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Quantitative carcinogenesis and dosimetry in rainbow trout for aflatoxin B_1 and aflatoxicol, two aflatoxins that form the same DNA adduct

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Abstract

Two exposure protocols were used to establish complete dose-response relationships for the hepatic carcinogenicity and DNA adduction in vivo of aflatoxin B_1 (AFB_1) and aflatoxicol (AFL) in rainbow trout. By passive egg exposure, AFL was taken up less well than AFB_1, but was more efficiently sequestered into the embryo itself, to produce an embryonic DNA binding curve that was linear with carcinogen dose and with a DNA binding index three-fold greater than AFB_1. Both aflatoxins produced the same phenotypic response, predominantly mixed hepatocellular/cholangiocellular carcinoma. Tumor responses as logit [incidence] vs. ln [dose] were parallel-offset, non-linear responses showing a three-fold greater carcinogenic potency for AFL at all doses examined (i.e. 3 times more AFB_1 than AFL required to produce an equivalent liver tumor incidence). By molecular dosimetry analysis (logit [incidence] vs. ln [DNA adducts]), the two data sets were coincident, indicating that, per DNA adduct formed in vivo in total embryonic DNA, these two aflatoxins were equally efficient in tumor initiation. By dietary fry exposure, both carcinogens produced linear DNA binding dose responses in liver, but with an AFL target organ DNA binding index only 1.14 times that of AFB_1 by this exposure route. The tumor dose-response curves also did not exhibit the three-fold difference shown by embryo exposure, but were closely positioned non-linear curves. Since the DNA binding indices differed by only 14%, the resulting molecular dosimetry curves for AFL and AFB_1 by dietary exposure were similar to the tumor response curves. These results indicate that differing exposure routes produced differing relative carcinogenicity estimates based on doses applied, as a result of protocol-dependent differences in AFL and AFB_1 pharmacokinetic behaviors, but that potency comparisons based on molecular dose received were similar for the two protocols. By comparison with standard DNA adducts produced in vitro using the dimethyloxirane-produced 8,9-epoxides of AFB_1 and AFL, we conclude that > 99% of AFL-DNA adducts produced in vivo were identical to those produced by AFB_1. Thus similar molecular dosimetry responses should be expected under all exposure protocols in which the two parent carcinogens do not exhibit differing toxicities to the target organ.

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1. Introduction

Aflatoxin B_1 (AFB_1) is one of the most potent experimental hepatocarcinogens known, and is the only naturally occurring dietary carcinogen currently recognized by the International Agency for Cancer Reseach (IARC, 1993) as carcinogenic to humans. The principal phase I metabolites in various species are aflatoxin M_1 (AFM_1), aflatoxicol (AFL), aflatoxins P_1 and Q_1, aflatoxicol M_1 ($AFLM_1$), and the hypothesized reactive intermediate AFB_1-8,9-oxide. Phase II metabolites include the 8,9-dihydrodiol, the glutathione conjugate of AFB_1 epoxide, and the glucuronides of AFL and $AFLM_1$. Human exposure to aflatoxins occurs through AFB_1-contaminated corn, other grains and nuts, from AFM_1 in the milk of humans or animals consuming moldy foods and feeds, and from primary metabolites such as AFL intracellularly. Under the influence of certain drugs or dietary factors, the phase I and phase II enzymes that catalyze conversion of AFB_1 to AFM_1, AFB_1-glutathione conjugate, and other metabolites can be dramatically altered. For example, exposure to Ah agonists such as the model agonist β-naphthoflavone or the ubiquitous environmental contaminant polychlorinated biphenyls decreases AFL production, dramatically increases production of AFM_1, $AFLM_1$, and $AFLM_1$-glucuronide, and reduces AFB_1 hepatocarcinogenicity in trout and rats (Loveland et al., 1983; Shelton et al., 1986; Goeger et al., 1988; Gurtoo et al., 1985). The apparent toxicity to an individual exposed to AFB_1 will thus reflect the pattern of metabolites produced in vivo in that individual as well as the dose. However, current understanding of these exposures and dietary modulations is limited through lack of information of the metabolism, adduct formation, adduct structures and relative carcinogenicities of the various AFB_1 primary metabolites.

On the basis of limited data, AFL appears to be the most carcinogenic AFB_1 metabolite, with a potency reportedly approaching AFB_1 in rats (Nixon et al., 1981) and trout (Schoenhard et al., 1981). However, the comparisons are only approximate because full dose-response studies have not been conducted. The observation has been made that species producing AFL as a major AFB_1 metabolite tend to be most susceptible to AFB_1 carcinogenicity (Salhab and Edwards, 1977; Schoenhard et al., 1981), suggesting that AFL may be a reservoir for subsequent DNA damage. Support for this hypothesis comes from studies of AFL metabolism in isolated rainbow trout hepatocytes, in which AFL is more slowly metabolized than AFB_1 and appears to be largely oxidized to AFB_1 prior to DNA adduction (Loveland et al., 1987, 1988). However, AFL derived DNA adducts remain to be fully characterized. The present study quantitatively establishes the relative carcinogenic potencies of AFL and AFB_1 using two exposure protocols in the rainbow trout model, describes the DNA adducts formed, and establishes the relationship between tumor response and molecular dose received (DNA adduction) for these two aflatoxins. A preliminary account of some of these results has appeared (Dashwood et al., 1992).

2. Materials and methods

Animals

Fertilized eggs and fry from Shasta strain rainbow trout (*Oncorhynchus mykiss*) were obtained from the brood stock maintained by our laboratory (Wales et al., 1978). Except during dietary exposure to aflatoxins, fish were maintained on a semi-purified control Oregon Test Diet (OTD) containing casein and gelatin as the protein source (Lee et al., 1991).

Chemicals: source and purity

Non-radioactive AFB_1 and AFL were purchased (Sigma Chemical Company, St. Louis, MO) and determined to be usable without further purification. Purity of each compound with respect to other aflatoxins was assessed by thin-layer chromatography (TLC) with densitometry,

or by high performance liquid chromatography (HPLC) with liquid scintillation counting of collected fractions. Detection limits and recoveries were verified by addition of known amounts of aflatoxin standard to a portion of the analytical sample. AFL contained $< 0.09\%$ AFB_1 and the 'unnatural' isomer was estimated to be $< 0.25\%$. Generally tritiated AFL was biosynthesized from generally tritiated AFB_1 (Moravek Biochemicals, Inc., Brea, CA) and purified as previously described (Marien et al., 1987; Loveland et al., 1988). By this procedure, [^{3}H]AFB_1 (17.4 ± 0.4 Ci/mmol) gave [^{3}H]AFL (16.0 ± 0.2 Ci/mmol). Aflatoxin concentrations were measured by UV spectrophotometry and scintillation counting in a manner previously described (Loveland et al., 1988).

Synthesis of aflatoxin epoxides and DNA adduct standards

AFB_1, AFL, and AFM_1 were individually reacted with a 1.5 molar excess of dimethyldioxirane as previously described by Baertchi et al. (1988). The resultant individual epoxides were then reacted with calf thymus DNA (3.0 mg/ml) dissolved in water. This reaction produced primarily the respective aflatoxin-N^7-guanyl adducts following hydrolysis in 0.15 N HCl for 15 min at 95°C. The AFB_1 and AFM_1 adducts (data not shown) were identical to those previously characterized (Kensler et al., 1985; Groopman et al., 1981). The acid hydrolyzed, AFL-epoxide modified DNA produced one major adduct, which was more lipophilic than the AFB_1-N^7-guanine (Fig. 1) and had a UV spectrum identical to the parent AFL (max. 325 nm). Treatment of the AFL modified DNA by the basic conditions described by Groopman et al. (1981) produced two new HPLC products tentatively identified as the expected major and minor formamido-pyrimidine derivatives characteristic of aflatoxin-N^7-guanyl adducts (data not shown). The yields for adducts from AFM_1 and AFL epoxides were approximately 1/3 that from AFB_1 epoxide.

