Utilization of Labeled Thymidine in DNA Synthesis: Studies for PET

Anthony F. Shields, Kap Lim, John Grierson, Jeanne Link, and Kenneth A. Krohn

Departments of Medicine and Radiology (Nuclear Medicine), University of Washington, Seattle; VA Medical Center; and Fred Hutchinson Cancer Research Center, Seattle, Washington

Carbon-111-thymidine and positron emission tomography (PET) have the potential for noninvasively measuring DNA synthesis in tumors and tissues. In order to validate this potential, one needs to construct accurate biochemical models that reflect the metabolism of thymidine, including its uptake and degradation as well as its incorporation into DNA. Our studies employed thymidine labeled in the methyl position using ^{11}C, ^{14}C, and ^{3}H. Studies with rapidly proliferating tissues of mice demonstrated that most of the activity, 60 min after injection of labeled thymidine, was present in DNA, with smaller amounts found in metabolites. Studies in dogs, however, reveal that more activity was present in metabolites, rather than in the DNA of tumors and spleen tissue. HPLC analysis of canine blood after the injection of thymidine revealed rapid conversion of thymidine to thymine and other metabolites. We have found significant differences in the metabolism of [^{3}H]thymidine versus thymidine labeled with radioactive carbon. These differences, which were not found when comparing ^{14}C and ^{11}C, indicate that [^{3}H]thymidine is not an appropriate standard for comparison with PET studies employing [^{11}C] thymidine. To accurately interpret images of [^{11}C]thymidine as representations of DNA synthesis we are developing kinetic models that take into account the metabolism of thymidine and the contribution of degradation products to the ^{11}C images.

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Labeled thymidine is widely used in biologic research to measure the growth of tissues and tumors, both in cell culture and in vivo. The various in vivo measurements, which can be performed using labeled thymidine have been limited by the necessity of obtaining tissue samples at biopsy or autopsy to detect the beta emitting isotopes. More recently, carbon-11 (^{11}C) thymidine has been developed for use in positron emission tomography (PET), allowing one to obtain noninvasive measurements of thymidine uptake and retention in tissues (1–3). Each of these studies has demonstrated increased retention of labeled thymidine in rapidly growing tissues and tumors. PET measures the total radioactivity retained in each tissue at any point in time, regardless of the chemical species with which the isotope is associated. Thus, to interpret the images obtained using PET, one must have a detailed knowledge of the biochemistry of thymidine metabolism and know the proportion of the activity found in the various chemical species. In particular, the relative amounts of activity incorporated into DNA compared with those appearing as degradation products needs to be known. When human tumor cell lines were labeled in vitro with hydrogen-3- (^{3}H) thymidine between 18% to 100% of exogenously-supplied thymidine that was taken up was incorporated into DNA; the rest being metabolized (*4*). The contribution of the degradation pathways to activity retained in vivo must also be known in order to interpret PET images. We have conducted studies of the metabolism of thymidine in mice and dogs in order to validate biochemical and kinetic models needed for interpreting PET images of [^{11}C]thymidine as representative of DNA synthesis.

MATERIALS AND METHODS

Radiochemistry. These studies employed [^{3}H]thymidine (Fig. 1) labeled in the methyl position (1-2-deoxy-β-D-ribofuranosyl)-5-[H-3]methyluracil) (70 Ci/mmol; New England Nuclear, North Billerica, MA); [C-14]thymidine (also labeled in the methyl position; 20 mCi/mmol; Research Products International); for each study the radiochemical purity was >98% as assayed by high performance liquid chromatography (HPLC) (see below). No carrier added [^{11}C] thymidine (labeled in the methyl position) was prepared just prior to use according to the method of Sundoro-Wu et al. with the inclusion of hexamethylphosphonic triamide as a reaction rate accelerator (two equivalents per equivalent of lithiated precursor) (*5*). The radiochemical purity of HPLC isolated material was >95% and the specific activity was 8–30 Ci/mmol decay corrected to the time of injection. The entire preparation of the [^{11}C] thymidine from the [^{11}C] CO_2 required 60–80 min.

