Biochemical Studies on ω-Carboxyl Groups of Aspartic Acid and Glutamic Acid

MAKOTO KITO

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ABBREVIATIONS

ADP	Adenosine diphosphate
Ammediol	2-Amino-2-methylpropane-1 : 3-diol
ASA	Aspartic β-semialdehyde
ATP	Adenosine triphosphate
EDTA	Ethylenediaminetetraacetic acid
Km	Michaelis constant
NAD	Nicotine adenine dinucleotide
$NADH_2$	Reduced nicotine adenine dinucleotide
NADP	Nicotine adenine dinucleotide phosphate
$NADPH_2$	Reduced nicotine adenine dinucleotide phosphate
PCMB	*p*-Chloromercuribenzoate
Pi	Orthophosphate
Tris	Tris (hydroxymethyl) aminomethane

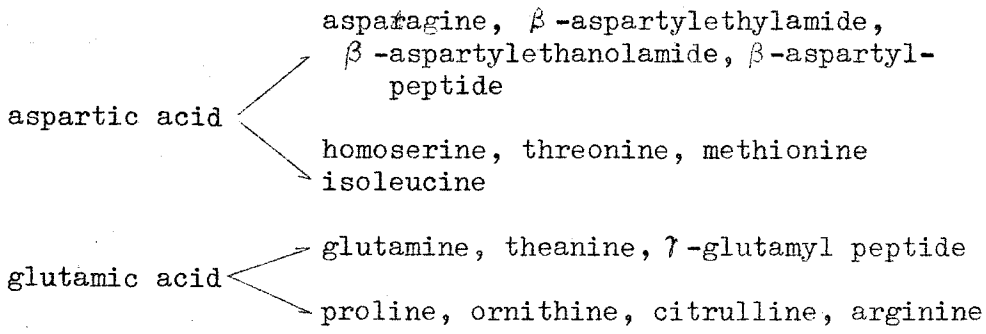
CONTENTS

CHAPTER I

INTRODUCTION

Many studies[1)] have shown that the monoaminodicarboxylic acids, particularly aspartic acid and glutamic acid, occupy a key position in the metabolic transformations of nitrogenous substances, and are regarded to be the primary amino acid in nitrogen metabolism.

They are converted to various amino acids, amides and peptides through the reactions of their ω-carboxylic acids, as follows;



Among those biochemical reactions, aspartic β-semialdehyde dehydrogenase reaction lies on the pathway in which aspartic acid is converted to the other amino acids, such as homoserine, threonine, isoleucine and methionine, through the activation of its β-carboxyl group, and catalyzes the

formation of acylphosphate from the aldehyde, that is, the trapping of orthophosphate on substrate level (substrate level phosphorylation). Therefore, it is a unique reaction in the field of enzyme chemistry that this enzyme has the dual role of oxidizing the aldehyde and producing the acylphosphate.

In the present studies, from the point of view described above, it was attempted to obtain the highly purified enzyme from pea seedlings, to confirm its various properties, and to assume its reaction mechanism.

On the other hand, the biosynthesis of amides and peptides through the activation of their ω-carboxyl groups attracts our interests.

Recently, many β-aspartyl and γ-glutamyl derivatives have been found in Nature[2,3], and elucidation of their significances has been expected. However, few biochemical studies on those matters have been performed.

Theanine was first found in tea leaves by Sakato[4]. This compound was also found Xerocomus badius[5].

From the point of view that theanine (γ-glutamylethylamide) has a structure analogous to glutamine and -glutamyl dipeptides, it was undertaken to clarify the mechanism of theanine biosynthesis.

In the present studies, biosynthesis of theanine in tea seedlings, by tea seedling homogenate, in pea seedlings and by pea and pigeon liver acetone powder extracts were elucidated.

In the present paper, the detailes of the results are described.

CHAPTER II

Aspartic β-Semialdehyde Dehydrogenase in Pea Seedlings[a,b]

1. INTRODUCTION

Aspartic β-Semialdehyde dehydrogenase, which was found for the first time in yeast[6], catalyzes the following reaction, and plays a role in homoserine biosynthesis.

$$\beta\text{-Aspartyl phosphate} + NADPH_2 \rightleftharpoons ASA + NADP + Pi$$

In this chapter, detailed properties of this enzyme from pea seedlings[7] are described.

2. MATERIALS AND METHODS

Pea Seedlings

Pea seeds (pisum sativum var. Usui) were germinated in the dark at 20°C.[8]

Preparation of Enzyme

Four-day pea seedlings were used for the preparation of the enzyme. The isolation procedures are as follows. One hundred and sixty grams of decotylized seedlings were homogenized with 160 ml of water in a Waring blendor. The homogenate was squeezed through gauze, and then centrifuged at 10,000 xg for thirty minutes. To 224 ml of this extract, an equal volume of saturated ammonium sulfate solution (adjusted to pH 7.0 with 5 N NH_4OH) was added. This solution was allowed to stand for thirty minutes, and then

the precipitate was removed by centrifugation (10,000 x g, for thirty minutes). The supernatant solution was brought to 75 per cent saturation with the further addition of saturated ammonium sulfate solution. After having been stood for thirty minutes, the precipitate was collected by centrifugation and was dissolved in water. To 15 ml of this solution, 10 ml of acetone was added at $-10^{\circ}C$, the precipitate formed was removed by centrifugation (4,000xg, for five minutes, at $-10^{\circ}C$) and discarded. The clear supernatant solution (22.6 ml) was further treated with 11.3 ml of acetone. The precipitate was collected by centrifugation and was dissolved in water. This enzyme solution was dialyzed against water for several hours with stirring, and small amounts of precipitate formed during this dialysis were removed by centrifugation (Enzyme 1, this enzyme solution contained 52μg/ml of Pi, as determined by the method of Chen[9] et al.).

Enzyme I was further purified by vertical zone electrophoresis by means of methanolized cellulose power column[10].

The enzyme solution was dialyzed against phosphate buffer (pH 7.6, μ=0.112), and the precipitate formed was removed by centrifugation (10,000xg, for thirty minutes).

The dialyzate was applied on a methanolized cellulose powder column (3 x 50 cm) and electrophoresis was carried out in phosphate buffer (pH 7.6, μ=0.112) at 200V, 28 mA for 45.5 hours. After run, the zones separated (anodic) were eluted with the same buffer, and the eluate was collected in a series of 3.0 ml fractions. The enzyme appeared mainly in No. 6-8 fractions (Enzme II).

All procedures described above were carried out at a low temperature (below 5°C).

The purification procedures are summarized in Table 1.

Table 1. Summary of Purification Procedure

Fraction	Total volume ml	Total protein mg	Total activity	Activity per mg of protein	Recovery per cent
Pea extract*	224	1,410.0	55,000**	39	100
Ammonium sulfate fraction, 0.50 - 0.75	15	444.0	84,800	191	154
Acetone fraction, 40 - 60%	6	50.3	49,100	976	90
Dialysis, pH 7.6	6.5	45.6	46,800	1,030	85
Zone electrophoresis	9	1.6	31,800	19,900	58

Activity: Optical density change at 340 mμ per minute x 10^3.

* Centrifuged at 59,000xg for thirty minutes to determine the enzyme activity and concnetrations of protein.

** The activity of the first extract was usually low, probably owing to side reactions during the measurement.

This enzyme is very stable. There was no loss of activity, when kept at 6°C for 24 hours at pH 5.8-7.6, and could be stored at -20°C for at least 6 months without loss of activity.

Standard Assay Conditions

The enzyme activity was determined by the reverse reaction of the equation described above, measuring the rate of reduction of NADP with a Beckman model DU spectrophotomoter (light path, 1 cm) at a room temperature. The complete system contained 300 μ moles of Tris (pH 8.7), 30μ moles of K_2HPO_4, 0.37μ mole of NADP, 4μ moles of DL-ASA, 3μ moles of sodium thioglycolate, and definite amounts of the enzyme in a total volume of 3.0 ml. The reaction was started by the addition of ASA. The changes in optical density at 340 mμ were measured every fifteen seconds. The increment in optical density between fifteen and forty-five seconds of the reaction, multiplied by 2, was taken as the enzyme activity per minute. The amounts of the enzyme used in the tests were adjusted so that the optical density change per minute did not exceed 0.120.

Under these conditions, the reaction proceeded linearly for the initial one minute, and the initial rate was pro-

portional to the enzyme concentration, as shown in Fig. 1.

The reason of the addition of thioglycolate in the assay system will be described in RESULTS.

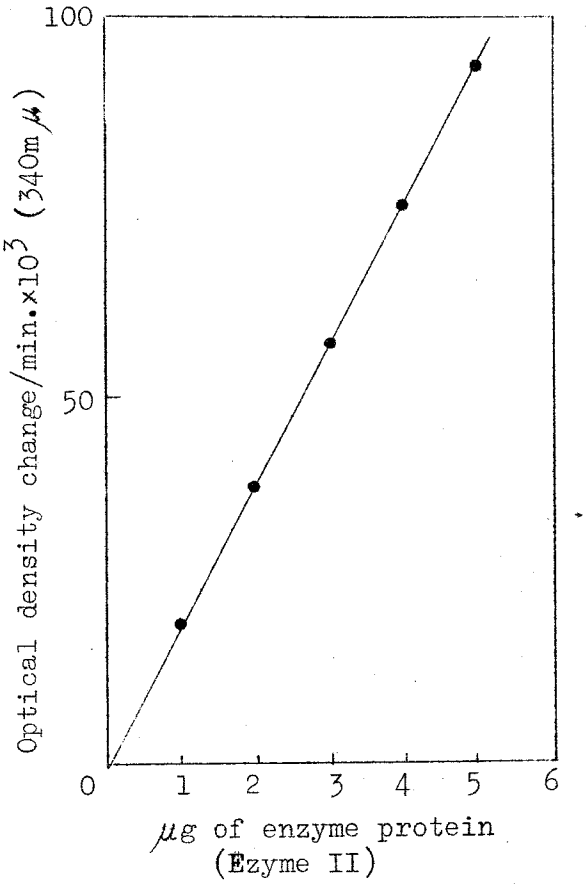


Fig. 1. Effect of Enzyme Concentration on the Reaction Rate.

Method for The Determination of Km in The Reaction Containing Three Substrates.

As is well known, Km can be determined under the Michaelis and Menten[11] or Briggs and Haldene[12] steady

state. However, only in the case of the reaction containing one or two substrates, mathematical analysis have been clarified[13)]

As partic β-semialdehyde dehydrogenase reaction has three substrates. Therefore, the author intended to perform the theoretical assumption of the reaction mechanism in which three substrates are present.

In this case, the enzyme reaction can be represented by the following reaction sequences, according to the Michaelis and Menten steady state theory.

$$E + S_1 \rightleftharpoons ES_1 \qquad Ks_1$$

$$E + S_2 \rightleftharpoons ES_2 \qquad Ks_2$$

$$E + S_3 \rightleftharpoons ES_3 \qquad Ks_3$$

$$ES_2 + S_1 \rightleftharpoons ES_1S_2 \qquad Ks1'$$

$$ES_1 + S_2 \rightleftharpoons ES_1S_2 \qquad Ks2'$$

$$ES_1 + S_3 \rightleftharpoons ES_1S_3 \qquad Ks3'$$

$$ES_3 + S_1 \rightleftharpoons ES_1S_3 \qquad Ks1''$$

$$ES_3 + S_2 \rightleftharpoons ES_2S_3 \qquad Ks2''$$

$$ES_2 + S_3 \rightleftharpoons ES_2S_3 \qquad Ks3''$$

$$ES_2S_3 + S_1 \rightleftharpoons ES_1S_2S_3 \qquad Ks1'''$$

$$ES_1S_3 + S_2 \rightleftharpoons ES_1S_2S_3 \qquad Ks2'''$$

$$ES_1S_2 + S_3 \rightleftharpoons ES_1S_2S_3 \qquad Ks3'''$$

$$ES_1S_2S_3 \xrightarrow{k} E + \text{Products}$$

where, E is enzyme, S_1 S_2 and S_3 are substrates, ES_1 is the enzyme to which S_1 is bound and Ks_1 is the corresponding dissociation constant; similarly, Ks_2, Ks_3, $Ks_{1'}$, $Ks_{2'}$, $Ks_{3'}$, $Ks_{1''}$, $Ks_{2''}$, $Ks_{3''}$, $Ks_{1'''}$, $Ks_{2'''}$, and $Ks_{3'''}$, are the corresponding dissociation constants. $ES_1S_2S_3$ is the active quarternary complex, and k the rate limitting step of the reaction.

The total amount of the enzyme, $[E]_0$, is as follows;

$$[E]_0 = [E]_{free} + [ES_1] + [ES_2] + [ES_3] + [ES_1S_2] + [ES_2S_3] + [ES_1S_3] + [ES_1S_2S_3]$$

where the concentrations are those in equilibrium.

The reaction velocity is expressed in

$$v = k[ES_1S_2S_3]$$

When $Ks_1 \doteq Ks_{1'} \doteq Ks_{1''} \doteq Ks_{1'''}$

$Ks_2 \doteq Ks_{2'} \doteq Ks_{2''} \doteq Ks_{2'''}$

$Ks_3 \doteq Ks_{3'} \doteq Ks_{3''} \doteq Ks_{3'''}$

and

$Ks_2 \ll [S_2] \doteq$ constant

$Ks_3 \ll [S_3] \doteq$ constant

The equation is rearranged to

$$\frac{[E]_0}{[ES_1S_2S_3]} = \frac{V}{v} = \frac{Ks_{1'''}}{[S_1]} + 1$$

where, $[S_1]$ is the substrate (S_1) concentration and V the maximum velocity

Here, the familiar Michaelis and Menten equation is given.

