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INTERACTIONS OF SULFITE AND MANGANOUS ION WITH PEROXIDASE OXIDATION PRODUCTS OF INDOLE-3-ACETIC ACID*

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Abstract—Chemical and spectral data were used to study the fate of IAA during the first minute of its peroxidase catalysed oxidation. In the first 30 sec of the reaction, a hypsochromic shift of 2 nm was detected in the UV spectrum of IAA. In the presence of sulfite, the rate of spectral shift is enhanced. As the oxidation of IAA proceeds, the effect of sulfite diminishes, being ineffective when added to the reaction after 20–30 sec. The loss of the sulfite effect coincides with a hyperchromic shift at 264 nm. This is not observed when sulfite is added at the beginning of the oxidation reaction. The rate of the hypochromic shift at 282 nm is greatly enhanced when sulfite is added at the start of the reaction, but is ineffective when added after 30–40 sec. The initial effect of sulfite is attributed to a chain reaction of unstable IAA-free radicals and sulfite oxidation, and this is further enhanced by manganous ions, which function in electron transfer between sulfite ions and IAA-free radicals.

INTRODUCTION

THE OXIDATION of indole-3-acetic acid (IAA) by peroxidase enzymes indigenous to lower and higher plants has received much attention and the reader is referred to excellent reviews by Galston and Hillman[1] and Galston and Davies.[2] Most authors consider the enzymic oxidation of IAA synonymous with IAA destruction. The concept of IAA destruction, as first conceived more than 30 yr ago,[3] finds support primarily from the distribution of IAA-oxidase activity in etiolated pea seedlings,[4] in light-grown cotton,[5] and in lentil seed and carrot roots.[6, 7]

By contrast, other investigators found a converse relationship between IAA-oxidase activity and age of tissue. Briggs *et al.*[8] found, for instance, that IAA-oxidase activity is low in dormant leaves of *Osmunda*, rises to a peak during early stages of growth and decreases as the leaves become mature. Similarly, Jacobson and Caplin[9] found that young carrot root phloem tissues respond to IAA more than other tissues, and that the IAA-oxidase activity was higher in young phloem tissues than in other tissues.

More recently, Ockerse *et al.*[10] reported that apices of tall, dark-grown pea seedlings contain 35 times higher IAA-oxidase activity than buds of dwarf peas. Furthermore, they

* Part II in the series "Oxidation of Indole-3-acetic acid by peroxidase enzymes". For Part I see Ref. 14

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showed that gibberellic acid increases the IAA-oxidase activity in dwarf peas to a level equal to that of tall pea seedlings. This relationship between tissue expansion and peroxidase activity also occurred with tobacco tissue; young actively expanding leaves contained higher IAA-oxidase activity than mature leaves.[11]

In addition, several investigators have shown that oxidation products of IAA promote growth and that the biological activity of these products was greater than that obtained with IAA.[12,13] The enzymic oxidation of IAA therefore assumes a new significance. The oxidation of IAA may not only lead to its ultimate destruction, but more importantly, IAA may attain its activity as a growth hormone via such oxidative transformations.

This concept prompted us to reinvestigate the oxidation reactions of IAA with particular attention to the early oxidation products. In the first paper of this series,[14] we described a condensation reaction between an intermediate product of IAA (an indolenine) and *p*-dimethylaminocinnamaldehyde. In the present paper emphasis is placed on the effect of sulfite ions, a sensitive detector of free radicals, and of Mn^{2+} on the oxidation of IAA.

