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The Action of Aflatoxin B_1 on the Rat Liver

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The administration of a single dose of aflatoxin B_1 to the rat (7mg./kg. body wt.) results in the slow development of a periportal necrosis. Hepatic enzymes are released into the serum in the second 24hr. of the poisoning, closely preceding the onset of the necrosis, which is followed by a rise in serum alkaline-phosphatase activity and bilirubin concentration. Aflatoxin B_1 has been detected in the nucleus of the poisoned liver cell and *in vitro* it has been shown to interact with DNA. The toxin inhibits the production of nuclear RNA, probably by preventing the transcription of DNA by the RNA polymerase. It is proposed that the interaction of the toxin with DNA gives rise to its inhibitory action on mitosis and its necrogenic action.

Aflatoxin is the metabolite of certain strains of *Aspergillus flavus* that grow on groundnuts. It was the toxic agent present in feed mixes responsible for the major outbreak of turkey 'X' disease in England in 1960 and for subsequent minor outbreaks in ducklings and pheasants. This disease was not limited to poultry and has been reported in pigs (Loosmore & Harding, 1961) and calves (Loosmore & Markson, 1961). In all these cases there was pathological evidence of acute liver necrosis.

Aflatoxin is a mixture of closely related compounds, the structure of which was elucidated by Asao *et al.* (1963) and Van Dorp *et al.* (1963). Aflatoxin B_1 is the most toxic member to 1-day-old ducklings (Nesbitt, O'Kelly, Sargeant & Sheridan, 1962), and Butler (1964) demonstrated that in rats a single dose of aflatoxin B_1 produced a periportal liver necrosis. This necrosis develops comparatively slowly, reaching a maximum at 48–72 hr. after poisoning. In the rat maximum concentrations of the toxin occurred in the liver within 1 hr. after poisoning (Butler & Clifford, 1965). It is the purpose of the present investigation to determine the mechanism of action whereby aflatoxin B_1 produces the liver necrosis.

Some of the results obtained have been reported in a preliminary communication (Clifford & Rees, 1966).

MATERIALS AND METHODS

Animals. Male Wistar albino rats fed on the M.R.C. 41 diet without restriction (150g. body wt.) and bred from the same colony were used throughout this investigation.

Treatment of animals. Aflatoxin B_1 (unless otherwise indicated) was given at a dose of 7mg./kg. body wt. This dose had been previously shown by Butler (1964) to be the LD_{50}. The appropriate quantity of aflatoxin B_1 was dissolved in 0·2ml. of dimethylformamide and administered by gastric intubation. Control animals were given dimethylformamide alone.

DL-Ethionine was dissolved in 0·9% NaCl and the solution (25mg./ml.) maintained at 37°. It was administered intraperitoneally in two equal doses at zero time and 1hr. to give a total dose of 1g./kg. body wt. The control animals were given intraperitoneal injections of 0·9% NaCl of the same volume as given to the experimental animals.

Carbon tetrachloride was administered by gastric intubation at a dose of 1·25ml./kg. body wt. and was given as a 1:4 (v/v) solution in liquid paraffin. The control animals were similarly treated with liquid paraffin.

Hydrocortisone succinate, L-tryptophan and puromycin hydrochloride dissolved in 0·9% NaCl were administered intraperitoneally at a dose rate of 2, 136 and 3·5mg./100g. body wt. respectively.

The animals were killed by cervical dislocation at the appropriate times after dosage. For the measurement of serum enzymes the animals were anaesthetized with ether and the blood was collected from the carotid arteries into tubes. After the blood had clotted, the tubes were centrifuged and the serum was separated.

Chemicals. Cytochrome *c*, ATP and AMP were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Puromycin hydrochloride was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

L-Tryptophan was obtained from British Drug Houses Ltd., Poole, Dorset.

DL-Ethionine was supplied by Calbiochem, Los Angeles, Calif., U.S.A., and was chromatographically pure.

Phosphoenolpyruvate and pyruvate kinase were supplied by C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Aflatoxin B_1. Pure aflatoxin B_1 was a gift from Dr de Iongh, Unilever Ltd., Vlaardingen, The Netherlands.

A crude mixture of aflatoxins was a gift from the Medical

Research Council and aflatoxin B_1 was separated from this mixture by a thin-layer chromatography technique devised by W. Lijinsky (personal communication).

Determinations. The accumulation of soluble DL-[1-^{14}C]-leucine in liver slices was determined by the method of Manchester & Young (1960), who followed the accumulation of amino acids in rat diaphragm.

Ion movements by liver slices were measured by the method described by McLean (1960).

Serum and liver enzyme determinations (glutamate dehydrogenase, isocitrate dehydrogenase and malate dehydrogenase) were measured by the method described by Rees & Sinha (1960). Bilirubin was determined by the method of Haslewood & King (1937). Alkaline phosphatase was determined by the method of King & Armstrong (1934).

The induction of tryptophan pyrrolase was assayed by the method of Feigelson & Greengard (1961). The μmoles of kynurenine produced/g. wet wt. of liver was plotted against incubation time and the slope of the line during the linear phase gave the enzyme activity.

Total lipid extraction was by the method of Folch, Lees, & Sloane-Stanley (1957).

RNA was determined by the orcinol–$FeCl_3$ method of Greenbaum & Slater (1957).

ATP determinations were made by a fluorimetric technique described by Dydynska & Wilkie (1966).

Adenosine triphosphatase was measured by the method described by Recknagel & Anthony (1959).

Oxidative phosphorylation was measured by the method described by Judah & Williams-Ashman (1951).

Preparation of tissue fractions. Mitochondria were isolated from 10% liver homogenates in 0·25 M-sucrose solution by the method of Schneider (1948).

