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γ-GLUTAMYL TRANSPEPTIDASE IN PLANTS*

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Abstract—In a search for the mode of synthesis of γ-glutamyl dipeptides in plants, a γ-glutamyl transpeptidase has been demonstrated in several legume tissues and in other higher plants. Evidence for the presence of this enzyme was obtained by the release of cysteinyl-glycine from glutathione, by the formation of γ-glutamyl dipeptides and by the release of aniline from the synthetic substrate, γ-glutamyl aniline. Purified transpeptidase from kidney bean fruit (*Phaseolus vulgaris*) was used in most of these studies.

γ-Glutamyl-S-methyl cysteine was identified as the product of the reaction between S-methyl cysteine and either glutathione or γ-glutamyl aniline by chromatography, sulfur tests and hydrolysis. The enzyme was characterized by a pH optimum of about 9·5, by a two to threefold activation with sodium citrate, by the large number of amino compounds which would act as glutamyl acceptors and by its inhibition by carbonate and borate buffers. The transpeptidase was inhibited by bromcresol green in the presence of sodium citrate but not in its absence. The bean enzyme was notably different from the kidney enzyme in being soluble, in its inhibition by borate, in lacking a magnesium requirement and in its low activity towards glycylglycine.

INTRODUCTION

THE ISOLATION and identification of nine γ-glutamyl dipeptides from plants[1] in recent years prompted a search for their mode of formation. Our initial studies and unpublished work of Webster[2] indicated that none of these dipeptides is formed from the appropriate amino acids and ATP as is γ-glutamyl cysteine.[3,4]

$$\text{Glutamic acid} + \text{cysteine} + \text{ATP} \longrightarrow \gamma\text{-glutamyl cysteine} + \text{ADP} + \text{Pi}$$

While trying other mechanisms of γ-glutamyl dipeptide synthesis, we obtained evidence for a γ-glutamyl transpeptidase in several plant tissues which may be responsible for the formation of naturally occurring γ-glutamyl dipeptides.

$$\text{Glutathione} + \text{amino acid} \rightleftharpoons \gamma\text{-glutamyl dipeptide} + \text{cysteinylglycine}$$

This paper reports the partial purification of a γ-glutamyl transpeptidase from kidney bean fruit and a determination of some of its characteristics.

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[1] J. F. THOMPSON, C. J. MORRIS, W. N. ARNOLD and D. H. TURNER, *Amino Acid Pools*, p. 54, edited by J. T. HOLDEN, Elsevier Press, Amsterdam (1962).
[2] G. C. WEBSTER, private communication.
[3] G. C. WEBSTER and J. E. VARNER, *Arch. Biochem. Biophys.* **52**, 22 (1954).
[4] S. MANDELES and K. BLOCH, *J. Biol. Chem.* **214**, 639 (1955).

RESULTS

Detection of Transpeptidase Activity in Plants

After an early indication of transpeptidase in aqueous extracts of dry kidney bean seeds and kidney bean fruit, several plant tissues were assayed for γ-glutamyl transpeptidase activity by the method of Goldbarg *et al.*,[5] in which γ-glutamyl aniline is the substrate.

γ-glutamyl aniline + amino acid $\rightleftharpoons$ γ-glutamyl dipeptide + aniline

TABLE 1. TRANSPEPTIDASE ACTIVITY IN DIFFERENT PLANT TISSUES*

Plant tissue	Transpeptidase activity†
Phaseolus vulgaris—Red Kidney Bean	
Whole Fruit	38
Immature Seed	12
Leaves	16
Mature Seed	4
Glycine max—Soy Bean	
Immature Seed	25
Green Pod	24
Phaseolus limensis—Lima Bean	
Immature Seed	61
Green Pod	24
Pisum sativum—Pea	
Mature Seed	23
Raphanus sativus—Radish	
Storage Root	7
Iris xithium × *Iris tingitana* (Wedgewood Iris)	
Bulb	1·5
Allium cepa—Onion	
Bulb	0·0
Brassica rapa—Turnip	
Storage Root	0·0

* Fresh tissues were ground in a blender with an equal weight of cold 0·05 M $NaHCO_3$ for 2 min. After centrifugation, 1 ml of the supernatant solution was incubated at 37° C for 2 hr with 2·25 μmoles of γ-glutamyl aniline, 11·25 μmoles of S-methyl-L-cysteine,[9] and 100 μmoles of sodium veronal buffer at pH 9·0 in a total volume of 3 ml.

† mμmoles aniline formed/g fresh weight/hr.

