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THE BIOGENESIS OF γ-CONICEINE IN HEMLOCK (*CONIUM MACULATUM* L.)

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Abstract—Feeding of uniformly-labelled L-lysine-^{14}C, DL-α-aminoadipic acid-6-^{14}C and uniformly-labelled Δ^1-piperideine-^{14}C to young plants of Hemlock leads to incorporation of radioactivity into γ-coniceine (2-*n*-propyl-Δ^1-piperideine), the major alkaloid of the vegetative parts of the plant. Δ^1-Piperideine is regarded as the immediate precursor of the ring structure of γ-coniceine and α-aminoadipic acid-δ-semialdehyde (Δ^1-piperideine-6-carboxylic acid) appears to be a key intermediate in the biogenesis of γ-coniceine from lysine and α-aminoadipic acid.

Feeding of cadaverine-1-5-^{14}C and uniformly-labelled α-keto-ε-amino caproic acid-^{14}C (Δ^1-piperideine-2-carboxylic acid) also leads to incorporation of radioactivity into γ-coniceine but it is not yet known whether these compounds are natural precursors of the alkaloid. Incorporation of radioactivity into γ-coniceine also takes place after feeding with sodium propionate-2-^{14}C and it is thought that the side-chain of the alkaloid may be derived from this compound.

INTRODUCTION

PREVIOUS work on the distribution of the alkaloids in tissues of Hemlock in various stages of development has shown that γ-coniceine (2-*n*-propyl-Δ^1-piperideine) is the major alkaloid in seedlings, in the vegetative parts of mature plants and in the early stages of fruit formation.[1–3] Mann and Smithies[4] have shown the presence of a diamine oxidase in pea seedlings which catalyses the oxidative deamination of cadaverine and lysine with the production of Δ^1-piperideine and Δ^1-piperideine-6-carboxylic acid respectively. By use of this enzyme, Hasse and Berg[5] and Mothes and his co-workers[6] showed that Δ^1-piperideine resulting from the oxidative deamination of cadaverine is the precursor of the piperideine ring of anabasine. Leete[7,8] obtained evidence of the incorporation of radioactivity into anabasine after feeding cadaverine-1-5-^{14}C and L-lysine-2-^{14}C to plants of *Nicotiana glauca* and Schiedt and Höss[9] claim to have obtained radioactive coniine from leaves of Hemlock plants which were fed with uniformly-labelled L-lysine-^{14}C. In the present investigation a study has been made of the pathways leading to the biogenesis of γ-coniceine in young plants of Hemlock by feeding possible ^{14}C-labelled precursors of the alkaloid.

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RESULTS AND DISCUSSION

TABLE 1. INCORPORATION OF ^{14}C INTO γ-CONICEINE FROM POSTULATED PRECURSORS FED TO YOUNG PLANTS OF *Conium maculatum* L

Experiment No. and date	Precursor and where fed	Wt. of precursor fed (mg)	Total activity of precursor (cpm $\times 10^{-4}$)	Time of expt (days)	Total activity γ-coniceine (cpm $\times 10^{-4}$)	% Incorporation of radioactivity into γ-coniceine ($\times 10^{2}$)
1: 21.6.62	Lysine-HCl-^{14}C (U)	1.0	30	2	22	74
	Excised leaves	1·0	28	4	7·8	28
		1·0	30	6	21	70
2: 21.6.62	Lysine-HCl-^{14}C (U)	1·0	23	2	14	60
	Excised leaves	1·0	30	4	4·6	20
		1·0	23	6	18	80
3: 8.7.62	Lysine-HCl-^{14}C (U)	1·0	33	6	2·8	8·0
	Via roots	1·0	33	4	2·8	8·0
		1·0	33	2	0·36	1·0
4: 7.8.62	Lysine-HCl-^{14}C (U)	0·129	26	1	0·13	5·0
	Excised leaves	0·129	38	2	0·57	1·5
		0·129	41	3	1·6	40
		0·129	41	4	1·7	41
5: 21.8.62	Lysine-HCl-^{14}C (U)	0·021	27	2	0·60	2·4
	Via roots	0·021	39	4	1·2	4·5
		0·021	35	6	0·96	3·0
		0·021	32	8	0·48	1·3
6: 9.8.63	Lysine-HCl-^{14}C (U)	0·052	58	2	38	60
	Via petiole	0·052	58	4	29	52
		0·052	58	6	24	42
		0·052	58	8	13	22
7: 7.8.63	α-Aminoadipic acid-6-^{14}C	1·0	9·2	4	1·4	27
	Via petiole	1·0	9·2	2	1·5	16
		1·0	9·2	6	5·0	54
		5·0	46	2	7·33	16
		5·0	46	6	0·60	1·3
8: 20.8.63	α-aminoadipic acid-6-^{14}C	5·0	7·8	4	8·7	110
	Via petiole	5·0	7·8	5	7·0	90
		5·0	7·8	6	2·9	37
		5·0	7·8	7	6·6	85
9: 10.9.63	Cadaverine-1-5-^{14}C dihydrochloride	5·0	5·9	2	1·5	25
	Via petiole	5·0	5·9	4	3·3	50
		5·0	5·9	6	2·9	46
		5·0	5·9	8	0·84	13
10: 16.10.63	Cadaverine-1-5-^{14}C dihydrochloride	4·0	4·8	2	0·69	14
	Via petiole	4·0	4·8	4	0·73	15
		4·0	4·8	6	1·7	36
11: 17.8.62	Na-propionate-2-^{14}C	0·11	28	1	4·2	19
	Excised leaves	0·11	25	2	6·0	23
		0·11	27	3	12	43
		0·11	29	4	37	130

