# The Regulation of Activity of the Enzymes Involved in the Assimilation of Nitrate by Higher Plants

By J. INGLE*
*Department of Botany, Purdue University, Lafayette, Ind., U.S.A.*
and K. W. JOY† and R. H. HAGEMAN
*Department of Agronomy, University of Illinois, Urbana, Ill., U.S.A.*

(*Received* 8 *February* 1966)

1. Possible mechanisms regulating the activities of three enzymes involved in nitrate assimilation, nitrate reductase, nitrite reductase and glutamate dehydrogenase, were studied in radish cotyledons. 2. Nitrate-reductase and nitrite-reductase activities are low in nitrogen-deficient cotyledons, and are induced by their substrates. 3. Glutamate dehydrogenase is present regardless of the nitrogen status, and the enzyme can be increased only slightly by long-term growth on ammonia. 4. Although nitrate is the best inducer of nitrate reductase, lower levels of induction are also obtained with nitrite and ammonia. The experiments did not distinguish between direct or indirect induction by these two molecules. 5. Nitrite reductase is induced by nitrite and only indirectly by nitrate. 6. The induction of both nitrate reductase and nitrite reductase is prevented by the inhibitors actinomycin D, puromycin and cycloheximide, indicating a requirement for the synthesis of RNA and protein. 7. The decay of nitrate reductase, determined after inhibition of protein synthesis, is slower than the synthesis of the enzyme. Nitrite reductase is much more stable than nitrate reductase. 8. The synthesis of nitrate reductase is not repressed by ammonia, but is repressed by growth on a nitrite medium. 9. There is no inhibition of nitrate reductase, nitrite reductase or glutamate dehydrogenase by the normal end products of assimilation, but cyanate is a fairly specific inhibitor of nitrate reductase.

The primary pathway for the assimilation of nitrate by plant tissues is considered to be:

$$NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+ \rightarrow \text{glutamic acid}$$

The first stage of this pathway, the reduction of nitrate to nitrite by the enzyme nitrate reductase (NADH–nitrate oxidoreductase, EC 1.6.6.1) is well established (Evans & Nason, 1953; Candela, Fisher & Hewitt, 1957; Afridi & Hewitt, 1964; Beevers, Flesher & Hageman, 1964; Sanderson & Cocking, 1964). [As a result of the initial work with soya bean (Evans & Nason, 1953) nitrate reductase was classified as NAD(P)H–nitrate oxidoreductase (EC 1.6.6.2). Subsequent work (Beevers *et al.* 1964) has shown nitrate reductase of many plants to have a requirement specific for NADH, hence the classification EC 1.6.6.1.] The further reduction of nitrite was formerly thought to involve a series of two electron shifts via hyponitrite and hydroxylamine to ammonia (Kessler, 1964). Recent studies, however, suggest that in higher plants the nitrite may be reduced directly to ammonia by the enzyme nitrite reductase without the occurrence of the free intermediates (Hageman, Cresswell & Hewitt, 1962; Cresswell, Hageman, Hewitt & Hucklesby, 1965). The incorporation of the ammonia into glutamic acid, by the enzyme glutamate dehydrogenase (L-glutamate–NAD oxidoreductase, EC 1.4.1.2), may be considered as the major route for the final assimilation of the nitrogen into organic compounds (Yemm & Folkes, 1958).

The adaptive nature of nitrate reductase has been demonstrated both in micro-organisms (Nason & Evans, 1953; Morton, 1956; Kinsky, 1961; Morris & Syrett, 1963*a*) and in higher plants (Tang & Wu, 1957; Hageman & Flesher, 1960; Afridi & Hewitt, 1964; Beevers, Schrader, Flesher & Hageman, 1965). However, the induction of enzyme activity does not appear to be completely specific for nitrate as the inducer molecule. With *Neurospora* (Nason & Evans, 1953; Nicholas, Nason & McElroy, 1954) and

* Present address: Department of Botany, University of Edinburgh.
† Present address: Department of Botany, University of Toronto, Toronto, Canada.

with cauliflower (Candela *et al.* 1957) nitrate-reductase activity was approximately the same for material cultured on nitrate or nitrite. Although the addition of $NH_4^+$ ions to fungi cultured on a glucose–nitrate medium repressed nitrate-reductase activity by 70% within 2hr., fungi cultured exclusively on ammonia for a long period contained appreciable activity. Ammonia similarly repressed the synthesis of nitrate reductase in *Chlorella* (Morris & Syrett, 1963*a*); cells cultured on ammonium nitrate and on ammonium sulphate contained respectively 50 and 1·5% as much activity as those grown on potassium nitrate. These workers also showed that *Chlorella* cells cultured for 50hr. on glycine, urea, arginine and alanine media developed 51, 22, 7 and 6% as much nitrate reductase as cells cultured on nitrate. They suggested that a more rapid hydrolysis of arginine and alanine released $NH_4^+$ ions, which repressed enzyme formation. Candela *et al.* (1957) found that cauliflowers grown on an ammonium medium, under non-sterile conditions, had appreciable nitrate-reductase activity. However, in subsequent studies with plants grown on ammonium sulphate or glutamic acid under sterile conditions, nitrate-reductase activity was extremely low (Afridi & Hewitt, 1964). Activity could be induced in such sterile tissue with nitrate, but not with nitrite or ammonia. Studies with higher-plant tissues indicate that nitrate reductase is not repressed by ammonia (Candela *et al.* 1957; Beevers *et al.* 1965). However, recent studies with cultured tobacco cells have shown that the induction of nitrate reductase by exogenous nitrate was repressed by growing the cells in a complete mixture, or in a variety of individual amino acids (Filner, 1965). Consequently, although the inducible nature of nitrate reductase has been well demonstrated, the specificity for the inducer molecule and the possible repression or inhibition mechanisms regulating the activity of this enzyme are not clear. Regulation of the reduction of nitrite to ammonia has received little consideration in the past, owing to the lack of information on the mechanism of this reduction and to the lack of a convenient assay system. Although there has been no direct evidence for the induction of nitrite reductase, the fact that nitrite does not accumulate to any large extent in tissues induced with nitrate suggests either that nitrite reductase is also induced or that the tissue initially contains enough of this enzyme activity to handle the increased production of nitrite. By using the methods described by Joy & Hageman (1966) it has been possible to study the regulation of nitrite reductase.

The present investigation was undertaken to study the regulatory mechanisms involved in the assimilation of nitrate by radish cotyledons. The three enzymes involved in this pathway, nitrate reductase, nitrite reductase and glutamate dehydrogenase, were studied to determine the effect of substrate on the induction of enzyme activity. Studies were undertaken to determine whether induction of the assimilation pathway was under operon control, whereby all the enzymes were controlled by a single de-repressor, or whether each was controlled independently. Other enzyme activities, glyceraldehyde 3-phosphate dehydrogenase (D-glyceraldehyde 3-phosphate–NADP oxidoreductase, EC 1.2.1.9), aldolase (ketose 1-phosphate aldehyde-lyase, EC 4.1.2.7) and glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate–NADP oxidoreductase, EC 1.1.1.49), and respiration were monitored in these experiments to determine whether the initial assimilation of the nitrogen source had any major effect on the general metabolism of the tissue. The possible roles of end-product repression of enzyme synthesis and of feedback inhibition of enzyme activity were investigated in relation to the overall regulation of the pathway.