RESULTS

UV Spectral Shifts During Oxidation of IAA

As shown earlier,[12] the UV spectrum of IAA, with its characteristic indolic triple absorption maxima in the 280 nm region, undergoes several changes during the course of oxidation. The first is a rapid 2 nm hypsochromic shift of the triple absorption maxima, and this is succeeded by a less rapid increase in absorbance at 265 nm. The spectrum continues to change at a very slow rate for another 24–36 hr, this being due to the slow accumulation of an oxindole derivative, indicated by the increase of absorbance at 245 and 255 nm and the concomitant decrease of absorbance in the 280 nm region.[12,15]

The relative rate of the initial two changes can be reduced from a series of repetitive scans of the difference spectrum shown in Fig. 1. The decrease of absorbance at 280 nm and 290 nm delineates the 2 nm hypsochromic shift and the increase in absorbancy at 255 nm corresponds to the increase of absorbance at 260 nm of the initial IAA spectrum. Changes of absorbancy at the longer wavelength (280 and 290 nm) reach a maximum when the change at the shorter wavelength has reached about one-half its maximum. In the presence of high IAA concentrations this incipient reaction (change at 290 nm) is inhibited.

In the plot of 1/v against S (Fig. 2), points for high IAA concentrations lie on a straight line, denoting substrate inhibition of the reaction. The upward deviation represents points for low substrate concentrations.[16] This reciprocal relationship is also shown when the same data are presented in a plot of 1/v against 1/S. In this graph, data for high substrate concentrations deviate upward from the straight line and data for IAA concentrations of less than 0·2 mM fit the line. Similar results were obtained when the effect of substrate concentrations on the oxidation of IAA was assayed with Salkowski reagent. In this test, the oxidation of IAA was determined as a function of residual concentration of IAA measured at 525 nm (Fig. 2). Subsequent shifts of the 251 nm absorption band adhere closely to the color complex formation with *p*-dimethylaminocinnamaldehyde (DMACA).

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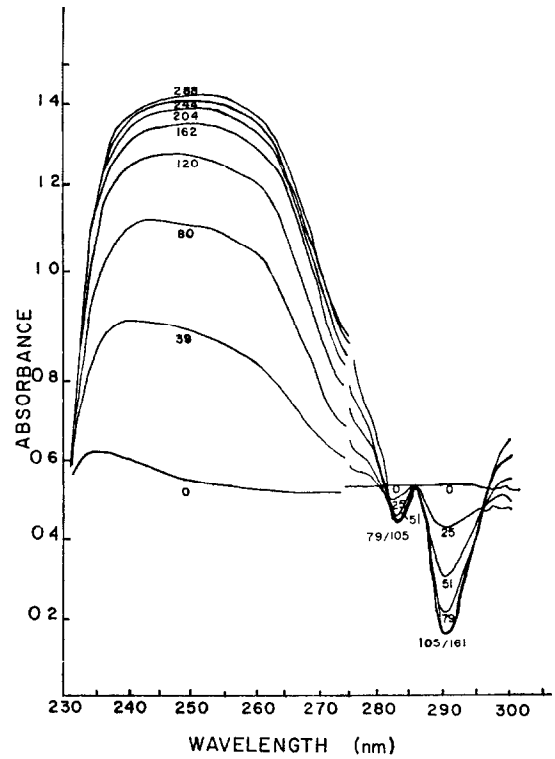

FIG. 1. REPETITIVE SCAN OF AN OXIDATION REACTION MIXTURE CONTAINING 0·2 mM IAA, 0·1 mM DCP, 0·1 mM $MnCl_2$, AND 0·1 μM HRP.

Scans were made at times (in seconds) indicated. Reference cell contained reaction mixture less HRP.

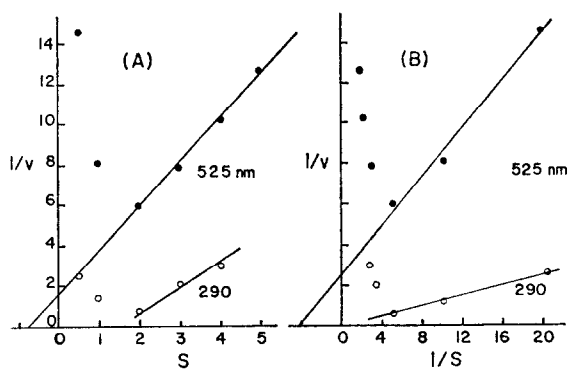

FIG. 2. SUBSTRATE INHIBITION BY IAA IN THE PRESENCE OF 0·1 μM HRP, 0·1 mM DCP, AND 0·1 mM $MnCl_2$ AS ASSAYED WITH SALKOWSKI REAGENT (525 nm) AND AS A FUNCTION OF CHANGE OF ABSORBANCE AT 290 nm.

