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AN *S*-ADENOSYL-L-METHIONINE; CONIINE METHYLTRANSFERASE FROM *CONIUM MACULATUM*

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Key Word Index—*Conium maculatum*; Umbelliferae; hemlock; *S*-adenosyl-L-methionine; coniine methyltransferase; alkaloid biosynthesis.

Abstract—Evidence is presented for a cell free system from *Conium maculatum* which catalyses the transfer of a methyl group from *S*-adenosyl-L-methionine to coniine with the formation of *N*-methyl coniine. Maximum enzyme activity which occurred in the unripe fruits was enhanced by dithiothreitol, and evidence for the role of sulphydryl groups of the enzyme was obtained from inhibition with *p*-CMB, iodoacetamide and *N*-methyl maleimide. A divalent metal cation dependency was not detected.

INTRODUCTION

RESEARCH on *N*-methyltransferases in plants is limited, although alkaloids containing methylated nitrogen are widely distributed. However, recent work with radioactive tracers and cell free systems has indicated that the donor of the *N*-methyl group is L-methionine usually in the form of *S*-adenosyl-L-methionine (SAM).

Cell free systems have been isolated from *Pisum sativum*[1] and castor bean seedlings[2] which convert nicotinic acid to trigonelline in the presence of SAM, and a similar system exists in barley roots[3] catalysing the formation of methyltyramine and hordenine from tyramine, and gramine from 3-aminomethylindole via 3-methylaminomethylindole.[4] Further work with barley roots[5] showed that the appearance of tyramine methyltransferase activity during germination is strikingly coincident with the accumulation of its product *N*-methyltyramine. More recently[6] a putrescine *N*-methyltransferase has been isolated from tobacco roots, also with a requirement for SAM as the methyl group donor, and it is suggested that the presence of this enzyme in tobacco roots indicates the probable involvement of *N*-methylputrescine as an intermediate in nicotine biosynthesis. The present work sets out further evidence for the participation of SAM in the *N*-methylation of alkaloids and strengthens the generally accepted hypothesis[7] for the biosynthesis of the hemlock alkaloids.

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[7] ROBERTS, M. F. *Phytochemistry* (1974) **13,** 1841.

RESULTS

The occurrence of N-*methyltransferase and distribution within the plant*

Cell free systems were prepared from a number of varieties of *Conium maculatum* and in general *N*-methyltransferase could be detected only in parts of the plant where methyl coniine occurred. In consequence the most active *N*-methyltransferase preparations were obtained from the unripe fruits of *C. maculatum* cv. Bowles.[8] Enzyme activity was also detected in the leaves of flowering plants of *C. maculatum* cv. Bowles and cv. Messina. In all cases enzyme preparations were capable of methylating coniine in the presence of *S*-adenosyl-L-methionine (SAM) (Table 1). With cell free preparations from unripe fruits of cv. Bowles, methylation of coniine also occurred to a lesser degree in the presence of L-[methyl-^{14}C]-methionine and ATP (Table 1). However, no enzyme activity could be detected in the older unripe fruits (4–5 weeks from petal opening).

TABLE 1. THE OCCURRENCE OF CONIINE *N*-METHYLTRANSFERASE IN *Conium maculatum*

Variety of *C. maculatum*	Substrate	Sp. act. of methylconiine
cv. Bowles unripe fruits		
1 week from petal fall	SAM	10200
2 weeks from petal fall	SAM	21300
	L-methionine + ATP	670
3 weeks from petal fall	SAM	17800
cv. Bowles leaves from plants at flowering	SAM	220
cv. Messina leaves from plants at flowering	SAM	900

Sp. act. = dpm methylconiine formed/mg protein/hr. SAM-*S*-adenosyl-L-[^{14}C methyl]-methionine.

