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AGMATINE IMINOHYDROLASE IN MAIZE

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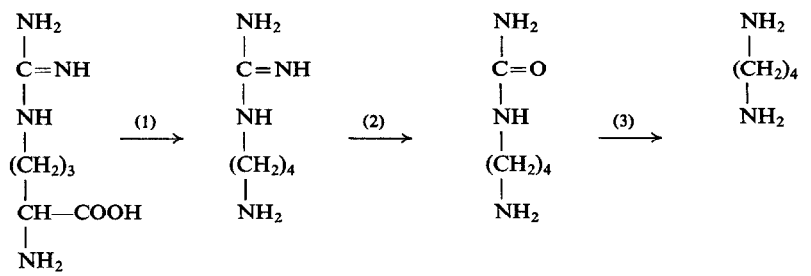
Abstract—Agmatine iminohydrolase, which converts agmatine to *N*-carbamylputrescine and ammonia, was found in extracts of the leaves and seed of maize, and in sunflower seedlings. The energy of activation of the maize leaf enzyme was 19·0 kcal/mole and the energy of activation for the denaturation of the enzyme was 27·8 kcal/mole. The optimum pH lay in the range 6·5–7·5. The enzyme was inhibited by *N,N'*diguanidinobutane and *p*-chloromercuribenzoate, and it was highly substrate specific. Potassium-deficient maize leaves were found to possess about thirty times more putrescine than normal maize leaves after 35 days' growth. However, at this age no significant effect of potassium nutrition on agmatine iminohydrolase activity could be demonstrated.

INTRODUCTION

ON THE basis of feeding experiments[1,2] it has been shown that the diamine putrescine is formed from arginine in barley leaves by the pathway:



Arginine — Agmatine — *N*-Carbamylputrescine — Putrescine

The enzymes arginine carboxy-lyase (1) and *N*-carbamylputrescine amidohydrolase (3) are active in barley leaf extracts and have been partially characterized[3,4] but agmatine iminohydrolase (2) could not be detected in extracts from barley leaves, even though feeding experiments indicated that this enzyme is active in the intact plant. A survey of higher plants showed that agmatine iminohydrolase is particularly active in extracts of maize leaves, and the present study was conducted in order to investigate some of its properties. Also, since arginine carboxy-lyase and *N*-carbamylputrescine amidohydrolase activities are increased in potassium-deficient barley leaves,[3,4] it was of particular interest to determine the effects of potassium deficiency on agmatine iminohydrolase.

[1] T. A. SMITH and F. J. RICHARDS, *Biochem. J.* **84**, 292 (1962).
[2] T. A. SMITH and J. L. GARRAWAY, *Phytochem.* **3**, 23 (1964).
[3] T. A. SMITH, *Phytochem.* **2**, 241 (1963).
[4] T. A. SMITH, *Phytochem.* **4**, 599 (1965).

RESULTS

Agmatine Iminohydrolase Activity in Maize and Other Plants

In plants other than maize, agmatine iminohydrolase activity was found in extracts prepared from the cotyledons of sunflower seedlings, the leaves of oat seedlings, and the outer leaves of mature cabbage and brussels sprouts plants. However, activity was obtained consistently only in maize and sunflower tissue. No activity could be detected in extracts of the leaves of barley, rye, wheat or pea seedlings or of leaves from mature apple trees, nor could any activity be found in extracts of maize roots. Maize seeds soaked for 18 hr in distilled water had an activity of 0·48 μmole/hr/g fresh weight. Duplicate extracts of the leaves of 14-day-old maize seedlings had an activity of 1·25 and 1·28 μmoles/hr/g fresh weight, and seeds taken from these plants had an activity of 0·59 μmole/hr/g fresh weight. The agmatine iminohydrolase activities of normal and potassium-deficient mature maize plants are given in Table 1.

TABLE 1. AGMATINE IMINOHYDROLASE ACTIVITY OF POTASSIUM-DEFICIENT AND NORMAL MAIZE LEAVES

	Agmatine iminohydrolase activity in μmoles/hr		
	Normal	Potassium deficient	Standard error
per g fresh weight	1·41	1·24	0·16
per g protein	100·4	97·6	10·3
per g protein nitrogen	1104	1151	68

The results are the means of two separate experiments from plants sown on 11 September and 21 October 1968, both being sampled in duplicate after 5 weeks' growth. The activities are expressed as μmoles/hr per g fresh weight, per g protein, or per g protein nitrogen.

