Enzymic Assimilation of Nitrate in Tomato Plants
I. Reduction of Nitrate to Nitrite [1]

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It has been established with considerable certainty that nitrate is assimilated via ammonia in higher plants. Evidence for this comes from several different investigations in which N^{15}-labeled compounds have been utilized. Firstly, it has been shown that nitrate is reduced to ammonia (7, 17). Secondly, it has been shown that nitrate and ammonia have essentially the same effect on the production of free amino acids and proteins (17, 26). Finally, ammonia has been shown to be directly incorporated into organic nitrogenous substances via glutamate and glutamine (4, 26).

It has come to be generally accepted that nitrate reductase is the first enzyme to act on nitrate during its assimilation in higher plants (8). However, the physiological importance of this enzyme must rest, ultimately, on the demonstration that enzymes capable of reducing nitrite to the level of amino-nitrogen, also exist in higher plants (20).

In this investigation a study was made of the enzymic systems responsible for the entire process of nitrate assimilation in tomato plants. The present paper describes the properties of the nitrate reductase found in tomato and its distribution within the plant.

Materials and Methods

Culture of Plant Material. Tomato plants (*Lycopersicon esculentum,* Mill., var. Sutton's Best of All) were raised from seed, and grown in sand for varying lengths of time. Watering by tap water was carried out until the cotyledons were above the level of the sand. After this, watering was continued with Long Ashton nutrient solution (12), in which the nitrate content had been doubled. The final nitrate concentration in this nutrient solution was 20 mM. Supplementary illumination was provided to ensure an intensity of 1000 ft-c on the surface of the plants over a fourteen hour period each day.

In a short survey for the presence of nitrate reductase in plant material, 6 additional species of plants were grown under identical conditions to those for tomato. These plants were apple (*Pyrus malus,* L.), barley (*Hordeum vulgare,* L.), corn (*Zea mays,* L), kidney bean (*Phaseolus vulgaris,* L.) potato (*Solanum tuberosum,* L.), an ornamental flower (*Nicotiana affinis,* T. Moore), and wheat (*Triticum vulgare,* Vill.).

In studies on excised tomato roots, seeds were germinated on moistened filter paper at 27°. After 4 days, approximately 2 cm of the root tips were excised. These roots tips were grown for 7 days at 27° in 50-ml culture flasks closed by means of nylon wrapped cotton plugs. Each flask contained 25 ml of White's nutrient solution (25) modified to contain copper and molybdenum (1) and FeEDTA in place of ferric citrate. The nitrate content of this solution, which was designated "Normal NO_3–Level Solution," was 3.2 mM. One set of cultures was grown in the above nutrient solution modified by increasing the level of nitrate containing salts sixfold. The concentration of nitrate in these cultures, which was designated "6 × NO_3–Level," was 19.2 mM. In these studies the seeds were sterilized before germination and all subsequent manipulations were carried out under aseptic conditions.

Preparation of Enzyme. Plant tissue was ground in 4 times its weight of 0.1 M Tris • HCl buffer (pH 7.5) containing 10^{-3} M cysteine. Grinding was done with a cold mortar and pestle containing approximately as much cold acid washed sand by weight as plant material. The macerate was pressed through cheese cloth and the filtrate was centrifuged at 1750 × *g* for 20 minutes. The supernatant solution was either used directly as the crude enzyme preparation or after purification by Sephadex treatment.

Treatment with Sephadex was carried out by passing 4.0 ml of crude enzyme preparation through a column containing 6.0 g of Sephadex G-25 (A. B. Pharmacia, Uppsala, Sweden). Elution was carried out with 0.01 M Tris • HCl buffer (pH 7.5) containing 10^{-3} M cysteine. From 80 % to 100 % of the enzyme was normally recovered virtually free of nitrate in the macerate in the 6 ml of eluate following the first 12 ml. The enzyme containing eluate was used as the purified enzyme preparation. All of the above operations were carried out at 0° to 4° using cold materials and reagents.

