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The Short-Term Effects of Manganese Toxicity on Ribulose 1,5 Biophosphate Carboxylase in Tobacco Chloroplasts

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The Short-Term Effects of Manganese Toxicity on Ribulose 1,5 Bisphosphate Carboxylase in Tobacco Chloroplasts

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by

Kerrie Lynn Elliott

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The Short-Term Effects of Manganese Toxicity on Ribulose 1,5 Bisphosphate Carboxylase in Tobacco Chloroplasts

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The Short-Term Effects of Manganese Toxicity on Ribulose 1,5 Bisphosphate Carboxylase in Tobacco Chloroplasts

Kerrie Lynn Elliott **May 1990** **28 pages**

Directed by: Dr. Frank Toman, Dr. Valgene Dunham, and Dr. Claire Rinehart

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The short-term effects of manganese toxicity on ribulose 1,5 bisphosphate carboxylase (Rubisco) activity and concentration in tobacco chloroplasts were examined. The activity of the enzyme from both manganese-treated and control plants was determined 6, 12, 18, 24 , and 48 h after introduction of manganese (80 mg/L) . Enzyme activity was determined by monitoring rates of radioactive CO_2 fixation into acid stable products. A slight stimulation of the enzyme's activity was noted in experimental plants after 18 h of exposure to manganese as compared with control plants. A decrease in the enzyme's activity in experimental plants was noted after 48 h of exposure. Visible symptoms such as chlorosis and decreased leaf size were also observed after 48 h of manganese exposure in experimental plants. Using Rocket Immunoelectrophoresis, no appreciable difference between Rubisco concentration levels of the experimental plants and the control plants was noted after 6, 12, 18, 24, and 48 h of manganese exposure indicating that the effect on Rubisco activity is a post-translational phenomenon and that Rubisco is not being degraded at an accelerated rate. Even after 7 d of exposure to high manganese concentrations, when visible symptoms such as chlorosis and necrotic lesions were very evident, the level of Rubisco in the manganese-treated plants varied little from the levels in the control plants. Manganese accumulated in the experimental plants to concentrations as high as 3282 μg/g dry wt as determined by atomic absorption spectrophotometry. A shuttling mechanism for manganese between young and old leaves

was indicated by an observed decrease in the concentration of manganese in the young leaf tissue between 12 and 18 h after treatment .

INTRODUCTION AND LITERATURE REVIEW

Manganese is a widely distributed element which is essential to plant systems. It serves as a transfer agent in photosystem II, the oxygen evolution system of photosynthesis (Packham and Barber, 1984). In addition, manganese is important in the proper functioning of enzymes such as catalase, peroxidases, and superoxide dismutase (Leidi et al., 1987). Recently it has been speculated that a manganese/pyridoxal phosphate system in the presence of peroxide and phenol may be important in the oxidative decarboxylation of amino acids (Smith and Marshall, 1988). Thus a certain amount of manganese is necessary for plant growth and proliferation. When manganese concentrations become greater than the desired optimal level, deleterious effects may result . This condition is known as manganese toxicity (Sigel, 1986). As industrial technology develops, numerous conditions could result in increased manganese in soils including acid rain (Foy et al., 1978; Rechcigl et al., 1987), and the use of industrial and private sewage on agricultural land as fertilizer. This sludge can increase the manganese in the soil to concentrations exceeding 700 mg/kg, a level which produces toxic effects in plants (Hue et al., 1988). As a result, decreased dry matter accumulations were observed in field grown burley tobacco as Mn concentrations increased. These decreased accumulations occurred when Mn concentrations reached about 400 μg/g, a concentration which would not result in the visible symptoms associated with manganese toxicity (Miner and Sims, 1983).

The visible symptoms of manganese toxicity vary and may be observed as early as 24 h after contamination (Nable et al., 1988). These symptoms include necrotic spots in barley (Rechcigl et al., 1987) and lettuce leaves (Ohki, 1984), puckering, distortion of leaves, mottled chlorosis and necrotic lesions in cotton (Foy et al., 1978; Sirkar and Amin, 1974). Brown spots, chlorosis, and leaf shedding are characteristic symptoms of manganese toxicity in cowpea (Horst, 1983; Wissemeier and Horst, 1987). Similar

symptoms are seen in soybeans and wheat such as crinkle leaf and necrotic spots (Heenan and Campbell, 1981; Rufty et al., 1979). In tobacco, chlorosis leads to necrotic lesions. Toxicity is usually seen in the young leaves first and affects the lamina of the leaves before affecting the midvein. Reduced yields may result from manganese toxicity in tobacco (Ohki, 1984; Rufty et al., 1979).

Manganese is normally present in the soil at levels between 500-900 mg/kg. Manganese in the divalent form is most accessible to plants (Sigel, 1986). Low pH and flooding conditions facilitate the reduction of Mn^{+4} and Mn^{+3} to the available divalent form (Patrick and Turner, 1968; Sigel, 1986). There is evidence that a plant's ability to tolerate wet soil conditions coincides with the plant's tolerance of excess manganese. The tolerance of rice, for example, is attributed to the ability of rice roots to oxidize manganese and limit concentrations of the accessible divalent form. In more alkaline pH conditions, microbial reduction of manganese to the divalent form also produces an environment favorable for manganese toxicity (Foy et al., 1978).

In addition to the availability of manganese, other factors influence manganese toxicity. One factor is the genotypic differences between plants. Manganese tolerance in cowpeas is more related to the genotypic variability within the species than the actual concentration of the manganese available to the plant (Horst, 1983). Temperature is another factor affecting manganese toxicity. Tobacco plants grown in warmer temperatures were more tolerant to high tissue levels of manganese. This was attributed to the increased rate of leaf expansion and vacuolar capacity in the warmer climate (Rufty et al., 1979).

Though the actual biochemical mechanism of manganese toxicity in plants is not fully understood, several ideas have been proposed. One hypothesis is that manganese, because of its multiple oxidation states, may allow the thylakoid membrane to become more permeable to O_2 which could cause lipid peroxidation. This peroxidation would cause the disorganization of membrane structure and the loss of photochemical activities (Panda et

al., 1987). Another proposal is that excess manganese could cause indoleacetic acid (auxin) to be destroyed (Foy et al., 1978; Ohki, 1984). Enzymatic activity changes were noted when cotton plants were exposed to manganese concentrations between 81-200 mg/L . First, manganese accumulated in the leaf tissue. After this accumulation, a rise in respiration was noted followed by the stimulation of polyphenoloxidase. It was at this point that the first visible symptoms of manganese toxicity were observed in the cotton plants. After this series of changes, it was noted that ethylene was evolved followed by the stimulation of peroxidase activity. Severe symptoms of manganese toxicity appeared in the cotton plants at this point. After the severe symptoms had become evident, terminal oxidase activity and respiration both were noticeably depressed. The final morphological changes which were observed were the abscission of the growing tip and the proliferation of the stem tissue (Sirkar and Amin, 1974).