## MATERIALS AND METHODS

### *Plant material*

Radish seeds (*Raphanus sativus* L., var. Cherrybelle) were surface-sterilized in 1% hypochlorite solution for 10min., rinsed and distributed in a 3-quart Pyrex dish containing four layers of cheese cloth and 100ml. of N-deficient growth medium (Table 1). The seeds were covered with 800ml. of Krum, an inert soil conditioner prepared from natural volcanic ash (Ryolex Corp., Champaign, Ill., U.S.A.), watered with 200ml. of the required growth medium (Table 1) and then the dish was covered with Saran Wrap (Dow Chemical Co., Midland, Mich., U.S.A.) perforated for aeration. The initial 100ml. of N-deficient growth medium was necessary to prevent the inhibition of germination caused by the $NH_4^+$ and $NO_2^-$ growth media. The seeds were germinated in a controlled environment chamber having a 16hr. photoperiod of 1200ft.-candles at 22·5°. Each dish was watered daily with 100ml. of the appropriate nutrient growth medium (Table 1) for the next 4 days. On the fourth day the Saran Wrap was removed. A 200ml. volume of the appropriate nutrient growth medium was given on subsequent days.

### *Induction of enzyme activities in excised cotyledons*

Each sample consisted of cotyledons from 15 uniform seedlings. The cotyledons were excised under water, rinsed, drained and transferred as quickly as possible to a 50ml. Erlenmeyer flask containing 1mM-potassium phosphate buffer, pH 5·5, the inducer substrate and inhibitor in a final volume of 7ml. Inducer substrates were generally used at a final concentration of 10mM; exceptions are detailed in the Results section. The inhibitors, actinomycin D (Merck, Sharp and Dohme Inc., Rahway, N.J., U.S.A.), puromycin (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) and cycloheximide (Nutritional Biochemicals Corp.), were used at 20, 200 and 2$\mu$g./ml. respectively. After the required

Table 1. *Media used for growing radish seedlings*

The solutions, prepared fresh every few days, were made up in tap water, and adjusted to pH 7·4.

| | Concn. (mM) | | | | |
|---|---|---|---|---|---|
| Salt | N-deficient medium | $NO_3^-$ medium | $NH_4^+ + NO_3^-$ medium | $NH_4^+$ medium | $NO_2^-$ medium |
| $KH_2PO_4$ | 1 | 1 | 1 | 1 | 1 |
| $MgSO_4$ | 2 | 2 | 2 | 2 | 2 |
| $FeSO_4$ | 0·03 | 0·03 | 0·03 | 0·03 | 0·03 |
| $CaCl_2$ | 5 | — | 5 | 5 | 5 |
| $K_2SO_4$ | 3 | — | 3 | — | — |
| $Ca(NO_3)_2$ | — | 5 | — | — | — |
| $KNO_3$ | — | 6 | — | — | — |
| $NH_4NO_3$ | — | — | 8 | — | — |
| $NH_4Cl$ | — | — | — | 6 | — |
| $(NH_4)_2SO_4$ | — | — | — | 5 | — |
| $KNO_2$ | — | — | — | — | 10 |
| $NaNO_2$ | — | — | — | — | 6 |

induction period at 30° with constant shaking in the dark, the cotyledons were removed from the medium, thoroughly washed with deionized water and homogenized for the determination of enzyme activity, protein and nitrate or nitrite content. Duplicate or triplicate samples were used in all experiments, and each experiment was repeated at least twice. The results presented, unless otherwise stated, are the average values from at least four separate samples.

### *Enzyme assays*

All enzyme preparations were carried out in the cold (0–4°).

*Nitrate reductase.* The cotyledons (15 pairs) were homogenized in 10ml. of 10mM-potassium phosphate–10mM-EDTA–5mM-cysteine (pH 8·2) medium for 30sec. in a VirTis homogenizer at medium speed. After filtration through four layers of cheese-cloth the homogenate was centrifuged for 15min. at 20000*g*. Nitrate-reductase activity was determined in the supernatant by the colorimetric estimation of the nitrite produced under the assay conditions described by Hageman & Flesher (1960), and expressed in milliunits (mμmoles of nitrite produced/min.). Since the protein content of the excised cotyledons did not change significantly during treatment, activity was expressed on the basis of a pair of cotyledons rather than on mg. of protein.

*Nitrite reductase.* Cotyledons were homogenized in acetone [at a final concentration of 75% (v/v), containing Cleland's (1964) reagent (1mM)]. The precipitate, after being washed with acetone, was dried under vacuum. The acetone-dried powder was resuspended in 0·12M-sodium phosphate buffer, pH 7·5, centrifuged, and the supernatant was assayed by the method of Joy & Hageman (1966), with dithionite and benzyl viologen as electron donors. The activity was expressed in milliunits/pair of cotyledons.

*Glutamate dehydrogenase.* The acetone-dried powder, prepared as described above, was resuspended in 0·1M-sodium phosphate buffer, pH 7·4, containing 0·1% of OPC.45 (a non-ionic detergent; Petro-Chemical Industries, Manchester). After centrifugation the supernatant was assayed at room temperature (approx. 20°) by the oxidation or reduction of NAD or NADP. The respective incubation media contained (*a*) 0·2ml. of 0·2M-α-oxoglutarate, 0·2ml. of 0·2M-$(NH_4)_2SO_4$, 0·5ml. of 1mM-NADH (or NADPH), 0·1ml. of enzyme and 2·0ml. of 0·2M-tris buffer, pH 8·1, and (*b*) 0·2ml. of 0·2M-glutamate, 0·5ml. of 1mM-$NAD^+$ (or $NADP^+$), 0·5ml. of enzyme and 1·8ml. of 0·2M-tris buffer, pH 8·1. The activity was expressed in milliunits (mμmoles of NAD or NADP oxidized or reduced/min.)/pair of cotyledons.

*Aldolase.* Activity was measured on the acetone-dried extract, as prepared for nitrite reductase, by following, at room temperature, the hydrazone formation at 240mμ. A modification of the method of Jagannathan, Singh & Damodaran (1956), as described in the *Worthington Enzyme Manual* (1965), was used. A hydrazine–dihydroxyacetone phosphate-plus-glyceraldehyde 3-phosphate (triose phosphate esters; Calbiochem Corp., Los Angeles, Calif., U.S.A.) standard curve was used to convert the $\Delta E_{240}$ into mμmoles of fructose diphosphate cleaved, and the activity was expressed in milliunits.

*Glyceraldehyde 3-phosphate dehydrogenase.* Activity was measured on the acetone-dried extract, as prepared for nitrite reductase, by following the reduction at room temperature of $NADP^+$, as described by Gibbs & Turner (1964), and expressed in milliunits (mμmoles of $NADP^+$ reduced/min.)/pair of cotyledons.

