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# Detection of DNA Synthesis in Intact Organisms with Positron-Emitting [*Methyl*-$^{11}$C]Thymidine

**(cyclotron/short-lived isotopes/liver/spleen/mice)**

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**ABSTRACT** **$^{11}CO_2$ produced in the Brookhaven 152-cm cyclotron was converted to formaldehyde, which in turn was used for the enzymatic conversion of deoxyuridine-5′-phosphate to [$^{11}$C]thymidylate. Enzymatic treatment of the nucleotide with alkaline phosphatase gave [$^{11}$C]thymidine.**

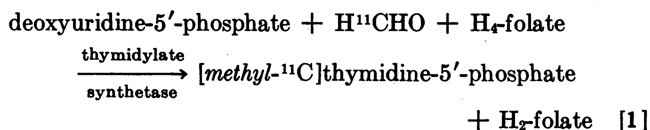
**The preparation of [$^{11}$C]thymidine from cyclotron-generated $^{11}CO_2$ required 110 min (about 5 half-lives): 35 min for the synthesis of H$^{11}$CHO, 25 min for the enzymatic conversion to [$^{11}$C]thymidylate, 20 min for column chromatography, 5 min for phosphatase treatment, 10 min for evaporation, 2 min for filtration through an anion-exchange resin, and 13 min for miscellaneous manipulations.**

**Positron-emitting [$^{11}$C]thymidine and [$^{11}$C]thymidylate were used for *in vivo* tracer studies of DNA synthesis in mice for periods of up to 3 hr. Findings with carbon-11 were consistent with earlier studies in which carbon-14 and tritium-labeled thymidine were used. For example, 3 hr after injection of [$^{11}$C]thymidine, spleen DNA was labeled to a much greater extent than was liver DNA.**

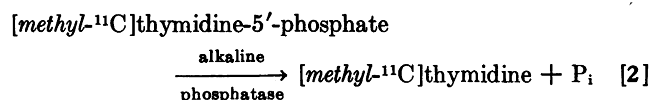
Before the advent of $^{14}$C as a tracer, $^{11}$C was used in many animal and plant studies involving carbon dioxide and simple carbon compounds (1); however, the short half-life of $^{11}$C (20.4 min) and the requirement for a cyclotron installation have severely limited its widespread use in biological research. New interest in the use of the short-lived positron-emitting isotopes, such as $^{11}$C, $^{13}$N, $^{15}$O, and $^{18}$F, has been aroused because of the advantages in detection, the unique occurrence of these elements in biological molecules, and the lower dose delivered to patients during *in vivo* tests. The emission of two photons separated by 180° that results from positron annihilation makes possible high-resolution and high-sensitivity positional information about such isotopes.

Despite advances in the detection of positron emitters, the short half-life limits the time available for inclusion of the isotope into labeled compounds. This time can be shortened in some cases by use of specific enzymes for rapid biosynthesis from relatively simple precursors. An example of this approach is the use of thymidylate synthetase for the incorporation of [$^{11}$C]formaldehyde into the methyl group of thymidylic acid (Eq. [1]), followed by phosphatase treatment, thus affording a simple means of converting the nucleotide to [$^{11}$C]thymidine (Eq. [2]).

Abbreviations: H$_4$-folate, tetrahydropteroyl-L-glutamate; H$_2$-folate, dihydropteroyl-L-glutamate.



$$\text{deoxyuridine-5'-phosphate} + \text{H}^{11}\text{CHO} + \text{H}_4\text{-folate} \xrightarrow[\text{synthetase}]{\text{thymidylate}} [methyl\text{-}^{11}\text{C}]\text{thymidine-5'-phosphate} + \text{H}_2\text{-folate} \quad [1]$$



$$[methyl\text{-}^{11}\text{C}]\text{thymidine-5'-phosphate} \xrightarrow[\text{phosphatase}]{\text{alkaline}} [methyl\text{-}^{11}\text{C}]\text{thymidine} + \text{P}_i \quad [2]$$

This study describes the preparation of these labeled compounds, as well as experiments in which they were injected into mice and the distribution of radioactivity was determined in various tissues.

