*Phytochemistry*, 1975, Vol. 14, pp. 2393–2397. Pergamon Press. Printed in England.

# γ-CONICEINE REDUCTASE IN *CONIUM MACULATUM*

MARGARET F. ROBERTS

Department of Pharmacognosy, The School of Pharmacy, University of London, 29/39, Brunswick Square, London WC1N 1AX, England

(*Received* 11 *March* 1975)

**Key Word Index**—*Conium maculatum*; Umbelliferae; hemlock; γ-coniceine reductase; alkaloid biogenesis.

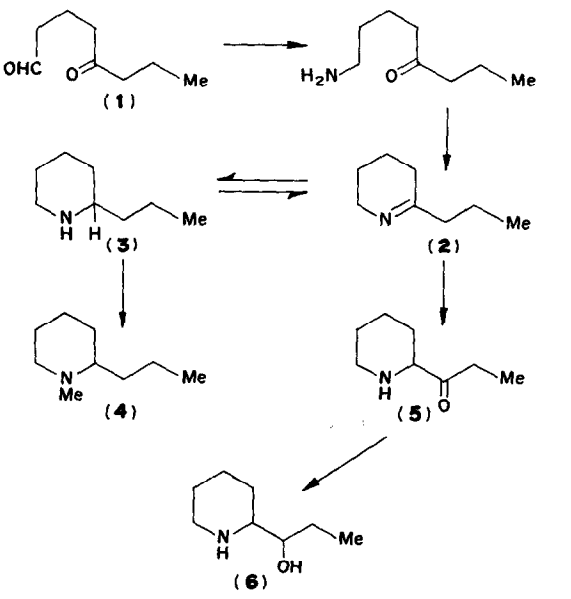
**Abstract**—γ-Coniceine reductase, isolated from leaves and fruits of a number of *Conium maculatum* cultivars, was NADPH dependent. The hydride ion was transferred to the double bond from the B(pro-S) side of the pyridine nucleotide to yield coniine.

## INTRODUCTION

The route of biogenesis of the hemlock alkaloids from acetate via 5-ketooctanal (**1**) and transamination utilising L-alanine has been established [1–3]. In all cultivars of *C. maculatum* investigated, the major route from the first formed alkaloid γ-coniceine (**2**) is via reduction to coniine (**3**) and subsequent methylation with *S*-adenosyl-L-methionine to methyl coniine (**4**) [4–8]. However the oxidation of γ-coniceine to conhydrinone (**5**) with subsequent reduction to conhydrin (**6**) also occurs as an alternative and, in general, minor pathway.

Fairbairn and Suwal [9] have shown that γ-coniceine and coniine are rapidly inter-converted with dramatic fluctuations in the amounts of these alkaloids occurrring in a single day. These interconversions have been further investigated using $^{14}C$ labelled γ-coniceine and coniine [5,8,9] and the results of these experiments indicate that the reduction of γ-coniceine to coniine occurs more readily than the reverse reaction in which (+) coniine is the preferred isomer [5].

The ready interconversion of γ-coniceine to (+) coniine in *C. maculatum* may have an important role in the regulation of alkaloid biogenesis and it has been suggested by other researchers that these alkaloids may be involved in an oxidation-reduction system which is of biological importance to the plant as a whole [5,9]. The present work aims to establish the presence of γ-coniceine reductase and to determine the requirement for reduced pyridine nucleotides.




