

## The Occurrence of Nitrate Reductase in Apple Leaves<sup>1</sup>

Lowell Klepper and R. H. Hageman

Department of Agronomy, University of Illinois, Urbana, Illinois 61801

Received September 11, 1968.

**Abstract.** Nitrate reductase utilizing NADH or reduced flavin mononucleotide (FMNH<sub>2</sub>) as electron donor was extracted from the leaves, stems and petioles, and roots of apple seedlings. Successful extraction was made possible by the use of insoluble polyvinylpyrrolidone (Polyclar AT) which forms insoluble complexes with polyphenols and tannins. The level of nitrate reductase per gram fresh weight was highest in the leaf tissue although the nitrate content of the roots was much higher than that of the leaves. Nitrite reductase activity was detected only in leaf extracts and was 4 times higher than nitrate reductase activity. Nitrate was found in all parts of young apple trees and trace amounts were also detected in mature leaves from mature trees. Nitrate reductase was induced in young leaves of apple seedlings and in mature leaves from 3 fruit-bearing varieties. An inhibitor of polyphenoloxidase, 2-mercaptobenzothiazole was used in both the inducing medium and the extracting medium in concentrations from 10<sup>-3</sup> to 10<sup>-5</sup> M with no effect upon nitrate reductase activity.

Classically it has been held that the seat of formation (23) of amino acids in apple trees is the root. This view was supported by the observation that nitrate could be detected only in the youngest root tips. Other workers have concluded that reduction of nitrate is largely confined to the roots of apple trees (2) and other members of the family Rosaceae (3). This conclusion was based on the fact that only trace amounts of nitrate were found in the tracheal sap and leaves, both of which contained abundant organic nitrogenous metabolites. Similar conclusions can be drawn from the work of Wallace and Pate (24) with field peas. These workers have proposed that the roots were capable of reducing the absorbed nitrate until a "threshold" external level (5-20 ppm—a concentration low enough to limit growth for many plants) of nitrate was obtained and only then was nitrate transported upward and reduced in the leaves.

Although much of the initial work on characterizing nitrate reductase from higher plants was done with extracts from soybean leaves, Evans and Nason (7) also reported low levels of nitrate reductase in roots. Most of the subsequent work on nitrate reductase has been on leaf tissue because leaves contain much more enzyme than does root tissue (5, 10), and it is easier to obtain the tissue. The existence of

nitrate reductase in the roots of various plants was shown by Sanderson and Cocking (20).

Recently Miflin (17) reported that roots of young barley seedlings had higher levels of both nitrate and nitrite reductase per unit of extracted protein than did leaf tissue from the same plants. However, activities were not presented on a per unit of fresh weight or plant part basis. Wallace and Pate (24) found that field pea leaves had a greater amount of nitrate reductase per unit fresh weight than did the roots. Since this disparity increased with plant maturity, they suggested that the site of nitrate reduction may shift to the leaves with plant maturation. This concept is consistent with the high activities found in the leaves of corn (21, 25) and wheat (6) at the onset of the reproductive phase.

With the exception of apple trees and related species the documented evidence suggests that leaf tissue has a greater capacity to reduce nitrate than has root tissue. Recently Grasmanis and Nicholas (8) extracted from apple roots a nitrate reductase that requires benzyl viologen rather than NADH as the electron donor. Although it is difficult to extract enzymes from apple leaves because of the high content of polyphenols and related substances, Jones *et al.* (11, 12), using soluble polyvinylpyrrolidone, obtained enzyme activities from plant parts rich in phenolics and tannins.

The objectives of this experiment were to show: A) the existence and relative amounts of nitrate reductase in root and leaf tissue of apple trees, B) that apple seedlings transport nitrate to the leaves when given adequate amounts of nitrate, and C) that nitrate reductase is induced in mature leaves provided with nitrate.

<sup>1</sup> This work was supported in part by federal funds provided to the Department of Agronomy, and by Grant GB-3750 from the National Science Foundation.