Agmatine Degradation in vivo

Agmatine was fed to the cut shoots of 14-day-old maize seedlings for 24 hr, and ethanol extracts of the shoots were chromatographed in the butanol–ketone solvent. Large amounts of *N*-carbamylputrescine and smaller amounts of putrescine were found in the leaves which had been fed. By comparison, control shoots possessed no detectable *N*-carbamylputrescine and only traces of putrescine. No putrescine or *N*-carbamylputrescine could be found in the residual feeding solutions. The putrescine and agmatine concentrations in the shoots fed with L-arginine were similar to those in the controls, and no *N*-carbamylputrescine could be detected in extracts of shoots which had been fed with L-citrulline.

Agmatine, N-*carbamylputrescine and Putrescine in Maize Leaves*

In 2-week-old seedlings the putrescine content was about 0·25 μmole/g fresh weight and agmatine about 0·1 μmole/g fresh weight. No *N*-carbamylputrescine could be detected (lower limit 3 mμmoles/g fresh weight) in extracts of 3-week-old potassium-deficient or normal maize leaves. The putrescine content of potassium-deficient and normal maize leaves was determined at intervals after sowing, and the results are given in Table 2.

TABLE 2. PUTRESCINE CONTENT OF NORMAL AND POTASSIUM-DEFICIENT MAIZE LEAVES EXPRESSED AS μmoles/g FRESH WEIGHT

Sowing date	Age in days	Normal	Potassium deficient
1 November 1968	28	0·15, 0·13	1·01, 0·85
17 December 1968	35	0·04, 0·06	1·30, 1·79
11 September 1968	43	0·19, 0·29	0·48, 0·45
17 December 1968	49	0·37, 0·22	1·40, 1·09

Determinations were made in duplicate.

Properties of the Agmatine Iminohydrolase of Maize

Effect of temperature. From the results for the activity of the enzyme determined at 25° and 0° the energy of activation was calculated to be 19·0 (s.e. 0·2) kcal/mole. The regression on the proportion of activity lost in 15 min at temperatures varying from 35 to 55° was used to determine the energy of activation for the denaturation of the enzyme. This value was found to be 27·8 (s.e. 2·6) kcal/mole. The agmatine iminohydrolase activity in these extracts was reduced by 50 per cent on heating for 15 min at 43°. In extracts of the leaves of brussels sprouts this enzyme was apparently less stable, 50 per cent loss of activity being found after heating for 15 min at about 33°.

Inhibition. The results are given in Table 3. Arcain (*N,N'*diguanidinobutane), which is a potential analogue of agmatine, inhibited the enzyme activity by 50 per cent at 1 mM. The sensitivity of the enzyme to *p*-chloromercuribenzoate indicates that sulphydryl groups are important for activity. No evidence was obtained for product inhibition by *N*-carbamylputrescine, or by putrescine, which is the product of the enzymatic hydrolysis of *N*-carbamylputrescine.

TABLE 3. EFFECT OF INHIBITORS ON AGMATINE IMINOHYDROLASE ACTIVITY

Inhibitor	Final concentration (mM)	Percentage inhibition
p-Chloromercuribenzoate	0·075	50
Sodium cyanide	40	20
Semicarbazide	10	50
N-Carbamylputrescine	5	0
Putrescine	5	0
Arginine	5	0
Guanidine	5	0
Arcain	1	50

The enzyme preparation (0·5 ml) with the inhibitor (0·5 ml) was incubated for 18 hr with water or with agmatine (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 concentration required to produce 50 per cent inhibition was obtained by interpolation of the points plotted graphically, relating activity with a series of inhibitor concentrations.

Optimum pH. Samples of maize leaf extract (10 ml) were dialysed at 2° in the presence of toluene for 48 hr (two changes) against a series of buffers with pH ranging from 4·5 to 10·5, using phosphate buffers at pH 8·5 and below, and carbonate buffers above this value. The volume of the samples was measured after dialysis and the enzyme activity measurements were corrected for the dilution occurring during dialysis. Estimations of pH were made on the extracts after dialysis. The results are given in Fig. 1. The optimum was found to lie between pH 6·5 and 7·5.

