ACTION OF α-AMANITIN *IN VIVO* AND *IN VITRO*

C.E. SEKERIS and W. SCHMID

Institut für Physiologische Chemie der Universität Marburg, 355 Marburg/Lahn, Lahnberge, BRD

Received 11 August 1972

1. Introduction

Since the basic work of Stirpe and Fiume [1] on the action of α-amanitin on RNA synthesis of mammalian cells, and after the demonstration that this toxic octapeptide product of *Amanita phalloides* acts on RNA polymerase of eukaryotes and not of bacteria [2] a great deal of work has been amassed concerning the mechanism of action of this substance (for a review see [3]). A significant observation was that α-amanitin preferentially inhibits RNA polymerase B [1, 5] the enzyme presumably responsible for the synthesis of DNA-like RNA, having no action on RNA polymerase A, the enzyme localized in the nucleolus [5], which synthesizes ribosomal RNA. This finding raised hopes that the long sought specific inhibitor of DNA-like RNA was found, which could be of great importance for studies of control of RNA synthesis in higher organisms. However, *in vivo* experiments demonstrated that α-amanitin impairs both DNA-like RNA as well as ribosomal RNA [6, 7] showing less inhibitory action on low molecular RNA. The impairment of ribosomal RNA synthesis after blocking of DNA-like RNA synthesis suggested [6, 7] that the continuous synthesis of DNA-like RNA is necessary for the unimpaired functioning of the ribosomal RNA producing apparatus. The experiments described below were designed to test this hypothesis.

2. Materials and methods

[^{3}H]UTP (specific activity 1 Ci/mmole), [^{3}H]orotic acid (specific activity 20–25 Ci/mmole) and [^{3}H]phenylalanine (specific activity 1 Ci/mmole) were obtained from the Radiochemical Centre, Amersham. The non-labelled nucleoside triphosphates were obtained from Boehringer, Mannheim. DE-32 cellulose (Whatman) was purchased from Hormuth and Vetter, Heidelberg. All other reagents were of analytical grade. Wistar BR II male rats weighing 100–120 g, kept under standard conditions, were used throughout.

Rat liver nuclei were prepared according to Chauveau et al. as described in [8]. Nucleoli were prepared according to Bush [9].

Extraction of RNA polymerase from nuclei and chromatography on DEAE-cellulose was as described in [10]. The *in vitro* RNA synthesizing system is described in [2]. The incorporation of [^{3}H]phenylalanine in nucleolar proteins was determined according to [11].

DNA was determined according to Burton [12].

3. Results

In a first series of experiments we examined the effects of administration of α-amanitin on the *in vivo* labelling with RNA precursors of nuclear, nucleolar and extranucleolar RNA. As seen from fig. 1 there is within 15 min after the administration of α-amanitin a significant decrease in the labelling of both extranucleolar and nucleolar RNA. The specific activity of the RNA of the nuclear fractions continues to decrease at 30 min and tends to level at 60 min. One possibility for the impairment of nucleolar RNA synthesis could be that α-amanitin *in vivo* is converted to a metabolite acting on RNA polymerase A. We therefore extracted and separated on DEAE-cellulose columns the RNA polymerases from liver nuclei of control and α-amanitin treated rats and quantitated the activity of RNA poly-

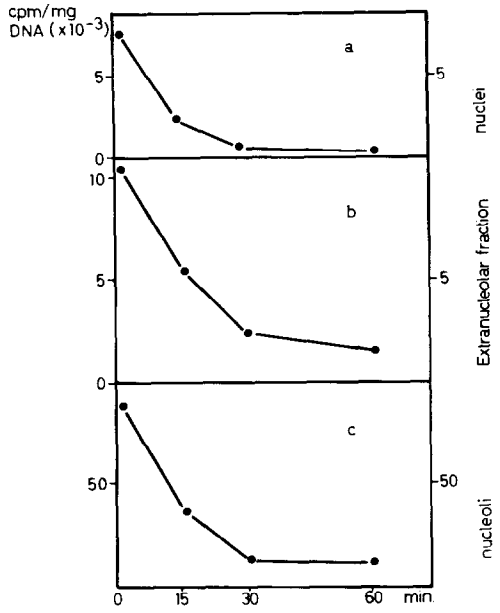

Fig. 1. Effects of α-amanitin on the incorporation of [^{3}H]orotic acid into nuclear, nucleolar and extranucleolar RNA. α-amanitin (1 μg/g rat) was administered intraperitoneally, the animals killed at the time denoted in the ordinate of the figure after having received previously 15 min pulses of 25 μCi [^{3}H]orotic acid per rat. Nuclei, nucleolar and extranucleolar fractions were prepared and the incorporation of orotic acid into RNA measured. a) Nuclei; b) extranucleolar fraction; c) nucleoli.