One of the most widely cited explanations of manganese toxicity is the inhibition of other cations such as magnesium and iron, by manganese absorption. Because manganese and magnesium are both divalent ions and are of similar atomic radii, the decrease of magnesium has been attributed to ion absorption competition. Since chlorosis is not only a symptom of manganese toxicity but is also a symptom of magnesium deficiency, this proposal is widely accepted (Allen and Robinson, 1980; Heenan and Campbell, 1981). Manganese may block the access of the iron ions to the functional sites of the magnesium branch of the tetrapyrrol synthesis pathway. Because of this blockage, iron deficiency may result (Csatorday et al., 1984). Disagreement with this hypothesis was registered in a study of tobacco which found only a 25% decline in iron levels relative to controls in plants on which manganese treatments were conducted. Concentrations of iron remaining in the plant still remained greater than the amount required for the maximal growth of tobacco leaves. Iron requiring enzymes such as catalase, peroxidase, and respiratory complex enzymes were not greatly affected throughout the development of manganese toxicity (Nable et al., 1988).

In the phytoene biosynthesis system, geranylgeranyl pyrophosphate (GPP) was diminished with increasing amounts of manganese. Since GPP is an intermediate in gibberellic acid synthesis, growth reductions could occur with toxic levels of manganese (Clairmont et al., 1986; Wilkinson and Ohki, 1988).

Another proposal for the mechanism of manganese toxicity is that manganese affects photosynthesis. Since manganese is required for isoprenoid synthesis which produces many of the essential elements of photosynthesis such as carotenoids, chlorophyll and sterols, an excess of manganese could have a detrimental affect on the production of these compounds. Increased production of polyphenoloxidase activity could inhibit photosynthesis. If reduced polyphenoloxidase leaked through a ruptured tonoplast, the phenolics produced could inhibit photosynthesis (Nable et al., 1988).

A photosynthetic enzyme which has been studied in regard to its reaction to manganese toxicity is ribulose 1, 5 bisphosphate carboxylase / oxygenase (Rubisco). This enzyme was called Fraction I protein due to its electrophoretic homogeneity by Wildman and Bonner who found it to be the major protein constituent of green leaves (Wildman and Bonner, 1947). Since that time it has been shown that the enzyme catalyzed the initial steps in both the carbon reduction cycle of photosynthesis and the oxygenation of ribulose bisphosphate in photorespiration (Chapman et al., 1986; Lundquist and Schneider, 1989).

The Rubisco molecule is shaped like a barrel and is composed of 16 subunits, 8 large and 8 small. A fourfold molecular axis runs down a central aqueous channel (Chapman et al., 1986). The functional unit of carboxylase activity is a dimer of large subunits. The active site is located in the parallel *alpha*/*beta* barrel domain (Lundquist and Schneider, 1989). The height of the molecule is 10.5 nm and the diameter at the widest point is approximately 13.2 nm (Chapman et al., 1986). The sequence of the small subunit (*ssu*) is composed of 145 amino acids containing only three cysteine molecules per subunit. The *ssu* also contains a high proportion of aromatic residues, a core of four antiparallel *beta*- strands, and three *alpha*-helices . The large subunit (*lsu*) is composed of 475 amino

acids (Knight et al., 1989). In higher plants the *lsu* and *ssu* are coded for by the respective genes *rbcl* and *rbcs* which are located in different subcellular compartments. The *rbcl* is transcribed and translated in the chloroplast yielding the 53-kDa polypeptide. The rbcs is encoded in the nucleus, transcribed into a 20-kDa precursor polypeptide which must be transported into the chloroplast, a process requiring a transit peptide. The mature *ssu* contains highly conserved sequences of 16 amino acids which may constitute the domains needed to facilitate the assembly of the holoenzyme (Wasmann et al., 1989).

In order for the carboxylation and oxygenation reactions to occur, the enzyme must be activated. First the *epsilon*-amino group of a conserved lysine side chain must react with a carbon dioxide molecule forming a carbamate. This carbamate is stabilized by a Mg^{+2} ion. The ternary complex consisting of the enzyme-CO_2-Mg^{+2} is now activated and ready to catalyze both the oxygenation and carboxylation of ribulose bisphosphate. The better understood carboxylation reaction consists of three distinct partial reactions. The first partial reaction is the enolization of ribulose bisphosphate followed by the hydrolysis of the stable six carbon intermediate, 2-carboxy-3-keto-D-arabinitol 1,5 bisphosphate. These two initial reactions are both catalyzed by the activated Rubisco enzyme. The third reaction is the decarboxylation of the six carbon intermediate which leads to the enediolate form of ribulose 1,5 bisphosphate. This third step which is catalyzed by the nonactivated enzyme is mechanistically different from the true reverse reaction of the carboxylation step. The third reaction differs from the true reverse carboxylation step in that it lacks both a metal ion and a carbamate and in that it can be inhibited by a transition state analogue, 2-carboxy-D-arabinitol-1,5 bisphosphate. Since in the activated enzyme the 2-carboxyl group is ligated with a metal ion, the metal seems to play an integral role in catalysis. In fact, x-ray crystallographic studies of Rubisco and its transition state analogue indicate that the metal not only is essential for catalysis, but it may also play a role in the proper positioning of the substrate (Lundquist and Schneider, 1989).

Stromal protein-protein interactions may exist between Rubisco and

phosphoribulokinase. This enzyme has been found to copurify with Rubisco unless treated with high salt concentrations. This binding specificity between the kinase and the carboxylase showed enhanced stability during catalysis and could have some kinetic benefits to the carboxylation reaction (Sainis et al., 1989).

Since Rubisco is an abundant enzyme comprising 65% of the soluble leaf protein (Salvucci et al., 1986), and because of its importance in photosynthesis, it was chosen as the object of study in the investigations of the short-term effects of manganese toxicity on tobacco chloroplasts. Both enzyme activity and enzyme concentrations in tobacco tissue were measured and compared.