Dog Studies. Five dogs weighing from 19 to 23 kg were used for studies involving blood clearance and metabolism of thymidine (two normal and three with untreated lymphoma). Each dog was anesthetized and arterial and venous catheters placed in the groin. Carbon-11-thymidine (2.4–7.9 mCi) was mixed with [^{14}C] thymidine (34.4–58.8 μCi) in 10 to 20 ml of

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For reprints contact: Dr. Anthony Shields, Seattle VA Medical Center, Mail Stop (111), 1660 S. Columbian Way, Seattle, WA 98108.

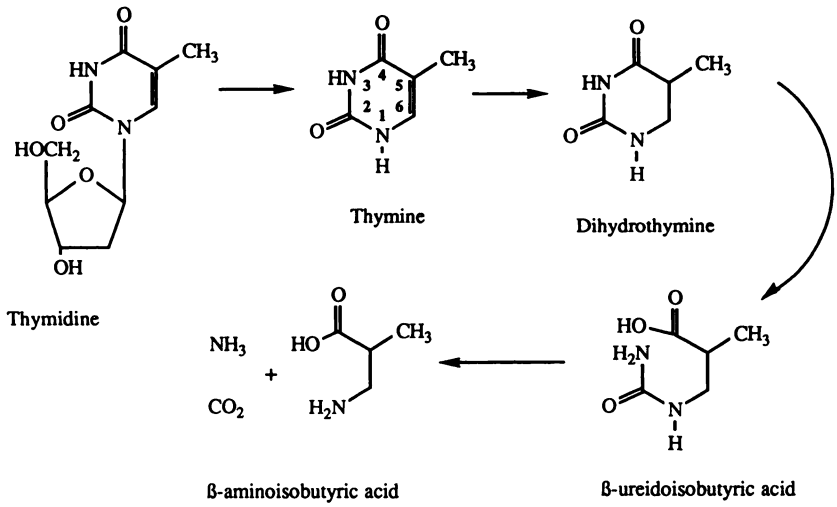

FIGURE 1
Structure of thymidine at its major metabolites.

physiologic saline and infused intravenously over 60 sec using a syringe pump. Addition of [^{14}C]thymidine facilitated a more complete analysis of the products of thymidine degradation than was possible using the shorter-lived ^{11}C radionuclide. Blood samples (1.5 ml) were drawn from the arterial line using a manifold and a series of syringes. Sampling was performed at ~10-sec intervals over the first minute after injection, then 20-sec intervals over the second minute, then at 3, 4, 5, 7, 9, 11, 13, 15, 18, 21, 24, 27, 30, 35, 40, 45, 50, 55, and 60 min. Blood was drawn into heparinized syringes containing about 30,000 cpm of [^{3}H] thymidine to act as an internal standard for thymidine degradation that occurred during processing. Aliquots (0.2 ml) of blood were then spotted onto gauze pads and immediately placed into the gamma counter to measure ^{11}C activity. These pads were then dried and burned in an oxidizer (Packard Instrument Co., Downers Grove, IL) and ^{3}H and ^{14}C quantitated by scintillation spectrometry. All scintillation counts were corrected for quenching by the external standard method and results were expressed as a percentage of injected dose per gram. Results for ^{11}C were decay corrected to a standard time.

To quantitate the amounts of activity in blood present as thymidine and its metabolites, 1 ml of whole blood was mixed immediately after withdrawal with three volumes of methanol:acetonitrile (3:1). After centrifugation, the supernatant was removed and frozen for HPLC analysis. The samples were dried under a stream of nitrogen gas and then resuspended in water for HPLC analysis. The samples were analyzed on a reverse-phase column (Spherisorb ODS2, 5-micron particle size, 250 × 4.6 mm) with a guard column of LC-18 pellicular packing (50 × 4.6 mm). The mobile phase was 1% or 2% acetonitrile and water. The retention times for thymidine and thymine standards (Sigma Chemical Co., St. Louis, MO) were 8.3 and 5.7 min, respectively. In each case, we routinely separated and collected three fractions from the eluate of the HPLC; thymidine, thymine mixed with dihydrothyamine, and rapidly eluting material near the void volume (other metabolites) (Fig. 1). The other metabolites, which include β-aminoisobutyric acid (BAIB) and β-ureidoisobutyric acid, are not incorporated in to DNA to any significant extent (*6*). The HPLC fractions were quantitated by scintillation spectrometry as described above, and the percentage of activity in the fractions were determined.

