# ENZYMIC SYNTHESIS OF L-GLUTAMIC ACID–$^{15}$N AND 4-AMINO-BUTYRIC ACID–$^{15}$N AND THE PREPARATION OF L-PYROGLUTAMIC ACID–$^{15}$N

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**Received on March 28, 1975.**

SUMMARY

*The production of L-glutamic acid-$^{15}$N in good yield by direct enzymic synthesis from 2-oxoglutaric acid, ammonium chloride-$^{15}$N (95 atom % $^{15}$N) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) is described. The NADPH is continuously regenerated by coupling the first reaction to the oxidation of glucose-6-phosphate, which serves to drive the synthesis of L-glutamic acid to completion.*

*4-Aminobutyric acid-$^{15}$N is produced by incubation of L-glutamic acid-$^{15}$N with glutamic acid decarboxylase. $CO_2$ is lost from the reaction mixture and the conversion of glutamic acid to 4-aminobutyric acid proceeds to completion.*

*L-Pyroglutamic acid-$^{15}$N is produced by heating aqueous solutions of L-glutamic acid-$^{15}$N at 135$^{o}$ for 3 hours.*

## INTRODUCTION

L-Glutamic acid, produced by amination of 2-oxoglutaric acid, is the primary product of ammonia assimilation and the major source of α-amino nitrogen for other amino acids in many bacteria(1), fungi(2), plants(3), and mammals(4). Because of the potential application of L-glutamic acid-$^{15}$N in studies of amino acid metabolism we have designed an efficient enzymic synthesis of this compound. The synthesis resembles that of Pitts et al.(5) but whereas they use rat liver mitochondria as a source of enzymes we use only materials which are commercially available.

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L-Pyroglutamic acid (5-oxopyrrolidine-2-carboxylic acid) and 4-aminobutyric acid are two products of L-glutamic acid metabolism which have potential application in metabolic studies. We have therefore investigated the methods of synthesising these compounds and report procedures which are suitable for use with L-glutamic acid-$^{15}$N. The enzymic synthesis adopted for production of 4-aminobutyric acid is similar to that of Camien et al.[6] except that we use a commercial source of enzyme and prefer to use an ion-exchange technique for isolating the product. L-Pyroglutamic acid may be produced by heating L-glutamic acid in aqueous solution under pressure[7]. This method is suitable for synthesis using isotopic material.

## EXPERIMENTAL

Analytical techniques. Gas chromatographic analysis was performed with a Pye 104 temperature programmed FID gas chromatograph using a 1.5m x 4mm I.D. column containing 5% OV1 on 100-120 mesh Gas-Chrom Q. Amino acids were chromatographed both as trimethyl silyl and as N-dimethyl-aminomethylene methyl ester derivatives.

Optical rotation was measured with a Perkin-Elmer 141 polarimeter using a 10cm light path.

A VG Micromass 12B mass spectrometer interfaced via a glass jet separator to a Varian gas chromatograph was used to confirm isotopic composition. The ionizing current was 100μA, the electron energy was 25eV, the accelerating voltage was 2.5KV and the ion source temperature was 240$^{\circ}$. L-Glutamic acid and L-pyroglutamic acid were converted into glutamic acid diethyl esters and 4-aminobutyric acid was converted into the N-dimethylaminomethylene methyl ester.

Preparation of L-glutamic acid-$^{15}$N hydrochloride. In a 1 l. beaker were mixed 500 ml of 0.1M potassium phosphate buffer, pH 7.6 and 43 millimoles 2-oxoglutaric acid (Sigma, K salt) and the solution adjusted to pH 7.6 with 5M KOH. To the solution were then added 43 millimoles glucose-6-phosphate (Sigma, Na salt), 33 millimoles ammonium chloride-$^{15}$N (95 atom % $^{15}$N), 0.5 millimoles adenosine-5-diphosphate (Sigma, Na salt) and 70 micromoles NADP (Sigma, Na salt). Adenosine-5-diphosphate was added to stabilize the glutamic dehydrogenase subsequently added[8]. The mixture was adjusted to pH 7.6 with HCl or KOH as required and the total volume made up to 800 ml with 0.1M potassium phosphate buffer pH 7.6. Portions of the reaction mixture (200 ml) were placed in 200 ml volumetric flasks, and to each flask was added 100 enzyme units (I.U.) of glucose-6-phosphate dehydrogenase (Sigma type 15, bakers yeast, lyophilized) and 600 enzyme units (I.U.) of glutamic dehydrogenase (Sigma, bovine liver, type 2, free from ammonium ions). The four flasks were stoppered and incubated overnight (18 hrs) at 25$^{o}$ during which time all the nitrogen$^{-15}$ was incorporated into L-glutamate-$^{15}$N. After incubation, the reaction mix was boiled for 2 min and the coagulated protein removed by filtration through a sintered filter and washed with distilled water.

After cooling to room temperature the deproteinized solution (800 ml) was passed through a column of 200 g Amberlite IR-120 ion exchange resin ($H^+$ form) and the product was absorbed on the column. The glutamic acid was then eluted with M KOH, using the ninhydrin reaction to follow the elution pattern.

