Salvage of Circulating Pyrimidine Nucleosides in the Rat

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ABSTRACT

A new procedure was developed to measure uridine and cytidine in plasma. These nucleosides are present in micro-molar concentrations in the plasma of rats, mice, and humans. Inhibitors of pyrimidine synthesis de novo (pyrazofurin or N-phosphonacetyl-L-aspartate) produce only modest decreases in the concentration of circulating uridine or cytidine in the rat. Since both uridine and cytidine are rapidly cleared from the circulation of the rat, constant infusions of radiolabeled uridine and cytidine were used to establish a steady-state specific activity of circulating nucleoside without altering the normal endogenous concentration. These studies permitted an estimation of the contribution of circulating pyrimidine nucleoside to the nucleotide pools of various rat tissues. Most of the uridine entering the circulation (>70%) is catabolized rather than salvaged by formation of nucleotides. Cytidine in the circulation is much more efficiently utilized and is predominantly salvaged. The implication of these results for chemotherapy based on inhibition of pyrimidine synthesis de novo is discussed.

INTRODUCTION

Separate enzymatic pathways exist in mammalian cells to generate nucleotides by de novo synthesis or by salvage of preformed bases or nucleosides (12, 19). Although it has been suggested that bone marrow and intestinal mucosa are primarily dependent on the salvage pathway to obtain purine nucleosides from preformed bases or nucleosides (12, 19) and that the liver provides preformed purines and pyrimidines to other tissues through the circulatory system (15, 16, 23), the extent to which a particular tissue depends on each pathway has not been firmly established. A more complete understanding of the balance between alternate pathways would help guide the proper chemotherapeutic use of PF3 or PALA, inhibitors of the de novo pathway, and p-nitrobenzyl-thioinosine (31) or inhibitors of uridine kinase, which produce only modest decreases in circulating uridine (>70%) is catabolized rather than salvaged by formation of nucleotides. Cytidine in the circulation is much more efficiently utilized and is predominantly salvaged. The implication of these results for chemotherapy based on inhibition of pyrimidine synthesis de novo is discussed.
and cytosine, all <7.5 min; cytidine, 8.5 min; uridine, 14 min; deoxycytidine, 14 min; deoxyuridine, 23 min; thymidine, 35 min; and adenine, 30 min. Peak height was directly proportional to amount injected in the range of 0.1 to 1.0 nmol at a sensitivity of 0.005 absorbance units, full scale.

Analysis of RNA Hydrolysate by Anion Exchange. Incorporation of [5-3H]uridine into RNA was assayed by separation of the 2',3'-mononucleotides resulting from alkaline hydrolysis on a 25-cm BAX-4 (Benson Co., Reno, Nev.) column at 50° with a linear gradient from 0.6 to 1.0 M ammonium acetate, pH 8.4, for 35 min during a period of 20 min and subsequent isocratic elution with 1 M buffer at 2 ml/min.

Cation-Exchange Chromatography of Cytidine. A 25- x 0.46-cm column packed with BPAN6 resin (Benson Co.) was eluted at 50° with a flow of 1.5 ml/min with 0.3 M formic acid adjusted to pH 4.0 with ammonium hydroxide. The following retention times were observed: void, 2.0 min; uracil, 2.4 min; uridine, 2.6 min; guanosine, 5.8 min; adenosine, 15 min; cytidine, 24 min; adenine, 55 min; and cytosine, 57 min. For quantitation of unlabeled cytidine in extracts, a better resolution of the 2',3'-mononucleotides resulting from alkaline hydrolysis was obtained by isocratic elution with 1 M buffer at 2 ml/min.

Determination of Uridine and Cytidine in Plasma. Plasma from heparinized blood was mixed with an equal volume of ice-cold 1 M HClO4 and kept at 4° for 20 min. The precipitated protein was removed, and the supernatant was neutralized to pH 7 to 9 at 0° with 10 N KOH.

