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Analysis of the Stable End Products and Intermediates of Oxidative Decarboxylation of Indole-3-acetic Acid by Horseradish Peroxidase†

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ABSTRACT: A study of the in vitro oxidation of indole-3-acetic acid (IAA) by horseradish peroxidase (HRP) has been carried out by isolating and characterizing the products. Under our experimental conditions, indole-3-acetic acid was converted to oxindole-3-carbinol, 3-methyleneoxindole, indole-3-carbinol (IC) (novel product), and indole-3-carboxaldehyde at pH values between 5.0 and 7.0. The oxygen atom in indole-3-carbinol (IC) arises from O_2. A reaction sequence for peroxidase-catalyzed oxidation of IAA is proposed in which the peroxidase functions as a one-electron oxidizing agent and indol-3-ylmethyl hydroperoxide is the first product of the interaction between oxygen and IAA. Chemiluminescence produced in HRP-catalyzed oxidation of IAA at pH 7.0 is probably derived from the enzymatic degradation of indol-3-ylmethyl hydroperoxide to IC.

Activation of indole-3-acetic acid (IAA)[1] in plants involves two reactions: (1) the formation of physiologically active intermediate products via the oxidation of IAA by peroxidase and (2) the binding of an intermediate oxidation product of IAA to a receptor molecule in the plant cell (Meudt & Galston, 1962).

The oxidation of IAA by HRP can occur in the absence of added hydrogen peroxide and in the presence of catalytic amounts of catalase (Kenten, 1955; Ray, 1962; Fox et al., 1965). Little is known, however, of the detailed metabolic pathway of HRP-catalyzed oxidation of IAA, probably because of the instability of the metabolites and the lack of a precise detection method for the products. It has long been suggested that the initial step in the HRP-catalyzed reaction is a one-electron transfer from IAA to ferriperoxidase. The resulting IAA radical could then be attacked by O_2, producing a β-hydroperoxyindolenine analogue (Hinman & Lang, 1965; Morita et al., 1967; Ricard & Job, 1974). Hinman & Lang (1965) have proposed that β-hydroperoxyindolenine-3-acetic acid is nonenzymatically converted to two major end products, MOI and IA, via an indolenine epoxide. On the other hand, Nakajima & Yamazaki (1979) have recently suggested the formation of another hydroperoxide of IAA, probably indol-

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[1] Abbreviations: IAA, indole-3-acetic acid; MOI, 3-methyleneoxindole; OIC, oxindole-3-carbinol; IC, indole-3-carbinol; IA, indole-3-carboxaldehyde; DBAS, 9,10-dibromoanthracenesulfonate; PBN, α-phenyl-*N*-*tert*-butylnitrone; Me_3Si, trimethylsilyl; HRP, horseradish peroxidase; HPLC, high-performance liquid chromatography; GC, gas chromatography; TLC, thin-layer chromatography; UV, ultraviolet; IR, infrared; NMR, nuclear magnetic resonance; ESR, electron spin resonance; RQY, relative quantum yield; RTLI, relative total intensity; MS, mass spectrometry; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Me_2SO, dimethyl sulfoxide.

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3-ylmethyl hydroperoxide, which can be enzymatically converted to the corresponding alcohol.

The present work was undertaken to elucidate the metabolic pathway of HRP-catalyzed oxidation of IAA at two different pH values. HPLC was used for the isolation and identification of products, and the chemiluminescence reaction in the HRP–IAA system was followed spectrometrically.

Materials and Methods

Chemicals. The materials used in the present study were obtained from the following sources: IAA and D_2O (99.5%), Merck Co.; [2-^{14}C]IAA, Amersham; IA, *N*-acetyl-L-tryptophan, indole-3-propionic acid, and indole-3-butyric acid, Nakarai Chemical Ltd.; xanthene dyes (eosin Y, tetrachlorofluorescein, and rose bengal) and 2,5-di-*tert*-butylhydroquinone, Tokyo Kasei Co.; 99.1% ^{18}O (excess O_2) and 21.4% ^{18}O (excess water), British Oxygen Co. Ltd. *N*-Methylindole-3-acetic acid was kindly donated by Dr. T. Goto of Nagoya University. IC, obtained from Aldrich Chemical Co., was recrystallized twice from hot benzene before use. Luminol, obtained from Wako Pure Chemical Industries Ltd., was crystallized twice from ethanol. MOI (Hinman & Bauman, 1960), [*o*-(formylamino)benzoyl]acetic acid (Nakano & Sugioka, 1978), *o*-(formylamino)acetophenone (Nakano & Sugioka, 1979), and 9,10-dibromoanthracenesulfonate (Heilborn & Heaton, 1967) were prepared by the methods cited in the respective references.

Enzyme. Approximately 93% pure HRP was obtained from Boehringer Co. The enzyme (10 mg in 1 mL) in 50% $(NH_4)_2SO_4$ was dialyzed overnight at 4 °C against 3 L of 0.05 M sodium phosphate buffer (pH 7.0). Its concentration was then determined by its absorbancy at 403 nm ($\epsilon = 9 \times 10^4$ M^{-1} cm^{-1}) (Maehly, 1955; Takayama & Nakano, 1977). The peroxidase activity of the enzyme was measured spectrometrically at 485 nm in the presence of *p*-phenylenediamine and H_2O_2 (Lück, 1963).

Incubation Experiments. The standard incubation mixture consisted of 400 nM HRP, 200 μM IAA, and 50 mM acetate buffer (pH 5.0) or 50 mM sodium phosphate buffer (pH 7.0) in a total volume of 36 mL. The reaction mixture for analyzing the effect of deuterium was prepared by replacing most of the H_2O in the incubation mixture (more than 95%) with D_2O. The reaction mixture was adjusted to an appropriate pD (the pH meter reading + 0.4) (Glasoe & Lang, 1960; Nakano et al., 1975) with a minimum amount of concentrated HCl. In some cases, the following buffer solutions were used: citrate–HCl, pH 3.0; acetate, pH 4.0; sodium phosphate, pH 6.0. The total volume of the reaction mixture was 1 mL for the measurement of O_2 uptake and 3 mL for the detection of the chemiluminescence reaction. The reaction was initiated by the addition of IAA, and the incubation was carried out at 25 °C with vigorous shaking. For preparation of the HPLC samples, an aliquot of the reaction mixture was removed at the times indicated and mixed with 1.25 volume of ether; the compounds were then extracted into the ether layer. Extraction with ether was repeated twice; the pooled extracts were dried over Na_2SO_4, and then evaporated to dryness under reduced pressure at room temperature.

High-Performance Liquid Chromatographic Analysis. Compounds in the residue, obtained by the incubation experiment, were dissolved in methanol and analyzed by HPLC (Hitachi LC, 683-30) after the addition of *N*-acetyl-L-tryptophan as an internal standard. The effluent from a column was simultaneously monitored by both the light absorption at 254 nm (Hitachi 635-3088) and the light emission at 354 nm with excitation at 280 nm (Hitachi 650-105). The compounds separated were simply characterized by comparison with the authentic samples with respect to their chromatographic retention times. HPLC for determination of IA and MOI was carried out on a 2.6 × 500 mm column packed with Hitachi gel 3010, using 70% aqueous methanol at a flow rate of 0.4 mL/min, while that for determination of IAA and IC (fluorescent compounds) was carried out on the same size column packed with Hitachi gel 3050, using 50% aqueous methanol at a flow rate of 0.5 mL/min.