Two normal dogs were sacrificed 60 min after the injection of 50 μCi of [^{14}C]thymidine. The animals were anesthetized during the experiment and killed by a bolus injection of saturated KCl. Samples of the organs were immediately removed and dried for oxidation or frozen for extraction as previously described (*7*). At extraction, the amount of activity present in the acid soluble fraction (called small molecules), RNA, DNA, and protein and were quantitated. The protein fraction, which also contained lipid, was obtained by solubilization of the pellet after removal of the other fractions. We also obtained biopsy samples of lymph nodes from the necks of three dogs with lymphoma, at 60 min after the infusion of [^{14}C]- and [^{11}C]thymidine. Samples of the nodes were analyzed as had been done for the tissues of normal dogs.

Mouse Studies. All studies were done in normal 5–6 wk old Balb/C mice obtained from the Hutchinson Cancer Research Center. The different isotopic forms of thymidine were compared by injecting a mixture of the compounds, 1 μCi each of ^{3}H and ^{14}C, and ~25 μCi of ^{11}C into mice via a tail vein and followed in each case by cervical dislocation at 60 min postinjection. The organs were immediately removed, blotted dry, weighed, and rapidly measured in a gamma-counter (Packard, Downer's Grove, IL) if ^{11}C was employed, and then frozen for subsequent extraction or dried for oxidation (using a Packard Oxidizer; Downer's Grove IL) prior to liquid scintillation spectrometry to quantitate ^{3}H and ^{14}C content. A time-course study was also performed by killing mice at 2, 5, 20, and 60 min after the coinjection of [^{3}H]- and [^{14}C]thymidine. Tissue extraction was done as described above. For the data presented all small molecule fractions were dried to remove tritiated water.

RESULTS

In order to better understand the metabolism of labeled thymidine, its clearance was measured in dogs. Five dogs were infused with both [^{14}C]- and [^{11}C]thymidine over 60 sec, and the blood clearance of the two isotopes was determined. An example of one such study is shown in Figure 2. Carbon-11 content of the whole blood was measured in a gamma-ray spectrometer immediately after withdrawal and pipetting, while ^{14}C

content was measured after drying, oxidation, and scintillation counting. In order to determine if ^{14}C and ^{11}C were being cleared similarly, we compared the ratio of the integrated time-activity ratios for each isotope. No significant difference was found between the whole-blood content of the two isotopes at early time points (up to 20 min); however, after this initial period there was a small but significant difference ($p < 0.05$). The difference reached a mean of 13% by 60 min ($n = 5$). This is consistent with the losses of ^{14}C seen from drying the samples, as described below.

Extraction of the ^{14}C content from the whole blood and analysis by HPLC revealed rapid conversion of the labeled thymidine to thymine and other metabolites (Figs. 2 and 3). In fact, the total percentage of activity present in thymidine over the first 60 min after injection was on average ($n = 5$) only 23%, calculated using the area under the time-activity curve. Twenty percent of the activity was in thymine and 57% was in other metabolites. While the shape of the thymidine degradation curve was similar in these dogs, the percentage of activity as thymidine varied from animal to animal (Fig. 4). For example, at 2 min postinjection the percent of activity in thymidine ranged from 30% to 57%. HPLC analysis of multiple blood samples was, therefore, necessary for an accurate determination of the input function in any given individual.

We extracted and analyzed the ^{14}C content of two normal dog spleens and tumors from three dogs with lymphoma 60 min after the injection of labeled thymidine. Even in these rapidly proliferating tissues much of the activity was contained in small molecules (Table 1). Only 33%–49% of the activity was contained in DNA.

