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EFFECT OF PHENOBARBITAL PRETREATMENT ON THE ABILITY OF AFLATOXIN B_1 TO INHIBIT RIBONUCLEIC ACID SYNTHESIS IN RAT LIVER

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Abstract—Pretreatment of rats with phenobarbital conferred a significant degree of protection against the ability of aflatoxin to inhibit synthesis *in vivo* of nuclear and cellular RNA in liver. The increase in liver weight brought about by phenobarbital treatment appeared to result from cellular enlargement as opposed to cellular proliferation.

THE METABOLISM of aflatoxin B_1 in animals results in the production of metabolites whose chemical nature and relationship to liver damage remain largely to be explored. Although one such conversion, the hydroxylation of aflatoxin B_1 to M_1 by liver microsomes, appears to represent a step toward detoxification,[1] recent evidence raises the question of whether aflatoxin B_1 or a metabolite is the proximate toxin, at least in regard to the inhibition of RNA synthesis.[2,3] Since various pathways of metabolism by liver microsomes, including hydroxylation, are stimulated by phenobarbital,[4] it was of interest to determine what effect the induction of microsomal enzymes by phenobarbital would have on the hepatotoxic action of aflatoxin.

The present investigation reports changes brought about by phenobarbital treatment in the ability of aflatoxin to interfere with nucleic acid metabolism in rat liver *in vivo*. Some observations on changes induced by phenobarbital alone are included.

EXPERIMENTAL

Animals. Twenty-four male Wistar strain rats from our colony (192–210 g) were fed *ad lib.* Purina Laboratory Chow. One-half received daily intraperitoneal injections of sodium phenobarbital (75 mg/kg in 0·5 ml saline) for 5 days, and the other half received saline. After this pretreatment, the following procedure was repeated 3 days in succession with phenobarbital treatment continuing an additional 1 or 2 days for the rats used on the second or third day. Two animals from each pretreatment group (± phenobarbital) were fasted overnight and 4·5 hr before death were injected intraperitoneally with 1·5 mg/kg of crystalline aflatoxin B_1 dissolved in 0·2 ml propylene glycol–dimethylsulfoxide (2:1, v/v). Two additional animals from each group, also fasted overnight, were given solvent only. Fifteen min before death, all eight were injected intraperitoneally with 20 μc/kg of 6-^{14}C-orotic acid (18 mc/m-mole) in 0·2 ml saline. The animals were anesthetized with ether and exsanguinated. Livers were immediately removed and washed in cold saline for isolation of nuclei. This procedure when repeated twice resulted in four treatment groups of six animals each with the following variables: ± phenobarbital and ± aflatoxin.

Isolation of nuclei. Liver (2 g) was homogenized in approximately 20 vol. of ice-cold 2·2 M sucrose containing 3·3 mM $CaCl_2$ and centrifuged at 35,000 *g* and 3° for 1 hr.[5,6] The resulting nuclear pellet was washed by resuspension in 5 ml of 1 M sucrose containing 1 mM $CaCl_2$ followed by centrifugation for 5 min at 3000 *g*. The nuclei were finally resuspended in 1 ml of cold water and frozen until assayed. Tissue concentrations in the 2·2 M sucrose homogenates were determined on the basis of weight of homogenizing medium, since the high viscosity of 2·2 M sucrose precluded accurate volumetry.

Nucleic acid assay. RNA was extracted with perchloric acid after mild alkaline hydrolysis, according to the method of Munro and Fleck,[7] and determined spectrophotometrically at two wavelengths.[8] DNA was determined by the color reaction with diphenylamine.[9] Whole cell RNA and DNA were assayed on 25 per cent aqueous liver homogenates.

Measurement of radioactivity. Radioactivity was measured with the Packard Tri-Carb liquid scintillation spectrometer, model 3375. Total radioactivity present in liver was determined on 0·1 g of 2·2 M sucrose homogenate dissolved in 1 ml NCS solubilizer (Nuclear Chicago Corp.) and 10 ml toluene–fluor solution. For RNA extracts, 1-ml samples were neutralized with 1 M NaOH and dissolved in 16 ml of a naphthalene-based solvent.[10] Counting efficiencies were determined by external standardization with efficiency–external standard ratio curves prepared from appropriate unlabeled rat liver preparations internally standardized. Results are expressed as disintegrations per minute (dis./min).

Nitrogen determination. Liver nitrogen content was determined on 25 per cent aqueous homogenates by the Kjeldahl technique.

Statistical calculations. Results on the four treatment groups were compared by 2-way analysis of variance and on any pair of groups by Student's *t*-test.[11]

RESULTS

The effect of prior phenobarbital treatment on the concentration of RNA and DNA in livers of rats 4·5 hr after administration of aflatoxin is shown in Table 1. Without phenobarbital, marked reduction occurred in the level of nuclear RNA and to a slight extent in RNA of the cell as a whole. In rats treated with phenobarbital, a significant interaction took place regarding nuclear RNA, whereby the effect of aflatoxin was reduced more than 3-fold. This protective interaction is reflected in the nuclear RNA/DNA ratios. The response of cellular RNA concentration to aflatoxin was too small to permit observation of a similar interaction. DNA concentration, cellular or nuclear, was not significantly altered by aflatoxin.

