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L-ARGININE CARBOXY-LYASE OF HIGHER PLANTS AND ITS RELATION TO POTASSIUM NUTRITION

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Abstract—On a fresh weight basis the mean agmatine content was found to be eight times as high, and the mean L-arginine carboxy-lyase (arginine decarboxylase) activity twice as high in leaves of potassium-deficient barley plants as in leaves of normal barley plants. A range of other plant species was also shown to accumulate agmatine and putrescine when grown in conditions of potassium deficiency. The arginine decarboxylase of barley seedlings was concentrated and purified by freezing or acetone fractionation, followed in each case by ammonium sulphate precipitation. The pH optimum was broad, lying between 6·5 and 9·0 for a purified extract, and between pH 5·5 and 8·0 for crude extracts. In three purified preparations of the enzyme, the Michaelis constant was found to be 0·77, 0·74 and 0·45 mM respectively. The energy of activation was 13,200 (s.e. 950) cal/mol and the energy of activation for the denaturation of the enzyme was 26,800 (s.e. 1100) cal./mol. Out of seven L-amino acids the enzyme decarboxylated only L-arginine. The effects of some inhibitors were investigated. Preliminary attempts to resolve the arginine decarboxylase into apo- and co-enzyme were not successful.

INTRODUCTION

PREVIOUS work[1] suggested that the amines, putrescine and agmatine, which accumulate in the leaves of potassium-deficient barley plants, are derived from arginine. Evidence for this was provided by feeding experiments using excised leaves of barley seedlings. Agmatine and a smaller amount of putrescine were produced on supplying arginine, and with agmatine feeding the leaves accumulated putrescine. In potassium-deficient barley leaves, indirect evidence indicated that the increase in these amines probably resulted from an enhanced L-arginine carboxy-lyase (arginine decarboxylase) activity. The work now to be reported confirms this view. Attempts to purify the enzyme are also described, and the results of investigations into some of its properties are presented.

Although relatively large amounts of potassium are required for normal growth in plants, only few discoveries have been made which implicate this element in metabolic processes. Since arginine decarboxylase activity, at least in barley, is apparently influenced by potassium status, this enzyme is of considerable interest.

EXPERIMENTAL AND RESULTS

Agmatine and putrescine content of barley leaves in relation to potassium nutrition

Three varieties of barley (*Hordeum vulgare* L.), Proctor, HB 248/17/4 (mildew resistant), and Plumage Archer, were grown in sand culture in the open during the summer of 1962 with the ammonium nutrient solution of Richards and Berner.[2] Potassium was supplied in solution at two levels for each variety, corresponding to optimum and deficient conditions respectively, the "low-potassium" plants being provided with only one-sixteenth of the amount given to the "high potassium" plants.

The Proctor and Plumage Archer varieties were attacked by mildew (*Erysiphe graminis*) on the older leaves. However, the young leaves used in the experiments were almost

[1] T. A. SMITH and F. J. RICHARDS, *Biochem. J.* **84**, 292 (1962).
[2] F. J. RICHARDS and E. BERNER, *Ann. Botany, London* **18**, 15 (1954).

entirely free from infection. No infection was evident on the mildew resistant variety. At weekly intervals, starting from the eighth week after sowing, samples of the young leaves were analysed for agmatine content by the procedure described in the Methods section.

The results plotted in Fig. 1 represent the averages of two independent determinations. On a fresh weight basis the mean agmatine content of the deficient material was approximately eight times as high as that of the normal barley tissue. The potassium-deficient

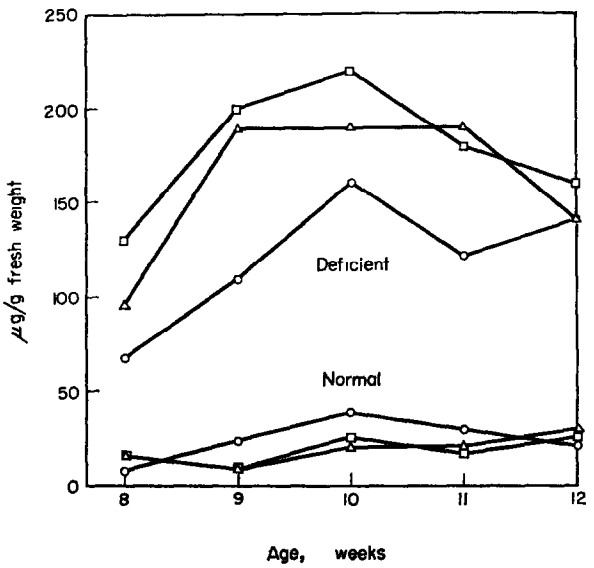

FIG. 1. AGMATINE PRESENT IN THE LEAVES OF THREE VARIETIES OF BARLEY, GROWN IN NORMAL AND POTASSIUM-DEFICIENT CONDITIONS.