*Glucose 6-phosphate dehydrogenase.* Cotyledons were homogenized in 50mM-tris buffer, pH 7·4, and, after centrifugation at 20000*g* for 15min., the supernatant was assayed at room temperature by following the reduction of $NADP^+$, as described in the *Worthington Enzyme Manual* (1965), based on the method of Kornberg (1950). The activity was expressed in milliunits (mμmoles of $NADP^+$ reduced/min.)/pair of cotyledons.

### *Respiration*

Four pairs of cotyledons were incubated at 30° in Warburg flasks containing 2·5ml. of 1mM-phosphate buffer, pH 5·5. Oxygen uptake was measured over a 2hr. period, and then

Table 2. *Growth of seedlings on different nitrogen sources*

Whole seedlings were harvested after 7 days' growth. The results given are averages of two experiments. In each experiment four replicate samples of 15 seedlings were used, two for dry weight and two for protein determinations. The values for fresh weight are expressed as means ± S.E.M. of eight samples.

| Nitrogen source | Fresh wt. (mg./seedling) | Dry wt. (mg./seedling) | Protein (mg./seedling) |
|---|---|---|---|
| N-deficient | 129 ± 6 (8) | 10·0 | 1·25 |
| $NO_3^-$ | 146 ± 9 (8) | 9·4 | 1·43 |
| $NH_4^+$ | 124 ± 11 (6) | 9·5 | 1·49 |
| $NH_4^+ + NO_3^-$ | 166 ± 15 (8) | 11·3 | 1·83 |
| $NO_2^-$ | 143 ± 14 (6) | 11·0 | 1·58 |

the inducer substrate was added to a final concentration of 10 mM from the side arm. Oxygen uptake was recorded for a further 2 hr., and this rate was expressed as a percentage of the initial rate.

### *Other determinations*

The nitrate and nitrite contents of the cotyledons were determined by a modification of the method described by Woolley, Hicks & Hageman (1960) on samples of the supernatant prepared for the nitrate-reductase assay, and expressed as mμmoles/pair of cotyledons. The smallest amounts detectable in the assay, when corrected for interfering substances present in the extract, were 15 and 4 mμmoles of nitrate and nitrite respectively, corresponding to 20 and 5 mμmoles/pair of cotyledons.

For the determination of protein content of the seedling tissue, portions of the initial homogenate were precipitated with trichloroacetic acid (final concn. 5%, w/v), washed with 5% trichloroacetic acid and defatted with ethanol–ether–chloroform (2:1:1, by vol.). The final residue was dissolved in 0·1 N-NaOH and assayed by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as the standard.

Protein synthesis was determined by the incorporation of [$^{14}$C]leucine into protein as described by Key (1964).

## RESULTS

### *Intact seedlings*

*Growth of seedlings on different nitrogen sources.* Radish seedlings grew well on the four nitrogen sources used (Table 1). After 7 days' growth the protein contents of $NO_3^-$-, $NH_4^+$-, $NH_4^+ + NO_3^-$- and $NO_2^-$-grown seedlings were higher than that of the comparable N-deficient seedlings (Table 2), although only the $NH_4^+ + NO_3^-$ and $NO_2^-$ growth media resulted in dry weights higher than the N-deficient control. These findings suggested that the radish seedlings were capable of utilizing $NO_3^-$, $NH_4^+$ or $NO_2^-$ as a primary nitrogen source.

*Activity and inducibility of nitrate reductase in the cotyledons.* The cotyledons of seedlings grown without exogenous nitrogen showed only small changes in nitrate-reductase activity with growth, and the activity was very low over the entire period (Fig. 1).

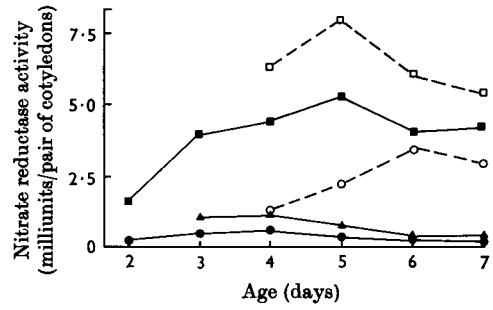


Fig. 1. Nitrate-reductase activity in cotyledons from seedlings grown on different nitrogen sources: ●, N-deficient; ▲, $NO_2^-$; ○, $NH_4^+$; ■, $NO_3^-$; □, $NH_4^+ + NO_3^-$.

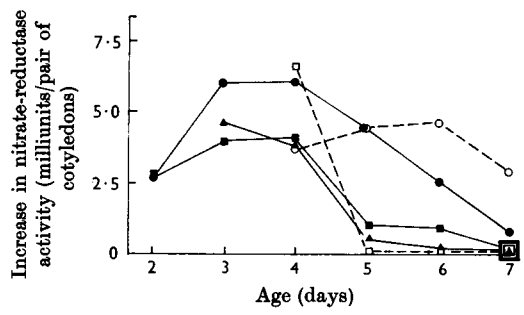


Fig. 2. Induction of nitrate-reductase activity, by incubation with $NO_3^-$, in cotyledons excised from seedlings grown on different nitrogen sources: ●, N-deficient; ▲, $NO_2^-$; ○, $NH_4^+$; ■, $NO_3^-$; □, $NH_4^+ + NO_3^-$. Cotyledons were incubated with $NO_3^-$ for 4 hr. The initial activities, as shown in Fig. 1, were subtracted from the total activities after incubation.

The activity in cotyledons from $NO_3^-$-grown seedlings was fairly constant after 3 days, and a higher activity resulted from growth on the $NH_4^+ + NO_3^-$ growth medium. With $NH_4^+$-grown seedlings the activity was low during the first few days, but by 6 and 7 days it was comparable with that of $NO_3^-$-grown seedlings. Low activity was

found in the cotyledons of the young $NO_2^-$-grown seedlings, and this activity decreased with growth.

All three nitrogen sources, $NO_3^-$, $NH_4^+$ and $NO_2^-$, appeared to stimulate the activity of nitrate reductase above that of the N-deficient control. Since the seedlings were not grown under rigidly sterile conditions, interconversions of the nitrogen supply by contaminating micro-organisms could not be ruled out. However, the seeds were initially surface-sterilized, the Krum was sterile and fresh growth media were used. Further, analysis of the $NH_4^+$ growth medium and the corresponding cotyledon tissue, after 7 days' growth, showed that the nitrate concentration in the medium was less than 0·05mM, and there were less than 20mμmoles of nitrate/pair of cotyledons. Such concentrations, if given as an exogenous $NO_3^-$ supply, would not give a measurable stimulation of nitrate-reductase activity. However, the possibility cannot be ruled out that small amounts of $NO_3^-$, below the level of detection, were active at the site of induction in the presence of $NH_4^+$.

The response of the excised cotyledons to a 4hr. incubation with exogenous $NO_3^-$ varied according to the growth conditions and the age of the seedlings. Incubation of cotyledons excised from 3- or 4-day seedlings produced a large increase in nitrate-reductase activity, irrespective of their growing conditions (Fig. 2). On subsequent days there was a linear decrease in the activity induced in cotyledons from N-deficient seedlings, a sharp fall of activity induced in cotyledons from $NO_3^-$-, $NH_4^+ + NO_3^-$- and $NO_2^-$-grown seedlings, whereas cotyledons from $NH_4^+$-grown seedlings maintained their ability for the induction of nitrate reductase.

