RNA SYNTHESIS IN CHINESE HAMSTER CELLS

I. Differential Synthetic Rate for Ribosomal RNA in Early and Late Interphase

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ABSTRACT

The incorporation of methionine-methyl-^{14}C into 18S ribosomal RNA of cultured Chinese hamster ovary cells in early and late interphase has been determined by zone-sedimentation analysis of phenol-extracted RNA preparations. Synchronized cell cultures were prepared for these studies by thymidine treatment and by mechanical selection of mitotic cells. The specific activity of 18S RNA labeled in late interphase was found to be 1.1–1.2 times that of 18S RNA labeled in early interphase. Upon correction for increase in RNA mass, the rate of methylation of 18S RNA in late interphase is about 1.9 times that in early interphase.

INTRODUCTION

DNA synthesis in the mammalian cell is confined to a discrete segment of the life cycle (the S phase), in contrast to bacterial systems in which DNA is synthesized throughout the interphase period. Thus there is a time (G_2) in the life cycle of mammalian cells during which the genome has been duplicated but cell division has not yet occurred. During this interval, direct or indirect gene-dosage effects on the rate of RNA synthesis should be detectable. In this connection, a number of investigators have determined the rate at which nucleoside RNA precursors are incorporated during the various phases of the mammalian life cycle. In the case of HeLa cells and various Chinese hamster cell lines, incorporation of labeled nucleoside is continuous throughout interphase, the rate of incorporation in late G_2 being as much as three times that obtained in early G_1 (1–4). During some stages of mitosis, however, there is no detectable incorporation (1, 3, 5, 6). Since such studies involve measurement of the rate of incorporation of a precursor into both stable (transfer, ribosomal) and unstable (messenger, nuclear-heterogeneous) (7) RNA, they do not necessarily reflect the rates at which ribosomal and transfer RNA are synthesized during the life cycle. That the rate of synthesis, as well as the change in rate, during early interphase are different for nucleolar and nonnucleolar RNA of kidney cells has been noted (8). Ribosomal RNA synthesis during G_1, S, and G_2 has been demonstrated by studying incorporation of nucleoside into 45S ribosomal precursor and ribosomal RNA in synchronized HeLa cells (2). However, these measurements do not permit quantitation of the relative rates of ribosomal RNA synthesis through the cycle.

In this paper we report the results of experiments designed to measure specifically the relative rate of ribosomal RNA synthesis (measured by its rate of methylation) in early and late interphase. We made use of the fact that there is detectable incorporation of ^{14}C from methionine-methyl-^{14}C into transfer and ribosomal RNA but not into less

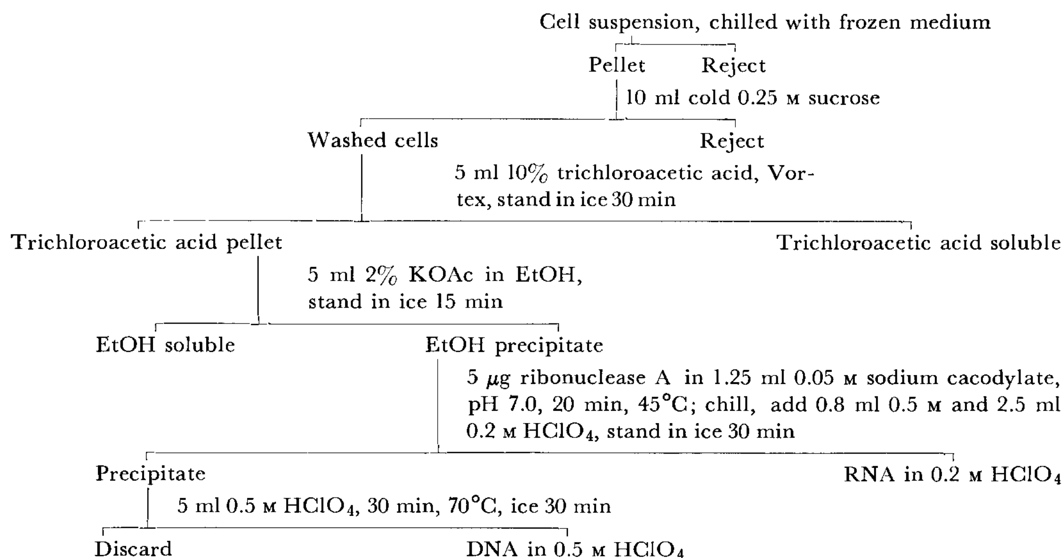

FIGURE 1 Modified Schmidt-Thannhauser procedure.

stable messenger or nuclear-heterogeneous RNA (9, 10). It is noted that such methyl-incorporation studies reflect the rate of ribosomal RNA synthesis only in the absence of pool effects and only if the degree of methylation is constant throughout the cycle. Also, results could conceivably be affected if conversion of the less methylated 45S precursor (11) occurs at different rates throughout interphase.

The results reported here demonstrate that the specific activities of 18S RNA from early and late interphase populations labeled with methionine-methyl-^{14}C are those expected if the rate of ribosomal RNA methylation in late G_2 is approximately twice that in early G_1.