MATERIALS AND METHODS

Plant Growth Conditions:

Nicotiana tabacum plants, variety KY 17, were grown from seeds in a growth chamber for 85-95 d on a 12/12 h light/dark photoperiod. Plants were then transplanted into individual pots to allow for root expansion. After 2 wk, the roots of the plants were washed to remove soil and the plants were transferred to tubs each containing 8 L of one-half strength Hoagland's solution (Hoagland and Arnon, 1950) which was aerated. Plants were allowed to adjust to the hypotonic solution for 7 d. After this time, 80 mg/L Mn as $MnCl_2 \cdot 4H_2O$ was introduced into the experimental tub. The photoperiod was then adjusted to 24 h of continuous light. The maintenance of activated Rubisco requires constant light (Portis et al., 1987).

Sampling:

Leaf samples were taken primarily from the young leaves at 6, 12, 18, 24, and 48 h after treatment with manganese. The leaves were analyzed for Rubisco activity, Rubisco concentration, total protein content, and manganese concentration at these time intervals both in control and experimental plants.

Rubisco Activity:

Rubisco activity was determined by a modified radioactive isotope technique (Boon-Long , 1980; Johnson et al., 1974; and Laing, et al., 1975). Leaf material (0.4 g) was macerated in 5 mL of extraction media [50mM Hepes, pH 8.1, 10 mM $MgCl_2$, 5 mM D-isoascorbate, 0.25 mM Na_2EDTA, 5 mM Dithiothreotol (DTT), and 0.025 mM polyvinyl-pyrrolidone (PVP)] and centrifuged in a Beckman Centrifuge at 17,000 x g for 20 min at 2-3 ^{o}C. One mL of assay media [50mM Hepes, pH 8.1, 5mM $MgCl_2$, 3 mM DTT, 0.1 mM

Na_2EDTA, 0.1 mM ribulose 1,5 bisphosphate (RUBP), 0.1 mM $NaH^{14}CO_3$, and 2.4 mM $NaHCO_3$] was added to a scintillation vial and incubated for exactly 4 min at 25°C. The reaction was initiated by the addition of 0.1 mL of cold crude enzyme extract and incubated 3 min. The reaction was stopped by the addition of 0.1 mL of 6N acetic acid. All samples, assayed in triplicate, were then allowed to air dry overnight in a hood to remove the excess $^{14}CO_2$ which was not fixed into acid stable 3-phosphoglycerate (PGA). The contents of the vials were then redissolved in 1 mL of deionized water and vortexed. After the addition of 10 mL of scintillation cocktail (18 mM 2,5-diphenyloxazole, 0.26 mM 1,4-bis[5-phenyl 1-2-oxazoly 1]-benzene, 33% Triton X-100, 68% toluene), the radioactivity was determined by a Packard Liquid Scintillation Counter .

Protein Analysis:

Protein determinations were performed using Biorad (Biorad Laboratories) protein reagent concentrate diluted 1:5 (dye to deionized water). Bovine serum albumin (BSA) was used as a standard protein (range of 0 to 200 µg protein/mL). One mL of diluted Biorad solution was added to 50 µL of sample and the absorbance monitored at 595 nm using a Shimadzu Research Spectrophotometer. This protein method uses the phenomenon that Coomassie Brilliant Blue G-250 dye absorbs maximally at 465 nm. When the dye is bound to protein, however, the maximal adsorption of the dye shifts to 595 nm. The method is sensitive and has fewer interferences than the more complicated Lowry method (Bell and Bell, 1988).

Rubisco Purification:

Rubisco was purified from tobacco by a modified procedure by Salvucci (Salvucci et al., 1986). Three hundred g of tobacco lamina with the mid-ribs removed were homogenized in 600 mL of extraction buffer (50 mM Tris-HCL pH 7.6, 20mM $MgCl_2$, 20 mM $NaHCO_3$, 0.1 mM EDTA, 10 mM DTT, 1% casein, 2% PVP, 10% glycerol) with a

chilled blender. The slurry was filtered through 8 layers of cheesecloth and 1 layer of miracloth which had been prerinsed with deionized water and chilled. The filtrate was then centrifuged at 9486 x g for 20 min in large screw-top centrifuge containers. The supernatant was decanted and the extraneous protein was precipitated out by bringing the salt concentration to 40% by the addition of saturated ammonium sulfate. The solution again was centrifuged at 9486 x g for 20 min. The supernatant was brought to 60% ammonium sulfate to precipitate Rubisco . Again the mixture was centrifuged at 9486 x g for 20 min. The pellet was dissolved in 20 mL of Sephacryl buffer (100mM Tris-HCL pH 7.6, 20mM $MgCl_2$, 20 mM $NaHCO_3$, 0.1 mM EDTA, 50 mM 2-methanol). The redissolved pooled pellets were then ultracentrifuged for 1 h at 45000 x g at 4°C. The clarified resuspended pellet was then loaded onto a Sephacryl S-300 (330 mL) column with a flow rate of 0.3 mL/min. Protein was monitored at 280 nm using an ISCO VA-5UV monitor with a 5 mm HPLC flow cell. One mL fractions were collected with an ISCO FOXY fraction collector. Fractions from tubes 14-18 were pooled and an estimation of the protein content was accomplished based on absorption at 280 nm. Additional purification of the enzyme was carried out by ion-exchange Fast Protein Liquid Chromotography on a Mono Q column. Ten mg of protein was loaded via a 10 mL superloop and protein was eluted with a KCl gradient. The elutant was again collected by the fraction collector and monitored at 280 nm. The tubes containing the Rubisco were pooled, brought to 60% ammonium sulfate saturation and allowed to settle. The Rubisco slurry was then dropwise added to liquid nitrogen to form "pearls". These were stored at -80°C.

To use the purified Rubisco from tobacco, a "pearl" was thawed and desalted with G-50 Sephadex column chromatography. The Rubisco was placed directly on the G-50 column and eluted using Immunoelectrophoretic Tricine IV Buffer, pH 8.6.

