AN ABSTRACT OF THE THESIS OF

Daniel Brock for the degree of Master of Science in Fisheries Science presented on October 2, 1990.

Title: Temperature Modulated Hepatic DNA Binding but not Biliary Metabolites of Aflatoxin B_1 in Rainbow Trout.

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Lawrence R. Curtis

Acclimation temperature alters the status of hepatic microsomal xenobiotic metabolizing enzymes in fish. This is most evident when microsomes from cold and warm acclimated fish are tested at a intermediate temperature in vitro. The effects of temperature acclimation and acute temperature shifts on the in vivo metabolism and toxicity of aflatoxin B_1 (AFB1) were studied here. Rainbow trout acclimated to 10°C or 18°C for 1 month were injected (ip) with 0.4mg [^{3}H]AFB1/kg and held at their respective acclimation temperature or acutely shifted to 14°C. The hepatic distribution of the [^{3}H]AFB1 at 16 hr was unaltered by temperature. At all temperature regimens except 18°C-14°C, the amount of hepatic DNA binding was proportional to liver [^{3}H]AFB1 concentrations and described by simple linear regression. No difference in hepatic DNA binding was seen between the 18°C-18°C and 10°C-10°C fish; however, the 10°C-14°C fish had significantly more hepatic DNA binding compared to the 18°C-14°C fish. No temperature effects were apparent in biliary metabolic

profiles and protein binding; however, elevated plasma levels of the enzymes GOT and GPT indicated soft tissue toxicity.

Temperature Modulated Hepatic DNA Binding but not Biliary Metabolites of Aflatoxin B_1 in Rainbow Trout.

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TEMPERATURE MODULATED HEPATIC DNA BINDING BUT NOT BILIARY METABOLITES OF AFLATOXIN B_1 IN RAINBOW TROUT

INTRODUCTION

Disposition and actions of metabolically activated toxicants are often altered by pretreatment of the animal with another chemical. For instance, pretreatment of rainbow trout with B-napthoflavone decreases the incidence of aflatoxin B_1 induced hepatocarcinomas (Nixon et al., 1984), probably via induction of the cytochrome P4501A1 which produces a metabolite (aflatoxicol-M1) much less mutagenic than aflatoxin B_1 (Loveland et al., 1983). On the other hand, pretreatment of rats with phenobarbital increases the toxicity of a subsequent dose of carbon tetrachloride by inducing the P450 isozyme which forms the reactive intermediate believed responsible for this haloalkane's toxicity (Hanzlik, 1981). Although certainly a valuable technique, chemical pretreatment may have significant biochemical and physiological effects aside from the specific chemical interaction of interest. This can confound mechanistic interpretation of the results. Temperature can be used to modulate the pharmacokinetics and metabolism of exogenous agents in fish (Curtis 1983; Kemp and Curtis 1987; Curtis et al., 1990) in lieu of chemical pretreatment. In addition to the potential use of temperature for basic research, it may help explain xenobiotic associated neoplasms found in wild fish populations as seen with English

sole (Malins et al., 1985).

Fish are poikilotherms (thermal conformers) and as such must maintain vital life functions within a narrow range in face of wide seasonal fluctuations in body temperature. Adaptations to such chronic temperature changes is known as thermal acclimation (Hazel and Prosser, 1974). Membrane lipid changes are a component involved in thermal acclimation. Hepatic membranes of rainbow trout acclimated to low temperatures have less saturated and significantly more polyunsaturated fatty acids compared to those trout acclimated to higher temperatures (Hazel, 1979). Such alteration of membrane lipids helps maintain similar physical states of the membrane despite marked differences in prevailing temperature, a process known as homeoviscous adaptation (Sinensky, 1974). For example, membrane viscosities of E. coli raised (acclimated) at different temperatures and tested at their respective acclimation temperatures are similar (Sinensky, 1974). Acute temperature shifts also directly effect the membrane physical state. An increase in temperature causes the membrane to become less ordered and more fluid; whereas, a decrease in temperature creates a more ordered crystalline membrane state. The rainbow trout thermal acclimation model is based upon temperature acclimation dependent differences in membrane compostion and acute, direct temperature effects on membrane physical state with subsequent effect on membrane bound processes.

Many cellular homeostatic processes are membrane bound or associated with the membrane. Indeed a variety of studies have shown that membrane associated processes are significantly affected by chronic and acute temperature shifts. Curtis (1983) found that *in vivo* glucuronidation was similar for cold and warm acclimated steelhead trout tested at their acclimation temperatures. Kiovusaari et al. (1981) found similar results for *in vitro* monooxygenase activity of temperature acclimated fish. This ideal temperature compensation suggested biotransformation enzymes maintained near constant activity although acclimation temperatures differed substantially. On the other hand, aryl hyrdocarbon hydroxylase activity in microsomes isolated from 10°C and 18°C acclimated trout and tested at a common temperature of 14°C demonstrated significantly different Km values although the Vmaxs were similar (Carpenter et al., 1990). It can be hypothesized that this was due to the fluidity differences of the respective microsomal vesicles. Such temperature induced differences in xenobiotic metabolism may influence xenobiotic related injury.

To investigate temperature induced differences in xenobiotic metabolism *in vivo*, we chose aflatoxin B_1 (AFB1) as our test chemical for use in the rainbow trout thermal acclimation model for a variety of reasons. First, AFB1 is a potent hepatocarcinogen, especially in rainbow trout, that is metabolically activated by the membrane bound cytochrome

P-450 LM2 isozyme (Williams and Buhler, 1983) to the putative ultimate carcinogen, the 2,3-epoxide (Wogan, 1973). Second, the amount of hepatic DNA binding of AFB1 correlates very well with the development of hepatic tumors (Dashwood et al., 1989). Third, in addition to the putative epoxide several other AFB1 metabolites are formed by trout (Fig. 1). In the liver, aflatoxicol and aflatoxin-M_1 are the primary and secondary metabolites formed respectively in vitro (Loveland et al., 1977). Aflatoxicol formation is catalyzed by the cytosolic enzyme NADPH-dependent 17-hydroxy-steroid dehydrogenase (Cambell and Hayes, 1976) and the aflatoxin-M_1 by the membrane bound cytochrome P450 system (Salhab and Edwards, 1977). The primary biliary metabolites formed are aflatoxicol glucuronide and aflatoxicol-M1 glucuronide (Loveland et al., 1984). Therefore, by monitoring hepatic DNA binding and profiles of biliary metabolites in temperature acclimated and acute temperature shifted fish, this study assessed the influence of temperature on the genotoxicity and metabolism of AFB1 in rainbow trout.

Figure 1. Routes of AFB1 metabolism in rainbow trout.

