

A COMPARATIVE STUDY OF NITRATE REDUCTION AND THE OXIDATION OF GLYCOLATE

D. KAPLAN AND S.H. LIPS

*Department of Biology and The Jacob Blaustein Institute for Desert Research,
Ben Gurion University of the Negev, Be'er Sheva 84105 Israel*

ABSTRACT

Activity of nitrate reductase and glycolate dehydrogenase in spinach leaves changes in identical patterns following changes in the levels of nitrate available in the nutrient solution of the plants. Replacing nitrate by ammonium ions caused loss of activity of both enzymes in cauliflower but this loss was less extensive in spinach plants. Glycolate dehydrogenase and nitrate reductase activities were negligible in Mo-deficient spinach and cauliflower leaves, but increased to identical extents upon addition of Mo to the nutrient medium. The activity of both enzymes was increased to levels found in control plants when tungstate was added to Mo-deficient plants. Phosphate buffer was found to be the best extraction medium for both enzymes, stimulating nitrate reductase by 15% and glycolate dehydrogenase 3-fold. Both enzymes showed a similar pH sensitivity but differed in their thermal denaturation activation energies. Gel chromatography indicated that glycolate dehydrogenase protein differs from that of nitrate reductase.

Nitrate reductase has a specific requirement for NADH in higher plants (Beevers & Hageman, 1969) although the metabolic source of the reducing equivalents used for the reduction of nitrate is uncertain. Klepper et al. (1971) suggested that the oxidation of glyceraldehyde-3-phosphate by its NADH-dependent dehydrogenase may supply the electrons required for the reduction of nitrate. Lips (1971) suggested that oxidation of glycolate could be a suitable source of electrons for this process. These alternative sources are not necessarily mutually exclusive.

Subsequent observations were made supporting the idea of the involvement of glycolate metabolism in nitrate reduction: (a) Conditions which would normally prevent induction of nitrate reductase by nitrate, such as darkness, permit induction of the enzyme upon supplementation of the induction medium with glycolate (Roth-Bejerano & Lips, 1973b); (b) Glycolate dehydrogenase, an enzyme whose capacity to oxidize glycolate and reduce NAD⁺ becomes evident under anaerobic conditions was found in higher plants (Roth-Bejerano & Lips, 1973a); (c) Glycolate dehydrogenase and nitrate reductase activities were both found in plants grown on nitrate and disappeared with nitrate depletion of the plants' growth medium (Roth-Bejerano & Lips, 1973a).

The present work compares the enzymes involved in glycolate oxidation and nitrate reduction.

Received June 23, 1983 and in revised form October 24, 1983.

Abbreviations used in text: GLDH – glycolate dehydrogenase, GO – glycolate oxidase, Cat – catalase, NR – nitrate reductase, NADH – reduced nicotinamide adenine dinucleotide, EDTA – ethylenediaminetetraacetate, NAD⁺ – nicotinamide adenine dinucleotide.

MATERIALS AND METHODS

Plant Material

Spinach (*Spinacea oleracea* L. cv. Noorman) was germinated and grown in a greenhouse for 6–8 weeks in nutrient sand cultures and irrigated with Long Ashton nitrate type nutrient solution (Hewitt, 1966). Some experiments were also performed with cauliflower (*Brassica oleracea* L. var. *botrytis* cv. Majestic) or barley (*Hordeum vulgare* L. cv. Dvir), 6–8 days old, grown under identical conditions. Molybdenum deficiency conditions were produced using the purified nutrient media of Hewitt (1966).

Enzyme Extraction

Fully expanded leaves of spinach plants, 6–8 weeks old, were detached, washed thoroughly in tap water, rinsed in deionized water, and immediately homogenized in 0.1 M phosphate buffer, pH 7.4, containing 10⁻³M EDTA. Homogenization was carried out using 3 ml medium per 1 g of tissue in an ice-cold mortar with a pre-chilled pestle. The homogenate was filtered through 2 layers of gauze and the filtrate was centrifuged for 15 min at 19,000g.

