

Stability of Glutamic Acid, Glutamine, and γ -Glutamyl Derivatives during Isolation from Natural Sources

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^{14}C -Labelled glutamic acid, glutamine, alanine, 5-oxoproline, and γ -glutamylalanine (in different experiments labelled in the different amino acid moieties) have been subjected to ion-exchange fractionations either in mixtures with pure amino acids or in mixture with an extract from seeds of *Fagus sylvatica* containing large amounts of γ -glutamyl derivatives. Limited degradation of glutamine into glutamic acid and 5-oxoproline was observed. When glutamic acid is applied in aqueous ethanol to a strongly acidic ion-exchange resin in the hydrogen form with subsequent elution with ammonia, partial transformation *via* the γ -ethyl ester to 5-oxoproline takes place. No production of γ -glutamyl derivatives by ring-opening of 5-oxoproline or by transpeptidation takes place. The results prove that γ -glutamyl derivatives cannot be produced chemically as artefacts during standard procedures for isolation of amino acids from natural material.

The transformation of glutamic acid into 5-oxoproline (pyroglutamic acid, 2-pyrrolidone-5-carboxylic acid) by heating in aqueous solution¹ may be responsible for the losses of glutamic acid on adsorption to strongly acidic ion-exchange resins in the H^+ -form with subsequent elution with aqueous ammonia. However, the greatest losses (up to 60 %) occur when the amino acid mixture is applied to the resin in aqueous ethanol, presumably involving the half ester as an intermediate.²⁻⁵

The amide bond in glutamine is much more labile towards acid hydrolysis than normal peptide bonds¹ and therefore substantial amounts of glutamine can be transformed into

glutamic acid in acid solution at room temperature. In neutral solution glutamine can be transformed into 5-oxoproline by gentle heating.^{1,6} Correspondingly, substantial losses of glutamine on passage through a strongly acidic ion-exchange resin have been reported.^{2,8}

Numerous γ -glutamyl derivatives of amines and amino acids and a number of tripeptides with a γ -glutamyl linkage are known from nature, especially from higher plants and mushrooms.⁷ These compounds are similar in properties to glutamine, undergoing easy acid hydrolysis and ring formation in neutral solutions. The rate constant for acid hydrolysis of the γ -glutamyl bond is about four times larger than the rate constant for acid hydrolysis of α -glutamyl bonds.⁸ The transformation of γ -glutamyl dipeptides into 5-oxoproline and the free second amino acid has been shown in many instances.⁹

On the other hand, it is known that the ring in 5-oxoproline can be opened by nucleophilic attack with amines to give γ -glutamyl derivatives.^{10,11} Furthermore, glutamic acid γ -ethyl ester can react with ammonia to give glutamine although ring formation to give 5-oxoproline is predominant.¹²

γ -Glutamyl derivatives in plants occur in widely varying concentrations. Thus, from 2.3 kg of *Fagus sylvatica* seeds we isolated 3 g of γ -glutamylphenylalanine but only 0.5 mg of γ -glutamylalanine.¹³ Furthermore, a few tripeptides with unusual structures have been isolated from plants, including γ -glutamylphenylalanylwillardiine¹⁴ and γ -glutamyl- γ -glutamylphenylalanine.¹⁵

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Table 1. Passage of ^{14}C -labelled compounds in artificial amino acid mixtures through ion-exchange resins. % of applied amount of radioactivity and compounds in different fractions.

^{14}C -Labelled compound ^a	Strongly acidic resin in H^+ -form			Strongly basic resin in AcO^- -form		
	1. Amino acid in H_2O		2. Amino acid in 80 % EtOH - H_2O	1. Amino acid in H_2O		2M AcOH eluate
	Effluate + washing water	NH_3 -eluate	Effluate	Washing water	Effluate + washing water	2M AcOH eluate
Glutamic acid (1)	1.9	97 (1)	1.7	0.1	1.9	96
					98	0.9
					87 (1)	
Alanine (2)	0.5	100 (2)	0.5	0.1	99 (2)	0.5
5-Oxoproline (3)	104	0.3	104	0.01	0.5	0.1
			97 (3)		99 (2)	0.2
			7 (6) ^b		0.3	99 (3)
			3.6 (3) ^c		97 (4)	2.6 (3) ^c
Glutamine (4)	5 (3) ^c	95		1.2	95	
		87 (4)			87 (4)	
		4 (1)			3 (1)	
		4 (3)			5 (3)	
γ -Glutamylalanine (5) ^d	0.5	99 (5) ^c	0.3	0.2	0.4	95 (5)
γ -Glutamylalanine (5) ^e	0.3	98 (5) ^c	0.2	0.6	1.0	98 (5)
					97 (5) ^c	1.4
					97 (5) ^c	0.8

^a In mixture with 1, 2, 3, 4 and in the last two cases with the acidic resins and in all cases with basic resin 5. ^b 5-Oxoproline ethyl ester. ^c And minor spots. ^d Labelled in glutamic acid moiety. ^e Labelled in alanine moiety.

