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METABOLISM OF δ-ACETYLORNITHINE IN TWO LEGUMINOUS SPECIES

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Abstract—Experiments in which radioactive amino acids were supplied to germinating mung bean (*Phaseolus aureus* Roxb.) and sainfoin (*Onobrychis viciifolia* Scop.) showed that [2-^{14}C]ornithine was converted into a number of amino acids, in particular acetylornithine (sainfoin) and proline (mung bean), whilst δ-acetyl-[2-^{14}C]ornithine was converted into α,δ-diacetylornithine in both species. Synthesis of δ-acetylornithine was demonstrated in cell-free extracts of both species; N^{α}-acetylated amino acids, acetylcoenzyme A and acetyl phosphate, but not N^{ω}-acetylated amino acids, were shown to be effective acetylating agents. Coenzyme A was not required for direct transacetylation from N^{α}-acetylated amino acids. In cell-free extracts δ-acetyl-[2-^{14}C]ornithine failed to act as either an acetyl donor or acceptor and no hydrolysis was detected. The discrepancies between *in vivo* and *in vitro* experiments are discussed.

INTRODUCTION

THE occurrence of a number of mono-acetylated amino acids has been reported in a variety of higher plants,[1,2] but reports of enzymic acetylation of amino acids have been confined to microbial and animal systems. Extracts of *Clostridium kluyveri* have been shown to be capable of synthesizing a wide range of acetylated amino acids.[3,4] The original reports suggested that acetyl phosphate was the donor compound in a direct acetylation reaction which was strikingly stimulated by the presence of cyanide. This organism is now known to contain a phosphotransacetylase enzyme capable of synthesizing acetylcoenzyme A from acetyl phosphate and coenzyme A[5] and later work has indicated that cyanide reacts with acetylcoenzyme A to form acetyl cyanide which, in the presence of suitable acceptors, promotes non-enzymic acetylation reactions.[6]

Of the two possible mono-*N*-acetyl derivatives of ornithine, δ-acetylornithine appears to be confined to higher plants whilst α-acetylornithine has been reported only in members of the Enterobacteriaceae.[1] Direct N^{α}-acetylation of ornithine has not been reported. α-Acetylornithine is derived from *N*-acetylglutamic acid in *Escherichia coli* and closely related species via the intermediate *N*-acetylglutamic-γ-semialdehyde.[7] The synthesis of *N*-acetylglutamic acid in *E. coli* requires glutamic acid and acetylcoenzyme A[8] (and not acetyl phosphate as the immediate acetyl donor), and acetylcoenzyme A is now considered to be the direct acetylating agent for many amino acids. However, Grisolia and Harmon[9,10]

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recently have reported the synthesis of δ-acetylornithine from acetyl phosphate and ornithine, using a partially-purified ornithine carbamyl transferase from frog liver. The reaction is analogous to the more usual one yielding citrulline and apparently does not require the presence of coenzyme A.

RESULTS AND DISCUSSION

Radioactive Feeding Experiments

Radioactive amino acids were provided in the water imbibed by germinating mung bean and sainfoin seeds. After a suitable period of growth, the distribution of radioactivity in the free amino acid fraction was determined by two-dimensional paper chromatography. No estimate was made of the conversion to protein-bound amino acids or other metabolites. The results obtained after feeding DL-[2-^{14}C]ornithine and δ-acetyl-DL-[2-^{14}C]ornithine are presented in Table 1.

TABLE 1. THE DISTRIBUTION OF RADIOACTIVITY IN AMINO ACIDS PRESENT IN SEEDLINGS OF SAINFOIN AND MUNG BEAN RECEIVING δ-ACETYL-[2-^{14}C]ORNITHINE OR [2-^{14}C]ORNITHINE