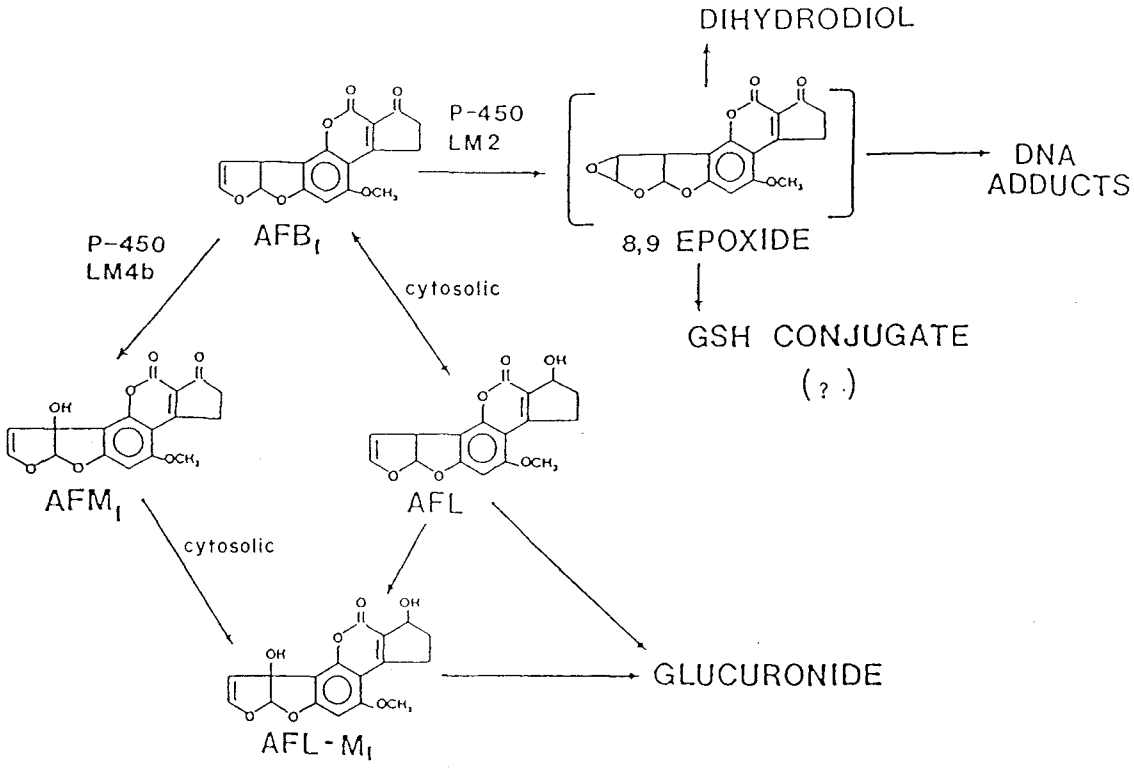
DIHYDRODIOL
P-450
LM2
DNA
ADDUCTS
AFB1
8,9 EPOXIDE
P-450
LM4b
cytosolic
GSH CONJUGATE
(?)
AFM1
AFL
cytosolic
GLUCURONIDE
AFL-M1

Figure 1.

MATERIALS AND METHODS

Animals

Sexually immature Shasta strain rainbow trout (130-250 g) were acclimated at 10°C or 18°C ±0.3 degrees for a minimum of 1 month in 300 l flow-through aquaria in a constant temperature room at Oak Creek Laboratory, Corvallis, Oregon. This time is sufficient to complete membrane changes associated with temperature acclimation (Hazel and Prosser, 1974). The fish were fed a ration of Oregon Test Diet (Sinnhuber et al., 1977) (2% body wt/day for 18°C and 1% body wt/day for 10°C) to maintain similar weight gains during the acclimation period. The photoperiod was held at 12 hr light and 12 hr dark throughout the acclimation period.

Chemicals

[^{3}H]AFB1 was obtained from Moravek Biochemicals (Brea, California) and unlabelled AFB1 from Sigma Chemical Company (St. Louis, MO). Purity was checked by TLC and HPLC. AFB1 biliary metabolite standards were a generous gift from Ms. Pat Loveland (Food Science, Oregon State University). Proteinase K, Tris, phenol, and SDS, utilized for DNA binding determinations were obtained from VWR (Philadelphia, PA). All other reagents were obtained from Sigma Chemical Company. All scintillation counting utilized protosol and aquasol (New England Nuclear, Wilmington, DE) and were counted on Packard Tricarb Counter (Model 1600).

Exposures

Fish were injected ip with [^{3}H]AFB1 (spec act: 17.66 or 12.38 nmols/uCi in ethanol or 34.23 nmols/uCi in salmon oil) at a dose of 0.4 mg AFB_1/kg fish after a 48 hr fast. Injections occurred at either the acclimation temperature (10°C or 18°C) or at an intermediate temperature of 14°C. Those injected at 14°C were shifted to that temperature 1 hr prior to injection from 10°C or 18°C. This time was previously shown to be adequate for the trout liver temperature to reach the environmental temperature (Kemp and Curtis, 1987). After injection, fish were held in static aerated aquaria with activated charcoal filters and water jacketed to maintain the desired temperature. All fish were killed 16 hr after injection (Shelton et al. 1986), dorsal vein blood samples taken with heparinized syringes, and the liver and gallbladder excised and separated.

Bile Metabolite Analysis

The gallbladder was placed in a preweighed beaker and the gallbladder broken and removed leaving the bile. The beaker and bile were weighed, aliquots taken for LSC, and the remainder frozen at -80°C until further analysis on a Spectra Physics HPLC equipped with a Phenomenex C18 reversed phase column. Bile was diluted 1:1 with MeOH, centrifuged to remove particulate, and injected directly onto the HPLC. A 45 min linear gradient of 10-60% methanol in 20mM potassium acetate (pH 5) was utilized. Because the metabolites did not absorb

ultraviolet light dependably, a uv detector was not employed in metabolite detection and quantification. Instead, one minute drop fractions were collected for LSC counting (quantification) and metabolites were identified by comparison with known radiolabeled standards.

Plasma Enzymes

Blood samples were centrifuged (500 g, 5min) and aliquots of plasma taken for LSC. In addition, aliquots were taken for measurement of the plasma enzymes glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT). Samples were kept in the refrigerator (maximum of 5 days) until analysis with Sigma transaminase kits. Uninjected, but acclimated and acute temperature shifted fish were utilized as controls.

Liver Analysis

Livers extracted from fish were weighed and homogenized in 25 ml buffer (0.25 M sucrose; 3 mM $MgCl_2$; 10 mM Tris, pH 7.2) with six strokes of a Potter Elvehjem homogenizer. The homogenate was centrifuged at 1000 g for 5 min to remove nuclei for DNA binding studies and the remaining supernatant centrifuged at 100,000 g for 90 minutes. The resulting pellet was resuspended in 2 mls of buffer (20% glycerol; 10 mM K_2EDTA; 0.1 M phosphate, Ph 7.25) and frozen at -80°C until protein binding analysis.

