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Studies on Auxin Protectors. IV. The Effect of Manganese on Auxin Protector-I of the Japanese Morning Glory

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Abstract. **Auxin protector-I of the Japanese morning glory is inactivated by manganese. Experiments carried out *in vitro* indicate that in the absence of oxygen the manganic, but not the manganous, ion rapidly inactivates the protector. It is clear from these, and other data described in this report, and the results of other workers, that in the presence of oxygen, manganese accelerates auxin inactivation by means of 2 separate and distinct mechanisms: 1) manganese catalyzes the oxidation of auxin protectors, and 2) following the inactivation of the protectors, or in the absence of protectors, accelerates the oxidation of indoleacetic acid by endogenous peroxidases.**

In a recent publication, Morgan *et al.* (6) provided evidence that the symptoms observed on plants exhibiting manganese toxicity can be explained in terms of the abnormally low levels of IAA brought about by a manganese-induced activation of IAA-oxidase. These authors suggested further that this activation was caused by a manganese-catalyzed destruction of IAA-oxidase inhibitor(s). We would like to support and expand this hypothesis by demonstrating that manganese brings about the *in vitro* inactivation of an auxin protector substance isolated from young, rapidly expanding Japanese morning glory leaves.

Morgan and Hall (5) had already reported earlier that aqueous extracts of various organs of cotton plants contain a heat-stable, dializable substance (or substances) which inhibits the enzymatic oxidation of IAA, primarily by causing a lag in the initiation of measurable oxygen uptake. Subsequently, Morgan (4) reported that the concentration of this inhibitor substance was greatest at the apex of the shoot and declined basipetally, while IAA-oxidase activity showed an inverse gradient, *i.e.,* IAA oxidation increased towards the base. We have also recently reported the existence of substances which show a similar gradient in inhibiting the enzymatic destruction of IAA in the leaves and stems of the Japanese morning glory (*Pharbitis nil,* Choisy), and which appear to possess important regulatory functions in stem elongation (9, 10, 12, 13). The major difference between the system described by Morgan and coworkers, and ours is this: the IAA-oxidase inhibitor in cotton plants appears to be a low molecular-weight compound such as gossypol or kaempferol. The inhibitors of IAA-oxidation in the Japanese morning glory, on the other hand, appear to be relatively heat-labile substances of large molecular weight. On the basis of gel filtration studies, we have identified at least 3 components of this auxin protection system: protector A (mol wt exceeding 200,000), protector I (mol wt *ca.* 8000), and protector II (mol wt *ca.* 2000) (9, 13). We feel that the system is not unique to the Japanese morning glory since Phipps (7, 8) has reported the presence of similar, relatively high molecular weight substances in *N. tabacum,* and we have obtained indications that such substances also exist in several other Nicotiana species, in sunflower seedlings, and in the liquid of the coconut (10, and unpublished observations).

Materials and Methods

Tissue Extract. Tissue extracts were obtained by collecting 5 g of young Japanese morning glory leaves (*Pharbitis nil* Choisy) less than 4 cm long at the midrib, and grinding them in 5 ml cold 20 mM phosphate buffer, pH 6.1, in a chilled mortar. The brei was strained through cheese cloth and centrifuged for 15 minutes at 10,000 r.p.m. with average force on the sample of approximately 6000 g. The supernatant (*ca.* 5 ml) was then passed through a dextran gel.

Dextran Gel Filtration. Columns for gel filtration were prepared as follows: 16.9 g of Sephadex G-50 powder was allowed to swell overnight in 225 ml 20 mM phosphate buffer, pH 6.1. After stirring, the slurry was poured into a 30 × 400 mm chromatographic tube (bed volume 66 ml), allowed to settle to a height of 350 mm and flushed with buffer at a rate of 1.7 ml/min. Five ml of tissue extract was applied to the top of the column. The column filtrate was collected serially in a fraction collector in 5 ml samples. Dextran Blue 2000 (Pharmacia Co.) and Pyronin Y (Allied

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Chemical) were used as dye markers to delimit the molecular weights (> 10,000 vs. 300). One ml of the former was added to the extract as a 0.15 % solution in buffer; the latter was added as 1 ml of an 0.005 % solution as soon as the extract had entered the gel.

Auxin Protector. The bulk of the auxin protector activity was found as protector I (Pr-I) in tubes number 17 to 19 (volume of effluent 85–100 ml). These 3 tubes, totaling 15 ml, were combined, thoroughly mixed, dispensed in 1 ml vials and frozen until used.

Reaction Mixture. The reaction mixture used to assay for auxin destruction consisted of a mixture of 2,4-dichlorophenol (Eastman Organic Chemicals), manganese chloride (Baker Analyzed), and IAA (Calbiochem), each at a concentration of 0.1 mM, and horseradish peroxidase (Calbiochem, RZ value 0.84) at a final concentration of 0.2 μg/ml. The final volume of the reaction mixture was 10 ml; all reactions were buffered at pH 6.1 with 20 mM potassium phosphate (Baker Analyzed). Destruction of IAA was followed by removing samples at various time intervals and assaying by means of Salkowski reagent; the optical density at 540 mμ was used to determine the amount of IAA in the reaction mixture.

