ENZYMATIC SYNTHESIS OF GAMMA-GLUTAMYL-CYSTEINE IN HIGHER PLANTS[1]

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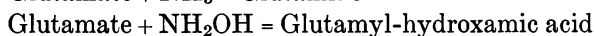
It has been demonstrated that enzyme preparations from higher plants catalyze the following syntheses in the presence of adenosine triphosphate (ATP) and magnesium ions (**2**, **8**):

Glutamate + NH_3 = Glutamine



Glutamate + NH_2OH = Glutamyl-hydroxamic acid

It therefore seemed possible that the synthesis of dipeptides involving the glutamyl moiety might occur by an analogous reaction. The glutamyl peptide, gamma-glutamyl-cysteinyl-glycine (glutathione), is known to occur generally in plants. The enzymatic synthesis of gamma-glutamyl-cysteine has therefore been first investigated since this compound constitutes a possible intermediate in the synthesis of glutathione. JOHNSTON and BLOCH (**4**) have already demonstrated a necessity of ATP for glutathione formation by liver preparations, and SNOKE and ROTHMAN (**7**) have reported the condensation of gamma-glutamyl-cysteine and glycine to form glutathione in the same system.

The enzyme system used in this work consisted of dialyzed extracts of acetone powders of bean (*Phaseolus vulgaris*) seedlings prepared as previously described (**8**). The enzyme was incubated for 60 minutes at 25° C in a reaction mixture consisting of: 0.05 M phosphate buffer (pH 7.0), 0.01 M glutamic acid-2-C^{14} (specific activity—0.1 mc./mM), 0.01 M cysteine, 0.002 M ATP, 0.005 M $MgSO_4$, and 0.005 M KCl in a total volume of 5 ml. The reaction was stopped by the addition of cold acetone, and the precipitated protein removed by centrifugation at 5000 × g. The supernatant solution was evaporated to dryness under a vacuum, and the residue was taken up in a small quantity of a solution of *N*-ethyl maleimide as described by HANES *et al.* (**3**). The components present in the solution were next separated by paper chromatography (**8**). Best separation was obtained with a butanol-acetic acid-water mixture (4 : 1 : 1) as the solvent. The presence of radioactive material on the chromatogram was indicated by radioautography (**1**), while non-radioactive amino acids and peptides were detected with ninhydrin. Table I gives R_f values obtained in a typical experiment. It is evident that a radioactive substance has been formed enzymatically which has an R_f identical to that of authentic gamma-glutamyl-cysteine prepared synthetically as described elsewhere (**9**). Further-

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TABLE I

RESULTS OF CHROMATOGRAPHIC SEPARATION OF THE COMPONENTS INVOLVED IN THE SYNTHESIS AND IDENTIFICATION OF GAMMA-GLUTAMYL-CYSTEINE.

Component	R_f			
	0.05	0.33	0.45	0.53
Cystine alone	N			
Glutamate alone		N		
Cysteine alone				N
Gamma-glutamyl-cysteine alone			N	
Glutamate-C^{14} + cysteine + boiled enzyme preparation		R		N
Glutamate-C^{14} + cysteine + active enzyme preparation		R	R	N
Products of acid hydrolysis of enzymatically formed peptide	N	R		
Products of acid hydrolysis of authentic gamma-glutamyl-cysteine	N	N		

All spots on the chromatogram corresponded to the above four R_f values. Values given for cysteine refer to its ninhydrin-reactive derivative with *N*-ethyl maleimide. Non-radioactive spots indicated by N; radioactive spots indicated by R.

more, when a portion of the reaction mixture was subjected to acid hydrolysis before chromatography, only glutamic acid and cystine were recovered (table I). Hydrolysis of a sample of authentic gamma-glutamyl-cysteine gave the same result. That the substance formed was glutamyl-cysteine rather than cysteinyl-glutamate, was indicated by treating a sample of the peptide (isolated by precipitation as the cadmium salt as described elsewhere (**9**)) with 2,4-dinitrofluorobenzene (**5**), and subjecting the resulting *N*-dinitrophenylpeptide to acid hydrolysis. Chromatography of the products of hydrolysis with tertiary amyl alcohol showed only *N*-dinitrophenylglutamate to be present, thus demonstrating that the amino position of the glutamyl residue is free in the original peptide. That the linkage was gamma, rather than alpha, was indicated by its ease of hydrolysis in dilute acid. It may, therefore, be concluded that the product of the enzymatic reaction studied here is gamma-glutamyl-cysteine.

A preliminary examination was made of the nature of the synthetic reaction. Quantitative estimation of the formation of glutamyl-cysteine

TABLE II

SYNTHESIS OF GAMMA-GLUTAMYL-CYSTEINE BY EXTRACTS OF ACETONE POWDERS OF BEAN SEEDLINGS.

System	Glutamyl-cysteine formed (µg./ml. enzyme)
Complete	141
No glutamate	4
No cysteine	5
No ATP	9
No ATP–adenosine monophosphate	10
No magnesium ion	17
No potassium ion	65
Plus coenzyme A	143

was made by radioactive assay (**9**). No evidence for a glutamyl exchange reaction (**9**) was found during the formation of glutamyl-cysteine. The data of table II indicate the degree to which the various components of the reaction system are essential to the progress of the reaction. It is evident that the presence of ATP and magnesium ions is necessary for the formation of the peptide, and ATP cannot be replaced by adenosine monophosphate. The presence of potassium ion also appears to be necessary for maximal activity of the enzyme system. That an absolute requirement for metal ions is not shown is probably due to our inability to completely free the enzyme protein of metals by dialysis. The addition of coenzyme A did not influence the course of the reaction under the present conditions, a characteristic also found for glutamine synthesis by plant systems (**8**). It appears probable then, that the formation of glutamyl-cysteine proceeds in a manner similar to the synthesis of glutamine as previously reported (**2, 8**).

As this investigation was being completed, SNOKE and BLOCH (**6**) reported a very similar enzymatic synthesis of gamma-glutamyl-cysteine by preparations from mammalian liver. It appears, therefore, that the formation of this peptide may be a feature common to various organisms.

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