Phytochemistry 1974 Vol. 13 pp. 1401 to 1406. Pergamon Press. Printed in England.

L-ALANINE AS A PRECURSOR OF ETHYLAMINE IN *CAMELLIA SINENSIS*

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(*Received* 5 *November* 1973)

Key Word Index—*Camellia sinensis*, Theaceane, tea, theanine, ethylamine, alanine, acetaldehyde, decarboxylation

Abstract—After absorption of ammonium nitrogen, nitrogen-deficient *Camellia sinensis* synthesized theanine following synthesis of glutamic acid and alanine The rate of incorporation of ^{14}C from L-alanine U-^{14}C into theanine was faster than from acetaldehyde 1-2^{14}C Incorporation of ^{14}C from L-alanine U-^{14}C into the ethylamide of theanine was prevented by adding an excess of ethylamine to the culture solution Green seedlings converted alanine to ethylamine more rapidly than did etiolated seedlings

INTRODUCTION

THEANINE (N^5-ethyl-L-glutamine) is synthesized from glutamic acid and ethylamine in the roots of the tea plant.[1,2] This synthesis is catalyzed by L-glutamate: ethylamine ligase which is peculiar to the tea plant and has a high affinity for ethylamine.[3]

The N-ethyl carbon atom of theanine is incorporated into the phloroglucinol nucleus of catechins in the *Camellia sinensis*.[4] Previously, alanine[5] and acetaldehyde[6] had been reported to be precursors of ethylamine in plant tissues. The origin of the ethylamine in theanine and the differences between theanine synthesis in green and etiolated *C. sinensis* have now been studied.

RESULTS AND DISCUSSION

Amino acid composition of C. sinensis *extracts*

Theanine accounted for about 80% of the amino acid content in extracts of the roots of seedlings grown either in the light or dark (Table 1). However, the roots of etiolated seedlings contained larger amounts of theanine than roots of green-seedlings. Extracts of etiolated shoots contained larger amounts of theanine which again accounted for about

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[3] SASAOKA, K, KITO, M and OHNISHI, Y (1965) *Agr Biol Chem* **29,** 984
[4] KITO, M, KOKURA, H, IZAKI, J and SASAOKA, K (1968) *Phytochemistry* **7,** 599
[5] CROCOMO, O J and FOWDEN, L (1970) *Phytochemistry* **9,** 537
[6] MEYER, H and REHM, H J (1967) *Naturwissenschaften* **54,** 370

80% of the amino acid content. The concentration of amino acids was much lower in green-seedling shoots than in etiolated shoots. Theanine accounted for only 65% of the amino acid content of green-shoot extracts.

TABLE 1 CONCENTRATIONS OF NH_3 AND AMINO ACIDS IN SEEDLINGS (expressed as mg%)

Fraction	Etiolated seedlings Root	Etiolated seedlings Leaf	Green seedlings Root	Green seedlings Leaf
Ammonia	11 6	14 9	11 9	6 4
Arginine	1440	739	422	trace
Aspartic acid	307	331	106	40 6
Glutamic acid	175	185	120	82 5
Alanine	8 5	33 4	31 4	9 3
Theanine	7590	4620	3010	284

Variation of amino acids in roots

Changes in the amounts of free ammonia and amino acids in *C. sinensis* seedling roots after ammonia feeding are given in Table 2. Supply of NH_3 for 1 hr resulted in a rapid increase of free NH_3 in the roots. However, the level of NH_3 fell to its original level during the 8 hr exposure to N-deficient medium following NH_3 feeding. Glutamic acid and alanine increased during the 8 hr period after NH_3 supply, but aspartic acid showed no significant change. Arginine and theanine decreased during the initial 3 hr of this period but then increased. The results indicated that arginine and theanine synthesis was induced after accumulation of precursors, possibly glutamic acid and alanine.

TABLE 2 VARIATIONS OF AMINO ACIDS IN THE ROOTS OF GREEN-SEEDLINGS (expressed as mg%, 10 seedlings 5 g)

Hours	0	1	2	3	8
Ammonia	7	15	10	11	6
Alanine	7	5	9	8	11
Glutamic acid	16	14	15	17	21
Aspartic acid	10	10	8	8	9
Arginine	140	110	120	90	150
Theanine	360	330	360	310	350

For the first hour the seedlings were fed with an ammonium medium, the seedlings were then transferred to a N-deficient medium

Incorporation of ^{14}C-alanine and ^{14}C-acetaldehyde into the ethylamine fraction from theanine

After ^{14}C-alanine and ^{14}C-acetaldehyde were absorbed by tea roots, 20–30% of the total radioactivity in the EtOH-soluble fraction was recovered as amino acids (Table 3). The main components containing ^{14}C in the amino acid fraction were alanine, glutamic acid, aspartic acid and theanine, indicating that absorbed alanine and acetaldehyde are transformed to glutamic acid and theanine The incorporation of ^{14}C into ethylamine was much

higher from ^{14}C-alanine than from ^{14}C-acetaldehyde. As shown in Table 3 about 4 and 40% of the radioactivity of theanine was detected in ethylamine from acetaldehyde- and alanine-treated roots, respectively.

