ were procured from Merck, India. Other chemicals were of analytical grade. The concentration of HRP and LPO were determined from $\epsilon_{403} = 102\ mM^{-1}\ cm^{-1}$ (*37*) and $\epsilon_{412} = 112\ mM^{-1}\ cm^{-1}$ (*38*), respectively. The possible contamination of catalase in SOD was checked by a catalase activity assay (*39*, *40*) with SOD while that of the SOD in catalase was checked by a SOD activity assay (*16*, *17*) with catalase. Neither SOD nor catalase used in this study showed any detectable contamination with the other.

Peroxidase-Catalyzed Oxidation of Epinephrine. Epinephrine oxidation by HRP (4 nM) or LPO (4 nM) was measured in an incubation mixture containing 640 μM epinephrine in 50 mM sodium phosphate buffer, pH 7.5, in the absence or presence of 200 μM H_2O_2 in a final volume of 1 mL. Enzyme was added last to start the reaction. The activity was measured spectrophotometrically by monitoring the time-dependent increase in absorbance at 480 nm due to formation of adrenochrome. The concentration of adrenochrome was determined from $\epsilon_{480} = 4000\ M^{-1}\ cm^{-1}$ (*41*).

Measurement of O_2 Consumption during Epinephrine Oxidation. Oxygen consumption during HRP-catalyzed epinephrine oxidation was measured in a Gilson oxygraph fitted with a Clark type electrode. The assay system contained in a final volume of 2 mL: 50 mM sodium phosphate buffer, pH 7.5, 640 μM epinephrine, and 7 nM HRP. The concentration of H_2O_2, when used, was 200 μM. The initial rate was determined from the linear part of O_2 consumption from the oxygraph tracing.

Kinetic Evidence for the Generation of o-Semiquinone Radicals during Epinephrine Oxidation. Spin stabilization is a technique by which *o*-semiquinone and related radicals in HRP-catalyzed epinephrine oxidation could be stabilized by chelation with diamagnetic divalent metal ions such as Zn^{2+} or Mg^{2+} (*11*−*13*). Thus its involvement in HRP-catalyzed epinephrine oxidation can be studied kinetically on adrenochrome formation in the presence of Zn^{2+} ion (*42*). Spin stabilization of *o*-semiquinones is usually carried out in Tris-HCl buffer, pH 7.5, instead of phosphate buffer which may interact with Zn^{2+}. Anhydrous zinc sulfate was dissolved in Tris-HCl buffer, and the solution was filtered to remove insoluble impurities.

Adrenochrome formation was measured at 480 nm in a reaction mixture containing 640 μM epinephrine in 50 mM Tris-HCl buffer, pH 7.5, and 4 nM HRP in the absence or presence of varying concentrations of Zn^{2+} ion. Involvement of free radicals was also studied kinetically by using PBN as a spin trap.

Reduction of Ferricytochrome c by Superoxide Generated during Epinephrine Oxidation. The reduction of ferricytochrome *c* by O_2^- generated during HRP-catalyzed epinephrine oxidation was monitored by measuring the increase in absorbance at 550 nm (*43*). The assay system contained in a final volume of 1 mL: 50 mM sodium phosphate buffer, pH 7.5, 4 nM HRP, 30 μM ferricytochrome *c*, and 640 μM epinephrine. The concentration of reduced cytochrome *c* was determined from $\Delta\epsilon_{550}[E_{Fe(11)cytc} - E_{Fe(111)cytc}] = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$.

Binding Studies by Optical Difference Spectroscopy. For measurements of the difference spectrum of the HRP−ligand complex versus HRP, both the reference and sample cuvettes were filled up with 1 mL of the enzyme solution (8 μM) for baseline tracing. This was followed by the addition of a small volume of the ligand to the sample cuvette with concomitant addition of an equal volume of solvent into the reference cuvette (*44*−*46*). The apparent equilibrium dissociation constant (K_d) for the complex formation was calculated from the following equation:

$$\frac{1}{[S]} = \frac{[E]\Delta\epsilon}{K_d \Delta A} - \frac{1}{K_d} \qquad (10)$$

where [S] and [E] are the concentrations of the ligand and the enzyme, respectively, ΔA is the change in absorbance between the peak and trough of the spectrum, and $\Delta\epsilon$ is the difference in molar absorptivity. K_d was calculated from the plot of $1/\Delta A$ versus 1/[S]. All kinetic and spectral studies were carried out in a Shimadzu UV-2201 computerized spectrophotometer at 28 ± 2 °C.

RESULTS

HRP-Catalyzed Epinephrine Oxidation. The kinetics of the oxidation of epinephrine to adrenochrome by HRP in the absence of added H_2O_2 is shown in Figure 1. The oxidation occurs almost linearly for 5 min and is completely blocked by CN^- indicating the involvement of heme iron in the oxidation process. The oxidation is associated with concur-

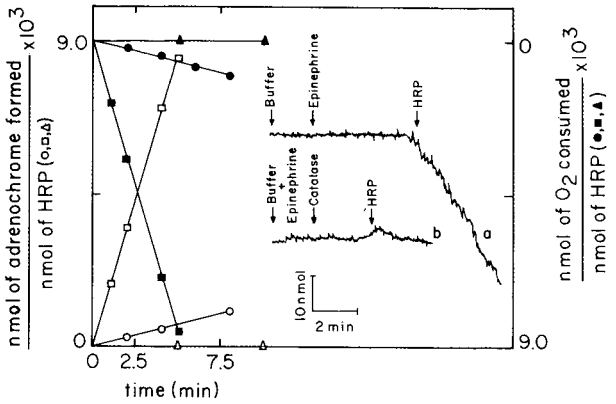


FIGURE 1: HRP-catalyzed epinephrine oxidation and O_2 consumption in the absence of added H_2O_2. The assay system has been described under Materials and Methods. The inset shows a typical tracing of O_2 consumption in HRP-catalyzed epinephrine oxidation. The arrows indicate the time of addition of each reactant: inset a, control; and inset b, in the presence of catalase. The concentrations of catalase and cyanide used were 150 nM and 1 μM, respectively: ■ or □ indicates control; ▲ or △ indicates control + CN^-; ● or ○ indicates control + catalase.

rent consumption of O_2 (inset a) which is also linear for 5 min and blocked by CN^- (Figure 1). The rate of oxidation is not significantly inhibited by EDTA (not shown), indicating no involvement of free metal ion in the oxidation process. Stoichiometry of oxidation indicates that one mole of O_2 is consumed per mole of epinephrine to form one mole of adrenochrome. The result (Figure 1) further shows that both the rate of epinephrine oxidation and the concurrent oxygen consumption (inset b) were significantly inhibited in the presence of catalase, indicating that a major part of epinephrine is oxidized by HRP in the presence of H_2O_2 generated during the reaction. However, inactive catalase prepared after heating at 100° C for 15 min could not inhibit adrenochrome formation and O_2 consumption (not shown). Thus, in the absence of added H_2O_2, HRP catalyzes epinephrine oxidation

