Chem. Pharm. Bull. 25(8)1970—1973(1977)

UDC 547.896.04 : 577.159.08

An S-Adenosyl-L-methionine; Cytisine Methyltransferase in *Thermopsis* Seedlings[1)]

Isamu Murakoshi, Akihiro Sanda, Joju Haginiwa,[2a)] Noboru Suzuki,[2b)] Shigeru Ohmiya, and Hirotaka Otomasu[2c)]

Faculty of Pharmaceutical Sciences, University of Chiba,[2a)] Shizuoka Eiwa Junior College,[2b)] and Hoshi College of Pharmacy[2c)]

(Received December 6, 1976)

Cytisine N-methyltransferase, a new enzyme catalyzing the formation of N-methylcytisine (II) from cytisine (I) and S-adenosyl-L-methionine, was found in the seedlings of *Thermopsis* species. 5-Methyltetrahydrofolic acid did not act as a methyl donor for the N-methylation of cytisine. A magnesium cation dependency was not detected. Some other properties of the enzyme are also described.

Keywords——enzyme; methyltransferase; alkaloid; cytisine; S-adenosyl-L-methionine; 5-methyltetrahydrofolic acid; leguminosae; *Thermopsis chinensis*; *Thermopsis fabacea*

We have previously reported the presence of N-formylcytisine, a new lupin alkaloid, in *Thermopsis chinensis* along with cytisine, N-methylcytisine, anagyrine, and lupanine.[3)]

In connection with the origin of formyl carbon of N-formylcytisine, variations in alkaloid content at various stages of seedling growth of *Thermopsis chinensis* were examined and cytisine derivatives were observed in varying concentration at different times in young seedlings: the concentration of N-methylcytisine increased rapidly throughout the early development of the seedlings on the contrary to the content of cytisine as shown in Fig. 2. The effect of daylight on production of lupin alkaloids was not observed during the first 3—12 day's growth of seedlings.

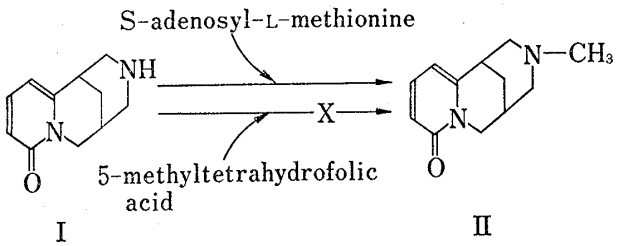

Fig. 1. Scheme for the Biosynthesis of N-Methylcytisine (II) by an Enzyme in *Thermopsis* Seedlings

The present paper describes a evidence for the participation of S-adenosyl-L-methionine in the N-methylation of cytisine(I) by an enzyme in the seedlings of *Thermopsis* species as shown in Fig. 1.

Materials and Methods

Plant materials——The seedlings of *Thermopsis chinensis* and *Thermopsis fabacea* were grown in moistened vermiculite in the dark for 4—5 days at 30°. In some case the immature seeds of *Lupinus luteus* (soft-greenish) were used. After harvest, the testas were removed and the seedlings were cooled at 0° for 30 min before extraction.

Chemicals——5-Methyltetrahydrofolic acid and S-adenosyl-L-methionine were purchased from Sigma Co. Cytisine (mp 154—155°, $(\alpha)_D^{19}$ −121°) was obtained from *Sophora* and *Thermopsis* species as described in previous papers.[3,4)]

1) This work was presented at the 20th Kanto Branch Meeting of the Pharmaceutical Society of Japan at Tokyo, November 27, 1976 (Meeting Abstracts, p. 11).
2) Location: *a) 1-33 Yayoi-cho, Chiba, 280, Japan; b) 1769 Ikeda, Shizuoka, 422, Japan; c) 2-4-41 Ebara, Shinagawa-ku, Tokyo, 141, Japan.*
3) S. Ohmiya, H. Otomasu, I. Murakoshi, and J. Haginiwa, *Phytochemistry*, **13**, 643 (1974).
4) S. Ohmiya, H. Otomasu, I. Murakoshi, and J. Haginiwa, *Phytochemistry*, **13**, 1016 (1974).

Enzyme Preparation——All operations involved in preparation of enzyme extracts were performed at 4°. Crude enzyme preparations were obtained by a modification on that described by Roberts[5] for coniine N-methyltransferase: the seedlings were homogenized in a cold mortar with a small amount of sand in the presence of 0.02 M K-phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol and 1 mM Na-metabisulfite using 0.5 ml buffer for each gram of plant materials. To the homogenate was added polyclar AT (0.1 g/g of tissue) and after stirring for 20 min the mixture was filtered through fine nylon and centrifuged at 25000 ***g*** for 30 min. The protein fraction precipitating between 10 and 75% with $(NH_4)_2SO_4$ was dissolved in a small amount of the grinding buffer and centrifuged at 25000 ***g*** for 15 min. The residual supernatant was desalted with a column of Sephadex G-25 (fine) equilibrated with the grinding buffer. The cloudy protein solution was used directly as the enzyme preparation. Unless otherwise specified, enzyme preparations from the seedlings of *T. fabacea* were used as the source of enzyme activity.

