TRITIUM DISTRIBUTION, ENANTIOMER FORMATION, AND GLYCINE PRODUCTION IN L-VALINE LABELED BY MICROWAVE DISCHARGE ACTIVATION OF TRITIUM GAS*

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SUMMARY

The labeling of L-valine by microwave discharge activation of tritium gas was found to be general (i.e., all positions were labeled) but non-random in distribution. The α, β, and γ positions contained 7.1%, 32.7% and 60.2% respectively. On a per-hydrogen basis the β-position was favored by more than a factor of 3, whereas the chiral α-position was the least labeled. Net retention of the L-configuration was observed at the chiral center with only 1.56% D-valine being formed. Small amounts of glycine were found to be produced by fragmentation of the alkyl side chain of valine.

Key Words: Tritium Labeling, [^{3}H]-L-Valine, Microwave Discharge Activation

INTRODUCTION

The increasing usefulness of tritium labeling by microwave discharge activation of tritium gas (1) of biologically active molecules for biological experimentation (2-5) has made it necessary to investigate the labeling characteristcs and patterns more closely. To this end we have labeled the amino acid L-valine, determined the tritium distribution at each position,

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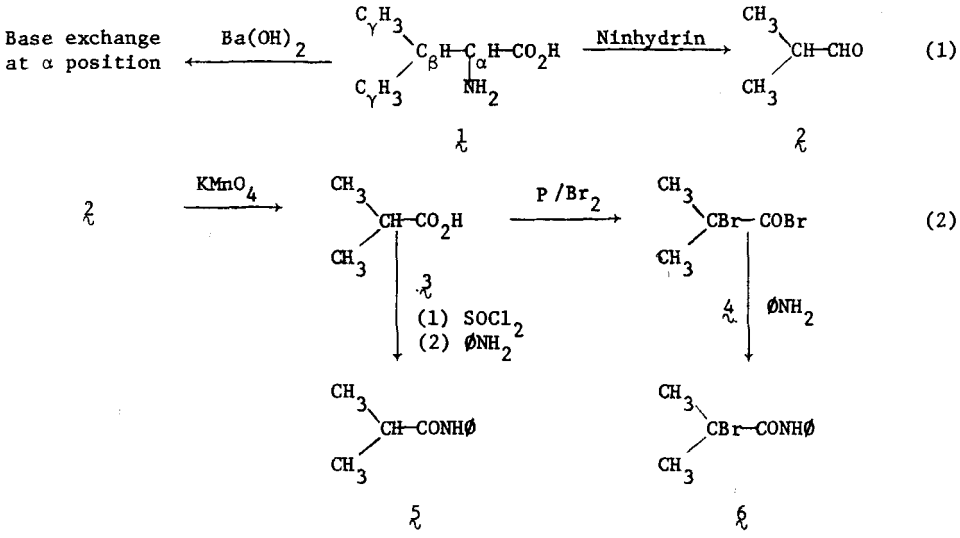
quantitated the amount of D-valine formed from the labeling reaction at the chiral α-carbon, and determined the production of labeled glycine formed by carbon-carbon bond cleavage during the labeling process.

The degradation scheme (see Scheme I) involves 1) oxidation of the valine (1) with ninhydrin to isobutyraldehyde (2) (6), 2) oxidation of the isobutyraldehyde to isobutyric acid (3) with $KMnO_4$ (6), and 3) bromination of the isobutyric acid to 2-bromoisobutyryl bromide (4) with P/Br_2 (7). For counting, and specific activity determinations both the isobutyric acid and the 2-bromoisobutyryl bromide were converted to their respective anilides (see Scheme I).

Scheme I



EXPERIMENTAL

(a) Materials

L-Valine, purchased from Schwarz-Mann, was determined both by optical rotation and glc analysis to contain 2.4% D-valine. [^{14}C]-glycine, purchased from New England Nuclear, was chromatographed on Dowex 50W-X8 prior to use. Tritium gas was purchased from Oak Ridge National Laboratory. Liquid scintillation counting was carried out on a Beckman LS-II counter in Aquasol counting solution (New England Nuclear). All counting data, when necessary, were corrected to DPM by use of internal standards. GLC analysis was carried out on a Varian Aerograph 1520 chromatograph.

(b) Labeling Apparatus

The labeling of L-valine was carried out using the microwave discharge activation of tritium gas in a reaction system previously described (1) with the following modification: light traps were placed at a right angle bend 5 cm above the reaction area to eliminate UV light from reaching the sample area (5,8).

(c) Labeling of L-valine (1)

L-valine was labeled in six batches (6.54, 6.06, 6.70, 7.41, 6.44 and 7.29 mg) totaling 40.44 mg. Each sample was labeled on an 8 x 10 mm glass tray for 5 min with liquid nitrogen cooling at a pressure of 4 mm Hg of tritium gas (~1 Ci). The tritium discharge was sustained by a microwave power of 20 watts at 2450 MHz. The crude samples were combined yielding 941 μCi/40.44 mg.

