Preparation of tritium labelled L-phenylalanine

Ch. GERDAY and W. G. VERLY
Biochemistry Department, University of Montreal (Canada)
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SUMMARY

Tritiated N-chloracetyl-DL-phenylalanine, obtained by reduction of the N-chloracetyl-dehydrophenylalanine with tritiated hydrogen, is hydrolysed by carboxypeptidase A. The method gives L-phenylalanine-2,3-^3H of high optical purity and with any specific activity up to 60 curies per millimole.

Most of the biological tracer works using labelled amino-acids ought to be done with the natural optical isomers. Those available commercially are usually prepared by biosynthesis; they are Carbon-14 generally labelled, very expensive and of rather low specific radioactivity. The few chemically prepared are of doubtful optical purity.

A method is described which enables to prepare tritium labelled L-phenylalanine with an optical purity greater than 99 %. It consists to reduce the N-chloracetyl-dehydrophenylalanine by tritium gas and to proceed to the stereospecific hydrolysis of the resulting tritiated N-chloracetyl-DL-phenylalanine with carboxypeptidase A. The free tritiated L-phenylalanine is separated from the unhydrolyzed N-chloracetyl-D-phenylalanine by passing through a weak base column of Rexyn RG3 (OH^-), and then further purified by passing through a weak acid column of Rexyn 102 (H^+).

The chemical purity is checked by paper chromatography and the optical purity by making a dipeptide with L-glutamic acid followed by chromatography [5].

METHODS

I. — SYNTHESIS OF TRITIATED N-CHLORACETYL-DL-PHENYLALANINE

(a) *N-chloracetyl-dehydrophenylalanine.*

The product is obtained by the method of Bergman *et al.* [1] from β-phenylserine (Nutritional Biochemicals Corporation).

(b) *Reduction of N-chloracetyl-dehydrophenylalanine with tritiated hydrogen.*

242 mg (1 mmole) of N-chloracetyl-dehydrophenylalanine is dissolved in 15 ml glacial acetic acid in a 100 ml Erlenmeyer flask, 100 mg of palladium black added and the flask connected to an all-glass hydrogenation apparatus. Tritium gas (6 mC; about 0.5 C/mmole) contained in an ampoule provided with a break-seal is admitted while the solution is stirred magnetically. The initial pressure is 690 mm Hg; after 20 min., it has fallen to 340 mm Hg. At this moment, ordinary hydrogen is admitted and the pressure maintained at 700 mm Hg until no more hydrogen uptake is observed (1 hr). The hydrogen excess is pumped off through a tube containing CuO heated to 450 °C and the residual tritium caught as water in a trap.

The flask content is filtered through a Whatman No. 2 paper, and the clear filtrate evaporated to dryness under vacuum. The oily residue is dissolved in a minimum of boiling water; after cooling, 141 mg of tritiated N-chloracetyl-DL-phenylalanine is collected (fraction I). 600 mg of inactive N-chloracetyl-DL-phenylalanine are added to the mother liquors and the crystallization process repeated, yielding a second crop of 570 mg of labelled compound (fraction II).

The radiochemical purities of the two fractions are checked by paper chromatography on Whatman No. 1 developped with butanol : acetic acid : water (4 : 1 : 1) for 8 hrs. The dried paper is sprayed with 0.04 % bromophenol blue in ethanol; only one yellow spot ($Rf = 0.83$) can be detected on a blue background. Scanning the paper chromatogram with a radioactivity detector (Nuclear Chicago-model Actigraph III 4π) shows that for each of the two fractions the radioactivity is entirely localized in the yellow spot.

The specific activities, measured by liquid scintillation counting, are : $1.33\ 10^{10}$ d/min.mmole for fraction I; $1.10\ 10^{9}$ d/min.mmole for fraction II.

II. Enzymatic hydrolysis of the tritiated N-chloracetyl-DL-phenylalanine.

6 mg (24 μmoles) ($3.60\ 10^{8}$ d/min) of N-chloracetyl-DL-phenylalanine are dissolved in 25 ml of LiOH 0.01 N; the pH is 7.5. The temperature is brought to 37° C, and 0.1 ml of a 0.1 % carboxypeptidase A solution (Mann-CAO.DFP) is added. The solution is maintained at pH 7.5 by continuous addition of LiOH 0.1 N using a pHstat. The hydrolysis is followed by ninhydrin measurements on 0.1 ml aliquots [2].

