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# Stimulation of Indoleacetic Acid Oxidase and Inhibition of Catalase in Cotton Extracts by Plant Acids

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*Abstract.* **Activity of indoleacetic acid oxidase in partially purified extracts from cotton is stimulated by small amounts of malate, succinate, fumarate, and other plant acids. The stimulation is apparently due to inhibition of catalase, which is detectable in certain preparations. The lag phase of indoleacetic acid oxidation by crude preparations is eliminated by steps in processing which conceivably either denatures or dilutes catalase, or concentrates inhibitors to catalase.**

IAA-oxidase is inhibited *in vitro* by catalase (4, 5, 11), polyphenols (1, 3, 7, 14), various inhibitors and substrates of peroxidase (6, 20), and by unidentified constituents of plant extracts (13, 19). A number of phenols act *in vitro* either as inhibitors or co-factors, and a rather extensive literature provides a tacit support for the function of phenols in these roles in the plant.

The possibility of regulation of IAA-oxidase by catalase *in vivo* has received surprisingly little emphasis. This has probably followed the observation of Goldacre *et al.* (6) that phenols stimulate IAA-oxidase of peas in the absence of catalase. In efforts to partially purify IAA-oxidase from cotton roots, Lane (12) found that the protein (enzyme) fraction was separated from certain plant acids with a complete loss of IAA-oxidase activity. Addition of a small amount of any one of several organic acids restored activity completely.

Noting that these same acids are inhibitors of catalase, we determined the effect of several acids on activities of catalase, peroxidase and IAA-oxidase in extracts of cotton.

## Results and Discussion

*Plant Culture.* Cotton plants (*Gossypium hirsutum* L., cultivar Stoneville 213) were grown in the greenhouse in aerated 5 liter cultures using standard Hoagland's solution plus chelated iron. Solutions were replaced with fresh material every 2 weeks. Tap water was added daily as needed. The plants were harvested shortly after appearance of flower buds.

*Extraction and Concentration of Homogenates.* Either roots or leaves were extracted by blending for 1 min with cold $H_2O$ or 0.05 M acetate buffer, pH 5.8, using 3 ml of liquid per gram tissue. The mixtures were filtered through 4 layers of cheesecloth and centrifuged 15 min at 17,000*g*. Suitable dilutions of the supernatant were taken for catalase assays and the remaining extract reduced 10 to 20 fold in volume by ultrafiltration (17). Further concentration was done by evaporation from bags of cellulose dialysis casing. All extractions and concentrations were carried out at 2°. Extracts processed by the foregoing procedure were labeled crude enzyme.

*Gel Filtration.* Aliquots of the crude enzyme were fractionated on a 5 × 55 cm column of Sephadex G-75 developed with water at 2°. The eluate was monitored at 280 nm. Recorder tracings of the protein elution pattern showed a shoulder past the peak. Eluate between the start of protein elution and the shoulder was caught, concentrated to the starting volume by evaporation from dialysis bags and labeled purified enzyme.

*IAA-oxidase.* Oxygen uptake was measured with a Warburg apparatus at 28° following a 30 min equilibration period. The volume of reaction mixture was either 3.3 or 3.4 ml and was contained in 22 ml reaction vessels. Control reaction mixtures included 0.05 M acetate buffer pH 5.8, 3 μmoles $Mn^{2+}$, 30 μmoles IAA, and enzyme. Other compounds tested were added at a concentration of 3 μmoles in 0.1 ml of water. Except when noted, 2,4-dichlorophenol was not used. All assays were conducted using 1 ml of enzyme solution, regardless of extent of concentration.

*Catalase.* Decomposition of $H_2O_2$ was monitored spectrophotometrically at 240 nm at 25°. The reaction mixture contained 2 ml plant extract at appropriate dilution and 1 ml 0.059 M $H_2O_2$ in 0.05 M phosphate buffer, pH 7.0.

For studies on the inhibition of catalase activity by organic acids and 2,4-dichlorophenol, 3 μmoles of these compounds were added to the above in 0.1 ml. Controls contained 0.1 ml water.

