A General Procedure for the Preparation of Deuterated and Tritiated Amino Acids by Incorporation of Solvent Isotope during Synthesis

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A convenient, specific, inexpensive, and general method for the preparation of α-deuterated and α-tritiated amino acids is described. The procedure involves incorporation of solvent isotopes of hydrogen into the α position of amino acids concomitant with decarboxylation of the substituted aminomalonate precursors, $NH_2CR(COOH)_2$. The potential use of α-tritiated amino acids to measure the extent of racemization during the synthesis of peptides is discussed. In addition, the possibilities for the specific labeling of amino acids by incorporation of hydrogen isotopes from solvent during syntheses are pointed out.

The *N*-acylaminomalonic ester synthesis (eq 1) is

$$\underset{\text{I}}{RCONHCH(COOR')_2} + R''X \longrightarrow \underset{\text{II}}{HX + RCONHCR''(COOR')_2} \xrightarrow[\Delta]{H^+} \underset{\text{III}}{NH_2CHR''COOH} + RCOOH + 2R'OH + CO_2 \quad (1)$$

where R = H, CH_3, C_6H_5, etc., and R' = CH_3 or C_2H_5; in addition, one or both of the carboalkoxy groups in I may be replaced by CN

easily the most widely used procedure for the laboratory preparation of DL amino acids.[1] A typical amino acid synthesis employing the scheme shown in eq 1 would involve generation of the carbanion of the starting material I in strongly basic media, such as ethanolic sodium ethoxide. The carbanion is then used to effect a nucleophilic displacement of the halide from an alkyl halide, R''X, to yield the key intermediate II. This intermediate is usually hydrolyzed, deacylated, and decarboxylated in one step in boiling acid to produce the desired amino acid III.[2]

Obviously, acid-catalyzed conversion of the substituted intermediate II to the amino acid III must proceed through a symmetrical, substituted aminomalonic acid derivative IV. Equally obviously, the decarboxylation of the sensitive intermediate IV to the desired amino acid end product must involve the incorporation of solvent hydrogen into the α position of the resulting amino acid. This fact has been utilized to produce DL amino acids which are specifically labeled in the α position with deuterium or tritium. The method is reported herein and is demonstrated to be a convenient, general, and inexpensive method for the preparation of high-purity α-deuterium- and α-tritium-labeled amino acids.

$$\underset{\text{IV}}{^+NH_3CR''(COOH)_2}$$

Experimental Section

Materials. α-Methylaminomalonic Acid.[4,5]—Diethyl α-methylacetamidomalonate was prepared by the conventional alkylation procedure used in the synthesis of amino acids. Thus, a warm solution of 54.25 g of diethyl acetamidomalonate (Aldrich, 0.250 mol) in 200 ml of absolute ethanol was added dropwise with magnetic stirring to 75 ml of ethanolic sodium ethoxide (6.33 g of sodium metal, 0.275 g-atom) in a 500-ml reaction flask fitted with a condenser and drying tube. Then a solution of 48.3 g of methyl iodide (Aldrich, 0.34 mol) in 25 ml of absolute ethanol was added dropwise with stirring. The reaction mixture was refluxed for 17 hr and the solvent was removed on a rotary evaporator, leaving a yellow oil containing white crystals (NaI). This was dissolved in 50 ml of hot water and refrigerated. The resulting crystals were collected and washed with cold water, yield 39.6 g (69% of theory).

Deacetylation and hydrolysis of this product (35.0 g, 0.151 mol) was effected by refluxing in 152 ml of 5 *N* KOH for 5 days. The desired α-methylaminomalonate was isolated as the monoammonium salt employing the ion-exchange procedure described by Thanassi[6] for the preparation of the ammonium salt of aminomalonic acid, yield 14.2 g (63% of theory).

For analysis, the product was converted to the free acid by addition of cold, concentrated HCl (1 equiv) to a concentrated aqueous solution of the ammonium salt. The resulting precipitate was recrystallized from warm water and dried *in vacuo* over P_2O_5 and KOH.

Anal. Calcd for $C_4H_7NO_4$ (133.11): C, 36.09; H, 5.30; N, 10.53. Found: C, 36.20; H, 5.18; N, 10.27.