These results (Table 1) showed that transpeptidase activity was relatively high in many legume tissues as compared with other tissues. The low enzymatic activities in the iris bulb and the lack of activity in the onion bulb were particularly surprising when their γ-glutamyl peptide contents are considered.[6–8] It was further determined that onion did not contain an

[5] J. A. GOLDBARG, O. M. FRIEDMAN, E. P. PINEDA, E. E. SMITH, R. CHATTERJI, E. H. STEIN and A. M. RUTENBURG, *Arch. Biochem. Biophys.* **91**, 61 (1960).
[6] C. J. MORRIS, J. F. THOMPSON, S. ASEN and F. IRREVERRE, *J. Biol. Chem.* **236**, 1181 (1961).
[7] C. J. MORRIS, J. F. THOMPSON, S. ASEN and F. IRREVERRE, *J. Biol. Chem.* **237**, 2180 (1962).
[8] A. I. VIRTANEN and E. J. MATIKKALA, *Suomen Kemistilehti.* **B 34**, 53 (1961).
[9] J. F. THOMPSON, C. J. MORRIS and R. M. ZACHARIUS, *Nature* **178**, 593 (1956).

inhibitor of the bean enzyme. Kidney bean fruit were chosen as a source of enzyme for further purification because of their high transpeptidase activity, ready availability and ease of handling.

Purification of Transpeptidase from Kidney Bean Fruit

Fresh green beans (200 g) were ground with 200 ml of 0·05 M $NaHCO_3$ (4°) in a blender at maximum speed for two minutes. This and all subsequent steps were performed at 0–7°. Large particles were removed by filtration through two layers of cheesecloth and solid ammonium sulfate was dissolved in the filtrate (29 g/100 ml). After clarification of the filtrate by centrifugation at 1500*g* for one hour, solid ammonium sulfate (12·5 g/100 ml) was added to the supernatant solution. The resultant precipitate was collected by centrifugation as before, and the supernatant solution was discarded. After dissolving the precipitate in 25 ml of water, the resultant solution was dialysed for 18 hr against 0·01 M potassium phosphate buffer at pH 6·8 containing 0·001 M mercaptoethanol. These procedures increased the activity of the enzyme from 12 to 165 mμmoles aniline/hr/mg protein. The purified enzyme could be lyophilized or kept at −20° for a month with no measurable loss in activity.

Evidence for γ-Glutamyl Transpeptidase Activity and Stoichiometry

Proof of the presence of a γ-glutamyl transpeptidase in a kidney been preparation was obtained in several ways. The formation of aniline from γ-glutamyl aniline[5] or cysteinyl

TABLE 2. EVIDENCE FOR γ-GLUTAMYL TRANSPEPTIDASE ACTIVITY

Experiment	Aniline μmoles
Complete*	0·327
Minus Methylcysteine	0·104
Minus γ-Glutamyl aniline	0·004
Boiled Enzyme	0·038
Minus Enzyme	0·027
Double amount of Enzyme	0·600
Double Enzyme—minus Methylcysteine	0·183
	Cysteinylglycine μmoles
Complete†	1·96
Minus Leucine	0·0
Minus Glutathione	0·0
Boiled Enzyme	0·19
Minus Enzyme	0·0

* The composition of the complete reaction mixture and the incubation conditions were as given in the section on materials and methods. The amounts of protein used were 105 and 210 μg. Enzyme solution was obtained from bean fruit as given in the text, and catalysed the formation of aniline at a rate of 1·11 μmoles/hr/mg·protein.

† The complete incubation mixture contained 2·5 μmoles glutathione, 25 μmoles methylcysteine, 500 μmoles *tris*, and 1·5 mmoles sodium citrate at pH = 9·4, and 780 μg of bean protein in a volume of 1·5 ml. Incubation was for 2 hr at 37° C. Enzyme preparation was made from bean fruit by the usual procedure and produced 0·35 μmoles of aniline/hr/mg protein.

glycine from glutathione was greater in the complete system than when any one component was omitted (Table 2). The formation of a γ-glutamyl dipeptide was demonstrated.

Evidence for the formation of γ-glutamyl-S-methylcysteine was obtained from a large scale incubation of S-methylcysteine with γ-glutamyl aniline or glutathione (Table 3). After purification[10] of amino acids and peptides, acidic compounds were separated[11] from neutral compounds and each group was chromatographed[12] on paper. Quantitative determination[12] of amino compounds was obtained from some chromatograms (Table 3). Other chromatograms were treated with iodoplatinate reagent[13] to locate γ-glutamyl-S-methylcysteine. After

