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N-CARBAMYLPUTRESCINE AMIDOHYDROLASE OF HIGHER PLANTS AND ITS RELATION TO POTASSIUM NUTRITION

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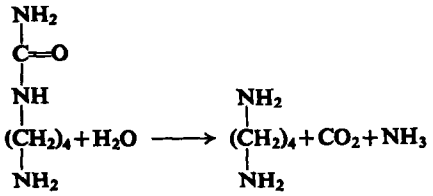
Abstract—Activity of the enzyme degrading N-carbamylputrescine to putrescine (N-carbamylputrescine amidohydrolase) is greater in leaves of potassium-deficient barley than in leaves of barley grown with a normal potassium status. The enzyme was detected in the leaves of seedlings of wheat, rye, oats, maize, pea, radish, sunflower and three varieties of barley. The pH optimum of the enzyme extracted from the leaves of barley seedlings was found to lie between 7 and 8. The energy of activation was 13·5 (s.e. 1·3)kcal/mole and the energy of activation for the denaturation of the enzyme was 32·5 (s.e. 1·7)kcal/mole. Half of the activity was lost on heating the enzyme for 15 min at 52°. The enzyme was strongly inhibited by *p*-chloromercuribenzoate, and this inhibition could be reversed with cysteine. Enzyme preparations did not degrade putrescine, allantoin, citrulline or N,N'-dicarbamylputrescine with the evolution of ammonia. The N-carbamylputrescine amidohydrolase was not responsible for the glutaminase or urease activity which could be detected in the barley leaf extracts.

INTRODUCTION

IN BARLEY, putrescine is formed from arginine by the following pathway; arginine→agmatine→N-carbamylputrescine (I)→putrescine (II).[1–3] Both N-carbamylputrescine and putrescine are produced on feeding agmatine to excised barley leaves, and entire leaves and barley leaf macerates hydrolyse N-carbamylputrescine to putrescine. Although it is known that N-carbamylputrescine may be degraded to putrescine by micro-organisms.[4,5] the enzyme effecting this step has not apparently been studied in detail; moreover it appears that this enzyme has not previously been reported in higher plants.

The work to be presented concerns some of the characteristics of this enzyme (N-carbamylputrescine amidohydrolase, to be termed the amidohydrolase).



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[3] T. A. SMITH and J. L. GARRAWAY, *Phytochem.* **3**, 23 (1964).
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Since it is known that putrescine accumulates in potassium-deficient barley leaves and that simultaneously the activity of L-arginine carboxy-lyase (arginine decarboxylase) is increased,[2] it was also of interest to discover whether a similar increase with potassium-deficiency might be found in the activity of the amidohydrolase.

EXPERIMENTAL AND RESULTS

Amidohydrolase Activity in Seedlings

A quantitative estimation of the amidohydrolase activity in Proctor barley seedlings was made on duplicate samples after 2 weeks' growth. For those grown with tap-water only, the activities were 50 and 58 μg NH_3/24 hr/g fresh weight. Seedling leaves of this variety grown with full nutrients gave values of 26 and 34 μg NH_3/24 hr g fresh weight. The putrescine level in the seedlings which had been grown in tap-water was estimated chromatographically and found to be 2–3 times that of the controls which had been grown with full nutrients.

In an attempt to detect amidohydrolase activity in other plants, seedlings of eight species were grown in tap-water. The enzyme was determined using the semiquantitative method (see Methods section). The enzyme was detected in barley (vars. Proctor, Mildew resistant and Plumage Archer), wheat, rye, oats, maize, pea, radish and sunflower.

Potassium Deficiency

Representative samples of the leaves from 11- and 14-week old normal and potassium-deficient barley plants were extracted (see Methods section), and the N-carbamylputrescine amidohydrolase and L-arginine carboxy-lyase activities were determined for each extract. The results are presented in Table 1. After 17 weeks' growth the putrescine concentration in the leaves of the potassium-deficient plants was estimated chromatographically and found to be approximately 250 μg/g fresh weight.