Agmatine was estimated on duplicate samples of the leaf material, which were dried and extracted with boiling water. The agmatine was adsorbed to IRC-50 resin (H^+ form) and after washing the resin with 4N ammonia, the agmatine was eluted with saturated ammonium carbonate solution. The ammonium carbonate was removed by boiling and the agmatine determined with the modified Sakaguchi method described in the text.

Δ, Proctor. O, Mildew resistant. □, Plumage Archer.

mildew resistant barley leaves had less ($P < 0{\cdot}1\%$) agmatine than either of the other varieties grown in the same conditions. The mean content of agmatine in the deficient leaf material of the three varieties was higher ($P < 0{\cdot}1\%$) on the tenth week than on the eighth week, and also higher ($P = 0{\cdot}1$ to 1%) than on the twelfth week; the mean value for the twelfth week was apparently higher ($P = 0{\cdot}1$ to 1%) than that for the eighth week.

The results of other agmatine and putrescine determinations are given in Table 1. In contrast with the potassium-deficient mature barley plants, the ratio of agmatine to putrescine was found to exceed unity in the seedlings. At this stage the concentration of agmatine in the leaves of the mildew resistant variety was higher than in the leaves of either the Plumage Archer ($P = 1$ to 2%) or the Proctor ($P = 0{\cdot}1$ to 1%) seedlings. On a dry weight basis the mean agmatine level is similar to that found in the mature potassium-deficient barley leaves. In terms of fresh weight, however, the mean value was intermediate between those found for the normal and potassium-deficient mature plants (Fig. 1). (The dry weights of the 10-day old seedling leaves were 11·5, 11·2, and 9·9 per cent of the fresh weights for the Proctor, mildew resistant, and Plumage Archer barley respectively.)

TABLE 1. AGMATINE AND PUTRESCINE CONTENT OF BARLEY LEAVES

Amine	Age weeks	K. level	Barley variety‡		
			Proctor	Mildew resistant	Plumage Archer
Agmatine	11–12	normal	84	93	87
Agmatine	11–12	deficient	830	630	970
* Putrescine	11–12	normal		140	180
* Putrescine	11–12	deficient		8000	5500
Agmatine	10 days	normal	540	910	650
† Putrescine	10 days	normal	<50	50–250	<50

Agmatine was determined on duplicate samples by the procedure used for obtaining the results given in Fig. 1.

Putrescine (*) was determined quantitatively in ethanol extracts of the barley leaves. These were applied to a column of Dowex-50 resin (H+ form) and the resin was washed with 4N ammonia. The putrescine was then eluted with 5% ammonium carbonate. After removal of the ammonium carbonate by boiling, the putrescine content was determined by the ninhydrin method of Yemm and Cocking.[3] The results given are means of replicate samples (unpublished data, C. Sinclair).

Other putrescine estimations (†) were made by chromatography of crude extracts in the butanol–ketone solvent (see Methods section). The putrescine spots obtained from the plant extracts were compared visually with spots resulting from known amounts of putrescine applied to the chromatogram.

‡ The concentration is expressed in terms of μg amine/g dry weight of plant material.

The occurrence of putrescine and agmatine in other higher plants in relation to potassium status.

Rough estimates of the contents of putrescine and agmatine in the leaves of twelve other species of plants were made from paper chromatograms, the amine spots obtained from the plant extracts being compared visually with spots resulting from known amounts of the substances applied to the same chromatogram. The results are shown in Table 2.

TABLE 2. OCCURRENCE OF AGMATINE AND PUTRESCINE IN LEAVES OF PLANTS GROWN UNDER NORMAL AND POTASSIUM-DEFICIENT CONDITIONS

Plant species	Age in weeks	Putrescine		Agmatine	
		Normal	Deficient	Normal	Deficient
Tomato (*Lycopersicon esculentum*, Mill.)*	12	+	++	+	+
Flax (*Linum usitatissimum* L.)*	11	+	+	+	+
Cabbage (*Brassica oleracea*, L. var. *capitata*, L.)*	9	++	+++	+	++
Radish (*Raphanus sativus*, L.)*	6	++	+++	+	+++
Lettuce (*Lactuca sativa*, L.)*	8	++	++++	+	+
Groundsel (*Senecio vulgaris*, L.)*	5	+	+	+	+
Pea (*Pisum sativum*, L.)	7	+	++	+	+
Clover (*Trifolium pratense*, L.)	12	+	+++	+	+
Beet (*Beta vulgaris*, L.)	12	+	++	+	+
Oat (*Avena sativa*, L.)	9	+	+++	+	++
Wheat (*Triticum aestivum*, L.)	9	++	+++	+	++
Rye (*Secale cereale*, L.)	9	+	+++	+	++

Key to symbols:
+: <50 μg/g dry weight ++: >50 and <250 μg/g dry weight
+++: >250 and <1000 μg/g dry weight ++++: >1000 μg/g dry weight
* Grown in glasshouse.

