SYNTHESIS OF RNA IN MAMMALIAN CELLS DURING MITOSIS AND INTERPHASE

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ABSTRACT

Chinese hamster cells in the mitotic and G_1 phases of the growth cycle were incubated for 30 or 60 min in suspension tissue culture and pulse-labeled with tritiated uridine. After appropriate chases, washes, and extractions, it was found that all incorporation into the nucleic acid may be accounted for by those cells in interphase. An average of 410 counts was found for incorporation into the cell population (approximately 2.0×10^5 cells) of which over 80% of the cells was initially in mitosis. The increasing number of cells leaving mitosis and entering interphase during the 30 min incubation was theoretically able to account for 470 counts. In addition, short-pulse labeling experiments have shown a consistent linear relationship between the percentage of cells in division and the incorporation of the isotope, which strongly suggests that, if 100% of the cells were in mitosis, the counts would be essentially zero. Thus, the entire label may be attributed to those cells in interphase where portions of the chromosomal material are known to be already extended.

INTRODUCTION

It has become generally accepted by cell biologists that the condensed chromosomes of mammalian mitotic cells are incapable of appreciable nucleic acid synthesis (2, 10–12, 24, 25, 35, 36). Reports, however, continue to persist of some minimal incorporation of isotopically labeled nucleotides into chromosomes during various stages of meiosis and mitosis. These reports of studies include investigations on tissue culture cells (17, 20), the spermatogenesis stage of the locust (9) and grasshopper (21), and on synchronized cultures of *Physarum polycephalum* (3, 18, 19). In addition, a few studies continue to show a low level of cytoplasmic RNA synthesis in the mitotic cell (8, 14, 22).

All of the previous studies with mammalian cells have utilized radioautographs in the investigation of incorporation of label into chromosomes. The development of techniques to synchronize mammalian cells, both with (34) and without the use of drugs (27, 36), has made it possible to study incorporation of nucleic acid precursors throughout the mitotic and interphase periods. Recent investigations have shown a general increase in uridine uptake in the G_1 period (31) and two maximum peaks in RNA synthesis in the G_1 and G_2 periods (3, 16, 19). One report has shown continuous increase in RNA synthesis through the S period with a constant level maintained throughout G_2 (6). In all these studies, the first determinations were made after the cells had proceeded into interphase.

It is the purpose of this paper to demonstrate the pattern of incorporation of labeled uridine into RNA in synchronized Chinese hamster cell populations, particularly in the short time period during and immediately following mitosis.

MATERIALS AND METHODS

Chinese hamster cells (*Cricetus griseus*) (Strain Dede, CHL-48, from T. C. Hsu), were grown at 37°C in a CO_2 incubator with Ham's F-10 medium (13) (Hyland Laboratories, Los Angeles, Calif.) enriched

with 3% newborn calf serum and 1% heart infusion broth. 24 plastic bottles (250 cc capacity) were seeded, each with 4×10^6 cells in 10 cc of medium. 20 hr later the medium was decanted, the monolayers were washed twice with saline, and 10 cc of calcium-free F-10 medium were added to each culture. After an additional 2 hr of incubation, the mitotic cells were shaken off the surface of the monolayers by the methods of Robbins and Marcus (27) and Terasima and Tolmach (36).

The released mitotic cells were centrifuged at 800 rpm for 5 min and the pellet was resuspended in 1 ml of prewarmed F-10 medium plus calcium at pH 7.4. Clumped cells were dispersed by repeated pipetting with a fine-bore pipette. One drop of the cell suspension was stained with aceto-orcein, and 300 cells were counted for a mitotic index. An additional drop was utilized for a cell count in a standard hemocytometer, and yields ranged from 7.5×10^5 to 3×10^6 cells per an average total of 24 bottles.