α-Deuterio-DL-alanine.—In order to remove exchangeable hydrogens, the ammonium salt of α-methylaminomalonic acid (0.50 g, 3.3 mmol) was first evaporated three times from 99.9% D_2O (Bio-Rad). Decarboxylation to the desired product was then effected by refluxing the starting material in 9.4 ml of D_2O and 0.6 ml of concentrated HCl for 3 hr. Paper chromatography showed only one ninhydrin positive spot corresponding to authentic alanine. The nmr spectrum of the product revealed that the quartet corresponding to the α hydrogen of alanine had disappeared and that the doublet for the hydrogens on the α-methyl group had merged into a singlet.

α-Deuterio-DL-phenylalanine.—2-Benzyl-2-acetamidomalonic acid was prepared according to the procedure of Albertson and Archer.[7] Deacetylation and decarboxylation to α-deuterio-DL-phenylalanine were accomplished by refluxing 2.1 g of the acetylated dicarboxylic acid precursor in 16 ml of D_2O and 0.80 ml of concentrated H_2SO_4 for 5 hr. The amino acid was isolated by isoelectric precipitation. Paper chromatography of the product in three different solvent systems showed only one ninhydrin positive spot, corresponding to authentic phenylalanine. An nmr spectrum indicated that the signals for the α hydrogen of unlabeled phenylalanine had essentially disappeared.

α-Tritio-DL-glutamic Acid.—This compound was prepared by condensing β-propiolactone (Eastman) and the sodio salt of diethyl acetamidomalonate, according to the one-step procedure described by Talbot, *et al.*,[8] except that deacetylation, hydrolysis, and decarboxylation of the oily intermediate, *N*-acetyl-2-carboethoxyglutamic acid diethyl ester (1.5 g), to the labeled amino acid were accomplished by refluxing the intermediate in 20 ml of 6 *N* HCl containing 2.5 mCi of tritiated water (New England Nuclear) for 5 hr. After removal of the radioactive solvent, the amino acid was refluxed for 95 hr in 32 ml of nonradioactive 6 *N* HCl. This was done in order to remove "semi-labile" tritium

(1) J. S. Meek, S. Minkowitz, and M. M. Miller, *J. Org. Chem.*, **24**, 1397 (1959).

(2) For detailed synthetic schemes employing this general procedure, and for numerous references to original articles, the reader is referred to Greenstein and Winitz.[3]

(3) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. 3, Wiley, New York, N. Y., 1961.

(4) J. W. Thanassi and J. S. Fruton, *Biochemistry*, **1**, 975 (1962).

(5) G. B. Bailey, O. Chotamangsa, and K. Vuttivej, *ibid.*, **9**, 3243 (1970).

(6) J. W. Thanassi, *ibid.*, **9**, 525 (1970).

(7) N. F. Albertson and S. Archer, *J. Amer. Chem. Soc.*, **67**, 308 (1945).

(8) G. Talbot, R. Gaudry, and L. Berlinguet, *Can. J. Chem.*, **34**, 1440 (1956).

introduced into the γ position of the glutamic acid.[9] The specific radioactivity of the resulting α-tritiated DL-glutamic acid was 28% of that expected based on the specific activity of the tritium in the solvent, indicating an isotope effect of 3.6. Paper chromatography of the product in three different solvent systems showed only one spot corresponding to authentic glutamic acid.

The position of the label was confirmed with the aid of L-glutamic–oxaloacetic transaminase.[11,12] Enzyme (0.4 mg) (Boehringer), neutralized α-tritiated DL-glutamic acid (14.6 mg, 0.1 mmol), and 14.6 mg (0.1 mmol) of neutralized α-ketoglutaric acid (Boehringer) were incubated at room temperature for 1 hr in 3.0 ml of water containing 0.3 mmol of trishydroxymethylaminomethane hydrochloride buffer at pH 8.25. At the end of this time, the reaction mixture was frozen and the water collected by lyophilization into a cold finger immersed in acetone–Dry Ice. The water contained 44% of the total counts introduced into the reaction mixture as tritiated DL-glutamate; theory for the complete stereospecific enzyme-catalyzed exchange of the tritium in the L isomer with the medium is 50% of the total counts in the racemate, assuming that all of the isotope is in the desired α position.

