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THE ROLE OF MOLYBDENUM IN THE SYNTHESIS OF NITRATE REDUCTASE IN CAULIFLOWER (*BRASSICA OLERACEA* L. VAR. *BOTRYTIS* L.) AND SPINACH (*SPINACEA OLERACEA* L.)

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SUMMARY

1. The ^{185}W analogue of nitrate reductase was produced in molybdenum-deficient cauliflower (*Brassica oleracea* L. var. *botrytis* L.) leaves following petiolar uptake of ^{185}W from a molybdenum-free medium. L-Azetidine-2-carboxylic acid in the nutrient solution had no effect on analogue formation, but puromycin decreased by 75% ^{185}W incorporation into protein in the fraction normally associated with nitrate reductase. The analogue co-purified with spinach (*Spinacea oleracea* L.) nitrate reductase.

2. An antiserum prepared against partially purified spinach nitrate reductase inhibited enzyme activity.

3. Using the antiserum a method was developed, based on 50% inactivation of a "standard" nitrate reductase preparation, to measure the amount of material in solution cross-reacting as nitrate reductase protein but having no enzyme activity. Crude extracts and solutions resulting after purification with respect to nitrate reductase were obtained from molybdenum-deficient spinach plants grown with nitrate either as the sole nitrogen source or supplemented with ammonium salt. These were assayed for their content of cross-reacting material.

4. The results indicate that approximately 30% of the normal complement of nitrate reductase protein found in extracts of healthy spinach plants was present in extracts of molybdenum-deficient spinach plants either as aponitrate reductase orits immunochemically cross-reacting sub-units. Purification of crude extracts with respect to nitrate reductase resulted in a preferential and substantial loss of cross-reacting material relative to the enzyme. It is suggested that breakdown of the apoprotein is rapid in vivo compared with that of the holoenzyme. Continued net increase of nitrate reductase resulting from metal incorporation into apoprotein is dependent on protein synthesis.

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INTRODUCTION

Nitrate reductase of higher plants (NADH:nitrate oxidoreductase, EC 1.6.6.1) is a nitrate-inducible [1, 2] molybdoprotein which also exhibits the following partial activities: NADH dehydrogenase, as shown by the reduction of numerous electron acceptors such as cytochrome *c* [3] or dichlorophenolindophenol [4] and the reduction of nitrate by reduced benzyl or methyl viologens [5, 6]. It has been shown by complementation studies using fungal mutants that these two partial activities reside in different polypeptides [7–12].

Infiltration of molybdenum into leaves of molybdenum-deficient plants grown with nitrate as a nitrogen source resulted in the formation of nitrate reductase [2]. Inhibitors of protein and nucleic acid syntheses infiltrated at the same time as the metal prevented enzyme formation [13, 14]. It was, therefore, concluded that induction of the enzyme in response to molybdenum was dependent on protein synthesis and not due to the rapid incorporation of the constitutive metal into pre-existing apoenzyme.

In *Neurospora crassa* [15] and *Chlorella fusca* [16] grown without molybdenum, an NAD(P)H cytochrome *c* reductase component of nitrate reductase having the same $s_{20,w}$ value and activity level as the complete enzyme was found. Addition of molybdenum to molybdenum-deficient *Chlorella fusca* in the presence of cycloheximide, to prevent de novo protein synthesis, resulted in limited formation of NADH nitrate reductase activity with the same $s_{20,w}$ value as the NADH cytochrome *c* reductase [16].

Tungsten is a competitive inhibitor of molybdenum in the formation of nitrate reductase activity in barley (*Hordeum vulgare* L.) [3], *Neurospora crassa* [15], *Chlorella fusca* [16] and spinach (*Spinacea oleracea* L.) [17] without affecting either the NADH dehydrogenase activity [3, 15–17] or the $s_{20,w}$ value of the whole NADH nitrate reductase complex [3, 15, 16]. This was shown to be due to the formation of a tungsten analogue of the nitrate reductase portion of the complex [17, 18] which is functional only with respect to NADH dehydrogenase activity.

Antibodies produced against NADPH nitrate reductase complex of *Aspergillus nidulans* have been shown to cross-react with proteins induced by nitrate in mutants of the fungus which contained NADPH dehydrogenase activity but not NADPH nitrate reductase activity [10]. Respiratory and assimilatory nitrate reductase systems from *Escherichia coli* react equally with antiserum prepared against the respiratory enzyme to produce a precipitate [19]. Enzyme activity, assayed by the benzyl viologen method [5], lost from solution after the antibody–enzyme interaction could be accounted for in the precipitate [19].

We report here a study of the role of molybdenum (or tungsten) in the synthesis of nitrate reductase (or analogue) from two higher plants using two different approaches, namely: (a) the effect of the proline analogue L-azetidine-2-carboxylic acid [20] and the peptide synthesis terminator puromycin [21] on the incorporation of ^{185}W into the nitrate reductase complex of molybdenum-deficient cauliflower (*Brassica oleracea* L. var. *botrytis* L.) leaves. Both L-azetidine-2-carboxylic acid and puromycin inhibit production of nitrate reductase activity in response to molybdenum [22]. (b) The use of an antiserum to a higher plant nitrate reductase, to test for the presence of serologically cross-reacting material in extracts from molybdenum-

deficient spinach plants which might provide an estimate of amounts of apoenzyme or other precursors of the enzyme. The results indicate that protein synthesis is required for substantial accumulation of the metal-containing nitrate reductase complex and that the full complement of aponitrate reductase is not present in molybdenum-deficient plants.

