# RNA SYNTHESIS IN CHINESE HAMSTER CELLS

## II. Increase in Rate of RNA Synthesis during $G_1$

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ABSTRACT

Cultures of mitotic Chinese hamster cells, prepared by mechanical selection, were pulse-labeled with methionine-methyl-$^{14}C$ or with uridine-$^{3}H$ at different stages in the life cycle. The rate of $^{14}C$ incorporation into 18S RNA was measured, as was the rate of uridine-$^{3}H$ incorporation into total RNA for both monolayer and suspension cultures. The rate of incorporation increased continuously throughout interphase in a fashion inconsistent with a gene-dosage effect upon RNA synthesis.

## INTRODUCTION

Measurement of the kinetics of RNA synthesis is of fundamental importance as a step to understanding regulatory processes involved in traverse of the life cycle. One elementary model of regulation states that the rate of RNA synthesis is controlled by genome availability, resulting in a doubling of synthetic rate following duplication of the genome during the S phase of the mammalian cell cycle (i.e., the gene-dosage effect). Evidence for a gene-dosage effect upon RNA synthesis in HeLa cells has been reported and reviewed by Pfeiffer and Tolmach (1, 2). Essentially the same system has been employed by Scharff and Robbins (3) who concluded, to the contrary, that the rate of RNA synthesis increased continuously throughout interphase.

Earlier studies from this laboratory (4) indicated that the rate of methylation of a stable RNA species (18S ribosomal RNA) in Chinese hamster cells was approximately twice as great in late interphase as in early interphase; however, insufficient data were presented to distinguish between a gene-dosage effect and a continuous increase in synthetic rate. In the present report we have extended our investigation of the RNA synthetic rate in cultures of mechanically selected (and, therefore, minimally perturbed) Chinese hamster cells by measuring the rate of methylation of 18S RNA and the rate of incorporation of uridine-$^{3}H$ into total RNA from cells labeled in different portions of the life cycle. Since the gene-dosage model predicts a uniform synthetic rate across $G_1$, in contrast to an increased synthetic capacity across $G_1$ for the continuously increasing synthesis rate model, special emphasis has been placed on measurements in $G_1$—a period which the cells traverse before appreciable decay of synchrony can occur. To ensure reproducible growth conditions, several fundamental parameters of cell growth and division were determined simultaneously with RNA synthetic rate.

Although the magnitude of increase in the rate of RNA synthesis was dependent on experimental conditions such as cell density, suggesting involvement of as yet undefined factors in the microenvironment, the rate of increase of synthesis was continuous across interphase in all instances—in a fashion inconsistent with a simple gene-dosage regulatory mechanism in the Chinese hamster cell.

## MATERIALS AND METHODS

### *Synchronization of Cell Growth*

Chinese hamster cells, line CHO (5), were grown in F-10 medium without added calcium, supplemented with 10% calf and 5% fetal calf sera, penicillin, and streptomycin. The cell line was monitored for PPLO contamination with the agar described by Chanock *et al.* (6); no PPLO were detected during the course of these experiments.

Mitotic CHO cells were collected with the aid of an oscillating mechanical shaker as described previously (7, 8). Cultures grown in Blake bottles (Hoffman Specialty Mfg. Corp., Indianapolis, Ind.) were shaken for 1–3 sec periods at 10-min intervals. The medium containing detached cells was decanted and replaced with a 25-ml aliquot of fresh medium. Material from the first six cycles was discarded, and only cells from the seventh and following separations (mitotic fractions of 0.9 or better) were used in experiments. The yield from six Blake bottles immediately after detachment was $1.5 \times 10^6$ cells, the majority of them being in late metaphase.

Two methods for accumulation of sufficient material were employed. The first method, referred to as "chill-accumulation," involved immediately chilling cells collected at 10-min intervals over a period of several hours. Upon resuspension in warm medium, the cells completed mitosis and divided synchronously (7, 8). The second method, devised to avoid chilling, involved two sets of Blake bottles harvested on alternate 10-min schedules so that the yield from three successive harvests (comprising one sample) was collected over a 10-min period, pooled, and resuspended in the appropriate concentration in fresh medium. This protocol is referred to as "continuous harvest."

