THE INCORPORATION OF TRITIUM FROM THYMIDINE INTO PROTEINS OF THE MOUSE

B. J. BRYANT

From the Division of Microbiology, Medical Research Center, Brookhaven National Laboratory, Upton, New York, and the Division of Biological and Medical Sciences, United States Naval Radiological Defense Laboratory, San Francisco, California

ABSTRACT

Tritium from methyl-H^3-thymidine was found to be incorporated into proteins in mice. This incorporation in the mouse as a whole represented between 1 and 10% of the injected tritium. Tritiated water was not an intermediate. Transmethylation reactions are proposed as a means whereby certain amino acids might have acquired the tritium from thymidine at some stage of its catabolism. The initial (2 hr) ratios of DNA to protein tritium activities per milligram of wet tissue ranged from 5 in two tissues of low DNA synthetic activity (pancreas, liver) to 35 to 40 in two tissues of high DNA synthetic activity (spleen, small intestine). Labeled nuclear protein was coincident with labeled DNA in nuclei, where it constituted less than 2.5% of the total tritium. The significance of the findings is discussed.

INTRODUCTION

In studies which required tritiated thymidine (H^3-TdR) in high dosages (2), routine sections of mouse liver and pancreas were found, in radioautograms, to possess discernible labeling in what seemed to be cytoplasmic proteins. This result was noted with several samples of methyl-H^3-TdR from one of the major manufacturers (Schwarz Bioresearch Labs., Inc., Mount Vernon, New York). Methyl-H^3-thymidine was therefore obtained from another source, and its possible lack of DNA specificity investigated more systematically.

MATERIAL AND METHODS

For H^3-TdR injections, random-bred male Swiss mice, aged 12 wk, were used. Methyl-H^3-TdR (2.0 c/mmole, New England Nuclear Corp., Boston, Lot 134-202) was diluted (1:1) to 500 μc/ml with triple-distilled water a few minutes before injection into a tail vein. For tritiated water (THO) injections, A/HeJ male mice, aged 10 wk, were used. THO (5.0 mc/g, New England Nuclear Corp., Boston, Lot 81-100) was diluted 1:5 with distilled water before intravenous administration.

LIQUID SCINTILLATION ANALYSIS: Groups of four mice were anesthetized with Nembutal at intervals of 2 hr, 1 to 6, 8, 10, and 16 days after injection of 2 μc of H^3-TdR per gram of body weight. About 1.0 ml of cardiac blood was withdrawn by heparinized syringe and centrifuged to sediment blood cells. The cell-free plasma was stored at −20°C until radiochemically analyzed. Pancreas, liver, small intestine and spleen were rinsed in distilled water, blotted, weighed to 0.1 mg and also stored at −20°C.

Thawed tissue samples weighing up to 100 mg were homogenized in 5.0 ml of ice cold 0.3 N trichloroacetic acid (TCA). Acid-soluble compounds were extracted with four 10-min washes with the cold TCA, after which no further acid-soluble activity could be extracted. The sediment was twice extracted for 25 min each with 5.0 ml of 0.6 N TCA at 90°C. The pooled supernatants contained the DNA-bound radioactivity, as evidenced by the absence of supernatant radioactivity when the extraction was repeated a third time. Plasma samples were taken directly to hot TCA, but the extracted activity was considered to represent acid-soluble compounds only. After the final TCA treatment, the sediments were dissolved in 90% formic acid and bleached, for liquid scintillation counting purposes, with the addition of 0.25 volume of 30% hydrogen

peroxide. After 40 min, the protein was precipitated with concentrated TCA, washed once with warm ethanol, twice with warm ethanol-ether (3:1), and finally with ether. The precipitate was then resuspended in ether, pipetted into tare-weighed liquid scintillation counting vials, allowed to dry overnight, and weighed. Protein thus determined usually amounted to 10 to 15 mg per sample and represented recovery of about 13% of the tissue wet weight.

