

HYDROGEN PEROXIDE IN THE ENZYMIC OXIDATION OF HETEROAUXIN

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Summary

A hydrogen peroxide-peroxidase system is essential to the enzymic oxidation of indole-3-acetic acid. Catalase and colloidal platinum inhibited strongly such oxidation, and extremely low concentrations of guaiacol competed with the I.A.A. for the I.A.A. oxidase.

Added low concentrations of hydrogen peroxide enhanced the enzymic oxidation of I.A.A. The endogenous hydrogen peroxide does not arise from external flavoprotein oxidations; it probably does not arise as a co-product in the oxidation of I.A.A. itself or of an oxidation product of I.A.A.

2,4-D opposed the effect of added catalase and inhibited pea epicotyl catalase approximately 30 per cent. at $10^{-3}M$ concentration. It had no influence on the peroxidase.

Boiled onion bulb juice strongly inhibited the peroxidase. Boiled pea epicotyl juice did not.

I. INTRODUCTION

An enzyme system from etiolated plant tissues capable of oxidizing and inactivating indole-3-acetic acid (heteroauxin, I.A.A.) has been described by Tang and Bonner (1947, 1948) and studied by Wagenknecht and Burris (1950). In an earlier communication (Goldacre 1949) it was shown that the activity of this system was enhanced by the presence of the synthetic plant growth regulator, 2,4-dichlorophenoxyacetic acid (2,4-D). It was of interest to examine the manner in which this stimulation arose. The possibility of 2,4-D behaving as an artificial redox carrier was considered, but the molecule is rather stable to oxidation and reduction, and no polarographic step could be obtained between applied voltages of +2.00 and -2.00 (*versus* the saturated calomel electrode) under a wide range of pH and with a variety of supporting electrolytes. This has also been the experience of Velstra (1944) using several other plant growth regulators.

The enzyme system is strongly inhibited by a heat-stable substance present in pea epicotyl tissue (Tang and Bonner 1948) and 2,4-D opposes this inhibition. It was suggested (Goldacre 1949) that 2,4-D may act by counteracting the effect of the natural inhibitor. However, it was subsequently found that after removing the inhibitor by precipitation of the enzyme with acetone, 2,4-D still increased the activity of the system. A study was therefore made into the nature of the I.A.A. oxidase system. This paper reports some of the information obtained.

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II. EXPERIMENTAL

(a) *Enzyme Preparations*

Seeds of green peas (variety William Massey) were stored dusted with "Spargon" (tetrahydroxybenzoquinone) to control the growth of moulds. Seeds were soaked in tap water for two hours, sown in sand in flats and incubated at 24°C. in complete darkness. After seven to ten days, when the epicotyls were 10-15 cm. long, they were harvested and ground for three minutes in approximately half their weight of ice-cold distilled water in an ice-jacketed metal Waring Blendor. The juice was squeezed through muslin and filtered in the cold. The filtrate was used as "crude I.A.A. oxidase" in many experiments.

To concentrate the enzyme system and free it from an inhibitor present in the tissue (Tang and Bonner 1948) 0.44 volumes of ice-cold acetone were added to the cold filtrate. The precipitate was centrifuged down, washed twice with cold 40 per cent. acetone, and shaken in a small volume of M/15 phosphate buffer, pH 6.64. This was centrifuged and the supernatant used as "acetone-precipitated" enzyme. Most experiments reported here have been carried out both with crude and acetone-precipitated enzyme.

(b) *Activity Measurement*

The activity of the I.A.A. oxidase was measured by determining the residual I.A.A. concentration after 20 minutes' incubation at 27.5°C. During this time progress curves were essentially linear. I.A.A. was determined by means of the $\text{FeCl}_3\text{-H}_2\text{SO}_4$ reagent of Tang and Bonner (1947). The intense cherry-red colour rises to a maximum optical density and then slowly fades. Not only the rate of development and fading but also the maximum intensity reached depend upon the room temperature (Goldacre, unpublished data). Readings were therefore made at a time after adding the reagent when, according to the room temperature, the colour would be at its maximum intensity. A series of standards was included in every run.

