ABERRANT INTRANUCLEOLAR MATURATION OF RIBOSOMAL PRECURSORS IN THE ABSENCE OF PROTEIN SYNTHESIS

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ABSTRACT

To help elucidate the role of protein in the maturation of ribosomal RNA in cultured L cells, we have studied the effects of cycloheximide upon the maturation process and upon the intranucleolar ribonucleoprotein particles containing the "preribosomal RNA's." Five parameters of these particles were analyzed: (*a*) extractability, (*b*) sedimentation characteristics in sucrose gradients, (*c*) RNA composition, (*d*) buoyant density in CsCl gradients, and (*e*) effects of increased ionic strength on the buoyant density. When protein synthesis is inhibited, the rate of conversion of the precursor 45S ribosomal RNA is rapidly diminished, falling to less than 30% of the control rate within 1 hr. Nevertheless, in terms of the first three parameters there is no difference between control and cycloheximide nucleolar particles. However, the cycloheximide particles have a lower and more heterogeneous buoyant density and a more variable response to increased ionic strength. The results imply that the protein composition of the cycloheximide particles is different from that of particles from control cells, and that the entire protein complement is not necessary for the first cleavages in the maturation process, although it is necessary for the normal rate of processing and for the eventual appearance of both 18S and 28S rRNA in mature ribosomes.

INTRODUCTION

Within the past decade, many of the over-all features of the maturation of ribosomal RNA (rRNA) in mammalian cells have been elucidated (see recent reviews by Perry, 1969; Maden, 1968; Darnell, 1968). The mature 18S and 28S rRNA's are synthesized first as one large continuous molecule (45S rRNA) which subsequently undergoes a series of specific cleavages. This process is not conservative in terms of molecular weight (over 40% of the original RNA is lost), but is conservative for the methylated bases present in the 45S rRNA. However, it is presently not clear how the cleavage sites are determined with such a high degree of specificity, and to what extent other components are required for the maturation process.

Since protein is the other major component of ribosomes, and because the nucleolar "preribosomal RNA's" exist as ribonucleoprotein complexes (e.g., Warner and Soeiro, 1967; Liau and Perry, 1969), an obvious candidate for a role in the maturation process is protein. Since in HeLa cells some rRNA is synthesized and processed when protein synthesis is inhibited with the drug cycloheximide (Warner et al., 1966), it was concluded that protein synthesis need not occur concurrently with rRNA maturation. Any immediate requirement for ribosomal protein during the rRNA maturation could be satisfied by a pool of preexisting ribosomal protein which has been estimated to be equivalent to that in 5–10% of the cell's ribosomes (Warner, 1966). Others report (Ennis, 1966; Yoshikawa-Fukada, 1967; Higashi et al., 1968; Willems et al., 1969) that inhibiting

protein synthesis, especially for longer times, does have an effect on rRNA maturation. It was found that when protein synthesis is inhibited, the maturation leading to 18S rRNA is aborted, and the rate of processing of the rRNA intermediates as well as the rate of synthesis of the 45S precursor is decreased. In these experiments, however, only the RNA was examined; and with the exception of the study of Willems et al. (1969), the maturation process within the nucleolus was not studied.

In the experiments reported in this paper, we have examined the intranucleolar ribonucleoprotein complexes containing the "preribosomal RNA's" under conditions in which protein synthesis is inhibited and the pool of ribosomal protein is diminished. The results show that these complexes (or particles) have an abnormal protein complement, and that the degree of association between the protein and the preribosomal RNA is altered. In addition, we have studied the early intranucleolar steps in the maturation of the ribosomal RNA. In agreement with the results of Willems et al. (1969), marked effects on the synthesis and rate of processing of the 45S precursor were observed when protein synthesis was inhibited.

MATERIALS AND METHODS

Culture Conditions, Radioactive Labeling, and Treatment with Inhibitors

The experiments were performed with mouse L-cell fibroblasts grown in suspension culture as described in Perry and Kelley (1968). Cycloheximide (Actidione, E. R. Squibb & Sons, New York) was added to the unconcentrated cultures at 50 or 100 μg/ml, a concentration that inhibits protein synthesis to greater than 98%. For short-term labeling of the RNA, the cells were concentrated 4-5-fold, and 3–5 μCi/ml of either uridine-^{3}H(generally labeled) or uridine-5-^{3}H(6–9 Ci/mmole and 26–28 Ci/mmole, respectively, New England Nuclear Corp., Boston, Mass.) was then added. Cells were incubated with $Na_3{}^{32}PO_4$ (Abbott Laboratories, N. Chicago, Ill.) at 1–3 μCi/ml in culture medium for 18–24 hr to provide steady-state labeled RNA and ribosomes. Actinomycin D, a gift of Merck, Sharpe and Dohme, West Point, Pa., was used at a concentration of 2 μg/ml.

