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AMINO ACID DECARBOXYLASES OF HIGHER PLANTS: THE FORMATION OF ETHYLAMINE

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Abstract—The enzymic decarboxylation of alanine has been demonstrated by production of ^{14}C-carbon dioxide from [1-^{14}C] alanine and of ^{14}C-ethylamine from [U-^{14}C] alanine using extracts from *Ecballium elaterium* and cucumber seedlings. Some properties of the decarboxylase enzymes are described. The same plant extracts catalysed the decarboxylation of serine at a slower rate.

INTRODUCTION

CERTAIN non-protein amino acids form characteristic constituents of some members of the family Cucurbitaceae,[1] and previous publications from this laboratory have described enzymic work concerned with the synthesis of β-pyrazol-1-ylalanine in cucumber seedling extracts[2] and of N^4-substituted asparagines in extracts of *Ecballium elaterium* seedlings.[3] N^4-Ethyl- and N^4-hydroxyethyl-asparagines appear confined to the two allied genera, *Ecballium* and *Bryonia*,[1] and are formed by an exchange reaction in which the amide-NH_2 group of asparagine is replaced by a residue of ethylamine or ethanolamine, respectively.[3] Although the amines required as substrates in these enzymic syntheses are present in low concentrations in *E. elaterium* plants,[4] their origins in *Ecballium* have remained unknown. However, ethanolamine was formed by decarboxylation of serine, which occurred incidentally to the main reaction forming β-pyrazol-1-ylalanine from pyrazole and serine in the cucumber seedling extracts.[2]

The experiments now presented show that a similar decarboxylation of serine is catalysed by extracts of *Ecballium*, and that these extracts also produce ethylamine by decarboxylation of alanine.

RESULTS AND DISCUSSION

The decarboxylation reactions were studied under anaerobic conditions using enzyme preparations obtained by adding ammonium sulphate (to 72 per cent saturation) to cell-free extracts of either *Ecballium* or cucumber seedlings.

It was essential initially to establish that ethylamine was produced from alanine. To achieve this, [U-^{14}C]-L-alanine was used as the substrate for an *Ecballium* preparation and, after appropriate reaction periods, any volatile amine formed was transferred to dilute hydrochloric acid using the Conway micro-diffusion technique. After evaporation, the

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radioactive residue was cochromatographed with authentic ethylamine hydrochloride; subsequent radioautography demonstrated an exact coincidence of radioactive and ninhydrin positive spots.

The properties of the enzymes responsible for the decarboxylation of alanine and serine were studied using carboxyl-labelled DL-racemates, reaction being measured by scintillation counting of the ^{14}C-carbon dioxide produced. The rate of $^{14}CO_2$ production from either labelled alanine or serine was linear with time over a 2-hr period, irrespective of whether *Ecballium* or cucumber extracts were used as the source of decarboxylases. In these experiments, the amounts of labelled substrates added to reaction mixtures would produce concentrations of approximately 5 μM alanine and 12 μM serine (based on the L-isomers); however, the final concentration of each amino acid was probably somewhat higher because some endogenous alanine and serine would be present in the enzyme preparations used.

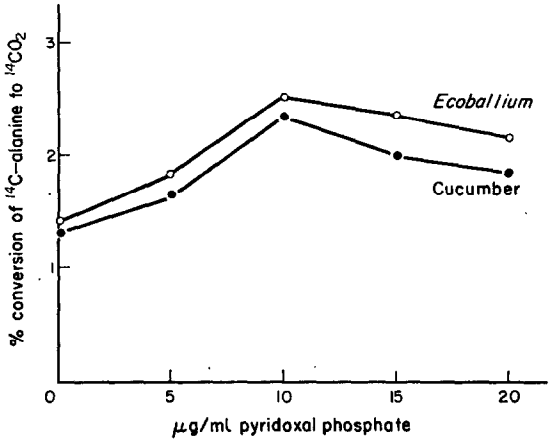

FIG. 1. THE EFFECT OF ADDED PYRIDOXAL PHOSPHATE UPON THE RATE OF $^{14}CO_2$ PRODUCTION FROM [1-^{14}C]ALANINE IN THE PRESENCE OF *Ecballium* AND CUCUMBER SEEDLING EXTRACTS.