One to 2 ml of the extracts were prechromatographed on 1 ml of borate affinity resin (5). The column was washed with 4 ml of 0.3 M ammonium acetate, pH 8.8, and eluted with 3 portions (1.5, 2, and 2 ml) of 0.1 N formic acid. The last 2 fractions, which contained the nucleosides, were lyophilized and reconstituted in 0.3 M ammonium acetate buffer for chromatography as described above. The overall recovery of uridine or cytidine determined by addition of internal standards of radiolabeled nucleoside was 92 ± 10% (S.D.) for uridine and 90 ± 6% for cytidine.

Acetylation of Uridine Peak. A derivatization procedure was performed to confirm the structure of the component identified as uridine. The uridine fraction from HPLC of rat plasma was lyophilized, and pyridine (100 µl) and acetic anhydride (50 µl) were added and allowed to react at room temperature for 1 hr to form the triacetate derivative (2). After removal of acetic anhydride and pyridine, the sample was reconstituted in 0.5 ml of 0.3 M sodium acetate (pH 5.0), adsorbed onto a 25-cm Lichrosorb 5-µm particle size C18 reverse-phase column, and eluted with 0.3 M sodium acetate (pH 5.0, 1 ml/min) for 10 min followed by a 15-min gradient to achieve a buffer: methanol mix of 3:1. A uridine standard derivatized as described above was converted to 2',3',5'-triacetyl uridine with 87% recovery; the uridine component obtained from rat plasma was converted in 84% yield.

Stability of [5-3H]Uridine in Blood. Tracer amounts of [5-3H]uridine (3.6 x 105 cpm/ml; 1.3 pmol) were added to blood from BALB/c x DBA/2 F1, (hereafter called CD2F1) mice or male Sprague-Dawley rats along with 10 µl of heparin (1000 units/ml) and incubated at 37° for 10 or 20 min. HClO4 (0.5 M) was added to stop the reaction. The extract was processed and chromatographed on the HAX-4 column as described above, and 1-ml fractions were collected and counted in 10 ml of Formula 963 (New England Nuclear).

Disposition of [5-3H]Uridine and [5-3H]Cytidine in the Rat. Male Sprague-Dawley rats (250 to 350 g) were anesthetized with methoxyflurane, and catheters were placed in the jugular vein and the carotid artery. [5-3H]Uridine (27.9 Ci/mmol) was prepared in sterile 0.9% NaCl solution, and 0.1 mCi was injected via the jugular vein in a volume of 0.2 ml. At the indicated times, 100 µl of blood from the carotid artery were mixed with 400 µl of ice-cold 0.5 M HClO4, and the resulting extract was processed and chromatographed sequentially on the borate affinity resin and on BAX-4 anion-exchange resin as described above. Radioactivity was determined in 10 ml of Formula 963 at 24% efficiency.

Similar experiments were performed with [5-3H]cytidine. The neutralized HClO4 extract of blood (50 to 200 µl) was chromatographed directly on a 25-cm BPAN6 cation-exchange column as described above. Fractions of 3 ml (2 min) were collected, and radioactivity was determined by scintillation spectrometry.

Infusion of [5-3H]Uridine or [5-3H]Cytidine. Catheters containing heparinized (100 units/ml) 0.9% NaCl solution were placed in the carotid artery and left jugular vein of a male Sprague-Dawley rat (250 g) that had been anesthetized with methoxyflurane. The catheters were protected by a spring and attached to swivels to permit sustained infusion in conscious animals. [5-3H]Uridine or [5-3H]cytidine (40 µCi/ml) was infused via the jugular vein at 2.3 ml/hr. Carotid blood was obtained at 30-min intervals for determination of radiolabeled nucleoside. At the end of the 4-hr infusion, tissue samples were frozen in liquid N2. Tissue samples were homogenized with 10 volumes of N HClO4 at 0°, and total acid-soluble uracil and cytosine nucleotides were determined after acid hydrolysis as above. The acid-insoluble fraction was washed 3 times with ice-cold 0.3 M HClO4, and the RNA was hydrolyzed and quantitated by the method of Blobel and Potter (1). The resulting oligonucleotides were further hydrolyzed in 0.5 M KOH at 37° overnight to produce a mixture of 2' and 3'-mononucleotides for chromatography as described above.

