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THE OXIDATIVE DECARBOXYLATION OF ORNITHINE BY EXTRACTS OF HIGHER PLANTS

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Key Word Index—*Triticum aestivum*; *Avena sativa*; Gramineae; wheat; oats; ornithine decarboxylase; monooxygenase; pyridoxal phosphate; 4-aminobutanamide; putrescine; manganese.

Abstract—Extracts of wheat leaves catalysed the oxidative decarboxylation of ornithine in the presence of pyridoxal phosphate, with the production of 4-aminobutanamide and 4-aminobutyric acid. In similar experiments, putrescine was not converted to the amide or acid suggesting it is not an intermediate. The optimum concentration of pyridoxal phosphate was 1 mM, and activity declined on increasing the concentration to 10 mM. The K_m for ornithine was 10 mM. The activity was found principally in the particulate fraction. Both the particulate and the soluble fractions showed two pH optima, at *ca* pH 9 and 10.5. Two pH optima were also found for ornithine decarboxylation in the particulate fraction of oat leaves: one at pH 6.5–7 and another at pH 9–9.5. Activity was lost on heating for 1 hr at 100°, though discontinuities in the thermal denaturation curve indicated a heterogeneous system. D- and L-Ornithine and L-lysine acted as substrates. 2,3-Diaminopropionic acid and 2,4-diaminobutyric acid were oxidized to ninhydrin-positive compounds which were presumably respectively 2-aminoacetamide and 3-aminopropanamide. Aminoguanidine (0.25 mM) inhibited ornithine decarboxylation by 30–40%, while difluoromethylornithine at 1 mM showed no inhibition. Dithiothreitol, dithioerythritol, cysteine, mercaptoethanol and glutathione inhibited the oxidation at 1 and 5 mM. Particulate preparations of barley, sugar beet and rape leaves also oxidized ornithine to 4-aminobutanamide and 4-aminobutyric acid. Gel filtration on Sephadex G-25 showed that the M_r of the major component causing oxidative decarboxylation of ornithine present in the wheat leaf supernatant was *ca* 4000. However another less active fraction was found to have a M_r of > 5000. Mn^{2+} ions at μM concentrations catalyse a similar decarboxylation of ornithine in the presence of pyridoxal phosphate with the production of 4-aminobutanamide, and may be responsible for the activity in the plant extracts. Mn^{2+} ions are known to associate with nucleic acids and may therefore simulate a complex catalyst of relatively large M_r. At high pH, Mn^{2+} ions exist as the unstable hydroxide which can be readily oxidized, explaining the apparent thermal denaturation, which appears to be accelerated in the presence of the plant extracts.

INTRODUCTION

Two major pathways of putrescine biosynthesis occur in plants [1]. Arginine may be decarboxylated to agmatine which is then hydrolysed to putrescine via *N*-carbamoylputrescine. Alternatively ornithine may be directly decarboxylated to putrescine by the enzyme ornithine decarboxylase (ODC), a route which is well established in animal tissues [2].

$$\underset{\text{Ornithine}}{NH_2CH(COOH)CH_2CH_2CH_2NH_2} \rightarrow CO_2 + \underset{\text{Putrescine}}{NH_2CH_2CH_2CH_2CH_2NH_2}$$

The putrescine which is formed by ODC has been identified on incubating ornithine with extracts of tobacco [3–5]. The hybridization of tobacco RNA with mammalian ODC cDNA also confirms the presence of a true ODC in tobacco [6]. An arginine decarboxylase from *Cucumis* is also a latent ODC, showing the latter activity only on extensive purification [7]. Putrescine was also found on incubating crude extracts of *Avena*, *Hordeum*, *Zea*, *Pisum*, *Lactuca* and *Heliotropium* with ornithine, though in much smaller amounts than would be expected on the basis of carbon dioxide release [8]. Many studies on ODC have depended on the release of $^{14}CO_2$ from [1-^{14}C]-ornithine, and it has been assumed that this is a measure of ODC activity leading to putrescine formation. Birecka *et al.* [9] indicated that in some plants the product of ornithine decarboxylation may not be putrescine, and they suggested that an oxidative process may be involved in the decarboxylation. They also drew attention to the possibility that the putrescine, if it was not found, could have been eliminated by amine oxidases present in the crude extracts, since these are widely distributed in higher plants [10]. In the present work it is shown that ornithine is oxidatively decarboxylated by extracts from a wide range of higher plants in the presence of pyridoxal phosphate to produce 4-aminobutanamide. This activity may be attributed to the presence of Mn^{2+} in the plant material.