Isolation of X_1 (OIC). A 1-L Erlenmeyer flask containing 15 mg of HRP in 800 mL of 50 mM acetate buffer (pH 5.0) was placed in a water bath at 25 °C. IAA (44 mg) in 2 mL of 99% ethanol was added to the HRP solution, and the reaction was allowed to proceed with vigorous agitation. The progress of the reaction was followed by withdrawing aliquots of the reaction mixture at 20–30-min intervals and measuring their ultraviolet absorption. Production of X_1 in the reaction mixture could be simply tested by the increase of the 250- to 280-nm absorption ratio, since X_1 has a higher absorbance at 250 nm (see Table I) compared to indolic compounds with absorbance maxima near 280 nm. When the 250- to 280-nm absorption ratio (A_{250}/A_{280}) rose to values greater than 1.7, 17.6 mg of IAA in 0.8 mL of 99% ethanol and 1 mg of HRP were added to the reaction mixture. IAA and HRP supplementation was performed 9 times. Such a supplementation of HRP is due to a possible denaturation of the enzyme during a long incubation period. Thus, a total of 220 mg of IAA and 25 mg of HRP was used in the experiment. The reaction mixture was incubated until the A_{250}/A_{280} ratio was greater than 1.7. At the end of the reaction, a dark red precipitate was removed by filtration, the clear solution was extracted 3 times with 200-mL aliquots of ethyl acetate, and the extracts were pooled. The organic solvent was removed under reduced pressure at 30 °C. Product X_1 in the residue was separated from other products by HPLC on an 8 × 500 mm column packed with Hitachi gel 3010 (Hitachi LC, 683-30) and eluted with 70% aqueous methanol at a flow rate of 2.5 mL/min. Removal of the solvent from the X_1-rich fraction gave 21 mg of a yellowish solid material. This material was analyzed by using UV, IR, and NMR spectroscopies.

Isolation of X_2 (IC). The X_2 product was isolated from a reaction mixture which was incubated at pH 7.0, the optimum pH for the production of X_2. An Erlenmeyer flask containing 1 L of 50 mM phosphate buffer (pH 7.0), 115 mg of IAA in 50 mL of 99% ethanol, and 10 mg of HRP was incubated with vigorous stirring in a water bath at 25 °C. At hourly intervals, 23 mg of IAA in 1 mL of 99% ethanol and 1 mg of HRP were added to the mixture as supplements. Supplementation was continued for 5 h. Thus, a total of 235 mg of IAA and 15 mg of HRP was used in the experiment. After the final addition of HRP and IAA, the incubation was continued for 24 h. The reaction mixture was then extracted 3 times with 100-mL aliquots of 1-butanol, the extracts were pooled, and the organic solvent was evaporated under reduced pressure at 40 °C.

Product X_2 in the residue was isolated by HPLC (Hitachi LC) as follows: the residue was dissolved in methanol, applied to an 8 × 500 mm column packed with Hitachi gel 3010, and eluted with 70% aqueous methanol at a flow rate of 2.7 mL/min. The crude X_2 fraction was further purified on a similar column packed with Hitachi gel 3050. Elution was carried out with 65% aqueous methanol at a flow rate of 2.0 mL/min. Removal of the solvent from the fraction containing X_2 gave 13.7 mg of a pale yellow material. This product was analyzed by UV, IR, and NMR spectroscopies.

Preparation of the Trimethylsilyl Analogue of IC. The sample containing approximately 1–2 μmol of IC was dissolved in 100 μL of acetonitrile and treated with 50 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide for 15 min at room temperature.

Incorporation of Isotope into IC and MOI. The preparation of IC and MOI was carried out in 0.05 M phosphate buffer at pH 7.0 and in 0.05 M acetate buffer at pH 5.0, respectively. The isotope incorporation experiments with $^{18}O_2$ gas were performed in a Thunberg-type vessel equipped with a stoppered side arm. The Thunberg vessel (50 mL) contained 400 nM HRP and 50 mM buffer in a total volume of 10 mL. The contents of the vessel were frozen in dry ice–acetone, and the vessel was evacuated (water aspirator) and then filled with 99.5% atomic ^{18}O, excess O_2. After the vessel stood for 30 min at room temperature, it was chilled, evacuated, and refilled with $^{18}O_2$. This procedure was repeated 3 times. The reaction was then initiated by injecting 200 μL of 20 mM IAA through the side-arm rubber stopper. Incubation was continued for 24 h at 25 °C with vigorous agitation. For the experiment with $H_2{}^{18}O$, the water in the reaction mixture containing 400 nM HRP and 200 μM IAA in a total volume of 10 mL was partially replaced with $H_2{}^{18}O$ (10.7% ^{18}O excess), and the incubation was carried out in air for 24 h at 25 °C with shaking. In both experiments, the reaction mixtures were extracted 3 times with 1.5 volumes of 1-butanol–acetoacetate (1:1 v/v), the pooled extracts were dried over Na_2SO_4, and the organic solvents were evaporated under reduced pressure at 40 °C. The resultant IC-containing product was analyzed by GC–MS after treatment with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide. The MOI fraction was analyzed without further treatment. The experiment for the incorporation of deuterium into the $-CH_2$ group of IC was carried out under the same reaction conditions as those used for $H_2{}^{18}O$, except that water in the reaction mixture was replaced by D_2O (95.5%).

The amount of ^{18}O incorporated from solvent, or gas, was calculated from the intensity ratio $(M + 2)/[M + (M + 2)]$ accumulated across a given chromatographic peak and was corrected for the natural-abundance $M + 2$ intensity determined in the unlabeled sample. The amount of deuterium incorporated into IC from the solvent was calculated in the same way as that for ^{18}O, except that the intensity ratio $(M + 1)/[M + (M + 1)]$ was used.

Gas Chromatography–Mass Spectrometry. The GC–MS analysis of the sample was performed on a JEOL JMS-300-(EL) gas chromatograph linked to a JMA-2000 computer system. Chromatography for MOI was performed on a column (2.0 mm × 1 m) packed with 3% PZ 179 on 80/100-mesh Unimport HP, using helium as a carrier gas (1.0 kg/cm^2). The injection and enricher temperatures were 280 and 290 °C, respectively. An ionization potential of 30 eV and an ionization current of 300 μA were employed. Chromatography of the trimethylsilyl derivative of IC was performed on a column (2.0 mm × 1.5 m) packed with 3% OV-1 on 100/120-mesh Supelcoport, using helium as a carrier gas (0.9 kg/cm^2). The injection and enricher temperatures were 280 and 290 °C, respectively. An ionization potential of 30 eV and an ionization current of 300 μA were employed.

