The Effect of Aflatoxin B₁ on Normal and Cortisol-Stimulated Rat Liver Ribonucleic Acid Synthesis

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1. Aflatoxin B₁, administered *in vivo*, inhibits the incorporation of [¹⁴C]orotic acid *in vivo* into rat liver nuclei, and also inhibits both Mg²⁺- and Mn²⁺-dependent RNA polymerase activities in nuclei assayed *in vitro*. 2. Aflatoxin B₁ inhibits the cortisol-induced increase in incorporation of [¹⁴C]leucine *in vivo*, but does not affect the control value of this activity. 3. Aflatoxin B₁ administered *in vivo* inhibits the increase in nuclear Mg²⁺-dependent RNA polymerase activity, assayed *in vitro*, which results from the treatment with cortisol. 4. Adrenalectomy causes a decrease in Mg²⁺-dependent RNA polymerase activity. The effect on this enzymic activity of adrenalectomy plus treatment with aflatoxin B₁ is no greater than that of treatment with aflatoxin B₁ alone. 5. These results suggest that the inhibition of cortisol-stimulated biochemical pathways by aflatoxin B₁ is due to an inhibition of cortisol-stimulated RNA synthesis. 6. The cytoplasmic action of aflatoxin is thought to be due to a competition for receptor sites on the endoplasmic reticulum between steroid hormones and aflatoxin B₁. No evidence was obtained for a similar competition for nuclear receptor sites between [³H]cortisol and aflatoxin B₁. 7. No differences were observed between the activities of RNA polymerase preparations solubilized from control or aflatoxin-inhibited nuclei. 8. No differences in 'melting' profiles were observed between DNA and chromatin preparations isolated from control nuclei or from aflatoxin-inhibited nuclei. 9. It is suggested that aflatoxin B₁ exerts its effect on RNA polymerase by decreasing the template capacity of the chromatin and that the aflatoxin 'target' area of the chromatin includes that region which is stimulated by cortisol. This process, however, does not involve inhibiting the movement of cortisol from the outside of the hepatic cell to the nuclear chromatin.

The administration of corticosteroids to rats results in a stimulation of the activities of several liver enzymes, including tryptophan pyrrolase (Feigelson *et al.*, 1962). Unlike the induction of tryptophan pyrrolase by its substrate, the induction by corticosteroids can be inhibited by actinomycin D (Green-gard & Acs, 1962), which selectively inhibits nuclear RNA synthesis. The induction of tryptophan pyrrolase by corticosteroids has therefore been presumed to occur as a result of an effect on RNA synthesis (Feigelson *et al.*, 1962). Like actinomycin D, the potent liver toxin and carcinogen aflatoxin B₁ also inhibits both the induction of tryptophan pyrrolase by corticosteroids (Wogan & Friedman, 1968) and rat liver RNA synthesis (Wogan, 1969; Clifford & Rees, 1967a), and is without effect on the induction of tryptophan pyrrolase by its substrate (Wogan & Friedman, 1968). Actinomycin D is known to exert its inhibitory effect on RNA synthesis by reacting with the DNA template (Reich & Goldberg, 1964) and the apparently close similarities between many of the biochemical effects of aflatoxin B₁ and actinomycin D have reasonably been presumed to be due

to similar mechanisms of action (Bernhard *et al.*, 1965; Sporn *et al.*, 1966). However, certain important differences do exist between the actions of aflatoxin B₁ and actinomycin D on liver, in particular the failure of actinomycin D to produce liver necrosis or hepatomas (Wogan, 1969). This suggests that the mechanism of action of aflatoxin B₁ on liver cells must differ in some respects from that of actinomycin D, which is further indicated by the failure of aflatoxin B₁ to inhibit RNA polymerase activity in isolated nuclei, in contrast to actinomycin D, which is an effective inhibitor of the activity both *in vivo* and *in vitro* (Edwards & Wogan, 1970). It thus appeared that the mechanism of action of aflatoxin B₁ in liver nuclei might be more complex than simple binding to DNA and that other possible mechanisms should be investigated.

Williams & Rabin (1969), who have been examining the 'degranulating' action of aflatoxin B₁ on rough endoplasmic reticulum, have suggested that this cytoplasmic action of aflatoxin B₁, in which polyribosomes are released from the membranes, is due to a competition for polyribosome-binding sites on

the membranes between steroid hormones and aflatoxin B₁. In support of this theory, they have pointed out that certain structural similarities exist between molecules of steroid hormones and aflatoxin B₁. If steroid hormones and aflatoxin B₁ can compete for binding sites on the endoplasmic reticulum it at least appears possible that a similar competition might occur within the cell nucleus and account for the inhibitory action of aflatoxin B₁ on the induction of tryptophan pyrrolase by corticosteroids. Similarly, competition between endogenously circulating steroids and aflatoxin B₁ might account for part at least of the inhibition of hepatic RNA synthesis in normal rats. The present paper deals with some experiments carried out to examine these possibilities.

