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4. The cells of 18-day-old embryos were seeded in plastic petri dishes containing cover slips (R. Consigli, H. Minocha, H. Abo-Ahmed, *J. Bact.*, in press). Unattached and dead cells were removed after eight hours. Cells labeled for 24 hours were therefore labeled in the period from 8 to 32 hours after seeding.
5. The specific activity of the H^3-thymidine was 5.8 c/mmole. Cells incorporated between 10 to 15 percent of the radioactivity in the medium during 24 hours of growth, after which time fresh, radioactive medium was added. Cells were seeded at different concentrations corresponding to the amount of growth desired. One generation of growth was calculated to be 24 hours.
6. See K. G. Lark and R. Bird (*1*). The radioautographs were stained with Giemsa. A background of between 1 to 2 grains per nucleus was *not* subtracted in the experiments described in Figs. 1, 2, and 3. Correction was made in Fig. 4 (*13*).
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9. B. M. Richard, P. M. B. Walker, E. M. Deeley, *Ann. N.Y. Acad. Sci.* **63**, 831 (1956); with H^3-thymidine labeling, we have substantiated the results of Richard *et al.* on our culture of mouse fibroblasts. Thus, we found that a pulse labels about 20 percent of the cells in a subcultured mouse fibroblast culture.
10. At different times after seeding, the fraction of cells incorporating thymidine was: 10 hours, 8 percent; 14 to 18 hours, 60 percent; and 24 hours, 30 percent.
11. Petri dishes (100 mm) were seeded with 2.5×10^6 cells. After 72 hours of incubation with H^3-thymidine, the cells were washed three times with phosphate-buffered saline (PBS) and digested with 0.1 percent trypsin in PBS for 10 minutes. Trypsin was removed by centrifugation and the cells were used as inocula to seed the cultures described in Figs. 3*B*, *C*, *D*, and *E*.
12. The G-3 strain of Chinese hamster cells isolated from strain B14FAF28 was obtained from Dr. J. H. Taylor, who in turn received it from Dr. T. C. Hsu. It is primarily diploid [T. C. Hsu and C. E. Somers, *Proc. Nat. Acad. Sci. U.S.* **47**, 396 (1961)]. Cells were grown in Ham's medium [R. G. Ham, *Exp. Cell Res.* **29**, 515 (1963)]. In one generation (20 hours) 10^5 cells removed less than 10 percent of the thymidine from the medium. Radioactive medium was added to start the experiment 20 hours after cells were seeded. An inoculum of 6×10^5 cells per petri dish (100 mm) was used for all of the samples, regardless of the duration of growth.
13. Fig. 4*C* was partially corrected for background grains in Fig. 4*D*. The background was 1 grain per nucleus. The observed number of cells with no grains is the intersection of two probabilities—that of obtaining no grains due to background, 37 percent, and that of obtaining no grains produced by radioactive decay (which is thus the observed fraction of cells with no grains, divided by 0.37). From this we calculated the frequency of cells which had received one, two, three (and so forth) grains from background alone and subtracted these from the original distribution. No correction for background was made in the case of cells with one or more grains due to radioactive decay since the error is compounded by previous calculations.
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Rapid and Marked Inhibition of Rat-Liver RNA Polymerase by Aflatoxin B_1

Abstract. *From 15 minutes to 2 hours after the administration of aflatoxin B_1 in vivo there is a 35- to 70-percent inhibition of DNA-directed RNA synthesis. The inhibition was reversed 12 and 24 hours later.*

Aflatoxin B_1 is a metabolite produced by certain strains of *Aspergillus flavus*, and its activity as a potent carcinogen for rat liver has been reviewed (*1*). Aflatoxin B_1 markedly inhibits hydrocortisone-induced synthesis of tryptophan pyrrolase (*2*). Other studies have shown that aflatoxin B_1 inhibits the incorporation in vivo of cytidine into rat-liver nuclear RNA and lowers the RNA content of the nucleus (*3*). In studying the mechanism of inhibition of enzyme induction and cytidine incorporation by aflatoxin B_1, we have found that soon after aflatoxin B_1 is administered there is a marked inhibition of DNA-dependent RNA polymerase in rat-liver nuclei. The inhibition was observed when 1 mg of aflatoxin per kilogram of body weight was administered, the same amount that prevents enzyme induction by hydrocortisone (*2*); but this dose is considerably lower than the LD_{50} (lethal dose, 50 percent effective) for the rat or the dose used to inhibit cytidine incorporation into RNA (*3*).

