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# Effects of Enzyme/Substrate Ratio and of Cofactors on the Oxidation Products of Indole-3-Acetic Acid Catalyzed by Peroxidase

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During the oxidation of indole-3-acetic acid catalyzed by peroxidase, the relative amounts of the products closely depends on the enzyme/substrate ratio.

In the absence of cofactors, high enzyme/substrate ratio induces a rise in the level of indole-3-aldehyde and indole-3-methanol, and a drop in that of oxindoles.

2,4-dichlorophenol, although a very efficient cofactor, promotes inhibition of the oxidation after a few minutes, presumably through the formation of a phenol-derivative inhibitor. 2-4-dichlorophenol also inhibits the production of oxindoles at all stages. Both inhibitory effects are abolished by a low concentration of enzyme.

$Mn^{2+}$, itself a weak inhibitor, synergizes the catalytic effect of 2,4-dichlorophenol, perhaps by preventing the formation of the inhibitor.

The results are discussed against more widely accepted mechanisms of indole-3-acetic acid oxidation.

Indole-3-acetic acid (IAA) is a characteristic plant hormone involved in the growth and many aspects of the differentiation of plant cells and tissues. The regulation of its concentration in the plant has, therefore, an undoubted physiological meaning and, besides the biosynthesis and conjugation, the biochemical process that directly influences the level of the hormone is its oxidation. This is catalyzed by peroxidases (E.C. 1.11.1.7) and the reaction has been the subject of many studies since KENTEN described it in 1955 (7). However the mechanism of the reaction remains still unsolved owing to its complexity and the fact that many factors can influence qualitative and quantitatively the results. Among those factors the concentration of enzyme and substrate and the effect of phenolic substances and other possible cofactors have been extensively studied (1-5, 8-13, 16) because they can obviously play a physiological role.

According to the mechanism proposed by RICARD and JOB (13) the relative

concentration of enzyme and substrate (E/S ratio) can induce different levels of the active forms of peroxidase and yield, consequently, different products. Thus, with equimolar concentrations of enzyme and substrate, Compound III is the predominant form of the enzyme and indole-3-aldehyde (IAld) the main product of the oxidation of IAA, whereas for lower E/S ratios Compound II is increasingly formed, oxindoles being now preferently produced. In this and other proposed mechanisms IAld and oxindoles are the products almost exclusively considered.

In the present paper the effect of different E/S ratios on the production of indole-3-methanol (IM) is studied and observed for the first time. Influence of the cofactors 2,4-dichlorophenol (DCP) and $Mn^{2+}$ on the products of the reaction have never been reported. We have also found and studied such influences.

## Materials and Methods

*Enzyme.* Lyophilized horseradish peroxidase (HRP), Calbiochem, Grade B, RZ = 0.71 was dissolved in phosphate buffer 0.1 M pH 6.3. The concentrations were calculated on the basis of a molecular weight of 40,000. The commercial enzyme contains 4 isoenzymes. Occasionally the enzyme type VI (two isoenzymes) from Sigma was used with the same results.

*Enzymatic reaction.* Was performed at 30° C with continuous stirring. The mixtures contained IAA $3 \times 10^{-4}$ M, phosphate buffer $6 \times 10^{-2}$ M, pH 6.3 and HRP in variable amounts. When DCP and $MnCl_2$ were used as cofactors, their final concentration was $10^{-4}$ M.

*Measure of $O_2$ uptake.* An oxygraph with Clark electrode was used. The reaction mixtures were as stated previously and the final volume, 3 ml. Before the addition of the enzyme the vessel was air saturated by bubbling at 30° C, so that the initial content of oxygen was 0.63 μmoles.

*Analysis of substrate and products.* For this purpose the volume of the reaction media used was 75 ml. The substances were extracted, concentrated, chromatographied and eluted as stated in a previous paper (14). For the quantitative measurements the following wavelengths were used: IAA, 280 nm ($\epsilon = 4{,}020\ M^{-1}\ cm^{-1}$); HMO, 250 nm ($\epsilon = 19{,}000\ M^{-1}\ cm^{-1}$); IAld, 295 nm ($\epsilon = 9{,}650\ M^{-1}\ cm^{-1}$) and IM, 280 nm ($\epsilon = 5{,}410\ M^{-1}\ cm^{-1}$).

## Results

*Whithout cofactors.* In the study of the products formed with different E/S ratios, media were used with the same concentration of substrate and variable concentrations of enzyme. Under those conditions, using similar time of reaction for the different media would not be comparative (the rates and products of reaction are very dissimilar) for which reason the media were rather allowed to react up to a fixed consumption of oxygen. Since the reactions will take place in open vessels, previous test were performed in oxygraph (closed vessel) to determine the correlations. When 50 % of the oxygen initially present in the vessel remains unreacted its concentration is not yet rate limiting. One can then measure graphycally the times needed to consume 50 % of the oxygen by reaction media with different concentration of the en-

*Abbreviations:* IAA, indole-3-acetic acid; IAld, indole-3-aldehyde; HMO, 3-hydroxymethyloxindole; IM, indole-3-methanol; DCP, 2,4-dichlorophenol; HRP, horseradish peroxidase; $Fe_p^{3+}$, ferriperoxidase; CoI, CoII, CoIII, intermediate active compounds of peroxidase; R'OOH, hydroperoxide form of skatole.