Enzymic Assay. Nitrate reductase was assayed using the following reaction mixture: 0.5 ml of 0.1 M potassium phosphate (pH 7.5), 10 μmoles KNO_3, 0.27 μmole DPNH, 0.05 to 0.20 ml enzyme preparation and distilled water to make a final volume of 0.8 ml. Incubations were for 20 minutes at 27°. Incubations were stopped by adding 0.2 ml M zinc acetate followed by 6.0 ml 95 % ethanol. The treated reaction mixtures were centrifuged at 1500 × *g* for 5 minutes. A suitable aliquot of the supernatant fraction, usually 2.0 ml, was removed for estimation of nitrite. One ml of 1 % (w/v) sulfanilamide in N HCl and 1.0 ml

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of 0.01 % (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride solution were added to the above aliquot and the solutions were thoroughly mixed. After standing for 30 minutes the optical density of these solutions at 540 mμ was determined with a Unicam SP 500 spectrophotometer. The nitrite content was obtained from a standard curve. No nitrite was lost from reaction mixtures under the conditions of the assay.

The specific activity is defined as the mμmoles of nitrite formed per mg protein per hour. Protein in enzyme preparations was estimated by the method of Lowry et al. (16).

Results

The Effect of Cysteine on the Extraction of Nitrate Reductase. The level of cysteine in the extraction media was varied during the extraction of nitrate reductase from the entire aerial portions of 33-day old tomato seedlings. The results of this study (fig 1) showed that the presence of 10^{-3} M cysteine in the extraction media resulted in maximal activity in enzyme preparations. This was repeated with extracts of leaves and roots of various ages, with the same results.

The Effect of Sephadex Treatment. Tests showed that crude enzyme preparations contained nitrate ions at a level which nearly saturated the nitrate reductase under the conditions of the standard assay.

Sephadex treatment was effective in removing this nitrate and establishing virtually complete dependence for enzyme activity on added nitrate (table I).

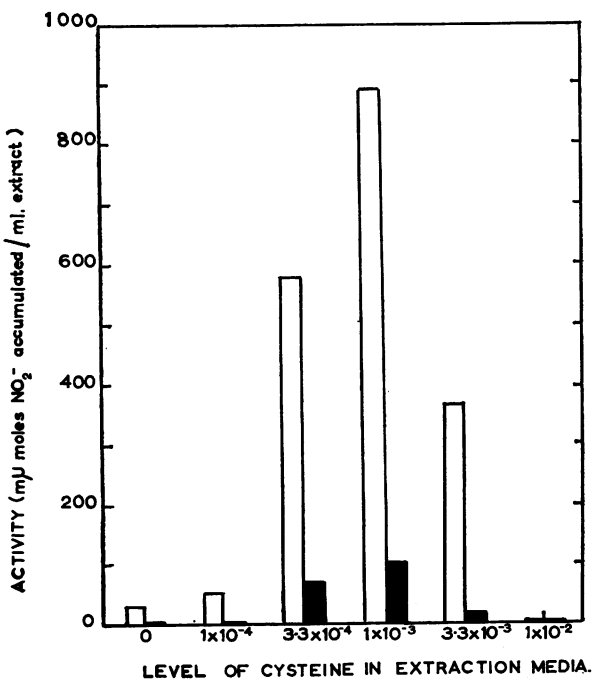

FIG. 1. The effect of cysteine in the extraction media on the level of nitrate reductase activity in tomato seedling shoot extracts. Extracts were made on tops of 33-day-old tomato seedlings. Open bars (fresh extracts) and solid bars (after 4 days storage at −15°).