Table 1: Efficiency of Adrenochrome Formation from Epinephrine by Different Hemoproteins in the Absence of Added H_2O_2[a]

system	k_{cat} (min^{-1}) (adrenochrome formation)
epinephrine + native HRP	$1.75 \pm 0.10 \times 10^3$
epinephrine + $FeCl_3$	$1.5 \pm 0.20 \times 10^{-2}$
epinephrine + hemin	$1.6 \pm 0.15 \times 10^{-2}$
epinephrine + apo-HRP	0
epinephrine + reconstituted HRP	$1.7 \pm 0.10 \times 10^3$
epinephrine + LPO	$2.5 \pm 0.25 \times 10^3$
epinephrine + ferricytochrome *c*	$5.0 \pm 0.05 \times 10^{-2}$

[a] HRP is converted to apo-peroxidase by the extraction of heme with distilled *n*-butanone at pH 2.0 at O °C (*64*). The reconstituted HRP was prepared by mixing hemin with the apo-HRP (1:1) in 50 mM Tris-HCl buffer, pH 8, by standard procedure (*65*).

through both oxidative and peroxidative reactions consuming oxygen. However, the rate of oxidation is further increased by 3-fold on addition of 200 μM H_2O_2 (Figure 2) due to peroxidative oxidation of epinephrine at an optimum rate. The consumption of O_2 as well as adrenochrome formation was significantly inhibited by SOD in the absence of added H_2O_2, indicating a vital role of O_2^- in HRP-catalyzed epinephrine oxidation. Boiled (100 °C for 15 min) SOD, however, had no such effect (not shown). The insets show the corresponding oxygraphic tracings in the presence of H_2O_2 (tracing a) or SOD (tracing b). Table 1 shows the efficiency of various hemoproteins on epinephrine oxidation. Ferric ion, often used as an inorganic catalyst in epinephrine oxidation (*4*−*5*), is at least 10^5 times less efficient than HRP in catalyzing adrenochrome formation. Free hemin, which is as active as Fe^{3+}, requires apoperoxidase (which itself is inactive) to generate full activity. LPO, on the other hand, catalyzes epinephrine oxidation at a rate 50% higher than that of HRP. However, ferricytochrome *c* oxidizes epinephrine at a very slow rate (approximately 3500 times lower than HRP). Table 2 shows the rate of adrenochrome

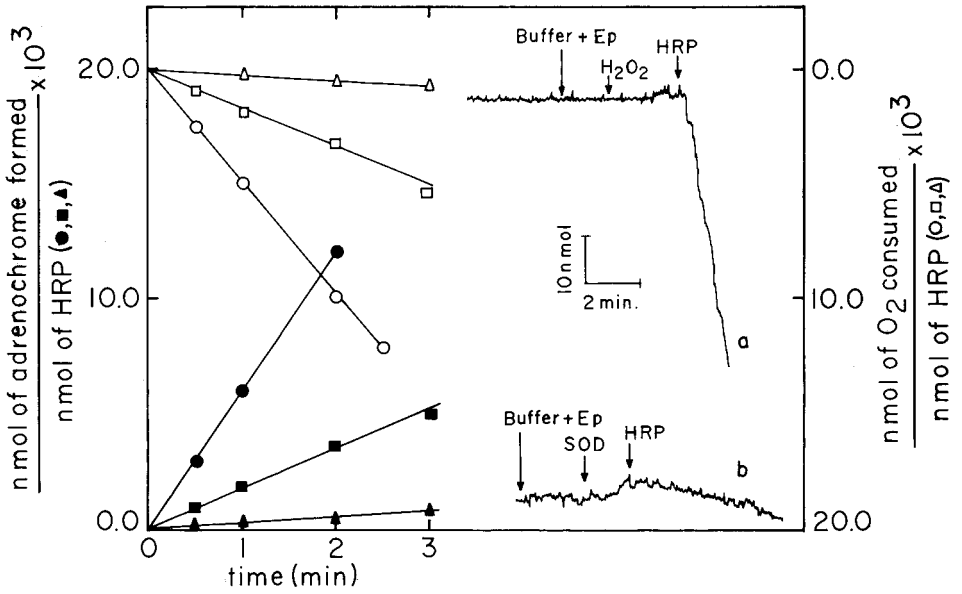


FIGURE 2: The effect of added H_2O_2 and superoxide dismutase in HRP-catalyzed epinephrine oxidation and O_2 consumption. The assay system has been described under Materials and Methods. The concentrations of H_2O_2 and SOD used were 200 μM and 140 nM, respectively. The inset shows a typical tracing of O_2 consumption in HRP-catalyzed epinephrine oxidation in the presence of added H_2O_2 (inset a) or SOD (inset b). The arrows indicate the time of addition of each reactant: □ or ■ indicates control; △ or ▲ indicates control + SOD; and ○ or ● indicates control + H_2O_2.

Table 2: Effect of Catalase and Superoxide Dismutase in HRP-Catalyzed Epinephrine Oxidation[a]

system	adrenochrome formation (nmol/nmol of HRP)	oxygen consumption (nmol/nmol of HRP)
epinephrine + HRP	$1.75 \pm 0.1 \times 10^3$	$1.70 \pm 0.15 \times 10^3$
epinephrine + HRP	$1.16 \pm 0.05 \times 10^2$	$1.1 \pm 0.1 \times 10^2$
epinephrine + HRP + catalase	$2.9 \pm 0.2 \times 10^2$	$2.8 \pm 0.3 \times 10^2$
epinephrine + HRP + catalase + SOD	$2.8 \pm 0.2 \times 10$	$2.7 \pm 0.2 \times 10$
epinephrine + HRP + boiled (catalase + SOD)	$1.65 \pm 0.15 \times 10^3$	$1.60 \pm 0.15 \times 10^3$

[a] The assay systems for the measurement of O_2 consumption and adrenochrome formation were described under Materials and Methods. Due to very low activity in the presence of catalase and SOD, the HRP used in this system was 60 nM instead of 4 nM used in other systems. The concentrations of catalase and SOD used were 300 and 350 nM, respectively.

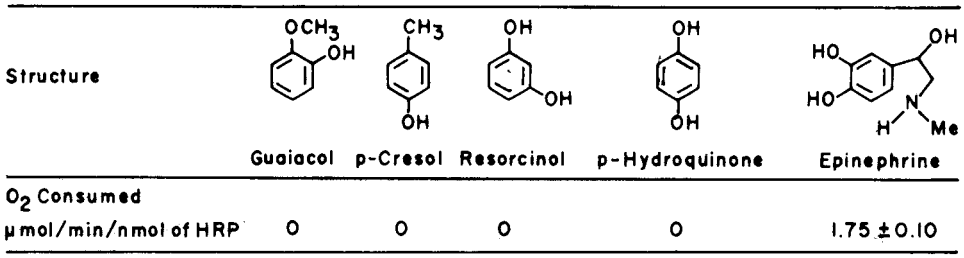


FIGURE 3: Oxidase activity of HRP in the presence of various electron donors HRP (7 nM) was incubated with 640 μM different electron donors in 50 mM sodium phosphate buffer pH 7.5. Oxygen consumption was measured as described under Materials and Methods.