Microsomal Protein Binding

The following procedure was adopted from Sun and Dent (1980). Protein contents for aliquots of resuspended microsomes from AFB1 injected fish were measured by the Bradford method (1976) and the remaining microsomal suspension made 2% SDS and boiled 10 min. After boiling, the suspension was transferred to Spectrapor 3 dialysis bags and exhaustively dialyzed against three 1000 ml volumes of dialysis buffer (0.01 M sodium phosphate, pH 7.0; 0.1% (w/v) SDS). The final volume of dialysis buffer contained negligable radioactivity.

DNA Binding

The liver nuclear pellet was resuspended in 1.5 ml of DNA buffer (0.1 M sodium chloride; 50 mM Sodium-4-aminosalicylate, pH 7.2) with six strokes of a Potter Elvehjem homogenizer. The homogenate was lysed by vortexing in 1.5 ml lysis buffer (2% SDS, 2% sodium chloride, 12% sodium-4-aminosalicylate, 12% 2-butanol). The lysate was shaken for 1 hr on a rotary shaker and an additional hour after the addition of 1.5 ml IAC (5% isoamyl alcohol, 96% chloroform) and 1.5ml of phenol which was presaturated with equal volumes of 1 M Tris and phenol saturation solution (300 mM NaCl; 100 mM Tris, pH 8.0). The resulting solution was centrifuged at 3000 g for 15 min and the upper aqueous layer aspirated and extracted twice with equal volumes of IAC. The DNA was precipitated with 0.4 ml of 5 M sodium perchlorate and 3 volumes cold ethanol and subsequently centrifuged at high speed for 15 min. The DNA

pellet was washed with ethanol. After being redissolved in 1.5 ml of DNA buffer for 24 hr, the nucleic acids and proteins were digested for 1 hr at 37°C with 100 ul RNase (RNase A 50mg/ml Sigma R-5000; RNase 5000 units/ml Sigma R-8251) and an additional 2 hrs at 37°C with 10 ul of proteinase k (20 mg/ml). The resulting mixture was extracted with 1.5 ml Tris saturated phenol and 1.5ml IAC, the DNA pelleted by high speed centrifugation for 3 min, and the lower phenol layer discarded. After extracting the remaining layer 3 times with IAC, the DNA was precipitated with 0.4 ml of 5M sodium perchlorate and 3 volumes cold ethanol and pelleted by centrifugation. The pellet was washed with 75% ethanol followed by 95% ethanol and redissolved in distilled water. Aliquots were hydrolyzed by heating 20 min in 1 M perchloric acid at 70°C, counted for radioactivity and analyzed for DNA by the method of Burton (1956). Briefly, the hydrolyzed DNA and DNA standards were incubated at 30°C in the dark for 18 hrs with 0.5 M perchloric acid and Burton reagent (diphenylamine, glacial acetic acid, concentrated sulfuric acid, 1.6% acetaldehyde 1.5/100/1.5/.5, w/v/v/v) then read at 600 nm on a Beckman 35 spectrophotometer.

Statistics

Except for DNA and protein binding all data was analyzed by Kruskal-Wallis analysis of variance by ranks (Conover, 1980) with the significance level set at $P \leq 0.05$. DNA and protein binding was analyzed by the F test (alpha = 95%).

Data analysis consisted of paired comparisons between the $10^{\circ}C$-$10^{\circ}C$ and $18^{\circ}C$-$18^{\circ}C$ temperature regimens to assess the notion of ideal temperature compensation and between $10^{\circ}C$-$14^{\circ}C$ and $18^{\circ}C$-$14^{\circ}C$ to determine the effect of acclimation temperature.

RESULTS

Vehicles

The UV and radiochemical purities of the AFB1 mixture (labelled and unlabelled) prior to dissolution in salmon oil were 99% and 92%, respectively. However, at a later date after injections, the AFB1 in salmon oil was found to have 78% UV purity and 74% radiochemical purity. All lost UV and radioactive purity was in 1 new peak not corresponding to aflatoxin M_1 or aflatoxicol. The AFB1 mixture in ethanol utilized for injections was of similar uv and radiochemical purity to the above (prior to dissolution in salmon oil) and eluted from the HPLC column as a single UV and radioactive peak. Therefore, the AFB1 in salmon oil was contaminated, but based on the similarities of the plasma enzyme and DNA binding data between the two vehicles, we feel the contamination in the salmon oil did not adversely influence our results (detailed below).

Distribution

Kruskal-Wallis analysis of variance by ranks indicated a vehicle dependent difference in plasma concentration of [^{3}H]AFB1 (Table 1) after 16 hrs ($P<0.05$) at all temperature regimens. The salmon oil exhibited a minimum two-fold elevation compared to ethanol at all temperature regimens. Temperature regimens did not effect plasma concentrations of [^{3}H]AFB1 with the exception of the 10°C-14°C ethanol vehicle

Table 1. Disposition of [3H]AFB1 in rainbow trout 16 hr after ip injection in different vehicles. Plasma values are statistically different between oil and ethanol injected fish at all temperature regimens ($P < .05$).

		Temperature Regimen[a]			
		18-18	*10-10*	*10-14*	*18-14*
PLASMA nmols AFB1/ ml plasma	Ethanol	0.14 ± 0.04 (3)	0.18 ± 0.03 (5)	0.17 ± 0.01* (3)	0.27 ± 0.02 (3)
	Oil	0.46 ± 0.06 (5)	0.46 ± 0.08 (3)	0.36 ± 0.12 (3)	0.54 ± 0.07 (5)
LIVER nmols AFB1/ g liver	Ethanol	16.23 ± 1.41 (3)	14.17 ± 1.05 (3)	13.50 ± 3.35 (3)	14.81 ± 2.83 (3)
	Oil	16.57 ± 2.05 (5)	18.69 ± 2.39 (4)	22.07 ± 2.48 (4)	22.70 ± 2.90 (5)
BILE nmols AFB1/ ml bile	Ethanol	38.45 ± 11.79 (3)	55.9 ± 5.27 (3)	50.69 ± 9.62 (3)	52.12 ± 4.40** (3)
	Oil	41.58 ± 9.74 (4)	64.32 ± 12.82 (5)	75.63 ± 9.82 (4)	84.22 ± 17.16 (5)

[a] Results are means ± SE for (n) individual fish for each temperature regimen.

*Statistically different than 18-14 ethanol injected fish ($P = 0.05$)

**Statistically different than 18-14 oil injected fish ($P = 0.05$)

fish having significantly less [^{3}H]AFB1 than the 18°C-14°C ethanol vehicle fish (P=0.05) (Table 1).

Concentrations of [^{3}H]AFB1 in the gallbladder bile varied between 42-76 nmols AFB1/ml bile; however, no temperature and vehicle effects were seen except in the 18°C-14°C fish where the bile concentration was higher in the oil injected fish compared to the ethanol injected fish (P=0.05) (Table 1). These data were not conclusive due to apparent gallbladder voiding in some fish. Gallbladder voiding may have been caused by an apparent loss of osmoregulatory control, as fish tended to gain weight after injection. Hepatic concentrations of [^{3}H]AFB1 also showed no temperature or vehicle effects varying between 15-20 nmols AFB1/g liver (Table 1).

