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STUDIES ON THE BIOGENESIS OF SOME SIMPLE AMINES AND QUATERNARY AMMONIUM COMPOUNDS IN HIGHER PLANTS.

ISOAMYLAMINE AND ISOBUTYLAMINE

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Abstract—*In vivo* infiltration of the inflorescences of *Sorbus aucuparia* and *Crataegus monogyna* with L-leucine-U-^{14}C and L-valine-U-^{14}C gave rise to radioactive isoamylamine and isobutylamine respectively. Simple enzyme preparations from the flowers of both species protected with polyvinylpyrrolidone (PVP) catalysed the decarboxylation of DL-leucine-1-^{14}C and L-leucine-U-^{14}C. The product of the decarboxylation was isoamylamine. The enzyme in both species was substantially inhibited by hydroxylamine. Extracts from *Crataegus monogyna* required the addition of the co-enzyme pyridoxal phosphate for maximum activity, whereas extracts from *Sorbus aucuparia* did not. The enzyme in *Sorbus aucuparia* was in the soluble cytoplasmic fraction and appeared to have an optimum pH in the region of 7·5.

INTRODUCTION

ISOAMYLAMINE (I) and isobutylamine (II) are widely distributed in the plant kingdom[1,2] and are particularly characteristic of the flowers of many species of the Rosaceae.[3] The simplest reactions which might bring about the formation of these amines would be the decarboxylation of the amino acids leucine and valine. Such reactions are well-known in micro-organisms[4,5] and have been studied recently in *Proteus vulgaris* Hauser.[6,7] However, our knowledge of amino acid decarboxylases in plants is very incomplete, and the scanty evidence for the existence of these enzymes in higher plants has been reviewed by Sanwal and Lata.[8] Several workers have been unable to demonstrate any decarboxylase activity when breis and extracts of various amine containing plant tissues were incubated with the amino acids L-leucine and L-valine.[9–11] More recently, however, attention has once more been directed to this method of biosynthesis of the amines in plants by the successful demonstration of a valine decarboxylase activity in homogenates of spadices of *Arum maculatum* L.[12]

* This paper constitutes a section of a Ph.D. thesis submitted to the Univesity of Hull in 1965.

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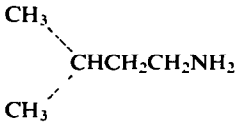
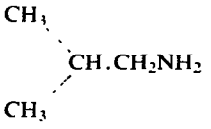
The biogenesis of amines via reversible transaminase reactions has been reported by Hasse and Schmid[13] in various higher and lower plants. These workers observed that a transaminase preparation from peas could bring about the transamination of glycollic aldehyde with either glutamic acid or alanine to produce the primary amine ethanolamine. These findings indicated the alternative possibility that isoamylamie (I) and isobutylamine (II) might arise from the transamination of an amino acid with isovaleric aldehyde (III) or isobutyric aldehyde (IV) respectively.



(I) Isoamylamine



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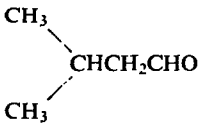
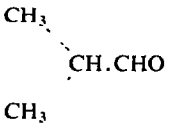


(III) Isovaleric aldehyde



(IV) Isobutyric aldehyde

This paper reports on the *in vivo* production of isoamylamine and isobutylamine from L-leucine-U-^{14}C and L-valine-U-^{14}C in the flowers of *Crataegus monogyna* Jacq. and *Sorbus aucuparia* L., and the presence of an enzyme in simple homogenates of the flowers of these species which catalyzed the decarboxylation of DL-leucine.

RESULTS AND DISCUSSION

Paper chromatography and ion-exchange column chromatography of the volatile amines isolated from inflorescences of *Crataegus monogyna* and *Sorbus aucuparia* by steam distillation revealed the presence of isoamylamine, isobutylamine, β-phenylethylamine and trimethylamine thus confirming the findings of previous workers.[1,3] The liberation of the volatile primary amines by both species was shown to be closely correlated with the stage of development of the flowers. Amines were not detected until the individual flowers of the inflorescence began to open. The greatest amounts (70–90 μg isoamylamine equivalents/g fresh wt.) were detected when nearly all the flowers were open. The production of amines, however, did not persist once this stage was reached and within a few days after flower opening was complete the concentration of amines fell quite rapidly. Similar fluctuations in the content of amines have been recorded in the flowers and fruits of *Sarothamnus scoparius* L., Koch[14] and in *Mercurialis annua* L.[3] The present results are in direct contrast to the observation that isoamylamine and isobutylamine were produced even in the immature green buds of the flowers of *Pyrus communis* L., *Crataegus tomentosa* L. and *Sambucus nigra* L.[3]

