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THE FORMATION AND REACTIVITY OF PEROXIDASE COMPOUND III

JACQUES RICARD AND JOANNÈS NARI
Laboratoire de Physiologie Végétale, Faculté des Sciences, Marseille (France)

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SUMMARY

1. Indoleacetic acid can, under certain conditions (acidic medium in particular), reduce horseradish peroxidase (donor:H_2O_2-oxidoreductase, EC 1.11.1.7). It can also reduce phenosafranine, the oxidation–reduction potentials of which, at various pH values, are very close to those of peroxidase. These results are obtained in the absence of peroxide in the medium. They confirm those described in an earlier report.

2. If ferriperoxidase is reduced by H_2 or by the semiquinone of methylviologen, the appearance of the Compound III spectrum may be recorded in the presence of O_2. Compound III obtained under these conditions is therefore identifiable with oxyferroperoxidase.

3. Oxyferroperoxidase appears to be capable of oxidizing indoleacetic acid and catechol. This last substance can also be destroyed by the ferriperoxidase–indoleacetate system. Catechol degradation is achieved under these conditions by 2 different mechanisms:

(a) A peroxidation reaction leading, probably, to the appearance of *o*-benzoquinone;

(b) A degradation reaction of a different type (perhaps an oxygenation) conditioned by the ferroperoxidase–oxyferroperoxidase system, insensitive to cyanide, and leading to the appearance of substances different from *o*-benzoquinone.

INTRODUCTION

It is generally accepted that horseradish peroxidase (donor:H_2O_2 oxidoreductase, EC 1.11.1.7) cannot act as an oxygenase[1]. The oxidation of various electron donors catalysed by this enzyme would, therefore, be peroxidation reactions involving the formation of reducing radicals which could react with oxygen[2–10].

However, KLAPPER AND HACKETT[12] found that peroxidase can induce the oxidation of certain quinols and naphthoquinols and concluded that the enzyme is itself

Abbreviations: E_m, midpoint potential; E_{m,pH_7}, midpoint potential at pH 7.

reduced and then reacts with O_2 forming a compound (Compound III) endowed with reactivity. Recently we proposed[13] a similar mechanism to explain indoleacetic acid destruction by horseradish peroxidase. The reactivity of Compound III has been described independently in the earlier work of YAMAZAKI[7,8].

We intend here to set out new experiment facts showing that, under certain experimental conditions, horseradish peroxidase is reduced by indoleacetic acid and then reacts with oxygen to form a compound endowed with reactivity.

METHODS

Peroxidase (Fluka, Boehringer or Nutritional Biochemical Corp.) was purified using PAUL's method[14]. Its RZ ('Reinheitszahl' $\varepsilon_{403\ m\mu}/\varepsilon_{280\ m\mu}$ ratio) varies between 2.8 and 3.2. The degree of purity of the enzyme does not, however, qualitatively influence the phenomena observed. The Soret spectra of peroxidase were recorded using a Spectronic 505 Bausch and Lomb spectrophotometer. The kinetics of the oxidation of indoleacetic acid and of catechol were recorded with a Jobin and Yvon spectrophotometer equipped with a Sefram recorder. This apparatus was modified so as to be capable simultaneously of rapidly injecting a reactant into the medium and recording the change in transmission at a fixed wavelength. Mixing was effected by a mechanical stirrer. The stirring and injection apparatus was positioned outside the light beam.

EXPERIMENTAL RESULTS

YAMAZAKI and his co-workers[6,9,10] have shown that, on reducing peroxidase by dithionite or by NADH, and by letting O_2 into the medium, they could record the formation of a compound spectroscopically identical to Compound III. On the other hand, CHANCE[15] was unable to obtain these results in the case of dithionite. We have been able, by a different method, to confirm the results of YAMAZAKI AND PIETTE[6]. If the peroxidase is reduced by hydrogen in the presence of platinized asbestos and phenosafranine, the ferroperoxidase Soret spectrum is obtained at first. Then, as O_2 enters the medium a substance is formed with a Soret spectrum identical to that of Compound III (Fig. 1). This substance can obviously only be oxyferroperoxidase. In the same way, if the semiquinone of methylviologen is injected into a peroxidase solution and if the change in transmission at 418 mμ or 580 mμ is simultaneously recorded, the formation of the Compound III (*i.e.* oxyferroperoxidase) absorption bands is seen (Fig. 1).