## METHODS

*Preparation of [$^{11}$C]Formaldehyde by $^{11}B(p,n)^{11}C$ or $^{14}N(p,\alpha)^{11}C$ Reactions.* $^{11}CO_2$ was produced either by 22 MeV proton irradiation of $B_2O_3$ or by 16 MeV proton irradiation of nitrogen gas. For $B_2O_3$, helium was used as the flow gas through the target, at a rate of about 100 cm$^3$/min. $B_2O_3$ (about 30 g, spread over 30 cm$^2$) was heated by the beam (30 $\mu$A of 22 MeV protons) to about 300°C and became sufficiently molten to allow the $^{11}$CO and $^{11}CO_2$ formed to escape into the helium atmosphere†. Before trapping, the gas was passed through a CuO furnace at 700°C, to oxidize the $^{11}$CO present to $^{11}CO_2$. An irradiation of 20 min (10 $\mu$A-hr total dose) produced a total activity of 250–300 mCi of $^{11}CO_2$ at the trap when a freshly prepared target was used.

In production of $^{11}$C by the (p,$\alpha$) reaction on gaseous nitrogen, the $N_2$ is flowed through a target vessel 1.14-m long, at a rate of about 500 cm$^3$/min. At the maximum current available in the deflected beam (average 10 $\mu$A), roughly 500 mCi can be trapped in a 30-min irradiation.

The trapped $^{11}CO_2$, produced mostly by the $B_2O_3$ procedure, was reduced to [$^{11}$C]methanol by 0.1 mmol of $LiAlH_4$ in 1 ml of tetrahydrofuran. This method resembles that for direct reduction of $CO_2$ to HCHO (4). However, no attempt was

† The production of $^{11}$C mainly as volatile oxides during bombardment—a property crucial to success in these experiments—was quantitatively established in the $^{10}$B(d,p)$^{11}$C reaction by Ruben, Kamen, and Hassid (2), while the appearance of some $^{11}$C as volatile oxides in this process was first noted by Yost, Ridenour, and Shinohara (3).



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made to stop the reduction at the aldehyde stage, because the stoichiometric ratios of reactants could not be controlled at the concentrations used. The tetrahydrofuran was removed by evaporation under reduced pressure, and the flask containing the residue was placed in a train in front of a ferric–molybdenum oxide catalyst tube heated to 580°C (5). The sample was hydrolyzed with 0.4 ml of water. The vapor formed by heating the liquid was pumped through the catalyst bed with a current of air (purified by passage through $H_2SO_4$, then KOH pellets), trapped with liquid nitrogen, and finally distilled to the desired receiver.

The usual amount of distillate recovered in the final receiver was 0.30–0.35 ml. One 10-$\mu$l aliquot was taken for assay of the total radioactivity, another was taken for Nash assay of the HCHO concentration (6), and a third 10-$\mu$l sample was added to 1 ml of a 1 M solution of HCHO in water, with subsequent precipitation as a dimedon derivative (7). The derivative was filtered, dried, and counted to determine the activity of [$^{11}$C]formaldehyde in the distillate. The total time from end of bombardment to delivery of $H^{11}CHO$ was about 35 min.

The total activity in the final distillate after a typical run was about 100 mCi (scintillation well counter, efficiency 70%) as of the end of bombardment, the time designated as $t_0$ in these runs. The HCHO assay was usually in the range of 2–10 $\mu$mol/ml, or 0.7–2 $\mu$mol in the solution delivered. As determined by the Nash HCHO assay and the radioactivity of the dimedon derivative, the best specific activity of the $H^{11}CHO$ produced was in the range of 15–30 Ci/mmol, and the fraction of the total activity present as $H^{11}CHO$ was usually in the range of 40–60%. It was found by radio-gas chromatography that the remainder of the activity was essentially all $^{11}CH_3OH$ that was not oxidized under these unusually rapid conditions. The highest specific activity attained during these experiments was 31.3 Ci/mmol at $t_0$.

