The Destruction of Indoleacetic Acid. III. Relationships between Peroxidase Action and Indoleacetic Acid Oxidation

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Received July 2, 1959

The indoleacetic acid (IAA)-oxidizing enzyme preparation from *Omphalia flavida* exhibits peroxidase activity on pyrogallol with an optimum at pH 3.5–3.7, like that for the IAA-oxidizing activity. It shows no polyphenolase activity. Peroxidase and IAA-oxidizing activities run parallel both during thermal inactivation and in course of purification of the enzyme, and are therefore considered to be due to one enzyme.

IAA inhibits peroxidation of pyrogallol by the enzyme, 50% inhibition being obtained at about $7 \times 10^{-6}\,M$. The inhibition appears to be specific to substrates of the oxygen-consuming activity of the enzyme. IAA destruction is inhibited at the same time, even though an excess of H_2O_2 is present. IAA is not destroyed at an appreciable rate in the absence of O_2, even if substrate amounts of H_2O_2 are present, whereas pyrogallol peroxidation is not influenced by the absence of O_2. IAA oxidation is light-reversibly inhibited by CO; the inhibition is affected by O_2 concentration. A possible interpretation of the results is that the reaction depends upon a free-radical mechanism involving the formation of ferrous peroxidase.

INTRODUCTION

The involvement of peroxidase in oxidation of indoleacetic acid (IAA) by extracts of plants was suggested by Goldacre (5). IAA-oxidizing activity has since been found generally to be associated with the presence of peroxidase. The principal exception appears to be the IAA-oxidizing activity in preparations from certain fungi such as *Polyporus versicolor* (1, 29) and *Lactarius* spp. (11, 12), in which it is thought that polyphenoloxidases (laccases), rather than peroxidase, are responsible for IAA oxidation. The present paper concerns the IAA-oxidizing enzyme of cultures of the fungus *Omphalia flavida* (*Mycena citricolor*), which is shown to depend upon peroxidase activity.

Existing views about the mechanism of peroxidase-catalyzed oxidations of IAA are rather widely divergent. Galston, Bonner,

and Baker (3, 4) concluded that IAA oxidation in pea homogenates results from the combined action of a peroxidase and a flavine enzyme, the latter enzyme reacting with oxygen to produce hydrogen peroxide, which is utilized by the former enzyme. This general type of reaction sequence can be formulated

$$IAA + H_2O_2 \xrightarrow{\text{peroxidase}} P_1 + 2H_2O \quad (1)$$

$$P_1 + O_2 \xrightarrow{\text{flavine}} P_2 + H_2O_2 \quad (2)$$
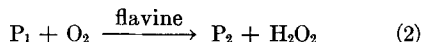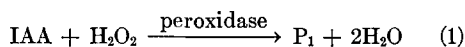
The involvement of a flavine enzyme was questioned by Kenten (9), who observed rapid IAA oxidation by purified horse-radish peroxidase preparations, and could find no evidence that a flavine was present. Stutz (24, 25) suggested that the purified IAA-oxidizing enzyme from *Lupinus albus* possesses separate peroxidase and dehydrogenase-like centers, the action of which is coupled through peroxides.

A major line of evidence for the occurrence of reaction (1) has been the inhibitory effect of polyphenols upon IAA oxidation, since

these are typical peroxidase substrates and would be expected to compete with IAA for H_2O_2. Only Platt (14a) has reported direct evidence for the occurrence of reaction (1). Waygood, Oaks, and Maclachlan (14, 31) concluded that in IAA oxidation a peroxide other than H_2O_2 serves as H acceptor for peroxidase, and that polyphenols inhibit by reacting with other components of the system than peroxidase. They formulated the reaction as an oxidation of IAA by Mn^{3+} generated indirectly by peroxidase, and obtained evidence that oxygen uptake results from a reaction between O_2 and radicals formed by the initial oxidation of IAA. This would mean that neither IAA nor O_2 reacts with an enzyme during IAA oxidation.

Yamazaki and Souzu (32) recently obtained evidence for the occurrence of the reaction

$$IAA + \tfrac{1}{2}H_2O_2 \xrightarrow{\text{peroxidase}} P\cdot + H_2O \quad (3)$$
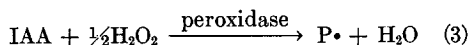
in which strongly reducing radicals (P·) are formed. He considered that H_2O_2 is probably regenerated in a reaction between P· and O_2, which would occur spontaneously.

The present experiments were conducted to investigate the relationship between peroxidase activity and IAA oxidation in *Omphalia* enzyme preparations. Attention was given to the question of how polyphenols inhibit, and indications were obtained that IAA reacts directly with peroxidase, although not in a simple peroxidation like Eq. (1). Inhibition by carbon monoxide was also studied.

MATERIALS

Enzymic reactions were conducted in sodium citrate buffer, pH 3.7, unless indicated otherwise. Buffers were prepared by adjusting a solution of the acid to the desired pH with NaOH. IAA (Hoffmann-LaRoche Ltd.) was dissolved in water by adding an equivalent amount of NaOH and warming gently. H_2O_2 solutions were prepared by dilution of Merck "Superoxol" H_2O_2 solution, and standardized against permanganate. Pyrex-redistilled water, and glassware rinsed in such water, was used for all solutions. Gas mixtures were prepared from commercial tank gases (purified grades), first passed through ascarite.

