

AN ABSTRACT OF THE THESIS OF

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Modification in Rainbow Trout (Salmo gairdneri)

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Aflatoxin B₁ (AFB₁) is a mold-produced toxin which has been shown to be a potent hepatocarcinogen in many animal species. Of the species studied thus far, rainbow trout have proven to be the most sensitive. Experiments were conducted to investigate various aspects of AFB₁ metabolism in this species, including in vitro mutagenesis, and effects of dietary modifiers of AFB₁ carcinogenesis on in vitro metabolism and mutagenesis. A comparative study of AFB₁ metabolism in two salmonid species was also conducted.

In the first study, the relative mutagenic potencies of several aflatoxin metabolites were evaluated using a trout liver fraction system. Preliminary studies characterizing trout liver fractions for use as an activation system were described. The results from comparative mutagenicity experiments demonstrated that in vitro mutagenic potencies qualitatively correlated with the in vivo carcinogenic activities of various aflatoxins in rainbow trout. The importance of these findings is discussed.

In the second study fish hepatocytes were characterized to examine possible differences in activation of AFB₁ to bacterial mutagens by hepatocytes from rainbow trout and coho salmon, two species which are known to differ markedly in sensitivity to the carcinogenic effects of AFB₁. Activation efficiency was approximately three times greater in hepatocytes from trout compared to salmon. A more marked difference was seen when S20 liver fractions from the two species were used. Analysis of unbound [³H]AFB₁ metabolites revealed that trout hepatocytes metabolized [³H]AFB₁ to a greater extent than salmon. The results accurately reflected in vivo carcinogenesis trends in salmonid fish.

Additional experiments were conducted to evaluate the effects of dietary modifiers of AFB₁ carcinogenesis on in vitro mutagenesis and metabolism of AFB₁:

Dietary β-naphthoflavone (β-NF) was shown to induce the production of a novel trout metabolite of AFB₁, aflatoxicol M₁ (AFL-M₁). AFL-M₁ exhibited a mutagenic potency less than AFB₁ or aflatoxicol (AFL), but greater than that of aflatoxin-M₁ (AFM₁). Dietary β-NF, however, appeared to have no effect on in vitro mutagenic activation of AFB₁ using hepatocytes or liver S20 fraction from trout.

Dietary PCBs (Aroclor 1254) was shown to significantly decrease in vitro mutagenesis of AFB₁, which reflected a similar PCB-mediated inhibitory effect on AFB₁ carcinogenesis in trout in vivo.

Cyclopropenoid fatty acids (CPFAs) present in the diet (0-600 ppm) were shown to have no effect on in vitro mutagenesis of AFB₁,

indicating CPFAs may not significantly alter in vivo initiation of AFB₁ carcinogenesis.

Aflatoxin Mutagenesis and Metabolism and their Dietary
Modification in Rainbow Trout (Salmo gairdneri)

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To Roger Nickolas and Rebecca

"And whatever you do in word or deed, do all in the name of the Lord Jesus.."

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LITERATURE REVIEW

Xenobiotic metabolism and carcinogenesis in fish

Until recently, it was generally regarded that aquatic species lacked the enzyme systems required for metabolizing xenobiotics (Brodie and Maickel, 1962). However, upon the discovery of bioaccumulation of xenobiotics and their metabolites in fish from diverse aquatic environments, much emphasis has been directed toward studying biotransformation in aquatic species. The extensive similarities of in vivo biotransformation reactions demonstrated in fish species relative to those in mammals have been thoroughly reviewed (Franklin, et al., 1980; Lech and Bend, 1980; Bend and James, 1979; Ahokas, 1977). These reactions include classical phase I conversions such as O and N-dealkylation, azo and nitro reductions, N-demethylation, epoxidation, hydroxylation and aryl hydrocarbon hydroxylase activity. Phase II synthetic reactions such as acetylations and methylations as well as glucuronide, glycine, sulfate, taurine and glutathione conjugations have also been demonstrated.

The impetus underlying the recent interest in aquatic toxicology has resulted in large part from epizootics of liver cancer in domesticated rainbow trout (Salmo gairdneri), especially those which

were later linked to aflatoxin (AF)*-contaminated rations (Rucker, et al., 1961). As a direct outgrowth of these outbreaks, and subsequent observations of the extreme sensitivity of this species to AFs, rainbow trout soon emerged as an animal model well suited for the study of chemical carcinogenesis (Sinnhuber et al., 1977; Hendricks, 1982).

