

Destruction of Indoleacetic Acid

IV. Kinetics of Enzymic Oxidation¹

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Kinetics of enzymic oxidation of indoleacetic acid (IAA) are interpreted as indicating that the reaction has an autocatalytic, cyclical mechanism involving unstable intermediates whose formation and disappearance are important in determining the over-all reaction rate. The kinetics do not support the idea that IAA oxidation occurs mainly by reaction with Mn^{+2} , nor that the reaction is an electron transfer from IAA to O_2 catalyzed by a peroxidase- H_2O_2 - Mn^{+2} complex, nor that Mn is essential to the reaction. H_2O_2 is probably not a major reaction intermediate. One-electron oxidation of IAA by peroxidase giving a free radical, followed by spontaneous reaction of the radical with oxygen to give a peroxy oxidant which can reoxidize the peroxidase to a peroxide complex, appears to be a likely mechanism.

INTRODUCTION

Oxidation of indoleacetic acid (IAA), catalyzed by enzyme preparations containing peroxidase, has been interpreted in terms of cyclical mechanisms involving the utilization and production of hydrogen peroxide or other intermediates (3, 4, 6, 9, 15, 19). This paper presents a kinetic study of the IAA-oxidizing reaction catalyzed by a peroxidase from the fungus *Omphalia flavida*. It considers the principles by which the rate of such a cyclical mechanism is determined and attempts to distinguish between different types of mechanisms which have been proposed for the reaction.

METHODS

The indoleacetic acid (IAA)-oxidizing enzyme of *Omphalia flavida* was prepared as described earlier (13). IAA oxidation was followed spectrophotometrically at 261 $m\mu$ (12), using a Beckman DU spectrophotometer or a Cary recording spectrophotometer equipped with a 0-0.1 optical density

range slidewire. The Salkowski reagent of Gordon and Weber (5) was used to determine IAA where indicated.

Assay for Mn was performed as follows [cf. (14)]. The sample was evaporated and ashed in a quartz crucible at red heat for 1.5 hr. in a muffle furnace. The slight residuc of white ash was taken up and evaporated to dryness three times in 0.3 ml. of conc. HNO_3 , and then dissolved in 0.2 ml. water. Duplicate drops were placed on a spot plate along with duplicate drops of $MnSO_4$ solutions between 10^{-6} and 10^{-5} M. To each, 1 drop of 5% $NaIO_4$ and 1 drop of 2 N acetic acid were added, the plate was shaken to mix the reagents, and after 5 min. 1 drop of 1% *p,p'*-methylenebis-*N,N*-dimethylaniline (Eastman) in acetone was added. A 10^{-6} M solution of $MnSO_4$ gave a detectable blue color immediately. The concentration of Mn in the sample drops was estimated by comparison with the standards.

Enzyme reactions were conducted at room temperature (about 23°C.), in 5 mM sodium citrate buffer, pH 3.7, unless indicated otherwise.

EXPERIMENTAL AND RESULTS

THE INDUCTION EFFECT

In manometric experiments with the *Omphalia* enzyme, a lag of about 5 min. after IAA was added preceded the attain-

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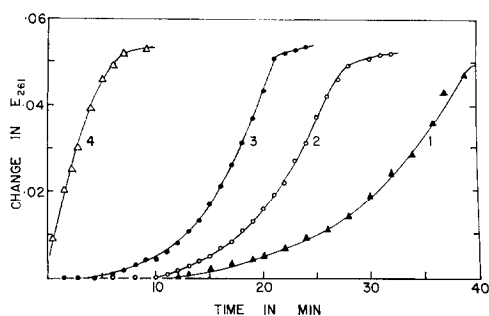


FIG. 1. Effect of enzyme concentration and H_2O_2 on induction phase. To 3 ml. citrate buffer containing $0.137 \mu\text{mole}$ IAA the following amounts of enzyme were added: curve 1, 0.12 ml. ; curve 2, 0.2 ml. ; curves 3 and 4, 0.3 ml. The sample in curve 4 contained, in addition, $8 \mu\text{moles}$ H_2O_2 .

ment of a steady rate of O_2 uptake and CO_2 production. Under the more dilute conditions used in spectrophotometric measurement, this induction phase lasted longer and could be followed more precisely. Figure 1 shows the progress of the reaction at three different enzyme concentrations (curves 1-3). The amount of IAA was small enough that a steady rate had not been reached by the time all the IAA had been oxidized.

The following observations indicate that the induction phase is not due to inhibitors present in the preparation [cf. (6, 10, 11, 18)]: (a) The induction phase was accelerated by increasing the enzyme concentration. (b) Enzyme preparations at all stages of purification (13) showed the induction effect. (c) Addition of boiled enzyme preparations did not extend the induction phase. (d) The induction phase was repeated when more IAA was added to a sample of enzyme after the initially added IAA had been oxidized.

