Plant Physiol. (1971) 47, 790–794

Intact Tissue Assay for Nitrite Reductase in Barley Aleurone Layers[1]

Received for publication October 14, 1970

THOMAS E. FERRARI[2] AND JOSEPH E. VARNER
Department of Horticulture and Michigan State University-Atomic Energy Commission Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48823

ABSTRACT

A method has been devised for the detection and measurement of nitrite reductase activity in intact barley (*Hordeum vulgare* L. cv. Himalaya) aleurone layers. The technique involves feeding aleurone layers nitrite and measuring nitrite disappearance after a given time period. The method also allows simultaneous determination of nitrite uptake by the tissue. Quantitative recovery of nitrite is obtained by rapid heating of tissue in the presence of dimethyl sulfoxide.

Using the procedure described, nitrite reductase activity in intact barley aleurone layers was determined. Enzyme activity was increased by prior incubation of the tissue with nitrate, but considerable activity was present in tissue incubated without nitrate. Nitrate-induced activity was inhibited by cycloheximide but not by actinomycin D. Enzyme activity in induced layers was inhibited by 2,4-dinitrophenol, and partially by antimycin A and 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide. Activity in noninduced tissue appeared to be less sensitive to these respiratory inhibitors. In contrast, both activities were inhibited more than 90% by anaerobiosis; but nitrate-induced and noninduced aleurone layers were able to reduce nitrite anaerobically when the concentration of substrate in the assay medium was reduced from 250 μM to 25 μM. Nitrite uptake was relatively insensitive to anaerobiosis and to the inhibitors tested.

Nitrite depletion from the medium by aleurone layers was rapid at pH 4.5 and negligible at pH 7.5. Nitrite accumulated at pH 4.5 under anaerobic conditions was rapidly released when the tissue was transferred to medium at pH 7.5. Nitrite release at pH 7.5 occurred whether the tissue was maintained under anaerobic or aerobic conditions.

The reduction of nitrate by intact plant tissues and the release of the reduced product, nitrite, occurs under anaerobic conditions (4, 6, 9). A similar reponse can be observed aerobically in the presence of respiratory inhibitors (4). Why nitrite leaks from the tissue is not known. Either the rate of nitrate reduction exceeds nitrite reduction, or nitrite reduction does not proceed under conditions in which nitrite release occurs. To test these possibilities requires that nitrite reductase activity be measured in the intact tissue under conditions similar to those in which nitrite release occurs. This report describes a method for the detection and measurement of nitrite reductase activity in intact barley aleurone layers, and the effects of conditions favoring nitrite release in the activity of the enzyme. The technique involves administering nitrite to aleurone layers and measuring nitrite disappearance with time. The method also allows simultaneous determination of nitrite uptake by the tissue.

MATERIALS AND METHODS

Tissue Preparation. Aleurone layers were isolated from barley (*Hordeum vulgare* L. cv. Himalaya, 1965 Harvest) seeds as described previously (4). Erlenmeyer flasks (50 ml) containing 50 aleurone layers, plus or minus 0.05 M KNO_3 and 50 μg of chloramphenicol in a total volume of 5 ml were stoppered with cotton plugs and placed at 23 C in a metabolic water bath shaker at 200 rpm. Tissue preparation and incubation were done under sterile conditions. After 6 to 12 hr of incubation, the tissue was rinsed with approximately 50 ml of distilled water, and the effect of various conditions on enzyme activity was determined.

Assay of Nitrite Reductase Activity. Unless otherwise indicated, nitrite reductase activity was assayed by measuring nitrite disappearance after placing 10 aleurone layers in 2 ml of medium containing 0.1 M potassium phosphate, pH 4.5, chloramphenicol (20 μg), nitrite, and the treatment solution as indicated in the text. Nitrite (final $NaNO_2$ concentration, 2.5×10^{-4} M) was added to start the assay. Two 0.1-ml aliquots were immediately removed to provide an accurate measure of the initial nitrite concentration of the medium with tissue present. After 40 min, two 0.1-ml aliquots of the medium were again removed for nitrite determination. The difference between the final and initial nitrite concentration of the medium indicates the amount of nitrite taken up by the tissue. DMSO[3] was then added to the medium (final concentration, 50% [v/v]), and the 25-ml Erlenmeyer flask and its contents were placed on a hotplate until the medium came to a boil (about 20–30 sec). Under these conditions the nitrite of the tissue rapidly leaked back into the medium. After cooling, two 0.2-ml aliquots were removed for nitrite determination. The difference between this nitrite concentration and the initial concentration measures the amount of nitrite reduced.