METHODS

ENZYME PREPARATION

The IAA-destroying enzyme of *Omphalia flavida* was obtained from cultures of the fungus on synthetic liquid medium, as previously described (20). The enzyme was purified from the culture medium by the following procedure, which was carried out at 3°C.

1. Exhaustive dialysis against glass-redistilled water, replaced five times over a period of 1 week.

2. Precipitation with saturated ammonium sulfate, followed by exhaustive dialysis of the redissolved orange precipitate against glass-redistilled water.

3. Adsorption on $Ca_3(PO_4)_2$ gel, prepared according to the method of Singer and Kearney (23), and elution with $1.5 \times 10^{-3} M$ Na_2HPO_4, followed by exhaustive dialysis against glass-redistilled water. The product was a clear watery solution with a weak absorption maximum at 410 mμ.

ASSAY OF IAA-OXIDIZING ACTIVITY

In measurement of enzyme activity, H_2O_2 was added in amount greater than $2 \times 10^{-6} M$. The purpose of this is to avoid induction effects and bring about a steady initial reaction rate capable of being accurately determined. The course of the reaction in the absence of added H_2O_2, and the effect of H_2O_2 on the reaction rate are considered in the following paper of this series (19). The amount of H_2O_2 added in assay was sufficient to make the rate independent of H_2O_2 concentration, but was much less than the amount of IAA oxidized during the experiment.

In the standard assay of IAA-destroying activity, the reaction was conducted for 6 min. at 25°C. in 1 ml. of solution containing enzyme, 0.09 μmole IAA, 0.005 μmole H_2O_2, and 0.05 mmole sodium citrate (pH 3.7). The reaction was stopped by adding 2 ml. of Salkowski reagent (6), and the remaining IAA was determined by measuring optical density at 530 mμ after 45 min. Activity was determined using an amount of enzyme which destroyed about one-third of the added IAA during the test.

IAA oxidation was measured spectrophotometrically according to Ref. (18).

ASSAY OF PEROXIDASE ACTIVITY

Measurement of pyrogallol peroxidation was performed spectrophotometrically by following the increase in optical density at 380 mμ.

Assay of peroxidase activity was carried out at 25°C. in 3 ml. of solution containing enzyme, 15 μmoles sodium citrate (pH 3.7), 0.6 μmole pyro-

gallol, and 0.9 μmole H_2O_2. After 6 min., 0.1 ml. of 3.75% H_2SO_4 was added to stop the reaction, and the optical density at 380 mμ, which was stable, was subsequently determined.

RESULTS

PEROXIDASE ACTIVITY OF THE OMPHALIA ENZYME

In the absence of added H_2O_2, no oxidation of polyphenols such as pyrogallol, catechol, or guaiacol took place in the presence of either crude or purified preparations of the *Omphalia* enzyme. They evidently contain no polyphenolase activity. If H_2O_2 were added, rapid formation of colored oxidation products occurred, in an amount dependent upon the amount of H_2O_2 added. The rate was proportional to enzyme concentration.

Table I presents final changes in optical density, from which the value 1.5 for the H_2O_2 to pyrogallol stoichiometry can be calculated. Although this figure corresponds to the formation of purpurogallin, this substance did not appear to be the principal product, because the absorption spectrum formed in the solution consisted of generally rising absorption into the ultraviolet, with a shoulder at about 260 mμ and a broad subsidiary maximum at 380 mμ, a spectrum unlike that of pure purpurogallin (7).

The effect of pH on the rate of pyrogallol peroxidation is shown in curve 1 of Fig. 1. The *Omphalia* peroxidase is unusual (although apparently not unique, cf. [16]) in exhibiting optimum activity at pH 3.5–3.7. Curve 2 of Fig. 1 shows an experiment in which the pH dependence of IAA oxidation by this enzyme preparation was determined; it is seen that the effect of pH on IAA oxidation is very similar to that on pyrogallol peroxidation.

Although the pH optimum of 3.5–3.7 for the *Omphalia* enzyme seems very distinctive, it appeared possible that it might be the result of the conditions employed. A similar optimum was obtained previously for IAA oxidation by the *Omphalia* enzyme in the absence of added H_2O_2 (20). IAA oxidation by pea homogenates has been reported by Reinert *et al.* (21) to show an optimum at

TABLE I
STOICHIOMETRY OF PYROGALLOL PEROXIDATION

Cuvette	1	2	3	4
Pyrogallol, μmoles	1.0	1.0	1.0	1.0
H_2O_2, μmoles	3.0	1.0	0.5	0.25
Final E_{380}	0.490	0.330	0.167	0.079
Pyrogallol oxidized[a]	1.0	0.67	0.34	0.16
Stoichiometry	—	1.5	1.5	1.6

[a] Calculated on the basis of complete oxidation in cuvette 1, which contained excess H_2O_2.

