THE SELECTIVE INTERRUPTION OF NUCLEOLAR RNA SYNTHESIS IN HELA CELLS BY CORDYCEPIN

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ABSTRACT

Cordycepin is an analogue of adenosine lacking the 3′-OH. When incorporated into a growing RNA molecule, cordycepin prevents further elongation, thus producing a prematurely terminated RNA molecule. When HeLa cells are exposed to low concentrations of cordycepin, DNA and protein synthesis are unaffected during short exposure periods. The synthesis of completed ribosomal and ribosomal-precursor (45S) RNA is significantly depressed. Partially completed 45S ribosomal precursor molecules accumulate in the nucleolus. 18S ribosomal RNA can be cleaved from these incomplete precursors, while 32S ribosomal precursor cannot be produced from partially snythesized 45S molecules. The synthesis of transfer RNA is also reduced in the presence of cordycepin. The synthesis of the nuclear heterogeneous RNA species is unaffected by the drug while the cytoplasmic heterogeneous RNA is slightly reduced.

INTRODUCTION

The nucleolus is a specialized and highly organized structure in the cell, whose principal and perhaps only function is the production of ribosomes (1–3). Nucleolar function can be easily disrupted by agents which interfere with nucleic acid or protein synthesis. Nucleolar synthesis is inhibited more rapidly and at lower doses of these various agents than the synthesis of other species of RNA (1, 4, 5). Thus, levels of actinomycin which have little effect on most cellular RNA metabolism can completely inhibit nucleolar synthesis. Mitomycin C, which inhibits RNA synthesis by a very different mechanism than actinomycin, also causes selective nucleolar inhibition (R. Laing and S. Penman. Unpublished observation).

Another class of agents is incorporated into nucleolar RNA and interferes with normal processing. Examples are: toyocamycin, tubercidin, and azaguanine (6, 7).

Cordycepin (3′ deoxyadenosine) is a unique agent which can interfere with the synthesis of complete molecules (8). Cordycepin triphosphate has been shown to cause premature termination of RNA synthesized in vitro by DNA-dependent RNA polymerase (9, 18).

The effects of cordycepin on HeLa cell RNA metabolism are described in this report. This drug is selective and principally affects nucleolar synthesis. Ribosomal precursor molecules are prematurely terminated. Apparently only 18S ribosomal RNA can be produced from these shortened molecules.

The principal heterogeneous high molecular weight species of RNA are relatively unaffected by the drug. However, tRNA[1] synthesis is also affected.

[1] The following abbreviations are used: TCA, trichloroacetic acid. SDS, sodium dodecyl sulfate.

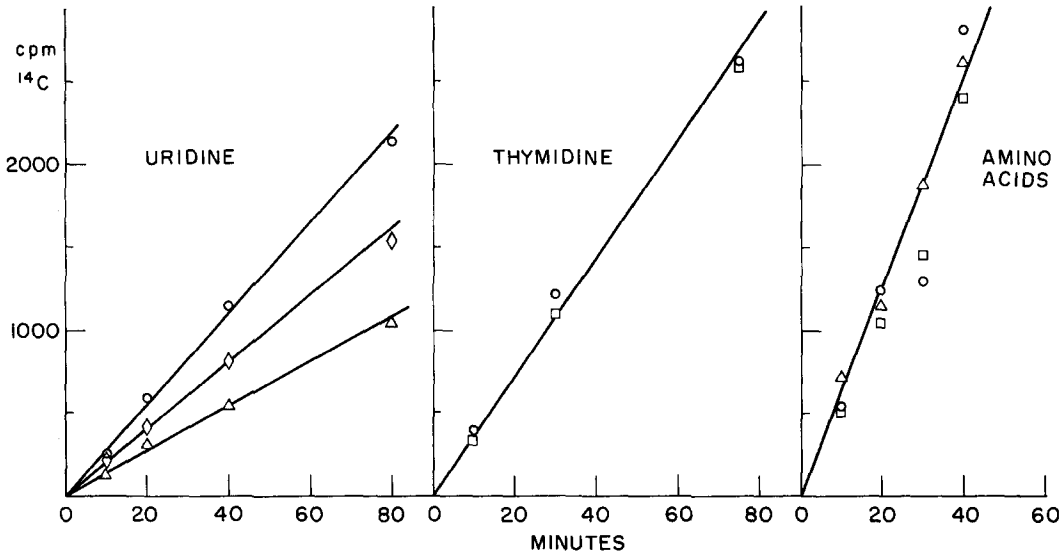

FIGURE 1 *The effect of cordycepin on macromolecular synthesis.* HeLa cells were concentrated to 2×10^6/ml and preincubated with cordycepin for 10 min. The labeled precursors were then added. At the indicated times, 0.2 ml samples were pipetted into 5 ml of cold Earle's saline and centrifuged. The cell pellet was resuspended in 0.2 ml of distilled water, precipitated with 2 ml of cold 10% TCA, collected on Millipore filters, and washed with 5% TCA. For the amino acid incorporation, this procedure was modified by the addition of 0.5 ml of 1 N KOH to the resuspended cell pellet. 15 min later, 1 ml of 20% TCA was added, and the samples were filtered and washed as before. Labeled isotopes were added as follows: uridine-^{14}C, 0.1 μc/ml; thymidine-^{14}C, 0.1 μc/ml; reconstituted protein hydrolysate-^{14}C, 0.5 μc/ml. ○, control; □, 50 μg/ml cordycepin; ◇, 2.5 μg/ml cordycepin; △, 20 μg/ml cordycepin.