MATERIALS AND METHODS

Synchronization of Cell Growth

The Chinese hamster ovary (CHO) cells employed in this study were aneuploid with a modal chromosome number of 21. Cells were maintained free of PPLO (pleuropneumonia-like organism) as suspension cultures and in bottles of F-10 medium (12) without added calcium, supplemented with 10% calf and 5% fetal calf sera, penicillin, and streptomycin. Cell growth was synchronized (13) by treatment of suspension cultures wtih 10 mM thymidine for 12 hr, followed by resuspension in normal medium. 8 hr later a second 10 mM thymidine blockade was applied and was reversed 13 hr later by again resuspending the cells in fresh medium.

TABLE I

Schmidt-Thannhauser Fractionation

Fraction	dpm per 0.5 ml aliquot	
	Uridine-^3H	Thymidine-^3H
Trichloroacetic acid	22,200	1210
Ethanol solution	15	2
RNA	13,500	0
DNA	320	2110

Populations of cells in mitosis were prepared by selectively detaching mitotic cells from monolayer cultures with a mechanical shaker (14). Six cultures, grown in Pyrex Blake bottles in the low calcium medium described above, were shaken simultaneously in a Precision equipoise reciprocating shaking machine (Precision Scientific Co., Chicago, Ill.) for 5 sec, after which the overlay liquid containing detached cells was removed, and an aliquot of 25 ml of fresh medium was added. This shake treatment was repeated at intervals of 10 min thereafter, and the material from the first seven shake treatments was discarded. Material from the eighth and subsequent shake treatments was used in experiments. The fraction of mitotic cells was always 0.9 or greater. Samples collected at 10-min intervals over a 4 hr period were chilled in an ice bath immediately after collection; upon subsequent resuspension in warm medium, the cells divided synchronously.

Cell concentrations were determined to a statistical

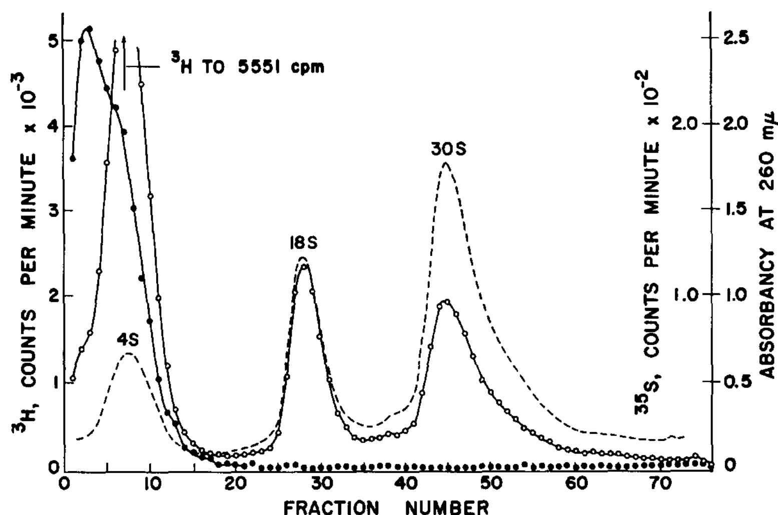

FIGURE 2 Chinese hamster ovary cells growing in normal F-10 medium were labeled for three generations with 0.25 μc/ml of methionine-methyl-^{3}H (New England Nuclear Corp., Boston, Mass., 0.77 mc/mg) plus 0.5 μc/ml of methionine-^{35}S (Schwarz Bio Research Inc., Orangeburg, N.Y., 47.3 mc/mmole < 2 wk before use). RNA was hot-phenol extracted, and zone-sedimentation analysis was performed on an aliquot. Absorbancy (----), ^{3}H (-O-O-), and ^{35}S (-●-●-).

precision of 1% or better with an electronic particle counter.

Modified Schmidt-Thannhauser Procedure

The procedure is summarized in Fig. 1. All precipitates were extracted a second time with 5 ml of the reagents specified. Supernatants were pooled and diluted to 10 ml in a volumetric flask. Radioactivity in aliquots of 0.5 ml was determined in a Packard Tri-Carb (Packard Instrument Co., Inc., Downers Grove, Ill.) liquid-scintillation spectrometer after the addition of 0.75 ml of water and 10 ml of Bray's scintillation fluid (15). Quenching corrections were made by using internal standards with suitable blanks obtained from cold cell extracts. Ultraviolet absorption spectra were obtained with a Unicam recording spectrophotometer. Ribonuclease A (EC 2.7.7.16) was purchased from Worthington Biochemical Corporation. Typical data for a 30-min incorporation of 1 μc/ml of uridine-^{3}H or thymidine-^{3}H into Schmidt-Thannhauser fractions of exponentially growing Chinese hamster ovary (CHO) cells are shown in Table I.

