Liver Microsomal Metabolism of Aflatoxin B₁ to a Reactive Derivative Toxic to Salmonella typhimurium TA 1530¹

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SUMMARY

A reduction in the survival of Salmonella typhimurium TA 1530 was observed when the bacteria were incubated with aflatoxin B₁, rat liver microsomes, and a reduced nicotinamide adenine dinucleotide phosphate-generating system. The lethality appeared to depend on the formation of a metabolite of aflatoxin B₁ by a mixed-function oxygenase system. The killing was very rapid; only 1% of the bacteria were able to form colonies after 2 min of incubation with large amounts of microsomes and aflatoxin B₁. Attempts to separate the toxic metabolite from the microsomal system have not been successful.

Toxic metabolites for S. typhimurium TA 1530 were also formed if aflatoxin B₁ was replaced by either aflatoxin G₁ or sterigmatocystin in the microsome-mediated toxicity assay. Except for aflatoxicol, the derivatives that were tested either had much less activity or were inactive.

The livers from a number of other species of rodents and a single autopsy sample of human liver also were active in the microsome-mediated aflatoxin B₁ toxicity assay. The addition of RNA or DNA to the incubation mixture inhibited the killing of the bacteria.

The RNA (which was reisolated after its incubation with aflatoxin B₁), liver microsomes, and a reduced nicotinamide adenine dinucleotide phosphate-generating system showed a low, broad absorption, with a maximum at 366 to 370 nm. This high wavelength absorption was not removed by Sephadex G-10 chromatography of the RNA or by extraction procedures and appeared to be attributable to covalently bound aflatoxin B₁ derivative(s). The formation of the conjugated RNA was dependent on reduced nicotinamide adenine dinucleotide phosphate and was inhibited by the addition of aniline; the amount formed was a function of the activity of the mixed-function oxygenases in the incubation mixture.

On the basis of the data presented, it is tentatively suggested that the derivative that is toxic to S. typhimurium TA 1530 and the one that reacts with nucleic acids are identical. The possible relationship of this derivative to the hepatocarcinogenicity of aflatoxin B₁ is discussed.

INTRODUCTION

Recent data from this and other laboratories have shown that many carcinogens require metabolic activation to the ultimate reactive and carcinogenic derivatives in vivo (28). Furthermore, a consideration of the structures of the ultimate carcinogenic derivatives, whether the carcinogens are administered in the ultimate form (e.g., alkylating agents) or are metabolized to the derivatives in vivo, suggests that most if not all of them are electrophilic reactants (28, 29). These electrophilic derivatives react with nucleophilic cellular constituents, and it is presumed that certain of these reactions are of critical importance in the induction of neoplasia.

The high hepatocarcinogenic activity of aflatoxin B₁ over a wide species range can be interpreted as evidence that the compound is carcinogenic per se. However, several factors, in addition to the lack of reactivity of aflatoxin B₁ with nucleophiles (5, 41), suggest that metabolic activation is important for its carcinogenic activity. With p.o. or i.p. administration, aflatoxin B₁ is for rats the most potent hepatocarcinogen known, but tumors develop less frequently at other sites (30, 43, 44); sarcomas can be induced by s.c. injection (10, 43). The carcinogenicity of aflatoxin B₁ in the liver varies considerably from species to species (30), and in rats it is greatly decreased by hypophysectomy (13). The unimpaired sensitivity of hypophysectomized rats to hepatic tumor induction by dimethylnitrosamine (22) suggests that their refractoriness to aflatoxin B₁ is not due to a reduction in the ability of their livers to develop neoplasia. Rather, the defect appears to be in the utilization of the aflatoxin for reactions required to initiate carcinogenesis. The metabolic conversion of aflatoxin B₁ to reactive intermediate(s) in vivo is suggested by the formation of nucleic acid- and protein-bound radioactivity after the administration of ³H-labeled aflatoxin B₁ (23). Only noncovalent interactions have been described for nonenzymatic in vitro systems (5, 41). Chromatin from the livers of rats treated with aflatoxin B₁ has impaired template activity for RNA polymerase, while the template activity of chromatin was not altered by the addition of aflatoxin B₁ to in vitro systems (11).

In an earlier communication, we presented evidence for the formation by rat liver microsomes of metabolite(s) of aflatoxin B₁ that were lethal to 2 strains of S. typhimurium (TA 1530 and TA 1531) (12). These 2 strains are histidine auxotrophs, with a major deletion extending through the galactose, chlorate resistance, histidine utilization, biotin A, and UV DNA repair B genes (1). Two related histidine auxotrophs without this major deletion were resistant to the...
Metabolic Activation of Aflatoxin B₁

toxic action of the aflatoxin B₁ metabolite(s). The rat liver enzymes were inducible by administration of phenobarbital and showed the characteristic properties of the mixed-function oxygenases. Further data on the formation of the aflatoxin metabolite(s) and on some of its structural properties are presented here.

