[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT AND THE UNIVERSITY OF ROCHESTER]

THE SYNTHESIS OF DL-LYSINE WITH C^{14} IN THE EPSILON POSITION[1]

PAUL OLYNYK, DAVID B. CAMP, ARVON M. GRIFFITH, SIEGFRIED WOISLOWSKI, R. W. HELMKAMP

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In the attempt to prepare DL-lysine labeled with C^{14} for use in metabolic studies, our initial efforts were directed toward the development of a method which would permit the radioactive carbon to be introduced at a late stage in the process so that a large percentage of the C^{14} used would appear in the final product. The value of such a procedure would be increased if an intermediate formed prior to the introduction of the C^{14} offered the possibility of resolution into optically active forms. In accordance with these requirements, we sought to synthesize an α-acetamido-δ-halovaleric acid. It was hoped that this compound could be transformed by the action of $KC^{14}N$ into α-acetamido-δ-cyanovaleric acid, which on reduction of the cyano group and subsequent hydrolysis of the acetamido group should yield DL-lysine with C^{14} in the epsilon position.

Our attempts to prepare the desired valeric acid were unsuccessful. When equimolecular amounts of diethyl acetamidomalonate (*cf.* 1) and sodium ethoxide were refluxed in anhydrous ethanol with (a) an equimolar amount of 1-bromo-3-chloropropane (in the absence and in the presence of sodium iodide), (b) an equimolar amount of 1-chloro-3-iodopropane, and (c) four molar equivalents of 1,3-dibromopropane, oils were obtained from which no crystalline product other than unchanged acetamidomalonic ester could be isolated. Refluxing sodioacetamidomalonic ester (*cf.* 2) for approximately twenty-four hours with three molar equivalents of 1,3-dibromopropane in dioxane solution resulted in a practically quantitative formation of sodium bromide. However, nearly all of the acetamidomalonic ester was recovered from the reaction mixture (*cf.* 3).

Treatment of acetamidomalonic ester with 1-bromo-3-phenoxypropane in sodium ethoxide solution gave the expected product, diethyl acetamido-(3-phenoxypropyl)malonate. This ester was transformed by alkaline hydrolysis into α-acetamido-δ-phenoxyvaleric acid, and by heating with 48% hydrobromic acid into α-amino-δ-phenoxyvaleric acid (*cf.* 4), but attempts to cleave the ether linkage by refluxing the ester in an acetic acid solution of hydrogen bromide and in constant boiling hydriodic acid gave no satisfactory result.

Meanwhile the great reduction in the price of C^{14} made it less important that the introduction of the radioactive carbon occur in a late step in the synthesis. The feasibility of adapting a known procedure for preparing lysine to the production of this compound labeled with C^{14} was therefore considered. Of the methods described in the literature (5, 6, 7, 8, 9, 10), only that of von Braun (7) as improved by Eck and Marvel (9) and more recently by Galat (10) has been found

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to give a satisfactory yield. However, this synthesis offers no opportunity for introducing C^{14}, since the starting material (cyclohexanone or ϵ-caprolactam) already contains the six-carbon chain which appears in the lysine prepared from it. Of the remaining methods, that of Fischer and Weigert (5) seemed to be the most promising, even though their over-all yield was not high and the isolation of the lysine dihydrochloride by way of the phosphotungstate and picrate is cumbersome. We have succeeded in modifying their procedure so that their reaction sequence forms now the basis of a satisfactory method for preparing this amino acid.[2]

Below are summarized the steps in the synthesis and the principal modifications made in the original procedure which resulted in the yields indicated.

A. $KCN + Cl(CH_2)_3Br \xrightarrow{75\text{–}82\%} Cl(CH_2)_3CN$. The procedure was essentially that given in Organic Syntheses (11). However, since the object was to obtain maximum conversion of $KC^{14}N$ into $Cl(CH_2)_3C^{14}N$, two molar equivalents of 1-bromo-3-chloropropane to one of potassium cyanide were used. In order to obtain a high yield of the nitrile, an exhaustive extraction of the aqueous alcohol solution with chloroform was necessary. In the distillation, a cut was taken over a rather wide temperature range in order that it include the γ-bromobutyronitrile formed in the reaction, since obviously this compound can be used jointly with its chloro analog in step B.

