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**The Isolation and Characterization of  $\gamma$ -L-Glutamyl-S-Methyl-L-Cysteine from Kidney Beans  
(*Phaseolus vulgaris*)<sup>1</sup>**

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INTRODUCTION

In recent times (+)S-methyl-L-cysteine sulfoxide has been isolated and characterized by Morris and Thompson (1) from turnips and by Synge and Wood (2) from cabbage. In a search for the precursor of this compound, Zacharius, Thompson, and Morris (3) succeeded in isolating and identifying the compound S-methyl-L-cysteine from the nonprotein nitrogen fraction of the kidney bean. Subsequently, the latter substance has been reported as a naturally occurring metabolite of some strains of *Neurospora crassa* (4).

At the time Zacharius *et al.* (3) reported S-methylcysteine in beans, they were aware through paper chromatographic studies (5) of the presence of this compound in some form of peptide in the same fraction. The isolation and identification of this peptide as  $\gamma$ -L-glutamyl-S-methyl-L-cysteine has been reported in a preliminary publication (6). The details of the original isolation and characterization of this compound including its synthesis are described in this paper. Because of the relatively small yield, an alternative method of isolation is described whereby a larger amount of the dipeptide was obtained.

<sup>1</sup> Presented before the Division of Biological Chemistry at the 132nd Meeting of the American Chemical Society, New York, N. Y., September 9, 1957.

## EXPERIMENTAL

*Isolation Procedure*

1. Red kidney bean seeds (10 lb.) were pulverized and extracted with 50% ethanol at room temperature. The soluble fraction was treated with mercuric acetate, and the resultant precipitate was centrifuged down and decomposed with  $H_2S$ . After removal of mercuric sulfide, the solution was passed through a column ( $100 \times 0.58$  cm.) of Dowex 50-X4 (200-400 mesh) in the hydrogen form so that the amino compounds were absorbed on the top third of the resin (7). The resin was washed with water and then eluted with 0.15  $N$   $NH_4OH$ , and the eluate was collected in 25-ml. fractions. The unknown was found to be in fractions 20-65 by two-directional paper chromatography and was contaminated with aspartic acid, glutamic acid, and *S*-methylcysteine. Fractions 30-50 were concentrated *in vacuo*, dissolved in 80 ml. of 25% ethanol, and neutralized to pH 7. This solution was placed on a column ( $245 \times 0.58$  cm.) of Dowex 50-X4 (200-400 mesh) in the sodium form. The solution was then washed through the column at a rate of 100 ml./hr. with 25% ethanol (8),<sup>2</sup> and 25-ml. fractions were collected. Fractions 71-77 contained the unknown uncontaminated with other ninhydrin-reactive substances; these were combined and concentrated by vacuum distillation.

Fractions 79-93, containing the unknown as well as glutamic acid and aspartic acid, were vacuum-concentrated and dissolved in a minimum volume of phenol-water (80:30). This solution was placed on a paper roll (9) and chromatographed with phenol-water. Twenty-milliliter fractions were collected, and the unknown was found in fractions 200-360 uncontaminated by other ninhydrin-reactive compounds. These fractions were combined and the unknown was extracted from phenol with water, and the excess phenol was removed from the water by extraction with ether. The aqueous solution was concentrated and combined with the solution obtained from fractions 71-77 from the column of sodium resin. This solution was decolorized by passage through a column ( $20 \times 1$  cm.) of Norit A and evaporated to dryness. The residue was dissolved in a minimum of water, and excess absolute alcohol added. The resultant amorphous precipitate was separated and dried; it weighed 200 mg. This had a light gray color and was not obtained in crystalline form.

An infrared curve and subsequent ash determination (6.95%) showed that the isolated compound was in the form of a sodium salt which probably resulted from exposure to the Dowex resin during purification. The compound was therefore stripped of the sodium by passage through a Dowex 1 column (acetate form) with 0.5  $N$  acetic acid (see below). The compound was recovered as its free acid.

2. Red kidney bean seeds (8 lb.) were pulverized, and the resultant powder was divided into three portions. Each portion was shaken with 4-6 l. of 60% ethanol and allowed to stand at 5°C. until the supernatant solution was clear. The supernatant was removed by siphoning, and the residue was twice re-extracted. The combined volume of extracts was 36-40 l. Eight-liter portions were passed through a column ( $4.6 \times 25$  cm.) of Dowex 50-X4 in the acid form at 5°C. The column was washed with 50% ethanol, and the resin was eluted with 5  $N$   $NH_4OH$  in 50% ethanol until the eluate gave only a weak ninhydrin-positive reaction. The ammonia eluates were concentrated by evaporation under reduced pressure to dryness. The residue was dissolved in a liter of water and was filtered to remove insoluble material.