MATERIALS AND METHODS

Animals and Tissue Preparation. The following young adult animals of both sexes were obtained from the sources given: CD random-bred rats and CD1 mice, Charles River Breeding Laboratory, Wilmington, Mass.; guinea pigs, M. R. O'Brien Co., Madison, Wis.; and Syrian golden hamsters, Con Olson Co., Madison, Wis. The mice, hamsters, and guinea pigs were fed a grain diet (32) which, for the guinea pigs, was supplemented with 1 g of ascorbic acid per kg. In comparative species studies, the rats were also fed the grain diet. In all other cases, only male rats (250 to 350 g) were used, and they were fed a purified diet containing 30% casein and 5% olive oil (27). As specified, some rats or hamsters also received phenobarbital sodium (1 mg/ml) in their drinking water for at least 5 days prior to the experiment (24). Hypophysectomies were performed by the Endocrine Laboratories, Madison, Wis. Sham-operated control rats either were restricted to 8 g of food per rat per day or were allowed to eat ad libitum.

The livers of decapitated animals were excised and washed in ice-cold 0.9% NaCl solution, and a 5-g portion of each liver was homogenized in 15 ml of 150 mM KC1 by 15 strokes of a Potter-Elvehjem-type homogenizer with a Teflon pestle. The postmitochondrial fraction was obtained by centrifugation of the homogenate at 9,000 X g for 10 min. The postmitochondrial fractions from 3 animals were pooled either for direct use in the lethality assay or for preparation of the microsomal fraction by centrifugation at 100,000 X g for 60 min. The microsomal pellet was resuspended in 150 mM KC1 so that 1 ml contained the microsomes from 250 mg of liver. All of the above procedures were carried out at 4°C.

Chemicals. Aflatoxins B₃, B₂, G₁, and G₂, as well as sterigmatocystin and bovine serum albumin, were purchased from Calbiochem, Los Angeles, Calif. NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase (Torula yeast), RNA (yeast tRNA, type III), salmon sperm DNA (highly polymerized), CMP, GMP, NADH, NADPH, pancreatic RNase (type A), and sodium borohydride were products of Sigma Chemical Co., St. Louis, Mo. AMP and TMP were purchased from P-L Biochemicals, Milwaukee, Wis., and sodium phenobartibal was obtained from Merck and Co., Inc., Rahway, N. J. Aflatoxin P₁ was kindly provided by Professor G. N. Wogan, Massachusetts Institute of Technology, Cambridge, Mass.

For the preparation of aflatoxicol, 10 mg of aflatoxin B₁ (32 μmoles) were dissolved in 2 ml of CHCl₃ and were reacted with 1.7 mg of NaBH₄ (45 μmoles) in 7.7 ml of isopropyl alcohol for 90 min at room temperature. After the reaction was stopped by the addition of 5 ml of 0.01 N acetic acid, 5 ml of CHCl₃ were added, the mixture was shaken, and the aqueous phase was discarded. The organic phase was streaked on silica gel thin-layer plates which were developed with ethyl acetate:CHCl₃ (2:1). The main fluorescent band with a Rₚ of 0.54 (Rₚ of aflatoxin B₁, 0.40) was eluted with methanol. The resulting product (10% yield) had the same UV and mass spectral properties as had aflatoxicol \(m^+ = 314; m^+ - 18 (H_2O) = 296\) (9). Most of the aflatoxin B₁ was converted to a nonfluorescent product with a Rₚ of about 0.

For preparation of the acetic acid ester, aflatoxicol was reacted with excess acetic anhydride and triethylamine at room temperature for 30 min and was purified by chromatography in the system described for aflatoxicol. Only traces of the aflatoxicol remained, and the main fluorescent band with a Rₚ of 0.87 was eluted with methanol. The identity of this product as the acetic acid ester of aflatoxicol was confirmed by its UV and mass spectra \(m^+ = 356\) (weak); \(m^+ - 43 (CH_3CO) = 313; m^+ - 59 (CH_3COO) = 297\). The amount of the ester was calculated from its absorption at 325 nm, on the assumption that its molar extinction coefficient was equal to that of aflatoxicol.

