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The Action of α-Amanitin *in vivo* on the Synthesis and Maturation of Mouse Liver Ribonucleic Acids

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α-Amanitin acts *in vitro* and *in vivo* as a selective inhibitor of nucleoplasmic RNA polymerases. Treatment of mice with low doses of α-amanitin causes the following changes in the synthesis, maturation and nucleocytoplasmic transfer of liver RNA species. 1. The synthesis of the nuclear precursor of mRNA is strongly inhibited and all electrophoretic components are randomly affected. The labelling of cytoplasmic mRNA is blocked. These effects may be correlated with the rapid and lasting inhibition of nucleoplasmic RNA polymerase. 2. The synthesis and maturation of the nuclear precursor of rRNA is inhibited within 30min. (*a*) The initial effect is a strong (about 80%) inhibition of the early steps of 45S precursor rRNA maturation. (*b*) The synthesis of 45S precursor rRNA is also inhibited and the effect increases from about 30% at 30min to more than 70% at 150min. (*c*) The labelling of nuclear and cytoplasmic 28S and 18S rRNA is almost completely blocked. The labelling of nuclear 5S rRNA is inhibited by about 50%, but that of cytoplasmic 5S rRNA is blocked. (*d*) The action of α-amanitin on the synthesis of precursor rRNA cannot be correlated with the slight gradual decrease of nucleolar RNA polymerase activity (only 10–20% inhibition at 150min). (*e*) The inhibition of precursor rRNA maturation and synthesis precedes the ultrastructural lesions of the nucleolus detected by standard electron microscopy. 3. The synthesis of nuclear 4.6S precursor of tRNA is not affected by α-amanitin. However, the labelling of nuclear and cytoplasmic tRNA is decreased by about 50%, which indicates an inhibition of precursor tRNA maturation. The results of this study suggest that the synthesis and maturation of the precursor of rRNA and the maturation of the precursor of tRNA are under the control of nucleoplasmic gene products. The regulator molecules may be either RNA or proteins with exceedingly fast turnover.

Selective inhibition of RNA synthesis and maturation may be an important tool in studying regulatory mechanisms of ribosome biogenesis and genetic information transfer (Hadjiolov, 1972). The *Amanita phalloides* toxic octapeptide, α-amanitin, is a strong inhibitor of RNA synthesis in eukaryotic cells (Fiume & Wieland, 1970). Initial studies have indicated that α-amanitin selectively inhibits nucleoplasmic RNA polymerase, although the nucleolar enzyme is not influenced (Stirpe & Fiume, 1967). These results were confirmed and extended in studies with purified enzymes and it was shown that α-amanitin interacts with the molecule of nucleoplasmic RNA polymerases (RNA polymerases B) without affecting the DNA or chromatin template. On the contrary, nucleolar RNA polymerases (RNA polymerases A) were found to be resistant to α-amanitin (Jacob *et al.*, 1970*a*; Kedinger *et al.*, 1970; Roeder & Rutter, 1970; Chambon *et al.*, 1972). However, administration of α-amanitin *in vivo* caused a strong inhibition of both nucleoplasmic and nucleolar RNA labelling (Jacob *et al.*, 1970*b*; Niessing *et al.*, 1970). The possibility that α-amanitin *in vivo* inhibits both RNA polymerases A and B is ruled out by studies which have shown that, under these conditions, only RNA polymerase B is affected by the drug (Tata *et al.*, 1972; Smuckler & Hadjiolov, 1972; Sekeris & Schmid, 1972). It was reported also that inhibition of nucleolar RNA labelling is preceded by a decrease in nucleoplasmic RNA polymerase activity (Tata *et al.*, 1972). These results suggest that *in vivo* the synthesis of rRNA is under the control of some product dependent on the activity of nucleoplasmic RNA polymerases. Unfortunately, the inadequate resolution of nucleolar RNA species attained impedes a more critical evaluation of the site of action of α-amanitin on rRNA synthesis and/or maturation. All but one (Stirpe & Fiume, 1967) of the studies *in vivo* were carried out with rat liver: since the rat is known to be partly resistant to α-amanitin (Fiume & Wieland, 1970), additional effects may distort the experimental findings and their interpretation.

We have studied the effect of α-amanitin on [^{14}C]-orotate incorporation *in vivo* into different mouse liver nuclear and cytoplasmic RNA species. The drug inhibits nucleoplasmic RNA polymerases and the

synthesis of pre-mRNA*. It is shown also that α-amanitin initially inhibits the early steps of 45S pre-rRNA maturation and subsequently (or simultaneously) the synthesis of 45S pre-rRNA. The latter effect is not due to an alteration of nucleolar RNA polymerase activity. Part of these results has been reported previously (Hadjiolov, 1972; Hadjiolov & Mackedonski, 1972).

Experimental

Reagents

Analytical-grade reagents were used throughout. α-Amanitin was a generous gift from Professor Th. Wieland, Max-Planck Institut für Medizinische Forschung, Heidelberg, Germany. Sodium deoxycholate, Brij 35, ATP, GTP and CTP were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.; acrylamide and *NN'*-methylenebisacrylamide were from BDH Chemicals Ltd., Poole, Dorset, U.K.; PPO (2,5-diphenyloxazole) and dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] were from Koch–Light Laboratories Ltd., Colnbrook, U.K.; Protosol was from New England Nuclear Corp., Boston, Mass., U.S.A., and 'stains all' was from Eastman Kodak Co., Rochester, N.Y., U.S.A. [^{3}H]UTP (ammonium salt) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.) and [6-^{14}C]orotate (sp. radioactivity 25mCi/mmol) from NAEC Institute for Isotopes, Budapest, Hungary.

Animals

Experiments were carried out with male albino mice weighing 20±2g and fed *ad libitum* on a standard laboratory diet. α-Amanitin and [^{14}C]orotate were administered intraperitoneally at doses and time-intervals as specified in the text. The animals were killed by cervical dislocation, the livers were carefully freed from adherent gall bladders, rinsed in cold 0.9% NaCl and immediately processed.

Preparation of nuclei

Purified nuclei were obtained by the method of Blobel & Potter (1966), modified to account for the partial degradation of some nuclear RNA species which occurs during isolation of nuclei by this procedure (Dabeva & Tsanev, 1968). The minced livers were homogenized with a glass–Teflon Potter–Elvehjem homogenizer in 2vol. of 0.25M-sucrose in TKM buffer (0.05M-Tris–HCl, pH7.8; 0.025M-KCl; 0.01M-$MgCl_2$) and filtered through four layers of cheesecloth or a nylon bolting-cloth (200 mesh). Then 2vol. of 2.3M-sucrose in TKM buffer was added and 28ml of the resulting mixture was layered over 10ml of 2.3M-sucrose in TKM buffer. Nuclei were sedimented for 40min at 25000rev./min in the SW27 rotor of a Beckman L 65B ultracentrifuge at 4°C and the resulting nuclear pellet was immediately processed.

Isolation and fractionation of nuclear RNA

Three fractions corresponding roughly to nuclear sap, nucleolar and heterogeneous nuclear RNA were extracted from purified nuclei by treatment with phenol at different temperatures (Mackedonski *et al.*, 1972; Markov & Arion, 1973). The nuclear pellet was suspended in an ice-cold 1:1 (v/v) mixture of 0.14M-NaCl and phenol, saturated with 0.14M-NaCl (pH6.0) containing 0.1% 8-hydroxyquinoline. The mixture was homogenized briefly in a glass–Teflon homogenizer, shaken for 15min at 4°C and centrifuged at 3000*g* in the cold. RNA extracted in the water layer was further deproteinized by three washings with cold phenol, then once with a 1:1 (v/v) mixture of phenol–chloroform containing 1% sodium dodecyl sulphate and was precipitated from the final water phase with 2vol. of 96% ethanol–1% potassium acetate. This fraction was designated as nuclear 4°C RNA and corresponded to nuclear sap RNA. The interphase layer was suspended in cold 0.14M-NaCl, mixed with an equal volume of phenol and extracted at 4°C as described above. The resulting interphase layer was suspended in a 1:1 (v/v) mixture of 0.14M-NaCl and phenol (as above) and shaken for 15min at 50°C, then chilled in ice and centrifuged as described above. The RNA extracted in the water phase was deproteinized and precipitated as described above and designated as nuclear 50°C RNA. It contains the bulk of nucleolar RNA components. The interphase layer was suspended in 0.14M-NaCl and extracted with an equal volume of phenol for 15min at 85°C in the presence of 0.5% sodium dodecyl sulphate. The mixture was chilled in ice and centrifuged (20min at 3000*g*) in the cold. The RNA recovered from the water phase was purified further by treatment with deoxyribonuclease I and passage through a Dowex 1 (formate form) column (Mackedonski *et al.*, 1972) and was designated as nuclear 85°C RNA. It corresponds to heterogeneous nuclear RNA (Markov & Arion, 1973).