Infiltration of inflorescences of both species with L-leucine-U-^{14}C under sterile conditions gave rise to ^{14}C-labelled isoamylamine in the flowers (Table 1). The radioactive amine was only produced by mature inflorescences in which most of the flowers were open and which were giving off the characteristic odour. Similarly labelled isobutylamine was isolated from the inflorescences after feeding with L-valine-U-^{14}C.

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TABLE 1. PRODUCTION OF RADIOACTIVITY LABELLED ISOAMYLAMINE FROM L-LEUCINE-U-^{14}C BY CUTTINGS OF THE INFLORESCENCES OF *Sorbus aucuparia* AND *Crataegus monogyna*

Plant species	Flowers open (%)	Duration of feeding (hr)	Uptake of radioactivity* (%)	Radioactivity in isoamylamine	
				count/min	As % of L-leucine
Sorbus aucuparia	0	48	32·47	0	0
	60	24	46·07	2,652	0·10
	100	24	41·33	10,890	0·48
Crataegus monogyna	0	36	22·50	253	0·02
	50–60	24	17·56	3,030	0·31
	100	24	27·71	1,300	0·08

* 2·5 μ of L-leucine-U-^{14}C were fed to each plant.

Initial attempts to demonstrate the presence of a leucine or valine decarboxylase in the flowers of *Crataegus monogyna* and *Sorbus aucuparia* by using simple homogenates and conventional manometric methods were unsuccessful. A rapid browning reaction was observed in many of the preparations used. It was thought that this browning was possibly due to the oxidative polymerization of the phenolic compounds which are abundant amongst members of the Rosaceae.[15] Previous workers[16–18] who have been unable to obtain active enzyme preparations from extracts of the fruit and flowers of various Rosaceae have suggested that the inhibition was probably due to the presence of certain phenolic compounds such as leucoanthocyanidins, catechins, quercetin and cyanidin glycosides. The incorporation of polyvinylpyrrolidone (PVP) in extraction media facilitated the preparation of highly active mitochondrial preparations from the peel and pulp of apple tissues[16] and from the petals of various flowers.[19,20] It has also been suggested[8] that the amino acid decarboxylases in higher plants might have a very weak activity such that assay methods of high sensitivity would be required for their detection.

In later experiments PVP was routinely incorporated into the extraction media and ^{14}C-labelled substrates used to increase the sensitivity of the assay methods. Under these conditions a leucine decarboxylase was demonstrated in simple homogenates of the flowers of both *Sorbus aucuparia* and *Crataegus monogyna* (Table 2).

The enzyme from both species was substantially inhibited by the carbonyl inhibitor hydroxylamine ($2{\cdot}5 \times 10^{-5}$ M). In the case of the leucine decarboxylase from *Sorbus aucuparia* the inhibition was almost complete whereas in those extracts from *Crataegus monogyna* only 69 per cent inhibition of activity occurred. Similar inhibition by hydroxylamine has been reported for the glutamic acid decarboxylases of several plant tissues,[21] the L-arginine decarboxylase of barley seedlings,[22] and the valine decarboxylase of *Arum maculatum*.[12] The greater inhibition of the leucine decarboxylase activity in extracts of *Sorbus aucuparia* flowers by hydroxylamine suggested that this enzyme system had a stricter requirement for the

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TABLE 2. DECARBOXYLATION OF DL-LEUCINE-1-^{14}C BY SIMPLE HOMOGENATES FROM THE FLOWERS OF *Sorbus aucuparia* AND *Crataegus monogyna*

Reaction mixture	Radioactivity in CO_2 released*† count/min/flask		
	Sorbus aucuparia		*Crataegus monogyna*
	(2 hr)	(3·5 hr)	(3 hr)
Complete system‡	955	3825	5530
Complete system (boiled enzyme)	224	355	726
—less enzyme	134	294	1005
—less pyridoxal phosphate	936	4006	4049
$+2{\cdot}5 \times 10^{-5}$ M hydroxylamine	309	529	2158

* Each value is the mean of three replicates.

