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ORIGIN OF THE METHYL CARBON OF METHYL CONIINE IN *CONIUM MACULATUM*

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Key Word Index—*Conium maculatum*; Umbelliferae; hemlock; alkaloid biogenesis; methyl coniine; L-methionine.

Abstract—Studies with [methyl-^{14}C]-L-methionine have established that the methyl carbon of L-methionine can act as a precursor of the *N*-methyl group of methyl coniine in *Conium maculatum*.

INTRODUCTION

TRANSMETHYLATION is well established in animals[1] and there is now evidence that L-methionine is the major donor of *O*- and *N*-methyl groups in plants.[2] In particular it has been established using radioactive tracer techniques that the *O*- and *N*-methyl groups of the piperidine and pyrrolidine alkaloids originate in this manner. The methyl carbon of L-methionine has been found to serve as a precursor of the *O*- and *N*-methyl groups of ricinine,[3] the activity of these two groups accounting, within the limits of experimental error, for all the activity in the ricinine molecule. The two groups also exhibited similar activity, indicating that the enzymes responsible for the transfer of the methyl group of L-methionine must in both instances be comparable. Similarly, it was found that the *N*-methyl of hyoscyamine in *Datura stramonium*[4] and the *N*-methyl of nicotine in *Nicotiana rustica*[5] originated from the methyl group of L-methionine. The present work investigates the further utilisation of L-methionine in the formation of methyl coniine in *Conium maculatum*.

RESULTS AND DISCUSSION

Since whole plants of *C. maculatum* cv. Bowles had amounts of methyl coniine which were easily extracted from the leaves at flowering (0·14 mg/g fr. wt), three plants were wick-fed with [methyl-^{14}C]-L-methionine and harvested after 4 or 6 days. Incorporation of radioactivity into the methyl coniine was found to be low (0·05%) and subsequent experiments with cut fruit panicles gave a much improved incorporation of radioactivity (1·5%). The unripe fruits from a number of varieties of *C. maculatum* were used for the experiments shown in Tables 1 and 2. At 9–21 days from flowering the different varieties had a similar

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concentration of total alkaloids per g, but the concentrations of the individual major alkaloids, coniine, methyl coniine and γ-coniceine varied considerably. As early as 9 days from flowering the major alkaloid in the Messina and Bowles fruits was methyl coniine, whilst in Minnesota and Chelsea the major alkaloids were coniine and γ-coniceine respectively. As the fruits ripened the only significant changes in alkaloid composition took place in the Chelsea fruits where at 3·5 weeks from flowering coniine accounted for some 66% of the total alkaloids and γ-coniceine for 32·5%, methyl coniine occurring only in trace amounts.

TABLE 1. THE INCORPORATION OF [METHYL-^{14}C]-L-METHIONINE INTO METHYL CONIINE IN THE FRUITS OF A NUMBER OF VARIETIES OF *Conium maculatum*

