THE EFFECT OF PUROMYCIN ON INTRANUCLEAR STEPS IN RIBOSOME BIOSYNTHESIS

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ABSTRACT

Inhibition of protein synthesis by puromycin (100 γ/ml) is known to inhibit the synthesis of ribosomes. However, ribosomal precursor RNA (45S) continues to be synthesized, methylated, and processed. Cell fractionation studies revealed that, although the initial processing (45S $\rightarrow$ 32S + 16S) occurs in the presence of puromycin, the 16S moiety is immediately degraded. No species of ribosomal RNA can be found to have emerged from the nucleolus. The RNA formed in the presence of puromycin is normal as judged by its ability to enter new ribosomal particles after puromycin is removed. This sequence of events is not a result of inhibition of protein synthesis, for cycloheximide, another inhibitor of protein synthesis, either alone or in combination with puromycin allows the completion of new ribosomes.

It has been found in cultured mammalian cells that, even after the inhibition by cycloheximide of more than 99% of cell protein synthesis, new ribosomal RNA can be synthesized and incorporated into ribosomes which enter the cell cytoplasm (Warner et al., 1966 *a*). These experiments indicate that in HeLa cells there exists a pool of ribosomal proteins which can be used to assemble ribosomes in the absence of continuing protein synthesis (Warner, 1966). In light of these findings it was somewhat surprising that puromycin, another agent which has been widely used as a suppressor of protein synthesis in both animal and bacterial cells, was found to have a drastically different effect from cycloheximide on the synthesis of ribosomes in HeLa cells (Latham and Darnell, 1965; Warner, et al., 1966 *a*). Although the synthesis of 45S ribosomal precursor RNA molecules appeared to continue after cells had been exposed to puromycin, none of this precursor RNA ever appeared in ribosomal subunits in the cell cytoplasm.

Newer cell fractionation techniques (Penman, 1966; Penman et al., 1966; Soeiro et al., 1966; Vaughan et al., 1967 *a*) have now allowed an examination of ribosome biosynthesis at several points between the synthesis within the nucleolus of the 45S ribosomal precursor RNA molecule and the eventual appearance of new cytoplasmic subribosomal particles. This report describes a detailed analysis of the effect of puromycin on the various stages of ribosome manufacture which can at present be observed.

METHODS AND MATERIALS

Cells and Labeling Procedures

The growth, labeling, and harvesting of suspension cultures of HeLa cells were performed as has been described previously. (Eagle, 1959; Warner et al., 1966 *b*).

SOLUTIONS: Composition and designation of solutions employed were as follows: RSB, reticulocyte standard buffer, 0.01 M NaCl, 0.01 M Tris (pH 7.4), 0.0015 M $MgCl_2$; HSB, high salt buffer, 0.5 M NaCl, 0.05 M $MgCl_2$, 0.01 M Tris (pH 7.4); SUT,

0.5% SDS, 0.5 M urea, 0.01 M Tris (pH 7.4); NETS 0.1 M NaCl. 0.01 M EDTA (pH 7.0), 0.01 M Tris (pH 7.4), 0.2% SDS.

Cell Fractionation

The preparation of cytoplasmic extracts in RSB, the detergent treatment of nuclei, and subsequent subfractionation of nuclei, for the yielding of a "pellet fraction" containing the nucleoli and a "nuclear supernatant" fraction, have been described previously (Penman, 1966; Soeiro et al., 1966).

Release and Sedimentation Analysis of RNA

RNA was released from cytoplasmic extracts by SDS–EDTA treatment as has been previously described (Girard, 1964). It was found that RNA in the nuclear pellet fraction could be quantitatively released by resuspension in SUT. Ribosomal RNA from nuclear supernatant preparations in HSB was quantitatively released in the presence of 1% SDS and 0.1 M EDTA (Vaughan et al., 1967 *a*). However, the quantitative release of heterogeneously sedimenting nuclear RNA from the nuclear supernatant requires deproteinization by hot phenol in the presence of SDS (Soeiro et al., 1966).

Isolated RNA was subjected to sedimentation analysis on sucrose gradients (15–30% w/w sucrose dissolved in NETS buffer) as has been previously described (Penman et al., 1964). Details are given in legends.

MATERIALS: Uridine-^{3}H, 20 mc/μmole, was purchased from Nuclear-Chicago Corporation, Des Plaines, Ill.; and methyl-^{14}C-labeled methionine, 10–50 μc/μmole; and uridine-^{14}C, 30 μc/μmole, from New England Nuclear Corp., Boston, Mass. Puromycin dihydrochloride was purchased from Nutritional Biochemicals Corporation, Cleveland, O. and cycloheximide (Actidione) from Upjohn Co., Kalamazoo, Mich.