Nitrite was determined colorimetrically by the Griess-Ilosvay method (10) by adding to the unknown 0.3 ml each of 1% sulfanilamide in 3 N HCl and 0.02% *N*-1-naphthylethyl-

[1] Research carried out under United States Atomic Energy Commission Contract AT (11-1)-1338.

[2] Present address: Biology Department, McGill University, Montreal, 110 Canada.

[3] Abbreviations: DMSO: dimethyl sulfoxide; DNP: 2,4-dinitrophenol; ioxynil: 3,5-diiodo-4-hydroxybenzonitrile; HOQNO: 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide.

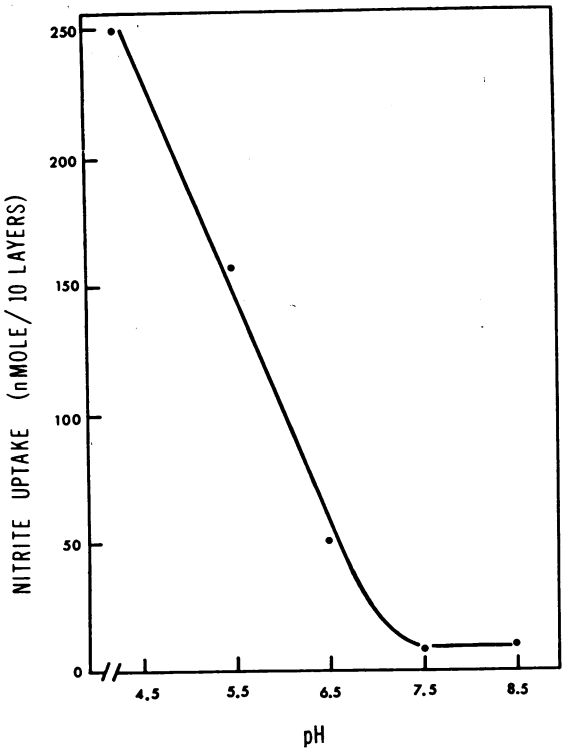

FIG. 1. Effect of pH on nitrite accumulation. Nitrite accumulation by 10 aleurone layers was determined over a 90-min period in 4 ml of medium containing 1.25 mM sodium nitrite, 20 μg of chloramphenicol, and 0.1 M phosphate buffer at the indicated pH.

enediamine dihydrochloride (w/v). Absorbance at 540 nm was determined after centrifugation at 2000g for 10 min.

RESULTS

Characteristics of Nitrite Uptake and Disappearance. Figure 1 shows the effect of pH on nitrite accumulation by aleurone layers. Accumulation decreased with increasing pH from pH 4.5 to pH 7.5, after which little or no depletion of nitrite from the medium occurred. Nitrite accumulation at pH 4.5 was rapid and was not affected by prior incubation of tissue in nitrate (Fig. 2). Within 20 min, approximately 50% of the added nitrite had been taken up by the tissue at 0.1 mM and 0.25 mM nitrite.

Nitrite reductase activity was increased by prior incubation with nitrate, but considerable activity was present in noninduced tissue (Fig. 3). It is not known whether nitrate or the product of nitrate reduction, nitrite, was responsible for the increased enzyme activity. The temperature coefficient (Q_{10}) of nitrite uptake was approximately 1.4, compared to 2.6 and 2.4 for nitrite disappearance with induced and noninduced tissue, respectively. Temperature coefficients were determined over a 15 to 30 C temperature range.

