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Synthesis of L-Glutamine-5-^{14}C, L-Glutamic Acid-5-^{14}C, and L-Ornithine-5-^{14}C

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SUMMARY

On alkylation of K^{14}CN with the methyl ester of L-α-benzyloxycarbonylamino-γ-bromobutyric acid, the methyl ester of L-α-benzyloxycarbonylamino-γ-cyano-^{14}C-butyric acid (II) was obtained The latter was submitted to mild alkaline hydrolysis to yield the free nitrilo acid III which on treatment with hydrogen bromide solution in acetic acid gave L-glutamine-5-^{14}C in one reaction step. L-Ornithine-5-^{14}C was prepared by catalytic hydrogenation of the nitrilo ester II, and L-glutamic-5-^{14}C acid was obtained on acid hydrolysis of the same compound (II). The activated ester (2, 4, 5-trichlorophenyl ester) of acid III may serve as a suitable intermediate for the preparation of glutamine or ornithine peptides. All prepared substances were optically active L-forms.

INTRODUCTION.

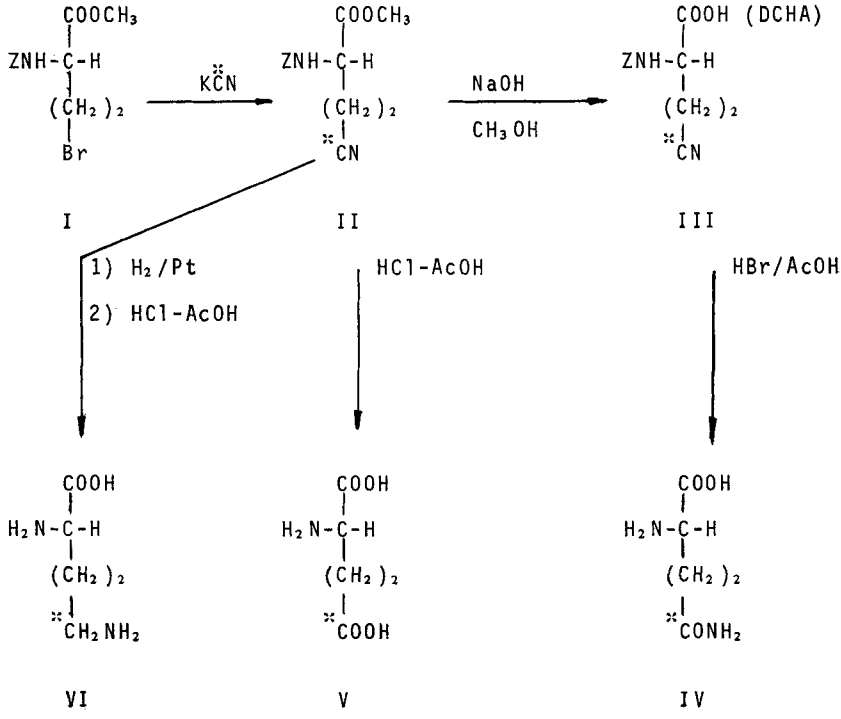
Most literature data on glutamine-^{14}C refer to universally labelled glutamine-^{14}C prepared from glutamic-^{14}C(U) acid [1,2,3,4]. Barry [5] prepared glutamine-^{14}C from glutamic-1-^{14}C acid, but only in the form of its racemate, in a 30 % yield, *via* the phthaloyl derivatives of glutamic acid and glutamine.

In this paper the preparation of specifically labelled glutamine-5-^{14}C of natural L-configuration is described, the starting radioactive precursor also being suitable for the preparation of other five-carbon-atom L-amino acids, as for example L-glutamic acid and L-ornithine, labelled specifically in the position 5. Most of the known syntheses of glutamine start from glutamic acid. They usually involve at least four steps with 40-60 % yields. One of the most successful seems to be the method of Rudinger and Zaoral [6], giving a 90-95 % yield (taking the starting N-tosyl-L-glutamic acid as the basis of calculation). However, for radioactive syntheses the yields may be smaller. In

addition, the starting glutamic-^{14}C acid is relatively expensive, accessible either biosynthetically (universally labelled, in L-form), or by a multistep chemical synthesis in the form of a racemate (For the sake of brevity we only refer to the monograph of Murray and Williams [7]).

