Journal of Labelled Compounds — January-March — Vol. II, No. 1

Isotopic discrimination between ^{3}H and ^{14}C *in vitro* during enzymatic phosphorylation of labelled thymidine

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Received on 2 January, 1966

SUMMARY

We compare the phosphorylation of 6-^{3}H-TdR and 2-^{14}C-TdR to TMP, TDP and TTP by tissue extracts and to TMP by purified kinase preparations. The rate of phosphorylation of 6-^{3}H-TdR is only 0.6 to 0.8 that of 2-^{14}C-TdR. The same isotopic discrimination exists between methyl-labelled thymidines. In mixtures of ^{3}H-TdR and ^{14}C-TdR incubated together with a liver extract, the isotopic ratio is lower in the products than in the substrate. Degradation products of ^{3}H-TdR do not seem to interfere with enzymatic activity. This isotopic discrimination is tentatively ascribed to an isotopic effect of ^{3}H which is mainly detectable at the high substrate/enzyme ratios prevailing in the in vitro *incubation conditions.*

INTRODUCTION

It is well known that tritium may be responsible for rather important isotopic effects [1, 2, 3].

During a study of the effects of X-irradiation on ATP : thymidine-5 phosphotransferases (EC 2.7.1. 21) in regenerating rat liver [4], the results of which will be published shortly, we noticed that the proportion of thymidine phosphorylated by a liver extract *in vitro* was not the same for ^{14}C or ^{3}H labelled thymidine (TdR). The present work started from that observation : a preliminary communication has already been published [5].

TECHNIQUES

1. *Liver extracts*

Male Wistar rats of 180 g are partially hepatectomized according to the technique of HIGGINS and ANDERSON [6] and are sacrificed by decapitation 36 to 40 hours later.

Regenerating livers are perfused in ice-cold 0.9% NaCl, then homogenized in cold distilled water pH 7 in a Potter tube. Concentration of the homogenate is 30%. Rat thymus has been used in similar conditions.

The homogenate is spun at 300.000 g for 60 minutes; the supernatant (20 to 30 mg protein per ml) is used as the active extract.

2. *Enzyme purification*

TdR-kinase was purified 150 to 200 fold from regenerating rat liver according to the technique described by WEISSMAN *et al.* [7] for ascites-tumour, with the exception that precipitation with $(NH_4)_2SO_4$ was done at 30% saturation instead of 35%. TMP- and TDP-kinase activity is lost during this purification procedure. In a recent article, however, GRAV and SMELLIE [8] managed to keep all three kinases active during purification.

3. *Enzymic assays*

The composition of the assay mixture is the following, in a final vol. of 0.7 ml :

Tris-HCl buffer pH 7.9	55 μ moles
ATP	5 μ moles
$MgCl_2$	2.5 μ moles
TdR	30 to 40 mμ moles
	1 μCi ^{14}C or 8 to 10 μCi ^{3}H
Active extract	2 to 5 mg protein
or purified kinase	30 to 600 μg protein.

Usually, after 30 min incubation at 37 °C, the reaction is stopped by cooling the mixture and adding 0.1 ml $HClO_4$ 10 N. The cold acid-soluble fraction is neutralized with 7 N KOH and submitted to chromatography on column or paper. The enzymatic activity is expressed from radioactivity measurements as the percentage of TdR converted to TMP, TDP and TTP, or as the amount of phosphorylated products formed.

Proteins are determined with the Folin-Ciocalteu reagent.

4. *Chromatography*

a) Column chromatography

Ecteola cellulose columns (Whatman ET 30 Powder) 0.5 meq/g are prepared according to the technique of GRAV and SMELLIE [9]. Half a ml of the acid-soluble fraction is put on the column (1 × 10 cm), and then eluted successively with 100 ml water, 50 ml 0.01 N HCl, 50 ml 0.02 N HCl, 100 ml 0.04 N HCl and 70 ml 0.1 N HCl.

Portions of 50 ml are collected and the radioactivity determined by liquid scintillation counting in a Packard Tri-Carb counter mod. 314.

To 1 ml of each fraction are added 10 ml of scintillating liquid composed of 10 g PPO, 250 mg POPOP, 100 g naphtalene, dioxane to make 1 liter, and 166 ml ethanol.