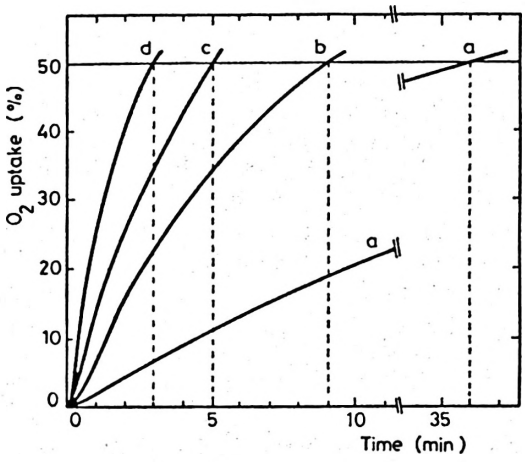


Fig. 1. *Polarographic measurements of oxygen uptake along the time by media with four concentrations of HRP.*
*a*) 0.4 μM; *b*) 1.6 μM; *c*) 2.6 μM; *d*) 5.3 μM. The values of oxygen are expressed as percentage of the initial content (0.63 μmoles) in the air saturated media. Times used to consume 50 % of oxygen are calculated graphycally.

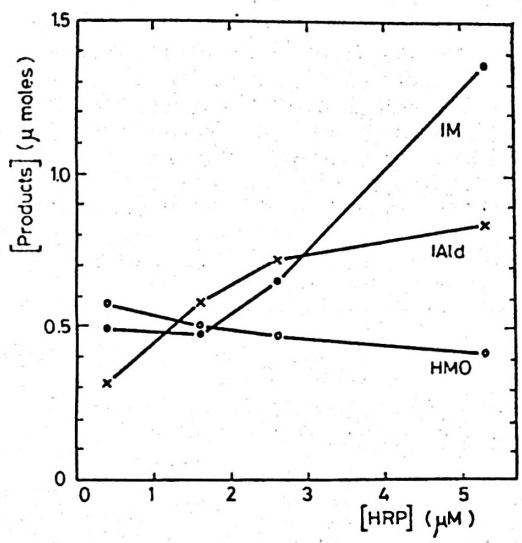


Fig. 2. *Products of the oxidation of IAA with four concentrations of HRP.*
Times of reaction were in each case the needed to consume 7.8 μmoles of oxygen. HRP concentrations were the shown in figure 1.

zyme. For the concentrations of enzyme used: 0.4, 1.6, 2.6 and $5.3 \times 10^{-6}$ M the times were, respectively 36, 9, 5 and 3 minutes.

Those were the conditions used for the reactions in open vessels. The results (figure 2) show that high concentrations of enzyme (and, therefore, the E/S ratio) increase the amount of IM and IAld produced, although in different form, and diminish that of 3-hydroxymethyloxindole (HMO).

*With cofactors.* Usual cofactors in the oxidation of IAA are DCP and $Mn^{2+}$. According to the results in table I, DCP inhibits the formation of oxindoles, specially when the E/S ratio is high. In spite of that, as expected, its presence lead to the oxidation of larger amounts of substrate. However the formation of IM and, in lesser extent, of IAld are favoured by the monophenol. $Mn^{2+}$ is itself an inhibitor, but reinforces the effects produced by DCP.

The results in table I correspond to measurements made after a time of reaction, 20 min, that for comparative purposes is common to all the six assays. The low levels of IAA at the end of the reaction in the media with DCP are due to its known role of cofactor, but the significant increase in the substrate consumed in C′ as compared to C reveals a possible inhibition of the effects of DCP when level of enzyme is high. Table II shows the results of the determination of substrate and products along the time in a reaction medium similar to C of table I. As can be seen the reaction takes place very quickly in the first few minutes but it fades even before the concentration of substrate become rate-limiting (addition of more substrate does not increase the rate of oxygen uptake).

Table I. *Effect of DCP and $Mn^{2+}$ on the oxidation products of IAA with two concentrations of enzyme.*
The concentrations of HRP were $4 \times 10^{-6}$ M in the media A, B, C and D, and $2 \times 10^{-7}$ M in the media C' and D'. Cofactors were used as follows: A = no cofactors; B = $Mn^{2+}$; C and C' = DCP; D and D' = DCP + $Mn^{2+}$. The time of reaction was 20 minutes in all cases. The rest of conditions as in Materials and Methods.

| Medium | HRP (μM) | IAA consumed (% of the initial) | Products (μmoles) HMO | IM | IAld |
|---|---|---|---|---|---|
| A | 4 | 70.2 | 0.60 | 0.20 | 0.26 |
| B | 4 | 49.6 | 0.27 | 0.00 | 0.11 |
| C | 4 | 83.2 | 0.00 | 1.51 | 0.59 |
| D | 4 | 96.9 | 0.00 | 10.35 | 0.27 |
| C' | 0.2 | 96.6 | 0.23 | 4.27 | 1.35 |
| D' | 0.2 | 100.0 | 0.20 | 5.90 | 1.12 |

