Synthesis of Ribonucleic Acid in L Cells during Inhibition of Protein Synthesis by Cycloheximide

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(Received July 8, 1966)

SUMMARY

The role of protein synthesis in ribonucleic acid (RNA) synthesis was studied using the antibiotic cycloheximide to dissociate the synthesis of these macromolecules in L cells growing in suspension culture. The experimental evidence indicated that uninterrupted synthesis of protein was required for normal ribosomal RNA formation and for normal transport of RNA from nucleus to cytoplasm.

INTRODUCTION

The RNA which is rapidly labeled in animal cells growing in culture is found in the nucleus as high molecular weight species which sediment at about 45 S[1] and 35 S (1–3). The heavy nuclear RNA is a precursor of the cytoplasmic ribosomal components (2–4). Transfer RNA (4 S) is also synthesized in the nucleus, but there is no evidence that this material is derived from the heavy RNA (2).

The interrelationships between RNA and protein synthesis have been studied by using puromycin to inhibit protein synthesis (5, 6). These investigations have indicated that the formation of 28 S and 16 S ribosomal RNA is dependent on continuous protein synthesis. Messenger and 4 S RNA synthesis, on the other hand, are under no such restriction (6, 7).

In the present investigation the antibiotic cycloheximide (8–11) was used to stop protein synthesis in L cells growing in suspension culture. Under these conditions normal 16 S ribosomal RNA is not observed. The results of this investigation suggest that during inhibition of protein synthesis by cycloheximide 16 S ribosomal RNA is either selectively inhibited or this species of RNA is selectively degraded.

[1] Since actual corrected sedimentation velocities were not obtained, *s* values have been used only as a convenient label for the main classes of RNA and ribosomal subunits, as described by others (13, 14).

MATERIALS AND METHODS

Cells. Suspension cultures of L cells with a doubling time of about 24 hr were grown at 37° in Eagle's MEM suspension medium (12) obtained from Microbiological Associates, Bethesda, Maryland. All experiments were performed with exponentially growing cells at a concentration of 4 to 5×10^5 cells/ml. The cultures were tested during this study for PPLO contamination, and none was found.

Radioisotopes and antibiotics. Uridine-2-^{14}C (30 mC/mmole) and L-leucine-1-^{14}C (23.3 mC/mmole) were purchased from New England Nuclear Corporation, Boston, Massachusetts, and were used at the concentrations indicated in each experiment. Cycloheximide (Acti-dione) was purchased from the Upjohn Company, Kalamazoo, Michigan, and actinomycin D

was a gift of Dr. A. E. Osterberg, National Cancer Institute, Bethesda, Maryland.

Measurement of incorporation of radioactivity into RNA, DNA, and protein. To measure leucine-^{14}C incorporation, the L cells were incubated in medium containing leucine-^{14}C, and 5-ml samples were taken at intervals. The cell suspensions were centrifuged at 1000 *g* for 5 min, washed once with Earle's salt solution, and suspended in 5% trichloroacetic acid; 1-ml samples were filtered and washed, in duplicate, on 0.45 μ (pore size) Millipore filters. The filters were mounted on aluminum planchets, dried, and counted with a Nuclear-Chicago thin-window low background counter (Nuclear-Chicago Corporation, Des Plaines, Illinois).

Uridine-^{14}C incorporation into both RNA and DNA was measured in the following way. Cells incubated with uridine-^{14}C were collected at intervals as above and suspended in 5 ml cold 0.5 N NaOH; 1-ml samples, in duplicate, were precipitated with cold trichloroacetic acid, filtered, and counted. This corresponds to the RNA plus DNA of the cell. The remainder of the sample in 0.5 N NaOH was incubated at 37° overnight; 1-ml samples were precipitated and counted. This corresponds to the DNA of the cell. The difference between the radioactivity in the RNA plus DNA and the DNA fractions gives the counts in the RNA.

Isolation of RNA. The cells were harvested by centrifugation at 1000 *g* for 5 min and washed once with Earle's salt solution; the RNA was extracted by the hot phenol method (1).

Cell fractionation. Cytoplasmic extracts to be analyzed on sucrose density gradients were prepared as previously described (13, 14).

Nuclear and cytoplasmic extracts used for isolation of purified RNA were prepared as previously described (13, 14) except that the nuclear fraction was further purified by washing with 0.2% citric acid.

Determination of radioactivity in the nuclear and cytoplasmic fractions of the cells was achieved by the citric acid method (15). Washed cells were treated with 0.2% citric acid, and the nuclear and cytoplasmic fractions were isolated. Cold trichloroacetic acid (final concentration of 5%) was added to each fraction, and the resulting precipitate was washed once with cold 5% trichloroacetic acid. The number of counts in RNA was determined as outlined above.

Density gradient centrifugation. Purified RNA was analyzed as previously described (1) using 5–20% (w/v) linear sucrose density gradients made in acetate buffer, 10^{-2} M, pH 5.1 containing NaCl 5×10^{-2} M, $MgCl_2$ 10^{-4} M. In all analyses 400 μg purified RNA in 1 ml was layered on a 25-ml gradient and centrifuged in the SW 25.1 rotor of the Spinco Model L-2 at 18,000 rpm for 16 hr at 3°.

Cytoplasmic components were separated by centrifugation using 15–30% (w/v) sucrose density gradients. The sucrose was dissolved in RSB as previously described (13, 14).

Analysis of gradients. Fractions of approximately 0.7 ml were taken by puncturing the centrifuge tube with a 20-gauge hypodermic needle and collecting 8 drops into individual tubes. The optical density at 260 mμ was determined with a Zeiss spectrophotometer. Carrier serum albumin (250 μg/tube) and cold trichloroacetic acid (final concentration 5%) were added. The resulting precipitate was filtered and washed on a 0.45 μ Millipore filter, pasted on an aluminum planchet, and counted.