Mention has already been made that we endeavoured to introduce a radioactive carbon atom into the position 5 of the carbon chain. A similar idea was also followed by Pichat and co-workers [8] who obtained on cleavage of DL-α-benzamido-γ-butyrolactone with potassium cyanide-^{14}C in dimethylformamide at 150° C the potassium salt of DL-α-benzamido-γ- cyanobutyric acid in 58 % yield. This N-benzoyl derivative is not suitable for the synthesis of glutamine-^{14}C, but it is a good starting material for the preparation of glutamic-5-^{14}C acid and ornithine-5-^{14}C and arginine-5-^{14}C. The obtained substances are again only racemates. The cleavage of α-phthalimido-γ-butyrolactone with potassium cyanide-^{14}C was studied by Vereš [9], but the yield of DL-glutamic acid obtained on hydrolysis of the crude nitrile was only 21 %.

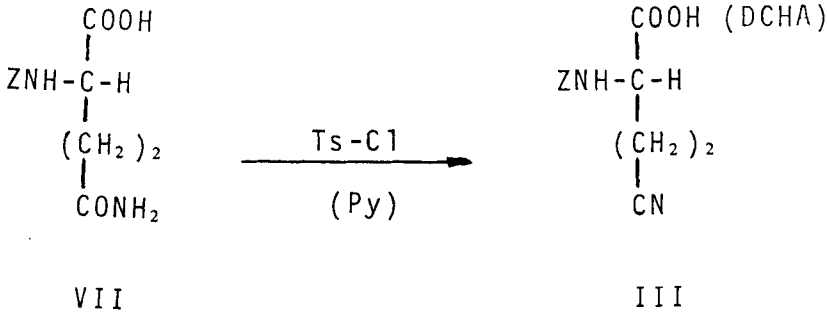
N-Substituted α-amino-γ-cyanobutyric acids are also accessible by other routes; the dehydration of N-substituted glutamines with various reagents (as e.g. tosyl chloride in pyridine [10], dicyclohexylcarbodiimide in pyridine [11], dimethylchloromethyleneammonium chloride [11], p-toluenesul-



fonic acid or phosphorus oxychloride [13], etc.), is the usual procedure. These procedures were not suitable for our purpose, although dehydration may be carried out under the preservation of the optical purity.

The basic compound of our procedure is the methyl ester of L-α-benzyloxycarbonylamino-γ-cyano-^{14}C-butyric acid (II) which was prepared on reaction of potassium cyanide-^{14}C, with methyl ester of L-α-benzyloxycarbonylamino-γ-bromobutyric acid (I) the preparation of which was described by Jošt and Rudinger [14] (We thank Dr Jošt for the samples of this substance and for giving us the detailed procedure for its preparation.). The alkylation takes place best in dimethylsulfoxide at room temperature and with a high chemical yield. The lower radiochemical yield is caused evidently by impurities in K^{14}CN. The benzoyloxycarbonyl substituent is designated by Z in the formulas.

