Effects of Cadmium on Carbonic Anhydrase and Activities Dependent on Electron Transport of Isolated Chloroplasts[1]

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ABSTRACT

Low concentrations of Cadmium inhibit the electron transport and CO_2 fixation reactions of isolated chloroplasts. CO_2 fixation is more sensitive to Cd than electron transport and dark pre-incubation increases the degree of toxicity to both. Carbonic anhydrase, an enzyme associated with CO_2 fixation, is very sensitive to Cd either when applied directly to partially purified preparations of the enzyme or when enzyme preparations are obtained from intact chloroplasts previously exposed to Cd. Strong inhibition occurs at Cd concentrations lower than those required to inhibit any of the electron transport dependent reactions studied. These results are interpreted as evidence that carbonic anhydrase is one of the most sensitive sites of Cd action in isolated chloroplasts.

INTRODUCTION

Cadmium, a non-essential element in plant nutrition, accumulates in different tissues and impairs plant growth. Recent evidence indicates that cadmium inhibits whole plant photosynthesis (1) and the electron transport activities of isolated chloroplasts (2). The early events in the oxidizing side of photosystem II are considered to be the most sensitive site of inhibition in the electron transport chain of isolated chloroplasts. Inhibition of whole plant photosynthesis occurs at concentrations lower than those required for strong inhibition of electron transport, indicating the possibility of additional sites of action. The present work presents evidence showing that in the isolated chloroplast, the activity of carbonic anhydrase, an enzyme associated with photosynthetic CO_2 fixation, is even more sensitive to Cd than electron transport.

MATERIALS AND METHODS

The bean plants (*Phaseolus vulgaris* L. cv. Jamapa) used in these experiments were grown under greenhouse conditions in 6-in diameter plastic pots containing sterilized soil. Chloroplasts were extracted from fully expanded trifoliolate leaves, when the plants were approximately 2 weeks old.

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Chloroplasts used in all the experiments were isolated by the method of Robinson and Stocking (10). Solutions A and B used contained 50 mM sorbitol, 40 mM isoascorbate, 40 mM $NaNO_3$, 20 mM $MnCl_2$, and 20 mM $MgCl_2$. In addition, solution A contained 20 mM disodium ethylenediamine tetraacetate (EDTA), 50 mM 2-(N-morpholino) ethanesulfonic acid (MES), and 20 mM $CaCl_2$ with a pH of 6.1, and solution B contained 50 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) and 20 mM NaCl with pH adjusted to 6.7.

Chlorophyll content was measured by the method of Arnon (1). The degree of inhibition of electron transport associated with PS II activity was determined by measuring changes in oxygen concentration polarographically as described by Cedeño-Maldonado et al. (2). The assay solution contained 100 ug chlorophyll, 50 mM HEPES, 1 mM KCN, and

TABLE 1.—*Effect of different concentrations of Cd on PS II activity of isolated chloroplasts*[1]

μM $CdCl_2$	Oxygen evolution	% Inhibition
0	50	0
100	50	0
200	43	13
250	35	30
350	30	39
500	24	52
650	21	58

[1] Assay medium contained: 50 mM HEPES, 0.4 mM $K_3Fe(CN)_6$, 10 mM KCN, and chloroplasts at a chlorophyll concentration of 100 μg/1.75 ml. Oxygen evolution is given in umoles 0_2/mg chlorophyll/hr.

0.4 mM $K_3Fe(CN)_6$. The preparations were uncoupled by adding 100 uM $(NH_4)_2SO_4$. The effect of Cadmium on PS II and in all the experiments discussed in this work were determined by adding different concentrations of $CdCl_2$ to the reaction mixtures.

To explore the possible participation of Cd in inhibition of CO_2 fixation, O_2 evolution was determined with preparations of intact chloroplasts. These chloroplasts were obtained from leaves which had been exposed to 24 hours of darkness before extraction. The reaction media in these experiments contained 100 μg chlorophyll, 100 mM K_2HPO_4, 5 mM $MgCl_2$, 10 mM KCN, and 50 mM HEPES. The mixture was saturated with gaseous CO_2 before oxygen evolution was measured.