*The Enzymatic Preparation of [$^{11}$C]Thymidylate and [$^{11}$C]-Thymidine.* A typical incubation mixture is given in Table 1. [2-$^{14}$C]Deoxyuridylate was present as a different and important label to assess the synthesis of thymidylate. All ingredients except the formaldehyde were combined before the addition of $H^{11}CHO$. After 25 min of incubation at 33–37°C, a 50-$\mu$l sample was removed for counting and the mixture was then applied to a 0.8 × 13 cm column of DEAE-cellulose [Whatman 22 advanced fibrous form, previously freed of fine particles, washed with 1 M $NH_4HCO_3$, and equilibrated with 1 mM $NH_4HCO_3$ (8)].

The incubation mixture and all subsequent fractions were forced through the column under pressure. After the column was washed with 1 mM $NH_4HCO_3$, elution was started with 250 mM $NH_4HCO_3$. The effluent, 12 ml of 1 mM $NH_4HCO_3$ wash, and the first 3 ml of 250 mM $NH_4HCO_3$ eluate were discarded. The next 10 ml, which exhibited a strong blue fluorescence under UV light, contained the thymidylate and deoxyuridylate. For biological experiments with [$^{11}$C]thymidylate, this fraction was evaporated under reduced pressure at 80–90°C. Small portions of water were added three times and evaporated in order to free the mixture of $NH_4HCO_3$.

In contrast to the above procedure, if [$^{11}$C]thymidine was wanted, the material from the column was collected in a tube containing 20 units of alkaline phosphatase from *Escherichia coli* that had been dialyzed against 0.1 M $NH_4HCO_3$. The mixture was incubated 5 min at 37°C to insure complete dephosphorylation of the nucleotides, and was then evaporated as described above. The slightly-yellow residue was dissolved in 1 ml of $H_2O$ and applied to a small column (Spin-Thimble, Terra-Marine Bioresearch, La Jolla, Calif.) containing 1-ml packed volume of the anion exchange resin, Biorad AG1X10, 200–400 mesh, that had been washed with $H_2O$ and absolute ethanol. The small column, resting in the mouth of a 12-ml graduated centrifuge tube, was dried by centrifugation for 1 min, and 0.8 ml of solution was collected. This preparation was used for injection into mice.

TABLE 1. *A typical incubation mixture for the conversion of deoxyuridine-5′-phosphate to [$^{11}$C]thymidylate*

| Stock solutions | Amount ($\mu$l) | Total ($\mu$mol) | Incubation concentration ($\mu$mol/ml) |
|---|---|---|---|
| 126 mM *dl*-$H_4$-folate in 1 M 2-mercaptoethanol* | 80 | 10 | 1.3 |
| 64 mM [$^{14}$C]Deoxyuridylate† (9.4 mCi/mol) | 150 | 9.6 | 1.28 |
| 10 mM [$^{11}$C]Formaldehyde‡ (22 Ci/mmol) | 240 | 2.4 | 0.32 |
| Crystalline thymidylate synthetase§ (1800 units/ml) | 50 | — | — |
| 14 M 2-Mercaptoethanol | 10 | 290 | 38.5¶ |
| 0.5 M $MgCl_2$ | 15 | 7.5 | 1.0 |
| 0.05 M Tris·HCl buffer (pH 7.4)–0.01 M 2-mercaptoethanol | 7000 | 350 | 46 |

* See refs. 9 and 10. Tetrahydrofolic acid was freeze-dried directly from glacial acetic acid without prior precipitation with diethyl ether. 100 mg of lyophilized powder was dissolved in 1 ml of 1 M 2-mercaptoethanol upon neutralization with $NH_4HCO_3$.

† Unlabeled dUMP, disodium salt (see ref. 11 for preparation of [2-$^{14}$C]dUMP from [2-$^{14}$C]dCMP).

‡ Specific activity at $t_0$ (end of cyclotron run). This preparation contained 33% [$^{11}$C]$CH_3OH$. The methanol is probably washed through DEAE-cellulose in the wash fractions (see *Methods*).

§ The crystalline enzyme from *Lactobacillus casei* (12) was prepared at Tufts University School of Medicine under the direction of Dr. Roy Kisliuk, to whom we are greatly indebted. The earliest preparations of [$^{11}$C]thymidylate involved the use of thymidylate synthetase from *E. coli* (13); however, the availability of crystalline enzyme of high specific activity from dichloroamethopterin-resistant *L. casei* makes this source preferable.

