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.).

Acknowledgment.—The authors wish to thank Prof. M. Calvin for his interest and assistance in this work.

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

BERKELEY, CALIFORNIA

[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]

BY ALEXANDER T. SHULGIN, OLIVER G. LIEN, JR., EMERY M. GAL AND DAVID M. GREENBERG

RECEIVED JANUARY 31, 1952

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).

threonine free from DL-allothreonine, the allothreonine fraction, nevertheless, contained DL-threonine. The chromatographic method described here separated both isomers quantitatively.

The complete separation of DL-threonine and DL-allothreonine was determined on several small samples and on a final sample of 500 mg. of the mixture. For the latter, an eight-foot by 12-mm. column of cation resin (Dowex 50, 250/500 mesh) in the hydrogen form worked satisfactorily. The extent of the separation was determined by collecting 1.2-ml. fractions on an automatic fraction collector and assaying aliquots of the individual fractions for radioactivity.

Experimental

Preparation of N^{15}-Labeled DL-Threonine.—3.5 g. of the low-melting isomer of α-bromo-β-methoxybutyric acid (m.p. 46–52°) was dissolved in 12 ml. of water in a special pressure flask (Fig. 1A), frozen with liquid nitrogen, attached to a vacuum manifold, and evacuated. Also attached to the manifold was the ammonia generator, consisting of a splash trap, a pressure-equalized separatory funnel, and a 200-ml. round-bottom flask (Fig. 1B). Into this 47.0 g. of ammonium nitrate was introduced containing 11.51% atom excess N^{15} in the NH_3. A solution of 41.1 g. of KOH in 41.1 ml. of water was placed in the separatory funnel and this system was evacuated.

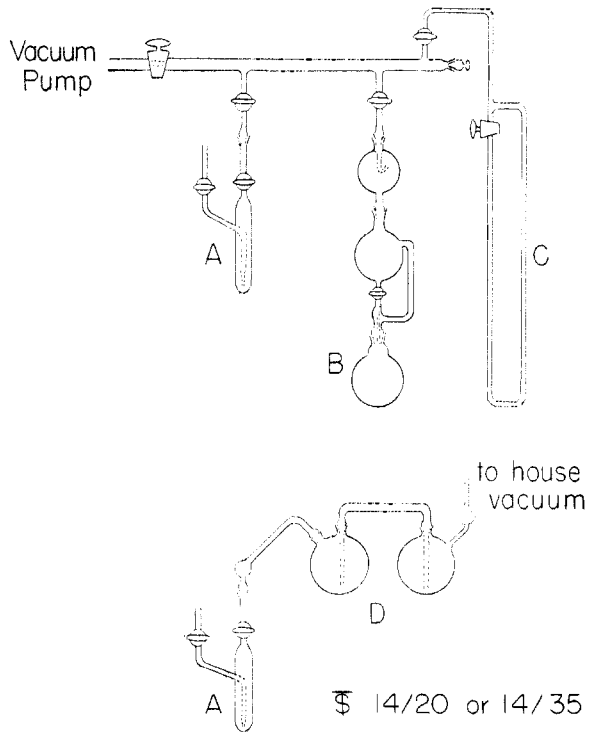


Fig. 1.—Assembly used for the synthesis of N^{15}-DL-threonine.

The KOH solution was then added to the ammonium nitrate at such a rate that the fall of the mercury column in the differential manometer (Fig. 1C) did not exceed 10 cm. The $N^{15}H_3$ was distilled into the reaction flask containing the α-bromo-β-methoxybutyric acid, which was cooled with liquid nitrogen. Finally flask B was heated with a flame until essentially all of the water was driven over. The system then was allowed to stand until the internal pressure, as measured by the manometer, achieved a minimum. Flask A was then closed, removed from the manifold, and allowed to warm slowly to room temperature. The total amount of distillate was slightly more than 30 ml.

The amination of the α-bromo-β-methoxybutyric acid was carried out by heating flask A in a water-bath at 80–85° for 10 hours.[7] Upon completion of the reaction there was present an excess of unreacted $N^{15}H_3$ and, in addition, N^{15} in the form of ammonium bromide and the ammonium salt of O-methyl-DL-threonine.

At the end of the heating period, flask A was equipped with a two-way trap[8] (Fig. 1D) which was attached to the house vacuum line. A mixture of 90 ml. of concentrated HNO_3 and 40 ml. of water was placed in one of the traps, the system was opened to the house vacuum, and, with the flask immersed in the hot water-bath, the excess ammonia was removed. Finally the capillary stopcock was opened to allow a slow stream of air to aid the removal of the water and ammonia until the residue was nearly dry. Flask A was filled with dry acetone[9] and the procedure from this point on, with the exception of the formylation step, was essentially that as outlined.[2] The crude yield was 0.71 g. which, after recrystallization, yielded 0.43 g. of pure white crystalline DL-threonine, m.p. 235–237°. The mother liquors gave an additional 0.21 g. of pure DL-threonine (m.p. 238–240°, sintering at 235°).