Rubisco Concentration:

Tobacco leaf lamina (0.4 g) was ground in 500 µL of extraction buffer (25 mM Hepes pH 7.5, 1 mM EDTA, 5 mM isoascorbate, 4 mM DTT) and microfuged (12000 x g) for 1.5 min to remove membrane fragments. Specific rabbit antibodies made to the holoenzyme were obtained from Cocalico Biological Inc. of Reanstown, Pennsylvania. Fifty µL of the antibody were incorporated into a 1% agarose gel (2 Biorad agarose immunoelectrophoretic tablets in 10 ml of Immunoelectrophoretic Tricine IV Buffer, pH 8.6) which was poured onto FMC GelBond agarose gel support medium. As the enzyme migrated in the electric field through the gel, it came into contact with the antibody. An antigen/antibody complex was formed in the shape of an ascending rocket. The height of the rocket was proportional to the amount of antigen placed in the well (Laurell and McKay, 1981). Immunoelectrophoresis Tricine IV Buffer, pH 8.6 was wicked onto the gel using Whatman #1 filter paper. Electrophoresis was run for 30 min at 35 V and then for 16 h at 80 V.

The gels were then pressed under Whatman #4 filter paper for 30 min, dried with a hair dryer, and stained for 15 min with Coomassie Blue Stain (1.0 g coomassie brilliant blue stain, 10 mL glacial acetic acid, 160 mL ethanol diluted to a L with deionized H_2O). After soaking the gels in destainer (10 mL glacial acetic acid, 160 mL ethanol diluted to a L with deionized H_2O) for 10 min, the gels were allowed to air dry.

Manganese concentrations:

Dried leaf material (0.1 g) was placed in a 25 mL Erlenmeyer flask. After adding 5 mL of 9:1 (v/v) nitric/perchloric acid solution, the samples were allowed to reflux for 12 h, after which they were evaporated to dryness. The ashed material was redissolved in 5 mL of 1N HCl and analyzed on a Perkin Elmer Atomic Absorption Spectrophotometer for manganese concentrations.

RESULTS AND DISCUSSION

When Rubisco activities of control plants were compared with those which had been exposed to manganese (80 mg/L), there was little difference between the two activities at 6 and 12 h after treatment (See Table I and Fig. 1). However, 18 h after treatment the activities of the experimental plants were slightly but consistently higher than the control plants. Twenty-four h after contamination, the activities of control and experimental plants were approximately equal. Rubisco activities in Mn treated plants were lower than the corresponding control plants after 48 h. These differences were not substantiated statistically, however, when subjected to a Student's t test (Steele and Torrie, 1980). These trends were maintained when Rubisco activities were calculated on a protein basis (See Table II and Fig. 2) using the Biorad protein technique previously described. When the data for the protein based activities were analyzed statistically, significant differences were noted at a confidence level of 0.05. After 6 h of exposure to manganese, the control was significantly higher than the manganese-treated plant. After 12 and 18 h the manganese treated plants had Rubisco activities significantly higher than the control plants. Twenty-four h after treatment, there was no significant difference between control and treated plants. After 48 h of high manganese the control plants had Rubisco activities significantly higher than the manganese-treated plants.

The trends for both the dry weight and the protein based activities of Rubisco from this short-term study were different from those observed over long term exposure experiments. Rubisco activity of tobacco chloroplasts treated with Mn was significantly lower than controls after a period of 24 h. This decrease in activity continued upon further exposure to Mn (Toman et al., 1984). The discrepancies for the 24 h time period could simply be due to the varying response of different varieties of tobacco to Mn toxicity.

Table I
Rubisco Activity Comparisons
(dry wt basis)

Time (h)	(# experiments)	Control (mg CO_2/g dry wt/h)	Manganese (mg CO_2/g dry wt/h)
6	(15)	6.08	6.02
12	(14)	12.58	13.33
18	(15)	10.56	13.42
24	(16)	10.40	10.98
48	(12)	11.84	8.72

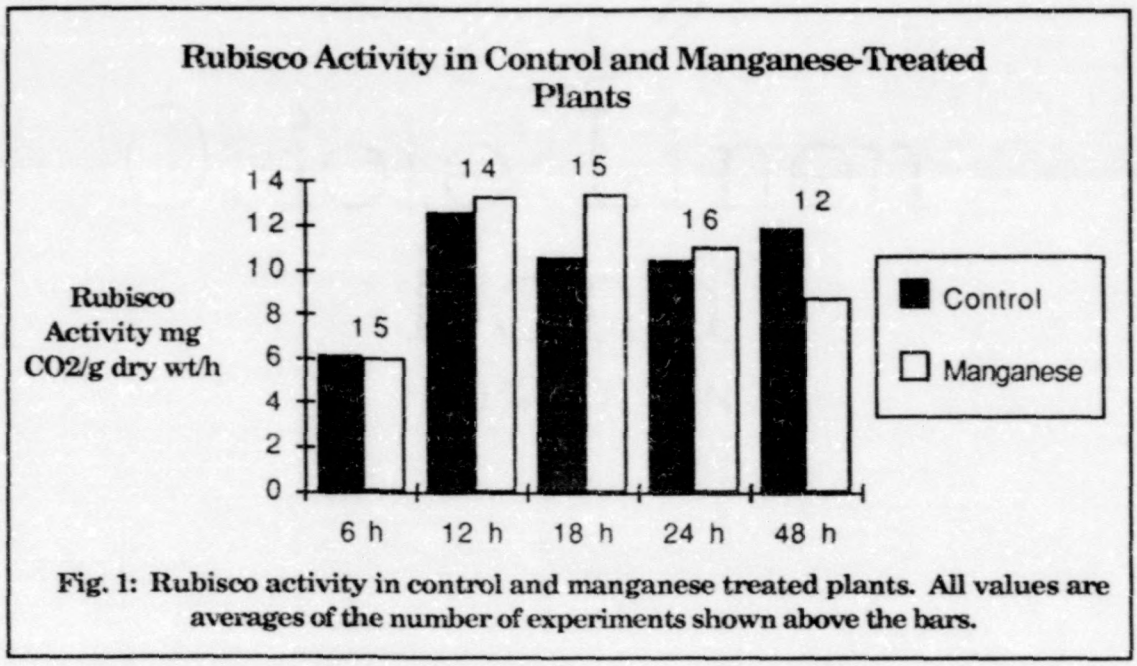

Fig. 1: Rubisco activity in control and manganese treated plants. All values are averages of the number of experiments shown above the bars.

