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In Vitro Metabolism of Aflatoxin B_2 by Animal and Human Liver[1]

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ABSTRACT

The metabolism of aflatoxin B_2 by postmitochondrial supernatant fractions of duck, rat, mouse, and human livers was studied in an *in vitro* system. Duck liver had a much higher level of activity than had tissues from other species. Postmitochondrial supernatant equivalent to 0.2 g whole liver metabolized 40 to 80% of the initial substrate in 30 min, compared to < 6% for the other species. Among several metabolites formed by duck liver, aflatoxin B_1 was produced in amounts equivalent to 2 to 8% of the initial substrate, and metabolites having chromatographic properties postulated for aflatoxicols 1 and 2 and aflatoxins M_1 and M_2 were also formed in small amounts. In contrast, rat, mouse, and human liver preparations produced no detectable aflatoxin B_1 and only small amounts of compounds thought to be aflatoxins Q_2 and P_2. The greater susceptibility of duck liver to the toxicity of aflatoxin B_2 may be attributable to its ability to form aflatoxin B_1, which could then be activated through further metabolism.

INTRODUCTION

We have been investigating the metabolism *in vitro* of aflatoxins by liver preparations from various experimental animals and from humans to provide information important for elucidation of mechanisms responsible for species differences in susceptibility to the toxic and carcinogenic actions of these compounds. Such information is relevant to pathways of metabolic activation and inactivation. Results of a previous study (11) of AFB_1[5] metabolism have been reported previously; the present report deals with similar studies on AFB_2 (dihydro-AFB_1).

These results are of particular interest in connection with the mechanisms involved in metabolic activation of aflatoxins. Current evidence strongly supports the postulate that activation of AFB_1 occurs through the formation of the 2,3-epoxide, which may represent the ultimate carcinogenic derivative (5, 12). Activation of AFB_2 could not take place directly through the same pathways, since the latter compound lacks the 2,3-double bond. That activation of AFB_2 does not readily occur in rats was in fact indicated by our earlier findings (14, 15) that its potency in that species is reduced by more than 150 times, compared to that of AFB_1. In contrast, AFB_2 is considerably more active in some other systems, including duck (in which its lethal potency is about one-fourth as great as that of AFB_1), chicken embryo, cell culture, and various subcellular biochemical model systems (13).

Therefore, it was of interest to determine the pathways through which AFB_2 was metabolized by duck liver, compared to those metabolized by rat, mouse, and human livers. Our results indicate that duck liver metabolizes AFB_2 much more actively than do the mammalian species and that a significant pathway in this transformation is 2,3-desaturation to form AFB_1. The latter compound would then be available for activation through the epoxidation pathway as in other species. We found no evidence for this transformation in rodent or human liver.

MATERIALS AND METHODS

Aflatoxins. Ring-labeled [^{14}C]AFB_2 was purified by preparative scale TLC from chloroform extracts of *Aspergillus parasiticus* cultures incubated with [1-^{14}C]acetate. Authentic samples of aflatoxins B_1, B_2, P_1, M_1, Q_1, and B_{2a} and aflatoxicol were available in our laboratory or provided by Dr. G. H. Büchi, Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Mass.

Radioactivity Measurements. A Packard Tri-Carb Model 2002 liquid scintillation spectrometer was used to measure radioactivity. [^{14}C]Toluene standard was used for internal standardization. Chloroform-insoluble samples were counted in Aquasol scintillation fluid, and chloroform-soluble aflatoxins were counted in toluene-based scintillation fluid (4 g PPO and 100 mg POPOP per liter reagent-grade toluene). All phosphors and standards were products of New England Nuclear, Boston, Mass.

Tissues. Livers were immediately excised from males of 3 species killed by decapitation: 4- to 7-day-old Pekin ducks (C and R Duck Farm, Long Island, N. Y.), 28-day-old Fischer CDF rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.), and 31-day-old White Swiss CFW mice (Carworth Farms, New City, N. Y.). For each species, livers from 3 to 9 animals were pooled to provide sufficient material for all subsequent assays.

Samples of human liver (5 to 20 g each) were obtained from cadavers of renal transplant donors through the cooperation of Dr. A. B. Cosimi, Assistant in Surgery, Massachusetts General Hospital, Boston, Mass. Donors were maintained by artificial respiration for varying lengths of time after 2 successive inactive electroencephalograms at 24-hr intervals. Liver samples were removed when kidney transplantation was performed. Pertinent information on the 2 individuals involved can be summarized as follows. Subject 1 was a 49-year-old woman who died of a brain abscess, having been hospitalized for 12 days during which

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[5] The abbreviations used are: AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; TLC, thin-layer chromatography; PMS, postmitochondrial supernatant.

