

# On the Role of Hydrogen Peroxide in Peroxidase Catalyzed Metabolism of Indole-3-acetic Acid

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Z. Naturforsch. 37 c, 884–888 (1982); received July 29, 1982

Indole-3-acetic Acid, IAA-Oxidase, Peroxidase

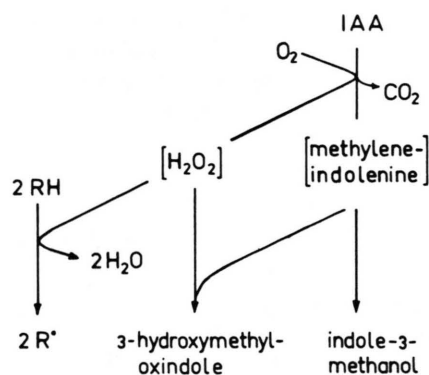
Formation of  $H_2O_2$  in the course of peroxidase catalyzed IAA metabolism was measured using the peroxide-dependent decarboxylation of [ $^{14}C$ ]glyoxylate and the oxidation of phenolic substrates giving rise to fluorescent products in IAA/HRP systems. The results agree with the recent concept of IAA metabolism which includes the formation of  $H_2O_2$  in the course of oxidase action on IAA and the consumption of this  $H_2O_2$  by subsequent peroxidase action in the presence of phenolic cosubstrates.

## Introduction

Existing assumptions about the mechanism of peroxidase catalyzed oxidation of indole-3-acetic acid (IAA) and the pathway of IAA metabolism are still controversial. Some workers have proposed a free-radical mechanism which involves a reaction of  $O_2$  with IAA free-radicals but not with peroxidase [1–3]. Others have proposed an oxygenase-type mechanism which involves a direct activation of  $O_2$  by peroxidase [4–8]. The main products of the IAA oxidation reaction were thought to be indole-3-aldehyde (IAI) and 3-hydroxymethyloxindole (HMOx) which is nonenzymatically converted into 3-methyleneoxindole (MOx).

Very recently a number of arguments were presented that the peroxidase catalyzed degradation of IAA results in the formation of indole-3-methanol (IM) in the presence of phenolic compounds or of HMOx in their absence [9]. The results were interpreted in favour of the assumption that the phenols compete with a methyleneindolenine intermediate for  $H_2O_2$  which is produced by oxidase action preceding the peroxidase action in the course of IAA degradation (Fig. 1). If this  $H_2O_2$  is consumed

by peroxidase action in the presence of phenols, then IM is virtually the only product which is eventually converted into the corresponding aldehyde (IAI). Additional results have indicated that such a cooxidation of IAA and phenols also plays a dominant role in wheat leaves (manuscript in prep.). Certainly, such a cooxidation of IAA and phenols coupled through the formation of  $H_2O_2$  by oxidase action of IAA may be related to the function of IAA and phenols in biological systems. In accordance with this view the "oxindole pathway" leading to HMOx would function to remove excess  $H_2O_2$  in case of low concentrations of phenols thus providing



**Abbreviations:** HMOx, 3-hydroxymethyloxindole; HPPA, 3-(*p*-hydroxyphenyl)propionic acid; HRP, horseradish peroxidase; IAA, indole-3-acetic acid; IAI, indole-3-aldehyde; IM, indole-3-methanol; MOx, 3-methyleneoxindole; TLC, thinlayer chromatography.

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0341-0382/82/1000-0884 \$ 01.30/0

Fig. 1. Peroxidase catalyzed metabolism of IAA (simplified version according to Grambow and Langenbeck [9]). This concept includes the production of  $H_2O_2$  in the course of oxidase action upon IAA, the consumption of this  $H_2O_2$  by peroxidase action in the presence of phenolic cosubstrates (RH), or, alternatively, the detoxification of this  $H_2O_2$  by reaction with a methyleneindolenine intermediate to form an oxindole in the absence or at low concentrations of the phenols.

a self-detoxifying property to the IAA-phenols co-oxidizing system.