DNA adducts produced from AFL in vivo were obtained by injecting rainbow trout (200–300 g) interperitoneally with generally tritiated AFL (10 μCi, 300 μg/kg body weight), isolating liver DNA

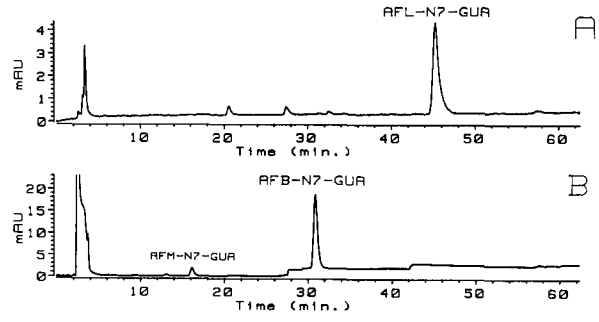

Fig. 1. Chromatographic resolution of DNA adducts from aflatoxicol. (A) Adducts from DNA treated in vitro with synthetic dimethyl oxirane generated AFL-8,9-epoxide; (B) Adduct from liver DNA of trout exposed to AFL in vivo.

48 h later, and hydrolyzing for HPLC analysis under the conditions used for the synthetic AFL-8,9-epoxide adduct.

HPLC conditions for aflatoxin-DNA adduct resolution

Nucleic acid hydrolysates were analyzed for specific adducts by reverse phase chromatography using a C18 ODS-Ultrasphere column (Rainin Inst. Co., Woburn, MA). Chromatography was performed at ambient temperature with a Beckman model 324 MP liquid chromatograph coupled to a Hewlett-Packard 1040A diode array detector scanning at 200–400 nm using a 25 min gradient of 10–18% ethanol in 20 mM triethylammonium formate (pH 3.0) at 1.0 ml/min.

Embryo exposure by bath treatment

Solutions of aflatoxins were prepared by adding measured amounts of non-radioactive and radioactive stock ethanol solutions to untreated hatchery well water. Twenty-one day old embryos were exposed to solutions of aflatoxins for 1 h as described (Hendricks et al., 1980, 1984). For tumor studies quadruplicate groups of 120 eggs each were submerged in individual 50 ml quantities of non-radioactive aflatoxin solutions. Following exposure, embryos were rinsed in water, placed in individual egg cups where hatching and yolk sac absorption occurred. At swimup 90 healthy fry from each of the treatment groups were started on OTD, with two exceptions. Both the 0.5 ppm AFB_1 and AFL doses resulted in

high initial mortalities (33% and 50%, respectively) so that only 80 and 60 healthy fry per group were available to start feeding for AFB_1 and AFL, respectively. For aflatoxin uptake, distribution, and DNA binding studies, groups of 12 eggs were treated in 5 ml of [^{3}H]aflatoxin solutions. After exposure, eggs were rinsed in water and placed in incubators for maintenance and rearing, or immediately frozen for later analysis. Nominal total concentrations of AFB_1 and AFL ranged from 0.01 to 0.5 μg/ml, based on measured ethanol stock solution concentrations. Solutions containing 1–2 μCi/ml tritiated aflatoxins were used for total egg uptake and distribution studies, and 8 μCi/ml was sufficient for embryo DNA adduction determinations.

Total uptake and distribution of aflatoxins

Treated eggs were weighed and left whole or dissected into shell, embryo and yolk. Yolks were absorbed on a piece of MF-Millipore membrane filter (Millipore Corp., Bedford, MA). Eggs or parts were digested with NCS Tissue Solubilizer (Amersham Corp., Arlington Heights, IL) and counted with OCS cocktail (Amersham Corp.).

Aflatoxin-DNA adduction in embryos

Eggs from bath treatments with radiolabeled aflatoxins were held in running water for 24 h to allow maximal aflatoxin-DNA adduction (Whitham et al., 1982), and frozen for later analysis. Embryos were dissected from the shell and yolk (two embryos per sample), digested in 200 μl lysis buffer (1% sodium dodecyl sulfate, 6% sodium 4-aminosalicylate, 1% NaCl, 6% 2-butanol) plus 0.1 mg proteinase K (P-0390, Sigma Chemical Co., St. Louis, MO) for 3 h at 37°C and stored under refrigeration until the embryos appeared dissolved. DNA was isolated and binding was determined as previously described, starting with addition of 5 M $NaClO_4$ (Nixon et al., 1984). The association of tritium from non-covalently bound aflatoxins with DNA was found to be insignificant (data not shown), as previously seen (Loveland et al., 1988).

Dietary exposure of fry

Test diets were prepared by measuring known quantities of stock solutions into the dietary oil component of the dry ingredients prior to final mixing of the diets. For the tumor-response experiment, fry were fed OTD containing 0, 4, 8, 16, 32, or 64 ng AFB_1 or AFL per g dry weight of diet from feeding onset through the next 2 weeks. They were then maintained on aflatoxin-free control diet until termination, 9 months after the onset of aflatoxin exposure. Each dietary treatment consisted initially of 400 fish distributed among four tanks for aflatoxin diets, and 200 fish distributed among two tanks for control diet.

Aflatoxin-DNA adduction in fry

For liver DNA adduction, 2 month old fry (average weight 1.5 g each) were fed OTD containing 0, 12.4, 25, 50, and 75 ng AFB_1 or AFL with 0.5 μCi tritiated aflatoxins per g dry weight of diet for 2 weeks, as for the tumor study. Each test treatment consisted of $n = 4$ or 8 pools of 15 fish each. At the end of the 2 weeks test feeding, the fish were killed, weighed, and livers removed for weighing and DNA binding measurement. DNA isolation and specific activity determinations were performed as previously described (Dashwood et al., 1988).

Tumor incidence and pathology

At necropsy for both experiments fish were anesthetized with tricaine methane sulfonate, weighed, and bled by cutting one or more gill arches. Livers were removed, weighed, inspected for tumors under a dissecting microscope, and fixed in Bouin's solution. All surface tumors were measured and marked for later retrieval. After 48 h in fixative, the livers were hand sliced with razor blades into 1 mm slices to detect any additional internal tumors and select tissue to embed for light microscopy. For the embryo exposure experiment, if a liver had only one or two tumors, those were embedded. When multiple tumors were present, only one slice/liver, having as many tumors as possible, was embedded. Thus, all tumors were not viewed microscopically but a representative sample of tumors from each tumored liver was diagnosed microscopically. Tumor classification was conducted as described by Hendricks (1994). For the dietary exposure experiment, tissues from all livers having tumors less than or equal to 2 mm were embedded for histo-