The counting efficiency is determined with the help of internal standards. The proportion of TdR converted to TMP and TTP is calculated from the ratio of the radioactivity in the respective fraction to the total activity eluted from the column.

b) Paper chromatography

50 µl of the acid-soluble fraction are put on a strip of diethylaminoethyl cellulose paper (Whatman DE 20), 3 cm wide, and submitted to ascending chromatography for 18 hours in a solvent made of formic acid 4 N and ammonium formate 0.1 M, in which the nucleotides have the following Rf : TTP, 0.04 ; TDP, 0.14 ; TMP, 0.47 ; TdR, 0.78. They differ only slightly from those obtained by IVES *et al.* [10] in descending chromatography. The radioactivity is measured in two ways.

Routinely, the radioactivity is automatically recorded in a Baird-Atomic strip scanner (4π counting). The total radioactivity usually present on the chromatogram is of the order of 0.1 µCi for ^{14}C and 1 µCi for ^{3}H. The surface of the recorded peaks corresponding to TTP, TDP, TMP, TdR is measured by planimetry and the enzymatic activity is expressed by the percentage of TdR converted to TMP, TDP and TTP. The validity of the planimetric method is checked by connecting the scanner to an automatic printer giving the activity in *cpm* for a preselected time of 40 seconds (which measured the radioactivity on 12 mm intervals). Those two ways of recording the activity of the chromatograms give indentical results.

In a second method, the paper is cut out in squares of 9 cm^2 and placed in counting vials. Each square is soaked with 1 ml HCl 0.1 N per vial for 12 hours, after which period 14 ml scintillating liquid are added. In this way, more than 90% of the radioactivity is eluted from the paper in the scintillating liquid.

Several acid-soluble fractions obtained after incubation of ^{3}H-TdR in the presence of liver extracts, have been analyzed by the three methods described above. It can be seen, from Table I, that the three methods give closely related values for ^{14}C-TDP and ^{14}C-TTP.

The value obtained for ^{3}H-TDP and ^{3}H-TTP are a little higher by the scanner method than by the two others ; nevertheless, the differences observed between phosphorylation of ^{3}H-TdR and ^{14}C-TdR remain significant even by the scanner method.

Products used :

All non labelled nucleotides were purchased from Sigma.

— TdR-6-^{3}H, specific activity 3 Ci/mmole was supplied by Schwartz or Amersham,

TABLE 1. Percentage of TdR phosphorylated by 3 liver extracts, measured by 3 different techniques

Extract number		^{3}H-TdR			^{14}C-TdR		
		Column chromatogr.	Paper chromatography		Column chromatogr.	Paper chromatography	
			Scanner	Liquid scintillat.		Scanner	Liquid scintillat.
1	TTP + TDP	36.2	40	32.6	87.8	78.2	72
	TMP	8.8	—	4.7	5.8	13	9
	TdR	54.9	60	62.6	6.3	8.7	8.9
2	TTP + TDP	33.7	36.3	32	87.9	83.5	86.1
	TMP	8.7	—	5.8	5.4	8.6	6.2
	TdR	57.5	63.6	62	6.5	7.8	7.6
3	TTP + TDP	36.2	44	37.4	51.2	54.7	53.1
	TMP	16.8	9.8	8.8	13.3	6.1	5.8
	TdR	46.8	46.2	53.6	35.9	39.2	41.0

— TdR-CH_2T, specific activity 2.7 Ci/mmole, from Amersham,
— TdR-2-^{14}C, specific activity 30 mCi/mmole, from Amersham,
— TdR-$^{14}CH_3$, specific activity 1.7 mCi/mmole, was synthesized at the C.E.N., in Mol, by Dr. M. Winand.

TdR and ATP are purchased from Sigma (Sigma grade); Tris « analar » is purchased from BDH.

RESULTS

Table 2 gives the percentages of phosphorylation of TdR obtained with 4 different liver extracts. In each case, equal amounts (33 mμ moles) of both labeled TdR have been used.