Nuclei were isolated from liver homogenates in 0·25 M-sucrose solution containing $CaCl_2$ (5 mM) by the procedure described by Rees & Rowland (1961).

For microsomes, the liver was homogenized in twice its weight of a medium containing $MgCl_2$ (10 mM), KCl (25 mM), tris buffer, pH 7·8 (35 mM), and sucrose (0·15 mM) (Rendi & Hultin, 1960). The homogenate was centrifuged for 10 min. at 10000*g* to remove cell debris, nuclei and mitochondria, leaving the 'mitochondria-free supernatant'. For the preparation of microsomes the supernatant was centrifuged in a Spinco model L preparative centrifuge (no. 40 rotor) for 50 min. at 105000*g*. After the supernatant had been decanted the microsomal pellet was resuspended in the initial preparative medium to give a final volume of 0·4 ml./g. wet wt. of liver.

Histology. A piece of liver from each animal used was fixed in 4% formaldehyde in 0·9% NaCl. Paraffin sections 5–7 μ thick were stained with Ehrlich's acid haematoxylin and eosin; frozen sections were stained with Sudan IV.

Radioactive-precursor-incorporation experiments. All the radioactive materials were obtained from The Radiochemical Centre, Amersham, Bucks. The specific activity of the DL-[1-^{14}C]leucine was 36·6 mc/m-mole, of the [6-^{14}C]-orotic acid monohydrate was 30·0 mc/m-mole and of the [^{32}P]phosphate was 7·0 mc/m-mole.

Incorporation *in vivo* of [6-^{14}C]orotic acid into the RNA of liver-cell nuclei and into the total liver nucleotides. Animals received a caudal-vein injection of [^{14}C]orotic acid (100 μc/kg. body wt.) dissolved in 0·2 ml. of 0·9% NaCl. After 15 min. the animals were killed and the livers removed. A small portion of the liver (about 300 mg.) was homogenized in ice-cold 0·6 N-$HClO_4$ and centrifuged at 0° in an MSE Major refrigerated centrifuge at 2000*g* for 2 min. The residue was re-extracted with 0·2 N-$HClO_4$ and the combined supernatant fluids were read at 260 mμ in the Unicam SP. 500 spectrophotometer. A portion of this sample was neutralized and the radioactivity was determined in a Nuclear–Chicago scintillation counter. The remainder of the liver was homogenized in a sucrose–$CaCl_2$ medium for the isolation of the nuclei according to the method of Rees & Rowland (1961). The nuclear fraction was extracted with $HClO_4$ for the determination of the RNA according to the method of Rees & Rowland (1961), and the resulting powder was then extracted in 2 ml. of 5% (w/v) trichloroacetic acid for 15 min. at 90° and centrifuged. The supernatant was divided into two equal parts. One was neutralized with 0·3 N-KOH and portions were taken to be counted in the Nuclear–Chicago scintillation counter (720 series) for two 2 min. periods. The results were corrected for background and then to 100% efficiency. The other half of the supernatant was diluted to 6 ml. and portions were taken for RNA estimations by the method of Greenbaum & Slater (1957), employing the correction for DNA.

Incorporation *in vivo* of DL-[1-^{14}C]leucine into liver and kidney proteins. Control and poisoned rats were given a caudal-vein injection of 0·2 ml. of 0·9% NaCl containing DL-[1-^{14}C]leucine (100 μc/kg. body wt.). After 15 min. the animals were killed, and the livers and kidneys were removed and homogenized in 0·25 M-sucrose. Portions of the tissue suspension were treated with an equal volume of 20% (w/v) trichloroacetic acid and the precipitated proteins were washed and dried by the method described by Rees & Rowland (1961).

Incorporation *in vitro* of ^{14}C-labelled amino acids into proteins and [6-^{14}C]orotic acid into the RNA of liver slices. Liver slices (total about 200 mg.) were incubated in 3 ml. of Krebs–Ringer phosphate (Umbreit, Burris & Stauffer, 1959) at 37° in Warburg flasks, the gas phase being oxygen. After 5 min. equilibration the labelled compound was added from the side arm of the flask (1·0 μc in 0·2 ml. of Krebs–Ringer phosphate). With the ^{14}C-labelled amino acid incorporation the reaction was stopped with 2 ml. of 20% (w/v) trichloroacetic acid and the protein extraction was carried out by the method described by Rees & Rowland (1961). With the [6-^{14}C]orotic acid incorporation the reaction was stopped with ice-cold 10% (w/v) $HClO_4$ and the RNA was extracted by the method described by Rees & Rowland (1961).

Incorporation *in vitro* of DL-[1-^{14}C]leucine into proteins of the mitochondria-free supernatant of microsomes. Each incubation tube contained ^{14}C-labelled amino acid (1 μc), 2 μmoles of ATP, 10 μmoles of phosphoenolpyruvate and 100 μg. of pyruvate kinase, to which was added either 0·7 ml. of the mitochondria-free supernatant or 0·4 ml. of resuspended microsomes and 0·3 ml. of final supernatant. The final volume was 1·5 ml., the gas phase was air and incubation was carried out at 37° for 20 min. The reaction was stopped with an equal volume of 20% (w/v) trichloroacetic acid and the proteins were extracted as described above for liver slices.

Preparation of dried protein residues for measurement of radioactivity. The protein residue was ground in a pestle and mortar and then compressed with a stainless-steel pestle on a planchet of 1 cm.^{2} surface area. All samples were counted for 5 min. in an end-window Geiger–Müller

counter (type EHM2/5; General Electric Co.). The results were corrected for background and adjusted for infinite thickness.