Enzyme assay

a) Nitrate reductase (EC 1.6.6.2) was assayed with NADH, measuring nitrite formation as previously described (Hewitt & Nicholas, 1964).

b) Glycolate oxidase (EC 1.1.3.1) was assayed following the method described by Hess and Tolbert (1967), measuring glyoxylate phenylhydrazone formation at 324 nm.

c) Glycolate dehydrogenase was assayed spectrophotometrically at 324 nm by following the rate of glyoxylate phenylhydrazone formation in the presence of NAD⁺ under anaerobic conditions using Thunberg cuvettes with an optical path length of 1 cm. The assay mixture contained 3.3 mM phosphate buffer, pH 7.4; 1 μM NAD⁺; 3.3 mM phenylhydrazine and 0.1 ml homogenate in 2.7 ml water and 0.3 ml of 0.1 M glycolate in the side arm. The cuvette was alternately evacuated and flushed with argon three times before tipping in the glycolate to start the reaction. Rates of glycolate dehydrogenase activity were expressed as μmoles of phenylhydrazone formed in the presence of NAD⁺, after subtracting the amount of phenylhydrazone formed by the control (without NAD⁺), using an extinction coefficient of 1.7 × 10⁴ l.mol⁻¹ cm⁻¹ (Dixon & Kornberg, 1959). Generation of NADH could not be followed with the crude homogenate due to its NADH-oxidizing capacity.

d) Product identification: For the identification of phenylhydrazones, one-dimensional paper chromatography was used following the procedure described by Cavallini et al. (1949). The assays for glycolate oxidase and glycolate dehydrogenase

were carried out in a water bath at 30°C for 5 min, and then boiled (assay mixture as described above). A sample containing the complete assay mixture that had been boiled at 0 time and glycolate phenylhydrazone prepared *in vitro* was used as control. Samples of 0.4 ml of each treatment were applied to Whatman No. 1 chromatography paper and run for 15 h in a pre-equilibrated chromatography tank. For radioactive identification $1\text{-}^{14}\text{C}$ glycolate from the Radioactive Centre, Amersham, was used and radioactivity was measured using a Packard Tri-carb liquid scintillation spectrometer 3380.

e) Catalase (EC 1.11.1.6) was assayed as described by Lück (1963).

f) Column chromatography of protein: After centrifugation at 19,000g the supernatant was applied to a Sepharose Cl-6B column (3 X 43 cm) pre-equilibrated with 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA. Elution of proteins from the column was carried out with the same buffer at a rate of $1.2\text{ ml}\cdot\text{min}^{-1}$, in 7 ml fractions.

RESULTS

Effects of Nutrient Media Composition

Spinach plants grown in complete nutrient medium were transferred to nitrate-free medium. Nitrate in the plants at the time of transfer was rapidly assimilated and the resulting shortage of nitrate became evident in the declining activity of nitrate reductase. Activity of NR, GLDH and GO (Fig. 1) was followed for 3 days during the period of nitrate depletion, and for a further 2 days in which nitrate was restored to the nutrient medium of the plants. Three such experiments were made over depletion periods of 3–4 days resulting consistently in identical changes in the activities of nitrate reductase and glycolate dehydrogenase. Glycolate oxidase was unaffected by changing nitrate levels in the medium.

Molybdenum was shown to be essential for nitrate reductase activity (Hewitt, 1975) and its replacement by tungstate caused inhibition of the enzyme (Heimer et al., 1969). Two types of experiment were performed in the present work to study the effect of MoO_4^{2-} and WO_4^{2-} on the enzymes under observation. In the first (Table I), increasing concentrations of Mo in the growth medium led to increased activity of the three enzymes, but to different extents. Increasing Na_2MoO_4 concentration in the nutrient medium of spinach plants from 0.05 to 48 $\mu\text{g/l}$ caused nitrate reductase activity to increase 55-fold and glycolate dehydrogenase 18-fold. In cauliflower leaves the increase was 81- and 65-fold, respectively. Glycolate oxidase and catalase in the same plants and under identical conditions increased only 5-fold in spinach and 2.5-fold in cauliflower.

The effect of MoO_4^{2-} supply and addition of WO_4^{2-} for different periods of time on the activity of NR, GLDH and GO was studied (Table II). Plants grown in the presence of low concentrations of MoO_4^{2-} were transferred to nutrient solutions containing WO_4^{2-} . The activity of GO after 8 days increased to about 90% of the level of control plants (adequate Mo concentration) but GLDH activity increased to only 50% and NR activity to only 40% of that found in the control plants in the same period.