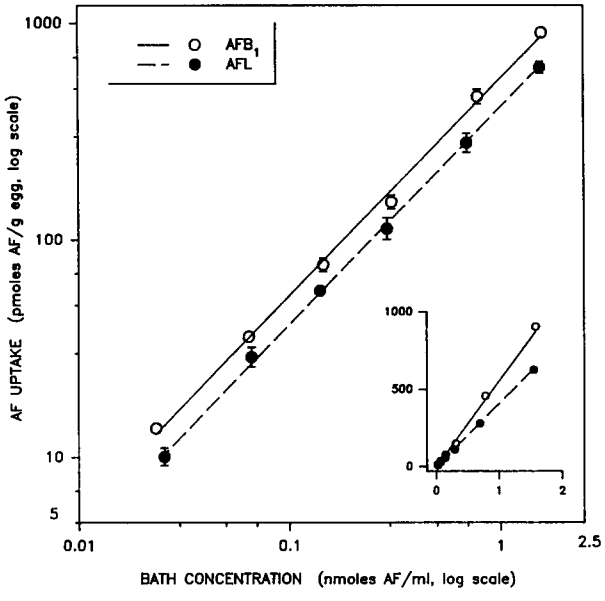

Fig. 2. Total uptake of [^{3}H]AFB$_1$ and [^{3}H]AFL in whole eggs at the end of a 1 h bath exposure of 21 day old embryos. Data points are geometric means obtained by backtransformation from analyses of the log-transformed data; error bars are 95% confidence intervals of these means. Fitted lines are based on geometric means of the individual slopes averaged over all doses (n = 36). The axes of the main graph are on a $\log_{10}$ scale with non-transformed tick values given. The inset graph shows the same means on a linear scale (error bars not depicted). The log of the slope of the lines on the linear inset graph correspond with the y-intercepts of the lines on the logarithmic (main) graph. The slopes of the lines differ significantly ($P < 0.0001$).

logical confirmation and classification. Only a representative sample of tumors from livers having tumors greater than 2 mm was processed for histology to reduce the work load. This resulted in about 50% of all tumored livers being diagnosed histologically.

Data analysis and statistics

Data for uptake and distribution of aflatoxins and DNA binding were analyzed in SAS (SAS Institute, 1993), using ANOVA models. Because the data often ranged over several orders of magnitude, were positively skewed, and had relatively constant coefficients of variation, all rates (i.e. response/dose) were transformed to the log scale for analysis. On the log transformed scale, homogeneity of variance was adequate for nearly all analyses (Bartlett's test, $P > 0.01$) based on the robustness of ANOVA tests (Milliken and Johnson, 1984). The single exception was embryo content of tritium at the end of a 1 h bath exposure, where the response for the fourth dose of AFB$_1$ was highly variable. Results given here include that dose because deleting it resulted in little change in either point estimates, confidence intervals or P-values. The estimates of rates (up-

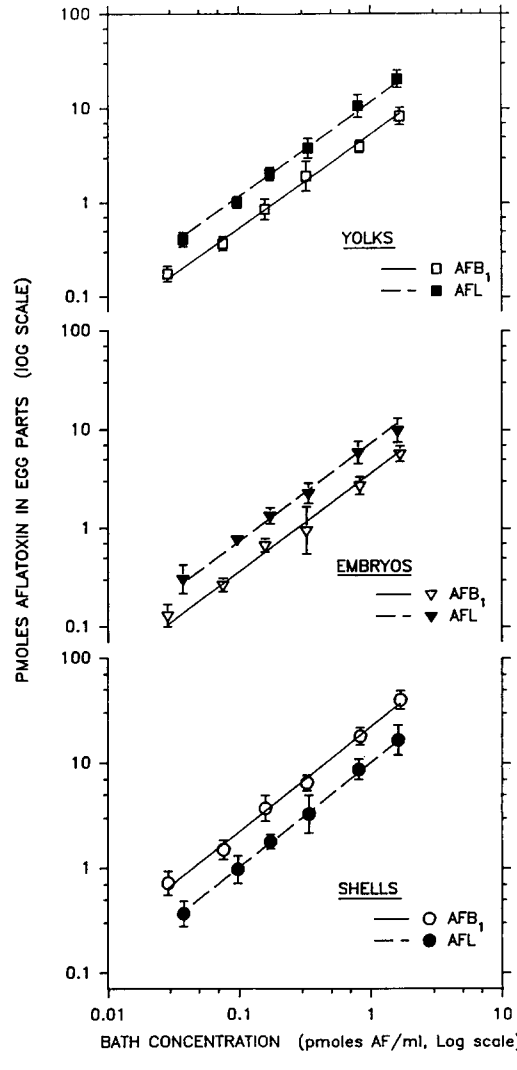

Fig. 3. Distribution of tritium into gross egg parts, shell, embryo and yolk, at the end of a 1 h bath soaking of 21 day old embryos. See Table 1 for further details.

take coefficients and DNA binding indices) presented here are the geometric means of the individual rates observed, averaging over all doses used. This is consistent with assuming a constant rate model (rate = response/dose = constant) also referred to as a straight line through the origin model (response = rate × dose) for all dose levels. More complex models allowing for rates to change with dose were also examined and for some responses provided improved fit, but are not further discussed because the estimates of differences between AFB_1 and AFL changed very little between the various models.

Tumor incidence data were analyzed using maximum likelihood estimation and likelihood ratio tests in the context of logistic regression of the data pooled across replicate tanks in GLIM (Numerical Algorithms Inc., release 3.77, Downers Grove, IL). Variation between replicate tanks was as expected under the standard binomial error assumption, with no evidence of over-dispersion.

Table 1
Distribution of tritium-labeled aflatoxins in parts of eggs following bath exposure [a]

Aflatoxin	(pmol AF per part)/(nmol/ml bath solution) [b,c,d]			
	embryo	yolk	shell	embryo + yolk
Immediately following exposure				
AFB_1	3.62 (3.38, 3.88)	5.32 (5.04, 5.63)	22.3 (20.9, 23.7)	9.09 (8.68, 9.52)
AFL	7.34 (6.85, 7.86)	11.7 (11.0, 12.4)	10.2 (9.5, 10.8)	19.2 (18.3, 20.1)
24 h after exposure				
AFB_1	1.12 (0.93, 1.33)	1.84 (1.63, 2.08)	0.503 [e] (0.366, 0.693)	3.05 (2.77, 3.37)
AFL	3.01 (2.52, 3.60)	5.33 (4.71, 6.02)	0.778 [e] (0.565, 1.07)	8.47 (7.67, 9.36)

[a] Experiment was conducted concurrently with the whole egg uptake experiment and the DNA binding measurement (Fig. 4).
[b] Values are geometric means averaging over all doses used; 95% confidence intervals are in parentheses.
[c] Confidence intervals were formed on the log scale and backtransformed. Sample sizes were 72 and 36 respectively for immediately following and 24 h after exposure.
[d] Significantly different from each other for the same egg part(s) except where noted otherwise ($P < 0.001$ for t-test comparisons).
[e] $P = 0.062$ for t-test comparison of AFB_1 vs. AFL. Log analysis may be conservative here (non-parametric Wilcoxon test, $P = 0.033$).

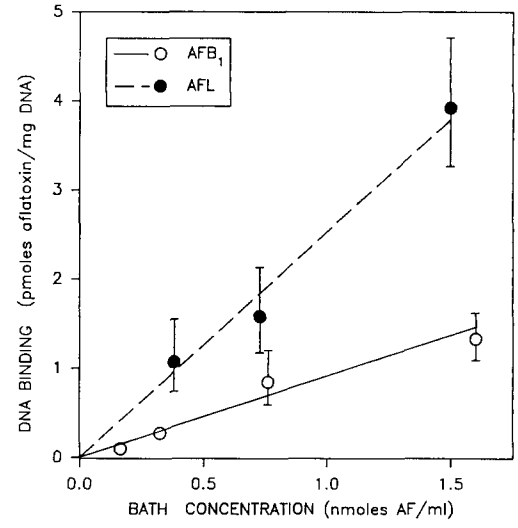

Fig. 4. DNA binding vs. bath concentration for AFB_1 and AFL in embryos 24 h after a 1 h bath exposure of eggs. The eggs were sampled from the same lot as those used for the distribution study (Table 1). Data points are geometric means ± sample SD for six replicate groups of three pooled embryos. Fitted lines were calculated by ANOVA from the data on a log scale, assuming a straight line through the origin model was appropriate on the original scale. The slopes of the lines, which were used to predict DNA binding at doses used for tumor studies (Fig. 1), were: AFB_1, 0.922; AFL, 2.53, in units of [pmol AF/mg DNA]/[nmol AF/ml bath] (significantly different, $P \leq 0.0001$).

