The Reaction of Coumarins with Horseradish Peroxidase

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ABSTRACT

The peroxidase catalyzed oxidation of indole-3-acetate is inhibited by naturally occurring coumarins such as scopoletin. This inhibition is due to the preferential reactivity of the coumarins with the peroxidase compounds I, II, and III. In view of the possible growth regulatory role of coumarins in plants, the mechanism of oxidation of scopoletin by horseradish peroxidase has been investigated.

Peroxidase catalyzed coumarin oxidation requires either an electron donor and molecular oxygen or hydrogen peroxide. If peroxide is present, the reaction is mediated by peroxidase compound II which reacts rapidly and stoichiometrically with scopoletin. Different oxidation products are formed, depending on whether IAA or hydrogen peroxide promotes the reaction. A scopoletin-free radical intermediate has been isolated from the peroxide reaction mixture but was not detected in the peroxide-free system.

When indole-3-acetate is the electron donor, reduced peroxidase combines with molecular oxygen to give peroxidase compound III. Added scopoletin is cooxidized with indole-3-acetate. Compared to the scopoletin peroxidation, this reaction is slower and yields fewer coumarin oxidation products. The differences observed between the two scopoletin oxidation pathways reflect: (a) the competition between indole-3-acetate and scopoletin for peroxidase compounds; (b) the lower reactivity of scopoletin with peroxidase compound III compared with peroxidase compound II. The peroxide-promoted reaction is eliminated by catalase, while the indole-3-acetate initiated oxidation is not affected by excess quantities of either catalase or superoxide dismutase.

Evidence is presented here for the oxidation state of the initial product and for possible structures of other products.

Coumarin oxidation can also proceed in the absence of H2O2. In this second pathway a reductant, IAA, and molecular oxygen initiate the oxidation of scopoletin. Moreover, it has been shown (8, 18) that the rapid reaction between phenolic inhibitors and an activated enzyme intermediate induces a lag in the course of IAA cooxidation. A previous study (18) of the rate of oxidation of IAA during the lag period showed that scopoletin competitively inhibits the catalytic oxidation of IAA even though IAA is required for initiation of the oxidation of the inhibitors. This inhibition was found to be of a non-linear type since the plots of reciprocal reaction velocity against reciprocal IAA concentration curved but intersected at the same maximum velocity regardless of the inhibitor concentration.

Protection against or control of IAA oxidation by coumarin compounds might be envisioned as having a regulatory role in plant growth. Suggestive evidence for an indirect connection of this type was obtained with an Avena coleoptile bioassay (17). For example, scopoletin enhances the stimulation of coleoptile segment elongation by exogenous IAA. Even in the absence of exogenous IAA, scopoletin can stimulate the elongation of apical segments, which contain endogenous IAA. This suggests that the inhibitor could also protect endogenous IAA against oxidation (J. C. Sirois, unpublished).

Peroxidase compounds I, II, and III are heme iron-oxygen compounds containing 2, 1, and 3 oxidizing equivalents, respectively, as documented throughout the literature of this versatile plant enzyme. Hence compound III may be reduced to compound I by donation of a single electron by IAA and compound I may in turn be reduced to compound II and then to the native ferric peroxidase by a similar process as proposed by Yamazaki and Yamazaki (19). The reduction of compound III to compound I provides a pathway for production of a heme-hydrogen peroxidase complex from O2 and native peroxidase which involves only enzyme bound intermediates. This pathway may be important in the catalase-insensitive oxidation of IAA.

The structures of the peroxidase compounds are beyond the scope of the present report. However, it is relevant to note that the formation of compound III requires molecular oxygen and a single reducing equivalent whereas compounds I and II form spontaneously on the addition of H2O2 to ferric peroxidase. Compound II is the most stable of the HRP compounds, persisting in solution at room temperature sufficiently long to permit the visible absorbance maximum to be recorded by conventional means.

The involvement of HRP compounds III, II, and I for initiation and catalysis of IAA degradation in the presence of O2 has been previously considered (7, 15, 18–20). Several reports (7, 8, 15, 19) have provided direct as well as indirect evidence that the three HRP compounds have a function in

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1 Contribution No. 789, Chemistry and Biology Research Institute; Contribution No. 483, Soil Research Institute.
2 Abbreviations: HRP: high spin ferric horseradish peroxidase; ESR: electron spin resonance.
the oxidase type reactions with IAA. A key requirement of these reaction pathways is a role for the peroxide intermediates which are inaccessible to catalase. Also, freely diffusing superoxide anion (univalently reduced O₂⁻) is not involved in the reactions since it will be shown that excess superoxide dismutase does not affect the rate of scopoletin oxidation in the presence of IAA. A possible peroxide intermediate which may result from the reaction of an IAA-free radical with molecular oxygen is IAA peroxide radical (9, 19).