Isolation and fractionation of cytoplasmic RNA

Fractionation of cytoplasmic ribonucleoproteins was essentially as described by Hadjiolov *et al.*

* Abbreviations: pre-rRNA and pre-tRNA, precursors of rRNA and tRNA; pre-mRNA, heterogeneous nuclear DNA-like RNA considered as precursor of mRNA; mRNA, heterogeneous cytoplasmic messenger-like RNA.

(1972). The pooled livers of ten mice were homogenized in 0.025M-Tris–HCl buffer (pH7.4)–0.005M-$MgCl_2$–0.05M-KCl–0.25M-sucrose. The 20% homogenate was centrifuged at 10000*g* for 30min at 4°C. All subsequent manipulations were at 4°C. The supernatant was layered on to ½vol. of 0.5M-sucrose in the same buffer and centrifuged for 60min at 50000$g_{av.}$. The sediment constituted the microsomal fraction. The microsomal fraction was suspended by gentle homogenization in 0.25M-sucrose in the same buffer and sodium deoxycholate and Brij 35 were added to final concentrations of 1 and 2% respectively. The suspension was layered on to ½vol. of 0.5M-sucrose and centrifuged for 120min at 105000*g*. The translucent pellet constituted the ribosome fraction. Sodium deoxycholate and Brij 35 were added to the supernatant of the microsomal fraction to final concentrations of 1 and 2% and the mixture was centrifuged for 240min at 105000*g*. The pellet obtained was designated as the postmicrosomal ribonucleoprotein fraction. The final supernatant contained only 4S RNA. The RNA from the three cytoplasmic ribonucleoprotein fractions was extracted with phenol saturated with 0.2M-sodium acetate buffer (pH6.0) in the presence of 0.5% sodium dodecyl sulphate and 0.005M-EDTA. Deproteinization was carried out at 4°C with an equal volume of phenol saturated with 0.2M-sodium acetate buffer (pH6.0). This step was repeated three times and RNA was precipitated from the final water phase with 96% ethanol. The RNA from the ribosome fraction was used to prepare the ribosomal low-molecular-weight RNA after precipitation of the high-molecular-weight RNA components with 2M-NaCl for 10h at −10°C.

Gel electrophoresis of RNA fractions

Agar-gel electrophoresis and radioautography were as described by Tsanev & Staynov (1964) under conditions specified by Mackedonski *et al.* (1972). Since a simple correlation exists between the sedimentation coefficient of RNA molecules and their mobility in agar gel (Hadjiolov *et al.*, 1966) the separate components are designated further in the text by their S values. In some experiments the dried agar-gel plates were cut in 1mm slices and treated with 0.4ml of 0.01M-Tris–HCl buffer (pH7.9)–0.14M-NaCl–0.01M-EDTA–0.5% sodium dodecyl sulphate at 60°C overnight in counting vials. Scintillation-counting medium (1vol. of Triton X-100 plus 2vol. of 5g of PPO and 0.25g of dimethyl-POPOP in 1 litre of toluene) was added and the samples were counted for radioactivity in a Packard Tri-Carb model 3320 liquid-scintillation counter.

The low-molecular-weight RNA was also analysed by polyacrylamide-gel electrophoresis (Loening, 1970). Horizontal gel plates (18cm×10cm) of 10% polyacrylamide cross-linked with 0.5% *NN'*-methylenebisacrylamide were used. The buffer contained 36mM-Tris–HCl (pH7.8), 30mM-NaH_2PO_4 and 1.0mM-EDTA. The gel was pre-run for 60min and fractionation of RNA carried out at 80–100V (about 30mA) for 6h at 15°C. The gel plates were stained overnight with 'stains all' (20mg of 'stains all' in 80ml of dimethylformamide diluted to 1 litre with water) and the excess of dye was removed with water (Dahlberg *et al.*, 1969). The gels were cut into 1mm slices, put into counting vials and the dye was bleached by direct exposure to light. The gel slices were incubated for 1h at 60°C in a mixture of toluene–water–Protosol® (10:1:9, by vol.), then cooled and counted in a toluene–PPO–dimethyl-POPOP medium prepared as described above.

Assay of nuclear RNA polymerase activity

Nuclei were suspended in 50mM-Tris–HCl buffer (pH7.9)–5mM-β-mercaptoethanol–20% (v/v) glycerol to a concentration of about 2mg of protein/ml. The incubation mixture contained: 0.5mg of nuclear protein/ml; 65mM-Tris–HCl (pH7.9); 0.8mM-ATP, -GTP and -CTP; 15μM-[^{3}H]UTP (sp. radioactivity 1mCi/μmol); 5% (v/v) glycerol; 1.25mM-β-mercaptoethanol; total volume 0.08ml. The samples contained also either 7mM-$MgCl_2$ or 2.5mM-$MnCl_2$ plus 112mM-$(NH_4)_2SO_4$, ionic conditions favourable for nucleolar and nucleoplasmic RNA polymerase respectively (Widnell & Tata, 1966; Roeder & Rutter, 1970). Enzyme activity was determined in the presence and absence of 2.5μg of α-amanitin/ml for 20min at 37°C. Samples (0.07ml) were pipetted on to Whatman no. 1 filter-paper discs, and radioactivity was determined as described previously (Smuckler & Hadjiolov, 1972).

Results

Nuclear RNA polymerases

The enzyme activity was determined in nuclei isolated at different time-intervals after administration of α-amanitin *in vivo*. As shown in Fig. 1, the activity of nucleoplasmic (α-amanitin-sensitive) RNA polymerase is markedly decreased at 30min after administration of α-amanitin, almost complete inhibition being attained at 60min. This effect is better manifested when RNA polymerase activity is determined at high ionic strength in the presence of Mn^{2+}, but similar results are obtained when the activity is measured at low ionic strength and with Mg^{2+}. In contrast with observations with rat liver (Tata *et al.*, 1972), the inhibition of nucleoplasmic RNA polymerase is not rapidly reversed and lasts at least up to 150min after administration of α-amanitin. This is probably due to the faster elimination of α-amanitin

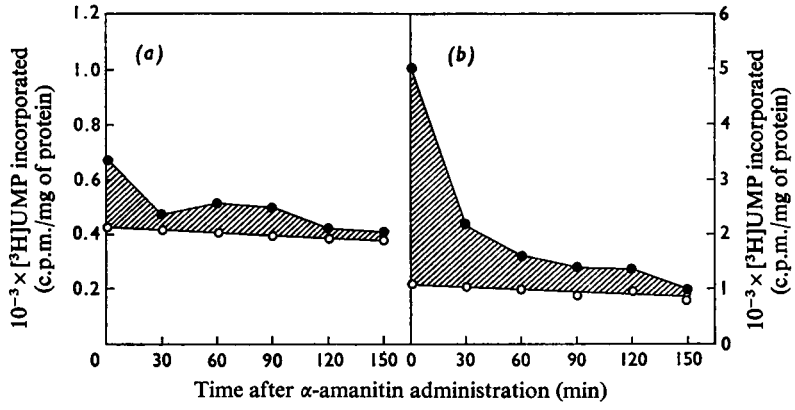


Fig. 1. *Action of α-amanitin in vivo on the activity of RNA polymerase of isolated mouse liver nuclei determined in the presence of (a) 7 mM-$MgCl_2$ or (b) 2.5 mM-$MnCl_2$ plus 112 mM-$(NH_4)_2SO_4$*

α-Amanitin at a dose of 0.20 μg/g body wt. was given intraperitoneally at different times before killing. The nuclei were isolated and their RNA polymerase was determined as described in the Experimental section, with (○) or without (●) 2.5 μg of α-amanitin/ml of incubation mixture. Each value is the mean of two independent experiments with three mice per experimental point. The shaded area shows the decrease of nucleoplasmic α-amanitin-sensitive RNA polymerase activity after α-amanitin administration *in vivo*.