The latter experiment is shown in Fig. 2. IAA oxidation was followed at $288.5 \mu\mu$, where extinction decreases as IAA is oxidized, so it was possible to make measurements after a second addition of IAA. When more IAA was added just before that originally present had been used up (curve 1), a rapid reaction continued; but when IAA was added several minutes after the first reaction had ceased, an induction phase rather similar to that which had occurred

initially was observed (curve 2). Had the first induction phase been due to progressive inactivation of an inhibitor, no induction phase should have been observed when more IAA was added.

The different behavior of samples 1 and 2 in Fig. 2 may be understood by considering that the increase in reaction rate during induction is due to a gradual increase in concentration of reaction intermediates upon which the rate of a cyclical mechanism depends. These intermediates were still present at the time more IAA was added to sample 1, but had largely disappeared by the time it was added to sample 2. The process by which the concentration of rate-controlling intermediates increases will be referred to as *initiation*.

That the reaction rate gradually approaches a steady value can probably be ascribed to processes which limit the concentration of active intermediates which can be attained in the system. There is indication in the experiment of Fig. 2 of reactions leading to the disappearance of these intermediates. When the rate of disappearance becomes equal to the rate of formation of intermediates, a "steady state" of constant IAA oxidation rate should ensue. Reactions leading to loss of intermediates will be called *termination* reactions.

EFFECT OF H_2O_2

In Fig. 1, curve 4 shows what happens if a small amount of H_2O_2 is added to the same system as in curve 3. A rapid reaction occurs immediately and proceeds to completion without increase in rate. H_2O_2 either is, or is convertible into, one of the reaction intermediates. Figure 3 shows how the initial reaction rate depended upon concentration of added H_2O_2 . The rate increased rapidly up to about $5 \times 10^{-6} M$, above which it reached a plateau and can be said to be saturated in respect to H_2O_2 . Rate-saturating concentrations of H_2O_2 caused negligible destruction of IAA in the absence of the enzyme.

The complex spectroscopic changes which accompany IAA oxidation (12) were closely similar in the presence and absence of added H_2O_2 . The amount of IAA destroyed when

H_2O_2 was added was many times greater than the amount of H_2O_2 added (see below), and the reaction required O_2 and consumed it stoichiometrically (12, 13). Therefore, the rapid reaction which occurs when H_2O_2 is added is not due to a distinct "peroxidative" reaction but is simply an increase in the reaction rate. Saturating amounts of H_2O_2 raise the rate to several times the steady-state value. However, amounts of H_2O_2 much in excess of saturation are inhibitory.

It was shown previously (13) that the IAA-oxidizing enzyme of *Omphalia flavida* is a peroxidase. Therefore, added H_2O_2 most likely promotes IAA oxidation by reacting with the enzyme. The H_2O_2 saturating rate is then determined by the rate of the enzyme-catalyzed step when the enzyme is saturated with H_2O_2 , the enzyme not being saturated in the steady state.

Addition of catalase inhibited IAA oxidation, as measured manometrically (steady state), but rather large amounts were required. For example, 0.4 mg. of Armour 30 catalase slowed the induction phase and reduced the steady state Q_{O_2} from 312 μ l./hr. to 161 μ l./hr. using 1 ml. of *Omphalia* enzyme and 12.8 μ moles IAA at pH 3.7 (catalase activity was shown to be stable under these conditions). The same amount of boiled catalase had no effect. However, much larger amounts of catalase did not completely suppress IAA oxidation, as one might have expected were H_2O_2 a reaction intermediate.

Although the results with catalase suggest that H_2O_2 may be involved in the reaction to some extent, neither they nor the promotive action of H_2O_2 itself allow one to conclude that H_2O_2 is a major reaction intermediate.

ENZYME AND SUBSTRATE CONCENTRATION

Both in the steady state and in the presence of a saturating amount of H_2O_2 , the relation between reaction rate and enzyme concentration was somewhat less than linear, but considerably more than in proportion to square root of enzyme concentration.

In the presence of added H_2O_2 , the reac-

tion rate was quite independent of IAA concentration down to about 4×10^{-5} M. In the absence of added H_2O_2 , increases in IAA above this concentration had a small effect on the reaction rate, as seen spectro-

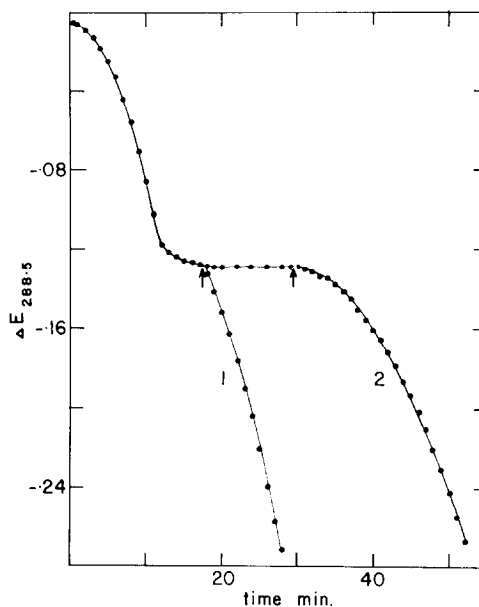


FIG. 2. Induction effect after cessation of IAA oxidation. At the start 0.2 ml. enzyme solution was added to 0.27 μ mole IAA in 3 ml. citrate buffer. At the arrows, 0.27 μ mole more IAA was added to cuvette 1 or 2. Optical density corrected for change upon adding IAA. Under the conditions, 288.5 $m\mu$ was a wavelength at which extinction ceased to change when the IAA was used up, so that IAA oxidation could be followed without interference from secondary reactions (12).

