AFLATOXIN B_1-2,3-OXIDE: EVIDENCE FOR ITS FORMATION IN RAT LIVER IN VIVO AND BY HUMAN LIVER MICROSOMES IN VITRO

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Received August 21,1974

SUMMARY

Injection of [^{3}H]aflatoxin B_1 into rats yielded covalently bound derivatives in hepatic DNA, rRNA, and protein. Mild acid hydrolysis of the DNA and rRNA adducts formed a derivative indistinguishable from 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1. The data indicate that approximately 60% of the nucleic acid adducts were derived from reactions in vivo with aflatoxin B_1-2,3-oxide. Acid hydrolysis of rRNA-[^{3}H]aflatoxin B_1 adduct formed by human liver microsomes in vitro also liberated the dihydrodiol in significant amount. The 2,3-oxide of aflatoxin B_1 is a probable ultimate carcinogenic metabolite.

Aflatoxin B_1, a potent hepatotoxic and hepatocarcinogenic fungal constituent, requires metabolic activation to exert its biological effects (see 1) and is covalently bound in the rat to hepatic DNA, RNA, and protein (2). Rodent liver microsomes convert AFB_1 to toxic and reactive derivative(s) (1,3). Although efforts to isolate such metabolite(s) have failed (1), they can be trapped in situ by reactions with added DNA, RNA, or protein (1,4,5). In this laboratory the isolation of the 2,3-dihydrodiol of AFB_1 after mild acid hydrolysis of the rRNA adduct provided strong evidence that AFB_1-2,3-oxide is a reactive intermediate in the microsomal binding of AFB_1 to rRNA (6). Other evidence has since been reported for the microsomal formation of this oxide (7). In this paper we show that AFB_1-2,3-oxide is apparently responsible for a major share of the covalent binding in vivo of AFB_1 to liver DNA and rRNA in the rat and that this oxide is formed by human liver microsomes in vitro.

MATERIALS AND METHODS

TLC was carried out on 0.5-mm layers of silica gel HF-254 (Merck, AG,

Abbreviations: AFB_1, aflatoxin B_1; AFB_2, 2,3-dihydro-aflatoxin B_1; AFB_{2a}, 2,3-dihydro-2-hydroxy-aflatoxin B_1; rRNA, ribosomal RNA; DMSO, dimethylsulfoxide; EDTA, ethylenediamine tetraacetic acid; TLC, thin layer chromatography.

Darmstadt, Germany) on glass or on Chromagram 13181 sheets of silica gel on plastic (Eastman Kodak Co., Rochester, N.Y.), with or without boric acid impregnation (6). The following development solvents were used: A, $CHCl_3$:CH_3OH (97:3); B, $CHCl_3$:CH_3OH (9:1); C, benzene:95% ethanol (8:3); D, ethyl acetate: CH_3OH (4:1); and E, ethyl acetate:CH_3OH:H_2O (80:10:2).

[^{3}H]AFB_1 (4.1 Ci/mmole; Nuclear Dynamics Corp., El Monte, Calif.) was stored at 0-4°C. in benzene:CH_3OH (1:1). The radiochemical purity at time of use was 96% as determined by TLC on silica in solvents A, C, and D. The remaining radioactivity was generally distributed over the plates. The use of excessive heat in spotting and drying the plates was avoided. Exchanges of radioactivity of 12% in 0.15 N HCl at 100°C. for 90 min and of 93% in 1.0 N NaOH at 100°C. for 100 min were noted. The latter finding indicates that most of the ^{3}H was on the α-carbon of the cyclopentenone ring.

An improved synthesis of 2,3-dihydro-2,3-dihydroxy-aflatoxin B_1 (6) (hereafter designated as the dihydrodiol) was employed. Ten mg AFB_1 was reacted with 15 mg OsO_4 in 15 ml dry pyridine for 3-7 days at 25°C. The solution of osmate ester was condensed to 5 ml and reductively hydrolyzed at 25°C. for 1 hr with a solution of 0.61 g $NaHSO_3$, 10 ml H_2O, and 6.8 ml pyridine. After the addition of 13 ml of glacial acetic acid, the solution was evaporated to near dryness under reduced pressure, diluted to 6 ml with H_2O, and applied to a 2.5 cm X 57 cm Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) column. The dihydrodiol was eluted with H_2O in the 5th exclusion volume. Overall yield (A_{360}) was 23%. AFB_{2a} was synthesized by the CF_3COOH-catalyzed hydration of AFB_1 (8).