Isolation of Nucleoli and Nucleolar Particles

The procedure used to isolate nucleoli was basically that of Penman (1966) with several modifications to reduce ribosomal contamination. The cells were harvested by centrifugation, washed with balanced salt solution, suspended in reticulocyte standard buffer (RSB) (0.01 M Tris, pH 7.4, 0.01 M NaCl, 0.0015 M $MgCl_2$) at a concentration of $\sim 2 \times 10^7$ cells/ml, and allowed to swell for 10 min. The cells were then opened by gentle homogenization with a Dounce homogenizer (Kontes, 0.0025 in. clearance), and the nuclei were pelleted by 2–5 min of centrifugation at 800 *g*. Nuclei from up to 2×10^8 cells were suspended in 20 ml of RSB. 3 ml of 6.7% Tween-40 (w/w): 3.3% deoxycholate (w/w) was added, and the suspension was briefly agitated on a vortex mixer, and then underlaid with 5 ml of 20% sucrose in RSB and centrifuged for 5 min at 2,000 *g*. The nuclei were suspended in high salt buffer (HSB) (0.01 M Tris, pH 7.4, 0.5 M NaCl, 0.05 M $MgCl_2$) at a concentration of $\sim 4 \times 10^5$ cells/ml, and the resulting gel was digested at room temperature with 50 μg/ml of electrophoretically purified DNase (Worthington Biochemicals, Freehold, N.J.) until all of the clumps were dispersed. The solution was made 50% in sucrose, and the nucleoli were pelleted for 30 min at 27,000 rpm in a SW27 swinging bucket rotor.

The nucleolar particles were isolated by the method of Warner and Soeiro (1967), and then analyzed on 36-ml linear 15–30% sucrose gradients in TEA-NEB buffer (0.01 M triethanolamine-HCl, pH 7.4, 0.01 M NaCl, 0.01 M EDTA) containing 0.001 M dithiothreitol (Calbiochem, Los Angeles, Calif.). This method involves the use of EDTA and yields derivative particles analogous to the derived 30S and 50S ribosomal subunits.[1]

Cytoplasmic 50S ribosomal subunits, labeled with ^{32}P, were prepared by the method of Warner (1966), except that the TEA-NEB buffer was used for the dissociation and for the sucrose gradients.

Analytical Techniques

Radioactivity was determined as described by Liau and Perry (1969). The buoyant density of these particles, before and after treatment with LiCl, was determined by using formaldehyde-fixation and subsequent CsCl density-gradient centrifugation as described by Perry and Kelley (1966 *a*). RNA was extracted as follows: after two extractions with phenol and 0.5% sodium dodecyl sulfate (SDS) at room temperature, the interphase was reextracted at 55°C with phenol-SDS and then with chloroform-isoamyl alcohol (Penman, 1966). The aqueous phases were combined and precipitated with ethanol (2–2½ volumes). The RNA was analyzed by electrophoresis in acrylamide gels as described by Loening (1967) and Weinberg et al. (1967).

[1] A recently devised method, which extracts nucleolar particles under nondissociative conditions (Liau and Perry, 1969), was not deemed suitable for the small amounts of material used in this study.

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RESULTS

Effect of Cycloheximide on the Maturation of rRNA Precursor

One of the first detectable effects of cycloheximide on RNA metabolism is a reduction in the conversion rate of 45S → 32S rRNA as shown in Fig. 1. In this experiment, cells were treated for various times with cycloheximide, labeled with uridine-^{3}H for 30 min, and then chased in the presence of 2 μg/ml of actinomycin D, which prevents further synthesis of 45S rRNA. From the exponential rate of disappearance of the 45S rRNA, an average "half-life" can be determined for each condition. Greater than 40% reduction of the conversion rate is produced by as little as 5 min of preincubation; and over 70% reduction is observed with 60 min of treatment. A graphic illustration of this effect may be seen in Figs. 2 *a* and *c*. In contrast to the situation for the control cells, the RNA of nucleoli from cells treated with cycloheximide has only a small proportion of the radioactivity in the 32S rRNA intermediate; most is still present in the 45S precursor.

The effect of cycloheximide on the amount of label incorporated into the 45S rRNA precursor may be seen by comparing the peak heights in Figs. 2 *a* and *c*. The extent of inhibition is dependent on the duration of pretreatment, as was first shown by Willems et al. (1969; see their Fig. 2 *a*) and confirmed by us. For treatment times of up to 30 min, this dependence is almost the same as that for the inhibition of the conversion rate, but for longer times the rate of synthesis is relatively more depressed. The synthesis of other species of RNA—such as heterogeneous nuclear or "transfer" RNA—is much less immediately affected, so that in terms of the total RNA of the cell, a pretreatment with cycloheximide for 10 min inhibits the incorporation of uridine into RNA by only 10% for 15 min labeling and 20% for 60 min labeling.

Effect on Nucleolar Particles

EXTRACTABILITY: As can be seen from a comparison of the levels of radioactivity in Figs. 2 *a* vs. 2 *b* and Figs. 2 *c* vs. 2 *d*, there is no significant difference between the control and cycloheximide-treated cells in terms of the proportion of labeled RNA in the nucleoli which can be extracted as particles; in both cases, it ranges from 75 to 85% of the total radioactivity. In both situations, the particle extracts are slightly deficient and the residues are only slightly enriched in the relative proportion of the 45S component.

SEDIMENTATION BEHAVIOR OF THE NUCLEOLAR PARTICLES: There is no qualitative difference in the sedimentation profiles of the nucleolar particles extracted from control and cycloheximide-treated cells. Both extracts contain two classes of particles which become labeled after 60 min of incubation with uridine-^{3}H. Since these particles contain the rRNA precursors (*vide infra*), they may be referred to as preribosomal particles. They are designated as "55S" and "80S" in Fig. 3.[2] In addition, both preparations contain particles which do not become labeled under these conditions (30 and 50S). Since our preparations contain significant quantities of unlabeled 28S and 18S rRNA, we assume that these two classes of unlabeled particles correspond to the mature ribosomal subunits.