Table II
Rubisco Activity Comparisons
(protein basis)

Time (h)	(# experiments)	Control (ng CO_2/μg protein/h)	Manganese (ng CO_2/μg protein/h)
6	(15)	3.01	2.50
12	(13)	3.90	6.30
18	(15)	7.50	9.80
24	(13)	3.59	3.59
48	(12)	4.58	1.01

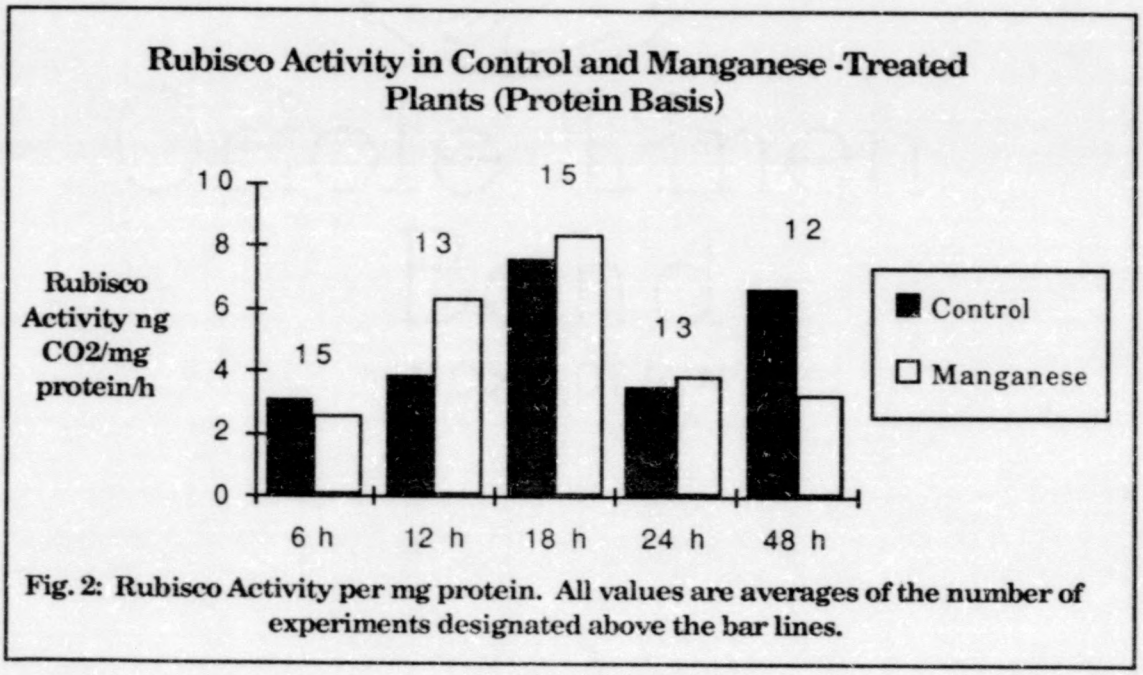

Fig. 2: Rubisco Activity per mg protein. All values are averages of the number of experiments designated above the bar lines.

However, it appears that manganese does not reduce the activity of Rubisco in the initial time after treatment as was noted after longer exposures to manganese.

To insure that manganese was indeed being absorbed by experimental plants, manganese determinations were performed. The manganese concentration in experimental plants was much higher than that in control plants (See Table III). However, after 12 h of treatment, a decrease appeared in the manganese concentration as compared to the concentration at 6 h. The concentration of manganese increased 387% between 12 and 24 h (See Fig. 3). Some plants have the ability to compartmentalize metal ions to protect themselves from possible interference of the ion with important cell processes. Other plants may combat excess manganese by shuttling the metal from the rapidly growing young leaves to the older leaves. The older leaves may then be shed, thus ridding the plant of the excess manganese (Bingham et al., 1986). Since the younger leaves were generally used in analysis, the shuttling hypothesis could apply. To test this hypothesis, both young and old leaves were sampled and the accumulation of manganese in the tissue was compared. The young leaves showed the characteristic decrease in concentration after 12 h, while the manganese concentration in the older leaves progressively increased (See Table IV and Fig. 4). These results indicated that a shuttling mechanism may exist between young and old leaves. Rubisco activity increases and decreases in light of the fluxing manganese concentrations, pose an interesting hypothesis. The Rubisco activity of the control and experimental plants seemed to be equal as long as the plant could combat the problem by shuttling the manganese out of the young leaf tissue as seen in both the 6 and 12 h data (See Table V). As soon as the manganese concentration became too great for the shuttling mechanism to control, there was a slight increase in the Rubisco activity. Then as manganese continued to accumulate, the Rubisco activity leveled off and decreased as compared to the control.

To examine the possibility that manganese affected the translation of the enzyme, Rubisco concentrations were determined by rocket immunoelectrophoresis.

Table III
Manganese Concentrations in Young Leaves
(All values are averages of 26 experiments)

Time (h)	Control (µg/g dry wt)	Manganese (µg/g dry wt)
6	178	768
12	165	311
18	151	1032
24	173	1890
48	NA	NA

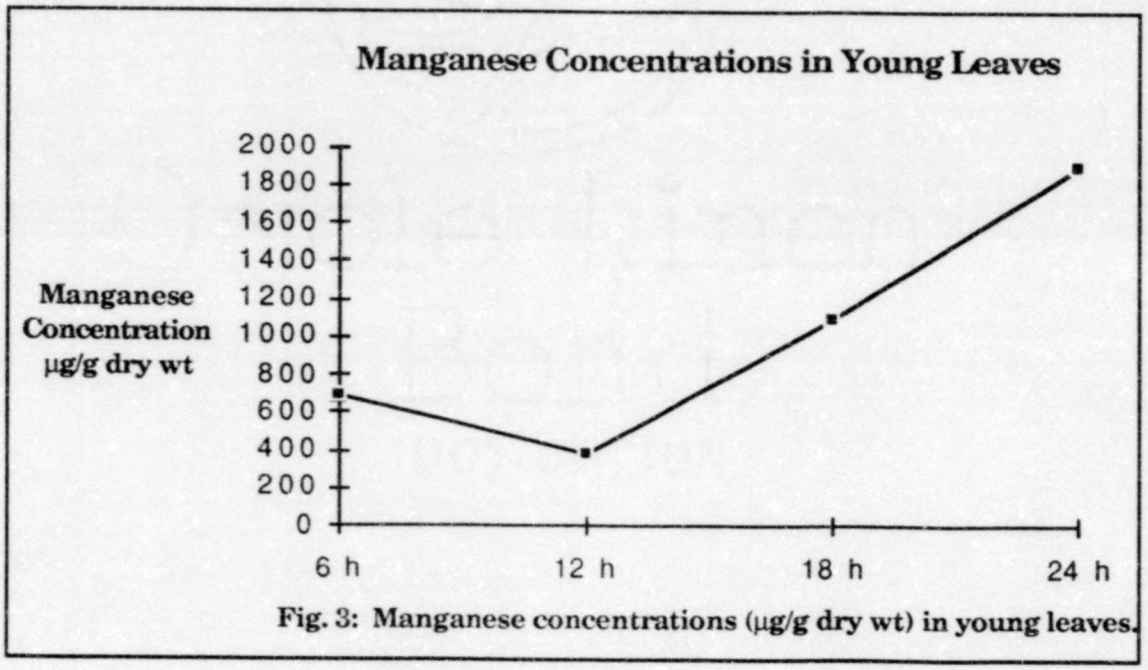

Fig. 3: Manganese concentrations (µg/g dry wt) in young leaves.

