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The Effect of the Aflatoxins B_1, G_1 and G_2 on Protein and Nucleic Acid Synthesis in Rat Liver

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A comparison has been made of the difference spectra obtained by causing various aflatoxins (B_1, G_1 and G_2) to interact with calf-thymus DNA. The effect of these toxins on RNA and protein synthesis by rat-liver slices has been measured. The extent of their inhibitory action on the synthetic reactions was proportional to the degree of spectral shift obtained with their interaction with DNA. It is proposed that their toxicity depends on this interaction. It was demonstrated that the RNA polymerase of nucleoli isolated from the livers of aflatoxin B_1-poisoned rats was inhibited. This finding is in agreement with the proposed mechanism for the hepatotoxic action of aflatoxin.

Aflatoxin is the metabolite of certain strains of *Aspergillus flavus* that grows on ground nuts. Clifford & Rees (1966, 1967) proposed that the biochemical changes underlying the development of liver necrosis in the rat after the administration of aflatoxin B_1 were initiated by the toxin interacting with the DNA. This interaction prevented the RNA polymerase transcribing the DNA and inhibited the formation of messenger RNA. A failure in messenger-RNA formation resulted in an inhibition in protein synthesis which they considered to be the cause of the liver-cell necrosis. Sporn, Dingman, Phelps & Wogan (1966) also suggested that the rapid and drastic alteration of nuclear RNA metabolism by aflatoxin B_1 might indicate that its ability to bind to DNA might be a crucial aspect of its toxic and carcinogenic properties.

There are a number of other toxic aflatoxins of very similar chemical composition which are produced by certain strains of the mould *Aspergillus flavus*. These aflatoxins have a wide range in toxicity as determined by their LD_{50} for 1-day-old ducklings (Chang, Abdel-Kadar, Wick & Wogan, 1963; Nesbitt, O'Kelly, Sargeant & Sheridan, 1962). It may well be that such variations in toxicity within a given species are a reflection of the ability of the toxins to interact with DNA.

In the present investigation the interaction of aflatoxin G_1 and G_2 with DNA is compared with that of aflatoxin B_1. Their effect on RNA and protein syntheses by rat-liver slices has been compared and the effect of aflatoxin B_1 on the RNA-polymerase activity of liver-cell nucleoli has been measured.

MATERIALS AND METHODS

Animals. Male Wistar albino rats fed on the M.R.C. 41B meal (Parkes, 1946), bred from the same colony, were used throughout this investigation.

Dosage. Aflatoxin B_1 was given at the dose 7 mg./kg. body wt. The appropriate quantity of the toxin was dissolved in 0·2 ml. of dimethylformamide and administered by gastric intubation.

Materials. ATP, CTP, UTP, GTP and calf-thymus DNA were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Phosphoenolpyruvate and pyruvate kinase were supplied by C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

A crude mixture of aflatoxins was a gift from the Medical Research Council. Aflatoxin B_1, G_1 and G_2 were separated by using thin-layer chromatoplates of silica gel with sulphate binder 1·0 mm. thick. The solvent used was acetic acid–ether–chloroform (1:2:2, by vol.). The bands were identified in u.v. light, removed from the plates and extracted with chloroform. The solvent was removed under reduced pressure in a Craig rotary evaporator.

Radioactive materials (supplied by The Radiochemical Centre, Amersham, Bucks.) were [6-^{14}C]orotic acid (sp. activity 30·0 mc./m-mole) and DL-[1-^{14}C]leucine (sp. activity 36·6 mc/m-mole). [^{3}H]UTP (sp. activity 3·6 c/m-mole) was obtained from Schwartz BioResearch Inc., Orangeberg, N.Y., U.S.A.

Tissue separations. Nuclei were prepared by the method of Rees & Rowland (1961). Erythrocytes were, however, removed from the preparation by freezing the liver to $-80°$ instead of perfusing the liver *in situ*.

Nucleoli were prepared from isolated nuclei by the method of Rees, Rowland & Varcoe (1963) and were washed free of other nuclear components with 0·25 M-sucrose instead of glass-distilled water as it was found that the nuclei tended to clump when water was used. All preparations of nuclei and nucleoli were always examined for purity by phase-contrast

microscopy and found to be free of any contamination. Occasional preparations were examined by electron microscopy and again no contamination of the preparations was observed.