(A)—Points on the straight line denote substrate inhibition of the reaction. Points deviating from the line denote reaction velocities for low substrate concentrations. (B)—Normalized plot for the same data in which points on the line correspond to low concentrations of IAA and the upward deviation from the straight line correspond to high concentrations of IAA. In both plots, S = 0·1 mM.

As reported elsewhere,[12] this reaction involves a condensation between an indolenine, conjectured to be an early oxidation product of IAA, and the aldehyde DMACA.[12]

Sodium metabisulfite greatly stimulates the initial oxidation of IAA and inhibits the subsequent accumulation of the 250 nm absorbing intermediate. As shown in Fig. 3, at constant substrate, enzyme and cofactor concentrations, the change of absorbance at

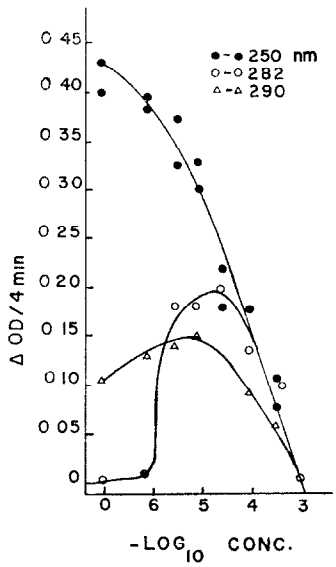

FIG. 3. CONCENTRATION RESPONSE CURVE FOR HSO_3^- ON THE OXIDATION OF 0·1 mM IAA IN THE PRESENCE OF 0 1 mM $MnCl_2$, 0 1 mM DCP, AND 0·1 μM HRP.
For comparison, changes at 282 nm are plotted as positive values.

282 nm increases to a maximum in the presence of 5×10^{-5}M HSO_3^-, while higher concentrations inhibit the oxidation of IAA. The absorbance at 250 nm, on the other hand, decreases logarithmically with increasing sulfite concentrations.

An inverse relationship occurs between the sulfite-induced increase of absorbancy at 282 nm and the concomitant decrease at 250 nm. The concentration of IAA was 0·1 mM and the maximum stimulation of the oxidation of IAA was obtained with 0·4 mM bisulfite. However, when $MnCl_2$ is omitted from the oxidation reaction mixture, the sulfite effect is then reduced and the maximum effect is obtained with 0·1–0·2 mM bisulfite. The catalytic effect of $MnCl_2$ was further substantiated by results which suggest that manganous ions function as an electron bridge between the intermediate and sulfite ions. In these experiments, the effect of $MnCl_2$ and sulfite was tested in reaction mixtures containing various concentrations of HRP. The sulfite was in excess of the amount required for a maximum response so that sulfite ions did not become a limiting factor. The mixtures contained similar amounts of IAA, which became the rate-limiting factor in both reactions. Without $MnCl_2$ and HSO_3^- the initial velocity of the oxidation of IAA was very low and increased at a slow rate as the peroxidase concentrations increased. When HSO_3^- was added, the HRP concentration response was more pronounced and the rate of oxidation increased proportionately with increasing peroxidase concentrations. The maximum rate was reached with 40·0 mμ M of HRP. In the presence of $MnCl_2$, the same maximum reaction rate was

