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SHORT COMMUNICATION

THE FORMATION OF γ-CONICEINE FROM 5-KETOOCTANAL BY A TRANSAMINASE OF *CONIUM MACULATUM*

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Abstract—A transaminase in hemlock (*Conium maculatum*) has been isolated which utilizes L-alanine to convert 5-ketooctanal to γ-coniceine.

INTRODUCTION

IN A series of recent papers, Leete[1–3] has shown that the biosynthesis of the hemlock alkaloids from acetate probably proceeds via the intermediates 5-ketooctanoic acid and 5-ketooctanal, and it is suggested that a transaminase could provide the required nitrogen and final cyclization of 5-ketooctanal to γ-coniceine. The present communication presents evidence for the existence of this enzyme in *Conium maculatum*.

RESULTS AND DISCUSSION

Young hemlock leaves were extracted with phosphate buffer pH 7·6 and the protein precipitated with ammonium sulphate. The active enzyme was found to reside in the protein fraction which precipitated with 40–70% ammonium sulphate saturation. This protein was resuspended in buffer, passed through Sephadex G15 and dialysed overnight. Experiments indicated that the enzyme might also be successfully isolated by first preparing an acetone powder prior to the extraction with phosphate buffer and that enzyme activity might be increased by the inclusion of 0·05 M mercaptoethanol in the buffer used for the extraction of the acetone powder (Table 1). However, it was found necessary to remove the mercaptoethanol during the overnight dialysis, as this substance interfered with the assay procedure for γ-coniceine.

The enzyme extracts showed considerable transaminase activity with 5-ketooctanal and L-alanine as the amino group donor and traces of activity when glutamic acid was used. No activity was observed with aspartic acid, glycine and phenylalanine as amino group donors. The results of the measurement of the formation of pyruvate and γ-coniceine when protein extracts from hemlock were used to convert 5-ketooctanal to γ-concieine using L-alanine are given in Table 1. The pyruvate was measured in the presence of 5-ketooctanal by the determination of the absorbance at 510 nm of the 2-4 dinitrophenylhydrazones in

[1] E. LEETE, *J. Am. Chem. Soc.* **92**, 3835 (1970).
[2] E. LEETE, *J. Chem Soc.* **D**, 1634 (1970).
[3] E. LEETE, *Acc. Chem. Res.* **4**, 100 (1971).

TABLE 1. ASSAYS FOR THE FORMATION OF PYRUVATE FROM L-ALANINE AND γ-CONICEINE FROM 5-KETOOCTANAL USING A TRANSAMINASE OF HEMLOCK

Preparation of enzyme extracts	Protein mg/ml of reaction mixture	μmoles of pyruvate formed from L-alanine Incubation time (hr) 1	2	3	4	5	6
I Preparation from fresh tissue	2·56	—	—	0·025	—	—	0·275
II Preparation from acetone powder	22·0	0 05	—	0 200	—	0 275	—
III Preparation as II + inclusion of 0·05 M mercaptoethanol in the buffer solution	1·32	—	0·320	—	0·320	—	0·310
IV Preparation as III after 24 hr and with dilution of the enzyme solution	0·54	0·05	0 290	0·330	0 330	0·330	—

	μmoles of γ-coniceine formed from 1 μmole of 5-ketooctanal Incubation time (hr) 1	2	3	4	5	6	% Conversion of 5-ketooctanal to γ-coniceine at the termination of the experiment
I	—	—	—	—	—	0·24	25 5
II	—	—	—	—	—	—	27·5
III	—	0·35	—	0·345	—	0·340	33 0
IV	—	—	—	—	0·34	—	33·5

