Plant Physiol. (1974) 53, 934–941

Effect of Carbon Dioxide on Nitrate Accumulation and Nitrate Reductase Induction in Corn Seedlings[1]

Received for publication November 7, 1973 and in revised form January 14, 1974

A. C. PURVIS,[2] D. B. PETERS, AND R. H. HAGEMAN
Department of Agronomy, University of Illinois, Urbana, Illinois 61801

ABSTRACT

Exposure of the leaf canopy of corn seedlings (*Zea mays* L.) to atmospheric CO_2 levels ranging from 100 to 800 μl/l decreased nitrate accumulation and nitrate reductase activity. Plants pretreated with CO_2 in the dark and maintained in an atmosphere containing 100 μl/l CO_2 accumulated 7-fold more nitrate and had 2-fold more nitrate reductase activity than plants exposed to 600 μl/l CO_2, after 5 hours of illumination. Induction of nitrate reductase activity in leaves of intact corn seedlings was related to nitrate content. Changes in soluble protein were related to *in vitro* nitrate reductase activity suggesting that *in vitro* nitrate reductase activity was a measure of *in situ* nitrate reduction. In longer experiments, levels of nitrate reductase and accumulation of reduced N supported the concept that less nitrate was being absorbed, translocated, and assimilated when CO_2 was high. Plants exposed to increasing CO_2 levels for 3 to 4 hours in the light had increased concentrations of malate and decreased concentrations of nitrate in the leaf tissue. Malate and nitrate concentrations in the leaf tissue of seven of eight corn genotypes grown under comparable and normal (300 μl/l CO_2) environments, were negatively correlated. Exposure of roots to increasing concentrations of potassium carbonate with or without potassium sulfate caused a progressive increase in malate concentrations in the roots. When these roots were subsequently transferred to a nitrate medium, the accumulation of nitrate was inversely related to the initial malate concentrations. These data suggest that the concentration of malate in the tissue seem to be related to the accumulation of nitrate.

The induction of NRA[3] is dependent on, and within limits, proportional to, the nitrate content of the tissue (1). However, tissue NO_3^- exists in "active" and "inactive" pools with only the active pool available for induction of NR and subsequent reduction (12). Recently, Meeker (20) observed that the NO_3^- content of the leaf blade of corn, as opposed to the total No_3^- content (blade plus midrib), was proportional to NR activity. Induction and maintenance of NR activity in *Perilla* leaves (15) and corn and pigweed leaves (17) have also been shown to be dependent on the presence of CO_2. Klepper *et al.* (17) also suggested that CO_2 fixation may be necessary to supply reducing power for NO_3^- reduction. Travis *et al.* (26) have shown a definite requirement for energy for polysome formation, and polysome formation was related to the subsequent increase in NR observed when plants were exposed to light. The present investigation was undertaken to: (*a*) determine the effect of ambient CO_2 concentrations ranging from 100 to 800 μl/l on NO_3^- accumulation and induction of NR; (*b*) distinguished between the CO_2 effect on NO_3^- uptake and utilization; and (*c*) establish how CO_2 levels affect NO_3^- uptake and metabolism.

MATERIALS AND METHODS

Plant Material. Corn (*Zea mays* L.) seeds were obtained from R. J. Lambert, University of Illinois, Urbana. Genotypes used were B37, B14 × B37, B14 × OH43, B14, OH43, B14 × R177, R177, and Dixie 82.

Vermiculite Culture. Seedlings were cultured in 10-quart plastic pans with perforated bottoms to permit subirrigation. Vermiculite (Zonolite, Co., Chicago) was used as a supporting medium and was subirrigated daily with a nutrient solution. The ammonia culture medium contained: 3 mM $MgSO_4$; 1 mM KH_2PO_4; 1 mM K_2SO_4; 3 mM $CaCl_2$; 5 mM $(NH_4)_2SO_4$; Fe^{3+} supplied as Chel-138, (Geigy Chem. Co., Ardsley, N. Y.), and micronutrients (25). When NO_3^- was the desired source of nitrogen the medium contained: 3 mM $MgSO_4$; 1 mM KH_2PO_4; 5 mM $Ca(No_3)_2$; 5 mM KNO_3; 2.5 mM $(NH_4)_2SO_4$; Fe^{3+} supplied as Chel-138, 0.05 mM; and micronutrients. The pH of the nutrient solution was adjusted to 4.5 before use. The containers were placed in a dark germinator at 30 C and 65% relative humidity for 2 days before being transferred to growth chambers.

Liquid Culture. The method of Meeker (20) was used for culturing seedlings in liquid. Seeds were surface-sterilized with 0.37% sodium hypochlorite and were germinated for 3 days on water-saturated paper towelling in Pyrex utility dishes in a dark germinator. The radicles of the 3-day-old seedlings were inserted directly through 2-mm holes drilled in a 120-mm × 120-mm blackened Plexiglas sheet which fitted into the top of a 2-quart freezer container. Nutrient solution was half-strength Hoagland's medium with respect to major elements, iron was increased to 0.45 mM supplied as Chel-330, and the concentration of other micronutrients were as described, except zinc was tripled. The changes in Fe and Zn concentrations were made to reduce chlorosis which developed in corn seedlings cultured in liquid at high light intensities. The pH of the solutions was adjusted daily and solutions were renewed every 2 days. All equipment was thoroughly washed and sterilized with a 0.37% sodium hypochlorite solution before each transplanting.

[1] This work was supported by Hatch Funds, Department of Agronomy, University of Illinois, Urbana, and by United States Department of Agriculture Cooperative Agreements Nos. 12-14-100-10-057, 12-14-100-2430(41), and by a Frasch Foundation grant.

[2] Present address: Department of Botany, North Carolina State University, Raleigh, N. C. 27607.

[3] Abbreviations: NRA: nitrate reductase activity; NR: nitrate reductase.

Growth Chambers. The CO_2 studies were conducted in airtight chambers covered with 100 μm polyethylene film. The chambers were lighted from two sides with lights external to the chamber. The light intensity (53,000 lux) was obtained from an array of four Power Gruve tubes (1.3 m), one Multi-Vapor lamp (1000 w), and six Lucalox bulbs (400 w) (General Electric, Schenectady, N. Y.), arranged on both sides of the chamber. Carbon dioxide concentration in each chamber was controlled by monitoring a continuous flow of gas from the chamber with a Beckman Model 215A infrared gas analyzer. The gas analyzer was fitted with dual output leads which permitted simultaneous use of a millivolt recorder (for measurement of CO_2 rate changes) and a double set point meter relay controller (for control of CO_2 concentration).