Table IV
Manganese Concentrations in Young and Old Leaves

Time (h)	Young Leaf (µg/g dry wt)	Old Leaf (µg/g dry wt)
6	525	322
12	504	661
18	1242	872
24	1906	1065
48	3282	2110

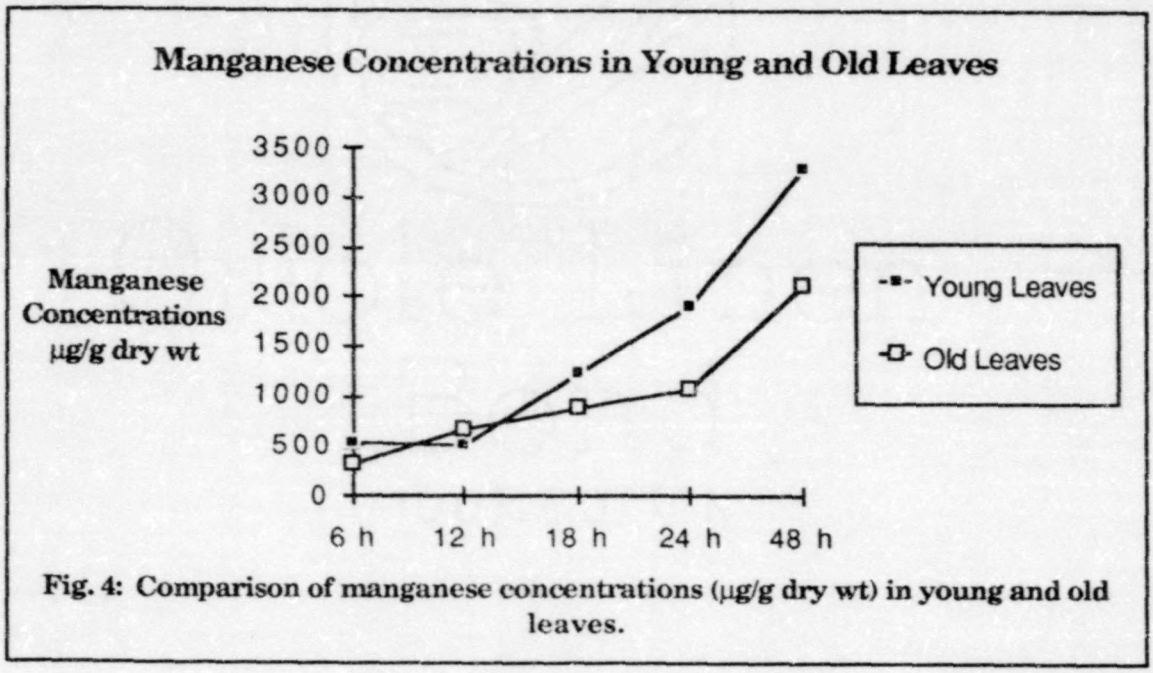

Fig. 4: Comparison of manganese concentrations (µg/g dry wt) in young and old leaves.

Table V
The Average Rubisco Activity of Control and Manganese-treated Plants Compared With the Concentration of Mn Accumulated in the Young Leaves

Time (h)	Control (mg CO_2/g dry wt/h)	Manganese (mg CO_2/g dry wt/h)	Young Leaf (μg/g dry wt)
6	6.08	6.02	525
12	12.58	13.33	504
18	10.56	13.42	1242
24	10.40	10.98	1906
48	11.84	8.78	3282

This method is made specific for Rubisco by placing an antibody to the enzyme in the agarose gel. The buffer must be wicked to the gel during this procedure so as not to solubilize the antibody in the buffer. By utilizing known amounts of Rubisco along with the unknown samples, a standard curve may be plotted and used to determine the enzyme concentration in the tobacco plant tissue (See Fig.5). A standard curve was determined for each rocket immunoelectrophoretic assay. However, since the antibody in the gel must be specific for tobacco Rubisco, the enzyme must first be purified from tobacco plants to be used as standards in the electrophoretic technique as well as to make the specific antibody against the enzyme. This purification was done by Fast Protein Liquid Chromatography (FPLC) which is similar to High Performance Liquid Chromatography in that pumps are used to pass a substance through an ion exchange column. In FPLC all of the columns, pumps, and other components that may come into contact with the protein are made of glass so as not to interfere with the activity of the protein being purified. The FPLC system may also be effectively utilized at cool temperatures which facilitates the maintenance of an enzyme's activity (Salvucci et al., 1986).

When Rubisco concentrations in the control were compared with concentrations in the experimental plants (See Fig. 6), no significant differences were noted as determined by the Student's t Test . In fact, the concentrations of Rubisco were almost identical between control and Mn treated plants for 6, 12, 18, 24, and 48 h. These results indicated that manganese affected the activity of the enzyme and not the actual production of the enzyme. Therefore translational or pretranslational processes of Rubisco production are likely not affected. These results also indicate that the degredation of Rubisco is not enhanced. One would expect to eventually see a decline in the Rubisco concentration after increased exposure to manganese, because of the appearance of the visible symptoms which indicate that the metabolic reactions within the plant are being affected. But even after 7 d of exposure to manganese toxicity, when the visual symptoms are very evident, the Rubisco