The reaction mixture contained M L-alanine (8·3 ml) 0·01 M 5-ketooctanal (1·3 ml); enzyme (2·6 ml); 5 μg/ml catalase (0·2 ml); 0·001 M pyridoxal phosphate (0·2 ml). Controls were (1) minus 5-ketooctanal, (2) with boiled enzyme, (a) with 5-ketooctanal and (b) minus 5-ketooctanal. Samples of 1 ml for the pyruvate assay and 3 ml for the γ-coniceine assay were taken. 1 ml of reaction mixture contained 1 μmole 5-keto-octanal. Indicates assays not done The % conversion to γ-coniceine is an average measurement taken from the two assays.

alkaline solution[4] and γ-coniceine was assayed using a spectroscopic assay with alkaline sodium nitroprusside.[5]

The γ-coniceine formed in these experiments was identified using paper[6] and thin layer chromatography. Since 5-ketooctanal gave a colour similar to that for γ-coniceine with alkaline sodium nitroprusside, all assays were rigorously checked by chromatography for

[4] S. REITMAN and S. FRANKEL, *Am. J. Clin. Path.* **28,** 56 (1957).
[5] M. F. ROBERTS, B. T CROMWELL and D. E. WEBSTER, *Phytochem.* **6,** 711 (1967).
[6] B. T CROMWELL, *Biochem. J.* **64,** 259 (1956)

contamination with 5-ketooctanal. γ-Coniceine was found only with the complete reaction mixture and was not found to be present in any of the control samples.

Experiments in which 5-ketooctanal was left at room temp. for up to 5 days with physiological amounts of ammonia showed that 5-ketooctanal would not undergo a non-enzymic reaction with ammonia to produce γ-coniceine.

The results for the formation of pyruvate and γ-coniceine show over 30% conversion of 5-ketooctanal to γ-coniceine, and this would favourably account for the high incorporation of radioactive 5-ketooctanal into coniine as observed by Leete,[2] since there is good experimental evidence that γ-coniceine is the first formed alkaloid[7,8] and is subsequently in some strains[9] of hemlock rapidly converted to coniine in the vegetative tissue. Further confirmation of the origin of the nitrogen of γ-coniceine is in progress utilizing DL-alanine-^{15}N.

EXPERIMENTAL

Enzyme preparations. Young actively growing hemlock leaves (100 g) were minced in a Waring Blender with 0·05 M phosphate buffer pH 7·6 (250 ml). After standing at 0° for 1 hr, the extract was pressed through cheese cloth to remove fibrous material and centrifuged at 6000 rev/min for 30 min to remove cell debris. The protein was precipitated from the resulting supernatant with $(NH_4)_2SO_4$ and the precipitate removed by centrifuging at 10,000 rev/min for 30 min, to give a fraction A at 30–40% saturation and a fraction B at 40–70% saturation. The protein fractions A and B were each resuspended in 6 ml of 0·05 M phosphate buffer pH 7·6 passed through a Sephadex G25 column (2 × 60 cm) and the resulting extracts (20 ml) dialysed overnight.

Acetone powders. Hemlock leaves (100 g) were made into an acetone powder at −10°, and a proportion of the acetone powder (4 g) extracted with 0·05 M phosphate buffer pH 7·6 for 1 hr. The procedure that followed was similar to that used for fresh material. Acetone powders were used within a week of their preparation.

In the experiments III and IV (Table 1) the 0·05 M phosphate buffer pH 7·6 used for extractions of the acetone powders contained 0·05 M mercaptoethanol and this buffer was also used for equilibration of the Sephadex G15 column, but was not added to the buffer used in the overnight dialysis. All extracts after dialysis were made up to 25 ml with 0·05 M phosphate buffer pH 7·6 and the protein content of the solution determined by UV measurements at 280 nm. These extracts were used as the enzyme preparations.