RESULTS

Effect of Cycloheximide on Protein, RNA, and DNA Synthesis

As we had reported previously (11), the addition of cycloheximide (10 μg/ml) to cultures of L cells growing in suspension resulted in the immediate and complete inhibition of incorporation of leucine-^{14}C into protein (Fig. 1). Protein synthesis was inhibited to the same extent by concentrations of cycloheximide as low as 2 μg/ml. Cycloheximide at 10 μg/ml was

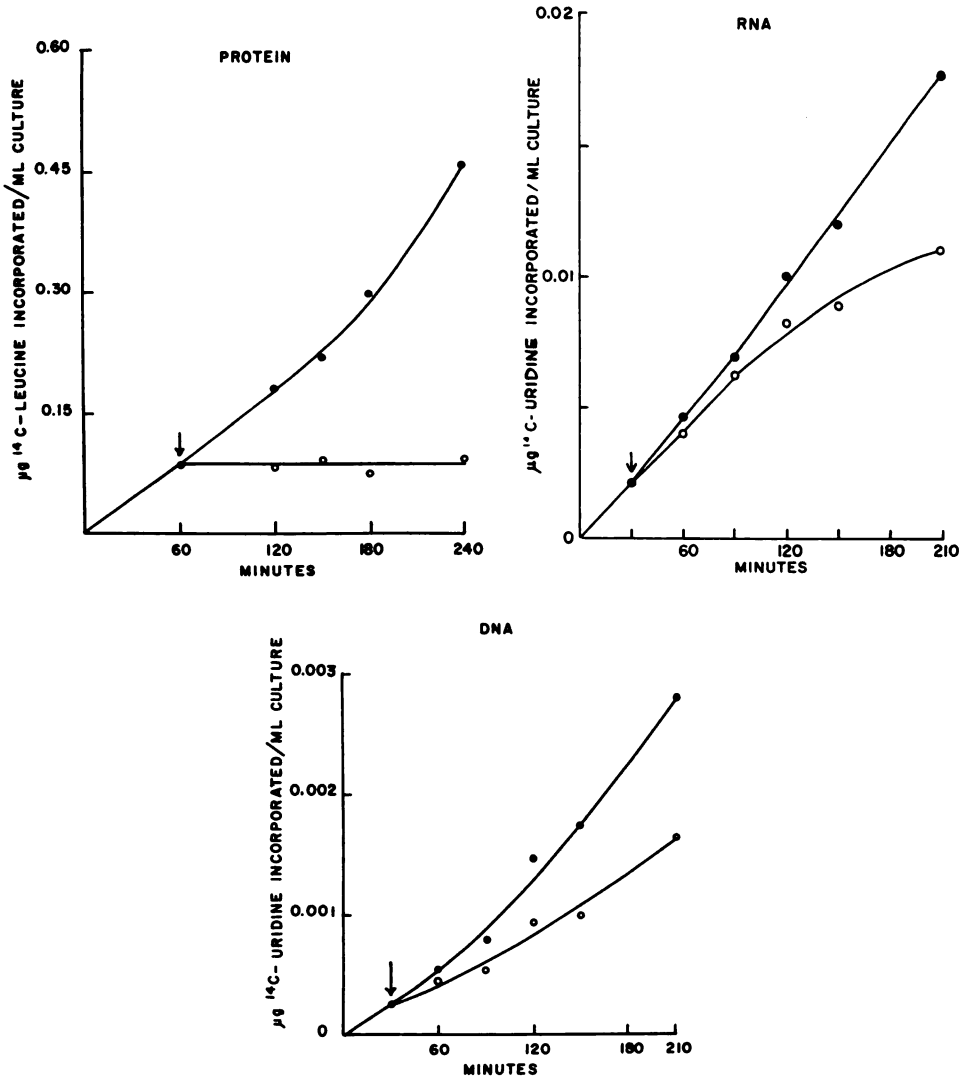

FIG. 1. *Effect of cycloheximide on protein, RNA, and DNA synthesis*

Four cultures, each containing 50 ml of 4×10^5 L cells/ml were prepared. To two cultures leucine-^{14}C (0.15 μC/ml, 52 μg/ml) was added, and to the other two cultures uridine-^{14}C (0.05 μC/ml, 0.41 μg/ml) was added. The cultures were incubated at 37°, and cycloheximide (10 μg/ml) was added to one flask in each group at the time indicated by the arrow. The incorporation of leucine-^{14}C into protein and uridine-^{14}C into RNA or DNA was determined as described in the Materials and Methods section. ●——●, Control, no antibiotic added. ○——○, Antibiotic added.

used in all experiments requiring this antibiotic.

Under the same conditions, the incorporation of uridine-^{14}C into RNA was much less affected. Depending on the experiment, the rate of RNA synthesis continued at about the same rate as observed in the uninhibited culture for 2–3 hr. Then the rate gradually decreased and the cells continued to incorporate radioactivity into RNA at this diminished rate for several more hours.

Uridine-^{14}C incorporation into deoxyribonucleic acid (DNA) was inhibited by 30–50% by the antibiotic.

In contrast to these results, the use of

puromycin to inhibit protein synthesis in this strain of L cells was unsatisfactory for two reasons. Inhibition of protein synthesis by 95% was obtained only by using high concentrations of the antibiotic (100 μg/ml), and at this concentration RNA synthesis was inhibited by 35% in a 60-min incubation. This marked inhibition of RNA synthesis by puromycin has also been observed by others (5–7).

The inhibition of protein synthesis by cycloheximide is readily reversible. Cells treated with cycloheximide for 3 hr were washed free of antibiotic and suspended in fresh medium lacking the drug. Protein, RNA, and DNA synthesis commenced immediately at a rate about equal to that of cells not pretreated with the antibiotic.

Because the antibiotic is a very potent inhibitor, is readily reversible, and has little effect on the rate of total RNA synthesis, as an inhibitor of protein synthesis in this strain of L cells it is superior to puromycin.

Synthesis of RNA during Inhibition of Protein Synthesis by Cycloheximide

The rates of synthesis of the various kinds of RNA were studied during normal growth and during inhibition of protein synthesis by cycloheximide. In these experiments total cell RNA was isolated from cultures of L cells incubated for 45, 120, and 240 min with uridine-^{14}C as the RNA precursor present for the entire time, in the absence or presence of cycloheximide. Figure 2 illustrates the results obtained in the uninhibited cultures. At 45 min the heavy nuclear 45 S and 35 S RNA predominated. Transfer RNA (4 S) was also made. At 120 min most of the label appeared in the 28 S and 16 S ribosomal RNA, and in the 4 S RNA. At this time, the heavy RNA was a small fraction of the total RNA. This was more evident at 240 min of incubation. This, then, is the normal sequence of RNA synthesis in L cells growing in suspension culture.

In the next experiment the effect of cycloheximide on the sequence of RNA synthesis was studied. The synthesis of the heavy RNA and transfer RNA did not appear to be affected after 45 min of treatment with the antibiotic (Fig. 3). However, as the incubation was continued, synthesis of 16 S ribosomal RNA was inhibited. Furthermore, even at 240 min the heavy RNA was still observed.

Additional evidence that 16 S RNA synthesis from heavy nuclear precursors was dependent on continued protein synthesis is given by the following experiment. A cell suspension was pulsed for 30 min with uridine-^{14}C (the presence or absence of cycloheximide during this 30-min pulse did not influence the results). Then half of the cell suspension (A) was washed and placed into medium lacking uridine-^{14}C but containing nonradioactive uridine (100 μg/ml) and cycloheximide and incubated at 37° for an additional 3 hr. The other half of the cell suspension (B) was washed in the same way and inoculated into fresh medium lacking both uridine-^{14}C and cycloheximide but containing nonradioactive uridine (100 μg/ml) and incubated for 3 hr. The total RNA was isolated from both cultures and subjected to sucrose density gradient analysis.