## Materials and Methods

**Seedling Culture.** Apple seeds of unknown variety were germinated in sand and transplanted at the cotyledonary stage into waxed paper cups containing 30 grams (dry) of Krum (an expanded silicate soil conditioner manufactured by the Ryolex Corporation, Champaign, Illinois). The seedlings grown in the greenhouse, were given a 16 hr day of supplemental light (10,000 lux fluorescent light). Two nutrient solutions were used for the plant culture. The first contained in mM concentrations,  $MgSO_4$ , 3;  $KH_2PO_4$ , 1;  $Ca(NO_3)_2$ , 5;  $KNO_3$ , 5;  $(NH_4)_2SO_4$ , 2.5;  $Fe^{+3}$ , 0.05 (as Chel-138, Geigy Agricultural Chemical Company, Yonkers, New York); in  $\mu M$  concentrations  $H_3BO_3$ , 23;  $MnCl_2$ , 46;  $ZnSO_4$ , 15;  $CuSO_4$ , 1.6; ammonium molybdate 0.7. Leaves from plants grown on this medium contained nitrate and nitrite reductase. The other nutrient solution was the same except that both the  $KNO_3$  and  $Ca(NO_3)_2$  were replaced by  $K_2SO_4$  and  $CaCl_2$  (2.5 and 5.0 mM respectively). Leaves from plants grown on this medium contained no nitrate and were used for the induction experiments. One hundred ml of the appropriate nutrient media were added twice weekly. Water was supplied as needed. When the seedlings were 15 to 30 cm tall the top and middle leaves were selected for use in the induction experiments. Whole plants were used in other experiments.

**Tree Culture.** Mature leaves were obtained from mature trees of Golden Delicious and Starkrimson varieties growing in the University of Illinois Department of Horticulture orchard. These trees had received the low annual amounts of nitrogen common with normal orchard maintenance. Leaves were also obtained from a mature tree of unknown early summer variety growing in a lawn adequately supplied with nitrogen. All trees were bearing fruit when the leaf samples were taken. Only leaves from the lower branches fully exposed to the sun were used in these experiments.

**Polyvinylpyrrolidone (Polyclar AT) Preparation.** Polyclar AT (General Aniline and Film Corporation, Dyestuffs and Chemical Division, 436 Hudson Street, New York, New York) was mixed with enough deionized water to form a thin slurry. The excess was removed from the Polyclar AT by manual squeezing through 3 layers of cheesecloth. This procedure was repeated 3 times. The repeated washings were necessary to hydrate the powder and to remove contaminating nitrite. The final Polyclar AT preparation (25–35% dry weight) was then stored in a sealed container at 2 to 3° until used.

**Enzyme Extraction.** The tissue was weighed, then cut into small (1 × 1 cm) pieces and mixed with 5 to 6 grams of the Polyclar AT preparation per gram of tissue and 6 to 10 volumes of grinding medium in a chilled mortar. Mature tissue required higher amounts of grinding medium and Polyclar AT. The grinding medium was a mixture of 50 mM potassium phosphate, 5 mM EDTA, and 10 mM

cysteine, and adjusted to pH 8.8 with KOH. Normally from 1 to 3 grams of tissue were used. The tissue was ground until a homogeneous slurry was obtained (usually 2–3 min). The slurry was squeezed through 2 layers of cheesecloth and centrifuged at 30,000g for 15 min. The clear light green supernatant was used for the enzyme assay. The temperature was kept at 0 to 3° throughout these operations. Mechanical homogenizers could be substituted for the mortar and pestle, but enzyme activity was decreased by 15 to 20%.

**Enzyme Induction.** The procedure described by Beevers *et al.* (1) was used except that 0.1 M  $KNO_3$  was used as the inducing medium and 0.1 M KCl as the control.