Solution of the protein was effected without difficulty in 2.0 ml of hyamine hydroxide (1 M in methanol, Packard Instrument Co., Chicago) by heating the closed vials in steam for 1 hr. 18.0 ml of toluene-based scintillation solution (3.0 g of 2,5-diphenyloxazole (PPO); 100 mg of 1,4-bis-(5-phenyl)-2-oxazolylbenzene (POPOP); 1.0 liter of toluene) were added to the hyamine vials and mixed. To count the aqueous fractions, 0.5 ml of TCA extract was added to duplicate vials containing 9.5 ml of dioxane-based scintillation solution (7.0 g of PPO; 50.0 mg of POPOP; 50.0 g of naphthalene; 1.0 liter of 1,4-dioxane). Counting was performed on a Packard liquid scintillation spectrometer (Model 4322) at 5°C. A minimum of 5000 counts above background per sample was recorded. Tritiated toluene was used as an internal standard.

RADIOAUTOGRAPHIC ANALYSIS: Ten mice were killed 60 min after injection of 4 μc of H^3-TdR per gram of body weight. Pancreas, liver, small intestine, and spleen were fixed in neutral formalin, sectioned at 4 μ in paraffin, cleared through xylol and alcohols, and dipped into liquid emulsion (NTB-3, Kodak, diluted 1:1 with distilled water). The radioautograms were exposed for 3 months in dry air at 4°C. Duplicate sections which had been digested with deoxyribonuclease (DNase) or with 0.6 N TCA (90°C, 30 min) were similarly radioautographed. The DNase digestion was carried out at 37°C, for 1 hr, in a 0.01% solution of the enzyme (Calbiochem, Los Angeles) in 0.003 M $MgSO_4$. The finished radioautograms were stained with hematoxylin and eosin.

Four mice were killed 60 min after injection of 4 μc of tritiated water per gram of body weight. The same organs were radioautographed as after H^3-TdR injection.

RADIOLYSIS OF STORED H^3-TDR: The routine paper chromatographic assay of the manufacturer for the tritiated thymidine used herein indicated 100% radiochemical purity. This assay employed butanol–acetic acid–water (80:12:30, v/v/v) as developing solvents. The H^3-TdR was used for the study about 2 months after this assay. An unused bottle was opened 4 months later, after 6 months of storage, and 0.05 ml (50 μc) of the contents spotted on Whatman No. 1 paper by multiple application of small volumes. Two chromatograms were prepared, using ethyl acetate–water–formic acid (60:30:10) as solvents (3, 5). The developed chromatograms were cut laterally into narrow strips and the radioactivity measured by immersion of the strips into vials containing 15.0 ml of toluene-based scintillation solution.

RESULTS

LIQUID SCINTILLATION COUNTING: Fig. 1 shows the ratios of DNA to protein tritium incorporations per milligram of wet tissue at intervals after injection of 2 μc of H^3-TdR per gram of mouse weight. The ratios were roughly constant and tissue-characteristic (liver and pancreas, 5:1 to 10:1, intestine and spleen, 20 to 40:1) throughout the experiment. Fig. 2 shows H^3 incorporation per milligram of protein in various tissues. The initial (2 hr) values were two and three times greater in intestine and spleen, respectively, than in liver and pancreas. Protein activity subsequently declined in all tissues, including plasma.

RADIOAUTOGRAPHY: The heavy deposits of silver grains in cytoplasmic areas of liver and pancreas, which were found after long radioautographic exposure (3 months) and high levels of administered H^3-TdR (4 μc per gram of body weight), are shown in Figs. 3 and 4. Cytoplasmic activity in spleen and intestine (Fig. 5) seemed less than in liver and pancreas, but nuclear activity was greater. Prior treatment of the sections with deoxyribonuclease or 0.6 N trichloroacetic acid at 90°C did not prevent subsequent radioautographic registration of the cytoplasmic activity (Fig. 6). Nuclear activity, however, while not affected by these treatments in terms of numbers of labeled nuclei, was greatly reduced in all tissues in terms of grains per labeled nucleus. The reduction could not be quantitated because of overexposure of the untreated nuclei. Organs from mice injected with radioequivalent amounts of tritiated water failed to produce radioautographic labeling.