Thin-Layer Chromatography. The reaction mixture consisted of 400 nM HRP, 200 μM IAA with tracer doses of ^{14}C-labeled IAA, and 0.05 M sodium phosphate buffer at pH 7.0 in a total volume of 10 mL. H_2O or D_2O (95.5%) was employed as a solvent for the above components. The reaction flask was shaken in air at 25 °C for 30 min. The reaction was terminated by addition of 1.5 volumes of precooled ether (−20 °C). Two phases were separated after mixing by centrifugation at 0 °C. This extraction procedure was repeated twice. The organic phase from each extraction was pooled and dried over Na_2SO_4. Under these conditions, 80–90% of the radioactivity in the reaction mixture was recovered by extraction. The organic solvent was removed under reduced pressure at 0 °C. At 4 °C, the dried residue was dissolved in a small volume of ethyl acetate and was placed on a silica gel G plate (Merck). The plate was developed in the chloroform–ethanol (99:1 v/v) for 60 min at −20 °C. After thin-layer chromatography, the compounds on the plate were visualized under UV light, after being sprayed with Van Urk's reagent (Kaldeway, 1969) or with acidic KI solution. The radioautogram was made the usual way with an unstained chromatoplate. The radioactivity on the plate was measured by the procedure described previously (Nakano & Sugioka, 1979).

Chemiluminescence Measurement and Chemiluminescence Spectrometry. Chemiluminescence intensity was measured in a luminescence reader (Aloka, BLD-101). The spectrum of chemiluminescence was recorded by using a spectrometer with a filter analyzer (Nakano et al., 1975).

Measurement of Relative Quantum Yield. The expression of the quantum yield (ϕ) in a chemiluminescent reaction is $\phi = N/(MN_0)$ where N is the total number of photons observed during the reaction, M is the number of moles of substrate initially added, an N_0 is Avogadro's number (6.02×10^{23} mol^{-1}). The total number of photons (N) is given by the following equation: $N = K\int_0^\infty I(t)\,dt = KA$, where $I(t)$ is the function of luminescence intensity expressed by counts of photons per minute, A is the area under the luminescence curve (proportional to the total number of photons), and K is an inherent constant for the instrument. A value of K was determined relative to the base-catalyzed oxidation of luminol in dimethyl sulfoxide as a standard reaction (Lee et al., 1966). With the assumption of 1.28×10^{-2} for ϕ of the luminol oxidation (Lee et al., 1966), we obtained 1.56×10^{12} for K, but the sensitivity to the emission wavelength of our instrument was not corrected. Therefore, a relative quantum yield (ϕ') with respect to the oxidation of IAA (B moles) was calculated by the following equation: $\phi' = (1.56 \times 10^{12})A/(BN_0)$.

ESR Spectrometry. One milliliter of the reaction mixture containing IAA in 50 mM sodium phosphate buffer or 50 mM acetate buffer was mixed with HRP and immediately transferred to an ESR cell (a quartz flat cell). For control experiments, either IAA or HRP was omitted from the reaction mixture. For the spin-trapping technique of free-radical estimation, 0.1 M α-phenyl-*N*-*tert*-butylnitrone (PBN) was added to the reaction mixture prior to the initiation of the reaction. ESR spectra were taken at room temperature by using a Varian E-109E X-band EPR spectrometer at a field modulation of 100 kHz, a magnetic field of 3360 G, a microwave power of 12 mW, a microwave frequency of 9.4 GHz, and a modulation width of 1.25 G using a time constant of 0.5 s. The g values of spin adducts were measured by means of Mn^{2+} in magnesium oxide in a capillary tube as a standard. The apparent g value of Mn^{2+} of $g_{app} = 2.0064$, which means the center of spectrum, was determined by comparing its g value with those of diphenylpicrylhydrazyl ($g = 2.0036$) and strong pitch ($g = 2.0023$). The effect of D_2O on the ESR signal intensity of the spin adducts and spectral patterns were examined in an incubation mixture containing D_2O (more than 95%) instead of H_2O.

Other Analytical Procedures. Oxygen consumption was measured with a Clark-type electrode (Instech oxygenometer)

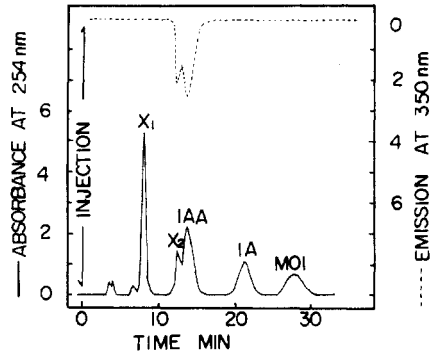

FIGURE 1: Chromatogram of IAA and products in the HRP–IAA system. The standard reaction mixture at pH 5.0 was incubated for 2 min at 25 °C, followed by extracting IAA and its products and analyzing them by HPLC as described in the text. HPLC was carried out on a 2.6 × 500 nm column packed with Hitachi gel 3010 by eluting the compounds with 70% aqueous methanol. The solid line was the chromatogram monitored by the absorption at 260 nm, and the dashed line was the chromatogram monitored by the emission at 350 nm with excitation at 280 nm. X_1, oxindole-3-carbinol; X_2, indole-3-carbinol.

Table I: UV, IR, and NMR Spectra of X_1

UV λ_{max} (ϵ_{max}) (EtOH)	280 sh (1060), 250 (8650)
IR ν (cm^{-1}) (CH_3CN)	3450, 3220, 3010, 2930, 1715, 1630
NMR δ (Me_2SO-d_6)	3.37 (1 H, t, J = 5 Hz), 3.83 (2 H, q, J = 5 Hz), 6.67–7.40 (4 H, m)

and calculated by using a value of 258 nmol/mL O_2 in the initial incubation mixture. The NMR spectrum was obtained by using a Varian A-60D spectrometer and the mass spectrum by using a JEOL D-100 instrument. The UV, IR, and fluorescent spectra were obtained with Hitachi 200-10, Hitachi EPI-G-3, and Hitachi 650-10S spectrometers, respectively.

Results

Properties of the Products. Figure 1 shows a chromatogram of the compounds in the reaction mixture obtained during 2-min incubation of IAA with HRP at pH 5.0. Under standard incubation conditions, MOI and IA could be clearly identified along with authentic standards by comparing their UV absorption spectra and retention times. In addition to these products, two unknown compounds, X_1 and X_2, were detected by UV absorption. Compound X_1 did not show the characteristic absorption band of the indolic compound (near 280 nm) and did not emit fluorescence when excited at 280 nm. However, X_2 was shown to be indolic in nature and to be fluorescent when excited at 280 nm. These four main products were also produced by HRP-catalyzed oxidation of IAA at pH 7.0.