MATERIALS AND METHODS

Plant culture

Molybdenum-deficient cauliflower and spinach plants were grown in purified sand culture [23] using nutrient salts purified with respect to molybdenum and with nitrate as the nitrogen source ($-MoNO_3$) [23]. After 27 days, $(NH_4)_2SO_4$ (4 mM) was added to the nutrient medium given to some spinach plants ($-MoNO_3NH_4$) for 10 days before sampling at 37 days. A minimal supplement of 0.2 nM molybdate was added to the nutrient medium from the outset in order to prevent premature plant death during 5–6 weeks growth. Healthy spinach plants ($+MoNO_3$) were grown for similar periods also in sand culture with a normal (0.5 μM) molybdate supply [23].

Assay methods

NADH nitrate reductase, plant protein and radioactivity were determined as before [17]. Where necessary, solutions were concentrated in an Amicon Diaflo cell using a PM 30 membrane. Serum protein concentrations were determined by 280-nm absorption with serum albumin as a standard. NADH nitrate reductase activity units are defined as nmoles NO_2^- produced/15 min per ml or per g fresh weight or per mg protein as relevant.

Induction and purification of enzyme and tungsten analogue

A preliminary experiment established that when petioles of 6-week-old molybdenum-deficient cauliflower leaves (approx. 50 g) were dipped into 200 ml of nutrient solution containing: (a) no molybdenum, or (b) 3 μg/ml molybdenum and kept at 25 °C under 11 000 lux white fluorescent light for 24 h. NADH nitrate reductase activity increased from an initial level of 42 enzyme units/g in crude extracts [24] to 48 or 588 units for (a) or (b), respectively.

Comparable cauliflower leaves were dipped into 200 ml molybdenum-free nutrient solution containing, 15 μCi ^{185}W and 24 μg unlabelled W either alone or with the addition of 300 mg L-azetidine-2-carboxylic acid or 400 mg puromycin. After 24 h in light the midribs were removed and the weighed lamina (26–35 g) mixed with 25 g healthy ($+MoNO_3$) 6-week-old spinach leaves. The mixed leaf material was extracted and purified with respect to nitrate reductase for spinach as previously described [24] using $Ca_3(PO_4)_2$ gel adsorption, $(NH_4)_2SO_4$ precipitation and Biogel A 0.5 m chromatography. Fractions from the Biogel A column having NADH nitrate reductase activity were bulked and concentrated before reassaying.

Preparation of antigen

NADH nitrate reductase was purified from approx. 200 g of $+MoNO_3$ spinach leaves as previously described [24], but repeating the Biogel chromatography stage once more. The final eluate was concentrated to a gross protein content of

between 4 and 5 mg/ml and sterilised by filtration through a Sartorius membrane filter 11307 (cellulose nitrate) 0.2 μm pore size via an Ultra-Asept syringe. Specific activities ranged from 1313 to 4709 enzyme units/mg protein, i.e. a purification factor of between 20 and 58.

Preparation of antisera

Guinea pigs (strain Dunkin Hartley, smooth-haired, aged 6 weeks at the start of the experiment) were initially injected subcutaneously with 1 ml (1.8 mg gross protein) of a 30 times purified nitrate reductase preparation emulsified with Freund's complete adjuvant and 3 times subsequently at 2 or 4 week intervals intravenously without adjuvant with 0.2 ml (0.78–1.5 mg gross protein) of enzyme purified 20–58 times. The approx. 6 ml of serum obtained from each guinea pig was sterilised by filtration and stored at −18 °C.

Enzyme antibody interactions

The presence of immunologically cross-reacting material in solutions obtained from molybdenum-deficient spinach plants was tested by the following method. NADH nitrate reductase purified from $+MoNO_3$ spinach leaves as for antigen preparation was diluted (usually 2–3-fold) with 0.1 M sodium phosphate, 1 nM EDTA (pH 7.5) to an activity of 2800 enzyme units/ml. Preparations with this level of activity were used as standards in the assays. Samples of this solution (0.15 ml) were mixed with equal volumes of immune serum previously diluted to varying degrees with the same phosphate buffer. After 1 h at 4 °C the mixtures were centrifuged at 14 000 $\times$ g for 5 min and residual NADH nitrate reductase activity determined in the supernatants. These activities were then calculated as percentages of a control in which serum was replaced by an equal volume of phosphate buffer and plotted against degree of immune serum dilution. From this curve was obtained the serum dilution required to cause 50% inactivation of NADH nitrate reductase in the test preparation.

Crude extracts from molybdenum-deficient plants, or solutions obtained by purifying the crude extracts with respect to NADH nitrate reductase [24], were concentrated about 10 times and filter-sterilised before being used as substitutes for the phosphate buffer to dilute the active NADH nitrate reductase preparation to 2800 enzyme units/ml.