The centrifuged mitotic cells were diluted in complete medium to a final cell concentration of 2×10^5 cells per cc, and the cells were divided into two groups: (*a*) the T_0 samples, in which greater than 80% of the cells was in mitosis and in which uridine incorporation was immediately measured (0.1 μc/ml uridine-^{3}H, specific activity 1.9 c/mmole, Schwarz BioResearch Inc., Orangeburg, N.Y. for 30 min incubation), and (*b*) the T_{30} samples, which were allowed to proceed through mitosis until over 80% of the cells was in interphase, at which time a similar incorporation study with uridine was carried out. After incubation with uridine, the cells were centrifuged, the isotope solution was removed, the cells were washed, and the pellet was resuspended in chase medium (1 mg/ml unlabeled uridine in F-10 medium) for an additional 30 min. At the end of the chase period the pellet was washed once with 1 mg/ml uridine in saline, twice with saline alone, and finally resuspended in 0.5 cc of cold 5% trichloroacetic acid and extracted overnight at 4°C. The following morning, the cells were washed twice in fresh, cold trichloroacetic acid, once in cold acetone, permitted to air-dry, and dissolved in 0.5 cc of Hyamine hydroxide (Packard Instrument Co., Inc., Downers Grove, Ill.). The Hyamine was dissolved in [9.5 cc 0.4%

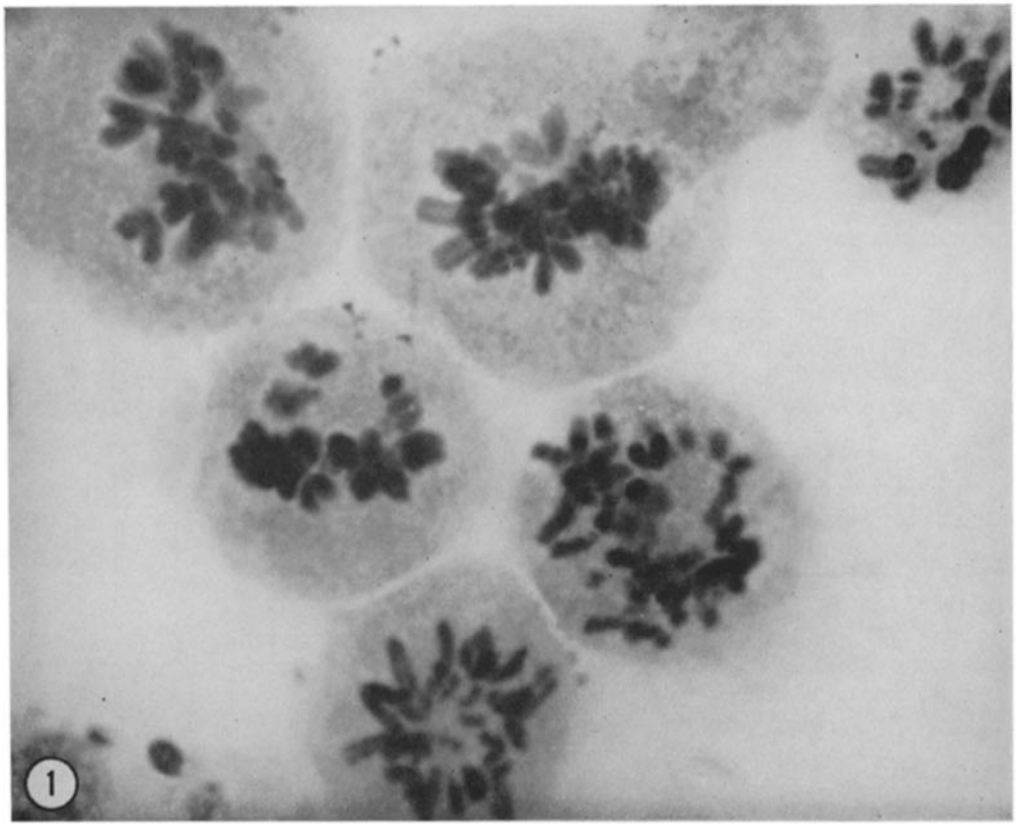

FIGURE 1 Synchronized Chinese hamster cells, 80–86% in mid-mitosis. Aceto-orcein. × 1650.