Hot Phenol–RNA Preparations

Hot phenol extraction of Chinese hamster cell RNA was performed as previously described (11). The procedure involves equilibration of a buffered cell suspension with phenol at 60°C in the presence of sodium dodecyl sulfate and bentonite, followed by two cold phenol and three ether extractions and ethanol-acetate precipitation. An addition to the procedure was made in that the ethanol-acetate precipitate, after dissolution in pH 7.0, 0.05 M sodium cacodylate buffer, was dialyzed against 500 volumes of cold 0.1 M NaCl, 0.05% sodium dodecyl sulfate for 18–24 hr before sedimentation analyses were performed. In some instances, the RNA preparations were digested with deoxyribonuclease I (EC 3.1.4.5, Worthington, DPFF) in the presence of 5 mM $MgCl_2$ prior to dialysis.

Zone Sedimentation

Zone sedimentation analyses of hot phenol-prepared RNA were performed as previously described (11).

RESULTS

Nature of Methionine-Methyl-^{14}C Incorporation

Transfer and ribosomal ribonucleic acids of bacterial and mammalian cells are methylated. In bacterial tRNA, methylation occurs at the polynucleotide level by transmethylation with *S*-adenosyl-L-methionine (16). In the case of CHO cells,' it has been shown that all isotope incorporated into ribosomal RNA after 2 hr exposure of the cells to methionine-methyl-^{14}C is susceptible

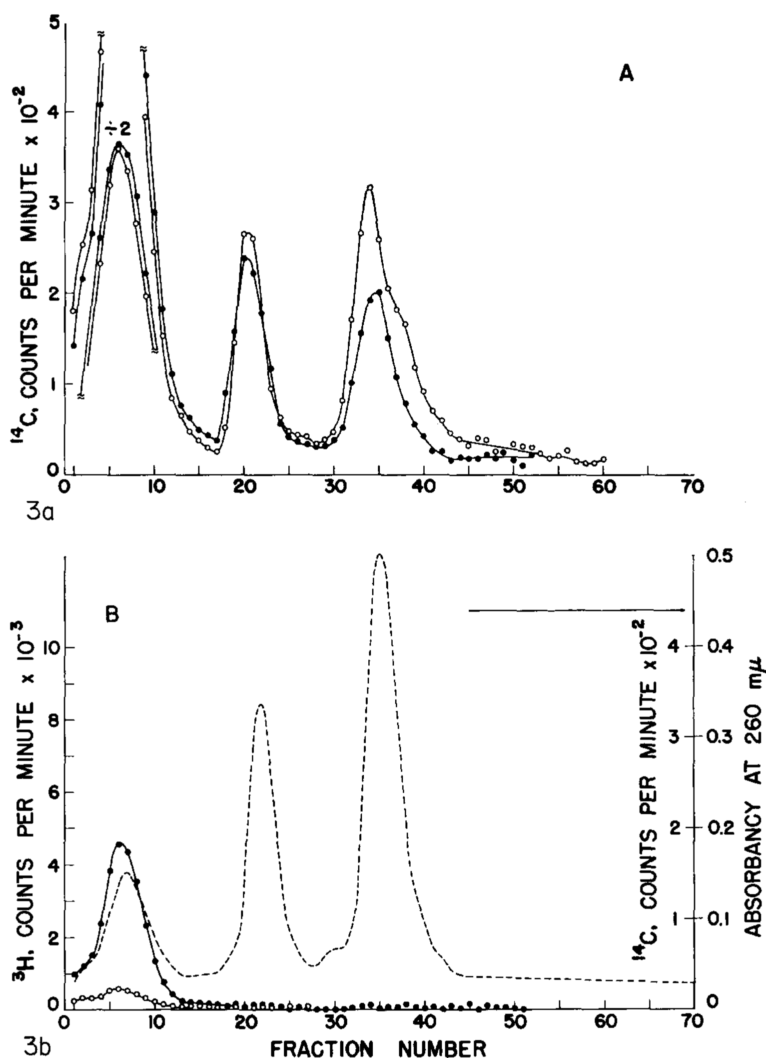

FIGURE 3 *a*, Chinese hamster ovary cells growing in methionine-free F-10 medium were pulsed with 0.3 μc/ml of methionine-methyl-^{14}C for 1 hr. Actinomycin D was then added to a concentration of 10 μg/ml; 7½ min after the addition of actinomycin, a sample was taken for hot-phenol preparation of the RNA, and 4 hr later another sample was taken. Zone-sedimentation analyses were performed, and the resulting data are plotted as counts per minute—normalized to the same absorbancy of RNA. The counts per minute for the first (pulse) sample are represented by the open circles (-○-○-), and those for the chase are shown by closed circles (-●-●-). *b*, In the same experiment an aliquot of cells was pretreated with actinomycin D for 7½ min prior to addition of 1 μc/ml of uridine-^{3}H and 0.3 μc/ml of methionine-methyl-^{14}C. RNA was extracted after labeling for 1 hr and 20 min. Absorbancy (----), ^{3}H counts per minute (-○-○-), and ^{14}C counts per minute (-●-●-).

to ribonuclease A (11). The data shown in Fig. 2 demonstrate that there is no detectable incorporation of methionine-^{35}S label into CHO ribosomal RNA.