Incorporation *in vitro* of [^{32}P]phosphate into RNA and phospholipid of liver slices. The procedure was as described for [1-^{14}C]leucine incorporation except that 0·1ml. of inorganic [^{32}P]phosphate (100μc/ml.) was put into the side arm of the Warburg flask. The extraction was carried out by the method of Davidson, Frazer & Hutchison (1951). The final extracts were counted in a liquid counter (thin-walled B^{12}; 20th Century Electronics Ltd.). Measurements were corrected for isotope decay, background and dead time of the instrument.

RESULTS

The histological changes in the livers of rats after poisoning with aflatoxin B_1 in the present investigation were similar to those described by Butler (1964). By 12hr. after poisoning there was an occasional parenchymal cell in the periportal zone showing histological change and at 24hr. in this region of the lobule necrotic cells were apparent. Finally by 72hr. there was a well-developed periportal necrosis.

After administration of aflatoxin B_1 the rats were killed at 24, 48 and 72hr. They were exsanguinated, the sera separated and the livers removed. Isocitrate dehydrogenase, glutamate dehydrogenase and malate dehydrogenase were determined in the sera and the results are given in Table 1. Up to 24hr. after poisoning there was little rise in the serum activities of these enzymes but in the following 24hr. a large increase was detected followed by a return to near-normal serum activities by 72hr. These changes in serum enzyme activities are in contrast with the results for other hepatotoxic agents, where a release of hepatic enzymes into the serum may be detected within 3hr. of poisoning (see Rees, 1964). The fall in the activities of these enzymes in the livers of the poisoned rats (Table 1) indicated that this was the source of the enzymes appearing in the serum.

It was observed that the serum of the poisoned animals showed a bilirubinaemia, and analysis (Table 2) revealed that by 96hr. after poisoning there was a rise in both the bilirubin concentration and alkaline-phosphatase activity. It was concluded that the necrotic cells in the periportal zone of the liver lobule had obstructed biliary flow.

Previous studies on the biochemical changes underlying the development of experimentally induced liver injury have revealed in some instances that damage to mitochondria and in others that failure in the enzyme systems involved in maintaining the ionic composition of the cells preceded the onset of the histological necrosis. Both these were examined in the livers of rats poisoned with aflatoxin B_1. Oxidative phosphorylation by mitochondrial preparations from the livers of poisoned rats was compared with that of those from control rats. Even up to 24hr. after poisoning, with a range of substrates no changes in respiration or P/O ratios could be detected. There was, however, a small fall (26 and 35% at 3 and 5hr. after poisoning respectively) in the ATP concentrations in the livers of poisoned rats. There was no alteration in adenosine-triphosphatase activity.

Table 2. *Alkaline-phosphatase activity and bilirubin concentration in the serum during aflatoxin B_1 poisoning*

The results are the means ± S.E.M. for three animals.

Time after poisoning (hr.)	Alkaline-phosphatase activity (King–Armstrong units/100ml. of serum)	Concn. of bilirubin (mg./100ml. of serum)
0	27·7±0·78	0
24	29·3±0·78	0
48	29·7±0·80	0
72	32·0±0·33	4·7±0·33
96	53·7±3·5	20±1·15

Table 1. *Activities of malate dehydrogenase, isocitrate dehydrogenase and glutamate dehydrogenase in liver homogenates and serum after aflatoxin B_1 poisoning*

Results for liver are expressed as μmoles of nicotinamide nucleotide reduced/g. wet wt. of liver/hr. and are the means ± S.E.M. of at least 12 control rats and the means ± S.E.M. of three rats at each time-interval. Results for serum are given as μl. of O_2/ml./hr. and are the means ± S.D. of four rats at each time-interval.

Time after poisoning (hr.)	Activity in homogenate			Activity in serum		
	Isocitrate dehydrogenase	Malate dehydrogenase	Glutamate dehydrogenase	Isocitrate dehydrogenase	Malate dehydrogenase	Glutamate dehydrogenase
0	838±31·0	558±17·5	258±10·3	3±0·8	3±0·9	3±0·8
24	796±20·1	606±21·2	205±21·0	5±2·4	4±0·8	2±0·9
48	800±20·8	549±35·8	197±26·0	158±21·0	60±0·9	41±4·8
72	583±12·0	420±28·9	145±7·5	10±1·6	18±2·2	5±1·7
96	410±11·5	380±11·5	125±18·6	10±1·6	20±2·6	5±2·4

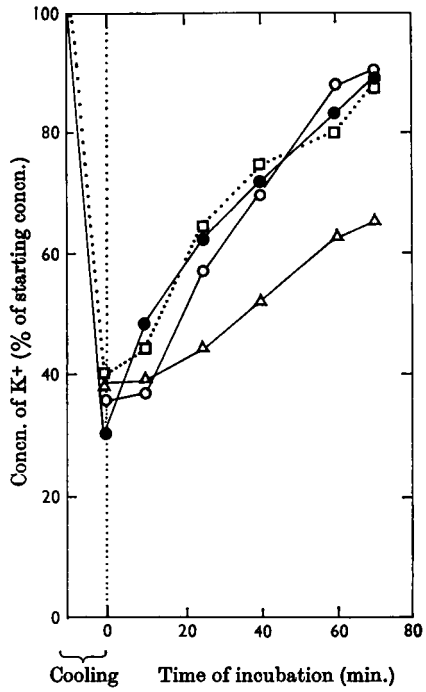

Fig. 1. Percentage reaccumulation of K^+ by liver slices after cooling for 35min. Experimental conditions and treatment of animals are as described in the Materials and Methods section. □, Control rat; ●, aflatoxin B_1-treated rat (3hr.); ○, aflatoxin B_1-treated rat (21hr.); △, ethionine-treated rat.