$$\underset{\text{Ornithine}}{NH_2CHCH_2CH_2CH_2NH_2} + O_2 \rightarrow CO_2 + H_2O + \underset{\text{4-Aminobutanamide}}{NH_2CCH_2CH_2CH_2NH_2}$$

RESULTS AND DISCUSSION

Identification of the decarboxylation products

The particulate fraction from leaves of dark-grown wheat seedlings 14–21-day-old was incubated with ornithine and pyridoxal phosphate (see Experimental). Electrophoresis of the extract showed an unknown ninhydrin-positive spot running slightly faster than ornithine, together with a spot identified from its mobility as 4-aminobutyric acid. On elution from the paper with water, and acid hydrolysis (11 M HCl, 100°, 1 hr), the unknown product was entirely converted to 4-aminobutyric acid. It therefore seemed likely that this unknown was 4-aminobutanamide. The presumed amide band obtained on preparative high voltage electrophoresis was eluted from the paper with water and passed through a column of IRA-400(OH^-) anion exchange resin which adsorbed the residual acetic acid from the buffer, together with contaminating ornithine. After concentration, the presumed amide, which was 95% pure on the basis of the ninhydrin reagent, was shown to be indistinguishable from synthetic 4-aminobutanamide by paper chromatography in three different solvents, and by paper electrophoresis with the ninhydrin reagent and the hydroxamic acid test for detection. The amide was also detected in incubates containing ornithine, pyridoxal phosphate and wheat leaf or root supernatants. No putrescine or pyrroline could be detected with the ninhydrin reagent. The amide was formed as rapidly from D-ornithine as from L-ornithine.

Incubation of the homologous diamino acids 2,3-diaminopropionic acid and 2,4 diaminobutyric acid gave products at positions expected for 2-aminoacetamide and 3-aminopropanamide on electrophoresis. L-Lysine showed a spot corresponding to 5-aminovaleric acid, though in this electrophoretic system the corresponding amide would probably coincide with the parent amino acid and would not be visible. No degradation products of arginine or citrulline could be detected on incubating these amino acids in the system, although it is possible that these products, if formed, could again coincide with the substrate spots on electrophoresis. However, absence of these products would be consistent with the finding by Smith [11] and Birecka *et al.* [8] that in many plants agmatine is formed in stoichiometric proportions with CO_2 release in arginine decarboxylase assays. Moreover, putrescine was not degraded by the wheat leaf extracts in the presence of pyridoxal phosphate in the present system, suggesting that this diamine is not formed as an intermediate in the oxidative decarboxylation of ornithine.

At least some of the pyridoxal phosphate was converted to pyridoxamine phosphate during the oxidative decarboxylation of ornithine. This could be associated with the conversion of the amide to 4-aminobutyric acid.

Activity of ornithine decarboxylation in wheat leaves (Table 1)

In all extracts, most activity was found in the particulate fraction, in which the activity was always greater at pH 10.3 than at pH 8.9 (see also Fig. 1). In the supernatant the activity at the higher pH was greater only in the seven-day-old plants. Activity in crude (unfractionated) extracts was about 60% of that in the supernatant and particulate fractions when assayed separately, indicating that the activity in the crude extracts is inhibited.

Table 1. Ornithine decarboxylation in extracts of the leaves of wheat plants (c.v. Timmo) grown in the dark or light at 23° in nutrient A (the activity was assayed at pH 9 and 10.5)

			Supernatant		Particulate	
Conditions	Age (weeks)	Extraction pH	Actual pH	Activity (pkat/g fr. wt)	Actual pH	Activity (pkat/g fr. wt)
Light	1	9	8.8	273	8.9	356
		10.5	10.0	281	10.3	690
	2	9.0	8.9	245	8.9	590
		10.5	10.0	194	10.3	840
	3	9.0	8.8	224	8.9	821
		10.5	10.0	198	10.3	919
Dark	1	9.0	8.8	265	8.9	537
		10.5	10.0	315	10.3	719
	2	9.0	8.8	198	8.9	502
		10.5	9.8	141	10.3	656

Table 2. Ornithine decarboxylation at pH 9 in extracts of the leaves of cereals and of dicotyledonous plants

Name	Species	Variety	Age (days)	Supernatant (pkat/g fr. wt)	Precipitate (pkat/g fr. wt)
Wheat	*Triticum aestivum l.*	Timmo	7	331 (331)	576
Durum	*Triticum durum l.*	Valdor	7	314 (296)	602
Triticale	*Triticale*	Torrs	7	175 (129)	417
Rye	*Secale cereale l.*	Rhiedor	7	41.2	555
Oats	*Avena sativa l.*	Trafalgar	7	457	538
Barley	*Hordeum vulgare l.*	Golden Promise	7	333 (253)	426
Maize	*Zea mays l.*	LG 11	7	236 (159)	197
Sugar beet*	*Beta vulgaris l.*	Hilleshog Samson	42	52	1490
Rape*	*Brassica napus l.*	Rafal	35	3	1080
Tomato*	*Lycopersicon esculentum* Mill.	Ailsa Craig	49	19	57
Tobacco*	*Nicotiana tabacum l.*	White Burley	91	229	133
Pea†	*Pisum sativum l.*	Meteor	8	38	59

The cereals were grown in the light in nutrient B. Activity of the supernatant after 4 days storage at 4° is shown in brackets. *Grown in a greenhouse; †grown in darkness at 23° in nutrient A.