In order to compare the uptake and retention of [^{11}C]-, [^{14}C]-, and [^{3}H]thymidine, we injected a mixture

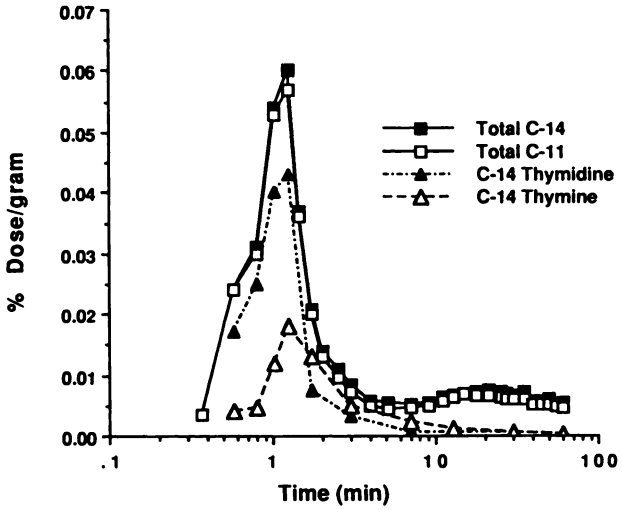

FIGURE 2
Blood concentration of [^{14}C]- and [^{11}C]thymidine in a single dog with lymphoma. Time is on a logarithmic scale to better view early time points.

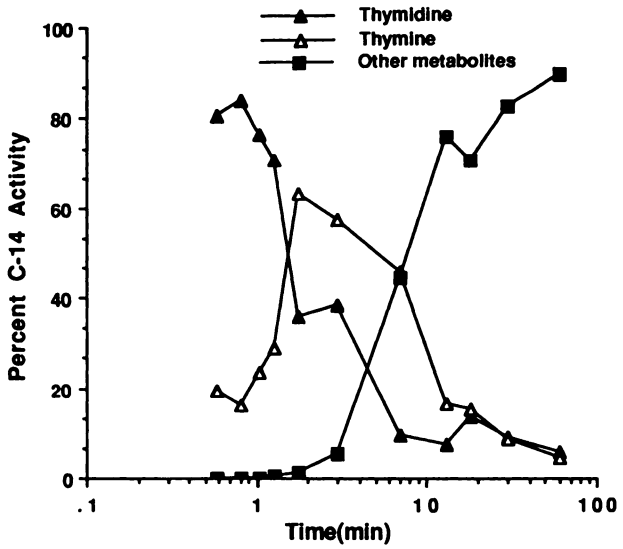

FIGURE 3
Time course of thymidine degradation in the same dog as shown in Figure 2.

of the three compounds intravenously into four mice and killed them 60 min later (Table 2). The ratio of retention of the different isotopes in each sample were compared using a paired t-test. Hydrogen-3 and ^{14}C uptake were significantly different for each organ when paired samples were analyzed ($p < 0.01$). In tissues with high rates of cell turnover, such as the femur (marrow), thymus, duodenum, and spleen, the ratio of total ^{3}H to ^{14}C retention ranged from 0.77 to 0.92 (Table 3). In organs with low rates of cell growth, such as the kidney, lung and heart, the ^{3}H to ^{14}C ratio ranged between 0.45 and 0.57, and in the liver this ratio was only 0.25.

In contrast to the marked differences seen between

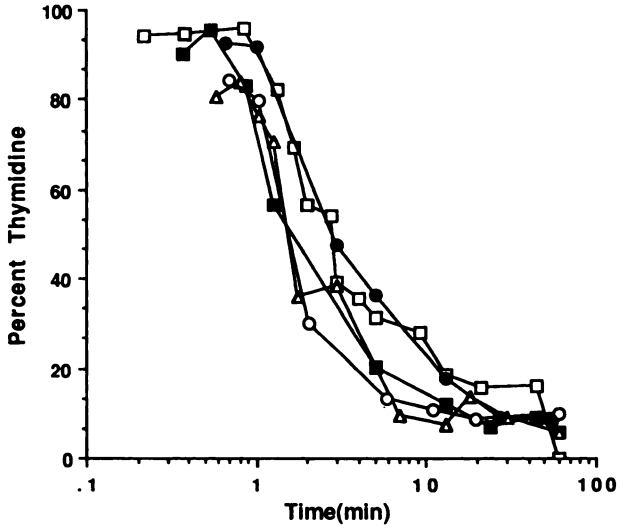

FIGURE 4
Percentage of blood activity in thymidine in five dogs infused with [^{14}C]thymidine. Samples were analyzed by HPLC to determine the fraction in thymidine.