RESULTS

Distribution and Stability of Uridine and Cytidine in Blood. The conversion of uridine to uracil was reported to occur in dog blood by Tseng et al. (29). To examine this possibility, whole rat and mouse blood was drawn and assayed for the degradation of uridine using HPLC as described in “Materials and Methods.” In mouse blood incubated at 37°, only 6 and 10% of the uridine was converted to uracil after 10 and 20 min, respectively. In rat blood, 10% degradation of added uridine occurred after 10 min and 18% after 20 min.

Analysis of rat blood adjusted to hematocrit values ranging between 0 and 69% and supplemented with 1 µM [5-3H]uridine showed that the plasma concentration was that predicted for a passive distribution into erythrocytes within 1 min at 37°. The plasma concentration of radioactivity did not change during a 30-min incubation at 37°; thus, erythrocytes do not concentrate uridine in nucleotide form to a significant extent. This is consistent with the low level of pyrimidine nucleotides in erythrocytes and with the lack of uridine kinase and phosphorylase activity reported by Tax et al. (28). Oliver and Paterson (21)
have reported previously that pyrimidine nucleosides rapidly diffuse into erythrocytes without further metabolism.

Similar incubations of [5-3H]cytidine in heparinized rat blood and in plasma were formed at 37° using 10 or 100 µM cytidine. Less than 10% deamination of cytidine could be observed by cation-exchange HPLC whether in blood or plasma after 30 min, and the concentration of cytidine in plasma from whole blood incubations was that predicted for passive diffusion and did not decrease during the incubation.

Determination of Uridine and Cytidine in Plasma. When 3 ml of blood were processed as described in "Materials and Methods," a totally resolved uridine peak was observed (Chart 1). The identity and purity of the UV-absorbing peak were established by several lines of evidence. This plasma component cochromatographs with uridine in the HAX-4 anion-exchange systems as described in "Materials and Methods" without increase of the width at half height and displays the same 280:254-nm absorbance ratio as a uridine standard. This component of rat plasma collected from anion-exchange chromatography shows the correct retention time and 280:254-nm absorbance ratio when rechromatographed on the Lichrosorb reverse-phase system described in "Materials and Methods." Derivatization of the collected peak from the anion exchange with acetic anhydride quantitatively produces a peak which has the retention time and absorbance ratio of 2',3',5'-triacetyl uridine as described in detail in "Materials and Methods."

Cytidine in plasma can also be measured as described in "Materials and Methods" and is well resolved (Chart 2). The component identified as cytidine cochromatographed with a cytidine standard and had an identical 280:254-nm absorbance ratio. In addition, this component was collected from rat plasma and was rechromatographed in 2 additional HPLC systems to confirm its identity. The first system consisted of a 25-x 0.45-cm BPAN6 column at 50° eluted with 0.5 M potassium phosphate, pH 3.0, at 1.0 ml/min. In this system, cytidine and adenosine had retention times of 45 and 42 min, respectively. The second system consisted of a 25-cm ODS-2 reverse-phase column (Whatman, Inc.) that eluted at 50° with a 98.2 mixture of 0.005 M sodium heptanesulfonic acid adjusted to pH 2.2 with phosphoric acid and methanol. At a flow rate of 1.0 ml/min, cytidine and adenosine had retention times of 28 and 48 min, respectively. The collected cytidine fraction of rat plasma quantitatively cochromatographed with a cytidine standard and gave the expected 280:254-nm ratio in both of these systems.

The concentrations of uridine and cytidine observed in rat, mouse, and human plasma, as well as in commercial horse serum, are given in Table 1. Since the horse serum was not...
freshly prepared, the value given here may reflect some degradation during shipment or storage at \(-20^\circ\) but may be of interest to those who use this serum in tissue culture. In all cases, the concentrations observed were in the low micromolar range. The combined concentration of both nucleosides was 3.7 to 5.4 \(\mu M\), but the ratio of uridine to cytidine varied widely and may reflect the relative activity of catabolic and anabolic enzymes in the animals.

Effect of PALA and PF on Circulating Pyrimidine Nucleosides. Since salvage of circulating uridine or cytidine might replete nucleotide pools of tissues blocked in de novo synthesis, the effect of treatment with PALA and PF on these circulating pools was examined. Neither PALA nor PF produced >40\% depletion of cytidine or uridine in Sprague-Dawley rats 24 hr after treatment (Table 2). In CD2F1 mice examined in a similar manner, plasma uridine decreased no more than 20\% relative to control animals 3 or 24 hr after treatment with PALA (500 mg/kg) or PF (100 mg/kg).