Pyruvate assay. For the assay of pyruvate the method of Reitman and Frankel[4] using the formation of the 2,4-dinitrophenylhydrazones of the keto acids and their measurement at 510 nm in alkaline solution was used. The basic reaction mixture contained M. L-alanine (8·3 ml); 0·01 M 5-ketooctanal (1·3 ml); enzyme solution (2·6 ml); 5 μg/ml catalase (0·2 ml); 0·001 M pyridoxal phosphate (0·2 ml). A 1-ml sample of the reaction mixture containing the equivalent of 1 μmole 5-ketooctanal was taken at intervals over 6 hr for the assay of pyruvate using phosphate buffer blanks. Control samples used were, (1) minus 5-ketooctanal, (2) with boiled enzyme—(a) with 5-ketooctanal and (b) minus 5-ketooctanal. Formation of pyruvate occurred only with the complete reaction mixture. Under these experimental conditions 1 μmole pyruvate had absorbance of 0·880 and 1 μmole of 5-ketooctanal an absorbance of 0·190.

γ-Coniceine assay. For the assay of γ-coniceine 3 ml of the reaction mixtures, as set up for the pyruvate assay, was made acid with 1% HCl (1 ml) and extracted with four aliquots of ether to remove 5-ketooctanal. The solution was then made to 5 ml with distilled water and 3 ml of this solution used for the assay of γ-coniceine using the method of Roberts, Cromwell and Webster[5] with alkaline sodium nitroprusside. Blanks used in this assay were the reaction mixture minus 5-ketooctanal. The remainder of the 5 ml solutions were used for the confirmation of the presence or absence of γ-coniceine by paper chromatography.[6]

5-Ketooctanal and ammonia. (a) 0·01 M 5-ketooctanal (1 ml) and 0·001 M ammonia (1 ml) in distilled water (20 ml) were left for periods of up to 5 days to check for non-enzymic formation of γ-coniceine. (b) 0·01 M 5-ketooctanal (1 ml) was added to a reaction mixture containing 0·05 M phosphate buffer pH 7·0 (1 ml), 0·01 M L-alanine (1 ml) and commercially prepared amino acid oxidase (0·5 mg). Samples were taken at hourly intervals and checked for the possible presence of γ-coniceine as a result of non-enzymic utilization of ammonia formed during the oxidation of L-alanine by the added amino acid oxidase.

[7] E. LEETE and N. ADITYACHAUDHARY, *Phytochem.* **6,** 219 (1967).

[8] S. M. C. DIETRICH and R. O. MARTIN, *J. Am. Chem. Soc.* **90,** 1921 (1968).

[9] Leete's strain of Hemlock has coniine as the major alkaloid of the vegetative tissue. Our English strains have γ-coniceine as the major alkaloid of the vegetative tissue with coniine or methyl coniine as the major alkaloid in the fruits only.

γ-Coniceine. The presence of γ-coniceine was authenticated by chromatography using a pure reference sample of γ-coniceine. Two systems were used (a) paper chromatography using the system devised by Cromwell,[4] and (b) TLC using silica gel plates and $CHCl_3$–MeOH (1:1). Dragendorff's reagent and alkaline sodium nitroprusside[4] were used as the spray reagents for both paper and thin layer chromatograms.

5-Ketooctanal. The substance was prepared by two methods: (a) 1-propylcyclopentene using the method of Leete[10] was converted to 1-propylcyclopentane-1,2-diol using osmium tetroxide followed by a sodium sulphite solution. The diol was finally converted to 5-ketooctanal with sodium metaperiodate. (b) 1-propylcyclopentene (450 mg) in dry CH_2Cl_2 (10 ml) and MeOH (1 ml) was converted to the ozonide which was then converted to 5-ketooctanal by stirring with Zn (4 g) and HOAc (12 ml) for 4 hr. The acid was neutralized (pH 7·0) with Na_2CO_3 and the 5-ketooctanal extracted with ether. The ether extracts were dried with anhydrous Na_2SO_4 and the ether evaporated. Yield 5-ketooctanal (350 mg) IR, GLC and the formation of the disemicarbazone m.p. 184–185° showed this material to be identical with that prepared by Leete's method.

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[10] E. LEETE, *J Labelled Compounds* (in press, 1971).