Materials and Methods

Animals

Male rats of the Wistar-derived Porton strain, weighing 140–160g, were maintained on MRC diet 41B (Bruce & Parkes, 1956). Aflatoxin B₁ was administered intraperitoneally in solution in dimethylsulphoxide ('special for spectroscopy' grade) at a dosage of 3.5mg/kg (unless otherwise stated). This is 50% of the oral LD₅₀ (Butler, 1964). The present studies were, in the main, carried out over short time-periods, and during this period of up to 6h after dosing the observable changes in liver structure are dislocation of ribosomes from the rough endoplasmic reticulum, vesiculation of the rough-endoplasmic-reticulum cisternae and the formation of nucleolar 'caps' (Butler, 1971). At the dosage used, periportal necrosis was evident from 12h after dosing, and occasional deaths were encountered in longer-term studies. Cortisol acetate suspended in 0.88% NaCl was administered intraperitoneally at a dosage of 50mg/kg. Control animals received injections of the appropriate vehicles. Bilateral adrenalectomies were carried out under ether anaesthesia, the skin incisions being closed with suture clips. Animals were given 1% NaCl as drinking water and used 5 days after the operations. Animals were killed by cervical dislocation, exsanguinated and the livers removed and rapidly chilled on ice.

Chemicals

Aflatoxin B₁ was separated from a mixture of aflatoxin B₁, B₂, G₁ and G₂ (supplied by the Microbiological Research Establishment, Porton, Wilts., U.K.) by a modification of the alumina column method of Steyn (1970) designed by Dr. J. B. Greig of this department. Purity was checked by chromatography on cellulose t.l.c. plates prepared in 10% (v/v) formamide in acetone. The plates were developed with benzene saturated with formamide, and afla-

toxins were located by their fluorescence on viewing the plates under an Hanovia u.v. Chromatolite.

[6-¹⁴C]Orotic acid (specific radioactivity 60.8mCi/mmol), [5-³H]UTP (specific radioactivity 1 Ci/mmol), L-[1-¹⁴C]leucine (specific radioactivity 62mCi/mmol), and [1,2-³H]cortisol (specific radioactivity 43Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. ATP, CTP, GTP, UTP, cortisol acetate and calf thymus DNA were obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K.

Chemical analyses

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. DNA was determined by the method of Giles & Myers (1965) with calf thymus DNA as standard. Portions (0.5ml) of homogenates or isolated nuclei were washed three times with ice-cold 5% (w/v) trichloroacetic acid. RNA was determined spectrophotometrically after hydrolysing the acid-insoluble residues with 1M-KOH at 37°C for 16h, acidifying with 0.5M-HClO₄ and clarifying by centrifuging. The extinction of the supernatant was measured at 260nm, the extinction coefficient used being 34.2mg of RNA·ml⁻¹·cm⁻¹ (Scott *et al.*, 1956).

Radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrometer, count rates being converted into d.p.m. by means of internal standardization.

Incorporation of [¹⁴C]orotic acid and [¹⁴C]leucine *in vivo*

Groups of three animals were used. In the [¹⁴C]-orotic acid-incorporation experiments, rats were injected intraperitoneally with 2.5μCi of [¹⁴C]orotic acid in 0.88% NaCl (1ml) 15min before being killed. Livers were removed and chilled on ice. All subsequent procedures were carried out at 0–4°C. Portions (4g) of pooled livers were homogenized in 10ml of 0.25M-sucrose–1mM-MgCl₂ by using a Potter–Elvehjem homogenizer fitted with a Teflon pestle (TRI-R instruments; Camlab, Cambridge, Cambs., U.K.). The homogenate was diluted with 2ml of water and a purified nuclear fraction was isolated essentially by the method of Widnell & Tata (1964). RNA present in residues after washing nuclei three times with ice-cold 5% trichloroacetic acid was hydrolysed in 1M-KOH as described above. Portions (100μl) of the hydrolysate were taken for spectrophotometric measurement of the RNA content. Other samples (200μl) were placed in radioactivity-counting vials and, after careful neutralization, 10ml of dioxan-based scintillator (Bray, 1960) was added. Radioactivity counting efficiency, determined by internal standardization, was 69%.

In the [^{14}C]leucine incorporation studies animals were injected intraperitoneally with L-[^{14}C]leucine ($2\mu\text{Ci}$) in 0.88% NaCl (1 ml) 1 h before being killed. Livers were removed, and from the pooled livers duplicate samples (2 g fresh wt.) were homogenized in 10 ml of ice-cold 0.25 M-sucrose–1 mM- MgCl_2 . Acid-insoluble material was dissolved overnight at 37°C in 1 ml of 1% Hyamine hydroxide in methanol. Dioxan-based scintillator was added to the digested samples and radioactivity was determined as described above. Counting efficiency, determined by using [^{14}C]toluene as an internal standard, was 67%. Protein was determined in samples ($50\mu\text{l}$) of the original homogenates. In the above experiments in which the incorporation of labelled orotic acid and leucine was examined, no inhibitory effect of aflatoxin B_1 on the uptake of label into the acid-soluble fraction of the homogenates was observed.

Tryptophan pyrrolase assay

Tryptophan pyrrolase activity in liver was assayed by the method of Feigelson & Greengard (1961) in unfractionated homogenates. Preliminary experiments were carried out to determine the time required to reach the linear phase of enzymic activity, and subsequent assays were carried out during the linear phase.