RESULTS

Formation of 45S RNA in Puromycin-Treated Cells

Considerable evidence from this and other laboratories (Scherrer et al., 1963; Perry, 1964; Rake and Graham, 1964; Girard et al., 1965; Penman, 1966; Penman et al., 1966; Warner et al., 1966 *b*) has provided the following scheme of the synthesis of ribosomal RNA and its assembly into ribosomal particles in cultured animal cells. This is summarized in Fig. 1, in which the operations necessary for examination of each cell fraction are also outlined. The synthesis within the nucleolus of a large molecule of RNA, the 45S ribosomal precursor RNA, is the first observable stage of rRNA (ribosomal RNA) manufacture. After addition of methyl groups to the 45S polynucleotide chain (Greenberg and Penman, 1967; Zimmerman and Holler, 1967), this molecule is cleaved and yields 32S and 16S moieties. The 16S species is rapidly assembled into a ribonucleoprotein subribosomal particle and in less than 5

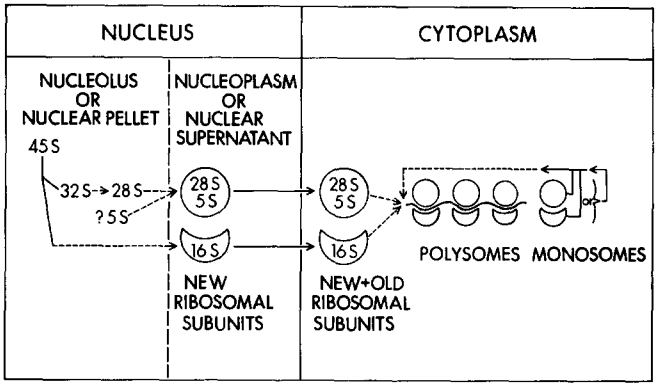

FIGURE 1 Scheme for biosynthesis of ribosomes in HeLa cells. The diagram presents the flow of rRNA molecules (labeled 45S, 32S, 28S, 16S, and 5S) from the point of synthesis in the nucleolus through the maturation process whereby rRNA becomes associated with protein to form particles. After cell rupture in hypotonic medium, the nucleus of the cell is isolated from the cytoplasm by centrifugation. The nucleus is subsequently treated with detergent, lysed in a high salt DNase mixture, and then divided by centrifugation into a nuclear pellet, containing the nucleoli, and a nuclear supernatant fraction. See text for details of methods and references.

min enters the cytoplasm as the smaller (45S), subribosomal particle. The 32S species is further modified so that it sediments as 28S rRNA which then appears in a larger subribosomal nuclear particle. A lag period of approximately 15–30 min exists before this larger (60S) subribosomal particle appears in the cytoplasm. As is shown in Fig. 1, both the large and small nuclear subribosomal particles are detected in the nuclear supernatant (Vaughan et al., 1967 *a*). Whether this is the true intranuclear distribution or represents loss of these particles from the nucleolus