TABLE 3. ANALYSIS OF VARIOUS INCUBATION MIXTURES

Incubation mixture	Aniline	γ-Glutamyl-methylcysteine	Glutamic acid	γ-Glutamyl-γ-glutamyl-aniline
	μmoles	μmoles	μmoles	μmoles
Complete*	28·7	20·9	2·3	1·6
Minus Methylcysteine	15·5	0·0	3·2	5·6
Minus γ-Glutamyl aniline	0·0	0·0	1·5	0·0
Boiled Enzyme	6·2	0·0	1·2	0·0
	Cysteinyl glycine μmoles	γ-Glutamyl-methylcysteine μmoles	Glutamic acid μmoles	
Complete*	16·7	17·0	2·2	
Minus Methylcysteine	0·0	0·0	0·84	
Minus Glutathione	0·0	0·0	1·3	
Boiled Enzyme	0·0	0·0	0·82	

* Bean enzyme preparation (1·09 mg protein) was incubated for 4 hr at 37° C with 500 μmoles of methylcysteine, 50 μmoles of glutathione or γ-glutamyl aniline, 2·5 mmoles of *tris*-acetate and 10 mmoles of sodium citrate at pH of 9·0 in a total volume of 11 ml.

the peptide was eluted from the paper, it was hydrolysed and fragments quantitatively determined. The peptide, which was only formed in the complete mixture (Table 3), was identified by the following criteria: (1) It behaved like an acidic compound by its retention on Dowex 1[14] in the acetate form.[11] (2) It cochromatographed in phenol–water (8:3) and butanol–acetic acid–water (9:1:2·5) with authentic γ-glutamyl-S-methylcysteine.[15] (3) It behaved like a reduced sulfur compound by its bleaching action on iodoplatinate. (4) On hydrolysis it yielded an amino acid which cochromatographed in two solvents with glutamic acid and a neutral compound that bleached iodoplatinate and cochromatographed in two solvents with methylcysteine. (5) Quantitative determination[12] of the hydrolytic products gave a molar ratio of glutamic acid to methylcysteine of 1·12 and showed that it was a

[10] J. F. THOMPSON, C. J. MORRIS and R. K. GERING, *Anal. Chem.* **31**, 1028 (1959).
[11] C. J. MORRIS and J. F. THOMPSON, *Biochem.* **1**, 706 (1962).
[12] J. F. THOMPSON and C. J. MORRIS, *Anal. Chem.* **31**, 1031 (1959).
[13] G. TOENNIES and J. J. KOLB, *Anal. Chem.* **23**, 823 (1951).
[14] Mention of trade products or companies in this paper does not imply that they are recommended or endorsed by the Department of Agriculture over similar products of other companies not mentioned. Trade names are used for convenience in reference only.
[15] R. M. ZACHARIUS, C. J. MORRIS and J. F. THOMPSON, *Arch. Biochem. Biophys.* **73**, 281 (1958).

dipeptide. (6) The peptide gave a test for an unsubstituted alpha amino acid grouping with pyridoxal[16] which indicated that both the alpha amino and carboxyl groups of glutamic acid were free and that the peptide was, therefore, a γ-glutamyl peptide.

Chromatography of the acidic amino compounds obtained with γ-glutamyl aniline in the complete incubation mixture, revealed an unknown ninhydrin reactive compound which was present in larger quantities in the mixture lacking γ-glutamyl methylcysteine. The characteristics of the unknown compound and its absence in the other two incubation mixtures suggested that the compound might be γ-glutamyl-γ-glutamyl aniline formed enzymatically.

$$2\gamma\text{-glutamyl aniline} \longrightarrow \gamma\text{-glutamyl-}\gamma\text{-glutamyl aniline} + \text{aniline}$$

The validity of the above suggestion was proven as follows: The unknown compound was located on a chromatogram by its absorption of ultraviolet light. The compound was eluted from the paper and hydrolysed. One product was shown to be glutamic acid by its acidic nature and its chromatographic characteristics. Another product behaved like aniline in dye formation and absorption spectrum of the dye. Analysis of the hydrolytic products gave a molar ratio of glutamic acid to aniline of 2·01.

The larger amount of γ-glutamyl-γ-glutamyl aniline in the incubation mixture lacking methylcysteine than in the complete incubation mixture showed that the latter compound competed with the γ-glutamyl acceptor as an acceptor of glutamyl groups. The formation of less γ-glutamyl-γ-glutamyl aniline in the presence of methylcysteine than in its absence means that the difference in aniline released with and without methylcysteine results in a low measure of transpeptidase activity. The error is not serious, in most cases, because the apparent activity represents approximately the same proportion of the true activity. However, the measurement of enzymatic activity with poor glutamyl acceptors requires the measurement of the dipeptide formed (*vide infra*).