The nitrile II obtained in this manner has the same optical rotation value as the substance prepared by Itoh [15] on dehydration of the methyl ester of benzyloxycarbonyl-L-glutamine with p-toluenesulfonyl chloride, but the melting point was 8° C higher. Alkaline hydrolysis of the ester group of nitrile II gave the free acid III which was isolated in the form of its dicyclohexylammonium salt. We also prepared this substance in its non-radioactive form for comparative purposes by dehydration of benzyloxycarbonyl-L-glutamine (VII) with p-toluenesulfonyl chloride in 69 % yield :



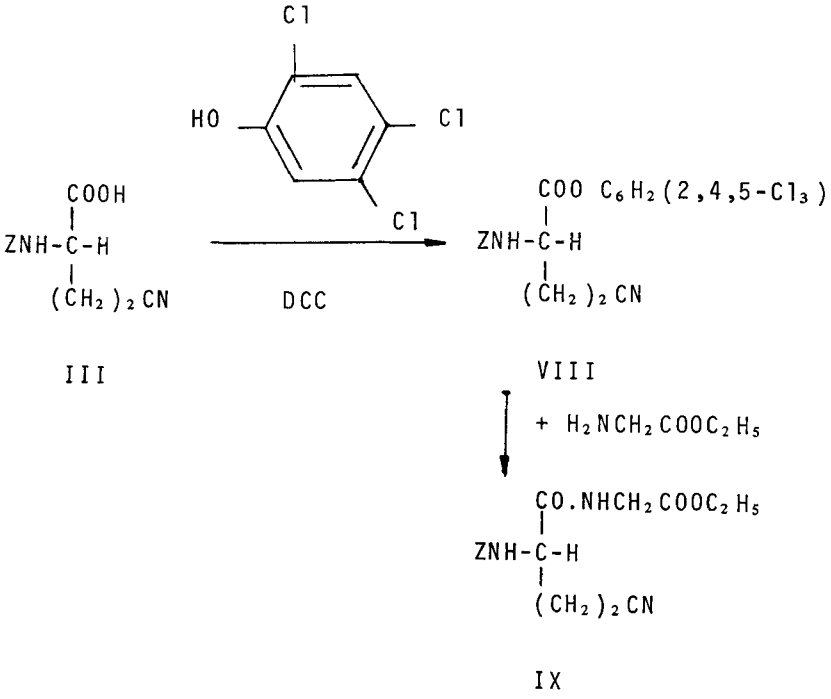
VII III

Using hydrogen bromide in acetic acid the free nitrilo acid III can be converted into L-glutamine by hydration of the nitrile group and the elimination of the protecting group on the α-nitrogen. During the work with radioactive nitrile III we obtained L-glutamine-5-^{14}C in 63 % chemical and 57 % radiochemical yield (nitrilo-ester II as the basis of calculation). Hydrogenation of nitrilo-ester II with Adams catalyst and subsequent acidolysis afforded L-ornithine-5-^{14}C in 26 % radiochemical yield. The same nitrilo-ester may serve as a suitable starting material for the synthesis of L-ornithine-(5,5-$^{3}H_2$). It would also be possible to prepare γ-cyano-^{14}C-L-α-aminobutyric acid from III on selective elimination of the carbobenzoxyl group [11] with sodium in anhydrous liquid ammonia.

On the other hand, a strong acid hydrolysis of the nitrilo ester (II) gave L-glutamic-5-^{14}C acid in 38 % radiochemical yield. Chemical yields of L-glutamic-5-^{14}C acid and L-ornithine-5-^{14}C were not calculated, because the available amount of final products was too small.

Recently Ressler and Giza [16] described a synthesis of L-β-cyanoalanine-4-^{14}C, L-asparagine-4-^{14}C, and L-aspartic-4-^{14}C acid based also on the alkylation of K^{14}CN with diethyl α-formamido-α-dimethylamino methylmalonate methiodide. Racemic intermediates were thus obtained which had to be resolved enzymatically.

The easy transformation of the nitrile group into the amide group or the aminomethyl group, and the easy elimination of the protecting groups offers the possibility of utilizing the nitrilo acid III for the synthesis of L-glutamine or L-ornithine-^{14}C. In order to check this possibility we prepared the 2, 4, 5-trichlorophenyl ester of L-α-benzyloxycarbonylamino-γ-cyanobutyric acid (VIII) which, being an activated ester, condenses easily with the free amino group of another amino acid.



