

THE ACTION OF α -AMANITIN ON RNA SYNTHESIS IN CHINESE HAMSTER OVARY CELLS

Ultrastructural and Biochemical Studies

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ABSTRACT

α -Amanitin acts in vitro as a selective inhibitor of the nucleoplasmic form B RNA polymerases. Treatment of Chinese hamster ovary (CHO) cells with this drug leads principally to a severe fragmentation of the nucleoli. While the ultrastructural lesions induced by α -amanitin in CHO cells and in rat or mouse liver are quite similar, the results diverge concerning the effect on RNA synthesis. It has been shown that in rat or mouse liver α -amanitin blocks both extranucleolar and nucleolar RNA synthesis. Our autoradiographic and biochemical evidence indicates that in CHO cells high molecular weight extranucleolar RNA synthesis (HnRNA) is blocked by the α -amanitin treatment, whereas nucleolar RNA (preribosomal RNA) synthesis remains unaffected even several hours after the inhibition of extranucleolar RNA synthesis. Furthermore, the processing of this RNA as well as its transport to the cytoplasm seem only slightly affected by the treatment. Finally, under these conditions, the synthesis of the low molecular RNA species (4-5S) still occurs, though less actively. The results are interpreted as evidence for a selective impairment of HnRNA synthesis by α -amanitin in CHO cells.

The toadstool *Amanita phalloides* produces a toxic octopeptide, α -amanitin (39), which strongly inhibits RNA synthesis in isolated nuclei of mouse liver cells (36). Several groups of investigators, using purified RNA polymerases, have shown clearly in vitro that α -amanitin inhibits DNA transcription by binding specifically to the extranucleolar form B RNA polymerases without affecting either the nucleolar or the extranucleolar form A RNA polymerases (7, 14, 16, 17). There seems to be some confusion regarding the action of the drug in vivo. When administered to rats or mice, α -amanitin has been shown to block the

synthesis of all types of nuclear RNA including nucleolar ribosomal precursor RNA (11, 14, 15, 23, 33, 37). On the other hand, autoradiographic studies on *Chironomus* salivary glands (2, 8, 34, 40) and biochemical studies on *Triturus* oocytes (3) and chick embryo fibroblasts (12) provide good evidence that nucleolar RNA synthesis is not affected by α -amanitin in agreement with data obtained in in vitro experiments.

In the present paper, we report combined ultrastructural, autoradiographic, and biochemical studies on the action of α -amanitin in cultured chinese hamster ovary (CHO) cells. Although

nucleolar fragmentation similar to that described in rat or mouse liver (9, 10, 18, 20, 27) is observed, nucleolar RNA synthesis, processing, and transport remain apparently unaffected, whereas extranucleolar high molecular weight RNA synthesis is strongly inhibited by α -amanitin action. Biochemical arguments in favor of this selective impairment of RNA metabolism are presented and discussed.

MATERIALS AND METHODS

Cell Cultures

CHO cells used in these experiments were a gift from Dr. L. Siminovitch. The cells were grown in prescription bottles in modified Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal calf serum (Flow Laboratories, Rockville, Md.). At confluence, the cells were trypsinized, pelleted by centrifugation, washed with a phosphate buffered saline (PBS), and either resuspended in Eagle's modified medium for monolayer subculture or transferred to a spinner flask for suspension culture. In the latter case, the cells were grown with α -medium lacking nucleosides and supplemented with L-glutamine and 10% fetal calf serum (Flow Laboratories). The cells were allowed to recover in this medium for at least 24 h. They were then diluted with the same medium to a concentration of $3-4 \times 10^5$ cells/ml. Under these conditions, the cells reached an exponential growth phase within 4-6 h at 37°C, and continued growing exponentially for at least 15 h (the doubling time was about 20 h). The drug, α -amanitin, at a concentration of 5 μ g/ml, was simply added to the culture medium.

Electron Microscopy

Small cell pellets (about 10^6 cells) were fixed for 30 min with buffered 2.5% glutaraldehyde followed by 2% osmium tetroxide. After ethanol dehydration, the specimens were embedded in Epon. Ultrathin sections were treated for 10 min with 5% uranyl acetate for contrast, followed by 10 min with lead citrate (29).

High-Resolution Autoradiography

Exponentially growing cells, treated or not with 5 μ g/ml of α -amanitin, were labeled for 1 h with 100 μ Ci/ml [$5\text{-}^3\text{H}$]uridine (26 Ci/mmol: Amersham/Searle Corp., Arlington Heights, Ill.). After the labeling period, the cells were quickly chilled with ice cold PBS, pelleted, and washed extensively with cold PBS containing 0.005% unlabeled uridine. Cell pellets were then fixed and embedded as indicated. Ultrathin sections were prepared for autoradiography with Ilford L4 Emulsion (Ilford Ltd., Ilford, Essex, England), were exposed at 4°C for 20 days, and then developed and stained essentially as described by Caro and van Tubergen (5).

Labeling Procedure for Biochemical Studies

Labeling experiments were done only with cells growing exponentially in suspension cultures. The cells were incubated with 0.05 μ Ci/ml of [$2\text{-}^{14}\text{C}$]uridine (56 Ci/mmol: New England Nuclear, Boston, Mass.) and/or with the indicated concentration of [$5\text{-}^3\text{H}$]uridine (20-30 Ci/mmol). At the end of the labeling period, the cells were quickly chilled with ice cold PBS, sedimented by centrifugation, washed once with 40 vol of cold PBS, and then frozen at -40°C .

