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MECHANISM OF CORN INDOLE-3-ACETIC ACID OXIDASE *IN VITRO**

J. N. BEMILLER and W. COLILLA

Department of Chemistry and Biochemistry, Southern Illinois University, Carbondale, IL 62901, U.S.A.

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Key Word Index—*Zea mays*; Gramineae; indole acetic acid oxidase; mechanism of action *in vitro*; isolation of intermediates.

Abstract—When indole-3-acetic acid (IAA) was oxidized in the presence of Mn^{2+} and 2,4-dichlorophenol by a crude corn extract shown to contain IAA oxidase and by non-enzymic oxidation with Mn^{3+}, 10 products of the reaction could be extracted by CH_2Cl_2 and separated by TLC. Four of these products, 3-hydroxymethyloxindole, 3-methyleneoxindole, indole-3-aldehyde, and the 3-indolylmethyl ester of indole-3-acetic acid had been previously identified as reaction products. In addition, 3,3'-di-indolylmethane and 3-methyleneindolenine were found. 3-Methyleneindolenine was readily and reversibly formed from 3-hydroxymethylindole. A reaction pathway involving a two-electron oxidation catalyzed by Mn^{3+} and 3-hydroperoxymethylindole as a key intermediate in oxindole formation is proposed.

INTRODUCTION

PLANT growth is believed to be controlled in part by the enzyme catalyzing indole-3-acetic acid (IAA) degradation[1,2] which has two activities—a peroxidase activity and an IAA oxidase activity.[3] The IAA oxidase of most plants needs obligate cofactors of Mn^{2+} and a monosubstituted phenol,[4,5] although some will oxidize IAA (especially with pure enzymes) in the absence of any cofactors.[6–8]

The IAA oxidizing enzyme contains heme as a prosthetic group.[7] However, Galston and Siegel[9] demonstrated that the apoenzyme of horse radish peroxidase (HPR) could oxidize IAA in the presence of Mn^{2+} and 2,4-dichlorophenol (DCP), although it had no peroxidase activity. Their work was challenged by Ku *et al.*[10] who used a mixture of an acid and butanone (instead of the acid-acetone of Galston and Siegel) for dissociation of the heme group from its apoenzyme and were not able to effect the oxidation of IAA in the presence of the apoenzyme alone or with Mn^{2+} and DCP as cofactors.

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IAA oxidation appears to require 1 mol of oxygen for every mol of IAA consumed; 1 mol of CO_2 is given off. The presently accepted final product of IAA oxidation under physiological conditions is 3-methyleneoxindole (I). This product was first demonstrated by Hinman *et al.*[11] as a product of the oxidation of IAA by HRP. Its identity has since been confirmed by chemical synthesis.[8]

Tuli and Moyed[12] were able to extract I from intact etiolated pea seedlings incubated with 0·1 mM IAA for 16 hr. They and others[13] further demonstrated that I arises from a spontaneous dehydration of 3-hydroxymethyloxindole (II). I was not formed at high concentrations of IAA (0·2 mM) using HRP; the product was instead a compound tentatively identified as the 3-indolylmethyl ester of IAA.[13] With purified Japanese-radish peroxidase at high concentrations of enzyme, indole-3-aldehyde (III) was indicated to be the dominant product from an examination of the UV spectrum of the reaction mixture. This compound was first reported to be a product of IAA oxidation by Racusen,[14] who isolated a very small amount of it from the reaction mixture of IAA and etiolated pea epicotyl preparations. III was also the final product when IAA oxidase from *Lupinus alba* was used together with cytochrome-*c* oxidase.[15] Wightman[16] reported the isolation of labelled indole-3-glycolic acid and indole-3-glyoxylic acid from whole tomato plants injected with labeled IAA. A hydroxylated derivative of *o*-aminoacetophenone[17] and a number of unidentified compounds[18,19] have also been reported as products.