The lower set point of the controller was operative during the light period when photosynthesis was acting to remove CO_2 from the chamber. When the concentration dropped to the set point, the controller activated a time delay relay which in turn opened a solenoid valve to allow injection of CO_2 into the chamber. The rate of CO_2 injection was controlled and monitored by a mass flowmeter. The duration of injection was controlled by the time delay relay which could be adjusted between 0 and 10 sec. This feature allowed control of the concentration in the chamber within any prescribed limit with a sensitivity of 5 μl/l. Following the period of injection, photosynthesis would again remove CO_2 from the chamber. This rate of change of concentration was monitored.

The upper set point of the controller was operative during the night when CO_2 was being evolved by the plants. When the concentration was above the set point the controller actuated a pump which removed air from the chamber and passed the air through a KOH scrubber for removal of CO_2. When the concentration returned to the set point the controller actuated a time delay relay which allowed the concentration in the chamber to increase for a preset time before actuation of the pump for removal of CO_2 from the air. The rate of change of CO_2 concentration was linear and calibrated for determination of CO_2 removal rate (photosynthesis) or addition rate (respiration).

Other studies were conducted in growth chambers with a light intensity of 27,000 lux (cool white fluorescent tubes supplemented with incandescent bulbs). In all cases, light-dark periods of 14 and 10 hr, and temperatures of 32 C and 27 C were used.

Inductions. Induction of NR with 8-day-old ammonia-cultured seedlings was initiated by adding KNO_3 (10 or 15 mM) to the culture medium. Preliminary experiments showed that flushing the supporting medium (vermiculite culture) with water before irrigation with the NO_3^- nutrient solution had no effect on subsequent NO_3^- accumulation and NR induction. Inductions were conducted in growth chambers at temperatures and light intensities used for growth.

Salt-induced Malate Synthesis. Seedlings were cultured (in liquid) on the ammonia medium previously described. On the 7th day, the roots were thoroughly rinsed in distilled water, the solution was replaced with 5 mM tris-KOH buffer, pH 9.5, containing the various concentrations of salts (1–10 mM K_2CO_3 and K_2SO_4), and seedlings were replaced in the containers. The high pH treatments caused no visible injury to the roots. Eighteen hr (4 light-10 dark-4 light) later the roots were thoroughly rinsed in distilled water, the tris-buffered salt solutions were replaced with a solution containing only 5 mM KNO_3, and seedlings were replaced in the containers. Root samples were taken at zero time for malate and after 3 hr for malate and NO_3^- determinations.

Plant Tissue. All expanded leaf tissue (with midrib) was stripped from 8-day-old plants which had received 4 hr illumination before harvesting, unless otherwise stated. The tissue was cut into small pieces and a random sample was taken for homogenization. Root tissue was rinsed for 2 min with running tap water and then placed in 0.5 mM $CaSO_4$, at 3 C, for 10 min. Tissue was then removed, blotted dry, and cut into small pieces before sampling.

Extraction and Assay for Enzymes. Tissue was homogenized by hand with a TenBroeck homogenizer in extraction media (10:1, v/w) containing 10 mM cysteine·HCl, 25 mM K_2KPO_4, and 5 mM EDTA at a final pH of 8.6. Homogenates were then centrifuged (15 min at 27,000*g*) to obtain supernatants used for NR assays, water soluble protein and NO_3^- determinations. Extraction was carried out at 0 to 3 C. *In vitro* nitrate reductase was assayed as described by Schrader *et al.* (25).

Chemical Assays. Nitrate was determined on the clarified enzyme extracts or water extracts by the method of McNamara *et al.* (19). Samples of the dry materials were extracted with deionized water (100:1, v/w) in a shaker water bath at 40 C for 4 hr, and NO_3^- determinations were made on filtered water extracts. Malate was extracted and assayed according to the method of Williamson and Corkley (29). Malate dehydrogenase (Sigma, No. 410-9) was diluted to provide 5 μM (Sigma) units per ml. Soluble protein in the clarified homogenates was determined by the Folin method (18) using bovine serum albumin, fraction V (Nutritional Biochemicals Corp.) as a standard. Total N determinations were made on material which had been dried in an oven at 70 C for 48 hr, ground, and passed through a 20-mesh screen according to the methods of Peterson and Chesters (24).

RESULTS

EFFECT OF CO_2

A. Induction of NR and Accumulation of NO_3^- in Intact Seedlings. As the CO_2 concentration was increased from 200 to 800 μl/l, the net accumulation of NO_3^- was decreased for the seven genotypes tested, as shown by typical patterns in Figure 1. The net induction of NR activity paralleled the NO_3^- concentrations for five of the genotypes (as illustrated in Fig. 1A), while two of the genotypes (OH43 and B14 × R177) exhibited the pattern shown in Figure 1B. All genotypes exhibited low endogenous levels of NO_3^- and NR activity before induction and these levels did not change during the 19 hr CO_2 pretreatment before addition of NO_3^-.

In a second experiment, lower CO_2 concentrations were used and the dark CO_2 pretreatment time was decreased to 2 hr (Fig. 2). The CO_2 effects were similar to those obtained in the first experiment. However, CO_2 concentrations of 200 μl/l and lower did not suppress NR induction in B14 × R177 and OH43 as in the previous experiment. Treatments of 100 and 150 μl/l of CO_2 caused high accumulation of NO_3^- in all genotypes, and the maximum accumulation of NO_3^- (15.2 and 15.7 μmoles g fresh wt^{-1} for B14 and B14 × R177, respectively) was obtained at 100 μl/l of CO_2. There seemed to be no effect of length of the dark pretreatment time with CO_2 on the subsequent accumulation of NO_3^- and NR induction.