MATERIALS AND METHODS

HeLa cells were grown in suspension culture as previously described (10).

Labeling and fractionation of cells were carried out as previously described (11). The subfractionation of the nuclei was carried out by the method described by Penman, Smith, and Holtzman (3). Zonal centrifugation for isolating nucleoli as described by Willems, Wagner, Laing, and Penman was used (12). RNA extraction was performed with the hot phenol-SDS method described by Scherrer and Darnell (13) which was modified by additional hot chloroform extraction (3).

The analysis of RNA was performed with SDS-sucrose as previously described (14) or by acrylamide gel electrophoresis first described by Loening (15) which was subsequently modified to include glycerol in the gels (16).

Uridine-^{14}C (43–50 μc/μmole), uridine-^{3}H (20 c/mmole), reconstituted protein hydrolysate-^{14}C and thymidine-^{14}C (49 μc/μmole) were purchased from Schwarz Bioresearch, Orangeburg, N.Y., and methylmethionine-^{3}H (2c/mmole) from Nuclear Chicago, DesPlaines, Ill. Cordycepin was a gift of the Cancer Chemotherapy National Service Center of the National Institutes of Health and was also purchased from Sigma Chemical Co., St. Louis. Actinomycin D was a gift of Merck, Sharpe, and Dohme, West Point, Pa.

RESULTS

Cordycepin (3′ dA) is an analogue of adenosine in which 3′OH is replaced by 3′H (8). It has been shown to possess some anti-tumor activity (17). Experiments on cells in culture have suggested that the drug may act principally as an inhibitor of RNA synthesis (8).

In vitro experiments with the DNA-dependent RNA polymerase from *Micrococcus lysodeikticus* have shown that 3′-deoxy-ATP is incorporated only in the terminal position of nascent RNA molecules (9). Because the analogue has an OH group in the

3′-dA, cordycepin. nRNA, nuclear heterodisperse RNA. tRNA, transfer RNA. rRNA, ribosomal RNA. PRPP, phosphoribosyl pyrophosphate. ATP, adenosine triphosphate. AMP, adenosine monophosphate. 3′-dATP, 3′-deoxyadenosine triphosphate. cRNA, cytoplasmic heterodisperse RNA.