If cross-reacting protein which could not be identified by the NADH nitrate reductase assay were present in preparations from the molybdenum-deficient plants, the amounts of serum needed to inactivate given amounts of nitrate reductase, when these preparations were used as diluants instead of phosphate buffer for producing the standard activity, would be increased. The characteristic titration curves would then be seen to be shifted towards smaller dilution values for the overall titration and the 50% inactivation value. By using control treatments described below to compensate for known amounts of active nitrate reductase in the molybdenum-deficient preparations it was possible to estimate the amount of cross-reacting protein expressed in terms of antigenic nitrate reductase as equivalent NADH enzyme activity units.

Solutions which had lost their NADH nitrate reductase activity during prolonged storage at 4 °C retained full antigenic activity. This was demonstrated by the

constancy of the amount of immune serum required to cause 50% inactivation of the NADH nitrate reductase activity in a fixed volume of solution, irrespective of the decay in enzyme activity of that solution with time. A cold storage deactivated nitrate reductase preparation diluted to various extents was used in place of phosphate buffer to cause shifts in the serum titration curves of a standard nitrate reductase preparation. From these shifts a calibration curve relating immune serum concentrations to units of antigenic nitrate reductase was prepared (Graf, L., Notton, B. A. and Hewitt, E. J., unpublished). The cold storage deactivated preparations were also used to provide the compensatory controls for effects of the small amounts of active NADH nitrate reductase originally present in the molybdenum-deficient preparations as follows: A purified NADH nitrate reductase preparation from healthy ($+MoNO_3$) spinach leaves, whose known original enzyme activity had been allowed to decay to zero at 4 °C, was diluted to an extent which, in terms of original NADH nitrate reductase activity, was equivalent to the level of NADH nitrate reductase activity found immediately after obtaining the molybdenum-deficient crude extracts of purified preparations. This control solution was used as an alternative diluant for the active NADH nitrate reductase preparation in place of either phosphate buffer or of extracts from molybdenum-deficient plants to produce a "standard" solution of 2800 enzyme units/ml. This "standard" activity was, therefore, obtained either with addition of no protein (phosphate buffer), or of an unknown amount of potential cross-reacting aponitrate reductase, or a control to compensate for the known small amount of holonitrate reductase. For the first experiment in Table II with purified preparations from molybdenum-deficient plants obtained by the procedure used for normal plants, the standard preparation obtained from 168 g of $+MoNO_3$ leaves had 7100 enzyme units/ml and required to be diluted with 2 vol. of phosphate buffer (Dilutant A) to produce 2800 enzyme/units/ml. The $-MoNO_3$ preparation obtained from 22 g leaves of 22-day-old plants had an activity of 4 enzyme units/ml in 7.8 ml (Dilutant B), and the $-MoNO_3NH_4$ preparation from 55 g leaves of 37-day-old plants had an activity of 100 enzyme units/ml in 10 ml (Dilutant C). The compensating controls for Dilutants B and C were obtained from a cold storage deactivated nitrate reductase preparation having an original activity of 8400 enzyme units/ml which was diluted either 2100 times as a control for Dilutant B (Dilutant D) or 84 times as a control for Dilutant C (Dilutant E).

For the second experiment in Table II with crude extracts from 37-day-old molybdenum-deficient plants the standard preparation obtained from 135 g of $+MoNO_3$ leaves had 5600 enzyme units/ml and was diluted with 1 vol. of phosphate buffer (Dilutant F). The $-MoNO_3$ crude extract from 27 g leaves had an activity of 110 enzyme units/ml when concentrated to 8 ml (Dilutant G) and the $-MoNO_3NH_4$ crude extract from 50 g leaves had an activity of 107 enzyme units/ml when concentrated to 13.5 ml (Dilutant H). The compensating control for both Dilutants G and H was obtained by diluting the cold storage deactivated preparation used in the first experiment by 76.5 times (Dilutant I).

When non-immune serum replaced immune serum in the phosphate buffer diluted series (Dilutant A) there was no decrease in the NADH nitrate reductase activity recovered irrespective of the serum concentration used. Complete enzyme inactivation occurred at immune serum concentrations which produced no visible precipitate.

RESULTS

Induction and co-purification of tungsten analogue with nitrate reductase

The addition of spinach leaves containing high nitrate reductase activity (2400 ± 400 enzyme units/g) to the molybdenum-deficient cauliflower leaves permitted the purification of protein from cauliflower which, as obtained from spinach, is associated with the concentration of nitrate reductase, and from which the overall purification could be calculated. The NADH nitrate reductase activity, radioactivity and protein were determined in samples taken at the various stages outlined in Materials and Methods, and relevant results are shown in Table I. It was expected

TABLE I

Effect of L-azetidine-2-carboxylic acid and puromycin on incorporation of ^{185}W into protein associated with nitrate reductase produced in molybdenum-deficient cauliflower leaves and co-purified with 25 g of healthy (+ $MoNO_3$) spinach leaves. Inducing medium: 15 μCi ^{185}W + 25 μg tungsten in 200 ml molybdenum-free nutrient.
Treatment A, control treatment with inducing medium without inhibitors. Weight of cauliflower leaves 35.4 g having total enzyme activity of 2336 units. Treatment B, inducing medium with 1.5 mg/ml L-azetidine-2-carboxylic acid. Weight of cauliflower leaves 33.4 g having total enzyme activity of 1803 units. Treatment C, inducing medium with 2 mg/ml puromycin. Weight of cauliflower leaves 26.0 g having total enzyme activity of 1872 units.