MATERIALS AND METHODS

Horseradish peroxidase, type VI, was obtained from Sigma Chemical Co. The concentrated enzyme was dissolved in 0.05 M NaH₂PO₄ and passed through a 2.5 × 15 cm column of Sephadex G-25 in order to remove fluorescent impurities. Ratio of the absorbance of the purified enzyme at 403 nm to the absorbance at 280 nm was 3.1. The enzyme was electrophoretically homogeneous. Indole-3-acetic acid and scopoletin were recrystallized from distilled water until a single UV-absorbing, fluorescent spot was obtained on TLC. A solvent consisting of benzene-methanol (95:5) was used in separating the less polar oxidation products of scopoletin oxidation by TLC on silica gel-coated glass plates which were supplied by EM Laboratories, New York. An alternative solvent, ammonia-isopropanol-water (80:10:10) separated the highly polar end products of coumarin degradation. GLC of scopoletin reaction products isolated from TLC plates was carried out with helium on an SE-30 medium with a temperature program starting at 100 C. Mass spectra were obtained at an ionization voltage of 70eV with the Du Pont Model 490 mass spectrometer. Absorbance spectra were recorded with the Perkin-Elmer Model 356 spectrophotometer. Fluorescence was measured with a Perkin-Elmer MPF-2A spectrofluorometer. ESR spectra were obtained at 77 K with a liquid nitrogen dewar in the cavity of the Varian Model E-3 ESR spectrometer.

RESULTS AND DISCUSSION

Reaction of Scopoletin with Compound II. Sirois and Miller (18) reported that addition of stoichiometric amounts of scopoletin to HRP (1–10 μM) caused a change in the absorbance spectrum of the enzyme. A minimum was observed at 418 nm in the difference spectrum of scopoletin-treated HRP recorded with a blank containing untreated enzyme. These experiments were carried out in 0.05 M citrate buffer at pH 5.0 and were interpreted as indicating the formation of a scopoletin-HRP complex. Further studies in our laboratory have now shown that this spectral shift does not occur in inorganic buffers or in the presence of excess catalase. The observed scopoletin-induced change was due to the presence of a small percentage of HRP compound II which is formed on reaction of HRP with peroxides present in air-equilibrated citrate buffers. The published spectral data are hence direct evidence for the reaction of scopoletin and compound II, since this is the only HRP peroxide-derived compound stable enough to observe by conventional spectroscopy (cf. ref. 19).

The magnitude of the spectral change on addition of scopoletin provides a measure of the original compound II concentration under these conditions. This concentration was calculated to be 0.15 μM in a 1 μM solution of HRP based on the spectral extinction coefficients for HRP and compound II used by Chance (4). In the previous report (18), a citrate buffer system containing this HRP compound II was reacted with at least a 10-fold excess of scopoletin, and the resulting absorbance change at 418 nm was, therefore, a pseudo-first order process with a rate constant of 10⁶ min⁻¹. This rate constant was calculated from stop-flow data obtained with 10 μM HRP (18).

The oxidation of scopoletin by compound II may also be monitored fluorometrically (1, 2, 18) or at concentrations above 5.0 μM, by absorbance spectrophotometry. This reaction is a complex process yielding several nonfluorescent coumarin-derived intermediates and products at pH 5. Compound II concentration may also be monitored by recording absorbance at 418 nm.

The stoichiometry between scopoletin and compound II was established spectrophotometrically and fluorometrically. An enzyme preparation in sodium phosphate at pH 5 containing 0.20 μM compound II caused a change in scopoletin concentration (initially 0.33 μM) of 0.17 μM when the reactants were mixed in the fluorometer. Likewise, when 0.30 μM compound II was treated with 0.17 μM scopoletin, the concentration of compound II was reduced by 0.20 μM as determined directly at 418 nm.

