THE SYNTHESIS OF ALANINE MULTILABELED WITH STABLE ISOTOPES

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SUMMARY

The work presented illustrates a practical sequence requiring relatively simple and inexpensive apparatus, and using the commercially available isotopic sources, to produce L-alanine from M+3 ($^{13}C_2$, ^{15}N) to M+12 ($^{13}C_3$, ^{15}N, D_4, $^{18}O_2$).

Key Words: Barium Carbide-$^{13}C_2$, acetylene-$^{13}C_2$, acetaldehyde-$^{13}C_2$, L-alanine-2,3-$^{13}C_2$-^{15}N, L-alanine-D_4, L-alanine-$^{18}O_2$.

INTRODUCTION

Amino acids labeled with stable isotopes (^{13}C, ^{15}N) have been used in the studies of metabolic disorders such as diabetes[1], methylmalonic acidemia[2] and the septic state[3]. The concern in this laboratory has been to facilitate the studies of the multiple organ failure syndrome by synthesizing labeled amino acids, and this study describes multilabeling of alanine. The use of multilabeled amino acids not only increases the sensitivity of detection of the traced biochemical, in spite of the background of natural ^{13}C [4-6], but also allows the followup of metabolic pathways by assay of secondary or tertiary metabolites.[7] Thus the use of ^{13}C, ^{15}N multilabeled alanine facilitated the assessment of conversion of this amino acid to pyruvate, lactate, glucose, the Krebs cycle intermediates, as well as to aspartic and glutamic acids, urea and ammonia. The availability of alanine-D_4 and alanine-$^{18}O_2$ allows the study of the alanine to lactate biochemical shunt, which circumvents the pyruvate-glucose glycolytic pathway.

DISCUSSION

Introduction of ^{13}C and ^{15}N into alanine has been accomplished by three different procedures. The biosynthetic procedure[8], which used green

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algae (Chlorella Pyrenoidosa-Sorokin strain) is unsuitable for the production of 10 g amounts of a specific amino acid because of inefficient ^{13}C incorporation into the desired product. The linked enzyme system procedure[9], requiring pyruvic-2,3-$^{13}C_2$ acid, has been used for ^{15}N labeling only but with poor isotopic efficiency. The synthesis via the Perkin reaction [10], though dependent on availability of acetic-$^{13}C_2$ acid, specialized equipment and rather exotic reagents, gave alanine in a high overall yield (37-38%).

The proposed synthesis of alanine-2,3-$^{13}C_2$-^{15}N from $Ba^{13}CO_3$ and $^{15}NH_4Cl$, by a procedure which may be extended to labellind the remaining atoms in the molecule, consisted of three steps: a) conversion of $Ba^{13}CO_3$ to $Ba^{13}C_2$, b) generation of acetylene-$^{13}C_2$ and its hydration to acetaldehyde-$^{13}C_2$ and c) production of triple labeled alanine by Strecker reaction.

Reduction of $BaCO_3$ to BaC_2 by heating with Mg in an atmosphere of hydrogen was first reported by Maquenne[11]. Subsequent publications[12-15] provide no practical details except for a brief description of 1 millimolar scale reaction by Abrams.[13] Assuming the following stoichiometry:

$$2BaCO_3 + Mg + 5H_2 \quad BaC_2 + BaO + Mg + 5H_2O$$

the reaction would evidently be promoted by using a large excess of both magnesium and hydrogen. Extensive preliminary experiments using $BaCO_3$ defined the reaction conditions, as well as the best ways to achieve intimate contact between $BaCO_3$ and Mg, and maximum exposure to H_2. The reaction is initiated at 600°, and being exothermic, procedes probably at 1107°C (b.p. of Mg) as is implied by sublimation of Mg to the cool zone of the reaction tube.