The yields obtained show that this route is promising, but a further elaboration of this method is out of the scope of this study.

EXPERIMENTAL.

The melting points were carried out predominantly on a Kofler block and are not corrected. Similarly as optical rotations, they were determined with non-radioactive compounds (For the measurement of optical rotations, our sincere thanks are due to Dr Frič of the Institute of Organic Chemistry and Biochemistry of the Czechoslovak Academy of Sciences, Prague.)

Radioactivity measurements were carried out on Mark I Liquid Scintillation Counter (Nuclear Chicago). Paper chromatography was carried out by descending technique on Whatman No. 1 paper, preparative chromatography on Whatman No. 3 paper.

Radioactive chromatograms and electrophorograms were scanned on Frieseke and Hoepfner apparatus.

L-α-Benzyloxycarbonylamino-γ-bromobutyric acid methyl ester (I).

Ester I was prepared from L-α-tosylglutamic acid by a six step synthesis making use to the procedure of Jošt and Rudinger [14], with practically the same yields. M.p. 60-61° C (capillary); $(\alpha)_D^{25}$ 40.1° (c 0.51; dimethylformamide).

Methyl L-α-benzyloxycarbonylamino-γ-cyanobutyrate (II).

A mixture of I (3.3 g; 10 mmole) and potassium cyanide (0.65 g; 10 mmole) in dimethylsulfoxide (12 ml) was stirred under nitrogen with a magnetic stirrer at room temperature for 72 hours (pH of the solution at the end of the reaction was 7). After the addition of water (15 ml) a continuous extraction with ether was carried out (3 hours). The ethereal solution was dried over magnesium sulfate and evaporated to dryness. The oily residue crystallised when exposed to a mixture of ether and light petroleum at a low temperature. The m.p. of the product was 59-60° C, yield 2.56 g (90 %). For analysis, the crystallisation was repeated from a mixture of ethyl acetate and light petroleum; For $C_{14}H_{16}N_2O_4$ calculated : 60.86 % C, 5.84 % H; found : 60.56 % C, 5.94 % H.

$(\alpha)_D^{25}$ —33.2° (c 0.50; methanol), Itoh [15] gives

$(\alpha)_D^{21}$ —34.5° (c 3; methanol).

Dicyclohexylammonium salt of L-α-benzyloxycarbonylamino-γ-cyanobutyric acid (III).

(*a*) Saponification of ester II.

A solution of methyl ester II (0.60 g; 2.17 mmole) in 4 ml of methanol and 1.4 ml of 2N—NaOH was stirred at room temperature for one hour. The solution was partly concentrated in vacuo and acidified with dilute hydrochloric acid (1 : 1) to pH approx. 2. The liberated acid was extracted with

ether (3 × 10 ml). The combined ethereal extracts were extracted with water and dried over sodium sulphate. After evaporation of the solvent the free acid III was obtained in the form of an oil (456 mg; 81 %). It was dissolved in 5 ml of ether and mixed with dicyclohexylamine (0.35 ml), affording a white crystalline precipitate which was allowed to stand in a refrigerator overnight. After filtration with suction and washing with ether it was crystallised from a mixture of ethyl acetate and light petroleum. Yield 64.8 mg (67 %), m.p. 155-6° C. For analysis it was crystallised from ethyl acetate, m.p. 156-8° C; For $C_{25}H_{37}N_3O_4$ calculated : 67.69 % C, 8.41 % H, 9.47 % N; found : 67.50 %C, 8.71 % H, 9.54 % N.
$(\alpha)_D^{25}$ + 2.2 (c 0.50; methanol).

(*b*) Dehydration of benzyloxycarbonyl-L-glutamine (VII).

