

## Pulse Labeling of RNA of Mammalian Cells\*

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*Communicated by Shields Warren, January 16, 1970*

**Abstract.** When cells from a hypotetraploid strain of Ehrlich ascites tumor are exposed to uridine-<sup>3</sup>H either *in vivo* or *in vitro*, the amount of radioactivity incorporated into RNA reaches a maximum within ten minutes, after which any further incorporation stops. <sup>3</sup>H-uridine triphosphate disappears from the acid soluble pool within 30 minutes and the findings indicate that the RNA of these cells can be pulse labeled without the use of any antibiotic or the need of a "chase." The stability of the pulse labeled RNA in the presence of pentobarbital (an inhibitor of RNA synthesis) indicates the virtual absence of RNA breakdown. However, actinomycin D, at a dosage of 250 µg/mouse *in vivo* and 10 µg/ml *in vitro* produces breakdown of labeled RNA, thus confirming earlier observations that the drug is not a suitable tool for RNA kinetics determinations. The pulse-labeled RNA leaves the nucleus slowly and some radioactive RNA is still present in the nuclear fraction after 24 hours. Radioactivity begins to appear in cytoplasmic ribosomal RNA after 20 minutes and continues to increase up to six hours.

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Pulse labeling of RNA is a necessary prerequisite for a proper study of the kinetics of RNA synthesis, intracellular distribution and turnover. However, pulse labeling of RNA in mammalian cells, either *in vivo* or *in vitro*, presents considerable difficulties. When mice or rats are injected with radioactive precursors of RNA, incorporation of radioactivity into RNA continues for at least several hours.<sup>1-3</sup> *In vitro* incorporation into RNA also continues for several hours after removal of the radioactive precursors as first demonstrated by Feinendegen *et al.*<sup>4</sup> in HeLa cells with cytidine-<sup>3</sup>H. Chasing of a radioactive precursor with cold precursor decreases the rate, but does not stop the incorporation of radioactivity into RNA,<sup>5-8</sup> even when the amount of cold precursor added is exceedingly high.<sup>5</sup> On the other hand, the use of actinomycin D to stop RNA synthesis is not acceptable as a method of pulse labeling, because the drug may cause breakdown of RNA.<sup>3</sup>

In the present communication we describe the pulse labeling of RNA in a strain of Ehrlich ascites cells either *in vivo* or *in vitro*, without drugs or chase and our preliminary studies on the rate of migration of the radioactivity from the nucleus to the cytoplasm.

**Methods and Materials. Materials:** Uridine-<sup>3</sup>H generally labeled (spec. act. 10.1 Ci/mmole), uridine-5-<sup>3</sup>H (spec. act. 25.9-28.3 Ci/mmole), adenine-<sup>3</sup>H (spec. act. 6.31 Ci/mmole), and cytidine-<sup>3</sup>H (spec. act. 5 Ci/mmole) were purchased from New England

Nuclear Corporation. Actinomycin D was purchased from Mann Research Laboratories, and pentobarbital (Nembutal Sodium) from Abbott Laboratories, North Chicago, Illinois. Triton X-100 and N-101 were kindly donated by Rohm and Haas, Philadelphia. All other chemicals were of reagent grade.

Ehrlich ascites tumor cells were maintained by weekly serial transfer through male Fels A mice bred in this laboratory. The tumor, a hypotetraploid subline, has been described previously in detail.<sup>9, 10</sup>

**In vivo experiments:** Fels A or Swiss male mice, purchased from Carworth Company, were inoculated with approximately  $12 \times 10^6$  tumor cells intraperitoneally. On the fifth day after inoculation they were injected with 50 to 100  $\mu\text{Ci}$  of uridine-5-<sup>3</sup>H dissolved in sterile aqueous solution. Actinomycin D was dissolved in sterile water and acetone (20:1), and injected intraperitoneally at 250  $\mu\text{g}/\text{mouse}$ . The animals were killed by cervical dislocation, and the tumor was collected in an ice bath.

**In vitro experiments:** 2–2.5  $\times 10^7$  Ehrlich ascites tumor cells, pooled from mice bearing 5-day-old tumors, were suspended in a 7:2 mixture of Earle's balanced salt solution and newborn calf serum containing 1  $\mu\text{Ci}/\text{ml}$  of radioactive precursor.

The cells were incubated at 37° in an Erlenmeyer flask in an atmosphere of 95% air and 5% CO<sub>2</sub> in a humid incubator. The incorporation was terminated by pipetting 0.5 or 1 ml of the incubation mixture into 1 ml of 1 N perchloric acid.