In this experiment all the radioactivity present in RNA at the end of the 3-hr incubation came from the RNA synthesized during the initial 30-min incubation with uridine-^{14}C and any radioactive precursors present in the pool at that time. No label was lost from the cell during the subsequent 3-hr chase with nonradioactive uridine. At 30 min most of the RNA was the heavy RNA and was found in the nucleus of the cell. (This can be seen in Fig. 2 and in Table 1, 0 hr sample.)

Figure 4 shows that the heavy nuclear RNA made during the 30-min pulse with uridine-^{14}C can be chased during subsequent growth in medium lacking cycloheximide into normal ribosomal RNA (culture B). On the other hand, this same heavy RNA, in the presence of cycloheximide, was chased primarily into 28 S RNA and very little appeared in 16 S RNA (culture A).

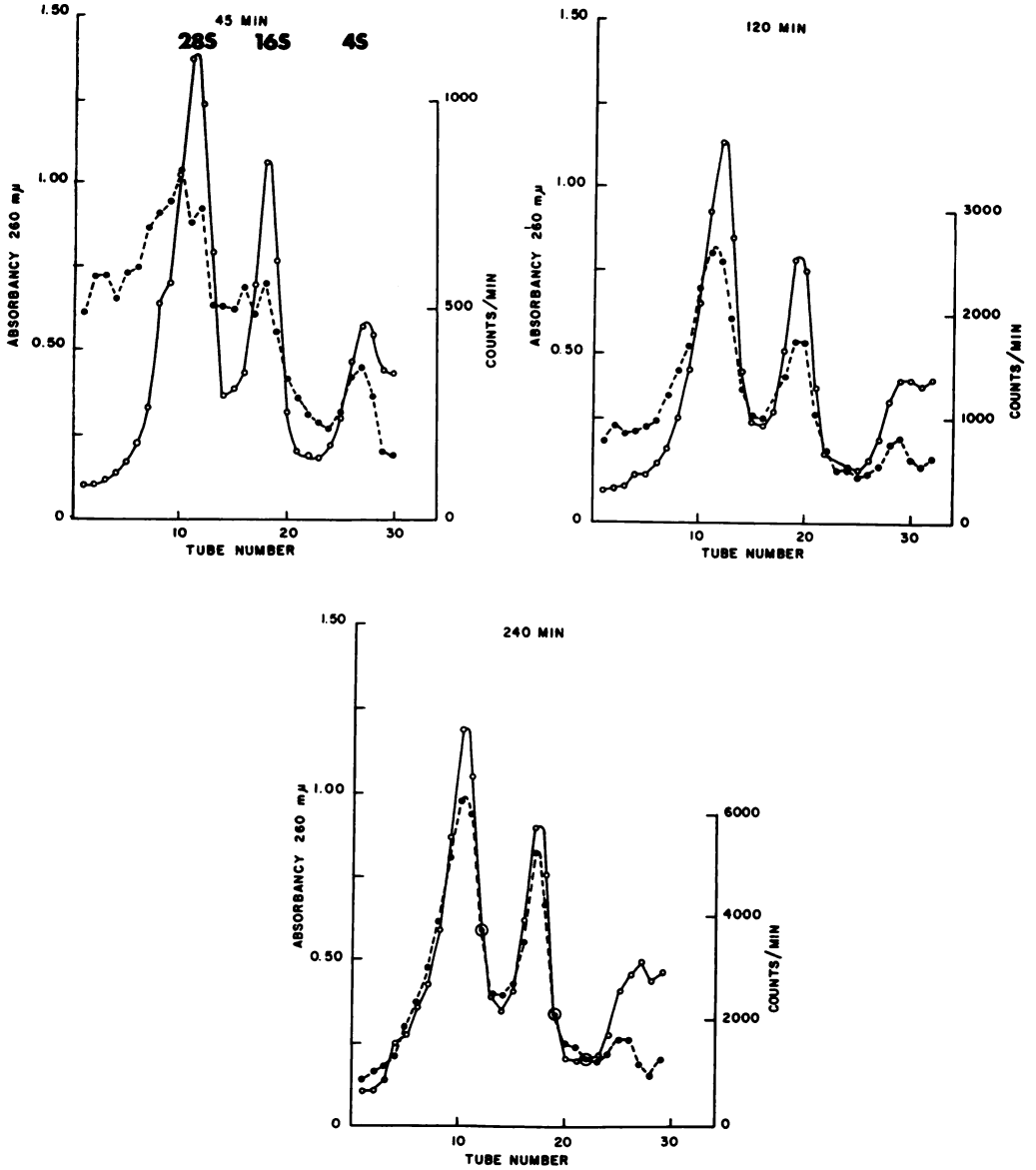

FIG. 2. *Synthesis of RNA in growing cultures of L cells*

To 300 ml of L cells growing in suspension at 5×10^5 cells/ml uridine-^{14}C (0.05 μC/ml, 0.41 μg/ml) was added and the cells were incubated at 37°. At 45, 120, and 240 min, 100-ml aliquots were taken. The cells were centrifuged and washed in Earle's salt solution. The total cell RNA was extracted and subjected to sucrose density gradient analysis as described in the Materials and Methods section. The optical density profile is an index of RNA in the cell before the addition of uridine-^{14}C; and the radioactivity profile is an index of the RNA made during the incubation in the presence of the radioactive precursor. ○——○ Absorbancy at 260 mμ. ●- - -● Counts per minute.