Ornithine decarboxylation in various species of cereals (Table 2)

In comparison with Table 1, the greater activity for the supernatant and particulate fractions in the wheat may be related to the different nutritional regimes in the two experiments. In all species about 20% of the activity in the particles was found in the supernatant after standing at 4° for 4 days, though a small portion of this may be attributed to supernatant remaining in the particulate fraction. Attempts were made to increase the rate of release of the activity from the particulate fraction. Sodium chloride (M) did not cause significant release, and inhibited activity to about 80%. Heating at 50° for 1 hr and treatment with Triton X-100 or Tween 20 were unsuccessful. However, ultrasonication for 10, 20 and 60 min increased the supernatant activity × 4.2, × 4.7 and × 5.6 respectively. The supernatant activity could be concentrated by freeze-drying or by rotary film evaporation below 40°.

Activity was found in ungerminated wheat seed (supernatant 216; precipitate 458 pkat/g fr. wt) and seed germinated for 3 days (supernatant 51; precipitate 816 pkat/g fr. wt). In roots of 17-day-old seedlings, activity in the supernatant and precipitate was respectively 490 and 689 pkat/g fr. wt.

Products of ornithine decarboxylation in other plants

Incubates of particulate fractions obtained from barley, sugar beet, rape, pea, tomato and tobacco leaves with ornithine and pyridoxal phosphate were also subjected to electrophoresis. 4-Aminobutanamide was found in the incubates of the barley, beet and rape, but not pea, tomato or tobacco. 4-Aminobutyric acid was found in incubates of barley, beet, rape and tomato. Putrescine could not be detected in any of the incubates. No 4-aminobutanamide, 4-aminobutyric acid or putrescine could be detected in the minus-ornithine or minus-extract controls. The decarboxylation activities expressed in pkat/g fr. wt for the species are given in Table 2.

Optimum pH

Two peaks of activity were found in the supernatant and particulate preparations from light-grown 14-day-old wheat leaves at *ca* pH 9 and *ca* pH 10.3 (Fig. 1). Similarly, two pH optima were found in the particulate preparations from oat leaves, one at pH 6.5–7 and another pH 9–9.5 (Fig. 2). In the wheat extracts the relative activities at the two pH optima were variable, and in some extracts the peaks of activity appeared to merge.

The variability of the activity is also demonstrated in Fig. 2 where in separate oat leaf extracts activity at pH 6.5–7 ranges from being barely significant, to being the largest of the two peaks. Large scale incubation showed that the products of the wheat leaf particulate preparations were identical at both pH optima. A similar dual pH

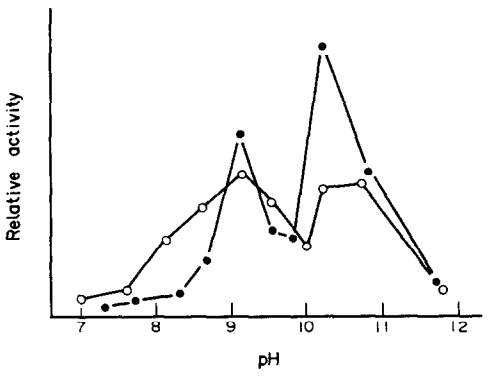

Fig. 1. Effect of pH on ornithine decarboxylation of the supernatant (open circles) and particulate (closed circles) fractions from leaves of light-grown wheat seedlings (2 weeks old).

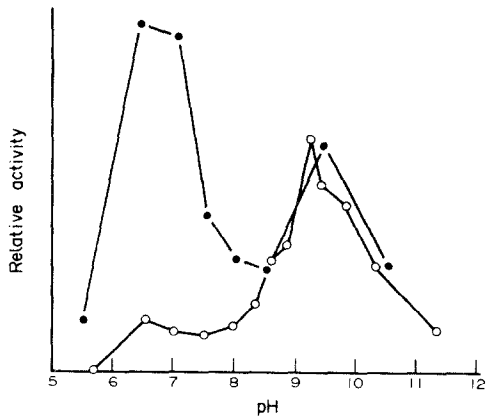

Fig. 2. Effect of pH on ornithine decarboxylation of the particulate fraction from light-grown oat leaves (2 weaks old). Open and closed circles represent two different experiments.

optimum with a single activity has been found for urease [12].