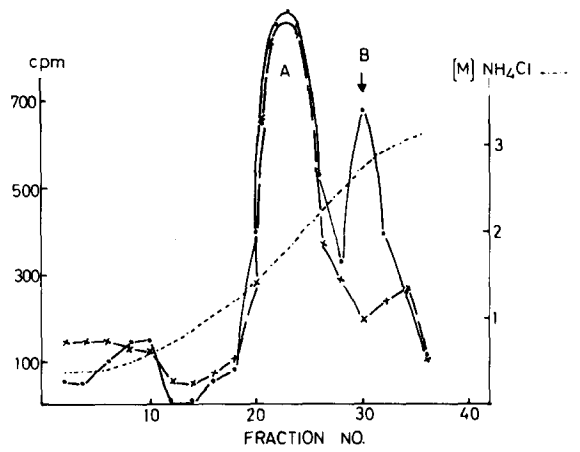

Fig. 2. DEAE-cellulose chromatography of DNA-dependent RNA polymerase from liver nuclei of control and α-amanitin treated rats. Amanitin was applied at a dose of 1 μg/g weight for 1 hr. Crude nuclei were prepared and the enzymes extracted and chromatographed as described by Seifart et al. [10]. Enzyme assay was performed as described by Seifart and Sekeris [2]. Control (•—•—•); amanitin treated (X —X —X).

merase A and B recovered. Fig. 2 shows the results of such an experiment. It is evident that the amount of RNA polymerase assayed in nuclei of rats treated for 1 hr with α-amanitin is the same as that found in control nuclei. Similar results have been obtained with nuclei from 2 hr treated animals. No RNA polymerase B activity can be detected in preparations from amanitin treated rats. This could be due either to the failure to extract the enzyme from nuclei or to the extraction of the enzyme in an inactive form due to its binding with α-amanitin.

In order to differentiate between these possibilities we incubated minced liver with ^{14}C-labelled-methyl α-amanitin, then extracted and chromatographed nuclear RNA polymerases on DEAE-cellulose columns and further i) tested for RNA polymerase activity and ii) measured the ^{14}C-radioactivity in the different fractions. It can be seen (fig. 3), that radioactivity is found in the fractions where enzyme B is normally isolated, which shows that the enzyme protein is present in the chromatograms but is inactive in the RNA synthesis assay for it is inhibited by bound α-amanitin.

We further tested the RNA synthetic capacity of isolated nuclei derived from control and α-amanitin treated animals in the presence or absence of α-amanitin and characterized RNA synthesis on the basis of its amanitin sensitivity. Fig. 4 shows that in contrast to *in vivo* findings, nuclei from amanitin treated rats do not show decreased capacity to synthesize RNA in comparison to the controls. Not RNA synthesis in the nuclei from amanitin treated rats is nearly totally amanitin resistant. We then fractionated the nuclei into a nucleolar and an extranucleolar fraction and measured the RNA synthetic capacity of the two fractions in an *in vitro* system. Fig. 5 shows that nucleolar RNA synthesis is inhibited in the α-amanitin treated preparations similary to the *in vivo* experiments, whereas extranucleolar RNA synthesis is greatly enhanced. Both nucleolar and extranucleolar activities are amanitin resistant and stimulated preferentially by Mg^{2+}. These results taken together with the morphological findings of nucleolar segregation after amanitin action