## RESULTS

### *The occurrence and distribution of γ-coniceine reductase in hemlock plants*

Cell free preparations were made using actively metabolising leaves or fruits at a development stage where coniine or methyl coniine occurred as the major alkaloids. Extractions using 0·1 M Tris buffer at pH 7·5 indicated that it was beneficial to include either dithiothreitol $10^{-3}$ M or

Table 1. The occurrence of γ-coniceine reductase in *Conium maculatum*

| Variety of *C. maculatum* | Protein (mg) | Coniine HCl (dpm) | Sp. act. |
|---|---|---|---|
| cv. *Minnesota* 1st yr leaves [*B*] fresh material | 15 | 608 | 40·5 |
| cv. *Minnesota* 2nd yr leaves [*A*] $Me_2CO$ powder | 17 | 381 | 22·4 |
| cv. *Minnesota* 2nd yr leaves [*B*] $Me_2CO$ powder | 6·2 | 116 | 19·0 |
| cv. *Bowles* 2nd yr leaves [*B*] $Me_2CO$ powder | 8·0 | 80 | 10·0 |
| cv. *Bowles*. Fruits 2 weeks from petal fall $Me_2CO$ powder | 15 | 86 | 5·3 |

The protein precipitated at 80% $(NH_4)_2SO_4$ saturation from a cell free extract of *C. maculatum* was incubated in 0·1 M Tris pH 7·5 with γ-coniceine HCl[U-$^{14}$C] (6 μmol sp. act. $3 \times 10^6$ dpm/mmol) in the presence of an NADPH generating system for 3 hr at 30°. Alkaloids were extracted and the coniine isolated by PC. Controls contained either (a) boiled enzyme or (b) no enzyme. The activity found in these controls was 12–15 dpm. The results shown are means of triplicate results from 2–3 experiments. sp. act. = dpm coniine formed/mg protein/3 hr. *A* = Young leaves (just expanded). *B* = Mature leaves.

2-mercaptoethanol $10^{-2}$ M with EDTA $10^{-3}$ M in the extracting medium and in preparations of fruits the high concentrations of phenolic material and the occurrence of phenolase made the inclusion of ascorbate $10^{-2}$ M in the initial extracting medium necessary.

Some preliminary unpublished work by Cromwell* suggested that in cell free preparations the conversion of γ-coniceine to coniine was slow and therefore unsuited to the existing assay methods for coniine [11]. An assay was therefore employed which utilised γ-coniceine-[U-$^{14}$C] (sp. act. $3 \times 10^6$ dpm/mmol) as substrate. This rendered possible the detection and confirmation of the formation of nmol amounts of coniine with relatively simple techniques. To facilitate the detection of γ-coniceine reductase activity utilizing a $^{14}$C labelled substrate of low sp. act., it was found advantageous to concentrate the active protein by ammonium sulphate precipitation and experiments showed that γ-coniceine reductase activity occurs in the precipitate obtained with 40–60% $(NH_4)_2SO_4$ saturation.

Initial experiments utilised cell free preparations of leaves and fruits from *C. maculatum* at various stages of development. These preparations incubated with γ-coniceine-[U-$^{14}$C] in the presence or absence of an NADPH generating system which consisted of glucose-6-phosphate, NADP, $MgCl_2$ and glucose-6-phosphate dehydrogenase formed nmol amounts of coniine which indicated the presence of a γ-coniceine reductase. The results given in Table 1 show that the most active preparations were obtained from cultivar Minnesota leaves. Despite variations in the sulphydryl and reducing agents added to the initial extracting medium and variations in the grinding process, the extraction of reductase activity from the fruits was poor compared with that from the leaf preparations. γ-Coniceine activity was not observed in samples which contained boiled hemlock enzyme or from which the NADPH generating system had been omitted.

*Reduced pyridine nucleotide requirement*

Leaf cell free preparations from cv. Minnesota were used to compare γ-coniceine reductase activities in the presence of NADPH and NADH generating systems. The results given in Table 2 show that γ-coniceine reductase has an absolute requirement for NADPH. Further, these experiments also show that in the conditions of the assay procedure, coniine was more efficiently produced using an NADPH generating system than with the direct inclusion of NADPH. However, activity in the latter instance was sufficient to

* B. T. Cromwell—private communication.