Table 1. *Action of α-amanitin on the incorporation of [^{14}C]orotate into mouse liver nuclear RNA fractions*

α-Amanitin was given at a dose of 0.20 μg/g body wt. followed after 30 min by 1 μCi of [^{14}C]orotate (sp. radioactivity 16.7 mCi/mmol)/g body wt. The labelling time was 2 h. Nuclear RNA fractions were isolated as described in the Experimental section.

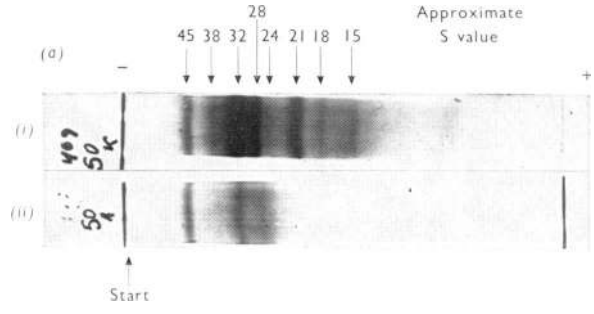
RNA fraction	Specific radioactivity (c.p.m./E_{260} unit of RNA) Controls	+α-Amanitin	Residual incorporation (%)
85°C RNA	82200	22850	27.8
50°C RNA	20100	5700	28.3
4°C RNA	2760	690	25.0
Low-molecular-weight 4°C RNA*	4500	3750	83.3

* The low-molecular-weight RNA fraction was obtained from 4°C RNA after precipitation of the high-molecular-weight RNA components with 2 M-NaCl.

by the rat and its relative resistance to this drug (Fiume & Wieland, 1970). Nucleolar (α-amanitin-resistant) RNA polymerase is, however, only slightly affected by α-amanitin. When determined either at low ionic strength in Mg^{2+} or at high ionic strength in Mn^{2+}, the activity of nucleolar RNA polymerase is decreased by no more than 10–20% even at 150 min after α-amanitin addition. The resistance of mouse liver nucleolar RNA polymerase to α-amanitin *in vivo* is in agreement with findings on the rat liver enzyme (Tata *et al.*, 1972; Smuckler & Hadjiolov, 1972; Sekeris & Schmid, 1972).

Labelling of nuclear high-molecular-weight RNA fractions

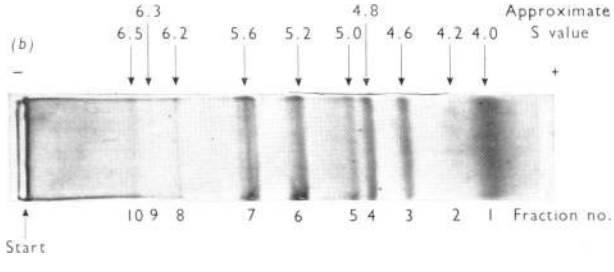
The action of α-amanitin administration on the overall [^{14}C]orotate-labelling of nuclear RNA fractions is given in Table 1. The results show that labelling of all RNA fractions obtained from isolated nuclei by phenol extraction at different temperatures is strongly inhibited by α-amanitin. It should be noted that labelling of the 85°C RNA and 50°C RNA fractions is inhibited to the same extent. These results reveal that both nucleoplasmic and nucleolar RNA



EXPLANATION OF PLATE 1(*a*)

Radioautograms of nuclear 50°*C RNA components from* (*i*) *control and* (*ii*) α*-amanitin-treated mice, fractionated by agar-gel electrophoresis*

α-Amanitin (0.20 μg/g body wt.) was given intraperitoneally followed after 30 min by 25 μCi of [^{14}C]orotate (sp. radioactivity 16.7 mCi/mmol)/mouse. Labelling time was 2 h. The approximate S values of the separate RNA components were assigned in relation to marker rat liver 28S and 18S rRNA. For details see the text.



EXPLANATION OF PLATE 1(*b*)

Polyacrylamide-gel electrophoresis fractionation of the low-molecular-weight RNA components of mouse liver nuclear 4°*C RNA fraction*

The isolation of 4°C RNA and conditions of low-molecular-weight RNA fractionation are described in the Experimental section. The polyacrylamide-gel plate was stained with 'stains all'. The approximate S values of the separate RNA components were assigned in relation to marker cytoplasmic 5S and 4S RNA of rat liver.

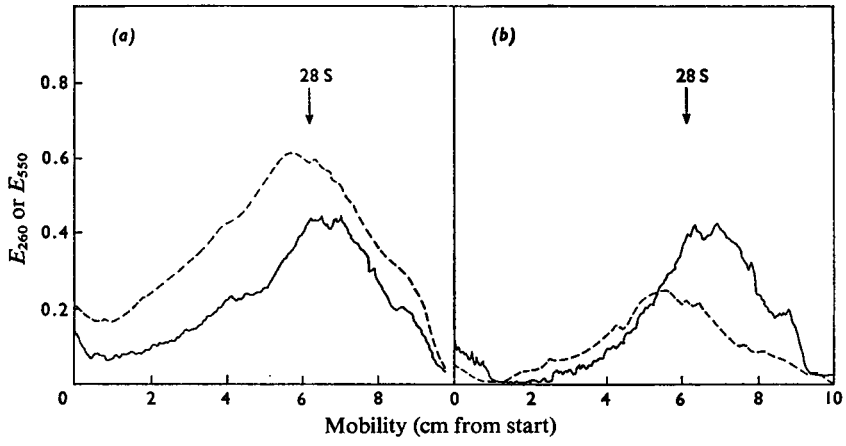


Fig. 2. *Agar-gel electrophoresis of liver nuclear 85°C RNA isolated from control (a) and α-amanitin-treated (b) mice*

The animals were given 0.10 μg of α-amanitin/g body wt. and 30 min later 25 μCi of [^{14}C]orotate (sp. radioactivity 16.7 mCi/mmol)/mouse. The labelling time was 2 h; ten mice were used for each experimental group. Nuclei were isolated and 85°C RNA was extracted and purified as described in the Experimental section. ——, E_{260}; ----, radioactivity recorded from the blackening of the radioautogram as E_{550}.

labelling is affected by the action of the drug *in vivo*. Further, fractionation of the nuclear 4°C RNA fraction by 2 M-NaCl precipitation reveals that the high-molecular-weight RNA components are much more strongly affected by α-amanitin than are the low-molecular-weight RNA constituents of this fraction. The site of action of α-amanitin on the synthesis and maturation of nuclear RNA was studied by gel electrophoresis of the separate RNA fractions.

Agar-gel electrophoresis and radioautography of nuclear 85°C RNA (Fig. 2) reveals that the bulk of this fraction consists of heterogeneous DNA-like RNA (Mackedonski *et al.*, 1972; Markov & Arion, 1973). The labelled RNA species in this fraction move slower than 28S rRNA and show a broad heterogeneous distribution typical of pre-mRNA. Treatment of mice with α-amanitin leads to a marked decrease in the labelling of all RNA components constituting pre-mRNA. Therefore it may be deduced that *in vivo* α-amanitin randomly inhibits the synthesis of pre-mRNA species.