"Crude" $Ba^{13}C_2$ thus produced (contaminated with Mg and BaO) is hydrolysed by gradual addition to water and liberates a mixture of

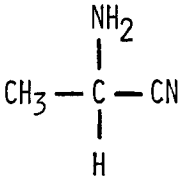
acetylene-$^{13}C_2$ ^{16} and hydrogen* as follows:

$$Ba^{13}C_2 + 2H_2O \quad Ba(OH)_2 + {}^{13}C_2H_2$$

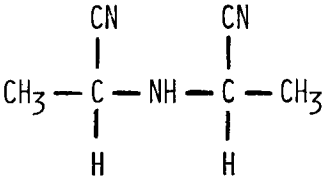
This reaction slows down as pH rises (due to buildup of $Ba(OH)_2$) but in a heated solution, it is believed to proceed in good yields. The system used for acetylene liberation and hydration shown in Fig. 1, is considered the best way to minimize losses in this stage of the overall synthesis.

Hydration of acetylene to acetaldehyde, although an established commercial process that has been studied in some detail, presented considerable difficulties. The hydration solution reported by Scharf[18] (2% $HgSO_4$ in 2N H_2SO_4) was shown (by G.L.C.) to give poor yields of acetaldehyde due to complex formation of both acetylene and acetaldehyde with the catalyst.[19] Changes to phosphoric acid[20] and to procedures mentioned by Lifson et. al. and Wood[15] were also found to be unsatisfactory. The modification of the hydration reaction used by Isagulyants and Govina[21] gave however, after steam distilation, the acetaldehyde-$^{13}C_2$, in 50% yield based on $Ba^{13}CO_3$

The modification of Strecker synthesis[22] in which acetaldehyde, ammonium chloride, and sodium cyanide are reacted under aqueous conditions, was studied by field ionization mass spectrometry (F.I.M.S.). The results of this and additional studies of the mechanism of Strecker reaction will be discussed in detail in a separate publication[23]. For the purposes of the present work it is pertinent to point out that the only two intermediates produced are the amine (I) and the imine (II):



(I)



(II)

*Addition of water to BaC_2, as suggested by Calvin et. al.[17], in spite of external cooling,, liberates enough heat to ignite the magnesium.

and that whereas the amine (I) hydrolyses to alanine, the imine gives rise to both alanine and lactic acid (thus reducing the total yield of alanine).

Following a desalting-isolation procedure using ion exchange chromatography D,L-alanine-1,2-$^{13}C_2$-^{15}N was acetylated by a modification of the standard procedure, since the latter was shown (by F.I.M.S.) to lead to considerable dimerization of the amino acid. Enzymatic resolution[24] of the acetylated enantęomers yielded pure L-antipode, the mass and n.m.r. spectra of which support the structure.

Should further labeling of the alanine molecule become necessary, deuteration could be carried out using D_2O/D_2SO_4 in the hydration of acetylene, carbonyl carbon labeling would result from the use of $K^{13}CN$ in the Strecker reaction step, and ^{18}O would be introduced when (I) + (II) are hydrolyzed with $H_2^{18}O$/HCl.

In order to evaluate the extension of the present procedure to deuterium and ^{18}O labeling, the isotopic exchange reactions were examined. Thus deuteration of L-alanine using glutamic-pyruvic transaminase as a catalyst, which has previously been demonstrated on a mg scale[24], was scaled up with considerable modification, and shown to be a viable process for generating L-alanine-D_4. Similarly, introduction of ^{18}O into amino acids by acid catalyzed exchange[25], after considerable modification gave L-alanine-$^{18}O_2$ in good yield on a gram scale with good isotopic enrichment and without racemization. The structure of both isotopically labeled compounds was confirmed by F.I.M.S.

EXPERIMENTAL

Materials and Methods: $Ba^{13}CO_3$ (90 atom % ^{13}C) was purchased from Monsanto Research Corporation, Miamisburg, Ohio; $^{15}NH_4Cl$ (85 atom % ^{15}N) and heavy water (98.4 atom % ^{18}O, 0.3 atom % ^{17}O, 91.5 atom % D) from YEDA Research and Development Corporation, Ltd. (at the Weizmann Institute of Science), Rehovat, Israel; and deuterium oxide (99.8 atom % D) from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin. Glutamic-pyruvic transaminase (from

Pig heart) and acylase (from hog kidney) were purchased from Sigma Chemical Co., St. Louis, Missouri.