3. Results

Uptake and distribution of aflatoxins in bath-treated eggs

Differing carcinogenicities may depend as much on uptake and distribution behavior as on metabolic characteristics. To examine this, we measured the amounts of tritiated aflatoxins taken up and retained by the whole egg and by gross egg parts under conditions used for the tumor study. Total aflatoxin content (as parent compound, unbound metabolites, forms bound to macromolecules) was determined by counting the total radioactivity in each egg. Data for the first of two experiments are shown in Fig. 2 as AF 'uptake' vs. bath concentration. (This is not a

true uptake estimate because some metabolism of parent aflatoxins and metabolite export will have occurred during the 1 h egg exposure. Because a relatively lengthy 24 h period is required for completion of AFB_1 and AFL DNA adduction, the tritium content of eggs immediately after exposure is expected to consist largely of parent compound and thus to provide a reasonable estimate of true 'uptake' coefficients.) The content of both aflatoxins was found to be linear with dose, with 'uptake' coefficients (intercepts of the log transformed data plots) of 552 (95% c.i. 535, 569) and 407 (95% c.i. 394, 419) pmol AF/g egg/μM AF for AFB_1 and AFL, respectively. By this estimate the relative total uptake efficiency of AFL immediately following exposure was 74% that of AFB_1; a second such experiment (data not shown) provided a relative estimate of 96%, for an average of 85%. However, 24 h following the 1 h exposure, the retention of AFL in the whole egg (166 pmol AFL/g egg/μM AFL, 95% c.i. 142, 195) exceeded AFB_1 (79 pmol AFB_1/g egg/μM AFB_1, 95% c.i. 68,92) by a factor of approximately 2.

In a separate experiment, we assessed the distribution of aflatoxins (measured as tritium label) into the shell, yolk, and embryo itself immediately following the 1 h initiation exposure. Again, tritium content in the three respective egg parts increased linearly with dose of each aflatoxin (Fig. 3A–C). In contrast to the total uptake experiments, however, the proportion of AFL tritium distributed into the embryo and the yolk sac reservoir immediately after exposure was 2.0 and 2.2 times, respectively, that from the AFB_1 treatment (Table 1). The distribution factors in Table 1 are derived from analysis of multi-dose data, where the relative distribution into various egg compartments did not change substantially with dose for either aflatoxin (data not shown). In eggs held in hatchery water for 24 h following exposure, the relative levels of tritium from AFL retained in the embryo and yolk were still 2.7 and 2.9 times those from AFB_1, respectively.

DNA adduction in bath-exposed embryos

Concurrently with the distribution experiment, additional eggs were bath-exposed to different concentrations of tritiated AFB_1 and AFL, and adduction to total embryo DNA was determined.

Table 2
Mortalities, body weights and hepatic tumor incidences in trout exposed to AFB_1 and AFL as embryos

Treatment (ppm)/1 h	Mortalities during growout (%) [a,b]	Average body weight (g) ± SD [b]	Hepatic tumor incidence [b]	Percent
Sham control	11 (3.1)	240 ± 67	1/349	0.3
0.01 AFB_1	14 (3.9)	248 ± 59	15/346	4.3
0.025 AFB_1	12 (3.3)	256 ± 63	59/348	17.0
0.05 AFB_1	5 (1.4)	254 ± 53	131/355	36.9
0.1 AFB_1	17 (4.7)	259 ± 54	191/343	55.7
0.25 AFB_1	13 (3.6)	251 ± 58	254/347	73.2
0.5 AFB_1	7 (2.2)	254 ± 57	252/313	80.5
0.01 AFL	13 (3.6)	249 ± 52	28/347	8.1
0.025 AFL	14 (3.9)	251 ± 45	157/346	45.4
0.05 AFL	7 (1.9)	248 ± 46	245/353	69.4
0.1 AFL	5 (1.4)	245 ± 47	276/355	77.8
0.25 AFL	22 (6.1)	246 ± 60	275/338	81.4
0.5 AFL	20 (8.3)	260 ± 64	148/220	67.3

[a] Mortalities occurring after the growout period was started with four groups of 90 fish except for 0.5 ppm AFB_1 (80 fish) and 0.5 ppm AFL (60 fish). Post-initiation mortalities were higher in those groups, leaving fewer fish to carry to final tumor, especially at the highest AFL dose (50% loss).

[b] Data for mortalities, average body weights ± SD, and final tumor incidence were combined for the quadruplicate groups at each dose level. There were no significant differences among groups in average body weights.

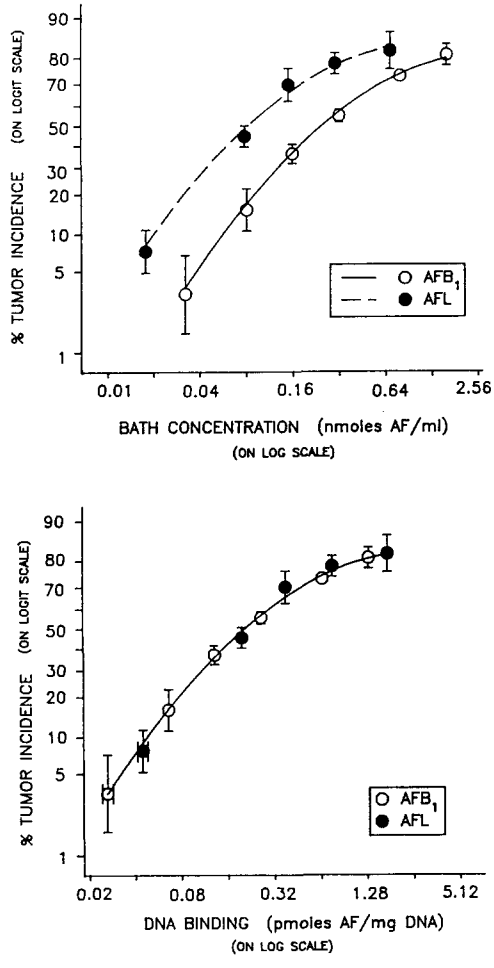

Fig. 5. Tumor incidence (means ± sample SD of logits for four replicate tanks of 100 fish) in trout exposed to AFB_1 or to AFL by bath treatment of 21 day old embryos (unhatched eggs). Corresponding tumor incidences are plotted on a logit scale as a function of: (upper) AF bath concentration (nmol AF/ml, log scale). The data for the two aflatoxins fit a model with a common quadratic coefficient but with different intercepts and linear coefficients (overall lack of fit, $P = 0.56$). This is consistent with the two curves being horizontally displaced from each other. * The highest AFL dose was not included in curve fitting due to excessive mortalities. (lower) DNA binding (pmol AF/mg DNA, log scale) predicted for the actual concentrations used in the tumor study. These DNA values were calculated from the slopes determined in the separate experiment in which DNA binding was measured as a function of bath concentration. Horizontal error bars for all points of the same AF are the same size, hence are shown only for the lowest doses of each aflatoxin; they were derived from standard errors of estimated slopes of the DNA binding vs. concentration lines (Fig. 4). The data for both aflatoxins fit a single quadratic curve, indicating no difference between aflatoxins (lack of fit, $P = 0.45$). * The highest AFL dose was not included in curve fitting due to excessive mortalities.