TABLE 1. N-CARBAMYLPUTRESCINE AMIDOHYDROLASE AND L-ARGININE CARBOXY-LYASE ACTIVITIES IN THE LEAVES OF NORMAL AND POTASSIUM-DEFICIENT PROCTOR BARLEY

Enzyme Activity	Age of plant in weeks	Type of plant: Normal	Type of plant: Potassium-deficient
N-carbamylputrescine amidohydrolase (μg NH_3/24 hr/g fresh weight)	11	18, 33, 27	89, 56, 64
	14	53, 58	115, 108
L-arginine carboxy-lyase (μg agmatine/hr/g fresh weight)	14	2·1, 3·2	33, 49

Amidohydrolase activity was determined by estimating the amount of ammonia produced on incubating the leaf extract with N-carbamylputrescine. L-Arginine carboxy-lyase activity was determined by measurement of the rate of agmatine production (see Methods section). The results given are those obtained in replicated determinations.

pH Optimum

The pH optimum for the amidohydrolase activity *in vitro* was estimated using extracts which had been dialysed against buffers of various pH values ranging from 4·5 to 10·0 (Fig. 1). No difference in pH optimum could be detected between an unpurified extract and an extract prepared by the acetone precipitation technique; in both cases the optimum was found to lie between pH 7 and 8. In subsequent experiments the standard assay was conducted at pH 7·2.

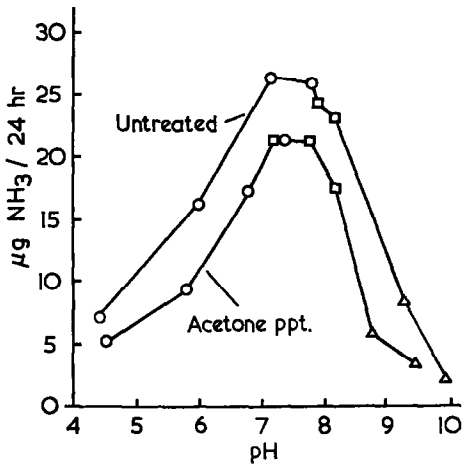

FIG. 1. THE EFFECT OF pH ON N-CARBAMYLPUTRESCINE AMIDOHYDROLASE ACTIVITY.

Samples (6 ml) of untreated barley leaf extracts and extracts prepared by the method of freezing followed by acetone precipitation were dialysed against a range of buffers for 48 hr at 2° with addition of toluene as antiseptic. Each enzyme solution was then made up to 7 ml and 1 ml samples were used for the estimation of amidohydrolase activity. The pH was determined on the residual solutions.

○ Sodium/potassium phosphate buffers (0·1 M)
□ Tris-hydroxymethylaminomethane/HCl buffers (0·05 M)
△ Sodium carbonate/bicarbonate buffers (0·1 M)

Effect of Temperature

From the results for the activity determined at 25° and 0°, the energy of activation was calculated to be 13·5 (s.e. 1·3) kcal/mole. Half of the amidohydrolase activity was lost on heating for 15 min at 52°. The regression on the proportion of activity lost in 15 min at temperatures ranging from 42·5° to 55° was used to determine the energy of activation for the denaturation of the enzyme. This value was found to be 32·5 (s.e. 1·7) kcal/mole.

Inhibition

The results are given in Table 2. The sensitivity of the enzyme to *p*-chloromercuribenzoate and the reversal of the inhibition by cysteine indicates that sulphydryl groups are important for activity.

The enzyme preparation (0·5 ml) with the inhibitor (0·5 ml) was incubated for 24 hr with water (0·25 ml) or with N-carbamylputrescine (0·25 ml of 25 mM) in Conway units. Simultaneous estimations of ammonia formation in the absence of inhibitor were made in separate Conway units. The inhibitor concentrations required to produce 50% inhibition were obtained by interpolation.

