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INHIBITION BY L-AZETIDINE-2-CARBOXYLIC ACID OF INDUCTION OF NITRATE REDUCTASE IN PLANTS AND ITS REVERSAL BY L-PROLINE

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Abstract—The effect of L-azetidine-2-carboxylic acid on the induction of nitrate reductase in leaf tissues of radish and cauliflower plants was studied. Plants were grown under conditions of nitrate starvation or with ammonium sulphate either in sterile culture, or in the presence of 2-chloro-6-(trichloromethyl)-pyridine ("N-Serve") or under conditions of molybdenum deficient sand culture. L-Azetidine-2-carboxylic acid severely inhibited nitrate reductase induction by nitrate in tissues with a low proline content. Inhibition was reversed by simultaneous infiltration of L-proline. Induction of the enzyme in response to molybdenum was less inhibited in tissues of molybdenum deficient plants which had a high endogenous proline content. No reversal was obtained with seventeen other normal protein amino acids.

INTRODUCTION

THE studies of Steward *et al.*,[1] Fowden[2] and Fowden and Richmond[3] showed that L-Azetidine-2-carboxylic acid (A-COOH), a proline analogue, was an inhibitor of growth in carrot tissue cultures, mung bean radicles and *E. coli*. The analogue was incorporated into the newly synthesized protein in place of L-proline and both incorporation and inhibition of growth were reversed by L-proline.[2] Petersen and Fowden[4] studied the enzymic reaction of A-COOH in prolyl-transfer-ribonucleic acid-transferase of mung bean, a species not producing A-COOH, and in *Polygonatum multiflorum*, a liliaceous species containing A-COOH, and showed that A-COOH is a competitive substrate for L-proline in the mung bean enzyme system.

Afridi and Hewitt[5] tested the effects of several synthetic and natural antimetabolites as inhibitors of induction of nitrate reductase in excised leaf tissues of cauliflower in response to nitrate or molybdenum as the inducing agent. Many of the compounds tested were known to have multiple or complex effects on the processes of protein or nucleic acid synthesis or growth and the interpretation of these effects was sometimes difficult. The specific action of A-COOH as a competitive analogue[4] of one essential amino acid, which is probably associated with the tertiary structure of proteins[3] and its known reactivity in protein synthesis by intact plant tissues, suggested it would be of particular interest to test its effect on the induction of a single plant enzyme: reduced-NAD: nitrate oxido-reductase. (EC 1. 6. 6.1). (Nitrate reductase).

RESULTS AND DISCUSSION

The effect of increasing concentrations of the natural L-isomer of A-COOH on the induction of the enzyme in nitrate-starved cauliflower leaves is shown in Fig. 1. A separate

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[2] L. FOWDEN, *J. Exp. Botany* **14**, 387 (1963).
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experiment illustrating the reversal of this inhibition by L-proline is also shown. The proline content of these tissues as measured in the extract used in the enzyme assays was 27·5 μg/g.

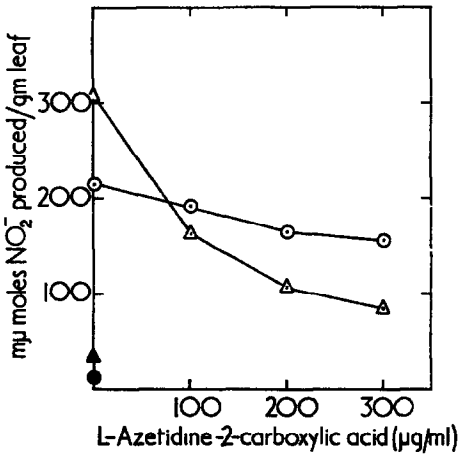

FIG. 1. THE EFFECT OF INCREASING CONCENTRATION OF L-AZETIDINE-2-CARBOXYLIC ACID ON THE INDUCTION OF NITRATE REDUCTASE BY NITRATE IN NITRATE-STARVED CAULIFLOWER LEAF TISSUE AND THE REVERSAL OF INHIBITION BY L-PROLINE.

