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

I. Racemization of L-valine during exposure to tritium gas

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SUMMARY

Optically pure L-valine was labelled with tritium by the Wilzbach method. Only 4 % of the activity of the radiochemically pure valine was found in D-valine. This figure was determined by reverse isotope dilution.

The suitability of this method for valine was tested, with good results, with DL-valine also labelled by the Wilzbach method.

INTRODUCTION

The Wilzbach method is frequently used for the labelling of peptides and proteins [1-9]. The solid materials (0.1-1 gram) are labelled with tritium by bringing them into contact with curie-quantities of tritium-gas.

Radiochemical impurities, caused by racemization during labelling might be important in this field, for 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.

This prompted us to study the racemization of some amino-acids and peptides under the influence of tritium-gas.

For the evaluation of traces of labelled D-amino-acids in the corresponding L-acids, we choose the reverse isotope dilution method [10]. The Wilzbach tritiated amino-acid is first purified to such an extent that is consists only of D- and L-acid.

If we call the amount of D-acid m_D, with a total activity A_D, and the amount of L-acid m_L, with an activity A_L, the reverse isotope dilution is performed as follows. We divide the mixture of L- and D-acid into three equal

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parts. To portion I we add an amount M_D of inactive D-acid, to portion II an amount M_L of inactive L-acid, and to portion III an amount M_{DL} of inactive DL-acid.

$$M_D = M_L = M_{DL},\ M_D \gg m_D + m_L$$

By repeated crystallizations we remove in portion I the activity due to L-acid, and in portion II the activity due to D-acid.

The specific activities of the three portions are :

$$a_D = \frac{A_D}{M_D + 1/3m_D},\ a_L = \frac{A_L}{M_L + 1/3m_L} \text{ and } a_{DL} = \frac{A_L + A_D}{M_{DL} + 1/3(m_D + m_L)}.$$

The percentages of activity present in D- and L-valine, thus obtained, are given in good approximation by

$$\frac{a_D}{a_{DL}} \times 100, \text{ and by } \frac{a_L}{a_{DL}} \times 100.$$

This method was applied first for amino-acids by RITTENBERG *et al.* in 1940 [11] and, later on, by several other authors [12, 13]. BAYLY in 1960 [14] drew again attention to the usefulness of the method. KÖGL, HALBERSTADT and BARENDREGT [15] used it successively for glutamic acid. In the case of lysine [16] however, it was demonstrated that the method must be used carefully, as had been pointed out also by GREENSTEIN and WINITZ [17]. Co-precipitation of traces of D-acid with the L-acid, and vice versa, may introduce considerable errors. To avoid this co-precipitation, a dilution factor has to be chosen which, is of the order of 100 [14].

In our case, co-precipitation can easily be detected. The sum of the activities of D- and L-acid (portions I and II) will then exceed the activity of DL-acid (portion III).

Moreover, we tested the reverse isotope dilution method on Wilzbach-tritiated DL-amino-acid. In this case, of course, the relations

$$\frac{a_D}{a_{DL}} \times 100 = \frac{a_L}{a_{DL}} \times 100 = 50 \text{ must hold.}$$

Another method for evaluating traces of a radioactive D-amino-acid in the corresponding L-acid appeared to be the use of the enzyme D-amino-acid oxidase. EVANS *et al.* [18], however, recently found that the α-hydrogen atom of a generally labelled, tritiated L-amino-acid is labilized in the presence of renal D-amino-acid oxidase. This finding precludes the use of the enzymatic method.

EXPERIMENTAL PART

1. — GENERAL OUTLINE OF PROCEDURE.

The inactive starting materials were checked on optical purity as described below (2.1). L-and DL-valine were tritiated by the Wilzbach method, and the labile tritium atoms were removed (2.2). To the tritiated L-valine an equal amount of inactive D-valine was added before the purification, in order not to remove traces of active D-valine by the crystallization procedure. After purification of the products by repeated crystallization (2.3), and applying preparative thin-layer chromatography (2.4.1), the end product was checked on radiochemical purity by paper-chromatography and by analytical thin-layer chromatography (2.4.2).

The purified product was dissolved in water, and this solution was divided into three equal parts. The reverse isotope dilution was carried out by adding to each of these three parts, respectively, a 100-fold excess of inactive L-valine, D-valine and DL-valine (2.5). These three portions were crystallized several times, and the specific activities were determined by counting the samples in a liquid scintillation counter (2.6).

2. — DETAILED DESCRIPTION OF EXPERIMENTS.

2.1. — *Optical purity of materials used.*

L-, D-, and DL-valine were obtained from Fluka (puriss-quality). Specific rotation of L-valine :

$$[\alpha]_D^{24} = +28.5^\circ \text{ (in 6 N HCl).}$$

[Reference 19 : $[\alpha]_D^{20} = +28.8^\circ$ (in 6 N HCl)].

