[CONTRIBUTION FROM THE RADIATION LABORATORY AND DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY][1]

Synthesis of Several Carbon-14 Labeled DL-Alanines

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The synthesis of the three carbon-14 singly labeled DL-alanines and necessary intermediates to prepare them *via* propionic acid is described. Small scale procedures were used throughout. Ion exchange columns and vacuum sublimation were used to give high purity amino acids in good yield on a small scale.

The synthesis of DL-alanine singly labeled with carbon-14 in each of the three positions was undertaken in conjunction with some of the biological studies of this group and of the Division of Biochemistry of the University of California.

Although both 1- and 2-labeled alanine have been prepared before[3-6] other methods with much lower yields have been used.

In the course of this work, procedures for the small scale synthesis of several important intermediates were developed, including the 2- and 3-labeled propionic acids. Carboxyl-labeled propionic acid was prepared by carbonation of ethylmagnesium bromide in the standard fashion. The 2- and 3-labeled propionates were prepared from the corresponding 1- or 2-labeled acetic acid according to the schemes

$$CH_3CO_2H \xrightarrow{LiAlH_4} CH_3CH_2OH \xrightarrow{PBr_3} CH_3CH_2Br \xrightarrow[CO_2,\ H^+]{Mg} CH_3CH_2CO_2H$$

Alanine was prepared by direct bromination and amination of the propionic acid.

It was observed that in the reduction of the acetic acid with lithium aluminum hydride in diethyl carbitol solvent[7] there occurred a marked and erratic dilution of the labeled ethanol with inactive material.

To ensure that the solvent itself was originally free from ethanol, it was treated with lithium aluminum hydride, filtered and fractionally distilled. The fraction boiling at 72° (10.5 mm.) was used for the syntheses and for all experimental work. Attempts to identify ethanol in the solvent after treatment with lithium aluminum hydride or in the $LiAlH_4$ itself were unsuccessful. The presence of carbon-containing compounds in the lithium aluminum hydride was demonstrated, but its analysis (C, 0.95, 0.59%) in the two samples used showed this source to be insufficient to account for the inactive halide formed in the subsequent reaction.

After treating the diethyl carbitol with phosphorus tribromide under the conditions of the bromination, 0.18 g. of product was isolated. When an identical amount of solvent was first treated with lithium aluminum hydride, then brominated, the product was 0.33 g. Even when the greatest of care was taken to exclude diethyl carbitol from the bromination of synthetic ethanol, yields in excess of theoretical were still found.

Methanol was prepared by lithium aluminum hydride reductions of $C^{14}O_2$ in a check reaction and converted to the iodide. Sodium acetate was prepared from the methyl iodide.[8] The equivalent weight of the acetates so prepared was as much as 8% high, whereas that of sodium propionate made from ethyl bromide prepared in the manner described above varied a maximum of 0.6% from theoretical in six runs. On the basis of this work, it was concluded that probably some lithium compound caused splitting of the diethyl carbitol under the conditions of the preparation to give ethanol and thus cause the effects described.

Since completion of the syntheses described herein, other workers[9] have reported a thorough investigation of the lithium aluminum hydride reduction of carbon dioxide and have isolated inactive ethanol in the methanol-C^{14} produced by this reaction. These workers have concluded that the ethanol is derived from the diethyl carbitol through splitting of the ether bonds.

The purification of the alanine was achieved by a novel combination of steps which gave a high purity material on a small scale with a minimum loss of material due to manipulation. In this procedure the alanine was first absorbed on a cation exchange resin and the anions (chloride, phosphate and bromide ions) washed out. The alanine was then eluted with ammonia to give an alanine–ammonia solution. When this eluate was evaporated to dryness, fairly pure, salt-free alanine was left. By high vacuum sublimation of this material a very pure product was obtained. There is no evidence for the formation of the diketopiperazine of alanine by this procedure. In similar experiments with glycine, a second radioactive, ninhydrin-negative compound was found in the sublimate, which disappeared upon hydrolysis with hydrochloric acid. It seems, therefore, likely that any diketopiperazine of alanine would have been seen on the chromatograms if present.