The formation of  $\text{H}_2\text{O}_2$  is critical to the concept cited above. In this paper some additional evidence and more detailed information is given concerning the formation of  $\text{H}_2\text{O}_2$  in peroxidase catalyzed IAA degradation.

### Materials and Methods

Purified HRP was obtained from Boehringer (HRP grade I; RZ approx. 3.0).  $[1\text{-}^{14}\text{C}]$ glyoxylic acid and  $[\text{U-}^{14}\text{C}]$ phenol were obtained from Amersham/Buchler. IAA was from Sigma, tyramine was from Serva, and HPPA was from Fluka. Hydrogen peroxide solution (Merck) was standardized by acid permanganate titration.

Hydrogen peroxide formation was monitored by trapping  $^{14}\text{CO}_2$  release from  $[1\text{-}^{14}\text{C}]$ glyoxylic acid [10] or by measuring fluorescence intensity of fluorescing products formed from HPPA or tyramine in  $\text{H}_2\text{O}_2$ /HRP-systems [11] and  $\text{H}_2\text{O}_2$ -generating IAA/HRP systems. Fluorescence was measured using a Jobin Yvon JY3D spectrofluorimeter at 414 nm with the excitation at 288 nm (data are uncorrected).

Enzymic reactions were performed in phosphate buffer, 0.05 M, pH 5.0. For the purpose of quantitative  $\text{H}_2\text{O}_2$  estimation by fluorescence measurements, the procedure was as follows: HPPA, tyramine,  $\text{H}_2\text{O}_2$ , or IAA were added from appropriate stock solutions to the reaction mixture (for concentrations see Results). The final volume was 4 ml. The reaction was started by the addition of HRP from a diluted stock solution at 22 °C. Fluorescence was measured after completion of the reaction (see Results).

To investigate the products formed from  $[\text{U-}^{14}\text{C}]$ phenol, the latter instead of HPPA was added to  $\text{H}_2\text{O}_2$ /HRP systems or to IAA/HRP systems. After 30 min 50  $\mu\text{l}$  aliquots were applied to 7 cm sections of TLC plates (KG F<sub>254</sub>; Merck) which had been marked before with an appropriate mixture of reference compounds. The solvent system was benzene/dioxane/acetic acid (90:25:4). For quantitative estimation the compounds were scraped of from the plates and mixed with scintillation reagent and thixotropic gelling agent. Radioactivity was measured using a Berthold BF 8000 scintillation counter programmed for automatic quench correction.

### Results and Discussion

Measuring the  $\text{H}_2\text{O}_2$ -dependent release of  $^{14}\text{CO}_2$  from radiolabelled  $\alpha$ -keto acids provides a sensitive method of determining  $\text{H}_2\text{O}_2$  [10]. When using this method to study the production of  $\text{H}_2\text{O}_2$  in the HRP/IAA system, a significant increase in the release of  $^{14}\text{CO}_2$  from  $[^{14}\text{C}]$ glyoxylate was observed in the absence of a phenolic factor (Fig. 2). On a molar basis, however, the amounts of  $^{14}\text{CO}_2$  released, *i.e.* the amounts of  $\text{H}_2\text{O}_2$  available for the decarboxylation reaction, were small compared to the amounts of IAA oxidized during the reaction period. When phenol was added to the system, then the availability of  $\text{H}_2\text{O}_2$  as indicated by the release of  $^{14}\text{CO}_2$  was drastically decreased. At a glance, such a result could be misleadingly interpreted to favour the assumption that  $\text{H}_2\text{O}_2$  production, though significant in the absence of phenols, does not play a major role in IAA degradation catalyzed by peroxidase.