TABLE I

Mitotic Index during the 1st Hr of Incubation

Each value represents a count of 300 cells. The time of incubation corresponds to the time after the cells were shaken off the monolayer, with a short lag during which the cells were centrifuged.

Time of incubation	Cells in mitosis	Cells in interphase
min	%	%
0	88	12
5	84	16
10	60	40
15	41	59
20	29	71
25	26	74
30	17	83
45	12	88
60	8	92

2,5-diphenyloxazole and 0.01% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene in toluene, scintillation counter fluid, and samples were counted for 10 min in a Packard Tri-Carb Model 3003 liquid scintillation spectrometer.

In a second series of experiments, mitotic cell populations obtained in the same manner were centrifuged at 2200 rpm for 1 min and the pellet was resuspended in prewarmed F-10 medium plus calcium at pH 7.4. The cell suspension was maintained at 37°C for 1 hr. At appropriate times (e.g., 0, 10, 20, and 60 min after synchronization), approximately 2×10^5 cell aliquots were removed and mitotic indices scored. These cells were then incubated in 1 ml of uridine-^{3}H (10 μc/ml, specific activity 1.9 c/mmole, Schwarz) for 60 sec. All subsequent centrifugations were done at 2200 rpm for 1 min at 4°C. The cells were washed twice with 10 cc of F-10 medium containing 1 mg/ml unlabeled uridine at 4°C, once with 10 cc of saline at 4°C, and once with 2 cc of cold saline, after which the pellet was extracted overnight in 5% cold trichloroacetic acid and counted as in the T_0 and T_{30} experiments.

RESULTS

Chinese hamster cells, synchronized in the described manner, yield cell populations with greater than 80% of the cells in the metaphase stage (Fig. 1). After returning the cells to complete medium in a CO_2 incubator at 37°C, the cells rapidly proceeded into the G_1 period of interphase. After 30 min, only 17% of the cells remained in mitosis and after 60 min only 8% of mitotic figures remained (Table I).

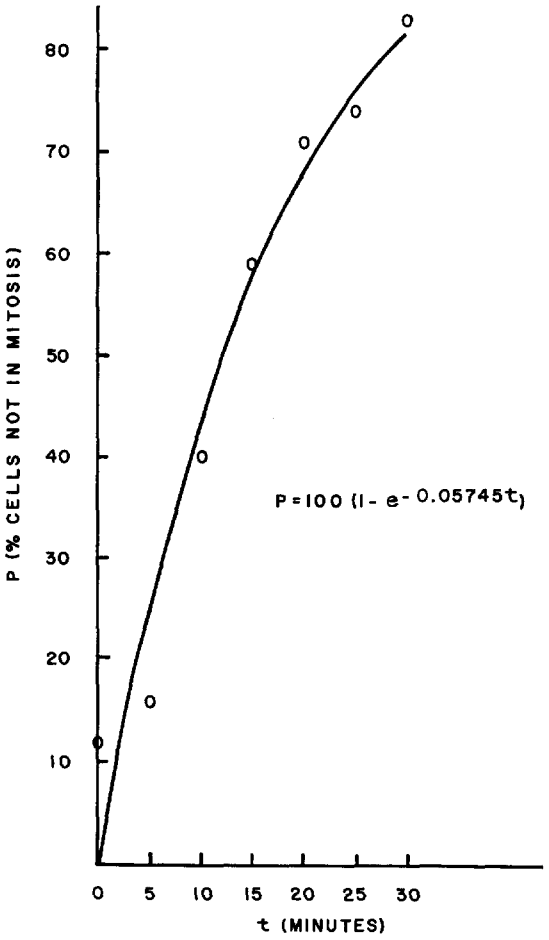

FIGURE 2 Mitotic index during initial (T_0) 30 min period of incubation.