Dowex 1-X2 (200-400 mesh) prepared in the acetate form was packed in a column

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<sup>2</sup> Also unpublished results of J. F. Thompson and C. J. Morris.

## $\gamma$ -L-GLUTAMYL-S-METHYL-L-CYSTEINE

(125  $\times$  4.5 cm.) in 5-10-cm. layers. The concentrated extract was fractionated in three portions on this column. A third of the solution was added to the top of the column at 5°C. and washed in. Water was passed through the column, and 160 fractions of 15 ml. each were collected. The column was then eluted with 0.5 *N* acetic acid, wherein the acid broke through around fraction 190. Glutamic acid appeared in fractions 216-270, aspartic acid in fractions 271-321, and the unknown was eluted in fractions 480-640.

The fractions containing the unknown combined and evaporated to dryness *in vacuo* yielded 13.05 g. Paper chromatography of this material showed the presence of a small amount of a substance which subsequently was shown to be the oxidized (sulfoxide) form of the peptide, but no other ninhydrin-reactive substances.

One gram of the isolated material was dissolved in 50 ml. of 70% ethanol and chromatographed with the same solvent on a paper roll (9) previously washed with 0.1 *N* HCl and 70% ethanol. The first 2 l. of 70% ethanol representing a single fraction was discarded. Thereafter 15-ml. fractions were collected. The unknown compound was found in fractions 55-115, and it still contained a small amount of the contaminant (sulfoxide). A weak peak at fractions 200-220 contained more of this contaminant. Fractions 55-115 were combined and evaporated to dryness *in vacuo* at room temperature. The residue was dissolved in a small amount of water and reprecipitated with acetone. The supernatant was decanted, and the residue was redissolved in a small amount of water. The solution was allowed to evaporate *in vacuo* over solid NaOH until a 1-ml. volume was reached. On cooling, crystals formed which after washing with cold water and 75% aqueous acetone and drying weighed 148 mg. This material was found to be homogeneous by paper chromatography.

*Anal.* Calcd. for  $C_9H_{16}N_2O_5S \cdot 2H_2O$ : C, 35.99; H, 6.71; N, 9.33; S, 10.68;  $H_2O$ , 12.00. Found: C, 35.75; H, 6.51; N, 9.20; S, 10.62;  $H_2O$ , 11.50.

The water of crystallization was removed on prolonged drying at 70°C. with a dry air stream at approximately 5 mm. pressure.

### *Identification of Unknown Compound*

Reaction of the isolated material with ninhydrin (10) showed the presence of 4.15% of primary amino nitrogen based on a color factor of 0.97. The amount of nitrogen determined did not vary with heating times of 10-30 min. indicating that hydrolysis did not occur during this reaction. The factor used is probably not wholly applicable for this compound, thus accounting for the difference between 4.15% and the subsequently established theoretical value of 5.3%. The unknown gave a positive test with iodoplatinate (11) and negative tests with nitroprusside and cyanide (11), indicating that the compound may contain sulfur but not in disulfide or sulfhydryl groups.

A biuret reaction and Rydon-Smith test (12) were positive, suggesting that the compound was a peptide or an amide. However, acid hydrolysis of the compound yielded two amino acids and no ammonia indicating that the substance was a peptide.

The isolated material was hydrolyzed with a 1:1 mixture of glacial acetic acid and 3 *N* HCl in the presence of  $H_2S$  in the autoclave at 15 lb.

pressure for 3 hr., and the excess acid was removed by drying over solid NaOH. The residue was redissolved in water, neutralized to pH 6.5, and passed through a column of Dowex 1-X2 in acetate form. The effluent fractions were combined and dried. The residue was recrystallized three times from aqueous methanol. Synthetic *S*-methyl-L-cysteine and the compound appearing in these fractions were found to be identical by matching infrared absorption spectra. The isolated fragment had an optical rotation  $[\alpha]_D^{23} -27.8^\circ$  ( $\text{H}_2\text{O}$ ,  $c = 2.0$ ) as compared to the synthetic compound  $[\alpha]_D^{23} -29.0^\circ$  ( $\text{H}_2\text{O}$ ,  $c = 2.0$ ).