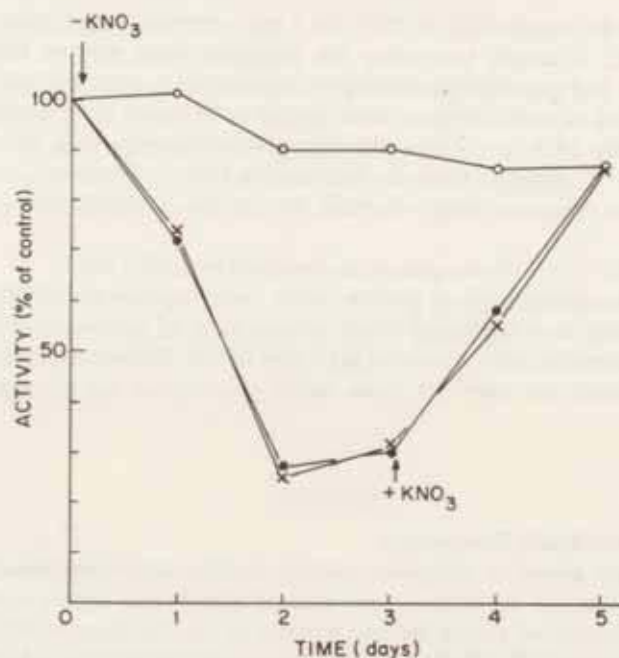


Fig. 1. Effect of nitrate content of nutrient medium on NR, GLDH and GO activities. NR, ●-● (100% activity = 7.6 $\mu\text{moles NO}_2^-$ formed/g/h); GLDH, x-x (100% activity = 7.3 $\mu\text{moles glyoxylate phenylhydrazone formed/g/h}$); GO, ○-○ (100% activity = 73.2 $\mu\text{moles glyoxylate phenylhydrazone formed/g/h}$). Data of representative experiment.

TABLE I

The effect of Na_2MoO_4 concentration in the nutrient supply of spinach and cauliflower leaves on the activities of nitrate reductase (NR), glycolate dehydrogenase (GLDH), glycolate oxidase (GO) and catalase (Cat)

Plant	Na_2MoO_4 $\mu\text{g/l}$	Activity ¹			
		NR	GLDH	GO	Cat
Spinach	48	6.60	5.03	67.8	31.2
	3.0	6.90	3.33	59.4	28.2
	0.2	0.68	0.83	21.8	11.5
	0.1	0.47	0.55	19.3	9.1
	0.05	0.12	0.28	13.0	5.8
Cauliflower	3.0	11.4	11.0	93.7	—
	0.05	0.14	0.17	36.6	—

¹ NR activity = $\mu\text{mol NO}_2^-$ formed/g fr. wt/h; GLDH activity = $\mu\text{mol glyoxylate-phenylhydrazone formed/g/h}$; GO activity = $\mu\text{mol glyoxylate-phenylhydrazone formed/g/h}$; Cat activity = $\text{mmol H}_2\text{O}_2$ oxidized/g fr. wt/h.

TABLE II
The effect of Na_2WO_4 added to the nutrient medium of spinach plants grown under conditions of Na_2MoO_4 deficiency on the activity of nitrate reductase (NR), glycolate dehydrogenase (GLDH) and glycolate oxidase (GO) extracted from leaves

Growth conditions	Activity ¹		
	NR	GLDH	GO
Standard medium (3 $\mu\text{g/l}$ Mo)	100 ²	100 ³	100 ⁴
Mo deficiency (0.05 $\mu\text{g/l}$)	7	8	28
Mo deficiency + 3 $\mu\text{g/l}$ Na_2WO_4			
for: 4 days	26	37	47
8 days	38	50	91
28 days	44	62	101

¹ Activities presented as % of that of control plants grown on standard medium.

² 6.78 $\mu\text{mol NO}_3^-$ formed/g fr. wt/h.

³ 6.68 μmol of glycolate-phenylhydrazone formed/g fr. wt/h.

⁴ 67.52 μmol of glyoxalate-phenylhydrazone formed/g fr. wt/h.

TABLE III
Interactions between nitrogen source and molybdenum supply on the activities¹ of nitrate reductase (NR), glycolate dehydrogenase (GLDH) and glycolate oxidase (GO) in spinach and cauliflower leaves

Plant	N supply	Mo supply ²	NR	% control	GLDH	% control	GO	% control
Spinach	NO_3^-	+	9.94	100	11.70	100	93.8	100
		-	0.67	7	0.83	7	20.9	22
	NH_4^+	+	8.04	80	3.54	30	49.8	53
		-	0.26	3	0.41	4	51.8	55
Cauliflower	NO_3^-	+	12.7	100	12.8	100	89.2	100
		-	0.21	2	0.25	2	23.4	26
	NH_4^+	+	0.18	3	0.34	3	56.1	63
		-	0.06	0.5	0.06	0.5	45.9	51

¹ Activities as $\mu\text{moles product/g fr. wt/h}$. Mean values of four experiments.

