α Amanitin, a specific inhibitor of transcription by mammalian RNA-polymerase *

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Evidence has been presented that α amanitin, the potent bicyclic polypeptide toxin of the toadstool amanita phalloides inhibits transcription by purified RNA polymerase from rat liver nuclei. This inhibition is highly specific for the mammalian enzyme, since it does not impede the same reaction catalyzed by the enzyme from E. coli thus rendering an interaction between the template and inhibitor very unlikely. α Amanitin is a very potent inhibitor and calculations show an approximate stoichiometry of 1 : 1 between enzyme and inhibitor. In addition, evidence is forthcoming suggesting that the inhibitor allows chain initiation to occur, but abruptly inhibits elongation of RNA chains.

α Amanitin is a potent toxin of the toadstool amanita phalloides. It was isolated by WIELAND et al. and its structure was determined as a bicyclic polypeptide by WIELAND. STIRPE and FIUME initially reported in 1967 that the toxin inhibits RNA-synthesis of isolated mouse liver nuclei both after in vivo and in vitro application. In contrast, the inhibitor has no influence on bacterial growth nor on the replication of a number of DNA- or RNA-viruses. The important role played by inhibitors of RNA synthesis in elucidating the mechanism of transcription as well as its control is generally acknowledged. The known inhibitors belong mainly to two categories: those interacting with the DNA template eg. actinomycin D and those reacting with the RNA-polymerase as has been shown for rifamyacin and its derivatives. The latter class of inhibitors, however, only influence bacterial RNA-polymerase and can therefore not be employed for investigations concerned with mechanisms and control of RNA-synthesis in higher organisms.

It has therefore been the aim of this investigation to study more closely the mechanism by which α amanitin exerts its inhibitory influence on RNA-synthesis. This attempt was greatly facilitated by the existence in our laboratory of a purified RNA-polymerase from rat liver nuclei.

Experimental Procedures

Materials

α Amanitin was a generous donation by Prof. Th. WIELAND (Heidelberg). [3H] UTP (1.5 C/mmole) was obtained from the Radiochemical Centre (Amersham); ATP, GTP, CTP, UTP, creatinphosphokinase from Boehringer (Mannheim); DEAE-Cellulose (microgranular, 1 mequiv/g) from Hormuth and Vetter (Heidelberg); Sepharose 4B from Pharmacia (Uppsala); Heparin (A-grade, Na-salt) from Sigma (St. Louis); salmon sperm DNA (highly polymerized A grade) from Calbiochem and nitrocellulose membrane filters (0.45 μm) from Sartorius (Göttingen) respectively.

Methods

Liver nuclei were isolated from male wistar BR II rats by detergent treatment as previously described. RNA-polymerase (E.C. 2.7.7.6.) was purified from frozen E. coli cells (K-12, 3/4 log; Fallek, New York) essentially as described by ZILLIG et al. RNA-polymerase from rat liver was extracted by ultrasonic treatment of isolated nuclei in high ionic strength (0.75 M NaCl) followed by purification of the enzyme as described by SEIFART and SEKERIS with the following modifications. During the extraction procedure the nuclei were sonified at 20 kHz, 125 Watt. When handling large amounts of starting material (± 500 g liver tissue) it was found necessary to insert a gel-filtration step through a 1.5 × 30 cm Sepharose 4B column after...
the DEAE-cellulose stage. The column was equilibrated and eluted with buffer containing 20% glycerol. Enzymatically active fractions were pooled, precipitated with crystalline \((\text{NH}_4)_2\text{SO}_4\) at 50% saturation, dialyzed against 1/10 of buffer containing 10% glycerol for 6 hours (3 changes) and further purified by density gradient centrifugation as described. RNA-polymerase activity was assayed as incorporation of \(^{3}H\)UTP into RNA. Unless otherwise stated, the in \textit{vitro} system was incubated for 40 min at 37°C and contained 0.2 \(\mu\)moles each of ATP, GTP, CTP; 1.0 \(\mu\)Ci \(^{3}H\)UTP; 10 \(\mu\)moles Tris-Cl, pH 7.9; 0.5 \(\mu\)mole Mn\(\text{SO}_4\); 1.5 \(\mu\)moles mercaptoethanol; 1.5 \(\mu\)moles creatine phosphate; 5 \(\mu\)g creatine phosphokinase; 10.0 \(\mu\)moles \((\text{NH}_4)_2\text{SO}_4\); 25 \(\mu\)g DNA and approximately 10 \(\mu\)g RNA-polymerase in a final volume of 0.150 ml. At the end of the incubation, an aliquot was pipetted onto filter paper discs (4 cm²; Schleicher and Schuell 2043 B), precipitated in ice cold 5% perchloric acid, washed with ethanol and diethyl ether and counted by liquid scintillation in toluene (5 g PPO; 200 mg POPOP/1).