Assay of Cytisine N-Methyltransferase Activity——The normal reaction mixtures contained cytisine (5 μmol), S-adenosyl-L-methionine or S-adenosyl-L-(methyl-^{14}C)-methionine (2 μmol, 2 μCi), dithiothreitol (4 μmol) and 0.5 ml of enzyme preparation which contained 3.5—4.0 mg of the soluble protein from about 3 g of the fresh weight of seedlings in a final volume of 0.8 ml. The pH of incubation mixtures was normally adjusted to pH 8.5 with 0.05 M Tris-HCl buffer. Incubations were conducted at 30° and terminated by addition of a few drops of conc. HCl. With each batch of samples, the control tube, from which S-adenosyl-L-methionine or cytisine had been omitted, or which contained a boiled enzyme preparation, were included. Occasionally, S-adenosyl-L-methionine was replaced in the incubation mixture with 5-methyltetrahydrofolic acid as a methyl donor for the further purified enzyme preparations, fractionated through a Sephadex G-50 column with 0.01 M K-phosphate buffer, pH 7.0, after the 85% $(NH_4)_2SO_4$ precipitation.

Identification of the Reaction Product as N-Methylcytisine (II)——The acidic reaction mixtures terminated with HCl were extracted twice with CH_2Cl_2 and then made alkaline with conc. K_2CO_3 solution. The alkaloids were extracted from the resulting solution with CH_2Cl_2. The formation of N-methylcytisine was demonstrated by subjecting the alkaloidal fractions to thin-layer chromatography (TLC) on Silica gel G (Merck, type 60) and paper chromatography: N-methylcytisine was detected with a Dragendorff's reagent or iodine vapor as chromogenic reagents.

The reaction product co-chromatographed with authentic N-methylcytisine in the following solvent systems: 1, $CHCl_3$–CH_3OH (5: 1, v/v); 2, CH_2Cl_2–CH_3OH–28%NH_4OH (90: 9 : 1, v/v); 3, pyridine–*n*-butanol–water (1: 1: 1, v/v). The *Rf* values for N-methylcytisine on TLC in solvent 1 and 2 were 0.88 and 0.68, respectively, whilst cytisine exhibited the following *Rf* data: 0.48 and 0.44, respectively. N-Methylcytisine and cytisine on PC moved at *Rf*'s of 0.80 and 0.61, respectively, in solvent 3.

The further identity of the reaction product as N-methylcytisine and the quantitative estimation of the amount of N-methylcytisine formed were established by the use of high-speed liquid chromatography (Kyowa Seimitsu, Model K-880) employing a monitoring flow system (310 nm) coupled to recorder, using LiChrosorb SI 100 (mean particle size 10 μm, Merck) as absorbent and 15% CH_3OH–$(C_2H_5)_2O$: H_2O:25% NH_4OH (500: 10: 1, v/v) as the developing solvent. Mass spectrometric determination of the reaction product as N-methylcytisine was performed by eluting the compound from a column of the high-speed liquid chromatography. The formation of N-methylcytisine was also determined by measuring the incorporation of radioactivity from S-adenosyl-L-(methyl-^{14}C)-methionine (2 μCi) into N-methyl-^{14}C-cytisine in reaction mixture which was otherwise as described in the above assay: labelled compound was separated by TLC in solvent 1 and radioactivity associated with Dragendorff's positive substances was measured using a gas flow radiochromatogram scanner as described in previous papers[6] (Fig. 3).

Protein was determined by the method of Lowry, *et al.*[7]

Results and Discussion

The activity of cytisine N-methyltransferase could be detected in 4—5 day's seedlings of *Thermopsis chinensis* under condition in which the concentration of N-methylcytisine increased rapidly as shown in Fig. 2, and of *Thermopsis fabacea* where N-methylcytisine occurs together with many other lupin alkaloids.

The specific activity of enzyme preparations from the seedlings of *T. fabacea* was approximately 2.5 fold greater than those from *T. chinensis*. However, the N-methyltransferase activity in the enzyme preparations obtained from the immature seeds of *Lupinus luteus* which are known as a rich source of lupin alkaloids was negligible, as shown in Fig. 4.

