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 [¹³C]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 (Greenberg & 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 of at least 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 140–160 g, 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 3.5 mg/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 6 h 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 12 h 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 50 mg/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 B₂, B₃, 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 acetonite. The plates were developed with benzene saturated with formamide, and aflatoxins were located by their fluorescence on viewing the plates under an Hanovia u.v. Chromatolite.

[6-¹⁴C]Orotic acid (specific radioactivity 60.8 mCi/ mmol), [5-³H]UTP (specific radioactivity 1 Ci/mmole), L-[¹⁴C]leucine (specific radioactivity 62 mCi/mmole), and [1,2-³H]cortisol (specific radioactivity 43 Ci/ mmole) 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 (0.5 ml) 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 1 M-KOH at 37°C for 16 h, acidifying with 0.5 M-HClO₄ and clarifying by centrifuging. The extinction of the supernatant was measured at 260 nm, the extinction coefficient used being 34.2 mg of 

\[ \text{RNA} \cdot \text{ml}^{-1} \cdot \text{cm}^{-1} \]

(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 2.5 μCi of [¹⁴C]orotic acid in 0.88% NaCl (1 ml) 15 min before being killed. Livers were removed and chilled on ice. All subsequent procedures were carried out at 0–4°C. Portions (4 g) of pooled livers were homogenized in 10 ml of 0.25 M-sucrose–1 mM-MgCl₂ by using a Potter–Elvehjem homogenizer fitted with a Teflon pestle (TRI-R instruments; Camlab, Cambridge, Camb., U.K.). The homogenate was diluted with 2 ml 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 1 M-KOH as described above. Portions (100 μl) of the hydrolysate were taken for spectrophotometric measurement of the RNA content. Other samples (200 μl) were placed in radioactivity-counting vials and, after careful neutralization, 10 ml of dioxan-based scintillator (Bray, 1960) was added. Radioactivity counting efficiency, determined by internal standardization, was 69%.
In the \[^{14}\text{C}]\text{leucine incorporation studies animals were injected intraperitoneally with }1\times[^{14}\text{C}]\text{leucine (2}\mu\text{Ci})\text{ in 0.88}\%\ \text{NaCl (1 ml) 1 h before being killed. Liver were removed, and from the pooled livers duplicate samples (2 g fresh wt.) were homogenized in 10 ml of ice-cold 0.25}\mu\text{m-sucrose--1}\mu\text{m-MgCl}_2.\text{ Acid-insoluble material was dissolved overnight at 37°C in 1 ml of 1}\%\ \text{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}\text{C}]\text{toluene as an internal standard, was 67}\%.\text{ 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\text{ } 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 unfraccionated 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 \[^{3}\text{H}]\text{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 2.2M-sucrose--10mm-magnesium acetate--0.25mm-spermine (Busch et al., 1967). The incubation medium contained, in a total volume of 0.75ml: calf thymus DNA, 50\mu g; tris--HCl buffer, pH 8.0, 40\mu mol; MgCl\(_2\), 2\mu mol; MnCl\(_2\), 1.5\mu mol; NaF, 12\mu mol; cysteine, 7\mu mol; spermine, 2\mu mol; ATP, 0.35\mu mol; CTP and GTP, 0.3\mu mol; UTP, 0.07\mu mol; \[^{3}\text{H}]\text{UTP, 0.2}\mu mol; saturated (NH\text{2})_2\text{SO}_4 soln., 15\mu l; solubilized polymerase enzyme, 200\mu l. Assays were preincubated in the absence of \[^{3}\text{H}]\text{UTP and (NH\text{2})_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 0.1ml of a solution containing 50nmol of unlabelled UTP and 0.1mg of bovine serum albumin, the nucleic acids and protein were precipitated by adding 3ml of ice-cold 10\% (w/v) trichloroacetic acid containing 0.04M-sodium pyrophosphate. The precipitate was centrifuged down, and washed with 3 x 3ml of ice-cold 5\% (w/v) trichloroacetic acid containing 0.04M-sodium pyrophosphate and then 2 x 3ml of ethanol--ether (3:1, v/v). Samples were hydrolysed in 1ml of 5\% (v/v) HClO\(_4\) at 90°C for 15 min. After cooling and centrifuging, samples (200\mu l) were placed in counting vials, carefully neutralized, and the dioxan-based scintillator (10ml) was added. Radioactivity was then determined as described above. Each enzyme preparation used was checked for saturation by the 50\mu g of DNA present in the assay medium.