**Determination of total, nuclear, and cytoplasmic RNA:** One milliliter of tumor was divided in two 0.5-ml fractions. One fraction was added to 2 ml of 0.3 N perchloric acid, and RNA and DNA in whole cells were extracted and determined by the method of Scott *et al.*<sup>11</sup> The second fraction was centrifuged at 4° in a refrigerated International centrifuge for 3 min at 1600  $\times g$ , the cells were resuspended for 10 min in 5 ml of ice cold distilled water, and the suspension centrifuged again at 1600  $\times g$  for 3 min in an International centrifuge. The supernatant (cytoplasmic fraction I) was saved and the pellet, resuspended in 5 ml of 1% Triton N-101, was kept in an ice bath for 10 min. A light-microscopic examination at this point revealed clean nuclei, which were collected by centrifugation in a refrigerated centrifuge at 1600  $\times g$  for 3 min. The supernatant (cytoplasmic fraction II) was combined with the first cytoplasmic fraction. RNA and DNA were extracted and determined from the combined cytoplasmic fractions and the nuclear pellet as described above. The radioactivity was determined in a Tricarb model 3380 (Packard), at an efficiency of 33%.

**Paper chromatography:** The acid-soluble pool was extracted from whole cells with 0.3 N perchloric acid: uracil, uridine, and uridine mono-, di-, and triphosphate, were separated by descending paper chromatography, using DEAE-cellulose paper and 4 N formic acid in 0.1 M ammonium formate as solvent.<sup>19</sup> The running time was 4 hr. With this method UTP remains at the origin and uridine travels with the solvent front. The paper was cut into 10-mm pieces that were placed in 10 ml of Liquifluor and counted in a Tricarb. Standard samples of UMP and uridine were run concomitantly to confirm the identification of the peaks.

**Preparation of cytoplasmic ribosomal RNA for gel electrophoresis analysis:** For the preparation of a cytoplasmic fraction the cells were fractionated using an association of ionic and nonionic detergent as described by Penman.<sup>12</sup> Tumor cells (1.5–3 ml), collected by centrifugation in a refrigerated International centrifuge (mod PR 4), were resuspended for 5 min in 5 ml of ice-cold distilled water.

Bentonite, at a final concentration of 6 mg/ml, was added, and the cells were homogenized with five strokes in a Dounce homogenizer, using a loose fitting pestle. The homogenate was centrifuged in the International centrifuge at 4° for 3 min at 1600  $\times g$ , and the supernatant (cytoplasmic fraction I) collected and kept in the cold at 4°. The pellet was resuspended in 3 ml of distilled water containing 1% Triton X-100 and 0.5% deoxycholate, shaken in a Vortex mixer for 20 sec, and centrifuged for 3 min at 4° at 1600  $\times g$ . The supernatant was added to the cytoplasmic fraction I and centrifuged again for 5 min at 4° at 1600  $\times g$  to eliminate any nuclear contamination.

To the combined cytoplasmic fractions, NaCl and sodium ethylenediaminetetraacetate

( $\text{Na}_2\text{-EDTA}$ ) were added to a final concentration of, respectively, 0.15 and 0.002  $M$  and the suspension was mixed with an equal volume of cold 90% phenol.<sup>13</sup> After stirring at 4° for 20 min and separation, by centrifugation, of the aqueous and phenol phases, the extraction was repeated three times. RNA was precipitated from the aqueous phase with 2 vol of cold absolute ethanol, and kept at -20° overnight in the freezer. The RNA was collected by centrifugation in a Sorvall refrigerated centrifuge at 10,000  $\times g$  for 15 min, and washed with 0.05  $M$   $\text{NaCl}$  and 0.002  $M$   $\text{Na}_2\text{EDTA}$ .

**Polyacrylamide gel electrophoresis:** Analysis of RNA on acrylamide gels was performed according to the method of Loening,<sup>14</sup> with some modifications introduced by others.<sup>15, 16</sup> The electrophoresis buffer described by Bishop *et al.*<sup>15</sup> containing 0.2% sodium dodecyl sulfate was used. One hundred micrograms of RNA were dissolved in 0.2 ml sucrose buffer (30% electrophoresis buffer, 10% sucrose), and layered on 8-cm long 2.7% polyacrylamide gels. Electrophoresis was then performed at room temperature for 3 hr at a potential gradient of 10 v/cm and 5 ma/gel. Gels were scanned in a Gilford modified spectrophotometer (model 2410), then frozen in a bath of hexane and cut in 2-mm slices by a multiblade razor (P. R. Perry, personal communication). The slices were dissolved in 0.3 ml of concentrate  $\text{NH}_4\text{OH}$ <sup>17</sup> and radioactivity was determined in Tricarb model 3380 (Packard), with a toluene-methoxyethanol-liquifluor mixture.<sup>18</sup>

**Results.** All experiments described in this paper were performed with Ehrlich ascites tumor cells on the fifth day after intraperitoneal inoculation into male mice.