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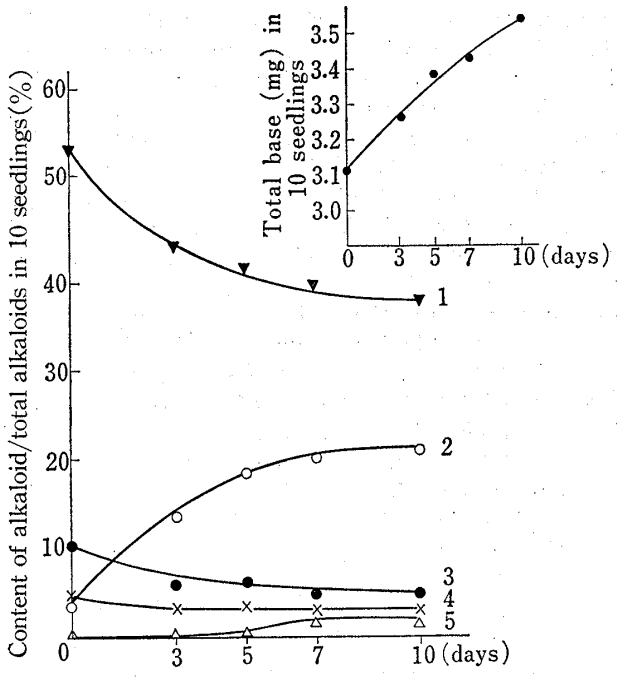

Fig. 2. Variations in Alkaloid Content at Various Stages of Seedling Growth of *Thermopsis chinensis*

Alkaloid content was quantitatively estimated by the high-speed liquid chromatography as described in the experimental: 1, cytisine; 2, N-methylcytisine; 3, anagyrine; 4, baptifoline; 5, N-formylcytisine.

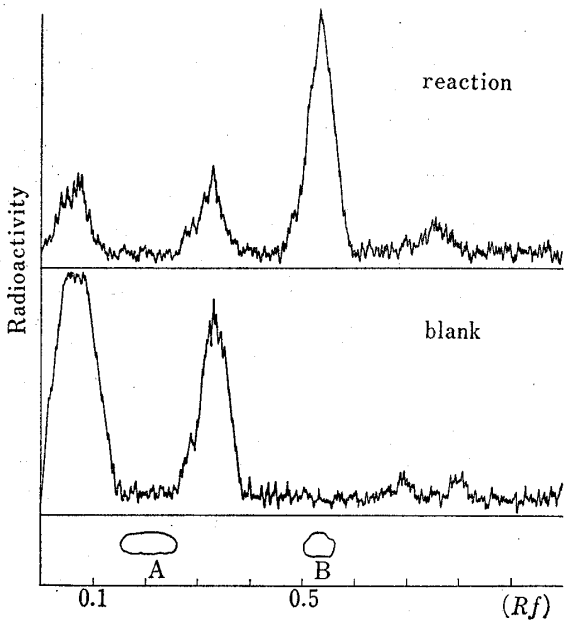

Fig. 3. The Profile of Carbon-14 Incorporation from S-Adenosyl-L-(methyl-^{14}C)-methionine into N-Methylcytisine (II) by an Enzyme in *Thermopsis* Seedlings

TLC were developed in solvent 1: A, cytisine; B, N-methyl cytisine.

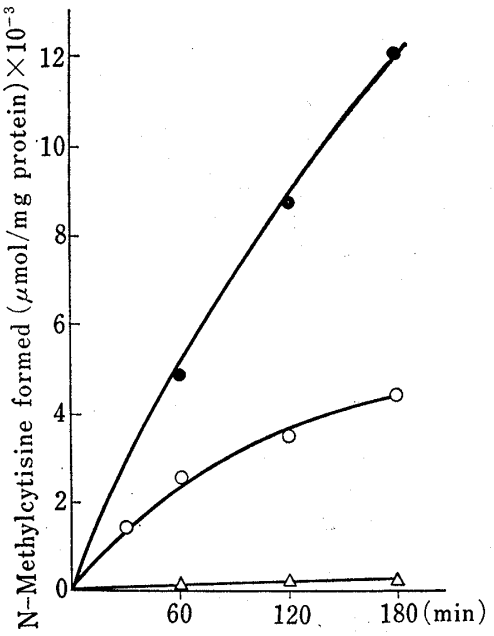

Fig. 4. Comparative Time Courses for the Synthesis of N-Methylcytisine (II) catalyzed by an Enzyme in *Thermopsis fabacea* (Closed Circles), *Thermopsis chinensis* (Opened Circles) and *Lupinus luteus* (Opened Triangles) Seedlings