To further characterize compound X_1 (obtained by the large-scale experiment; see Materials and Methods), UV, IR, and NMR analyses were carried out (Table I). The IR spectrum indicated that compound X_1 possesses a carbonyl group (1715 cm^{-1}), an indole NH group (3220 cm^{-1}), and a hydroxyl group (3450 cm^{-1}). UV spectra supported the assumption that the compound was OIC previously reported by Hinman & Lang (1965). In the NMR spectrum, the methine and methylene peaks were split into three and four parts, respectively. Even though such spin–spin coupling has not been completely analyzed, two protons of the methylene group could not be equivalent. The spin–spin coupling between methine and methylene groups in the compound X_1 is remarkably similar to that of 3-(phenylthio)methyloxindole (Hinman & Bauman, 1964), an analogue of OIC. Furthermore, we confirm that compound X_1 was converted to MOI in 50 mM acetate buffer at pH 5.0, as previously reported by Hinman & Lang (1965). From these results, it is reasonable to consider compound X_1 to be OIC.

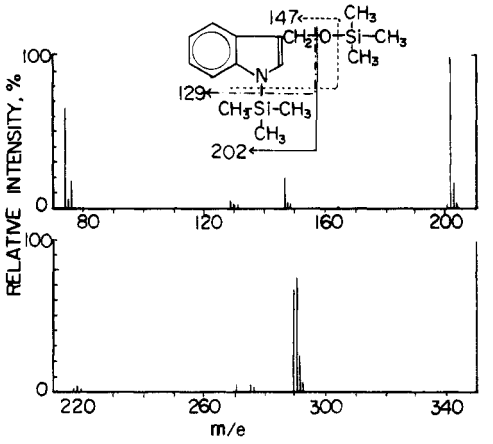

FIGURE 2: GC–MS of the trimethylsilyl derivative of X_2 (indole-3-carbinol). Assay conditions are described under Materials and Methods.

Table II: Incorporation of ^{18}O into Product, IC or MOI, from $^{18}O_2$ and $H_2{}^{18}O$[a]

source of ^{18}O	% of ^{18}O incorp into IC	MOI
$^{18}O_2$ (99.1%)	98.0	4.5
$H_2{}^{18}O$ (10.7%)	2.6	9.2

[a] The incubation was carried out for 18 h in the standard reaction mixture at pH 7.0 for the preparation of IC and for 24 h in the standard reaction mixture at pH 5.0 for the preparation of MOI.

To characterize the unknown product X_2, X_2 was prepared by a large-scale experiment (see Materials and Methods). This was analyzed by fluorescence, UV, IR, and mass spectrometries. The compound, X_2, fluoresced in methanol at 348 nm when excited at 288 nm. The UV absorbance spectrum in methanol showed peaks at 279 (maximum) and 288 nm with a shoulder at 275 nm. These spectra were essentially the same as those of authentic IAA and IC in methanol. Changing the solvent methanol to ethanol–0.05 M buffer (1:1 v/v) at pH 5.0 or at pH 7.0 caused only a 2–3-nm blue shift of the UV spectrum of the compound X_2, identical with that of authentic IC, but did not modify that of IAA. Thus, it is reasonable that many works have failed in the spectrophotometric identification of IC (X_2) as a product of HRP-catalyzed oxidation of IAA. The IR spectrum of X_2 in acetonitrile showed the following principal bands: 3510, 3380, 3030, 2900, 2825, 1640, 1545, 1330, 1245, 1230, 1090, 1065, 990, 820, 765, and 740 cm^{-1}, which are in complete agreement with those of authentic IC. Mass spectrometry of X_2 gave m/e 147 of parent ion (M), suggesting a compound with the formula C_9H_9NO. Thus, if X_2 is an indolic compound (C_8H_6N–X), an X group of CH_3O would be attached to C_3 on the indole ring. The CH_3O must imply the hydroxymethyl group (–CH_2OH) from the band at 3510 cm^{-1} in the IR spectrum.

Furthermore, X_2 contaminated with other products can easily be separated and characterized by GC–MS after treatment with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide. Gas chromatographic analysis of the trimethylsilyl derivative of X_2 showed a single sharp peak. The mass spectrum is given in Figure 2. Ions were present at m/e 291 (M), 202 [M – 80; loss of $(CH_3)_3SiO$], 147 [M – (2 × 73) + 2], 129, and 74 which were coincidental with those obtained with trimethylsilyl derivatives of authentic IC.

GC–MS was also used to determine the origin of the oxygen atom in IC or MOI by using $H_2{}^{18}O$ and $^{18}O_2$ instead of H_2O

Table III: Oxidations of Indole-3-acetic Acid Catalyzed by HRP[a]

run	[HRP] (nM)	[H_2O_2] (μM)	pH	reaction time (h)	no. of expt	yield of products (%) OIC	IC	IA	MOI
1	400		3	4	3	24	0	6	1
2	400		4	4	2	39	1	5	18
3	400		5	6	3	46	25	7	22
4	400		6	8	2	36	41	3	13
5	400		7	12	3	5	80	2	12
6	400	200	3	4	2	36	0	4	1
7	400	20	5	4	3	48	20	1	21
8	400	200	5	4	3	52	8	5	24
9	400	200	7	6	2	26	43	1	10
10	4000		3	4	2	31	1	9	1
11	4000		5	4	3	46	27	10	4
12	20000		5	4	2	10	69	13	2
13	4000		7	6	2	36	35	6	2

[a] The concentration of IAA was 200 μM. Reactions were carried out in 10 mL of a 50 mM buffered solution: citrate–HCl buffer at pH 3.0; acetate buffer at pH 4.0 and 5.0; or phosphate buffer at pH 6.0 and 7.0. The temperature was 25 °C. The yields, which were calculated on the basis of an internal standard (*N*-acetyl-L-tryptophan), were the average values of the number of experiments shown in the table.

and air, respectively, in the incubation mixture. As shown in Table II, the oxygen atom in the product IC was mainly derived from molecular oxygen, whereas that in MOI was from water.

When IC, produced in the HRP–IAA system at pH 7.0 or at pD 6.6, was analyzed as its trimethylsilyl analogue by GC–MS, the experimental evidence did not support the incorporation of deuterium into the $-CH_2$ group of IC (data not shown).

Effects of pH and Enzyme Concentration of Product Yields. Analysis of the compounds formed by HRP-catalyzed oxidation of IAA under various conditions was carried out by HPLC (Table III). Under standard incubation and assay conditions, approximately 80–99% of IAA metabolized at pH values higher than 5.0 and only 31–40% of IAA metabolized at pH values of 3.0 and 4.0 were recovered in the products, OIC, MOI, IC, and IA. Such a low recovery of products at pH values of 3.0 and 4.0, where HRP possesses maximum activity toward IAA oxidation, is due to nonenzymatic decomposition of both IC and OIC to compounds not detected in our assay systems. For example, IC produced in the HRP–IAA system at pH 7.0 decreased in yield by 20% when the reaction mixture was brought to a pH below 3.0 with 2 N HCl before extraction of the products into ether. OIC was also unstable in acidic media and converted to MOI and unknown products. Thus, both IC and OIC may dehydrate and polymerize to unknown compounds which are not detected by our HPLC analysis. In contrast to IC and oxindoles (MOI and OIC), IA was a minor product in the standard reaction mixtures at pH values between 3 and 7, and its yield was not significantly influenced by the acidity of the mixture.