Oxyferroperoxidase can be decomposed by indoleacetic acid and catechol (Fig. 1). Further on, it will be seen that this decomposition implies the destruction of these substances.

As will be seen later, these results suggest a reduction of peroxidase by indoleacetic acid followed by its oxygenation. It is interesting to note that indoleacetic acid can reduce (Fig. 2) various oxidation–reduction indicators, in particular methylene blue ($E_{m,pH7}$, 11 mV), Laught's violet ($E_{m,pH7}$, 64 mV) and phenosafranine ($E_{m,pH7}$, -252 mV). This reduction occurs at acid pH and when indoleacetic acid is in excess of the dye. The reduction does not occur if indoleacetate is replaced by indolepropionate or indolebutyrate. It is also important to note that the E_m values of phenosafranine and peroxidase are similar, except for very low pH.

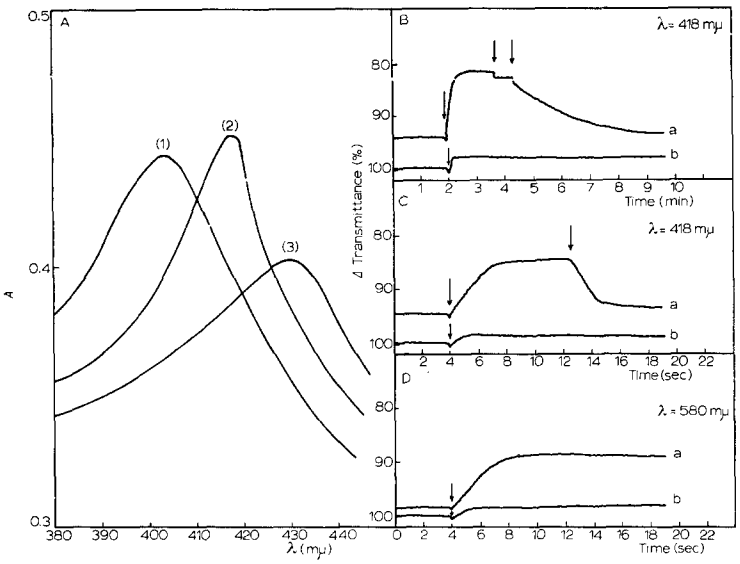

Fig. 1. A. Reduction of peroxidase by hydrogen and formation of Compound III. Curve (1), Soret spectrum of ferriperoxidase; Curve (2), spectrum of Compound III (oxyferroperoxidase) obtained after oxygenation of ferroperoxidase. Curve (3), spectrum of the ferroperoxidase obtained after bubbling of H_2; The absorbance values are artificially increased due to the presence of platinized asbestos in the medium. The pH is 6 (phosphate–citrate buffer). The quantities of peroxidase and phenosafranine are, respectively, $5 \cdot 10^{-3}$ μmoles and 10^{-2} μmoles. The volume of the reaction medium is 2 ml. At the concentration used, the phenosafranine does not absorb between 380 mμ and 440 mμ. B, C and D. Formation of Compound III and its decomposition. Curve B a, the first arrow represents the introduction of methylviologen semiquinone (1.25 μmoles) into a peroxidase solution; the second represents the introduction of water (which does not decompose the oxyferroperoxidase); the third, the introduction of indoleacetic acid (1.2 μmoles); Curve B b, injection of the methylviologen semiquinone (1.25 μmoles) into the buffer; Curve C a, the first arrow represents the injection of methylviologen semiquinone (1.25 μmoles) into a peroxidase solution; the second, the introduction of catechol (1.25 μmoles); Curve C b, injection of methylviologen semiquinone (1.25 μmoles) into the buffer; Curve D a, appearance of an absorption peak at 580 mμ of Compound III. The arrow represents the injection of methylviologen semiquinone (1.25 μmoles) into a peroxidase solution; Curve D b, injection of methylviologen semiquinone (1.25 μmoles) into the buffer. The amount of peroxidase is $8 \cdot 10^{-3}$ μmoles (B a, C a) or $4 \cdot 10^{-2}$ μmoles (D a). The final volume of each medium is 4 ml, the pH (phosphate–citrate buffer is 6. In the case of the Curves B a, C a and D a, the blank cuvettes of the spectrophotometer contain, respectively (for a 4-ml volume) $8 \cdot 10^{-3}$ μmoles (B a, C a) or $4 \cdot 10^{-2}$ μmoles (D a) of peroxidase.