† 10^3 count/min = 0·018 per cent of DL-leucine-1-^{14}C added.

‡ Complete system contained 1 ml of enzyme preparation, 0·5 ml of a solution containing 0·2 M Tris-HCl buffer, 0·01 M EDTA K_2 salt, 0·01 M KH_2PO_4 and H_3PO_4 at pH 7·4, 0·5 ml of a solution of pyridoxal phosphate (10 μg/ml) and 0·5 ml of a solution of DL-leucine containing 2·5 μc of DL-leucine-1-^{14}C. Gas phase was nitrogen. Temperature 27°. Other details described in Experimental.

coenzyme pyridoxal phosphate, but surprisingly the extracts from the flowers of *S. aucuparia* did not appear to require the addition of extra pyridoxal phosphate for maximum activity. On the other hand those extracts from *Crataegus monogyna* which were not inhibited to the same extent by hydroxylamine did require the addition of extra pyridoxal phosphate for maximum activity. The fact that decarboxylation of leucine occurred even in the absence of added pyridoxal phosphate in extracts of *Sorbus aucuparia* suggests that the co-factor is tightly bound to the enzyme in this species.

The enzyme in *Sorbus aucuparia* appeared to be in the soluble cytoplasmic fraction obtained by differential centrifugation rather than in the mitochondria (Table 3). In this

TABLE 3. DECARBOXYLATION OF DL-LEUCINE 1-^{14}C BY DIFFERENT CELLULAR FRACTIONS OF AN EXTRACT FROM THE FLOWERS OF *Sorbus aucuparia*

System	Radioactivity in CO_2 (count/min/flask)*†
Complete system‡	2726
—less enzyme	211
Boiled enzyme	205
Mitochondrial fraction	327
Supernatant (soluble cytoplasm)	1927

* Mean of 3 replicates.

† 10^3 count/min = 0·018% of the DL-leucine-1-^{14}C added.

‡ Complete system as in Table 2. Temperature 27°. Duration of experiment 3 hours.

respect the enzyme is similar in its intracellular distribution to the glutamic decarboxylase in squash,[11] the arginine decarboxylase of barley,[22] and the valine decarboxylase of the spadix of *Arum maculatum*.[12]

The optimum pH for the decarboxylation of DL-leucine by the extracts of *Sorbus aucuparia* was found to be in the region of 7·5 (Fig. 1). This is in contrast to the pH optimum of 5·0 observed for the decarboxylation of L-leucine by the mycelium of *Claviceps purpurea* (Fr.) Tulasne.[23] Also the majority of the other amino acid decarboxylases which have been studied in bacteria and higher plants have been found to possess either acid or neutral pH optima for

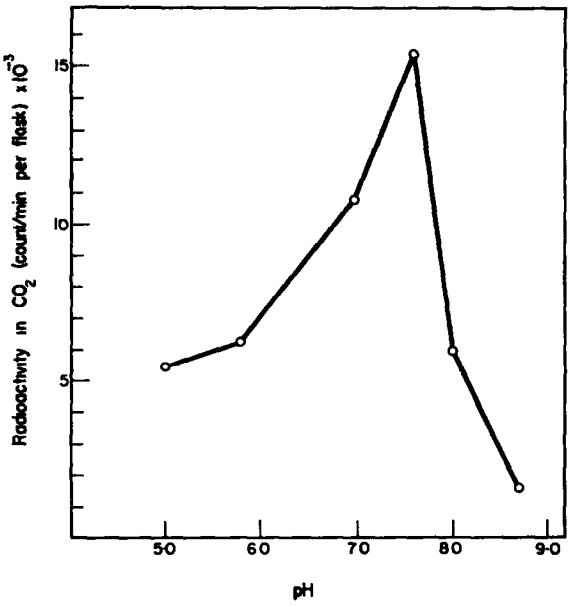

FIG. 1. EFFECT OF pH ON THE DECARBOXYLATION OF DL-LEUCINE BY EXTRACTS FROM INFLORESCNENCES OF *Sorbus aucuparia*

Duration of Assay 4 hr. Other details as described in Table 2 and the Experimental section.