Metabolism of Circulating Uridine. The kinetics of removal of uridine from blood was determined after tracer injection of \([5-^3H]\)uridine (Chart 3). Even after 0.7 min, a large percentage of radioactivity in acid extracts of blood was associated with metabolites such as uracil. By 2 min, distribution of total radioactivity was nearly complete, but the concentration of \([5-^3H]\)uridine continues to decrease with a \(t_{1/2}\) of \(-3\) min.

Salvage of Circulating Uridine. Sprague-Dawley rats received infusions for 4 hr with tracer amounts of \([5-^3H]\)uridine by the jugular vein, and a constant level of labeled uridine was achieved within 1 hr in arterial blood even though total circulating radioactivity increased linearly with time (Chart 4). At an infusion rate of 0.092 mCi/hr, the radioactivity associated with uridine was 4.5 \(\pm\) 1.3, 8.4 \(\pm\) 0.3, 9.8 \(\pm\) 0.5, and 12 \(\pm\) 0.8 \(\times\) 10^3 cpm/ml (mean \(\pm\) S.E. for 7 samples taken at 30-min intervals) in 4 rats examined. The plasma concentrations of endogenous uridine in these 4 rats at the conclusion of the infusion average 0.95 \(\mu M\), similar to the value obtained for the larger sample of rats examined for Table 1. The specific activity of circulating uridine was an average of 0.024 Ci/mm for the 4 rats, and from this it can be calculated that, in rats, approximately 95 \(\mu M\) of uridine enter and leave the circulation each day.

In the tissues of these animals, tritiated water was the major metabolite of \([5-^3H]\)uridine (Table 3). Ynger et al. (32) have also reported \(^3\)H_2O as a metabolite of \([5-^3H]\)uridine in the mouse. The lower levels of volatile radioactivity in bone marrow and intestine may reflect the washing these tissues receive
during the workup. Assuming equal distribution throughout body water, \( ^{3}H_{2}O \) comprises \( \approx 70\% \) of the infused dose at 4 hr. Much of the nonvolatile radioactivity found in tissues is not associated with nucleotides and probably represents intermediate degradation products such as \( \beta \)-alanine and \( \beta \)-ureidopropionate. These are ultimately catabolized further, and, at the conclusion of a 24-hr infusion, 85 to 99% of acid-soluble radioactivity in every tissue examined is present as \( ^{3}H_{2}O \). The mean circulating \([5-^3H]\)uridine concentrations obtained in these rats are given in the text. The efficiency of scintillation counting was 37%. The skeletal muscle was a portion of the abdominal wall. The mean circulating \([5-^3H]\)uridine concentrations obtained in these rats are given in the text. The efficiency of scintillation counting was 37%.