Table 2. The specificity of γ-coniceine reductase for NADPH and the efficiency of the NADPH generating system

| Source of reduced pyridine nucleotide | Coniine HCl formed dpm | Sp. act. |
|---|---|---|
| NADPH | 118 | 4 |
| NADPH formed by generating system | 672 | 22 |
| NADH formed by generating system | 25 | 1·6 |

*C. maculatum cv. Minnesota.* 2nd yr young leaves. Protein (8 mg) obtained by $(NH_4)SO_4$ fractionation (40–60% saturation) was incubated in 0·1 M Tris pH 7·5 total vol 5 ml, for 3 hr at 30° with γ-coniceine HCl [U-$^{14}$C] (6 μmol 1·8 × $10^4$ dpm) in the presence of either added NADPH or a generating system for NADPH or NADH. The NADPH generating system consisted of $MgCl_2$ (6 μmol) glucose-6-phosphate (23 μmol), NADP (12 μmol) and glucose-6-phosphate dehydrogenase (2·5 U). The NADH generating system consisted of L-glutamate (20 μmol) $MgCl_2$ (6 μmol), NAD (12 μmol) and L-glutamate dehydrogenase (9·0 U). The alkaloids were extracted, the coniine HCl isolated by PC and recrystallised to constant activity. Controls contained boiled enzyme and gave a background activity of 12–15 dpm. Results shown are means of triplicate results from 2 experiments. sp. act. = dpm coniine formed/mg protein/3 hr.

allow an investigation of the stereospecificity of the transfer of the hydrogen from the reduced pyridine nucleotide to be made with a view to the use of tritiated NADPH as an alternative to γ-coniceine-[U-$^{14}$C] in the assay for reductase activity.

*Stereospecificity of the hydrogen transfer from NADPH to the* Δ′ double bond of γ-coniceine

B-[4-$^3$H]-NADPH labelled according to Pastore *et al.* [12] and Palm *et al.* [13] by the combined hexokinase:glucose-6-phosphate dehydrogenase reaction with D-glucose-1-$^3$H as substrate had a sp. act. 4·1 × $10^6$ dpm/μmol. A-[4-$^3$H]-NADPH was labelled by a combined NAD-kinase:glucose-6-phosphate dehydrogenase reaction using 4(*n*)$^3$H-NAD as substrate and had a sp. act. 4·8 × $10^6$ dpm/μmol. These co-enzymes, used under the conditions described in the Experimental, showed that in the presence of γ-coniceine and the hemlock reductase preparation significant radioactivity in the product, coniine, only occurred with the use of B-[4-$^3$H]-NADPH. In two experiments with duplicate results A-[4-$^3$H]-NADPH yielded radioactive coniine HCl 100 and 150 dpm, whereas in experiments using B-[4-$^3$H]-NADPH, the radioactivity of the coniine HCl was 48600 and 50600 dpm. Therefore it must be concluded that the hydride transfer occurs stereospecifically from the B(pro-S) side of the pyridine nucleotide.

## DISCUSSION

The present study has substantiated the existence of a γ-coniceine reductase in the leaves and fruits of *C. maculatum* cv Minnesota and Bowles. The preparation of cell free systems from fresh leaves suggests that γ-coniceine reductase is a soluble enzyme. However, the low reductase activity isolated from young unripe fruits is at present difficult to explain, particularly since γ-coniceine-[U-$^{14}$C] fed to cut fruit particles [10] was rapidly converted into coniine. Furthermore previous work [7] using similar extraction methods showed that a soluble *N*-methyltransferase was readily isolated from the fruits. Further studies on the possible particulate nature of the γ-coniceine reductase may elucidate this problem.

The reduction of γ-coniceine requires NADPH rather than NADH and experiments indicate that the stereospecific removal of hydride from the B(pros) side of NADPH may be used as an alternative to the utilisation of γ-coniceine-[U-$^{14}$C] for the assay of reductase activity.