Radioactive chemical fed	δ-Acetyl-DL-[2-^{14}C]ornithine		DL-[2-^{14}C]ornithine	
Activity (μc)	0·478	0·319	0·225	0·170
Species	Mung bean	Sainfoin	Mung bean	Sainfoin
Seed weight (g)	0·267*	0·748†	0·250*	0·751†
Compound	Percentage of total activity in free amino acid fraction			
Aspartic acid	0·1	-	2·5	0·1
Asparagine	0·1	-	22·7	0·3
Glutamic acid	0·1	0·1	4·7	0·5
Glutamine	0·4	0·5	4·6	2·1
Ornithine	-	-	25·7	3·9
Citrulline	0·6	0·6	3·8	‡
Arginine	-	-	11·4	5·3
δ-Acetylornithine	95·6	94·6	-	78·4
α,δ-Diacetylornithine	1·5	1·1	-	1·5
Proline	0·2	2·1	40·4	5·3
γ-Aminobutyric acid		-	1·9	0·3
α-Alanine	-			0·2
β-Alanine		-		0·8
Serine	-	-	-	0·1
Ethanolamine	-	-	-	0·4
Valine	-	-	3·1	0·5
γ-Glutamyl dipeptides:				
S-Methylcysteine sulphoxide	-	-	0·8	
Phenylalanine	-	-	0·5	0·2
Tyrosine	-	-	0·1	-
Unidentified compounds (number of individual compounds in parentheses)	1·5 (5)	1·1 (3)	1·9 (2)	0·4 (2)

* Weight of dry seeds.
† Weight of seeds after aeration overnight.
‡ Glutamine and citrulline not clearly separated; total activity included as glutamine.

In both plant species ornithine was more readily converted into other amino acids than was δ-acetylornithine. Ornithine apparently was metabolized by conventional pathways to

yield arginine, proline, glutamic acid and amino acids derived from glutamic acid. In sainfoin, radioactivity was concentrated also in acetylornithine but in mung beans this compound was not labelled.

Material identified as α,δ-diacetylornithine was formed as by-product during the chemical synthesis of δ-acetyl-DL-[2-^{14}C]ornithine.[1] In the feeding experiments α,δ-diacetylornithine (identified by its chromatographic position) is considered to have been formed from δ-acetylornithine in both species and from ornithine in sainfoin. When labelled δ-acetylornithine was supplied, a number of amino acids, normally considered to be derived from ornithine, became labelled but no label was detected in ornithine. It is possible that these compounds were derived directly from acetylornithine by unidentified pathways but, since ornithine itself was converted readily to the same compounds, the failure to detect label in ornithine may have been the result of its high turnover rate.

Metabolism of δ-Acetylornithine in Extracts

Cell-free extracts were used to determine the ability of various compounds to donate their acetyl group to ornithine. Extracts from both sainfoin and mung bean seeds were

TABLE 2. THE SYNTHESIS OF δ-ACETYLORNITHINE FROM DL-[2-^{14}C]-ORNITHINE BY AN EXTRACT OF SAINFOIN SEED IN THE PRESENCE OF VARIOUS ACETYL DONORS

Reaction mixture* (additions in μmoles)	Percentage of total activity recovered in δ-acetylornithine (corrected for boiled control)
None	0
N-Acetylglutamic acid (0·053)	59·4
Acetylcoenzyme A (0·124)	15·4
N-Acetyldjenkolic acid (0·034)	0·2†
O-Acetylhomoserine (0·062)	0·3‡
γ-Acetyldiaminobutyric acid (0·061)	0·4‡

* All tubes contained sainfoin extract (1·84 mg protein/tube), DL-[2-^{14}C]-ornithine (0·02 μc), ATP (0·986 μmole) and $MgSO_4$ (0·10 μmole) in a final volume of 100 μl.

† Possibly α-acetylornithine.

‡ Possibly β-aminopiperidone.

shown to enzymically synthesize acetylornithine (Tables 2–4). Co-chromatography with authentic δ-acetylornithine was used to identify the isomer synthesized as δ- and not α-acetylornithine. A number of extracts formed a radioactive compound which was tentatively identified as β-aminopiperidone (formed by cyclization of ornithine)[11] on the basis of its chromatographic position in butan-1-ol–acetic acid–water. As the synthesis of β-aminopiperidone appeared to be enzymic and was usually associated with active acetylornithine synthesis it is suggested that the formation of β-aminopiperidone arises as a by-product of the acetylating enzyme.