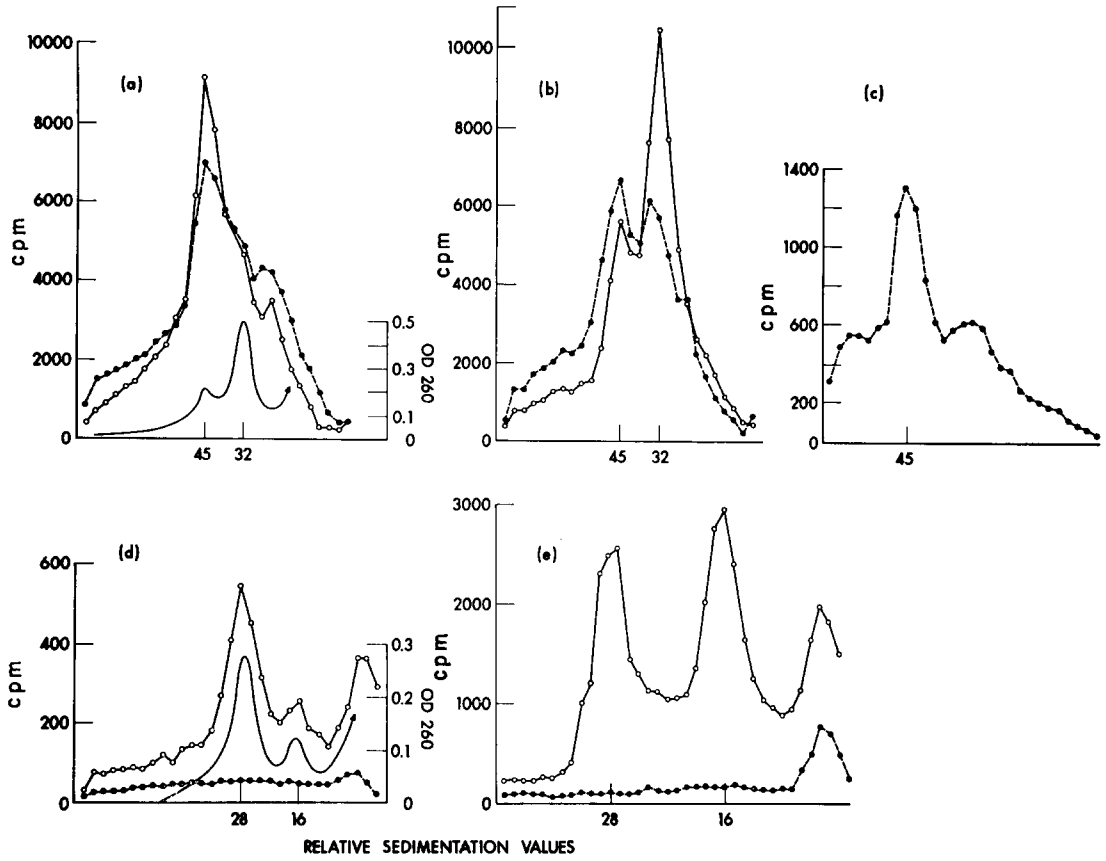

FIGURE 2 RNA synthesis in puromycin-treated cells. A growing culture of HeLa cells was divided (−5 min), and one-half was treated for 5 min with puromycin. At time 0, both cultures were labeled with uridine-^{3}H (0.5 μc ml, 20 mc/μmole) for 5 min followed by the addition of unlabeled nucleosides (final concentrations uridine 0.1 mM, cytidine 0.05 mM, and thymidine 0.05 mM). One sample (*a*) was taken at +15 min and another (*b, d, e*) at +75 min. The cell samples were then subjected to cell fractionation followed by RNA extraction and sedimentation analysis of the various subcellular fractions (See Methods and Materials). In all samples radioactive RNA from cell fractions of control cells is indicated by ○ and that from puromycin-treated cells by ●. The solid lines in *a* and *d* indicate the OD_{260} tracings of the control samples which were identical with those from the puromycin-treated samples. The positions of all peaks in this figure and subsequent figures was determined by comparing them with major peaks in the OD_{260} tracings. The OD_{260} profile, however, is only given in *a* and *d* of this figure, for the sake of simplicity. *a*, nuclear pellets +15 min; *b*, nuclear pellets +75 min; *d*, nuclear supernatant +75 min; *e*, cytoplasm +75 min.

For comparison of the over-all sedimentation profiles of the radioactive RNA in the nuclear pellet fractions (*a* and *b*) the cpm in the puromycin samples have been multiplied by four for *a* and by two for *b*. *c* represents an analysis of the RNA of the nuclear pellet of cells treated with puromycin for 60 min before a 40 min exposure to uridine-^{14}C (0.01 μc/ml; 30 μc/μmole).

during the nuclear fractionation has not been established. Also depicted in the diagram is the recent finding that 5S RNA, a newly discovered "structural" RNA (Rosset et al., 1964; Knight and Darnell, 1967), joins the larger ribosomal subunit before it leaves the nucleus.

Fig. 1 further illustrates that newly formed ribosomal subunits first enter the cytoplasm as subunits and then are found in polyribosomes. Later they are found in single ribosomes. Although it has been established that there is eventual equilibrium between free subunits (Vaughan et al., 1967 *a*), free 74S ribosomes, and the ribosomes in polysomes, the mechanism by which this equilibrium is accomplished is at present obscure.

The over-all process of ribosomal manufacture in HeLa cells, as tested by the appearance in the cytoplasm of subribosomal particles bearing newly formed rRNA, has been shown to be suppressed by puromycin treatment (Latham and Darnell, 1965; Warner et al., 1966 *a*). With newer techniques it is possible, as outlined above, to observe several stages in the biosynthesis of ribosomes prior to cytoplasmic appearance. Therefore, an attempt was made to locate the stage at which puromycin exerted its inhibition of the proposed scheme of ribosome synthesis.

Previous experiments have shown that, when actinomycin D is added to previously labeled cells at the time the 45S RNA is the dominant labeled RNA species, no further incorporation into RNA takes place and that the 45S RNA disappears while 32S (ultimately 28S) and 16S rRNA appear (Scherrer et al., 1963; Warner et al., 1966 *b*; Penman, 1966). In addition, the 45S molecule is the only rapidly labeled RNA species with the ap-