The identity and purity of the propionic acids was checked by equivalent weight determinations. The chemical identity and radiopurity of the derived amino acids was checked by two-dimen-

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(3) S. Gurin and D. W. Wilson, *Federation Proc.*, **1**, 114 (1942).

(4) R. B. Loftfield, *Nucleonics*, **1**, No. 3, 54 (1947).

(5) D. Frank, R. B. Loftfield and W. W. Miller, *Science*, **106**, 544 (1947).

(6) J. Baddiley and J. Ehrenvärd and H. Nilsson, *J. Biol. Chem.*, **178**, 399 (1949).

(7) R. F. Nystrom and W. G. Brown, THIS JOURNAL, **69**, 1197, 2548 (1947).

(8) B. M. Tolbert, *J. Biol. Chem.*, **173**, 205 (1948).

(9) J. D. Cox, H. S. Turner and R. J. Warne, *J. Chem. Soc.*, 3167 (1950).

sional paper chromatography. The two solvent systems used were phenol saturated with water and butanol–propionic acid–water (2:1:1.4), respectively.[10] After making radioautographs of the papers they were sprayed with a 0.1% solution of ninhydrin in 95% ethanol. These experiments showed that only one amino acid, alanine, was present and that the ninhydrin spot corresponded with the radioautograph spot.

The importance of this check can be shown by the following unsuccessful experiment. The preparation of alanine-3-C^{14} was first attempted by condensation of diethyl acetamidomalonate with labeled methyl iodide. It was found that about 10% of the product thus formed was present as a radioactive contaminant which had amino acid-like properties and could be separated from the alanine by fractional elution from an ion-exchange column. This impurity, believed to be sarcosine (N-methylglycine), would not be easily detected in alanine by ordinary analytical methods (elemental analyses, recrystallization, etc.) and indicates the need in the small-scale radiopreparations of special purity tests such as the paper chromatograms and radioautographs just mentioned.

It is interesting to note that small quantities of alanine may be decarboxylated by bacterial action if left standing in unsterilized solution at room temperature. Thus, 59 mg. of alanine-1-C^{14} was aerated for three days in aqueous solution and the carbon dioxide was trapped in sodium hydroxide and precipitated as barium carbonate. It was found that of the 2.43×10^6 dis./min. put into some 75 ml. of water only 1.69×10^6 dis./min. were found in the solution after aeration and 0.69×10^6 dis./min. were found in the barium carbonate precipitated from the sodium hydroxide bubblers.

Experimental

Sodium Propionate-1-C^{14}.—Thirty millimoles of ethylmagnesium iodide was carbonated with 20 millimoles of $C^{14}O_2$ (from 3.94 g. of $BaC^{14}O_3$, 5.02 μc./mg.).[11] The yield was 1.87 g. of sodium propionate-1-C^{14} (specific activity 10.4 μc./mg.) which is a 97.8% yield.

α-Bromopropionic-1-C^{14} Acid.—Dry sodium propionate (937 mg., 9.87 mc.) from the above preparation was placed in a gas-solid reactor.[12] This bulb was evacuated to about 10 microns pressure and filled with an excess (one-half atmosphere) of dry, purified hydrogen chloride. The solid sodium propionate was then heated gently over a Bunsen burner. After the exchange reaction was complete, the mixture of propionic acid and excess hydrogen chloride was distilled *in vacuo* into a large trap cooled with liquid nitrogen. The cooling bath was then changed to a Dry Ice–isopropyl alcohol mixture, and the excess hydrogen chloride pumped off. In preliminary runs it was established that the salt residue contained less than 0.1% of the initial activity and no demonstrable amounts of propionic acid. It was also shown that the propionic acid so prepared contained 3–5% water.