their action.[6,21,24–27] On the other hand, the arginine decarboxylase of crude extracts of barley seedlings possessed a pH optimum lying between 5·5 and 8·0, whilst the pH optimum of purified extracts lay between 6·5 and 9·0.[22]

The nature of the decarboxylation product was investigated by incubating L-leucine-U-^{14}C with PVP-protected extracts from the flowers of *Sorbus aucuparia* and *Crataegus monogyna* and then subjecting the reaction mixtures to micro-steam distillation. In all cases paper chromatography in a number of different solvent systems (Table 4) followed by radioautography revealed only a single radioactive area which coincided with the ninhydrin positive spot of isoamylamine. Co-chromatography of the volatile radioactive product with authentic samples of iso-amylamine confirmed its identity.

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[26] P. WEINBERGER and K. A. CLENDENNING, *Can. J. Bot.* **30**, 755 (1952).
[27] L. V. EGGLESTON, *Biochem. J.* **65**, 735 (1957).

TABLE 4. R_f VALUES OF VOLATILE AMINES FROM INFLORESCENCES OF *Sorbus aucuparia* AND *Crataegus monogyna*

Solvent system	R_f values: Isoamyl-amine	Isobutyl-amine	Trimethyl-amine	β-Phenyl-ethylamine
n-butanol:acetic acid:water (4:1:5)	0·70	0·59	0·31	0·74
n-butanol:conc HCl:water (7:2:1)	0·87	0 78	0·50	—
pyridine:*n*-pentanol:water (3:3:2)	0·72	0·64	0·55	0·75
phenol, saturated with water	0·84	0·75	0 80	0 78
n-butanol:acetic acid:water (50:1:49) (Paper pretreated with 0·2 M sodium acetate)	0·69	0·55	0·19	0 62

EXPERIMENTAL

Isolation of the Volatile Amines from Inflorescences of Sorbus aucuparia *and* Crataegus monogyna

The flowers (0·5–5 g) were ground up in a mortar with 10^{-3} N HCl (5 ml) and the resultant mash transferred to the flask of an all-glass semi-micro steam distillation apparatus. A saturated solution of NaCl (10 ml) was added, followed by 1–2 g of solid MgO. The receiving flask contained 0·1 N HCl (8 ml). Steam was passed into the apparatus for 30 min, during which time approximately 50 ml of distillate were collected.

The distillate containing the volatile amines was reduced to dryness *in vacuo* at 50°. In some experiments the residue was taken up in a few ml of distilled water and the total content of volatile primary and secondary amines and ammonia estimated spectrophotometrically with the 1-fluoro-2,4-dinitrobenzene reagent.[28] In other experiments the individual amines were separated from one another by band chromatography on Whatman No. 3 MM paper. Before application of the samples the papers were washed with 1 N HCl for 30 min, and then for 2–3 hr in distilled water. Band chromatograms were developed by ascending chromatography counter to the direction of machining with *n*-butanol:acetic acid:water (4:1:5, upper phase). The amines were located with I_2 in light petrol.[29] The iodine was removed by evaporation and the individual amines eluted from the paper in 50% ethanol (acidified with a few drops of 0·01 N HCl). The R_f values of these amines in this solvent and the other solvent systems used during this investigation are given in Table 4.

As isoamylamine and β-phenylethylamine were poorly separated from one another by paper chromatography, mixtures of the two were resolved by chromatography on a column (0·8 × 110 cm) of Zeo-Karb 226, resin (100 mesh beads, H form) buffered at pH 7·3 with 0·2 M phosphate-citrate buffer[30] and eluted with the same buffer at pH 5·3. Fractions (5 ml) were collected at a flow rate of 40–60 ml/cm²/hr. Isoamylamine was eluted as a sharp peak in fractions 32–38, and β-phenylethylamine as a broader peak in fractions 64–72.