**Pulse labeling of RNA:** When Ehrlich ascites tumor cells are exposed *in vivo* to or incubated *in vitro* with uridine-<sup>3</sup>H, incorporation of the radioactive precursor into total RNA ceases after a period of about 10 minutes. Table 1 shows the specific activity of RNA of Ehrlich ascites tumor cells growing in the peritoneal cavity of mice, between 1 and 30 minutes after an intraperitoneal injection of uridine-<sup>3</sup>H. Similar results were obtained when these tumor cells were incubated *in vitro* with uridine-<sup>3</sup>H: this is shown in Figure 1, which also demonstrates that other RNA precursors are incorporated for longer times than uridine: cytidine for at least 60 minutes and adenine for 45 minutes.

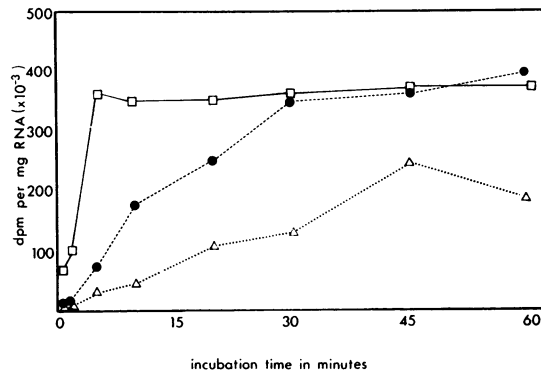
The different patterns of incorporation of cytidine and adenine and the fact that uridine incorporation *in vivo* also ceases after ten minutes indicate that this ten-minute pulse labeling is not due to cell damage. *In vitro* this was confirmed by experiments (not shown), in which Ehrlich ascites tumor cells, preincubated for four hours, were then exposed to uridine-<sup>3</sup>H: incorporation of the radioactive precursor again ceased after ten minutes at a level which approximated that seen in Figure 1.

TABLE 1. Incorporation of uridine-<sup>3</sup>H into RNA of Ehrlich ascites cells growing in the peritoneal cavity of mice.

Time after injection of uridine- <sup>3</sup> H (in min)	dpm in RNA/mg RNA		
	Animal no.		
	1	2	3
1	12,550	—	—
2	22,500	23,300	—
5	23,700	33,900	26,500
10	27,800	49,800	33,300
20	30,100	43,900	—
30	30,400	44,200	32,600

Fels A mice were inoculated intraperitoneally with  $8 \times 10^6$  tumor cells and injected with uridine-<sup>3</sup>H (10  $\mu\text{Ci}$  per mouse) on the fifth day after inoculation. Tumor samples were repeatedly taken from each animal by aspiration with a syringe. Specific activity of RNA was determined as described in the text. For later times, see Fig. 4.

FIG. 1.—*In vitro* incorporation of uridine-<sup>3</sup>H (□—□), cytidine-<sup>3</sup>H (●—●), and adenine-<sup>3</sup>H (Δ··Δ) into RNA of Ehrlich ascites tumor cells. Tumor cells were incubated as described in *Methods and Materials*, with 1 μCi/ml of uridine-<sup>3</sup>H, cytidine-<sup>3</sup>H, or adenine-<sup>3</sup>H. Samples (0.5 ml) were taken for RNA determinations at the intervals indicated on the abscissa.



**Fate of the radioactive precursor:** Table 2 indicates that after a 30-minute exposure period to uridine-<sup>3</sup>H either *in vitro* or *in vivo*, no more radioactive UTP can be found in the intracellular acid-soluble fraction of Ehrlich ascites tumor cells. Other experiments (not shown) demonstrated that a medium containing uridine-<sup>3</sup>H, and which had been previously in contact for 30 minutes with Ehrlich ascites tumor cells, did not any longer contain products that could be incorporated into the RNA of freshly incubated tumor cells.

