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SOME EFFECTS OF AFLATOXIN B_1 ON RNA SYNTHESIS IN RAT AND MOUSE LIVER

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Abstract—Rat liver Mn^{2+} and Mg^{2+} activated RNA polymerase activities, assayed *in vitro* are inhibited by aflatoxin B_1 administered *in vivo*, whereas the enzyme activities in mouse liver are not. [^{14}C]orotic acid incorporation *in vivo* into rat liver RNA is inhibited by aflatoxin B_1 administered *in vivo*, whereas incorporation into mouse liver RNA is not. Mg^{2+} activated RNA polymerase and [^{14}C]orotic acid incorporated into RNA in both rat and mouse liver are inhibited by incubating liver slices *in vitro* in the presence of aflatoxin B_1. No significant inhibition of hepatic Mg^{2+} activated RNA polymerase by aflatoxin B_1, administered *in vivo*, is observed when rats are pre-treated with phenobarbitone. These data demonstrate that RNA synthesis in mouse liver is potentially capable of being inhibited by aflatoxin B_1 and suggest that the level of microsomal metabolism is an important factor in determining the presence or absence of inhibition. Attempts, using SKF 525 A treated mice, to demonstrate inhibition by aflatoxin B_1 *in vivo* of mouse liver RNA polymerase were unsuccessful. Incubation studies *in vitro* however, indicated that SKF 525 A did not significantly affect the overall rate of metabolism of aflatoxin B_1 by liver slices or microsomal preparations.

AFLATOXIN B_1 is acutely toxic to a wide range of species of domestic and laboratory animals. The toxic action is associated with the appearance of histological liver damage. Nuclear RNA synthesis appears to be a primary target for aflatoxin action in liver, since it is, as far as is known to date, the biochemical process most rapidly affected by aflatoxin B_1, and also amongst the most sensitive.[1] The correlation between the early inhibition of liver RNA synthesis and the later appearance of histological hepatic damage has recently been further emphasized by the finding of Akao, Kuroda and Wogan[2] that resistance in mice to aflatoxin B_1 toxicity and absence of liver damage are accompanied by a lack of inhibition of liver nuclear RNA synthesis *in vivo* even after the administration of aflatoxin B_1 doses as high as 60 mg/kg. It was suggested by Akao *et al.*[2] that this might reflect a fundamental difference between the RNA synthesizing systems in rat and mouse liver concerning sites for toxin-chromatin interactions. We have also carried out some experiments relating to this topic, the results of which are presented in this paper.

MATERIALS AND METHODS

Animals. Mice used were males of the LAC "A" strain derived from CFW Swiss mice, 20–25 g in weight. Rats were males of a Porton Wistar derived strain weighing 140–160 g. Animals were killed by cervical dislocation, and exsanguinated before removal of the livers. Aflatoxin B_1 dissolved in dimethyl sulphoxide (DMSO) was injected intraperitoneally into the rats or mice usually in a total volume of 0·05 ml. Control animals received the vehicle alone. Injections were normally carried out

between 10 and 11 a.m. [^{14}C]orotic acid dissolved in 0·88% saline was injected intraperitoneally in a total volume of 1 ml. SKF 525 A (β-diethyl-aminoethyl diphenylpropyl acetate) was also injected intraperitoneally dissolved in saline (40 mg/kg 0·25 ml/mouse 3 ml/rat). Sodium phenobarbitone was given as a 0·1 per cent solution in the drinking water for 12 days prior to the rats being used in experiments.

Chemicals. Uridine-5[^{3}H]-5′ triphosphate (1 Ci/mMole) and orotic acid 6-[^{14}C](60·8 mCi/mMole) were obtained from the Radiochemical Centre, Amersham, England. Aflatoxin B_1 was separated from a mixture of aflatoxins B_1, B_2, G_1 and G_2 by a modification, devised by Dr. J. B. Greig of this department, of the alumina column method of Steyn.[3] DMSO used was the "special for spectroscopy" grade obtained from BDH. NADPH was obtained from Boehringer Corp.; ATP, GTP, CTP and UTP were obtained from Koch-Light Laboratories, Colnbrook. SKF 525 A was a gift from Smith, Kline & French Ltd. All other chemicals used were DBH "analar" grade.