The decreased accumulation of NO_3^-, affected by CO_2, noted in these experiments may be attributed to: (*a*) an increased assimilation of NO_3^- as CO_2 was increased in the presence of light (11, 17), which is unlikely because of the decrease in NRA; (*b*) a direct or indirect interference with NO_3^- uptake and translocation of NO_3^- to the leaf tissue as CO_2 concentration was increased; or (*c*) a combination of *a* and *b*. Increases in ambient CO_2 concentrations from 0 to 800 μl/l of CO_2 could decrease stomatal opening and transpiration, and thus

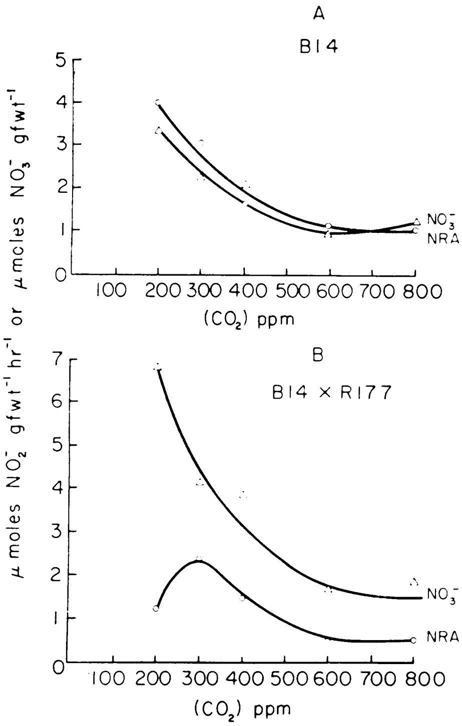

FIG. 1. Effect of ambient CO_2 (200–800 μl/l) on the net induction (final minus initial level) of nitrate reductase and nitrate accumulation in leaves of 8-day-old corn seedlings. Inductions were initiated by the addition of 15 mM KNO_3 to the root medium (vermiculite) of ammonia-cultured seedlings 19 hr (7 hr light-10 hr dark-2 hr light) after initiation of CO_2 treatments. Seedlings were induced for 5 hr. Initial levels of nitrate reductase and nitrate were 1.9 and 1.8 μmoles of NO_2^- produced hr^{-1} g fresh wt^{-1}, 0.3 and 0.4 μmole of NO_3^- g fresh wt^{-1} at the time of addition of nitrate for genotypes B14 and B14 × R177, respectively.

indirectly decrease NO_3^- accumulation. Work with corn (7, 21) and *Vicia faba* (23) has shown that such increases in CO_2 concentrations decrease transpiration up to 55%. Thus, changes in transpiration could contribute, but are insufficient in magnitude to account for the changes in NO_3^- accumulation observed, especially in CO_2 concentrations ranging from 100 to 400 μl/l.

B. Rate of NO_3^- Accumulation and Induction of NR in Leaf Tissue. The changes in NR (induction) and NO_3^- accumulated by the leaves of corn genotype B14 × B37, in response to varying ambient CO_2 concentrations and subsequent addition of NO_3^- to the root medium are shown in Figure 3. During the first hr, plants treated with 100 and 300 μl/l of CO_2 induced identical amounts of NR activity (1.3 μmoles of NO_2^- produced g fresh wt^{-1} hr^{-1}) but accumulated NO_3^- at different rates (2.3 and 0.7 μmoles g fresh wt^{-1}, respectively). In contrast, during the first hr, plants treated with 400 and 600 μl/l of CO_2 accumulated only 0.2 μmole of NO_3^- g fresh wt^{-1}, and there was no induction of NR activity. This suggests that an apparent threshold level of NO_3^- accumulation in the whole leaf must be reached before initiation of enzyme induction. However, these measurements did not distinguish between "mobile" and "storage" pools of NO_3^- as proposed by Heimer and Filner, (12).

After the first hr, plants treated with 100 μl/l of CO_2 accumulated NO_3^- in the leaf tissue in a linear manner. In contrast, plants treated with higher CO_2 concentrations accumulated NO_3^- in a parabolic manner. Plants treated with 400 and 600 μl/l of CO_2 had extremely low rates of accumulation during the first 3 hr of the experiment, and the rates of NR induction were also low.

The effects of CO_2 treatments on induction of NR activity are shown by the levels of enzyme activity at the end of the experiment. Plants exposed to 300, 400, and 600 μl/l of CO_2 had 83, 70, and 56%, respectively of the activity of plants treated with 100 μl/l of CO_2.

Because induction of NR activity normally follows the influx of NO_3^- into the tissue (12, 13), these data support the contention that increased levels of CO_2 in some way suppresses the movement of nitrate into the leaf. Conversely, it is possible that the higher CO_2 level enhanced assimilation rates and thus assimilation could be the major factor regulating NO_3^- accumulation. This seems unlikely, since all plants had nearly identical and low initial levels of NR activity. Thus, assuming that the influx of NO_3^- into the leaf was unaffected by CO_2 treatments, the *in situ* reduction of NO_3^- would have had to be negligible at 100 μl/l of CO_2 and drastically reduced at 300 μl/l of CO_2 to account for the differential levels of NO_3^- among the treatments at the end of the first hr. However, both the 100- and 300-μl/l CO_2 treatments had greater amounts of NR than the other CO_2 treatments.

C. NR Induction and NO_3^- Accumulation in Seedlings in Liquid Culture. In all previous experiments, plants were cultured in a vermiculite medium and the porous medium was exposed to various CO_2 concentrations. To determine whether the CO_2 was exerting a direct effect on NR induction and NO_3^-

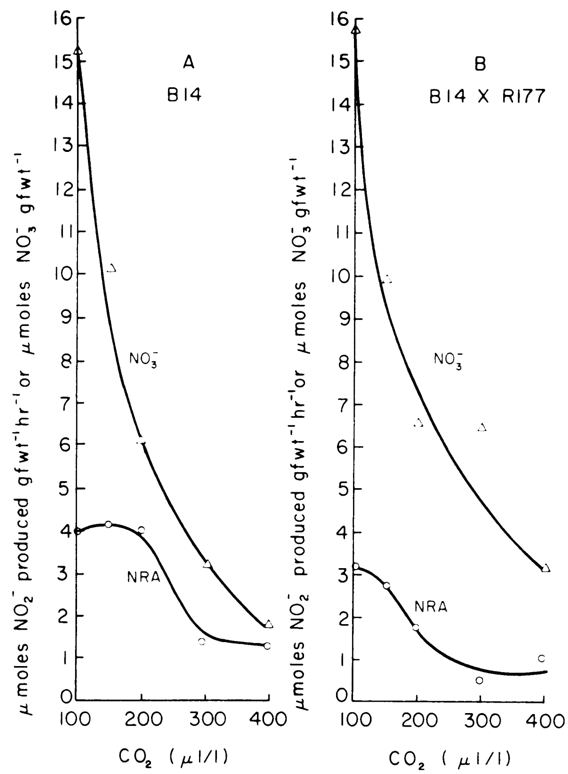

FIG. 2. Effect of ambient CO_2 (100–400 μl/l) on the net induction of nitrate reductase and nitrate accumulation in leaves of 8-day-old corn seedlings. Experimental details were as described for Figure 1, except that the CO_2 pretreatments were initiated 4 hr (2 hr dark, followed by 2 hr light) before addition of KNO_3. Initial levels of nitrate reductase and nitrate were 2.1 and 1.4 μmoles of NO_2^- produced hr^{-1} g fresh wt^{-1}, 0.4 and 0.4 μmole of NO_3^- g fresh wt^{-1} at the time of addition of nitrate for genotypes B14 and B14 × R177, respectively.