The ability of liver slices from control and aflatoxin B_1-poisoned rats to reaccumulate K^+ after cooling was examined. These experiments were to test the integrity of the sodium-pump mechanism (Judah, Ahmed & McLean, 1964). As there was a fall in the ATP concentrations of the liver of the aflatoxin B_1-poisoned rat, a comparison was made with slices prepared from the livers of rats poisoned with ethionine. Farber, Shull, Villa-Trevino, Lombardi & Thomas (1964) have shown that in ethionine poisoning there is a rapid fall in the liver ATP concentration. The results of these experiments are given in Fig. 1, which shows the loss of K^+ from the slices during the cooling period with the subsequent reaccumulation when the slices were transferred to the Krebs–Ringer phosphate containing glucose (100mg./100ml.) at 37°. In contrast with the slices prepared from the livers of rats poisoned with ethionine, those from the aflatoxin B_1-treated rats even after 21hr. of poisoning readily reaccumulated K^+. The resting liver concentrations of K^+ and Na^+ of the livers of the aflatoxin B_1-poisoned rats were of the same order as those of the controls, whereas with ethionine poisoning the K^+ concentration was lower and the Na^+ concentration higher than those of the controls.

Table 3. *Incorporation* in vitro *of* [^{14}C]*leucine into liver slices of rats poisoned with aflatoxin* B_1

The results are the means of two control and two treated rats at each time-interval.

Time after poisoning (hr.)	Radioactivity incorporated into liver protein (counts/min. at infinite thickness)		Inhibition (%)
	Control rat	Treated rat	
1	2444	1410	42
3	2196	1037	53
6	2573	1332	48
18	2347	1309	44
24	2119	1060	50
50	2161	865	60

From the results of these experiments it was concluded that the death of the liver cell in aflatoxin B_1 poisoning was not primarily due to an inhibition in energy production nor to a disturbance of ion-transport mechanisms. The poison might have a more specific point of action in the cell and it was decided to study its effects on synthetic processes.

The incorporation of [^{14}C]leucine into the proteins of liver slices prepared from the livers of poisoned rats was compared with that of similar preparations from control animals. The results given in Table 3 show that by 1hr. after poisoning there was a marked inhibition in the incorporation of the amino acid and further that this inhibition could be detected throughout the period preceding the development of necrosis. There was no generalized disturbance in synthetic reactions, as the incorporation of [^{32}P]phosphate into the phospholipids and the RNA of liver slices was not inhibited until later in the poisoning (Table 4).

As an inhibition in amino acid incorporation occurred so early in the poisoning a more detailed study of this aspect was undertaken. The addition of aflatoxin B_1 *in vitro* to liver slices prepared from the livers of control rats resulted in an inhibition of the incorporation of [^{14}C]leucine into the proteins of the liver slice (Table 5). At an aflatoxin B_1 concentration *in vitro* of 0·03mM the degree of inhibition was of the same order as for slices prepared from the livers of poisoned rats receiving the LD_{50} dose. Also included in Table 5 are the results for other amino acids: the incorporation of both [^{14}C]alanine and ^{14}C-labelled mixed amino acids into liver proteins was inhibited.

In view of the results of the experiments with liver slices it was expected that microsomal pre-

Table 4. *Incorporation of* [^{32}P]*phosphate into the RNA and phospholipid of liver slices prepared from the livers of rats poisoned with aflatoxin* B_1

The results are the means of two control and two treated rats at each time-interval.

Time after poisoning (hr.)	Radioactivity incorporated (counts/min./μg. of P)					
	RNA			Phospholipid		
	Control rat	Treated rat	Inhibition (%)	Control rat	Treated rat	Inhibition (%)
3	4·1	4·2	Nil	2·7	2·6	Nil
29	3·3	2·0	39	3·1	2·2	28
50	4·6	3·1	32	3·9	2·2	44

Table 5. *Effect of the addition of aflatoxin* B_1 in vitro *on the incorporation of* ^{14}C*-labelled amino acids into the proteins of liver slices from control rats*

Slices were incubated in Krebs–Ringer phosphate containing 1 μc of amino acid for 1 hr. at 37°. The gas phase was oxygen. The results are the means of two control and treated rats at each time-interval. Aflatoxin B_1 was dissolved in chloroform and portions were added to the reaction vessels. The chloroform was removed by maintaining the vessels at 37° in the dark. Control vessels had chloroform added and were treated in a similar manner.

Amino acid used	Concn. of aflatoxin B_1(mM)	Radioactivity incorporated into liver protein (counts/min. at infinite thickness)	Inhibition (%)
DL-[1-^{14}C]Leucine	0	2307	—
	0·03	1134	51
	0·06	543	76
L-[U-^{14}C]Alanine	0	581	—
	0·03	442	24
Mixed U-^{14}C-labelled amino acids (from *Chlorella vulgaris* hydrolysate)	0	1317	—
	0·03	737	44

Table 6. *Incorporation of* [^{14}C]*leucine into microsomal preparations from the livers of rats poisoned with aflatoxin* B_1 *or carbon tetrachloride*

Treatment of animals, isolation of microsomes and incubation medium are described in the Materials and Methods section. The results are the means of two control and two treated animals at each time-interval.