RNA polymerase assays. Nuclei were prepared from freshly excised livers and kidneys or incubated liver slices using the method of Widnell and Tata,[4] all operations being carried out at 0–4°. Nuclear preparations were always checked by phase-contrast microscopy for integrity of the nuclei and for freedom from contamination with other sub-cellular material. Both Mg^{2+} and Mn^{2+}-$(NH_4)_2SO_4$ stimulated RNA polymerase activities present in the isolated nuclei (approximately 250 μg DNA/assay tube) were assayed essentially by the method of Widnell and Tata.[5] Assay solutions were always freshly prepared immediately prior to use. This was done because it was found that if the 10 mM $MnCl_2$ and 0·25 M Tris–HCl buffer used in the Mn^{2+}-$(NH_4)_2SO_4$ activated assay system were prepared in one stock solution, and stored in the cold (4°), the solution progressively darkened. This was due to the formation of an oxidation product, presumably manganese dioxide, since the discolouration could be rapidly removed by the addition of thiol reagents such as β-mercaptoethanol or dithiotheitol. Similarly, storage of a stock Mg^{2+} assay solution, containing 0·25 M Tris–HCl pH 8·5 12·5 mM $MgCl_2$, 50 mM cysteine and 15 mM NaF, in the cold, was found to be unsatisfactory, rapidly resulting in much lower levels of polymerase activity being detected in the assays. It was observed that, after storage of the assay solution, nuclei placed in those assay tubes which contained ATP, GTP and CTP in addition to the Mg^{2+} assay solution rapidly aggregated whereas no clumping of the nuclei was seen in the control assays from which the triphosphates were omitted. It appeared therefore that during storage of this assay medium, the Mg^{2+} concentration in the solution was reduced by reaction with one of the other constituents, and that when used for RNA polymerase assays, removal of further Mg^{2+} by formation of a complex, presumably with ATP, resulted in a final concentration of Mg^{2+} below that necessary to prevent clumping of the nuclei. Incubations were carried out at 37° (20 min for Mg^{2+} stimulated, 30 min following 15 min pre-incubation in the absence of [^{3}H] UTP for Mn^{2+}-$(NH_4)_2SO_4$ stimulated activities. Incorporations were terminated by chilling the tubes and adding an equal volume of ice cold 10% TCA. After standing the tubes in ice for 15 min, the precipitates were centrifuged down and then washed three times with 3 ml cold 5% TCA followed by 3 ml ethanol–ether (3:1 v/v). RNA and DNA were hydrolysed by adding 1 ml 5% $HClO_4$, and heating at 90° for 15 min. Duplicate 0·2 ml samples were placed in radioactivity-counting vials, carefully neutralized with NaOH, and then 10 ml dioxan scintillator was added.[6] Radioactive

counting was carried out in a Packard Tri-Carb liquid scintillation counter. Counting efficiency for [^{3}H] using the above technique was 15 per cent, internal standardization being carried out in each experiment. Further 0·2 ml aliquots of the $HClO_4$ hydrolysates were taken for the estimation of their DNA content, using the Giles and Myers[7] modification of the diphenylamine method.[8] Polymerase activities were calculated as pmoles UMP incorporated/mg DNA.

Incubations in vitro. Liver slices (approx. 0·3 mm thick) were cut from freshly excised livers using a hand microtome.[9] Slices (200 mg fresh wt.) were incubated in 2·5 ml Krebs–Ringer phosphate medium plus 0·27% glucose, at 37° in a shaking incubator bath at approximately 100 oscillations per min, under an atmosphere of O_2. Aflatoxin B_1 was added in solution in DMSO, DMSO only being added to the control vessel. [^{14}C]-labelled orotic acid (2 μc) was added to the flasks and incorporation allowed to proceed for 30–45 min. At the end of the incubation period, the flasks were chilled in ice and 2·5 ml ice-cold 10% TCA was added. The tissue was then homogenized in a Potter homogenizer fitted with a teflon pestle (TRI-R instruments, obtained from Camlab, Cambridge, England). The homogenates were allowed to stand for 15 min in ice, and were then centrifuged at 4°. The pellets were washed three times with 3 ml cold 5% TCA. In initial experiments, these washings were followed by one wash with ethanol–ether (3:1 v/v). However, it proved difficult to spin down the precipitates after this washing, and since it did not appear to affect the results finally obtained, the ethanol–ether wash was omitted, and the residue from the third 5% TCA wash was immediately dissolved in 1 ml 1N KOH and hydrolysed at 37° for 16 hr.[10] After acidifying with 0·2 ml 6N HCl followed by 1 ml 5% $HClO_4$, the precipitate was removed by centrifuging. Duplicate 0·4 ml samples were carefully neutralized with NaOH and 10 ml dioxan scintillator added. The counting efficiency for [^{14}C] in this system was 70 per cent. The RNA content of diluted samples of the hydrolysate was also estimated spectrophotometrically at 260 nm, assuming an absorbance of 34·2/mg RNA/ml/cm[11] after confirming that the u.v. absorption spectrum indicated no gross contamination of the hydrolysed RNA with other u.v. absorbing material.