The stability of methionine-methyl-^{14}C-labeled 18S rRNA to actinomycin chase is shown in Fig. 3 *a*. The slight decrease in methylated 18S is within experimental error and, in a number of experiments involving pulse times of 45–120 min, there was no significant increase or decrease of

methylated 18S after 4 hr actinomycin chase. In the experiment described in the legend of Fig. 3 *a*, the durations of isotope incorporation and subsequent actinomycin treatment are such that, when uridine-^{3}H is used as the isotopically labeled RNA precursor, over 50% of the isotope incorporated into total RNA decays during the actinomycin treatment. That no ^{14}C is incorporated into 18S RNA after 7½ min pretreatment with actinomycin, the time at which the "pulse" sample is taken, is shown in Fig. 3 *b*. The lability of labeled RNA in the 30S–35S region may reflect the fact that the 30S ribosomal RNA and 35S ribosomal precursor RNA remain in the nucleus for an appreciable amount of time (compared to 18S which apparently exits immediately after formation) and that actinomycin causes nuclear RNA to remain in the nucleus and to suffer abnormal degradation (17–19).

That the specific activity of pulse-methylated 18S RNA does not decrease during actinomycin chase supports our contention that we are measuring the incorporation of isotope into only the relatively stable species which sediment in the 18S region. Presumably, such stability is a property of the rRNA and not of the messenger and nuclear-heterogeneous RNA.

The methionine-methyl-^{14}C pulses used in the experiments reported in this paper were administered to cells growing in normal F-10 medium possessing a full complement of methionine. Nonetheless, the possibility that cell growth would deplete the medium of enough methionine to increase the specific activity in a later pulse, compared to an earlier one, was investigated. A 1 hr pulse of 200 μc methionine-methyl-^{14}C was given to a 200 ml culture of Chinese hamster cells which had been resuspended in fresh F-10 medium for 1 hr. The cell concentration was 240,000 per milliliter halfway through the pulse. After 12 hr of growth, a second aliquot of the culture was similarly pulsed. Average cell density during the second pulse was 370,000 per milliliter. RNA was prepared from the pulsed cells, and zone sedimentation analyses were performed. The specific activities of the 18S peaks were measured and found to be 730 and 735 cpm per A_{260}. It was concluded that, with respect to the cell densities and times between pulses used in the experiments on synchronized cells, there would be at most a small percentage difference in the specific activities of the pulse-labeled RNA from early and late interphase cells that could be attributed to growth depletion of methionine in the medium.

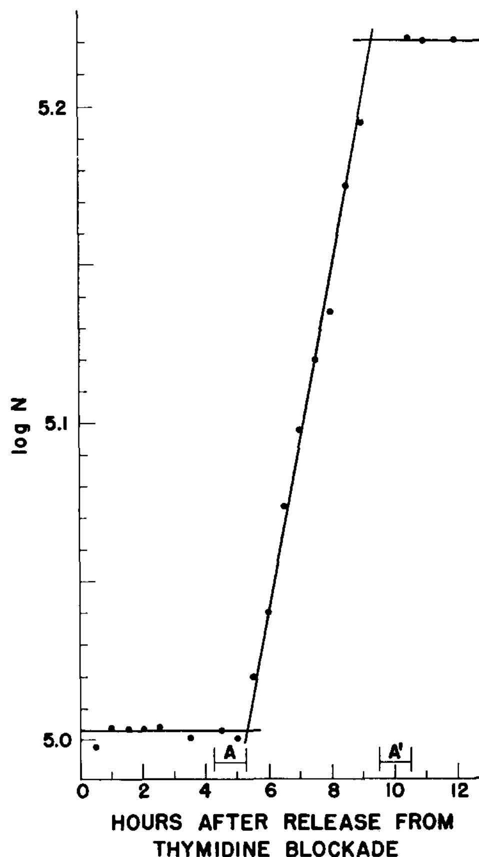

FIGURE 4 Cell concentration as a function of time after release from double thymidine blockade. *A* and *A'* represent labeling periods with methionine-methyl-^{14}C.

Isotope Incorporation during Early and Late Interphase

For the obtaining of populations rich in G_1 or G_2 phase cells, two methods were employed for synchronizing cell growth. In the first method, synchronous cell growth was chemically induced by treating the cells with 10 mM thymidine, and at the time of release from the second thymidine blockade all cells were located at the G_1/S boundary. Cell count data, together with the period of labeling with methionine-methyl ^{14}C, are shown in Fig. 4. The first labeling period had terminated just prior to the time at which the first cells commenced dividing; this indicates that there were no G_1 cells during the incorporation period. Because