Aflatoxin B₁ was prepared essentially according to the method of Ciegler and Peterson (4). Aflatoxin B₁ (10 mg) in 1 ml of dimethyl sulfoxide was refluxed for 4 hr with 40 ml of 0.1 N citric acid. The reaction mixture was extracted twice with 40 ml of CHCl₃. After removal of the CHCl₃, the residue was redissolved in CHCl₃ for preparative thin-layer chromatography on silica with CHCl₃:methanol (95:5). No aflatoxin B₁ was detected. The main fluorescent band with a Rₚ of 0.16 (Rₚ of aflatoxin B₁, 0.89) was extracted with methanol. The infrared and UV spectra of this product were identical to those of aflatoxin B₁ (4).

Aflatoxin M₁ was isolated from the urine of male rats (300 to 350 g), the bile ducts of which had been ligated 24 hr prior to being given an i.p. injection of 5 mg of aflatoxin B₁ per 0.5 ml of dimethyl sulfoxide per kg body weight. Preliminary experiments indicated that bile duct ligation increased the excretion of aflatoxin M₁ about 2-fold. The 24-hr urine sample was extracted 3 times with CHCl₃:methanol (85:15). The organic phase was extracted with 0.1 M potassium dihydrogen phosphate titrated to pH 5 with KOH and evaporated to dryness, and the residue was dissolved in CHCl₃ for chromatography on silica gel plates with CHCl₃:methanol (97:3). The identity of the aflatoxin M₁ was confirmed by UV and mass spectrum analyses \(m^+ = 328\) (16). Approximately 5% of the aflatoxin B₁ was recovered as aflatoxin M₁.

For preparation of its acetic acid ester, aflatoxin M₁ was reacted with acetic anhydride and triethylamine, and the ester was isolated by chromatography, as described for the purification of aflatoxin M₁. In this system, the Rₚ's of aflatoxin M₁, of its acetic acid ester, and of aflatoxin B₁ were 0.30, 0.81, and 0.80, respectively. The identity of the ester was confirmed by UV and mass spectrum analyses \(m^+ = 370; m^+ - 42 (CH_2CO) = 328\). The amount of acetyl aflatoxin M₁ was calculated on the assumption that its extinction coefficient was the same as that of aflatoxin M₁ at 357 nm (16).

All of the above-described chromatographic separations were carried out on 1-mm silica gel thin layers on glass plates (Silica Gel HF-254; Merck, AG, Darmstadt, Germany). The tanks were not lined with filter paper and were not previously equilibrated.

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Assay for Microsome-mediated Toxicity of Aflatoxins for S. typhimurium TA 1530. S. typhimurium TA 1530* was grown overnight in 10 ml of nutrient broth (Difco Laboratories, Inc., Detroit, Mich.), collected by centrifugation, and resuspended in 3 ml of 0.9% NaCl solution (3 to 6 × 10^8 bacteria/ml).

All of the flasks were prepared in duplicate. Each flask contained 100 μmoles KCl, 20 μmoles glucose-6-phosphate, 25 μmoles MgCl₂, 300 μmoles disodium hydrogen phosphate titrated to pH 7.4 with HCl, 1.5 μmoles NADP, 0.1 ml bacterial suspension, 0.6 to 200 μmoles aflatoxin B₁ or other test compound dissolved in 0.03 ml of dimethylsulfoxide (or the solvent alone), and the specified amount of postmitochondrial liver supernatant fraction in a total volume of 3 ml. In studies in which the microsome fraction replaced the postmitochondrial fraction, 1 unit of glucose-6-phosphate dehydrogenase was also added. In some experiments, the assay volume was 0.75 or 1.5 ml, with a proportionate reduction in the amounts of all of the ingredients. Incubations were carried out in the dark in 10-ml flasks at 37° for 20 min (unless otherwise specified) in a reciprocating shaker (100 strokes/min). The flasks were then cooled in ice, and aliquots were serially diluted and plated on minimal Davis agar for determination of the number of surviving bacteria (i.e., those capable of forming colonies) (12).

For the experiments on the rate of loss of viability of the bacteria, the flasks were set up as just described, except that they were incubated at 37° for 3 min before the addition of the aflatoxin B₁. Aliquots were removed at 15-sec intervals for determination of the number of surviving bacteria.

The results are expressed as 100 times the number of surviving bacteria in the assay flasks containing aflatoxin B₁ or other test compound, divided by the number of surviving bacteria in comparable flasks containing all of the assay ingredients except the test compound.