The labelling of the 50°C RNA fraction obtained from purified nuclei shows a more complex pattern of α-amanitin action. The bulk of this fraction consists of nucleolar RNA components. The densitometer tracings at 260 nm reveal that 28S and 18S rRNA predominate, but the peaks of 32S and 45S pre-rRNA are also clearly delineated. The labelled components of nuclear 50°C RNA form several distinct bands with mobilities corresponding to 45S, 38S, 32S, 28S, 21S, 18S and 15S RNA (Plate 1). In addition, a faint radioactive band can be seen at about 24S, and a broad band in the zone of 4–7S RNA. Treatment with α-amanitin causes characteristic changes in the pattern of labelled components in 50°C RNA. The 45S pre-rRNA band has an intensity comparable with that measured in controls. The labelling of 32S and 21S pre-rRNA is markedly decreased, and that of 28S and 18S rRNA is almost completely blocked. The labelling of the minor fractions, 38S and 15S RNA, is also strongly inhibited, but the 24S RNA band is well delineated and becomes even more prominent in α-amanitin-treated mice.

The quantitative estimate of the changes in [^{14}C]-orotate incorporation into the separate components of the nuclear 50°C RNA fraction is shown in Fig. 3. The labelling of 45S pre-rRNA is only slightly decreased by treatment with low doses of α-amanitin. The labelling of all other RNA components is more strongly inhibited by α-amanitin. Thus about 80% inhibition is found for 32S and 21S pre-rRNA as well as for 28S and 18S rRNA. Labelling of the 24S RNA fraction is inhibited less (about 50%), so that this peak becomes more prominent in the radioactivity tracings. It should be noted that α-amanitin not only causes typical changes in the labelling of nucleolar RNA components, but also alters their amount. Thus the E_{260} peaks of 45S, 28S and 18S RNA are clearly delineated in α-amanitin-treated mice, but the peak of 32S RNA is markedly decreased.

Further information about the action of α-amanitin on rRNA labelling was obtained by agar-gel-electrophoretic analysis of the nuclear 4°C RNA fraction. Figs. 3(*c*) and 3(*d*) show that this fraction contains

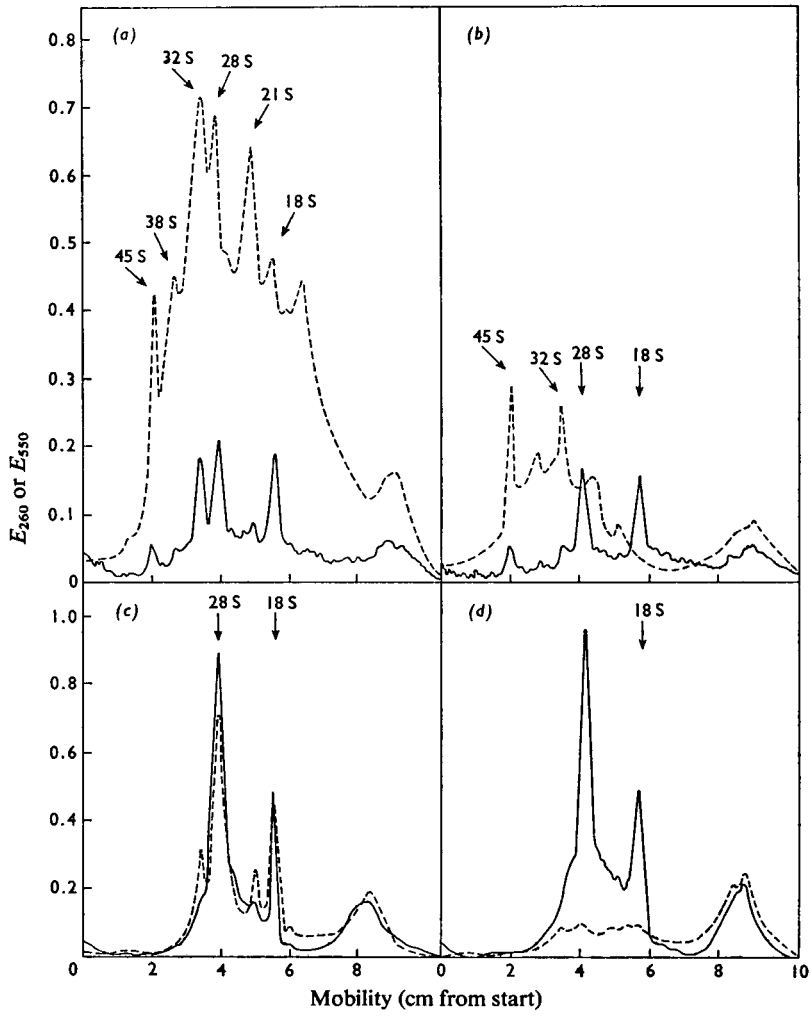


Fig. 3. *Agar-gel electrophoresis of liver nuclear 50°C RNA (a) and (b) and 4°C RNA (c) and (d) isolated from control (a and c) and α-amanitin-treated (b and d) mice*

Mice were given 0.1 μg of α-amanitin/g body wt. and 30 min later 25 μCi of [^{14}C]orotate (sp. radioactivity 16.7 mCi/mmol)/mouse. The labelling time was 2 h. The 50°C RNA and 4°C RNA fractions from purified nuclei were isolated as described in the Experimental section. The arrows indicate the S values and the position of the main peaks determined with rat liver 28S and 18S rRNA as internal markers (Hadjiolov *et al.*, 1966). ——, E_{260}; – – – –, radioactivity recorded from the blackening of the radioautogram as E_{550}.

the bulk of the nuclear 28S and 18S rRNA, probably obtained from ribosomal subparticles in the nuclear sap *en route* to the cytoplasm. Administration of α-amanitin almost completely abolishes (80–90% inhibition) the labelling of 28S and 18S rRNA in nuclear 4°C RNA.

Since the labelling (and the amount) of 32S and 21S pre-rRNA is strongly affected and that of 28S and 18S rRNA almost completely blocked by α-amanitin whereas the synthesis of 45S pre-rRNA is inhibited much less, it is likely that the drug preferentially inhibits the early steps of 45S pre-rRNA maturation.

Time-course of α-amanitin action on nuclear pre-rRNA synthesis and maturation

As shown above, α-amanitin appears to inhibit both the synthesis and maturation of pre-rRNA. The

succession of these two effects of α-amanitin has been further investigated by experiments in which the drug was given at different time-intervals before the animals were killed. Short-term labelling with [^{14}C]orotate (20min) was used; at this time of labelling, the label is located mainly in 45S pre-rRNA and its early maturation products, i.e. 32S and 21S pre-rRNA. The RNA from purified nuclei was extracted directly at 50°C. This nuclear RNA fraction includes both nucleolar and nuclear-sap RNA components. Fig. 4 shows that administration of α-amanitin causes a rapid inhibition of short-term labelling of nuclear RNA components. This effect is fully developed within 30min (about 80% inhibition) and lasts up to 150min after administration of α-amanitin. Agar-gel electrophoresis of short-term-labelled nuclear RNA shows that in control mice the ^{14}C label is located mainly in 45S, 38S, 32S and 21S pre-rRNA, whereas 28S and 18S rRNA remain unlabelled (Fig. 5). At 30min after α-amanitin administration *in vivo* the labelling of 45S pre-rRNA is decreased by about 30% by comparison with controls. In contrast, the labelling of 32S and 21S pre-rRNA is much more strongly inhibited, 75–80% inhibition being recorded for 32S pre-rRNA. At longer periods of α-amanitin action the labelling of 45S pre-rRNA decreases gradually to about 80% inhibition at 120 or 150min. These results confirm that the initial effect of α-amanitin *in vivo* is a strong inhibition of the early steps of 45S pre-rRNA maturation, i.e. its conversion into 32S and 21S pre-rRNA. The synthesis of 45S pre-rRNA is more slowly affected, but the inhibition is durable and deepens with time of α-amanitin action.