The addition of H_2O_2 to the HRP–IAA system increased the yield of oxindoles with a concomitant decrease of IC accumulation, but did not influence the yield of IA. On the other hand, Ricard & Job (1974) have reported that the yield of IA decreased with increasing amounts of H_2O_2 added to a HRP–IAA system at pH 4.0.

With a fixed IAA concentration, increasing HRP concentrations increased the yield of IC with a concomitant decrease in the yield of oxindoles at pH 5.0, whereas an inverse relationship was observed between IC and oxindoles in their yields at pH 7.0. Morita et al. (1962) and Ricard & Job (1974) have reported that with Japanese radish peroxidase, HRP-P_2, and turnip peroxidase P_1 and P_7, the major end product of IAA oxidation is MOI for a high [IAA]/[peroxidase] ratio, whereas IA is formed in appreciable amounts only when this ratio is decreased. These results, however, were obtained only by spectrophotometric assay of the products in the reaction mixture. Thus, if one can presume IA in these reports to really be IC, the experimental results obtained here would be in close agreement.

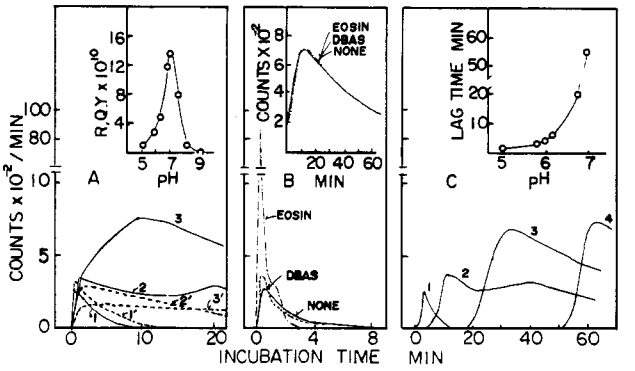

FIGURE 3: Chemiluminescence produced in the HRP–IAA system. The reaction mixture contained 400 nM HRP and 200 μM IAA in the buffer indicated. (A) Effect of D_2O on chemiluminescence: curve 1, pH 5.0 (50 mM acetate buffer); curve 1′, pD 4.6 (50 mM acetate buffer in D_2O); curve 2, pH 6.0 (50 mM sodium phosphate buffer); curve 2′, pD 5.6 (50 mM sodium phosphate buffer in D_2O); curve 3, pH 7.0 (50 mM sodium phosphate buffer); curve 3′, pD 6.6 (50 mM sodium phosphate buffer in D_2O). Inset: Relative quantum yield, estimated by the procedure described under Materials and Methods. The standard reaction mixture with various buffers (50 mM acetate buffer at pH 5.0, 50 mM sodium phosphate buffer at pH values between 6.0 and 7.6, and 50 mM sodium borate buffer at pH 9.0) was used. (B) Effect of dyes on chemiluminescence intensities in the HRP–IAA system at pH 5.0 and at pH 7.0 (inset). Each dye (5×10^{-5} M) was added to the standard reaction mixture prior to the initiation of the reaction, and chemiluminescence was measured by a chemiluminescence reader. (C) Effect of 2,5-di-*tert*-butylhydroquinone on the appearance and intensity of chemiluminescence in the HRP–IAA system. The incubation conditions were essentially the same as those in (A), except that 19 μM 2,5-di-*tert*-butylhydroquinone was added prior to the initiation of the reaction. Curve 1, pH 5.0; curve 2, pH 6.0; curve 3, pH 6.8; curve 4, pH 7.0. Lag times of the appearance of the luminescences were calculated from the curves in (C) and those obtained from the additional experiments against pHs (inset).

Chemiluminescence. The time course of chemiluminescence produced in HRP-catalyzed oxidation of IAA is shown in Figure 3A. In all experiments with fixed concentrations of HRP (400 nM) and IAA (200 μM), but with varied pH, the luminescence appeared just after the addition of IAA without a lag period and increased rapidly. At pH 5.0, chemiluminescence reached a maximum within 30 s after the addition of IAA, decreased exponentially, and disappeared within 6 min. A period of emission (Figure 3A) and the relative

quantum yield (Figure 3A, inset) were prolonged and elevated, respectively, with increasing pH of the reaction mixture up to pH 7.0. When the pH increased above 7.0, relative quantum yields decreased sharply. As shown in the same figure, the second peak emission, which appeared at pH values greater than 6.0, was significantly suppressed by the replacement of H_2O in the reaction mixture with D_2O; however, the first peak emission was not affected. No deuterium effect on the oxygen consumption of the HRP–IAA system was, however, observed (data not shown).

In contrast to the chemiluminescence, oxygen consumption showed a lag time in all cases. If the energy of the excited species generated in the HRP–IAA system (especially in the triplet state) is transferred to a common triplet sensitizer, then light emission in the visible region should be augmented by a sensitized reaction. Vidigal et al. (1975) have reported that 9,10-dibromoanthracenesulfonate and eosin Y enhance the luminescence intensity of a HRP–IAA system at pH 5.0. As shown in Figure 3B and its inset, no significant difference in both the peak intensity and the time-dependent curve of luminescence was found in the presence of 9,10-dibromoanthracenesulfonate, either at pH 5.0 or at pH 7.0. On the other hand, eosin Y caused a significant 20-fold increase in the peak intensity at pH 5.0 (but not at pH 7.0) and shortened the original emission period with its fading. Similar results were obtained with other xanthene dyes such as tetrachlorofluorescein and rose bengal. These results strongly indicate that the apparent dye-sensitized emission involves a radical-mediated oxidation of the xanthene dye (Kamiya & Iwaki, 1966) rather than an energy transfer of the excited species to the dye.

When 2,5-di-*tert*-butylhydroquinone (19 μM), a radical scavenger, was added to the HRP–IAA systems at pH values between 5.0 and 7.0, it caused a delay in the appearance of chemiluminescence at all pHs tested but did not interfere with the time-dependent curve of light intensity seen after a lag period (Figure 3C). The lag period induced by the radical scavenger increased significantly with increasing pH (Figure 3C and its inset), suggesting the production of fewer radical species able to initiate the luminescence at higher pHs.

Of the indole analogues tested, *N*-methylindole-3-acetic acid, as well as IAA, caused light emission and oxygen consumption when it was incubated with HRP at pH 5.0 and 7.0, but others, such as indole-3-propionic acid, indole-3-butyric acid, and IC, did not.