Effect of potential inhibitors (Table 3)

Aminoguanidine (AG) has been investigated as a possible inhibitor of amine oxidases in earlier attempts to reduce possible enzymic oxidation of putrescine during ODC assays [9]. Indeed AG was shown to reduce the decarboxylation of ornithine in their experiments. In the present work AG inhibited decarboxylation of ornithine by 50% at *ca* 0.3 mM.

Difluoromethylornithine (DFMO) was completely without effect on either the supernatant or particulate fractions at 1 mM. Birecka *et al.* [8] found that DFMO significantly reduced the production of putrescine in extracts of various plants, though it had little effect on the rate of ornithine decarboxylation. Moreover, DFMO had no effect on ornithine decarboxylation in oat leaf extracts [13]. However, in tobacco, DFMO inhibited cell division causing cell enlargement [14]. It also retarded tobacco fruit development and this could be reversed on application of putrescine [15]. Moreover, DFMO inhibited growth of *Helianthus* explants grown *in vitro* [16]. This suggests that a true ODC is present in these plants.

Inhibition by EDTA of decarboxylation (4.5% of the particulate activity and 29% of the supernatant activity remaining with 100 μM EDTA) suggests that a metal ion is involved in the reaction.

Effect of pyridoxal phosphate (Table 4)

Maximal activity in the particulate preparation of wheat was found with 1 mM pyridoxal phosphate. Increasing the concentration to 10 mM resulted in a loss of *ca* 50% of the activity. A small but significant decarboxylation was found in the absence of pyridoxal phosphate. Activity shown by the supernatant fraction was also increased about 50-fold on adding 1 mM pyridoxal phosphate. For the supernatant (pH 9) of light-grown oat leaves (8 days old) activity with 200 μM pyridoxal phosphate was about half of that at 1 mM.

Aerobic nature of the decarboxylation

On deoxygenation by purging with nitrogen every 30 min for the 2 hr incubation period during assay, only 12% of the particulate activity and 1.2% of the supernatant activity could be detected, indicating that oxygen is required for the decarboxylation. Purging assay tubes with oxygen increased activity by only 5%.

Effect of sulphydryls (Table 5)

All sulphydryls tested were inhibitory. This is compatible with the decarboxylation being dependent on an oxidation.

K_m *for ornithine*

The K_ms for ornithine decarboxylation by the light-grown leaf particulate preparation of wheat and oat were 10 and 5 mM respectively. The ornithine concentration (5 mM) used in the assay was therefore suboptimal.

Effect of temperature

Samples of the particulate fraction at pH 9 from both oat and wheat leaves were heated for 1 hr at various

Table 3. Effect of various compounds on ornithine decarboxylation (% of activity remaining) in the supernatant and particulate fractions of extracts of light-grown wheat leaves (15 days old)

Compound	Concentration (mM)	Supernatant	Precipitate
Aminoguanidine	0.01	96	98
	0.1	60	71
	0.25	60	71
	1.0	13	11
	2.5	4	2
Difluoromethyl ornithine	1.0	101	95
D-Ornithine	1.0	120	123

The inhibitors were pre-incubated with the plant extract and pyridoxal phosphate for 45 min before initiating the assay by adding substrate.

Table 4. Effect of pyridoxal phosphate on ornithine decarboxylation (relative to 1 mM pyridoxal phosphate = 100%) by 17-day-old dark grown wheat leaf particulate preparations

Pyridoxal-phosphate concentration	Dark-grown	Light-grown
0.0	0.8	2.4
50 μM	1.4	6.0
1 mM	100	100
2 mM	93.8	—
5 mM	84.4	—
10 mM	42.9	54.6

Table 5. Effect of sulphydryls at 1 or 5 mM final concentration on ornithine decarboxylation (% activity remaining) in the particulate fraction from wheat and oats

Sulphydryl	Oats 1 mM	Oats 5 mM	Wheat 1 mM	Wheat 5 mM
Dithiothreitol	77	2.5	47	21
Dithioerythritol	87	0.4	68	43
Cysteine	47	0	58	26
Mercaptoethanol	16	0	—	—
Glutathione	86	4	—	—

The pH for the assay was 6.8 for the oats and 9.5 for the wheat.
—Not determined.