about pH 4 only if high concentrations of Mn^{++} are added; however, no Mn was added in the pH experiments with the *Omphalia* enzyme mentioned above. Maclachlan and Waygood (13) stated that citrate inhibits IAA oxidation above pH 4 but not at more acidic pH, so it seemed possible that the pH optimum might depend upon the buffer used. The effect of pH on IAA oxidation was therefore examined using as buffers propionate and phosphate, which would not be expected to show the chelating properties to which
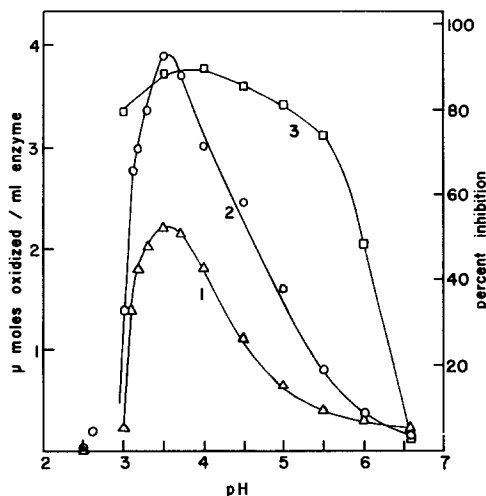

FIG. 1. Curve 1, effect of pH on peroxidation of pyrogallol by *Omphalia* enzyme (left ordinate); 2×10^{-4} M pyrogallol and 3×10^{-4} M H_2O_2. Curve 2, effect of pH on IAA oxidation; 9×10^{-5} M IAA and 4×10^{-6} M H_2O_2. Curve 3, effect of pH on inhibition of pyrogallol peroxidation by 5×10^{-5} M IAA (right ordinate); conditions as in curve 2. All experiments done in 5×10^{-3} M Na citrate buffers.

inhibition by citrate is ascribed. Each was employed over the pH range in which it constitutes an effective buffer. The results, given in Fig. 2, show that the IAA oxidation rate responded to pH in a qualitatively similar manner regardless of buffer used; the optimum lay on the acid side beyond the buffering limit of either buffer. Addition of promoting concentrations of 2,4-dichlorophenol also did not qualitatively alter the pH response, but since dichlorophenol promotion appeared at lower concentration at the higher pH values (19), some alteration in the proportions of the curve resulted.

To test whether different enzymes might be responsible for pyrogallol peroxidation and IAA oxidation, an *Omphalia* enzyme preparation was subjected to thermal inactivation at 70°C., samples being withdrawn at intervals and subsequently assayed for IAA-oxidizing and pyrogallol-peroxidizing activities. The results are given in Table II; they show a close proportionality between the two activities even after activity had been reduced to about 3% of that initially present.

In Table III the change in IAA-oxidizing and pyrogallol-peroxidizing activity is compared in the course of purification by the procedure given above. The ratio of the two activities remained substantially constant,
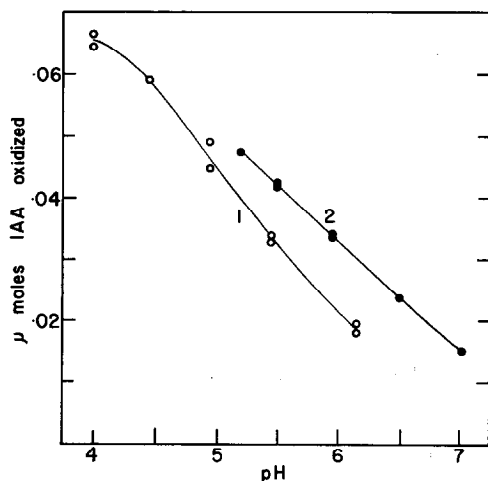

FIG. 2. Effect of pH on IAA oxidation by *Omphalia* enzyme in (curve 1) sodium propionate buffers, and (curve 2) sodium phosphate buffers; concentration of buffers, 0.05 M.

TABLE II
THERMAL INACTIVATION OF OMPHALIA ENZYME
Activities expressed as μmoles oxidized/ml. of enzyme solution/min.

Min. at 70 ± 1°C.	0	6	15	30
IAA-oxidizing activity	0.150	0.070	0.027	0.0049
Pyrogallol-peroxidizing activity	0.132	0.058	0.020	0.0037
Activity ratio	1.1	1.2	1.3	1.3
Per cent of initial activity	100	44	18	3.3

TABLE III
FRACTIONATION OF OMPHALIA ENZYME

Preparation step	1	2	3
Vol. of solution. $ml.^a$	245	32.1	46
Dry weight. $mg./ml.$	0.51	0.85	0.08
$E_{280}{}^b$	1.18	3.66	0.23
IAA oxidation^c	0.127	0.667	0.270
Pyrogallol peroxidation	0.147	0.736	0.287
Activity ratio	0.86	0.90	0.94
Relative IAA activity (dry wt. basis)	1	3.1	11
Per cent recovery of activity	100	69	40

^{a} Corrected for removal of samples for assay.
^{b} Determined on appropriately diluted sample.
^{c} Micromoles oxidized/min./ml. enzyme solution.

even after the loss of activity which occurred in the final step.

From these findings it seems probable that IAA oxidation and pyrogallol peroxidation by the *Omphalia* enzyme preparations are due to one and the same enzyme. In the inactivation and fractionation experiments, no indication was obtained that the preparations contain any separable factors, or any enzymes other than peroxidase, involved in the IAA-oxidizing reaction.