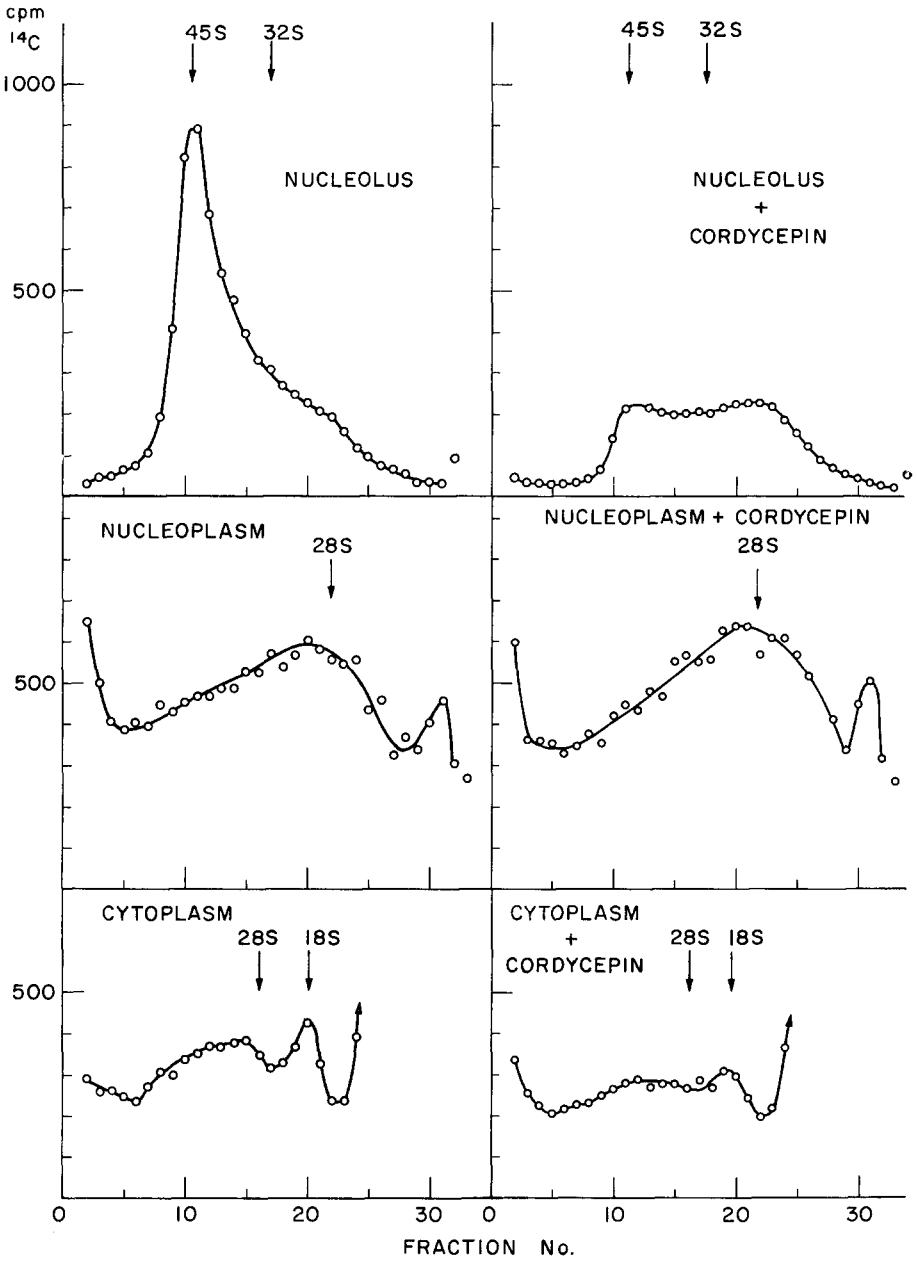

FIGURE 2 *Sedimentation analysis of RNA from cordycepin-treated cells.* 20 ml of cells at a concentration of 2×10^6/ml were incubated for 10 min with 10 μg/ml cordycepin and then labeled with uridine-^{14}C at a concentration of 0.1 μc/ml for 10 min (nucleolus, nucleoplasm) or 30 min (cytoplasm). The extracted RNA was analyzed on 15–30% SDS-sucrose gradients as described in Materials and Methods, and the results compared with RNA from untreated cells. Nucleolar RNA was centrifuged at 24,300 rpm for 12.5 hr, and nucleoplasmic RNA at 19,000 rpm for 15 hr in the SW 25.3 rotor. Cytoplasmic RNA was centrifuged in the SW 40 rotor at 40,000 rpm for 3.75 hr.

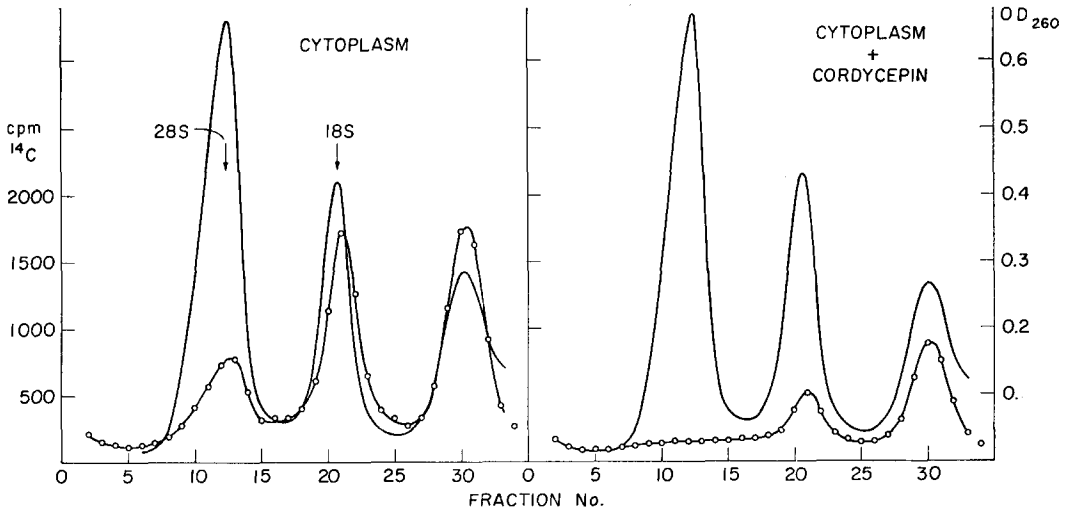

FIGURE 3 *The suppression of labeled cytoplasmic ribosomal RNA in cordycepin-treated cells.* 10 ml of cells at 2 × 10^6/ml per sample were treated with 20 μg/ml cordycepin for 10 min and then labeled for 60 min with uridine-^{14}C as described in Fig. 2. The extracted RNA was analyzed on 15–30% SDS-sucrose gradients spun in the SW 25.3 rotor for 18 hr at 25,000 rpm.

2′ position, it is apparently accepted by the polymerase as a ribonucleotide. A phosphodiester bond is formed joining the 5′ position in 3′dA to the previously incorporated nucleotide. However, no further bonds can be formed to the 3′ position and the RNA molecule is terminated.

In this report, the effects of 3′dA on HeLa cell RNA metabolism are studied. In vivo, premature termination of RNA occurs in the nucleolus, and shortened ribosomal precursor molecules are formed. The other cellular RNA species, except for tRNA, are relatively insensitive to the drug, and premature termination has been detected only in the ribosomal precursor species.