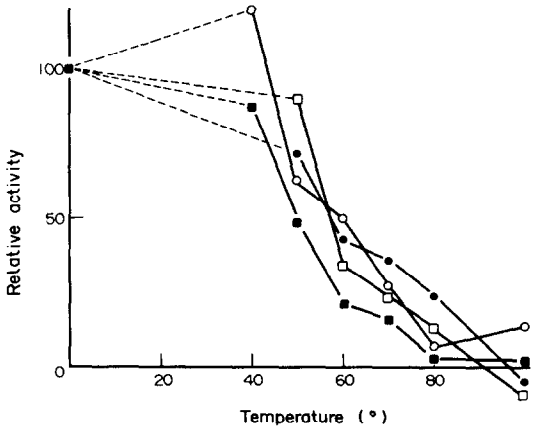

Fig. 3. Effect of heating for 1 hr at the stated temperatures on ornithine decarboxylation activity for the supernatant fraction (open circles) and particulate fraction (closed circles) of 17-day-old wheat leaves, and the supernatant from 8-day-old oat leaves (open squares) and 11-day-old wheat roots (closed squares).

temperatures and activity was determined and compared with that of unheated samples (Fig. 3). A proportion of the activity (*ca* 60%) was lost rapidly on increasing the temperature from 40 or 50 to 60°. However, the activity remaining was not completely lost unless the extract was heated for 1 hr at 100°. A similar pattern for denaturation was shown by both the oat and wheat leaf extracts. No ninhydrin-positive products could be found in the particulate fraction which had been boiled for 1 hr prior to incubation. Activity at 30° was linear for the entire 2 hr incubation period. At incubation temperatures of 30, 40 and 50° relative activities were 100, 229 and 260%. No loss of activity could be detected on storage of the wheat leaf particulate fraction in pH 9.5 buffer for 4 weeks at −15° and for the oat leaf particulate fraction 65% of the activity could be recovered after storage for 8 weeks at −5°. Wheat leaves frozen for two days at −15° showed no significant loss of activity.

M_r *of the factor present in the plant extracts causing the oxidative decarboxylation of ornithine*

Preliminary experiments with the soluble supernatant fraction of the wheat leaf extracts indicated that the factor was dialysable, suggesting that the M_r was less than 10000. Gel filtration on a Sephadex G-25 column confirmed this. With V_t of 74 ml and V_0 of 32 ml *ca* 15% of the activity was eluted at 34 ml (near to V_0). However, most of the activity was eluted with a peak at 47 ml, suggesting a M_r of *ca* 4000. Total activity was increased 2- to 3-fold after gel filtration, again indicating the presence of an inhibitor in the crude supernatant.

Oxidative decarboxylation of ornithine by manganous ions

Ornithine (5 mM), pyridoxal phosphate (1 mM) and $MnCl_2$ (250 μM) were incubated in 0.1 M (pH 9.3) sodium borate. After 4 hr at 40° with shaking, electrophoresis showed the presence of 4-aminobutanamide, 4-aminobutyric acid, pyridoxamine phosphate, and some residual ornithine. Although the smell of pyrroline was very strong during the incubation, this cyclic amine could not be detected after electrophoresis, probably because of the high volatility of the free base at the pH of the reaction. Hill and Mann [17] suggested that peroxidase and phenol were factors in the oxidative decarboxylation of amino acids by manganous ions at pH 7. However, in our experiments at pH 9.3, addition of peroxidase had no apparent effect on the course of oxidation; nor did peroxidase by itself cause amide formation; nor did phenol appear to potentiate the oxidation.

The reaction mechanism

A model system depending on Mn^{2+}, pyridoxal phosphate and peroxidase [18] has been shown to form 3-methylthiopropanamide from methionine and the oxidative decarboxylation of various amino acids by this system has been studied also by Hill and Mann [17]. A reaction mechanism for the oxidative deamination of alanine by pyridoxal and Mn^{2+} to produce pyruvate has been proposed by Hamilton and Revesz [19].

Although Mn^{2+} was not normally added to the incubates in the present study, Mn^{2+} is undoubtedly present in the plant extracts and the EDTA inhibition of the reaction would be compatible with this mechanism. In preliminary experiments we have shown that Mn^{2+} in the presence of pyridoxal phosphate will catalyse the oxidative decarboxylation of ornithine to form 4-aminobutanamide, and the fact that both the D- and L-forms of ornithine are attacked by the plant system suggests a 'chemical' rather than a 'biochemical' mechanism. The

activity was also found to be dialysable, indicating that a small factor is responsible. However, gel filtration showed that the M_r was *ca* 4000 and this appeared to preclude Mn^{2+} *per se* as the active component.