Protein Determination

Protein concentrations were determined spectrophotometrically according to Warburg and Christian[14].

Reagent

DL-ASA was prepared from DL-allylglycine according to the method of Black and Wright[6]. ASA was determined enzymatically.[7] NADP and NAD were determined enzymatically using pig heart isocitric dehydrogenase[15] and yeast alcohol dehydrogenase[16], respectively. DL-Glyceraldehyde 3-phosphate was determined by muscle glyceraldehyde 3-phosphate dehydrogenase[17]. In the experiments in which glyceraldehyde was used, the aqueous solution of the aldehyde was treated according to Velick[18] to convert the aldehyde to the monomeric form.

DL-Allylglycine, NADP, NAD, DL-glyceraldehyde 3-phosphate diethylacetal (barium saalt), yeast alcohol dehydrogenase and muscle glyceraldehyde 3-phosphate dehydrogenase were purchased from the Sigma Chemical Company (U.S.A.).

3. RESULTS

Effect of pH

The optimum pH for pea ASA dehydrogenase (reverse reaction) is shown in Fig. 2. In these experiments, Tris-HCl buffer and Ammediol-HCL buffer (μ=0.05) were used. A sharp maximum was obtained at pH 8.76 in both buffers, though the enzyme was a little more active in the latter buffer than in the former one.

Effect of Substrate Concentration

Effects of substrate concentrations on the reaction rate were examined under the standard assay conditions except that the concentrations of ASA, NADP or orthophosphate were varied. Km values for the substrates were calculated from Lineweaver-Burk plots[19]. In these experiments, Enzyme II was used. And, in the determination of Kpi value (Fig. 5), the concentrations of pre-existing

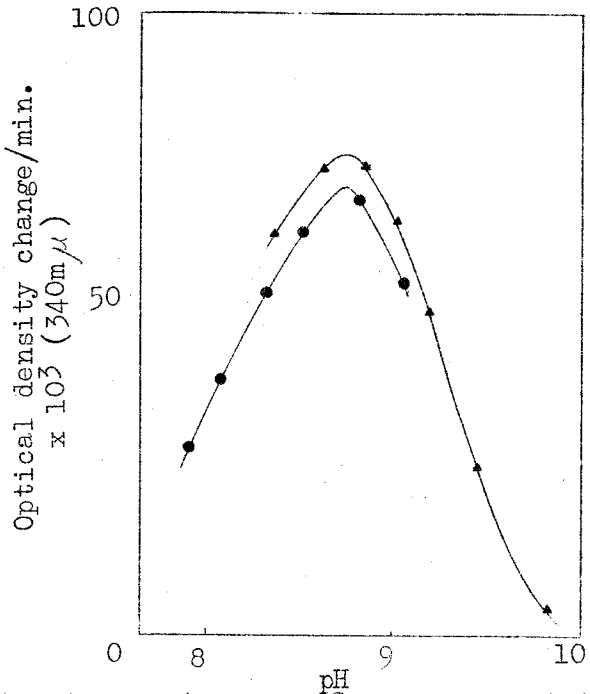


Fig. 2. pH-Activity Curve of Pea ASA Dehydrogenase.

The standard assay procedures were carried out at varied pH values as indicated. Enzyme II was used.

—●— Tris-HCl buffer (μ = 0.05)

—▲— Ammediol-HCl buffer (μ = 0.05)

orthophosphate in the enzyme solution were corrected.

Though this dehydrogenase has three substrates, ASA, NADP and orthophosphate, the Km value of one substrate (S_1) can be calculated by the method of Lineweaver and Burk, because the following equation can be obtained, when the concentrations of the other two substrates (S_2 and S_3) are large enough and are kept constant as described above.

$$\frac{V}{v} = \frac{Ks'''}{[S_1]} + 1$$

v: Velocity, V: maximum velocity, S_1 : concentration of substrate, Ks''': dissociation constant in the equation, $ES_2S_3 + S_1 \rightleftharpoons ES_1S_2S_3$.

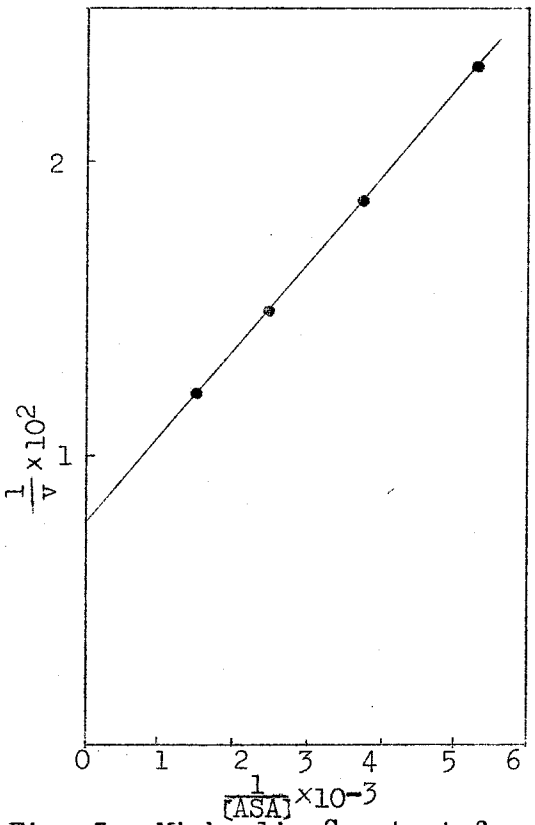


Fig. 3. Michaelis Constant for ASA.

$K_{ASA} = 4.0 \times 10^{-4}$ M

v : Optical density change at 340 mμ per minute x 10^3.

[ASA] : Concentration of L-ASA (M).

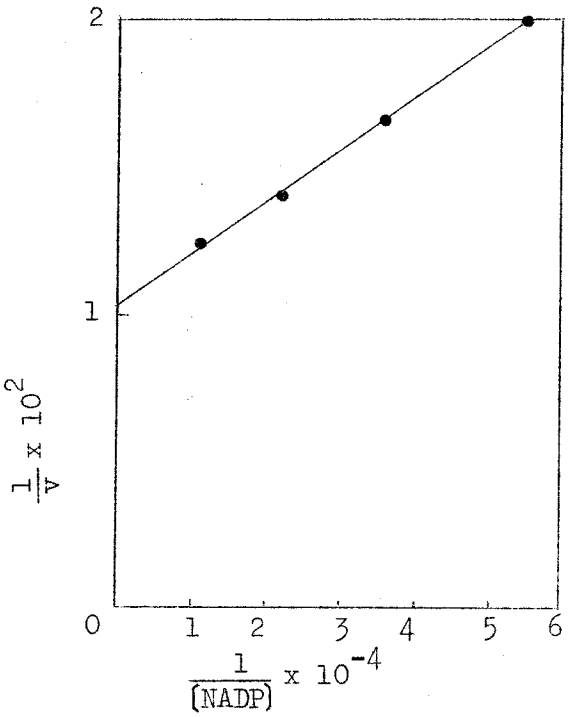


Fig. 4. Michaelis Constant for NADP

$K_{NADP} = 1.6 \times 10^{-5}$ M

v: Optical density change at 340 mμ per minute x 10^3.

[NADP]: Concentration of NADP (M).

As shown in Figs. 3, 4, and 5, the Km values for ASA, NADP and orthophosphate were 4 x 10^{-4}M, 1.6 x 10^{-5}M and 2.7 x 10^{-3}M, respectively. The Km value for ASA of pea ASA dehydrogenase was approximately 10 times lower than that of the yeast enzyme[6], but those for NADP and orthophosphate were the same order.

Arsenate could be substituted for orthophosphate in the reaction, and the Km value for arsenate was 9 x 10^{-3}M (Fig. 6).

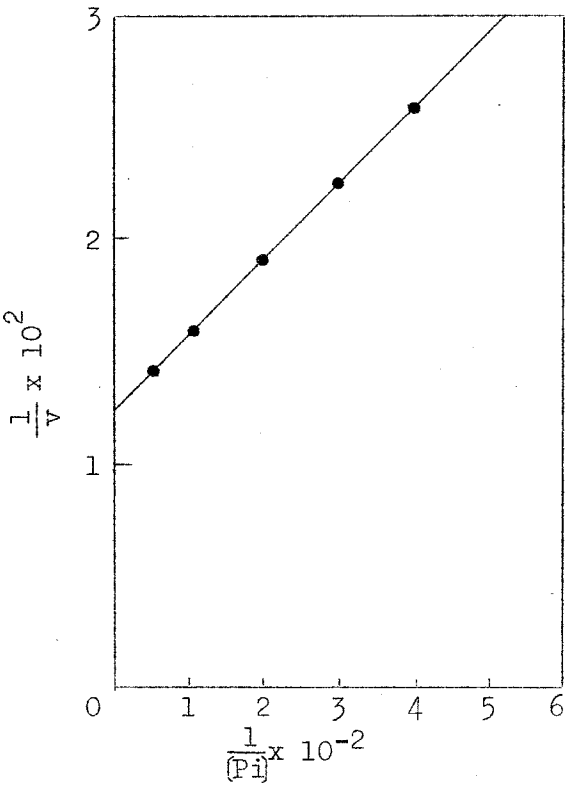


Fig. 5. Michaelis Constant for Orthophosphate.

$$K_{Pi} = 2.7 \times 10^{-3} M$$

v: Optical density change at 340 m μ per minute x 10^3.

[Pi] : Concentration of orthophosphate (M).

The participation of orthophosphate in this reaction shows the formation of β-aspartyl phosphate. The formation of this compound was detected by the hydroxamate method of Lipmann and Tuttle[20], as shown in Table II.

In this table, in the complete system, $NADPH_2$ and hydroxamate were clearly formed. In the control experiments in which either NADP or ASA was omitted from the

reaction mixture, or heated enzyme was used, neither $NADPH_2$ nor hydroxamate was formed. When K_2HPO_4 was omitted, there was a little formation of $NADPH_2$, probably because of the preexisting orthophosphate in the enzyme solution as described above, but hydroxamate could not be detected owing to the sensitivity of this method.

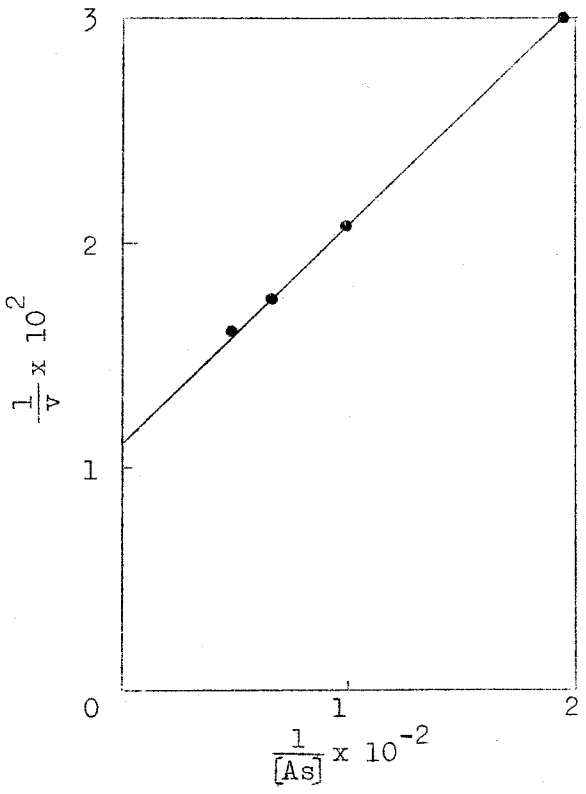


Fig. 6. Michaelis Constant for Arsenate.

$K_{As} = 9.0 \times 10^{-3}M$

v: Optical density change at 340 mμ per minute x 10^3

[As] : Concentration of Na_2HAsO_4 (M).

Table II. Formation of β-Aspartohydroxamate

	$NADPH_2$ formed ΔE_{340}	Hydroxamate formed ΔE_{500}
Complete	2.44	0.074
-NADP	0	0
-ASA	0	0
-Phosphate	0.21	0
Heated Enzyme*	0	0

300 μmoles of Tris (pH 8.7), 30 μmoles of K_2HPO_4, 12 μmoles of NADP, 4 μmoles of DL-ASA, 3 μmoles of thioglycolate and 1.0 mg of Enzyme I in a total volume of 3.0 ml.

The reaction was followed spectrophotometrically for five minutes, and then 1 ml of 2 M hydroxylamine (pH 6.4) was added to 2ml of the reaction mixture. The hydroxamate formed was determined according to the method of Lipmann and Tuttle.

* In a boiling water bath for five minutes.

Paper chromatographic tests[21] of the samples prepared according to the method of Stadtman and Barker[22] from the reaction mixtures similar to those described in Table II showed the formation of a spot identical with authentic β-aspartohydroxamate prepared by the method of Roper and McIlwain[23].

Substrate and Coenzyme Specificity

Table III. Substrate Specificity

Substrate	Concentration (M)	Activity per cent
DL-Aspartic β-semialdehyde	1.3×10^{-3}	100
Acetaldehyde	6.6×10^{-4}	0
"	3.3×10^{-2}	0
Propionaldehyde	6.6×10^{-4}	0
"	3.3×10^{-2}	0
n-Butyraldehyde	6.6×10^{-4}	0
"	3.3×10^{-2}	0
DL-Glyceraldehyde	1.3×10^{-3}	0
"	3.3×10^{-2}	0
DL-Glyceraldehyde 3-phosphate	1.3×10^{-3}	0
"	6.6×10^{-3}	0

ASA was replaced by the aldehydes listed under the standard assay conditions (Enzyme II).

