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ACTIVATION AND DEACTIVATION of AFLATOXIN B_1 IN ISOLATED RAT HEPATOCYTES

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Isolated rat hepatocytes took up [^{3}H]-aflatoxin B_1 during incubation with fifty percent of the aflatoxin B_1 covalantly bound to cellular macromolecules. The amount of bound-aflatoxin B_1 was proportional to the medium concentration of aflatoxin B_1. The specific radioactivity (pmole/mg) of aflatoxin B_1 found in the DNA fraction was 20 fold greater than that associated with protein. Metyrapone (0.75 mM) inhibited significantly the uptake and binding whereas 1,2-epoxy-3,3,3-trichloropropane (0.5 mM) enhanced 2-3 fold both the uptake and binding. Glutathione (0.25 mM) reduced these processes. Results indicate that a transformation of aflatoxin B_1 is catalyzed by cytochrome P-450 mixed function oxidase and aflatoxin B_1-2,3-epoxide so formed is primarily deactivated by epoxide hydrolase. In the isolated hepatocyte depletion of the epoxide by glutathione apparently has an insignificant role in aflatoxin detoxication.

Aflatoxin B_1 (AFB_1), produced by strains of Aspergillus flavus, is a potent hepatotoxin and hepatocarcinogen in experimental animals and requires metabolic activation to exert its biological effects (1). According to current concepts, AFB_1 must be activated by the liver cytochrome P-450 mixed function oxidase to form AFB_1-2,3-epoxide (2,3). Isolation of AFB_1-2,3-epoxide has been attempted but unsuccessful. Formation of the reactive electrophile is postulated by the identification of the 2,3-dihydrodiol derivative of AFB_1 and 2,3-dihydro-2-(N^7-guanyl)-3-hydroxy-AFB_1 as hydrolysis products from an AFB_1-nucleic acid adduct (2-5) and AFB_1-protein adduct (6). The deactivation of the epoxide is belived to involve the formation of AFB_1-dihydrodiol (7) and an AFB_1-glutathione (GSH) conjugate (8) catalyzed by epoxide hydrolase and GSH-S-transferase respectively. Since the AFB_1-2,3-epoxide can

Abbreviations: AFB_1, Aflatoxin B_1; BSA, bovine serum albumin; GSH, glutathione; TCA, trichloroacetic acid; TCPO, 1,2-epoxy-3,3,3-trichloropropane.

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be trapped in situ by forming adducts with endogenous (DNA, RNA or protein) or added nucleophiles determination of AFB_1-macromolecule adducts is routinely used to indicate the existence of AFB_1-2,3-epoxide (9). Because of the varibility in activity of the enzymes involved in activation and deactivation (10), level of endogenous or added nucleophiles, and rates of individual reactions, the extent of AFB_1 activation and the competition between detoxifying pathways is still unclear. Utilizing inhibitors of microsomal enzymes, 2-methyl-1,2-di-3-pyridyl-1-proponone (Metyrapone) and 1,2-epoxy-3,3,3-trichloropropane (TCPO), and addition of GSH in vitro with isolated hepatocytes we show that the activation of AFB_1 is sensitive to metyrapone and the major detoxifying pathway is the formation of AFB_1-dihydrodiol catalyzed by the epoxide hydrolase.

Materials and Methods

Swim's S-77 medium and trypan blue stain were purchased from the Grand Island Biological Co.; Metyrapone, TCPO and GSH were supplied by the Sigma Chemical Co.; collagenase (Type III) was obtained from Worthington Biochemicals. Crystalline bovine serum albumin (BSA) was supplied by Miles Laboratories. [^{3}H]-Aflatoxin B_1 (13-14 Ci/mmole) was obtained from Moravek Biochemicals, Inc. Rats (250-300 g, adult male Wistar) were obtained from West Jersy Farm Division of the Parco Scientific Co. and were fed water and Ziegler laboratory chow ad lib.

Hepatocytes were prepared as published elsewhere (11). In the present study, 1% BSA was used in liver perfusion and the final hepatocyte suspension contained 0.2% BSA in Swim's S-77 medium. The cell viability was measured by trypan blue exclusion (0.27% final trypan blue concentration) and was 94%. Hepatocyte suspensions ($0.55 \pm 0.08 \times 10^6$ cells/ml) in a final volume of 3-5 ml were incubated in polycarbonate vials at 37° C, under a constant stream of 95% O_2-5% CO_2, with constant shaking (72-80 rpm) for 15 min before addition of [^{3}H]-AFB_1 and/or other agents.