Because a previous study has shown that the maximum concentration of DNA adducts in AFB_1-treated eggs is reached approximately 24 h after exposure (Croy et al., 1980), eggs in the present study were held in running water for 24 h before DNA binding analysis. From ANOVA of the DNA adduction/dose ratios, AFL adduction was 2.74 times that of AFB_1 (Fig. 4). Taken together, these results indicate that AFL and AFB_1 have significantly different pharmacokinetic and metabolic properties by this exposure route, leading to less efficient egg uptake of AFL from solution but much more extensive distribution, metabolism and adduction within the embryo for that proportion of dose taken up by the whole trout egg, compared to AFB_1.

Tumor response by embryo bath exposure

Tumor response was determined 13 months after a 1 h immersion exposure of 21 day old trout embryos to varying concentrations of AFB_1

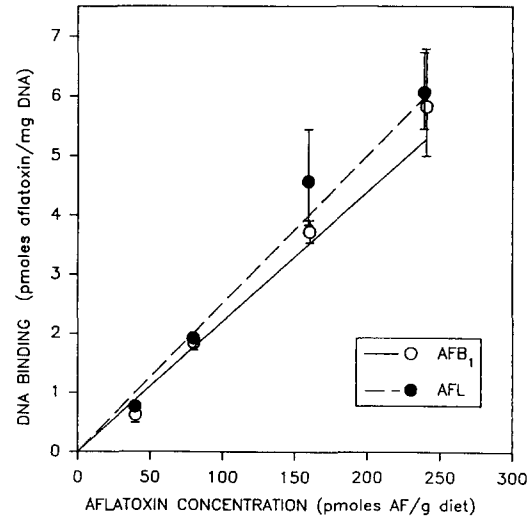

Fig. 6. DNA binding in livers vs. concentration of AFB_1 and AFL in the diets fed to trout fry for 2 weeks prior to measurement. Each data point is a mean from four (for lower two doses) or eight (for higher two doses) replicate groups of 15 pooled fish; error bars are ± sample SD. Fitted lines were derived by calculation of a mean ratio for DNA binding/concentration by using ANOVA of the data on a log scale. DNA binding for AFL is 1.14 times that of AFB_1 (95% confidence intervals are 1.06–1.23, AFL significantly different from AFB_1, $P < 0.01$).

or AFL. Increasing post-treatment mortalities were observed for the higher AFB_1 and AFL doses in the initial exposure of 120 embryos (data not shown). Mortalities at the highest AFL dose were extensive (50%) (Table 2), and we do not consider the tumor datum from this group in our further analyses because of probable bias (e.g. death of the most susceptible individuals). After the initial treatment related mortalities, survival was comparable in most of the groups, although mortalities were slightly higher in the two highest AFL dose groups (Table 2). Subsequent growth was unaffected by the embryo exposures. Average body weight was actually higher than the control level in all groups, though not significantly so.

Histological examination revealed that mixed hepatocellular/cholangiocellular carcinomas were the predominant tumor type. For all the AFB_1 doses, 1510 tumors were examined. Of these 46.7% were mixed carcinomas (MC), 41.1% were hepatocellular carcinomas (HCC), 3.0% were cholangiocellular carcinomas (CCC), 0.1% were mixed adenomas, 6.0% were hepatocellular adenomas (HCA) and 3.1% were cholangiomas (CH). For all the AFL doses, 2071 tumors were examined. These consisted of 60.0% MC, 25.6% HCC, 0.3% MA, 2.6% CCC, 5.4% HCA, and 6.1% CH. The distribution of tumor types was independent of dose for both carcinogens.

The tumor responses for AFB_1 and AFL defined complete dose-response relations plotted as logit incidence vs. $\log_n$ of exposure concentration (Fig. 5, upper). Throughout most of the concentration range tested, AFL was a more potent carcinogen than AFB_1, based on dose applied, though at the highest dose tested, the observed AFL response (see Table 2; data point not shown) dropped below that for AFB_1, which we presume due to selective mortalities. At 50% tumor incidence, AFL was over three times more potent (i.e. three-fold greater AFB_1 concentration was needed to elicit the same tumor response). Both curves showed negative departure from linearity in this study. When analyzed in the form of a molecular dosimetry plot (logit incidence vs. $\log_n$ embryo DNA binding; Fig. 5, lower), the data for AFB_1 and AFL are essentially coincident. Since DNA adduction (molecular dose received) was linear with dose for both aflatoxins (Fig. 4), the tumor response would be expected to remain non-linear with molecular dose received.

DNA adduction of AFB_1 and AFL in fry liver by dietary exposure

We were interested to determine if the molecular dosimetry relationship derived from embryonic exposure would hold at later life stages or other exposure routes, where pharmacokinetic

Table 3
Mortalities, body weights and hepatic tumor incidences in trout exposed to dietary AFB_1 and AFL as fry

Dietary dose ppb (pmol/g)	Mortalities (%) [a,b]	Average body weight (g) ± SD [b]	Hepatic tumor incidence [b]	Percent
0 (0)	8 (4.0)	195 ± 44	0/192	0
4 (12.8) AFB_1	18 (4.5)	208 ± 56	25/382	6.5
8 (25.6) AFB_1	13 (3.3)	205 ± 51	98/387	25.3
16 (51.3) AFB_1	11 (2.8)	200 ± 55	194/389	49.9
32 (102.6) AFB_1	11 (2.8)	203 ± 54	287/389	73.8
64 (205.1) AFB_1	17 (4.3)	202 ± 52	302/383	78.9
4 (12.7) AFL	10 (2.5)	200 ± 50	57/390	14.6
8 (25.5) AFL	7 (1.8)	195 ± 50	143/393	36.4
16 (50.9) AFL	14 (3.5)	199 ± 51	183/386	47.4
32 (101.9) AFL	17 (4.3)	200 ± 50	255/383	66.6
64 (203.8) AFL	10 (2.5)	200 ± 53	291/390	74.6

[a] Mortalities recorded from the start of feeding aflatoxin-containing diets until termination at 9 months.
[b] Data for mortalities, body weights ± SD, and final tumor incidence were combined for the quadruplicate groups at each dose level. Average body weight did not differ significantly among groups.

differences between AFB_1 and AFL may vary. An additional point of interest is that dosimetry studies using eggs, though extremely sensitive, provide quantification of DNA damage averaged over all cells in the embryo and not in the actual target organ. Thus such studies implicitly assume that the relative binding of the two carcinogens to embryonic liver parallels their relative binding in the total embryo. To address these issues, trout fry were fed diets containing varying doses of tritiated AFB_1 or AFL for 2 weeks, livers excised, and DNA adduction levels compared. By ANOVA of DNA adduction/dose ratios, DNA adduction increased linearly with dietary aflatoxin dose (Fig. 6), with a DNA binding index for AFL ((0.0250 pmol adduct/mg DNA)/(pmol aflatoxin/gram diet), 95% confidence limits 0.0231, 0.0271) that was 1.14 times that for AFB_1 (0.0220, 95% confidence limits 0.0203, 0.0238). Since aflatoxin-DNA adducts are poorly repaired in trout liver (Bailey et al., 1988), such adducts do not reach steady state during chronic exposure but accumulate linearly with time of exposure as well as dose (Dashwood et al., 1989). Thus the relative adduction levels in Fig. 6 approximate the total relative liver DNA adduction accumulated from the two aflatoxins during the 2 week period of tumor initiation.