formation and O_2 consumption in the presence of catalase or SOD or both. Catalase is a more effective inhibitor than SOD while both can cause 98% inhibition of epinephrine oxidation. No significant inhibition was observed with boiled catalase or SOD or by both, indicating that the effect is really due to the generation of H_2O_2 and O_2^-. Figure 3 shows the ability of HRP to catalyze the oxidation of various aromatic electron donors containing the phenolic group in the absence of added H_2O_2. Guaiacol, which is easily peroxidized to form tetraguaiacol in the presence of added H_2O_2, shows neither peroxidation nor O_2 consumption in the absence of H_2O_2. Similar is the case with other phenolic electron donors having one or two hydroxyl groups such as *p*-cresol, resorcinol, or *p*-hydroquinone. Only epinephrine, having two hydroxyl groups with one in the ortho position, is oxidized at a high rate with the consumption of O_2. Thus, among various aromatic electron donors used, only epinephrine has the unique ability to be oxidized by HRP in the absence of exogenous H_2O_2.

pH Dependence in HRP-Catalyzed Epinephrine Oxidation. HRP-catalyzed epinephrine oxidation to adrenochrome as well as O_2 consumption is dependent on pH as shown in Figure 4. The optimum activity was observed at pH 7.5. At both sides of the optimum pH, the oxidation rate sharply decreases. At pH values at or below 6, there is very little oxidation.

Spectral Evidence for Epinephrine Oxidation. Figure 5A shows the spectrum obtained during the initial oxidation of epinephrine by HRP. The native ferric enzyme shows the Soret peak at 403 nm (trace a). Addition of epinephrine to the native enzyme produces a hump at 480 nm (trace b) which increases with time (traces c and d) due to the formation of adrenochrome. However, the Soret peak at 403 nm did not show any shift, indicating that, during initial state of oxidation, HRP remains in the ferric state. However, when the oxidation was allowed to continue for 60 min to reach nearly steady state, the enzyme still shows the ferric state (inset). In the presence of catalase (Figure 5B), when the

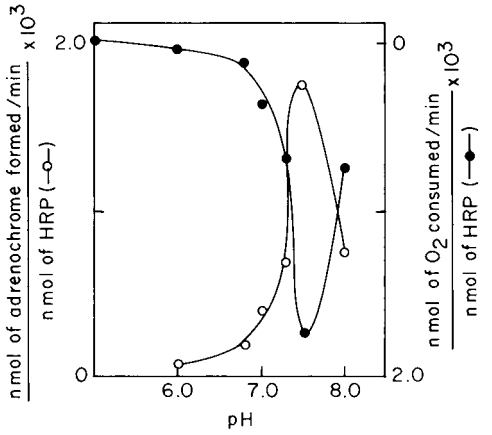


FIGURE 4: pH dependence of adrenochrome formation and O_2 consumption during epinephrine oxidation by HRP. For epinephrine oxidation, 50 mM sodium phosphate buffer, pH 6−8, was used along with 640 μM epinephrine and 4 nM HRP. Enzyme activity was measured as an increase in absorbance at 480 nm due to the formation of adrenochrome after a correction for the nonenzymatic rate, if any. The procedure of O_2 consumption was as described under Materials and Methods.

enzyme shows oxidase activity, the enzyme again shows the ferric state which is not altered even when the oxidation was continued for 60 min (inset). However, the time-dependent increase in absorption of the enzyme in the Soret region is the reflection of the absorption of adrenochrome at 480 nm.

Involvement of o-Semiquinone-Related Radical Intermediates: Effect of Zn^{2+} or PBN on HRP-Catalyzed Epinephrine Oxidation. Zn^{2+} ion acts as a stabilizer of *o*-semiquinone-related radicals in the enzymatic oxidation of epinephrine by the HRP−H_2O_2 system, and the ESR spectrum of the stabilized zinc−semiquinone complex has already been demonstrated (*11*, *12*). To investigate whether epinephrine oxidation to adrenochrome by HRP in the absence of added H_2O_2 is mediated through intermediate formation of *o*-semiquinone and related radicals, the effect of Zn^{2+} on this process was investigated. The result (Figure 6A) shows that Zn^{2+} can significantly block both adrenochrome formation and O_2 consumption in a concentration-dependent manner,

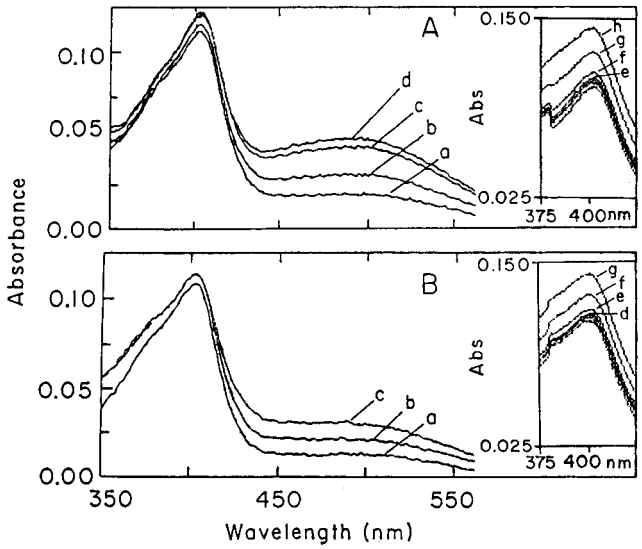


FIGURE 5: Spectral evidence for the oxidation state of HRP during epinephrine oxidation in the absence (A) or presence (B) of catalase. (A) Trace a is the Soret spectrum of 1 μM HRP; traces b−d are spectra obtained 0.5, 1.5, and 2.0 min after the addition of 10 μM epinephrine to the native HRP in 50 mM phosphate buffer, pH 7.5. The inset shows the Soret absorption of HRP when the oxidation was continued beyond 2 min, that is, traces e−h, 5, 10, 30, and 60 min, respectively. (B) Trace a is the Soret spectrum of 1 μM HRP in the presence of 150 nM catalase in 50 mM phosphate buffer, pH 7.5; traces b and c were obtained at 2 and 4 min, respectively, after the addition of 10 μM epinephrine to the solution. The inset shows the Soret absorption of the HRP when the reaction was continued beyond 4 min, that is, traces d−g, 5, 10, 30, and 60 min, respectively.

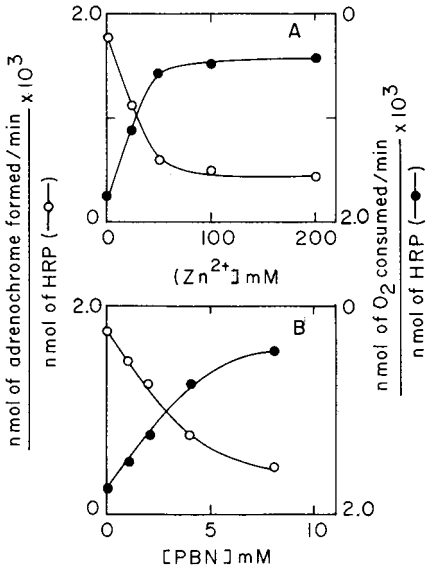


FIGURE 6: Effect of varying concentrations of spin stabilizer Zn^{2+} ion [A] and free radical scavenger, PBN [B], in HRP-catalyzed adrenochrome formation and O_2 consumption. The procedure was the same as that described under Materials and Methods.