Treatment	Fraction	Enzyme activity		Radioactivity		
		(total units/mg protein)		(total cpm	cpm/g cauliflower	cpm/mg protein)
A	Crude extract	46 240	54.4	$1.13 \cdot 10^6$	$3.16 \cdot 10^4$	$1.3 \cdot 10^3$
	$Ca_3(PO_4)_2$ gel supernatant	760	1.4	$7.52 \cdot 10^5$	$2.12 \cdot 10^4$	$1.3 \cdot 10^3$
	Biogel A eluate	3 956	1041	$6.63 \cdot 10^2$	18.6	$1.7 \cdot 10^2$
B	Crude extract	56 880	62.3	$4.31 \cdot 10^5$	$1.31 \cdot 10^4$	$4.7 \cdot 10^2$
	$Ca_3(PO_4)_2$ gel supernatant	368	0.9	$2.98 \cdot 10^5$	$8.05 \cdot 10^3$	$7.1 \cdot 10^2$
	Biogel A eluate	3 963	1246	$6.58 \cdot 10^2$	19.7	$2.1 \cdot 10^2$
C	Crude extract	52 374	50.9	$2.35 \cdot 10^5$	$9.04 \cdot 10^3$	$2.3 \cdot 10^2$
	$Ca_3(PO_4)_2$ gel supernatant	2 964	4.4	$1.78 \cdot 10^5$	$6.84 \cdot 10^3$	$2.7 \cdot 10^2$
	Biogel A eluate	4 797	1518	$1.14 \cdot 10^2$	4.38	$0.37 \cdot 10^2$

that the tungsten analogue would be produced in cauliflower in the same manner as that shown for spinach by various criteria of protein purification including electrophoresis in polyacrylamide gel [17] and pH focusing [24].

The peak of NADH nitrate reductase activity from the Biogel A column, which originated from the spinach, occurred in the same region as the peak of radioactivity originating from the cauliflower, but the lability of the active enzyme, especially when relatively dilute in the off-peak regions, resulted in changing proportionality between enzyme activity and radioactivity (Fig. 1). Neither peak was associated with the bulk of gross protein as measured by 281-nm absorption. Polyacrylamide gel electrophoresis of the combined peak fractions showed comparable association of radioactivity and enzyme activity to that already described for spinach [17].

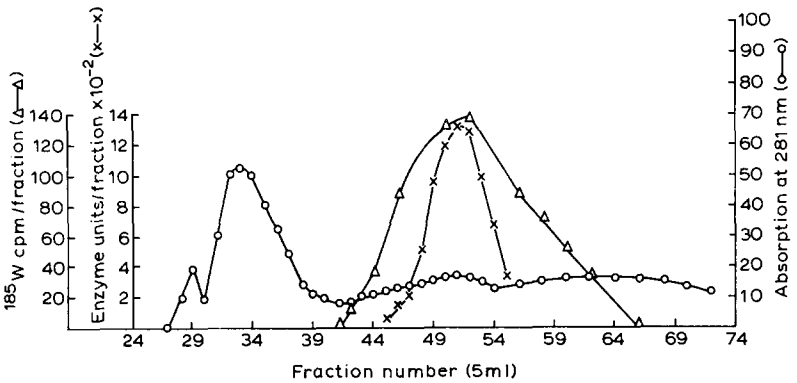

Fig. 1. Elution profiles from Biogel A 0.5 m gel chromatography of NADH nitrate reductase activity originating from $+MoNO_3$ spinach leaves (×—×), radioactivity originating from molybdenum-deficient cauliflower leaves fed with ^{185}W (△—△), and 281-nm absorption (○—○).

The slight skew distribution of the ^{185}W from the column indicates either that some breakdown was occurring [16] or that a lower molecular size species was also present. However, there was no evidence for in vitro metal exchange in similar preparations [25].

Inhibitor studies

The incorporation of tungsten into protein co-purified with nitrate reductase was compared in relation to the absence or presence of L-azetidine-2-carboxylic acid or puromycin which inhibit formation of nitrate reductase activity in cauliflower [22]. Results in Table I show that the L-azetidine-2-carboxylic acid treatment resulted in Biogel A eluate values almost identical to the control in terms of radioactivity per g cauliflower leaf or per mg purified proteins. On the other hand, puromycin treatment produced a Biogel A eluate fraction which contained only 23% of the radioactivity per g cauliflower leaf and showed a 79% reduction in the radioactivity per mg purified proteins in spite of a higher degree of purification of the associated NADH nitrate reductase.

Although the addition of L-azetidine-2-carboxylic acid or puromycin depressed the total uptake of ^{185}W by 60% or 72%, respectively, to that of the control, the amount of ^{185}W taken up by the tissue was in excess of the amount needed for metal protein synthesis. This is based on the facts that the supernatant fractions from the first purification step ($Ca_3(PO_4)_2$ gel) contained 69% and 75% (1000-fold excess) of the total radioactivity in the crude extracts in comparison with 66% for the control.