B. $Cl(CH_2)_3CN + CH_2(COOC_2H_5)_2 \xrightarrow{74\%} NC(CH_2)_3CH(COOC_2H_5)_2$. The γ-chlorobutyronitrile was allowed to react with sodio-malonic ester (prepared by the action of alcohol-free sodium ethoxide on the ester) in an excess of the ester as solvent,[3] first at room temperature and finally at that of the steam-bath.[4]

C. $NC(CH_2)_3CH(COOC_2H_5)_2 + C_2H_5ONO + NaOC_2H_5 \xrightarrow{70\text{–}83\%} NC(CH_2)_3C(=NOH)COOC_2H_5$. The alcohol was not removed, as in the procedure of Fischer and Weigert, by evaporation in a vacuum desiccator (*cf.* 12). Instead, the reaction mixture was diluted with ice-water and maintained at as near 0° as possible by further addition of ice during the subsequent extraction with ether, acidification, and reextraction with ether. It was found that washing the ether solution of the oximino ester with a solution of sodium bicarbonate removed most of the colored impurities, so that an almost white, crystalline product was obtained on evaporation of the ether.

D. $NC(CH_2)_3C(=NOH)COOC_2H_5 \xrightarrow{73\%} NH_2(CH_2)_4CHNH_2COOH \cdot HCl$ (recrystallized). The oximino ester was reduced catalytically (PtO_2) in acetic

[2] In a private communication, Professor A. J. Haagen-Smit has recently informed us that the synthesis of DL-lysine labeled with C^{14} in the epsilon position has also been accomplished by the Fischer and Weigert method at the California Institute of Technology.

[3] The use of malonic ester as the solvent in this reaction was suggested to us by Dr. N. F. Albertson of the Winthrop Chemical Company.

[4] The attempt was made to prepare γ-cyanopropylmalonic ester by inverting steps (A) and (B): $Cl(CH_2)_3Br \rightarrow Cl(CH_2)_3CH(COOC_2H_5)_2 \rightarrow NC(CH_2)_3CH(COOC_2H_5)_2$. While γ-chloropropylmalonic ester was obtained in good yield by the method of Fischer and Bergmann [*Ann.*, **398**, 120 (1913)], the nitrilation proceeded slowly and was accompanied by other reactions.

anhydride solution at 50–60° under a hydrogen pressure of 50–60 lbs./sq. in. The acetylated lysine ester was not isolated but was hydrolyzed directly to lysine dihydrochloride by refluxing it in hydrochloric acid solution. Conversion of the dihydrochloride into the monohydrochloride and recrystallization of the latter were carried out according to the directions in Organic Syntheses (13). The identity of the product was confirmed by its transformation into the dibenzoyl derivative.

To produce radioactive lysine monohydrochloride, $HC^{14}N$ liberated on acidification of $KC^{14}N$ [prepared by the method of Cramer and Kistiakowsky (14, 15, 16; *cf.* 17] was absorbed in excess potassium hydroxide solution. After its cyanide content had been determined by titration with silver nitrate, the solution was acidified, the cyanide precipitated as silver cyanide, and the latter dissolved in a water-alcohol mixture containing a known amount of potassium cyanide. This solution was then used in the preparation of radioactive γ-chlorobutyronitrile. The over-all yield of the recrystallized lysine monohydrochloride, based on the cyanide used, was 28.4%; however, its activity per millimole was found to be only about 85% of the calculated.

EXPERIMENTAL

The preparation of $KC^{14}N$. The tubes used in the preparation of the radioactive potassium cyanide were heavy-walled Pyrex combustion tubes approximately 25 cm. long and 22 mm. o.d., drawn down to 4 mm. i.d. at each end. To connect the tube to the vacuum line in such a way that it could be rotated into either a horizontal or vertical position, a ground glass joint was sealed to one end at an angle of 120°. The other end was sealed into a test tube, 16 mm. o.d. to form a T. Into the latter was introduced potassium which had been weighed under toluene. After the toluene had been pumped off, the open end of the test tube was sealed, and the bomb tube, held horizontally, was evacuated with a mercury diffusion pump. The potassium, heated by a small direct flame, was gradually distilled into the bomb. From one to two grams of potassium could be deposited as a mirror on the wall of the bomb; if more was used in a run, it was collected in a small heap near the middle of the tube. The test tube was sealed off and discarded.