### Table 3

Salvage of infused \([5-^3H]\)uridine

Sprague-Dawley rats (~325 g) received infusions via the jugular vein with \([5-^3H]\)uridine at 0.092 mCi/hr as described in “Materials and Methods.” After 4 hr of infusion, the rats were anesthetized with sodium pentobarbital and rapidly dissected. Radioactivity in the acid-soluble and RNA fractions was measured as described in “Materials and Methods.” The skeletal muscle was a portion of the abdominal wall. The mean circulating \([5-^3H]\)uridine concentrations obtained in these rats are given in the text. The efficiency of scintillation counting was 37%.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Total</th>
<th>Uracil nucleotides</th>
<th>Cytosine nucleotides</th>
<th>Volatile</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>470 ± 66</td>
<td>160 ± 21</td>
<td>15 ± 4</td>
<td>210 ± 40</td>
<td>160 ± 38</td>
</tr>
<tr>
<td>Intestine</td>
<td>480 ± 56</td>
<td>84 ± 22</td>
<td>12 ± 2</td>
<td>310 ± 40</td>
<td>57 ± 10</td>
</tr>
<tr>
<td>Kidney</td>
<td>1500 ± 75</td>
<td>310 ± 45</td>
<td>17 ± 4</td>
<td>790 ± 80</td>
<td>52 ± 16</td>
</tr>
<tr>
<td>Liver</td>
<td>1300 ± 130</td>
<td>44 ± 4</td>
<td>10 ± 3</td>
<td>730 ± 120</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>Lung</td>
<td>1100 ± 60</td>
<td>22 ± 50</td>
<td>12 ± 2</td>
<td>660 ± 50</td>
<td>67 ± 10</td>
</tr>
<tr>
<td>Muscle</td>
<td>750 ± 39</td>
<td>10 ± 1</td>
<td>5</td>
<td>670 ± 40</td>
<td>2</td>
</tr>
<tr>
<td>Spleen</td>
<td>1500 ± 160</td>
<td>500 ± 100</td>
<td>26 ± 8</td>
<td>680 ± 50</td>
<td>110 ± 10</td>
</tr>
<tr>
<td>Thymus</td>
<td>650 ± 130</td>
<td>44 ± 14</td>
<td>5</td>
<td>540 ± 300</td>
<td>13 ± 2</td>
</tr>
</tbody>
</table>

\* Mean ± S.E. from 4 rats.

### Table 4

Acid-soluble pyrimidine nucleotide and RNA pyrimidine content of rat tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cytosine nucleotides (µmol/g)</th>
<th>Uracil nucleotides (µmol/g)</th>
<th>RNA pyrimidine (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>0.11 ± 0.005*</td>
<td>0.50 ± 0.04</td>
<td>7.8</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.13 ± 0.006</td>
<td>0.78 ± 0.02</td>
<td>6.4</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.12 ± 0.0007</td>
<td>0.75 ± 0.02</td>
<td>5.4</td>
</tr>
<tr>
<td>Liver</td>
<td>0.12 ± 0.01</td>
<td>1.4 ± 0.1</td>
<td>10.0</td>
</tr>
<tr>
<td>Lung</td>
<td>0.12 ± 0.007</td>
<td>0.52 ± 0.04</td>
<td>3.8</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.05 ± 0.001</td>
<td>0.21 ± 0.05</td>
<td>1.8</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.20 ± 0.01</td>
<td>0.82 ± 0.04</td>
<td>9.1</td>
</tr>
</tbody>
</table>

\* Mean ± S.E. for tissues from 7 rats.

Minimal activity. In general, the degree of incorporation into RNA was a reflection of the incorporation into acid-soluble nucleotides. In the bone marrow and intestine, both of which contain a significant proportion of dividing cells, the amount of label in RNA equaled that in acid-soluble pools after 4 hr of infusion, whereas, in all other tissues, the fraction of radioactivity incorporated into RNA was much smaller.

**Metabolism of Circulating \([5-^3H]\)Cytidine.** As a preliminary to infusion of \([5-^3H]\)cytidine, the clearance of a single bolus i.v. injection of \([5-^3H]\)cytidine was examined (Chart 5). In contrast to uridine, clearance is slower and produces much smaller amounts of catabolites. Both total radioactivity and \([5-^3H]\)cytidine continue to decrease throughout the 60-min course of the experiment. Although the turnover of cytidine \( (t_{1/2} \sim 20 \text{ min}) \) appeared slower than that of uridine \( (t_{1/2} \sim 3 \text{ min}) \), it was sufficiently rapid to attain a steady state in subsequent infusion experiments.

**Salvage of Circulating Cytidine.** To estimate the degree of salvage of endogenous cytidine from the circulation, a steady-state specific activity was established by constant infusion of \([5-^3H]\)cytidine. The concentrations of total radioactivity and tritiated cytidine in blood obtained by this procedure are shown in Chart 6. After 30 min, the concentration of \([5-^3H]\)cytidine was somewhat lower than at subsequent times, but between 1 and 4 hr, the concentration of \([5-^3H]\)cytidine was nearly constant, while the blood concentration of total radioactivity increased about 2-fold. In 3 experiments, the steady-state blood concentrations of \([5-^3H]\)cytidine were \( 91 \pm 6 \times 10^{3}, 120 \pm 7 \times 10^{3} \), and \( 120 \pm 7 \times 10^{3} \text{ cpm/ml} \) (mean ± S.E., \( n = 7 \) samples) at an infusion rate of 0.092 mCi/hr. This radioactivity can be considered a tracer amount since it represents a concentration of \( 5 \times 10^{-8} \text{ M} \) \([5-^3H]\)cytidine, and plasma concentration averaged 3.3 µM. Thus, the specific activity was about 0.04 Ci/µmol, and based on steady-state calculations, 50 µmol of cytidine transverse the circulation each day. The 10-fold-higher blood concentration of \([5-^3H]\)cytidine obtained in comparison with that achieved on infusion of \([5-^3H]\)uridine is consistent with the slower clearance of cytidine seen in the experiments using a single injection.

