DISTRIBUTION OF TRITIUM LABEL IN DL-[G-3H] PHENYLALANINE

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Received on April 23, 1975.

SUMMARY

The position of the tritium label in DL-[G-3H] phenylalanine prepared by platinum catalysed exchange in tritiated water at elevated temperatures has been determined by chemical degradation and by tritium nuclear magnetic resonance spectroscopy. There is good agreement between the results of the two methods : analysis of the product of two separate preparations showing 73 ± 3% of the activity in the aromatic nucleus and 27 ± 3% in the side chain.

INTRODUCTION

L-Phenylalanine, an essential amino acid, is involved in many metabolic pathways. For example, it is converted to tyrosine by the enzyme phenylalanine-4-hydroxylase and subsequently to dopa, dopamine and norepinephrine or to L-thyroxine and 3,3',5-tri-iodothyronine. Also phenylalanine may be oxidised *in vivo* to p-hydroxyphenylpyruvic acid then *via* homogentisic acid to fumaric and acetoacetic acid.

Such biochemical pathways are often studied by the use of isotopically labelled substrates such as tritium labelled phenylalanine. However, as tritium may be lost from certain positions during the substitution and degradation reactions it is important to know the position of the tritium atom(s) within the molecule.

This paper describes the determination of the distribution of the tritium label in DL-[G-3H]phenylalanine by chemical methods and by the use of tritium nuclear magnetic resonance spectroscopy.

EXPERIMENTAL

DL-[G-3H]Phenylalanine (The Radiochemical Centre Limited, catalogue code number TRK.30) was prepared by heating together a mixture of DL-phenylalanine (0.5g), reduced Adams catalyst (0.2g) and tritiated water (1-2 ml containing approximately 300 Curies) in a sealed tube at 135° for 18 hours. After removal of catalyst and labile tritium the DL-[G-3H]phenylalanine was purified by paper chromatography. Two separate batches of DL-[G-3H]phenylalanine with specific activities of 8.2 and 4.8 Ci/mmol were prepared and used at the specific activities for the T.n.m.r. studies. For the chemical degradation studies the specific activities were reduced by the addition of unlabelled DL-phenylalanine (Sigma Chemical Co.).

Paper chromatography (PC) was performed on 2" strips of Whatman No. 1 paper which were developed by descending chromatography. Thin-layer chromatography (TLC) was performed on silica gel plates of 0.25mm thickness (E. Merck, Darmstadt, Germany). The following solvent systems were used:

A n-Butanol:glacial acetic acid:water (12:3:5)

B Ethanol:ammonia (0.880):water (80:4:16)

C n-Butanol:pyridine:water (1:1:1)

D n-Butanol:ethanol:ammonia (18%) (60:15:75)

E Chloroform:cyclohexane:glacial acetic acid (80:20:10)

F Benzene:methanol:acetic acid (90:16:8)

The radioactivity on the chromatograms was measured in a windowless gas flow counter and non-radioactive markers visualised by ninhydrin (amino acids) or 0.1% ethanolic dichlorophenol-indophenol (carboxylic acids).

Specific activites were determined by oxidation of 2-3 mg samples by the Schöniger method and the tritiated water counted in a Nuclear Chicago Mk 1 scintillation counter using Triton X100/PPO/POPOP as the scintillant.

Tritium nuclear magnetic resonance spectra (T.n.m.r.) were measured in D_2O at the University of Surrey using a Bruker-WH90 instrument operating by the pulse Fourier-transform method at a frequency of 96.02 MHz.

For the chemical determination of the distribution of the tritium label, the reaction scheme shown in figure 1 was used.