TABLE 2. Percentage of TdR phosphorylated by four aqueous liver extracts
Incubation : 30 min
TdR-2-^{14}C : 33 mμ moles — 1 μCi
TdR-6-^{3}H : 33 mμ moles — 10 μCi

	TdR Phosphorylated (%)			
	6-^{3}H-TdR		2-^{14}C-TdR	
	TMP	TDP + TTP	TMP	TDP + TTP
1	4.0	38.3	4.2	57.2
2	4.7	17.1	3.4	28.9
3	17.4	42	11.2	65.7
4	13.2	34	11.8	38

The enzymatic activity varies from one extract to the other according to individual and seasonal factors [11]. Nevertheless, in all cases the phosphorylation of 6-^{3}H-TdR to TTP is only 0.6 to 0.8 that of 2-^{14}C-TdR. The same difference also exists when the level of phosphorylation of TdR is measured in relation to the amount of substrate (Fig. 1), or to the amount of phosphate donor, ATP (Fig. 2), or to the time of incubation (Fig. 3).

As seen in Table 2 and in figures 1 and 3, the difference between ^{14}C and ^{3}H compounds is greater at the triphosphate level than at the monophosphate level. In cases where ^{3}H-TMP is produced in higher amounts than ^{14}C-TMP (Table 2) it must be realized that more ^{3}H-TdR than ^{14}C-TdR remains at the end of the incubation and that, therefore, the equilibrium constant for TMP production, given by the ratio TMP/TdR is higher for ^{14}C-TMP than for ^{3}H-TMP. This will show clearly from the results with purified kinase. When ^{3}H-TdR and ^{14}C-TdR are incubated together with the same liver extract in one test tube,

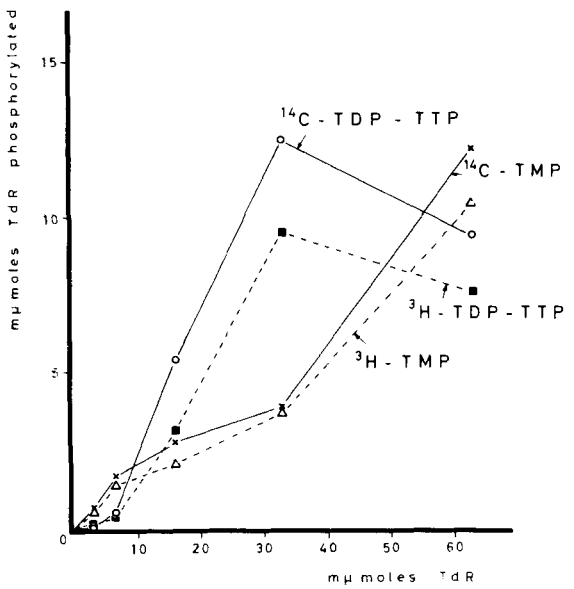

FIG. 1. — Amount of TdR phosphorylated (in mμ moles) by a regenerating liver extract, in relation to the amount of TdR incubated.
The ratio ATP/TdR is kept constant (= 154).
Incubation time : 30 minutes.

the isotopic ratio $^{3}H/^{14}C$ in the products is lower than in the substrate (fig. 4, part A).

The autoradiolysis products of ^{3}H-TdR samples, separated in the solvents used by APELGOT and EKERT [12] and by EVANS and STANFORD [13], represent between 10 and 20% of the total radioactivity. Since, in our enzymatic assays, the ^{3}H-TdR is diluted 10 times with cold TdR, the actual small decrease in the amount of ^{3}H-TdR in the stock solution due to the presence of radioactive degradation products cannot have any influence on the phosphorylation rate*.

But degradation products might influence the enzyme activity. This possibility was tested by submitting to the action of the same liver extract 50 mμ moles of ^{3}H-TdR and ^{14}C-TdR separately and in mixture. The chromatogram paper strips are cut in squares and the radioactivity is measured by liquid scintillation at two different high tensions (800 V and 1200 V) in a Packard Tri-Carb counter Mod 314. With the help of internal standards, the radioactivity due to ^{3}H and ^{14}C can be measured in a mixture of both isotopes. Figure 4 shows the

* For instance, the 33 mμmoles of ^{3}H-TdR incubated are made up of 30 mμmoles unlabeled TdR and 3 mμmoles ^{3}H-TdR. Assuming that 33% of the labeled TdR has been degraded, then 2 mμmoles ^{3}H-TdR instead of 3 are used, but the total amount of TdR — which determines the rate of the enzymatic reaction — is 32 mμmoles instead of 33: the influence of such a small difference on the reaction rate is hardly detectable.