In isolation of γ -glutamyl derivatives from natural material losses may occur because of degradation. γ -Glutamyl derivatives might also be produced at least in small amounts as artefacts by reaction of amines or amino acids with 5-oxoproline of glutamic acid γ -ethyl esters, especially under the ill-defined conditions on ion-exchange resins. The tripeptides might be formed by chemical transpeptidations although a number of experiments have been performed to exclude this possibility.¹⁴ To disprove or establish these possibilities recovery and stability experiments have been undertaken with ¹⁴C-labelled glutamic acid, glutamine, 5-oxoproline, and γ -glutamylalanine labelled either in the glutamic acid moiety or in the alanine moiety. The syntheses of the labelled dipeptides are described.

RESULTS

A mixture of 5-oxo-L-proline, glycine, and aqueous ammonia was heated to 65°C. Samples were withdrawn at intervals and inspected by paper chromatography (PC) and high voltage paper electrophoresis (HVE) at pH 3.6.¹⁵ No traces of γ -glutamylglycine or glutamic acid could be found after 60 min of reaction. Trace amounts of glutamic acid could be identified after 90 min of reaction. Trace amounts of γ -glutamylglycine may have been formed after 180 min of reaction but the amounts were too small to permit identification.

A mixture of 5-oxo-L-proline and 2-aminoethanol in water was heated to 120°C. Trace amounts of glutamic acid and of *N*⁵-(2-hydroxyethyl)glutamine after 1 h were observed by PC and HVE. Previously the production of *N*⁵-(2-hydroxyethyl)glutamine by reaction between 5-oxoproline and 2-aminoethanol at 37°C for several weeks has been reported.¹¹

In Table 1 are recorded the results obtained by passage of labelled compounds in artificial mixtures through acidic and basic ion-exchange resins. The amount of radioactivity in the various fractions was measured by liquid scintillation counting. Furthermore, the fractions were subjected to paper chromatography followed by scanning with a flow-counter and by autoradiography. The last method is very sensitive and in a number of cases trace amounts of unidentified compounds were observed. However, none of these compounds were γ -glutamyl derivatives.

In Table 2 are recorded the results obtained by passage through ion-exchange resins of labelled compounds in an extract from seeds of *Fagus sylvatica*. These seeds contain high concentrations of γ -glutamyl peptides, especially γ -glutamylphenylalanine, and of various amino acids.¹³ Again, the various fractions were investigated by paper chromatography and autoradiography. No labelled γ -glutamyl peptides have been formed in any of the experiments. The recoveries reported in Table 2 are in most experiments quite different from 100%. How-

Table 2. Passage of ¹⁴C-labelled compounds in extracts from seeds of *Fagus sylvatica* + γ -glutamylalanine through strongly acidic and subsequently strongly basic ion-exchange resins. % of applied amount of radioactivity and compounds found in different fractions.^{a,b}

¹⁴ C-labelled compound	Strongly acidic resin in H ⁺ -form		Strongly basic resin in AcO ⁻ -form		
	Effluete + washing water	NH ₃ -eluate	Effluete + washing water	0.2 M AcOH eluate	2 M AcOH eluate
Glutamic acid (1)	1.9	104	2.4 (1) ^c	92 (1)	0.4
Alanine (2)	0.4	122	96	0.4	0.1
5-Oxoproline (3)	109 (3)	8.5 (3)	2.4	0.7	87 (3)
Glutamine (4)	7	107 ^d	88 (4) ^e	4 (1)	6 (3)
γ -Glutamylalanine (5) ^f	0.8	104 (5) ^e	23 (5) ^c	71 (5)	1.7
γ -Glutamylalanine (5) ^g	0.5	109	16 (5) ^c	80 (5)	0.6

^a Determination of counting efficiencies is difficult because of coloured solutions. ^b The percentage for passage through the basic resin is calculated on the basis of the amount of radioactivity applied to the basic resin, not on basis of the total amount of radioactivity. ^c Overflow. ^d Several compounds. ^e And minor spots. ^f Labelled in glutamic acid moiety. ^g Labelled in alanine moiety.

