Rates of rat liver RNA degradation *in vivo* as determined from cytidine release during brief cyclic perfusion *in situ*

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The breakdown of RNA and of long-lived proteins in rat liver is believed to occur largely within the lysosomal–vacuolar system. Both processes are induced by amino acid lack and suppressed by insulin, and in all circumstances a consistent lag of 15–20 min was observed between the introduction of a physiological regulator and onset of the degradative response. This lag has allowed us to determine rates of liver RNA degradation *in vivo* during brief cyclic perfusions, as was done previously for long-lived-protein breakdown [Hutson & Mortimore (1982) *J. Biol. Chem.* **257**, 9548–9554]. Degradation was measured from the release of [¹⁴C]cytidine in livers of rats previously labelled *in vivo* with [6-¹⁴C]orotic acid. Release was linear and unaffected by physiological regulators between 2 and 12 min of perfusion. In contrast with protein breakdown, no short-lived component was observed. In animals trained to feed between 16:00 and 20:00 h, the content of liver RNA fell at an average rate of 0.26 mg/h per 100 g initial body wt. between 07:00 and 16:00 h, a loss that was within 9% of that predicted from the net release (total release minus reutilization) of cytidine *in vivo*. In addition, the total rate of RNA degradation determined at the end of the meal was only 12% of that at the start of the post-absorptive period 14 h later (2.1 versus 17.1%/day). This finding is fully consistent with a lysosomal mechanism for RNA degradation, since autophagy is strongly suppressed by food intake. This approach provides a comparatively simple means of approximating moment-to-moment rates of RNA degradation in the rat liver *in vivo*.

INTRODUCTION

The RNA content of liver is not constant; it increases and decreases rather strikingly in parallel with the supply of nutrients (Kosterlitz & Cramb, 1943; McNurlan *et al.*, 1979; Conde & Franze-Fernández, 1980). Although alterations in the rate of RNA synthesis are indispensable in RNA homeostasis (Hirsch & Hiatt, 1966; Kawada *et al.*, 1977; Lewis & Winnick, 1979; Conde & Franze-Fernández, 1980; Ashford & Pain, 1986), regulation is also achieved through modifications of the rate of RNA degradation (Enwonwu & Munro, 1970; Stenram & Nordgren, 1970; Kawada *et al.*, 1977; Conde & Franze-Fernández, 1980; Ashford & Pain, 1986).

Early methods for evaluating RNA degradation *in vivo* involved measuring the decay in the specific radioactivity of radioactive RNA over several days (Loeb *et al.*, 1965; Hirsch & Hiatt, 1966; Wilson & Hoagland, 1967; Blobel & Potter, 1968; Enwonwu & Munro, 1970; Nikolov *et al.*, 1983). Such studies, however, were difficult to interpret because of label reutilization. An improved approach, in which the rate of RNA degradation was estimated from the difference between the rate of ribosome synthesis and the change in ribosome content (Conde & Franze-Fernández, 1980; Ashford & Pain, 1986), circumvents the problem of reutilization, but requires a relatively long time base. For this reason the latter procedures would not be useful for measuring acute changes in rate.

More recently, hepatic RNA degradation was measured in the perfused rat liver by monitoring the release of [¹⁴C]cytidine from livers of rats prelabelled *in vivo* with [¹⁴C]orotic acid (Lardeux *et al.*, 1987). Since labelled cytidine was released in easily measurable amounts, derived exclusively from RNA, and neither

degraded nor reutilized in the presence of 0.5 mM unlabelled cytidine, it appeared to be a valid marker. RNA degradation was found to have several features in common with resident-protein degradation, a process believed to be lysosomal in nature (for review see Mortimore & Pösö, 1987). This led to the conclusion that both processes are carried out in the same lysosomal compartment. For example, both are inhibited in parallel by amino acids and insulin (Lardeux & Mortimore, 1987), and both are suppressed by the lysosomotropic agent chloroquine (Sameshima *et al.*, 1981; Lardeux & Mortimore, 1987). In addition, degradable pools of each constituent have been identified in hepatocyte lysosomes (Mortimore & Ward, 1981; S. J. Heydrick, B. R. Lardeux & G. E. Mortimore, unpublished work).