indicating that adrenochrome formation is mediated through intermediate formation of *o*-semiquinone and related radicals consuming oxygen. HRP-catalyzed epinephrine oxidation was further studied in the absence or presence of the spin trap, PBN, as shown in Figure 6B. Similar to Zn^{2+}, PBN also blocks both adrenochrome formation and O_2 consumption in a concentration-dependent manner, indicating the involvement of free radicals as intermediates in the oxidation process. As *o*-semiquinone and similar free radicals have

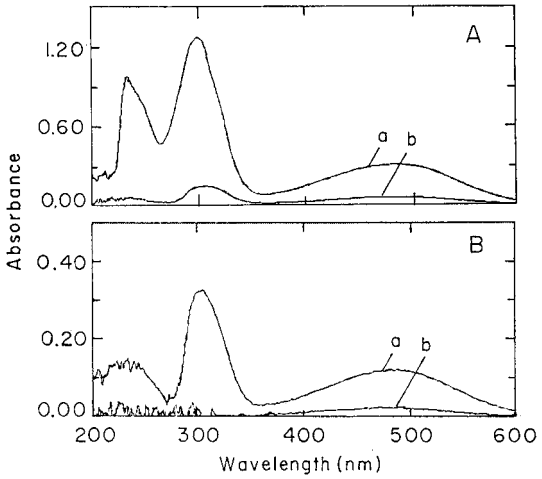


FIGURE 7: Spectral evidence for the inhibition of adrenochrome formation by Zn^{2+} [A] and PBN [B]. (A) Trace a represents the spectrum of epinephrine oxidation products in absence of Zn^{2+}. Both the reference and experimental cuvettes contain 640 μM epinephrine in 50 mM Tris-HCl buffer, pH 7.5. The spectrum was taken 7 min after the addition of 9 nM HRP in the experimental cuvette. Trace b represents the spectrum of epinephrine oxidation products in the presence of 0.4 M Zn^{2+}. (B) Traces a and b represent the spectra of epinephrine oxidation products in the absence and presence of PBN (8 mM), respectively.

already been demonstrated in the HRP−H_2O_2 system by the ESR spin-stabilization technique using Zn^{2+} (*11*, *12*), ESR studies were not presented.

Spectral Evidence for the Inhibition of Epinephrine Oxidation by Zn^{2+} and PBN. To study the mechanism of epinephrine oxidation by HRP in the absence of H_2O_2, spectral studies were carried out in the presence of the spin trap, Zn^{2+} or PBN. Figure 7A shows the spectrum of the epinephrine oxidation product in the absence (spectrum a) and presence of Zn^{2+} (spectrum b). In the absence of Zn^{2+}, the UV and visible bands at 234, 300, and 480 nm for epinephrine oxidation products were significantly decreased in the presence of Zn^{2+}. This indicates that Zn^{2+} blocks the formation of epinephrine oxidation product(s) by stabilizing the intermediate radicals. Similar to Zn^{2+}, PBN also prevents oxidation of epinephrine as evidenced by the significant decrease of the peaks at 234, 300, and 480 nm (Figure 7B). Thus epinephrine oxidation to adrenochrome by HRP is mediated through the formation of the free radical intermediates similar to the HRP−H_2O_2 system (*11*, *12*).

Spectral and Kinetic Evidence for the Generation of Superoxide during Epinephrine Oxidation. Superoxide generation during epinephrine oxidation can be detected by the reduction of ferricytochrome *c*. When epinephrine was incubated with ferricytochrome *c* the in absence of HRP (Figure 8 inset, trace a), epinephrine itself reduced ferricytochrome *c* and a characteristic peak of ferrocytochrome *c* appeared at 550 nm. However, in the presence of HRP, cytochrome *c* reduction was further increased (trace b). The rate of ferricytochrome *c* reduction in the absence or presence of HRP is shown in Figure 8. The rate is increased by HRP and is almost completely blocked by SOD, indicating that O_2^- is produced during epinephrine oxidation by HRP.

Binding of Epinephrine to HRP as Revealed by Optical Difference Spectroscopy. As epinephrine is rapidly oxidized by HRP at pH 7.5, binding studies were carried out at pH

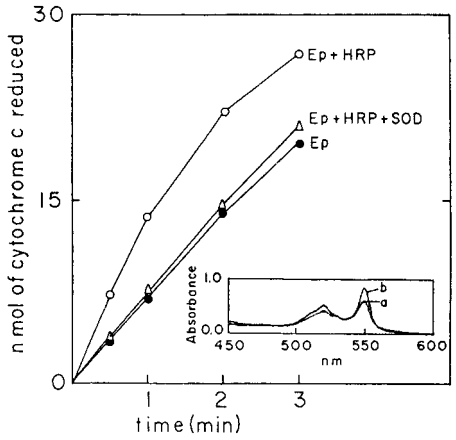


FIGURE 8: The reduction of ferricytochrome *c* in the epinephrine−HRP system. The concentrations of HRP, SOD, epinephrine, and ferricytochrome *c* were 4 nM, 350 nM, 640 μM, and 30 μM, respectively. The reduction of ferricytochrome *c* was measured by the increase in absorbance at 550 nm. The inset shows the visible spectrum of reduced cytochrome *c* obtained after the addition of both epinephrine and HRP. Both spectra were obtained 1 min after the addition of epinephrine (trace a) or epinephrine plus HRP (trace b) to the ferricytochrome *c* solution.

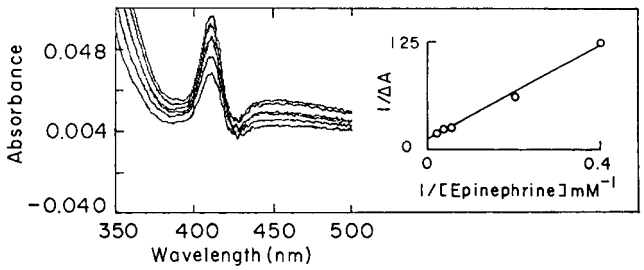


FIGURE 9: Epinephrine binding on HRP by optical difference spectroscopy. Difference spectra of HRP−epinephrine versus HRP at pH 4.5 were obtained with 8 μM HRP using different concentrations of epinephrine (20, 25, 30, 35, and 40 mM). The inset shows the plot of $1/\Delta A$ versus 1/[Epinephrine] to calculate the K_d of epinephrine.

4.5 where oxidation is very little. The binding of epinephrine gives a characteristic difference spectrum of the HRP−epinephrine complex versus HRP, having a maximum at 411 nm and a minimum at 427 nm (Figure 9). The apparent equilibrium dissociation constant, K_d, for the HRP−epinephrine complex as calculated from the plot of $1/\Delta A$ versus 1/[Epinephrine] (inset of Figure 9) was 20 mM. When binding was studied in the presence of cyanide, epinephrine did not interact with the HRP−CN^- complex, showing no characteristic difference spectra at the Soret region (not shown). The result suggests that CN^- binding prevents epinephrine binding at a site close to the heme iron. This is further substantiated by the finding (Table 3) that the binding of CN^- to the HRP ($K_d = 2.0$ μM) is significantly decreased ($K_d = 11$ μM) in the presence of epinephrine. Interestingly epinephrine binding is also altered in the presence of SCN^- showing a K_d value of 67 mM (Table 3). This indicates that epinephrine interacts at a site close to the SCN^- (inorganic anion) binding site (*50*). This is further supported by the finding that the K_d of the HRP−SCN^- complex (110 mM) is significantly increased (250 mM) in the presence of epinephrine. As epinephrine is an aromatic electron donor, its binding was also studied in the presence of guaiacol. Epinephrine binds to the HRP−guaiacol complex with the K_d value of 50 mM (Table 3). Conversely the K_d of the HRP−guaiacol complex (14 mM) is also increased (25 mM) in the presence of epinephrine. These data also suggest that epinephrine binds close to the aromatic donor binding site also.