TABLE 1
Extraction of Dog Tissues 60 Min Postinjection of [^{14}C] thymidine

	Lymphoma			Spleen	
% ^{14}C activity	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5
DNA	33	40	37	49	41
Small molecules	49	45	52	41	49
RNA	6	3	3	2	3
Protein	12	12	8	8	8

TABLE 2
Ratio of Retention of ^{14}C-, ^{11}C-, and [^{3}H]Methyl Thymidine at 60 Min in the Organ of Mice

Organ	H-3/C-14	^{11}C/^{14}C
Spleen	0.92	1.00
Thymus	0.80	1.05
Duodenum	0.85	1.01
Femur	0.77	1.11
Heart	0.57	1.30
Lung	0.49	1.35
Kidney	0.45	1.24
Liver	0.25	1.07

The sd of the ratios varied from 0.003 to 0.07, n = 4.

^{3}H and ^{14}C retention, smaller differences were found between ^{14} and ^{11}C (Table 2). The relative retention of [^{11}C]- and [^{14}C]thymidine were not significantly different in the spleen, thymus or duodenum, but significant differences were seen in the other organs. The ratio of ^{11}C/^{14}C activity was highest in the lung (1.35). Since the ^{14}C samples were dried prior to oxidation, and the ^{11}C content was measured while the samples were fresh, we compared the retention of ^{14}C in separate experiments where samples were oxidized with or without prior drying. Drying resulted in significant loss of ^{14}C in the kidney (between 10%–20% in different experiments) and in the lung (10%–31%).

Because of the marked variability between ^{3}H and ^{14}C retention, the percentage of radioactivity in nonacid precipitable counts, RNA, DNA and protein were compared for each tissue and fraction (Table 3). In organs with rapid cellular proliferation, such as the thymus, spleen, and duodenum, most of the radioactivity was present in the DNA fraction. In organs with low rates of cell turnover, such as the heart and kidneys, most of the activity was present in nonacid precipitable molecules (labeled small molecules). In the liver, much of the activity was present in protein and nonacid precipitable fractions. Analysis of the extracts demonstrated that in the thymus, spleen and duodenum the ratio of ^{3}H/^{14}C was <1, because there was less tritium in dried, nonacid precipitable counts and protein (Table 4). The ratio of ^{3}H/^{14}C in DNA in these rapidly proliferating organs varied from 0.90 to 0.97. In the heart, lung, and kidney there was a marked increase in ^{14}C activity in small molecules (nonacid precipitable counts) compared with ^{3}H. The liver was unique in demonstrating increased retention of ^{14}C in protein as well as nonacid precipitable counts.

A time-course study of the incorporation of labeled thymidine into the mouse spleen showed that the ratio of ^{3}H/^{14}C activity in DNA remained relatively constant and was not significantly different than unity (range 0.93–1.01, s.d. 0.08). On the other hand, the ratio of ^{3}H/^{14}C activity in nonvolatile, small molecules steadily fell over time from 0.94 at 2 min to 0.22 at 60 min. A plot of the time-course of activity in the ^{14}C fractions displays a rapid rise in the ^{14}C content of DNA, while the activity in small molecules is slowly falling (Fig. 5).

DISCUSSION

Carbon-111-thymidine can be used to image tumors as demonstrated in early work in mice (*2*) and more recently in man (*3*). One must understand thymidine's metabolism, however, in order to interpret the information obtained using [^{11}C]thymidine and PET. We have already addressed a number of problems associated with interpreting PET images of thymidine, including the contributions to thymidine metabolism of intracellular pools, reutilization, and blood flow (*7–9*). Our results show that one can calculate the effective specific activity of the intracellular pool, that reutilization should not interfere with the uptake of labeled thymidine, and that metabolism rather than perfusion is the rate limiting step in thymidine retention.