CHROMATOGRAPHY: Radioactivity in the chromatographed samples of H^3-TdR used in this study was found to occupy three peaks. The major peak was assumed to represent thymidine and contained 96% of the total activity. The other two peaks were assumed to represent decomposition products and contained 1.5 and 2.5% of the total activity. No activity was found at the origin.

DISCUSSION

EXTENT OF TRITIUM INCORPORATION INTO PROTEINS: Tritium incorporation into

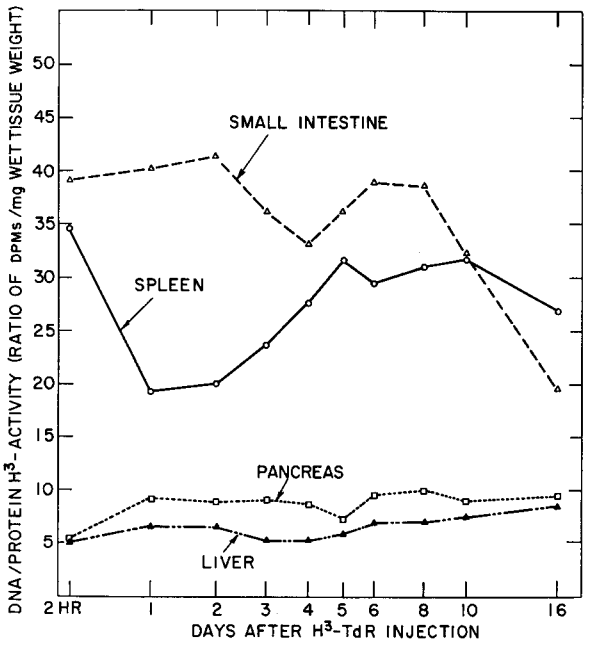


FIGURE 1 Ratios of DNA to protein H^3 activities per milligram of wet tissue at intervals after injection of 2 μc of methyl–H^3-TdR per gram of mouse weight. Each point represents the mean of four mice. Values were usually within 10% of the mean.

DNA has been estimated to represent 50% of the administered H^3-TdR (11). The initial (2 hr) ratios of DNA to protein H^3-activities found herein (Fig. 1) ranged from 5 in two tissues of low DNA synthetic activity (liver, pancreas) to 35 to 40 in two tissues of high DNA synthetic activity (spleen, small intestine). If these tissues are representative, H^3-incorporation into proteins in the mouse as a whole must have amounted to somewhere between 1 and 10% of the injected tritium.

Some data which suggest a lack of DNA specificity with H^3-TdR labeling of animal cells may be found in at least two previous reports. Marsh and Perry (10), using a suspension of human leukemic cells, recovered about 1% of H^3-TdR activity in protein and 2% in RNA after 1 hr. Fox and Prusoff (7) found in mice, which had received multiple injections of H^3-TdR over 2 days, tritium protein values comparable, in some tissues, to those reported here.

PATHWAY OF TRITIUM INCORPORATION INTO PROTEINS: The incorporation of tritium into proteins was presumably mediated through (*a*) in vivo degradation products of methyl–H^3-TdR, or (*b*) in vitro degradation products, formed prior to use, in the stored H^3-TdR.

The available data indicate that methyl–H^3-thymidine, specific activity above 1 c/mmole, stored at 2 to 4°C, decomposes at a rate of 0.5 to 1.0% per month (1, 4). Chromatographic results on the stored methyl–H^3-TdR in this experiment were within this range (4% in 6 months).