*Induction of enzyme activities in excised cotyledons*

Since the growth of seedlings on $NO_3^-$, $NH_4^+$ and $NO_2^-$ growth media resulted in increased nitrate-reductase activity, these substrates were tested for their ability to stimulate activity in cotyledons excised from seedlings grown on the N-deficient growth medium for 5 days. A summary of the changes in enzyme activities in response to a variety of possible inducer substrates is given in Table 3. The enzyme activities after a 4hr. incubation with $Cl^-$ were very similar to the initial values, and hence represent the constitutive activities of the tissue. There were large increases in the activities of nitrate reductase and nitrite reductase in response to their substrates, whereas glutamate-dehydrogenase activity remained constant. The activities of the other enzymes, glyceraldehyde 3-phosphate dehydrogenase, aldolase and glucose 6-phosphate dehydrogenase, and respiration showed only small changes, indicating that the incubations were not changing the general metabolism of the tissue to any large extent.

*Nitrate reductase.* With the range of ions used significant stimulations of nitrate-reductase activity were obtained in response to short periods of exposure to $NO_3^-$, $NH_4^+$ and $NO_2^-$ (Table 3). The relative activities induced with these three substrates were quite reproducible, $NO_2^-$ being 50–70% and $NH_4^+$ 20–30% as effective as $NO_3^-$. The

Table 3. *Enzyme activities in excised cotyldons in response to inducer substrates*

Cotyledons from 5-day N-deficient seedlings were incubated for 4hr. in media containing the inducer substrate at a final concentration of 10mM. The activity after 4hr. incubation with $Cl^-$ represents the constitutive activity of the tissue.

| | Enzyme activity (milliunits/pair of cotyledons) | | | | | | |
|---|---|---|---|---|---|---|---|
| Inducer substrate | Nitrate reductase | Nitrite reductase | Glutamate dehydrogenase* | Glyceraldehyde phosphate dehydrogenase | Aldolase | Glucose 6-phosphate dehydrogenase | $O_2$ uptake (% of initial value) |
| $Cl^-$ | 0·35 | 0·27 | 20·6 | 62·1 | 30·0 | 29·0 | 90 |
| $NO_3^-$ | 4·98 | 0·79 | 21·9 | 81·1 | 27·6 | 32·4 | 95 |
| $NH_4^+$ | 1·45 | 0·13 | 21·9 | — | 32·8 | — | 88 |
| $NO_2^-$ | 3·04† | 1·59 | 20·6 | 66·0 | 25·1 | — | 108 |
| $NH_4^+ + NO_3^-$ | 9·35 | — | — | — | — | — | — |
| $NO_3^- + NO_2^-$ | 3·80† | — | — | — | — | — | — |
| $NH_4^+ + NO_2^-$ | 3·50† | — | — | — | — | — | — |
| $PO_4^{3-}$ | 0·67 | — | — | — | — | — | — |
| $SO_4^{2-}$ | 0·52 | — | — | — | — | — | — |
| $CO_3^{2-}$ | 0·38 | — | — | — | — | — | — |
| Citrate | 0·43 | — | — | — | — | — | — |
| Acetate | 0·52 | — | — | — | — | — | — |

* NADH-dependent (see Table 5).

† $NO_2^-$ was used at a final concentration of 4mM.

Table 4. *Effect of inhibitors on the induction of nitrate reductase and nitrite reductase in excised cotyledons*

Cotyledons were pretreated with the inhibitor (see the Materials and Methods section for concentrations) for 1 hr. before the addition of inducer substrate for a further 3·5 hr. For each inducer an appropriate control without inhibitor was prepared.

| | Activity (% of control) | | |
|---|---|---|---|
| Inhibitor ... ... | Puromycin | Cycloheximide | Actinomycin D |
| Nitrate reductase in response to: | | | |
| $NO_3^-$* | 11 | 6 | 21 |
| $NH_4^+$ | 21 | — | 27 |
| $NO_2^-$ | 23 | — | 39 |
| Nitrite reductase in response to: | | | |
| $NO_2^-$ | 0 | — | 22 |

* The $NO_3^-$ contents of these cotyledons were 89, 116 and 103% of the control for puromycin, cycloheximide and actinomycin D respectively.

activity attained during the 4 hr. incubation of excised cotyledons with $NO_3^-$ was comparable with the activity normally present in cotyledons of $NO_3^-$-grown seedlings. The striking synergistic response with $NH_4^+ + NO_3^-$ (Table 3) could not be explained by increased uptake of $NO_3^-$, since the nitrate contents of cotyledons after incubation with $NO_3^-$ and $NH_4^+ + NO_3^-$ were 230 and 220 mμmoles/pair of cotyledons respectively.

As reported by Beevers *et al.* (1965) the induction of nitrate-reductase activity in response to exogenous $NO_3^-$ was prevented by the inhibitors of protein and RNA synthesis, puromycin and actinomycin D. Induction of activity in response to $NH_4^+$ and $NO_2^-$ was also inhibited under these conditions (Table 4). Since these inhibitors did not decrease the uptake of $NO_3^-$ into the tissue (Table 4), and had no effect on the enzyme assay (Table 9), it appeared that the increase in enzyme activity, in response to $NO_3^-$, $NH_4^+$ and $NO_2^-$, required the synthesis of new protein and RNA.

*Nitrite reductase.* The incubation of cotyledons from N-deficient seedlings with $NO_3^-$ or $NO_2^-$ increased the activity of nitrite reductase. $NH_4^+$ had no such stimulatory effect (Table 3). Activity after a 4 hr. incubation with $NO_2^-$ was about half of that measured in cotyledons from $NO_2^-$-grown seedlings (Tables 3 and 8). A similar period of incubation with $NO_3^-$ resulted in a lower activity (Table 3), but as shown in Fig. 3 there was a lag of 3 hr. before any increase in nitrite-reductase activity in response to $NO_3^-$ was detectable. The induction of nitrite reductase by $NO_2^-$ began almost immediately. The kinetics of the induction of nitrate reductase and nitrite reductase, in response to $NO_3^-$ (Fig. 3), suggested that the primary effect of $NO_3^-$ was on the induction of nitrate reductase, and that the subsequent production of $NO_2^-$ in the tissue in turn induced nitrite reductase. It should be noted that,

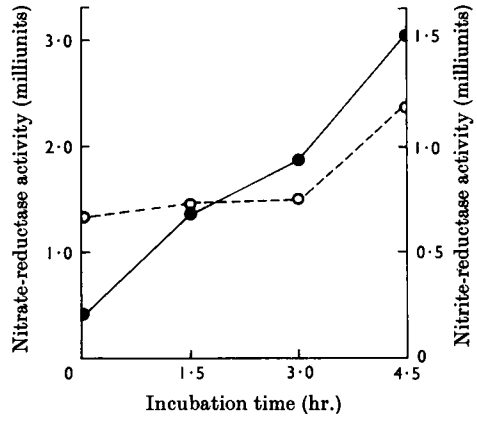


Fig. 3. Time-course of induction of nitrate reductase (●) and nitrite reductase (○) in response to $NO_3^-$. Cotyledons from N-deficient seedlings were incubated with $NO_3^-$.

under the conditions of the experiments, assays showed no detectable $NO_2^-$ in the tissue; however, this does not prove that the concentration of $NO_2^-$ needed for induction was not available at the actual site of induction.