Radioactive experiments. 1. The method used to estimate RNA polymerase was based on that of Villalobos, Steele & Busch (1964). The nucleoli isolated from a single rat were suspended in 1·0 ml. of 0·25 M-sucrose and incubated at 37° for 30 min. with 1·0 ml. of a medium containing 10 μmoles of phosphoenolpyruvate, 100 μg. of pyruvate kinase, 50 μmoles of tris–HCl buffer, pH 8·0, $MgCl_2$ (5 μmoles), ATP (2 μmoles), CTP (0·25 μmole), [^{3}H]UTP (0·14 mμmole; 0·5 μC). After incubation the reaction was stopped by the addition of 0·1 ml. of a solution of 1 mg. of unlabelled UTP/ml. and then 2 ml. of 20% (w/v) trichloroacetic acid. The tubes were centrifuged and the residue was washed once with 10% (w/v) trichloroacetic acid and twice with 5% (w/v) trichloroacetic acid. The lipids were extracted with acetone followed by two extractions with chloroform–ethanol (2:1, v/v) and once more with acetone. The RNA was then extracted by the Schmidt & Thannhauser (1945) procedure, the precipitate being incubated overnight with 2 ml. of 1·0 N-NaOH at 37°. The semi-digested protein and undigested DNA were precipitated with 1 ml. of 5% (w/v) trichloroacetic acid and 1 ml. of conc. H_2SO_4. After centrifugation the ribonucleotide content of the supernatant was determined by the $FeCl_3$ and orcinol method of Mejbaum (1939) as modified by Slater (1956). A standard curve was prepared with a purified sample of RNA (8% phosphorus content obtained from the Sigma Chemical Co.). The radioactivity of the supernatant was determined by using a Packard model 526 liquid-scintillation spectrometer. A portion (0·5 ml.) of the supernatant was added to a phial, followed by 19·5 ml. of a mixture of 4 g. of 2,5-bis-(5-tert.-butylbenzoxazol-2-yl)-thiophen (BBOT, from Ciba Ltd., Duxford, Cambs.), 80 g. of naphthalene and 36 g. of Aerosil Standard Silica in 400 ml. of methylCellosolve and 600 ml. of toluene. The mixture was shaken vigorously and then slowly to remove air bubbles. The samples were counted for 20 min. by using 50% gain and a 50–1000 window. Quenched samples were prepared by using standardized [^{3}H]hexadecane, up to 0·5 ml. of chloroform, 0·5 ml. of water and 19·5 ml. of scintillation mixture. From the graph of the percentage efficiency and external standard counts (5% gain, 1000–00 window) the disintegrations per minute of the samples were calculated and from this the specific activity of the RNA was determined.

2. The incorporation of [^{14}C]leucine into proteins *in vitro* and [^{14}C]orotic acid into the RNA of liver slices *in vitro* was carried out as described by Clifford & Rees (1967). The preparation of the dried residues for the measurement of the protein and nucleic acid was as described in that paper.

Spectral determinations. The difference spectra were studied with a Cary model 14 (serial no. 273) recording spectrophotometer with light-path 1 cm. Solutions of the aflatoxins B_1, G_1 and G_2, and of calf-thymus DNA, were prepared in 0·01 M-tris–HCl, pH 7·4, containing 0·01 M-NaCl.

RESULTS

In previous investigations (Clifford & Rees, 1966; 1967; Sporn *et al.* 1966) it was found that the addition of DNA to aflatoxin B_1 resulted in the production of a difference spectrum. This was considered to indicate that the aflatoxin B_1 was interacting with the DNA. Since all the aflatoxins studied in the present investigation are closely related in structure it was expected that they would interact with DNA in a similar manner to aflatoxin B_1. In Fig. 1 are shown the difference spectra produced by mixing a given concentration of DNA with equimolecular portions of B_1, G_1 and G_2. It may be seen that the changes in the spectra of G_1 and G_2 are considerably smaller than those with B_1.

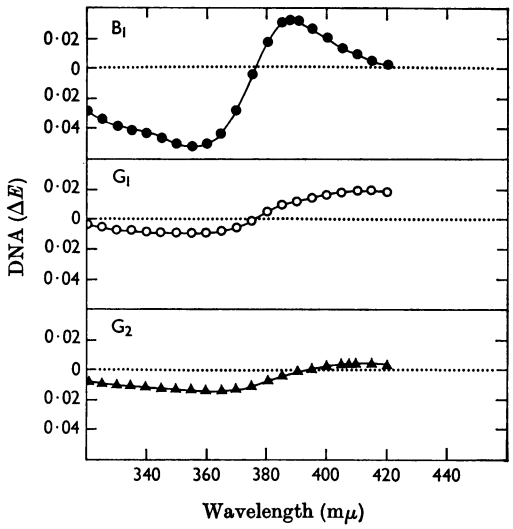

Fig. 1. Difference spectra of aflatoxins B_1, G_1 and G_2 with calf-thymus DNA. Concentration of each toxin was 64·0 μM and of the DNA 1·99 mM. The experimental conditions are described in the Materials and Methods section.