The increase in nitrite reductase activity produced by incubation with nitrate was completely inhibited by cycloheximide and enhanced slightly by actinomycin D (Table I). Nitrite disappearance in noninduced tissue was not affected by either inhibitor (Table I). Enzyme activity in induced layers was inhibited by three uncouplers of oxidative phosphorylation: DNP, pentachlorophenol (7), and ioxynil (3) (Table II). Two inhibitors of mitochondrial electron transport, antimycin A and HOQNO, partially inhibited nitrite disappearance in induced tissue (Table II); however, activity in noninduced tissue was unaffected by these two chemicals (Table II). Anaerobiosis (medium gassed with nitrogen for 1 min) strongly depressed nitrite disappearance in both induced and noninduced tissue (Table II). Nitrite uptake was relatively unaffected by the inhibitors and anaerobiosis (Table II).

Nitrite Release by Aleurone Layers. The following experiment was performed to determine if aleurone layers could retain nitrite under conditions where nitrite release occurs during anaerobic nitrate reduction (4). No nitrite is taken up by the tissue at pH 7.5 (Fig. 1), the pH of the anaerobic nitrate reduction medium (4). Therefore, tissue was first preloaded with nitrite at pH 4.5, under anaerobic conditions to prevent its reduction. Layers were then placed in nitrite-less medium at pH 7.5 to measure nitrite release. Nitrite levels of the medium at the start of the pH 4.5 preloading period and after 40 min of incubation (before and after DMSO extraction) are shown in Table III. Though the tissue accumulated approximately 300 nmoles of nitrite, no nitrite was reduced during the 40-min uptake period. This was indicated by its recovery in the medium

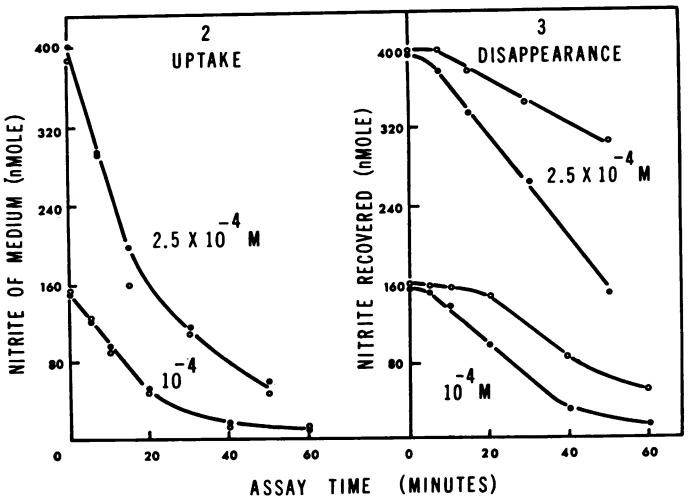

FIG. 2. Kinetics of nitrite accumulation. Prior to determination of nitrite uptake, tissue was incubated 12 hr in the presence or absence of 0.05 M potassium nitrate. Accumulation was measured with 10 aleurone layers incubated in 2 ml of medium containing either 0.1 mM or 0.25 mM sodium nitrite and 0.1 M phosphate buffer, pH 4.5. Open and closed circles represent noninduced and nitrate-induced tissue, respectively.

FIG. 3. Kinetics of nitrite disappearance. Assay conditions were as described in Figure 2. Nitrite disappearance was measured as described in "Materials and Methods." Open and closed circles represent noninduced and nitrate-induced tissue, respectively.

Table I. *Effect of Protein Synthesis Inhibitors on Uptake and Disappearance of Nitrite in Noninduced and Nitrate-induced Aleurone Layers*

Tissue was incubated 7 hr in the presence or absence of 0.05 M potassium nitrate, cycloheximide (10 μg/ml), or actinomycin D (10 μg/ml). Then, after the medium was discarded, nitrite uptake and disappearance were measured as described in "Materials and Methods."