Serological studies

Results of the first experiment with purified preparations from the molybdenum-deficient plants obtained by the procedures used for obtaining nitrate reductase from normal plants are shown in Fig. 2 and Table II. The curves relate residual enzyme activity as a percentage of the control, in which serum was replaced by an equal volume of phosphate buffer to the degree of immune serum dilution. The degree of serum dilution which caused 50% inactivation of the NADH nitrate reductase activity

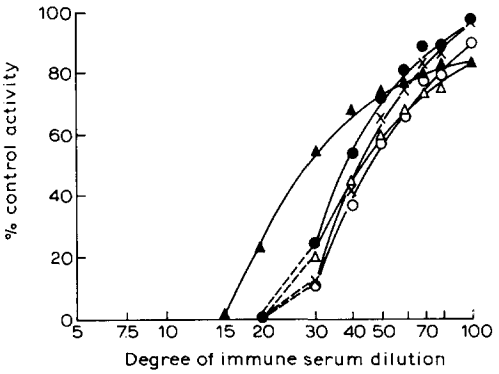

Fig. 2. Effect of dilutions of immune serum (protein content 73.6 mg/ml) on the nitrate reductase activity of a purified spinach preparation (activity 7100 units/ml) diluted to an activity of 2800 units/ml with the following: phosphate buffer (○—○); a purified nitrate reductase preparation from 22 g of leaves from $-MoNO_3$ plants (△—△); a purified nitrate reductase preparation from 55 g of leaves from $-MoNO_3NH_4$ plants (▲—▲); a cold storage (4 °C) deactivated enzyme diluted 2100 times (×—×) and 84 times (○—○).

TABLE II

ANTIGENIC NITRATE REDUCTASE IN $-MoNO_3$ AND $-MoNO_3NH_4$ EXTRACTS

Degree of dilution and protein content of immune serum required to cause 50% inactivation of "standard" (diluted to 2800 enzyme units/ml) nitrate reductase preparations. Standard enzyme preparations were obtained by dilution of the concentrate either with phosphate buffer (Dilutants A, F) or with purified (Dilutants B, J) or crude (Dilutant G) extracts of fresh or frozen leaves of $-MoNO_3$ plants or with purified (Dilutant C) or crude (Dilutant H) extracts of $-MoNO_3NH_4$ plants or with compensatory controls obtained by dilution with preparations of cold storage deactivated nitrate reductase as explained in Materials and Methods (Dilutants D, E and I). Differences in immune serum requirements between phosphate buffer and other dilutants for 50% inactivation were related to equivalent concentrations of antigenic nitrate reductase in extracts.

Dilutant		Immune serum		Antigenic nitrate reductase (units/ml)
		Dilution for 50% enzyme inactivation	Protein content (μg/ml)	
A	Phosphate buffer	45.2	1628	—
B	$-MoNO_3$ purified	42.7	1724	40
C	$-MoNO_3NH_4$ purified	26.3	2798	794
D	Control for Dilutant B	42.7	1724	40
E	Control for Dilutant D	38.0	1937	200
F	Phosphate buffer	17.4	4230	—
G	$-MoNO_3$ crude	12.0	6133	2150
H	$-MoNO_3NH_4$ crude	9.2	8000	5770
I	Control for Dilutants G and H	16.4	4488	150
A	Phosphate buffer	44.0	1559	—
B	$-MoNO_3$ purified	44.0	1559	0
J	$-MoNO_3$ purified	41.0	1673	50

varied with the solutions (Dilutants A–E) used to dilute the concentrated active enzyme preparation to the standard value (Table II).

Using the calibration curve, the increases in serum protein required for 50% enzyme inactivation when Dilutants B–E replaced phosphate buffer (Dilutant A) were related to antigenic nitrate reductase protein content (Table II). The $-MoNO_3NH_4$ preparation (Dilutant C) and its control (Dilutant E) contained 794 and 200 units antigenic nitrate reductase/ml, respectively. On a fresh weight basis (Table III) Dilu-

TABLE III

Immunologically cross-reacting material in crude extracts and solutions purified as for nitrate reductase obtained from $-MoNO_3$ and $-MoNO_3NH_4$ spinach plants compared with similar solutions obtained from $+MoNO_3$ spinach plants.

Sample	Active enzyme (units/ml)	Total cross-reacting material (units/g)	% of $+MoNO_3$ plants cross-reacting material	
			Total	Less active enzyme
$+MoNO_3$ crude	2400 ± 400		100	100
$-MoNO_3$ crude	31.9	854	35.6	34.3
$-MoNO_3NH_4$ crude	24.5	899	37.5	36.4
$+MoNO_3$purified	617 ± 150		100	100
$-MoNO_3$ purified	1.4*	14	2.3	2.0
	0 **	5.3	0.8	0.8
$-MoNO_3NH_4$ purified	18	144	23.3	20.4

* Fresh leaves.
** Leaves stored at −20 °C.

tant C was equivalent to 144 units of antigenic nitrate reductase/g, only 18 of which could be accounted for by the observed NADH nitrate reductase activity. The increase in serum protein requirement as a result of the $-MoNO_3$ (Dilutant B) treatment and its control (Dilutant D) were both equivalent to 40 units of antigenic nitrate reductase/ml (Table II; 14 units/g fresh weight, Table III). Comparable purified $+MoNO_3$ preparations had an active enzyme content of approx. 617 ± 150 enzyme units/g fresh weight (for 6 preparations).