Figure 1 shows the spectrophotometric kinetics of the peroxidation of scopoletin. On mixing scopoletin, H₂O₂, and potassium phosphate, pH 5, with HRP a blue, colloidal intermediate product having an absorbance maximum at 560 nm appears immediately. The formation of this intermediate is accompanied by a complete loss of scopoletin fluorescence within 10 sec. The rate of formation of the blue compound is shown at 560 nm. Disappearance of the 560 nm absorbance maximum begins after 10 min. The original absorbance of scopoletin at 345 nm decreases initially and concomitantly

![Fig. 1. Kinetic course of the oxidation of scopoletin by compound II. Scoptoletin, final concentration 16 μM, H₂O₂, 16 μM, and HRP, 0.52 μM, were mixed in 30 mM potassium phosphate, pH 5. The H₂O₂ was added last and mixing required 2 sec. Two different time scales were used in the figure. To the left of the vertical dashed line the time scale marks on the abscissa represent 10-sec intervals; on the right-hand side the marks represent 120-sec intervals. The traces were recorded at the three indicated wavelengths. The loss of scopoletin absorbance at 345 nm accompanied the appearance of the new absorbance maximum at 560 nm. Part of the initial absorbance increase at 418 nm is due to conversion of HRP to compound II by H₂O₂.](image)
with the formation of the blue compound (560 nm). An enzyme species, presumably HRP compound II, absorbing at 418 nm is generated during this initial period. The 418 nm absorbance maximum is superimposed on the absorbance of scopoletin and scopoletin-derived oxidation products as shown in the 3-min spectrum in Figure 2. The enzyme compound disappeared when the added H₂O₂ had been consumed. It should be noted that in the previously reported experiments (18) no excess H₂O₂ was present, hence scopoletin reacted immediately with all of the compound II which was present. This fully accounts for the loss of absorbance when scopoletin was added to HRP containing some compound II. In the experiment of Figure 2, excess H₂O₂ is present and so compound II persists until H₂O₂ is consumed. The scopoletin-derived products of the reaction show an absorbance maximum at 385 nm with shoulders throughout the visible region. Reigh et al. (14) reported following the peroxidation of scopoletin at 450 nm. Our results will show that the yellow oxidation products are an unstable mixture.

During the first 10 min of the reaction, a free radical compound detectable by electron spin resonance spectroscopy at 77 K, was present (Fig. 3). The concentration of the free radical peak height of first derivative of microwave absorbance intensity. See Fig. 3 for reaction mixture composition and ESR parameters.

<table>
<thead>
<tr>
<th>Reaction Time</th>
<th>Scopoletin Conc</th>
<th>Signal Magnitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>µM</td>
<td>units</td>
</tr>
<tr>
<td>1</td>
<td>167</td>
<td>367</td>
</tr>
<tr>
<td>10</td>
<td>167</td>
<td>38</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
<td>111</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>26</td>
</tr>
<tr>
<td>6</td>
<td>167</td>
<td>0²</td>
</tr>
<tr>
<td>1</td>
<td>167</td>
<td>160³</td>
</tr>
</tbody>
</table>

³ 80 µM IAA included in reaction mixture.

Table I. Dependence of Transient-free Radical Signal from Oxidized Scopoletin on Scopoletin Concentration, Reaction Time, and IAA

**Fig. 2.** Spectral changes due to oxidation of scopoletin. Reaction mixtures were prepared as detailed for Fig. 1. The spectrum of scopoletin plus HRP (---) shows both the absorbance maximum of the coumarin at 345 nm and the shoulder at 403 nm due to native peroxidase. There is no apparent reaction between these components in the absence of H₂O₂ in this system. The enzyme was converted to compound II as evidenced by the peak at 418 nm in the spectrum recorded 3 min after mixing with H₂O₂ (---). In this spectrum, scopoletin absorbance at 345 nm has decreased and a broad new absorbance band at 560 nm has appeared. The blue product of scopoletin oxidation was colloidal and was slowly converted to the yellow end-products which absorb throughout the visible region with a maximum at 385 nm.