TABLE 1—*continued*

Experiment No. and date	Precursor and where fed	Wt. of precursor fed (mg)	Total activity of precursor (cpm $\times 10^{-4}$)	Time of expt (days)	Total activity γ-coniceine (cpm $\times 10^{-4}$)	% Incorporation of radioactivity into γ-coniceine ($\times 10^2$)
12: 21.8.62	Na-propionate-2-^{14}C	0·113	26	4	0·48	1·9
	Via roots	0·113	26	8	0·42	1·8
13: 24.9.62	Na-propionate-2-^{14}C	0·113	26	3	0·42	1·7
	Via roots	0·113	26	4	0·35	1·4
		0·113	26	6	0·42	1·7
14: 3.10.62	Na-propionate-2-^{14}C	0·113	26	2	3·6	14
	Via petiole	0·113	26	2	3·0	11
	1–2 year plants					
15: 16.10.62	Na-propionate-2-^{14}C	0·113	14	4	2·1	15
	Via roots	0·113	15	6	1·7	11
16: 15.8.63	Na-propionate-2-^{14}C	0·560	84	3	3·8	4·5
	Via petiole	0·560	84	7	5·3	6·2
		0·560	84	9	3·5	4·1
17: 14.11.62	Δ^1-piperideine-^{14}C (U)	0·367	9·5	6	180	1860
	Via petiole	0·367	9·5	6	160	1700
		0·367	9·5	6	120	1300
18: 19.2.63	Δ^1-piperideine-^{14}C (U)	0·596	7·3	4	30	400
	Via roots	0·596	7·3	8	110	1500
19: 19.2.63	α-keto-ε-amino-caproic acid-^{14}C (U)	0·128	1·3	4	12	890
	Via petiole	0·128	1·3	6	28	2300
		0·128	1·3	8	29	3700

Note: In experiments 1, 2 and 3, Lysine-HCl-^{14}C (U) was diluted × 10 with unlabelled lysine.

The results of the present series of experiments (Table 1) show that feeding of Δ^1-piperideine-^{14}C and α-keto-ε-aminocaproic acid-^{14}C (Δ^1-piperideine-2-carboxylic acid) into young Hemlock plants leads to a high degree of incorporation of radioactivity into γ-coniceine isolated from the tissues. In the light of recent work[10] on the structure of γ-coniceine, which is now represented as 2-*n*-propyl-Δ^1-piperideine, these findings suggest that Δ^1-piperideine is the immediate precursor of γ-coniceine in the plant. The work of Mann and Smithies[4] has shown that the amine oxidase system of pea seedlings catalyses the oxidative deamination of cadaverine and lysine with the formation of 5-aminopentanal and α-aminoadipic acid-δ-semialdehyde which undergo spontaneous cyclization with the formation of Δ^1-piperideine and Δ^1-piperideine-6-carboxylic acid respectively. Since feeding of both cadaverine-1-5-^{14}C and universally-labelled L-lysine-^{14}C to Hemlock plants leads to incorporation of radioactivity into γ-coniceine it is probable that oxidative deamination of one of the amino groups of cadaverine and the ε-amino group of lysine is the first step in the biogenesis of γ-coniceine from these compounds. However, work in this laboratory has so far failed to provide conclusive evidence for the presence of an amine oxidase in the tissues of Hemlock which catalyses the oxidation of cadaverine and lysine. The presence in young plants of an enzyme which catalyses a slow transamination of an amino group from