TABLE 2. Absence of labeled uridine triphosphate in the acid-soluble fraction of Ehrlich ascites cells after a short exposure to uridine-<sup>3</sup>H.

Time of exposure to uridine- <sup>3</sup> H (in min)	Percentage of Total Radioactivity in Peaks			
	1	2	3	4
<i>in vivo</i> 2	2.9	8.4	13.1	75.6
10	1.4	4.2	5.6	88.9
30	None	8.6	12.9	78.4
<i>in vitro</i> 30	None	8.7	29.4	61.9

Acid-soluble fractions were prepared and chromatographed as described in *Methods and Materials*. Peak 1 was identified as containing uridine triphosphate. Each value is the average of three animals.

**Possibility of breakdown of RNA and reutilization of radioactive products:** Although the specific activity of RNA does not increase further after a ten-minute exposure to uridine-<sup>3</sup>H, it is possible that some of the newly synthesized radioactive RNA may be broken down and its radioactive constituents rapidly and efficiently reutilized. This possibility was tested by the following experiments.

Ehrlich tumor cells were treated *in vitro* with two inhibitors of RNA synthesis, actinomycin D and pentobarbital.<sup>20</sup> Both these inhibitors, added *in vitro* 15 minutes before uridine-<sup>3</sup>H, at a concentration of 10 μg/ml and 1.5 mg/ml, respectively, inhibited incorporation of the radioactive precursor by 97 per cent. If actinomycin D or pentobarbital were added *in vitro* 30 minutes after uridine-<sup>3</sup>H, a difference was observed in the effect of the two inhibitors. While pentobarbital did not affect the specific activity of RNA in the subsequent two hours of incubation, actinomycin D reduced the specific activity of RNA by 40 to 45 per cent (Table 3). Two conclusions can be drawn from these experiments: (1) since there is no decrease in RNA specific activity after pentobarbital (which effectively inhibits any further RNA synthesis) no appreciable breakdown of RNA must occur *in vitro* for at least two hours; and (2) actinomycin D at the

TABLE 3. *Effect of pentobarbital sodium and actinomycin D on the specific activity of RNA of Ehrlich ascites cells labeled with uridine-<sup>3</sup>H.*

Time after addition of inhibitor	cpm/mg RNA ( $\times 10^{-3}$ )		
	Controls	Actinomycin D	Pentobarbital
0	16-15-19 (16)		
15 min	18-17-16 (17)	12-12-11 (12)	16-16-16 (16)
30 min	17-16-16 (16)	11-12-12 (11)	17-17-16 (17)
1 hr	18-17-14 (16)	11- 9-10 (10)	16-16-16 (16)
2 hr	17-17-14 (16)	9-10-10 (10)	15-14-16 (15)

Cells were incubated *in vitro* with 1  $\mu$ Ci/ml of uridine-5-<sup>3</sup>H (spec. act. 28.3 Ci/mmmole). After 30 min, pentobarbital (1.5 mg/ml) or actinomycin D (10  $\mu$ g/ml dissolved in 20:1 distilled water acetone mixture) were added to the incubation mixtures (solvent was added to the control mixtures). Specific activity of RNA was determined from 1.0 ml aliquots. 0 time represents the values at 30 min after incubation with uridine-<sup>3</sup>H. Mean in parentheses.

concentration used causes breakdown of recently synthesized RNA in Ehrlich ascites tumor cells. *In vivo* experiments with actinomycin D at a dose of 250  $\mu$ g/mouse (not shown) confirmed these data.

**Fate of pulse-labeled RNA:** The fate of pulse-labeled RNA has been followed for a 24-hour period (Fig. 2). The specific activity of total RNA in whole cells increased for about ten minutes and then remained essentially constant for at least four hours. Between 4 and 6 hours the specific activity decreased about 50 per cent and then remained constant up to 24 hours. The specific activity of cytoplasmic RNA increased almost linearly for the first 2 hours, reached a maximum at 4 hours, and then remained constant between 4 and 24 hours.

Notice that the percentage of total cellular radioactivity remaining in the nuclei decreased from 92.3 per cent in the first 5 minutes to 22.2 per cent at 6 hours, and to 11 per cent at 24 hours.