Expt. no.	Dates (a) flowering (b) feeding	Var. of hemlock fruits	No. of panicles and type	Fr. wt (g)	Feeding time (hr)	[Methyl-^{14}C]-L-methionine administered Total activity (dpm × 10^6)	Incorpn. of ^{14}C into total alkaloids (dpm × 10^4)	% Incorpn.	Yield of total alkaloids (mg)	Yield of individual alkaloids (a) coniine (b) Me coniine (c) γ-coniceine (mg)	Sp. act. (dpm/mmol × 10^5)
1	(a) 7/6/73 (b) 21/6/73	Messina	11 MP	25·9	24	24·2	43·0	1·8	152	(a) 2·0 (b) 150·0 (c) 0·0	0·0 5·3 0·0
2	(a) 7/6/73 (b) 21/6/73	Messina	12 MP	22·1	48	13·2	15·0	1·25	106	(a) 9·0 (b) 97·0 (c) 0·0	0·0 3·5 0·0
3	(a) 7/6/73 (b) 21/6/73	Messina	12 MP	26·1	48	26·4	32·0	1·3	121	(a) 9·2 (b) 109·0 (c) 0·0	0·0 5·3 0·0
4	(a) 7/6/73 (b) 21/6/73	Bowles	12 MP	26·1	48	13·2	19·0	1·5	116	(a) 9·6 (b) 106·4 (c) 0·0	0·0 3·5 0·0
5	(a) 7/6/73 (b) 21/6/73	Bowles	10 MP	25·5	48	22·0	20·0	0·9	64	(a) 12·4 (b) 51·6 (c) 0·0	0·0 7·0 0·0
6	(a) 21/6/73 (b) 2/7/73	Bowles	13 MP	33·0	24	14·3	1·8	0·15	124	(a) 10·8 (b) 114·0 (c) 0·0	0·0 0·18 0·0
7	(a) 21/6/73 (b) 2/7/73	Bowles	12 MP	34·0	24	26·4	4·2	0·20	112·4	(a) 4·2 (b) 98·0 (c) 0·0	0·0 0·7 0·0
8	(a) 7/6/73 (b) 16/7/73	Messina	13 MP	15·0	48	14·3	3·5	0·24	32·7	(a) 1·7 (b) 31·0 (c) 0·0	0·0 1·9 0·0
9	(a) 9/7/73 (b) 16/7/73	Chelsea	13 SP	13·0	48	14·3	0·1	0·07	95·8	(a) 0·43 (b) 0·29 (c) 95·0	0·0 6·2 0·0
10	(a) 9/7/73 (b) 18/7/73	Minnesota	11 SP	19·7	6	12·1	1·5	0·13	130	(a) 128·0 (b) 2·1 (c) 0·0	0·0 12·5 0·0
11	(a) 9/7/73 (b) 18/7/73	Minnesota	11 SP	20·5	12	12·1	1·6	0·13	118	(a) 115·0 (b) 2·5 (c) 0·0	0·0 11·6 0·0
12	(a) 9/7/73 (b) 18/7/73	Minnesota	11 SP	16·9	24	12·1	1·6	0·13	92	(a) 89·7 (b) 2·0 (c) 0·0	0·0 14·5 0·0
13	(a) 16/7/73 (b) 25/7/73	Minnesota	11 SP	15·9	24	24·2	3·7	0·28	72·2	(a) 69·1 (b) 1·5 (c) 1·6	0·0 43·7 0·0

SP = Single panicle of fruits.
MP = Multiple panicle composed of three panicles of fruits.

Incorporation of radioactivity from [methyl-^{14}C]-L-methionine into the total alkaloids of the hemlock fruits was variable, depending on the stage of development of the whole plants and fruits. The highest incorporation of radioactivity (1·8%) was obtained in the early summer (prior to leaf senescence) with multiple fruit panicles, at *ca* 1·5 weeks from flowering (Exps. 1–4). At this stage the administration of double the concentration of

[methyl-^{14}C]-L-methionine (i.e. 26·4 × 10^6 cpm) doubled the incorporation of radioactivity into the alkaloid fraction and harvesting after 24 hr as opposed to 48 hr (Exps. 1, 6, 7 and 13) suggests that there is no advantage to be gained by harvesting panicles after a longer period of time. Indeed Exps. 10 and 11 suggest that maximal incorporation is obtained in the first 6 hr. Since preliminary experiments had not indicated that such a short time was required for maximal radioactivity in the alkaloids it is suggested that the hot sunny conditions during the period used for the experiments recorded in Table 1 brought about an early cessation in metabolism in the cut fruit panicles. The [methyl-^{14}C]-L-methionine was normally administered in 0·05 ml/per panicle of culture solution buffered to pH 7·0 and these solutions were taken up by the fruit panicles in 30–60 min. The flowering process with *C. maculatum* normally covers several weeks and it was also found that the Bowles and Messina varieties flowered some weeks earlier than the Minnesota and Chelsea varieties. However, with the later flowering panicles (fed at 1·5 weeks from flowering) it was observed that there was a sharp decrease in the incorporation of radioactivity into the alkaloid fraction by a factor of 5–10 which may in part be due to the early senescence resulting from the dry hot conditions.