Cell Fractionation

ISOLATION OF NUCLEI: Nuclei were prepared according to a modification (42) of a standard technique based on the use of nonionic detergents (41). The frozen cell pellet (ordinarily 2×10^7 cells) was thawed in 10 ml of 10 mM Tris (pH 7.4) containing 0.25 M sucrose, 2.5 mM MgCl_2 , 0.1 mM CaCl_2 , and 200 μ g of polyvinylsulfate. After thawing, the cells were suspended in the presence of 6 mg of collagenase (Calbiochem, Los Angeles, Calif.), 0.05% Celanol 251 (vol/vol), and 0.26% Cemulsol NPT 10 (vol/vol). Celanol and Cemulsol were obtained from Melle-Bezons (Neuilly-sur-Seine, France). This suspension was gently homogenized with an ultra-Turrax (Janke and Kunkel, Staufen, W. Germany), at low speed. Nuclei were then pelleted at 800 g for 10 min. The supernate (cytoplasmic fraction) was used for ribosomal RNA preparations. The nuclear pellet was resuspended and washed in the same solution but without detergents and collagenase.

PREPARATION OF RIBOSOMES: The cytoplasmic fraction was centrifuged for 20 min at 18,000 g and the postmitochondrial supernate, made 0.8% with Celanol 251, was recentrifuged for 2 h at 260,000 g. The ribosomal pellet was resuspended in 8 ml of 20 mM Tris (pH 7.4) containing 1 M NH_4Cl and 10 mM MgCl_2 . After 1 h at 0°C , the ribosomes were sedimented at 260,000 g for 2 h.

Extraction and Analysis of RNA

(a) Total cellular RNA was extracted according to a method derived from that of Scherrer and Darnell (31). The cells (ordinarily 10^7 cells) were suspended in 3 ml of 10 mM Tris (pH 7.4) containing 0.5 M KCl and 2 mM MgCl_2 and were disrupted by sonication (5-10 s) with an MSE 100W ultrasonic disintegrator (Measuring and Scientific Equipment Ltd., London, England). A 0.75-ml aliquot of this homogenate was removed from DNA content estimation (4). To the remaining 2.25 ml of homogenate, 50 μ g of RNase-free DNase I (Worthington Biochemical Corp., Freehold, N.J.) were added. After a 30-min incubation at 0°C , EDTA and sodium dodecyl sulfate (SDS) were added to final concentrations of 5 mM and 0.5%, respectively. RNA was then extracted at 55°C for 10 min with 1 vol of water-saturated

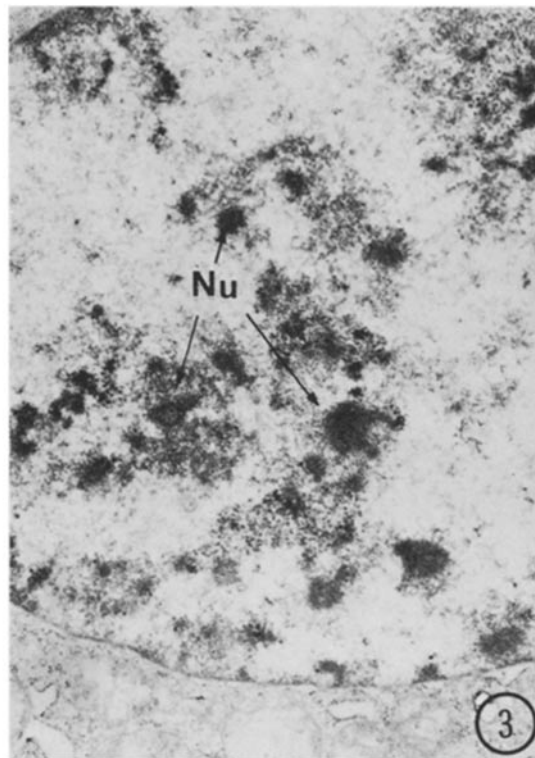
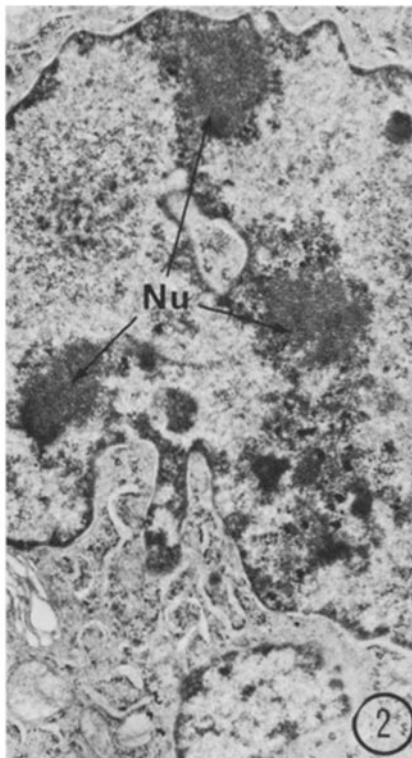
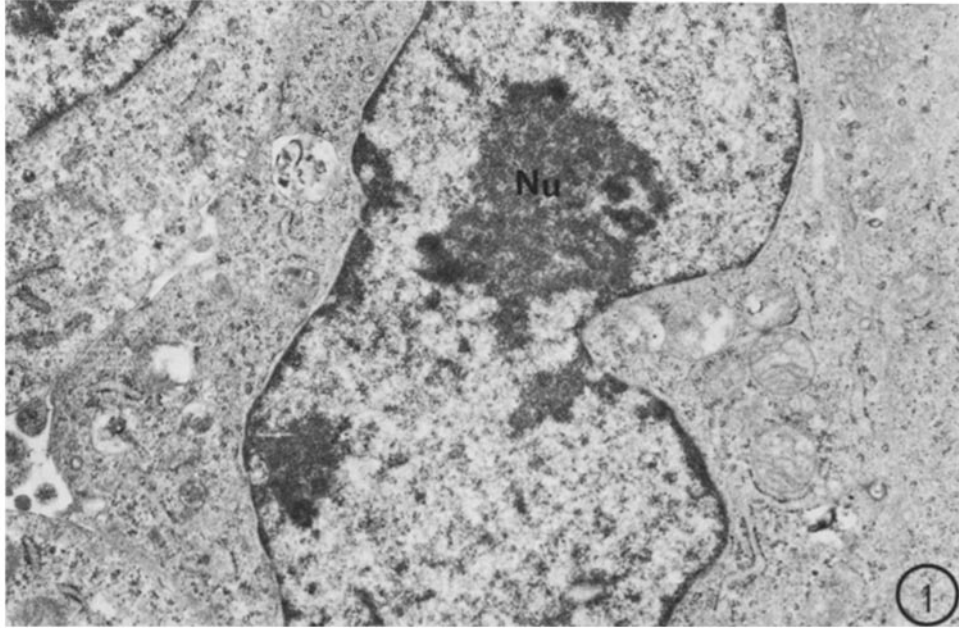


