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THEANINE, A PRECURSOR OF THE PHLOROGLUCINOL NUCLEUS OF CATECHINS IN TEA PLANTS

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Abstract—The *N*-ethyl carbon of theanine was significantly incorporated into the phloroglucinol nucleus of catechins in tea shoots. This incorporation was controlled by light. Acetate-1-^{14}C was also incorporated into the phloroglucinol nucleus, but most of its activity was randomly distributed in other compounds, and light had no effect. The physiological significance of theanine in catechin biosynthesis in tea plants is discussed.

INTRODUCTION

THEANINE (γ-glutamylethylamide) is a major constituent of the soluble nitrogen fraction of tea leaves (*Thea sinensis*),[1] and has an important relationship to the taste of Japanese green tea. This amide has also been reported in *Xerocomus badius*.[2]

The authors have studied the metabolism of theanine, because of its structural similarity to glutamine and the γ-glutamyl peptides which occur in plants.[3] The biosynthesis of theanine in tea seedlings has been reported[4,5] and a theanine synthesizing enzyme which catalyses the synthesis of theanine from L-glutamic acid and ethylamine in the presence of ATP has been isolated.[6,7] However, its physiological significance in tea plants has remained unsolved. In this paper, the metabolic fate of isotopically labelled *N*-ethyl carbon of theanine is described and shown to be a significant precursor of the phloroglucinol nucleus of tea catechins.

RESULTS

Incorporation of Radioactivity from N-Ethyl-^{14}C-Theanine into Catechins in Tea Seedlings

Almost all the radioactivity is extracted by 75 per cent ethanol from the tea seedlings, and this radioactivity remains in the whole seedlings throughout the incubation period, as shown in Table 1. This table also shows that the radioactivity adsorbed on the Amberlite IR-120 column decreases and that not adsorbed on Amberlite IR-120 increases with time (see Experimental). All the radioactivity adsorbed on the Amberlite IR-120 column corresponds only to theanine even after 28 days, as shown by paper chromatography and radioautography. Therefore, theanine is metabolized to some compounds not adsorbed on the Amberlite IR-120 column.

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TABLE 1. FATE OF *N*-ETHYL-^{14}C-THEANINE IN TEA SEEDLINGS*

Days	Radioactivity in the ethanol extract† (cpm × 10^{-5}/seedling)	Amberlite IR-120 column: Adsorbed (%)‡	Amberlite IR-120 column: Not adsorbed (%)‡
0	9·1	99	1
7	10·1	97	3
14	11·3	96	4
21	8·3	82	18
28	10·3	49	51

* Seedlings were grown under 4000 lx.
† An average of $11{\cdot}3 \times 10^5$ cpm each was initially contained at 0-day per seedling.
‡ The radioactivity of the ethanol extract was regarded as 100 per cent.

Table 2 shows the distribution of radioactivity in 28-day-old seedlings. In the roots and cotyledons, most of the radioactivity exists in theanine, whereas, in shoots, 90 per cent of the radioactivity is present in the fraction not adsorbed on Amberlite. Figure 1 shows a paper chromatogram of the Amberlite IR-120 eluate and a radioautogram prepared from that chromatogram. The radioactivity exists in catechins and in an unidentified compound, *A*. In this case, the radioactivity of catechins in this fraction is 90 per cent and the remaining

TABLE 2. DISTRIBUTION OF RADIOACTIVITY FROM *N*-ETHYL-^{14}C-THEANINE IN 28-DAY-OLD SEEDLINGS*

Radioactive compounds	Roots (cpm × 10^{-5}†	Roots %	Cotyledons (cpm × 10^{-5}†	Cotyledons %	Shoots (cpm × 10^{-5}†	Shoots %
Total	2·00	100	3·61	100	5·72	100
Theanine	1·64	82	3·46	96	0·57	10
Compounds not adsorbed on Amberlite IR-120	0·36	18	0·15	4	5·15	90

* Grown under 4000 lx.
† Per seedling.

10 per cent is in compound *A*, which is not adsorbed on an Amberlite IR-45 column and which gives no reaction with ammoniacal silver nitrate. This compound is also formed during the anaerobic alkaline degradation of the radioactive catechins, and may be an artifact derived from catechins. No radioactivity was observed in the sugar and organic acid regions of the chromatogram. By the anaerobic alkaline degradation of the radioactive catechins, phloroglucinol, protocatechuic acid and gallic acid were obtained, and the radioactivity was found only in the phloroglucinol.