Attempts to Separate the Toxic Metabolite from Liver Microsomes. The 3 separation processes used were as follows: (a) Flasks containing 0.2 μmole of aflatoxin B₁, 250-mg rat liver-equivalents of pooled postmitochondrial fraction from 3 animals, and the other ingredients for the toxicity assay (except the bacteria) were incubated for various times (2 to 20 min) at 37°. The microsomes were then removed by centrifugation at 4°, the supernatant solution was incubated for various times (2 to 20 min) at 37°, and the numbers of surviving bacteria were then determined. (b) Flasks were prepared as in Process a, except that 0.1 mille of bacterial suspension in a small sack prepared from dialysis membrane (104-16; 6-mm dialysis tubing; LaPine Scientific Co., Chicago, Ill.) was incubated in each flask for 60 min at 37°. The numbers of surviving bacteria in the dialysis sacks were determined. (c) The lethality assay medium (30 ml) was prepared as described above, with the substitution of Tris-HCl buffer, pH 7.5 (23°), for the phosphate buffer. The bacteria and aflatoxin B₁ were omitted, and microsomes equivalent to 80 mg of fresh liver from phenobarbital-treated hamsters were added. The mixture was placed in a Model 52 ultrafiltration cell (with either a PM-10 or a XM-100 membrane and a magnetic stirrer; Amicon Corp., Lexington, Mass.) and was stirred vigorously. The liquid was forced through the membrane with air pressure (55 psi). The filtrate was collected in a side-arm tube containing 0.1 ml of bacterial suspension for 5 min. Aflatoxin B₁ (2 μmoles in 0.3 ml of dimethyl sulfoxide) was then added, the side-arm tube was replaced with another tube containing 0.1 ml of bacterial suspension, and the filtrate was collected for another 5 min. After the tube was changed, a third 5-min collection was made. The number of viable bacteria was determined for each collection tube.

Reaction of Aflatoxin B₁ Metabolite with RNA. To 3-ml aliquots of the lethality assay medium were added 1.3 μmoles of aflatoxin B₁ in 40 μl of dimethyl sulfoxide, 4 mg of RNA, and microsomes equivalent to 12.5 to 500 mg of liver from pooled samples obtained from 2 animals. The bacterial suspension was omitted. After incubation at 37° for 60 min, 0.5 ml of 0.1% sodium lauryl sulfate was added, and the medium was extracted with an equal volume of Kirby phenol mixture (0.05 g of 8-hydroxyquinoline, 555 ml of liquefied phenol, and 50 ml of m-cresol) (20). The aqueous layer was reextracted with 1.5 volumes of the phenol mixture, and the combined phenol extracts were then extracted with 3 ml of 10 mM Tris-HCl buffer, pH 7.4. This aqueous extract was combined with the original aqueous layer, extracted 3 times with an equal volume of water-saturated ethyl ether, and adjusted to 100 mM with 0.9% NaCl solution. The extractions were carried out at 4°. The RNA, precipitated by the addition of 3 volumes of ethanol and by storage overnight at −25°, was redissolved in 4 ml of 10 mM Tris-HCl buffer, pH 7.4, and centrifuged at 100,000 × g for 20 min to remove the glycoprotein. UV absorption spectra of the RNA solutions were determined on a Beckman DB spectrophotometer equipped with a Sargent SR recorder; the concentrations were approximately 1 mg/ml for spectra from 320 to 420 nm and 30 μg/ml for spectra from 240 to 320 nm.

For facilitation of removal of any adsorbed aflatoxin derivatives, the RNA solution in the Tris buffer was passed through a 2-× 10-cm column of Sephadex G-10 that had been previously equilibrated with 10 mM Tris-HCl buffer, pH 7.4. The RNA was precipitated with 0.9% NaCl solution and ethanol, as described above, and was dissolved in the Tris-HCl buffer for spectral analysis.

As a further test for the presence of noncovalently bound metabolites of aflatoxin B₁ which were tightly associated with the RNA, 4 mg of tRNA that had been incubated with 500-mg liver equivalents of microsomes from phenobarbital-treated rats and with aflatoxin B₁ were digested in 2 ml of Tris-HCl buffer, pH 7.5, with 6 μg of pancreatic RNase for 10 min. After the addition of 0.6 ml of 25% perchloric acid, the solution was extracted twice with 5 ml of water-saturated CHCl₃ and then was subjected to UV analysis.

RESULTS

As described previously the incubation of S. typhimurium TA 1530 with aflatoxin B₁, rat liver postmitochondrial supernatant or rat liver microsomes, and a NADPH-generating system caused a marked reduction in the survival of the
bacteria, whereas no toxicity was observed if either the aflatoxin or the liver preparation was omitted. Survival of the bacteria decreased exponentially as a linear function of the liver concentration (12). The logarithm of the percentage of surviving bacteria also decreased nearly linearly with increasing concentrations of aflatoxin B1 from 2 to 12 μM (Chart 1). However, this line did not extrapolate to 100% survival at zero concentration. Limited data suggest that a line of different slope extrapolating to 100% survival at zero concentration was obtained when concentrations below 2 to 3 μM were used. The survival curve may thus be biphasic between 0 and 12 μM concentrations of aflatoxin B1. With the postmitochondrial fraction from 250 mg of liver and with an incubation period of 20 min, 1.6 μM aflatoxin B1 (0.5 μg/ml) reduced the survival to 30%; with 6.4 μM aflatoxin B1, the survival was 4%. The critical reaction leading to loss of viability was extremely rapid. After incubation with saturating concentrations of aflatoxin B1 and liver postmitochondrial supernatant for 2 min, only 1% of the bacteria were able to form colonies (Chart 2).