The stoichiometry of the reaction was good with glutathione as the substrate (Table 3), and reasonably good with γ-glutamyl aniline as the substrate, provided the formation of γ-glutamyl-γ-glutamyl aniline was considered. The data in Table 3 show that methylcysteine inhibited the formation of γ-glutamyl-γ-glutamyl aniline and the concomitant release of aniline (see equation). When this inhibition is taken into account, the increased aniline arising from the addition of methylcysteine was essentially equivalent to the γ-glutamyl methylcysteine formed as required by theory. The greater amount of glutamic acid in the presence of γ-glutamyl aniline than in its absence indicates some enzymatic hydrolysis of γ-glutamyl aniline.

$$\gamma\text{-glutamyl aniline} + H_2O \longrightarrow \text{glutamic acid} + \text{aniline}$$

The data of Table 3 show that glutathione and γ-glutamyl aniline were comparable in their ability to donate glutamyl groups.

Effects of Buffers and other Salts on Enzymatic Activity

In the determination of the effect of pH on enzymatic activity, it was found that carbonate and borate were inhibitory (Table 4), and that this effect was not due to effects on the assay system. Differences in enzyme activity with *tris* buffers prepared with various acids led to the finding that sodium citrate and sodium ethylene diaminetetraacetic acid (EDTA) in relatively high concentrations (> 1 M) increased enzymatic activity severalfold (Table 4). Both cation and anion moieties were involved in the enhancement of enzymatic activity. Since sodium

[16] G. D. KALYANKAR and E. E. SNELL, *Nature*, **180**, 1069 (1957).

citrate was easier than EDTA to handle, it was routinely included in assay mixtures to increase sensitivity.

TABLE 4. THE EFFECT OF BUFFERS AND OTHER SALTS ON TRANSPEPTIDASE ACTIVITY*

Buffer	Buffer conc. (molarity)	Added salt	Salt conc. (molarity)	Relative activity
tris-Acetate	0·5	None	—	100
Sodium Carbonate	,,	,,	—	43
Sodium Borate	,,	,,	—	5
tris-Acetate	,,	Sodium Citrate	1·0	289
,,	,,	Sodium EDTA	0·5	238
,,	,,	Potassium Citrate	1·0	250
,,	,,	Lithium Citrate	1·0	60
,,	,,	Sodium Acetate	1·0	135
,,	,,	Sodium Chloride	1·0	56

* All conditions, except for buffers and added salts, were as given in the section on materials and methods. Final pH in all treatments was 9·4.

Effect of pH, Substrate Concentration and Enzyme Concentration on Transpeptidase Activity

The determination of pH optimum was difficult because in the pH range of 9–10, the best buffers (carbonate and borate) were inhibitory (see Table 4). A satisfactory curve (Fig. 1) for

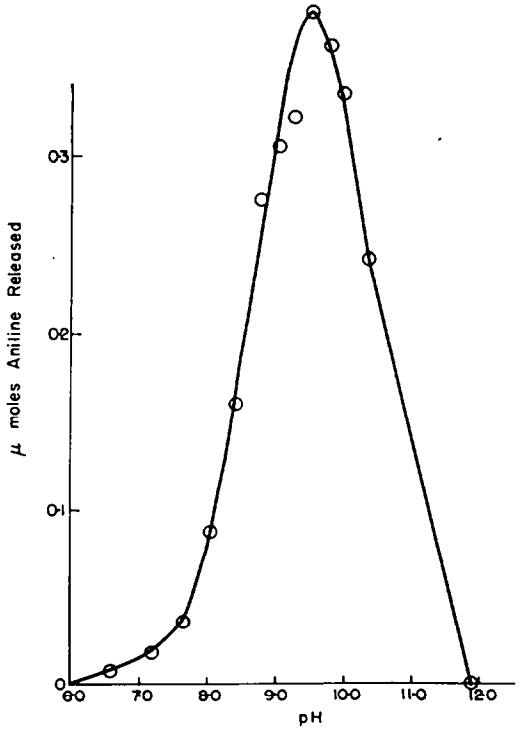

FIG. 1. THE INFLUENCE OF pH ON γ-GLUTAMYL TRANSPEPTIDASE ACTIVITY. STANDARD CONDITIONS WERE USED AS GIVEN IN THE TEXT.

the effect of pH on enzymatic activity was obtained in a solution containing *tris*-acetate plus sodium citrate by measuring the pH of the complete incubation mixture rather than the pH of the buffer.

