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^{15}N-labed glycine synthesis

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ABSTRACT

This work describes a method for ^{15}N-isotope-labeled glycine synthesis, as well as details about a recovery line for nitrogen residues. To that effect, amination of α-haloacids was performed, using carboxylic chloroacetic acid and labeled aqueous ammonia ($^{15}NH_3$). Special care was taken to avoid possible $^{15}NH_3$ losses, since its production cost is high. In that respect, although the purchase cost of the ^{13}N-labeled compound (radioactive) is lower, the stable tracer produced constitutes an important tool for N cycling studies in living organisms, also minimizing labor and environmental hazards, as well as time limitation problems in field studies. The tests were carried out with three replications, and variable $^{15}NH_{3\,(aq)}$ volumes in the reaction were used (50, 100, and 150 mL), in order to calibrate the best operational condition; glycine masses obtained were 1.7, 2, and 3.2 g, respectively. With the development of a system for $^{15}NH_3$ recovery, it was possible to recover 71, 83, and 87% of the ammonia initially used in the synthesis. With the required adaptations, the same system was used to recover methanol, and 75% of the methanol initially used in the amino acid purification process were recovered.

Key words: stable isotope, glycine, amino acid.

INTRODUCTION

Nitrogen cycle studies can be carried out with tracers consisting of radioactive (^{13}N) or stable (^{14}N/^{15}N) nitrogen isotopes. Papers involving ^{13}N applications in biology have been presented in the literature (Krohn and Mathis 1981, Cooper et al. 1985). However, the main inconvenience of using the radioisotopic technique, especially in biological researches, is represented by the time factor, since the radioisotope with the longest half-life is ^{13}N, with only 9.97 minutes (Lide 1997).

With regard to the purchase cost of these products, the price of the labeled compound is lower for the radioisotope (Hauck and Bremner 1976). However, experiments where ^{13}N is used are extremely difficult to conduct, and the results obtained may be misleading (Knowles and Blackburn 1993). When ^{15}N is used, it is possible to develop studies without time limitations, and the material being experimented is not exposed to radiation, making it unnecessary to adopt any safety measures because

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of radioactivity (Trivelin et al. 1979, 2002, Eriksen 1996, Máximo et al. 2000). In studies developed for the isotopic separation of ^{34}S, Bendassolli et al. (1997) mentioned that the use of stable isotopes is an international current trend, and is especially encouraged in field researches. This trend, together with the possibility of obtaining refined information on the nitrogen cycle, reinforce the reasons for the continued growth of use of the isotopic technique in applied researches.

Although ^{15}N is considered an important tracer in biochemical and agronomic studies, and has been used for almost 7 decades (Shoenheimer et al. 1937, Vickery et al. 1940), it is correct to make the statement that the use of ^{15}N-labeled compounds (especially highly-labeled ones) in applied researches is still limited, especially because of their high price.

Until recently, some nitrogen compounds, among them ^{15}N amino acids, were not produced in South America, due to difficulties of a methodological nature, and had to be imported from the United States of America, Europe, or Asia. The production of ammonia ($^{15}NH_3$) is crucial in obtaining a number of nitrogen compounds. The Stable Isotopes Laboratory of Centro de Energia Nuclear na Agricultura of Universidade de São Paulo (LIE-CENA/USP) dominates the method for obtaining ammonium ($^{15}NH_4^+$) ion with enrichment in the range of up to 90% ^{15}N atoms (Máximo et al. 2000).

Several nitrogen compounds can be produced from labeled ammonium, among which are: anhydrous ammonia (Bendassolli et al. 1988a); nitric acid, from ammonia-^{15}N combustion; urea (Bendassolli et al. 1988b), and ^{15}N-uran (Bendassolli et al. 1989).

A highly important nitrogen compound is glycine, prominent for being a simple amino acid, and one of the most soluble in water. In living organisms, it is frequently added to other molecules to make them more soluble, so they can be excreted in urine (Campbell 2000). This trait allows the nitrogen cycle to be elucidated in the metabolic medium. Stack et al. (1989) used ^{15}N amino acids to evaluate human nutritional status. Dichi et al. (1996), Marchini et al. (1993), and Schelp et al. (1995) conducted metabolism studies in ^{15}N-labeled proteins. These compounds have provided a safe method in such studies, eliminating invasive procedures and patient exposure to radioactivity, which makes their application extremely favorable in this area (Klein and Klein 1987).