RNA FROM NUCLEOLAR PARTICLES: Fig. 4 illustrates the types of RNA found in the various nucleolar particles from the experiment shown in Fig. 3. In the majority of experiments, there was no significant difference between the control cells and those pretreated with cycloheximide in terms of the RNA profiles from each type of nucleolar particle. The 55S particles contained predominantly 32S rRNA with a smaller amount of 28S rRNA, and often a slight amount of 36S RNA appearing as a shoulder on the 32S peak (Figs. 4 *a*, *d*). Of the total radioactive RNA extracted from the 55S particles, the proportion present as 36S + 32S + 28S components was the same in control and cycloheximide-treated cells, suggesting that, in cycloheximide-treated cells, essentially all of the RNA in the 55S particles has undergone the normal first stages of maturation. The 45S component was found in the 80S particles, although other size classes of RNA including all of the ribosomal RNA intermediates, and some smaller heterogeneous RNA were also present (Figs. 4 *b*, *e*). Particles greater than 80S had RNA pro-

[2] If one assumes values of 30S and 50S for the sedimentation coefficients of the derived ribosomal subunits, then the values for the labeled nucleolar particles, when calculated by proportionality, are 58S and 72S. However, since linear 15–30% sucrose gradients are not strictly isokinetic (Noll, 1967), and since these values are not exact, we refer to them as 55S and 80S in conformity with the original designations of Warner and Soeiro (1967).

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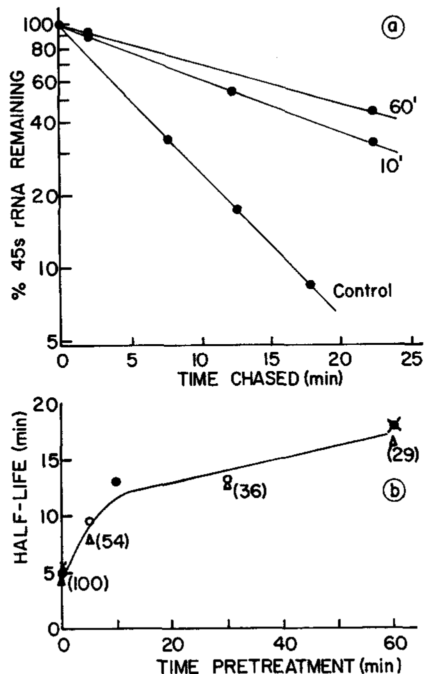

FIGURE 1 Effect of cycloheximide on the half-life and conversion rate of 45S ribosomal RNA. Cultures of L cells were concentrated threefold, treated with cycloheximide (150 μg/ml) for various time periods, labeled for 30 min with 3–5 μCi/ml or uridine-^{3}H, and then chased with a 10,000-fold excess of cold uridine in the presence of 2 μg/ml of actinomycin D. Nucleolar RNA was extracted from cells and analyzed by electrophoresis in acrylamide gels. In one experiment, steady-state ^{32}P-labeled cells were mixed with the ^{3}H-labeled cells for monitoring the recovery of the 45S rRNA. The amount of 45S rRNA remaining after a given chase interval was expressed as a percentage of that present at the end of the labeling period, and plotted semilogarithmically (Fig. 1 *a* represents one experiment in which control cells are compared with cells pretreated for 10 and 60 min with cycloheximide). From these and other similar data, we calculated the half-lives (time for 50% conversion) and the conversion rate (slopes), and then plotted these parameters as a function of the time of pretreatment with cycloheximide (Fig. 1 *b*). The average percentage of the control conversion rate is shown in parentheses for each time of pretreatment. Each of the four symbols represents an independent experiment.

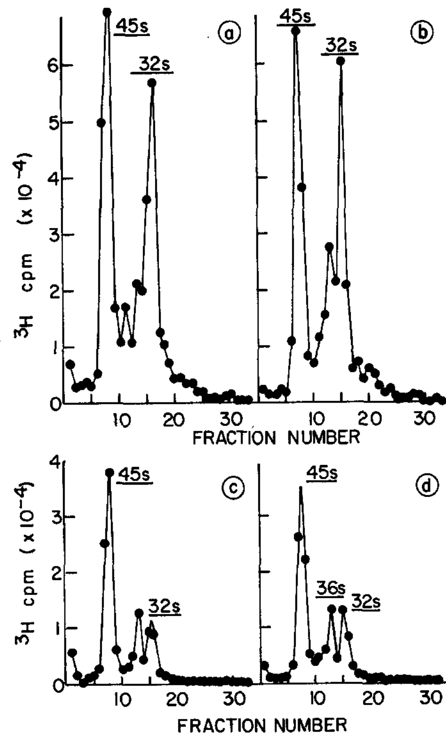

FIGURE 2 RNA from the nucleoli and from the nucleolar particle extract of control cells and of cells pretreated with cycloheximide. Fig. 2 *a:* control nucleoli; Fig. 2 *b:* control nucleolar particle extract; Fig. 2 *c:* cycloheximide nucleoli; Fig. 2 *d:* cycloheximide nucleolar particle extract. A 950 ml culture was divided into two equal parts, cycloheximide at 100 μg/ml added to one, and both were incubated for 60 min. After fivefold concentration, both cultures were then labeled with 5 μCi/ml of uridine-5-^{3}H for an additional 60 min. Nucleoli were isolated, and particles were extracted as described in the Methods section. Aliquots equivalent to 36 ml of culture were taken from the nucleolar suspension and from the extract of nucleolar particles. The RNA was extracted and analyzed by electrophoresis in 2.7% acrylamide gels (5 ma for 4.75 hr; 1 fraction is equivalent to 2 mm of gel).

files quite similar to those of the 80S particles (Figs. 4 *c*, *f*), suggesting that these larger particles are not unique, but rather are aggregates of 80S units. These results are similar to those of Warner and Soeiro (1967) who used sucrose gradients instead of acrylamide gels, and to those of Liau and Perry (1969) who used a different technique for isolation of the particles.