α-Tritio-DL-aspartic Acid.—The procedure of Galat[13] was followed except that diethyl acetamidomalonate was used instead of the formamido derivative.[14] In addition, the oily intermediate, *N*-acetyl-2-carbethoxyaspartic acid diethyl ester, was hydrolyzed, deacetylated, and decarboxylated in tritiated HCl. An isotope effect of approximately 2.4 was found. Paper chromatography in three different solvent systems showed that the desired compound had been obtained. The position of the label was confirmed enzymatically as described above. All conditions were identical except that the glutamic acid was replaced by an equivalent amount of α-tritiated DL-aspartic acid. The counts released into the water amounted to 45% of the total introduced into the solution.

α-Tritio-DL-alanine and Tritioglycine.—The ammonium salts of the dicarboxylic acid precursors were dissolved in tritiated water containing 2 equiv of HCl. Decarboxylation was effected by heating at 100° for 3 hr. An isotope effect of approximately 3 was found in the decarboxylation of α-methylaminomalonic acid to α-tritio-DL-alanine.

Methods. Paper Chromatography.—The solvent systems employed were 1-butanol–acetic acid–water (4:1:1), ethanol–concentrated ammonia–water (6:3:1), and pyridine–1-butanol–water (1:1:1). Whatman No. 3 MM filter paper was used in an ascending fashion. Amino acids were visualized by spraying the paper with a ninhydrin solution in acetone.

Nmr Spectra.—These were taken on a Varian Model A-60 or a Perkin-Elmer Model R-12 nmr spectrometer; the solvent was D_2O.

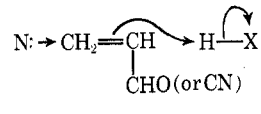
Isotope Counting.—Samples were counted in 16 ml of a solution composed of 1.0 ml of water and 15 ml of a cocktail containing, per 1200 ml, 8 g of butyl-PBD, 0.5 g of PBBO, 200 ml of Bio-Solv BBS-3, and 1000 ml of scintillation grade toluene. All materials were from Beckman Instruments, Inc. The counting efficiency for tritium was approximately 33%; counting was done on a Beckman Model LS-250 liquid scintillation counter.

Kinetics of Decarboxylation of α-Methylaminomalonic Acid.—The rate of decarboxylation of α-methylaminomalonic acid was followed by measuring the incorporation of carbon-bound tritium into the α position of the product, alanine. Aliquots (0.25 ml) of a 0.05 *M* solution of α-methylaminomalonic acid in 1.0 *M* HCl, containing tritiated water, were dispensed into 1.0-ml break-seal ampoules. The sealed ampoules were immersed in a 70° bath. After the ampoules had come to temperature, individual samples were removed at the desired times and the reaction was quenched by immersing the ampoule in ice–water. A 0.2-ml aliquot of the reaction solution was transferred to a counting vial and taken to dryness in an evacuated desiccator containing P_2O_5 and KOH pellets. Drying was repeated twice more after the addition of 0.25 ml of nonradioactive water each time in order to remove exchangeable counts. The residue was then taken up in the counting solution and the radioactivity determined. Rate constants were calculated using the conventional first-order rate equation, $k = 2.3/t \log [(\mathrm{cpm}_\infty - \mathrm{cpm}_0)/(\mathrm{cpm}_\infty - \mathrm{cpm}_t)]$, where cpm represents the observed counts per minute.

Results and Discussion

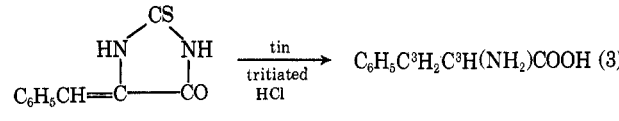
Radioactive and stable isotopes have revolutionized biological research and considerable effort and ingenuity have gone into the synthesis of compounds labeled in specific positions for use as biological tracers.[15] Inspection of the literature reveals that most, if not all, of the amino acids of biological interest can be synthesized *via* α-substituted aminomalonyl derivatives[3] and it follows that many amino acids can therefore be specifically labeled in the α position with solvent deuterium or tritium, employing procedures analogous to those presented in the Experimental Section of this paper. Thus, decarboxylation of aminomalonate precursors of amino acids in tritiated or deuterated water is a general method with wide potential use. The amount of isotope introduced into the α position during decarboxylation is directly proportional to the amount of isotope in the medium and can therefore range, depending on the medium, from a very small amount to essentially 100%, the latter figure being approached with a deuterium oxide solvent. Since tritiated water is readily available, one can synthesize in the laboratory highly radioactive amino acids for use as biological tracers at little cost.

