TRITIUM NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY PART 12. PATTERNS OF LABELLING IN TRITIATED FOLIC ACID AND METHOTREXATE

E. Anthony Evans, J.Philip Kitcher and David C. Warrell
The Radiochemical Centre, Amersham, Bucks

John A. Elvidge, John R. Jones and Robert Lenk
Department of Chemistry, University of Surrey, Guildford, Surrey

Key Words: Folic acid, methotrexate, pteroyl-glutamates, tritium, halogen-tritium exchange, tnmr spectrometry, specificity of labelling

SUMMARY

Labelling patterns in tritiated folic acid and methotrexate have been examined by tritium nuclear magnetic resonance spectrometry. The results indicate that oxidative degradation with permanganate can give a false tritium distribution.

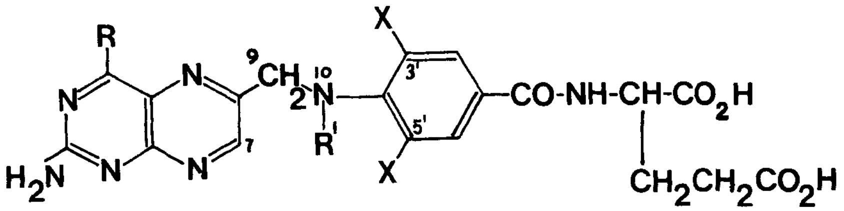
INTRODUCTION

Tritiated folic acid and its 4-amino-N^{10}-methyl analogue, methotrexate are widely used in biomedical research. For studies in which the tritium is used as a tracer to follow the fate of the intact molecule, the specificity of tritium labelling is normally unimportant. However, in studies where the possibility of metabolism or chemical alteration of the compound arises, it is very important to know the distribution of tritium in the tracer compound (1).

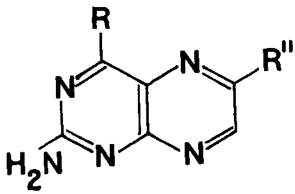
Folic acid (I; R=OH, R^1=H, X=H) and methotrexate (I; R=NH_2 R^1=Me, X=H) are labelled with tritium by catalytic halogen-tritium replacement in 3',5'-dibromofolic acid (I; R=OH, R^1=H, X=Br) and 3',5'-dichloro-methotrexate (I; R=NH_2, R^1=Me, X=Cl) respectively using tritium gas (2,3). Labelling with tritium is also achieved by a procedure using catalysed exchange between tritium gas and the compound in solution (2,3,4).

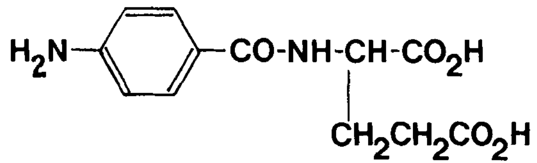
0362-4803/79/0516-0697$01.00
©1979 by John Wiley & Sons, Ltd.

Received November 20, 1978
Revised December 6, 1978



(I)





(II)

(III)

Previous studies (2) using chemical oxidative and enzymatic methods to establish the patterns of labelling have indicated that the 9-methylene position is labelled with tritium during either catalysed halogen-tritium replacement or catalysed hydrogen-tritium exchange. Oxidative degradation of pteroyl glutamates (folic acid and methotrexate) with alkaline permanganate cleaves the C^9-N^{10} bond giving the corresponding pteridine-carboxylic acid (II; R=OH or NH_2, R"=COOH) and 4-aminobenzoyl-glutamic acid (III). Tritium in the 9-methylene group is oxidised to tritiated water under the experimental conditions (2). A recent publication by Maruyama et al. (5) describes a detailed study of the oxidative cleavage of folates and that the 4-aminobenzoyl-glutamic acid formed is also prone to further oxidation to unidentified products. This further degradation of the 4-aminobenzoyl-glutamic acid results in the formation of tritiated water from tritium atoms in the 3',5'-positions of the folic acid or methotrexate which invalidates this chemical oxidation procedure for establishing the patterns of tritium labelling in these compounds.

Tritium nuclear magnetic resonance spectroscopy has been established (6,7) as a routine non-destructive method for determining patterns of labelling in tritiated compounds. Figure 1 shows the proton decoupled triton spectrum of folic acid prepared by catalytic halogen-tritium replacement, and Figure 2 the proton decoupled triton spectrum of folic acid labelled by catalytic hydrogen-tritium exchange. Figures 3 and 4 shows the corresponding spectra for methotrexate.

