Aflatoxin B$_1$-2,3-oxide as a Probable Intermediate in the Covalent Binding of Aflatoxins B$_1$ and B$_2$ to Rat Liver DNA and Ribosomal RNA in Vivo

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SUMMARY

Administration of [3H]aflatoxin B$_2$ (2,3-dihydro aflatoxin B$_2$) (AFB$_2$) to male rats resulted in levels of hepatic DNA- and ribosomal (r)RNA-aflatoxin adducts that were about 1% of those for rats given [3H]aflatoxin B$_1$ (AFB$_1$). The levels of hepatic protein-aflatoxin adducts were 35 to 70% as great for AFB$_2$-treated as compared to AFB$_1$-treated rats.

Mild acid hydrolysis of hepatic DNA and rRNA from rats given [3H]AFB$_1$ or [3H]AFB$_2$ yielded 2,3-dihydro-2,3-dihydroxy aflatoxin B$_1$ and two other major tritiated products that were separable by high-pressure liquid chromatography. With 2-hr hydrolyses approximately 77 and 54% of the $^3$H from the hepatic DNA of rats given [3H]AFB$_1$ or [3H]AFB$_2$, respectively, and 40 and 32%, respectively, of the $^3$H from the rRNA of rats given [3H]AFB$_1$ or [3H]AFB$_2$ were isolated as these products. One of the tritiated products was converted to 2,3-dihydro-2,3-dihydroxy-aflatoxin B$_1$ on further mild acid hydrolysis. The high levels of the dihydriodiol and its precursor in the hydrolysates, especially when the 35% loss of added 2,3-dihydro-2,3-dihydroxy aflatoxin B$_1$ under the hydrolysis conditions is taken into account, indicate that aflatoxin B$_2$-2,3-oxide is a major ultimate precursor of the nucleic acid-bound derivatives formed from both AFB$_1$ and AFB$_2$ in rat liver. From these data, AFB$_2$ appears to be converted to AFB$_1$ in the rat liver in vivo to an extent (about 1%) that is comparable to the ratio of hepatocarcinogenicities of these compounds in this species.

The levels of hepatic DNA-bound adducts from [3H]AFB$_1$ were about one-half of the control level in hypophysectomized rats, while the levels of the rRNA- and protein-aflatoxin adducts were similar to those of the controls. The levels of the hepatic macromolecule-bound adducts were 10 to 30% of the control levels in phenobarbital-treated rats. The levels of macromolecule-bound adducts of AFB$_1$ in kidney, spleen, and small intestine were much lower than those of liver. The inhibitory action of phenobarbital on AFB$_1$-induced hepatocarcinogenesis was confirmed; administration of benz(a)anthracene or high levels of ascorbic acid with AFB$_1$ did not alter the tumor incidence.

INTRODUCTION

The aflatoxins are difuranocoumarins that are metabolites of certain strains of Aspergillus flavus (35). The major metabolite, AFB$_1$, is hepatotoxic and hepatocarcinogenic in several animal species at very low doses; its metabolism in relation to its biological activities has recently been critically reviewed by Campbell and Hayes (7). Much evidence (7, 10—12, 14, 30, 32—35) indicates that this mycotoxin requires metabolic activation to exert its biological activities. AFB$_1$-2,3-oxide was first suggested (31) as an ultimate carcinogenic metabolite of AFB$_1$, primarily by analogy to the metabolic activation of the polycyclic aromatic hydrocarbons. Indirect evidence for the importance of epoxidation for the biological activity of AFB$_1$ has been provided by the formation of a toxic, reactive, and mutagenic metabolite from AFB$_1$, via a cytochrome P-450-dependent microsomal oxidation in which the 2,3-double bond of AFB$_1$ was required for expression of these properties (9, 11—13, 23, 30, 33). Furthermore, the much lower carcinogenicity of AFB$_2$ (2,3-dihydro aflatoxin B$_1$) in the rat and rainbow trout as compared to that of AFB$_1$ (3, 36) suggested that the 2,3-double bond is essential for the biological activity of AFB$_1$.

The 1st definitive evidence for the metabolic transformation of AFB$_1$-2,3-oxide was obtained with the isolation by Swenson et al. (33) of 2,3-dihydro-2,3-dihydroxy-AFB$_1$ (also referred to herein as the dihydrodiol) from an acid hydrolysate of the rRNA-aflatoxin adduct formed by hepatocellular oxidation of AFB$_1$, in the presence of rRNA. Related but less direct evidence was reported by Garner (9). Similarly, a $^3$H derivative indistinguishable by thin-layer chromatography from the dihydrodiol was released from the hepatic DNA and rRNA of rats given [3H]AFB$_1$; this finding provided strong evidence for the epoxidation of AFB$_1$ in vivo (32). Since attempts to synthesize AFB$_1$-2,3-oxide chemically did not succeed, AFB$_1$-2,3-dichloride was synthesized as a model of the epoxide (34). AFB$_1$-2,3-dichloride displayed the reactivity and mutagenicity characteristic of the metab
Macromolecular Binding of AFB₁ and AFB₂

Macromolecular binding of aflatoxins AFB₁ and AFB₂ has been studied extensively, particularly in relation to their carcinogenic potential. This paper presents data on the comparative binding of AFB₁ and AFB₂ to rat liver DNA and RNA in vivo and on the release by acid hydrolysis of these nucleic acids of the dihydrodiol and 2 other major aflatoxin derivatives. The data provide evidence that AFB₂-2,3-oxide is an intermediate in the formation of the nucleic acid-bound derivatives of both AFB₁ and AFB₂ in vivo and indicate that AFB₂ is hepatocarcinogenic via dehydrogenation to AFB₁. Data are also presented on (a) the levels of the macromolecule-bound AFB₂ derivatives in several tissues and at several times after administration of AFB₁ to normal, phenobarbital-treated, or hypophysectomized rats, and (b) the inhibitory effect of phenobarbital on the hepatocarcinogenicity of AFB₁.