To inhibit IAA destruction, the amounts of Pr-I usually added were such as to cause a lag (in IAA destruction) which varied from 0.5 to 3 hours. The Pr-I was added first to the buffer, then DCP, Mn, enzyme, and last IAA. Reactions were carried out at 32° on a Dubnoff shaker equipped to permit carrying out reactions under nitrogen. Many of the details of these techniques have been described previously (9).

In the experiments illustrated in table I, Protector-I was preincubated with $MnCl_2$ in buffer for the periods indicated; at the end of this period, DCP, peroxidase, and IAA were added. The flasks which had been kept under nitrogen during preincubation, were returned to air just prior to adding the other reagents. In these experiments no extensive precautions were taken to avoid contamination by air. In contrast, the experiments (table II) with MnF_3 (City Chemical), involved extensive precautions to avoid exposing dissolved MnF_3 to air, since the manganese would precipitate very rapidly upon exposure to only small, contaminating quantities of air. To prevent the formation of this precipitate, the MnF_3 was dissolved in freshly boiled buffer after the buffer had cooled in an ice bath while nitrogen was bubbled through. The concentration of manganese in this stock solution was 0.15 mM. By means of a pipette, 6.7 ml of the stock solution were added to the preincubation reaction mixture which consisted of boiled buffer, cooled to 32° under nitrogen, and maintained under nitrogen except for a brief exposure to air at the time the Pr-I or Mn was added. The final concentration of manganese in the preincubation reaction mixture was 0.1 mM. Control mixtures contained 0.1 mM MnF_2, or 0.1 mM MnF_2 supplemented with 50 μM NaF to check the possibility that the effects observed were caused by the increase in fluoride ion.

Results

Figure 1 shows the typical oxidation of IAA when exposed to the action of horseradish peroxidase in the presence of an electron acceptor such as dichlorophenol. As is well known, the addition of manganous chloride speeds up this reaction. In contrast, in the presence of protector-I, the oxidation of IAA was initially completely inhibited, but then proceeded normally following a lag which could extend for a period of many hours, depending on the quantity of Pr-I present. The presence of manganese chloride greatly reduced the Pr-I-induced lag.

Table I shows that upon preincubation of the protector with $MnCl_2$, the lag was dramatically reduced when compared to the controls (protector shaken in buffer). In fact, in this experiment, preincubating the protector with $MnCl_2$ for 3 hours in air inactivated it almost completely (5-min lag).

When Pr-I was incubated in the Dubnoff shaker with Mn^{2+} under an atmosphere of nitrogen rather than air, the inactivation of Pr-I by the manganous ions proceeded more slowly (table I). However, Pr-I by itself (in buffer) also became inactivated more slowly when shaken under nitrogen. Preincubating Pr-I in nitrogen under conditions more carefully controlled to minimize contamination by air, caused virtually no inactivation of the protector, even in the presence of Mn^{2+} (see table II).

Because we were unable to obtain $MnCl_3$, we used MnF_3. However, we could detect no appreciable difference between $MnCl_2$ and MnF_2 at a concentration of 0.1 mM. That is, both the enzy-

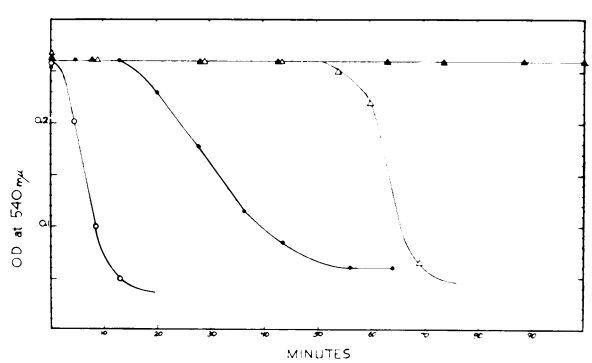

FIG. 1. Destruction of IAA by horseradish peroxidase in the presence and absence of manganese and in the presence and absence of protector-I (Pr-I): (○) +Mn, —Pr-I; (●) —Mn, —Pr-I; (△) +Mn, +Pr-I; (▲) —Mn, +Pr-I. (See also text).

Table I. *Effect of Shaking Pr-I in the Presence and Absence of Mn^{2+} in an Atmosphere of Air or of Nitrogen*

Preincubation mixture	Preincubation time	Length of Pr-I induced lag		
		When Pr-I not preincubated	When Pr-I preincubated in nitrogen	When Pr-I preincubated in air
	min	*min*	*min*	*min*
...	0	120	...	...
Pr-I	180	...	95	45
Pr-I + $MnCl_2$	180	...	35	5
Pr-I + $MnCl_2$	90	...	55	10

Table II. *Effect of Shaking Pr-I with Mn^{3+} as Compared with Mn^{2+} in an Atmosphere of Nitrogen*

Preincubation mixture	Preincubation time	Length of Pr-I induced lag	
		When Pr-I not preincubated	When Pr-I preincubated as indicated in column I
	min	*min*	*min*
...	0	20	...
Pr-I	30	...	20
Pr-I + $MnCl_2$	30	...	20
PR-I + MnF_2 + NaF[1]	30	...	20[2]
Pr-I + MnF_3	30	...	0

[1] In addition to 0.1 mM MnF_2, 50 μM NaF was added to assure that the concentration of F^- would equal that supplied 0.1 mM MnF_3.