Reaction mixtures contained: 2 ml enzyme preparation (containing 18 and 62 mg protein per ml for *Ecballium* and cucumber respectively), 2 ml Na_2HPO_4–citrate buffer (pH 5·5), and 0·5 ml ^{14}C-alanine 0·5 μc). Gas phase: nitrogen. Reaction measured for 2 hr at 25°.

The optimum pH for alanine decarboxylation by *Ecballium* extracts was about 5·5. The pH-activity curve exhibited a sharp peak at this pH, and activity measured at either pH 5·0 or 6·0 was only about 20 per cent of that at the optimum. An optimum pH of 6·5 was found for the serine decarboxylase from *Ecballium* seedlings: the decrease of activity at pH's removed from the optimum was less marked than with the alanine decarboxylase. The serine decarboxylase from cucumber seedlings was shown earlier[2] to have an optimum pH of about 7.

Addition of pyridoxal phosphate to reaction mixtures (10 μg/ml) produced an approximate 1·8-fold stimulation of alanine decarboxylase activity, irrespective of the source of enzyme (see Fig. 1). Higher pyridoxal phosphate concentrations were less effective in stimulating decarboxylation. The same conclusion was reached in replicate experiments with each type of seedling extract. The reaction rates measured in these experiments suggest that *Ecballium* seedlings contain higher levels of alanine decarboxylase (per mg soluble protein) than do cucumber seedlings. The same tentative conclusion was reached in all other experiments permitting this type of species comparison.

As in previous studies with glutamic decarboxylase,[5, 6] the activity of alanine decarboxylase was inhibited by semicarbazide. This compound caused 59 and 64 per cent inhibition of decarboxylation when present at 10^{-3} and $2{\cdot}5 \times 10^{-3}$ M, respectively, in reaction mixtures containing *Ecballium* enzyme.

The formation of ethylamine by enzymic decarboxylation of alanine seems not to have been demonstrated previously, although the amine forms a constituent of many plants. In addition to its presence in *Ecballium* plants,[4] Tunmann and Linde[7] reported this amine to occur in the roots of the related plant, *Bryonia dioica*. Ethylamine also occurs in seeds of the tea plants,[8] a species synthesizing N^5-ethylglutamine from ethylamine.[9] Other plants reported to contain ethylamine include *Vicia faba* and *Zea mays*,[10] *Erodium cicutarium*,[11] the fruits of apple[12] and *Crataegus*,[13] and the flowers of *Sambucus nigra* and *Arum italicum*.[14] Certain of these plants possess decarboxylases active upon other monoamino–monocarboxylic acids, e.g. enzymic decarboxylation of valine has been described by Simon[15] using extracts of *A. maculatum* spadix, while Richardson[16] found decarboxylase activity for both valine and leucine to be present in extracts of flowers of *Sorbus aucuparia* and *Crataegus monogyna*.

Amination of acetaldehyde has been suggested as an alternative method of ethylamine formation in apple fruits.[17] Increased levels of the amine resulted when apple fruits were incubated with acetaldehyde for 48 hr, but amination of acetaldehyde could not be detected in homogenates, press-juice or cell-free extracts of apples. Probably *in vivo* amination is catalysed by a transaminase system similar to that described for *Mercurialis perennis* and *Nicotiana*;[18] extracts of these two plants converted a range of aliphatic aldehydes, including acetaldehyde, into their corresponding monoamines provided a suitable amino acid (but not ammonia) was present as amino donor.