Initial rates of reaction were plotted from optical density readings taken at 10 sec intervals for 2 min.

*Peroxidase.* Appearance of 8-aminoquinoline oxidation product was monitored spectrophotometri-



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cally at 485 nm at 25°. The reaction mixture contained 1 ml plant extract at appropriate dilution, 2 ml 0.015 M 8-aminoquinoline in 0.05 M phosphate buffer pH 7.0, and 0.1 ml 0.01 M $H_2O_2$.

For studies on the inhibition of peroxidase activity by organic acids and 2,4-dichlorophenol, 3 μmoles of these compounds were added to the above in 0.1 ml. Controls contained 0.1 ml water.

Recording and plotting of data was carried out in the same manner as for catalase activity.

The hydrogen donor for the peroxidase reaction was 8-aminoquinoline since most of the extracts exhibited sufficient phenolase activity to render an assay using other hydrogen donors unreliable.

## Results

*IAA-Oxidase Activity in Crude Extracts.* A characteristic feature of IAA-oxidation by plant extracts is a lag in reaction (8). A lag of approximately 25 min was exhibited in assays performed with a crude extract of cotton roots taken directly from the refrigerator (fig 1). However, the lag was all but eliminated if the enzyme solution was warmed to room temperature or heated to 60° for 15 min prior to assay (fig 1). The lag was sharply reduced by repeated freezing and thawing (results not shown), and others (13, 19, 20) have reduced the length of lag phase considerably simply by dilution of the enzyme.

Preparations which exhibited no lag phase were also obtained from extracts of cotton. This was accomplished either by prolonged washing in the ultrafilter, or by evaporation of extract to a paste and dissolving in water. The crude enzyme used to obtain the data recorded in figure 2 was prepared by evaporation.

*Restoration of IAA-Oxidase Activity of "Purified" Enzyme by Plant Acids.* The IAA-oxidase activity of preparations showing little or no lag was lost after filtration with Sephadex G-75. Typical before and after results are shown in figure 2. IAA-oxidase activity was restored upon addition of 3 μmoles of malic acid to the reaction mixture (fig 2).

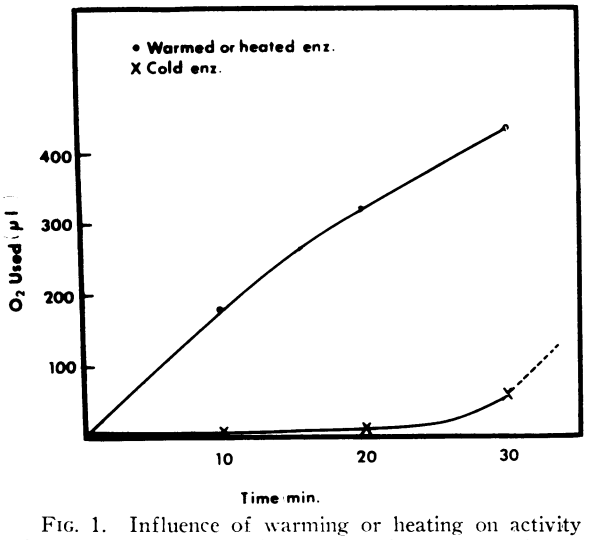


FIG. 1. Influence of warming or heating on activity of IAA-oxidase in crude extracts from cotton leaves or roots.

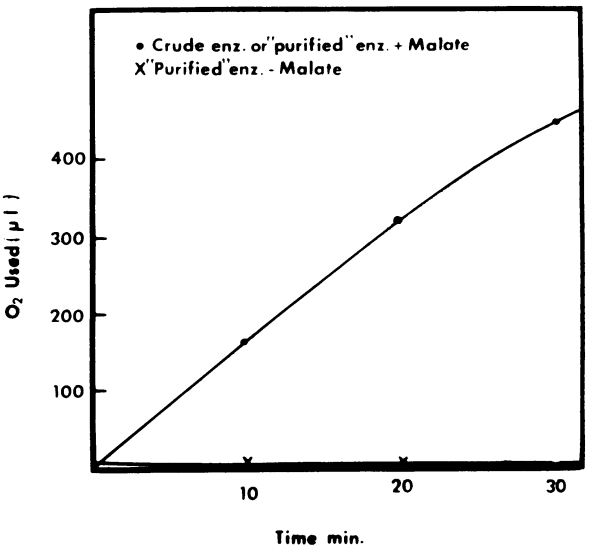


FIG. 2. Effect of gel filtration on activity of IAA-oxidase in crude extracts, and restoration of activity by purified enzyme with the addition of 3 μmoles of malic acid.