Glycine can be synthesized by means of the Strecker, Hoffmann syntheses, and also by a modification of Gabriel's synthesis for amine, using potassium phthalimide. The latter presents high yield (85%) and allows easy purification (Morrison and Boyd 1973). Although having lower yields (in the order of 40 to 60%), amination of α-haloacids can also be used. In this reaction, a chlorinated or bromated carboxylic acid at α position is submitted to direct ammonolysis, with a great excess of concentrated ammonia solution (Morrison and Boyd 1973). Displacement of the halogen by ammonia forms the amine salt (amino acid).

In the present work, we studied labeled-glycine synthesis, using the α-haloacid amination method. The selection of this method is conditioned to the use of ammonia, the main raw material from the synthesis reaction, already produced at LIE_CENA/USP. Thus, the novelty presented by this work is the use of a previously labeled reagent (^{15}N-ammonia) in the proposed synthesis, paying special attention to the recovery of the previously labeled ammonia reagent; this is crucial due to the high added value of the compound (U$ 200 per gram of the isotope).

Within this context, as this method for obtaining ^{15}N-glycine becomes further developed, it is now possible to contribute for the advancement of research in the biological and biomedical fields, and an important tool is offered that could be used in studies of this nature.

MATERIALS AND METHODS

In the glycine synthesis reaction described by Vogel (1980), carboxylic chloroacetic acid, ammonium

carbonate, and concentrated (23–25% m/v) aqueous ammonia (excess) are used, and the reaction can be represented according to equation 1.

$$2CH_2ClCOOH + 2^{15}NH_3 + 1(NH_4)_2CO_3 \rightarrow 2CH_2NH_2COOH + 2NH_4Cl + H_2CO_3 \quad (1)$$

The proposed method was modified in relation to that indicated by Vogel (1980), notably for the exclusion of ammonium carbonate from the synthesis process, considering that the use of this compound with natural isotopic abundance (0.366% ^{15}N atoms) would cause isotopic dilution with the highly enriched aqueous ammonia (50 to 90% ^{15}N atoms), although the reaction yield would be increased to up to 62%.

The tests were carried out with three replicates, in which we varied the amount of ammonia (0.73, 1.46, and 2.20 mols), corresponding to 50, 100, and 150 mL of 25% m/v aqueous ammonia, in order to obtain the best economic and operational condition for glycine synthesis. Initially, a chloroacetic acid solution (25 g chloroacetic acid dissolved in 25 mL water) was added slowly under agitation into an adapted 250 mL volumetric flask (two inlets, with a Teflon valve attached to one of them) containing concentrated ammonia. A 1 mL aliquot was taken from the final volume containing ammonia and chloroacetic acid and the initial N content (N_o) in the reaction volume was quantified (Malavolta et al. 1997). The closed flask remained resting for a period of 24 hours at room temperature.

After the rest period, an aliquot was again taken to quantify the N present in excess in the solution (N_e). During the concentration of the solution, the excess nitrogen, in the form of aqueous ammonia, was recovered in two compartments. The first compartment consisted of traps, 9 mm external diameter and 6 mm internal diameter, inserted into three 24 / 40-ground-joint borosilicate tubes 35 cm in height, 36 mm external diameter, and 32 mm internal diameter, containing 6 mol L^{-1} sulfuric acid. The first was connected to a second compartment consisting of a MARCONI model MA-120v vacuum rotary evaporator, with an evaporation and recovery flask that operates at reduced pressure, at a temperature of 60°C. In this second compartment, ammonia traces were retained in a 6 mol L^{-1} sulfuric acid solution in the system's recovery flask. The nitrogen fraction retained in the compartments was quantified and designated as recovered nitrogen (N_r). After 40 minutes of concentration, new nitrogen determinations were made. The nitrogen present in the form of ammonium chloride (NH_4Cl), the main impurity formed during the process, was quantified in the concentrated solution. In the final step, 100 mL methanol (CH_3OH) were added to the solution to cause glycine crystallization and thus separate it from the ammonium chloride soluble in methanol. The purification procedure was repeated three times.