FIGURE 1 Untreated CHO cell. The nucleolus (*Nu*) is compact and the granular and fibrillar components are distributed in anastomosed molecular structures forming a meshwork. The chromatin is condensed on the nuclear membrane and around the nucleolus. Scattered granules can be identified in a finely fibrillar nucleoplasmic matrix. All micrographs represent cells fixed with glutaraldehyde, postfixed in OsO_4 , Epon embedded, and stained with uranyl acetate and lead citrate. $\times 12,000$.

FIGURE 2 CHO cell treated with α -amanitin for 8 h; the nucleolus has broken up in small fragments, one of them being surrounded by a large number of electron-dense granules. The fragments contain both the granular and fibrillar components. $\times 12,000$.

FIGURE 3 CHO cell treated with α -amanitin for 24 h. Fragmentation of the nucleolus is complete and numerous small nucleolar remnants can be identified. The chromatin is redistributed in small clumps. $\times 12,000$.

phenol with constant shaking. The aqueous phase plus the buffer-phenol interphase layer were reextracted twice for 10 min at room temperature, once with 1 vol phenol, and once with 1 vol chloroform containing 1% isoamyl alcohol. Finally, the aqueous phase alone was reextracted with 1 vol of the chloroform-isoamyl alcohol mixture, and RNA was precipitated overnight at -20°C by the addition of 2 vol of 95% ethanol to this aqueous phase.

(b) The nuclear RNA extraction procedure was derived from that of Tiollais et al. (38). Nuclei prepared from 2×10^7 cells were suspended in 3 ml of 20 mM sodium acetate (pH 5) containing 0.5% SDS and 5 $\mu\text{g}/\text{ml}$ polyvinylsulfate. An equal volume of water-saturated phenol containing 0.1% 8-hydroxyquinoline was then added. The mixture was vigorously shaken at 55°C for 10 min. The aqueous phase and the interphase layer were reextracted together at 4°C for 30 min with 0.5 vol of phenol. The RNA solution was brought to 0.2 M NaCl and precipitated with 2 vol cold 95% ethanol at -20°C overnight.

(c) Ribosomal RNA extraction from the ribosome pellet was performed using the nuclear RNA extraction procedure, but omitting the heating step. RNA concentration was estimated by the orcinol method (19).

The extracted RNAs were run on 11-cm long composite gels of 2.3% acrylamide-0.5% agarose according to Peacock and Dingman (25). After migration, gels were cut into 2- or 3-mm slices which were then treated at room temperature overnight in 0.7 ml NH_4OH 6 N. 7 ml Aquasol (New England Nuclear) were added for counting in a Packard Tricard liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

RESULTS

Electron Microscope Observations

Chinese hamster fibroblasts (CHO) in exponential growth are elongated cells with large nuclei and compact nucleoli. The chromatin is condensed on the nuclear membrane and around the nucleolus. Nucleolar granules and fibrils are mixed and distributed throughout the nucleolus as in most other mammalian cells in culture. Perichromatin granules are rare, but the 200–250 \AA

interchromatin granules are sparsely distributed in a finely fibrillar nucleoplasmic matrix (Fig. 1).

When the cells are treated with α -amanitin at a concentration of 5 $\mu\text{g}/\text{ml}$, no significant modifications can be observed during the first hours. After 6–8 h, the normal contour of the nucleolus begins to distort and then breaks up into small roundish masses. The fragmented remnants still contain the granular components. In addition, small spherical electron-opaque masses appear in the vicinity of the nucleolus. These structures are composed of granules of 400 \AA , reminiscent of perichromatin granules. One single nucleolus can become four to five fragments (Fig. 2).

As the time of treatment increases from 8 to 24 h, the fragments get smaller and numerous. Some of them are mostly fibrillar, while others are both fibrillar and granular. Chromatin aggregation is more prominent around the nuclear membrane as well as in the area adjacent to the nucleolus. Clumping of interchromatin granules appears in the cytoplasm. These alterations can be seen in nearly all nuclei, though minor differences in the timing of appearance is observed from cell to cell (Fig. 3). Preferential staining for ribonucleoproteins is strongly positive over the nucleolar fragments and the clumps of interchromatin granules.