Tumor response in trout fry by dietary exposure to AFB_1 and AFL

The results of the dietary exposure tumor study are presented in Table 3. There was no effect of the AFB_1 or AFL exposure on either mortalities or body weights. In this experiment, a total of 1119 tumors from the AFB_1 exposed fish and 1247 tumors from the AFL exposed fish were examined microscopically. As in the embryo exposure experiment, MC were the predominant tumor type (61.9% of all the AFB_1 initiated tumors observed and 56.9% of the AFL initiated tumors). Most of the tumors developed in this experiment were small (≤ 2 mm), and there were more HCA (13.8% for AFB_1 exposed fish, 11.2% for AFL exposed fish) and fewer HCC (11.8% and 17.9% for AFB_1 and AFL, respectively) than in the embryo exposure study. Cholangiomas (5.5% AFB_1, 8.5% AFL) were also slightly more prevalent than CCC (4.6% AFB_1, 3.9% AFL). The remaining tumors were mixed adenomas (2.4%, AFB_1; 1.6%, AFL). We believe that the

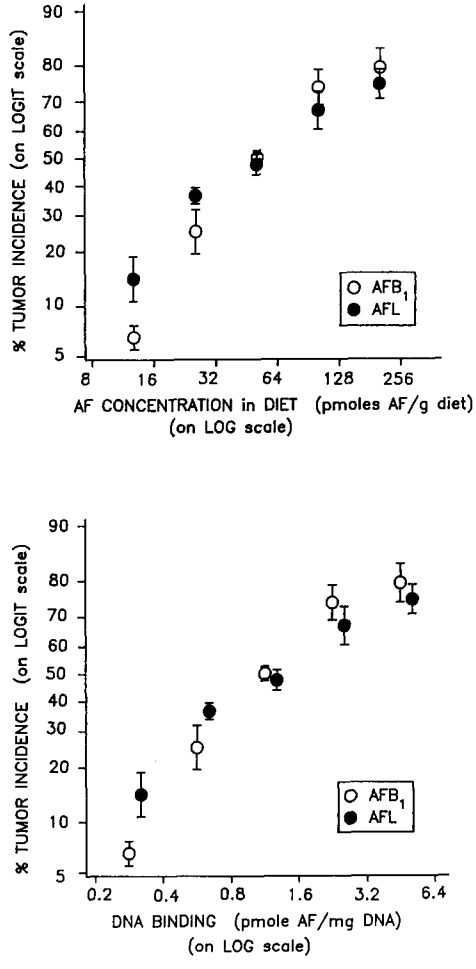

Fig. 7. Tumor incidence (means ± sample SD of logits for four replicate tanks containing nominally 100 fish) in trout exposed to AFB_1 or AFL by feeding of fry for 2 weeks. Incidences are plotted on a logit scale as a function of: (upper) AF dietary concentration (pmol AF/g diet, on a logarithmic scale). Completely separate quadratics describe the data for the two aflatoxins (lack of fit, $P = 0.11$), indicating that they are not just shifted horizontally from each other, but also differ in the degree of curvilinearity. (lower) DNA binding (pmol AF/mg DNA, on a logarithmic scale) predicted for the actual concentrations used in the tumor study; they were calculated from the slopes determined in the separate experiment which measured DNA binding as a function of dietary concentration (Fig. 6). Completely separate quadratics describe the data for the two aflatoxins (lack of fit, $P = 0.11$).

adenomas are precursors to malignant forms in most cases which would explain these observations. As for the embryo exposure, the distribution of tumor types was independent of dose of AFB_1 or AFL.

Tumor dose-response characteristics for the two aflatoxins when fed in the diet to hatched fry were non-linear (Fig. 7, upper), as also seen with the passive embryo exposure protocol. Unlike the embryo exposure, the dietary response functions plotted as logit vs. ln dose were not clearly separated. The TD_{50} values for AFB_1 and AFL were nearly equal at about 50 pmol/g dietary concentration, and not three-fold different as for embryo treatment. By statistical examination the responses were not entirely coincident (significant lack of fit, $P < 0.001$), with AFB_1 appearing in this study to be slightly less potent than AFL at lower doses but more potent at higher doses. Most of the evidence for non-coincidence comes from the two lower doses, where there is greatest separation. Because the DNA binding index for AFL was only 14% greater than for AFB_1, the molecular dosimetry analysis (Fig. 7, lower) can result in only a small horizontal shift in the curves which does not significantly change the general relationship between the two aflatoxins by dietary treatment.

4. Discussion

DNA adduction by AFB_1 and AFL

Adducts formed from AFB_1 in trout embryos and fry have been previously described (Croy et al., 1980), and consist almost entirely of 8,9-dihydro-8-[N^7-guanyl]-9-hydroxyaflatoxin B_1 (AFB_1-N^7-guanine) and its ring-opened formamidopyrimidine derivatives also predominant in mammalian species. With regard to AFL adducts, our previous studies on the metabolism of C-1 tritiated AFL in isolated trout hepatocytes indicated that the great majority of recovered AFL derived DNA adducts were identical to the AFB_1 derived species, as a result of AFL cyclopentenol oxidation prior to cytochrome P450 catalyzed 8,9-oxidation (Loveland et al., 1987). However, no direct control was then available for the recovery and HPLC behavior of any potential AFL-8,9-oxide adducts, and the possibility remained that an undetected adduct with significant biological potency for tumor initiation might be generated in vivo from P450 catalyzed epoxidation of AFL at the 8,9 double bond. The present results do not support this possibility. Treatment of DNA in vitro with activated AFL produced one major, readily detectable acid hydrolysis product that, on the basis of behavior after alkaline hydrolysis, we tentatively identify as AFL-N^7-guanine. This finding suggests that the presence of the non-planar cyclopentenol moiety of AFL does not preclude relatively efficient DNA adduction in vitro and thus interferes little if at all in the precovalent intercalation association important in the interaction between AFB_1-8,9-oxide and double-stranded DNA (Gopalakrishnan et al., 1989). By contrast, HPLC analysis of DNA derived from trout treated with AFL in vivo showed no trace of the adduct derived from AFL-8,9-oxide in vitro, only adducts derived from AFL conversion to AFB_1, and secondarily to AFM_1. On the basis of Fig. 1B (14000 dpm in the major AFB_1-N^7-guanine peak derived from AFL exposure in vivo; a base-line of 15 dpm in the region of the AFL-N^7-guanine standard; an assumed ability to detect three-fold activity over background; 33% yield of any AFL-N^7-guanine adduct), we calculate that $< 1\%$ of AFL adduction in vivo can be from direct adduction via an AFL-8,9-oxide metabolite.

In separate studies we have analyzed the spectrum of Ki-ras proto-oncogene mutations induced in trout hepatic tumors by AFB_1, AFL, and two additional aflatoxins. We find that AFL induces exclusively codons 12 and 13 G-T transversions in 70–80% of hepatic tumors examined (unpublished results). This profile is indistinguishable from that produced by AFB_1 in the trout (Chang et al., 1991), and suggests a predominant role for guanine adduction in AFL tumor initiation. Although other workers have provided evidence for AFB_1 adduction at adenine residues in vitro (D'Andrea and Haseltine, 1978; T.M. Harris, personal communication), we have yet to find any indication of transforming adduction at Ki-ras codon 61 adenine in vivo by any aflatoxin. We

have also previously examined the interaction of AFL-8,9-dichloride with specific gene sequences in vitro, and found that its efficiency for interacting with double-stranded DNA and its sequence-context profile for interactions with guanines along these sequences were indistinguishable from AFB_1-8,9-dichloride (Marien et al., 1987). On the basis of these results we conclude that, within the limits of detection in these experiments, AFB_1 and AFL form the same major DNA adduct in vivo, and that essentially 100% of AFL derived adducts reflect prior oxidation of AFL to AFB_1. Although a minor AFL-8,9-epoxide adduct ($< 1\%$) in vivo cannot be excluded, there is no suggestion that such an adduct, if formed, has sequence selectivity or transforming efficiency significantly different from AFB_1-N^7-guanine.