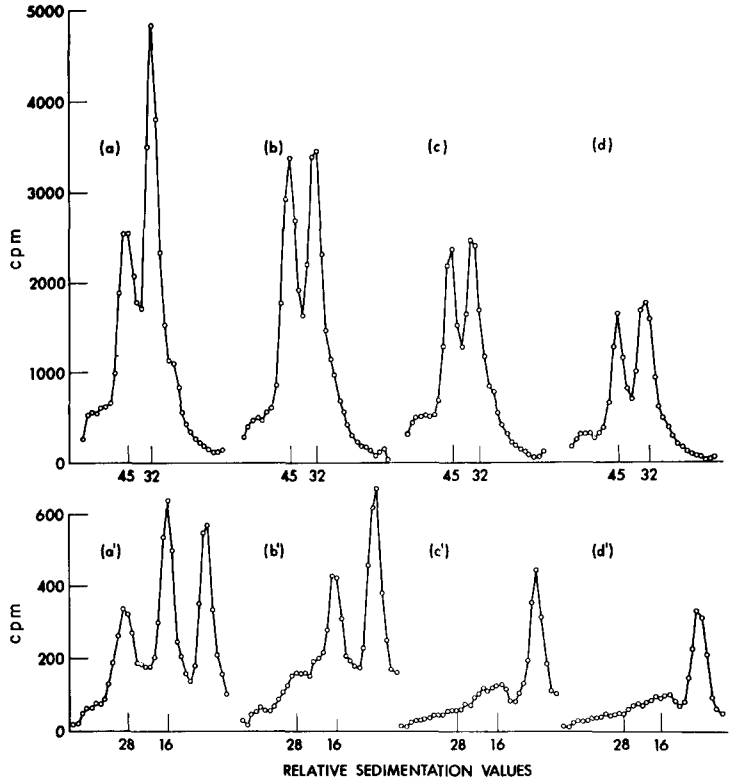

FIGURE 3 Dose dependence of puromycin inhibition of ribosome formation. Logarithimically growing HeLa cells were preincubated for 5 min with puromycin at final concentrations of 0 (*a*, *a'*), 5 (*b*, *b'*), 20 (*c*, *c'*), or 100 γ/ml (*d*, *d'*), and then (in the continued presence of the drug) labeled for 75 min with uridine-^{3}H as in Fig. 2. Each culture was fractionated, and the newly synthesized radioactive RNA in the nuclear pellet (*a*, *b*, *c*, *d*) and "cytoplasmic" (*a'*, *b'*, *c'*, *d'*) fractions analyzed as in Fig. 2. In this and all subsequent experiments nuclear pellet RNA labeled with a uridine precursor was released with SUT and the nuclear supernatant and cytoplasmic fractions with SDS (○, cpm).

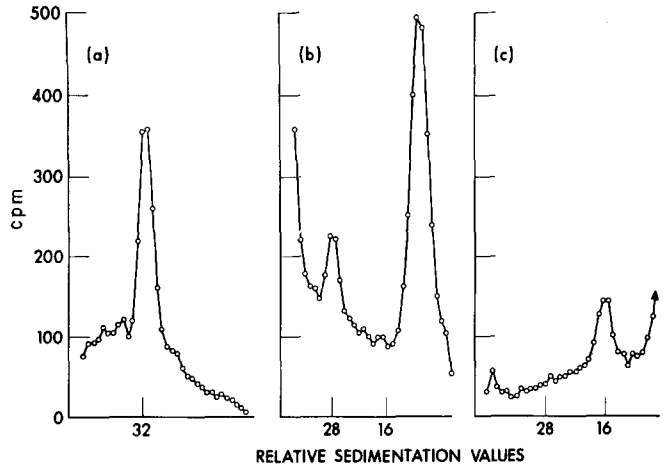

FIGURE 4 Fate of RNA accumulated in the presence of puromycin after removal of drug. HeLa cells were preincubated for 5 min with puromycin 100 γ/ml and then labeled with uridine-^{14}C (30 μc/μmole; 0.02 μc/ml) in the presence of the drug. After 30 min, actinomycin D (5 γ/ml) was added to the culture and the cells were washed free of puromycin by centrifugation (800 *g* for 3 min at 37°C) and resuspension in fresh medium containing actinomycin D. After 60 min of further incubation the cells were fractionated and the radioactive RNA was analyzed as before. *a*, nuclear pellet; *b*, nuclear supernatant; *c*, cytoplasm.

propriate, high guanosine plus cytosine content to serve as a precursor to ribosomal RNA (Soeiro et al., 1966). It was therefore concluded earlier that 45S RNA is a precursor to both 28S and 16S rRNA. Fig. 2 shows an experiment in which cells had been preincubated for 5 min with puromycin (100 γ/ml) and then labeled with uridine-^{3}H (5 min pulse followed by a "chase" with unlabeled nucleosides). RNA from various cell fractions was then examined by sedimentation analysis. The nuclear pellet or nucleolar fraction of cells exposed to uridine-^{3}H for a total of 20 min contained newly synthesized RNA which sedimented as a sharp peak at 45S just as did RNA released from normal cells (Fig. 2 *a*). Furthermore (Fig. 2 *b*), after a 75 min exposure to uridine-^{3}H both labeled 45S and 32S ribosomal precursor RNA were found in the nucleolus of puromycin-treated cells, but, unlike the control culture, 28S or 16S RNA was unlabeled in both the nuclear supernatant (Fig. 2 *d*) and the cytoplasm (Fig. 2 *e*). When the 32S RNA appeared in the control culture, radioactive RNA totaling about one-third the number of counts in the 32S peak was observed in the 16S RNA of the cytoplasm and the nuclear supernatant. In the puromycin-treated culture, no radioactive 16S RNA could be detected in any of the cell fractions. This finding suggests that any 16S RNA formed is rapidly degraded in the presence of the drug.

Fig. 2 *e* shows that incorporation into ribosomal precursor molecules continued even after 60 min of exposure to puromycin although the appearance of 32S was slowed compared to normal cultures (Scherrer et al., 1963; Warner et al., 1966 *b*).

It is obvious from the results in Fig. 2 that, while puromycin causes no great qualitative changes in the types of RNA being formed in the nucleus of treated cells, there is a sharp decrease in the rate of total incorporation. This is probably not due to a real decrease in the rate of RNA synthesis but to the lower specific activity attained in the acid-soluble pool of nucleotides when puromycin-treated cells are exposed to uridine-^{3}H, a phenomenon to be described elsewhere (Soeiro, R., and S. Vaughan. Data in preparation.). However, Fig. 3 shows that the defect in the synthesis of ribosomes which is imposed by puromycin is separable from its effect on total uridine incorporation into RNA. Cells were treated with three concentrations of puromycin (5, 20, and 100 γ/ml) before being labeled with uridine-^{3}H and then were fractionated as described in Methods and Materials. It can be seen that cells treated with 5 γ/ml of puromycin incorporate almost normal (>90% of control) amounts of uridine-^{3}H into ribosomal precursor RNA (Fig. 3 *a* and *b*) and that cells treated with 20 γ/ml of puromycin incorporate about one-half as much as control (Fig.