Preparation of DL-Threonine and DL-Allothreonine (1,2-C^{14}).—3.6 g. of C^{14}-labeled crotonic acid[10] was converted

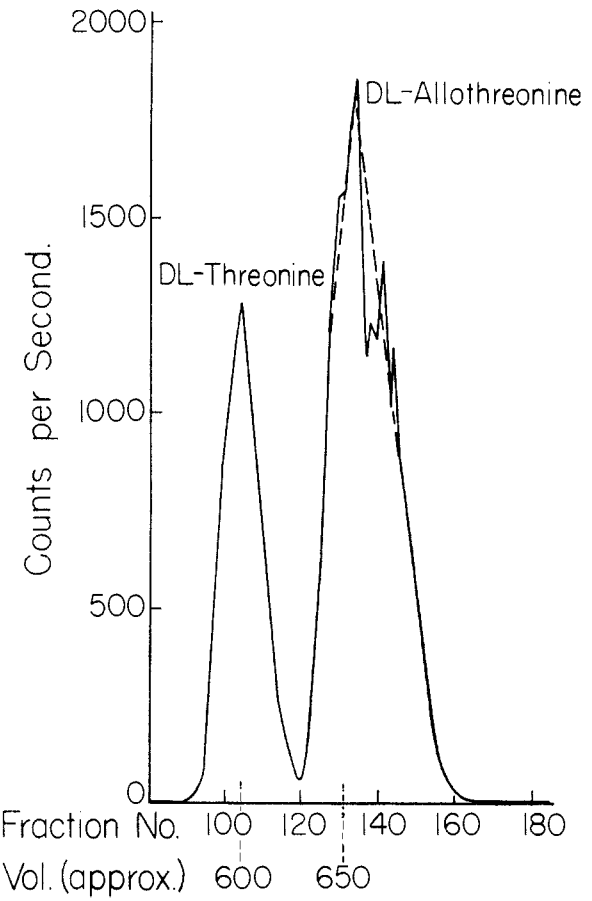


Fig. 2.—Column chromatographic separation of DL-threonine and DL-allothreonine.

(7) There was an invariable loss of ammonia through the stopcocks if the temperature of the bath was allowed to exceed 90°.

(8) Two 300-ml. round-bottom flasks with a bridge tube extending nearly to the bottom of each flask were used.

(9) The ammonium nitrate–nitric acid solution obtained was transferred with ample washing to a porcelain dish, and heated on a steam-bath to dryness. In this manner 41.6 g. of dry ammonium nitrate was recovered, representing a recovery efficiency with respect to the unreacted $N^{15}H_3$ of 97.3%.

(10) The radioactivity of the crotonic acid was 4.3 μc/mg. determined as its sodium salt. The purity was determined by titration, comparing it with a pure sample of Eastman Kodak Co. crotonic acid and also with a sample of crotonic acid synthesized from malonic acid (Eastman Kodak C.P.). Since the yield of our crotonic acid was 93% and the purity was found to be 91%, our final yield was 84% of pure crotonic acid.

into a mixture of DL-threonine and DL-allothreonine.[2] The N-formyl-O-methyl derivative that separated out from the first crystallization (m.p. 140–158°, sintering at 136°) was worked up yielding 0.58 g. of a white material, with a m.p. 213–216°. The final yield of recrystallized material was 0.443 g. (m.p. 232–234°) having an activity of 3.1 μc./mg. The material which was obtained in this manner on large scale runs[2] was reported to be essentially pure DL-threonine; however, under the present conditions, the material thus obtained assayed microbiologically only 53% of DL-threonine. On this scale it was further found that recrystallization of the water-insoluble N-formyl-O-methylthreonine isomer yields a product with a melting point of 164–170° (sintering at 147°) which, after hydrolysis and isolation as above, contains much less of a DL-allothreonine contamination. However, the over-all yield is reduced by 50% as a consequence of this second recrystallization.

DL-Allothreonine was isolated from the filtrate remaining after crystallization of the formyl derivatives according to the same technique as described above for DL-threonine. 0.294 g. of DL-allothreonine (m.p. 239–241°) was obtained. This material was combined with the filtrates from the threonine preparations, and worked up by the chromatographic method described below.