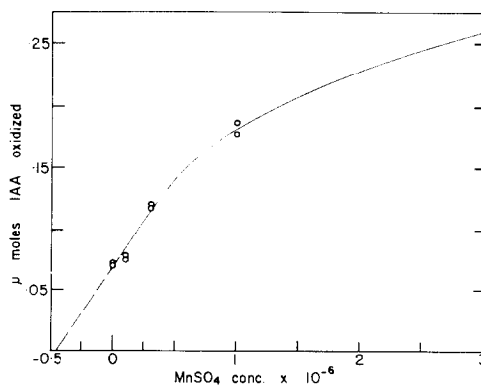


FIG. 3. Effect of added H_2O_2 on rate of IAA oxidation. Initial rate of increase in optical density at 261 $m\mu$ shown on ordinate.

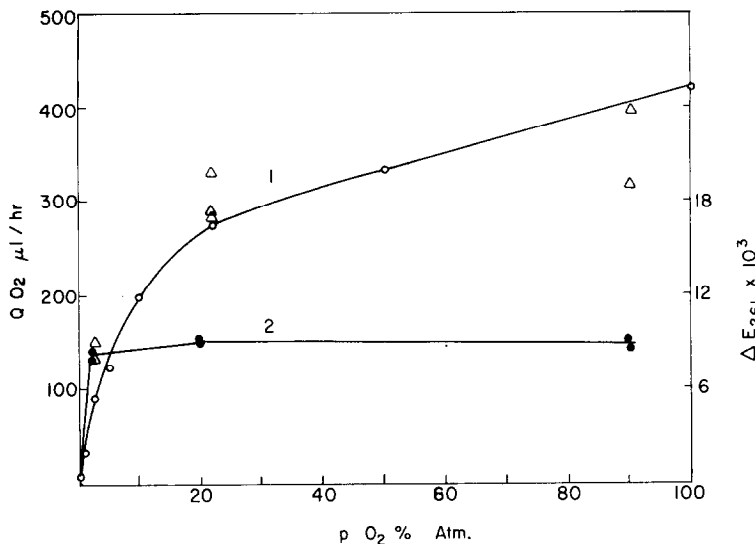


FIG. 4. Effect of oxygen concentration on IAA oxidation. Curve 1 (open circles, left-hand ordinate), Q_{O_2} measured manometrically (19 μ moles IAA, 2.2 ml. of 0.05 M citrate containing 1 ml. enzyme). Triangles, increase in $E_{261} \times \frac{1}{2}$ (right-hand ordinate) in first 15 min. of induction phase (0.3 μ mole IAA, 0.1 ml. enzyme, in 3 ml. of 5 mM citrate). Curve 2 (right-hand ordinate), increase in E_{261} per min., under conditions similar to the preceding, but 15 $m\mu$ moles H_2O_2 added. Vessels gassed with appropriate mixtures of O_2 and N_2 before addition of enzyme.

photometrically. In manometric experiments strong dependence of reaction rate on IAA at much higher concentrations was usually observed. Although the results of some experiments corresponded well with Michaelis-Menten kinetics, different experiments were not quantitatively consistent, and it is felt that the manometric results do not afford a measure of the "affinity" of the system for IAA. They may be due to sec-

ondary effects upon the steady state, or to effects on initiation.

The pronounced effect of oxygen concentration on the steady state, measured manometrically, is shown in curve 1 of Fig. 4. Increase in oxygen concentration accelerated the rate also under the conditions used in spectrophotometric measurement (triangles in Fig. 4), but the effect was not as strong. Curve 2 of Fig. 4 shows that the reaction rate was independent of oxygen concentration over a wide range when a saturating concentration of H_2O_2 was added, even though oxygen was required.