Liver Activity as a Function of Species, Sex, and Hypophysectomy. In a comparison of the livers of male rodents in the toxicity assay, the microsomes from hamsters and mice were more active than those from rats, while guinea pig liver microsomes were a little less active (Table 1). No sex difference was noted for the livers from hamsters, mice, or guinea pigs, but the microsomes from female rat livers appeared to be less active than those from male rat livers. However, because the difference in activity was not large and because there was some variation from assay to assay in the susceptibility of the bacteria, the difference in the activities of male and female rat liver microsomes was apparent only on comparison of assays carried out in the same experiment.

A single sample of male human liver, obtained 6 hr after the patient's death during heart surgery, had an activity similar to that of mouse or hamster livers (Table 1). Two other male human liver samples were inactive in this assay at levels up to 20 mg of liver per assay tube; these samples were obtained 9 to 12 hr after the death of chronically ill patients.

Livers from adult male rats hypophysectomized 1 or 8 weeks prior to the assays had essentially the same activities in the toxicity assays as those of sham-operated or of both sham-operated and food-restricted controls. These assays were carried out as described in the legend to Table 1.

Comparative Activities of Aflatoxin B1 and Related Compounds in the Toxicity Assay. Aflatoxins B1 and G1 and sterigmatocystin had similar high toxicities for S. typhimurium TA 1530 when incubated with the postmitochondrial fraction of rat liver (Table 2). The 2 aflatoxins are produced by Aspergillus flavus, while sterigmatocystin is a metabolite of Aspergillus versicolor. The 2 aflatoxins and sterigmatocystin are structurally similar in that all 3 compounds contain 2 fused...
The activities of the livers from various species for the production of the metabolite of aflatoxin B1, toxic to S. typhimurium TA 1530

Aliquots of pooled postmitochondrial fraction from the livers of 3 animals were incubated in the usual assay medium with S. typhimurium TA 1530 and 0.2 µmole of aflatoxin B1 for 20 min at 37°. Each liver sample was assayed in duplicate at 4 levels from 5- to 40-mg liver equivalents. Only the data for the 5- and 10-mg liver equivalents are presented in the table. A different subculture of bacteria was used for each experiment.

<table>
<thead>
<tr>
<th>Activity [no. of viable bacteria (treated)/no. of viable bacteria (control) X 100] after incubation with</th>
<th>Male liver</th>
<th>Female liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>Experiment</td>
<td>5 mg</td>
</tr>
<tr>
<td>Rat</td>
<td>1</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>34</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>4</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>75</td>
</tr>
<tr>
<td>Mouse</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Hamster</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Human (postmortem)</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

a The active human liver sample was obtained 6 hr after the death of a 59-year-old Caucasian male during heart surgery. This sample was assayed at 4 levels from 10- to 250-mg liver equivalents. With 25-mg liver equivalents, the viability was 0.06%. Two other liver samples obtained 9 to 12 hr after the death of chronically ill male patients showed no activity.

Toxicities of aflatoxins and related compounds for S. typhimurium TA 1530 in the rat liver microsome system

Flasks containing 250-mg liver equivalents of pooled postmitochondrial fraction from 3 rats were incubated in a 3-ml volume with various concentrations of the test compounds and S. typhimurium TA 1530 for 20 min at 37°. For the assays with aflatoxins M1 and P1, and acetyl aflatoxin M1, the incubation volume was 0.75 ml with a proportionate reduction in each of the constituents. Each of the values given is the average result for 2 independent experiments.

<table>
<thead>
<tr>
<th>Values obtained [no. of viable bacteria (treated)/no. of viable bacteria (control) X 100] with the following toxin concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
</tr>
<tr>
<td>----------------------------------------------------------</td>
</tr>
<tr>
<td>Aflatoxin B1</td>
</tr>
<tr>
<td>Aflatoxin G1</td>
</tr>
<tr>
<td>Sterigmatocystin</td>
</tr>
<tr>
<td>Aflatoxicol</td>
</tr>
<tr>
<td>Acetyl aflatoxicol</td>
</tr>
<tr>
<td>Aflatoxin M1</td>
</tr>
<tr>
<td>Acetyl aflatoxin M1</td>
</tr>
<tr>
<td>Aflatoxin B2</td>
</tr>
<tr>
<td>Aflatoxin G2</td>
</tr>
<tr>
<td>Aflatoxin P1</td>
</tr>
<tr>
<td>Aflatoxin B2a</td>
</tr>
</tbody>
</table>

furan rings, one of which is also fused to a benzene ring and the other of which has an isolated double bond at position 2,3 (Chart 3). Aflatoxins B2 and G2 were inactive in the assay; these compounds, which are also produced by A. flavus, are 2,3-dihydro aflatoxins B1 and G1, respectively.