Occasionally, some of the cycloheximide particles sedimenting in the 55S region contained some 45S rRNA. This occurred when the sucrose gradient profile of the nucleolar particles was more flattened and when the 80S peak tended to merge into the 55S peak. However, this was not observed with 55S particles from control cells processed in

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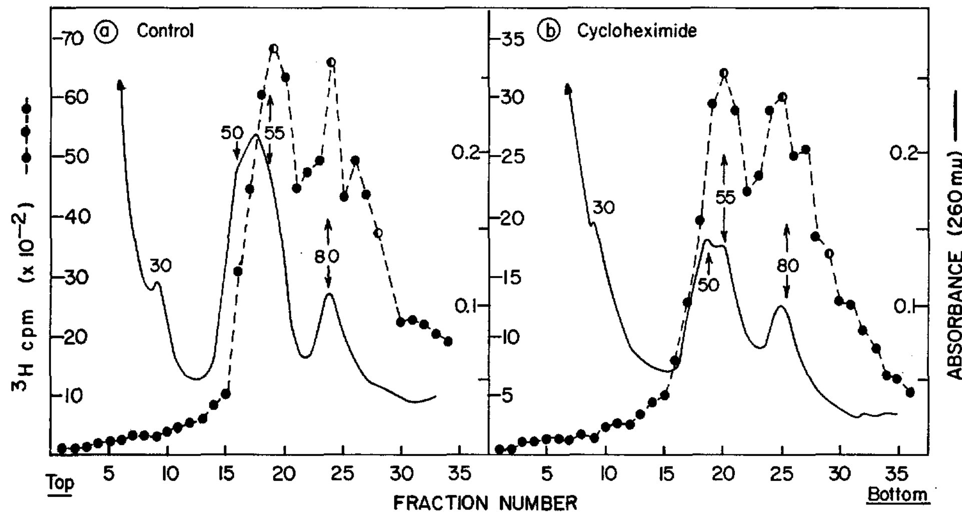

FIGURE 3 Sedimentation profiles of nucleolar particles from control cells and from cells pretreated with cycloheximide. Fig. 3 *a:* control particles; Fig. 3 *b:* cycloheximide particles. Nucleolar particles isolated in the experiment described in Fig. 2 were centrifuged through linear 35-ml 15–30% sucrose gradients for 13 hr at 24,000 rpm in a Spinco SW27 rotor and then collected through the flow cell of a Gilford recording spectrophotometer. Aliquots equivalent to 2.5% of the total material were taken for assay or radioactivity from each fraction of 1 ml. Fractions (◐) were used for the analysis of the RNA constituents of the particles (Fig. 4). The numbers associated with the curves are the approximate sedimentation coefficients. ——, optical density at 260 mμ. --●--●--●--, radioactivity.

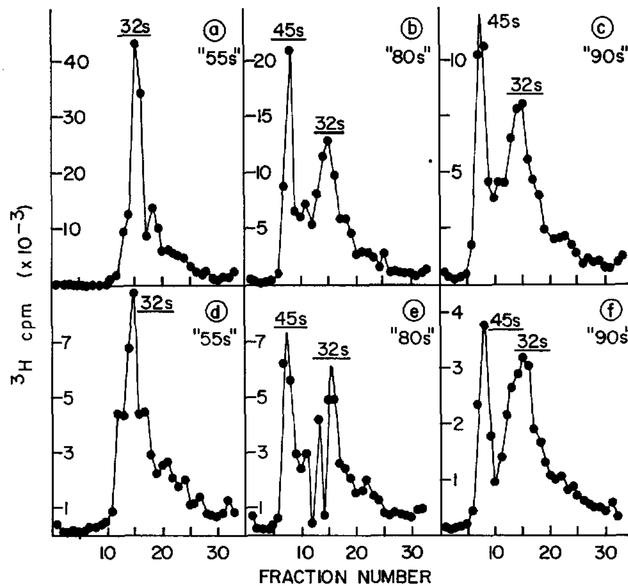

FIGURE 4 RNA constituents of the various nucleolar particles from control cells and from cells pretreated with cycloheximide. Fig. 4 *a–c:* RNA from 55S, 80S, and 90S particles, respectively, from control cells. Fig. 4 *d–f:* RNA from 55S, 80S, and 90S particles from cells pretreated with cycloheximide. RNA was extracted from the indicated fractions of the sucrose gradients shown in Fig. 3, and analyzed by electrophoresis in 2.7% acrylamide gels (5 ma for 4.75 hr).

parallel. One might interpret this to mean that occasionally some of the cycloheximide particles containing 45S rRNA undergo a conformational change or degradation which causes them to sediment in the 55S region.