Table I

The Dependence of Nitrate Reductase Activity in a Tomato Leaf Extract on Added Nitrate before and after Sephadex Treatment

Enzyme preparation	Reaction mixture	mμmoles nitrite accumulated/mg protein hr
Crude leaf extract	Complete	178
	−nitrate	124
Sephadex fraction	Complete	404
	−nitrate	4

The Effect of Buffer Composition. In order to test the effect of buffer composition on enzymic activity, sodium pyrophosphate buffer (pH 7.5, 0.1 M) or Tris • HCl buffer (pH 7.5, 0.1 M) was substituted for the sodium phosphate buffer (pH 7.5, 0.1 M) in the standard nitrate reductase assay of a crude leaf extract. This study showed that maximal activity occurred in reaction mixtures containing phosphate buffer.

The quantitative nature of the stimulation of nitrate reductase activity by phosphate was shown by adding graded amounts of sodium phosphate buffer (pH 7.5, 0.1 M) to reaction mixtures. The pH was controlled by adding enough Tris • HCl buffer (pH 7.5, 0.1 M) to reaction mixtures so that a total of 0.5 ml of buffer was present in every case. The results showed that maximal activity was obtained in the standard assay when the molarity of the phosphate buffer was 0.05 M.

The Effect of pH. The pH of the standard assay was varied in order to study the effect of pH on nitrate reductase activity. This study showed that pH 7.5 was the optimal pH for this enzyme (fig 2), with DPNH as coenzyme.

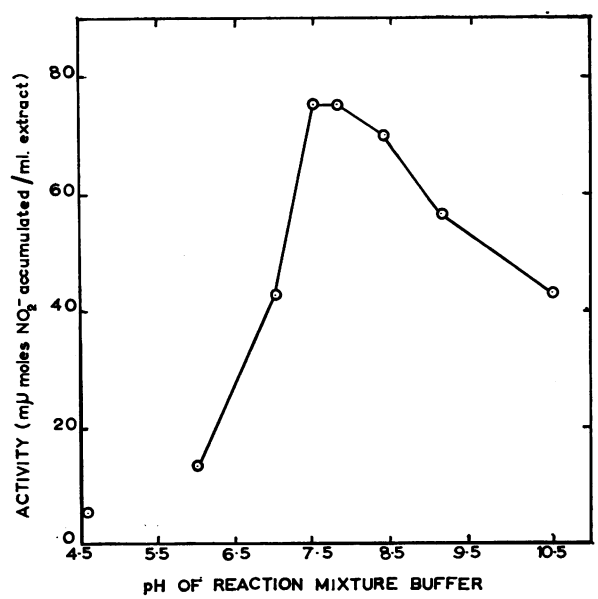

FIG. 2. The pH dependance of nitrate reductase in tomato root extracts.

Cofactor Requirements. The following cofactors were added to standard assay reaction mixtures with Sephadex-treated enzyme preparation: 0.1 μmole FMN, 0.1, μmole FAD, 10^{-3} μmole sodium molybdate and 0.2 μmole $MnCl_2$. In every case the reaction rate was either unaltered or slightly depressed.

The Effect of Nitrate Concentration. The effect of varying the concentration of nitrate is shown in figure 3. Using a reciprocal plot of the data, the K_m