Differences in pH of the complete incubation mixture and the mixture lacking methylcysteine were avoided by utilizing proline which had the same buffering action as methylcysteine but had no effect on enzymatic activity (*vide infra*). The pH optimum was about 9·5, a value somewhat higher than those reported for the kidney enzyme.[5,17,18]

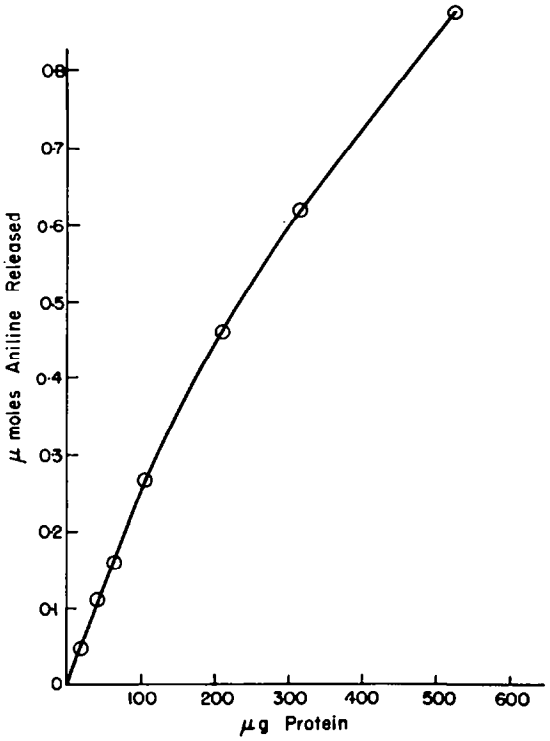

FIG. 2. THE RELATIONSHIP BETWEEN ENZYME ACTIVITY AND AMOUNT OF ENZYME. CONDITIONS OF ASSAY ARE GIVEN IN THE TEXT.

Figure 2 shows that the enzymatic activity is well correlated with the amount of enzyme over a wide range of enzyme concentrations.

After determining the effect of concentration of methylcysteine and γ-glutamyl aniline on the rate of the transfer reaction under the conditions described in the experimental section, Lineweaver–Burk plots[19] gave *Km* values of $1{\cdot}1 \times 10^{-2}$ M for methylcysteine and $1{\cdot}1 \times 10^{-4}$ M for γ-glutamyl aniline.

Specificity of Transpeptidase for Glutamyl Acceptors

A number (about eighty) of amino compounds (mostly amino acids and related compounds) were tested for their ability to accept glutamyl groups. Data from a few representative

[17] F. BINKLEY, *J. Biol. Chem.* **236**, 1075 (1961).
[18] P. J. FODOR, A. MILLER and H. WAELSCH, *J. Biol. Chem.* **202**, 551 (1953).
[19] H. LINEWEAVER and D. BURK, *J. Am. Chem. Soc.* **56**, 658 (1934).

TABLE 5. ACTIVITY OF VARIOUS AMINO COMPOUNDS IN THE TRANSPEPTIDASE SYSTEM

Glutamyl acceptor*	Relative activity†
Sulfur Amino Acids	
S-methyl-L-cysteine	100
L-methionine	148
D-methionine	26
DL-homomethionine	134
L-ethionine	136
S-ethyl-L-cysteine	96
S-methyl-L-cysteine sulfoxide	68
S-methyl-L-cysteine sulfone	55
L-cysteine	2
L-homocysteine	15
Acidic Amino Acids and Related Compounds	
L-aspartic acid	2
L-glutamic acid	17
DL-α-aminopimelic acid	55
L-asparagine	43
D-asparagine	3
L-glutamine	30
Neutral Amino Acids and Related Compounds	
glycine	20
DL-alanine	0
L-α-amino-*n*-butyric acid	36
β-amino-*n*-butyric acid	0
γ-amino-*n*-butyric acid	7
DL-α-aminoisobutyric acid	−14‡
DL-β-aminoisobutyric acid	1
β-alanine	0
DL-homoserine	28
L-valine	53
L-leucine	84
DL-isoleucine	53
isoamylamine	26
L-phenylalanine	92
L-tyrosine	57
L-proline	0
Miscellaneous Compounds	
L-ornithine	39
δ-acetyl-DL-ornithine	73
α-acetyl-L-ornithine	5
glycylglycine	13
β-aminopropionitrile	80
glutathione	−9‡
cysteinylglycine (disulfide form)	0
ammonia	0

* The amount of each amino compound was 25 μmoles for the D, L or optically inactive forms, and 50 μmoles for the DL mixtures.

† Incubation conditions were as given in the text. S-methylcysteine caused the release of 0·13 μmoles of aniline/hr in the presence of 105 μg of protein.