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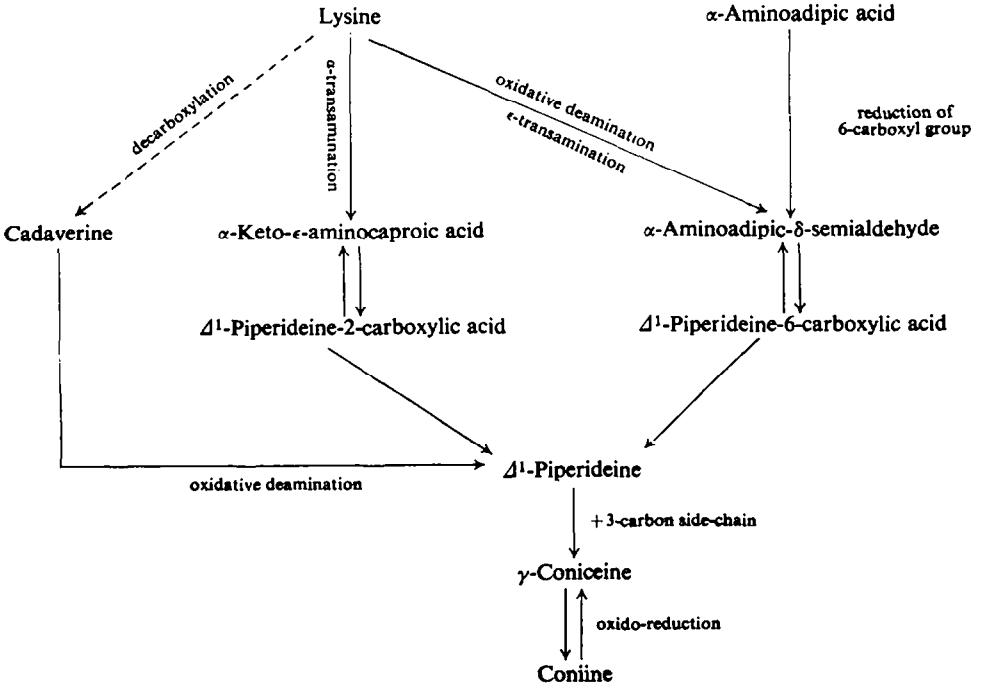
lysine to α-ketoglutaric acid with the formation of glutamic acid has been established. It is not yet known whether this transamination involves the α- or the ε-amino group of lysine. Fincham[11] has shown the presence of an enzyme in *Neurospora crassa* which catalyses the transfer of the δ-amino group of L-ornithine to α-ketoglutaric acid with the formation of glutamic acid and glutamic-γ-semialdehyde. If the lysine transaminase of Hemlock is similar to the *Neurospora* enzyme, the ε-amino group of lysine will be transferred to α-keto glutaric acid with the formation of α-aminoadipic-δ-semialdehyde (Δ^1-piperideine-6-carboxylic acid). Alternatively, if the α-amino group of lysine is transferred to α-ketoglutaric acid, α-keto-ε-



SCHEME 1. PATHWAYS OF BIOGENESIS OF γ-CONICEINE.

amino caproic acid (Δ^1-piperideine-2-carboxylic acid) will be formed. By use of ^{15}N-labelled lysine it is hoped to discover which of the two amino groups of lysine participates in the transamination reaction. Incorporation of radioactivity from α-aminoadipic acid into γ-coniceine suggests that reduction of the 6-carboxyl group of α-aminoadipic acid takes place with the formation of α-aminoadipic-δ-semialdehyde (Δ^1-piperideine-6-carboxylic acid) as the intermediate compound. An enzyme catalysing the reduction of α-aminoadipic acid has been prepared from yeast by Sagisaka and Shimura[12,13] and by Larson, Sandine and Broquist.[14] An attempt is now being made to obtain an enzyme preparation from the tissues of Hemlock which will catalyse this reaction. In Scheme 1 the postulated pathways leading to the biogenesis of γ-coniceine are summarized.