**Subcellular distribution of \[^{3}\text{H}]\text{cortisol**}

Animals were injected intraperitoneally with \[^{3}\text{H}]\text{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 0-4°C. Portions of liver (10g fresh wt.) were homogenized in 2vol. of SKMT buffer (0.25M-sucrose--0.025M-KCl--0.01M-MgCl\(_2\)--0.05M-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 600g spin was centrifuged at 110000g for 90 min in a Spinco 40 angle rotor. The supernatant from this spin is referred to as the cytosol fraction. Portions (5ml) of the cytosol fraction were subjected to gel filtration on columns (2cm x 80cm) of Sephadex G-25 previously equilibrated with SKMT buffer, elution being carried out with the same solution. Fractions (3ml) were collected at a flow rate of 1ml/min. The extinction of the eluate at 254nm was continuously monitored by using an LKB Infracord instrument. The radioactivity of the eluted fractions was determined by placing 0.5ml samples directly into 10ml of the dioxan-based scintillator.

Portions (0.5ml) 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 (5ml, 5-20\% (w/v) sucrose) were prepared by hand in 1mm-\beta-mercaptoethanol--1mm-EDTA--0.5mM-MgCl\(_2\)--0.01mM-tris--HCl buffer, pH 8.0 (MEMT buffer). After samples (0.5ml) of the labelled macromolecular cytosol fraction had been layered on top of the gradient, the gradients were centrifuged at 36000rev./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 \(^{3}H\) 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 20 000 g 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 M 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 M 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**

**Trypottan pyrrolyase activity**

Although the inhibitory effect of aflatoxin B\(1\) 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 B\(1\) administered in vivo (3.5 mg/kg injected intraperitoneally in 0.05 ml of dimethyl sulphoxide).

**RNA polymerase activity assayed in vitro**

In an initial experiment the effect of various dosages of aflatoxin B\(1\) on Mg\(^{2+}\)- and Mn\(^{2+}\)-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).

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![Graph](image-url)  
**Fig. 1. Effect of various dosages of aflatoxin B\(_1\) on hepatic nuclear Mg\(^{2+}\)- and Mn\(^{2+}\)-(NH\(_4\))\(_2\)SO\(_4\)-dependent RNA polymerase activities**

- •, Mg\(^{2+}\) assays; ○, Mn\(^{2+}\)-(NH\(_4\))\(_2\)SO\(_4\) assays. Aflatoxin B\(_1\) 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.

1972
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

![Graph](image)

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

Animals received 3.5 mg 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 dimethyl sulphoxide 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.

<table>
<thead>
<tr>
<th>Hormonal status</th>
<th>Treatment</th>
<th>RNA polymerase activity (pmol of UMP incorporated/mg of DNA)</th>
<th>Mg²⁺-activated</th>
<th>Mn²⁺-(NH₄)₂SO₄-activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>Dimethyl sulphoxide and saline</td>
<td></td>
<td>819±22 (100%)</td>
<td>2701±131 (100%)</td>
</tr>
<tr>
<td></td>
<td>Aflatoxin B₁ and saline</td>
<td></td>
<td>372±6 (45%)</td>
<td>1391±75 (48%)</td>
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<tr>
<td></td>
<td>Dimethyl sulphoxide and cortisol</td>
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<td>1219±25 (149%)</td>
<td>2539±97 (94%)</td>
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<tr>
<td></td>
<td>Aflatoxin B₁ and cortisol</td>
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<td>448±16 (55%)</td>
<td>1134±52 (42%)</td>
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<tr>
<td>Adrenalectomized</td>
<td>Dimethyl sulphoxide and saline</td>
<td></td>
<td>533±20 (65%)</td>
<td>411±25 (66%)</td>
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<td></td>
<td>Aflatoxin B₁ and saline</td>
<td></td>
<td>383±11 (47%)</td>
<td>193±11 (44%)</td>
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<tr>
<td>Sham-operated</td>
<td>Dimethyl sulphoxide and saline</td>
<td></td>
<td>852±24 (104%)</td>
<td>345±14 (42%)</td>
</tr>
<tr>
<td></td>
<td>Aflatoxin B₁ and saline</td>
<td></td>
<td>345±14 (42%)</td>
<td>193±11 (44%)</td>
</tr>
</tbody>
</table>

Vol. 130
Table 2. Effect of aflatoxin B, on the incorporation of [14C]orotic acid into nuclei in vivo

Groups of three rats were injected with 2.5μCi of [14C]orotic acid 15min 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 (0.5h, dimethyl sulphoxide and saline) nuclei was 226000 d.p.m./mg of DNA.