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she received massive antibiotic and steroid therapy. Histopathological examination revealed the presence of cirrhosis and lipid infiltration, associated probably with alcoholism. Subject 2 was a 38-year-old man who died of traumatic head injury and was maintained for 48 hr before a sample was obtained. Histopathological examination revealed milk lipid infiltration and fibrosis. Both subjects were Caucasian.

All excised liver samples were immediately placed in ice-cold potassium phosphate buffer (pH 7.4; 50 mM).

Subcellular Fractionation. Within 30 min after removal of the liver, a 25% homogenate was prepared in an all-glass Dounce homogenizer with 2.7 mM KCl:50 mM potassium phosphate buffer (pH 7.4). The PMS of a 20-min centrifugation at 9000 × *g* was used in the *in vitro* studies. All procedures were performed at 0–4°.

***In Vitro* Metabolism of AFB_2.** PMS aliquots equivalent to 0.2 g whole liver were incubated with [^{14}C]AFB_2 (0.04 to 0.08 μCi) in a medium consisting of potassium phosphate buffer (pH 7.4; 50 mM):KCl (2.7 mM, glucose 6-phosphate (2.4 mM), $NADP^+$ (0.1 mM), and $MgCl_2$, (1.0 mM). The total volume per flask was 5.0 ml (1, 3). After a 5-min incubation period to generate NADPH, approximately 50 μg AFB_2 in 0.05 ml reagent-grade methanol was added, initiating metabolism at a final AFB_2 concentration of approximately 30 μM. For Human 1 and Human 2 incubations, 130 μg and 207 μg AFB_2, respectively, were added as described above. These higher levels were added to facilitate the identification of AFB_1 as an intermediate. Absorbance values of aliquots of the methanolic AFB_2 solution at 362 nm provided an accurate measure of the actual quantity added (9). Duplicate flasks were shaken aerobically at 37° for 5, 10, 15, and 30 min. The results were compared to those obtained with AFB_2 incubated with PMS inactivated by heating at 100° for 10 min. The addition of 5 ml cold methanol terminated the incubations. The flasks were stored no longer than 48 hr at −20° before extraction.

Extraction of Aflatoxins from the Incubation Medium. The methanolic incubation medium was extracted 5 times successively with 25-ml aliquots of reagent-grade chloroform. The combined chloroform extracts (125 ml) were concentrated to 2 ml under vacuum at 40°. The chloroform-insoluble fraction was quantitatively recovered. This fraction included a clear supernatant phase and denatured cellular constituents. Aliquots of both fractions were taken for determination of radioactivity.

TLC. The chloroform-soluble aflatoxins were separated by TLC on 0.25-mm plates of Adsorbosil-1 (Applied Science Labs., Inc., State College, Pa.) in chloroform:acetone:water (88:12:1.5). For resolution of AFB_2 and aflatoxicol, which cochromatograph in the above solvent system, a second system of ethyl acetate/chloroform (2:1) was used. Chromatography with known aflatoxins provided identification of most metabolites. Unknown AFB_2 metabolites and regions of the chromatogram showing no fluorescence were demarcated in reference to the R_F of the standards. All portions of the chromatogram were scraped into vials for liquid scintillation counting. For each duplicate flask at each incubation time, 2 TLC plates were developed. AFB_2 and its chloroform-soluble metabolites were identified by their UV fluorescence on the silica gel plates.

Other Assays. The protein content of the PMS was determined by the method of Lowry *et al.* (8). Microsomal enzymatic activity of the PMS was measured by the *O*-demethylation of *p*-nitroanisole (7). The UV fluorescence of the aflatoxins on the TLC plates was photographed with Kodachrome 25 film (Eastman Kodak Co., Rochester, N. Y.).

Calculations. Rates of AFB_2 metabolism and the quantity of the metabolites formed were calculated in relation to the weight of the whole-liver equivalent and as a function of the protein content of the PMS fraction. In the chloroform:acetone:water solvent system, AFB_2 and aflatoxicol cochromatograph; hence the amounts of these 2 aflatoxins present were determined by chromatography in the second solvent system (ethyl acetate:chloroform), in which they were satisfactorily resolved.

RESULTS

Total conversion of AFB_2 to all metabolities by liver preparations of 4 species is summarized in Chart 1. Data for duck and human liver preparations are from duplicate experiments, whereas only single experiments with rat and mouse livers were performed because replicate samples produced virtually identical values in preliminary experiments. AFB_2 at <7% was metabolized by rat, mouse, and human livers. Duck liver was capable of extensive metabolism, converting 40 to 60% of the substrate to metabolites.