In the perfused liver, a 15–20 min lag generally occurs between the introduction of a physiological regulator of lysosomal function and the autophagic response to that regulatory agent (Khairallah & Mortimore, 1976; Hutson *et al.*, 1982; Lardeux *et al.*, 1987). In a previous study, we took advantage of this delayed response by determining rates of proteolysis *in vivo* from the accumulation of valine during the first 15 min of cyclic perfusion (Hutson *et al.*, 1982). The aim of the present study is to use similar brief cyclic perfusions to determine the rate of RNA degradation *in vivo* from the accumulation of labelled cytidine during similar short cyclic perfusions.

EXPERIMENTAL

Animals and RNA labelling

Male rats of the Lewis strain from Microbiological Associates (Walkersville, MD, U.S.A.), weighing 120–

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140 g at the time of perfusion, were maintained under controlled lighting (off 19:00 to 07:00 h) and allowed to drink water *ad libitum*. Two feeding regimens were used. One group was given normal commercial laboratory chow *ad libitum* (normal-fed). The other group was fed on a powdered synthetic diet between 16:00 and 20:00 h (meal-fed) for 2 weeks before perfusion. The synthetic diet was essentially the same as that reported by Altman & Dittmer (1972), except for the following modifications: 35% casein, 37% corn starch, 15% sucrose and 8% corn oil. Growth rates were within 85% of those of normal-fed animals. Perfusions of livers from normal-fed rats were started at 10:00 h. Perfusions of livers from meal-fed rats were started either immediately after eating (20:00 h) or 14 h later (10:00 h). Livers were perfused 60 h after the last of three daily intraperitoneal injections of 5–7 μ Ci of [6- 14 C]orotic acid (45–60 mCi/mmol; Research Products International, Mount Prospect, IL, U.S.A.) as previously described (Lardeux *et al.*, 1987). Additional groups of six unlabelled meal-fed rats were killed every 3 h during the postabsorptive period (07:00–16:00 h) in order to determine changes in liver RNA content during this time.

Liver perfusion

Livers were cyclically perfused *in situ* (Mortimore & Mondon, 1970; Mortimore *et al.*, 1972) at a flow rate of 10 ml/min. The perfusion medium was composed of Krebs–Ringer bicarbonate buffer, pH 7.4, containing 4% (w/v) bovine serum albumin (Pentex, fraction V; Miles Laboratories, Naperville, IL, U.S.A.) and 0.27% (v/v) washed bovine erythrocytes (Schworer & Mortimore, 1979). Unlabelled cytidine (0.5 mM) was added in the perfusate to prevent the reutilization of radioactive cytidine, except as indicated in Table 1. Other additions to the perfusate are indicated in the Figure legends. After 45 s washout, the perfusate was recycled and samples were taken every 2 min between 2 and 12 min for the measurement of labelled cytidine release. At the end of perfusion, livers were quickly excised and frozen between aluminium blocks previously cooled in liquid nitrogen.

Determination of unidirectional and net rates of RNA degradation

Unidirectional rates of RNA degradation were assessed in perfused livers from the accumulation of radioactive cytidine in the perfusate plus total liver water when reutilization is prevented by the presence of 0.5 mM unlabelled cytidine. This procedure has been described in detail by Lardeux *et al.* (1987). Labelled cytidine was quantified by dividing the radioactivity (d.p.m.) in [14 C]cytidine by the specific radioactivity (d.p.m./ μ mol) of 3'-CMP in the liver RNA. From these values, rates of release were computed by least-squares regression. Rates of RNA degradation were calculated by dividing the above by the quantity of CMP in liver RNA, 0.87 μ mol/mg of RNA (Lardeux *et al.*, 1987). Fractional rates of RNA degradation were obtained by dividing rates of RNA degradation by the hepatic RNA content at the end of perfusion.

Net rates of RNA degradation were determined in a similar manner from the accumulation of labelled cytidine in the absence of unlabelled cytidine. Since label is normally reutilized in this situation, these net rates would represent the difference between RNA degradation and synthesis.