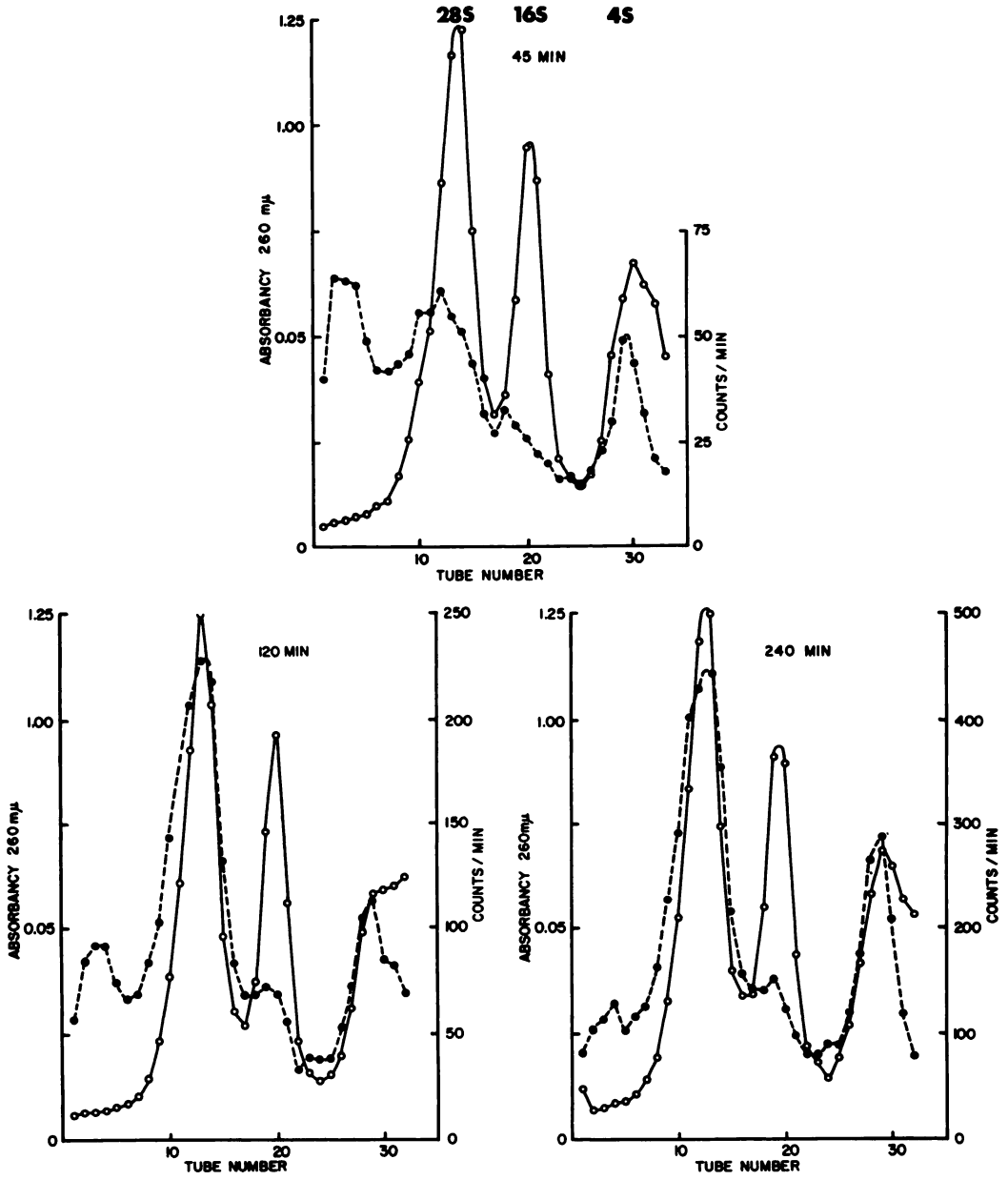

FIG. 3. *Synthesis of RNA in cultures of L cells inhibited by cycloheximide*

The experiment was performed in the same way as that outlined in Fig. 2 except that cycloheximide (10 μg/ml) was present throughout the incubation. ○——○, Absorbancy at 260 mμ. ●- - -●, Counts per minute.

Thus, in the absence of protein synthesis the maturation of the heavy nuclear RNA into 16 S ribosomal RNA was inhibited. This effect apparently was due to inhibition of protein synthesis by cycloheximide and not by another direct effect of the antibiotic, because similar results were obtained with high concentrations of puromycin, which also inhibited protein synthesis in these cells.

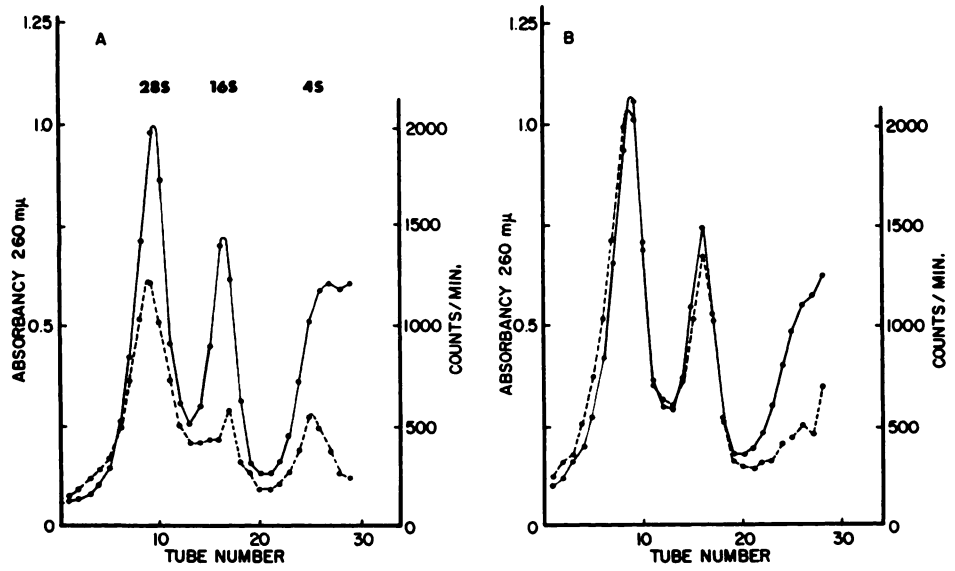

FIG. 4. *Inhibition of the synthesis of 16 S RNA by cycloheximide*

Uridine-^{14}C (0.05 μC/ml, 0.41 μg/ml) was added to 200 ml of L cells growing in suspension at 5×10^5 cells/ml, and the culture was incubated at 37° for 30 min. Then the cells were washed, suspended in fresh medium, and divided into two equal portions. To (A) cycloheximide (10 μg/ml) and nonradioactive uridine (100 μg/ml) were added. To (B) only nonradioactive uridine was added. Growth of the cells occurs only in culture (B). Both cultures were incubated for an additional 3 hr and the cells were harvested. The RNA was extracted from the cells and subjected to sucrose density gradient analysis. O——O, Absorbancy at 260 mμ. ●----●, Counts per minute.

Requirement for Protein Synthesis for Movement of RNA from Nucleus to Cytoplasm

L cells were pulsed for 30 min with uridine-^{14}C either in the absence (A) or presence (B) of cycloheximide. The cells pulsed in the absence of cycloheximide were then washed and resuspended in fresh medium containing nonradioactive uridine (100 μg/ml) but no cycloheximide. The cells pulsed in the presence of cycloheximide were washed and resuspended in the same medium, but containing cycloheximide. Samples were taken at intervals, nuclei and cytoplasm were isolated, and the amount of radioactivity in the RNA in each fraction was determined. The results are summarized in Table 1.

As others have reported (1–4), most of the RNA synthesized during the 30-min pulse (0 hr samples) was found in the nucleus. During normal growth in nonradioactive medium, this was chased out of the nucleus (A). However, when cycloheximide was present during the chase (B), the movement of labeled RNA out of the nucleus was inhibited. Labeled RNA was not degraded during the chase either in the presence or the absence of cycloheximide. At 4.5 hr about three times more RNA was transported out of the nucleus of untreated cells as compared to antibiotic-treated cells.