There are practical difficulties performing biochemical studies with [^{11}C]thymidine imposed by its 20.4-min half-life, which are solved by using longer-lived isotopes for nonimaging studies. Thymidine is commercially available with ^{3}H or ^{14}C in the methyl position, the same position that we label with ^{11}C. While [^{14}C]thymidine should behave identically to its ^{11}C counterpart, [^{3}H]thymidine is significantly less costly than [^{14}C]thymidine and has a much higher specific activity, allowing one to use more [^{3}H]thymidine in a given experiment. For this reason, it might be the tracer of choice for metabolism studies. However, the data presented here reveal large differences between the tritium and radiocarbon labeled products of thymidine metabolism. Previous work demonstrated differences in the behavior between thymidine labeled in the methyl position and the ring 2-position (*6,10*), however, there have been no direct comparisons of thymidine labeled with ^{3}H and ^{14}C on the methyl position. Our data demonstrate that [^{14}C]- and [^{11}C]thymidine behave similarly, as expected.

The differences between the retention of [^{3}H]thymidine and thymidine labeled with radiocarbon, appears to be the result of tritium exchange occurring within the organs. There appears to be reutilization of the carbon from the methyl group of thymidine, which is

TABLE 3
Percentage of Activity in Each Fraction in the Organs of Mice at 60 Min Postinjection of a Mixture [^{3}H]$^-$ and [^{14}C] thymidine

	Organs with Rapid Cellular Proliferation					
	Spleen		Thymus		Duodenum	
Fraction	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
DNA	92.5	85.9	91.0	70.1	86.6	77.1
Small molecules	2.4	8.0	1.0	20.3	4.3	12.0
Protein	3.3	4.7	3.9	7.9	5.6	8.8
RNA	1.7	1.4	4.1	1.7	3.5	2.1
Total % dose/g	9.0	10.3	3.9	5.2	5.8	7.2

	Organs with Low Rates of Cell Turnover							
Fraction	Liver		Heart		Lung		Kidney	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
DNA	15.1	12.7	13.0	17.3	54.7	44.8	21.7	20.5
Small molecules	37.7	32.4	63.6	64.7	22.6	29.5	50.4	52.4
Protein	36.8	46.7	1.3	8.3	3.8	16.2	2.6	15.2
RNA	10.4	8.3	22.1	9.8	18.9	9.5	25.2	11.9
Total % dose/g	1.1	3.1	0.8	1.3	0.5	1.1	1.2	2.1

The small molecules fraction was dried prior to counting. N = 8 to 10.

present in metabolites and incorporated into protein, while the tritium is lost, presumably in the form of tritiated water. Since we dried both the oxidized and extracted samples, the tritiated water is lost in processing. When wet samples were analyzed, significantly more activity was seen in tritium (data not shown). In the rapidly proliferating organs of the mouse, where most of the activity is incorporated into DNA, the end result is that smaller differences between the total tissue activity of ^{3}H and ^{14}C are apparent. At the other extreme is the liver, where almost all of the thymidine is degraded, magnifying the differences between the handling of ^{14}C and ^{3}H. In the liver, little activity ends up in DNA.

Metabolite information is important in interpreting studies of thymidine uptake. It is clear from our results that one cannot use [^{3}H]thymidine to predict the behavior of [^{14}C]- or [^{11}C]thymidine, even though all three are labeled at the methyl position. These results demonstrate that most of the activity retained after 1 hr in rapidly growing tissues of the mouse, such as the spleen, is incorporated into DNA. Nevertheless, some thymidine is degraded and ends up as nonacid precipitable or protein label. Our data clearly indicate that total thymidine uptake in slowly replicating tissues, such as the liver and heart, is not going to predict cell growth.

The metabolism of thymidine in the dog is somewhat different from that in the mouse. Most of the activity in the rapidly proliferating organs of the mouse is contained within DNA. In the dog, a large fraction of the activity appears as metabolites of thymidine, even in rapidly proliferating tissues and tumors. Furthermore, the blood time-activity curves are dominated by the presence of the metabolites of thymidine, rather than thymidine itself. Preliminary evidence suggests that the metabolism of thymidine in humans will more closely resemble that in the dog. For example, the kidney retains large amounts of the metabolites of thymidine in man and in the dog, but not the mouse (*3,6*).