Inhibitor	Disappearance		Uptake	
	Nitrate-induced	Noninduced	Nitrate-induced	Noninduced
	nmoles/40 min·10 layers			
None	170	68	344	388
Cycloheximide	66	68	322	311
Actinomycin D	207	65	336	312

Table II. *Effect of Respiratory Inhibitors on Uptake and Disappearance of Nitrite by Aleurone Layers*

After 9 to 12 hr of incubation with nitrate, nitrite uptake and disappearance (in the presence of inhibitor) were determined as described in "Materials and Methods." Unless otherwise indicated, inhibitors were dissolved in acetone (final concentration, 1%). Acetone was added to controls. With HOQNO and antimycin A treatments, tissue was preincubated for 30 min in 0.1 M phosphate buffer, pH 7.5, and in the presence of inhibitor dissolved in ethanol (final ethanol concentration, 5%). Nitrite uptake and disappearance were measured after the preincubation medium was discarded and the tissue was rinsed two times with 4 ml of 0.1 M phosphate, pH 4.5.

Treatment	Nitrite Disappearance		Nitrite Uptake	
	Induced	Non-induced	Induced	Noninduced
	% of control			
2,4-DNP, 10^{-4} M	10	40	75	65
2,4-DNP, 10^{-5} M	25	65	60	80
Pentachlorophenol, 10^{-3} M	25	55	90	85
Pentachlorophenol, 10^{-4} M	60	65	90	85
Ioxynil, 10^{-4} M	15	35	75	75
Ioxynil, 10^{-5} M	75	70	85	85
N_2 atmosphere	0	5	95	75
HOQNO, 0.25 mM	75	95	80	70
Antimycin A, 0.5 mM	70	90	80	75

Table III. *Nitrite Accumulation by Aleurone Layers at pH 4.5*

Nitrate-induced or noninduced tissue was incubated in a N_2 atmosphere (medium with tissue gassed with N_2 for 1 min) for 40 min with 2 ml of medium containing 0.1 M phosphate buffer, pH 4.5, 0.25 mM sodium nitrite, and 20 μg of chloramphenicol. Nitrite of the medium and tissue was determined as described in "Materials and Methods."

KNO_3 Induction	Nitrite of Medium at Start of Uptake Period	Nitrite of Medium at End of Uptake Period		Nitrite of Tissue[1]
		Before DMSO extraction	After DMSO extraction	
	nmoles/2 ml			*nmoles/10 layers*
+	429	139	452	313
−	436	135	427	292

[1] Nitrite of tissue equals nitrite of medium after DMSO extraction minus nitrite of medium before DMSO extraction. Uptake period was 40 min.

after extraction from the tissue. When the tissue was subsequently washed and placed in nitrite-less media at pH 7.5 for an additional 40 min, nitrite leaked from the tissue whether the latter was incubated under aerobic or anerobic conditions (Table IV). At pH 7.5, considerable nitrite disappeared when tissue was maintained under aerobic conditions. Anaerobic conditions inhibited this disappearance (Table IV). Kinetics of nitrite release and disappearance at pH 7.5 for aerobic conditions are indicated in Figure 4. Approximately 50% of the nitrite accumulated in the tissue during preincubation with nitrite at pH 4.5 leaked into the nitrite-less medium during the first 5 min of incubation at pH 7.5.

Anaerobic Nitrite Disappearance at Low Nitrite Concentrations. Nitrite was found to disappear under anaerobic conditions when noninduced tissue was incubated in 25 μM nitrite (Table V), a 10-fold lower concentration than that used in previous experiments. DNP partially inhibited this disappearance (Table V).

On the other hand, nitrate-induced tissue showed no apparent ability to reduce nitrite when incubated with low levels of the latter (Table V). Unexpectedly, when DNP was included in the assay medium, nearly 2.5 times more nitrite was re-

Table IV. *Release of Accumulated Nitrite under Aerobic and Anaerobic Conditions at pH 7.5*

Nitrite of tissue at 40 min was taken from Table III. Experimental procedure is described in Figure 3 and in the text.

