Effects of the Hepatotoxic Agents Retrorsine and Aflatoxin B₁ on Hepatic Protein Synthesis in the Rat

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1. Aflatoxin and the pyrrolizidine alkaloid retrorsine inhibited the incorporation of labelled amino acids into rat liver and plasma proteins *in vivo*. Inhibition was greater and detected earlier with retrorsine (1 hr.) than with aflatoxin (3 hr.). 2. Both toxins affected the liver ribosomal aggregates, causing increases in the proportion of monomers plus dimers. The effect of retrorsine was greater than that of aflatoxin. 3. Incorporation of labelled amino acids into proteins of cell-free preparations of liver from rats treated with aflatoxin was lower than in control preparations. The main site of inhibition appeared to be the ribosomes. 4. Both toxins inhibited the incorporation of orotate into liver nuclear RNA 1 hr. after administration.

Retrorsine, a pyrrolizidine alkaloid, and aflatoxin B₁ are hepatotoxic and carcinogenic (Cook, Duffy & Schoental, 1950; Butler & Barnes, 1964; Butler, 1966). Within 48 hr. after a single oral LD₅₀ dose of retrorsine, rats develop severe liver damage, characterized by centrilobular necrosis and haemorrhage. These changes are very similar to those produced by dimethylnitrosamine, which is also hepatotoxic and carcinogenic (Barnes & Magee, 1954; Magee & Barnes, 1956). Svoboda & Saga (1966) reported that lasiocarpine, a pyrrolizidine alkaloid related to retrorsine, induced nuclear changes and a decrease in liver RNA concentration 30 min. after administration; total liver protein was lower than the control value 15 hr. after treatment with the alkaloid, suggesting impairment of hepatic protein synthesis.

In contrast with the pyrrolizidine alkaloids, aflatoxin B₁ induces in rats acute periportal liver necrosis, which is preceded by progressive swelling and degranulation of the endoplasmic reticulum of the parenchymal cells (Butler, 1966). Similar ultrastructural changes were reported to follow the administration of ethionine (Baglio & Farber, 1965), dimethylnitrosamine (Emmelot & Benedetti, 1960) and carbon tetrachloride (Smuckler & Benditt, 1965).

Inhibition of hepatic protein synthesis by aflatoxin B₁ was shown in enzyme-induction studies (Wogan & Friedman, 1965), in experiments

on the incorporation of labelled amino acids *in vivo* (Shank & Wogan, 1964) and with tissue slices and cell-free preparations (Smith, 1963, 1965). Aflatoxin B₁ also inhibits RNA synthesis *in vivo* (Sporn, Dingman, Phelps & Wogan, 1966; Lafarge, Frayssinet & De Recondo, 1965) and in tissue slices (Clifford & Rees, 1966).

The present paper describes changes in hepatic ribosomal aggregates after administration of aflatoxin B₁ or retrorsine to rats, and the effects of these compounds on incorporation of amino acids into liver and plasma proteins and on incorporation of orotate into liver nuclear RNA.

MATERIALS AND METHODS

Retrorsine and aflatoxin. Crystalline retrorsine was a gift from Dr A. R. Mattocks. Aflatoxin B₁ was prepared from crude aflatoxin (supplied by the Medical Research Council) by the method of Lijinsky & Butler (1966).

Isotopically labelled compounds. L-[1-¹⁴C]Leucine (specific radioactivity 8.5 mc/m-mole) and [6-¹⁴C]orotic acid (specific radioactivity 30 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.

Animals. Male Wistar-derived rats of the Porton strain, weighing 150–200 g., were maintained on M.R.C. diet 41B (Bruce & Parkes, 1956). They were kept without food overnight to decrease glycogen, which interferes with the preparation of ribosomes for the cell-free system and with the preparation of ribosome patterns. The hepatotoxins were given by stomach tube. Retrorsine (40 mg./kg. body wt.) was administered as a solution in 0.9% NaCl, and aflatoxin B₁ (7 mg./kg. body wt.) as a solution in dimethylformamide.