The production of more than one product in IAA oxidation was first intimated by Morita *et al.*[20] when PC of their reaction mixture revealed at least 8 or 9 components. They identified III and proposed that two other bands were Galston's hydroxylated derivative of *o*-aminoacetophenone and Hinman and Lang's ester. I and II have been demonstrated to be potent growth inhibitors of fungi and higher plants at high concentrations and growth promoters at low concentrations.[21]

The mechanism of IAA oxidase action is yet unknown, but a number of proposals have been put forth. The earliest proposal[22] suggested that IAA oxidase was a combination of enzymes—a light activated flavoprotein and a peroxidase. Kenten[23] and Kenten and Mann[24–26] proposed a Mn^{2+}–phenol–peroxide–peroxidase system. MacLachan and Waygood[27,28] went one step further and proposed that Mn^{3+} was the oxidant. Sacher,[29] on the basis of inhibitory effects of polyphenols, proposed a free radical system, which is supported by more

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[12] V. Tuli and H. S. Moyed, *Plant Physiol.* **42,** 425 (1967).
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[27] G. A. Maclachan and E. R. Waygood, *Plant Physiol.* **31** (suppl), xxvi (1956).
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[29] J. A. Sacher, *Plant Physiol.* **37,** 74 (1962).

recent work.[30–32] These proposals were all formulated before I was identified as the oxidation product. After its identification as such, Hinman and Lang[13] proposed a pathway for its formation. All proposals to date involve the iron atom of the heme group as an activator of the oxygen molecule.

RESULTS

Enzyme preparations were prepared from corn (*Zea mays* L.) seedlings by the method of van Jaarsveld and Meynhardt[33] because, according to them, it is the only preparation that does not exhibit a lag time before the reaction. The enzyme preparations rapidly destroyed IAA, as determined by the disappearance of the pink color with Salkowski reagent, when added to a reaction mixture consisting of 0·3 mM IAA, 1 mM $MnSO_4$, 0·001 mM DCP, and 10 mM KH_2PO_4 adjusted to pH 3·5. Activity could be abolished by heating the preparation in a boiling water bath, excluding oxygen or adding cyanide, behavior characteristic of IAA oxidase.[7] The pH optimum of IAA oxidase of corn seedlings was indicated to be 3·5.

3-Hydroxymethyloxindole (II) and 3-methyleneoxidinole (I) were identified as reaction products as follows. When 10 ml of reaction mixture and 0·15 ml of enzyme preparation were incubated and the progress of the reaction was followed by means of UV spectrophotometry, a decrease in absorbance was found at about 280 nm and an increase around 250 nm. After 18 hr, the twin peaks at 248 and 254 nm characteristic of I could be easily seen. When a 20-hr reaction-enzyme mixture was concentrated under diminished pressure and analyzed by PC with isopropanol–water (1:19), several UV absorbing bands were present; one at R_f 0·74 had a particularly strong absorbance. When extracted with water, a solution was obtained which, when boiled (10 min), gave the characteristic spectrum of I. This agrees with Hinman and Lang's[13] observation of easy conversion of II to I. I and II could be separated by extracting the unboiled solution with ether. The water solution gave a spectrum of II, and the ether extract a spectrum of I.[13,14,34]

When the reaction-enzyme mixture was extracted with 2 vols. of CH_2Cl_2 after 5 min, and the organic layer was concentrated and investigated by TLC using chloroform–ethyl acetate (2:3) several bands which reacted with both Salkowski and Ehrlich reagents were obtained (Table 1). In the reaction mixture described here, the formation of these products was pH dependent. The enzyme extract destroyed IAA up to pH 7·0. At pH 2·8, band 3 was greatly diminished and band 6 was absent; at pH 4·9, band 6 was slightly increased and band 4 was absent. No reactions above pH 7·0 were done.

Following TLC on silica gel H, six bands were revealed (pH 3·5 reaction mixture) by the Salkowski reagent. Ehrlich reagent revealed three more bands. Band 2 could be resolved into one pink (2a) and one yellow (2b) component. Band 6 was a composite of a red–purple (6a) and a yellow band (6b). A colorless (decolorizing) band (7) at R_f 0·75 was also observed.

When a pH 3·5 reaction mixture (without DCP or Mn^{2+}) was incubated with HRP, the subsequent chromatogram was almost identical to that obtained with the crude corn enzyme extract, although band 2 was more prominent. Since peroxidase is known to oxidize phenolic compounds,[35] a reaction mixture was made up without IAA and allowed to

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[32] E. V. PARUPS, *Can. J. Biochem.* **47,** 220 (1969).
[33] P. P. VAN JAARSVELD and J. J. MEYNHARDT, *S. Afr. J. Agric. Sci.* **10,** 901 (1967).
[34] T. T. FUKUYAMA and H. S. MOYED, *J. Biol. Chem.* **239,** 2392 (1964).
[35] W. W. WESTFIELD and C. LOWE, *J. Biol. Chem.* **145,** 463 (1962).

react with enzyme extract. Chromatograms of a CH_2Cl_2 extract contained no Ehrlich reagent-positive bands.

