

AN ABSTRACT OF THE THESIS OF

Carlos Wickliff for the degree of Doctor of Philosophy
in Botany and Plant Pathology presented on July 13, 1977

Title: THE EFFECTS OF CADMIUM ON THE NITROGEN

FIXATION SYSTEM IN ALNUS RUBRA

Abstract approved: Redacted for privacy
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Alnus rubra (Bong.) seedlings were grown in sand culture and irrigated with nutrient solution containing CdCl_2 ranging from 5 μg to 100 mg per liter. Treatment of A. rubra seedlings for 4 weeks with 50 and 100 mg CdCl_2 per liter of nitrogen-free nutrient solution decreased in situ nitrogenase activity 93 and 99%, respectively, when compared to controls. Nitrogen fixation was decreased 32 and 65% at CdCl_2 concentrations of 50 and 100 mg per liter, respectively. Growth was decreased to about the same extent as nitrogen fixation. Cadmium concentrations in the organs of A. rubra increased with increasing CdCl_2 concentrations in the nutrient solution and increasing duration of treatment.

Treatment of A. rubra seedlings for 11 weeks with CdCl_2 concentrations ranging from 0.1 to 25 mg per liter of nitrogen-free nutrient solution decreased in situ nitrogenase activity 25 to 89% when compared to controls. These treatments resulted in nitrogen fixation

decreases of 23 to 98% and the number of nodules per plant decreased 29 to 74%. Similar reductions were observed in plant growth. Treatment with lower cadmium concentrations (0.01 to 0.1 mg CdCl_2 per liter) decreased nitrogenase activity 6 to 31%. Nitrogen fixation was not significantly reduced in the 0.01 to 0.1 mg CdCl_2 per liter treatment range and treatments in this range resulted in an increased number of nodules formed. Cadmium concentrations in the plant organs decreased with increasing CdCl_2 concentration in the nutrient solution.

Either the Mo-Fe or the Fe protein component of nitrogenase was pre-incubated with CdCl_2 and then combined with the other non-incubated component to reconstitute the enzyme. Pre-incubation of the Mo-Fe protein with 136 μM CdCl_2 decreased in vitro nitrogenase activity to a minimum of 45% of the control assay without cadmium. Incubation of the Fe protein with cadmium non-specifically increased activity up to 175% (at 27 μM CdCl_2). Activity then decreased to 70% of control at 136 μM CdCl_2 . When the two proteins were combined and then pre-incubated with CdCl_2 the results were similar to those obtained by pre-incubating the Fe protein.

A. rubra seedlings without nodules were inoculated at the start of the experiment. The growth period prior to apparent nodulation increased from 5 to 8 weeks as the CdCl_2 concentration increased from 10 to 100 μg per liter of nitrogen-free nutrient solution. Nitrogen fixation decreased 52 and 89%, when compared to control plants,

at 10 and 20 $\mu\text{g CdCl}_2$ per liter, respectively. No detectable nitrogen fixation was observed at higher cadmium concentrations. Decreases in plant growth from CdCl_2 treatment were roughly parallel to decreases in nitrogen fixation. When seedlings without nodules were given 12 mM NH_4NO_3 nitrate reductase activity in the roots decreased 22 to 25% as the CdCl_2 concentration increased from 10 to 100 μg per liter. Nitrogen gain and growth were not decreased from this range of CdCl_2 treatments. When the seedlings were given 6 mM concentrations of either NH_4NO_3 or $\text{Ca}(\text{NO}_3)_2$ nitrate reductase activity decreased 22 and 24%, respectively, at 100 $\mu\text{g CdCl}_2$ per liter. Nitrogen gain and growth were decreased in plants supplied with $\text{Ca}(\text{NO}_3)_2$ and 50 and 100 $\mu\text{g CdCl}_2$ per liter. Cadmium concentrations in the plant organs increased as CdCl_2 in the nutrient solution increased.

The ultrastructure of root and nodule cells was investigated by electron microscopy. Spaces lacking cristae were observed in mitochondria and endophyte resorption advanced as the cadmium concentration increased. The number of starch grains in root xylem parenchyma cells increased as the CdCl_2 concentration increased from 20 to 100 μg per liter. Nucleoli increased in prominence and mitochondrial cristae became less well defined over the same range of CdCl_2 concentrations.

These results indicate that cadmium in nutrient media inhibits nitrogenase activity, and therefore nitrogen fixation in Alnus rubra.

Growth, nodulation, and nitrate reductase activity were inhibited by the element. Observations of root and nodule cell ultrastructure suggest that cadmium exerts a portion of its effect by influencing the structure of organelles.

The Effects of Cadmium on the Nitrogen
Fixation System in Alnus rubra

by

Carlos Wickliff

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1978

APPROVED:

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Date thesis is presented July 13, 1977

Typed by Opal Grossnicklaus for Carlos Wickliff

ACKNOWLEDGEMENTS

The author wishes to express his sincere thanks to his co-major professors, Drs. Harold J. Evans and Donald B. Zobel, for their helpful suggestions and efforts to design a good graduate program. Dr. Evans not only suggested the area of research and provided equipment and facilities, but also maintained a keen interest throughout my Ph. D. program. Dr. Zobel maintained a keen interest in my Ph. D. program and was available for advice during the more difficult phases. Dr. Geoffrey W. Harvey substituted for Dr. Zobel during the final oral examination. I wish to express thanks to Dr. David P. Moore for his helpful suggestions and efforts to design certain phases of my research and a good graduate program. I also wish to thank Drs. Ralph S. Quatrano and Leo W. Parks for their helpful suggestions and efforts to design a good graduate program.

I wish to acknowledge the help of Mr. Sterling A. Russell, Mr. Kevin R. Carter, and others of Dr. Evans' laboratory for donating time and effort in certain areas of my research. I wish to express my appreciation to Mr. Rudolph B. Addison and Mr. Alfred H. Soldner for assistance in electron microscopy and to Dr. Fred R. Rickson for assistance in interpretation of electronmicrographs. I also wish to thank Dr. David T. Tingey for making helpful suggestions on conducting the research and the Laboratory for Analytical Support for assistance in certain analytical procedures.

The research was supported by the U.S. Environmental Protection Agency, Ecological Effects Research Division, Terrestrial Ecology Branch.

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LIST OF ABBREVIATIONS

A	amyloplast
ATP	adenosine triphosphate
c	capsular material
C	host cytoplasm
cm	endophyte cell membrane
cw	endophyte cell wall
CW	host cell wall
D	dictyosome
DEAE-cellulose	diethylaminoethyl-cellulose
h	hypha
m	mesosome
M	mitochondrion
mg	milligram
Mg ⁺⁺	magnesium ions
μg	microgram
μM	micromolar
n	nuclear material
N	nucleus
NM	nuclear membrane
NP	nucleopore
NRA	nitrate reductase activity
NU	nucleolus

P	plasmalemma
PPB	parts per billion
ppm	parts per million
RNA	ribonucleic acid
rv	partially resorbed vesicle
s	septum
S	starch grain
ss	side septum
unk	unknown
v	vesicle
V	vacuole

THE EFFECTS OF CADMIUM ON THE NITROGEN FIXATION SYSTEM IN ALNUS RUBRA

INTRODUCTION

Several investigators have reported the accumulation of cadmium by plants from contaminated soils and nutrient solutions (4, 18, 20, 21). Other investigators have published reports indicating that cadmium in nutrient media affects plant metabolism. Cadmium has been reported to increase respiration rates (27), affect mitochondrial oxidation-reduction reactions (5), and inhibit photosynthesis and nitrogen fixation (17). Since these processes are critical to normal plant development it would be expected that growth would be adversely affected by cadmium absorption. Growth reduction has indeed been shown to be a response to cadmium treatment (17, 35). These studies have demonstrated the toxicity of the element to living systems.

Although knowledge concerning the cycling of cadmium in the environment is incomplete, the presence of the element in mineral deposits, and water are known (10). Cadmium enters the environment from both natural and man-made sources. The abundance of cadmium in natural sources usually does not exceed 0.22 parts per million (ppm), but uptake from man-made sources may reach considerably greater levels. For example, a cadmium concentration in the soil as high as 1750 ppm has been measured within 1 km of a zinc smelter (7). A cadmium concentration of 1.45 ppm was measured

in roadside soil and the accumulation decreased with increasing distance from the road (25). This pattern indicated that traffic was the source of the element. The cadmium contents of lubricating oils have been estimated to range from 0.20 to 0.26 ppm, and those of motor vehicle tires range from 20 to 90 ppm (10, 25).

Man-made sources of cadmium are many and these apparently account for most of the element found in the environment (10). Cadmium concentrations in the soil of relatively unpolluted areas average about 0.06 ppm and have a range of 0.01-7.0 ppm (1). The element does not appear to be required by plants as a nutrient. The presence of cadmium and its toxicity to living systems make further studying of its effects desirable.

Alnus species are well known for their contribution of nitrogen to soils. Alnus rubra (Bong.) is a hardwood species and has commercial uses in the production of furniture and charcoal. Alnus rubra is widely distributed in the Douglas fir (Pseudotsuga menziesii) region of the Pacific Northwest and has been shown to enhance stand development of the fir and improve fertility of the forest soil (38). Soils under Alnus rubra-Pseudotsuga menziesii mixed stands show a significant increase in soil nitrogen and organic matter when compared to those under pure stands of fir (38). The wide distribution of Alnus species in the Northwest, the widespread occurrence of environmental pollution, and the importance of Alnus species in soil fertility served

as a basis for this investigation. The purpose of this research was to obtain formulating data on the extent to which cadmium would affect the nitrogen fixation system of Alnus rubra.

REVIEW OF THE LITERATURE

Absorption of Cadmium by Plants

Most studies of cadmium absorption are concerned with root uptake. Many metallic cations, however, are absorbed by the leaves of plants, which is termed foliar absorption. Monovalent cations absorbed by plant leaves include potassium, sodium and rubidium; divalent cations so absorbed include calcium, magnesium, strontium, and barium. Nutrient elements required in trace amounts and absorbed through the foliage include iron, manganese, zinc, copper, molybdenum, and cobalt (43). There is general agreement that foliar absorption rates for most nutrients are greater for young leaves than for old leaves. Calcium and magnesium in addition to ions that are mobile in the phloem (42) may be leached from old leaves. Minerals leached from leaves may be taken up by other leaves resulting in a circulation of minerals.

Although radiotracers are available there seems to have been a relatively small number of published reports dealing with detailed mechanisms of foliar absorption and transport of minerals. Since cadmium is released into the environment through volatilization in the air, particulate emission, and washing followed by solubilization (10), foliar absorption of this element from air-borne sources (18) must be considered.

In contrast to foliar absorption, there are many reports concerning absorption of cadmium from soil by plant root systems. John et al. (22), studied factors that affect plant uptake and phytotoxicity of cadmium added to soils. These factors include the relative capacity of the soil to adsorb cadmium, the presence of other metallic cations in the soil, and the soil organic matter content. Haghiri (15) studied the effects of cation exchange, organic matter, and soil temperature on cadmium accumulation by oat and soybean plants. Bittell and Miller (6) determined selectivity coefficients for lead, cadmium and calcium and concluded that cadmium may compete on a more or less even basis with calcium for clay adsorption sites. Santillan-Medrano and Jurinak (37) conducted equilibrium experiments with samples of soil to obtain solubility data on lead and cadmium in soils. They concluded that precipitation of cadmium as carbonate and/or orthophosphate regulated cadmium solubility at high cadmium concentrations.

There have been several reports published concerning absorption of cadmium from nutrient media. Page et al. (35) reported the use of solution culture to study the response of corn, turnips, beets, beans, tomatoes, cabbage, lettuce, green peppers, and barley to cadmium levels in solution in the range of 0.1 to 10 mg/l. Growth reduction occurred in all species tested. Cadmium concentrations in plant leaves were measured and found to increase as the amount of cadmium added to the substrate increased. Jarvis et al. (20)

examined the uptake of cadmium by the roots of plants and its transport to the shoots using solution culture and concluded that the roots of several species take up large quantities of cadmium from solutions, but there were mechanisms which may restrict the movement of the element through plants.

Foliar Absorption. Lagerwerff (24) published data indicating that a fraction of the metal content of plants is derived from the air and that this fraction decreased as soil metal content increased. In tests of cadmium, lead and zinc uptakes by leaves of radish only zinc was translocated to the roots to a significant extent. Both cadmium and lead were slowly translocated from plant tops to roots. Cadmium deposited on leaves from airborne sources may simply adhere to the leaf surface rather than be absorbed (36). In such cases the metal may be easily rinsed off the leaves with water or a dilute acid solution. For this reason, foliar cadmium concentrations given in some reports may be deceptively high. Foliar absorption of cadmium in many cases may be insignificant in comparison to uptake by root systems.

Absorption from Soil. John et al. (22) concluded that the cadmium content of plants grown in contaminated soil is related to the amount of exchangeable cadmium in that soil. The quantity of exchangeable cadmium is in turn related to the capacity of the soil to adsorb cadmium, the soil pH, and the organic matter content. Plant

cadmium levels were inversely related to the ability of the soil to adsorb cadmium and to the organic matter content of the soil. Highest levels of cadmium in plants were associated with increased soil acidity.

Haghiri (15) harvested the shoots of oat and soybean plants following growth in soil to which cadmium had been added. The cadmium concentrations in these materials were determined and it was reported that cadmium in oat shoots decreased with increasing cation exchange capacity (CEC) of the soil. Except for its effect on CEC, organic matter was not shown to influence the concentration of cadmium in oat shoots. The cadmium concentration in soybean shoots was found to increase as soil temperature increased. When 10 ppm (parts per million) cadmium were added to the soil, the addition of zinc at a concentration range from 5-50 ppm resulted in a significant increase in the cadmium content of soybean shoots. It was concluded that this increase was primarily due to decreased plant growth. Depression of cadmium uptake began to occur when the concentration of zinc applied to the soil reached 100 ppm.

Absorption from Nutrient Media. Page et al. (35) cultured several plant species in solutions containing the essential nutrients and with variable cadmium concentrations. For example, the leaves of the red beet accumulated cadmium to an extent of 280 μg per gram of dry matter when grown for three weeks at a concentration of 0.1 mg/l of solution. Bean leaves from plants grown in solutions of the

same concentration accumulated only 9 μg of the element per gram of dry matter. Intermediate were corn and turnip which accumulated cadmium in leaves at concentrations of 90 and 160 μg per gram of dry matter, respectively. When these same species were grown at a solution concentration of 1 mg/ml, turnip accumulated 429 μg of cadmium per gram of dry matter. Red beet, corn and bean leaves accumulated the element at concentrations of 326, 35, and 227 μg per gram of dry leaf material, respectively. The data obtained from this research also showed that cadmium absorption by all species tested increased as the solution concentration increased.

Cutler and Rains (9) have used barley seedlings and excised barley roots in their investigation of cadmium uptake. The cadmium content of roots and shoots of intact barley seedlings grown in solution culture, containing 1 mg of radioactive cadmium per liter, increased with increasing time. Cadmium accumulation continued for 28 days, or until the contents were greatly in excess of solution concentration of cadmium. In order to elucidate the mechanism responsible for the retention and accumulation of cadmium, these investigators conducted experiments with excised barley roots. These were exposed to labelled cadmium and then transferred to desorption solutions containing unlabelled cadmium, zinc, copper or mercury. They (9) found that a large fraction of labelled cadmium was displaced from the roots within 30 minutes. In another experiment in which the

concentration of unlabelled cadmium in the desorption solution was varied, complete desorption was observed only with cadmium concentrations of 50 mg per liter or above. That fraction taken up but not desorbed was found to be a linear function of temperature in the range from 3 to 50 C and to be decreased by low oxygen concentration and by the presence of 2,4-dinitrophenol. The investigators concluded that a large fraction of the cadmium taken up by excised barley roots was the result of exchange absorption and was displaced by subsequent desorption with divalent cations. The fraction that was not displaced by desorption in the desorption solution resulted from strong, possibly irreversible binding of cadmium at specific sites in the roots. These workers suggested that diffusion of cadmium followed by sequestering may account for the accumulation in barley plants.

Jarvis et al. (20) studied the uptake of cadmium by 23 plant species grown 7 days in $0.09 \mu\text{M CdCl}_2$. They found that cadmium concentration ranged from 1.8 to 21.1 ppm in shoots and 4.9 to 163.8 in roots. In all species the cadmium concentrations in the roots were greater than those in the shoots, and fibrous roots contained much higher concentrations than the corresponding storage roots. In 17 of the 23 species studied retention of cadmium in the roots exceeded 65% of the total uptake, indicating restricted transport of cadmium from roots to shoots. Cadmium transport ceased when cadmium was made unavailable to the roots by removing the element from the nutrient

solution and the addition of phosphate. The investigators suggested that the transport and mobility of cadmium within plants may be related to the solubility of cadmium phosphate.

In a separate experiment, Jarvis et al. (20) exposed ryegrass to cadmium for 3 days, after which the nutrient solution containing the element was removed and replaced with one lacking cadmium. The concentrations of cadmium were found to decrease in both shoots and roots due to plant growth. The distribution of the element within the plant, however, remained constant. Cadmium uptake was not restricted to living roots and was even enhanced when roots of ryegrass were killed by immersion in boiling water. Short term uptake by the living roots of this plant was decreased by the addition to the nutrient solution of calcium alone or calcium plus zinc or manganese. These investigators suggested that calcium may depress cadmium uptake by competing for exchange sites and by influencing cell membranes. Manganese and zinc in their opinion may depress uptake by competing for exchange sites.

Physiological Effects of Cadmium on Plants

Cadmium accumulation in the environment poses a threat to plant life because of the phytotoxicity of excesses of the element. Barber and Brennan (2) exposed bush bean, lettuce, and tomato to 1 ppm cadmium in sand culture. Bush bean was the most sensitive

of the plants investigated and showed injury after 3 weeks of exposure. Lettuce and tomato exhibited less injury than bush bean although the foliar cadmium accumulations were respectively 1000 and 3000 times greater than those of bush bean. The presence of cadmium in the nutrient solution also greatly increased the uptake of other mineral elements. Accumulation of cadmium in the chloroplasts was also reported.

Page et al. (35) listed the symptoms of visual injury associated with concentrations of cadmium in solution that produced 50% growth reduction. Chlorosis was observed in the majority of plant species investigated. Sweet corn, however, displayed a reddish-orange coloration at leaf margins as well as chlorosis and necrosis. Red beets and field beans wilted as a result of excess cadmium. John et al. (22) exposed radish and lettuce plants to 100 ppm cadmium in surface soils and reported that both species exhibited a reduction in growth. Radish leaves also became chlorotic.

Miller et al. (33) found that growth of soybean shoots was generally depressed when tissue concentrations of cadmium reached 3 to 5 μg per g dry weight. Cadmium uptake was correlated with the ratio of added cadmium to the cadmium adsorptive capacity of the soil, which is controlled primarily by the soil cation exchange capacity. Soil pH exerted a significant influence on cadmium uptake.

Imai and Siegel (18) excised the embryos from red kidney bean

seeds and cultured them in the presence of the chlorides, sulfates, or nitrates of heavy metals. They found that cadmium salts inhibited growth of the embryos at concentrations in excess of 3 mg/l. Growth inhibition was accompanied by inhibition of greening and induction of acute curvature in the young shoots.

Effects on Nitrogen Fixation. Some of the effects of cadmium on nitrogen fixation by soybean have been reported by Huang et al. (17). These investigators cultured nodulated soybean in a sand-vermiculite mixture which was irrigated when nutrient solution containing the following concentrations of cadmium: 0, 0.018, 0.09, 0.45 and 0.90 mM. Of all the parameters that were measured, cadmium had the most dramatic effect on nitrogenase activity. A concentration of 0.018 mM caused a 71% decline in activity. Photosynthesis was decreased 17% at this concentration. Cadmium inhibited nodule weight, nitrogenase activity, nodule ammonia concentration, nodule protein, rate of photosynthesis, nodule carbohydrate content, and weights of roots and leaves at all concentrations tested. These investigators concluded that cadmium inhibited plant metabolism generally and that key processes affected were photosynthesis and nitrogen fixation which are interdependent.

Effects on Photosynthesis. Bazzaz et al. (3) observed that photosynthesis was reduced in sunflower by 50% of maximum when the cadmium concentration in leaf tissue was 193 ppm. Both

photosynthesis and transpiration were inhibited and it was concluded that the primary mode of action was interference with stomatal function. The degree of stomatal opening decreased log-linearly with increasing concentrations of cadmium. This effect would be expected to inhibit gas exchange and also transpiration and photosynthesis. The reduction in net photosynthesis and transpiration in plants treated with higher concentrations of the element caused a bending of the petiole until leaves were in a horizontal position.

Huang et al. (17) found that photosynthesis of soybean exposed to 0.90 mM cadmium was reduced to a non-detectable rate. Cadmium concentrations of 0.0 to 0.45 mM reduced photosynthesis from 60.5 to 40.0 $\mu\text{g CO}_2$ fixed per gram dry leaf per hour. Cadmium was a more effective inhibitor of photosynthesis than lead. When the rate of photosynthesis was depressed by cadmium to approximately 60% of control carbohydrate failed to accumulate in the nodules. The decrease in carbohydrate level was considered sufficient to limit nitrogen fixation by the nodules.

Hampp et al. (16) isolated chloroplasts from spinach leaves and incubated them in 0.001-1.0 mM cadmium chloride and $\text{H}^{14}\text{CO}_3^-$. They found that the $^{14}\text{CO}_2^-$ fixation was inhibited by cadmium at all concentrations investigated. A concentration of 0.0035 mM cadmium salt caused a half maximum decrease of photosynthetic carbon dioxide fixation as compared to controls. The effect of cadmium was more

pronounced than that of zinc. Lineweaver-Burke plots of the processes of carbon dioxide fixation indicated non-competitive inhibition by cadmium. Hill activity was measured by the photosystem II reduction of dichlorophenol-indophenol (DCIP) in fragmented chloroplasts. The rate of DCIP reduction was reduced 22% by a cadmium concentration of 0.05 mM. Increasing the concentration of cadmium resulted in increasing inhibition.

Hampp et al. (16) also investigated the effects of a mixture of cadmium and zinc on $^{14}\text{CO}_2$ fixation and Hill activity. An additive effect of zinc on carbon dioxide fixation at lower concentrations of these elements was observed, but at higher concentrations of zinc the inhibiting effect was less than that of the cadmium solution alone. Pronounced inhibition of the Hill reaction occurred only at concentrations higher than 0.1 mM. Mixtures of the two elements resulted in greater inhibition of the Hill reaction than comparable reactions containing single solutions of either cadmium or zinc. These data indicate that mixtures of cadmium and zinc at fairly high concentrations cause an inhibition pattern that is synergistic.

Effects on Respiration. The effects of cadmium on isolated corn shoot mitochondria were investigated by Miller et al. (32). In the absence of phosphate, cadmium stimulated the rate of oxidation of exogenous NADH at concentrations between 6.8×10^{-3} mM and 6.8×10^{-2} mM. Stimulation was maximal at 2.5×10^{-2} mM.

Cadmium concentrations greater than 0.1 mM inhibited the oxidation rate. When phosphate was present, the cadmium stimulation of the rate of exogenous NADH oxidation was not observed, but inhibition was still apparent at concentrations of 1.0 mM and above. In the absence of phosphate, 50% inhibition of the rates of oxidation of succinate and a combination of malate and pyruvate were observed when the cadmium concentration was approximately 0.05 mM. The point of 50% inhibition was shifted to higher cadmium levels in the presence of phosphate. The DCIP reduction effected by succinate was inhibited 50% at a cadmium concentration of 0.05 mM. That effected by the malate and pyruvate combination was inhibited 50% at 0.5 mM cadmium. It was also reported that the ratio of ADP phosphate per oxygen taken up was reduced by cadmium. These data suggested that the site of cadmium effect likely is in the early stages of electron transport.

Miller et al. (32) observed that cadmium caused an increase in the passive swelling of corn mitochondria, but the increase did occur when phosphate was present. The effect of cadmium on swelling was more dramatic when the mitochondria had contracted and were oxidizing a substrate. When succinate or NADH was the substrate a pronounced swelling occurred at cadmium concentrations as low as 0.05 mM. A 2.0 mM phosphate concentration was required to protect NADH contracted mitochondria from cadmium induced swelling.

When dithiothreitol, a sulfhydryl protector was present, cadmium failed to cause swelling or to inhibit respiration. On the basis of these observations these investigators suggested that sulfhydryl groups were likely involved in a cadmium-membrane interaction.

Lee et al. (27) found that soybean seedlings cultured in 0.9 mM cadmium exhibited a 63% increase in respiration rate. There were also increases in the rate of activity of several important enzymes.

Lindegren and Lindgren (29) grew yeast cells in nutrient broth to which cobalt, cadmium, or thallium had been added. Cells were then fixed for electron microscopy. The endoplasmic reticulum of cells grown with 10 ppm cadmium was a conspicuous cytoplasmic component, but the endoplasmic reticulum was rarely stained in cells grown in a normal broth without cadmium. Most of the mitochondria in cells grown with cadmium were so modified that no trace of cristae could be seen. More than 30% of the cells recovered from broth with added cadmium were respiratory deficient.

Bittell et al. (50) conducted experiments with corn mitochondria in which respiration, phosphorylation, and swelling-contraction were measured. The results obtained were similar to those previously reported by Miller et al. (32). The effects of lead and zinc on mitochondria from corn were found to be similar to those of cadmium.

Effects on Specific Enzymes. Lee et al. (27) extracted six enzymes from the leaves of soybean seedlings following culture in

nutrient solution containing 0.0, 0.45, 0.90, and 1.35 μM cadmium. The plants were cultured 10 days, and then were sampled for assay. At a cadmium concentration of 0.90 μM the activities of malate dehydrogenase, ribonuclease, and acid phosphatase increased 75%, 102%, and 11% respectively. At the same concentration of cadmium the activities of deoxyribonuclease and peroxidase increased 200% and 291% respectively, and activity of carbonic anhydrase decreased 21%. The activity of carbonic anhydrase was decreased at each of the other concentrations investigated. Since the activity of carbonic anhydrase is zinc dependent, it is possible that cadmium interacts antagonistically with zinc, possibly displacing it and reducing activity. These investigators also discussed the possibility that cadmium toxicity in soybean seedlings is expressed as a stress factor which caused an appearance of general senescence.

Mathys (31) investigated effects of metal addition on several enzymes of heavy metal resistance and non-resistant populations of Silene cucubalus. The effects of copper, zinc, cadmium, nickel, cobalt, and manganese on nitrate reductase, malate dehydrogenase, isocitrate dehydrogenase, and glucose-6-phosphate dehydrogenase were studied. In the in vitro studies, enzymes of resistant and non-resistant ecotypes show a similar sensitivity to all metals investigated and no resistant enzyme could be detected. In the in vivo experiments, some of the enzymes from the heavy metal resistant

ecotype could be activated by addition of the metal. The activities of enzymes from the heavy metal resistant ecotype remained high while those of the non-resistant ecotype were inhibited. The existence of resistance apparently was dependent upon an intact cell since loss of resistance followed structural damage to the cell. Addition of cadmium at 1 mM to the incubation medium containing extracted nitrate reductase, glucose-6-phosphate dehydrogenase, and malate dehydrogenase resulted in 80%, 70%, and 70% inhibition of activities, respectively.

Hadwiger et al. (14) investigated the effects of 5×10^{-4} M cadmium on the synthesis of isoflavonoid, pisatin, and the activity of phenylalanine ammonia lyase (PAL) induced in the excised pods of Alaska pea. Synthesis of isoflavonoid and pisatin were increased. The increased syntheses were suppressed by application of an RNA synthesis inhibitor. Cycloheximide also blocked the response when it was applied within 6 hours after cadmium application. There was an increase in the rate of synthesis of all sizes of RNA as well as an increase in template activity and dye binding capacity of the chromatin.

Morphological Effects of Cadmium on Plants

The most observed symptom of cadmium injury was chlorosis (27, 32, 35). Other symptoms included wilting and necrosis of the

leaves (35). The upper leaves of soybean seedlings treated with cadmium not only were chlorotic, but also were curled and tended to absciss easily (27). Barber and Brennan (2) observed that cadmium toxicity symptoms, in addition to chlorosis, included wrinkling of leaves and appearance of a red pigment. Imai and Seigel (18) observed that the excised embryo of red kidney bean showed acute curvature of the embryonic axis as a result of cadmium toxicity. In all studies cadmium toxicity caused growth inhibition.

MATERIALS AND METHODS

Culture of *Alnus rubra* Seedlings

In the various experiments to be described, polyethylene containers of 3.78 liter capacity were used as culture vessels. In one series of culture vessels two holes, one cm in diameter and six cm apart, were punched in the bottoms of the vessels. In each of these holes was fitted a piece of polyethylene tubing two cm in length which facilitated the drainage of solution from the container (Fig. 1). A second set of polyethylene containers without holes in the bottoms were used as receiving containers for the nutrient solution which drained from the vessels containing the plants. Each container was washed with 3N HCl and then thoroughly rinsed with distilled water. The bottoms of the containers in which drain tubes were installed were covered with a thin layer of glass wool and these were placed upon supports. The unmodified containers were placed directly beneath those fitted with drainage tubes and served for collection of nutrient solution (Plate 1). The upper containers were then filled with 20 mesh silica sand to a distance of 3 cm from the top. The sand was thoroughly rinsed in each container by successive flushings with distilled water. Two seedlings of *Alnus rubra* were transplanted into the sand of each container and the containers were then supplied with nitrogen-free nutrient solution or with a nutrient solution

Figure 1. Cross-section of polyethylene jar for culture of Alnus rubra seedlings. The supports were constructed of 2 in. \times 4 in. sections of wood blocks. One supported the front and another the rear of the jar.

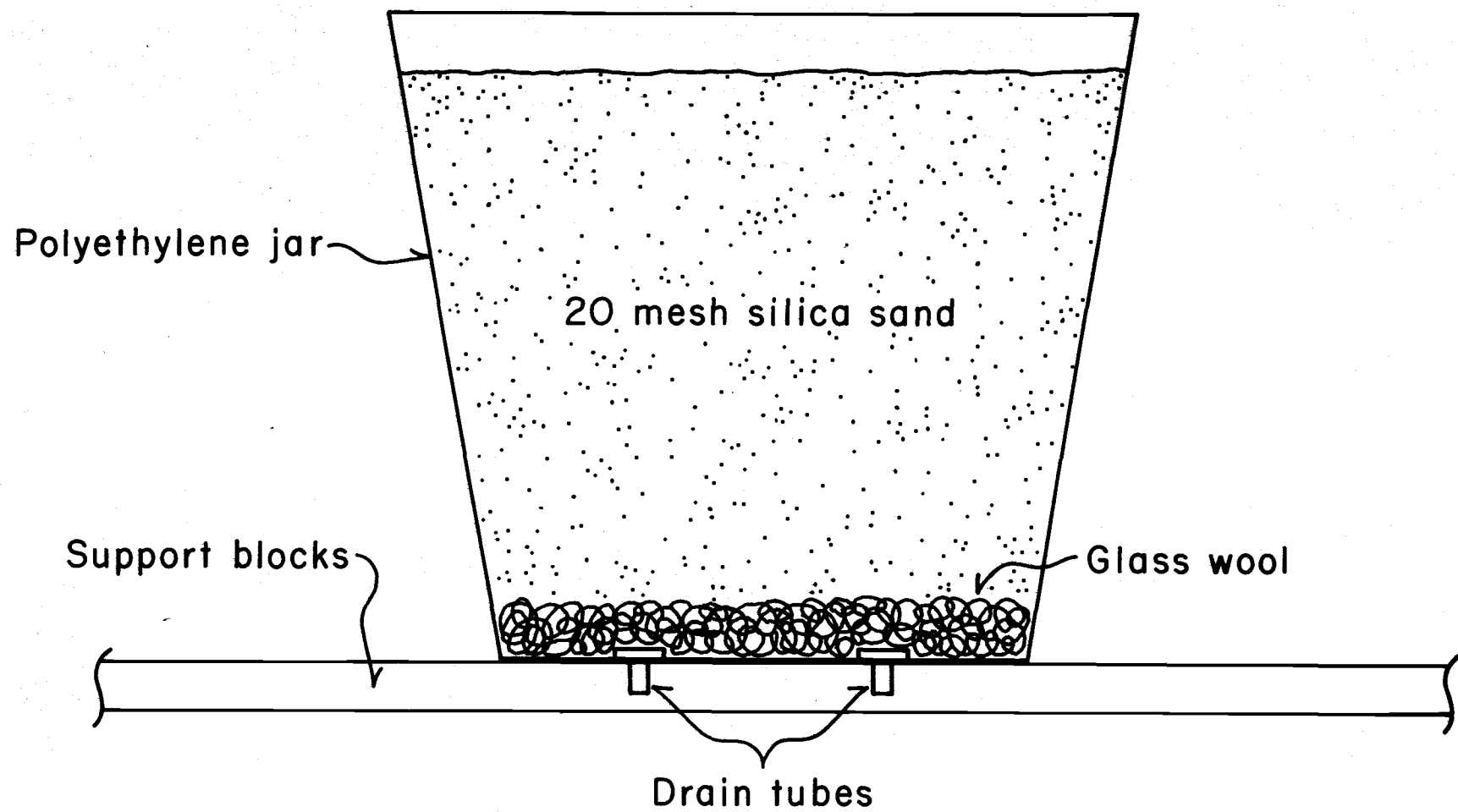


Figure 1

Plate 1. Culture of Alnus rubra seedlings in the greenhouse. The upper containers were fitted with drain tubes and contain 20 mesh silica sand. Seedlings were grown in the sand and irrigated with nutrient solution from the lower containers. The CdCl_2 concentrations were indicated on the containers. PPB = μg per liter.



PLATE 1

containing fixed nitrogen compounds (Table 1).

In the initial investigations of the effects of cadmium of plants that were supplied with nitrogen gas only, nodulated seedlings 7 to 18 cm in height above the ground were harvested from an alder stand near Yachats, Oregon. The soil was washed from the roots and the seedlings were transplanted into the sand culture where they were maintained with nitrogen-free nutrient solution. In the preparation for other experiments, seed of Alnus rubra were collected from trees along the Oregon Coast and stored in a refrigeration room at 0 to 4 C. The seeds were germinated in moist vermiculite and inoculated with a suspension of crushed alder nodules taken from the roots of mature trees. The seedlings were maintained in the vermiculite for three months and then transplanted into the sand culture as described above. In the investigation of the effects of cadmium on plants grown with fixed nitrogen, seeds were sterilized by moderate agitation in a 5.25% solution of NaClO (commercial bleach) for a period of 45 minutes and then were thoroughly rinsed with sterile distilled water. The sterilized seeds were germinated with autoclaved vermiculite which had been moistened with sterile distilled water and maintained in a growth chamber. These were supplied with autoclaved nutrient solution containing fixed nitrogen as described in Table 1. After a three month growth period the seedlings were transplanted into sand culture as described above and supplied with nutrient solution

Table 1. The composition of the nutrient solutions used for culture of Alnus rubra seedlings. Nutrient solution B becomes a nitrogen-free nutrient when NH_4NO_3 is omitted. Each solution was diluted 1:4 before addition to plant culture media.

Nutrient Solution A		Nutrient Solution B	
Macronutrients	Conc. (M)	Macronutrients	Conc. (M)
K_2SO_4	0.00158	K_2SO_4	0.00158
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.002	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.002
KH_2PO_4	0.001	KH_2PO_4	0.001
$\text{Ca}(\text{NO}_3)_2$	0.006	$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$	0.006
CaCl_2	0.0005	CaCl_2	0.0005
		NH_4NO_3	0.006

The following micronutrient solutions were used with either nutrient solution A or B

Micronutrients	Conc. Stock Soln. (mg/l)	Vol. Added (ml/l)
H_3BO_3	250	1.00*
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	250	
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	50	
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	20	
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	10	
FeEDDHA^{**}	2000	2.00
CoCl_2	360	0.14

*One ml of combined stock solution was added to nutrient solution A or B.

**Sodium ferric ethylenediamine-di-O-hydroxyphenylacetate.

containing fixed nitrogen (Table 1). In these experiments all containers utilized for media or other purposes were washed with 5.25% NaClO and thoroughly rinsed with sterile distilled water to prevent contamination with the alder endophyte.

Greenhouse temperatures were maintained at 60° F at night and 70° F during the day. During the months of July and August, however, the day temperatures in some cases approached 100° F. Natural light was supplemented with fluorescent lamps about 500 ft-c at three feet and a 16-hour photoperiod was maintained. The intensity of natural light varied greatly during the year being much lower in winter than in summer months.

Plant Maintenance. Seedlings of Alnus rubra were germinated in moist vermiculite and irrigated twice weekly with nutrient solution. After the seedlings were grown in vermiculite for 3 months, those of uniform appearance were transplanted into sand culture for experimentation. These plants were irrigated daily with 3.2 liters of nutrient solution. The daily irrigation was continued for 6 days each week and on the 7th day plants were irrigated thoroughly with distilled water to prevent metal accumulation in the sand. Each week the nutrient solution was discarded and replaced with fresh solution. The procedure was continued throughout the duration of the experiment.

Cadmium Treatment. Initially cadmium concentrations of

0, 50, and 100 mg/l applied as CdCl_2 were added to the test plants by appropriate dilution of a 20 g/l stock solution of CdCl_2 . Prior to the application of cadmium the plants were allowed a one-week period to recover from effects of transplanting. There were four replicate cultures of each treatment and plants were harvested 13, 28, and 42 days following the first cadmium application. Measurements were taken as described under the heading "Assay Procedures." In all subsequent investigations there were six replicate cultures and cadmium applications were continued for 11 weeks. Concentrations in the range of 0 to 25 mg/l CdCl_2 were applied to test plants for investigations of the effects of cadmium on plants fixing nitrogen. Concentrations in the range of 0-100 $\mu\text{g/l}$ were utilized in all other greenhouse investigations.

Assay Procedures

The activities of two enzymes, namely nitrogenase and nitrate reductase, were measured. Nitrogenase was assayed in intact nodules and also in a crude enzyme preparation eluted from a DEAE-cellulose column. The type of preparation will be specified in the various experiments to be described. Nitrate reductase activity in freshly harvested leaves and roots was measured by an in vivo method. Nitrogenase activity of nodules was determined by use of the acetylene reduction assay.

Nitrogenase. Nitrogenase activity was assayed in Alnus rubra nodules by the method described by Fishbeck et al. (12). The root of each plant was severed from the stem and placed in a 250 ml wide-mouth Erlenmeyer flask which was then sealed with a serum stopper. Sufficient acetylene was added by use of a hypodermic syringe to obtain 0.1 atmosphere and the sample was incubated for a period of 30 to 60 minutes.

All attempts to obtain an active cell-free nitrogenase preparation from the nodules of Alnus rubra failed and therefore soybean nodules were used as a source of enzyme. A preparation from soybean nodules was made by use of the method described by Koch et al. (23). Nitrogenase was extracted from the bacteroids, partially purified using DEAE cellulose chromatography and assayed by the method of Israel et al. (19). In each reaction mixture 0.10 ml of enzyme extract was added. Activity of the enzyme was measured by the rate of acetylene reduction using the method of Fishbeck (12). The protein samples were determined by the method of Lowry et al. (30). Appropriate cadmium concentrations in reaction mixtures were obtained by the addition to the reaction mixture of an appropriate concentration of a cadmium chloride stock solution containing 200 micrograms of CdCl_2 per milliliter. Purified nitrogenase preparations were incubated for 20 minutes at room temperature with cadmium in the buffer used for the reaction medium prior to the addition of sodium dithionite

and initiation of the reaction by the addition of acetylene.

The effect of cadmium on the activity of nitrogenase was investigated using sodium dithionite (Table 2). A purified preparation of the enzyme was incubated for 20 minutes at room temperature in buffer containing cadmium. Some of the pre-incubation media contained sodium dithionite and the other did not. In those pre-incubation media lacking sodium dithionite, this compound and acetylene were added to initiate the nitrogenase reaction.

A crude preparation of nitrogenase was heat treated as described by Israel et al. (19) and portions of the preparation used to investigate the effect of cadmium on nitrogenase activity in reactions containing final concentrations of 2.0, 6.7, and 20 mM MgCl_2 (Table 3). The enzyme preparations were incubated 20 minutes at room temperature with cadmium prior to initiation of the reaction.

Nitrate Reductase. For investigation of the effects of cadmium on Alnus rubra supplied with fixed nitrogen, seeds were subjected to the disinfection procedures that were described under Materials and Methods, subheading "Culture of Alnus rubra Seedlings." Two series of plants were grown, one of which was supplied with ammonium nitrate in a nutrient solution and the second set was supplied with calcium nitrate as the only source of fixed nitrogen. Cadmium concentrations in the range of 0 to 100 micrograms per liter were applied added as cadmium chloride, described above. At the completion of

the eleven week growth period plants were harvested and nitrate reductase activities in roots assayed with the method of Li et al. (28). Nitrate reductase in leaves was assayed by the same method after removal of leaf discs 5 mm in diameter for use in the procedure. One gram of tissue or less was used for the assays.

Determination of Total Nitrogen

Total nitrogen was determined by the Kjeldahl method as described in the Laboratory for Analytical Support, Corvallis Environmental Research Laboratory. Plants used in the initial investigations were separated only into tops and roots with attached nodules. All plants other than those used in the initial investigations were separated into the various organs: namely, leaves, stem, root, and where applicable, nodules. The organs were dried for 48 hours in a 70 C oven and weighed. The leaf and root sections used for the nitrate reductase activities were dried after completion of the assay and weights of these added to those of the respective leaves and roots. These samples were not used in the determination of total nitrogen because KNO_3 was added as a substrate for the nitrate reductase assay. The dried plant material was ground in a Wiley mill prior to analysis. Samples of 10-25 mg were used in the Kjeldahl analyses. The selenium modification of Nelson and Sommers (34) was used for total nitrogen determination on plants grown with a fixed nitrogen

source. Appropriate standards of ammonium nitrate were prepared and included in each digestion. The ammonia produced was measured by the alkaline phenol and sodium hypochlorite method in a Technicon auto-analyzer (13).

Determination of Total Cadmium

Approximately 1.0 g or less of the dry ground plant material remaining after the determination of the total nitrogen was used for determination of total cadmium. Plant material from the initial investigations was placed in 30 ml Kjeldahl flasks and digested 4 hours in 4 ml of concentrated HNO_3 . The digested material was placed in 125 ml Erlenmeyer flasks and another 6 ml portion of HNO_3 was added. The partially digested material was then transported to the laboratories of the Soil Science Department, Oregon State University, and further digested with 3 ml perchloric acid until a clear digestate resulted. This digestate solution was filtered, made to 100 ml volume with distilled water, and analyzed for cadmium by atomic absorption spectrometry in the Laboratory for Analytical Support. Appropriate concentrations of CdCl_2 in the acid digestate were also prepared as cadmium standard solutions. Cadmium determinations for the later investigations were conducted in the laboratory for Analytical Support where digestion tubes and a heating block replaced the Kjeldahl apparatus.

Electron Microscopy

Segments of nodules and secondary roots of Alnus rubra were thinly sectioned with a razor blade and fixed in 5% glutaraldehyde solution in 0.1 M Sorensens monobasic-dibasic phosphate buffer, pH 7.0. The segments were then extensively washed in the buffer alone and treated with 0.1% OSO_4 until blackening occurred. The segments were dehydrated by washing for 15 minutes in 20, 50, 70% acetone in distilled water. Uranyl acetate in 70% acetone was used to stain the segments and they were further dehydrated by immersion for 15 minutes in 100% acetate. After dehydration the segments were placed in a 2:1 plastic:acetone mixture. The plastic consisted of 53 ml dodecenyl succinic anhydride (DDSA), 35 ml Araldite 6005 and 12 ml Epon 812 resin. The segments were allowed to stand in the plastic-acetone mixture 24 hours after which they were embedded in plastic containing one drop of the catalyst (benzyl dimethylamine or trimethylaminomethyl phenol) per ml. The blocks formed were then cured 24 hours at 60-70 C and were trimmed with a clean razor blade and sectioned with a glass knife. The sections were mounted on copper grids coated with Formvar (0.5% polyvinyl formal in ethylene dichloride). The specimen produced were observed with a Phillips EM 300 (Botany and Plant Pathology Department, Oregon State University) or a Phillips EM 200 (Forestry Sciences Laboratory, USDA).

Analysis of Data

The data in Figures 2-47 were subjected to analysis of variance and the means were plotted on the graphs. The standard errors of the means are indicated by error bars. The data were reported as significant when the bars did not cross. Each data point is mean \pm standard error of the mean.

RESULTS

Initial Investigations

Initially it was necessary to determine whether or not cadmium affected the nitrogen fixation system of Alnus rubra. The rates of acetylene reduction by nodulated plants, which is a measure of nitrogenase activity by the nodules of Alnus rubra, decreased with increasing cadmium concentration and duration of treatment (Fig. 2). Shoots continued some growth between two and four weeks following treatment initiation then declined thereafter (Fig. 3). Root growth was not significantly reduced by 50 and 100 mg CdCl_2 per liter of nutrient solution for a four week period (Fig. 4). There was a decrease in dry weight of the roots following six weeks of exposure to 100 mg CdCl_2 per liter. The dry weight of the nodules did not change significantly in six weeks as a result of the 50 and 100 mg CdCl_2 per liter treatments (Fig. 5). The amount of nitrogen fixed in the control plants increased approximately linearly during the six week growth period (Fig. 6). Treatment with CdCl_2 at 50 and 100 mg per liter resulted in significantly less total nitrogen. The greater effect was observed at the higher cadmium treatment.

The cadmium concentration increased in both shoots and roots of plants treated with 50 and 100 mg CdCl_2 per liter during the first four weeks of the experiment (Figs. 7 and 8). Plants without added

Figure 2. Acetylene reduction by the nodules of Alnus rubra following treatment with cadmium in the nutrient solution. Rates are based on the dry weights of the nodules.