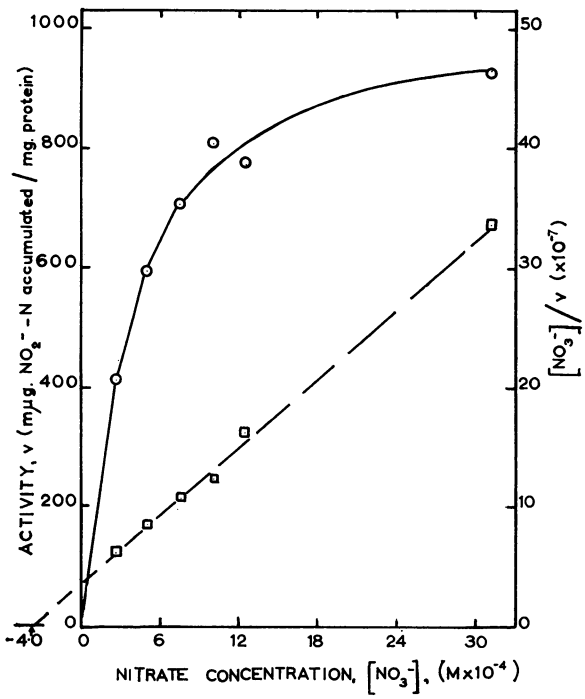

FIG. 3. The effect of nitrate concentration on nitrate reductase activity in a tomato leaf enzyme preparation and determination of the Michaelis constant. Curve O has activity, V, as ordinate and nitrate concentration, $[NO_3^-]$, as abscissa. Line □ has $[NO_3^-]/V$ as ordinate and nitrate concentration $[NO_3^-]$ as abscissa.

for NO_3^- was 4.0×10^{-5} M. The concentration of nitrate used in the standard assay, 1.25×10^{-4} M, supported maximal activity.

The Effect of Enzyme Concentration. The effect of varying the enzyme concentration is shown in figure 4. The rate of activity was proportional to the enzyme concentration in the standard assay, up to 0.1 ml of enzyme preparation.

Coenzyme Specificity and the Effect of Coenzyme Concentration. DPNH and TPNH were tried as electron donors for nitrate reductase and it was found that the enzyme in tomato plants is specific for DPNH (fig 5) at pH 7.5. A reciprocal plot of the data showed that the K_m for DPNH was 2.3×10^{-5} M.

The Effect of the Light Regime of the Plant on the Extractable Nitrate Reductase. In order to study the effect of the light regime of the plant on the level of extractable nitrate reductase, plants which had

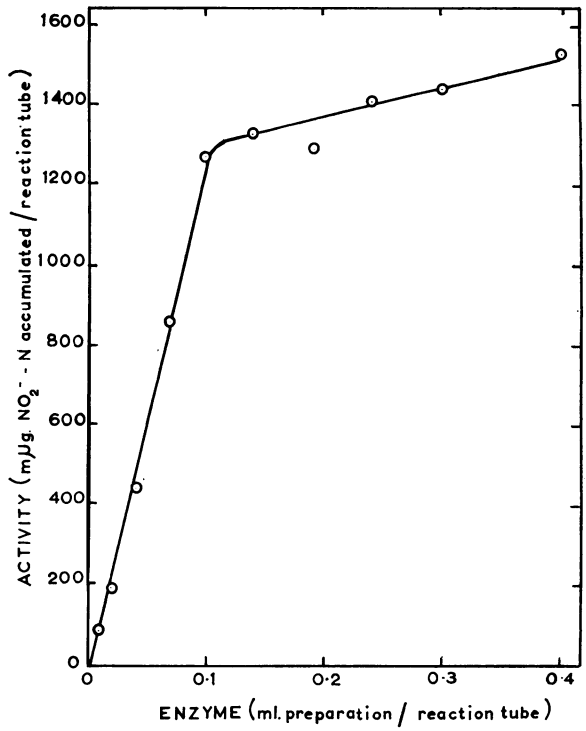

FIG. 4. The effect of enzyme concentration on rate of nitrate reductase activity.

been grown under the prevailing greenhouse light conditions were transferred when 35 days old to the following 3 light regimes: Dark–under a loosely fitting tent of black cloth, plants in darkness; Shade–under a wooden cover about 12 inches high with sides open, about 20 ft-c of light incident on leaves; Light–fully exposed under a light frame, about 1000 ft-c of light incident on leaves. After 24 hours under the above