The stimulation of nitrite-reductase activity was prevented by puromycin and actinomycin D, indicating a requirement for the synthesis of protein and RNA for the induction of enzyme activity (Table 4).

*Glutamate dehydrogenase.* The synthesis of glutamic acid by radish glutamate dehydrogenase was fairly specific for NADH, and the ratio of the activities with NADH and NADPH varied only slightly in cotyledons from seedlings grown on different nitrogen sources (Table 5). The oxidative deamination of glutamic acid was similarly $NAD^+$-

dependent, although this activity was extremely low relative to the synthesis of glutamic acid (Table 5). The activity of glutamate dehydrogenase in cotyledons from N-deficient seedlings was as high as that from cotyledons of $NO_3^-$-grown seedlings. Incubation of cotyledons from N-deficient seedlings with $NO_3^-$, $NH_4^+$ or $NO_2^-$ resulted in no change in activity (Table 3), suggesting that the initial amount of this enzyme was sufficient to handle the $NH_4^+$ produced by the stimulation of $NO_2^-$ or $NO_3^-$ assimilation. However, growth of seedlings on $NH_4^+$ growth medium for 6 days did result in a higher activity (Table 5).

*Stability of nitrate reductase and nitrite reductase*

Since large increases in enzyme activity occurred in response to substrate, mechanisms should exist for the loss of this activity when the substrate is no longer present. The increase in activity required the synthesis of new protein, so that it appeared probable that a loss of activity would result when the rate of decay of the enzyme was greater than its rate of synthesis, which could be controlled by the removal of substrate or by the addition of inhibitors. The nitrate-reductase activity decreased by about 20% when cotyledons from $NO_3^-$-grown seedlings were incubated with $Cl^-$ for 6hr. (Table 6). Although the $NO_3^-$ content of the cotyledons decreased by 70% during this period, the nitrate remaining in the tissue (480mμmoles/pair of cotyledons) was still twice as much as that taken up during the incubation of cotyledons from N-deficient seedlings with $NO_3^-$. Consequently there was still plenty of $NO_3^-$ present for the continual induction of activity. Inhibition of protein synthesis during the 6hr. incubation, by cycloheximide, puromycin or actinomycin D, resulted in larger decreases in activity, indicating the continual turnover of the enzyme. Under similar conditions, however, there was very little decrease in the activity of nitrate reductase, indicating the relative stability of this enzyme (Table 6).

Since the cotyledons from $NO_3^-$-grown seedlings were not readily depleted of their nitrate, cotyledons from N-deficient seedlings were used to show the decrease in nitrate-reductase activity in response to the removal of inducer. The changes in activity in cotyledons that were incubated with $NO_3^-$ for 1hr., then washed and transferred to a $Cl^-$ medium for a further 4hr., are shown in Fig. 4.

*Repression of nitrate-reductase induction*

The possible repression of nitrate-reductase synthesis by a variety of individual (or a mixture of) amino acids was studied by pretreating cotyledons, excised from N-deficient seedlings, for various times with the amino acid, then adding $NO_3^-$ to the medium to induce activity. A 2hr. pretreatment with glutamine, aspartic acid, alanine or leucine had no effect on subsequent induction (Table 7). Asparagine and glutamic acid caused a 20–30% decrease in induction, but this effect was not specific for the naturally occurring L-isomers, nor was the repression increased by longer pretreatments (Table 7). A mixture of amino acids, including asparagine and glutamic acid, had no effect on induction. Thus there was only indication of slight

Table 5. *Activity of glutamate dehydrogenase in cotyledons from seedlings grown on different nitrogen sources for 6 days.*

The enzyme was assayed with $NAD^+$, $NADP^+$, NADH and NADPH, as described in the Materials and Methods section.

| Nitrogen source | Glutamate-dehydrogenase activity (milliunits/pair of cotyledons) | | | |
|---|---|---|---|---|
| Coenzyme... | NADH | NADPH | $NAD^+$ | $NADP^+$ |
| N-deficient | 20·6 | 2·7 | 1·7 | 0·1 |
| $NO_3^-$ | 21·2 | 3·2 | — | — |
| $NH_4^+$ | 34·8 | 5·3 | 2·7 | 0·2 |

Table 6. *Stability of nitrate reductase and nitrite reductase in excised cotyledons*

Cotyledons from $NO_3^-$-grown seedlings, with high nitrate content and high nitrate-reductase and nitrite-reductase activities, were incubated with $Cl^-$ with and without inhibitors (see the Materials and Methods section).

| Treatment ... | $Cl^-$ | $Cl^-$ + cycloheximide | $Cl^-$ + puromycin | $Cl^-$ + actinomycin D |
|---|---|---|---|---|
| Nitrate reductase after 6hr. (% of initial activity) | 82 | 52 | 65 | 64 |
| $NO_3^-$ content after 6hr. (% of initial content) | 32 | — | — | — |
| Protein synthesis* during 6hr. (% of $Cl^-$ incorporation) | 100 | 28 | 70 | 81 |
| Nitrite reductase after 4hr. (% of initial activity) | 105 | 98 | — | — |

* See the Materials and Methods section.

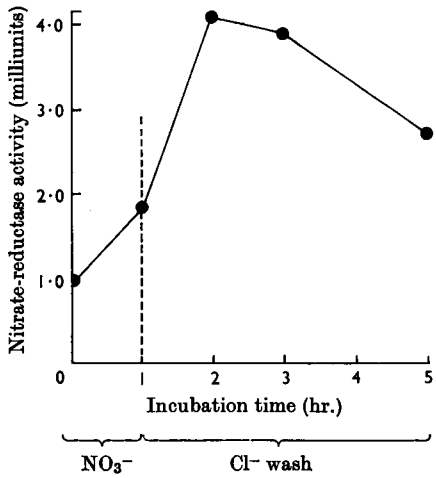


Fig. 4. Changes in nitrate-reductase activity after removal of the inducer. Cotyledons from N-deficient seedlings were incubated with $NO_3^-$ for 1 hr., then removed, washed, and incubated in $Cl^-$ for a further 4 hr.

Table 7. *Repression of nitrate-reductase induction in excised cotyledons*

Cotyledons from N-deficient seedlings were incubated in the supplemented medium (additions were 10 mM final concentration) for various times, after which $NO_3^-$ or $Cl^-$ was added to a final concentration of 5 mM. Enzyme activity was then determined after a further 3·5 hr., and the difference between the $NO_3^-$ and $Cl^-$ treatments expressed as the induction.

| Supplement to incubation | Pretreatment time (hr.)* | Activity (% of control) |
|---|---|---|
| L-Glutamine | 2 | 95 |
| L-Asparagine | 2 | 72 |
| | 6 | 100 |
| | 18 | 110 |
| | 24 | 134 |
| D-Asparagine | 2 | 72 |
| L-Glutamic acid | 2 | 67 |
| | 6 | 77 |
| | 18 | 95 |
| | 24 | 51 |
| D-Glutamic acid | 2 | 83 |
| L-Aspartic acid | 2 | 93 |
| L-Alanine | 2 | 94 |
| L-Leucine | 2 | 105 |
| Mixture: L-asparagine (2·8 mM) + L-glutamine (1·4 mM) + L-alanine (1·4 mM) + L-glutamic acid (2·8 mM) + L-aspartic acid (1·4 mM) | 2 | 102 |

* For pretreatments longer than 2 hr., the incubation solutions were changed frequently and contained a low concentration of chloramphenicol (40 μg./ml.) to decrease bacterial contamination.

repression of synthesis by the possible end-product amino acids under the conditions of these experiments.