A mixture of VII (2.8 g; 10 mmole), *p*-toluenesulphonyl chloride (2.16 g; 12.5 mmole), and 18.5 ml of pyridine was kept at 50° C for 30 minutes. Pyridine was distilled off in vacuo and the syrupy residue was dissolved in water (12 ml). The resulting solution was acidified with dilute hydrochloric acid to pH approx. 2 and extracted with ethyl acetate. The combined ethyl acetate extract was washed with water, dried over sodium sulphate, filtered and concentrated under reduced pressure to half of its volume. Dicyclohexylamine (2.04 ml; 10 mmole) was then added to this solution and allowed to stand overnight in a refrigerator. A white substance crystallised out, yield 3.23 g (73 %), m.p. 146-150° C. After crystallisation from a mixture of abs. ethanol and light petroleum the yield was 3.05 g (69 %), m.p. 155-7° C. The mixture melting point with a product prepared as under (*a*) was undepressed, $(\alpha)_D^{25}$ + 3.2° (c 1.10; methanol).

L-Glutamine (IV).

Dicyclohexylammonium salt of L-α-benzyloxycarbonylamino-γ-cyanobutyric acid (III) (220 mg; 0.5 mmole) was suspended in 1.4 ml of 0.5N-NaOH and then diluted with 4 ml of water. The liberated dicyclohexylamine was extracted with ether (8 ml) and the aqueous layer was acidified with 1N — HCl to pH 2. The product was extracted twice with 10 ml of ether and the combined extracts were washed with water, dried over sodium sulphate, and concentrated. The free acid was obtained in the form of an oil. This was dried overnight in a dessicator over P_2O_5 and KOH. It was then mixed with 1 ml of an approximately 35 % hydrogen bromide solution in acetic acid. After 10 minutes 5 ml of dry ether were added and the precipitated crude product was decanted several times with ether. The filtered product was dissolved in 1 ml of water and the formed solution was extracted twice with 0.5 ml of ether. The pH of the aqueous layer was adjusted to 6.5 on addition of 25 % ammonium hydroxide and the mixture was diluted with 2.5 ml of methanol. After standing in a refrigerator overnight the product crystallised out which was

recrystallised from water and ethanol. Yield 42 mg (57.5 %) of a chromatographically and electrophoretically pure product (buffer of pH 5.7); $(\alpha)_D^{25}$ + 5.9 (c 0.49; water). For $C_5H_{10}N_2O_3$, calculated : 41.09 % C, 6.90 % H; found : 41.00 % C, 6.79 % H.

L-Glutamic acid (V).

A mixture of methyl L-α-benzyloxycarbonylamino-γ-cyanobutyrate (II) (300 mg; 1.09 mmole), 8 ml of acetic acid, and 4 ml of conc. hydrochloric acid was refluxed on an oil bath at 125-135° C for 6 hours. The reaction mixture was evaporated to dryness in vacuo on a rotatory evaporator, water was added to the residue and again evaporated. This procedure was repeated several times in order to eliminate completely volatile acids. The transformation of the hydrochloride of glutamic acid to the free acid was carried out on a Dowex 50 W × 8 column (10 × 1 cm). L-Glutamic acid was eluted with 2N-NH_4OH. After the evaporation of the eluate to dryness in vacuo 155 mg (98 %) of chromatographically almost pure L-glutamic acid were obtained (solvent isopropanol-ammonia 7 : 3). After crystallisation from aqueous methanol and acetone the yield was 95 mg (60 %); For $C_5H_9NO_4$ calculated : 40.81 % C, 6.17 % H, 9.52 % N; found : 40.99 % C, 6.30 % H, 9.38 % N. $(\alpha)_D^{25}$ + 30.2° (c 0.25; 6N — HCl).

L-Ornithine (VI).