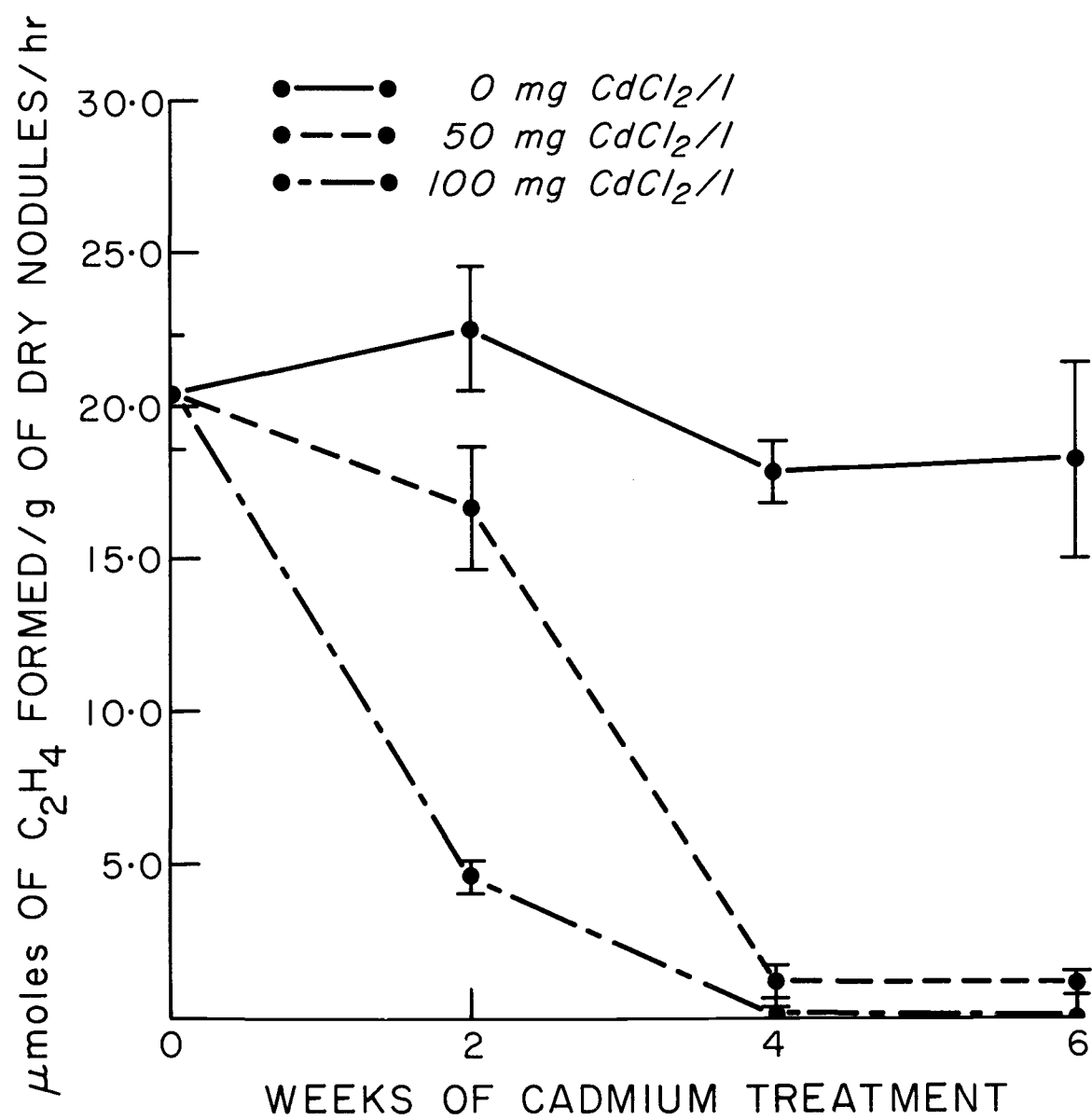


Figure 2

Figure 3. Dry weight of the shoots of Alnus rubra following treatment with cadmium in the nutrient solution.

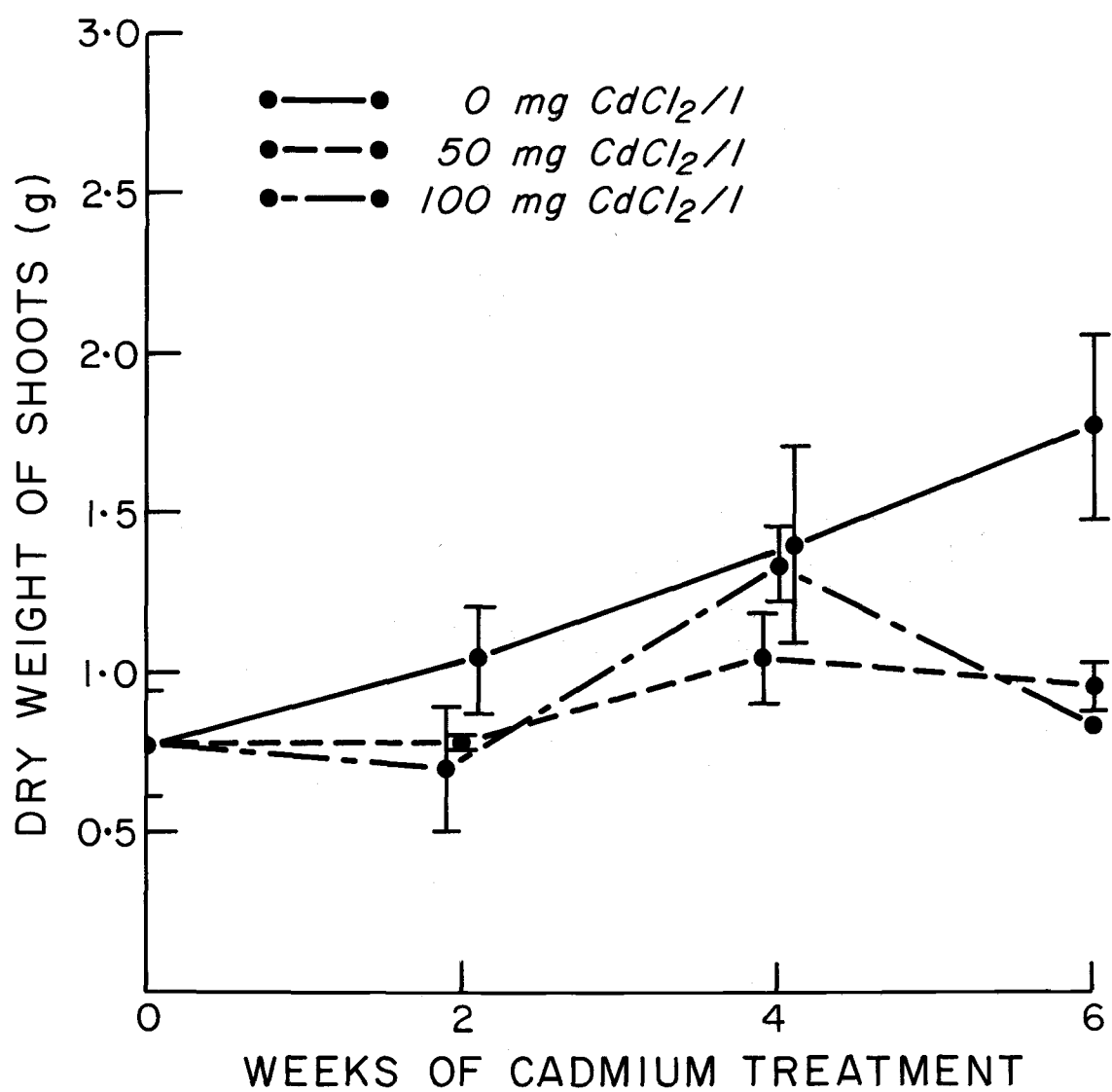


Figure 3

Figure 4. Dry weight of the roots of Alnus rubra following treatment with cadmium in the nutrient solution.

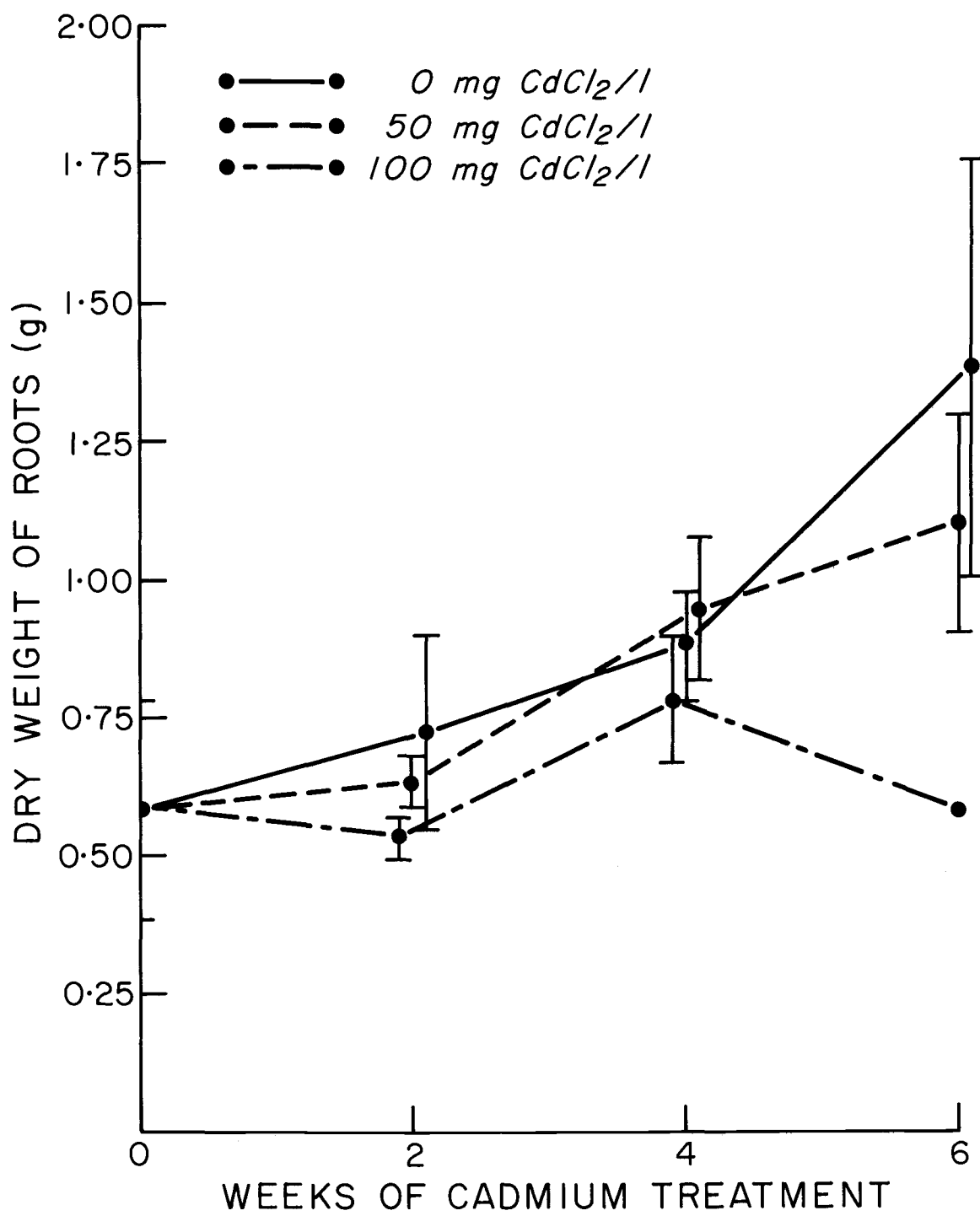


Figure 4

Figure 5. Dry weight of the nodules of Alnus rubra following treatment with cadmium in the nutrient solution.

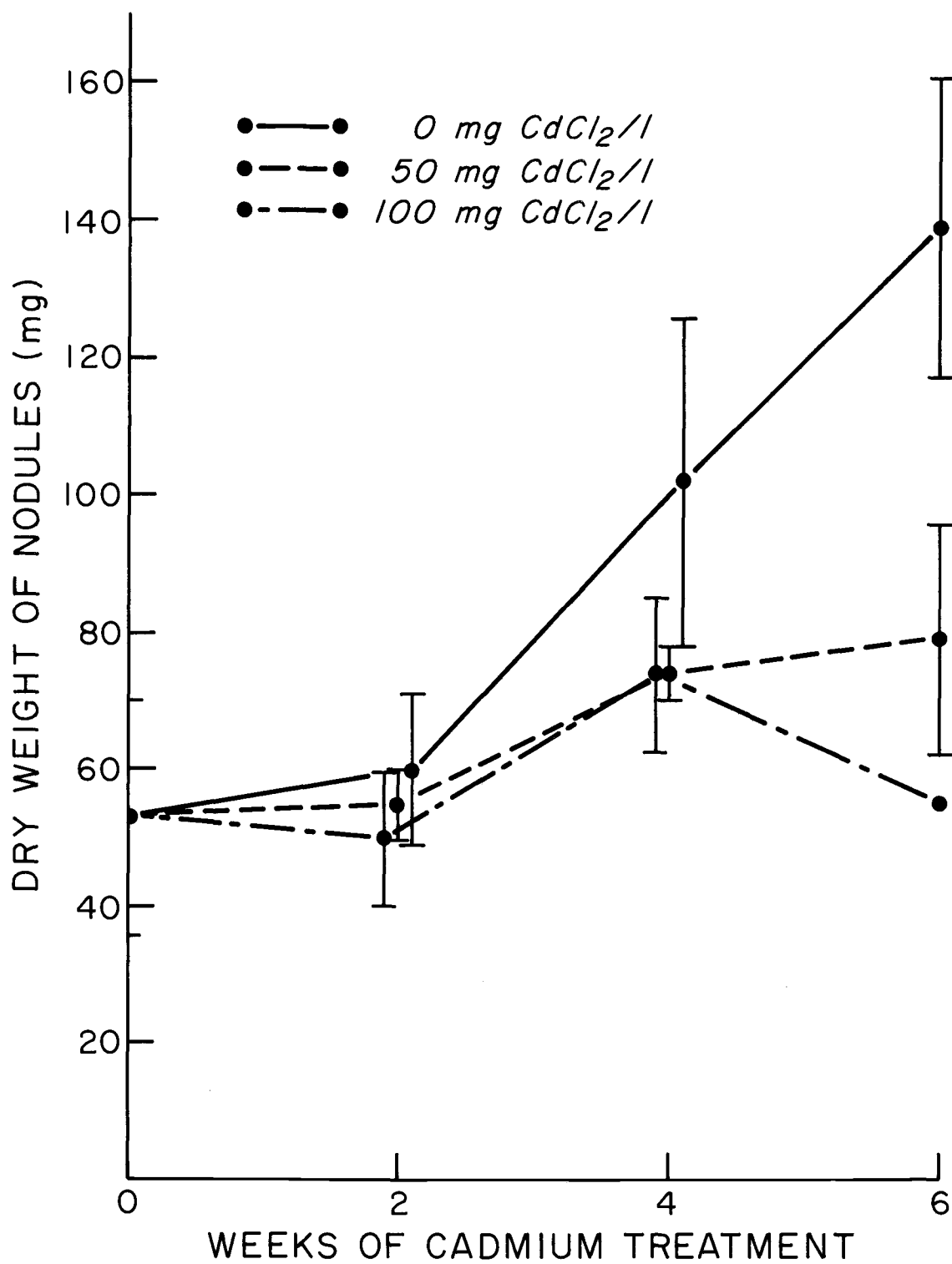


Figure 5

Figure 6. Nitrogen fixation in Alnus rubra following treatment with cadmium in the nutrient solution. Nitrogen fixed = final total nitrogen - initial total nitrogen.

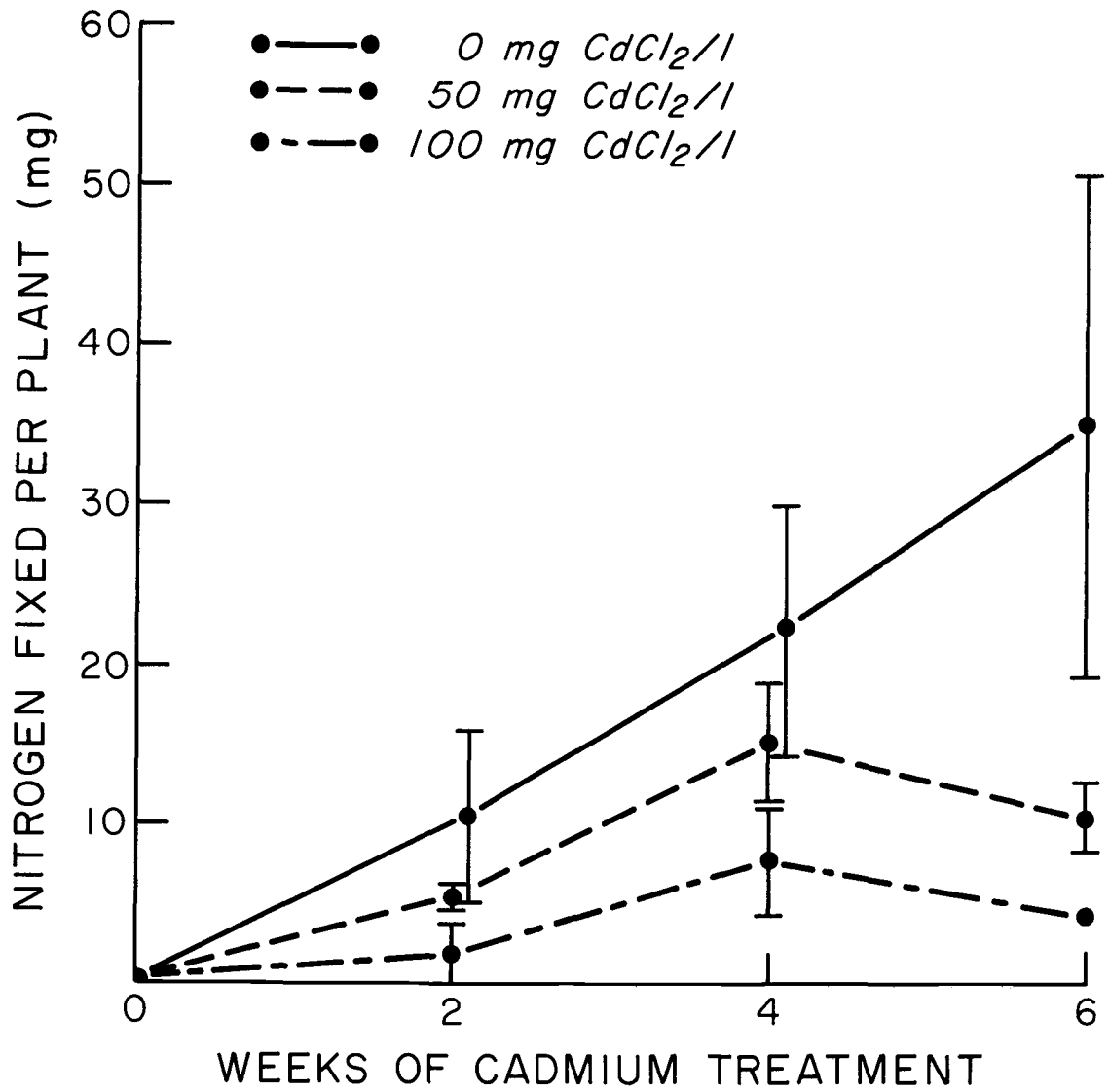


Figure 6

Figure 7. Cadmium concentration in the shoots of Alnus rubra following treatment with the element in the nutrient solution.

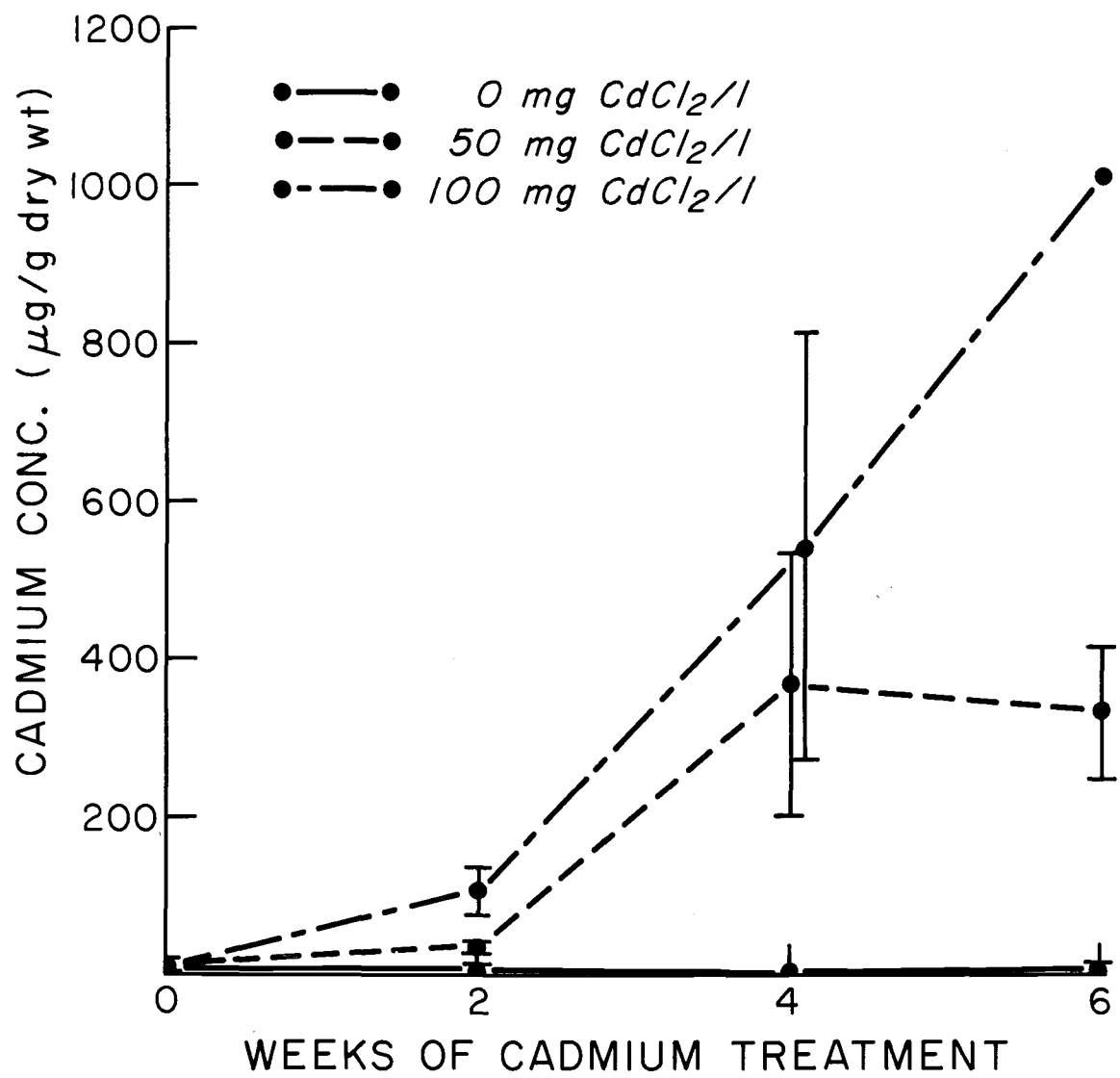


Figure 7

Figure 8. Cadmium concentration in the roots of Alnus rubra following treatment with the element in the nutrient solution.

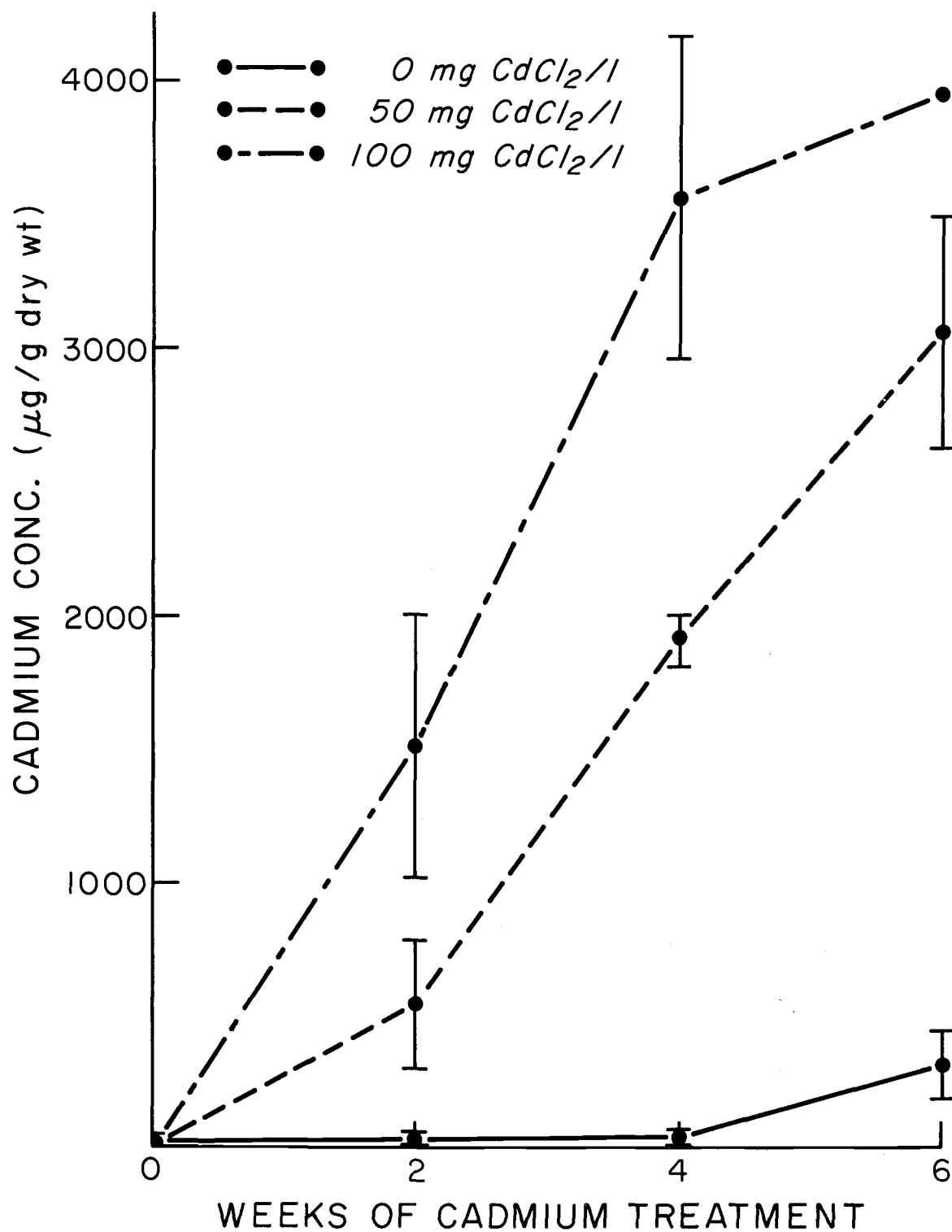


Figure 8

cadmium contained only small amounts of the element in their tissues.

Effects of Cadmium on Plants Fixing Nitrogen

Alnus rubra seedlings were treated with 2 to 25 and 0.1 to 5.0 mg CdCl_2 per liter of nutrient solution in the greenhouse during the periods of natural summer and winter illumination, respectively. Seedlings were treated with 5 to 200 μg CdCl_2 per liter during the period of natural spring illumination in the greenhouse. The effect of light intensity on the experimental results will be discussed more fully in the discussion of the results.

Plant Growth. The dry weight of the organs of Alnus rubra decreased as cadmium increased in the range of 2 to 25 mg CdCl_2 per liter of nutrient solution applied for 11 weeks (Figs. 9 and 10 and plates 2 and 3). Cadmium caused chlorosis of the leaves as shown in plate 4. The greatest reductions in dry weight occurred in the range between 2 and 10 mg per liter. The decreases in growth were less apparent in experiments where 0.1 to 5.0 mg CdCl_2 per liter were applied for 11 weeks (Figs. 11 and 12). Growth reductions were not apparent where 5 to 100 μg CdCl_2 per liter were applied for 11 weeks (Figs. 13 and 14). The dry weights of the leaves of plants given 0.2 to 25 mg CdCl_2 per liter were significantly reduced by these cadmium additions (Figs. 9 and 11).

Nitrogenase Activity. The rate of nitrogenase dependent

Figure 9. Dry weights of the leaves and stems of Alnus rubra following treatment with 0 to 25 mg CdCl₂ per liter of nutrient solution for 11 weeks.

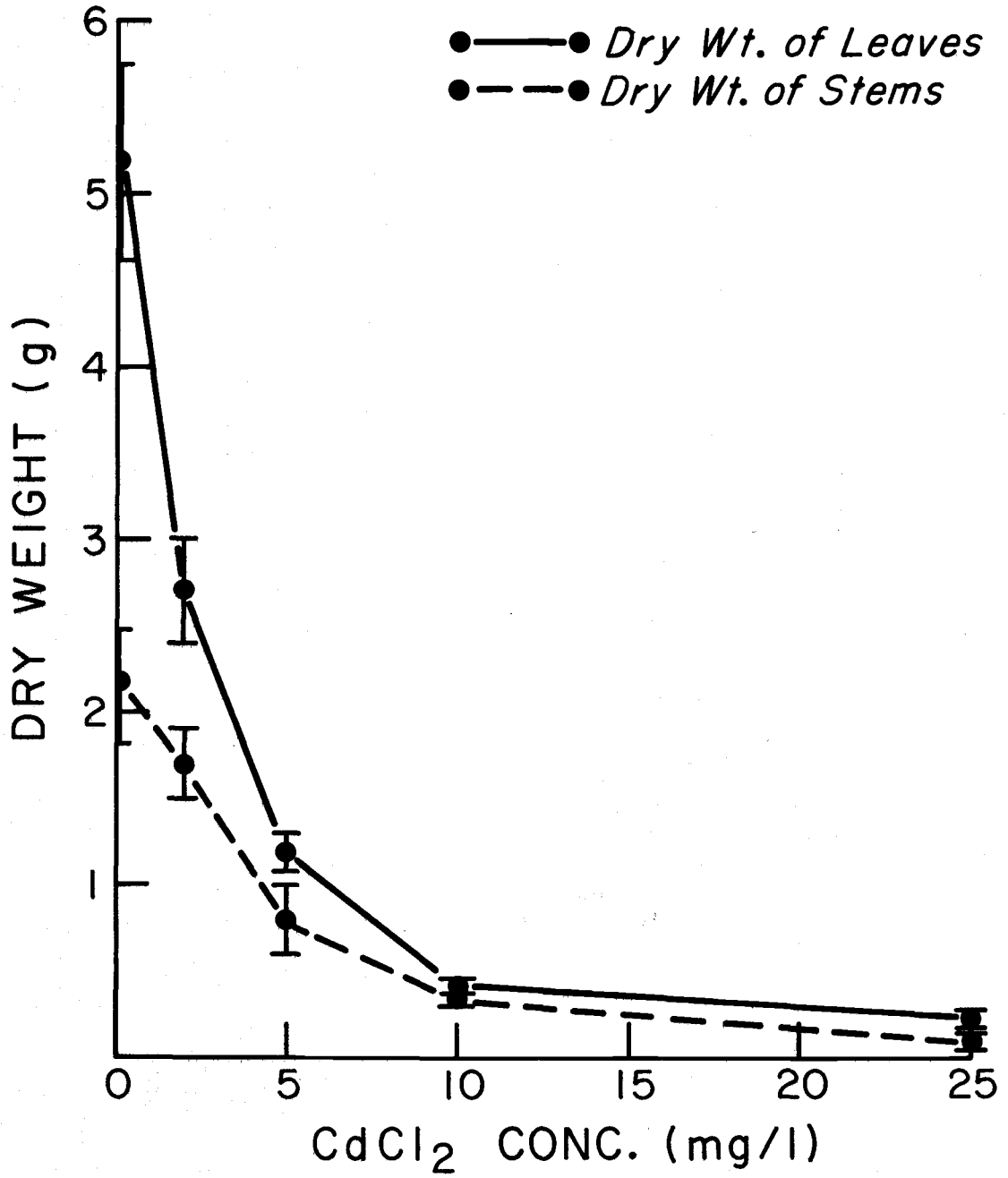


Figure 9

Figure 10. Dry weights of the roots and nodules of Alnus rubra following treatment with 0 to 25 mg CdCl_2 per liter of nutrient solution for 11 weeks.

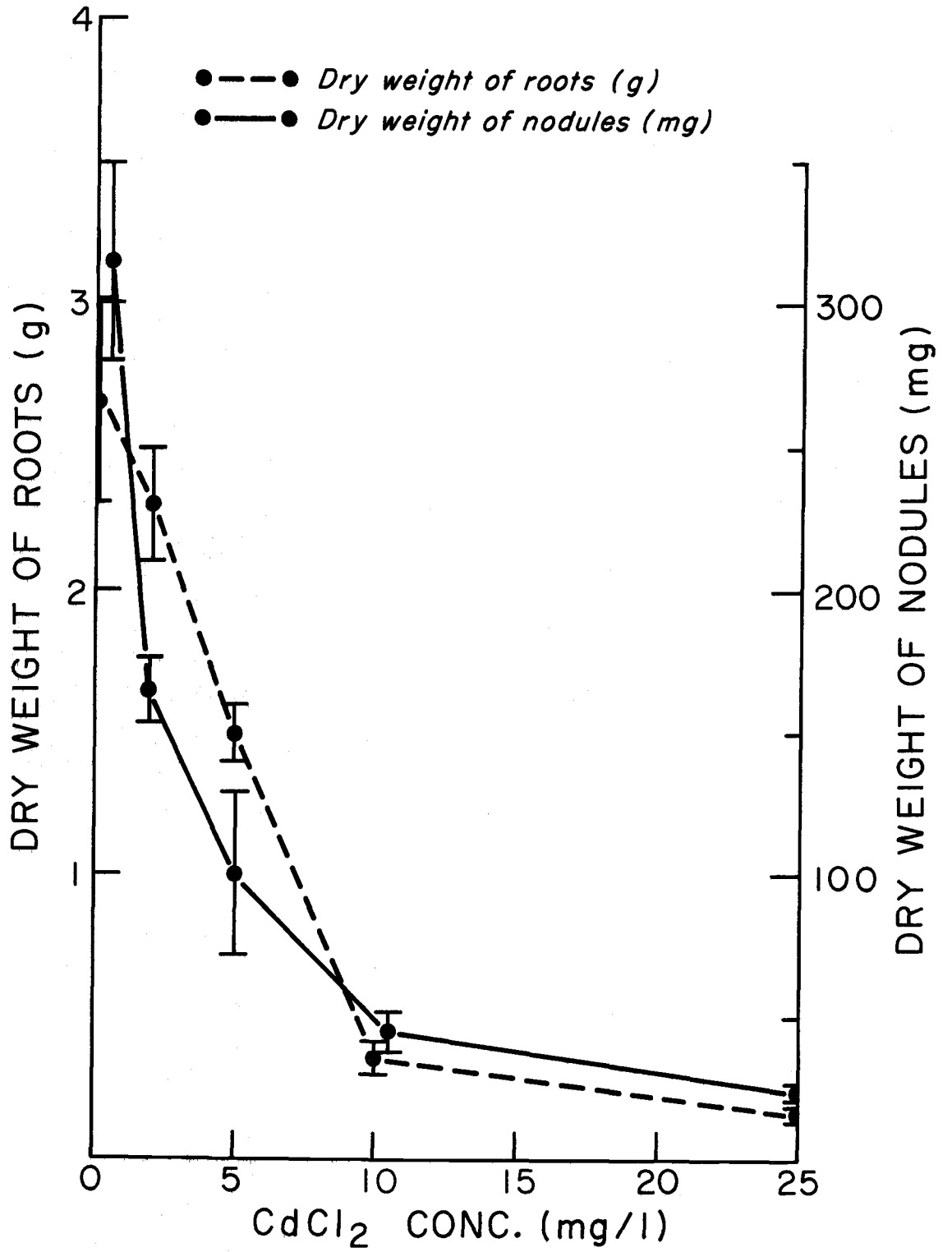


Figure 10

Plate 2. Effect of cadmium on plant growth. Plant size decreased as the CdCl_2 concentration increased (Left to right: 0, 10, and 25 mg CdCl_2 per liter of nutrient solution).



PLATE 2

Plate 3. Effect of cadmium on plant growth. Plant size decreased as the CdCl_2 concentration increased (Left to right: 5, 2, and 0 mg CdCl_2 per liter of nutrient solution).



PLATE 3

Plate 4. Foliar injury resulting from cadmium treatment. The leaves of the plants on the right were chlorotic as a result of treatment with 50 $\mu\text{g CdCl}_2$ per liter of nutrient solution for 11 weeks. The plants on the left were not given cadmium.

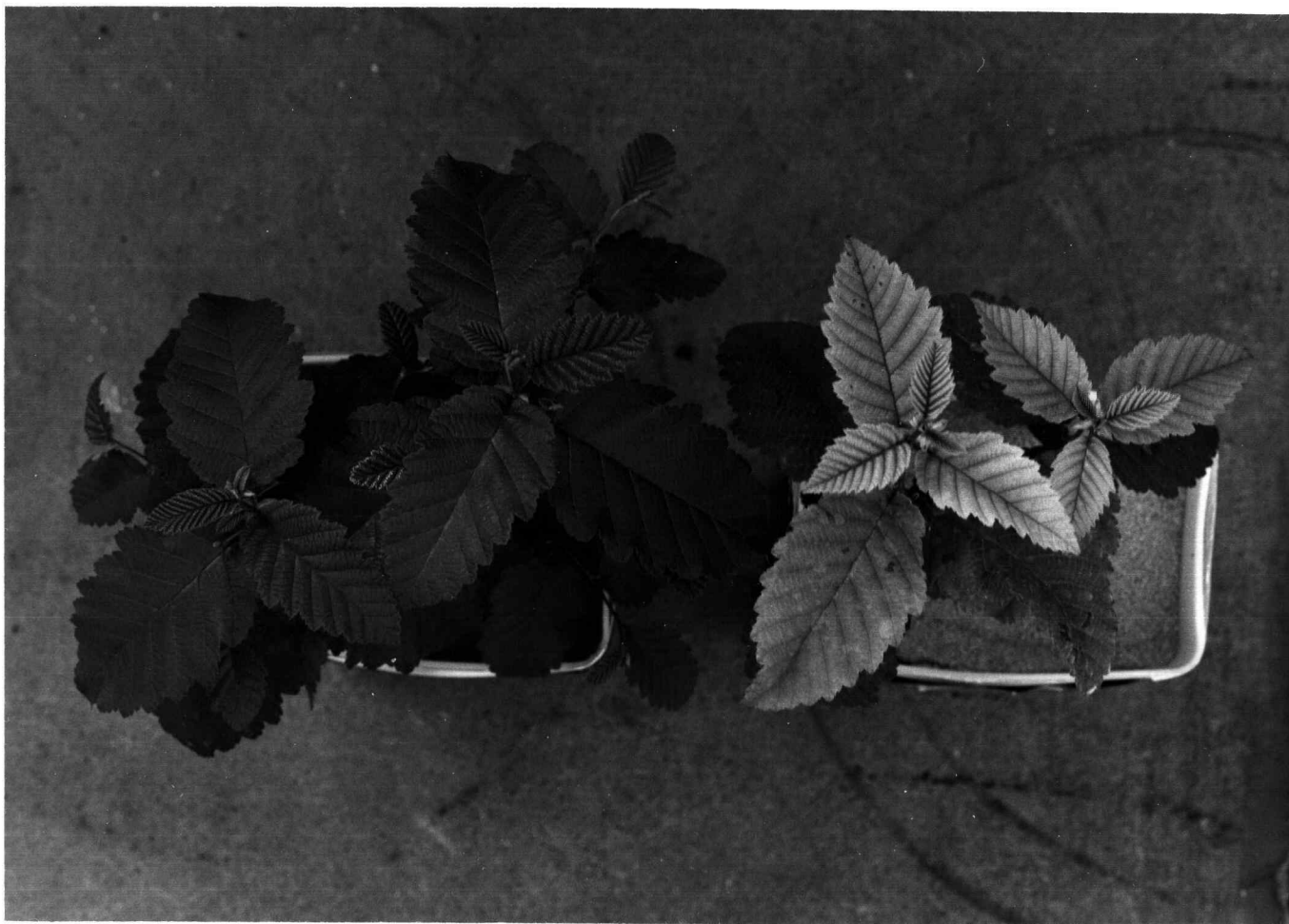


PLATE 4

Figure 11. Dry weight of the leaves and nodules of Alnus rubra following treatment with 0 to 5.0 mg CdCl_2 per liter of nutrient solution for 11 weeks.

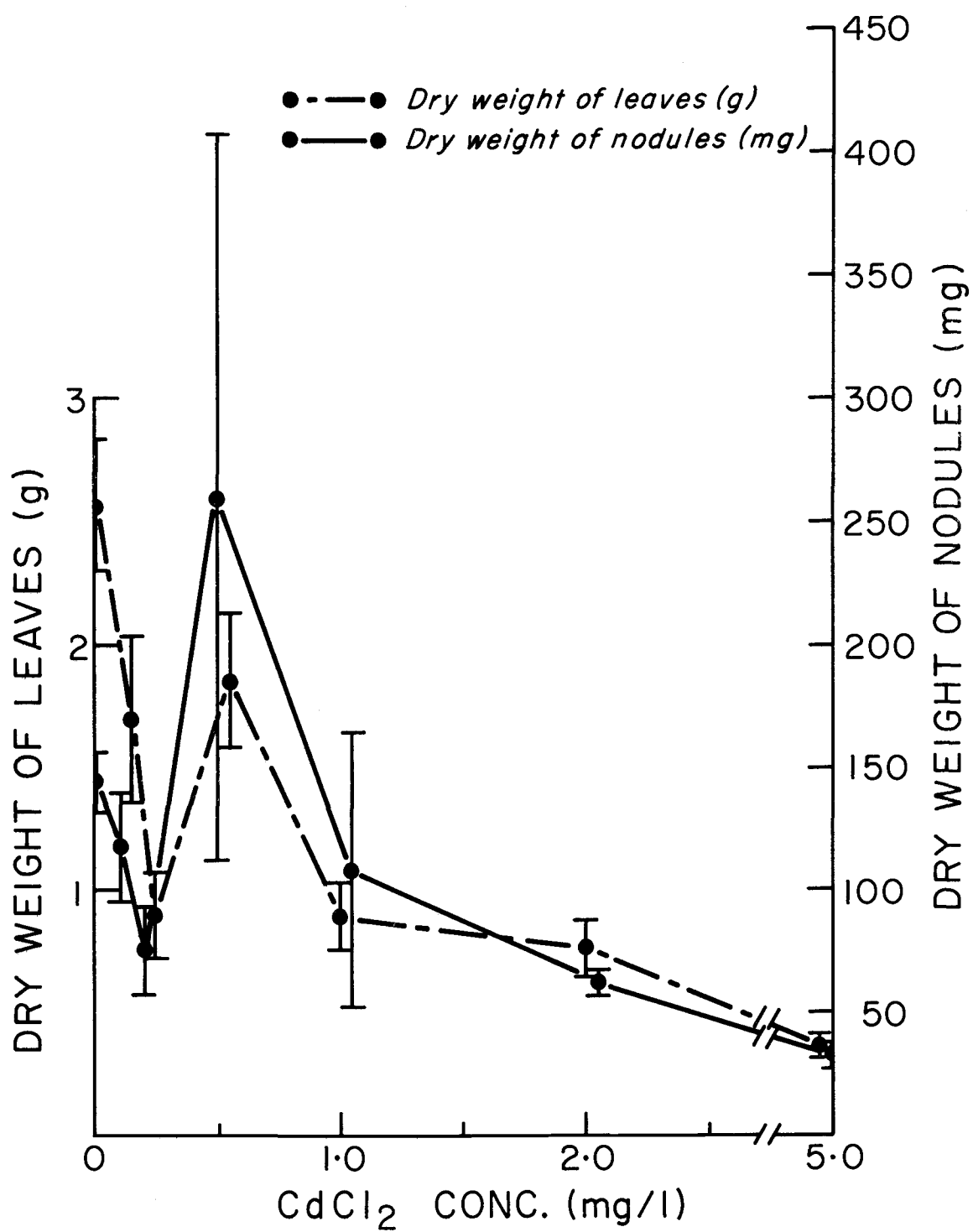


Figure 11

Figure 12. Dry weights of the stems and roots of Alnus rubra following treatment with 0 to 5.0 mg CdCl_2 per liter of nutrient solution for 11 weeks.

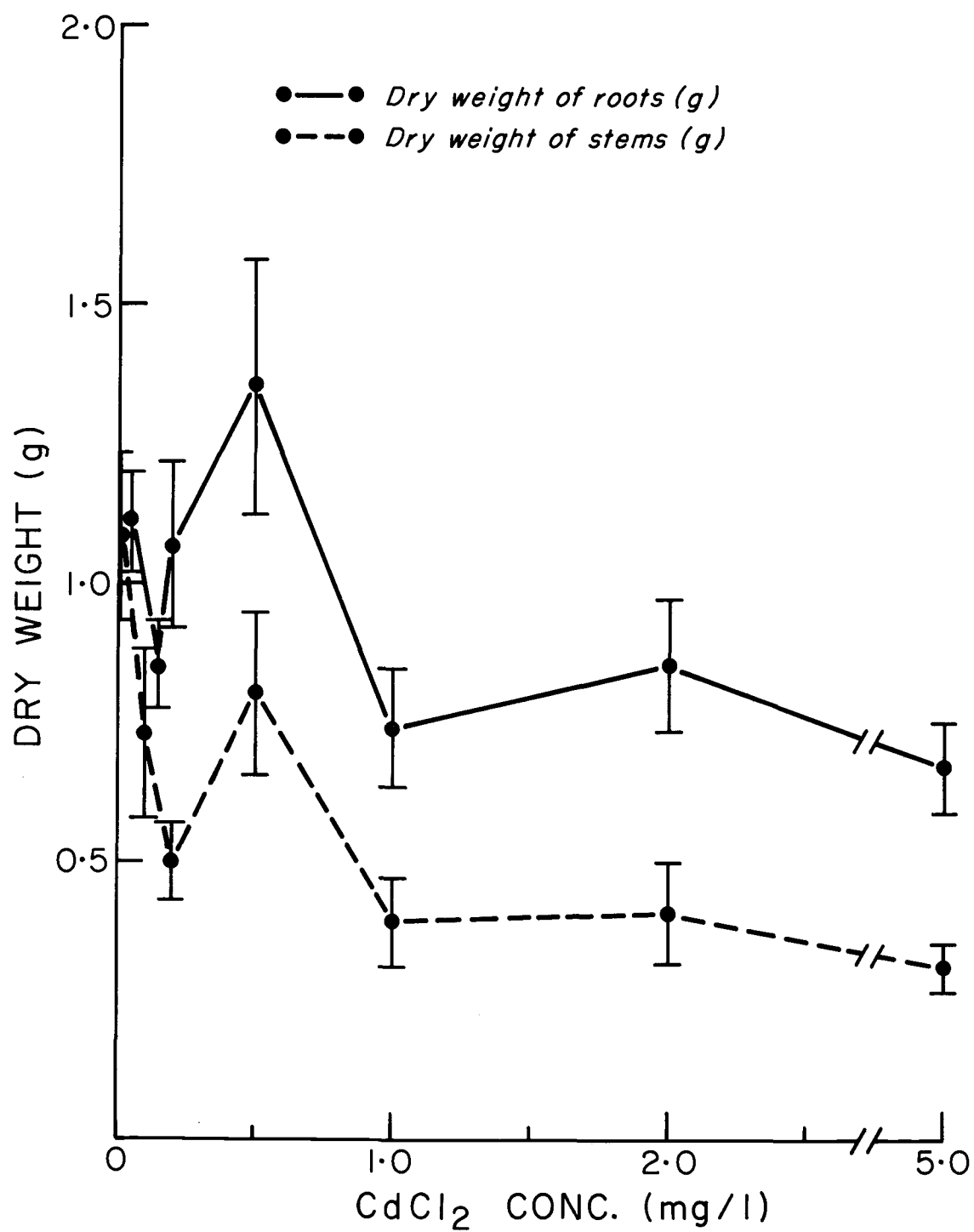


Figure 12

Figure 13. Dry weights of the leaves and nodules of Alnus rubra following treatment with 0 to 200 $\mu\text{g CdCl}_2$ per liter of nutrient solution for 11 weeks.

