PLANT PHYSIOLOGY

Vol. 38 No. 4

July 1963

# Indoleacetic Acid Oxidizing Enzyme & Inhibitors from Light-Grown Cotton [1, 2]

Page W. Morgan & Wayne C. Hall

Department of Plant Sciences, Agricultural & Mechanical College of Texas, College Station, Texas

As one aspect of a study of abscission in the cotton plant, we have been interested in the possible presence of an indoleacetic acid (IAA) oxidase in the aerial portions of the green plant. The need for investigation was indicated by the possibility that IAA oxidase could affect the decline in detectable auxin in cotton which occurs just prior to leaf fall (1, 2).

A study was initiated after noting that although there is evidence for the occurrence of IAA in cotton (3, 12), a preliminary attempt to demonstrate IAA oxidase was unsuccessful (13). Furthermore, information on the occurrence of the enzyme in other green tissues was relatively limited and an unequivocal function of the enzyme in higher plants has not been clearly established.

This paper presents evidence for the occurrence of an IAA oxidase in cotton and some characteristics of the enzyme and an inhibitor(s).

## Methods & Materials

*Plant Culture.* Upland Cotton (Deltapine 15 & Empire) was grown in the greenhouse in an inert medium (4). The plants were watered with a soluble fertilizer (4) or Hoagland's nutrient solution (15) modified to include $7.16 \times 10^{-5}$ M iron as the sodium ferric diethylenetriamine pentaacetate chelate (Geigy Sequestrene).

*Extraction and Purification.* Samples of leaves and other parts were collected, weighed, and either extracted immediately or stored at −20° until used. All subsequent manipulations were carried out in a 2° cold room. Samples were crushed while frozen and homogenized with a blender in 3 ml of water per gram of fresh tissue. The brei was squeezed through four layers of cheese cloth and centrifuged at 22,000 × *g* for 20 minutes at 0°. This centrifugation was assumed sufficient to remove mitochondria and all larger particles.

The resultant particle-free liquid (hereafter termed extract) was either dialyzed or treated with acetone. Dialysis was conducted in the cold in 10 liter batches of deionized water changed daily for at least 3 days. At the end of the dialysis period the extract was centrifuged as before. Purification by acetone precipitation was carried out according to the method of Galston and Baker (9). The precipitate was redissolved in water, dialyzed 24 hours, centrifuged as before and assayed immediately or stored in the dark at −20° until used. The activity of such preparations declined slowly in the cold room but it was still measurable after 60 days.

All of the experiments reported here in tabular or graphic form, except figures 2, 5 and 6 were conducted using acetone-purified enzyme. Tests for occurrence of the enzyme in the species and races of cotton and the experiments illustrated in figures 2, 5, and 6 were conducted using a dialyzed extract for the enzyme source.

*IAA Oxidase Assay.* A manometric assay system was used. Each flask contained 30 μmoles of IAA in the side arm; 0.2 ml of 20 % KOH in the center well with a filter paper wick; and 1 ml of 0.4 M $Na_2HPO_4$-$NaH_2PO_4$ buffer (pH 5.8), 3 μmoles of 2,4-dichlorophenol, 3 μmoles of $MnCl_2$, 0.0319 μmole of riboflavin, enzyme (usually 1 ml) and water in the main compartment to give a total volume of 3.3 ml. The assay was conducted at 25° following a 15 minute equilibration period. Unless otherwise noted, assays were carried out in a lighted room. Blanks containing riboflavin but no enzyme yielded no measurable oxygen uptake at the light intensity involved here (<80 ft-c). Duplicate flasks were prepared in all cases.

[1] Received Sept. 26, 1962.

[2] Supported in part by a grant from the Foundation for Cotton Research and Education, National Cotton Council. Published with the approval of the Director, Texas Agricultural Experiment Station. A portion of the work described here was taken from a dissertation submitted by Page W. Morgan for the Ph.D. degree at the A & M College of Texas, August, 1961.