The new compound 2,3-dihydro-3-hydroxy-2-methoxy-AFB_1 was prepared by refluxing the dihydrodiol in 0.1 N methanolic HCl for 5 min. After evaporation and TLC in solvent B a blue-fluorescent product (λ_{max} in CH_3OH = 362 nm; R_F = 0.62, R_F of AFB_{2a} = 0.55) was obtained in 58% yield. The structure of this glycoside-like product was shown by its mass spectrum (6) (m/e = 360 (M^+), 342, 331, 327, 312, 299, 283, and 271) and the failure of alkali to cause a

bathochromic shift in the UV absorption spectrum. This shift is characteristic of the 2-hydroxy aflatoxins (6,8).

Male Fischer rats (Charles River Breeding Laboratory, Wilmington, Mass.), 175-190 g, were fed Wayne Breeder Blox (Allied Mills, Inc., Chicago, Ill.) and tap H_2O ad libitum. Four rats were each given 0.95 mCi (72 µg) [^{3}H]AFB_1 in 0.25 ml 50% DMSO by i.p. injection and sacrificed 14 hours later. The liver DNA and rRNA were isolated from each rat by a phenol extraction procedure (9), and the protein was precipitated by addition of 1.5 volumes of acetone to the phenol extracts. The protein was weighed and the nucleic acids determined by UV spectrometry. ^{3}H was determined in a Packard Tri-Carb scintillation spectrometer. The nucleic acids were dissolved in H_2O and counted in Scintisol (Isolab, Inc., Akron, Ohio), while the protein samples were dissolved in Soluene (Packard Instrument Co., Downers Grove, Ill.) and counted in RPI Scintillator PPO-POPOP (Research Products International, Elk Grove Village, Ill.). The aflatoxin derivatives were dissolved in CH_3OH and counted in Scintisol.

The DNA- and rRNA-[^{3}H]AFB_1 adducts were hydrolyzed in 0.15 N HCl as previously described (6) after the addition of unlabelled carrier dihydrodiol. The neutralized hydrolysates were chromatographed on 1.5 X 22 cm Sephadex LH-20 columns with H_2O as the eluant, and the dihydrodiol was purified by TLC with solvent B. The specific activity of the dihydrodiol was measured, more carrier dihydrodiol added, and the specific activity of the dihydrodiol monitored during consecutive TLC's in solvents C, B, and E. The dihydrodiol was converted to the 2-O-methyl derivative and its specific activity measured after TLC in solvents B and C.

A sample of human liver was obtained from a biopsy on a 48-year old female suspected of having Hodgkin's disease. The fresh liver (0.80 g) was homogenized in a Polytron homogenizer (Kinematica GMBH, Lucerne, Switzerland) in 25 ml 0.25 M sucrose- 1 mM Tris-HCl, pH 8.0. A microsomal pellet was obtained by centrifugation (1) and resuspended in 2 ml of the sucrose-Tris buffer. Five 5-ml reaction flasks each contained: 3.3 mg microsomal protein (biuret

assay); [^{3}H]AFB_1 (2.64 X 10^8 dpm, 9 μg) in 0.4 ml DMSO; 8 mg rat liver rRNA; 0.5 ml 1 M sodium phosphate buffer, pH 7.4; 5 μmoles EDTA; 3 μmoles NADP; 25 μmoles glucose-6-phosphate; and 5 units of glucose-6-phosphate dehydrogenase (ZF, Worthington Biochemical Corp., Freehold, N.J.). The flasks were incubated and the rRNA recovered as previously described (6). The rRNA was hydrolyzed in the presence of carrier dihydrodiol and the hydrolysate chromatographed on Sephadex LH-20 in H_2O. The dihydrodiol was chromatographed on silica and the specific activities were determined as described above.