observed at a concentration of HRP 10 times less than when $MnCl_2$ was omitted. Auto-oxidation of IAA was also observed provided that $MnCl_2$ and HSO_3^- were present. Under auto-oxidative conditions, the UV spectral changes of IAA are accompanied by two well-defined isosbestic points which are less defined in the repetitive scans obtained from peroxidase catalyzed reactions (Fig. 4). The presence of isosbestic points indicates that under auto-oxidative conditions IAA is simply oxidized without the formation of intermediate products, as observed when the oxidation reaction of IAA is catalysed by peroxidase. It is conceivable that under auto-oxidative conditions, the oxidation of IAA is initiated by sulfite ions and that $MnCl_2$ acts as a catalyst as observed in enzymic oxidation reactions. In the presence of enzymes, the sulfite-induced chain reaction is, however, initiated by an intermediate oxidation product of IAA. On addition of bisulfite at the start of the enzymic oxidation reaction, the rate of oxidation of IAA is maximal. The sulfite effect is diminished as the addition of sulfite ions is delayed. When added after 20 sec sulfite ions have no effect.

DISCUSSION

The presence of two enzyme sites each combining with a particular part of the IAA molecule may be deduced from the results reported here. At high substrate concentrations, IAA molecules will compete for available enzyme sites and the enzymic oxidation of IAA will be inhibited.

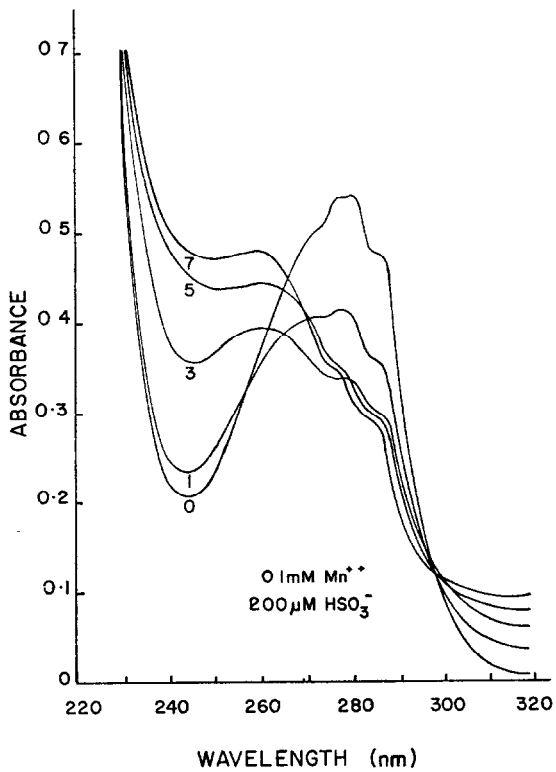

FIG. 4.(a). SPECTRAL CHANGES OF 0·1 mM IAA DURING ENZYMIC OXIDATION IN THE PRESENCE OF 200 μM SULFITE.

Number under the scans designates time in minutes.

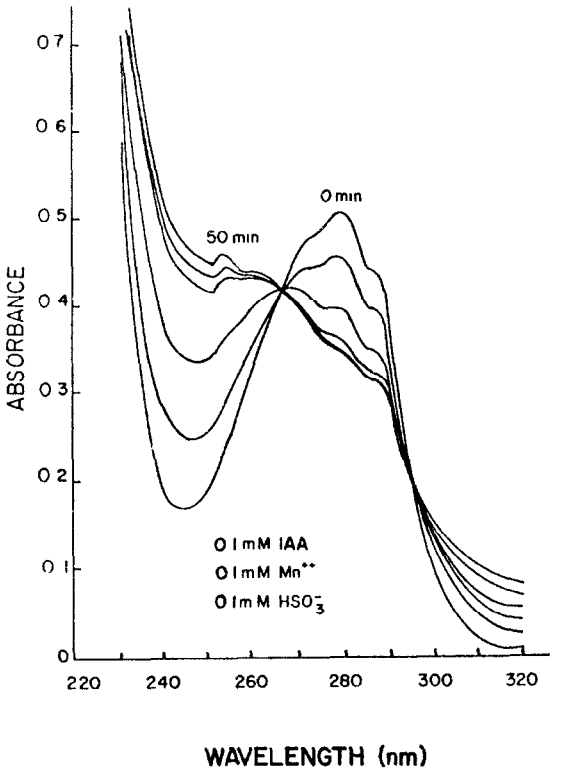

FIG. 4(b). CHANGE OF ABSORBANCE DURING AUTO-OXIDATION OF 0·1 mM IAA IN THE PRESENCE OF 0·2 mM HSO_3^-, AND 0·1 mM $MnCl_2$ AT pH 6 1.