[2] Shape of curve somewhat altered. See text.

matic oxidation of IAA in the absence of Pr-I, and the inactivation of the protector when preincubated with manganese, were unaffected by the change in the anion. (We also checked on a second variable, viz., the fact that a solution of 0.1 mM MnF_3 supplies 50 % more fluoride ions to the solution than does MnF_2. For this reason we added 50 μM NaF. This did not appreciably affect the protector, since lag times remained essentially the same. However, the presence of NaF did depress the rate of IAA oxidation in the absence of protector.) The manganic fluoride, on the other hand, rapidly and completely inactivated Pr-I, even under nitrogen. This total inactivation could take place in a matter of minutes (table II).

The effect of the manganic ion on the protector was very dramatic, and if adequate precautions were taken to exclude oxygen, easily repeatable. The effect of the Mn^{3+} ion on the oxidation of IAA by peroxidase in the absence of protector substances was more difficult to reproduce, but it did appear that whereas with 0.1 mM MnF_2 (or $MnCl_2$) there could frequently be observed a 1 to 3 minute lag prior to decline in IAA; with MnF_3, no such lag could ever be ascertained: The oxidation of IAA in the presence of MnF_3 initially proceeded so rapidly, that we were unable to obtain reliable readings at t_0.

Discussion

The role of manganese as a cofactor in the oxidation of IAA was first reported by Wagenknecht and Burris (11) and has since been observed by numerous workers (*e.g.*, as reviewed by 2, 6). The specific role of manganese in the oxidation of IAA by horseradish peroxidase has been investigated in detail by Kenten (3). This author reported that the effect of Mn^{2+} was greatest at about pH 6, that there was an optimum concentration (at about 33 μM in his system), and that the effect of Mn^{2+} on IAA oxidation was least with the most highly purified preparations of the enzyme used. Furuya and Galston (1) have reported that preincubating pea homogenates with manganese increased IAA-oxidase activity by inactivating an oxidase inhibitor (a glucoside of quercitin).

In the Japanese morning glory system, preincubating manganese with Pr-I *in vitro* results in a dramatic decrease in the lag time prior to the onset of IAA-oxidation in the presence of the protector. This decrease in lag implies an inactivation of Pr-I by manganese. The data illustrated in table I show that Pr-I also becomes inactivated spontaneously when shaken by itself in buffer in an atmosphere of air. In contrast, under nitrogen this inactivation is eliminated when adequate precautions are taken to avoid contamination by small quantities of air. Even in the presence of 0.1 mM Mn^{2+}, Pr-I is not appreciably inactivated under nitrogen (see table II). However, under nitrogen, 0.1 mM Mn^{3+} causes a total, and very rapid, inactivation of Pr-I. Thus the data suggest that in the presence of oxygen, the manganous ion is converted to the manganic ion, which in turn oxidizes the protector. Although not as clear-cut, the data also suggest that the Mn^{3+}

ion is responsible for, or at least more efficient in, catalyzing the oxidation of IAA by peroxidase.

Protector-I exerts its protecting effect by virtue of its ability to act as a reducing agent (unpublished observations). The presence of the manganese ion therefore probably has a 2-fold effect in reducing the efficiency of Pr-I: First, as discussed above, the manganese interacts directly with Pr-I and oxidizes it. Second, as indicated in figure 1 and as has been shown by many others, in the presence of peroxidase, manganese speeds the transfer of electrons from IAA to the electron acceptor (*e.g.* DCP). Since Pr-I compensates for the electrons lost by IAA, it becomes depleted of electrons more rapidly, and the period of auxin protection is reduced still further.

Morgan *et al.* (6) have shown that in cotton plants there exists a direct correlation between high (toxic) levels of nutrient manganese, and both tissue manganese and IAA-oxidase activity. These workers reported an inverse correlation between high manganese and IAA-oxidase inhibitor (auxin-protector) activity and that the degree of oxidase stimulation was directly related to the severity of symptoms. The authors concluded that high levels of manganese in the tissues catalyzed the destruction of IAA-oxidase inhibitors (auxin-protectors), thus allowing the enzyme present to function.

We have no data on the *in vivo* effect of high levels of manganese on IAA destruction in the Japanese morning glory. However, it is very clear from the data presented in this communication that *in vitro,* manganese, specifically Mn^{3+}, not only speeds up the oxidation of IAA by peroxidase, but also causes the inactivation of auxin protector substances. These findings strongly support the concepts put forth by Morgan and coworkers for cotton plants, and Furuya and Galston for pea seedlings. The evidence further indicates that this manganese-induced inactivation of the protector is the primary effect, since IAA oxidation, and hence the catalytic effect of manganese on peroxidase activity, cannot manifest itself until the protector system has been inactivated.

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