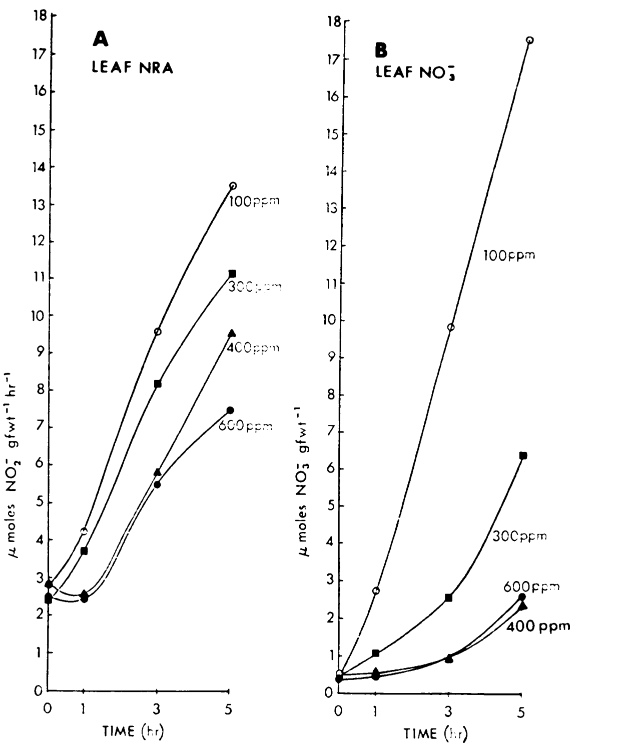

FIG. 3. Effect of ambient CO_2 (100–600 μl/l) on the rates of nitrate reductase induction and nitrate accumulation in leaves of intact 8-day-old B14 × B37 corn seedlings. Inductions were initiated by the addition of 15 mM KNO_3 to the root medium (vermiculite) of ammonia-cultured seedlings and the equilibration (30 min dark) of CO_2 concentrations made after a 10-hr dark period. Each plotted point represents the average values of four separate samples obtained from two experiments. The two experiments were nearly identical with minimal variation.

accumulation in the leaf or an indirect effect via root absorption, plants were cultured in aerated nutrient media and roots of all treatments were aerated with air from a common source. Positive pressure within the root container and sealing of the container except for a small outlet minimized direct CO_2 contact with the nutrient solution. The results of these experiments (Fig. 4) show that exclusion of roots from exposure to the various aerial CO_2 concentrations did not alter the treatment effects on NR induction and NO_3^- accumulation by the leaves. Increasing CO_2 concentrations from 100 to 600 μl/l of CO_2 caused a progressive decrease in accumulation of NO_3^- in expanded leaf tissue and "stem" (unexpanded leaf and stalk tissue above the root node) and in induction of NR in leaf tissue with two exceptions. These exceptions were: the low level of NRA induced in the leaves at 100 μl/l of CO_2; and the higher amount of NO_3^- in the stems at 600 μl/l of CO_2. The failure to attain induction of NR in relation to NO_3^- accumulation at low levels of CO_2 was noted earlier, where variation was observed among experiments (*cf.* Fig. 1B with Fig. 2B), with the same genotype. It is suggested that the metabolic status, specifically the carbohydrate supply from the diminishing endosperm reserve, of these 8-day-old seedlings, may be responsible for the variation in induction of NR activity at the lower CO_2 concentration (17).

D. Changes in NRA, NO_3^- and Water Soluble Protein of Seedlings. To distinguish further between the effects of CO_2 treatments on uptake, transport, and assimilation of NO_3^-, attempts were made to estimate assimilation by following changes in water soluble protein (5, 8, 9). The effects of CO_2 treatments on net changes of NR activity, NO_3^-, and water soluble protein in seedlings that initially contained high levels of NR activity (8.8 μmoles NO_3^- reduced g fresh wt^{-1} hr^{-1}) and NO_3^- (32.2 μmoles g fresh wt^{-1}) are shown in Figure 5, A and B. At the end of the 4 hr treatment, the actual NO_3^- concentrations in the leaves ranged from 52 to 24 μmoles g fresh wt^{-1} for the highest and lowest CO_2 treatments (100 and 600 μl/l). As shown in Figure 5B, the plants exposed to 100 μl/l of CO_2 exhibited a net increase of 20 μmoles NO_3^- g fresh wt^{-1}, while the plants exposed to 600 μl/l of CO_2 exhibited a net decrease of 8 μmoles g fresh wt^{-1}. Thus NO_3^- accumulation by the leaves was affected by the CO_2 treatments in a similar manner regardless of whether the plants had been grown with or without NO_3^- before treatment (*cf.* patterns of NO_3^- in Figs. 2 and 4 with 5B). While CO_2 treatments of 200, 300 and 400 μl/l of CO_2 caused substantial net increases in NR activity, the increase in NR activity with both 100 and 600 μl/l of CO_2 was much smaller (Fig. 5A). These variations in NR activity with CO_2 treatments were paralleled by net changes in water soluble protein concentrations (Fig. 5C). That these parallel changes in NRA and protein are a reflection of *in situ* NO_3^- assimilation is supported by recent work (4) which shows a high degree of correlation ($r = 0.98$) between daily changes in NRA and total reduced-N. While the decrease in net change of NRA and protein observed, as CO_2 treatments were increased from 200 to 600 μl/l, can be attributed to decreased transport of NO_3^- into the leaves (Fig. 5), this explanation will not hold for the 100 μl/l treatment. As previously noted, the amount of induc-