In those experiments measuring the incorporation of radioactivity on membrane filters, aliquots of the incubation mixture were pipetted into 5 ml ice cold 2× SSC-buffer (0.3 M NaCl, 0.03 M Na-citrate, pH 7.0). The filters were moistened with distilled water and subsequently soaked in buffer for 2 hours. The entire mixture was then filtered by gentle suction and washed with 100 ml 2× SSC-buffer. The filter was subsequently air-dried and counted as described above.

Normally, the reaction was started by the addition of \(^{3}H\)UTP to a system containing all other components. Where the action of inhibitors was studied, the order of mixing the components was varied and solutions of the inhibitor in Tris-buffer were added as appropriately described in the legends to the figures. Heparin (MW ± 12,500) and \(\alpha\) amanitin (MW ± 1000) were added at approximately equimolar concentrations.

Protein and DNA determinations were conducted according to the methods of Lowry et al. and Burton respectively.

**Results**

The results obtained with isolated rat liver nuclei (Table 1) clearly demonstrate, that \(\alpha\) amanitin greatly inhibits RNA-synthesis as was shown before by Stirpe et al. for mouse liver nuclei. The inhibition, although observed with both Mn\(^{2+}\) and Mg\(^{2+}\), was less pronounced with the latter cation. In the presence of \((\text{NH}_4)_2\text{SO}_4\) the relative inhibition is potentiated for Mn\(^{2+}\) due to the stimulation of synthesis by \((\text{NH}_4)_2\text{SO}_4\) in the control group. If isolated RNA-polymerase from rat liver nuclei is employed it is evident (Fig. 1), that \(\alpha\) amanitin inhibits RNA-synthesis with both Mn\(^{2+}\) and Mg\(^{2+}\) if conducted at constant ionic strength. The relative inhibition seen with Mn\(^{2+}\) is higher than that with Mg\(^{2+}\) due to the lower rate of RNA-synthesis in the

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<th>RNA polymerase activity (cpm/mg protein)</th>
<th>with ((\text{NH}_4)_2\text{SO}_4) (ionic strength = 0.4)</th>
<th>without ((\text{NH}_4)_2\text{SO}_4) (ionic strength = 0.05)</th>
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<td>control amanitin inhibition [%]</td>
<td>control amanitin inhibition [%]</td>
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<tr>
<td>Mn(^{2+}) 8150</td>
<td>3958</td>
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<td>Mg(^{2+}) 2925</td>
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Table 1. Effect of \(\alpha\) amanitin on the RNA polymerase activity of isolated rat liver nuclei in the presence or absence of \((\text{NH}_4)_2\text{SO}_4\). For the determination of RNA polymerase activity, 50 \(\mu\)l of nuclear suspension were incubated for 10 minutes in an \textit{in vitro} system as described in Methods with the following modifications: The incubation flasks (150 \(\mu\)l final volume) contained either 1 \(\mu\)mole Mn\(\text{SO}_4\) or 1 \(\mu\)mole Mg\(\text{SO}_4\) in the presence or absence of \((\text{NH}_4)_2\text{SO}_4\) (ionic strength = 0.4). Where appropriate, \(\alpha\) amanitin was added (0.25 \(\mu\)g/ml final concentration) before start of the reaction with the nuclear suspension.