TABLE 2. EFFECT OF INHIBITORS ON N-CARBAMYLPUTRESCINE AMIDOHYDROLASE ACTIVITY

Compound	Final concentration (mM)	Inhibition %
Putrescine dihydrochloride	10	0
N,N'-dicarbamylputrescine	4	0
Iodoacetate	30	50
Potassium cyanide	50	50
Semicarbazide hydrochloride	40	50
p-Chloromercuribenzoate	1	100
	0·1	60, 75*
Cysteine	50	50
	2	0
p-Chloromercuribenzoate and cysteine	0·1	24, 33*
	2	

* Duplicate determinations.

Specificity

From the results presented in Table 3 it appears that the hydrolysis of glutamine and urea was not effected by the N-carbamylputrescine amidohydrolase.

The protein in the extracts is probably hydrolysed enzymatically, giving rise to free glutamine and asparagine. These amides may then be hydrolysed by L-glutamine or L-asparagine amidohydrolase resulting in the observed increase in ammonia in the absence of the normal substrate (Fig. 2).

TABLE 3. THE SPECIFICITY OF N-CARBAMYLPUTRESCINE AMIDOHYDROLASE

Substrate	Concentration (mM)	Relative activity
Putrescine dihydrochloride	5	0
Allantoin	5	0
Citrulline	5	0
N,N'-dicarbamylputrescine	4	0
Asparagine	5	37
Glutamine	10	39
	5	34
N-carbamylputrescine	10	25
	5	28
N-carbamylputrescine and glutamine	5	56
	5	
Urea	10	11
	5	12
N-carbamylputrescine and urea	5	37
	5	

Activity was determined by estimating the amount of ammonia produced on incubating the enzyme preparation with and without substrate.

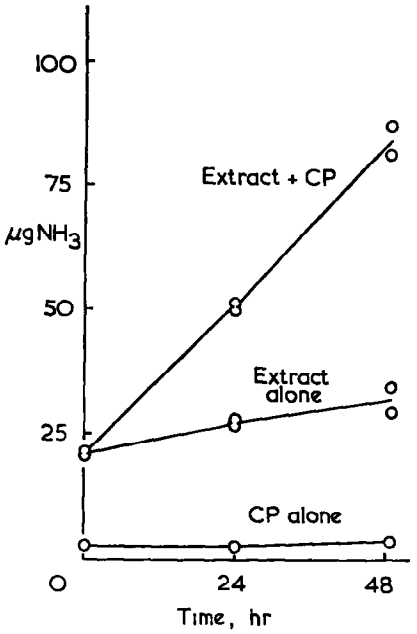

FIG. 2. THE RATE OF AMMONIA FORMATION BY A BARLEY LEAF EXTRACT WITH AND WITHOUT THE ADDITION OF N-CARBAMYLPUTRESCINE.

Samples of untreated dialysed barley leaf extract (1 ml) were incubated with water (0·25 ml) or with 25 mM N-carbamylputrescine hydrochloride (CP) (0·25 ml) in Conway units. After 0, 24 and 49 hr, the ammonia formed was released by addition of saturated potassium carbonate and trapped in 0·01 N HCl. The ammonia was determined by Nessler's reagent.

Subcellular Distribution

On separating the particulate fraction of the leaves of barley seedlings grown without nutrients in 0·5 M sucrose by centrifuging at 12,000 *g* for 15 min, all the amidohydrolase activity was found to be associated with the supernatant.

Stoichiometry

To investigate the stoichiometry of the amidohydrolase reaction, the enzyme was permitted to hydrolyse a known amount of N-carbamylputrescine to completion, the resulting amounts of putrescine and ammonia estimated, and the molar proportions of these three components compared.