EXPERIMENTAL

Labelled Amino Acids

[U-^{14}C]-L-Alanine (14·2 mc/mmole), [1-^{14}C]-DL-alanine (23·4 mc/mmole) and [3-^{14}C]-L-serine (4·3 mc/mmole) were obtained from the Radiochemical Centre, Amersham. [1-^{14}C]-DL-Serine (5 mc/mmole) was purchased from New England Nuclear Corp.

Plant Materials

Ecballium elaterium seeds were soaked in water with aeration for 3 days, freed from the mucillaginous layer covering the testa by scouring with sand, and planted in moist vermiculite at 25°. After a further 3 days, the seedlings were harvested, washed and extracted. Cucumber seeds (var. Best of All, Carters Tested Seeds Ltd., London, S.W.20) were aerated in water overnight and grown at 25° for 3 days. Protein was determined colorimetrically by the method of Lowry *et al.*[19]

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Preparation of Enzyme Extracts

Washed seedlings were ground with a little sand and ice-cold water (about 4 ml/g fresh wt. seedlings). The macerate was pressed through muslin, and the extract centrifuged at 30,000 *g* for 15 min. $(NH_4)_2SO_4$ (5 g/10 ml) was added to the supernatant and, after standing for 15 min, precipitated protein was sedimented by centrifuging at 16,000 *g* for 15 min. The protein pellet was redissolved in cold distilled water to give the final enzyme preparations used in the decarboxylation studies, and a portion was boiled to give a control value in all enzymic assays.

Decarboxylase Experiments

(*a*) *^{14}C-Ethylamine production from [U-^{14}C]alanine.* Reaction mixtures containing 1 ml enzyme preparation (containing about 14 mg protein) from *Ecballium* seedlings, 0·5 ml citric acid–Na_2HPO_4 buffer, pH 5·5, and 0·5 ml [U-^{14}C]-L-alanine (2·5 μc) were placed in the outer annulus of Conway units, which were flushed with N_2 before sealing. The units were incubated at 25° for periods up to 24 hr. Then 0·1 N-HCl (1 ml) was introduced into the centre well, and reaction was stopped by addition of 5 N-NaOH (1·5 ml) to each outer annulus. The units were resealed and again incubated overnight to ensure the complete diffusion of volatile ethylamine released from reaction mixtures into the centre wells. The contents of each centre well were removed, evaporated to dryness, and any residual amine hydrochloride was transferred, together with added authentic ethylamine hydrochloride, to a paper chromatogram run in butan-1-ol–acetic acid–water (90:10:29, v/v). The chromatogram was scanned with a Packard Radiochromatogram Scanner, developed with ninhydrin, and subsequently radioautographs were prepared to ensure exact coincidence of radioactive and ninhydrin-positive areas.

(*b*) *Assays based on ^{14}C-carbon dioxide production.* Reaction mixtures containing 2 ml enzyme preparation, 2 ml citrate–phosphate buffer of appropriate pH and 0·5 ml labelled amino acid (1 μc of [1-^{14}C]-L-alanine of 0·5 μc of [1-^{14}C]-L-serine) were shaken at 25° under N_2 in the main chamber of Katz flasks. After appropriate times, 0·1 ml hyamine hydroxide (M-solution in methanol, Nuclear Enterprises Ltd.) was added to a glass-fibre disc (Whatman GF/A, 2·1 cm dia.) contained in the centre wells, and 2 N-H_2SO_4 (2 ml) was injected into the reaction mixture. Shaking was continued for a further hour to effect complete absorption of $^{14}CO_2$ by the hyamine-treated glass-fibre discs. The discs were transferred to vials containing 10 ml scintillator solution (6 g butyl-PBD, Ciba Ltd., in 800 ml toluene and 200 ml methanol) and, after standing and mixing overnight, radioactivity was counted using a Packard Tri-Carb scintillation counter.

In some experiments, pyridoxal phosphate or semicarbazide were added in 0·1 ml solution to reaction mixtures of this type.

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