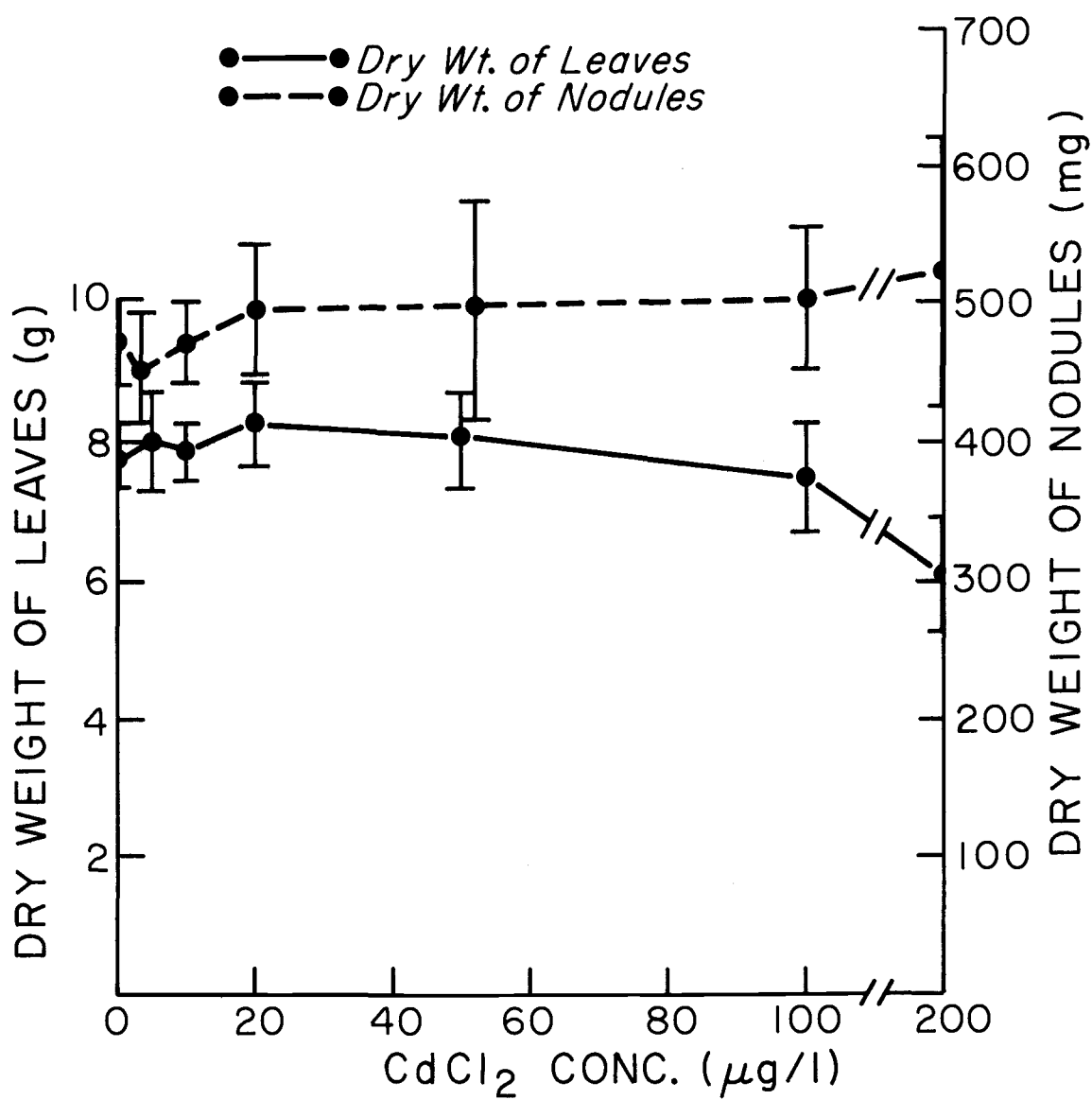


Figure 13

Figure 14. Dry weights of the stems and roots of Alnus rubra following treatment with 0 to 200 $\mu\text{g CdCl}_2$ per liter of nutrient solution for 11 weeks.

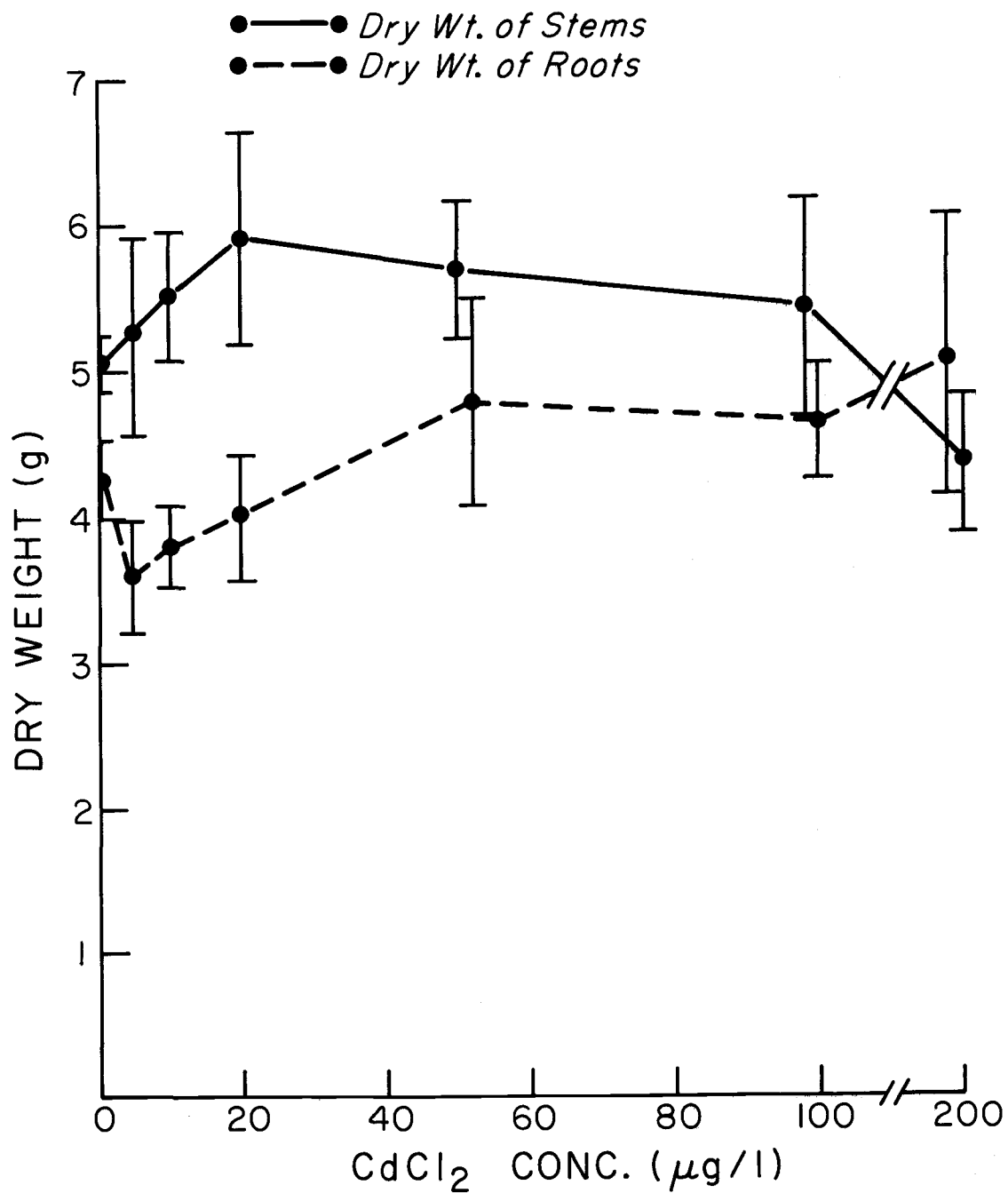


Figure 14

acetylene reduction was decreased in the nodules of plants receiving 2 to 25 mg CdCl_2 per liter of nutrient solution (Fig. 15). The decrease was pronounced between 2 and 5 mg per liter. As shown in Figure 16, increasing the CdCl_2 concentration up to 5 mg per liter caused a decrease in the nitrogenase activity regardless of whether the results were expressed on a fresh or dry weight basis. Increasing the CdCl_2 in solution from 5 to 50 μg per liter caused a decrease in the nitrogenase activity in the nodules (Fig. 17).

Nitrogen Fixation. The amount of nitrogen fixed by Alnus rubra decreased as cadmium concentration increased from 0.2 to 25 mg CdCl_2 per liter of nutrient solution (Figs. 18-20). Nitrogen fixation did not decrease significantly in the range of 5 μg to 0.2 mg CdCl_2 per liter (Fig. 20).

Nodulation. The number of root nodules decreased following growth on nutrient solutions containing cadmium in the range from 2 to 25 mg CdCl_2 per liter (Figs. 21 and 22). However, the weight per nodule decreased most drastically between at 10 and 25 mg per liter (Fig. 21). The number of nodules increased significantly in the cadmium concentration range of 20 to 100 μg CdCl_2 per liter (Fig. 23).

Cadmium Absorption. The cadmium concentrations in the organs of Alnus rubra increased with increasing concentrations of cadmium in the nutrient solution applied for 11 weeks (Figs. 24-28). The roots accumulated greater amounts of cadmium than the leaves,

Figure 15. Nitrogenase activity in the nodules of Alnus rubra following treatment with 0 to 25 mg CdCl_2 per liter of nutrient solution for 11 weeks.

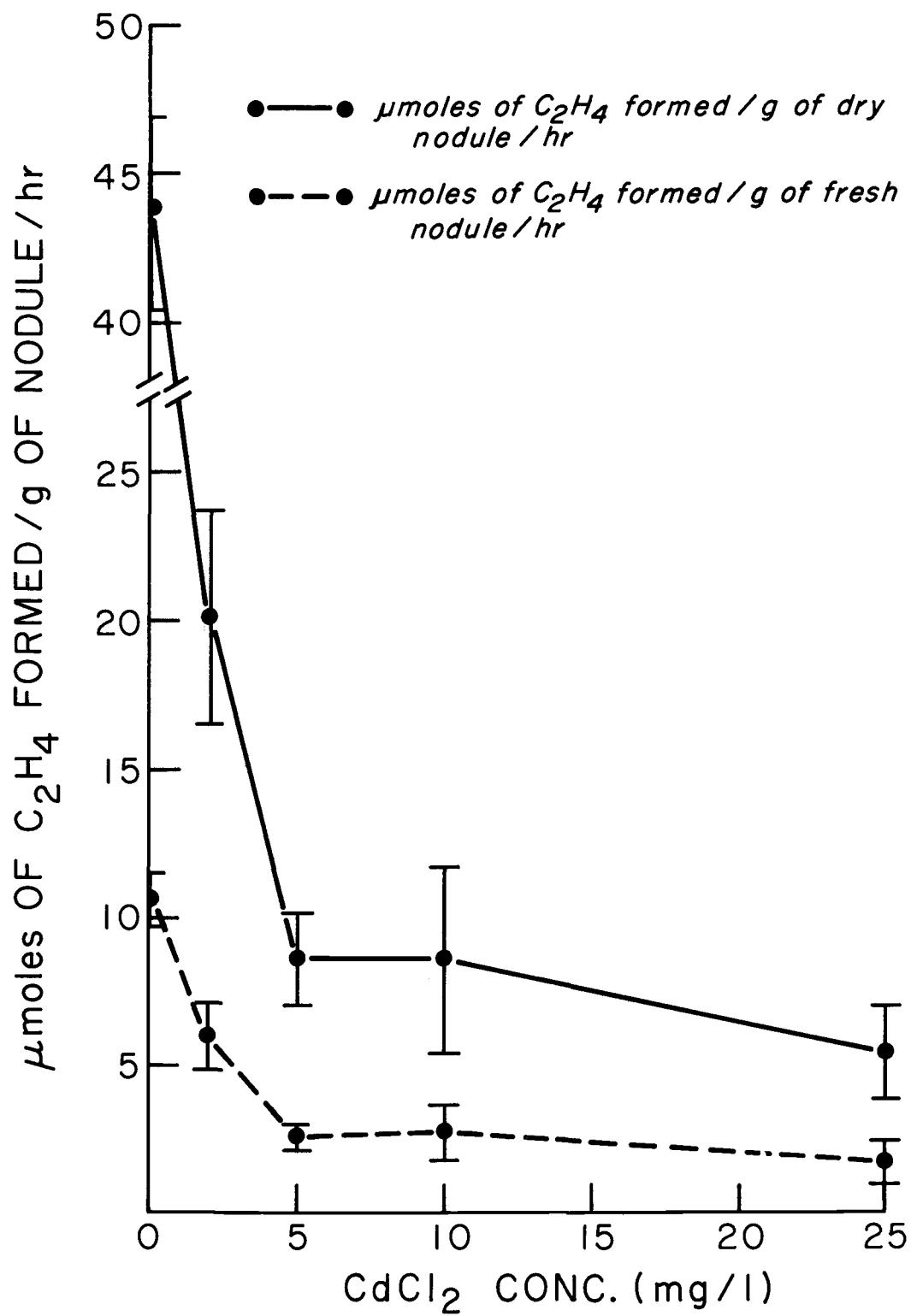


Figure 15

Figure 16. Nitrogenase activity in the nodules of Alnus rubra following treatment with 0 to 5.0 mg CdCl_2 per liter of nutrient solution for 11 weeks.

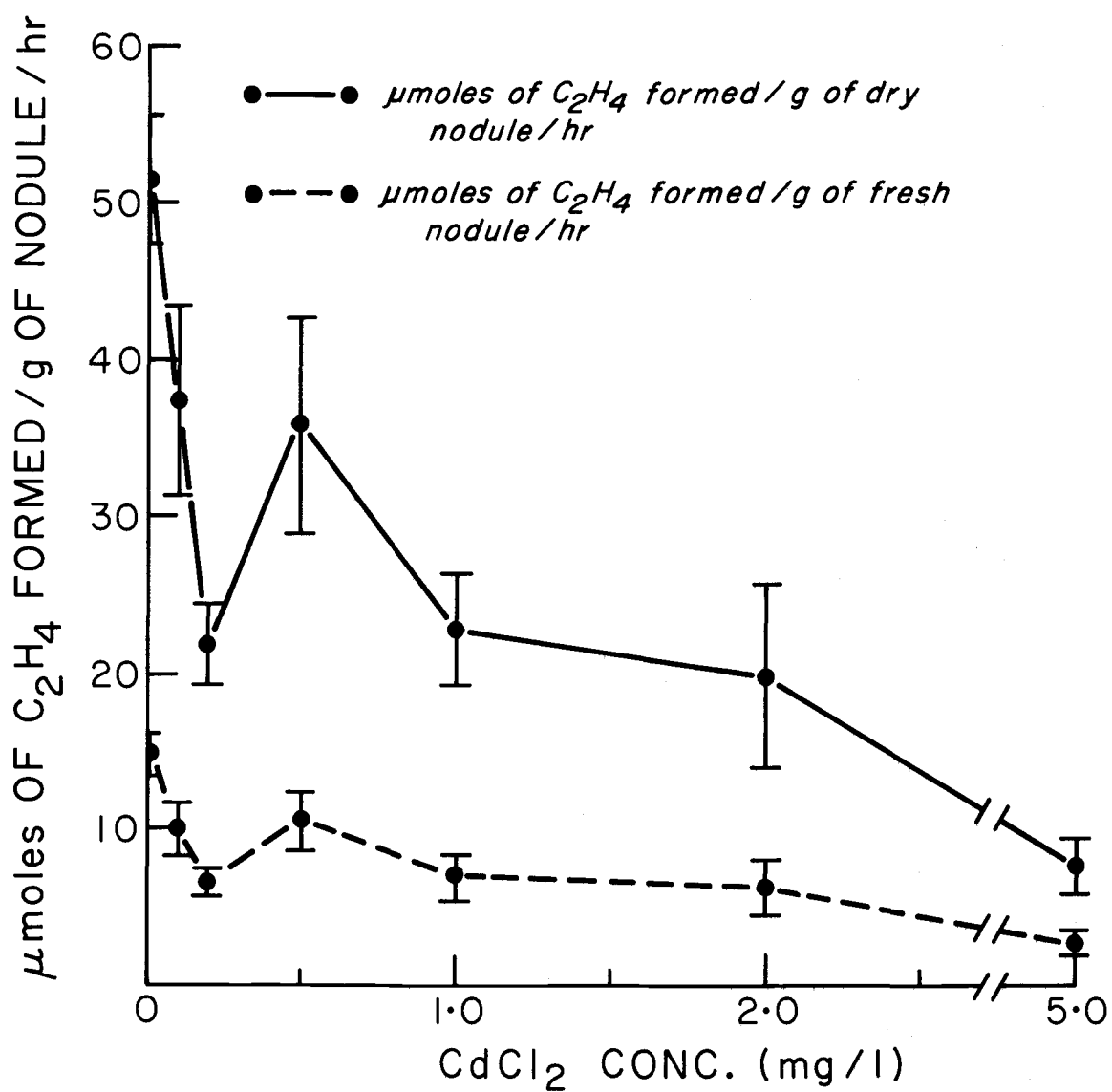


Figure 16

Figure 17. Nitrogenase activity in the nodules of Alnus rubra following treatment with 0 to 200 $\mu\text{g CdCl}_2$ per liter of nutrient solution for 11 weeks.

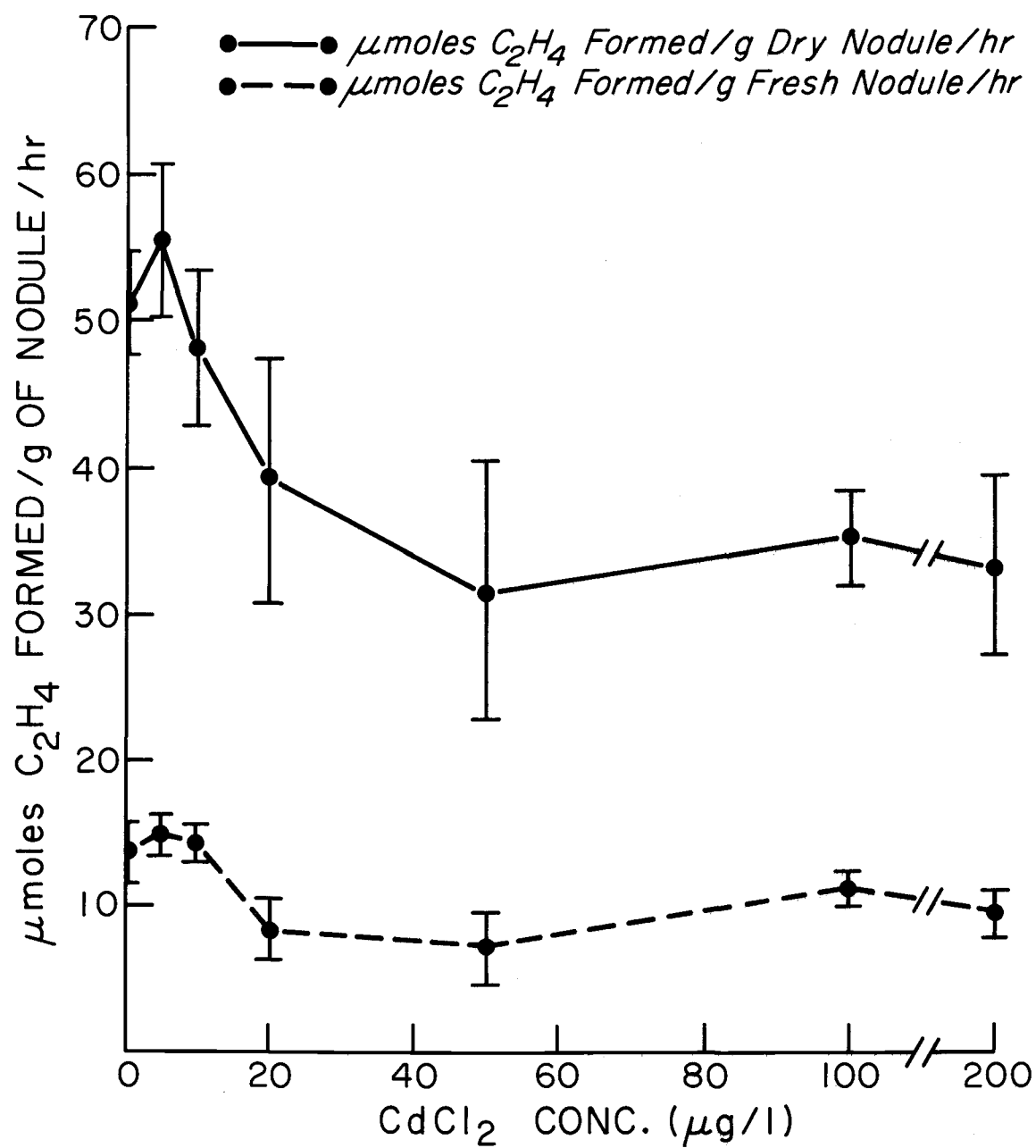


Figure 17

Figure 18. Total nitrogen and nitrogen fixation in Alnus rubra following treatment with 0 to 25 mg CdCl_2 per liter of nutrient solution for 11 weeks. Nitrogen fixed = final total nitrogen - initial total nitrogen.

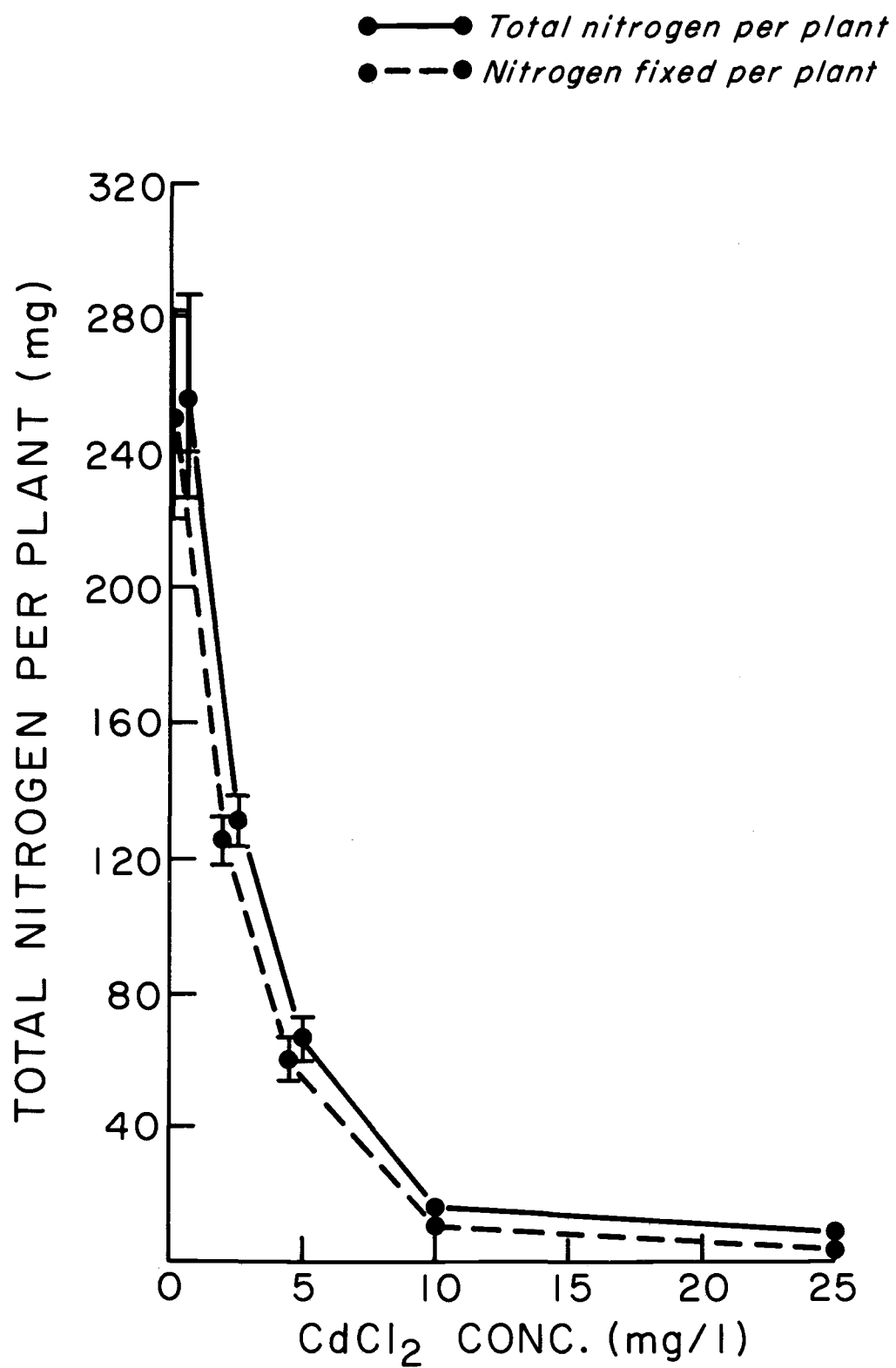


Figure 18

Figure 19. Total nitrogen and nitrogen fixation in Alnus rubra following treatment with 0 to 5.0 mg CdCl_2 per liter of nutrient solution for 11 weeks. Nitrogen fixed = final total nitrogen - initial total nitrogen.

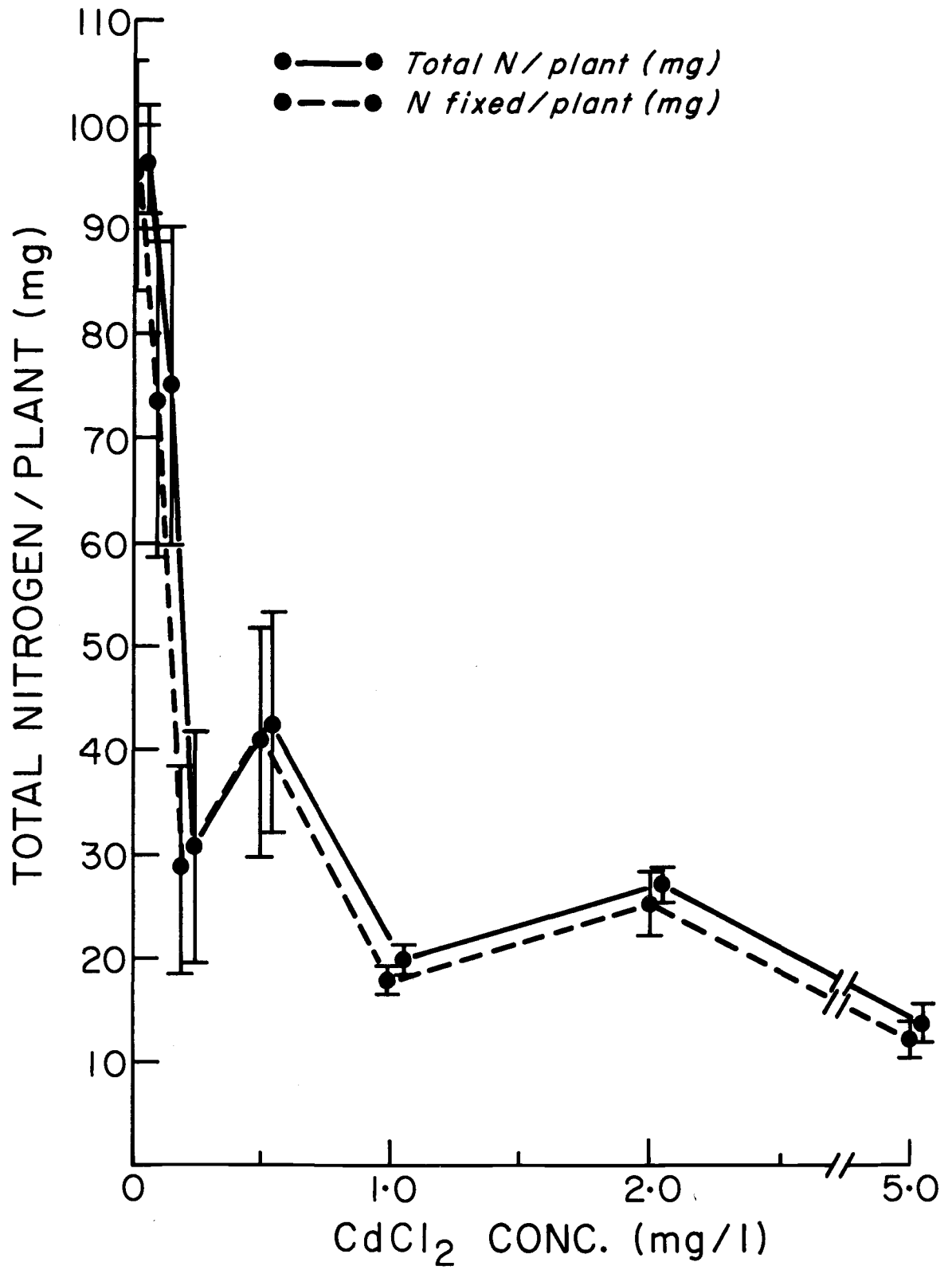


Figure 19

Figure 20. Total nitrogen and nitrogen fixation in Alnus rubra following treatment with 0 to 200 $\mu\text{g CdCl}_2$ per liter of nutrient solution for 11 weeks. Nitrogen fixed = final total nitrogen - ~~initial~~ total nitrogen.

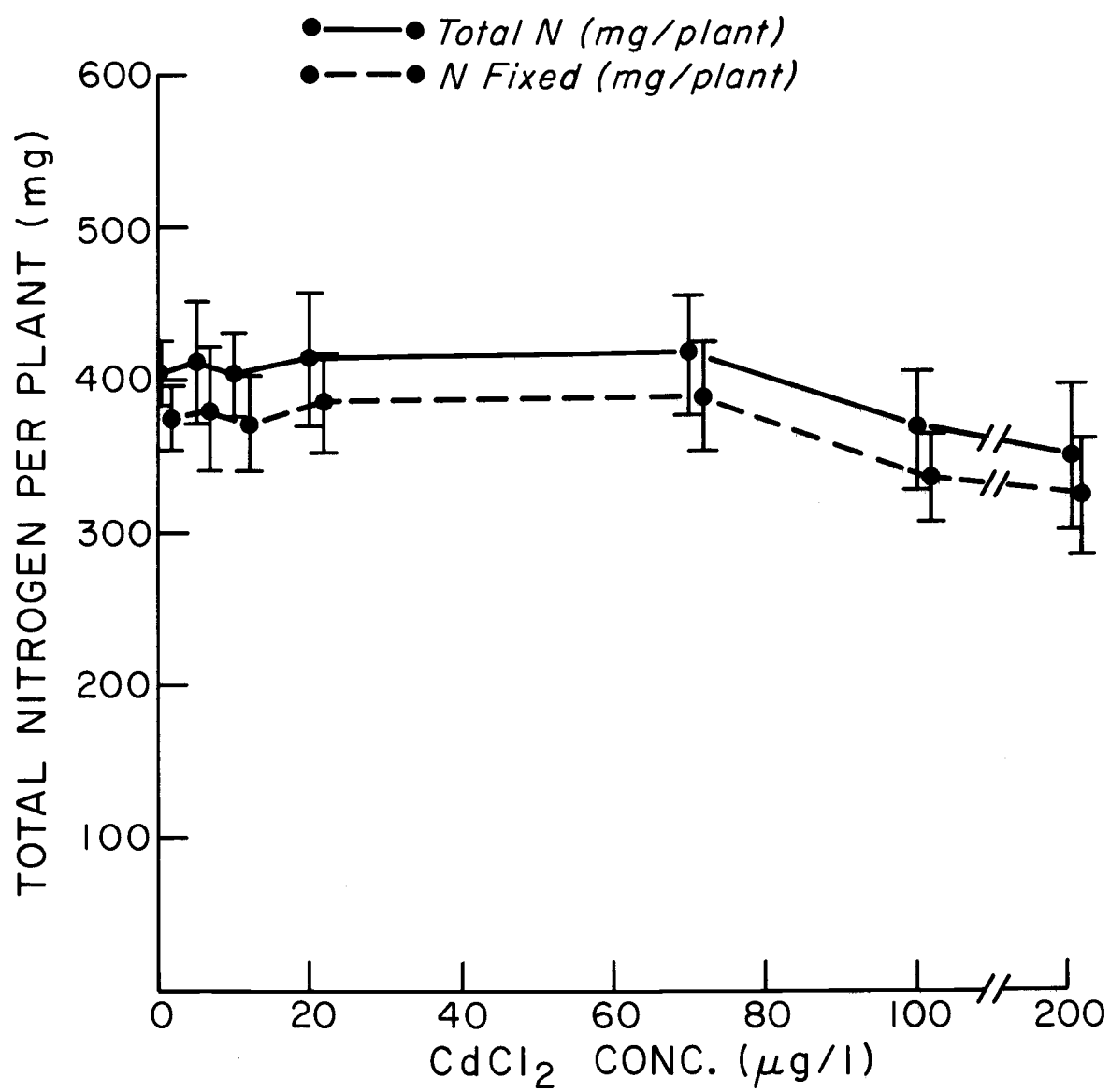


Figure 20

Figure 21. The number of nodules per plant of Alnus rubra and the weight per individual nodule following treatment with 0 to 25 mg CdCl_2 per liter of nutrient solution for 11 weeks.

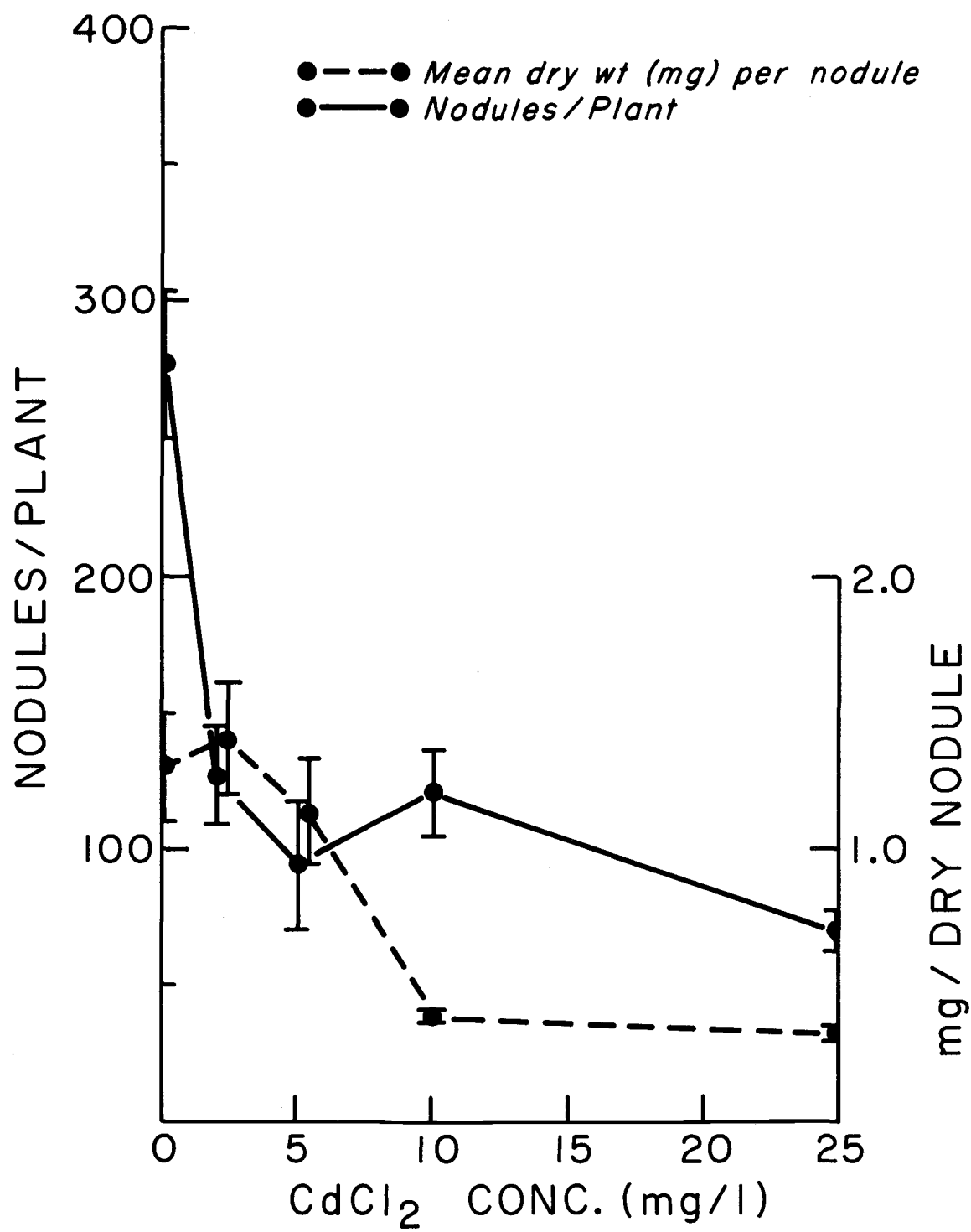


Figure 21

Figure 22. The number of nodules per plant of Alnus rubra and the weight per individual nodule following treatment with 0 to 5.0 mg CdCl₂ per liter of nutrient solution for 11 weeks.

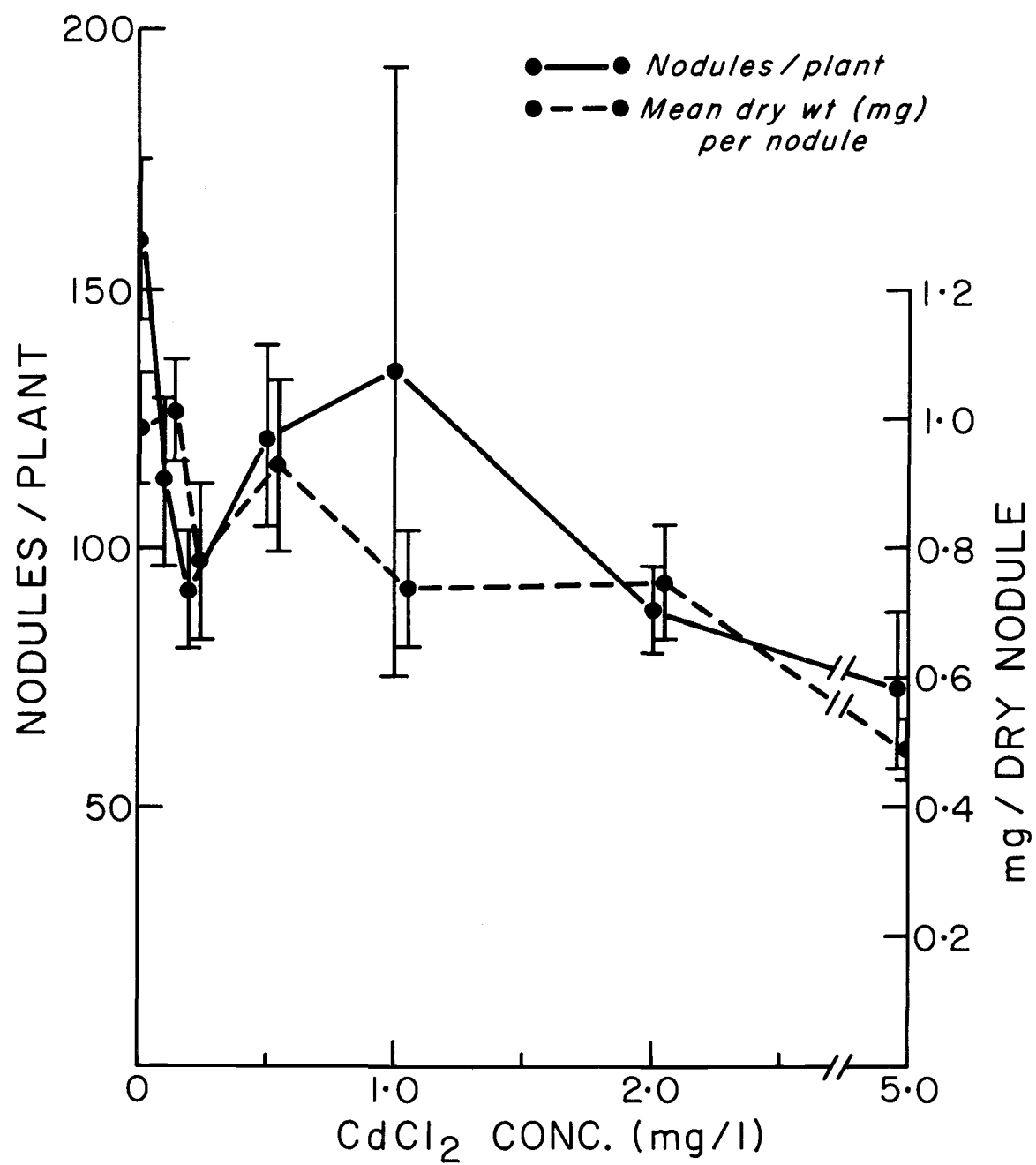


Figure 22

Figure 23. The number of nodules per plant of Alnus rubra and the weight per individual nodule following treatment with 0 to 200 $\mu\text{g CdCl}_2$ per liter of nutrient solution for 11 weeks.

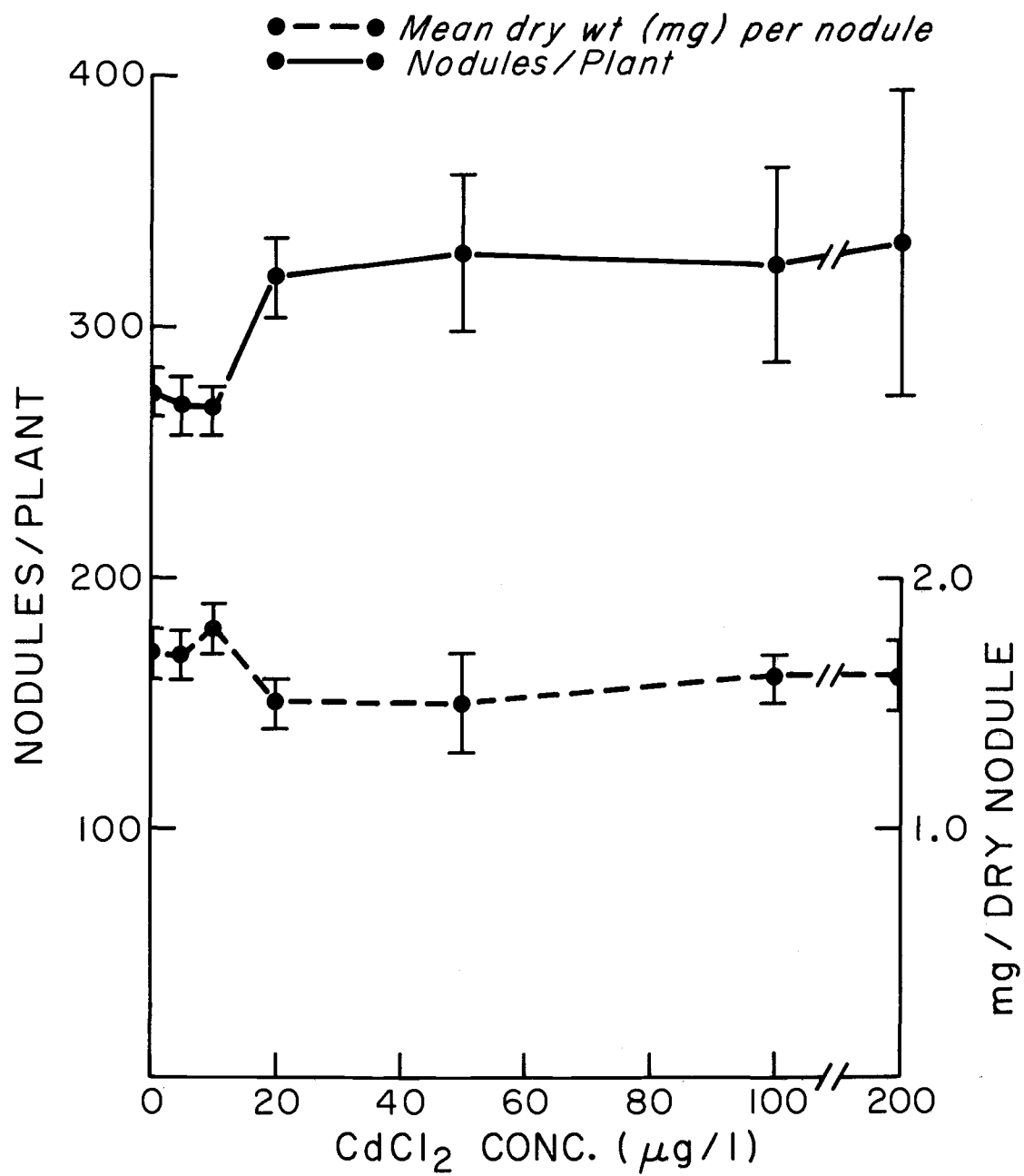


Figure 23

Figure 24. Cadmium concentration in the organs of Alnus rubra following treatment with 0 to 25 mg CdCl₂ per liter of nutrient solution for 11 weeks.