With the bomb tube in an almost vertical position, one millimole of carbon dioxide and two millimoles of ammonia per gram of potassium were frozen into it by use of liquid nitrogen. The quantities of gases were measured with a calibrated flask and a manometer attached to storage flasks. The carbon dioxide was generated by addition of concentrated sulfuric acid to the radioactive carbonate. The ammonia was cylinder ammonia passed over sodium hydroxide pellets.

The bomb tube was sealed off from the vacuum line, allowed to warm up for five minutes, then heated, with frequent rotation, for twenty minutes in a combustion furnace (30 cm. long), whose mid-point temperature was maintained at 630°. After the tube had been removed from the furnace and had cooled to room temperature, it was connected by wide-bored suction tubing to a dropping-funnel and a condenser attached to the vacuum line through a ground glass joint. The space to the bomb tube was evacuated, the tip of the bomb was broken off in the rubber tubing, and carbon dioxide-free water was allowed to drip into the tube from the dropping-funnel. After the excess potassium had reacted, the tube was removed from the vacuum line, and its contents were rinsed into a flask.

The determination of the cyanide in the solution could not be carried out directly by titration with silver nitrate because of the formation of a small amount of black precipitate (apparently free silver), which obscured the end-point. The solution was therefore acidified with dilute nitric acid, and the liberated hydrogen cyanide and carbon dioxide boiled

out and absorbed in 50% carbonate-free potassium hydroxide solution. To recover the carbonate, the alkaline solution was drained into saturated barium nitrate solution, and the precipitated barium carbonate filtered and washed in a carbon dioxide-free atmosphere. The cyanide in the filtrate was determined by titration with silver nitrate in the presence of ammonia and 0.1 millimole of potassium iodide as indicator. The solution was then acidified with nitric acid, and the cyanide precipitated by further addition of silver nitrate.

The barium carbonate used had an activity of 211 μc. per millimole. The yields of cyanide in three runs with 1.06, 5.89, and 5.40 millimoles of carbonate were 70, 61 and 75% respectively. The average yield accordingly was 68%, and the total yield 8.40 millimoles. Approximately 18% of the radioactivity was recovered as barium carbonate.

Preparation of $Cl(CH_2)_3C^{14}N$. The precipitated radioactive silver cyanide obtained from the three runs mentioned above was filtered on a sintered glass funnel and as much as possible transferred with a spatula to a three-necked flask containing 18 g. of 95% potassium cyanide. The funnel and the spatula were then rinsed, first with a solution of 2 g. of 95% potassium cyanide in 20 ml. of water, and then with 10 ml. of water. Addition of these washings to the reaction flask produced a solution of 0.3 mole of cyanide in 30 ml. of water. After the solution had stood overnight, 100 g. of 1-bromo-3-chloropropane (0.6 mole) and 110 ml. of ethanol were added, and the mixture was refluxed for three hours. It was then treated with 120 ml. of water and 35 ml. of chloroform, and the aqueous layer was extracted six times with 40-ml. portions of chloroform. The combined chloroform extracts were repeatedly washed with 50-ml. portions of 20% calcium chloride solution and dried over calcium chloride. After decantation from the calcium chloride (which was then washed with additional chloroform), the solution was fractionally distilled, first at atmospheric pressure until a temperature of 120° was attained, and then under a pressure of 26 mm. The fractions boiling at 60–82° and 82–105° at the latter pressure were redistilled, and the product with the boiling range 90–100° collected; yield 23.4 g. (75%). In a preliminary run in which the cyanide used was non-radioactive, a yield of 82% was obtained.

Preparation of $NC^{14}(CH_2)_3(COOC_2H_5)_2$. A solution of 5.2 g. of sodium in 75 ml. of dry ethanol (18) was heated under diminished pressure on a steam-bath until no more solvent could be removed. Atmospheric pressure was then restored by permitting illuminating gas to enter the system. The white sodium ethoxide so obtained was cooled in ice-water, 127.6 g. of diethyl malonate (0.79 mole) was added, and the mixture was then shaken until complete solution of the ethoxide had occurred. By means of 75 ml. of dry ether, the 23.4 g. of radioactive γ-chlorobutyronitrile (0.226 mole) was rinsed into the reaction flask, and the mixture was allowed to stand overnight at room temperature. It was then refluxed on a steam-bath until evening, when the ether was removed as far as possible by distillation on a steam-bath. To ensure completeness of the reaction, the mixture was gently warmed under a reflux condenser on a steam-bath until the following morning. The precipitated sodium halides were taken up in ice-water, and the mixture was thoroughly extracted with ether. The ethereal solution was dried over sodium sulfate, concentrated on a steam-bath, and the residual oil was then subjected to fractional distillation under reduced pressure. After removal of the excess malonic ester, the distillation temperature rose rapidly to the boiling point of the product, which distilled at 138–143° at 1.1 mm., leaving only a small amount of colored material in the distilling flask; yield 38 g. (74%).