## Methods

Nitrate reductase was assayed as previously described (10) with NADH as the electron donor and by the procedure of Paneque *et al.* (18) except that the FMNH<sub>2</sub> concentration was increased to 2.4 mM, and 0.01 M potassium phosphate buffer, pH 7.5, was substituted for the sodium bicarbonate buffer of the dithionite solution. Nitrite reductase was assayed as described by Joy and Hageman (13). Protein in the extracts was precipitated by an equal volume of 10% trichloroacetic acid; the precipitate was then removed by centrifugation and dissolved in KOH 0.1 N for assay. Protein assays were either by the method of Lowry *et al.* (16) or with the biuret reagent (9). Kjehldahl procedures were not used because some Polyclar AT precipitates with the protein. Nitrate was measured by the procedure of Lowe and Hamilton (15). A Beckman model DB spectrophotometer was used to determine the absorption spectra of extracts.

## Results and Discussion

**Nitrate Reductase in Apple Leaves.** Substantial amounts of nitrate reductase can be found in apple leaves when the enzyme is properly protected during extraction (fig 1). Past efforts to detect this and other enzymes in this tissue have probably failed not only because the polyphenols and related substances inactivated the enzymes but because most of the soluble protein was precipitated (fig 2). Estimates of protein content were erratic (by both methods) when Polyclar AT additions were less than 2 grams per gram fresh weight of tissue.

Although maximum levels of activity obtained from the apple leaves with these procedures are reasonably high, the activity is only 15 to 20% of that observed for cereal crops. It is not known whether optimal concentrations of Polyclar AT provide complete recovery of the enzyme or if a certain proportion is always denatured during extraction.