Metabolites produced by the different liver preparations are summarized in Table 1. With respect to chloroform-soluble derivatives, livers of the mouse and of 1 human produced barely detectable amounts of substances with chromatographic and fluorescence properties expected for aflatoxins Q_2 and P_2, although these compounds were not specifically identified (11). All species except humans produced blue fluorescent compounds that migrated in the aflatoxin M_1 to M_2 aflatoxin region but that were also unidentified. Both samples of duck liver also produced compounds with chromatographic properties expected for aflatoxicols 1 and 2.

Duck liver preparations also produced a blue fluorescent metabolite with chromatographic properties of AFB_1. This metabolite was found to cochromatograph with authentic AFB_1 in the 2 TLC system described in "Materials and Methods" and also in a third solvent system, chloroform:methanol (19:1).

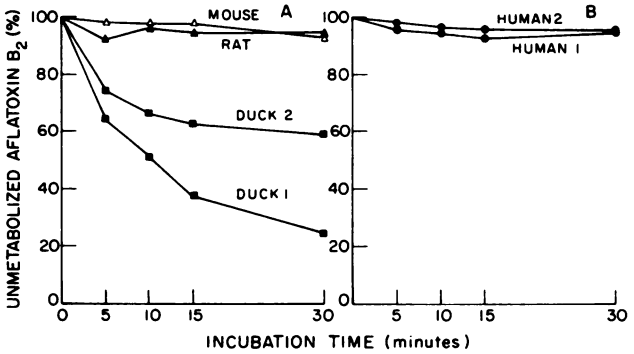

Chart 1. Total metabolism of AFB_2 by PMS preparations of mouse, rat, duck, and human livers. Each incubation mixture contained PMS equivalent to 0.2 g whole liver; each *point* represents the mean of 4 values (duplicate chromatograms of 2 incubation flasks; in all cases the S.D. < 6%).

Table 1

AFB_2 metabolites after a 15-min incubation

The identity of metabolites is based on chromatographic properties analogous to metabolites produced from AFB_1 (11).

Aflatoxin fraction	nmol aflatoxin/mg protein/15 min					
	Duck 1	Duck 2	Rat	Mouse	Human 1	Human 2
B_1	0.5 ± 0.0[a]	0.2 ± 0.1	ND[b]	ND	ND	ND
Aflatoxicol 1	0.1 ± 0.1	ND	ND	ND	ND	ND
Aflatoxicol 2	0.5 ± 0.1	0.5 ± 0.1	ND	ND	ND	ND
Q_2	ND	ND	ND	0.0 ± 0.1	0.3 ± 0.1	ND
P_2	ND	ND	ND	0.0 ± 0.2	0.1 ± 0.1	ND
M_1 or M region	0.3 ± 0.1	0.3 ± 0.1	0.0 ± 1.0	0.3 ± 0.4	ND	ND
M_2	0.9 ± 0.1	0.4 ± 0.1				
O+	0.4 ± 0.1	ND	ND	ND	ND	ND
Origin	0.3 ± 0.0	0.4 ± 0.2	0.0 ± 1.0	0.2 ± 0.2	0.2 ± 0.1	ND
Chloroform-insoluble	1.6 ± 0.3	0.8 ± 0.1	1.0 ± 1.0	0.0 ± 0.1	0.3 ± 0.1	0.0 ± 0.0
Total conversion	4.2 ± 0.2	3.4 ± 0.3	2.0 ± 0.2	0.8 ± 0.4	1.2 ± 0.2	2.0 ± 0.0

[a] Mean ± S.D.
[b] ND, none detected.

The kinetics of formation of this metabolite, presumed to be AFB_1, are shown in Chart 2. Both duck liver PMS preparations produced this derivative, which accumulated during the early portions of the incubation period. After 5 to 15 min, its concentration declined, possibly reflecting further metabolism of AFB_1 through pathways described previously (11).

As an independent indication of mixed-function oxygenase activity of the liver preparations, the activity of marker enzyme *p*-nitroanisole *O*-demethylase was measured. The activity of this enzyme for each species is summarized in Chart 3. Rat, mouse, and human livers showed greater activity than did duck liver, but all species clearly contained active mixed-function oxygenases.