The barium carbonate and magnesium were pressed into pellets using a specimen mount hydrolic press and mould assembly from Buehler Ltd., Evanston, Illinois. The reduction to barium carbide was carried out in a Thermolyne tube furnace (Model F21125), from Thermolyne Corp., Dubuque, Iowa.

Mass spectra were determined on a non-commerical, double focussing, reverse geometry instrument operating in the field ionization (F^{τ}) mode and which was equipped with a Finnigan Incos Model 2400 data system. The instrument was operated in a mass range of 0-400 and the magnet was scanned from 1-450 amu in 12 sec (with 4 sec. magnet field stabilization interval), the probe temperature was programmed to increase from 30^{o} - $130^{o}C$ in 4 min. Gas chromatography was performed on Varian Series 2100 (flame ionization detector) instrument, using nitrogen (20 ml/min.) as carrier gas, a glass column (6 mm O.D., 3 mm I.D. 45 cm length) packed with Tenax GC 60/80 (Altech Associates) temperature gradient of 50^{o} to $200^{o}C$ (at 20^{o}/min.), an injection port temperature $180^{o}C$ and detector temperature $220^{o}C$. Optical rotations were measured on Perkin-Elmer (Model 141) polarimeter and melting points were taken on a Fischer-Johns apparatus and are uncorrected.

N.m.r. spectra were determined on a Brucker spectrometer model W.P. 200, operating in ^{13}C frequency sweep mode (at $30^{o}C$), on deuterium oxide solutions using p-doxane as internal standard.

Barium Carbide - $Ba^{13}C_2$: In a typical preparation $Ba^{13}CO_3$ (1.97 g, 10 mmol), and powdered magnesium metal (1.92g, 80 mmol), were mixed thoroughly and pressed into a pellet (25 mm diameter and 4 mm thick approx.). Five such pellets contained in a stainless steel wire mesh cage (4 x 20 cm approx.) were located in the heated portion (50 cm) of the reaction tube (54" long), which was in turn contained within the 2" electrical tube furnace.

The reaction tube was then closed off at both ends and all the joints sealed with rubber cement. The tube was then purged with dry argon (5 l/min for 10 min), and then with hydrogen (5 l/min for 15 min). The furnace was then switched on, the temperature allowed to rise rapidly to 500°C (indicated temperature) and the hydrogen flow increased (10 l/min). At 600°C the reaction occurred, as indicated by a drastic momentary decrease in hydrogen flow rate, and the furnace was then switched off. When the temperature began to drop, the tube was carefully and gradually withdrawn from the oven, while maintaining reduced hydrogen flow (5 l/min), and air cooling the exposed portions. At 300°C (indicated temperature), purging gas was changed to argon (5 l/min) and cooling continued to room temperature. The reaction product (a dark grey-black flakey solid) was then removed from the cage and any material deposited on the wire mesh or the inner walls of the tube (mostly sublimed Mg) scraped off. The product thus obtained was a mixture of $Ba^{13}C_2$, BaO and Mg, and was stored in sealed vials. The reaction tube was cleaned with dilute acid after every experiment.

In the interest of safety the importance of (a) checking for tight seals particularly when hydrogen is being passed through the heated tube; (b) making sure that the seals are not broken when the tube is being withdrawn for cooling; and (c) venting the gases away from the hot furnace, cannot be emphasized strongly enough.

Acetaldehyde-$^{13}C_2$: Generation and hydration of acetylene-$^{13}C_2$ were carried out as a continuous process in the apparatus shown in Fig. 1. The hydrolysis flask, the burette and the hydration vessel

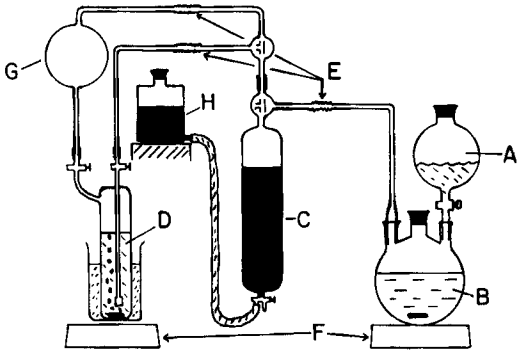


Fig I.