(c) *Oxidation of I.A.A.*

According to Tang and Bonner (1947) each mole of I.A.A. consumes one mole of oxygen and produces one mole of carbon dioxide, the indole nucleus remaining intact. The overall equation fitting these observations is shown in Figure 1.

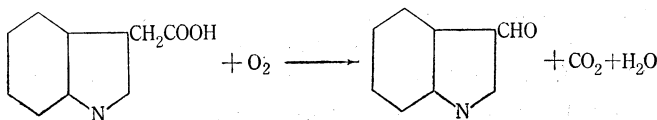
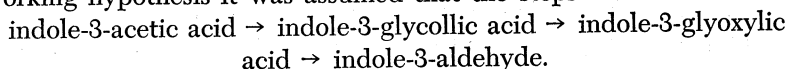


Fig. 1

It is possible to prepare the 2,4-dinitrophenylhydrazone of a neutral, ether-extractable substance which increases in concentration as the reaction proceeds and which is presumed to be indole-3-aldehyde. It is usual for biological

oxidations to proceed by two hydrogen equivalents per step so it is probable that two oxidations and one decarboxylation are involved in this conversion. As a working hypothesis it was assumed that the steps involved are:



Felber (1948) demonstrated cytologically that protruberances induced on bean tissue by treatment with 2,4-D had greater peroxidase activity than untreated tissue. It has also been shown (Goldacre 1949) that 2,4-D increases the *in vitro* activity of I.A.A. oxidase. Further, Tang and Bonner (1947) indicated that the I.A.A. oxidizing system may contain a haem enzyme, and the system is poisoned by peroxidase inhibitors such as cyanide, azide, and hydroxylamine. These facts suggested that one of the oxidation steps in the I.A.A. oxidase system may be a peroxidase. Both crude and acetone-precipitated enzyme preparations do in fact have strong peroxidase activity, and in the presence of 0.05M hydrogen peroxide vigorously oxidized solutions of guaiacol, guaiacum resin, benzidine, pyrogallol, *p*-phenylene diamine, *o*-cresol, *m*-cresol, and *p*-cresol. Pea epicotyl brei gave the red colour of tetraguaiacoquinone on adding guaiacol even in the absence of added hydrogen peroxide.

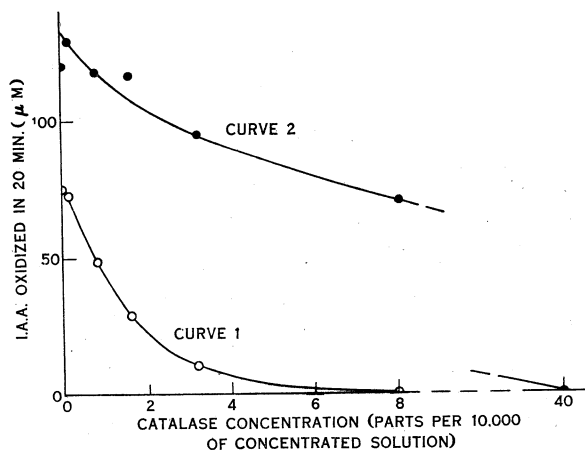


Fig. 2.—Effect of catalase on the I.A.A. oxidase (curve 1) and the influence of 2,4-D on this effect (curve 2). Tubes loaded with 0.5 ml. M/15 phosphate buffer, pH = 6.64; 0.5 ml. crude enzyme; 0.5 ml. 10^{-3} M I.A.A.; catalase; 2×10^{-3} M 2,4-D; and distilled water to a total volume of 2.5 ml. Incubated 20 min. at 27.5°C.