L-α-Benzyloxycarbonylamino-γ-cyanobutyrate (II) (300 mg; 1.09 mmole) dissolved in acetic acid was hydrogenated on Adams catalyst (100 mg Pt) at normal pressure and room temperature for 24 hours. The catalyst was filtered off and the reaction mixture acidified with 3 ml of hydrochloric acid and hydrolysed for 3 hours under reflux. After concentration in vacuo, L-ornithine hydrochloride was transformed to free ornithine on Dowex W × 8. Hydrochloric acid was eluted first with water, and the amino acid with 2N — NH_4OH. The eluate was evaporated to dryness under reduced pressure and the residue (145 mg) was dissolved in 10 ml of water and filtered over charcoal. The free ornithine was transformed to monohydrochloride by adjusting the pH to 3 with 0.1N-HCl. Crystallisation was carried out from methanol-acetone. Yield 124 mg (68 %). The product was chromatographically pure. $(\alpha)_D^{25}$ + 16.9° (c 0.47; water).

2, 4, 5-Trichlorophenyl ester of L-α-benzyloxycarbonylamino-γ-cyanobutyric acid (VIII).

The free acid was liberated from the dicyclohexylammonium salt of III (222 mg; 0.5 mmole) in the same manner as described for the preparation

of L-glutamine (IV). The dried acid was dissolved in 1.5 ml of acetonitrile and the resulting solution was transferred into a centrifuge tube (5 ml) containing a solution of 2, 4, 5-trichlorophenol (99 mg; 0.5 mmole) in 1 ml of acetonitrile. A solution of dicyclohexylcarbodiimide (102 mg; 0.5 mmole) in 0.7 ml of acetonitrile was added to it under cooling with ice-water. The mixture was shaken and allowed to stand in a refrigerator overnight. The precipitated dicyclohexylurea was centrifugated and washed twice with ether acetate. The combined supernatants were concentrated in vacuo to dryness and the residue was crystallised from ethyl acetate—light petroleum mixture. After 3 hours standing in refrigerator 142.3 mg (64.5 %) of the colourless product crystallised out, melting point 158-159° C. After repeated crystallisation the m.p. remained unchanged. For $C_{19}H_{15}N_2O_4Cl_3$ calculated : 51.66 % C, 3.42 % H, 6.34 % N; found : 51.43 % C, 3.67 % H, 5.98 % N.

(L-α-Benzyloxycarbonylamino-γ-cyanobutyryl) glycine ethyl ester (IX).

To a solution of VIII (580 mg; 1.33 mmole) in 17 ml of dioxane 3.4 ml of a chloroform solution of freshly distilled glycine ethyl ester (453 mg dissolved in 10.0 ml), i.e. 1.49 mmole, were pipetted and the mixture was allowed to stand at room temperature overnight. The solvent was evaporated under reduced pressure to dryness and the residue was diluted with light petroleum. After prolonged standing (overnight) the crude product crystallised out which was filtered and washed with light petroleum. Yield 280 mg (61 %), m.p. 116-9° C. After crystallisation from ethanol and water the m.p. did not increase. For $C_{17}H_{21}N_3O_5$ calculated : 58.78 % C, 6.09 H; found : 59.08 % C, 6.06 % H.

RADIOACTIVE SYNTHESES.

Methyl L-α-benzyloxycarbonylamino-γ-cyano-^{14}C-butyrate (II).

A mixture of L-α-benzyloxycarbonylamino-γ-bromobutyric acid methyl ester (I) (330 mg; 1 mmole), potassium cyanide (60 mg; 0.92 mmole), and potassium cyanide-^{14}C (10 mg; 0.15 mmole) of declared activity 1480 μCi (according to the certificate of the producer : Central Institute of Nuclear Research, Dresden-Rossendorf, German Dem. Republic; the sample was 4 years old) was stirred under the same conditions as given for the non-radioactive synthesis of II for 72 hours. Further working up of the reaction mixture was also the same. Radiochemical yield was 595 μCi, i.e. 40 % of the declared radioactivity of K^{14}CN. Chemical yield of the dried product was 270 mg (98 %). (The substantial difference between the chemical and the radiochemical yields was caused evidently by the unsatisfactory quality of the radioactive cyanide.) Specific activity was 0.61 mCi/mmole.