![Fig. 1. Influence of amanitin on the rate of transcription by purified RNA polymerase from rat liver nuclei. Where appropriate, \(\alpha\) amanitin was added at a final concentration of 0.25 \(\mu\)g/ml and Mn\(\text{SO}_4\) (A) was replaced by 10 mM Mg\(\text{SO}_4\) (B). All other components were as described in Methods. The order of mixing was: enzyme, amanitin and then all other components. The reaction was started by the addition of \(^{3}H\)UTP.](image)

presence of Mg\(^{2+}\) with the enzyme preparation under consideration.

Employing the same template and RNA-polymerase isolated from different organisms, it can be shown that α-amanitin greatly impedes RNA-synthesis by the enzyme from rat liver while transcription by \textit{E. coli} RNA-polymerase remains completely unimpaired (Fig. 2). This observation is not due to a difference in sensitivity of the two enzymes to the inhibitor, nor can it be explained by a different enzyme-inhibitor stoichiometry, since the construction of a dose-response curve reveals (Fig. 3), that the bacterial enzyme is not inhibited even at very high concentrations of α-amanitin. In contrast, the mammalian enzyme is almost maximally inhibited at a concentration of about 0.03 μg/ml incubation volume. Taking into consideration the amount of RNA-polymerase protein added (60 μg/ml incub. vol.) and assuming a molecular weight of 1000 for α-amanitin and approximately 400,000 per active RNA-polymerase molecule, a stoichiometric ratio of 1 RNA-polymerase to 1.5 α-amanitin molecules has been calculated. Since the highest possible ratio is below 2 it is very likely, that the true stoichiometry between enzyme and inhibitor is 1:1.

From the studies concerning the inhibition of transcription of \textit{E. coli} polymerase by rifamycin and its derivatives,\(^5\) it was known that only free enzyme not bound to DNA is inhibited by the antibiotic.

The order of mixing the components of the incubation system was therefore modified as outlined

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\textbf{Fig. 2.} Influence of α-amanitin on the activity of purified RNA polymerase from rat liver nuclei (A) and \textit{E. coli} (B). The conditions of the assay were as described in Fig. 1. To account for the higher activity of the bacterial enzyme, the specific activity of [\(3\)H] UTP was lowered to 5 mCi/mmol by the addition of unlabeled UTP and the incubation period was reduced to 10 min in the assay of \textit{E. coli} RNA polymerase.

\textbf{Fig. 3.} Dose-response curve between concentration of α-amanitin and transcription catalyzed by purified RNA polymerase from rat liver nuclei (A) and \textit{E. coli} (B). Incubation conditions were as described in Fig. 2.

\textbf{Fig. 4.} Effect of adding α-amanitin at different stages of the incubation on the resulting rate of transcription by purified RNA polymerase from rat liver nuclei. The basic conditions were as described in Methods and the legend to Fig. 1 but the order of adding the inhibitor was varied. Control values contained no inhibitor (△ ——— △). In one treatment group α-amanitin (0.25 μg/ml final concentration) was preincubated with the free enzyme for 10 min at 37°C (○——○) before addition of all other components and start of the reaction with [\(3\)H] UTP. In a third group the enzyme was mixed with all other components before start of the reaction with premixed α-amanitin and [\(3\)H] UTP (x ——— x).
in Fig. 4, to clarify whether an analogous mechanism could be established for amanitin. The results of this study show, however, that the toxin exerted its inhibitory effect regardless of whether it was preincubated with the free enzyme, or whether it was added after binding of the enzyme to DNA had already occurred. It has been observed that a residual nucleoside triphosphate incorporation of approximately 15% occurred in either case which could not be blocked by amanitin.