(d) Effect of Hydrogen Peroxide-Consuming Systems

A highly active purified catalase preparation was made from sheep's livers according to Von Euler and Josephson (1927) and had a Katalasefähigkeit (K.f.) value of approximately 4100. This proved to be highly inhibitory to the I.A.A. oxidase system, even in low concentrations. This is shown in Figure 2, curve 1. 2,4-D reversed the inhibitory effect of catalase (Fig. 2, curve 2).

Boiling the catalase solution for 15 minutes to destroy its peroxide-splitting activity also destroyed its inhibiting effect.

In addition, a suspension of colloidal platinum, high in H_2O_2 -splitting activity, was prepared by arcing under distilled water two stout platinum electrodes using 240 volts A.C. and a 1000 watts series resistor in circuit. This suspension also inhibited the I.A.A. oxidase system strongly (Fig. 3).

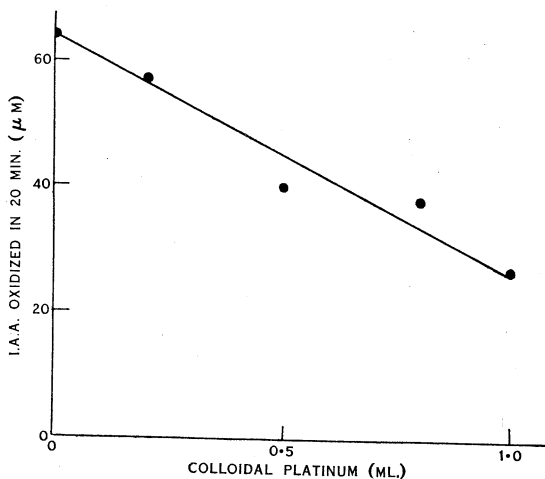


Fig. 3.—Effect of colloidal platinum on I.A.A. oxidase. Colloidal platinum prepared by Bredig's method. Tubes loaded with 0.5 ml. M/15 phosphate buffer, pH = 6.64; 0.5 ml. crude enzyme; 0.5 ml. 10^{-3}M I.A.A.; colloidal platinum as shown; and distilled water to a total volume of 2.5 ml. Incubated 20 min. at 27.5°C . Series of standards contained concentrations of colloidal platinum to take into account photo-absorption due to this substance.

This is strong evidence that hydrogen peroxide, which is specifically and rapidly decomposed by catalase and by colloidal platinum, is essential to the enzymic oxidation of I.A.A. Confirmatory evidence was obtained by observing strong inhibition of I.A.A. oxidation by extremely low concentrations of guaiacol, a substrate specific for peroxidases. The Michaelis-type curve relating activity to substrate (I.A.A.) concentration was converted in the presence of $5 \times 10^{-6}\text{M}$ guaiacol to a sigmoid curve typical of the competitive type of inhibition (Fig. 4). When plotted as reciprocals (Fig. 5) two straight lines were obtained having significantly different slopes, indicating that the $K_s/(S)V$ term of the equation relating reaction velocity to substrate concentration,

$$\frac{1}{v} = \frac{1}{(S)} \cdot \frac{K_s}{V} + \frac{1}{V},$$

where v = observed initial velocity, (S) = substrate concentration, K_s = Michaelis constant, and V = limiting velocity, has been increased and hence that the inhibitor is competing with the substrate.

We may therefore conclude that H_2O_2 -peroxidase system is essential to the enzymic oxidation of I.A.A.