INHIBITION OF PYROGALLOL PEROXIDATION BY IAA

Addition of low concentrations of IAA to mixtures of a polyphenol, H_2O_2, and the *Omphalia* enzyme was found to depress the rate of polyphenol oxidation markedly. Fig-

ure 3 shows progress curves for the reaction, with pyrogallol as substrate, obtained at a series of IAA concentrations. An inhibitory effect is obtained well within what is regarded as the physiological range of IAA concentration. Curve 1 of Fig. 4 shows the inhibition of peroxidase activity as a function of IAA concentration, and compares it with the inhibition by HCN (curve 2). IAA, giving 50% inhibition at less than 10^{-5} M, proved to be more than ten times as powerful an inhibitor as HCN under these conditions.

Inhibition was obtained if IAA were added to the reaction mixture when pyrogallol peroxidation was already proceeding. If IAA were added before pyrogallol, and enough time allowed for the IAA to be destroyed by the enzyme before pyrogallol was added, then peroxidation of pyrogallol proceeded at the uninhibited rate. Since inhibition is obtained with amounts of IAA much smaller than the amount of pyrogallol which undergoes oxidation at the inhibited rate, it appears unlikely that interaction of IAA with intermediate products of pyrogallol peroxidation can explain the inhibition.

Inhibition of horse-radish peroxidase by IAA was previously reported by Siegel (22). In the present work, a horse-radish peroxidase preparation was studied under the conditions used in the experiments with the *Omphalia* enzyme and was found to be considerably less sensitive to IAA, as shown in curve 3 of Fig. 4. The different behavior of the different enzymes suggests that inhibition by IAA is due to interaction of IAA with the enzyme.

Curve 3 of Fig. 1 shows how the per cent inhibition of peroxidation caused by 5 × 10^{-5} M IAA varied with pH. A conspicuous decline occurred above pH 5.5, although even at pH 6.6 30% inhibition was observed at a tenfold greater IAA concentration.

Table IV shows the effect of some other indole derivatives and acetic acid derivatives on peroxidation of pyrogallol by the *Omphalia* enzyme. The inhibitory effect is not a general property of either class of compounds. Besides IAA, only indole-3-isobutyric acid and 5-hydroxyindoleacetic acid, among the compounds tested, gave any de-
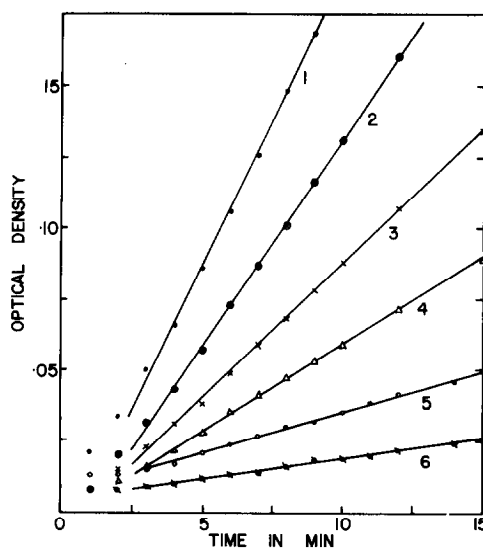

FIG. 3. Effect of IAA on the course of pyrogallol peroxidation followed at 380 mμ. Pyrogallol and H_2O_2 (0.6 μmole each) in 3 ml. Na citrate buffer containing enzyme, with IAA concentrations as follows: Curve 1, 0; curve 2, 2.5 × 10^{-6} M; curve 3, 7.5 × 10^{-6} M; curve 4, 1.5 × 10^{-5} M; curve 5, 3.5 × 10^{-5} M; curve 6, 7 × 10^{-5} M.
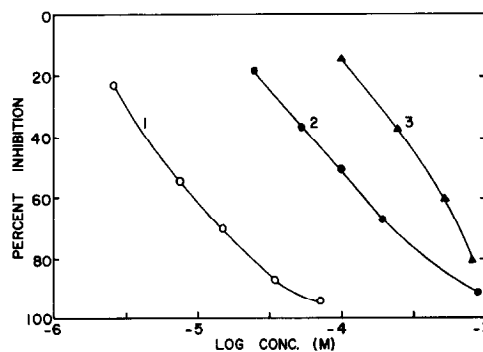

FIG. 4. Inhibition of peroxidases by IAA and HCN. Reaction conditions as in Fig. 3. Curve 1, effect of IAA on *Omphalia* peroxidase. Curve 2, effect of HCN (added as KCN) on *Omphalia* peroxidase. Curve 3, effect of IAA on horse-radish peroxidase.

tectable inhibition. These two compounds are known to be substrates of the oxygen-consuming activity of the enzyme, whereas the remaining compounds are not substrates (20). The inhibition thus appears to be associated with the activity of the enzyme toward oxidation of IAA and related compounds.