The activity of RNA polymerase was determined in nuclei isolated from livers of male Sprague-Dawley rats (200 g) that were fasted 20 to 24 hours before the experiment. Two animals per group were injected intraperitoneally with either 0.1 ml of triethylene glycol or 0.1 ml of triethylene glycol containing 0.2 mg of aflatoxin B_1 (extinction coefficient, E_{360}, is equal to 2.11×10^4 in methanol). Animals were killed by exsanguination 2 hours after aflatoxin treatment, the livers were rapidly removed and chilled, and nuclei were isolated from 8 g of liver by the method of Barondes, *et al.* (*4*), modified to include 0.002*M* $MgCl_2$ in all solutions. The nuclei were centrifuged (International T-60 ultracentrifuge) in 2.1*M* sucrose at 23,500 rev/min for 0.5 hour at 4°C. Nuclei were washed and brought to 2.0-ml volume with homogenizing sucrose. Preparations were assayed in duplicate at each interval, with 0.32*M* $(NH_4)_2SO_4$, 0.64*M* $(NH_4)_2SO_4$, and without any $(NH_4)_2SO_4$. The standard assay mixture for nuclear RNA polymerase was as follows: 100 μmole of tris, *p*H 8.0; 10 μmole of cysteine; 3 μmole of $MgCl_2$; 0.5 μmole each of adenosine, uridine, and guanosine triphosphates, respectively (ATP, UTP, GTP); 0.04 μmole of tritiated cytidine triphosphate (CTP-H^3) (2×10^7 count/min per micro-mole); 160 μmole of $(NH_4)_2SO_4$ and nuclei in a final volume of 0.5 ml. Each assay tube was incubated for 10 minutes at 37°C. Carrier RNA (2.0 mg) from yeast in 0.2*M* EDTA (ethylenediaminetetraacetate) *p*H 7.4 was added, and the reaction was immediately stopped with 2.5 ml of 10 percent perchloric acid which contained 40 μmole of sodium pyrophosphate and 10 μmole of EDTA. The mixture was allowed to stand 10 minutes and was then centrifuged at 2000 rev/min for 10 minutes. The pellet was successively washed: once with a mixture of 5 percent perchloric acid and 0.1*M* sodium pyrophosphate (4:1), twice with 5 percent perchloric acid, once with ethanol, and once with a mixture of ethanol, chloroform, and ether (2 : 2 : 1). The pellet was dried overnight. The precipitate was hydrolyzed with 1.0 ml of 5 percent perchloric acid at 90°C for 15 minutes; the hydrolyzate was cooled and centrifuged.

Table 1. RNA polymerase from liver of rats treated for 2 hours with aflatoxin B_1.

Additions to complete system		Deletions	Incorporation of CTP-H^3 (mμ mole/mg of DNA)	
Substance	Amt (μg)		Control	Treated
None		None	4.52	2.36
None		$(NH_4)_2SO_4$	2.00	.68
None		ATP,UTP,GTP	.24	.16
Deoxyribonuclease*	200	None	.24	.16
Ribonuclease	200	None	.36	.32

* Deoxyribonuclease was incubated with nuclei for 10 minutes at 37°C prior to the addition of CTP-H^3.

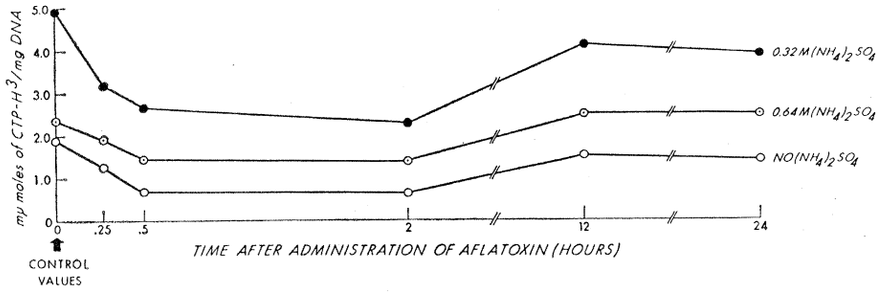

Fig. 1. Rat-liver RNA polymerase after aflatoxin B_1 treatment. The complete assay system is the same as for Table 1. The animals were injected with aflatoxin B_1 (1 mg/kg) at 0.25, 0.5, 2, 12, or 24 hours before they were killed. The control animals received 0.1 ml of triethylene glycol at 24 hours. All animals were fasted for 24 hours before the experiment was begun.

