RNA Degradation in Perfused Rat Liver as Determined from the Release of \([^{14}C]\)Cytidine*

Bernard R. Lardeux, Stanley J. Heydrick, and Glenn E. Mortimore
From the Department of Physiology, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033

The degradation of RNA in the cyclically perfused rat liver was determined from the release of labeled cytidine from RNA that had been previously labeled with \([6-{^{14}}C]\)orotic acid in vivo. Because cytidine is not appreciably degraded in rat liver (its deamination to uridine is virtually nil) or produced in significant amounts from free 5'-nucleotides, its release will directly reflect net RNA breakdown. This conclusion was substantiated by the fact that the specific radioactivity of released cytidine equaled that of CMP in RNA and remained unchanged for 180 min of perfusion. The initial rate of \([^{14}C]\)cytidine accumulation was slow, but after 10–20 min it increased abruptly by more than 4-fold and remained virtually constant. The addition of 0.5 mM unlabeled cytidine effectively prevented the reutilization of label and increased the rate of labeled cytidine release by an amount representing 13% of the maximal rate of cytidine accumulation. Rates of RNA degradation, measured between 20 and 60 min in the presence of 0.5 mM unlabeled cytidine, averaged 1.00 ± 0.05 mg h\(^{-1}\) liver\(^{-1}\) (100-g rat), the equivalent of 65% of total RNA per day. This accelerated value, which was about 4-fold larger than the initial rate, is believed to be the direct consequence of amino acid deprivation since, in separate experiments, the increase was completely suppressed by the addition of plasma amino acids (Lardeux, B. R., and Mortimore, G. E. (1987) J. Biol. Chem. 262, 14514–14519). These findings demonstrate the potential value of cytidine as a marker for following moment-to-moment regulatory alterations in RNA degradation in the isolated liver or hepatocyte preparation.

Current methods for determining RNA degradation employ either the fall in the specific radioactivity of RNA as a measure of its turnover under steady state conditions (1–5) or the difference between ribosomal RNA synthesis and the net change of cellular RNA (6). These procedures possess at least two inherent disadvantages, (i) it is not possible to assess the reutilization of label for RNA synthesis and (ii) since a time base of several days is needed for a single rate determi-

* This work was supported by United States Public Health Service Grant DK-21624 (to G. E. M.) and funding from Centre National de la Recherche Scientifique (France) (to B. R. L.). A preliminary report was presented at the 69th annual meeting of the Federation of American Societies for Experimental Biology, Anaheim, CA, April 21–26, 1985. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** Present address: Laboratoire de Biologie Cellulaire, Faculté de Médecine, Xavier Bichat, 16, Rue Henri Hochard, 75018 Paris, France.

** Portions of this paper (including "Materials and Methods," Table 1, and Figs. 2 and 5–8) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 87M-1163, cite the authors, and include a check or money order for $4.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

(Received for publication, April 15, 1987)
material as determined by its binding to Dowex 50. The release, which was initially slow, increased abruptly after 10-20 min. Thereafter, the accumulation was constant except for a slight decrease at 90 min. As shown in Fig. 4A, almost all cationic label was eluted as a single band with a retention time equal to that of cytidine. We have confirmed the identity of this peak as cytidine by several analytical procedures (see Miniprint Section), including anion exchange chromatography.

**Fig. 1.** Scheme of pyrimidine metabolism in liver showing pathways relating to RNA metabolism. The broken arrows indicate reactions catalyzed by cytidine deaminase, the activity of which is virtually absent in rat liver. The catabolism of β-alanine is not shown; as mentioned in the text, this species is rapidly oxidized to ammonia, CO₂, and acetyl-CoA.

**Fig. 3.** Cumulative release of radioactivity from livers previously labeled with orotic acid in vivo. Livers from nonfasted rats that had received [6-¹⁴C]orotic acid as in Fig. 2 were cyclically perfused for the determination of released label as follows: total plasma radioactivity, ○—○; plasma cationic radioactivity eluted from Dowex 50 as described under “Materials and Methods,” ⌂—○; cationic radioactivity released from liver in the presence of 0.5 mM unlabeled cytidine, △—△; the effect of 0.5 mM unlabeled cytidine, ↓—△. Values are means of five experiments.