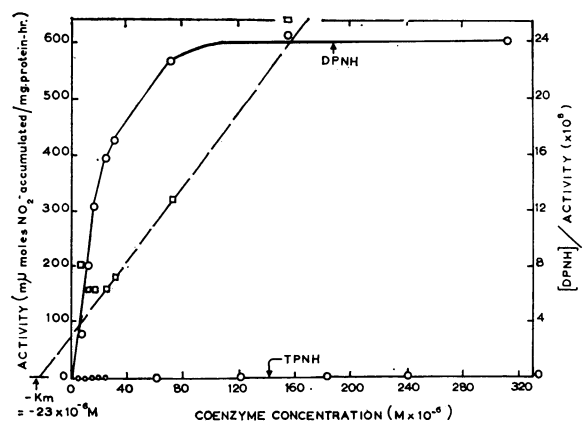

FIG. 5. The effect of coenzyme concentration on nitrate reductase activity in a tomato leaf enzyme preparation and determination of the Michaelis constant. Curve O has activity as ordinate and coenzyme concentration, [DPNH] or [TPNH] as abscissa. Line □ has [DPNH]/activity as ordinate and [DPNH] as abscissa.

light regimes, the leaves were removed and were extracted and assayed as soon as possible. The data showed that light did have a pronounced effect on the level of extractable nitrate reductase which varied directly with the stimulus (fig 6).

The Effect of Nitrate Feeding of Plant on the Level of Extractable Nitrate Reductase. Plants grown until 75 days old with nitrogen supplied as nitrate were used to study the effect of nitrate feeding on the level of extractable nitrate reductase. At this time a number of plants were removed for assay and the remaining plants were thoroughly leeched with tap water. Leeching was followed by irrigation with nutrient solution in which nitrate-nitrogen was replaced by an equimolar amount of ammonium-nitrogen (12). After 5 days the normal feeding with nitrate containing nutrient solution was resumed. The level of extractable nitrate reductase was followed during this time by removing plants at random from among the test plants from time to time and assaying them using the complete reaction mixture of the standard assay. A check on the level of nitrate within the plant tissues was also made using the reaction mixture of the standard assay from which the nitrate had been omitted. The results, showed that the level of enzymic activity dropped rapidly, within 2 days, on cesssation of nitrate feeding to a level about one-fifth of the original level and this low level of activity persisted over the 5 days of feeding with ammonium-nitrogen. On renewal of feeding with nitrate containing nutrient solution the level of activity rose rapidly, within 2 days, to the original level.

These results support the contention that nitrate reductase is an adaptive enzyme (13, 22, 23) but because of the residual nitrate present throughout the study, as shown by enzymic assay, no definite conclusions can be drawn.

Nitrate Reductase in Excised Roots Cultured in Sterile Nutrient Solutions. The properties of nitrate reductase described above were studied first in tomato leaf extracts and then in tomato root extracts. In every case enzymic activity was found in the root extracts at from one-fourth to equal levels of activity as found in leaf extracts. In order to demonstrate with certainty that this activity was due to enzymic activity endogenous to the roots, excised roots grown in sterile nutrient solution culture for 7 days were extracted and assayed. The results showed clearly that tomato roots do contain nitrate reductase and that the level of activity is dependent on the level of nitrate supplied during culture (fig 3). Furthermore, the specific activities found, i.e., 190 and 330 for normal and 6 × nitrate level cultured roots respectively, (based on 10 minute assays) were equal to those found in roots of intact plants (fig 7).

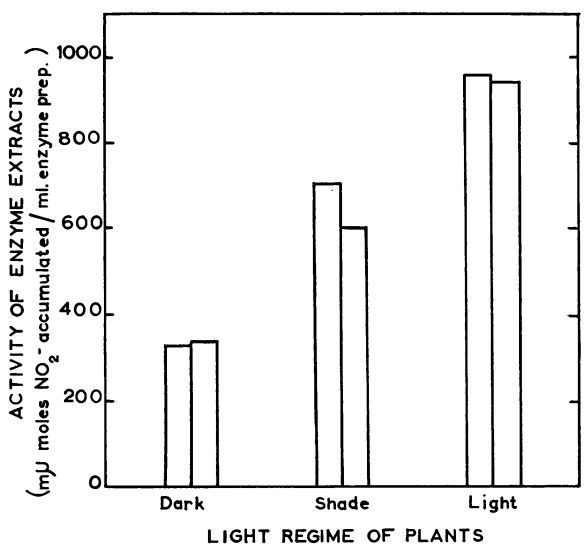

FIG. 6. The effect of the light regime of the tomato plant on the level of nitrate reductase extractable from leaflets. Results of duplicate assays are shown as adjacent bars. The incident light intensities were 0 ft-c (dark), 20 ft-c (shade), and 1000 ft-c (light).