In those experiments in which nuclei were prepared from liver tissue following incubation *in vitro*, 1 g fresh wt. of tissue slices were incubated in 25 ml of the Krebs–Ringer phosphate–glucose solution for 30 min under an atmosphere of O_2 with rapid shaking. Aflatoxin B_1 dissolved in DMSO was placed in the medium at the same concentration as that used in the [^{14}C]orotic acid incorporation studies previously mentioned. At the end of the incubation period the tissue was chilled, rinsed in cold 0·25 M sucrose containing 1 mM $MgCl_2$, and nuclear fractions prepared and RNA polymerase activity assayed by the methods already described.

RNA synthesis in vivo. The incorporation of [^{14}C]orotic acid into nuclear RNA of rat and mouse liver at various times after dosing with aflatoxin B_1 was determined. Groups of 2 animals were injected intraperitoneally with either aflatoxin B_1 in DMSO (0·05 ml, rat 7 mg/kg, mouse 60 mg/kg) or DMSO alone. At various times after these injections, animals were injected intraperitoneally with 2·5 μc [^{14}C]orotic acid in 0·88% saline (0·5 ml). Incorporation was allowed to proceed for 15 min. The animals were then killed and the livers rapidly removed. Nuclei were isolated as already described. After washing three times with cold 5% TCA, and once with ethanol–ether (3:1 v/v), RNA was hydrolysed with KOH and incorporation determined by the method described under the *in vitro* incubation section. Determinations of levels of

soluble radioactivity were carried out routinely to eliminate errors due to changes in uptake of the precursor into liver.

Metabolism of aflatoxin B_1 in vitro. Mouse liver slices (2·0 g wet wt) were incubated in air in 10 ml 0·25 M sucrose, 0·025 M KCl, M $MgCl_2$ and 0·05 M Tris–HCl pH 7·6 (SKMT buffer) at 37° for 1 hr in conical flasks in a shaking incubator bath.[17] Aflatoxin B_1 (64 μg) dissolved in DMSO was added to the flasks. In some experiments, microsomal suspensions (equivalent to 2 g fresh wt. of tissue), were incubated *in vitro*. In these experiments 1 μmole NADPH was added to the incubation medium. Microsomal fractions were prepared by homogenizing the tissue in 2·5 vol. of SKMT buffer. The homogenate was centrifuged at 20,000 *g* for 20 min, and then the supernatant centrifuged at 110,000 *g* for 90 min, in a Spinco model L centrifuge. The microsomal pellet was resuspended in 10 ml SKMT and resedimented at 110,000 *g* for 60 min. The microsomal preparation was then suspended in SKMT at the required level. Boiled tissue controls were included in each experiment. At the end of the incubation period, aflatoxin was extracted and separated on TLC plates. In initial experiments, the extraction technique of De Iongh, Van Pelt, Ord and Barrett[12] was used. Frequently, however, fluorescent spots other than B_1, were observed on the TLC plates, in control incubations from which tissue was omitted. Further experiments indicated that these fluorescent breakdown products were formed at the methanol extraction stage. It was found that no such difficulties were encountered if the methanol extraction was replaced by aqueous acetone. The procedure ultimately adopted, which also gave less lipid material in the final extract, was as follows: liver tissue was homogenized in the 10 ml SKMT buffer incubation solution. Acetone was added to the homogenate at the level 2·3 vol. acetone to 1 vol. homogenate. The suspension was thoroughly mixed, stood in the cold (4°) for 30 min, and then centrifuged at 2000 rev/min in an MSE Mistral 4L. The pellet was washed once with acetone–water 70:30 v/v and centrifuged. The supernatant was extracted twice with 0·5 vol. of 40/60 petrol, the petrol layer being discarded. The solution was then extracted twice with 1 vol. of chloroform, the aqueous phase then being discarded. The chloroform extract was evaporated to a low volume in a stream of nitrogen. It was then transferred to a small stoppered tube, evaporated to dryness, and dissolved in a small volume of $CHCl_3$. Measured volumes were applied to Kieselgel G TLC plates (Merck) using Drummond microcaps. 0·05–0·5 μg aflatoxin B_1 spots were applied as standards. The plates were developed in equilibrated tanks in $CHCl_3$–MeOH (97:3 v/v). A development time of 35 min allowed the solvent front to advance approximately 14 cm. Aflatoxin B_1 and its fluorescent metabolites were located by viewing the plates under a Hanovia Chromatolite u.v. lamp. Approximate quantitation of B_1 was achieved by eluting aflatoxin B_1 containing zones with $CHCl_3$, measuring their extinction at 362 nm in a Unicam SP 500 and comparing them with the extinctions of the standards.