Poison	Time after poisoning (hr.)	Radioactivity incorporated into microsomal protein (counts/min. at infinite thickness)		Inhibition (%)
		Control rat	Treated rat	
Aflatoxin B_1	3	196	205	Nil
	5	117	127	Nil
	16	137	152	Nil
Carbon tetrachloride	5	149	86	42

parations from the livers of poisoned rats would show a similar inhibition in amino acid incorporation. In Table 6 are given the results of incorporation experiments with microsomal preparations, and for purposes of comparison results are included for similar preparations from the livers of rats poisoned with carbon tetrachloride. The preparations from the livers of the aflatoxin B_1-poisoned

rats showed no inhibition in amino acid incorporation, in contrast with those from the livers of carbon tetrachloride-poisoned rats. Various concentrations of aflatoxin B_1 (0·015–0·3mM) were added *in vitro* both to the microsomal preparations and to mitochondria-free supernatants prepared from the livers of control rats, but no inhibitions in amino acid incorporation were detected.

Experiments with hepatotoxic agents such as carbon tetrachloride, ethionine and dimethylnitrosamine have shown that, when a slice prepared from the liver of the treated rat had a lowered amino acid incorporation, there was an inhibition in the incorporation *in vivo* of amino acids into liver proteins (Rees & Shotlander, 1963). With aflatoxin B_1 there was no inhibition in the incorporation *in vivo* of amino acids into liver proteins (Table 7). Also included in Table 7 are results for incorporation of amino acids into kidney proteins. The values were the same for control and poisoned rats, indicating that all the rats had received the same dose of ^{14}C-labelled amino acid. Experiments *in vivo* were not carried out in the second 24hr. period of the poisoning, as it was considered that the occurrence of necrotic cells in the liver of the poisoned rat would not permit an interpretation of the results. For comparison, experiments were also repeated with carbon tetrachloride-poisoned rats and the results are included in Table 7.

The results of these experiments *in vivo* were in complete contrast with the findings obtained with liver slices. In both these procedures incorporation of amino acid had been taken as a measure of protein synthesis. Therefore the effect of aflatoxin B_1 on the induction of a specific protein such as tryptophan pyrrolase was studied. Two methods of induction have been described, by administration of either substrate or cortisone. In the latter case the mechanism proposed is that cortisone stimulates nuclear RNA synthesis, in particular of m-RNA,* which in turn stimulates cytoplasmic protein synthesis (Greengard & Feigelson, 1962). The results of these experiments (Table 8) show that, whereas aflatoxin B_1 poisoning resulted in an inhibition in the cortisone-induced tryptophan pyrrolase, the substrate-induced enzyme was unaffected. These results are analogous to those obtained for actinomycin D (Greengard & Feigelson, 1962) and suggest that aflatoxin B_1 could be acting by inhibiting the production of m-RNA.

* Abbreviation: m-RNA, messenger RNA.

Table 7. *Incorporation* in vivo *of* [^{14}C]*leucine into liver and kidney proteins in rats poisoned with aflatoxin* B_1 *and into liver proteins of rats poisoned with carbon tetrachloride*

[^{14}C]Leucine was administered in 0·2ml. of 0·9% NaCl via the caudal vein. The animals were killed 15min. later, and the liver and kidney were removed and homogenized. Two samples were taken and the proteins were prepared for counting as described in the Materials and Methods section. The results are the means of the duplicate samples for two rats in each group.

		Radioactivity incorporated into protein (counts/min. at infinite thickness)			
		Liver		Kidney	
Poison	Time after poisoning (hr.)	Control rat	Treated rat	Control rat	Treated rat
Aflatoxin B_1	3	373	402	359	405
	18	408	422	260	280
Carbon tetrachloride	3	460	282	—	—

Table 8. *Effect of aflatoxin* B_1 *poisoning on the induction of tryptophan pyrrolase*

The results are expressed as μmoles of kynurenine formed/g. wet wt. of liver/hr. and are the means ± S.E.M. of three experiments of duplicate samples on the pooled livers of four rats. The rats were killed 3hr. after poisoning and the method of induction and enzyme assay are given in the Materials and Methods section.

		Induced liver activity		
Method of enzyme induction	Basal liver activity	Control rat	Treated rat	Inhibition (%)
Substrate	2·2±0·17	5·9±0·29	6·2±0·19	Nil
Cortisone	1·7±0·09	5·6±0·17	2·4±0·09	57

To examine this point further, the time-scale of the inhibition of incorporation of [^{14}C]leucine into liver slices from the livers of control rats was examined in the presence of aflatoxin B_1. The results of this experiment (Fig. 2) show that the inhibition took some 15min. to develop. That this effect was not due to an inhibition of the entry of amino acid into the slice in the presence of aflatoxin B_1 was shown by directly measuring this parameter by the method of Manchester & Young (1960). In contrast with the delayed onset of inhibition in amino acid incorporation, there was an immediate inhibition in the incorporation of [^{14}C]orotic acid into the RNA of the liver slice in the presence of aflatoxin B_1 (Fig. 3). Experiments (not reported in detail here) had shown that in this type of experiment most of the [^{14}C]orotic acid (more than 80%) was incorporated into the nuclear RNA. That nuclear RNA synthesis was inhibited by aflatoxin was demonstrated by measuring the incorporation *in vivo* of [^{14}C]orotic acid into the nuclear RNA of the liver of control and aflatoxin B_1-poisoned rats. There was no inhibition in the incorporation of orotic acid into the nucleotide pool. In two experiments, however, the inhibition of incorporation of the nucleotide precursors into nuclear RNA of the livers of aflatoxin-poisoned rats compared with the controls was 86 and 88% respectively.