However, according to the IAA-phenol cooxidation model cited in Fig. 1, it would be expected that

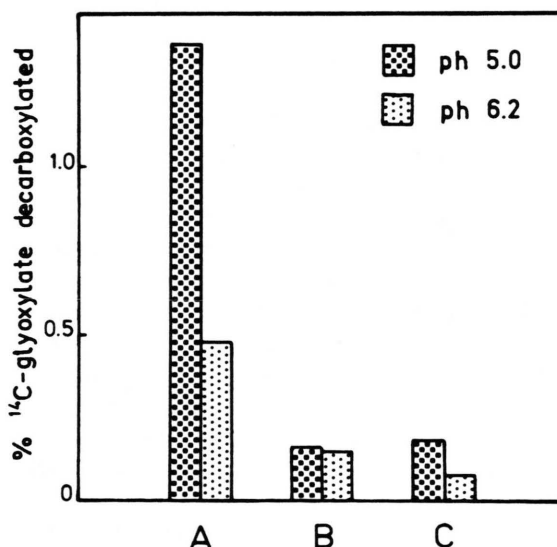


Fig. 2. Decarboxylation of  $[^{14}\text{C}]$ glyoxylate in HRP systems containing: A: IAA ( $4 \times 10^{-6}\text{ M}$ );  $^{14}\text{C}$ -glyoxylic acid ( $10^{-5}\text{ M}$ ); and HRP ( $10^{-8}\text{ M}$ ); B: same as A, but phenol ( $10^{-4}\text{ M}$ ) added; C: same as A, but IAA omitted. Reaction time was 1 h. Under the conditions of this test (at pH 5) about 90% of the IAA were consumed as well in the presence as in the absence of phenol (not shown in figure). While the amounts of  $[^{14}\text{C}]$ glyoxylate decarboxylated were significantly increased in experiment A compared to B and C, the percentage of glyoxylate decarboxylated was still very low.

equimolar amounts of  $\text{H}_2\text{O}_2$  are produced in the course of IAA oxidation and that this  $\text{H}_2\text{O}_2$  is consumed by peroxidase activity in the presence of phenols or by reaction with the methyleneindole-nine intermediate. Since it is possible that the  $\text{H}_2\text{O}_2$  is not readily released from the protein or that its consumption by the reactions described above is fast compared to its reaction with the  $\alpha$ -keto acid, one would suspect that  $\text{H}_2\text{O}_2$  production during the peroxidase-catalyzed IAA metabolism cannot be reliably determined using the test system described above.

A different approach to this problem has now led to results which are in full agreement with the model cited above. It has been previously shown [9] that no specificity exists concerning the phenolic cosubstrates in the peroxidase-catalyzed IAA degradation. Consequently, it should be possible to measure the  $\text{H}_2\text{O}_2$ -dependent formation of fluorescent products from suitable phenolic substrates, *e.g.* HPPA or tyramine [11]. The results obtained using such systems containing IAA, peroxidase, and HPPA or tyramine are presented in Fig. 3. As judged on the basis of fluorescence measurements, it is evident from the " $\text{H}_2\text{O}_2$  curves" (A I, B I) and the "IAA curves" (A II, B II) that considerable amounts of  $\text{H}_2\text{O}_2$  must have been produced during IAA oxidation under the conditions of the test. The discrepancy between curves I and II cannot be fully explained on the basis of the available results. It does not necessarily mean that less than equimolar amounts of  $\text{H}_2\text{O}_2$  are produced from IAA at the higher concentrations. Instead, some additional experiments (not described in detail) indicated that the observed discrepancy is the result of opposing effects upon the fluorescence intensities caused by the various factors present in the complex systems. Thus, indolic products of IAA oxidation appear to contribute to increased intensity of fluorescence at low concentrations of IAA and to increased quenching of fluorescence at the higher concentrations of IAA metabolites when in combination with high concentrations of phenolic products. So far however, the complexity of the systems rendered it difficult to apply standard techniques for correction of fluorescence data.