In the assay procedure which involved the use of *S*-adenosyl-L-[methyl-^{14}C]-methionine (100 nmol, $1{\cdot}1 \times 10^5$ dpm) coniine HCl (3·0 nmol) and enzyme protein (150–250 μg), the formation of *N*-[methyl-^{14}C]-coniine, determined hourly over the first 3 hr of incubation was 14500, 28300 and 34800 dpm/mg protein respectively. Increases in concentration of *S*-adenosyl-L-[methyl-^{14}C]-methionine did not significantly affect the formation of *N*-[methyl-^{14}C]-coniine over the 3 hr period. The standard assay procedure therefore used 100 nmol SAM and a 1 hr incubation period. Under these conditions *N*-methyltransferase activity was linear with respect to protein concentration over the range 0–550 μg and the optimal rate of conversion was 140 nmol coniine/hr/mg protein. The formation of *N*-[methyl-^{14}C]-coniine was established using paper chromatography[9] followed by repeated recrystallization with non-radioactive methyl coniine. Initial experiments showed that the total radioactivity in the alkaloid fraction extracted from the incubation media was due to *N*-[methyl-^{14}C]-coniine.

Extraction and partial purification

During the initial extraction of the enzyme the presence of a strong reducing agent (sodium metabisulphite) together with Polyclar AT and dithioreitol (DTT) was essential

[8] FAIRBAIRN, J. W. and CHALLEN, S. B. (1959) *Biochem. J.* **72**, 556.
[9] CROMWELL, B. T. (1956) *Biochem. J.* **64**, 259.

for the maintenance of activity, and the inclusion of DTT in the extraction medium was maintained throughout the subsequent purification steps. Further purification was effected by ammonium sulphate precipitation of the active protein followed by ion-exchange separation to yield a highly labile active protein fraction. A summary of the enzyme purification and yield in each step is given in Table 2.

TABLE 2. PARTIAL PURIFICATION OF CONIINE *N*-METHYLTRANSFERASE

Purification steps	Total protein (mg)	Sp. act.	Apparent recovery of activity
Crude extract	132·6	17900	100
Ammonium sulphate saturation 40–80%	64·0	47800	78
DEAE Sephadex A25 chromatography	5·8	57300	10·9

Sp. act. dpm methylconiine formed/mg protein/hr.

pH optimum

The effect of pH on the rate of *N*-methylation of coniine by the enzyme was investigated using a phosphate–citrate–borate buffer.[10] It was found to have maximal activity at pH 8·2. Activity was half maximal at pH 7·4 and 9·2.

The sulphydryl requirement

In assays with the ammonium sulphate precipitated protein, optimal activity was exhibited at the relatively high concentration of 6–12 mM DTT. Inhibitor experiments with the ammonium sulphate protein precipitate further demonstrated sulphydryl group involvement in *N*-methyltransferase activity. Complete inactivation of the enzyme was achieved with *N*-methyl maleimide, *p*-CMB and iodoacetamide in experiments where DTT was omitted from the pre-incubation media, whereas inclusion of DTT at this stage prevented complete inactivation (Table 3). In the latter experiments the maintenance of

TABLE 3. THE INHIBITION OF CONIINE *N*-methyltransferase

Inhibitor	Conc. ($M \times 10^{-3}$)	Relative act. (%)
None		100
KCN	7	100
N-Methylmaleimide	1·4	63
	1·4*	0
p-Chloromercuribenzoate	0·3	25
	0·3*	0
Iodoacetamide	1·4	20

Protein obtained from $(NH_4)_2SO_4$ fractionation (250 μg) was incubated for 5 min at 32° with phosphate buffer (30 μmol) at pH 8·2 containing 4 μmol DTT and indicated amounts of inhibitor. The conc. of each inhibitor refers to conc. during preincubation. Then coniine HCl (3·0 μmol) and SAM (100 μmol 12·1 $\times$ 10^6 dpm) were added. The mixture 0·5 ml was incubated for 1 hr.