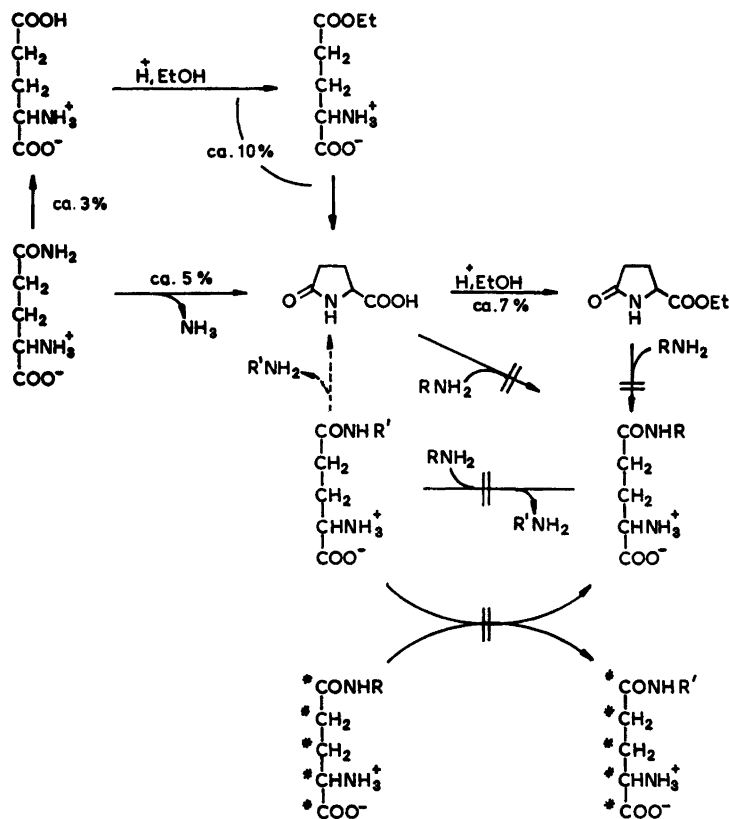


Fig. 1. Transformations of glutamic acid, glutamine, 5-oxoproline, and γ -glutamyl derivatives during standard isolation procedures for amino acids from natural material.

ever, measurement of counting efficiency in the solutions slightly coloured from the seeds is not precise. It is evident that the basic ion-exchange resin has been overloaded in some of the experiments. It is also apparent from the table that 5-oxoproline to a certain degree is adsorbed to the resin and only eluted with NH_3 . This is in contrast to the behaviour in the mixture of pure compounds.

DISCUSSION

Fig. 1 contains a summary of the various reactions taking place or excluded during standard procedures for isolation of amino acids from natural material.

When glutamic acid is applied in aqueous ethanol into an acidic resin with subsequent

washing with water and elution with aqueous ammonia a substantial fraction is found in the ammonia eluate as 5-oxoproline. This must be a two-step reaction involving the γ -ethyl ester because no transformation takes place when glutamic acid is applied in aqueous solution. This result is in agreement with previous investigations although the values for percentage of transformation differ in the various reports.²⁻⁵ Complete recovery of glutamic acid can, however, be obtained when the combination of a strongly acidic ion-exchange resin in the H^+ -form and aqueous ethanol is avoided.

Again, 5-oxoproline can be partly transformed to the ethyl ester by the combination of a strongly acidic ion-exchange resin and aqueous ethanol. 5-Oxoproline may also be found in an amino acid fraction due to adsorp-

tion to resins but does not give rise to any γ -glutamyl derivatives or other artefacts under the various procedures.

Glutamine undergoes partial decomposition to glutamic acid and 5-oxoproline on the acidic resin both with and without ethanol in agreement with previous reports.^{2,3} 5-Oxoproline identified in the experiment with the basic resin is probably due to an impurity in the sample of glutamine used (cf. Experimental).

No degradation of γ -glutamylalanine has taken place under the varying treatments.