Prior to purification of the valine, unlabeled glycine (10.0 mg) and [^{14}C]-glycine (4.96 μCi) were added.

Purification was carried out by ion exchange chromatography on Dowex 50W-X8 using 0.1 M pyridine/acetate buffer at pH 3.1.

After initial chromatography on a 1.45 x 50 cm column at room temperature the partially purified valine was divided into four equal batches and chromatographed on a 0.9 x 100 cm Dowex column with the same buffer at 37°.* Using [^{14}C]-glycine as a marker the glycine portion was separated from the majority of the valine and saved for future glycine analysis (see f in experimental).

(d) Degradation of Valine

Prior to degradation the valine was diluted with inactive carrier valine to a specific activity of 0.98 μCi/mmol).

The oxidation of valine to isobutyraldehyde (2) with ninhydrin and subsequent permanganate oxidation of the aldehyde to isobutyric acid (3) were carried out as reported by Crawhall and Smyth (6). Bromination of the isobutyric acid with P/Br_2 was as reported by Smith and Norton (7). The anilides (5, 6, Scheme I) were prepared by standard methods, and were the samples that were finally counted for specific activity determinations. Both of the anilides were chromatographed

*The valine thus purified contained 56.76 μCi/17.97 mg (3.16 μCi/mg, 370 μCi/mmol).

on silica gel using hexane/ether prior to specific activity determinations. After chromatography the melting points and nmr's were found to be consistent with the expected structures. Further purity was checked and confirmed by tlc on silica gel using 50/50 hexane/ether, and on glc using a 1-meter column of 11% SE-30 on chromosorb-W(HMDS) at 150° with a flow of 50 cc/min. Under these conditions the retention times for both anilides was less than 10 min.

(e) Base Exchange

Base exchange, which results in racemization and exchange of the α-carbon hydrogen (or tritium) was carried out as reported by Blackburn (9). Samples were chromatographed on Dowex 50W-X8 prior to counting and specific activity determinations. Completeness of the exchange was confirmed by observing complete racemization of the valine by glc of the diastereomeric amides (see below).

(f) Determination of [^{3}H]-D-valine

The amounts of D and L-valine were determined by glc separation of the diastereomeric N-(L-2-chloropropionyl)-valine methyl esters of the D,L-valine mixture. Preparation and glc analysis of these diastereomers has been reported elsewhere (8,10-16). After preparation of the diastereomeric mixture, samples were injected into the glc. The separated LD and LL diastereomers were collected separately and counted in a liquid scintillation counter. Corrections were made both for the amount of D-valine (2.4%) in the original and labeled sample as well as the amount of D-2-chloropropionyl chloride (3.75%) found in the resolving reagent (8,16).

(g) Glycine Formation

The glycine, which was separated during the initial purification of the crude valine, was derivatized in the same way as the above valine to the N-(L-2-chloropropionyl)-glycine methyl ester. Glc analysis of this sample (as well as the valine) is carried out on a 1/8" x 12' carbowax 20M/chromosorb w (HMDS) 100-200 mesh at 170° and a flow rate of 38 cc/min. Under these conditions the glycine derivative had a retention time of 17 min.

Once the glycine derivative was prepared the sample was injected and collected from the glc and counted in a liquid scintillation counter to determine the

amounts of tritium and ^{14}C found in the glycine sample. Since a known amount of [^{14}C]-glycine (4.96 μCi) was added immediately following the initial labeling, the amount of [^{3}H]-glycine formed from the labeling can then be determined directly by multiplying the $^{3}H/^{14}C$ ratio (from glc analysis) by the amount of [^{14}C]-glycine initially added to the crude reaction mixture.

RESULTS AND DISCUSSION

Valine Degradation

The distribution of tritium in the labeled valine is shown in Table I.

Table I. Distribution of tritium in microwave discharge activation of tritium gas labeling of L-valine.[a]

Position	% Tritium/Position	% Tritium/C-H bond[b]	% lost during Base Exchange[c]
α	7.1	7.1	9.1
β	32.7	32.7	---
γ	60.2	10.0	---

a. Initial specific activity of valine 0.98 μCi/mmol, isobutyranilide 0.91 μCi/mmol, 2-bromoisobutyranilide 0.59 μCi/mmol.

b. Calc. as % per position divided by number of equivalent hydrogens in that position. Random labeling would predict 12.5%/C-H.

c. 4N $Ba(OH)_2$ for 72 hrs at 110°. Sample was shown to be completely racemized by glc. This represents % α-carbon labeling.