TABLE IV

INHIBITION OF PYROGALLOL PEROXIDATION

Substance	Per cent inhibition at	
	$5 \times 10^{-5} M$	$1.8 \times 10^{-4} M$
Indoleacetic acid	85	97
Indoleacetic acid methyl ester	—	4
Indole-3-propionic acid	—	3
Indole-3-isobutyric acid	28	54
5-Hydroxyindoleacetic acid	41	65
Indole-3-carboxaldehyde	—	0
Skatole	—	0
Imidazole-4-acetic acid	—	0
Benzofurane-3-acetic acid	—	0
2,4-Dichlorophenoxyacetic acid	—	2
Naphthalene-1-acetic acid	—	0

The inhibition of peroxidation caused by $8 \times 10^{-6} M$ IAA was determined at pyrogallol concentrations varying from 2×10^{-4} to $1.25 \times 10^{-3} M$, as shown in Fig. 5. With increasing pyrogallol concentration, the uninhibited reaction rate increased more than in proportion to the IAA-inhibited rate, so that per cent inhibition actually increased, i.e., increase in pyrogallol concentration did not reverse the inhibition. The results, therefore, are not an instance of kinetically "competitive" inhibition.

INHIBITION OF IAA OXIDATION BY POLYPHENOLS

Oxidation of IAA by the *Omphalia* enzyme is, like other IAA oxidations, strongly inhibited by *o*- and *p*-diphenols and polyphenols, such as catechol, hydroquinone, and pyrogallol, at concentrations of about $10^{-5} M$. When followed manometrically this inhibition appeared as a depression of the rate of O_2 uptake, without much increase in the short induction period which always precedes attainment of a steady rate of oxygen consumption. This differs from the inhibitions reported for horse-radish peroxidase (9), *Lupinus* enzyme (24), and wheat-leaf enzyme preparation (31), where polyphenols brought on an extended lag in the beginning of O_2 uptake. Whether this difference is due to the more acidic pH used for the present enzyme or to other causes is undetermined.

When pyrogallol peroxidation is occurring

in the presence of IAA, the inhibition by IAA does not diminish as the reaction progresses, as can be seen in Fig. 3. Consequently, it would appear that IAA is not being destroyed in the course of the experiment. This may be thought surprising, in view of the small amount of IAA which is present, the excess of H_2O_2, and the considerable inhibition of pyrogallol peroxidation suggestive of an interaction of IAA with the enzyme. Since colorimetric determination of IAA in the presence of the amounts of polyphenol and H_2O_2 used in such experiments is very unreliable (15), two physical methods were employed to ascertain how much IAA destruction was actually occurring in the presence of pyrogallol and excess H_2O_2.

To assay IAA at low concentrations, in the range giving partial inhibition of pyrogallol peroxidation, advantage was taken of the fact that the products of IAA destruction are neutral. IAA labeled with C^{14} in the 2-carbon of the indole ring was used, and the amount of acidic ether-soluble label (residual IAA) was determined. With $5 \times 10^{-6} M$ IAA, which gave 51% inhibition of pyrogallol peroxidation, IAA destruction appeared to be at least 95% inhibited over that in the absence of pyrogallol.

At IAA concentrations above about $4 \times 10^{-5} M$, its oxidation may be followed spectrophotometrically at 261 mμ (18). Peroxidation of pyrogallol causes increases in optical density at 261 mμ and also at 380 mμ, a
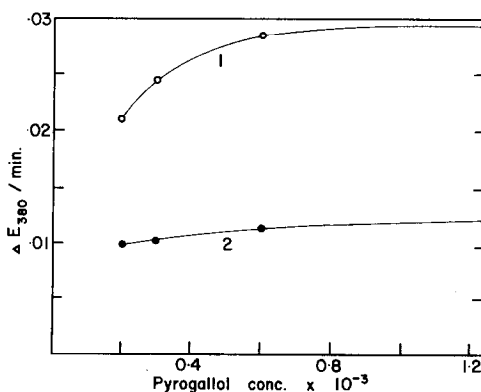

FIG. 5. Effect of pyrogallol concentration on inhibition of peroxidation by IAA. Curve 1, control rate; curve 2, rate in presence of $8 \times 10^{-6} M$ IAA. Reaction conditions as in Fig. 3.

TABLE V

INTERACTION BETWEEN IAA AND PYROGALLOL OXIDATIONS

Rates of oxidation measured spectrophotometrically for IAA or pyrogallol separately in the presence of the *Omphalia* enzyme and indicated concn. of H_2O_2, and determined for samples containing both IAA and pyrogallol by the two-component method described in the text.

Expt. No.	Substrates, $M \times 10^{-5}$			IAA oxidation		Pyrogallol peroxidation		Per cent inhibition	
	IAA	Pyrogallol	H_2O_2	alone	w/pyrogallol	w/IAA	alone	IAA oxidation	Pyrogallol peroxidation
				$m\mu moles/min.$		$m\mu moles/min.$			
1	6.0	0.65	1.6	25	4.0	2.4	a	84	a
2	6.4	3.3	6.6	9.5	0.4	2.8	21	95	85
3	5.9	6.6	3.3	16.5	1.1	4.3	32	94	87
4	6.5	6.6	13.4	10.3	0.2	3.5	43	98	91

a Not determined.

wavelength at which IAA oxidation causes negligible changes at the concentration used. If the relationship between optical changes at 261 and 380 mμ due to pyrogallol peroxidation is first determined, then a two-component analysis of mixtures of IAA and pyrogallol, both undergoing oxidation, may be made by following extinction simultaneously at 261 and 380 mμ. The results of several experiments with this method are given in Table V. In all cases the rates of both IAA and pyrogallol oxidation were strongly inhibited when the two substrates were present together. The figures for pyrogallol peroxidation are given in terms of the equivalent amount of H_2O_2 consumed. The amount of H_2O_2 used in peroxidation of pyrogallol, in the presence of IAA, was considerably less than the amount by which IAA oxidation was being inhibited. The inhibition of IAA oxidation cannot, therefore, be explained by saying that H_2O_2 is diverted from Eq. (1) to peroxidation of the phenol, or attributed to a competition between phenol and IAA for H_2O_2. The results suggest that in the presence of both substrates an inactive form of the enzyme, incapable of oxidizing either of them, accumulates.