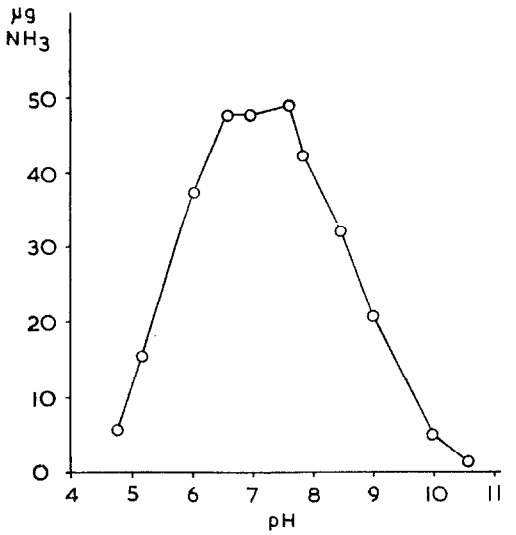


FIG. 1. THE EFFECT OF pH ON AGMATINE IMINOHYDROLASE ACTIVITY.
Activity is expressed in terms of μg of ammonia formed from agmatine during an 18-hr incubation period.

Specificity. No detectable activity, as determined by ammonia formation, was found with arcain, guanidine, arginine, or *N*-carbamylputrescine as the substrates of the maize leaf enzyme preparation. The limit of the detection of activity using these compounds was 1 per cent of the activity obtained with agmatine as substrate.

Subcellular Distribution. On separating the particulate fraction of mature maize leaves by extracting in 0·5 M sucrose and centrifugation at 15,000 *g* for 15 min, all the agmatine iminohydrolase activity was found to be associated with the supernatant phase.

Stoicheiometry. To investigate the stoicheiometry of the reaction, the iminohydrolase was permitted to hydrolyse a known amount of agmatine to completion, the resulting *N*-carbamylputrescine and ammonia estimated, and the molar proportions of these three components compared.

Samples (1 ml) of the enzyme preparation were placed in tubes and incubated with or without 0·25 ml of 25 mM agmatine. Agmatine was also incubated without the enzyme preparation in a separate tube. Each tube was replicated three times, 0·1 ml of toluene was added to each and the tubes were stoppered. After 24 hr at 25°, a 0·5 ml sample was with-

drawn from each for estimation of ammonia by the Conway method. Further samples (0·2 ml) were withdrawn, diluted to 5 ml, and 1 ml of the diluted incubate was used for the estimation of *N*-carbamylputrescine.

The recoveries (taking agmatine as 100 per cent) were ammonia 92, 93 and 92 per cent, and *N*-carbamylputrescine 101, 101 and 100 per cent. In a further experiment, in which only ammonia was determined, the recovery was 107, 107 and 106 per cent.

DISCUSSION

Agmatine has been found to be degraded in maize and other higher plants by an enzyme, agmatine iminohydrolase, with the formation of *N*-carbamylputrescine and ammonia. This reaction is analogous to that effected by arginine iminohydrolase, which converts arginine to citrulline, an enzyme already characterized in extracts of *Chlorella*.[5] Agmatine iminohydrolase activity has been found in micro-organisms.[6] These were also capable of degrading arcain by an analogous route, the enzyme for which is absent from maize leaf extracts.

Increased putrescine content is a characteristic of potassium deficiency in the leaves of many higher plants[3] and since two of the enzymes concerned in the formation of putrescine in higher plants have been shown to have increased activity in potassium-deficient barley leaves,[3, 4] the effect of potassium deficiency on agmatine iminohydrolase was of considerable interest.

Although there is evidence for an effect of both season and age on putrescine content of potassium-deficient maize leaves (Table 2) the major factor is potassium status. The leaves of the potassium-deficient maize plants contained 5·8 times the putrescine content of the normal leaves on average expressed in terms of the fresh weight. By comparison, potassium-deficient barley leaves contained on average about fifty times the amount of putrescine found in normal barley leaves.[7]

Determinations of agmatine iminohydrolase activity were made on leaves from maize plants which had been grown for 5 weeks. Plants of this age possessed about thirty times more putrescine in the potassium-deficient leaves than in the normal leaves. From the results given in Table 1, no significant effect of potassium deficiency could be detected on the agmatine iminohydrolase activity, when this was expressed per g fresh weight, per g protein, or per g protein nitrogen. Since, by comparison with barley, the increase of putrescine with reduced potassium status in maize is only small, a proportionately smaller change in the enzyme activities leading to putrescine formation might be expected. To establish a possible increase in activity of the agmatine iminohydrolase with potassium deficiency, it would be necessary to increase the replication of the enzyme determinations.