Since aflatoxin B_1 inhibits the incorporation of the nucleotide precursors into the nuclear RNA there are two possible methods for its action: either by direct inhibition of the DNA-dependent RNA polymerase, or by an interaction of the toxic agent with the DNA, thus preventing the RNA polymerase transcribing the DNA. It has been shown that actinomycin D binds to the DNA and thus inhibits the DNA-dependent RNA synthesis (Reich & Goldberg, 1964). If such a reaction were to occur DNA would be expected to alter the characteristic absorption spectrum of aflatoxin B_1. We therefore examined the effects on the absorption spectrum of aflatoxin B_1 of adding various concentrations of calf-thymus DNA: the difference spectrum (Fig. 4) was similar to the difference spectrum given by actinomycin D in the presence of calf-thymus DNA (Reich & Goldberg, 1964). The aflatoxin and DNA were dissolved in 0·01 M-tris–hydrochloric acid buffer, pH 7·4, containing sodium chloride (0·01 M). In this medium aflatoxin B_1 has an absorption maximum of 370mμ (which is at 362mμ in ethanol). The final concentration of aflatoxin B_1 was 64·0 μM and the two concentrations of DNA used for curves *1* and *2* in Fig. 4 were 397·5 and 662·5 μM respectively with respect to DNA phosphorus. The maximum decrease in

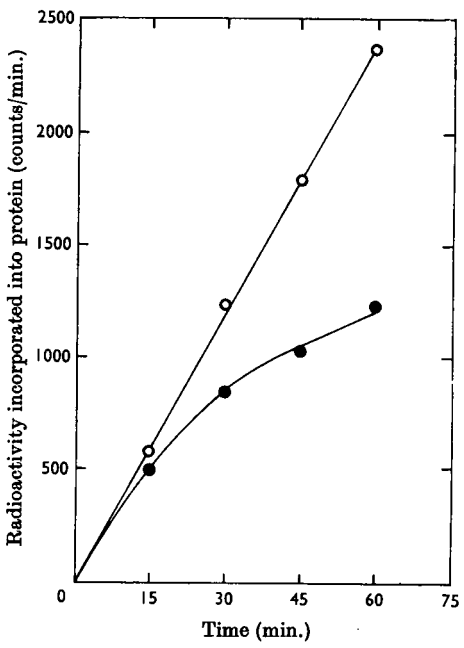

Fig. 2. Time-curve of the incorporation of [^{14}C]leucine into the proteins of liver slices. Experimental conditions are as described in the Materials and Methods section. ○, Control rat; ●, aflatoxin B_1-treated rat.

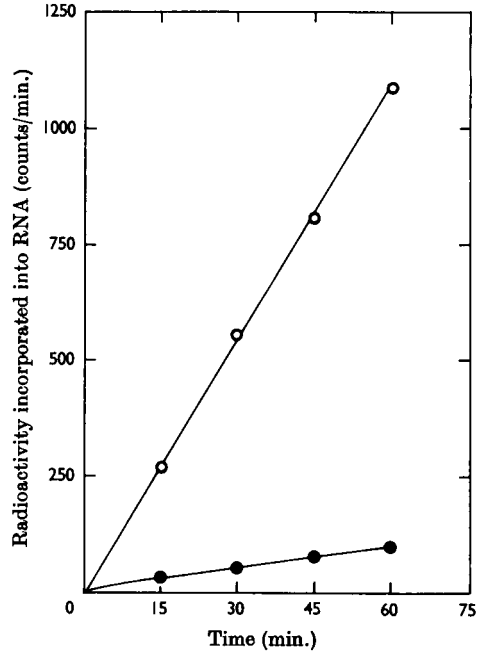

Fig. 3. Time-curve of the incorporation of [6-^{14}C]orotic acid into the RNA of liver slices. Experimental conditions are as described in the Materials and Methods section. ○, Control rat; ●, aflatoxin B_1-treated rat.

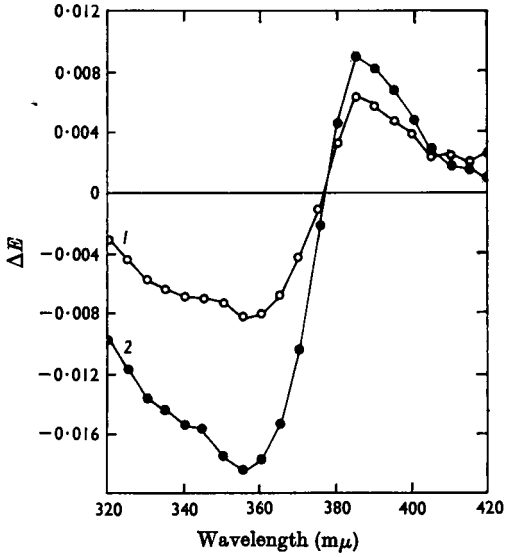

Fig. 4. Effect of calf-thymus DNA on the spectral properties of aflatoxin B_1, measured in a Cary model 14 (serial no. 273) recording spectrophotometer. Experimental conditions are as described in the text. The concentration of aflatoxin B_1 was 64·0 μM. Concentrations of DNA (with respect to DNA phosphorus) were: curve *1* (○), 397·5 μM; curve *2* (●), 662·5 μM.

absorption occurs at 355mμ. Similar results have been reported by Sporn, Dingman, Phelps & Wogan (1966).

To determine whether it was possible to detect aflatoxin B_1 *in vivo* in the nucleus of the liver cell, three male rats (150g. body wt.) were poisoned with 3mg. of aflatoxin B_1. After 30min. they were killed and the livers homogenized in 0·25 M-sucrose. The combined liver homogenates were then fractionated to yield nuclear, mitochondrial, microsomal and cell-sap fractions as described in the Materials and Methods section. The tissue fractions were extracted and the extracts were separated by thin-layer chromatography as described by de Iongh, van Pelt, Ord & Barrett (1964). The aflatoxin B_1-containing fractions were removed and assayed by the extinction at 362mμ. The results (Table 9) show that the nuclear fraction contained the second largest concentration of aflatoxin B_1.