If the phenolic substrates were added after consumption of the IAA, then clearly reduced yields of  $\text{H}_2\text{O}_2$  could be detected on the basis of fluorescence measurements (Fig. 3; A III, B III). This result can

easily be explained on the basis of the model cited above (Fig. 1) assuming that the  $\text{H}_2\text{O}_2$  produced during IAA oxidation is consumed by oxindole formation under these conditions.

It is also evident that the fluorescence intensity, *i.e.* the amount of  $\text{H}_2\text{O}_2$  generated by oxidation of IAA, is not significantly affected by changing the relative concentrations of IAA and the enzyme, *i.e.* the IAA/HRP ratio (Fig. 4). Such a result is important since it may help to clarify some earlier observations, namely that the nature of products formed from IAA varies with the substrate/enzyme ratio [1, 12]. Apparently, such findings are at variance with the results presented above. As cited in Fig. 1, the nature of the products formed from IAA is correlated with the mode of  $\text{H}_2\text{O}_2$  consumption, *i.e.* with the availability of phenolic cosubstrates. In accordance with this model, the nature of products varies with the IAA/phenol ratio [9]. Correspondingly, even small amounts of phenolic impurities

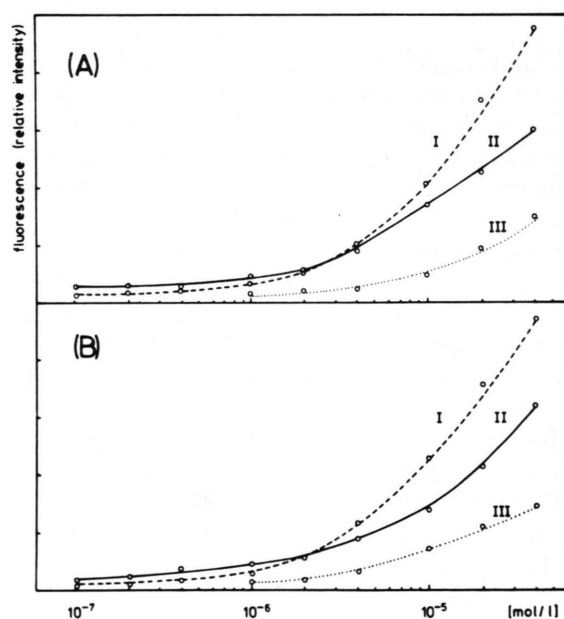


Fig. 3. Fluorescence intensities as a function of different concentrations of  $\text{H}_2\text{O}_2$  or of IAA in the following systems: A I)  $\text{H}_2\text{O}_2$ /HPPA/HRP; A II) IAA/HPPA/HRP; A III) IAA/HRP; HPPA added after 1 h; B I)  $\text{H}_2\text{O}_2$ /tyramine/HRP; B II) IAA/tyramine/HRP; B III) IAA/HRP; tyramine added after 1 h. Concentrations of  $\text{H}_2\text{O}_2$  and IAA are indicated on the abscissa. Concentrations of HPPA or tyramine were  $4 \times 10^{-4}$  mol/l. Concentration of HRP was  $2 \times 10^{-7}$  mol/l. Fluorescence was measured 2 h after starting the reaction.

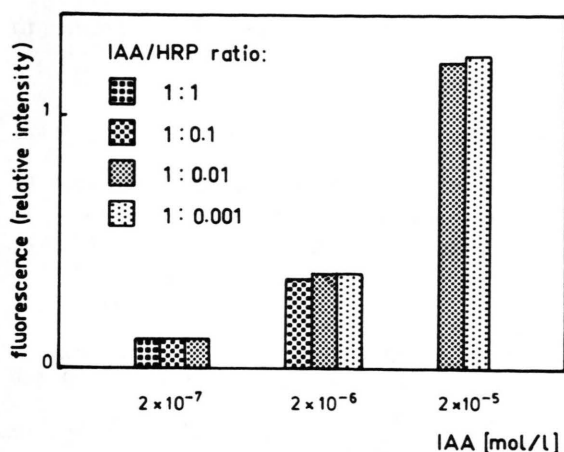


Fig. 4. Fluorescence intensities in IAA/HPPA/HRP systems. The molar ratio of IAA/HPPA was kept constant (1:20) while the molar ratio of IAA/HRP varied from 1:1 to 1:0.001 as indicated. Measurements were performed after completion of the reaction (5 h at the highest substrate/enzyme ratio).