The addition of aflatoxin B_1 (0·03 M final concentration) to rat-liver slices *in vitro* had been found to produce an immediate inhibition in the incorporation of [^{14}C]orotic acid into the RNA of the slice (Clifford & Rees, 1967). The choice of this concentration was based on the assumption that the whole of the LD_{50} of the toxin (7 mg./kg. body wt.) was concentrated in the liver of the poisoned rat. It was of interest to determine whether there was a correlation between the interaction of the aflatoxins with DNA and their inhibitory action on RNA synthesis.

The effect of a range of concentrations of the various aflatoxins was tested on the incorporation of [^{14}C]orotic acid into the RNA of the liver slices prepared from the livers of control rats. From the results of these experiments, which are given in Fig. 2, it may be seen that B_1 produced a much greater inhibition than G_1 and G_2.

Such an inhibition in RNA synthesis by aflatoxin B_1 in rat-liver slices had preceded an inhibition in the incorporation of amino acids into the proteins of

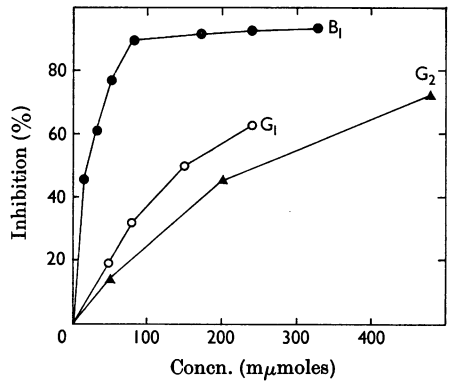

Fig. 2. Inhibitory action of the aflatoxins on the incorporation of [^{14}C]orotic acid into the RNA of rat-liver slices. Experimental conditions were as described by Clifford & Rees (1967). The medium was Krebs–Ringer phosphate, pH 7·4 (Umbreit, Burris & Stauffer, 1959), containing glucose (100 mg./100 ml.); the gas phase was oxygen. Incubation was carried out at 37° in Warburg flasks and the [^{14}C]orotic acid was added from the side arm after 5 min. equilibration. Concentrations of the toxins are given in mμmoles present in the 3 ml. of medium in which the 200 mg. of rat-liver slices was incubated.

Table 2. *Action of aflatoxin B_1 on nucleolar RNA polymerase*

Experimental conditions are described in the Materials and Methods section. Each result is the value from the total nucleoli isolated from 1 rat liver. The amount of aflatoxin B_1 given in Expt. 1 is the quantity added to the 2 ml. incubation medium. In Expt. 2, treated rats were given 14 mg. of aflatoxin (dissolved in dimethylformamide/kg. body wt. and killed 90 min. later; the controls received dimethylformamide alone as described in the Materials and Methods section.

Expt. no.	Aflatoxin B_1	Total liver nucleolar disintegrations/min.	Total nucleolar RNA P (μg. of P)	Sp. activity of nucleolar RNA (disintegrations/min. /μg. of RNA P)
1	Nil	19836	15·6	1267
	Nil	24000	15·5	1540
	30 μg.	21184	16·3	1298
	300 μg.	23266	16·5	1410
	700 μg.	22210	16·1	1383
2	Control	19485	11·5	1696
	Control	18909	9·4	2020
	Treated	3771	8·3	454
	Treated	3501	15·3	230

Table 1. *Effect of various aflatoxins on the incorporation of* DL-[1-^{14}C]*leucine into the proteins of rat-liver slices*

Slices were incubated in Krebs–Ringer phosphate containing 1 μc of DL-[1-^{14}C]leucine for 1 hr. at 37°. The gas phase was oxygen. In each experiment the results are the mean of two slices for each determination and are prepared from the same liver. The aflatoxins were dissolved in chloroform and the appropriate portions were added to each vessel. The chloroform was removed by maintaining the vessel at 37° in the dark. Control vessels had chloroform added and were treated in a similar manner.