The reaction was dependent on IAA since no $O_2$ uptake was observed with malic acid as substrate in the absence of IAA. IAA-oxidase activity was also restored by 2,4-dichlorophenol, but that compound was much less stimulatory than several organic acids. The order of effectiveness of several compounds tested was as follows: malate = fumarate = oxalate = succinate > citrate = cis-aconitate > 2,4-dichlorophenol. α-Ketoglutarate, oxaloacetate, and oxalosuccinate were ineffective.

*Catalase Activity of Extracts.* Figure 3 shows catalase activity in the initial dilute extracts. Catalase could not be detected in active IAA-oxidase preparations. Spectrophotometric assay of catalase using the purified enzyme preparation was unsuccessful due to high ultraviolet absorption and possibly poor recovery from the Sephadex column, but visual detection of $O_2$ release was made upon addition of $H_2O_2$ to all the undiluted purified enzyme preparations. The visible release of $O_2$ after gel filtration indicates that catalase was present in active IAA-oxidase preparations, but was apparently inhibited by the presence of certain organic acids. Considerable quantities of fumarate, malate, and succinate were found in the small molecule fraction from the Sephadex column (12).

Catalase activity in fresh extracts diminished rapidly, particularly if the solutions were allowed to warm, but was detectable for several days in extracts kept cold.

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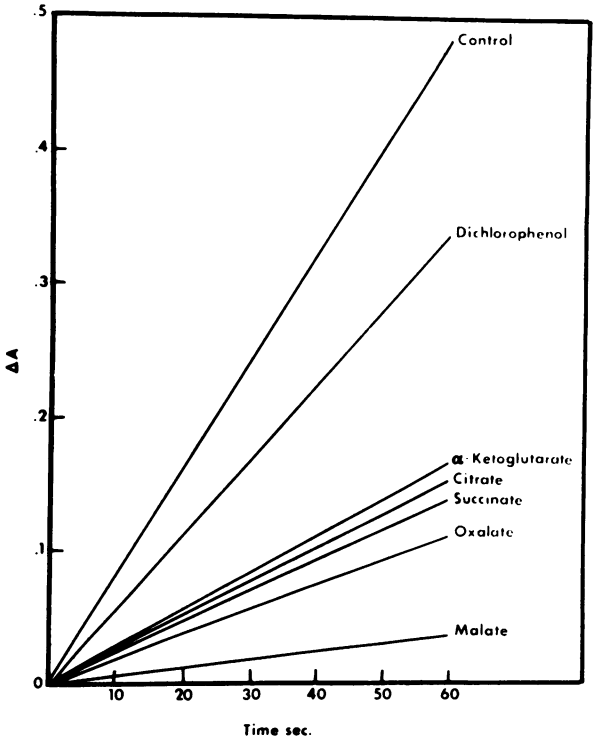


FIG. 3. Inhibition of catalase in crude cotton extracts by organic acids and 2,4-dichlorophenol. Initial reaction rates.