*Anal.* Calcd. for  $\text{C}_4\text{H}_9\text{NO}_2\text{S}$ : C, 35.53; H, 6.71; N, 10.36; S, 23.72. Found: C, 35.04; H, 6.47; N, 10.09; S, 23.40.

The Dowex 1 column was eluted with 1 *N* HCl, and the eluate was concentrated by evaporation at reduced pressure. The residue was twice recrystallized from water. The second hydrolytic product was found to be glutamic acid by matching infrared absorption spectra with the known compound. The isolated residue had an optical rotation  $[\alpha]_D^{23} +30.7^\circ$  (5 *N* HCl,  $c = 1.0$ ) as compared to  $[\alpha]_D^{23} +31.5^\circ$  (5 *N* HCl,  $c = 1.0$ ) for known glutamic acid.

*Anal.* Calcd. for  $\text{C}_5\text{H}_9\text{NO}_4$ : C, 40.81; H, 6.17; N, 9.52. Found: C, 40.74; H, 6.21; N, 9.26.

The hydrolytic products were also shown to be glutamic acid and *S*-methylcysteine by cochromatography on paper with authentic compounds in water-saturated phenol, *n*-butanol-acetic acid-water (9:1:2.5), 70% ethanol, collidine-lutidine-water (3:1:1), and methanol-water-pyridine (80:20:4). Employing the quantitative paper chromatographic procedure of Thompson *et al.* (13) modified, and using as the development solvent *tert*-butanol-benzyl alcohol-water (5:5:2), the molar ratio of glutamic acid to *S*-methylcysteine in four determinations ranged from 0.92 to 1.05. The amount of amino acid recovered was 96–101% of the total weight, showing that there were no other constituents in the molecule.

The dinitrophenyl derivative of the unknown was prepared (14) and hydrolyzed. The products were separated by two-directional paper chromatography with a modified "toluene" solvent and 1.5 *M* aqueous  $\text{Na}_2\text{HPO}_4$  described by Levy (15). The dinitrophenyl group was attached to the glutamic acid moiety proving that the glutamic acid had the free amino group. From the amount of free amino nitrogen found in the peptide, the compound could only be the dipeptide glutamyl-*S*-methylcysteine. The linkage was tentatively assumed to be through the  $\gamma$ -carboxyl of the glutamic acid because of the similarity of this compound with the glutamyl cysteine moiety of glutathione, and the Van Slyke  $\text{NH}_2\text{-N}$  values yielded by the isolated peptide (see below).

*Synthesis of  $\gamma$ -L-Glutamyl-S-methyl-L-cysteine*

*S*-Methylglutathione was prepared by the methylation of glutathione according to the procedure of Kermack and Matheson (16). Recrystallized *S*-methylglutathione (405 mg.) was incubated with 8.37 mg. carboxypeptidase (3 $\times$  crystallized) in a 20-ml. solution of pH 8.5 phosphate buffer at 37°C. in a manner similar to the enzymic cleavage of glycine from glutathione reported by Grassmann *et al.* (17). Trial runs with aliquots of the enzyme and substrate demonstrated the splitting off of the glycine moiety which was followed by paper chromatography at intervals of 6, 21, 48, and 72 hr. The reaction was found to be completed within 48 hr. since no further increase in glycine could be determined at the end of 72 hr. Fractionation of the main reaction mixture at the end of 72 hr. was carried out on a Dowex 1-X8 resin (200-400 mesh) column (17  $\times$  0.9 cm.) in the acetate form. The column was eluted with 0.5 *N* acetic acid and collected in 1-ml. fractions at the rate of 12 ml./hr.

Glycine appeared in the first 18 fractions and was recovered in a yield of 88% as determined by the ninhydrin color method (10). The unhydrolyzed tripeptide appeared through fractions 35-68 and accounted for 16% of the initial *S*-methylglutathione. Fractions 69-125 contained  $\gamma$ -L-glutamyl-*S*-methyl-L-cysteine. The dipeptide fractions were combined and concentrated under reduced temperature and pressure. When this fraction was chromatographed on the same resin column to check its homogeneity, two small peaks consisting of glutamic acid and *S*-methylcysteine separated; these probably resulted from acid hydrolysis of the dipeptide. On concentration of the dipeptide fraction to dryness, 240 mg.  $\gamma$ -L-glutamyl-*S*-methyl-L-cysteine was obtained as an amorphous white solid. The residue was dissolved in a minimum of water and reprecipitated with an excess of absolute ethanol. The highly deliquescent solid was further dried by trituration with absolute ethanol and evaporation of the solvent *in vacuo*. The dipeptide crystallized as rosettes of white needles from a saturated aqueous solution in the cold. On a micro hot stage it softened 74°, liquified 77°, darkened 183°; by capillary, softened 116°, liquified 121°, darkened 175°.