¶ Final concentration from all sources.

*Analysis of a Preparation from a Typical Experiment.* Thymidine and deoxyuridine in a 0.1-ml sample were separated by ascending paper chromatography (16–18 hr on Whatman no. 1 paper) with $H_2O$-saturated *n*-butyl alcohol–15 M $NH_4OH$ 100:1 (14). By the end of the chromatography all of the $^{11}$C had decayed, but $^{14}$C was present as a tracer.

Two spots that quenched fluorescence under 255-nm light, corresponding to thymidine ($R_F$ = 0.57) and deoxyuridine ($R_F$ = 0.38) were eluted; their spectra were observed, and their $^{14}$C activity was counted. Thymidine $A_{268}$ = 0.135 $\mu$mol and 1.2 nCi; deoxyuridine $A_{260}$ = 0.59 $\mu$mol and 5.4 nCi; nonspecific $^{14}$C activity (from $R_F$ = 0–0.38) was 0.3 nCi or 4.9% of the total activity. The conversion of [$^{14}$C]dUMP to [$^{14}$C]-dTMP was calculated as 18%. $\{100[1.2 \text{ nCi}/(1.2 \text{ nCi} + 5.4 \text{ nCi})]\}$.

This chromatographic procedure was used for a series of preparations, as summarized in Table 2.

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TABLE 2. *Summary of various [¹¹C]thymidine preparations*

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|
| | Specific activity of $H^{11}CHO$, Ci/mmol at $t_0$ | Incubation components | | | | | | | | |
| Run no. | | $H^{11}CHO$, μmol | [¹⁴C]-dUMP, μmol | *dl*-$H_4$-folate μmol | Enzyme, units | Percent conversion to dTMP* | [¹⁴C]dT μmol† | [¹¹C]dT μmol‡ | Percent recovery of thymidine based on ¹⁴C§ | Percent recovery of thymidine based on ¹¹C¶ |
| 20 | 9.1 | 1.6 | 8.6 | 9.0 | 70 | 27 | 0.88 | 0.65 | 38 | 28 |
| 24 | 2.2 | 0.44 | 9.5 | 10 | 180 | 13 | 0.67 | 0.16 | 54 | 13 |
| 25A | 19 | 0.12 | 4.8 | 4.9 | 9 | 2 | 0.034 | 0.0038 | 35 | 4.0 |
| 25B | 19 | 0.12 | 4.8 | 5.0 | 90 | 13 | 0.38 | 0.042 | 61 | 6.7 |
| 26 | — | None | 4.8 | 5.0 | 90 | 7.4 | 0.20 | None | 56 | — |
| 27 | 22 | 2.3 | 9.6 | 10 | 90 | 18 | 0.96 | 0.59 | 56 | 34 |
| 28 | 4.6 | 6.0 | 9.6 | 9.5 | 90 | 27 | 1.6 | 2.0 | 62 | 77 |
| 29 | 15 | 2.0 | 9.6 | 9.5 | 90 | 21 | 0.96 | 0.38 | 48 | 19 |
| 16 | 5.3 | 19 | 7.8‖ | 12 | 72 | — | None | 3.2 | — | — |

* This value [100 × dT/(dT + dU)] is based on ¹⁴C by chromatography, as described in *Methods*.
† Based on ¹⁴C in the nucleoside fraction, specific activity of [¹⁴C]dUMP, and percent conversion to dTMP.
‡ Based on ¹¹C in the nucleoside fraction and specific activity of [¹¹C]formaldehyde.
§ 10⁴ [col. 8/(col. 7 × col. 4)].
¶ 10⁴ [col. 9/(col. 7 × col. 4)].
‖ dUMP not labeled with ¹⁴C in this case.

