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In Vitro Effects of Aflatoxin B_1 on the Uptake of ^{14}C-orotic Acid Into Kidney, Liver and Muscle Tissue of the Mongolian Gerbil, *Meriones unguiculatus*

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Aflatoxins are a mixture of highly toxic metabolites produced by certain strains of the mold *Aspergillus flavus*, a contaminant of groundnuts and other grains. The chemical structures for these potent hepatotoxic and hepatocarcinogenic agents have been studied extensively and aflatoxin B_1 (AFB_1) has been found to be the most abundant and toxic metabolite (BUTLER 1966).

A major area of study involving the aflatoxins has dealt with their mechanism of action. Often severe inhibition of protein synthesis by the cell can be measured by uptake and incorporation of various labeled precursors of protein and nucleic acids (SMITH 1963, CLIFFORD & REES 1966, 1967). One investigator reported inhibition of nuclear and nucleolar RNA synthesis (30% and 85%, respectively) as measured by ^{14}C-orotic acid incorporation in rat livers (LEFARGE et al. 1966). Other work with rats showed no inhibition of incorporation of ^{14}C-orotic acid in in vivo studies of the nucleotide pool, but immediate inhibition in in vitro studies. However, the authors did find inhibition of nucleotide precursors into nuclear RNA and postulated inhibition of RNA polymerase as a possible explanation (CLIFFORD & REES 1967). Later work subsequently explored this hypothesis corroborating it for RNA polymerase and implicating metabolites of AFB_1 as the active intermediate forms responsible for the inhibition (AKINRIMIST et al. 1974). A metabolite of AFB_1 was also suggested as the actual inhibitor of RNA synthesis in another work where the incorporation of RNA precursors, ^{14}C-orotic acid and ^{3}H-orotic acid, was markedly inhibited by low levels of AFB_1 and AFG_1, but not AFB_2 and AFG_2 (MCINTOSH et al. 1976).

Most of the literature involving measurement of uptake and incorporation of precursors has dealt mainly with the effects on rats and few recent studies have used ^{14}C-orotic acid. It was the purpose of this study to determine the effects of AFB_1 on the uptake of ^{14}C-orotic acid into kidney, liver and muscle tissue of the Mongolian gerbil, *Meriones unguiculatus*. This animal appears to be less sensitive to the toxin than the rat (GODY & NEAL 1976, LLEWELLYN & THOMEN 1978).

EXPERIMENTAL PROCEDURE

Adult male and female Mongolian gerbils were taken from the stock colony at Virginia Commonwealth University. Until sacrificed,

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they had been maintained on commercial laboratory block feed and housed in pairs in plastic cages with plated steel wire lids containing wood chip litter (not cedar). Their feed contained no detectable amounts of aflatoxin.

An initial study was performed to determine the uptake of ^{14}C-orotic acid at various incubation times in gerbil kidney, liver and muscle tissue. Kidney and muscle tissues were incubated for 30, 60 and 120 min while liver was incubated for 15, 30, 45, 60 and 120 min. Incubation medium was prepared by diluting the stock ^{14}C-orotic acid solution to obtain a 25 ug-mL solution in normal saline. Radioactive ^{14}C-orotic acid was obtained from Calbiochem, La Jolla, CA with a specific activity of 37 mCi/mM. "Cold" orotic acid hydrate was used as a carrier, the final specific activity being 3.32 uCi/mg or 7.38 x 10^6 dpm/mg, in a stock solution of 1 mg/mL in H_2O. Sufficient NaCl was added to the incubation medium to adjust for the dilution of the saline resulting from the H_2O in the ^{14}C labeled stock solution.

Blank tissues were run in normal saline solution. Four mL of the incubation medium or blank solution were placed in each 25 mL Erlenmeyer flask used for incubation. The flasks were then placed on ice, and 0.05 mm slices of tissue, sliced by hand microtome, from freshly sacrificed gerbils (one male and one female) were placed in each after weighing quickly in plastic disposable weighing boats. Sample weights ranged from 50 to 150 mg. Samples were then transferred from the ice to an Eberbach water bath shaker set at 37°C and a shaking rate of 120 cycles/min and incubated for the appropriate time.