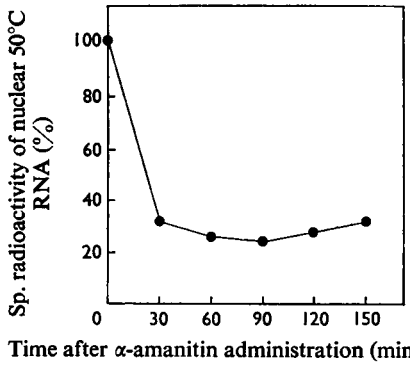


Fig. 4. *Time-course of the action of α-amanitin in vivo on the labelling of total nuclear 50°C RNA from mouse liver*

The mice were given 0.20μg of α-amanitin/g body wt. The labelling in all experimental groups was with 20μCi of [^{14}C]orotate (sp. radioactivity 8.5mCi/mmol)/mouse administered 20min before death. Three mice were used for each experimental point, the results being the mean of two independent series of experiments. The total nuclear 50°C RNA was extracted by direct treatment of purified nuclei with phenol at 50°C under conditions specified in the Experimental section. Here the nuclear 50°C RNA is a mixture of nuclear-sap and nucleolar RNA. Portions of the 50°C RNA fractions were precipitated with cold 5% trichloroacetic acid, filtered through Whatman GF/C glass-fibre discs, washed with cold 5% trichloroacetic acid, ethanol–ether (3:1, v/v), and finally ether, and counted for radioactivity with the toluene–PPO–dimethyl-POPOP medium in a liquid-scintillation counter. The specific radioactivity of total nuclear 50°C RNA from control mice was 5020c.p.m./E_{260} unit and was taken as 100%.

Ultrastructural changes of the nucleolus

In order to compare the effects of α-amanitin *in vivo* on the synthesis and maturation of pre-tRNA with the alteration of nucleolar ultrastructure, we carried out electron-microscopic studies of purified nuclei isolated at different times after α-amanitin administration. These studies were done in collaboration with Dr. I. Emanuilov and Mrs. R. Nikolova. The main conclusions are summarized as follows. The overall picture of nucleolar lesions caused by α-amanitin is in good agreement with previous findings (Fiume *et al.*, 1969; Petrov & Sekeris, 1971). The time-course of nucleolar alterations after a single dose of 0.25μg/g body wt. is as follows. At 15 and 30min no alterations in nucleolar ultrastructure can be detected by standard electron microscopy. At 60 and 90min initial fragmentation of nucleoli is observed, and the number of altered nuclei increases with time. Nuclei with completely fragmented nucleoli appear at 60min and their number gradually increases to reach about 80% of the total population at 150min. Consequently the inhibition of 45S pre-rRNA maturation and synthesis observed at 30min after α-amanitin administration precedes the alterations in nucleolar ultrastructure.

Labelling of nuclear low-molecular-weight RNA fractions

The effect of α-amanitin on the separate low-molecular-weight RNA components of nuclear 4°C RNA and 50°C RNA fractions was studied further by polyacrylamide-gel electrophoresis. The separate low-molecular-weight RNA components observed in nuclear 4°C RNA and their estimated sedimentation coefficients are represented in Plate 1(*b*). A similar pattern is found for the low-molecular-weight RNA components of the nuclear 50°C RNA fraction, although the relative amounts of the separate RNA components are different. These results are in good general agreement with previous studies on rat liver nuclear low-molecular-weight RNA (Moriyama *et al.*, 1969; Prestayko *et al.*, 1971). They show that nuclear low-molecular-weight RNA contains at least

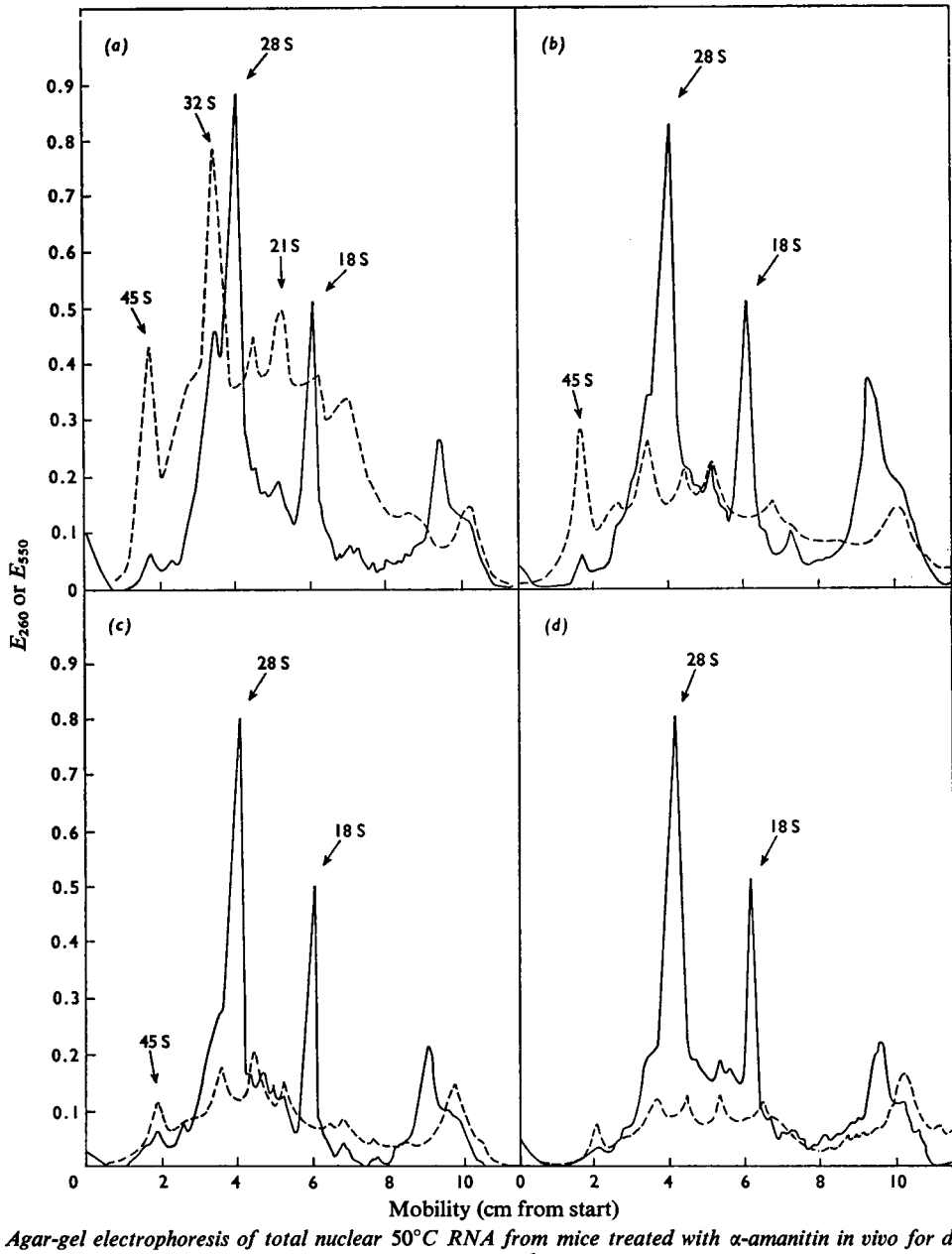


Fig. 5. *Agar-gel electrophoresis of total nuclear 50°C RNA from mice treated with α-amanitin in vivo for different time-intervals*

Treatment with α-amanitin, labelling with [^{14}C]orotate (20min) and isolation of total nuclear 50°C RNA were as described in the legend to Fig. 4. (*a*) Control, (*b*) 30min, (*c*) 60min and (*d*) 120min of α-amanitin treatment. ——, E_{260}; – – – –, radioactivity recorded from the blackening of the radioautogram as E_{550}.