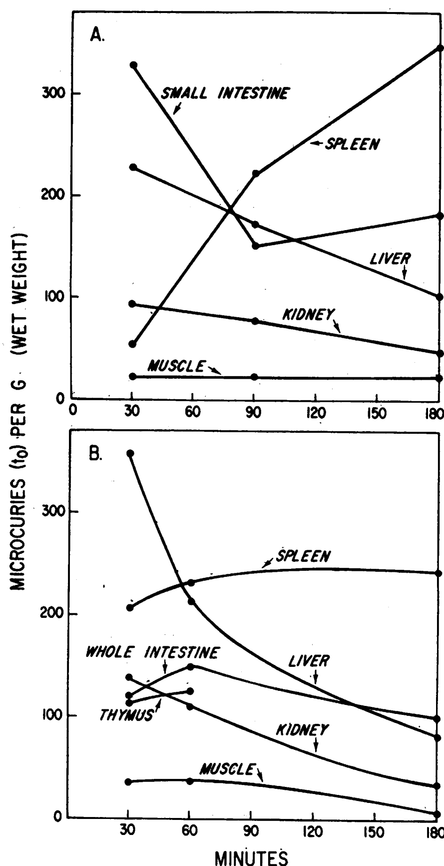


FIG. 1. Incorporation of ¹¹C into various tissues of mice. Each point represents the total activity per g of tissue, and is for a separate mouse injected intraperitoneally. A tabulation of the amount of tracer at various times is presented in Table 3. (*A*) Experiment with [¹¹C]thymidine, *run 27*. (*B*) Experiment with [¹¹C]thymidylate, *run 16*.

*Estimation of ¹¹C in Mouse Tissues.* At various times after intraperitoneal injection of 0.2-ml solutions of either [¹¹C]-thymidine or [¹¹C]thymidylate into white Swiss male mice (BNL), the animals were killed by overdosage with diethyl ether. Weighed tissues were counted in a Picker well counter, homogenized with 7 volumes of ice-cold $H_2O$ in a Ten-Broeck glass homogenizer, treated with 2 volumes of 70% perchloric acid, homogenized again, and centrifuged. The solids were twice suspended in 10 volumes of ice-cold 6% perchloric acid and centrifuged. Cold acid-soluble radioactivity was the sum of the activity in the first supernatant fluid plus the two washes. The residue was suspended in 10 volumes of 6% perchloric acid, heated for 20 min at 80–90°C and centrifuged. The hot acid-soluble fraction and residue (hot acid-insoluble) were counted separately.

## RESULTS AND DISCUSSION

A vast literature has accumulated on the use of thymidine containing ¹⁵N, ¹⁴C, and ³H (15). Tritium labeling made possible not only a tracer compound of high specific activity, but also provided a most effective procedure for the intracellular localization of newly synthesized DNA by autoradiography. The use of the thymidine analogue iododeoxyuridine, containing either ¹²⁵I or ¹³¹I (16), provides a means of scanning intact animals, as well as a valuable approach for estimation of cellular turnover, since salvage of iododeoxyuridine-labeled DNA is almost completely avoided. The present investigation with ¹¹C-labeled thymidine opens a new dimension for the assessment of cell kinetics. Although all measurements in this study were made with an ordinary scintillation well-counter, localization of rapidly dividing cells in intact organisms should be demonstrable with the positron camera, a detector whose potential sensitivity remains relatively undeveloped (17). To demonstrate the feasibility of using [¹¹C]thymidine as a tracer, various experiments were done with mice.

The kinetics of thymidine utilization and degradation are well established from a wealth of data obtained with ¹⁴C- and