Substantial salvage of endogenous cytidine by conversion to nucleotides and incorporation into RNA was seen after 4 hr (Table 5). In all tissues except muscle, the nucleotides and RNA comprised \( \geq 65\% \) of the total radioactivity in the tissue.
The remaining acid-soluble radioactivity was found in the void volume of the chromatographic system used for analysis of UMP and CMP. More radioactivity was present in cytosine nucleotides than in uracil nucleotides except in the liver, although both pools showed incorporation of tritium. Most notable is the >7-fold-greater labeling of RNA by cytidine compared to uridine. The more efficient incorporation of [5-3H]cytidine into RNA recommends it for use in estimating RNA synthesis in vivo.

In tissues other than the kidney and liver, the radioactivity present in RNA exceeded that in acid-soluble nucleotides. This suggests that these pools turn over in less than 4 hr. Calculations from the data of Tables 4 and 5 reveal that, in no case, however, does the specific activity of the cytosine nucleotide pool exceed 0.008 Ci/mmol. Since the specific activity of circulating cytidine during the infusion was 0.045 Ci/mmol, no more than 18% of the cytosine nucleotide pool in any tissue was derived from circulation during the 4-hr course of the infusion. In bone marrow, spleen, and thymus, comparison of the specific activity of the cytosine nucleotide pool to that of the circulating cytidine reveals that approximately 7% of this pool was derived from the circulation in 4 hr.

From the specific activity of circulating uridine and cytidine during the infusions and the incorporation of radioactivity into acid-soluble nucleotides and nucleic acids, it is possible to estimate the contribution of circulating pyrimidine nucleosides to the nucleotide pool and RNA of each tissue (Table 6). Since the precise pyrimidine nucleotide requirement of a tissue has never been established, direct calculation of the percentage of contribution of circulating pyrimidines to the total requirement is not possible. However, the relationship of this amount to the turnover of RNA pyrimidines in a nondividing tissue or the minimum amount required to double RNA and acid-soluble pyrimidine nucleotide pools in a rapidly growing tumor can be estimated. In the liver, it has been reported that the pyrimidines of RNA turn over with a t₁ of ~5 days (1). Since this tissue contains 7 mg of RNA per g and since 45% of this is pyrimidine ribonucleotide by weight, the daily requirement would be 1.2 μmol/g/day or about 3 times the calculated daily contribution from circulating uridine and cytidine.

### Table 5

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Acid soluble (cpm/g x 10⁻³)</th>
<th>RNA (cpm/g x 10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Uracil nucleotides</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>670 ± 60</td>
<td>65 ± 21</td>
</tr>
<tr>
<td>Intestine</td>
<td>780 ± 20</td>
<td>100 ± 17</td>
</tr>
<tr>
<td>Kidney</td>
<td>2700 ± 40</td>
<td>610 ± 100</td>
</tr>
<tr>
<td>Liver</td>
<td>1800 ± 300</td>
<td>570 ± 220</td>
</tr>
<tr>
<td>Lung</td>
<td>800 ± 80</td>
<td>130 ± 12</td>
</tr>
<tr>
<td>Muscle</td>
<td>330 ± 30</td>
<td>50</td>
</tr>
<tr>
<td>Spleen</td>
<td>1200 ± 30</td>
<td>120 ± 19</td>
</tr>
<tr>
<td>Thymus</td>
<td>730 ± 30</td>
<td>35 ± 9</td>
</tr>
</tbody>
</table>

 Mean ± S.E. from 3 rats.