‡ α-aminoisobutyric acid and glutathione apparently interfered with the formation of γ-glutamyl-γ-glutamyl aniline (see discussion of assay method).

compounds are given in Table 5. Several generalizations that were obvious from the complete set of data are illustrated in Table 5: (1) The enzyme catalysed the transfer of glutamyl groups to many compounds. (2) The higher homologs in a series had more activity than the lower ones. (The exception seemed to be glycine and alanine.) (3) L-Amino acids were much more active than their D-isomers. (4) α-Amino acids were better glutamyl acceptors than the corresponding β- and γ-amino acids. (5) α-Amino acids were more active than their decarboxylation products. (6) The addition of a polar group (e.g., hydroxyl or carboxyl) decreased ability to accept glutamyl groups. (7) Amino acids with a thioether group were more active than closely related amino acids with a hydrocarbon side chain. (8) Sulfhydryl amino

TABLE 6. PRODUCTION OF γ-GLUTAMYL PEPTIDES OF SELECTED AMINO ACIDS

Amino acid	γ-Glutamyl peptide formed*
	μmoles
S-methylcysteine	3·1
α-aminoisobutyric acid	0·0
β-aminoisobutyric acid	0·42
β-alanine	0·13
L-proline	0·0

Purified bean protein (1·2 mg) was incubated with 250 μmoles *tris* acetate buffer, 750 μmoles sodium citrate, 5 μmoles γ-glutamyl aniline and 50 μmoles of amino acid at pH 9·4 for 10 hr at 37° C. The dipeptides were determined by the methods used for obtaining the data in Table 3.

* The identity of the peptides was determined by cochromatography with authentic peptides, and hydrolysis to amino acids with appropriate chromatographic characteristics.

acids are much poorer glutamyl acceptors than the corresponding thioethers. (9) Oxidation of a thioether reduced its activity.

The glutamyl acceptors listed in Table 5 include nine amino acids (methylcysteine, methionine, leucine, valine, tyrosine, phenylalanine, isoleucine, β-alanine and β-aminoisobutyric acid) which have been found to occur naturally as γ-glutamyl peptides.[1, 6–8, 11, 15, 20, 21] Of this group, seven were good glutamyl acceptors, while two compounds β-aminoisobutyric acid and β-alanine, showed no activity. The latter two compounds, plus α-aminoisobutyric acid and proline, were tested further for ability to accept glutamyl groups by measuring the dipeptide formed (Table 6). γ-Glutamyl peptides of the β-amino acids were formed in small amounts, but none was formed from α-aminoisobutyric acid and proline. It was also noted that proline and β-alanine did not interfere with the formation of γ-glutamyl-γ-glutamyl aniline, although α-aminoisobutyric acid and β-aminoisobutyric acid inhibited its formation.

[20] A. I. VIRTANEN and E. J. MATIKKALA, *Z. Physiol. Chem., Hoppe-Seyler's.* **322**, 8 (1960).
[21] A. I. VIRTANEN and E. J. MATIKKALA, *Suomen Kemistilehti.* **B 33**, 83 (1960).

The latter observation explains the apparent negative activity of α-aminoisobutyric acid as a glutamyl acceptor (Table 5).

Inhibition and Activation

The common enzyme inhibitors (iodoacetate, *p*-chloromercuribenzoate and cyanide) did not seriously depress activity (< 20 per cent) at 10^{-3} M.

Binkley[17] found that bromcresol green (0·10 mg/ml) completely inhibited the kidney transpeptidase. Bromcresol green showed an unusual inhibition of the bean enzyme by inhibiting in the presence of sodium citrate but not in its absence (Table 7).

TABLE 7. THE EFFECT OF BROMCRESOL GREEN ON BEAN TRANSPEPTIDASE AS INFLUENCED BY THE PRESENCE OF SODIUM CITRATE

Concentration of bromcresol green (mg/ml)	Sodium citrate*	Relative activity
0	−	35
0·5	−	31
0·1	−	33
0	+	100
0·5	+	1
0·1	+	21
0·05	+	79

Assay conditions were the same as given in the text.

* Final concentration was 1·0 M.

The activation of the transpeptidase by sodium citrate was tested to see whether the activation was due to; (1) prevention of inactivation[22] during incubation, or (2) removal of an inhibitor. The results (Table 8) of these experiments indicate that neither of these alternatives can explain the sodium citrate activation. Although the kidney transpeptidase requires magnesium ions, the bean enzyme shows no requirement as would be assumed from its activation by high concentrations of sodium citrate (Table 4).

Localization of the Enzyme

The ease of extraction and handling of the bean enzyme indicated that it was not associated with the microsomal fraction as is the kidney enzyme.[17] This indication was verified by extract fractionation. Fresh bean fruit were ground with sand in a mortar in an equal volume of cold 0·10 M potassium phosphate buffer (pH 6·8) containing 0·25 M sucrose and 2·5% Ficoll[23] (synthetic polysaccharide from Pharmacia, Uppsala, Sweden[14]). After filtering the suspension through two layers of cheesecloth, the sand and cell debris were removed by centrifugation at 200*g* for 2 min. The chloroplasts, mitochondria and microsomes were separated by

[22] J. B. SUMNER, *The Enzymes*, edited by J. B. SUMNER and K. MYRBÄCK. Vol. I, part 1, p. 24, Academic Press, New York (1950).