The mixed-function oxidase system

Although some carcinogens are potent alkylating or acylating agents and can, without metabolic conversions, react directly with cellular macromolecules, procarcinogens require metabolic transformation to the active compound, the ultimate carcinogen (Miller, 1970; Heidelberger, 1973). Procarcinogens, as well as many drugs, xenobiotics and endogenous steroids are metabolized by the hepatic mixed-function oxidase system (MFO), also known as the drug metabolizing enzyme system or the monooxygenase system. The mammalian MFO system was elucidated about 25 years ago, when initial studies on hepatic microsomes revealed a CO-binding pigment (Klingenberg, 1958) which was later shown to be responsible for the hydroxylation of steroids and the oxidative demethylation and hydroxylation of drugs (Estabrook et al., 1963; Omura et al., 1965). This pigment, cytochrome P-450, which is identified spectrally as the CO complex, is an

*Abbreviations: AFs, aflatoxins; AFB₁, AFG₁, AFB₂, AFQ₁, AFM₁, aflatoxins B₁, G₁, B₂, Q₁, and M₁, respectively; AFL, aflatoxicol; AFL-M₁, aflatoxicol M₁; MFO, mixed function oxidase; PCBs, polychlorinated biphenyls; PAHs, polynuclear aromatic hydrocarbons; B(α)P, benzo (α)pyrene; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; CPFAs, cyclopropanoid fatty acids.

integral component of the NADPH-dependent O_2 -requiring MFO system. This system contains two other elements known to be required for metabolic activity: a) phosphatidylcholine, whose function is yet to be determined; and b) cytochrome P-450 reductase, which is thought to be the rate-limiting component (Campbell and Hayes, 1975).

Aflatoxins and aflatoxin metabolism

Aflatoxins (series B and G), which are a group of mycotoxins produced by strains of Aspergillus flavus and parasiticus, are procarcinogens, and as such, are metabolically activated by the MFO system. AFB₁, the most biologically active, hence the most widely studied, has been shown to be a potent inducer of liver carcinomas in rainbow trout as well as in rat (Wogan and Newberne, 1967), and duck (Carnaghan, 1965) and monkey (Cuthbertson et al., 1967), although the latter species is comparatively resistant.

AFB₁ also induces acute liver cirrhosis in many domestic and laboratory animals (Newberne and Butler, 1969). Although the liver appears to be the main target organ, AFB₁ also induces carcinomas in other tissues (Wogan and Newberne, 1967), presumably because MFO activity is present extrahepatically or by extrahepatic circulation of reactive metabolites (Umbenhauer and Pegg, 1981). There exists some epidemiological evidence which implicates AFB₁ as a possible source of human hepatocellular carcinomas (Peers and Lindsell, 1973). Other mold-produced AFs include AFB₂, AFG₁ and AFG₂. AFG₁ is a carcinogen in rainbow trout (Halver et al., 1967) and in rats (Butler et al., 1969), but to a lesser degree than AFB₁. Studies performed in rats

indicate that AFB₂ is either noncarcinogenic (Butler et al., 1969) or very mildly carcinogenic (Wogan et al., 1971).

AFB₁ is converted to a variety of metabolites; all but one, AFL are produced by the MFO system. AFM₁, the 4-hydroxylated derivative of AFB₁, has been found in milk, urine, and tissues of animals that have consumed AFB₁. Other metabolites formed in tissues or under in vitro conditions with liver sub-cellular fractions include AFQ₁, AFP₁, AFB_{2a}, AFH₁ and AFL-M₁. These metabolites are much less biologically active than AFB₁ and, as such, are considered to be detoxification products, although Sinnhuber et al. (1974) have reported that AFM₁ had some carcinogenic activity in rainbow trout. AFL, the most toxic of the stable metabolites, is formed by a reduction mediated by cytosolic enzymes. Since this reaction is reversible, AFL has been proposed to provide an intracellular reservoir for AFB₁ (Patterson, 1974). AFL has also been shown to be highly carcinogenic in rats (Nixon et al., 1981) and in trout (Schoenhard et al., 1981). For these reasons, reduction of AFB₁ to AFL is a questionable detoxification step.