An exponential graph of the percentage of cells not in mitosis versus time was plotted from the values obtained (Table I) for the region of interest, 0–30 min after shaking. The equation for this curve is $P = 100\ (1 - e^{-0.05745t})$ where P represents the percentage of cells not in mitosis and t, time. With this graph, the percentage of cells not in mitosis at any point in the 30 min time period may be easily estimated (Fig. 2).

The incorporation of uridine into Chinese hamster cells in mitosis and interphase is recorded in Table II. Each experiment represents a single population of cells, half of which were incubated immediately with uridine-^{3}H for 30 min (1 *a*–6 *a*), and half of which were allowed to progress into interphase before the 30 min incubation with isotope was initiated (1 *b*–6 *b*). It was seen in six ex

TABLE II

Incorporation of Tritiated Uridine into Synchronized Chinese Hamster Cells during (T_0) and after (T_{30}) Mitosis

Chinese hamster cells were incubated at 37°C for 30 min either immediately (1 *a*–6 *a*), or after a 30 min incubation period (1 *b*–6 *b*), with 0.1 μc/ml uridine-^{3}H specific activity 1.9 c/mmole, Schwarz BioResearch. The cells were washed, chased for 30 min with 1 mg/ml unlabeled uridine, washed again, and extracted in 5% TCA at 4°C. The extracted material was washed twice in fresh 5% TCA, once in acetone, dissolved in Hyamine hydroxide, and counted for 10 min in a Packard Tri-Carb scintillation counter. Values represent counts per minute per 2 × 10^5 cells.

Exp. No.	Cells in division	Cells in interphase	No. of cells (×10^5)	cpm per 2 × 10^5 cells
	%	%		
T_0 experiments (80–86% cells initially in mitosis)				
1 *a*	80	20	1.9	439
2 *a*	86	14	2.0	420
3 *a*	86	14	2.0	427
4 *a*	80	20	2.0	366
5 *a*	81	19	2.1	405
6 *a*	85	15	2.0	403
Average	83	17	2.0	410
T_{30} experiments (80–87% cells in interphase)				
1 *b*	19	81	1.9	761
2 *b*	13	87	2.0	806
3 *b*	—	—	2.0	699
4 *b*	14	86	2.0	924
5 *b*	20	80	2.1	758
6 *b*	15	85	2.0	701
Average	16	84	2.0	775
Control incubations (4°C for 0 and 30 min respectively)				
7	2	98	2.8	13
8	2	98	2.8	28

TABLE III

Theoretical Calculations of Counts of Tritiated Uridine Incorporation into Nucleic Acid of Interphase Cells

Time	Estimated % not in mitosis at end of time period	Estimated average % not in mitosis*	Estimated count for constant % not in mitosis‡	Estimated contribution to count during 5-min period§	Cumulative contribution to count (C)‖
min					
0–5	25	13	114	22	22
5–10	43	35	308	51	73
10–15	59	51	449	77	150
15–20	68	64	564	96	246
20–25	76	72	634	107	353
25–30	82	79	696	117	470
29–30		82	722		
30–60		88	775		

* Each value represents the estimated *average* number of cells which have proceeded out of mitosis and into the G_1 period of interphase in any given 5 min time period. See Fig. 2 and Table I.

‡ The estimated counts for the initial 30 min time period have been extrapolated, using as the reference value the experimental datum that 88% of cells in interphase incorporated 775 cpm. See Tables I and II.

§ The estimated contribution to count during each 5 min time period was also calculated from the reference value of 775 cpm for 88% of cells not in mitosis. 88 × 30 (min) = 2640 units, with each unit worth a count of 0.29356 (775/2640). The integration of $P = 100\ (1 - e^{-0.05745t})$ between 0 and 30 min, yielded 1601 units which converted to a final theoretical count of 470 (1601 × 0.29356).