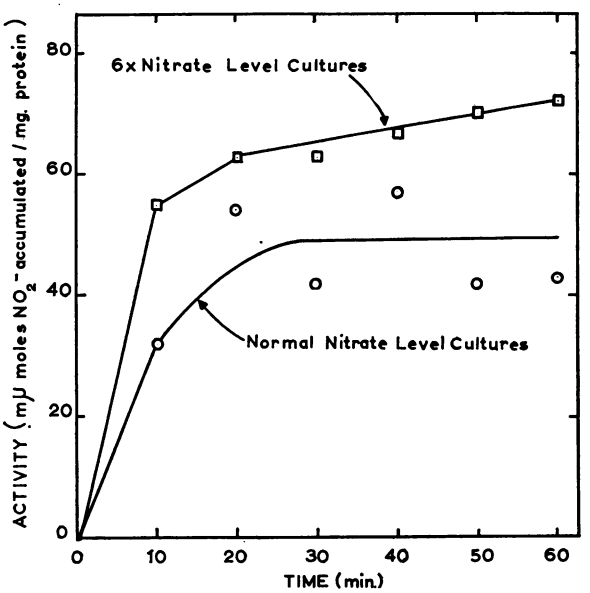

FIG. 7. Nitrate reductase activity in extracts of excised tomato roots grown in sterile nutrient solution culture at 2 levels of nitrate.

A Survey of Nitrate Reductase in Extracts of 7 Plant Species, and the Effect of Cysteine on the Extraction of this Enzyme. Seven species of plants were grown under the conditions adopted for the study of nitrate reductase in tomatoes. Leaves and roots were extracted using 1×10^{-3} M cysteine, and assayed for nitrate reductase activity. Control experiments did not contain cysteine. The results are shown in table II. Data for tomatoes are shown for comparison.

Enzyme activity was found in all species tested except apple. Since only in the case of tomato were the optimum extraction conditions worked out, it is probable that the failure to detect nitrate reductase in apple extracts is due solely to the use of unsatisfac-

Table II

Nitrate Reductase Activity in 8 Species of Higher Plants and the Effect of Cysteine on the Extraction of this Activity

Plant species	Age of plants	Level of cysteine in extraction medium	Roots				Leaves			
			Protein	Activity: Length of assay (min)			Protein	Activity: Length of assay (min)		
				10	20	40		10	20	40
	(days)	(M)	(mg/ml extract)	(mμmole NO_2^- accumulated/ml extract)			(mg/ml extract)	(mμmole NO_2^- accumulated/ml extract)		
Apple	101	0	2.3	0	0	0	12.7	0	0	0
		1×10^{-3}	3.7	0	0	0	12.0	0	0	0
Barley	54	0	1.2	34	47	53	1.8	0	0	0
		1×10^{-3}	1.3	43	51	55	5.8	516	701	770
Corn	53	0	1.5	13	19	20	3.8	38	63	65
		1×10^{-3}	1.4	4	5	...	5.0	167	300	424
Kidney bean	33	0	...*	...	...	...	...	...	...	...
		1×10^{-3}	...	11	38	43	...	75	121	129
Potato	42	0	0.7	8	13	34	5.2	105	110	116
		1×10^{-3}	0.8	19	39	44	5.4	385	641	710
Tobacco	75	0	1.4	0	0	0	2.7	13	22	26
		1×10^{-3}	1.5	28	55	71	2.7	145	278	512
Tomato roots	72	0	...	50	77	112	...	...	27	...
leaves	33	1×10^{-3}	...	85	160	267	...	...	887	...
Wheat	33	0	...	...	...	...	...	...	...	...
		1×10^{-3}	...	16	49	81	...	3	8	38

* ... No determination carried out.

tory conditions for this plant material. The marked effect of cysteine on the level of activity extracted from the organs tested in this survey is evidence of the importance of the extraction conditions on the results obtained.