Radioautographs of cells labeled with 1 $\mu$c/ml of thymidine-$^3$H (6 c/mM, Schwarz Bio Research Inc., Orangeburg, N. Y.) were prepared by the method of Puck and Steffan (9) except that cells were stained after development. Cell volume distribution spectra were determined with the electronic particle counting apparatus described previously (10). Cell concentrations were measured with an electronic particle counter.

### *Analysis of Rate of Methionine-methyl-$^{14}$C Incorporation into 18S RNA*

The nature of methionine-methyl-$^{14}$C incorporation, the procedure for RNA extraction with hot phenol, and the technique of zone sedimentation analysis of extracted RNA's have been described (4, 11). Hot phenol extractions were performed at 58°C rather than at 60°C.

RNA losses during phenol extraction and subsequent preparation were variable. To compensate, methionine-methyl-$^{14}$C-labeled cells were mixed with replicate amounts of uridine-5-$^3$H-labeled cells[1] before phenol extraction, and measurement of the $^{14}$C/$^3$H ratio in the 18S peak of the zone sedimentation analysis was then used as a measure of the relative rate of methionine-methyl-$^{14}$C incorporation (cf. reference 4 for zone sedimentation analyses of methyl-labeled Chinese hamster RNA). In all instances, between 100 and 150 ml of synchronized cells at a density of approximately 150,000/ml were pulsed with 1.5 $\mu$c/ml of methionine-methyl-$^{14}$C (52 mc/mmole) for 1 hr.

Pulse-labeling was terminated by pouring the culture over 50 g of crushed, frozen F-10 medium before centrifugation and subsequent washing with cold 0.25 M sucrose. The washed cells were quantitatively rinsed onto a pellet of uridine-$^3$H-labeled cells before phenol extraction.

### *Cell Fractionation*

Aliquots of labeled cells or of labeled cells plus carrier cells containing $2\text{–}5 \times 10^7$ cells were centrifuged, and the cells were washed with 0.25 M sucrose and then with 0.25 M sucrose containing 5 mg of bentonite. The final cell-bentonite pellet was suspended in 1 ml of NCM buffer (0.13 M NaCl; 0.01 M sodium cacodylate, pH 7.4; 1.5 mM $MgCl_2$) and was shell-frozen in dry ice-ethanol. Upon thawing, 0.1 ml of 10% Nonidet P40 (12) was added, and the suspension was agitated with a Vortex mixer (Scientific Industries, Inc., New York) at full speed for 10 sec. After standing for 10–15 min at 4°C, 0.1 ml of 5% sodium deoxycholate was added; the mixture was resuspended and, after an additional 10–15 min at 4°C, was agitated again before centrifugation for 10–15 min at 800–1000 *g*. The pellet was then washed

[1] The uridine-$^3$H-labeled cells were prepared as follows. Exponentially growing Chinese hamster cells were labeled with 0.1 $\mu$c/ml of uridine-5-$^3$H (4.0 c/mmole) for a period equivalent to the culture doubling time, centrifuged, and suspended in cold 0.25 M sucrose. Aliquots containing $2.5 \times 10^7$ cells were dispensed into centrifuge tubes; the cells were centrifuged, the sucrose solution was decanted, and the cell pellets were frozen.



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twice with Tween-DOC in RSB (13). Nuclei thus obtained were digested with 100 μg of DNase (DPFF, Worthington Corporation, Harrison, N.J., E.C. 3.1.4.5) in HSB (0.5 M NaCl; 0.05 M $MgCl_2$ ; 0.01 M Tris, pH 7.4) or HSB × 1.6 (14) for 1–1½ hr at 4°C. If clumps were present, pipetting and agitation were employed to obtain a uniform suspension. This suspension was centrifuged for ½ hr at 800–1000 *g*, and the supernatant (nucleoplasm) and pellet (nucleoli) were saved for analysis of labeled RNA content.

### *Determination of Uridine Incorporation into RNA*

Suspension cultures containing 35–90 ml of mechanically synchronized cells at concentrations of 1.5–2.5 × $10^5$/ml were labeled with 2–5 μc/ml of uridine-5-$^3$H (4 c/mmole). Labeling was terminated by pouring aliquots over frozen medium. 5 or 10 ml were usually harvested for determination of uridine-$^3$H incorporation into total RNA (2–4 × $10^7$ chilled, unlabeled cells added for carrier). The remaining cells were harvested for fractionation and determination of incorporation into nuclear pellet (nucleoli) and into the nuclear supernatant (nucleoplasm).

