The Use of an Enzyme Electrode in the Analysis of Indole-3-acetic Acid Oxidase Activity in Avena

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ABSTRACT
A flexible analytical system which allows for the continuous potentiometric monitoring of the disappearance of an electrochemical species, ferrocyanide, by the peroxidase enzyme is described. The ability of peroxidase to mediate the oxidation of indole-3-acetic acid is followed by observing the competition of indole-3-acetic acid with ferrocyanide for the peroxidase enzyme. This is accomplished by examining potentiometrically the decrease in the rate of ferrocyanide oxidation with increasing indole-3-acetic acid concentration. Homogenates of Avena sativa coleoptiles are investigated for both peroxidase and indole-3-acetic acid oxidase activity. Observations are made with respect to H2O2 and ferrocyanide in the presence and absence of indole-3-acetic acid and naphthalene acetic acid and several interpretations of the reaction kinetics are postulated. Solutions previously assayed for indole-3-acetic acid oxidase activity, when dialyzed and reassayed for peroxidase activity, demonstrated an unimpaired ability to oxidize ferrocyanide peroxidatively, suggesting interpretations of the bisubstrate situation which differ slightly from interpretations given in the literature.

Since 1934, when Thimann (15) first reported the inactivation of native auxins by plant tissue homogenates, sufficient evidence has been collected to define the major substance responsible for auxin activity as IAA and to attribute the oxidative destruction of IAA within the plant, primarily, to a peroxidase-based enzyme system generally known as IAA oxidase (2, 4, 5, 7, 9, 13). It has been suggested that the IAA-oxidase system may function as a plant growth regulating mechanism because of its role in limiting the supply of IAA within the plant.

Quantitative determinations of IAA oxidase activity have generally been limited to three basic assay approaches for IAA: (a) physiological response to IAA in decapitated Avena coleoptiles (13), (b) absorbance changes at 261 nm (11), and (c) colorimetric determination of indoles with Salkowski reagent (6, 14). The latter technique depends on the development of a pink colored complex with Fe++ in the presence of a concentrated mineral acid. However, Perley and Stowe (10) emphasized that the Salkowski reagent is not reliable in crude homogenates where interfering oxidizing or reducing substances may be present, nor can it measure individual indoles in the presence of others.

Ray (11) has postulated that the relationship between IAA and other substrates of peroxidase is not one simply of substrate competition. He observed that the addition of physiological concentrations of IAA to mixtures which were reacting peroxidatively resulted in a marked depression of the rate of peroxidation. It was found that the rates of both IAA oxidation and electron donor (AH) peroxidation were strongly inhibited when the two substrates were present together. He concluded that the inhibition of peroxidation could not be explained by saying that H2O2 has been diverted from the reaction

$$H_2O_2 + 2AH \xrightarrow{ \text{peroxidase} } 2A + 2H_2O$$  (1)

where: AH = donor in reduced form, A = donor in oxidized form to the oxidation of IAA by the reaction

$$IAA + H_2O_2 \xrightarrow{ \text{peroxidase} } IAA_{\text{oxidized}} + H_2O$$  (2)

or attributed to a competition between IAA and the electron donor species (AH) for H2O2. According to Ray, the results suggested that in the presence of both substrates, an inactive form of the enzyme, incapable of oxidizing either of them, accumulates.

We report here a slightly different interpretation of the interactions of the peroxidase and IAA oxidase systems, based on observations made by means of a new continuous flow, potentiometric technique developed first by R. I. Porterfield and C. L. Olson of the Ohio State University (personal communication) employed in this laboratory to study the peroxidase-IAA oxidase systems. The advantages and problems posed by this new analytical system, as well as several further applications, are discussed.

MATERIALS AND METHODS

Plant Culture and Homogenate Preparation. Oats (Avena sativa cv. Victory) were grown on water-saturated cotton in complete darkness at 23 C. At from 8 to 18 days, the etiolated coleoptiles and primary leaves were cut off in the light at 5 C, weighed, placed in 0.15 M potassium phosphate buffer (pH 6.20), and homogenized for 3 min in a Waring Blender. The brei was then pressed through four layers of cheesecloth, centrifuged at 25,000 g for 15 min at 5 C, and the supernatant decanted. This supernatant, which contained the plant enzyme studied, was used immediately without further purification or separation.