RESULTS

Effect of aflatoxin B_1 on [^{14}C]orotic acid incorporation in vivo. The level of incorporation of [^{14}C]orotic acid into rat and mouse liver nuclear RNA at various times after an intraperitoneal injection of aflatoxin B_1 was determined, and the results are illustrated in Fig. 1. DMSO treatment in rat or mouse did not produce any inhibition of incorporation compared with the level of incorporation into saline treated animals.

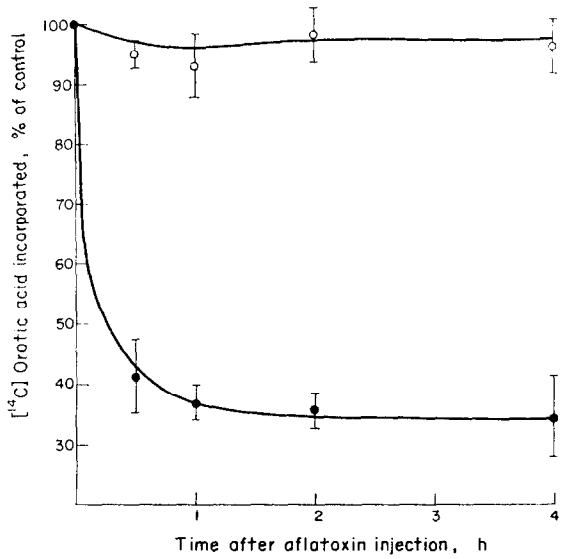

FIG. 1. Effect of aflatoxin B_1 on [^{14}C]orotic acid incorporation into mouse and rat liver nuclear RNA *in vivo*. ○——○ mouse liver ●——● rat liver. Groups of 2 animals injected with DMSO (0·05 ml) or aflatoxin B_1 (Rat 7 mg/kg, mouse 60 mg/kg) in DMSO. At various times after injections, animals (aflatoxin B_1 or DMSO treated) injected with 2·5 μc [^{14}C]orotic acid. Animals killed 15 min later and incorporation into nuclear RNA determined. Triplicate hydrolyses carried out on aliquots of duplicate samples. Results are incorporations into aflatoxin B_1 treated livers as per cent of incorporation into controls ±S.E. (vertical bars).

No significant effect of the aflatoxin treatment on the incorporation into mouse liver RNA was detected, in contrast to the rapid and extensive effect on rat liver RNA synthesis.

Effect of aflatoxin B_1 administered in vivo *on RNA polymerase activity assayed* in vitro. The effect of aflatoxin B_1, administered *in vivo* on rat and mouse liver nuclear Mg^{2+} and Mn^{2+}-$(NH_4)_2SO_4$ stimulated RNA polymerase activities assayed *in vitro* is summarized in Table 1. Also included is the effect of the aflatoxin treatment on rat and mouse kidney Mg^{2+} stimulated RNA polymerase activity. The data concerning the rat and mouse liver activities are in agreement with the results of Akao *et al.*[2] and confirm the lack of inhibition of the mouse liver system. The results indicate that the inhibition in mouse kidney noted by Akao *et al.*[2] also occurs in the rat kidney. During preliminary experiments it was consistently found that the final levels of inhibition in the Mg^{2+} and Mn^{2+} activated systems, achieved 10–15 min after administration of the toxin, were similar. In subsequent experiments it was decided therefore that only the Mg^{2+} activated system would be studied.