Table IV. Coenzyme Specificity

Coenzyme	Concentration (M)	Activity per cent	Km
NADP	9×10^{-5}	100	1.6×10^{-5} M
NAD*	9×10^{-5}	0	
"	10^{-3}	5	1.4×10^{-3} M
"	2.3×10^{-3}	11	

* NADP was replaced by NAD under the standard assay conditions (Enzyme II).

The substrate specificity for various aldehydes was examined. As shown in Table III, all aldehydes used could not be substituted for ASA under the test conditions.

Though pea ASA dehydrogenase showed negligible activity when NAD was used instead of NADP as coenzyme under the standard assay conditions, a little activity was detected in high concentrations of NAD, as presented in Table IV. The approximate Km value for NAD calculated from the results in Table IV was 1.4×10^{-3}M. This value is 100 times higher than that for NADP. These results suggest that this enzyme is specific to NADP.

Effect of Thiol Compound.

Table V. Activation by Thiol Compound

Thiol	Concentration (M)	Activity per cent
Thiol omitted*	--	3
Thioglycolate	10^{-2}	85
"	10^{-3}	100**
"	10^{-4}	95
L-Cysteine	10^{-3}	100
Glutathione (reduced)	10^{-3}	100

* Thioglycolate was omitted from the standard assay system. (Enzyme 1).

** The value obtained with 10^{-3}M thioglycolate was regarded as 100.

Thioglycolate and other thiol compounds activated pea ASA dehydrogenase predominantly, as shown in Table V. 10^{-3}M and 10^{-4}M thioglycolate showed nearly the same degree of activating effect, though this compound was slightly inhibitory at 10^{-2}M. Therefore, 10^{-3}M thioglycolate was used in the standard assay of the enzyme. Yeast ASA dehydrogenase does not require such thiol compounds[6].

Effect of Chelating Agent.

EDTA and o-phenanthroline showed no effect on the enzyme reaction, independently of preincubation with the enzyme, as shown in Table VI.

These results suggest that the activation by thiol compounds mentioned above may not be due to the chelating action of these thiol compounds, but due to the regeneration of essential -SH groups in the enzyme protein.

This assumption may be confirmed by the experiments described in Fig. 7. In this figure, the reaction proceeded for the initial three minutes without any addition of the chelating agent or thioglycolate. At the point indicated, EDTA was added,but no increase of the reaction rate was observed. Whereas, the enzyme was highly activated by the further addition of thioglycolate.

Table VI Effect of Chelating Agent

Compound	Concentration (M)	Preincubation time (min.)	Activity per cent
Control	--	0	100
"	--	20	100
EDTA	5×10^{-3}	0	100
"	"	20	100
O -Phenanthroline	5×10^{-3}	0	100
"	"	20	100
"	"	40	100

Various chelating agents were added under the standard assay conditions (Enzyme I). The activity was measured immediately after the addition of the chelating agents or after preincubation at a room temperature.

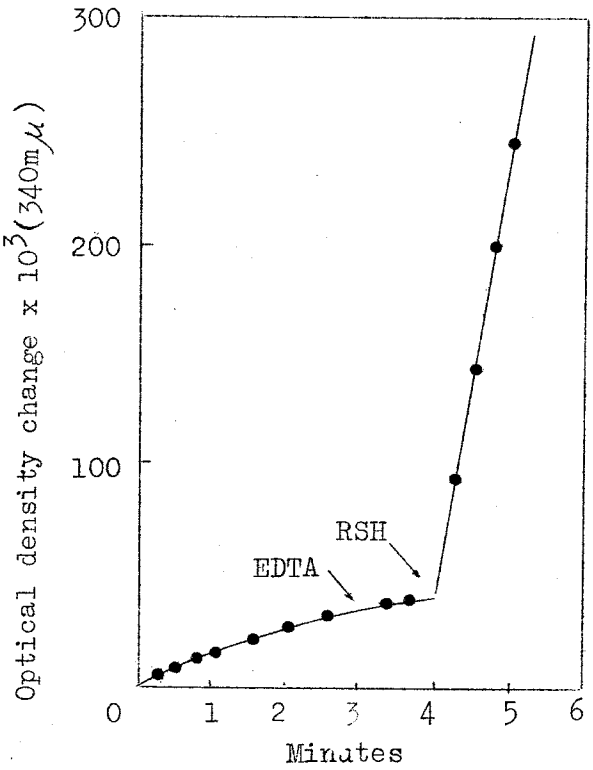


Fig. 7. Activation by Thioglycolate.

The reaction (Enzyme I) proceeded for the initial three minutes without an addition of the chelating agent or thioglycolate. EDTA (5×10^{-3} M) and thioglycolate (RSH, 10-3 M) were added at the points indicated.

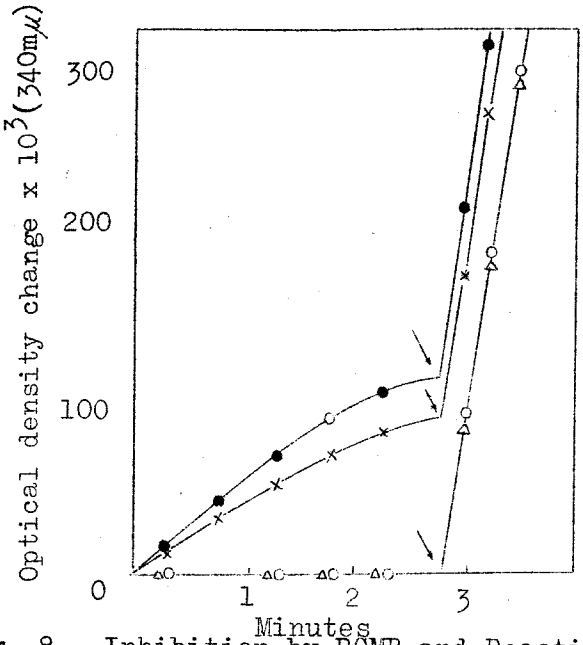


Fig. 8. Inhibition by PCMB and Reactivation by Thioglycolate.

These experiments were performed under the conditions similar to those of the standard assay except that large amounts of the enzyme (Enzyme I) were used and thioglycolate was omitted from the initial reaction mixture. Thioglycolate was added at the points indicated.

—●— Control —○— 10^{-4} M PCMB
—△— 10^{-5}M PCMB —×— 10^{-6} M PCMB

Effect of PCMB

The results described above suggest this dehydrogenase to be a -SH enzyme. PCMB was used to examine this problem. The results obtained are shown in Fig. 8. In this case, thioglycolate was omitted from the standard assay system and large amounts of the enzyme were used.

10^{-4}M and 10^{-5} M PCMB inhibited the enzyme completely,

and 10^{-6}M PCMB showed 20% inhibition.

This inhibition was completely removed by the addition of 3.3×10^{-3} M thioglycolate. The rate of the reaction after the reactivation by thioglycolate was the same order with that of the complete system determined under the standard assay conditions (control, in Fig. 8).

These results show that this enzyme is very sensitive to the -SH reagent, and suggest that -SH groups in the enzyme protein is closely related to the enzyme action. The role of -SH groups in the glyceraldehyde 3-phosphate dehydrogenase reaction has been well studied[24].

Effect of Aldehyde

Effects of several aldehydes, structurally related compounds to ASA, were examined, as shown in Table VII.

Though the aldehydes listed in Table VII could not be substrates for pea ASA dehydrogenase, as described above, they revealed inhibitory action. Among the aldehydes, glyceraldehyde and glyceraldehyde 3-phosphate showed comparatively strong inhibition.

These facts suggest an affinity between this enzyme and the aldehyde group. Hereupon, the behaviour of glyceraldehyde toward the enzyme reaction was studied as a

representative of the aldehydes. And the results in Fig. 9 were obtained.

Table VII. Effect of Aldehyde

Compound	Concentration (M)	Activity per cent
Control	--	100
Acetaldehyde	5×10^{-3}	100
"	10^{-2}	87
Propionaldehyde	5×10^{-3}	100
"	10^{-2}	80
n-Butyraldehyde	5×10^{-3}	100
"	10^{-2}	84
DL-Glyceraldehyde	5×10^{-3}	80
"	10^{-2}	70
DL-Glyceraldehyde 3-phosphate	5×10^{-3}	30
"	10^{-2}	20

Aldehydes were added under the standard assay conditions (Enzyme I).

Though this dehydrogenase reaction involves three substrates, the Lineweaver-Burk plots[19] in the presence of the inhibitor can give ordinary inhibition curves under the conditions described before. When competitive inhibition occurs, the following equation can be obtained.

$$\frac{V}{v} \doteqdot \frac{Ks'''}{[S_1]}\left(1 + \frac{[I]}{Ki'''}\right)$$

[I]: Concentration of Inhibitor, Ki''': Dissociation constant of the ES_2S_3I complex ($ES_2S_3 + I \rightleftharpoons ES_2S_3I$) The other abbreviations are the same with those described before.

Therefore, this enzyme may have a site at which glyceraldehyde competes with ASA.

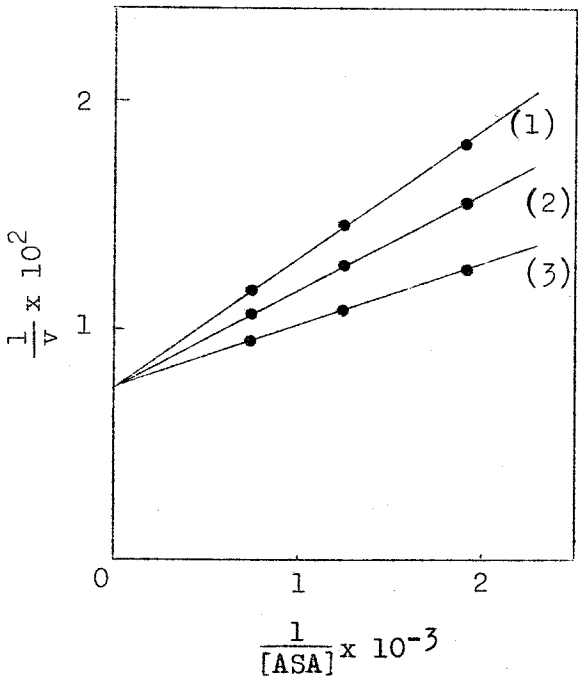


Fig. 9 Competitive Inhibition by Glyceraldehyde.

The experiments were carried out under the standard assay conditions with the modification that the concentration of ASA was varied and the definite concentration of the inhibitor was added.

v : Optical density change at 340 mμ per minute x 10^3

[ASA]: Concentration of L-ASA(M).

[I] : Concnetration of DL-glyceraldehyde (M).

(1) [I] = 10^{-2} M, (2) [I] = 6.7 x 10^{-3}M

(3) [I] = 0

Effect of Amino Acid.

Effects of various amino acids were examined (Table VIII).

Aspartic acid, asparagine, homoserine, threonine, isoleucine and methionine, structurally or metabolically related amino acids, had no effect on the enzyme reaction. However, serine and glycine inhibited the enzyme. The mode of the inhibition by serine is competitive with the substrate as shown in Fig. 10. These facts show that the enzyme may have another site (or sites) except the site for aldehyde mentioned above

Table VIII. Effect of Amino Acid

Compound	Concentration (M)	Activity per cent
Control	--	100
L-Aspartic acid	10^{-2}	100
L-Asparagine	10^{-2}	100
DL-Homoserine	2×10^{-2}	100
L-Threonine	10^{-2}	100
L-Serine	10^{-2}	60
"	5×10^{-3}	80
L-Methionine	10^{-2}	100
L-Isoleucine	10^{-2}	100
Glycine	10^{-2}	80
DL-Alanine	2×10^{-2}	100
DL-α-Amino-n-butyric acid	10^{-2}	100
L-Lysine	10^{-2}	100

These experiments were performed under the standard assay conditions (Enzyme I).

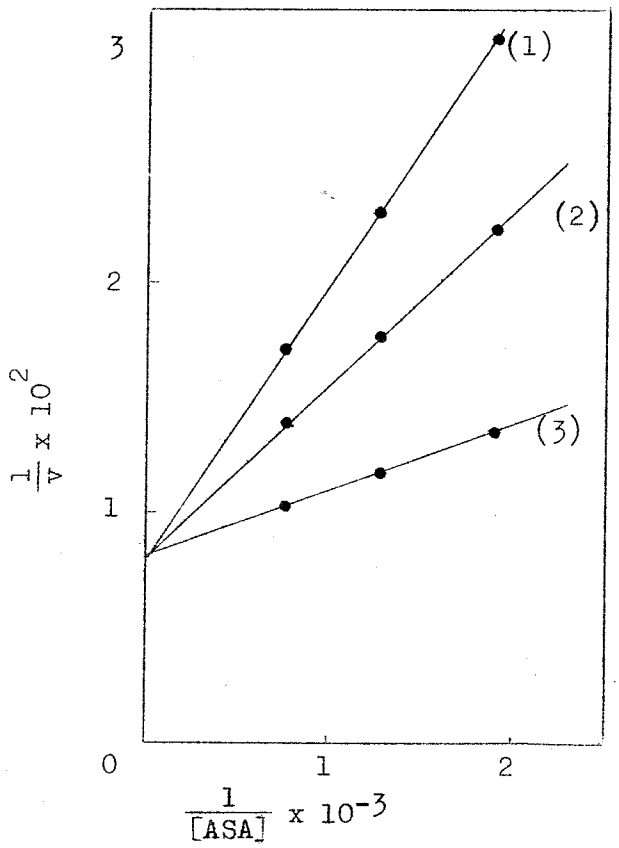


Fig. 10. Competitive Inhibition by Serine.