[23] S. I. HONDA, personal communication.

TABLE 8. THE EFFECT OF VARIOUS TREATMENTS ON THE ACTIVITY OF BEAN TRANSPEPTIDASE

Treatment of enzyme prior to incubation	Sodium citrate present during incubation	Relative activity
1. Standard*	−	31
2. Standard	+	100
3. 2 hr at 37° without citrate	+	97
4. 2 hr at 37° with citrate	+	98
5. Dialysed 90 hr with dilute buffer*	−	20
6. Dialysed 90 hr with dilute buffer	+	62
7. Dialysed 45 hr against 1·5 M Na citrate pH = 7·0, and 24 hr with dilute buffer†	−	32
8. Dialysed 45 hr against 1·5 M Na citrate pH = 7·0, and 24 hr with dilute buffer†	+	89

* Standard treatment included an 18 hr dialysis at 0° against a solution of 10^{-3} M mercaptoethanol in 10^{-2} M potassium phosphate buffer pH = 7·0 (dilute buffer of treatments 5–8).

† 10^{-2} M potassium phosphate pH = 7·0.

differential centrifugation[24,25] and washed. The various fractions were tested for transpeptidase activity and essentially all of the enzyme was found to be soluble (Table 9) in contrast to the kidney enzyme.

TABLE 9. LOCALIZATION OF ENZYME OF KIDNEY BEAN AND RAT KIDNEY

Fraction	Percent of original activity: Kidney bean fruit*	Rat kidney†
Nuclei	—	30
Chloroplasts	3·2	—
Mitochondria	2·6	58
Microsomes	1·0	100
Soluble	102	7
Total	109	195

* Incubation mixture includes in 3 ml, 500 μmoles of *tris*-acetate at pH = 9·0, 25 μmoles of methylcysteine and 2·25 μmoles of γ-glutamyl aniline. The mixture was incubated 2 hr at 37°.

† Data of Binkley.[17]

[24] B. AXELROD, *Methods in Enzymology*, edited by S. P. COLOWICK and N. O. KAPLAN. Vol. I, p. 19, Academic Press, Inc., New York (1955).

[25] P. GORHAM, *Methods in Enzymology*, edited by S. P. COLOWICK and N. O. KAPLAN. Vol. I, p. 22, Academic Press, New York (1955).

DISCUSSION

Virtanen and Berg[26] suggested that they had a γ-glutamyl transpeptidase in peas but their evidence was scanty. Virtanen and Matikkala[20] found no evidence for this transpeptidase in onion leaves, dormant onion bulbs or sprouting bulbs. These results agree with our observations (Table 1) but are unexpected in view of the numerous γ-glutamyl peptides in onions.[8] Our paper presents evidence for a γ-glutamyl transpeptidase in legumes and other species. This transpeptidase probably accounts for the biosynthesis of the numerous γ-glutamyl dipeptides in legumes[1,11,15,27] because of the high activity of methylcysteine, methionine, leucine, phenylalanine and tyrosine as γ-glutamyl acceptors (Table 5). The role of transpeptidase in peptide formation in other tissues[6–8,20,21] is not so clear. The iris bulb had low transpeptidase activity (Table 1). The low enzymatic activity in iris bulbs and the absence of activity in onions suggests that the transpeptidase is not responsible for dipeptide biosynthesis in these plants but alternative explanations are possible. It is reasonable that

TABLE 10. CONTRASTS BETWEEN BEAN AND KIDNEY TRANSPEPTIDASES

Bean	Kidney
Soluble	Occurs mainly in the microsomes[17]
No magnesium requirement	Requires magnesium[17]
Glycylglycine is a poor substrate	Glycylglycine is the best substrate[5,17]
Not inhibited by bromcresol green in the absence of sodium citrate	Inhibited by bromcresol green[17]
Inhibited by borate	Not inhibited by borate[5]
Catalyses the formation of γ-glutamyl-γ-glutamyl aniline	Does not catalyse the formation of γ-glutamyl-γ-glutamyl aniline[5]
Activated by sodium citrate and sodium EDTA	Not activated by salts[17]

γ-glutamyl-β-amino-propionitrile, the toxic factor in sweet peas[28] (lathyrus factor), is formed by glutamyl transfer, since β-amino-propionitrile is an active glutamyl acceptor (see Table 5).