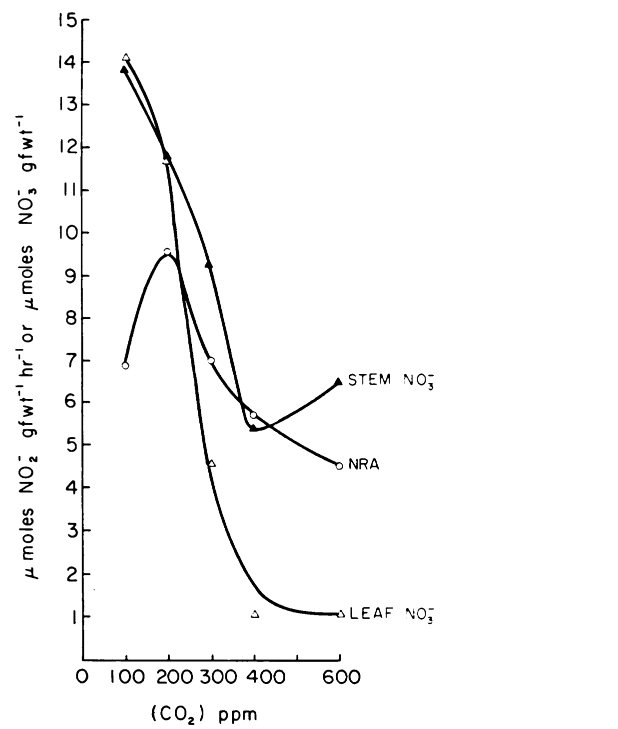

FIG. 4. Effect of ambient CO_2 on the induction of nitrate reductase in leaves and nitrate accumulation in leaves and "stems" of intact 8-day-old B14 × B37 corn seedlings in liquid culture. Inductions were initiated by the addition of 10 mM KNO_3 to the root medium of ammonia-cultured seedlings after a 10-hr dark period. CO_2 treatments were started simultaneously. Treatments lasted for 4 hr. Initial nitrate reductase was 2.1 μmoles of NO_2^- produced g fresh wt^{-1} hr^{-1} and initial levels of nitrate for leaves and "stems" were 0.4 and 1.4 μmoles g fresh wt^{-1}, respectively.

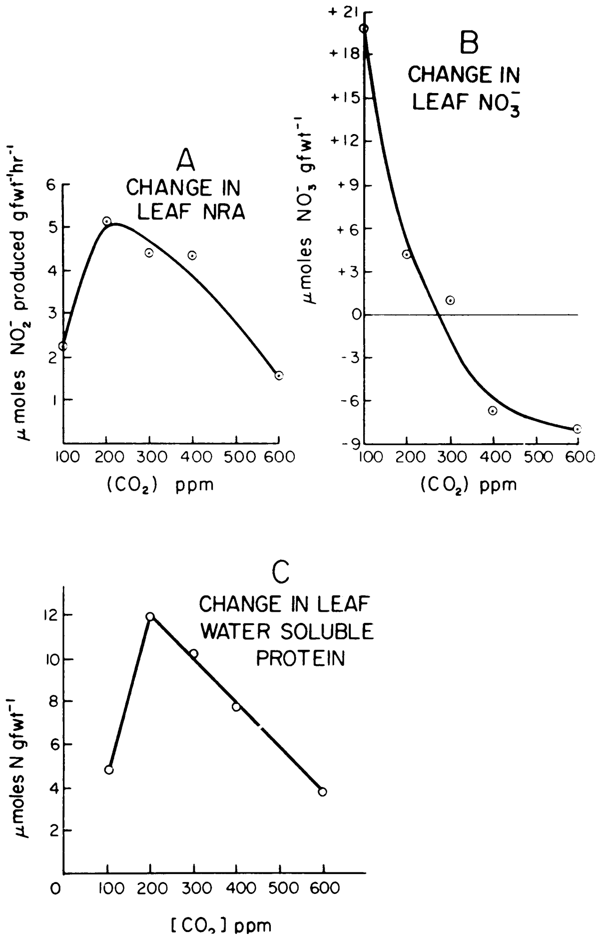

FIG. 5. Effect of variation in ambient CO_2 concentrations on the net changes in nitrate reductase activity, nitrate accumulation, and water soluble protein in leaves of 8-day-old B14 × B37 corn seedlings during a 4-hr light treatment. CO_2 treatments were initiated after a 10-hr dark period, 30 min before illumination. The plants had been cultured in vermiculite for 8 days on a 15 mM nitrate nutrient solution and were subirrigated with the nutrient solution at the beginning of the dark period. Endogenous nitrate reductase, nitrate, and water soluble protein were 8.8 μmolar units, 32.2 and 164.6 μmoles g fresh wt^{-1}, respectively just before CO_2 treatment.

tion of NR by plants treated with low levels of CO_2 varied from experiment to experiment (Figs. 1 and 2).

These data support the concept that increasing CO_2 concentrations affect the movement of NO_3^- into the leaf. Assuming that CO_2 concentrations do not affect the movement of NO_3^- into the leaf, data of Figure 5B show that plants treated with 600 μl/l of CO_2 should have assimilated 28 μmoles of NO_3^- g fresh wt^{-1} (increase of 20 of μmoles NO_3^- g fresh wt^{-1} at 100 μl/l of CO_2 plus the decrease of 8 μmoles of NO_3^- at 600 μl/l of CO_2). Assimilation rates of this magnitude are inconsistent with net increase of 3.7 μmoles of N as water soluble protein (Fig. 5C). Thus, the small increase in water soluble protein observed with the 600-μl/l CO_2 treatment suggests that the lack of an adequate supply of NO_3^- at the induction and assimilation sites is responsible for the small increases in both NR activity and water soluble protein. However, it is conceivable that CO_2 or a product of CO_2 metabolism is directly affecting protein synthesis.

These data show that increasing CO_2 concentrations are decreasing the transport of NO_3^- into the leaf and NO_3^- assimilation as reflected by changes in water soluble protein of the 200-, 300-, 400-, and 600-μl/l CO_2 treatments. Hardy *et al.* (10) suggested that increases in ambient CO_2 under field conditions suppress the utilization of soil nitrogen by soybean plants.

E. Long Term Experiments. Exposure of 8-day-old corn seedlings, previously cultured on NO_3^-, to 100, 300, 400, and 600 μl/l of CO_2 for a 72-hr period (14 hr light, 10 hr dark, with daily samplings made 4 hr into the light period) established 100 μl/l of CO_2 was inadequate for normal plant growth, and the plants became flaccid after 36 hr. Photosynthesis rates of 40, 130, 138 and 184 μmoles of CO_2 fixed g fresh wt^{-1} (leaf tissue) hr^{-1} were noted during the second day for the 100-, 300-, 400-, and 600-μl/l CO_2 treatments, respectively. These rates were reflected in net changes in dry weight of 75, 110, 118, and 130 mg shoot^{-1} over the 72 hr period. Considering only the three highest CO_2 treatments, there was no effect on NO_3^- concentration in the leaf tissue sampled at 24, 48, or 72 hr. With these three treatments, NO_3^- decreased from the initial level of 50 μmoles g fresh wt^{-1} to 25 to 30 μmoles g fresh wt^{-1} after 24 hr and to 17 to 25 μmoles g fresh wt^{-1} after 72 hr. In contrast, plants treated with 100 μl/l of CO_2 maintained the initial level of NO_3^- during the first day and decreased to 42 μmoles g fresh wt^{-1} by the end of the experiment.