Some divergence exists in the literature as regards the identity of the decomposition products. Thymine has been identified by some workers as the major decomposition product (3), by others as the minor product (1, 4). The other decomposition product has been identified as dihydrothymine by some (3), while others (1) have reported 5,6-dihydroxythymidine (thymidine glycol). No attempt was made in this study to identify the two decomposition peaks; presumably one of them was thymine. The presence of thymine, dihydrothymine, or other thymidine catabolites in the stored H^3-TdR is without significance for the present interpretation since these compounds are formed in quantity in vivo. At the time the H^3-TdR solution was used (2 months of storage), the total decomposition products presumably amounted to significantly less than the 4% found at 6 months of storage. Furthermore, only part of the decomposition products may have been a compound (thymi-

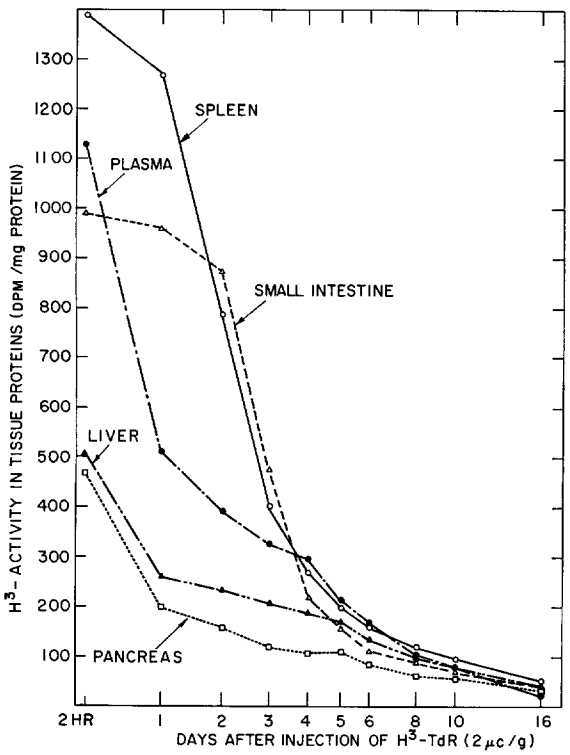


FIGURE 2 H^3 activity per milligram tissue protein at intervals after injection of 2 μc of methyl-H^3-TdR per gram of mouse weight. Each point represents the mean of four mice. Values were usually within 10% of the mean.

dine glycol) not normally a thymidine catabolite. It seems unlikely that the protein labeling, representing between 1 and 10% of the injected tritium, could have arisen substantially from this source. The mechanism of the protein labeling is indeed unknown, but transmethylation reactions form a general class of reactions whereby certain amino acids might have acquired the methyl group from thymidine at some stage of its catabolism.