The specific activities of nuclear and cellular RNA after incorporation of orotic acid-^{14}C are presented in Table 2, on an absolute basis and after an adjustment was made with respect to the concentration of total radioactivity present in the liver. Incorporation rates within a treatment group were found to be dependent, to an extent, on the concentration of radioactivity in the liver, which in turn was inversely related to liver weight. Thus, the latter presentation removes variation due to differences in liver weight (shown by increased *t* values for aflatoxin treatment) and reduces the apparent effect of phenobarbital by itself on incorporation.

As a result of aflatoxin poisoning, incorporation rates of orotic acid into nuclear and

TABLE 1. EFFECT OF AFLATOXIN B_1 ON THE CONCENTRATION OF LIVER RNA AND DNA IN RATS TREATED WITH PHENOBARBITAL

	Aflatoxin	Phenobarbital		Student's t*		F† interaction
		−	+	Aflatoxin	Phenobarbital	
RNA (mg/g)						
Cellular	−	6·30	6·33	3·483	0·154	0·37
	+	5·82	6·00			
Reduction (%)		7·6	5·2			
Nuclear	−	0·230	0·197	7·811	6·981	21·02
	+	0·165	0·180			
Reduction (%)		28·3	8·6			
DNA (mg/g)						
Cellular	−	2·80	2·15	0·884	5·575	0·19
	+	2·69	2·12			
Reduction (%)		3·9	1·4			
Nuclear	−	1·15	0·91	1·343	4·872	1·22
	+	1·24	0·91			
Reduction (%)		(0)	0			
RNA/DNA						
Cellular	−	2·26	2·95	1·077	6·222	0·00
	+	2·17	2·86			
Reduction (%)		4·0	3·1			
Nuclear	−	0·201	0·217	6·587	1·514	16·69
	+	0·133	0·197			
Reduction (%)		33·8	9·2			

* Mean of untreated rats compared with that of rats receiving either aflatoxin or phenobarbital alone; $t_{0.05} = 2{\cdot}228$, $t_{0.01} = 3{\cdot}169$, $t_{0.001} = 4{\cdot}587$.

† F value of interaction between phenobarbital and aflatoxin derived from 2-way analysis of variance; $F_{0.05} = 4{\cdot}35$, $F_{0.001} = 14{\cdot}82$.

TABLE 2. EFFECT OF AFLATOXIN B_1 ON INCORPORATION OF OROTIC ACID-^{14}C INTO LIVER RNA IN RAT TREATED WITH PHENOBARBITAL

Specific activity	Aflatoxin	Phenobarbital		Student's t*		F* interaction
		−	+	Aflatoxin	Phenobarbital	
(dis.min/μg RNA)						
Cellular	−	11·22	6·87	15·611	6·194	28·40
	+	3·06	3·64			
Inhibition (%)		72·7	47·0			
Nuclear	−	170·3	116·2	14·002	5·501	15·98
	+	59·4	73·8			
Inhibition (%)		65·1	36·5			
(dis.min/μg RNA adjusted)†						
Cellular	−	13·00	10·65	16·367	2·661	25·25
	+	3·72	6·84			
Inhibition (%)		71·4	35·8			
Nuclear	−	197·2	178·6	22·432	2·850	34·68
	+	71·9	135·9			
Inhibition (%)		63·5	23·9			

* Statistical evaluation is the same as described in Table 1.

† Dis./min/μg RNA (adjusted) = dis./min/μg RNA × 10^6/(total dis./min/g liver).

cellular RNA were severely inhibited. From the adjusted specific activities of Table 2, the extent of this inhibition was reduced approximately 2·7-fold for nuclear and 2-fold for cellular RNA in animals pretreated with phenobarbital. These reductions, as indicated by the statistical interactions, were highly significant.

It can be shown from the relative concentrations and specific activities of nuclear and cellular RNA (Tables 1 and 2) that an average of 55 per cent of the total radioactivity incorporated into cellular RNA was located in the nuclear fraction. Thus, it was of interest to determine whether the effect of aflatoxin on incorporation into cellular RNA was largely just a reflection of incorporation rates into nuclear RNA. However, when orotic acid incorporation into cytoplasmic RNA was estimated by subtraction, it was found to be inhibited to the same extent as was that into cellular RNA. The reduction of this inhibition brought about by phenobarbital treatment was also similar to that of cellular RNA.