Selectivity

The high molecular weight RNA synthesized in the nucleolus is the 45S ribosomal precursor (13, 3). This molecule has a lifetime of 16–20 min (19). It is then cleaved to form several short-lived intermediates (16) and finally 32S RNA, which remains in the nucleolus for further processing, and 18S RNA which is exported rapidly to the cytoplasm. When radioactive uridine is incorporated for a time less than the lifetime of 45S RNA (e.g 10 min), the radioactivity in the nucleolus is found associated principally with completed 45S RNA and a small population of incompletely synthesized molecules (20).

Fig. 1 shows the effect of various concentrations of 3′dA on the incorporation of labeled precursors into whole cell TCA-precipitable material. A concentration of 25 μg/ml depresses uridine incorporation (total RNA synthesis) by 50%, while thymidine incorporation (DNA synthesis) and amino acid incorporation (protein synthesis) are unaffected at 50 μg/ml. The insensitivity of DNA and protein synthesis shows that 3′dA does not directly interfere with general cell metabolism.

The principal site of action of 3′dA is the nucleolus. Cells were pretreated with 3′dA for 10 min and then labeled with uridine-^{14}C for 10 or 30 min as indicated. RNA was extracted from the principal cell fractions and analyzed on sucrose gradients. The results are shown in Fig. 2. The effect on nucleolar RNA synthesis is dramatic. Both the amount and size distribution of nucleolar RNA synthesized are markedly affected by 3′dA. In contrast, nucleoplasmic heterodisperse RNA is unaffected in size or amount. The labeling of cytoplasmic cRNA is slightly depressed, but the RNA synthesized in the presence of 3′dA appears to have a normal size distribution.

The inhibition of nucleolar synthesis results in suppression of ribosomal RNA entering the cytoplasm in 3′dA-treated cells. This is shown clearly (Fig. 3) when cells are labeled for 60 min. The amount of labeled 18S has decreased to 25% of