The results reported in Tables 2 and 3 indicate that no endogenous acetylating agent was present in the dialysed extracts of sainfoin. These extracts could synthesize acetylcoenzyme A from coenzyme A and pyruvate (Table 3) and this compound has been shown to be capable of acetylating the δ- and not the α-amino group of ornithine. However *N*-acetylglutamic

[11] C. T. G. VAN DER HORST, *Nature* **196**, 147 (1962).

acid is an appreciably better acetyl donor than is acetylcoenzyme A (Table 2). α-Acetylornithine also donates its acetyl group to ornithine to give the δ-acetyl derivative but amino acids acetylated elsewhere than on the α-amino group, e.g. *O*-acetylhomoserine, *N*-acetyldjenkolic

TABLE 3. THE EFFECT OF VARIOUS ACETYL DONORS ON δ-ACETYLORNITHINE SYNTHESIS FROM DL-[2-^{14}C]-ORNITHINE BY AN EXTRACT OF SAINFOIN SEEDS*

Donor compound	Additions to basal reaction mixture†		
	None	Coenzyme A	Acetylcoenzyme A
α-Acetylornithine	74·3 (1·6)	68·3 (2·5)	71·9‡ (4·5)
N-Acetylglutamic acid	73·8 (N.D.)	68·3 (5·9)	67·4‡ (5·1)
δ-Acetylornithine	N.C. (N.C.)	N.C (N.C.)	22·4‡ (N.D.)
Pyruvate	N.C. (N.C.)	2·0 (N.D.)	28·9 (2·2)

* The radioactivity determined in δ-acetylornithine formed is expressed as a percentage of total activity recovered from the chromatograms (figures in parenthesis are for β-aminopiperidone).

† All tubes contained sainfoin extract (1·64 mg protein/tube), DL-[2-^{14}C]-ornithine (0·02 μc), ATP (0·020 μmole) and $MgSO_4$ (0·10 μmole) in a final volume of 100 μl. Reaction additions consisted of *N*-acetylglutamic acid (0·053 μmole), α- or δ-acetylornithine (0·057 μmole), acetylcoenzyme A (0·007 μmole), coenzyme A (0·008 μmole) and pyruvate (1·817 μmole).

‡ Results uncorrected for boiled control.

N.C. = Not counted and probably not detectable. N.D. = Not detectable although possibly included in the acetylornithine spot.

TABLE 4. THE EFFECT OF pH AND THE NATURE OF THE ACETYL DONOR ON δ-ACETYLORNITHINE SYNTHESIS FROM DL-[2-^{14}C]-ORNITHINE IN EXTRACTS OF MUNG BEAN SEEDS

Reaction mixture*		Percentage of total recovered activity		
pH	Additions	Glutamic acid	δ-Acetyl-ornithine	β-Amino-piperidone
7·4	None	0	2·6	1·1
7·4	δ-Acetylornithine	0	2·1	0
7·4	*N*-Acetylglutamic acid	0·7	36·0	1·8
7·4	α-Acetylornithine	0	39·7	0
8·6	None	0·3	6·9	0
8·6	δ-Acetylornithine	0·5	5·8	0
8·6	*N*-Acetylglutamic acid	2·0	18·4	0
8·6	α-Acetylornithine	2·0	40·0	0

* All tubes contained mung bean extract (5·37 mg protein/tube, at pH 7·4; 3·74 mg protein/tube at pH 8·6) and DL-[2-^{14}C]ornithine (0·02 μc) in a final volume of 100 μl. Reaction additions consisted of *N*-acetylglutamic acid (0·053 μmole), α- or δ-acetylornithines (0·057 μmole).

acid, γ-acetyldiaminobutyric acid (Table 2) and δ-acetylornithine (Table 3) appear to be incapable of participating in δ-acetylornithine synthesis. The failure of added acetylcoenzyme A or coenzyme A to stimulate acetylornithine synthesis from either *N*-acetylglutamic acid or α-acetylornithine (Table 3) suggests that synthesis is by direct acetyl transfer from the amino acid and not by the intermediary formation of acetylcoenzyme A.