An enzyme catalysing the oxidative decarboxylation of lysine (lysine 2-monooxygenase: EC 1.13.12.2) has been purified from a *Pseudomonas* [20–22] and an enzyme catalysing the hydrolysis of 5-aminovaleramide, the product of lysine breakdown by this enzyme, has been characterised in this microorganism [23]. Moreover an enzyme effecting the oxidative decarboxylation of arginine (EC 1.13.12.1) has been purified from *Streptomyces griseus* [24] and has also been found in the pond snail *Limnaea stagnalis* [25]. This enzyme may also function in higher plants since 4-guanidinobutanamide has been detected in several tree species [26]. In addition D-lysine may be a true metabolite of higher plants in which it is the preferred precursor of pipecolic acid [27].

Hill and Mann [17] have shown that peroxidase will potentiate the Mn^{2+}-catalysed oxidation of amino acids by eliminating a lag period. This is unlikely to be the explanation for the thermolabile nature of the present reaction since horseradish peroxidase added to the boiled extract without Mn^{2+} would not restore the activity; nor could any activity be demonstrated after a lag period of up to 5 hr on boiling the plant extract. However, circumstantial evidence gives strong support to the possibility that Mn^{2+} ions are responsible for the decarboxylation. Mn^{2+} is known to have a high affinity for nucleic acids [28]. This would explain the finding that the M_r of the catalytic factor is much greater than that of free Mn^{2+} ions on gel filtration, but not as great as the smallest known enzyme (M_r *ca* 10000). It would also account for the apparent heterogeneity of the catalytic complex, both in its pH optima and in the appearance of several peaks on gel filtration. Moreover, the apparent loss on heating to 100° for 1 hr could be explained by the formation of the unstable hydroxide at high pH [29] which can readily undergo oxidation with elimination of Mn^{2+} ions. This could lead to the loss of catalytic activity as shown in Table 6. This reaction appears to be significant even at pH 9.3, and seems to be accelerated by the presence of the plant material. Mn^{2+} is active as a catalytic factor in the decarboxylation of ornithine even at 4 μM, a concentration probably approaching that found in the plant extracts.

Whether or not the activity associated with the plant extracts described in the present work is an artefact, the oxidation of ornithine with the release of CO_2 from the carboxyl group is effected in conditions similar to those used conventionally for the assay of ornithine decarboxylase (ODC), the enzyme forming putrescine, and for other amino acid decarboxylases (notably those for lysine, arginine and *S*-adenosylmethionine decarboxylase) in crude plant extracts. ODC activity measurements are usually made on the supernatant only, at pH values ranging from 7.2 to 8.5. Although ODC assays have been conducted with concentrations of pyridoxal phosphate as low as 25 μM rather than those used in the present work (1 mM), there is no reason to suppose that the mechanism at the lower concentrations would favour ODC activity. ODC activity measurements are often made in the presence of sulphydryls (dithiothreitol, up to 10 mM) which may in theory reduce the oxidative decarboxylation (Table 5). The EDTA which is frequently added would also have the effect of reducing oxidative decarboxylation. However, the potential occurrence of the oxidative decarboxylation of amino acids may necessitate modification to these assays in order to obtain results which would indicate the true rate of non-oxidative decarboxylation. The realization that ornithine decarboxylation can give rise to products other than putrescine [30] has already led to the development of more specific means of ODC measurement. Since the factor forming the amide has a small M_r, it is unlikely to be precipitated by ammonium sulphate. This step [31] may therefore be used to eliminate the oxidative ornithine decarboxylation prior to ODC assay.

Table 6. Loss of ornithine decarboxylation activity on heating a wheat leaf particulate preparation with $MnCl_2$

$MnCl_2$ (μM)	Minus particles		Plus particles	
	Unheated	Heated	Unheated	Heated
0	—	—	54	1
4	165	25	135	2
20	274	28	259	3
100	245	103	278	15

Before assay for decarboxylation, incubates at pH 9.3 were preincubated for 1 hr at 0° (unheated) or at 100° (heated). Results are expressed as dpm/100.

EXPERIMENTAL

Plants. Cereals (listed in Table 2) were grown at 23° (14 hr day; 250 μmol/sec/m^2) or in darkness at 23°, in a mixture of sand and perlite (1:1) watering as required with a nutrient soln composed of (final concn in parentheses) (A) NH_4NO_3 (8 mM); $CaCl_2$ (4 mM); $MgSO_4$ (1 mM); $NH_4H_2PO_4$ (1 mM); KCl (6 mM); FeEDTA (0.1 mM) and micronutrients: or (B) KNO_3 (4 mM); $MgSO_4$ (1.5 mM); NaH_2PO_4 (1.33 mM); Ca $(NO_3)_2$ (4 mM); FeEDTA (0.01 mM) and micronutrients. Beet, rape, tomato, and tobacco were grown in a greenhouse in compost. Pea seedlings were grown in darkness in the conditions given above for the wheat.