Analytical System. The experimental apparatus consisted of four components and plastic tubing (Tygon through the pump, Teflon elsewhere) connecting the various components: a thermostatically controlled water bath (Precision Scientific Co., Chicago; Model 83) maintained at 29.0 ± 0.3 C, a variable speed peristaltic pump (Harvard Apparatus Co., Dover, Mass.: Model 500-1200) set at 30 rpm, a set of glass-proportioning tees and magnetic mixers (2 × 8 mm Teflon-covered stir bars.
driven by a variable speed motor with magnet head, and the electrode proper. Reagents were continuously pumped from the water bath through the mixing and proportioning components and into the electrode. The arrangement of the components is given in Figure 1.

In-stream dilution at the proportioning tees resulted in a halving of the absolute concentrations of lines 1 and 4, and a quartering of the absolute concentrations of lines 2 and 3. The absolute concentrations of reagents therefore had to be adjusted accordingly so that uniform in-stream dilution of the reagents prior to entering the electrode resulted.

The purpose of the pumping and proportioning components of the apparatus was to set up two solutions, reference and sample, in which all of the experimental variables, except the one variable under consideration, were identical. The mixture of reagents from lines 2 and 3 contained all the reactants necessary for the enzymatic reaction (ferrocyanide, ferricyanide, and enzyme), except for the reaction variable under study, and this mixture was split equally between the reference and sample solutions. Addition to the sample solution of the reaction variable (H₂O + IAA) by means of line 1 resulted in an active enzymatic reaction and a continuous shift in reactant and product concentrations. The reference solution therefore acted as a reagent blank for the reaction, cancelling the effects of dilution, ionic strength and activity, pH, temperature, interfering species, and any nonenzymatic reactions that might have been occurring.

For the determinations of the oxidation of IAA, the two halves of the tubular carbon electrode were separated, and the solutions were allowed to drip into two test tubes each containing 5 ml of Salkowski reagent (6) until a total volume of 8.5 ml was reached in each tube. This served to stop the reaction and to initiate color development. The color development was read at 535 nm using a Bausch and Lomb Spectronic 20 spectrophotometer. For the determinations of the oxidation of IAA by Avena, the crude homogenates were preincubated with 9.5 μg/ml IAA for 100 min.

**Enzyme Electrode.** The electrode assembly was made up of two identical half-cells, each consisting of three portions: a smooth bored section in which laminar solution flow was established to help stabilize the half-cell potential, a tubular carbon electrode in which the half-cell potential of the solution passing through it was sensed electrochemically, and a central collecting chamber in which electrical contact was made between reference and sample half-cell solutions, and from which excess solution dripped into a waste receptacle (Fig. 2).

Each half-cell assembly was constructed by gluing a one-quarter inch long segment of three-sixteenth inch diameter ceresin wax-impregnated spectral grade graphite rod (National Spectrographic Laboratories, Cleveland) into a three-sixteenth inch diameter hole, drilled one inch into a one and seven-eighths inch piece of three-quarter inch diameter Teflon rod with epoxy. A Teflon plug was then glued into the remainder of that hole in a similar manner. A one-sixteenth inch diameter hole was then drilled through the entire half-cell assembly, resulting in one continuous bore throughout. This bore was then cleaned with acetyl acetone to remove the wax surface from the inside of the tubular carbon electrode. A small hole was drilled part way into the tubular carbon electrode through the side of the Teflon rod, and a pointed piece of platinum was inserted to provide electrical contact with the tubular carbon electrode. The two half-cells thus assembled were then joined by means of a short segment of Tygon tubing (3/16 inch i. d.) with a hole cut into its side as a solution waste exit, and Teflon lines (3/16 inch o. d.) were glued into each end of the electrode assembly. These lines were then attached to the pumping and proportioning components of the experimental apparatus as indicated in Figure 1.

The tubular carbon electrode leads were attached by means of shielded cable to the ground and positive (input) terminals of a strip chart recording potentiometer (Heath Kit, Benton Harbor, Mich.; Model EVW-20A); a 55 μfarad capacitor was attached between the terminals to partially dampen signal noise. The range of the recorder could be varied from 10 to 250 mv full scale deflection, depending on the voltage being sensed. Between assays, the electrode was stored in the oxidation reduction couple (ferrocyanide-ferricyanide) solution.