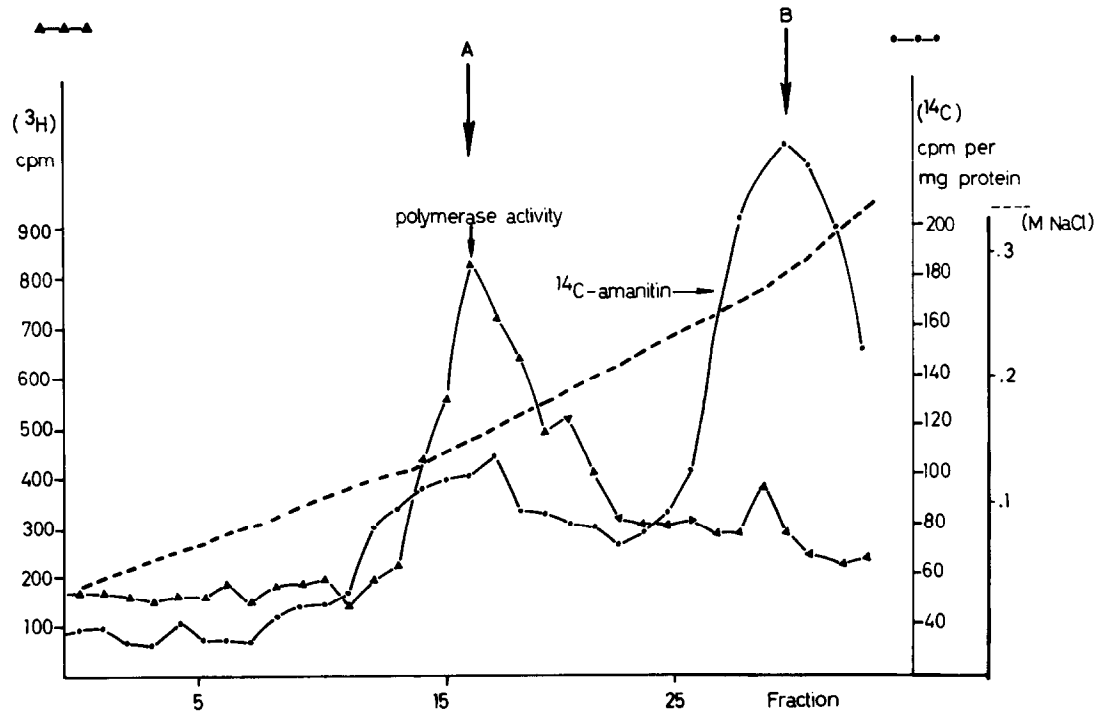

Fig. 3. Binding of [^{14}C]methyl-amanitin to rat liver nucleolar proteins. Rat liver was finely minced and incubated at with 1 μg/g tissue [^{14}C]methyl-amanitin (specific activity 45.3 μCi/mg) for 20 min at 22° in buffer. Crude nuclei were then prepared and RNA polymerase extracted and separated on DEAE-cellulose as described by Seifart et al. [10]. ^{14}C-radioactivity as well as the incorporation of [^{3}H]UTP in a standard RNA synthesizing mixture (Seifart and Sekeris, 1968) was measured in aliquots of the fractions.

[13, 14] suggested to us that polymerase A has leaked into the extranucleolar space.

The impairment of ribosomal RNA synthesis could be due to the lack of a special DNA-like RNA essential for nucleolar structure and RNA synthesis or indirectly to the lack of the translation product of the RNA. The first hypothesis is favoured by the very rapid and extensive inhibition of nucleolar RNA synthesis which is seen 15 min after α-amanitin application. The second hypothesis was suggested from data on the incorporation of labelled amino acids into nucleolar proteins. In table 1 the results of such an experiment is shown. [^{3}H]phenylalanine was injected to control and α-amanitin treated rats and the specific activity of proteins, of nuclei and nuclear fractions determined. Although general cytoplasmic and nuclear protein synthesis is not influenced at all [15] incorporation of the amino acid into nucleolar proteins is significantly inhibited.

4. Discussion

The experiments described above were performed to explain some of the apparent inconsistencies between the *in vivo* and *in vitro* results obtained with α-amanitin. The main conclusions to be drawn is that RNA polymerase A is itself not impaired. However, *in vivo*, the incorporation of labelled precursors into both extranucleolar and nucleolar RNA is inhibited, whereas *in vitro* extranucleolar RNA synthesis is enhanced and gets qualities which are characteristic for synthesis by RNA polymerase A. Nucleolar RNA synthesis is inhibited as well as *in vivo*. It is conceivable that *in vivo* under the action of amanitin RNA polymerase leaks from the nucleoli into the extranucleolar space and that the polymerase in this case is either not active in RNA synthesis or the RNA synthesized will be immediately digested by the nuclear nucleases.