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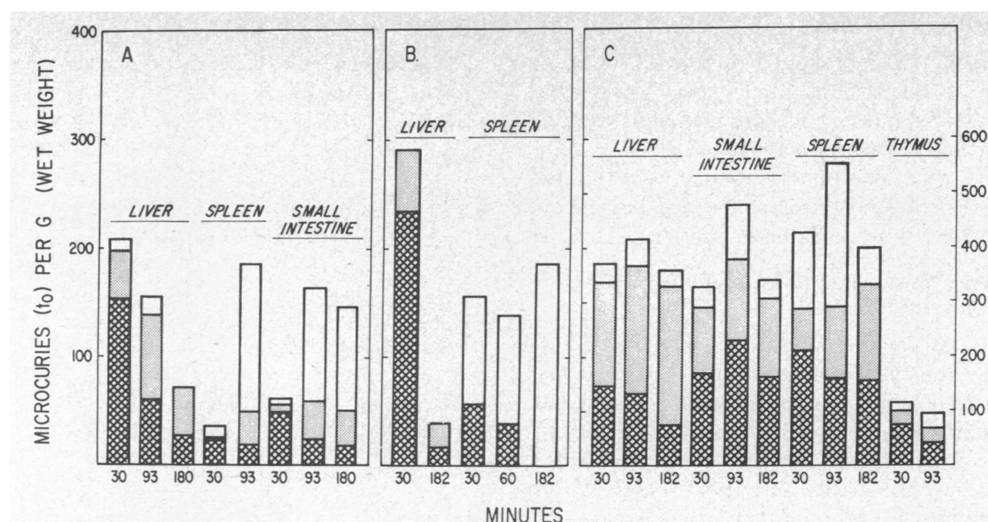


FIG. 2. Distribution of $^{11}$C in various fractions of tissues from mice injected intraperitoneally. All values less than 0.8 times above the background (120 cpm in these experiments) were not considered significant and were, therefore, not included. (*A*) [$^{11}$C]thymidine injected (same tissues as Fig. 1*A*, *run 27*). (*B*) [$^{11}$C]thymidylate injected (same tissues as Fig. 1*B*, *run 16*). (*C*) [$^{11}$C]formaldehyde injected. 0.19 μmol, 220 μCi/g of mouse at $t_0$. This preparation contained 55.7% H$^{11}$CHO. Gas chromatography of a similar sample indicated that the other major component was [$^{11}$C]methanol. □, hot acid-soluble; ■, cold acid-soluble; ▩, hot acid-insoluble.

$^3$H-labeled thymidine. The findings with $^{11}$C-labeled thymidine presented in Figs. 1*A* and 2*A* are consistent with earlier results from many laboratories (15, 18). Thus, in a tissue such as the liver in an adult animal, the synthetic pathway for the conversion of thymidine to nucleotides by a sequence of phosphorylation steps does not result in any major incorporation of thymidine into DNA. Meanwhile the degradative pathway, via conversion of thymidine to β-aminoisobutyric acid, decreases the pool of labeled intermediates. In contrast to the liver in an adult animal, a tissue such as mouse spleen, with its high rate of DNA replication, should, and does, exhibit a marked incorporation of labeled thymidine into DNA.

TABLE 3. *Radioactivity of $^{11}$C at various stages of experiments described in Fig. 1A and B*

| Experiment* | Amount of tracer injected, nmol per g of mouse | Radioactivity of $^{11}$C, μCi per g of mouse: At $t_0$ | At time of injection | At time of counting tissues |
|---|---|---|---|---|
| [$^{11}$C]Thymidine, Fig. 1*A* | | | | |
| 30 min | 4.5, 6.9† | 97 | 2.16 | 0.47 |
| 93 min | 5.7, 8.8 | 123 | 2.74 | 0.077 |
| 182 min | 5.5, 8.5 | 119 | 2.64 | 0.0037 |
| [$^{11}$C]Thymidylate, Fig. 1*B* | | | | |
| 30 min | 21 | 108 | 1.54 | 0.42 |
| 60 min | 23 | 121 | 1.78 | 0.127 |
| 182 min | 23 | 121 | 2.88 | 0.0036 |

* Each experiment is for a separate mouse. Time indicated is the period between injection of tracer and administration of ether.

† The first number given is based on specific activity of [$^{11}$C]-formaldehyde in the incubation mixture, the second number is based on specific activity of [$^{14}$C]dUMP in the incubation mixture.

Whereas the total amount of $^{11}$C in liver decreases markedly during a 3-hr period due to dissipation of the cold acid-soluble pool, the radioactivity in spleen and intestine decreases much less with time (Table 4 and Figs. 1 and 2).