‖ The cumulative contribution to count (C) was found from the normalized integral $C = 26.1163\ [t + 17.4064(e^{-0.05745t} - 1)]\ 0 < t < 30$ The cumulative contribution for the 30 min period is equal to 470, corresponding to the experimental average of 410. See Table II.

periments that the initial percentage of cells in mitosis varied between 80 and 86%. After 30 minutes' incubation the percentage of cells in mitosis varied between 13 and 20%. The number of counts per minute per constant number of cells in each particular period of mitosis and interphase was remarkably similar. The control experiments, in which cells were incubated at 4°C for 0 and 30 min to ascertain the degree of nonspecific absorption, showed negligible quantities of isotope bound to the samples.

It was found that the average number of counts incorporated into cells of the mitotic sample was 410 cpm, while the average number of counts in the interphase cell sample was 775 cpm (Table II). The initial percentage of cells in mitosis was 80–86%; however, during the 30 min incubation

TABLE IV

Incorporation of Tritiated Uridine into Synchronized Chinese Hamster Cells Entering G_1 Period of Interphase

Synchronized Chinese hamster cells were incubated, as they proceeded into G_1, at 37°C for 60 sec with 10 μc/ml uridine-^{3}H specific activity 1.9 c/mmole, Schwarz BioResearch. The cells were washed twice with unlabeled uridine in F-10 medium, once with unlabeled uridine in saline, and once with saline alone; all washes were done at 4°C. After washing, the cells were extracted in 5% trichloroacetic acid at 4°C. The extracted material was washed twice in fresh 5% trichloroacetic acid the following day, once in acetone, dissolved in Hyamine hydroxide and in 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene in toluene, and counted for 10 min in a Packard Tri-Carb scintillation counter.

Exp. No.	Time after shake	Cells in division	No. of cells ($\times 10^5$)	cpm per sample	cpm per 2×10^5 cells
	min	%			
1	15	80	2.3	138	120
	40	27	2.3	704	612
2	15	79	2.1	134	128
	30	56	2.1	274	261
3	10	60	2.25	254	226
	20	47	2.25	337	300
	60	12	2.25	1066	948
4	10	59	2.2	316	287
	20	47	2.2	326	296
5	10	68	2.2	211	192
	20	51	2.2	355	323
	60	14	2.2	908	825
6	10	67	2.1	274	261
	20	34	2.1	598	570
	50	15	2.1	863	822
7	10	61	1.9	238	251
	20	45	1.9	296	311
	60	11	1.9	926	975
8	0	84	1.9	108	114
	10	62	1.9	256	269
	20	46	1.9	382	402
9	0	86	1.9	103	108
	10	74	1.9	190	200
	30	51	1.9	358	377

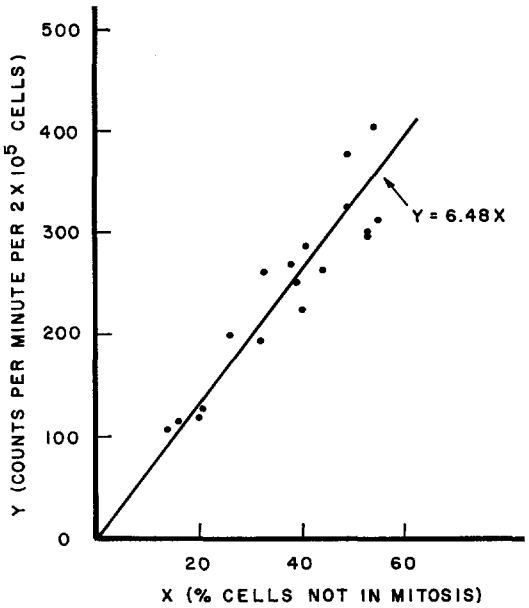

FIGURE 3 Incorporation of uridine-^{3}H during initial 30 min period of incubation. 60-sec pulses at 10-min intervals as synchronized cells proceeded out of mitosis and into G_1.

with uridine, increasing numbers of cells progressed out of mitosis into interphase. At the end of 30 min, only 17% remained in mitosis (Table I). The average number of counts for incorporation during the interphase period of incubation was 775 cpm (Table II), and this was correlated with an average 12% of cells still in mitosis, i.e., 16% at 30 min and 8% at 60 min. With this value of 775 cpm for 12% of cells in mitosis, as well as the exponential graph of the cells as they progressed out of mitosis (Fig. 2), it was possible to examine theoretically the number of counts contributed by the cells per unit time as they proceeded into interphase and increased their uridine incorporation (Table III).