DISAPPEARANCE OF ADDED H_2O_2

When an amount of H_2O_2 is added which gives a rate greater than the steady-state value, the initially rapid rate declines with time. This appears to be due to loss of H_2O_2 from the system, for the rapid rate can be restored by adding more H_2O_2 . In Fig. 5 the sample in curve 1 received no H_2O_2 ; that in curve 3 received an amount several times in excess of saturation; while that in curve 2 received slightly less than the saturating amount. Its rate fell gradually to

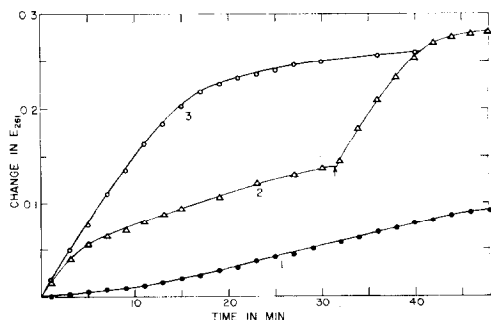


FIG. 5. Changes in reaction rate after H_2O_2 is added. Amounts added initially: curve 1, no H_2O_2 ; curve 2, 10 $m\mu$ moles H_2O_2 ; curve 3, 50 $m\mu$ moles H_2O_2 . At arrow, 50 $m\mu$ moles H_2O_2 added to sample 2.

nearly the same value as was reached, after induction, in curve 1. Addition of more H_2O_2 at the arrow brought back the saturating rate.

The behavior noted in Fig. 5 would be expected if the steady value which the rates in curves 1 and 2 both approach represented, as suggested previously, a "steady state" which arises when the rate of formation of active intermediates (initiation) is equal to the rate at which they are disappearing (termination). Above this reaction rate, termination exceeds initiation, and below it initiation exceeds termination. Because the loss of H_2O_2 observed when it is added may be caused by the same termination process, it was thought important to examine the loss of H_2O_2 quantitatively.

The rate at which H_2O_2 disappeared when IAA oxidation was proceeding at H_2O_2 saturation was determined by the kind of experiment shown in Fig. 6. The more the amount of H_2O_2 which was added, the longer the saturating reaction rate continued. The point at which the rate began to decline rapidly can be considered the time at which the H_2O_2 concentration had fallen below the saturating value. By dividing the amount of IAA destroyed in the interval between the bends in curves 1 and 2 by the difference in amount of H_2O_2 added to the two samples, we obtain the relative rates of IAA and H_2O_2 disappearance. This ratio was approximately 9 in the experiment of Fig. 6.

Table I shows the influence of varying the enzyme concentration upon the ratio of IAA oxidation to H_2O_2 disappearance. Several-fold differences in enzyme concentration and reaction rate had very little effect on this ratio. This fact is useful in arriving at a kinetically suitable termination process.

KINETICS OF THE REACTION COURSE

During the early part of the induction phase, for example in Fig. 1, there was indication that acceleration increased as the reaction rate itself increased. This suggested that the initiation process depended upon the IAA-oxidizing reaction, so that the reaction as a whole is autocatalytic.

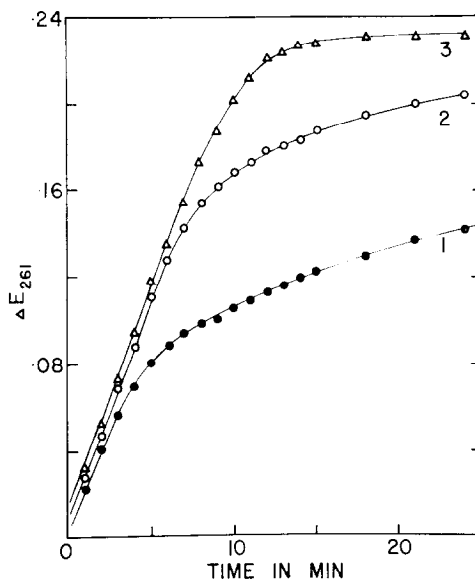


FIG. 6. Method for measuring loss of H_2O_2 during reaction. Enzyme and $0.53 \mu\text{mole}$ IAA in 3 ml. containing: curve 1, 20 $\text{m}\mu\text{moles}$ H_2O_2 ; curve 2, 40 $\text{m}\mu\text{moles}$ H_2O_2 ; curve 3, 60 $\text{m}\mu\text{moles}$ H_2O_2 . In sample 3 reaction ended when all IAA was oxidized.

The steady state which follows the induction phase could be the result of termination increasing more than in proportion to initiation as the reaction rate increases, until the rate of termination becomes equal to the rate of initiation and the concentration of active intermediates becomes constant. It is interesting to consider the kinetics of a reaction whose steady state is determined in this manner.

There are reasons for thinking that some of the intermediates in IAA oxidation may be free radicals. In this case the principal

TABLE I
EFFECT OF ENZYME CONCENTRATION ON RATIO OF
IAA OXIDATION TO H_2O_2 DISAPPEARANCE

Expt. No.	Enzyme soln.	Initial ΔE_{261} min.	$\frac{\Delta \text{IAA}}{\Delta \text{H}_2\text{O}_2}$
	<i>ml.</i>		
1	0.33	.013	9.7
	0.90	.029	9.0
2	0.33	.011	10.7
	1.00	.031	10.4

termination process may well be recombination of radicals, which would be of the second order.

