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In Vivo Nitrate Reduction in Relation to Nitrate Uptake, Nitrate Content, and *in Vitro* Nitrate Reductase Activity in Intact Barley Seedlings[1]

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ABSTRACT

A study was done to relate the *in vivo* reduction of nitrate to nitrate uptake, nitrate accumulation, and induction of nitrate reductase activity in intact barley seedlings (*Hordeum vulgare* L. var. 'Numar'). The characteristics of nitrate uptake in response to both time and ambient concentration of nitrate regulated reduction and accumulation. Uptake, accumulation, and *in vivo* reduction achieved steady state rates in 3 to 4 hours, whereas extractable (*in vitro*) nitrate reductase activity was still increasing at 12 hours. *In vivo* reduction of nitrate was better correlated exponentially than linearly over time with *in vitro* activity of nitrate reductase. A similar relationship occurred over increasing concentration of nitrate in the ambient solution. The results suggest that the rate of *in vivo* reduction of nitrate in barley seedlings may be regulated by the rate of uptake at the ambient concentrations of nitrate employed in the study.

The assimilation of nitrate from uptake through reduction includes several inducible processes including an apparent induction of a root transport system and root and leaf reductases. Uptake of nitrate is characterized initially by an exponential rate followed by a linear rate, a typical two-phase pattern already reported for several plants (11, 13, 19) and *Neurospora* (21). The exponentially developing time-course curve for uptake is indicative of an inducible transport system. Development of the accelerated rate is counteracted by inhibitors of RNA (13, 21), protein synthesis (13, 21), and low temperatures (13). In *Neurospora,* the nitrate transport system is induced only in the presence of nitrate or nitrite, and its activity declines as nitrate is depleted in the medium (21). Evidence indicates that the half-life of the *Neurospora* transport system is about 3 hr.

Much work has been done to define the activity of nitrate reductase as related to nitrate concentration (3, 17), light (2-4, 10, 24, 25), photosynthesis, respiration, and carbohydrate supply (2). Very good information has appeared recently on the development of *in vivo* nitrate reduction and its relationship to uptake and accumulation with time in wheat seedlings (1). Still lacking is a determination of the interrelationship of *in vivo* reduction to nitrate uptake and accumulation, and induction of nitrate reductase with respect to nitrate concentration and time.

Various proposals (5, 6, 20, 26) suggest that *in vivo* reduction of nitrate has a regulatory role on nitrate uptake. Also, the activity of nitrate reductase as determined *in vitro* is often taken as an estimate of its *in vivo* activity. Lack of information on the development of the *in vivo* activity of nitrate reductase in relation to nitrate absorption and induction of nitrate reductase in response to both time and concentration of nitrate has prevented establishment of meaningful correlations. This work was undertaken to describe further the relationships of the processes included in nitrate assimilation after exposure of barley seedlings to nitrate.

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MATERIALS AND METHODS

Barley seeds (*Hordeum vulgare* L. var. 'Numar') were placed for 1 day in aerated H_2O then put on screens on 0.2 mM $CaSO_4$ and kept in darkness for 5 more days in a growth chamber at 24 C and 48% relative humidity. The $CaSO_4$ solutions were regularly renewed. On the 6th day, uniform plants were selected, again placed in 0.2 mM $CaSO_4$ and put into light (500 μeinsteins cm^{-2} sec^{-1}) for 24 hr. On the 7th day, roots were rinsed and seedlings were transferred to treatment solutions.

Nitrate Uptake. Eight seedlings per treatment were placed in volumes of KNO_3 solutions varying from 30 to 190 ml to supply sufficient nitrate during the time course, yet deplete nitrate about 20%. Nitrate uptake was measured as that disappearing from the substrate solution with time. Nitrate depletion in the treatment solutions was monitored by reading an aliquot in a spectrophotometer at A_{210} (7). In the time-course experiment, the solutions were replaced every hr. Three replicate samples were harvested at each time period, and the experiment was repeated 3 times.

In the experiments where nitrate was varied from 0.1 to 1 mM, solutions were monitored at A_{210} as above. Solutions were renewed at 1, 3, 5, 7, and 9 hr, and the plants were harvested at 9 hr. The cumulative uptake was determined from a line of best fit of uptake against time. This experiment was repeated 3 times with three replicate plant samples per treatment.

All the treatment solutions contained 5 mM $CaSO_4$, 15 μg/ml of chloramphenicol to retard bacterial growth, and 5 mM K-phosphate buffer, pH 6.2, in addition to the nitrate.