Since arginine carboxy-lyase and *N*-carbamylputrescine amidohydrolase activities are increased in barley leaves with potassium deficiency[3, 4] or reduced pH,[8] it is possible that the genes for the enzymes converting arginine to putrescine might lie within one potassium or pH controlled operon. In view of the difficulty of demonstrating an effect of potassium deficiency on the activity of agmatine iminohydrolase in maize leaves, and since neither arginine carboxy-lyase nor *N*-carbamylputrescine amidohydrolase can be detected easily in extracts of mature maize leaves, this plant may not be suitable for the further investigation of this possibility.

[5] J. SHAFER, JR., and J. F. THOMPSON, *Phytochem.* **7**, 391 (1968).
[6] F. LINNEWEH, *Hoppe-Seyler's Z. Physiol. Chem.* **205**, 126 (1932).
[7] C. HACKETT, C. SINCLAIR and F. J. RICHARDS, *Ann. Botany* **29**, 331 (1965).
[8] T. A. SMITH and C. SINCLAIR, *Ann. Botany* **31**, 103 (1967).

The *N*-carbamylputrescine, which accumulates in sugar cane tissue grown *in vitro*,[9] has been shown to arise from arginine. However, in this material, it is possible that the immediate precursor is citrulline, which may be decarboxylated to give *N*-carbamylputrescine. In the present work, no evidence could be found for the decarboxylation of citrulline in maize tissue.

EXPERIMENTAL

Plant Material

Maize seed (*Zea mays* L. variety Hy2 × 07, Illinois Seed Producers Association, Champaign, Illinois, U.S.A.) was grown in a greenhouse in 24-cm black polythene pots provided with drainage holes in the base, each containing 10 kg of washed Bedfordshire silver sand. Nine seeds were sown in each pot and these were watered daily with rain-water containing the following nutrients: $MgSO_4$, 1·5 mM; $CaCl_2$, 4·0 mM; $(NH_4)_2HPO_4$, 0·07 mM; $(NH_4)H_2PO_4$, 1·33 mM; NH_4NO_3, 6·0 mM. The solution also contained the micronutrients: Mn, Cu, Zn, B, Mo, Na, and Fe (as ferric EDTA). For normal growth the nutrient solution contained K_2SO_4 at 2 mM and for K deficiency this was omitted.

The following plants were also investigated: sunflower (*Helianthus annuus* L. var. Sutton's Giant Yellow); oats (*Avena sativa* L. var. Black Supreme); cabbage (*Brassica oleracea* L. var. *capitata* L.); brussels sprouts (*B. oleracea* var. *gemmifera* Zenker); barley (*Hordeum vulgare* L. var. Zephyr); rye (*Secale cereale* L.); wheat (*Triticum aestivum* var. Atle); pea (*Pisum sativum* L. var. Meteor); apple (*Malus sylvestris* Mill. var. Lord Lambourne). Seedlings were grown in sand in the greenhouse, and watered with the nutrient medium to which potassium had not been added. They were used after growing for 14 days. Mature plants were grown under field conditions.

Chromatography

TLC of the amines was effected on MN 300 cellulose (Camlab Ltd., Cambridge, U.K.) spread in layers 300μ thick on 20 × 20 cm glass plates. The solvent was butanol/ethyl methyl ketone/ammonia/water in the proportions 5:3:1:1 by vol.[10] After running, the plates were dried, heated to 100° for 15 min, and then cooled to room temperature. For the detection of putrescine (R_f 0·7) and *N*-carbamylputrescine (R_f 0·6), ninhydrin (0·2% in acetone) was used after spraying the plates with HOAc. Ehrlich's reagent was used as a more selective spray for *N*-carbamylputrescine, and agmatine (R_f 0·3) was detected with the Sakaguchi reagent.

Estimation of N-*Carbamylputrescine and Putrescine*

The diacetylmonoxime/*p*-aminodiphenylamine method of Hunninghake and Grisolia[11] was used for the quantitative determination of *N*-carbamylputrescine in the stoicheiometry experiment. A colour suppression of 5 per cent was found on estimating *N*-carbamylputrescine added to the plant extract, and standards were therefore prepared in the presence of this extract. The putrescine content of the maize leaves was estimated by gas chromatography.[12] The results presented have not been corrected for experimental losses.