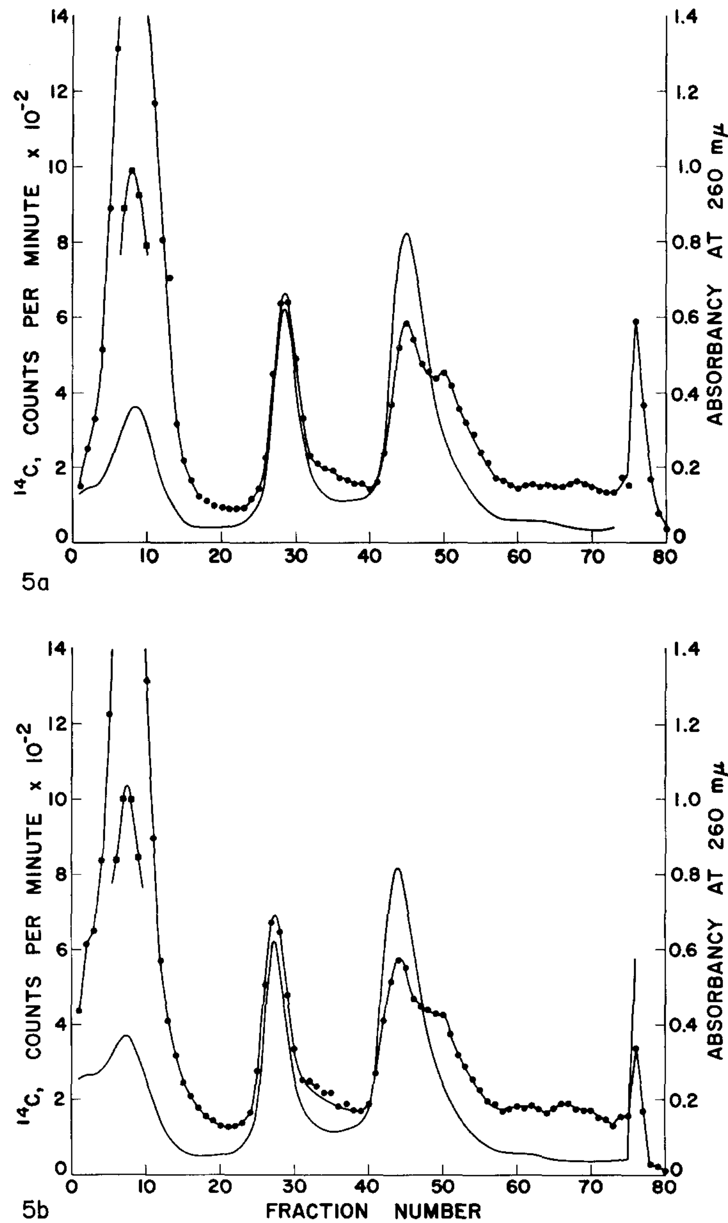

FIGURE 5 Chinese hamster cells were synchronized by using the thymidine block procedure. 4¼ hr after release from the second thymidine block, a 200 ml aliquot was pulsed (late interphase, *A* of Fig. 4) with 200 μc of methionine-methyl-^{14}C (Schwarz Bio Research Inc., Orangeburg, N.Y. 49 mc/mmole) for 1 hr, and the RNA was extracted. An early interphase (*A'* of Fig. 4) pulse was performed 9½–10½ hr after release. The methionine-labeled RNA samples were treated with DNase, dialyzed, and sedimented through sucrose gradients. *a*, Results for the early interphase sample, and *b*, results for the late interphase sample. Absorbancy (——), and counts per minute (-●-●-).

the width of the division wave was 4 hr, and since the G_2 period is 2.7 hr in the CHO cell (19), the fraction of cells in G_2 during the first labeling period is 2.7/4.0 or approximately 70%, with about 25% of the remaining cells in the S phase and 5% mitotic cells. Approximately 5 hr elapsed between the time at which the first cells commenced dividing (Fig. 4) and the time at which the second

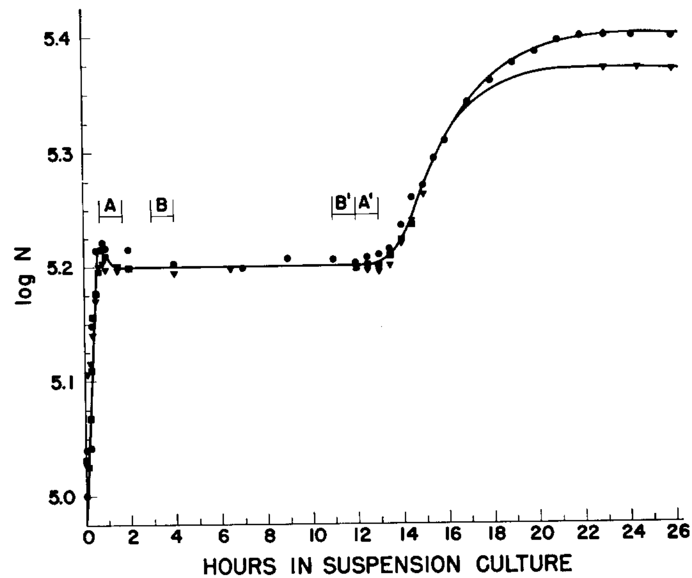

FIGURE 6 Normalized composite cell count data from three cultures synchronized by the mechanical selection technique. Large quantities of cells were obtained by the chill-accumulation protocol described in Materials and Methods. *A* and *A'*, *B* and *B'* represent labeling periods with methionine-methyl-^{14}C in two separate experiments. Actual cell concentrations during labeling periods *A* and *B* were 180,000 per ml and 195,000 per ml, respectively. The third culture was utilized for determination of the mass and rate of uridine-^{3}H incorporation into total RNA (Table II).

labeling period was completed. Since the duration of G_1 in the CHO cell is 4.8 hr (20), essentially all of the cells are located in the G_1 phase during the second labeling period.