BUOYANT DENSITY OF NUCLEOLAR PARTICLES: In order to study further the properties of the nucleolar particles, we determined their buoyant densities in CsCl density gradients. As was shown in previous studies (Spirin et al., 1965;

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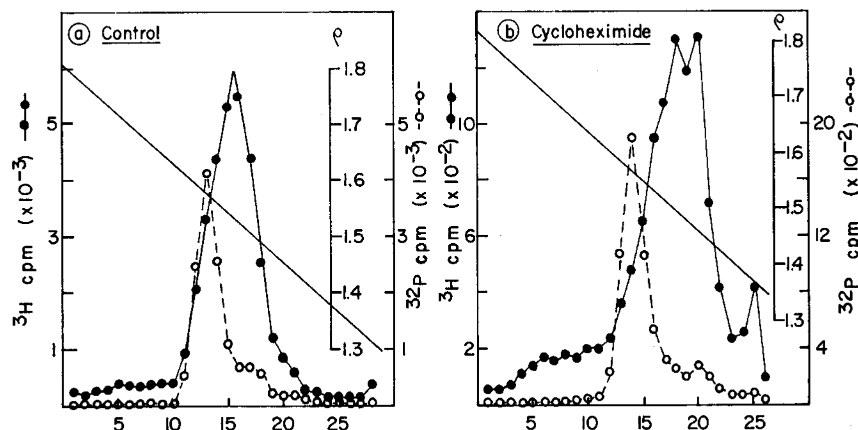

FIGURE 5 Buoyant density of the 80S nucleolar particles from control cells and from cells pretreated with cycloheximide. 80S nucleolar particles were isolated from a sucrose gradient such as shown in Fig. 3, mixed with a preparation of ^{32}P-labeled 50S ribosomal subunits, fixed, and centrifuged in a preformed CsCl density gradient (16 hr at 35,000 rpm in a Spinco SW50.1 rotor).—●—●—●— ^{3}H-labeled nucleolar particles; --○--○--○-- ^{32}P-labeled 50S ribosomal subunits; —— buoyant density (g/cm^3).

Perry and Kelley, 1966 *b*), the buoyant density of ribosomal particles primarily reflects their protein/RNA ratio. The 80S nucleolar particles from control cells have an average buoyant density of about $\rho = 1.52$ and a wide band width compared with that of a sample of mature 50S ribosomal subunits ($\rho = 1.57$) run in the same gradient (Fig. 5 *a*). This implies that this nucleolar particle has a higher protein content and is more heterogeneous than the 50S subunit. The 80S particles from cells pretreated with cycloheximide for 60 min before labeling (Fig. 5 *b*) have an even greater band width, often with two adjacent bands, and a lower average density ($\rho \simeq 1.49$), implying that they are still more heterogeneous and have a higher protein content than the control 80S nucleolar particles.

The control 55S nucleolar particles also exhibit a lower average density ($\rho = 1.56_5$) and a wider band width than the mature 50S subunit (Fig. 6 *a*). However, the 55S nucleolar particles are less heterogeneous and have less protein per unit amount of RNA than the 80S nucleolar particles. The 55S particles from cycloheximide-treated cells (Fig. 6 *c*) are lower in average buoyant density ($\rho \simeq 1.52$) and have a greater range of densities than the control 55S particles, indicating that they, like the 80S cycloheximide particles, have a higher average protein/RNA ratio and a greater variability in this ratio.

EFFECT OF LICL ON THE NUCLEOLAR PARTICLES: It was surprising to find that the nucleolar particles from cells pretreated with cycloheximide had a higher protein/RNA ratio than did the nucleolar particles from control cells, especially since in the cycloheximide-treated cells protein synthesis had been inhibited for over an hour before labeling. Since it was demonstrated that corresponding particles from both types of cells had the same RNA components, it necessarily follows that the cycloheximide particles have a higher protein content. It was suspected that some, or all, of this increased protein content might be due to nonspecific interactions of the RNA with species of protein other than ribosomal. To examine the specificity of the protein-protein and protein-RNA interactions, we exposed the particles to increasing ionic strength (up to 0.6 M LiCl) and monitored the effect on the buoyant densities (Perry and Kelley, 1968). As shown below, LiCl treatment is effective in removing a certain quantity of protein from the purified preribosomal particles. The presence of this protein in the particles is apparently not eliminated by the 0.5 M NaCl used in the isolation of nucleoli. However, exposure to 0.01 M EDTA during the extraction of the preribosomal particles presumably causes sufficient alterations in these structures so that the removal of protein upon subsequent treatment with LiCl is facilitated (Spitnik-Elson and Atsmon, 1969).