### Table 6

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Uridine (μmol/g/day)</th>
<th>Cytidine (μmol/g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>0.13±0.02</td>
<td>0.37</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.06</td>
<td>0.22</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.17</td>
<td>0.41</td>
</tr>
<tr>
<td>Liver</td>
<td>0.03</td>
<td>0.37</td>
</tr>
<tr>
<td>Lung</td>
<td>0.13</td>
<td>0.14</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.23</td>
<td>0.29</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.03</td>
<td>0.14</td>
</tr>
</tbody>
</table>

 Mean for 4 uridine or 3 cytidine infusions.

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From the specific activity of circulating uridine and cytidine during the infusions and the incorporation of radioactivity into acid-soluble nucleotides and nucleic acids, it is possible to estimate the contribution of circulating pyrimidine nucleosides to the nucleotide pool and RNA of each tissue (Table 6). Since the precise pyrimidine nucleotide requirement of a tissue has never been established, direct calculation of the percentage of contribution of circulating pyrimidines to the total requirement is not possible. However, the relationship of this amount to the turnover of RNA pyrimidines in a nondividing tissue or the minimum amount required to double RNA and acid-soluble pyrimidine nucleotide pools in a rapidly growing tumor can be estimated. In the liver, it has been reported that the pyrimidines of RNA turn over with a t₁ of ~5 days (1). Since this tissue contains 7 mg of RNA per g and since 45% of this is pyrimidine ribonucleotide by weight, the daily requirement would be 1.2 μmol/g/day or about 3 times the calculated daily contribution from circulating uridine and cytidine.

### Table 5

Salvage of infused [5-3H]cytidine

Sprague-Dawley rats (~250 g) were given infusions via the jugular vein with [5-3H]cytidine at 0.092 mCi/hr as described in "Materials and Methods." At 4 hr, the animal was anesthetized with sodium pentobarbitol and quickly dissected. Radioactivity in the acid-soluble and RNA fractions was measured as described in "Materials and Methods." The musculature was a portion of the abdominal wall. The mean blood levels of radiolabeled cytidine obtained in these 3 rats are shown in Chart 6.

### Table 6

Contribution of circulating pyrimidine nucleosides to the nucleotide pools and RNA pyrimidines of rat tissues

The radioactivity in pyrimidine nucleotides and RNA was converted to pmol by use of the specific activity of circulating uridine and cytidine obtained in the infusion.

### Chart 5

Metabolism of circulating cytidine. Sprague-Dawley rats (~250 g) received 0.15 mCi of [5-3H]cytidine by bolus injection into the jugular vein. Blood samples (100 μl) were drawn from the carotid artery at the indicated times, and total radioactivity and [5-3H]cytidine were measured in the acid-soluble extract as described in "Materials and Methods." Data from 3 experiments are combined. Points, mean for 3 experiments; bars, S.E.
Blood flow has been measured in a number of experimental tumors of rats by Gullino and Grantham (7), who found that these tumors are much less well vascularized than liver. At an average blood flow of 10 ml/hr/g, only 1 µmol of pyrimidine per g could be supplied per day assuming 100% clearance and conversion to nucleotide. We calculate that this is far less than the estimated 10 µmol/g/day required for those tumors to grow with the doubling times (<2 days) observed (7).

**DISCUSSION**

Enzymes and transport systems for utilization of extracellular nucleosides have been demonstrated in numerous cells and tissues. However, very few studies have examined the dynamics of salvage of nucleic acid precursors in vivo (11, 15, 17). The current study examines the concentration of circulating pyrimidine nucleosides available for salvage, the turnover of these nucleosides, and their conversion to nucleotides by selected tissues of the rat.