The method of labeling amino acids by incorporation of solvent hydrogen isotopes during synthesis also has potential use in the labeling of amino acids in a number of other positions. For example, the preparations of the following amino acids can involve additions to acrolein or acrylonitrile: lysine,[16] methionine,[17] proline,[18] and ornithine[19] (eq 2). Solvent tritium incorporation



(2)

into the 2 carbon of the starting material would occur in each case.[20] Similarly, solvent–tritium incorporation could be used to prepare DL-phenylalanine labeled in the α and β positions employing the procedure of Johnson and coworkers[22,23] except that tritiated HCl would be used in the tin–HCl reduction of 5-benzalthiohydantoin to DL-phenylalanine (eq 3).



(3)

(9) Ratner, *et al.*,[10] have shown that the hydrogens on the γ carbon of glutamic acid are "semi-labile," *i.e.*, will exchange with solvent hydrogen in boiling 20% HCl, whereas the α hydrogen is stable to this treatment.

(10) S. Ratner, D. Rittenberg, and R. Schoenheimer, *J. Biol. Chem.*, **135**, 357 (1940).

(11) W. T. Jenkins and I. W. Sizer in "Methods in Enzymology," Vol. 5, S. P. Colowick and N. O. Kaplan, Ed., Academic Press, New York, N. Y., 1962, p 677.

(12) W. T. Jenkins and I. W. Sizer, *J. Biol. Chem.*, **234**, 1179 (1959).

(13) A. Galat, *J. Amer. Chem. Soc.*, **69**, 965 (1947).

(14) F. H. MacMillan and N. F. Albertson, *ibid.*, **70**, 3778 (1948).

(15) A. Murray and D. L. Williams, "Organic Synthesis with Isotopes," Interscience, New York, N. Y., 1958.

(16) O. A. Moe and D. T. Warner, *J. Amer. Chem. Soc.*, **70**, 2763 (1948).

(17) E. Rothstein, *J. Chem. Soc.*, 1560 (1940).

(18) N. F. Albertson and J. L. Fillman, *J. Amer. Chem. Soc.*, **71**, 2818 (1949).

(19) N. F. Albertson and S. Archer, *ibid.*, **67**, 2043 (1945).

(20) Frequently a nonaqueous protic solvent such as ethanol is required in these syntheses. Radioactive ethanol could easily be obtained by preparing anhydrous ethanol from a mixture of ethanol and tritiated water.[21]

(21) L. F. Fieser, "Experiments in Organic Chemistry," 3rd ed, D. C. Heath, Boston, Mass., 1957, p 285.

(22) T. B. Johnson and B. H. Nicolet, *J. Amer. Chem. Soc.*, **33**, 1973 (1911).

(23) T. B. Johnson and W. B. O'Brien, *J. Biol. Chem.*, **12**, 205 (1912).