DNA and Protein Binding

DNA and protein binding were dependent upon the hepatic concentration of [^{3}H]AFB1 which was highly variable. Therefore, DNA and protein binding were plotted against [^{3}H]AFB1 liver concentration for individual fish, a simple linear regression line was fitted to the data, and the equation of the line used for statistical comparison utilizing the F test statistic. The correlation coefficients for the regression lines of the 10°C-10°C and 18°C-18°C fish were 0.87 and 0.73 respectively (Fig. 2). Statistical comparison of these two lines indicate they are not different (F=0.76). The 10°C acclimated fish shifted to 14°C showed very good linearity between hepatic concentration of [^{3}H]AFB1 and DNA binding

Figure 2. DNA binding vs. hepatic AFB1 concentration. Upper panel represents those fish acclimated and tested at 18°C and lower panel those acclimated and tested at 10°C. Each point indicates 1 fish. Each line was calculated by least squares estimation.

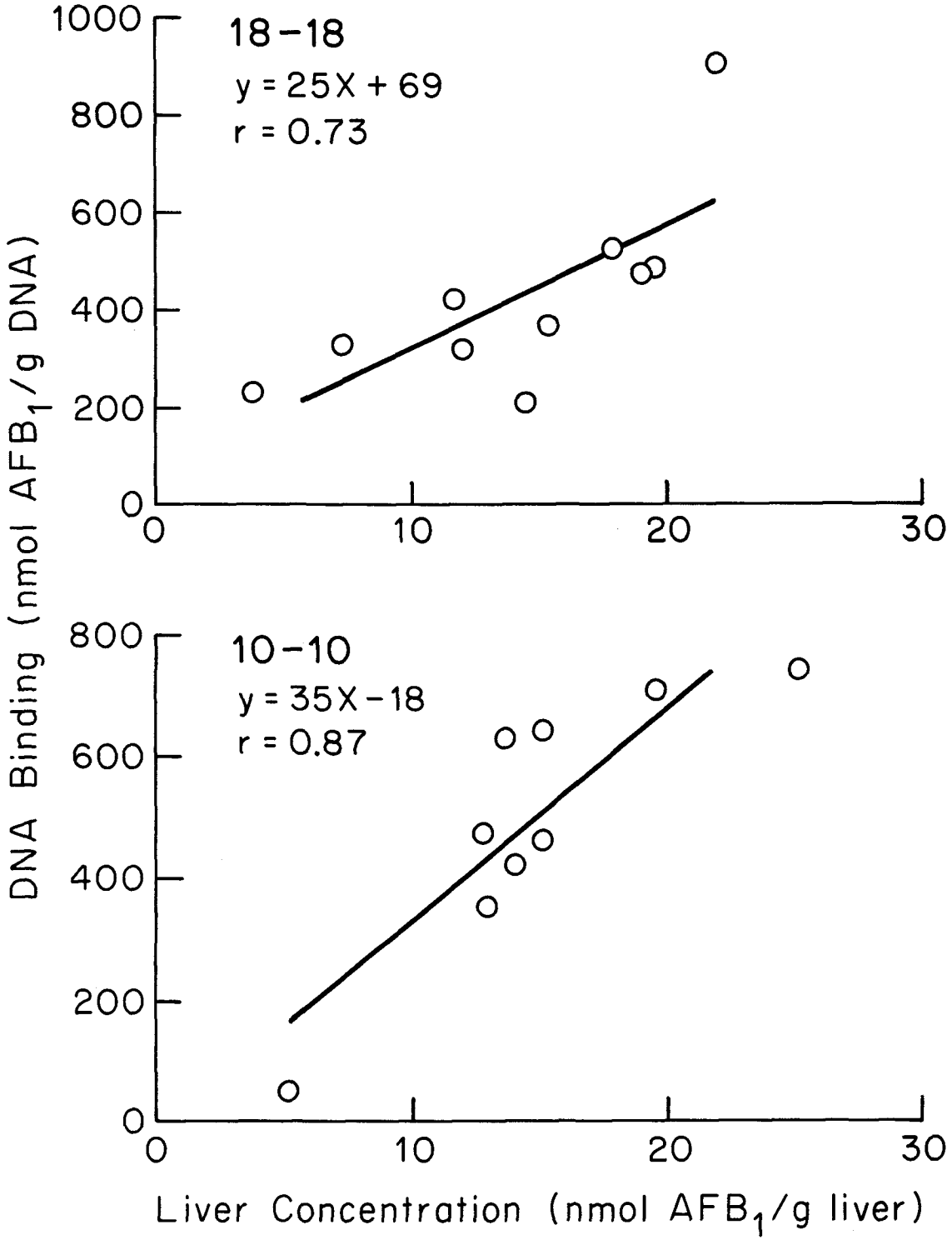
18-18
y = 25X + 69
r = 0.73
10-10
y = 35X - 18
r = 0.87
DNA Binding (nmol AFB1/g DNA)
Liver Concentration (nmol AFB1/g liver)
0
200
400
600
800
1000
10
20
30

Figure 2.

(r=0.95); however, the 18°C acclimated fish shifted to 14°C showed very poor correlation between hepatic concentration of [^{3}H]AFB1 and DNA binding (r=0.34)(Fig. 3). We interpreted these results as an indication that DNA binding is different between these two groups and statistical analysis of the representative lines supported this contention (F=3.89).

At all temperature regimens the correlation coefficients for the regression lines between hepatic concentration and protein binding were greater than r=0.65 (Table 2). No difference in protein binding between the 18°C-18°C and 10°C-10°C fish was seen (F=0.59); in addition, the temperature shifted fish showed no difference in protein binding (F=0.78) unlike DNA binding.

Biliary Metabolic Profiles

The two primary biliary metabolites seen were aflatoxicol glucuronide and aflatoxicol-M1 glucuronide and an unknown metabolite (Fig. 4). Each metabolite had a respective capacity factor (k') of 4.25 (unknown), 6.00 (aflatoxicol-M_1), and 8.50 (aflatoxicol glucuronide).

No temperature regimen significantly altered the biliary profile of AFB1 metabolites (Fig. 5). Aflatoxicol glucuronide made up between 59-69% of the total metabolites recovered, aflatoxicol-M_1 glucuronide 22-29%, and the unknown metabolite between 9-16%. Typically, 90% of the radioactivity introduced onto the HPLC was recovered in the fractions collected.

Figure 3. DNA binding vs. hepatic AFB1 concentration. Upper panel represents the fish acclimated at 18.0°C and shifted to 14.0°C 1 hr. prior to injection and the lower panel represents the fish acclimated at 10.0°C and shifted to 14.0°C 1 hr. prior to injection. Each point indicates 1 fish. Each line was calculated by least squares estimation.