*Inhibitor Assay.* (The term "inhibitor" is used for convenience, but does not imply that the inhibition is necessarily caused by a single substance). The solution to be tested for inhibitor content, appropriately diluted, was added in an 0.1 ml aliquot to the standard IAA-oxidase assay. A control with water instead of inhibitor was run with each assay. Oxygen uptake vs. time was plotted and the initial linear portion of the reaction curve was extrapolated to the base line to indicate the length of the lag period (example, fig 2 & 6).

*Peroxidase Assay.* Relative peroxidase activity was assayed according to the chronometric-colorimetric method of Maehly and Chance (17).

## Results

*Characteristics of Enzyme and Inhibitor.* Initially, the earlier finding that IAA-oxidase activity could not be demonstrated in crude extracts of cotton leaves (13) was confirmed. The preparation of an acetone powder, when redissolved and dialyzed, yielded an enzyme solution which only became active in the manometric assay system after a lag period of 70 to 120 minutes. As the pH of the assay medium was decreased, the lag decreased (fig 1). The reduced lag could have been caused by an acid-catalyzed inactivation of an inhibitor or activation of a co-factor(s). However, the more acidic conditions were suboptimal for the enzyme since the total oxygen uptake decreased as the pH decreased (fig 1).

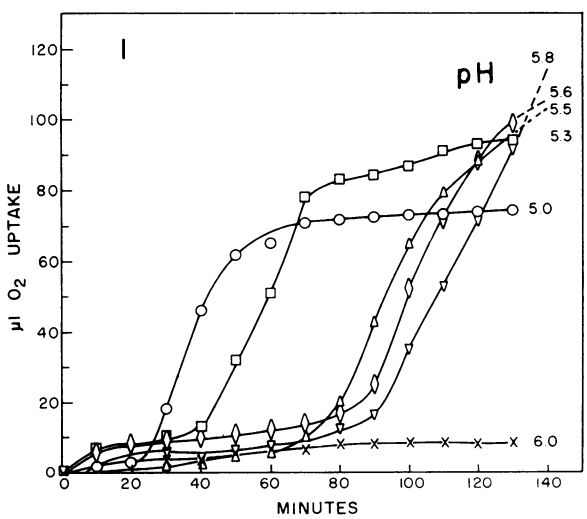


FIG. 1. Effect of assay pH (phosphate buffer) on the activity of IAA oxidase from cotton leaves.

The manometric assay system was shown to be specific for destruction of IAA catalyzed by the enzyme. There was no significant oxygen uptake if either substrate, enzyme, $MnCl_2$, or 2,4-dichlorophenol was omitted. Boiling the enzyme for 3 minutes removed all of the activity.

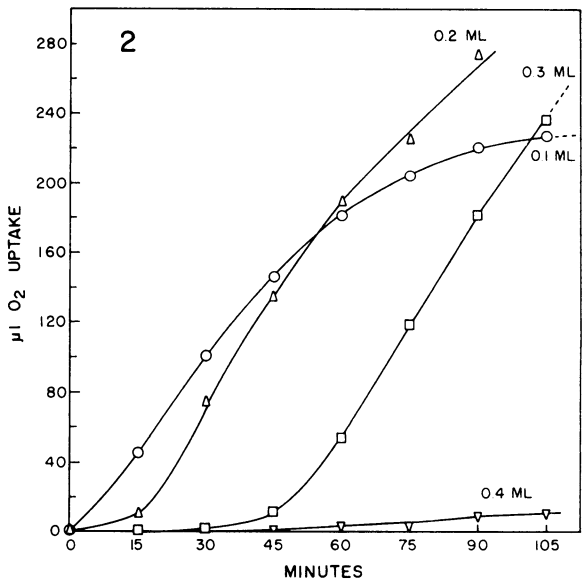


FIG. 2. Effect of enzyme concentration (dialyzed cotton leaf extract) on the lag in oxygen uptake.

Dialyzed extracts, inactive at higher concentrations, did have IAA-oxidase activity when properly diluted (fig 2). The lag was extended by increasing the enzyme concentration, which indicated the presence of an inhibitor. Inhibitor activity was confirmed when the characteristic lag, removed by purification, was returned by addition of boiled, non-dialyzed extract (fig 3).

