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THE PREPARATION OF LEUCYLALANINE-2-C^{14}

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Rosemarie Ostwald

May 10, 1956

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ABSTRACT

L-Leucyl-DL-alanine-2-C^{14} with a specific activity of 2.5 μc/mg was prepared in an over-all yield of 22% from alanine-2-C^{14}. Thirty-three percent of the starting activity was recovered as DL-alanine-2-C^{14}. A byproduct of the reaction in a yield of 22% was tentatively identified as an intra- or intermolecular anhydride of the peptide.

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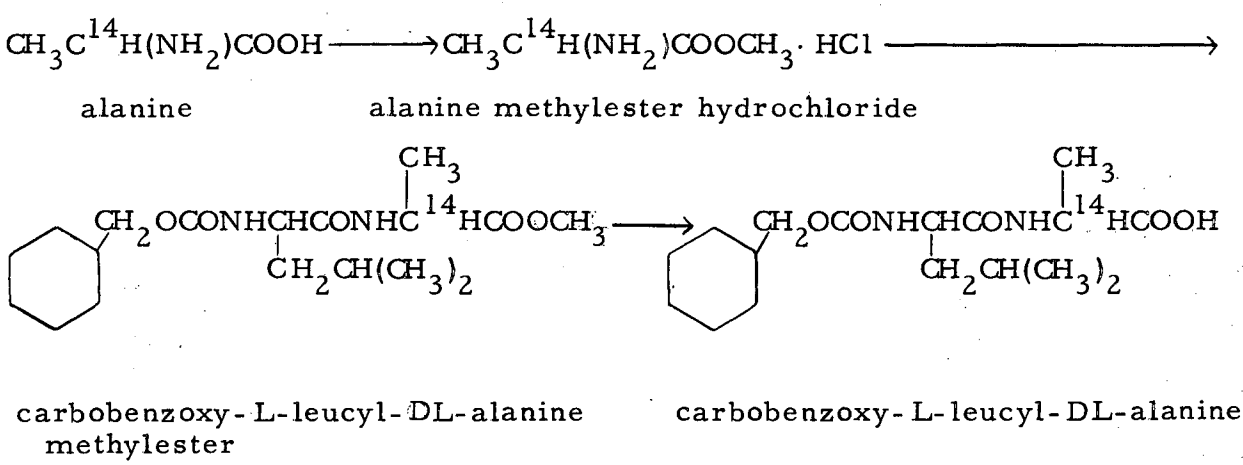
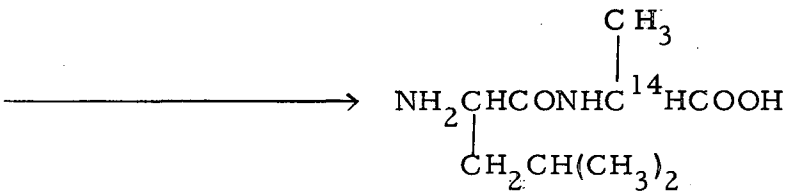
INTRODUCTION

L-Leucyl-DL-alanine-2-C^{14} was prepared as a potentially useful compound for the investigation of the mechanism of protein synthesis in living organisms.

The following sequence of reactions was used in the preparation:





L-leucyl-DL-alanine

Alanine-2-C^{14} was prepared as described previously.[1, 2] The peptide was prepared by an adaptation of the method described by Boissonas.[3] The overall yield of pure peptide from alanine-2-C^{14} was 22%. In addition, 34% of the starting activity was recovered as alanine.

The crude peptide contained about 50% of a radioactive compound which was identified as an intra- or intermolecular anhydride. It was formed during the hydrogenation of carbobenzoxy-leucylalanine from carbobenzoxy-leucylalanine methylester that had remained unsaponified in the preceding step. The same compound has been found to be formed during chromatography of the pure peptide with phenol as solvent. Leucylalanine was obtained as a chromatographically pure compound by the use of an ion-exchange resin.

EXPERIMENTAL

DL-Alanine-2-C^{14} methylester hydrochloride

DL-Alanine-2-C^{14} was dissolved in an excess of absolute methanol that had been saturated with gaseous hydrogen chloride at 0°C. The solution was kept in the refrigerator overnight and the solvent distilled off *in vacuo*. The process was repeated twice. The residue was triturated with ether, filtered, and dried. From 1.34 g alanine containing 10.8 mc, 2.2 g alanine methylester hydrochloride containing 8.2 mc was obtained. This is a radioactive yield of 76%. The ester contained about 15% free alanine and was used without further purification.

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[1] D. E. Pack and B. M. Tolbert, The Reduction of C^{14}-Labeled Carbon Dioxide and Fatty Acids with Lithium Aluminum Hydride, UCRL-1957, Sept. 1952.