As already shown[13], cyanide, which is an inhibitor when present in the medium before the reaction starts, has practically no inhibitory effect when added to the medium after the reaction has started. Under these conditions, the rate of indoleacetic acid destruction depends on the enzyme concentration in the medium.

It is possible to obtain catechol degradation by a mixture of peroxidase and indoleacetic acid in the absence of peroxide. The spectrum of the degradation products obtained under these conditions is not identical to that of the catechol peroxidation product (*o*-benzoquinone). Its shape varies during the reaction (Fig. 3) and shows several absorption peaks in the visible region; one is localized just below 400 mμ, another at about 590 mμ. The kinetics of the appearance of the 2 peaks are entirely different. Whereas the first forms very rapidly after introduction of the catechol into the medium, the formation of the second is much slower (Fig. 4). Moreover, the 2 peaks

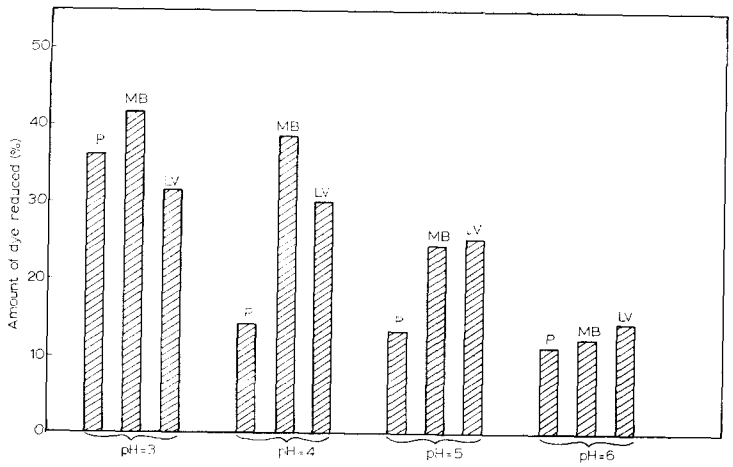

Fig. 2. Reduction of phenosafranine (P), methylene blue (MB) and Laught's violet (LV) by indoleacetic acid at various pH values. In the Thunberg cuvettes, under vacuum, 0.1 μmole of dye (phenosafranine, methylene blue or Laught's violet) and 30 μmoles of indoleacetic acid are mixed together. One determines the absorbance of the medium under these conditions after 30 min, and then after opening the cuvettes and re-oxygenation. The difference between the 2 values obtained enables one to calculate the quantity of dye reduced by indoleacetic acid (expressed in % of the quantity initially present in the medium). The buffers used are phosphate–citrate.