Cells washed with 0.25 M sucrose or cell fractions prepared as described above were suspended in 10% trichloroacetic acid (TCA) for ½ hr in an ice bath. The TCA-insoluble material was sedimented, washed with ethanol containing 2% potassium acetate (ethanol:acetate solution), and drained for 20–30 min in the cold. The pellets were then suspended in 1.25 ml of sodium cacodylate buffer (pH 7.0, 0.05 M) containing 25 μg of ribonuclease A (Worthington, E.C. 2.7.7.16) for 30 min at 48–50°C or were suspended in 1 ml of 0.3 M KOH for 18 hr at 37°C; the digests were then acidified with 0.8 or 1.7 ml of 0.5 M perchloric acid, respectively. After standing at 0° for 30 min the acidified digests were clarified by centrifugation, 0.5 ml of the supernatant was mixed with 0.75 ml of $H_2O$ plus 10 ml of Bray's scintillation mixture, and the radioactivity was determined in a liquid scintillation spectrometer.

To determine the rate of incorporation of uridine-$^3$H into monolayers, aliquots of mitotic cells in 2.0–4.5 ml of fresh medium were placed directly into 25-cm$^2$ plastic tissue culture flasks (Falcon Plastics, Los Angeles, Calif.) immediately after determination of the cell concentration. The number of cells per flask was varied from $10^4$ to $10^6$ per flask in different experiments. At different times throughout the life cycle, uridine-$^3$H was added. Incorporation was terminated by decanting the supernatant medium and adding 5 ml of cold 10% TCA. After 30 min at 0°, the TCA was decanted and the bottles were rinsed with an additional 5 ml of 10% TCA. 5 ml of cold ethanol: acetate solution were added, and after 20–30 min at 0° the bottles were rinsed with 5 ml of the same solution. After draining, 4.25 ml of 0.05 M sodium cacodylate[2] (pH 7.0) buffer containing 25 μg/ml of ribonuclease A were added. After 1 hr at 37°c, the material was decanted and 5 ml of 0.2 M perchloric acid were added. Following incubation at 4°C for 20 min, the ribonuclease and perchloric extracts were pooled and diluted to 10 ml. An aliquot of 1.25 ml was added to Bray's scintillation fluid, and the radioactivity of the sample was determined.

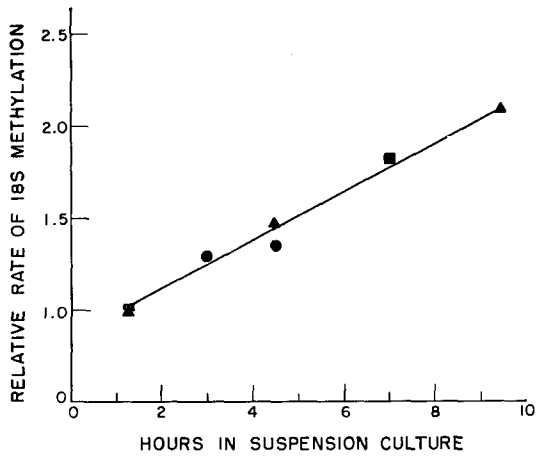


FIGURE 1 Rate of methylation of 18S ribosomal RNA species in the CHO cell throughout interphase. Aliquots of 150 ml of cell suspension (1.9 × $10^5$ cells/ml), prepared by the chill-accumulation technique, were pulse-labeled with 1.5 μc/ml of methionine-methyl-$^{14}$C for 1-hr periods. Before RNA extraction the cells were mixed with uridine-5-$^3$H-labeled cells (prepared as described in Materials and Methods) for a comparison of the efficiency of recovery. Data points are plotted as the midpoint of a 1-hr labeling period, and all rates are normalized to a relative rate of 1.0 for a common culture, in the three experiments, labeled during the interval 0.75–1.75 hr after resuspension in warm medium. As shown previously (4), samples collected through 3 hr consisted exclusively of cells in $G_1$. The 4.5-hr sample contained 96% of cells in $G_1$ and 4% in S; the 7-hr sample contained 68% in $G_1$ and 32% in S; and the 9.5-hr sample contained 31% in $G_1$, 59% in S, and 10% in $G_2$.