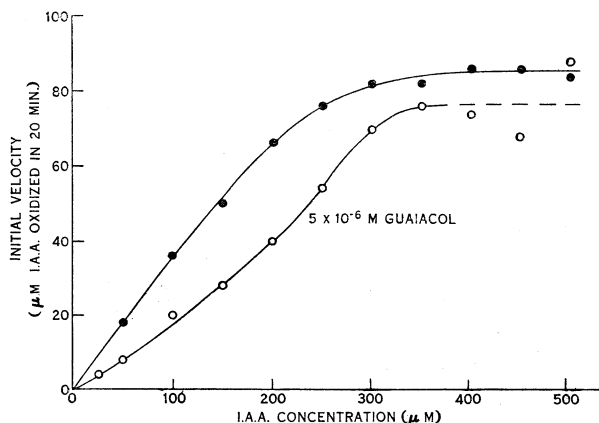


Fig. 4.—Competitive inhibition of I.A.A. oxidase by guaiacol. Tubes loaded with 0.5 ml. M/15 phosphate buffer, pH = 6.64; 0.5 ml. crude enzyme; I.A.A. in concentrations shown; 5×10^{-6} M guaiacol for the curve indicated; and distilled water to a total volume of 2.5 ml. Incubated 20 min. at 27.5°C . Guaiacol in this concentration had no influence on the chemical determination of I.A.A.

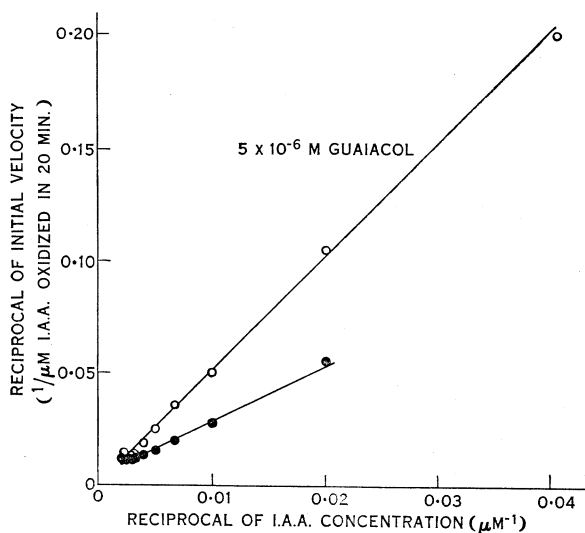


Fig. 5.—The data of Figure 4 plotted as reciprocals.

(e) Effect of Added Hydrogen Peroxide

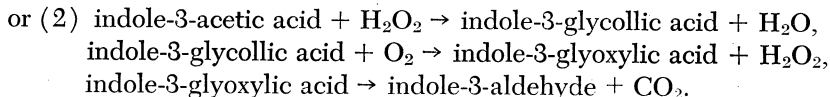
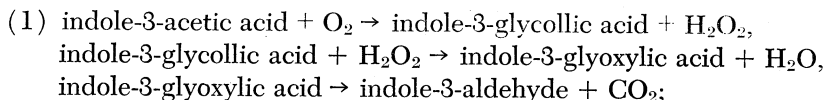
Hydrogen peroxide, like most strong oxidizing agents, rapidly destroys I.A.A. However, at concentrations below 0.02N the non-enzymic oxidation of

I.A.A. during 20 minutes incubation at 27.5°C. is negligible. In the presence of crude pea epicotyl enzyme, the activity was increased 28 per cent. by 0.0014N added H_2O_2 and 61 per cent. by 0.014N added H_2O_2 .

(f) *The Source of Endogenous Hydrogen Peroxide*

Hydrogen peroxide is known to arise from molecular oxygen during oxidation by specific flavoprotein enzymes of substrates such as xanthine, aldehydes, *D*-amino acids, reduced coenzyme 1, uric acid, glucose, etc. The addition of $1.25 \times 10^{-3}\text{M}$ xanthine, $1.25 \times 10^{-3}\text{M}$ hypoxanthine, $1.25 \times 10^{-3}\text{M}$ adenine, 10^{-2}M glucose or $2.5 \times 10^{-3}\text{M}$ *D,L*-alanine to an acetone-precipitated I.A.A. oxidase preparation resulted in no increase in the rate of enzymic oxidation of I.A.A. Benzoate (10^{-2}M), an inhibitor for *D*-amino acid oxidase, showed no inhibition. It appears that no enzyme capable of utilizing these substrates to produce H_2O_2 is present in the preparation, though the preparation has the capacity to oxidize I.A.A. by means of H_2O_2 .