Table II. *Products along the time.*
The reaction media were identical to C in table I.

| Time of reaction (min) | IAA consumed (% of the initial) | Products (μmoles) HMO | IM | IAld |
|---|---|---|---|---|
| 3 | 68.0 | 0.00 | 0.72 | 0.52 |
| 5 | 75.3 | 0.00 | 0.85 | 0.53 |
| 10 | 77.7 | 0.00 | 0.97 | 0.55 |

## Discussion

The increase in the production of IAld and the decrease of HMO when E/S ratio is high agree well with the results obtained by MORITA *et al.* (11) and can be satisfactory explained in the context of the mechanism proposed by RICARD and JOB (13) which stablishes two different routes for the production of, respectively, IAld and oxindoles. However the accumulation of IM under the same conditions of high E/S ratio could not be found by MORITA nor explained by RICARD (the latter do not include IM in their proposed mechanism) althouhg it happens to be even more pronounced than that of IAld. Thus IM can be situated in the route of formation of IAld, as differentiated from that of oxindoles and reinforces the view of the authors (14) that IM is an intermediary: IAA → IM → IAld. This view is supported by the fact that the levels of IM and IAld in figure 2 show complementary evolutions.

The use of cofactors is physiologically meaningfull because both, monophenols and manganese are normal constituents of plant cells.

The lack of measurable amounts of HMO when DCP is present and the concentration of enzyme is high (table I, C and D) shows that DCP, not only speeds the oxidation of IAA (that has been known for long) but also influences the route of oxidation. If we assume that DCP reduces the active forms of the enzyme (FOX and PURVES [1] demonstrated that it accelerates the transformation of Compound I into Compound II) the consequences, expressed in terms of the mechanism of IAA oxidation proposed by NAKAJIMA and YAMAZAKI (12), would be the following:

*a*) A rise in the level of the ferric form of the enzyme:

$$CoI + DCP \rightarrow CoII + DCP^{\cdot}$$
$$CoII + DCP \rightarrow Fe_p^{3} + DCP^{\cdot}$$

*b*) A rise in the level of free radicals of IAA:

$$DCP^{\cdot} + IAA \rightarrow DCP + IAA^{\cdot}$$

and consequently, of hydroperoxide

$$IAA^{\cdot} + O_2 \rightarrow R'OO \cdot + CO_2$$
$$R'OO^{\cdot} + IAA \rightarrow R'OOH + IAA^{\cdot}$$

A higher level of hydroperoxide could yield more oxindoles through a slow, non enzymatic, process, but the presence of large amounts of ferric enzyme and the high value of $K_1$ allows that the reaction

$$Fe_p^{3+} + R'OOH \xrightarrow{K_1} CoI + R'OH\ (IM)$$

compete in advantage for the hydroperoxide.

Thus, the presence of the monophenol would produce the same results as a high concentration of the enzyme, as it is the case.

A high concentration of IM formed makes possible (but not necessary) a rise in the production of IAld, as has been proposed recently (14).

According to the results in table II the oxidation of IAA in the presence of DCP takes place rapidly at the beginning but it slows after few minutes. This effect appears before the concentration of IAA becomes rates limiting and is not observed in the absence of DCP. Although it has not been studied, one should expect that DCP, as a free radical, not only react with IAA, but also undergo some other transformations that result in the formation of an inhibitor (the corresponding diphenol could be such an inhibitor). The effect of $Mn^{2+}$ may be interpretated in this context. It does not accelerate the oxidation of IAA in the absence of DCP (it rather inhibit it: see table I-B) but it overcomes the inhibitory effect of DCP, presumably by preventing the formation of the inhibitor (6, 15). As can be seen in table I the IAA consumed in D approaches 100 %.

## Resumen

Durante la oxidación del ácido indol-3-acético catalizada por peroxidasa las cantidades relativas de los productos formados dependen de la relación enzima/sustrato.

En ausencia de cofactores, una relación enzima/sustrato alta induce un aumento en el nivel de indol-3-aldehído e indol-3-metanol y un descenso en el de oxindoles.

El 2,4-diclorofenol, aunque es un cofactor muy eficiente, da lugar a una inhibición en la oxidación al cabo de pocos minutos, posiblemente por medio de la formación de un derivado suyo con carácter inhibidor. El 2,4-diclorofenol inhibe, además, la formación de oxindoles. Ambos efectos inhibitorios desaparecen con bajas concentraciones de enzima.

El $Mn^{2+}$, que por sí mismo es un inhibidor débil, sinergiza el efecto catalítico del 2,4-diclorophenol, quizá evitando la formación de inhibidor.

Los resultados se discuten atendiendo a los mecanismos más aceptados para la oxidación del ácido indol-3-acético.

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