In a second experiment, crude extracts obtained from leaves of 37-day-old $-MoNO_3$ and $-MoNO_3NH_4$ spinach plants were used. The purified active nitrate reductase obtained from $+MoNO_3$ plants used to prepare the "standard" in this second experiment contained considerable amounts of denatured or inert enzyme when compared to the equivalent preparation used in the previous experiment as indicated by the increase in immune serum protein required to cause 50% nitrate reductase inactivation (Table II, 4230 μg as compared to 1628 μg). Differences of this nature are often large and are minimized by standardization of plant culture and sampling conditions. All three dilutants (Dilutants G, H and I) of the active NADH nitrate reductase preparation caused a shift in the inactivation curve (Fig. 3) compared with a phosphate buffer dilution. The increases in immune serum protein required for 50% enzyme inactivation (Table II) were equivalent to 150 antigenic nitrate reductase units/ml for the control based on inactive enzyme (Dilutant I) and a further

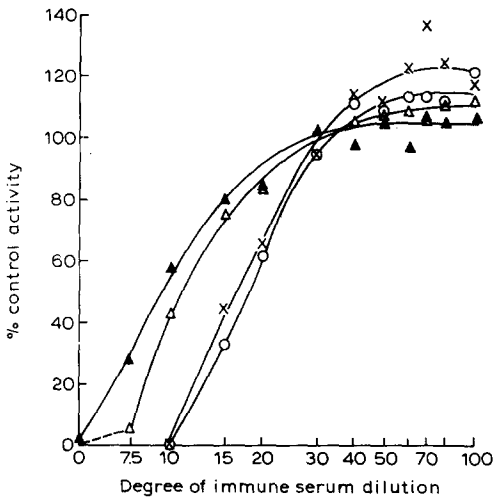

Fig. 3. Effect of dilutions of immune serum (protein content 73.6 mg/ml) on the nitrate reductase activity of a purified spinach preparation (activity 5600 units/ml) diluted to an activity of 2800 units/ml with the following: phosphate buffer (○—○); a crude extract from 27 g of leaves from $-MoNO_3$ plants (△—△); a crude extract from 50 g of leaves from $-MoNO_3NH_4$ plants (▲—▲); a cold storage (4 °C) deactivated enzyme diluted 76.5 times (×—×).

2000 antigenic nitrate reductase units/ml for the $-MoNO_3$ preparation (Dilutant G) and 5620 antigenic nitrate reductase units/ml for the $-MoNO_3NH_4$ preparation (Dilutant H). On a fresh weight basis (Table III) these values were equal to 854 and 899 units/g for Dilutants G and H, respectively, indicating a negligible increase in antigenic nitrate reductase cross-reacting material as a result of the $(NH_4)_2SO_4$ nutrient. Crude extracts of $+MoNO_3$ spinach plants had active NADH nitrate reductase contents of approx. 2400 ± 400 enzyme units/g (for 6 preparations).

The total antigenic nitrate reductase cross-acting material (Table III) expressed as a percentage of the active NADH nitrate reductase found in an equivalent healthy $+MoNO_3$ spinach preparation was 2.3% for $-MoNO_3$ and 23.3% for $-MoNO_3NH_4$ in purified preparations (Expt 1) but was 34% for the $-MoNO_3$ and 36% for the $-MoNO_3NH_4$ treatments in crude extracts (Expt 2).

In a third experiment designed to test the validity of the extremely low antigenic nitrate reductase content of the $-MoNO_3$ purified solution (14 units/g), 91 g of leaves taken from 6-week-old $-MoNO_3$ spinach plants and stored −20 °C were extracted and protein was purified as for nitrate reductase. The purified solution was concentrated to 9.6 ml and was devoid of nitrate reductase activity. This solution (Dilutant J); the solution (Dilutant B) obtained from 22 g of $-MoNO_3$ spinach and used in the first experiment; and phosphate buffer were used to dilute 3-fold, from 9100–2800 enzyme units/ml, a purified nitrate reductase preparation obtained from 160 g of $+MoNO_3$ spinach leaves closely comparable with those used in the first experiment. Serum inactivation curves were prepared as before (Fig. 4). 50% enzyme inactivation occurred at 44 times immune serum dilution (1559 μg serum protein) for solutions (Dilutant B) and phosphate buffer treatments, and 41 times immune serum dilution (1673 μg serum protein) for solution (Dilutant J) treatment (Table II).

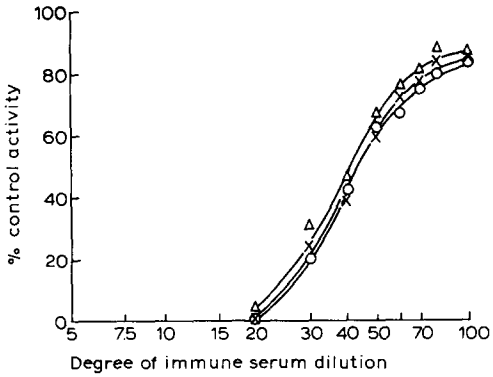

Fig. 4. Effect of dilutions of immune serum (protein content 73.6 mg/ml) on the nitrate reductase activity of a purified spinach preparation (activity 9100 units/ml) diluted to an activity of 2800 units/ml with the following: phosphate buffer (○—○); a purified extract from 22 g of leaves from fresh $-MoNO_3$ plants (×—×); a purified extract from 91 g of leaves which had been stored at −20 °C from $-MoNO_3$ plants (△—△).