Moreover, one mole of I.A.A. consumes one mole of oxygen and produces one mole of carbon dioxide (Tang and Bonner 1947). Thus if oxygen were first utilized by an external flavoprotein oxidase system to form hydrogen peroxide which in turn oxidized I.A.A., *two* moles of oxygen would be required. The possibility was considered that hydrogen peroxide may be produced in one of the oxidation steps of the I.A.A. oxidase system and subsequently utilized in the other step, e.g.



However, it is known that if I.A.A. is left for long enough in contact with the enzyme it becomes *totally* oxidized. Either of the above schemes would require that hydrogen peroxide formed in one reaction be *quantitatively* available for the second. Owing to the instability of hydrogen peroxide in tissue extracts containing traces of catalase it seems unlikely that this condition should ever be achieved. The origin of the hydrogen peroxide is at present being investigated.

(g) *Effect of 2,4-D on Catalase*

Crude and acetone-precipitated I.A.A. oxidase preparations each contained weak catalase activity. Since hydrogen peroxide is essential to the enzymic oxidation of I.A.A. and since 2,4-D opposes the inhibitory effect of catalase on the system (Fig. 2), it was reasonable to suppose that the accelerating effect due to 2,4-D may be attributed to its inhibition of the catalase present in the I.A.A. oxidase preparations.

This was tested in the following manner. Catalase activity was measured by adding a volume of diluted enzyme to 10 ml. M/15 phosphate buffer, pH

6.8, 25 ml. approximately 0.1N H_2O_2 , addenda, and water to a total volume of 41 ml. in an ice bath at 0°C . At time intervals 4 ml. aliquots were pipetted into 2 ml. 2N H_2SO_4 and titrated with 0.05N KMnO_4 . Typical logarithmic decay curves were obtained. Monomolecular velocity constants were determined for each time interval using the relationship

$$k = \frac{1}{t} \log_{10} \frac{a}{a-x}$$

where a = original concentration of hydrogen peroxide, x = concentration of H_2O_2 decomposed in t minutes, and extrapolated to zero time. This value was used as a measure of the catalase activity.

Using first the catalase concentrate prepared from sheeps' livers, the concentration range in which activity is proportional to enzyme concentration was first established. This is a prerequisite to testing for non-competitive inhibition. Within this range, concentrations of 2,4-D up to $1.25 \times 10^{-3}\text{M}$ produced a maximum of 30 per cent. inhibition.

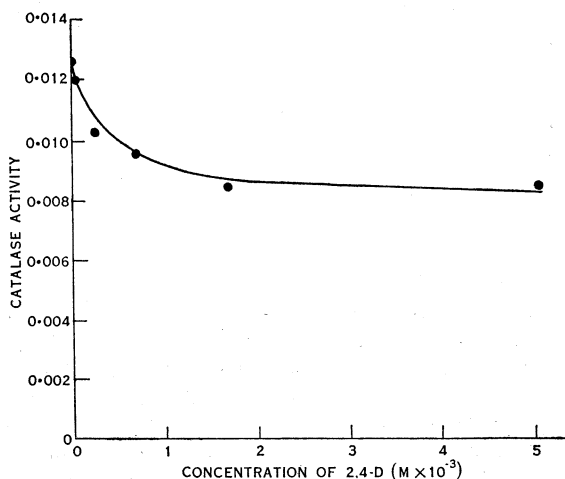


Fig. 6.—Effect of 2,4-D on the catalase activity of the acetone-precipitated I.A.A. oxidase preparation from etiolated pea epicotyls. Catalase activity is given by $\log a/(a-x)$ per min. at zero time, where a = original conc. of H_2O_2 , x = conc. of H_2O_2 decomposed in t minutes.