Of the other aflatoxin derivatives studied, aflatoxicol, formed by reduction of the cyclopentenone keto group of aflatoxin B1 to an alcohol, and the corresponding acetic acid ester were the most active, but these compounds were less active than aflatoxins B1 or G1 or sterigmatocystin. Aflatoxin M1 (4-hydroxy aflatoxin B1) had low activity, while its acetyl derivative, aflatoxin P1 (desmethyl aflatoxin B1), and aflatoxin B2a were essentially inactive.

None of the compounds mentioned above were active in the lethality assay in the absence of rat liver postmitochondrial fraction, nor were they active if heat-denatured postmitochondrial fraction was used.

Inability to Separate the Toxic Metabolite from the Incubation Mixture. Probably because of its reactivity, we were unsuccessful in separating the toxic metabolite from the microsomal system. In one type of experiment, the microsomal system was incubated with aflatoxin B1, and the microsomes were then removed by centrifugation. The resulting supernatant solution had no toxicity for S. typhimurium TA 1530.

If bacteria in a dialysis bag were placed in the assay medium containing 0.64 µmole of aflatoxin B1 and 250-mg liver equivalents of postmitochondrial fraction and were incubated at 37° for 60 min, the recovery of the bacteria was 105%.

Chart 3. The structures of the aflatoxins and sterigmatocystin. All of these compounds are shown for aflatoxin B1.
Survival of the bacteria was less than 1% in flasks that were identical, except that the bacteria were added directly to the medium.

Similarly, the eluate from an ultrafiltration cell that contained phenobarbital-treated hamster liver microsomes and aflatoxin B₁ in the usual assay medium was not toxic to *S. typhimurium* TA 1530 (Table 3). Microsomes from the livers of phenobarbital-treated hamsters are very active in the production of the toxic metabolite of aflatoxin B₁.

**Reduction of Lethality on Addition of Certain Compounds to the Assay Medium.** If the toxic product formed from aflatoxin B₁ by liver microsomal oxygenases is an electrophilic reagent, the addition of nucleophiles to the incubation mixture might reduce the concentration of metabolite available for reaction with the bacteria. Accordingly, the effects of various nucleophilic compounds on the survival of *S. typhimurium* TA 1530 were determined when the bacteria were incubated for 20 min in the usual medium containing the postmitochondrial supernatant fraction from 6 mg of rat liver and 0.11 or 0.22 mM aflatoxin B₁. Since aflatoxin B₁ binds to nucleic acids with weak noncovalent bonds (5, 41), the high level was used to ensure that the amount of available aflatoxin was not rate limiting for the microsomal system. Under these conditions, the addition of 0.7 mg of RNA or DNA per ml increased the survival of the bacteria approximately 6-fold. The bacterial survival was approximately 6, 3, or 2 times the control level when 10 mM CMP, GMP, or AMP was added to the incubation mixture. The survival was not increased by the addition of 10 mM TMP. At 5 mM concentrations, CMP approximately doubled the survival, but the other nucleotides had little effect. The survival was twice the control level when 1.3 mg of serum albumin per ml were added to the incubation mixture. The survival in the control flasks ranged from 0.4 to 17%. The bacteria were plated at 2 dilutions, and the significance of the colony counts with low survivals was similar to that of colony counts with higher survivals.

**Evidence for Covalent Binding of an Aflatoxin B₁ Metabolite to tRNA.** The inhibition by the nucleic acids of the toxicity of the aflatoxin B₁ metabolite in the liver microsome-mediated system suggested that RNA might covalently bind the metabolite. For a test of this possibility, RNA was incubated with aflatoxin B₁, liver microsomes, and a NADPH-generating system, and the RNA was then reisolated and passed through a column of Sephadex G-10 to remove any adsorbed aflatoxins. When liver microsomes from male hamsters or phenobarbital-treated male rats were used, the reisolated RNA showed increased high wavelength absorption with a broad maximum at 366 to 370 nm (Chart 4). When liver microsomes from control male rats were used, a smaller increase in high wavelength absorption was seen. This increase in the absorption above 300 nm was not observed when the RNA was incubated with aflatoxin in the absence of liver microsomes or with liver microsomes in the absence of aflatoxin. For a further test to determine the possible presence of adsorbed aflatoxins, 2 RNA samples, after passage through Sephadex, were digested with RNase and then were extracted with CHCl₃. The A₃₆₈:A₅₅₈ ratio was reduced about 10% by this treatment.