**Fig. 4.** Polyacrylamide gel chromatography of labeled cationic material released from orotic acid-labeled livers. Livers were labeled as in Fig. 2 and cyclically perfused for 180 min. Final samples of plasma were applied to columns of Dowex 50 and eluted; the eluates then were rechromatographed on Bio-Gel P-2 (see “Materials and Methods”). Fractions were collected every minute and counted for radioactivity. Elution times for cytidine (Cyd), cytosine (Cyt), and β-alanine (β-Ala) were previously established with authentic standards. A, no additions to the perfusion medium. B, addition of 0.5 mM unlabeled β-alanine during perfusion.

The absence of labeled β-alanine in the perfusate was unexpected since earlier observations had indicated that its oxidation in liver was relatively slow (29). However, after loading with 10 mM β-alanine, a labeled peak appeared (Fig. 4B), indicating that β-alanine is rapidly oxidized. This was confirmed in experiments in which unlabeled livers were perfused with β-[¹³C]alanine. As seen in Fig. 9, radioactivity completely disappeared from the perfusate plasma within 20 min. Because less than 1% of the initial radioactivity was recovered in the liver after 180 min of perfusion (data not shown), the loss was almost certainly the result of the decarboxylation of β-alanine. The addition of unlabeled β-alanine reduced considerably the catabolism of radioactive β-alanine, but did not suppress it completely. Thus, the use of β-alanine...
as a marker was not considered feasible for studies employing the intact liver. Stabilility of Cytidine—The alternative marker, cytidine, was more promising since it accumulated steadily in the medium over the course of perfusion (Fig. 3). Its conversion to uridine, catalyzed by cytidine deaminase, has been reported to be virtually nonexistent in rat liver (18, 19), as demonstrated by the low rate of cytidine oxidation in this species. This was indirectly assessed in cyclically perfused rat livers from the recovery of radioactivity following a single addition of \([U-14C]\) cytidine. In the absence of unlabeled cytidine, 60% of the label was removed from the perfusate by the liver after 60 min of perfusion (Fig. 10A), and it was distributed equally between the acid-soluble and RNA fractions (Table II). If significant amounts of cytidine had been converted to uridine, the recovery of radioactivity would have been incomplete since uridine is rapidly degraded via \(\beta\)-alanine to radioactive \(\text{CO}_2\) and acetyl-CoA. Some uridine, though, might have been phosphorylated and reincorporated into RNA. However, anion exchange chromatography of 24-h alkaline hydrolysates of RNA indicated that all detectable radioactivity was associated with 3'-CMP (data not shown). These results confirm the expected stability of cytidine in the rat liver, a conclusion supported by findings of Moyer et al. (30) and by Holstege et al. (31).

Reutilization of Cytidine—At its lowest concentration, cytidine is phosphorylated rather efficiently as demonstrated by the relatively high incorporation (56%) of \([1^4C]\)cytidine into nucleotides and RNA (Table II). The addition of 0.5 mM unlabeled cytidine prevented almost completely the disappearance of total radioactivity and labeled cytidine from the perfusate (Fig. 10, A and B) and decreased the incorporation of labeled cytidine to 7.4% of the initial label (Table II). These effects were the result of isotopic dilution at sites of nucleotide and RNA synthesis. Since little additional suppression of radioactive cytidine incorporation was observed when the concentration of unlabeled cytidine was doubled (1.0 mM), the dilution effect at 0.5 mM was considered maximal.

As might be predicted from the foregoing, the addition of 0.5 mM unlabeled cytidine during the cyclic perfusion of livers previously labeled in vivo with \([6-14C]\)orotic acid increased the accumulation of \([14C]\)cytidine in the perfusate plasma as a consequence of the decrease in label reutilization (Fig. 3). No additional effect was observed at 1.0 mM and higher concentrations of unlabeled cytidine (data not shown). The reutilization of labeled cytidine was highly constant over 150 min of perfusion as determined from the difference in \([14C]\)cytidine release in the presence and absence of 0.5 mM cytidine (Fig. 3). The average rate of reutilization was calculated to be 0.17% of the initial liver radioactivity per h, a value representing 13.2% of the maximal rate of labeled cytidine release between 20 and 60 min of perfusion.

