Journal of Labelled Compounds — October-December — Vol. II, No. 4

Tritium labelling of amino-acids and peptides by the Wilzbach method

II. Racemization of amino-acids during exposure to tritium gas*

J. H. PARMENTIER

Centraal Laboratorium TNO, Delft, Netherlands

Received on 27th July 1966

SUMMARY

Optically pure amino-acids were labelled with tritium by the Wilzbach method. The percentage of radioactivity found in the opposite enantiomer was determined by reverse dilution analysis. After tritiation of L-phenylalanine, D-phenylalanine, L-tyrosine, L-proline, D-proline, L-glutamic acid and L-alanine respectively 2.7, 2.2, ⩽ 1.1, 4.6, 4.0, 11.9 and 19.3 of the activities of the radiochemically pure amino-acids were found in D-phenylalanine, L-phenylalanine, D-tyrosine, D-proline, L-proline, D-glutamic acid and D-alanine.

Wilzbach labelling of phenylalanine caused the formation of a considerable amount of a radiochemical impurity that was identified as β-cyclohexylalanine.

INTRODUCTION

Radiochemical impurities in peptides and proteins, labelled by the Wilzbach method and caused by racemization, may be important, as little is known about the effectiveness of the usual purification methods for the removal of traces of highly active, diastereo-isomeric forms of these peptides and proteins. The possible presence of a considerable amount of labelled material with an unnatural configuration might reduce the value of a biological tracer substance very seriously. It would therefore be desirable to have more information about the amount of radiochemical impurities caused by racemization under the influence of tritium gas, in amino-acids, peptides and proteins.

In this work, results obtained with several amino-acids are given. Traces of labelled D-amino-acids in the corresponding L-acids, and vice versa, were

* This investigation was supported by Euratom under contract 078-64-RISN

determined by a reverse isotope dilution method. For the principle of this method, and for a discussion of the factors that can influence the results, we refer to a previous publication [1].

EXPERIMENTAL PART

1. GENERAL OUTLINE OF PROCEDURE

Inactive starting materials were checked on optical purity as described in 2.1. below. After the Wilzbach labelling of the amino acids (2.2.), labile tritium atoms were removed.

To the tritiated amino-acid, an equal amount of the inactive, opposite enantiomer was added before the purification in order not to remove the traces of this active enantiomer by the crystallization procedure. After purification of the product by repeated crystallization (2.3.), it was purified further by preparative thin-layer chromatography (2.4.). (In the case of phenylalanine, β-cyclohexylalanine could be isolated and identified as a radiochemical impurity (2.5.).

After the chromatographic purification, the product was dissolved and the solution was divided into three equal parts. The reverse dilution analysis was carried out by adding to each of these three parts respectively a 100-fold excess of inactive L-amino-acid, D-amino-acid and DL-amino-acid (2.6.). These three portions were crystallized several times, and the specific activities were determined by counting the samples in a liquid scintillation counter (2.7.).

2. DETAILED DESCRIPTION OF EXPERIMENTS

2.1. *Optical purity of materials used*

Phenylalanine. L-, D- and DL-phenylalanine were obtained from Fluka (puriss-quality).
L-phenylalanine : $[\alpha]_D^{24} = -33.2°$, $c = 1 - 2$, in water.
D-phenylalanine: $[\alpha]_D^{24} = +33.7°$, $c = 0.8 - 2.2$, in water.

Tyrosine. L-, D-, and DL-tyrosine were obtained from Fluka.
L-tyrosine : $[\alpha]_D^{20} = -8.8°$, c = 4.44 in 6.3 N HCl.
D-tyrosine : $[\alpha]_D^{20} = +7.8°$, $c = 3.59$ in 6.3 N HCl.

Proline. L-, D- and DL-proline were obtained from Calbiochem (puriss-quality).
L-proline : $[\alpha]_D^{20} = -83.6°$, $c = 1$ in water.
D-proline : $[\alpha]_D^{20} = +82.9°$, $c = 1$ in water.

Glutamic acid. L-, D- and DL-glutamic acid were obtained from Fluka (puriss-quality).

L-glutamic acid : $[\alpha]_D^{24} = +31.7°$, $c = 2.7$ in 1.73 N HCl.
D-glutamic acid : $[\alpha]_D^{24} = -31.6°$, $c = 2.7$ in 1.73 N HCl.

Alanine. L-, D- and DL-alanine were obtained from Calbiochem (puriss quality).