They were then removed, washed with saline, placed in glass liquid scintillation vials and digested overnight in 2.0 mL hydroxide of hyamine 10-x (Packard).

Twenty mL of KPPO, a liquid scintillation counting cocktail in routine use at A.H. Robins Research Laboratories, Richmond, VA, were then added to the samples in the scintillation vials. KPPO consists of the following: toluene, 800mL; p-dioxane, 800 mL; absolute ethanol , 480 mL, added to 160 g napthalene and 10 g PPO in a volumetric flask for a 2-L final volume. The samples were counted for 10 min in a liquid scintillation spectrometer set at 6°-7°C, utilizing the factory pre-set ^{14}C channel.

The samples were fortified with 0.1 mL ^{14}C-benzoic acid standard used as an internal standard, mixed in toluene to obtain 2000-3000 dpm/0.1 mL and then recounted for 10 min for quench correction calculations. Only one sample of each tissue was run at each time interval in the initial study.

After the initial study of uptake of ^{14}C-orotic acid was completed the effects of 1.25 ug/mL and 2.50 ug/mL AFB_1 on uptake of the acid were examined. Aflatoxin B_1, grade A, dried *in situ* (Calbiochem) was first dissolved in acetone (10 mg/mL). This solution was added to 500 mL of sterile water and the latter was

heated and stirred gently for several hours to remove the acetone and aid in mixing. This solution was designated as the stock solution and it was stored at 10 C in darkness. All experimental dilutions were made from this source containing 6.25 ug AFB_1/mL of H_2O.

Aliquots of the stock solution were tested for the level of AFB_1 and other metabolites. Twenty mL samples were spotted on silica gel tlc plates. Fluorescent spots were compared visually to reference samples spotted simultaneously using the official AOAC procedure (HORWITZ et al. 1975). All toxin analyses were completed at the Mycotoxin Laboratory of the Virginia Division of Consolidated Laboratory Services, Richmond, VA.

Kidney, liver and muscle from one male and one female gerbil were incubated for 75 min in blank Tyrode's solution, or Tyrode's solution with 25 ug/mL ^{14}C-orotic acid or Tyrode's solution with 25 ug/mL of ^{14}C-orotic acid and 1.25 ug/mL AFB_1. Tissues from another male and female gerbil were incubated as above, except that the last solution contained 2.50 ug/mL AFB_1. Stock Tyrode's solution consisted of 80g, NaCl; 2 g, KCl; 1g, $CaCl_2$, 0.575 g, $NaH_2PO_4 \cdot H_2O$; 2.14 g, $MgCl_2 \cdot 6H_2O$ in 500 mL H_2O. This was diluted 1:20 to obtain the physiology solution. Volumes and analytical techniques were the same as for the initial study. Blanks were run in duplicate and all others in triplicate. Tissue weights incubated ranged from 22 to 168 mg. Using this method, each animal's tissues served as its own blanks and controls.

RESULTS

In the preliminary study there was a drop in the level of ^{14}C-orotic acid in the tissues between 45 and 60 min and a gradual increase after 60 min. Subsequent studies utilized a longer incubation time. There is apparently no significant difference due to sex on uptake and incorporation of ^{14}C-orotic acid when incubated for 75 min.

In the tissues treated with 1.25 ug/mL AFB_1, there is no clear effect of the toxin on uptake in kidney or muscle when comparing individual animal tissues. The liver ^{14}C-orotic acid uptake seems to be depressed, more in the male, than the female (Table 1). At the 2.50 ug/mL of AFB_1, uptake of ^{14}C-orotic appears to be clearly depressed in all tissues of the female, while in the male the results are ambiguous, and differences between toxin treated and untreated tissues are insignificant, but certain trends are evident. The results of both sexes were combined to give the analysis of variance results shown in Table 2.