Production of 4-aminobutanamide from ornithine. Leaves (15 g) were ground with 15 g of acid washed quartz sand in 80 ml of pH 9.3 Na borate (0.1 M). After centrifuging (14000 *g*, 15 min) the supernatant was discarded and the particulate fraction was resuspended by grinding in a total vol. of 70 ml of pH 9.3 Na borate buffer (0.1 M). To this was added 20 ml of 25 mM L-ornithine hydrochloride and 10 ml of 10 mM pyridoxal phosphate. After incubation for 4 hr at 40° in darkness with shaking, 2.5 g of trichloroacetic acid (TCA) was added and the ppt. was removed by centrifuging at 5000 *g* for 5 min. The TCA was extracted with Et_2O (2 vol × 2) and the residue was concd to 10 ml under vacuum at 40°. The soln was cooled to crystallize the Na borate which was removed by decantation.

Assuming that both D- and L-ornithine are attacked, oxidative decarboxylation measured by assay with D,L-[1-^{14}C]-ornithine was at most 10 to 20% of the amount of ornithine added. Since 4-aminobutyric acid and 4-aminobutanamide were produced in almost equal amounts, the anticipated yield of the latter in one incubation was at most about 3 mg.

Assay for ornithine decarboxylation. Leaves (1 g samples) were extracted in a pestle and mortar with 1 g acid washed sand and 9 ml of Na borate buffer pH 9.3 (0.1 M). On centrifuging

(14000 *g*, 15 min) the ppt. was made up to 10 ml with pH 9.3 Na borate buffer and dispersed with a glass homogeniser. Aliquots of this ppt were taken for the assay after gentle agitation to resuspend the ppt. Aliquots of the orginal supernatant were also removed for assay.

The incubation was conducted in 22 × 75 mm glass tubes with internally fitted polythene stoppers. The rim of the stopper retained a piece of folded filter paper (1 × 4 cm) to which 25 μl of NCS (Amersham) was applied [32]. The reaction mixture (total vol. 250 μl) consisted of 150 μl of plant extract, 25 μl of 10 mM pyridoxal phosphate, and 75 μl of DL-[1-^{14}C] ornithine HCl, (Amersham) (0.05 μCi, 1850 Bq) in 16.7 mM ornithine monoHCl (BDH) (final concn 5 mM). After incubating the mixture for 2 hr at 30°, 0.2 ml of M HCl was added with a syringe through a plastic tube which passed through the stopper, 1 hr was allowed for distillation of the $^{14}CO_2$. (Distillation was virtually complete 15 min after addition of the HCl.) The paper was removed and placed in 4 ml of Optisorb 2 (LKB) before counting for 5 min. The mean of 3 counts was used for calculating the enzyme activity, which is expressed in pkat/g fr. wt.

The amount of NCS was critical. On reducing the vol. to 5 μl, only 45% of the CO_2 was recovered. If the vol. was in excess of 25 μl, the filter paper wicks became saturated and some of the NCS was lost as a film on the polythene stoppers. All solns containing pyridoxal phosphate were kept dark to avoid photolysis [33]. Blanks consisting of pyridoxal phosphate, ornithine and buffer, but with no plant extract were routinely included and usually gave counts which were less than 1% of the test incubates. No decarboxylation was effected on incubation with the sand alone.

pH optimum. In order to determine the pH optimum of the wheat leaf enzyme, the leaves (1 g) were extracted with sand into a range of buffers (9 ml) and the pH re-determined after extraction. For the particulate enzymes in oat and wheat, extraction was effected in 0.1 M pH 6.8 Na–Pi buffer or 0.1 M pH 9.3 Na borate buffers respectively (10 g leaves/90 ml buffer). The extract was equally dispensed into centrifuge tubes (10 ml each), centrifuged (20000 *g*, 15 min) and the supernatant discarded. The ppts were resuspended into a series of buffers using a glass homogeniser. The following 0.1 M buffers were used: Na-Pi pH 5.5–7.5, Na borate–HCl pH 8.1–9.3, Na borate–NaOH pH 9.3–10.7 and Na_2HPO_4–NaOH pH 10.8–11.8.

Electrophoresis. The buffer (pH 4.6) was prepared from 20 ml pyridine and 25 ml HOAc made up to 1 l. Separations were effected at 1500 V on Whatman 3MM papers 56 × 23 cm. After electrophoresis, the paper was dried at 100° and the amino compounds detected with ninhydrin (0.1% in acetone) used as a dip followed by heating at 100° for 10 min. The mobilities of 4-aminobutanamide and related compounds are given in Table 7.