Discussion

This investigation has shown that an active nitrate reductase is present in the tomato plant. Nitrate reductase has previously been characterized to some extent in 5 other species of higher plants, namely in soybean leaves (8), in cauliflower leaves (3), in wheat embryos (21), in corn leaves (11), and in marrow leaves (5). The properties of the enzyme from the 6 species of plants are shown in table III for comparison.

The similarity in the properties of the reported nitrate reductases is considerable. Firstly, the pH optima of the enzymes are generally near pH 7.5. The soybean leaf enzyme is a notable exception with an optimum at pH 6.0.

In general, the enzymes showed a specific requirement for the coenzyme DPNH. However, the enzymes extracted from soybean leaves and marrow leaves were reported to be operative with both DPNH and TPNH. While this could be an indication that these enzymes are in fact different from the others, it is also possible that these results are due to the operation of coenzyme transhydrogenase which could transfer electrons from added TPNH to endogenous DPN. The latter enzyme has been shown to be present in spinach leaves (14).

A requirement for P_i has been found in every instance that has been investigated, except in marrow leaves. It is noteworthy that phosphate buffers have been used in the assay reaction mixtures of all the investigations compared in table III except the one on soybean leaves where pyrophosphate buffers were used. Kinsky and McElroy (15) carried out a detailed study of the effect of P_i on nitrate reductase from Neurospora and postulated that the role of P_i in stimulating this enzyme was due to the existence of a phosphomolybdate complex in the enzyme which renders the enzymic molybdate much more reactive than it is in the free state. It is plausible that this phenomenon operates in higher plants.

The beneficial effect of cysteine in the extraction media is almost certainly due to the protection given by this substance to sulfhydryl groups on the enzyme which are required for enzymic activity as has been shown for Neurospora nitrate reductase (18).

The flavin appears to be rather firmly bound to the enzyme, and only in 2 cases (8, 21) has a flavin requirement been demonstrated. In these 2 cases, however, the enzymes have been shown to be FAD-specific.

The Michaelis constants for coenzyme (DPNH or TPNH) and nitrate ion agree reasonably well with those published for other nitrate reductases (table III). The variation found in the Michaelis constants

Table III

Summary of Properties of Tomato Nitrate Reductase and Nitrate Reductases Reported for 5 Other Species of Higher Plants

Plant material (investigation)	Property of enzyme						
	pH optimum	Coenzyme	Requirement for inorganic phosphate	Effect of cysteine extraction	Flavin requirement	Michaelis Constants	
						K_m (coenzyme)	K_m (NO_3^-)
						M	M
Tomato leaves (the present investigation)	7.5	DPNH specific	yes	Beneficial	...	23×10^{-6}	4.0×10^{-4}
Tomato roots (the present investigation)	7.5	DPNH specific	yes	Beneficial	...	6×10^{-6}	2.3×10^{-4}
Soybean leaves (8)	6.0	DPNH or TPNH	...	...	FAD-specific	30×10^{-6}	75×10^{-4}
Cauliflower leaves (3)	7.2	DPNH	...	Beneficial	...	...	...
Wheat embryos (21)	7.4	DPNH specific	yes	Beneficial	FAD-specific	8×10^{-6}	3.8×10^{-4}
Maize leaves (11)	...	DPNH specific	...	Beneficial	...	...	...
Marrow leaves (5)	7.5	DPNH or TPNH	...	Beneficial	...	...	1.8×10^{-4}

for coenzyme is probably explicable to the inhibitory effects of DPNH and TPNH analogs which have been shown to be generally present in preparations of these reduced coenzymes (6, 9). Failure to use the initial rate of reaction in these studies would also lead to variations in the Michaelis constants determined and it is doubtful whether any of the investigations compared in table III are entirely free of this error.