Preparation of $NC^{14}(CH_2)_3C(=NOH)COOC_2H_5$. A solution of 37.9 g. of radioactive γ-cyanopropylmalonic ester (0.167 mole), 90 ml. of dry ethanol, and approximately 15 g. of ethyl nitrite (0.2 mole) was cooled in an ice-salt mixture contained in a large Dewar flask. An ice-cold solution of sodium ethoxide, prepared by dissolving 3.83 g. of sodium in 75 ml. of dry ethanol, was added, the Dewar flask was covered, and the mixture was allowed to stand for twelve hours. After addition of 500 ml. of ice-water, the solution was extracted three times with ice-cold ether (total volume 300 ml.), and the ether extracts in sequence washed with ice-water, which was then added to the original solution. While kept cold by immersion in an ice-bath, the aqueous solution was made acid to Congo Red by addition of 3 *N* hydrochloric acid. It was then extracted six times with ether (total volume 900 ml.).

The ether extracts were washed twice with a saturated solution of sodium bicarbonate and twice with water, and then combined. After the ethereal solution had been dried over sodium sulfate, it was concentrated by distillation on a steam-bath. The residue in the distilling flask was then rinsed with a small amount of ether into a crystallizing dish, warmed for a short time on a steam-bath, transferred to a vacuum dessicator, and the remaining solvent pumped off as the oximino ester crystallized. The product was then remelted on a steam-bath and again allowed to crystallize in the evacuated desiccator; yield 21.5 g. (70%); activity 4.6 μc. per millimole. Yields of 83, 82, and 77% were obtained in three preceding runs in which the γ-cyanopropylmalonic ester was nonradioactive. In each of these runs the total volume of ethanol used (about 100 ml.) was less than in the run involving the radioactive ester, and the reaction mixture was kept not quite so cold and was allowed to stand for a somewhat longer period.

Preparation of $NH_2C^{14}H_2(CH_2)_3CHNH_2COOH \cdot HCl$. A mixture of 11.1 g. of the radioactive oximino ester, 60 ml. of acetic anhydride, and 0.3 g. of platinic oxide (Adams' catalyst) was shaken at 50° with hydrogen, the pressure of which was maintained at 50–60 lbs./sq. in. After about 65% of the amount of hydrogen corresponding to complete reduction of the cyano and oximino groups to amino groups had been absorbed, another portion of catalyst was added, and the temperature increased to 60°. When reduction was 85% complete, 20 ml. of acetic anhydride and a third portion of catalyst were added, the temperature was raised to 65°, and the shaking was continued until the calculated absorption of hydrogen had occurred.

After the acetic anhydride had been decomposed by treatment with cold water, the catalyst was filtered on a sintered glass funnel and washed with water. To hydrolyze the acetylated lysine ester, the filtrate was refluxed over night with one and one-half volumes of concentrated hydrochloric acid. The solution was then concentrated to a yellow syrup by heating under reduced pressure on a steam-bath. A small amount of concentrated hydrochloric acid was added to the residue, the solvent again evaporated, and this step repeated. The separation of the lysine dihydrochloride, its conversion into the monohydrochloride, and the recrystallization of the latter were carried out according to the procedures in Organic Syntheses (13); yield of recrystallized lysine monohydrochloride 8.0 g. (73%); m.p. 264–266° with decomposition; activity 5.0 μc. per millimole.

It is important in the reduction of the oximino ester that the theoretical amount of hydrogen be absorbed. It was found that at 50° approximately 70% of the calculated decrease in pressure occurred usually within fifteen hours. However, for complete reduction three to five days were required, during which the temperature was increased to 65° and additional portions of catalyst were added. When the hydrogenation was discontinued after absorption had reached 80–85% of the theoretical, poor yields of lysine monohydrochloride were obtained.