Nuclear and Cytoplasmic Localization of Normal RNA and RNA Made in the Absence of Protein Synthesis

The localization of the RNA made in the absence of protein synthesis was compared to that made during normal growth. In this experiment the cells were pulsed for 3 hr with uridine-^{14}C either in the presence or absence of cycloheximide. The nuclei and cytoplasm were isolated, mixed with whole unlabeled cells to provide an optical density marker, and the

TABLE 1

Distribution between nucleus and cytoplasm of RNA made during a 30-min pulse with uridine-^{14}C in the presence or absence of cycloheximide, and a subsequent chase with nonradioactive uridine

L cells were pulsed with uridine-^{14}C (0.05 μC/ml, 0.41 μg/ml) for 30 min in the absence (A) or presence (B) of cycloheximide (10 μg/ml). The cells pulsed in the absence of cycloheximide were washed and resuspended in fresh medium containing nonradioactive uridine (100 μg/ml) but no cycloheximide. The cells pulsed in the presence of cycloheximide were resuspended in the corresponding medium containing cycloheximide. Samples were taken at intervals, the nuclei and cytoplasm isolated, and the amount of radioactivity in RNA in each fraction was determined. The incubation time refers to the hours of incubation of the cells in the nonradioactive uridine containing media. The method for the isolation of nuclei and cytoplasm is given in the Material and Methods section.

Time after resuspension in nonradioactive medium (hr)	Counts/min/ml culture in		
	Nuclear RNA	Cytoplasmic RNA	Nuclear: cytoplasmic
A. Cycloheximide absent			
0	1068	92	11.6
2	767	477	1.6
4.5	498	603	0.8
B. Cycloheximide present			
0	806	60	13.4
2	612	177	3.5
4.5	497	233	2.1

RNA was isolated and subjected to sucrose density gradient analysis.

The distribution of RNA made in the uninhibited culture is given in Fig. 5. The nucleus contained several species of RNA: RNA heavier than 28 S, probably 28 S RNA (but this was obscured by overlapping with the heavy RNA), and a small amount of 16 C and 4 S RNA. The cytoplasm contained about an equal amount of radioactivity in 28 S and 16 S RNA and a large amount of 4 S material.

Penman (16) has indicated that no 16 S RNA was found in HeLa cell nuclei highly purified by detergents. However, Roberts (17) found 16 S RNA in the nuclei of Ehrlich ascites tumor cells isolated by the same citric acid method (15) used in the present investigation. The appearance of 16 S RNA in the nucleus therefore may be a function of the cell type or the method of isolation of nuclei. The cytoplasmic extracts were not contaminated by nuclei because no DNA was found.

The RNA from the nuclei of cycloheximide-treated cells contained the heavy RNA and probably some 28 S RNA, but no 16 S and 4 S RNA (Fig. 6). The cytoplasm contained, in contrast to what was found in the normally growing cultures, about twice as many counts in 28 S RNA as in 16 S RNA, and a relatively larger amount of 4 S RNA.

Synthesis of Cytoplasmic Ribosomes and 4 S RNA in the Absence of Protein Synthesis

The synthesis of cytoplasmic ribosomes was studied. Extracts of the cytoplasm of normally growing cells incubated for 4 hr with uridine-^{14}C showed ribosome monomers (74 S) and the 60 S and 45 S subunits (Fig. 7). Cells handled in a similar way but treated with cycloheximide showed a limited, but definite, synthesis of cytoplasmic ribosomes. This occurred whether or not the cells were pretreated for 15 min with cycloheximide before addition of uridine-^{14}C and occurred in the absence of detectable protein synthesis.

The results obtained in the experiment of Fig. 7 are also summarized in Table 2. The major portion of the RNA appearing in the cytoplasm of cycloheximide-inhibited-cells sedimented in the 4 S region. A large amount was found associated with the polyribosomes and was perhaps messenger RNA (7, 13, 18, 19). The amount of radioactivity in the antibiotic-treated cells found in the 4 S region was about the same as that found in the equivalent extract from normally growing cells. Therefore, it appears that there is no requirement for protein synthesis for the synthesis and appearance in the cytoplasm of 4 S RNA. Although the results are given for one experiment, this has been repeated four times with the same results.

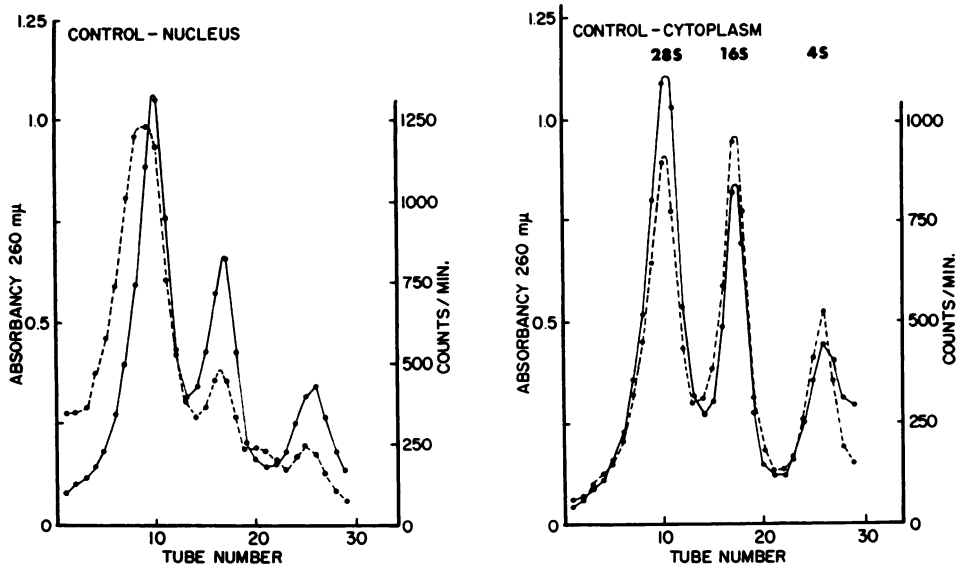

FIG. 5. *Distribution of RNA in nucleus and cytoplasm of growing L cells*

^{14}C-Uridine (0.5 μC/ml, 0.41 μg/ml) was added to 100 ml of L cells growing in suspension at 5×10^5 cells/ml, and the culture was incubated for 3 hr at 37°. The cells were then harvested, and the nuclear and cytoplasmic fractions were isolated as described in the Materials and Methods section. To each fraction whole unlabeled cells were added for an optical density marker, and the RNA was extracted and subjected to sucrose density gradient analysis. ○——○, Absorbancy at 260 mμ. ●- - - -●, Counts per minute.