**Fig. 3.** Electron spin resonance spectrum of scopoletin-free radical. Scopoletin, 0.17 mM, hydrogen peroxide, 0.20 mM, and HRP, 1.7 µM, were mixed in 0.017 M potassium phosphate buffer, pH 5. After 1 min of reaction time (upper spectrum) or 10 min of reaction time (lower spectrum) the mixture was frozen at 77 K in liquid N₂. ESR spectra were recorded at this temperature. Spectrometer parameters for Fig. 3 and Table I were: modulation amplitude, 5 gauss; microwave frequency 9.1319 G dz; microwave power, 32 mw; receiver gain, 5 × 10⁴ (upper spectrum) 3.2 × 10⁴ (lower spectrum). Receiver gain for data of Table I was 3.2 × 10⁴.
radical was dependent on the initial scopoletin concentration as was the rate of decay of the radical as illustrated in Table I. Inclusion of 0.5 mole of IAA per mole of scopoletin in the reaction mixture caused a 50% reduction in the observed free radical signal. A stoichiometric amount of IAA caused only a further 10% decrease in this signal. Centrifugation of the reacting mixture under the conditions of Figure 3 caused a total removal of both the blue intermediate compound and the free radical signal from suspension. Therefore, both the radical and the long wavelength absorbance are associated with a colloidal compound or complex. The final (17 hr) yellow colored (385 nm and longer wavelength-absorbing) reaction products were water-insoluble and were collected by centrifugation. In view of the low solubility of the oxidation products, the data of Figure 2 do not represent true solution spectra. The products are soluble in base (0.01 N KOH). Notable features of the free radical intermediate are its insolubility in phosphate buffers, failure to yield an ESR spectrum at room temperature and lack of hyperfine splitting interactions in the 77 K ESR spectrum. Lower modulation and microwave power levels reduced the intensity of the signal but did not reveal any hyperfine interactions.

**Isolation of Some Oxidation Products of Scopoletin.** The transient, insoluble intermediate with a 560 nm absorbance maximum may represent a charge transfer complex between two scopoletin moieties, one molecule in an oxidized form and one in the semiquinone form (Scheme I). It is probable that the initial product of scopoletin oxidation is a univalently oxidized coumarin having an unpaired electron (as indicated by ESR data) and that this blue complex is formed subsequently. As mentioned above, the blue intermediate was isolated by centrifugation and washed with distilled H2O. On resuspension of this material in H2O, yellow products were spontaneously formed as before.

Formation of this unstable species could account for the long wavelength absorbance and lead to subsequent opening of the lactone ring or replacement of the methoxy group with a hydroxyl group as outlined below. Polymerization of free radicals also might be expected.

**TLC chromatography of the scopoletin-compound II reaction mixture after 5 hr yielded four major fluorescent areas having Rf values of 0.04, 0.28, 0.87, and 0.93 in the benzene-methanol solvent as shown in Table II. The spots were reextracted from the silica gel with methanol and purified by gas chromatography. Table II shows the general lack of coincidence in two solvent systems of scopoletin oxidation products from the peroxide and IAA reaction mixtures. In the benzene-methanol system there is essentially no coincidence while in the basic solvent where more spots are resolved, three are coincident. An appreciable proportion of the products of the H2O2 reaction mixture did not migrate in either solvent. This material was complex and contained high mol wt compounds as determined by mass spectrometry.

**Ionization of Scopoletin and Oxidation Products in Mass Spectrometer.** Mass spectral analysis of scopoletin showed that the molecular ion is stable (M/e = 192, structure I), that the methoxy group is readily replaced by a hydroxyl (M/e = 177, II) and that opening of the lactone ring produces an ion lacking CO2 and having a mass to charge ratio of 164. Reclosure of the five membered ring is likely to yield the benzofuran compound (III) (3). The ion with M/e = 149 (IV) is identified as a benzofuran compound lacking the methoxy group as well as CO2. Possible structures of these ions are illustrated below:

![Scheme I](image)

<table>
<thead>
<tr>
<th></th>
<th>M/e 192</th>
<th>M/e 177</th>
<th>M/e 164</th>
<th>M/e 149</th>
<th>M/e 167</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
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<td></td>
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<tr>
<td>II</td>
<td></td>
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</tr>
<tr>
<td>III</td>
<td></td>
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<tr>
<td>IV</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td></td>
<td></td>
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</tbody>
</table>

[Organic structures I-V]

A pathway involving peroxidatic cleavage of the carbon-carbon double bond of the lactone ring could yield ions of M/e 167-170 such as V. Evidence for the presence of this compound in the insoluble oxidation products is given in Table III.

A trimethylsilyl derivative of scopoletin was prepared by the method of Morita (12), and the mass spectrum of this derivative was determined (Table III). The scopoletin derivative yielded a molecular ion of M/e = 264 indicating addition of the silyl group (mol wt = 73) at the single available hydroxyl group of coumarin. The reoccurrence of major peaks at M/e values of 147 and 149 in products of scopoletin oxidation suggests that orthoquinones corresponding to structure IV above may represent products of scopoletin oxidation by HRP compound II. Quinones would not be expected to react with the trimethylsilyl reagent whereas hydroquinones would do so.