MATERIALS AND METHODS

Instrumentation and General Procedures. High-pressure liquid chromatography was accomplished with a µBondapak C₁₈ reverse-phase column or a µPorasil column on a Waters ALC 201 liquid chromatograph equipped with a Model U6K injector system and a Model 600 solvent programmer (all products of Waters Associates, Milford, Mass.) and an SF 770 variable-wavelength photometer set at 360 nm (Schoeffel Instrument Corp., Westwood, N. J.). UV absorptions and spectra were determined on a Beckman DB spectrophotometer equipped with a Sargent recorder (Beckman Instruments, Inc., Fullerton, Calif., and E. H. Sargent Co., Chicago, III., respectively). ³H was determined in Packard Tri-Carb (Packard Instrument Co., Inc., Downers Grove, Ill.) or Isocap/300 (Nuclear Chicago, Inc., Chicago, Ill.) scintillation spectrometers. Eluates from liquid chromatograms and the nucleic acid samples (0.01 to 0.20 mg/0.5 ml water) were dissolved in 5 to 10 ml of Scintisol (Isolab, Inc., Akron, Ohio) for determination of ³H. Protein samples (5 to 10 mg) were dissolved in 2 ml of Soluene (Packard Instrument Co.), and 0.1- to 0.2-ml aliquots were dissolved in 10 ml of a toluene:PPO:POPOP scintillation cocktail (Research Products International, Elk Grove Village, Ill.) for determination of ³H.

Disposable vinyl gloves were used in the handling of the aflatoxins. Procedures such as thin-layer chromatography which could result in the formation of dusts containing aflatoxin were carried out in a chemical hood, and the operator was protected with a dust mask. Aflatoxin residues on equipment and in solutions were decontaminated with 5% sodium hypochlorite (15).

Chemicals. Dimethyl sulfoxide (99+% pure) and sodium 4-aminosalicylate were obtained from Aldrich Chemical Co., Milwaukee, Wis. AFB₁ and AFB₂ were purchased from Calbiochem, La Jolla, Calif. [³H]AFB₁, 4.1 Ci/m mole, and [³H]AFB₂, 0.35 Ci/m mole, were prepared by a ³H exchange process carried out by Nuclear Dynamics Corp., El Monte, Calif.; they were stored in benzene: methanol (1:1) at 0-4° [³H]AFB₁ was purified by chromatography on 0.5-mm layers of silica gel H₅₀₄ (Brinkman Instruments, Inc., Des Plaines, Ill.) in chloroform:methanol (97:3) and then in benzene:95% ethanol (8:3) to remove polar contaminants. Liquid chromatographic analysis on µPorasil eluted with 7% acetone in methylene chloride at 2.0 ml/min showed that approximately 2% of the ³H that was injected onto the column chromatographed with AFB₁; overall radiochemical purity was at least 96%. [³H]AFB₂ was similarly purified by thin-layer chromatography in chloroform:methanol (97:3) and chloroform:acetone (93:7). Thin-layer chromatographic analysis in chloroform:acetone (19:1) showed that <0.06% of the ³H chromatographed with AFB₁, and 99.5% chromatographed with AFB₂.

2,3-Dihydro-2,3-dihydroxy-AFB₁ was prepared as described previously (34). [³H]Dihydrodiol was prepared similarly from [³H]AFB₁ (34) or was isolated by high-pressure liquid chromatography from nucleic acid hydrolysates. The dihydrodiol is presumably a racemic mixture in view of the equilibrium in neutral aqueous media between the fused tetrahydronaphane: dihydrofuran and acyclic dialdehyde phenol tautomers of the 2-hydroxyaflatoxin B₃ derivatives (2, 5, 18, 27, 33, 34).

Treatment of Rats with [³H]AFB₁ and [³H]AFB₂ and Isolation of Tissue Macromolecules. Young adult male Fischer rats, from the Charles River Breeding Laboratories, Wilmington, Mass., or from ARS-Sprague-Dawley, Inc., Madison, Wis., were maintained on an 18% casein semipurified diet (1). Where indicated, the rats received 0.1% sodium phenobarbital (J. T. Baker Co., Phillipsburg, N. J.) in the drinking water from 1 week before injection of [³H]AFB₁ until the experiment was terminated. Some rats were hypophysectomized by Endocrine Laboratories, Inc., Madison, Wis., 3 weeks prior to the injection of AFB₁; the latter rats were maintained on the semipurified diet and sucrose supplements.

Two experiments were carried out for comparison of the bound aflatoxin derivatives in the nucleic acids and proteins of the livers of rats given [³H]AFB₁ as compared to [³H]AFB₂. In the 1st experiment, 0.1 mCi of tritiated compound was administered i.p. at a dose of 0.52 mg per 1.25 ml 50% dimethyl sulfoxide per kg body weight, and the animals were killed at 18 hr. In the 2nd experiment, the dose was 2.1 mg/kg body weight, and the rats were killed at 6 hr. As a control for the latter experiment, 2 rats were given nonradioactive AFB₂ to which had been added sufficient AFB₁ and [³H]AFB₂ so that the amount and specific activity of the added AFB₁ were equal to the maximum that could be present as an impurity in the [³H]AFB₂.

In the 1st experiment on comparative levels of [³H]AFB₁ macromolecule adducts in tissues of control, hypophysectomized, and phenobarbital-treated rats 18 hr to 63 days after administration of AFB₁, each rat received an i.p. injection of 76 μg (1 mCi) of [³H]AFB₁ in 0.25 ml of 50% dimethyl sulfoxide in water. This corresponds to 0.52 mg/kg body weight for the control and phenobarbital-treated rats (140 to 170 g) and 0.62 mg/kg for the hypophysectomized rats (123 to 152 g). For comparison of the hepatic macromolecule-bound AFB₁, 2 to 10 hr after injection, 0.52 mg of [³H]AFB₁ was administered per kg; for comparison of the binding as a function of dose, the rats received 0.26 to 0.78 mg/kg. The

* The retention time given for the dihydrodiol in Ref. 34 was 8.5 min. With the µBondapak column used in the present work the retention time was 10.5 min.
doses for the last 2 experiments contained 0.67 mCi of \(^3H\) per kg body weight.