Data obtained from zone sedimentation analyses of 18S RNA extracted from the two cultures of thymidine-synchronized CHO cells pulsed with methionine-methyl-^{14}C (Figs. 5 *a* and 5 *b*) yielded specific activity values of 1235 cpm per A_{260} for the culture in which 70% of the cells were located in G_2 during the labeling period and 1109 cpm per A_{260} for the predominantly G_1 population of cells. The ratio of specific activities of 18S RNA between cells labeled late in interphase and cells pulsed in early interphase was 1.11.

Because of the possibility of thymidine-induced biochemical changes, cell growth was also synchronized by mechanical selection of mitotic cells (14). Cell count data for three cultures synchronized by mechanical selection, as well as labeling periods with methionine-methyl-^{14}C for two of the cultures, are shown in Fig. 6. The high degree of reproducibility among the three experiments is readily apparent. In all three cultures, the initial fraction of mitotic cells (0.9 or better) dropped to less than 0.05 in approximately 20 min. The cell number rose by only about 60% during each division wave, but nearly all of this reduced yield of cells could be accounted for by incomplete separation of daughter cells, since the cell concentration was determined with an electronic particle counter. (The counter enumerates two nonseparated cells as a single event.)

For the obtaining of information on cell progress around the life cycle, aliquots were removed at varying times from a suspension culture (set up from mitotic cells) containing thymidine-^{3}H, and the fraction of labeled cells was determined radioautographically. The fraction of labeled cells can be used as a measure of the cumulative fraction of the population which has entered S at any given time (Fig. 7). The duration of the S phase in the CHO cell is 4.1 hr (20); it is possible, therefore, to approximate the fraction of cells which have completed DNA synthesis, i.e. cells which are leaving this phase, by constructing a curve parallel to, but displaced by 4.1 hr from, the curve representing entry into S. Also shown in Fig. 7 are cell count data, normalized by setting the

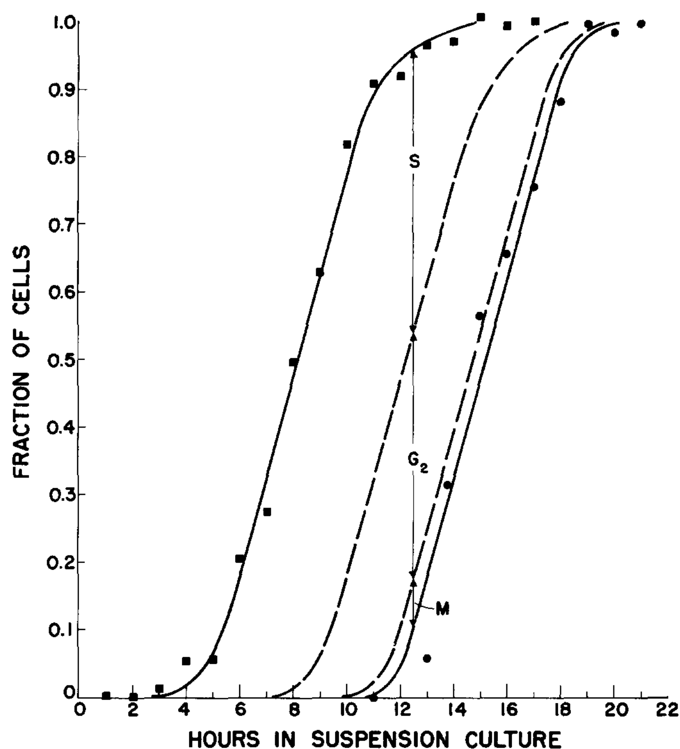

FIGURE 7 Approximate fraction of cells in different phases of the life cycle at different times after setting up a suspension culture of mechanically selected mitotic cells. The squares represent data obtained for the cumulative fraction of cells incorporating thymidine-^{3}H (0.1 μc/ml, 6 c/mM, Schwarz BioResearch Inc., Orangeburg, N.Y.), and the circles represent the fraction of cells dividing in the second round of division (see text for details of normalization). The dotted lines from left to right represent, respectively, the fraction of cells leaving the DNA-synthetic phase and the fraction of cells entering mitosis, based upon estimates of the duration of the S and M phases in the CHO cell.

maximum observed fraction of dividing cells equal to 100%. Normalization is justified by the fact that all the cells in the population completed mitosis, but the cell-number estimation was low because of unseparated daughter cells. From the value for the duration of M in the CHO cell (20), another curve was constructed parallel to the cell division curve in Fig. 7, and it was then possible to approximate the distribution of cells among the various phases of the life cycle during the labeling periods.