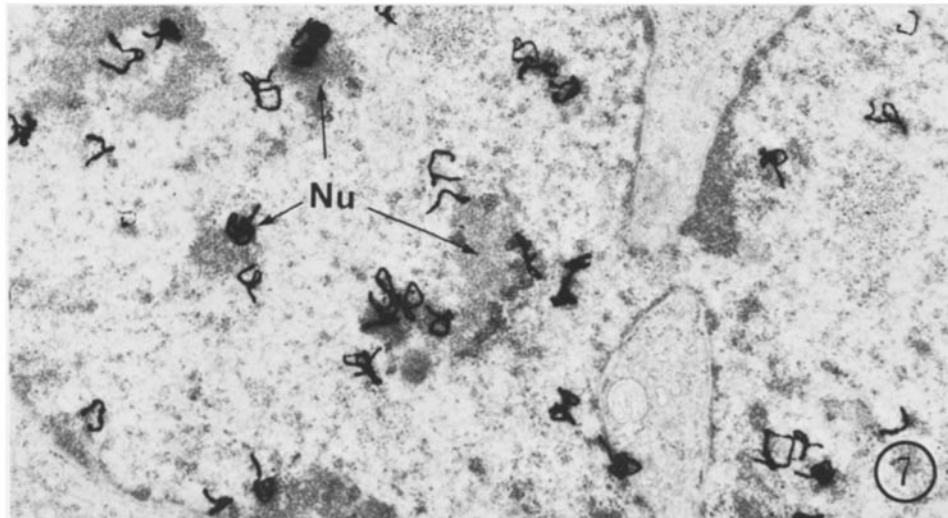
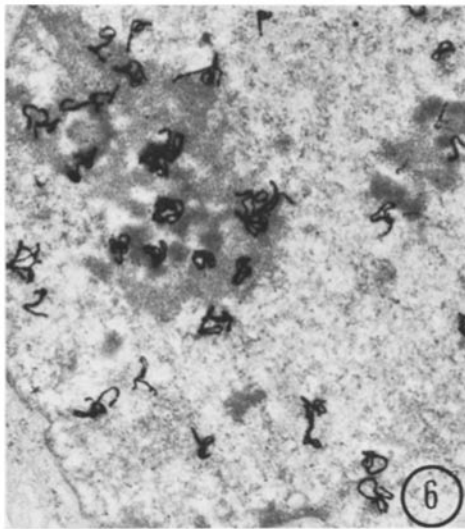
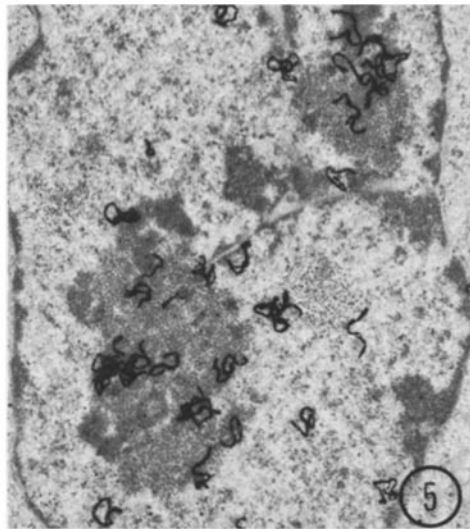
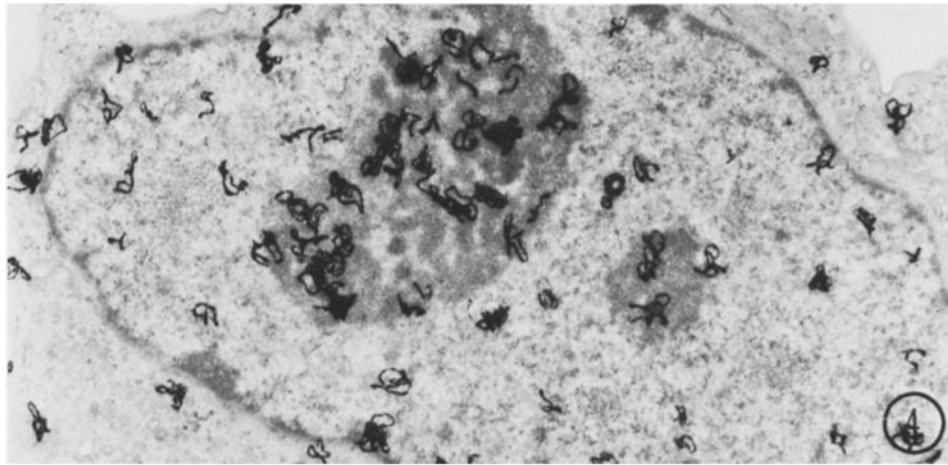
Autoradiography

