TRITIUM NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY. PART IV[ref. (1)]. DISTRIBUTION OF TRITIUM IN [G-^{3}H] PHENYLALANINE AND OTHER AMINO ACIDS

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SUMMARY

The usefulness of ^{3}H n.m.r. spectroscopy as an analytical tool for the determination of tritium distribution in both generally and specifically labelled compounds is illustrated by reference to the results for phenylalanine and several other amino acids.

Key words:- Tritium n.m.r., phenylalanine, tyrosine, tryptophan, proline

INTRODUCTION

Tritium-labelled amino acids find wide application in both chemistry and biochemistry(2) and are therefore in much demand. They are usually prepared by catalytic reduction of unsaturated precursors(3), catalytic halogen-tritium replacement(4), or metal-catalysed heterogeneous exchange(5). The first two methods usually lead to specific labelling and the third to a generally labelled product. For many purposes it is necessary to know the distribution of the label in the compound before embarking on e.g. biochemical experiments, and hitherto this has required stepwise degradation and counting of the isolated products. Not only is the process tedious and time consuming but it is only as reliable as the integrity of the reactions employed. ^{3}H N.m.r.(6) however provides a rapid, accurate and non-destructive method of determining both positions and extent of tritium labelling. This is because

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both chemical shifts and intensities of signals have the same significance as in ^{1}H n.m.r. spectroscopy(7) and the wealth of information available from the latter field is immediately applicable to the interpretation of ^{3}H n.m.r. spectra. Here we report our detailed findings on [G-^{3}H] phenylalanine and other amino acids.

RESULTS AND DISCUSSION

Phenylalanine

[G-^{3}H]Phenylalanine, prepared by platinum catalysed exchange of phenylalanine with tritiated water at 135°C for 18h, has long been available. The pattern of labelling has been established by degradation and counting, which incidentally took ca. 1 man-month(8). Recently(9) it was reported that the tritium in this product was confined to the aromatic ring, with 45% in the para-position and the rest in the ortho-positions. This conclusion is now shown to be erroneous(8).

The ^{3}H n.m.r. spectrum of the [G-^{3}H]phenylalanine (Figure 1), recorded with ^{1}H decoupling, shows a tritium line from each position in the molecule (excluding the carboxyl and amino groups) and hence that the label is indeed generally distributed. The assignments follow from the established ^{1}H spectrum of phenylalanine(10) and the results are summarised in Table 1. It is apparent that on average 26% of the label is in the side chain, mostly at the β-positions, and 74% in the ring, fairly equally distributed. The total time taken for a determination (as given in Table 1) was little more than the spectral acquisition time of 8.5h for a 54 mCi sample. It is noteworthy that the chemical shifts of the two anisochronous β-methylene hydrogens are available by inspection as also are those of the ring hydrogens. These latter shifts have not been derived before, even from the ^{1}H spectrum of phenylalanine(11) measured at 220 MHz, although this did indicate that the o-hydrogens gave rise to the highest field of the ring signals.

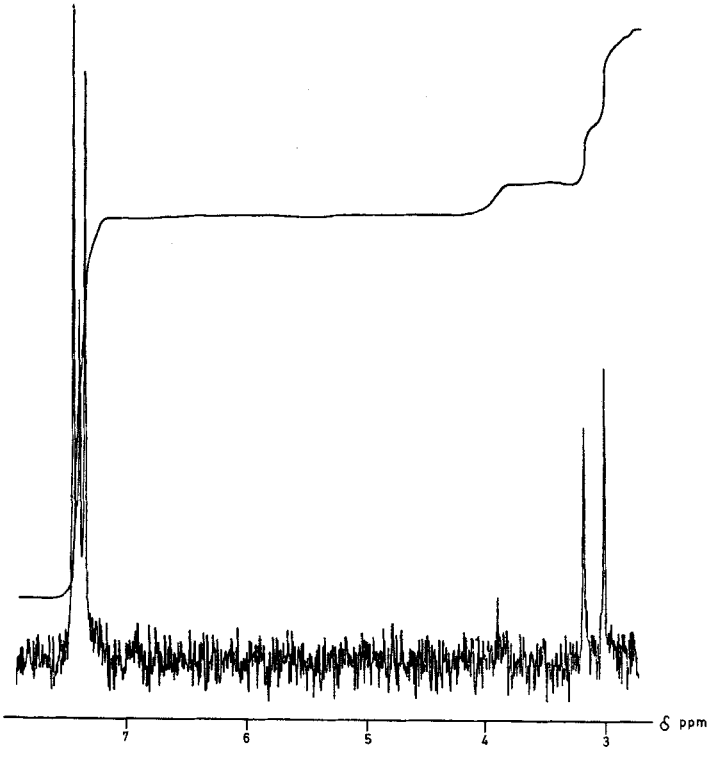


Figure 1

Table 1. ^{3}H N.m.r. results for [G-^{3}H]phenylalanine in d_6-DMSO at 96 MHz with ^{1}H decoupling