Animal tissues have an active transpeptidase which has been implicated in glutathione degradation,[17] but the advantage of forming one γ-glutamyl peptide at the expense of another is unknown. The transpeptidase in plants must function in the synthesis of some of the dipeptides, although the *raison d'être* of the dipeptides is obscure. The striking differences in properties of the plant and animal enzymes (Table 10) suggests a difference in function. A suggested role of γ-glutamyl peptide in protein synthesis[29] has not been proven.[30]

Most of the data in this paper were obtained with γ-glutamyl aniline as a substrate, but glutathione was active and is presumably the natural substrate. Virtanen and Matikkala[20] obtained no evidence that an onion tripeptide (S-β-carboxy-*n*-propyl glutathione) could donate glutamyl groups using kidney transpeptidase.

[26] A. I. VIRTANEN and A. M. BERG, *Acta. Chem. Scand.* **8**, 1089 (1954).
[27] C. J. MORRIS and J. F. THOMPSON, *J. Biol. Chem.* **238**, 650 (1963).
[28] E. D. SCHILLING and F. M. STRONG, *J. Am. Chem. Soc.* **77**, 2843 (1955).
[29] C. S. HANES, F. J. R. HIRD and F. A. ISHERWOOD, *Biochem. J.* **51**, 25 (1952).
[30] R. W. HENDLER and D. M. GREENBERG, *Biochem. J.* **57**, 641 (1954).

Goldbarg *et al.*[5] report no formation of γ-glutamyl-γ-glutamyl aniline with a kidney preparation so this may be a special property of the plant enzyme. It is a factor to be considered in using the synthetic substrate for analysis of glutamyl acceptors with low activity.

Bromcresol green had an unusual effect on the bean transpeptidase by causing inhibition only in the presence of sodium citrate. Possibly the salt expanded the transpeptidase molecule allowing the relatively large indicator molecule access to an active site.

This explanation may also apply to the activating effect of sodium citrate. Binkley[17] reported inhibition of the kidney enzyme by several salts but no activation. Our results showed that the sodium citrate activation was not due to a reduction of inactivation or a reversal of inhibition. Citrate activation of cystathionase[31] is due to its inhibition of phosphatases, which inactivate the cofactor, pyridoxal phosphate. Since no cofactor requirement has been found for the bean transpeptidase, this explanation also appears unlikely. A loosening of the protein molecule to permit a more ready access of substrate to active site is possible.

MATERIALS AND METHODS

Substrates

γ-Glutamyl acceptors were obtained from commercial sources with the exception of homomethionine[32] which was a gift from Prof. A. Kjaer, and α-acetylornithine which was prepared in our laboratory.[33]

γ-Glutamyl aniline was prepared from the α-benzyl ester of N-carbobenzyloxy-glutamic acid (Cyclo Chemical Company, Los Angeles, California), and aniline by the method of Sachs and Brand.[34] The product was finally purified by two recrystallizations from water. The yield from 0·0105 moles of the glutamic acid derivative was 35 per cent of theory.

Purification and Analysis of Peptides and Amino Acids

In those experiments where the peptides were measured, the incubation mixtures were acidified to pH 4·5 with acetic acid and heated to boiling. After removal of the protein precipitate, the amino compounds were purified on ion-exchange resins.[10] Acidic peptides were separated by absorption on Dowex 1 in the acetate form and subsequent elution.[11] Quantitative determination of amino compounds was made by reaction with ninhydrin after separation on filter paper.[12]

Assay of γ-Glutamyl Transpeptidase

Transpeptidase activity was usually determined by a procedure based on that of Goldbarg *et al.*[5] in which aniline released from γ-glutamyl aniline is measured colorimetrically. A typical incubation mixture contained 1·5 ml of 0·5 M *tris* buffer in 1·5 M sodium citrate, adjusted to pH 9·6 with acetic acid; 0·5 ml of 0·0045 M γ-glutamyl aniline in equimolar NaOH; 0·5 ml of 0·050 M S-methyl-L-cysteine in *tris*-citrate buffer; and 0·5 ml of enzyme solution. After incubation at 37° for 2 hr, 4 ml of 40% trichloracetic acid was added. The uncombined aniline was measured by the method of Goldbarg *et al.*[5] with this exception; the N-(l-naphthyl) + ethylenediamine concentration was increased to 3 mg/ml. Transpeptidase

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[32] A. KJAER and S. WAGNER, *Acta. Chem. Scand.* **9**, 721 (1955).
[33] J. F. THOMPSON and R. K. GERING, *Arch. Biochem. Biophys.* **99**, 326 (1962).
[34] H. SACHS and E. BRAND, *J. Am. Chem. Soc.* **75**, 4608 (1953).

activity was measured by the difference between aniline formed in the presence and in the absence of a glutamyl acceptor.

When glutathione was employed as a substrate, cysteinylglycine was measured by the method of Sullivan and Hess.[35]

Proteins were measured by the method of Lowry *et al.*[36]

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[35] M. X. SULLIVAN and W. C. HESS, *J. Biol. Chem.* **116**, 221 (1936).
[36] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).