RNA polymerase assay

DNA-dependent RNA polymerase activity (nucleoside triphosphate–RNA nucleotidyltransferase, EC 2.7.7.6), both that activated by Mg^{2+} and that activated by Mn^{2+} – $(\text{NH}_4)_2\text{SO}_4$, were assayed in intact nuclei essentially by the method of Widnell & Tata (1966), with [^3H]UTP as the labelled substrate. The nuclei were isolated from liver homogenates by the method described above. Each nuclear preparation was checked for freedom from gross contamination with other subcellular components by phase-contrast microscopy.

RNA polymerase activity was also assayed after solubilization from isolated nuclei by the method of Jacob *et al.* (1968). In these experiments the nuclei were isolated by homogenizing the liver in 2.2 M-sucrose–10 mM-magnesium acetate–0.25 M-spermine (Busch *et al.*, 1967). The incubation medium contained, in a total volume of 0.75 ml: calf thymus DNA, $50\mu\text{g}$; tris–HCl buffer, pH 8.0, $40\mu\text{mol}$; MgCl_2 , $2\mu\text{mol}$; MnCl_2 , $1.5\mu\text{mol}$; NaF, $2\mu\text{mol}$; cysteine, $7\mu\text{mol}$; spermine, $2\mu\text{mol}$; ATP, $0.35\mu\text{mol}$; CTP and GTP, $0.3\mu\text{mol}$; UTP, $0.07\mu\text{mol}$; [^3H]UTP, 0.2nmol ; saturated $(\text{NH}_4)_2\text{SO}_4$ soln., $15\mu\text{l}$; solubilized polymerase enzyme, $200\mu\text{l}$.

Assays were preincubated in the absence of [^3H]UTP and $(\text{NH}_4)_2\text{SO}_4$ for 4 min at 37°C . After addition of these substances, the incubations were continued

for 20 min. The incubations were terminated by cooling the tubes in ice, and after addition of 0.1 ml of a solution containing 50 nmol of unlabelled UTP and 0.1 mg of bovine serum albumin, the nucleic acids and protein were precipitated by adding 3 ml of ice-cold 10% (v/v) trichloroacetic acid containing 0.04 M-sodium pyrophosphate. The precipitate was centrifuged down, and washed with $3 \times 3\text{ml}$ of ice-cold 5% (w/v) trichloroacetic acid containing 0.04 M-sodium pyrophosphate and then $2 \times 3\text{ml}$ of ethanol–ether (3:1, v/v). Samples were hydrolysed in 1 ml of 5% (v/v) HClO_4 at 90°C for 15 min. After cooling and centrifuging, samples ($200\mu\text{l}$) were placed in counting vials, carefully neutralized, and the dioxan-based scintillator (10 ml) was added. Radioactivity was then determined as described above.

Each enzyme preparation used was checked for saturation by the $50\mu\text{g}$ of DNA present in the assay medium.

Subcellular distribution of [^3H]cortisol

Animals were injected intraperitoneally with [^3H]cortisol. After the appropriate interval, the animals were killed and bled, and livers removed and placed on ice. The subsequent procedures were based on those of Beato *et al.* (1969) and were all carried out at $0\text{--}4^\circ\text{C}$. Portions of liver (10 g fresh wt.) were homogenized in 2 vol. of SKMT buffer (0.25 M-sucrose–0.025 M-KCl–0.01 M- MgCl_2 –0.05 M-tris–HCl, pH 7.5). The homogenate was filtered through four layers of cheesecloth and a purified nuclear pellet was subsequently obtained as described above.

The supernatant from the 600 g spin was centrifuged at 110000 g for 90 min in a Spinco 40 angle rotor. The supernatant from this spin is referred to as the cytosol fraction. Portions (5 ml) of the cytosol fraction were subjected to gel filtration on columns (2 cm $\times$ 80 cm) of Sephadex G-25 previously equilibrated with SKMT buffer, elution being carried out with the same solution. Fractions (3 ml) were collected at a flow rate of 1 ml/min. The extinction of the eluate at 254 nm was continuously monitored by using an LKB Infracord instrument. The radioactivity of the eluted fractions was determined by placing 0.5 ml samples directly into 10 ml of the dioxan-based scintillator.

Portions (0.5 ml) of the column-excluded labelled fractions, obtained after passage of cytosol through Sephadex G-25 columns, were subjected to centrifugation on sucrose density gradients. Linear sucrose gradients [5 ml, 5–20% (w/v) sucrose] were prepared by hand in 1 mM- β -mercaptoethanol–1 mM-EDTA–0.5 mM- MgCl_2 –0.01 M-tris–HCl buffer, pH 8.0 (MEMT buffer). After samples (0.5 ml) of the labelled macromolecular cytosol fraction had been layered on top of the gradient, the gradients were centrifuged at 36000 rev./min in a Spinco SW 39 rotor at 4°C for

16.5 h. After removal from the rotor, the bottoms of the tubes were pierced and fractions collected directly in radioactivity-counting vials by means of counting drops. Some 10–12 equal fractions were collected, and their radioactivity was determined after addition of 10 ml of dioxan-based scintillator.