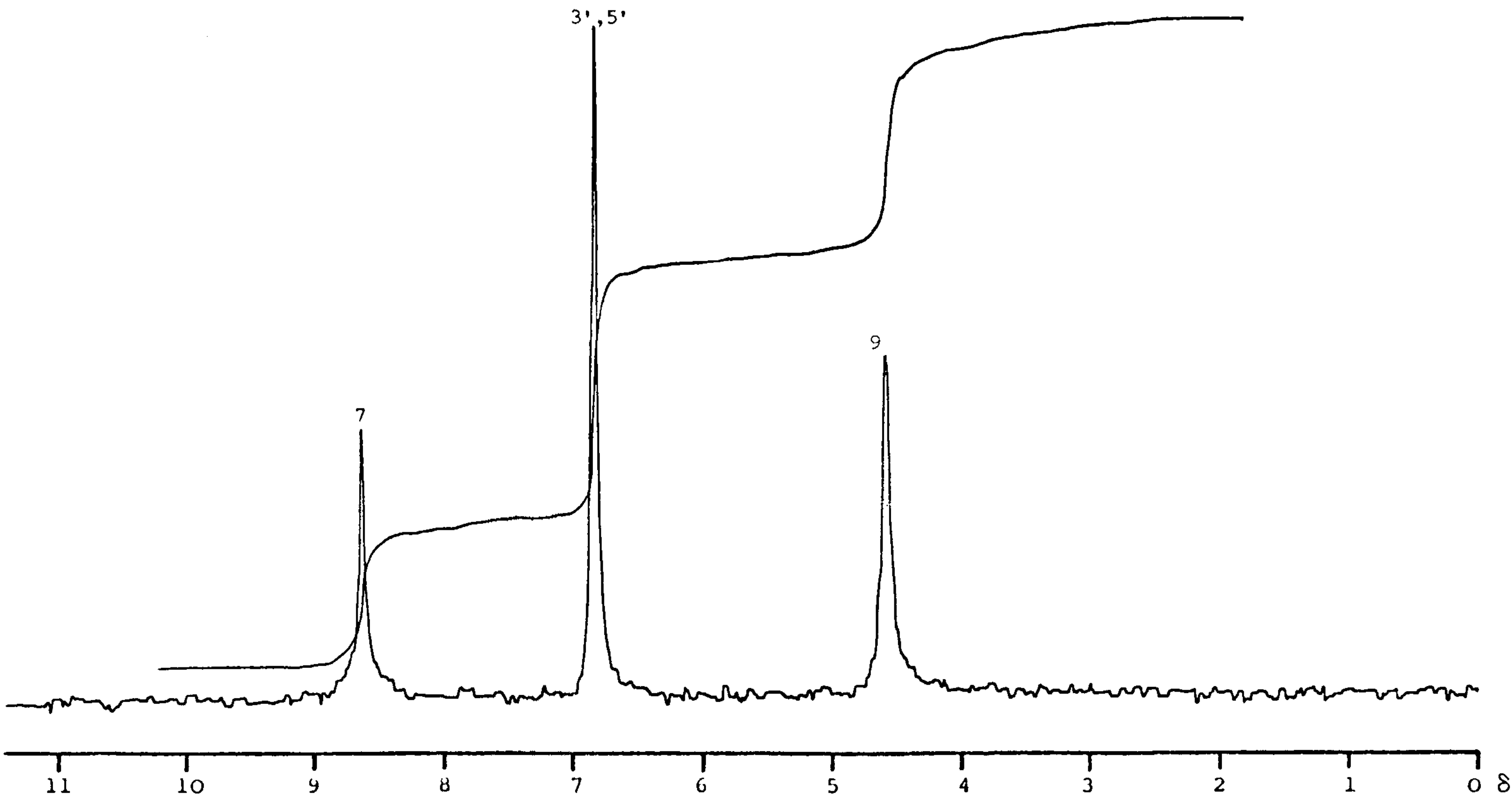


FIGURE 1 - ^{1}H decoupled tnmr spectrum of [3',5',7,9-^{3}H]folic acid

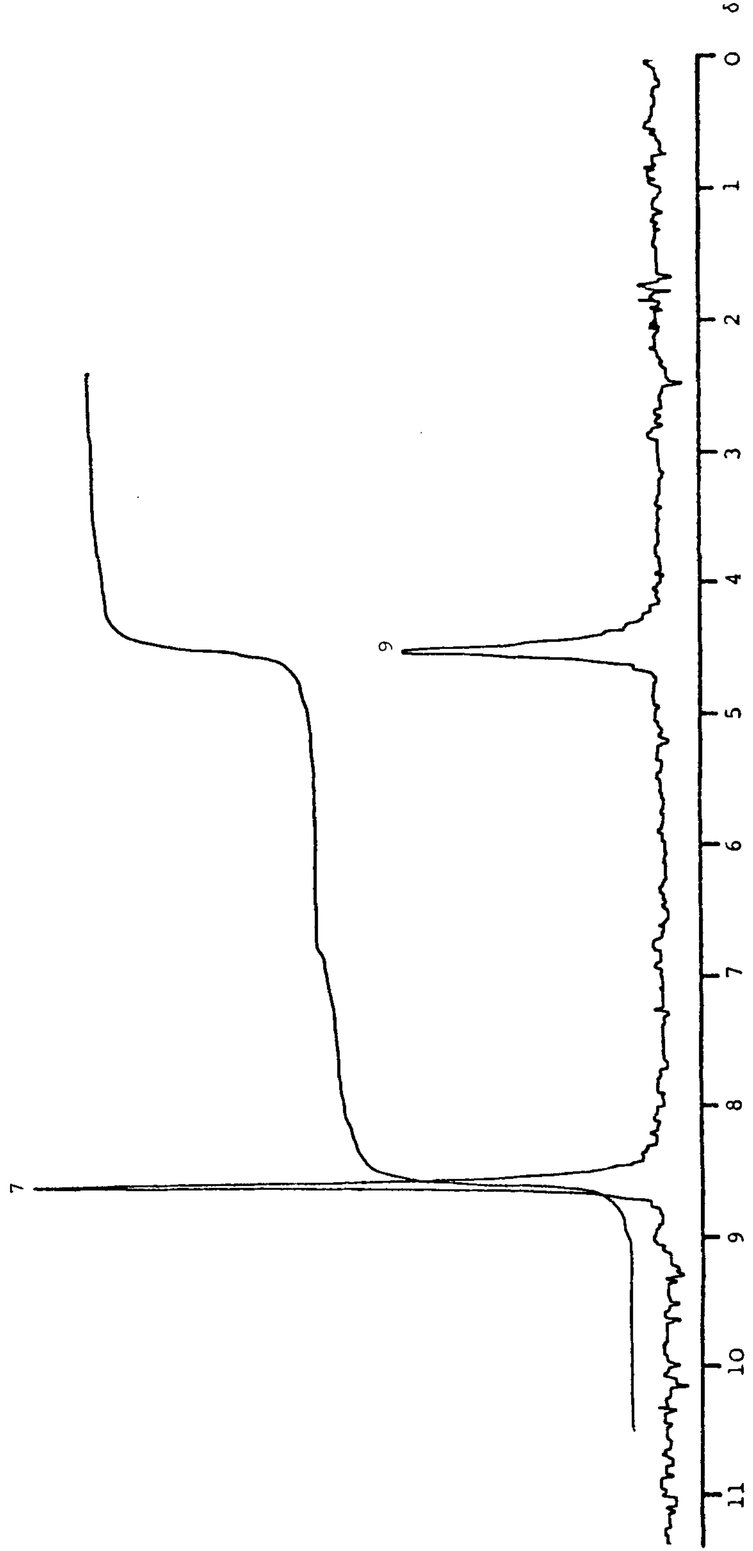


FIGURE 2 - ^{1}H decoupled tnmr spectrum of [7,9-^{3}H]folic acid

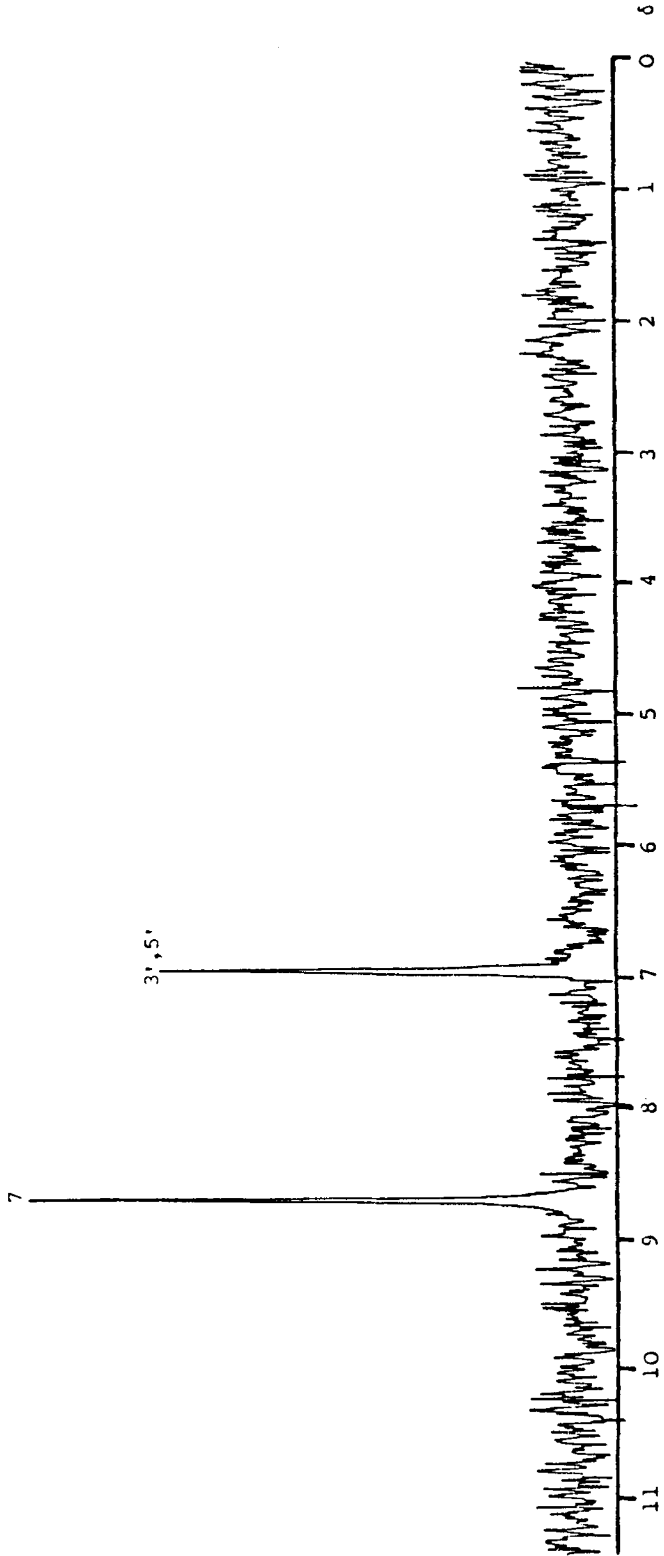


FIGURE 3 - ^{1}H decoupled tnmr spectrum of [3',5',7-^{3}H]methotrexate

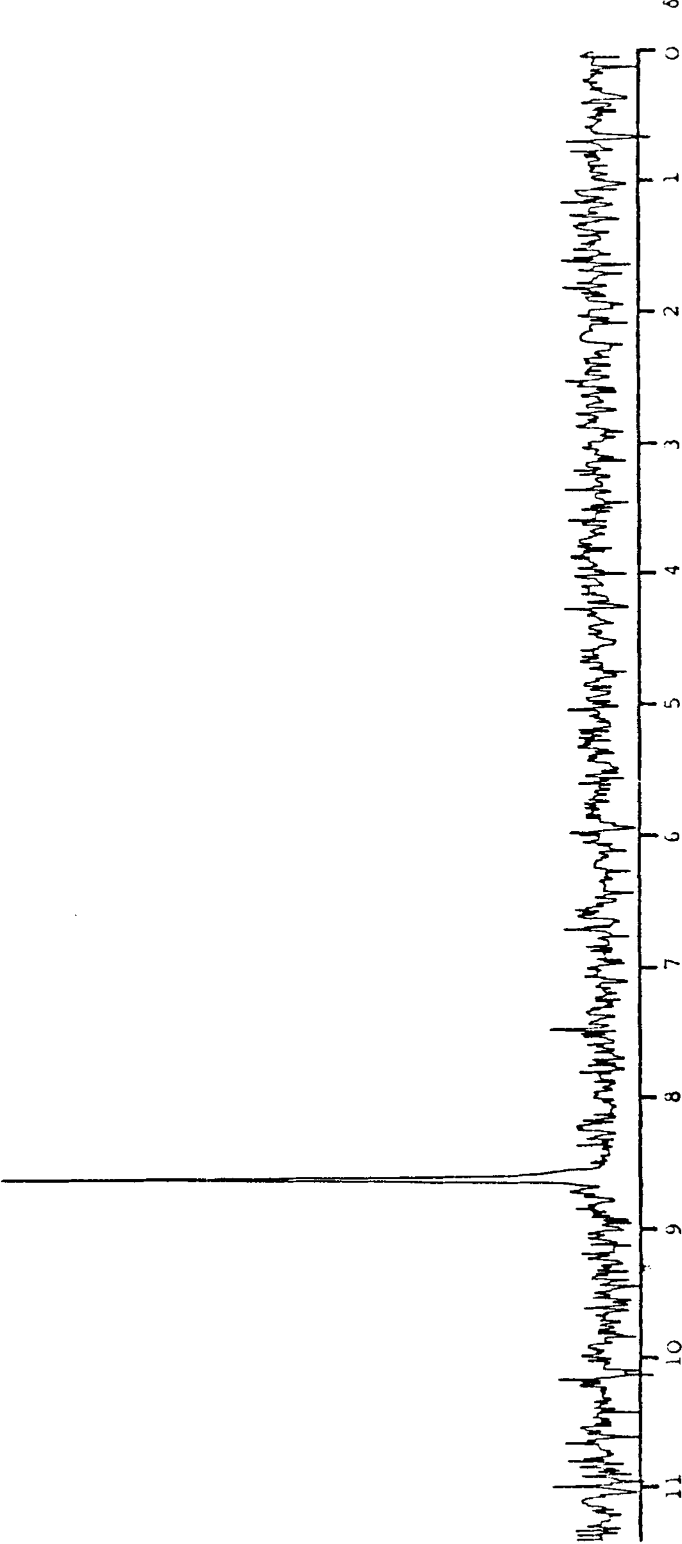


FIGURE 4 - ^{1}H decoupled tnmr spectrum of [7-^{3}H]methotrexate

EXPERIMENTAL