One of the potential problems in labeling amino acids by solvent–isotope incorporation is the danger of nonspecific incorporation into positions other than the ones desired. For example, in experiments designed to measure the extent of racemization of amino acids during hydrolysis of peptides by incorporation of solvent tritium into the α position, Manning[24] has found that nonspecific incorporation of tritium occurs, particularly in the case of glutamic and aspartic acids, histidine, phenylalanine, and tyrosine.[25] This does not appear to be a particular problem under our conditions; a stereospecific enzyme exchange assay, using L-glutamic-oxaloacetic transaminase (see Experimental Section) indicates that 90% of the radioactivity is found in the desired α position of DL-aspartic and -glutamic acids. It is probable that the relatively short reaction times used in these experiments minimize the amount of nonspecific incorporation into the product amino acids; whereas the experiments by Manning[24] involved a 22-hr heating time at 110°, the reaction times for decarboxylation in our experiments have never exceeded 5 hr. In Figure 1 are presented the rate data for the decarboxylation of α-methylaminomalonic acid in 1.0 M HCl and a reaction temperature of 70°. Under these conditions the rate constant for decarboxylation is 3.63×10^{-3} min^{-1}, corresponding to a half-time of 190 min. Assuming an increase in rate by a factor of 2 with every 10° rise in temperature, it can be calculated that the half-life of decarboxylation at 110° would be 11.9 min. Thus the decarboxylation in 1.0 M HCl at 110° would be over in about 2 hr. Preliminary experiments in this laboratory indicate that the pH–rate profile for the decarboxylation of α-methylaminomalonate resembles that of unsubstituted aminomalonate (see Thanassi[6]) and that the most sensitive species is the neutral species, $NH_3^+CCH_3(COO^-)COOH$, rather than the positively charged species, $NH_3^+CCH_3(COOH)_2$. Hence, by carrying out the decarboxylation in a buffer favoring the neutral species, the reaction time might be further decreased by a factor of 2 or 3.

(24) J. M. Manning, *J. Amer. Chem. Soc.*, **92**, 7449 (1970).

(25) Manning[24] found an isotope effect of 3.5 during the racemization of L-alanine in tritiated HCl. Similarly, Donkewalter, *et al.*,[26] found an isotope effect of 4.6 in the incorporation of tritium into the α position of amino acids during peptide synthesis. These compare with isotope effects of 2.4–3.6 found in the present study.

(26) R. G. Denkewalter, H. Schwam, R. G. Strachan, T. E. Beesley, D. F. Weber, E. F. Schoenewaldt, H. Barkemeyer, W. J. Paleveda, Jr., T. A. Jacob, and R. Hirschmann, *J. Amer. Chem. Soc.*, **88**, 3163 (1966).

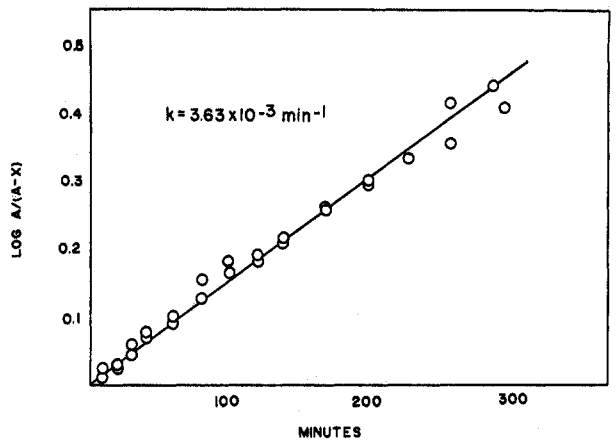


Figure 1.—First-order plot for decarboxylation of α-methylaminomalonic acid in 1.0 M HCl (T = 70°).

In addition to their use as metabolic tracer compounds, readily available, highly radioactive, α-tritiated amino acids can be of great help in the determination of racemization of amino acids during synthesis of peptides. The procedure would be essentially the converse of that employed by Denkewalter, *et al.*[26] That is, instead of measuring the incorporation of solvent tritium *into* the amino acids of the peptide, one would simply measure the amount of isotope coming *out* of the amino acids into the solvent. This has a considerable technical advantage in that no separations are required except the removal of the solvent in order to count it. Also the optically active L isomers of the amino acids need not be used in control syntheses carried out in order to test for the extent of racemization since those experimental conditions causing racemization of amino acids operate equally on the D and L isomers and hence will cause the release of tritium from the α positions of both isomers of the racemate into the medium.

Registry No.—α-Methylaminomalonic acid, 26767-88-4; α-deuterio-DL-alanine, 31024-91-6; α-deuterio-DL-phenylalanine, 14246-24-3; α-tritio-DL-glutamic acid, 24125-49-3; α-tritio-DL-aspartic acid, 31024-94-9; α-tritio-DL-alanine, 31024-95-0; tritioglycine, 22712-83-0.

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