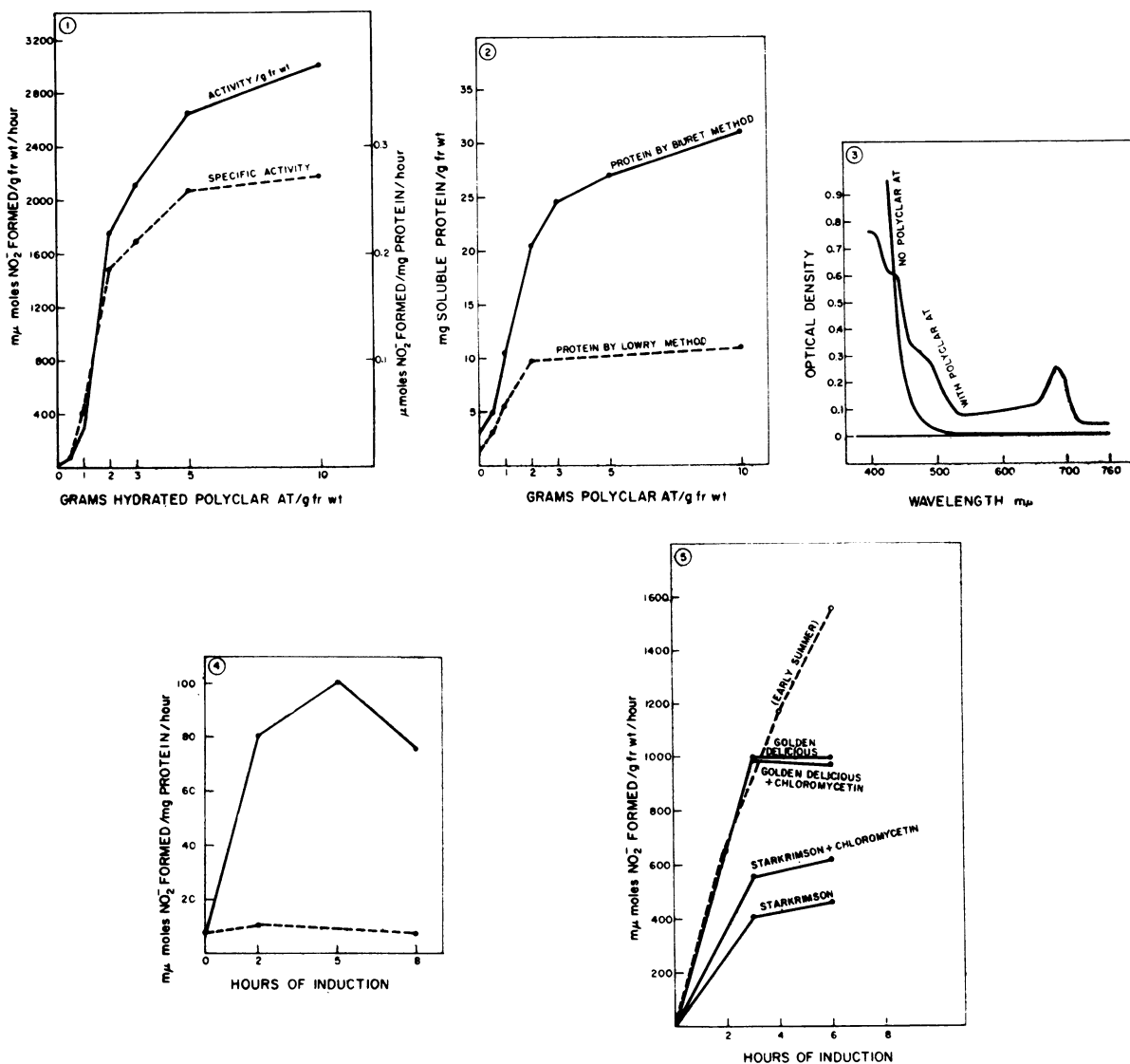


FIG. 1. The effect of increasing amounts of insoluble polyvinylpyrrolidone (Polyclar AT) on the extraction of nitrate reductase from apple seedling leaves. Enzyme activity was determined using NADH as electron donor. Protein for specific activity values was determined by the method of Lowry *et al.* (16). All values represent the average of 3 replicate samples.

FIG. 2. The effect of increasing amounts of hydrated Polyclar AT on the extraction of soluble protein from apple seedling leaves. All values represent the average of 3 replicate samples.

FIG. 3. The effect of Polyclar AT as shown by the absorption spectra of apple seedling leaf extracts. Three gram samples were ground in a mortar with 30 ml grinding medium with and without Polyclar AT (15 g). Each cuvette (1  $\times$  1 cm) contained 0.25 ml of extract and was made up to 3 ml with grinding medium. Polyclar AT in solution did not exhibit any absorption at the wavelengths used.

FIG. 4. Induction of nitrate reductase by apple seedling leaves. (— Treatment, 0.1 M KNO $_3$ ; - - - - control, 0.1 M KCl). Enzyme activity was determined using NADH as electron donor. All values are averages of 3 replicate samples.

FIG. 5. Induction of nitrate reductase by mature leaves from 3 varieties of mature fruit-bearing apple trees. Nitrate reductase was extracted with a mechanical homogenizer (5 g tissue, 30 ml grinding medium and 40 g hydrated Polyclar AT). Chloromycetin was used at a concentration of 30  $\mu$ g/ml in the induction medium. Enzyme activity was determined using NADH as electron donor. Values are expressed as net induction (total induction minus control value). Controls exhibited no induction and are not shown. All values are averages of 2 replicate samples.

*Absorption Spectra of Extracts.* The absorption spectra of apple leaf extracts prepared with or without Polyclar AT are shown in figure 3. The reddish brown supernate obtained when Polyclar AT was omitted during extraction, exhibited intense absorption at 475  $m\mu$  and lower, which is characteristic of phenolics and related compounds. In contrast, the greenish supernate obtained when Polyclar AT was present during extraction, exhibited less absorbancy in the 400 to 475  $m\mu$  region and more absorbancy in the 475 to 700  $m\mu$  region. The greater absorbancy in the 475 to 700  $m\mu$  region for the extract prepared with Polyclar AT is attributed to chlorophyll, protein and other components that are otherwise precipitated by the phenolics and tannins.

*Nitrate Reductase and Nitrate Content of Various Parts of Apple Seedlings.* The data of table I show that nitrate reductase and nitrate are found in all parts of the apple seedlings. The highest amount of nitrate reductase was found in the leaf when computed on a per unit of fresh weight or plant part basis. Although nitrate content was highest in root tissues, appreciable amounts were found in the leaf tissue in spite of the higher level of enzyme activity concentrated there. The enzyme from all parts of the plant had electron donor requirements similar to those of other plant species. The concentration of FMNH<sub>2</sub> required for highest activity was 4 and 12-fold greater than that required by the enzyme from corn (22) and spinach (18), respectively. No credence is given to the differences between levels of activity attained with NADH and FMNH<sub>2</sub> in table I, for in other experiments the activities with the 2 electron donors were similar. According to the work of Schrader *et al.* (22), nitrate reductase of corn can utilize either cofactor with equal efficiency.