The lack of differences in leaf NO_3^- accumulated by the end of the experiment, among the 300-, 400-, and 600-μl/l CO_2 treatments, suggests that NO_3^- assimilation decreases with increases in CO_2 concentration. This assumes that in long term as well as short term experiments, the transport of NO_3^- to the leaf decreases with increases in CO_2 levels. Other evidence that NO_3^- assimilation decreases with increases in CO_2 concentration were also noted. The first was that plants treated with 300 μl/l of CO_2 had more NR activity in leaf tissue than those treated with 400 and 600 μl/l of CO_2 when sampled at 24, 48, and 72 hr. Plants exposed to 300, 400, and 600 of μl/l CO_2 for 72 hr had NR activities of 12, 9, and 7.5 μmoles of NO_2^- produced g fresh wt^{-1} hr^{-1}, respectively. Other work (12, 13, 20) suggests that the level of activity is a reflection of the rate of NO_3^- movement into the leaf. The second was that the net change in total reduced N per shoot decreased slightly with increasing CO_2 concentrations during the 72 hr period. The changes were 4.3, 3.8, and 3.8 mg N shoot^{-1} for CO_2 treatments of 300-, 400-, and 600-μl/l of CO_2, respectively. This is in contrast to the net increases in dry weight of the same plants.

The changes in NRA, NO_3^-, and reduced N associated with increases in CO_2 concentrations from 300 to 600 μl/l of CO_2 during the 72-hr period were comparable to changes observed in the short term experiment (Fig. 5). The major exception was that plants treated with 600 μl/l exhibited a net decrease in NR activity from the initial level (9.7 μmoles of NO_2^- produced g fresh wt^{-1} hr^{-1}) for all samplings.

F. NO_3^- Accumulation and NR of Seedlings in the Dark. Since short term exposures of seedlings to increasing CO_2 concentrations in the light period caused marked differences in NR induction and NO_3^- accumulation, assessment of exposure of plants to various levels of CO_2 in the dark, was undertaken. During the 4-hr dark treatment period, there was no change in levels of NRA, and the small increase in NO_3^- concentrations in the leaves was independent of the CO_2 treatments (75–600 μl/l of CO_2) imposed (data not shown).

Malate as a Possible Factor Mediating the CO_2 Effect. That malate and NO_3^- metabolism are interrelated is suggested by the observations that malate is synthesized and catabolized to maintain pH and charge balance with respect to ion flux and NO_3^- assimilation (3, 6, 16, 18, 27). It has also been suggested that malate oxidation plays a key role in NO_3^- uptake in the root (2, 3). *In vitro* studies with phosphoenolpyruvic carboxylase from *Kalanchoe* leaves have shown that increases in CO_2 concentrations increases malate production (28), and malate concentrations vary diurnally in corn leaves with highest levels occurring during the light period (unpublished). Accordingly, experiments were undertaken to explore the possibility that malate was involved in mediating the CO_2 effect on NO_3^- accumulation.

Effect of CO_2 on Accumulation of Malate and NO_3^- in Leaves of Corn Seedlings. The hourly changes in concentration of malate and NO_3^- in the leaf tissue are shown in Figure 6. The trend in malate accumulation with respect to the CO_2 treatments was opposite that of NO_3^-. Because the rates of accumulation of malate and NO_3^- were different within each CO_2 treatment, it does not seem that the total metabolic processes of malate synthesis and utilization are directly coupled to NO_3^- accumulation and assimilation.

While these data can be used to support the hypothesis that malate is accumulated to maintain pH and charge balance following NO_3^- reduction (3, 6, 16), previous experiments have shown that assimilation of NO_3^- does not account for differences in levels of accumulated NO_3^- at the various CO_2 concentrations. In addition, there was a rapid increase in malate immediately after initiation of treatments and before increases in NR activity were noted (*cf.* Figs. 3 and 6). Therefore, the possibility exists that the concentration of malate in the tissue has a more direct effect on the accumulation of NO_3^-.

Relationship of Concentrations of Malate and Nitrate. The relationship between the concentrations of malate and NO_3^- in the leaf tissue of eight corn genotypes grown under normal conditions is shown in Figure 7. Malate and NO_3^- accumulations are negatively correlated with a significant r value of -0.747 (OH43 omitted). It could be argued that the reduction of NO_3^- enhances malate synthesis and accumulation, and decreases the NO_3^- content of the tissue, but a plot of NR activity and malate content of each genotype failed to establish such a relationship.

The absence of malate in the OH43 genotype (Fig. 7) is considered to be an artifact arising from the experimental condi-

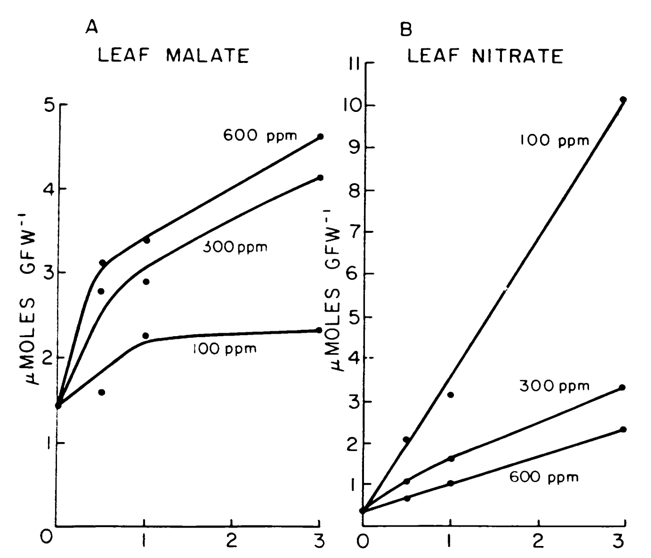

FIG. 6. Effect of variations in ambient CO_2 concentrations on accumulation of malate and nitrate in leaves of intact B37 × B14 corn seedlings. Carbon dioxide and nitrate treatments were initiated on 8-day-old ammonia-cultured seedlings (in vermiculite) at the end of a normal 10-hr dark period. The ammonia medium was displaced by subirrigation with the nitrate medium.