The propionic-1-C^{14} acid was distilled into the bromination vessel[13] which contained 0.04 g. of red phosphorus, 0.02 g. of iodine and 0.2 ml. of propionyl chloride. The mixture was allowed to reflux on the steam-bath for one-half hour to destroy the water, 1.5 ml. of bromine was added dropwise and refluxing was continued for three hours.[14] The low temperature condenser was kept at Dry Ice–isopropyl alcohol temperature.

After sweeping out the excess bromine with a slow stream of air, the mixture of α-bromopropionic-1-C^{14} acid and α-bromopropionyl-1-C^{14} bromide was cooled and hydrolyzed by slow addition of 2 ml. of water.

Alanine-1-C^{14}.—The amination was performed in a three-necked flask by slow addition of α-bromopropionic-1-C^{14} acid to a mixture of 6 g. of ammonium carbonate and 15 ml. of concentrated ammonium hydroxide.[15,16] The reaction mixture was kept at 60° for six hours and then distilled to dryness *in vacuo*. The distillate contained 1–2% of the starting activity.

The crude alanine-1-C^{14} was purified by (a) passage through an ion-exchange resin column and (b) high vacuum sublimation.

A glass column (30 cm. × 2 cm. o.d.) filled with 60 cc. of Dowex-50 resin (20–40 mesh) was treated by cycling it to exhaustion three or four times with 2 *N* sodium hydroxide solution and 2 *N* hydrochloric acid, respectively, ending with the acid. All the excess acid was washed out thoroughly with water, and 600–900 mg. of crude alanine-1-C^{14}, dissolved in a minimum amount of water, was added to the column, followed by 500 ml. of water to wash out the anions. The alanine was eluted with 250 ml. of 1.5 *N* ammonium hydroxide, followed by 250 ml. of water. The resin was regenerated with 2 *N* hydrochloric acid. The water effluate, ammonium hydroxide eluate and hydrochloric acid regeneration solution were each evaporated to dryness and were found to contain 3, 88 and 5%, respectively, of the radioactivity put on the resin.

In order to remove traces of ammonia the dry residue of the ammonium hydroxide eluate was made slightly alkaline with sodium hydroxide and evaporated to dryness. After redissolving and adjusting the *p*H to 6.8 the solution was transferred to the lower part of a sublimation apparatus with a 1-cm. sublimation gap, evaporated to dryness and sublimed at one micron pressure and 160–200° for two hours with the cold finger at liquid nitrogen temperature. The sublimation residue was redissolved (*p*H 8.5), adjusted to *p*H 6.8, dried and resublimed. This final residue contained 4% of the initial crude alanine activity.

The yield of alanine-1-C^{14} was 802 mg. with a specific activity of 9.9 μc./mg. (theoretical, 9.11 μc./mg.). This represents a radiochemical yield of 80.5% based on sodium propionate-1-C^{14} used and a chemical yield of 76%.

Ethyl-1-C^{14} Bromide.—Acetic-1-C^{14} acid was prepared from 1.602 g. (29.2 mc.) of sodium acetate-1-C^{14} [8] using dry hydrogen chloride as in the previous procedure for preparing propionic acid. The product was distilled *in vacuo* into a liquid nitrogen-cooled dropping funnel which contained 5 ml. of repurified diethyl carbitol. The funnel was attached to a three-necked conical flask which contained 1.16 g. of lithium aluminum hydride in 35 ml. of diethyl carbitol and to which was also attached a U-trap immersed in a Dry Ice bath.[7,17] The acid solution was dropped slowly onto the lithium aluminum hydride solution with occasional shaking and cooling. After addition of the acid was completed, the dropping funnel was rinsed with several small portions of the solvent and the solution was allowed to stand with occasional shaking for 30 minutes. The lithium aluminum hydride solution was decomposed by the slow addition of 50 ml. of *n*-butyl carbitol and the system swept free of hydrogen by a slow nitrogen stream.