The most striking feature (Table I) is the non-randomness of the tritium distribution in the labeled valine. The tertiary β hydrogen was shown to contain nearly three times the 12.5% tritium predicted from a purely random labeling process.

In contrast, labeling at the chiral center is very low, accounting for only 7.1% of the tritium. The lack of significant α-carbon labeling was also confirmed by base exchange of the α-tritium, which showed 9.1% tritium in the α-CH position (see Table I). This trend for high specificity of labeling of the β-tertiary position and low specificity at the α-position has also been

observed in the labeling of L-isoleucene and L-alloisoleucine (8) and may be attributable to bond energy differences and energy of activation differences for hydrogen abstraction at these positions.

Production of D-valine

Due to the importance of chiral integrity in biologically important molecules, the amount of D-valine formed from the labeling of L-valine was determined. Glc analysis of the N-(L-2-chloropropionyl)-D,L-valine methyl ester showed that only 1.56 $\pm$ 0.51% D-valine was produced (see Table II). This lack of labeling at the α-carbon as well as the high degree of retention of the L-configuration is in agreement with other amino acids labeled by the microwave discharge technique (8).

Glycine Formation

The fragmentation of the alkyl side chain from the α-amino carbon of valine was observed by the formation of carrier-free tritium labeled glycine.

Glycine was identified both by its ion exchange properties (chromatography on Dowex 50W-X8) as well as by glc of its N-(L-2-chloropropionyl)-glycine methyl ester. The amount of glycine formed was found to be 13.5 μCi. This corresponds to only about 10% of the valine activity (128 μCi), and thus represents only a minor labeled product (see Table II).

Table II. Amount of each identified product from the labeling of L-valine.

Compound	μCi Produced	% Each Compound Formed	μCi Formed/mg Valine labeled[a]
L-valine	126	89.1	3.12
D-valine	2.0	1.4	0.05
glycine	13.5[b]	9.5	0.33

a. 40.44 mg valine was labeled.

b. This corresponds to ~30 μg glycine, assuming one tritium/molecule.

CONCLUSION

The biological implications of the valine labeling are favorable. The

majority of the valine labeled remains as L-valine. Greater than 92% of the tritium label predominates in biologically unreactive positions (β,γ) and only a small amount of D-valine, by inversion of the chiral carbon, is formed. Glycine, by side chain fragmentation, was also shown to be a minor product. These trends are currently being investigated in peptides labeled by the microwave discharge activation of tritium gas technique.

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REFERENCES

1. Hembree, W. C., Ehrenkaufer, R. E., Lieberman, S., and Wolf, A. P. - J. Biol. Chem. 248, 5532 (1973)
2. Fishman, J., Norton, B. I., and Hembree, W. - J. Labelled Cmpds. 9, 562 (1973)
3. Puszkin, E., Puszkin, S., Lo, K. W., and Tannenbaum, S. W. - J. Biol. Chem. 248, 7754 (1973).
4. Hembree, W. C., Ehrenkaufer, R. L. E., and Wolf, A. P. - Unpublished results.
5. Hembree, W. C., Wolf, A. P., Ehrenkaufer, R. E., and Lieberman, S. - "Tritium Labeling of Amino Acids in Polypeptide Hormones" in Methods in Enzymology, Vol. 37B, O'Malley and Hardman, Eds., Academic Press, New York, 1975, pp. 313-321
6. Crawhall, J. C. and Smyth, D. G. - Biochem. J. 69, 280 (1958).
7. Smith, C. W. and Norton, D. G. - Org. Synth. Coll. Vol. 4, 348 (1963)
8. Ehrenkaufer, R. L. E., Hembree, W. C., Lieberman, S. and Wolf, A. P. - Manuscript submitted.
9. Blackburn, S. - Amino Acid Determination. Methods and Techniques, Marcel Decker, New York, 1968, pp. 23-24
10. Renard, M. - Bull. de la Soc. de Chem. Biol. 28, 497 (1946)
11. Fu, S-C. J., Birnbaum, S. M., and Greenstein, J. P. - J. Amer. Chem. Soc. 76, 6058 (1954).
12. Brown, H. C. - J. Amer. Chem. Soc. 60, 1325 (1938)
13. Fickett, W., Gardner, H. K., and Lucas, H. J. - J. Amer. Chem. Soc. 73, 5063 (1951)
14. Halpern, B. and Westley, J. W. - Chem. Comm. 246 (1965)
15. Westley, J. W. and Weinstein, B. - Nature 210, 837 (1966).
16. Pettijohn R. - "Part A: Radiative Neutron Capture Induced Reactions of Iodine-128, with Gaseous Ethylene and Propylene. Part B: Stereochemistry of the Cl-for-Cl Substitution Activated by Nuclear Transformations in Enantiomeric Chlorocarbons", Dissertation, University of Nebraska, 1973, pp. 131-158 (University Microfilms No. 74-13,011)