Chromatography. The following solvents were used with Whatman 3MM paper (ascending): (1) *n*-BuOH–HOAc–H_2O (12:3:5); (2) *n*-BuOH–Pyr–H_2O (1:1:1); (3) EtOH–NH_4OH (15 M)–H_2O (20:1:4). The hydroxamic acid test [34] gave mauve spots on a pale yellow background with 4-aminobutanamide. The R_f values for the amide were respectively: solvent (1) 0.37, (2) 0.42, and (3) 0.54. The R_f value for pyrrolidinone in solvent 3 was 0.85.

Synthesis of 4-aminobutanamide. Ethyl 4-aminobutyrate hydrochloride (2 g) and 15 M NH_4OH (40 ml) was left for 24 hr at room temp. After concn to dryness at 40° the residue was taken up in 10 ml of H_2O and passed through a column (2 cm diam. × 5 cm) of IRA-400 (OH^- form) to remove 4-aminobutyric acid which is also formed. After concn to dryness the product was dissolved in dry CH_2Cl_2, and dry HCl gas (generated from NaCl and 36 N H_2SO_4) was used to ppt. the hydrochloride. The solvent was removed and the product (yield 38%) was recrystallized from EtOH–Et_2O mp 134°–135° (lit. 126–129°, [35]; 135–138°, [36].

Table 7. Electrophoretic mobilities on Whatman 3 MM paper of 4-aminobutanamide and related compounds

Compound	cm/hr	Ninhydrin colour
Putrescine	8.1	Pink
Agmatine	7.0	Pink
Na^+*	6.0	—
2-Aminoacetamide†	5.9	Pink
3-Aminopropanamide†	5.4	Beige/Pink
Pyrroline	5.4	Brown
4-Aminobutanamide	4.9	Pink
Diaminopropionic acid	4.5	Dark pink
Diaminobutyric acid	4.3	Pink
Ethyl 4-aminobutyrate	4.1	Pink
Ornithine	3.8	Pink
N-Carbamoylputrescine	3.8	Pink
Arginine	3.7	Purple
Lysine	3.6	Pink
Pyridoxamine	3.6	Orange
4-Aminobutyric acid	2.0	Pink
5-Aminovaleric acid	1.9	Pink
2-Pyrrolidinone‡	0.9	—
Pyridoxamine phosphate	0.65	Orange
Citrulline	0.6	Purple
Glutamic acid	−1.0	Pink
Methyl Green	3.6	—

Methyl Green is a basic dye which serves as a visual mobility reference. All mobilities (in cm/hr) are normalized for 10 V/cm. The buffer was 0.1 M pyridine/acetic acid at pH 4.6.

*Detected by Bromocresol Green spray.

†Presumed structure.

‡Detected with the hydroxamic acid test.

Mass spectra (probe) of the free base were determined on a Kratos MS80 RFA instrument. EI, 70 eV *m/z* (rel. int.) 85 [M − 17]$^+$ (100), 56 (12), 44 (77), 41 (62). The amide was apparently completely pyrolysed to 2-pyrrolidinone (*m/z* 85). *FAB* The amide was bombarded with xenon (6.5 kV) from a saddle field neutral beam gun in a glycerol–thioglycerol matrix, scanning at 30 sec/decade. *m/z* 178 [pyrrolidinone + glycerol]$^+$ (10), 103.0958 [M + H]$^+$ (34) (deviation -84ppm), 87.0616 [pyrrolidinone + H]$^+$ (43) (deviation -78ppm). ^{1}H NMR. 400 MHz. D_2O, dioxane as int. std δ1.663 (*br, s*), 1.694 (*m*, J = 7.6), 2.147 (*t*, J = 7.4), 2.776 (*t*, J = 7.7), 3.172 (*t*, J = 7.1). ^{13}C NMR: δ23.61, 24.18, 31.07, 32.61, 39.66, 178.66, 182.5.

The product was ninhydrin positive and may therefore be distinguished from 2-pyrrolidinone which is ninhydrin negative. Both give a mauve colour with the $FeCl_3$–NH_2OH reagent, and the properties of the two compounds are otherwise similar. Unsuccessful attempts were made to derivatize the synthetic 4-aminobutanamide by dansylation [37] and for GC with pentafluorobenzoyl chloride [38].

Gel filtration. The extract of 5 g of 2-week old dark-grown wheat leaves with 20 ml of Na borate (0.1 M) was centrifuged at 14000 *g* for 15 min. The supernatant (5 ml) was applied to a column of Sephadex G-25 (40 × 1.5 cm) pre-equilibrated with Na borate (0.1 M). Separation was effected with the same buffer at 1 ml/min, collecting 2 ml fractions.

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