Effect of aflatoxin B_1 on orotic acid incorporation in vitro. In view of the lack of effect of aflatoxin B_1 on mouse liver RNA synthesis *in vivo*, assayed by [^{14}C] labelled orotic acid incorporation, it was of interest to examine its effect on incorporation *in vitro*. Liver slices, prepared from freshly excised livers, were incubated in Krebs–Ringer phosphate–glucose medium, as detailed in the Materials and Methods section. The results of a typical experiment are given in Fig. 2. Radioactivity determinations on washed slices did not indicate any effect of aflatoxin B_1 on orotic acid uptake, and

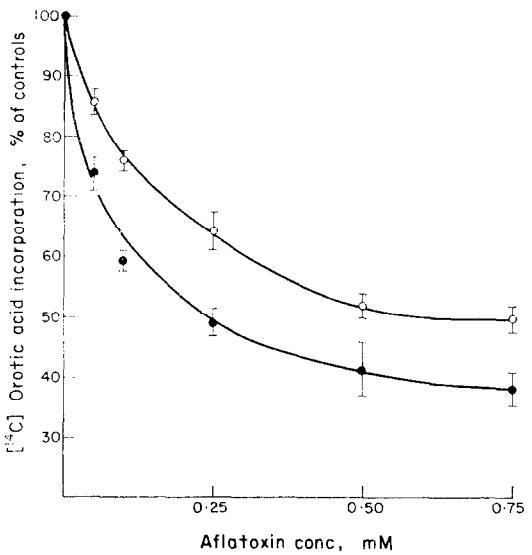

FIG. 2. Effect of aflatoxin B_1 on [^{14}C]orotic acid incorporation into RNA *in vitro* by rat and mouse liver slices. ○——○ Mouse liver, ●——● rat liver. 200 mg fresh wt of liver slices (0·3 mm thick) incubated in 2·5 ml Krebs–Ringer phosphate medium plus 0·27% glucose at 37° in a shaking incubator bath (approx. 100 oscillations/min) under O_2 [^{14}C]orotic acid (2 μc) added to each flask. Aflatoxin B_1 added in DMSO. Final DMSO concentration in all flasks = 1·25 per cent. Each point is the mean of triplicate assays on duplicate incubations for 45 min. Tissue processed as in Materials and Methods section. Duplicate RNA and radioactivity assays carried out on each sample. S.E.s of the means are indicated by vertical bars. Levels of incorporation in absence of aflatoxin B_1, rat liver slices 9060 ± 98 dis./min/mg RNA, mouse liver slices—7970 ± 40 dis./min/mg RNA.

TABLE 1. THE EFFECT OF AFLATOXIN B_1, ADMINISTERED *in vivo*, ON NUCLEAR RNA POLYMERASE ACTIVITY ASSAYED *in vitro*

Animal	Organ	Treatment	RNA polymerase (pmoles UMP incorporated/mg DNA)	
			Mg^{2+} activated	Mn^{2+}-$(NH_4)_2SO_4$ activated
Rat	Liver	DMSO	836 ± 24 (100%)	2620 ± 140 (100%)
		Aflatoxin B_1	226 ± 8 (27%)	786 ± 76 (30%)
	Kidney	DMSO	272 ± 15 (100%)	
		Aflatoxin B_1	148 ± 11 (54%)	
Mouse	Liver	DMSO	1041 ± 25 (100%)	2983 ± 135 (100%)
		Aflatoxin B_1	984 ± 18 (95%)	2790 ± 98 (94%)
	Kidney	DMSO	376 ± 6 (100%)	
		Aflatoxin B_1	173 ± 14 (46%)	