For assaying carbonic anhydrase activity, chloroplast preparations suspended in solution B were centrifuged and re-suspended in 20 mM sodium diethyl barbiturate, pH 8.0, to break the chloroplasts and liberate the enzyme. The lysed chloroplasts were then centrifuged for 15 min at 4,000 g. The supernatant (enzyme extract) was used to obtain a partially

purified enzyme preparation by the method described by Everson (4). Carbonic anhydrase activity was measured by the method described by Chang (3).

Since carbonic anhydrase is associated with the stroma, intact chloroplasts had to be employed to determine whether Cd was reaching the enzyme by penetration through the chloroplast membranes. Chloroplasts at a chlorophyll concentration of 100 mg were suspended in test tubes containing solution B and 50 mM $CdCl_2$. The tubes were kept in darkness from 0 to 30 min., and then centrifuged for 2 min. at 2,000 g. The resultant pellet was washed with cadmium-free medium and centrifuged again. The remaining pellet was then resuspended for 15 min. in 5 ml veronal buffer to rupture the chloroplasts and release the enzyme, and then centrifuged for 15 min at 4,000 g. The supernatant (enzyme extract) was used for assaying carbonic anhydrase activity.

TABLE 2.—*Effect of dark incubation on inhibition by Cd of PS II activity in isolated chloroplasts*[1]

μM $CdCl_2$	O_2 Evolved		% Inhibition	
	0 min	10 min	0 min	10 min
0	37.5	34.3	0	0
100	37.5	37.5	0	0
250	27.0	17.9	28.1	47.8
350	20.9	15.8	44.3	54.0
500	21.7	11.9	42.0	65.2

[1] Assay medium contained: 50 mM HEPES, 0.4 mM $K_3Fe(CN)_6$, 10 mM KCN, and chloroplasts at a chlorophyll concentration of 100 μg/1.75 ml. Oxygen evolution is given in umoles O_2/mg/chlorophyll hr.

RESULTS AND DISCUSSION

The effect of cadmium on oxygen evolution associated with PS II activity was determined by the use of $K_3Fe(CN)_6$ as electron acceptor. Results shown in table 1 demonstrate the degree of inhibition produced in this photosystem. Inhibition was observed at $CdCl_2$ concentrations exceeding 200 μM, and concentrations exceeding 500 μM were required to obtain over 50% inhibition of PS II electron flow activity. Willing (12) reported 35% inhibition with 300 μM Cd; and Li and Miles (9) found 20% inhibition with concentrations between 100 and 300 μM Cd, which are comparable to the results presented in table 1.

Since previous studies with heavy metals (2) have shown that exposure of preparations to periods of dark pre-incubations could change the pattern of toxicity, an experiment was performed to reveal the behavior of Cd in this respect. In darkness the chloroplast samples were pre-incubated for 10 min at 25 C with preparations containing Cd before

being assayed. The effect of dark pre-incubation on PS II activity is shown in table 2. Dark incubation had considerable effect on the degree of inhibition in this assay system, especially at high Cd concentrations. Apparently, some time is required for Cd to reach the sensitive sites of PS II and to exert its inhibitory action. In this respect the action of Cd is different from that of Cu, which exerts a maximum inhibitory action immediately after its addition to chloroplast preparations (2).

A procedure for the determination of CO_2-supported O_2 evolution by intact chloroplasts has been recently described (5). With this procedure, experiments were performed to explore the effects of cadmium on CO_2 fixation. The results shown in table 3 indicate that CO_2 fixation by intact chloroplasts is inhibited by Cd even to a greater degree than electron transport, a fact that points to the reactions involved in CO_2 fixation as sensitive sites to the action of Cd.