Leucine incorporation into proteins *in vivo*. At 1 hr. before the rats were killed 1 ml. of an aqueous solution containing 1 µC of [1-¹⁴C]leucine was injected intraperitoneally into

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each. The rats were anaesthetized with Nembutal (60mg./kg. body wt.) and the livers and samples of aortic blood were taken. Each liver was homogenized in 25ml. of 10% (w/v) trichloroacetic acid and centrifuged, and 1ml. of the supernatant was taken for determination of radioactivity in the acid-soluble fraction. Plasma and liver proteins were prepared and assayed for radioactivity as described by Villa-Treviño, Farber, Staehelin, Wettstein & Noll (1964).

Leucine incorporation into proteins in vitro. Ribosomes and pH5 fractions were prepared as described by Villa-Treviño, Shull & Farber (1963). The pellets were resuspended in water and adjusted to pH 7.6, and preparations having $E_{260}^{1\text{cm}}$ 100 and $E_{260}^{1\text{cm}}$ 35 were used as ribosomal and pH5 fractions respectively. Each millilitre of incubation mixture contained 0.2ml. of the ribosomal fraction, 0.3ml. of the pH5 enzyme fraction, 10 μ g. of pyruvate kinase, 1 μ C of [^{14}C]leucine, ATP (1mM), GTP (0.4mM), phosphoenolpyruvate (10mM), mercaptoethanol (8.5mM), NH_4Cl (75mM), magnesium acetate (6mM) and tris-HCl buffer, pH 7.4 (15mM). The samples were incubated for 1 hr. at 25°

before addition of 0.2ml. of a solution containing 10mg. of albumin/ml. in N-NaOH , and then incubated for a further 15 min. Then 10% (w/v) trichloroacetic acid was added and the precipitate was washed twice with propan-2-ol and assayed for radioactivity. This preparation incorporated linearly for at least 1 hr. (Villa-Treviño *et al.* 1963).

Orotate incorporation into RNA. At 15 min. before the rats were killed 0.5ml. of an aqueous solution containing 2.5 μ C of [^{14}C]orotate was injected via the portal vein under Nembutal anaesthesia (60mg./kg. body wt.). A purified fraction of liver nuclei was isolated and RNA extracted by a sodium dodecyl sulphate-phenol method as reported by Villa-Treviño, Shull & Farber (1966). RNA was purified and assayed for radioactivity as described by Villa-Treviño (1967); a solution of 1mg. of RNA/ml. was assumed to have $E_{260}^{1\text{cm}}$ 20.

Analysis of ribosomal aggregates. This was carried out by zone centrifugation as described by Villa-Treviño (1967). The sedimentation patterns were plotted (Figs. 1 and 2) and the ratio of the area of the monomers plus dimers

Table 1. *Effect of aflatoxin and retrorsine on incorporation of [^{14}C]leucine into liver proteins in vivo*

Rats were killed at increasing time-intervals after the administration of aflatoxin (7mg./kg. body wt.) or retrorsine (40mg./kg. body wt.). Incorporation of [^{14}C]leucine into total liver proteins was measured as described in the text. The numbers of rats per group are given in parentheses. Specific radioactivities are given as means $\pm$ s.e.m. *P* values were determined by Student's *t* test.