Chemical shifts δ ppm	Assignment	Relative Intensity % (different samples)
3.06	β-CH_2	14.4, 12.7, 11.3, 13.0
3.22	β-CH_2	12.5, 12.1, 7.4, 11.4
3.90	α-CH	2.3, 1.8, 3.6, 1.1
7.34	o-H	26.6, 27.4, 77.7, 74.5
7.39	p-H	14.9, 17.5,
7.43	m-H	29.3, 28.5,

[4-^{3}H]Phenylalanine

This specifically labelled compound was prepared by catalytic tritiodehalogenation of p-chlorophenylalanine and examined as before, but using a deuterium oxide solution. The ^{3}H spectrum showed a strong line at δ7.39 from the p-^{3}H, together with minor lines at δ7.25 and 6.86 from unknown impurities. At a similar concentration in deuterium oxide, the ^{3}H spectrum of [G-^{3}H]phenylalanine provided lines at δ7.36, 7.40, and 7.44 from the o-, p-, and m-^{3}H respectively, confirming the deductions.

p-Fluoro[G-^{3}H]phenylalanine

The ^{3}H spectrum (with ^{1}H decoupling) (Figure 2) resembled that of [G-^{3}H]phenylalanine in the aliphatic region but showed four lines

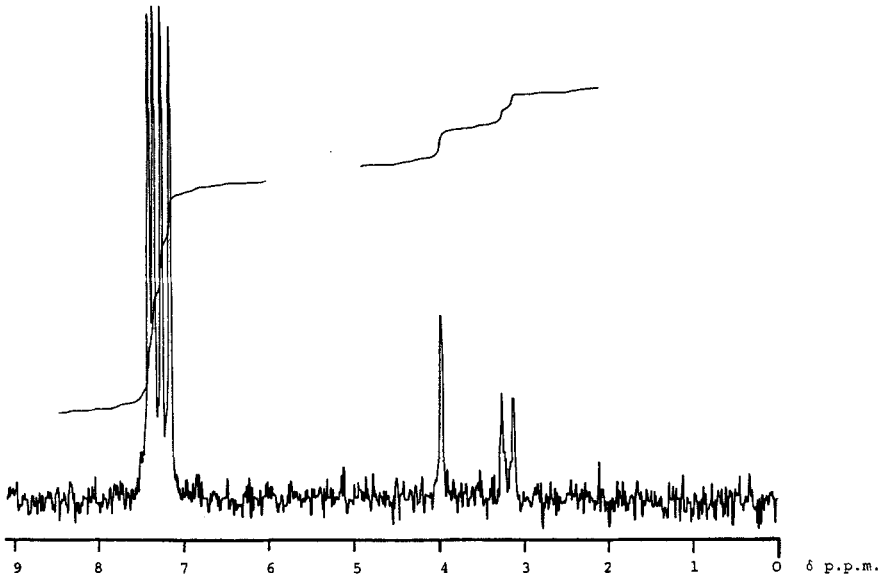


Figure 2

of roughly equal intensity in the aromatic region. Since on average J(o-^{1}HF) is ca. 8.5 Hz and J(m-^{1}HF) is ca. 7.0 Hz(12), and knowing that J(^{3}HX) = 1.06664 J(^{1}HX) (1), it was possible to assign the higher field pair of lines (centred at δ7.17) with

J 9.7 Hz to the (ortho) 3,5-^{3}H, and the other doublet with J = 5.3 Hz to the 2,6-^{3}H. No other pairing gave reasonable values for the coupling constants. The results are summarised in Table 2. Again it is noteworthy that the chemical shifts could not be extracted from the ^{1}H spectrum measured at the same field strength (90 MHz).

Table 2. ^{3}H N.m.r. results for p-fluoro[G-^{3}H]phenylalanine in D_2O at 96 MHz with ^{1}H decoupling

Chemical shifts δ ppm	Assignment	Relative intensity %
3.09 3.22	β-CH_2	11.3 (equally distributed)
3.93	α-CH	9.4
7.17 (doublet, J 9.7 Hz)	3,5-H	37.8
7.33 (doublet, J 5.3 Hz)	2,6-H	41.5

[2,6-^{3}H]Tyrosine

With ^{1}H decoupling, the ^{3}H spectrum showed a single line at δ7.20, in accord with the labelling expected from tritiodeiodination of 2,6-di-iodo-tyrosine.

[3,5-^{3}H]Tyrosine

Measured as in the previous case, the ^{3}H spectrum showed a singlet at δ6.90 from the more shielded tritons ortho to the hydroxyl group. Again, the complete integrity of the labelling method - tritiodeiodination of 3,5-di-iodotyrosine - was demonstrated unequivocally.