The structure of γ-coniceine and its formation from 5-ketooctanal with subsequent reduction to coniine may be compared with the formation of the structurally similar proline and pipecolic acid. The pyridine nucleotide dependent reduction of glutamic semi-aldehyde or its cyclization products (Δ′pyrroline-5-carboxylic and -2-carboxylic acids) recognised as precursors of proline, and the reduction of the α-keto analogue of lysine or its cyclized product (Δ′-piperidine-2-carboxylic acid) to pipecolic acid have been studied in animals [14–16], plants [14,18–23] and bacteria [14,17]. Experiments show that in proline and pipecolic acid metabolism the requirement for $NAD^+$ or $NADP^+$ or their reduced analogues may vary and is dependent on the source of the enzyme. In cell free extracts from germinating peanut seedlings proline dehydrogenase required NAD and with the same extract Δ-pyrroline-5-carboxylic acid reductase required NADPH [18,19]. This suggests that the dehydrogenase and reductase are separate enzymes. However, experiments with

wheat germ [20] indicate that proline dehydrogenase and $\Delta^1$-pyrroline-5-carboxylic acid reductase have a requirement for $NAD^+$ and the reduced analogue respectively, with $NADP^+$ and NADPH as powerful inhibitors, and to explain these results it was postulated that two binding sites for the substrate exist on the same protein. Since $\gamma$-coniceine reductase has an absolute requirement for NADPH, the requirements of coniine dehydrogenase would be of interest in considering the possible involvement of separate proteins for the reductase and dehydrogenase activities, or a single protein with more than one substrate binding site.

Radiotracer experiments have shown the reduction of $\gamma$-coniceine to be reversible with coniine converted to $\gamma$-coniceine in the vegetative parts of the cv. Chelsea [5,9] though this was not observed in the fruits [9]. The percentage incorporation into $\gamma$-coniceine was found to be 0·26 for the natural (+)-coniine-[2-$^{14}$C] using the cv. Chelsea compared with 3·28 for the conversion of $\gamma$-coniceine to coniine in the cv. Minnesota, and these results together with the fact that coniine and methylconiine generally accumulate in mature plants particularly at flowering, would suggest that the reaction catalysed by $\gamma$-coniceine reductase is dominant. If the maintenance of a balance between $\gamma$-coniceine and coniine is important at any particular stage in the development of the plant, then the low rate of conversion of coniine into $\gamma$-coniceine is unexpected, since the presence of a large excess of coniine might be expected to stimulate coniine dehydrogenase. It is of interest that at germination cotyledons contain $\gamma$-coniceine [10] and a consideration of whether this alkaloid is formed *de novo* or as a result of coniine dehydrogenase activity in the germinating seed would be of interest particularly as coniine is still present in appreciable amounts in the remains of the seed.

Obviously factors other than substrate concentration are involved if the maintenance of a balance between $\gamma$-coniceine and coniine is of importance in alkaloid biogenesis.

## EXPERIMENTAL

*Plant material. C. maculatum* cv. Minnesota and Bowles were used. In expts (A) young leaves (leaf blade *ca* 50% expanded) or (B) mature leaves were used together with fruits at *ca* 1–3 weeks from petal opening.

*Preparation of a cell free extract.* Fresh leaves 26–50 g were ground to a fine powder with liq. $N_2$ and extracted further with 0·1 M Tris buffer pH 7·5 containing mercaptoethanol $10^{-2}$ M, EDTA $10^{-3}$ M and glutathione $10^{-3}$ M. Cell debris was removed by squeezing through cheese cloth and centrifugation at 39000 g/10 min. To concentrate the protein, the supernatant was brought to 40% saturation with $(NH_4)_2SO_4$, centrifuged at 39000 g/10 min and the inactive protein discarded. $\gamma$-Coniceine reductase was finally precipitated at 40–60% saturation $(NH_4)_2SO_4$ and isolated by centrifugation at 39000 g/10 min. The active ppt was dissolved in 1 ml of 0·1 M Tris buffer pH 7·5 containing mercaptoethanol $10^{-2}$ M EDTA $10^{-3}$ M and desalted by elution from a column of Sephadex G25 (1·5 × 30 cm), to give an enzyme preparation (5–10 ml) which was used in assays for the determination of $\gamma$-coniceine reductase activity.