Little evidence was obtained for a significant amount of oxidative coupling of two intact scopoletin molecules to give a stable dimer. Ions with mass to charge ratios of 363 and 348 were found in the mass spectrum of the products of scopoletin peroxidation which did not migrate in the organic chromatographic solvent systems. These masses would correspond to dimers of the products similar to II or a dimer consisting of equimolar amounts of I and II above. It is clear that peroxidative degradation of scopoletin yields a complex mixture of unstable products which include higher mol wt polymers.

**IAA-dependent Oxidation of Scopoletin.** Previous reports indicated that IAA acts as a reductant for HRP with the
sequential formation of ferroperoxidase, oxyferroperoxidase (HRP compound III) (15, 16, 18) and compounds I and II (7, 19). The formation of compound III (and the subsequently formed enzyme compound II) requires molecular oxygen and is insensitive to the presence of excess quantities of catalase and superoxide dismutase (18, 19). Evidence that IAA oxidation exhibits a lag phase in the presence of scopoletin is provided by curves 3 and 4 of Figure 4. The lag phase of the inhibited reaction has a measurable initial rate which is dependent on both the inhibitor (scopoletin) and substrate concentrations as previously reported (18). During this initial phase, the scopoletin is oxidized in a reaction whose rate is nearly time independent when followed fluorometrically (curve 5, Fig. 4).

Table II. Thin Layer Chromatography of Scopoletin Oxidation Products

<table>
<thead>
<tr>
<th>Isopropanol-Ammonia-Water (80:10:10)</th>
<th>Benzene-Methanol (95:5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rp</td>
<td>HzO₂</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>0.08</td>
<td>−</td>
</tr>
<tr>
<td>0.13</td>
<td>−</td>
</tr>
<tr>
<td>0.28</td>
<td>+</td>
</tr>
<tr>
<td>0.40</td>
<td>+</td>
</tr>
<tr>
<td>0.41</td>
<td>+</td>
</tr>
<tr>
<td>0.45</td>
<td>−</td>
</tr>
<tr>
<td>0.69²</td>
<td>−</td>
</tr>
<tr>
<td>0.82</td>
<td>+</td>
</tr>
<tr>
<td>0.90</td>
<td>+</td>
</tr>
</tbody>
</table>

¹ Considerable insoluble material remained at the origin of the peroxide reaction mixture with both solvent systems.
² Residual scopoletin.
³ Spots having closely similar Rp values were distinguishable by fluorescence intensity or color. For example, in the isopropanol solvent the spots at Rp = 0.9 exhibited blue fluorescence when HzO₂ was the oxidant while the fluorescence was yellow when IAA promoted the reaction.

When the inhibitor is fully oxidized at the time indicated in curve 3 by the second vertical arrow, the rate of IAA oxidation returns to that observed in the absence of scopoletin (latter part of curve 3 compared to curve 1). During the slow phase of IAA oxidation, oxidizing equivalents for scopoletin must come from molecular oxygen mediated by prior reaction with ferroperoxidase (which in turn originates through reduction of HRP by IAA). The fluorometric data of Figure 4 establish that while 1.8 \( \mu \)M scopoletin is completely oxidized, more than four times this amount of IAA is consumed during the first (slow) phase of the IAA oxidation. Hence, IAA serves to initiate coumarin oxidation. The oxidation of additional molecules of IAA by the HRP compounds then competes with scopoletin oxidation. The latter reaction is kinetically favored over IAA oxidation but can be retarded by an excess concentration of IAA (18).

During the initial inhibitory phase, IAA oxidation is required in order to keep the peroxidase in the active forms (compounds III, II) in the absence of free HzO₂. Ferulic acid has also been reported to induce a lag in IAA oxidation but, in this case, no IAA oxidation was detected until all of the inhibitor had been completely oxidized (8). It is likely that IAA was oxidized during the lag phase but this was not detected by the analytical procedure which was employed.

An outstanding aspect of the IAA-promoted oxidation of scopoletin is the lack of effect of catalase and superoxide dismutase. Figure 4 shows that at no stage are the oxidations of either IAA or scopoletin affected by several micrograms of pure catalase or superoxide dismutase (curve 1 versus curve 2, curve 3 versus curve 4). The insensitivity to these enzymes virtually rules out the participation of either freely diffusing hydrogen peroxide or superoxide anion in the reactions. However, there is an absolute requirement for IAA and molecular oxygen. One interpretation of these facts is that the indole provides the reducing equivalents which afford formation of the peroxidase compounds II and III in the presence of O₂ (15, 16). It is these peroxidase forms which catalyze the oxidation of scopoletin as well as IAA.