At the times indicated, the rats (usually 3 per group) were decapitated, and the livers were removed and frozen in liquid nitrogen. In the time course experiment, the small intestines (after being split lengthwise and rinsed in 6% sodium 4-aminosalicylate to remove fecal matter), spleens, and kidneys for each group and time point were pooled and frozen immediately. The tissues were stored at \(-20^\circ\) C. DNA and rRNA were isolated by a phenol extraction procedure (19). The protein was precipitated by addition of 1.5 volumes of acetone to the phenol layers, collected by centrifugation, washed by successive suspension and recentrifugation in 95% ethanol and ethyl ether, and dried over CaCl\(_2\).

Total rRNA (4) and DNA (6) were determined on aliquots of the liver homogenates, and the total protein was estimated from its dry weight after recovery from the phenol. The nucleic acids isolated from the livers were dissolved in 0.01 M sodium phosphate buffer, pH 7.4, and were quantitated by their absorbance at 260 nm; 49 \(\mu g/ml\) (DNA) or 50 \(\mu g/ml\) (rRNA) were taken to have an absorbance of 1.0.

Hydrolysis of Nucleic Acid-[\(^3H\)]Aflatoxin Adducts. Samples of hepatic rRNA or DNA (0.3 to 2 mg) in 4 ml of 0.15 M \(NH_3\) were heated for 2 hr in a boiling water bath. The samples were then neutralized with 0.38 ml of 3.0 M sodium acetate, filtered through a 0.45-\(\mu\)m Millipore filter (Millipore Corp., Bedford, Mass.), and mixed with nonradioactive dihydrodiol for chromatography of 100-\(\mu l\) aliquots on a Bonnelapak C\(_18\) column. The column was eluted with 30% methanol:0.01% acetic acid in water with a flow rate of 1.0 ml/min for 2 min. The flow rate was then changed to 2.0 ml/min with a linear 20-min solvent gradient that increased the methanol concentration to 90%. Fractions collected at 0.25-min intervals were dissolved in 5 ml of Scintisol for determination of \(^3H\).

Products I, II, and III (see "Results"), separated by high-pressure liquid chromatography of 0.15 \(N\) \(HC1\) hydrolysates of the hepatic rRNA and DNA from rats given [\(^3H\)]AFlB\(_1\), were collected by pooling the appropriate fractions from successive chromatographies. In some tests these fractions were lyophilized, rehydrolyzed in 0.15 \(N\) \(HC1\) for 2 hr, and neutralized with sodium acetate. The above hydrolysates were chromatographed in admixture with 0.15 \(N\) \(HC1\) hydrolysates of DNA that had been recovered after incubation with NADPH-fortified microsomes from phenobarbital-treated hamsters (0.1% in the drinking water for 1 to 2 weeks) and AFB\(_1\), in vitro. The latter hydrolysate contained aflatoxin products apparently identical to those obtained by hydrolysis of the adducts formed in vivo (32)\(^7\) and was used as a UV marker for the location of the products in the eluates. Fractions were collected for scintillation counting as described above.

The 2-hr hydrolysis time was chosen on the basis of preliminary studies with rRNA-AFB\(_1\), adducts that showed that the yield of the dihydrodiol was nearly maximal at this time. The recoveries of added [\(^3H\)]dihydrodiol were 100, 91, 84, 66, and 35% after 0.5-, 1-, 1.5-, 2-, and 3-hr hydrolyses, respectively. About 15% of the \(^3H\) in the [\(^3H\)]dihydrodiol apparently underwent exchange during a 2-hr hydrolysis in acid, since it was liberated as a volatile product that eluted in the 1st column volume and was presumed to be tritiated water. The major share of the \(^3H\) appears to be located alpha to the cyclopentenone carbonyl group of the aflatoxins (32); it is very readily exchanged in alkaline solutions.

Modification of the Carcinogenicity of AFB\(_1\), by Phenobarbital or Other Dietary Additives. Male Fischer rats (Charles River Breeding Laboratory) with an average initial weight of 120 g were fed a purified diet based on that used by Wogan and Newberne (37). The composition per kg was: purified casein (Teklad Test Diets, Madison, Wis.), 220 g; Phillips-Hart mineral mix (Teklad Test Diets), 40 g; glucose monohydrate (Cerelose; CPC International, Inc., Englewood Cliffs, N. J.), 639 g; corn oil (Mazola; CPC International, Inc.), 100 g; choline chloride, 1 g; vitamin B\(_6\), 0.05 mg; biotin, 0.5 mg; folic acid, 2.0 mg; Menadione, 5 mg; thiamine chloride, 10 mg; riboflavin, 20 mg; niacinamide, 50 mg; pyridoxine hydrochloride, 10 mg; p-aminobenzoic acid, 100 mg; calcium pantothenate, 50 mg; inositol, 200 mg; retinyl acetate, 1.5 \(\times\) 10\(^2\) units; vitamin D\(_3\), 0.047 mg; and tocopherol acetate, 42 mg. AFB\(_1\) was added to the diet in acetic acid solution at a level of 0.3 mg/kg; benz(a)anthracene (Eastman Organic Chemicals, Rochester, N. Y.) was added in acetic acid solution at a final concentration of 70 mg/kg, and ascorbic acid (Sigma Chemical Co., St. Louis, Mo.) was added at a level of 25 g/kg (instead of glucose). The basal diet was placed in plastic bags, the additives were distributed over the diets, and the solvents were evaporated in a hood. Then the bags were closed tight, and the diets were mixed by kneading. The diets were mixed weekly and stored at 4\(^\circ\). Sodium phenobarbital was dissolved in the drinking water (1 g/liter); the water bottles were changed 3 times/week. All personnel who worked with these animals wore laboratory coats, plastic gloves, and filter masks.