It has been suggested that the sensitivity of a species to AFB₁ relates to the species' ability to reduce AFB₁ to AFL (Edwards, 1975). However, recent evidence from experiments in AFB₁-resistant and sensitive lines of Japanese quail show that AFL cannot be used as a sole indicator of species sensitivity (Rice et al., 1981). In general, most evidence favors the hypothesis that the biological activity of AFB₁ is related to its overall metabolic fate in several competing pathways present in target tissues (Hsieh et al., 1977; Campbell and Hayes, 1977). A summary of known and proposed metabolic conversions

of AFB₁ in rainbow trout as well as in other species is presented in Figure 1.

AFB₁-2,3 epoxide: the active AFB₁ metabolite

Early findings by Garner and others (1971) showed that an AFB₁ metabolite formed from a rat liver microsomal activation system, which required NADPH and O₂, was lethal to two strains of Salmonella typhimurium and could also covalently bind to DNA and RNA which were added to the microsomal system. It was also demonstrated in this work that unsaturation at the 2,3 position on the terminal furan ring was required for its formation. Working in the same laboratory, Swenson et al. (1973) later reported that when an RNA complex of this metabolite was hydrolyzed, 2,3-dihydro-2,3-dihydroxy-AFB₁, commonly referred to as the AFB₁-diol, was recovered as a product. Swenson (1973) concluded that the reaction of cellular nucleophiles by the hypothetical AFB₁-epoxide was through the highly electrophilic C-2 position. The basis for this conclusion was established by Schoental (1970) who suggested that AFB₁, like several carcinogenic K-region containing polycyclic aromatic hydrocarbons (PAHs), may undergo epoxidation at the 2,3 unsaturated position of the molecule. The 2,3 epoxide has yet to be isolated, presumably due to its extreme reactivity and short half-life. However, much indirect evidence points to its existence and to its involvement in the biological activity of AFs (Swenson, 1973). Evidence includes observations that the addition of the epoxide hydrase inhibitor 1,1,1-trichloropropane-2,3 oxide (TCPO) to microsomal preparations greatly increased AFB₁-induced mutagenicity

(Ong, 1978), ostensibly by preventing loss of the 2,3 epoxide by enzymatic hydrolysis. Furthermore, reduction of the 2,3 double bond yielding 2,3 dihydro AFB₁ (AFB₂) results in a 200-500 fold decrease in mutagenicity and a 150-fold decrease in carcinogenicity (Stark, 1980). The presence of a 2,3 double bond does not appear to be the sole determinant in either carcinogenic or mutagenic potential, since metabolically derived alterations occurring elsewhere in the molecule normally result in the reduction of toxicity. The presence of substitutions in the cyclopentenone ring such as a 7-hydroxylation (AFQ₁) or a reduction of the keto group (AFL) or its conversion to a lactone ring (AFG₁) result in a significant reduction of mutagenic potential, despite unsaturation at the 2,3 position (Wong and Hsieh, 1976).

Interactions between AFB₁ and DNA have been extensively studied. Investigations of acid hydrolysates of covalent AFB₁-DNA adducts in tissues from mouse, rat, and human demonstrated the predominant adduct to occur at the N⁷ atom of guanine moieties as evidenced by the liberation of 2,3-dihydro-2-(N⁷-guanyl)-3-hydroxyl AFB₁ (AFB₁-N⁷-GUA) (Essigman et al., 1977; Lin et al., 1977; Croy et al., 1978). Recently, Croy and colleagues (1980) identified AFB₁-N⁷-GUA as the major adduct in DNA from rainbow trout embryos and liver treated with AFB₁. The N⁷ atom of guanine is a prime target for AFB₁ and other bulky mutagens and carcinogens, presumably due to steric availability (Stark, 1980). Binding of the N⁷ site induces a positive charge in the purine rendering the C-1 pentose, N-1 purine bond labile to acid hydrolysis (Singer, 1975). There is evidence, however, that for

methylating and ethylating agents, adduct persistence at the O⁶ site of guanine may have a stronger correlation with mutagenesis and carcinogenesis than the N⁷ position (Singer, 1975).