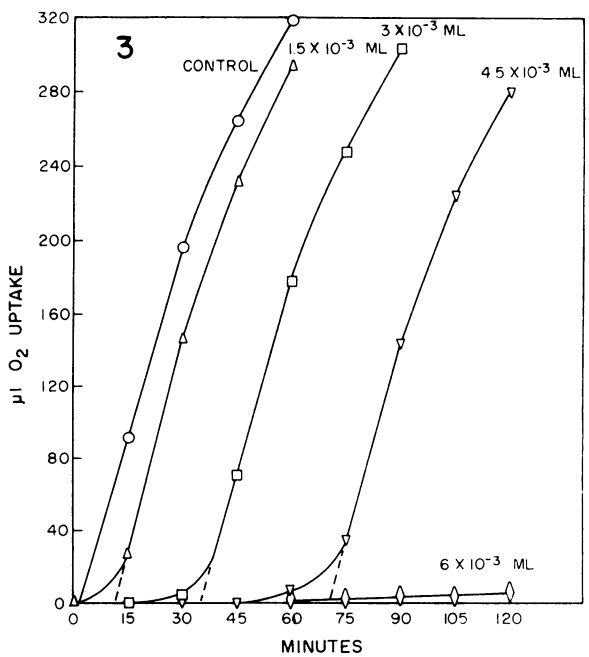


FIG. 3. Induction of a lag in oxygen uptake by addition of boiled cotton-leaf extract to IAA oxidase from similar tissue.

**Table I**

*Effect of Gossypol on the Activity of Cotton IAA Oxidase*

Gossypol was dissolved in absolute ethanol. Controls with ethanol or water but no gossypol were included in each experiment and there was no measurable lag due to the ethanol (0.1 ml per flask).

| Gossypol concentration | Length of lag period-minutes* | |
|---|---|---|
| | Dark-Without Riboflavin | Light-With Riboflavin |
| $1 \times 10^{-4}$ M | >240 | 26.5 |
| $5 \times 10^{-5}$ M | 140 | 12.5 |
| $1 \times 10^{-5}$ M | 13 | 0 |
| $5 \times 10^{-6}$ M | 6.5 | 0 |
| $1 \times 10^{-6}$ M | 0 | 0 |

* The lag was measured under conditions most favorable (dark-without riboflavin) and most unfavorable (light-with riboflavin) for expression of inhibitor activity.

Riboflavin (0.0319 μmole or $1 \times 10^{-5}$ M) in the reaction mixture reduced the lag period of the first enzyme preparations as much as 60 minutes but had little effect on the rate or stability of the reaction. Subsequently, the degree to which riboflavin reduced the lag was shown to be related to the inhibitor concentration (also demonstrated with gossypol, table I). The effect of several flavins on the inhibitor-induced lag in enzymatic destruction of IAA is shown in figure 4 (all materials were Nutritional Biochemicals Corp. products, FAD was 80–90 % purity). The effects of riboflavin and FMN were approximately equal, but FAD was less effective. Based on these findings, riboflavin was added as a standard practice to all assay systems.

The action of riboflavin (fig 4) was concluded to be the inactivation of an inhibitor and not photooxidation of IAA (7) because there was no measurable oxygen uptake when controls were run with riboflavin and enzyme was omitted. The fact that riboflavin only served to speed the initiation of a reaction which would start without riboflavin also indicates that the bulk of the oxygen uptake was not due to photooxidation.