TABLE 3.—*Effect of different concentrations of Cd on CO_2 supported oxygen evolution by intact chloroplasts*[1]

μM $CdCl_2$	Oxygen evolution	% Inhibition
0	7.7	0
25	5.1	33
50	4.6	38
100	4.2	44
200	4.2	44
350	3.9	52
450	1.7	77

[1] Assay mixture contained: 100 mM K_2HPO_4, 5 mM $MgCl_2$, 10 mM KCN, 50 mM HEPES and chloroplasts (100 μg chlorophyll/1.75 ml). CO_2 gas was bubbled to saturate the medium. Oxygen evolution is given in umoles O_2/mg chlorophyll/hr. Assay mixture was incubated in darkness for 10 min before the assay.

To obtain additional illustrative information on the possible mechanism of inhibition of CO_2 fixation, pre-incubation experiments similar to these previously described were performed. Results are illustrated in figure 1. A concentration of 350 μM $CdCl_2$ was employed since it conveniently produced about 50% inhibition after 10 min of incubation. The degree of inhibition obtained was time dependent, increasing with incubation time. This is an indication that the sensitive sites are located inside the chloroplasts. Since all these experiments were conducted with intact chloroplasts in which CO_2 is the electron acceptor, the results are indicative of the dark reactions of photosynthesis as being very sensitive to Cd.

According to Li and Miles (9), the ultimate site of cadmium inhibition rests directly on PS II photoreaction, but Willing (12) mentioned the possibility that the primary inhibitory effects of Cd may not be due to

effects on the electron transport system but rather to inhibition of some of the reductase enzymes involved in CO_2 fixation. In whole plants Cd inhibits growth and photosynthesis at very low concentrations, an indication that inhibition of other photosynthetic reactions could be more sensitive to Cd than electron transport. Our preliminary examination of some of the enzymes involved in CO_2 fixation showed that carbonic anhydrase was the most sensitive. Therefore, the effects of Cd on this enzyme were examined in more detail.

The effects of Cd on carbonic anhydrase activity were determined with enzyme preparations obtained as previously described by the electrometric method as described by Chang (3). Results are presented in table 4.

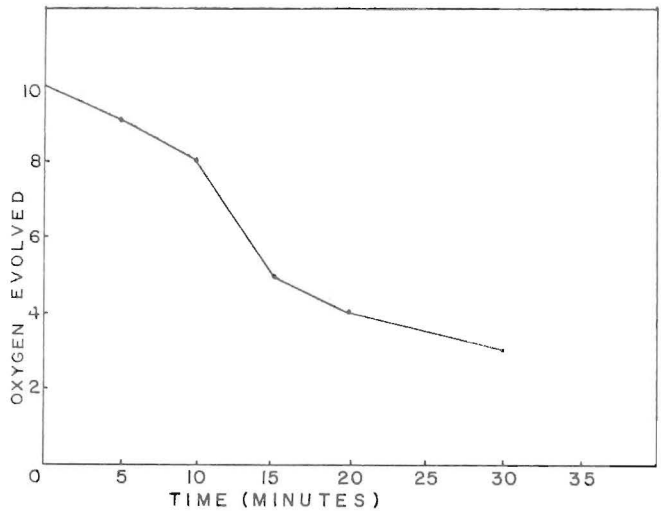

FIG. 1.—Relationship between dark pre-incubation and cadmium inhibition of CO_2 supported oxygen evolution.

Carbonic anhydrase activity was strongly inhibited by Cd. Concentrations of 30 μM $CdCl_2$ produced over 50% inhibition, and complete inhibition of enzyme activity was obtained at approximately 100 μM $CdCl_2$. Lee et al. (8) also found that carbonic anhydrase in soybean seedlings was drastically affected with low concentrations of $CdSO_4$.

To determine whether carbonic anhydrase activity was inhibited with Cd applied to intact chloroplasts, as was the case of inhibition of electron transport, we performed experiments in which the intact chloroplasts were incubated in 50 μM $CdCl_2$ (which in preliminary experiments produced approximately 50% inhibition) prior to the assay. The chloroplasts were removed from the cadmium-containing solution and washed in

cadmium-free assay medium prior to enzyme extraction. Figure 2 shows that Cd could readily penetrate the chloroplast membranes and inhibit carbonic anhydrase, a stromal enzyme. As in the case of electron transport, inhibition of carbonic anhydrase was time dependent, with approximately 10 min required for 50% inhibition. In contrast to electron transport, inhibition of this enzyme was observed at very low $CdCl_2$ concentrations. These results point to the fact that carbonic anhydrase is one of the most important sites of action of Cd.