The methanol used for purification was recovered by fractionated distillation, using a rotary evaporator at reduced pressure at a temperature of 30°C.

In order to verify the presence of glycine, the synthesized samples were submitted to thin-layer chromatography (TLC) analysis, by reaction with ninhydrin (Vilella 1976) and high performance liquid chromatography (HPLC).

In the TLC analyses, 10 mg from each sample were weighed and solubilized in 1 mL water. Next, the samples were applied with a 10μl automatic pipettor to the silica plate, which was placed in a laboratory bowl containing a mobile phase, consisting of butanol, acetic acid, and water at a 4:1:1 rate, and developed in a 0.2% (m/v) ninhydrin solution.

For HPLC determinations, 10 mg from each sample were needed, solubilized in 1 mL purified water (18 MΩ.cm); 100 μL were taken from the final solution and added of 900μL water. Next, the sample was centrifuged and filtered (0.2μm micropore filter). A 10μL sample was taken for analysis from the final volume and mixed into 30μL o-phthaldehyde (OPA), in order to derivatize the amino acid, enabling it to be detected by HPLC. The amino acid was determined in a C18 Superpac ODS-2 4 $\times$ 250 mm reverse phase column, 0.8 ml.min^{-1} flow (Supelco), at room temperature,

having as mobile phase a phosphate buffer (Buffer A) with pH 7.25 corrected with glacial acetic acid, consisting of 50 mM NaOAc, 50 mM Na_2HPO_4, 20 mL tetrahydrofuran, 20 mL methanol, and a (Buffer B) consisting of 65% HPLC-degree methanol prepared with purified, degassed water, and vacuum-filtered through a Millipore HVPL 047 membrane. After derivatization of the amino acid, the derivatives were detected by fluorescence with a Shimatsu RF 551 fluorescence detector, adjusted for excitation (λ = 250 nm) and emission (λ = 480 nm).

The isotopic (% ^{15}N atoms) and N content (%) determinations in samples with natural isotopic abundance and in the enriched amino acid (glycine) were performed by mass spectrometry (model ANCA-SL 20-20 mass spectrometer by PDZ Europe). A solution containing 535.3 mg glycine in 10 ml (synthesized and Sigma standard) was prepared for the isotopic and N content determinations in glycine. The use of 10 μL of this solution containing 100 μg N-glycine was sufficient for good analytical precision.

RESULTS AND DISCUSSIONS

The nitrogen balance (N recovery in the process) and glycine amino acid production results (gravimetric determination) as a function of 25% (m/v) aqueous ammonia volume (50, 100, or 150 mL) are presented in Table I. The table shows data corresponding to Nitrogen recovery (N_r) in the amino acid concentration system from the excess N (N_e), glycine production, and N mass in the amino acid (N_G). The N_e was obtained as described in item 2.

Based on the data from Table I it can be determined that N losses in the global process (reaction and amino acid concentration line) were in the order of 3.6 $\pm$ 0.3 g N ($(N_o - (N_r + N_G))$). Nitrogen losses were in gas form (NH_3) and especially due to the reagent handling process, were practically independent from the amount of ammoniacal solution employed. With regard to the N_e recovery system (special line at reduced pressure) excess nitrogen, it can be determined, from the data in Table I, that N losses (%) ($(N_r/N_e).100$) were reduced, since about 94 $\pm$ 1% of the excess nitrogen (N_e) present at the end of the reaction were recovered. These data demonstrate the effectiveness of the excess ammonia recovery stage, and confirm that the greatest ^{15}N losses are related to the initial stage (handling, reaction system, reagent transfers). The reduced-scale (micro scale) amino acid synthesis and the elimination of the nitrogen quantification stage (N_o and N_e) as a routine may bring advantages to the process, especially by reducing ammonia losses at the reaction stage (handling, and volatile reagent transfers, among others).