EFFECT OF OXYGEN

Substrate amounts of H_2O_2 were added to IAA and the enzyme, in the presence and absence of oxygen, and IAA destruction followed spectrophotometrically (thus avoiding the interfering effect of substrate quan-

tities of H_2O_2 on the colorimetric determination). The measured rate of IAA destruction in the absence of oxygen was always less than 10 % of that in air, and the amount of IAA destroyed never reached more than a small fraction of the amount of H_2O_2 added. Table VI gives results of one such experiment. This contrasts sharply with pyrogallol peroxidation, which, as can be seen in curves 1 and 2 of Fig. 6, is quite unaffected by the presence or absence of oxygen. It would appear that a peroxidative reaction like reaction (1) should also be insensitive to oxygen.

Although peroxidation of pyrogallol was indifferent to oxygen, the inhibitory effect of IAA on peroxidation of the phenol was markedly increased in the absence of oxy-

TABLE VI

EFFECT OF O_2 ON IAA OXIDATION

Evacuated Thunberg tubes contained 0.15 μmole IAA, and H_2O_2, in 3.5 ml. buffer, with enzyme in side arm; air was admitted to appropriate tubes. Figures show difference between extinctions at 261 and 272 mμ, determined against a blank containing buffer and IAA, 30 min. after contents of tubes and side arms were mixed. Each figure is the average of two determinations; zero value 0.006.

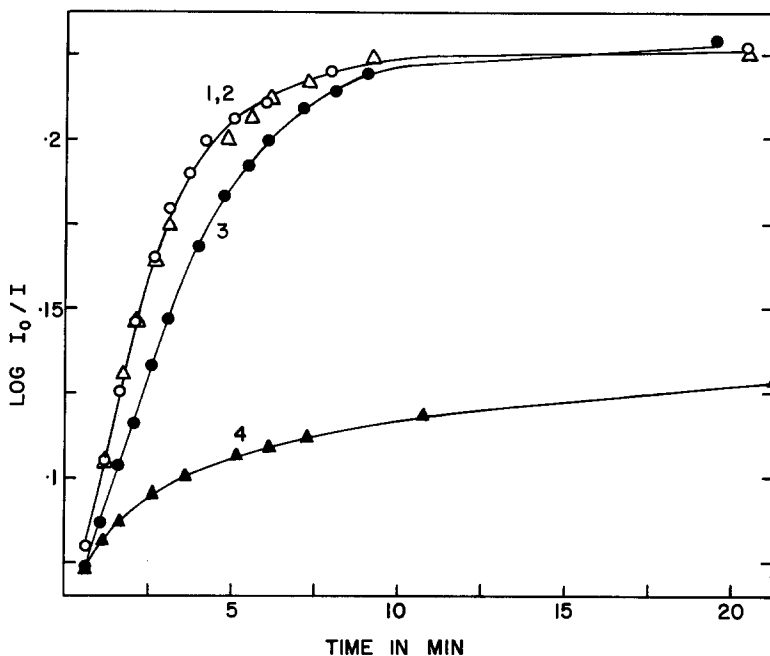| Micromoles H_2O_2	$E_{261} - E_{272}$	
	Air	Vacuum
0.01	0.098	0.006
0.10	0.077	0.004

FIG. 6. Effect of oxygen on pyrogallol peroxidation in absence and presence of 5×10^{-6} M IAA Curve 1 (open circles), control in air; curve 2 (open triangles), control *in vacuo*; curve 3, with IAA, in air; curve 4, with IAA, *in vacuo*. Thunberg tubes contained 6.6×10^{-5} M pyrogallol and 10^{-4} M H_2O_2; enzyme solution was held in side arm while tubes were evacuated, air was readmitted to tubes 1 and 3, and all side arms were then tipped. Absorption was followed at 380 mμ versus air.

gen. Figure 6 shows an experiment in which the effect of 5×10^{-6} M IAA on pyrogallol peroxidation was tested in the presence and absence of air. Inhibition in air was 28%; in the absence of air it was 60% initially and increased further as the reaction progressed. It seems unlikely that the stronger inhibition observed anaerobically could be due simply to conservation of IAA through prevention of its destruction, since, as was brought out above, even in air at most only a small fraction of the IAA added undergoes destruction in this type of experiment. Oxygen appears rather to protect the enzyme partially against inhibition by IAA.