Contribution by the Dephosphorylation of Free Nucleotides to Cytidine Release—RNA degradation is the most likely source of the steady accumulation of cytidine that was observed in this study. Nevertheless, owing to the existence of phosphatase activity in liver (32), one cannot totally exclude the possibility that a significant proportion was generated at the expense of free nucleotides in the cell. If so, one would expect to see a measurable decrease in the nucleotide pool over the course of perfusion. Results in Table III show that

![Figure 10](https://example.com/fig10.png)
TABLE II

<table>
<thead>
<tr>
<th>Cytidine additions</th>
<th>None</th>
<th>0.5 mM</th>
<th>1.0 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>dpm × 10⁻³/liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perfusion plasma</td>
<td>111.5 ± 15.8</td>
<td>236.8 ± 2.6</td>
<td>239.9 ± 3.2</td>
</tr>
<tr>
<td>Total liver</td>
<td>165.0 ± 9.3</td>
<td>49.4 ± 2.4</td>
<td>43.9 ± 2.7</td>
</tr>
<tr>
<td>Sum</td>
<td>276.4 ± 15.8</td>
<td>286.2 ± 2.9</td>
<td>282.9 ± 2.5</td>
</tr>
<tr>
<td>Percent recovery (A)</td>
<td>99.7 ± 5.7</td>
<td>103.7 ± 1.1</td>
<td>102.1 ± 0.9</td>
</tr>
<tr>
<td>RNA</td>
<td>78.2 ± 5.0</td>
<td>6.2 ± 0.2</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>Acid-soluble nucleotides</td>
<td>68.7 ± 4.5</td>
<td>14.3 ± 0.9</td>
<td>12.0 ± 0.5</td>
</tr>
<tr>
<td>Liver cytidine</td>
<td>11.6 ± 2.6</td>
<td>30.3 ± 2.4</td>
<td>26.1 ± 1.0</td>
</tr>
<tr>
<td>Sum</td>
<td>158.6 ± 9.8</td>
<td>50.7 ± 3.3</td>
<td>42.0 ± 1.5</td>
</tr>
<tr>
<td>Percent recovery (B)</td>
<td>96.8 ± 2.1</td>
<td>102.4 ± 3.2</td>
<td>96.4 ± 3.0</td>
</tr>
</tbody>
</table>

TABLE III

Stability of the acid-soluble nucleotide pool during liver perfusion

Livers from rats previously labeled with 15 μCi of [6-¹⁴C]orotic acid were cyclically perfused for 180 min in the presence of 0.5 mM unlabeled cytidine; plasma cytidine was extracted as in Fig. 3A. Acid-soluble nucleotide radioactivity was determined at the beginning (from the excised caudate lobe) and at the end of perfusion as described under "Materials and Methods." The radioactivity distribution ratio: caudate lobe/remaining liver was 1.13 ± 0.04 (n = 12), and the data were corrected accordingly. Values are means ± S.E. of five to six perfusions.

<table>
<thead>
<tr>
<th>Labeled fraction</th>
<th>Initial (A)</th>
<th>Final (B)</th>
<th>A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleotide pool</td>
<td>149.1 ± 12.4</td>
<td>161.3 ± 11.3</td>
<td>1.102 ± 0.052</td>
</tr>
<tr>
<td>Cytidine release</td>
<td>0</td>
<td>155.1 ± 13.8</td>
<td></td>
</tr>
</tbody>
</table>

TABLE IV

Comparison of the specific radioactivities of 3'-CMP residues in RNA and cytidine released from livers previously labeled with orotic acid in vivo

Livers were cyclically perfused 60 h after three daily intraperitoneal injections of [6-¹⁴C]orotic acid (A) or 16 h after a single injection of the label (B); the doses were the same as those in Table I. No additions were made to the medium. After 180 min, plasma cytidine was extracted as in Fig. 3A, and the specific radioactivity was determined by C18 reversed-phase HPLC. Similar measurements of 3'-CMP residues from RNA were carried out with anion exchange chromatography. The specific radioactivity ratio: Cyt/3'-CMP was computed for each perfusion. Values are means ± S.E. for five to six experiments.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Specific radioactivity</th>
<th>Specific activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytidine</td>
<td>3'-CMP (Cyt/3'-CMP)</td>
</tr>
<tr>
<td>A</td>
<td>55.7 ± 2.2</td>
<td>54.2 ± 2.3</td>
</tr>
<tr>
<td>B</td>
<td>140.2 ± 12.7</td>
<td>147.0 ± 14.4</td>
</tr>
</tbody>
</table>

Because cytidine is neither deaminated in liver nor produced in significant amounts by the depletion of pre-existing nucleotides, we can conclude that the release of [¹⁴C]cytidine in the presence of 0.5 mM unlabeled cytidine directly reflects the breakdown of RNA. The fact that the specific radioactivity of cytidine in perfusate plasma equals that of 3'-CMP in RNA (Table IV) and is constant over the course of perfusion (data not shown) suggests that cytidine is randomly lost from RNA during the breakdown process. These findings are of additional interest in that they make it possible to calculate rates of RNA degradation in absolute terms.