(A) Feeding Funnel, (B) Hydrolysis Flask, (C) Burette, (D) Hydration Vessel, (E) Flexible Joints, (F) Magnetic Stirrers, (G) Gas-overflow Bulb, (H) Mercury Reservoir.

were evacuated, and the latter isolated from the burette. Crude $Ba^{13}C_2$ (approx. 17 g, 25 mmol assuming quantitative conversion from $Ba^{13}CO_3$) was then added slowly, and with vigorous stirring from the feeding funnel to 750 ml of distilled water. The mixture of $^{13}C_2H_2$ and H_2 was allowed to fill up the burette, and the latter was then discharged into the hydration solution (1% $HgSO_4$ in 30% v/v aqueous H_2SO_4, 200 ml) which was maintained at 0^o - 5^oC. Undissolved or unreacted gases were collected in the overflow bulb (500 ml capacity) and recirculated through the hydration solution. When the addition of $Ba^{13}C_2$ was complete, the hydrolysis flask was heated to complete the reaction and expell the dissolved gases. The above procedure was repeated (3 x 17g of $Ba^{13}C_2$), using fresh water (3 x 750 ml) each time, but retaining the same hydration solution. At this point hydrogen had to be released due to back pressure build up. Fresh hydration solutions

(3 x 200 ml) were used to process another 150 mmol of $Ba^{13}C_2$ (approx.) in the manner described above. The combined hydration solutions (4 x 200 ml) were diluted, with cooling, and the acetaldehyde steam distilled[21] to give approx. a 10% aqueous solution. The yield of acetaldehyde was determined by G.L.C. to be 5.7g, 48% based on $Ba^{13}CO_3$.

D,L-Alanine-2,3-$^{13}C_2{}^{15}N$: $^{15}NH_4Cl$ (6.54 g, 120 mmol) was dissolved in a cold (-5°C) mixture of water (50 ml) and ether (50 ml), containing acetaldehyde-1,2-$^{13}C_2$ (4.7 g, 100 mmol). While maintaining the temperature at -5°, a cold solution of NaCN (5.6 g, 110 mmol in 50 ml of water) was added to the reaction mixture with stirring over a period of 50 min. After stirring at 0-5°C for 18 h, F.I.M.S. on an acidified sample showed that α-aminoacetonitrile (I) and the imine (II) were the only products. The reaction mixture was carefully acidified (fume hood - HCN is evolved), then made 6N with conc. HCl, and the ether layer was blown off with N_2. The remaining solution was heated at 120°C (oil bath) and when about 50 ml distilled over a further 20 ml of conc. HCl were added to the distillation flask. The residue remaining after complete evaporation was stirred thoroughly with ethanol (95%, 100 ml), the salts removed by filtration and washed with a further 100 ml of 95% ethanol. The combined filtrate and washings were evaporated to dryness, the residue dissolved in ethanol (50 ml, 98%) ether (20 ml) mixture. The resulting solution was filtered, evaporated to dryness, the residue dissolved in water (20 ml), and the solution passed through BioRad AG3-X4A exchange resin in the OH^- form (50 ml resin bed has sufficient capacity to remove all Cl^-). The eluant was collected until neutral and evaporated to a syrupy solid which crystallized from ethanol (95%). The labeled D,L-alanine was recrystallized from ethanol (95%) to give 4.6 g of product (50%).