***In Vivo* Reduction.** *In vivo* reduction was determined by subtracting the total amount of nitrate in both roots and shoots from total uptake of nitrate at each assay period.

Nitrate Analysis. Nitrate was monitored at A_{210} in a spectrophotometer (7) and was also determined in each substrate solution by the phenoldisulfonic acid method (16). When the solutions were changed every hr, the spectrophotometric method (A_{210}) compared closely with the chemical method. With intervals longer than 4 hr between solution changes, differences between the chemical and spectrophotometric methods occurred, presumably due to excretion of organic compounds from the roots. When uptake was compared against time and ambient

nitrate concentrations, the solutions were changed each hr for the former, and every 2 hr for the latter. In our hands, reading at A_{210} was not suitable for assaying the plant tissue content of nitrate. For consistency, the data for uptake and tissue content are reported from the chemical method.

At each harvest period, the seedlings were rinsed, separated into roots and shoots at the scutellar node, and placed in an oven at 70 C for 12 hr. The plant parts were then weighed, placed in 50-ml test tubes in water at 85 to 90 C and allowed to wet for 2 hr; they were then boiled for 10 min to complete the extraction of nitrate. We found that equal amounts of nitrate were extracted from unground and ground (finely divided powder with mortar and pestle) leaf and root tissue by this method. Extraction of unground tissue was much easier and faster. Nitrate was assayed by the phenoldisulfonic acid method (16).

To illustrate the contribution of roots and shoots to the whole plant, the data for each were calculated on the basis of the proportion each made up of 1 g dry weight of the whole plant. The roots and shoots respectively contributed about 44 ± 0.71% and 56 ± 0.71% of 1 g dry weight of a whole plant. The results are computed from the individual weights of roots and shoots for each replicate sample, not from the overall average of all the plants used in each experiment. The concentration of nitrate in roots or shoots was calculated on the basis of 1 g dry weight and then multiplied by the proportional weight that each contributed to 1 g dry weight of the total plant. In this presentation, the sum of the nitrate in the roots and in the shoots equals the amount in 1 g dry weight of the whole plant.

Enzyme Assay. Nitrate reductase was assayed as before (2) by following the conversion of nitrate to nitrite by the method of Schrader *et al.* (23), in which reduced riboflavin-5-P was the reducing agent. Nitrite was determined by adding 1.5 ml of a solution containing 0.75 N HCl, 0.5% (w/v) sulfanilamide, and 0.01% (w/v) N-(1-napthyl)ethylenediamine dihydrochloride. After 15 min, absorbance was read at 540 nm. In agreement with others (27), we found that induction of NADH and $FMNH_2$ nitrate reductase activities in barley leaves were quantitatively closely similar. We also did not detect significant differences between the two activities using a nitrate reductase system extracted from barley roots. Protective agents to preserve nitrate reductase activity were added as described by Schrader *et al.* (22), but these were ineffective on the system from young barley seedlings.

To compare these analyses with uptake, accumulation, and *in vivo* reduction, the data were converted from activity/g fresh weight·hr to activity/g dry weight·hr. Roots and shoots at 7 days contained 93 and 80% moisture, respectively, and these values were used to convert to plant dry weight. The results were converted as above to show the distribution of nitrate reductase activity between roots and shoots such that their sum shows the amount in a whole plant on a basis of g dry weight.

RESULTS

Nitrate uptake was characterized initially by an exponential rate up to about 4 hr, followed by linearity (Fig. 1A). The time course was determined for 9 hr to be certain that uptake had achieved a linear rate. Figure 1B shows actual distribution of nitrate in the roots and shoots during the time course in relation to the whole plant. The accumulation of nitrate in roots and shoots showed patterns similar to those for uptake, achieving linearity after about 3 to 4 hr. *In vivo* reduction of nitrate also showed an initially slow rate, followed by a large increase in rate (Fig. 1C). A balance sheet for the comparative rates of the assimilatory processes was determined from curves shown in Figures 1 and 2 after steady state levels were reached (Table I). The rates of nitrate accumulation by the whole plant, roots, and shoots were 63, 46, and 17%, respectively, of the uptake rates. *In vivo* nitrate reduction was 35% of the uptake rate.