This difference was equivalent to 50.2 antigenic nitrate reductase units/ml or 5.3 units/g fresh weight. The similarity between the dilution of immune serum required for 50% enzyme inactivation of the control (phosphate diluted) treatments in the first and third experiments (Table II, 45.2 and 44 times) reflect the value of standardization of plant growth and sampling. The low antigenic nitrate reductase content of the $-MoNO_3$ solution (Dilutant J) is similar to that obtained from Solution B in the first experiment (Table III) and the difference is probably within the limits of experimental error. Frozen leaves from healthy ($+MoNO_3$) spinach have been found to retain high levels of active nitrate reductase; it would, therefore, seem unlikely that degradation of antigenic nitrate reductase would take place during storage under these conditions, particularly as the antigen remains substantially unchanged in cross-reacting value after denaturation of the active enzyme occurs during storage at 4 °C for several weeks.

DISCUSSION

The results obtained using puromycin indicate that protein synthesis is required for maximal production of holoprotein. This was shown by a decrease in tungsten labelling of protein in the eluate from Biogel A chromatography associated with active nitrate reductase. However, L-azetidine-2-carboxylic acid caused no interference in metal–protein formation at a concentration which inhibits production of nitrate reductase activity by molybdenum in molybdenum-deficient cauliflower leaves [22]. Two explanations appear possible: (a) the full complement of aponitrate reductase is already present in the absence of molybdenum and the metal induces the formation of a molybdenum transferase protein; (b) the net level of aponitrate reductase is the limiting factor. Puromycin would inhibit synthesis of either or both proteins; L-azetidine-2-carboxylic acid which, by replacement of proline, alters the tertiary structure of enzymes is generally responsible for catalytic properties [26]. However, transferase activity might be impaired by the distortion in tertiary structure.

The serological experiments which reveal low concentrations of cross-reacting material in molybdenum-deficient plants support the second hypothesis. Plants grown without molybdenum have impaired protein synthesis when nitrate is the only nitrogen source. Ammonium compounds provide available nitrogen for protein synthesis under these conditions [27]. Addition of $(NH_4)_2SO_4$ to the nutrient supplied to $-MoNO_3$ spinach plants for 10 days almost doubled the extracted crude protein; from 3.1–5.9 mg/g fresh weight. A comparable $+MoNO_3$ spinach plant would contain 20 mg/g fresh weight. However, whereas the nitrate reductase activity actually decreased from 31.9–24.5 enzyme units/g during the $(NH_4)_2SO_4$ treatment compared to 2400 enzyme units/g for $+MoNO_3$ spinach plants, material cross-reacting as nitrate reductase protein was similar for both the molybdenum-deficient treatments and it was equivalent to approx. 36% of normal nitrate reductase activity of $+MoNO_3$ spinach plants. The doubling in the amount of extractable protein as a result of the $(NH_4)_2SO_4$ treatment made it seem unlikely that formation of cross-reacting material was limited to the observed extent by available nitrogen supply. In a separate experiment, purification of crude extracts resulted in a decrease in cross-reacting material when compared to similarly treated nitrate reductase from $+MoNO_3$ preparations. This effect was most pronounced for the $-MoNO_3$ plants compared to the $-MoNO_3NH_4$ plants (Table III).

As nitrate reductase systems are thought to comprise aggregates of multiple sub-units having NAD(P)H dehydrogenase activity [3, 10, 15, 28], the relative losses of cross-reacting material during purification designed to isolate nitrate reductase protein might well be explained by the differences in molecular size between sub-units and whole protein. The existence of a small molybdenum binding or transferase protein able to cross-react with anti-nitrate reductase would produce similar results. It is possible that the presence of ammonium compounds favours the state of aggregation of the sub-units, perhaps by reducing the rate of disaggregation, thereby causing the bulk of the total cross-reacting material to be in the aponitrate reductase form. In contrast with our findings, the concentration of aponitrate reductase in *Neurospora crassa* [15] or *Chlorella fusca* [16] may attain up to 90% of the holoenzyme level. It is proposed that in higher plants breakdown of the apoprotein in vivo is rapid compared to that of the holoenzyme, which, though also subject to turnover [29], is relatively stabilised by molybdenum or tungsten. Decreased stability of apoprotein in the absence of a prosthetic metal is similarly inferred for apoferritin or its sub-units [30] or apoceruloplasmin [31] which in copper-deficient rats attained about 25% of the normal holoprotein level [32].

Apoceruloplasmin of rats (approx. 160 000 daltons) cross-reacts identically with antiserum to the holoprotein [32]. Apoferredoxin of *Clostridium pasteurianum* (approx. 6000 daltons) also cross-reacts with its holoprotein antiserum, but the extent is inconsistent and may be diminished [33] or enhanced [34]. In *Aspergillus nidulans* mutants having NADPH dehydrogenase activity but lacking nitrate reductase (approx. 250 000 daltons) nevertheless contain proteins which cross-react with antiserum to the whole enzyme [10]. Making the basic assumption that aponitrate reductase would cross-react to a similar extent to holoenzyme, it would seem that only a small proportion, between 2 and 20% depending on nitrogen source treatment, of the total complement of apoenzyme of full molecular size is present in purified extracts of molybdenum-deficient plants.