In the first 30 min incubation period, as the cells proceeded from a minimum of 2 to 79 average per cent not in mitosis (Fig. 2), the estimated contribution to the count during each 5 min period varied between 22 and 117 cpm, and the total contribution to counts totaled 470 cpm (Table III). This equates well with the experimental value of 410 cpm (Table II) and strongly suggests that all of the counts incorporated into the nucleic acid can be accounted for by cells already in or just entering the interphase period.

The results of short-pulse labeling experiments with synchronized hamster cells are shown in Table IV. The cell population had been incubated with uridine-^{3}H (100 times the concentration used in the T_0 and T_{30} experiments) for 60-sec pulses at 10-min intervals during the first 60 min after synchronization. The fitting of a general model for these data was accomplished by the least squares procedure with untransformed counts, where Y equals the observed count and X equals the percentage of cells not in mitosis. During the first 30 min period, a linear relationship through the origin $Y = 6.48X$ was found between the percentage of cells in mitosis and the incorporation of isotope (Fig. 3). Theoretically then, if 100% of the cells was in mitosis, one would expect the counts incorporated into nucleic acid to be nearly 0.

Additional data analysis, utilizing a breakdown in the mitotic indices into mid-mitotic (true metaphase) and late-mitotic (anaphase and telophase) values (Table V), gave no indication that differences in mitotic stages would suggest any other interpretation of the data.

Thus, the evidence from these short-pulse labeling experiments also strongly suggests that all of the incorporation into nucleic acid can be accounted for by cells in the interphase period.

TABLE V

Mitotic Indices during the 1st Hr of Incubation

Chinese hamster cells were synchronized in calcium-free F-10 medium by an adaptation of centrifugal force shaking techniques (27, 36). Cells were stained with aceto-orcein and mitotic indices were scored at 10- and 20-min intervals during the 1st hr of incubation. Each value represents 200–300 cells scored.

Exp. No.	Time after shake	Cells in meta-phase	Cells in ana-telo-phase	Cells in inter-phase	Cells in division
	min	%	%	%	%
1	0	65	20	15	85
	10	61	5	34	66
	20	44	4	52	48
	40	13	5	82	18
	60	6	4	90	10
2	0	62	25	13	87
	10	62	6	32	68
	20	37	8	55	45
	40	22	3	75	25
	60	10	2	88	12
3	0	66	20	14	86
	10	61	4	35	65
	20	41	5	54	46
	40	40	3	57	43
	60	28	5	67	33
4	0	63	18	19	81
	10	59	2	39	61
	20	41	4	55	45
	40	32	2	66	34
	60	18	3	79	21
5	0	62	16	22	78
	10	48	4	48	52
	20	35	3	62	38
	40	33	2	65	35
	60	22	3	75	25

DISCUSSION

The growth pattern in mammalian cells has been divided into four phases of activity, i.e., RNA and protein synthesis most prominent during G_1 and G_2, nuclear DNA synthesis only during the S period, and cessation or depression of all synthetic activity during the short period of actual mitosis, M. The period of mitosis (approximately 30 min in many mammalian cells) is one in which the chromosomes are coiled and contracted to their greatest extent (26). Although Cooper has provided evidence that heterochromatic regions of chromosomes in *Drosophila* may not be entirely inert (5), it has been strongly suggested, from studies of Diptera and the lampbrush loops of the newt oocyte, that a state of flux exists between the contracted, relatively inert state and the extended active state of the helical DNA strands. The chromosomal material of phage and bacteria may be physically extended at all times and thus potentially capable of activity, although other control mechanisms in these instances may or may not allow the genetic activity to be expressed.