At 15 months the livers of all of the rats were examined by laparotomy, and the rats were then fed the basal diet without additions until the experiment was terminated at 20 months. On death each rat was given a routine gross examination, which included the ear duct glands and the organs of the abdominal and thoracic cavities. Each tumor and all grossly abnormal tissues were fixed in 10% formalin, sectioned at 5 to 6 \(\mu\)m, and stained with hematoxylin and eosin.

RESULTS

Comparative Macromolecular Binding of [\(^3H\)]AFB\(_1\), and [\(^3H\)]AFB\(_2\), in Rat Liver. In 2 experiments, the levels of aflatoxin residues bound to hepatic DNA and rRNA from AFB\(_2\) were 0.6 to 1.4% of those bound after administration of AFB\(_1\) (Table 1). The levels of protein-bound aflatoxin residues from AFB\(_1\) in the same livers were 35 to 73% of those obtained from AFB\(_1\). Approximately 1 AFB\(_1\), residue was bound per 20,000 nucleotide residues in DNA or rRNA in Experiment 1; in Experiment 2 there was approximately 1 AFB\(_1\), residue bound per 2000 nucleotide residues. The much higher levels of bound aflatoxin residues in the 2nd experiment were presumably due to the higher dose (4 times that used for the 1st experiment) and the earlier time.

\(^7\) J.-K. Lin, J. A. Miller, and E. C. Miller, manuscript in preparation.
Hepatic macromolecule-bound aflatoxin in rats given \(^{3}H\)AFB\(_1\) or \(^{3}H\)AFB\(_2\)

In each experiment 3 male Fischer rats, 180 to 200 g, received injections i.p. of \(^{3}H\)AFB\(_1\) or \(^{3}H\)AFB\(_2\) at the doses indicated. The liver macromolecules were isolated according to the procedure of Irving and Veazey (19). For further details, see "Materials and Methods."

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Dose of (^{3}H)AFB(_1) or (^{3}H)AFB(_2) (mg/kg body wt)</th>
<th>Time rats killed (hr after injection)</th>
<th>Macromolecule isolated</th>
<th>(\mu)oles aflatoxin bound/mg macromolecule (x 10(^{-6}))</th>
<th>Ratio of bound products (AFB(_2):AFB(_1))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.52</td>
<td>18</td>
<td>DNA</td>
<td>130 ± 20(^a) 1.4 ± 0.1</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>rRNA</td>
<td>140 ± 5 2.0 ± 0.2</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Protein</td>
<td>11 ± 1 8.3 ± 0.4</td>
<td>0.73</td>
</tr>
<tr>
<td>2</td>
<td>2.08</td>
<td>6</td>
<td>DNA</td>
<td>1700 ± 100 12 ± 3</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>rRNA</td>
<td>1900 ± 200 13 ± 3</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Protein</td>
<td>150 ± 60 52 ± 3</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>0.0012 (^{3}H)AFB(_2) (^b) + 2.08 AFB(_2) (non-radioactive)</td>
<td>6</td>
<td>DNA</td>
<td>(\leq0.4), (\leq0.4)</td>
<td>(\leq0.01), (\leq0.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>rRNA</td>
<td>(\leq0.2)</td>
<td>(\leq0.02)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Protein</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Average ± S.D. for 3 rats

\(^b\) This amount of AFB\(_1\) and its tritium content were the maximum that could have been present as an impurity in the \(^{3}H\)AFB\(_2\).

\(^c\) Data for 2 rats.

At which the rats were killed (6 hr as compared to 18 hr for the 1st experiment; see below).

As a control, 2 rats in the 2nd experiment received injections of a mixture of \(^{3}H\)AFB\(_1\) and nonradioactive AFB\(_2\). The amount and specific activity of the AFB\(_2\) were the maximum that could have been obtained from the \(^{3}H\)AFB\(_2\) used for Experiment 2, based on the assumption that the specific activity of any contaminating AFB\(_1\) (determined to be \(<0.06\%) was the same as that of the AFB\(_2\). The levels of \(^3\)H derivatives bound to the hepatic nucleic acid or protein from these rats were insignificant in comparison to those obtained from the \(^{3}H\)AFB\(_2\)-injected rats (Table 1).

**Release of 2,3-Dihydro-2,3-dihydroxy-AFB\(_1\) and of 2 Other Major Aflatoxin-containing Derivatives on Acid Hydrolysis of the Hepatic Nucleic Acids.** High-pressure liquid chromatography of mild acid hydrolysates (0.15 N HCl, 100\(^\circ\), 2 hr) of the DNA from rats given \(^{3}H\)AFB\(_1\), showed, in addition to the material eluting within the 1st column volume, 3 major products (Products I, II, and III) that contained \(^3\)H from \(^{3}H\)AFB\(_1\), and several minor peaks of \(^3\)H-containing material (Chart 1A). Similar radioactive profiles were obtained on chromatography of acid hydrolysates of rRNA from the livers of AFB\(_1\)-treated rats or the DNA or rRNA from the livers of rats given \(^{3}H\)AFB\(_2\) (Chart 1B, C, and D), except that a relatively smaller amount of Product III was obtained from the rRNA hydrolysates. The data in Chart 1 were obtained by elution with a 30 to 90% methanol gradient in aqueous 0.01% acetic acid. Identity between the retention times of the peaks obtained from the \(^{3}H\)AFB\(_1\) and \(^{3}H\)AFB\(_2\)-nucleic acid adducts was also obtained by high-pressure liquid chromatography with an isocratic system [95% ethanol:water:glacial acetic acid (5:15:0.001, by volume)].