² + = 48 $\mu\text{g/l}$; - = 0.1 $\mu\text{g/l}$ for spinach and <0.05 $\mu\text{g/l}$ for cauliflower.

The interactions between the effects of different nitrogen sources in the medium and Mo requirements were examined using ammonium sulphate instead of potassium nitrate with spinach and cauliflower plants (Table III). Decreasing Mo supply caused loss of NR and GLDH with either nitrate or ammonium treatments in spinach plants. Only low GLDH and NR activities were found in cauliflower with Mo supplied in the presence of ammonium ions (by contrast with spinach plants), but Mo deficiency caused further decrease of enzyme activity. Replacement of nitrate by ammonium

TABLE IV
Activities of nitrate reductase (NR), glycolate dehydrogenase (GLDH) and glycolate oxidase (GO) in extracts obtained from spinach leaves, at different pH (0.1 mM phosphate buffer + 1 mM EDTA)

pH	Activity ¹		
	NR	GLDH	GO
7.0	3.75	2.10	43.50
7.5	7.05	7.05	73.00
7.8	7.00	8.80	75.00
8.2	6.40	6.30	78.00
8.5	6.05	5.00	77.50

¹ Activities as μ moles product/g fr. wt/h.

TABLE V
The effect of different extracting buffers (pH 7.5) on the activities of nitrate reductase (NR), glycolate dehydrogenase (GLDH) and glycolate oxidase (GO) of spinach leaves grown under standard conditions

Buffer	Activity ¹		
	NR	GLDH	GO
0.02 M tris-HCl + 1 mM EDTA	4.8	0.8	12.3
0.10 M tris-HCl + 1 mM EDTA	5.0	1.3	11.7
0.10 M potassium phosphate buffer + 1 mM EDTA	5.7	4.5	12.8

¹ Activities as μ moles product/g fr. wt/h.

caused similar decreases of GO (45–55%) in both spinach and cauliflower given Mo, but there was little or no further decrease of GO with Mo deficiency in ammonium, while activities in this treatment were more than twice those in the corresponding nitrate treatment.

Biochemical Properties

The pH optima of the three enzymes were determined (Table IV). The results indicate a broad pH optimum (7.5–8.5) for GO, a narrow one (pH 7.5–7.8) for NR, while that for GLDH is 7.8.

Homogenization of the tissue in different buffers did not affect the activity of GO (Table V) but the activity of GLDH dehydrogenase was significantly greater in phosphate buffer than in tris-HCl. Phosphate buffer also yielded higher activity of NR, as would be expected with this enzyme (Hewitt and Nicholas, 1964; Lips, 1975).

We also studied thermal inactivation of the enzymes (Fig. 2). Nitrate reductase is the most sensitive of the three enzymes tested, GLDH is slightly more stable and GO is a relatively stable enzyme at higher temperatures.

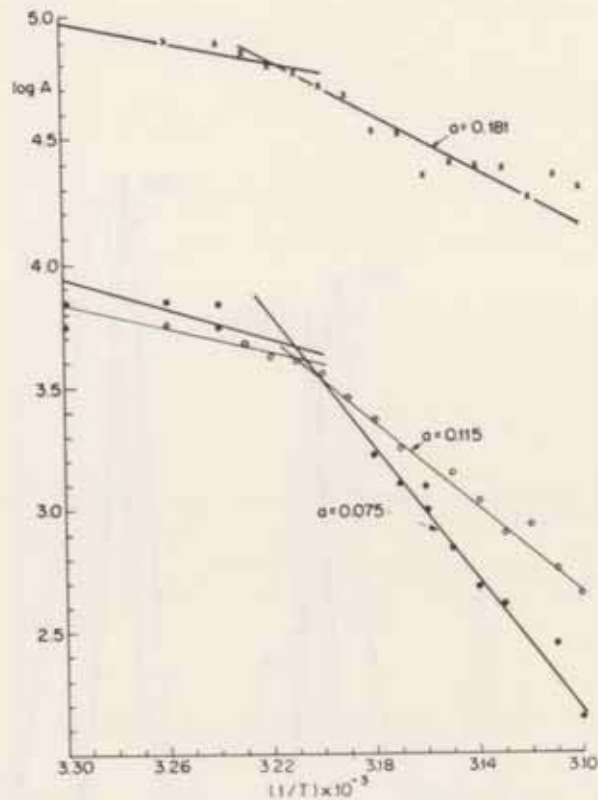


Fig. 2. Heat inactivation of NR, GLDH and GO activities in extracts of spinach leaves. NR, ●—●; GLDH, x—x; GO, ○—○.