**Time of appearance of ribosomal RNA in the cytoplasm:** Gel electrophoretic analysis of cytoplasmic RNA extracted at 4° indicated that for the first ten minutes the radioactivity was mostly in the region of transfer RNA and pre-transfer RNA,<sup>21</sup> and to a less extent in the region of heterodispersed RNA. At this time it was impossible to observe any clear peak referable to 28S, or 18S

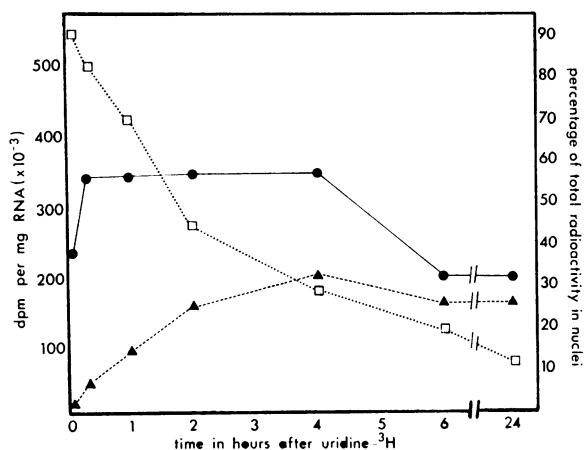


FIG. 2.—*In vivo* incorporation of uridine-<sup>3</sup>H into RNA of Ehrlich ascites tumor cells. Specific activity of total RNA (●—●), and cytoplasmic RNA (▲---▲), and percentage of total radioactivity in the nuclear fraction (□···□). 50  $\mu$ Ci of uridine-5-<sup>3</sup>H were injected intraperitoneally. Cellular fractions were prepared as described in *Methods and Materials*. Each point represents the average of four mice.

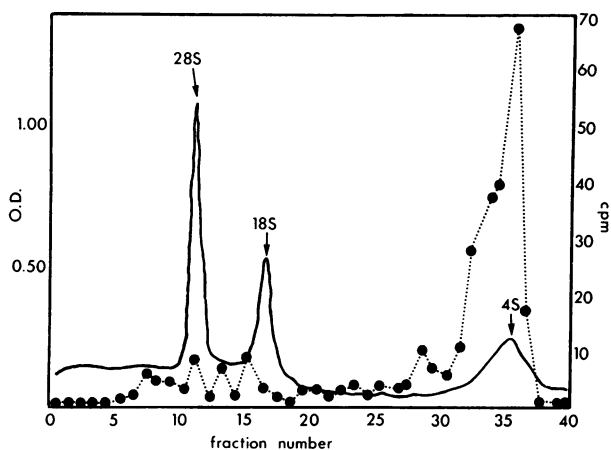


FIG. 3.—Polyacrylamide gel electrophoresis of cytoplasmic RNA, 10 min after injection of 100  $\mu$ Ci/mouse of uridine-5- $^3$ H. Optical density (—), radioactivity (●...●).

ribosomal RNA (Fig. 3). At 20 minutes after exposure to uridine- $^3$ H two identifiable peaks of radioactivity appeared in the regions of 28S and 18S. The specific activity was higher in the 18S peak than in the 28S peak. At later times the radioactivity in the 18S and 28S peaks was more pronounced than in any other region (Fig. 4). Note that the radioactivity scales in Figures 3 and 4 are different. Figure 5 shows the specific activities of the 18S and 28S peaks at various times after uridine- $^3$ H.

**Discussion.** Our results show that the incorporation of uridine- $^3$ H into RNA ceases after 10 minutes of exposure to the radioactive precursor. Pentobarbital, although inhibiting RNA synthesis, does not inhibit the uptake and phosphorylation of nucleosides,<sup>20</sup> and our experiments with pentobarbital indicate that there

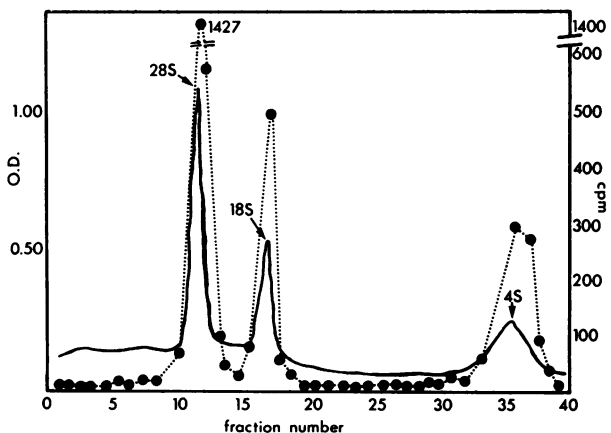


FIG. 4.—Polyacrylamide gel electrophoresis of cytoplasmic RNA 4 hrs after injection of uridine-5- $^3$ H; optical density (—), radioactivity (●...●).