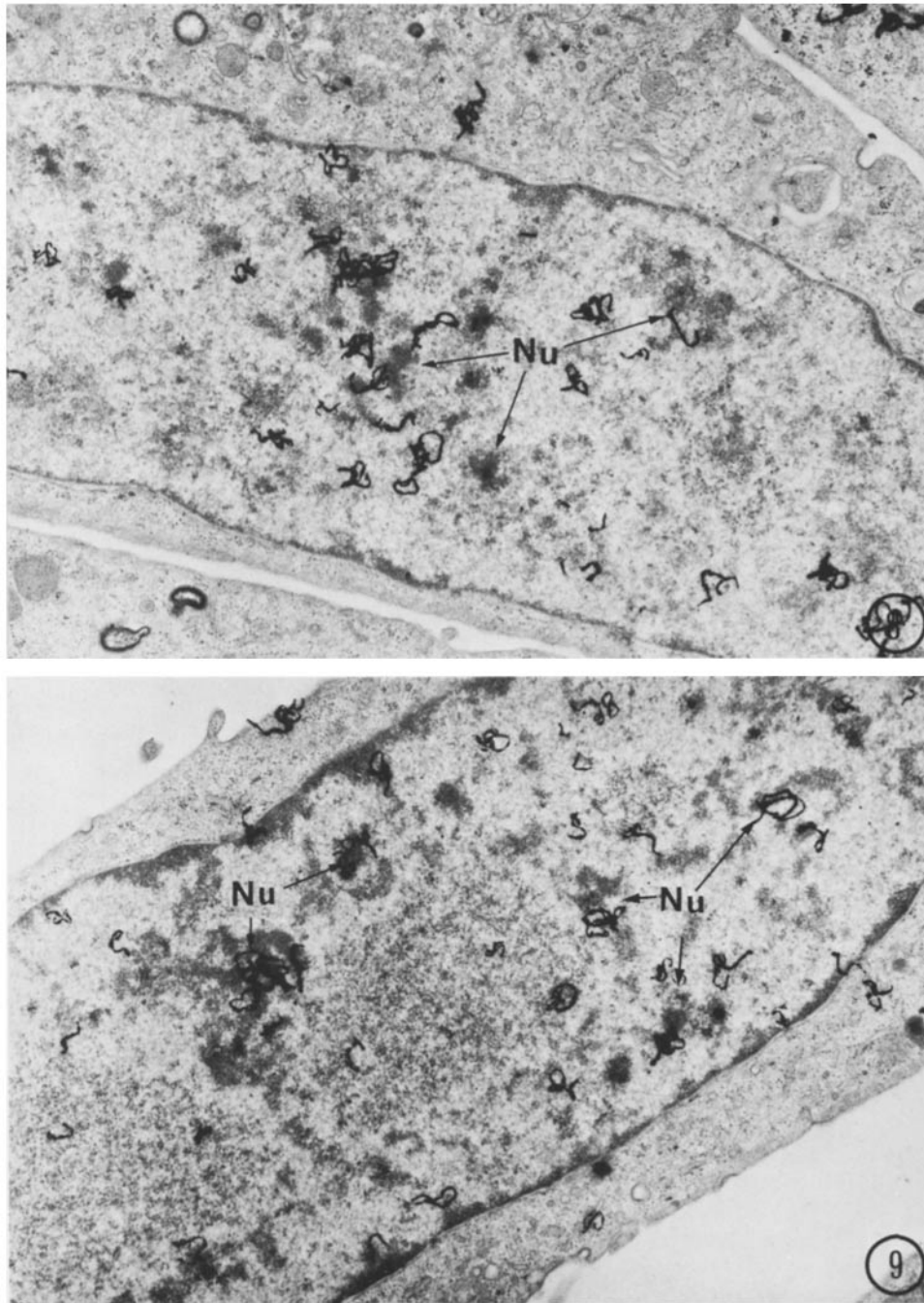
Incorporation of [^3H]uridine into CHO cells occurs mostly in the nucleus during a 1-h pulse. The nucleolus is heavily labeled on both the granular and fibrillar portions, while incorporation is less evident in the extranucleolar regions of the nucleus. An approximate grain count per unit area reveals a ratio of labeling of 4 to 1 between the nucleolar and nonnucleolar regions of the nucleus (Fig. 4). After 2–4 h of treatment with α -amanitin, no noticeable modification in the distribution of silver grains can be observed (Figs. 5 and 6);

FIGURE 4 Autoradiography of a control cell pulse labeled with [^3H]uridine for 1 h. The radioactivity is located mostly over the nucleolus but the extranucleolar portion of the nucleus is also quite heavily labeled. A few Silver grains can be seen in the cytoplasm. All autoradiographs were processed with Ilford L4 emulsion. $\times 12,000$.

FIGURES 5 and 6 Cells treated with α -amanitin for 2 h (Fig. 5) and 4 h (Fig. 6) and pulse labeled with [^3H]uridine for 1 h. The same distribution of radioactivity can be observed over the nucleoli and nuclei. $\times 13,000$.

FIGURE 7 α -Amanitin 8 h followed by [^3H]uridine 1 h. The radioactivity is mostly associated with the nucleolar fragments (*Nu*). The extranuclear labeling is markedly reduced as compared with Fig. 4. $\times 13,000$.





FIGURES 8 and 9 α -Amanitin 12 h (Fig. 8) and 24 h (Fig. 9) followed by [3 H]uridine for 1 h. In both cases fragmentation of the nucleolus (*Nu*) is severe and only small nucleolar remnants can be identified. Most of the silver grains are however associated with the fragments. The extranucleolar labeling is strongly reduced. Clumps of interchromatin granules can be identified in Fig. 9. $\times 14,000$.

however, after 8 h of α -amanitin treatment, nucleolar fragmentation is evident, and the large majority of the silver grains are located over the nucleolar fragments, while most of the extranucleolar labeling is either abolished or the grains are so close to nucleolar fragments that a high probability exists that they result from β -particles emitted by a tritium source located in the fragments (Fig. 7). The fragmentation is more complete after 12 h (Fig. 8) and 24 h (Fig. 9), but here again the silver grains are associated with or located over nucleolar fragments.

Biochemical Observations

The best way to confirm the autoradiographic results would be to fractionate the nuclei of prelabeled cells into a nucleolar and extranucleolar fraction and then to compare the RNA content of these subfractions in either untreated or α -amanitin-treated cells. But owing to the severe nucleolar fragmentation induced by α -amanitin, it proved difficult to prepare clean, noncross-contaminated nucleolar and extranucleolar fractions. Therefore, the effects of α -amanitin on total RNA

synthesis were examined first. As seen in Fig. 10 *a*, there is a 20% decrease in the labeling of total RNA after 9 h of α -amanitin treatment, and this decrease remains more or less constant up to 18 h after α -amanitin addition to the culture media.

On the other hand, when the cells are labeled under the same conditions but in the presence of a low concentration of actinomycin D, an inhibition of about 80–90% of [3 H]uridine incorporation into residual RNA is observed after 9–12 h of α -amanitin treatment as compared with the labeling of non α -amanitin-treated cells (Fig. 10 *b*). The concentration of actinomycin D chosen for this experiment (0.1 μ g/ml) results in specific inhibition of nucleolar RNA synthesis (26, 43). Therefore, the inhibition due to α -amanitin treatment in this experiment is most probably a reflection of blocking by α -amanitin of extranucleolar RNA synthesis.