Nuclei, isolated from livers of animals treated with [^3H]cortisol, were extracted with MEMT buffer. MEMT buffer (2 ml) containing 0.6 M-NaCl was added to the nuclear pellet, and the suspension was stirred for 1 h in an ice bath. The viscous solution was centrifuged for 30 min at 20000g in a Spinco SW 39 rotor at 4°C. The supernatant was carefully removed, and portions (1 ml) were applied to a column (2 cm $\times$ 20 cm) of Sephadex G-25 that had previously been equilibrated with the MEMT buffer containing 0.3 M-NaCl. The column was developed with the same solution. A flow rate of 1 ml/min was maintained and 3 ml fractions were collected. The extinction at 254 nm of the eluate was continuously monitored by using an LKB Infracord instrument. The radioactivity of the eluted fractions was determined after addition of portions (0.5 ml) to 10 ml of dioxan-based scintillator.

Determination of thermal hyperchromicity (T_m) of chromatin and DNA

Chromatin was prepared from isolated nuclear preparations by a modification of the method detailed by Mainwaring *et al.* (1971), all procedures being carried out at 0–4°C. Nuclei (isolated from 4 g of tissue) were washed successively, once with 15 ml of 0.2% Triton X-100, twice with 15 ml of 80 mM-NaCl–20 mM-EDTA adjusted to pH 6.3 with sodium citrate, and once with 15 ml of 1.5 mM-NaCl–15 mM-sodium citrate buffer, pH 7.0. The final chromatin pellet was dispersed in water by gently homogenizing by hand in a Dounce all-glass homogenizer. Determinations of thermal hyperchromicity were carried out as described by Geiduschek (1962) for the 'd' assay (an assay which indicates the dissociation of base pairs). Assays were carried out in a Unicam SP.800 spectrophotometer fitted with an electrically heated cell-holder. Samples' temperatures were measured by means of a thermocouple inserted into the spectrophotometer cell. The chromatin solutions were prepared in 0.25 mM-EDTA, pH 8.0. This low-ionic-strength solution was used to magnify the difference in 'melting' profile between free DNA and chromatin (Bonner *et al.*, 1968). Purified DNA was prepared from the chromatin essentially by the method of Marmur (1963). The DNA finally obtained was dissolved in 0.25 mM-EDTA, pH 8.0. Determinations of thermal hyperchromicity were carried out on the DNA solutions as described by Geiduschek (1962) for both the 'd' and 'i' assays (the 'i' assay gives a measure of the irreversibility of denaturation).

Results

Tryptophan pyrrolase activity

Although the inhibitory effect of aflatoxin B₁ on the induction of tryptophan pyrrolase by cortisol is well authenticated (Wogan & Friedman, 1968; Clifford & Rees, 1967a), in view of its importance in the present work it was considered necessary to demonstrate the inhibition in the strain of rat to be used in this study. An experiment was therefore carried out to demonstrate this inhibition. The results indicated that in contrast to the control enzyme activity the cortisol-induced enzyme activity was strongly inhibited (>80%) by aflatoxin B₁ administered *in vivo* (3.5 mg/kg injected intraperitoneally in 0.05 ml of dimethylsulphoxide).

RNA polymerase activity assayed *in vitro*

In an initial experiment the effect of various dosages of aflatoxin B₁ on Mg²⁺- and Mn²⁺-activated RNA polymerase activity was determined. The results of this experiment are given in Fig. 1. Maximal inhibition (approx. 70%) was obtained with dosages of 2.5 mg/kg and above. The rate of development of the inhibition was also determined (Fig. 2).

Fig. 1. Effect of various dosages of aflatoxin B₁ on hepatic nuclear Mg²⁺- and Mn²⁺-(NH₄)₂SO₄-dependent RNA polymerase activities

●, Mg²⁺ assays; ○, Mn²⁺-(NH₄)₂SO₄ assays. Aflatoxin B₁ was administered in a total volume of 0.1 ml of dimethyl sulphoxide, and the animals were killed 3 h after dosing. Nuclei were prepared from pooled livers from groups of three animals.

The effect of administration of aflatoxin B₁ on the RNA polymerase activities in control and cortisol-stimulated animals was examined. Both Mg²⁺- and Mn²⁺-(NH₄)₂SO₄-stimulated RNA polymerase activities were assayed. The RNA polymerase activities found in the nuclei are indicated in Table 1. The results indicate that increased RNA synthesis, resulting from the administration of cortisol, is observed in the Mg²⁺-stimulated assays but not in the Mn²⁺-(NH₄)₂SO₄-stimulated assays. They show that the stimulated Mg²⁺-activated RNA synthesis resulting from cortisol administration is also inhibited by aflatoxin B₁. Also included in Table 1 are the results of experiments carried out on adrenalectomized animals. Adrenalectomy decreased the activity of hepatic nuclear Mg²⁺-activated RNA polymerase, but this enzymic activity in adrenalectomized animals treated with aflatoxin B₁ was not significantly different from that in intact animals treated with the toxin.