[3] E. W. YEMM and E. C. COCKING, *Analyst* **80**, 209 (1955).

Most of the plants were found to accumulate putrescine in conditions of potassium deficiency; agmatine increased under such conditions only in members of the Cruciferae and Gramineae studied. In all cases the content of putrescine was as great or greater than the content of agmatine. In earlier work, potassium deficiency had been found to increase both agmatine and putrescine on a dry weight basis in flax, tomato and clover.

The arginine decarboxylase level of barley in relation to potassium nutrition

Arginine decarboxylase activity was estimated from the amount of agmatine produced in 2 hr, when arginine was incubated at 30° with leaf extracts which had been centrifuged and dialysed against 0·1 M phosphate buffer (pH 6·3). The results given in Table 3 were obtained with young leaves taken from the mature barley plants used for the agmatine determinations (Fig. 1). They are the means of two values given by different samples of plant material.

TABLE 3. L-ARGININE CARBOXY-LYASE ACTIVITY IN THE LEAVES OF THREE VARIETIES OF BARLEY GROWN UNDER NORMAL AND POTASSIUM-DEFICIENT CONDITIONS

Variety	Age in weeks	μg agmatine produced/hr/g fresh weight of barley leaf	
		Normal	Deficient
Proctor	6	62	172
	9	62	146
Mildew resistant	7	19	36
	10	25	72
Plumage Archer	8	78	101
	11	62	146

The activity was determined by measurement of the rate of agmatine production. Extracts were prepared by grinding 3 g fresh weight of the leaves with 6 ml 50 mM Na_2HPO_4, centrifuging, and dialysing against 0·1M phosphate buffer (pH 6·3). Samples (2 ml) of these enzyme solutions were incubated with 0·5 ml 25 mM L-arginine hydrochloride under toluene at 30°. Agmatine estimations were made on 0·5 ml samples taken after 0, 2 and 4 hr. Activity measurements were based on the increase in agmatine over the first 2 hr period.

The arginine decarboxylase was between two and five times as active ($P < 0·1\%$ for differences) in the potassium-deficient Proctor and Plumage Archer as in the potassium-deficient mildew resistant barley leaves. This may explain the relatively lower agmatine content of the potassium-deficient mildew resistant barley leaves (Fig. 1). For each variety, arginine decarboxylase activity in the leaves of the potassium-deficient plants was higher ($P < 0·1\%$) than in the leaves of plants of the same variety which had been grown with adequate potassium.

Arginine decarboxylase activity and distribution in seedlings

The means of two estimations of arginine decarboxylase activity in the leaves of 10-day old barley seedlings, as indicated by the method of agmatine production, were 220, 140, and 180 μg agmatine/hr per g fresh weight for Proctor, mildew resistant, and Plumage Archer respectively, the varietal differences not being statistically significant. In each variety, the relatively high activities in the seedlings appear to resemble more closely those

of the potassium-deficient mature barley plants than those of the mature plants grown with a normal potassium status.

The arginine decarboxylase activity in the Proctor seedlings was found predominantly in the leaves, none being detected in the roots, and only slight activity in the grain. On separating the particulate fraction of these leaves in 0·5 M sucrose by centrifuging at 16,000 × g for 15 min all the activity was found to be associated with the supernatant.

A survey for L-arginine carboxy-lyase activity in other members of the Gramineae indicated that seedlings of *Avena sativa* L., *Secale cereale* L., *Poa trivialis* L. and *Festuca rubra* L. subsp. *rubra* possessed the enzyme, but seedlings of *Triticum aestivum* L., *Zea mays* L., *Dactylis glomerata* L., *Lolium multiflorum* Lam., *Phleum pratense* L. and *Festuca arundinacea* Schreb. had no activity which could be detected by the method used.