These experiments were carried out by the same way as described in Fig. 3.

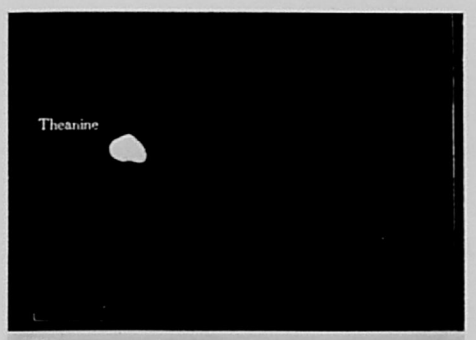
v : Optical density change at 340 mμ per minute x 10^3
[ASA] : Concentration of L-ASA(M).
[I] : Concentration of L-serine (M).

(1) $[I] = 10^{-2}M$, (2) $[I] = 5 \times 10^{-3}M$
(3) $[I] = 0$

4. DISCUSSION

The kinetic studies on this enzyme reaction suggest that the reaction proceeds under the Michaelis and Menten steady state, that is, an enzyme-substrate-quarternary complex is the active intermediate. Therefore, the mode of aspartic β-semialdehyde dehydrogenase reaction seems to be a little different from that of triose phosphate dehydrogenase[24].

Paperchromatograms and Radioautograms.



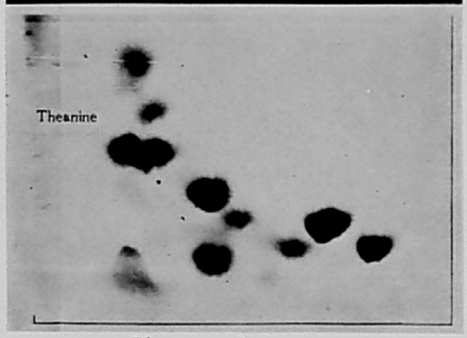


Photo A

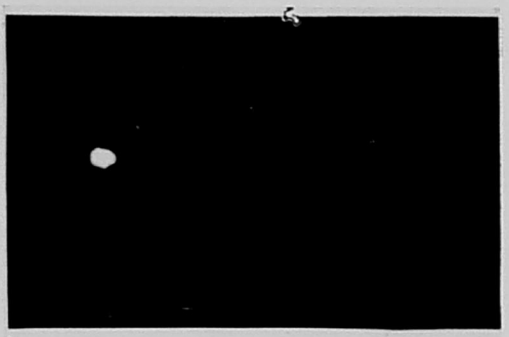
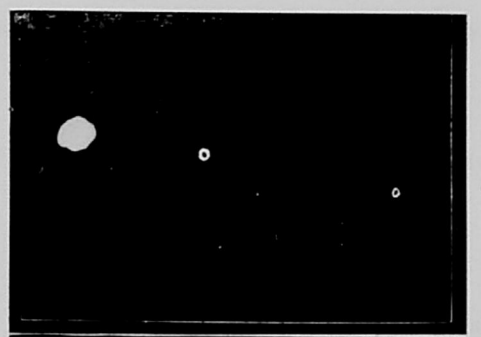


Photo B



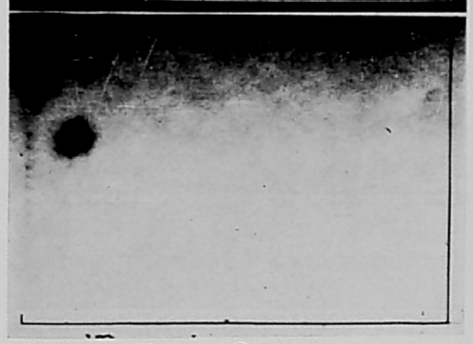
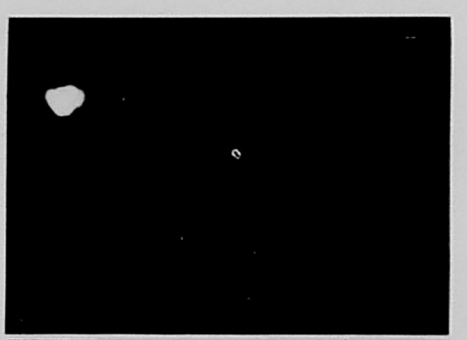


Photo C



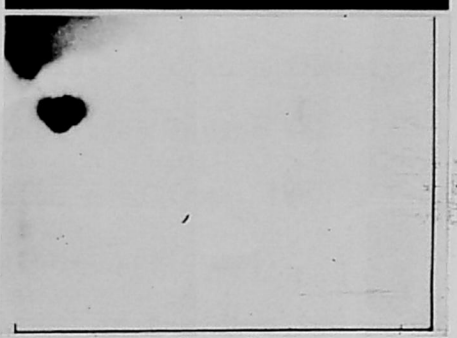


Photo D

Solvent used:

Phenol-water (4 : 1 NH_3) firstly;

n-Butanol-acetic acid-water (4 : 1 : 1) secondly.

a) Radioautogram

b) Paperchromatogram

CHAPTER III.

Biosynthesis of Theanine

Theanine was first found in tea leaves by Sakato[4]. This compound was also found in Xerocomus badius[5].

The author has been interested in the biochemical significance of ω-carboxyl groups of monoaminodicarboxylic acids which are metabolically related to various amino acids[25], amides[26, 27, 28, 29] and peptides[2, 3,]. Theanine is one of derivatives of γ-carboxyl group of glutamic acid, and has an intermediate structure between glutamine and γ-glutamyl dipeptides which have been succesively found in plants[2]. Therefore, the author has undertaken to clarify the mechanism of theanine biosynthesis.

SECTION I.

Biosynthesis of Theanine in Tea Seedlings

1. Incorporation of Glutamic Acid-l-^{14}C into Theanine[c]

Theanine is formed in large quantities during the germination of tea seeds. Using these seedlings, the following isotopic experiments have been performed.

Seven day-seedlings were incubated in a medium (pH 5.0) containing DL-glutamic acid-I-^{14}C (1.8×10^{-3}M,

1.6μ c/ml) and ethylamine-HCl (10^{-2} M); Sample A, or DL-glutamic acid-I-^{14}C (1.8 x 10^{-3}M, 1.6μ c/ml) alone; Sample B, in the dark at 20°C for three days. Amino acid fractions were prepared from both incubated seedlings by the use of Amberlite -IR 120 column[8]. Radioactive theanine was formed remarkably from glutamic acid-I-^{14}C. Distribution of radioactivities in amino acids was determined by a G.M counter and is shown in Table IX.

Table IX. Distribution of Radioactivities

	Sample A		Sample B	
	cpm	%	cpm	%
Theanine	470	43	60	17
Glutamine	130	12	80	22
Glutamic acid	170	15	150	42
Total activity	1,110		360	

Radioactivities in amino acids were determined by a G.M counter on two-dimensional chromatograms obtained as described in Fig. 1 (Definite amounts of each sample (25μl) prepared by the same way were applied). The total activity was measured at the starting point before development.

The above results clearly show that theanine is derived from glutamic acid-I-^{14}C. Moreover, the addition of ethylamine into the incubation medium increased the incorporation of racioactivity into theanine predominantly. This result may suggest the participation of ethylamine

in theanine biosynthesis, though other physiological factors should be considered. It is interesting that ethylamine was found in higher plants[30].

2. Incorporation of Ethylamine-I-^{14}C into the Ethylamine Part of Theanine[d]

In SECTION I-1, the incorporation of glutamic acid-I-^{14}C into theanine in tea seedlings was described, and the participation of ethylamine in the biosynthesis of theanine was suggested from the fact that the addition of ethylamine into the incubation medium increased the above incorporation. Incorporation of ethylamine-I-^{14}C into the ethylamine part of theanine will be described.

Four grains of tea seedlings (eleven-day-old) were incubated in a medium containing 3.0 μ moles of L-glutamic acid and 3.5 μ moles of ethylamine-I-^{14}C (6.5×10^{-4} cpm, G.M counter) in a total volume of 3.5 ml (pH 5.6) in the dark at 20°C for three days. The amino acid fraction was prepared from the incubated seedlings by a method similar to that described above. This fraction contained approximately 65 per cent (4.2×10^4 cpm, G.M counter) of the radioactivity used for the incubation.

The radioautogram and paper chromatogram obtained

with the above amino acid fraction were shown in photo A. As shown in the figure, the formation of radioactive theanine was observed, and almost all of the activity of the amino acid fraction was found in theanine.

The distribution of radioactivity in theanine molecule was examined after hydrolysis.

In this experiment, theanine was isolated from the amino acid fraction by two dimensional paper chromatography (2 : 4-lutidine-water (7 : 3) firstly, n-butanol-acetic acid-water (4 : 1 : 1) secondly), and was hydrolyzed in 6 N HCl for twenty four hours at 100°C. The hydrolysate was concentrated to dryness in vacuo, and the residue was dissolved in water. Paper electrophoresis was carried out with this hydrolysate. After run, the dried strips were cut into 1-cm sections and the radio-activity was measured by a gas flow counter. The other strips were treated with ninhydrin.

As shown in Fig. 11, the radioactivity of the hydrolysate was quantitatively found in ethylamine. The same distribution pattern was observed in the paper chromatographic test shown in Table X. These results show that ethylamine was incorporated into the ethylamine part of theanine.

The above results indicate the participation of ethylamine in the biosynthesis of theanine in tea seedlings.

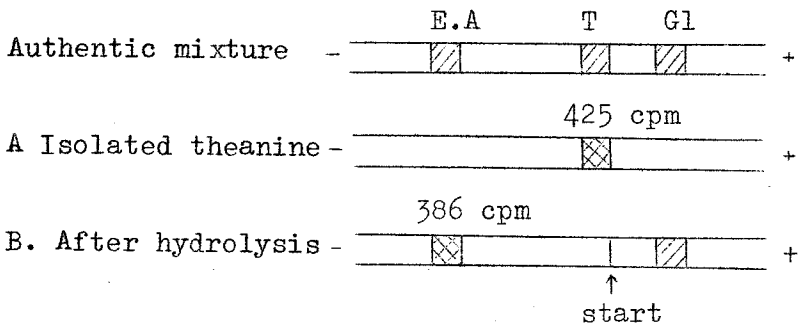


Fig. 11. Distribution of Radioactivity in Theanine. (Paper Electrophoresis)

Radioactivity on the starting point before electrophoresis: A, 426 cpm; B, 384 cpm. Electrophoresis : Phosphate buffer, pH 6.0, μ = 0.04; 7.6 v/cm; sixty minutes; Toyo filter paper No. 51 (2 x 25 cm).

Abbreviations : T, Theanine ; E.A, Ethylamine; Gl, Glutamic acid.

: Ninhydrin positive.

: Ninhydrin positive and radioactive.

Table X. Distribution of Radioactivity in Theanine (Paper Chromatography)

	cpm
Hydrolysate used	7020
Ethylamine	6540
Glutamic acid	< 10

The radioactivity was measured by a gas flow counter.
Development : n-Butanol-acetic acid-water (4 : 1 : 1).
Elution from the chromatogram : Ethylamine, with 0.01 N HCl; Glutamic acid, with water.

SECTION II.

Synthesis of Theanine by Tea Seedling Homogenate[e,f)]

1. INTRODUCTION

The incorporation of glutamic acid-1-^{14}C and ethylamine-1-^{14}C into theanine in tea seedlings have been described in SECTION I.

The synthesis of theanine by a homogenate of tea seedlings will be described here.

2. MATERIALS AND METHODS

Tea Seedlings

Tea seeds (Thea sinensis) were sterilized by a 0.02 per cent solution of benzalkonium chloride after elimination of shells. The sterilized seeds were soaked in running water overnight, and germinated on moist absorbent cotton covered with filter paper, in the dark at 20°C.

Preparation of Homogenate

Decotylized tea seedlings (eleven-day-old) were ground in a chilled mortar having been kept at -20°C with double the amounts of 0.1 M potassium phosphate buffer (pH 7.35). After thawing the frozen homogenate, the residue was removed. The homogenious solution thus obtained was immediately used for the experiments.

Reaction

The homogenate obtained as described above was incubated with 75μ moles of potassium phosphate buffer (pH 7.35), 25μ moles of L-glutamic acid (sodium salt), 25μ moles of ethylamine-1-^{14}C (HCl salt; 1.4 x 10^4 cpm/μmole, gas flow counter), 8μ moles of ATP, 27μ moles of creatine phosphate, 500μ g of creatine phosphokinase 25μ moles of $MgCl_2$ and 35μ moles of KCN (total volume 1.0 ml). The reaction was carried out for sixty minutes at 35°C. ATP and KCN were neutralized before use.

Measurement

After the incubation, 28μ moles of DL-theanine were added, and the reaction was stopped by the addition of 3 volumes of 99.5 per cent alcohol. The precipitate formed was removed by centrifugation, and was washed twice with 5 ml of 75 per cent alcohol. The supernatant solution was combined with the washings, and then was applied on an Amberlite IR-120 column (H^+-form, 1 x 17 cm). After washing the column with water, the adsorbed amino acids were eluted with 100 ml of 1 N NH_4OH. The eluate was concentrated in vacuo for 2.5 hours at 50°C, and the concentrated solution was kept in a vacuum desiccator over conc. H_2SO_4 overnight

at 20°C. The dried residue was dissolved in definite amounts of water (this fraction will be called amino acid fraction). The radioactivity of this fraction was measured by a gas flow counter. Free ethylamine was completely removed during the preparation of this fraction, and all of the radioactivity of this fraction was attributable to theanine formed (every sample was checked by paper chromatography). The mean value of theanine recovered by this procedure was approximately 92 per cent (Fig. 12).