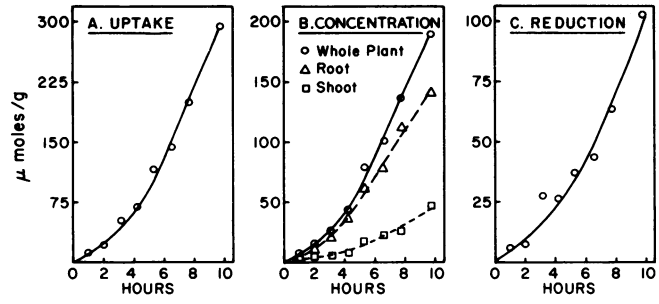

FIG. 1. Time course of nitrate uptake (A), accumulation (B), and *in vivo* reduction (C) in 0.5 mM KNO_3, 5 mM $CaSO_4$, 5 mM phosphate buffer, pH 6.2, and 15 μg/ml of chloramphenicol. Uptake was determined as the nitrate disappearing from the solution and reported on a g dry weight basis. Nitrate concn of the solutions was monitored by taking aliquots and measuring the A_{210} change in a spectrophotometer. The solutions were changed each hr. Nitrate depletion was determined also by the phenoldisulfonic acid method, and these values are shown in the figure. *In vivo* reduction was determined by subtracting the total amount of nitrate in both roots and shoots from the total uptake of nitrate at each assay period. The contents of roots and shoots were proportionally adjusted such that their sum shows the nitrate concentration in a whole plant (*i.e.* μmoles/g dry weight). LSD 0.05, 0.01 (uptake): 17, 24; (concn): root, 32, 45; shoot, 34, 47; whole plant, 10, 13; (reduction): 19, 26.

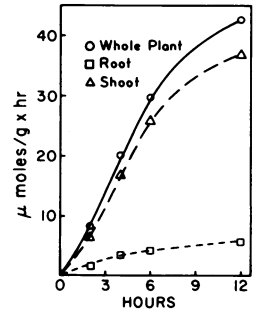

FIG. 2. Appearance of nitrate reductase activity *in vitro* with time. The activities of roots and shoots were proportionally adjusted such that their sum shows the nitrate reductase activity in a whole plant, *i.e.*, μmoles/g dry weight·hr.

Table I. *Comparison of Rates of Processes of Nitrate Assimilation after Linearity is Achieved*

The values were calculated from slopes of curves from Fig. 1. The *in vitro* activity of nitrate reductase was taken from Fig. 2 at 6.5 and 9.8 hr for comparison.

	Rates μmoles/g x hr	
Nitrate uptake	42.3	
Nitrate accumulation		
Whole plant	26.6	
Root	19.4	
Shoot	7.3	
In vivo reduction of nitrate		
Whole plant	14.7	
% reduction ($\frac{\text{rate of reduction}}{\text{rate of uptake}} \times 100$)	35%	
In vitro activity of nitrate reductase		
	μmoles/g x hr	
	6.5 hr	9.8 hr
Whole plant	31.8	39.5
Root	4.5	5.5
Shoot	27.5	34.5

In vitro nitrate reductase activity in the shoots and roots was determined separately and then summed for whole plant (Fig. 2). The enzymic activity in the shoots increased linearly between 2 and 6 hr and then began to level off. Activity in the roots showed a similar response, the linear phase occurring during the first 4 hr. Activity after 12 hr was about one-sixth as much in the roots as in the shoots. The accumulative *in vivo* reduction of nitrate was plotted against the appearance of *in vitro* nitrate reductase activity during the time course (Fig. 3). Each assay of extractable nitrate reductase activity can be considered as an indication of the amount of nitrate reductase accumulated up to that time. The *in vivo* reduction of nitrate was better correlated exponentially than linearly with the measured *in vitro* activity (Fig. 3, Table II).

The *in vitro* activity was sufficient in the shoot but not in the root to account for the observed *in vivo* reduction of nitrate by the whole plant (Table I). On the basis of our results, the majority of the reduction may indeed occur in the shoot.

Effect of Nitrate Concentrations. Nitrate uptake increased as the concentration of nitrate in solution was increased from 0.1 to 0.5 mM and leveled off between 0.5 and 1 mM (Fig. 4A). The distribution of nitrate in response to ambient concentrations was determined as above such that the sum of the root and shoot shows the amount in a whole plant on a g dry weight basis (Fig. 4B). The accumulation of nitrate in roots and shoots showed response curves to increasing concentrations of nitrate similar to those for uptake. Again, the roots accumulated about three times as much nitrate as did the shoots over the concentration range. *In vivo* reduction of nitrate also showed the same response pattern to increasing concentrations of nitrate in the ambient solution as did uptake (Fig. 4C). The curve was drawn in this manner since the reduction values above 0.5 mM were not significantly different.