If air were introduced into a mixture containing enzyme, H_2O_2, and IAA, which had been incubated anaerobically for several minutes, a rapid reaction would ensue only if more enzyme were added, suggesting that the original enzyme had been inactivated in some way not directly reversible by oxygen. This introduces a difficulty into the attempt to demonstrate reaction (1), because it is not

known whether the absence of reaction is due primarily to lack of oxygen or to inactivation of enzyme. What can be pointed out is that peroxidation of pyrogallol shows no such effect of oxygen, indicating an involvement of oxygen in IAA oxidation more direct than is implied in separate reactions (1) and (2). Likewise, admission of air to an evacuated system containing enzyme, H_2O_2, IAA, and pyrogallol would only partially reverse the inhibition of pyrogallol peroxidation.[2]

INHIBITION OF IAA OXIDATION BY CO

A possible interpretation of the interaction between IAA oxidation and pyrogallol peroxidation is that in the presence of IAA, a form of the enzyme appears which is inactive in peroxidation but may possibly be involved in a reaction with oxygen. On this

[2] Yamazaki and Souzu (32) recently reported inhibition of turnip peroxidase when IAA and H_2O_2 were incubated with it anaerobically. They attributed it to reaction of P· [Eq. (3)] with the enzyme.
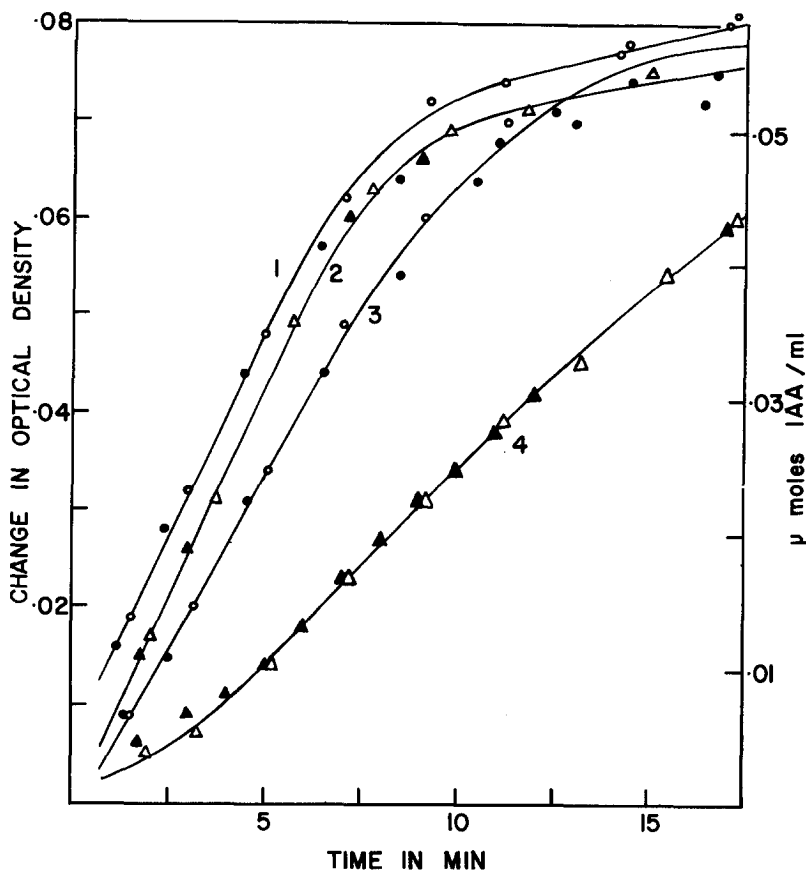
FIG. 7. Effect of carbon monoxide and light on IAA oxidation followed at 261 mμ. Curve 1, 5% O_2/ 95% N_2 in light; curve 2, same in dark; curve 3, 5% O_2/95% CO, in light; curve 4, same in dark. Cuvettes contained 0.18 μmole IAA and 0.03 μmole H_2O_2 in 2.9 ml., and were gassed for 5 min. Enzyme solution was evacuated separately, and 0.15 ml. was introduced into cuvette, under a stream of the gas mixture, at zero time. Illumination from two 15-w. daylight fluorescent lamps at a distance of 15 cm. Solid and open symbols show duplicate experiments.

account it was especially interesting to find that IAA oxidation by the *Omphalia* enzyme showed definite light-reversible inhibition by carbon monoxide. Curve 4 of Fig. 7 shows the inhibitory effect of 95% CO/5% O_2 in the dark, against a control of 95% N_2/5% O_2 (curve 2). Curve 1 is the same control run in the light, indicating no effect of light on the reaction in the absence of CO. With CO, however, curve 3 was obtained in the light and shows almost complete reversal of inhibition. Inhibition by CO was also observed in manometric experiments, in which H_2O_2 was not added. Using gas mixtures similar to those in Fig. 7, the same degree of inhibition (about 50%)

was observed, and reversal by light was complete.

Figure 8 shows how the rate of IAA oxidation depended upon CO pressure at constant O_2 tension. Inhibition reached 50% at about 0.5 atm. CO, and could not be increased much by further increase in CO pressure, except at the beginning of the reaction. At higher CO pressures the initially slow rate rose during several minutes to a steady value shown by solid circles in Fig. 8. The open circles in Fig. 8 show an estimate of the initial rates. Below 0.5 atm. CO, the rates were steady from the first observation.