Rates of RNA Degradation Induced by Amino Acid Depetration in the Cyclically Perfused Rat Liver—The net accumulation of cytidine induced by amino acid deprivation during 180 min of cyclic perfusion was calculated by dividing the total radioactivity in cytidine by the specific activity of 3'-CMP residues in RNA. The results of these calculations are presented in Fig. 11. The mean rate of cytidine release during the initial 10-min period was 3.6 ± 0.3 nmol min⁻¹ liver⁻¹ (100-g rat). Between 20 and 60 min, the rate increased abruptly by more than 4-fold, averaging 14.5 ± 0.7 nmol min⁻¹ per liver. Thereafter, the rate decreased until a new steady state, 11.1 ± 0.5 nmol min⁻¹, was reached between 90 and 180 min of perfusion.

For the purpose of calculating absolute rates of RNA degradation, we determined the amount of CMP in liver RNA and obtained a mean of 0.87 ± 0.01 μmol per mg of RNA for five samples. This value agreed closely with results of Loeb et al. (33) who reported 0.86 and 0.88 μmol per mg of RNA from free ribosomes and membrane-bound ribosomes, respectively. Values obtained from the primary structure or sequence of 5.8 S (34), 18 S (35), and 28 S RNA (36) were slightly higher.
Cytidine Release as Marker of RNA Degradation

0.91 \mu mol/mg, while the determinations of De Lamirande et al. (37), based on total liver RNA, were 0.95 \mu mol/mg RNA.

Taking our value of 0.97 \mu mol/mg for the quantity of CMP in RNA, we have calculated absolute and fractional rates of RNA degradation from the rate of cytidine release. During cyclic perfusions with the basic medium, rat livers exhibited high rates of RNA degradation, giving absolute and fractional rates averaging, respectively, 1.00 \pm 0.15 \mathrm{mg}^{-1} \mathrm{h}^{-1} (100-g rat) and 64.8 \pm 2.6\% per day between 20 and 60 min. This accelerated value, which was about 4-fold larger than the initial rate, appeared to be directly related to amino acid deprivation since the increase was strongly inhibited by the addition of amino acids to the perfusate (38). A similar acceleration was observed in vivo during short-term starvation (39, 40).

The initial 10-20-min delay in the accelerated release of cytidine is similar to that previously reported for the release of valine in protein breakdown (20). If RNA is degraded largely within macroautophagic vacuoles as findings elsewhere suggest (38), then the delay could be explained by the time required to transform newly formed autophagosomes into digestive autophagic vacuoles (14). In fractional terms, the rate of RNA degradation in the first 10-20 min extrapolates to 16.3 \pm 1.4\% day^{-1}. This value is in close agreement with the majority of estimates in vivo, which range from 11.2 to 16.8\% day^{-1} (1-4, 22, 40, 42). It is thus possible that the rate of cytidine release during this initial period reflects RNA breakdown occurring in vivo.

The slight decrease in the rate of [6-\mathrm{H}]cytidine release observed after 90 min of perfusion is also consistent with these observations since leucine, a strong inhibitor of autophagy, is known to increase steadily in the medium during cyclic perfusion (41). The regulation and mechanism of RNA degradation will be dealt with in the accompanying paper (38). It should be pointed out that some RNA is likely to be broken down by cytosolic ribonucleases to 5'nucleoside monophosphates (see Fig. 1). Even in the presence of unlabeled cytidine, the resulting 5'-CMP may be rapidly rephosphorylated to CDP and CTP with little or no production of cytidine, leading to an underestimation of RNA degradation. However, the production of 5'-CMP in the cytoplasm appears to be small compared to that of 3'-CMP (and ultimately cytidine), since Lardeux et al. report a close agreement between measured changes in total liver RNA and those predicted from the difference between RNA synthesis and degradation.

Acknowledgments—We express our thanks to John J. Wert, Jr., Catherine E. Adams, and Dean Stossel for their excellent technical assistance, to Dr. Reidar Wallin for HPLC facilities, and to Maxine L. Gerberich and Connie Marko for typing the manuscript.