A similar specificity for the acetyl-donor amino acid was shown by dialysed extracts of mung bean (Table 4). Again δ-acetylornithine appears incapable of transferring its acetyl group to ornithine. There is a suggestion that different pH optima exist for acetylornithine synthesis when *N*-acetylglutamic acid or α-acetylornithine act as donor compounds, the latter functioning better at slightly higher pH values (Table 4).

The synthesis of δ-acetylornithine from ornithine and acetyl phosphate is catalysed by the ornithine carbamyl transferase from frog liver[9, 10] (see Introduction). Several extracts of sainfoin were shown to effect citrulline synthesis from ornithine and carbamyl phosphate. A similar synthesis of δ-acetylornithine from ornithine and acetyl phosphate was observed using these extracts but, whereas citrulline synthesis was abolished when boiled extracts were used, considerable amounts of δ-acetylornithine still were formed under these conditions. In this series of enzymic experiments, reactions were terminated by addition of ethanol to a final concentration of 75%: later experiments established that in aqueous ethanolic solutions, synthesis of δ-acetylornithine from acetyl phosphate and ornithine occurred in the absence of seed extracts. In the absence of ethanol Grisolia and Harmon[9, 10] were unable to demonstrate a similar chemical formation of acetylornithine, although their actual experimental controls consisted of the complete reaction mixture minus ornithine (Grisolia, personal communication). In a further enzymic experiment (using the dialysed mung bean extract referred to in Table 4), aliquots of the reaction mixture were spotted directly (without addition of ethanol) onto a chromatogram which was developed in the usual way to separate δ-acetylornithine. Acetylornithine synthesis from [2-^{14}C]ornithine (0·02 μc) and acetyl phosphate (1·6 μmoles) was observed in this experiment at both pH 7·4 and pH 8·6; the acetylornithine contained 34 and 66 per cent respectively of the total activity recovered. In the presence of ATP (0·4 μmole) slightly less acetylornithine was synthesized, e.g. pH 7·4, 23 per cent and pH 8·6, 58 per cent. When these results are taken in conjunction with Grisolia's failure to observe chemical synthesis of acetylornithine under aqueous conditions, it seems likely that acetyl phosphate can serve as a donor in the enzyme-catalysed acetyl group transfer in mung bean extracts.

The ability of the sainfoin extract (referred to in Table 2) to metabolize δ-acetyl-DL-[2-^{14}C]ornithine (0·016 μc), was tested both alone and with various additions. No labelled ornithine was produced in the absence of additional compounds (i.e. hydrolysis) or in the presence of glutamic acid (0·068 μmole), ornithine (0·076 μmole) or coenzyme A (0·006 μmole) (i.e. transacetylation with δ-acetylornithine as donor). Diacetylornithine was not formed on incubation with α-acetylornithine (0·057 μmole), acetylglutamic acid (0·053 μmole) or acetylcoenzyme A (0·124 μmole) (i.e. transacetylation with δ-acetylornithine as acceptor). Similar stability of δ-acetylornithine in mung bean extracts was noted in other experiments.

Comparison of these *in vivo* and *in vitro* experiments indicated that mung beans are potentially capable of synthesizing δ-acetylornithine but that *in vivo* this reaction is inactive for some reason. In sainfoin no such blockage of δ-acetylornithine synthesis was observed. In both species limited metabolism of δ-acetylornithine was observed *in vivo* but none could be demonstrated *in vitro*. A distinctive feature of both species was their similar order of conversion of δ-acetylornithine to α,δ-diacetylornithine. Assuming that both amino groups are acetylated by the same donor compound, the failure of mung bean seeds to synthesize δ-acetylornithine may be due to the acetylating reaction being a poor competitor for available ornithine and not to their lack of a suitable endogenous acetylating agent.

Another feature common to both plant species was their ability to convert considerably more than half of the labelled ornithine supplied to them into other radioactive compounds. This occurred even though the ornithine used was a DL-racemate, indicating that the seedlings either were able to directly metabolize the D-isomer by normal intermediary pathways or, more probably, contained an active racemase for the D-ornithine.