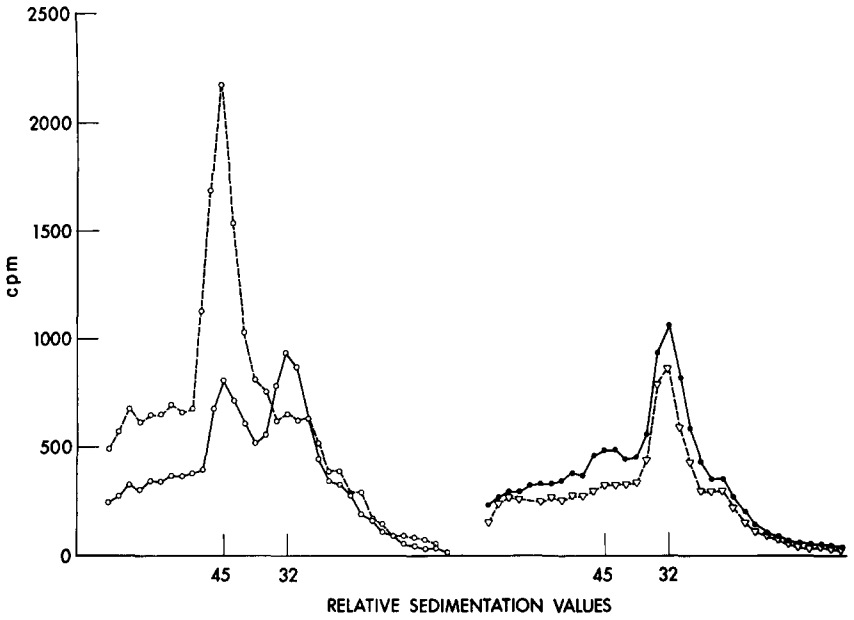

FIGURE 5 Fate of puromycin RNA in presence of the drug. HeLa cells were preincubated with puromycin (100 γ/ml) for 5 min and then labeled with uridine-^{14}C for 30 min (0 time). An aliquot was removed, and the remaining culture was treated with actinomycin D (5 γ/ml) and allowed to incubate in the presence of both drugs. After a further 30, 60, or 90 min, samples were taken and fractionated. The radioactive RNA of the nuclear pellet was analyzed as in the previous experiments. 0 time (--○--); 30 min (—○—); 60 min (—●—); and 90 min (--▽--).

3 *c*). It is also apparent that treatment of cells with 20 or 100 γ/ml of puromycin completely prevented the cytoplasmic appearance of 16S or 28S rRNA (Fig. 3 *c'* and *d'*). Even 5 γ/ml of puromycin decreased the appearance of 28S rRNA (Fig. 3 *b'*). Thus, puromycin produces a specific block(s) in ribosomal manufacture past the stage of transcription of 45S ribosomal precursor RNA.

Fate of 45S RNA in Puromycin-Treated Cells

The next series of experiments was designed to determine whether the 45S RNA formed in the presence of puromycin could be utilized to form ribosomes when puromycin was removed. This can be accomplished through the use of both puromycin and actinomycin D, a drug which stops further RNA synthesis within 1–2 min (Reich et al., 1961; Warner et al., 1966*b*).

Fig. 4 shows an experiment in which puromycin-treated cells were labeled for 30 min to accumulate radioactive 45S RNA. Actinomycin D was then added to the culture. The cells were washed free of puromycin, but actinomycin was maintained in the medium. After 60 min the cells were fractionated, and it was found that 16S rRNA had appeared in the cytoplasm and that 28S rRNA could be detected in the nuclear supernatant. In separate experiments performed exactly as that shown in Fig. 4, it was found that both the 16S and 28S rRNA observed in the cytoplasm or nuclear supernatant can in fact be recovered from subribosomal particles. In this experiment, no 28S rRNA had entered the cytoplasm because of the defective exit of larger nuclear subribosomal units in the presence of actinomycin (Girard et al., 1964). Thus the 45S formed in the presence of puromycin can, when puromycin is removed, give rise to 28S and 16S rRNA without any further RNA synthesis.

As has been suggested earlier, since no 28S and 16S rRNA appear in the cytoplasm in the presence of puromycin, they may be destroyed in the nucleus soon after formation. This possibility was also tested with the aid of actinomycin. Cells which had been labeled in the presence of puromycin were further treated with actinomycin D to arrest RNA synthesis. The fate of the 45S-labeled RNA