*Anal.* Calcd. for  $C_9H_{16}N_2O_5S \cdot 2H_2O$ : C, 35.99; H, 6.71; N, 9.33; S, 10.68;  $H_2O$ , 12.00. Found: C, 35.85; H, 6.78; N, 9.14; S, 10.68;  $H_2O$ , 11.59. Removal of the water of crystallization required prolonged drying at 70°C. with a dry air stream at approximately 5 mm. pressure.

*Comparison of the Natural and Synthetic Peptides*

The natural peptide chromatographed on the Dowex 1 (acetate) column has the same effluent volume as that of the synthetic compound.

Infrared curves were made of the natural compound and the synthetic preparation of  $\gamma$ -L-glutamyl-S-methyl-L-cysteine, both as the free peptide and sodium salt. The curves for the sodium salts were identical. The curves of the free synthetic and natural (from the first isolation) peptides differed only in the appearance of a small characteristic sulfoxide peak at  $1020\text{ cm.}^{-1}$  on the isolated peptide curve. The sulfoxide component was estimated from the absorbancies to be approximately 30% of the sample. This was corroborated by quantitative paper chromatography (28.5%). That oxidation of the isolated compound had occurred subsequent to the isolation (and some time before or during sodium removal) was clear from the fact that the sulfoxide peak was absent from the original infrared curve of the sodium salt before opportunity had been provided for autoxidation. The compound from the second isolation was found to be identical with synthetic  $\gamma$ -L-glutamyl-S-methyl-L-cysteine by matching infrared curves (Fig. 1) using the KBr disk technique. Moreover, the crystalline natural and synthetic peptides yielded matching x-ray diffraction patterns.

The isolated and synthetic compounds were found to be indistinguishable from each other on paper chromatograms employing water-saturated phenol ( $R_f$  0.47) and *n*-butanol-acetic acid-water (9:1:2.5) ( $R_f$  0.18 based on 0.27 for *S*-methylcysteine). The two peptides had very similar diffuse decomposition points and optical rotations  $[\alpha]_D^{23} -19.6^\circ$  (water,  $c = 2.20$ ) for the synthetic and  $[\alpha]_D^{23} -20.5^\circ$  (water,  $c = 2.22$ ) for the isolate.

Sachs and Brand (19) have shown that  $\gamma$ -glutamyl dipeptides with a free amino and carboxyl group yield 2 moles  $\text{NH}_2\text{-N}$  per mole of compound

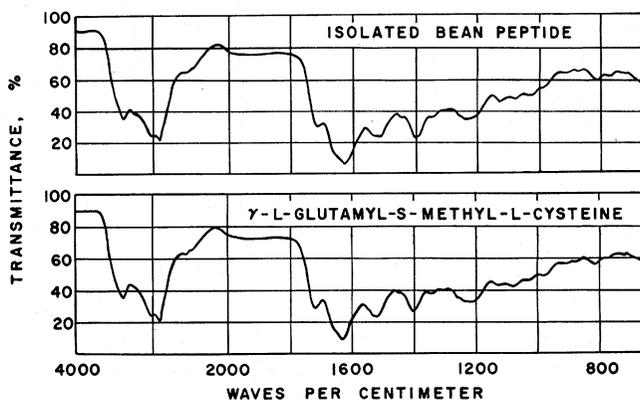


FIG. 1. Infrared curves of synthetic  $\gamma$ -L-glutamyl-S-methyl-L-cysteine and the isolated peptide.

TABLE I  
*Carboxyl and Amino Nitrogen as Related to Total Nitrogen*

Compound	% total-N	% CO <sub>2</sub> H-N	$\frac{\text{CO}_2\text{H-N}}{\text{total N}}$	% NH <sub>2</sub> -N	$\frac{\text{NH}_2\text{-N}}{\text{total N}}$
L-Leucine	10.68 <sup>a</sup>	10.25	0.96	10.65	1.0
Glutathione	13.68 <sup>a</sup>	4.55	0.33	9.98	0.73
S-Methylglutathione	13.08 <sup>a</sup>	4.25	0.33	—	—
$\gamma$ -L-Glutamyl-S-methyl-L-cysteine	10.40 <sup>b</sup>	4.50	0.44	9.10	0.88
Isolated bean peptide	10.32 <sup>b</sup>	4.94	0.48	9.05	0.88
Alanylmethionine	12.71 <sup>b</sup>	0.024	0.002	12.81	1.01

<sup>a</sup> Theoretical value.