During the labeling periods, 0.75–1.75 hr and 12–13 hr (*A* and *A′* in Fig. 6), all of the cells during the initial labeling period were in G_1, while the percentages of cells in the G_1, S, G_2, and M phases at the midpoint of the second labeling period were 14, 42, 37, and 7%, respectively. Similarly, all cells in the culture labeled during the interval 3–4 hr ((*B* in Fig. 6) were G_1 cells, while the distribution of cells among G_1, S, G_2, and M at the midpoint of the labeling period of 11–12 hr (*B′* in Fig. 6) was 10, 52, 35, and 3%, respectively.[1] The zone-sedimentation patterns for RNA extracted from cells pulsed during 3–4 and 11–12 hr intervals are shown in Figs. 8 *a* and 8 *b*. The ratio of specific activity of the 18S RNA species between *A′* and *A* is 1.17 and that between *B′* and *B* is 1.21. Thus, it is apparent that the ratio of specific activities of the 18S RNA species in late and early

[1] That the fraction of G_1 cells is greater in the culture labeled between 12 and 13 hr than in the population labeled between 11 and 12 hr is due to the fact that there are two different sources of G_1 cells. Both slowly moving cells which have not yet begun to synthesize DNA and rapidly moving cells which have traversed the entire life cycle and reentered the G_1 phase (Fig. 7) are considered in the estimate of the G_1 phase.

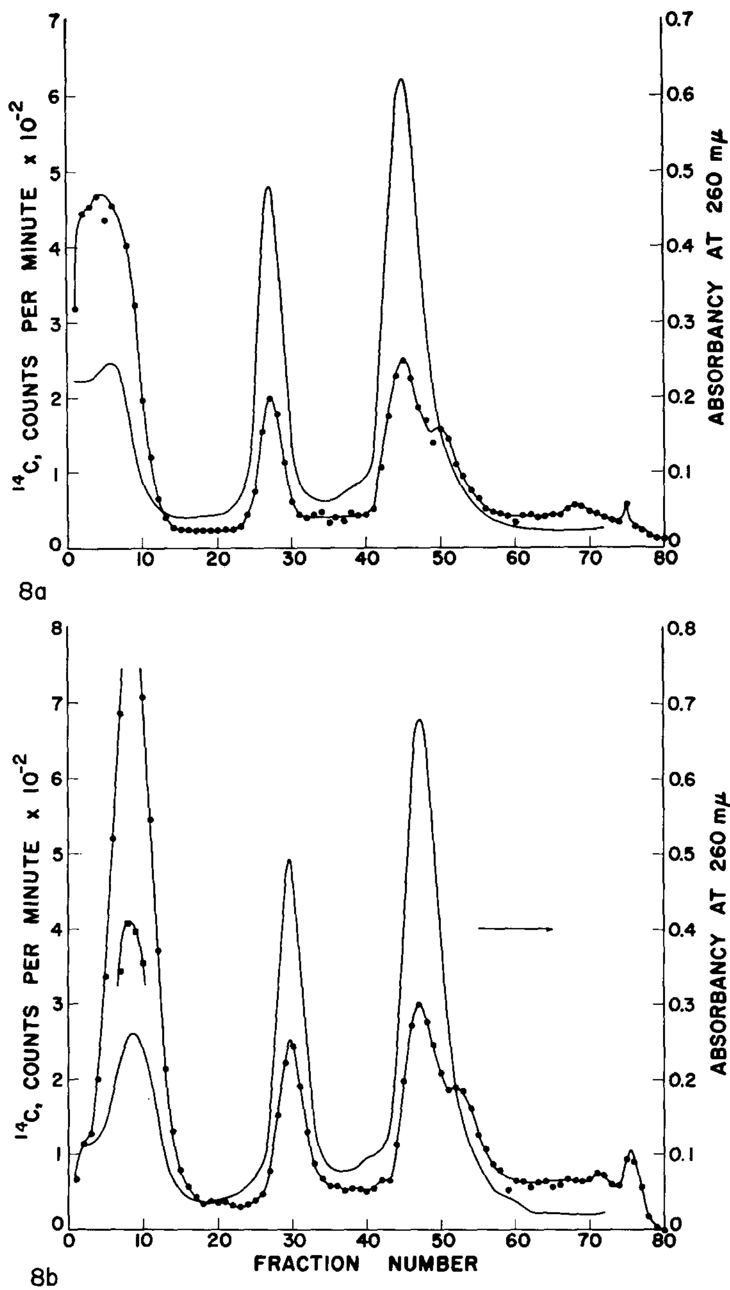

FIGURE 8 Cells were synchronized by the shake technique. The methionine-methyl-^{14}C (1 μc/ml) pulses were done with 160 ml of cells (195,000/ml) at 3–4 hr (*B* of Fig. 6) and 11–12 hr (*B'* of Fig. 6) after release. RNA preparations were treated with DNase prior to dialysis and sedimentation analysis. *a*, Early interphase, and *b*, late interphase. Absorbancy (——), and counts per minute (-●-●-).

interphase is comparable in cultures prepared by either thymidine synchronization or mechanical selection.