Reagents

Ethylamine-1-^{14}C (HCl salt) was purchased from N. V. Philips-Duphar (Holland) and was purified by the micro-diffusion method[31]. DL-Glutamic acid-1-^{14}C was obtained from the Daiichi Pure Chemicals Company (Japan). ATP (disodium salt) was purchased from the Sigma Chemical Company (U. S. A.). Creatine phosphate (disodium salt) and rabbit muscle creatine phosphokinase (16 units/mg of protein) were the products of Carbiochem (U. S. A.). L-Theanine was a gift from Dr. Sakato.

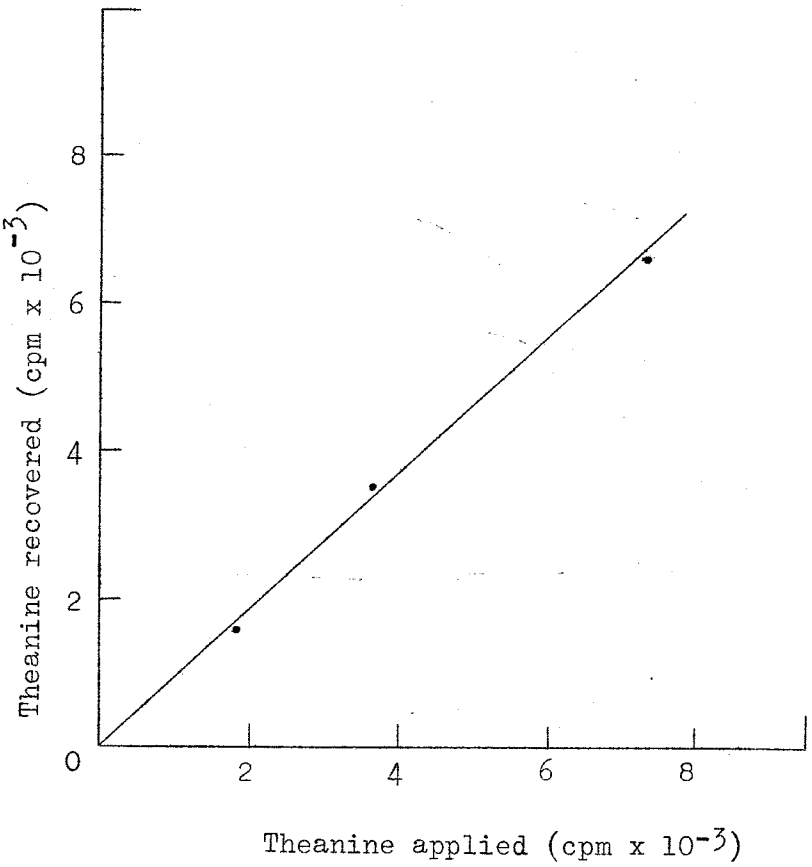


Fig. 12. Recovery of Theanine in Amino Acid Fraction

3. RESULTS

Formation of Radioactive Theanine from Ethylamine-^{14}C.

As shown in Table XI, radioactive theanine was formed from glutamic acid and ethylamine-1-^{14}C in the presence of ATP. When glutamic acid was omitted from the reaction system, approximately 25 per cent of theanine of the complete

system was formed. This may be due to the pre-existing glutamic acid in the homogenate. The necessity of glutamic acid in the reaction was further confirmed by the use of glutamic acid-1-^{14}C, as described below. Creatine phosphate and creatine phosphokinase were used as an ATP generating system. Elimination of this system decreased the theanine formation in spite of the presence of ATP. When ATP and the ATP generating system were omitted, or a heated homogenate was used instead of the native one, theanine formation was negligible. Little formation of theanine was observed in the absence of Mg^{++}.

Table XI. Synthesis of Theanine by Tea Seedling Homogenate

	Radioactive Theanine formed cpm
Complete system	5480
minus L-Glutamic acid	1380
minus ATP	850
minus Creatine phosphokinase system	1880
minus ATP and creatine phosphokinase system	230
minus $MgCl_2$	220
minus KCN	3910
Heated homogenate *	10

* A heated homogenate (in a boiling water bath, five minutes) was used instead of the native one.

This fact suggests the necessity of this cation in the synthesis of theanine, though creatinephosphokinase also requires Mg^{++} [32]. The necessity of divalent cations in the theanine synthesizing reaction will be studied with a purified enzyme. KCN tended to increase the theanine formation.

For the identification of the reaction product, the following experiments were performed.

After the incubation was carried out, as described above, the reaction was stopped by alcohol without the addition of carrier theanine. From this alcohol solution, the amino acid fraction was prepared. Theanine was isolated[33] from this fraction by paper chromatography, using 2 : 4 -lutidine-water (7 : 3) and n-butanol-acetic acid-water (4 : 1 : 1). Photo B shows an example of chromatograms and radioautograms obtained with the isolated theanine. The same chromatogram and radioautogram were obtained in cochromatography with authentic theanine.

Hydrolysis of the isolated theanine (6 N HCl, twenty four hours, 100°C) gave glutamic acid and ethylamine and radioactivity was found in ethylamine, as shown in Table XII (Sample A).

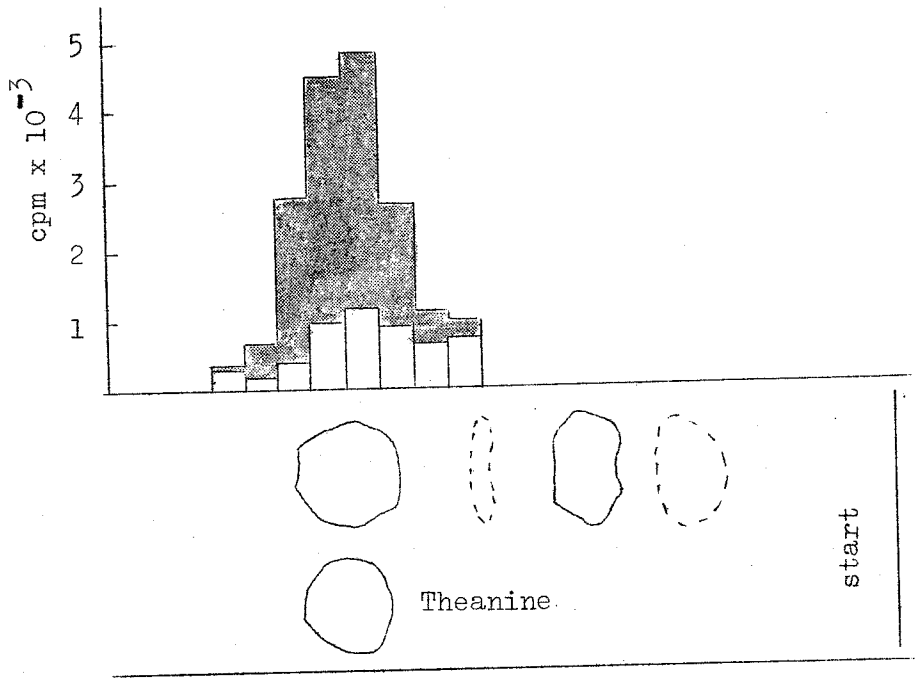


Fig. 13. Synthesis of Radioactive Theanine from Glutamic acid-1-^{14}C

Complete system Ethylamine omitted

A : Sample B : Authentic theanine

Table XII. Distribution of Radioactivity in Theanine

Hydrolysate of theanine	Ethylamine cpm	Glutamic acid cpm
Synthesized from ethylamine-1-^{14}C (212 cpm)	204	0
Synthesized from glutamic acid-1-^{14}C (1360 cpm)	0	1258

Sample A : Hydrolysate of radioactive theanine prepared from non-isotopic glutamic acid and ethylamine-1-^{14}C. The radioactivity applied was 212 cpm.

Sample B : Hydrolysate of radioactive theanine prepared from glutamic acid-1-^{14}C and non-isotopic ethylamine. The radioactivity applied was 1,360 cpm.

Glutamic acid and ethylamine were separated by paper chromatography, using n-butanol-acetic acid-water (4 : 1 : 1), and were eluted from the chromatograms (glutamic acid, with water; ethylamine, with 0.01 N HCl).

Formation of Radioactive Theanine from Glutamic Acid-1-^{14}C

When glutamic acid-1-^{14}C was used as the radioactive substrate instead of ethylamine-1-^{14}C, radioactive theanine was also formed.

In these experiments, 8.6 μ moles of DL-glutamic acid-1-^{14}C (8.7×10^5 cpm/μ mole, gas flow counter) and 25 μ

moles of non-isotopic ethylamine were incubated in the same way as described above. After the incubation, the amino acid fraction was prepared without the addition of carrier theanine. One-dimensional chromatography was carried out with this amino acid fraction, using n-butanol-acetic acid-water (4 : 1 : 1). The chromatogram was cut into 5 mm sections, and the radioactivity was measured by a liquid scintillation counter. As shown in Fig. 13, radioactive theanine formation was observed in the complete system. When ethylamine was omitted from the reaction system, a little formation of radioactive theanine was detected. This may be due to the occurrence of ethylamine in the homogenate.

The reaction product in these experiments was further confirmed by two-dimensional chromatography, after isolation from the amino acid fraction by paper chromatography in the same way as described above. In this case, the radioactivity was found in the glutamic acid part of theanine, as shown in Table XII (Sample B).

4. DISCUSSION

As to the mechanism of theanine biosynthesis, several kinds of reactions may be presumed. The most probable

reactions among them are: (a) a synthetase reaction as seen in glutamine synthetase[26] and γ-glutamylcysteine synthetase,[34,35] (b) a transfer reaction of γ-glutamyl residue to ethylamine, and (c) decarboxylation of γ-glutamylalanine. Decarboxylation of γ-glutamylalanine was speculated by Tanaka[36], though it could not be proved. According to this speculation, decarboxylation of γ-glutamyl-β-alanine found in iris bulbs[37] may be possible[2]. But the results in SECTION I suggest that the reaction a or b is more probable. And the present results suggest that theanine is synthesized by a manner similar to glutamine synthetase and γ-glutamylcysteine synthetase reactions. More detailed mechanism of this reaction will be sutdied with a purified enzyme. As other mechanism of the theanine biosynthesis, some of γ-glutamyl transfer reactions[38, 39, 40, 41, 42] from γ-gluamyl donors may also have to be considered. Recently, Thompson[43] observed that ethylamine was not nearly as good an acceptor as other amino acids, in γ-glutamyl transpeptidation in plants.

SECTION III

Synthesis of Theanine by Pea Seed Acetone Powder Extract[g)]

1. INTRODUCTION

In SECTION II, the synthesis of theanine by a homogenate of tea seedlings from glutamic acid and ethylamine in the presence of ATP was described. This reaction seems to be similar to those of glutamine synthetase[44,45)] and γ-glutamylcysteine synthetase[35,46)].

In the preliminary experiments, radioactive theanine formation was observed by incubating pea seedlings in a medium containing L-glutamic acid and ethylamine-1-^{14}C. The radioactivity was found in the ethylamine part of theanine. From this observation, the author examined theanine synthesis by an acetone powder extract of pea seeds.

2. MATERIALS AND METHODS

Preparation of Acetone Powder Extract

Pea seed (Pisum sativum, var. Alaska) were soaked in running water overnight. The soaked seeds were ground in a chilled mortar, and then added into 10 volumes of acetone (-10°C) with stirring. Acetone was removed by vacuum filtration. The residue was washed with 8 volumes of chilled acetone. After removal of acetone by filtration, the residue was dried in vacuo. The dried acetone powder was extracted with 5 parts of 0.1 M $NaHCO_3$ with stirring for twenty minutes, and the precipitate was removed by centrifugation (10,000xg, thirty minutes). The clear supernatant solution was dialyzed against 0.15 M NaCl-0.05 M $NaHCO_3$ for twenty hours. All these operations were

performed below 5°C. The dialyzed extract contained approximately 16 mg of protein per ml, according to the spectrophotometric method of Warbug and Christian[14], and actively formed hydroxamate[20] from glutamic acid and hydroxylamine in the presence of ATP. This extract was used as the enzyme solution.

Standard Assay Conditions

The complete system contained 100 μ moles of Tris-HCl buffer (pH 8.3), 50 μ moles of L-glutamic acid (sodium salt), 50 μ moles of ethylamine-1-^{14}C (HCl salt; 8 x 10^3 cpm/μmole, gas flow counter), 2.5 μ moles of ATP, 27 μ moles of creatine phosphate, 500 μg of creatine phosphokinase, 10 μ moles of $MgCl_2$ and definite amounts of the enzyme (usually 8 mg) in a total volume of 1.0 ml. The reaction was carried out forty five minutes at 35°C. After the incubation, 28 μ moles of DL-theanine was added as the carrier, and the reaction was stopped by the addition of 3 volumes of 99.5 per cent alcohol. After centrifugation, the alcohol solution was treated with Permutit as described in the next section, and radioactive theanine formed was determined by a gas flow counter. Under the conditions described above, the reaction proceeded linearly at least

for sixty minutes (Fig. 14), and the radioactive theanine formation was proportional to the amounts of the enzyme (Fig. 15).

Measurement of Radioactive Theanine

Radioactive theanine formed was measured after the following treatment (this method will be called Permutit-method).

Three ml of the alcohol solution prepared after the incubation was applied on a Permutit column (0.8 x 10 cm, washed with a 10 per cent solution of NaCl).[47] After the sample sank into the column, the column was washed with water. The flow-rate was 1-1.5 ml per minute. By this procedure, ethylamine was completely adsorbed by the column and theanine appeared in the 3-13 ml fraction as shown in Fig. 16. Therefore, the 3-13 ml fraction (this fraction will be called Permutit-effluent) was collected and the radioactivity in this fraction was messured by a gas flow counter.