When glycine masses obtained with 50 and 100 mL of aqueous ammonia are compared, it can be observed that the yield increase in amino acid synthesis was not proportional to the mass of aqueous ammonia used, since doubling the mass of ammonia reagent provided an increase of only 17.6% in the glycine mass obtained.

Considering the stoichiometry of the glycine synthesis reaction (equation 1) and the amino acid production data presented in Table I, it was possible to determine that synthesis yield was in the order of 8.7; 10.3; and 16.4% when volumes of 50, 100, and 150 mL aqueous ammonia were used, respectively. In a single test utilizing 50 mL ammonia labeled with 1.0% ^{15}N atoms, it was possible to synthesize 1.7 g glycine with the same isotopic labeling (1.0% ^{15}N atoms), demonstrating that no isotopic fractionation occurs in the proposed process. Thus, the final amino acid labeling is a function of the isotopic abundance of the ammonia used initially.

In tests (three replicates) in which 50 mL ammonia were used, 1.7g glycine could be obtained, on average, with N losses (global) in the order of 2.9 g (Table I). Under these conditions and using ammonia with a 95% abundance in ^{15}N atoms (50 mL volume), it can be estimated that the glycine production costs (fixed and variable) with the same degree of enrichment was in the order of R$ 360.00 (Three hundred and sixty reals – 1 US$ = R$ 3.0) per gram of the amino acid. Ammonia losses in the synthesis system represent about 85%

of amino acid production costs when the methodology is used. These data show that, using the proposed method to obtain ^{15}N-glycine, the final cost was about 5% lower than the FOB price in the international market (without taking into account fees related to import, transport, storage, and others, in case the compound is imported).

The synthesis of organic molecules, especially in the pharmaceutical, agrochemical, and fine chemical industries, among others, many times involves several reaction steps, usually leading to very low yields. Many times, depending on the process, a 10% yield may be considered satisfactory from an economic point of view, but not from an ecological perspective, since byproducts and/or residues are generated. In the ^{15}N-glycine synthesis process (high ^{15}N enrichment), the economic aspect is also relevant (due to the ^{15}N isotope), especially when conversion of reagents into the product of interest is low. For this reason, an effective excess ammonia recovery system becomes important, as well as the ecological aspect given by the generation of residues (methanol and ammonium chloride).

The synthesized glycine was submitted to TLC, HPLC, and MS analyses, as described in item 2, with the objective of verifying its purity.

Figure 1 presents the results obtained by TLC for the 3 synthesized glycine samples (A, B, and C) with natural ^{15}N abundance (0.366% atoms), a ^{15}N-enriched sample (D) (1.0% ^{15}N atoms), and two samples (E and F) of the p.a. standard amino acid (Sigma).

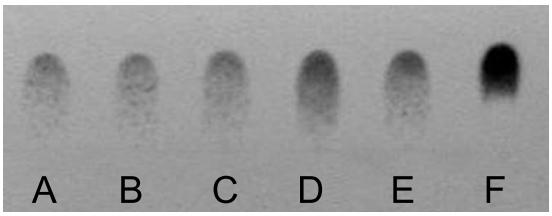

Fig. 1 – TLC plate developed in ninhydrin for glycine analysis. A, B, and C are glycine samples synthesized from 50, 100, and 150 ml ammonia, respectively. Sample D refers to ^{15}N-enriched glycine. E and F correspond to the glycine p.a. standard.

As it can be seen in Figure 1, the synthesized samples, according to the procedure proposed in this work, indicated the presence of the amino acid glycine in comparison with the Sigma standard.

In the HPLC determinations, with the objective of detecting possible impurities, it was observed that the chromatogram relative to the synthesized sample (Figure 2a) presents the same characteristics as the standard (Figure 2b), with a retention time of approximately 26 minutes for both the sample and the standard. According to the chromatograms, no impurity was measured.