Time (hr.) ...	Specific radioactivity (disintegrations/min./mg.)				
	0.5*	1	3*	6	8
Control	193 $\pm$ 17 (5) (100%)	303 $\pm$ 21 (5) (100%)	171 $\pm$ 7 (4) (100%)	393 $\pm$ 47 (4) (100%)	450 $\pm$ 22 (5) (100%)
Aflatoxin	162 $\pm$ 53 (4) (84%)	297 $\pm$ 22 (5) (98%)	97 $\pm$ 22 (4) (57%) <i>P</i> < 0.05	235 $\pm$ 31 (4) (60%) <i>P</i> < 0.05	275 $\pm$ 26 (5) (61%) <i>P</i> < 0.001
Retrorsine	152 $\pm$ 38 (4) (79%)	84 $\pm$ 11 (5) (28%) <i>P</i> < 0.001	62 $\pm$ 9 (4) (36%) <i>P</i> < 0.001	128 $\pm$ 14 (5) (33%) <i>P</i> < 0.001	111 $\pm$ 9 (5) (25%) <i>P</i> < 0.001

* The groups measured at 0.5 hr. and at 3 hr. received half the dose of labelled amino acid received by the other groups.

Table 2. *Effect of aflatoxin and retrorsine on incorporation of [^{14}C]leucine into plasma proteins in vivo*

The results are from the same animals as in Table 1. Specific radioactivities are given $\pm$ s.e.m., with the numbers of animals per group in parentheses.

Time (hr.) ...	Specific radioactivity (disintegrations/min./mg.)				
	0.5*	1	3*	6	8
Control	106 $\pm$ 33 (5) (100%)	154 $\pm$ 17 (5) (100%)	71 $\pm$ 15 (4) (100%)	200 $\pm$ 43 (4) (100%)	171 $\pm$ 41 (5) (100%)
Aflatoxin	90 $\pm$ 17 (4) (85%)	145 $\pm$ 26 (5) (95%)	21 $\pm$ 4 (4) (30%) <i>P</i> < 0.05	80 $\pm$ 12 (4) (40%) <i>P</i> < 0.05	101 $\pm$ 15 (5) (59%) <i>P</i> < 0.05
Retrorsine	100 $\pm$ 24 (4) (95%)	37 $\pm$ 4 (5) (24%) <i>P</i> < 0.001	13 $\pm$ 3 (4) (18%) <i>P</i> < 0.01	47 $\pm$ 8 (5) (23%) <i>P</i> < 0.01	38 $\pm$ 7 (5) (22%) <i>P</i> < 0.02

* The groups measured at 0.5 hr. and at 3 hr. received half the dose of labelled amino acid received by the other groups.

(hatched) to that of the larger aggregates was determined by cutting out and weighing the sections, the results being expressed as percentages of the total area.

Radioactivity assays. These were carried out by scintillation counting, as described by Villa-Treviño (1967).

RESULTS

After the oral administration of aflatoxin B₁ there was inhibition of incorporation of [¹⁴C]leucine into total liver and plasma proteins at 3 hr., and this inhibition persisted until 8 hr., the longest period tested. At 0.5 hr. and 1 hr. after treatment there was no significant inhibition (Tables 1 and 2). In contrast, retrorsine inhibited leucine incorporation into total liver and plasma protein markedly at 1 hr. after oral administration (Tables 1 and 2). The radioactivity of the acid-soluble fraction was always higher in the livers of the treated rats, suggesting that the labelled amino acid reached the intracellular pools at least as well in the treated as in the control groups.

Table 3. *Effect of aflatoxin in vivo on incorporation of [¹⁴C]leucine into proteins by a liver system in vitro*

Rats received 7 mg. of aflatoxin/kg. body wt. Details of the liver ribosomal pH 5 enzyme system are given in the text. In the experiments on the effect of aflatoxin, each result represents the mean of triplicate determinations in three experiments.