The experiments of Walter et al. demonstrated, that the polyanion heparin binds with the free enzyme from *E. coli* and thereby completely eliminates subsequent synthesis. Fig. 5, demonstrates that this fact also applies to the mammalian enzyme, and supports the assumption that the action of heparin and amanitin is clearly not identical.

![Fig. 5. Effect of a amanitin and heparin on RNA synthesis by purified RNA polymerase from rat liver. The basic incubation conditions were as described in Methods and Fig. 1. The free enzyme was preincubated with either buffer (▲—▲), a amanitin (O—O; 0.25 µg/ml final concentration) or heparin (x—x; 10 µg/ml final concentration) for 10 min at 37°C. After addition of all other components, the reaction was started by the addition of [3H] UTP.](image)

Fig. 5. Effect of a amanitin and heparin on RNA synthesis by purified RNA polymerase from rat liver. The basic incubation conditions were as described in Methods and Fig. 1. The free enzyme was preincubated with either buffer (▲—▲), a amanitin (O—O; 0.25 µg/ml final concentration) or heparin (x—x; 10 µg/ml final concentration) for 10 min at 37°C. After addition of all other components, the reaction was started by the addition of [3H] UTP.

As has been shown for RNA polymerase from mammalian and a variety of other sources, optimal synthesis occurs at an elevated ionic strength, presumably due to a dissociation of the DNA-enzyme-RNA complex and a higher degree of reinitiation. A comparison of the effect of amanitin at low and high ionic strength reveals, that the rate of synthesis in the control groups is markedly elevated at an ionic strength of 0.2, but that the residual synthesis seen in the presence of a amanitin is similar in both cases (Fig. 6). Among other possibilities it could be postulated, that the inhibitor permits binding of the enzyme to the DNA and initiation to occur but prevents chain elongation. Under this assumption reinitiation would be prevented which could account for the data of Fig. 6.

![Fig. 6. Effect of a amanitin on the kinetics of RNA-synthesis by purified rat liver RNA polymerase in the presence (A) or absence (B) of (NH₄)₂SO₄. Incubation conditions were as described in Methods and Fig. 1. The ionic strength was 0.20 and 0.05 in the presence and absence of (NH₄)₂SO₄ respectively. The enzyme was preincubated with buffer (▲—▲) or amanitin (O—O; 0.25 µg/ml).](image)

Fig. 6. Effect of a amanitin on the kinetics of RNA-synthesis by purified rat liver RNA polymerase in the presence (A) or absence (B) of (NH₄)₂SO₄. Incubation conditions were as described in Methods and Fig. 1. The ionic strength was 0.20 and 0.05 in the presence and absence of (NH₄)₂SO₄ respectively. The enzyme was preincubated with buffer (▲—▲) or amanitin (O—O; 0.25 µg/ml).

It was attempted to assess chain initiation by a modification of the method proposed by Van der Helm and Zillig employing a preincubation period with an incomplete assay mixture containing [3H] UTP, but lacking one nucleoside triphosphate (CTP). While permitting initiation, this technique prevents normal chain elongation, which can be restored by the addition of CTP. Incorporation was measured on nitrocellulose membranes without prior acid precipitation as described in Methods.

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The results obtained by this method show that the lack of CTP does not completely arrest chain elongation in the control group, but allows a limited amount of residual synthesis to occur (Fig. 7). The lack of a clear-cut plateau in this system unfortunately prevents the exact measurement of initiation and limits the conclusions which can be drawn. Addition of CTP, however, results in a greatly enhanced and linear synthesis allowing an unequivocal distinction between the two phases. Compared to the control group, preincubation of the enzyme with 3-amanitin results in a similar degree of synthesis in the first minutes of incubation in the system lacking CTP, although a more complete plateau is reached in the latter case. Addition of CTP to this system results in no additional synthesis, indicating that chain elongation has been arrested. Preincubation of the enzyme with heparin (Fig. 7) results in elimination of synthesis at all stages, indicating that the enzyme has been completely inactivated.