The incorporation of tungsten by molybdenum-deficient cauliflower in the presence of puromycin to the extent of 24% compared with control or L-azetidine-2-carboxylic acid treatments is consistent with the maximal level of apoprotein deduced serologically for the spinach plants. The inability of higher plants to produce normal levels of nitrate reductase in response to addition of molybdenum in vivo in the presence of protein synthesis inhibitors [13, 14] is, therefore, attributed to the sub-optimal level and presumed high turnover rate of the apoprotein.

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REFERENCES

1 Hewitt, E. J. and Afridi, M. M. R. K. (1956) Nature 183, 57–58
2 Afridi, M. M. R. K. and Hewitt, E. J. (1964) J. Exp. Bot. 15, 251–271
3 Wray, J. L. and Filner, P. (1970) Biochem. J. 119, 715–725
4 Oji, Y. and Izawa, G. (1969) Plant Cell Physiol. 10, 734–749
5 Cresswell, C. F., Hageman, R. H., Hewitt, E. J. and Hucklesby, D. P. (1965) Biochem. J. 94, 40–53
6 Ramirez, J. M., Del Campo, F. F., Paneque, A. and Losada, M. (1964) Biochem. Biophys. Res. Commun. 15, 297–302
7 Sorger, G. J. (1963) Biochem. Biophys. Res. Commun. 12, 395–401
8 Sorger, G. J. (1964) Nature 204, 575–576
9 Sorger, G. J. (1966) Biochem. Biophys. Acta 118, 484–494
10 Pateman, J. A., Cove, D. J., Beevers, B. M. and Roberts, D. B. (1964) Nature 201, 58–60
11 Nason, A., Antoine, A. D., Ketchum, P. A., Fazier, III, W. A. and Lee, D. K. (1970) Proc. Natl. Acad. Sci. U.S. 65, 137–144
12 Nason, A., Lee, K.-Y., Pan, S. S., Ketchum, P. A., Lamberti, A. and De Vries, J. (1971) Proc. Natl. Acad. Sci. U.S. 68, 2242–2246
13 Afridi, M. M. R. K. and Hewitt, E. J. (1965) J. Exp. Bot. 16, 628–645
14 Hewitt, E. J., Notton, B. A. and Afridi, M. M. R. K. (1967) Plant Cell Physiol. 8, 385–397
15 Subramanian, K. N. and Sorger, G. J. (1972) Biochim. Biophys. Acta 256, 533–543
16 Vega, J. Ma., Herrera, J., Aparicio, P. J., Paneque, A. and Losada, M. (1971) Plant Physiol. 48, 294–299
17 Notton, B. A. and Hewitt, E. J. (1971) Biochem. Biophys. Res. Commun. 44, 702–710
18 Paneque, A., Vega, J. Ma., Cardenas, J., Herrera, J., Aparicio, P. J. and Losada, M. (1972) Plant Cell Physiol. 13, 175–178
19 Murray, E. D. and Sanwal, B. D. (1963) Can. J. Microbiol. 9, 781–790
20 Fowden, L. (1963) J. Exp. Bot., 14, 387–398
21 Yarmolinsky, M. B. and De La Habba, G. L. (1959) Proc. Natl. Acad. Sci. U.S. 45, 1721–1729
22 Hewitt, E. J. and Notton, B. A. (1967) Phytochemistry 6, 1329–1335
23 Hewitt, E. J. (1966) Sand and Water Culture Methods Used in The Study of Plant Nutrition, 2nd edn, Commonwealth Bureau of Horticulture, Tech. Commun. 22, Farnham Royal, England

24 Notton, B. A., Hewitt, E. J. and Fielding, A. H. (1972) Phytochemistry 11, 2447–2449
25 Notton, B. A. and Hewitt, E. J. (1971) FEBS Lett. 18, 19–22
26 Fowden, L., Lewis, D. and Tristram, H. (1967) Advances in Enzymology (Nord, F. F., ed.), Vol. 29, pp. 89–163, Interscience, Publ., New York
27 Hewitt, E. J., Agarwala, S. C. and Williams, A. H. (1957) J. Hort. Sci. 32, 34–48
28 Notton, B. A. and Hewitt, E. J. (1971) Plant Cell Physiol. 12, 465–477
29 Zielke, H. R. and Filner, P. (1971) J. Biol. Chem. 246, 1772–1776
30 Drysdale, J. W. and Munro, H. N. (1966) J. Biol. Chem. 241, 3630–3637
31 Holtzman, N. A. and Gaumnitz, B. M. (1970) J. Biol. Chem. 245, 2354–2358
32 Holtzman, N. A. and Gaumnitz, B. M. (1970) J. Biol. Chem. 245, 2350–2353
33 Hong, J. S. and Rabinowitz, J. C. (1970) J. Biol. Chem. 245, 2995–3000
34 Nitz, R. D., Mitchell, B., Gerwing, J. and Christensen, J. (1969) J. Immunol. 103, 319–323