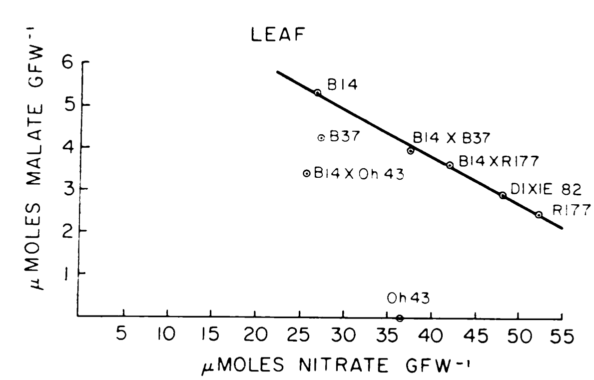

FIG. 7. Relationship between the concentration of malate and nitrate in the leaf tissue of eight genotypes of corn. All eight genotypes were grown in a common container. The growth medium was vermiculite subirrigated with a 15 mM NO_3^- nutrient solution. Leaf tissue was harvested after 4 hr of illumination on the 8th day after sowing.

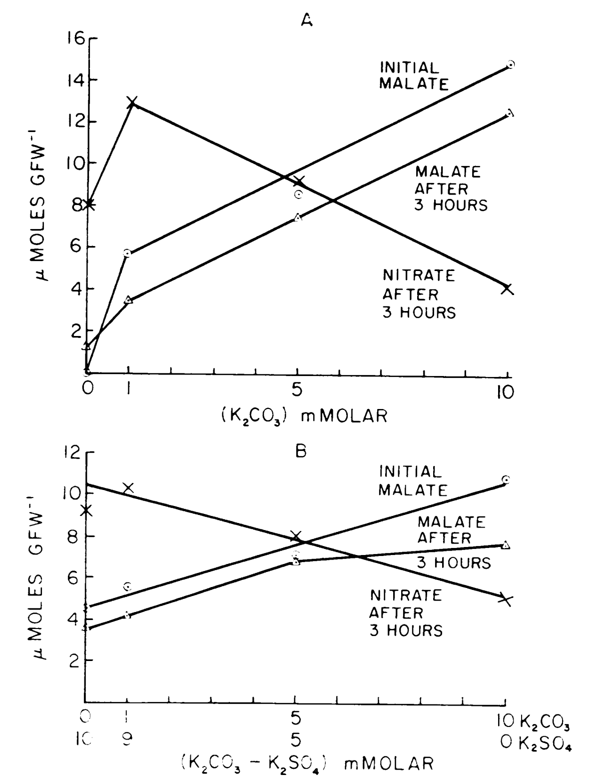

FIG. 8. Relationship between salt-induced malate accumulation in the roots of intact B37 × B14 corn seedlings and the subsequent accumulation of nitrate when the roots were transferred from the salt-inducing to a nitrate medium.

tions or seed source rather than a metabolic abberation. The OH43 seedlings were less vigorous and developed more slowly than the other genotypes. The extracts from OH43 did not contain an inhibitor of malate dehydrogenase.

Effect of Salt-induced Malate Accumulation on the Subsequent Uptake of Nitrate by the Root System of Corn Seedlings. Roots of intact seedlings treated with increasing concentrations of K_2CO_3, with or without K_2SO_4, accumulated malate in a linear manner (Fig. 8—initial malate). When such roots were transferred to a NO_3^- medium, the subsequent accumulations of NO_3^- were inversely related to the malate concentrations of the roots (Fig. 8). Thus, high concentrations of malate in the root seem to suppress rather than enhance NO_3^- uptake, as proposed by Ben-Zioni *et al.* (2, 3). These data also suggest that there is no stoichiometry between malate metabolism and NO_3^- accumulation, as the roots of all seedlings lost similar amounts of malate regardless of the amount of NO_3^- accumulated.

Although malate and NO_3^- concentrations in the roots were negatively correlated ($r = 0.99$) when the K_2CO_3 treatments ranged from 1 to 10 mM, the control (buffer only) treatment (no initial malate and 1.4 μmoles of malate g fresh wt^{-1} and 8 μmoles of NO_3^- g fresh wt^{-1} after 3 hr) did not fit this pattern (Fig. 8A). This exception raises the possibility that low levels of malate may be stimulating NO_3^- accumulation or that the relationship between malate and NO_3^- is indirect and complicated by K_2CO_3 effects.

Attempts to obtain varying levels of malate in root tissue by incubation of the roots in varying concentrations of malate were unsuccessful.

DISCUSSION

Increasing ambient CO_2 concentrations from 100 μl/l to 800 μl/l depressed NO_3^- accumulation and subsequent induction of NR in corn seedlings in the light. Increased assimilation of NO_3^- at high CO_2 concentrations (11) would not account for differences in NO_3^- accumulation at the various CO_2 concentrations. Concentrations of CO_2 above 200 μl/l seem to decrease NO_3^- assimilation in the leaves. Consequently CO_2 was directly or indirectly interfering with NO_3^- uptake and translocation into the leaves. There is no doubt that increasing levels of CO_2 increase stomatal resistance (7, 21, 23); hence transpiration and ion transport would be reduced. However, the 55 to 60% decreases in transpiration affected by increasing CO_2 concentrations from 100 to 800 μl/l (7, 21, 23) seem to be of insufficient magnitude to account for the differences (5- to 10-fold) in NO_3^- accumulation observed when CO_2 concentrations were increased from 100 to 400 μl/l.

Three sets of indirect evidence were obtained that suggest that malate could be a factor in mediating the CO_2 effect (decreased NO_3^- accumulation). First, increasing CO_2 concentrations around illuminated leaves increased malate and decreased NO_3^- concentrations in the leaves. Second, malate and NO_3^- concentrations in leaf tissue of seven corn genotypes (grown under comparable and standard environmental conditions) were negatively correlated. Third, roots containing varying levels of malate (induced by salt treatments) accumulated NO_3^- inversely to their malate concentration when transferred to a NO_3^- medium.

If NO_3^- absorption or accumulation or both are controlled by total ion and charge balance, it is possible that the negative relation between malate and NO_3^- observed, while valid under these experimental conditions, may not hold under all experimental conditions. These data in no way invalidate the well established principle that synthesis and catabolism of malate maintain pH and charge balance with respect to ion flux including NO_3^- assimilation (3, 6, 14, 27). Assuming that malate is the principal organic acid of fluctuation in maintaining pH and charge balance, one would anticipate a stoichiometric relationship between NO_3^- assimilation and malate synthesis. However, if NADH for NO_3^- reduction is generated by the oxidation of malate, as can be deduced from the work of Mulder *et al.* (22), the stoichiometry would be negated. It is also doubtful if a strict stoichiometry between NO_3^- uptake or assimilation, or both, and malate metabolism can be obtained *in situ*, especially with the whole plant over an extended period of time, for the following reasons. First, malate metabolism is involved in several metabolic systems that are probably compartmentalized (30). Second, the oxidation of 3-P-glyceraldehyde has also been proposed as a source of NADH for NO_3^- reduction (16). Third, the stoichiometry proposed between malate oxidation for HCO_3^- generation for NO_3^- uptake (2, 3) would hold only if other carbohydrates or organic acids were not catabolized. This does not imply that stoichiometry between NO_3^- and malate metabolism cannot be obtained in an isolated system.