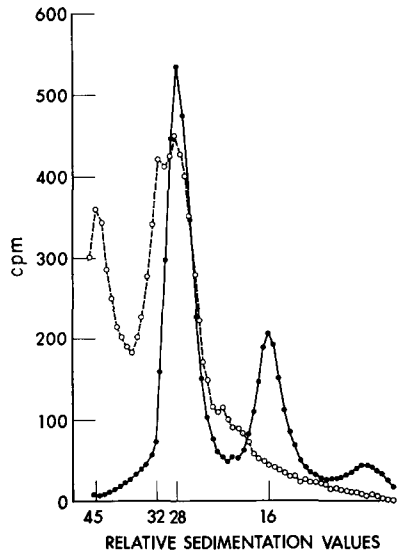

FIGURE 6 Analysis of puromycin 32S RNA. A culture of HeLa cells was preincubated for 5 min with puromycin (100 γ/ml) and then labeled continuously with uridine-^{14}C for 2 hr in the presence of the drug. The radioactive puromycin RNA was extracted from the nuclear pellet and cosedimented with normal cytoplasmic RNA labeled with ^{3}H for 16 hr. The uridine-^{3}H–labeled sample served as a radioactive marker for normal cytoplasmic 28S RNA.

molecules was then studied after an additional 30, 60, and 90 min (Fig. 5). Radioactivity in 45S RNA decreased although considerably more slowly than has previously been observed in normal cells (Warner et al., 1966*b*). There was at first a slight increase in "32S" RNA, but no 16S rRNA was detected. Thus there must be degradation of new 16S rRNA in cells continually treated with puromycin, since the experiment of Fig. 4 shows definitely that 16S molecules can, upon the removal of puromycin, derive from 45S RNA formed in the presence of puromycin.

So that nominal 32S RNA, the first larger rRNA product of 45S cleavage, could be distinguished from nominal 28S rRNA, the ultimate product found in the ribosomes, a precise sedimentation analysis was then carried out on the 32S RNA in the nucleolus which accumulated in the presence of puromycin. For this purpose, cytoplasmic RNA from growing cells labeled overnight with uridine-^{3}H was mixed with the nucleolar RNA from puromycin-treated cells which had been labeled for 2.5 hr with uridine-^{14}C. Fig. 6 shows that some RNA-^{14}C cosediments with 28S cytoplasmic RNA. This implies that puromycin does not completely block the development of 28S RNA. Since the puromycin-treated cells continue to make 45S, 32S, and probably 28S RNA without making subribosomal particles, perhaps the 28S RNA, like the 16S, is degraded.

Lack of Effect of Puromycin on RNA Methylation

In light of the knowledge that methyl groups are incorporated into the 45S ribosomal precursor RNA (Greenberg and Penman, 1967; Zimmerman and Holler, 1967) prior to its cleavage into 32S and 16S RNA, it was possible that puromycin exerted its blocking effect on proper ribosomal maturation by affecting methylation of 45S RNA. Therefore, cells which had been preincubated for 5 min with puromycin (100 γ/ml) were labeled with methyl-^{14}C–labeled methionine for 20 min. The degree of labeling in 45S RNA of (Fig. 7) the puromycin-treated cells was the same as that of the control cells. Two conclusions can be made

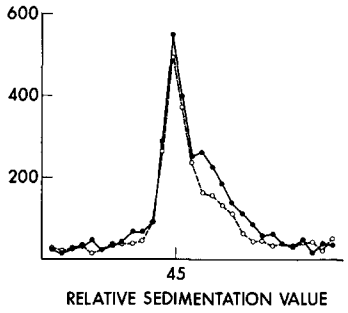

FIGURE 7 Labeling of 45S RNA with methyl-^{14}C–labeled methionine in the presence of puromycin. A culture of HeLa cells which had been grown in Eagle's medium containing adenosine 10^{-4} M was centrifuged and resuspended in methionine-free Eagle's medium (5% dialyzed horse serum, adenosine 10^{-4} M). The adenosine is used to prevent utilization of the CH_3 groups of methionine in purine ring synthesis (Greenberg and Penman, 1967). After 5 min at 37°C the culture was recentrifuged and suspended in methionine-free Eable's medium (plus adenosine 10^{-4} M). The culture was then divided, and one-half was incubated with puromycin (100 γ/ml). After 5 min each culture was exposed to methyl-^{14}C–labeled methionine (0.5 μc/ml; 15 μc/μmole) for 20 min. After 10 min of incorporation, an additional 5% of undialyzed horse serum was added to prevent methionine depletion. The nuclear pellet RNA was extracted with hot phenol and was then analyzed as in Fig. 2. ● control; ○ puromycin treated.

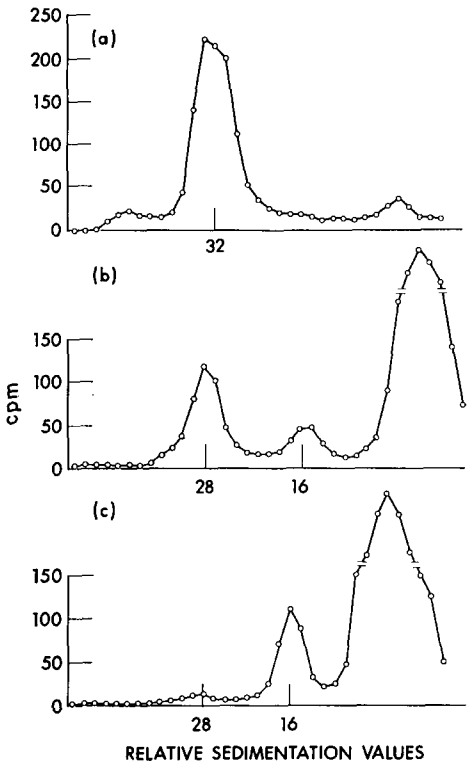

FIGURE 8 Fate of methyl-^{14}C-labeled puromycin RNA after removal of drug. Cells were labeled with methyl-^{14}C methionine as in the experiment of Fig. 7. After a 30 min period of labeling, further incorporation was stopped by the addition of actinomycin D (5 γ/ml); the cells were washed free of the puromycin, and then incubated in the presence of actinomycin D for an additional 60 min. RNA was analyzed after phenol extraction from (*a*) nuclear pellet, (*b*) nuclear supernatant, (*c*) cytoplasm.