[2] R. Ostwald, P. T. Adams, and B. M. Tolbert, J. Am. Chem. Soc. 74 2425 (1952).

[3] R. A. Boissonas, Helv. Chim. Acta 34, 874 (1951).

Carbobenzoxy-L-leucyl-DL-alanine-2-C^{14} methylester hydrochloride

The preparation of the mixed anhydride from carbobenzoxy-L-leucine (commercially obtained) and ethylchloroformate, as well as the condensation of this compound with alanine methylester, was performed in the apparatus shown in Fig. 1. Carbobenzoxy-L-leucine (16 mM) in 18 ml anhydrous toluene was placed in the flask A. DL-Alanine-2-C^{14} methylester hydrochloride (16.2 mM) dissolved in 8 ml toluene and 16.2 mM tri-n-butylamine was placed in the bulb B. All glassware had been thoroughly dried. After the apparatus had been closed, both flasks were chilled to -15°C. In order to form the mixed anhydride 16.2 mM tri-n-butylamine was added to flask A with a syringe and stirred for 15 min. Ethylchloroformate (16.2 mM) was added and the stirring continued for 15 min. The carbobenzoxy-peptide ester was formed by adding the contents of bulb B to the anhydride by turning the bulb around the connecting joint. The bulb was washed by swishing the contents of the apparatus back and forth. The carbon dioxide evolved in the reaction was removed by insertion of a syringe needle at intervals through the syringe bottle stopper. This procedure permits manipulations and the addition of reagents to be performed under anhydrous conditions at low temperatures. The reaction was allowed to proceed with stirring for 90 min at -20°C, for 2 hours at -15 to -5°C and overnight at room temperature.

The carbobenzoxy-peptide methylester was isolated by a series of extractions of the toluene solution. Unreacted alanine and alanine methylester were removed by shaking three times with 1 N hydrochloric acid; unreacted carbobenzoxy-leucine by shaking three times with 10% sodium bicarbonate and once with saturated sodium carbonate. After the toluene solution had been dried with sodium sulfate, the carbobenzoxy-peptide ester was crystallized by the addition of petroleum ether. Carbobenzoxy-peptide ester (8.5 mM) with a melting point of 125°-126°C and containing 5.95 mc C^{14} was obtained. This represents 72% of the radioactivity of the alanine ester used. It proved to be chromatographically pure (0.6% of a radioactive impurity).

Thirty-four percent of the alanine ester activity used was recovered as pure alanine from the acid extract of the toluene solution. The alanine ester was hydrolyzed, the alanine isolated by means of a Dowex-50 column, and purified by sublimation. It had the same specific activity as the starting alanine.

Carbobenzoxy-L-leucyl-DL-alanine-2-C^{14}

The saponification of the carbobenzoxy-peptide ester was performed in 60 ml acetone with 9 ml of 1 N sodium hydroxide at room temperature for 1 hour. After acidification and removal of the solvent by vacuum distillation, the carbobenzoxy-peptide was extracted from the dry residue with acetone, which left the inorganic salt behind. The acetone was distilled off *in vacuo* and the residue taken up in 30 ml methanol.