Incorporation of [¹⁴C]orotic acid and [¹⁴C]leucine in vivo

The effect of aflatoxin B₁ on the incorporation of [¹⁴C]orotic acid and [¹⁴C]leucine *in vivo* into an acid-insoluble form in the livers of both control and cortisol-treated rats was determined. The results of the experiments with [¹⁴C]orotic acid are given in Table 2. The effect of aflatoxin B₁ on orotic acid

Fig. 2. Development of inhibition of Mg²⁺-dependent RNA polymerase after administration of aflatoxin B₁ *in vivo*

Animals received 3.5mg of aflatoxin B₁/kg at zero time. Nuclei were prepared from pooled livers from groups of three animals.

Table 1. Effect of aflatoxin B₁, cortisol and adrenalectomy on nuclear RNA polymerase activity

Groups of three rats were injected intraperitoneally with (a) 0.05 ml of dimethylsulphoxide and 1 ml of saline, (b) aflatoxin B₁ (3.5 mg/kg) in 0.05 ml of dimethyl sulphoxide and 1 ml of saline, (c) 0.05 ml of dimethyl sulphoxide and cortisol acetate (50 mg/kg) in 1 ml of saline, or (d) aflatoxin B₁ (3.5 mg/kg) in 0.05 ml of dimethyl sulphoxide and cortisol acetate (50 mg/kg) in 1 ml of saline. Animals were killed and livers removed 4 h after injection. Duplicate nuclear preparations were obtained from pooled livers. Assays of polymerase activity were carried out in triplicate on each nuclear preparation. Dependence on the presence of all four nucleotides was checked in each experiment by omission of ATP, GTP and CTP. Activity in assays in which these nucleotides were omitted was less than 10% of that with the complete system. For details of assay systems and adrenalectomies see the Materials and Methods section. Values are means ± S.E.M. for six observations.

Hormonal status	Treatment	RNA polymerase activity (pmol of UMP incorporated/mg of DNA)	
		Mg ²⁺ -activated	Mn ²⁺ -(NH ₄) ₂ SO ₄ -activated
Intact	Dimethyl sulphoxide and saline	819 ± 22 (100%)	2701 ± 131 (100%)
	Aflatoxin B ₁ and saline	372 ± 6 (45%)	1391 ± 75 (48%)
	Dimethyl sulphoxide and cortisol	1219 ± 25 (149%)	2539 ± 97 (94%)
	Aflatoxin B ₁ and cortisol	448 ± 16 (55%)	1134 ± 52 (42%)
Adrenalectomized	Dimethyl sulphoxide and saline	533 ± 20 (65%)	
	Aflatoxin B ₁ and saline	383 ± 11 (47%)	
Sham-operated	Dimethyl sulphoxide and saline	852 ± 24 (104%)	
	Aflatoxin B ₁ and saline	345 ± 14 (42%)	

Table 2. *Effect of aflatoxin B₁ on the incorporation of [¹⁴C]orotic acid into nuclei in vivo*

Groups of three rats were injected with 2.5 μ Ci of [¹⁴C]orotic acid 15 min before being killed. Duplicate nuclear preparations were obtained from pooled livers. Animals were treated with aflatoxin and cortisol as described in Table 1. For details of assays see the Materials and Methods section. Incorporation into control (0.5 h, dimethyl sulphoxide and saline) nuclei was 226 000 d.p.m./mg of DNA.

Time after injections (h)	Treatment ...	Incorporation of label (% of corresponding dimethyl sulphoxide- and saline-treated control)		
		Aflatoxin B ₁ and saline	Dimethylsulphoxide and cortisol	Aflatoxin B ₁ and cortisol
0.5		37	84	48
1		30	96	43
2		25	106	35
4		37	133	46

Table 3. *Effect of aflatoxin B₁ on the incorporation of [¹⁴C]leucine into liver protein in vivo*

Groups of three rats were injected with aflatoxin B₁ and cortisol as described in Table 1. Then 2.5 h later all rats were injected with 2 μ Ci of [¹⁴C]leucine. Animals were killed 1 h later and incorporation into acid-insoluble material was determined. Duplicate 2 g amounts of pooled liver were homogenized and triplicate samples assayed from each homogenate as described in the Materials and Methods section. Values are means $\pm$ s.e.m. for six observations.

Treatment	Incorporation of [¹⁴ C]leucine (d.p.m./10 mg of protein)
Dimethyl sulphoxide and saline	1399 $\pm$ 75 (100%)
Aflatoxin B ₁ and saline	1285 $\pm$ 49 (92%)
Dimethyl sulphoxide and cortisol	1948 $\pm$ 58 (139%)
Aflatoxin B ₁ and cortisol	1471 $\pm$ 36 (105%)

incorporation *in vivo* is similar to that of the toxin on Mg²⁺-activated RNA polymerase assayed *in vitro*. The incorporation of orotic acid into control livers is decreased by approx. 70% by pretreatment with aflatoxin B₁, and the cortisol-induced increase in orotic acid incorporation is inhibited by the simultaneous administration of the toxin. The results of the experiment with [¹⁴C]leucine are given in Table 3. The cortisol-induced increase in leucine incorporation is also sensitive to inhibition by aflatoxin B₁, but in contrast to the effect of the toxin on the orotic acid incorporation, the control value for leucine incorporation appears to be resistant to the action of aflatoxin.