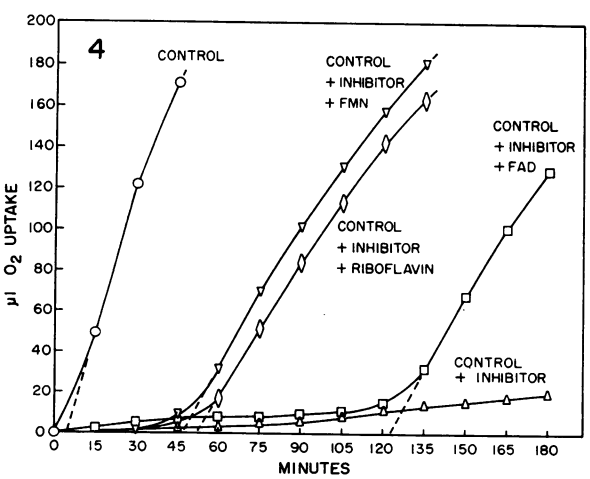


FIG. 4. Effect of various flavin compounds ($1 \times 10^{-5}$ M) on an inhibitor-induced lag in IAA-oxidase activity. Control was enzyme in standard system without flavin or inhibitor. Inhibitor was a boiled leaf extract.

The inhibitor concentration was reduced by dialysis, but the process was not completely effective since dialysis, for up to one week usually resulted in enzyme preparations which still had a measurable lag in the absence of riboflavin. However, dialysis for 3 days and dilution of the preparations to the linear reaction range allowed a fairly rapid process for evaluating the IAA-oxidase activity of a large number of samples. In actual practice such assays showed a very short lag when riboflavin was included. Some enzyme preparations purified by the acetone procedure contained inhibitor (fig 1). The inhibitor content varied with age of plants, tissue, and season of harvest.

*Enzyme Occurrence.* Following the initial experiments, the enzyme was demonstrated repeatedly in extracts of leaves, petioles, stems, and roots of the upland cotton varieties used. The enzyme was found in the dialyzed extracts of leaves of vegetative plants of the following races of *Gossypium hirsutum*: latifolium (two collections), marie-galante, morrilli, palmeri, and punctatum (two collections). In addition, the enzyme was present in the dialyzed extracts of mature leaves of the following species of Gossypium: anomalum, areysianum, aridum, australe, herbaceum, incanum, lobatum, somalense, tomentosum, and triphyllum. This group includes at least one species from each of the genome groups of Gossypium.

*Inhibitor.* We have demonstrated the inhibitor in aqueous extracts of leaves, petioles, stems, roots, squares, flowers, and bolls of several commercial upland cotton varieties. In addition, it was present in all of the previously mentioned species of Gossypium and races of *G. hirsutum*. The lag-inducing substance was also extracted from whole leaves by boiling them in water for 20 minutes.

Light was necessary for the riboflavin-mediated reduction of inhibition. Assay of an inhibitor-containing enzyme preparation in the dark resulted in extension of the lag period until the room lights were turned on (30–80 ft-c at the bath surface) (fig 5). Without riboflavin, the lag period extended over 60 minutes beyond the end of the dark period. The result was obviously the inactivation of the inhibitor instead of light-activation of an enzyme, since the lag can be added to an active enzyme preparation with an enzyme-free, boiled extract (fig 3). Results shown in figures 3 and 5 have been repeated several times.

The major effect of the inhibitor was to cause a lag in the start of rapid oxygen absorption during IAA oxidation; but measurements indicated that there was also an effect upon the rate of the subsequent reaction. In one test 48 samples of leaves

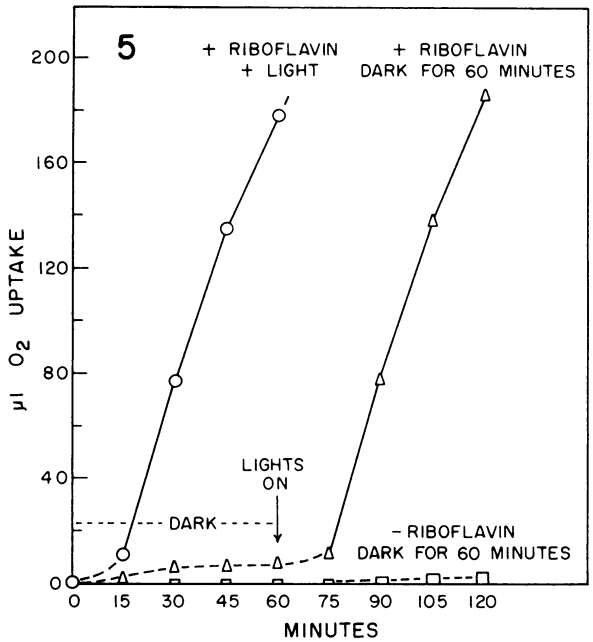


FIG. 5. Effect of light and riboflavin on an inhibitor induced lag in IAA-oxidase activity.