Increasing the O_2 pressure decreased the per cent inhibition due to CO and tended
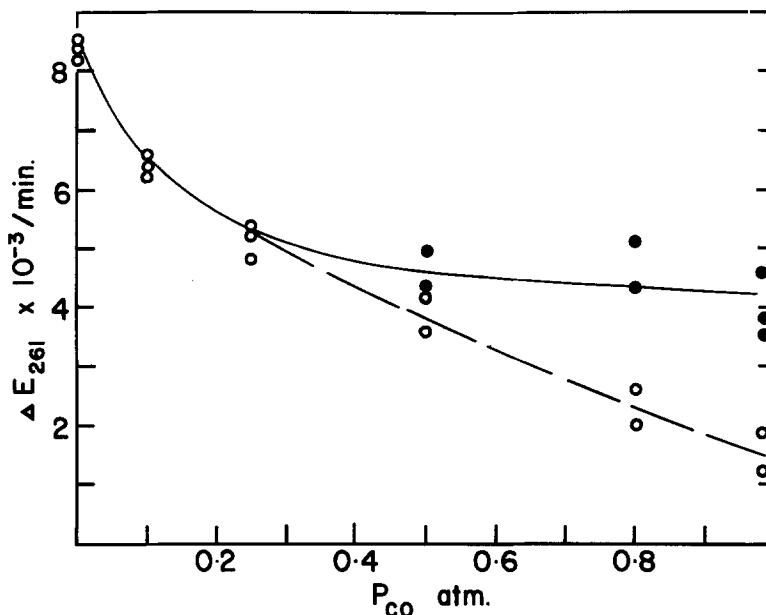
FIG. 8. Effect of CO pressure on oxidation of IAA in the dark. Conditions similar to those of Fig. 7. All gas mixtures contained 2% O_2, and were made to 100% with N_2. Open symbols show initial rates of reaction; solid symbols show the steady rate eventually reached, in cases where it differed from the initial rate.

TABLE VII

EFFECT OF O_2 ON CO INHIBITION OF IAA OXIDATION

CO, atm.	Oxygen, atm.		
	0.02	0.20	0.90
	Per cent inhibition[a]		
0.10	29.5	—	19
0.80	55	30	—

[a] Per cent inhibition over O_2/N_2 mixture of same P_{O_2}; each determination in duplicate. Reaction conditions similar to those in Fig. 7.

to eliminate the induction effect caused by CO. The results of some experiments at different oxygen concentrations are given in Table VII. Increases in O_2 concentration did not reverse the inhibition to the extent which might have been expected if O_2 and CO were competing in the same reaction, and rather suggest that the effect of O_2 on the CO inhibition may be indirect.

DISCUSSION

The light-reversible inhibitory effect of CO suggests that ferrous peroxidase may be

formed during the oxidation of IAA, since ferrous peroxidase is known to give a light-dissociable CO complex (8). Formation of ferrous hemoprotein has been proposed, on the basis of inhibition by CO, for several analogous oxygen-consuming reactions: the oxidation of dihydroxyfumaric acid by horse-radish peroxidase (26), the oxidation of tryptophan by tryptophan peroxidase (10), and the oxidation of azide by catalase (28). Tang and Bonner (27) previously reported inhibition of IAA oxidation by CO, but, as pointed out by Wagenknecht and Burris (30), their interpretation is rendered doubtful because of the control used. It is felt that the present experiments demonstrate genuine light-reversible inhibition by CO.

Could ferrous peroxidase be the inactive form of the enzyme which appears in the presence of IAA, and accounts for the inhibition of peroxidation by IAA? Reconversion of ferrous to ferric form or a peroxide complex thereof might then be the step involving oxygen indicated by the effect of oxygen on IAA oxidation, on CO inhibition, and on IAA inhibition of peroxidation. The excessively inhibitory effect of polyphenols on IAA oxidation suggested (17) that a free-

radical process could be involved, since polyphenols act as antioxidants by combining with free radicals (2). If a free-radical mechanism were the basis for reaction of oxygen with the system, as is considered to be the case in many organic autoxidations, it would be apparent why polyphenols inhibit reconversion of the inactive (ferrous) to the active (ferric) form, and inhibition of both peroxidation and IAA oxidation results.

Although reaction sequences can be written for IAA oxidation based on a free-radical mechanism involving ferrous peroxidase, their relation to existing evidence would be largely speculative. Moreover, it is not established that inhibition of peroxidation by IAA and inhibition of IAA oxidation by CO involve the same form of the enzyme. The effect of CO on the inhibition of pyrogallol peroxidation by IAA was tested, in the expectation that if the same form of the enzyme were involved, CO should increase the inhibition. The per cent inhibition by IAA was found not to be markedly affected by CO; in fact, CO caused a small but definite inhibition of pyrogallol peroxidation alone. Neither is the formation of ferrous peroxidase considered proved by the inhibitory effect of CO. Spectroscopic evidence is needed to show what form of the enzyme does accumulate when CO is present, or when pyrogallol and IAA are present together; the *Omphalia* enzyme preparations were not obtained in concentrated enough form for such observations.

Whatever the sequence of reactions which actually occurs during IAA oxidation, it seems probable that an important part of the inhibitory effect of polyphenols on it is due to interference with a mechanism not adequately described by Eq. (1) and (2). This mechanism appears likely to be a free-radical one, as Maclachlan and Waygood (14) and Yamazaki and Souzu (32) have concluded. The nature of the interactions is such as to make difficult a decision as to whether H_2O_2 is a reaction intermediate. H_2O_2 can certainly enter into the reaction, since it exerts strong effects upon the reaction rate. These are considered in the next paper of this series (19).

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