TABLE VI
Paper chromatography of the products of the glycolate dehydrogenase (GLDH) reaction. The experiments were performed on green barley seedlings

Assay conditions	R_f	Radioactivity in spot (DPM) ¹
+O ₂	0.78	5038
-O ₂	0.79 ²	106
-O ₂ + NAD ⁺	0.79	2227
Glyoxylate phenylhydrazine	0.78	-

¹ Neither UV fluorescence nor radioactivity were found along the chromatogram except the ones shown.

² A very pale spot.

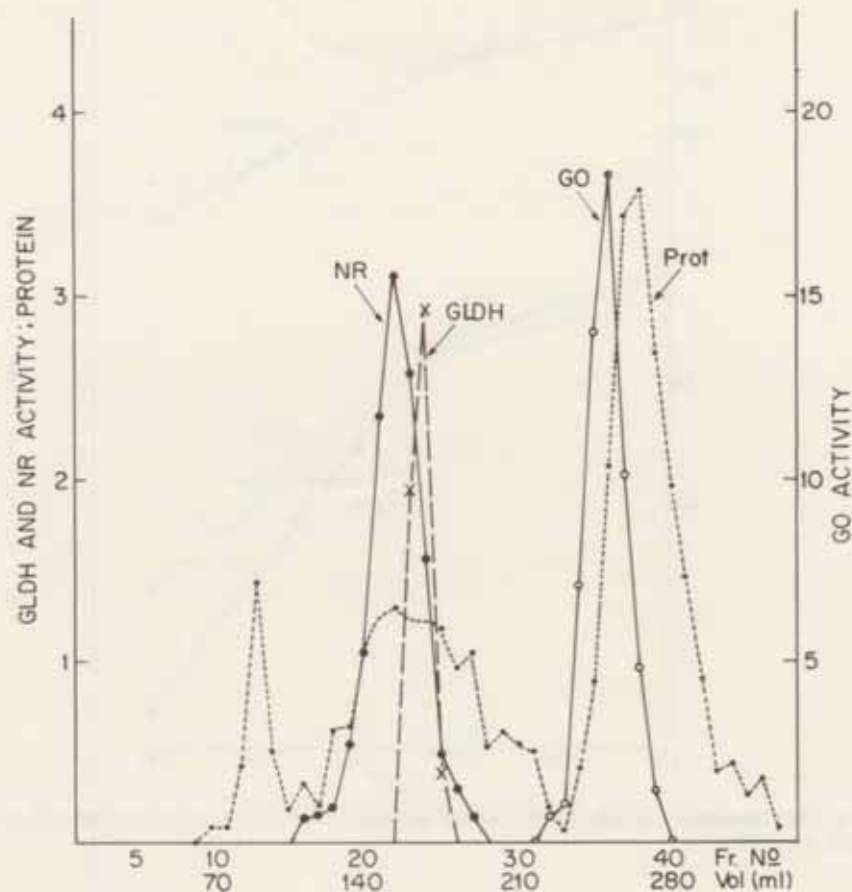


Fig. 3. Gel chromatography of GLDH, NR and GO on Sepharose Cl-6B. Activities expressed in moles product/fraction/h, and protein in mg/fraction. NR, ●-●; GLDH, x-x; GO, ○-○.

The occurrence of GLDH in higher plants has been shown in previous work (Roth-Bejerano & Lips, 1973a). The reaction product of GLDH *in vitro* was identified in two ways: (a) fluorescence after UV irradiation of the chromatogram; (b) using $1\text{-}^{14}\text{C}$ glycolate as the substrate of the reaction and determining location of radioactivity in the chromatogram. Radioactivity was found only in one spot of the chromatogram (other than the origin), and coincides with that found by UV irradiation (Table VI). The results obtained by two independent methods show that the products of glycolate dehydrogenase and glycolate oxidase are identical, most probably being glyoxylate.