After two crystallizations from water-ethanol :

$$[\alpha]_D^{24} = +28.3^\circ \text{ (in 6 N HCl).}$$

The optical purity of L-valine was also checked by enzymatic analysis with L-amino acid oxidase [1]. This analysis indicated an D-amino-acid content of less than 0.5 %. Specific rotation of D-valine :

$$[\alpha]_D^{21} = -29.3^\circ \text{ (in 6 N HCl).}$$

After two crystallizations from water-ethanol :

$$[\alpha]_D^{21} = -29.2^\circ \text{ (in 6 N HCl).}$$

[Reference 19 : $[\alpha]_D^{20} = -29.04^\circ$ (in 6 N HCl)],

L, D, and DL-valine were used as delivered by Fluka, without further purification.

[1] These determinations were performed under the supervision of Ir. B. C. GOVERDE, of N. V. Organon, Oss, The Netherlands.

2.2. — *Wilzbach labelling.*

Apparatus.

The exposure to tritium-gas was performed in an apparatus, described by WENZEL [20], equipped with an automatic Toepler pump. A modification was used, in which the uranium tube was replaced by an ampoule filled with molecular sieve type 4A [21]. The tritium-gas can easily be stored in this molecular sieve for short periods [1] and, eventually, removed by sealing the ampoule.

Tritium-gas

The tritium-gas was delivered in 3C portions by the Radiochemical Centre, Amersham, in break-seal ampoules.

The isotopic purity of the gas was 98 %, the volume 1.2 ml at NTP. Each 3C portion of the gas was used only twice.

Exposure of L-valine.

100.2 mg finelly powdered and thoroughly outgassed L-valine was exposed for 8 days to 3C tritium-gas, at a pressure of 200 mm Hg, at room temperature.

Exposure of DL-valine.

102.5 mg finely powdered and thoroughly outgassed DL-valine was exposed for 9 days to 3C tritium-gas at a pressure of 215 mm Hg at room temperature.

2.3. — *Purification by crystallization.*

To remove labile bound tritium atoms, the labelled substances were dissolved in an excess of water. The water was removed under diminished pressure.

To L-valine an exactly equal portion of inactive D-valine was added, and to DL-valine about the same amount of inactive DL-valine was added. The product was crystallized four times by times by adding about 8 times its weight of hot water, followed by an equal volume of hot ethanol. The solution was filtered over a G-3 glass filter and cooled slowly to about 5° C. The crystals were filtered off and washed with alcohol. The activities of the several recrystallization products are given in Table 1. They were determined as described in 2.6.

[1] Curie quantities of tritium, in our laboratory stored for some years in ampoules with this molecular sieve, had exchanged to a very considerable extent with the hydrogen, bound in the molecular sieve.

TABLE 1. Purification of labelled L- and DL-valine. Yields of the crystallizations and specific activities of the several fractions.

Number of crystallizations	L-valine labelled (D-carrier added)		DL-valine labelled (DL-carrier added)	
	Yield (mg)	Spec. act. (cpm/mg)	Yield (mg)	Spec. act. (cpm/mg)
1	108.2	11.3×10^5	131.9	5.14×10^5
2	85.4	7.47×10^5	102.7	4.48×10^5
3	58.5	6.42×10^5	83.6	4.14×10^5
4	39.0	6.02×10^5	59.2	4.04×10^5

The effect of this purification procedure was also followed by paper chromatography (2.4.2). In Fig. 1 a paper chromatogram is given of tritiated L-valine before the first crystallization.

2.4.1. — *Purification by preparative thin-layer chromatography.*

After four crystallizations, about 5 mg of the product was purified further by thin-layer chromatography. Thin-layer plates (20 × 30 cm) with silica-gel H