The analytical method described here, together with the rigorous confirmation of the identity of the nucleosides measured, has established that low micromolar concentrations of uridine and cytidine are available for salvage from the plasma (Table 1). The uridine concentrations reported here are similar to those reported earlier for sheep plasma (2 µM, Ref. 8) and dog plasma (0.4 µM, Ref. 25) as determined by a multistep separation by thin-layer chromatography. The uridine concentration found in human plasma is slightly higher than that reported for human serum by Hartwick et al. (3.2 µM, Ref. 10). One earlier report had suggested a much higher concentration of uridine in rat plasma (32 µM, Ref. 25), perhaps because of incomplete resolution from another UV-absorbing component. In addition, the cytidine concentration was 10 µM, rather higher than that found in this study. The reason for this discrepancy is not clear, although different extraction procedures were used, and the rats examined in Ref. 25 were female. It is possible that nutritional factors may influence the circulating levels, particularly since Tseng et al. (29) have shown that the concentration of uridine rises several fold in dogs fed 2 pounds of meat. Obviously, meat would be a food high in nucleic acid compared to commercial animal chow. The influence of diet is unclear and requires further study. On the other hand, a recent study by Sonoda and Masamiti (27) indicates very poor utilization of 14C-labeled RNA fed to mice except by the intestinal mucosa.

The kinetics of uridine clearance after i.v. injection (Charts 3 and 5) confirms an earlier report (4), which showed an extremely rapid clearance of a single injection of this nucleoside but a slower clearance of cytidine in the rat. The current infusion experiments using an infusion of radiolabeled uridine or cytidine to steady-state levels reveal that approximately 140 µmol of pyrimidine nucleosides enter the circulation of a 300-g rat in a day. Earlier studies by Tseng et al. (29) using different techniques had indicated that a similar rapid flux of uridine and cytidine occurred in the circulation of the dog. The current infusion experiments have also permitted measurement of nucleoside salvage from the circulation.

In addition to providing an estimate of the daily contribution of blood-borne pyrimidine nucleosides to tissue nucleotide pools (Table 6), the data of Tables 3 and 5 allow several qualitative statements. (a) Greater than 70% of the uridine that enters the plasma pool in the rat is catabolized rather than salvaged. (b) Although cytidine turnover is less rapid, the circulating cytidine is more efficiently converted to tissue nucleotides. This fact, together with the differences in nucleotide pool sizes (Table 4), accounts for the observation of Hammarsten et al. (9) that cytidine was much more effective in labeling RNA in vivo than was uridine. The difference in salvage of cytidine compared to uridine by the rat may not be seen in other species, since the rat is notably deficient in cytidine deaminase but has somewhat higher uridine phosphorylase activity relative to other mammals (3, 24).

The failure of PF or PALA to substantially deplete plasma concentrations suggests that the source is not very sensitive to these inhibitors of pyrimidine synthesis de novo. Recently, a determination of circulating uridine concentrations in patients treated with PALA was reported to be slightly higher than that reported here, but relatively little change was observed after a 5-day course of therapy (14), consistent with the inability of PALA to decrease circulating levels in mice or rats given toxic doses of this inhibitor of de novo synthesis.

The source of circulating nucleotides is currently not known. The diet, intestinal flora, or "feeder" organs are all possibilities. Possibly, constant leakage from acid-soluble nucleotide pools in a wide range of tissues occurs at a rate influenced by the concentration of monophosphate and the relative activities of phosphorylating (kinase) and dephosphorylating enzymes. Recently cultured cells have been shown to exclude small amounts of uridine, cytidine, and pseudouridine into the medium, generating micromolar concentrations, particularly in the plateau phase (30). Ongoing studies in this laboratory are directed toward understanding the factors controlling the concentrations and flux of circulating pyrimidine nucleosides, with particular focus on the essentially complete clearance of uridine in the portal vein by the liver and its replacement by uridine derived from de novo synthesis (5).

Although only a small fraction of the pyrimidine nucleotide pools in tissues is derived from the circulation, during chemotherapy with inhibitors of pyrimidine synthesis de novo, this may change markedly. The failure of PALA or PF to greatly deplete circulating levels in treated animals suggests that salvage should be considered as an ongoing process during therapy. Further studies on salvage by tumors both sensitive and resistant to PALA and PF are required for a more complete understanding of the role of nucleoside salvage. Similar studies extending this work to the salvage of thymidine and deoxycytidine would be essential in experiments with the several chemotherapeutic drugs that inhibit the production of the corresponding deoxynucleotides.

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Salvage of Circulating Pyrimidine Nucleosides in the Rat

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