No increased absorption above 300 nm was observed if the phenobarbital-treated rat liver microsomes and a NADPH-generating system were incubated with aflatoxin B₁ for 60 min, the microsomes were removed by centrifugation at 4°, and the RNA was then incubated with the supernatant solution for 60 min at 37°. This observation is consistent with the lack of toxicity of such supernatant solutions for *S. typhimurium* TA 1530 after preincubation of aflatoxin B₁ with rat or hamster liver preparations.

The formation of the aflatoxin-conjugated RNA appears to require the mixed-function oxygenase systems. Thus, when aflatoxin B₁ was incubated with the microsomal system described in Chart 4, the ratio of the absorbance at 368 nm to that at 258 nm of the RNA isolated after incubation in the

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**Table 3**

<table>
<thead>
<tr>
<th>Collection period (min)</th>
<th>Aflatoxin B₁ (64 μM)</th>
<th>Values obtained (no. of viable bacteria (treated)/no. of viable bacteria (control) × 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>0–5</td>
<td>–</td>
<td>(100)</td>
</tr>
<tr>
<td>5–10</td>
<td>+</td>
<td>93</td>
</tr>
<tr>
<td>10–15</td>
<td>+</td>
<td>107</td>
</tr>
</tbody>
</table>

*a Control.

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**Chart 4.** The absorption spectra of RNA after its incubation with liver microsomes and a NADPH-generating system, with or without aflatoxin B₁. Flasks containing 4 mg of RNA were incubated with 500-mg liver equivalents of pooled microsomes from 2 livers from control rats, or hamsters, or phenobarbital (PB)-treated rats with or without 0.43 mM aflatoxin B₁, for 60 min at 37°. The RNA was recovered and chromatographed on Sephadex G-10 as described in "Materials and Methods." 30X, 30-fold; B₁, aflatoxin B₁.
absence of any added pyridine nucleotide was 0.003 (equivalent to the value obtained if all of the ingredients except the aflatoxin B1 were added). The addition of 6 μmoles of NADH or NADPH or of the NADPH-generating system described in “Materials and Methods” increased this ratio to 0.008, 0.008, or 0.010, respectively. If aniline (8 mM) was also added to the incubation mixture containing the NADPH-generating system, the binding of the aflatoxin to RNA was inhibited (A368:A258 = 0.004).

In the above studies, the RNA extracted from the incubation mixtures was a mixture of the added tRNA and liver microsomal RNA. In subsequent studies (D. H. Swenson, J. A. Miller, and E. C. Miller, unpublished observations), the microsomal and tRNA have been extracted separately. These RNA's were obtained in a ratio of about 1:2, and their absorption spectra were very similar.

The data in Chart 4 indicate that as much as 1 mole of aflatoxin derivative may be bound per 300 moles of nucleotide in the RNA. For this calculation, it was assumed that the average molar absorbance of the nucleotides in the RNA was 7,000 at 260 nm and that the absorbance of the aflatoxin portion was equal to that of aflatoxin B1 [21,800 (36)] at 360 nm. These incubations were carried out with very large amounts of both liver microsomes and aflatoxin B1.

DISCUSSION

The data in the present paper extend our previous observations (12) on the reduction in survival of S. typhimurium TA 1530 incubated with aflatoxin B1 and a liver microsomal mixed-function oxygenase system. From the present data, the toxic factor appears to be a previously unreported metabolite of aflatoxin B1. The relationship of this toxic factor to the cytotoxic derivative of aflatoxin B1 produced by rat liver cells in the study by Scaife (37) is not clear. The factor reported by Scaife may differ from that of our study, since he reported accumulations in tissue culture fluid, while we obtained no evidence for an accumulation of toxin in our liver microsome system.

None of the known metabolites of aflatoxin B1 were as active as the parent compound in the microsomal-mediated assay, and neither aflatoxin B1 nor any of the aflatoxin metabolites or other derivatives studied was active in the absence of the mixed-function oxygenase system. The activities of the compounds that have been tested in the toxicity assay suggest that the toxic metabolite is activated at the 2,3-double bond and that the structure of the coumarin portion of the molecule is not critical. Thus, aflatoxin B1, aflatoxin G1, and sterigmatocystin, the most active compounds in the microsome-mediated system, all contain the 2,3-double bond, but they differ with respect to the structure of the distal portion of the molecule. The 2,3-dihydro derivatives (aflatoxins B2 and G2) and the hydrated derivative of aflatoxin B1 (aflatoxin B2a) had little or no activity in the bacterial assay.