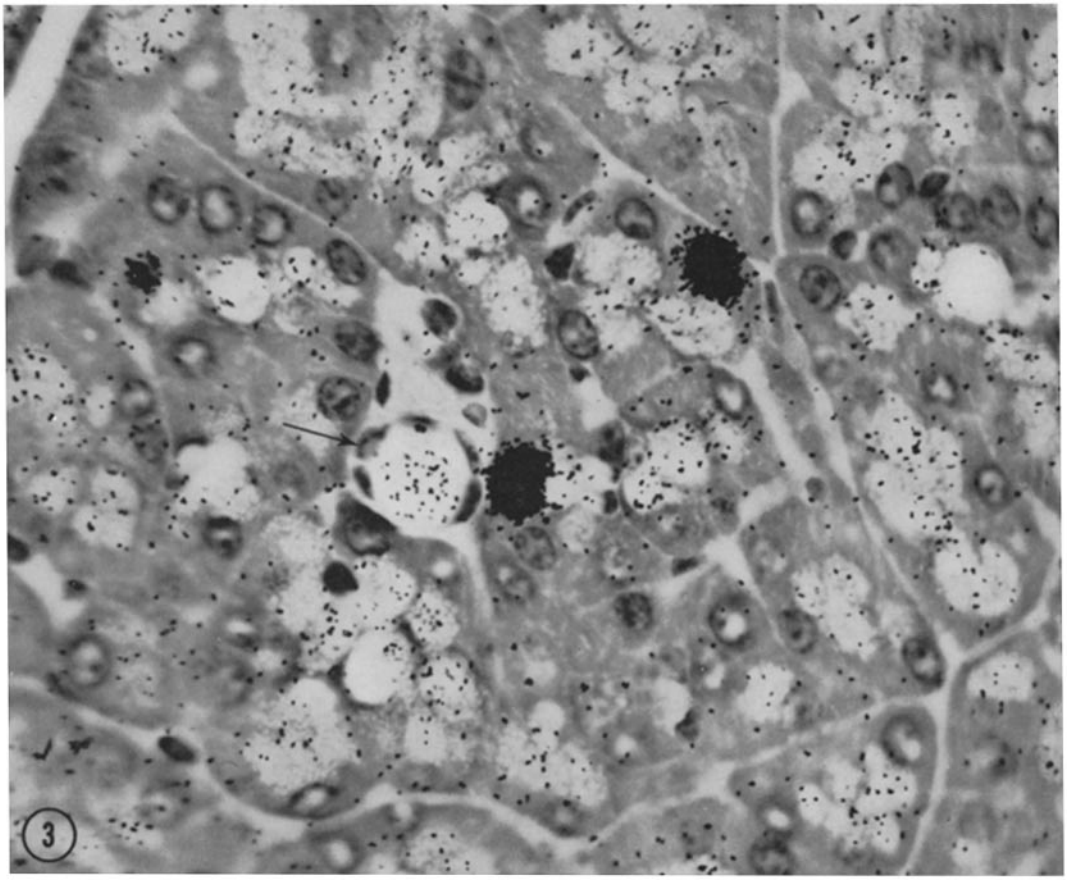
The failure to find labeled protein after administration of radioequivalent amounts of tritiated water agrees with previous reports (cf. reference 12) and indicates that THO, the major labeled catabolite of H^3-TdR (11), was not a significant intermediate in this system.

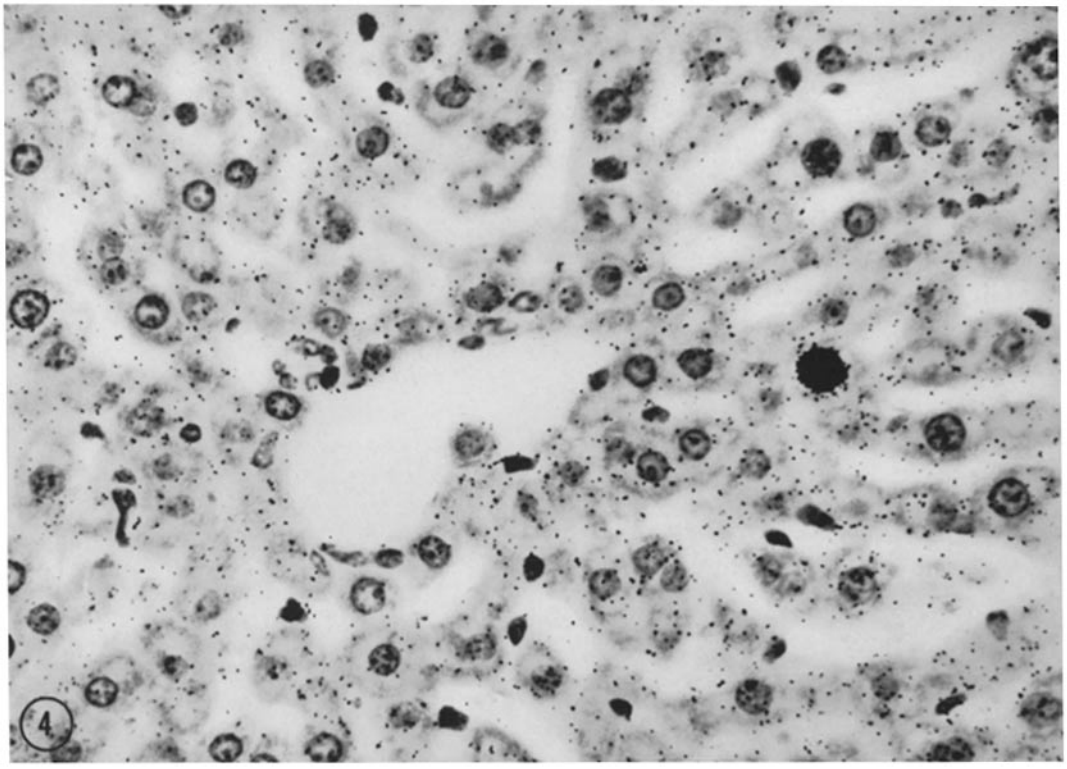
That the intermediates responsible for the protein labeling were thymidine catabolites helps to explain the otherwise anomalous correlation found between initial (2 hr) protein activity and organ DNA synthetic activity (Fig. 2). Thymidine uptake per milligram of organ may have been related to organ DNA synthetic rate (7); conse-