were extracted, aliquots of each extract were heated on a boiling water bath for 20 minutes and dilutions subsequently assayed in combination with acetone-purified enzyme from leaves. These "inhibitor samples" reduced the initial reaction rates by 4 to 46 % while causing lag periods ranging from 3 to 160 minutes. Increasing the inhibitor concentration increased the lag to a much greater degree than it

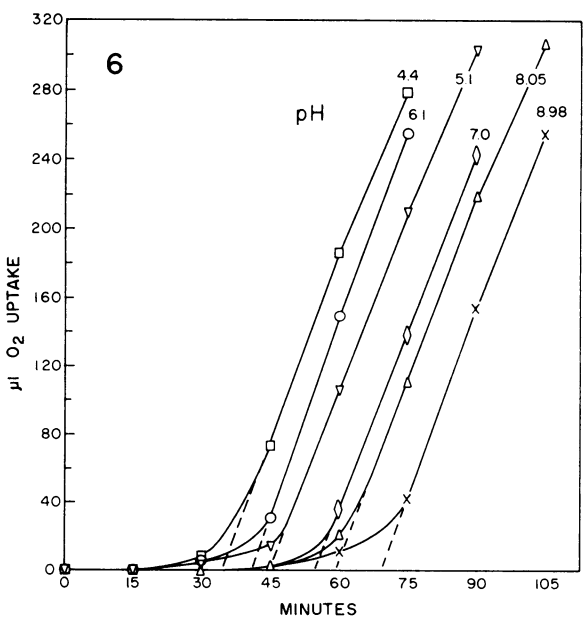


FIG. 6. Effect of pH of extraction medium on IAA-oxidase activity of subsequently dialyzed extracts. One ml of each preparation was assayed in the standard system.

decreased the rate. The rate-repression may simply be a non-specific effect associated with crude, boiled extracts, but the possibility of the existence in cotton of a competitive type of inhibitor is being studied.

Several experiments were conducted where phosphate buffers (0.2 M) over the range of pH 4.4 to 8.9 were used to extract homogenous samples of cotton leaves. There was no effect of pH on the amount of enzyme recovered. When aliquots of the extract were boiled and centrifuged free of denatured protein, assay revealed that as the pH decreased the inhibitor content increased. The remainder of each extract was dialyzed. Assay of these enzyme preparations revealed the opposite results, as the pH decreased the inhibitor content also decreased (fig 6). Apparently, the inhibitor dialyzes more readily from those extracts prepared with the more acidic buffers; therefore, there may be some type of bond between the inhibitor and the leaf protein which is broken more readily under acid conditions. All of the samples had reached a pH equilibrium prior to assay.

*Other Inhibitors.* Gossypol, a phenolic substance found in cotton seed, leaves, and roots (21), has certain structural similarities (5) to an IAA-oxidase inhibitor in peas (19). Gossypol acted as a potent inhibitor of the cotton leaf enzyme (table I). It caused a lag in oxygen uptake which was reversed by light and riboflavin in the same manner (fig 7)

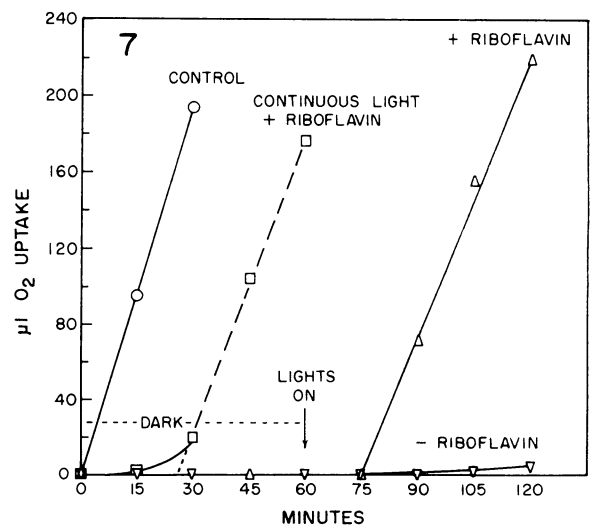


FIG. 7. Effect of light and riboflavin on gossypol-induced lag in IAA-oxidase activity. All except control contained $1 \times 10^{-4}$ M gossypol. All except continuous light were in initial 60 minute dark period.