L-alanine : $[\alpha]_D^{23,5} = +14.4°$, $c = 5$, in 1 N HCl.
D-alanine : $[\alpha]_D^{23,5} = -14.3°$, $c = 5$, in 1 N HCl.

The optical purity of L-phenylalanine, L-tyrosine and L-glutamic acid was also checked by enzymatic analysis with L-amino oxidase (LAO) *. L-tyrosine was completely digested ; in L-phenylalanine this analysis indicated a D-amino-acid content of less than 0.5%. L-glutamic acid was not completely digested by LAO ; 4.1% was found after the digestion. In a separate, identical experiment with an optically very pure standard sample of L-glutamic acid, 4.1% of this standard sample was not digested in the reaction time that was chosen. We conclude that the optical purity of the L-glutamic acid used in our experiments was better than 99.5%, and that of L-tyrosine better than 99.9%. Estimated from the optical rotations, the optical purities of L- and D-proline and L- and D-alanine were at least 99%.

The amino-acids were used without further purification ; they were all chromatographically homogeneous.

2.2. *Wilzbach labelling*

The exposure to tritium-gas was performed in an apparatus described by Wenzel [2], equipped with an automatic Toepler pump and slightly modified [1]. The tritium gas was obtained in 3 C portions from the Radiochemical Centre, Amersham (England), in break-seal ampoules. The isotopic purity of the gas was about 98%, the volume 1.2 ml at N.T.P. Each 3 C portion of the gas was used only two or three times. About 100 mg of the finely powdered and thoroughly outgassed amino-acid was exposed for 7-8 days to 3 C tritium gas, at a pressure of about 20 cm Hg, at room temperature.

2.3. *Purification by crystallization*

Labile bound tritium atoms were removed by dissolving the tritiated product in an excess of water. In the case of tyrosine, 6 N HCl was used. The solvent was removed under vacuum. To the labelled L-amino-acids an exactly equal portion of inactive D-amino-acid (for tyrosine : D-tyrosine-HCl) was added, and to the labelled D-amino-acid an exactly equal amount of inactive L-amino-acid. The products were crystallized as follows :

Phenylalanine : 4 × from ethanol-water,
Tyrosine : 1 × from 6 N HCl, 3 × from water,
Proline : 1 × from ethanol-ether,

* These determinations were carried out under the supervision of Ir. B.C. Goverde of N.V. Organon, Oss, (The Netherlands).

Glutamic-acid : 6 × from water,
Alanine : 2 × from ethanol-water.

The number of crystallizations depended on the ease, with which the bulk of the radiochemical impurities could be removed, as indicated by radiochromatography (L-glutamic acid was obtained with a radiochemical purity of 97% and was not further purified by chromatography, see 2.4).

2.4 *Purification by preparative thin-layer chromatography*

Thin-layer plates (20 × 30 cm) with silica-gel H were prepared (thickness 1 mm) and dried overnight, then 1 h at 110°C.

Per plate, 5 mg of an amino-acid was dosed in a band. The amino-acid was dissolved in 0.5 ml of water, or, in the case of tyrosine, in 0.5 ml 1 N HCl.

Chromatograms were developed with butanol/acetic acid/water 3 : 1 : 1, and then dried at a temperature of at least 50°C. The distribution of the radioactivity over the chromatogram was determined with a Berthold scanner (see 2.7.). The amino-acids were removed by extracting the SiO_2 with hot water; the water was removed under diminished pressure. In the case of tyrosine, the extraction was done with 40 ml of hot water, acidified with 5 ml 0.1 N HCl.

Depending on the purity that was obtained, the isolated products were chromatographed a second or a third time; in most of these cases we started the first chromatografic purification with 10 mg of substance, dosed on two plates. After the first chromatogram, the extracts were added together. L-glutamic acid was purified only by crystallization from water, (6 ×), as we had the impression that purification over SiO_2 caused the formation of additional impurities.

Radiochemical purities of the final products were better than 99%, except in the case of D-phenylalanine and that of L-glutamic acid, where radiochemical purities were 99% and 97% respectively. These figures were estimated from the second or third preparative chromatogram, or from an analytical thin-layer or paper chromatogram.

These paper and thin-layer chromatograms were also developed with butanol/acetic acid/water 3 : 1 : 1 (ascending technique; Whatman paper no. 1, or a silica-gel H thin layer of 0.20 mm thickness). In the case of paper chromatograms, the radioactivity distribution was determined by cutting the paper in pieces of ½ cm and counting in a liquid scintillation counter (2.7.). From the thin-layer plate, the silica-gel was removed in bands of ½ cm, and the activity of each band was counted (2.7.).