No evidence for heterogeneity of any of these 3 products has been obtained on rechromatography of each product on the \(\mu\)Bondapak column with 6 different isocratic systems. These solvent systems contained various proportions of water and an alcohol (methanol, ethanol, or 1-propanol) with or without small amounts of acetic or formic acid; retention times of approximately 8 to 16, 9 to 20, and 12 to 16 min were obtained.
D. H. Swenson et al.

26 min were obtained for Products I, II, and III, respectively.

Product II (Chart 1) cochromatographed with synthetic 2,3-dihydro-2,3-dihydroxy-AFB₂; this finding was in agreement with our previous thin-layer chromatographic data (32, 33) on the presence of the dihydromel in mild acid hydrolysates of DNA- or rRNA-AFB₂ adducts formed in vivo or rRNA-AFB₂ adducts formed in vitro. Examination of the amounts of the products as a function of the time of hydrolysis suggested that Product I might hydrolyze to the dihydromel (Chart 2). Thus, the amount of Product I was high at early times and decreased in amount as the amount of the dihydromel increased. These hydrolysis curves did not provide any evidence for a precursor-product relationship between Product III and the dihydromel; the former product was released rapidly, and the amount remained relatively constant with longer hydrolysis periods.

When Product I was collected from the eluates and rehydrolyzed, it yielded a product chromatographically indistinguishable from the dihydromel. Thus, after ³H-labeled Product I was heated at 100° in 0.15 N HCl for 2 hr, the hydrolysate contained 34% of the original amount of Product I; 38% of the ³H was in a product with the retention time of the dihydromel, and 19% of the ³H eluted in the 1st column volume (presumably as [³H]H₂O). Product III was essentially stable under these conditions; 70% of the ³H was recovered at the original retention time, 18% eluted in the 1st column volume, and only 9% eluted at the retention time of the dihydromel. Further data on the structures of Products I and III will be presented separately. The recovery of ³H from the [³H]dihydromel at the retention time of the dihydromel under these mild acid hydrolysis conditions was 66%. In these studies hydrolysates of DNA- or rRNA-AFB₂ adducts formed by incubation of AFB₂ with phenobarbital-induced hamster liver microsomes in a NADPH-generating system (33) were cochromatographed with the radioactive products to provide UV identification of Products I to III. The products formed by hydrolysis of these in vitro adducts appear to be identical to those obtained by hydrolysis of the adducts formed in vivo.⁷

On an average these 3 products accounted for 77 and 54% of the ³H in the hepatic DNA from rats given [³H]AFB₁ and [³H]AFB₂, respectively. Likewise, these products accounted for 40 and 32%, on the average, of the ³H in the hepatic rRNA from the rats of these 2 groups (Table 2). As is evident from the table, the S.D.’s for the analyses are quite large. A number of factors probably contributed to the observed variations. More recent studies have shown that the amounts of Product III are decreased and those of Product I are increased if the nucleic acids are kept at pH's above 7 prior to hydrolysis.⁷ The destruction of the dihydromel during the hydrolysis, the slow exchange of ³H under the hydrolysis conditions, and the incomplete hydrolysis of the rRNA in 0.15 N HCl (as evidenced by the occasional formation of a precipitate on neutralization of the RNA hydrolysate) may have further contributed to the observed variations.

Hepatic Macromolecule-bound Derivatives of [³H]AFB₁ in Phenobarbital-treated or Hypophysectomized Rats. The body weights of the control rats and of those given phenobarbital for 1 week were similar, while those that had been hypophysectomized 3 weeks previously were 17% lighter (Table 3). As expected from the literature, the liver weights, calculated as a percentage of body weight, were similar for the controls and the hypophysectomized rats, while those from the phenobarbital-treated rats were about 50% heavier. In spite of the differences in wet weight, the contents of DNA per liver were very similar for all 3 groups; the amounts of rRNA and, especially, of protein reflected the differences in total liver weights.

At 18 hr after the injection of [³H]AFB₁, the earliest time point at which the effects of the treatments were compared, the specific activities of the hepatic rRNA and crude protein were similar for the control and hypophysectomized rats. The specific activity of the hepatic DNA from the hypophysectomized rats was about one-half that from the control.

### Table 2

<table>
<thead>
<tr>
<th>Product</th>
<th>DNA-AFB₁</th>
<th>DNA-AFB₂</th>
<th>rRNA-AFB₁</th>
<th>rRNA-AFB₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>32 ± 12</td>
<td>28 ± 7</td>
<td>17 ± 7</td>
<td></td>
</tr>
<tr>
<td>II (dihydromel)</td>
<td>22 ± 12</td>
<td>19 ± 6</td>
<td>13 ± 4</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>19 ± 7</td>
<td>15 ± 3</td>
<td>6 ± 4</td>
<td>8 ± 3</td>
</tr>
</tbody>
</table>

* No corrections were made for recoveries. The recovery of [³H]dihydromel hydrolyzed under these conditions was 66% with 15% of the ³H apparently being released as [³H]H₂O. See “Results.”

* Average ± S.D. for analyses on samples from 3 livers.

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The fractions of ³H in the hepatic DNA and rRNA from rats given injections of [³H]AFB₁ or [³H]AFB₂, released as dihydromel and Products I and III on mild acid hydrolysis.

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The DNA and rRNA from the livers of rats given injections of [³H]AFB₁, or [³H]AFB₂ (Table 1, Experiment 2) were hydrolyzed for 2 hr in 0.15 N HCl in a boiling water bath. After being neutralized, the hydrolysates were analyzed by high-pressure liquid chromatography (see "Materials and Methods").