The ethanol-1-C^{14} thus produced was distilled into the cold trap using a nitrogen sweep. Alternating periods of boiling were used to ensure complete exchange between the excess *n*-butyl carbitol and the lithium ethanolate. Prolonged boiling without gas sweep may produce enough hydrogen to cause an explosion. The non-volatile distillation residue contained 2.9% of the initial activity.

The cold trap containing the ethanol and about 5 ml. of the solvent was removed from the reaction flask, attached

(10) A. A. Benson, *et al.*, THIS JOURNAL, **72**, 1710 (1950).

(11) Calvin, *et al.*, "Isotopic Carbon," John Wiley and Sons, Inc., New York, N. Y., 1949, pp. 178–179.

(12) Calvin, *et al.*, *ibid.*, p. 162.

(13) B. M. Tolbert, F. C. Christenson, F. N. H. Chang and P. P. T. Sah, *J. Org. Chem.*, **14**, 528 (1949).

(14) J. C. Eck and C. S. Marvel, "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1943, p. 74.

(15) N. D. Cheronis and K. H. Spitzmueller, *J. Org. Chem.*, **6**, 349 (1941).

(16) J. M. Orton and R. W. Hill, "Organic Syntheses," Coll. Vol. I John Wiley and Sons, Inc., New York, N. Y., 1941, p. 300.

(17) R. F. Nystrom, W. H. Yanko and W. G. Brown, THIS JOURNAL **70**, 441 (1948).

to the vacuum line, and subjected to two vacuum distillations to reduce the amount of carbitol present. The ethanol was then distilled into a small flask and converted to ethyl-1-C^{14} bromide with phosphorus tribromide.[13]

The chemical yield of purified ethyl bromide was 2.49 g., representing 116% of the theoretical yield. Specific activity of the product was not measured, but the specific activity of the sodium propionate prepared from this ethyl bromide would indicate a 26% dilution of the active halide with the inactive bromide produced from solvent breakdown.

Sodium Propionate-2-C^{14}.—The ethyl-1-C^{14} bromide thus prepared was converted to the Grignard reagent and this compound carbonated with inactive carbon dioxide.[3] The yield of dry sodium propionate-2-C^{14} was 1.58 g. (19.8 mc.) of specific activity 12.5 μc./mg. or a radiochemical yield of 68%.

Alanine-2-C^{14}.—Sodium propionate-2-C^{14} (0.935 g., 9.7 millimoles) with a specific activity of 4.66 μc./mg. was converted to alanine as previously described. The chemical yield (0.689 g.) was 67% based on the propionate and propionyl chloride used. The radiochemical yield was 63% and the specific activity of the product was 4.08 μc./mg. (theoretical 4.21 μc./mg.).

Sodium Propionate-3-C^{14}.—Sodium acetate-2-C^{14} was prepared[8] from methyl-C^{14} iodide which had been obtained *via* high pressure hydrogenation of carbon dioxide[18] in order to avoid the contamination found in the methyl iodide obtained *via* the lithium aluminum hydride reduction. As previously described, 1.229 g. (13.2 mc.) of sodium acetate-2-C^{14} was reduced with lithium aluminum hydride in diethyl carbitol soution and the alcohol thus produced converted to the bromide to give 1.92 g. of ethyl-2-C^{14} bromide. This labeled ethyl bromide was then converted to the Grignard reagent and carbonated. In this manner, 1.225 g. (6.51 mc.) of sodium propionate-3-C^{14} was produced, which represented a radiochemical yield of 49.5% based on the acetate used to begin the synthesis.

Alanine-3-C^{14}.—1.11 g. of sodium propionate-3-C^{14} with a specific activity of 5.32 μc./mg. was converted to alanine as previously reported. The chemical yield (0.965 g.) based on the propionate and propionyl chloride used was 77%. The radiochemical yield was 74%. The specific activity of the product was 4.53 μc./mg. (theoretical 4.75 μc./mg.).