The three enzymes were separated on a Sephadex-Cl-6B column (Fig. 3) indicating that they are three different proteins.

DISCUSSION

Glycolate dehydrogenase and nitrate reductase seem to have several common characteristics (Fig. 1). The extractable activities of both enzymes depended similarly and very sensitively on nitrogen nutrition during growth of the plants (Fig. 1), even when different species behave differently with respect to forms of nitrogen supplied. The activity of glycolate oxidase, on the other hand, was less affected by nitrogen depletion, or (in cauliflower) by replacing nitrate by ammonium.

It seems unlikely that the activity of GLDH described here is related to a NR sub-unit or apoprotein having NADH dehydrogenase activity with cytochrome *c* as an acceptor which is induced by nitrate together with NR (Wray & Filner, 1970; Hewitt, 1975). While GLDH disappeared in the plants lacking Mo (where little active NR was detected), cytochrome *c* reductase occurred abundantly in similarly treated plants (Hewitt et al., 1977). Plants grown on a range of concentrations of Mo showed close correlation between the amounts of the dehydrogenase and reductase activities and these were decreased to 2–6% when the plants were deprived of Mo, indicating that Mo may in some way be necessary for the activity, production or stability of GLDH. Glycolate oxidase and catalase were less sensitive to Mo level in the nutrient medium, but also decreased to about one-third of control values, although only when nitrate was used as the nitrogen source (Table I).

These observations were supported by the effects of WO_4^{2-} (Table II) in Mo-deficient plants. Similar changes were observed for both NR and GLDH, which increased slowly and incompletely but more rapidly initially than GO. Nitrate reductase contains Mo, but giving WO_4^{2-} to Mo-deficient plants slowly restored NR activity although W by itself was unable to activate the enzyme. This apparent anomaly may possibly be explained by the fact that small amounts of W improve incorporation of Mo into NR (Notton et al., 1979). The WO_4^{2-} enhancement of NR activity also extended to GLDH activity and the slow response may indicate a structural change or repair of the enzymes accompanied by enhanced Mo incorporation into the protein.

The subcellular site of nitrate reductase is unknown although it has been suggested that it is on the single membrane of microbody-like organelles or the outer membrane of chloroplasts (Hewitt, 1975; Lips, 1975). Glycolate dehydrogenase may also be on microbody membranes (Lips, 1971, 1975) while glycolate oxidase and catalase have been established as microbody stroma enzymes (Tolbert, 1971). The loss of NR and GLDH from association with microbodies occurred very easily during rapid acceleration in a centrifuge (Lips, 1975). Mo deficiency caused severe breakdown of chloroplast membranes (Notton, 1972; Fido et al., 1977), which has been ascribed to possible superoxide damage caused by excessive cytochrome *c* reductase activity in nitrate-induced systems. Damage in this case was mitigated by the supply of tungstate or by replacing nitrate with ammonium. We found a close correlation between the effects of nitrogen nutritional conditions on NR and GLDH activities, but not on that of GO. The various biochemical tests indicate that NR and GLDH activities are independent of GO and that they are associated with different proteins.

The very close relationships between NR and GLDH activities are compatible with previous suggestions (Kaplan et al., 1974; Lips, 1979) that glycolate can provide the reductant of NR. This view has received support from *in vivo* tests on *Lolium perenne* using glycolate (James & Smith, 1979), which stimulated NR activity. Inhibitors of glycolate dehydrogenase decreased NR activity and glyceraldehyde-3-phosphate did not promote NR activity. We have so far been unable to couple NR with GLDH *in vitro* using crude extracts of spinach containing both systems with (or without) added phenylhydrazine under anaerobic conditions. Purified NR did not appear to catalyze glycolate oxidation in the presence of NAD⁺ and phenylhydrazine (unpubl. work with D.P. Hucklesby). Although the reduction of NAD⁺ by glycolate is thermodynamically very unfavourable the overall reaction between glycolate and nitrate should be feasible and phenylhydrazone formation would be expected to promote the reaction. The evidence for *in vivo* coupling in *Lolium* implies that conditions may nevertheless be appropriate in intact cells for achieving the reaction.

ACKNOWLEDGEMENTS

We thank Dr. E.J. Hewitt FRS, for much helpful discussion and laboratory facilities.