Histological examination of the livers of male rats poisoned with aflatoxin B_1 showed little fat accumulation until the onset of necrosis. The total fat was extracted from the livers of the poisoned rats at 24hr. periods up to 4 days and there was a small transient rise at 72hr. The total lipids as a percentage of the wet weight of the liver rose from 3·7 to 5·9. Compared with the effects of an agent such as carbon tetrachloride (Rees & Shotlander, 1963) this was quite a small accumulation of fat.

Table 9. *Distribution of aflatoxin B_1 within the liver cell 30min. after poisoning*

The results are the means of duplicate analyses on the pooled livers of three poisoned animals. Dosage and methods of analysis are as described in the Materials and Methods section.

	Distribution of aflatoxin B_1	
	(μg./g. wet wt. of liver)	(% of that in homogenate)
Homogenate	0·52	(100)
Nuclear fraction	0·15	29
Mitochondria	0·05	10
Microsomal fraction	0·12	23
Cell sap	0·22	42

DISCUSSION

Aflatoxin B_1 is readily absorbed from the gut of the rat and can be detected in the liver of the poisoned rat within 30min. (Butler & Clifford, 1965). The results described in the present paper show that almost 30% of the aflatoxin B_1 in the liver cell has reached the nucleus after 30min. The aflatoxin B_1 will bind *in vitro* with DNA in a manner similar to that of actinomycin D. It has been suggested (Reich & Goldberg, 1964) that the structure of actinomycin D is such that it wedges into a groove of the DNA helix, with the quinoidal oxygen and amino group of the chromophore undergoing hydrogen-bonding. A consideration of the structure of aflatoxin B_1 (Asao *et al.* 1963; Van Dorp *et al.* 1963), with its five rings and groups capable of hydrogen-bonding, suggested that it might bind to DNA in a similar manner.

It would be expected that, if such an interaction of aflatoxin B_1 occurred with the DNA *in vivo*, it would result in an inhibition of the RNA polymerase and subsequently an inhibition in the synthesis of nuclear RNA. The experiments *in vivo* have revealed that at 3hr. after the administration of aflatoxin B_1 there is a large inhibition in the incorporation of nucleotide precursors into nuclear RNA. Similarly the addition of aflatoxin B_1 *in vitro* immediately inhibits the incorporation of orotic acid into the RNA by liver slices. It has been found by subcellular fractionation of the liver slices after orotic acid incorporation *in vitro* that most of the orotic acid is incorporated into the nuclear RNA.

Two experiments indicate that there is an

inhibition of m-RNA production after aflatoxin B_1 poisoning. There is not an immediate inhibition in the incorporation *in vitro* of leucine into protein on the addition of aflatoxin B_1 to the liver slice, but it takes some 15min. to develop. This is not due to a time-lag in the entry of aflatoxin B_1 as its effect on RNA synthesis is immediate, nor is it the result of a decreased entry of amino acid into the liver slice.

The second experiment indicating that aflatoxin B_1 was inhibiting the production of m-RNA was the study on the induction of tryptophan pyrrolase. Two methods of induction have been described, by substrate or cortisone. In the former case the mechanism of induction is now in dispute, and it has been suggested that this is not a true induction of new enzyme but rather an inhibition in the breakdown of the basal liver content of the enzyme (Schimke, Sweeney & Berlin, 1964). With cortisone induction of tryptophan pyrrolase it has been proposed that this is the result of the cortisone stimulating the production of m-RNA by the nucleus. This m-RNA in turn stimulates the synthesis of new enzyme. Both types of induced enzymes are inhibited by puromycin, whereas only the cortisone-induced tryptophan pyrrolase is inhibited by actinomycin D (Greengard, Smith & Acs, 1963). Likewise in the present investigation it was found that only the cortisone-induced enzyme production was inhibited by aflatoxin B_1.

The interaction of aflatoxin B_1 with DNA might also be expected to inhibit DNA polymerase in a similar manner to that in which the RNA polymerase is inhibited. If this should be the case it might explain the results of Legator, Zuffante & Harp (1965), namely that the addition of aflatoxin B_1 to cultured heteroploid embryonic lung cells inhibited DNA synthesis and mitosis and increased the formation of giant cells, and those of Lilly (1965), namely an inhibition in mitosis in the roots of the seedlings of *Vicia faba* after treatment with aflatoxin. De Recondo, Frayssinet, Lafarge & Le Breton (1965) found that the administration of aflatoxin B_1 to rats during the period of liver regeneration after partial hepatectomy resulted in an inhibition in DNA synthesis in the liver. This was not the result of an inhibition of the enzymes concerned with DNA synthesis, and De Recondo, Frayssinet, Lafarge & Le Breton (1966) concluded that the inhibition in DNA synthesis was caused by a failure of the DNA polymerase to transcribe the DNA in the livers of the poisoned rats.

There are many similarities between the biochemical actions of actinomycin D and aflatoxin B_1. Both compounds react with DNA and similar spectral changes are observed as a result of this interaction. The result of this interaction with both compounds is the decreased nuclear RNA synthesis and inhibition of m-RNA with associated inhibition of the cortisone induction of tryptophan pyrrolase. Both compounds fail to inhibit amino acid incorporation *in vivo* into liver proteins (Greengard & Acs, 1962; Greengard, *et al.* 1963). Actinomycin D, like aflatoxin B_1, produces a marked inhibitory effect on DNA synthesis in regenerating liver after partial hepatectomy (Hackmann, 1954; Philips, Schwartz, Sternberg & Tan, 1960).