Cell free extracts from fruits were in some experiments prepared as above with the inclusion of ascorbate $10^{-2}$ M in the initial extracting medium since fruits but not leaves were found to have a high concentration of phenolic material and polyphenoloxidase. In other experiments a dyno mill was used as previously described [7]. $Me_2CO$ powders of leaves and fruits were also prepared and 2–4 g samples extracted using the method described above.

*Preparation of substrates.* $\gamma$-Coniceine HCl-[U-$^{14}$C] was prepared in the following manner. Young plants of *C. maculatum* cv. Chelsea were grown for 1 week in a chamber with an atmosphere rich in $^{14}CO_2$. The plants were harvested and the alkaloids (90% $\gamma$-coniceine) extracted as previously described [6]. The $\gamma$-coniceine was purified by PC [11] and TLC [7]. The radiochemical purity was checked by autoradiography. The $\gamma$-coniceine prepared in this manner had a sp. act. of 3 × $10^6$ dpm/mmol. Coniine HCl was prepared from commercially available coniine base.

*Preparation of A-[4-$^3$H]-NADPH.* 4(*n*)$^3$H-NAD (sp. act. 50 mCi/mmol) diluted with NAD (18 $\mu$mol sp. act. 6·0 × $10^6$ dpm/$\mu$mol) was incubated for 1 hr at 37° in 0·1 M Tris pH 7·5 with ATP (24 $\mu$mol) $MgCl_2$ (12 $\mu$mol) and chicken liver NAD-kinase (30 U). To this mixture was added glucose-6-phosphate (23 $\mu$mol) and glucose-6-phosphate dehydrogenase (2·5 U) to give a final vol. of 3 ml. The formation of NADPH at 30° was followed at A 340 nm. An overall conversion of 80% was obtained. Calc. sp. act. of A-[4-$^3$H]-NADPH was 4·8 × $10^6$ dpm/$\mu$mol. The mixture was boiled for 2 min and the insoluble protein removed by centrifugation with very little loss in activity of the radioactive NADPH. The soln was divided into 3 (1 ml) portions for use with the hemlock reductase preparation.

*Preparation of B-[4-$^3$H]-NADPH.* [12,13] D-glucose-[1-$^3$H] (sp. act. 3·4 Ci/mmol) diluted with D-glucose (7 $\mu$mol sp. act. 1·5 × $10^7$ dpm/$\mu$mol) was incubated for 15 min at 30° in 0·1 M Tris buffer pH 7·5 with ATP (24 $\mu$mol) $MgCl_2$ (12 $\mu$mol) and yeast hexokinase (22 U). To this mixture was added glucose-6-phosphate dehydrogenase (2·5 U) and NADP (12 $\mu$mol) to give a final vol of 3 ml. The formation of NADPH at 30° was followed at A 340 nm. An overall conversion of 83% was obtained. NADPH (12 $\mu$mol) was then added. Calc. sp. act. for the B-[4-$^3$H]-NADPH 4·1 × $10^6$ dpm/$\mu$mol. The mixture was boiled for 2 min, the insoluble protein removed by centrifugation and the resultant soln divided into 3 parts for use with the hemlock and coniceine reductase preparation.