Fig. 1. Effect of perfusate amino acids and insulin on the linearity of initial release of cytidine during cyclic perfusion of the rat liver

Livers from normal-fed rats previously labelled *in vivo* with [6- 14 C]orotic acid were cyclically perfused *in situ* with a mixture of 20 amino acids at 4 times the normal plasma concentrations along with the infusion of insulin at 2.4 mg/h (○), or without amino acids and insulin (●) (Lardeux *et al.*, 1987). Cytidine release was determined from the accumulation of labelled cytidine in the presence of 0.5 mM unlabelled cytidine as described in the Experimental section. Results are means from two to six livers.

Analytical procedures

Radioactive cytidine and the specific radioactivity of 3'-CMP residues in RNA were assayed as previously described (Lardeux *et al.*, 1987). Liver RNA content was measured after alkaline hydrolysis for 4 h as detailed by Munro & Fleck (1966) and corrected for amino acid contamination by taking dual-wavelength (260 and 232 nm) absorbance readings (Ashford & Pain, 1986).

RESULTS AND DISCUSSION

RNA and protein degradation share many common features. Both are strongly inhibited by amino acids, insulin and chloroquine, and both show a 15 min lag between the introduction of a physiological regulatory agent and the response to that agent. These similarities led us to believe that the rate of RNA degradation *in vivo* could be determined during the first 15 min of cyclic perfusion, since an analogous procedure had been successfully used to determine rates of proteolysis *in vivo*

Table 1. Predicted and observed loss of liver RNA in meal-fed rats

Perfusions of livers from meal-fed rats started at 10:00 h. The net rates of cytidine release (nmol/min per 100 g initial body wt.) were determined by least-squares regression from the accumulation of labelled cytidine between 2 and 12 min of perfusion in the absence of unlabelled cytidine (A). The predicted loss of liver RNA (B) was calculated from the rate in line A as described in the Experimental section. The observed mean loss of liver RNA (C) was computed by least-squares regression from the change in liver RNA content (mg of RNA/100 g initial body wt.) between 07:00 and 16:00 h as described in the Experimental section. Results represent six livers in each group; values are given as means $\pm$ S.E.M.

(A) Cytidine release (nmol/min per 100 g initial body wt.)	3.39 ± 0.48
RNA loss (mg/h per 100 g initial body wt.)	
(B) Predicted	-0.234 ± 0.033
(C) Observed	-0.257

(Hutson *et al.*, 1982). RNA degradation was evaluated by the release of [14 C]cytidine from livers that had been previously labelled *in vivo* with [14 C]orotic acid, in a manner similar to the evaluation of proteolysis by the release of valine. Fig. 1 shows that the initial release (2–12 min) of labelled cytidine from perfused livers of normal-fed rats (normal commercial diet *ad libitum*) was both linear and unaffected by physiological regulators. Thereafter, the release changed abruptly; the rate decreased with insulin and $4\times$ amino acids (4 times normal plasma concns.) and increased in their absence. These observations confirm our previous results (Lardeux *et al.*, 1987; Lardeux & Mortimore, 1987). The initial release of labelled cytidine from livers of rats adapted to meal feeding (meal-fed; see under 'Animals and RNA labelling') also displayed this linearity and initial insensitivity to physiological regulators. Unlike the initial release of the proteolytic marker valine, which is characterized by the presence of both short- and long-lived components (Hutson *et al.*, 1982), the initial release of labelled cytidine appears to be derived from a single long-lived source. Since cytidine is neither synthesized nor degraded during perfusion *in situ* (Lardeux *et al.*, 1987), we hypothesized that the initial rate of labelled cytidine release in the presence of 0.5 mM unlabelled cytidine directly reflects the rate of hepatic RNA degradation existing *in vivo* at the start of the perfusion in both normal- and meal-fed rats.