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(18) B. M. Tolbert, THIS JOURNAL, **69**, 1529 (1947).

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[CONTRIBUTION FROM THE DIVISION OF BIOCHEMISTRY, SCHOOL OF MEDICINE, UNIVERSITY OF CALIFORNIA, BERKELEY]

Synthesis and Chromatographic Separation of Isotopically Labeled DL-Threonine and DL-Allothreonine[1]

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The synthesis of isotopically labeled DL-threonine and its alloform has been carried out. It was found that chemical methods on a small scale will not serve for a complete separation of the isomers, which is also true for paper chromatography. Quantitative separation of the isomers was effected by the use of a cation exchange column (Dowex 50). Microbiological techniques confirmed the efficiency of this technique.

For the purpose of evaluating the chromatographic separation, the general procedure set forth by Carter[2] was followed in the synthesis, suitably modified because of the reduced scale on which the reactions had to be carried out. It was found, however, that the method of Carter and Zirkle[3] coupled with that of Pfister, *et al.*,[4] was more suitable from a purely preparative standpoint, although involving additional steps.

In the preparation of N^{15}-labeled DL-threonine, separation of the alloform was avoided by the use of the low-melting isomer of α-bromo-β-methoxybutyric acid[5] which led to DL-threonine directly.

For the preparation of C^{14}-labeled DL-threonine and DL-allothreonine, labeled crotonic acid was prepared from malonic acid[6] in good yields and converted to the N-formyl-O-methyl derivatives of DL-threonine and DL-allothreonine. After one recrystallization, the N-formyl-O-methyl-DL-threonine yielded upon hydrolysis a mixture of nearly equal amounts of DL-threonine and DL-allothreonine (as determined by microbiological assay) rather than pure DL-threonine, as has been reportedly obtained in large scale runs.[2] It was found, however, that mixtures of the two isomers could be separated on a small scale, either by fractionation of the sodium salts with ethanol[4] or by a chromatographic separation on a cation resin. Paper chromatography failed to separate DL-threonine from DL-allothreonine.

Although the sodium salt method yielded DL-

TABLE I

EFFICIENCY OF RESOLUTION OF DL-THREONINE AND DL-ALLOTHREONINE AS DETERMINED BY MICROBIOLOGICAL ASSAY[a]

No.	Compound	Method of resolution	% DL-threonine[c]
1	DL-Threonine—80% DL-Allothreonine—20%	Synthetic mixture	80
2	DL-Threonine—57% DL-Allothreonine—43%	Synthetic mixture	53, 54
3	DL-Threonine sodium salt	Sodium salt fractionation from No. 2 above	97, 86[b]
4	C^{14} DL-Threonine	Fractionation of N-formyl-O-methyl deriv. (one crystallization)	53, 53
5	DL-Threonine	Cation exchange resolution (Fig. 2)	98
6	DL-Allothreonine	Cation exchange resolution (Fig. 2)	0
7	N^{15}-DL-Threonine	None	106, 110, 110

[a] We are indebted to Dr. Ethelda Norberg for running the microbiological assays. [b] Values for DL-threonine are calculated as double the values of L-threonine found. D-Threonine is inactive for *S. faecalis*, [c] Values for DL-threonine sodium salt are calculated from assays *vs.* DL-threonine standard. They are recorded as their DL-threonine equivalent.

(1) This investigation was supported in part by a research grant from the National Cancer Institute, of the National Institutes of Health, U. S. Public Health Service.

(2) Carter and West, *Org. Syntheses*, **20**, 101 (1940).

(3) Carter and Zirkle, *J. Biol. Chem.*, **178**, 709 (1949).

(4) Pfister, Robinson, Shabica and Tishler, THIS JOURNAL, **71**, 1101 (1949).

(5) Generously provided by Dr. Max Tishler of Merck & Co., Inc., Rahway, N. J.

(6) Gal and Shulgin, THIS JOURNAL, **73**, 2938 (1951).