as the inhibitor in our aqueous extracts (fig 5). The identity of the natural inhibitor and its distribution are being further investigated.

Kaempferol (Mann Research Laboratories), a component of IAA-oxidase inhibitors (at $10^{-4}$ M) identified in peas (6, 19), functioned in our assay system in a manner identical to gossypol and the natural inhibitor from cotton (fig 5 & 7). It caused

a marked lag period at $1 \times 10^{-4}$ M and was reversed by light and riboflavin, either of which alone had no noticeable effect.

*Peroxidase.* Due to the widely accepted role of peroxidase in the destruction of IAA, the relationship of peroxidase and IAA-oxidase activity in cotton leaves was determined. All preparations active as IAA oxidase have also had peroxidase activity. However, the converse was not true. Crude homogenates of cotton leaves have immediate peroxidase activity, yet rarely have such preparations oxidized IAA without some type of purification. Even with samples of lowest inhibitor content (winter-grown seedlings) peroxidase activity was detected with less difficulty than IAA-oxidase activity.

We have found no significant stimulation of IAA oxidase of cotton leaves with hydrogen peroxide (Baker Chemical Co.). A typical experiment is shown in figure 8.

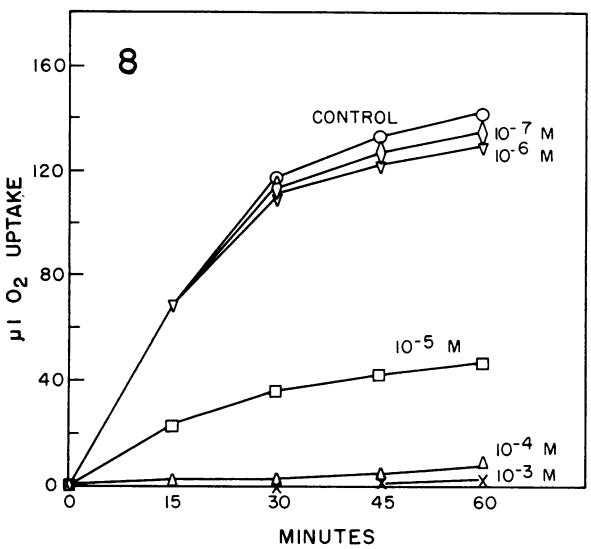


FIG. 8. Effect of hydrogen peroxide concentration on the activity of cotton-leaf IAA oxidase. Assay conducted in the dark (< 1 ft-c at bath surface) without riboflavin.

## Discussion

The results of this study show that an IAA-destroying enzyme system is present in extracts of the various parts of the light-grown cotton plant and that this system is apparently distributed throughout the genus Gossypium. Therefore, the participation of the enzyme in physiological processes in green tissue is a possibility.

The natural inhibitor of IAA oxidase in cotton is not unique (9, 11, 18, 19, 22, 24, 25, 26), but its in vivo status is critical to the physiological significance of the enzyme in green tissue. Evidence has been presented that such inhibitors may regulate enzymic destruction of IAA (22). In this connection, the interconversion of inhibitors and cofactors of IAA oxidase by a red-near infrared light mechanism has been proposed (8, 14, 19). Also, inhibitor content is known to vary with day length (8) and season of the year (18, 26, present paper).