Determination of Protein Nitrogen

Samples (5 ml) of the enzyme extracts were added to 5 ml of 10% trichloroacetic acid and after standing for 2 hr, the precipitate was collected by centrifuging. It was then washed in water, and dried in an oven at 100° for 18 hr and weighed. The *N* content of these samples was determined after digestion in H_2SO_4, using a Se catalyst. The ammonia was distilled in Conway units[13] and estimated with Nessler's reagent. The mean N content of the trichloroacetic acid precipitate was only 9 per cent of the dry weight, indicating that this precipitate was not composed entirely of protein.

Preparation of Extracts

Leaves from 6- to 8-week-old maize plants grown in a nutrient solution containing K at the level needed for normal growth were frozen for at least 24 hr and macerated with 0·1 M Na_2HPO_4 (two vols.) in an ice-cooled blender. The resulting slurry was filtered through muslin, dialysed for 24 hr at 2° against two changes of 0·1 M, pH 7·0, phosphate buffer, centrifuged at 3000 *g* for 5 min and frozen for storage. Extracts prepared in this way were used for investigating the properties of the enzyme. On storage at −10°, 46 per cent of the activity was found to be lost in 11 weeks. For the determination of the activity of the enzyme in the leaves,

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[10] M. WOLFE, *Biochim. Biophys. Acta* **23**, 186 (1957).
[11] D. HUNNINGHAKE and S. GRISOLIA, *Anal. Biochem.* **16**, 200 (1966).
[12] T. A. SMITH, *Anal. Biochem.*, in press.
[13] E. J. CONWAY, *Microdiffusion Analysis and Volumetric Error*, Crosby Lockwood, London (1962).

10 g of pre-frozen leaf tissue was macerated in 40 ml of 0·05 M Na_2HPO_4 and the extract centrifuged. Samples were taken for assay after dialysis against 0·1 M, pH 7·0, phosphate buffer.

Concentration of the Enzyme

Attempts were made to purify and concentrate the agmatine iminohydrolase. $(NH_4)_2SO_4$ at 50 per cent saturation precipitated the enzyme, but provided little purification, and the assay was complicated by the presence of the ammonium ion. Cold acetone (two vols.) also precipitated the enzyme without increasing the purity, and about 50 per cent of the activity was lost. About 70 per cent of the activity was recovered after freeze-drying, but again purification was not obtained. Some purification was achieved by adsorption of inert protein to alumina $C\gamma$ or $Ca_3(PO_4)_2$ gel, the purification factor varying between two and five times in terms of protein nitrogen.

Quantitative Estimation of Agmatine Iminohydrolase

This method was based on the estimation of ammonia released from agmatine by the enzyme. Conway units were used for the enzyme incubation and for the subsequent ammonia distillation, and the ammonia was determined with Nessler's reagent. The agmatine sulphate (R. N. Emanuel Ltd., London, U.K.) was used without further purification, since the m.p. and i.r. spectrum were correct, and no impurities could be demonstrated by TLC. The enzyme preparation (normally 1 ml at pH 7·0) was placed in the outer compartment of a Conway unit together with 0·25 ml of 25 mM agmatine and 0·1 ml of toluene. The central compartment contained 2 ml of 0·01 N HCl. The units were sealed and normally incubated for 18 hr at 25°. At the end of this period, 1 ml of saturated K_2CO_3 was added to the outer compartment and, after standing for a further 3 hr at room temperature, the ammonia which had distilled to the HCl in the inner compartment was determined with Nessler's reagent. Conway units with enzyme alone, or substrate alone, were included routinely.

The rate of ammonia formation from agmatine in the presence of maize leaf extract was constant on incubation for 48 hr at 25°. During this period no increase in ammonia could be detected in the extracts to which substrate had not been added. No significant difference in the rate of ammonia release could be detected on reducing the concentration of agmatine from 5 mM to 1 mM. The final concentration of substrate used in each assay was 5 mM, corresponding to a maximum of 106 μg of ammonia. No hydrolysis of the agmatine by the saturated K_2CO_3 solution was detected during the 3-hr period of distillation.

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