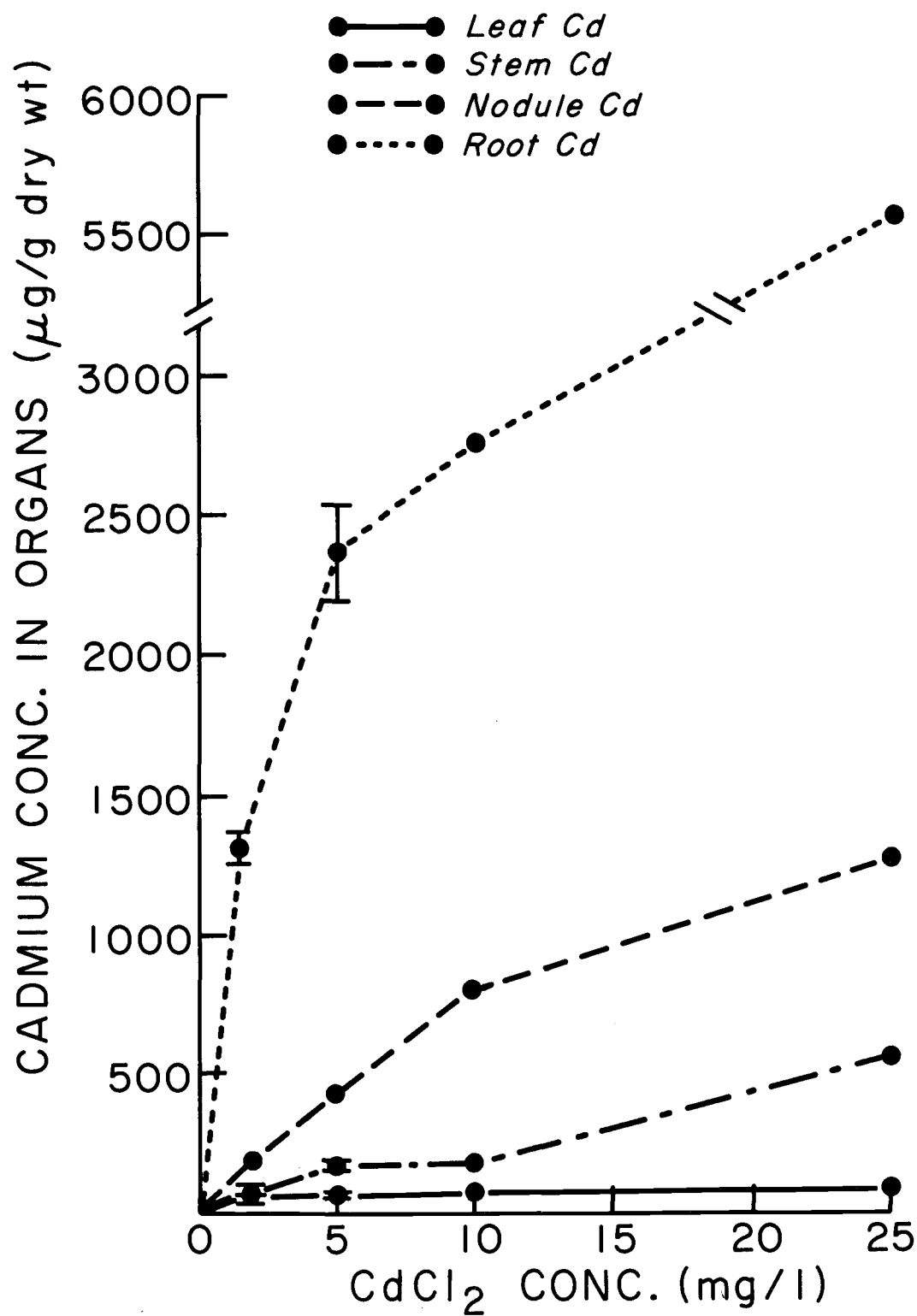


Figure 24

Figure 25. Cadmium concentrations in the stem, leaves, and nodules of Alnus rubra following treatment with 0 to 5.0 mg CdCl_2 per liter of nutrient solution for 11 weeks.

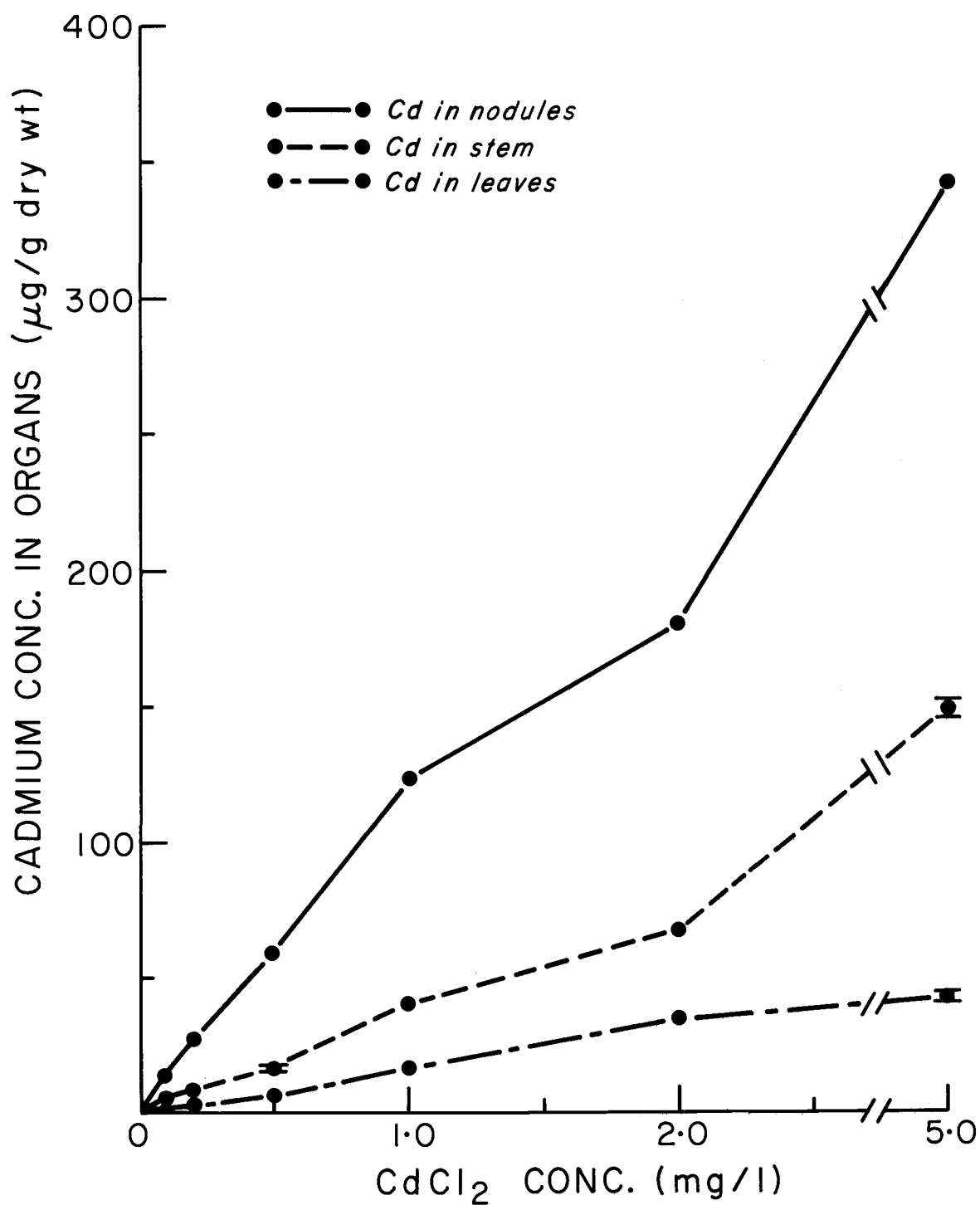


Figure 25

Figure 26. Cadmium concentration in the roots of Alnus rubra following treatment with 0 to 5.0 mg CdCl₂ per liter of nutrient solution for 11 weeks.

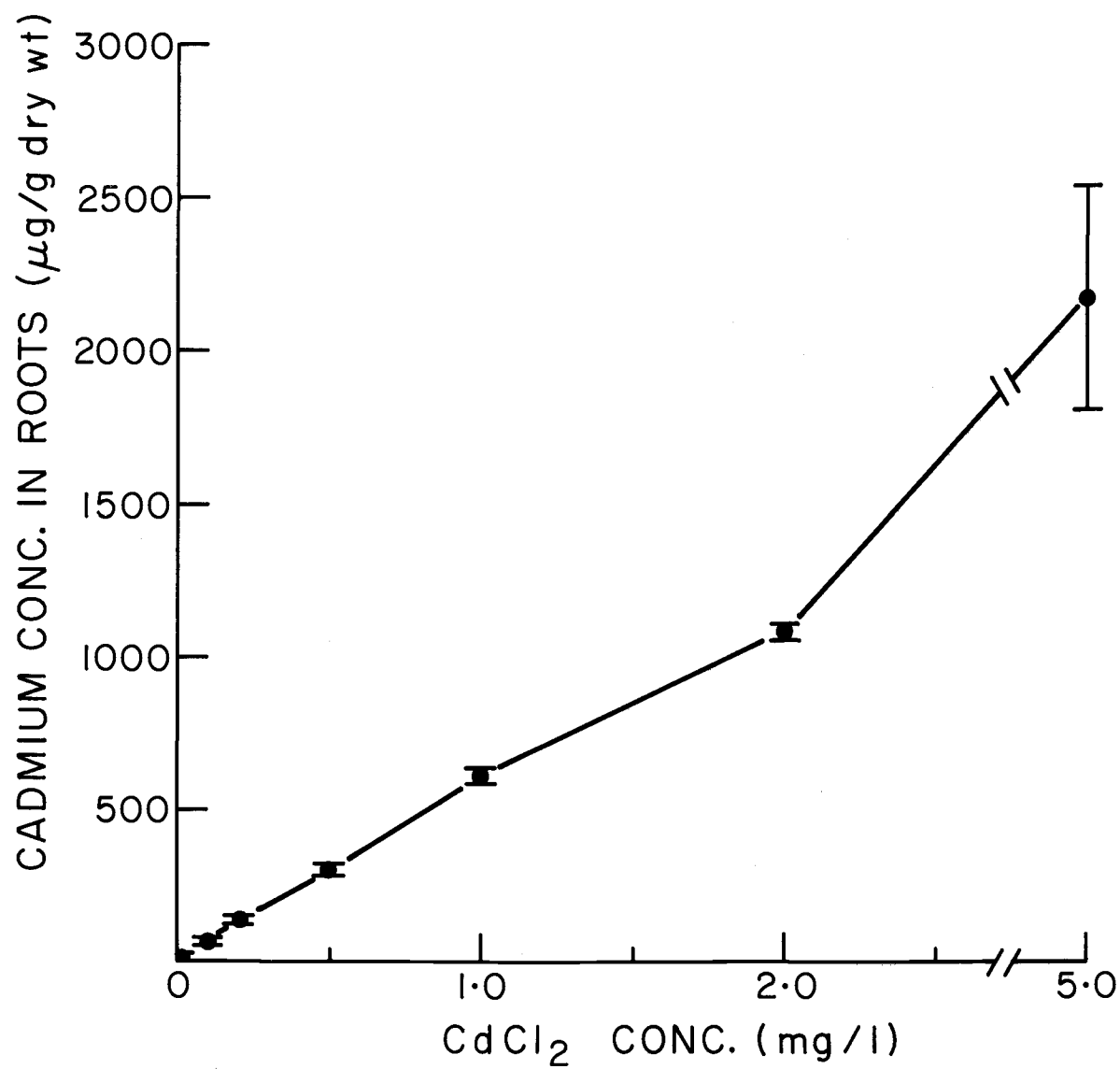


Figure 26

Figure 27. Cadmium concentrations in the leaves, stems, and nodules of Alnus rubra following treatment with 0 to 200 $\mu\text{g CdCl}_2$ per liter of nutrient solution for 11 weeks.

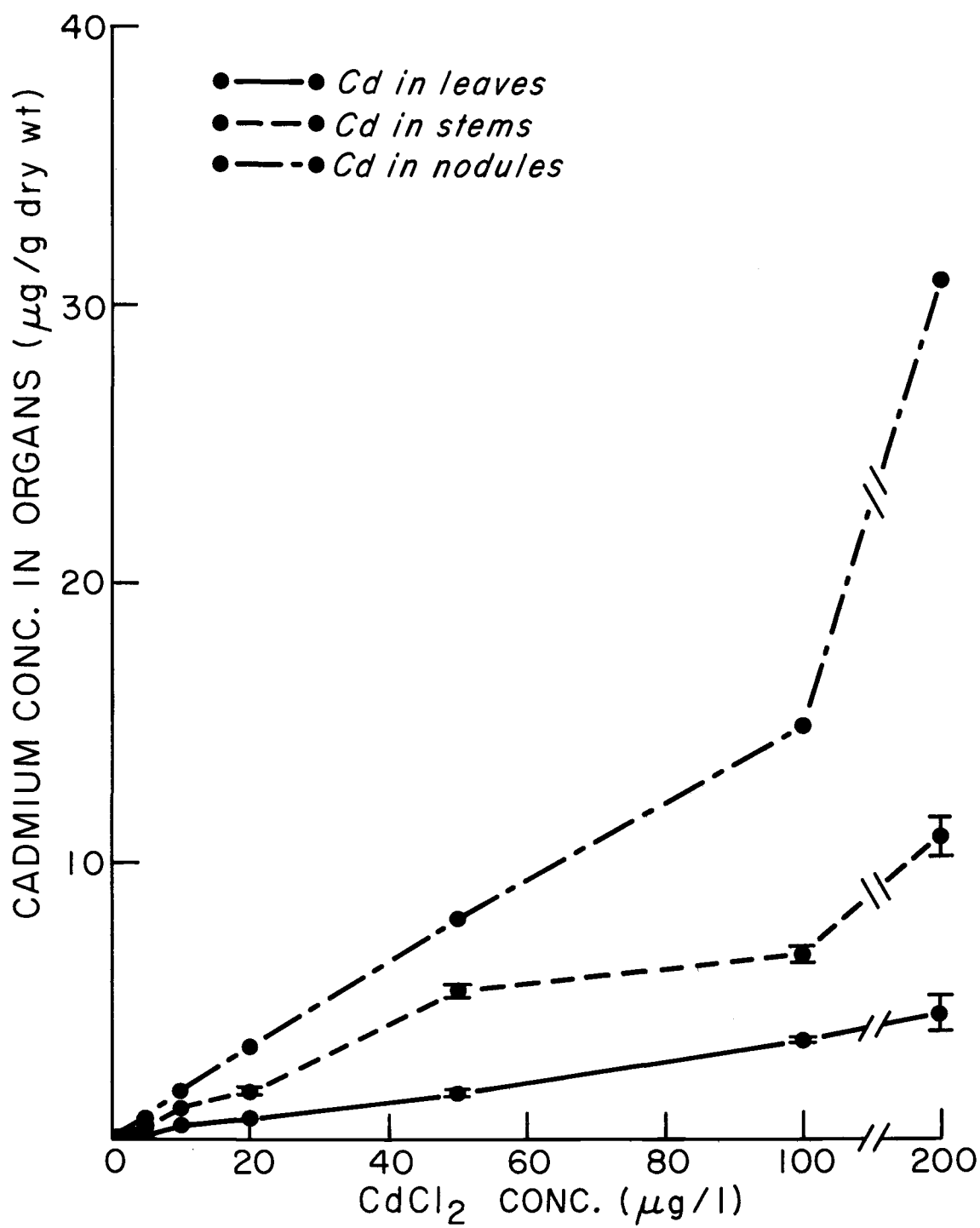


Figure 27

Figure 28. Cadmium concentration in the roots of Alnus rubra following treatment with 0 to 200 $\mu\text{g CdCl}_2$ per liter of nutrient solution for 11 weeks.

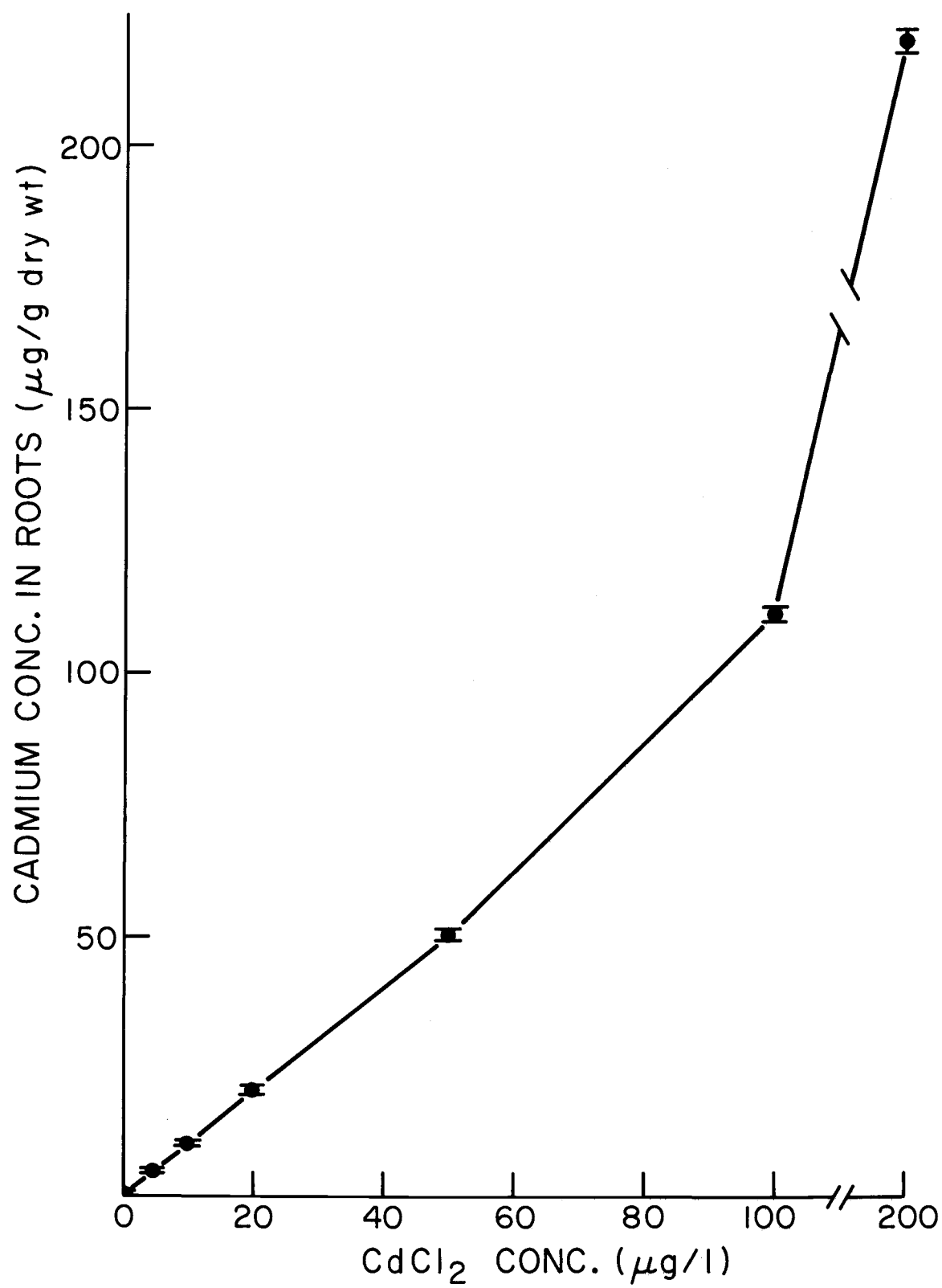


Figure 28

stems, and nodules.

Effects of Cadmium on Plants without Nodules and Inoculated

Plant Growth. Plants lacking nodules that were inoculated at the start of the experiment failed to grow as rapidly as plants that were nodulated prior to the initiation of the experiments. The dry weights of the plant organs decreased as cadmium concentration increased (Figs. 29-31 and Plates 5-9). The number of nodules per plant and the dry weights of the nodules decreased as cadmium increased from 0 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution (Fig. 30). The extent of greening of the leaves indicated that nodulation had occurred and the plants were receiving sufficient nitrogen for growth. Plants without nodules did not grow appreciably (Plate 9). The growth period prior to apparent nodulation increased as cadmium concentration increased (Fig. 31). These results indicate that cadmium inhibits the initiation of nodulation.

Nitrogen Fixation. Nitrogenase dependent acetylene reduction did not decrease following treatment of Alnus rubra with cadmium ranging from 10 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution (Fig. 32). Nitrogenase activity was absent at the initiation of cadmium treatment, as expected, because the roots were not nodulated at this time. Nitrogenase activity was detected later in the growth period when nodulation became apparent.

Figure 29. Dry weights of the roots and stems of Alnus rubra following treatment with 0 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution for 11 weeks.

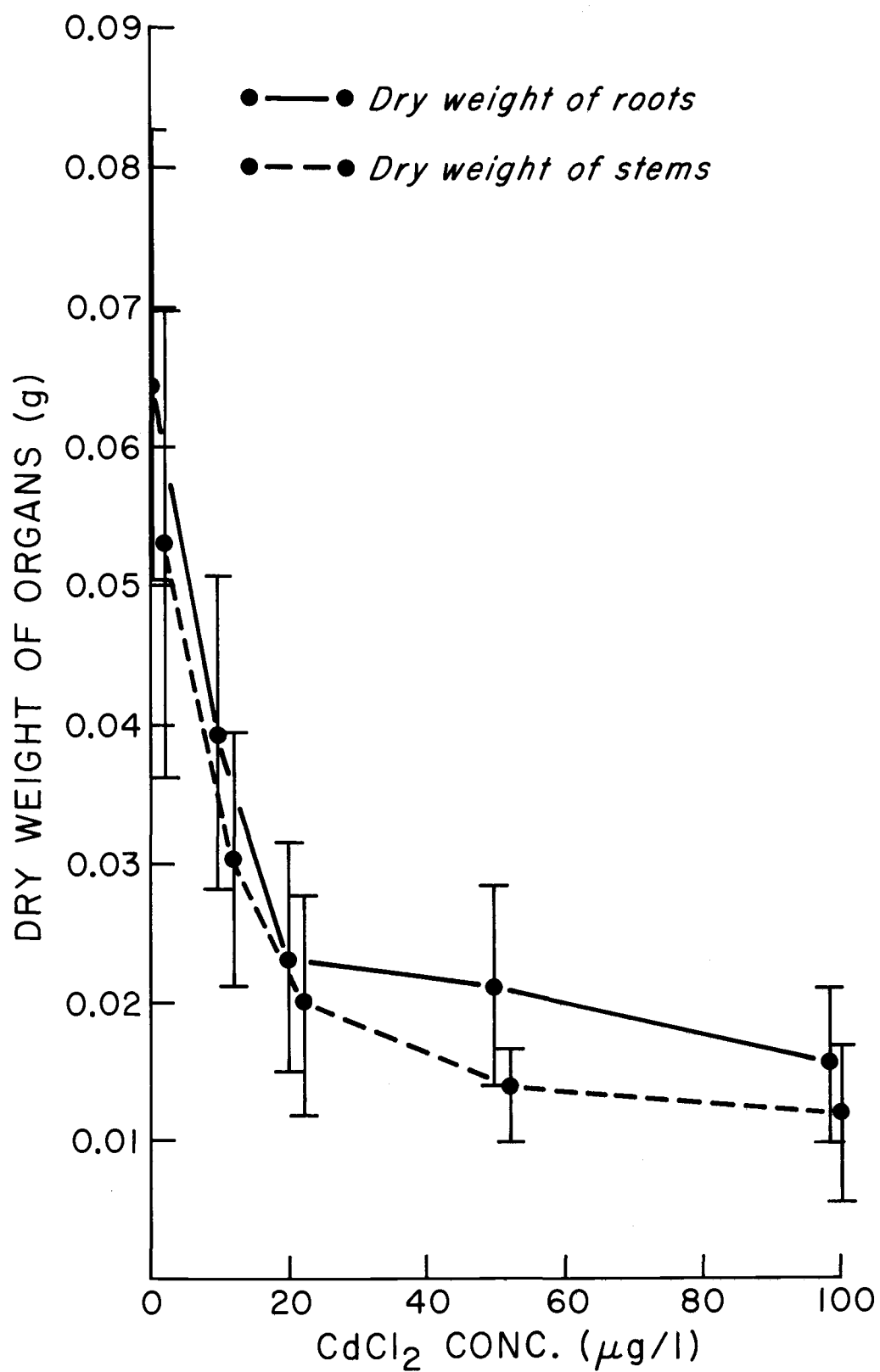


Figure 29

Figure 30. The number of nodules per plant of Alnus rubra and the dry weight per individual nodule following treatment with 0 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution for 11 weeks.

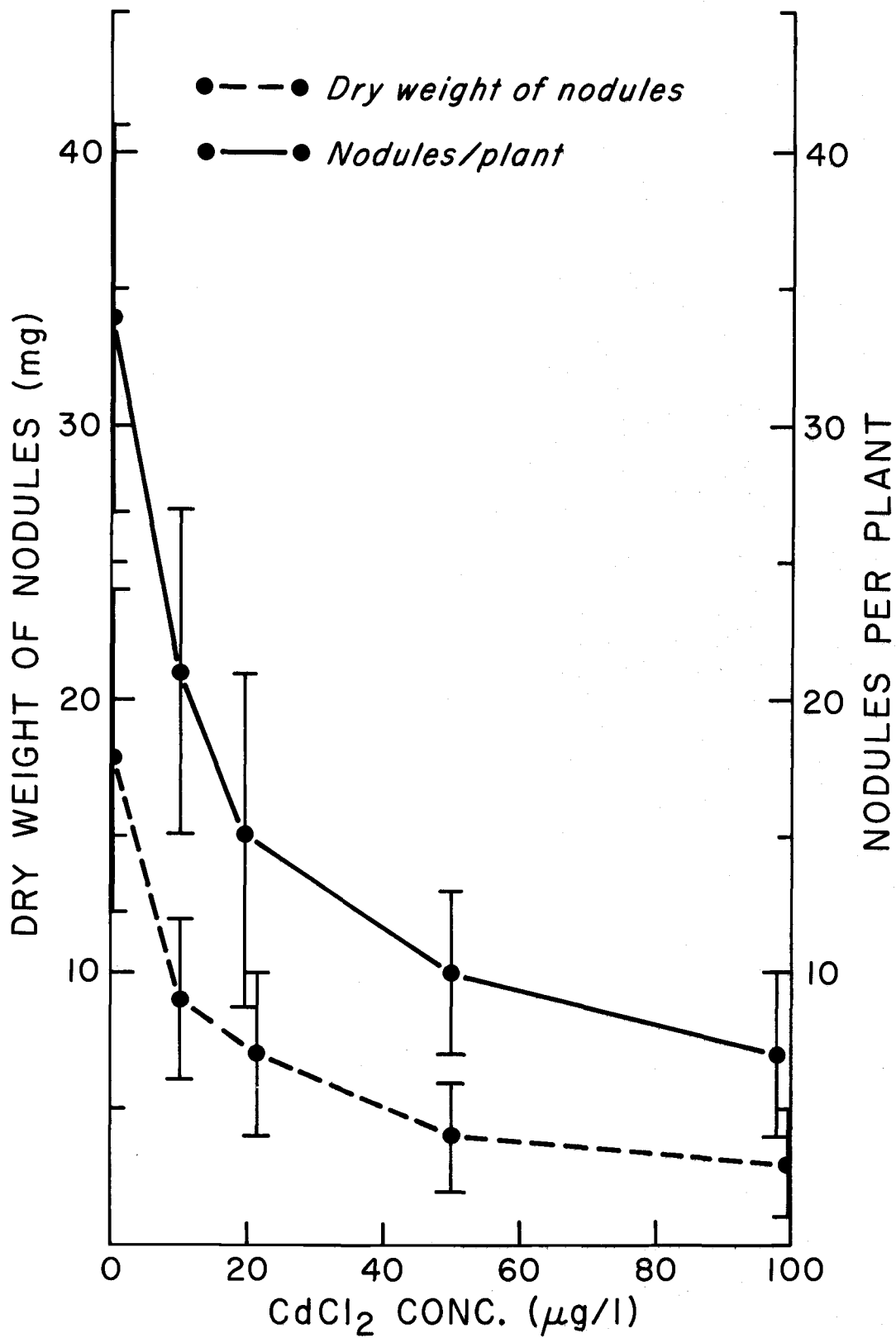


Figure 30

Figure 31. The growth period prior to apparent nodulation and the dry weight of the leaves of Alnus rubra following treatment with 0 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution for 11 weeks.

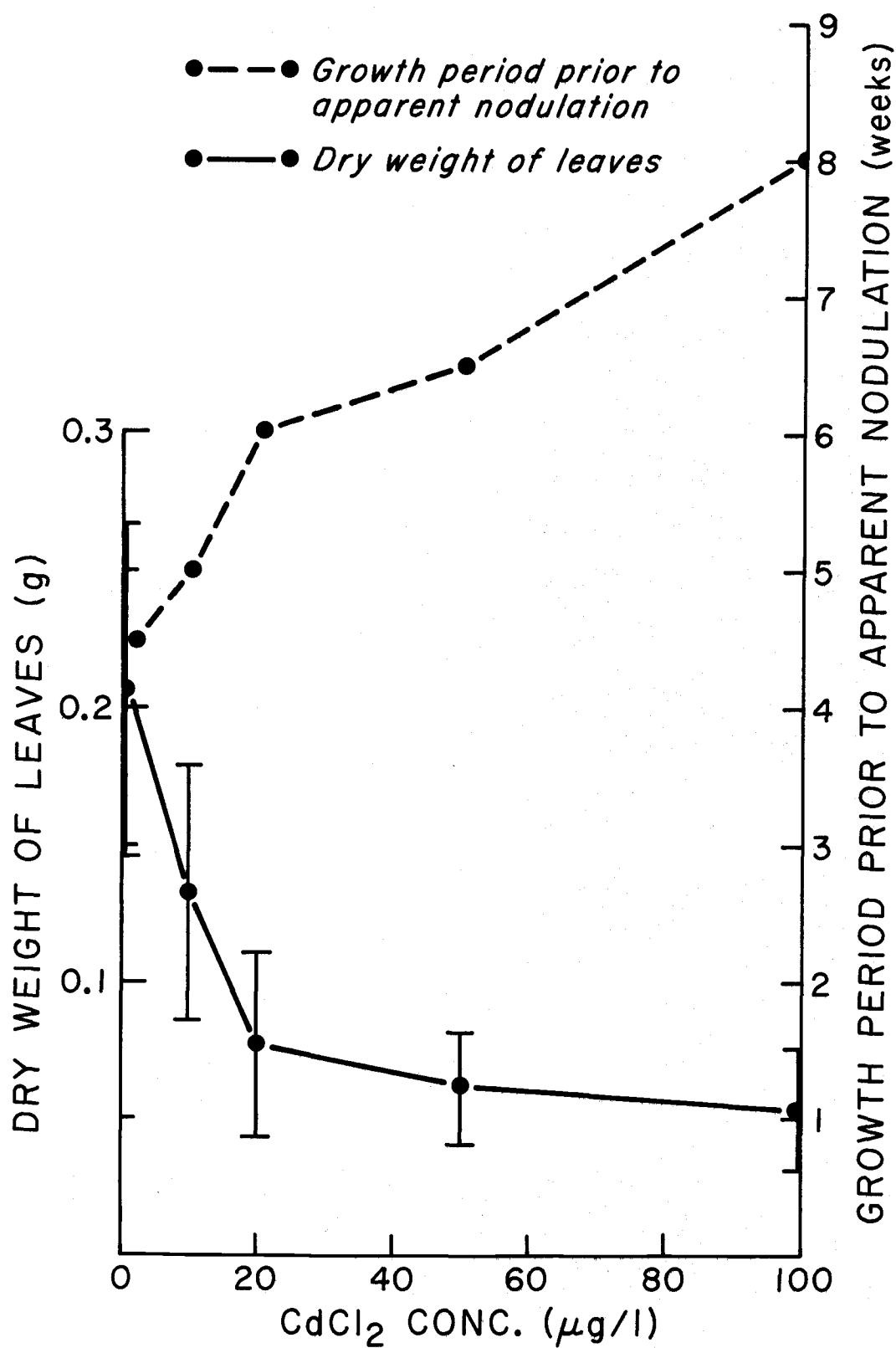


Figure 31

Plates 5-9. Effect of cadmium on the growth of plants without nodules and inoculated. Plant size decreased as the CdCl_2 concentration increased. A scale in centimeters was included for comparison. The number of $\mu\text{g CdCl}_2$ in a liter of nutrient solution are indicated on the tags attached to the plants. The plants in each photograph are replicates of the same treatment. The differences in size within treatment are likely due to random differences in time for the root to contact the infecting microorganism and effective nodulation to occur.



PLATE 5

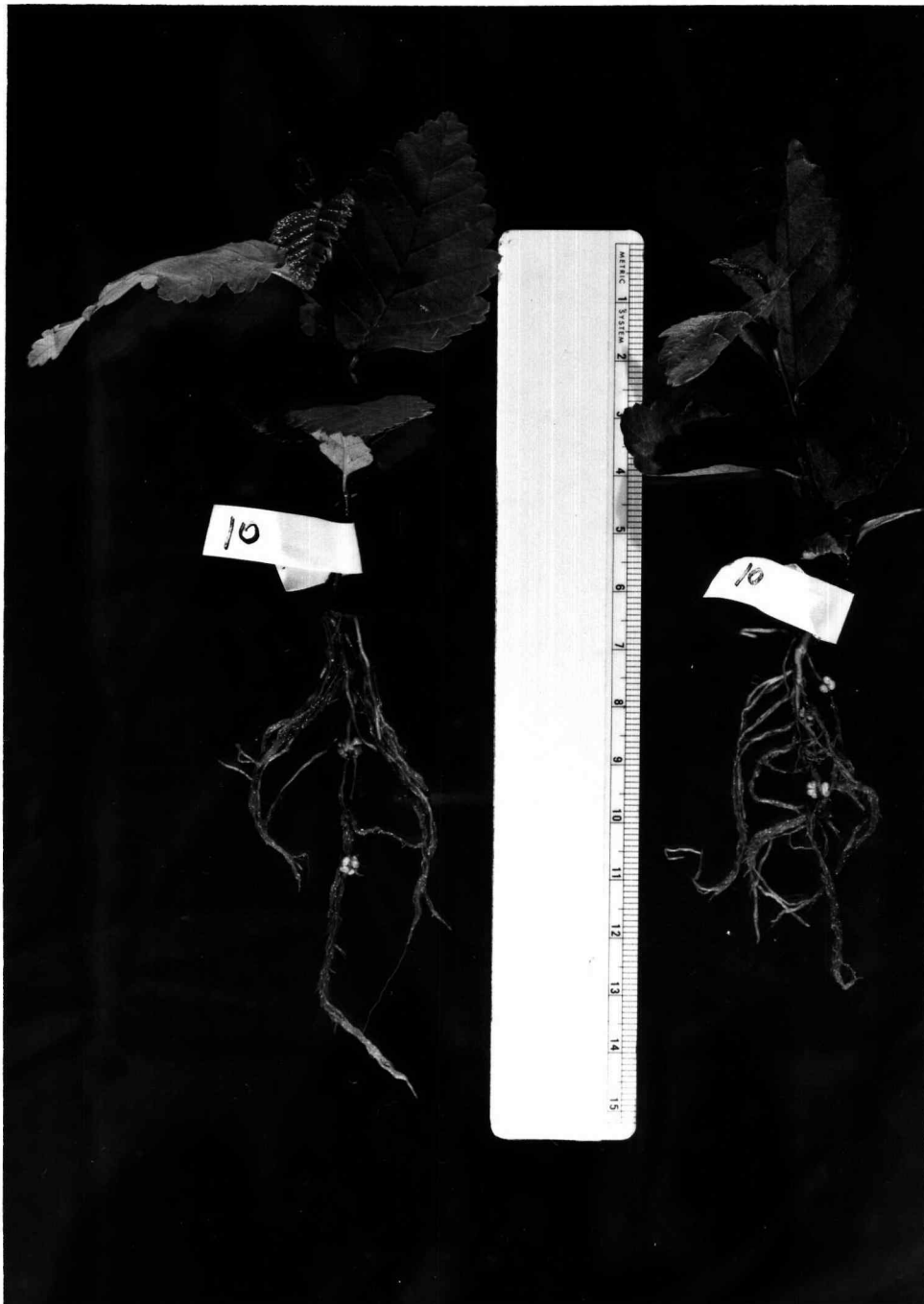


PLATE 6

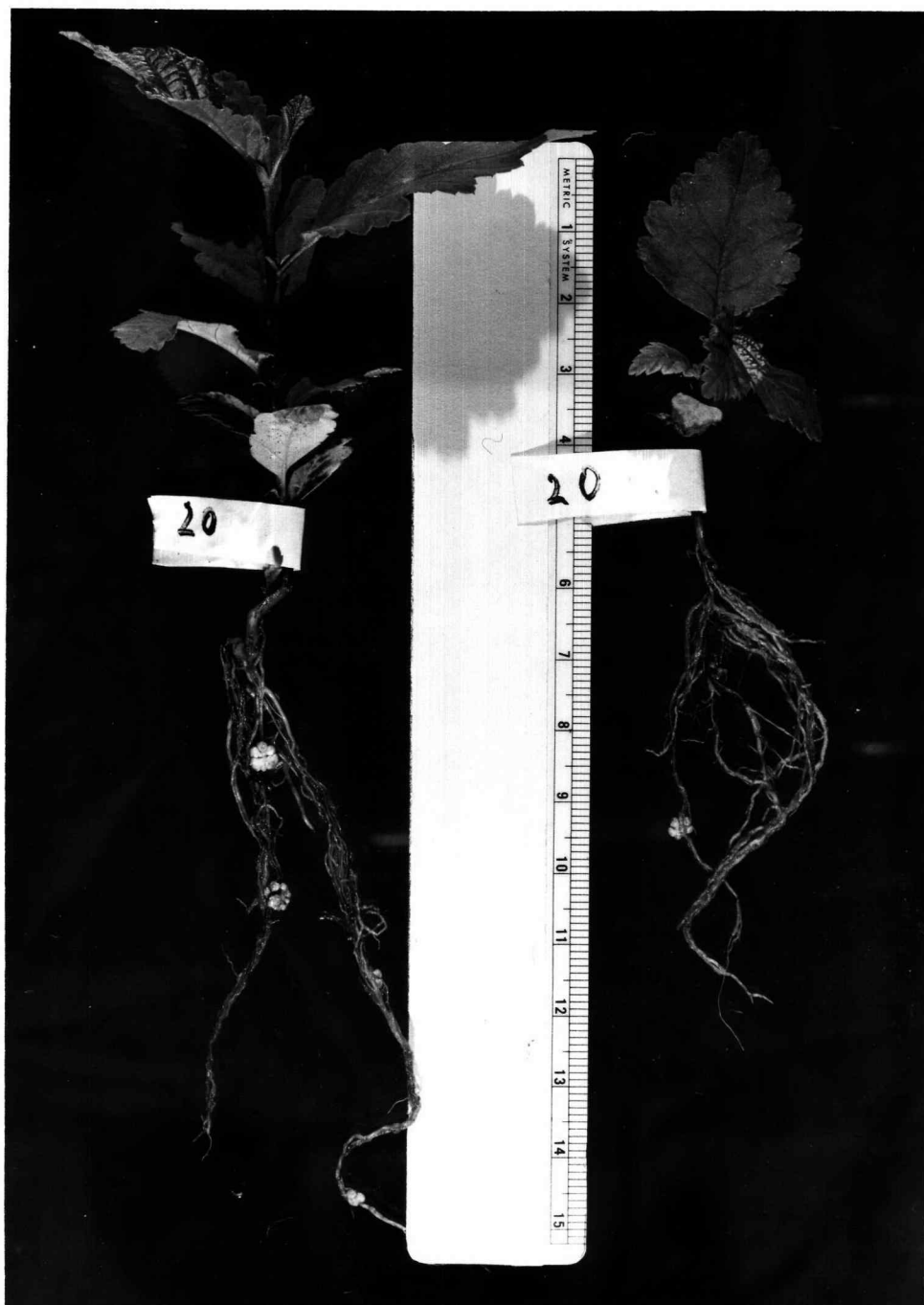


PLATE 7



PLATE 8

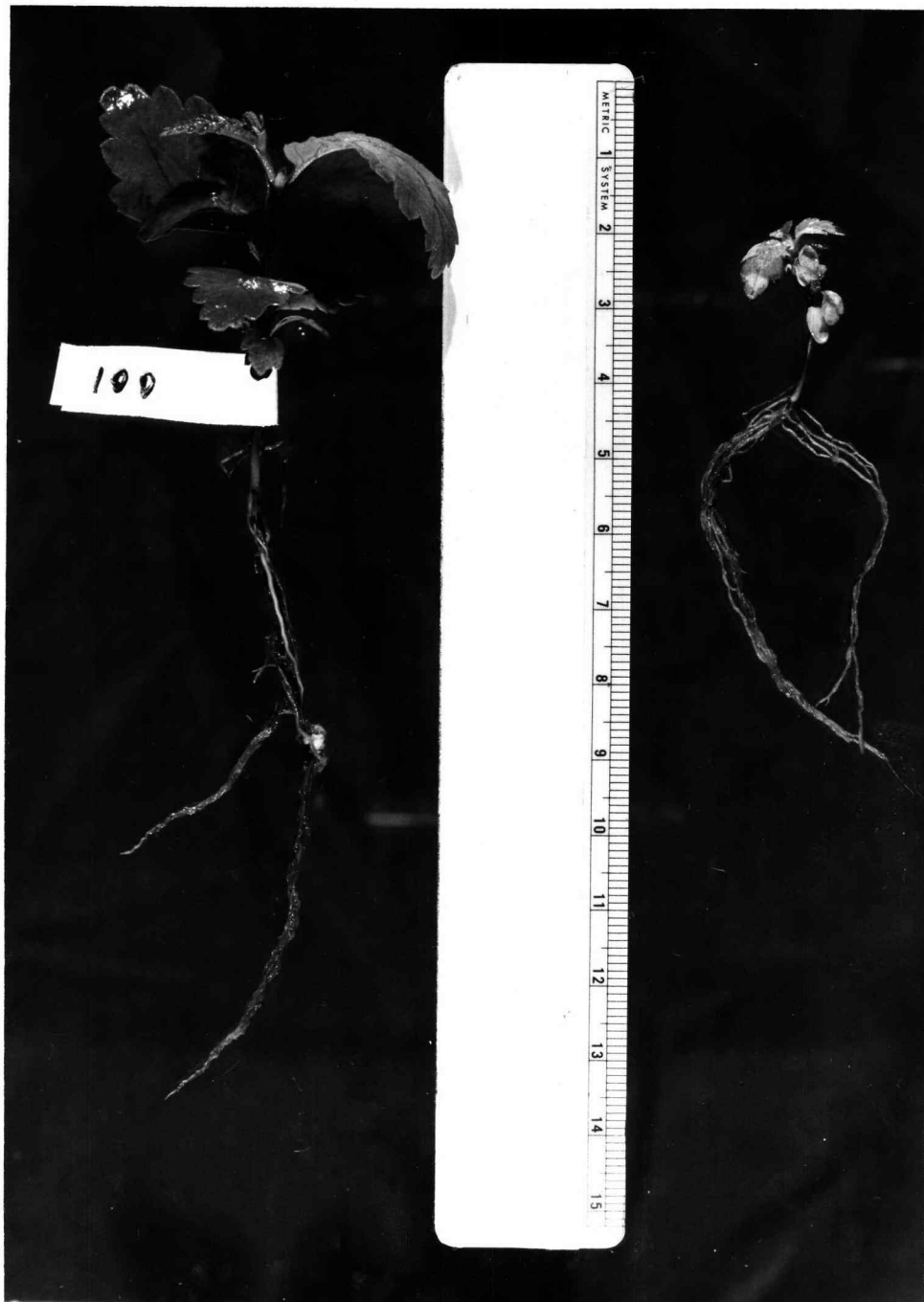


PLATE 9

Figure 32. Nitrogenase activity in the nodules of Alnus rubra following treatment with 0 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution for 11 weeks.