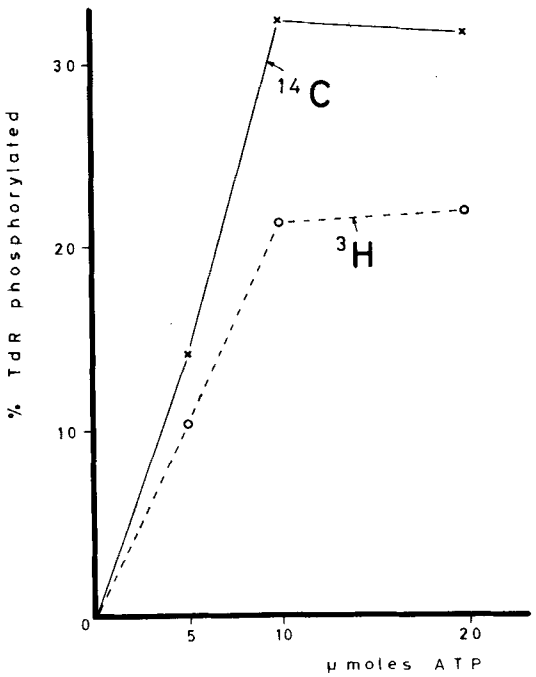

FIG. 2. — Percentage of TdR phosphorylated by a liver extract in relation to the amount of ATP. Incubation time : 30 minutes.
2-^{14}C-TdR : 33mμ moles — 1 μCi 6-^{3}H-TdR : 33 mμ moles — 10 μCi

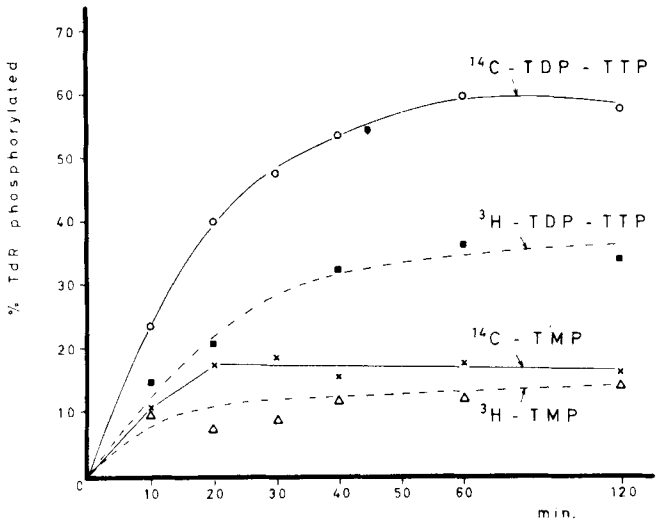

FIG. 3. — Phosphorylation of TdR by a rat thymus extract, in relation to time of incubation.
2-^{14}C-TdR : 33 mμ moles — 1 μCi 6-^{3}H-TdR : 33 mμ moles — 10μCi

results of this experiment, repeated twice with similar results. The same liver extract was used for both parts of the experiment: that is, for measuring phosphorylation of 50 mμ moles of ^{3}H-TdR and 50 mμ moles of ^{14}C-TdR incubated in separate tubes, and for measuring phosphorylation of both thymidines incubated in the same tube. Part B of figure 4 shows that ^{14}C counts in TMP and TDP + TTP are the same in the presence or in the absence of ^{3}H-TdR. Therefore, kinase activity does not seem to be inhibited by degradation products from ^{3}H-TdR.