L-Alanine-2,3-$^{13}C_2$,-^{15}N: The labeled D,L-alanine (3.2 g) was added to a hot (110°C) mixture of glacial acetic acid (50 ml) and acetic anhydride (3.2 ml). Following complete dissolution (2-3 min), the reaction was quenched by the addition of ice and water. The resulting solution was evaporated to dryness, and the residue was co-evaporated with several portions of water, benzene and finally water. The residue was then dissolved in hot anhydrous acetone, filtered and evaporated to a crystalline solid which was dissolved in distilled water (50 ml). The pH of the solution was adjusted to 7.4 with dilute ammonia, acylase (Sigma grade 1, 20 mg) added, and the incubation carried out for 24h at 38°C. The resulting solution was then treated with charcoal, boiled, filtered through celite, and the combined filtrate and washings evaporated under reduced pressure. The residue was co-evaporated twice with ethanol (80%), and the pure L-alanine (1.5 g, 93%) was crystallized from ethanol, sublimed at 248°C, $[\alpha]_D^{27}$ + 13.8° (5% in 6N HCl) ^{13}C.m.r. (D_2O) δ 50.34 (m, CH_3, $J_{^{13}C-H}$ 127.4 Hz, $J_{^{13}C-C-H}$ 3.9 Hz), δ 16.59 (m, CH, $J_{^{13}C-H}$ 143.7 Hz, $J_{^{13}C-C-H}$ 3.98 Hz), $J_{^{13}C-^{13}C}$ 35.8 Hz). FIMS: (M + 3) + H at mass no. 93.

L-Alanine-α, β-D_4: L-alanine (4.5 g., 50 m.moles) powdered and dried over P_2O_5 in vacuum, was dissolved in D_2O which was made 0.08 M in phosphate by addition of NaH_2SO_4 and the pH adjusted to 7.0 using NaOH in D_2O. Following addition of α-ketoglutaric acid (50 mg), pyridoxal phosphate (100 mg), glutamic-pyruvic transaminase (500 units, approx. 5 mg) and toluene (2-3 drops) the reaction was incubated for 2 days at 38°C. A further 500 units of enzyme were then added and the incubation allowed to continue for 2 more days. FIMS examination of an aliquot from the reaction, back exchanged with water, showed a deuteration equilibrium at approx. 85% M + 4 and 15% M + 3. The solution was then treated with charcoal, boiled, filtered, and the combined filtrate and washings combined and evaporated to dryness. The residue was dissolved in water (10 ml) and the solution passed through BioRad AG3-4XA (OH^- form) (25 ml resin bed). The column was eluted with water

until the washings were neutral and the resulting solution evaporated to dryness and the residue dried thoroughly. The incubation was then repeated as described above but with 1000 units of enzyme for 5 days at 38°C. Following the same isolation procedure as above L-Alanine- α, β -D_4 (3.6 g, 80%) was obtained by crystallization from aqueous ethanol. FIMS showed the final product to be 93% (M + 4) + H at mass no. 94, and 7% (M + 3) + H at mass no. 93, (a total deuterium content of 98.25%), m.p. sublimes at 248-250°C, $[\alpha]_D^{27}$ + 13° (5% in 6N HCl).

L-Alanine-$^{18}O_2$: L-alanine (2.67 g, 30 moles) which had been powdered and dried thoroughly over P_2O_5 and in vacuum, was added to $D_2^{18}O$ (10 ml) and the mixture made 5 M with dry HCl gas. The thick wall pyrex glass reaction tube was capped and heated at 90-100°C (oil bath) for 7 days. After freeze drying the residue is dissolved in dilute ammonia and co-evaporated with it several times. The residue was finally dissolved in water and passed through BioRad AG3-X4A resin (OH^-form) (15 ml resin bed) and the column eluted with water until eluents were neutral. The combined eluents were evaporated to dryness and the product was obtained by crystallization from 95% ethanol. It was shown by FIMS to be 70% (M+4) + H at mass no. 94 and 27% (M+2) + H at mass no. 92 (a total ^{18}O content of 83.5%), yield 2.0 g (71.4%), m.p. sublimes at 248-257°C $[\alpha]_D^{27}$ + 13.5° (5% in 6N HCl).

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