Infiltration by nitrate at 4 mM. Endogenous proline 27·5μg/g fresh wt. of leaf. Time of incubation 5 hr.

△—NO_3^- + L-Azetidine-2-carboxylic acid; ⊙—NO_3^- + L-Azetidine-2-carboxylic acid + 50 μg/ml L-proline; ▲ and ● Nitrate reductase activity at 0 hr incubation.

Changes in enzyme activity with time are shown in Fig. 2. Nitrate induced considerable activity after 5½ hr. A-COOH prevented this induction and proline reversed the effect of

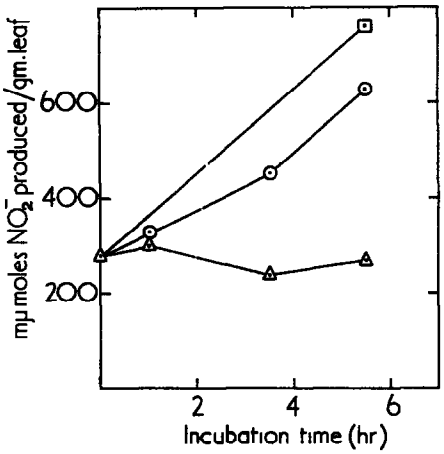

FIG. 2. THE EFFECT OF TIME OF INCUBATION ON THE INDUCTION OF NITRATE REDUCTASE BY NITRATE IN RADISH LEAF TISSUE GROWN WITH AMMONIUM NITROGEN AND "N-SERVE" 10 p.p.m. IN SOLUTION SHOWING INHIBITION OF INDUCTION BY L-AZETIDINE-2-CARBOXYLIC ACID AND REVERSAL BY L-PROLINE.

Infiltration by Nitrate at 4 mM; L-Azetidine-2-carboxylic acid at 1 mg/ml; L-proline at 1 mg/ml. Endogenous proline 126 μg/g fresh wt. of leaf.

—NO_3^- only; △—NO_3^- + L-azetidine-2-carboxylic acid; ⊙—NO_3^- + L-azetidine-2-carboxylic acid + L-proline.

the A-COOH. The tissue used in this experiment was radish grown with an ammonium nitrogen source in non-sterile cultures in the presence of "N-serve" (2-chloro-6-(trichloromethyl) pyridine), to prevent nitrate production by nitrification. (See Methods.) An adequate supply of nitrogen, provided up to the time of sampling, was reflected in a proline content of 126 μg/g.

Table 1 shows that the D-isomer of A-COOH had no effect on the induction of nitrate reductase when infiltrated together with nitrate into nitrate-starved tissue or together with molybdate into molybdenum deficient cauliflower tissues.

TABLE 1. EFFECT OF D-AZETIDINE-2-CARBOXYLIC ACID ON THE INDUCTION OF NITRATE REDUCTASE IN MOLYBDENUM DEFICIENT AND NITRATE STARVED CAULIFLOWER LEAVES

	Nitrite produced as mμM NO_2 produced/g fresh wt. of leaf in 15 min after 5 hr incubation					
Infiltration Treatments	H_2O	D-Azetidine 2-carboxylic acid	Mo	Mo + D-Azetidine 2-carboxylic acid	NO_3^-	NO_3^- + D-Azetidine 2-carboxylic acid
Molybdenum deficient	9	12	198	216	—	—
Nitrate starved	70	73	—	—	169	156

NO_3^- at 4 mM, Mo at 3 μM, D-azetidine-2-carboxylic acid at 100 μg/ml in infiltrating solutions.

In order to show that the effects observed with proline shown in Figs. 1 and 2 were caused by its competition with A-COOH and not the result of independent stimulation by limiting amounts of an essential amino acid, 1-g samples of radish leaves, grown with ammonium nitrogen and "N-Serve" were infiltrated with nitrate, proline and A-COOH each alone and together. The results, in Table 2, indicate that proline alone is not responsible for the increase in enzyme induction when infiltrated with the inducer and the inhibitor.