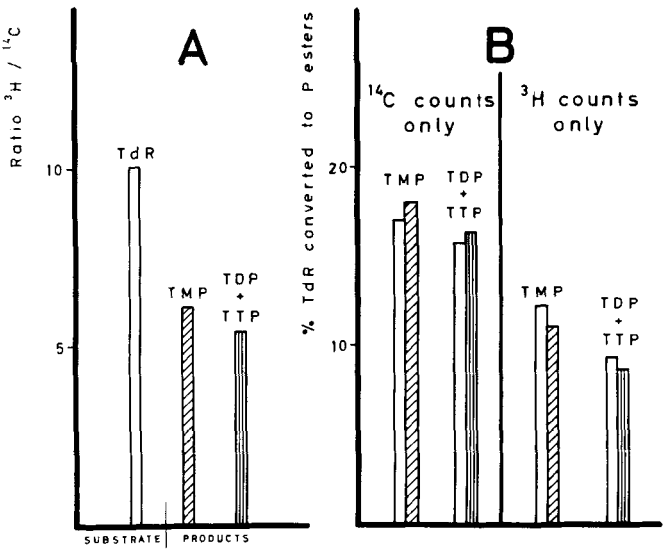

FIG. 4. — Phosphorylation of 10 μCi ^{3}H-TdR and 1 μCi ^{14}C-TdR (50 mμ moles) by a liver extract.

A. The two thymidines mixed together in the same test tube. Isotopic ratio in the substrate (TdR) and in the products (TMP and TDP + TTP).

B. Proportion (in %) of TdR converted to P esters. White bars : ^{14}C-TdR incubated alone (left) or ^{3}H-TdR incubated alone (right)
Hatched bars : ^{14}C-TdR and ^{3}H-TdR incubated together :
left : ^{14}C counts only
right : ^{3}H counts only.

The same isotopic discrimination between ^{3}H and ^{14}C is also observed with methyl-labelled TdR (Table 3). High amounts of ^{14}C-methyl-TdR had to be used in view of the low specific activity of the compound.

Purified kinase preparations

The kinetics of TMP formation are shown in figure 5 where different amounts of TdR kinase have been used. Two main differences between ^{14}C

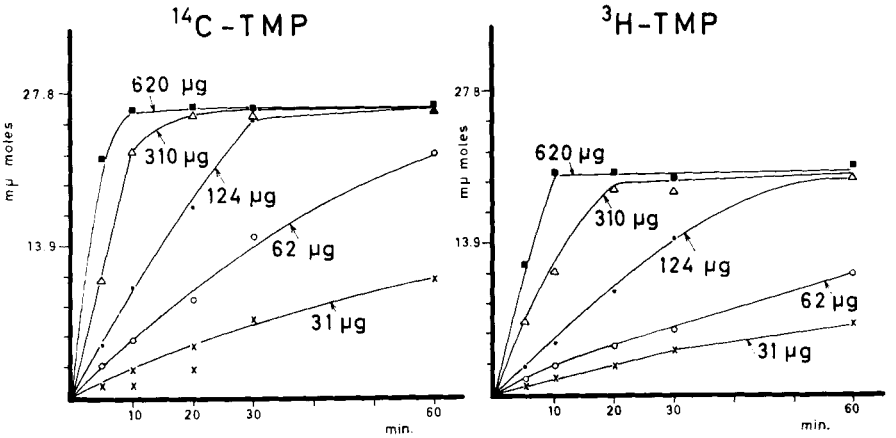

FIG. 5. — Phosphorylation of 27.8 mμ moles of ^{14}C-TdR and ^{3}H-TdR by different amounts (in μg protein) of the same purified kinase preparation.
Abscissae : time of incubation. Ordinates : mμ moles TMP formed.