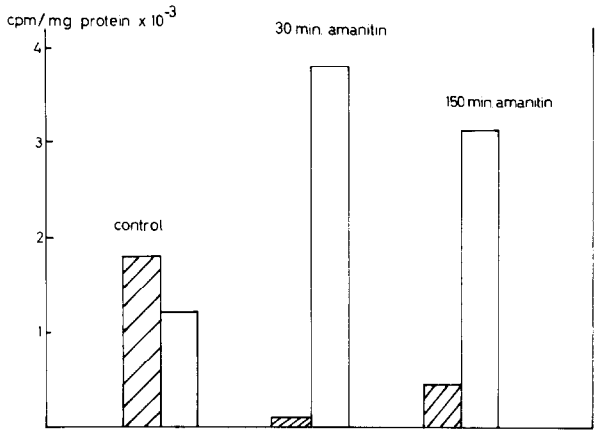

Fig. 4. Effect of α-amanitin applied *in vivo* on RNA synthesis of isolated rat liver nuclei. Animals were treated with 1 μg/g α-amanitin for different time periods. Rat liver nuclei were then prepared according to [8] and their RNA synthetic capacity measured as described by Seifart and Sekeris [2] in the presence 1.5 μg/ml and absence of α-amanitin. ▨: Amanitin sensitive cpm; □: amanitin resistant cpm.

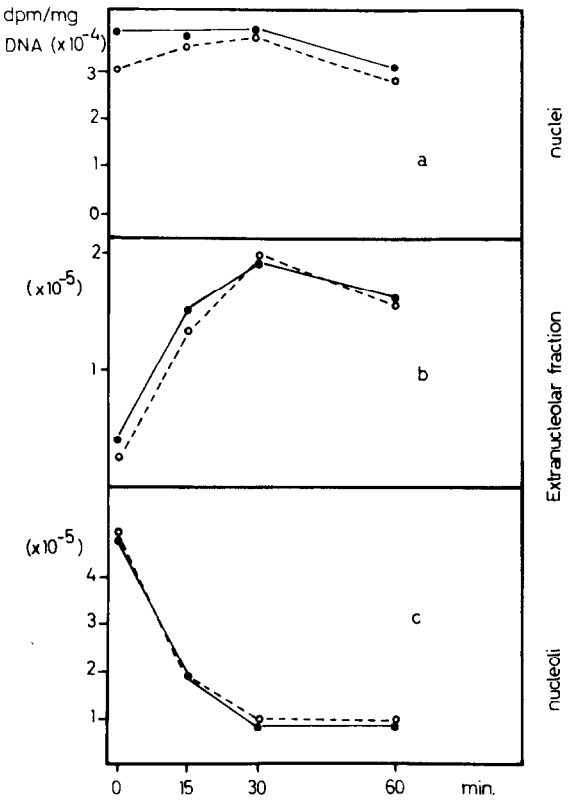

Fig. 5. Effect of α-amanitin applied *in vivo* on RNA synthesis of isolated nuclei, extranucleolar fraction and nucleoli. Rats were treated with 1 μg/g α-amanitin for different time periods. Rat liver nuclei, nucleoli and extranucleolar fraction were prepared and their RNA synthetic capacity determined in the presence of 5 μg/ml (○- -○- -○) and in the absence (●—●—●) of α-amanitin. The incubation system was as described in [2] but instead of Mn, 19 mM $MgCl_2$ was used. a) Nuclei; b) extranucleolar fraction and c) nucleoli.

Table 1
Effect of α-amanitin on the incorporation of [^{3}H]phenylalanine into nuclear and cytoplasmic proteins.

		Incorporation	
		(cpm/mg protein)	(cpm/mg DNA)
Nuclei	Control	3200	6240
	α-Amanitin	3580	5860
Nucleoli	Control	1790	71400
	α-Amanitin	1200	32500
Extranucleolar fraction	Control	4200	9150
	α-Amanitin	4770	9850
Post-mitochondrial fraction	Control	11000	–
	α-Amanitin	13000	–

Rats were injected 100 μCi/100 g body weight [^{3}H]phenylalanine 50 min before sacrifice followed by either 1 μg/g body weight α-amanitin in saline or saline alone 30 min before sacrifice. Nuclei, nuclear fractions and post mitochondrial supernatant were prepared and the incorporation of [^{3}H]phenylalanine in total proteins determined.