* In these assays DTT was omitted from the preincubation mixture.

[10] *Geigy Scientific Tables*, 6 edn, Geigy p. 34.

some *N*-methyltransferase activity was presumably due to the protecting effect of the DTT. KCN at concentrations of 1×10^{-3} M and 7×10^{-3} M did not inhibit methylation.

The effect of divalent cations

A number of methyltransferases require divalent metal ions but in the present experiments activity was neither enhanced by 2 mM Mg^{2+} or 0·5 mM Mn^{2+} nor inhibited by 1 mM EDTA.

Kinetic studies

Substrate saturation was observed for coniine HCl and a Lineweaver–Burke Plot was made. From the latter a K_m value for coniine HCl of 1·55 mM was obtained.

DISCUSSION

A methyl coniine forming enzyme designated coniine *N*-methyltransferase has been isolated from *C. maculatum*. Enzyme preparations containing coniine *N*-methyltransferase activity were only achieved using mature 2nd yr plants at flowering and young unripe fruits where methyl coniine occurred in amounts which were easily extracted (0·03–0·14 mg/g fr. wt) and this, together with the low K_m and high maximal rate of activity, suggests that an *N*-methyltransferase which is specific for coniine may be operative. By comparison with *N*-methyltransferases isolated from pea seedlings[1] and tobacco roots,[6] coniine *N*-methyltransferase exhibits a sharp pH maximum and has maximal activity which is greater than that for tyramine *N*-methyltransferase by a factor of 100, and that for methylation of nicotinic acid and putrescine by a factor of 3. Unlike the methylation of nicotinic acid, methylation of coniine appears to have a requirement for additional sulphydryl groups in a manner similar to that observed in some instances for *O*-methyltransferase activity.[11] However, as with trigonelline formation[1] by pea seedling preparations, crude enzyme preparations of hemlock fruits will catalyse the methylation of coniine utilizing L-methionine and ATP although this activity appears to be lost with further purification.

The occurrence of coniine *N*-methyltransferase in *C. maculatum* and the variations in methyl coniine in the fruits are of particular interest since despite the extreme variations in methyl coniine content in cvs Bowles, Messina, Minnesota and Chelsea, all cultivars appear to show similar levels of *N*-methyltransferase activity in that the percentage incorporation of the methyl group of L-[methyl-^{14}C]-methionine into methyl coniine is similar in all varieties.[7]

The generally accepted pathway for the biogenesis of the hemlock alkaloids is via the initial formation of γ-coniceine from 5-keto-octanal[12] by a transaminase which utilized L-alanine as the amino group donor.[13] It is assumed from the work of Fairbairn and Challen[8] and Dietrich and Martin[14] that γ-coniceine is reduced to coniine in a reversible reaction followed by the formation of methyl coniine in certain parts of the plant. The present work shows quite conclusively that an *N*-methyltransferase system exists in *C. maculatum* which will methylate coniine and which is most active in the parts of hemlock where methyl coniine is found.

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[12] LEETE, E. (1970) *J. Chem. Soc. D*. 1637.
[13] ROBERTS, M. F. (1971) *Phytochemistry* **10**, 3057.
[14] DIETRICH, M. C. and MARTIN, R. O. (1969) *Biochemistry* **8**, 4163.

EXPERIMENTAL

Plant material Conium maculatum L cv. Bowles,[8] Chelsea,[8] Minesota and Messina[15] were used. In most of the experiments young unripe fruits were taken except in the preliminary experiments (Table 1) in which leaves from whole plants at flowering were studied.