To determine the uptake of [^{3}H]-AFB_1 duplicate samples (25 µl) were removed, added to 10 ml cold incubation medium and filtered immediately with glass fiber disc (Whatman GF/C). The cells collected on the filter disc were washed gently with two 5-10 ml portions of cold incubation medium and then the filters transferred to counting vials. For total macromolecule bound AFB_1, the hepatocytes collected on the filter were further washed twice with distilled H_2O and 5% cold trichloroacetic acid (TCA). The acid-insoluble materials collected on the filter were then transferred to counting vials. To determine AFB_1 bound to proteins, the TCA insoluble materials collected on the filter were treated with HCl (0.15 N) to hydrolyze AFB_1 bound to nucleic acid. With this procedure greater than 90% of AFB_1 covalently bound to RNA and DNA was found to be released (3,4). To determine the amount of AFB_1 bound to DNA, the TCA insoluble material collected on the filter was treated with KOH (0.3 N) at 37° C. The hydrolysates were rapidly cooled on ice, neutralized with HCl (3 N), and, after adding 1.2 ml TCA (50%), the insoluble materials were collected on glass fiber before transferring to counting vials. The alkaline treatment hydrolyzed the AFB_1-RNA adduct (4) and AFB_1 bound to proteins (6) without affecting AFB_1-DNA (5). The non-specific binding of AFB_1 was corrected by samples taken at zero time in each experiment followed by treatments described above.

Radioactivity was determined in a Beckman LS-3100 liquid scintillation system; cpm were computed to dpm using external standardization. The counting error was 5% or less. Tritium lose and/or exchange due to NIH shift (12) was not corrected. Proteins were determined by the method of Lowry et al.,(13). DNA was determined by the diphenylamine reaction as described previously (14).

Results and Discussion

As depicted in Fig 1, during 4 h incubation of hepatocytes with [^{3}H]-AFB_1 (0.71 μM) maximal uptake and binding occurred by the end of the second h. The formation of the AFB_1-protein adduct exhibited almost a linear rate during the first 90 min of incubation whereas the formation of AFB_1-DNA adduct reached a maximum level between 2-3 h. The uptake and binding of [^{3}H]-AFB_1 was both time and concentration dependent. At 0.5 μM AFB_1, the maximum uptake and binding was reached by 60 min. The amount of AFB_1 bound to cellular macromolecules increased with increasing AFB_1 concentrations (0-1.5 μM). The correlation coefficient was found to be 0.996 (Table 1). Demonstration of the formation of covalently bound [^{3}H]-AFB_1 in the present study indicates that indeed freshly isolated hepatocytes from normal rats contain sufficient activity of the cytochrome P-450 mixed function oxidase

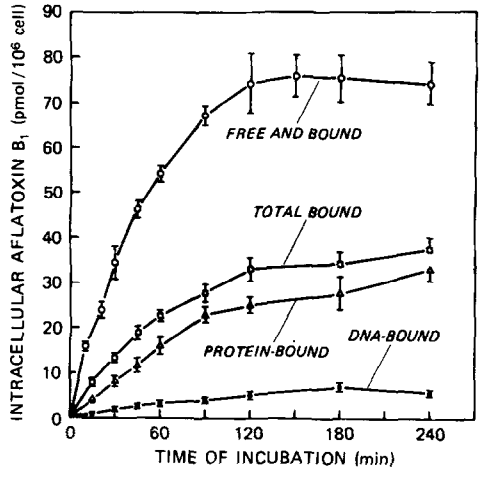

Figure 1 Binding of Aflatoxin B_1 to cellular Macromolecules. Incubation of hepatocytes and binding of [^{3}H]-AFB_1 to cellular macromolecules were conducted as described under "Methods". The concentration of AFB_1 in the incubation medium was 0.71 uM. Data are given as mean + S.E.M. for duplicate determinations from 6 experiments.

Table 1. Binding of [^{3}H]-AFB_1 During Incubation[a]

[^{3}H]-AFB_1 added (μM)	0.07	0.21	0.36	0.51	0.71	1.02	1.42
	(Mean ± S.E.M.)						
[^{3}H]-AFB_1 bound (pmole/10^6cell)	2.2 ±0.2 (11)	9.6 ±0.5 (4)	11.7 ±1.8 (8)	23.3 ±1.9 (12)	35.1 ±1.9 (12)	55.2 ±3.7 (8)	69.3 ±6.3 (8)

[a]Hepatocytes were incubated with [^{3}H]-AFB_1 (13 Ci/mmole) for 180 min. Correlation coefficient calculated from the mean values was 0.996 from number of experiments given in parenthesis.

such that pretreatment of rats with inducers of cytochrome P-450 was unnecessary (15).