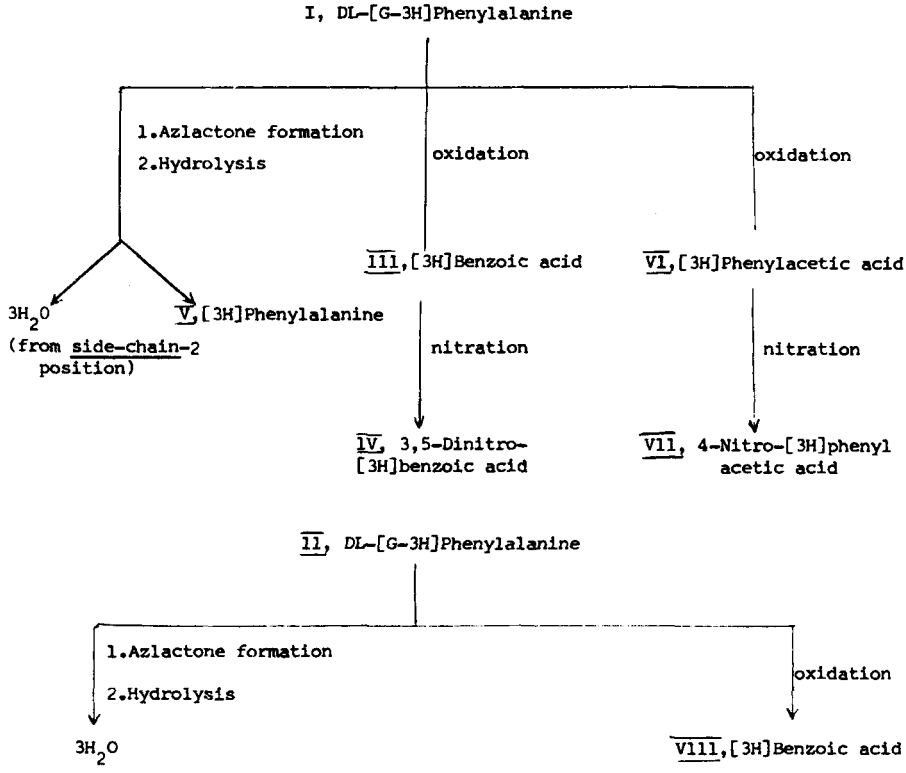


Figure 1

Oxidation of DL-Phenylalanine to Benzoic acid

A mixture of DL-[G-3H]Phenylalanine, I (422 mg, 2.56 mmol, 87.1 mCi/mmol), finely powdered potassium permanganate (1.5 g), anhydrous sodium carbonate (1.2 g) and water (30 ml) was heated under reflux for 20 hours. The cooled reaction mixture was acidified with 2N sulphuric acid to pH 2 and

extracted with chloroform (4 x 75 ml). The organic phase was dried ($MgSO_4$) and the solvent removed *in vacuo* to give crude [3H]benzoic acid (294 mg). Crystallisation from hexane gave [3H]benzoic acid, III (202 mg), mp 122°C, sp.act. 61.1 mCi/mmol; radiochemically pure by PC (solvents C and D) and by TLC (solvents E and F).

Similarly, DL-[G-3H]phenylalanine, II (395 mg, 2.39 mmol, 71.6 mCi/mmol) gave [3H]benzoic acid, VIII (119 mg), mp 122°C, sp.act. 51.3 mCi/mmol; radiochemically pure by PC (solvents C and D) and by TLC (solvents E and F).

Nitration of [3H]Benzoic acid, III to 3,5-Dinitro-[3H]benzoic acid, IV

[3H]Benzoic acid, III (150 mg, 1.23 mmol, 61.1 mCi/mmol) was nitrated to give 3,5-dinitro-[3H]benzoic acid, IV (75 mg), mp 208°C, sp.act. 36.6 mCi/mmol; radiochemically pure by PC (solvents C and D) and by TLC (solvents E and F).