We shall represent with $[Z]$ the concentration of active intermediates upon which the reaction rate depends, and assume that IAA oxidation (governed by k_1) and initiation (governed by k_2) are proportional to $[Z]$; k_3 is the rate coefficient for termination. The difference between initiation and termination is $d[Z]/dt = k_2[Z] - k_3[Z]^2$. This can be integrated to give

$$[Z] = \frac{k_2 e^{k_2 t}}{k_3 e^{k_2 t} + k_3 D} \quad (1)$$

The factor D is $(k_2 - k_3[Z_0])/k_3[Z_0]$, in which $[Z_0]$ is the concentration of active intermediates at zero time.

Since the reaction rate is practically independent of IAA concentration ($[S]$), it may be written $d[S]/dt = -k_1[Z]$. Substitution for $[Z]$ and integration leads to

$$[S_0] - [S] = \frac{k_1}{k_3} \ln(e^{k_2 t} + D) + \text{const.} \quad (2)$$

This expression has the form $a \ln(b +$

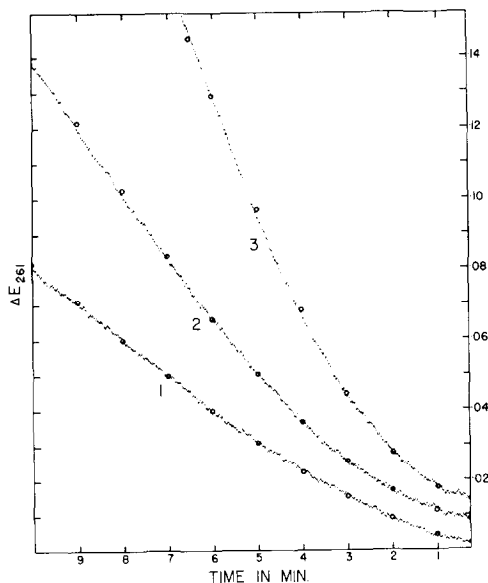


Fig. 7. Induction phase and steady state. Cary spectrophotometer records made with $0.46 \mu\text{mole}$ IAA in 2.7 ml. of $0.05 M$ citrate, pH 3.7. Curve 1, 0.45 ml. enzyme; curve 2, 0.9 ml.; curve 3, 1.8 ml. Circles are points calculated as described in text.

TABLE II

VALUES OF COEFFICIENTS IN EQ. (9)

Figures are in optical density units ($261 \mu\mu$), and minutes, and apply to data of Fig. 11.

Expt. No.	Enzyme	Steady state rate	a	b	c
	ml.	min. ⁻¹			
1	0.45	.011	.025	2.8	.46
2	0.90	.019	.031	6.3	.62
3	1.80	.034	.035	10.3	.95

e^{ct}) + const. One can determine whether Eq. (2) is consistent with the reaction kinetics by seeing whether values for a , b , and c can be found which reproduce the experimentally observed reaction course. Figure 7 shows records of the induction phase and steady state obtained at three enzyme concentrations. The circles shown in this figure represent values of Eq. (2) computed for each curve using the coefficients listed in Table II, which were obtained by a trial and error method. A rather close fit with the experimental curves was obtained, suggesting that Eq. (3) may come near to describing the actual kinetics. While this does not prove that the reaction rate is determined in the manner suggested, it does seem striking that such a close correspondence with expectation could be obtained.

KINETIC ROLE OF THE ENZYME

Acceleration of the induction phase and steady state by the enzyme nearly in proportion to its concentration could be due to catalysis either of initiation or of the main oxidation. A direct test of whether the enzyme catalyzes initiation or the main reaction can be made by allowing the reaction to proceed through induction into the steady state, and then increasing the enzyme concentration by adding more enzyme. It can be shown from Eq. (2) that if changing the enzyme concentration affects k_2 , the rate coefficient for initiation, and not k_1 , the rate coefficient for the main reaction, then the time required for the reaction rate to increase to a new steady-state value (for example, to double) should be similar to the time taken for the rate to increase comparably (to double) during the later part

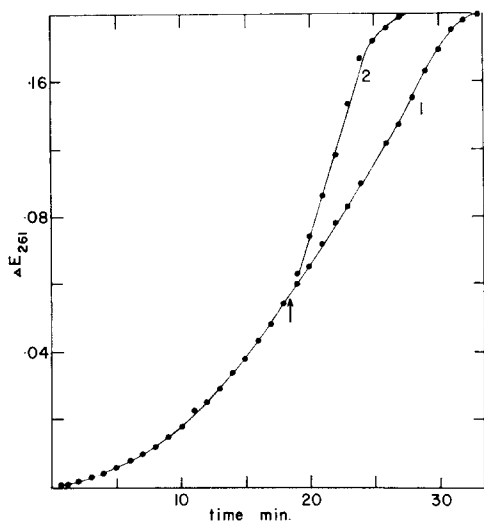


FIG. 8. Effect of changing the enzyme concentration. Induction was followed using 0.1 ml. enzyme in 3 ml. buffered 10^{-4} M IAA. At arrow, 0.1 ml. more enzyme added to sample 2.

of the induction phase. If k_1 is increased, on the other hand, the increase in reaction rate should be immediate.