<table>
<thead>
<tr>
<th>Time after injections (h)</th>
<th>Treatment ...</th>
<th>Aflatoxin B, and saline</th>
<th>Dimethyl sulfoxide and cortisol</th>
<th>Aflatoxin B, and cortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td></td>
<td>37</td>
<td>84</td>
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<td>106</td>
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<tr>
<td>4</td>
<td></td>
<td>37</td>
<td>133</td>
<td>46</td>
</tr>
</tbody>
</table>

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

Groups of three rats were injected with aflatoxin B, and cortisol as described in Table 1. Then 2.5h later all rats were injected with 2μCi of [14C]leucine. Animals were killed 1h later and incorporation into acid-insoluble material was determined. Duplicate 2g amounts of pooled liver were homogenized and triplicate samples assayed from each homogenate as described in the Materials and Methods section. Values are means ± S.E.M. for six observations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incorporation of [14C]leucine (d.p.m./10mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl sulfoxide and saline</td>
<td>1399±75 (100%)</td>
</tr>
<tr>
<td>Aflatoxin B, and saline</td>
<td>1285±49 (92%)</td>
</tr>
<tr>
<td>Dimethyl sulfoxide and cortisol</td>
<td>1948±58 (139%)</td>
</tr>
<tr>
<td>Aflatoxin B, and cortisol</td>
<td>1471±36 (105%)</td>
</tr>
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</table>

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 B, 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 B, 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 B, on the uptake of [3H]-cortisol into liver tissue was examined. Animals were injected with [3H]cortisol after receiving injections of aflatoxin B, 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 B, 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 subcellular 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 (5ml) of the cytosol fractions was applied directly to the column, with the result given in Fig. 3. When portions (0.5ml) 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 (1ml) of the
Two experiments were carried out on two groups of rats. The first group was injected with 0.05 ml of dimethyl sulphoxide and the second group was injected with aflatoxin B₁ (3.5 mg/kg) dissolved in 0.05 ml of dimethyl sulphoxide. Then 2h later all rats were injected with 25 μCi of [³H]cortisol. Animals were killed 20 min after the cortisol injections, and the livers were perfused in situ with 20 ml 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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytosol (c.p.m./mg of protein)</th>
<th>Nuclear (c.p.m./mg of DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl sulphoxide</td>
<td>3631±372</td>
<td>1464±212</td>
</tr>
<tr>
<td>Aflatoxin B₁</td>
<td>3867±438</td>
<td>1298±182</td>
</tr>
</tbody>
</table>

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 adminstration 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 (o-○-○) 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 B₁ or cortisol

Four groups of four rats were used. Injections of dimethyl sulphoxide, saline, cortisol or aflatoxin B₁ were carried out as in Table 1. Rats were killed 3.5h 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 ± S.E.M. for six observations.

<table>
<thead>
<tr>
<th>Assay conditions</th>
<th>Treatment</th>
<th>Dimethyl sulphoxide and saline</th>
<th>Dimethyl sulphoxide and cortisol</th>
<th>Aflatoxin B₁ and saline</th>
<th>Aflatoxin B₁ and cortisol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td></td>
<td>1617 ± 31</td>
<td>1990 ± 71</td>
<td>1525 ± 22</td>
<td>2021 ± 8</td>
</tr>
<tr>
<td>- DNA</td>
<td></td>
<td>84 ± 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Aflatoxin B₁</td>
<td>(120 μM)</td>
<td>1730 ± 25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Actinomycin D</td>
<td>(25 μg)</td>
<td>174 ± 17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- ATP, GTP, CTP</td>
<td></td>
<td>155 ± 12</td>
<td></td>
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</table>

Discussion

The inhibitory action of aflatoxin B₁ 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 B₁ 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 Mg²⁺-activated RNA polymerase activity was assayed, the increase in RNA synthesis which resulted from the administration of cortisol was inhibited by aflatoxin B₁, although in all experiments carried out
Fig. 5. 'Melting' characteristics of chromatin samples prepared from nuclei of dimethyl sulfoxide- and aflatoxin B₁-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-Mn²⁺-activated assays, although inhibited by aflatoxin B₁, 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 B₁ 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 B₁ 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 Mg²⁺-dependent RNA polymerase activity being observed in the isolated nuclei, but this decrease and the inhibitory effect of aflatoxin B₁ were not additive. It therefore appeared that the part of the RNA-synthesizing mechanism that was inhibited by aflatoxin B₁ 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 B₁. 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 B₁ 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 B₁ 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 B₁ 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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References


1972
EFFECT OF AFLATOXIN ON RNA SYNTHESIS

629


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Vol. 130