The results demonstrate that γ -glutamyl derivatives cannot be formed as artefacts during normal isolation procedures. This conclusion is especially justified by the high sensitivity of the autoradiography used. A hypothetical route from glutamic acid or glutamine to 5-oxoproline followed by ring opening by nucleophilic attack with the amino group in an amino acid or amine is ruled out. Chemical transpeptidation is ruled out by the experiments with labelled alanine and γ -glutamylalanine. On the other hand, the results indicate that the combination of ethanol and acidic resin must be avoided. Losses of glutamine are inevitable but can be reduced to insignificant amounts by use of low temperature³ and fast procedures. Again, decomposition of γ -glutamyl derivatives must in principle occur but at a slower rate than for glutamine and insignificant even at room temperature provided the operations are performed reasonably fast

EXPERIMENTAL

Radioactive compounds. [^{14}C]-L-Glutamic acid, [^{14}C]-L-glutamine, and [^{14}C]-L-alanine were purchased from Amersham. The specific activities were 270, 40 and 164 $\mu\text{Ci}/\mu\text{mol}$, respectively. [^{14}C]5-Oxo-L-proline was purchased from New England Nuclear with a specific activity of 260 $\mu\text{Ci}/\mu\text{mol}$. Purity was checked by PC followed by scanning and autoradiography. The sample of glutamine contained a significant amount of 5-oxoproline, a contamination which probably is responsible for the observation of this compound in the experiment with passage of glutamine through a strongly basic resin (cf. Discussion). Other unidentified radioactive impurities were present but in amounts less than 1% of the main compound.

γ -[^{14}C]-L-Glutamyl-L-alanine. *N*-Benzyloxy-carbonyl-[^{14}C]-L-glutamic anhydride (0.14

mmol, 7×10^7 dpm) derived from [^{14}C]-L-glutamic acid by the traditional method¹⁶ was condensed with L-alanine (0.17 mmol) in the presence of triethylamine (0.45 mmol) in H_2O (70 μl) for 20 min at room temperature. The protective group was removed by hydrogenation with palladium black and the two isomeric glutamyl derivatives formed were separated and isolated by adsorption to a strongly acidic ion-exchange resin in the H^+ -form (Dowex 50), elution with aqueous ammonia, concentration to dryness, solution in H_2O , adsorption to a strongly basic ion-exchange resin in the AcO^- -form (Dowex 1 \times 8, 1 ml), and elution with 0.2 M AcOH. Fractions of 1 ml were collected and γ -glutamylalanine was found in fractions 8–14. Concentration provided a crystalline residue (0.04 mmol, 2×10^7 dpm), pure according to PC and autoradiography.

γ -L-Glutamyl-[^{14}C]-L-alanine. This compound was produced in a similar way using [^{14}C]-L-alanine (0.15 mmol, 100 μCi). The product (0.05 mmol, 7×10^7 dpm) was pure according to PC and autoradiography.

Ion-exchange fractionation procedures

1. *Passage through a strongly acidic resin, application in aqueous ethanol* The radioactive compound (ca. 1×10^6 dpm) together with L-alanine (4.5 mg), L-glutamic acid (4.7 mg), L-glutamine (8.6 mg), and 5-oxo-L-proline (4.4 mg) was dissolved in 80% EtOH (5 ml) and applied to a Dowex 50 (H^+) column (5 ml). The column was washed with 80% EtOH (20 ml) and H_2O (30 ml) and eluted with 2 M NH_3 .aq. (50 ml). The flow rate was 20 ml/h. In the experiments with labelled γ -glutamylalanine also 1.9 mg of unlabelled γ -glutamylalanine was added. After counting the different fractions were taken into dryness and subjected to PC.

2. *Passage through a strongly acidic resin, application in H_2O* The experiments were performed as under 1 with the exception that the amino acids were dissolved in H_2O (1 ml) and that the column was washed with H_2O (50 ml).

3. *Passage through a strongly basic resin* The same mixture as under 1, however, always with γ -glutamylalanine, dissolved in H_2O (1 ml) was applied to a Dowex 1 \times 8 (AcO^-) column (2 ml). The column was washed with water (20 ml), and eluted with 0.2 M AcOH (20 ml), and 2 M AcOH (20 ml).

4. *Fractionation of a natural extract* The labelled compound (again ca. 1×10^6 dpm) was added to an 80% ethanolic extract of seeds of *Fagus sylvatica* (10 ml, corresponding to 4 g seeds and about 60 mg amino acids and peptides). After concentration of the mixture the residue was dissolved in H_2O (1 ml) and subjected to the procedure described under 2.

The NH_3 -eluate was evaporated to dryness, dissolved in H_2O and subjected to the procedure described under 3.

Counting, PC, autoradiography. Radioactivity in solution was determined by liquid scintillation counting using a Packard 3320 instrument and Turner's solution.¹⁷ Counting efficiency was determined by use of an external standard. PC was performed on Whatman No. 1 in $\text{BuOH}-\text{AcOH}-\text{H}_2\text{O}$ (12:3:5). Radioactivity on paper chromatograms was measured by use of a Berthold Dünnschicht Scanner II. Autoradiography was performed exposing the paper chromatograms to Kodax X-ray films for weeks or months.

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