Groups of four rats or six mice killed 2 hr after i.p. injection of DMSO (0·05 ml) or aflatoxin B_1 (7 mg/kg rat, 60 mg/kg mouse) in DMSO. Livers pooled and duplicate 4 g portions used to prepare nuclear samples. RNA polymerase activity assayed in triplicate. Assays minus GTP, CTP and ATP included in all experiments, incorporation < 10 per cent of complete system. Mg^{2+} conc. = 5 mM, Mn^{2+} conc. = 4 mM. Results are means of triplicate assays on duplicate samples ±S.E. Figures in parentheses are the aflatoxin B_1 results expressed as percentages of the corresponding DMSO controls.

the previous report of a lack of effect of the toxin on orotic acid entry into the nucleotide pool,[13] was confirmed. However, aflatoxin B_1 was clearly capable of inhibiting [^{14}C]orotic acid incorporation into RNA *in vitro*, by both rat and mouse liver slices, although inhibition of the rat system was still more marked than that of the mouse.

Effect of aflatoxin B_1 administered in vitro *on nuclear RNA poylmerase activity assayed* in vitro. Rat and mouse liver slices were incubated in Krebs–Ringer phosphate–glucose medium. Some of the incubation vessels, which contained 200 mg wet wt tissue slices were used to determine [^{14}C]orotic acid incorporation, as in Fig. 2. Other vessels contained 1 g wet wt. tissue slices and nuclei were isolated from the tissue at the end of the incubation period. Mg^{2+} stimulated RNA polymerase activity was

TABLE 2. EFFECT OF AFLATOXIN ON [^{14}C]OROTIC ACID INCORPORATION AND Mg^{2+} ACTIVATED RNA POLYMERASE OF MOUSE AND RAT LIVER *in vitro*

Animal	Treatment	[^{14}C]orotic acid incorporation	
		dis./min/mg RNA	% of DMSO treated tissue
Rat	DMSO	13,970 ± 130	100
	Aflatoxin B_1	2930 ± 76	21
Mouse	DMSO	9033 ± 153	100
	Aflatoxin B_1	3212 ± 66	36
		Mg^{2+} activated RNA polymerase	
		pmole UMP incorporated/mg DNA	% of DMSO treated tissue
Rat	DMSO	625 ± 49	100
	Aflatoxin B_1	369 ± 56	59
Mouse	DMSO	443 ± 41	100
	Aflatoxin B_1	332 ± 15	75

Orotic acid incorporations: Vessels contained 2·5 ml Krebs–Ringer phosphate plus glucose. 2 μc [^{14}C]orotic acid and 200 mg liver slices per flask. Aflatoxin (final conc. 0·5 mM) added in DMSO, DMSO only added to controls. Triplicate incubations carried out for 30 min under O_2 with rapid shaking at 37°. For DNA and radioactivity assays see Materials and Methods section. Results are means ±S.E. RNA polymerase assays. Tissue slices (1 g/25 ml incubation medium) incubated as for the orotic acid incubation. Nuclei isolated at end of incubation period and polymerase assays carried out in triplicate.

then assayed. The results of these experiments are given in Table 2. In these experiments it was consistently observed that although aflatoxin B_1 inhibited both rat and mouse liver nuclear RNA polymerase activities, the inhibitions were not so great as those found when [^{14}C]orotic acid incorporations were examined. Experiments were carried out in which groups of 6 rats were injected intraperitoneally either with aflatoxin B_1 (7 mg/kg in 0·05 ml DMSO) or with the DMSO alone. The animals were killed 2 hr after the injections, the livers removed and pooled and the pool divided into two portions. One was used for the preparation of nuclear fractions, whose Mg^{2+} activated RNA polymerase activity was then assayed, the other was used for the preparation of liver slices, which were then incubated (60 min) with [^{14}C]orotic acid as before, and the total incorporation of [^{14}C]orotic acid into RNA determined. The results of 2 experiments are given in Table 3.

TABLE 3. EFFECT OF AFLATOXIN B_1 ADMINISTERED *in vivo* ON RNA POLYMERASE AND [^{14}C]OROTIC ACID INCORPORATION OF RAT LIVER ASSAYED *in vitro*

	Treatment	
	DMSO	Aflatoxin B_1
[^{14}C] Orotic-acid incorporation (dis./min/mg RNA)	12,772 ± 310 (100%)	4712 ± 125 (37%)
Mg^{2+} Activated RNA polymerase (pmole UMP incorporated/mg DNA)	1007 ± 34 (100%)	403 ± 14 (40%)

Groups of six rats injected i.p. with DMSO (0·05 ml) or aflatoxin B_1 (7 mg/kg) 2 hr before killing and removal of livers. Livers from each group divided into 2 pools of 3 livers. Slices for incubation and nuclear preparations made from each pool. [^{14}C]orotic acid incorporations, tissue incubated for 1 hr in presence of 1 μc [^{14}C]orotic acid in 3 ml incubation medium. Assay details as in Tables 1 and 2.