Comparative carcinogenesis and dosimetry for AFB_1 and AFL

The results show that different exposure routes produce different relative carcinogenic potency estimates for these two aflatoxins. AFL was three times more potent than AFB_1 by passive embryo uptake over all doses examined, but about as potent (see also below) as AFB_1 by dietary exposure. Thus it is clear that exposure route can have significant influence on the ranking of relative carcinogenic hazards derived from animal bioassay data.

That the two protocols should produce different relative potencies should not be surprising, and appears to reflect differing pharmacokinetic behavior of the two aflatoxins in the two exposure systems. AFB_1 was more efficiently taken up by trout eggs, but much less efficiently sequestered into the embryo and metabolized into embryonic DNA binding species, compared to AFL. The net result was a superior tumorigenic response to AFL, when expressed in terms of exposure concentrations. By comparison, total AFL derived tritium content in livers in the dietary study was determined to be 52% higher than for AFB_1 treated fish at the end of the exposure period, even though DNA adduction levels were more nearly equal. This is consistent with previous findings that, at equivalent concentrations, AFL is metabolized by isolated trout hepatocytes to DNA binding species only 60% as rapidly as AFB_1 (Loveland et al., 1988), and may thus tend to bioaccumulate more extensively in liver in vivo pending metabolism to polar or binding species.

Since the two aflatoxins give the same DNA adduct, molecular dosimetry plots should give coincident data sets regardless of exposure protocol, unless there were significant differences in toxicity between the two aflatoxins. The data in Figs. 5 and 7 are largely consistent with this expectation and do not suggest substantial differences in toxicity for AFB_1 and AFL under these conditions. A limitation in this conclusion is that dosimetry in embryo exposure protocols is of necessity based on carcinogen binding to total embryonic DNA and not solely to embryonic liver, the target organ. We have recently completed quantitative dosimetry and tumorigenesis studies for four aflatoxins, including AFB_1 and AFL, by direct microinjection into trout embryos, a procedure that bypasses uptake pharmacokinetic differences. In those experiments, the dose-related comparison of AFL and AFB_1 carcinogenicities differed substantially from the three-fold ratio seen in the present experiment, yet the molecular dosimetry curves remained coincident (unpublished results). This lends confidence that the relative DNA adduction of AFL and AFB_1 to whole embryonic DNA does not differ markedly from their relative binding in embryonic liver cells.

Non-linearities and potency estimations

Statistical modeling of the embryo and fry exposure results shows a significant lack of fit to straight-line relationships for all four tumor curves. Linearity could not be restored by deletion of one or two data groups from the analysis of any curve except Fig. 7, AFL. In the embryo exposure results, a quadratic relationship fits the data for each carcinogen, based on deviance (lack of fit) statistics and examination of residuals. A model with a single, common quadratic exponent but with intercepts and linear coefficients differing for the two carcinogens fits the data quite well (overall lack of fit, $P = 0.56$). This indicates horizontally displaced quadratic curves which, in practical terms, means that AFL was three-fold

more potent than AFB_1 over the entire dose range examined.

The results for dietary exposure were more complex. These two curves were also statistically non-linear, but the data suggest two closely placed quadratics crossing at a common TD_{50} value (50 ppm), not simple horizontally shifted quadratic dose responses. As part of a larger study we have repeated this comparison, and saw no evidence of such divergence (manuscript in preparation). This suggests the non-parallel behavior, particularly the low-dose divergence, to be an anomaly of this particular experiment. Our overall conclusion is that these two aflatoxins have very similar if not identical dose-response carcinogenicities in rainbow trout by dietary exposure, in terms of both dose given to the organism and molecular dose received in the target organ.

We have no entirely satisfactory mechanistic explanation for the non-linearities observed in the two dose-response tumor studies reported here. Since the DNA binding responses were dose-linear, the non-linearities in tumor response were not due to dose-dependent uptake (embryo) or consumption (diet) efficiencies, target organ distribution, saturation of carcinogen-activating enzymes, DNA adduction, or activation of DNA repair pathways. Although dose-dependent target organ toxicities have been invoked to explain non-linearities in *N*-nitrosodiethylamine hepatocarcinogenesis (Swenberg et al., 1991) and 2-acetylaminofluorene bladder carcinogenesis (Poirier et al., 1991; Cohen and Ellwein, 1991), these effects produce positive departures from linearity rather than the negative departures observed here. Negative departures from linearity might be ascribed to selective mortalities of the most sensitive individuals with increasing carcinogen dose. We believe this contributed greatly if not entirely to the non-linear response observed among the survivors from embryo treatment seen in Fig. 5, but there is no direct evidence that this is so. For the dietary treatment, previous (Dashwood et al., 1989) and subsequent studies (unpublished results) of AFB_1 dose-response carcinogenesis have not indicated non-linearity. The apparent non-coincidence between AFL and AFB_1 and the apparent non-linear response may both simply represent anomalously low responses for the two lowest AFB_1 doses in this particular dietary exposure study.

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References

Baertschi, S.W., K.D. Raney, M.P. Stone and T.M. Harris (1988) Preparation of the 8,9-epoxide of the mycotoxin aflatoxin B1: the ultimate carcinogenic species, J. Am. Chem. Soc., 110, 7929–7931.

Bailey, G.S., D.E. Williams, J.S. Wilcox, P.M. Loveland, R.A. Coulombe and J.D. Hendricks (1988) Aflatoxin B1 carcinogenesis and its relation to DNA adduct formation and adduct persistence in sensitive and resistant salmonid fish, Carcinogenesis, 9, 1919–1926.

Chang, Y.-J., C. Mathews, K. Mangold, K. Marien, J. Hendricks and G. Bailey (1991) Analysis of *ras* gene mutations in rainbow trout liver tumors initiated by aflatoxin B1, Mol. Carcinogen., 4, 112–119.

Cohen, S.M., and L.B. Ellwein (1991) Genetic errors, cell proliferation, and carcinogenesis, Cancer Res., 51, 6493–6505.

Croy, R.G., J.E. Nixon, R.O. Sinnhuber and G.N. Wogan (1980) Investigation of covalent aflatoxin B_1-DNA adducts formed in vivo in rainbow trout (*Salmo gairdneri*) embryos and liver, Carcinogenesis, 1, 903–909.

D'Andrea, A.D., and W.A. Haseltine (1978) Modification of DNA by aflatoxin B1 creates alkali-labile lesions in DNA at positions of guanine and adenine, Proc. Natl. Acad. Sci. USA, 75, 4120–4124.

Dashwood, R.H., D.N. Arbogast, A.T. Fong, J.D. Hendricks and G.S. Bailey (1988) Mechanisms of anti-carcinogenesis by indole-3-carbinol: detailed in vivo DNA binding dose-response studies after dietary administration with aflatoxin B_1, Carcinogenesis, 9, 427–432.