RESULTS

Studies with rat liver. Nine percent of the ^{3}H injected as [^{3}H]AFB_1 was recovered covalently bound to the liver macromolecules. The specific activities of the DNA and rRNA were 15 and 20 times, respectively, those of the protein (Table 1). Compared to results with other chemical carcinogens the relatively high selectivity of AFB_1 toward nucleic acid binding in rat liver *in vivo* is unusual (10). This finding is in strong contrast to a previous report (2) in which the binding of AFB_1 to liver protein appeared to far exceed that of the nucleic acids. The reason for this large variance in results is not known, but may be related to the differences in sex and strain of rat employed, to the very large dose (900 μg) of AFB_1, or more likely, to the high level of radioimpurity in the [^{3}H]AFB_1 employed in the previous work (2).

The dihydrodiol was identified as a tritiated hydrolysis product of the nucleic acid adducts by its chromatography with essentially constant specific

Table 1. Binding of [^{3}H]AFB_1 to Rat Liver Macromolecules *In Vivo*.

	Specific Activity ± S.D.* dpm/mg (X 10^{-5})
DNA	22.9 ± 3.0
rRNA	15.0 ± 1.8
Protein	1.01 ± 0.28

*Data from 4 rats.

Table 2. Constancy of Specific Activity of Dihydrodiol Released from Nucleic Acids of Rats Injected with [^{3}H]AFB$_1$.

Determination after	R_F	Specific Activity dpm/A_{360} (X 10^{-4})	
		DNA	rRNA
TLC, solvent C	0.47	4.2	11
TLC, solvent B	0.26	4.1	11
TLC, solvent E	0.45	4.0	11
Methylation		3.6*	10*
TLC, 2-O-methyl derivative, solvent B	0.63	3.7	8.9
TLC, 2-O-methyl derivative, solvent C	0.65	3.7	8.4

*Decreases apparently due to an increase in extinction coefficient after methylation.

activity with carrier dihydrodiol and, upon methylation, with the 2-O-methyl derivative as carrier (Table 2). Methylation by brief exposure to hot 0.1 N HCl in methanol is highly specific for hemiacetal hydroxyl groups. The recoveries of ^{3}H and A_{360} from each TLC were 60-80%. Boric acid-impregnation of plastic-backed silica plates reduced the R_F in solvent C of the [^{3}H]dihydrodiol and of carrier dihydrodiol by 40%, as is characteristic for vic-diols (6).

Calculations indicate that more than one-half of the ^{3}H bound to the nucleic acids was released as the dihydrodiol. Unlabelled dihydrodiol (15.0 A_{360} units) was added to 2.3 mg of the rat liver DNA (3.3 X 10^6 dpm). After acid hydrolysis and chromatography, first on Sephadex LH-20 and then on silica in solvent B, 3.84 A_{360} units of dihydrodiol containing 4.93 X 10^5 dpm were recovered. Thus the recovery of ^{3}H in the dihydrodiol was 15% without correction for losses encountered in the isolation. A ^{3}H exchange of 13% occurred during a comparable acid hydrolysis of 2,3-dihydro-3-hydroxy-2-meth-

oxy-[^{3}H]AFB$_1$ and as noted in Materials and Methods an exchange of 12% was found for [^{3}H]AFB$_1$ under these conditions. After correction for this exchange of ^{3}H and for recovery (26%) of the carrier dihydrodiol, the amount of ^{3}H in the DNA which was released as dihydrodiol was calculated to be 67%.

Similarly, when 20 mg of rRNA containing 3.7 X 10^7 dpm plus 14.2 A_{360} units of carrier dihydrodiol was treated in the same manner, 3.01 A_{360} units of dihydrodiol containing 3.78 X 10^6 dpm ^{3}H were recovered. Thus, a minimum of 10% of the ^{3}H in the rRNA was recoverable as dihydrodiol. After correction for exchange of ^{3}H and recovery (21%) of carrier dihydrodiol, the percent of the rRNA-bound ^{3}H in products hydrolyzable to dihydrodiol would be 56%.