Diethyl acetamido-(3-phenoxypropyl)malonate. This compound was prepared by refluxing a solution of 1 part of sodium, 9.35 parts of acetamidomalonic ester, and 10 parts of 1-bromo-3-phenoxypropane in 60 parts of absolute ethanol; yield of recrystallized product (from alcohol-water solution) 60.6%; m.p. 78.5°.

Anal. Calc'd for $C_{18}H_{25}NO_6$: C, 61.3; H, 7.2.
Found: C, 61.8; H, 7.0.

α-Acetamido-δ-phenoxyvaleric acid. A mixture of 3.2 g. of diethyl acetamido-(3-phenoxypropyl)malonate and 10 ml. of 20% sodium hydroxide solution was refluxed for two and one-half hours. It was then treated with 3.3 ml. of concentrated hydrochloric acid, refluxed for another hour, and filtered while hot. The product which separated on cooling was recrystallized from water; fine needles, melting at 150°.

Anal. Calc'd for $C_{13}H_{17}NO_4$: C, 62.1; H, 6.8.
Found: C, 62.3; H, 6.8.

α-Amino-δ-phenoxyvaleric acid. This compound was formed when diethyl acetamido-(3-phenoxypropyl)malonate was refluxed in 48% hydrobromic acid (1 g. of ester to 4.5 ml. of acid). Although it is not very soluble in water, it can be recrystallized from this solvent.

It dissolves readily in both alkali and acid, and when treated with ninhydrin solution, gives the color reaction obtained with α-amino acids. It melts with decomposition *ca.* 255°.

Anal. Calc'd for $C_{11}H_{15}NO_3$: C, 63.1; H, 7.2; N, 7.1.
Found: C, 63.7; H, 7.1; N, 6.9.

The radioactivity determinations were made by the method developed by Dr. William Bale and associates in the Radiology Department. The authors wish to acknowledge their indebtedness to Dr. Raymond Masters and his co-workers for carrying out these determinations.

SUMMARY

Improvements in the Fischer and Weigert synthesis of DL-lysine are described. DL-Lysine with C^{14} in the epsilon position has been prepared by this modified procedure.

ROCHESTER, N. Y.

REFERENCES

(1) SNYDER, SHEKLETON, AND LEWIS, *J. Am. Chem. Soc.*, **67**, 310 (1945).
(2) SNYDER AND SMITH, *J. Am. Chem. Soc.*, **66**, 351 (1944).
(3) DUNN AND SMART, *J. Biol. Chem.*, **89**, 45 (1930).
(4) ALBERTSON AND ARCHER, *J. Am. Chem. Soc.*, **67**, 309 (1945).
(5) FISCHER AND WEIGERT, *Ber.*, **35**, 3772 (1902).
(6) SÖRENSEN, *Compt. rend. trav. Lab. Carlsberg*, **6**, 1 (1903).
(7) VON BRAUN, *Ber.*, **42**, 839 (1909).
(8) SUGASAWA, *J. Pharm. Soc. Japan*, **550**, 1044 (1927).
(9) ECK AND MARVEL, *J. Biol. Chem.*, **106**, 387 (1934).
(10) GALAT, *J. Am. Chem. Soc.*, **69**, 86 (1947).
(11) *Organic Syntheses*, Coll. Vol.I, 2nd ed. (John Wiley and Sons, New York, **1941**), p. 156.
(12) SHIVERS AND HAUSER, *J.Am. Chem. Soc.*, **69**, 1265 (1947).
(13) *Organic Syntheses*, Coll. Vol. II (John Wiley and Sons, New York, **1943**), p. 374.
(14) CRAMER AND KISTIAKOWSKY, *J. Biol. Chem.*, **137**, 549 (1941).
(15) LOFTFIELD, *Nucleonics*, **1**, 54 (1947).
(16) FRANTZ, LOFTFIELD, AND MILLER, *Science*, **106**, 544 (1947).
(17) ADAMSON, *J. Am. Chem. Soc.*, **69**, 2564 (1947).
(18) *Organic Syntheses*, Coll. Vol. II (John Wiley and Sons, New York, **1943**), p. 155.