Triton and proton nuclear magnetic resonance spectra were obtained using a Bruker WH90 spectrometer operating in the Fourier transform mode. Aqueous samples of [3',5',7,9-^{3}H]folic acid K salt (50mCi;58Ci/mmol), [7,9-^{3}H]folic acid K salt (50mCi;9Ci/mmol), [3',5',7-^{3}H]methotrexate Na salt (50mCi;6Ci/mmol), [7-^{3}H]methotrexate Na salt (50mCi;11Ci/mmol) and [7-^{3}H]pteridine-6-carboxylic acid K salt (25mCi;20Ci/mmol) were frozen and lyophilised. Each sample was redissolved in deuterium oxide (105μl) (to provide for field frequency locking) and a trace of sodium 4,4-dimethylsilapentanesulphonic acid (DSS) added to provide the ^{1}H reference. The solutions were sealed in cylindrical microcells (100μl; Wilmad) and inserted into standard n.m.r. tubes (5mm) which were capped. Triton spectra (with ^{1}H decoupling) were recorded at 96 MHz and 25°C as previously (8,9).

[3',5',7,9-^{3}H]Folic acid - Prepared as previously described (2) from 3',5'-dibromofolic acid by halogen-tritium replacement in 4 per cent potassium hydroxide solution with tritium gas using 10 per cent palladium on calcium carbonate as catalyst.

[7,9-^{3}H]Folic acid - Prepared by hydrogen-tritium exchange (1hr) as previously described (2).

[3',5',7-^{3}H]Methotrexate - Prepared by catalysed halogen-tritium replacement (overnight) from 3',5'-dichloromethotrexate as for folic acid (2).

[7-^{3}H]Methotrexate - Prepared by catalytic hydrogen-tritium exchange as for [7,9-^{3}H]folic acid (2).

[7-^{3}H]Pteridine-6-carboxylic acid - Pterin-6-carboxylic acid (13.1mg) in 4% potassium hydroxide (2ml) were stirred with tritium gas (20Ci) for 2.5hr in the presence of 10% palladium on charcoal (11.8mg).