We sought to confirm the validity of the above hypothesis by using short cyclic perfusions to predict the decrease in the content of hepatic RNA in meal-fed rats between periods of food intake. These perfusions were carried out in the absence of unlabelled cytidine, allowing labelled cytidine to be reutilized. Since reutilization has been shown to remain constant for up to 180 min (Lardeux *et al.*, 1987), the net release of labelled cytidine in these experiments should therefore reflect the balance between RNA synthesis and degradation at the time of perfusion (see Fig. 3). In Table 1, the loss of RNA predicted from the net release of cytidine in meal-fed rats perfused at 10:00 h was 0.23 mg of RNA/h per liver per

Fig. 2. Effect of feeding on the initial rate of cytidine release

Livers from prelabelled meal-fed rats (meals given between 16:00 and 20:00 h) were perfused either at 20:00 h (○) or at 10:00 h the next morning (●). Cyclic perfusion was carried out for 12 min with perfusate containing no amino acids or insulin. The release of labelled cytidine was determined in the presence of 0.5 mM unlabelled cytidine as described in the Experimental section. Values are means $\pm$ S.E.M. for five to six livers.

100 g initial body wt. (13.4%/day). In a parallel group of unlabelled rats, hepatic RNA was observed to decrease linearly between 07:00 and 16:00 h at an average rate of 0.26 mg of RNA/h per liver per 100 g initial body wt. (14.8%/day), a value within 10% of our prediction. Both rates were similar to the average rate observed in the rat by McNurlan *et al.* (1979) over 48 h of starvation (17.5%/day).

Effects of feeding on RNA degradation in livers of meal-fed rats

It is well established that degradation of both resident protein and RNA in liver is strongly decreased after food intake, particularly in nutritionally deprived animals (Conde & Scornik, 1976; Millward, 1980; Conde & Franze-Fernández, 1980; Hutson & Mortimore, 1982). As mentioned above, more recent evidence has attributed this to a common lysosomal mechanism (Lardeux *et al.*, 1987; Lardeux & Mortimore, 1987). In meal-fed rats proteolysis begins to increase 2 h after the end of feeding and continues to rise until the next meal (Millward, 1980). A similar time course would be expected for RNA degradation. In view of this observation, we decided to demonstrate the usefulness of short cyclic perfusion by using it to confirm the inhibition of RNA degradation induced by feeding. The times chosen for the start of perfusion, 20:00 h and 10:00 h the next day, corresponded to the initial rise in RNA content after feeding and the start of the decline after peak values respectively (Kosterlitz & Cramb, 1943; Waterlow *et al.*, 1978; Millward, 1980). These events presumably represent the start of the absorptive and post-absorptive periods. As

Table 2. Correlation of RNA degradation with feeding in livers of normal and meal-fed rats

Meal-fed rats were allowed to eat between 16:00 and 20:00 h, whereas normal-fed rats were allowed to eat *ad libitum* as described in the Experimental section. Livers from meal-fed rats were perfused as in Fig. 1 either immediately after the meal (20:00 h, day 1) or 14 h later (10:00 h, day 2); normal-fed rats were perfused only at 10:00 h. Rates of labelled cytidine accumulation were determined as in Table 1. Absolute (mg of RNA/h per 100 g initial body wt.) and fractional (%/day) rates of RNA degradation were calculated as described in the Experimental section. Values are means $\pm$ S.E.M. Numbers of animals are given in parentheses.

Condition	Day	Time of day	RNA degradation	
			Absolute (mg of RNA/h per 100 g initial body wt.)	Fractional (%/day)
Meal-fed				
Absorptive (5)	1	20:00 h	0.035 ± 0.003	2.1 ± 0.2
Post-absorptive (6)	2	10:00 h	0.291 ± 0.017	17.1 ± 1.4
Normal fed (8)	2	10:00 h	0.258 ± 0.030	14.8 ± 1.7

seen in Fig. 2 and Table 2, the unidirectional rate of RNA degradation *in vivo* just after feeding was only 12% of that during the post-absorptive period (2.1 versus 17.1%/day), a finding consistent with the aforementioned studies. Thus, as expected, RNA degradation is strongly influenced by feeding.

It is noteworthy that the fractional unidirectional rate of hepatic RNA degradation in normal-fed rats (14.8%/day), determined at 10:00 h, was in close agreement with a number of previous estimates of the fractional turnover of hepatic RNA in rats on a similar diet, which range from 12 to 22%/day (see the Introduction for references). It must be emphasized, however, that the present results are from determinations of the instantaneous rate of RNA degradation, whereas the previous data were derived from studies where rate measurements required a minimum of 24 h.