ten distinct RNA components. Among these, 5S and 4S RNA are extracted almost completely in the 4°C RNA fraction. Most of the nuclear low-molecular-weight RNA components display low labelling at 2h after [^{14}C]orotate and therefore the effect of α-amanitin cannot be evaluated. Consequently our interest was mainly confined to the zone between 4S and 5.5S RNA, where three fractions (4S, 4.6S and 5S RNA) show sufficient [^{14}C]orotate labelling to allow more correct estimates. As shown in Fig. 6, α-amanitin

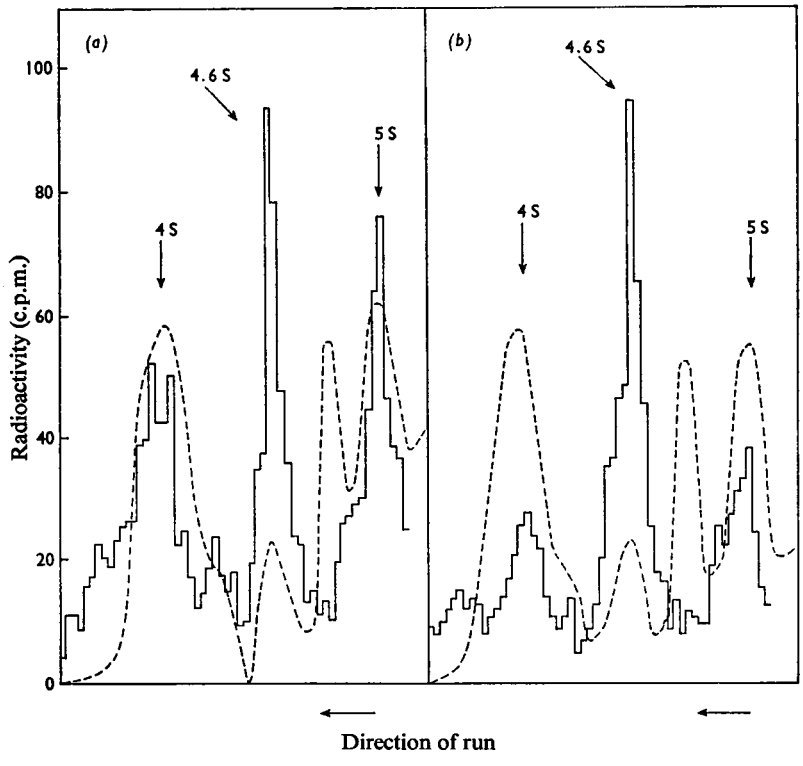


Fig. 6. *Effect of α-amanitin in vivo on the labelling of liver nuclear low-molecular-weight RNA components from (a) control and (b) α-amanitin-treated mice*

α-Amanitin (0.20 μg/g body wt.) was given 30 min before 20 μCi of [^{14}C]orotate (sp. radioactivity 8.5 mCi/mmol)/mouse. Labelling time was 45 min. For each experimental group 12 mice were used. The low-molecular-weight RNA components from the 4°C RNA fraction of purified nuclei were fractionated by polyacrylamide-gel electrophoresis as described in the Experimental section. ----, Chromoscan (in arbitrary units) of the gel stained with 'stains all'; ——, radioactivity of the separate polyacrylamide-gel slices. Only the radioactivity of the zone between 4S and 5S RNA was determined, since the labelling of the slower-moving nuclear low-molecular-weight RNA components (Plate 1*b*) was too low to permit correct estimates.

Table 2. *Action of α-amanitin on the incorporation of [^{14}C]orotate into mouse liver cytoplasmic RNA fractions*

α-Amanitin was given at a dose of 0.20 μg/g body wt. followed after 30 min by 1.25 μCi of [^{14}C]orotate/g body wt. The labelling time was 2 h. Isolation of RNA from cytoplasmic ribonucleoprotein fractions was as described in the Experimental section.

Cytoplasmic fraction source of RNA	Specific radioactivity (c.p.m./E_{260} unit of RNA)		Residual incorporation (%)
	Controls	+α-Amanitin	
Ribosomes	544	70	12.9
Microsomal fraction	1120	170	15.2
Postmicrosomal ribonucleoproteins	1720	280	16.4
Final supernatant (4S RNA)	910	580	63.0

treatment causes about 50% inhibition of the labelling of 4S and 5S RNA. However, the labelling of 4.6S RNA is practically unaltered by α-amanitin. Since the latter fraction has been identified as pre-tRNA (Burdon, 1971; Choe & Taylor, 1972), our results indicate that α-amanitin does not inhibit transcription of tRNA genes, but interferes with the processing of pre-tRNA to mature tRNA.

Labelling of cytoplasmic RNA fractions

The effect of α-amanitin on [^{14}C]orotate labelling of RNA from different cytoplasmic fractions is given in Table 2. The results show that α-amanitin strongly inhibits the labelling in all cytoplasmic particulate components studied. Since we have shown (Hadjiolov *et al.*, 1972) that at the times of labelling studied the label is equally distributed between rRNA and mRNA, the observed 80–90% inhibition indicates that both cytoplasmic RNA species are inhibited by α-amanitin. The labelling of cytoplasmic t-RNA is also inhibited by the drug (about 50% inhibition).

The effect of α-amanitin on the labelling of cytoplasmic RNA components was studied further by agar-gel electrophoresis (Fig. 7). The inhibition of cytoplasmic RNA labelling by α-amanitin reflects the changes in the labelling of nuclear RNA species. The labelling of 28S and 18S rRNA is almost completely abolished, not only in the microsomal fraction and purified ribosomes, but also in the postmicrosomal ribonucleoprotein fraction constituted mainly by nascent ribosomal subparticles and mRNA (Nikolaev *et al.*, 1972). As expected, the labelling of mRNA in the microsomal fraction and postmicrosomal ribonucleoproteins is also blocked by α-amanitin, the inhibition being of the same order as that of nuclear pre-mRNA.

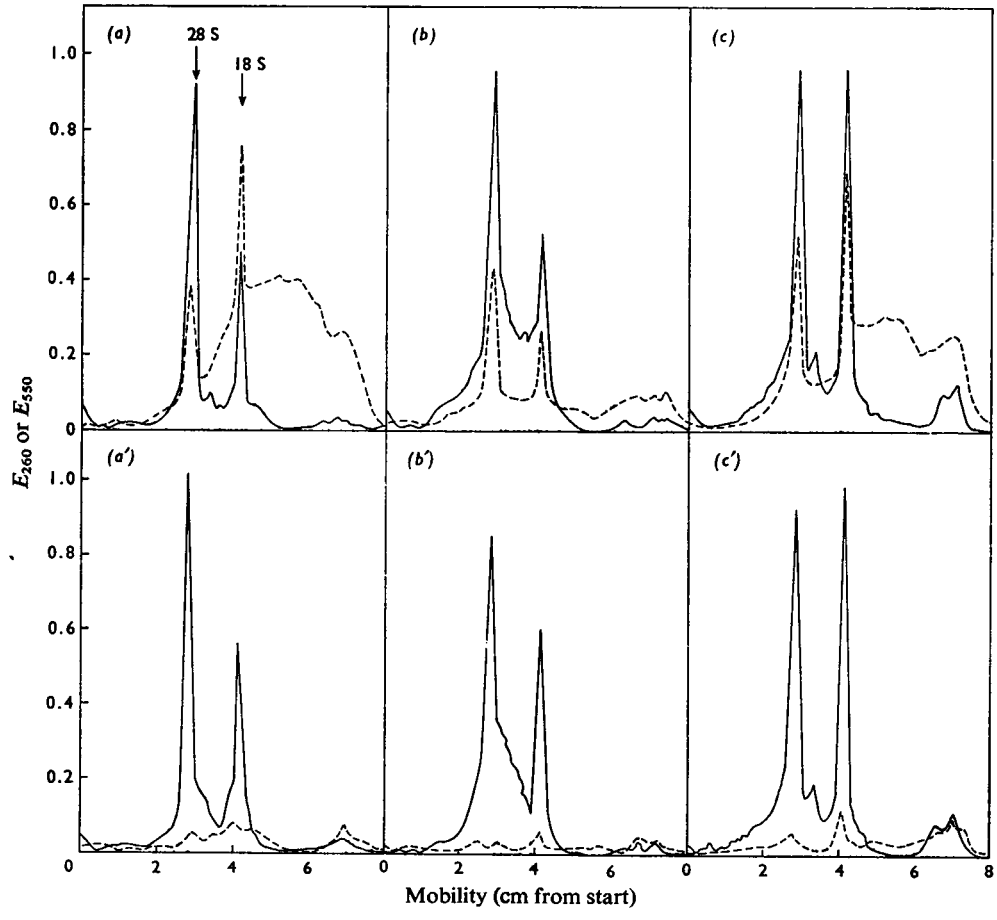


Fig. 7. *Agar-gel electrophoresis of liver RNA isolated from microsomal fraction (a and a'), ribosomes (b and b') and post-microsomal ribonucleoproteins (c and c') isolated from control (a, b and c) and α-amanitin-treated mice (a', b' and c')*

