MECHANISM OF THE IN VITRO INHIBITION OF TRANSCRIPTION BY PATULIN, A MYCOTOXIN FROM *BYSSOCHLAMYS NIVEA*

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1. Introduction

Patulin, a mycotoxin synthesized by *Byssochlamys nivea* and several strains of *Penicillium* and *Aspergillus,* induces pathological lesions in various species [1]. It inhibits growth of bacterial and eucaryotic cells and its toxicity for animals is clearly established: the LD_{50} is 4.6 mg/kg for adult male Wistar rats and 8.2 mg for adult male Swiss mice, by intraperitoneal injection [2].

The mechanism of the toxic effect of patulin is still unknown. However, alterations of some metabolic pathways have been demonstrated, and it is considered that these biochemical lesions may account for the short-term action of the mycotoxin. The present results report the inhibition of the transcription process by patulin and attempt to elucidate the molecular mechanism of this effect.

2. Materials and methods

Patulin was isolated from cultures of *Byssochlamys nivea* according to a procedure previously described [3]; the purity of the mycotoxin was checked by thin-layer chromatography.

Liver nuclei were isolated from male Wistar rats which were fasted 15 h before decapitation. Livers were homogenized in 9 vol. of 2.2 M sucrose (d = 1.28) containing 1 mM Mg^{2+}. The homogenate was centrifuged for 80 min at 19 000 rev./min (Beckman centrifuge J 21, rotor JA 20) according to Chauveau et al. [4]. The pellets of nuclei were usually kept at −30°C without enzymatic inactivation before being tested.

Nucleoli were isolated by the procedure described by Muramatsu et al. [5]. The preparations were checked for purity by electron microscopy and the nucleoli were kept at −30°C.

Determination of the RNA polymerase activity of isolated nuclei was carried out as follows: pellets of nuclei were resuspended in 10 mM Tris–HCl, pH 7.8 (1.6 ml/pellet of nuclei isolated from 5 g of liver). The standard system contained: 40 μmol Tris–HCl buffer, pH 7.9, 2 μmol $MgCl_2$, 0.25 μmol each of three unlabelled nucleoside-5′-triphosphates and 0.25 μmol of a ^{14}C-labelled nucleoside-5′-triphosphate corresponding to 0.1 μCi (NEN Chemicals GmbH, Dreieichenhain, FRG). The reaction was started by the addition of 0.2 ml of a suspension of nuclei (30–40 μg DNA.P). The final volume was 0.25 ml unless otherwise stated. Ammonium sulphate was added as indicated in the tables. The incubations were run in duplicate for 10 min at 37°C, and the samples were processed according to the procedure previously described [6]. The radioactivity was counted in a liquid scintillation spectrometer (Intertechnique, Paris, France).

The standard assay for the determination of the bacterial RNA polymerase activity contained in 0.25 ml: 10 μmol Tris–HCl buffer, pH 7.9, 0.25 μmol $MnCl_2$, 1.25 μmol $MgCl_2$, 3 μmol β-mercaptoethanol, 80 μg calf thymus DNA (unless otherwise stated),

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0.025 μmol each of three unlabelled nucleoside-5′-triphosphates (ATP, GTP, CTP), 0.025 μmol [2-^{14}C] UTP corresponding to 0.09 μCi, 5.8 units (unless otherwise stated) of bacterial RNA polymerase purified from *E. coli* K12 (Miles Laboratories Inc., Elkhard, Indiana, USA). The incubations were run for 10 min at 37°C and the samples processed as previously described [6].

3. Results and discussion

Addition of patulin to a cell-free system functioning with isolated liver nuclei inhibited synthesis of RNA at low ionic strength (table 1) i.e., synthesis of ribosomal-type RNA promoted by enzyme A (see the review by Chambon [7]). The dose–response curve showed that 50 μg of patulin (200 μg/ml incubation medium) produced about a 50% inhibition as compared with the control value (fig.1).