2.5. *Isolation and identification of a radioactive impurity in tritiated phenylalanine*

In the case of tritiated L- and D-phenylalanine, the amount of a radiochemical impurity difficult to remove, was considerable (see fig. 1). We assumed

that this impurity might be β-cyclohexylalanine, formed from phenylalanine by hydrogenation. To check this assumption, the impurity was isolated from the preparative chromatogram by elution with water. After evaporation of the water, the residue, mainly SiO_2, was extracted with ethanol. β-Cyclohexylalanine was added as a carrier and a two-dimensional thin-layer chromatogram was made. About 50 μg substance was dosed on a thin-layer plate of 20 × 20 cm, with a thickness of 0.25 mm.

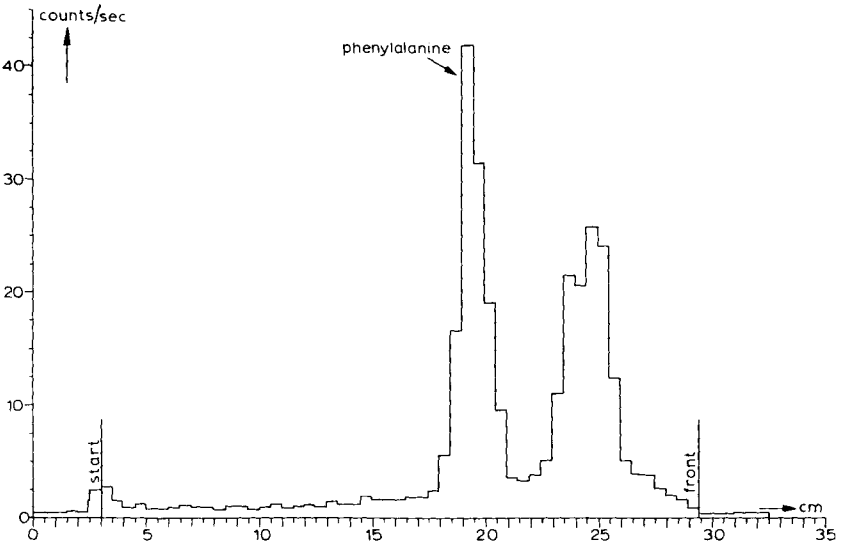

FIG. 1 — *Paper chromatogram of L-phenylalanine tritiated by means of the Wilzbach method, after four crystallizations.*

Development in the first direction occurred with pyridine/water/tert amylalcohol 105/90/105, in about 5 h. After drying, development in the second direction took place with butanol/acetic acid/water 3 : 1 : 1 in 4½ h.
After drying, the activity distribution over the chromatogram was determined by scanning, and next the plate was sprayed with ninhydrin-reagent. Only one coloured spot was visible, which coincided exactly with the radioactive spot.

2.6. *Reverse dilution analysis*

The amino-acids, obtained after the chromatographic purification, were dissolved in about 6.5 ml of water. In the case of tyrosine, 0.1 N HCl was used. If necessary, this solution was filtered over a G-3 glass filter, and divided in three portions of about 2 ml. These three portions were weighed, and a weighed amount of respectively L-, D-, and DL-amino-acid was added as a carrier (about 200 mg; in the case of proline about 300 mg). The fractions

were crystallized till the sum of the specific activities (corrected for slightly different dilutions) of the D- and the L-fractions was equal to the specific activity of the DL-fraction; then the fraction with the lowest specific activity was crystallized further to prove its constant specific activity.

Crystallization procedure :

Phenylalanine : 3 — 5 × from ethanol-water.
Tyrosine : 6 × from water.
Proline : 12 × from absolute ethanol.
Glutamic acid : 6 × from water.
Alanine : 2 × from ethanol-water.

Given is the number of crystallizations that was necessary to make the sum of the specific activities of the D- and the L-fraction approximately equal to the specific activity of the DL-fraction.

2.7. *Radioactivity measurements*

The amino-acids were measured by dissolving 0.5-2 mg of the acid in 1 ml of a Hyamine hydroxide 1.0 M solution in methanol (obtained from Nuclear Enterprises Ltd., Edinburgh, Scotland); when the amino-acid was completely dissolved, 6 ml scintillation liquid was added. (Composition : 200 ml ethanol p.a., 800 ml toluene p.a., 5 gr 2.5 diphenyl-oxazol, (PPO) and 0.5 g 2.2′ *p*-phenylen-bis-(5-phenyloxazol), (POPOP)). The measurements were performed in an automatic liquid scintillation counter. (Tritomat 6020, Isotope Development Limited.)