The N-carbamylputrescine used in this assay was purified by paper chromatography according to the method of Smith and Garraway.[3] Samples (1 ml) of the enzyme preparation were placed in the outer compartments of six Conway units. Water (0·25 ml) was added to three of these and N-carbamylputrescine (0·25 ml of 25 mM) to the other three. After 48 hr, samples (62·5 μl) were withdrawn from the outer compartments of the Conway units and applied to chromatography paper as described under putrescine estimation (see Methods section). The ammonia was then determined by Nesslerization after distillation. The recoveries (N-carbamylputrescine = 100%) were putrescine 94, 95, 97%; ammonia 103, 104, 104%.

Effect of Phosphate and Potassium Ions on Amidohydrolase Activity

As it is known that phosphorolysis is involved in the enzymatic degradation of citrulline[6, 7] it was considered that phosphate might be required by N-carbamylputrescine amidohydrolase. Also, since the amidohydrolase activity in potassium-deficient barley leaves was greater than in barley grown with a normal potassium status, the possibility of inhibition of the enzyme by potassium ions was investigated.

After dialysing a sample of a barley leaf extract against distilled water, half of this extract was dialysed against 0·1 M tris(tris-hydroxymethylaminomethane)-hydrochloric acid buffer (pH 7·2) and the remainder was dialysed similarly against 0·1 M phosphate buffer (pH 7·2). The activities of the two enzyme samples did not differ significantly, although the concentration of the phosphate ion was found to differ by a factor of 1000.

Potassium chloride was added to a sample of the tris·HCl dialysed extract. The amidohydrolase activity of the sample with potassium at a low concentration did not differ significantly from the activity of the sample to which potassium was added and in which the potassium concentration was increased by a factor of 6000. The sample of N-carbamylputrescine used as the substrate contained no detectable potassium or phosphate.

DISCUSSION

The present investigation represents the first record of the existence of N-carbamylputrescine amidohydrolase in higher plants. Like the L-arginine carboxy-lyase previously characterized[2] the activity of the amidohydrolase was also found to be higher in potassium-deficient barley leaves. These increased activities are associated with higher concentrations of agmatine and putrescine in the deficient plants, these substances being the products of the two enzyme reactions. N-carbamylputrescine, the intermediate in the pathway between agmatine and putrescine, does not occur at concentrations high enough for its detection by the methods used, in extracts of either normal or potassium-deficient barley.[3] The activity of the amidohydrolase in the leaves of barley seedlings grown in the absence of nutrients is also higher than the activity of those grown with a defined nutrient solution.

In later work on the enzyme (involving the freeze-drying technique) barley seedlings which had been grown in tap-water were used as the source, since on a fresh weight basis they possessed an amidohydrolase activity which was almost double that of the leaves which had been provided with defined nutrients.

N-Carbamylputrescine is structurally the decarboxylation analogue of citrulline. Since it has been shown that neither *Streptococcus faecalis*[6] nor extracts of mammalian liver[7] degrade citrulline to ornithine in the absence of phosphate (or arsenate), it was of interest that phosphate does not appear to be essential for the activity of the N-carbamylputrescine amidohydrolase from barley. Potassium ions have apparently no inhibitory effect on the enzyme and it is probable that there is a greater absolute amount of the enzyme in potassium-deficient than in normal barley plants. Alternatively the difference in activity may be due to the presence of an enzyme activator in the potassium-deficient plants or of an enzyme inhibitor in the normal plants, both inhibitor and activator being non-dialysable.

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METHODS

Barley Material

Proctor barley seed was grown, nine seeds in 10 in. glazed pots, during the third week of December in a glasshouse maintained at about 15°. All nutrients were supplied in one application, one week after sowing. Each pot, containing approximately 15 kg washed silver sand, received; NH_4NO_3, 4 g; $(NH_4)_2HPO_4$, 0·05 g; $NH_4H_2PO_4$, 0·77 g; $CaCl_2.6H_2O$, 0·5 g; $MgSO_4.7H_2O$, 1·25 g; $MnSO_4.4H_2O$, 0·01 g. In addition 1·76 g K_2SO_4 was supplied for optimum potassium status and 0·11 g for deficiency. After 2 weeks the plants were thinned to three per pot, and the leaves sampled after 11 and 14 weeks.