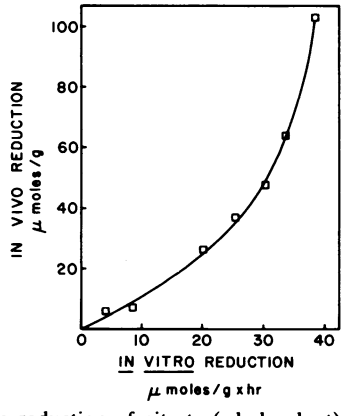

FIG. 3. *In vivo* reduction of nitrate (whole plant) *versus* extractable *(in vitro)* nitrate reductase activity (whole plant). See Table II for correlation coefficients.

Table II. *Correlation of in Vivo Reduction of Nitrate to in Vitro Activity of Nitrate Reductase for Whole Barley Seedlings*

Correlations							
With time				With concn			
Fig. 1C vs. Fig. 2				Fig. 4C vs. Fig. 5			
Linear		Exponential		Linear		Exponential	
r	F	r	F	r	F	r	F
0.91	31.5	0.96	68.9	0.75	10.2	0.82	16.8
	r.05	0.71				0.63	
	.01	0.83				0.77	
	F.05	5.99				5.32	
	.01	13.75				11.26	

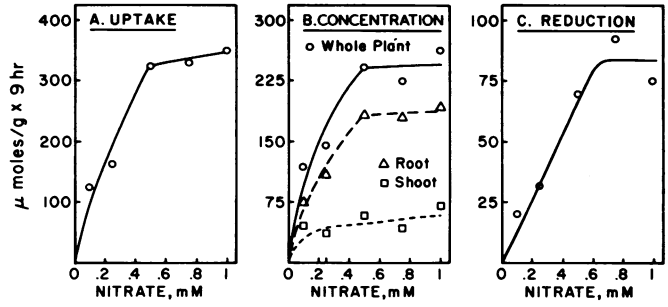

FIG. 4. Nitrate uptake (A), accumulation (B), and *in vivo* reduction (C) as a function of concentration of substrate solution during incubation for 9 hr. All solutions contained 5 mM $CaSO_4$, 5 mM phosphate buffer, pH 6.2, and 15 μg/ml chloramphenicol. To maintain depletion of less than 20%, the solutions were renewed at 1, 3, 5, 7, and 9 hr and the accumulative uptake was determined from a line of best fit of uptake *versus* time. *In vivo* reduction was determined by subtracting the total amount of nitrate in both roots and shoots from the total uptake of nitrate at each assay period. The contents of roots and shoots were proportionally adjusted such that their sum shows the nitrate concn in a whole plant, *i.e.*, μmoles/g dry weight. LSD 0.05, 0.01 (uptake): 15, 20; (concn): root, 60, 94; shoot, 17, 27; whole plant, 72, 113; (reduction): 30, 47.

In vitro nitrate reductase activity (Fig. 5) showed a different response to increasing concentration of nitrate in the external medium than did *in vivo* activity. The results were adjusted such that the sum of the root and shoot shows the amount of nitrate reductase activity in a whole plant on a g dry weight basis. While the *in vivo* reduction resembled that of uptake, the development of *in vitro* activity increased smoothly with increasing nitrate in the ambient solution. *In vitro* activity leveled off above 0.1 mM in roots, but in shoots was just beginning to level off above 0.75 mM. The *in vivo* reduction of nitrate was better correlated exponentially than linearly with *in vitro* activity of nitrate reductase across the concentration range (Table II).

DISCUSSION

The kinetic properties for the uptake of nitrate by whole plants seemed to set the pattern for both its accumulation and *in vivo* reduction. Each process was characterized initially by an exponential rate followed by a linear rate. This typical two-phase pattern for uptake of nitrate is already documented for barley (11, 19), corn (13), cotton (14), tobacco seedlings (14), cultured tobacco cells (12), and *Neurospora* (21). Nitrate assimilation processes in the barley seedlings responded to time similarly to those in wheat reported by Ashley *et al.* (1). The proportions of nitrate reduced by barley and wheat were similar at the end of a 9-hr time course, respectively, 35 and 36%.