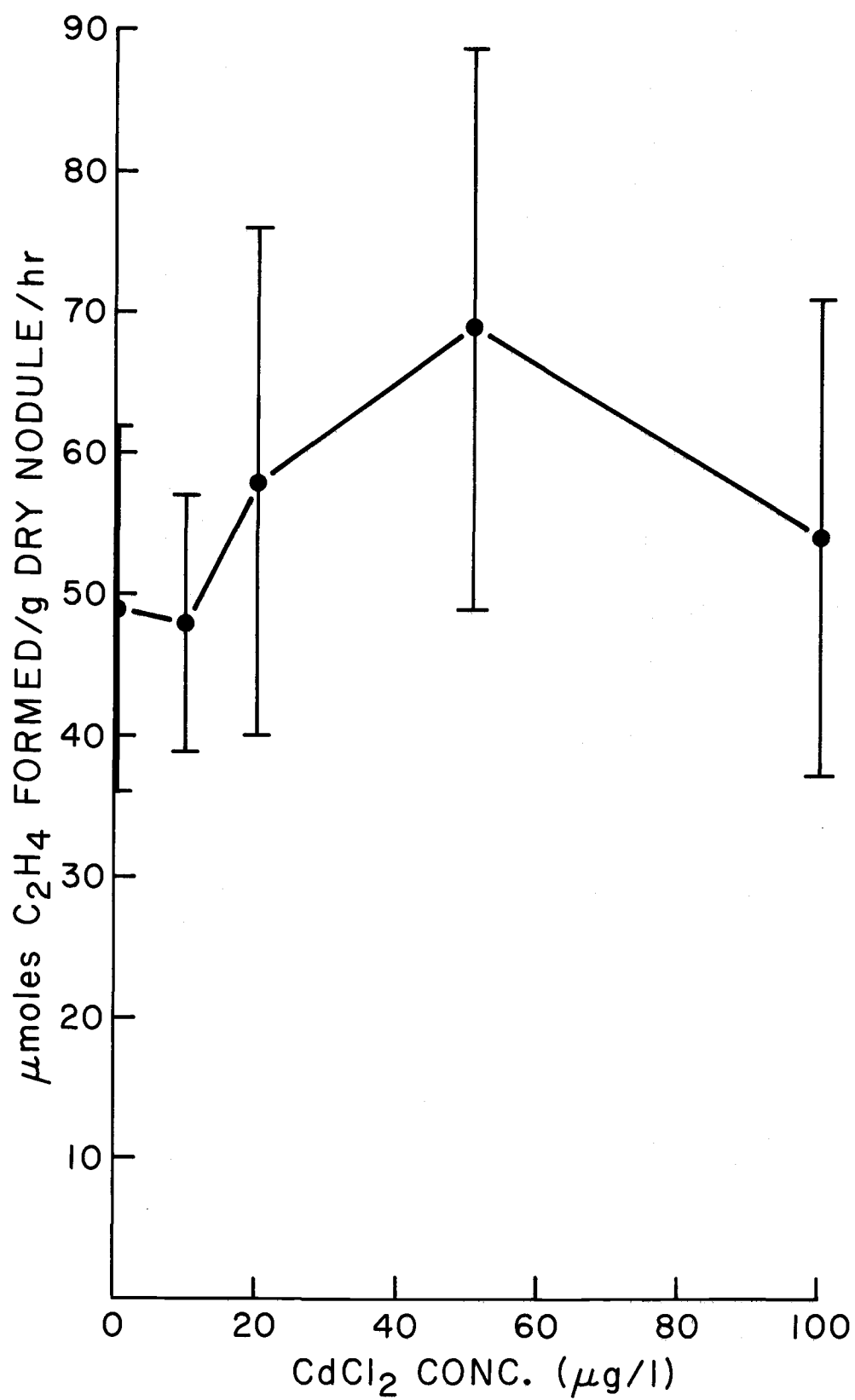


Figure 32

Total nitrogen and nitrogen fixation decreased as cadmium concentration increased (Fig. 33). One plant failed to nodulate at 50 μg per liter. Nitrogen fixation was below detectable range at these concentrations (Fig. 33).

Cadmium Absorption. Cadmium concentrations of the plant organs increased with increasing CdCl_2 content of the nutrient solution (Figs. 34 and 35). The roots accumulated greater amounts of the element than the leaves, stems, and nodules.

Effects of Cadmium on Plants Supplied with Fixed Nitrogen

Experiment 1. Initially Alnus rubra was cultured in nutrient solution containing 12 mM NH_4NO_3 . No nodules were apparent under these conditions. Nitrate reductase activity, expressed on a dry weight basis, was depressed at cadmium concentrations of 20 to 100 μg CdCl_2 per liter of nutrient solution (Fig. 36). Plant growth, total nitrogen, and nitrogen gain were not decreased, however, by the cadmium treatments (Figs. 37 and 38).

Experiment 2. Plants were cultured in nutrient solution containing 6 mM nitrogen as either ammonium or calcium nitrate (Table 1). Those plants supplied with ammonium nitrate were larger than those that received calcium nitrate (Plate 10). Nodules did not occur on the roots of either group of plants (Plates 11-14). The dry weights of the plant organs decreased with increasing cadmium concentration and

Figure 33. Total nitrogen and nitrogen fixation in Alnus rubra allowed to initiate nodulation in the presence of cadmium. Fixed nitrogen = final total nitrogen - initial total nitrogen.

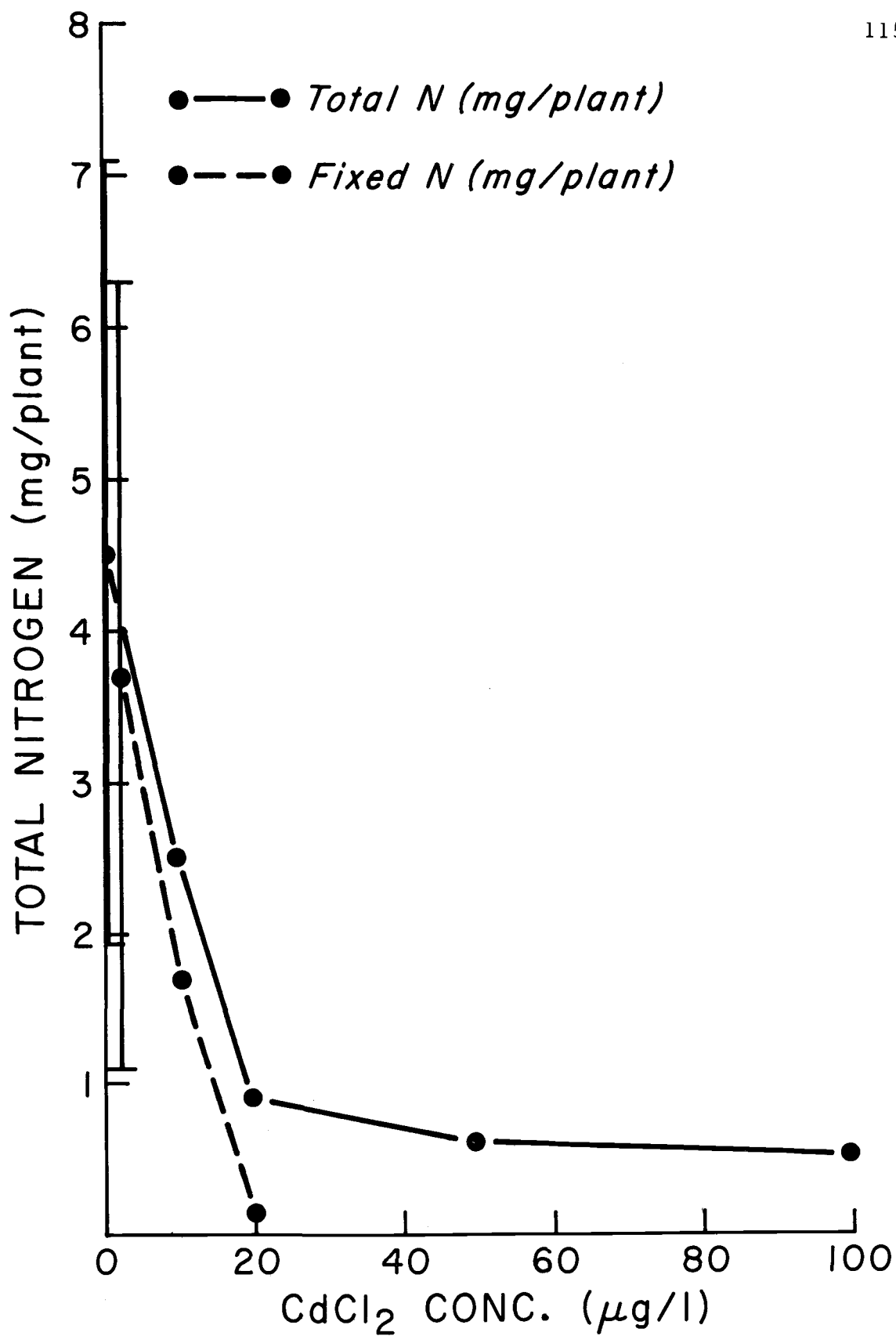


Figure 33

Figure 34. Cadmium concentration in the leaves and stems of Alnus rubra following nodulation in the presence of 0 to 100 μg CdCl_2 per liter of nutrient solution for 11 weeks.

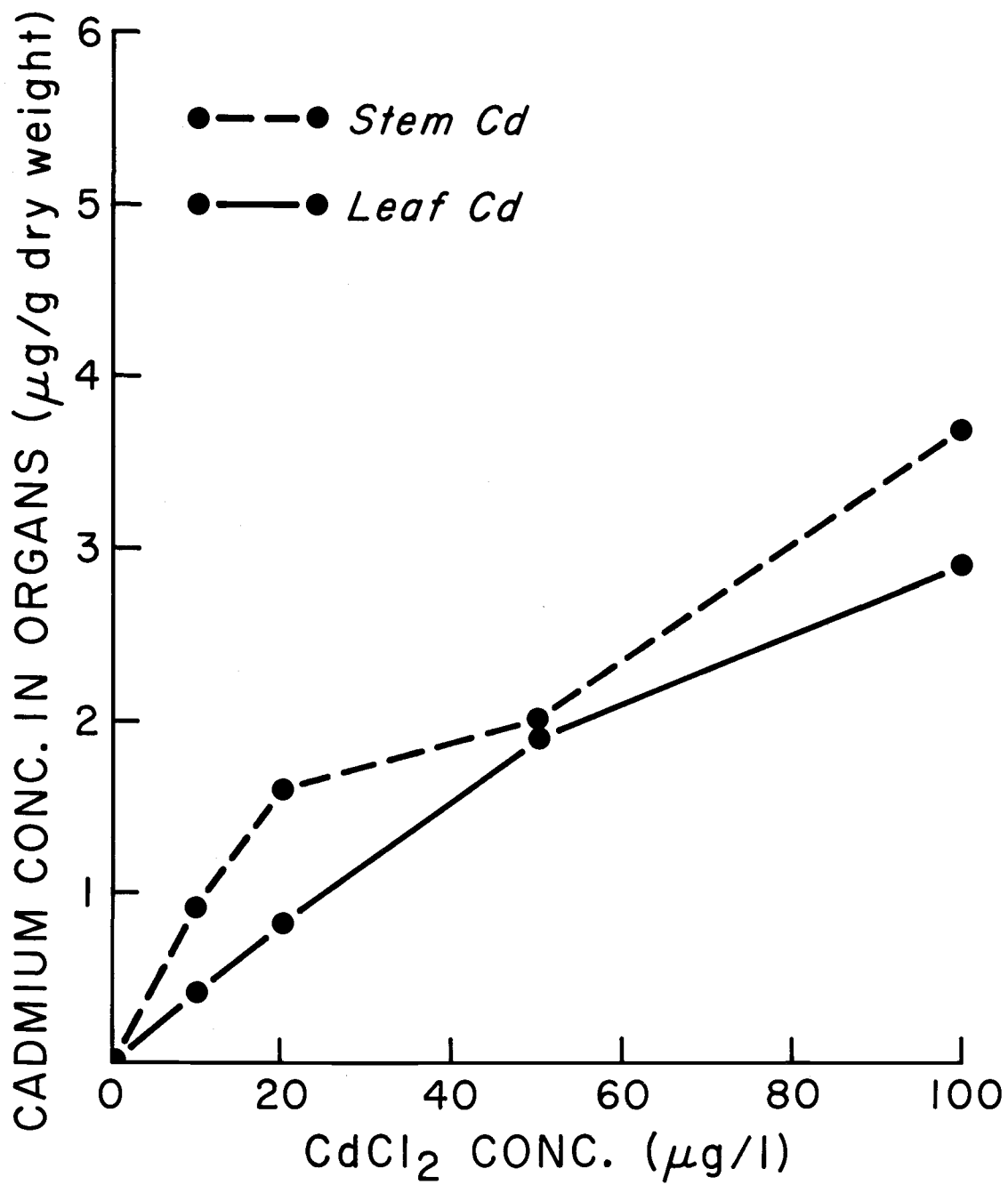


Figure 34

Figure 35. Cadmium concentration in the roots and nodules of Alnus rubra following nodulation in the presence of 0 to 100 μg CdCl_2 per liter of nutrient solution given 11 weeks.

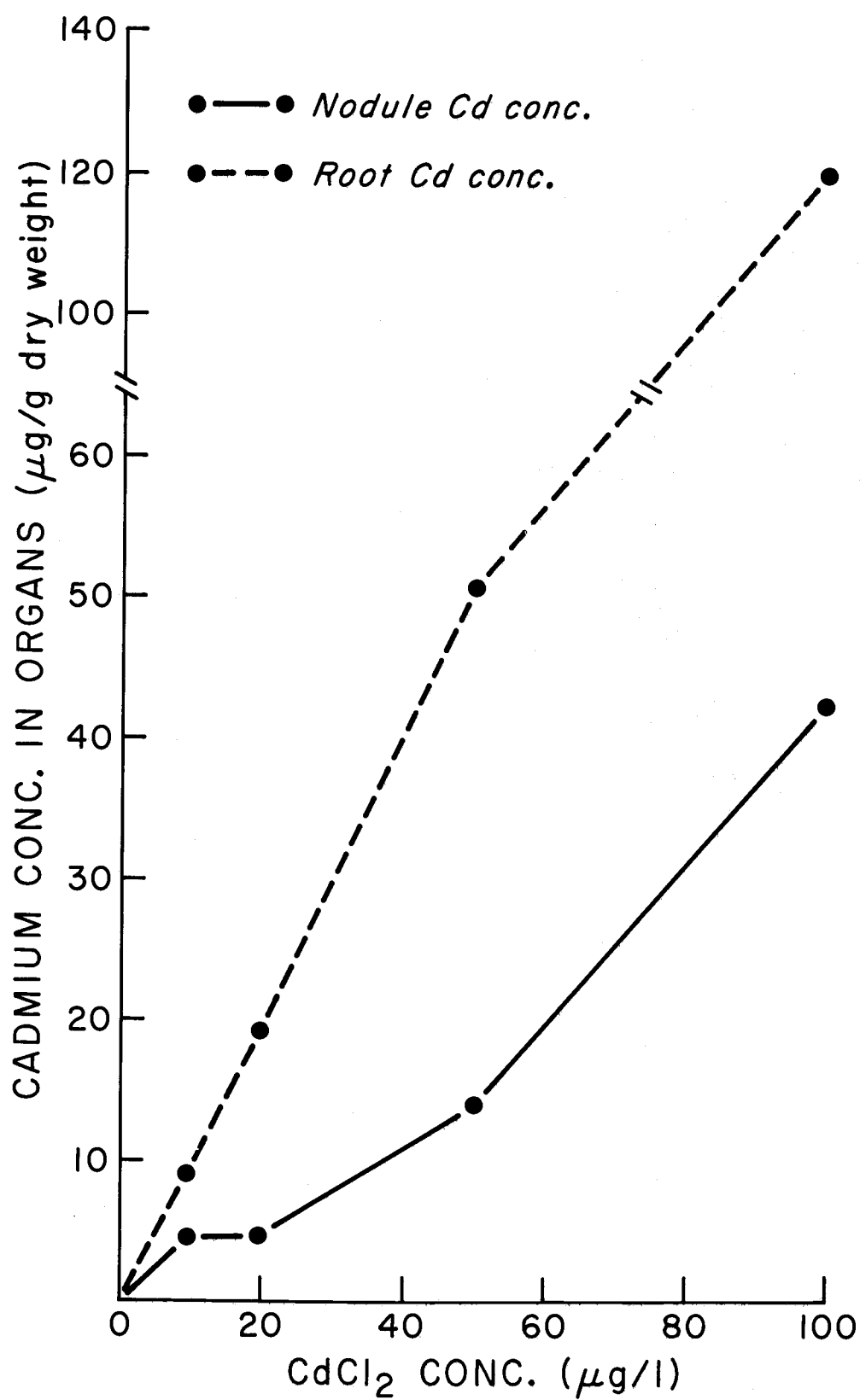


Figure 35

Figure 36. Nitrate reductase activity in the roots of Alnus rubra following treatment with 0 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution containing NH_4NO_3 for 11 weeks.

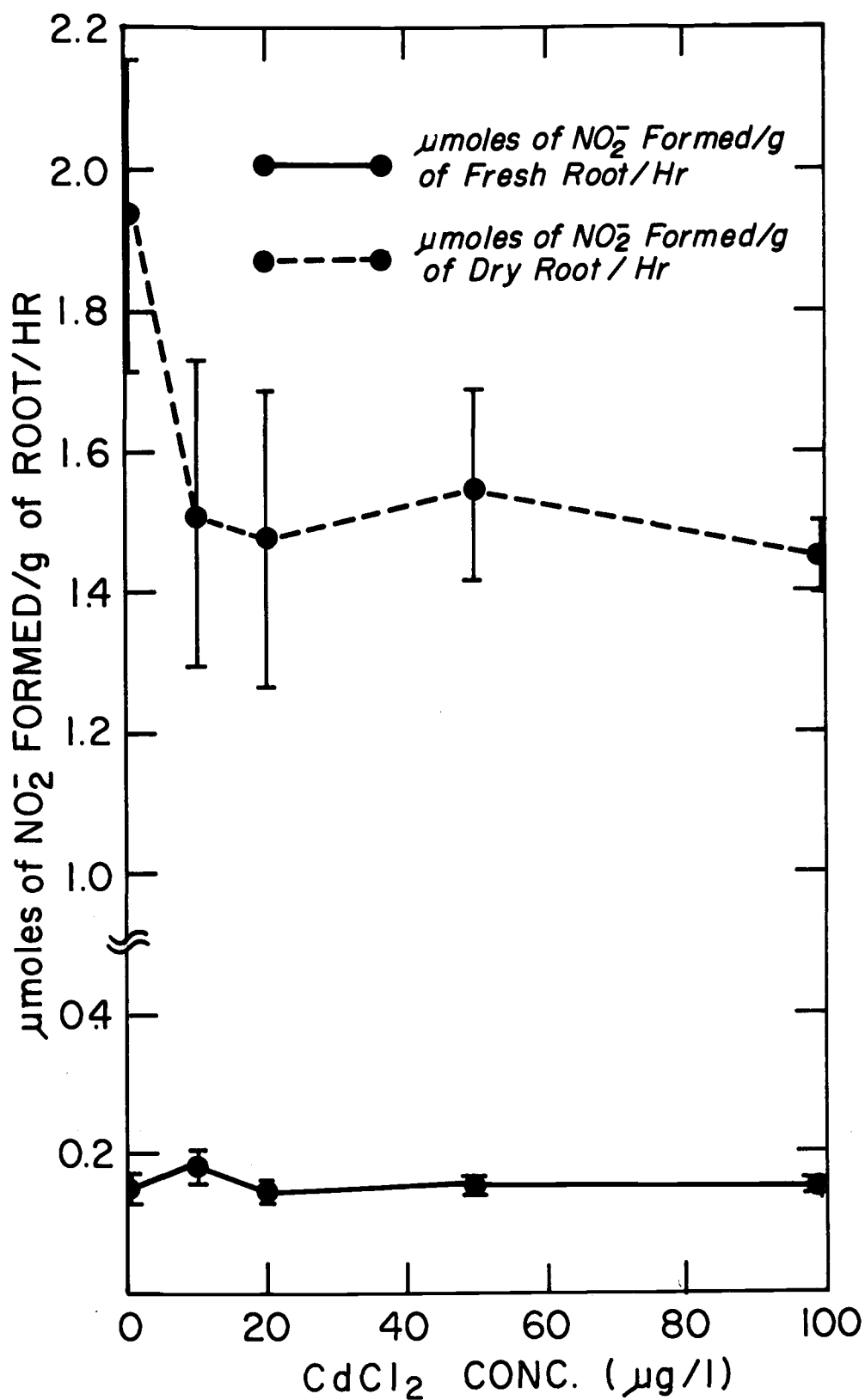


Figure 36

Figure 37. Dry weights of the organs of Alnus rubra following treatment with 0 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution containing NH_4NO_3 for 11 weeks.

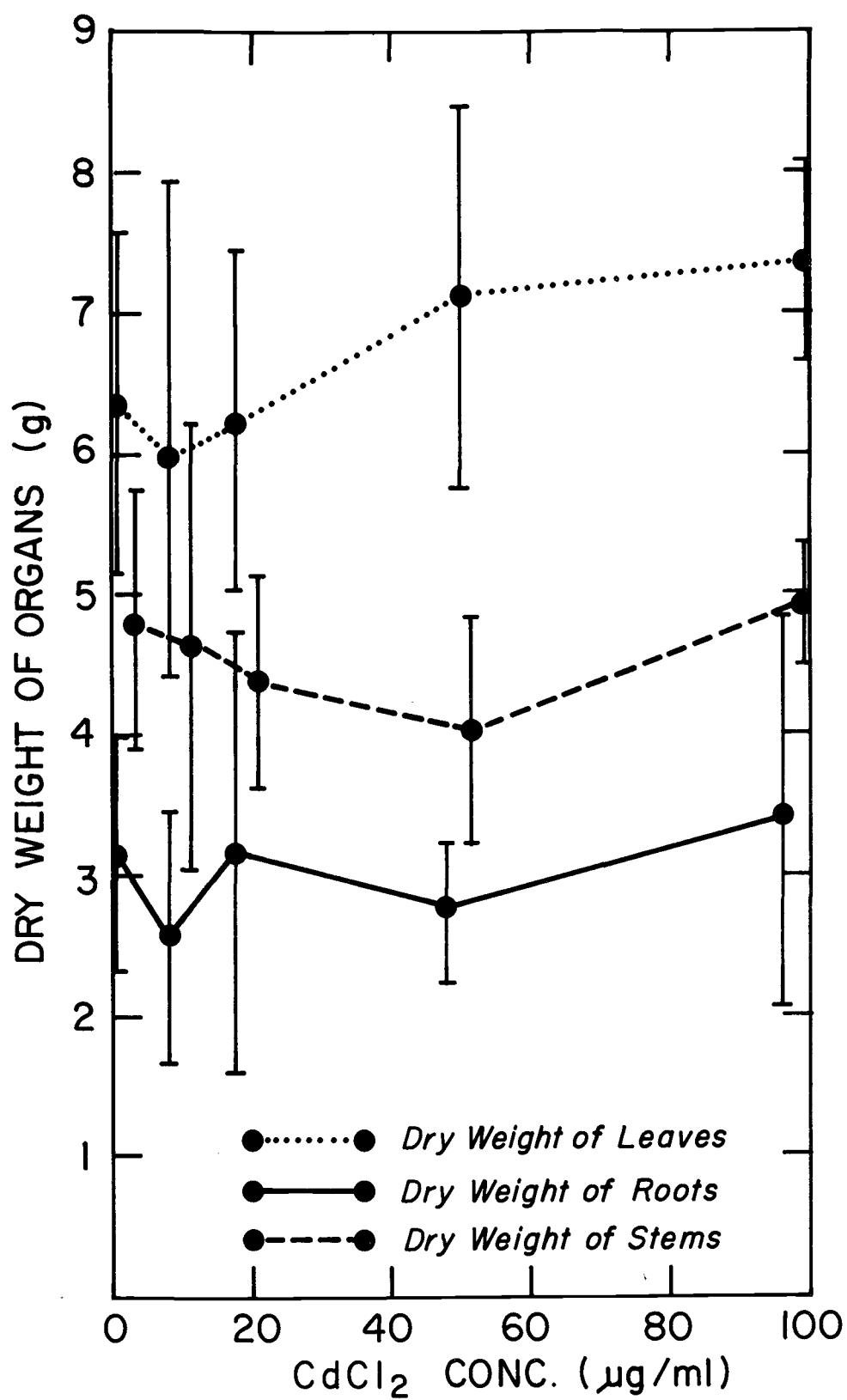


Figure 37

Figure 38. Total nitrogen and nitrogen gain in Alnus rubra following treatment with 0 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution containing NH_4NO_3 for 11 weeks. Nitrogen gain = final total nitrogen - initial total nitrogen.

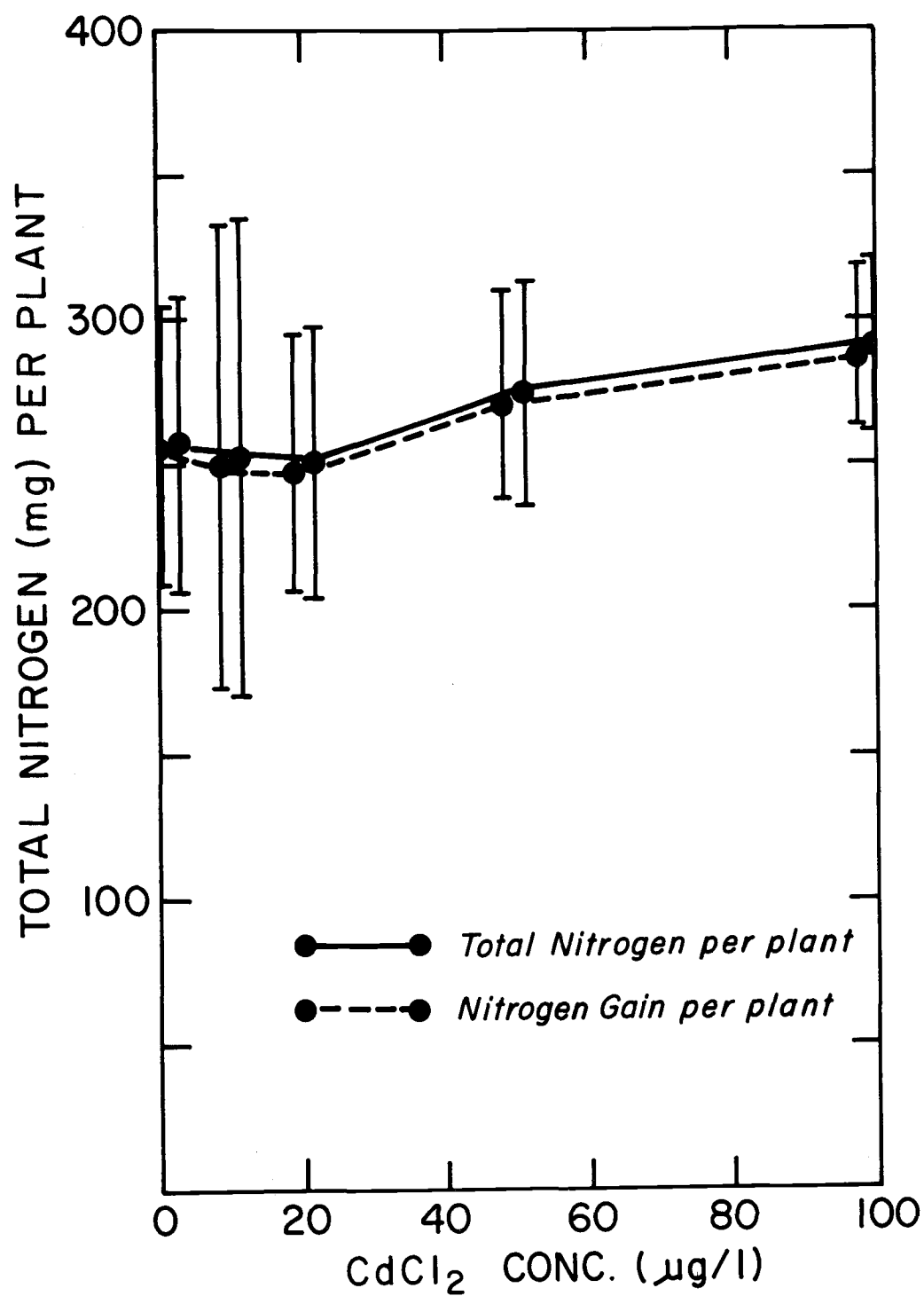


Figure 38

Plate 10. Effect of cadmium on the growth of plants supplied with fixed nitrogen. The plants on the left were supplied with 6 mM NH_4NO_3 as a source of reduced nitrogen. Those on the right were supplied with 6 mM $\text{Ca}(\text{NO}_3)_2$. The second and fourth plants from left were given 20 μg CdCl_2 per liter of nutrient solution. PPB = μg per liter.



PLATE 10

Plates 11 and 12. The roots of plants supplied with 6 mM NH_4NO_3 as a source of reduced nitrogen. There was more secondary root growth on these roots than on those supplied with 6 mM $\text{Ca}(\text{NO}_3)_2$ (Compare with plates 13 and 14).



PLATE 11

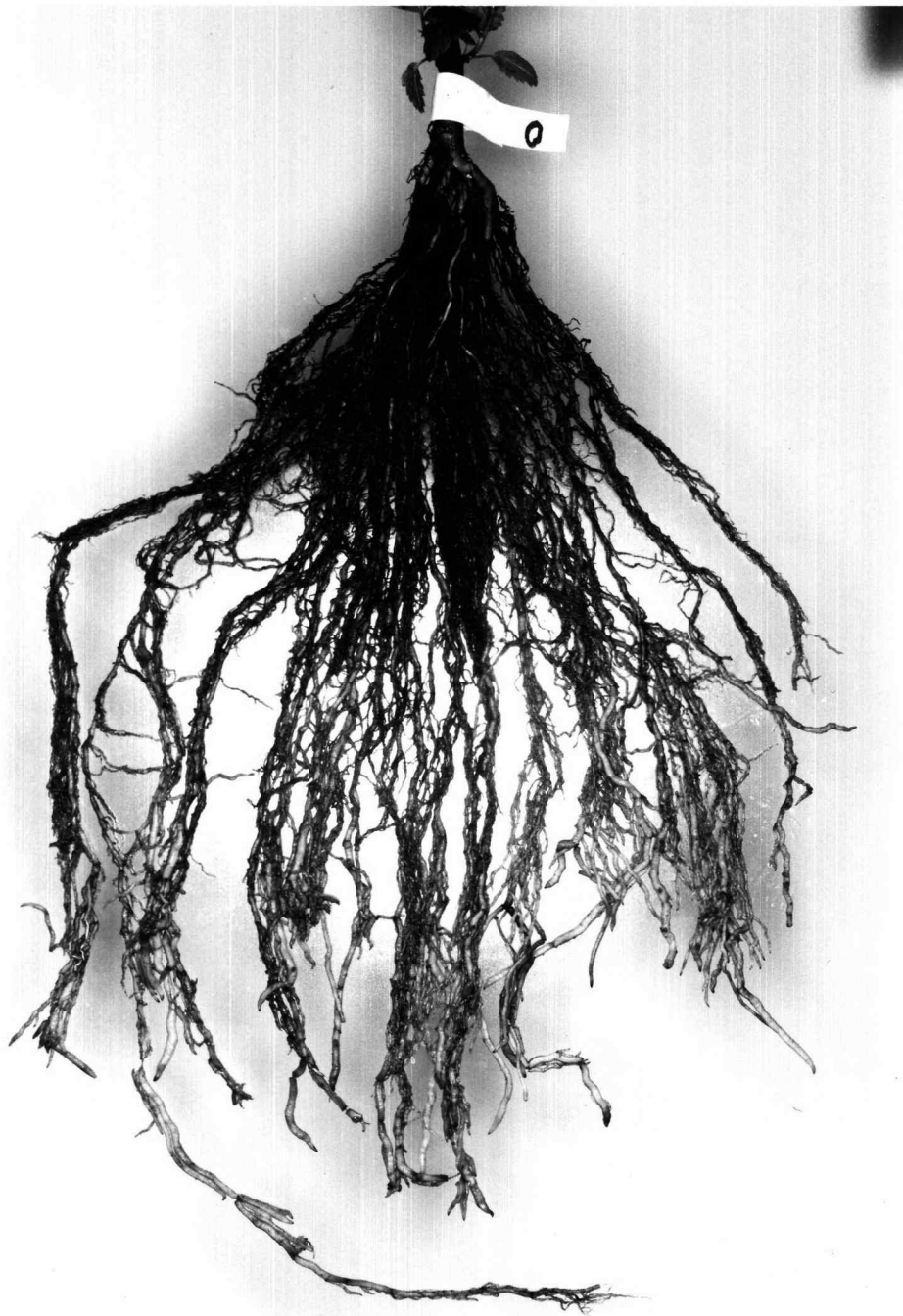


PLATE 12

Plates 13 and 14. The roots of plants supplied with 6 mM $\text{Ca}(\text{NO}_3)_2$ as a source of reduced nitrogen. There was less secondary root growth on these roots than on those supplied with 6 mM NH_4NO_3 (Compare with plates 11 and 12).

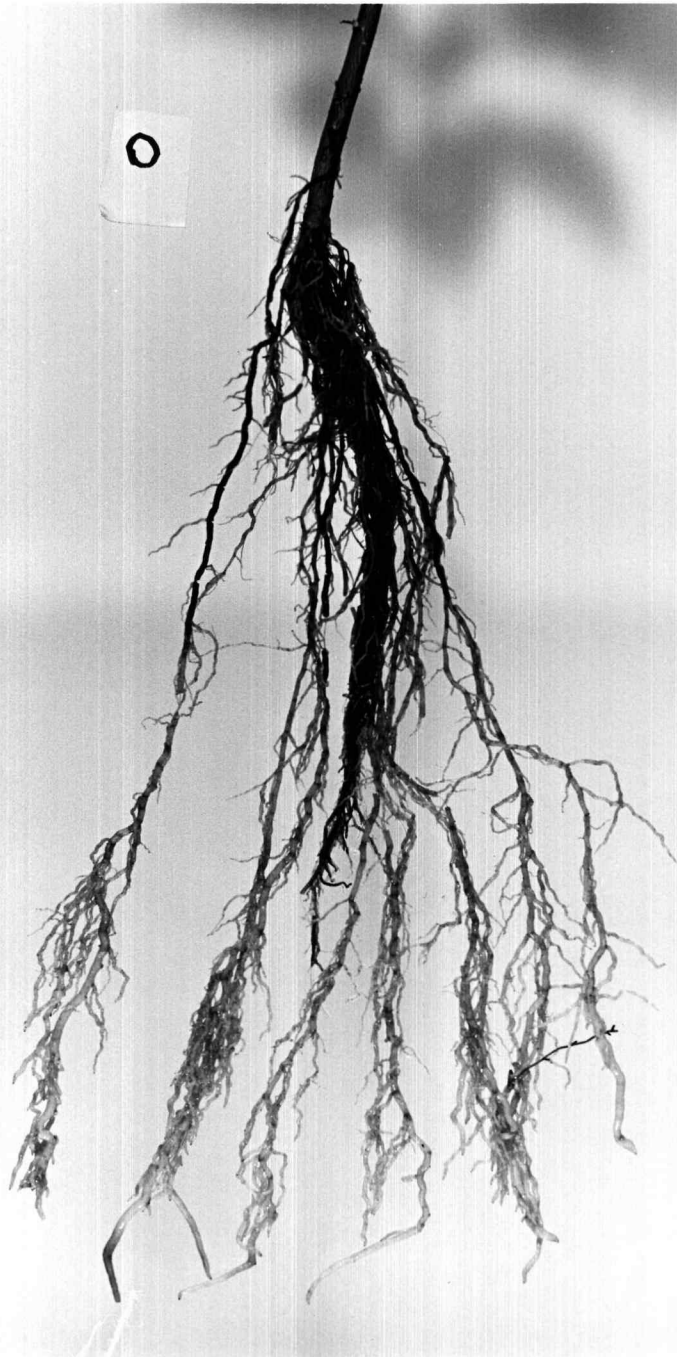


PLATE 13

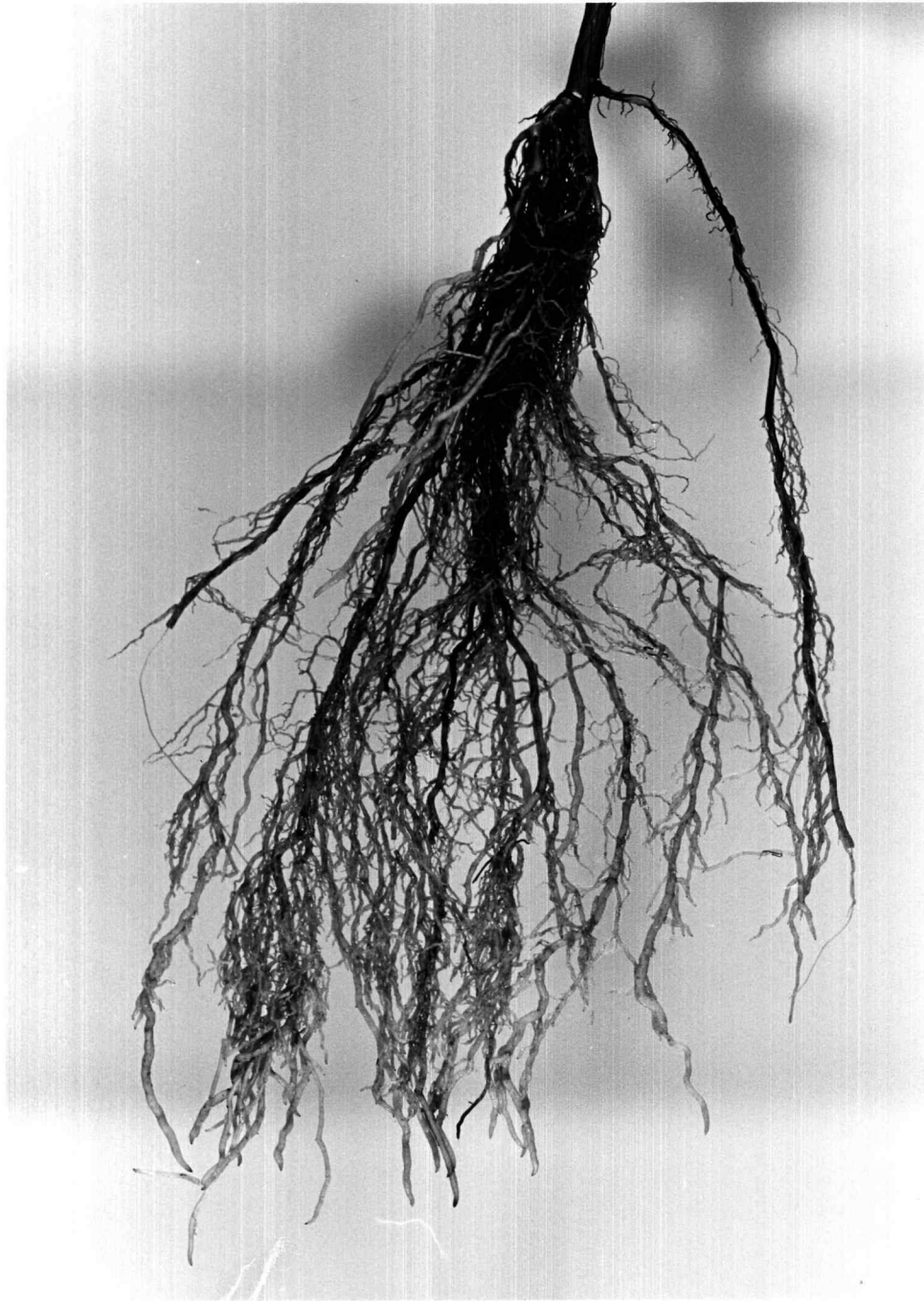


PLATE 14

the decreases were significant at all concentrations investigated when calcium nitrate was the nitrogen source (Fig. 39). There were significant decreases in the dry weights at 50 and 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution when ammonium nitrate was the nitrogen source although these decreases were not visible during the experiment (Fig. 40).

The nitrate reductase activities in the leaves and roots of plants given 100 $\mu\text{g CdCl}_2$ per liter were significantly decreased regardless of whether ammonium or calcium nitrate was the fixed nitrogen source (Fig. 41). The nitrogen gained during the growth period also was significantly decreased at all cadmium concentrations when calcium nitrate was the nitrogen source (Fig. 42). Decreases occurred at 50 and 100 $\mu\text{g CdCl}_2$ per liter when ammonium nitrate was the nitrogen source.

The cadmium concentrations in the plant organs increased as the concentration of CdCl_2 in the nutrient solution increased (Figs. 43 and 44). The roots accumulated more cadmium than the leaves and stems.

Effects of Cadmium on Nodule and Root Ultrastructure

Nodule Ultrastructure. The ultrastructure of nodules from control plants without cadmium are shown in Plates 15-19. The hyphal and vesicular endophyte forms were surrounded by a cell wall

Figure 39. Dry weights of the organs of Alnus rubra following treatment with 0 to 100 μg CdCl_2 per liter of nutrient solution containing $\text{Ca}(\text{NO}_3)_2$ as the nitrogen source for 11 weeks.

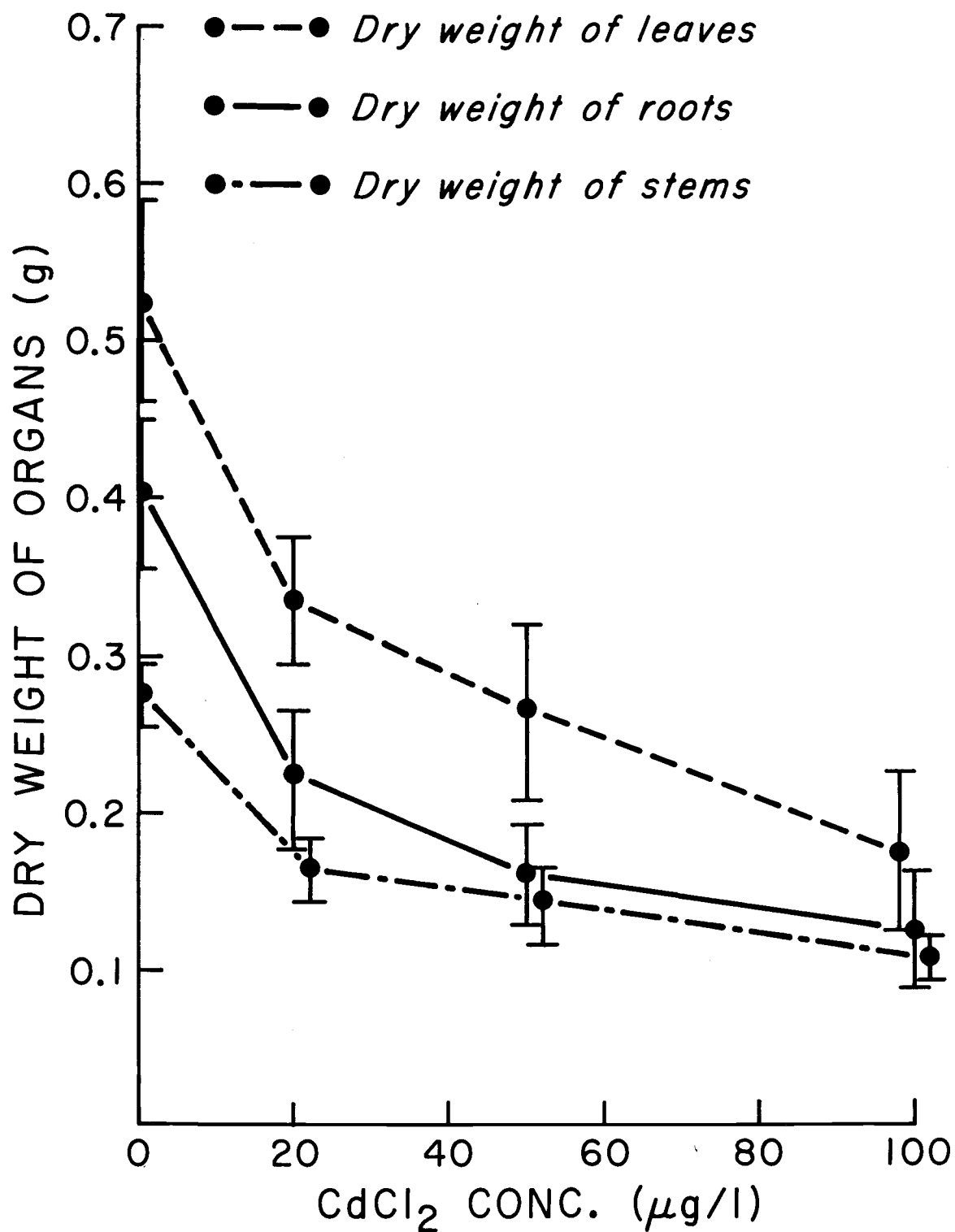


Figure 39

Figure 40. Dry weights of the organs of Alnus rubra following treatment with 0 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution containing NH_4NO_3 for 11 weeks.

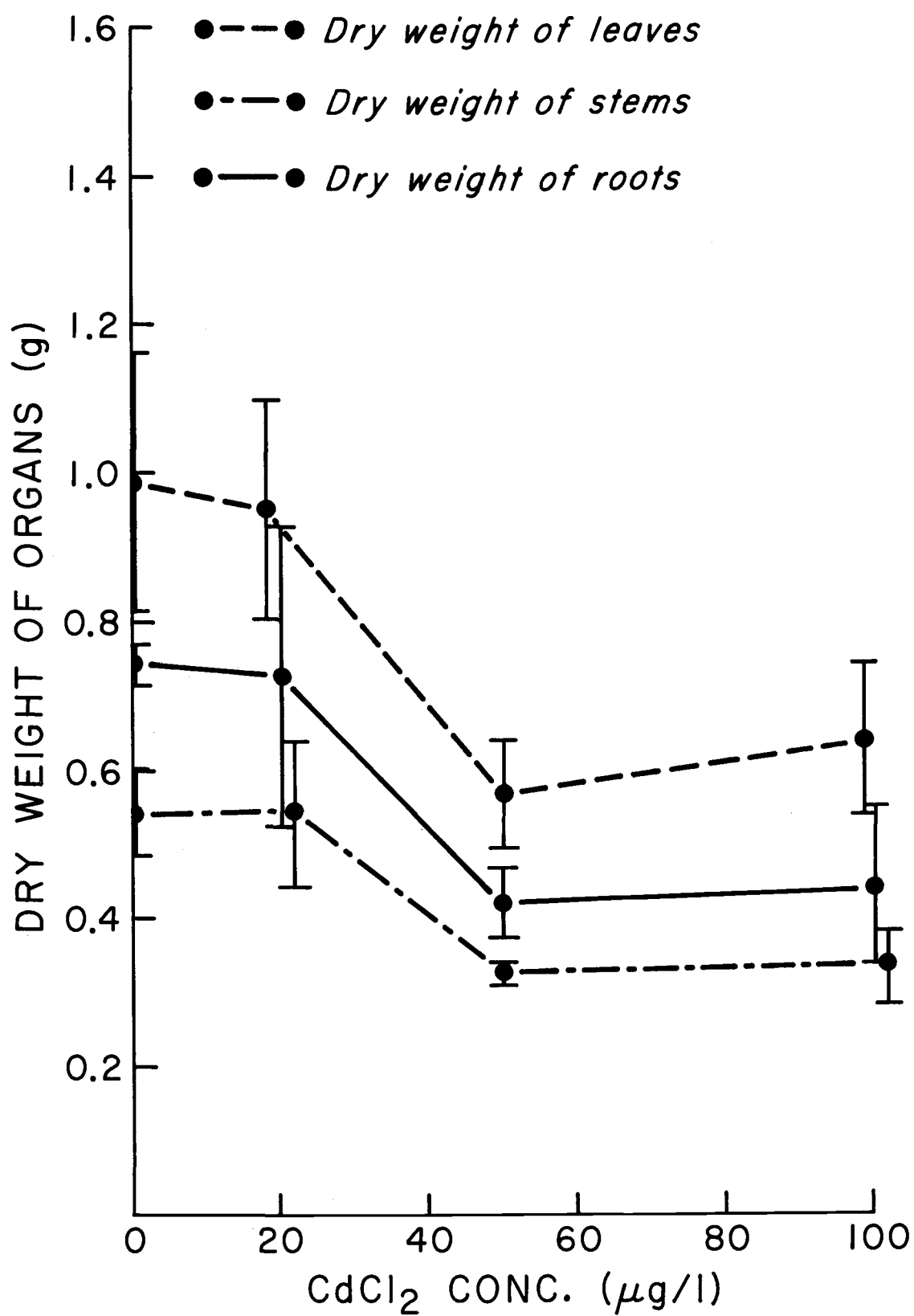


Figure 40

Figure 41. Nitrate reductase activity in Alnus rubra following treatment with 0 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution containing either NH_4NO_3 or $\text{Ca}(\text{NO}_3)_2$ for 11 weeks. NRA = nitrate reductase activity.