TABLE 2. THE EFFECT OF INFILTRATING PROLINE, NITRATE AND L-AZETIDINE-2-CARBOXYLIC ACID ON THE INDUCTION OF NITRATE REDUCTASE IN RADISH LEAVES (NH_4 "N-SERVE")

Nitrite produced as mμM NO_2 produced/g fresh wt. of leaf in 15 min after 5 hr incubation					
H_2O	Proline	NO_3^-	NO_3^- + Proline	NO_3^- + A-COOH	NO_3^- + Proline + A-COOH
192	264	577	577	277	613

NO_3^- at 4mM, proline at 1 mg/ml, "A-COOH" at 1 mg/ml in infiltrating solutions. Proline content of tissue 126 μg/g.

A similar experiment with two concentrations of A-COOH was carried out with cauliflower leaf tissues. These were plants grown with nitrate and then deprived of nitrate until endogenous nitrate reductase activity had decreased to a low level and had a lower endogenous proline content. The results are shown in Table 3.

TABLE 3. THE EFFECT OF "A-COOH" ON THE INDUCTION OF NITRATE REDUCTASE IN NITROGEN STARVED CAULIFLOWER LEAVES AND THE REVERSAL BY PROLINE

Nitrite produced as mμM NO_2 produced/g fresh wt. of leaf in 15 min after 5 hr incubation					
		NO_3^- + 2 mg/ml A-COOH		NO_3^- + 3 mg/ml A-COOH	
H_2O	NO_3^-	alone	+ 2 mg/ml proline	alone	+ 2 mg/ml proline
10	201	8	129	0	108

NO_3^- at 4 mM. Proline content of tissue 50 μg/g.

Cauliflowers were grown in sterile culture with ammonium sulphate as a source of nitrogen in order to test the effect of A-COOH on cauliflower leaves which had an adequate supply of nitrogen and therefore a normal proline content but had been exposed to a minimal concentration of nitrate during growth. The results are shown in Table 4.

TABLE 4. THE EFFECT OF INFILTRATING PROLINE, NITRATE AND "A-COOH" INTO CAULIFLOWER LEAVES GROWN IN STERILE CULTURE WITH AN AMMONIUM NITROGEN SOURCE ON THE INDUCTION OF NITRATE REDUCTASE

Nitrite produced as mμM NO_2 produced/g fresh wt. of leaf in 15 min after 5 hr incubation					
		NO_3^- + 500 μg/ml "A-COOH"		NO_3^- + 1 mg/ml "A-COOH"	
H_2O	NO_3^-	alone	+ 1 mg/ml proline	alone	+ 1 mg/ml proline
50	225	106	181	73	142

NO_3^- at 4 mM. Proline content of tissue 88·5 μg/g.

Cauliflower plants grown in molybdenum deficient nutrients appeared to accumulate proline, 250 μg/g being found in the leaf possibly accounting for the relative ineffectiveness of A-COOH observed. Table 5 shows four separate experiments where concentrations up to

TABLE 5. EFFECT OF INFILTRATING INCREASING CONCENTRATIONS OF "A-COOH" INTO MOLYBDENUM DEFICIENT CAULIFLOWER LEAVES ON THE INDUCTION OF NITRATE REDUCTASE

Nitrite produced mμM NO_2 produced/g fresh wt. of leaf in 15 min after 5 hr incubation						
		Mo + "A-COOH"				
H_2O	Mo	100μg/ml	200μg/ml	300μg/ml	500μg/ml	750μg/ml
8	970	—	900	—	804	717
8	303	291	315	301	—	—
		1mg/ml	2mg/ml	3mg/ml	5mg/ml	10mg/ml
8	481	324	—	—	324	330
0	1081	928	918	930	—	—

Mo at 3 μM. Proline content of tissue 250 μg/g.

10 mg/ml were used, the analogue sometimes failed to reduce the level of nitrate reductase formation induced by molybdenum or else inhibition was much less than that produced with tissues from nitrate starved plants and there was little or no response to increasing concentrations of A-COOH. Where an effect was found, this could be reversed with L-proline (Table 6). The effect of molybdenum on enzyme formation with time, already shown,[5] was confirmed as described in the experimental section.