Study with human liver. rRNA incubated with human liver microsomes and [^{3}H]AFB$_1$ produced an adduct (67% recovery of rRNA) with a specific activity of 2.0 X 10^6 dpm/mg. This is a recovery of 4% of the [^{3}H]AFB$_1$ in the incubation mixture. Upon hydrolysis in acid in the presence of carrier dihydrodiol a constant specific activity of 5.4-5.6 X 10^5 dpm/A_{360} was noted for the dihydrodiol during successive TLC's in solvents B, C, and E and, after methylation, for the 2-O-methyl derivative upon TLC in solvents B and C. After correction for recovery of the carrier dihydrodiol and for ^{3}H exchange the percent of the rRNA-bound ^{3}H in products hydrolyzable to dihydrodiol was 29%.

DISCUSSION

The data presented show that a large share of the [^{3}H]AFB$_1$ bound to DNA and rRNA in rat liver *in vivo* and to rRNA by human liver microsomes *in vitro* can be hydrolyzed under mildly acidic conditions to yield a compound indistinguishable from 2,3-dihydro-2,3-dihydroxy-AFB$_1$. These results strongly support the concept that these covalently bound forms of AFB$_1$ result from reactions of the metabolically generated electrophile AFB$_1$-2,3-oxide with nucleophilic nitrogen and oxygen atoms in the DNA and rRNA (Fig. 1), probably largely in guanine residues (4).

The ultimate carcinogenic metabolites of most, if not all, chemical carcinogens appear to be strong electrophiles which bind covalently to nucleophil-

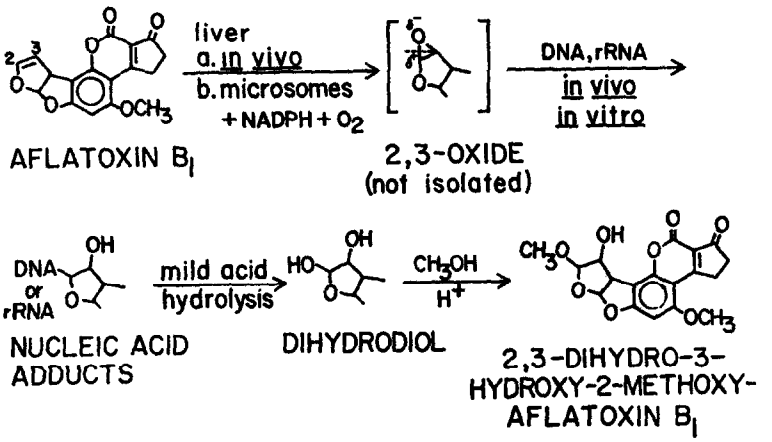

Fig. 1 Hepatic metabolism of AFB_1 to form nucleic acid-bound derivatives and the hydrolysis of these adducts to 2,3-dihydro-2,3-dihydroxy-AFB_1.

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ic sites in macromolecules *in vivo* (11). The electrophilic 2,3-oxide of AFB_1 also appears to be an ultimate carcinogenic metabolite, especially since the hepatocarcinogenicity of AFB_1 is greatly reduced when its 2,3-double bond is hydrogenated to form AFB_2 (12), a compound not capable of being directly converted to the 2,3-oxide. Other possible electrophilic metabolites of AFB_1, such as 2,3-oxides of the known metabolites aflatoxin M_1 (13), aflatoxin Q_1 (14), and aflatoxicol (15), may also exist and contribute to the *in vivo* hepatic macromolecular binding, carcinogenicity, and toxicity associated with AFB_1 administration.

Acknowledgements. This work was supported by grants CA-07175, CA-15785, and CRTY-5002 of the National Cancer Institute, USPHS. We thank Mrs. Dorianne Coughtry for excellent technical assistance. Drs. Joseph L. Skibba and Gerald M. Lower, Jr. of the University of Wisconsin Medical Center kindly supplied the sample of human liver.

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