DISCUSSION

Some salient points of the present studies are the following: (a) HRP can catalyze the oxidation of epinephrine to adrenochrome at physiological pH with the consumption of oxygen. (b) The oxidation is initiated by the oxidase activity of the HRP and proceeds mainly by its peroxidase activity through the intermediate formation of H_2O_2. In presence of catalase, oxidase activity continues to oxidize epinephrine at a very slow rate. (c) Oxidation occurs through sequential one-electron transfer forming *o*-semiquinone and subsequent radical intermediates which can be scavenged by Zn^{2+} or PBN to inhibit adrenochrome formation and concurrent O_2 consumption. (d) O_2^- is an obligatory intermediate which when removed by SOD significantly inhibits adrenochrome formation and O_2 consumption. (e) During oxidation in oxidative and peroxidative cycles, HRP remains in the ferric state. (f) For efficient electron transfer, epinephrine binds to HRP at a site very close to the heme iron but not far away from the aromatic donor or inorganic anion binding site.

The catalytic turnover of epinephrine by HRP was found to be fifteen-fold lower when oxidized in the presence of catalase. This low activity can be assigned to the basal oxidase activity of the HRP. However, in absence of catalase, H_2O_2 is generated in the system which can stimulate oxidation by peroxidative mechanism. In the presence of added H_2O_2, peroxidative oxidation is further increased by 3-fold with a further increase in O_2 consumption. This

Table 3: Diffference Spectral Characterization and Apparent Equilibrium Dissociation Constant (K_d) of HRP−Ligand Complexes

enzyme	ligand	spectrum of complex (nm) max	spectrum of complex (nm) min	K_d	$\Delta\epsilon_{peak-trough}$ (mM^{-1} cm^{-1})
HRP	EP	411	427	20.0 mM	11.4
HRP−CN^-	Ep				
HRP	CN^-	423	393	2.0 μM	102.0
HRP−Ep	CN^-	423	393	11.0 μM	103.0
HRP−SCN^-	Ep	411		67.0 mM	11.6
HRP	SCN^-	420	399	110.0 mM	20.0
HRP−Ep	SCN^-	420	399	250 mM	20.0
HRP−guaiacol	Ep	411		50.0 mM	11.4
HRP	guaiacol	409		14.0 mM	7.0
HRP−Ep	guaiacol	409		25.0 mM	7.0

stimulated peroxidation of epinephrine was shown earlier by Kalyanaraman and co-workers, but O_2 consumption was not demonstrated (*11*−*13*). Several questions can be asked on the mechanistic aspects on (i) how HRP oxidizes epinephrine with the consumption of O_2 in absence of added H_2O_2, (ii) how the HRP−epinephrine system produces O_2^- and H_2O_2, and (iii) how endogenous O_2^- and H_2O_2 stimulate epinephrine oxidation as well as O_2 consumption. In the case of indoleacetic acid (IAA) and NADH oxidation by HRP, IAA or NADH directly reduces ferric HRP to the ferrous form which, being highly unstable because of its very high affinity for O_2 (*27, 47*), gives rise to compound III (*26, 27, 29, 32, 35*). The mechanism of oxidation of IAA is extremely complex because of its dependence on pH and on the enzyme/substrate ratio (*32*). At pH 5 with a high enzyme/substrate ratio, compound III is formed mostly by oxygenation of ferrous HRP formed with the initial reduction of ferric enzyme with IAA (*32*). At pH 7, compound II has been shown to be the key intermediate in the oxidase−peroxidase reaction, presumably formed from ferrous HRP and H_2O_2 (*32*). Compound III formed from oxidation of ferroperoxidase has some interesting properties worth mentioning in explaining epinephrine oxidation. Compound III, an oxyperoxidase, can undergo spontaneous decay to ferriperoxidase (*47*) which is stimulated in the presence of electron donors such as IAA, catechol (*48*), or NADH (*35*). In fact, the oscillatory oxidation of NADH with the consumption of O_2 is due to the oscillation of the enzyme between compound III and ferriperoxidase (*35*). Phelps et al. (*49*) have further reported that compound III can decay to ferriperoxidase with the generation of O_2^-, and in the presence of ferroperoxidase in the system, compound III acts as an oxidase since it accelerates the formation of ferriperoxidase. As our spectral studies indicate the presence of ferric HRP during the oxidation of epinephrine both in the absence and in the presence of catalase, we suggest that epinephrine (AH_4), being a strong reducing agent, initially reduces ferric HRP to the ferrous form with the formation of the *o*-semiquinone radical ($AH_3^\bullet$) and the ferrous HRP is then oxidized by the molecular O_2 to form compound III in a manner similar to the oxidation of IAA (*29, 32*). Compound III is then reduced to form ferric HRP and O_2^-, the rate being stimulated in the presence of ferroperoxidase (*49*) or epinephrine or its subsequent oxidation product (*50*). The following reactions may thus occur for the initial oxidase activity:

$$\mathrm{HRP(Fe^{3+})} + \mathrm{AH_4} \rightarrow \mathrm{HRP(Fe^{2+})} + \mathrm{AH_3^\bullet} + \mathrm{H^+} \qquad (11)$$

$$\mathrm{HRP(Fe^{2+})} + \mathrm{O_2} \rightarrow \text{compound III} \qquad (12)$$

$$\text{compound III} \rightarrow \mathrm{HRP(Fe^{3+})} + \mathrm{O_2^-} \qquad (13)$$

Reactions 11 and 12 are well-known oxidase reactions (*24, 26, 27, 29, 32*). Ferrous HRP could not be demonstrated because of its immediate oxidation to compound III. The reductive decomposition of compound III (reaction 13) has been demonstrated and described (*29, 49, 51*). That O_2^- is formed from reaction 13 has also been demonstrated (*50*). Although O_2^- can dismutate to form H_2O_2, due to the presence of high concentrations of AH_4 or $AH_3^\bullet$ in the system, it is reduced to H_2O_2 in the following reactions:

$$\mathrm{AH_4} + \mathrm{O_2^-} + \mathrm{H^+} \rightarrow \mathrm{AH_3^\bullet} + \mathrm{H_2O_2} \qquad (14)$$

$$\mathrm{AH_3^\bullet} + \mathrm{O_2^-} + \mathrm{H^+} \rightarrow \mathrm{AH_2} + \mathrm{H_2O_2} \qquad (15)$$

where AH_2 is *o*-quinone. The plausible interaction of O_2^- with ferro or ferriperoxidase is prevented by high concentrations of AH_4 in the system. However, as soon as H_2O_2 is formed in this system, peroxidative oxidation of epinephrine starts due to higher affinity of ferric HRP toward H_2O_2. Compound III in this oxidase reaction is transiently formed and cannot build up sufficiently to be detected spectrally because of its rapid reaction with epinephrine and the forward pull of reaction 13 due to the reduction of O_2^- to H_2O_2 in the subsequent reactions. However, ferric enzyme was detected because of the slow reductive reaction of ferric HRP by AH_4 (reaction 11).