As illustrated in Figs. 6 *a*, *b*, and in Table I, treatment of ribonucleoprotein particles from

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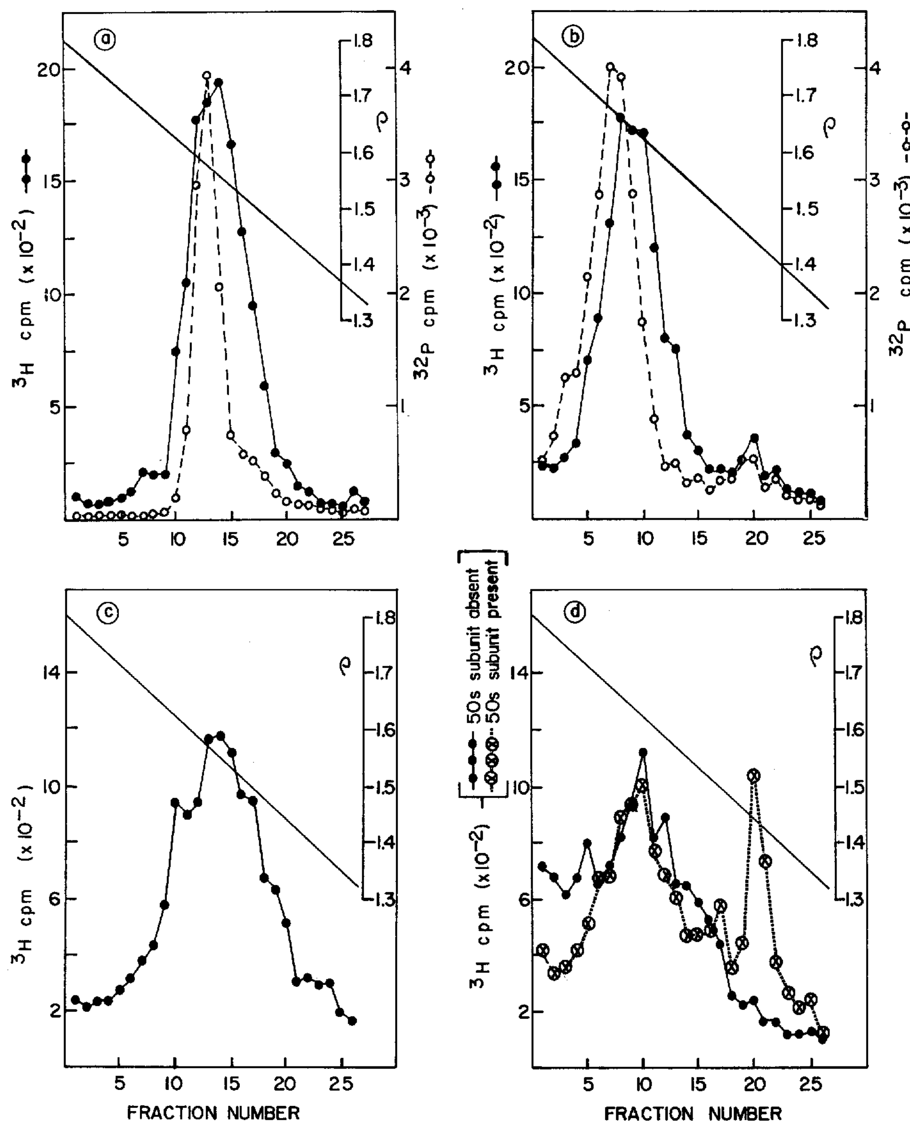

FIGURE 6 Effect of LiCl on the buoyant density of 55S nucleolar particles from control cells and from cells pretreated with cycloheximide. Fig. 6 *a*, *b*: buoyant density of 55S control particles before and after exposure to LiCl. Fig. 6 *c*, *d*: buoyant density of 55S cycloheximide particles before and after treatment with LiCl. 55S nucleolar particles were isolated from a sucrose gradient and either fixed immediately with formaldehyde or treated with 0.45 M LiCl in the presence of 0.045 M $MgCl_2$ for 30 min on ice, and then fixed. Particles were centrifuged in preformed CsCl density gradients as in Fig. 5. A fourfold excess of ^{32}P-labeled 50S ribosomal subunits was mixed with the control nucleolar particles before fixation (*a*) and before salt treatment (*b*). The salt-treated cycloheximide particles (*d*) were divided into two parts: a portion was mixed with 50S subunits (⊗) and a portion was not (●). The profiles of the ^{32}P subunit for panels (*c*) and (*d*) are identical to those of panels (*a*) and (*b*), respectively, and have been omitted for simplicity. —●—●—●—, ^3H radioactivity of the 55S nucleolar particle; --○--○--○--, ^{32}P radioactivity of the 50S ribosomal subunit; ||⊗||⊗||⊗||, ^3H radioactivity of the cycloheximide nucleolar particle exposed to LiCl in the presence of 50S ribosomal subunits; ——, buoyant density (g/cc^3).

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Table I

Comparative Effect of LiCl on the Buoyant Densities of Ribosomal and Preribosomal Particles

	Buoyant density (ρ)			$\Delta\rho \times 10^3$		
[LiCl]	50S subunit	55S	80S	50S	55S	80S
0	1.57_0*	1.56_5	1.53_0*			
0.3 M	1.60_0	1.58_0	1.55_5	30	15	25
0.6 M	1.74_0	1.69_0	1.61_5	170	125	85

* Shown in Fig. 5 *a*.

Nucleolar particles (^{3}H) from control cells were isolated from sucrose gradients as described in Materials and Methods and then divided into three portions. Each portion was treated with LiCl at the indicated concentration and $MgCl_2$ (at one-tenth the LiCl concentration) for 30 min on ice, fixed, and subsequently centrifuged in a CsCl density gradient. The ^{32}P 50S ribosomal subunits were mixed with the nucleolar particles before the exposure to LiCl. Other experiments showed that their presence did not affect the response of the control nucleolar particles to LiCl. The values represent the average buoyant density (g/cc^3) and the change in this density after salt treatment. $\Delta\rho$ represents the difference in buoyant density between untreated and salt-treated particles.