Properties of the arginine decarboxylase of barley seedlings

pH optimum. Activity was again estimated by measuring the rate of agmatine production, since at pH levels above 6·5, carbon dioxide retention makes manometric determinations unreliable. The results, which are given in Fig. 2, were obtained with a prefrozen crude extract, and an extract purified by freezing followed by ammonium sulphate precipitation. The arginine decarboxylase of the crude extract possessed a pH optimum lying between 5·5 and 8·0. The pH optimum of the purified extract lay between 6·5 and 9·0. A similar shift in pH optimum on purification has been found in canine gastric lipase by

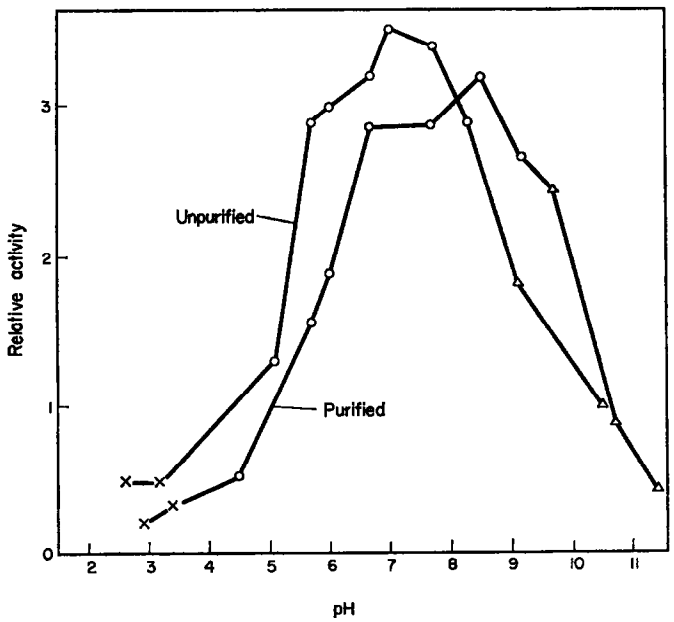

FIG. 2. THE EFFECT OF pH ON L-ARGININE CARBOXY-LYASE ACTIVITY.

Unpurified extracts, and extracts purified by the method of freezing, followed by ammonium sulphate precipitation, were dialysed exhaustively against distilled water at 2°, and 2 ml samples were added to 1 ml 25 mM L-arginine hydrochloride dissolved in water, together with 2 ml buffer. Arginine decarboxylase activity was measured by determining the amount of agmatine produced on incubation for 2 hr. at 30°. Measurements of pH were made at the end of the incubation period.

×—sodium formate/formic acid Final conc. 0·04 M., ○—sodium/potassium phosphate Final conc. 0·08 M., △—sodium carbonate/bicarbonate Final conc. 0·08M.

Willstätter, Haurowitz and Memmen.[4] In both preparations of the barley enzyme the pH optimum contrasts with that of the arginine decarboxylase of *Escherichia coli*, which Taylor and Gale[5] found to be most active at pH 5·25, only slight activity remaining at pH 6·0.

In most experiments in the present work arginine decarboxylase activity was measured at pH 6·3, this being within the pH range for optimum activity for crude extracts. In the case of purified preparations, activity determinations were made mainly by measuring carbon dioxide release in the Warburg apparatus. At pH values within the optimum for the activity of the purified extract, carbon dioxide retention makes such estimations unreliable. A pH of 6·3 was therefore also adopted for activity determinations with purified preparations, although this value was apparently below the optimal range.

Michaelis constant. The reaction velocity was determined for a range of substrate levels in 0·1 M phosphate buffer (pH 6·3) at 30°, using three preparations of arginine decarboxylase. The Michaelis constant was obtained by extrapolating the regression of the reciprocal of velocity on the reciprocal of substrate concentration.[6] Two preparations of the arginine decarboxylase, made using the purification scheme described in the Methods section, gave Km values of 0·77 and 0·74 mM (a third preparation, in which the protein in the initial macerate was precipitated by one volume of cold acetone, and then dialysed against 0·1 M phosphate buffer (pH 6·3), without further purification, gave a value of only 0·45 mM). In order to attain saturation of the enzyme, the activity determinations were made with 10 mM or 5 mM L-arginine. The bacterial enzyme studied by Taylor and Gale[5] had a Michaelis constant of 0·75 mM, similar to the values found for the barley enzyme.

Effect of temperature. The energy of activation over the range 15° to 45° in 0·1 M phosphate buffer (pH 6·3) was 13,200±950 cal/mol. After maintaining the enzyme solution for 5 min at a series of temperatures ranging from 70° to 90° in 0·1 M phosphate buffer (pH 6·3), activity determinations showed that 50 per cent loss occurred at 83°. The energy of activation over the range 70° to 90° for the denaturation of the arginine decarboxylase was 26,800±1,100 cal/mol.