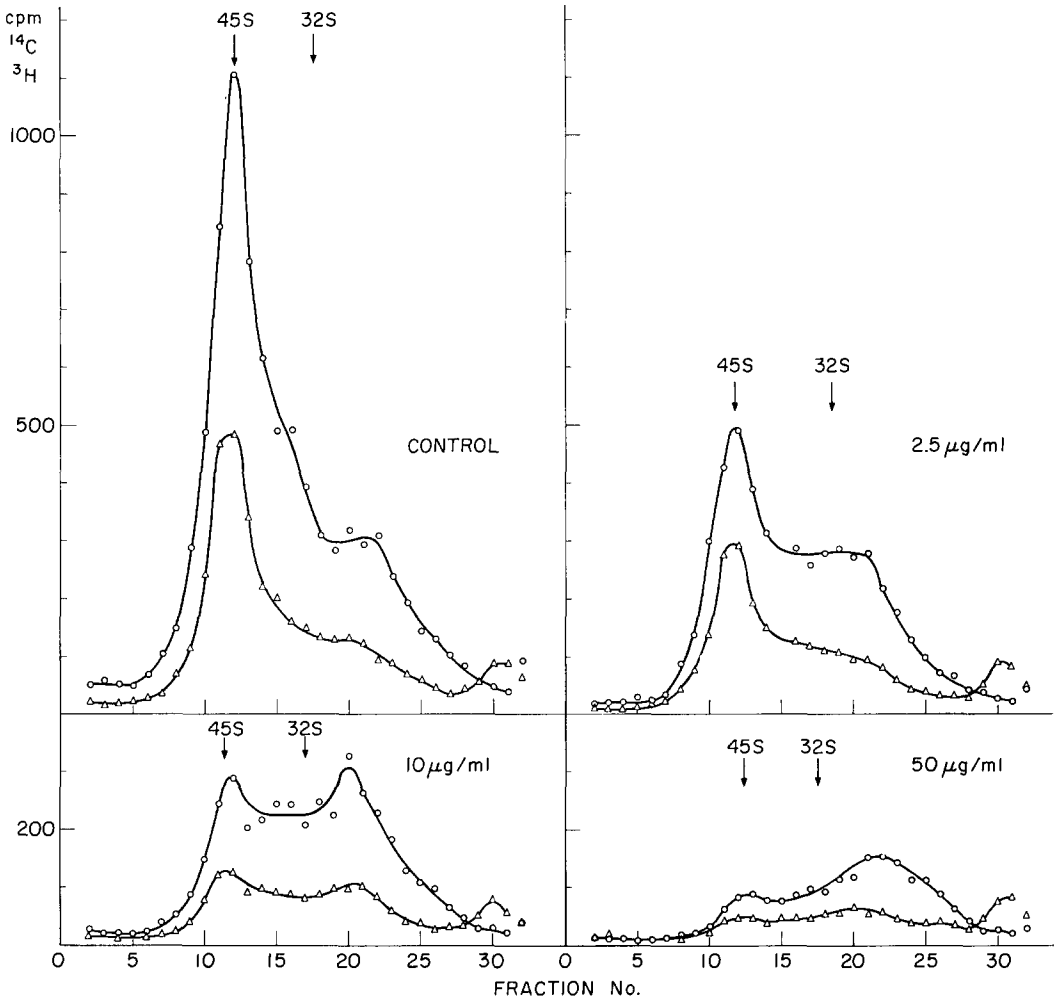

FIGURE 4 *Sedimentation analysis of nucleolar RNA synthesized at various concentrations of cordycepin.* 20-ml cell samples at a concentration of 2×10^6/ml were suspended in methionine-free medium containing adenosine and guanosine at a concentration of 2×10^{-5} M and treated with cordycepin at the indicated concentrations. [Methyl-^{3}H]-methionine was added at a concentration of 10 μc/ml, and 1 min later uridine-^{14}C was added for 10 min as in Fig. 2. —○—○—, ^{14}C; —△———△—, ^{3}H. 15–30% SDS-sucrose gradients of nucleolar RNA were centrifuged in the SW 25.3 rotor at 25,000 rpm for 11.3 hr.

control, and the production of 28S has been completely suppressed. This relative sensitivity of two species will be considered in detail later. The partial suppression of tRNA synthesis is also apparent in the drug-treated cells.

Effect on Nucleolar RNA

The effect of 3′dA on nucleolar rRNA synthesis was examined more closely. Increasing the concentration of 3′dA decreases the size of the species labeled in a 10-min pulse as measured by sedimentation (Fig. 4).

The appearance of small ribosomal precursor molecules is due to either premature chain termination or decrease in the rate of transcription. A comparison of a short and long nucleolar labels shows that the amount of partial ribosomal precursor molecules increases with the length of labeling time for at least 30 min (40 min after addition of 3′dA), but there is no formation of a distinct