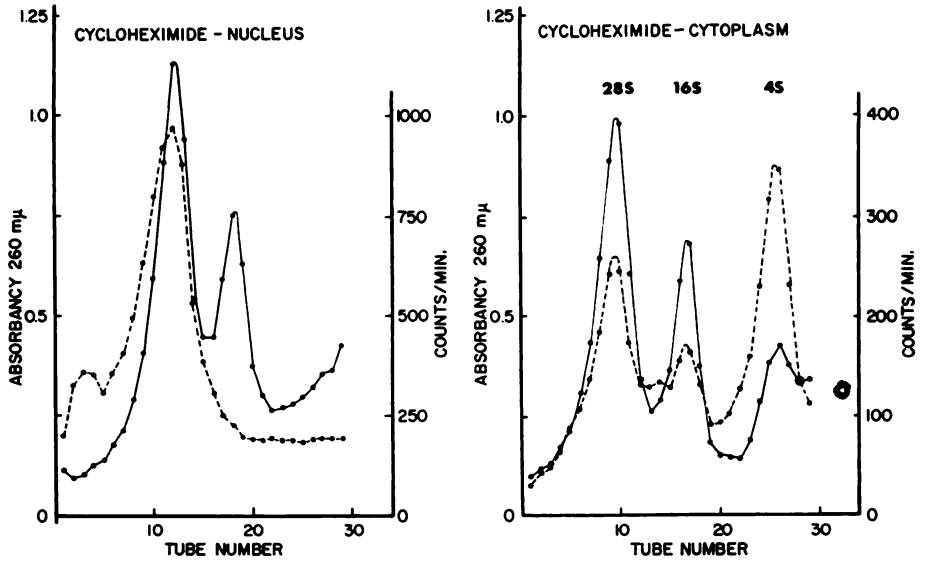

FIG. 6. *Distribution of RNA in nucleus and cytoplasm of L cells inhibited by cycloheximide*

This experiment was performed as outlined in the legend to Fig. 5 except that cycloheximide (10 μg/ml) was present throughout the 3-hr incubation. ○——○, Absorbancy at 260 mμ. ●- - - -●, Counts per minute.

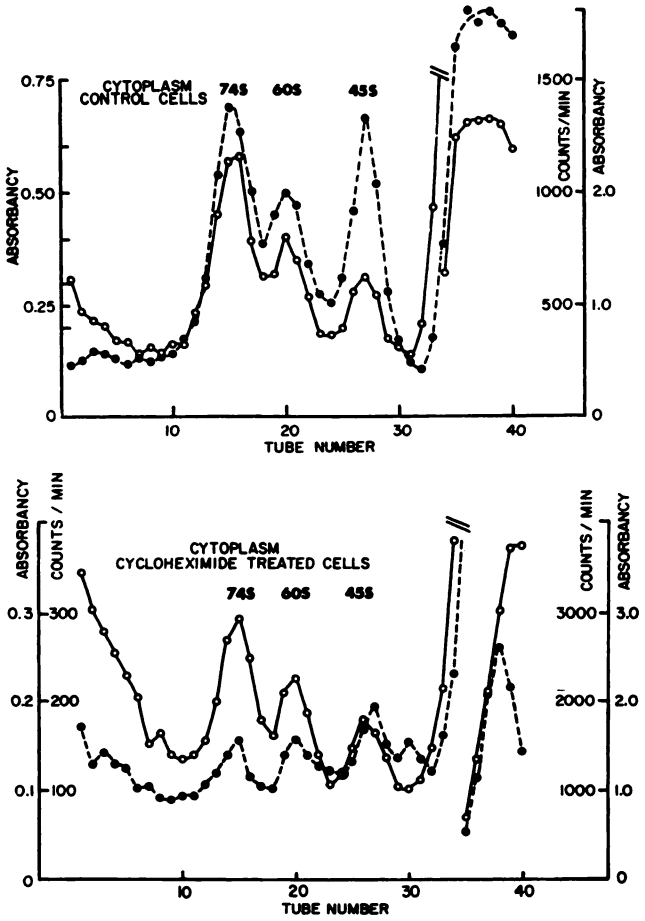

FIG. 7. *Synthesis of cytoplasmic ribosomes in the absence and presence of cycloheximide*

Two 100-ml cultures of L cells containing 5×10^5 cells/ml were prepared. To one, ^{14}C-uridine (0.05 μC/ml, 0.41 μg/ml) was added, and to the other ^{14}C-uridine plus cycloheximide (10 μg/ml). The cultures were incubated for 4 hr at 37°. The cells were then harvested, and the cytoplasm was isolated and immediately subjected to sucrose density gradient analysis (15–30% sucrose). The polyribosomes sedimented in this gradient. O——O, Absorbancy at 260 mμ. ●----●, Counts per minute.

In these experiments the same amount of cytoplasmic RNA (as measured by optical density at 260 mμ) was layered on each gradient. As can be seen, there was about one-half the amount of single ribosomes and subunits in the antibiotic-treated cells as that found in the control. Perhaps cycloheximide treatment resulted in greater polyribosome formation or stability, and thus fewer single ribosomes were seen [see also Wettstein *et al.* (20)].

In HeLa cells (7), no ribosomal RNA, other than that found associated with or in ribosomes or ribosome subunits, was found in the cytoplasm of either normally growing cells or in cells in which protein synthesis was inhibited. This was shown in the present investigation in cycloheximide-inhibited cells by centrifuging the cytoplasmic extracts through a 5–20% sucrose gradient to sediment the 45 S particle. Thus, the region from 45 S to 4 S was spread out over the entire gradient. No RNA other than in the 4 S region was found in particles sedimenting at less than 45 S, either in cytoplasmic extracts

TABLE 2

Distribution of RNA in various fractions of the cytoplasm of normally growing and cycloheximide-inhibited cells

The data are summarized from the results obtained in the gradient described in Fig. 7. The polyribosome region was the pellet at the bottom of the centrifuge tubes; the cytoplasmic ribosomes were tubes 10–30; and the 4 S region from tubes 31–40. Data are given in counts per minute.

Cytoplasmic fraction	Cell treatment	
	None (control)	Cycloheximide
Polyribosome region	17,000	5,150
Cytoplasmic ribosome region	16,600	2,785
4 S region	11,990	9,570

from normal or cycloheximide-inhibited cells.

Stability of RNA during Treatment with Actinomycin D

The rapidly labeled heavy nuclear RNA of animal cells is degraded when cells are placed in medium containing actinomycin D (4, 17, 21–23). In contrast, RNA in ribosomes and transfer RNA are relatively stable during this treatment. We used this property of these RNA's to study the synthesis of the various types of RNA and their distribution within the cell.

The total RNA of cells pulsed for 30 min with uridine-^{14}C either in the presence or absence of cycloheximide and then treated with actinomycin D was rapidly degraded to acid-soluble products (Table 3). Approximately 75% of this RNA was unstable in this experiment.