FIGURES 3 to 6 Radioautograms of organs of mice which had received 4 μc of methyl-H^3-TdR per gram of body weight 1 hr before death. The very dense clusters of silver grains represent overexposed radioactive nuclei, 3 months' exposure. Hematoxylin and eosin stain.

FIGURE 3 Pancreas. Cytoplasmic labeling is primarily over the zymogen granules. An interacinar ductule (arrow) contains radioactive protein, consistent with the known turnover time of exportable mouse pancreatic protein, estimated at 47 min (13). × 1000.

FIGURE 4 Liver. The cytoplasmic activity is diffuse. × 600.


3


4

quently more H^3-TdR may have been degraded and incorporated into protein, as well as incorporated into DNA, in organs of high DNA synthetic activity. Usually, after administration of labeled amino acids (9), protein specific activity in liver and pancreas versus spleen and intestine is higher than reported herein.

In rat liver, Fink et al. (5) found C^{14} from thymine, the first step in thymidine catabolism (8), to be much more efficiently converted to CO_2 and urea when the label was on the 2-position than on the methyl group. Conversely, labeled alanine and glucose were identified as catabolites of only the methyl-labeled thymine. These data suggest that 2-C^{14}-thymidine may possess greater DNA specificity than methyl-H^3-thymidine.

SITES OF PROTEIN LABELING: The stability of cytoplasmic labeling to digestion with DNase or 0.6 N TCA at 90°C (Fig. 6) indicates that this labeling was primarily in protein rather than cytoplasmic DNA. The lability of the nuclear labeling, however, suggests that labeling in this site was primarily in DNA. The 2-hr ratios of DNA to protein tritium activities for intestine and spleen (Fig. 1) indicate that the acid-insoluble protein activity amounted to about 2.5% of the DNA activity. If cytoplasmic activity is assumed negligible, the 2.5% would then represent the relative nuclear activities. Since the assumption is clearly more true for DNA than protein, the estimate of 2.5% for nuclear protein to DNA activities is maximal, but probably is not in error by more than a factor of 2 since the bulk of the protein labeling in these tissues was nuclear (Fig. 6).

The labeling of pancreatic protein at 1 hr (Fig. 3) is consistent with the estimate of 47 min for the turnover time of exportable mouse pancreatic proteins by Warshawsky, Leblond, and Droz (13). The rapid formation and release of labeled protein, from liver and elsewhere, is indicated by the high activity of plasma proteins at 2 hr (Fig. 2). The subsequent declines in tissue protein specific activities presumably reflected such factors as the decline of labeled precursor availability, protein turnover and export, cell death and migration.