This experiment, shown in Fig. 8, gave the latter result. It thus appears that the enzyme catalyzes the main reaction, and not initiation.

MANGANESE AND PHENOLS

With the purified *Omphalia* enzyme, Mn^{+2} did not promote the reaction unless a monophenol such as resorcinol or 2,4-dichlorophenol was also added. Figure 9A shows that when H_2O_2 was not added, neither Mn nor phenol promoted by itself, but strong promotions could be obtained by combinations of the two. Promotive combinations accelerated both in the induction phase and in the steady state.

By contrast, when the effects of Mn and phenols were tested in the presence of a rate-saturating concentration of H_2O_2 , it was found (Fig. 9B) that the phenol promoted substantially by itself, while Mn did not promote at any phenol concentration but had only an inhibitory action.

The concentration range in which 2,4-dichlorophenol gave promotion depended upon pH. As shown in Fig. 10, the optimal con-

centration was about two orders of magnitude lower at pH 6.5 than at pH 3.7.

That promotion by Mn is obtained only in the induction phase and steady state, and not at H_2O_2 saturation, suggests two possible explanations:

(a) Mn^{+2} promotes the reaction with O_2 , or some further step in the regeneration of the primary oxidant, which is not rate-limiting at H_2O_2 saturation. Such explanations have been proposed previously (7, 15, 19).

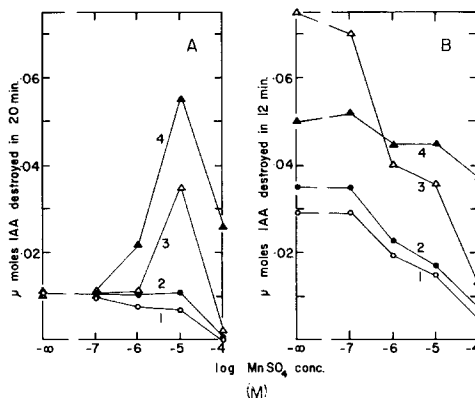


FIG. 9. Effects of Mn^{+2} and 2,4-dichlorophenol without (A) and with (B) added H_2O_2 . Samples contained 90 μ moles IAA and 5 μ l. enzyme in 1 ml. of 0.05 M citrate pH 3.7; in Fig. 9B, also 6 μ moles H_2O_2 . Concentrations of 2,4-dichlorophenol: curve 1, none; curve 2, 10^{-5} M; curve 3, 10^{-4} M; curve 4, 8×10^{-4} M. IAA determined with Salkowski reagent.

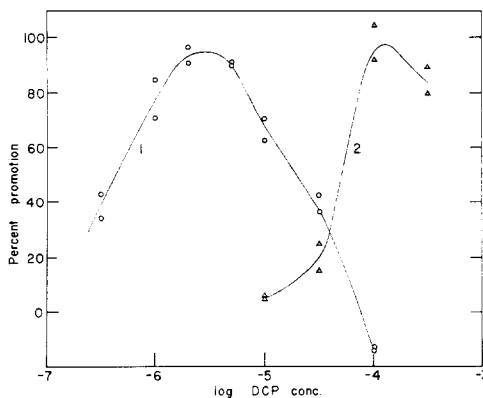


FIG. 10. Effect of 2,4-dichlorophenol in presence of H_2O_2 . Curve 1, in 0.05 M sodium phosphate, pH 6.5. Curve 2, in 0.05 M sodium citrate, pH 3.7.

(b) Mn^{+2} promotes initiation; the reaction rate should not depend upon initiation when the enzyme is H_2O_2 -saturated. Optimal Mn-phenol combinations raised the steady-state rate nearly to that which could be obtained by adding H_2O_2 ; this suggests that they increase the concentration of active intermediates nearly to the H_2O_2 saturation level, by promoting initiation.

Maclachlan and Waygood (9) interpreted the promotive effects of Mn and phenols on enzymic IAA oxidation, and other evidence, as indicating that IAA is oxidized not by the enzyme but by Mn^{+3} , which is formed as a result of oxidation of a phenol by peroxidase. Kinetic analysis of the reaction sequence they proposed indicates that if it were operating, addition of Mn^{+2} could promote in the steady state but not at H_2O_2 saturation if and only if the concentration of phenol were large compared with the concentration of peroxy intermediate required to give saturation of the enzyme.

Since phenol concentrations effective in bringing on promotion by Mn at pH 3.7 (Fig. 9) were large compared with the rate-saturating concentration of H_2O_2 , it was felt that the Mn oxidation proposal could be tested more critically by examining the effect of Mn in the presence of 10^{-6} M 2,4-dichlorophenol at pH 6.5. This low phenol concentration, less than the 2×10^{-6} M

H_2O_2 required for rate saturation, made possible large promotions by 10^{-6} and 10^{-5} M $MnSO_4$ in the absence of added H_2O_2 at pH 6.5. However, not the slightest promotion was obtained, when H_2O_2 was present, by any Mn^{+2} concentration between 10^{-7} and 10^{-3} M.