The results summarized in Table 3 show that almost all the RNA incorporated during the 30-min pulse was found in the nucleus (0 hr samples). In this experiment all the RNA that was degraded was nuclear. Little of the RNA moved from the nucleus to the cytoplasm. However, this type of experiment cannot indicate whether cytoplasmic RNA degraded and was replaced by an equal amount of RNA from the nucleus during the actinomycin chase. Under these circumstances, the total number of counts in the cytoplasm would have remained constant. However, results obtained by sucrose density gradient analysis of RNA isolated from the cytoplasm of these cells did not support this latter idea. Little increase in the amount of 28 and 16 S RNA in the cytoplasm was observed.

The results were different if cells were pulsed with uridine-^{14}C for 30 min as described before, and then a large excess of nonradioactive uridine was added as a chase and the cultures were incubated for

TABLE 3

Stability of nuclear and cytoplasmic RNA synthesized during a 30-min pulse with uridine-^{14}C in the absence or presence of cycloheximide

L cells were pulsed with uridine-^{14}C (0.05 μC/ml, 0.41 μg/ml) for 30 min in the absence (A) or presence (B) of cycloheximide (10 μg/ml). The cells were washed and resuspended in fresh medium containing nonradioactive uridine (100 μg/ml) and actinomycin D (5 μg/ml). Samples were taken at the time the cells were resuspended in the fresh medium (0 hr) and 2 hr later, and the radioactivity in the nuclear and cytoplasmic fractions was determined. The method for the isolation of nuclei and cytoplasm is given in the Materials and Methods section.

Time after addition of actinomycin D (hr)	Counts/min/ml culture in			Per cent stable RNA
	Nuclear RNA	Cytoplasmic RNA	Total RNA	
A. Cycloheximide absent				
0	615	44	659	—
2	106	42	148	22
B. Cycloheximide present				
0	489	32	521	—
2	113	34	147	28

TABLE 4

Stability of nuclear and cytoplasmic RNA synthesized during a 30-min pulse with uridine^{14}C in the presence or absence of cycloheximide and subsequent 4-hr chase with nonradioactive uridine in the presence or absence of cycloheximide

L cells were pulsed with uridine-^{14}C (0.05 μC/ml, 0.41 μg/ml) for 30 min in the absence (A) or presence (B) of cycloheximide (10 μg/ml). At 30 min nonradioactive uridine (200 μg/ml) was added to each culture and the cultures were incubated at 37° for an additional 4 hr. The cells were then washed and resuspended in fresh medium containing actinomycin D (5 μg/ml) and nonradioactive uridine (100 μg/ml) without cycloheximide and incubated at 37° for 2 hr. Samples were taken at the time the cells were resuspended in the fresh medium (0 hr) and 2 hr later, and the radioactivity in the nuclear and cytoplasmic fractions was determined. The method for the isolation of nuclei and cytoplasm is given in the Materials and Methods section.

Time after addition of actinomycin D (hr)	Counts/min/ml culture in			Per cent stable RNA
	Nuclear RNA	Cytoplasmic RNA	Total RNA	
A. Cycloheximide absent				
0	2090	1452	3542	—
2	1090	1606	2696	76
B. Cycloheximide present				
0	1709	501	2210	—
2	731	588	1319	60

an additional 4 hr. The data in Table 4 show that only about 35% of this RNA was unstable in the presence of actinomycin. Thus, a large amount of the RNA made during the 30-min pulse was capable of becoming stable to actinomycin treatment. It is important to note that the RNA became stable whether or not protein synthesis occurred during the 4-hr chase.

Similar results were obtained if the cells were incubated for 4 hr with uridine-^{14}C except that in this experiment about 55% of the RNA was unstable.

Only about 10% of the RNA made during a 30-min pulse was found in the cytoplasm (Tables 1 and 3). In contrast to this finding, about one-third to one-half of the RNA made during the 30-min pulse and subsequent chase for 4 hr with nonradioactive uridine was found in the cytoplasm (4.5-hr sample, Table 1 and 0-hr samples, Table 4). Again, as in the experiment summarized in Table 3, all the RNA which was unstable to actinomycin treatment was found in the nucleus (although all the nuclear RNA was not unstable). However, in this experiment some of the nuclear RNA appeared to be chased into the cytoplasm by treatment with actinomycin (Table 4, 2-hr samples).

The fraction of stable RNA remaining in the nucleus increased during the 4-hr chase, either in the presence or absence of protein synthesis. It was about 20% (Table 3) immediately after the 30-min pulse, and increased to about 45% (Table 4) after the 4-hr chase in nonradioactive medium. This indicated that a large portion of the pulse-labeled nuclear RNA could become stable without being transported out of the nucleus, and without simultaneous protein synthesis.

Sucrose density gradient analyses of RNA extracted from the nucleus and cytoplasm of these cells treated with actinomycin showed that little of the heavy RNA was stable to this treatment. Mostly ribosomal and 4 S RNA were observed in these gradients.

DISCUSSION

The antibiotic cycloheximide is a potent inhibitor of protein synthesis in animal cells growing in culture (11). The drug is a notable addition to the group of inhibitors of protein synthesis in animal cells which have proved useful in studying the synthesis of macromolecules. In the present investigation this antibiotic was used to study some interrelationships

between protein and RNA synthesis in animal cells growing in culture.

The most important observation noted in this investigation is that normal synthesis of 16 S ribosomal RNA does not occur in the presence of cycloheximide. This finding can be explained in two ways. One explanation is that 16 S ribosomal RNA synthesis is selectively inhibited during inhibition of protein synthesis by cycloheximide. Another equally appealing hypothesis is that 16 S RNA is synthesized during this time but that this species of RNA is unstable, perhaps due to a requirement for the continued synthesis of a specific protein for stability. The end result is the same in both cases, 16 S RNA does not accumulate during treatment of L cells with cycloheximide.

The synthesis of heavy nuclear RNA (45 S and 35 S), 28 S ribosomal RNA, and transfer RNA (4 S) did not seem to require continuous protein synthesis. Synthesis of 16 S ribosomal RNA, however, is dependent on simultaneous protein synthesis, and the formation of this component stops early during treatment with cycloheximide. The transport of ribosomal RNA out of the nucleus into the cytoplasm also requires protein synthesis. On the other hand, in agreement with previous work (6), 4 S RNA is synthesized and transported out of the nucleus as well in cycloheximide-inhibited as in uninhibited cultures. In this regard Latham and Darnell (7) showed that HeLa cell messenger RNA synthesis was also unaffected by inhibition of protein synthesis by puromycin.