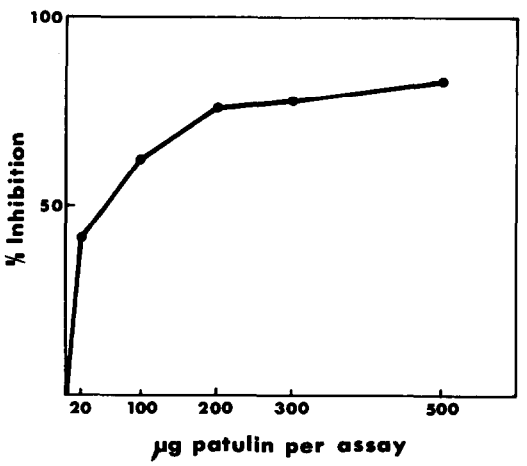

Fig.1. Dose–response curve of the inhibition induced by patulin on in vitro RNA synthesis carried out by isolated rat nuclei at low ionic strength.

When the assays were carried out at high ionic strength (conditions allowing the latent enzyme B activity to be revealed) the extent of inhibition was much smaller (table 1). These results indicated that either enzyme B activity was resistant to the action of patulin or the toxin was ineffective at high ionic strength. In fact, the following experiments demonstrated the sensitivity of enzyme B activity to the mycotoxin and thereby explained the overall decrease of the transcription inhibition in the presence of salt.

By using α-amanitin, a mycotoxin which specifically inhibited enzymes B (and C) but not enzyme A (see Chambon [7]) it was possible to calculate the activity of enzyme B (table 2). The results show a significant inhibition (25%) of enzyme B activity by patulin. In addition, they clearly demonstrate a net decrease in the inhibition induced by patulin when salt was added to the incubation mixture. Thus, in vitro ribosomal RNA synthesis was inhibited to an extent of 68% in the absence of $(NH_4)_2SO_4$ whereas the inhibition was lowered to 14% at high ionic strength (table 2).

Experiments carried out with isolated nucleoli confirmed these findings. Isolated liver nucleoli synthesized only one type of RNA, i.e., ribosomal RNA whatever the ionic strength of the medium and the nature of the divalent cation used [8]. A pro-

Table 1
Effect of patulin on the in vitro transcription carried out by isolated liver nuclei

Incubation medium	Patulin (80 μg/ml)	CMP incorporated (pmoles)	% Inhibition
Low ionic strength	–	1438	
	+	717	50
High ionic strength	–	2796	
	+	2305	18

The isolation of liver nuclei and the composition of the assay are described in Materials and methods. The reaction was started by the addition of 0.2 ml of a suspension of nuclei (30–40 μg DNA.P). Patulin was dissolved in distilled water. The high ionic strength corresponded to a final concentration of 280 mM $(NH_4)_2SO_4$.

Table 2
Inhibition of RNA polymerase B activity by patulin

Experimental conditions	Patulin (200 μg/ml)	UMP incorporated (pmoles)		
		α-Amanitin		Enzyme B (activity calculated)
		No	0.2 μg/ml	
Low ionic strength	–	529	480	
	+	166	153	
% Inhibition		69	68	
High ionic strength	–	1854	367	1487
	+	1425	316	1109
% Inhibition		23	14	25

The experimental conditions are described in Materials and methods. High ionic strength corresponded to a final concentration of 280 mM $(NH_4)_2SO_4$. The activity of enzyme B corresponded to the α-amanitin-sensitive activity at high ionic strength.

gressive increase in the concentration of $(NH_4)_2SO_4$ in the assay determined a progressive decrease in the percent of patulin-induced inhibition on in vitro nucleolar transcription (table 3). The shift down was particularly noticeable up to 70 mM $(NH_4)_2SO_4$. This effect was not specific to ammonium sulphate but was shared by various salts. Figure 2 shows that the

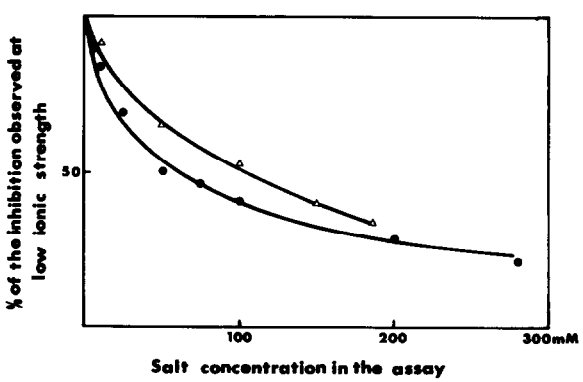

Fig.2. Effect of the addition of salt on the patulin-induced inhibition of in vitro transcription carried out by isolated nuclei. The inhibition observed in the absence of salt equaled 100%. The patulin concentration was 200 μg/ml. (△) Addition of Na_2SO_4, (●) addition of $(NH_4)_2SO_4$.