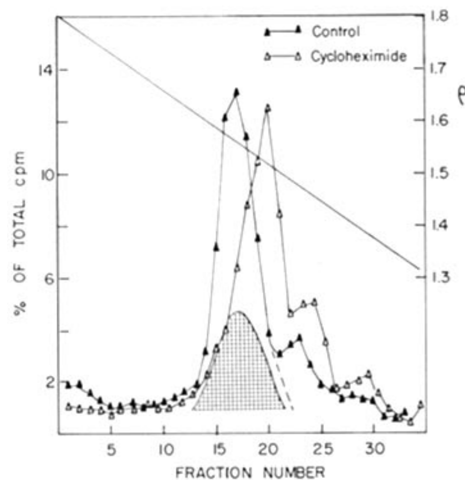

FIGURE 7 Comparison of buoyant density profiles of 55S particles from control and cycloheximide-treated cells. Buoyant density data from an experiment similar to that shown in Fig. 6*a* and *c* is plotted in terms of per cent of the total material on the gradient for control (▲) and cycloheximide-treated (△) cells. The area under each curve is thus equal to 100%. The maximum proportion of the cycloheximide particles possessing density characteristics equivalent to those of control particles is estimated by drawing the maximum Gaussian curve in the position of the principal density band of control particles which subtends the curve for cycloheximide particles. The ratio of the ordinate of this Gaussian curve to that of the principal band (assuming that it is also approximately Gaussian) is the desired proportion. In this case, it amounts to approximately one-third. The total area of overlap between the control and cycloheximide curves, including base line and other material outside of the principal density band, is about 74%.

control cells with increasing concentrations of LiCl causes a progressive increase in the buoyant density of the particles, implying that increasing amounts of protein are being removed. The 80S nucleolar particles appear to be less susceptible to salt dissociation than the 55S particles, which, in turn, are less sensitive than 50S ribosomal subunits derived from monoribosomes with EDTA. The 55S nucleolar particles from cells pretreated with cycloheximide exhibit slightly different behavior under the same conditions (Fig. 6 *d*). When treated with LiCl they tend to increase in buoyant density as do the control 55S particles, but they also become much more heterogeneous and do not form a well-defined band in CsCl. Thus, in contrast to the proteins of ribosomal particles and control nucleolar particles, the proteins of cycloheximide particles are much less uniform in their ability to be displaced under high ionic strength conditions. In addition, when the cycloheximide particles are treated with LiCl in the presence of a 3-4-fold excess of 50S ribosomal subunits, some of them actually decrease in buoyant density, as if they have adsorbed some of the protein lost from the 50S subunits. These results suggest that cycloheximide particles, when treated with LiCl, are more capable of binding additional protein than control particles. Both of these observations strongly suggest that some of the protein originally bound to cycloheximide particles is actually nonribosomal.

In order to investigate whether or not this seemingly less specific protein forms complexes with nucleolar RNA during the extraction of the nucleolar particles, and in order to see if this protein is present only in nuclei from cycloheximide-treated cells, labeled nucleolar RNA was added to unlabeled isolated nucleoli just before extraction of the particles. When the particle extract was centrifuged through a sucrose gradient and then

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analyzed in CsCl gradients, most of the RNA sedimented to the bottom of the CsCl gradient, as one would expect for a mixture of free RNA and RNA complexed with only a few protein molecules. In similar experiments with nucleoli from cycloheximide-treated cells, even more of the RNA sedimented to the bottom of the CsCl gradient. From these experiments, it seems clear that during the extraction of particles from nucleoli of cycloheximide-treated cells, little protein is made available for complexing with free RNA. Thus, the cycloheximide nucleolar particles are not produced by the adventitious binding of protein to free nucleolar RNA during the extraction of the particles with EDTA and dithiothreitol. They must have been formed earlier, either in vivo or during one of the earlier fractionation steps.

MAXIMUM PROPORTION OF NORMAL 55S PARTICLES IN CYCLOHEXIMIDE-TREATED CELLS: Buoyant density data may be used to estimate the maximum proportion of the 55S particles in cycloheximide-treated cells which possess a normal complement of protein. Fig. 7 illustrates data from an experiment similar to that shown in Figs. 6 *a*, *c* in which a comparison is facilitated by plotting the profiles in terms of the per cent of the total material on the gradient. When one confines the analysis to the principal density band, it is readily seen that, at most, about a third of the cycloheximide particles (shaded area) might have the same buoyant density as the control particles. Similar comparisons from four other experiments yield an average value of 40% for the maximum proportion of normal 55S particles in the cycloheximide-treated cells.

DISCUSSION

From the experiments reported in this paper, it is clear that the normal maturation of ribosomal RNA is not independent of protein synthesis. It would appear from the time-course of the cycloheximide effect that one or more proteins with relatively short half-lives are necessary for normal maturation. Whether these proteins are structural or enzymatic or both is not known. Thus, preventing the synthesis of these proteins with cycloheximide causes several effects, three of which are discussed in this paper: an inhibition of the conversion rate of 45S rRNA to 32S rRNA, an inhibition of the synthesis of the 45S precursor rRNA, and an alteration in the properties of the nucleolar precursor particles. A further effect of cycloheximide—inhibition of the appearance of 18S rRNA—has been studied by others (Ennis, 1966; Higashi et al., 1968; Willems et al., 1969). Willems et al. (1969) also described the inhibition of the synthesis and conversion rate of 45S rRNA caused by cycloheximide.