The individual alkaloids were determined quantitatively using GLC, and individual alkaloids isolated using PC followed by recrystallisation to constant activity. The results in Table 1 show that all the radioactivity was confined to the alkaloid methyl coniine in all varieties of hemlock fruits and since the generally accepted biosynthetic pathway for these alkaloids is γ-coniceine→coniine→methyl coniine[6,7] it is reasonable to assume that the radioactivity is confined to the *N*-methyl group of the methyl coniine molecule. The large variations in the specific activity of the methyl coniine reflect the varietal differences in methyl coniine content at the commencement of the experiments since with Bowles and Messina varieties large amounts of methyl coniine are already present, whereas Minnesota and Chelsea varieties contain only small amounts of this alkaloid. However, comparison of the results from Exps. 6–13 for the per cent incorporation into the alkaloid fraction suggest a methylating enzyme in the fruits of all the hemlock varieties which is capable of similar activity, so that it may be assumed that these results are either a direct response to an excess of the precursor of the *N*-methyl of methyl coniine or that in some varieties under normal growing conditions the methyl coniine formed is more rapidly metabolised to other substances; a consideration which deserves further investigation.

To further confirm that the whole of the radioactivity of methyl coniine resided at the *N*-methyl carbon, methyl coniine from three separate experiments, after purification by recrystallization to constant activity, was degraded using a variation of the Pregl method[8] for the estimation of methyl- and ethyl-imino groups.

The *N*-methyl of methyl coniine was obtained as triethyl–methyl–ammonium iodide which was then converted to the reineckate salt for determination of radioactivity. The results in Table 2 show that the recovery of the methyl coniine radioactivity as the methyl group of the quaternary iodide ranged from 94 to 101% indicating that, within experimental error, all the activity is localized on the *N*-methyl carbon of methyl coniine as had been anticipated by the results given in Table 1.

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[7] DIETRICH, M. C. and MARTIN, R. O. (1969) *Biochemistry* **8,** 4163.
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TABLE 2. LOCATION OF RADIOACTIVITY IN THE METHYL CONIINE MOLECULE AFTER ADMINISTERING [METHYL-^{14}C]-L-METHIONINE TO HEMLOCK FRUITS

Experiment no.	Hemlock variety	Sp. act. dpm/mmol: Methyl coniine hydrochloride ($\times 10^5$)	Sp. act. dpm/mmol: *N*-methyl as triethyl methyl ammonium reineckate ($\times 10^5$)
3	Messina	5·3	5·0
4	Bowles	3·5	3·3
14	Bowles	2·24	2·27

Experiments 3 and 4 appear in Table 1. Exp. 14 consisted of single panicles (30) which were fed 24.6.73. The incorporation into methyl coniine was 0·22%.

EXPERIMENTAL

Plant material. Conium maculatum cvs. Chelsea, Bowles, Messina and Minnesota* were grown at Myddelton House gardens, Enfield.

Administration of [methyl-^{14}C]-L-methionine (Radio-Chemical Centre, Amersham, Bucks). (A) Whole plants of *C. maculatum* variety Bowles were fed with [methyl-^{14}C]-L-methionine ($2·2 \times 10^8$ dpm, 320 μg) via a cotton wick placed in the stem 40 cm from ground level. Plants (*ca* 2 kg) were harvested after 4 or 6 days. (B) Fruit panicles were dated at flowering. Experiments used cut single or multiple (3) panicles in batches of 10–13, each single or multiple panicle being placed in an Eppendorf tube and administered [methyl-^{14}C]-L-methionine (sp. act. 0·5 μCi/mmol) in 0·05 ml of culture soln buffered at pH 7. After uptake (30–60 min) of the soln two further aliquots of culture soln (0·05 ml) were added and after uptake of these the tubes were topped up with culture soln. After not more than 48 hr, the fruits were removed from the panicles, weighed and stored at −20°.