Dietary modification of AFB₁ carcinogenesis; promotion and inhibition

Many naturally occurring and synthetic compounds, which are not themselves carcinogenic, alter tumor development when present in the diet or applied to the skin of an animal exposed to a carcinogen. Compounds applied after carcinogen exposure which elevate tumor responses via alterations in post-initiation processes are termed promoters, a classical example of which are phorbol esters. When repeatedly applied to the skin of laboratory animals subsequent to exposure to PAHs, these promoters enhance PAH-initiated skin tumors with respect to incidence and time to onset (Berenblum, 1954; reviewed Blumberg, 1980; 1981). Prompted by observations that many promoters, when administered at adequate doses for prolonged periods, are actually carcinogenic, Williams (1981) proposed that promoters be redefined based on their ability, or lack thereof, to induce genetic damage. These were termed genotoxic and epigenetic carcinogens of the promoter class, respectively. There also exists much evidence that many promoters may also act as cocarcinogens (Vose et al., 1981), compounds which increase the overall carcinogenic process when administered simultaneously with the carcinogen.

Although phorbol esters remain a popular experimental model, a number of promoters of cancer in other organ systems have been dis-

covered, including phenobarbital (Peraino et al., 1971) and DDT (Peraino et al., 1975), which promote hepatocarcinogenesis.

Various promoters have been shown to alter intercellular transmission of regulatory factors (Williams, 1981), induce γ -glutamyl transpeptidase activity (Williams et al., 1980), activate specific gene sequences (Boutwell and Verma, 1979; Weeks and Slaga, 1979) and inhibit DNA repair (Gaudin et al., 1972). However, the relationship between these observations and actual mechanisms underlying promoter action remain obscure.

Evidence of a promotional effect on AFB₁-induced carcinogenesis in rainbow trout was first seen when tumor incidences in AFB₁ studies were noted to be greater using cottonseed-containing fish diets than with cottonseed-free diets. Investigation of this effect led to the finding that naturally occurring cyclopropenoid fatty acids (CPFAs; malvalic and sterculic acids) present as triglycerides in cottonseed oil, meal, and flour, were the agents responsible for the promoting effect. Levels of sterculic acid from 10-200 ppm in the diet with AFB₁ at a few ppb cause a striking increase in the incidence and growth of liver cancer in trout (Sinnhuber et al., 1977). A similar, although less striking, effect on AFB₁ carcinogenesis has also been noted in rats (Lee et al., 1969). In addition, studies conducted using rainbow trout fed cottonseed oil and glandless cottonseed kernels containing 90 ppm and 250 ppm sterculic acid, respectively, indicated CPFAs to be carcinogenic, but this observation may have been due to the presence of synergists or other carcinogens (Hendricks et al., 1980).

Confusion concerning whether CPFAs are primarily cocarcinogenic or promotional exists in the literature and much evidence points to both possibilities. Experiments demonstrating that CPFAs increase the carcinogenic response when given subsequent to a subcarcinogenic dose of AFB₁ have yet to be conducted, thus in a strict sense, it is premature to label these compounds as promoters. However, Hendricks (1981) showed that dietary CPFAs promoted hepatocarcinogenesis in trout which were previously exposed to solutions of AFB₁ as embryos.

Recent studies have indicated that dietary CPFAs alter protein composition in liver microsomal membranes (Selivonchick et al., 1981), as well as alter hepatocyte membrane and mitochondrial function (Nixon et al., 1974). These findings suggest that CPFAs may exert effects on cell membranes in a similar manner to other compounds with demonstrated promotional activity (Williams, 1981).

CPFAs have also been shown in in vitro studies to significantly decrease levels of cytochrome(s) P-450, NADPH-cytochrome c reductase and inhibit detoxification of AFB₁ to AFM₁ in trout (Loveland et al., 1979) while increasing benzo(α)pyrene hydroxylase activity (Eisele et al., 1978). These findings, indicating induction as well as inhibition of various MFO activities, and decreased detoxification, suggest that CPFAs may affect the overall pharmacokinetics of AFB₁. Recent work has indicated that dietary CPFAs alter AFB₁ metabolism and reduce the number of AFB₁-DNA lesions in isolated trout hepatocytes (Bailey et al., 1982b). These authors theorized that CPFAs may enhance AFB₁ carcinogenesis by reducing initial genetic damage by AFB₁, but later markedly promoting the probability of neoplastic

transformations from those reduced numbers of lesions. This work is in contrast to the finding that dietary CPFAs have no effect on AFB₁-induced mutagenesis in S. typhimurium using liver S20 fractions from rainbow trout fed CPFAs (Eisele, Coulombe et al., 1982), which indicate CPFAs have no effect on initiation of AFB₁ mutagenesis. Similarly, other work has shown that dietary CPFAs have no effect on AFB₁ adduct formation in trout liver DNA when [³H]AFB₁ is administered by i.p. injection (Whitham et al., 1982).