TABLE 3 INCORPORATION OF ^{15}C INTO AMINO ACIDS

^{14}C Compound Fraction	^{14}C-Alanine Root	^{14}C-Alanine Leaf	^{14}C-Acetaldehyde Root	^{14}C-Acetaldehyde Leaf
Absorbed total ^{14}C in seedlings*	2·6 × 10^6 cpm		7·7 × 10^6 cpm	
Alcohol extracted fraction	345000	107200	367000	195200
Amino acid fraction	70000	10000	113000	11300
Alanine	24900	1050	52100	1000
Glutamic and aspartic acids	3100	1750	11600	3000
Theanine	9550	800	26300	2650
Ethylamine fraction of theanine	3810 (40%)†	544 (68%)	975 (4%)	800 (30%)

* Each experiment 10 seedlings (5 g) Values are means of duplicate lots
† Ratio of cpm of ethylamine fraction of theanine to that of theanine fraction

The activity of the ^{14}C-ethylamine of the theanine per μmol theanine was about 5× greater in alanine-treated roots than in acetaldehyde-treated roots (Table 4). Hence, acetaldehyde and alanine can act as precursors of theanine in tea roots, but alanine is closer to theanine on the biosynthetic pathway than is acetaldehyde.

TABLE 4 SPECIFIC ACTIVITY OF ^{14}C IN ETHYLAMINE DERIVED FROM THEANINE

Treatment	^{14}C-Alanine	^{14}C-Acetaldehyde
Theanine* in 5 g tissue	34 μg	23 μg
Sp act theanine	82 cpm/μmol	90 cpm/μmol
Sp act ethylamine†	41 cpm/μmol	9 cpm/μmol

* Determined by the colorimetric analysis using ninhydrin.
† Ratio of cpm of ethylamine fraction/μmol purified theanine in root sample.

Conversion of ^{14}C-alanine in roots incubated with excess ethylamine

The incorporation of ^{14}C into the ethylamine fraction of theanine from ^{14}C-alanine in roots was effectively interrupted by adding excess amounts of ethylamine to the culture soln. As shown in Table 5, the incorporation rate of ^{14}C into the ethylamine fraction of

theanine was reduced by about 50% in the ethylamine-fed roots as compared with the control roots Furthermore the ratios of the radioactivity of the ethylamine fraction of theanine to that of theanine showed 13 2 and 76 9% in both treatments, respectively On the other hand, the incorporation of ^{14}C in theanine of the ethylamine-fed roots was 3× greater than that of the non-fed roots However, the specific activities of $^{14}C/\mu mol$ of theanine showed about the same value in each treatment Therefore, it was considered that the conversion of L-alanine to ethylamine in roots was inhibited by excess ethylamine in the culture soln, and fed L-alanine was transformed into glutamic acid through the Krebs-cycle after deamination, and then incorporated into theanine

TABLE 5 CONVERSION OF ^{14}C-ALANINE INTO ETHYLAMINE FRACTION OF THEANINE

Treatment	Ethylamine-fed *C. sinensis**	Control roots†
Radioactivity in theanine	2340 cpm/g‡	695 cpm/g
Sp act theanine	49 9 cpm/μmol	47 8 cpm/μmol
Radioactivity in ethylamine fraction	296 cpm/g	534 cpm/g
Ratio of cpm of ethylamine fraction of theanine to that of theanine	13 2%	76 9%
Ratio of incorporation into ethylamine from ^{14}C-alanine	0 55	1

* Ten roots were dipped in 150 ml of a solution containing 50 μmol of L-alanine, 5 μCi of ^{14}C-alanine with 50 μmol of ethylamine pH 6

† Ten roots were treated as before but without ethylamine

‡ Fresh weight Values are means of duplicate lots

From the above results, it is proposed that glutamic acid and alanine are synthesized after ammonium nitrogen absorption, alanine is converted to ethylamine by alanine decarboxylase and ethylamine reacts with glutamic acid in the presence of L-glutamate ethylamine ligase to produce theanine. Theanine synthesis is accelerated when excess ammonium nitrogen is supplied to the *C. sinensis* [7]

Development of the root system and biosynthesis of theanine

Etiolated seedlings have only a thick white main root while green seedlings possess many developed rootlets on a brown main root The change in root form induced by light was accompanied by functional changes in the biosynthesis of theanine As shown in Table 6, ^{14}C from absorbed ^{14}C-alanine in the roots was incorporated into theanine However, in roots of green seedlings the incorporation was higher than in etiolated seedlings Also the radioactivity of the ethylamine fraction was 10× greater in green seedlings than in