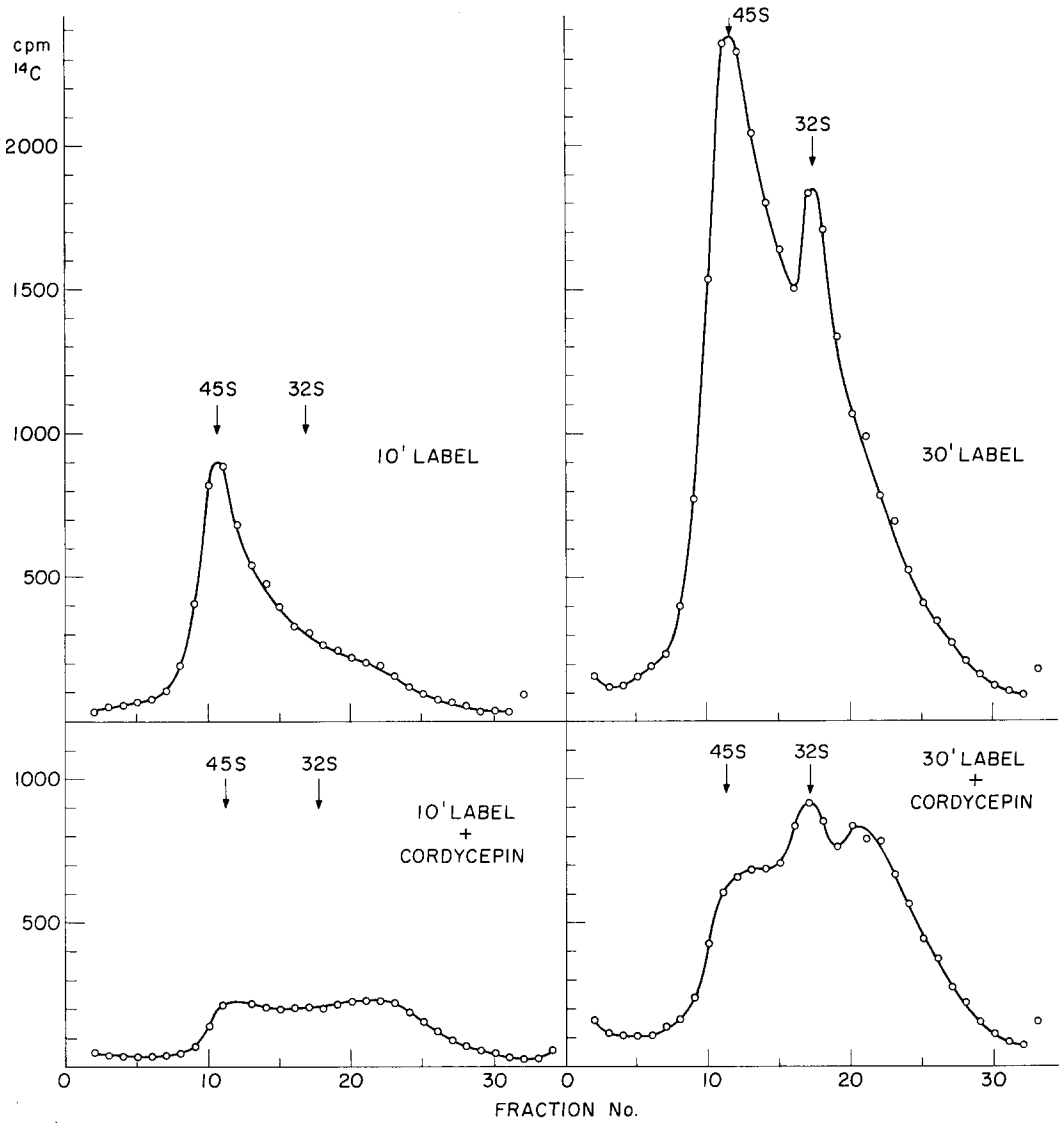

FIGURE 5 *Long vs. short label of nucleolar RNA in untreated and cordycepin-treated cells.* Cordycepin was at a concentration of 10 μg/ml. Samples of 20 ml of cells at a concentration of 2×10^6 cells/ml were labeled as in Fig. 2. 15–30% SDS-sucrose gradients of nucleolar RNA were centrifuged in the SW 25.3 rotor at 24,300 rpm for 12.5 hr.

peak of completed 45S molecules (Fig. 5). Therefore, the short chains seen in a 10-min pulse do not elongate to become 45S RNA molecules. It appears that these RNA molecules are prematurely terminated and are not precursors to 45S RNA.

Fig. 6 shows that there is no cumulative effect of 3′dA on the nucleolar rRNA-synthesizing machinery. 10-min pulses at various times up to 1 hr after 3′dA addition give virtually identical results. Therefore, the result of the long incorporation in the presence of 3′dA shown in Fig. 5 is not due to progressive deterioration in nucleolar synthesis.

The experimental results presented here are consistent with the presumed role of 3′dA as a chain terminator. Incomplete rRNA precursor molecules are made at a constant rate, and their size is inversely related to 3′dA concentration.

The nucleolar RNA shown in Fig. 4 was also

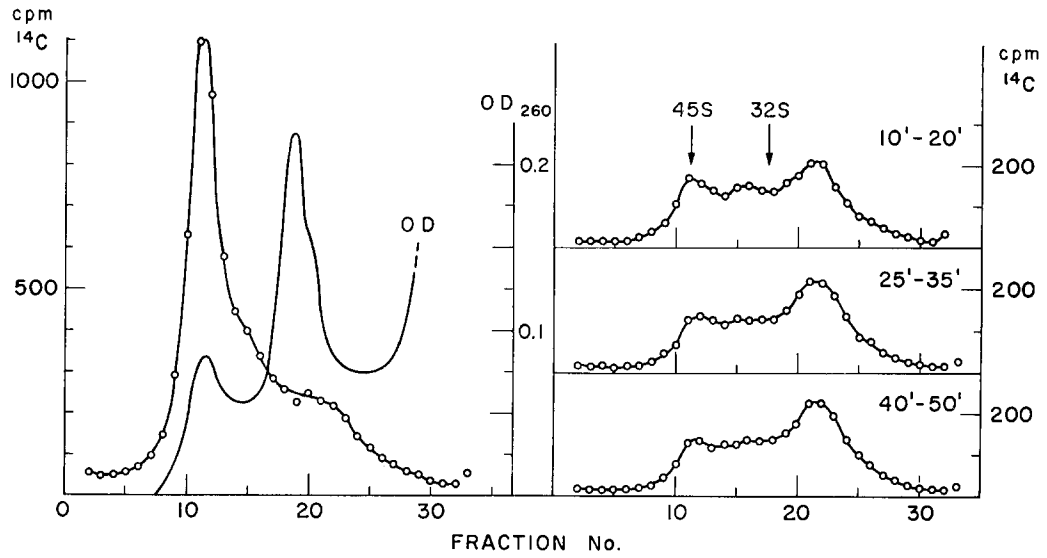

FIGURE 6 *Labeling of nucleolar RNA at various times after addition of cordycepin.* 20 ml of cells (2×10^6 cells/ml) per sample were treated with 10 μg/ml of cordycepin and labeled, as in Fig. 2, with uridine-^{14}C for 10-min periods at different times after addition of cordycepin. 15–30% SDS-sucrose gradients of nucleolar RNA were centrifuged in the SW 25.3 rotor for 16 hr at 21,000 rpm.

labeled with methionine. The methylation of ribosomal precursor RNA has been previously shown to occur shortly after synthesis (20). The amount of methyl label relative to uridine label in the 3′dA produced chains is similar to that in normal 45S precursor.

Processing of Partial 45S Molecules

The fate of incomplete 45S molecules was investigated. Cordycepin-treated cells were labeled for 10 min with uridine-^{3}H. A high concentration of actinomycin was added so as to prevent additional synthesis, and incubation was continued so as to allow processing. An aliquot of cells was removed before incubation in actinomycin, after 25 min and after 90 min in actinomycin. Another culture was labeled with uridine-^{14}C and treated similarly except that cordycepin was omitted. At each time point, ^{3}H- and ^{14}C-labeled samples were mixed and harvested. Fig. 7 shows nucleolar RNA after the 10-min label, nucleolar RNA after 25-min incubation in actinomycin, and cytoplasmic RNA after 90-min incubation in actinomycin. The amount of label in 45S, 32S, and 18S species of RNA was estimated, and the results are presented in Table I.