Uptake of [³H]cortisol

The effect of aflatoxin B₁ on the uptake of [³H]-cortisol into liver tissue was examined. Animals were injected with [³H]cortisol after receiving injections of aflatoxin B₁ solution or dimethyl sulphoxide alone, as detailed in the Materials and Methods section. Nuclear and cytosol fractions were prepared, and

their radioactive contents determined (Table 4). It appeared that aflatoxin B₁ did not affect the total uptake of radioactivity into the cytosol or nuclear fractions. It was possible, however, that the toxin could have affected the extent of macromolecular binding of the [³H]cortisol in either of these two sub-cellular fractions, without altering the total uptake. The labelled nuclear and cytosol fractions were therefore examined further by using gel filtration on Sephadex G-25 columns. A portion (5 ml) of the cytosol fractions was applied directly to the column, with the result given in Fig. 3. When portions (0.5 ml) of the column-excluded fractions were subjected to centrifugation in sucrose density gradients, as described in the Materials and Methods section, the peak in radioactivity was close to the position of the 4S marker (bovine serum albumin) and no differences were observed between the fractions prepared from control or aflatoxin-treated tissues. A preparation of isolated labelled nuclei (Table 4) was extracted with MEMT-buffered saline as described in the Materials and Methods section, and a portion (1 ml) of the

Table 4. Uptake of label into hepatic nuclear and cytosol fractions after administration of [³H]cortisol *in vivo*

Two experiments were carried out on two groups of two rats. The first group was injected with 0.05ml of dimethyl sulphoxide and the second group was injected with aflatoxin B₁ (3.5mg/kg) dissolved in 0.05ml of dimethyl sulphoxide. Then 2h later all rats were injected with 25 μCi of [³H]cortisol. Animals were killed 20min after the cortisol injections, and the livers were perfused *in situ* with 20ml of ice-cold SKMT buffer. Separation into cytosol and nuclear fractions was carried out on duplicate portions of pooled liver as detailed in the Materials and Methods section. Radioactivity determinations were carried out in triplicate; results are means ± s.e.m. for six observations.

Treatment	Radioactivity present in fractions	
	Cytosol (c.p.m./mg of protein)	Nuclear (c.p.m./mg of DNA)
Dimethyl sulphoxide	3631 ± 372	1464 ± 212
Aflatoxin B ₁	3867 ± 438	1298 ± 182

Fig. 3. Fractionation of labelled cytosol fraction on Sephadex G-25

Groups of two animals were injected either with aflatoxin B₁ (3.5mg/kg) or dimethylsulphoxide. After 2h rats were injected with 25 μCi of [³H]cortisol. Animals were killed 20min later and the cytosol fractions prepared as described in the Materials and Methods section. Portions of cytosol fractions from control (○—○) and aflatoxin-treated animals (●—●) with equal radioactive contents were subjected to gel filtration on Sephadex G-25. Fractions (3ml) were collected and the radioactivity was determined on 0.5ml portions of each fraction.

extract was passed through a Sephadex G-25 column (Fig. 4). In view of the results of these experiments it appeared that aflatoxin B₁ at a dosage that maximally inhibited RNA synthesis neither affected the total uptake of [³H]cortisol into the nuclear or cytosol fractions, nor the extent of macromolecular binding of radioactivity within these fractions.

Solubilized RNA polymerase activity

RNA polymerase activity was solubilized from isolated control hepatic nuclei, and the activity was compared with that of enzyme solubilized from nuclei from rats pretreated with aflatoxin B₁ and/or cortisol acetate (Table 5). It did not appear that the activities of the salt-extractable RNA polymerase enzymes reflected the inhibitions observed in the RNA polymerase activities of intact nuclei after treatment with aflatoxin. Addition of aflatoxin B₁ to the assay system, in contrast to actinomycin D, was without effect on the enzyme activity.

‘Melting’ profiles of chromatin and DNA

The ‘melting’ profiles of chromatin and DNA, prepared from hepatic nuclei isolated from control and aflatoxin-treated rats, were examined by using the techniques described in the Materials and Methods section. The ‘melting’ profiles observed with the chromatin fractions are given in Fig. 5. It appeared that the thermal ‘melting’ characteristics of chromatin were not altered by prior administration of a dose of aflatoxin B₁ that would maximally inhibit nuclear RNA synthesis. The ‘melting’ profiles of the DNA similarly showed no change caused by the administration of aflatoxin B₁. The ‘melting’ profiles of the DNA solutions were similar to those reported by Bonner *et al.* (1968).

Fig. 4. Fractionation of labelled nuclear extract on Sephadex G-25

Labelled nuclei were prepared after treatment with [^3H]cortisol as described in Fig. 3. Nuclei were extracted with buffered saline as detailed in the Materials and Methods section and portions of extracts, prepared from nuclei isolated from control (○—○) and aflatoxin-treated animals (●—●) with equal radioactive contents, were applied to Sephadex G-25 columns. Fractions (3 ml) were collected and the radioactive content of 0.5 ml portions of the fractions was determined.