After removal of labile tritium the crude tritiated product was purified by paper chromatography (Whatman 3MM paper, descending mode) firstly with 0.5% w/v aqueous potassium carbonate for 4-6hr and secondly with pyridine: n-butanol:water (1:1:1) overnight. In each case the radioactive band corresponding to the authentic marker (visualised under u.v) was extracted into water. The yield of radiochemically pure [7-^{3}H]pteridine-6-carboxylic acid was 29mCi at 20Curies/mmol. The specific activity was calculated using the weight determined by u.v. spectroscopy (0.1M sodium hydroxide solution, ϵ 364nm = 11.5 x $10^3 M^{-1} cm^{-1}$). The proton decoupled ^{3}H NMR spectra of the [7-^{3}H]pteridine-6-carboxylic acid is shown in Figure 5.

Alkaline permanganate oxidation procedure - A solution of the radioactive pteroyl-glutamate (2ml at 1mCi/ml) was adjusted to pH 9 by the addition of equal amounts of potassium carbonate and bicarbonate. To the solution was added potassium permanganate (ca. 5mg). The oxidation mixture was left at room temperature (20°C) for folic acid (1hr), methotrexate (16hr overnight), pteridine-6-carboxylic acid (1.5hr) and 4-aminobenzoic acid (4hr). After this time the solution was frozen and the labile tritium as tritiated water lyophilised and measured by liquid beta scintillation counting.

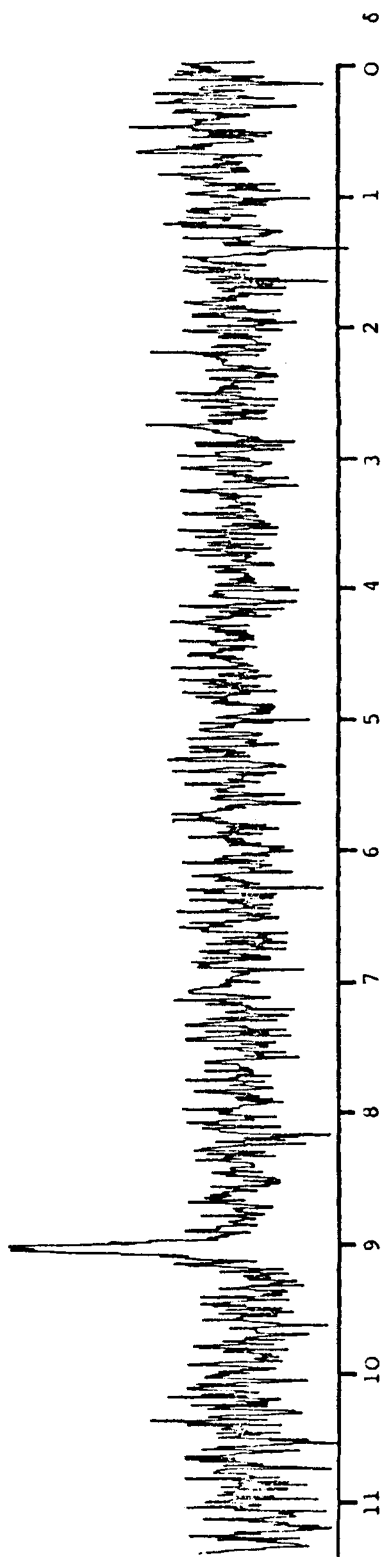


FIGURE 5 - ^{1}H decoupled tnmr spectrum of [7-^{3}H]pteridine-6-carboxylic acid

RESULTS AND DISCUSSION

The tritium nuclear magnetic resonance spectrum of tritiated folic acid prepared by the catalytic halogen-tritium replacement method indicated that the tritium was present in the 3',5'-positions (42.5%), 7-position (25.5%) and the 9-methylene group (32%). The percentage of tritium in the 9-position is considerably lower than the percentage of labile tritium produced during the permanganate oxidation of the compound, as seen from the table (I). This of course is now known to be due to labile tritium arising from the 3',5'-positions as well as from the 9-position during the oxidation reactions. The tritium nmr spectrum of [7,9-^{3}H]folic acid prepared in solution by catalysed exchange with tritium gas shows extensive labelling in the 7-position (59%) as well as in the 9-methylene group (41%). Note that in this case the labile tritium formed by permanganate oxidation arising from the 9-position (42.4%) is in very close agreement with the tnmr spectrum results, as expected.