The experimental apparatus thus assembled held constant the time from the initial mixing of the enzyme and its substrate to the reading of the resultant potential (E₉₀), and thus allowed the continuous potentiometric monitoring at some time, (0.5 min at 30 rpm), of the disappearance of an electrode-sensitive species from the reaction under study. Since the reference solution acted as a blank for the reaction and the fixed flow rate held the reaction time constant, the potential difference (E₉₀) of the half-cells as sensed between the identical indicator electrodes was therefore a function only of the concentration and activity of the variable under study.

**IAA-Oxidase and Peroxidase Assays.** The materials involved in studies of the IAA-oxidase and peroxidase activities of the plant homogenate included purified IAA (CalBioChem, San Diego), naphthalene acetic acid (Nutritional Biochemicals Corp., Cleveland), potassium ferrocyanide (Fisher, Pittsburgh), potassium ferricyanide (Fisher), and hydrogen peroxide (Fisher, 30% v/v), in addition to purified horseradish peroxidase (Nutritional Biochemicals Corp., 400 IUB unit/mg.).

Table I indicates the experimental variations employed.

**Dialysis of Recovered Reaction Solutions.** The waste solu-
Table 1. Experimental Conditions for Studies of IAA-Oxidase Reaction

<table>
<thead>
<tr>
<th>Reaction Variable</th>
<th>Line 1</th>
<th>Line 2</th>
<th>Line 3</th>
<th>Line 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen peroxide</td>
<td>$H_2O_2$, 1.0 $\times 10^{-4}$ M to $1.0 \times 10^{-3}$ M</td>
<td>Fo/Fi, 1 $\times 10^{-2}$ M Fo and $1 \times 10^{-3}$ M Fi</td>
<td>Plant homogenate (0.025 g eq. fresh wt/ml) or horseradish peroxidase (0.025 mg/ml)</td>
<td>Buffer (potassium phosphate, pH 6.2) 0.15 M</td>
</tr>
<tr>
<td>IAA</td>
<td>$H_2O_2 +$ IAA, 1.0 $\times 10^{-4}$ M $H_2O_2$ and 1.0 $\times 10^{-3}$ M IAA</td>
<td>Same as above</td>
<td>Same as above</td>
<td>Same as above</td>
</tr>
<tr>
<td>Naphthalene acetic acid</td>
<td>$H_2O_2 +$ NAA, 1 $\times 10^{-4}$ M $H_2O_2$ and 1 $\times 10^{-3}$ M NAA</td>
<td>Same as above</td>
<td>Same as above</td>
<td>Same as above</td>
</tr>
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</table>

![Graph](https://example.com/graph.png)

**Fig. 3.** Influence of hydrogen peroxide concentration on the kinetics of the peroxidase reaction. ○: Avena homogenate; ●: commercial horseradish peroxidase (Nutritional Biochemistry). $[H_2O_2]$ is the final in-stream molar concentration of hydrogen peroxide, and $[Fo/s]/[Fo/r]$ is the relative concentration of ferrocyanide of the reference solution to that of the sample solution.

Addition of IAA to the sample solution (along with $H_2O_2$, Fo, Fi, and enzyme) consistently yielded a linear decrease in the amount of Fo consumed with increasing IAA (Fig. 4). For both the Avena homogenate and purified HRP, a linear decrease in $(Fo)_{sample}/(Fo)_{reference}$ (1/antilog $(E_{00} / 59mv)$) was observed, caused by the increasing interference of IAA in the Fo reaction in the range of $1 \times 10^{-4}$ M to $1 \times 10^{-2}$ M IAA. Substitution of NAA for IAA resulted in no change in $E_{00}$, and correspondingly, no change in $(Fo)_{sample}/(Fo)_{reference}$.