Table 5. Activity of nuclear RNA polymerase solubilized from livers of rats treated with aflatoxin B_1 or cortisol

Four groups of four rats were used. Injections of dimethylsulphoxide, saline, cortisol or aflatoxin B_1 were carried out as in Table 1. Rats were killed 3.5 h after injection, and RNA polymerase was solubilized by the method of Jacob *et al.* (1968). Assays were carried out in triplicate as detailed in the Materials and Methods section. Results are means $\pm$ S.E.M. for six observations.

Assay conditions	Treatment	RNA polymerase activity (d.p.m. incorporated/20 min per 100 μg of protein)			
		Dimethyl sulphoxide and saline	Dimethyl sulphoxide and cortisol	Aflatoxin B_1 and saline	Aflatoxin B_1 and cortisol
Complete		1617 $\pm$ 31	1990 $\pm$ 71	1525 $\pm$ 22	2021 $\pm$ 8
– DNA		84 $\pm$ 5			
+ Aflatoxin B_1 (120 μM)		1730 $\pm$ 25			
+ Actinomycin D (25 μg)		174 $\pm$ 17			
– ATP, GTP, CTP		155 $\pm$ 12			

Discussion

The inhibitory action of aflatoxin B_1 on the induction of tryptophan pyrrolase in rat liver by cortisol, previously reported by Clifford & Rees (1967a) and Wogan (1969), was confirmed in the present study. The possible correlation between the effect of aflatoxin B_1 on enzyme induction by cortisol

and on cortisol-stimulated RNA synthesis was examined. Both *in vivo*, when the incorporation of labelled orotic acid was studied, and *in vitro*, when Mg^{2+} -activated RNA polymerase activity was assayed, the increase in RNA synthesis which resulted from the administration of cortisol was inhibited by aflatoxin B_1 , although in all experiments carried out

Fig. 5. 'Melting' characteristics of chromatin samples prepared from nuclei of dimethyl sulphoxide- and aflatoxin B_1 -treated rats

Chromatin samples were dissolved in 0.25 mM-EDTA, pH 8.0. ○---○, Control; ●—●, aflatoxin-treated.

RNA synthesis in nuclei isolated from animals treated with cortisol and aflatoxin was greater than that in nuclei isolated from animals treated with the toxin alone (Tables 1 and 2). The activity in the high-salt- Mn^{2+} -activated assays, although inhibited by aflatoxin B_1 , was not stimulated by the treatment with cortisol *in vivo*. Increased RNA polymerase activity in low-salt, but not in high-salt, assay systems is a commonly observed feature in nuclei of target organs isolated from hormonally stimulated animals (Tata, 1967). The inhibitory effect of aflatoxin B_1 on the cortisol-induced increase in tryptophan pyrrolase activity, but not on the control value of the enzyme activity, is paralleled by the effect of the toxin on the incorporation of labelled leucine *in vivo*. In summary these results could be interpreted as indicating that aflatoxin B_1 inhibits the stimulatory action of cortisol on hepatic nuclear RNA synthesis, and that consequent to this initial action there is an inhibition of cortisol-stimulated protein synthesis, of which synthesis of the enzyme tryptophan pyrrolase is a component. The lack of effect of the toxin on control values of amino acid incorporation may indicate that the latter process is dependent either on the aflatoxin-resistant RNA synthesis for a supply of the necessary ribonucleic acids or that longer-lived messenger RNA is involved. The relationship between aflatoxin action and the stimulation by cortisol of hepatic nuclear RNA synthesis was further indicated by the results of the experiment with adrenalectomized animals. Adrenalectomy resulted in a decreased Mg^{2+} -dependent RNA polymerase activity being observed in the isolated nuclei, but this decrease and the inhibitory effect of aflatoxin B_1 were not additive. It therefore appeared that the part of the RNA-

synthesizing mechanism that was inhibited by aflatoxin B_1 included the sites that were normally activated by endogenous corticosteroid. The extent of maximal inhibition of RNA polymerase activity consistently observed in the present study (70%) is close to that observed by Pong & Wogan (1970). This result indicates that approx. 30% of the RNA-synthesizing mechanism in rat liver nuclei is resistant to the action of aflatoxin B_1 . Whether this result is due to the total inhibition of RNA synthesis in 70% of the nuclei present in the nuclear preparation, or is due to a 70% inhibition of the enzymic activity in individual nuclei (or something in between these two extremes) is obviously of great importance.