Therefore, the kinetics of Mn^{+2} action on the present system do not support the mechanism proposed by Maclachlan and Waygood (9). However, it seems possible that some of the reactions they suggested may be involved in the promotive effect of Mn^{+2} on initiation.

One can test kinetically whether Mn is essential to the reaction, by whatever mechanism. If Mn were essential, then the reaction which is observable in the absence of added Mn must be due to endogenous Mn in the system, and a concentration of added Mn^{+2} which promotes only slightly must be presumed to be less than the endogenous.

No Mn was detected either in the enzyme preparation, the IAA, or the citrate buffer, by spectrographic analyses performed through the courtesy of Dr. C. A. Price and Dr. B. L. Vallee.

In the following experiment the effect of added Mn^{+2} was compared directly with the Mn content of a particular reaction system, assayed by a very sensitive but less specific method. As shown in Fig. 11, an insignificant promotion was obtained at 10^{-7} M $MnSO_4$, and the apparent endogenous Mn concentration, assuming Mn is essential, appears from back extrapolation of the data to have been about 4×10^{-7} M. A 225-ml. sample of the same reaction components was evaporated, ashed, and tested for Mn by the procedure described under *Methods*. The color reaction obtained corresponded to $1-2 \times 10^{-6}$ M Mn in the final 0.2-ml. sample or to $1-2 \times 10^{-9}$ M in the original reaction mixture.

Therefore, the endogenous Mn concentration was much too low to account for the threshold for promotion by added Mn; one must conclude that the reaction in the absence of added Mn does not depend upon endogenous Mn and that Mn is not essential.

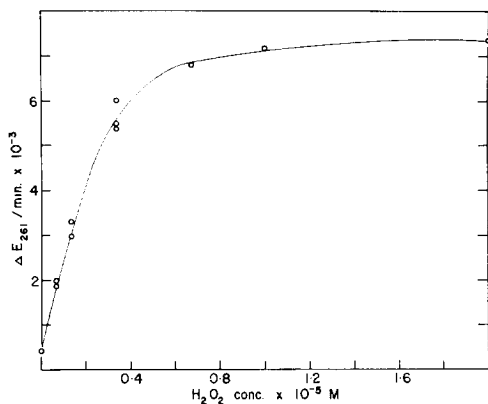


Fig. 11. Determination of threshold for Mn^{+2} promotion. Samples contained 0.3 ml. enzyme, $0.45 \mu\text{mole}$ IAA and $0.5 \mu\text{mole}$ 2,4-dichlorophenol in 5 ml. of 5 m.M ammonium citrate buffer pH 3.7. IAA determined by Salkowski assay after 12 min. of reaction.

DISCUSSION

The bearing of the kinetics on the principal types of mechanisms which have been suggested for IAA oxidation and related oxygen-consuming reactions catalyzed by peroxidases, other than the Mn oxidation hypothesis considered in the last section, will now be discussed. These are:

(a) Coupled action of a peroxidase and an oxidase, the latter forming H_2O_2 (or some other peroxide) from O_2 , and the former using this peroxide as substrate (3, 15).

(b) One-electron oxidation of IAA by peroxidase giving a radical, which reacts with oxygen non-enzymically to regenerate H_2O_2 (19).

(c) Oxygen-consuming reaction catalyzed by the H_2O_2 complex of peroxidase, activated by Mn^{+2} (1, 2).

(d) Oxidation by addition of O_2 , catalyzed by ferrous peroxidase, and not involving H_2O_2 (formation of ferrous from ferric peroxidase "activates" the enzyme and depends upon H_2O_2) [cf. tryptophan pyrrolase (16)].

The effects observed with Mn^{+2} are considered inconsistent with the type of mechanism proposed in (c). According to it, Mn^{+2} should interact with the H_2O_2 complex of peroxidase to bring about the oxygen-consuming reaction, whereas Mn^{+2} did not promote when H_2O_2 was added, even though the oxygen-consuming reaction occurred. The effect of H_2O_2 on the oxygen kinetics is also contrary to (c).

The idea (a) that the reaction depends upon more than one enzyme (oxidase and peroxidase), or on different catalytic centers of one enzyme which act at different steps in the reaction sequence, is not very attractive for several reasons. In the kinetics of such reaction sequences the rate coefficients for successive steps are generally to be multiplied, so that if the enzyme acted at two steps the reaction would be expected to be proportional to the square of the enzyme concentration, which it definitely is not. Further, no evidence of a requirement for enzyme activity different from peroxidase was detected in purification and thermal inactivation experiments (13).

That oxygen-consuming oxidation of IAA need not involve reaction of O_2 with any enzyme is shown by the initiation of such oxidation by Mn^{+3} (8) and by nitrite (17) in the absence of enzymes.