Table 3
Influence of ionic strength on the extent of patulin-induced inhibition of in vitro transcription by isolated nucleoli

Patulin (200 μg/ml)	UMP incorporated (pmoles)			
	Low ionic strength	Concentration in $(NH_4)_2SO_4$		
		70 mM	140 mM	280 mM
–	408	207	260	396
+	134	164	222	330
% Inhibition	67	21	15	17

The experimental conditions are described in Materials and methods. The nucleoli isolated from 24 g of liver were resuspended in 3.5 ml of 10 mM Tris–HCl buffer, pH 7.8.

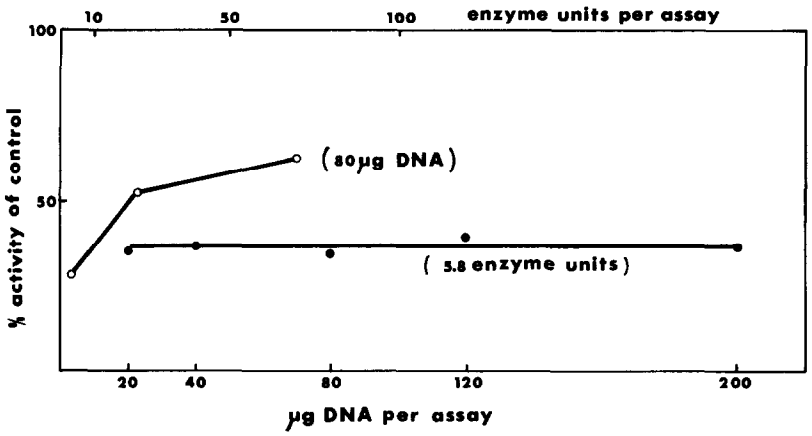

Fig.3. Effect of increasing amounts of either DNA or *E. coli* RNA polymerase on the inhibition of in vitro transcription by patulin. (○) Each assay of this series contained 80 μg DNA and an amount of *E. coli* RNA polymerase as indicated on the graph (2.9–70.2 units). (●) Each assay of that series contained 5.8 units of RNA polymerase and an amount of DNA as indicated on the graph (20–200 μg). Both series contained 150 μg patulin/assay.