## RESULTS AND DISCUSSION

### *Methionine-methyl-$^{14}$C Incorporation into 18S RNA*

Three rate experiments were performed. A methionine-methyl-$^{14}$C pulse was introduced at

[2] When $10^4$ cells were labeled, only 1.25 ml of RNase solution was used and 1.0 ml was taken for counting without a perchloric acid rinse.



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0.75–1.75 hr after resuspension in a spinner flask in each instance. The data were normalized to give this sample a value of 1.0 in each experiment. The composite data for rates of synthesis across $G_1$ thus obtained are presented in Fig. 1. It is apparent that the rate of methylation increased continuously during the first 10 hr of interphase.

### *Comparison of Chilled and Nonchilled Cultures*

The data in Fig. 1 provide evidence that the rate of methylation of 18S RNA increases continuously across $G_1$ in the Chinese hamster cell. Two possible objections might be raised to comparing these results with data of Pfeiffer and Tolmach (1, 2) and Scharff and Robbins (3) obtained with HeLa cells: 1. the cells employed in these methylation studies were chilled before utilization, which could conceivably introduce biochemical perturbation; and 2. the data pertaining to HeLa cells involved incorporation of uridine-$^3$H into various RNA fractions rather than methylation. To avoid these objections, cultures prepared by chill-accumulation and continuous harvest were compared by measurement of fundamental parameters relating to cell growth and division. We had previously demonstrated that cells stored at 0° for prolonged periods and subsequently resuspended in warm medium were able to complete mitosis in a manner identical to that for nonchilled cultures (7). The data in Fig. 2 provide evidence that the pattern of cell division,[3] volume increase, and rate of entry into the DNA-synthetic period are indistinguishable in cultures prepared by both methods. Dispersion reflected by the reduced rate of entry into S and completion of mitosis in the second round of division are also evident in Fig. 2, emphasizing that the reliability of measurements is greatest in early interphase (4). It should also be noted from the data in Figs. 1 and 2 that the increasing rate of RNA synthesis and volume growth began in early $G_1$ under these conditions, indicating that there is no appreciable delay in initiating $G_1$ traverse. One of the above parameters (usually volume growth), in addition to RNA synthetic rate, was measured in all experiments.

[3] The cell number rose by approximately 60–75% in the division wave, but most of the reduction in cell yield could be accounted for by incomplete separation of daughter cells (the particle counter enumerates two nonseparated cells as a single event) and cell adhesion to the walls of the spinner flask.

### *Uridine Incorporation*

Representative data from experiments in which uridine incorporation (15–min pulses) into cells obtained by either continuous harvest or chill-accumulation techniques are shown in Fig. 3. Data for pulses beginning 1 and 4 hr into interphase are averages of duplicate determinations. As was shown in Fig. 2, the pulse which began 4 hr into interphase was given at a time when most of the population is still in $G_1$. There is approximately a 60% increase in rate from the 1st to the 4th hour of $G_1$ traverse, whereas no increase in RNA synthetic rate should have occurred if the gene-dosage model were correct. Note also that the synthetic rate by the 8th hour increased to a much greater extent than the factor of two predicted by the gene-dosage model. Furthermore, there is no indication in Figs. 1 and 3 of an abrupt change in RNA synthetic rate at the time at which genome duplication begins. Thus, the gene-dosage model may not be invoked to explain the regulation of RNA synthesis in the Chinese hamster cell.

The increase in rate of methylation of 18S RNA from early to late interphase (Fig. 1) is significantly less than the increase in rate of uridine incorporation into total RNA (Fig. 3). Methylation of 18S RNA may not reflect the rate of stable RNA synthesis because of possible differences in extent of methylation of RNA species in different portions of the life cycle, ribosomal precursor effects, and other factors.