Very little nitrate-reductase activity could be induced in cotyledons excised from seedlings grown for 5 or more days on $NO_3^-$, $NH_4^+ + NO_3^-$ or $NO_2^-$ growth medium (Fig. 2). Cotyledons from $NO_3^-$- and $NH_4^+ + NO_3^-$-grown seedlings had a high initial activity (Fig. 1). In contrast, cotyledons from $NO_2^-$-grown seedlings had very low activity (Fig. 1). With cotyledons from 6-day $NO_2^-$-grown seedlings, the activity induced on incubation was only 10% of that induced in comparable cotyledons from N-deficient seedlings, although the uptake of $NO_3^-$ into the tissue was only slightly less (Table 8). This was not a general toxicity effect since the $NO_2^-$-grown seedlings were growing very well (Table 2), and there was high nitrite-reductase activity, which could be further increased by incubation with $NO_2^-$ (Table 8). The magnitude of the repression of nitrate-reductase synthesis depended on the amount of nitrite supplied to the seedlings over the growth period (approx. 15 m-moles/dish in 6 days).

No analogous effects were observed with the induction of nitrite reductase. Although cotyledons from 6-day $NO_2^-$-grown seedlings had a high initial activity, further activity, comparable with that with tissue from N-deficient seedlings, was induced by incubation of the excised cotyledons with $NO_2^-$. Cotyledons from $NH_4^+$-grown seedlings, which had low initial activity, similarly showed good induction on incubation with $NO_2^-$ (Table 8).

### *Inhibition of nitrate-reductase, nitrite-reductase and glutamate-dehydrogenase activities*

Nitrate-reductase activity was not affected by the presence of the metabolic inhibitors, actinomycin D, puromycin or cycloheximide, in the assay (Table 9). Neither was there significant inhibition of nitrate-reductase, nitrite-reductase or glutamate-dehydrogenase activity when any of the normal end products of nitrate assimilation, such as nitrite, ammonia, glutamic acid and aspartic acid or their corresponding amides, alanine, leucine or arginine, was added to the assay. However, cyanate inhibited nitrate-reductase activity by 80% when used at a concentration half that of the substrate, but inhibited nitrite-reductase activity by only 25% when present at 17 times the substrate concentration, and glutamate dehydrogenase activity by 5% when present at substrate concentration (Table 9).

## DISCUSSION

The activities of the first two enzymes involved in the assimilation of nitrate, nitrate reductase and nitrite reductase, increased several-fold in response

Table 8. *Induction of nitrate reductase and nitrite reductase in cotyledons excised from seedlings grown on different nitrogen sources for 6 days*

| Nitrogen source | Nitrate reductase (milliunits) | | | Nitrite reductase (milliunits) | |
|---|---|---|---|---|---|
| | Initial activity | Increase in activity after 4hr. induction in $NO_3^-$ | Increase in $NO_3^-$ content after 4hr. induction in $NO_3^-$ (mμmoles/pair of cotyledons) | Initial activity | Increase in activity after 4hr. induction in $NO_2^-$ |
| N-deficient | 0·17 | +2·60 | +240 | 0·72 | +2·41 |
| $NO_2^-$ | 0·25 | +0·25 | +200 | 3·82 | +2·08 |
| $NH_4^+$ | 3·41 | +6·00 | +254 | 0·67 | +2·15 |

Table 9. *Inhibition of nitrate-reductase, nitrite-reductase and glutamate-dehydrogenase activities*

The normal nitrate-reductase preparation of cotyledons from $NO_3^-$-grown seedlings was desalted on a column of Bio-Gel P-10. Additions to the assay medium were at substrate concentration (10mM) unless otherwise shown. Nitrite reductase was purified from spinach (Joy & Hageman, 1966). Additions to the assay medium were at substrate concentration (0·3mM) unless otherwise shown. Glutamate dehydrogenase was prepared from cotyledons of $NH_4^+$-grown seedilngs, and assayed with NADH. Assays with NADPH gave similar results. Additions to the assay medium were at substrate concentrate (13mM).

| Addition to assay medium | Activity (% of control) | | |
|---|---|---|---|
| | Nitrate reductase | Nitrite reductase | Glutamate dehydrogenase |
| $NO_3^-$ omitted | 0 | — | — |
| Actinomycin D (2μg./ml.) | 100 | — | — |
| Puromycin (20μg./ml.) | 99 | — | — |
| Cycloheximide (0·2μg./ml.) | 95 | — | — |
| $NH_4^+$ | 90 | 103 | — |
| L-Glutamine | 91 | 101 | 100 |
| L-Asparagine | 92 | 101 | 108 |
| L-Glutamic acid | 89 | 105 | 92 |
| L-Aspartic acid | 87 | 100 | 98‡ |
| L-Alanine | — | 99 | 98 |
| L-Leucine | 97 | — | 98 |
| L-Arginine | — | — | 100 |
| Nitrite (40mM) | 96* | — | — |
| Cyanate | 20† | 75† | 95 |

* Reaction mixtures minus enzyme, rather than minus NADH, were used as the blank to correct for the interference of NADH with the nitrite–diazo complex (Medina & Nicholas, 1957).

† Final concentration of cyanate was 5mM.

‡ Reaction mixtures minus $NH_4^+$, rather than minus α-oxoglutarate, were used as the blank to correct for the oxidation of NADH resulting from contaminating transaminase and malate-dehydrogenase activities.

to their respective substrates. The activity of glutamate dehydrogenase, the final enzyme considered in this pathway, was normally high in the tissue and was only slightly increased by growth on $NH_4^+$. Consequently, radish seedlings were able to use $NO_3^-$, $NO_2^-$ or $NH_4^+$ as a primary nitrogen source.

The cotyledons of radish seedlings grown under non-sterile conditions on a $NO_3^-$ or $NH_4^+ + NO_3^-$ growth medium developed (in 2–3 days) and maintained a high nitrate-reductase activity throughout the experimental period. In contrast, cotyledons of $NH_4^+$-grown seedlings did not develop any appreciable activity until day 5, and only after this time was the activity comparable with that of the $NO_3^-$-grown seedlings. Candela *et al.* (1957) reported that nitrate-reductase activity in cauliflowers grown under non-sterile conditions was 50% that of the $NO_3^-$-grown controls, and in this case the activity was thought to result from contamination with, or conversion into, $NO_3^-$ or $NO_2^-$. Corn seedlings also developed a high nitrate-reductase activity when grown on $NH_4^+ + NO_3^-$ growth medium. When $NH_4^+$ was the source of nitrogen the activity in the seedling was only 15–20% of the $NO_3^-$-grown control; however, the addition of traces of $NO_3^-$ (5–10p.p.m.) to the $NH_4^+$ growth medium increased the enzyme activity to 60% of that of the $NO_3^-$-grown seedlings (L. E. Schrader, unpublished work). Thus the

synthesis of nitrate reductase in higher plants is not repressed by $NH_4^+$, which contrasts with the regulatory mechanisms functioning in fungi (Nason & Evans, 1953; Morton, 1956; Kinsky, 1961) and *Chlorella* (Morris & Syrett, 1963*a*).