If 3-amanitin is added to a system 10 min after active transcription has commenced, the inhibitor instantaneously causes an almost complete arrest of chain elongation, whereas addition of heparin under these conditions allows a certain amount of subsequent synthesis to progress (Fig. 8). This is in agreement with the finding of Fuchs et al.15, showing that heparin does not inhibit synthesizing enzyme to the same extent as free enzyme. In addition these results further support the conclusion that 3-amanitin and heparin do not act by similar mechanisms.

Discussion

The results presented confirm the observations of Stirpe and Fiume3 that 3-amanitin inhibits RNA-synthesis in isolated rat liver nuclei. In contrast to the findings of the above mentioned authors, however, inhibition of nuclear RNA-synthesis was evident both in the presence of either Mn2+ or Mg2+ although the inhibition was much less pronounced with Mg2+ (Table 1). The relative inhibition with Mn2+ was potentiated by (NH4)2SO4, due to the stimulation of RNA-synthesis in the control group. The recent data of Roeder and Rutter20 indicate that two types of RNA-polymerase exist in the nucleus. In view of the relative resistance of the

Mg\(^{2+}\) dependent reaction of the nuclei to α amanitin it could be postulated, as already proposed by Stirpe and Fiume\(^3\) that one of the polymerases, mainly Mn\(^{2+}\) dependent, is responsible for the synthesis of DNA-like RNA\(^{21}\) while the other, mainly Mg\(^{2+}\) dependent, catalyses the synthesis of ribosomal RNA and is insensitive to the toxin. This interesting hypothesis must await experimental proof by the measurement of the sensitivity of different types of polymerases toward α amanitin after extensive purification of these enzymes.

The experiments conducted with the enzyme purified from rat liver nuclei clearly show that α amanitin greatly inhibits RNA-synthesis catalyzed by the mammalian enzyme while transcription by the enzyme from E. coli remains unaltered. This finding renders unlikely any effects of the toxin on the DNA-template and focuses attention on the interaction of the enzyme protein with the inhibitor. This represents the first case of a specific inhibitor for mammalian RNA-polymerase, analogous to the well-known compounds, eg. rifamycin, selectively acting on the bacterial enzyme.

It is interesting to note, that the enzymes from bacteria and mammals, although catalyzing basically the same reaction with very similar requirements, are apparently structurally different enough to warrant a very high degree of specificity toward certain inhibitors. It is well conceivable that more precise knowledge of the exact mechanism of transcription by the different enzymes may reveal, that these structural differences are also expressed in subtle functional variations.

α Amanitin is a very potent inhibitor and from the dose-response relationship it can be calculated that one molecule of enzyme interacts with 1.5 molecules of inhibitor, making at 1 : 1 stoichiometry very likely. The availability of radioactively labeled amanitin should render studies on the exact localization of the binding sites feasible.

The results obtained by experiments in which the influence of the order of addition of inhibitor was studied definitely show, that α amanitin does not selectively act on the free enzyme as is the case for heparin and as has been shown for rifamycin in E. coli, but also inhibits enzyme already bound to DNA. In order to investigate more directly the mechanism of this inhibition, it was attempted to measure chain initiation by the method of Maitra and Hurwitz\(^{22}\) employing the dual incorporation of [γ-\(^{32}\)P] ATP and [\(^{3}H\)] UTP. Results of these investigations revealed that the incorporation of [\(^{3}H\)] UTP was greatly inhibited by α amanitin while leaving unaltered the incorporation of [γ-\(^{32}\)P] ATP. It was found however, that this method cannot be employed to measure chain initiation in this particular case, since the mammalian enzyme alone binds considerable amounts of radioactivity derived from [γ-\(^{32}\)P] ATP. This results in high background-values which mask the activity incorporated into 5' terminal residues. The enzyme-associated radioactivity is not acid hydrolyzable (12% TCA; 95°; 20 min) and therefore probably reflects phosphorylation of the protein. This phenomenon is not seen with [\(^{14}\)C] or [γ-\(^{32}\)P] ATP nor with [\(^{32}\)P] orthophosphate, thus excluding unspecific attachment of radioactivity. It remains to be seen whether this phosphorylation of the polymerase protein serves a functional role since it cannot be completely excluded at the present time that it reflects phosphorylation of some minor contaminating component.