As an alternate method of estimating synthesis of relatively stable species, uridine-$^3$H incorporated into total RNA's after 1–hr pulses and into the nuclear pellet (nucleolar fraction) after either 15–min or 1–hr pulses was measured. Measurement of uridine incorporation into nucleoli after 15–min pulses at various times during interphase provided the data in Fig. 4. The rate of uridine incorporation increased continuously throughout interphase, but the magnitude of the increase more closely approximated that for uridine incorporation into total RNA after 15–min pulses than that for 18S methylation or uridine incorporation into total RNA after 1–hr pulses, which are quite similar in magnitude (Tables I and II). It appears that part of the nucleolar species labeled during 15–min pulses is unstable,



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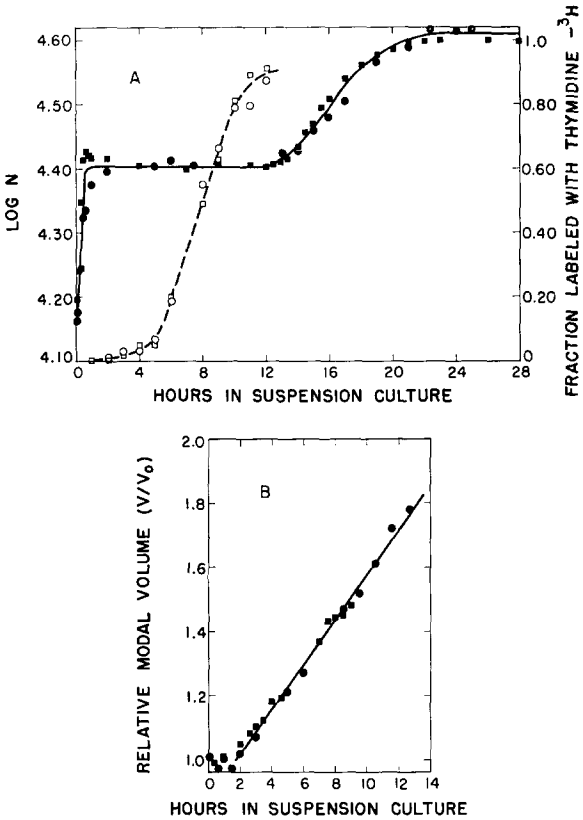


FIGURE 2 Comparison of rates of cell division, volume growth, and entry into the DNA-synthetic period in cultures of CHO cells prepared by the chill-accumulation and continuous harvest techniques. The circles represent nonchilled cultures, and the squares represent cultures which were chilled during the collection period. (A) Cell concentrations are represented by the solid figures, and the open figures represent the fraction of cells incorporating thymidine-$^3$H (1 $\mu$c/ml administered to the culture at T = 30 min), and (B) data points represent values for the population modal volume as a function of time after resuspension of cells in a spinner flask (10).

suggesting the need for caution in equating nucleolar (the site of ribosomal RNA synthesis is the nucleolus) and ribosomal synthetic rates.

Such a suggestion is in accord with Steele's observation (15) that RNA cistrons of rat liver nucleoli account for only 0.6% of the total nucleolar DNA. However, additional work will be required for adequate interpretation of our results concerning types of RNA synthesized by nucleoli. Of particular importance is the method of purification of nucleoli. Vesco and Penman (16) have demonstrated that the partition of 60S nuclear particles or nuclear heterodisperse RNA between the nucleolar and nucleoplasmic fractions is a function of the salt concentration used during isolation of the nucleoli. They conclude that "the demands of nucleolar purity and nucleolar integrity are conflicting and that a compromise must be made which depends on the purpose of the experiment." Accordingly, studies on the kinds of newly synthesized RNA's present in Chinese hamster cell nucleoli prepared by a variety of techniques are in progress (in collaboration with R. A. Walters and A. G. Saponara). To date we have noted that low-molecular-weight, methylated RNA's (17) of the type termed "U-rich, 4–7S nuclear" by the workers in Busch's laboratory (18, 19) or "small molecular weight monodisperse nuclear RNA" as termed by Weinberg and Penman (20) are present in our nucleoli preparations when the procedure employing bentonite described in this paper is utilized. These same species are present in the nucleoplasm (and, to some extent, in the cytoplasm) when polyvinyl sulfate (PVS) is substituted as a nuclease inhibitor. Further, the PVS nucleoli show the classic pattern of high-molecular-weight RNA species (14, 21, 22), including the presence of 45, 32, and 28S RNA and the absence of 18S RNA. On the other hand, nucleoli prepared without PVS contain 18 and 28S with little or no 45S RNA, suggesting that conversion of precursor had occurred. Such conversion would be in accord with the observations of Liau et al. (23).