from this experiment. First, puromycin does not affect rRNA maturation by grossly disturbing the extent of methylation. Second, the rate of 45S RNA synthesis is not impaired by puromycin, even though, as has been discussed previously, uridine incorporation is lowered by the drug. This conclusion is based on the facts that *only* the new 45S RNA molecules are receptors for methyl groups and that the time for the entire process of methylation and cleavage of 45S requires only 10–15 min. Thus, when a culture treated with puromycin for a total of 25 min was found to have exactly the same amount of radioactive methyl groups in 45S RNA as the control, it could be concluded that continuing 45S RNA synthesis was not impaired by the drug.

A second experiment demonstrates that the same methyl-labeled rRNA precursor molecules which are formed in puromycin-treated cells can in fact be utilized in the construction of ribosomes when puromycin is removed. Puromycin-treated cells were labeled with methyl-^{14}C-labeled methionine for 30 min; then actinomycin was added, and the cells were washed free of the puromycin. Subsequently, radioactivity from the methyl-labeled 45S RNA molecules, which had accumulated prior to addition of actinomycin, appeared in 16 and 28S rRNA (Fig. 8). It has been shown that within 5 min of the addition of actinomycin D there is no further methyl-^{14}C labeling of 45S RNA (Zimmerman and Holler, 1967). Therefore in the experiment of Fig. 8 extensive methylation of RNA after removal of puromycin could not have occurred.

This experiment demonstrates that at least some 45S molecules formed in puromycin-treated cells not only have the normal number of methyl groups but probably also have the right pattern of methyl groups since it appears from other studies on ribosome formation in methionine-deficient cells (Vaughan, et al. 1967*a*) that proper methylation is a prerequisite for ribosomal maturation.

Cycloheximide vs. Puromycin

The final series of experiments to be described in this paper was undertaken to elucidate the basis of the puromycin-induced block of rRNA maturation. Even when protein synthesis is reduced to less than 1% of the control rate by cycloheximide, HeLa cells are still capable of initiating ribosomal RNA synthesis and completing the manufacture of ribosomes from a preexisting pool of protein (Warner et al., 1966 *a*). Thus the defect imposed by puromycin on rRNA maturation cannot be due to simple inhibition of protein synthesis or to a depletion of proteins essential to rRNA maturation. The action of puromycin on protein synthesis differs from cycloheximide in a very important respect. Peptide-bond formation continues in the presence of puromycin and results in the formation of acid-soluble peptidyl–puromycin products (Nathans, 1964; Smith et al., 1965) while cycloheximide acts by drastically slowing the rate of peptide-bond formation (Ennis and Lubin, 1964; Siegel and Sisler, 1964; Wettstein et al., 1964). Prior treatment of cells with cycloheximide might therefore be expected to inhibit the formation of these puromycin peptides. It was reasoned that,

if these compounds play a role in the puromycin-induced block in rRNA maturation, perhaps a prior treatment with cycloheximide might allow ribosome maturation to occur even in the presence of puromycin. HeLa cells which had received cycloheximide for 20 min and puromycin for 15 min were "pulsed" with uridine-^{3}H and chased with ribonucleosides in the presence of the two drugs. 150 min after the chase nucleosides had been added, cytoplasmic RNA in this and in two similar cultures which had received only a single drug was examined as before (Fig. 9). It is clear that the addition of cycloheximide allowed substantial 28S and 16S RNA to emerge into the cytoplasm of puromycin-treated cells.

The prevention of the puromycin blockage of rRNA maturation by cycloheximide is even more pronounced if normal RNA synthesis is allowed to occur for 15 min (at which time the only labeled RNA related to ribosomes will be the 45S RNA)

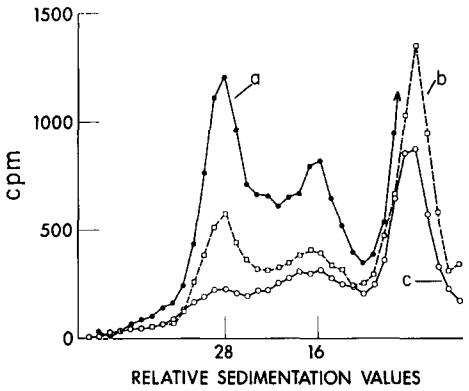

FIGURE 9 Effect of cycloheximide on the capacity of puromycin to block ribosome formation. Logarithmically growing HeLa cells were preincubated with *a*, cycloheximide (cyclo), 100 γ/ml for 20 min; *b*, cycloheximide for 20 min and puromycin (puro), 100 γ/ml for 15 min; or *c*, puromycin for 15 min before the addition of uridine-^{3}H. After 10 min of labeling chase nucleosides were added, and cultures were incubated a further 140 min at which time cytoplasmic RNA was examined.