A few percent labile tritium may arise from the 7-position during the oxidation as indicated by the experiments with [7-^{3}H]methotrexate and [7-^{3}H]pteridine-6-carboxylic acid, shown in the table I. Complete labilization of tritium occurs from the 3,5-positions during oxidation of 4-amino-[3,5-^{3}H]benzoic acid. The tritium nmr spectrum of tritiated methotrexate prepared by catalysed halogen-tritium replacement indicates that no detectable exchange has occurred into the 9-position probably due to the steric hindrance effect of the N^{10}-methyl group. This is also supported by the tritium nmr spectrum of tritiated methotrexate labelled by catalysed hydrogen-tritium exchange, by which method labelling in the 7-pteridine ring only occurs.

Although tritium labelling by catalysed exchange in solution with tritium gas is not suitable for the labelling of benzene (3,4) it was surprising to find that the heterocyclic pteridine ring was extensively labelled (in the 7-position) during this procedure, albeit not so readily as say the 9-position of folic acid. These results clearly show the advantages of the non-destructive tritium nmr spectroscopic technique for the determination of patterns of labelling.

TABLE I

Labile tritium by oxidation of tritiated folic acid and methotrexate

COMPOUND	DISTRIBUTION OF LABEL BY TNMR SPECTROSCOPY			LABILE TRITIUM (%) AFTER OXIDATION
	3',5'	7	9 (%)	
[7,9-^{3}H]Folic acid	-	59	41	42.4
[3',5',7,9-^{3}H]Folic acid	42.5	25.5	32	41.8
[7-^{3}H]Methotrexate	-	100	-	8
[3',5',7-^{3}H]Methotrexate	43	57	-	21.2
[7-^{3}H]Pteridine-6-carboxylic acid	-	100	-	8
4-Amino-[3,5-^{3}H]benzoic acid	100	-	-	100

In the table II are the results obtained using different batches of catalyst and the effect on the distribution of label in [3',5',7,9-^{3}H]folic acid and [3',5',7-^{3}H]methotrexate the reaction time being kept constant.

TABLE II

Distribution of tritium label as a function of catalyst batch

COMPOUND	DISTRIBUTION OF LABEL BY TNMR SPECTROSCOPY (%)		
	3',5'	7	9
[3',5',7,9-^{3}H]Folic acid	42.5	25.5	32.0
	43.2	23.6	33.2
	58.0	16.5	25.5
	73.1	2.8	24.1
[3',5',7-^{3}H]Methotrexate	37.0	63.0	
	46.4	53.6	

As might be predicted from a tritium labelling procedure in which two different reactions are occurring (*viz* hydrogen-tritium exchange and halogen-tritium replacement), the relative distribution of the tritium label can vary greatly depending on the activity of the catalyst, time of reaction etc.

REFERENCES

1. EVANS, E.A., Chap.6, p.734 et seq., in Tritium and Its Compounds, 2nd edition Butterworths, London 1974.

2. ZAKRZEWSKI, S.F., EVANS, E.A. and PHILLIPS, R.F., Analyt.Biochem., 36, 197(1970).

3. EVANS, E.A., Chap.4, p.310 et seq., in Tritium and Its Compounds, 2nd edition Butterworths, London, 1974.

4. EVANS, E.A., SHEPPARD, H.C., TURNER, J.C., and WARRELL, D.C., J.Label.Compounds, 10, 569(1974).

5. MARUYAMA, T., SHIOTA, T. and KRUMDIECK, C.L., Analyt.Biochem., 84, 277(1978).

6. BLOXSIDGE, J., ELVIDGE, J.A., JONES, J.R. and EVANS, E.A., Org.Magnetic Resonance, 3, 127(1971).

7. CHAMBERS, V.M.A., EVANS, E.A., ELVIDGE, J.A. and JONES. J.R. Tritium Nuclear Magnetic Resonance (tnmr) Spectroscopy, Review No.19 The Radiochemical Centre, Amersham, Bucks, England 1978 (68pp).

8. AL-RAWI, J.M.A., BLOXSIDGE, J.P., O'BRIEN, C. CADDY, D.E., ELVIDGE, J.A., JONES, J.R. and EVANS, E.A., J.C.S.Perkin II; 1635(1974).

9. AL-RAWI, J.M.A., BLOXSIDGE, J.P., ELVIDGE, J.A., JONES, J.R., CHAMBERS, V.M.A. and EVANS, E.A., J.Label.Compounds Radiopharmaceuticals, 12, 293(1976).

ACKNOWLEDGEMENTS

Robert Lenk is a recipient of a CASE studentship. The authors thank Dr. W.P. Grove, Managing Director, The Radiochemical Centre Ltd for permission to publish the results.