The animals were given 0.20 μg of α-amanitin/g body wt. and 30 min later 25 μCi of [^{14}C]orotate (sp. radioactivity 8.5 mCi/mmol)/mouse. The labelling time was 2 h. The isolation of the separate cytoplasmic ribonucleoprotein fractions and the extraction of their RNA is described in the Experimental section. ——, E_{260}; – – –, radioactivity recorded from the blackening of the radioautogram as E_{550}.

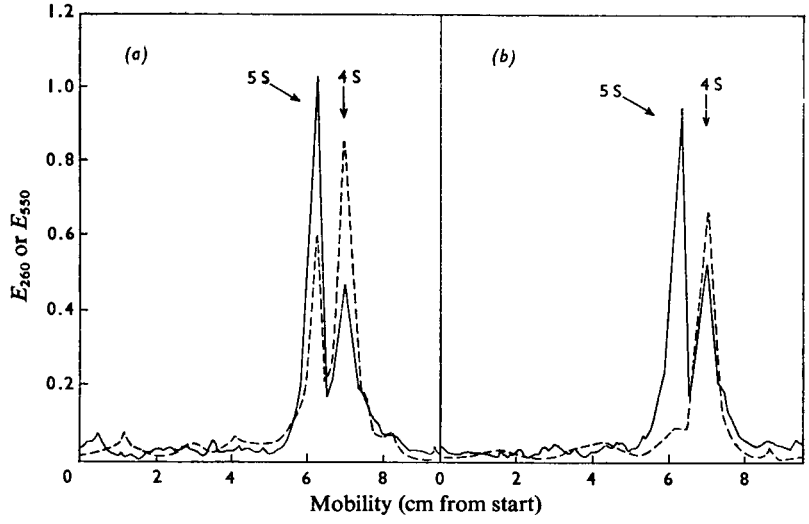


Fig. 8. *Agar-gel electrophoresis of low-molecular-weight RNA extracted from purified ribosomes of (a) control and (b) α-amanitin-treated mice*

Treatment with α-amanitin *in vivo* and labelling with [^{14}C]orotate were as described in Fig. 7. The low-molecular-weight RNA was obtained from the RNA of purified ribosomes by precipitation with 2M-NaCl. Agar-gel electrophoresis was in 2.5% agar gel instead of the standard 1.25% gel used in the fractionation of high-molecular-weight RNA. The arrows indicate the position of marker 5S and 4S RNA from liver. ——, E_{260}; ----, radioactivity recorded from the blackening of the radioautogram, E_{550}.

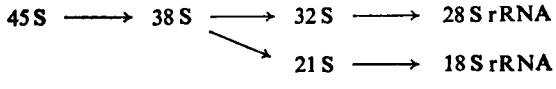
The action of α-amanitin on the labelling of cytoplasmic low-molecular-weight RNA components was of particular interest to us and we have studied these RNA components extracted from ribosomes or from the supernatant remaining after sedimentation of the postmicrosomal ribonucleoprotein fraction. The only RNA found in this supernatant is 4S RNA. The observed 50% inhibition of cytoplasmic 4S RNA is identical with that obtained for nuclear 4S RNA. For 4S and 5S RNA components extracted from purified ribosomes (Fig. 8) our results show that α-amanitin treatment inhibits the labelling of 5S rRNA more strongly than that of 4S RNA. Labelling of 5S rRNA is decreased to about 20% of that in control mice, which corresponds to the inhibition for the other two rRNA species. Since the labelling of nuclear 5S rRNA is less affected by α-amanitin than is 5S rRNA from cytoplasmic ribosomes, these results indicate that the nucleocytoplasmic transfer of 5S rRNA in liver cells requires the synchronous formation of mature 28S and 18S rRNA in nuclei.

Discussion

According to current concepts, there are four main products of transcription in eukaryotes, namely pre-mRNA, 45S pre-RNA, 5S rRNA and 4.6S pre-tRNA. At least three different RNA polymerases are involved in the transcription of these RNA species. Our results reveal that α-amanitin inhibits *in vivo* the synthesis of pre-mRNA and the activity of nucleoplasmic RNA polymerase. These results are in agreement with studies on rat liver, which showed that both *in vitro* (Jacob *et al.*, 1970*a*) and *in vivo* (Tata *et al.*, 1972; Smuckler & Hadjiolov, 1972; Sekeris & Schmid, 1972) α-amanitin acts as a highly selective inhibitor of nucleoplasmic RNA polymerase. Unlike the transient effect observed in rat liver (Tata *et al.*, 1972) our results show that in mice α-amanitin causes a rapid and lasting inhibition of nucleoplasmic RNA polymerase even at relatively low doses of the drug (Fiume & Wieland, 1970). It has been shown (Chambon *et al.*, 1972) that ^{3}H-labelled amanitin binds selectively and stoicheiometrically to nucleoplasmic RNA polymerase molecules. This is true not only for the purified enzyme, but also for crude tissue homogenates. Thus all the reported findings indicate that the action of α-amanitin *in vivo* is due to its selective inhibitory effect on nucleoplasmic RNA polymerases. There are not, at present, any indications about the occurrence of metabolic conversions of α-amanitin *in vivo* or *in vitro*. The effect of α-amanitin on RNA species other than pre-mRNA will therefore be considered as a consequence of its selective action on nucleoplasmic RNA polymerase. However, a

direct and independent action of α-amanitin cannot be ruled out.

Our results show that α-amanitin *in vivo* inhibits the labelling of mouse liver rRNA, as found previously for rat liver (Jacob *et al.*, 1970*b*; Niessing *et al.*, 1970; Tata *et al.*, 1972). We have specified further that α-amanitin initially inhibits the maturation, and subsequently the synthesis, of 45S pre-rRNA. If one considers the most popular scheme of pre-rRNA maturation (Maden, 1971) adapted to the actual S values found in our experiments:



our results show that α-amanitin strongly inhibits the early steps of rRNA maturation, i.e. the conversions 45S → 38S → 32S+21S pre-rRNA. This is shown by both the markedly decreased labelling of 32S and 21S pre-rRNA at the initial stages of α-amanitin action (while the labelling of 45S pre-rRNA remains practically unaltered) and the decreased amount of 32S pre-rRNA at longer times of action of the drug when the E_{260} peak of 45S pre-rRNA is still unaltered. The inhibition of nuclear and cytoplasmic 28S and 18S rRNA labelling is obviously a consequence of the block in 45S pre-rRNA maturation. How does one explain this early block in rRNA maturation caused by a specific inhibitor of nucleoplasmic RNA polymerase and pre-mRNA synthesis?