Determination of Tritium in the Side-chain-2 Position of DL-[G-3H]Phenylalanine

A mixture of DL-[G-3H]phenylalanine I (97 mg, 0.59 mmol, 87.1 mCi/mmol), acetic anhydride (6 ml) and water (1 ml) was heated under reflux for 3 hr. The reaction mixture was cooled and the solvent removed by short path distillation. An assay showed the distillate to contain 2.41 mCi tritium *i.e.* 4.7% of the tritium had been labilized.

The dry residue of N-acetyl-DL-[3H]phenylalanine was hydrolysed with 5N-hydrochloric acid (reflux; 2.5 hr) to give, after chromatographic purification, DL-[3H]phenylalanine, V (28 mg), sp.act 84.0 mCi/mmol; radiochemically pure by PC (solvents A,B and C). Thus 3.6% of the tritium had been labilized.

Similarly, DL-[G-3H]phenylalanine, II (135 mg, 0.82 mmol, 71.6 mCi/mmol)

gave a distillate containing 2.18 mCi tritium i.e. 3.7% of the tritium had been labilized.

Oxidation of DL-[G-3H]Phenylalanine, I to [3H]Phenylacetic acid, VI

DL-[G-3H]Phenylalanine, I (381 mg, 2.31 mmol, 87.1 mCi/mmol) was oxidatively deaminated[1] with chloramine T at pH 4.7 to give [3H]phenylacetic acid, VI (121 mg), mp 76-77°, sp.act. 62.7 mCi/mmol; radiochemically pure by TLC (solvents C,D and E).

Nitration of [3H]Phenylacetic acid, VI to 4-Nitro-[3H]phenylacetic acid, VII

Concentrated sulphuric acid (2.8 ml) was added slowly to [3H]phenylacetic acid, VI (73 mg, 0.40 mmol, 62.7 mCi/mmol) contained in a flask fitted with a reflux condenser and cooled in an ice bath. The mixture was stirred magnetically and when all the solid had dissolved concentrated nitric acid (0.8 ml) was added dropwise during ten minutes. Stirring was continued for one hour at +4°C and then the reaction mixture was added to crushed ice (25 g). The crystals which formed were filtered off and recrystallised from 70% ethanol to give 4-nitro-[3H]phenylacetic acid, VII (16.6 mg), mp 154°C, sp.act 34.0 mCi/mmol: radiochemically pure by TLC (solvents C,E and F).

RESULTS

The results of the chemical degradation experiments are summarised in Table 1. As will be seen, the amount of tritium in the ring as determined by oxidation of the DL-[G-3H]phenylalanine (sample 1) either to [3H]benzoic acid or to [3H]phenylacetic acid (taking account of the ready labilisation of tritium from the benzylic position[1] during the latter reaction) are in close agreement. Tritium in the ring-3,5 and 4 positions was found by conversion of the two acids to 3, 5-dinitro-[3H]benzoic acid and 4-nitro-

[3H]phenylacetic acid respectively. Tritium in the 2-and 6- positions was calculated by difference.

Tritium in the side-chain-2 position was determined by labilisation during azlactone formation[2], the amount being calculated in two ways: by measurement of the tritiated water produced and from the change in specific activity of the [3H]phenylalanine remaining at the end of the reaction. Tritium in the side-chain-3 position was calculated by difference.

In order to test the reproducibility of labelling by catalysed exchange

TABLE 1 RESULTS OF CHEMICAL DEGRADATION EXPERIMENTS

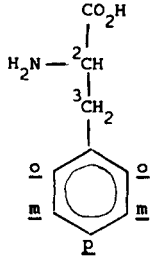
Compound	Specific activity found mCi/mmol	Percentage of total activity in compounds (DL-[G-3H]phenylalanine = 100%) (%)	Calculated percentage of activity in specific position of DL-[G-3H]phenylalanine (%)
I DL-[3H]Phenylalanine	87.1	100	-
III [3H]Benzoic acid	61.1	70.1	ring 70.1 side-chain 29.9
IV 3,5-Dinitro-[3H]benzoic acid	36.6	42.0	ring-3,5 28.1
V DL-[3H]Phenylalanine	84.0	96.4	side-chain-2 3.6
Labile tritium from side-chain	-	4.7	side-chain-2 4.7
VI [3H]Phenylacetic acid	62.7	72.0	ring 72.0 side-chain 28.0
VII 4-Nitro-[3H]phenyl-acetic acid	34.0	39.0	ring-4 33.0 ring-2,6 9.0
II DL-[G-3H]Phenylalanine	71.6	100	-
VIII [3H]Benzoic acid	51.3	71.6	ring 71.6 side-chain 28.4
Labile tritium from side-chain	-	3.7	side-chain-2 3.7