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Hepatic Macromolecule-bound Derivatives of [³H]AFB₁ in Phenobarbital-treated or Hypophysectomized Rats.
The specific activities of the DNA, rRNA, and protein for the livers of phenobarbital-treated rats were only 11 to 31% those of the control rats (Table 3). The latter data confirm a report by Garner (10), who showed that phenobarbital administration decreased the levels of hepatic nucleic acid-AFB₁ adducts. Garner, however, found no decrease in the level of hepatic protein-bound AFB₁ in phenobarbital-treated rats. At 18 hr, the specific activities of the rRNA's were 2 to 3 times those of the DNA, while the specific activities of the crude proteins were only 7 to 14% those of the rRNA. The overall percentages of the injected dose of [³H]AFBI bound to the hepatic macromolecules at 18 hr were 4, 6, and 10% for the phenobarbital-treated, hypophysectomized, and control rats, respectively. As noted in "Materials and Methods," the amounts of AFB₁ administered in this experiment were not adjusted for differences in body weight, so the hypophysectomized rats received about 20% more carcinogen per kg body weight than did the control or phenobarbital-treated rats.

In the above experiment, the earliest determinations of aflatoxin binding to tissue macromolecules were made at 18 hr in view of an earlier report (21) which indicated that the binding was maximal and similar at 6 and 18 hr. However, the high levels of macromolecule-bound aflatoxin at 18 hr as compared to the values at later times (Chart 3) suggested that the bound metabolites might have reached maximum values at an earlier time. In fact, data from a 2nd experiment (see Chart 3, inserts) demonstrated that for control rats the binding to each class of hepatic macromolecules was maximal by 2 hr. At this time the specific activities of the DNA and rRNA were approximately equal, while the specific activity of the crude protein was only 3% that of the nucleic acids. At 2 hr, 11% of the dose was bound to the hepatic macromolecules; in these rats, the amount bound to protein was somewhat lower, even at 2 hr, than for the rats killed at 18 hr in the larger experiment (Chart 3).

By 18 hr the specific activities of the hepatic macromolecules of the control rats had decreased to 38 to 66% of the values observed at 2 hr. The rates of loss of [³H] from the macromolecules isolated from the livers of the control, phenobarbital-treated, and hypophysectomized rats were generally similar. The rate of loss of the DNA-bound [³H] residues appeared to decrease after the 3rd day, while the rate of loss of the rRNA- and protein-bound residues did not change markedly. None of the hypophysectomized rats survived to the 63-day analysis point.

Over the range of doses studied (0.26 to 0.78 mg/kg body weight) the specific activities of the hepatic macromolecules from the control rats increased with increasing doses (Chart 4). For the macromolecules from the phenobarbital-treated rats there was little increase in the specific activities of protein and DNA on increasing the dose from 0.52 to 0.78 mg/kg.

Preliminary studies on the amounts of the dihydriodiol that could be released from these hepatic nucleic acids were carried out before detailed studies showed the presence of Products I and III in the hydrolysis mixtures and under conditions that were inadequate for the separation of Product III from the dihydriodiol. Insufficient amounts of these nucleic acid samples were available for further analyses after the chromatography and quantitation of Products I and III and the dihydriodiol had been refined. Nevertheless, these analyses, in which the products measured were the dihydriodiol and most or all of Product III, indicate that the proportions of the bound products in the livers from phenobarbital-treated and hypophysectomized rats were similar to those in the livers of control rats given AFB₁. The analyses were carried out on pooled nucleic acid samples from rats killed at the times indicated. The percentages of the DNA- and rRNA-bound [³H] recovered as dihydriodiol + Product III were 34 to 43 and 29 to 37%, respectively, for the control rats killed at 2 hr to 7 days; the values for the phenobarbital-

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### Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Body wt (g)</th>
<th>Liver wt (g)</th>
<th>mg macromolecule/liver</th>
<th>μmoles bound aflatoxin/mg macromolecule (x10⁻³)</th>
<th>Total μmoles bound aflatoxin/liver (x10⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>155 ± 2</td>
<td>5.5 ± 0.1</td>
<td>DNA 12 ± 1</td>
<td>130 ± 30</td>
<td>1.6</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>rRNA 38 ± 5</td>
<td>260 ± 20</td>
<td>9.9</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Protein 790 ± 70</td>
<td>16 ± 2</td>
<td>12.7</td>
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<tr>
<td>Phenobarbital-treated</td>
<td>155 ± 4</td>
<td>8.3 ± 0.4</td>
<td>DNA 14 ± 1</td>
<td>15 ± 5</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>rRNA 51 ± 4</td>
<td>35 ± 9</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Protein 1660 ± 100</td>
<td>5 ± 1</td>
<td>7.9</td>
</tr>
<tr>
<td>Hypophysectomized</td>
<td>129 ± 8</td>
<td>4.0 ± 0.3</td>
<td>DNA 13 ± 1</td>
<td>68 ± 8</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>rRNA 23 ± 2</td>
<td>210 ± 20</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Protein 620 ± 70</td>
<td>15 ± 2</td>
<td>9.3</td>
</tr>
</tbody>
</table>

* The binding to DNA at 18 hr in normal rats given AFB₁ at 0.52 mg/kg body weight varied little in 4 experiments (Tables 1 and 3; Charts 3 and 4). However, the level of binding to rRNA ranged from about 1 to 2 times that for the DNA. The cause of this variation is unknown.

* See text for details.

** Average ± S.D. for 3 rats.
treated rat livers were 34 to 38 and 32 to 33%, and for the hypophysectomized rat livers the values were 29 to 39 and 30 to 31%, respectively.

**Macromolecule-bound Derivatives of [³H]AFB₁ in Extrahepatic Rat Tissues.** The extrahepatic tissues studied contained much less of the macromolecule-bound aflatoxin derivatives than did liver. In most cases the specific activities of the kidney macromolecules from the AFB₁-treated rats were 10 to 20% those of the liver macromolecules (Chart 3). The specific activities of the macromolecules from spleen and small intestine were lower; for the control rats the values were generally 1 to 5% those obtained for the hepatic macromolecules. On comparison of the control, phenobarbital-treated, and hypophysectomized rats, the differences in specific activity were generally less for the nonhepatic than for the hepatic macromolecules. Garner (10) has likewise noted that phenobarbital-treated rats given injections of [³¹C]AFB₁ had lower levels of DNA and rRNA-aflatoxin adducts in the kidneys than did control rats. The percentages of the DNA- and rRNA-bound [³H] recovered as dihydrodiol + Product III for the kidneys, spleens, and small intestines of the control rats killed at 18 hr were 27 and 27, 35 and 34, and 32 and 34%, respectively.