Repeated with an acetone-precipitated I.A.A. oxidase preparation showing weak catalase activity, very similar results were obtained. Figure 6 shows up to 32 per cent. inhibition for concentrations of $2 \times 10^{-3}\text{M}$ 2,4-D and higher. Increasing the concentration results in no greater inhibition. When catalase activity was measured manometrically under the same conditions of temperature and pH (27.5°C ., pH 6.64) as those under which the stimulation in the rate of I.A.A. oxidation was observed, essentially similar results were obtained.

From this information it cannot be predicted with certainty whether the measured inhibition of catalase by 2,4-D is sufficient to account for the observed increase in the rate of I.A.A. oxidation, or whether 2,4-D may in other ways influence the availability of hydrogen peroxide. Kinetic treatment involves a knowledge of the H_2O_2 concentration during the course of the reaction, and since this work was done, Galston, Bonner, and Baker (1951) indicate that light and possibly other factors may influence the production of H_2O_2 . However, it is clear from Figure 2 that the rate of reduction of I.A.A. oxidase activity with increasing catalase concentration is markedly diminished in the presence of 2,4-D, indicating that this substance in some way makes more H_2O_2 available for the I.A.A. oxidation reaction.

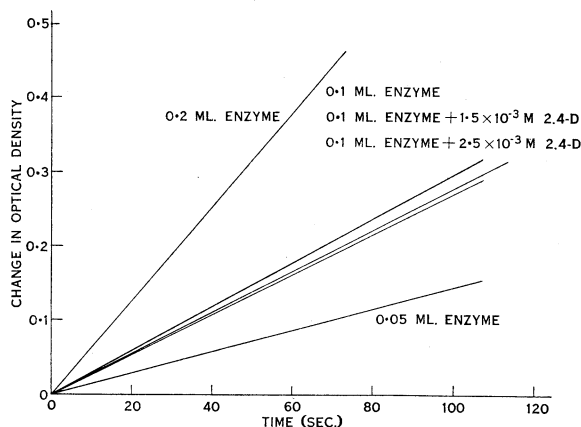


Fig. 7.—Progress curves for activity determination of peroxidase by the method of Ponting and Joslyn (1948). Temperature 19°C . Crude blended pea epicotyls diluted 1 in 10. Spectrophotometer cuvettes loaded with 5 ml. 0.2M acetate buffer, pH 5.0; 1 ml. approximately 0.1N H_2O_2 ; 1 ml. 0.04M guaiacol; the volume of diluted enzyme shown; and water to a total volume of 20 ml. Time rate of increase in optical density measured at 425 $\text{m}\mu$.

(h) Effect of 2,4-D on Peroxidase

Since I.A.A. is oxidized by a non-specific peroxidase capable of oxidizing guaiacol in the presence of H_2O_2 , a convenient way of studying separately the peroxidase fraction of the I.A.A. oxidase system proved to be the method of Ponting and Joslyn (1948), which depends on measuring the time rate of increase in optical density at a wavelength of 425 $\text{m}\mu$, using 0.002M guaiacol and 0.05N H_2O_2 as substrates for the enzyme. Guaiacol becomes oxidized to the red tetraguaiacoquinone. Progress curves were precisely linear for long periods and the slopes of these curves ($\log I_0/I_t$ per sec.) were used as a measure of the reaction velocities.

This method was used to examine whether 2,4-D had any direct stimulatory effect on the peroxidase itself. Using diluted whole pea epicotyl cytoplasm, the range of rate-limiting peroxidase concentrations was determined (Fig. 7), and within this range, concentrations of 2,4-D up to $2.5 \times 10^{-3}M$ had no effect.

However, it should be remembered that in these experiments H_2O_2 is present in excess while the concentration during the *in vitro* oxidation of I.A.A. is very low and possibly at times rate-limiting. Existing methods do not permit the measurement of catalase and peroxidase activity under these conditions.