in tritiated water, a second batch of DL-[G-3H]phenylalanine (sample II) was prepared. Oxidation to [3H]benzoic acid and labilisation of tritium from the side-chain-2 position showed that the distribution of tritium closely resembled that of the first batch of DL-[G-3H]phenylalanine.

Thus, the chemical degradation experiments indicated that there was 71 ± 1% of tritium in the ring with 29 ± 1% in the side-chain, of which 4% was in the side-chain-2 position.

Figure 2 shows an example of the tritium n.m.r. spectrum of DL-[G-3H]phenylalanine (sample I). This clearly indicates the distribution of the tritium in the ring and side-chain positions and the results obtained with both batches of DL-[G-3H]phenylalanine are summarised in Table 2 together with the results of the chemical degradation studies. These show that for DL-[G-3H]phenylalanine prepared by platinum catalysed exchange in tritiated water 73 ± 3% of the activity is in the ring and 27 ± 3% is in the side-chain. Of the activity in the ring most is in the m- and p- positions (of the 70% of the activity in the ring, 33% is in the 4-position, 28% in the 3,5-positions and 9% in the 2, 6-positions).

TABLE 2 DISTRIBUTION OF THE TRITIUM LABEL IN DL-[G-3H]PHENYLALANINE

		DL-[G-3H]Phenylalanine I			DL-[G-3H]Phenylalanine II	
		degradation %		T.n.m.r. %	degradation %	T.n.m.r. %
	2	4		2	4	4
	3	26 *		25	24 *	20
	o	9	70	73	72	76
	m	28				
	p	33				