KNO_3 Induction	Nitrite of Tissue			Nitrite of Medium			Nitrite Disappearance[1]	
	Incubation time (min)			Incubation time (min)				
	40	80 Air	80 N_2	40	80 Air	80 N_2	Air	N_2
	nmoles/10 layers			*nmoles/2 ml*			*nmoles/10 layers*	
+	313	24	60	5	204	220	90	38
−	292	17	65	6	207	215	74	18

[1] Nitrite disappearance equals total (medium and tissue) nitrite at 40 min minus total nitrite at 80 min.

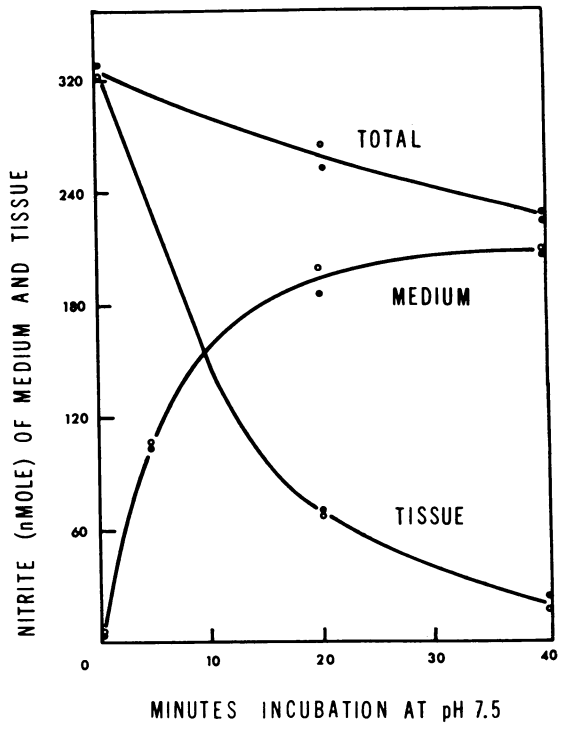

FIG. 4. Release of accumulated nitrite by aleurone layers. Tissue was induced in the presence or absence of 0.05 M potassium nitrate for 9 hr. Tissue was then rinsed three times with 40 to 50 ml of distilled deionized water, then preloaded with nitrite by incubation in a N_2 atmosphere for 40 min in 2 ml of medium containing 0.1 M phosphate buffer (pH 4.5), 0.25 mM sodium nitrite, and 20 μg of chloramphenicol. Tissue was next rinsed three times with 10 ml of cold phosphate buffer (pH 7.5), 0.1 M, and placed in 2 ml of nitrite-less media containing 0.1 M phosphate buffer, pH 7.5, and 20 μg chloramphenicol. Nitrite content of the medium and tissue was determined at the times indicated, as described in "Materials and Methods." ○: noninduced tissue; ●: nitrate-induced tissue.

covered at the end of the assay than was present at the start (Table V). Since little or no nitrite was present in induced tissue at the start of the assay, it appeared that nitrite was being produced from the anaerobic reduction of nitrate which had accumulated during the 9-hr nitrate incubation phase of the experiment. To test this, nitrate-induced tissue was assayed in the absence of nitrite. Under these conditions, 21 nmoles of nitrite were produced (Table V), and DNP increased considerably (3.5-fold) the amount of nitrite accumulated. Therefore, as evidenced by the additional accumulation of nitrite in the presence of DNP, nitrite disappearance was occurring in induced tissue as well as in noninduced tissue.

Effect of DNP on Nitrate Reduction. The effect of DNP on the anaerobic intact tissue assay (4) for nitrate reductase is shown in Table VI. When the assay was done in the absence of ethanol, DNP increased nitrite recovery by 65%; however, DNP had little or no effect on ethanol-enhanced nitrite production.