The low activity of nitrate reductase in cotyledons of $NO_2^-$-grown seedlings is in contrast with the results with cauliflower (Candela *et al.* 1957), and is difficult to reconcile with the good stimulation of nitrate-reductase activity resulting from the incubation of cotyledons excised from N-deficient seedlings with $NO_2^-$. The low activity in the intact seedlings could not be due to lack of $NO_2^-$ transported into the cotyledons, since a high nitrite-reductase activity was present (Table 8).

The stimulation of nitrogen assimilation by $NO_3^-$, $NO_2^-$ or $NH_4^+$ in the excised cotyledons had, during the relatively short periods studied, little effect on the general metabolism of the tissue, as measured by the activities of enzymes involved in glycolysis (aldolase, glyceraldehyde 3-phosphate dehydrogenase), the pentose monophosphate pathway (glucose 6-phosphate dehydrogenase) and respiration. The induction of nitrate-reductase activity in response to $NO_2^-$ and $NH_4^+$ was observed in a variety of tissues in addition to radish cotyledons. Stimulation of activity by $NO_2^-$ occurred with maize, marrow and cucumber tissues, and smaller stimulations by $NH_4^+$ were observed with cotyledons of marrow and cucumber, but not with maize or soya bean (K. W. Joy, unpublished work). Rigidly sterile techniques were not used in the present investigations, and interconversions of the nitrogen source by contaminating micro-organisms were possibly the cause of the effects of $NH_4^+$ and $NO_2^-$ on nitrate-reductase induction. However, certain results suggest that large-scale microbial conversion of $NO_2^-$ or $NH_4^+$ into $NO_3^-$ was not the cause of nitrate-reductase induction by these two substrates. (*a*) Portions of media plated out on malt-extract–agar at the end of the induction studies revealed that contaminants, mainly bacterial, were only about 1000/pair of cotyledons. (*b*) Studies showed that (exogenous) nitrate concentrations must exceed 1mM to induce appreciable activity in seedlings, and 0·5mM for excised cotyledons, yet no nitrate was detectable in the cotyledons, induction media (after 4hr.) or growth media (after 7 days) in studies with $NH_4^+$. (*c*) With both intact seedlings and excised cotyledons the effect of $NH_4^+ + NO_3^-$ was greater, in the latter case synergistic, than that of $NO_3^-$ alone (Fig. 1 and Table 3), indicating that the $NH_4^+$ was not acting indirectly after conversion into $NO_3^-$, which was present in excess in both cases. (*d*) $NH_4^+$ stimulated nitrate reductase in both seedlings and excised cotyledons, but did not give the stimulatory effect on nitrite reductase that would be expected if $NO_3^-$, produced from $NH_4^+$, were causing the induction (Tables 3 and 8). (*e*) No lag time was observed in the induction of nitrate reductase in response to $NH_4^+$ or $NO_2^-$, suggesting that these molecules did not have to be converted first into $NO_3^-$. (*f*) The conversion of $NH_4^+$ or $NO_2^-$ into $NO_3^-$ by micro-organisms may be expected to be more sensitive to metabolic inhibitors than would plant metabolism, and hence the induction of nitrate reductase by $NH_4^+$ or $NO_2^-$ should be more sensitive to inhibitors than induction by $NO_3^-$: no differences in sensitivity were in fact detected (Table 4).

Alternatively, the radish tissue itself may be responsible for the conversion of $NH_4^+$ and $NO_2^-$ into $NO_3^-$, for example by catalase oxidation of $NO_2^-$ to $NO_3^-$ (Heppel & Porterfield, 1949). However, certain of the arguments against microbial conversions, as outlined above, would similarly apply to such conversion *in vivo*.

Such considerations suggest that $NH_4^+$ and $NO_2^-$ may have direct effects on the induction of nitrate reductase. It is of course possible that under certain conditions, such as in the presence of other ions like $NH_4^+$ or $NO_2^-$, traces of $NO_3^-$ may be sufficient to induce nitrate reductase. Work in the Urbana Laboratory (L. E. Schrader, unpublished work) has shown that with corn seedlings $NH_4^+$ caused only a small induction of activity, but this induction was markedly increased when low concentrations of $NO_3^-$ (10p.p.m.) were added with the $NH_4^+$. The stimulation of nitrate-reductase induction in excised radish cotyledons by $NH_4^+$ does not appear to be due simply to an unspecific increase in the nitrogen supply for general protein synthesis. $NH_4^+$ did not greatly stimulate the induction of nitrite reductase, either by itself or in the presence of $NO_2^-$, nor did it increase the activities of glutamate dehydrogenase, aldolase or respiration (Table 3), suggesting that nitrogen deficiency was not limiting protein synthesis. In addition, RNA synthesis, which is often markedly limited under nitrogen-deficient conditions (Ingle, 1961), was not stimulated when cotyledons were incubated with $NH_4^+$ (J. Ingle, unpublished work).

Nitrite reductase showed a more specific requirement for the inducer molecule. $NH_4^+$ did not stimulate the activity, and the induction by $NO_3^-$ appeared to be a secondary effect in response to the $NO_2^-$ that was formed after the primary induction of nitrate reductase.

The inhibitor studies indicate that synthesis of protein and RNA is required for the induction of nitrate reductase and nitrite reductase. The regulation is therefore at the DNA-to-RNA transcription level. The mechanisms of induction of these enzymes may therefore be similar to the model described for bacterial systems by Jacob & Monod (1961). However, the interpretation of these

experiments is complicated by the use of true rather than gratuitous inducer substrates. Although $NO_3^-$ induces both nitrate reductase and nitrite reductase, the two enzymes do not appear to be operon-controlled since kinetic studies show that the two activities are not initiated simultaneously. The results suggest rather that sequential inductions occur. Such a sequential pattern of enzyme induction has been reported for mandelate catabolism in *Pseudomonas* (Stanier, Hegeman & Ornston, 1963). However, it does not seem possible to induce nitrite reductase without nitrate reductase.

The maintenance of a high nitrate-reductase activity requires both the presence of the inducer substrate and the ability of the tissue to synthesize RNA and protein. When exogenous $NO_3^-$ is removed from induced tissue the enzyme activity may decrease only slightly at first, owing to the difficulty of depleting the tissue of endogenous $NO_3^-$. Inhibition of protein synthesis, however, results in more immediate loss of activity. Such loss of activity by the decay of enzyme appears to be considerably slower than the rate of synthesis, but it may be that the inhibitors used, puromycin and cycloheximide, affect not only the synthesis but also the decay of protein. Nitrite reductase is not as labile as nitrate reductase, and shows very little turnover under the conditions used.