Although the effects of CPFAs have generated much interest, other factors, such as high dietary protein (Lee et al., 1978), beet containing diets (Boyd et al., 1982), vitamin A and lipotrope-deficient diets (Newberne and Rogers, 1971) have been demonstrated to increase the incidence of AFB₁-induced hepatocellular carcinomas in rainbow trout (Lee et al., 1978) and rats (Boyd et al., 1982; Newberne and Rogers, 1971). Stott and Sinnhuber (1978) demonstrated that high dietary protein decreased levels of epoxide hydrase, glutathione transferase, and increased cytochrome(s) P-450, indicating high dietary protein may increase activation while decreasing detoxification of AFB₁.

In contrast to promoters, some compounds can reduce or inhibit the carcinogenic response if present before or during carcinogen exposure. Examples of inhibitors include phenolic antioxidants (Wattenberg, 1973), some vitamins (Sporn et al., 1976), selenium salts (Shamberger, 1970), organic isothiocyanates and thiocyanates (Wattenberg, 1970) and certain MFO inhibitors and inducers (Gelboin et al., 1970; Wattenberg, 1970).

As with promoters, the amount of phenomenologic data is considerable, and more studies on mechanisms of inhibition are needed. Proposed mechanisms, however, include decreased activation or increased detoxification of procarcinogen, direct scavenging of reactive intermediates, and competitive inhibition of MFO enzymes (Wattenberg, 1978). Considerable research has centered on the effects of phenolic antioxidants, BHA and BHT, which have been shown to inhibit neoplasms induced by several carcinogens (Wattenberg, 1978). Although BHA and BHT were presumed to exert a scavenging effect on reactive electrophilic intermediates, recent evidence indicates phenolic antioxidants induce activities of both epoxide hydrase and glutathione-S-transferase, thereby possibly facilitating detoxification (Lam et al., 1981). Salocks et al. (1981) has shown that dietary BHT decreases metabolism and covalent binding of AFB₁ in primary rat hepatocyte cultures. In addition, an increased output of nontoxic, water soluble AFB₁ metabolites were seen relative to hepatocytes from control rats (Salocks et al., 1981).

Vegetables of the cabbage family, Cruciferae, have been shown to inhibit AFB₁-induced hepatocarcinomas in rats, as well as reducing levels of plasma α -fetoprotein (AFP) (Boyd et al., 1981; Boyd and Stoewsand, 1981). High plasma AFP is often associated with hepatotoxicity, hepatocarcinoma or the presence of preneoplastic lesions in the liver. The active components in these vegetables include benzyl thiocyanate, benzyl isothiocyanate, and phenyl isothiocyanate, which have also been shown to inhibit PAH-induced breast tumors in rats (Wattenberg, 1977). Studies conducted with related thiono-

sulfur-containing compounds, including 1-naphthyl-isothiocyanate, suggest these compounds may alter tumor development by lowering levels of cytochrome(s) P-450 (DeMatteis, 1974; Hunter and Neal, 1975). However, inhibition has also been demonstrated when these compounds were given one week subsequent to carcinogen exposure (Wattenberg, 1981), indicating a role of inhibitors in post-initiation processes.

Several studies have shown that MFO inducers such as PAHs, phenobarbital (PB), PCBs, and flavones can confer protection against chemical carcinogenesis upon certain exposure protocols. McLean and Marshall (1971) reported that AFB₁-induced tumors in rats were reduced by a pretreatment with PB. "PB-type" inducers, which also include PCBs, induce cytochrome P-450-linked microsomal activity, responsible for converting AFB₁ to AFQ₁ and activating AFB₁ to the AFB₁-2,3-epoxide (Gurtoo and Dahms, 1979). It has been postulated that these inducers elicit a burst of the activated species, thereby reducing the probability of the presence of the epoxide at critical periods in the cell cycle (Wattenberg, 1978).