The other two suggested mechanisms (b) and (d) contrast sharply, for (b) presumes a peroxidative action of the enzyme and the existence of peroxy intermediates, whereas in (d) the features of the reaction which have been ascribed above to the occurrence of catalytic intermediates must be interpreted at least in part in terms of the appearance of an "activated" (ferrous) form of the enzyme, and H_2O_2 is supposed not to be a reaction intermediate. The principal kinds of evidence (16) supporting mechanism (d) for tryptophan pyrrolase were: (i) catalase inhibited much more strongly at the start than when the reaction was under way; (ii) HCN inhibited strongly at first, but if added later caused only gradual inhibition, suggesting that the ferric form of the enzyme was no longer present; (iii) the ferrous enzyme was formed in the presence of tryptophan and H_2O_2 .

While there was evidence in previously reported experiments (13) that a different (possibly ferrous) form of the IAA-oxidizing enzyme might be appearing in the presence of IAA, it is difficult to exclude the possibility that the results were due to experimental treatment (polyphenols or CO) rather than a reflection of the IAA oxidation mechanism. Cyanide ($10^{-3} M$) caused complete inhibition within 1 min. when added after the start of the reaction [(12), Table II].

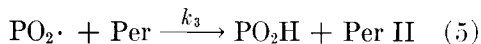
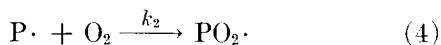
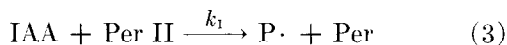
In manometric experiments it was found that catalase inhibited IAA oxidation more strongly when added initially than after the steady state had been reached. The difference was not as great, however, as found for tryptophan pyrrolase (16); for example, 0.5 mg. catalase inhibited 45% when added initially and 13% when added after 20 min.

We do not consider the results with catalase to be evidence against the existence of a peroxy intermediate other than H_2O_2 . They do suggest that H_2O_2 itself is not a major intermediate. It was not found possible to derive kinetics consistent with those

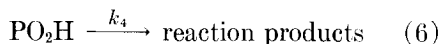
observed, on the assumption that H_2O_2 is a principal intermediate.

Since Yamazaki [(19) and unpublished experiments] has demonstrated single electron oxidation of IAA by peroxidase and the resulting formation of free radicals, and since the existence of free radical intermediates makes explicable a variety of properties of the system, as well as its kinetics, we favor the type of mechanism indicated under (b). If special forms of the enzyme are involved, we presume that their formation and disappearance is included in the kinetic description of the system discussed above.

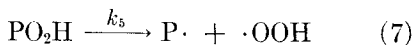
The following reaction sequence of type (b) would exhibit kinetics comparable with those observed in IAA oxidation, and is presented as a possible mechanism. Per represents peroxidase and Per II its peroxide complex II.



PO_2H is assumed to be an unstable hydroperoxide:

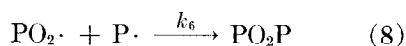


but it also dissociates to some extent to yield radicals which can participate in the reaction, thus leading to initiation:



The peroxy radical may disappear by side reactions, may react with the enzyme to give a peroxide complex, or may react with IAA to give $\text{P}\cdot$ and H_2O_2 ; any H_2O_2 formed will react with the enzyme to give a peroxide complex. These reactions result in initiation, and the possibility that H_2O_2 participates to some extent is thus apparent. However, to simplify analysis we assume that these reactions are relatively unimportant in initiation compared with reaction (7) followed by reaction (4).

The principal termination process is assumed to be



Reactions (3–8) can be shown to give the steady-state kinetics

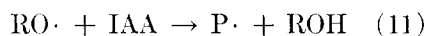
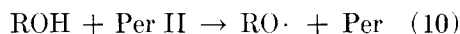
$$v = \frac{k_1 k_2 k_3 k_5 \cdot f(\text{IAA}) [\text{O}_2] [\text{E}]}{2k_1 k_4 k_6 \cdot f(\text{IAA}) + k_2 k_3 k_5 [\text{O}_2]} \quad (9)$$

in which [E] represents the concentration of peroxidase, and $f(\text{IAA})$ the dependence of reaction (3) on IAA concentration.

When the second term in the denominator of Eq. (9) is large compared to the first term, the rate is limited by reaction (3). This corresponds to the situation under H_2O_2 saturation, when almost all the peroxidase has been converted to Per II. In the ordinary steady state, in which the rate is considerably less, the second term cannot be large compared with the first term.

Although Eq. (9) corresponds pretty well with the observed kinetics, it is doubtful (but difficult to test) whether increasing the O_2 concentration would raise the rate to the H_2O_2 saturating value. However, this could easily be due to the occurrence of other termination reactions, for example between two $\text{PO}_2\cdot$ radicals.

Promotion by phenol under H_2O_2 saturation suggests that the following reaction sequence, in which ROH represents the phenol and $\text{RO}\cdot$ its oxidation product,



goes more rapidly than reaction (3).

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