TABLE 6. EFFECT OF INFILTRATING "A-COOH" INTO MOLYBDENUM DEFICIENT CAULIFLOWER LEAVES ON THE INDUCTION OF NITRATE REDUCTASE AND REVERSAL OF THIS EFFECT BY PROLINE

Nitrite produced as mμM NO_2 produced /g fresh wt. of leaf in 15 min after 5 hr incubation				
	Mo + "A-COOH" (250μg/ml)			
Mo	Alone	+250μg/ml proline	+500μg/ml proline	+1mg/ml proline
660	322	373	573	576

Mo at 3 μM "A-COOH" at 250 μg/ml in infiltrating solutions. Proline leaf content 250 μg/g.

The specificity of L-proline for reversal of the inhibitory effect of A-COOH was tested using equimolar concentrations of seventeen other L-amino acids as shown in Table 7. None of those tested, except proline, caused appreciable reversal in tissues of radish leaves grown with an ammonium nitrogen source in the presence of "N-Serve".

TABLE 7. EFFECT OF PROLINE AND OTHER COMMON PROTEIN AMINO ACIDS ON INHIBITION BY "A-COOH" OF NITRATE REDUCTASE INDUCTION IN RADISH LEAF TISSUES

Nitrite produced as mμM NO_2 produced/g fresh wt. of leaf in 15 min after 5 hr incubation				
H_2O	NO_3^-	NO_3^- + "A-COOH"	NO_3^- + "A-COOH" + amino acid	L-Amino acid
202	720	294	231	Glycine
			264	Alanine
277	811	463	433	Serine
			309	Valine
378	685	381	409	β-phenylalanine
			324	Aspartic acid
465	829	526	429	Arginine
			519	Histidine
			490	Tyrosine
126	643	262	247	Isoleucine
			286	Lysine
			307	Threonine
			274	Ornithine
75	547	177	189	Leucine
			223	Citrulline
178	564	175	188	Tryptophan
			216	Glutamic acid
			421	Proline

NO_3^- at 4mM. "A-COOH" at 1 mg/ml infiltrating solution. Proline leaf content 120 μg/g.

The previously reported effects[1-4] of L-Azetidine-2-carboxylic acid as an inhibitor of growth and as a competitive substitute for L-proline in protein synthesis in plants have now been shown to operate during the formation of a specific enzyme protein: nitrate reductase.

Plant tissues vary greatly in their concentration of endogenous proline as shown here and in other unpublished work with J. F. Thompson. Molybdenum deficient plants grown with nitrate usually have low concentrations of most free amino acids,[6,7] proline apparently being an exception, whereas in plants grown with ammonium sulphate, most amino acids are present at high concentrations[7] and nitrogen starved plants are deficient in most amino acids including proline.[8] Differences between the degree of inhibition produced in different tissues, especially when grown without molybdenum as contrasted with any other growth condition can therefore be most readily explained by differences in endogenous proline concentrations or possibly by the balance between proline and other free amino acids. This result is in complete accord with similar observations on the effects of endogenous proline concentrations of different species reported by Fowden.[2] If this conclusion is correct, the results for effects of the analogue on induction of nitrate reductase either by nitrate or by molybdenum as separate essential factors (Afridi and Hewitt)[9] indicate that proline incorporation is dependent equally on derepression by the substrate (nitrate) and the presence of the prosthetic metal. In unpublished work we have confirmed the observations of Beevers *et al.*[10] that puromycin inhibits the induction of nitrate reductase in response to nitrate and have extended this to show that puromycin inhibits to a comparable extent, formation of the enzyme in response to molybdenum.

Whereas puromycin terminates peptide chain synthesis, L-azetidine-2-carboxylic acid interferes in the incorporation of a single amino acid at the earlier stage of t-RNA-amino acid transfer. It therefore appears that both nitrate and molybdenum are equally necessary for the *de novo* synthesis of the enzyme. Further experiments are now in progress to test this conclusion.