Inhibition. The effect on the enzyme of a number of inhibitors was investigated, and the results are given in Table 4. All determinations were made in 0·1 M phosphate buffer

TABLE 4. EFFECTS OF INHIBITORS ON L-ARGININE CARBOXY-LYASE ACTIVITY

Inhibitor	Final concentration M	Percentage inhibition
Agmatine sulphate	0·02	<10
Ammonium sulphate *	0·6	50
Potassium cyanide	$1·6 \times 10^{-3}$	50
p-Chloromercuribenzoate	$1·6 \times 10^{-3}$	<2
Hydroxylamine hydrochloride *	7×10^{-5}	50
Semicarbazide	6×10^{-4}	50

After incubating 1 ml of the enzyme preparation at pH 6·3 with 1 ml of the inhibitor for 15 min at 30° in a Warburg flask, L-arginine hydrochloride (0·5 ml, 50 mM) was added from the side-arm and carbon dioxide output was measured manometrically. For inhibitors marked * the extract used was purified by the method of freezing, followed by ammonium sulphate precipitation; other inhibitors were tested on an extract prepared by acetone fractionation and ammonium sulphate precipitation. The concentration required to produce 50 per cent inhibition was obtained by interpolation.

[4] R. WILLSTÄTTER, F. HAUROWITZ and F. MEMMEN, *Z. physiol. Chem. Hoppe-Seyler's* **140**, 203 (1924).
[5] E. S. TAYLOR and E. F. GALE, *Biochem. J.* **39**, 52 (1945).
[6] H. LINEWEAVER and D. BURK, *J. Am. Chem. Soc.* **56**, 658 (1934).

(pH 6·3). The partially purified barley arginine decarboxylase was more stable to these inhibitors than the bacterial enzyme of Taylor and Gale.[5] However, it is possible that this difference could have resulted from protection afforded by impurities in the present enzyme preparation. For the barley enzyme, the absence of inhibition by 1·6 mM *p*-chloromercuribenzoate suggests that sulphydryl groups may not be important for activity. Sher and Mallette[7] have shown similarly that activity of the arginine decarboxylase of *E. coli* does not depend on free sulphydryl groups.

To investigate the possible effect of potassium ions on the enzyme, one sample was dialysed against phosphate buffer (pH 7·0) containing 50 m-equiv. of potassium per l., and another sample of the same preparation against 50 mM tris(hydroxymethyl)aminomethane–hydrochloric acid buffer (pH 7·0), in the absence of potassium. After dialysis for 24 hr no difference was found between the activities.

Specificity. The activities of two enzyme preparations, purified by the acetone and ammonium sulphate precipitation method, were determined with seven L-amino acids in 0·1 M phosphate buffer (pH 6·3). The assays were made in the Warburg apparatus, the amino acids being added from the side-arm to give a final concentration of 10 mM, except in the case of L-tyrosine which was at a final concentration of 2·5 mM.

One preparation decarboxylated L-arginine hydrochloride and L-glutamic acid at approximately the same rates, and slight activity was also found with L-tyrosine. The second preparation decarboxylated only L-arginine hydrochloride, no activity being found with the other compounds. The first preparation was apparently contaminated with L-glutamate 1-carboxy-lyase and L-tyrosine carboxy-lyase, both of which are known to occur in barley tissue[8,9], these being eliminated in the second preparation. In neither extract was activity found with L-ornithine hydrochloride, L-citrulline, L-lysine dihydrochloride or L-histidine.

A preliminary investigation of the stereospecificity of the enzyme using DL-arginine hydrochloride as substrate indicated that D-arginine is not attacked and, indeed, probably acts as a competitive inhibitor.

Effect of pyridoxal-5-phosphate. Taylor and Gale[5] have shown that pyridoxal-5-phosphate is the coenzyme for the arginine decarboxylase of *E. coli.* They were able to remove this coenzyme from the bacterial arginine decarboxylase preparations by precipitation with two volumes of saturated ammonium sulphate containing 10% ammonia (sp. gr. 0·88) by vol. The same procedure also inactivated the barley enzyme, but the activity was not restored with 0·16 mM pyridoxal-5-phosphate (Roche Products Ltd.).

A further sample of a barley leaf preparation was dialysed for 24 hr against distilled water at 2°. The arginine decarboxylase activity was found in the precipitate which formed during this process. On dissolving in 0·1 M phosphate buffer (pH 6·3) the enzyme actively decarboxylated L-arginine hydrochloride, and its activity was not stimulated significantly by pyridoxal-5-phosphate.

DISCUSSION

Decarboxylase activity has previously been observed in barley tissue for L-glutamic acid[8] and L-tyrosine.[9] The discovery of agmatine in barley suggested that L-arginine carboxylyase was present, and since the agmatine content was found to be increased with

[7] I. H. Sher and M. F. Mallette, *Arch. Biochem. Biophys.* **53**, 370 (1954).
[8] H. Beevers, *Biochem. J.* **48**, 132 (1951).
[9] G. Rabitzsch, *Planta med.* **7**, 268 (1959).

potassium deficiency, the arginine concentration being scarcely affected, it was anticipated that the activity of this enzyme might be greater in potassium-deficient plants.