**Inhibition of AFB₁-induced Hepatic Carcinogenesis by Coadministration of Phenobarbital.** All of the male Fischer rats fed a diet that contained AFB₁ at 0.3 mg/kg for 15 months developed hepatocellular carcinomas by the termination of the experiment at 20 months; the hepatic carcinoma incidence was 61% at 15 months (Table 4). On the other hand, rats that were fed this level of AFB₁ and that simultaneously received 0.1% of sodium phenobarbital in the drinking water had hepatic carcinoma incidences of only 11 and 67% at 15 and 20 months, respectively. This inhibitory effect of phenobarbital was in agreement with the slower rate of hepatic tumor induction by an AFB₁-contaminated peanut meal when it was administered to phenobarbital-treated rats (24). Administration of benz(a)anthracene at 70 mg/kg had no demonstrable effect on the development of hepatocarcinomas from AFB₁ (Table 4); when fed at a diet level of 28 mg/kg, this hydrocarbon markedly reduced the hepatocarcinogenic effect of 3'-methyl-N,N-dimethyl-4-aminoazobenzene (25). Supplementation of the diet with
Male Fischer rats (average initial weight, 120 g) were fed a purified diet (see "Materials and Methods"). AFB\textsubscript{1} was added to the diet at a level of 0.3 mg/kg, benz(a)anthracene was added at 70 mg/kg, and ascorbic acid was added at 25 g/kg. Sodium phenobarbital was added to the drinking water (1 g/liter). At 15 months the livers of all of the rats were examined by laparotomy, and the rats were then fed the basal diet without additions until the termination of the experiment at 20 months.

No. of rats with hepatocellular carcinomas  
No. of rats alive  
Av. wt gain (g) at  
2 mos. 4 mos. 

<table>
<thead>
<tr>
<th>Test compounds</th>
<th>No. of rats in group</th>
<th>Av. wt gain (g) at 2 mos.</th>
<th>Av. wt gain (g) at 4 mos.</th>
<th>No. of rats with hepatocellular carcinomas by 15 mos.</th>
<th>No. of rats alive without liver carcinomas by 20 mos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB\textsubscript{1}</td>
<td>18</td>
<td>135</td>
<td>188</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>AFB\textsubscript{1} + phenobarbital</td>
<td>18</td>
<td>138</td>
<td>179</td>
<td>2</td>
<td>12</td>
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<tr>
<td>AFB\textsubscript{1} + benz(a)anthracene</td>
<td>18</td>
<td>135</td>
<td>184</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td>AFB\textsubscript{1} + ascorbic acid</td>
<td>18</td>
<td>143</td>
<td>194</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>18</td>
<td>134</td>
<td>173</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Benz(a)anthracene</td>
<td>15</td>
<td>140</td>
<td>192</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>15</td>
<td>142</td>
<td>191</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>6</td>
<td>139</td>
<td>190</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* One rat developed an s.c. sarcoma.  
* One rat each had a carcinoma of the ear duct gland and a reticulum cell sarcoma; 5 rats had interstitial cell tumors of the testes.  
* One rat had a carcinoma of the small intestine, 1 had a papilloma on the lip, and 2 had interstitial cell tumors of the testes.  
* One rat had a transitional cell carcinoma of the urinary bladder.  
* One rat had a malignant lymphoma, and 4 rats had interstitial cell tumors of the testes.  
* One rat had an s.c. sarcoma, and 5 rats had interstitial cell tumors of the testes.  
* Four rats had interstitial cell tumors of the testes.  
* Three rats had interstitial cell tumors of the testes.

DISCUSSION

The levels of the DNA- and rRNA-aflatoxin adducts formed in vivo correlated quite well in several respects with the likelihood of tumor formation from AFB\textsubscript{1} or AFB\textsubscript{2}. These correlations include: (a) the relative specificity of AFB\textsubscript{1} for the induction of tumors in the liver of the rat (Table 4; Ref. 36) and for forming nucleic acid adducts in the liver as compared to other tissues (Chart 3), (b) the inhibition of both hepatocarcinogenicity and hepatic nucleic acid-binding of AFB\textsubscript{1} by phenobarbital administration (Chart 3; Table 4; Refs. 10 and 24), (c) the inhibition of both liver tumor induction and DNA-aflatoxin adduct formation in hypophysectomized rats (Chart 3; Table 4; Ref. 16), (d) and the much higher levels of nucleic acid-bound derivatives and of tumor formation in the livers of rats given AFB\textsubscript{1} as compared to AFB\textsubscript{2} (Table 1; Ref. 36). Garner and Wright (14) have similarly shown that the livers of hamsters, which appear to be more resistant to hepatocarcinogenesis by AFB\textsubscript{1} than are those of rats, formed lower levels of hepatic AFB\textsubscript{1}-nucleic acid adducts. In several of the above situations, correlations were also observed between the levels of protein-aflatoxin adducts and susceptibility to tumor formation from AFB\textsubscript{1}. However, 3 exceptions have been noted, and these exceptions suggest that nucleic acid-aflatoxin derivatives may be of greater significance in the induction of hepatocarcinogenesis by AFB\textsubscript{1} than are the protein-AFB\textsubscript{1} derivatives. These exceptions are: (a) the lack of correlation between the level of hepatic protein-aflatoxin adducts and susceptibility to hepatic carcinogenesis of rats given AFB\textsubscript{1} or AFB\textsubscript{2} (Table 1), (b) the similar levels of protein-AFB\textsubscript{1}, adducts in the livers of rats and hamsters (14), and (c) the similar levels of protein-AFB\textsubscript{1} adducts in the livers of control and hypophysectomized rats (Table 3; Chart 3). It is recognized, how-
ever, that the overall levels of DNA, rRNA, or protein binding of carcinogens have limited usefulness in specifying critical targets, since induction of tumors may result from only 1 kind of interaction of the ultimate carcinogen with the critical target [e.g., the correlations between O-6 as compared to N-7 alkylation of guanine residues in DNA in relation to tumor induction by alkylating agents (17, 20, 22, 26)].