In an alternative interpretation, Yamazaki and Yamazaki (19) postulated the formation of IAA-peroxide radicals from IAA free radicals. This peroxide then could react directly with HRP to give compound III. In a subsequent reduction of compound III to compounds I and II, IAA would donate additional electrons. However, the production of IAA peroxide radicals or hydroperoxides thus requires the prior single electron oxidation of IAA or a direct reaction between IAA and another peroxide.

Scopoletin may inhibit IAA oxidation at several points. One
point involves compound II which will be removed rapidly and quantitatively as established previously (2) and above. Another point occurs at compound I which is also a likely active oxidant of coumarins (8) and hence scopoletin will also be expected to remove this enzyme intermediate from the catalytic sequence.

Three additional observations remain to be explained in the peroxide-free system. (a) the 1:5 stoichiometry between the amounts of coumarin and IAA oxidized; (b) the competitive nature of the inhibition of IAA oxidation by scopoletin (18); (c) the differences in the products of scopoletin oxidation depending on whether stoichiometric amounts of H$_2$O$_2$ or excess IAA is the promoter of the reaction. The excess of IAA oxidized over scopoletin oxidized might be expected if scopoletin is oxidized in a one electron step by the activated forms of HRP but must compete with additional molecules of IAA for these forms.

The competition between scopoletin and IAA for compounds I, II, and III also explains the requirement for high IAA concentrations to partially overcome the inhibition due to scopoletin.

**CONCLUSION**

We have compared the oxidation of scopoletin by a stoichiometric H$_2$O$_2$-peroxidase system with a system containing IAA and O$_2$ but no H$_2$O$_2$. Differences have been found in the following.

1. **Rate of Scopoletin Oxidation.** By comparing the kinetic data of Figures 1 and 4, it may be seen that the compound II catalyzed oxidation of the coumarin had a half-time of about 5 sec for the initial loss of scopoletin absorbance at 345 nm, while in the IAA-promoted reaction, the half-time was 40 sec for loss of scopoletin fluorescence. The rate-limiting step for the IAA-promoted reaction is the initial reduction of HRP to form compound III (16, 18). The most compelling evidence for the formation of compound III from ferroperoxidase and O$_2$ has come from stopped flow spectral data previously reported which showed that a 430-nm absorbing species (ferroperoxidase) and a 418-nm absorbing species (compound III) were both present in HRP and IAA containing reaction mixtures 300 msec after initiating the reaction (11). Ricard and Job (15) reported unequivocal evidence that at pH 4 compound III is the major enzyme intermediate which participates in indole oxidation. On the other hand, for the compound II catalyzed reaction, the formation of compound II from HRP and peroxide is known to be far faster and hence not rate-limiting. It should be noted that in our experiments the HRP concentration was 3-fold lower for the peroxidatic reactions illustrated by Figure 1. Hence, the difference in the rates of scopoletin oxidation in the two systems is even greater than the above figures indicate.

2. **Appearance of Free Radical and Colored Transient Intermediates in Compound II Catalyzed Reaction.** No blue intermediate or free radical compound was found in the IAA-containing reaction mixtures. If free radical intermediates were formed in the IAA reaction, they were not present in sufficient concentration for detection.

A mechanism for the single electron oxidation of scopoletin by compound II in the presence of H$_2$O$_2$ is indicated by the spectroscopic results. The first intermediate in the reaction is the monophenol radical followed by formation of a charge transfer complex. Further oxidation of the coumarin structure may lead to the formation of compounds with a degraded lactone ring such as benzofuranins, quinones and polymers.

3. **Final Products of Scopoletin Oxidation.** The final prod-
ucts depended on the reaction conditions as evidenced by the lack of formation of water-insoluble pigments in the case of the IAA-promoted reaction. The appearance of pigments and polymerized products of scopoletin oxidation indicates vigorous oxidation by hydrogen peroxide and HRP compound II.

It is a fact of the chemistry of the coumarin molecule that in order to become oxidized beyond the free radical stage, either lactone ring opening and rearrangement or replacement of the methoxy group by an oxidizable hydroxyl group must occur. Due to the complex chemistry of these reactions, it has not been possible to fully characterize the major stable products of scopoletin peroxidation.

LITERATURE CITED


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