Despite the weight of evidence that contracted chromosomes incorporate no DNA precursors and little RNA or amino acid components, there are several reports which provide controversy. In some instances the radioautographic experiments may be disregarded because of insufficient acid washes to remove soluble pools of radioactive nucleotides. In others, the opposite may obtain—acid extraction may remove low molecular weight RNA synthesized on mitotic chromosomes. Konrad's careful study on hamster cells (17) continued to show a minimal incorporation of uridine-^{14}C (13–16% of the interphase counts) during mitosis. Other studies, reporting the sedimentation pattern of labeled RNA in synchronized myxo-

mycetes, show a definite minimal incorporation of uridine-^{3}H into RNA during mitosis, albeit on the lighter side of the gradient (3, 19). The latter authors suggest that the shift may be due to continued synthesis of soluble RNA. The studies on spermatogenesis in the locust (9) and grasshopper (21) are also inexplicable in terms of complete cessation of RNA synthesis.

In addition to the problem of direct synthesis of RNA on the contracted DNA, there has arisen the question of RNA synthesis in the nucleolus. Hsu has demonstrated the persistence of nucleoli during mitosis in 90–98% of mitotic cells in three strains of Chinese hamster cells (15), and ultrastructural studies have shown such persistent nucleolar masses to be less compact and to possess more widely dispersed granules than nucleoli of interphase cells (4). Arrighi, however, has shown in short-pulse labeling experiments that Chinese hamster metaphase and anaphase cells are completely free of label, despite the presence of persistent nucleoli. He has concluded that it is highly unlikely that synthesis of new nucleolar material takes place during the mid-mitotic stages (1).

Radioautographic studies comparing the rates of RNA synthetic activity in the chromatin and nucleolar fractions during mitosis have been done in root tips of *Allium* and *Nigella*, in which there are no persistent nucleoli (7). While the activity decreased in the condensing chromatin during prophase, the rate of synthesis in nucleoli remained constant until they disappeared in late prophase; in addition, synthetic activity resumed faster in the pronucleolar bodies than in the uncoiling chromatin of late telophase.

Polyribosomes, in association with messenger RNA molecules, are the site of nascent protein synthesis in many cell systems (33). In the mammalian metaphase cell, in vitro studies have shown that a rate-limiting component in protein synthesis involves the degree of aggregation of the ribosomal population. It is possible to effect a marked suppression of protein synthesis, as duplicated in an in vitro amino acid incorporating system, when ribosomes from metaphase cells are substituted for interphase ribosomes (29). Recent studies have shown that Chinese hamster cells form ribosomal-RNA via 35S and 50S precursors, with the 50S precursors incorporating uridine-^{3}H most rapidly (30). Electron microscope studies and gradient analyses have demonstrated the disaggregation of polyribosomes during meta-anaphase in normal and colchicine-treated cells (28, 32). Associated with this breakdown is a corresponding increase in the number of single ribosomes. This polyribosomal disaggregation cannot be explained by the absence of RNA synthesis, since messenger RNA in these cells has a half-life of 3–4 hr, significantly in excess of the duration of mitosis (23).

Polyribosome reaggregation and a twofold increase in ribosome-associated protein synthesis occur within 45 min after metaphase, when 90% of the synchronized cells has proceeded into G_1 (32). Such data are in agreement with the increase in RNA synthesis reported here and imply the absence of any significant time lag in these synchronized mammalian cells.

The pattern of DNA, RNA, and protein synthesis in relation to the cell cycle is becoming clear in a variety of cells. Of great interest for future studies will be a correlation of the fine structure of chromosomes with their functional activity. In these studies we have shown that all the incorporation of tritiated uridine into cells of the mitotic and G_1 phase of the cell cycle may be attributed to those cells in interphase. None of the usual explanations for nucleotide incorporation into the nucleic acids of mitotic cells need be invoked, i.e., RNA synthesis on partially uncoiled DNA chromosomal strands or synthesis in the nucleoli or mitochondria.

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