Tobin (11) reported 50% inhibition of carbonic anhydrase with 50 μM sodium azide; Chang (3) reported complete inhibition with 200 μM iodine; and Kisiel and Graf (7) obtained complete inhibition of this enzyme with

TABLE 4.—*Effect of different concentrations of Cd on carbonic anhydrase activity of isolated chloroplasts*[1]

μM $CdCl_2$	Units of activity	% Inhibition
0	136	0
8	112	18
15	77	43
29	62	54
57	29	79
83	19	86
108	2	99

[1] Reaction mixture contained: 3 ml 20 mM veronal buffer, 0.1 ml partially purified enzyme extract, and 3.5 ml CO_2- saturated water in a total volume of 6.6 ml. Enzyme activity is given in units of activity/mg chlorophyll.

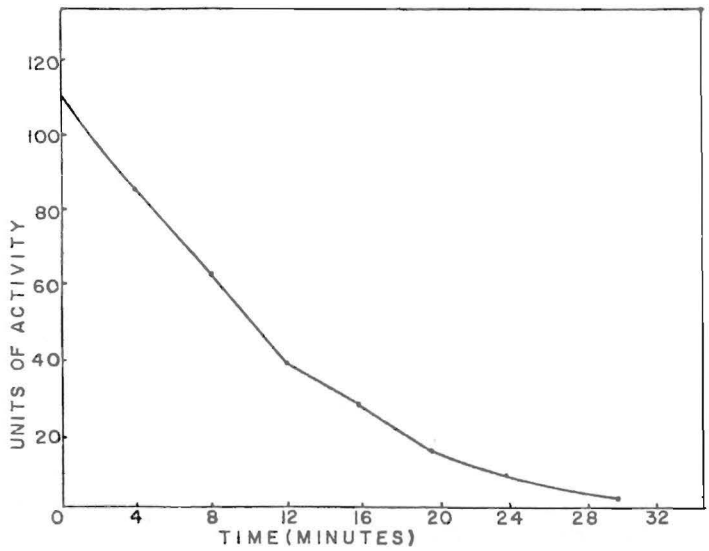

FIG. 2.—Effect of cadmium on carbonic anhydrase activity of intact chloroplasts.

500 μM mercury after 10 min of incubation. The experiments of the present study, in which 50% inhibition was obtained with 50 μM $CdCl_2$ and complete inhibition was obtained with 100 μM $CdCl_2$, indicate that Cd is one of the most potent inhibitors of plant carbonic anhydrase. Since this enzyme is probably one of the key enzymes involved in regulating the levels of CO_2 inside the chloroplast, its inhibition would create a disturbance of the normal metabolism of CO_2. At the whole plant level this would result in an inhibition of photosynthesis which in turn could contribute to the drastic reduction in plant growth and productivity obtained when plants are exposed to low concentrations of Cd.

RESUMEN

Concentraciones bajas de cadmio inhiben las reacciones envueltas en el transporte de electrones y la fijación de CO_2 por cloroplastos aislados. La fijación de CO_2 es más sensitiva al Cd que el transporte de electrones. La incubación previa en oscuridad aumenta el grado de toxicidad del Cd a ambas reacciones. La anhidrasa carbónica, enzima asociada con la fijación de CO_2 es muy sensitiva al Cd cuando éste se aplica directamente a preparaciones parcialmente purificadas de la enzima o cuando se obtienen preparaciones de la enzima de cloroplastos intactos previamente expuestos a Cd. A concentraciones bajas de Cd el grado de inhibición es mucho mayor que el observado en las otras reacciones estudiadas, dependientes del transporte de electrones. Se interpretan estos resultados como evidencia de que la anhidrasa carbónica es uno de los sitios más sensitivos a la acción del Cd en cloroplastos aislados.

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