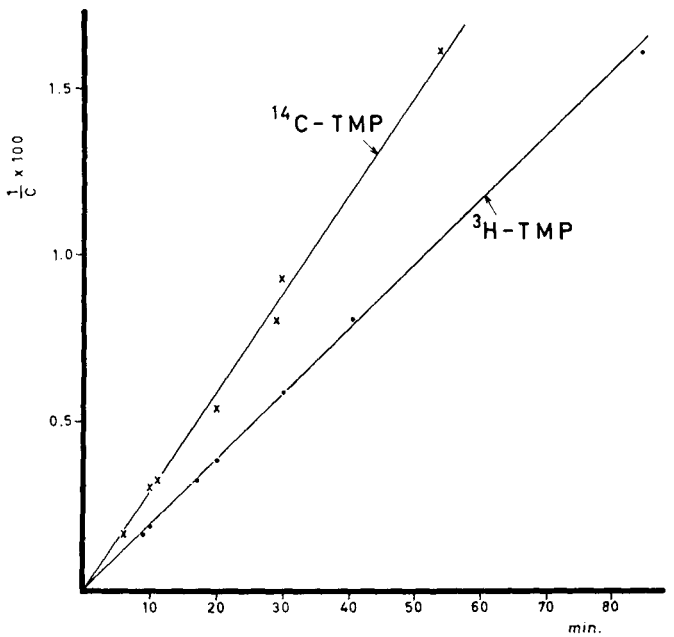

FIG. 6. — Phosphorylation of 27.8 mμ moles of ^{14}C-TdR and ^{3}H-TdR by different amounts of purified kinase.
Abscissae : time taken to reach the maximum phosphorylation level (calculated from the initial velocity).
Ordinates : reciprocal of the enzyme protein amount.

TABLE 3. Amounts of TMP and TDP + TTP formed (in mμ moles) by two regenerating liver extracts, with TdR labelled on the ring or on the methyl group. Incubation : 30 minutes

	1				2				
Position and nature of label	2-^{14}C	$^{14}CH_3$	6-^{3}H	$CH_2{}^{3}H$	2-^{14}C		$^{14}CH_3$	6-^{3}H	$CH_2{}^{3}H$
mμ moles TdR incubated	34	754	32.5	33.7	34	384	377	32.6	33.7
mμ moles									
TMP	2.2	17.4	1.75	2.4	2.6	6.9	6.0	1.2	1.75
TDP + TTP	12.5	15.1	6.8	7.8	8.5	11.5	11.7	6.2	5.9

and ^{3}H-TMP are noticeable : the maximum amount of TMP formed is smaller for ^{3}H-TMP and the rate for reaching the plateau region is also lower for ^{3}H-TMP than for ^{14}C-TMP. This last fact is clearly seen in figure 6 where the time taken to reach the plateau (calculated from the initial velocity) is plotted against the reciprocal of the amount of enzyme added. Two straight lines are obtained, with different slopes for ^{14}C-TMP and ^{3}H-TMP.

Discussion

In a private communication, Shooter [14] recently confirmed the differences we have described between phosphorylation of ^{14}C and ^{3}H-TdR.

As we have shown, autoradiolysis products of ^{3}H-TdR do not seem to interfere with the enzyme activity.

TdR is catabolized mainly by the liver [15, 16], but since the same isotopic discrimination is observed with thymus extracts (Fig. 3), which do not catabolize TdR, and with purified kinase preparations, it does not seem that catabolism of TdR plays any role in the discrimination observed. If catabolism occurs during incubation with liver extracts and results in some loss of total radioactivity, it seems that the specific activity of TdR is maintained. This is consistent with the observations of Evans [17] and of Winand and Gouverneur [18] who did not find any decrease in the specific activity of TdR during transribosylation of ^{3}H-thymine incubated with a rat or horse liver extract.

There remains the possibility of an isotope effect. As is expected from the theory, indeed, the heavier isotope reacts more slowly than the lighter one [1]. This is, of course, particularly marked for hydrogen isotopes, especially in cases where hydrogen removal is involved in the chemical or enzymological reaction [1].

The case of TdR-phosphorylation is different, because the enzymatic reaction takes place on C 5 of the deoxyribose ring, not on C 6 or CH_3 of the pyrimidine ring where the tritium is located.

The existence on the active center of TdR kinase, of two different binding sites, one for the esterification of deoxyribose and one for the thymine moiety [10] could provide a possible hypothesis of the mechanism of the isotope effect. One might consider, although very speculatively, that the presence of ^{3}H on the thymine moiety interferes with the binding of the thymine to or with its release from the active center of the enzyme.