Prior to studying the detoxifying pathways, the inhibition of AFB_1 activation by metyrapone was investigated. As shown on Table 2, with untreated hepatocytes incubated for 60 min with 0.51 μM [^{3}H]-AFB_1, the specific radioactivity of [^{3}H]-AFB_1 found in the protein fraction was 7.4 pmole/mg and in the DNA fraction 143.4 pmole/mg. The ratio of AFB_1-DNA/AFB_1-protein of 19.4 is similar to the ratio of 22.6 reported by Swenson et al. (3). This finding is important with respect to: (a) the establishment of a close correlation between the *in vivo* study conducted be Swenson et al. (3) and our study using *in vitro* hepatocyte suspensions; (b) the isolation of nucleic acid and protein by the phenol extraction procedure can be avoided; direct analysis of small sample volumes (25 μl cell suspension) as described in this report is rather simple, rapid and accurate; and (c) exchange and/or loss of radioactivity during mild alkaline hydrolysis employed in this study is not excessive, otherwise a greater decrease of the ratio would have been observed. Metyrapone is known to decrease the activity of drug-metabolizing enzymes in liver; with isolated hepatocytes, 0.5 mM metyrapone decreased covalent binding of radiolabeled bromobenzene (16), and in a cell free system 1.0 mM metyrapore inhibited AFB_1 activation by 71% (15). In this study, metyrapone at 0.75 mM inhibited the binding of [^{3}H]-AFB_1 to DNA and protein by 83% and 86% respectively. The inhibition also caused a similiar decrease of

Table 2. The Influence of Metyrapone, TCPO and GSH on the Uptake and Binding of [^{3}H]-AFB_1[a]

Incubation Condition	Intracellular [^{3}H]-AFB_1		Specific Radioactivity of [^{3}H]-AFB_1	
	Total	Bound	DNA-Bound	Protein-Bound
	(pmole/10^6 cell)		(pmole/mg)	
Control	52.3±6.7(9)	26.1±1.7(6)	143.4±8.1(10)	7.4±0.3(10)
+ Metyrapone (0.75 mM)	10.1±1.0(3)*	4.4±0.3(3)*	23.8±1.1(3)*	1.0±0.1(3)*
+ TCPO (0.5 mM)	119.6±23.5(4)*	78.1±19.8(4)*	315.9±94.5(4)*	18.8±4.0(4)*
+ GSH (0.25 mM)	36.9±3.0(4)	17.4±2.0(4)*	114.9±9.7(4)	6.1±1.0(4)

[a]The concentration of AFB_1 in the incubation medium was 0.5 μM and data presented are mean ± S.E.M. from number of experiments given in parenthesis.

*Indicates P value < 0.05 compared to control values.

AFB_1 (free and bound) found in the hepatocyte. These results further suggest that hepatocyte suspensions can be employed to study AFB_1 metabolism instead of cultured hepatocytes which require enriched medium to maintain cytochrome P-450 levels (17). The decreased binding of [^{3}H]-AFB_1 to cellular DNA and protein seen in metyrapone treated cells (Table 2) supports previous findings that AFB_1-2,3-epoxide is the reactive metabolite.

The overall toxicity and carcinogenicity of AFB_1 depends on the steady-state concentration of AFB_1-2,3-epoxide. Unknown at this time is the extent of which pathway, that is the formation of AFB_1-dihydrodiol or AFB_1-GSH conjugate, is the major detoxifying mechanism. TCPO (0.5 mM) an inhibitor of the epoxide hydrolase activity, stimulated 2-2.5 fold the [^{3}H]-AFB_1 bound to both DNA and protein (Table 2). GSH (.25 mM), which should enhance the depletion of AFB_1-2,3-epoxide, reduced the bindings by 20%. In all cases, when the level of bound AFB_1 was low there was a decrease of the total intracellular AFB_1 whereas the level was high when there was an increase of intracellular AFB_1. These results suggest that the rate of uptake and formation of covalently bound AFB_1 depends on it's activation and deactivation and each process is important in expressing the biological effects of the

toxin. The 20% decrease observed in cells treated with GSH suggests that detoxication of AFB_1-2,3-epoxide by GSH is not highly significant. This is in agreement with Emerole et al. (18) and others (19) who suggest that GSH was a less efficient nucleophile for AFB_1 metabolites than for other chemical carcinogens. The statistically significant ($p<0.05$) increase of [^{3}H]-AFB_1 binding to cellular DNA and protein observed in TCPO treated cells (Table 2) indicates strongly that the major detoxifying pathway is the formation of AFB_1-dihydrodiol via the reaction catalyzed by epoxide hydrolase.

With intact animals, cultured hepatocytes and cell-free systems efforts to correlate impairments of cellular functions, acute toxicity and tumor susceptibility caused by AFB_1 have not been very successful since these effects are known to be dose-and-time dependent, and can be modulated by nutritional, hormonal and/or metabolic factors (20-24). Attempts to isolate, identify and characterize the toxic effects of AFB_1 have been made but the results have not been consistent presumably due to the lack of understanding of how AFB_1 is transported, and thus, metabolized. In the present study, not only the formation of AFB_1-2,3-epoxide is confirmed but also its major detoxifying pathway identified. Investigations are currently in progress to assess the effect of metyrapone, TCPO and GSH on AFB_1-induced cytotoxicity and AFB_1-inhibited transcriptional activity. Future experiments employing hepatocyte suspensions should yield information concerning the molecular mechanism of initiation of carcinogenesis.

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