The recovery of theanine by this procedure was examined as shown in Fig. 17. In this figure, a solution having the same constituents with the reaction mixture and containing definite amounts of authentic radioactive

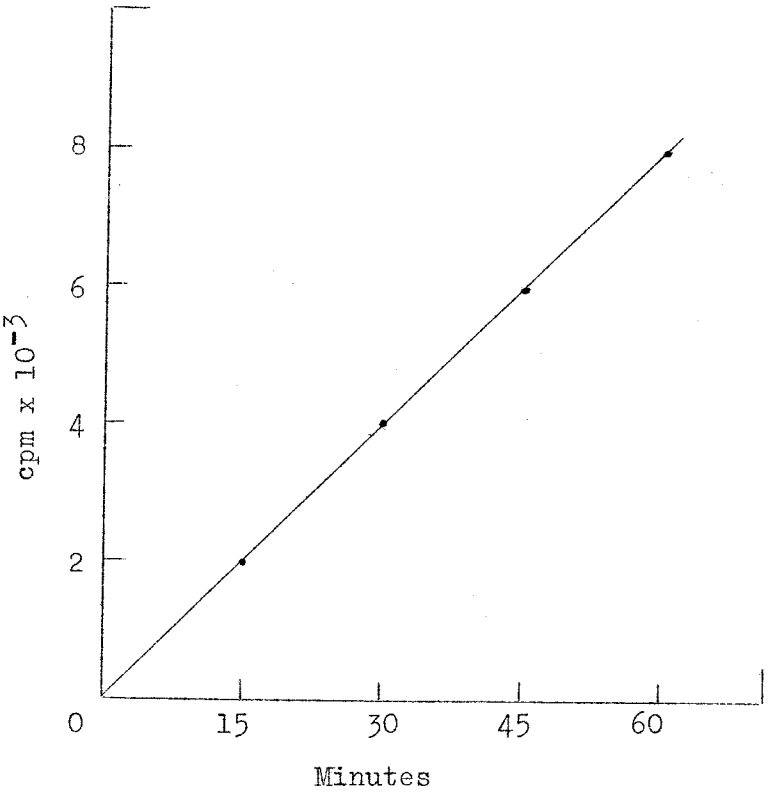


Fig. 14. Time-Course of the Reaction

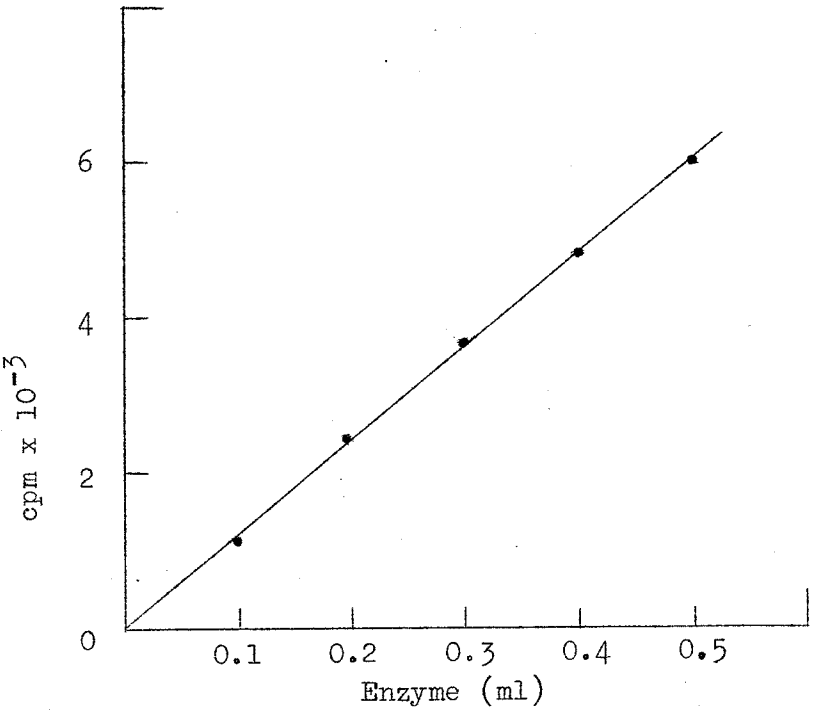


Fig. 15. Effect of Enzyme Concentration.

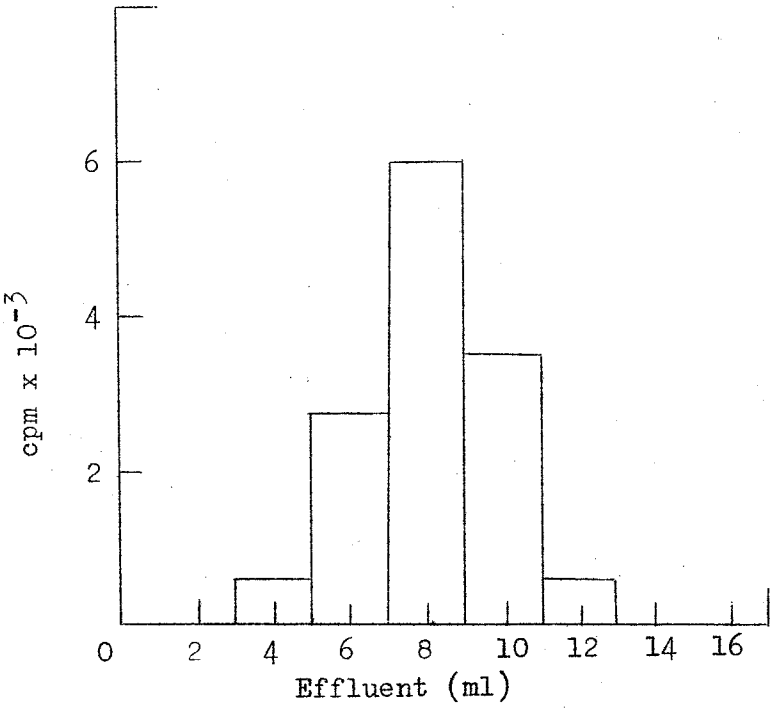


Fig. 16. Behavior of Theanine in the Permutit-Method.

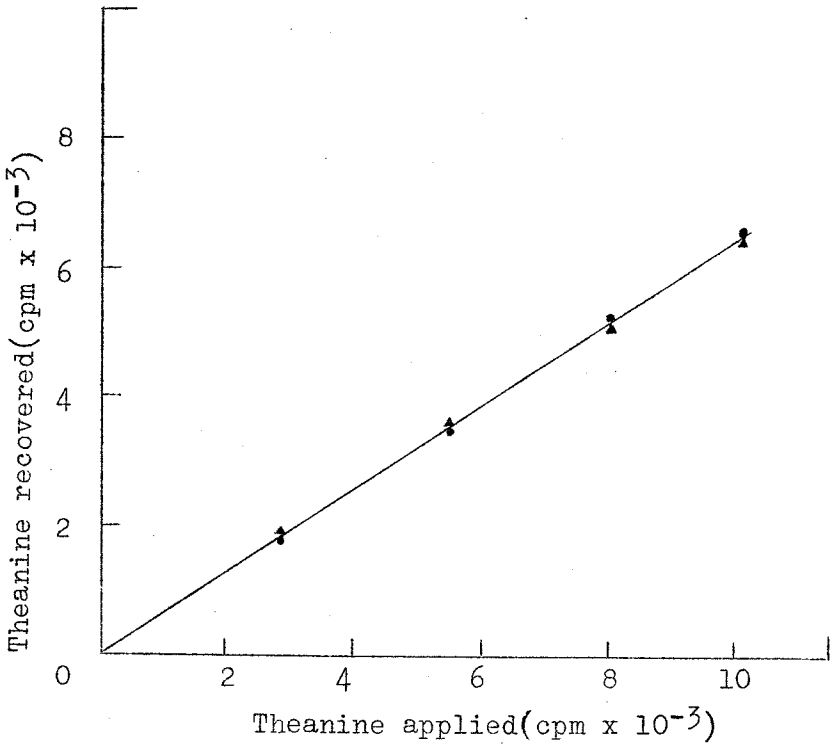


Fig. 17. Recovery of Theanine in the Permutit-Method

●——● Theanine recovered.

▲——▲ The experiment performed to correct for self-absorption.

theanine was treated as described above, and the radioactivity in the Permutit-effluent was measured. To correct self-absorption in the measurement of radioactivity, another solution having the same constituents with the reaction mixture without authentic radioactive theanine was treated by the same way, and to the Permutiteffluent obtained definite amounts of authentic radioactive theanine was

added, and the radioactivity was measured. As shown in the figure, both values were identical. This fact shows that the recovery of theanine was quantitative.

It is clear from the following facts that all of the radioactivity in the permutit-effluent prepared from the reaction mixture was attributable to radioactive theanine formed. The radioactive compound in this fraction was paper electrophoretically neutral. And the radioactivity was quantitatively transferred from this fraction into the amino acid fraction prepared by the treatment of Amberlite IR-120, as shown in Table XIII. The radioactivity in this amino acid fraction was always accompanied with theanine, as shown in the section of the experiments on the reaction product.

Reagents

Permutit was a commercial product used for the determination of Vitamin B_1. The other reagents were the same as described in SECTION II.

3. RESULTS

Synthesis of Theanine by Pea Seed Acetone Powder Extract

As shown in Table XIV, radioactive theanine was formed in the complete system. When either glutamic acid

Table XIII. Preparation of Amino Acid Fraction from Permutit-Effluent

	Radioactivity cpm	Recovery %
Permutit-effluent	72920	—
Amino acid fraction	65030	89.2

Table XIV. Synthesis of Theanine by Pea seed Acetone Powder Extract

	Radioactive theanine formed cpm
Complete system	5920
minus L-Glutamic acid	0
minus ATP	0
minus Creatine phospho-kinase system	3820
minus ATP and creatine phosphokinase system	0
minus $MgCl_2$	0
Heated enzyme*	0

* A heated extract (in a boiling water bath, five minutes) was used instead of the native one.

or ATP was omitted from the reaction system, theanine formation was not observed. In the absence of creatine phosphokinase system which was used for ATP generation, theanine formation was decreased to approximately 65 per cent of that of the complete system. The reaction did not occurred, when Mg^{++} was eliminated. This suggests the necessity of this cation in the theanine synthesizing reaction, though creatine phosphokinase also requires this cation[32]. More detailed experiments on this problem were carried out in the later section. A heated extract used instead of the native one could not form theanine. These results show that the pea seed acetone powder extract can synthesize theanine in the same manner with the homogenate of tea seedlings.

Reaction Product

A reaction was carried out under the standard assay conditions, except that ethylamine-1-^{14}C having high specific activity (8×10^4 cpm/μmole) was used. The reaction was stopped by the addition of alcohol. Carrier theanine was not added in this case. From this alcohol solution, a Permutit-effluent was prepared. An amino acid fraction was obtained from this permutit-effluent by the

treatment with Amberlite IR-120. Theanine was isolated from this amino acid fraction by paper chromatography, using 2 : 4 lutidine-water (7 : 3) and n-butanol-acetic acid-water (4 : 1 : 1). During these isolation precedures, radioactivity was always accompanied with theanine.

A two-dimensional chromatogram and a radioautogrm obtained with the isolated theanine were shown in Photo C. The complete identity of the radioactivity with authentic theanine was observed in cochromatography.

The distribution of radioactivity in theanine was examined after hydrolysis of the isolated theanine (6 N HCl, twenty four hours, 100°C). Glutamic acid and ethylamine were formed from theanine by hydrolysis, and the radioactivity was found in ethylamine. The same distribution pattern was also observed by paper electrophoresis. These results show the incorporation of radioactivity into the ehtylamine part of theanine.

Effect of pH

In Fig. 18, pH-activity curve of the reaction was shown. In these experiments, Tris-HCl buffer and Ammediol-HCl buffer were used. The final concentrations of both buffers were 0.1 M. The maximum was obtained at pH 8.6.

The enzyme was a little more active in Tris-HCl buffer than in Ammediol-HCl buffer.

Effect of Substrate Concentrations

In table XIV, glutamic acid ethylamine and ATP were shown to be the substrates of the reaction. Effects of the concentrations of these substrates on the reaction velocity were examined under the standard assay conditions, except that the concentrations of the substrates were varied (Table XV, XVI, XVII). As shown in the tables, the reaction velocity depended upon the concentrations of the substrates. Apparent Km values calculated according to Lineweaver and Burk[19] were 5.0×10^{-2}M for L-glutamic acid, 6.3×10^{-2}M for ethylamine and 4.6×10^{-5}M for ATP.

The effects of the concentrations of Mg^{++} on the reaction velocity were shown in Fig. 19. The velocity depended on the concentrations of Mg^{++} even in the absence of the creatine phosphokinase system. This result shows the necessity of this cation in the theanine synthesizing reaction. Mg^{++} in high concentrations tented to inhibit the reaction. In low concentrations of Mg^{++}, the activity in the absence of creatine phosphokinase system was higher than that in the presence of the ATP generating system,

probably because elimination of effective Mg^{++} from the reaction system by complex formation[48] with creatine phosphate influenced extensively the activity in low concentrations of this cation.