The determinations corresponding to the N content in synthesized samples were carried out by ANCA-SL (Automatic Nitrogen Carbon Analyzer, Solid and Liquid) mass spectrometry, from PDZ Europe. The mean results (mg L^{-1} N) for all synthesized glycine samples (solutions prepared from the amino acid produced) as well as for the Sigma standard can be observed in Table II. These data provide evidence that the N content in the samples are compatible with those in the p.a. Sigma standard, and the amino acid purity is in the same order of magnitude as the standard utilized. The isotopic determinations showed that no fractionation occurred in the synthesized samples, considering that the isotopic value of the ammonium sulfate used for aqueous ammonia production may vary from –10‰ (0.363% ^{15}N atoms) to 0‰ (0.366% ^{15}N atoms) (Kreitler et al. 1978, Heaton 1986). In order to make the use of synthesized glycine viable in biomedical assays, supplementary tests should be carried out (pyrogenicity and toxicity). The ^{15}N-glycine thus obtained can be used for the synthesis of other amino acids, as well as in the synthesis pathway of ^{15}N-glyphosate, one of the most used herbicides worldwide.

With regard to the process aimed at the recovery of methanol, the data indicate that it was possible to recover 75% of the solvent used in the assays, on average. The methanol purity obtained in the process was compatible with the requirements for the p.a. product employed in the glycine purification line. The same recovered methanol can

TABLE I
Excess nitrogen (N_e) recovery and glycine production as a function of volume of aqueous ammonia (25% m/v NH_3(aq) solution).

$NH_{3(aq)}$ (mL)	N_o (g)	N_e (g)	N_r (g)	N_G (g)	Recovered Nitrogen (%) *	Glycine Production (g) **
50	9.5 ± 0.3	8.4 ± 0.3	8.1 ± 0.2	0.32 ± 0.01	85 ± 2	1.71 ± 0.02
100	19.94 ± 0.2	17.3 ± 0.6	16.6 ± 0.5	0.37 ± 0.03	86 ± 2	2.0 ± 0.2
150	30.63 ± 0.1	27.7 ± 1	26 ± 2	0.61 ± 0.01	86 ± 6	3.2 ± 0.1

N_o (initial N); N_e (excess N after reaction); N_r (Nitrogen recovery), N_G (**N** incorporated in Glycine); *Effectiveness (%) of the recovery process = $[(N_r + N_G)/N_o].100$; ** Synthesized glycine mass.