Under certain conditions the synthesis of nitrate reductase appears to be repressed. Very little additional nitrate-reductase activity could be induced in cotyledons excised from seedlings grown on $NO_3^-$ or $NH_4^+ + NO_3^-$ growth medium for 5 or more days. Such cotyledons contained a high activity (5–7 milliunits), which was possibly near the full capacity for nitrate reduction of the tissue at that stage of development. The cotyledons showed a rather narrow range of maximal activity at days 3 and 4, when total activities as high as 13 milliunits were obtained (Figs. 1 and 2). Very little activity could be induced in cotyledons from seedlings grown on $NO_2^-$ growth medium for 5 or more days, but in this case the cotyledons contained very little nitrate-reductase activity, only just higher than the N-deficient control. Growth on $NO_2^-$ growth medium appeared to repress nitrate reductase fairly specifically, since the seedlings grew well on this medium, and they contained high nitrite-reductase activity. It appears unlikely that $NO_2^-$ itself was responsible for the repression, since a short incubation of cotyledons from N-deficient seedlings with $NO_2^-$ greatly stimulated nitrate-reductase activity (Table 3). Such a repression may be of use in balancing the different degrees of induction of nitrate reductase (10–20-fold) and nitrite reductase (5–10-fold). Any build up of $NO_2^-$, resulting from the large increase in nitrate-reductase activity, may (indirectly) repress further synthesis of this enzyme until the flow of nitrogen through the whole system is balanced. Such a regulatory system may also account for the lack of induction of further nitrate-reductase activity in cotyledons from $NO_3^-$-grown seedlings older than 5 days. Amino acids, either individually or as a mixture, have only small effects on induction. The report of Filner (1965) that nitrate reductase is repressed by certain amino acids in cultured tobacco cells, and our own limited observations, suggest that under certain conditions amino acids may exert some control over nitrate reductase. As discussed above $NH_4^+$ did not repress nitrate reductase, and no repression of nitrite reductase has been observed.

There appears to be very little regulation of this assimilation pathway by feedback inhibition of enzyme activity. None of the normal end products have any inhibitory action on nitrate-reductase, nitrite - reductase or glutamate - dehydrogenase activity, as determined in rather crude enzyme extracts. Purified enzymes may behave differently. Nitrate reductase is, however, inhibited by cyanate, which, as discussed by Morris & Syrett (1963*b*), may be formed *in vivo* from carbamoyl phosphate, and thus be considered as an end product of nitrate assimilation. The inhibition is fairly specific for nitrate reductase (Morris & Syrett, 1963*b*) (under comparable conditions nitrite reductase and glutamate dehydrogenase activities are only slightly affected) and consequently may be an example of feedback inhibition, where the end product of a metabolic pathway inhibits specifically the first enzyme of that pathway.

The availability of $NO_3^-$ therefore represents the primary regulation of $NO_3^-$ assimilation in radish cotyledons, via the sequential induction of the enzymes nitrate reductase and nitrite reductase. Further regulation of the assimilation in plant tissues differs from that described for micro-organisms in that the synthesis of nitrate reductase is not repressed by $NH_4^+$. However, the cofactor requirements of the enzymes from micro-organisms are very different from those of higher plants, suggesting that different proteins may function in the different classes of organisms.

The technical help of Mrs D. Flesher and Mrs J. Bunning is gratefully acknowledged. Actinomycin D was kindly supplied by Merck, Sharp and Dohme Inc. The work was supported by a National Science Foundation Grant GB 3750 and a U.S. Department of Agriculture Co-operative State Research Service Grant, 427-15-6 (Illinois), and an Atomic Energy Commission Contract AT (1101) 125 (Purdue).

## REFERENCES

Afridi, M. M. R. K. & Hewitt, E. J. (1964). *J. exp. Bot.* **15**, 251.

Beevers, L., Flesher, D. & Hageman, R. H. (1964). *Biochim. biophys. Acta*, **89**, 453.

Beevers, L., Schrader, L. E., Flesher, D. & Hageman, R. H. (1965). *Plant Physiol.* **40**, 691.
Candela, M. C., Fisher, E. G. & Hewitt, E. J. (1957). *Plant Physiol.* **32**, 280.
Cleland, W. W. (1964). *Biochemistry*, **3**, 480.
Cresswell, C. F., Hageman, R. H., Hewitt, E. J. & Hucklesby, D. P. (1965). *Biochem. J.* **94**, 40.
Evans, H. J. & Nason, A. (1953). *Plant Physiol.* **28**, 233.
Filner, P. (1965). *Plant Physiol.* **40** (Suppl.), iii.
Gibbs, M. & Turner, J. F. (1964). In *Modern Methods of Plant Analysis*, vol. 7, p. 534. Ed. by Linskens, H. F., Sanwal, B. D. & Tracey, M. V. Berlin: Springer-Verlag.
Hageman, R. H., Cresswell, C. F. & Hewitt, E. J. (1962). *Nature, Lond.*, **193**, 247.
Hageman, R. H. & Flesher, D. (1960). *Plant Physiol.* **35**, 700.
Heppel, L. A. & Porterfield, U. T. (1949). *J. biol. Chem.* **178**, 649.
Ingle, J. (1961). Ph.D. Thesis: University of Bristol.
Jacob, F. & Monod, J. (1961). *J. molec. Biol.* **3**, 318.
Jagannathan, V., Singh, K. & Damodaran, M. (1956). *Biochem. J.* **63**, 94.
Joy, K. W. & Hageman, R. H. (1966). *Biochem. J.* **100**, 263.
Kessler, E. (1964). *Annu. Rev. Plant Physiol.* **15**, 57.
Key, J. L. (1964). *Plant Physiol.* **39**, 365.
Kinsky, S. K. (1961). *J. Bact.* **82**, 898.
Kornberg, A. (1950). *J. biol. Chem.* **182**, 806.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Medina, A. & Nicholas, D. J. D. (1957). *Biochim. biophys. Acta*, **23**, 440.
Morris, I. & Syrett, P. J. (1963*a*). *Arch. Microbiol.* **47**, 32.
Morris, I. & Syrett, P. J. (1963*b*). *Biochim. biophys. Acta*, **77**, 649.
Morton, A. G. (1956). *J. exp. Bot.* **7**, 97.
Nason, A. & Evans, H. J. (1953). *J. biol. Chem.* **202**, 655.
Nicholas, D. J. D., Nason, A. & McElroy, W. D. (1954). *J. biol. Chem.* **207**, 341.
Sanderson, G. W. & Cocking, E. C. (1964). *Plant Physiol.* **39**, 416.
Stanier, R. Y., Hegeman, G. D. & Ornston, L. N. (1963). *Proc. Centr. nat. Rech. Sci. Symp., Marseilles*, p. 231.
Tang, P. S. & Wu, H. Y. (1957). *Nature, Lond.*, **179**, 1355.
Woolley, J. T., Hicks, G. P. & Hageman, R. H. (1960). *J. agric. Fd Chem.* **8**, 481.
*Worthington Enzyme Manual* (1965). Freehold, N.J.: Worthington Biochemical Corp.
Yemm, E. W. & Folkes, B. F. (1958). *Annu. Rev. Plant Physiol.* **9**, 245.