By contrast, "3MC-type" (3-methylcholanthrene type: PAHs, PCBs and β-naphthoflavone) preferentially induce cytochrome P-448 associated enzymes which mediate, among other reactions, the conversion of AFB₁ to AFM₁ (Gurtoo and Dahms, 1979). Therefore, in the case of AFB₁, 3-MC inducers may act to cause a relatively greater proportion of the carcinogen to be detoxified rather than activated to an active metabolite. This theory is supported by the observations that β-naphthoflavone, a potent inducer of P-448-type activity in

trout (Franklin et al., 1980), protects against AFB₁-induced hepatocellular carcinomas (Bailey et al., 1982b). Pretreatment with β -naphthoflavone has also recently been shown to decrease overall AFB₁-DNA binding in rainbow trout liver in vivo (Whitham et al., 1982) and in trout hepatocytes in vitro (Bailey et al., 1982b), further supporting the role of 3MC inducers in reducing initiation events in favor of detoxification pathways.

PCBs exert a strong inducing effect on P-450 associated enzymes as well as those associated with P-448, and are hence termed "mixed inducers". Poland and Glover (1977) have suggested that this mixed activity is due to specific PCB isomers inducing specific enzymes. The nonplanar isomers appear to induce cytochrome(s) P-450, while planar isomers are P-448 inducers. Bend (1977) postulated that PCBs exert their effect via competitive inhibition of the MFO system, thereby decreasing formation of the AFB₁-2,3 oxide. Supportive of this finding is that dietary Aroclor 1254, a mixture of PCB isomers, significantly reduced AFB₁-mutagenesis in S. typhimurium TA 98 using trout liver preparations (Shelton, Coulombe et al., 1982). A similar inhibition of mutagenesis was noted when Aroclor 1254 was added to the activation system in vitro (Shelton, Coulombe et al., 1982). Hendricks et al. (1977) have shown that Aroclor 1254 exhibited an inhibitory effect on AFB₁ hepatocarcinogenesis in rainbow trout when the two compounds were present simultaneously in the diet, decreasing both the tumor incidence and sizes of tumors. In addition to the above mechanisms, it is also tenable that PCBs may modify the growth of AFB₁-induced tumors. Kerkvliet and Kimeldorf (1977) reported that

Arochlor 1254 significantly reduced the transplantability and growth of the Walker 256 carcinosarcoma in rats. These authors speculated this effect may have been due to altered immune response of the host, resulting in heightened recognition of neoplastic cells.

It is clear that the mechanisms underlying the action of the foregoing modulators are complex. Identification and characterization of all steps involved in the development of carcinogenesis, especially those which are rate-limiting in initiation and post-initiation phases, will need to occur before the mechanisms are fully understood.

AFB₁ Mutagenesis in *S. typhimurium*; Background

Pioneering work by P.E. Hartman and others in the genetic mapping of the enteric bacterium *Salmonella typhimurium* made possible the selection, by B.N. Ames and colleagues, of a set of point mutations in the his operon that demonstrated a low rate of spontaneous mutation and the ability to be reverted to the wild type (his⁺) upon exposure to known chemical carcinogens (Ames et al., 1973a). In early work with *E. coli*, Benzer (1961) demonstrated that certain chemicals elicited specific somatic mutations; N-methylnitrosoquanidine and other compounds induced base-pair substitutions, while others such as 9-aminoacridine induced addition or deletion mutations (also called frameshift mutations). Using *S. typhimurium* strain LT-2, Ames developed three tester strains. Strain TA 1535 is a mutant in the hisG gene coding for phosphoribosyl ATP synthetase, an enzyme necessary for histidine synthesis. The mutation, designated his G46, confers histidine auxotrophy to the organism and is susceptible to

base-pair mutations. These mutations revert TA 1535 to the wild phenotype, enabling their enumeration on histidine-free microbiological medium (Ames et al., 1975). Strains TA 1537 and TA 1538 detect various kinds of frameshift mutagens.

Frameshift mutations are so named since they shift the "reading frame" of the translation template when the addition or deletion of base pairs not divisible by three occurs in a specific location within the gene. Organisms especially susceptible to frameshift mutations frequently possess a mutational "hot-spot", a sequence of identical bases (ie., CCCC), or a repetitive sequence of identical bases (ie., GCGC) in mutable genes (Okada et al., 1972).

Strain TA 1537 contains a run of cytidine residues in the sequence coding for histidine aminotransferase (Ames et al., 1973a), a mutation designated his C3076. Similarly, strain TA 1538 has a repetitive GC sequence in the structural gene for histidine dehydrogenase (Isono and Young, 1974), the mutation being termed his D3052.