Extraction of the alkaloids. The whole plants (2 kg) or fruits (15–35 g) were minced in MeOH in a blender, left 18 hr, filtered and the marc extracted with two further aliquots of MeOH. After evaporation to dryness of the acidified MeOH extracts, the residue was dissolved in H_2O $CHCl_3$ (1:1) and the resulting aq. layer further extracted with $CHCl_3$ until all pigments were removed. The aq. extract was then made alkaline using 10% Na_2CO_3 and the alkaloids extracted 3 × with $CHCl_3$. After washing with H_2O the alkaloids were extracted from the $CHCl_3$ into a small vol. of 10% HCl (2 ml) and the aq. acid soln evaporated to dryness to yield relatively pure samples of the total alkaloids as their hydrochlorides.

Separation and further purification of the alkaloids. The alkaloids were separated using PC[9] and where applicable methyl coniine, coniine and γ-coniceine isolated for the determination of radioactivity. Samples were recrystallised to constant activity.

Quantitative determination of alkaloids. Since some losses were experienced with PC, quantitative estimations of the individual alkaloids were made using GLC. Complete resolution of the major alkaloids was achieved on a 5 mm i.d. 1 m glass column containing 8% Carbowax 20 M, 2% KOH on silanized Chromosorb W, AW, DMCS 80–100 mesh conditioned at 130° (10 ml/min N_2 flow). The column was run at 80° with N_2 flow rate of 30 ml/min. *N,N*-Dimethylaniline was used as internal standard. A 95% recovery was obtained.

Degradation of methyl coniine. The adaptation of the Herzig and Meyer technique[8,10] as given by Brown and Byerrum[5] for the estimation of *N*-methyl and *N*-ethyl groups was employed to obtain a solid derivative of the methyl coniine methyl group for counting. MeI was liberated with HCl reacted with triethylamine to yield triethylmethyl ammonium iodide. The de-methylation apparatus was similar to that described by Pregl.[8] Ground glass joints were introduced to facilitate disassembly of the apparatus. Methyl coniine (50 mg) was placed in the reaction vessel together with PhOH (50 mg) and propionylanhydride (5 drops) and heated gently to dissolve. NH_4I (30 mg) 5% $HAuCl_4$ (2 drops) HI (s.g. 1·7, 3 ml) were then added. The gas washing vessel contained $CdSO_4$/$Na_2S_2O_3$ (5 ml) and the two receivers containing $(Et)_3N$ were cooled in a solid CO_2/methyl cellosolve bath. The reaction vessel through which a slow stream of N_2 was passed was heated using a Woods metal bath, the temp. rising to 200° in 30 min and then rapidly to 360° where it was held for 1 hr. The heat was removed and the N_2 stream continued until the reaction vessel had cooled. The receiving tubes were then stoppered tightly. After 18 hr, crystals of triethyl methyl ammonium iodide separated and were filtered. Recovery was 55–70% of the theoretical yield based on methyl coniine (*Anal.* Calcd. for $C_7H_{18}NI$: C, 84·08; H, 18·14; N, 14·008. Found: C, 83·42 H, 18·8; N, 13·89%.)

* From seed kindly supplied by Professor F. Leete.

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[10] HERZIG, J. and MEYER, H. (1900) *Monatsh.* **15**, 613.

Formation of triethyl-methyl-ammonium reineckate. Triethylmethyl ammonium iodide was dissolved in MeOH and the reineckate salt formed using ammonium reineckate. The salt was purified to constant radioactivity by repeated decomposition and re-precipitation of the reineckate in H_2O according to Kapfhammer and Bischoff.[11]

Determination of radioactivity. The alkaloids were dissolved in MeOH (50 mg/ml) and 0·1 ml taken for radioactive determinations using scintillation counting (efficiency 85%). The radioactivity of the reineckates was determined by taking a known amount and using Cab-*O*-Sil (0·5 mg) to maintain suspension in the scintillation fluid since the reineckate of the quaternary ammonium compound came out of soln on cooling.

[11] KAPFHAMMER, J. and BISCHOFF, C. (1938) *Z. Phys. Chem.* **191**, 179.