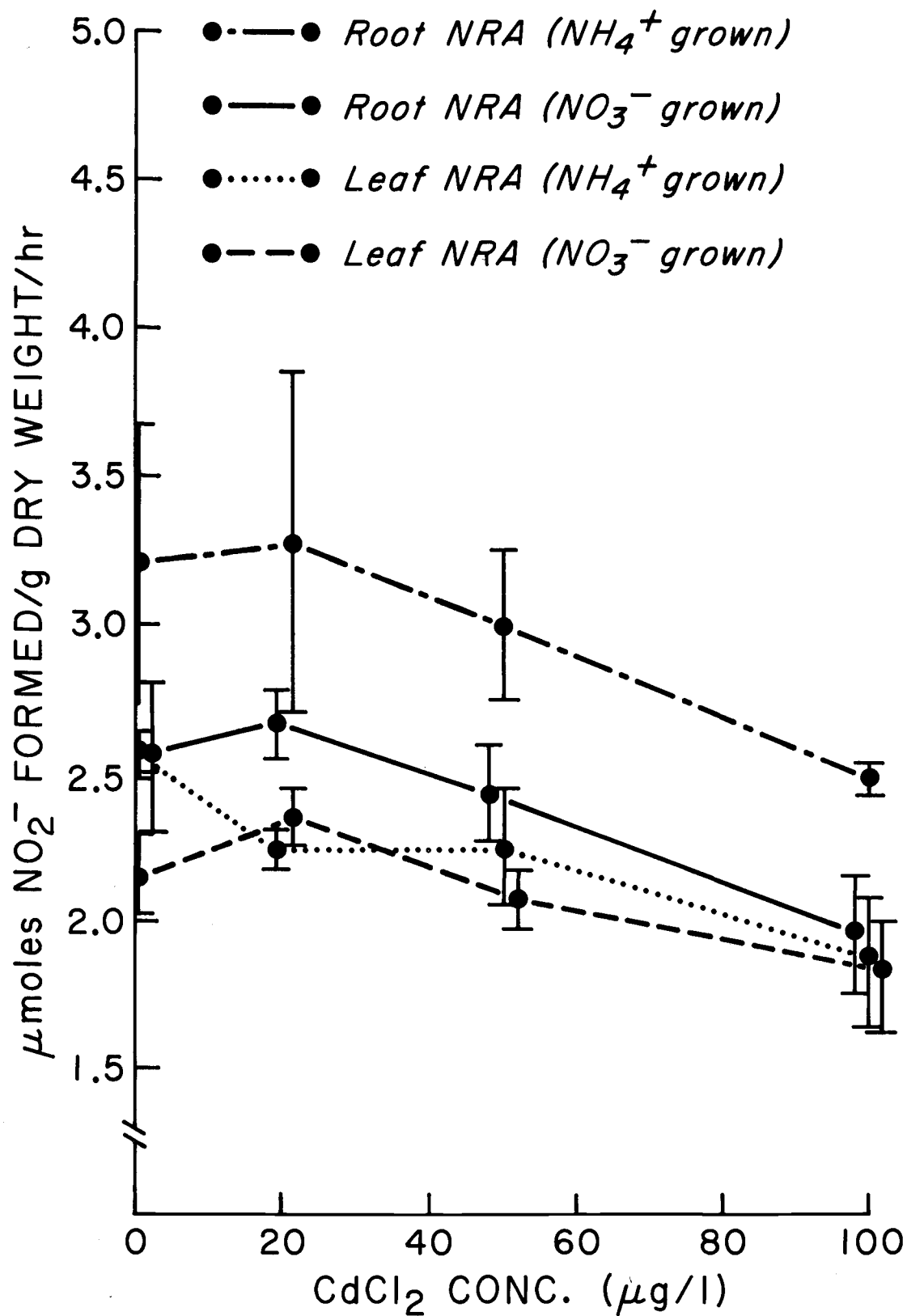


Figure 41

Figure 42. Total nitrogen and nitrogen gain in Alnus rubra following treatment with 0 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution containing 6 mM ammonium or calcium nitrate for 11 weeks. Nitrogen gain = final total nitrogen - initial total nitrogen.

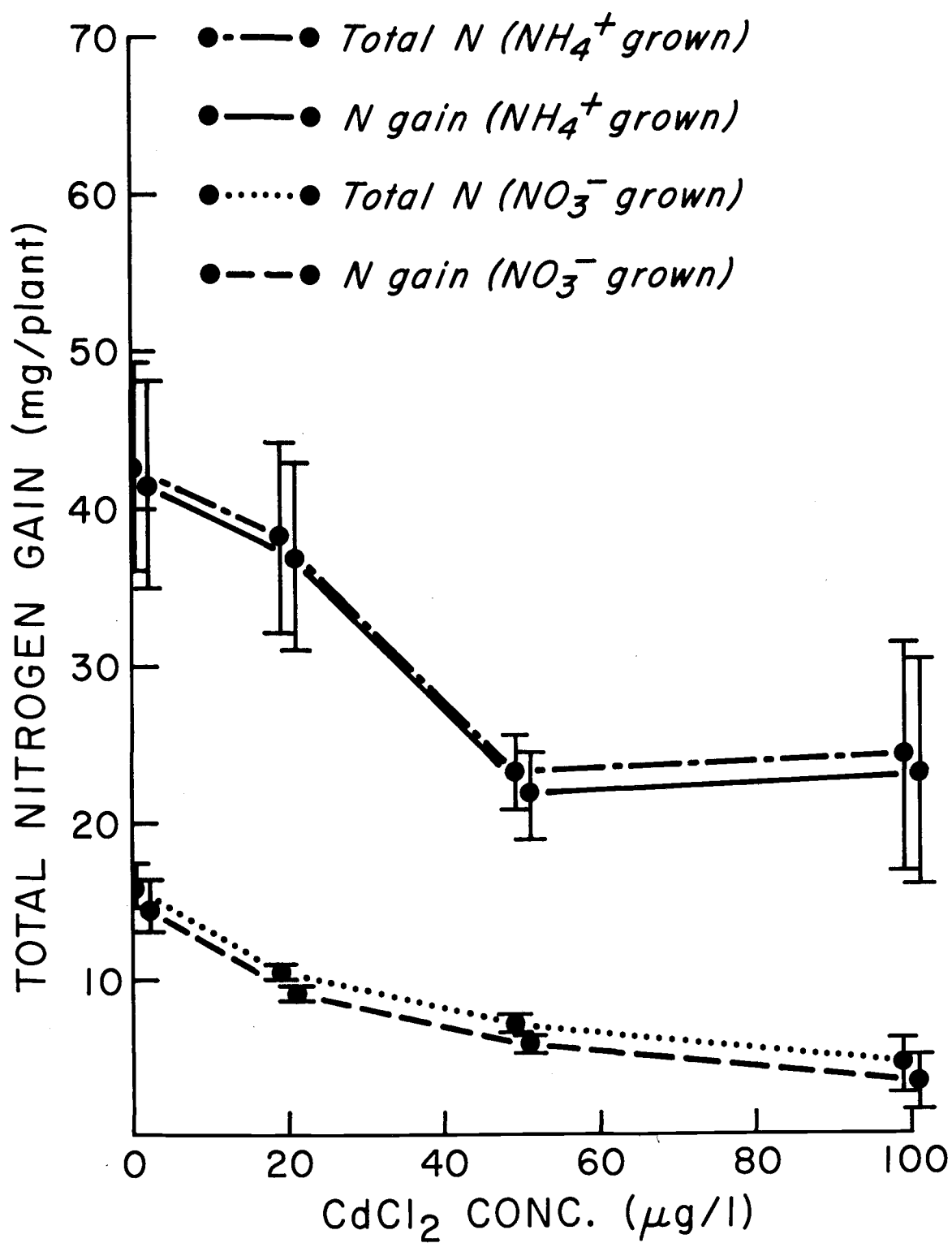


Figure 42

Figure 43. Cadmium concentration in the leaves and stems of Alnus rubra following treatment with 0 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution containing 6 mM ammonium or calcium nitrate for 11 weeks.

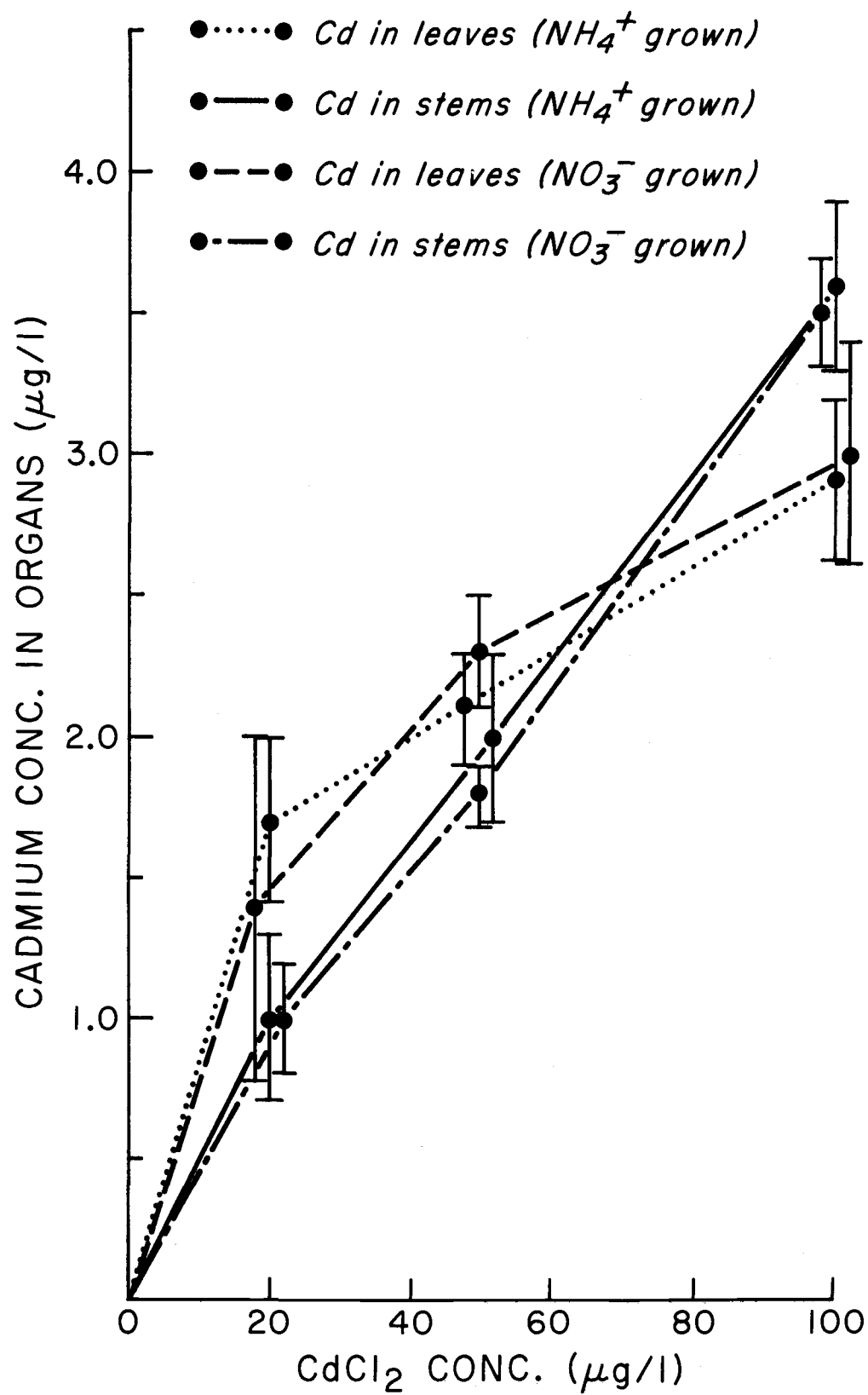


Figure 43

Figure 44. Cadmium concentration in the roots of Alnus rubra following treatment with 0 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution containing 6 mM ammonium or calcium nitrate for 11 weeks.

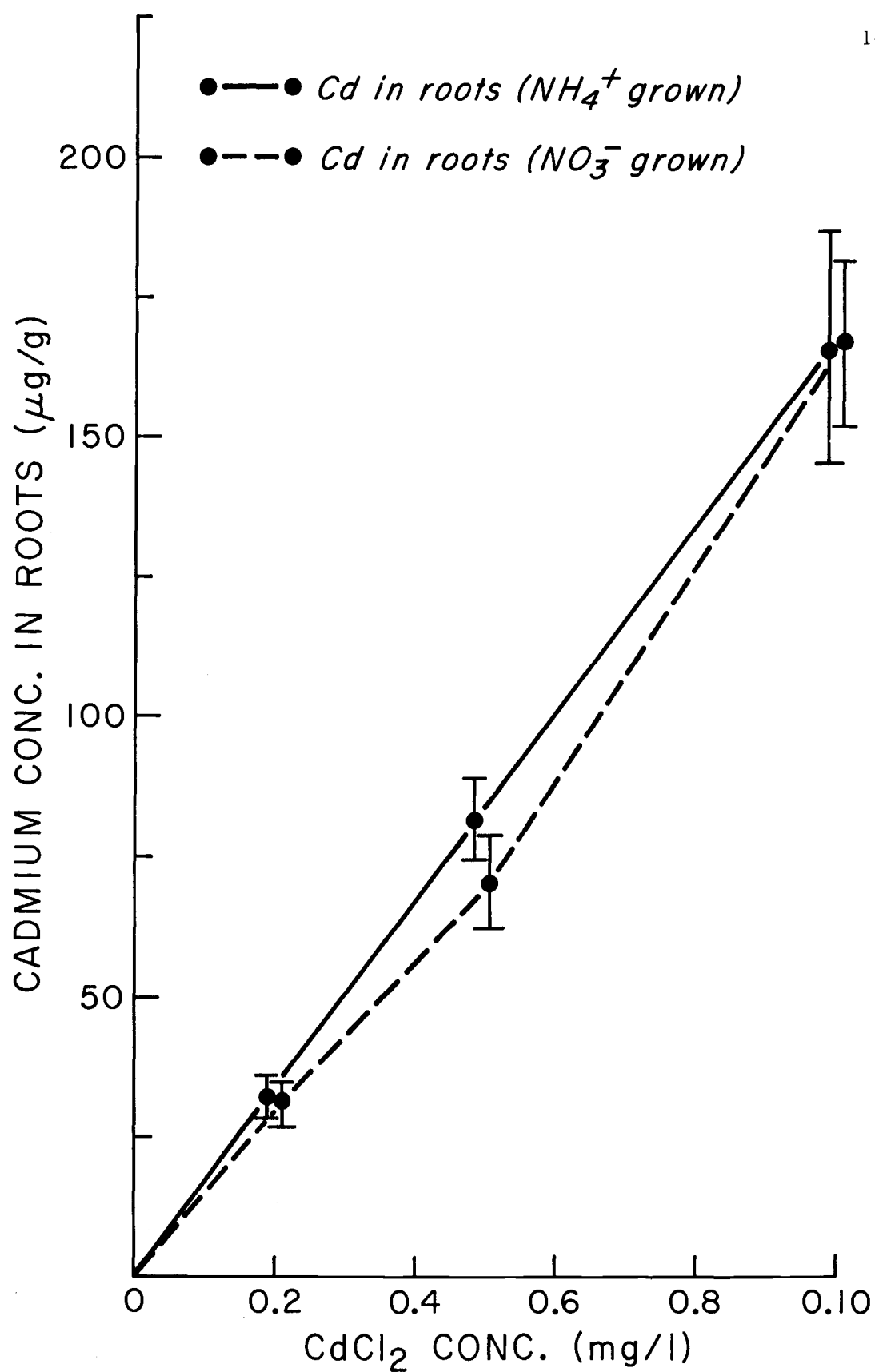


Figure 44

Plate 15. Endophyte vesicle (v) and hyphae (h) from the nodules of control plants given no cadmium. Structures labeled "unk" were not observed by Lalonde and Knowles (26) and are unknown. Magnification 8,000 X.

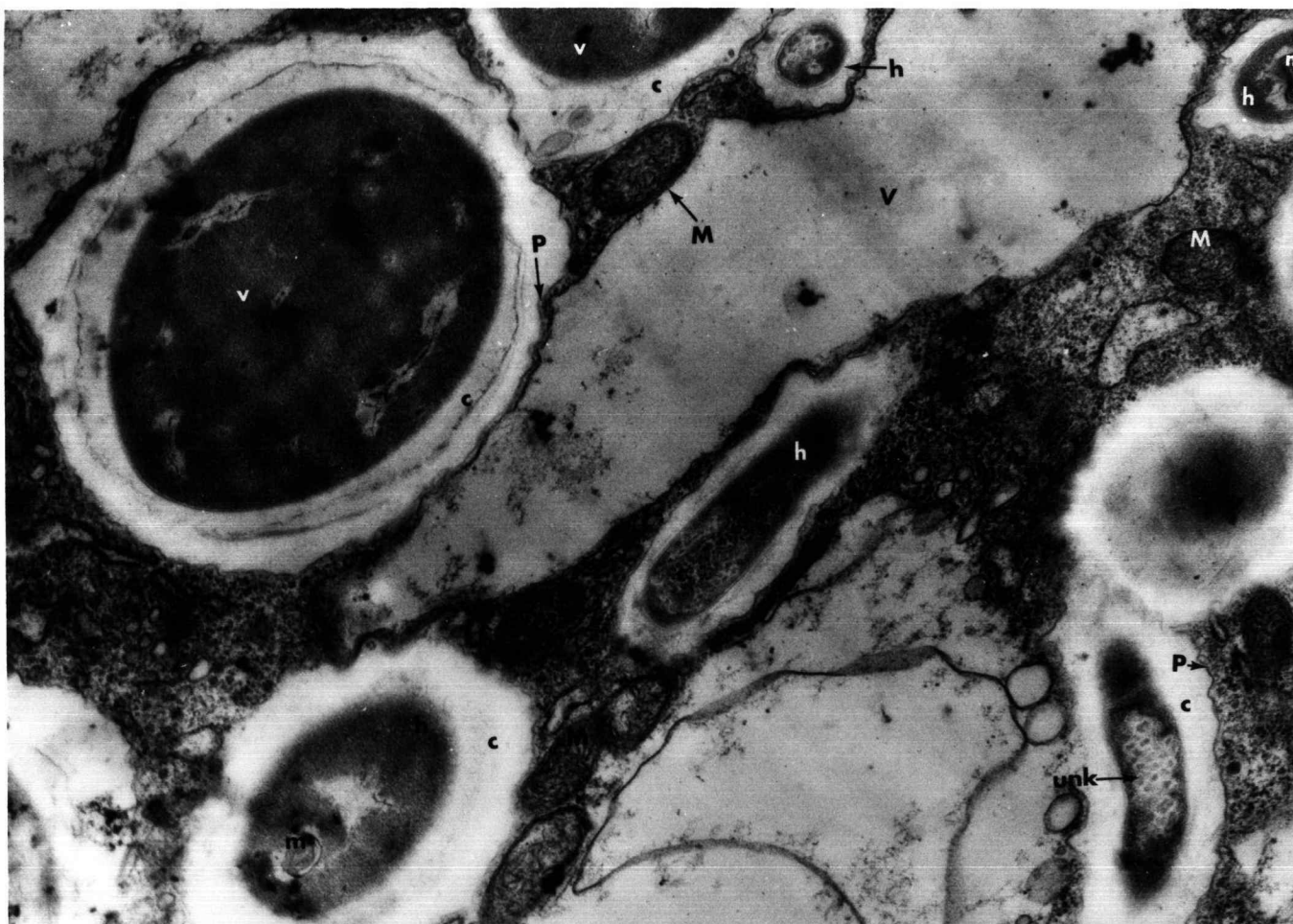


PLATE 15

Plate 16. Endophyte vesicle (v) continuous with hypha (h). The specimen was taken from the nodules of control plant given no cadmium. Magnification 15,000 X.

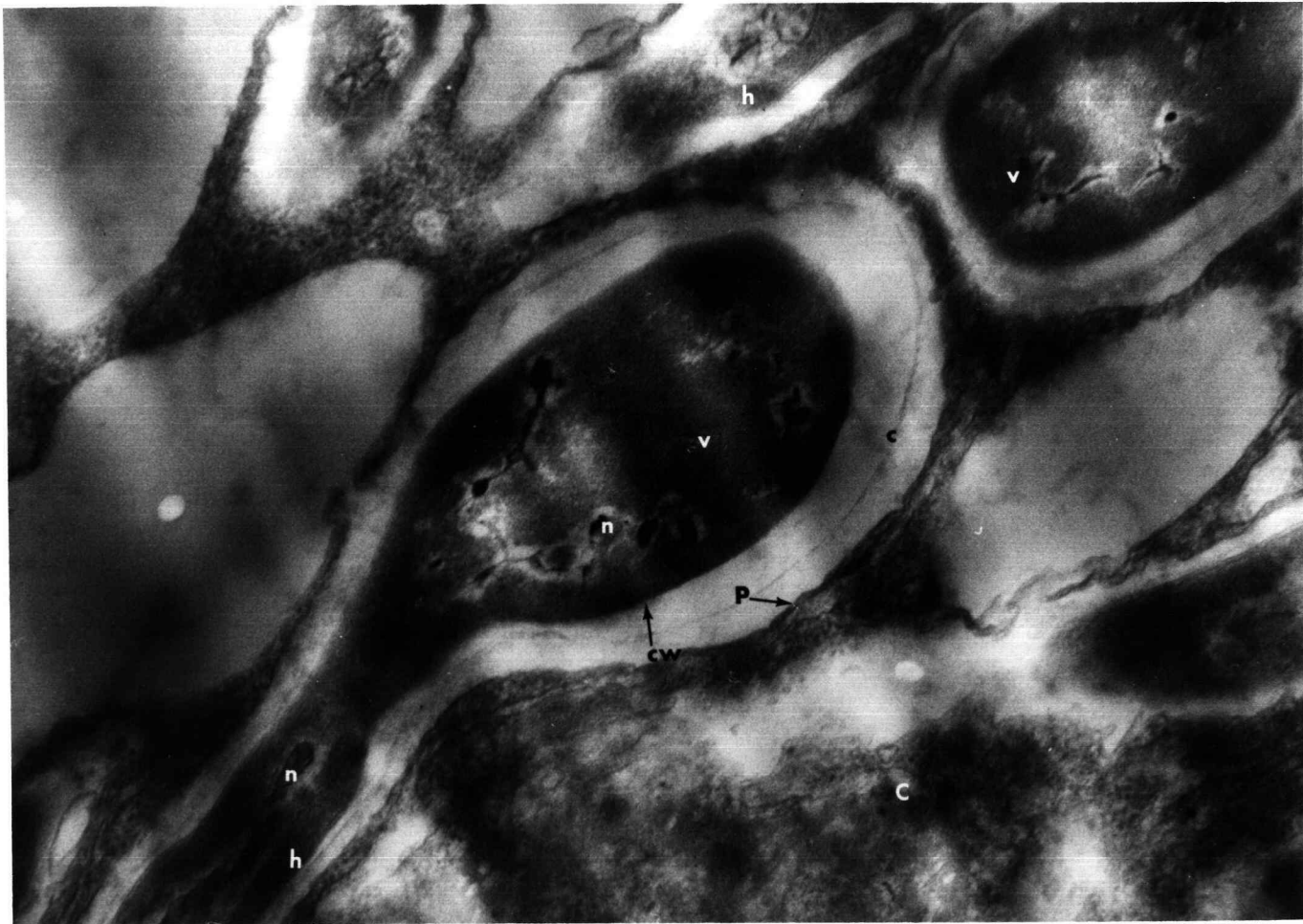


PLATE 16

and enclosed in a capsular material surrounded by the plasmalemma (Plates 17 and 18). Fine threads of what appear to be nucleic acid polymers attached to dense bodies were seen inside the endophyte (Plates 15 and 17). The hyphae were divided across their widths by septa which were also seen in some vesicles (Plates 16 and 19). Branched hyphae penetrated the cell wall, and grew intercellularly (Plates 19 and 20). The vesicles and hyphae contained mesosomes as described by Lalonde and Knowles (24) (Plate 17), but longitudinal sections of hyphae contained circular structures that were not described by them (Plate 15). The identity of these structures is unknown. Mitochondrial cristae were observed although organelles from control plants did not stain as densely as those from plants which received cadmium (Plate 18).

The ultrastructure of the endophyte and host cells from the nodules of plants given 10 and 20 $\mu\text{g CdCl}_2$ per liter of nutrient solution did not differ from that of the control (Plates 20-24). Numerous septa were seen within vesicles from plants given 50 $\mu\text{g CdCl}_2$ per liter (Plates 25 and 26). It was also observed that the vesicles and host cell cytoplasm appeared to be collapsing (Plate 26). The nuclei became more prominent at 50 $\mu\text{g CdCl}_2$ per liter and spaces lacking cristae were observed in the mitochondria (Plate 27). Septa were also numerous in the endophyte vesicles of plants given 100 $\mu\text{g CdCl}_2$ per liter (Plates 28 and 29), and spaces lacking cristae were observed

Plate 17. Endophyte vesicle (v) surrounded by capsular material (c) and host plasmalemma (P). The specimen was taken from the nodules of control plants given no cadmium. Magnification 34,000 X.

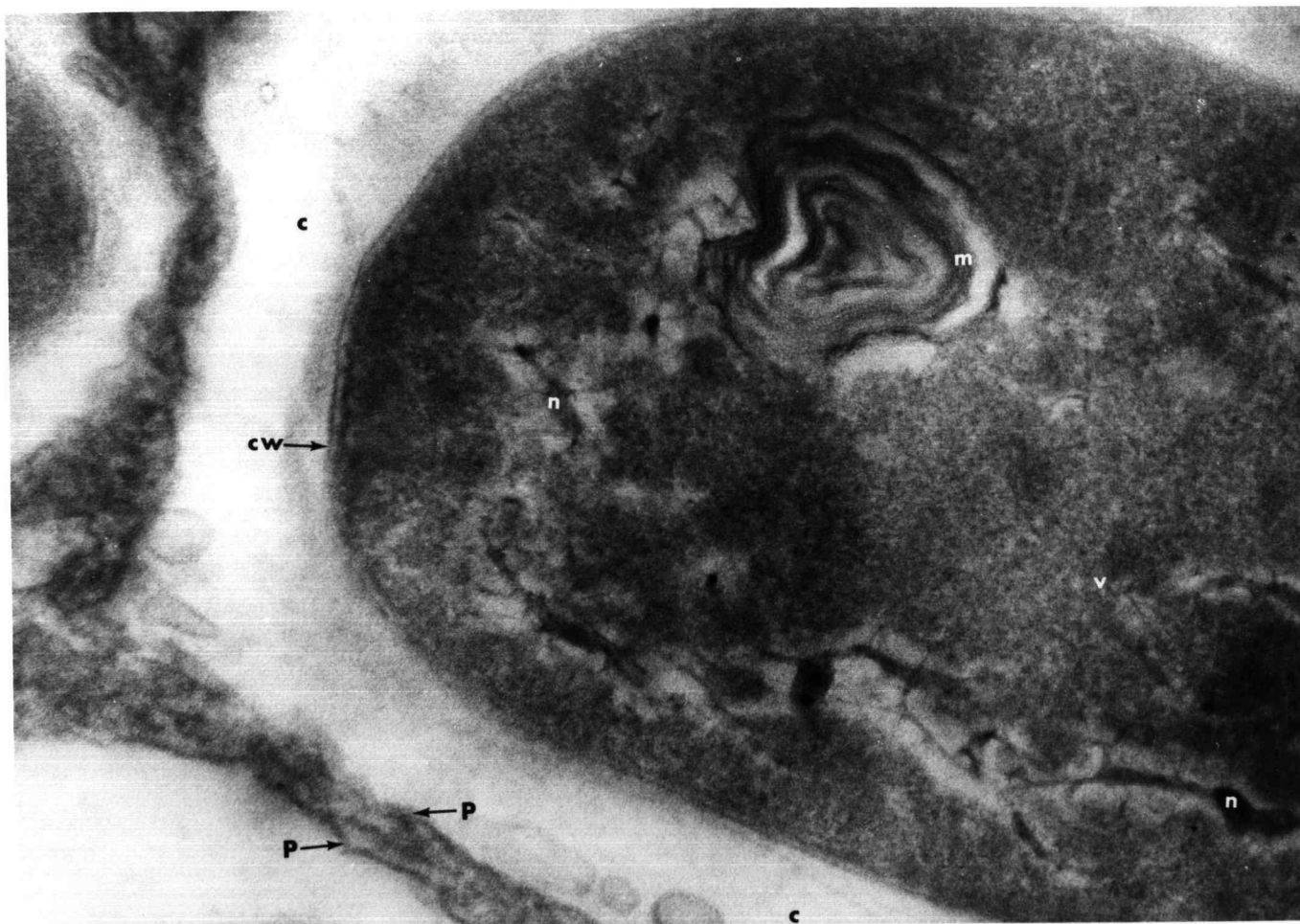


PLATE 17

Plate 18. Endophyte hyphae (h) surrounded by capsular material (c) and host plasmalemma (P). The specimen was taken from the nodules of control plants given no cadmium. Magnification 40,000 X.

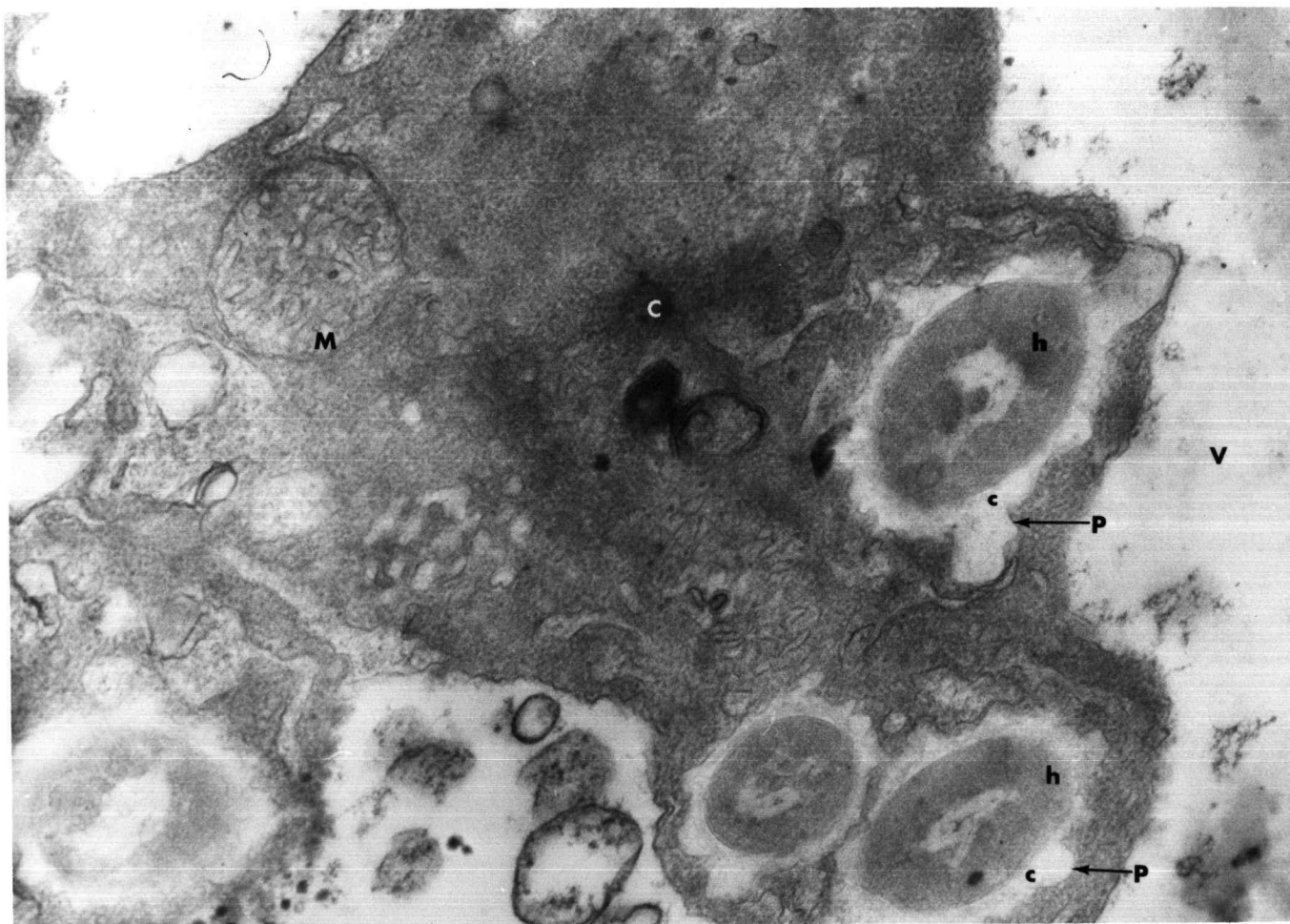


PLATE 18

Plate 19. Endophyte hypha (h) penetrating host cell wall (CW).
Specimen was taken from the nodules of control plants given no
cadmium. Magnification 4,750 X.

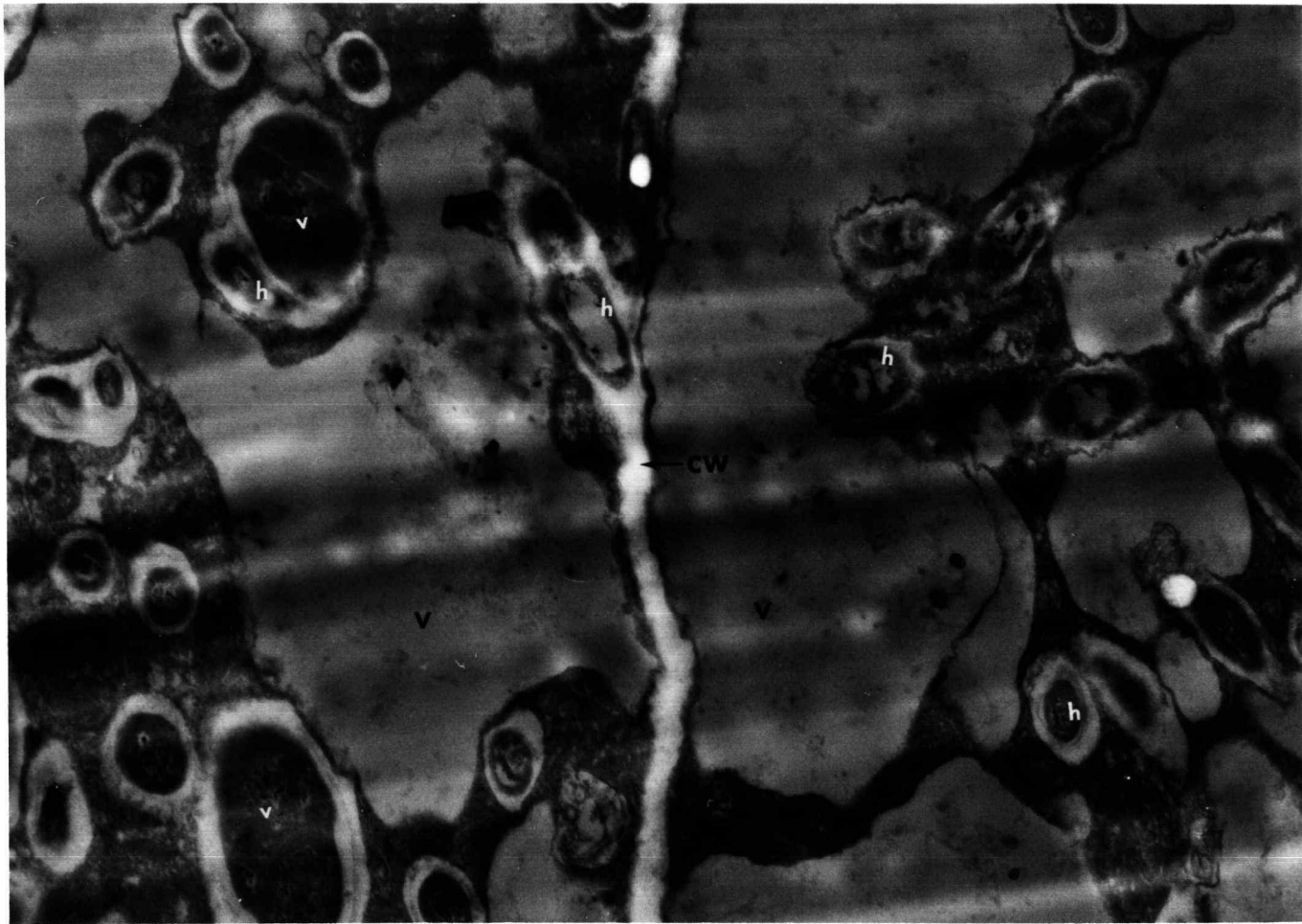


PLATE 19

Plate 20. Branched endophyte hypha (h) from the nodules of plants given 10 $\mu\text{g CdCl}_2$ per liter of nutrient solution. Magnification 8,000 X.

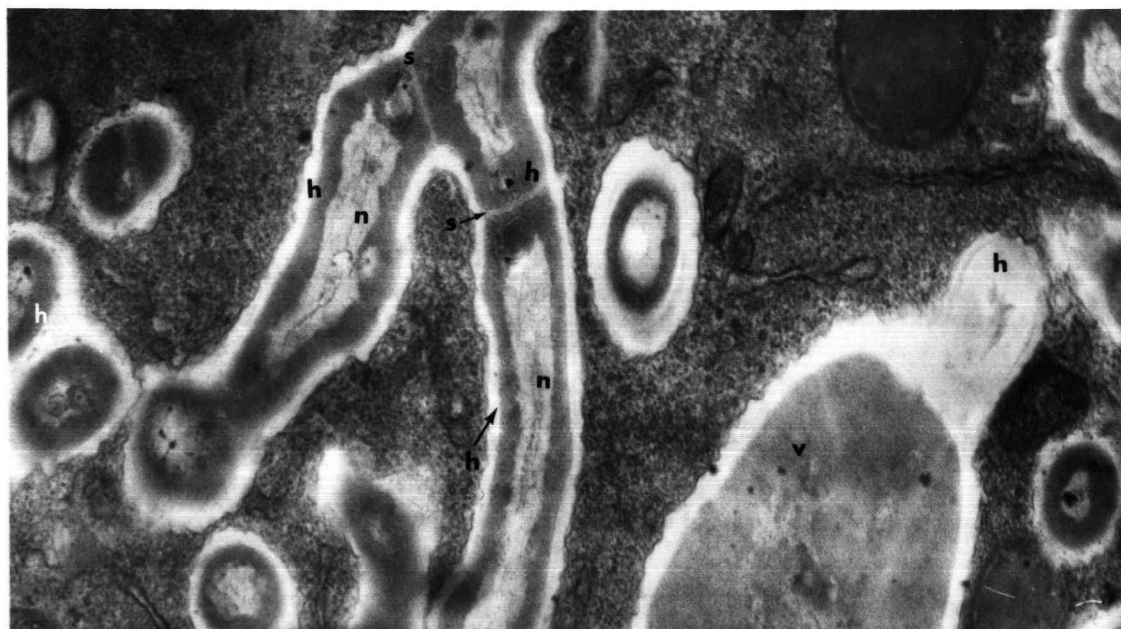


PLATE 20

Plate 21. Endophyte vesicle (v) surrounded by a cell wall (cw) and enclosed in capsular material (c). Specimen was taken from the nodules of plants given cadmium at a concentration of $10\text{ }\mu\text{g CdCl}_2$ per liter of nutrient solution. Magnification 34,000 X.

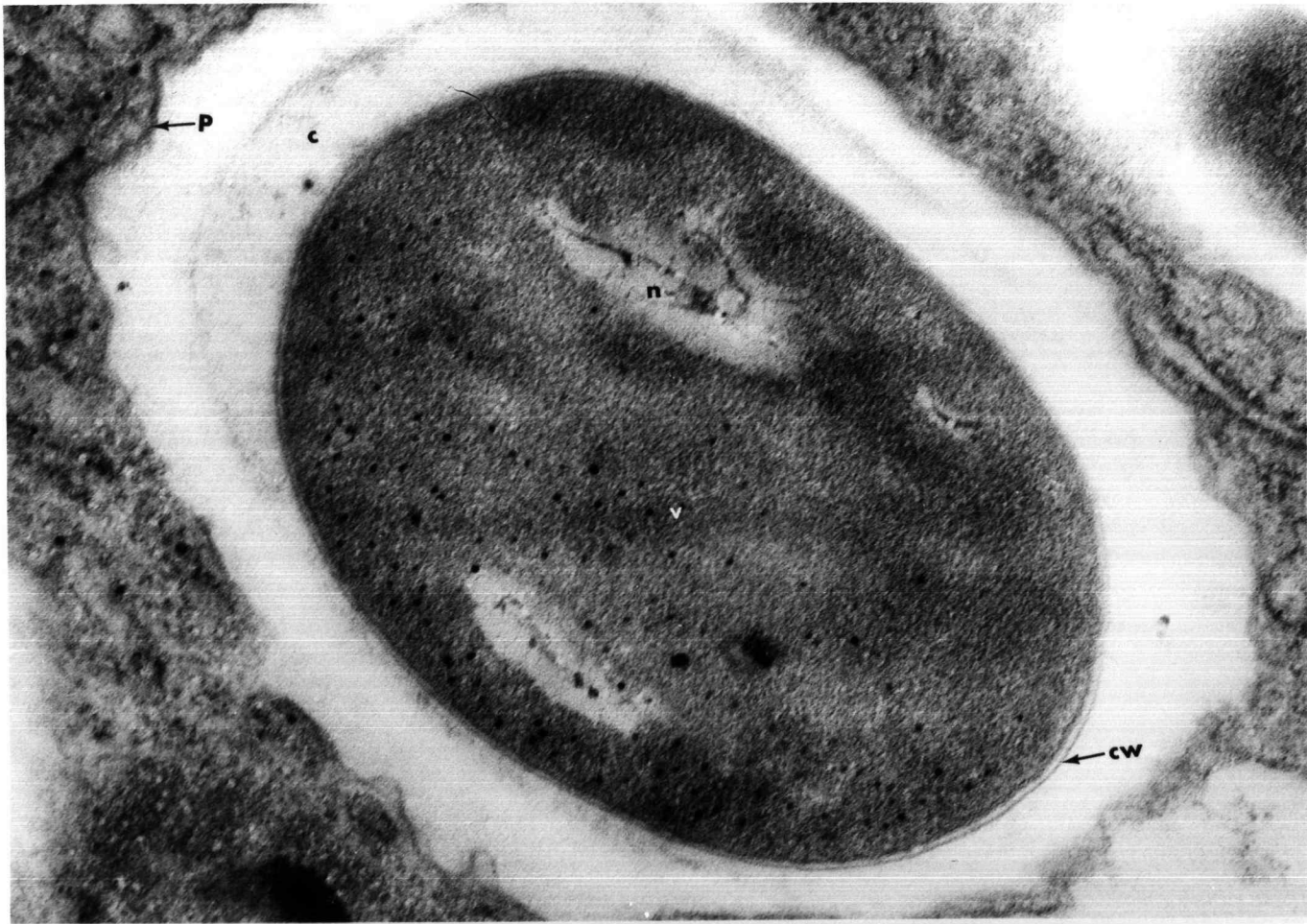


PLATE 21

Plate 22. Mitochondria (M) and endophyte hyphae (h) from the nodules of plants given cadmium at a concentration of $10\text{ }\mu\text{g CdCl}_2$ per liter of nutrient solution. Magnification 34,000 X.