Each of the above tester strains contain two additional mutations that greatly increase their sensitivity to mutagens: uvrB, which eliminates the DNA excision repair system that normally removes UV-induced thymine dimers from S. typhimurium; the "deep-rough" mutation, designated rfa, eliminates the polysaccharide side chain of the lipopolysaccharide (LPS) cell wall that coats the bacterial surface, making the cells more permeable, and thus more sensitive to, chemical mutagens (Ames et al., 1973a). The latter mutation also renders the bacterium completely nonpathogenic.

Working in Ames' laboratory, McCann and colleagues (1975) enhanced the sensitivity of existing tester strains by inserting a plasmid, pkm 101, which reportedly carries an error-prone DNA repair system (McCann et al., 1975). Insertion of pkm 101 to strain TA 1535 resulted in strain TA 100 and addition to TA 1538 created strain TA 98. The plasmid-containing strains have been demonstrated to be magnitudes more responsive to the mutagenic action of carcinogens that were previously weakly detected, such as AFB₁, sterigmatocystin, furylfuramide, benzo(α)pyrene, and methylmethane sulfonate (Ames et al., 1975).

The molecular mechanisms underlying AFB₁-induced mutagenesis in S. typhimurium have yet to be identified. In addition, a conclusive cause and effect relationship between DNA adduct formation by AFB₁ and mutagenesis has not been established (Stark et al., 1979). It is surprising to note that AFB₁ is mutagenic to TA 98 as well as TA 100 (Ames et al., 1975) indicating that AFB₁ is a base pair substitution mutagen in addition to a frame-shift mutagen.

D'Andrea and Hesselstine (1978) suggested that AFB₁-induced frame-shift mutations might arise via breakage of the phosphodiester bond at the site of AFB₁-DNA modification, leading to the elimination (ie., deletion) of the modified guanine residue. This conclusion was reached from experiments using ³²P-end labelled, 168 kb DNA restriction fragments of known sequence which were reacted in vitro with activated AFB₁, followed by alkali treatment and analysis using gel electrophoresis. Resulting cleavage products were identical in length to those isolated from alkali hydrolysis of the same DNA which

had been modified with dimethylsulfate. Other evidence indicates, however, that spontaneous depurination or enzymatic excision are the processes primarily responsible for AFB₁-DNA adduct removal (Groopman et al., 1981). It is possible that either deletions or base-pair substitutions may result as a consequence of pkm 101-induced repair errors during or after these events.

AFB₁ may also perturb pools of nucleotides or nucleotide precursors, an event which has shown to result in mutations in mammalian cells (Kaufman and Davidson, 1978). It is known that one mechanism for mutation in S. typhimurium involves loss of xanthine phosphoribosyltransferase (XPRT) (Benson and Gots, 1975). Stark et al. (1979) noted that AFB₁ induced overproduction of purine bases in S. typhimurium TM 677, presumably as a consequence of XPRT inhibition. Such a condition would provide a likely explanation for AFB₁-mediated base-pair or base substitution mutation.

Hepatic activation systems

Hepatic activation systems, requiring cofactors such as NADPH, and glucose-6-phosphate were first used by Ames et al. (1973a) to affect activation of the promutagens AFB₁, benzo(α)pyrene and others, thereby incorporating an important aspect of metabolism into the in vitro mutagen assay. Hepatic microsomes from a variety of sources have been used, including mouse, hamster, dog, pig, monkey (Muller et al., 1980) human (Ames et al., 1973a) and trout (Ahokas et al., 1976; Stott and Sinnhuber, 1978; Coulombe et al., 1982), although PCB-induced rat is the most widely used (Ames et al., 1973a).

Research in a number of laboratories (Ames 1973a, 1973b, 1975; McCann, 1975; Hsieh and Wong, 1976; Coulombe et al., 1982) have established a strong correlation between carcinogenic and mutagenic potency from a myriad of compounds, which has lent strong support for the theory that somatic mutations play a key role in chemically induced carcinogenesis (Burdette, 1955). In addition, in vitro mutagenesis has permitted the rapid screening of chemicals for potential carcinogenicity. This system also appears to lend itself to the evaluation of factors that alter metabolic activation of pro-carcinogens, namely inducers and inhibitors (Ames et al., 1975; Stott and Sinnhuber, 1978; Buening et al., 1981).