present in the test system would affect the nature of the products. Since extremely high peroxidase concentrations were used in the earlier investigations, and since it is difficult to prepare proteins of plant origin definitively free of phenolic material, a possible alteration of the IAA/phenol ratio and, consequently, of the nature of the products formed cannot be fully excluded because of phenolic contamination.

According to widely accepted assumptions, phenolic substrates act in peroxidase-catalyzed reactions by single-electron transfers on  $\text{H}_2\text{O}_2$  to produce  $\text{H}_2\text{O}$  and free radicals which finally combine to form coupled products. The results shown in Fig. 5 indicate that the same pattern of products is obtained in HRP/IAA/phenol or in HRP/ $\text{H}_2\text{O}_2$ /phenol systems. These results provide another strong argument that the oxidation of IAA is coupled to the oxidation of the phenolic substrate by  $\text{H}_2\text{O}_2$  produced by oxidase action on IAA.

The formation of *o,o'*-biphenol in an HRP/ $\text{H}_2\text{O}_2$ /phenol system has been described [13]. Under the conditions of this study, however, a characteristic product from phenol was apparently *p,p'*-biphenol instead of *o,o'*-biphenol as was demonstrated by comparison with authentic standards using TLC.

As far as HPPA is concerned, purified products resulting from HPPA in IAA/HRP systems or

$\text{H}_2\text{O}_2$ /HRP systems exhibited identical fluorescence excitation and emission spectra (results not shown in detail). This provides further evidence that the same type of products is formed in the presence of IAA or of  $\text{H}_2\text{O}_2$ .

Observed effects of  $\text{H}_2\text{O}_2$  on the peroxidase catalyzed metabolism of IAA have often been discussed in relation to peroxidative inactivation of inhibitory polyphenolic compounds [e.g. 14]. On the other hand, the observation that IAA added to pea stem sections promoted pyrogallol oxidation was taken as an indication that  $\text{H}_2\text{O}_2$  could have been produced during IAA oxidation in the system [15]. Owing to some inconsistencies the latter interpretation has been critically reviewed [16]. Further substantiation comes from a notice showing that there were some amounts of  $\text{H}_2\text{O}_2$  found in a crude corn extract/IAA system [17]. In addition, production of  $\text{H}_2\text{O}_2$  by oxidase action on IAA and its involvement in subsequent processes during the course of IAA metabolism was indicated by the effect of catalase and  $\text{H}_2\text{O}_2$  on the type of products formed in IAA/