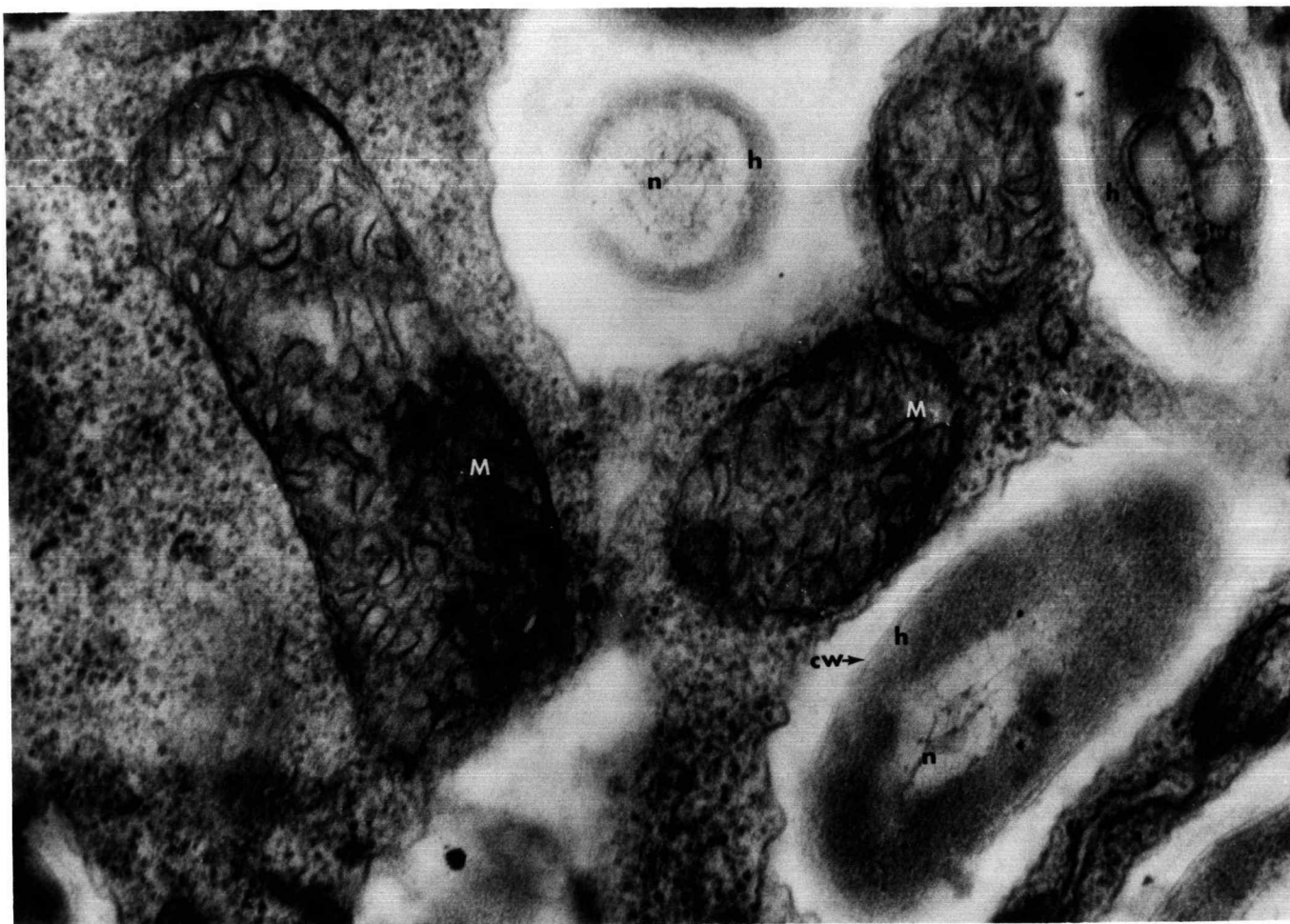


PLATE 22

Plate 23. Endophyte vesicles (v) and hyphae (h) from the nodules of plants given cadmium at a concentration of 20 $\mu\text{g CdCl}_2$ per liter of nutrient solution. Magnification 8,000 X.

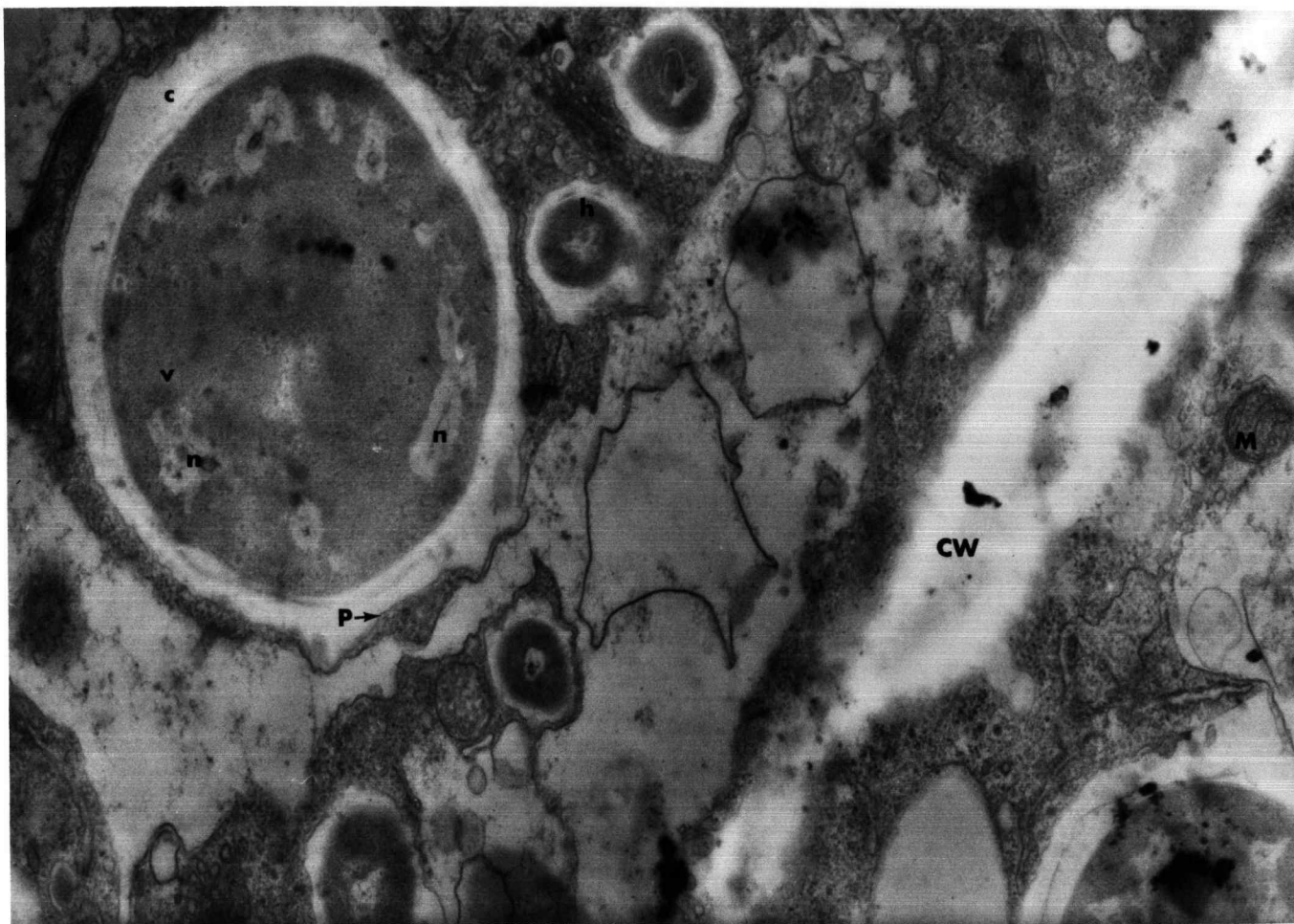


PLATE 23

Plate 24. Endophyte vesicle (v) containing what appear to be nucleic acid polymers attached to dense bodies. This is the nuclear material (n) of the endophyte. The cell membrane (cm) is also visible in this electron micrograph. Specimen was taken from the nodules of plants given cadmium at a concentration of 20 $\mu\text{g CdCl}_2$ per liter of nutrient solution. Magnification 34,000 X.

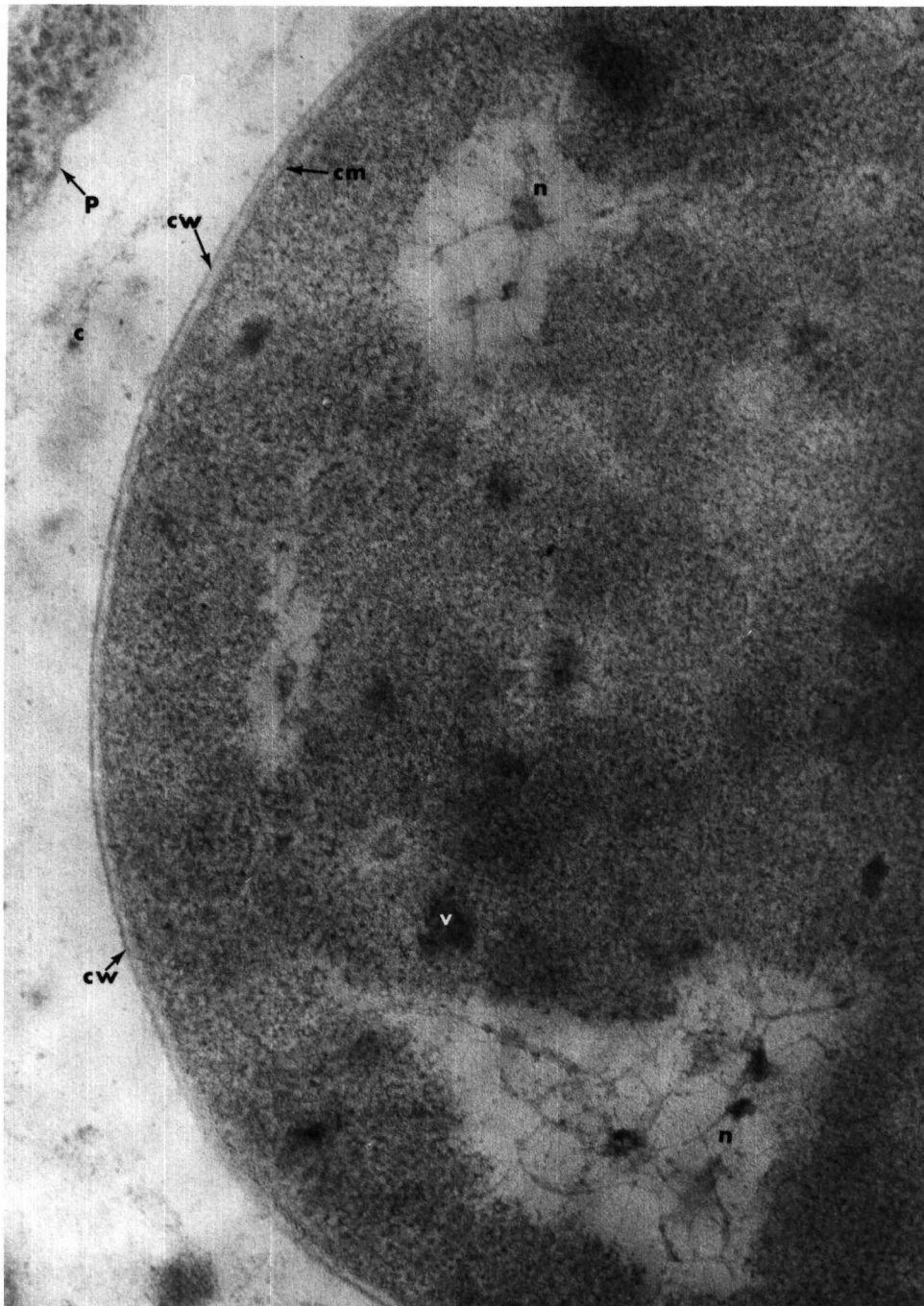


PLATE 24

Plate 25. Endophyte vesicle with septa (s) and side septum (ss).

Specimen was taken from the nodules of plants given cadmium at a concentration of 50 $\mu\text{g CdCl}_2$ per liter of nutrient solution.

Magnification 25,000 X.

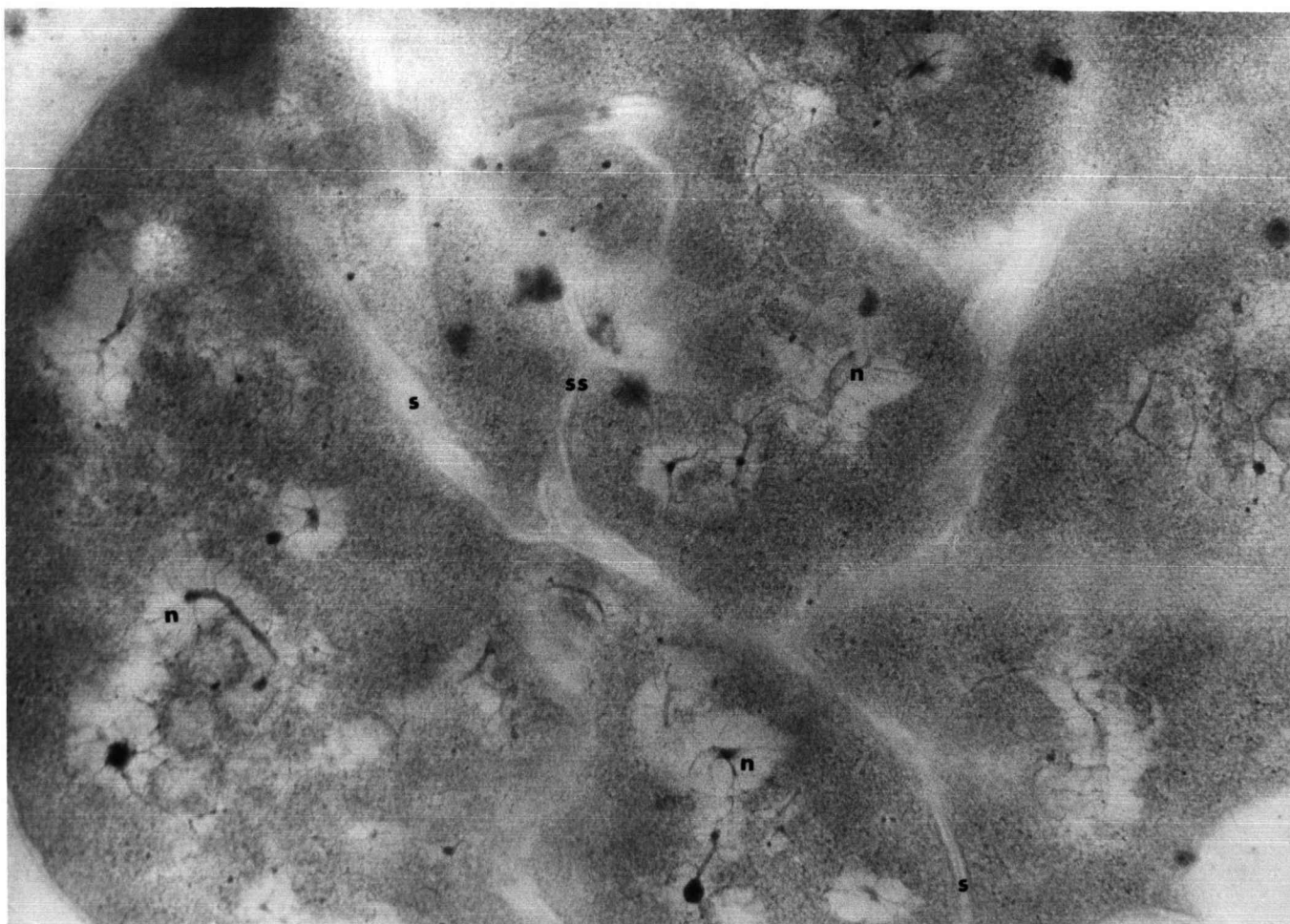


PLATE 25

Plate 26. Early stages of resorption of the endophyte. The vesicles (v) and host cytoplasm (C) appear to be collapsing. Specimen was taken from the nodules of plants given cadmium at a concentration of 50 $\mu\text{g CdCl}_2$ per liter of nutrient solution. Magnification 8,000 X.



PLATE 26

Plate 27. Hypha (h) and mitochondrion (M) from the nodules of plants given cadmium at a concentration of $50 \mu\text{g CdCl}_2$ per liter of nutrient solution. The center of the mitochondrion lacks cristae. Magnification 34,000 X.

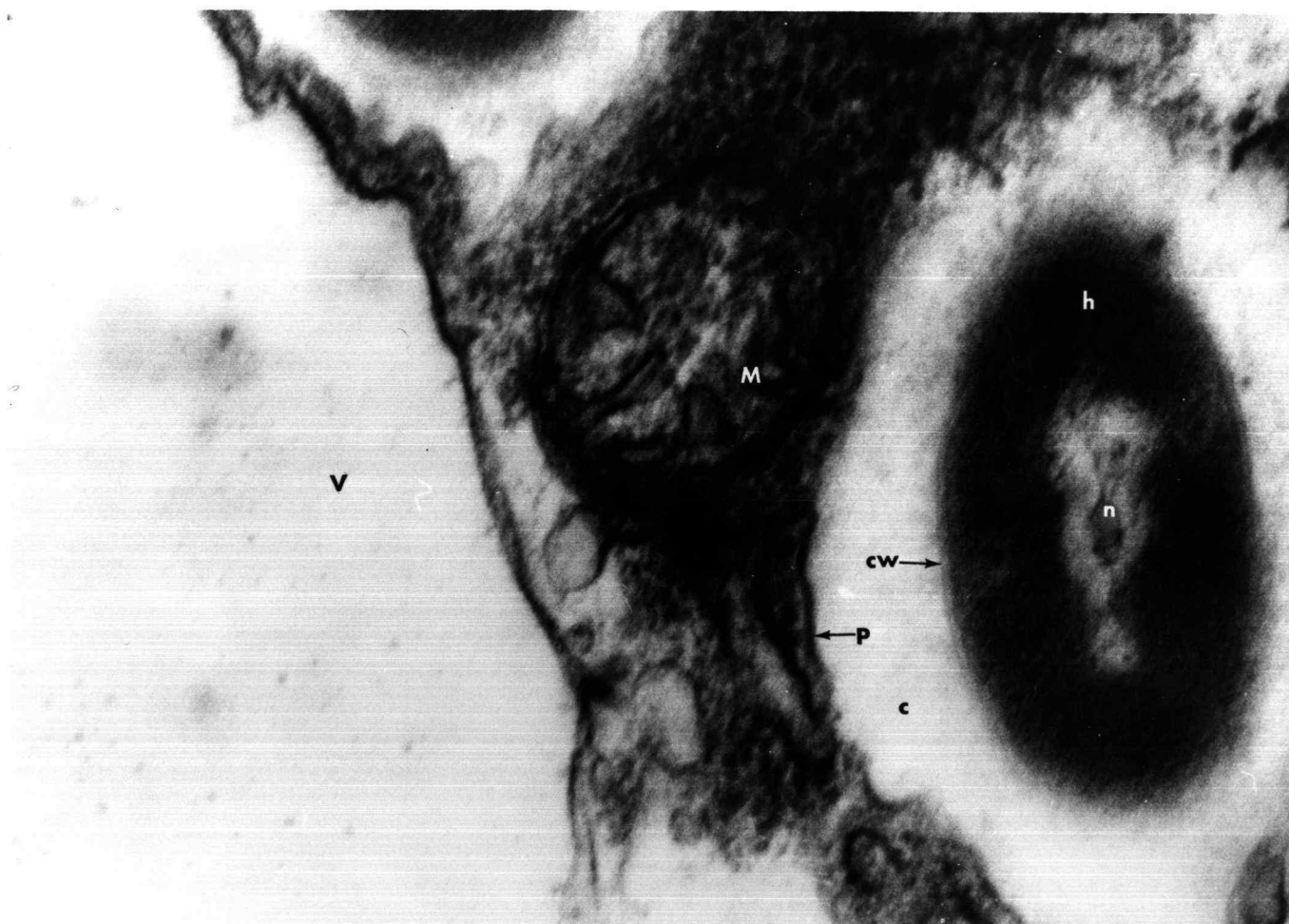


PLATE 27

Plate 28. Vesicle (v) and hyphae (h) from the nodules of plants given cadmium at a concentration of $100\text{ }\mu\text{g CdCl}_2$ per liter of nutrient solution. Wide septa (s) were observed in the vesicles at this concentration. Magnification 8,000 X.



PLATE 28

Plate 29. Vesicle (v) from the nodules of plants given cadmium at a concentration of 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution.

Septa (s) were numerous in endophyte vesicles (v) at this concentration. Magnification 15,000 X.

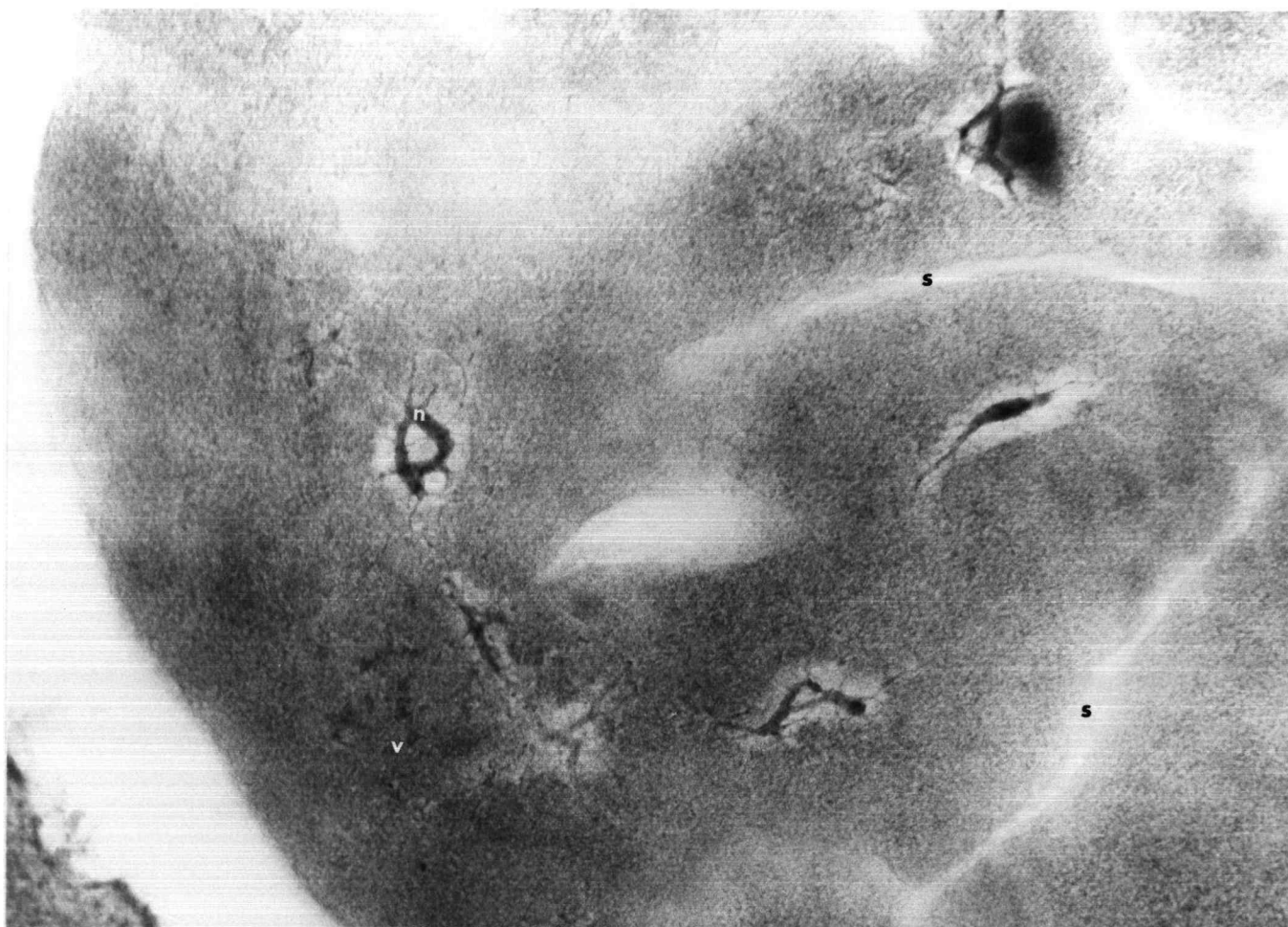


PLATE 29

in mitochondria (Plate 30). Resorption¹ of endophyte, explained by Lalonde and Knowles (26), was not seen at cadmium concentrations of 0 to 20 $\mu\text{g CdCl}_2$ per liter of nutrient solution, but the early and later stages of endophyte resorption were observed in specimen taken from the nodules of plants treated with 50 and 100 $\mu\text{g CdCl}_2$ per liter, respectively (Plates 26 and 31). Resorption was observed to be nearly complete in Plate 31.

Root Ultrastructure. Xylem parenchyma cells were observed in secondary roots collected from plants treated with cadmium. The mitochondria from control plants had well-defined cristae, inner, and outer membranes (Plate 32). Nucleoli could not be identified in the nuclei despite several observations (Plates 33 and 34). The cells of plants given 10 $\mu\text{g CdCl}_2$ per liter of nutrient solution did not differ from those of the control plants, but nucleoli and starch grains appeared when the CdCl_2 concentration was raised to 20 μg per liter (Plate 35). As the cadmium concentration increased to 50 $\mu\text{g CdCl}_2$ per liter cristae became less well defined as compared to similar preparations from the controls (Plate 36). Spaces lacking cristae also appeared. Nuclei did not appear in these sections, but they were observed in sections taken from roots of plants given 100 $\mu\text{g CdCl}_2$ per liter (Plate 37). The nucleus and endoplasmic reticulum were

¹The resorption phenomenon is characterized by progressive lysis of endophyte cytoplasm. The result is collapse of endophyte structures culminating in "ghost" vesicles and hyphae which lack protoplasts.

Plate 30. Mitochondria (M) from the nodules of plants given cadmium at a concentration of 100 $\mu\text{g CdCl}_2$ per liter on nutrient solution. Both organelles had clear spaces lacking cristae. Magnification 34,000 X.

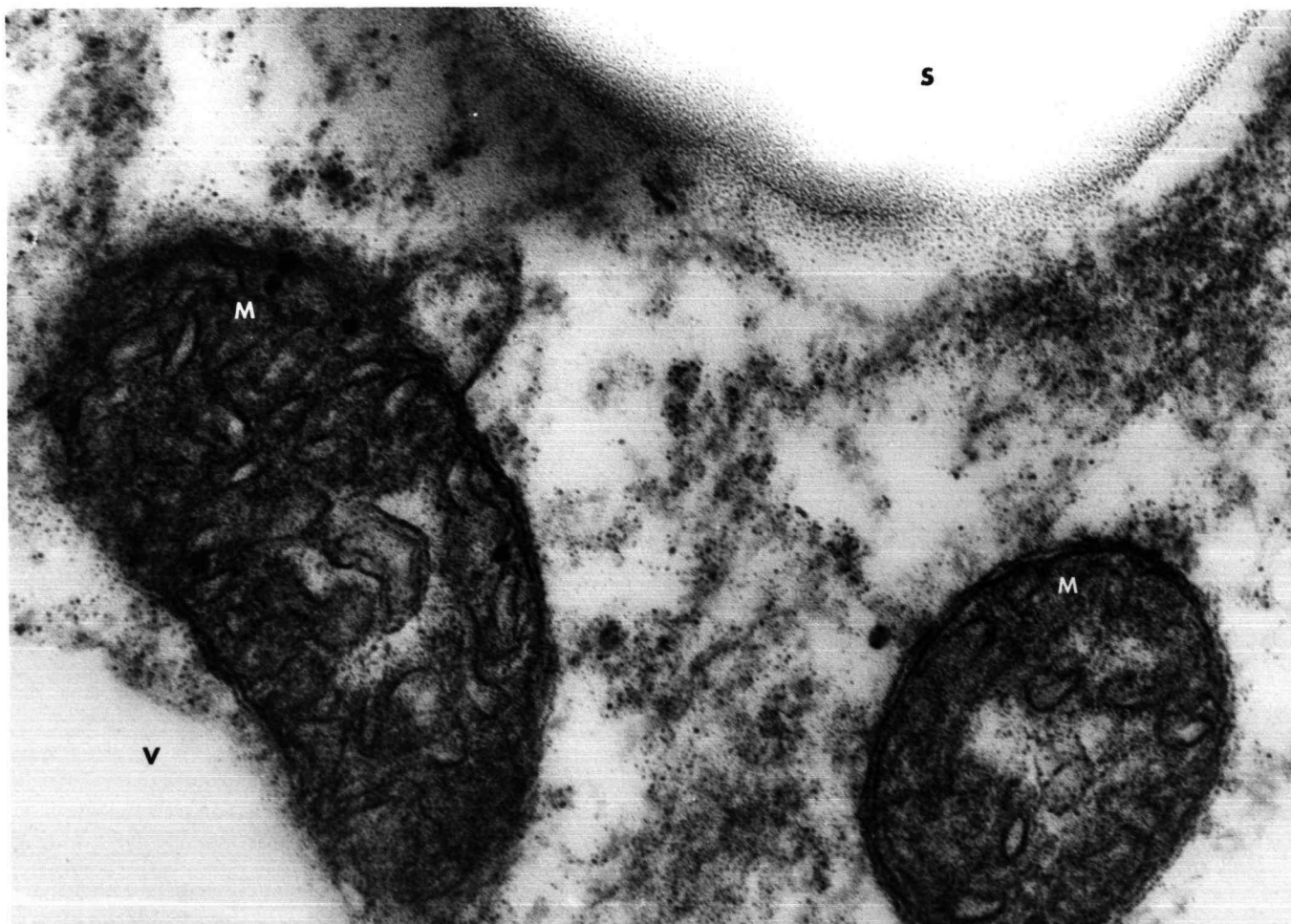


PLATE 30

Plate 31. Later stages of endophyte resorption. Partially resorbed vesicles (rv) and ghost hyphae (gh) were observed. Specimen were taken from the nodules of plants given cadmium at a concentration of $100\text{ }\mu\text{g CdCl}_2$ per liter of nutrient solution. Magnification 8,000 X.

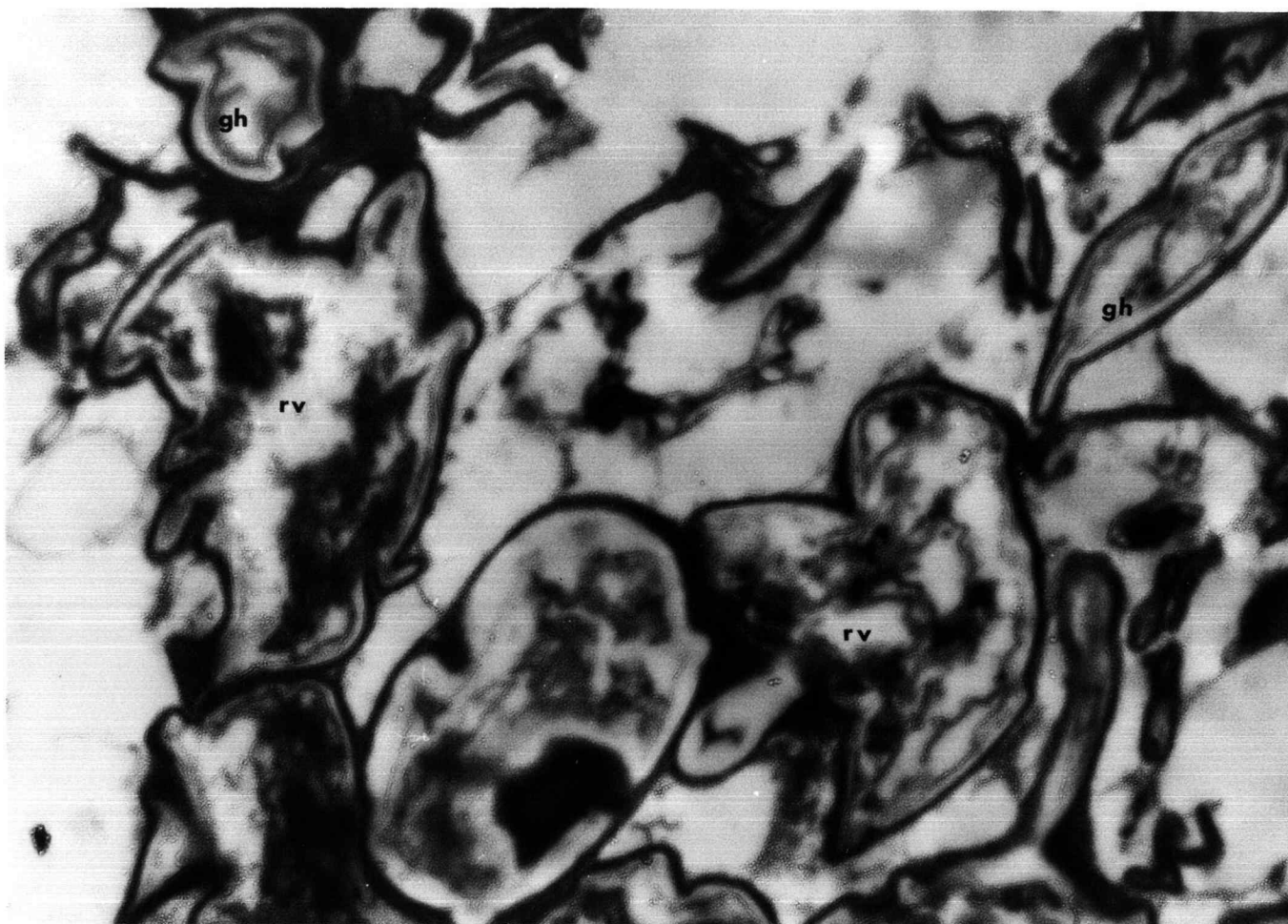


PLATE 31

Plate 32. Mitochondrion (M) from root xylem parenchyma cells of control plants given no cadmium. Magnification 46,000 X.

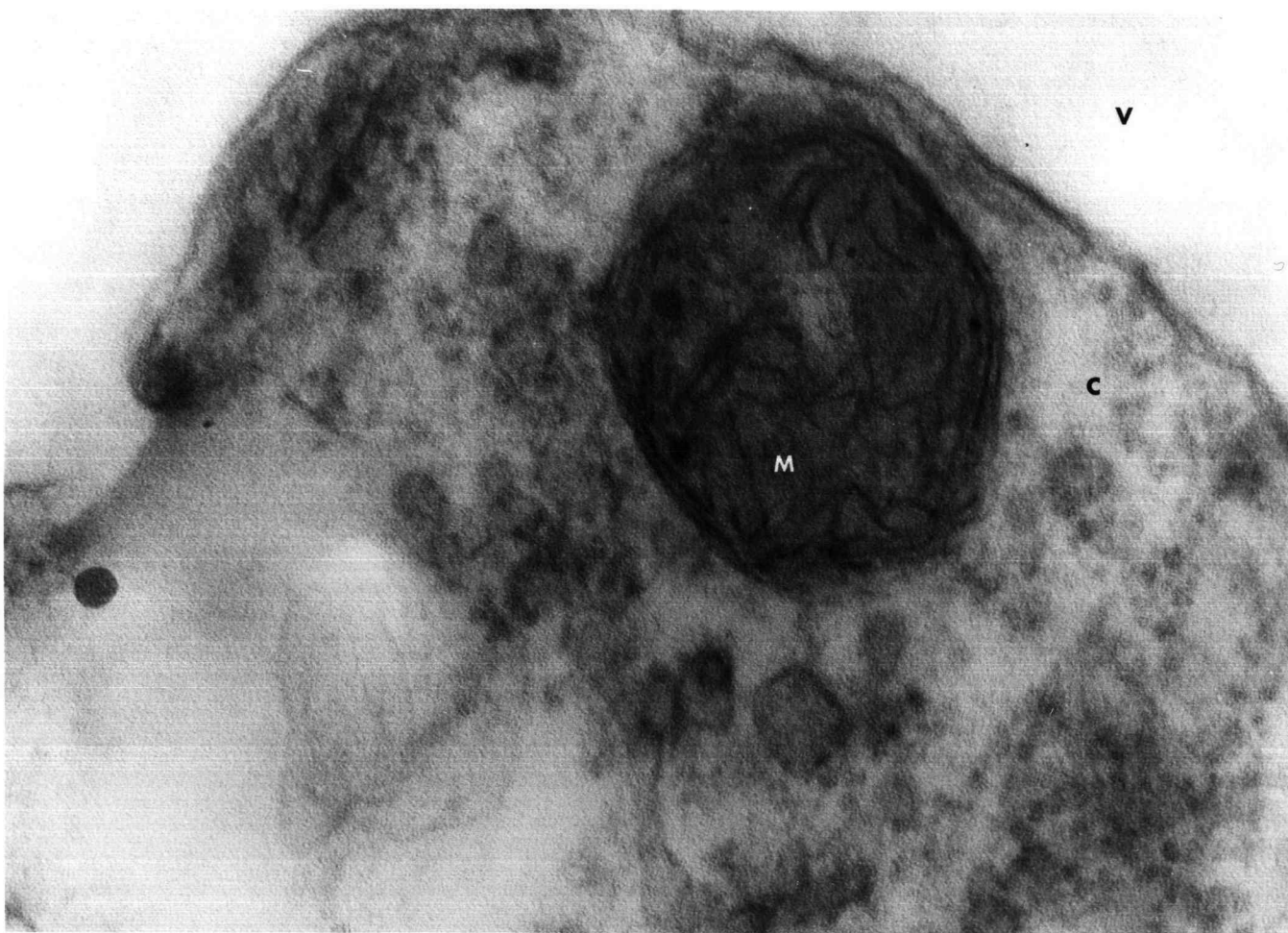


PLATE 32

Plates 33 and 34. Nuclei (N) from root xylem parenchyma cells of control plants given no cadmium. Nucleoli could not be identified in the nuclei despite several observations. Magnification 25,000 X and 19,000, respectively.

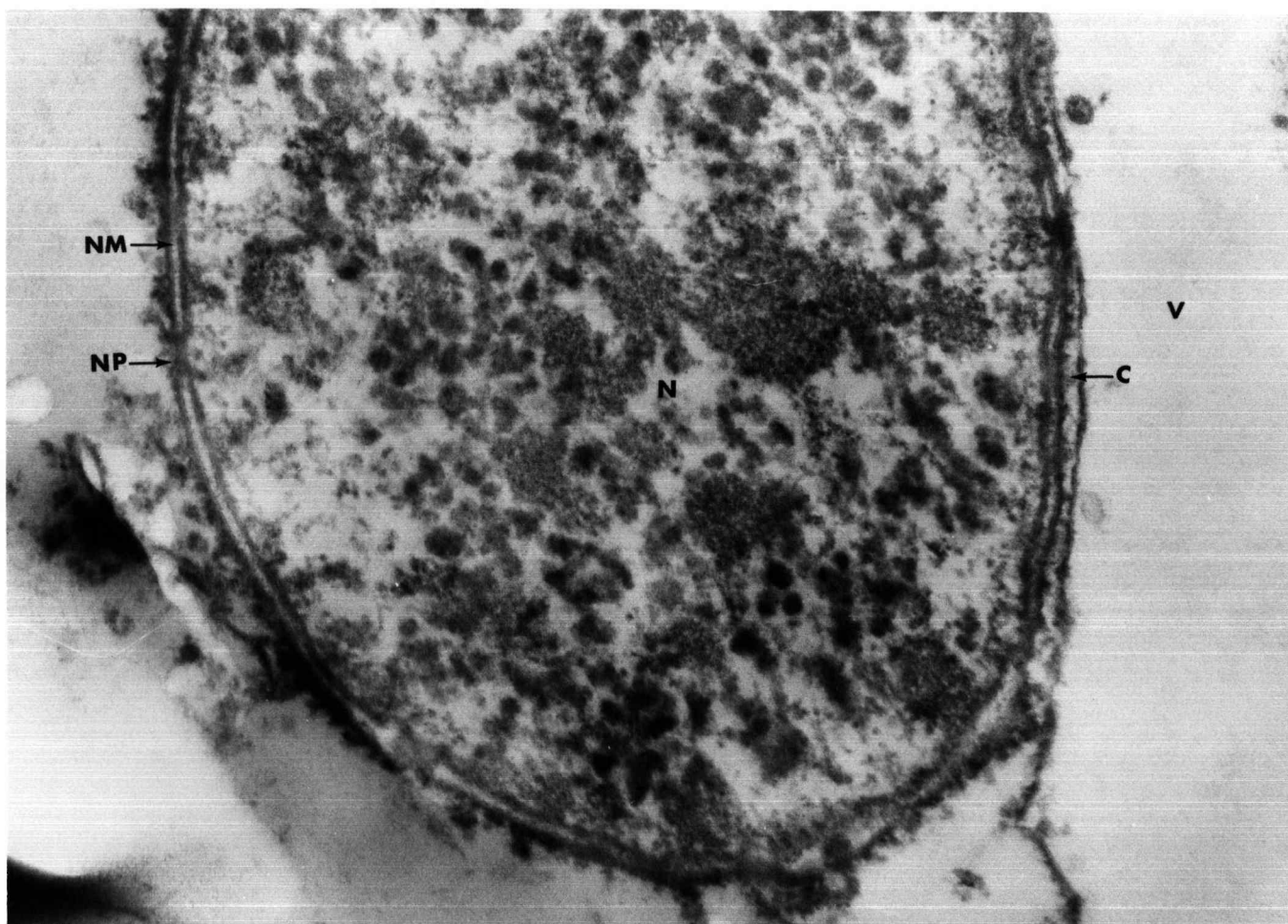


PLATE 33

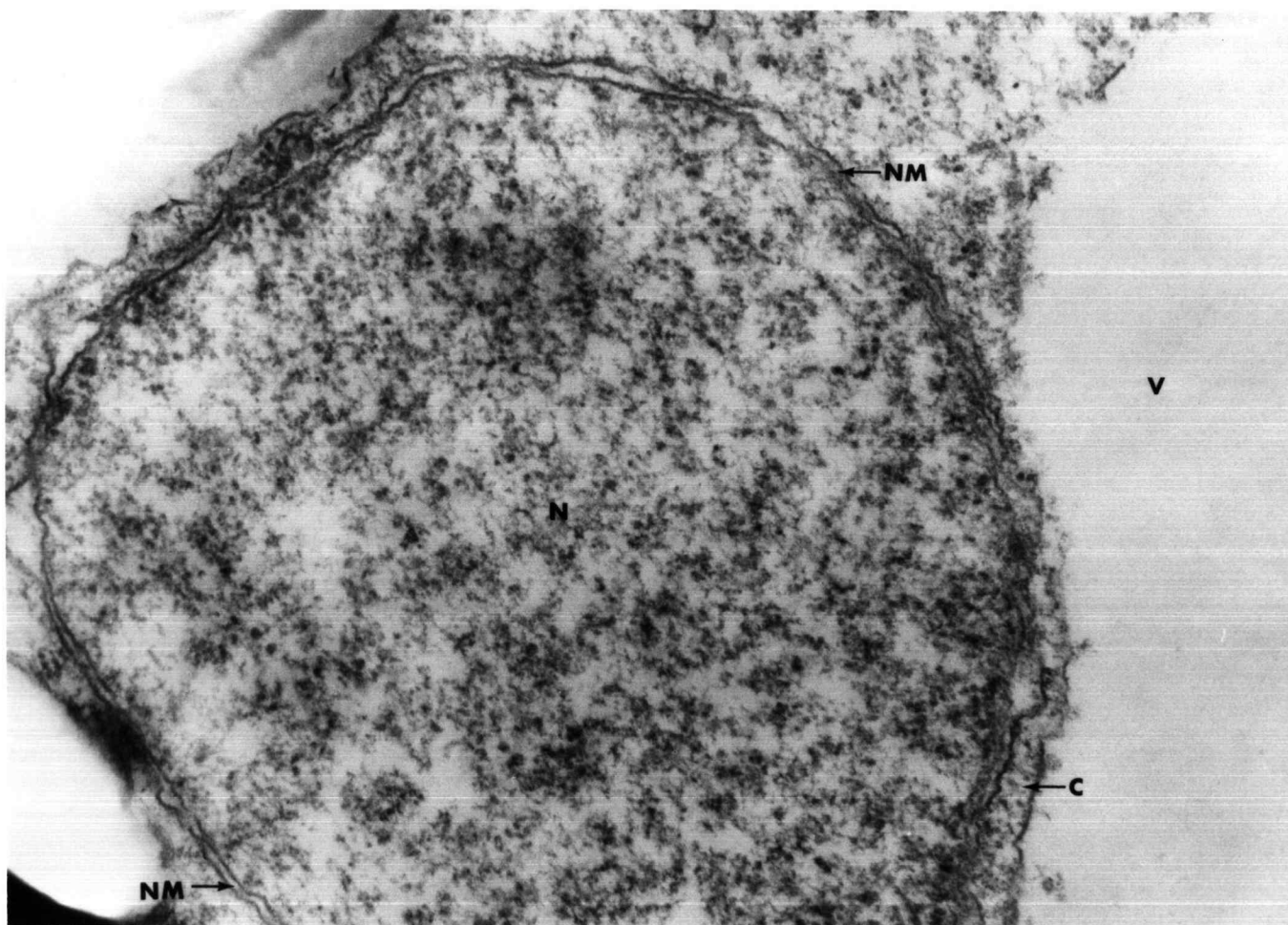


PLATE 34

Plate 35. Root xylem parenchyma cell from plant given cadmium at a concentration of 20 $\mu\text{g CdCl}_2$ per liter of nutrient solution. Nuclei (N) with nucleoli (NU) were observed at this concentration. Magnification 4,750 X.

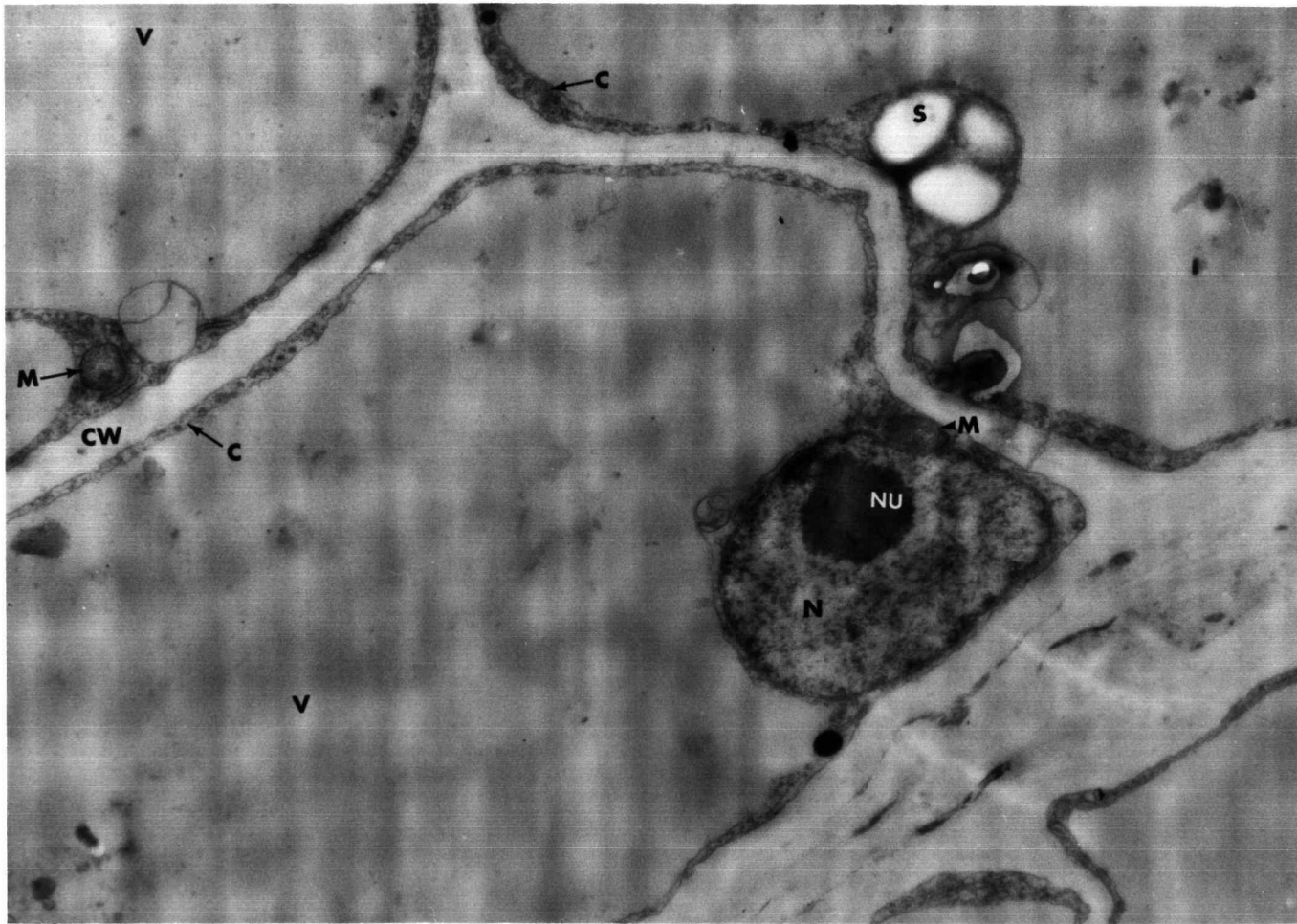


PLATE 35

Plate 36. Mitochondria (M) from root xylem parenchyma cells of plants given cadmium at a concentration of 50 $\mu\text{g CdCl}_2$ per liter of nutrient solution. Cristae were less well defined as compared to similar preparations from the controls. Magnification 60,000 X.