The activity distribution on paper chromatograms was determined by cutting the chromatogram in strips of ½ cm. These strips were put into the measuring bottles, as far as possible in the same positions, and covered with 5 ml of a scintillation mixture (composition : 60 g naphthalene p.a., 4 g PPO, 0.2 g POPOP, 100 ml ethanol, 20 ml ethylene glycol; added up to a volume of 1000 ml with *p*-dioxane p.a.).

Activities on analytical thin-layer chromatograms were measured by removing the silica gel H in bands of ½ cm, covering the gel with 5 ml of the dioxane-scintillation mixture and counting in the scintillation counter.

The activity distribution on preparative thin-layer chromatograms was determined with the Berthold « Dünnschicht-scanner LB 2720 ». Activities, measured on chromatograms by one of the methods described above, give only approximate estimations of radiochemical purities.

RESULTS

TABLE 1. Results of the reverse dilution analysis, with the estimated maximum deviations

Amino-acid that was tritiated	Carrier added	Percentage of activity	
L-phenylalanine	DL-phenylalanine	[100]	
	L-phenylalanine	96.6 ± 3	99.3
	D-phenylalanine	2.7 ± 0.5	
D-phenylalanine	DL-phenylalanine	[100]	
	L-phenylalanine	2.2 ± 1	99.7
	D-phenylalanine	97.5 ± 3	
L-tyrosine	DL-tyrosine	[100]	
	L-tyrosine	96.3 ± 3	97.4
	D-tyrosine	1.1 ± 0.4	
L-proline	DL-proline	[100]	
	L-proline	92.6 ± 3	97.2
	D-proline	4.6 ± 1	
D-proline	DL-proline	[100]	
	L-proline	4.0 ± 1	101.1
	D-proline	97.1 ± 3	
L-glutamic acid	DL-glutamic acid	[100]	
	L-glutamic acid	88.9 ± 3	100.8
	D-glutamic acid	11.9 ± 1	
L-alanine	DL-alanine	[100]	
	L-alanine	78.5 ± 3	98.5
	D-alanine	19.3 ± 2	

DISCUSSION

1. The largest uncertainties in Table 1. are caused by the uncertainties in the optical purities of the amino-acids, used for the Wilzbach tritiation. The value for tyrosine (1.1 ± 0.4) has to be considered as a maximum; after crystallizing one time from 6 N HCl (2.3.), it was realized that tritium might be lost from places ortho to the -OH-group by this procedure; thereafter only water was used for crystallizing [4, 5].
2. As a tentative conclusion we may say that the low percentages of the radioactive, opposite enantiomer in the cases of phenylalanine and tyrosine are presumably due to a high tritium content of the aromatic nucleus. As

aromatic amino-acids in peptides are preferentially tritiated by the Wilzbach method [3], we may assume that radioactive, diastereo-isomeric impurities will not be very important in peptides with a high percentage of aromatic amino-acids.

However, radioactive impurities due to hydrogenation of aromatic nuclei may be very important.

ACKNOWLEDGEMENT

I wish to thank Prof. Dr. E. Havinga for his stimulating interest in this work, Ir. B. Goverde of the N.V. Organon for the enzymatic control of the amino-acids and Miss M.J.Th. Toby, Mr. Th.J.G. Ambaum, Mr. D. Oudijn, Mr. Th. van Veen and Mr. L. Teljeur for their valuable technical assistance.

REFERENCES

1. PARMENTIER, J.H. — « Tritium labelling of amino-acids and peptides by the Wilzbach method. I. Racemization of L-valine during exposure to tritium gas ». Journal of Labelled Compounds, **1**, 93 (1965).
2. WENZEL, H., and SCHULZE. P.E. — « Tritium Markierung », Berlin 1962, p. 22.
3. VON HOLT, C., VOELKER, I., and VON HOLT, L. — « Markierung von Insulin mit Tritium », *Biochem. Biophys. Acta*, **38**, 88 (1960).
4. HILL, J., and LEACH, S.J. — « Hydrogen exchange at carbon-hydrogen sites during acid or alkaline treatment of proteins ». *Biochemistry*, **3**, 1814 (1964).
5. RITTENBERG, D., *et al.* — « Deuterium as an indicator in the study of intermediar metabolism. XIII. The stability of hydrogen in amino-acids ». *J. Biol. Chem.*, **125**, 1 (1938).