*Assay for $\gamma$-coniceine reductase. I. Experiments using $\gamma$-coniceine [U-$^{14}$C].* The assay mixture consisted of $\gamma$-coniceine-[U-$^{14}$C] (6 $\mu$mol) NADPH or NADH (12 $\mu$mol) or an NADPH or NADH generating system in 0·1 M Tris buffer pH 7·5 to

which was added a hemlock cell free preparation (8–15 mg protein) to give a total vol. of 5 ml. Mixtures were incubated for 3 hr at 30° followed by protein ppt with 3% sulphosalicylic acid (3 ml). After removal by centrifugation of the protein a soln containing coniine HCl (2 mg) was added. Alkaloids were extracted and the coniine isolated by PC as previously described [7]. Control experiments in which boiled hemlock enzyme protein was used had values for radioactivity which were in most cases $< 10\%$ of the normal γ-coniceine reductase activity. The identity of the radioactive product was further established by TLC [7] and recrystallisation to constant activity. For example, in one experiment the radioactivity of the 2nd, 3rd and 4th recrystallizations was 227, 237 and 235 dpm/mg respectively. Protein was determined using the method of Lowry *et al.* [23].

*Generating system for NADPH.* This consisted of $MgCl_2$ (6 μmol) glucose-6-phosphate (23 μmol) NADP (12 μmol) and glucose-6-phosphate dehydrogenase (2·5 U) in 0·1 M Tris buffer pH 7·5. Total vol. (3 ml).

*Generating system for NADH.* This consisted of L-glutamate (20 μmol) $MgCl_2$ (6 μmol) NAD (12 μmol) and L-glutamate dehydrogenase (9 U) in 0·1 Tris buffer pH 7·5. Total vol. (3 ml).

II. *Experiments using tritiated NADPH.* Assay mixture in these experiments was given in (I) except that cold γ-coniceine HCl was used and the soln containing either A-[4-$^3$H]-NADPH or B-[4-$^3$H]-NADPH were added in place of the previously described NADPH generating system. In experiments where incorporation of tritium was observed the coniine HCl was diluted with cold coniine HCl and recrystallized to constant activity. For example the radioactivity in one experiment for the 4th, 5th and 6th recrystallization was 3133, 3330 and 3396 dpm/mg respectively.

## REFERENCES


1. Leete, E. (1964) *JACS*, 2509.
2. Leete, E. (1970) *J. Chem. Soc. D.* 1637.
3. Roberts, M. F. (1971) *Phytochemistry* **10**, 3057.
4. Dietrich, M. C. and Martin, R. O. (1969) *Phytochemistry* **8**, 4163.
5. Leete, E. and Olson, J. O. (1972) *JACS* **94**, 5472.
6. Roberts, M. F. (1974) *Phytochemistry* **13**, 1841.
7. Roberts, M. F. (1974) *Phytochemistry* **13**, 1847.
8. Leete, E. and Adityachaudury, N. (1967) *Phytochemistry* **6**, 219.
9. Fairbairn, J. W. and Suwal, P. N. (1961) *Phytochemistry* **1**, 38.
10. Roberts, To be published.
11. Cromwell, B. T. (1956) *Biochem. J.* **64**, 259.
12. Pastore, E. J. and Friedkin, M. (1961) *J. Biol. Chem.* **236**, 2314.
13. Palm, D., Rambeck, W. and Simon, H. (1968) *Z. Naturforsch.* **236**, 881.
14. Meister, A., Radhakrishnan, A. N. and Buckley, S. D. (1957) *J. Biol. Chem.* **229**, 789.
15. Smith, N. E. and Greenberg, D. M. (1956) *Nature* **177**, 1130.
16. Adams, E. and Goldstone, A. (1960) *J. Biol. Chem.* **235**, 3499.
17. Yura, T. and Vogel, H. J. (1955) *Biochim. Biophys. Acta* **17**, 582.
18. Mazelis, M. and Fowden, L. (1969) *Phytochemistry* **8**, 801.
19. Mazelis, M. and Creveling, R. K. (1974) *Phytochemistry* **13**, 559.
20. Splittstoesser, S. A. and Splittstoesser, W. E. (1973) *Phytochemistry* **12**, 1565.
21. Sisler, E. C. (1974) *Tobacco Sci.* **13**, 96.
22. Noguchi, M., Koiwai, A. and Tamaki, E. (1966) *Agr. Biol. Chem.* **30**, 452.
23. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.