Accumulation and *in vivo* reduction also mimicked uptake in response to ambient concentrations of nitrate (Fig. 4). Uptake showed a typical saturation of the first mechanism, as reported by Rao and Rains (19).

Determining the rate of *in vivo* reduction of nitrate by our method very likely accounts for the total *in vivo* activity of nitrate reductase. Evidence in the literature (3) indicates that nitrate reductase is the rate-limiting enzyme in the reduction of nitrate to ammonia. Nitrate can accumulate to massive amounts in plants, whereas nitrite and ammonia concentrations are usually very low, further supporting the supposition. Extractable

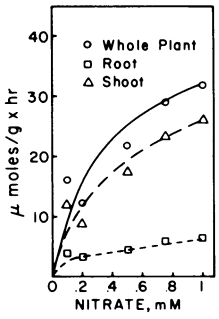

FIG. 5. Appearance of nitrate reductase activity *in vitro* in response to ambient nitrate concentration. The activities from roots and shoots were proportionally adjusted such that their sum shows the nitrate reductase activity in a whole plant, *i.e.* μmoles/g dry weight·hr.

nitrite reductase activity is usually much greater than *in vitro* nitrate reductase activity (9, 18), which in turn is greater than the *in vivo* reduction we have observed.

Extractable nitrate reductase activity *(in vitro)* is often taken as an indication of its *in vivo* activity. Differences between *in vivo* and *in vitro* rates have recently been reported for *Anthriscus sylvestris* (15). Our results show that the relation between the two is complex. When compared against time, *in vivo* reduction reached a steady state rate many hours before nitrate reductase was fully induced (compare Fig. 1C and 2). With time, and at a near constant concentration of ambient nitrate, an exponential relationship fit the data better than did a linear, even though both relationships were statistically significant (Fig. 3 and Table II). Comparison against the ambient concentrations illustrating mechanism 1 (19) of uptake showed significant correlations both exponentially and linearly with the former showing a better fit (Table II). Under the conditions of our experiments, the induction or appearance of nitrate reductase did not seem to limit the rate of *in vivo* reduction of nitrate. The rate of *in vivo* nitrate reduction seemed more a function of rate of uptake which supplies nitrate than of the amount of nitrate reductase present.

Some investigators have proposed a regulatory effect of *in vivo* reduction of nitrate on nitrate uptake. One hypothesis proposes that secondary products of nitrate reduction may regulate uptake rate (5, 6). In short term experiments, a stoichiometric relation was observed between nitrate reduction and malate synthesis. Postulated as the regulatory step for nitrate uptake was the synthesis of malate in the shoot in response to nitrate reduction, its subsequent translocation to the root, where it may be decarboxylated, and the exchange of HCO_3^- for nitrate in the external solution (6). Since hydroxyl ion is one of the products of nitrate reduction, the synthesis of malic acid could also serve as a buffer against large scale increases in pH. Results of a study of nitrate assimilation by *Ankistrodesmus braunii* were interpreted as evidence that nitrate uptake was dependent upon its *in vivo* reduction (26). Rao and Rains (20) recently reported that illumination of barley plants increased nitrate absorption. It was not clear whether the increased absorption was due to greater energy supply or an interaction of increased nitrate reductase activity, or both.

On the other hand, the development of the root transport system for nitrate has been separated from that of the induction of nitrate reductase by two methods. With one, treatments with tungstate or vanadium allowed the nitrate transport system to develop but prevented the appearance of nitrate reductase activity in XD tobacco cells (12), barley seedlings (12, 20), and *Neurospora* (21). With the other method, *Neurospora* mutants lacking a viable nitrate reductase still developed a normally functioning nitrate transport system (21).

The close similarity in kinetic patterns between uptake, accumulation, and *in vivo* reduction of nitrate with time and across the concentrations of the first absorption mechanism suggests that the rate of uptake has a strong regulatory role on the assimilation processes in barley seedlings. Hence, uptake may regulate *in vivo* activity by controlling the supply of nitrate, rather than *in vivo* activity regulating uptake. Furthermore, *in vivo* reduction seemed more a function of rate of uptake than of the extractable *(in vitro)* nitrate reductase activity in the seedlings.