It may be noted in Tables 1 and 2 that the unidirectional rate of RNA degradation in normal-fed rats and the net rate (degradation minus synthesis) in meal-fed rats were virtually identical at 10:00 h. This apparent agreement was entirely coincidental. Nevertheless, in view of the fact that reutilization is low relative to RNA turnover at this time, it is not unreasonable to have expected some similarity.

These findings demonstrate the potential value of cytidine as a marker of RNA degradation in the liver *in vivo*. There is considerable evidence that the bulk of the hydrolytic cleavage of RNA molecules in the cytoplasm produces nucleotides bearing a phosphate group in the 3' position (Sierakowska & Shugar, 1977; Lardeux *et al.*, 1987). These are subsequently degraded to nucleosides. It is, however, probable that some nucleotides bearing a phosphate in the 5' position are formed by the sequential actions of neutral endoribonucleases and 3'-hydroxy-

Fig. 3. Scheme showing major pathways of cytidine metabolism involved in RNA turnover

RNA is synthesized directly from nucleoside triphosphates. It is subsequently degraded to 3'-nucleotides in lysosomes (solid arrows) or 5'-nucleotides outside of the lysosome (broken arrows), giving 3'- and 5'-CMP as cytosine-containing products. 5'-CMP is rapidly rephosphorylated to CTP. 3'-CMP is further degraded to cytidine within the lysosome. It is then either rephosphorylated to CTP in the cytosol or transported out of the hepatocyte.

specific phosphodiesterases (Sierakowska & Shugar, 1977). Additionally, it is known that there is further RNA degradation within the nucleus, resulting from the processing of RNA, which undoubtedly produced 5'-nucleotides (Sierakowska & Shugar, 1977). Since 5'-nucleotides are rapidly rephosphorylated to nucleoside triphosphates, they are not likely to be degraded further to nucleosides such as cytidine. For this reason, we most probably underestimated the rate of RNA degradation in Fig. 2 and Table 2.

Despite the probable underestimation of the total rate of RNA degradation, a reasonable assessment of the net loss of RNA was obtained from the loss of cytidine from the hepatocyte. As depicted in Fig. 3, this most probably arises from the differential metabolism of 3'-CMP and 5'-CMP. Cytidine and 5'-CMP represent the cytosine-bearing end products of the degradation of RNA to 3'- and 5'-nucleotides respectively. Cytidine cannot be further degraded because cytidine deaminase, the enzyme which catalyses the first step in cytidine catabolism, is virtually inactive in rat liver (Camiener & Smith, 1965; Rothman *et al.*, 1971). 5'-CMP is not degraded further because it is rapidly rephosphorylated to CTP (Bucher & Swaffield, 1968). Of these two end products, only cytidine freely permeates the plasma membrane. For these reasons, cytidine efflux from hepatocytes would be the only means by which the total cellular cytosine pool in RNA, nucleotides and nucleosides can decrease during starvation. As shown in Fig. 3, any cytidine that is not transported out of the cell would be phosphorylated to 5'-CMP, allowing it also to be rephosphorylated to CTP and subsequently used in RNA synthesis. Since the size of the acid-soluble pool remains relatively constant during perfusion (Lardeux *et al.*, 1987), the net release of cytidine would directly reflect the loss of RNA.

The brief cyclic perfusion described in the present paper appears to be a promising method for determination of the instantaneous rate of RNA degradation in the rat liver. This technique has already been used successfully in the mouse to determine the rate of hepatic protein degradation *in vivo* from the rate of valine release in the perfusate (Hutson & Mortimore, 1982; Hutson *et al.*, 1982). Its usefulness in measuring rates of proteolysis *in vivo* is, however, limited, because the net

accumulation of valine reflects the degradation of at least three different classes of protein: short- and long-lived resident proteins and endocytosed proteins. Its application in determining the rate of RNA degradation shows more promise, since the kinetics of the release of labelled cytidine, unlike valine, are consistent with a single long-lived source. The degradation of tRNA and rRNA, which together comprise over 90% of the cytoplasmic RNA (Hirsch, 1967; Gunning *et al.*, 1981), is likely to contribute most of this label, since these species appear to turn over at a similar rate (Blobel & Potter, 1968; Gunning *et al.*, 1981).

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