In non α -amanitin-treated cells, the amount of radioactivity incorporated in the presence of the actinomycin D is just 20% of the amount incorporated in control cells (see legend to Fig. 10). Thus the inhibition of extranucleolar RNA synthesis obtained after α -amanitin addition (Fig. 10 *b*)

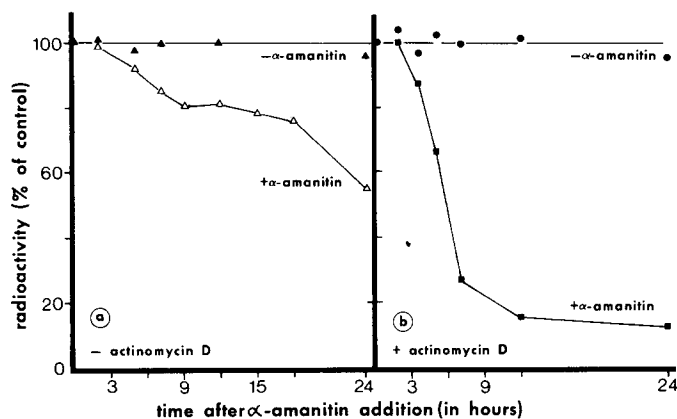


FIGURE 10 Effect of α -amanitin treatment on cellular RNA synthesis. Exponentially growing CHO cells were treated with 5 μ g/ml α -amanitin added to the culture medium at time 0. In parallel, untreated control cultures were also included in the experiment. At 30 min before the indicated times, 30-ml aliquots of cell suspension (about 10^7 cells) were labeled for 20 min with 2 μ Ci/ml [3 H]uridine either in absence (*a*) or in presence (*b*) or 0.1 μ g/ml actinomycin D (Merck and Co. Inc., West Point, Pa.) added 30 min before the radioactive precursor. The cells were then harvested and total cellular RNA was extracted as described in Materials and Methods. Aliquots of the extracted RNA were precipitated with trichloroacetic acid (TCA), filtered on Millipore HA filters (Millipore Corp., Bedford, Mass.) and washed by 20 ml TCA. The filters were dried and the retained radioactivity was counted in a toluene-based scintillation fluid. The values were corrected for an identical DNA concentration in each sample. In a typical experiment, the values at time 0 were 10.5×10^5 cpm in the absence of actinomycin D and 1.8×10^5 cpm in the presence of actinomycin D for a sample of 10^7 cells. Fig. 10 *a*: labeling in absence of actinomycin D without α -amanitin (control) (— \blacktriangle —), and with α -amanitin treatment (— \triangle —). Fig. 10 *b*: labeling in presence of actinomycin D without α -amanitin (control) (— \bullet —), and with α -amanitin treatment (— \blacksquare —).

accounts well for the 20% decrease in the labeling of total RNA after a 12-h treatment with α -amanitin (Fig. 10 a). Therefore, nucleolar RNA synthesis does not seem to be affected by α -amanitin, at least during the first 18 h of treatment.

Fig. 11 represents the electrophoretic migration pattern of total RNA extracted from cells labeled in presence of actinomycin D (0.1 μ g/ml) either after a 12-h α -amanitin treatment or after no treatment. In both cases, nucleolar RNA synthesis is inhibited by the low concentration of actinomycin D present in the culture medium. In the absence of α -amanitin, the labeling is found mainly in high molecular weight RNA species, ranging from 18S to more than 45S, and small RNA species migrating as molecules having a sedimentation coefficient of 4–5S. Most probably, the high molecular weight RNA species represent the so-called heterogeneous nuclear RNA (HnRNA), and 4–5S species consist of mixture of

tRNA, pre-tRNA, and 5S ribosomal RNA molecules which are not resolved on this type of gel. After the α -amanitin treatment, while the labeling of the HnRNA fraction is completely abolished, the small RNA species (4–5S) are still labeled. This residual RNA synthesis accounts well for the 10–15% RNA synthesis resistant to both α -amanitin and actinomycin D treatments (Fig. 10 b).

In order to confirm the previous conclusion that nucleolar RNA synthesis does not seem to be affected by α -amanitin, polyacrylamide gel electrophoresis was carried out on nuclear RNA extracted from cells treated for various times with α -amanitin. In these experiments, the labeling time was increased to 1 h in order to decrease the relative labeling of the rapidly labeled extranucleolar RNA versus nucleolar RNA. Furthermore, the RNA was extracted from purified nuclei, thus avoiding in our preparation a contamination by the rapidly labeled cytoplasmic messenger RNAs. Under these conditions, incorporation of precursor into HnRNA should not exceed 5% of the total. The results shown in Fig. 12 indicate clearly that total nucleolar RNA synthesis proceeds normally during α -amanitin treatment. Only after 24 h of treatment does total nucleolar RNA synthesis decrease significantly as also shown in Fig. 10 a.

This experiment not only confirms that, in presence of α -amanitin, nucleolar RNA is synthesized continuously even in the absence of HnRNA synthesis, but also suggests that the maturation of nucleolar RNA occurs normally. Indeed, as shown in Fig. 12 by the radioactivity profiles, the various nucleolar RNA species, pre-rRNA, and intermediates being processed are present at each time after α -amanitin treatment and in the same relative amounts as in the control cells. This observation indicates that the intranucleolar processing of ribosomal RNA precursors is not affected by α -amanitin, at least during the first 12–18 h of treatment.