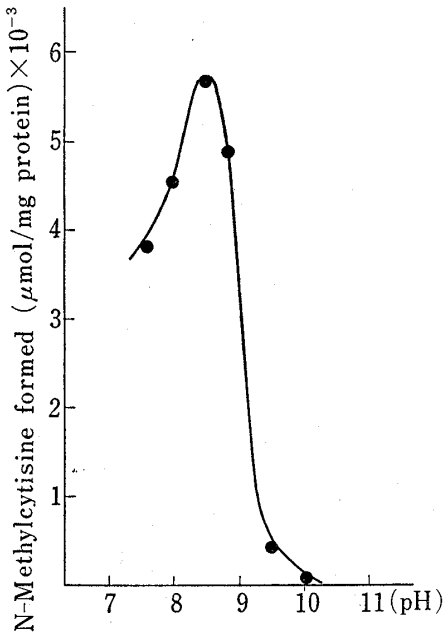

Fig. 5. Effect of pH on the Synthesis of N-Methylcytisine (II) catalyzed by an Enzyme in *Thermopsis* Seedlings

In all cases enzyme preparations were capable of methylating cytisine in the presence of S-adenosyl-L-methionine as a methyl donor. No enzyme activity was detectable toward the 5-methyltetrahydrofolic acid known as a methyl donor for various biogenetic amines.

The optimum pH values for the rate of N-methylation of cytisine by the enzyme were 8.5, using 0.05M Tris-HCl buffer, as shown in Fig. 5.

The formation of N-methylcytisine from cytisine and S-adenosyl-L-methionine was proportional to time for at least 100 min, but the reaction systems further continued to synthesize N-methylcytisine for 180 min tested, as shown in Fig. 4.

Enzyme activity slowly decreased during storage at 0° in the dark by 42—45% in 25 hr even under the conditions in the presence of SH reagents. The N-methyltransferase activity was inhibited by about 35% when the enzyme preparations were preincubated prior to the addition of cytisine and S-adenosyl-L-methionine for 5 min at 30° with N-ethylmaleimide at 1.5 mM under the same conditions containing dithiothreitol (DTT) at 4.5 mM as those described in the experiment.

A number of methyl-transferase, particularly O-methyltransferase,[8] require divalent metal ions but Mg ion at 2.5 and 5.0 mM caused 17 and 31% inhibition of the cytisine N-methyltransferase activity, respectively; maximum reaction rates were observed in a mixture containing no added Mg ion as shown in Fig. 6.

The fact that N-methylation of cytisine by an enzyme in *Thermopsis* seedlings is derived from not 5-methyltetrahydrofolic acid but S-adenosyl-L-methionine as a methyl donor is quite similar to those obtained with the N-methyltransferase of coniine,[5] tyramine,[9] putrescine,[10] and nicotinic acid.[11] At present, it is not certain whether the N-methylation of these compounds *in vivo* and *vitro* is catalyzed by the same enzyme. The formation of N-formylcytisine from cytisine by a 10-formyltetrahydrofolic acid generating system in *Thermopsis* species could not be demonstrated. A study on the origin of formyl-carbon in N-formylcytisine biosynthesis is currently under investigation in our laboratories.

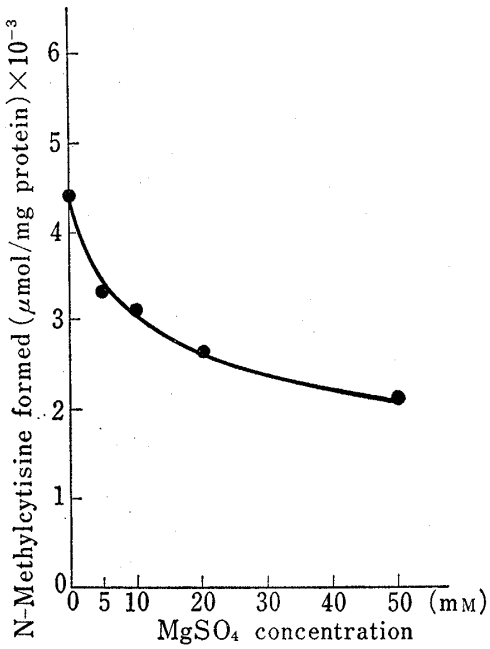

Fig. 6. Effect of Increasing Magnesium Sulfate Concentration upon the Cytisine N-Methyltransferase Activity

Acknowledgement We are grateful to Dr. M. F. Roberts, School of Pharmacy, University of London, for kindly providing polyclar AT and her kind informations concerning the coniine N-methyltransferase, and to Dr. J. M. Smith, Director, Process and Analytical Research Section, Lederle Laboratories, American Cyanamide Co. for a gift of leucovorin Ca-salt. We also are indebted to Mr. T. Ikeda and his students in the scientific groups, Okinoerabu High School, Kagoshima, for cordially supplying *Thermopsis* seeds.

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