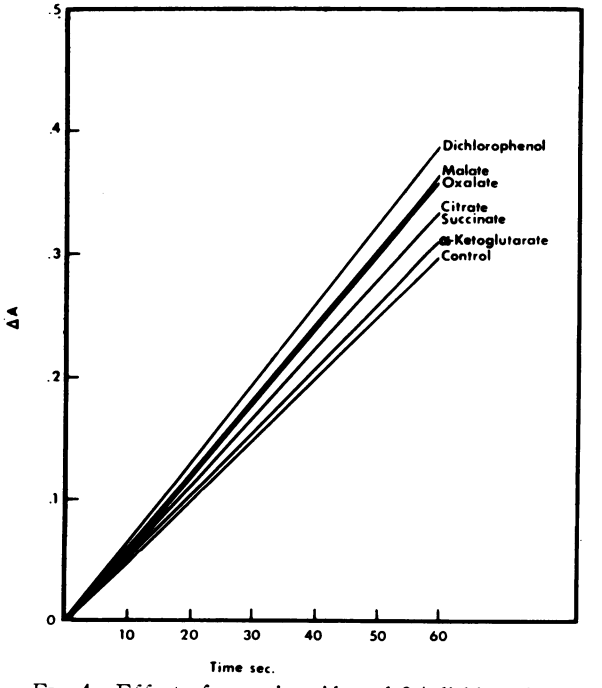


FIG. 4. Effect of organic acids and 2,4-dichlorophenol on peroxidase in crude or purified cotton extracts. Initial reaction rates.

*Effect of Plant Acids on Catalase and Peroxidase.* Catalase was strongly inhibited by several of the acids tested (fig 3). The results show that the acids most stimulatory to IAA-oxidation were most inhibitory to catalase. Nearly all acids tested were more effective catalase inhibitors and IAA-oxidase stimulators than 2,4-dichlorophenol. None of the compounds tested had a significant effect on peroxidase (fig 4). In addition, none of the acids which stimulated IAA-oxidase reacted with peroxide. α-Ketoglutaric acid, oxaloacetic acid, and oxalosuccinic acid reacted non-enzymically with $H_2O_2$.

## Discussion

We have shown in this paper that A) catalase is present in extracts of cotton, B) gel filtration of an active IAA-oxidase solution results in complete loss of activity, C) activity is fully restored by the addition of a small amount of any one of several organic acids, and D) those same acids inhibit catalase.

We conclude the stimulation of IAA-oxidase in cotton extracts by dicarboxylic acids (12) results from inhibition of catalase by these acids. The inhibition of catalase takes place in a classical IAA-oxidizing system. No $O_2$ uptake was noted in the absence of IAA. The system is therefore not the same as the one described by Kenten and Mann (10) in which certain dicarboxylic acids were oxidatively decarboxylated by a system involving peroxidase and $Mn^{2+}$. In general, the acids which gave negative results in their system were most effective in stimulation of IAA-oxidase.

The lag phase of IAA-oxidation may be explained in terms of a slow denaturation of catalase at reaction temperature. Further, the lag phase can be eliminated by various treatments of the enzyme solution (heating, freezing and thawing, complete evaporation, prolonged washing or dialysis, or dilution) which conceivably cause denaturation or dilution of catalase, or alterations in relative concentrations of peroxidase, catalase, and their inhibitors present in extracts. Morgan and Hall (13) described inhibitors which were extracted from green cotton tissues with boiling water, but questioned whether such inhibitors are specific for IAA-oxidase. The inhibitor in our extracts is heat labile and bears some semblance to an auxin protector of *Pharbitis* which has been described by Stonier *et al.* (18).

A scheme for regulation of IAA-oxidation *in vivo* involving catalase and plant acids is plausible, assuming that $H_2O_2$ is involved in the reaction mechanism (15). Certain dicarboxylic acids are known to accumulate under conditions leading to protein breakdown (16). These conditions occur in aging tissues which are considered to be low in auxin. We suggest that this acid buildup in aging tissue may play a regulatory role by inhibiting

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catalase, thus permitting $H_2O_2$ availability for initiation of the IAA-oxidizing system.

However, the results and interpretation presented in this paper cannot be reconciled with the results of studies with purified horseradish peroxidase (2, 9). Purified horseradish peroxidase catalyzes the oxidation of IAA without $Mn^{2+}$ or other co-factors and the reaction is not inhibited by catalase. Peroxides were not detected in the reaction mixture (2, 9).

Whether or not purified horseradish peroxidase is unique in catalysis of IAA-oxidation requires further studies with peroxidase or IAA-oxidase preferably purified from other sources. With few exceptions, the IAA-oxidase system in plant extracts requires co-factors and is inhibited by catalase.

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