<sup>b</sup> Determined value.

TABLE II  
*Comparison of Carboxyl and Amino Nitrogen*

Compound	Moles/mole of compound	
	CO <sub>2</sub> H-N	NH <sub>2</sub> -N
L-Leucine	0.96	1.0
Glutathione	1.0	2.2
S-Methylglutathione	0.98	—
$\gamma$ -L-Glutamyl-S-methyl-L-cysteine	0.88	1.8
Isolated bean peptide <sup>a</sup>	0.95	1.8
Alanylmethionine	0.0	1.0

<sup>a</sup> Assuming the compound is  $\gamma$ -glutamyl-S-methylcysteine.

by the Van Slyke nitrous acid method. By comparison,  $\alpha$ -linked glutamyl-peptides produce 1 mole NH<sub>2</sub>-N per mole compound. Both the natural and synthetic peptides were found to have a ratio of amino nitrogen to total nitrogen of 0.88 (Table I) or yield 1.8 moles of Van Slyke nitrogen based on the molecular structure of  $\gamma$ -glutamyl-S-methylcysteine (Table II). In addition, when the ninhydrin method of Van Slyke *et al.* (20) was employed, the isolated peptide yielded a ratio of carboxyl nitrogen to total nitrogen of 0.48 (Table I). This is 95% of the theoretical carboxyl nitrogen for  $\gamma$ -glutamyl-S-methylcysteine (Table II), thereby establishing the  $\gamma$ -glutamyl linkage.

Furthermore, the ratio (0.48) of carboxyl nitrogen to the total nitrogen established the fact that the compound is a dipeptide inasmuch as only one structure could be conceived involving a mole each of glutamic acid and S-methylcysteine to provide this ratio.

Moreover, a comparison of the infrared curves of the isolated peptide,

synthetic  $\gamma$ -L-glutamyl-S-methyl-L-cysteine, and S-methylglutathione reveals that the following ratio:

$$\frac{\text{absorbance at } \sim 1525 \text{ cm.}^{-1}}{\text{absorbance at } \sim 1450 \text{ cm.}^{-1}}$$

is greater for the tripeptide (by approximately 50%) than for the dipeptide. This ratio should increase with the ratio of the number of peptide groups per  $\text{COO}^-$  group. The isolated peptide closely resembles the synthetic dipeptide in this ratio, and the tripeptide is distinctly different. The above ratio would be even greater for a peptide of four or more amino acid components.

*Synthesis of  $\gamma$ -L-Glutamyl-S-methyl-L-cysteine Sulfoxide  
and Oxidation of the Isolated Peptide*

Twenty milligrams of synthetic  $\gamma$ -L-glutamyl-S-methyl-L-cysteine was dissolved in 0.1 ml. water, and 0.1 ml. of 3%  $\text{H}_2\text{O}_2$  was added. After mixing the reactants with a vibrator, the solution was allowed to stand at room temperature for 24 hr., and the liquid was removed with a gentle stream of air. The white solid was dried at  $65^\circ\text{C}$ ., and an infrared curve was prepared using the KBr disk technique. Twenty-two milligrams of the isolated compound was peroxidized in the same manner, and an infrared curve was made of the resulting product. The oxidized isolated compound was found to be identical with  $\gamma$ -L-glutamyl-S-methyl-L-cysteine sulfoxide by matching infrared curves (Fig. 2). The two sulfoxides yielded ninhydrin-positive spots with identical  $R_f$  values of 0.39 and 0.04 in the above-described phenol and acid butanol solvents, respectively.

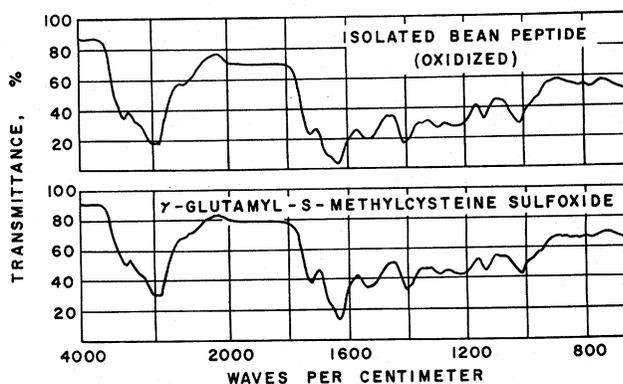


FIG. 2. Infrared curves of  $\gamma$ -L-glutamyl-S-methyl-L-cysteine sulfoxide and the isolated peptide oxidized.