Band 1 (Table 1) was identified as indole-3-acetate by chromatography against a standard. When the two yellow bands (2b and 5) were eluted with methanol, their UV spectra corresponded exactly to the spectra of II and I respectively.

Table 1. Products identified after incubation of IAA with corn IAA oxidase

Band	Average R_f*	Color with Salkowski reagent	Color with Ehrlich reagent	Identity	Remarks
1	0·05	Purple	Blue	Indole-3-acetate	Minor
2a	0·16	Pink	Pink		Minor
2b	0·16	Pink	Yellow	II	Major
3a	0·30	Pink	Red–pink	IV	Major above pH 3; somewhat unstable
3b	0·30		Pink	III	Minor
4	0·39	Brown–pink	Green–purple	Could be a phenolic ether of V	Major; slow reactions
5a	0·47		Yellow	I	Major
5b	0·47		Pink	Hinman and Lang's ester (?)	Minor
6a	0·65	Pink	Pink changing to purple on standing	VI	Major
6b	0·65	Pink	Yellow		Major; positive test for peroxide
7	0·75	Colorless	Colorless		Minor; positive test for peroxide

* On a TLC plate of silica gel H irrigated with $CHCl_3$–EtOAc (2:3) to a height of 10 cm.

The major component of band 3 was tentatively identified as 3-methyleneindolenine (IV). A solution of band 3 decolorized both bromine and potassium permanganate solutions, indicating unsaturation. 3-Methyleneindolenine, an α,β-unsaturated imine, should add nucleophilic reagents rather easily across its carbon-carbon double bond. When band 3 was allowed to react with an excess of KCN and the CH_2Cl_2 extract was investigated by TLC in chloroform–ethanol, five Ehrlich reagent-positive bands were obtained. The most concentrated band was a gray one at R_f 0·30. The methanolic extract of the band gave a spectrum that corresponded well to the reported spectrum of 3-indoleacetonitrile.[36] Furthermore, when band 3 was isolated, dissolved in a few drops of acetone and investigated by MS, a parent ion peak of 129, the MW of IV, was observed.

Band 3a was somewhat unstable on chromatograms. One new product formed from it reacted with both Ehrlich and Salkowski reagents to give a pink to red color, and upon elution and purification by rechromatography, gave a UV spectrum similar to that of an authentic sample of 3-hydroxymethylindole (V). In fact if band 3 was rechromatographed on silica gel H, two bands were obtained. One Salkowski or Ehrlich reagent-positive band corresponded closely in R_f to that reported for V.[37] However, when an authentic sample of V was chromatographed, its R_f in chloroform–ethyl acetate (2:3) was 0·52, substantially different from that of band 3.

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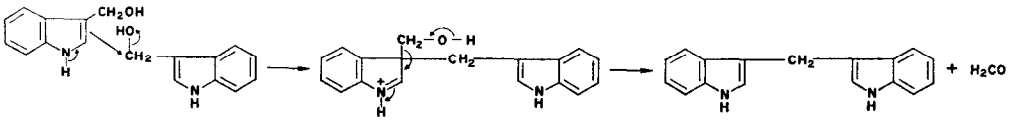
[37] E. Stahl and H. Kaldewey, *Z. Physiol. Chem.* **323,** 183 (1961).

To determine if V could be formed from IV, band 3 was eluted and treated with 10% KOH overnight; authentic V was similarly treated. The extracts were investigated by TLC on silica gel H in $CHCl_3$–ETOAc (2:3). Band 3 gave two Ehrlich reagent-positive bands (a new purple-red band at R_f 0·65 and the original pink band), while V gave three Ehrlich reagent-positive bands [a similar purple–red band at R_f 0·65, a pink band at R_f 0·52 (V), and IV]. It is known that V, when treated with hot alkali, gives VI.[38] Hence, VI was synthesized and shown to be identical in its UV spectrum and R_f to the product at R_f 0·65 (band 6a). When band 3 was eluted from the TLC plate with CH_2Cl_2 and the solvent was evaporated, the dry compound decomposed completely to VI.