The above observations lead us to suggest that the active metabolite may be a 2,3-epoxide. A number of unsaturated compounds are readily epoxidized by the mixed-function oxygenases of liver microsomes (18, 19, 39, 45). The epoxides of the carcinogenic polycyclic hydrocarbons are highly reactive, toxic, and mutagenic, and they also transform cells in culture (6, 14, 15, 17). The possibility that the 2,3-epoxide is an important metabolite of aflatoxin B1 was suggested by Schoental (38), but without supporting data. Activation does not appear to proceed via esterification of an allylic alcohol, a possible activation mechanism by analogy with the pyrrolizidine alkaloids (25). Thus, the acetic acid esters of aflatoxicol and aflatoxin M1, both of which are esters of allylic alcohols, had only low activities in the toxicity assay.

There appears to be a close relationship between the metabolite that is toxic to the bacteria and that which binds covalently to nucleic acids. The activity of aflatoxin B1 in both the toxicity assay and in the binding to RNA is a function of the activity of the mixed-function oxygenase system. Furthermore, the reduced toxicity of aflatoxin B1 in the microsome-mediated bacterial assay on the addition of nucleic acids suggests that the same metabolite can react either with the nucleic acids or with sites on or in the bacteria. The maximum at 366 to 370 nm of the high wavelength absorption of the treated RNA suggests that the coumarin portion of the molecule is relatively unaltered and, by implication, suggests the possible importance of the 2,3-double bond in this reaction. The binding of an aflatoxin B1 metabolite to RNA in the NADPH-fortified microsomal system is of interest in view of its possible relationship to the RNA- and DNA-bound tritium found in the livers of rats after the administration of tritiated aflatoxin B1 (23).

The relationship between the carcinogenic activity of aflatoxin B1 and the metabolite toxic to S. typhimurium TA 1530 is not known. Most of the structural data are consistent with the possibility that the new metabolite could have a role in the hepatocarcinogenicity of aflatoxin B1. Thus aflatoxins B1 and G1 and sterigmatocystin, the compounds which are most toxic in the bacterial assay, all are strong hepatocarcinogens in rats. However, aflatoxin B1 appears to be more hepatocarcinogenic than either of the other compounds (3, 35, 43). The dihydro derivative aflatoxin B2 is much less carcinogenic for rat liver, and both aflatoxin B2 and aflatoxin G2 are much less acutely toxic in the rat than their 2,3-ununsaturated derivatives (3, 43). These dihydro derivatives were not active in our microsome-mediated bacterial assay. Aflatoxin M1 was toxic in the microsome-mediated bacterial assay, but much less so than aflatoxin B1. Aflatoxin M1 showed similar acute toxicity to that of aflatoxin B1 in rats and ducklings (33, 34). Aflatoxin M1 is carcinogenic for trout liver (2), but its carcinogenicity for rat liver has not been reported. Aflatoxicol was also active in the microsome-mediated bacterial assay. The latter compound is produced by duckling liver from aflatoxin B1 (31) and is less active than aflatoxin B1 in the production of bile duct hyperplasia in ducklings (8, 9). Aflatoxin P1, a metabolite of aflatoxin B1 in monkeys (7), was not toxic in our microsome-mediated bacterial system.

The toxicity for S. typhimurium TA 1530 of aflatoxin B1, on incubation with liver microsomes from a number of rodent species, is consistent with the wide species range of hepatocarcinogenicity of aflatoxin B1 (30). The high activity of the livers of hypophysectomized rats and the even higher activity of livers from phenobarbital-treated rats in the
bacterial assay are at variance with the protective effects of both of these treatments on aflatoxin B₁-induced carcinogenesis (13, 26). A possibly important difference between the in vivo and in vitro systems is that the amount of aflatoxin B₁ is in excess in the in vitro system but is probably limiting in vivo. Thus, competitive metabolic pathways between activation and deactivation may be much more important in determining the outcome in the in vivo system.

The activity of human liver in the microscope-meditated bacterial assay will be of particular importance if the derivative that is toxic to the bacteria and the one that is involved in carcinogenesis should prove to be identical. Aflatoxins B₁ and G₁, as well as sterigmatocystin, are known to have a variety of fungi than are the aflatoxins. Aflatoxins B₁ and G₁ are known to have carcinogenic activity. Chemical Mutagens. Principles and Methods for Their Detection, Vol. 1, pp. 267–282. New York: Plenum Press, 1971.

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Metabolic Activation of Aflatoxin B₁


Liver Microsomal Metabolism of Aflatoxin B₁ to a Reactive Derivative Toxic to *Salmonella typhimurium* TA 1530

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