Meister, Radhakrishnan and Buckley[15] have shown that Δ^1-piperideine-2-carboxylic

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acid is reduced to pipecolic acid (piperideine-2-carboxylic acid) by a pyridine-nucleotide-dependent enzyme present in the tissues of *Pisum sativum* L. and *Phaseolus Mungo* L. var. *radiatus* and the work of Fowden[16] provides evidence that in *Acacia* phyllodes, pipecolic acid is formed primarily from lysine via α-aminoadipic-δ-semialdehyde and Δ^1-piperideine-6-carboxylic acid. In mutants of *Aspergillus nidulans* (Eidam) Wint., α-aminoadipic acid is the major precursor of pipecolic acid which is regarded as an intermediate in the biosynthesis of lysine from α-aminoadipic acid.[17] Pipecolic acid is of widespread occurrence in plants and in some plants appears to be a major constituent of the soluble nitrogen fraction. The presence of pipecolic acid in the tissues of young plants of Hemlock could not be established by paper chromatography but it has been discovered in small amount in mature plants.[18] Unsaturated alkaloids which appear to be closely related to γ-coniceine have been found by us in seedlings and young plants of *Punica granatum* L. (unpublished work) but so far as is known, Hemlock is the only plant which produces γ-coniceine in quantity. It is probable therefore that the sequence of reactions leading to the biogenesis of γ-coniceine is peculiar to Hemlock and that the critical factor is the extent to which reduction of one or both of the Δ^1-piperideine carboxylic acids to pipecolic acid takes place. If reduction is slow the Δ^1-piperideine nucleus would become available for the production of γ-coniceine. No evidence has yet been obtained for the presence in Hemlock tissues of an enzyme which catalyses the decarboxylation of the Δ^1-piperideine-carboxylic acids, and attempts to show the presence of lysine decarboxylase which would lead to the formation of cadaverine have failed. It is unlikely therefore that cadaverine is a natural precursor of γ-coniceine. The incorporation of radioactivity into γ-coniceine after feeding with propionate-2-^{14}C suggests that the propyl side-chain of the alkaloid might be derived directly from propionate. If this is so, radioactivity should be found principally in the side-chain. On the other hand, if propionate is metabolized through the Krebs cycle via acetate, a known precursor of lysine,[19] labelling should occur in the ring system of γ-coniceine. The position of labelling in γ-coniceine isolated from the plants after feeding with propionate-2-^{14}C is now being investigated by degradation of the alkaloid and the results of this work should indicate the extent to which propionate contributes to the side-chain.

EXPERIMENTAL

Materials and Methods

The plants of Hemlock used in these experiments were obtained from seed sown in coarse sand in Buchner funnels. After a period of 5–6 weeks the seedlings were transferred to small plant pots containing acid-washed Bedford sand and grown on in a cool greenhouse with supplementary lighting from Masda MBTR/U 200-W reflector lamps for a period of 3–5 weeks. During this period the plants were watered with culture solution twice a week and on intervening days with distilled water. Feeding of the postulated precursors of γ-coniceine was carried out on young plants which had produced 5 or 6 leaves, either through the root system or via cut petioles.

For root feeding, the young plants were removed from the pots, the roots washed free from sand and rinsed with a 2% v/v solution of Dettol (Reckitt & Colman Ltd.). They were

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then placed in 180-ml sterilized bottles and the roots covered with approximately 100 ml of distilled water containing 30 mg/l. of aureomycin to check bacterial growth. The radioactive substances were added as required and the plants placed under 200-W Mazda lamps in a gentle current of air maintained by a fan. During the course of the experiment the solutions were aerated continuously. After a period of 2 days, most of the radioactive material was taken up by the root system and the plants were then transferred to culture solutions for periods varying from 2 to 8 days before harvesting. Any radioactivity remaining in the residual solution was measured before the transfer of the plants to the culture solution.

Feeding via the petioles was carried out as follows. The leaf blade of one of the lower leaves of young plants growing in sand was removed with a sharp razor and the petiole introduced into a small tube (1 ml) containing 0·1–0·2 ml of distilled water containing the labelled compounds. After uptake of the solution was complete, the cut ends of the petioles were placed in distilled water to ensure that the labelled compounds were completely drawn into the plant. Depending upon the conditions of the experiment, uptake of solution was complete in 3–48 hr. When weather conditions permitted, experiments were made out-of-doors. On a sunny day with a gentle breeze, uptake of the solution was usually complete in 6 hr. Under glasshouse conditions with supplementary lighting and in a current of air, uptake of solution was usually complete within 48 hr. A third series of experiments was carried out using detached leaves of mature plants, the petioles of which were placed in small bottles (20–30 ml capacity) each containing 20 ml of solution of the labelled compounds. During the experimental period the solutions were aerated continuously. Uptake of the solution was usually rapid and, when complete, the leaves were placed in culture solution for a period of up to 4 days before harvesting.