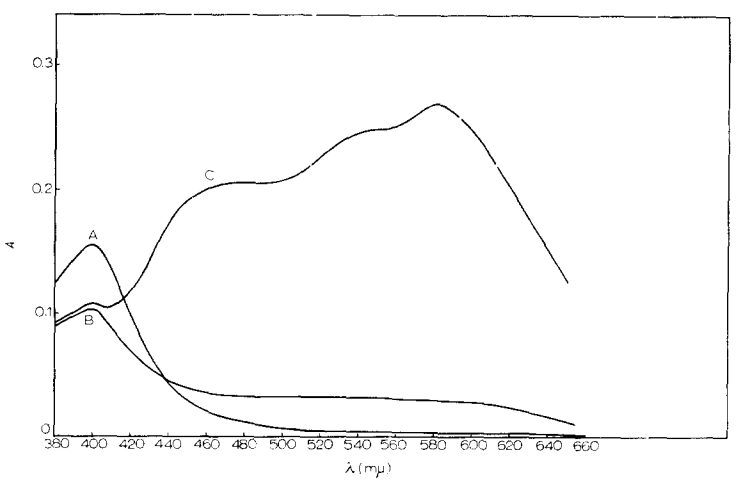

Fig. 3. Spectra of the catechol degradation products. A. Spectrum of the catechol peroxidation product (*o*-benzoquinone). The quantities of peroxidase, H_2O_2 and catechol in the medium are, respectively, 10^{-3} μmole, 30 μmoles and 6 μmoles. B. Spectrum of the products of catechol degradation by the peroxidase–indoleacetic acid system. The quantities of peroxidase, indoleacetic acid and catechol in the medium are, respectively, 10^{-3} μmole, 144 μmoles and 6 μmoles. Catechol is introduced into the medium last, 30 sec after indoleacetic acid. The spectrum is recorded 15 sec after the beginning of the reaction. C. Spectrum of the products of catechol degradation by the peroxidase–indoleacetic acid system. The experimental conditions are identical to those corresponding to Curve B but the recording is made 5 min after the beginning of the reaction. In every case the pH of the medium is 3 (phosphate–citrate buffer) and its final vol. is 2.9 ml.

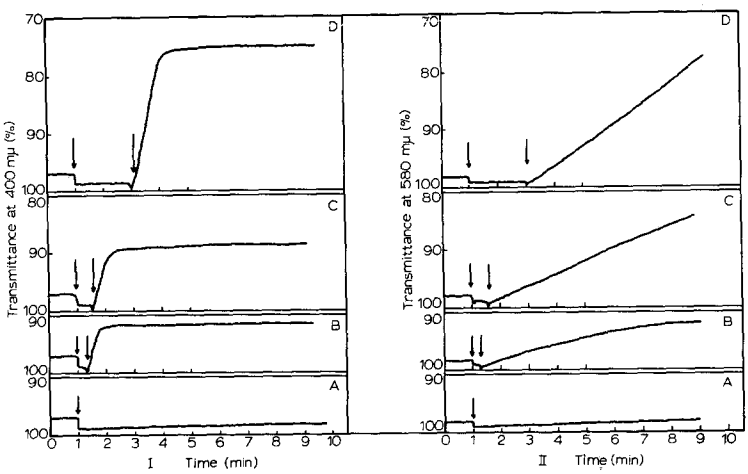

Fig. 4. I. Indoleacetic acid degradation kinetics recorded at 400 mμ. For all the curves, the first arrow corresponds to the introduction of indoleacetic acid into a peroxidase solution and the second to the introduction of catechol. In the case of Curve A, indoleacetic acid and catechol are introduced simultaneously into the medium. In the case of Curves B, C and D, the time which separates the injection of indoleacetic acid from that of catechol is increased. The respective amounts of peroxidase, indoleacetic acid and catechol in the media are $1.2 \cdot 10^{-3}$ μmole, 0.6 μmole and 2.5 μmoles. The pH is 3 (phosphate–citrate buffer); the final volume of each medium is 4 ml. II. Kinetics of the degradation of indoleacetic acid recorded at 580 mμ. The experimental conditions are identical to these described for Fig. 4 I.

only appear if the catechol is introduced into the medium after indoleacetic acid and peroxidase (Fig. 4). It is important to note that the appearance of the peak at 400 mμ is blocked by cyanide introduced into the medium after the reaction has started, whereas the peak at 580 mμ does appear under these conditions (Fig. 5). The rate of catechol destruction, recorded at 580 mμ in the presence of cyanide, also depends on the enzyme concentration. The concentrations of cyanide which block the appearance of the peak at 400 mμ are also those that block catechol peroxidation.