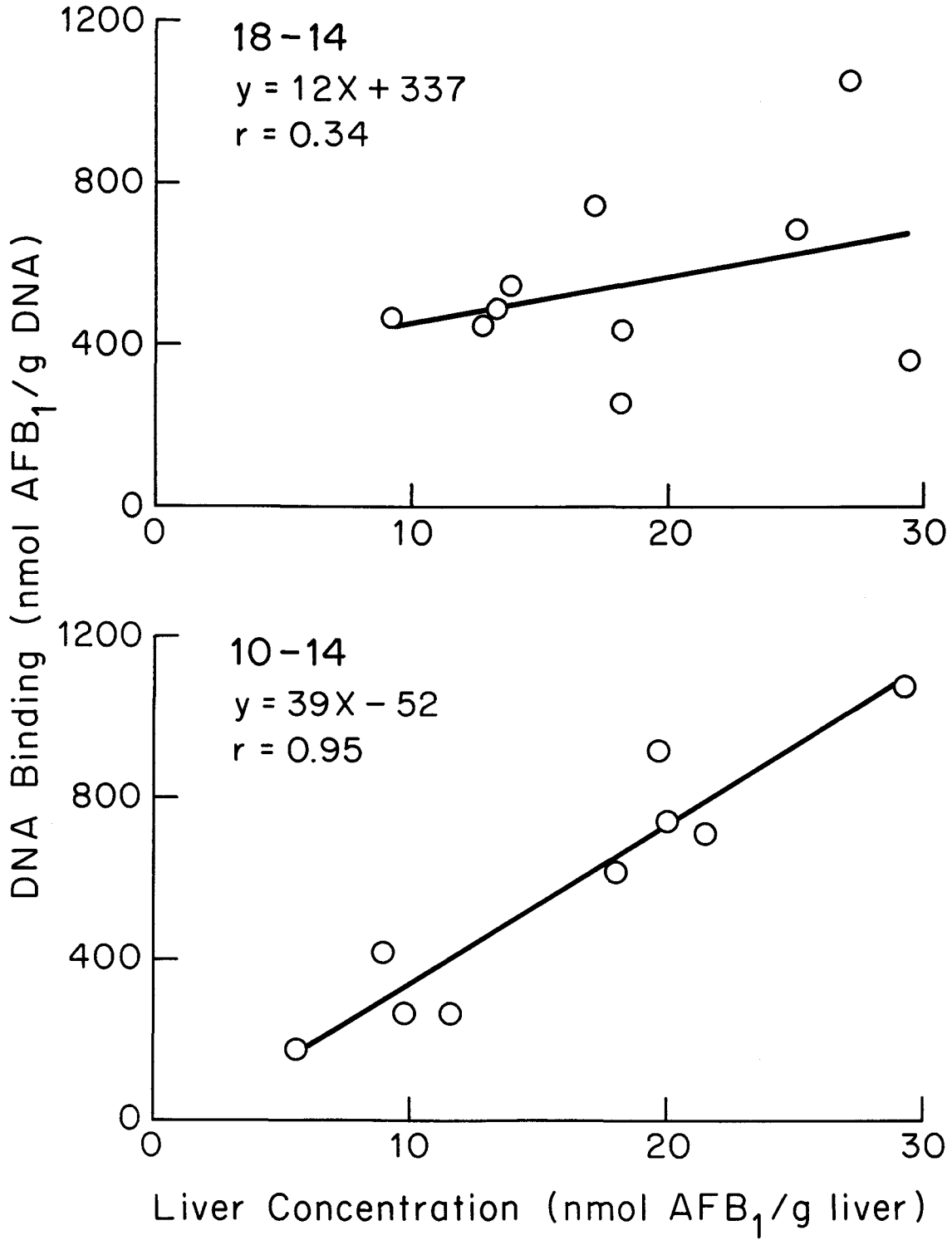
18-14
y = 12X + 337
r = 0.34
10-14
y = 39X - 52
r = 0.95
DNA Binding (nmol AFB1/g DNA)
Liver Concentration (nmol AFB1/g liver)
0
400
800
1200
0
10
20
30

Figure 3.

Table 2. Linear regression equations and correlation coefficients for hepatic AFB1 concentrations vs. protein binding. No statistical differences were seen between the lines. N = 3 for each temperature regimen.

Temperature Regimen

	18-18	*10-10*	*10-14*	*18-14*
Linear Regression Equation	y=5.8X+18.8	y=9.2X-57.4	y=6.6X+20.9	y=6.7X-12.1
Correlation Coefficient (r)	.65	.98	.83	.77

Figure 4. Representative AFB1 biliary metabolic profile from injected fish. Each point represents a 1 min fraction collected and counted by LSC. Metabolites identified by comparison with known standards.

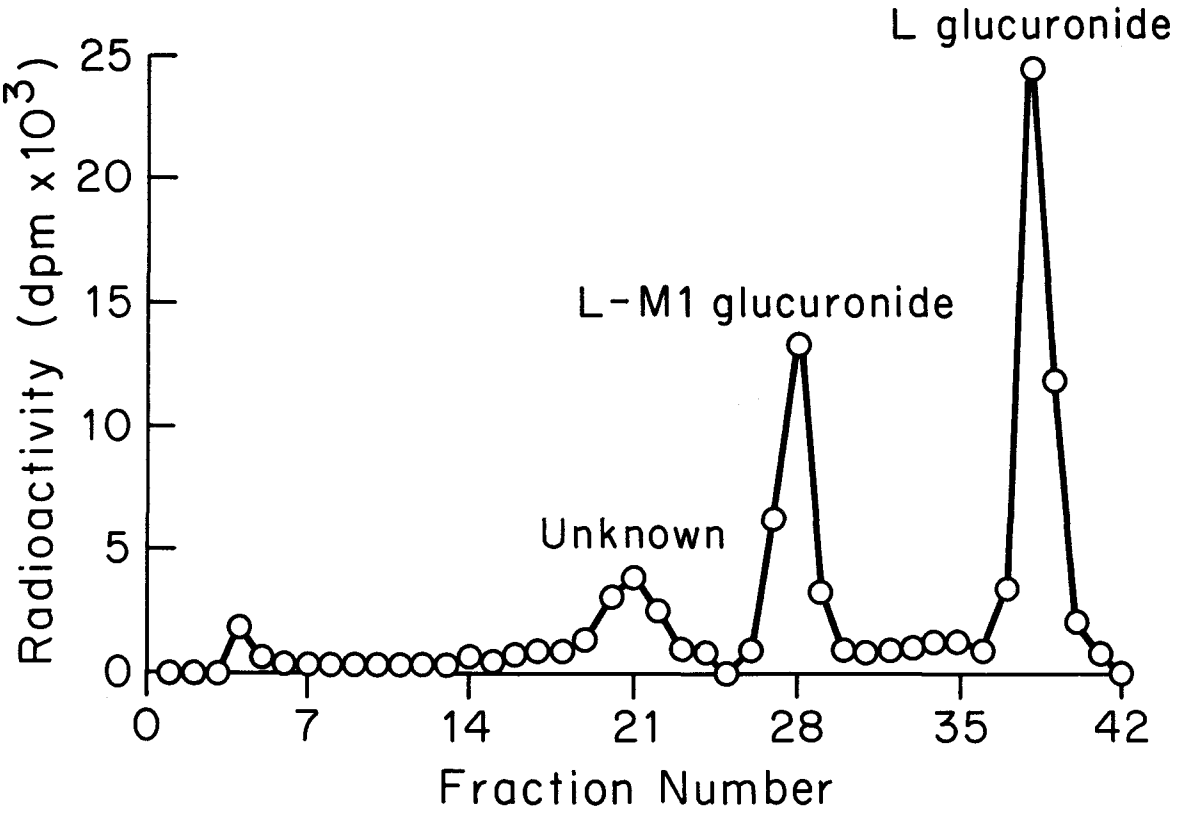
L glucuronide
L-M1 glucuronide
Unknown
Radioactivity (dpm x10³)
25
20
15
10
5
0
0
7
14
21
28
35
42
Fraction Number