In Fig. 13, the labeling rate of cytoplasmic ribosomal RNA was studied. It can be seen that in the α -amanitin-treated cells, the specific activity of the RNA extracted from the cytoplasmic ribosomal fraction increases up to about 90% of the value reached in nontreated cells after a 15 h labeling period. This RNA was fractionated into its 28S, 18S, and 5S RNA components by gel electrophoresis, and no difference in the relative labeling of these species was observed in the presence or absence of α -amanitin. Therefore, the

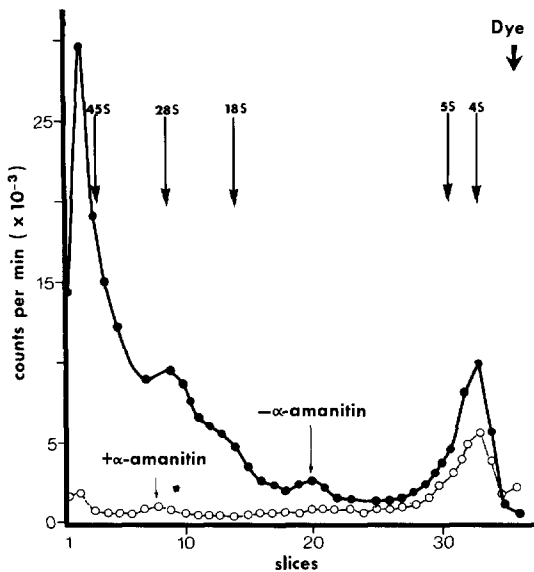


FIGURE 11 Effect of a 12-h α -amanitin treatment on the cellular RNA synthesized in presence of a low dose of actinomycin D. CHO cells, whether untreated or treated with 5 μ g/ml α -amanitin for 12 h, were labeled in the presence of 0.1 μ g/ml actinomycin D as in Fig. 10. Total cellular RNA was extracted, electrophoresed for 2 h at 10 V/cm and the gels were cut into 3-mm slices as described elsewhere. Arrows indicate the relative migration of marker RNA molecules run on a parallel gel. Radioactivity extracted from non α -amanitin-treated cells (—●—); radioactivity extracted from α -amanitin-treated cells (—○—).

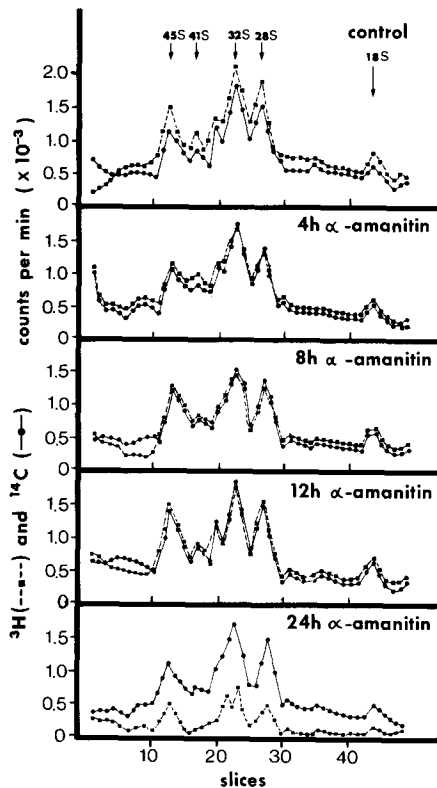


FIGURE 12 Effect of α -amanitin treatments on nucleolar RNA synthesis. CHO cells, whether untreated or treated with $5 \mu\text{g/ml}$ α -amanitin. Equal amounts of ^{14}C -labeled and ^3H -labeled CHO cells were mixed before nuclei preparation and extraction of RNA. The labeled RNA was then electrophoresed for 4 h at 10 V/cm and gels were cut into 2-mm slices. ^{14}C radioactivity of untreated cells labeled for 3 h with $0.05 \mu\text{Ci/ml}$ [^{14}C]uridine (●—●—●); ^3H radioactivity of either control or α -amanitin-treated cells ($5 \mu\text{g/ml}$ α -amanitin added for various times to the culture medium), labeled for 1 h with $0.5 \mu\text{Ci/ml}$ [^3H]uridine just before the end of the treatment (—■—■—■). Arrows indicate approximate sedimentation constant values.

discrepancy between the labeling kinetics of the cytoplasmic ribosomal RNA in control and α -amanitin-treated cells might be due to a slight impairment of nucleolar RNA transport to the cytoplasm, since nucleolar RNA does not appear to be significantly affected by α -amanitin after a 15-h treatment (Figs. 10 and 12).

DISCUSSION

Our autoradiographic experiments demonstrate clearly that inhibition of RNA synthesis by α -amanitin in cultured CHO cells is restricted to the

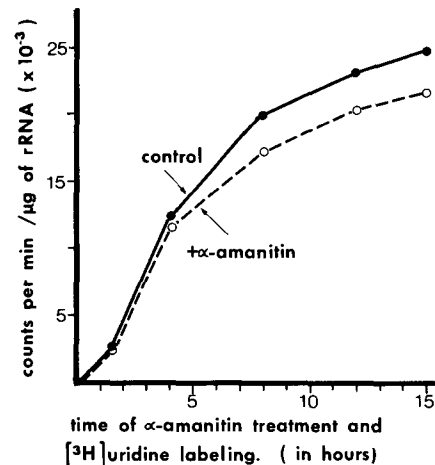


FIGURE 13 Effect of α -amanitin on the time course of cytoplasmic ribosomal RNA labeling. Exponentially growing CHO cells were given $2 \mu\text{Ci/ml}$ [^3H]uridine and either no α -amanitin or $5 \mu\text{g/ml}$ α -amanitin. After incubation at 37°C , samples of about 10^7 cells were taken; the ribosomes were prepared and ribosomal RNA extracted as described. TCA-precipitable radioactivity in the extracted RNA was measured as in Fig. 10. Specific activity of ribosomal RNA extracted from nontreated cells (control) (—●—); specific activity of ribosomal RNA extracted from α -amanitin-treated cells (—○—).

extranucleolar compartment of the nuclei. Nucleolar RNA synthesis occurs normally even in the small nucleolar fragments which proceed from the nucleolar fragmentation induced by the α -amanitin treatment.