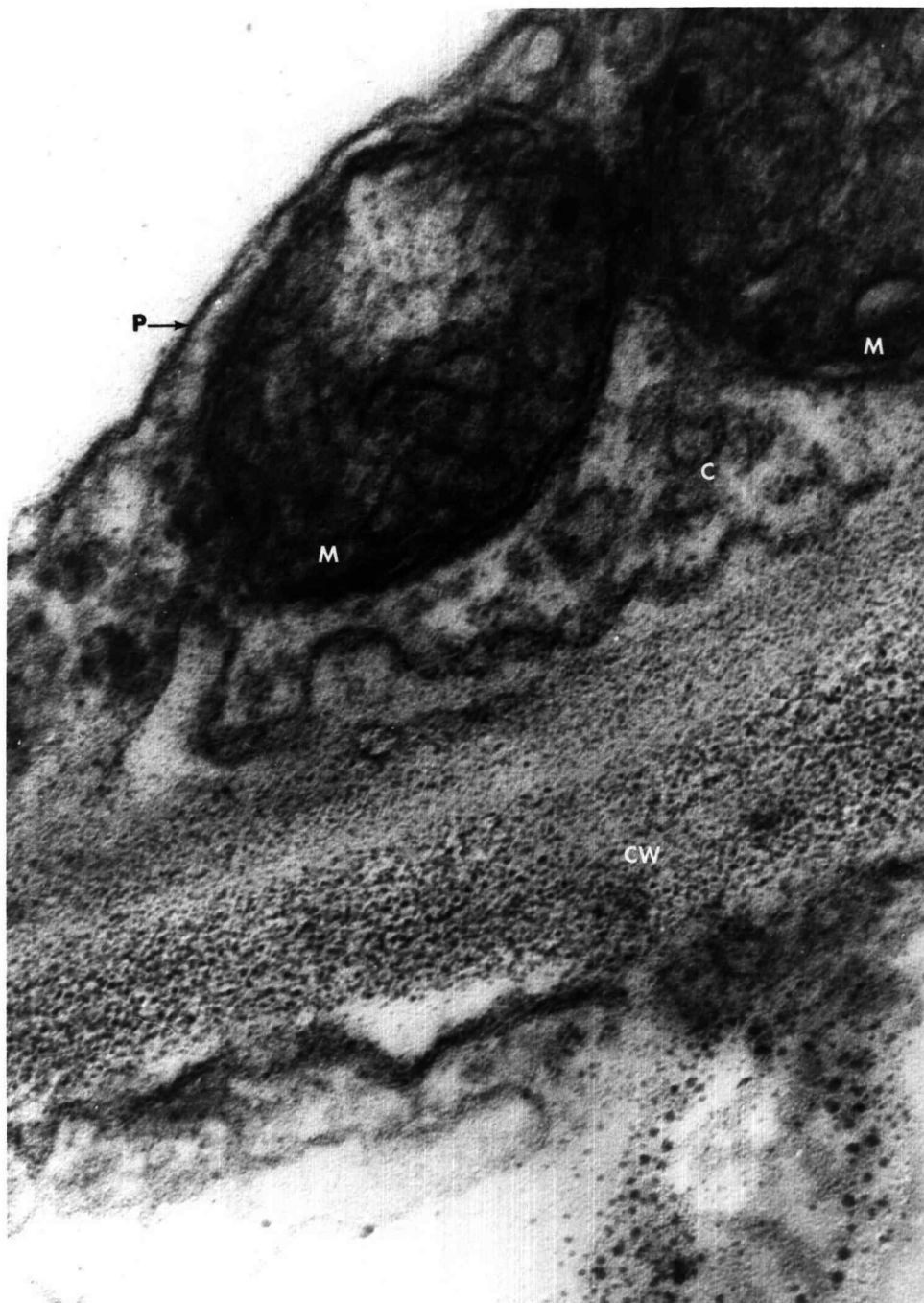


PLATE 36

Plate 37. Nucleus (N) and nucleolus (NU) from the root xylem parenchyma cells of plants given cadmium at a concentration of $100\text{ }\mu\text{g CdCl}_2$ per liter of nutrient solution. Magnification 8,000 X.

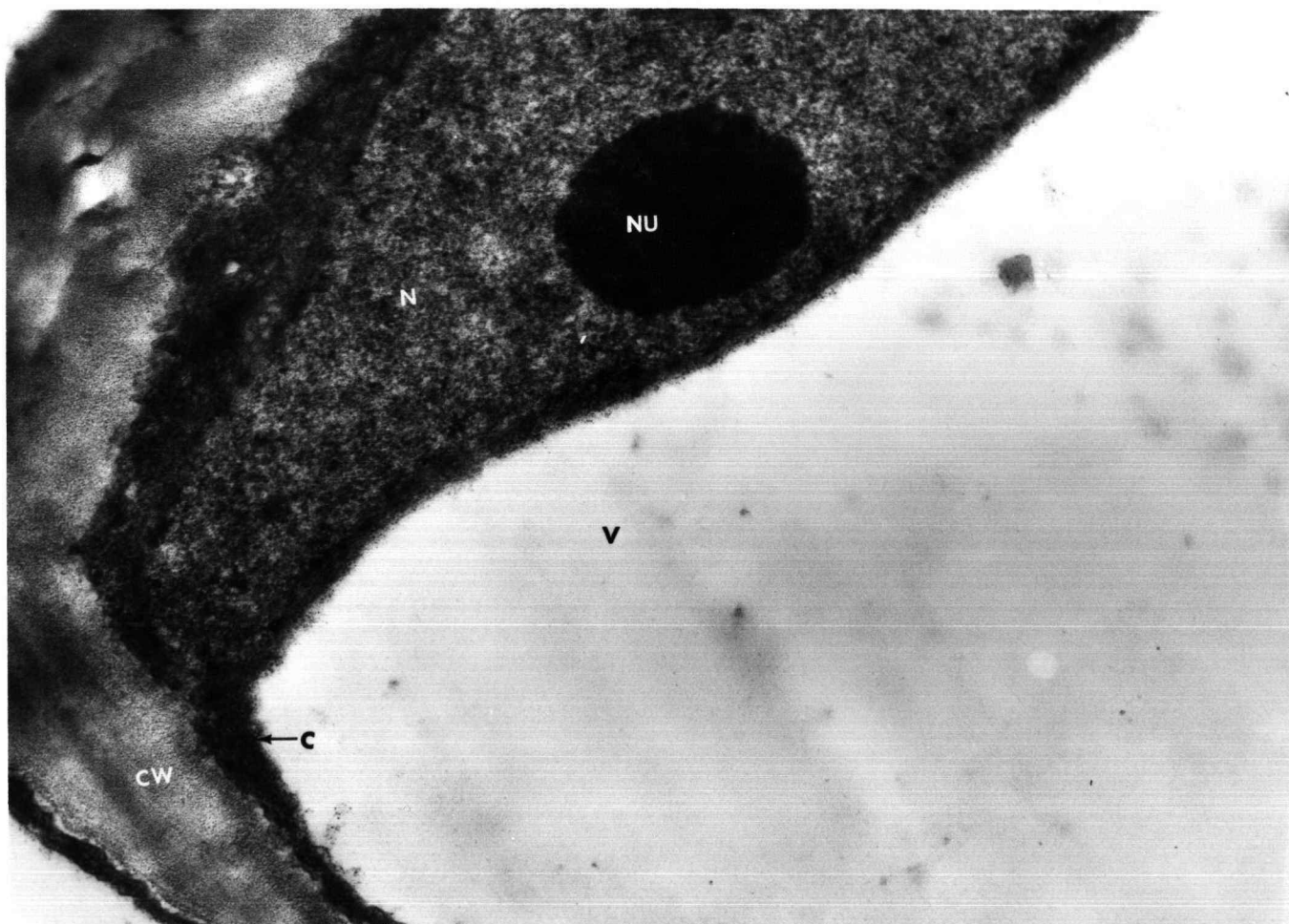


PLATE 37

densely stained and a prominent nucleolus appeared in the nucleus at this concentration (Plates 37 and 39). Starch grains were more numerous at $100\text{ }\mu\text{g CdCl}_2$ per liter and appeared in nearly every cell observed (Plate 38). Cristae in these cells were less well defined than those of the sample from control plants (Plate 39).

Effects of Cadmium on *in vitro* Nitrogenase Activity

Low concentrations of cadmium enhanced *in vitro* nitrogenase dependent acetylene reduction by a crude preparation of the enzyme. Significant increases in activity of the nitrogenase were observed at cadmium concentrations ranging from 11 to $54\text{ }\mu\text{M CdCl}_2$ (2 to 10 mg/l) in the reaction mixture (Fig. 45). The activity decreased below the control rate at $272\text{ }\mu\text{M CdCl}_2$ (50 mg/l).

DEAE cellulose purified components were pre-incubated for 20 minutes with varying amounts of CdCl_2 in a reaction buffer medium. After this the Mo-Fe (fraction I) and Fe (fraction II) protein components of nitrogenase were combined and allowed to catalyze acetylene reduction. There was a decrease in the activity of the combined components when fraction I alone was pre-incubated with cadmium (Figs. 46 and 47). Significant decreases in nitrogenase activity were observed when the CdCl_2 concentrations ranged from 11 to $136\text{ }\mu\text{M}$ (2 to 25 mg/l) in the reaction mixture. The activity of the combined components was significantly enhanced at $27\text{ }\mu\text{M CdCl}_2$ (5 mg/l)

Plate 38. Root xylem parenchyma cells from plants given cadmium at a concentration of 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution. Starch grains (S) appeared in nearly every cell observed at this concentration. Magnification 4,750 X.

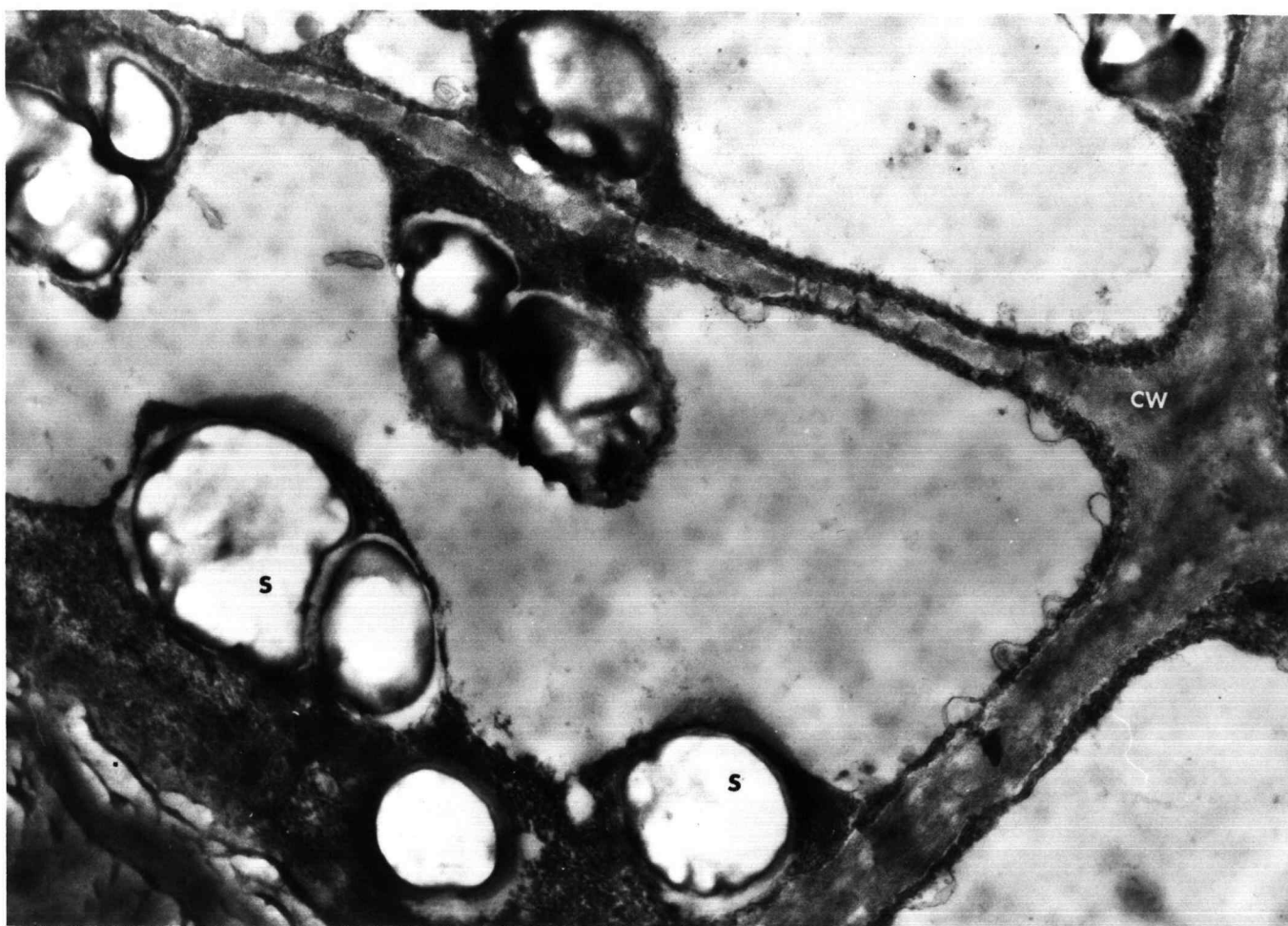


PLATE 38

Plate 39. Mitochondria (M) from root xylem parenchyma cells of plants given cadmium at a concentration of 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution. Magnification 8,000 X.

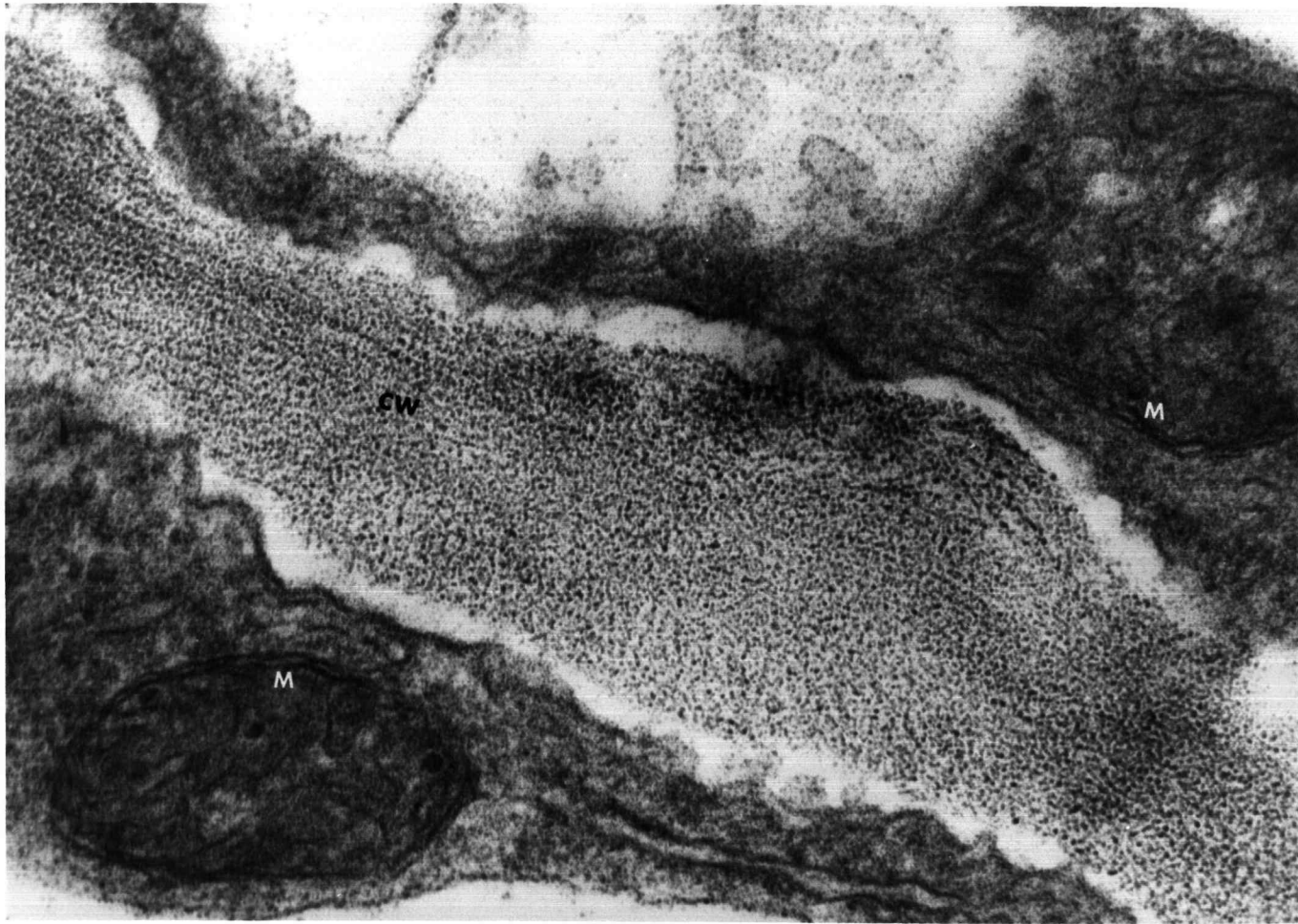


PLATE 39

Figure 45. The effect of cadmium on the activity of a crude preparation of nitrogenase. Cadmium chloride was present in the incubation mixtures in concentrations of 0, 11, 27, 54, 136 and 272 μM (equivalent to 0, 2, 5, 10, 25, and 50 mg per liter, respectively).

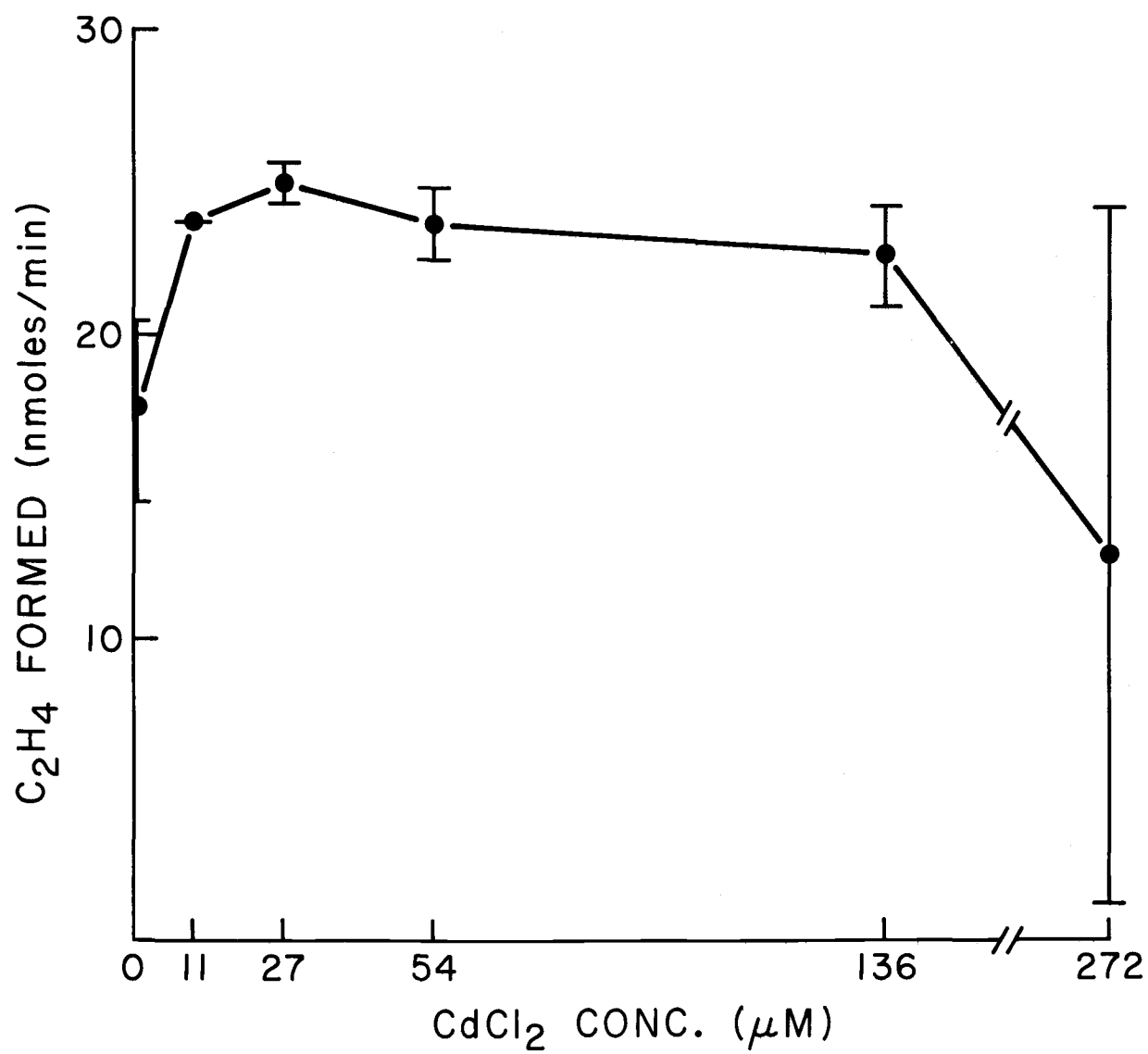


Figure 45

Figure 46. The effect of cadmium on the activity of a purified nitrogenase from soybean nodules. Cadmium chloride was present in the incubation mixtures at concentrations of 0, 11, 27, 54, and 136 μM (equivalent to 0, 2, 5, 10 and 25 mg per liter, respectively).

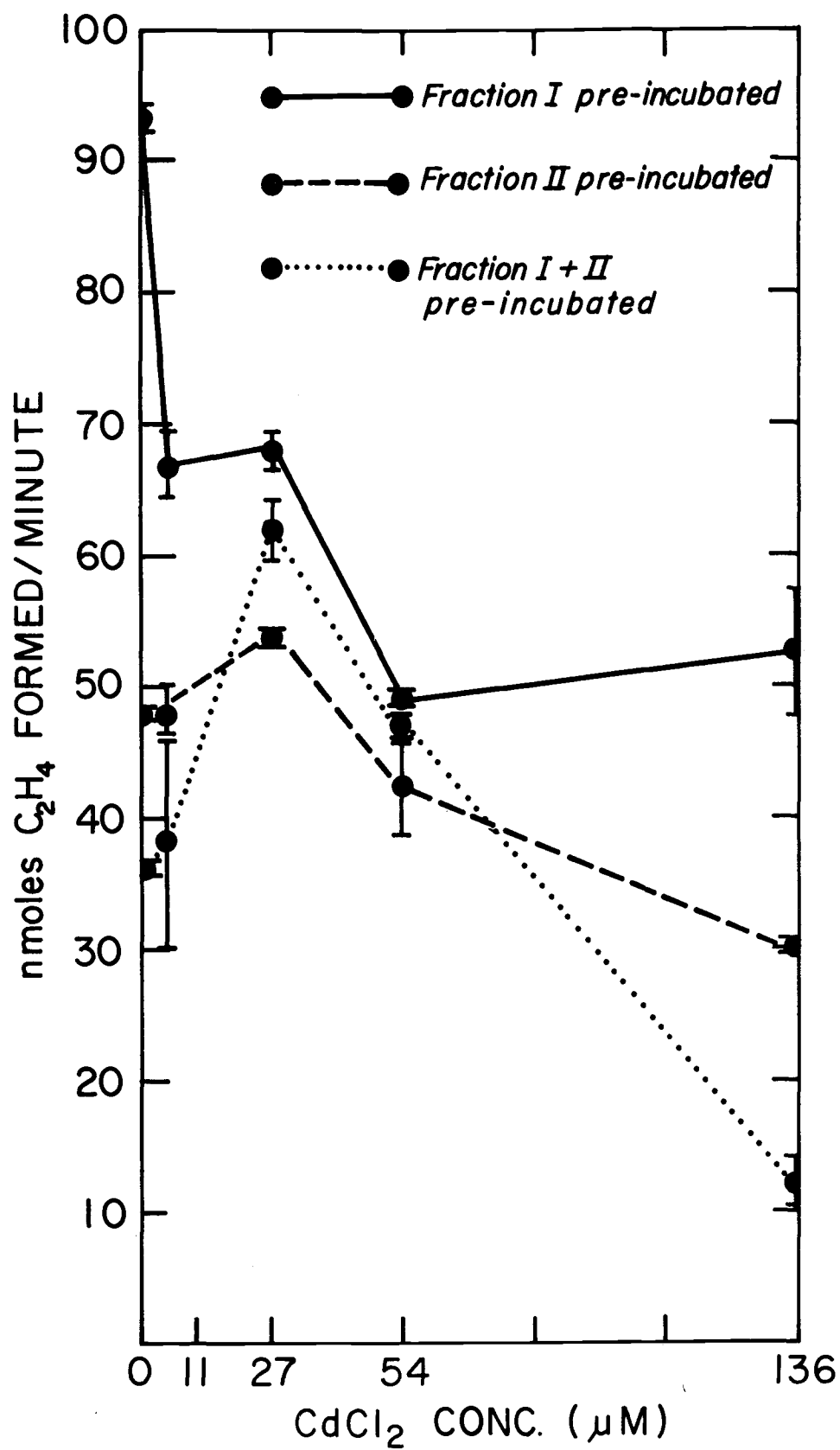


Figure 46

Figure 47. The effect of cadmium on the activity of a purified nitrogenase from soybean nodules. Cadmium chloride was present in the incubation mixtures at concentrations of 0, 11, 27, 54 and 136 μ M (equivalent to 0, 2, 5, 10, and 25 mg per liter, respectively). This was a replication of Figure 46.

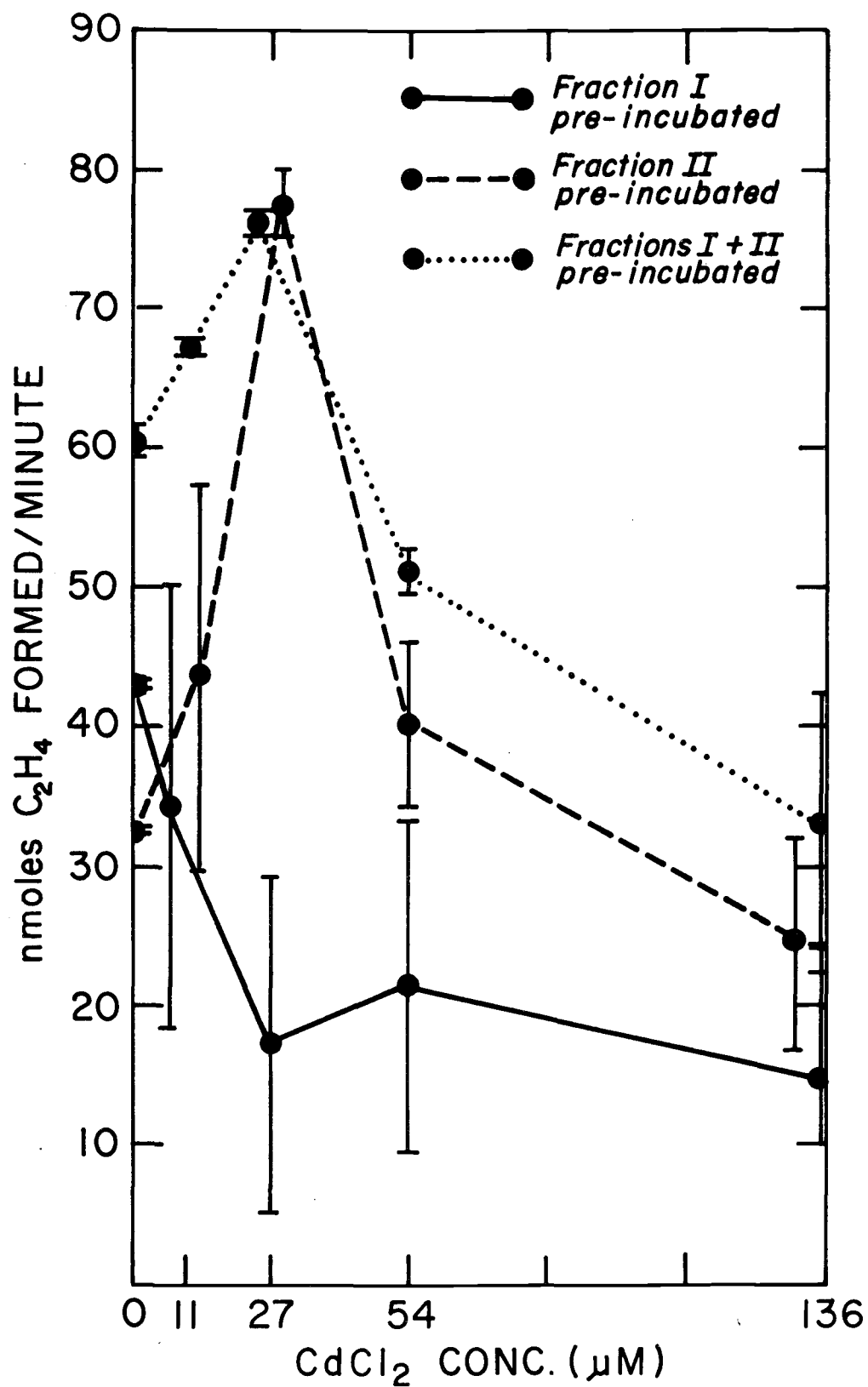


Figure 47

when fraction II alone was pre-incubated with cadmium (Figs. 46 and 47). Nitrogenase activity increased significantly at $54\text{ }\mu\text{M CdCl}_2$ (10 mg/l) when fractions I and II were combined and pre-incubated with cadmium (Figs. 46 and 47), but activity was significantly decreased at $136\text{ }\mu\text{M CdCl}_2$ (25 mg/l).

Sodium dithionite, a reducing agent, prevented the cadmium induced decline in nitrogenase activity when fraction II was pre-incubated with CdCl_2 (Table 2). The data show greater activity in the absence of the reducing agent, although the previous experiments showed a consistent decline in activity when fraction I was pre-incubated (Figs. 46 and 47). When fractions I and II were combined and pre-incubated there was a 31% decline in activity at the CdCl_2 concentration of $54\text{ }\mu\text{M}$ (10 mg/l) in the reaction mixture. The loss of activity was prevented by including sodium dithionite (Table 2). There was a 42% decline in activity at $136\text{ }\mu\text{M CdCl}_2$ (25 mg/l) which was not prevented by sodium dithionite.

The nitrogenase activity of a heat-treated crude preparation of the enzyme decreased 36% in the presence of $136\text{ }\mu\text{M CdCl}_2$ (25 mg/l) in the reaction mixture when the MgCl_2 concentration was optimum (6.7 mM) (Table 3). The activity declined 71% when the MgCl_2 concentration was lowered to 2.0 mM in the absence of cadmium. There was a 46% decrease in activity from the addition of $136\text{ }\mu\text{M}$ (25 mg/l)

Table 2. The effect of sodium dithionite on the inhibition of nitroge-
nase by cadmium. A DEAE cellulose purified preparation
was pre-incubated with CdCl_2 in a buffer with and without
20.0 μmoles sodium dithionite in 1.5 ml reaction mixture.
The assay system by Israel et al. (19) was used.

Fraction Pre-incubated with Cadmium	Cadmium Conc. (μM)	Percent of Control Activity	
		Dithionite Absent	Dithionite Present
Fraction I	0	100	100
Fraction I	54	144	52
Fraction I	136	66	46
Fraction II	0	100	100
Fraction II	54	70	109
Fraction II	136	57	70
Fractions I and II	0	100	100
Fractions I and II	54	69	107
Fractions I and II	136	58	45

Table 3. The effect of magnesium on the inhibition of nitrogenase by cadmium. A heat treated crude enzyme preparation was pre-incubated with CdCl_2 in buffer before magnesium addition. The assay system by Israel et al. (19) was used.

Conc. MgCl_2 (mM)	Percent of Control Activity	
	0 μM CdCl_2	136 μM CdCl_2
2.0	29	54
6.7	100	64
20.0	82	82

CdCl_2 at the same MgCl_2 concentration. Nitrogenase activity was not changed by cadmium when the MgCl_2 concentration was 20 mM.

DISCUSSION

The experiments reported in this investigation provide data on the extent to which cadmium affects the nitrogen fixation system of Alnus rubra. Relatively high concentrations of cadmium were given to plants in the initial investigation. The plants responded to cadmium treatment in a manner similar to that reported by many previous investigators (2, 17, 22, 29). The responses included growth reduction in all organs, wilting, loss of nitrogenase activity, and accumulation of cadmium to very high concentrations. Although plants are very seldom exposed to the range of cadmium concentrations used in the initial investigations data established that cadmium was toxic to Alnus rubra and its nitrogen fixation system. Also it was established that the intensity of toxicity responses increased as exposure to cadmium continued. It was also shown that Alnus rubra accumulated cadmium at progressively increasing concentrations as exposure to the element was continued.

Cadmium concentrations were lowered in additional investigations in which the duration of the treatments were increased from 6 to 11 weeks. Plants did not wilt when cadmium concentrations were 25 mg CdCl_2 per liter or less. Those plants grew at a very reduced rate indicating cadmium has a general depressing effect on plant metabolism. Chlorosis of the leaves at the lower concentrations showed that chlorophyll biosynthesis was inhibited or otherwise

impaired. Photosynthesis therefore would be expected to be inhibited which would lead to a lower level of substrate for carbohydrate metabolism. ATP is required for nitrogenase activity and is provided by oxidative phosphorylation coupled to electron transport in the cells of the endophyte (40, 41). The electrons for nitrogenase activity are generated by oxidation of carbohydrate in cells to carbon dioxide and water. An insufficient concentration of chlorophyll in the leaves would limit the availability of carbohydrate due to an insufficient rate of carbon dioxide fixation. This in turn would be expected to limit nitrogen fixation in the nodules of Alnus rubra and other nitrogen fixing species.

Cadmium has been shown to be translocated from the roots to the leaves of plants. Thus, the element may inhibit photosynthesis by binding to and reducing the activity of enzymes involved in the dark reactions of photosynthesis. Cadmium also was found to be present in the other plant organs. The element may inhibit the respiratory metabolism of cells by binding to the enzymes involved in intermediary metabolism, electron transport, oxidative phosphorylation, and other processes. The effects would decrease the concentration of ATP available for nitrogenase activity.

A more direct effect of cadmium on the nitrogen fixation system might involve an inhibition of the activity of the nitrogenase system. Incubation of the Mo-Fe component of nitrogenase with cadmium

caused loss of activity of the reconstituted nitrogenase. The binding of cadmium to the nitrogenase in the endophyte cells would be expected to reduce its activity similarly. There was non-specific metal activation of protein when the Fe component and the reconstituted nitrogenase were incubated with cadmium. Nitrogenase activities increased to maxima which occurred at $27 \mu\text{M CdCl}_2$ (Figs. 46 and 47). Activities then decreased with increasing cadmium concentration to minima which occurred at $136 \mu\text{M CdCl}_2$. An alternative explanation for the increased activities is cadmium binding of ADP which is a reaction product and inhibitor of nitrogenase (11). At higher concentrations cadmium would inhibit nitrogenase more than it would prevent inhibition by ADP. However, a cadmium ADP complex has not been demonstrated and further research is required.

When the Mo-Fe component was incubated with sodium dithionite and $54 \mu\text{M CdCl}_2$ (10 mg/l) nitrogenase was inhibited (Table 2). When the Fe component and the reconstituted nitrogenase were incubated with dithionite and $54 \mu\text{M CdCl}_2$ the enzyme was not inhibited. The opposite effect was observed when dithionite was omitted from the incubation mixture. Nitrogenase was not inhibited when the Mo-Fe component was incubated without dithionite, but the enzyme was inhibited when the Fe component and the reconstituted nitrogenase were incubated without dithionite. Also, there was a 44% increase in activity when the Mo-Fe component was incubated with $54 \mu\text{M CdCl}_2$

without dithionite. All reactions were decreased when the CdCl_2 concentration was $136 \mu\text{M}$ (25 mg/l). These results indicate that cadmium alters sodium dithionite reduction of the enzyme. This effect has not been studied extensively and further research is required.

Metal interaction or competition was implicated by the results showing that cadmium partially restored the in vitro activity of a crude nitrogenase preparation to which was added a limiting concentration of magnesium (Table 3). The presence of cadmium did not affect nitrogenase activity when the magnesium was supraoptimal, but there was reduction of activity at the optimum magnesium concentration. This indicates a competition between cadmium and magnesium in the nitrogenase reaction. Cadmium may reduce the rate of hydrolysis of ATP by forming a cadmium ATP complex instead of a magnesium ATP complex which is considered necessary for activity (8, 40).

At cadmium concentrations in the range of 2 to 25 mg CdCl_2 per liter of nutrient solution major effects on physiological processes occurred (Figs. 9, 10, 15, 18, and 21). At cadmium concentrations in the range of 0.1 to 5.0 mg CdCl_2 per liter the trends were variable and difficult to interpret (Figs. 11, 12, 16, 19, and 22). The metabolic processes that are affected in a plant depend upon the genetics of each plant and the environmental factors. The ecological tolerances of

each plant should be considered, however, the change in environmental conditions within the experiment would not be expected to be great. Slight differences in medium temperature, medium moisture, lighting, and nutrient supply, however, would be expected. Thus, cadmium might not be the only factor affecting growth. Since the data are highly variable, only a few definite conclusions can be drawn. Despite this variability it is evident that cadmium is detrimental to the nitrogen fixation system of Alnus rubra in the concentration range of 0.1 to 5.0 mg CdCl₂ per liter of nutrient solution.

Cadmium in the concentration range of 5 to 200 µg CdCl₂ per liter of nutrient solution did not significantly alter the growth and nitrogen accumulation of Alnus rubra. Nitrogenase activity, however, was decreased between the 20 and 200 µg CdCl₂ per liter treatments when expressed on a dry weight basis (Fig. 17). It was also observed that nodulation increased with increasing cadmium in this concentration range. The reduced enzyme activity was apparently compensated by an increased number of nodules on the roots. Although nitrogenase activity was inhibited, few of the other plant processes were affected significantly in the concentration range of 5 to 100 µg CdCl₂ per liter of nutrient solution. These data indicate that cadmium selectively inhibited the nitrogen fixation system of Alnus rubra in the concentration range of 20 to 100 µg CdCl₂ per liter.

Three ranges of cadmium concentrations, namely: 2 to 25 mg per liter; 0.1 to 5.0 mg per liter; and 5 to 200 μg per liter, were given to plants at different times of the year. Thus, the effects of cadmium on plants grown at one time during the year are not comparable with effects during another growth period. The most important environmental variables are light intensity, photoperiod, temperature and concomitant relative humidity, and medium moisture variations. There was little variation in the water or dilute acid extractable cadmium in the culture solution. The concentrations of cadmium in the plants grown at different periods showed relatively little variability.

The data in Figures 30 and 31 show that initiation of nodulation decreased with increasing cadmium. Although nodulation was stimulated by the presence of cadmium in previously nodulated plants, it would be expected to await root growth in non-nodulated plants from which a source of fixed nitrogen had been removed. Addition of cadmium decreased root growth and delayed nodulation (Figs. 29 and 31). As shown by the data in Figure 33 addition of 0 to 100 μg CdCl_2 per liter of nutrient solution delayed nitrogen fixation until near the end of the experiment. An insignificant amount of nitrogen was fixed although nitrogenase activity was detected.

When Alnus rubra plants were cultured in nutrient solution containing 12 mM NH_4NO_3 growth was not inhibited by cadmium

although a decrease in nitrate reductase activity was observed. These results suggest that the rates of nitrate reduction were not growth limiting. Alternately, ammonia might have been used as nitrogen for plant growth. Assuming that ammonia was utilized in this experiment, ammonia utilization apparently was not affected by the concentrations of cadmium that were investigated.

When the NH_4NO_3 concentration was reduced to 6 mM, nitrogen was growth limiting (Fig. 40). The plants were about one-fourth the size of those provided with 12 mM ammonium nitrate (Figs. 37 and 40). These results indicate that growth responses of Alnus rubra to ammonia nitrogen were greater than with nitrate. The differences in responses to forms of nitrogen complicated the experiment and obscured any cadmium effects on growth. It is concluded, however, that cadmium inhibited nitrate reductase activity and growth of non-nodulated Alnus rubra.

Studies of the ultrastructure of the nodules of Alnus rubra indicate that endophyte resorption is increased by the addition of cadmium to the nutrient medium. The presence of cadmium in some unknown way possibly influences some regulatory physiological process that controls resorption. Endophyte resorption in the nodules of Alnus spp. has not been studied extensively and the mechanisms that are involved in stimulation of the process by cadmium are not known. Further research is required.

Increasing prominence of membranes and cell organelles in yeast cells exposed to cadmium had been reported (29). Cadmium apparently alters the characteristics of membranes resulting in heavy staining of electron dense material. The prominent nucleoli in the root cells of Alnus rubra treated with cadmium may also be explained in terms of altered structural characteristics that permit dense staining. These postulations do not account for the failure to observe nucleoli in the root cells of control plants. Studies of the ultrastructure of the roots of nodulated Alnus spp. have not been previously reported. Nucleoli are indicative of resting cells and are less likely to be observed in rapidly dividing cells. It may be suggested that cells exposed to cadmium were dividing more slowly than corresponding control cells. Therefore nucleoli remained visible longer and were more likely to be observed in cells from plants given cadmium.

The mitochondria of yeast cells exposed to cadmium have been reported to lack cristae (29). The electron micrographs of mitochondria of Alnus root and nodule cells indicate that cadmium treatment caused some decrease in the occurrence of well defined cristae (Plates 27, 30, 36, and 39). The observations were not consistent, however, in all samples, and further research in this area is needed for more definitive results.

Starch grains were apparent in the root cells of plants exposed to cadmium, but were not obvious in cells of comparable control

plants. Inhibition of cellular growth by cadmium could lead to the formation of starch. This is based upon the assumption that cellular growth utilized a major part of the carbohydrate reserves.

SUMMARY

These investigations show that excessive cadmium in tissues inhibited nitrogen fixation and growth of Alnus rubra. A summary of the investigations of the effects of cadmium on the nitrogen fixation system in Alnus rubra and on the activity of nitrogenase preparations from soybean nodules is presented:

1. It was determined that in situ nitrogenase activity of the nodules of Alnus rubra was decreased by increasing cadmium concentrations in nutrient media and by increasing duration of treatments. The weights of shoots, roots, nodules and the fixation of atmospheric nitrogen were decreased by increasing cadmium concentrations in the range 0.2 to 25 mg CdCl_2 per liter of nutrient solution.
2. Increasing amounts of cadmium were accumulated in the plant organs with increasing concentrations of the element (range 5 μg to 25 mg CdCl_2 per liter) in the nutrient solution. Cadmium accumulation also increased with duration of treatments.
3. Plant growth and fixation of atmospheric nitrogen (as determined by total N fixed) were decreased by addition to nutrient solutions of cadmium concentrations in the range 0.2 to 25 mg CdCl_2 per liter. No significant decreases occurred below this range.
4. In the concentration range of 20 to 100 μg CdCl_2 per liter, extent

of nodulation of A. rubra roots increased. This increase compensated to some extent for the effects of cadmium on nitrogenase activity.

5. Cadmium concentrations in the range of 10 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution inhibited the initiation of nodulation of Alnus rubra.
6. The growth of plants with fixed nitrogen (without nodules) was reduced as the cadmium concentration increased from 20 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution. Under these conditions nitrogen was growth limiting. Growth was not reduced by cadmium when ammonium nitrate was added to the nutrient solution at double the normal concentration.
7. Treatment with 50 and 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution depressed in vivo nitrate reductase activity of the roots of non-nodulated Alnus rubra.
8. On the basis of electron micrographs of nodules the amount of endophyte protoplast decreased at cadmium concentrations of 50 and 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution. Also clear areas appeared in the host cell mitochondria at cadmium concentrations of 50 and 100 $\mu\text{g CdCl}_2$ per liter.
9. An increase in the prominence of nucleoli and occurrence of starch grains were observed in the root xylem parenchyma cells of Alnus rubra as the cadmium concentration increased

from 20 to 100 $\mu\text{g CdCl}_2$ per liter of nutrient solution. Also there was an increase in the number of apparent clear spaces in the mitochondria.

10. A decrease was observed in the in vitro activity of nitrogenase extracted from soybean root nodules when the Mo-Fe protein was pre-incubated with CdCl_2 . An increase in the activity of nitrogenase at CdCl_2 concentrations of 11 and 27 μM was observed when the Fe protein and combined proteins were pre-incubated.
11. Increasing the sodium dithionite concentration reversed the cadmium induced decline of nitrogenase activity that was observed when the Fe protein and the combined fractions were pre-incubated with CdCl_2 at 136 μM concentration.
12. Evidence was obtained that cadmium may compete with and partially replace the role of magnesium in the nitrogenase system at the CdCl_2 concentration of 136 μM .

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