Figure 4.

Figure 5. Percentage of each AFB1 biliary metabolite at each temperature regimen. No temperature dependent differences were seen in metabolite production. Values are mean$\pm$SE with N as follows: 10.0°C-10.0°C (6); 18.0°C-18.0°C (5); 18.0°C-14.0°C (4); 10.0°C-14.0°C (8).

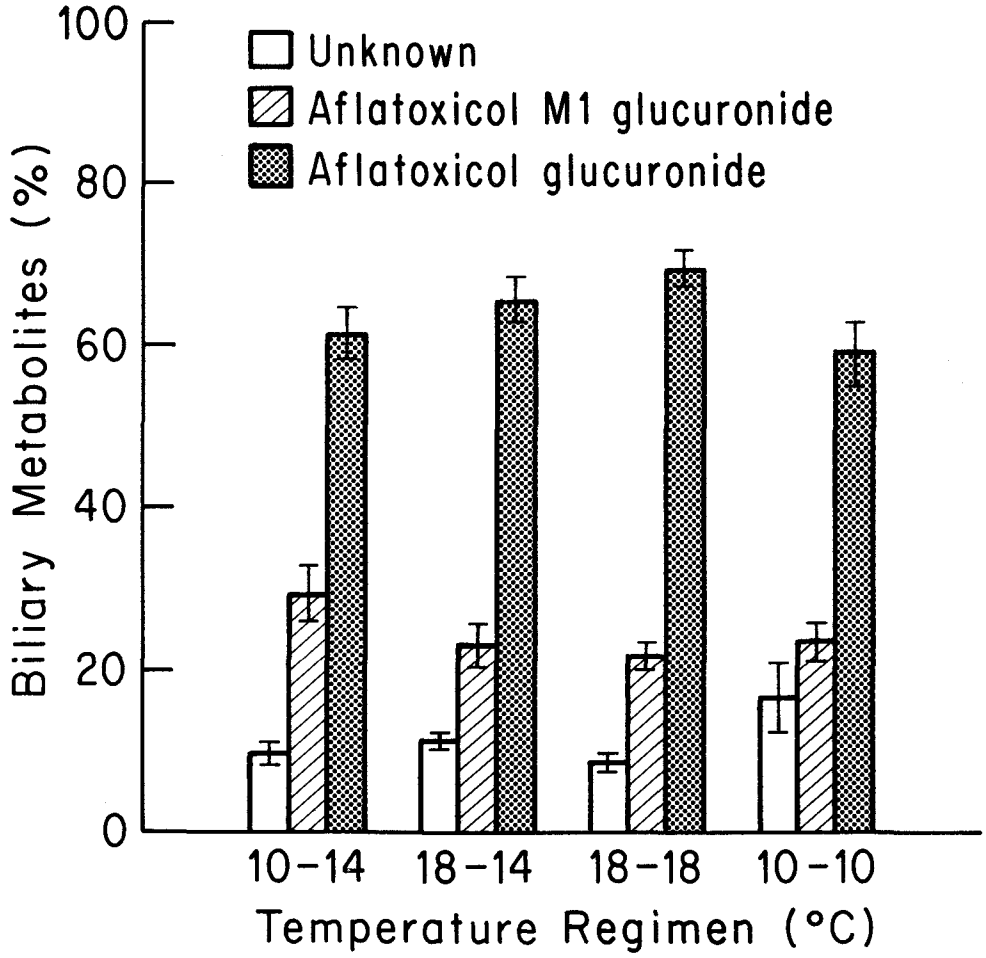
100
80
60
40
20
0
Biliary Metabolites (%)
Unknown
Aflatoxicol M1 glucuronide
Aflatoxicol glucuronide
10-14
18-14
18-18
10-10
Temperature Regimen (°C)

Figure 5.

Plasma Transaminases

Both GOT and GPT plasma levels were significantly higher in AFB1 injected fish than controls at all temperature regimens (GOT: $0.01>P>0.001$; GPT: $0.008>P>0.003$) regardless of vehicle.(Table 3). Because acclimation temperature and acute temperature shifts had no effect on plasma enzyme control levels the data was pooled. Similar levels of both enzymes were seen in the plasma of fish shifted from 10°C to 14°C and fish shifted from 18°C to 14°C. The fish acclimated and tested at their respective acclimation temperature (10.0°C or 18.0°C) also showed similar levels, to each other, of both enzymes in the plasma.

Table 3. Plasma GOT and GPT activities 16 hr after ip injection of [3H]AFB1 in various temperature regimens. All GOT values statistically higher than controls (.01>P>.002). All GPT values statistically higher than controls (.008>P>.003).

	Temperature Regimen[a]				
	Control	*18-18*	*10-10*	*10-14*	*18-14*
GOT	121 ± 13 (6)	188 ± 12 (9)	195 ± 14 (5)	208 ± 5 (9)	206 ± 5 (7)
GPT	10 ± 2 (6)	33 ± 9 (7)	22 ± 3 (6)	27 ± 1 (6)	28 ± 4 (7)

[a] Results are means ± SE for (n) individual fish for each regimen.

DISCUSSION

A vehicle dependent difference in AFB1 plasma concentrations occurred 16 hr after injection of the fish. Similar increases in the hepatic and biliary concentrations of AFB1 were also seen in the oil injected fish compared to the ethanol injected fish; however, statistically significant differences were seen only in the AFB1 biliary concentrations in 18°C acclimated fish shifted to 14°C. This vehicle dependent difference in distribution was probably due to slower absorption of salmon oil than ethanol from the peritoneal cavity. Salmon oil perhaps acted as a reservoir for the AFB1, although time course studies were not conducted. In addition, this difference was possibly related to the presence of the AFB1 complex or reaction product present in the salmon oil formulation (Results).