An important consideration for future investigations is that scanning devices located outside the body cannot distinguish between a tissue with a pool of radioactive nucleotides and one in which $^{11}$C has been incorporated into DNA. If the scanning procedure with [$^{11}$C]thymidine is to have any significant usefulness for assessing the kinetics of DNA replication, the sensitivity of detection must be sufficient to detect $^{11}$C in DNA after a period long enough to allow the pool of radioactive nucleotides to dissipate.

The incorporation into newly formed DNA of labeled thymine from metabolites of thymidine or by reutilization from dying cells (19), although probably slight for short periods of time, remains to be determined.

A rapid separation of [$^{11}$C]thymidine from deoxyuridine and [$^{11}$C]dTMP from dUMP has not been achieved in this study. Although the amounts of deoxyuridine or dUMP injected are not excessive (Table 2, column 7), the possible effects on body pool sizes remain uncertain and should be further investigated.

TABLE 4. *Comparison of $^{11}$C uptake into intestine, liver, and spleen*

| Minutes | Number of mice | Tissue uptake*: Intestine Mean | Intestine SE† | Liver Mean | Liver SE | Spleen Mean | Spleen SE |
|---|---|---|---|---|---|---|---|
| 30 | 8 | 1.52 | 0.23 | 2.32 | 0.18 | 2.20 | 0.33 |
| 60 | 5 | 1.42 | 0.24 | 1.46 | 0.10 | 2.68 | 0.29 |
| 180 | 6 | 1.03 | 0.13 | 0.70 | 0.07 | 2.03 | 0.16 |

* [μCi/g tissue, wet weight]/[μCi injected/g animal].

† Standard error of the mean.

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### [11C]Thymidylate tracer experiments

The monophosphate of [11C]thymidine can be used for synthesis of DNA (Figs. 1*B* and 2*B*). In a single experiment, [11C]-thymidylate, administered intravenously or intraperitoneally, was incorporated to the same extent into various mouse tissues at 60 min. Although prior dephosphorylation *in vivo* may be required for use of the 11C-labeled compound, the possibility of entry of intact nucleotide into cells has not been ruled out.

### The presence of extra nonlabeled formaldehyde in the preparation of [11C]thymidine

As shown in Table 2, the amount of thymidine found based on 14C was greater than the amount based on 11C (except in run 28). In run 26, in which *no* [11C]formaldehyde was added, 0.2 μmol of [14C]thymidine was recovered. This result indicated that *extra unlabeled formaldehyde must have been present in the incubation mixture.* Since the specific activity of [14C]dUMP is not affected by the presence of unlabeled formaldehyde, the amount of thymidine formed is more accurately assessed based on 14C rather than on 11C.

The presence of a formaldehyde-like compound in tetrahydrofolate preparations has been reported by other investigators (20). In our laboratory, preliminary spectrophotometric studies indicate that prior incubation of 0.6 mM tetrahydrofolate at 55°C for 10 min at pH 3–7 favors the generation of a substance that can substitute for formaldehyde in the overall thymidylate synthetase reaction.

### Contrast between H11CHO and [11C]thymidine as tracers

Earlier experiments with 14C-labeled formaldehyde helped to develop the concept of one-carbon metabolism (21). The profile of incorporation of radioactivity into various cell components is much broader and less specific with H11CHO as a tracer than with [11C]thymidine. This can be easily seen by comparison of Fig. 2*A* and *C*. With H11CHO as a tracer, the hot acid-soluble and hot acid-insoluble fractions of liver are much more prominent than with [11C]thymidine. This finding is consistent with one-carbon labeling of RNA via *de novo* purine synthesis, as well as of protein via serine and methionine.

### Metabolism of [11C]thymidine

In a preliminary experiment, it was found 180 min after intraperitoneal injection of [11C]thymidine (89 nmol, 1290 μCi) into a 24-gram mouse that about 13% of the radioactivity was excreted [feces, 111 μCi; urine, 52 μCi, and $CO_2$, 4.3 μCi (all $t_0$ values)].

### Conclusions

These experiments have demonstrated the utility of rapid preparation of important biological precursors labeled with isotopes of short half-life. The preparation of [11C]thymidine required about 2 hr. It should be possible by automated techniques, as well as by use of immobilized enzymes, to shorten this time even further. Many novel applications in the biomedical sciences should be feasible.

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