When V was dissolved in a few drops of 95% ethanol, and the resulting solution was added to 0·1 M KH_2PO_4 adjusted to pH 3·5, IV was formed. The addition of IAA did not alter this result; neither did DCP or IAA and DCP. At low concentrations of V, very little of it remained; IV was the major product and VI was the minor product.

It has been postulated that VI is formed as a result of V dissociation into indole and formaldehyde and condensation of indole with V. Supporting evidence was the synthesis of VI by the reaction of indole and formaldehyde.[38] The 3-position of indole, the site of high electron density,[13] was proposed as the attacking species. We suggest that another mechanism may be involved (Scheme 1). Internal decomposition could be aided by the unbounded pair of electrons on the indolenine ring nitrogen. Upon standing, V did give a positive test with chromotropic acid indicating the release of formaldehyde.



SCHEME 1. PROPOSED MECHANISM FOR THE CONVERSION OF V INTO VI.

To determine if V is an intermediate in the formation of I, a reaction mixture, made up with V substituting for IAA as the substrate, was treated with an enzyme extract. The reaction was monitored spectrophometrically to determine if I was obtained. Precipitation of VI occurred rapidly; no I was observed after 24 hr. Thus, it seems that V, formed reversibly from IV, is not a primary intermediate in the formation of I.

Indole-3-aldehyde (III), a second component of band 3, was identified as follows. A CH_2Cl_2 extract of a reaction-enzyme mixture at pH 2·6 was concentrated and investigated by TLC in chloroform–ethyl acetate (2:3). The band at R_f 0·30, the usual R_f value of band 3, which turned pink with Ehrlich reagent, gave a UV spectrum in methanol corresponding to III rather than band 3. To determine if III was formed at other pHs, advantage was taken of the instability of band 3 at the dry state. Band 3 was eluted with CH_2Cl_2, the organic solvent was evaporated, and the residue was allowed to stand for 3 hr. A few drops of CH_2Cl_2 were added to the dry residue, and the solution was transferred to silica gel H plates for TLC in the same solvent. Bands identified as VI and III were obtained. Thus, band 3 has a small amount of III in it at the other pHs as well.

When a reaction mixture was allowed to react with Mn^{3+} anaerobically and band 3 was isolated and decomposed as above, the subsequent chromatogram of the CH_2Cl_2

[38] E. LEETE and L. MARION, *Can. J. Chem.* **31**, 775 (1953).

extract did not indicate the presence of III. It thus seems that III could only be formed aerobically.

When band 6 was eluted from the plate and rechromatographed by TLC with chloroform–ethanol (99:1), it was separated into two bands; one at R_f 0·55 was identified as VI. The other along with band 7, gave a positive reaction to the KI-starch test for peroxides.

TLC with chloroform–ethyl acetate (2:3) of a CH_2Cl_2 extract of band 4 treated with 10% KOH for 8 hr revealed an Ehrlich-reagent positive band with R_f and color corresponding to VI and a phenolic band that has the R_f of DCP. A DCP ether of V (2,4-dichlorophenyl skatyl ether) is a possibility. However, when cresol was used as a cofactor, the same bands as those obtained with DCP were found. Visually, the concentrations of the various bands appeared to be the same whether DCP or cresol was used as the cofactor.

Band 5a, when sprayed with Ehrlich reagent on TLC, gave a yellow color, but it was noticed that band 5 invariably had a pink tinge. Another band with the same R_f could be seen on the TLC plate of an extract of an anaerobic reaction-oxidant mixture, where I is not produced. When this minor band was isolated and treated with 10% KOH for 8 hr, the TLC plate of a CH_2Cl_2 extract gave two Ehrlich reagent-positive bands with R_f (0·05 and 0·63) and colors corresponding to potassium indole-3-acetate and VI. Thus, this product could be Hinman and Lang's ester.[13]

To determine if H_2O_2 was present in the reaction-enzyme mixture, peroxidase and a chromagen (*o*-toluidine) were used. The comparative absorbances at 425 nm against controls were 0·47 and 0·06 for 2 mM H_2O_2 and 2 mM IAA in the peroxidase-chromagen solution, respectively. As the absorbance of the IAA solution was 13% of that of the H_2O_2 solution, even though IAA oxidase was acting under suboptimal conditions for the reaction was buffered at pH 7, there must have been at least 0·26 mM H_2O_2 produced in the IAA reaction solution. Thus, when IAA was oxidized, H_2O_2 was produced.