Table 3 shows that phenobarbital treatment by itself brought about a marked increase in liver weight in which total DNA (nuclear or cellular) remained essentially unchanged. This suggests that liver growth resulted mainly from a process of cellular enlargement as opposed to cellular proliferation. Keeping pace with liver growth was the production of total cellular RNA, as well as protein synthesis, indicated by constant liver nitrogen concentrations. The increase in total nuclear RNA was less than proportional to liver growth.

TABLE 3. EFFECT OF PHENOBARBITAL TREATMENT ON RAT LIVER

	Phenobarbital		Student's t*
	−	+	
Liver wt. (g)	6·22	8·37	9·271
Liver nitrogen (mg/g)	34·3	33·5	0·869
Total DNA (mg/liver)			
Cellular	17·4	18·0	0·713
Nuclear	7·18	7·59	0·943
Total RNA (mg/liver)			
Cellular	39·2	53·0	7·085
Nuclear	1·43	1·66	5·842
Dis./min/μg nuclear DNA†			
Cellular RNA	71·5	74·6	0·444
Nuclear RNA	39·7	38·9	0·397

* Significance of difference between means; $t_{0.05} = 2{\cdot}228$, $t_{0.001} = 4{\cdot}587$.

† Radioactivity from orotic acid -^{14}C incorporated into RNA per unit of nuclear DNA.

The specific activity of both nuclear and cellular RNA was reduced as a result of phenobarbital treatment (Table 2). When orotic acid incorporation into RNA was expressed as radioactivity per microgram of nuclear DNA, which is an estimate of the rate of incorporation per cell, nearly identical results were obtained with or without phenobarbital treatment (Table 3).

DISCUSSION

Aflatoxin poisoning. The effects of aflatoxin poisoning on liver RNA/DNA ratios and orotic acid incorporation observed in the present work were remarkably similar,

quantitatively, to those reported by Lafarge *et al.*,[12] even though the latter work was performed upon rats whose livers were undergoing active regeneration after partial hepatectomy. Confirming the observation of Lafarge *et al.*[12] was the relative stability of cellular RNA levels concurrent with severe inhibition of cellular RNA synthesis. This observation suggests that cytoplasmic RNA possesses a considerably slower turnover rate than does nuclear RNA, since inhibition of synthesis took place in the cytoplasm as well as in the nucleus. The concentration of cytoplasmic RNA, which is nearly equivalent to that of cellular RNA since nuclear RNA is a minor constituent, decreased only slightly in the presence of aflatoxin.

The relationship between susceptibility to aflatoxin poisoning and the metabolism of the toxin by liver is little understood at present. Portman *et al.*[1] have suggested that the metabolism of aflatoxin B_1 via a hydroxylated derivative (aflatoxin M_1) represents a detoxification pathway and have associated the greater resistance of mice, compared to that of rats, to a much faster rate of conversion by liver microsomes. Patterson *et al.*[13] have compared the metabolism of aflatoxin in several resistant and susceptible species. In the duckling and chick, which are regarded as susceptible, they found aflatoxin to be transformed almost quantitatively *in vitro* to nonfluorescent compounds by an NADPH-dependent liver microsomal enzyme system. They have suggested this conversion may be of particular importance to an animal's susceptibility. In the present work, phenobarbital treatment was shown to reduce a specific toxic effect of aflatoxin in liver, namely the inhibition of RNA synthesis. The extent of this reduction of inhibition was similar to the stimulation induced by phenobarbital of the hydroxylation *in vitro* of aflatoxin B_1 reported by Schabort and Steyn.[14] They found 2·3 times greater activity for this reaction in liver microsomal preparations from induced rats compared to untreated controls. Thus, our results indicate that NADPH-dependent microsomal enzymes, which act upon aflatoxin and are induced by phenobarbital, convert aflatoxin to inactive or less toxic metabolities *in vivo* and, indeed, represent a pathway of detoxification. The existence of a metabolite of aflatoxin, which itself is the active inhibitor of RNA synthesis, as proposed by Roy,[3] has of course not been ruled out.

Effects of phenobarbital. Consistent with observations on mice,[15] stimulation of liver growth by phenobarbital appeared to be predominantly the result of cellular enlargement accompanied by increased total RNA and protein per liver, which were maintained at nearly constant concentrations. Since total DNA per liver (nuclear or cellular) remained essentially unchanged, there was little indication of increased numbers of cells. This contrasts with reports that liver growth in rats stimulated by phenobarbital results from accelerated mitosis and cell division,[16,17] but agrees with recent studies by Stäubli *et al.*[18] based on morphometric techniques.

Despite increased total nuclear or cellular RNA in the livers of phenobarbital-treated rats, it was not possible to demonstrate increased rates of RNA synthesis as a reflection of increased specific activity from incorporation experiments. Incorporation rates per cell were nearly identical to those of untreated rats. Similar results have been reported for treatment with the microsomal enzyme inducer, 3-methylcholanthrene.[19] Others, however, after administering 3-methylcholanthrene have measured increased rates of orotic acid incorporation into nuclear RNA.[20,21]

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