To determine the properties of the excited species generated in HRP-catalyzed oxidation of IAA at pH 5.0 and 7.0, luminescence spectra in the region between 300 and 700 nm were examined by using a filter-type spectrometer (Nakano et al., 1975). However, both low intensity and short duration of luminescence at pH 5.0 made it difficult to characterize the spectral patterns. The spectrum, however, was found to have at least one main peak around 460 nm. On the other hand, the luminescence spectrum at pH 7.0 showed a prominent peak at 410 nm with shoulders near 460 and 480 nm. Such a spectral pattern is similar to that obtained from tryptophan or structurally related compounds in alkaline ice at 77 K when excited at 280–290 nm (Santus et al., 1971, 1972). These spectra show a peak at about 400 nm (fluorescence) and shoulders near 440 and 475 nm (phosphorescence) but are different from the emission spectrum obtained by excitation of IAA or IC at 288 nm in Tris-HCl buffer at pH 7.0 at room temperature (Figure 4). The spectrum is different from the luminescence spectra of the excited carbonyl (in singlet states) generated by a cleavage of the 1,2-dioxetane analogues of

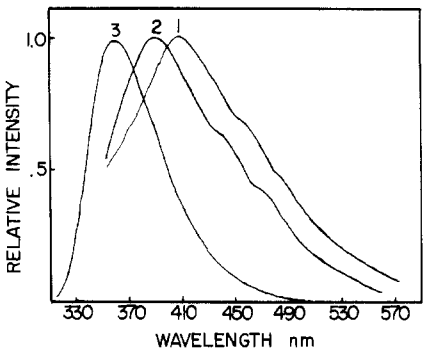

FIGURE 4: Chemiluminescence spectrum obtained in HRP–IAA at pH 7.0 and its comparison with others. The standard incubation mixture in a total volume of 15 mL was used. The luminescence spectrum was taken just after the addition of IAA and continued for 15 min. Curve 1, chemiluminescent spectrum in the HRP–IAA system at pH 7.0; curve 2, emission spectrum of tryptophan in alkaline ice at 77 K with excitation of 290 nm; curve 3, emission spectrum of IC or IAA in sodium phosphate buffer at pH 7.0 with excitation at 288 nm and at 25 °C.

indolic compounds in solution near neutral pH (Walrent & Santus, 1974; Nakano & Sugioka, 1978) or in strong alkaline solutions (Sugioka et al., 1967). It is also different from the spectra for an excited indolic compound (in a triplet state) produced by the interaction of an indolic cation radical with a solvated electron in solution (Vladimirov et al., 1970; Kobayashi et al., 1980; Yoshimoto et al., 1980) in that these spectra show their maximum peak emissions at more than 430 nm.

Production of Free Radicals. ESR spectrometry was applied to determine the formation of free-radical intermediates in the HRP-catalyzed oxidation of IAA. However, no ESR signal was detected in the standard reaction mixture or other mixtures in which the concentration of IAA or HRP was varied at pH 5.0–7.1 and at pD 4.6–6.7. If radicals are produced in the HRP–IAA system, then they should be trapped by the spin trap PBN to give ESR signals of the PBN–radical adducts which have long lifetimes.

When 8 μM HRP was added to either the H_2O system (pH 5.0) or the D_2O system (pD 4.6) which contained 8 mM IAA, 0.1 M PBN, and 50 mM acetate buffer, ESR signals of PBN–radical adducts appeared promptly and were detected by ESR spectrometry. The species and signal intensities of PBN–radical adducts in the H_2O system were essentially the same as those in the D_2O system, but in the latter system, a sharper and a better resolved ESR spectrum could be obtained (Figure 5A, spectrum a). The spectrum can be defined as a superposition of two sets of a doublet of triplets. The observed hyperfine splitting constants were a^N = 15.9 G and a_β^H = 4.5 G for spin adduct 1 and a^N = 16.1 G and a_β^H = 2.3 G for spin adduct 2, but both adducts had the same g value of 2.0056. Signals for spin adducts 1 and 2 decayed during the ESR measurements, but with different rates; i.e., spin adduct 2 decayed more rapidly than spin adduct 1, and only spin adduct 1 was detected at 30 min after initiation of the reaction. However, omission of the enzyme (Figure 5A, spectrum b) or IAA (Figure 5A, spectrum c) from the incubation mixture did not yield an ESR signal.

On the other hand, only one PBN–radical adduct with hyperfine splitting constants of a^N = 15.9 G and a_β^H = 4.5 G and a g value of 2.0056, corresponding to spin adduct 1, was observed under the same conditions, in which phosphate buffer at pH 7.1 was used instead of acetate buffer at pH 5.0. The replacement of H_2O with D_2O had no affect on the ESR signal intensities, but the sharpness and resolution of the

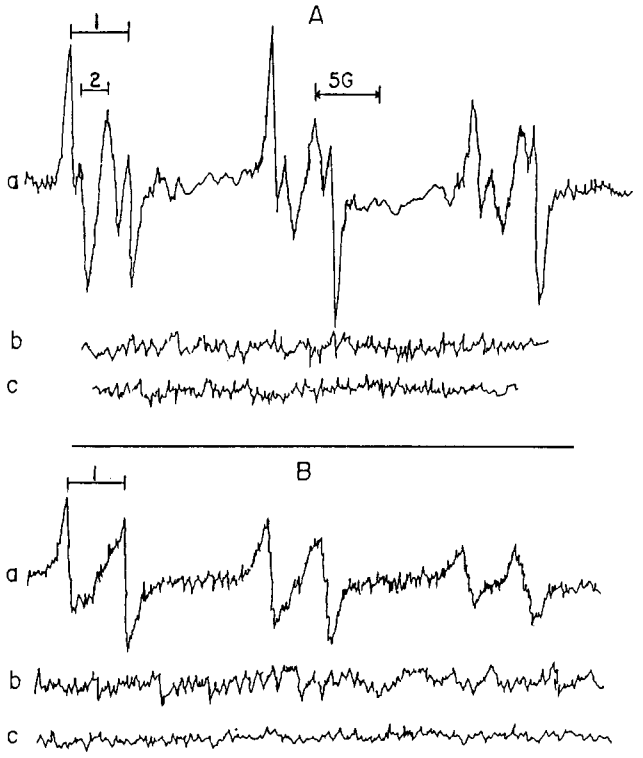

FIGURE 5: ESR spectra of PBN–radical adducts (1 and 2). (A) Spectrum a, the system contained 8 μM HRP, 8 mM IAA, 0.1 M PBN, and 50 mM sodium acetate in D_2O (pD 4.6); spectrum b, the system of spectrum a minus IAA; spectrum c, the system of spectrum a minus HRP. (B) Spectrum a, the system contained 8 μM HRP, 8 mM IAA, 0.1 M PBN, and 50 mM sodium phosphate in D_2O (pD 6.7); spectrum b, the system of spectrum a minus IAA; spectrum c, the system of spectrum a minus HRP.

spectrum involved (Figure 5B, spectrum a). Omission of the enzyme (Figure 5B, spectrum b) or IAA (Figure 5B, spectrum c) from the incubation mixture did not yield an ESR signal.