DISCUSSION

YAMAZAKI AND PIETTE[6] have demonstrated, under experimental conditions different from those used in the present work, the appearance of carboxyferroperoxidase in the presence of indoleacetic acid. They consider that traces of peroxide would lead to the formation of indoleacetic acid free radicals. These radicals, in the absence of O_2, would reduce peroxidase (appearance of carboxyferroperoxidase if the medium contains some CO). According to YAMAZAKI AND PIETTE[6], peroxidase reduction would precede its destruction. As we have already noted, this interpretation cannot be applied to our experimental results. In fact, if peroxidase were reduced by strongly reducing radicals, one should be able to obtain the appearance of ferroperoxidase on mixing (in the absence of O_2) ferriperoxidase and indoleacetic acid. This result has never been observed[13]. Similarly, in the decomposition of carboxyferroperoxidase by light, one should obtain the spectrum of the ferroperoxidase whereas it is the ferriperoxidase one which is obtained[13]. Finally, under our experimental conditions, the

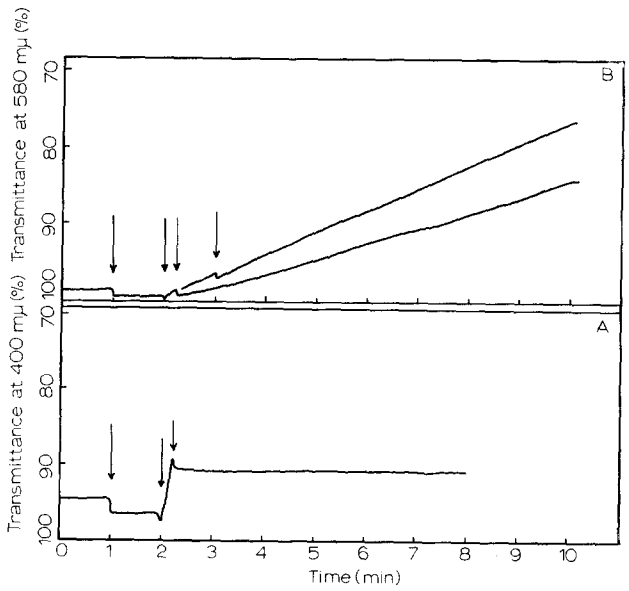

Fig. 5. Action of cyanide on the degradation of catechol (recorded at 400 mμ and 580 mμ) by the peroxidase–indoleacetic acid system. The first arrow corresponds to the introduction of indoleacetic acid into the peroxidase solution, the second to the introduction of catechol. The cyanide (12.5 μmoles) is introduced into the medium either 15 sec (3rd arrow) or 1 min after the beginning of the reaction (4th arrow, B). The cyanide introduced into the medium 1 min after the beginning of the reaction does not inhibit the formation of the products absorbing at 580 mμ (B, upper curve). This substance possesses a slight inhibiting effect if it is introduced into the medium 15 sec after the beginning of the reaction (B, lower curve). It blocks, however, the appearance of the products absorbing at 400 mμ. The quantities of peroxidase, indoleacetic acid and catechol are, respectively, $0.8 \cdot 10^{-3}$ μmole, 0.6 μmole and 2.5 μmoles. The pH of each medium is 3 (phosphate–citrate buffer), its final vol. 4 ml.

appearance of carboxyferroperoxidase does not precede the enzyme destruction[13].