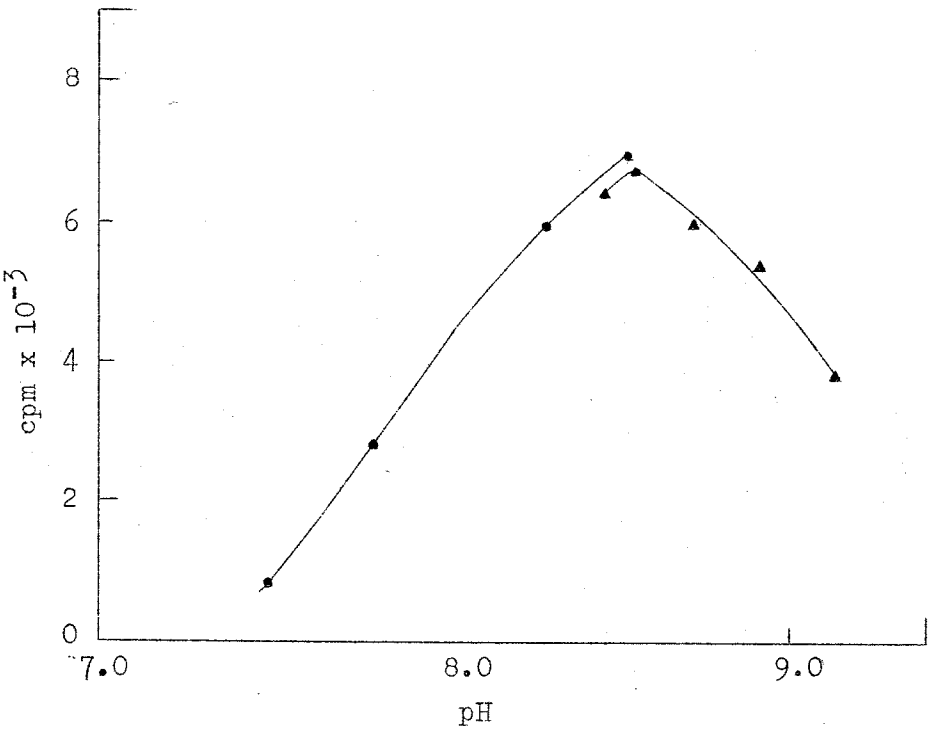


Fig. 18. Effect of pH

●——● Tris-HCl buffer.

▲——▲ Ammediol-HCl buffer.

The standard assay procedures were carried out at varied pH values. The pH Values are those measured at 35°C.

Table XV. Effect of Glutamic Acid Concentration

Concentration (M)	Radioactive theanine formed (cpm)
6.25×10^{-3}	1,340
8.33×10^{-3}	1,570
1.25×10^{-2}	2,420
2.50×10^{-2}	3,960
5.00×10^{-2}	5,920

Table XVI. Effect of Ethylamine Concentration

Concentration (M)	Radioactive theanine formed (cpm)
8.33×10^{-3}	1,580
1.25×10^{-2}	2,130
1.67×10^{-2}	2,700
2.50×10^{-2}	4,090
5.00×10^{-2}	5,920

Table XVII Effect of ATP Concentration

Concentration (M)	Radioactive theanine formed (cpm)
3.33×10^{-5}	2,510
5.00×10^{-3}	3,460
1.00×10^{-4}	4,080
2.00×10^{-4}	5,130
2.50×10^{-3}	5,920

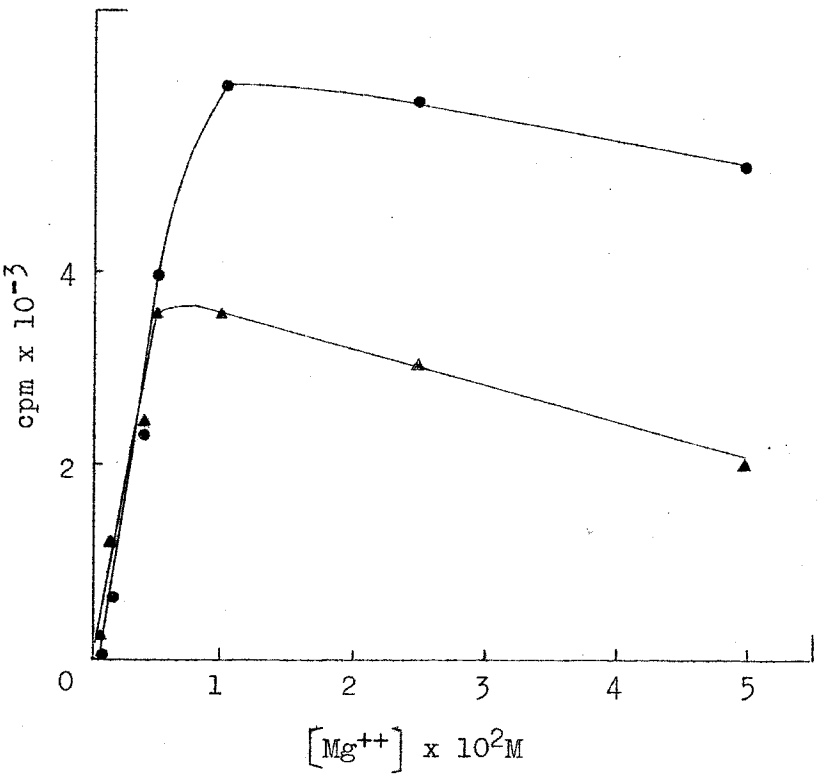


Fig. 19. Effect of Mg^{++} Concentration.

●——● in the presence of creatine phosphokinase system.

▲——▲ in the absence of creatine phosphokinase system.

4. DISUCSSION

The author observed that incubation of pea seedlings in a medium containing ethylamine-1-^{14}C produced radioactive theanine. And synthesis of theanine by an acetone powder extract of pea seeds from glutamic acid and ethylamine in the presence of ATP was proved. Pea seeds contain glutamine synthetase[49] and γ-glutamyl-cysteine synthetase[46]. Speck[44] observed orthophosphate liberation from ATP in the presence of methylamine by Pigeon liver glutamine synthetase preparation which had some activity also to cysteine. Though this result was questioned by Elliott[45] with the sheep brain enzyme, Meister[39] described the formation of γ-glutamylmethylamide by pea glutamine synthetase without giving experimental details, and he speculated[26] that theanine might be formed by an analogous reaction. It has been independently expected, from the observation of Speck[44], that theanine may be synthesized in a similar manner by enzymes from organisms other than the tea plant. The synthesis of theanine by a pea seed acetone powder extract described here may be due to a non-specific reaction of glutamine synthetase. But the possibility of participation of γ-glutamylcysteine synthetase may also have to be considered.

SECTION IV.

Synthesis of Theanine by Pigeon Liver Acetone Powder Extract[h)]

1. INTRODUCTION

In SECTION II and III, synthesis of theanine by a homogenate of tea seedlings was reported, and it was proved that a pea seed acetone powder extract synthesized theanine by an analogous reaction.

This SECTION will present that an extract of pigeon liver acetone powder can also synthesize theanine from glutamic acid and ethylamine in the presence of ATP.

2. MATERIALS AND METHODS

Preparation of Pigeon Liver Acetone Powder Extract

Pigeon liver acetone powder was stirred with 11 parts of 0.15 M NaCl-0.036 M $NaHCO_3$ for sixty minutes. After filtration of this dispersion through gauze, the filtrate was centrifuged at 10,000xg for thirty minutes. The clear supernatant solution was dialyzed against 0.15 M NaCl-0.005 M $NaHCO_3$ for twenty hours. These operations were carried out below 5°C. The solution thus obtained contained approximately 32 mg of protein per ml, according to the spectrophotometric method of Warburg and Christian[14)],

and formed hydroxamate[20] actively from glutamic acid and hydroxylamine in the presence of ATP. For the measurement of theanine formation, this solution was diluted with 3 parts of 0.15 M NaCl.

Standard Assay Conditions

The complete system contained 100 μ moles of Tris-HCl buffer (pH 8.3), 50 μ moles of L-glutamic acid, (sodium salt), 50 μ moles of ethylamine-1-^{14}C (HCl salt; 7 x 10^3 cpm/μ mole, gasflow counter), 2.5 μ moles of ATP, 27 μ moles of creatine phosphate, 500 μg of creatine phosphokinase, 10 μ moles of $MgCl_2$ and definite amounts of the enzyme (usually 1.6 mg) in a total volume of 1.0 ml. The reaction was carried out for forty five minutes at 35°C. After the incubation, 28 μ moles of DL-theanine was added as the carrier and the reaction was stopped by the addition of 3 volumes of 99.5 per cent alcohol. The precipitate formed was removed by centrifugation. The supernatant solution was treated with Permutit, as described in SECTION III, and the radioactivity in the Permutit-effluent obtained was measured by a gas flow counter. It was proved that the Permutit-method could be completely applied also in these experiments.

Under the standard assay conditions described above, the reaction proceeded linearly at least for sixty minutes (Fig. 20), and the radioactive theanine formation was proportional to the amounts of the enzyme (Fig. 21).

Reagents

Pigeon liver acetone powder was purchased from the Sigma Chemical company (U. S. A.). The other reagents used were the same as described in SECTION II.

3. RESULTS

Synthesis of Theanine by Pigeon Liver Acetone powder Extract

Table XVIII shows the radioactive theanine formation by a pigeon liver acetone powder extract. Glutamic acid and ATP were necessary for the reaction. Elimination of creatine phosphokinase system which was used as an ATP generating system, reduced the theanine formation. In the absence of Mg^{++}, the reaction did not occur. The necessity of this cation in the theanine synthesizing reaction was further proved below. When the native extract was replaced by the heated one, there observed no theanine formation.

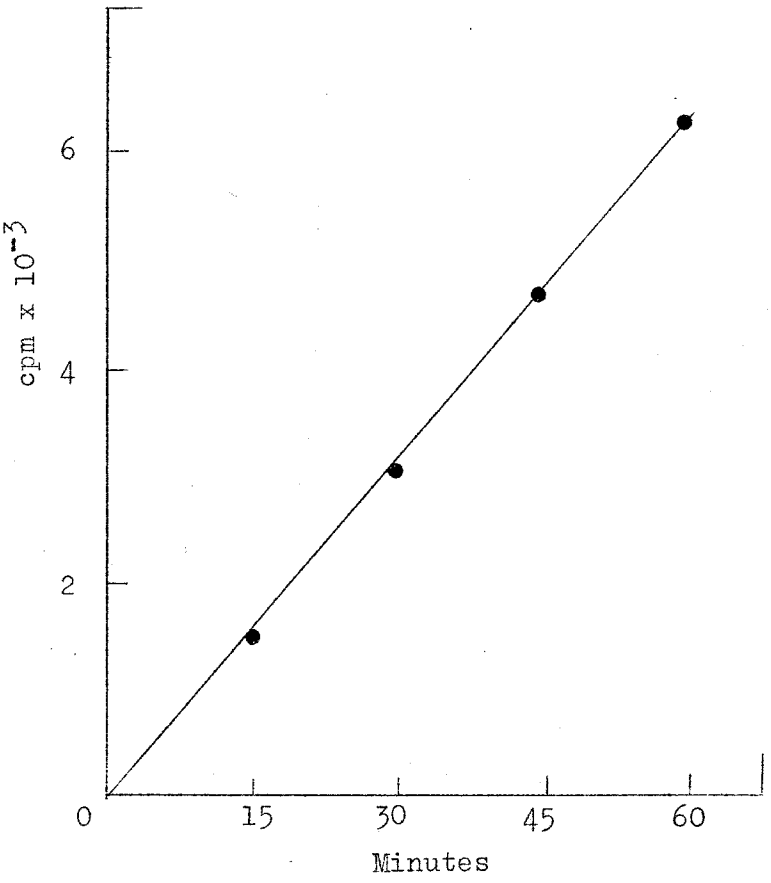


Fig. 20. Time-Course of the Reaction

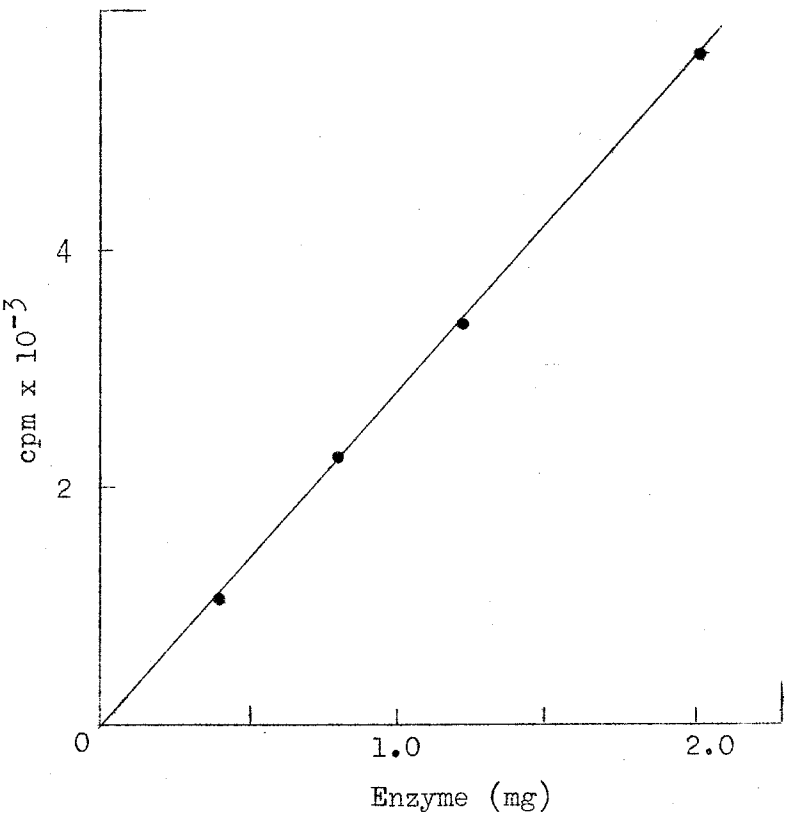


Fig. 21. Effect of Enzyme Concentration

Table XVIII. Synthesis of Theanine by Pigeon Liver Acetone Powder Extract

	Radioactive theanine formed cpm
Complete system	4610
minus L-Glutamic acid	0
minus ATP	0
minus Creatine phospho-kinase system	3310
minus ATP and creatine phosphokinase system	0
minus $MgCl_2$	0
Heated enzyme*	0

* A heated extract (in a boiling water bath, five minutes) was used instead of the native one.