[5-^{3}H]Tryptophan

As expected for specific labelling at the 5-position,

achieved by tritiodebromination of 5-bromotryptophan, the ^{3}H spectrum showed a singlet at δ7.01 when measured with ^{1}H decoupling. Without ^{1}H decoupling, the signal appeared as a (distorted) triplet, as a result of o-coupling of the triton to a proton on each side at the 4- and 6-position, with J ca. 7 Hz. There were signs of further doublet splitting from m-coupling to the 7-proton, with J ca. 2 Hz. Thus the assignment of the triton signal was fully confirmed.

[G-^{3}H]Tryptophan

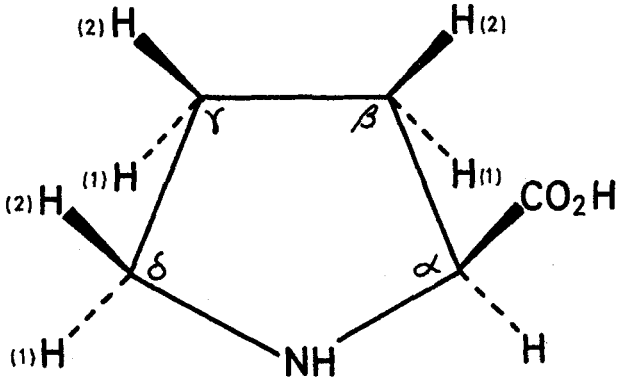
The ^{1}H decoupled ^{3}H n.m.r. spectrum of this sample in d_6-DMSO showed signals in the region δ2.29 to 3.48 from the labelled C-H s of the side chain amounting to 8.25% of total tritium content. Unfortunately the spectral acquisition time had to be curtailed and so the intensity was too low for the lines to be properly resolved from base-line noise. At lower field, there was a line from label at the 2-position of the ring, with δ6.65 (6.6%), and then four lines in the aromatic region, as expected, with δ7.07 (18.1%), 7.16 (19.8%), 7.30 (17.0%), and 7.44 (22.0%). Finally to lower field at ca. δ8.3 was a broadened signal, presumably from labelled NH (8.25%).

[3,4-^{3}H]Proline

This compound was prepared by catalytic reduction of $\Delta^{3,4}$-dehydroproline with tritium and hydrogen gas, a procedure which might be expected to yield a cis-3,4-ditritiated proline. However, experience with e.g. the hydrogenation with tritium of crotonic acid (13) or of 4-isopropylidene-2-phenyloxazolone (for labelled valine) (14) suggested that some non-specific labelling might be encountered.

The ^{3}H n.m.r. spectrum of the labelled proline in deuterium oxide, observed with ^{1}H decoupling, showed strong doublets with

J 8.2 Hz at δ1.96 and 2.32, chemical shifts consistent with a doubly-labelled cis [3,4-$^3H_2$]proline. In addition, there were single lines at each origin position, evidently from tritons present in singly-labelled [3-^{3}H] and [4-^{3}H] proline species. There were also two weak lines at δ3.24 and 3.32 from label in the 5-methylene group - in the δ_1 and δ_2 positions as indicated by (I) (see Ref. 15). In the precursor dehydroproline the 5-methylene group is an allylic methylene and so subject to exchange.



(I)

The ^{3}H n.m.r. spectrum indicated that this exchange is non-stereospecific and only takes place to a small extent. Interestingly there is no labelling at the 2-(α) position in the tritiated proline. Comparison with the data for the fully analysed ^{1}H spectrum of proline at various pH in deuterium oxide, measured at 250 MHz (16), facilitated the assignments given in Table 3. The results demonstrate that the hydrogenation of $\Delta^{3,4}$-dehydroproline under specified conditions occurs on the face of the molecule opposite to the carboxyl group and that side reactions do not proceed extensively. Percentage labelling results (b) in Table 3 were obtained after the sample solution at high radioactive concentration has been kept at 2°C for 1 month. On storage, self-decomposition may cause

the label to be lost slowly from the 3,4-positions equally with the tritium appearing as an unknown impurity (δ2.52).

Table 3. ^{3}H N.m.r. results for [3,4-^{3}H]proline in D_2O at 96 MHz with ^{1}H decoupling

Chemical shifts δ ppm	Assignment in (I)	Relative intensity, % (a)	(b)
1.96	γ_1	48.6	43.9
2.32	β_1	45.7	41.3
3.24	δ_1	4.3	4.6
3.32	δ_2		4.1
2.52	unknown	1.4	6.1

EXPERIMENTAL

The samples, mostly 25-50 mCi of high specific activity (from the Radiochemical Centre), were transferred to the stated deuterated solvent (to provide the lock) containing TMS or DSS as appropriate (7), and sealed in cylindrical microcells (100 µl; Wilmad) which were then inserted into standard 5mm. n.m.r. tubes: caps were added. The ^{3}H spectra were obtained at 25±1° with a Bruker WH90 pulse spectrometer operating at 96 MHz. The pulse width was usually 2.5 µs, and the repetition interval 1.7s. 10^4 - 4×10^4 Transients were acquired into 8K channels and Fourier transformed to provide a spectral display of width 1280 Hz.

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