The reaction sequence shown in Scheme 1 (*8*) indicates that oxidation of epinephrine to adrenochrome occurs via the intermediates of *o*-semiquinone ($AH_3^\bullet$) and o-quinone (AH_2) which undergoes rapid cyclization to form leucoadrenochrome (AH_2). Leucoadrenochrome is further oxidized to adrenochrome (A) through the intermediate formation of leucoadrenochrome radical ($AH^\bullet$). The sequence of the reaction is based on the earlier identification of some of the intermediates by a fast sweep electrochemical technique (*7*) and ESR spin stabilization with Zn^{2+} (*11, 12*). It is interesting to note that the rate of adrenochrome formation and oxygen consumption in HRP-catalyzed epinephrine oxidation is significantly inhibited in the presence of SOD. This indicates that endogenous O_2^- plays a vital role in adrenochrome formation and oxygen consumption. Therefore O_2^- must react with the intermediate for the reaction to proceed in the forward direction. Epinephrine and leucoadrenochrome (having similar structures) are substrates for HRP. Thus HRP can oxidize directly both epinephrine and leucoadrenochrome to form the *o*-semiquinone epinephrine radical ($AH_3^\bullet$) and the *o*-semiquinone leucoadrenochrome radical ($AH^\bullet$), respectively. The conversions of the *o*-semiquinone epinephrine radical to leucoadrenochrome and the *o*-semiquinone leucoadrenochrome radical to adrenochrome are nonenzymatic and may be mediated either by the disproportionation of the reaction between two same radicals or by the reduction of either O_2^- or molecular oxygen. From the SOD-sensitive adrenochrome formation and O_2 consumption, it is logical to suggest that the first radical ($AH_3^\bullet$) reaction (reaction 15) is O_2^--mediated while the last radical ($AH^\bullet$) reaction (reaction 18) is oxygen-mediated. Had the first radical reaction been oxygen-mediated, then SOD could not inhibit the O_2 consumption. Thus the O_2-consuming reaction should be in the latter step, that is, reaction 18. In presence of catalase, H_2O_2 generated from O_2^- in the reaction 15 is decomposed, thereby stopping the peroxidative oxidation leaving the oxidase reaction to continue at a very slow rate. The entire sequence of HRP-catalyzed epinephrine oxidation in the

presence of catalase may thus be represented as follows:

$$HRP(Fe^{3+}) + AH_4 \rightarrow HRP(Fe^{2+}) + AH_3^{\bullet} + H^+ \quad (11)$$

$$HRP(Fe^{2+}) + O_2 \rightarrow \text{compound III} \quad (12)$$

$$\text{compound III} \rightarrow HRP(Fe^{3+}) + O_2^- \quad (13)$$

$$AH^{\bullet}_3 + O_2^- + H^+ \rightarrow AH_2 + H_2O_2 \quad (15)$$

$$H_2O_2 + \text{catalase} \rightarrow H_2O + {}^1/_2O_2 \quad (16)$$

$$HRP(Fe^{3+}) + AH_2 \rightarrow HRP(Fe^{2+}) + AH^{\bullet} + H^+ \quad (17)$$

$$HRP(Fe^{2+}) + O_2 \rightarrow \text{compound III} \quad (12)$$

$$\text{compound III} \rightarrow HRP(Fe^{3+}) + O_2^- \quad (13)$$

$$AH^{\bullet} + O_2 \rightarrow A + O_2^- + H^+ \quad (18)$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (19)$$

$$H_2O_2 + \text{catalase} \rightarrow H_2O + {}^1/_2O_2 \quad (16)$$

$$\text{Sum: } AH_4 + O_2 \rightarrow A + 2H_2O$$

In the absence of catalase, molecular O_2 acts as an initiator of epinephrine oxidation (reactions 11 and 12). When H_2O_2 is generated in this system (reaction 15), HRP oxidizes epinephrine through a peroxidation reaction at a rapid rate with the intermediate formation of compounds I and II. As a result, the oxidase activity of HRP stops and the peroxidation reaction continues as it is more favorable than the oxidase reaction. The peroxidation reactions as shown below (reactions 20−22, 24, and 25) oxidize epinephrine and leucoadrenochrome in two consecutive cycles to form leucoadrenochrome radicals which are finally oxidized by molecular O_2 to form adrenochrome (reaction 26).

$$HRP(Fe^{3+}) + H_2O_2 \rightarrow \text{compound I} + H_2O \quad (20)$$

$$\text{compound I} + AH_4 \rightarrow \text{compound II} + AH_3^{\bullet} \quad (21)$$

$$\text{compound II} + AH_4 \rightarrow HRP(Fe^{3+}) + AH_3^{\bullet} + H_2O \quad (22)$$

$$2AH_3^{\bullet} + 2O_2^- + 2H^+ \rightarrow 2AH_2 + 2H_2O_2 \quad (23)$$

$$HRP(Fe^{3+}) + H_2O_2 \rightarrow \text{compound I} + H_2O \quad (20)$$

$$\text{compound I} + AH_2 \rightarrow \text{compound II} + AH^{\bullet} \quad (24)$$

$$\text{compound II} + AH_2 \rightarrow HRP(Fe^{3+}) + AH^{\bullet} + H_2O \quad (25)$$

$$2AH^{\bullet} + 2O_2 \rightarrow 2A + 2O_2^- + 2H^+ \quad (26)$$

$$\text{Sum: } 2AH_4 + 2O_2 \rightarrow 2A + 4H_2O$$

$$\text{or } AH_4 + O_2 \rightarrow A + 2H_2O$$

In presence of SOD, both O_2 consumption and adrenochrome formation through oxidase and peroxidase activities of HRP are significantly inhibited. Superoxide anions produced in the initial oxidase reaction (reaction 13) are dismutated to form H_2O_2 by SOD thereby bypassing reaction 23. Epinephrine is now oxidized by the peroxidase reaction in the presence of generated H_2O_2 to form *o*-semiquinone radicals (reactions 21 and 22). However, due to absence of O_2^- in the system in the presence of SOD, *o*-semiquinone radicals slowly disproportionate to form leucoadrenochrome and epinephrine. Thus O_2^- appears to play a regulatory role in epinephrine oxidation by HRP. If O_2^- is formed in the system (reactions 11−13), peroxidation reactions with O_2 consumption (reactions 20−26) are highly favored leading to adrenochrome formation. If O_2^- is removed by SOD (reaction 30), epinephrine is oxidized slowly by the relatively slow disproportionation reaction (reaction 31). As slightly higher O_2 consumption is noticed in the presence of SOD compared to catalase (Table 2), we suggest that reaction 26 is more O_2-consuming than the disproportionation reaction. The reaction sequence in the presence of SOD may thus be represented as follows:

$$2HRP(Fe^{3+}) + 2AH_4 \rightarrow 2HRP(Fe^{2+}) + 2AH_3^{\bullet} + 2H^+ \quad (27)$$

$$2HRP(Fe^{2+}) + 2O_2 \rightarrow 2\text{compound III} \quad (28)$$

$$2\text{compound III} \rightarrow 2HRP(Fe^{3+}) + 2O_2^- \quad (29)$$

$$2O_2^- + 2H^+ + SOD \rightarrow H_2O_2 + O_2 \quad (30)$$

$$HRP(Fe^{3+}) + H_2O_2 \rightarrow \text{compound I} + H_2O \quad (20)$$

$$\text{compound I} + AH_4 \rightarrow \text{compound II} + AH_3^{\bullet} \quad (21)$$

$$\text{compound II} + AH_4 \rightarrow HRP(Fe^{3+}) + AH_3^{\bullet} + H_2O \quad (22)$$

$$2AH_3^{\bullet} + 2AH_3^{\bullet} \rightarrow 2AH_2 + 2AH_4 \quad (31)$$

$$HRP(Fe^{3+}) + H_2O_2 \rightarrow \text{compound I} + H_2O \quad (20)$$

$$\text{compound I} + AH_2 \rightarrow \text{compound II} + AH^{\bullet} \quad (24)$$

$$\text{compound II} + AH_2 \rightarrow HRP(Fe^{3+}) + AH^{\bullet} + H_2O \quad (25)$$

$$2AH^{\bullet} + 2O_2 \rightarrow 2A + 2O_2^- + 2H^+ \quad (26)$$

$$2O_2^- + 2H^+ + SOD \rightarrow H_2O_2 + O_2 \quad (30)$$

$$\text{Sum: } AH_4 + O_2 \rightarrow A + 2H_2O$$

In the presence of SOD and catalase, the rates of both adrenochrome formation and O_2 consumption are 98%

inhibited. As both H_2O_2, and subsequent O_2-mediated oxidations are blocked, oxidation of epinephrine proceeds very slowly through the initial oxidase reactions (reaction 11 to 13) followed by disproportionation reactions only. The reaction sequence may thus be represented as follows:

$$2HRP(Fe^{3+}) + 2AH_4 \rightarrow 2HRP(Fe^{2+}) + 2AH_3^{\bullet} + 2H^+ \quad (27)$$

$$2HRP(Fe^{2+}) + 2O_2 \rightarrow 2\text{compound III} \quad (28)$$

$$2\text{compound III} \rightarrow 2HRP(Fe^{3+}) + 2O_2^- \quad (29)$$

$$2O_2^- + 2H^+ + SOD \rightarrow H_2O_2 + O_2 \quad (30)$$

$$H_2O_2 + \text{catalase} \rightarrow H_2O + {}^1/_2O_2 \quad (16)$$

$$2AH_3^{\bullet} \rightarrow AH_2 + AH_4 \quad (32)$$

$$HRP(Fe^{3+}) + AH_2 \rightarrow HRP(Fe^{2+}) + AH^{\bullet} + H^+ \quad (17)$$

$$HRP(Fe^{2+}) + O_2 \rightarrow \text{compound III} \quad (12)$$

$$\text{compound III} \rightarrow HRP(Fe^{3+}) + O_2^- \quad (13)$$

$$AH^{\bullet} + O_2 \rightarrow A + O_2^- + H^+ \quad (18)$$

$$2O_2^- + 2H^+ + SOD \rightarrow H_2O_2 + O_2 \quad (30)$$

$$H_2O_2 + \text{catalase} \rightarrow H_2O + O_2 \quad (16)$$

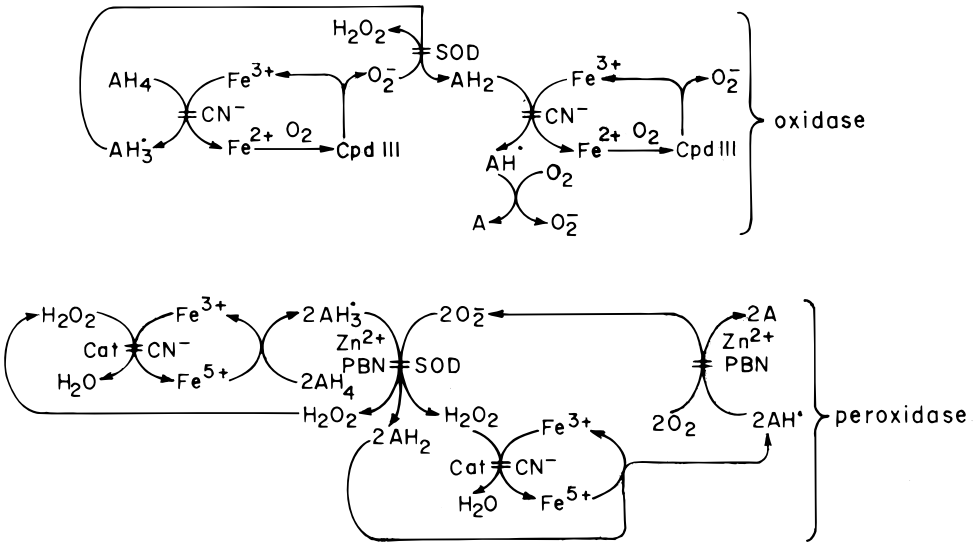
$$\text{Sum: } AH_4 + O_2 \rightarrow A + 2H_2O$$

From the reaction sequences described so far, it is clear that heme iron of HRP undergoes different redox states during epinephrine oxidation. Cyanide completely blocks the reaction by preventing the interaction of heme iron with AH_4, O_2, or H_2O_2. Ferric HRP not only goes to the transient ferrous and compound III states during the oxidase reaction but also goes to compound I and compound II states in the presence of generated H_2O_2 for the usual peroxidative reaction (*12*, *13*, *21*). However, similar to compound III, compound I and compound II could not be detected spectrally due to their very quick turnover to ferric HRP following very rapid reduction by epinephrine or leucoadrenochrome in the peroxidative cycles. Besides, O_2^-, H_2O_2, *o*-semiquinone, and leucoadrenochrome radicals are also generated as intermediates. Sensitivity to SOD and reduction of ferricytochrome *c* indicate the generation of O_2^- in the system. Catalase sensitivity proves the involvement of H_2O_2. The generation of *o*-semiquinone and related free radical intermediates in the HRP−H_2O_2 system has already been demonstrated by the ESR spin-stabilization technique with Zn^{2+} (*12*, *13*) and is further supported by our kinetic evidence for the significant decrease of adrenochrome formation and O_2 consumption when the radicals are allowed to make adducts with Zn^{2+} or PBN. Although no direct evidence for leucoadrenochrome formation in this system has been presented, it is already known that it is formed from *o*-semiquinone through the intermediate formation of *o*-quinone (*7*, *8*, *11*−*13*).