Experimental Plan

Time, *Min*... −20	−15	0	+10	150
a Cyclo.....				
b Cyclo.....	puro	..uridine-^{3}H..	chase..	sample
c	puro			

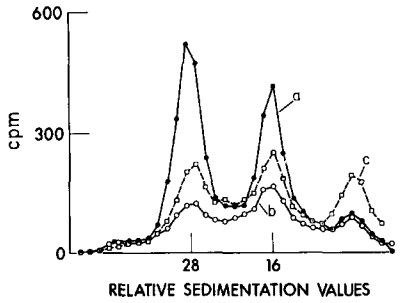

FIGURE 10 The effect of puromycin on the formation of ribosomes from normal RNA in the presence of cycloheximide. HeLa cells were labeled with uridine-^{14}C for 8 min before the addition of chase nucleosides. After an additional 10 min, the culture was divided and puromycin (100 γ/ml) or cycloheximide (150 γ/ml) plus puromycin were added according to the experimental plan outlined above. The cultures were incubated a further 72 min before newly labeled cytoplasmic RNA was examined.

Experimental Plan

Time, *Min*.. 0	8	18	90
Uridine-^{14}C.. chase..	*a* Control.....		sample
	b puro.....		
	c puro + cyclo.		

before the addition of the two drugs. Fig. 10 shows such an experiment in which cycloheximide plus puromycin allow 28S and 16S RNA to exit to the cytoplasm in almost one-half the control amount.

DISCUSSION

With the availability of techniques which allow the observation of several stages in ribosomal RNA biosynthesis and maturation, a re-examination of the puromycin-induced block of ribosome manufacture was in order. The experiments to be described in a separate paper (Soeiro, R., M. H. Vaughan, and J. E. Darnell. Data in preparation.) indicate that puromycin quickly depresses vhe rate of uridine incorporation into HeLa cell RNA largely by preventing the entry of uridine into the nucleotides of the acid-soluble pool. However, in spite of this pool effect, the same types of RNA become labeled in the puromycin-treated cell as in normal cells. Furthermore, labeling of 45S RNA with methyl-^{14}C-labeled methionine indicates that ribosomal precursor RNA is actually made at the same rate as control cells.

The fate of 45S RNA labeled in the presence of puromycin has been studied in several experiments by preventing further RNA synthesis with actinomycin. After removal of puromycin, the 45S RNA labeled in the presence of the drug either by uridine or by methyl-^{14}C–labeled methionine can be cleaved to 32S and 16S RNA. Moreover 16S RNA, derived from the 45S RNA made in the presence of puromycin, enters the cytoplasm where it may be found in ribosomal particles. The 32S RNA also enters the normal maturation process appearing as 28S RNA in the nuclear supernatant particles. These experiments indicate not only that 45S RNA is transcribed, but also that the stages of maturation associated with methylation, the cleavage of 45S RNA and the alteration of 32S to 28S rRNA, can proceed normally in the presence of puromycin.

The defect imposed by puromycin therefore seems to reside at the level of conversion of a cleavage product of ribosomal precursor RNA into a stable ribosomal particle. Furthermore, the data indicate that in the absence of such a maturation step the RNA is degraded. This conclusion follows from the finding that the puromycin-treated cell continues to synthesize ribosomal RNA without accumulating any of the RNA species. Whether rRNA degradation is an abnormal accompaniment of blocked ribosomal maturation imposed by this drug or the cell normally has a mechanism for degradation of unused or unwanted ribosomal RNA is impossible to answer at this time. It is pertinent to point out, however, that continued synthesis and destruction of ribosomal precursor RNA rather than its normal utilization have been found to occur in HeLa cells deprived of methionine (Vaughan et al., 1967*a*). It is possible therefore that over-all ribosome synthesis in these cells is regulated not at the level of transcription of the 45S ribosomal RNA precursor, but at the level of the utilization of the products of this molecule.

The final point of interest in these experiments relates to the mechanism whereby one inhibitor or protein synthesis, puromycin, effectively blocks ribosome maturation while another, cycloheximide, does not. The outstanding, known difference between cells treated with these two agents is that in puromycin-treated cells small peptidyl–puromycin complexes are continually being generated while in cycloheximide-treated cells the machinery for making proteins is simply slowed by a factor of 50–100. If the synthesis of the peptidyl–puromycin complexes is halted by the simultaneous presence of cycloheximide, rRNA maturation can proceed in the presence of puromycin. That cycloheximide does not block the entry of puromycin into the cell is suggested by the fact that polyribosomes, normally stable when inhibited by cycloheximide, are slowly disaggregated when puromycin treatment is superimposed (Columbo et al., 1965; Soeiro, R. Unpublished results). These results suggest that there is no direct involvement of the puromycin molecule itself in blocking ribosomal maturation but that a cellular product made in the presence of puromycin could be the responsible agent. Since the only known products of puromycin within cells are puromycin peptides or inactive degradation products of puromycin itself, it is tempting to conclude that puromycin–peptides combine with ribosomal precursor RNA or newly generated 28S and 16S rRNA in such a way that its proper union with ribosomal protein is inhibited and destruction of the RNA follows.

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