A study of environmental effects on the level of extractable nitrate reductase in tomato plants revealed additional similarities. The increase in activity associated with increased illumination of tomato plants was similar to the effect of light on this enzyme in cauliflower leaves (3) and in corn leaves (11). The action of light is not understood but it is probably not merely an effect on the level of reduced coenzymes present as these substances are added to assay reaction mixtures at saturating levels.

The tomato leaf enzyme showed a marked response to nitrate feeding which is also in agreement with the findings with cauliflower leaves (3) and corn leaves (11).

The comparisons discussed above make it clear that the enzyme nitrate reductase present in higher plants has very similar, if indeed not actually the same, properties from plant to plant.

During this investigation, considerable attention was directed to nitrate reduction in roots. The evidence in favor of nitrate reduction occurring in roots of plants is considerable. The findings of Bollard (2) that, at most, only traces of inorganic nitrogen could be detected in the xylem sap of a wide range of plants, and the demonstration that excised tomato roots thrive in sterile nutrient solution with nitrate as the sole source of nitrogen are but two of several relevant experimental findings. Nevertheless, the presence of nitrate reductase in roots has never before been demonstrated satisfactorily.

Evans and Nason (8) reported extremely low rates of activity in extracts of roots of 3 species of plants, namely, wheat roots, corn roots, and soybean roots where activity was about 6, 1, and 2 mμmoles nitrite formed per milligram dry weight of tissue per hour, respectively. The levels of activity in cauliflower roots (3) and in corn roots (11) were simply reported as being very low. Vaidyanathan and Street (24) reported barely perceptible rates (about 7–9 mμmoles nitrite formed per gram fresh weight of tissue per hour) in aqueous extracts of excised tomato roots. Cresswell (5) examined root extracts of several species of plants and found either low rates of activity, notably in marrow root, which he felt could be accounted for by the presence of denitrifying bacteria, or no activity at all.

In the present investigation, nitrate reductase was found in tomato root extracts when favorable extraction conditions were employed (table II). The properties of tomato root nitrate reductase are summarized in table III and they are very similar to the properties of the leaf enzyme. Most likely these enzymes are in fact identical, the differences in properties found being due to factors discussed above.

The finding of nitrate reductase in excised tomato roots grown in sterile nutrient solution culture at levels comparable to those found in intact roots confirms the contention that the enzyme studied in extracts of roots from intact plants was an endogenous

enzyme. The negligible rates of activity found by Vaidyanathan and Street (24) in extracts of similar material is undoubtedly due to their failure to appreciate the requirement for cysteine in the extraction media for the extraction of this enzyme in an active state.

Summary

Nitrate reductase has been extracted from tomato leaves and roots and its properties studied. It is apparently a sulfhydryl containing enzyme since cysteine is required for its extraction in an active state. Endogenous nitrate was removed from the enzyme preparation by treatment with Sephadex. Nitrate reductase in this enzyme preparation had a pH optimum of 7.5 and was dependent on the presence of 0.05 M phosphate for maximal activity; at pH 7.5 the enzyme was specific for reduced dephosphopyridine nucleotide.

The presence of this enzyme in tomato roots was confirmed by the extraction of an active nitrate reductase from excised tomato roots grown in sterile nutrient solution culture. The level of activity in excised roots has been shown to be comparable to that found in roots of intact plants.

The ubiquitous presence of nitrate reductase in higher plants is suggested from the finding of the enzyme in 7 of the 8 species tested. Failure to account for the sensitivity of this enzyme to environmental factors such as light and nitrate supply and to extraction and assay procedures are probably responsible for any inability to detect the enzyme.

Acknowledgments

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