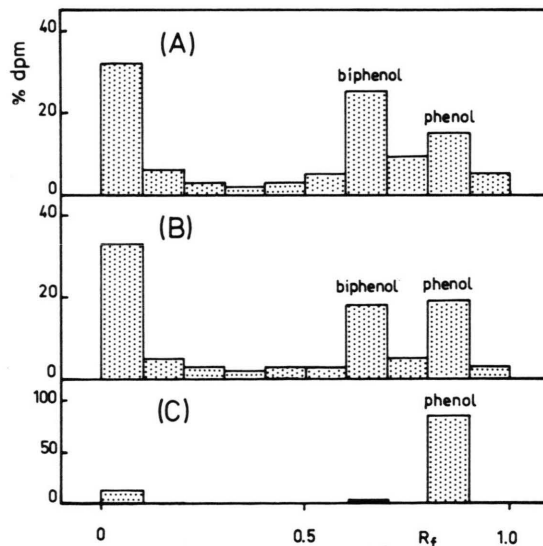


Fig. 5. Incorporation of radioactivity from  $[\text{U}-^{14}\text{C}]$ phenol into *p,p'*-biphenyl and unknown products in the following systems: A:  $[\text{U}-^{14}\text{C}]$ phenol ( $2 \times 10^{-6}$  M); IAA ( $2 \times 10^{-6}$  M); HRP ( $2 \times 10^{-8}$  M); B:  $[\text{U}-^{14}\text{C}]$ phenol ( $2 \times 10^{-6}$  M);  $\text{H}_2\text{O}_2$  ( $2 \times 10^{-6}$  M); HRP ( $2 \times 10^{-8}$  M). C:  $[\text{U}-^{14}\text{C}]$ phenol ( $2 \times 10^{-6}$  M); HRP ( $2 \times 10^{-8}$  M). Reaction time was 1 h. Products were separated by TLC. Apparently similar patterns of labelled products were obtained in systems A and B.

HRP systems [9]. Finally, the results also agree well with earlier observations that the oxidation of IAA by peroxidase proceeds with the consumption of 1 mol O<sub>2</sub>/mol IAA oxidized, and the formation of 1 mol CO<sub>2</sub>/mol O<sub>2</sub> consumed [e.g. 14].

Summarizing, clear evidence is presented that H<sub>2</sub>O<sub>2</sub> is produced in an oxidase step during the course of IAA oxidation and that this H<sub>2</sub>O<sub>2</sub> is consumed by subsequent peroxidase action in the

presence of phenolic cosubstrates according to Fig. 1.

#### Acknowledgement

I am grateful to Prof. H. J. Reisener and Prof. R. C. Staples for discussing the manuscript, to G. E. Grambow for assistance, and to the Deutsche Forschungsgemeinschaft for financial support.

- [1] R. L. Hinman and J. Lang, *Biochem.* **4**, 144 (1965).
- [2] R. Nakajima and I. Yamazaki, *J. Biol. Chem.* **254**, 872 (1979).
- [3] I. Yamazaki, M. Tamura, and R. Nakajima, *Mol. Cell. Biochem.* **40**, 143 (1981).
- [4] L. R. Fox, W. K. Purves, and H. I. Nakada, *Biochem.* **4**, 2754 (1965).
- [5] R. W. Miller and E. V. Parups, *Arch. Biochem. Biophys.* **143**, 276 (1971).
- [6] D. A. Gelinas, *Plant Physiol.* **51**, 967 (1973).
- [7] J. Ricard and D. Job, *Eur. J. Biochem.* **44**, 359 (1974).
- [8] J. Macháčkova, K. Gančeva, and Z. Zmrhal, *Phytochemistry* **14**, 1251 (1975).
- [9] H. J. Grambow and B. Langenbeck-Schwich, *Planta*, submitted.
- [10] E. F. Elstner and A. Heupel, *Planta* **130**, 175 (1976).
- [11] K. Zaitse and Y. Ohkura, *Anal. Biochem.* **109**, 109 (1980).
- [12] Y. Morita, Y. Kominato, and K. Shimizu, *Mem. Res. Inst. Food Sci. Kyoto Univ.* **28**, 1 (1967).
- [13] D. J. Danner, P. J. Brignac, D. Arceneaux, and V. Patel, *Arch. Biochem. Biophys.* **156**, 759 (1973).
- [14] R. H. Kenten, *Biochem. J.* **59**, 110 (1955).
- [15] S. M. Siegel and A. W. Galston, *Arch. Biochem. Biophys.* **54**, 102 (1955).
- [16] P. M. Ray, *Annu. Rev. Plant Physiol.* **9**, 81 (1958).
- [17] J. N. BeMiller and W. Colilla, *Phytochemistry* **11**, 3393 (1972).