The experiment was designed to determine whether partially completed ribosomal precursor molecules could give rise to either 18S or 32S products. The use of double label permits a comparison of cordycepin-treated with untreated cells which is independent of recovery. The use of high-resolution acrylamide gel analysis permits an estimate of the amount of completed 45S precursor formed in the drug-treated cells.

The amount of each RNA species in cordycepin-treated cells was compared to that in untreated cells, and the results are presented in Table II as the ratio of ^{3}H cpm (treated) to ^{14}C cpm (untreated). This ratio gives the relative amounts of each species and will be the same for 45S, 32S, and 18S if 32S and 18S in cordycepin-treated cells arise from whole 45S molecules, as is the case in untreated cells. Clearly, the relative amounts of 32S RNA are the same as for 45S. Thus, radioactivity present in 32S RNA can be accounted for by the 45S molecule present before incubation in actinomycin. A very different result is obtained for 18S RNA. Here, there is more than twice as much 18S RNA in the cordycepin-treated cells than can be derived from complete 45S molecules present before incubation in actinomycin. It is concluded that incomplete precursor molecules can give rise to 18S, while only whole (or almost whole) 45S molecules serve as precursor to 32S.

Cytoplasmic RNA was analyzed after 90 min of

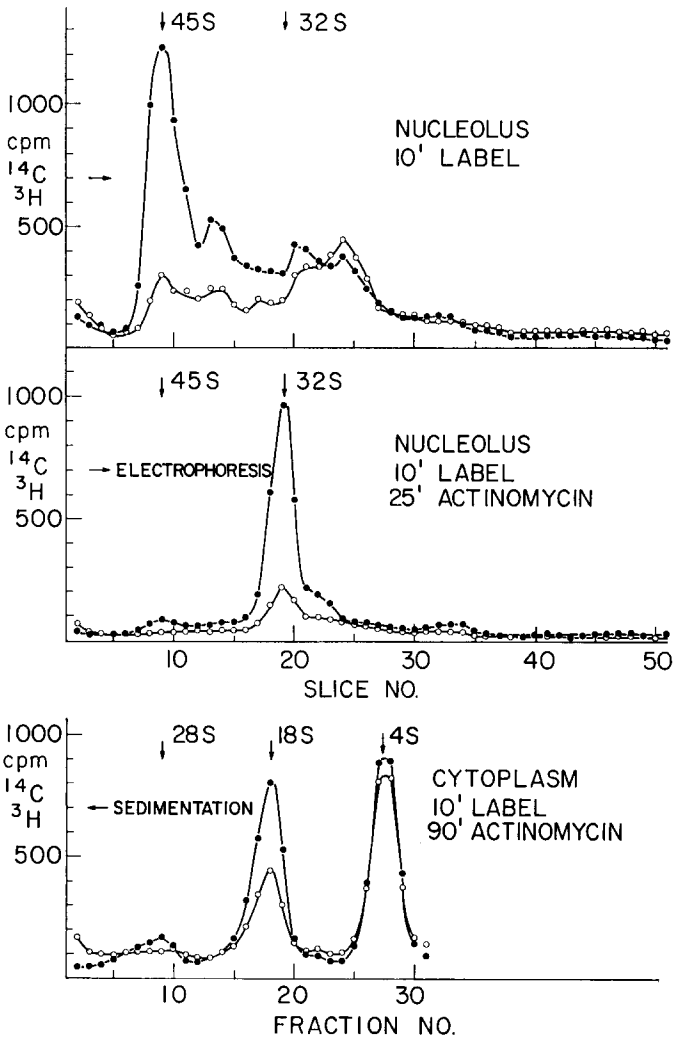

FIGURE 7 *Fate of incomplete ribosomal precursor molecules made in the presence of cordycepin.* 30 ml of cells (2×10^6 cells/ml) were incubated with 20 μg/ml cordycepin for 10 min. Uridine-^{3}H was then added at a concentration of 5 μc/ml. 10 min later, actinomycin was added at a concentration of 10 μg/ml, and the incubation was continued. Another culture was labeled with 0.2 μc/ml uridine-^{14}C and was similarly treated except that cordycepin was omitted. 10-ml samples from each of the two cultures were removed immediately after addition of actinomycin, after 25-min incubation in actinomycin, and after 90-min incubation in actinomycin. Simultaneous samples from the ^{3}H-labeled culture (cordycepin treated) and from the ^{14}C-labeled culture (untreated) were mixed before harvesting. Nucleolar RNA was analyzed by electrophoresis on 2.6% acrylamide gels as described in Materials and Methods. Electrophoresis was done at 10 v/cm for 4 hr. Cytoplasmic RNA was analyzed on 15–30% SDS-sucrose gradients centrifuged at 25,000 rpm for 17.5 hr in the SW 25.3 rotor. ——○——○, ^{3}H; ——△——△, ^{14}C.

incubation in actinomycin, because 25 min is not sufficient time for all of the 28S RNA to leave the nucleus in the presence of actinomycin.