The simplest idea which may provide an explanation of the results observed consists in admitting that indoleacetic acid can directly reduce ferriperoxidase:

$$\text{indoleacetic acid} + \text{ferriperoxidase} \rightleftharpoons \text{indoleacetic acid free radical} + \text{ferroperoxidase} \quad (1)$$

The direct interaction between indoleacetic acid and ferriperoxidase, postulated in reaction (I), is also in perfect agreement with the results of HINMAN AND LANG[17]. This interpretation is very much like that proposed by FEIGELSON, ISHIMURA AND HAYAISHI[18] for the case of tryptophan pyrrolase. However, the equilibrium postulated in Reaction (I) would normally be moved towards the left, so that, in the absence of CO or O_2 (able to react with ferroperoxidase), no reduction of peroxidase by indoleacetic acid would be observed. On the other hand, in the presence of CO or O_2, the Equilibrium (I) would be moved towards the right and the formation of carboxyferroperoxidase or oxyferroperoxidase would be observed. In postulating the existence of Equilibrium (I) normally moved towards the left, it is seen that the carboxyferroperoxidase photodissociation, in the absence of O_2, does not cause the accumulation of ferroperoxidase but of ferriperoxidase[13].

However, the peroxidase midpoint potential is so low (−270 mV at pH 7) that one may wonder whether the difference in the E_m values, for the 2 couples indoleacetic acid free radical/indoleacetic acid and ferriperoxidase/ferroperoxidase, is not such as to prohibit any reduction of ferriperoxidase by indoleacetic acid even in the presence of CO. First of all, it may be seen that the appearance, in large quantities, of carboxyferroperoxidase is only observed at a pH between 3 and 4. For these pH values, the peroxidase E_m is nothing like so negative as at pH 7 (ref. 19). Moreover, it has been seen (Fig. 2) that indoleacetic acid (but not indolepropionic acid and indolebutyric acid) can reduce various oxidation–reduction indicators, in particular phenosafranine, the potential of which is near to that of peroxidase. These reduction reactions are also much more significant at acidic pH. Since indoleacetic acid can reduce phenosafranine it is therefore not surprising that it can also reduce ferriperoxidase.

Two groups of results show that ferroperoxidase and oxyferroperoxidase take part in a degradation cycle of indoleacetic acid, catechol and probably also of other electron donors.

Certain aspects of the action of cyanide on the destruction of indoleacetic acid by peroxidase can only be explained by admitting that oxyferroperoxidase takes part in the reaction. Cyanide, even at high concentrations, cannot block the destruc-

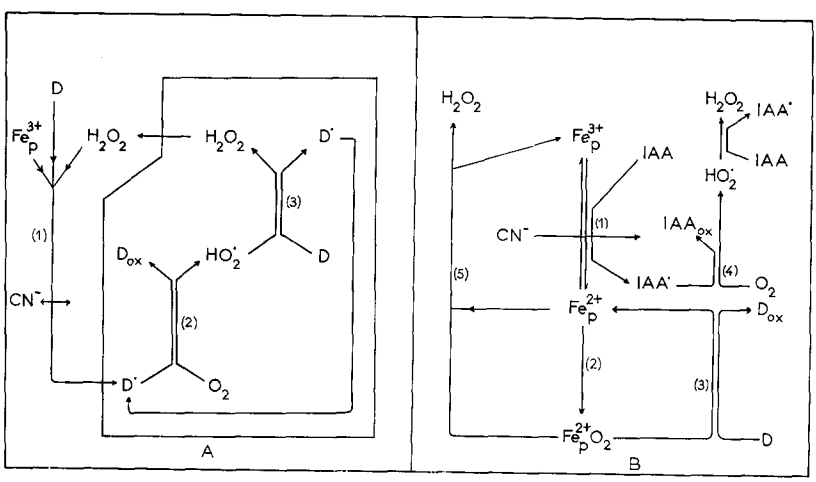

Fig. 6. Model, according to YAMAZAKI[4], for the mechanism of the oxidation of an electron donor, D, by the peroxidase–peroxide system. The enclosed part of the model corresponds to the propagation of the chain of free radicals. B. Model proposed to explain the mechanism of degradation of catechol D by the peroxidase–indoleacetic acid system. Abbreviations: $Fe^{3+}{}_p$, ferriperoxidase; $Fe^{2+}{}_p$, ferroperoxidase; IAA, indoleacetic acid.

tion of indoleacetic acid when it is added in the course of the reaction. Two interpretations can be given to this result: (1) indoleacetic acid is not destroyed by a peroxidation reaction, but by a degradation reaction (perhaps oxygenation) bringing into play the ferroperoxidase–oxyferroperoxidase system insensitive to cyanide; (2) ferriperoxidase is blocked by cyanide, and indoleacetic acid is destroyed solely by a non-enzymatic process corresponding to the simple propagation of a free radical chain.