The eluted fractions containing the amino acid were passed through a column of 100 g Amberlite IRA-400 ion

exchange resin ($OH^-$ form), and the absorbed glutamate eluted with 0.2M HCl. The fraction containing glutamic acid (c. 250ml) was concentrated _in vacuo_ to 20-30 ml. This solution was saturated with dry HCl gas, and pure L-glutamic acid-$^{15}N$ precipitated overnight at 5$^o$ as the hydrochloride. The hydrochloride was collected by filtration, washed with cold conc. HCl and then with cold absolute ethanol. Yield 4.8g (80% of theory).$[\alpha]_D^{20^o}$ + 18.9 (c. 1 in $H_2O$). Found, C, 32.51; H, 5.61; N, 8.19; Cl, 19.19. Calc. for $C_5H_9{}^{15}NO_4HCl$ (95 atom % $^{15}N$), C, 32.45; H, 5.54; N, 8.08; Cl, 19.16. Mass spectrum m/e 130 (5), 131 (95). By gas liquid chromatography >99% pure.

_Preparation of L-glutamic acid-$^{15}N$._ The free acid was obtained by elution of the IRA-400 column described above with 0.2M acetic acid instead of 0.2M HCl. Concentration _in vacuo_ of the eluate to a small volume followed by crystallization overnight at 5$^o$ resulted in precipitation of free glutamic acid (70-80% of theory). The free acid may also be produced by neutralization of hydrochloride with NaOH, or by exchange of the $Cl^-$ on an anion exchange column ($OH^-$ form).

_Preparation of 4-aminobutyric acid-$^{15}N$._ 95 atom % of $^{15}N$ L-Glutamic acid (4.9g) was dissolved with heating and stirring in 400 ml 0.2M acetic acid/KOH buffer pH 5.0. The solution was adjusted to pH 4.7 with 5M KOH, 600 units of glutamic decarboxylase (Sigma, type 2) were added, and the solution, was shaken mechanically at 37$^o$ to aid evolution of $CO_2$ for 4 hrs. It was then boiled to coagulate enzyme protein, cooled and filtered.

The combined solutions were applied to a column of 130g Amberlite IRA-400 anion exchange resin ($OH^-$ form). The amino

acid was absorbed onto the column and subsequently eluted with 0.2M HCl. 5M HCl (20ml) was added to the fraction of the eluate containing the amino acid. The mixture was evaporated to dryness on a rotary evaporator at 60° under reduced pressure and further dried in vacuo over KOH to remove traces of free HCl. The solid was washed with 1:1 v/v mixture of absolute ethanol and ether, filtered off and dried in vacuo over calcium chloride. It was then dissolved in 50 ml of boiling absolute ethanol to which, after cooling, was added 50 ml of anhydrous ether. 4-Aminobutyric acid was crystallized overnight at 5°. The crystals were filtered off, washed with absolute ethanol/ether and dried in vacuo over calcium chloride. Yield 3.18g (68% of theory). Found, C, 34.67; H, 7.18; N, 10.65; Cl, 25.23. Calc. for $C_4H_9{}^{15}NO_2$ HCl (95 atom % $^{15}N$), C, 34.19; H, 7.17; N, 10.64; Cl, 25.23. Mass spectrum m/e 172 (4), 173 (96). By gas liquid chromatography >99% pure.

Preparation of L-pyroglutamic acid-$^{15}N$. Finely divided 95 atom % $^{15}N$ L-glutamic acid (1g), obtained by neutralization of glutamic acid hydrochloride, $[\alpha]_D^{20°}$ + 18.9 (c. 1 in $H_2O$), with NaOH, was added to each of 4 pyrex screw top tubes containing 15 ml distilled water. The tubes were sealed and heated at 135° in an oil bath for 3 hr. The solutions were then cooled and the contents of the tubes, together with rinsings, were evaporated in a rotary evaporator in vacuo to dryness. Absolute ethanol (100ml) was added to the residue in the flask, which was then rotated in a water bath for 15 min at 65° to dissolve L-pyroglutamic acid. The contents of the flask were filtered, unconverted glutamic acid being retained on the filter disc. The filtrate was concentrated in vacuo to 25 ml, 75 ml of warm

light petroleum (b.p. 40-60°) were added, and L-pyroglutamic acid crystallized overnight at 5°. The crystals were filtered off and dried over calcium chloride. Yield 2.18g (62% conversion). $[\alpha]_D^{20^\circ}$ -12.7 (c. 1 in $H_2O$). Found, C, 46.17; H, 5.54; N, 11.62. Calc. for $C_5H_7{}^{15}NO_3$ (95 atom % $^{15}N$), C, 46.17; H, 5.42; N, 11.50. Mass spectrum m/e 130 (5), 131 (95). By gas liquid chromatography $>$99% pure.

The ethanol insoluble residue (1.22g) was L-glutamic acid which contained less than 3% L-pyroglutamic acid. This was extracted with absolute ethanol and the L-glutamic acid recovered as the hydrochloride, $[\alpha]_D^{20^\circ}$ + 18.4 (c. 1 in $H_2O$).

ACKNOWLEDGEMENTS

The authors wish to thank Professor Sir Ewart Jones for providing facilities for optical rotation and the elemental analysis, Dr G. T. Young for helpful advice, Dr D. Harvey for performing Mass spectroscopic analysis and Prochem. Ltd for providing the $^{15}NH_4Cl$.

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