LITERATURE CITED

1. BEEVERS, L., L. E. SCHRADER, D. FLESHER, AND R. H. HAGEMAN. 1965. The role of light and nitrate in the induction of nitrate reductase in radish cotyledons and maize seedlings. Plant Physiol. 40: 691-698.
2. BEN-ZIONI, A., Y. VAADAI, AND S. H. LIPS. 1970. Correlations between nitrate reduction, protein synthesis and malate accumulation. Physiol. Plant. 23: 1039-1047.
3. BEN-ZIONI, A., Y. VAADIA, AND S. H. LIPS. 1971. Nitrate uptake by roots as regulated by nitrate reduction products of the shoot. Physiol. Plant. 24: 288-290.
4. BRUNETTI, N. AND R. H. HAGEMAN. 1973. A comparison of *in vivo* and *in vitro* assays of nitrate reductase activity in wheat (*Triticum aestivum* L.) seedlings. Plant Physiol. 51: S-33.
5. CROY, L. E. AND R. H. HAGEMAN. 1970. Relationship of nitrate reductase activity to grain protein production in wheat. Crop Sci. 10: 280-285.
6. DIJKSHOORN, W. 1961. Metabolic regulation of the alkaline effect of nitrate utilization in plants. Nature 194: 165-167.
7. DOMES, W. 1971. Unterschiedliche CO_2-Abhangigkeit des Gasaustausches beider Blättseiten von *Zea mays*. Planta 98: 186-189.
8. HAGEMAN, R. H. AND D. FLESHER. 1960. Nitrate reductase activity in corn seedlings as affected by light and nitrate content of nutrient media. Plant Physiol. 35: 700-708.
9. HAGEMAN, R. H., D. FLESHER, AND A. GITTER. 1961. Diurnal variation and other light effects influencing the activity of nitrate reductase and nitrogen metabolism in corn. Crop Sci. 1: 201-204.
10. HARDY, R. W. F. AND U. D. HAVELKA. 1973. Symbiotic N_2 fixation: multifold enhancement by CO_2-enrichment of field-grown soybeans. Plant Physiol. 51: S-35.
11. HATTORI, A. 1962. Light-induced reduction of nitrate, nitrite, and hydroxylamine in a blue-green alga, *Anabaena cylindrica*. Plant Cell Physiol. 3: 355-369.
12. HEIMER, Y. M. AND P. FILNER. 1971. Regulation of the nitrate assimilation pathway in cultured tobacco cells. III. The nitrate uptake system. Biochem. Biophys. Acta 230: 362-372.
13. JACKSON, W. A., D. FLESHER, AND R. H. HAGEMAN. 1973. Nitrate uptake by dark-grown corn seedlings: some characteristics of apparent induction. Plant Physiol. 51: 120-127.
14. JACOBY, B. AND G. C. LATIES. 1971. Bicarbonate fixation and malate compartmentation in relation to salt-induced stoichiometric synthesis of organic acid. Plant Physiol. 47: 525-531.
15. KANNANGARA, G. C. AND H. W. WOOLHOUSE. 1967. The role of carbon dioxide, light, and nitrate in the synthesis and degradation of nitrate reductase in leaves of *Perilla fructescens*. New Phytol. 66: 533-567.
16. KIRKBY, E. A. AND K. MENGEL. 1967. Ionic balance in different tissues of the tomato plant in relation to nitrate, urea, or ammonium nutrition. Plant Physiol. 42: 1-5.
17. KLEPPER, L. A., D. FLESHER, AND R. H. HAGEMAN. 1971. Generation of reduced nicotinamide adenine dinucleotide for nitrate reduction in green leaves. Plant Physiol. 48: 580-590.
18. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.
19. MCNAMARA, A. L., G. B. MEEKER, P. D. SHAW, AND R. H. HAGEMAN. 1971. Use of a dissimilatory nitrate reductase from *Escherichia coli* and formate as a reductive system for nitrate assays. Agr. Food Chem. 19: 229-231.
20. MEEKER, G. B. 1972. The regulation of the seasonal cycles of nitrate reductase activity in corn (*Zea mays* L.) leaves by nitrate. Ph.D. thesis. University of Illinois, Urbana-Champaign.
21. MOSS, D. N. 1963. The effect of environment on gas exchange in leaves. Conn. Agr. Exp. Sta. Bull. 664: 87-99.
22. MULDER, E. G., R. BOXMA, AND W. L. VAN VEEN. 1959. The effects of molybdenum and nitrogen deficiencies on nitrate reduction in plant tissue. Plant Soil 10: 335-355.
23. PALLAS, J. E. AND B. G. WRIGHT. 1973. Organic acid changes in the epidermis of *Vicia faba* and their implication in stomatal movement. Plant Physiol. 51: 588-590.
24. PETERSON, L. A. AND G. CHESTERS. 1964. A reliable total nitrogen determination on plant tissue accumulating nitrate nitrogen. Agron. J. 56: 89-90.
25. SCHRADER, L. E., G. L. RITENOUR, G. L. EILRICH, AND R. H. HAGEMAN. 1968. Some characteristics of nitrate reductase from higher plants. Plant Physiol. 43: 930-940.

26. Travis, R. L., R. C. Huffaker, and J. L. Key. 1970. Light-induced development of polyribosomes and the induction of nitrate reductase activity in *Hordeum vulgare*. Plant Physiol. 23: 678–685.
27. Ulrich, A. 1941. Metabolism of nonvolatile organic acids in excised barley roots as related to cation-anion balance during salt accumulation. Amer. J. Bot. 28: 526–537.
28. Walker, D. A. and J. M. A. Brown. 1957. Physiological studies on acid metabolism. 5. Effects of carbon dioxide concentration on phosphoenolpyruvic carboxylase activity. Biochem. J. 67: 79–83.
29. Williamson, J. R. and B. E. Corkley. 1969. Assays of intermediates of the citric acid cycle and related compounds by fluorometric enzyme methods. Methods Enzymol. 13: 434–513.
30. Zschoche, W. C. and I. P. Ting. 1973. Malate dehydrogenases of *Pisum sativum*. Plant Physiol. 51: 1076–1081.