With regard to mechanisms by which aflatoxin B_1 could possibly inhibit the stimulatory action of cortisol on hepatic nuclear RNA synthesis, the work of Williams & Rabin (1969) has shown that at the level of the endoplasmic reticulum, competition for binding sites can take place between aflatoxin B_1 and certain steroid hormones. A similar mechanism could account for the inhibition by the toxin of cortisol-stimulated nuclear events. It should be pointed out that Williams & Rabin (1969) found cortisol to be non-effective in their system, but this could presumably indicate a predominantly nuclear role for this steroid hormone. The mechanism of action of corticosteroids in hepatic cells has been extensively studied, and, in common with the mechanism of action of other steroid hormones in their respective target organs, it has been suggested that corticosteroids enter the nucleus in two stages, first by attachment to a macromolecular receptor in the cytosol fraction, and then, presumably by transfer of this complex through the nuclear membrane, a macromolecular-corticosteroid complex is subsequently observed in the nucleus (Beato *et al.*, 1969). These workers report that although cortisol is extensively metabolized in rat hepatic tissue, the fraction that enters the nucleus is almost exclusively unmetabolized cortisol. In the present study no attempt was made to characterize the labelled compounds present in the fractions after the administration of [3H]cortisol. One mechanism by which certain antagonists of steroid-hormone action have been shown to exert their anti-hormonal effect in the target organ is by attachment to steroid receptor macromolecules, either in the cytosol or nucleus, thereby blocking the uptake of the hormone into the chromatin and preventing the increased synthesis of RNA which normally accompanies hormonal stimulation (Belham & Neal, 1971). In the present study, however, no effect on the macromolecular binding of label after the administration of [3H]cortisol was observed in the nuclear or cytosol fractions when animals were previously given a dose of aflatoxin B_1 that was known to result in a maximal suppression of RNA synthesis. It is concluded therefore that the inhibitory

action of aflatoxin B₁ on cortisol-stimulated processes is unlikely to be due to an exclusion of cortisol or an active metabolite of cortisol from binding sites present on soluble receptor substances.

The results obtained with solubilized RNA polymerase (Table 5) indicate that the effect of the toxin is not on the activity of the polymerase enzyme itself, and by inference it would appear probable that its action is on the chromatin template. Dissociation of an enzyme-inhibitor complex could possibly have occurred during extraction of the enzyme, but the stability of the inhibition in disrupted nuclei even after prolonged dialysis (G. E. Neal, unpublished work) indicates that this is an unlikely explanation. Edwards & Wogan (1970) have also produced evidence indicating that the activities of RNA polymerase enzymes present in aflatoxin-inhibited rat liver nuclei are not inhibited. These workers have also produced evidence which they claim demonstrates a decreased chromatin template capacity after pretreatment of the animals with aflatoxin. However, it would appear that this claim could be open to two possible objections. First, the RNA polymerase enzyme that they used (similar to that in the present study) was solubilized by extracting the nuclei in low-salt conditions, and this has been shown by Chesterton & Butterworth (1971) to result almost exclusively in the solubilization of type I (nucleolar) enzyme, which Butterworth *et al.* (1971) have shown to be virtually inactive in the transcription of mammalian chromatin. Secondly, the assay conditions used by Edwards & Wogan (1970) included high-salt concentrations, and these would result in endogenous RNA polymerase present in the chromatin templates used being maximally active. It would therefore appear that further experimentation examining the priming by chromatin templates of the activities of the individual species of RNA polymerase enzymes under a variety of assay conditions is necessary before it can be concluded with certainty that the toxin affects the template capacity of chromatin.

With regard to the mechanism of action of the cortisol, there has been a considerable diversity of opinion as to whether the stimulatory action of corticosteroids on RNA synthesis in hepatic nuclei is due to an effect of the hormone on the activity of polymerase enzymes, template capacity or both (Dahmus & Bonner, 1965; Yu & Feigelson, 1971). Blatti *et al.* (1970) have demonstrated an increase in the activity of nucleolar polymerase enzyme after the administration of glucocorticoid to adrenalectomized rats. In the present study, an increased solubilized polymerase activity was observed when nuclei from cortisol-treated rats were used (Table 5), and in view of the solubilization technique used, this was presumably due to an increased activity of nucleolar enzyme. This increased polymerase activity was still evident after aflatoxin B₁ administration. If, as

discussed above, the inhibitory effect of aflatoxin B₁ is predominantly on the template, then that fraction of the enhanced RNA synthesis which is due to increased activity of polymerase enzyme should not be inhibited by the toxin. This could account for the fact that Mg²⁺-activated RNA polymerase activity in intact nuclei isolated from rats treated with cortisol and aflatoxin B₁ was always greater than that in nuclei isolated from rats treated with aflatoxin B₁ alone.

If aflatoxin B₁ administered *in vivo* affects the capacity of the chromatin template for RNA synthesis, then the experiments carried out in the present study indicate that this does not involve changes readily detected by 'melting'-profile assays. Binding of aflatoxin B₁ to DNA *in vitro* has been observed (Sporn *et al.* 1966; Clifford & Rees, 1967b), but the binding is weak, and in contrast to the binding of actinomycin D to DNA, does not survive passage through a gel-filtration column. Also, if simple binding of aflatoxin B₁ to DNA accounted for its inhibitory action on RNA synthesis, then it is difficult to explain the non-effectiveness of aflatoxin B₁ when added to RNA-synthesizing systems *in vitro*, either intact nuclei (Edwards & Wogan, 1970) or isolated RNA polymerase enzyme-DNA mixtures (King & Nicholson, 1967; see also Table 5). Clearly the uptake and distribution of aflatoxin B₁ within the hepatic cell requires further detailed study before its inhibitory action on RNA synthesis can be fully explained.

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