DISCUSSION

Cordycepin (3′-deoxyadenosine) resembles the normal ribonucleoside sufficiently so that it is transported across the cell membrane and phosphorylated by the cellular enzymes (21). In this report, it is shown that cordycepin apparently causes the nucleolar polymerase to produce prematurely terminated RNA chains.

Other workers have suggested additional possible sites of inhibition by the drug cordycepin.

TABLE I

Labeling and Processing of Ribosomal RNA in the Presence and Absence of Cordycepin

	RNA Species	^{14}C (Untreated)	^{3}H (3′-dA Treated)
Nucleolus 10′ Label	45S	4213	799
Nucleolus 10′ Label 25′ Actinomycin	32S	2407	464
Cytoplasm 10′ Label 90′ Actinomycin	18S	2257	1057

TABLE II

Ratio of Labeling of Ribosomal RNA Species in the Presence and Absence of Cordycepin

	45S	32S	18S
$\frac{^{3}H(3'\text{-dA treated})}{^{14}C\text{ (untreated)}}$	0.19	0.19	0.47

3′-dATP has been shown to inhibit ribose phosphate phosphokinase which catalyzes the reaction ribose-5-P + ATP → PRPP + AMP (22, 30). Cordycepin has also been shown to be an inhibitor of phosphoribosylphosphate amidotransferase which catalyzes the reaction PRPP + glutamine → 5-phosphoribosylamine + glutamate (23). Both of these enzymes are involved in the initial steps in purine biosynthesis. Thus, there is the possibility that, in addition to the effects described here, cordycepin might cause a partial inhibition of the synthesis of endogenous purines. This does not appear to happen in HeLa cells with the concentrations of drug used in these experiments. The rate of DNA synthesis, as measured by the incorporation of radioactive thymidine, is unaffected by cordycepin for at least 85 min. In addition, except for the nucleolar and tRNA, the species of RNA examined show very little inhibition by cordycepin. Both these findings suggest that no significant reduction in supply of available purines has occurred under the experimental conditions used. The insensitivity of amino acid incorporation to cordycepin suggests that energy metabolism is unaffected at these concentrations.

The most striking finding is the effect of cordycepin on nucleolar synthesis. Almost no complete ribosomal precursor molecules are formed in the presence of the concentrations of the drug used in these experiments. In contrast, the synthesis of high molecular weight heterodisperse RNA is affected slightly, if at all. The simplest explanation for this selectivity is the existence of two distinct types of polymerase, one in the nucleolus, sensitive to the drug, and another in the nucleoplasm, insensitive to the drug. There is some evidence that two distinct RNA polymerases may, in fact, exist in the nucleolus (24–28). One such polymerase activity, activated by magnesium, produces a reaction product the base composition of which is similar to that of ribosomal RNA. The magnesium-activated activity has been shown by radioautography to be localized in the nucleolus. A distinct polymerase activity is activated by manganese. The reaction product resembles DNA in base composition, and it has been localized in the nucleoplasm by radioautography. The explanation of the results of this investigation would be that the magnesium-activated, nucleolar-localized enzyme, is sensitive to cordycepin. The suppression of tRNA synthesis by cordycepin suggests the possibility of a third distinct polymerase.

There are at least two possible explanations for the relative insensitivity of the nonnucleolar RNA polymerases to the drug. These are: (*1*) The nucleoplasmic RNA polymerase can distinguish between a true ribonucleotide and the compound used here which has a reduced 3′ position. (*2*) The lack of sensitivity may be only apparent. If the extranucleolar enzymes can complete a 5′ → 2′ phosphodiester linkage, then the cordycepin molecule may be incorporated internally, and this incorporation would result in an apparently normal rate of synthesis. The drug will, of course, have activity only in a polymerase system in which the incoming nucleotide is joined by its 5′ position to the preexisting polynucleotide.

The experiment shown in Fig. 7 indicates that the ribosomal precursor apparently must be nearly complete before it can give rise to a 32S molecule. In contrast, 18S molecules clearly are derived from incomplete precursor molecules. One possible explanation is that the 32S region is located near the 3′ end of the 45S molecule and is completed only when 45S RNA is nearly completed. The 18S region is apparently located in a portion of the

precursor which is closer to the 5′ end and is synthesized first. However, completion of the 45S molecule may be prerequisite for the production of 32S RNA, irrespective of the location of the 32S region on the precursor molecule.

It is interesting to note that the results presented here explain an earlier observation of Frederiksen and Klenow (29). They observed that crodycepin suppressed the labeling of RNA in one of two nuclear fractions and in the cytoplasm. Apparently, their inhibited nuclear fraction corresponds to the nucleolar fraction. They also used relatively long labeling times, and most of the labeled RNA in the cytoplasm would be ribosomal. The inhibition of nucleolar synthesis would account for the suppression of cytoplasmic labeling which they found.

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