The structures of spin adducts 1 and 2 are at present unknown. It seems likely, however, that spin adduct 1 can be assigned to a carbon-centered radical adduct rather than oxygen-centered radical adducts since oxygen-centered radical adducts give relatively small a_β^H values, i.e., <3.12 G for the HO· adduct (Harbour et al., 1974; Janzen et al., 1978), <4.03 G for the HO_2· adduct (Harbour et al., 1974; Janzen et al., 1978), <1.7 G for alkylperoxy adducts (Janzen et al., 1978), and <4.0 G for alkoxy adducts (Ledwith et al., 1973). Furthermore, the spin adduct is not likely to be the benzoyl adduct (a^N = 16.0 G, a_β^H = 4.3 G, and g = 2.0055) (Tero-Kubota et al., 1982), which can be formed by the oxidative decomposition of PBN, followed by an interaction of the degradation product with intact PBN. Judging from the ESR parameters, spin adduct 2 is not considered to be HO·, HO_2·, alkoxy, and benzoyl adducts. Under the experimental conditions described, the first signal height of the PBN–radical adduct 1 at pD 4.6 was approximately twice that obtained at pD 6.7 at about 2 min after initiation of the reaction.

Time Course of Product Accumulation. The time course of the accumulation of the products in the HRP–IAA system at pH 5.0 is shown in Figure 6A,B. OIC, which is a major product at this pH, increased after both oxygen consumption (Figure 6C) and chemiluminescence (Figure 3A) had completely ceased. It then reached a maximum and gradually decreased. When the ether extract, which had been obtained from a 2-min incubation of IAA with HRP at pH 5.0, was allowed to stand for 40 min at 4 °C and analyzed by HPLC, the yield of OIC was found to increase by 65%. This indicates

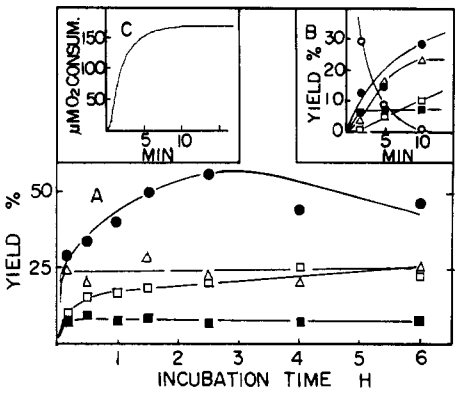

FIGURE 6: Time course correlation of product accumulation (A and B) and oxygen consumption (C) in the HRP–IAA system at pH 5.0. The standard incubation mixture was used. The compounds in the mixture were analyzed by HPLC: (○) IAA; (●) OIC; (△) IC; (□) MOI; (■) IA.

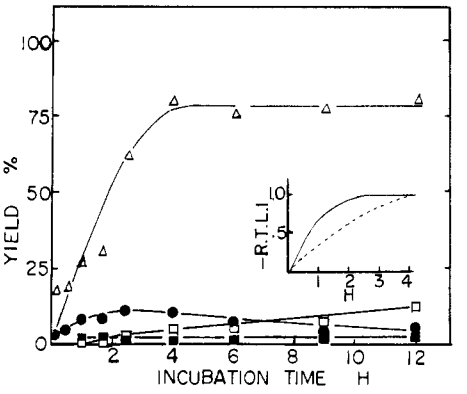

FIGURE 7: Time course of product accumulation in the HRP–IAA system at pH 7.0. The standard reaction mixture was used: (●) OIC; (△) IC; (□) MOI; (■) IA. Inset: Comparison of relative total light intensity (—) and relative IC accumulation (- - -). Each value at 4 h was taken as unity.

that a precursor of OIC, which could be nonenzymatically converted to OIC, is present in the reaction mixture. Even though a precursor of OIC has been suggested to be an indolenine epoxide (Hinman & Lang, 1965), this compound was not characterized under our experimental conditions. When the curves showing the accumulation of OIC are compared with that of MOI, it seems likely that OIC is a precursor of MOI (Figure 6A). In contrast to OIC and MOI, accumulation of IC and IA reached their maxima within 10 min (Figure 6B), suggesting that both IA and IC are not produced via the oxindole–methyleneoxindole pathway.

In the reaction mixture at pH 7.0, IC, a major product of HRP-catalyzed IAA oxidation, was accumulated almost linearly with increasing time up to 3 h (Figure 7). The total light emission in this system (integrated light intensity) was not parallel to the accumulation of IC, but luminescence lasted as long as 2.5 h (Figure 7 inset). The accumulation of other products in the reaction mixture at pH 7.0 was much lower than that found at pH 5.0, but their patterns were essentially the same.

A Precursor of IC. To investigate the possible formation of a hydroperoxide analogue of IAA, the substrate IAA, with tracer doses of [2-^{14}C]IAA, was incubated with HRP in Tris-HCl buffer at pH 7.1 or pD 6.7 for 30 min at 25 °C (standard incubation) and extracted with ether at low temperature: compounds in the ether layer were analyzed by TLC as described under Materials and Methods. Under these reaction conditions, 80–88% of the radioactivity in the reaction mixture was extracted in the ether layer. Even though the

Table IV: Deuterium Effect on the HRP-Catalyzed Reaction[a]

	H_2O	D_2O
IC yield (nM/30 min)		
before $NaBH_4$ treatment	20.8	9.6
after $NaBH_4$ treatment	38.0	30.0
O_2 consumption[b] (nM/30 min)	43	43
p-phenylenediamine oxidation[c] (ΔA_{485}/2 min)	0.135	0.090

[a] The reaction and assay conditions were as described under Materials and Methods (Thin-Layer Chromatography). The standard reaction mixture was used. [b] Oxygen consumption in the standard reaction mixture was measured by an oxygenometer. [c] The reaction mixture contained 0.7 nM HRP, 1×10^{-4} M H_2O_2, and 0.03% *p*-phenylenediamine (w/v) in 6.2×10^{-3} M phosphate buffer at pH 7.1 or pD 6.7. *p*-Phenylenediamine oxidation was monitored by an increase of A_{485}.

solvent system employed for TLC does not resolve all components of the mixture, both IC and IAA were clearly separated from each other and other compounds present. No compound on the TLC plate gave the positive hydroperoxide test with acidic KI solution. Furthermore, *o*-(formylamino)acetophenone and [*o*-(formylamino)benzoyl]acetic acid, which could be derived from cleavage of the 1,2-dioxetane analogue of IAA, were not detected on the chromatoplate under UV light.

The effects of D_2O on the production of IC in the HRP–IAA system and on *p*-phenylenediamine oxidation in the HRP–H_2O_2 system are summarized in Table IV. The formation of IC from IAA was halved by replacement of H_2O in the incubation mixture with D_2O. A similar deuterium effect was observed with *p*-phenylenediamine oxidation. Borohydride reduction of the ether-extractable products in the H_2O system resulted in a 2-fold increase in the yield of IC, whereas the same treatment of ether-extractable products in the D_2O system gave a 3-fold increase in the yield of IC. From these results and measurement of O_2 consumption in the H_2O and D_2O systems, respectively, it can be concluded that nearly 90% and 70% of the O_2 consumed were incorporated into a compound (which can be reduced to IC with $NaBH_4$) and into IC.