Comparison of data from the preceding experiments employing long and short pulses with



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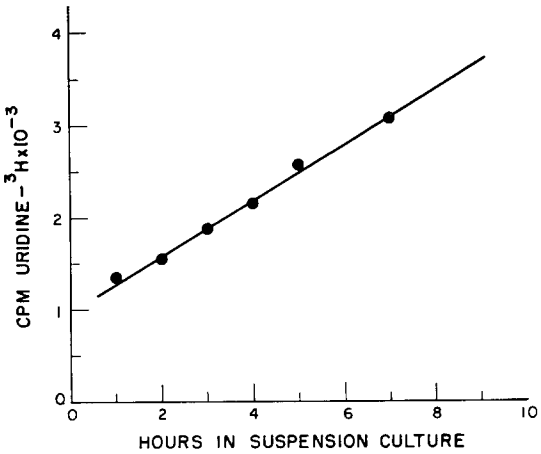


FIGURE 3 Incorporation of uridine-$^3$H into total RNA during $G_1$ and S. Aliquots of 40 ml, prepared by the continuous harvest technique, were pulse-labeled with 200 $\mu$c of uridine-5-$^3$H for 15-min periods throughout early interphase. Samples of 10 ml were then removed and nonlabeled carrier cells were added. The amount of label in total RNA was determined as described in Materials and Methods. The approximate concentration in each aliquot was $10^5$ cells/ml.

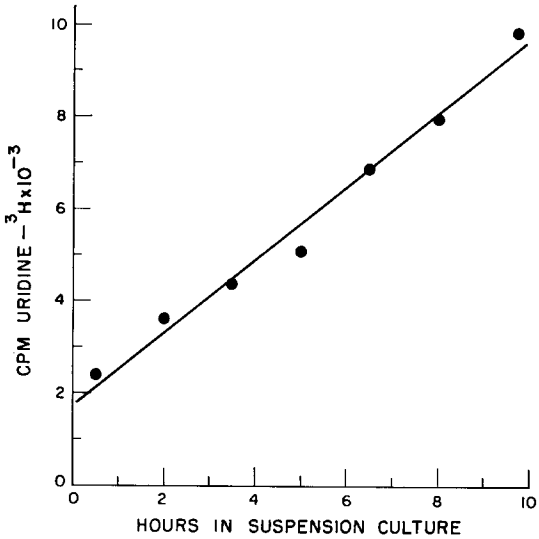


FIGURE 4 Incorporation of uridine-$^3$H into nucleoli throughout interphase. Aliquots of 35 ml of cells prepared by the chill-accumulation method were pulse-labeled for 15-min periods with 175 $\mu$c of uridine-5-$^3$H, and the nucleolar RNA was extracted as described in Materials and Methods. The approximate concentration in each aliquot was $10^5$ cells/ml.

TABLE I

*Uridine-$^3$H Incorporation into Chinese Hamster Cells at Different Times in Interphase*

| RNA sample | Incorporation period (hr) | Incorporated |
|---|---|---|
| | | *cpm* |
| Total | 2–2¼ | 2,860 |
| TCA | 11–11¼ | 9,490 |
| Precipitable | 2–3 | 15,170 |
| RNA | 11–12 | 36,070 |
| Nuclear | 2–3 | 13,010 |
| Supernatant | 11–12 | 47,360 |
| Nuclear | 2–3 | 186,740 |
| Pellet | 11–12 | 430,110 |

Aliquots of 90 ml of cells (approximately 3 × $10^5$ cells/ml) prepared by the chill-accumulation technique were pulse-labeled with 200 $\mu$c of uridine-5-$^3$H. 5-ml aliquots were removed at 15 and 60 min for determination of incorporation into total RNA. Incorporation after 60 min into nuclear supernatant and nuclear pellet fractions was then determined for the remaining 80 ml as described in Materials and Methods.

TABLE II

*Relative Rates of Isotope Incorporation into Chinese Hamster Cells for Late and Early Interphase*

| Length of exposure to isotope | Ratio of 11-hr value to 2-hr value: Uridine-$^3$H into total RNA | Uridine-$^3$H into nuclear supernatant | Uridine-$^3$H into nuclear pellet | Methionine-methyl-$^{14}$C into 18S |
|---|---|---|---|---|
| (*min*) | | | | |
| 15 | 3.3 | — | 3.0 | — |
| 60 | 2.4 | 3.6 | 2.1 | 2.1 |

The labeling protocol is described in Table I.