DISCUSSION

In the initial study of incorporation of ^{14}C-orotic acid into the tissues of the gerbil, the drop in the level of ^{14}C-orotic

TABLE 1. Mean and Standard Deviations for ^{14}C-Orotic Acid Uptake in Control (C) and Experimental (E) Tissues Treated With Aflatoxin B_1

Concentration of Aflatoxin B_1 ug/mL	Kidney				Liver				Muscle			
	Male		Female		Male		Female		Male		Female	
	C	E	C	E	C	E	C	E	C	E	C	E
1.25	19.3 ±2.8	21.6 ±0.6	23.4 ±2.5	23.4 ±3.2	19.6 ±2.1	15.4 ±1.0	19.3 ±1.1	18.1 ±2.0	24.7 ±2.4	20.2 ±5.0	22.9 ±8.4	24.5 ±5.4
2.50	22.6 ±5.4	21.6 ±6.3	30.6 ±3.0	24.4 ±1.0	___[a]	___[a]	___[a]	___[a]	21.0 ±2.7	21.3 ±1.9	27.6 ±2.7	17.5 ±1.3

	Combined Sexes		Combined Sexes		Combined Sexes	
	C	E	C	E	C	E
1.25	21.3 ±3.4	22.5 ±2.3	19.5 ±1.5	16.7 ±2.0	23.8 ±5.6	22.3 ±5.2
2.50	26.6 ±5.9	23.0 ±4.3	___[a]	___[a]	24.3 ±4.4	19.4 ±2.5

[a]Liver data not used due to technical problems.

TABLE 2: Analysis of Variance for Combined Sexes Evaluating ^{14}C-Orotic Acid Uptake

Tissue	AFB_1 Concentration (ug/mL)	Calculated F Value[a]	Significance
Kidney	1.25	1.66	Not significant
	2.50	2.49	Not significant
Liver	1.25	4.15	Significant
	2.50	___[b]	_____[b]
Muscle	1.25	0.37	Not significant
	2.50	10.74	Very significant

[a]Probability of larger F	Tabular F Value (with numerator df=3, denominator df=8)
0.10	2.92
0.05	4.07
0.01	7.59

[b]Liver data not used due to technical problems

acid at 45-60 minutes after initiation of the incubation seems to be a real event, not due to procedural techniques. However, since the cause was not known a 75 min incubation time was chosen for the remainder of the study to get past this period of lowered uptake levels.

It is apparent from this study that the ^{14}C-orotic acid is readily absorbed by the cells of all of the tissues used, whether or not treated with AFB_1. No clear differences due to sex emerge from the results of this study. For better statistical analysis the data for both sexes were combined. For the 1.25 ug/mL level of AFB_1, the combined results show that kidney uptake is not inhibited, liver inhibition is approximately 14% and muscle inhibition is 6%. Liver is the most severely depressed tissue. This tendency to be the most responsive tissue is in agreement with the general conclusion that liver is the target organ for aflatoxins. At the 2.50 ug/mL AFB_1 level, uptake of label into the kidney is 14% inhibited and muscle is 20% inhibited. The liver data is not available at this level, but would be expected to be more severly depressed at this higher level of treatment as seen in other and previous studies.

Overall sensitivity to AFB_1 of the tissues in this study appears to be: liver > muscle ≥ kidney. Under the conditions of this experiment the gerbil tissues used are not very sensitive to AFB_1 and compare to the about 10% inhibition of uptake of ^{14}C-orotic acid in mouse livers treated with AFB_1 (GODY & NEAL 1976). In the same study rat livers which are much more sensitive to AFB_1, exhibited inhibitions of uptake of ^{14}C-orotic acid of over 50%. The results of the present study also confirm previous work indicating relative insensitivity of gerbils to AFB_1 (LLEWELLYN & THOMEN 1978).

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