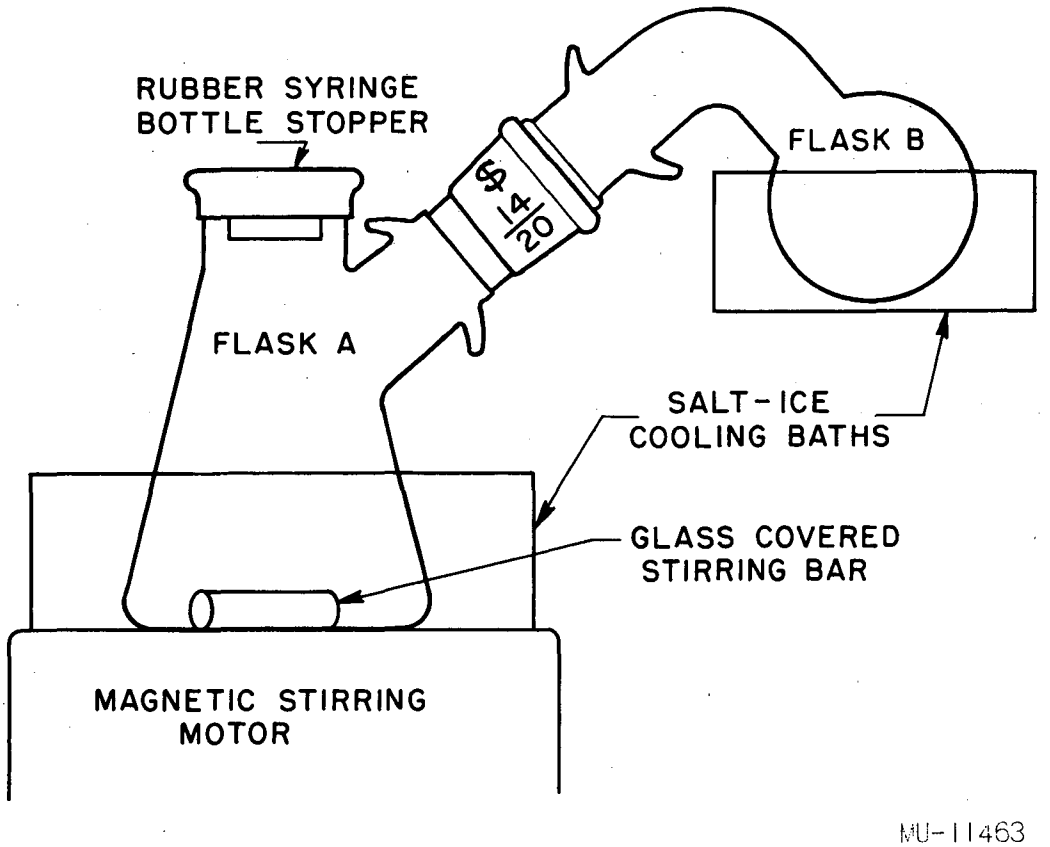

Fig. 1. Reaction Apparatus.

This solution, containing 5.8 mc C^{14}, was used directly for the next step. Subsequent chromatography of an aliquot showed that the saponification in this instance had gone to only 50% completion. In previous experiments this step had gone to about 90% completion.

L-Leucyl-DL-alanine-2-C^{14}

Removal of the carbobenzoxy group was accomplished by hydrogenation of the carbobenzoxy peptide for 3 hours in acidic methanol (30 ml methanol, 1 ml water, and 1 ml acetic acid) at room temperature and atmospheric pressure with palladium on charcoal (0.3 g) as catalyst. The progress of the reaction was followed by trapping the evolved carbon dioxide in a calcium hydroxide solution. After the reaction had gone to completion the catalyst was filtered off and washed with water and methanol. The combined filtrates were evaporated to dryness. The residue contained 4.75 mc C^{14}. This represents 57% of the activity of the alanine ester used. It proved to consist of about 50% peptide and 50% of an intra- or intermolecular anhydride arising during the hydrogenation from the unsaponified carbobenzoxy-peptide ester.

The peptide was obtained chromatographically pure by passage through a Dowex-2 column (in the hydroxyl form) and elution with 2 N acetic acid. The over-all yield from alanine-2-C^{14} to peptide was 22%.

Characterization of the intra- or intermolecular anhydride

The crude peptide obtained as described above showed--upon paper chromatography with either one of the three solvents phenol, butanol-propionic acid, and propanol-water (80-20)--a radioactive contaminant, containing about 50% of the activity applied to the paper. Its R_F value in phenol (0.85) is similar to the R_F of leucine (0.80) and leucylalanine (0.77); in propanol-water its R_F (0.73) is greater than leucylalanine (0.59). Both the peptide and the "unknown" gave, upon hydrolysis with concentrated hydrochlorid acid, leucine and radioactive alanine in about equal amounts. Neither one showed a peak in its ultraviolet spectrum characteristic for the carbobenzoxy group. (The molar extinction coefficient of carbobenzoxy leucine at 257.5 mμ is 1.68×10^3.) Both showed a characteristically different color from leucine when sprayed with ninhydrin containing 10% collidine. Both showed a positive reaction when sprayed with a potassium iodide-starch solution after the paper had been chlorinated, a test for amino- or amido nitrogen.[4] In contrast to the peptide, the "unknown" showed no ninhydrin color (or only at very much higher concentrations), and it contained no carboxyl group as shown by titration. Its analysis (C, 57.02%; H, 8.61%; N, 14.05% did not allow a positive identification. The analysis for leucylalanine would be C, 53.8%; H, 8.93%; N, 13.9%; for leucylalanine anhydride the figures would be C, 58.69%; H, 8.69%; N, 15.2%.

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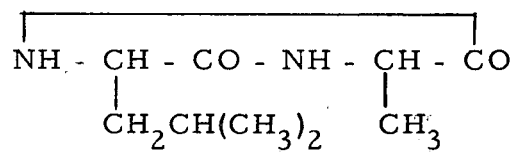
[4] H. Rydon, Nature 169, 923 (1952).

When the "unknown" was cochromatographed with synthetic unlabeled leucylalanine anhydride in propanol-water or butanol–propionic acid, the radioactivity coincided exactly with the potassium iodide color. From these observations it seems likely that the "unknown" is an intra- (or possibly an inter-) molecular anhydride of the following constitution:



A compound of very similar properties is formed when pure leucylalanine is chromatographed with phenol. This solvent is therefore to be avoided.

ACKNOWLEDGMENTS

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