Administration of Radioactive Compounds to Inflorescences

Known amounts of L-leucine-U-^{14}C and L-valine-U-^{14}C (2·5 μc) in small volumes (3–5 ml) of sterile distilled water were pipetted into 1 in. diameter glass feeding vials which had been previously sterilized. Each vial was fitted with a rubber bung through which passed a glass

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[30] T. C. MCILWAINE, *J. Biol. Chem.* **49**, 183 (1921).

aerating tube, an air-leak, and a glass sleeve for the plant. Each of the sleeves was fitted with sterile plugs of cotton wool. The stems of cut inflorescences were trimmed under sterile distilled water and then allowed to stand for 1 min with the cut end in a 1% Dettol solution (a phenolic disinfectant, Reckitt & Coleman Ltd., Hull). Finally the stems were again trimmed under sterile distilled water and the inflorescences inserted in the feeding vials. The inflorescences were placed in a forced draught and given supplementary illumination for up to 48 hr. Sterile distilled water was added as required. The sterility of each remaining solution was tested at the conclusion of the feeding time. At the end of the feeding period the volatile amines were isolated as described previously.

Measurement of Radioactivity

Sample aliquots (0·1 ml) were mixed with absolute ethanol (5 ml) and 5 ml of scintillator solution [6 g of 2,5-diphenyloxazole, Nuclear Enterprises Ltd., Edinburgh, and 0·6 g of 1,4-bis 2-(4-methyl-5-phenyloxazolyl)-benzene. Thorn Electronics Ltd., in 1 l. of toulene A.R.] and the radioactivity determined using a Packard Tri-carb liquid scintillation spectrometer. Corrections were made for background, and also for quenching, by means of an improvement on the channels ratio method (Baillie[31, 32]) to give absolute count rates.

Radioautographs of all paper chromatograms of ^{14}C-labelled compounds were made with Ilford Industrial G X-ray films.

Preparation of Enzyme

Flowers (10–20 g) were placed in 40 ml of the extraction medium which contained one per cent (w/v) polyvinylpyrrolidone (Kollidon 25, MW 28,000 Badische Anilin Soda Fabriken, Germany), 0·4 M sucrose, 0·2 M tris-(hydroxymethyl) aminomethane (Fluka A.G., Buchs S.G., Switzerland), 0·01 M ethylene diamine tetra-acetic acid di-potassium salt (Sequestrene K_2, pharmaceutical grade, Geigy Co. Ltd., Manchester), 0·01 M KH_2PO_4 and 0·02 M citric acid. Prior to the addition of the flowers the pH was adjusted to the required value by the addition of H_3PO_4. All media were previously chilled and the preparative operations carried out in a cold room (2–4°). The flowers were then vacuum infiltrated with this solution for 15 min before being ground up in a mortar with acid washed sand. The macerated material was strained through 3 layers of muslin into a further 20 ml of the extraction medium. In some experiments this crude homogenate was used without further purification, in others it was first fractionated into mitochondrial and supernatant fractions by differential centrifugation.[20]

Assay

Warburg flasks which were fitted with two side-arms were used. The normal outlet to the manometer was closed with a rubber bung through which passed a glass tube fitted with a glass stop-cock. Each flask received 1 ml of enzyme preparation, and 0·5 ml of 0·2 M tris-HCl buffer, which also contained 0·01 M EDTA K_2 salt, 0·01 M KH_2PO_4, and H_3PO_4 to adjust the pH to the required value. Each flask also recived 5 ml. of a solution of pyridoxal phosphate (10 μg/ml). The flasks were flushed with nitrogen for 10 min and 0·5 ml of a solution of DL-leucine containing 2·5 μc of DL-leucine-1-^{14}C was tipped in from the side-arm. The final concentration of the leucine was 0·0125 M. In some experiments the DL-leucine-1-^{14}C was replaced by L-leucine-U-^{14}C of the same final concentration. The centre well

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[32] G. A. BRUNO, and J. E. CHRISTIAN, *Analyt. Chem.* **33**, 650 (1961).

contained 0·2 ml. of 1 N sodium hydroxide to trap the CO_2 evolved. The flasks were shaken gently in a water bath at 27° for 2–4 hrs. At the end of this period 0·3 ml of 5 N H_2SO_4 was tipped in from the second side-arm and the shaking continued for a further 1 hr. The contents of the centre well were removed by means of a micro-syringe, and the radioactivity of the dissolved CO_2 determined by liquid scintillation counting.

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