DISCUSSION

As with nitrate reductase before (4), nitrite reductase activity has been measured in intact barley aleurone layers. Methods are now available which permit a comparison of the properties of two adjacent enzymes in a metabolic pathway within intact tissue. Unlike nitrate reductase (Ref. 4 and Table II), nitrite reduction was inhibited by inhibitors of electron transport and oxidative phosphorylation. When nitrate-induced tissue was incubated anaerobically in the presence of nitrate, nitrite leaked from the tissue (4, 6, 9). In this study, nitrite leakage was found to be dependent upon pH and occurred under aerobic as well as anaerobic conditions.

Comparisons of enzyme activity in the intact tissue with *in vitro* enzyme activity, as with those made for nitrate reductase (4), were not made for nitrite reductase. Thus, the intact tissue assay for nitrite reductase provides at present only a qualitative measure of enzyme activity. The possibility exists that the assay might underestimate enzyme activity because of a rate-limiting process, such as in the production of reductant, or because of the simultaneous production of nitrite (by nitrate reductase) from nitrate which accumulated during the induction phase of the experiment.

The production and release of nitrite from nitrate under anerobic conditions at pH 7.5 were enhanced considerably by ethanol (4). DNP, an inhibitor of nitrite reduction in higher and lower plants (Refs. 1, 5, 11, Tables II and V), failed to increase ethanol-enhanced nitrite production (Table VI). This indicated that the inhibition of nitrite reduction by anaerobiosis was complete. However, DNP did increase nitrite accumulation in tissue assayed in the absence of ethanol (Table VI). The additional nitrite accumulated may represent inhibition by DNP of the tissue's ability to reduce low levels (25 μM) of nitrite under anaerobic conditions (Table V). However, one cannot exclude the possibility that DNP might also be enhancing nitrate reduction, for example, by increasing the amount of nitrate available for reduction. In any case, the reduction of nitrite under anaerobic conditions at pH 7.5 is impaired (Table IV), and because nitrite cannot be retained by the tissue it leaks into the medium.

In the intact tissue assay for nitrite reductase, nitrite disappearance did not occur under anaerobic conditions in the presence of high levels of nitrite (250 μM). This might be due to the accumulation of toxic levels of nitrite within the cell, or due to the insufficient production of energy required for nitrite reduction during anaerobiosis. Assuming 0.01 ml of water and 30 μmoles of nitrite accumulated per layer (Table III), the concentration of nitrite within an aleurone layer would be approximately 3 mM. Such concentrations are toxic to

Table V. *Anaerobic Nitrite Production and Disappearance by Nitrate-induced and Noninduced Tissue Assayed in Presence or Absence of Low Nitrite Concentration*

After incubation in the presence of potassium nitrate for 9 hr, tissue was rinsed 3 times with approximately 50 ml of distilled water. Ten aleurone layers were then incubated in 2 ml of media containing ± sodium nitrite (25 μM), phosphate buffer (0.1 M, pH 4.5), and 20 μmoles of chloramphenicol. Tissue was added to the incubation medium just prior to deaeration for 1 min with N_2. DNP was 0.1 mM.

Assay Condition	Nitrite of Medium					
	Noninduced tissue			Induced tissue		
	At start of assay (0 min)	At end of assay (40 min)		At start of assay (0 min)	At end of assay (40 min)	
		Before DMSO extraction	After DMSO extraction		Before DMSO extraction	After DMSO extraction
	nmoles/2 ml					
+ nitrite	44	6	10	42	13	45
+ nitrite, + DNP	41	11	21	41	28	112
− nitrite	0	2	2	1	5	21
− nitrite, + DNP	0	2	3	2	18	74

Table VI. *Effect of DNP on Nitrate Reduction*

After 4 hr of induction with nitrate, tissue was placed in 2 ml of medium containing 0.1 M phosphate buffer (pH 7.5), 0.1 M potassium nitrate, and N_2 atmosphere. Nitrite of medium at 30 min (after DMSO extraction) was determined as described in "Materials and Methods." DNP was 0.1 mM and ethanol was 5%.

Assay Condition	Nitrate Reduction
	nmoles nitrite produced/layer·hr
− ethanol, − DNP	4.0
− ethanol, + DNP	6.6
+ ethanol, − DNP	25.6
+ ethanol, + DNP	27.0

Anabaena cylindrica (2) and cultured tobacco cells (H. Kelker, personal communication).