After 8 hrs. the amount of ninhydrin-reacting substance does not increase anymore and nearly 50 % of the N-chloracetyl-DL-phenylalanine is hydrolyzed, the reaction mixture is acidified to pH 5 with glacial acetic acid and, after addition of celite, the suspension filtered on Whatman No. 1. The clear filtrate is passed through a small column of Rexyn RG 3 (OH^-); the ion-exchanger is washed with water until the eluate, which contains the

tritiated phenylalanine, reaches 50 to 60 ml. In order to remove the Li^+ ions, the collected fraction is passed through a small column of Rexyn 102 (H^+); the phenylalanine is washed out with water, whereas Li^+ ions are retained on the column.

The final radioactive eluate is evaporated to dryness in vacuum leaving a residue of 1.8 mg. This product is identified, and its radiochemical purity checked, by paper chromatography on Whatman No. 1 with butanol : acetic acid : water (4 : 1 : 1) : one single radioactive and ninhydrin coloured spot is detected ($Rf = 0.49$).

III. — Optical purity.

A dipeptide is prepared with the radioactive product diluted with inactive DL-phenylalanine, and L-glutamic acid prepared by hydrolysis of N-acetyl-L-glutamic acid (Nutritional Biochemicals Corporation) with acylase I (Nutritional Biochemicals Corporation) [3]*. The method already described [5] is followed except that N-carbo-benzoxy-phenylalanine is coupled directly with the ethyl diester of L-glutamic acid using N,N'-dicyclo-hexylcarbodi-imide [4]. The diasteroisomers L-phenyl-alanyl-L-glutamic and D-phenyl-alanyl-L-glutamic acids are separated by column chromatography and the eluate radioactivity followed by liquid scintillation counting. All the radio-activity is in the L-phenylalanyl-L-glutamic acid peak, except for 0.84 % which is found in the position of the D-phenylalanyl-L-glutamic acid (Fig. 1).

Discussion

We have demonstrated experimentally that L-phenylalanyl-D-glutamic acid is eluted at the same place as D-phenylalanyl-L-glutamic acid, so that the chemical nature of the contaminating compound, seen in the analytical chromatography and containing 0.84 % of the total radioactivity, is ambiguous.

It seems that the stereospecificity of carboxypeptidase A cannot be suspected because the relative amount of contamination of the dipeptide synthesized from the liberated phenylalanine remains constant whatever the the moment when the sample is taken in the course of hydrolysis, or if the time of the enzyme action is lengthened up to 20 hrs. This argument favors the hypothesis that the prepared tritiated phenylalanine is 100 % in the L form.

A similar argument with acylase I leads to the conclusion that the L-glutamic acid used to synthesize the dipeptide must also be optically 100 % pure.

* Commercial L-glutamic acid contains as much as 3 % of the D isomer.

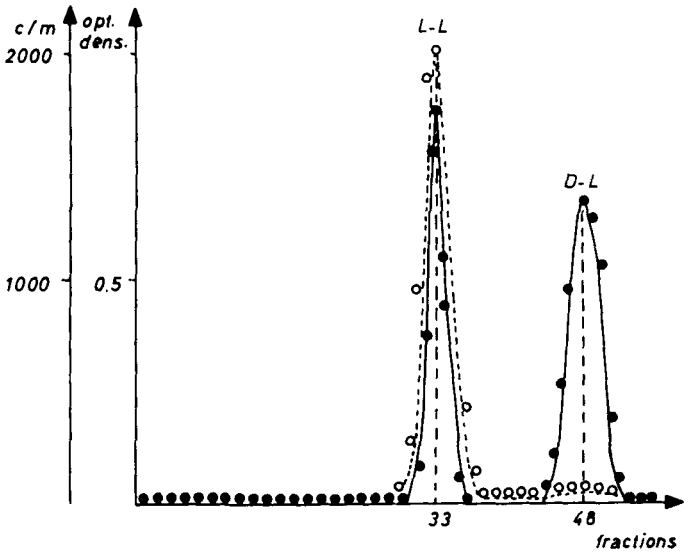

FIG. 1. Optical purity of a preparation of L-phenylalanine-2,3-^{3}H. First peak, L-phenylalanyl-L-glutamic acid; second peak, D-phenylalanyl-L-glutamic acid. Black spots : ninhydrin determinations; white spots : radioactivity.

What is suspected is the analytical method employed to check the purity of the optical isomer. A small degree of racemization must occur during the synthesis of the dipeptide; it likely does not involve the phenylalanine which is protected by a carbobenzoxy group, but rather the glutamic acid moiety. Although not perfect, the analytical method applied to the labelled amino-acids has such a high sensitivity that it cannot be replaced by more classical ones, such as polarimetry or determination of the oxygen uptake during the action of aminoxidases.

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