At our present state of knowledge it is difficult to offer an explanation for the finding that slices from the livers of aflatoxin B_1-poisoned rats show a decreased amino acid incorporation into proteins, whereas *in vivo* it has not been possible to show this inhibition. The addition of aflatoxin B_1 *in vitro* to liver slices produced an inhibition in amino acid incorporation into proteins that was secondary to the inhibition in RNA synthesis. This might indicate that in the liver slices, in contrast with the liver *in vivo*, a far greater percentage of amino acid incorporation is dependent on newly formed m-RNA. Thus it would appear that it is not possible to compare the alterations occurring in amino acid incorporation into proteins in a slice with those of the intact liver where the poison is acting indirectly on the system. On the other hand, comparisons would be possible in situations where the poison is having a direct effect on the cytoplasmic ribosomes, such as in carbon tetrachloride and dimethylnitrosamine poisoning (Smuckler & Benditt, 1965; Emmelot & Benedetti, 1960).

Several different effects may be observed with these agents. First, liver microsomal preparations from rats poisoned with actinomycin D have a decreased ability to incorporate amino acids, whereas those from aflatoxin B_1-poisoned rats show no inhibition. With actinomycin D, Staehelin, Wettstein & Noll (1963) have shown that there is a decrease in the average size of ribosomal aggregates that can be isolated from liver of the poisoned rat, and it was considered that this is the direct result of a decreased m-RNA synthesis, m-RNA being essential for the integrity of the polyribosomes. It would thus be expected that microsomal preparations from such livers would have diminished amino acid incorporation. W. H. Butler (personal communication) has observed the presence of many free ribonucleoprotein particles in the cytoplasm of the liver cells by 6hr. after poisoning with aflatoxin B_1. It may be that these ribonucleoprotein particles are still in a form capable of incorporating amino acids. In this context Breuer & Davis (1964) have observed decreases in the average size of ribosomal aggregates isolated from the livers of adrenalectomized rats and these preparations are more active in cell-free

amino acid incorporation than those from unoperated rats. Further, this change, observed by W. H. Butler (personal communication), appears to be similar to that occurring after administration of carbon tetrachloride. Previous studies on biochemical changes associated with the rough endoplasmic reticulum had revealed marked changes in the livers of the rats poisoned with carbon tetrachloride, whereas preparations from rats poisoned with aflatoxin B_1 for a comparable time (5hr.) gave no detectable change (Clifford & Rees, 1966). It is thus concluded that alterations in morphological appearance of the polyribosomes will not necessarily indicate that a decreased amino acid incorporation will occur in experiments *in vitro*.

A further difference in action between these agents lies in their effect on the intact liver in the poisoned rat. Only the LD_{50} dose of aflatoxin B_1 produces a liver necrosis, which reaches its maximum by 3 days and is the cause of the death of the animal, whereas the LD_{50} dose of actinomycin D results in the death of the rat before 2 days. Death in this case appears to result from haemorrhages secondary to the injury to rapidly proliferating cells in the gut wall. It may be that under 2 days is too short a time to permit the biochemical lesion in the liver to manifest itself. In the regenerating liver, with its rapid turnover in cells, actinomycin D does cause cytological changes (Schwartz, Sodergren, Garofalo & Sternberg, 1965) similar to those in regenerating liver after poisoning with aflatoxin B_1 (Bernhard, Frayssinet, Lafarge & Le Breton, 1965).

From the results described in the present paper coupled with the studies on regenerating liver it would appear that the nucleus and in particular the DNA is the primary site of attack of aflatoxin B_1. There is no evidence that injury to mitochondria plays a major role in the necrotic action of the toxin, since both biochemically and morphologically (W. H. Butler, personal communication) no changes can be detected in these organelles in the early stages of the poisoning. On the other hand, the results obtained from studies with slices prepared from the livers of aflatoxin B_1-poisoned rats indicate that there is some disturbance in protein synthesis throughout the course of the poisoning. One may speculate whether such an inhibition could play a role in the cellular necrosis. Previous studies with hepatotoxic agents have shown that, where there is an inhibition in overall protein synthesis, necrosis does not necessarily develop, e.g. with ethionine (Robinson & Harris, 1961), carbon tetrachloride in rats treated with promethazine hydrochloride (Rees & Shotlander, 1963) and *tert.*-butylmethylnitrosamine (Rees & Shotlander, 1964). In these instances the inhibition in protein synthesis is non-specific in that there has been a large decrease in the bound cytoplasmic ribosomes resulting in an overall inhibition in protein synthesis.

With aflatoxin B_1 poisoning there is no inhibition in overall protein synthesis. However, at least as long as the interaction with the DNA persists there will be an inhibition in the synthesis of proteins that rely at that time on the formation of new m-RNA. There is no information as to the nature of these proteins. There is, however, evidence that alterations in cellular membranes play a role in the development of the histological necrosis in aflatoxin B_1 poisoning. This is indicated by the leakage of hepatic enzymes into the serum preceding the necrosis, as reported in the present paper, and by the finding of Theron (1965) that treatment of the poisoned ducklings with promethazine hydrochloride diminishes the degree of liver necrosis. This agent has previously been shown to inhibit permeability changes and necrosis due to the action of a number of toxic agents (Rees & Spector, 1961). It might be that it is the formation of proteins essential for the integrity of cellular membranes that has been completely blocked by the action of aflatoxin B_1.

The active constituents of aflatoxin are of different toxicities within a single species, and different species show a wide degree of susceptibility. It may well be that such variations in toxicity within a given species are a reflection of the ability of the toxins to interact with DNA, and and that the susceptibility of a species resides in the extent to which its particular type of DNA will bind with the aflatoxins.

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