Reaction Product

To confirm the reaction product, a reaction mixture containing the same constituents with those of the standard assay conditions, except that ethylamine-1-^{14}C having high specific activity (7×10^4 cpm/μ mole) was used, was incubated for forty five minutes at 35°C. The reaction was stopped by the addition of alcohol. In this case, carrier theanine was not added. This alcohol

solution was treated with Permutit and Amberlite IR-120, and from the amino acid fraction prepared, theanine was isolated paper chromatographically, in the same way as described in SECTION III. During the paper chromatographic isolation, radioactivity was always accompanied with theanine.

Two-dimensional chromatography was carried out with this isolated theanine. Photo D shows a chromatogram and a radioautogram obtained. The same chromatogram and radioautogram were obtained in cochromatography with authentic theanine.

Hydrolysis (6 N HCl, twenty four hours, 100°) of the isolated theanine produced glutamic acid and ethylamine, and radioactivity was found in ethylamine. The same distribution pattern was also observed paper electrophoretically.

Effect of pH

Effect of pH on the reaction velocity was examined as shown in Fig. 22, using Tris-HCl and Ammediol-HCl buffers (final concentrations, 0.1 M). The pH optimum was found at 8.3. It was observed that the enzyme was more active in Ammediol-HCl buffer than in Tris-HCl buffer.

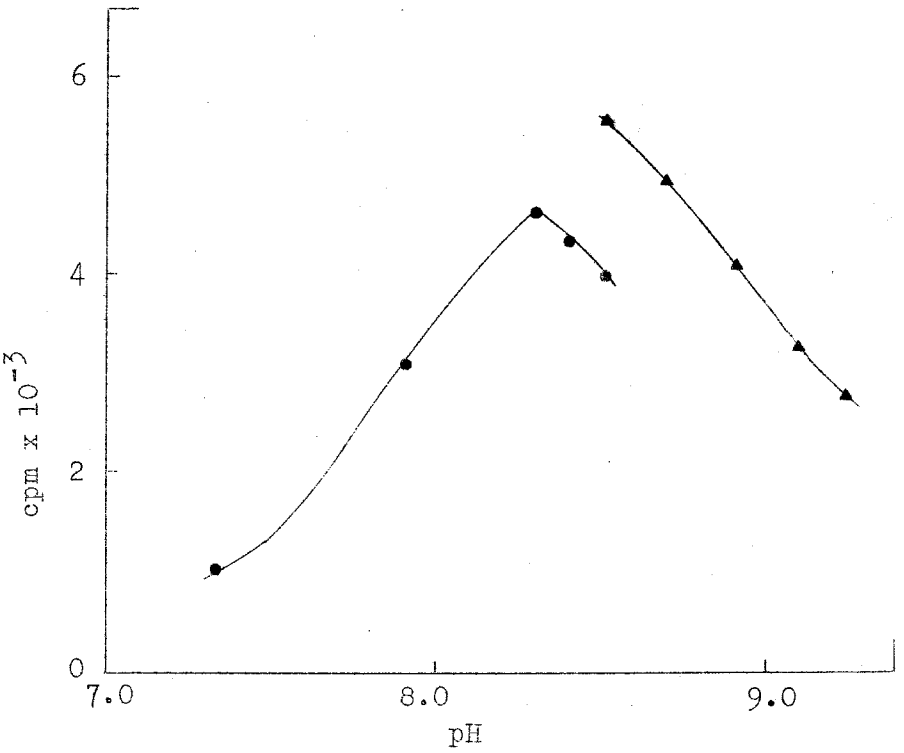


Fig. 22. Effect of pH.

●——● Tris-HCl buffer.

▲——▲ Ammediol-HCl buffer.

The standard assay procedures were carried out at varied pH values. The pH values were those measured at 35°C.

Effect of Substrate Concentration.

Effects of the concentrations of glutamic acid, ethylamine and ATP on the reaction velocity were examined under the standard assay conditions, except that the concentrations of the substrates were varied (Table XIX, XX, XXI). The reaction velocity dependedon the concentrations

Table XIX. Effect of Glutamic Acid Concentration

Concentration (M)	Radioactive theanine formed (cpm)
1.25×10^{-2}	2,110
1.65×10^{-2}	2,600
2.50×10^{-2}	3,260
3.13×10^{-2}	3,910
5.00×10^{-2}	4,610

Table XX. Effect of Ethylamine Concentration

Concentration (M)	Radioactive theanine formed (cpm)
1.25×10^{-2}	1,550
1.65×10^{-2}	1,950
2.50×10^{-2}	2,570
3.13×10^{-2}	3,540
5.00×10^{-2}	4,610

Table XXI. Effect of ATP Concentration

Concentration (M)	Radioactive theanine formed (cpm)
1.25×10^{-4}	1,960
1.67×10^{-4}	2,390
2.50×10^{-4}	2,810
5.00×10^{-4}	3,700
2.50×10^{-3}	4,610

of the substrates. Apparent Km values for L-glutamic acid, ethylamine and ATP calculated from the tables, according to Lineweaver and Burk[19] 3.3×10^{-2}M, 9.0×10^{-2}M and 1.8×10^{-4}M, respectively.

The effects of the concentrations of Mg^{++} on the reaction velocity were shown in Fig. 23. The dependence of the reaction velocity on the concentrations of this cation in the absence of creatine phosphokinase system shows the necessity of Mg^{++} in the theanine synthesizing reaction. In low concentrations of Mg^{++}, the activity in the absence of creatine phosphokinase system was higher than that in the presence of the ATP generating system, as in the pea enzyme. High concentrations of Mg^{++} tended to inhibit the enzyme.

4. DISCUSSION

This SECTION presents evidences that not only a pea seed acetone powder extract, but also a pigeon liver acetone powder extract can synthesize theanine from glutamic acid and ethylamine in the presence of ATP. Pigeon liver acetone powder was used as an enzyme souce of glutamine synthetase by Speck[44], and orthophosphate liberation from ATP in the presence of methylamine was observed, as described in SECTION III. The theanine

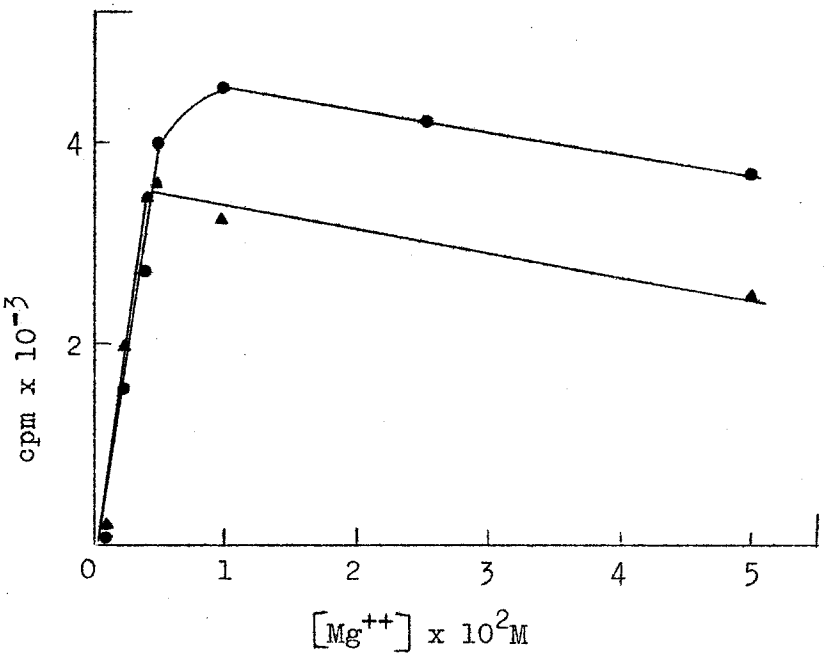


Fig. 23. Effect of $[Mg^{++}]$ Concentration.

●——● in the presence of creatine phosphokinase system.

▲——▲ in the absence of creatine phosphokinase system.

synthesis by the pigeon liver acetone powder extract described here seems to be due to a non-specific reaction of this enzyme. Pigeon liver was also used in the studies on glutathione synthesis[50], therefore, the possibility of the participation of γ-glutamylcysteine synthetase reaction in the synthesis of theanine may also be considered.

Purified enzymes prepared from acetone powder extracts of pea seeds and pigeon liver may be used as enzyme models

in studying and characterizing the enzyme found in tea seedlings.

SUMMARY

Among the many reactions of ω-carboxyl groups of aspartic acid and glutamic acid, aspartic β-semialdehyde dehydrogenase reaction and biosynthetic reaction of theanine are clarified.

These results are summarized as follows.

In CHAPTER II, highly purified aspartic β-semialdehyde dehydrogenase was prepared from pea seedlings. The optimum pH and Km values for the substrates were determined, and substrate and coenzyme specificities of this enzyme were elucidated. Moreover, effects of various compounds on this enzyme were examined. From the behaviours towards thioglycolate and PCMB, this enzyme was concluded to be a SH enzyme. The inhibitory effects and mechanism of aldehydes and amino acids were also clarified, and then the reaction mechanism was discussed.

In CHAPTER III, the precursors of theanine in tea seedlings were examined (SECTION I) by the use of glutamic acid-1-^{14}C and ethylamine-1-^{14}C. And biosynthesis of theanine from glutamic acid and ethylamine in the presence

Table XIX. Effect of Glutamic Acid Concentration

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of the substrates. Apparent Km values for L-glutamic acid, ethylamine and ATP calculated from the tables, according to Lineweaver and Burk[19] 3.3×10^{-2}M, 9.0×10^{-2}M and 1.8×10^{-4}M, respectively.

The effects of the concentrations of Mg^{++} on the reaction velocity were shown in Fig. 23. The dependence of the reaction velocity on the concentrations of this cation in the absence of creatine phosphokinase system shows the necessity of Mg^{++} in the theanine synthesizing reaction. In low concentrations of Mg^{++}, the activity in the absence of creatine phosphokinase system was higher than that in the presence of the ATP generating system, as in the pea enzyme. High concentrations of Mg^{++} tended to inhibit the enzyme.

4. DISCUSSION

This SECTION presents evidences that not only a pea seed acetone powder extract, but also a pigeon liver acetone powder extract can synthesize theanine from glutamic acid and ethylamine in the presence of ATP. Pigeon liver acetone powder was used as an enzyme souce of glutamine synthetase by Speck[44], and orthophosphate liberation from ATP in the presence of methylamine was observed, as described in SECTION III. The theanine

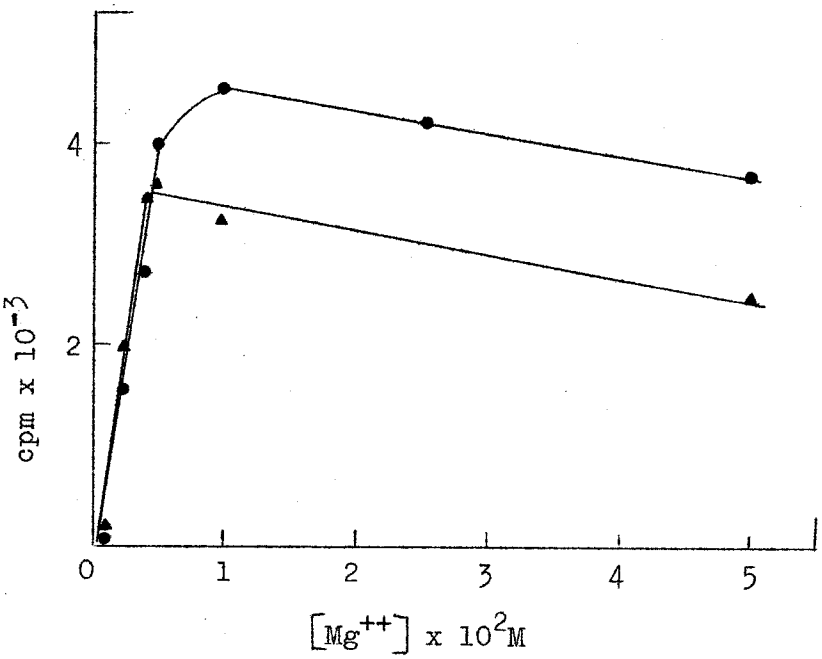

Fig. 23. Effect of $[Mg^{++}]$ Concentration.

●——● in the presence of creatine phosphokinase system.

▲——▲ in the absence of creatine phosphokinase system.

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Purified enzymes prepared from acetone powder extracts of pea seeds and pigeon liver may be used as enzyme models

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In CHAPTER III, the precursors of theanine in tea seedlings were examined (SECTION I) by the use of glutamic acid-1-^{14}C and ethylamine-1-^{14}C. And biosynthesis of theanine from glutamic acid and ethylamine in the presence

of ATP was observed by the use of a homogenate of tea seedlings. The mechanism of theanine synthesis was discussed (SECTION II).

A convenient method for the measurement of radioactive theanine formation was devised. Theanine synthesis by a pea seed acetone powder extract from glutamic acid and ethylamine in the presence of ATP was proved. Some properties of this reaction were clarified, and the mechanism was discussed (SECTION III). Theanine synthesis by a pigeon liver acetone powder extract was studied. Some properties of this reaction were elucidated (SECTION IV).

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