* by difference

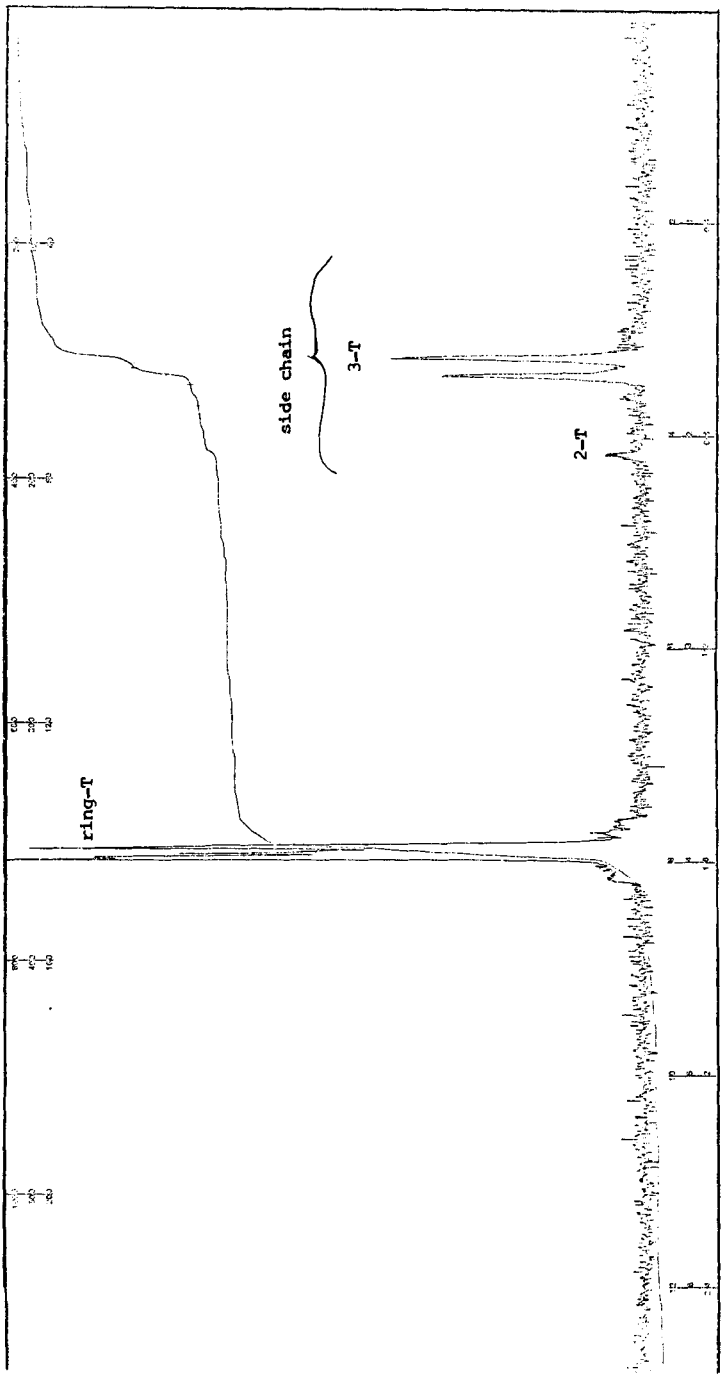


FIGURE 2

Fourier-transform tritium n.m.r. spectrum of [G-3H]phenylalanine in [$^2H_2$]water at 25°, 3.03×10^4 pulses at 1.7s intervals

DISCUSSION

The results above are in agreement with published work[3-8] on catalysed isotopic exchange of similar organic compounds. For example, the work of Garnett[3] indicates that heterogeneous catalysed exchange of phenylalanine with deuterated water at elevated temperature produces [2H]phenylalanine in which the isotope is distributed generally over the molecule. Aromatic compounds containing bulky side chains give labelled compounds in which there is little or no exchange in the ortho-positions [4,5,6] (ortho deactivation) and there is more exchange in the para-position than in the meta-position[7]. Heterogeneous and also homogeneous[6] catalysed exchange of aromatic compounds containing alkyl side-chains produce compounds labelled to a greater extent in the benzylic (α) positions than in the β positions.

Thus, one would expect the tritium label in DL-[G-3H]phenylalanine to be both in the ring and side-chain; that in the ring most of the activity would be in the m- and p- positions and that in the side-chain there would be more activity in the α-position than in the β-position. These expectations are confirmed by our experimental data. An indication that approximately 30% of the activity in DL-[G-3H]phenylalanine is in the side-chain was given in earlier experiments using crude amino acid oxidases.[9]

A recent report [10] on the distribution of the tritium label in DL-[G-3H]phenylalanine prepared under the same conditions as that studied by ourselves suggested that there was negligible radioactivity in the side-chain and that in the ring the radioactivity was confined to the o- and p- positions (52% and 45% respectively). This result is quite unexpected in view of the published work discussed above. It also conflicts with our chemical degradation results which are supported by tritium n.m.r. spectral evidence. This application of tritium n.m.r. is a good example of this direct and non-destructive technique[11] which can now be used with advantage to determine the pattern of labelling of tritium labelled compounds.

ACKNOWLEDGEMENTS

The authors thank Mr. R. Jarman for technical assistance and Dr. W. P. Grove (Managing Director, The Radiochemical Centre Ltd) for permission to publish this paper.

We thank Prof. J. A. Elvidge and staff of the Chemistry Department, University of Surrey, Guildford, for their collaboration in the tritium nuclear magnetic resonance spectroscopy investigations.

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