It was alternatively attempted to measure chain initiation by a slight modification of the method employed by Zillig and coworkers\(^{19}\) which utilizes a preincubation period in the presence of all components and three nucleoside triphosphates, thus allowing chain initiation but limiting chain elongation which can only progress freely after the addition of the fourth nucleoside triphosphate (CTP). The limitation of this system is, at least with the polymerase preparation employed, the lack of a clear-cut initiation plateau in the absence of CTP, which can probably be attributed to erroneous synthesis excluding CTP.

As nearly as can be ascertained by this method, however, preincubation of the enzyme with α amanitin allows a large portion of normal chain initiation to occur, but almost completely prevents chain propagation upon the addition of CTP. Similar conclusions are reached if α amanitin is added to a system which is fully engaged in transcription since this results in an instantaneous arrest of almost all subsequent synthesis. Very similar conclusions have been reached by Schleif\(^{23}\) concerning the mecha-

nism of action of streptolydigin in the E. coli system. It remains to be seen whether the very small degree of synthesis observed in the presence of amanitin is meaningful and possibly reflects an amanitin resistant component.

The addition of heparin to the system under similar sets of conditions yields completely different results and suggests that this inhibitor acts preferentially on the free enzyme, thus showing that the binding sites for heparin and amanitin to mammalian RNA-polymerase are probably not the same.

In can be concluded that very low concentrations of amanitin specifically inhibit transcription by mammalian RNA-polymerase. Evidence is presented supporting the hypothesis that the inhibitor allows initiation to occur but arrests chain elongation by a mechanism not fully understood at present but certainly not involving the template. Further knowledge concerning the possible sub-unit structure of the mammalian enzyme may advance the understanding of this mechanism and relevant experiments are in progress.

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Regulation glykolytischen Umsatzes durch Synthese und Abbau von Enzymen

Regulation of Glycolysis by the Synthesis and Degradation of Enzymes

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Differential derepression of the genome of potato tuber cells causes the onset of a vigorous metabolic activity, which is initiated by rapid synthesis of different RNA species, various proteins and phospholipids. Consequently enhanced respiration and the build up of cell compartments such as ribosomes and mitochondria as well as the performance of cell divisions and suberization of new-formed cell walls occur. Although there is an activation of metabolism in general with a concomitant rise in concentration of most glycolytic metabolites — as was proved for fructose-1,6-diphosphate, dihydroxyacetone, glyceraldehyde-3-phosphate, phosphoenolpyruvate and pyruvate — the activities of the corresponding enzymes do not reflect these uniform metabolic changes. Aldolase and in a pronounced manner enolase and glutamate—pyruvate—transaminase lower their activities suddenly after derepression. The activity of triosephosphateisomerase remains constant. In contrast phosphoglyceromutase, pyruvate kinase and to a lower extent malic enzyme enhance their action during the same time.

Without doubt, differential lowering and enhancing the activity of glycolytic chain constituents at the same time is an important regulatory mechanism of the cell. The activation represents de novo synthesis of the protein concerned whereas the inactivation depends largely on protein synthesis. This is clearly shown by experiments with inhibitors of protein synthesis.

It is proposed that this differential synthesis and degradation represent a "long-time-regulation" of enzymatic activity of the cell in contrast to the known "short-time-regulation" by feedback or competition.

In einer früheren Mitteilung 1 konnte dargelegt werden, daß nach einer differenziellen Genaktivierung durch Verwundung von Kartoffelknollen-Parenchym (Solanum tuberosum L.) der Stärkekatabolismus vorübergehend aktiviert wird. Bei diesem Prozeß sind im einzelnen noch unbekannte Regelmecha-