These results suggest that the *N*-ethyl carbon of theanine is specifically incorporated into the phloroglucinol nucleus of catechins in tea shoots. This incorporation is greatly affected by illumination, as shown in Table 3. As can be seen, theanine is transported from roots and cotyledons to shoots in the 28-day period. In shoots, theanine is more rapidly converted under 4000 lx than under 150 lx. For example, in the shoots of the 28-day-old seedlings grown under 4000 lx, 90 per cent of the radioactivity appears in catechins and the

TABLE 3. EFFECT OF ILLUMINATION ON THE CONVERSION OF *N*-ETHYL-^{14}C-THEANINE TO CATECHINS

Days	Lx	Distribution of radioactivity in whole seedlings (%)*		Distribution of radioactivity in shoots (%)†	
		Roots and cotyledons	Shoots	Theanine	Catechins and compound *A*
7	3000	99	1	71	29
14	4000	95	5	47	53
	150	96	4	78	22
21	4000	77	23	33	67
	150	93	7	76	24
28	4000	48	52	10	90
	150	83	17	83	17

* All the radioactivity in whole seedlings ($7{\cdot}0 \times 10^5$ cpm per seedling, in average) was regarded as 100 per cent.

† All the radioactivity in shoots was regarded as 100 per cent.

compound *A*, whereas, in those grown under 150 lx, the radioactivity in this fraction is only 17 per cent, and 83 per cent of the radioactivity still remains in theanine.

Incorporation of the Radioactivity of N-Ethyl-^{14}C-Theanine and Acetate-1-^{14}C into Catechins in Young Tea Shoots from Adult Trees

N-Ethyl-^{14}C-theanine and acetate-1-^{14}C with the same specific activity were fed through the freshly cut end of young tea shoots taken from adult trees. As shown in Table 4, when *N*-ethyl-^{14}C-theanine is fed to the shoots, all the radioactivity absorbed into the shoots was extracted by 75 per cent ethanol, and the radioactivity is distributed between theanine, catechins and compound *A*. The radioactivity in catechins under 4000 lx is $0{\cdot}268 \times 10^5$ cpm,

TABLE 4. INCORPORATION OF RADIOACTIVE CARBON OF *N*-ETHYL-^{14}C-THEANINE AND ACETATE-1-^{14}C INTO CATECHINS

Lx	Radioactive compounds	Radioactivity absorbed in young shoots* (cpm × 10^{-5})	Radioactivity in the ethanol extract			
			Total (cpm × 10^{-5})	Theanine (cpm × 10^{-5})	Catechins (cpm × 10^{-5})	Miscellaneous (cpm × 10^{-5})
4000	*N*-Ethyl-^{14}C-theanine	4·55	4·58	4·24	0·268	0·068†
	Acetate-1-^{14}C	8·98	3·05	0	0·560	2·49‡
100	*N*-Ethyl-^{14}C-theanine	2·84	2·97	2·61	0·034	Trace†
	Acetate-1-^{14}C	8·86	2·17	0	0·699	1·47‡

* Difference between the initial radioactivity in the incubation medium and the remaining radioactivity after the incubation.

† Compound *A*.

‡ Amino acids, sugars, organic acids, compound *A* and others.

which corresponds to $4{\cdot}29 \times 10^5$ cpm, since *N*-ethyl-^{14}C-theanine absorbed is diluted 16-fold by endogenous theanine in the shoots. The radioactivity in catechins only appears in the phloroglucinol nucleus. The rate of this incorporation into catechins under 4000 lx is 5-fold that under 100 lx. The same results were obtained when theanine was fed to tea seedlings under the same conditions.

In the case of acetate, $8{\cdot}98 \times 10^5$ cpm of the radioactivity is absorbed in the shoots under 4000 lx, but only $3{\cdot}05 \times 10^5$ cpm is found in the 75 per cent ethanol extract after the incubation. This may be due to its release as $^{14}CO_2$ from leaves. $0{\cdot}56 \times 10^5$ cpm is incorporated into catechins and the radioactivity again appears only in the phloroglucinol nucleus. However, unlike the case of *N*-ethyl-^{14}C-theanine, 90 per cent of the radioactivity absorbed in the shoots is randomly distributed in amino acids, sugars, organic acids and others besides compound *A*. In the shoots incubated under 100 lx, the distribution pattern of the radioactivity is similar to that under 4000 lx. Thus, light has no effect upon the incorporation of the radioactivity into catechins from acetate-1-^{14}C.

DISCUSSION

The refined Japanese green tea (Gyokuro) is manufactured from shaded young tea leaves. By shading tea leaves, large quantities of theanine accumulate (1–2% dry weight),[1] but, by contrast, the catechin content is lower than that in unshaded leaves.[8] In this paper, the authors have shown that the *N*-ethyl carbon of theanine is converted to the phloroglucinol nucleus of catechins in tea shoots, and this conversion is controlled by light. These results may well explain the relationship between theanine and catechin content in shaded and unshaded tea leaves.