The results show that although the enzyme has not previously been recognized in higher plants, it is indeed present in barley leaves, and moreover that its activity is significantly greater in potassium-deficient than in normal plants. The mechanism of its control *in vivo* by the potassium status is at present obscure. Potassium ions do not appear to have a direct effect on its activity, and it seems likely that there is a greater absolute amount of the enzyme in the leaves of the potassium-deficient plants.

As suggested by Coleman and Richards[10] the accumulation of the amines in potassium-deficient barley is probably homeostatic, the inorganic cation, potassium, being replaced by basic organic compounds. Putrescine was detected in a number of unrelated plant species when grown in potassium-deficient conditions and it is therefore probable that this mechanism is widespread in the plant kingdom.

In comparison with the bacterial arginine decarboxylase described by Taylor and Gale[5] the enzyme from barley leaves is less sensitive to pH and has a higher pH optimum; it is more stable to inhibitors; and also, using methods applied with success to the bacterial enzyme, the barley arginine decarboxylase could not be shown to require pyridoxal-5-phosphate as coenzyme; however, the Michaelis constant is similar for the enzymes from the two sources.

METHODS

Barley material

In order to study the effect of potassium status on the activity of arginine decarboxylase and on agmatine and putrescine in the three barley varieties, the seed was sown (nine seeds/10 in. glazed pot) during the first week of May and the nutrients were applied 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.

For experiments with barley seedlings the same three varieties were grown in a glasshouse during September and October 1962; the temperature varied from 16–26° during the days and from 9–14° in the nights. In experiments on both older plants and seedlings 1 g samples of the young leaves were collected at the desired growth stages; they were dried at 95° for 24 hr and extracted by boiling with two 25 ml portions of water. The volume of the combined extracts was reduced to approximately 2 ml by boiling.

Agmatine determination

The extracts were transferred to 13 ml tubes containing 0·5 g wet weight of the H^+ form of Amberlite IRC-50 resin (analytical grade), and the volume was made up to 10 ml with 4N ammonia. The tubes were then stoppered and shaken for 30 min, during which time the agmatine was adsorbed to the resin. The resin was then washed in three portions of 10 ml 4N ammonia for 30 min each. Saturated ammonium carbonate (10 ml) was added to the resin, and the tubes were shaken for a further 30 min, the agmatine now being desorbed. The ammonium carbonate solution, together with washings, was evaporated to 1 ml.

[10] R. G. COLEMAN and F. J. RICHARDS, *Ann. Botany, London* **20**, 393 (1956).

In order to convert the relatively insoluble agmatine carbonate to a more soluble salt, 0·1 ml N H_2SO_4 was added to each flask. The solutions were then cooled to room temperature and samples were transferred to colorimeter tubes.

The agmatine determination was made by a modification of the method of Sakaguchi.[11] The following solutions were used: 0·2% urea; 9N NaOH; 3% EDTA in 0·2 N NaOH; 0·05% 8-hydroxyquinoline potassium sulphate; and sodium hypobromite, made by dissolving 0·3 ml bromine in 100 ml 0·5N NaOH. The EDTA was included in order to dissolve the calcium hydroxide precipitate which appeared occasionally after adding the sodium hydroxide solution.

To each colorimeter tube, containing 5–50 μg of agmatine, 1 ml of each reagent was added in the order given, the sodium hypobromite being added forcibly. The solution was made up to 10 ml with water and the light absorption was determined 5 min later in the EEL Portable Colorimeter, using filter 623. The colour was stable for approx. 30 min. A standard, containing 50 μg agmatine, and a blank were included in each set of determinations.

In eight determinations the recovery from the resin of a known amount of agmatine was found to average 84 per cent with a standard error of 7·5 per cent. The experimental values were corrected for this loss.

Other Sakaguchi-positive substances, behaving similarly to agmatine on the IRC-50 resin, were not found in the barley extracts or incubates in amounts sufficient to give significant errors in the determination of agmatine. Arginine, although present, is not adsorbed to the resin in the presence of 4N ammonia.