Dashwood, R.H., D.N. Arbogast, A.T. Fong, C. Pereira, J.D. Hendricks and G.S. Bailey (1989) Quantitative inter-relationships between aflatoxin B1 carcinogen dose, indole-3-

carbinol anti-carcinogen dose, target organ DNA adduction and final tumor response, Carcinogenesis, 10, 175–181.

Dashwood, R.H., K. Marien, P.M. Loveland, D.E. Williams, J.D. Hendricks and G.S. Bailey (1992) Formation of aflatoxin-DNA adducts in trout and their use as molecular dosimeters for tumor prediction, in: D. Bhatnagar, E.B. Lillehoj and D.K. Arora (Eds.), Handbook of Applied Mycology, Volume 5: Mycotoxins in Ecological Systems, Dekker, New York.

Goeger, D.E., D.W. Shelton, J.D. Hendricks, C. Pereira and G.S. Bailey (1988) Comparative effect of dietary butylated hydroxyanisole and β-naphthoflavone on aflatoxin B1 metabolism, DNA adduct formation, and carcinogenesis in rainbow trout, Carcinogenesis, 9, 1793–1800.

Gopalakrishnan, S., S. Byrd, M.P. Stone and T.M. Harris (1989) Carcinogen-nucleic acid interactions: equilibrium binding studies of aflatoxin B1 with the oligonucleotide $d(ATGCAT)_2$ and with plasmid pBR322 support intercalative association with the B-DNA helix, Biochemistry, 30, 726–734.

Groopman, J.D., R.G. Croy and G.N. Wogan (1981) In vitro reactions of aflatoxin B1-adducted DNA, Proc. Natl. Acad. Sci. USA, 78, 5445–5449.

Gurtoo, H.L., P.L. Koser, S.K. Bansal, H.W. Fox, S.D. Sharma, A.I. Mulhern and Z.P. Pavelic (1985) Inhibition of aflatoxin B_1-hepatocarcinogenesis in rats by β-naphthoflavone, Carcinogenesis, 6, 675–678.

Hendricks, J.D. (1994) Histopathology of hepatocellular neoplasms and related lesions in teleost fish, in: C.J. Dalve (Ed.), An Atlas of Neoplasms and Related Disorders in Fishes, Academic Press, New York, in press.

Hendricks, J.D., J.H. Wales, R.O. Sinnhuber, J.E. Nixon, P.M. Loveland and R.A. Scanlan (1980) Rainbow trout (*Salmo gairdneri*) embryos: a sensitive animal model for experimental carcinogenesis, Fed. Proc., 39, 3222–3229.

Hendricks, J.D., T.R. Meyers, J.L. Casteel, J.E. Nixon, P.M. Loveland, and G.S. Bailey (1984) Rainbow trout embryos: advantages and limitations for carcinogenesis research, Natl. Cancer Inst. Monogr., 65, 129–137.

IARC (1993) Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Aflatoxins, Vol. 56, International Agency for Cancer Research, Lyon, pp. 245–396.

Kenseler, T.W., P.A. Enger, M.A. Trush, E. Bueding and J.D. Groopman (1985) Modification of aflatoxin B1 binding to DNA in vivo in rats fed phenolic antioxidants, ethoxyquin, and a dithiothione, Carcinogenesis, 6, 759–764.

Lee, B.C., J.D. Hendricks and G.S. Bailey (1991) Toxicity of mycotoxins to fish, in: J.E. Smith (Ed.), Mycotoxins and Animal Foods: Natural Occurrence, Toxicity and Control, CRC Press, Boca Raton, FL, pp. 607–626.

Loveland, P.M., R.A. Coulombe, L.M. Libbey, N.E. Pawlowski, R.O. Sinnhuber, J.E. Nixon and G.S. Bailey (1983) Identification and mutagenicity of aflatoxicol-M_1 produced by metabolism of aflatoxin B_1 and aflatoxicol by liver fractions from rainbow trout (*Salmo gairdneri*) fed β-naphthoflavone, Food Chem. Toxicol., 21, 557–562.

Loveland, P.M., J.S. Wilcox, N.E. Pawlowski and G.S. Bailey (1987) Metabolism and DNA binding of aflatoxicol and aflatoxin B_1 in vivo and in isolated hepatocytes from rainbow trout (*Salmo gairdneri*), Carcinogenesis, 8, 1065–1070.

Loveland, P.M., J.S. Wilcox, J.D. Hendricks and G.S. Bailey (1988) Comparative metabolism and DNA binding of aflatoxin B_1, aflatoxin M_1, aflatoxicol and aflatoxicol-M_1 in hepatocytes from rainbow trout (*Salmo gairdneri*), Carcinogenesis, 9, 441–446.

Marien, K., R. Moyer, P. Loveland, K. van Holde and G. Bailey (1987) Comparative binding and sequence interaction specificities of aflatoxin B1, aflatoxicol, aflatoxin M1, and aflatoxicol M1 with purified DNA, J. Biol. Chem., 262, 7455–7462.

Milliken, G.A., and D.E. Johnson (1984) Analysis of Messy Data. Vol. I: Designed Experiments, Van Nostrand Reinhold, New York.

Nixon, J.E., J.D. Hendricks, N.E. Pawlowski, P.M. Loveland and R.O. Sinnhuber (1981) Carcinogenicity of aflatoxicol in Fischer 344 rats, J. Natl. Cancer Inst., 66, 1159–1163.

Nixon, J.E., J.D. Hendricks, N.E. Pawlowski, C.B. Pereira, R.O. Sinnhuber and G.S. Bailey (1984) Inhibition of aflatoxin B_1 carcinogenesis in rainbow trout by flavone and indole compounds, Carcinogenesis, 5, 615–619.

Poirier, M.C., N.F. Fullerton, T. Kinouchi, B.A. Smith and F.A. Beland (1991) Comparison between DNA adduct formation and tumorigenesis in livers and bladders of mice chronically fed 2-acetylaminofluorene, Carcinogenesis, 12, 895–900.

Salhab, A.S., and G.S. Edwards (1977) Comparative in vitro metabolism of aflatoxicol by liver preparations from animals and humans, Cancer Res., 37, 1016–1021.

SAS Institute (1993) SAS System Under Windows, Release 3.77, Cary, NC.

Schoenhard, G.L., J.D. Hendricks, J.E. Nixon, D.J. Lee, J.H. Wales, R.O. Sinnhuber and N.E. Pawlowski (1981) Aflatoxicol-induced hepatocellular carcinoma in rainbow trout (*Salmo gairdneri*) and the synergistic effects of cyclopropenoid fatty acids, Cancer Res., 41, 1011–1014.

Shelton, D.W., D.E. Goeger, J.D. Hendricks and G.S. Bailey (1986) Mechanisms of anti-carcinogenesis: The distribution and metabolism of aflatoxin B_1 in rainbow trout fed Aroclor 1254, Carcinogenesis, 7, 1065–1072.

Swenberg, J.A., D.G. Hoel and P.N. Magee (1991) Mechanistic and statistical insight into the large carcinogenesis bioassays on *N*-nitrosodiethylamine and *N*-nitrosodimethylamine, Cancer Res., 51, 6409–6414.

Wales, J.H., R.O. Sinnhuber, J.D. Hendricks, J.E. Nixon and T.A. Eisele (1978) Aflatoxin B_1 induction of hepatocellular carcinoma in the embryos of rainbow trout (*Salmo gairdneri*), J. Natl. Cancer Inst., 60, 1133–1139.

Whitham, M., J.E. Nixon and R.O. Sinnhuber (1982) Liver DNA bound in vivo with aflatoxin B1 as a measure of hepatocarcinoma initiation in rainbow trout, J. Natl. Cancer Inst., 68, 623–628.