REFERENCES


Continued on next page.
Cytidine Release as Marker of RNA Degradation

SUPPLEMENTAL MATERIAL TO RNA Degradation in Eubacteria as a Marker of RNA Degradation from the End of the Last Injection

BY DONALD R. LINDSAY, ERIC J. HEITLING, AND JEREMIAH W. MORTON

MATERIALS AND METHODS

Holdahl-Malz media of the Lewis strain (Rb. Biochemicals, Madison, Wis.) was used as a culture medium. The cultures were grown in a 20:1 medium:water ratio in a 100-ml Erlenmeyer flask at 37°C in a shaking water bath. The cells were harvested by centrifugation and resuspended in 0.1 M sodium phosphate buffer, pH 7.4. After a 2-hour preincubation at 37°C, the cells were exposed to 1% sodium dodecyl sulfate (SDS) for 10 minutes, and the cell suspensions were then centrifuged at 10,000 g for 10 minutes. The supernatant was then collected and used for the measurement of cytidine release.

For the measurement of cytidine release, the supernatant was mixed with an equal volume of 0.1 M sodium phosphate buffer, pH 7.4, and heated at 100°C for 5 minutes. After cooling, the samples were analyzed for cytidine content using high-performance liquid chromatography (HPLC).

The results were expressed as the percentage of cytidine released relative to the total cytidine content of the cell suspension before heating.

TABLE 1

<table>
<thead>
<tr>
<th>Number of Injections</th>
<th>Time after last injection</th>
<th>Acidity</th>
<th>DNA</th>
<th>Recovery, %</th>
<th>Protein</th>
<th>DNA - Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (23)</td>
<td>18</td>
<td>22.4 ± 0.7</td>
<td>76.5 ± 0.3</td>
<td>1.9 ± 0.1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>2 (76)</td>
<td>60</td>
<td>4.2 ± 0.1</td>
<td>92.0 ± 0.2</td>
<td>1.9 ± 0.1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3 (7)</td>
<td>108</td>
<td>4.2 ± 0.1</td>
<td>92.0 ± 0.2</td>
<td>3.2 ± 0.6</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Fig. 2. Separation of nucleotides from RNA hydrolases by ion exchange chromatography. The reaction mixture (500 ml) was passed through a column of DEAE Sephadex A-50 equilibrated with 0.02 M Na citrate. The column was then eluted with a linear gradient of NaCl in 0.02 M Na citrate. The fractions containing RNA hydrolases were identified by the presence of activity after treatment with 0.02 M Na citrate. The fractions were collected and assayed for RNA hydrolase activity. The results are expressed as the percentage of the total activity recovered in each fraction. The data are representative of three separate experiments.
Cytidine Release as Marker of RNA Degradation

Fig. 1. Separation of cytidine from perfusate plasma by anion exchange chromatography.
Cytidine in the first peak of Fig. 1 was concentrated by pooling the fractions over 2000 as described in "Materials and Methods." After elution the material was taken to dryness and then dissolved in 2N HCl-1 N NaCl buffer, pH 2.2. The separation of cytidine from other nucleosides was performed by anion exchange chromatography using Bio-Rex 70.2

Fig. 2. Effect of acid hydrolysis on the positive peak of radioactive cytidine isolated from perfusate plasma.
The first peak of radioactivity (-----) represents the positive cytidine fraction that was eluted from Bio-Rex 7-2 in Fig. 1. Aliquots of this fraction were hydrolyzed in 0.3 N HCl for 1 and 10 mins at 100°C, respectively, followed by HCl-NaCl pH 8.7, and finally passed through a Bio-Rex 7-2 column as described in "Materials and Methods." (-----).

Fig. 3. UV spectral analysis of plasma cytidine.
A 100 ml sample of the positive cytidine peak, prepared as described in Fig. 1, was mixed with 20 ml of 10 N HCl and incubated at pH 2.2. (-----) at pH 8.2 (-----) and at pH 5.6 (-----). Absorptions were measured from 200 to 300 nm and expressed as a percentage of maximal. At pH 2.2, absorbance ratios at 260/290, 260/280, and 260/250 were 1.34, 1.25, and 1.92, respectively, for plasma-derived cytidine. For cytidine standard the equivalence values were 0.82, 0.55, and 1.50.

Fig. 4. Relative accumulation of cytidine in perfusate plasma as determined by reversed phase HPLC.
Livers previously labeled with [14C]-cytidine acid were perfused for 180 min. perfusate samples were taken at intervals for the chromatographic determination of cytidine. Plasma was passed through a reversed-phase column (Waters S7) equilibrated with monobasic potassium phosphate, pH 5.6, and a 20 A aliquot injected on a reversed-phase column (Waters S7, short-throw, as described in "Materials and Methods." The elution times of uridylic acid, cytidine, and cytidine were measured with a mixture of these standards at 10 min each per 20 A.

14513