DNP inhibition of nitrite reduction under aerobic conditions in aleurone layers, as well as in intact cells of *Chlorella* (5) and *Ankistrodesmus* (1), suggests that nitrite reduction is dependent on oxidative phosphorylation. The ability to inhibit nitrite disappearance by other uncouplers of oxidative phosphorylation (Refs. 1, 5 and Table II) with and without -NO_2 functional groups argues against the possibility that DNP is alternatively accepting reducing equivalents, as has been shown to be the case with isolates of *Pseudomonas denitrificans* (8) and reductants generated by spinach chloroplasts (12).

Pentachlorophenol and ioxynil inhibited nitrite disappearance in induced and noninduced tissue equally at the lower concentrations, but not at the higher concentrations (Table II). The reason for this observation is not known. The differential sensitivity of induced and noninduced nitrite disappearance to all inhibitors tested suggests that two enzymes might be responsible for nitrite reduction or, alternatively, that there is one enzyme present but it is localized in different cellular compartments.

Nitrite disappearance in both induced and noninduced tissue is evidently enzymatically mediated. This is suggested by

its temperature coefficients and its sensitivity to anaerobiosis and uncouplers of oxidative phosphorylation. The ability to inhibit nitrate-induced nitrite reductase activity with cycloheximide argues for a requirement for protein synthesis in the induced system.

Acknowledgment—The authors thank Dr. R. A. Nilan, Agronomy Department, Washington State University, for donating the Himalaya barley seeds used in this and previous studies.

LITERATURE CITED

1. Ahmad, J. and I. Morris. 1968. The effects of 2,4-dinitrophenol and other uncoupling agents on the assimilation of nitrate and nitrite by *Chlorella*. Biochim. Biophys. Acta 162: 32–38.
2. Brownell, P. F. and D. J. D. Nicholas. 1967. Some effects of sodium on nitrate assimilation and N_2 fixation in *Anabaena cylindrica*. Plant Physiol. 42: 915–921.
3. Ferrari, T. E. and D. E. Moreland. 1969. Effects of 3,5-dihalogenated 4-hydroxybenzonitriles on the activity of mitochondria from white potato tubers. Plant Physiol. 44: 429–434.
4. Ferrari, T. E. and J. E. Varner. 1970. Control of nitrate reductase activity in barley aleurone layers. Proc. Nat. Acad. Sci. U. S. A. 65: 729–736.
5. Kessler, E. and W. Bucher. 1960. Über die Wirking von Arsenat auf Nitratreduktion, Atmung und Photosynthese von Grünalgen. Planta 55: 512–524.
6. Kumada, H. 1953. The nitrate utilization in seed embryos of *Vigna sesquipedalis*. J. Biochem. 40: 439–450.
7. Moreland, D. E. 1967. Mechanisms of action of herbicides. Annu. Rev. Plant Physiol. 18: 365–386.
8. Radcliff, B. C. and D. J. D. Nicholas. 1968. Some properties of nitrite reductase from *Pseudomonas denitrificans*. Biochim. Biophys. Acta 153: 545–554.
9. Randall, P. J. 1969. Changes in nitrate and nitrate reductase levels on restoration of molybdenum to molybdenum-deficient plants. Aust. J. Agr. Res. 20: 635–642.
10. Snell, F. D. and C. T. Snell. 1949. Colorimetric Methods of Analysis, Vol. II, Ed. 3. Van Nostrand, Princeton, N. J. p. 804.
11. Voskresenskaya, N. P. and G. S. Grishina. 1962. The significance of light in nitrite reduction in the green leaf. Fiziol. Rast. 9: 7–15. (Translated in Soviet Plant Physiol. 1962. 9: 4–10).
12. Wessels, J. S. C. 1965. Mechanism of the reduction of organic nitro compounds by chloroplasts. Biochim. Biophys. Acta 109: 357–371.