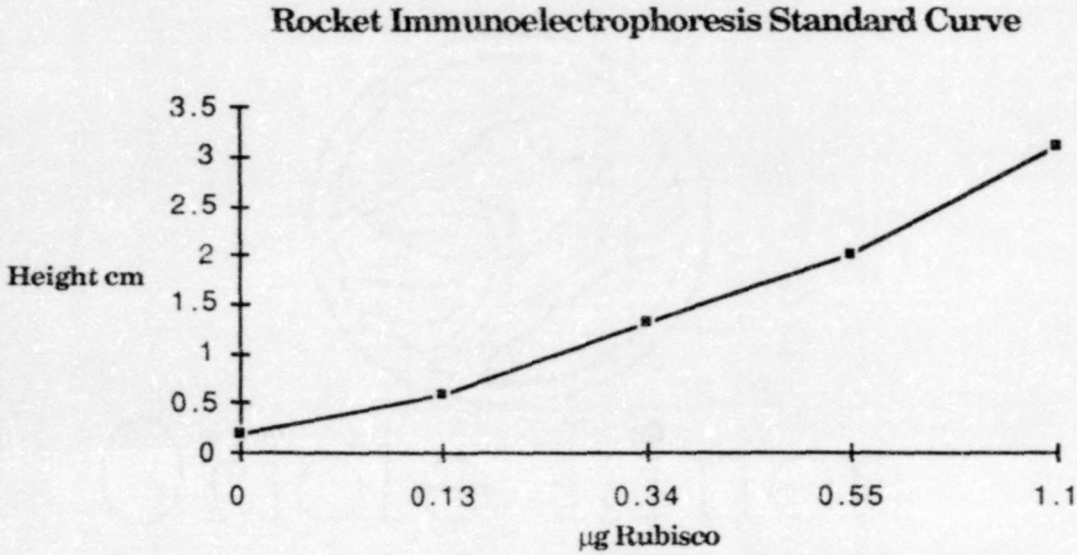

Fig. 5: Standard curve for Rubisco concentration assay.

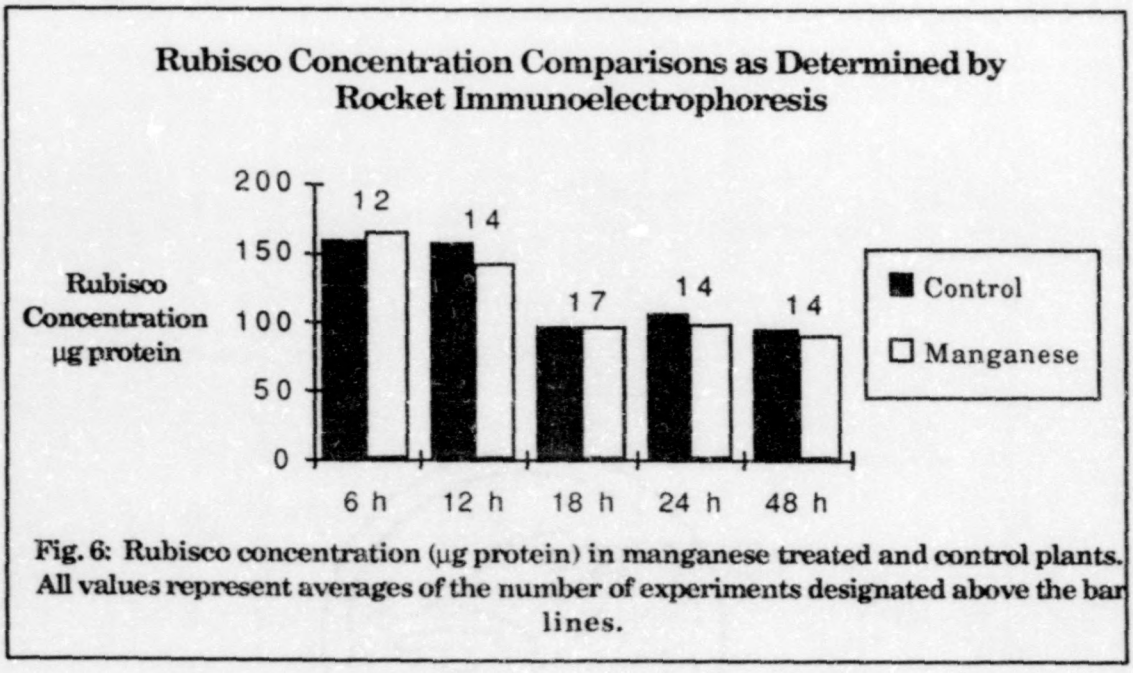

Fig. 6: Rubisco concentration (µg protein) in manganese treated and control plants. All values represent averages of the number of experiments designated above the bar lines.

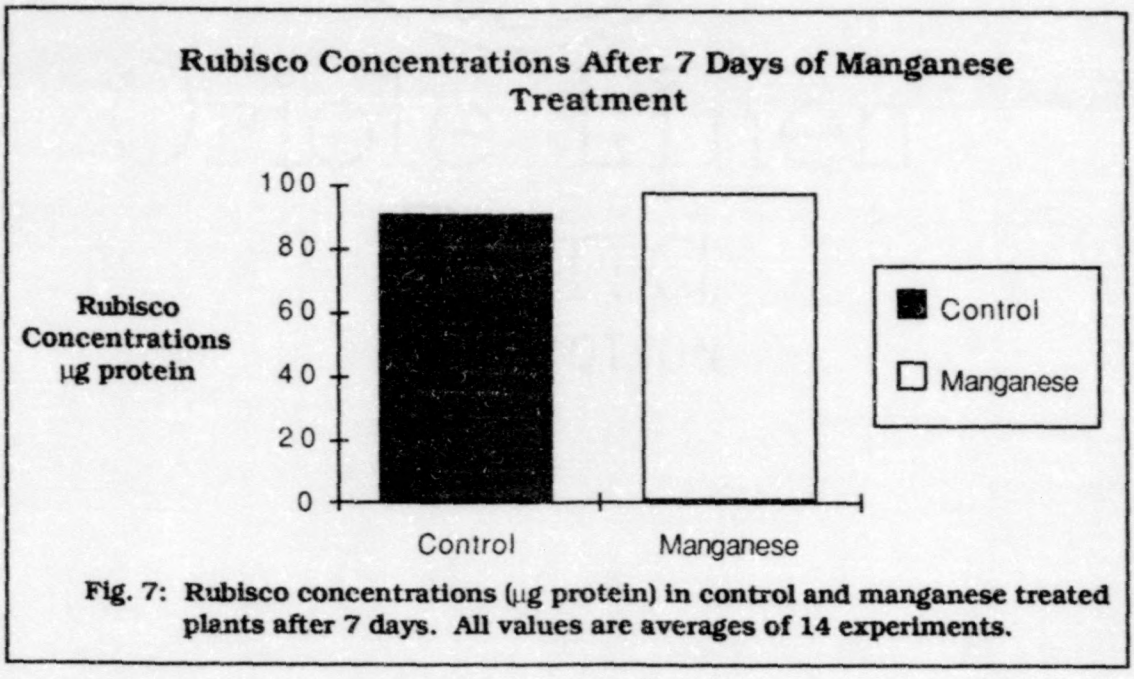

Fig. 7: Rubisco concentrations (µg protein) in control and manganese treated plants after 7 days. All values are averages of 14 experiments.

levels between the control and experimental plants remain relatively similar (See Fig. 7). Thus the marked decline in Rubisco activity after prolonged manganese exposure cannot be attributed to a decrease in the production of or increase in the degredation of the enzyme protein.