SIGNIFICANCE OF THE FINDINGS: Tritium incorporation into proteins was roughly an order of magnitude less than the incorporation into

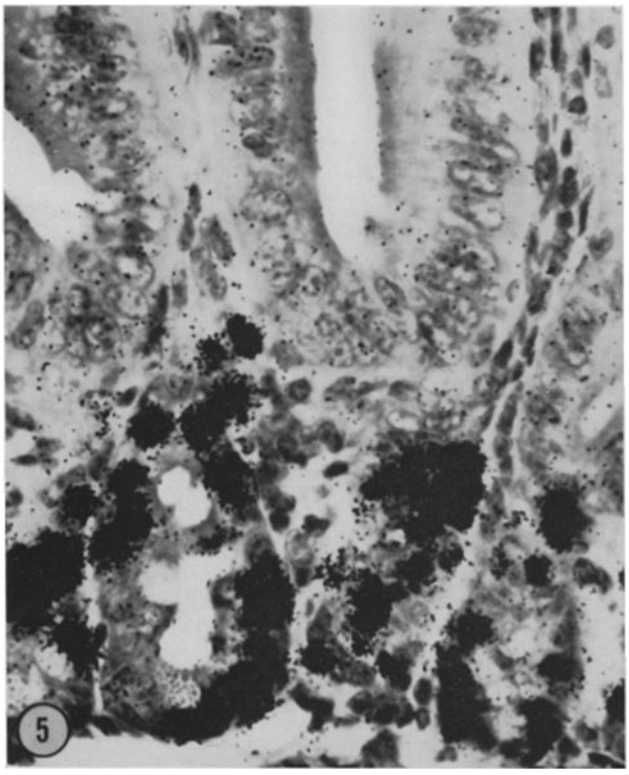


FIGURE 5 Small intestine. Diffuse light labeling is apparent over cytoplasmic areas of epithelial cells, and heavy labeling over crypt nuclei. × 625.

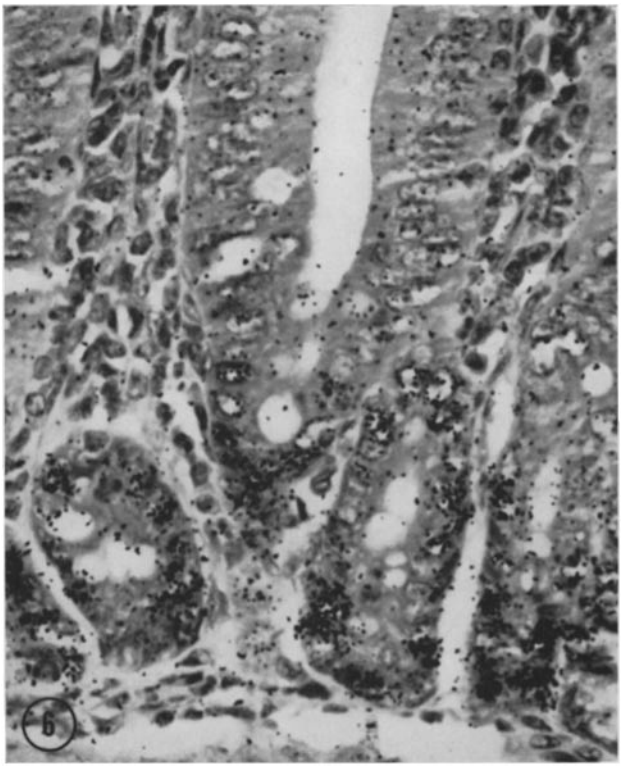


FIGURE 6 Small intestine. Section digested with 0.6 N trichloroacetic acid (90°C, 30 min) before radioautography. As with other tissues, this treatment greatly reduced nuclear activity but not cytoplasmic activity. Only the eosin component stained the section. × 625.