Commercial indole-3-glycolic acid was converted to indole-3-aldehyde by the crude enzyme extract in the presence of cofactors. Lupine IAA oxidase catalyzes the same reaction.[15] No skatole, skatole dimers or free radical signals were found.

When IV was reacted with H_2O_2 in buffer (pH 3·5), I and II were formed. When commercial skatole was treated with H_2O_2 in buffer, a minor UV absorbing band formed, which after elution, gave a spectrum whose absorbing maxima matched those given by Hinman and Bauman[8] for I. However, the major product, R_f 0·60, had the UV spectrum of *o*-aminoacetophenone, a product not found in the enzyme-catalyzed reaction.

Mn^{3+} was prepared as its pyrophosphate salt in solution by the combined procedures of Attenburrow *et al.*[39] and Kenten and Mann.[40] An IAA solution was prepared without cofactors. To 10 ml of this solution was added 0·15 ml of Mn^{3+} solution. The CH_2Cl_2 extract, obtained after 5 min of reaction, was concentrated and examined by TLC with chloroform–ethyl acetate (2:3). Two principal bands were revealed with Ehrlich reagent: a pink band at R_f 0·32 and a yellow band at R_f 0·26. In addition, I was detected by its characteristic absorbtion at 254 and 248 nm.

When a reaction mixture containing IAA and DCP was reacted with a Mn^{3+} solution, there was an instantaneous decolorization of the burgundy color of Mn^{3+}, and subsequent TLC revealed the same products as those obtained using the corn extract. The reaction proceded at various pHs up to pH 7·6. No higher pHs were tested.

[39] J. Attenburrow, A. F. B. Cameron, J. H. Chapman, R. M. Evans, B. A. Hems, A. B. A. Jansen and T. Walker, *J. Chem. Soc.* 1094 (1952).
[40] R. H. Kenten and P. J. G. Mann, *Biochem. J.* **61**, 279 (1955).

To determine if oxygen is required in the non-enzymic reaction, both the reaction containing IAA and DCP and the Mn^{3+} solution were purged for 5 hr with oxygen-free nitrogen; then the two solutions were allowed to react for 10 min. No absorption in the oxindole region was observed. A CH_2Cl_2 extract chromatographed (TLC) displayed the usual six Ehrlich reagent-positive bands with band 3 being particularly prominent. Neither did an anaerobic reaction mixture (without Mn^{2+} or DCP) containing HRP added as a dry powder show any oxindole absorption.

DISCUSSION

From the results reported here and those previously published, it seems obvious that decarboxylation of IAA occurs before oxidation of the indole ring to the oxindole; for if the oxindole was first formed, V could not have been easily obtained. Moreover, Hinman and Lang[13] have shown that oxindole-3-acetic acid cannot act as a substrate of HRP. With the elimination of indole-3-glycolic acid and III as intermediates in the formation of I, a logical alternative appears to be 3-hydroperoxymethylindole (VII).