Extraction and Estimation of γ-Coniceine

After the experimental period, the plants were killed by boiling in ethanol for 1 min. The ethanol was decanted off and the plant material ground in a mortar with a further small portion of ethanol which was then removed after the cell debris had been centrifuged down. The residue was extracted with three further portions of ethanol and the ethanol extracts combined. The combined extracts were acidified with 2–3 drops of 10% v/v hydrochloric acid and the ethanol completely removed on a water bath at 50°. Two methods were used for the removal of γ-coniceine from the residue.

In the first method chromatographic separation of γ-coniceine was carried out by extracting the residue with water and applying the solution in a band on Whatman No. 3MM paper. The chromatogram was run in the solvent system *tert.*-pentanol–*tert.*-butanol–N HCl (9:3:2 by volume) and the γ-coniceine located by the application of a 0·2% w/v solution of iodine in light petroleum. The band was cut out and the alkaloid eluted with water. The eluate was made to volume (25 or 10 ml) and the solution used for the determination of the content of γ-coniceine and estimation of radioactivity. In the second method the residue was extracted with water and the solution shaken with chloroform to remove colouring matter. The chloroform was run off, the aqueous solution made alkaline with sodium carbonate solution and shaken out with chloroform. The alkaloids were removed from the chloroform by extraction with three portions of N hydrochloric acid. The acid solution was evaporated to dryness on a water bath at 60°, the residue dissolved in distilled water (25 or 10 ml) and the solution used for estimation of γ-coniceine and radioactivity. Estimation of γ-coniceine was carried out by using the nitroprusside method of Cromwell.[1] The colour was measured at 490 mμ in a Unicam SP 600 spectrophotometer.

Measurements of Radioactivity

Measurements of ^{14}C were made by use of an I.D.L. Counter 1700 with a quench time of 400 μsec, 650 V and total disc bias. A Geiger tube MX 123, of window thickness 2·5 mg/cm^2 and dead time 85 μsec was used. To eliminate any errors due to self absorption the same weights of precursors and of alkaloid (50–150 μg) were placed on each planchet. To facilitate uniform spreading of the radioactive solutions on the planchets, a drop of soap solution (slightly acidified with dilute hydrochloric acid) was placed on the planchets prior to the addition of the solutions to be counted. Planchets were dried on a warm plate at 40°. For substances of high activity the necessary correction for the dead time of the instrument was made by use of the formula $N = n/(1-nt)$ where N = corrected count rate, n = observed count rate/sec, t = quench time in seconds. Background counts were made on each set of samples counted. For samples of low activity, the counting errors were reduced to *ca.* 1% by estimating the time taken for 10^4 counts.

Radioactive Compounds

L-Lysine-^{14}C (U) monohydrochloride (specific activity, 38·9 μc/mg) and sodium propionate-2-^{14}C (specific activity 38·5 μc/mg) were obtained from the Radiochemical Centre, Amersham. α-Aminoadipic acid-6-^{14}C was prepared by the method of Borsook *et al.*[20] from potassium cyanide-^{14}C (specific activity, 262 μc/mg) obtained from the Radiochemical Centre, Amersham. Cadaverine-1-5-^{14}C-dihydrochloride was prepared from 1-3-dibromopropane and potassium cyanide-^{14}C (specific activity 262 μc/mg) by the method of Leete.[8] Δ^1-Piperideine-^{14}C was prepared from L-lysine-^{14}C (U) monohydrochloride by the method of Jackoby and Fredericks.[21] α-Keto-ε-aminocaproic acid was prepared from L-lysine-^{14}C (U) monohydrochloride by the method of Aspen and Meister.[22]

Culture Solution

The composition of the culture solution used throughout the foregoing experiments was as follows: 1. Stock solution:—$Ca(NO_3)_2.4H_2O$ 5 g; KNO_3, 20 g; $MgSO_4.7H_2O$, 10 g; $NH_4H_2PO_4$, 4 g; ferric chloride (anhydrous), 0·01 g; distilled water to 2 l. 2. Micronutrient solution:—Boric acid, 0·60 g; $MnCl_2.4H_2O$, 1·81 g; $ZnSO_4.7H_2O$, 0·11 g; $CuCl_2.2H_2O$, 0·05 g; $Na_2MoO_4.2H_2O$, 0·025 g; distilled water to 1 l.

For use, 50 ml of stock solution and 0·5 ml of micronutrient solution were diluted to 1 l. with distilled water.

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