## $\gamma$ -L-GLUTAMYL-S-METHYL-L-CYSTEINE

*Anal.* Calcd. for  $C_9H_{16}N_2O_6S$ : C, 38.56; H, 5.75; N, 10.00. Found for the synthetic: C, 38.31; H, 5.56; for the isolate: C, 38.46; H, 5.62; N, 9.97.

### *$\gamma$ -Glutamyl-S-methylcysteine Sulfoxide in the Bean*

Paper chromatograms of the nonprotein nitrogen fractions of snap and red kidney bean seeds indicate the presence of small amounts of the sulfoxide of  $\gamma$ -glutamyl-S-methylcysteine.

### DISCUSSION

Uncombined *S*-methylcysteine had earlier been observed to be easily oxidized (1) as is methionine, particularly under acid conditions. Evidence has now been presented which indicates that  $\gamma$ -L-glutamyl-S-methyl-L-cysteine is also somewhat easily oxidized by atmospheric oxygen. Since the dipeptide is rather deliquescent and of an acidic nature, conditions permitting a combination of these properties would likely promote autoxidation of the compound to  $\gamma$ -L-glutamyl-S-methyl-L-cysteine sulfoxide. This could explain the 28.5% conversion of the first isolated dipeptide to its sulfoxide which occurred on prolonged storage.

Paper chromatograms of the nonprotein nitrogen fraction of the bean showed the presence of small amounts of  $\gamma$ -glutamyl-S-methylcysteine sulfoxide. However, the sulfoxide form of the dipeptide may not be indigenous to the bean seed but an artifact arising on extraction or during the chromatographic separation of the nitrogenous constituents employing the acid-butanol system. This possibility is supported by the fact that pure (as determined by infrared) synthetic  $\gamma$ -L-glutamyl-S-methyl-L-cysteine yielded almost 10% of the peptide sulfoxide on the papergram with this same solvent system. Moreover, the presence of free *S*-methyl-L-cysteine (3) and the absence of its sulfoxide in the same bean seed would suggest the lack of an active oxidative system for the former compound and presumably its combined form.

$\gamma$ -L-Glutamyl-S-methyl-L-cysteine accounts for a relatively large proportion (34%) of the nonprotein amino nitrogen of the mature red kidney bean seed; it is found in similar quantities in the mature snap bean (var. tendergreen) seed, and its presence may well be expected in other leguminous seeds. Recently this dipeptide has been reported isolated from lima beans (21).

The high concentration of  $\gamma$ -glutamyl-S-methylcysteine in the seed and its structural relationship to an established precursor of glutathione suggest a possible significant role for the compound in seed metabolism. Demethylation of the dipeptide would produce  $\gamma$ -glutamylcysteine. The enzymic formation of the latter compound from glutamic acid and cysteine in wheat germ (22) and bean extracts (23) has been demonstrated. An

enzyme from hog liver (24) has also been found to catalyze the formation of  $\gamma$ -glutamylcysteine from its amino acid components. Furthermore, an enzyme has been isolated from wheat germ (25), pigeon liver (26), and yeast (27) which catalyzes the condensation of  $\gamma$ -glutamylcysteine and glycine to form glutathione.

It is also interesting to note that Price (28) has reported a new uncharacterized sulfhydryl compound present as the major nonprotein thiol in the vegetative parts of *Phaseolus vulgaris* and certain other legumes. When present, glutathione occurs at a very much lower concentration.

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#### SUMMARY

A dipeptide of the newly reported sulfur-containing amino acid, *S*-methyl-L-cysteine has been found in the nonprotein nitrogen fraction of kidney bean seeds.

The compound was isolated and purified by two methods involving ion-exchange chromatography, ion exclusion, and partition chromatography on a paper roll.

On characterization, the compound was conclusively shown to be  $\gamma$ -L-glutamyl-*S*-methyl-L-cysteine. The structure of the new dipeptide was confirmed by synthesis from glutathione. *S*-Methylated glutathione was treated with carboxypeptidase whereby the glycine moiety was preferentially removed.

$\gamma$ -L-Glutamyl-*S*-methyl-L-cysteine represents a substantial part of the nonprotein nitrogen fraction of the mature bean seed.

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