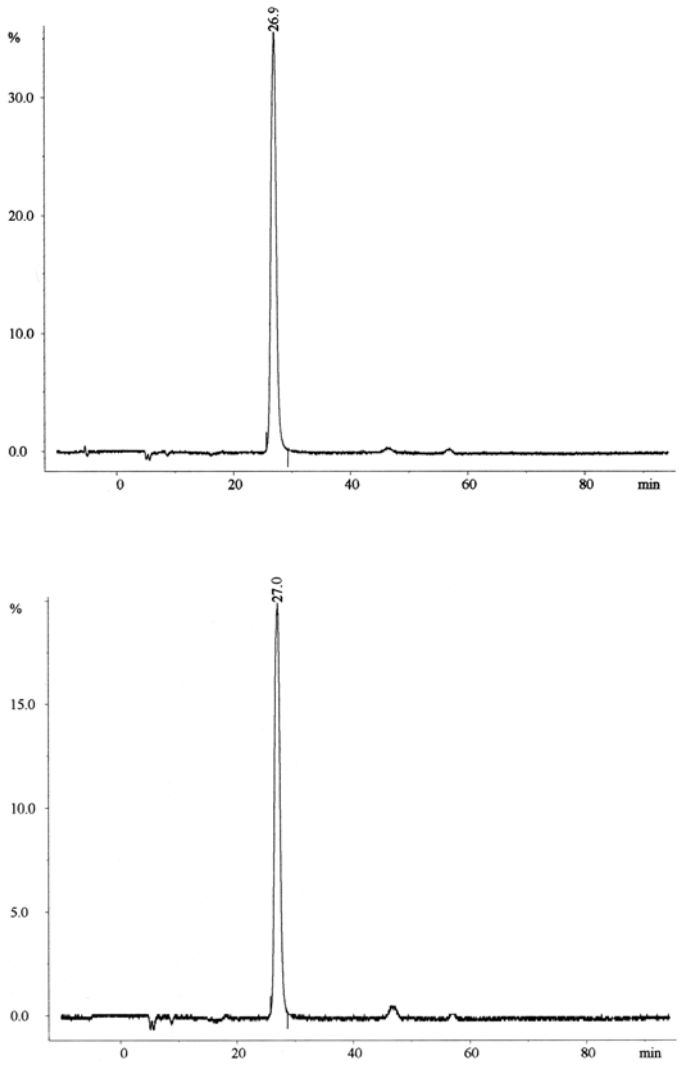

Fig. 2 – Chromatogram (HPLC) corresponding to the synthesized sample (top) and Sigma standard (botton), respectively.

TABLE II
Nitrogen content (mg L^{-1}) and isotope abundance data (% ^{15}N atoms) for synthesized glycine samples and for the p.a. Sigma standard.

Glycine Sample	Nitrogen Content (mg L^{-1} glycine N)	Isotopic abundance (% ^{15}N atoms)
T1	0.98 ± 0.01	0.363 ± 0.002
T2	0.96 ± 0.02	0.363 ± 0.002
T3	0.98 ± 0.01	0.363 ± 0.001
T4	0.98	1.0
Sigma Standard	0.98 ± 0.00	0.366 ± 0.001

T1, T2, and T3 = mean and standard deviation of the mean for samples synthesized from 50, 100, and 150 mL $NH_{3(aq)}$, respectively; T4 = single test with the use of 50 mL $^{15}NH_{3(aq)}$ enriched with 1.0% ^{15}N atoms.

be used in the ^{15}N-urea synthesis line (Bendassolli et al. 1988b) and reused in the glycine purification process.

CONCLUSION

The assays allowed to attest the viability of producing this amino acid, despite the fact that yield for the synthesis reaction was low (lower than 20%), regardless of the amount of ammonia used.

The additional use of ammonium carbonate would not be feasible, since it would produce isotopic dilution.

Due to the high effectiveness (94%) observed in the recovery system for excess labeled-ammonia in the reaction, the production cost could be 25% lower than the foreign FOB price.

The analyses performed by TLC, HPLC, RMN, and IR-MS attested the high degree of purity of the synthesized glycine. However, additional assays (pyrogenicity and toxicity) are required if it is to be used in the biomedical field. Another possible use would be as a precursor in the synthesis of other ^{15}N-labeled amino acids, as well as in the synthesis of ^{15}N-glyphosate, which is one of the most important herbicides used worldwide.

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RESUMO

Este trabalho descreve um método para a síntese de glicina marcada no isótopo ^{15}N, bem como detalhes da linha de recuperação dos resíduos nitrogenados. Para isso, foi realizada uma aminação de α-haloácidos, empregando-se o ácido carboxílico cloroacético e amônia aquosa marcada ($^{15}NH_3$). Especial cuidado foi tomado para evitar possíveis perdas de $^{15}NH_3$, uma vez que o custo de produção é elevado. A esse respeito, embora o custo de aquisição do composto marcado em ^{13}N (radioativo) seja inferior, o traçador estável produzido se configura numa importante ferramenta em estudos de ciclagem de N em seres vivos, minimizando também os riscos ocupacionais e ambientais, bem como os problemas de limitação de tempo em estudos de campo. Os testes foram realizados em triplicata variando-se o volume de $^{15}NH_{3(aq)}$ utilizado na reação (50, 100 e 150 mL), com a finalidade de aferir a melhor condição de trabalho, sendo as massas de glicina obtidas de 1,7, 2 e 3,2 g, respectivamente. Com o desenvolvimento do sistema para recuperação de $^{15}NH_3$, foi possível recuperar 71, 83 e 87% da amônia inicialmente

utilizada na síntese. Com as adaptações necessárias, o mesmo sistema foi utilizado na recuperação de metanol 75% do metanol utilizado inicialmente no processo de purificação do aminoácido.

Palavras-chave: isótopo estável, glicina, aminoácido.

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