For experiments with barley seedlings, Proctor barley was also grown in sand culture in a glasshouse. The first leaves were harvested when they were approximately 6 cm high (2–3 weeks in winter, 1–2 weeks in summer). Unless stated otherwise nutrients were supplied at optimal level. For later experiments in which leaf extracts were freeze-dried, tap water alone was given, since the amidohydrolase activity of seedlings grown in these conditions was greater than in those given nutrients at optimal level. Seedlings of other species were grown similarly, no nutrients being supplied.

Preparation of Extracts

In order to investigate the properties of the enzyme concentrated extracts were prepared. Approximately 100 g of the leaves of seedlings were frozen and macerated with 200 ml of 0·1 M Na_2HPO_4 in a blender which was cooled with iced water. The resulting slurry was filtered through nylon cloth and frozen at −15° overnight. After thawing and centrifuging the supernatant was cooled to 0°. Acetone (1 volume) at −15° was added with stirring and the precipitate was filtered on a Buchner funnel at −15°. Although it was found that most of the activity is recovered in the precipitate formed at between 33 and 50% (by vol.) acetone, fractional precipitation by acetone was not attempted and the entire precipitate was used. The precipitate was washed with acetone, dried and taken up in a small volume of 0·1 M phosphate buffer (pH 7·2). After dialysis against this buffer for 48 hr at 2° and centrifugation, the preparation was ready for use. One sample of the enzyme, concentrated in this way and tested after storage at −15° for 3 months was found to have lost only 30% of its initial activity. Since the activity of the enzyme preparation obtained by this method was rather variable, alternative procedures for concentrating the enzyme were investigated.

Freeze-drying was found to be satisfactory for this purpose. The leaves of seedlings which had been grown in tap-water without added nutrients were macerated in a cooled blender. The resulting extract was filtered through nylon cloth and dialysed exhaustively against distilled water. The powder obtained after freeze-drying was extracted with 0·1 M phosphate buffer (pH 7·2) and centrifuged. With this freeze-drying technique about 50–60% of the activity was regularly recovered, although a larger proportion (about 70%) of the total dry matter redissolved, and led to a corresponding loss in specific activity. From an extract of 100 g fresh weight of leaves which has been concentrated by the freeze-drying method, about 1 g soluble material was obtained, which was taken up in about 25 ml of buffer. Extracts prepared by this method were used to investigate the effect of temperature, inhibition, specificity and stoichiometry.

In experiments to determine the relative enzyme activities in normal and potassium-deficient barley leaves, the material was pre-frozen and samples (30 g) were macerated in 60 ml of 0·1 M Na_2HPO_4, centrifuged and dialysed against 0·1 M phosphate buffer (pH 7·2)

at 2° for 24 hr. The volumes of all the extracts increased by no more than 5% over this period. Samples were used to determine L-arginine carboxy-lyase and N-carbamylputrescine amidohydrolase.

Quantitative Estimation of Amidohydrolase Activity

This method was based on the estimation of ammonia released from N-carbamylputrescine in the presence of the enzyme. Conway microdiffusion units[8] were used for the enzyme incubation and the subsequent ammonia distillation, the ammonia being determined with Nessler's reagent.

The N-carbamylputrescine used as the substrate was prepared by the method of Smith and Garraway[3] but the chromatographic purification was omitted. The preparation therefore still contained some putrescine dihydrochloride (about 3%) and N,N'-dicarbamylputrescine (about 2%) but neither impurity affected the amidohydrolase and neither was hydrolysed by the enzyme preparations. These contaminants were detected on chromatograms run in an ascending solvent composed of *n*-butanol: ethyl methyl ketone: aq. ammonia (sp. gr. 0·88): water (5:3:1:1: by vol)[9] (the butanol-ketone solvent). In this solvent putrescine has R_f 0·6. N-carbamylputrescine R_f 0·5 and N,N'-dicarbamylputrescine R_f 0·4. Their proportions were estimated by comparing the intensity and size of their spots with those of known standards, using the ninhydrin reagent for putrescine, and *p*-dimethylaminobenzaldehyde·HCl reagent[10] for the N,N'-dicarbamylputrescine.