EXPERIMENTAL

Plant material. Sainfoin seeds germinated poorly overnight when planted in moist vermiculite but the percentage germination was increased by aerating seeds in distilled water overnight at room temperature. Material treated in this way was used for all experiments. In contrast mung bean seeds show nearly 100 per cent germination overnight so that labelled compounds were supplied during the initial stages of water imbibition. Seeds aerated overnight in distilled water were used for the preparation of enzyme extracts. Protein concentrations were determined by the method of Lowry *et al.*[12]

Radioactive compounds. DL-[2-^{14}C]-ornithine (1·93 mc/mM) was obtained from Volk Radiochemical Co., Ill. From this, δ-acetyl-DL-[2-^{14}C]-ornithine was synthesized by the method of Neuberger and Sanger[13] and isolated from the reaction mixture by chromatography in butan-1-ol–acetic acid–water.[1]

Chromatography methods. The labelled products formed in the feeding experiments were separated on two-dimensional paper chromatograms run on Whatman No. 3MM filter paper using 75% (w/w) phenol in an atmosphere of ammonia as first solvent, followed by butan-1-ol–acetic acid–water (90:10:29, by vol.). In all other experiments, separations were performed on one-dimensional chromatograms (Whatman No. 4 filter paper) developed in butanol–acetic acid–water. Radioactive compounds were located on radioautographs (Kodirex X-ray film) after their exposure to the chromatograms for periods of 1–3 weeks. Radioactivity in each compound was determined by Geiger scanning of chromatograms (efficiency about 1%).

Radioactive feeding experiments. Radioactive amino acids were supplied in limited volumes of water imbibed during the onset of germination of either aerated sainfoin or untreated mung bean seeds. The previously aerated sainfoin seeds were allowed to develop for a further 24 hr at 28° and mung bean seeds were grown for a total of 48 hr at 28°. At the end of the growth period, the seeds were washed four times with 5 ml water and partially dried by blotting with filter paper. The seeds were then ground in 75% (v/v) ethanol (20 ml), shaken for 24 hr and filtered. The clear filtrate and washings were then applied to small ZeoKarb 215 columns (10 × 0·8 cm, H^+ form). Each column was washed with 75% (v/v) ethanol (150 ml) and then the amino acids were eluted with 50 ml of 35% (w/v) ammonia–ethanol (1:3, v/v). The eluate was evaporated at laboratory temperature, and the whole of the radioactive residue was used for chromatography.

Cell-free enzymic experiments. Seeds were precooled and all subsequent operations were performed at 0–4°.

Aerated sainfoin seeds (25 g) were ground by hand in 50 ml of 0·1 M-phosphate buffer (KH_2PO_4 adjusted to pH 7·4 with KOH), filtered through muslin, and centrifuged for 30 min at 26,000 *g*. The resulting supernatant was brought to 60 per cent saturation by addition of solid $(NH_4)_2SO_4$; precipitated protein was sedimented by centrifugation for 30 min at

[12] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[13] A. NEUBERGER and F. SANGER, *Biochem. J.* **37**, 515 (1943).

26,000 *g* and then redissolved in 0·1 M-phosphate buffer (2 ml). After dialysis against 0·1 M-phosphate buffer overnight, the extract was again centrifuged for 30 min at 26,000 *g*, to yield the supernatant used in the enzymic studies.

Aerated mung bean seeds (50 g) were ground by hand in 50 ml 0·1 M-tris–0·1 M-sodium citrate buffer (adjusted to pH 9·9 with HCl), filtered through muslin, and centrifuged for 30 min at 26,000 *g*. Portions of the supernatant (1 ml) were adjusted to either pH 7·4 or pH 9·0 with 0·1 N HCl and dialysed overnight against 0·1 M-phosphate buffer, pH 7·4, or 0·1 M-tris–HCl buffer, pH 9·0, respectively. The dialysed extracts (measured pH 7·4 and 8·6 respectively) were used directly in the enzymic experiments.

All reaction mixtures (compositions as in Tables 2–4) were incubated for 3 hr at 28° and, unless otherwise stated, the reaction was terminated by the addition of three volumes of ethanol. After sedimentation of the protein by centrifugation, three-quarters of each sample was used for chromatography.

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