If inhibitors do play an important role in an in vivo auxin destruction system, our finding that riboflavin will mediate the light-activated reversal of several IAA-oxidase inhibitors may have significance in the same system. An alternative view, that "riboflavin may continuously exert a check on IAA destruction in vivo" (27) was based on the finding that FMN ($10^{-4}$ M) retards the rate of enzymatic destruction of IAA in vitro (26, 27). This view does not consider the effect of riboflavin on lag-inducing inhibitors of IAA oxidase. The protective effect of riboflavin was reversed by light, was insignificant at $2 \times 10^{-5}$ M (double the concentration used here) and was demonstrated with enzyme preparations essentially free of inhibitors (27). Although our findings are also in vitro observations, they do indicate the possibility that the predominant role of flavins in light-grown plants containing both IAA oxidase and inhibitors may be to encourage destruction of rather than protection of IAA (27).

The reversal of IAA-oxidase inhibition by light, without added flavins, has been noted previously (9, 11). Evidence was obtained in one study (9) indicating that the photoreceptor involved was a flavoprotein.

Two different light reactions could be involved in the regulation of IAA oxidase-inhibitor levels. Thus red light stimulates the synthesis of inhibitors (kaempferol &/or others in peas; 14, 19), but riboflavin and light (presumably blue, see 9) accelerates the inactivation of inhibitors (cotton inhibitor, gossypol, & kaempferol; this paper).

The similarity between the inhibitory effect of gossypol and the unidentified inhibitor present in tissue extracts suggests the possible existence in cotton of IAA-oxidase inhibitors with considerable differences in chemical structure from those already identified in peas (6, 19).

The significance of the absence of a hydrogen peroxide-stimulation (fig 8) cannot be determined without further study. A similar report has been made by Sacher (23), but the stimulatory effect of hydrogen peroxide in other systems is well-known (10, 16, 18, 20).

## Summary

An enzyme capable of oxidizing IAA in the presence of $MnCl_2$ and 2,4-dichlorophenol was demonstrated in dialyzed extracts and redissolved acetone powders of light-grown cotton leaves, stems, petioles, and roots. The enzyme is also highly active in leaves of several other species of Gossypium and races of *Gossypium hirsutum*.

Aqueous extracts of cotton leaves, stems, roots, flowers, and bolls also contain a heat-stable, dialyzable substance which inhibits the enzymic oxidation of IAA. The major effect of the inhibitor is to produce a lag in the initiation of measurable oxygen uptake.

The inhibitor-induced lag is reduced significantly or apparently eliminated by light and riboflavin. Neither light (30–80 ft-c) or riboflavin significantly reduced the lag period alone.

Gossypol and kaempferol both inhibit cotton-leaf IAA oxidase in a manner similar to the natural inhibitor. Inhibition by both is reversed by light and riboflavin. There was no stimulation of IAA oxidase activity with hydrogen peroxide.

## Acknowledgments

We are grateful to Dr. D. R. Ergle for the sample of gossypol used in this study. Dr. J. E. Endrizzi kindly supplied seed for the races of *Gossypium hirsutum* and samples of leaves from the species of Gossypium. The technical assistance of Mrs. Luanne Waters is gratefully acknowledged.