Since there was no deuterium effect on O_2 consumption by HRP-catalyzed oxidation of IAA, D_2O would mainly inhibit the conversion of an IC precursor to IC. IA, one of the products, is also converted to IC by borohydride reduction. IA which could be formed in the standard reaction mixture at pH 7.0 (Table IV) was, however, present in a negligible amount. Thus, the IC precursor is probably indol-3-ylmethyl hydroperoxide which would decompose during TLC and HPLC treatment.

Discussion

The present work describes analysis of the compound produced in the HRP–IAA system at pH values between 5.0 and 7.0. In addition to two oxindoles and IA, which have already been identified as products of peroxidase-catalyzed IAA oxidation (Hinman & Lang, 1965), IC was isolated and characterized as a novel and important product in the metabolic pathway of IAA.

The most plausible pathway for IAA oxidation by HRP is shown in Figure 8. Pathways 1a and 1b can be inferred from the work of Ray (1956) using omphalia peroxidase, which has a close similarity to the HRP–IAA system with respect to IAA oxidation, CO_2 evolution, and O_2 uptake. Pathway 1b would be more favorable to the decarboxylation of IAA, since there is little or no enzymatic oxidation toward indole-3-propionic,

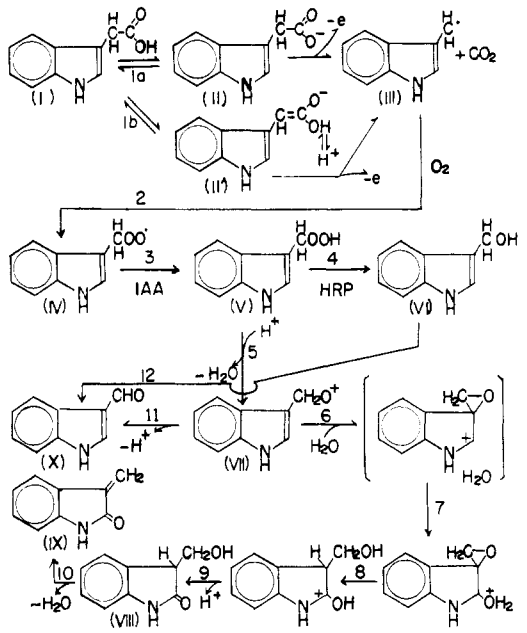

FIGURE 8: Plausible metabolic pathway of IAA oxidation by HRP. Compound I, IAA; compound II or II', conjugated base of IAA; compound III, indole-3-methyl radical; compound IV, indole-3-methylperoxy radical; compound V, indol-3-ylmethyl hydroperoxide; compound VI, IC; compound VII, an intermediate to IA or to OIC; compound VIII, OIC; compound IX, MOI; compound X, IA.

indole-3-butyric, and α,α'-dimethylindole-3-acetic acids at pH 5.0 (Hinman & Lang, 1965). However, on the basis of the finding that deuterium is not incorporated into the $-CH_2$ group of IC from D_2O, IAA may undergo a one-electron abstraction by HRP through pathway 1a rather than 1b. It seems likely, therefore, that IAA could save its corresponding conjugate base (compound II) in a greater amount, compared with other indole-3-alkylcarboxylic acids at the same pH. A number of metal oxidants such as Pb^{4+}, Ag^{2+}, Mn^{3+}, Co^{3+}, Co^{4+}, and Ta^{3+} acetates are known to be capable of affecting the decarboxylation of carboxylic acids (Kochi, 1973). The conversion of IAA to MOI by Fe^{3+} at pH 5.0, even though at a slower rate, has been observed by Hinman & Lang (1965). Furthermore, Kenten (1955) has reported that the oxidation of IAA by HRP is significantly stimulated by Mn^{2+} at about pH 6.0 (only in the absence of citrate) and emphasized that the Mn^{3+} species is involved in the stimulation.

Radicals produced during the enzymatic oxidation of IAA are not well characterized at present. However, a radical which could be trapped by PBN and detected as a PBN–radical adduct 1 is here considered to be a carbon-centered radical, probably indole-3-methyl radical (compound III). Attempts to isolate and identify indol-3-ylmethyl hydroperoxide (compound V) by thin-layer chromatography with low temperatures were unsuccessful, probably because of its high instability. However, evidence for the conversion of IAA to IC and the incorporation of O_2 into IC would support the production of compound V as a precursor of IC. If β-hydroxyindolenine, instead of compound V, is formed in the HRP–IAA system, as suggested by Hinman & Lang (1965), it would be easily converted to the 1,2-dioxetane analogue of IAA at neutral pH, which would then be cleaved to produce excited [*o*-(formylamino)benzoyl]acetic acid in a way similar to that described for O_2-dependent IAA oxidation (Nakano & Sugioka, 1978).

In this study, the luminescence spectrum of the HRP–IAA system at pH 7.0, which is probably a mixture of fluorescence and phosphorescence originating from excited indolic com-

pound(s), is different from that for [*o*-(formylamino)-benzoyl]acetic acid, or its decarboxylated compound, in a singlet excited state (Nakano & Sugioka, 1978). No decomposition product of the dioxetane analogue was observed in this system. The luminescence is also different from that observed in the HRP–IAA system at pH 5.0 in that the latter has no deuterium effect and shows a peak emission near 460 nm.

The deuterium effect on the accumulation of IC and on the chemiluminescence intensity in the HRP–IAA system at pH 7.0 may indicate that conversion of compound V to IC produces excited species. Changing the product ratio of IC to oxindoles by the addition of H_2O_2 to IAA–HRP system indicates that compound V and H_2O_2 compete with each other for reaction with HRP, thereby suppressing the enzymatic conversion of compound V to IC and inversely increasing the oxindole production in the nonenzymatic process. On the basis of the observation that the product ratio of IC to IA is higher than 1 at all pHs examined, it seems unlikely that the proposed precursor of compound V, namely, indole-3-methylperoxy radical (compound IV), is dimerized to a tetraoxide analogue, which splits into IC and IA in equimolar concentration according to Russell's mechanism (Russell, 1955; Howard & Ingold, 1968). Compound IV could readily react with IAA to produce indol-3-ylmethyl hydroperoxide and indole-3-methyl radical (compound III), as suggested by Nakajima & Yamasaki (1979).

The conversion of indol-3-ylmethyl hydroperoxide to IA (pathways 5 and 11), OIC (pathways 5–9), or MOC (pathways 5–10) by a nonenzymatic process can be explained by analogy to the degradation of cumene hydroperoxide to acetone and phenol in acidic media (Seubold & Vaughan, 1953). The present work confirms and extends the experimental work of others (Hinman & Lang, 1965) in showing that OIC is a product of the HRP-catalyzed oxidation of IAA and a precursor to MOI. The low product ratio of IC to oxindole in acidic media can further be explained by the assumption that pathway 5 predominates over pathway 4 at lower pH.

Acknowledgments

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Registry No. HRP, 9003-99-0; IAA, 87-51-4; OIC, 2005-90-5; MOI, 1861-29-6; IC, 700-06-1; IA, 487-89-8.

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