	Incorporation	
	(%)	(μ moles of leucine/mg. of protein)
Complete system	100	143
Minus ATP	9	13
Minus ribosomes	3	4
Minus pH 5 fraction	17	22
Zero time	1	< 1
Control	100	185
Aflatoxin (1 hr.)	74	135
Control	100	252
Aflatoxin (3 hr.)	53	134

Table 4. *Incorporation of [¹⁴C]leucine in vitro into proteins of interchanged liver preparations from rats treated with aflatoxin in vivo*

The experimental conditions were as in Table 3.						
Fraction	...	...	Source of fraction		Incorporation	
			Ribosomes	pH 5 fraction	(%)	($\mu\mu$ moles of leucine/mg. of protein)
			Control	Control	100*	251.3
			Aflatoxin	Aflatoxin	53*	133.7
			Control	Aflatoxin	84†	209.4
			Aflatoxin	Control	59†	148.2

* Mean of determinations made in three animals, each determination in triplicate.

† Mean of determination in triplicate from pooled livers of three animals.

Table 5. *Effect of aflatoxin on liver ribosomal aggregates*

The dose of aflatoxin was 7 mg./kg. body wt. and the experimental conditions and the calculations of the ratios of polyribosomes to monomers plus dimers were as described in the text. The numbers of animals per group are given in parentheses. Values for the percentage of area under the curve are given as means $\pm$ S.E.M. *P* values were determined by Student's *t* test.

	% of area under the curve		Increase in proportion of monomers plus dimers (%)
	Polyribosomes	Monomers plus dimers	
Control	71 $\pm$ 1	29 $\pm$ 1 (5)	—
Aflatoxin (1 hr.)	64 $\pm$ 2	36 $\pm$ 2 (5) (<i>P</i> = 0.01)	24
Aflatoxin (3 hr.)	60 $\pm$ 3	40 $\pm$ 3 (5) (<i>P</i> < 0.02)	39
Aflatoxin (6 hr.)	50 $\pm$ 4	50 $\pm$ 4 (5) (<i>P</i> < 0.001)	72

Fig. 1. Ribosomal patterns from post-mitochondrial supernatant fractions of livers from rats killed before (a; control) and after treatment with aflatoxin (7mg./kg. body wt.) for 1 hr. (b), 3 hr. (c) and 6 hr. (d). The hatched areas represent monomers plus dimers.

Fig. 2. Ribosomal patterns from post-mitochondrial supernatant fractions of livers from rats killed before (a; control) and after treatment with retrorsine (40mg./kg. body wt.) for 1 hr. (b), 3 hr. (c) and 6 hr. (d). The hatched areas represent monomers plus dimers.

Treatment of the rats with aflatoxin also caused a progressive inhibition of incorporation of [14 C]-leucine into proteins of the cell-free system (Table 3). The magnitude of this inhibition was about the

same as that observed at 3 hr. *in vivo*. When the ribosomal and pH 5 fractions from the control and aflatoxin-treated livers were interchanged the main site of inhibition appeared to be the ribosomes (Table 4).

The effects of aflatoxin on the ribosomal aggregates are shown in Fig. 1 and Table 5. There was some increase in the proportion of monomers plus dimers at 1 hr., when no inhibition of amino acid incorporation was found (Table 1). This increment (24%) was small, however, and it did not become much larger at 3 hr. (Table 5). With retrorsine (Fig. 2) the increments in the monomers plus dimers were 72%, 72% and 107% at 1, 2, and 6 hr. respectively. Hence the alkaloid had considerably greater effects at earlier times on the ribosomal aggregates than aflatoxin.

However, with both hepatotoxins the incorporation of orotate into nuclear RNA was significantly diminished 1 hr. after administration (Table 6).