There are some salient points which are worth mentioning while interpreting our observations on epinephrine oxidation at pH 7.5. Misra and Fridovich (*17*), while studying the autoxidation of epinephrine, have postulated that, at pH values lower than 8.5, the radicals ($AH_3^{\bullet}$ and $AH^{\bullet}$) mainly disproportionate (reactions 7 and 9) without any O_2 consumption and O_2^- generation while O_2 consumption and generation are evident (reactions 2 and 4) at pH values above 8.5. Besides, in the former, H_2O_2 is not generated whereas, in the latter, H_2O_2 is formed (reactions 3 and 5). While comparing the mechanism at pH values less than 8.5 (*17*) with HRP-catalyzed oxidation at pH 7.5 (current work), it is evident that O_2^- is generated in the enzymatic oxidation due to the reduction of compound III to ferriperoxidase. Second, O_2^- is the source of H_2O_2 which is formed by the reduction of O_2^- by $AH_3^{\bullet}$ (reaction 15). Hydrogen peroxide may be formed by the reduction of O_2^- with epinephrine or leucoadrenochrome (reactions 5 and 4, respectively) as postulated at pH values above 8.5 (*17*), but reactions between radicals (reaction 15) would be more favored. This reaction is supposed to be vital for subsequent peroxidative oxidation of epinephrine by HRP. Third, as there is very high O_2 consumption sensitive to Zn^{2+} or PBN, which is further stimulated by the addition of H_2O_2, we propose that the second radical ($AH^{\bullet}$) reaction is not the disproportionation reaction (*13*), but it is an O_2-consuming reaction (reaction 18) producing O_2^- which is utilized to generate H_2O_2 (reaction 23) for subsequent peroxidative reactions. Fourth, when compared with the IAA oxidation at neutral pH with a 10−100-fold excess IAA over HRP (*32*), the epinephrine oxidation system does not produce inactive P-670 which is evident in the IAA oxidation system (*32*). Fifth, in IAA oxidation at pH 7 where IAA oxidation is 10 times slower than the reaction at pH 5, compound II was detectable (*32*). In our system where epinephrine oxidation is much favored at slightly alkaline conditions, compound II was not detectable possibly because of its transient formation in the peroxidative cycles.

It is intriguing why HRP catalyzes oxidation of epinephrine by molecular O_2 while it is unable to oxidize other phenolic electron donors such as guaiacol or *p*-cresol or other dihydric phenolic compounds such as resorcinol or *p*-hydroquinone (which are known to bind HRP) in the absence of added H_2O_2. This might be explained as being due to the fact that while the standard reduction potential of phenols is near 900 mV (*52*), that of the epinephrine is variously quoted as 150−300 mV which makes it easier than other phenolic substrates to be oxidized by the enzyme. Moreover, the preferential binding pattern of epinephrine in the heme distal pocket due to the presence of its hydroxyl group in the ortho position may be another reason. Recently, NMR studies indicate that HRP binds aromatic donors at a site away from the heme iron center but close to the exposed heme edge at $C18H_3$ where Phe 179 takes part in the hydrophobic interaction (*53*) while Arg 38 appears to be essential for the stabilization of the OH ligand (*54*). Recent crystal structure of HRP C has also supported this (*55*). Our binding data (Table 3) indicate

Scheme 2. Proposed Mechanism of Oxidation of Epinephrine by HRP



[a] Fe^{3+}, native HRP; Fe^{2+}, ferrous HRP; AH_4, epinephrine; $AH_3^{\bullet}$, *o*-semiquinone epinephrine radical; AH_2, leucoadrenochrome; $AH^{\bullet}$, *o*-semiquinone leucoadrenochrome radical; A, adrenochrome; = indicates block by the reagent shown.

that epinephrine interacts at a site close to the cyanide binding site. Moreover, this site is close to the binding site of SCN^- (*56, 57*) and guaiacol (*53, 54*). Its interference with the binding of SCN^- and guaiacol might be explained as being due to a multiple hydrogen-bonding network formed by epinephrine with the nearby amino acid residues. However, the presence of a side chain of epinephrine may also hinder the binding of other electron donors in the adjacent site. This preferential binding of epinephrine close to the heme iron of the native HRP is suitable for the initiation of electron transfer to the heme iron for the oxidase reaction. As other aromatic donors bind away from the heme iron center (*53, 54*), the distance may not be suitable for electron transfer for the oxidase activity until and unless the ferric heme goes to the higher oxidation state (compound I/II) to act as a driving force for electron transfer in the peroxidative reaction. However, further NMR data are required to find out the precise location of the binding site of epinephrine to the HRP. From the evidence presented in this study as well as from the findings reported earlier (*7, 8, 11*−*13*), we now propose the mechanism of oxidation of epinephrine by HRP as shown in Scheme 2. Epinephrine is thus oxidized by the oxidase and peroxidase functions of HRP where the oxidase initiates the oxidation while the peroxidase continues the cycle at a very high rate through the intermediate formation of O_2^- and H_2O_2. The stoichiometry of oxidation indicates that one mole of adrenochrome should be formed from one mole of epinephrine with the consumption of one mole of O_2, which is evident in Figure 1. In other words, one mole of epinephrine is oxidized to form one mole of adrenochrome by the transfer of four electrons to one mole of O_2 to produce two moles of H_2O.

Although in the midsixties, the oscillatory reactions of NADH with O_2 catalyzed by HRP and compound III at pH values below 6 were reported (*35, 58*), renewed interest has been developed in recent years to understand the dynamics of the peroxidase−oxidase oscillatory reaction (*59*). Recently, the role of several naturally occurring phenols in inducing oscillations in the peroxidase−oxidase reactions has been studied (*52*). Although the structural features of the phenolic compounds have no significant effect on the dynamics, the latter is mainly influenced by the half-wave potential ($E_{1/2}$) for phenoxyl radical formation (*52*). The influence of the phenolic compounds on the dynamics of the peroxidase−oxidase reaction was evident at pH 5.1 and 6.3 (*52*). These phenolic compounds with a standard reduction potential below 900 mV can readily react with compounds I and II showing interesting dynamics (*52*). Epinephrine, having a standard reduction potential lower than other phenolic compounds (*52*), is thus more rapidly oxidized by compounds I and II, which also acounts for little steady-state generation of these intermediates to be detected spectrally during oxidation. It will be interesting to investigate whether epinephrine oxidation occurring optimally at pH 7.5 by the peroxidase−oxidase system undergoing various redox states shows any dynamic oscillatory reaction (*52, 59*).

Peroxidases are abundant in both animal and plant systems (*60*). Epinephrine and related catecholamines are also important constituents in plants and animals. Epinephrine acts on target tissues through circulation in high concentrations during stress to provide a rapid metabolic adjustment to emergencies. After functioning, epinephrine is quickly metabolized to maintain the normal physiological condition. Besides monoamine oxidase, cellular and extracellular soluble peroxidases (LPO, salivary peroxidase, lacrimal peroxidase, etc.) also play a vital role in epinephrine metabolism. Both HRP and LPO can catalyze epinephrine oxidation at a very high rate in the presence of H_2O_2 (*11*−*13, 22*). In normal physiological conditions where endogenous H_2O_2 level is very low, these enzymes may take part in oxidative catabolism of epinephrine through the peroxidase−oxidase mechanism (as shown in this study) and protect the physiological systems from the deleterious actions of higher concentrations of the hormone. However, secondary oxidative damage (*61*−*63*) caused by the free radicals or by the stable oxidation product of epinephrine cannot be excluded.

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