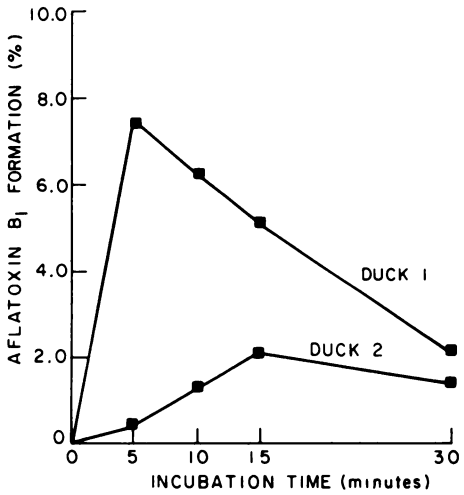

Chart 2. Formation of AFB_1 from AFB_2 by duck liver PMS equivalent to 0.2 g whole liver. Experimental conditions are the same as those described in Chart 1.

DISCUSSION

The data in Chart 1 and Table 1 show that AFB_2 was metabolized only to a small extent by rat, mouse, and human liver preparations, but duck liver actively transformed this compound to a series of metabolites. Low levels of activity in the other preparations cannot be attributed to an overall lack of mixed-function oxygenase activity, since the preparations with lower capability for AFB_2 conversion had higher levels of the microsomal marker enzyme than had duck liver preparations.

Duck liver preparations metabolized AFB_2 differently than did those from rat, mouse, and human liver. In addition to more extensive overall metabolism, 2 to 8% of the AFB_2 was initially metabolized to a compound with chromatographic properties identical with AFB_1. For duck liver preparations the acute AFB_2 dose that killed 50% of the animals was more than 170 times less than AFB_1 dose (14). If duck liver synthesized AFB_1 from AFB_2 *in vivo* at rates comparable to its *in vitro* synthesis, the relationship between the doses that killed 50% of the animals for the compounds in duck liver could be explained. However, evidence for this metabolic pathway in rat, mouse, and human liver was not found in these experiments.

These findings are pertinent to elucidation of the mechanisms of action underlying aflatoxin carcinogenesis and toxicity. Earlier experiments demonstrated that AFB_2 was weakly carcinogenic to rat liver; the effective dose was 150 times greater than that for AFB_1 (14, 15). The formation of small amounts of AFB_1 was suggested as a possible basis for the carcinogenicity of AFB_2. Direct evidence in support of that suggestion has recently been provided by Swenson *et al.* (12) who showed that AFB_2 forms nucleic acid adducts in the liver of rats dosed with the compound and, further, that aflatoxin B_1-2,3-oxide is an intermediate in the formation of those adducts, as indicated by their chromatographic identity to adducts formed from AFB_1. Transformation of AFB_2 to aflatoxin B_1-2,3-oxide would presumably occur through the intermediate formation of AFB_1 by desaturation at the 2,3-carbons of AFB_2. Interestingly, the ratio of nucleic acid adducts formed from the 2 compounds was very similar to the ratio of their carcinogenic potencies, approximately 1:100 (AFB_2:AFB_1).

It is difficult to compare quantitatively the metabolism of AFB_1 and AFB_2 by rat and mouse liver because so little total metabolism occured in either case. However, some interesting structure-activity relationships based on *in vivo* ob-

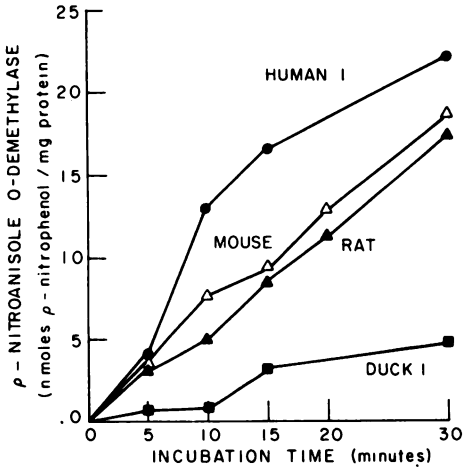

Chart 3. Activity of microsomal marker enzyme *p*-nitroanisole *O*-demethylase in liver preparations used in experiments depicted in Charts 1 and 2. Experimental conditions are the same as those described in Chart 1.

servations are worth recounting. Edwards *et al.* (4) noted that rats dosed i.p. with [^{14}C]AFB_1 and [^{14}C]AFB_2 retained less AFB_2 than AFB_1 in the liver. AFB_1 also inhibited rat RNA polymerase, whereas, at 200 times the quantity of AFB_1, AFB_2 had no effect (5). In a microsomal-mediated system, AFB_2 was 100 times less toxic to bacteria (6); a similar relationship was found with respect to the mutagenicity of *Salmonella typhimurium* (16).

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