Influence of metabolism of aflatoxin B_1 on inhibition of RNA synthesis. Groups of 3 rats were given phenobarbitone in the drinking water as detailed in the Materials and Methods section. Control rats received water. Groups of control and phenobarbitone treated rats were injected with either aflatoxin B_1 (1·8 mg/kg) dissolved in DMSO or DMSO alone. Livers were excised 2 hr later and nuclear fractions prepared from the pooled livers from each group. Mg^{2+} dependent RNA polymerase activity was assayed with the results given in Table 4. It appeared that the treatment with phenobarbitone had virtually eliminated the inhibition of RNA polymerase caused by aflatoxin B_1. The effect of SKF 525 A on the inhibition of RNA polymerase by aflatoxin B_1 was also examined. Groups of 3 rats and mice were injected intraperitoneally with either SKF 525 A in saline or saline alone. Aflatoxin B_1 was administered to

TABLE 4. EFFECT OF PHENOBARBITONE ADMINISTRATION ON THE INHIBITION OF RAT LIVER RNA POLYMERASE BY AFLATOXIN B_1 *in vivo*

		Mg^{2+} activated RNA polymerase activity	
Pre-treatment	Injection	pmoles UMP incorporated/mg DNA	% of Control DMSO activity
Control	DMSO	1089 ± 88	100
Control	Aflatoxin B_1	326 ± 6	29
Phenobarbitone	DMSO	1241 ± 30	114
Phenobarbitone	Aflatoxin B_1	1067 ± 72	98

Groups of three rats given 0·1% sodium phenobarbitone in drinking water for 12 days before experiment. Controls received water. Groups of control and phenobarbitone treated rats injected i.p. with either DMSO alone or aflatoxin B_1 (1·8 mg/kg). Livers removed 2 hr later. Nuclei prepared and RNA polymerase assayed as in Materials and Methods section. Results are means of duplicate assays using duplicate nuclear preparations obtained from pooled liver tissue from three rats ±S.E.

rats 30 min after the SKF 525 A, and to the mice 40 min after the SKF 525 A. Animals were killed 2 hr after the aflatoxin B_1 injections, nuclei isolated and Mg^{2+} stimulated RNA polymerase activity assayed. It was found that the level of mouse liver RNA polymerase activity following treatment with aflatoxin B_1 was similar in the controls

and SKF 525 A treated animals. The inhibition in the case of the rat also appeared to be unchanged by pretreatment with SKF 525 A.

Metabolism of aflatoxin B_1 by mouse liver. Experiments were carried out in which mouse liver slices and mouse liver microsomal fractions were incubated *in vitro* in the presence or absence of SKF 525 A as detailed in the methods section. SKF 525 A was added to appropriate vessels at a final concentration of 5 mM.[14] At the end of the incubation period the flasks were cooled in ice and the liver homogenized before extracting the aflatoxin, the microsomal incubations were extracted directly. Disappearance of aflatoxin B_1 was estimated from replicate TLC plates by comparison with standards as detailed in the Materials and Methods section. It was found that the addition of SKF 525 A was without effect on the disappearance of aflatoxin B_1 from the incubations.