Scans were made at 10-min intervals.

When bisulfite, a sensitive detector of free radicals, is added to the oxidation reaction mixture at the beginning of the enzymic reaction, the oxidation of IAA is greatly enhanced. The interaction of sulfite ions with free radicals is based on the initiation of aerobic oxidation of sulfite by free radicals,[17] and can be induced by peroxidizable substrates.[18] The free radical induced oxidation of sulfite, once initiated, is maintained as long as highly reactive intermediates are present. The decrease of the sulfite-induced enhancement of the rate of IAA oxidation, as the addition of sulfite ions is delayed, is because the oxygenation of sulfite ions and of IAA-free radicals is competitive. Both radicals compete for oxygen, the common component essential for the propagation of the chain reaction. The IAA-free radicals must, therefore, be formed prior to the oxygen incorporation and not, as suggested by Fox *et al.*[19] after the enzyme becomes oxygenated. This is substantiated by results of a reciprocal experiment in which the sulfite effect is still noted when IAA is added at various times after bisulfite is mixed with the enzyme.

Thus, it appears that formation of free radicals does not depend on the presence of oxygen and the interpretation of our results agree with the concepts proposed by Hinman

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and Lang.[15] The results also show that Mn^{2+} enhances the propagation of the sulfite-induced chain reaction during the enzymic- and auto-oxidation of IAA. Under auto-oxidative conditions, enhancement induced by $MnCl_2$ is much less than when HRP is present. A comparison of the change of UV absorption of IAA during its oxidation under both conditions suggests that the auto-oxidation of IAA proceeds without the formation of detectable intermediate products (Fig. 4).

The catalytic action of Mn^{2+} on the auto-oxidation of IAA in the presence of sulfite ions may arise either from the step-wise oxidation of sulfite, or from a step-wise reduction of oxygen, both inducible by Mn^{2+}.[18,20,21]

The strong absorbance of the auto-oxidation product of IAA at 255 nm suggests the presence of an oxindole.[15] The formation of oxindole was observed only after several hours of enzymic oxidation of IAA and after the production of an intermediate product which absorbs at 260 nm. The 260 nm absorbing intermediate product does not react with bisulfite solutions and is slowly converted to the 240–255 nm absorbing product. The 260 nm absorbing product reacts with the modified Ehrlich reagent,[14] and compares with published UV spectra for indolenine derivatives.[22–25] The formation of indolenines, characteristic to IAA-oxidation, was observed during peroxidase-catalysed oxidation of tryptophan,[26] and of indoles reacted with peracids.[27]

EXPERIMENTAL

The horseradish peroxidase (HRP) had a specific activity of 2900 μ/mg protein (Worthington Biochemical Corporation). Unless otherwise indicated, oxidation reaction mixtures contained 0·1 mM IAA, 0·1 mM $MnCl_2$, 0·1 mM 2,4-dichlorophenol (DCP), 0·1 μM HRP, and 1·0 mM pH 6·1 phosphate buffer. The components were mixed directly in 3 ml cuvettes of 1 cm light path, placed in a double-beam recording spectrophotometer. The enzyme was added last with a syringe. The reactions were assayed by the changes of absorbancy at 255, 280, and 290 nm, this being determined against a mixture of IAA and cofactors DCP and $MnCl_2$ in the reference cell. The Salkowski and Ehrlich reagents were used for the colorimetric analysis.[14]

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