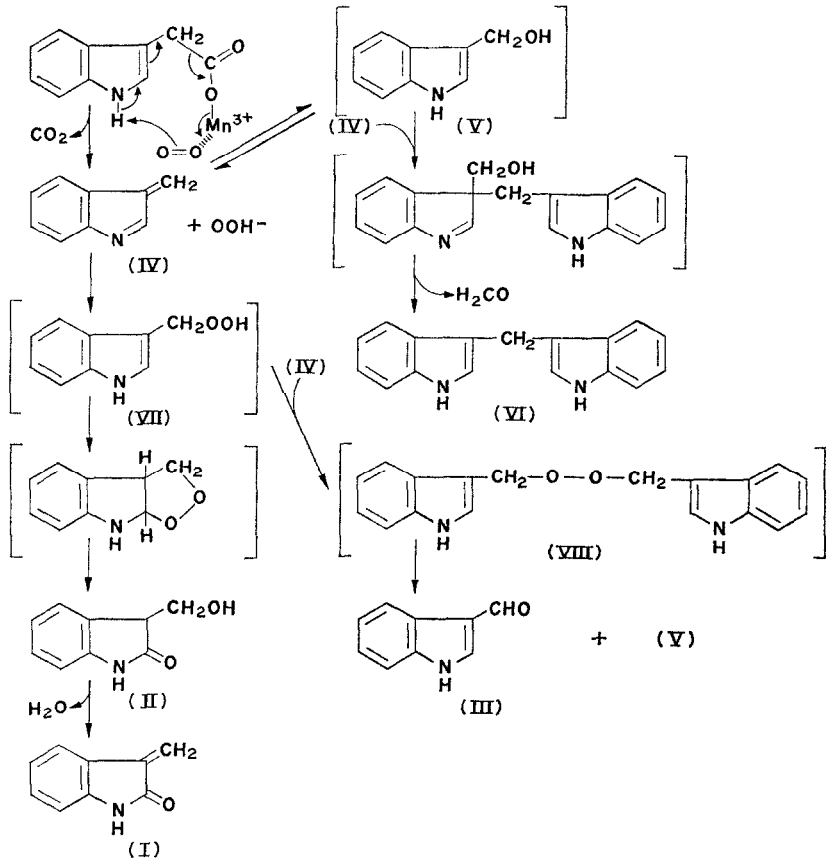
As MacLachlan and Waygood[27,28] pointed out, IAA may not be directly oxidized by an IAA oxidase, but indirectly by Mn^{3+} formed in the mixture. Using a manganese^{3+}–EDTA solution; they were able to decarboxylate IAA. Earlier, Kenten[23] had demonstrated that peroxidase could oxidize Mn^{2+} to Mn^{3+} in the presence of phenols. This oxidation was due to the reaction of some intermediate with Mn^{2+}, as the final solution of peroxidase-oxidized phenol did not oxidize Mn^{2+} to Mn^{3+}. It was found here that Mn^{3+} in the presence of oxygen not only destroys IAA but gives reaction products identical to those of the enzymic reaction. Mn^{3+} also effects the same reactions under anaerobic conditions but in a slower reaction. Why this happens is not clear, but Mn^{3+} could itself accept a pair of electrons to form Mn^{+} which could disproportionate with Mn^{3+} to form Mn^{2+}.

The pathway proposed here (Scheme 2) requires as a key step a two-electron oxidation catalyzed by a metal ion, be it the iron of heme (HRP) or Mn^{3+}, similar to the reactions proposed by Hamilton.[41] It is proposed that the enzyme oxidizes Mn^{2+} to Mn^{3+} and that IAA is subsequently oxidized non-enzymically by Mn^{3+} to 3-methyleneindolenine (IV) with the kind and amount of subsequent products being determined by the pH and composition of the reaction mixture. The pathway was proposed on the basis of the results obtained, viz. that IAA + IAA oxidase or HRP + Mn^{2+} + DCP (aerobically) and IAA + Mn^{3+} (aerobically) gave the products listed in Table 1; IAA + Mn^{3+} (anaerobically) gave only IV and some unidentified products. All the *in vitro* products can be explained by this scheme. Indoleglycolic and indoleglyoxylic acids were isolated by *in vivo* experiments with whole plants[16] and might not be produced by a degradative pathway. The reaction scheme does not require DCP for, as found by Hinman and Lang,[13] both IAA oxidase and HRP can oxidize IAA without its aid.

The first step is analogous to those reactions cited by Hamilton[41] to produce IV or VII. These two products could be in equilibrium since peroxide reactions with enamines are known.[42] The cyclic 5-membered peroxide could then rearrange to a more stable β-keto alcohol, which has been demonstrated to convert to I spontaneously.[13] In support of the proposed pathway is the reaction of IV with H_2O_2 to give II and subsequently I.

[41] G. A. HAMILTON, *Advan. Enzymol.* **32,** 55 (1969).
[42] A. REICHE, E. SCHMITZ and E. BEYER, *Chem. Ber.* **92,** 1212 (1959).

Also, in support of this pathway is the absence of skatole (3-methylindole) when the reaction occurs anaerobically. The conversion of IV into skatole would require hydride addition. The absence of skatole rules out MacLachlan and Waygood's scheme.[27] Neither has an EPR spectrum been found for peroxidase-catalyzed degradation of IAA. Only acidic decomposition of IAA with Ce^{4+} ions has given an EPR spectrum.[31]



SCHEME 2. PROPOSED PATHWAY FOR THE *in vitro* DEGRADATION OF IAA BY CORN IAA OXIDASE. UNBRACKETED SUBSTANCES HAVE BEEN IDENTIFIED IN THIS INVESTIGATION.