$^3$H-uridine indicate that the extent of increase in synthetic rate around the life cycle is different for stable and unstable RNA species. Our data do not indicate whether all species of stable (i.e., ribosomal, transfer, methylated low-molecular-weight monodisperse) RNA increase to the same extent throughout interphase, nor do they indicate whether all unstable (i.e., messenger, nuclear heterogeneous, cytoplasmic heterogeneous, etc.) species increase in rate in identical fashion across the life cycle. Further work will be required in order to resolve this problem and to determine whether or not pool sizes vary at different times



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in interphase, thereby contributing to variations in synthetic rates.

The above results were obtained when mitotic cells were seeded directly into spinner flasks immediately after collection. Similar results (continuous RNA synthetic rate) were obtained by Scharff and Robbins (3) for cultures of S3 HeLa cells also allowed to continue growth in suspension culture. Pfeiffer and Tolmach (1, 2), in essentially similar experiments except that the mitotic S3 HeLa cells were placed directly onto petri dishes, observed a sudden increase in RNA synthetic rate at the time of genome duplication. To investigate the possibility that monolayer cells yield a different pattern of RNA synthesis than suspension cells, mitotic Chinese hamster cells were placed in plastic tissue culture flasks, and the rate of uridine incorporation was determined throughout interphase. Fig. 5 presents data for RNA synthetic rates at three cell densities ranging from $10^4$ to $10^6$ cells per flask (25 $cm^2$). As in suspension cultures, the rate of RNA synthesis was continuous across interphase for monolayer cells, although the magnitude of the rate increase was greater at the higher cell densities, and the slope of the rate increase was the same in suspension and in monolayer cultures seeded at $10^6$ cells per flask. Thus, the differences in synthetic rate obtained with HeLa cells (1–3) rre probably not due to the mode of culture asuspension vs. monolayer) but instead may be (elated to undefined nutritional factors or intrinsic cell differences. "Intrinsic differences" need not imply profound biochemical differences. For example, the RNA synthetic rate in Chinese hamster cells could be regulated by the amount of ribosomal coat protein or messenger-ribonucleoprotein available such that the amount of rate-limiting compound would increase as the protein-synthetic capacity of the cell increased during interphase. Other similar, testable *ad hoc* models may also be envisioned. In any event, it appears

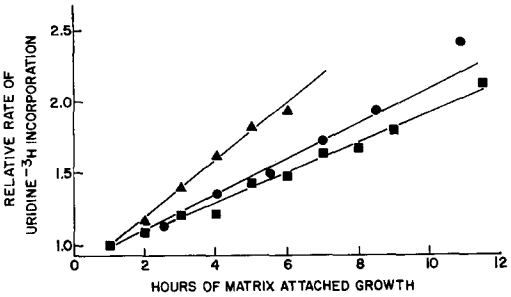


FIGURE 5 Incorporation of uridine-$^3$H into total RNA in matrix-attached cells. The material was centrifuged immediately after mechanical selection, and the cell pellets were appropriately diluted to yield concentrations of $10^6$ (triangles), $10^5$ (circles), or $10^4$ (squares) cells in 25-$cm^2$ plastic flasks. Cultures were pulse-labeled with uridine-5-$^3$H for 15-min periods with 11 $\mu$c/ml in flasks containing $10^6$ cells, with 12.5 $\mu$c/ml in flasks containing $10^5$ cells, and with 25 $\mu$c/ml in flasks containing $10^4$ cells. RNA was extracted as described in Materials and Methods. Activity of samples labeled from 1.0 to 1.25 hr ranged from 1.5 to 25 $\times$ $10^3$ cpm/aliquot for the various cell densities. The data were normalized to a value of 1.0 for cultures labeled during the interval 1.0–1.25 hr after detachment.

that RNA synthesis in the cultured mammalian cell can follow patterns other than that predicted by a model for a gene-dosage effect, indicating that control of RNA synthesis need not merely be restricted to simple regulation by the amount of genome present.

The authors wish to thank Dr. E. C. Anderson for providing the volume determinations and also wish to thank Dr. D. F. Petersen for helpful comments during preparation of the manuscript. The excellent technical assistance of Mrs. Evelyn Campbell and Mr. John Hanners is gratefully acknowledged.

This work was performed under the auspices of the U. S. Atomic Energy Commission.

*Received for publication 26 December 1968, and in revised form 14 February 1969.*

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