During the isolation of the nuclei this restriction of RNA synthesis could be lost so that *in vitro* RNA synthesis in the extranucleolar space by RNA polymerase A can be detected.

Our results do not prove whether the effect of α-amanitin on nucleolar RNA synthesis is exerted by the inhibition of the synthesis of a yet unknown DNA-like RNA which is an integral part of the nucleolus or whether amanitin acts by inhibiting transcription of the messenger RNA(s) of some essential nucleolar protein(s) with a very short half life time. That protein synthesis is needed for the continuous synthesis of

ribosomal RNA has already been reported in experiments using cyclohexomide [16–18] as inhibitor of protein synthesis.

α-Amanitin has been used lately as a specific inhibitor of DNA-like RNA synthesis in studies dealing with hormonal stimulation of RNA synthesis and on this basis [19] the hypothesis has been advanced that the stimulation of ribosomal RNA synthesis induced by hormones is a consequence of stimulation of synthesis of DNA-like RNA. This may well be so and is in fact favoured by our results showing a sequential stimulation by cortisol first of extranucleolar followed by nucleolar RNA synthesis in rat liver [20, 21]. However according to the data presented above caution should be applied in the interpretations of the results using α-amanitin, as its action is to block *in vivo* both DNA-like and ribosomal RNA which makes α-amanitin, in end effect, a rather unspecific inhibitor of RNA synthesis.

Acknowledgements

We thank Mrs. Ch. Pfeiffer, Miss G. Froehlich and Mrs. F. Seifart for excellent technical assistance. The kind donation of α-amanitin and [^{14}C]methyl-amanitin by Prof. Wieland, Heidelberg is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft.

References

[1] F. Stirpe and L. Fiume, Biochem. J. 105 (1967) 779.
[2] K.H. Seifart and C.E. Sekeris, Z. Naturforsch. 24b (1969) 1538.
[3] C. Fiume and Th. Wieland, FEBS Letters 8 (1970) 1.
[4] C. Kedinger, M. Gniadowski, J.L. Mandel. F. Gissinger and P. Chambon, Biochem. Biophys. Res. Commun. 38 (1970) 165.
[5] S.T. Jacob, E.M. Sajdel and H.N. Munro, Biochem. Biophys. Res. Commun. 38 (1970) 765.
[6] S.T. Jacob, W. Muecke, E.M. Sajdel and H.N. Munro, Biochem. Biophys. Res. Commun. 40 (1970) 334.
[7] J. Niessing, B. Schnieders, W. Kunz, K.H. Seifart and C.E. Sekeris, Z. Naturforsch. 25b (1970) 1119.
[8] M. Beato, J. Homoki and C.E. Sekeris, Exptl. Cell Res. 55 (1969) 55.
[9] H. Busch, in: Methods of Enzymology XII, eds. L. Grossman and K. Moldave (Academic Press, New York and London, 1967) p. 448.
[10] K.H. Seifart, B. Benecke and P. Juhasz, Arch. Biochem. Biophys, in press.
[11] N. Lang and C.E. Sekeris, Z. Physiol. Chem. 339 (1964) 238.
[12] K. Burton, Biochem. J. 62 (1956) 315.
[13] V. Marinozzi and C. Fiume, Exptl. Cell Res. 67 (1971) 311.
[14] P. Petrov and C.E. Sekeris, Exptl. Cell Res. 69 (1971) 393.
[15] C.E. Sekeris, J. Niessing and K.H. Seifart, FEB Letters 9 (1970) 103.
[16] K. Higashi, T. Matsuhisa, A. Kitao and Y. Sakamoto, Biochem. Biophys. Acta 166 (1968) 388.
[17] M. Willems, M. Penman and S. Penman, J. Cell Biol. 41 (1969) 177.
[18] A.J. Rizzo and T.E. Webb, European J. Biochem. 27 (1972) 136.
[19] E.E. Baulieu, Nature 236 (1972) 236.
[20] C.E. Sekeris and W. Schmid, Proc. IV. Intern. Congr. Endocr. (1972).
[21] W. Schmid and C.E. Sekeris, FEBS Letters 26 (1972) 109.