Effects on the Synthesis and Processing of rRNA

The inhibition of 45S rRNA synthesis may be closely coupled with the effect on the conversion rate, since both processes are initially inhibited to the same extent by a given treatment with cycloheximide. It is possible, as first suggested by Willems et al. (1969), that the effect on 45S rRNA synthesis is a secondary result of the slower conversion reducing the amount of available DNA template. Estimates based on the RNA content of L cells, the steady-state proportion of 45S rRNA in a cell (Darnell, 1968), and the number of 45S rRNA cistrons in the genome (see Perry, 1969) indicate that greater than 90% of the 45S rRNA molecules are not complexed with DNA, and, therefore, such a postulated reduction by physical blockage would have to involve only that small proportion of nascent 45S precursor molecules which are still attached to the DNA. Certain proteins might be necessary for release of the nascent 45S RNA from the DNA, and these could be sufficiently few in number and have sufficiently rapid turnover rates so that when protein synthesis is blocked, their level is rapidly reduced and 45S RNA can no longer be released.

It appears that in eukaryotes the more important aspect of the over-all regulation of ribosome biosynthesis occurs at the processing level rather than at the synthesis level. This hypothesis is supported by experiments of others in which the entire process has been perturbed: e.g., puromycin treatment (Soeiro et al., 1968), reduction in the level of methylation by starving for methionine (Vaughan et al., 1967), depletion of other amino acids (Maden et al., 1969), and infection with poliovirus (Weinberg et al., 1967). In all of these cases, there was some residual synthesis of the 45S precursor, even though the processing was affected to such an extent that little or no mature ribosomal RNA was produced. The effects on the synthesis of 45S RNA probably occur as a secondary consequence as we have discussed above.

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Effect on Nucleolar Particles

There are several types of experiments described in this paper which strongly suggest that a substantial proportion of the nucleolar particles from cells pretreated with cycloheximide differ in their protein complement from particles extracted from control cells. First, the buoyant density of the cycloheximide particles is always lower and more heterogeneous than that of the control particles. This implies that although protein synthesis has been inhibited for an hour, these particles are associated with more protein than control particles, and that the particles are less uniform with respect to their protein/RNA ratios. Second, the response to exposure to high ionic strength of the cycloheximide particles is more variable. When treated with LiCl in the presence of an excess of mature 50S ribosomal subunits, some of the cycloheximide particles actually assumed still higher protein/RNA ratios, presumably by binding some of the protein lost by the subunit; this was not observed for control particles. Finally, the cycloheximide nuclear particles are more susceptible to a conformational change or to degradation such that when a change of this type has occurred, the particles containing 45S rRNA would be found in the 55S region of the sucrose gradients even though normally (or in control extracts) they had sedimentation coefficients of 80S or greater.

The exact difference in the protein complement of these cyloheximide nucleolar particles is not yet completely understood, but our tentative explanation is that in the treated cells the nucleolar RNA is only partially complexed with protein, and that during the procedures leading to the isolation of nucleolar particles the RNA binds other nonribosomal, but basic, protein so as to form artificial complexes. From the results of mixing experiments, it appears that this binding occurs before the actual extraction of the particles from the isolated nucleoli. Two possible situations for artificial complexing would be: (*a*) during the initial rupture of the cell with its attendant drastic effects on internal ionic strength(s) and concentrations of various components (see Baltimore and Huang, 1970); and (*b*) during the disruption of the nuclei with high salt and deoxyribonuclease at room temperature. This explanation for the origin of the cycloheximide nucleolar particles is directly analogous to that given by Yoshida and Osawa (1968) and by Schlief (1968) for the origin of chloramphenicol particles in *Escherichia coli*. With several types of experiments, they showed that these CM particles are artificial complexes of rRNA and nonribosomal basic protein.

General Hypothesis of Cycloheximide Effects

Our hypothesis for the effect of inhibiting protein synthesis on the maturation of rRNA is as follows: When cells are treated with cycloheximide, the pools of ribosomal proteins (and/or other necessary proteins that are part of the nucleolar particles) become greatly reduced. The result of this would be that some or all of the nascent 45S rRNA would lack its normal complement of protein. Since all of the RNA in the 55S cycloheximide particles is apparently normal, and yet not more than half of these particles could have a normal protein complement, it follows that at least some of the 32S and 36S rRNA molecules which arise from the 45S rRNA are in protein-deficient particles, and hence that the first cleavages in the maturation process can occur without a full complement of protein; whether any protein is required remains to be established. However, the maturation *rate* depends on the supply of protein, and the process slows down as the effective concentration of ribosomal or other needed proteins becomes low. The 18S rRNA product would be unstable because not enough of the appropriate proteins are available to form a particle resistant to nuclease action. In reference to turnover, the 18S rRNA would resemble the nuclear heterogeneous RNA, most of which has a short half-life and never enters the cytoplasm. The fact that at least some of the 32S rRNA eventually matures into 28S rRNA and appears in the cytoplasmic 50S subunits means that most of its proteins must become available, either from the small pool of ribosomal protein which would persist to some extent because of slow utilization, or from another source such as turnover of preexisting ribosomes.

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