Up to 1 ml of the enzyme preparation in a final volume of 1 ml at pH 7·2 (unless otherwise stated) was placed in the outer compartment with 0·1 ml of toluene as antiseptic (volumes above 0·1 ml were found to impede the diffusion of ammonia). The central compartment contained 2 ml of 0·01 N HCl. N-Carbamylputrescine hydrochloride (0·25 ml of 25 mM) was added to the outer compartment. The units were incubated at 25°, normally for 24 hr, and saturated potassium carbonate (1 ml) was then added to the outer compartment. After 3 hr at room temperature (about 20°) the amount of ammonia was determined colorimetrically with Nessler's reagent. The activity of the amidohydrolase remained constant for 48 hr at 25° (Fig. 2). No difference in the rate of ammonia release could be detected on reducing the concentration of N-carbamylputrescine from 5 mM to 1 mM.

Toluene did not interfere in the determination but traces of acetone were found to give rise to considerable turbidity in the final mixture and it was thus necessary to dialyse acetone treated extracts exhaustively.

No hydrolysis of N-carbamylputrescine by the saturated potassium carbonate solution was detected, even after 36 hr at 20°. Ammonia formed from the extract in the absence of added substrate was routinely subtracted.

Putrescine, which reacts with Nessler's reagent, was found not to distil into the acid, even on incubating for 36 hr at 20° with the saturated potassium carbonate solution.

Semi-Quantitative Estimation of Amidohydrolase Activity

For the purpose of estimating amidohydrolase activity in preliminary experiments, a semi-quantitative method was employed. The leaves were frozen and 3 g extracted with 6 ml water in a cooled blender. After centrifuging, 0·5 ml samples were incubated with water (0·1 ml) or 25 mM N-carbamylputrescine (0·1 ml) under toluene. After 48 hr at 20°, 50 μl was

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[9] M. WOLFE, *Biochim. Biophys. Acta* **23**, 186 (1957).
[10] I. SMITH, *Nature*, **171**, 43 (1953).

removed from each incubate and chromatographed using butanol/ketone solvent. The amidohydrolase activity was assessed by determining the amount of putrescine formed, estimated by the intensity and size of spot after spraying with ninhydrin.

Estimation of Putrescine for Determination of Stoichiometry

Putrescine was separated by paper chromatography in the butanol-ketone solvent and after elution from the paper with 0·1 N NaOH was estimated using a modification of the method of Yemm and Cocking.[11] Duplicate estimations of the recovery of a known amount of putrescine dihydrochloride (50 μg) using this method gave values of 80 and 83%.

Phosphate and Potassium Estimation

Total phosphate was determined by the method of Kuttner and Lichtenstein;[12] potassium was estimated on the EEL flame photometer.

L-*Arginine Carboxy-Lyase Activity*

This was estimated quantitatively by the method of Smith[2] with the following modifications. The pH was increased from 6·3 to 7·2 and the temperature reduced from 30° to 25°. The leaf extract (3 ml) was incubated under these conditions with 0·75 ml of 25 mM L-arginine under 1 ml of toluene and 1 ml samples were withdrawn after 0, 2 and 4 hr. The agmatine was adsorbed to IRC 50 resin (H^+ form), and after washing with 4 N ammonia the agmatine was eluted with saturated ammonium carbonate solution. The ammonium carbonate was removed from the eluate by boiling, and agmatine determined by a modification of the method of Sakaguchi.[13] The amounts of agmatine produced during the initial 2 hr period were used in determining the arginine decarboxylase activities in these extracts.

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