Expt. no.	Aflatoxin added	Concn. of toxin (mμmoles in 3 ml.)	Liver protein (counts/min. at infinite thickness)	Inhibition (%)
1	Nil	—	1680	—
	B_1	20	1145	32
	G_1	150	1087	35
	G_2	230	1035	38
2	Nil	—	1574	—
	B_1	40	710	55
	G_1	300	598	62
	G_2	460	324	83

the slice. To determine whether G_1 and G_2 produced a similar inhibitory action, the concentration at which the aflatoxins produced a 50% inhibition in incorporation of orotic acid was tested against incorporation of amino acid by liver slices. In Table 1 are given the results of this experiment and the inhibitory effects of higher concentrations of the aflatoxins. It may be seen that different concentrations of the toxins employed produced an inhibition of the same order in amino acid incorporation into the proteins of the liver slice.

In view of the interaction of the aflatoxins with DNA a possible mechanism whereby RNA synthesis was inhibited would lie in an inhibition of the DNA by the RNA polymerase. The action of aflatoxin B_1 was therefore tested in an RNA-polymerase system *in vitro*. Two types of experiments were undertaken and the results are given in Table 2. Each reaction tube contained all the nucleoli isolated from one rat liver and it may be seen that such a preparation gave an active incorporation of nucleotide into the nucleolar RNA. In the first experiment the addition of several concentrations of B_1 to the nucleolar preparation *in vitro* was without effect. In the second experiment rats were poisoned with twice the LD_{50} of aflatoxin B_1 dissolved in dimethylformamide, by gastric intubation, and were killed 90 min. later. Control rats received dimethylformamide alone. The livers were removed and the nucleoli isolated. In this experiment there was a marked inhibition in the RNA polymerase of the nucleoli prepared from the livers of the poisoned rats.

DISCUSSION

The aflatoxins have a wide variation in their toxicity. Chang *et al.* (1963) found that the LD_{50} of B_2 was ten times that of B_1 to 1-day-old ducklings. Nesbitt *et al.* (1962) and Asao *et al.* (1963) reported that the LD_{50} of G_1 was three times that of B_1 to 1-day-old ducklings. Zuckerman, Rees, Inman & Petts (1967) showed that the necrotizing dose of G_1 was three times that of B_1 in human-liver cells grown on culture. Certain of the aflatoxins are carcinogenic and there is a variation in their carcinogenicity. Dickens & Jones (1963, 1965) produced local sarcomas in rat by injection of the aflatoxins and found that the induction period with B_1 was approximately one-third of the time required by G_1.

Notwithstanding this wide range in toxicity there is a close similarity in chemical structure. The only difference between B_1 and G_1 is that the dihydrofuran ring of the former is replaced by a lactone ring. In B_2 and G_2 one of the dihydrofuran rings of B_1 and G_1 respectively is hydrogenated (Nesbitt *et al.* 1962; Asao *et al.* 1963; Van Dorp *et al.* 1963; Chang *et al.* 1963; Cheung & Sim, 1964).

A difference spectrum indicates solely that molecular interaction has occurred and the magnitude of the changes are not necessarily a measure of the extent of the interaction. In the present investigation there was found to be a correlation between the magnitude of the spectral changes obtained in the DNA–aflatoxin interaction and the inhibition of RNA synthesis in the liver slices. In view of this it may be presumed that the magnitude of the spectral changes do give an indication of the degree of interaction. Coupled with the studies on protein synthesis it is proposed that the toxic action of the aflatoxins is related to their interaction with DNA and that their degree of interaction determines their degree of toxicity. It is not certain to what extent this DNA interaction plays a role in their carcinogenicity but Dickens & Jones (1965) drew attention to the fact that the carcinogenic dose and the LD_{50} of B_1 and G_1 were proportional.

The demonstration of the inhibition of RNA polymerase after aflatoxin B_1 poisoning gives further support to proposals that the inhibition of nuclear RNA synthesis in aflatoxin B_1 poisoning was the result of the toxin interacting with the DNA and thereby preventing a transcription of the DNA. The failure of the addition of aflatoxin B_1 to inhibit the polymerase *in vitro* may be e.g. the result of an alteration in the permeability of the isolated nucleoli to the toxin as opposed to the nucleolus within the intact cell or an alteration in the conformation of the DNA arising from the isolation procedures.

Because of their varying toxicity and carcinogenicity and yet closely related structures it would seem that the aflatoxins present an excellent opportunity for correlating biological activity with molecular structure.

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