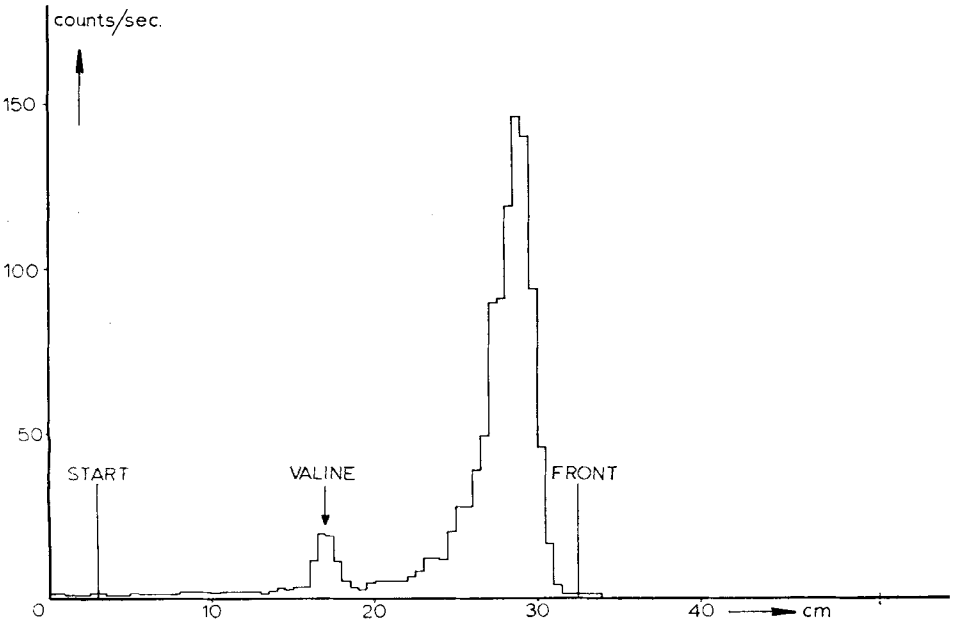

FIG. 1. — Paper chromatogram of tritiated L-valine before purification.

(thickness 1 mm) were prepared and dried overnight at room temperature, then 1 h at 110° C. About 5 mg valine in 0.5 ml water dosed in a band on the chromatogram. This was developed with butanol-acetic-water 3 : 1 : 1 in about 6 hours, and then dried at 80° C.

The silica-gel H was removed in bands of 1 cm and a small part of each band was measured in a liquid scintillation counter (2.6). The valine was eluted with 25 ml hot water ; the water was removed under diminished pressure.

In Fig. 2, the distribution of the radioactivity over the preparative thin-layer chromatogram of L-valine is given; the hatched part of the chromatogram was removed and eluted with water.

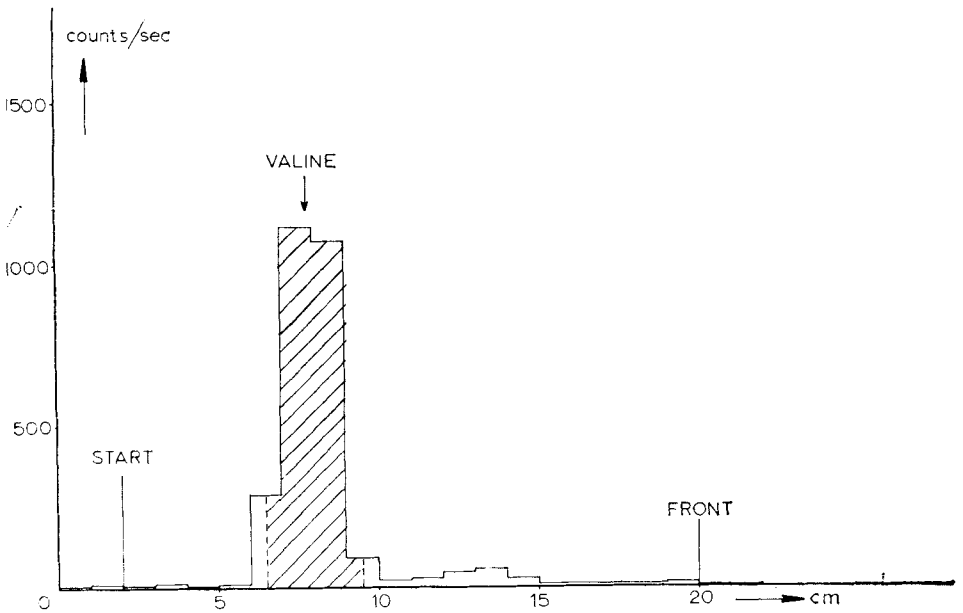

FIG. 2. — Preparative thin-layer chromatogram of tritiated L-valine recrystallized four times. The hatched part of the chromatogram was removed and eluted with water.

2.4.2. — *Control of products by analytical chromatography.*

The product, obtained as described in 2.4.1., was checked by analytical thin-layer chromatography on silica-gel G with phenol-water 3 : 1.

The silica-gel was removed in bands of ½ cm, and each band was measured in a liquid scintillation counter (2.6).

In Fig. 3, the distribution of the activity over the thin-layer chromatogram of purified L-valine is given.

Paper chromatograms were made of the product before and after the preparative thin-layer chromatography.

The chromatograms were developed with butanol-acetic acid-water 3 : 1 : 1. The strips were cut in pieces of ½ cm, and counted in a liquid scintillation counter (2.6).