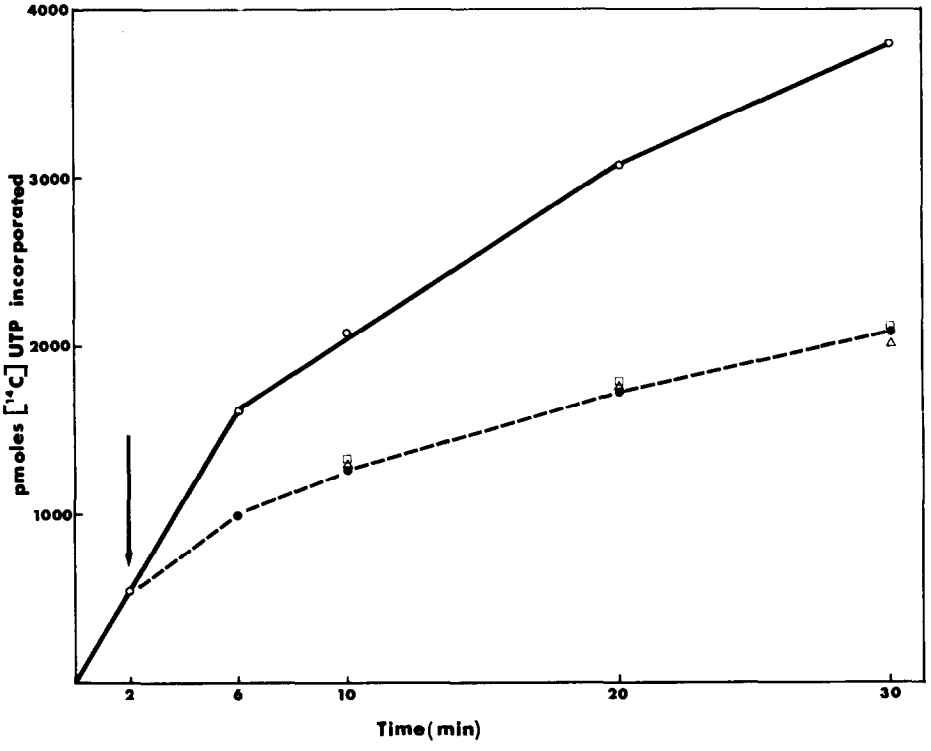

Fig.4. Effect of the addition of patulin on the time course incorporation of [^{14}C]UTP in a bacterial RNA polymerase system. At 2 min (arrow) i.e., after initiation of the reaction, the different toxins were added and the assays further incubated as indicated on the graph. (○——○) Control, (●——●) 300 μg of patulin/assay, (△) 300 μg of patulin and 5 μg of rifampicin/assay, (□) 5 μg of rifampicin/assay.

addition of Na_2SO_4 produced effects similar to those promoted by $(NH_4)_2SO_4$; other salts (NaCl, KCl, NH_4Cl, $NaCH_3COO$, NH_4CH_3COO) gave identical curves (results not shown). Thus, an increasing ionic strength severely impaired the overall capacity of patulin to inhibit the transcriptional process. It must be pointed out that similar results were observed with other mycotoxins such as PR toxin and PL toxin ([9] and results to be published).

Further experiments were performed in order to determine the molecular mechanism of patulin action, using a system functioning with *E. coli* RNA polymerase. Figure 3 shows that the extent of inhibition was not affected by adding increasing amounts of DNA template to the assay regardless of whether DNA was a limiting (20 μg/assay) or a non-limiting ($\geqslant$ 40 μg/assay) factor for the reaction. By contrast, addition of higher concentrations of RNA polymerase (2.9–70.2 units/assay) progressively relieved the inhibitory effects on RNA synthesis. These data suggest that patulin impairs in vitro transcription by alteration of the bacterial enzyme itself. It might be hypothesized that salt added in the reaction might act by preventing the toxic action on rat liver RNA polymerases.

The results indicate that inhibition concerns primarily the initiation step of the transcriptional process since addition of patulin 2 min after the start of the reaction did not block the further elongation of chains initiated before action of the toxin (fig.4). These data have been confirmed by the following experiments: simultaneous addition of rifampicin, a drug which selectively blocks RNA chain initiation by binding to the enzyme (see the review by Chamberlin [10]) and of patulin 2 min after the reaction had started did not modify the results observed with either patulin or rifampicin alone. These results indicate that the two drugs act on the same step of the reaction and that patulin can completely block the initiation process. It would be of interest to determine whether these data may be extended to patulin-induced inhibition of transcription in mammalian systems. This problem could be solved by using purified liver or thymus RNA polymerases.

Acknowledgements

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References

[1] Ciegler, A., Detroy, R. W. and Lillehoj, E. B. (1971) in: Microbial toxins (Ciegler, A., Kadis, S. and Ajl, S. J. eds) Vol. 6, pp. 409–434, Academic Press, New York.
[2] Escoula, L., Moré, J. and Baradat, C. (1976) Ann. Rech. Veter. in press.
[3] Escoula, L. (1974) Ann. Rech. Veter. 5, 423–432.
[4] Chauveau, J., Moulé, Y. and Rouiller, C. (1956) Exp. Cell Res. 11, 317–321.
[5] Muramatsu, M., Hodnett, J. L., Steele, W. J. and Busch, H. (1966) Biochim. Biophys. Acta 123, 116–125.
[6] Moulé, Y. (1970) Eur. J. Biochem. 17, 544–551.
[7] Chambon, P. (1974) in: The enzymes (Boyer, P. D. ed) Vol. 10, pp. 261–331, Academic Press, New York.
[8] Laval, M., Bouteille, M. and Moulé, Y. (1976) Exp. Cell Res. 102, 365–375.
[9] Moulé, Y., Jemmali, M. and Rousseau, N. (1976) Chem. –Biol. Interactions 14, 207–216.
[10] Chamberlin, M. J. (1974) in: The enzymes (Boyer, P. D. ed) Vol. 10, pp. 333–374, Academic Press, New York.