L-α-Benzyloxycarbonylamino-γ-cyano-^{14}C-butyric acid (III), dicyclohexylammonium salt.

A solution of nitrile II (0.75 g; 2.7 mmole) of activity 502 μCi in 5.5 ml of methanol and 1.48 ml of 2N-NaOH was stirred at room temperature for one hour. Further working up was analogous to that described for inactive synthesis. Yield 850 mg (71 %) of crystalline product of activity 346 μCi (69 %). Specific radioactivity was 0.18 mCi/mmole.

L-Glutamine-5-^{14}C (IV).

(*a*) Radioactive synthesis from II.

Methyl L-α-benzyloxycarbonylamino-γ-cyano-^{14}C-butyrate (II) (0.75 g; 2.7 mmole of 502 μCi activity was stirred at room temperature with 6 ml of methanol and 1.5 ml of 2N-NaOH for 1 hour. The reaction mixture was partially concentrated under reduced pressure and acidified with dilute hydrochloric acid to pH 2. The product was extracted with ether, the combined extracts were washed with water, and dried over sodium sulphate. Ether was evaporated and the residue dried in a vacuum dessicator. Approx. 35 % hydrogen bromide in anhydrous acetic acid (3 ml) was added to the residue and after 10 minutes standing dry ether was added. The precipitated crude product was decanted several times with ether. It was then dissolved in 3.5 ml of water and the pH value was adjusted on addition of 25 % NH_4OH to 6.5, the mixture was diluted with 9 ml of methanol and a few drops of acetone and allowed to stand overnight in a refrigerator. The crystalline product was recrystallised from aqueous ethanol. Yield 249 mg (63 %) of 286 μCi (57 %) radioactivity. Specific radioactivity 0.17 mCi/mmole. The product when submitted to chromatographic and electrophoretic control contained only traces of glutamic acid.

(*b*) In preliminary experiments the preparation of L-glutamine-5-^{14}C from II in one step was also investigated :

Hydration and hydrolysis of methyl L-α-benzyloxycarbonylamino-γ-cyano-^{14}C-butyrate was carried out in 35 % hydrogen bromide in acetic acid at 60° C for 15 hours. After concentration and further working up (chromatographic separation on Dowex 50 W × 9) the reaction mixture was purified by preparative paper chromatography (isopropanol-ammonia 7 : 3). Radiochemical yield was 28 % of L-glutamine-5-^{14}C. L-Glutamic-5-^{14}C acid was isolated as a by-product in a 6.4 % yield.

L-Glutamic-5-^{14}C acid (V).

Methyl L-α-benzyloxycarbonylamino-γ-cyano-^{14}C-butyrate (II) (95 mg; 0.34 mmole) of 210 μCi activity was submitted to hydrolysis in a mixture of acetic acid and hydrochloric acid (2 : 1). The working up of the reaction

mixture was carried out as in the section on the preparation of inactive L-glutamic acid. Free L-glutamic-5-^{14}C acid was obtained on chromatography on Dowex 50 W. The product was purified by preparative chromatography. The radiochemical yield of chromatographically and electrophoretically purified product (V) was 80 μCi (38 %).

L-Ornithine-5-^{14}C (VI).

A mixture of radioactive nitrilo ester II (6 mg; 0.028 mmole) of 8.5 μCi activity and inactive nitrilo ester (140 mg; 0.505 mmole) was hydrogenated in acetic acid on platinum catalyst (90 mg) prepared according to Adams, at normal pressure and room temperature, for 24 hours. The consumption of hydrogen was also theoretical. The catalyst was filtered off and the filtrate was submitted to acidolysis with 3 ml of hydrochloric acid. Further working up was carried out as in the case of inactive L-ornithine synthesis. Radiochemical yield 2.2 μCi (26 %) of a chromatographically pure product.

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