The biochemical evidence presented in this paper is in good agreement with these observations. Using the differential effect of low actinomycin D doses on nucleolar and extranucleolar RNA synthesis, we have shown that α -amanitin prevents HnRNA synthesis, whereas it does not affect nucleolar RNA synthesis, and only slightly affects the 4–5S RNA synthesis. Furthermore, our results suggest strongly that α -amanitin does not induce any significant impairment in the processing of pre-rRNA and that the transport of the ribosomal RNA species to the cytoplasm is only slightly slackened, at least during the first hours after HnRNA synthesis inhibition. Whether the preribosomal (nucleolar) and the cytoplasmic ribosomal particles of these cells present a normal or an altered protein content after α -amanitin treatment remains to be established.

The differential sensitivity to α -amanitin of the

above mentioned types of RNA in CHO cultured cells corroborates well previous studies concerning the existence and localization of different forms of RNA polymerase: the nucleolus has been shown to contain at least one α -amanitin-resistant RNA polymerase, enzyme AI; at least one other α -amanitin-resistant polymerase, enzyme AIII, has been found in the extranucleolar portion of the nucleus where are also localized the α -amanitin-sensitive RNA polymerases, enzymes B. Several lines of evidence, confirmed by the present results, indicate that enzyme AI is involved in ribosomal RNA synthesis, whereas enzymes B are responsible for HnRNA synthesis (for references see 6 and 13). All of the evidence concerning the possible role of enzyme AIII is circumstantial, but several observations (28, 43) suggest that this enzyme might catalyze the synthesis of a small fraction of the HnRNA as well as 4 and 5S RNAs whose genes are localized outside the nucleolus (1, 24, 30). Our results, showing that the labeling of 4-5S RNA was only moderately reduced in CHO cells after the α -amanitin treatment, are in agreement with the conclusion that the genes of 4 and 5S RNA are transcribed by an α -amanitin-resistant polymerase.

The different effects of α -amanitin on CHO cultured cells, *Chironomus* salivary glands (2, 8, 34, 40), *Triturus* oocytes (3), and chick embryo fibroblast cells (12) on one hand, and on rat or mouse liver (11, 14, 15, 23, 33, 37) on the other hand, are difficult to explain. In the latter systems, with the exception of the 4-5S RNA (11, 21), all types of nuclear RNA are blocked by α -amanitin, and the RNA inhibition occurs within 1 h after the treatment, while it takes 8 h in the presence of α -amanitin to stop HnRNA synthesis in CHO cells. Similarly, nucleolar fragmentation occurs 1 and 8 h after the treatment, respectively, in rat or mouse liver and in CHO cells. This discrepancy is likely due to unequal permeabilities of liver and CHO cells to α -amanitin or to a detoxication mechanism leading to very high α -amanitin concentrations in the liver compared to the concentration of 5 μ g/ml used in our experiments.

This possible difference in permeability to α -amanitin and/or in the local concentrations of the drug in liver and CHO cells may also explain its conflicting effects on RNA synthesis in these two systems. The biochemical changes induced by α -amanitin occur much faster in liver cells as compared to CHO cells: nucleolar RNA synthesis

begins to be inhibited only after 18 h of treatment in CHO cells, while both HnRNA and nucleolar RNA are completely inhibited after 1 h in rat or mouse liver. Another possible explanation for the α -amanitin effects in rat liver may be that α -amanitin inhibits the synthesis of a messenger RNA(s) continuously required for the synthesis of a rapidly renewed protein(s) which could be essential for normal nucleolar RNA synthesis by acting, for example, on the nucleolar polymerase as an initiation factor (22). Since in CHO cells nucleolar RNA synthesis is not affected by α -amanitin, at least several hours after HnRNA inhibition, one must assume according to this hypothesis either that this messenger RNA(s) turns over more slowly in CHO cells than in rat liver, or that the protein(s) coded by this RNA has a longer half-life in CHO cells. It has also been suggested that in rat liver, α -amanitin may block the synthesis of some species of DNA-like RNA affecting nucleolar RNA transcription (32). If that is true in CHO cells as well, one has to assume that the lifetime of this RNA species is much longer in CHO cells than in rat liver.

Finally, a surprising observation in the present study is the fact that the integrity of the nucleolar ultrastructure is not required for the conduct of its major function, the synthesis, and processing of nucleolar preribosomal RNA: the nucleolar fragments observed after α -amanitin treatment are still able to carry on these functions as shown by the combined autoradiographic and biochemical experiments. Although an explanation for the fragmentation is still lacking, in the present case the mechanism appears quite different than in the case of toyocamycin, ethionine, and adenosine. Those drugs induce a similar fragmentation of the nucleolus, but they are known to interact in some way with the nucleotide pool. Moreover, toyocamycin and ethionine interfere with the processing of preribosomal RNA (see 35 for a review). Hopefully, the further study of the pathology of the lesions induced by α -amanitin may provide new insight into the structural organization of the nucleus and nucleolus in relation to their related functions and also into the response pattern of these organelles to a selective metabolic injury.

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Note Added in Proof. In a recent paper, Weinmann and Roeder (1974. *Proc. Natl. Acad. Sci. U. S. A.* **71**:1790-1794.) demonstrate the sensitivity of enzyme AIII to higher concentrations of α -amanitin: in mouse myeloma cells, concentrations as high as 200 $\mu\text{g}/\text{ml}$ result in complete inhibition of RNA polymerase AIII. Their results could well explain the partial inhibition of the synthesis of the small RNA species (4-5S) observed at 5 $\mu\text{g}/\text{ml}$ as shown in Fig. 11 of this paper.

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