Currently, chemical carcinogenesis is believed to be a multistage process including initiation, promotion, and progression. Initiation includes the covalent binding of reactive compounds (xenobiotics) to sites on the DNA complex altering the genomic sequence for specific proteins. The expression of the genomic abberations and appearance of tumors encompasses the promotion and progression phases of carcinogenesis (Farber, 1982). AFB1 is a potent hepatocarcinogen in rainbow trout and is activated to a reactive epoxide intermediate (Wogan, 1973) by the P-450 LM2

isozyme (Williams and Buhler, 1983) which binds to specific sites on DNA (Marien et al., 1987). We found that fish acclimated and tested at their respective acclimation temperatures showed no difference in DNA binding thus exhibiting ideal temperature compensation. However, thermal acclimation followed by acute temperature shifts altered the amount of hepatic DNA binding of AFB1. As liver concentration of AFB1 increased, DNA binding increased linearly with a steep slope in fish acclimated at 10°C and shifted to 14°C. Whereas, DNA binding in fish acclimated at 18°C and shifted to 14°C demonstrated no linear increase in DNA binding with increasing hepatic concentration. We interpret this as a protective effect of this temperature regimen on DNA binding. In other words, more [^{3}H]AFB1 adduction to hepatic DNA occurred after acute temperature shift from 10°C to 14°C. Such data, taken in the context of molecular dosimetry (Dashwood et al.,1989), would suggest increased incidence of hepatic tumors in fish shifted from 10°C to 14°C compared to fish shifted from 18°C-14°C. This may partially explain the apparent temperature dependent increase of hepatocarcinomas in medaka (Oryzias latipes) (Kyono-Hamaguchi, 1984).

Increased DNA binding suggests a greater quantity of AFB1 being converted to the reactive epoxide by the LM2 isozyme. Due to the highly reactive nature of the epoxide we could not study it's production directly in intact animals, so binding

to cellular macromolecules were utilized as surrogates. In addition, biliary metabolites were studied to compare activities of AFB1 detoxication pathways in temperature acclimated and shifted fish.

Similar to that reported by Loveland et al. (1984), the major biliary metabolites we found were aflatoxicol glucuronide and aflatoxicol-M_1 glucuronide. The metabolic profiles of [^{3}H]AFB1 in the trout we studied indicate that the different temperature regimens did not effect production of these two metabolites. This was somewhat unexpected based on previous work. Hepatic microsomes from cold acclimated fish have greater aryl hydrocarbon hydroxylase activity (largely P4501A1 activity) at a common intermediate in vitro assay temperature than those from warm acclimated fish (Carpenter et al., 1990). In addition, immunoquantitation indicates that 10.0°C acclimated fish have about one-third more P4501A1 than 18.0°C acclimated fish (Carpenter et al., 1990). Since aflatoxicol-M_1 production is partially dependent on P4501A1 activity we would expect more aflatoxicol-M_1 glucuronide in the bile of 10°C acclimated fish compared to 18°C acclimated fish. This contradiction between in vivo and in vitro results has two possible explanations.

The major AFB1 metabolites produced by trout liver are aflatoxicol (Schoenhard et al., 1976) and aflatoxin-M_1 (Loveland et al., 1979). As mentioned previously, the two major metabolites in the bile of trout are glucuronides of

aflatoxicol-M_1 and aflatoxicol (Loveland et al., 1984). Aflatoxin-M_1 apparently is not a good substrate for UDP-glucuronosyltransferase (UDPGT) (Loveland et al., 1984) and does not occur in the bile. Therefore, both biliary metabolites require the enzymatic activity of a cytosolic dehydrogenase to form the aflatoxicol moiety (Fig. 1) and any temperature dependent changes in the formation of aflatoxin-M_1 (membrane bound P4501A1 activity) may be masked.

Unpublished results from this lab indicate that UDPGT has a higher specific activity in fish acclimated at 18°C versus 10°C when tested at 14°C *in vitro*, and Kiovusaari et al. (1981) report hepatic microsomal UDPGT activity from fish acclimated at cold temperatures and tested *in vitro* at that temperature have far less specific activity compared to those that are warm acclimated and tested at their acclimation temperature. In the current study; however, *in vivo* glucuronidation was neither affected by acclimation temperature or acute temperature shifts. This may indicate that *in vivo* glucuronidation was operating well below maximal rates as is not the case in *in vitro* assays where substrate concentrations may be much higher. Also, since the enzyme doesn't follow ideal temperature compensation patterns it may be masking any temperature dependent differences in aflatoxin-M_1 formation.

Two explanations for our results are then plausible: either temperature has no effect on the *in vivo* metabolic pathways studied, in which case *in vitro* results should be

carefully extrapolated to intact animals, or the cytosolic dehydrogenase and/or UDPGT activity is masking any temperature dependent differences in biliary metabolite formation.

Elevated plasma levels of GOT and GPT are strong indicators of soft tissue damage. Increased GPT levels indicate liver necrosis (Routh, 1970) while elevated GOT levels dictate other soft tissue damage including myocardial infarction (Chinsky et al., 1956). Both transaminases were significantly elevated in fish at all temperature regimens compared to controls; however, proportionally GOT had greater increases than GPT. This may mean that soft tissue other than the target organ for AFB1, the liver, may recieve greater acute damage. Histopathology of the kidneys 16 hr after AFB1 injections indicate no frank necrosis at any temperature regimen (data not shown); however, frank liver necrosis is also absent at this time (Nunez, 1990). In addition, Lorenzana et al. (1988) demonstrated the presence of the cytochrome P-450 LM2 isozyme (metabolic activation of AFB1) in the kidney, presumably capable of forming reactive epoxides. This evidence leads us to speculate that AFB1 induced nephrotoxicity may contribute to plasma enzyme elevation although it is unconfirmed.

We have shown that temperature acclimation followed by acute temperature shifts of rainbow trout alters the degree of hepatic DNA binding of an administered dose of AFB1. Although biliary metabolic profiles did not indicate any

temperature dependent differences in AFB1 detoxication pathways, the temperature dependent differences in DNA binding is probably due to different rates of metabolic activation of AFB1 to the reactive epoxide. All data from fish exposed at their respective acclimation temperature (10°C or 18°C) were similar supporting the theory of ideal temperature compensation in poikilotherms. Although we were unable to resolve the mechanism by which acclimation temperature and acute temperature shifts alter AFB1 genotoxicity , the rainbow trout thermal acclimation model is a useful nonmammalian approach for studying xenobiotic action without the use of chemical pretreatment.

BIBLIOGRAPHY

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72,248-254.

Burton, L. (1956). A study of the conditions and mechanisms of the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62,315-322.

Cambell, T.C., and Hayes, J.R. (1976). The role of aflatoxin metabolism in its toxic lesion. Toxicol. and Appl. Pharmacol. 35, 199-222.