[7] ISIGAKI, K unpublished

etiolated seedlings. The ratio of the radioactivity in the ethylamine fraction of theanine to the total activity of theanine was about twice as high in roots of green seedlings as in roots of etiolated seedlings. Hence, the results indicate a functional difference between roots of green and etiolated seedlings. Alanine may be utilized rapidly as a precursor of ethylamine in the roots of green seedlings while roots of etiolated seedlings are inferior in this biosynthetic ability

TABLE 6 DEVELOPMENT OF ROOTS AND CHANGES IN THEANINE BIOSYNTHESIS

Fraction	Etiolated seedlings*	Green seedlings*
EtOH-soluble fraction	165900 cpm	333000 cpm
Total amino acids	56000	111000
Alanine	7240	12400
Glutamic and aspartic acids	24600	39300
Theanine	12600	44200
Ethylamine from theanine	2520 (20%)†	21200 (48%)

* Values are means of duplicate lots of 10 seedlings

† Ratio of cpm of ethylamine fraction of theanine to that of the theanine fraction

EXPERIMENTAL

Camellia sinensis Tea seeds (*C. sinensis*, L.) were sterilized in 0.025% soln mercury ethylphosphate after removal of the testus. The sterilized seeds were washed in H_2O, soaked overnight, and germinated in a moist sand bed for 30 days in the dark at 20–25°. Part of the 30-day seedlings were grown in the greenhouse and part were kept in the dark for another 30 days.

Treatment with $(NH_4)_2SO_4$ On removal of the cotyledons, green seedlings were grown for 21 days under N-deficient conditions in H_2O culture. The seedlings were then grown on an ammonium medium (pH 5.5) containing 1 mM K_2HPO_4, 0.5 mM $MgSO_4$, 0.1 mM $FeSO_4$, 4.0 mM $CaCl_2$, 1.2 mM K_2SO_4, 1056 mg/l of $(NH_4)_2SO_4$ and 254 mg/l of NH_4Cl. After 1 hr the seedlings were transferred to a N-deficient medium for 8 hr.

Treatment with labeled compounds The roots of 10 cotyledon-free seedlings (green or etiolated) were dipped in 150 ml of either a soln containing 0.01 M phosphate buffer (pH 6) 50 μmol L-alanine, and 5 μCi L-alanine U-^{14}C/ (130mCi/μmol) with or without 50 μmol ethylamine, or a phosphate buffer soln containing 50 μmol MeCHO, 5 μCi of MeCHO 1-2^{14}C (23 μCi/mg) and 250 ppm of $(NH_4)_2SO_4$. The seedlings were then exposed to continuous illumination at an intensity of approx. 10000 lx at 30° for 24 hr.

Isolation and purification of ^{14}C-theanine and ^{14}C-ethylamine Tissue samples (5 g) were macerated in a mortar with 50 ml EtOH. The EtOH-soluble fraction was applied to a cationic resin column (Amberlite IR-120, H^+-form), and the adsorbed fraction (amino acid) was eluted with 2 N NH_4OH. The effluent was dried at 40° under red pres. and dissolved in 5 ml distilled H_2O. Neutral amino acids were fractionated by column chromatography using 100 × 1 cm column of Dowex 50W-X4 (Na-type), 200–300 mesh. Each amino acid was eluted by sodium–citrate buffer soln (pH 3.8) at 45° and detected by the ninhydrin reaction. The theanine fraction was concentrated under red. pres. and re-chromatographed by TLC on silica gel phenol–H_2O (8:2). The ^{14}C-theanine fraction was eluted with hot H_2O and hydrolyzed in a sealed tube with 6 N HCl at 100° for 24 hr. ^{14}C-Ethylamine was recovered in 1 N HCl after liberation from the hydrolysate by 5 N NaOH in a Conway diffusion apparatus.

Determination of radioactivity Samples in silica gel powder from TLC and ^{14}C-ethylamine-HCl soln were transfered to vials containing 10 ml of a dioxan based scintillator soln (4 g PPO, 0.2 g POPOP, 60 g of naphthalene, 100 ml MeOH, and 20 ml ethylene glycol in 1 l. dioxan). Measurements were made in an Aloka LSC-502 liquid scintillation counter.

Assay of amino acids *C. sinensis* were separated into roots and shoots and these were lyophilized. Samples were ground and extracted 3× with boiling H_2O. Catechins in the extracts of root samples were removed using

basic lead acetate soln and excess Pb was then precipitated with H_2S. Catechins in shoot extracts were removed with EtOAc. Catechin-free extracts were concentrated and adjusted to pH 2.2 with sodium citrate–HCl buffer. Amino acids and amides were determined with a JEOL JLC-5AH automatic amino acid analyzer.

Acknowledgement—I thank Dr. J. E. Baker (USDA, Agriculture Marketing Research Institute, Beltsville) for helpful advice on this manuscript.