REFERENCES

- Beevers, L. and R.H. Hageman. 1969. Nitrate reductase in higher plants. *Annu. Rev. Plant Physiol.* 20: 495-522.
- Cavallini, D., N. Frantali and G. Toschi. 1949. Determination of keto acids by partition chromatography on filter-paper. *Nature* 163: 568-569.
- Dixon, G.H. and H.L. Kornberg. 1959. Assay methods for key enzymes of the glyoxylate cycle. *Biochem. J.* 72: 3.
- Fido, R.J., C.S. Guncry, E.J. Hewitt and B.A. Notton. 1977. Ultrastructural features of molybdenum deficiency and whiptail of cauliflower leaves: Effects of nitrogen source and tungsten substitutions for molybdenum. *Aust. J. Plant Physiol.* 4: 675-689.
- Heimer, Y.M., J.L. Wray and P. Filner. 1969. The effect of tungstate on nitrate assimilation in higher plant tissues. *Plant Physiol.* 44: 1197-1199.
- Hess, E.J. and N.E. Tolbert. 1967. Glycolate pathway in algae. *Plant Physiol.* 42: 371-379.
- Hewitt, E.J. 1966. Sand and Water Culture Methods used in the Study of Plant Nutrition. 2nd edn. Commonwealth Bureau of Horticulture, Commonwealth Agricultural Bureau, Farnham Royal, UK. pp. 430-435.
- Hewitt, E.J. 1975. Assimilatory nitrate-nitrite reduction. *Annu. Rev. Plant Physiol.* 26: 73-100.
- Hewitt, E.J. and D.J.D. Nicholas. 1964. Enzymes of inorganic nitrogen metabolism. In: *Modern Methods of Plant Analysis*. H.F. Linskens, R.D. Sanwal and M.V. Tracey, eds. pp. 67-122.
- Hewitt, E.J., B.A. Notton and G.J. Rucklidge. 1977. Formation of nitrate reductase by recombination of apoprotein fractions from molybdenum deficient plants with a molybdenum-containing complex. *J. Less Common Met.* 54: 537-553.
- James, D.B. and S.M. Smith. 1979. Glycolate as a reductant source for nitrate reductase activity. In: *Nitrogen Assimilation of Plants*. E.J. Hewitt and C.V. Cutting, eds. 6th Long Ashton Symp. Sept. 1977. Academic Press, London, New York and San Francisco. pp. 579-581.
- Kaplan, D., N. Roth-Bejerano and S.H. Lips. 1974. Nitrate reductase as a product inducible enzyme. *Eur. J. Biochem.* 49: 393-398.
- Klepper, L., D. Flesher and R.H. Hageman. 1971. Generation of reduced nicotinamide adenine dinucleotide for nitrate reductase in green leaves. *Plant Physiol.* 48: 580-590.

- Lips, S.H. 1971. Photorespiration and nitrate reduction. 2nd Int. Congr. Photosynthesis. G. Forti, M. Avron and A. Melandri, eds. Stressa 2241-2249.
- Lips, S.H. 1975. Enzyme content of plant microbodies as affected by experimental procedures. *Plant Physiol.* 55: 598-601.
- Lips, S.H. 1979. Photosynthesis and photorespiration in nitrate metabolism. In: Nitrogen Assimilation of Plants. E.J. Hewitt and C.V. Cutting, eds. 6th Long Ashton Symp. Sept. 1977. Academic Press, London, New York and San Francisco. pp. 445-450.
- Lück, H. 1963. Catalase. In: Methods of Enzymatic Analysis. H.U. Bergmeyer, ed. Academic Press, New York. 885 pp.
- Notton, B.A. 1972. Ph.D. thesis. Bristol University, Long Ashton Research Station, England.
- Notton, B.A., R.J. Fido, D.M. Watson and E.J. Hewitt. 1979. Annu. Rep. Long Ashton Research Station.
- Roth-Bejerano, N. and S.H. Lips. 1973a. Glycolate dehydrogenase activity in higher plants. *Isr. J. Bot.* 22: 1-7.
- Roth-Bejerano, N. and S.H. Lips. 1973b. Induction of nitrate reductase in leaves of barley in the dark. *New Phytol.* 72: 253-257.
- Tolbert, N.E. 1971. Microbodies - peroxisomes and glyoxysomes. *Annu. Rev. Plant Physiol.* 22: 45-74.
- Wray, J.L. and P. Filner. 1970. Structural and functional relationships of enzyme activities induced by nitrate in barley. *Biochem. J.* 119: 715-725.