Carpenter, H.M., Fredrickson, L.S., Williams, D.E., Buhler, D.R., and Curtis, L.R. (1990). The effect of thermal acclimation on the activity of arylhydrocarbon hydroxylase in rainbow trout (Oncorhynchus mykiss). Comp. Biochem. Physiol. In press.

Chinsky, M., Shmagranoff, G.L., Sherry, S. (1956). Serum transaminase activity. J. Lab. Clin. Med. 47, 108-113.

Conover, W.J. (1980). Practical nonparametric statistics. 2nd ed. John Wiley and sons, New York, NY.

Curtis,L.R. (1983). Glucuronidation and biliary excretion of phenolphthalein in temperature-acclimated steelhead trout (Salmo gairdneri). Comp. Biochem. Physiol. 76C, 107-111.

Curtis, L.R., Fredrickson, L.K., and Carpenter, H.M. (1990). Biliary excretion appears rate limiting for hepatic elimination of benzo[a]pyrene by temperature acclimated rainbow trout. Fund. Appl. Toxicol. In press.

Dashwood, R.H., Arbogast, D.N., Fong, A.T., Pereira, C., Hendricks, J.D., and Bailey, G.S. (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.

Farber, E. (1982). Chemical carcinogenesis, a biological perspective. Am. J. Pathol. 106,271-296.

Hanzlik, R.P. (1981). Reactivity and toxicity among halogenated methanes and related compounds. Biochem. Pharmacol. 30, 3027-3030.

Hazel, J.R., and Prosser, C.L. (1974). Molecular mechanisms of temperature compensation in poikilotherms. Physiol. Rev. 54,620-677.

Hazel. J.R. (1979). Influence of thermal acclimation on membrane lipid composition of rainbow trout liver. Am. J. Physiol. 236,R91-R101.

Kemp, C.J. and Curtis, L.R. (1987). Thermally modulated biliary excretion of [^{14}C]taurocholate in rainbow trout (Salmo gairdneri) and the Na^+,K^+-ATPase. Canadian L. of Fish. and Aqua. Sci. 44, 846-851.

Kiovusaari, U., Harri, M., Hanninen, O. (1981). Seasonal variation of hepatic biotransformation in female and male rainbow trout (Salmo gairdneri). Comp. Biochem. Physiol. 70C, 149-157.

Kyono-Hamaguchi, Y. (1984). Effects of temperatures and partial hepatectomy on the induction of liver tumors in Oryzias latipes. Natl. Cancer Inst. Monogr. 65,337-344.

Lorenzana, R.M., Hedstrom, O.R., and Buhler, D.R. (1988). Localization of cytochrome P-450 in the head and trunk kidney of rainbow trout (Salmo gairdneri). Toxicol. and Appl. Pharmacol. 96,159-167.

Loveland, P.M., Sinnhuber, R.O., Berggren, K.E., Libbey, L.M., Nixon, J.E., and Pawlowski, N.E. (1977). Formation of aflatoxin B_1 from aflatoxicol by rainbow trout (Salmo gairdneri) liver in vitro. Res. Comm. in Chem. Path. and Pharm. 16,167-170.

Loveland, P.M., Nixon, J.E., Pawlowski, N.E., Eisele, T.A., Libbey, L.M., and Sinnhuber, R.O. (1979). Aflatoxin B_1 and aflatoxicol metabolism in rainbow trout (Salmo gairdneri) and the effects of dietary cyclopropene. J. Envir. Pathol. Toxicol. 2,707-718.

Loveland, P.M., Coulombe, R.A., Libbey, L.M., Pawlowski, N.E., Sinnhuber, R.O., Nixon, J.E., and Bailey, G.S. (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 B-naphthoflavone. Food Chem. Toxicol. 21,557-562.

Loveland, P.M., Nixon, J.E., and Bailey, G.S. (1984). Glucuronides in bile of rainbow trout (Salmo gairdneri) injected with [^{3}H]aflatoxin B_1 and the effects of dietary B-naphthoflavone. Comp. Biochem. Physiol. 78C,13-19.

Malins, D.C., Krahn, M.M., Brown, D.W., Rhodes, L.D., Myers, M.S., McCain, B.B., and Chan, Sin-Lam. (1985). Toxic chemicals in marine sediment and biota from Mukilteo, Washington:relationships with hepatic neoplasms and other hepatic lesions in english sole (Parophrys vetulus). J. Natl. Cancer. Inst. 74,487-494.

Marien, K., Moyer, R., Loveland, P., Van Holde, K., and Bailey, G. (1987). Comparative binding and sequence interaction specificities of aflatoxin B_1, aflatoxicol, aflatoxin M_1, and aflatoxicol M_1 with purified DNA. J. Biol. Chem. 262,7455-7462.

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

Nunez, O. (1990). The progression of aflatoxin B_1 hepatocarcinogenesis in rainbow trout (Oncorhynchus mykiss): 1. AFB_1 metabolism and cytotoxicity affecting hepatocarcinogenesis. 2. Ultrastructure of hepatocellular neoplasms. Masters Thesis, Oregon State University.

Routh, J.I. (1970). Liver function tests. In Fundamentals of Clinical Chemistry. NW Tietz, editor, Saunders, Philadelphia. 788.

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

Schoenhard, G.L., Lee, D.J., Howell, S.E., Pawlowski, N.E., Libbey, L.M., and Sinnhuber, R.O. (1976). Aflatoxin B_1 metabolism to aflatoxicol and derivatives lethal to Bacillus subtilis GSY 1057 by rainbow trout (Salmo gairdneri) liver. Cancer Res. 36,2040-2045.

Shelton, D.W., Goeger, J.D., Hendricks, J.D., and Bailey, G.S. (1986). Mechanisms of anti-carcinogenesis: the distribution and metabolism of aflatoxin B_1 in rainbow trout fed aroclor 1254. Carcinogenesis. 7,1065-1071.

Sinensky, M. (1974). Homeoviscous adaptation - A homeostatic process that regulates the viscosity of membrane lipids in Escherichia coli. Proc. Nat. Acad. Sci. 71,522-525.

Sinnhuber, R.O., Hendricks, J.D., Wales, J.H., and Putnam, G.B. (1977). Neoplasms in rainbow trout, a sensitive animal model for environmental carcinogenesis. Ann. N.Y. Acad. Sci. 298,389-408.

Sun, J.D., and Dent, J.G. (1980). A new method for measuring covalent binding of chemicals to cellular macromolecules. Chem. Biol. Interactions. 32,41-61.

Williams, D.E. and Buhler, D.R. (1983). Purified form of cytochrome P-450 from rainbow trout with high activity toward conversion of aflatoxin B_1 to aflatoxin B1-2,3-epoxide. Cancer Res. 43,4752-4756.

Wogan, G.N. (1973). Aflatoxin carcinogenesis methods. Cancer Res. 7,309-344.