LITERATURE CITED

1. ASHLEY, D. A., W. A. JACKSON, AND R. J. VOLK. 1975. Nitrate uptake and assimilation by wheat seedlings during initial exposure to nitrate. Plant Physiol. 55: 1102-1106.
2. ASLAM, M., R. C. HUFFAKER, AND R. L. TRAVIS. 1973. The interaction of respiration and photosynthesis in induction of nitrate reductase activity. Plant Physiol. 52: 137-141.
3. BEEVERS, L. AND R. H. HAGEMAN. 1969. Nitrate reduction in higher plants. Annu. Rev. Plant Physiol. 20: 495-522.
4. 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.
5. BEN-ZIONI, A., Y. VAADIA, AND S. H. LIPS. 1970. Correlations between nitrate reduction, protein synthesis and malate accumulation. Physiol. Plant. 23: 1039-1047.
6. 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.
7. CAWSE, P. A. 1967. The determination of nitrate in soil solutions by ultraviolet spectrophotometry. Analyst 92: 311-315.
8. EPSTEIN, E. 1961. The essential role of calcium in selective cation transport by plant cells. Plant Physiol. 36: 437-444.
9. GRANT, B. R., C. A. ATKINS, AND D. T. CANVIN. 1970. Intracellular location of nitrate reductase and nitrite reductase in spinach and sunflower leaves. Planta 94: 60-72.
10. 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.
11. HEIMER, Y. M., J. L. WRAY, AND P. FILNER. 1969. The effect of tungstate on nitrate assimilation in higher plant tissues. Plant Physiol. 44: 1197-1199.
12. HEIMER, Y. M. AND P. FILNER. 1971. Regulation of the nitrate assimilation pathway in cultured tobacco cells. III. The nitrate uptake system. Biochim. 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. JACKSON, W. A., R. J. VOLK, AND T. C. TUCKER. 1972. Apparent induction of nitrate uptake in nitrate-depleted plants. Agron. J. 64: 518-524.
15. JANIESCH, P. 1973. Contribution on the physiology of nitrophytes nitrate accumulation and nitrate assimilation of *Anthriscus sylvestris* Hoffm. Flora 162: 479-491.
16. JOHNSON, C. M. AND A. ULRICH. 1950. Determination of nitrate in plant material. Anal. Chem. 22: 1526-1529.
17. MEEKER, G. B., A. C. PURVIS, C. A. NEYRA, AND R. H. HAGEMAN. 1974. Uptake and accumulation of nitrate as a major factor in the regulation of nitrate reductase activity in corn (*Zea mays* L.) leaves: effects of high ambient CO_2 and malate. *In:* R. L. Bielski, A. R. Ferguson, and M. M. Cresswell, eds., Mechanisms of Regulation of Plant Growth. The Royal Society of New Zealand, Wellington. pp. 49-58.
18. MIFLIN, B. J. 1967. Distribution of nitrate and nitrite reductase in barley. Nature 214: 1133-1134.
19. RAO, K. P. AND D. W. RAINS. 1976. Nitrate absorption by barley. I. Kinetics and engergetics. Plant Physiol. 57: 55-58.
20. RAO, K. P. AND D. W. RAINS. 1976. Nitrate absorption by barley. II. The influence of nitrate reductase activity. Plant Physiol. 57: 59-62.
21. SCHLOEMER, R. H. AND R. H. GARRETT. 1974. Nitrate transport system in *Neurospora crassa*. J. Bacteriol. 118: 259-269.
22. SCHRADER, L. E., D. A. CATALDO, AND D. M. PETERSON. 1974. Use of protein in extraction and stabilization of nitrate reductase. Plant Physiol. 53: 688-690.
23. 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.
24. TRAVIS, R. L., R. C. HUFFAKER, AND J. L. KEY. 1970. Light-induced development of polyribosomes and the induction of nitrate reductase in corn leaves. Plant Physiol. 46: 800-805.
25. TRAVIS, R. L., W. R. JORDAN, AND R. C. HUFFAKER. 1970. Light and nitrate requirements for induction of nitrate reductase activity in *Hordeum vulgare*. Physiol. Plant. 23: 678-685.
26. ULLRICH-EBERIUS, C. I. 1973. Beziehungen der Aufnahme von Nitrat, Nitrit und Phosphat zur photosynthetischen Reduktion von Nitrat und Nitrit und zun ATP-speigel bei *Ankistrodesmus braunii*. Planta 115: 25-36.
27. WRAY, J. L. AND P. FILNER. 1970. Structural and functional relationships of enzyme activities induced by nitrate in barley. Biochem. J. 119: 715-725.