(i) *Effect of Etiolated Pea Epicotyl Inhibitor and of Onion Bulb Inhibitor on Peroxidase*

It is of interest to determine whether the strong inhibitions produced by substances present in plant tissues (Tang and Bonner 1948; Goldacre 1949) occur at the peroxidase step. Boiled inhibitor solutions were prepared by blending the tissue in a minimum volume of distilled water, boiling for 15 minutes, and filtering. Peroxidase activity was measured by the method of Ponting and Joslyn (1948), working in the rate-limiting range of enzyme concentration.

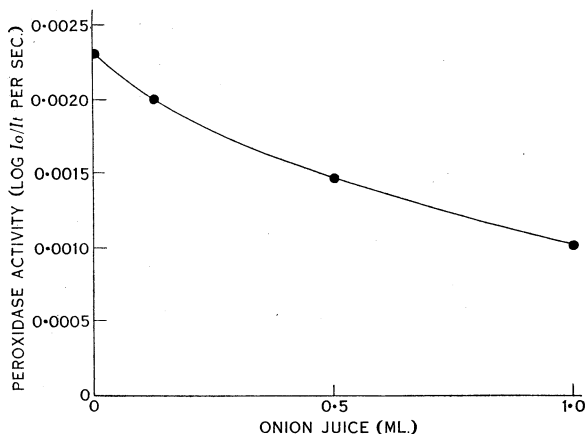


Fig. 8.—Effect of boiled onion juice on the peroxidase. Activity measured by method of Ponting and Joslyn (1948). Crude blended pea epicotyls diluted 1 in 10 (0.1 ml.) were used in a total volume of 20 ml. Temperature 19°C.

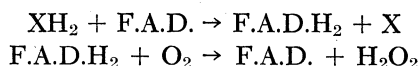
With pea inhibitor, no influence on the activity was obtained with concentration up to 100 times that associated with the enzyme in the tissue. Onion juice, however, produced strong inhibitions, shown in Figure 8.

Thus the inhibitory nature of these two plant extracts is not the same. Pea extracts may be concerned with the hydrogen peroxide-producing reaction while onion juice inhibits the peroxidase.

III. DISCUSSION

It has been shown that hydrogen peroxide-consuming systems strongly inhibit the enzymic oxidation of I.A.A. Catalase and colloidal platinum, which catalyse the dismutation of hydrogen peroxide, depress the rate of oxidation of I.A.A., presumably by reducing the availability of peroxide for this reaction. Guaiacol, a substrate requiring hydrogen peroxide plus peroxidase for its oxidation, produces marked inhibition in a competitive fashion of I.A.A. oxidation. Thus it is concluded that a hydrogen peroxide-peroxidase system is essential to the enzymic oxidation of I.A.A. Added low concentrations of H_2O_2 enhance the rate of oxidation.

The source of the hydrogen peroxide is not known, but reasons have been advanced to show that it does not arise from an external flavoprotein oxidation:



and probably not from the intermediary oxidation of I.A.A. or a product from it. Galston, Bonner, and Baker (1951) present evidence suggesting that peroxide formation is a function of light, and this aspect is now under examination.

2,4-D markedly diminishes the effect of catalase, particularly at relatively higher catalase concentrations (Fig. 2), in some way making available more peroxide for the I.A.A. oxidation reaction. 2,4-D inhibits both liver catalase and pea epicotyl catalase to the extent of about 30 per cent., but it is not yet known whether this is sufficient to account for the effect of 2,4-D on the oxidation of I.A.A. This must await further information on the source and concentration of peroxide in the reaction.

The oxidation products of I.A.A. are not yet known. It seems probable that the end product is indole-3-aldehyde but the intermediates have not yet been isolated or identified; as a working hypothesis indole-3-glycollic and indole-3-glyoxylic acids have been suggested. Nothing is yet known of the decarboxylation reaction.

IV. REFERENCES

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