Occurrence of putrescine and agmatine in other higher plants

Twelve other plant species were grown in sand in the normal and low-potassium media used for the barley. After 5 to 12 weeks' growth the leaves were harvested and dried at 95° for 24 hr. Extracts were prepared by boiling 2 g in water, centrifuging and making up to 10 ml. Portions (100 μl) of these were chromatographed on 20 cm squares of Whatman No. 1 paper, using an ascending solvent composed of n–butanol–ethyl methyl ketone–aq. ammonia (sp. gr. 0·88)–water (5 : 3 : 1 : 1 by vol.),[12] i.e. the butanol–ketone solvent. Agmatine (Rf 0·2) was detected by the Sakaguchi reagent[13] and putrescine (R_f 0·6) with ninhydrin. The agmatine and putrescine concentrations were determined semi-quantitatively from the sizes and intensities of the spots.

Determination of L-*carboxy-lyase activity.*

(*a*) *By agmatine production.* Freshly-cut barley leaves (3 g) were ground in acid-washed sand with 6 ml 50 mM Na_2HPO_4. After centrifuging, a 2 ml sample of the supernatant was dialysed against 0·1 M phosphate buffer (pH 6·3) for 20 hr at 2°. During this period the volume increased by no more than 5 per cent. Toluene (1 ml) and 25 mM L-arginine hydrocloride in 0·5 ml 0·1 M phosphate buffer (pH 6·3) were added, and a 0·5 ml asmple of the aq. layer was transferred to a 13 ml tube containing 4N ammonia (9ml) and 0·5 g IRC-50 resin (H^+ form). The remainder was incubated at 30° and further 0·5 ml samples were withdrawn after 2 and 4 hr, these being similarly transferred to tubes containing 4N ammonia and resin. The agmatine adsorbed to the resin from the ammoniacal solutions was estimated

[11] S. SAKAGUCHI, *J. Biochem., Tokyo* **37**, 231 (1950).
[12] M. WOLFE, *Biochim. biophys. Acta* **23**, 186 (1957).
[13] J. B. JEPSON and I. SMITH, *Nature, Lond.* **172**, 1100 (1953).

as described above. The addition of EDTA was found to be unnecessary in this assay.

In unpurified extracts of leaves of both seedlings and mature plants, the apparent activity as estimated by agmatine production during 4 hr was approximately 80 per cent of that obtainable after 2 hr, probably due to enzyme denaturation. It was anticipated, therefore, that the 2 hr determination would also underestimate the initial activity, though to a lesser extent. However, since the activity could not be shown to diminish significantly during the initial 2 hr period of assay (Fig. 3), the amounts of agmatine produced during that time were taken to represent the arginine decarboxylase activities in these extracts.

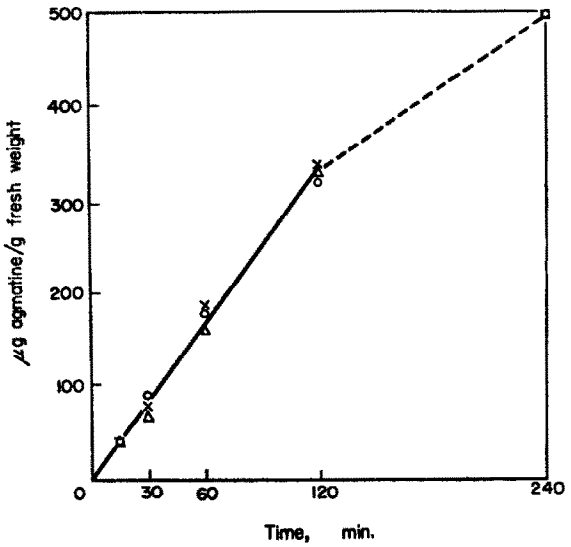

FIG. 3. RELATION BETWEEN TIME AND TOTAL AMOUNT OF AGMATINE FORMED BY L-ARGININE CARBOXY-LYASE.

Three tubes, each containing 4 ml crude barley leaf extract, buffered at pH 6·3, 1 ml 25 mM L-arginine hydrochloride, and 1 ml toluene were incubated at 30°. Samples were taken at intervals and analysed for agmatine. The results have been adjusted to agree at the mean of the values obtained after 4 hr. The regression line is given for the results obtained during the first 2 hr period.

The toluene, which was added as an antiseptic, had no apparent effect on the activity of the enzyme. Possible degradation of agmatine during the assay was investigated by paper chromatography. Using the butanol-ketone solvent it was found that less than 2 per cent of the agmatine was converted to mono-carbamyl putrescine (R_f 0·5) or putrescine. Although amine oxidase is quite active in legumes, it has not as yet been detected in barley.[14] Paper chromatography of barley incubates in a solvent composed of n-butanol–acetic acid–water (4 : 1 : 5 by vol.) did not reveal the presence of a spot (R_f 0·7) corresponding to the major Sakaguchi-positive substance (probably γ-guanidinobutyraldehyde) produced by incubating a pea seedling extract for 2 days at 20° with 10 mM agmatine at pH 7·6 under toluene.