Chromatographic Separation of DL-Threonine and DL-Allothreonine. Preparation of the Column.—A section of Pyrex tubing 16 mm. by 50 in. was constricted at one end in such a manner as to have a small bulb 1 cm. in diameter and an outlet tip 4 mm. in diameter. The bulb was loosely packed with glass wool and another 50-in. section of Pyrex tubing was attached to this by means of a short sleeve of rubber tubing. This column was mounted in position, temporarily closed at the bottom, and filled with water. A 6-in. funnel was attached to the top of the column and sufficient resin to fill the column was added as a slurry in 1.5 *N* HCl. This was permitted to stand until the resin settled to a constant level (40 hours) after which water was allowed to run through the column by gravity flow until the *p*H of the effluent water was 6 (universal *p*H indicator paper).

The excess resin and water in the funnel and the upper 10 cm. of the column were removed with a pipet. The funnel and rubber tubing were detached and as soon as the resin stirred up by the above operation had settled, the water above the resin was removed.

Separation Procedure.—The C^{14}-labeled DL-allothreonine and concentrated DL-threonine mother liquors as described above were dissolved in 6 ml. of water, forced into the column under 18 cm. pressure and followed with 5 ml. washings of water, each washing being forced into the column by pressure. Elution with 1.5 *N* HCl under 18 cm. pressure with the HCl reservoir level with the top of the column was then started. This gave an average flow rate of 5 ml. per hour.

After a forecut of 470 ml. had been collected, a fraction collector designed[11] to collect 15-min. samples was placed under the column and used for the remainder of the separation; 17- by 50-mm. shell vials were used as the receivers.

Analytical Procedure.—The location of the peaks of radioactivity was roughly determined by sampling and counting every tenth fraction. Then in the region containing the activity, 25-microliter aliquots were taken from each fraction and dried on polyethylene counting planchets, which were then counted for radioactivity. The results (Fig. 2)[12] indicate that a very nearly complete separation was effected.

Microbiological assays with *Streptococcus faecalis*, according to the method of Henderson and Snell,[13] indicated that the first compound eluted was DL-threonine and the second was the DL-allothreonine.

(11) Lien, Peterson and Greenberg, *Anal. Chem.* (in press).

(12) Since previous small scale separations of DL-threonine and DL-allothreonine gave regular, symmetrical peaks, the irregularities in the trailing edge of the allothreonine peak are not regarded as significant but are believed to be a result of either overloading of the column or counting irregularities resulting from an extremely high counting rate.

(13) Henderson and Snell, *J. Biol. Chem.*, **172**, 15 (1948).

BERKELEY, CALIFORNIA

[CONTRIBUTION FROM DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

Exchange Reactions of γ-Pyrone and Synthesis of Deuterated Pyrones

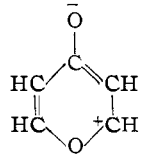
BY R. C. LORD AND W. D. PHILLIPS[1]

For spectroscopic purposes γ-pyrone, γ-pyrone-α-d_2, γ-pyrone-β-d_2 and γ-pyrone-d_4 have been prepared by synthesis and exchange. Exchange between γ-pyrone and deuterium oxide occurs readily at the α-position at *p*H 7. Lower *p*H retards the exchange and at higher *p*H γ-pyrone is unstable. The β-position shows no exchange under the same conditions. γ-Pyrone-d_4 was synthesized by the procedure of Claisen from the appropriate deuterated starting materials. γ-Pyrone-α-d_2 and γ-pyrone-β-d_2 were made by exchange from γ-pyrone and γ-pyrone-d_4, respectively.

γ-Pyrone and its three totally symmetrical deuterium derivatives γ-pyrone-α-d_2, γ-pyrone-β-d_2 and γ-pyrone-d_4 have been prepared to facilitate the analysis of the vibrational spectrum of γ-pyrone. For brevity, these compounds will on occasion be referred to as pyrone, α-d_2, β-d_2 and d_4, respectively. The infrared and Raman spectra and the spectroscopic interpretation will be published elsewhere.

Preparation of the d_4-compound was first attempted by exchange with deuterium oxide at *p*H 7, but only half the hydrogen atoms were found to exchange with appreciable rapidity. To determine whether these were the α- or β-hydrogens, exchange studies were then undertaken with 2,6-dimethylpyrone, in which the α-positions are blocked by methyl groups. It was presumed that the methyl hydrogens would not exchange under these conditions, and this presumption was verified. Furthermore the β-positions showed no exchange, from which the conclusion was drawn that in pyrone itself the α-positions are the active ones. Decrease in *p*H slowed down the rate of exchange.

These results are surprising, in that the usual exchange mechanisms would lead one to expect exchange in the β-position and increased rate of exchange at lower *p*H. It is possible that the mechanism is simply an ionic one in which the α-hydrogens are removed by direct attack by hydroxyl ion, followed by immediate replacement from the solvent molecules. The difference in rate of exchange between the α- and β-positions might then be due to greater positive charge on the α-carbon atom. Positive charge at the α-position would be expected from contribution to the electronic structure of pyrone by the resonance structure



(1) Chemical Department, E. I. du Pont de Nemours and Co., Wilmington, Del.