In the case of samples *A* and *A'*, a common spinner culture of shake-synchronized cells was employed, and the RNA preparations were dialyzed against a common dialyzate and sedimented through identical sucrose solutions. The same

TABLE II

Schmidt-Thannhauser Determination of Uridine-^{3}H Incorporation

Sample	Pluse time after release	RNA fraction	RNA fraction	$\frac{cpm}{A_{max}}$
	hr	*cpm/ml*	A_{max}	
A	1.75–2.00	530	0.20	2650
A′	12.25–12.50	2584	0.33	7830
B	3.75–4.00	662	0.20	3310
B′	11.50–11.75	2574	0.30	8580

Samples A and A′ were withdrawn from a common spinner culture. B and B′ were withdrawn from a parallel culture set up from the same batch of shake-synchronized cells as A and A′.

TABLE III

Methionine-methyl-^{14}C Incorporated into 18S RNA

Sample	Pulse time	Specific activity of 18S peak
	hr	
A	0.75–1.75	646
A′	12.00–13.00	758
B	3.00–4.00	427
B′	11.00–12.00	517

Methionine-methyl-^{14}C incorporation, RNA extraction, and analysis conditions were identical for A and A′ and for B and B′; A or A′ specific activity data are not to be compared with *B* or *B′* data.

batch of scintillation fluid was mixed with the sucrose gradient fractions, and the same amount of time elapsed between addition of fluid and determination of counts per minute in the scintillation counter. These steps were needed to make data from analysis of samples *A* and *A′* directly comparable. Since *B* and *B′* were prepared at a later date, different media, reagents, etc., were employed. The conditions of sample preparation and analysis and, therefore, specific activity of the pulses and the efficiency of counting were not identical with those used with *A* and *A′*; data from *A* and *A′*, therefore, are not to be compared with data from *B* and *B′*.

In a parallel shake-synchronization experiment, aliquots were removed for determination of RNA mass and radioactivity (modified Schmidt-Thannhauser procedure) after 15-min pulses with uridine-^{3}H. The results are shown in Table II. A summation of the data on incorporation of methionine-methyl-^{14}C into 18S RNA of cultures prepared by mechanical selection of mitotic cells, is shown in Table III. One may note from the data in Table II that in the CHO cell, as in other Chinese hamster cell lines investigated and HeLa cells (1–4), the rate of nucleoside incorporation into RNA of cells in late interphase is more than three times that of cells in early interphase.

DISCUSSION

The data obtained from zone-sedimentation analyses of methyl-labeled, hot phenol-prepared RNA are expressed in terms of the specific activity of 18S ribosomal RNA rather than as a rate of methylation, because the numerous and variable volume losses occasioned during the phenol extractions, ethanol precipitation, and dialysis steps do not permit quantitative recovery of the RNA. However, one may combine the data for increase of RNA mass with the (separately obtained) data on the specific activities of methionine-methyl-^{14}C– labeled 18S RNA to estimate the rate of methylation of 18S RNA in early and late interphase. Thus, we multiply the specific activities (18S) by the absorbancies (total RNA) to obtain approximate rates:

$$\frac{^{517cpm/A}18S\ (11\text{–}12\ hr)}{^{427cpm/A}18S\ (3\text{–}4\ hr)} \times \frac{^{0.30A}total\ (12\ hr)}{^{0.20A}total\ (4\ hr)} \cong \frac{\text{late interphase rate}}{\text{early interphase rate}} = 1.8.$$

Similarly,

$$\frac{^{758cpm/A}18S\ (12\text{–}13\ hr)}{^{646cpm/A}18S\ (0.75\text{–}1.75\ hr)} \times \frac{^{0.33A}total\ (12\tfrac{1}{2}\ hr)}{^{0.20A}total\ (2\ hr)} \cong \frac{\text{late interphase rate}}{\text{early interphase rate}} = 1.9.$$

The approximate nature of these estimates derives from the fact that insufficient shake-synchronized cells were produced to allow us to make simultaneously a determination of the specific activity of 18S RNA by zone sedimentation analyses of phenol-prepared methionine-methyl^{14}C–labeled RNA and a determination of total RNA mass by the modified Schmidt-Thannhauser method. Also, the accuracy of such estimates depends on how closely the increase in 18S RNA parallels the increase in total RNA mass.

The data on relative rates of methylation in the G_2 and G_1 portions of the life cycle obtained to date are inconsistent with a doubling of rRNA synthesis rate (per aliquot of cell culture) across M. That is, the rate of rRNA synthesis measured by rate of 18S rRNA methylation in Chinese hamster ovary cells is not a constant function of cell number but is almost twice as great in late G_2 as in early G_1.

Data from late G_2 and early G_1 samples agree with predictions of a model in which the rate doubles across S. However, the earlier G_2 and later G_1 set show a somewhat lower differential (1.8) than expected for an abrupt doubling in rate sometime in S. Theoretically, one could determine the abruptness of the rate doubling by performing the type of analysis reported in this paper on cells from increasingly later G_1 and earlier G_2 populations, obtaining G_2/G_1 specific activity ratios that increase markedly in the case of an abrupt doubling. The dispersion of synchrony one encounters experimentally (see Fig. 7), however, makes such an approach unfeasible with the techniques currently available. By measuring the absolute rate of rRNA synthesis throughout G_1, it may be possible to define further the nature of the change in synthesis rate during interphase.

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