## Literature Cited

1. Addicott, F. T., J. B. Storey, & D. S. Louie. 1955. Auxin, auxin gradient, & leaf abscission in cotton. Proc. Cotton Defoliation Conf. 10: 41–5.
2. Addicott, F. T. & R. Lynch. 1955. Physiology of abscission. Ann. Rev. Plant Physiol. 6: 211–38.
3. Carns, H. R. 1958. Present status of the abscission accelerator from young cotton bolls. Proc. Cotton Defoliation & Physiol. Conf. 13: 39.
4. DeWerth, A. F. & R. E. Odom. 1960. A standard light-weight growing medium for horticultural specialty crops. Texas Agr. Expt. Sta. Misc. Publ. 420: 11 p.
5. Edwards, J. D., Jr. 1958. Total synthesis of gossypol. J. Am. Chem. Soc. 80: 3798–99.
6. Furuya, M., A. W. Galston, & B. B. Stowe. 1962. Isolation from peas of co-factors and inhibitors of indolyl-3-acetic acid oxidase. Nature 193: 456–57.
7. Galston, A. W. 1949. Riboflavin-sensitized photoöxidation of indoleacetic acid & related compounds. Proc. Nat. Acad. Sci. U.S. 35: 10–17.
8. Galston, A. W. 1959. Studies on indoleacetic acid oxidase inhibitor & its relation to photomorphogenesis. In: Photoperiodism & Related Phenomena In Plants & Animals, R. B. Withrow, ed. Amer. Assoc. Adv. Sci., Washington, D. C. pp 137–57.
9. Galston, A. W. & R. S. Baker. 1951. Studies on the physiology of light action. III. Light activation of a flavoprotein enzyme by reversal of a naturally occurring inhibition. Am. J. Botany 38: 190–95.
10. Goldacre, P. L. 1951. Hydrogen peroxide in the enzymatic oxidation of heteroauxin. Australian J. Sci. Research Ser. B. 4: 293–302.
11. Gortner, W. A. & M. Kent. 1953. Indoleacetic acid oxidase & an inhibitor in pineapple tissue. J. Biol. Chem. 204: 593–603.
12. Hacskaylo, J. & H. R. Carns. 1955. Quantitative & qualitative methods of identifying indole compounds. Proc. Cotton Defoliation & Physiol. Conf. 9: 61–3.
13. Herrero, F. A. & W. C. Hall. 1960. General effects of ethylene on enzyme systems in the cotton leaf. Physiol. Plantarum 13: 736–50.
14. Hillman, W. S. & A. W. Galston. 1957. Inductive control of indoleacetic acid oxidase activity by red & near infrared light. Plant Physiol. 32: 129–35.
15. Hoagland, D. R. & D. I. Arnon. 1938. The water culture method for growing plants without soil. Calif. Agr. Expt. Sta. Circ. 347.
16. Lockhart, J. A. 1955. The role of 2,4-dichlorophenol in the destruction of indoleacetic acid by peroxidase. Plant Physiol. 30: 86–8.
17. Maehly, A. C. & B. Chance. 1954. The assay of catalases & peroxidases. In: Methods of Biochemical Analysis, D. Glick, ed. vol I, pp 357–424. Interscience Publ., New York.
18. Mudd, J. B., B. G. Johnson, R. H. Burris, & K. P. Buchholtz. 1959. Oxidation of indoleacetic acid by quackgrass rhizomes. Plant Physiol. 34: 144–148.
19. Mumford, F. E., D. H. Smith & J. E. Castle. 1961. An inhibitor of indoleacetic acid oxidase from pea tips. Plant Physiol. 36: 752–56.
20. Ray, P. M. 1961. The interpretation of rates of indole-3-acetic acid oxidation. In: Plant Growth Regulation, R. M. Klein, ed. Iowa State Univ. Press, Ames. pp 199–203.
21. Royce, H. D., J. R. Harrison, & E. R. Hahn. 1941. Cottonroot bark as a source of gossypol. Oil & Soap 18: 27–9.
22. Sacher, J. A. 1961. An IAA oxidase-inhibitor system in bean pods. I. Physiological significance & source of the inhibitor. Am. J. Botany 48: 820–28.
23. Sacher, J. A. 1962. An IAA oxidase-inhibitor system in bean pods. II. Kinetic studies of oxidase & natural inhibitor. Plant Physiol. 37: 74–82.
24. Stutz, R. E. 1957. The indole-3-acetic acid oxidase of *Lupinus albus* L. Plant Physiol. 32: 31–9.
25. Tang, Y. W. & J. Bonner. 1948. The enzymatic inactivation of indoleacetic acid. II. The physiology of the enzyme. Am. J. Botany 35: 570–78.
26. Waygood, E. R., A. Oaks, & G. A. Maclachlan. 1956. The enzymically catalyzed oxidation of indoleacetic acid. Canad. J. Botany 34: 905–26.
27. Waygood, E. R. & G. A. Maclachlan. 1956. The effect of catalase, riboflavin, & light on the oxidation of indoleacetic acid. Physiol. Plantarum 9: 607–17.