However, commercial skatole was also oxidized with H_2O_2 to I. It thus seems possible that there can be a direct oxidation of the C-2 position. However, the major product in the above reaction was one of ring opening as the band of highest concentration at R_f 0·60 had the UV spectrum of *o*-aminoacetophenone, a product not found in the enzyme-catalyzed reaction.

Indole 3-aldehyde, which is only formed in high yields at high concentrations of Japanese radish peroxidase in the presence of H_2O_2,[6] could be formed by the addition of VII across IV to form skatyl peroxide (VIII), which could then break down to give III and V. V could then be converted into IV and VII which could then form more skatyl peroxide and subsequently more III, making it the dominant product under these conditions. This scheme

also explains the ability of α-methyl-IAA and α,α-dimethyl-IAA to act as substrates for HRP, while indole-3-propionic acid and indole-3-butyric acid cannot.[13]

It has been suggested by Meudt[18] that some of the true biological activity might be due to an intermediate which decays to I *in vitro*, but which could retain its activity by binding to macromolecules present in *in vivo* systems. IV could be such a compound.

EXPERIMENTAL

Corn seedlings. Corn (*Zea mays* L.) (single cross WF9 X 38–11) kernels were germinated in a growth chambers in a Vermiculite bed kept moist with distilled H_2O. The growth chamber was set for 14 hr of daylight at 32° and 10 hr of darkness at 23°. The av. ht of the seedlings was *ca.* 15 cm at the time of collection (10 days after planting).

Crude enzyme preparation. Corn extract was prepared by a slight modification of the procedure of van Jaarsveld and Meynhardt.[33] 10-day-old seedlings (100 g) were fragmented with 200 ml of distilled H_2O in a Waring Blendor. To this mixture was added 600 ml of cold acetone (chilled with dry ice). The mixture was filtered by suction, and the residue was washed with another 200 ml of cold acetone. The dry powder was left at room temp. until it was free of acetone and then resuspended in 250 ml of 0·1 M KH_2PO_4 solution. The resulting suspension was stirred 5 hr at 4° and centrifuged. The dissolved protein was precipitated from the centrifugate by addition of $(NH_4)_2SO_4$ to 75% saturation and stirring the mixture overnight at 4°. Protein was then removed by centrifugation at 10 000 *g* for 20 min at 4°. The pellet was dissolved in 1 ml of 0·1 M KH_2PO_4 for each g of fresh tissue used, and the solution was recentrifuged at 10 000 *g* for 20 min at 4°. The supernatant was kept frozen and used as the enzyme preparation. Protein concentration was determined with Folin reagent.

Reaction mixture. Unless otherwise stated, 'reaction mixture' refers to a solution of 0·2 mM IAA, 0·2 mM DCP, 0·2 mM $MnSO_4$, and 0·1 M KH_2PO_4 adjusted to pH 3·5. 'Reaction-enzyme mixture' consists of the above reaction mixture with enzyme extract added (0·15 ml/10 ml of reaction mixture).

Assay of enzyme activity. Activity of the enzyme preparation was determined by either disappearance of substrate (IAA) or appearance of product (I) in the reaction mixture. The disappearance of IAA was followed with Salkowski reagent according to Perley and Stowe.[36] 2 ml of reaction enzyme mixture was treated with 2 ml of Salkowski reagent; the solution was allowed to stand for 90 min at room temp., and the absorbance at 550 nm was determined and compared to a standard curve covering the range of 5–100 mg of IAA per 2 ml of sample. The amount of I was determined from the absorbance at 254 nm and the molar absorptivity (23 500).[8] The reaction-enzyme mixture was allowed to stand for 24 hr at room temp., and the absorbance at 254 nm was determined. As acid solutions cause a slight amount of IAA to be transformed to I, appropriate blanks were carried along all the steps.

Determination of pH optimum. Duplicate samples of the reaction mixture (no DCP) at various pHs were prepared. 10 ml of these reaction mixtures were allowed to react with 0·3 ml of enzyme preparation for 30 min. Then 0·2 ml of 0·42 mM KCN (final concentration 8 mM KCN)[13] was added to each tube. The contents were boiled for 20 min to convert II to I, and the absorbances at 254 nm were determined.