Preparation of a cell free extract. Plant material (50 g) was ground to a fine powder with liq. N_2, placed in the glass cell of a dyno mill (Bachofen Machinfabrik base) with glass balls (0·25–0·5 mm dia. 270 ml) and 20 mM phosphate buffer pH 7·5 (250 ml) containing Na metabisulphite 1 mM and DTT 10 mM, and ground for 5 × 10 sec at 6000 rpm, the cell temp. being maintained at 10°. To the mixture of cell material and buffer was added Polyclar AT (20 g) and after stirring (15 min) the mixture was squeezed through cheese cloth and centrifuged 10 min at 18000 rpm. Part of the supernatant (1 ml) was desalted with a column of Sephadex G25 (9 mm × 30 cm) and used as the crude enzyme in preliminary experiments. The remainder of the supernatant was brought to 40% saturation with $(NH_4)_2SO_4$, centrifuged 10 min at 18000 rpm and the inactive protein discarded. The methyltransferase activity was finally precipitated at 80% saturation $(NH_4)_2SO_4$, isolated by centrifugation for 10 min at 18000 rpm and stored for 18 hr to 1 week at −20°. Further purification yielding a highly labile protein was achieved using columns of either DEAE cellulose or DEAE Sephadex A25 and 2 mM Sørensen's phosphate buffer pH 8·2[10] containing DTT 10^{-4} mM. A quarter of the 80% $(NH_4)_2SO_4$ ppt was dissolved in buffer (1 ml) and desalted with a Sephadex G25 column (9 mm × 30 cm) and the active eluate (4 ml) applied to an ion exchange column (9 mm × 30 cm). With these columns a discontinuous NaCl gradient was used. Active protein was eluted from the DEAE Sephadex A25 column prior to the application of the NaCl gradient and from the DEAE-cellulose column with 50 mM NaCl. The transmethylase activity of these fractions was lost with freezing unless stabilized by the addition of 5 mM mannitol.

Assay for coniine methyltransferase activity. Unless otherwise specified, assay mixtures contained in a final vol. of 0·5 ml buffer pH 8·2, 30 μmol; coniine HCl, 3 μmol; *S*-adenosyl-L-[methyl-^{14}C]-methionine (SAM), 100 μmol ($1·1 \times 10^5$ dpm); DTT, 4 μmol; and enzyme. The mixture was incubated at 32° for 1 hr. After incubation MeOH (1 ml) containing methyl coniine HCl (0·5 mg) was added to the mixture. In the control samples boiled enzyme was used or SAM was added after incubation and the addition of the MeOH soln. Samples were left 18 hr prior to extraction of the alkaloids. Samples were diluted with H_2O 100-fold and made alkaline with 10% Na_2CO_3. The alkaloids were extracted from the resulting soln with $CHCl_3$ (2 × 15 ml) and the bulked $CHCl_3$ extracts washed with H_2O (4 × 8 ml). Finally, the alkaloids were extracted from the $CHCl_3$ with 10% HCl (2 × 4 ml) and the acid soln evaporated to dryness *in vacuo*. The alkaloid residue was dissolved in MeOH (1 ml) and 0·2 ml taken for radioactive determinations (efficiency 80–85%). Using the above extraction procedure the control samples had values for radioactivity, 10% of the normal activity with methyltransferase. Isolation of radioactive methyl coniine HCl from a few samples of the total alkaloids using PC[9] showed negligible variations in radioactivity. The identity of the radioactive product as *N*-[methyl-^{14}C]-coniine HCl was further established by the addition of 10 mg methylconiine HCl and crystallizing to constant activity. For example, in one experiment the radioactivity of the 2nd, 3rd and 4th recrystallizations were 300, 314 and 310 dpm ($5·5 \times 10^4$ dpm mM) respectively. The data reported represents the average of duplicates from at least 2 separate preparations. Control samples were prepared with each group of assays as previously indicated. *N*-methyl transferase activity was measured as the amount of methyl coniine found (dpm) per mg of protein per hr. Protein was determined using the method of Lowry *et al.*[16]

[15] Seed obtained originally from Messina in Sicily and grown for some years at Myddelton House. The variety Minnesota was grown from seed kindly supplied by Professor E. Leete.

[16] LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193**, 265.