The use of [^{11}C]thymidine with PET requires a thorough understanding of the metabolism of thymidine. The incorporation of this biochemical information into the kinetic model is critical to quantitatively interpret-

TABLE 4
Mean Ratios of ^{3}H and ^{14}C Activity (as % dose/g) in Each Fraction in the Organs of Mice at 60 Min Postinjection of a Mixture [^{3}H]- and [^{14}C]thymidine

Fraction	Spleen	Thymus	Duodenum	Liver	Heart	Lung	Kidney
DNA	0.94	0.97	0.90	0.41	0.50	0.62	0.57
Small molecules	0.27	0.04	0.29	0.39	0.57	0.39	0.53
Protein	0.60	0.36	0.50	0.26	0.15	0.12	0.09
RNA	1.08	1.77	1.39	0.45	0.83	1.11	1.15
Total	0.70	0.80	0.77	0.33	0.54	0.51	0.56

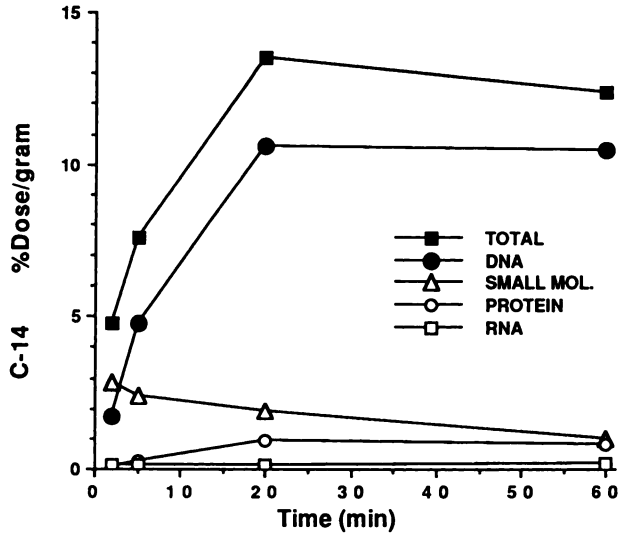

FIGURE 5
Time course of [^{14}C]thymidine activity in the mouse spleen. The data is presented as the mean percentage of activity in the indicated fractions (n = 3 except for the 60-min time point where n = 6).

ing the images as indicative of DNA synthesis. A recently published article demonstrates some of the pitfalls that can be encountered if the metabolism of thymidine is neglected (*3*). The authors showed that ^{11}C from labeled thymidine was readily taken up in the tumors of patients with non-Hodgkin's lymphoma, but the interpretation of the images and models overlooked some important elements in the metabolism of thymidine. The study explicitly assumed that [^{11}C]thymidine was not degraded in the blood during the experiment, yet thymidine rapidly undergoes metabolism while incubated in blood (*11*). It reported that metabolites of thymidine did not appear in the blood until 10 min after injection, but the assay used to separate thymidine from its metabolites did not separate thymidine from thymine. Our results in the dog demonstrate that most of the thymidine is converted to thymine within 2 min after injection and we have obtained similar results in humans (data not shown). No provision was made for the presence of metabolites within the tumor and it was assumed that all metabolites were cleared from the blood by 1 hr after injection. Previously published work (*6*), as well as our current data, shows that this is largely true in mice, but definitely not true in dogs and unlikely in humans. The importance of these points is demonstrated by the results of our extractions of tumor biopsies; approximately half of the activity contained within untreated tumor was in metabolites. Thus, if one assumed that all the activity was in DNA one could overestimate the DNA synthetic rate by a factor of 2 (Table 1). Overestimates of the DNA synthetic rate may be further magnified after therapy, when the DNA synthetic rate may be even lower and the proportion of activity in metabolites higher. It is for these reasons that we have extensively studied thymidine's metabolism and are incorporating our findings into kinetic models to image DNA synthesis instead of just the uptake [^{11}C] thymidine labeled in the methyl position. Furthermore, analysis of the images may be simplified by the use of thymidine labeled in the 2-carbon position (Fig. 1), which is rapidly degraded to labeled CO_2 rather than labeled BAIB and subsequent metabolites (*6,12,13*).

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