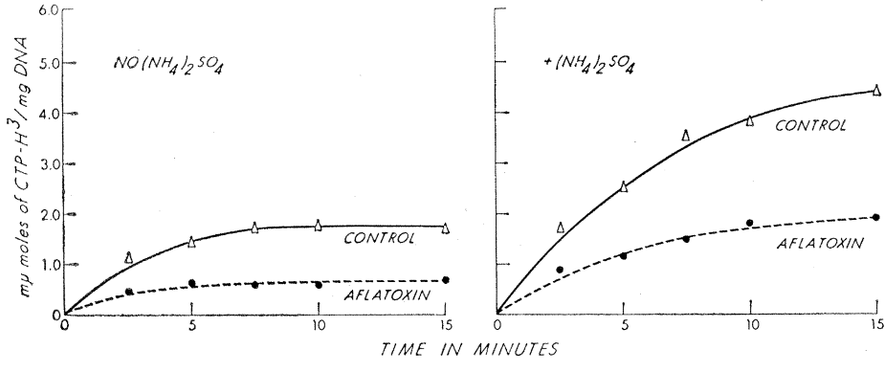

Fig. 2. The effect of aflatoxin on the kinetics of RNA polymerase activity. The complete assay system is the same as for Table 1. The animals were treated with aflatoxin B_1 (1 mg/kg) 2 hours before they were killed. All animals were fasted for 24 hours before the experiment was begun.

Portions (200 μl) of the supernatant were added to Bray's solution (*5*) and rapidly neutralized; and the radioactivity was determined in a Packard Tri-Carb liquid-scintillation counter. Another portion was used for DNA determination (*6*).

Incorporation of CTP into acid-insoluble material is greatest in the presence of ammonium sulfate, and in its absence less than half the maximum activity is observed (Table 1). In addition, CTP incorporation requires all four nucleotide triphosphates and is largely abolished in the presence of deoxyribonuclease or ribonuclease. The CTP incorporation in nuclei of aflatoxin-treated rats was less than that of the control preparations, showed the same requirements for optimum activity, and was also inhibited by the presence of deoxyribonuclease and ribonuclease.

We found that CTP incorporation was optimum in the presence of 0.32*M* ammonium sulfate (Fig. 1). In the presence of 0.64*M* ammonium sulfate, CTP incorporation was somewhat inhibited. As soon as 15 minutes after intraperitoneal administration of aflatoxin, the nuclei from the treated rats showed less than 40 percent of the activity shown by the nuclei from the untreated rats, when assayed in the absence of ammonium sulfate. Maximum inhibition of 70 percent was observed at 30 minutes and persisted for 2 hours. At 12 and 24 hours after aflatoxin treatment a 10-percent inhibition was observed. The maximum inhibition after each period of aflatoxin treatment was observed in the absence of ammonium sulfate. In the presence of 0.32*M* ammonium sulfate (the optimum salt concentration for CTP incorporation) inhibition by aflatoxin was also observed but was somewhat less than that observed in the absence of ammonium sulfate. In the presence of 0.64*M* ammonium sulfate, a concentration which gave less than maximum CTP incorporation, inhibition of the RNA polymerase was observed at 15 and 30 minutes and 2 hours, but no inhibition was observed 12 and 24 hours later.

In the absence of ammonium sulfate, incorporation proceeded for about 5 minutes before stopping; while in its presence CTP incorporation continued for 10 minutes and at a greater initial rate (Fig. 2). The nuclei from aflatoxin-treated rats showed 50 percent less CTP-incorporating activity than that of the control preparation in both the presence and absence of ammonium sulfate. The function of ammonium sulfate in stimulating RNA synthesis is not clear, but several explanations are possible. Goldberg (*7*) suggested that high ionic strength possibly affects the physical state of the polymerase–nucleic acid complex or has an inhibitory effect on degradative enzymes. It is also possible that the salt causes a derepression of RNA synthesis by dissociating a repressor from the DNA template (*8*). Our data showing an aflatoxin B_1 inhibition of RNA synthesis in the presence of an optimum concentration of ammonium sulfate suggests that aflatoxin is not operating by causing an increased repression but may be interacting with DNA in a manner which interferes with polymerase activity. Another possibility is that this drug inhibits the enzyme itself.

Thus, aflatoxin B_1 interferes with gene transcription soon after its administration. The inhibition of DNA-dependent RNA synthesis is to be expected in that aflatoxin B_1 is reported to lower the ratio of RNA to DNA of the nucleus and inhibits the incorporation of cytidine into RNA in vivo (*3*). The inhibition may be related to the binding of aflatoxin B_1 to DNA in vitro (*3*). The relationship, if any, between the carcinogenicity of aflatoxin B_1 and its interference with gene transcription is not yet clear.

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