According to the results of the analytical thin-layer and paper chromatography our products were radiochemically pure (purity better than 99 %) after the preparative chromatography.

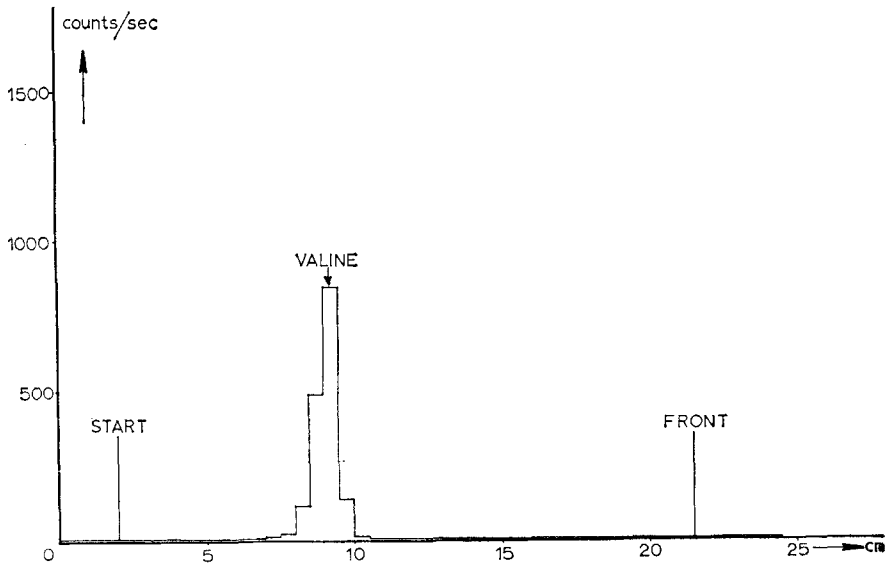

FIG. 3. — Thin-layer chromatogram of tritiated L-valine after purification.

2.5. — *Reverse isotopic dilution.*

The valine obtained after the chromatographic purification, was dissolved in about 6.5 ml water. This solution was filtered over a G-3 glass filter, and divided in three portions of 2 ml. These three portions were weighed, and a weighed amount of, respectively, L-, D-, and DL- (about 200 mg) valine was added. The valine was crystallized as described in 2.3, and the activities of the fractions were measured (2.6).

2.6. — *Radio-activity measurements.*

All measurements were performed in an automatic liquid scintillation counter made by Isotope Development Limited.

Valine was measured by dissolving about 1 mg (weighed on a Cahn electrobalance) in 200 mg water, and by adding 12 ml scintillation liquid. (Composition : 200 ml ethanol p. a., 800 ml toluene p. a., 5 gr PPO (2,5-diphenyloxazol) and 0.5 gr POPOP (2.2'*p*-phenylen-bis-(5-phenyloxazol)).

At room temperature, this mixture can just dissolve about 1 mg valine. The statistical error of the measurements was always less than 1 %.

The paper strips cut from the chromatograms, and the silica-gel from the thin-layer chromatogram were put into the measuring bottles, and covered with 6 ml of the same scintillation mixture.

Results and discussion

In Table 2, the results of the reverse isotope dilution analysis are given.

Table 2. Results of the reverse isotope dilution analysis with D, L and DL-valine. All figures are corrected to the same dilution factor.

	Carrier added	Percentage of activity present after two crystallizations	Percentage of activity present after four crystallizations
DL-valine tritiated	DL-valine	[100]	—
	L-valine	50.6	—
	D-valine	50.9	—
L-valine tritiated	DL-valine	[100]	[100] [1]
	L-valine	98.1	95.4
	D-valine	4.2	4.2

[1] After three crystallizations.

The results can be influenced by

1. The D-valine content of the L-valine that is to be tritiated.
2. Radiochemical impurities in the labelled valine that is used for the isotopic dilution.
3. Chemical and optical impurities in the inactive D-, L- and DL-valine, used in the isotopic dilution.
4. Incomplete separation of the optical isomers in the isotopic dilution.
5. The accuracy of the dilution and counting procedure.

The combined influence of factors 2, 3, 4 and 5 is reflected in the deviation of the sum of the specific activities of D- and L-valine from the specific activity of DL-valine.

Errors caused by factors 2, 3 and 4 tend to make this sum higher than the specific activity of DL-valine.

By far the largest uncertainty is caused by D-valine already present in L-valine that is to be tritiated. The maximum D-valine content of the L-valine used lies below 0.5 %. This means that the activity of D-valine, formed in L-valine that is tritiated by the Wilzbach method, lies between 3.7 and 4.2 % of the total activity, present as valine.

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