The results of experiments conducted to determine the actual oxidation of IAA by both HRP and Avena homogenates are found in Table II. The preincubation of Avena homogenates with a small quantity of IAA (9.5 $\mu$g/ml) was necessary to observe either the oxidation of IAA spectrophotometrically or the expected interference in the rate of Fo consumption in

**RESULTS**

Before investigating the action of the plant enzyme as an IAA-oxidase, preliminary studies of peroxidase reaction rate with respect to $H_2O_2$ were conducted in order to characterize the plant enzyme found in the Avena homogenate as a peroxidase (Fig. 3). The rate of peroxidation of Fo by the purified HRP was found to increase sharply between $2 \times 10^{-4}$ M and $4 \times 10^{-4}$ M $H_2O_2$, while the increase in peroxidative activity had resulted.

![Graph](https://example.com/graph2.png)

**Fig. 4.** IAA oxidase activity as measured by the analytical system. What is observed in the presence of IAA is a decrease in the observed potential which has been represented here as an increase in the $(Fo)_{sample}/(Fo)_{reference}$ ratio. ○: Avena homogenate; ●: commercial horseradish peroxidase (Nutritional Biochemistry). IAA and NAA are given in final in-stream molar concentrations and $(Fo)_{sample}/(Fo)_{reference}$ is the relative concentration of ferrocyanide of the sample solution to that of the reference solution.

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1 Abbreviations: HRP: horseradish peroxidase (purified); NAA: naphthalene acetic acid; Fo: ferrocyanide; Fi: ferricyanide.
response to IAA. These observations indicate the existence of an induction phase in the *Avena* enzyme similar to that observed by Ray (11) in the corresponding enzyme from *Omphalia*. These results indicate that IAA oxidation is occurring both in the case of HRP and *Avena* homogenates in the experimental system.

Dialysates of solutions which have previously been assayed for IAA-oxidase activity and shown to have a linear decrease in the rate of Fo consumption in response to IAA demonstrated uniform peroxidase activity when reasayed in the presence of H$_2$O$_2$, Fo, and Fi (Table III). Because of the quartering of the enzyme concentration by in-stream dilution, the $E_{oa}$, values obtained for the reasay of the sample solutions after dialysis were, as expected, approximately one-fourth of those obtained for the predialysis sample solution containing no IAA.

**DISCUSSION**

The assay of IAA oxidase activity with the analytical system depends upon two basic relationships; first, upon the Nernstian logarithmic relationship between $E_{oa}$ and the concentrations of electroactive oxidation reduction species (Fo-Fi), and second, upon the competition of IAA and other substrates such as ferrocyanide (Fo) for plant peroxidase.

The chemical constituents of the reaction system were established by the mixing and proportioning components of the apparatus (Fig. 1). The sample and reference solutions differed in only one aspect: the reference solution contained buffer (dilution blank) instead of the reaction substrate, H$_2$O$_2$. The peroxidase reaction under study was therefore occurring only in the sample solution, according to the equation:

$$\text{H}_2\text{O}_2 + \text{Fo} \xrightarrow{\text{peroxidase}} 2\text{Fi} + 2\text{H}_2\text{O}$$

(3)

In the presence of peroxidase and an excess of H$_2$O$_2$, ferrocyanide is oxidized to ferriyanide at a rate that is dependent upon the concentration and activity of peroxidase. The shift in ferriyanide concentration can be followed electrochemically according to a form of the Nernst equation which eliminates variables controlled by the analytical system.

$$E_{obs} = (59 \text{ mV}) \log \left( \frac{(\text{Fo})_r}{(\text{Fo})_s} \right)$$

where Fo = ferrocyanide; $r$ and $s$ indicate reference and sample solutions. Since no reaction is occurring in the reference solution, $(\text{Fo})_r$ is constant, and $E_{obs}$ is therefore a function only of $(\text{Fo})_s$. Sample ferrocyanide concentration is dependent upon the rate at which Fo is converted to Fi by the peroxidase reaction (reaction 3) in the sample solution. $E_{obs}$ therefore provides a measure of the rate of peroxidation of Fo in the sample solution.

The data presented in Figures 3 and 4 is plotted linearly; that is either $(\text{Fo})/r/(\text{Fo})_s$ (Fig. 3) or its inverse, $(\text{Fo})/r/(\text{Fo})_s$ (Fig. 4) versus concentration. Values for these ratios are obtained directly by taking the antilogarithm of $(E_{oa}/59 \text{ mV}$), an operation which removes the interfering logarithmic term that is implicit in the Nernst equation. In order to present the data in a form which represents IAA oxidase activity, rather than a decrease in $E_{oa}$, the data in Figure 4 are presented as the inverse function, $(\text{Fo})/(\text{Fo})_s$.