Three possibilities may be envisaged: (a) α-amanitin may inhibit the synthesis of 5S rRNA needed for the normal processing of 45S pre-rRNA; (b) some regulatory nucleoplasmic RNA molecule may be involved in pre-rRNA maturation; (c) some of the proteins involved in pre-rRNA maturation (endo- or exonucleases, methylases, ribosomal proteins etc.) might have an exceedingly high turnover rate and can easily become rate-limiting. The first possibility is unlikely, since our results have shown that labelling of nuclear 5S rRNA is inhibited to a much lesser extent than that of either pre-rRNA or mature rRNA species. Further, it was shown that 5S rRNA synthesis *in vitro* is resistant to α-amanitin (Price & Penman, 1972). At present it is impossible to decide between possibilities (b) and (c). It should be pointed out that an early inhibition of pre-rRNA maturation was found on cycloheximide inhibition of protein synthesis in HeLa cells (Willems *et al.*, 1969) and regenerating rat liver (Rizzo & Webb, 1972), but not in normal rat liver (Muramatsu *et al.*, 1970). These results indicate the participation of some unstable protein in the regulation of pre-rRNA maturation. For α-amanitin it should be stressed that the observed inhibition of 45S pre-rRNA maturation is almost fully developed within 30min, which suggests that the postulated regulator molecule (RNA or protein) is characterized by an exceedingly fast turnover.

The gradual inhibition of 45S pre-rRNA synthesis caused by α-amanitin *in vivo* is also difficult to explain. It is not due to a decreased activity of nucleolar RNA polymerase, since our results and those of others (Tata *et al.*, 1972; Sekeris & Schmid, 1972) have shown that this enzyme is little affected by α-amanitin. Since α-amanitin blocks the synthesis of mRNA, our results do not corroborate the assumption, deduced from experiments with cycloheximide and actinomycin D, that liver nucleolar RNA polymerase and its mRNA are characterized by an unusually fast turnover (Yu & Feigelson, 1972). More recently it was reported that rat liver nucleolar RNA polymerase is resistant to cycloheximide treatment *in vivo* (Benecke *et al.*, 1973). As shown in the present work, the inhibition of 45S pre-rRNA precedes the fragmentation of nucleoli caused by α-amanitin. Therefore the decreased synthesis of 45S pre-rRNA cannot be due to fragmentation of nucleoli, although the possibility of some more subtle alteration in the interaction between nucleolar RNA polymerase and rRNA genes is not unlikely. The discrepancy between the observed inhibition of 45S pre-rRNA synthesis by α-amanitin *in vivo* and the unaltered activity of nucleolar RNA polymerase indicates that the assay for this enzyme does not reflect accurately its function *in vivo*. Thus it is possible that the presumed regulator molecule which controls 45S pre-rRNA synthesis is involved in determining the correct initiation of transcription of rRNA genes. Since the inhibition of 45S pre-rRNA synthesis *in vivo* seems to be preceded by an inhibition of its processing, it may also be envisaged that transcription of rRNA genes is modulated by feedback-control mechanisms. Whatever the explanation, further elucidation of the mechanism of action of α-amanitin on pre-rRNA synthesis and maturation will help in the understanding of the regulation of these processes.

Finally, we would like to stress that maturation of 4.6S pre-tRNA may be also under nucleoplasmic control. As shown, α-amanitin inhibits the formation of mature nuclear and cytoplasmic tRNA, although the labelling of pre-tRNA remains unaltered. The effect of α-amanitin on pre-tRNA maturation is less pronounced and slower than its action on pre-rRNA. Therefore further studies are needed in order to elucidate its mechanism.

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References

Benecke, B. J., Ferencz, A. & Seifart, K. H. (1973) *FEBS Lett.* **31**, 53–58

Blobel, G. & Potter, V. R. (1966) *Science* **154**, 1662–1665
Burdon, R. H. (1971) *Progr. Nucl. Acid Res. Mol. Biol.* **11**, 33–79
Chambon, P., Gissinger, F., Kedinger, C., Mandel, J. L., Meilhac, M. & Nuret, P. (1972) *Gene Transcription in Reproductive Tissue; Karolinska Symp.* **5**, 222–246
Choe, B. K. & Taylor, M. W. (1972) *Biochim. Biophys. Acta* **272**, 275–287
Dabeva, M. D. & Tsanev, R. G. (1968) *Bull. Biochem. Res. Bulg. Acad. Sci.* **3**, 49–56
Dahlberg, A. E., Dingman, C. W. & Peacock, A. C. (1969) *J. Mol. Biol.* **41**, 139–152
Fiume, L. & Wieland, Th. (1970) *FEBS Lett.* **8**, 1–5
Fiume, L., Marinozzi, V. & Nardi, F. (1969) *Brit. J. Exp. Pathol.* **50**, 270–276
Hadjiolov, A. A. (1972) *Stud. Biophys.* **31/32**, 405–415
Hadjiolov, A. A. & Mackedonski, V. V. (1972) *Abstr. FEBS Meet. 8th* Abstr. no. 494
Hadjiolov, A. A., Venkov, P. V. & Tsanev, R. G. (1966) *Anal. Biochem.* **17**, 263–267
Hadjiolov, A. A., Nikolaev, N. & Shulga, A. (1972) *Int. J. Biochem.* **3**, 509–517
Jacob, S. T., Sajdel, E. M. & Munro, H. N. (1970*a*) *Biochem. Biophys. Res. Commun.* **38**, 765–770
Jacob, S. T., Muecke, W., Sajdel, E. M. & Munro, H. N. (1970*b*) *Biochem. Biophys. Res. Commun.* **40**, 334–342
Kedinger, C., Gniazdowski, M., Mandel, J. L., Gissinger, F. & Chambon, P. (1970) *Biochem. Biophys. Res. Commun.* **38**, 165–171
Loening, U. E. (1970) *Methods Med. Res.* **12**, 359–368
Mackedonski, V. V., Nikolaev, N., Sebesta, K. & Hadjiolov, A. A. (1972) *Biochim. Biophys. Acta* **272**, 56–66
Maden, B. E. H. (1971) *Progr. Biophys. Mol. Biol.* **22**, 127–176
Markov, G. G. & Arion, V. (1973) *Eur. J. Biochem.* **35**, 186–200
Moriyama, Y., Hodnett, J. L., Prestayko, A. W. & Busch, H. (1969) *J. Mol. Biol.* **39**, 335–349
Muramatsu, M., Shimada, N. & Higashinakagawa, T. (1970) *J. Mol. Biol.* **53**, 91–106
Niessing, J., Schnieders, B., Kunz, W., Seifert, K. H. & Sekeris, C. E. (1970) *Z. Naturforsch. B* **25**, 1119–1125
Nikolaev, N., Welfle, H. & Hadjiolov, A. A. (1972) *FEBS Lett.* **27**, 235–239
Petrov, P. & Sekeris,C. E. (1971) *Exp. Cell Res.* **69**, 393–398
Prestayko, A. W., Tonato, M., Lewis, B. C. & Busch, H. (1971) *J. Biol. Chem.* **246**, 182–187
Price, R. & Penman, S. (1972) *J. Mol. Biol.* **70**, 435–450
Rizzo, A. J. & Webb, T. E. (1972) *Eur. J. Biochem.* **27**, 136–144
Roeder, R. G. & Rutter, W. J. (1970) *Proc. Nat. Acad. Sci. U.S.* **65**, 675–681
Sekeris, C. E. & Schmid, W. (1972) *FEBS Lett.* **27**, 41–45
Smuckler, E. A. & Hadjiolov, A. A. (1972) *Biochem. J.* **129**, 153–166
Stirpe, F. & Fiume, L. (1967) *Biochem. J.* **105**, 779–792
Tata, J. R., Hamilton, M. J. & Shields, D. (1972) *Nature (London) New Biol.* **238**, 161–164
Tsanev, R. G. & Staynov, D. Z. (1964) *Biokhimiya* **27**, 1126–1131
Widnell, C. C. & Tata, J. R. (1966) *Biochim. Biophys. Acta* **123**, 478–492
Willems, M., Penman, M. & Penman, S. (1969) *J. Cell Biol.* **41**, 177–187
Yu, F. L. & Feigelson, P. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 2833–2837