It is interesting to note that the isotopic discrimination described here *in vitro* does not seem to show up in whole animals. Indeed, neither in the incorporation rate of thymidine into DNA [19], nor in the reutilization of thymidine from labelled DNA [20] can one detect any significant difference between ^{3}H or ^{14}C labelled TdR. But, of course, there is a vast difference between conditions of *in vitro* incubations and normal working conditions of an enzyme *in vivo*. In the first case, excess of substrate is usually supplied for the sake of an easier study of enzyme kinetics, and the final products of the reaction accumulate

in the test tube. In a living cell, on the contrary, substrate is handled as soon as it is produced and final products themselves are taken up at once for DNA synthesis without accumulating (except in pathological conditions where DNA synthesis is impaired). Moreover, DNA labelling experiments in mammals utilize relatively small amounts of TdR.

Therefore, it is improbable that the high substrate/enzyme ratios at which isotopic discrimination is observed *in vitro*, will be met *in vivo*.

The only case where the possibility of an isotope effect of ^3H *in vivo* has been suggested is the observation of RUBINI *et al.* [21] that bone-marrow cells *in vitro* take up only a limited amount of ^3H-TdR during a rather short time into their DNA, although in this case other explanations are also proposed.

ACKNOWLEDGEMENT

We are grateful to Dr. S. Apelgot (Institut du Radium, Paris), Dr. K. V. Shooter (Chester Beatty Research Institute, London) and Dr. E. A. Evans (The Radiochemical Center, Amersham) for helpful discussions and advice.

This work has been performed with the help of Euratom-C.E.N. contract No. 014-62-1 BIAB and the Fonds de la Recherche Scientifique Fondamentale Collective.

REFERENCES

1. BRODA, E. — Radioactive Isotopes in Biochemistry, Elsevier Publish. Co., 1960, p. 48.
2. ISBELL, H.S., FRUSH, H.L. and SNIEGOSKI, L.T. — Tritium in the Physical and Biological Sciences, Vol. II, 93. International Atomic Energy Agency,'Vienne, 1962.
3. KLEIN, P.D., SIMBORG, D.W. and SZCZEPANIK, P.A. — *Pure and Applied Chemistry*, **8**, (1964) 357.
4. GOUTIER, R., BAUGNET-MAHIEU, L. and SEMAL, M. — *Arch. intern. Physiol. Biochim.*, **71**, (1963) 130.
5. BAUGNET-MAHIEU, L., GOUTIER, R. and SEMAL, M. — *Arch. intern. Physiol. Biochim.*, **72**, (1964) 312.
6. HIGGINS, G.M. and ANDERSON, R.M. — *A. M. A. Arch. Pathol.*, **12**, (1931) 186.
7. WEISSMAN, S.M., SMELLIE, R.M.S. and PAUL, J. — *Biochim. Biophys. Acta*, **45**, (1960) 101.
8. GRAV, H.J. and SMELLIE, R.M.S. — *Biochem. J.*, **94**, (1965) 518.
9. GRAV, H.J. and SMELLIE, R.M.S. — *Biochem. J.*, **89**, (1963) 486.
10. IVES, D.H., MORSE, P.A. and POTTER, V.R. — *J. Biol. Chem.*, **238**, (1963) 1467.
11. MCDONALD, R.A., ROGERS, A.E. and PECHET, G.S. — *Ann. N. Y. Acad. Sci.*, **111**, (1963) 70.
12. APELGOT, S. and EKERT, B. — *J. Chim. Phys.*, **60**, (1963) 505.
13. EVANS, E.A. and STANFORD, F.G. — *Nature* (*London*),**197** (1963) 551.
14. SHOOTER, E.V. — Personal communication.
15. FINK, K., CLINE, R.E., HENDERSON, R.B. and FINK, R.M. — *J. Biol. Chem.*, **221**, (1956) 425.

16. RUBINI, J.R., CRONKITE, E.P., BOND, V.P. and FLIEDNER, T.M. — *J. Clin. Invest.*, **39**, (1960) 909.

17. EVANS, E.A. — Personal communication.

18. WINAND, M. and GOUVERNEUR, L. — *Proc. Conf. on Meth. of preparing and storing Marked Molecules*, Euratom 1625 e., Brussels, 1147 (1963).

19. GERBER, G. — Personal communication.

20. FEINENDEGEN, L.E. — Personal communication.

21. RUBINI, J.R. and CRONKITE, E.P. — *Comptes Rendus du 8e Congrés de la Soc. Européenne d'Hématologie*, S. Karger, Basel — New York, p. 64 (1962).