Zaprometov[9] reported the incorporation of radioactive acetate into the phloroglucinol nucleus of catechins in tea leaves. In our present experiments, similar incorporation of acetate-1-^{14}C into catechins was observed, but the greater part of the radioactivity was found to be randomly distributed in amino acids, organic acids and other compounds, and light was found to have no effect on the rate of incorporation of the radioactivity into catechins. The fact that theanine activity appears only in catechins and compound *A* suggests the existence of a restricted site of theanine degradation, where the *N*-ethyl carbon of theanine may be converted to the phloroglucinol nucleus of catechins probably by the condensation mechanism via acetyl-CoA.[10] Light may be needed for the degradation of theanine in that site. Zaprometov[9] described that the condensation of the acetate residues was the limiting factor in the synthesis of the phloroglucinol nucleus. But the low rate of the phloroglucinol synthesis in his experiments may have been due to the dilution of the labelled acetate residue by the cold acetate residue derived from endogenous theanine.

EXPERIMENTAL

Incubation of Tea Seedlings

Twenty seedlings (11-day-old) were incubated in a medium containing 100 μmoles of L-glutamic acid and 33·4 μmoles of ethylamine-1-^{14}C ($4{\cdot}72 \times 10^7$ cpm[11]) in a total volume of 34 ml (pH 5·6 with 3×10^{-2} M potassium phosphate buffer) in the dark at 25° for 2 or 3 days. By this incubation, radioactive theanine was synthe-

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[11] Radioactivity was determined by a gas flow counter.

sized in the seedlings, and all the radioactivity incorporated existed only in the ethylamine part of theanine, as described previously[5] (i.e. the radioactive theanine thus synthesized is *N*-ethyl-^{14}C-theanine). The seedlings with the labelled theanine in their roots and cotyledons at this stage were called 0-day-old seedlings, and then further grown on moistened cotton at 25° under fluorescent light. After these seedlings were grown under 3000 lx for the first 7 days, they were divided into two groups under different illumination (4000 and 150 lx). Two seedlings were used for each analysis.

Incubation of Tea Shoots from Adult Trees

Two young tea shoots from adult trees (one bud and two leaves) were incubated in a medium containing *N*-ethyl-^{14}C-theanine ($6{\cdot}0 \times 10^5$ cpm, 1·41 μc/μmole) or acetate-1-^{14}C ($9{\cdot}0 \times 10^5$ cpm, 1·41 μc/μmole) in a total volume of 0·2 ml at 25° for 1·8 hr, and then the shoots were transferred to 30 ml of deionized water and the incubation was continued for 22 hr under the appropriate illumination (4000 or 100 lx). *N*-Ethyl-^{14}C-theanine of high specific activity used in this incubation was enzymatically prepared, using a pigeon liver acetone powder extract,[12] from L-glutamic acid and ethylamine-1-^{14}C.

Analytical Methods

Seventy-five per cent ethanol extracts of tea seedlings or tea shoots were fractionated on an Amberlite IR-120 (H^+) column (1 × 17 cm) and an Amberlite IR-45 (OH^-) column (1 × 17 cm), previously equilibrated with 75 per cent ethanol. The cationic fraction was eluted with 1 N ammonia from the Amberlite IR-120 column and refractionated by two-dimensional paper chromatography, using first, phenol-water (4:1) then *n*-butanol–acetic acid–water (4:1:1). The amount of theanine in this fraction was quantitatively determined by an amino acid analyzer.[13] The anionic fraction was eluted with 2 N $(NH_4)_2CO_3$ from the Amberlite IR-45 column.

The radioactivity of catechins was determined by the following two methods: (1) the fraction not adsorbed on the Amberlite IR-120 column was concentrated in a rotary evaporator below 40°, and then separated by paper chromatography in 2 per cent acetic acid, and the radioactivity was measured by the use of a strip counter: (2) in these experiments, autoradiographic analyses showed that the radioactive compounds which were adsorbed on the Amberlite IR-45 column and could not be eluted with 2 N $(NH4)_2CO_3$, were only catechins, therefore the radioactivity adsorbed irreversibly on the Amberlite IR-45 column was regarded as that of catechins.

Anaerobic Alkaline Degradation of Catechins

Catechins were extracted with small amounts of ethyl acetate from the Amberlite IR-120 eluate, which had been concentrated and dissolved in water, and were anaerobically degraded according to Zaprometov.[9]

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