DISCUSSION

The absence of an effect of aflatoxin B_1 *in vivo* on RNA polymerase activity in mouse liver nuclei and incorporation of orotic acid into RNA is in agreement with the results of Akao *et al.*[2] and points to a link between inhibition of RNA synthesis and subsequent histological liver damage. The finding of Akao *et al.*[2] that kidney damage in mice is accompanied by an inhibition of RNA synthesis in the organ was also confirmed, and a similar inhibition of RNA synthesis was found in rat kidney, in which aflatoxin damage has been observed.[15] However some substances which inhibit RNA synthesis do not cause liver necrosis e.g. actinomycin D. The *in vitro* incubation studies showed that RNA synthesis in mouse liver could be almost as susceptible to inhibition by aflatoxin B_1 as rat liver RNA synthesis. This seems to exclude the possibility that no suitable receptors for the toxin exist in mouse liver chromatin. Although inhibition of Mg^{2+} activated RNA polymerase was demonstrated in nuclei isolated from both rat and mouse liver tissue after incubation *in vitro* with aflatoxin B_1, the inhibition was smaller than that reflected in orotic acid incorporation into RNA (Table 2). This difference may indicate that the nuclei were adversely affected by the prolonged incubation and subsequent isolation procedures and some evidence has been obtained that the activity of control nuclei was adversely affected to a greater extent than that of aflatoxin-treated nuclei (Neal, in preparation). The fact that the levels of RNA polymerase observed in nuclei isolated from incubated tissue were only 40–60 per cent of those normally found in nuclei isolated from freshly excised tissue (see Tables 1 and 2) is consistent with this hypothesis. The high tissue/medium ratio used in the experiments might also have had a bearing on this result, but the volume of medium could not be increased without increasing the amount of aflatoxin B_1 required for the experiments (only a limited amount of the toxin being available), and the amount of tissue could not be reduced if a satisfactory nuclear preparation was to be obtained. The orotic acid incorporation into RNA in tissue slices *in vitro* (Table 3) probably reflects the degree of inhibition of RNA polymerase in nuclei isolated from freshly excised livers. Clearly the differences in response to aflatoxin B_1 between the RNA synthesis seen in rat and mouse liver *in vivo* are almost eliminated when the aflatoxin is applied *in vitro*.

Since aflatoxin B_1 is capable of inhibiting mouse liver RNA production, why does it fail to do so *in vivo*, even at high dose levels? Aflatoxin B_1 is extensively metabolized

in the livers of both rat and mouse. There are differences between both the quantitative[16,17] and the qualitative[18] aspects of this metabolism. The differences in rates of metabolism are quite small ($< 1:1{\cdot}25$)[16,17] and would not be expected to be of great significance when comparing the aflatoxin B_1 doses employed in the present study. The role of metabolism in the inhibition of RNA polymerase is indicated in Table 4. Patterson and Roberts[19] have shown that metabolism of aflatoxin B_1 in rat liver by the microsomes is stimulated almost 10-fold by pretreatment with phenobarbitone. This presumably accounts for the loss of inhibition of the RNA polymerase in rats given phenobarbitone. This indicates that it is aflatoxin B_1 and not a metabolite (or at least not a metabolite whose formation is stimulated by phenobarbitone) which is responsible for the inhibition of RNA synthesis and secondly, that despite the normally high level of metabolism, changes in this level have a profound effect on inhibition of cellular processes. This result could have some relevance to the finding of McLean and Marshall[20] that pretreatment of rats with phenobarbitone resulted in a reduced induction of hepatocarcinoma by aflatoxin B_1.

Rat liver RNA polymerase responds to aflatoxin B_1 more like mouse liver if the microsomal enzyme systems are stimulated *in vivo* with phenobarbitone. The converse might follow the use of an inhibitor of microsomal enzymes which catalyse a wide range of reactions.[21–24] Schabort and Steyn[25] have shown that the aflatoxin B_1-4 hydroxylase activity, located in the microsomes of rat liver, is readily induced by phenobarbitone, and it appeared possible that SKF 525 A would have the reverse effect to that of phenobarbitone and cause mouse liver RNA polymerase activity to be inhibited by aflatoxin B_1 *in vivo*. This was not achieved in the present study. However SKF 525 A did not significantly affect the overall rate of metabolism of aflatoxin B_1 by mouse liver slices or microsomes *in vitro*, as judged by the disappearance of aflatoxin B_1 over a 1 hr incubation period. The concentration of SKF 525 A used (5 mM) was twice that reported as inhibiting the hydroxylation of acetanilide by 50 per cent.[14]

It is apparent that further detailed study of the mechanism of the uptake of aflatoxin into the nuclei of rat and mouse liver is necessary for an understanding of the differences in susceptibility of the two species to the toxin. The division into susceptible and non-susceptible species may well depend on fairly small differences in the balance of various metabolic pathways, rather than fundamental differences in the chemistry of the chromatins. It appears possible that by applying appropriate stimulators or inhibitors, a member of a susceptible species might become non-susceptible and vice versa.

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