(b) Manometrically

The activities of concentrated preparations of arginine decarboxylase were determined by measuring the rates of CO_2 evolution using the Warburg apparatus. The main compart-

[14] R. H. KENTEN and P. J. G. MANN, *Biochem. J.* **50**, 360 (1951).

ment of each flask contained a 2 ml sample of the enzyme in 0·1 M phosphate buffer (pH 6·3), this volume representing the extract from approximately 8 g fresh weight of barley seedling leaves. After equilibration at 30°, 0·5 ml of 50 mM L-arginine hydrochloride in pH 6·3 buffer was added from the side-arm, and readings were taken at 5 or 10 min intervals for 1 hr. Flasks with potassium hydroxide solution in the centre well showed no increase in volume. The data given for activity at pH 6·3 are corrected for CO_2 retention by the liquid, which accounted for approx 10 per cent of that produced.

Chromatography of the fluids in the flasks indicated that less than 2 per cent of the agmatine produced was converted into other ninhydrin- or Sakaguchi-positive products. Three experiments designed to investigate the stoichiometry between CO_2 production and arginine supplied gave CO_2 recoveries equivalent to 86, 98, and 98 per cent. Two comparisons between the amounts of CO_2 evolved and agmatine produced gave recoveries equivalent to 96 and 109 per cent.

Arginine decarboxylase purification

Proctor barley was grown in a glasshouse for 2–3 weeks in the winter, or 1–2 weeks in the summer, in sand, supplied with full nutrient solution. Approximately 100 g of the leaves were macerated with 2 vol 50 mM Na_2HPO_4 in a cooled blender and the slurry filtered through nylon. After cooling to 0°, 0·5 vol. of acetone at −5° was added with stirring and the green precipitate which formed was removed by centrifuging at 4000 × g for 5 min. A further 0·5 vol of cold acetone was added to the supernatant and the precipitate obtained was dissolved in 50 ml 0·1M phosphate buffer (pH 6·3). The precipitate which formed in this solution on adding 2 vol. saturated ammonium sulphate (adjusted to neutrality with ammonia), was collected by centrifuging at 16,000 × g for 15 min and dispersed in 25 ml 0·1 M phosphate buffer (pH 6·3). After dialysis against 0·1 M phosphate buffer (pH 6·3) the solution was centrifuged to remove insoluble material and stored at

TABLE 5. PURIFICATION OF L-ARGININE CARBOXY-LYASE FROM BARLEY

	Non-dialysable material μg/ml	Activity (original extract = 100)	Purification factor
First preparation:			
original extract	8·8	100	
second acetone ppt	2·0	42	1·9
ammonium sulphate ppt	0·28	37	11·6
Second preparation:			
original extract	10·6	100	
second acetone ppt	0·44	33	8·0
ammonium sulphate ppt	0·38	23	6·4

The leaves of 10-day old Proctor barley seedlings were macerated with 2 vol. 50 mM Na_2HPO_4 and the extract treated with 0·5 vol. cold acetone. The precipitate was centrifuged down and discarded. A further 0·5 vol. cold acetone was added and the precipitate centrifuged and redissolved in buffer (second acetone ppt). Two volumes saturated ammonium sulphate solution were added and the precipitate centrifuged and redissolved in buffer. Samples of the original extract, the second acetone precipitate, and the ammonium sulphate precipitate, were dialysed exhaustively against distilled water at 2°, dried at 90° and weighed. Further samples were used for arginine decarboxylase assay, by the method of measuring agmatine production described in the text.

−5° until required. Under these conditions arginine decarboxylase activity showed no loss for periods up to 12 weeks.

In two such experiments (Table 5), the arginine decarboxylase in the barley leaf macerates was found to be purified approximately twelve- and six-fold on the basis of non-dialysable dry matter, 37 and 23 per cent of the initial activity present in the macerates being recovered. Most of the loss in activity occurred during the acetone precipitation. In other preparations involving acetone fractionation, recovery of the arginine decarboxylase present in the seedlings, as determined by carbon dioxide evolution in the Warburg apparatus was very variable, being 60 per cent on one occasion, but more usually being below 30 per cent.

In later work (the determination of the pH optimum and the effect of certain inhibitors) the acetone fractionation step was replaced by a method in which the crude extract was frozen for 24 hr at −10°. Much of the inactive protein was found to be denatured on thawing and could be removed by centrifuging. Ammonium sulphate was then dissolved in the supernatant at the rate of 50 g/100 ml. The resulting precipitate was centrifuged down, dissolved in buffer and dialysed against 0·1 M phosphate buffer (pH 6·3) prior to use. It appears that this method may give better yields than that involving acetone fractionation.

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