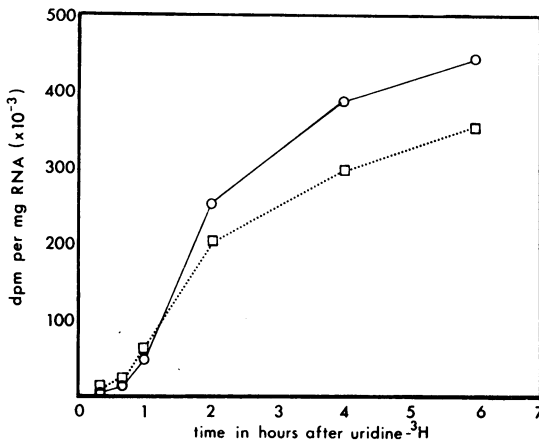


FIG. 5.—Specific activity of cytoplasmic ribosomal RNA at various intervals after a pulse label of Ehrlich ascites tumor cells with uridine- $^3\text{H}$ ; 28S (O—O); 18S (□···□).

is no significant breakdown of newly synthesized RNA (and therefore reutilization of its components) for at least two and a half hours after labeling with uridine- $^3\text{H}$ . The disappearance of labeled UTP from the acid-soluble intracellular fraction within 30 minutes is also indicative of the fact that radioactive precursor is no longer available for incorporation into RNA. On the other hand, *in vitro* or *in vivo* exposure to actinomycin D produces a rapid breakdown of newly synthesized RNA. These findings confirm in Ehrlich ascites tumor cells the observation of Stewart and Farber<sup>3</sup> in rat liver that actinomycin D produces RNA breakdown.

To our knowledge, this is the first time that a pulse labeling of RNA has been accomplished in mammalian cells without the use of drugs or high concentrations of cold precursors that can affect cellular function, especially the synthesis of macromolecules. The possibility of pulse labeling mammalian cell RNA could conceivably clarify the kinetics of RNA synthesis and migration, which, up to this time, have been confused and conjectural. According to Harris and his co-workers,<sup>6, 22</sup> a sizable fraction of newly synthesized RNA is broken down without even reaching the cytoplasm. Harris' views have now gained general acceptance,<sup>23–26</sup> but kinetic studies on mammalian RNA are necessarily based on the use of actinomycin D which clearly introduces an artifact in the previous studies. Similarly, use of a chase, while introducing another possible artifact, has not reduced the labeling period to a short pulse.<sup>4, 7</sup> In addition, a recent report by Church *et al.*<sup>27</sup> has shown that the transfer of RNA to the cytoplasm is most efficient in rapidly growing tumor cells and least efficient in highly differentiated cells.

We do not know why this subline of Ehrlich ascites tumor cells can be pulse labeled so sharply, but presumably it may be due to the fact that certain sublines of these cells have a very high uridine phosphorylase activity.<sup>28, 29</sup>

Some interesting conclusions can already be drawn from the preliminary experiments reported in this paper on the migration of pulse labeled RNA to the cytoplasmic compartments, namely: (1) there is a moderate loss of radioactive RNA between four and six hours after pulse labeling with uridine- $^3\text{H}$ . Part of the decrease can be accounted for by a decrease in labeling of transfer RNA

(unpublished observations), but part of it may represent the rapidly labeled RNA of nuclei that is particularly sensitive to a specific exonuclease;<sup>30, 31</sup> (2) although radioactive RNA is synthesized for a brief period of only ten minutes, the radioactivity in cytoplasmic ribosomal RNA continues to increase between one and six hours. This raises the question of why some rRNA molecules may take such a long time in leaving the nucleus, while others are much faster; (3) the early appearance in the cytoplasm of radioactive 18S RNA is in agreement with the observations of Perry<sup>32</sup> and Girard *et al.*,<sup>24</sup> but in our cells radioactive 28S RNA appears more rapidly in the cytoplasm than with HeLa cells;<sup>33</sup> and (4) even 24 hours after pulse labeling there is still some radioactive RNA in the nuclei. Studies are in progress to determine whether this RNA is the low molecular weight nuclear RNA, relatively stable, that has been recently described in HeLa cells by Weinberg *et al.*<sup>34</sup>

The results, even at this early stage, indicate that the possibility of obtaining a pulse labeling of RNA in mammalian cells may disclose totally new approaches for investigating the kinetics of RNA synthesis and migration.

\* This work was supported by grant 1019 from the Damon Runyon Memorial Fund.

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