Other evidence for the relative importance of nucleic acid-binding in aflatoxin carcinogenesis has been obtained through studies on AFB1-2,3-dichloride, a model for AFB1-2,3-oxide (34). In these studies, the nucleic acid-bound products from AFB1-2,3-dichloride appeared to result from direct reaction of the nucleic acids with the dichloride derivative, while the protein-bound products appeared to result from reaction with hydrolysis products of AFB1-2,3-dichloride, i.e., 3-chloro-2,3-dihydro-2-hydroxyafatoxin B2 and the dihydrodiol. The latter 2 products were very reactive toward proteins but were only slightly reactive toward nucleic acids; these products did not initiate skin papillomas in mice. AFB1-2,3-dichloride, which was very reactive toward both nucleic acids and proteins, was a very potent initiator of skin papillomas.

The release of the dihydrodiol by mild acid hydrolysis of the nucleic acids from the livers of AFB1-injected rats has provided strong evidence that AFB1-2,3-oxide is a reactive metabolite of AFB1. Additional evidence for the quantitative importance of the 2,3-oxide as a reactive intermediate has come from the finding that Product I, another hydrolysis product of the DNA- and rRNA-AFB1 adducts, can be further hydrolyzed to the dihydrodiol.

The release of the dihydrodiol and of Product I on acid hydrolysis of the AFB1-nucleic acid adducts from rat liver indicates that AFB1-2,3-oxide is also an intermediate in the formation of the nucleic acid-aflatoxin adducts from AFB1 (Chart 5). Transformation of AFB2 to AFB1-2,3-oxide would presumably occur via the intermediate formation of AFB1 by desaturation of the 2,3-carbons of AFB2. The formation of small amounts of AFB1 from AFB2 has previously been suggested as the basis for the carcinogenic activity of AFB2 in rat liver (36). Direct evidence for the formation of AFB1 from AFB2 by duck liver homogenates was provided by Roebuck and Siegel (28); they were unable to detect this transformation with rat or mouse liver homogenates. The ratio of the amounts of the nucleic acid-aflatoxin adducts formed from AFB1 and AFB2 in our experiments was very similar to the ratio of their hepatocarcinogenic activities in rats (36), and the formation of the nucleic acid-AFB1 adducts released as the dihydrodiol and Product I were two-thirds as great as for the AFB2-nucleic acid adducts. All of these data support the likelihood that AFB1-2,3-oxide is a major ultimate carcinogenic metabolite of both AFB1 and AFB2 in rat liver.

No direct evidence is available on the mechanism by which the binding of AFB1 to protein occurs in vivo. However, our studies on the binding of AFB1-2,3-dichloride to protein (34) suggest that the dihydrodiol, formed by hydrolysis of AFB1-2,3-oxide, may be a key intermediate in the formation of the AFB1-protein adducts. The much higher levels of the protein-AFB1 adducts formed in vivo as compared to the nucleic acid-AFB1 adducts suggest that the majority of the hepatic protein adducts formed from AFB2 are not derived from derivatives of AFB1-2,3-oxide. A candidate for the precursor of the protein-bound derivatives of AFB2 is AFB2a. Dann et al. (8) have reported that this hemiacetal is a major metabolite of AFB2 in rats; also, AFB2a and other 2-hydroxyafatoxin B2 derivatives react readily with proteins in vitro at neutrality (2, 18, 27, 34). These reactions appear to result primarily from Schiff base formation between free amino groups and the dialdehyde phenolate tautomers of these hemiacetals (2, 27).

The highly probable formation of AFB1-2,3-oxide in vivo and its expected properties appear relevant to the possible nature of the microsome- and NADPH-dependent metabolite of AFB1, described by Patterson and Roberts (27) and by Gurtoo and Campbell (18). This metabolite, which was not isolated, was designated as AFB2a from the UV spectral properties of a protein-bound derivative of the metabolite. A requirement for NADPH for hydration of the 2,3-double bond to form AFB2a is not consistent with the other known reactions of this type, and other authors (29) have reported that NADPH is not required for the hydration of AFB1. The NADPH- and microsome requirements would be consistent with the formation of the 2,3-oxide, which could then react with water to yield the dihydrodiol. The UV spectra of AFB2a and the dihydrodiol are indistinguishable (33), and the 2 compounds react with protein in the same fashion to yield similar products (34).

The results from the present studies on the in vivo binding of [3H]AFB1 to tissue macromolecules of rats differ from those in a previous report by Lijinsky et al. (21) in several important respects. Thus, the ratio of protein-bound to nucleic acid-bound aflatoxin derivatives in the liver and extrahepatic tissues was much higher in the study of Lijinsky et al. (21) than has been observed by us (this paper; Ref. 32) or by Garner (10). In the present studies approximately one-half of the total macromolecule-bound 3H in the liver was
associated with the nucleic acids as compared to about 0.1% of the \(^3\)H in the study of Lijinsky et al. (21). The specific activities of the macromolecules of the extrahepatic tissues, as compared to those of liver, were relatively higher in the study of Lijinsky et al. (21) than in this study, and the liver macromolecules had reached their maximum specific activities by our earliest analysis (2 hr) as compared to 6 to 18 hr in their study. Although a number of factors may have contributed to the differences in the results of the 2 studies, the major difference was probably the low radiochemical purity of the aflatoxin preparations used by Lijinsky et al. (21).

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Aflatoxin B₁-2,3-oxide as a Probable Intermediate in the Covalent Binding of Aflatoxins B₁ and B₂ to Rat Liver DNA and Ribosomal RNA \textit{in Vivo}


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