DISCUSSION

The ultrastructural changes induced by aflatoxin (Butler, 1966) and the present results (Table 4) suggest that the defect in protein synthesis induced by this compound is in the ribosomes. An inhibitory effect of aflatoxin on amino acid incorporation in cell-free preparations has been reported by Smith (1965), who suggested an action on the activation of amino acids. This effect was prevented by thiol compounds and was not similar to the inhibition described above, since the system used by us *in vitro* contained 8.5 mM-mercaptoethanol (see the Materials and Methods section), which is sufficient to prevent the effects reported by Smith (1965). There is evidence that suggests that the mechanism of inhibition of protein synthesis by aflatoxin is similar to that of actinomycin D *in vivo* (Lafarge *et al.* 1965; Sporn *et al.* 1966; Clifford & Rees, 1966) and in cell culture (Legator, Zuffante & Harp, 1965). The early inhibition of orotate incorporation into RNA observed (Table 6) was not as marked as that reported by other workers (Sporn *et al.* 1966; Clifford & Rees, 1966), but it did precede the inhibition of incorporation of leucine into proteins. The effect of aflatoxin on the ribosomal aggregates was similar to that described for actinomycin by Staehelin, Wettstein & Noll (1963) and may indicate a similar mechanism of action involving inhibition of messenger-RNA synthesis with consequent failure of formation of the aggregates. However, the morphological changes induced in the liver by aflatoxin differ from those due to actinomycin D, indicating that there must be underlying biochemical differences as well as the similarities discussed above.

Table 6. *Effect of aflatoxin and retrorsine on the incorporation of [¹⁴C]orotate into liver nuclear RNA in vivo*

Rats were killed at increasing time-intervals after administration of aflatoxin (7 mg./kg. body wt.) or retrorsine (40 mg./kg. body wt.). Incorporation of [¹⁴C]orotate into nuclear RNA was measured as described in the text. The numbers of rats per group are given in parentheses and the differences of the treated from the control groups are expressed as percentages. Specific radioactivities are given as means $\pm$ s.e.m. *P* values were determined by Student's *t* test.

Time (hr.)	...	10 ⁻³ $\times$ Radioactivity in nuclear RNA (disintegrations/min./20 E units)		
		0.5	1	3
Control		74.3 $\pm$ 8.1 (4)	62.1 $\pm$ 4.9 (5)	74.4 $\pm$ 5.2 (4)
Aflatoxin		61.1 $\pm$ 6.8 (4) (81%)	38.8 $\pm$ 2.6 (6) (62%) (<i>P</i> < 0.01)	48.1 $\pm$ 8.4 (5) (64%) (<i>P</i> < 0.05)
Retrorsine		58.2 $\pm$ 10.8 (4) (78%)	31.7 $\pm$ 3.7 (5) (51%) (<i>P</i> < 0.01)	48.0 $\pm$ 6.8 (5) (65%) (<i>P</i> < 0.02)

The morphological resemblances between the acute liver damage induced by the pyrrolizidine alkaloids and that induced by dimethylnitrosamine suggest a similar biochemical mechanism. Retrorsine does resemble dimethylnitrosamine more than aflatoxin in its earlier and greater inhibition of amino acid incorporation (Tables 1 and 2) and in its considerably greater effect on the ribosomal aggregates (Figs. 1 and 2). It has been suggested that methylation of messenger RNA may occur in the livers of rats treated with dimethylnitrosamine and that this methylation may be related to the inhibition of hepatic protein synthesis (Mizrahi & de Vries, 1965; Villa-Treviño, 1967). Culvenor, Dann & Dick (1962) have proposed that the pyrrolizidine alkaloids might act as alkylating agents *in vivo*. If this is so, the inhibition of hepatic protein synthesis by retrorsine (Table 1) might also follow alkylation of messenger RNA.

However, inhibition of amino acid incorporation does not precede inhibition of nuclear RNA synthesis by retrorsine (Table 6), and in this way the alkaloid resembles aflatoxin (Table 6) and actinomycin D rather than dimethylnitrosamine. Svoboda & Saga (1966) noted similarities between the early ultrastructural changes induced in rat liver by the pyrrolizidine alkaloid lasiocarpine and by actinomycin D. Assuming that lasiocarpine behaves like retrorsine, our results (Table 1) support their prediction that the alkaloid would inhibit protein synthesis. The mechanism of inhibition by retrorsine is far from clear and appears to have features in common with inhibition both by aflatoxin and by dimethylnitrosamine.

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