This second interpretation is in perfect agreement with the ideas of YAMAZAKI[6,7] and MASON[11] on the mechanism of peroxidase reactions. It is set out in Fig. 6A.

In such a model, cyanide added during the reaction will not have a large inhibiting effect (FOX, PURVES AND NAKADA[21]) if the rate of non-enzymatic appearance (Reaction 3) of indoleacetic acid free radicals is very much greater than their enzymatic production rate (Reaction I). Under these conditions, the rate of indoleacetic acid degradation measured after introduction of cyanide into the medium should be independent of the enzyme concentration, but this is not the case. We have, therefore, to admit the participation of oxyferroperoxidase in the destruction of indoleacetic acid. If, as has been seen, Compound III can destroy catechol and indoleacetic acid, these substances should decompose oxyferroperoxidase. The expected result has been observed (Fig. 1).

Confirmation of this participation has been provided by the study of catechol degradation through the peroxidase–indoleacetic acid system (Fig. 4). It is not possible to identify this phenomenon simply with a peroxidation reaction. In fact, cyanide, at a concentration sufficient to block the peroxidation of catechol, also blocks the appearance of the substance (or substances), absorbing at 400 mμ in the peroxidase–indoleacetic acid system, but not the formation of the substance (or substances) absorbing at 580 mμ. It is not possible to interpret these results in terms of the progressive transformation of one substance (absorbing at 400 mμ) into another (absorbing at 580 mμ). If this were the case, when introducing cyanide into the medium immediately after the beginning of the reaction one would have not only to block the formation of the peak at 400 mμ, but also to obtain an absorption decrease at that wavelength corresponding to the transformation of the substance absorbing at 400 mμ into a substance absorbing at 580 mμ. This is not the case (Fig. 4). It is therefore extremely tempting to admit that these 2 substances (or these 2 groups of substances) must be formed in the course of independent reactions: a peroxidation reaction (formation, probably, of *o*-benzoquinone absorbing at 400 mμ) and a degradation reaction of a different type, perhaps an oxygenation, conditioned by the ferroperoxidase–oxyferroperoxidase system insensitive to cyanide. These results do not enable one to state whether the substance absorbing at 580 mμ is formed directly under the action of oxyferroperoxidase, or whether it appears secondarily in the medium as a consequence of the non-enzymatic transformation of catechol degradation products. This second hypothesis seems more likely, especially if one considers the idea of catechol oxygenation by oxyferroperoxidase. It would be tempting to compare this particular action of peroxidase with that of pyrocatechase and metapyrocatechase[22–27].

The overall interpretation proposed is depicted in Fig. 6B. According to this model, the introduction of indoleacetic acid into a peroxidase solution would produce 2 types of phenomenon: a peroxidation reaction, due to the formation of H_2O_2 in the medium (Reactions 4 and 5) and a degradation reaction of a different type (perhaps an oxygenation) conditioned by the ferroperoxidase–oxyferroperoxidase system (Reaction 3). These experimental results, which have been expanded, do not show the intervention of Reactions 4 and 5. However, the work of YAMAZAKI[6,7] and GEORGE AND STRATMANN[28,29] render their existence most probable.

It thus appears that the results presented are in perfect agreement with the interpretation previously proposed, according to which ferriperoxidase would be reduced by indoleacetic acid, which would cause (in the presence of O_2) the formation of oxyferroperoxidase endowed with reactivity towards indoleacetic acid, catechol and probably other substances.

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