To explore other possible explanations for the proposed stimulation of Rubisco activity after 18 h of contamination and the more substantiated decline in activity after 48 h, one must consider the role of the metal ion in the activity of the enzyme. A divalent metal ion, usually Mg^{+2}, is required to form the active enzyme-CO_2-Mg^{+2} complex of Rubisco (Pierce and Reddy, 1986). The activating CO_2 binds to the *epsilon*-amino group of lys-201 of the large subunit. The carbamate that is formed is stabilized by Mg^{+2} (Yokota and Kitaoka., 1989; Lorimer et al., 1989). The complex may then catalyze either the carboxylation or the oxygenation of ribulose 1,5 bisphospate (RUBP) (Pierce and Reddy, 1986). Since Mg^{+2} and Mn^{+2} are both divalent and of similar atomic radii (Mg^{+2} ionic radius = 0.072 nm, Mn^{+2} ionic radius = 0.083 nm) and since Rubisco has a greater affinity for manganese than magnesium, manganese could substitute for magnesium in the active enzyme (Bingham et al., 1986; Houtz et al., 1988). When a series of metals were used to activate the enzyme, only Mg^{+2} and Mn^{+2} allowed for the catalysis of both the carboxylase and the oxygenase reactions of RUBP. However, the ratio of oxygenase to carboxylase activities was increased by the substitution of Mn^{+2} for Mg^{+2} (Pierce, 1986). This increase occurred because when the quaternary complex was formed with Mn^{+2} instead of Mg^{+2} [enzyme-CO_2-Mn^{+2}-carboxyarabinitol bisphosphate (CABP)], an exceedingly stable, tightly bound analogue of the carboxylated reaction intermediate was formed preventing a quick reaction between the bound RUBP and the active site (Pierce and Reddy, 1986; Lorimer et al., 1989). The resonance of the Mn^{+2} bound CABP and the carbamate molecules was obliterated as determined by Nuclear Magnetic Resonance (NMR). The loss of resonance was caused by the strong relaxation effects of the paramagnetic ion on the phosphorus atoms of CABP (Pierce, 1986).

The metal ion may also play a role in determining the relative substrate specificity of the enzyme (Pierce, 1988). Interactions of the divalent metal ion with the carbonyl group of the substrate would assist the placement of the substrate correctly in the catalytic site. Polarization of the carbonyl group by the metal ion could form an enediol by extracting a proton. Then the CO_2 or O_2 could react with the enediol consistently with the normal proposed kinetic pathway of the enzyme (Lorimer et al., 1989). Thus, substituting metal ions could alter the placement of the substrate in the active site. This substitution could enhance or decrease the reactivity of the enzyme based on the orientation in which the substrate was placed.

Kinetically, the enzyme is much more efficient when magnesium is used. For example, the ratio of V_{max}/K_m values, which is highly dependent on the nature of the activating metal, has a value of 80 in spinach with Mg^{+2} used as the activating ion, and a value of 3 when Mn^{+2} is used as the activating ion (Pierce, 1986). Therefore, when manganese bombards the plant's system, a substitution would decrease the kinetics of the carboxylase reaction thus lending insight to a possible explanation for the decrease in Rubisco activity after 48 h.

However, electron spin resonance studies suggested that Mn^{+2} could have a direct role in the catalysis of RUBP carboxylation. Chemically, a Mn^{+2} coordinated to the negatively charged acid would cause stabilization since it would serve as an electron sink (Miziorko and Sealy, 1984). If this is true, when the manganese first bombards the plant system after 18 h, the manganese could briefly enhance the carboxylation reaction.

Though Rubisco is known to have a potent nocturnal inhibitor, 2-carboxyarabinitol-1-phosphate, it has not been shown to be stimulated by manganese (Beck et al., 1989). Thus stimulation of the Rubisco inhibitor by manganese to decrease the enzyme's activity is unlikely.

The discovery of Rubisco Activase suggests another hypothesis for the effect of manganese on Rubisco activity. In most plants the activated Rubisco spontaneously forms

the carbamylated enzyme upon the addition of CO_2 and Mg^{+2} (Werneke et al., 1988). However, in 1982 a mutant strain of *Arabidopsis* did not maintain active Rubisco at atmospheric levels of CO_2. This strain was missing the soluble, chloroplast protein, Activase (Portis et al., 1987). Activase is thought to catalyze carbamylation, the coordination of the carbamate to the active site by the metal ion. Carbamylation ratios may be predicted by the activity ratios of Activase (Butz and Sharkey, 1989). Thus metal ion substitution could greatly alter this process. This enzyme has not been characterized sufficiently to substantiate this hypothesis.

Rubisco Activase *in vitro* was found to be dependent on ATP and is inhibited by ADP. Thus it may be regulated by ATP concentrations within the chloroplast. The Activase was labile and the ATPase activity required Mg^{+2}. Manganese caused slight stimulation of the ATPase activity which copurified with the Rubisco activase activity (Robinson and Portis, 1989). Since Activase was intrinsically involved in Rubisco activation and had a metal cofactor, the effects of manganese toxicity on Rubisco activity could be attributed to the effects of manganese toxicity on Rubisco Activase.

Though the actual mechanism of manganese toxicity on plants may be a syndrome instead of an isolated event, the effect on Rubisco activity probably plays a role in the overall result of manganese toxicity. Because of its importance in photosynthesis, a decline in Rubisco activity can cause a decline in the photosynthesis of a plant. The result of this study indicated that the mechanism of manganese toxicity on Rubisco was not a pretranslational or degredation event but may have involved cation substitution which would lower the kinetics of the carboxylase reaction or alter the substrate specificity. Rubisco Activase, an important enzyme in Rubisco activation, could also be affected by high levels of manganese since it, too, has a metal cofactor. This concept needs investigation. Whatever the mechanism may be, the plant attempts to combat the manganese toxicity by shuttling the ion to the older leaves away from the young growing

tissue. This shuttling mechanism, however, eventually is overcome and the inevitable post-translational decline in Rubisco activity occurs.

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