DNA. Effective demonstration of labeled protein in radioautograms required high dosages of H^3-TdR and long exposure. At the usual parameters employed in animal studies (0.5 to 1.0 μc/g, 1-month exposure), labeled protein would therefore seem to pose no significant difficulty for interpretations based on nuclear labeling. Labeled nuclear protein was restricted to DNA labeled nuclei, where it constituted somewhat less than 2.5% of the total tritium.

However, for critical work, these findings should be considered, particularly in systems where demethylation of the pyrimidine ring may be a major catabolic pathway (6), or when large dosages of methyl-H^3-TdR are used. Finally, the use of 2-C^{14}-thymidine for certain usages of methyl-H^3-thymidine may be indicated; it is more stable on storage than is methyl-H^3-TdR (4), and it may have greater DNA specificity, as described above.

The author is indebted to Messrs. J. Cassidy and L. Cook for preparation of the radioautograms. These studies have been supported by funds from the United States Atomic Energy Commission and the Bureau of Medicine and Surgery, United States Navy Department. The opinions and assertions herein are not to be construed as official or as reflecting the views of the Navy Department.

Received for publication 15 December 1965.

REFERENCES

1. APELGOT, S., and EKERT, B., Autodécomposition de thymidine tritée en solution aqueuse, *J. Chim Physic.*, 1963, **60**, 505.
2. BRYANT, B. J., The delayed uptake of ^{3}H-thymidine by Ehrlich ascites tumor cells, *Exp. Cell Research*, 1965, **37**, 490.
3. COOPER, E. H., and MILTON, J. D., The incorporation and degradation of pyrimidine DNA precursors by human leukocytes, *Brit. J. Cancer*, 1964, **93**, 701.
4. EVANS, E. A., and STANFORD, F. G., Stability of thymidine labeled with tritium or carbon-14, *Nature*, 1963, **199**, 762.
5. FINK, K., CLINE, R. E., HENDERSON, R., and FINK, R. M., Metabolism of thymine (methyl-C^{14} or -2-C^{14}) by rat liver *in vitro*, *J. Biol. Chem.*, 1956, **221**, 425.
6. FINK, R. M., and FINK, K., Relative retention of H^3 and C^{14} labels of nucleosides incorporated

into nucleic acids of Neurospora, *J. Biol. Chem.*, 1962, **237,** 2889.

7. Fox, B. W., and Prusoff, W. H., The comparative uptake of I^{125}-labeled 5-iodo-2′-deoxyuridine and thymidine-H^3 into tissues of mice bearing hepatoma-129, *Cancer Research*, 1965, **25,** 234.
8. Friedkin, M., and Roberts, D., The enzymatic synthesis of nucleosides. I. Thymidine phosphorylase in mammalian tissue, *J. Biol. Chem.*, 1954, **207,** 245.
9. Greenberg, D. M., and Winnick, T., Studies in protein metabolism with compounds labeled with radioactive carbon. II. The metabolism of glycine in the rat, *J. Biol. Chem.*, 1948, **173,** 199.
10. Marsh, J. C., and Perry, S., Thymidine catabolism by normal and leukemic human leukocytes, *J. Clin. Inv.*, 1964, **43,** 267.
11. Rubini, J. R., Cronkite, E. P., Bond, V. P., and Fliedner, T. M., The metabolism and fate of triatiated thymidine in man, *J. Clin. Inv.*, 1960, **39,** 909.
12. Thompson, R. C., and Ballou, J. E., Studies of metabolic turnover with tritium as a tracer V. The predominantly non-dynamic state of body constituents in the rat, *J. Biol. Chem.*, 1956, **223,** 795.
13. Warshawsky, H., Leblond, C. P., and Droz, B., Synthesis and migration of proteins in the cells of the exocrine pancreas as revealed by specific activity determination from radioautographs, *J. Cell Biol.*, 1963, **16,** 1.