EXPERIMENTAL

Plant material. Cauliflowers (*Brassica oleracea* var. Botrytis) commercial variety Tremendous were grown under three different conditions: in water culture under conditions of molybdenum deficiency for 6–8 weeks, Hewitt;[11] in sand culture with a limited period of nitrogen starvation of 7–10 days after a 6-week growth in complete nutrient; in sterile sand culture using ammonium as a source of nitrogen for 8 weeks.[9] Radish (*Raphanus sativus*) commercial variety Saxa were grown in sand culture for 4–6 weeks using ammonium as a nitrogen source and 10–20 p.p.m. "N Serve" (2-chloro-6-(trichloromethyl)pyridine) to inhibit nitrification (Goring[12]), as this procedure has been shown to be effective in production of plants having only low nitrate reductase activity (unpublished work of E. J. Hewitt, B. A. Notton and E. F. Watson).

Infiltration and incubation. L- or D-Azetidine-2-carboxylic acid, L-proline or other L-amino acids and molybdate (3μM) or nitrate (4 mM) were introduced into leaf tissues by vacuum infiltration.[9] All leaves were used from radish and from molybdenum-deficient and sterile-cultured cauliflower plants and medium-aged leaves were used from the nitrate-starved cauliflower plants for the tests on enzyme induction.

After removal of midribs, leaves were cut into small pieces and well-mixed 1-g samples were infiltrated in a total volume of 10 ml by three successive evacuations and restoration of normal atmospheric conditions. Tissues were then either processed at once or incubated at 25° in 11,000 lux white fluorescent light for a known period. This was usually 5 hr and based on previous results.[5,9]

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Enzyme assay. The leaf tissue was ground in a pre-chilled mortar with 3 ml potassium phosphate buffer (0·1 M pH 8·5) and activity was assayed using 0·5 ml of the centrifuged extract by incubating for 15 min at 17° in the presence of NADH (0·2 mM), NO_3^- 6 mM) and potassium phosphate buffer (0·06 M pH 7·2 in a total volume of 4 ml. The reaction was stopped by oxidative removal of NADH with acetaldehyde (1·5 ml of M) and alcohol dehydrogenase (ADH 0·1 ml containing 10^3 units) and mixing with the Griess–Ilosvay reagents to determine nitrite produced by measuring light extinction at 540 nm[13]. An assay control was obtained by omitting NADH from a similar reaction mixture and enzyme activity was determined from the difference in nitrite produced during incubation with or without NADH, when expressed as mμ moles/15 min/g fresh wt.

In most experiments the standard infiltration treatments comprised a control with water for comparison with effects of incubation with nitrate for the prescribed period, a combined treatment with the inducer (nitrate or molybdenum) together with L-azetidine-2-carboyxlic acid and with or without L-proline, and a treatment with the analogue alone. In a few tests one or more of these treatments were given at zero time immediately before assaying the samples. However experience in this work, in precisely similar experiments of Hewitt and Notton[14] and in other unpublished work by the writers as well as in the earlier experiments of Afridi and Hewitt[5,9] showed that measurements of zero time enzyme activities on all samples was unnecessary for the purposes of this work. The results of 7 "zero time" measurements given below for molybdenum show nevertheless that there is *no immediate* restoration of activity when the element enters the tissues; zero time, H_2O: 42 ± 17; zero time, Mo: 53 ± 20; after 5 hr, Mo: 463 ± 207.

Proline was estimated using 1 ml of the enzyme extract and the acid ninhydrin (pH about 1) reagent of Chinard[15] as advised by Fowden[2] in a personal communication. Recovery tests adding proline to ground leaf tissues were carried out and showed that valid results were obtainable.

Sources of chemicals—L- and D-azetidine-2-carboxylic acid were obtained from the California Corporation for Biochemical Research, NADH and ADH from Sigma Chemical Coporation, "N-Serve", as a gift, from the Dow Chemical Company, Seal Beach, California, L-proline from B.D.H. Poole, England and other chemicals were analytical reagent grade.

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