Examination of intermediates and products. The reaction-enzyme mixture was either directly concentrated under diminished pressure or first extracted with 2 vols. CH_2Cl_2, which was then concentrated. The concentrated solution was analyzed by either TLC (one or two-dimensional) or PC. TLC was done on silica gel H at a thickness of 25 μ using (A) $CHCl_3$; (B) $CHCl_3$–EtOAc (1:1); (C) $CHCl_3$–EtOAc (2:3); (D) $CHCl_3$–HOAc (19:1);[37] (E) MeOAc–*iso*-PrOH–25% NH_4OH (9:7:4);[37] (F) $CHCl_3$–EtOH (99:1).[43] PC was done by the descent on Whatman No. 1 paper using (G) *iso*-PrOH–H_2O (1:19);[33] (H) *iso*-PrOH–conc. NH_4OH–H_2O (10:1:1).[43] Detection was by UV irradiation or by spraying with Ehrlich[37] or Salkowski[44] reagents. Peroxides or hydroperoxides were detected according to the method of Stahl.[45]

Anaerobic reactions. For reaction under anaerobic conditions, both the reaction mixture and the oxidant (enzyme extract, etc.) were flushed for 5 hr with N_2 which was scrubbed twice with Fieser reagent[46] and once with a saturated solution of lead acetate. The two solutions were then allowed to react, and the resulting mixture was analyzed as described previously.

Preparation of manganese(III) *(mangani) pyrophosphate.* Hydrated MnO_2 was prepared according to Attenburrow *et al.*[39] The brown solid was dried in an oven at 100–120° and ground to a fine powder. Mn^{3+} was prepared as the pyrophosphate from hydrated MnO_2 and $MnSO_4$ according to the procedure of Kenten and Mann.[40] The presence of mangani pyrophosphate was determined by its absorbance at 258 nm.

[43] H. KALDEWEY, *Dunnschicht-Chromatographie* (edited by E. STAHL), p. 456, Springer, Berlin (1967).
[44] S. A. GORDON and L. G. PALEG, *Physiol. Plant.* **10**, 39 (1957).
[45] E. STAHL, *Arch. Pharm.* **293**, 531 (1960).
[46] L. F. FIESER, *J. Am. Chem. Soc.* **46**, 2639 (1924).

Preparation of 3,3′-*di-indolylmethane* (VI). This was prepared by the action of base on V and crystallized from benzene, m.p. 158–160°, reported[38] 163–164°. The spectrum was identical to that given for VI.[38]

Preparation of indole-3-*aldehyde*. This was prepared by the method of Stutz,[15] and checked by its UV spectrum.[47]

Synthesis of 3-*methyleneoxindole* (I) *from band* 3. Band 3 was isolated from a silica gel TLC plate and added to a pH 3·5 solution of H_2O_2 and allowed to react for 1 hr. The solution was extracted with 10 ml of CH_2Cl_2. The extract was concentrated and subjected to TLC with solvent C. Two UV absorbing spots were eluted with MeOH. The spectra were those of II and I.

Synthesis of 3-*indoleacetonitrile from band* 3. Band 3 was isolated from a silica gel TLC plate and added to 5 ml of 0·1 M KH_2PO_4 containing 0·5 g of KCN and adjusted to pH 3·5. The mixture was heated on a steam cone for 1 hr, cooled, and extracted with 20 ml of CH_2Cl_2. The concentrated extract was investigated by TLC with solvent F. Five Ehrlich reagent positive bands were obtained. When eluted with MeOH, the grey Ehrlich reagent-positive band at R_f 0·30 gave a spectrum identical to that of 3-indoleacetonitrile.[47]

Determination of the presence of hydrogen peroxide. 2 ml of 2 mM IAA solution was allowed to react for 3 hr with 2 ml of a reagent containing 0·5 mg HRP and 4·7 mg of *o*-toluidine in 0·1 M phosphate buffer, pH 7.0. 2 ml of 2 mM H_2O_2 solution was similarly tested, but the reaction time was 10 min. Individual blanks were prepared for each of the two reactions. The absorbance of each reaction mixture was determined against its blank at 425 nm in 10 mm silica cells.

[47] *Sadtler Research Laboratories*, Philadelphia, Pa. "Sadtler Standard UV Spectra" (1969).