This root enzyme appeared to be a typical pyridine nucleotide dependent nitrate reductase and thus differed markedly from the nitrate reductase from apple roots described by Grasmanis and Nicholas (8).

*Nitrite Reductase in Various Parts of the Apple Seedlings.* The level of nitrite reductase activity in the leaf tissues was routinely found to be 4 times higher than the nitrate reductase activity. The activity levels ranged from 5 to 12  $\mu$ moles NO<sub>2</sub><sup>-</sup> re-

duced per g fresh weight per hr. Although no nitrite reductase could be detected in root or stem and petiole tissue, this does not infer that this enzyme is absent in the *in vivo* tissue. Nitrite was not detected in any of tissue assayed.

*Induction of Nitrate Reductase in Leaves of Apple Seedlings.* When apple leaves from seedlings grown on a nitrate-free medium were excised and floated in KNO<sub>3</sub> medium there was a rapid induction of nitrate reductase (fig 4). The low initial level of nitrate reductase in the leaf tissue is attributed to low levels of nitrate present due to microbial conversion of the ammonium ion in the nutrient medium, as the cultures were not sterile. During the induction period, nitrate nitrogen content of the leaf tissue increased in a linear fashion from 15  $\mu$ g to 983  $\mu$ g per g fresh weight.

*Induction of Nitrate Reductase in Mature Apple Leaves.* Since the level of nitrate in the tissue appears to be a major factor limiting the level of nitrate reductase and apple leaves are reportedly devoid of nitrate (2, 23), induction experiments were carried out with mature leaves removed from mature apple trees in midsummer. The initial level of nitrate and nitrate reductase prior to incubation was different among the 3 varieties. In a typical experiment the levels of nitrate and enzyme activities were 1.4, 2.1, and 2.5  $\mu$ g NO<sub>3</sub><sup>-</sup>-N per g fresh weight and of 199, 358, and 623  $m\mu$ moles NO<sub>2</sub><sup>-</sup> formed per hr per g fresh weight for Starkrimson, Golden Delicious, and early summer varieties, respectively. Although good induction was observed with all 3 varieties (fig 5), the patterns and rates of induction were different for each. There was no difference in entry of nitrate into the leaves during induction among these varieties. No change in protein content was detected during the course of the experiment for any of the tissues. The addition of chloromycetin (30  $\mu$ g/ml) (4), to retard potential bacterial contamination, has no apparent effect on the induction rate. An inhibitor of polyphenoloxidase, 2-mercaptobenzothiazole, (19) was used in both the induction and extraction media at concentrations ranging from 10<sup>-3</sup> to 10<sup>-5</sup> M, with no effect on the induction or extraction of the enzyme.

Table I. *The Fresh Weight, Nitrate and Protein Content and Nitrate Reductase Activity With 3 Different Electron Donors of Various Parts of Young Apple Seedlings*

Plant part	Fr wt per plant <i>g</i>	Nitrate reductase activity <sup>1</sup>			Content of NO <sub>3</sub> <sup>-</sup> -N <sup>1</sup> <i>μg</i>	Protein <sup>1</sup> <i>mg</i>
		NADH	FMNH <sub>2</sub>	NADPH		
		<i>mμmoles NO<sub>2</sub><sup>-</sup> formed g fr wt<sup>-1</sup> hr<sup>-1</sup></i>				
Roots	1.03	351	571	0	298	7.2
Stems and petioles	1.34	809	628	0	21	8.3
Leaves	2.03	1312	711	0	45	17.5

<sup>1</sup> Expressed on a gram fresh weight basis.