Other investigators have studied RNA synthesis during inhibition of protein synthesis by puromycin. Tamaoki and Mueller (5) indicated that the synthesis of the heavy nuclear RNA is not inhibited in the absence of protein synthesis, but that the maturation of these into ribosomal RNA (28 S and 16 S) is prevented. In contrast, Holland (6) suggested that the synthesis of the ribosomal precursors is also inhibited by puromycin. However, as these authors have indicated, the concentrations of puromycin used were sufficient to markedly inhibit RNA synthesis, leading to inhibition of the synthesis of both ribosomal RNA components. Since cycloheximide does not inhibit RNA synthesis to as great an extent as puromycin, it was possible, in the present experiments, to follow RNA synthesis for a longer period of time than the previous workers who used puromycin. Thus, the maturation of the heavy RNA into the 28 S ribosomal RNA and the simultaneous inhibition of 16 S RNA synthesis was observed.

Consistent with the observation that little ribosomal RNA gets into the cytoplasm during inhibition of protein synthesis, is the finding that very few newly synthesized complete ribosomes or ribosome subunits are found in the cytoplasm [see also Latham and Darnell (7) using puromycin]. However, the more interesting observation is that *some* complete ribosomes and subunits are, in fact, synthesized during inhibition of protein synthesis (Fig. 7). This is in itself remarkable, because ribosomes contain a protein complement, and no new protein is synthesized during treatment with cycloheximide. The conclusion that we can draw from this finding is that protein, preexisting in the cell before treatment with the antibiotic, can be used for the synthesis of apparently normal ribosomes, an observation similar to what has been seen in bacterial systems (24).

It is tempting to speculate that this protein can therefore control the synthesis of at least one type of ribosomal RNA, the 16 S component. When this protein is exhausted, 16 S RNA synthesis stops. The hypothetical protein required for normal 16 S RNA synthesis does not need to be a ribosomal protein. It can perhaps be an enzyme necessary for 16 S RNA synthesis. The 28 S RNA is not under such a control because it can pile up in the nucleus without the concomitant synthesis of the 16 S component. The fact that 28 S RNA is synthesized quite well in the absence of protein synthesis may mean either (a) that the synthesis of this species of RNA is regulated by a mechanism other than the one controlling 16 S RNA

synthesis, or (b) that 28 S RNA is a direct precursor of the 16 S component and that protein synthesis is required for the conversion of 28 S to 16 S RNA (perhaps by an enzymic cleavage of 28 S RNA).

Recently Penman (16), in a kinetic study of RNA synthesis in HeLa cells, has suggested that the flow of synthesis of ribosomal RNA occurs as follows. First 45 S RNA is made in the nucleus. This is then converted into 35 S RNA (nuclear) and 16 S RNA (which immediately exists into the cytoplasm). The 35 S component is converted into 28 S RNA, which can then also enter the cytoplasm.

Similar studies have not been performed in L cells, and it is therefore impossible to directly relate the present findings to those obtained by Penman, who used HeLa cells. Keeping this precaution in mind, however, the following can be said. Penman's results indicate that there cannot be synthesis of 28 S RNA (and also of 35 S) without the concomitant synthesis of 16 S RNA, for, in order to get 28 S RNA the 45 S species must be converted to 35 S plus 16 S components. However, if the 16 S RNA were unstable, then the synthesis of 28 S RNA in the absence of 16 S accumulation would be observed.

The results of the present investigation show (Figs. 3, 4A, 6) that a large amount of 35 S and 28 S RNA can be synthesized at a time when very little 16 S is found. Furthermore, all the heavy nuclear RNA (30-min pulse) can be quantitatively chased, in the absence of protein synthesis, into ribosomal RNA composed of predominantly the 28 S species (Fig. 4A).

These data indicate that there may be another pathway of synthesis of ribosomal RNA in operation in L cells in addition to or in place of the scheme suggested by Penman (16) for HeLa cells; or that this scheme is in operation in L cells, but that 16 S RNA is unstable during synthesis in the presence of cycloheximide.

The remaining portion of this investigation is concerned with the stability and cellular distribution of RNA made in the presence and absence of protein synthesis. RNA made during a 30-min pulse with uridine ^{14}C is markedly unstable when incubated in growth medium containing actinomycin D, which is used to inhibit further RNA synthesis. Seventy-five per cent of this RNA degrades within 2 hr. All the unstable material is found in the nucleus (although not all the nuclear RNA is unstable), and there is little transport of the pulse-labeled RNA out of the nucleus during the actinomycin treatment. Others had previously shown that actinomycin interferes with the transfer of ribosomal RNA from the nucleus to the cytoplasm (16, 25–27).

In other experiments, L cells were pulsed for 30 min with uridine-^{14}C and then transferred to medium containing nonradioactive uridine and incubated for an additional 4 hr (either in the presence or absence of cycloheximide). The RNA made during the 30-min pulse (the heavy nuclear RNA) is, in this way, allowed to mature into other cell components during incubation in growth medium containing nonradioactive uridine. When actinomycin D is added to these cell suspensions, only 35% of the RNA is degraded. The results of these experiments therefore show that RNA made during the 30-min pulse can become stable if cells are incubated in fresh medium for a period of time before the addition of actinomycin. This can occur either in the presence or absence of cycloheximide, indicating that some RNA can become stable even though no protein synthesis occurs. All the unstable material is found in the nucleus (although not all the nuclear RNA is unstable). However, in this case, some RNA does leave the nucleus during treatment with actinomycin and enters the cytoplasm, although this is a small fraction of the total labeled RNA.

There is some disagreement concerning the stability of the 30-min pulse-labeled RNA. Scherrer *et al.* (4) and Girard *et al.* (25) indicate that only 30–40% of this material in HeLa cells degrades to acid-soluble material in the presence of actinomycin. The present results, which show that about 75% is unstable, are more in line with those observed by Harris (22)

and Lieberman *et al.* (23) and indicate a great degree of instability of pulse labeled RNA.

Girard *et al.* (25) explain this discrepancy by noting that it takes time for whole ribosomes to appear from 45 and 35 S RNA precursors and that this step is actinomycin sensitive. In their system 30 min is sufficient to allow newly formed RNA enough time to reach a stable configuration. If actinomycin is added before this time and the stable configuration is not achieved, the RNA is degraded by the cell nucleases. However, in this regard, in our L cell system, using exponentially growing cells RNA synthesized during a 4-hour pulse degrades by about 45% during incubation with actinomycin. This represents less stable material than the previous authors observed using only a 30-min pulse.

We can offer no explanation of differences in the results obtained in the present investigation and by Scherrer *et al.* (4). These workers used HeLa cells whereas L cells were used in this study, and perhaps there is some basic difference in the RNA metabolism of these two cell lines which could result in the different observations.

ACKNOWLEDGMENTS

I wish to thank Dr. A. E. Osterberg, National Cancer Institute, Bethesda, Maryland, for the gift of actinomycin D, and Mrs. R. Tirey for her excellent technical assistance.

This investigation was supported by Public Health Service Grant GM 12359-02 from the Division of General Medical Sciences, by Grant GB 3521 from the National Science Foundation, and by the American Lebanese Syrian Associated Charities (ALSAC).

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