Fowler and Morgan (1), working with cotton homogenates, found a very consistent, highly significant correlation between peroxidase activity and IAA-oxidase activity throughout their studies, supporting the assumption that the IAA-oxidase system is peroxidase based and, generally, measurement of one activity indicates the other (9, 11).

The study of the IAA oxidation by plant homogenates will be interpreted to be a substrate competition of IAA and Fo for the plant peroxidase, since addition of IAA does interfere consistently with peroxidation of Fo as revealed potentiometrically (Fig. 4). This interference appears to be specific for IAA, since the substitution of NAA, which is not a substrate of IAA oxidase (13), for IAA does not interfere with the peroxidation of Fo (causes no decrease in $E_{oa}$). Plant peroxidase can act either as an IAA oxidase, generating oxidized IAA (which is electrochemically inactive) according to reaction 2, or as a peroxidase, generating electrochemically measurable shifts in Fo according to reaction 3.

In the presence of an excess of H$_2$O$_2$ and a fixed concentration of Fo, the voltage $(E_{oa})$ was observed to vary as a linear function of added (IAA) (Fig. 4). This follows from the fact that with increasing IAA, there is an interference in the Fo/ peroxidase reaction by IAA. This in turn involves an increasing amount of the available plant peroxidase in the IAA oxidase reaction. The decrease in the rate of disappearance of (Fo) due to the competition of IAA with Fo as substrates of the plant peroxidase will be translated by means of the Nernst equation into a decrease in the observed voltage. Since $E_{oa}$ is a function only of (Fo) and not of oxidized IAA (IAA and oxidized IAA are an oxidation reduction couple but exhibit no potentiometrically measurable electroactivity), $E_{oa}$ therefore allows the continuous monitoring of the relative rate of peroxidation occurring in solutions in which the plant peroxidase is acting as both an IAA oxidase and a peroxidase simultaneously.

The difference in peroxidative characteristics of the *Avena*...
homogenate and purified HRP may be attributed to the presence of various inhibitors in the plant homogenate which are absent in the purified HRP. The data may also suggest that these two enzymes are not the same "peroxidase" and may act according to different mechanisms (2, 8, 16). Experiments are planned to clarify the basis for these differences in peroxidative characteristics.

Ray (11) observed that the addition of low ($10^{-3}$ M) concentrations of IAA to mixtures of pyrogallol (an electron donor), H$_2$O$_2$, and a peroxidase from Omphalia flava served to depress the rate of pyrogallol oxidation markedly. Furthermore, an inhibitory effect was obtained well within what is regarded as the physiological range of IAA concentration. Ray determined that the rates of both IAA and pyrogallol oxidation were strongly inhibited when the two substrates were present together.

Although the present experiments (Fig. 4) may be interpreted to support Ray's conclusion on the inhibitory effects of IAA, the postdialysis observations presented in Table III may be interpreted to disagree with his conclusion that the relationship between IAA and other substrates of plant peroxidase is one of enzyme inactivation. In this same paper (11), he concluded that in the presence of both substrates, the inhibition of peroxidation was due to the accumulation of an inactive form of the enzyme which was incapable of oxidizing either substrate. Our findings indicate that if his interpretation of the inhibition of peroxidation caused by an inactivation of the enzyme itself is correct, then at least peroxidase activity can be restored by dialysis (Table III). The data obtained can be interpreted to be in support of the model for the enzymatic oxidation of IAA recently proposed by Gelinis (3).

While in these experiments the enzyme electrode was employed to measure peroxidase activity directly and IAA-oxidase activity indirectly through interference with the electrochemically measurable shifts in (Fo), the analytical system is potentially useful in studying the activities of other plant enzyme systems and their substrates, both directly and indirectly. The applications of this technique to glucose oxidase and lactate dehydrogenase systems have been studied (R. I. Porterfield and C. L. Olson, personal communication) and investigation of amine oxidase and amino acid oxidase systems are under consideration.

The use of crude homogenates and the control, by means of the reference solution, of interfering reactions, inhibitors, and cofactors are among the advantages of the enzyme electrode approach to biochemical assays.

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LITERATURE CITED