## 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: 691-98.
2. BOLLARD, E. G. 1956. Nitrogenous compounds in plant xylem sap. *Nature* 178: 1189-90.
3. BOLLARD, E. G. 1957. Nitrogenous compounds in tracheal sap of woody members of the family *Rosaceae*. *Australian J. Biol. Sci.* 10: 281-91.
4. BROCK, T. D. 1961. Chloramphenicol. *Bacteriol. Rev.* 25: 32-48.
5. CRESSWELL, C. F. 1961. An investigation into the nitrate, nitrite and hydroxylamine metabolism in higher plants. Ph.D. thesis. University of Bristol.
6. CROY, L. I. 1967. Nitrate reductase in wheat (*Triticum aestivum* L.) and its relationship to grain protein and yield. Ph.D. thesis. University of Illinois, Urbana.
7. EVANS, H. J. AND A. NASON. 1953. Pyridine nucleotide nitrate reductase from extracts of higher plants. *Plant Physiol.* 28: 233-54.
8. GRASMANIS, V. O. AND D. J. D. NICHOLAS. 1967. A nitrate reductase from apple roots. *Phytochemistry* 6: 217-18.
9. GORNALL, A. G., C. J. BARDAWILL, AND M. M. DAVID. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* 177: 751-62.
10. HAGEMAN, R. H. AND D. FLESHER. 1960. Nitrate reductase activity in corn seedlings as affected by light and the nitrate content of the nutrient media. *Plant Physiol.* 35: 635-41.
11. JONES, J. D. AND A. C. HULME. 1961. Preparation of mitochondria from the peel of apples. *Nature* 191: 370-72.
12. JONES, J. D., A. C. HULME, AND L. S. C. WOOLTON. 1963. Mitochondrial preparations from the fruit of the apple. II. Oxidative phosphorylation. *Phytochemistry* 3: 201-12.
13. JOY, K. W. AND R. H. HAGEMAN. 1966. The purification and properties of nitrite reductase from higher plants, and its dependence on ferredoxin. *Biochem. J.* 100: 263-73.
14. LOOMIS, W. D. AND J. BATTAILE. 1966. Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry* 5: 423-38.
15. LOWE, R. H. AND J. L. HAMILTON. 1967. Rapid determination of nitrate in plant and soil extracts. *J. Agr. Food Chem.* 15: 359-61.
16. LOWRY, O. H., N. J. ROSEBOROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* 193: 265-75.
17. MIFLIN, B. J. 1967. Distribution of nitrate and nitrite reductase in barley. *Nature* 214: 1133-34.
18. PANEQUE, A., F. F. DELCAMPO, J. M. RAMIREZ, AND M. LOSADA. 1965. Flavin nitrate reductase from spinach. *Biochim. Biophys. Acta* 109: 79-85.
19. PALMER, J. K. AND J. B. ROBERTS. 1967. Inhibition of banana polyphenoloxidase by 2-mercaptobenzothiazole. *Science* 157: 200-01.
20. SANDERSON, G. W. AND E. C. COCKING. 1964. Enzymatic assimilation of nitrate in tomato plants. I. Reduction of nitrate to nitrite. *Plant Physiol.* 39: 416-22.
21. SCHRADER, L. E. AND R. H. HAGEMAN. 1967. Regulation of nitrate reductase activity in corn (*Zea mays* L.) seedlings by endogenous metabolites. *Plant Physiol.* 42: 1750-56.
22. SCHRADER, L. E., G. L. RITENOUR, G. L. EILRICH, AND R. H. HAGEMAN. Some characteristics of nitrate reductase from higher plants. *Plant Physiol.* In press.
23. THOMAS, W. 1927. The seat of formation of amino acids in *Pyrus malus* L. *Science* 66: 115-16.
24. WALLACE, W. AND J. S. PATE. 1965. Nitrate reductase in the field pea (*Pisum arvense* L.). *Ann. Botany* 29: 655-71.
25. ZEISERL, J. F., W. L. RIVENBARK, AND R. H. HAGEMAN. 1963. Nitrate reductase activity, protein content, and yield of four maize hybrids at varying plant populations. *Crop Sci.* 3: 27-32.