It is becoming evident, however, that the use of liver homogenates may not provide a realistic approximation of the metabolic behavior of intact liver (Thilly and Liber, 1980). In support of this contention, Bigger et al. (1980) demonstrated important differences between the metabolic activation products of 7,12-dimethylbenz(a)-anthracene in intact cellular systems and in liver homogenates from rat and human sources. The authors suggested that these differences may have been due to cellular organization and spatial orientation of relevant enzymes, as well as the presence of various cofactors which may be lost during fractionation.

Attempts have been made to duplicate conditions in intact hepatocytes by the addition of glutathione to S9 liver fractions (Booth et al., 1981). Studies have recently appeared which employ intact hepatocytes to affect metabolic activation of mutagens detectable by S. typhimurium. Glatt et al. (1981) reported an

improvement of the correlation of bacterial mutagenicity with carcinogenicity of benzo(α)pyrene and four of its major metabolites by activation with rat hepatocytes compared to rat liver S9 fractions. Hepatocyte activation of n-nitroso compounds (Raineri et al., 1981; Jones et al., 1981) and AFB₁ (Gayda and Pariza, 1981) has also been studied.

Despite the discussed attempts to improve correlation with carcinogenesis, it is essential to recognize that limitations exist in the use of bacterial mutagenesis systems, whether for screening or for evaluation of potential modifiers of carcinogens. Since somatic mutations do not provide the basis for all malignant transformations, microbial mutagen assays fail to detect epigenetic carcinogens, compounds which do not interact with DNA. In addition, some classes of compounds (ie., certain nitrosamines) often fail to elicit mutagenic responses, despite carcinogenic activity in vivo.

Comparative Mutagenicity of Aflatoxins using a Salmonella/
Trout Hepatic Enzyme Activation System

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ABSTRACT

A modification of the Ames assay using rainbow trout (Salmo gairdneri) liver postmitochondrial fraction (PMF) was developed to investigate the relative mutagenic potential of a series of aflatoxins (AFs). Preliminary experiments revealed that the 20,000 xg (S20) liver fraction contained a higher metabolic activity than either the S9 or S30 fractions, and that 5 mg of S20 protein per plate gave the highest mutagenic response. A 9-24 h preincubation period at 25°C was also required. The results from comparative mutagenicity experiments showed the following relative potencies: $AFB_1 > AFL > AFG_1 > AFM_1 > AFB_2 > AFP_1 > AFQ_1$. The relative potencies observed with this in vitro system qualitatively correlated with the in vivo carcinogenic activity seen in trout, indicating that this assay is of value in predicting the carcinogenic potential of mycotoxins in this species.

INTRODUCTION

Aflatoxins (B and G series) are a group of mycotoxins produced by strains of Aspergillus flavus and parasiticus. AFB₁*, the most biologically active and hence the most widely studied, has been shown to be a potent cause of liver carcinomas in rainbow trout (Sinnhuber et al., 1977), rat (Wogan and Newberne, 1967), duck (Carnaghan, 1965) and monkey (Cuthbertson et al., 1967). AFB₁ also causes acute cirrhosis of the liver in many domestic and laboratory animals (Newberne and Butler, 1969). Although the liver appears to be the main target organ, AFB₁ also induces carcinomas in other tissues (Wogan and Newberne, 1967). There exists some epidemiological evidence which implicates AFB₁ as a possible cause of human liver cancer (Peers and Linsell, 1973). AFG₁ is also a carcinogen in rainbow trout (Halver et al., 1967) and rats (Butler et al., 1969), but to a lesser degree than AFB₁. Studies performed in rats indicate that AFB₂ is either noncarcinogenic (Butler et al., 1969) or very mildly carcinogenic (Wogan et al., 1971).

AFB₁ is converted to a variety of metabolites; all but one, AFL, are produced by NADPH-dependent O₂-requiring microsomal enzymes in the liver as well as in other tissues. AFM₁, the 4-hydroxylated derivative of AFB₁, has been found in the milk,

*Abbreviations: AFs, aflatoxins; AFB₁, aflatoxin B₁; AFB₂, aflatoxin B₂; AFG₁, aflatoxin G₁; AFL, aflatoxicol; AFQ₁, aflatoxin Q₁; AFP₁, aflatoxin P₁; PMF, postmitochondrial fraction.

urine, and tissues of animals that have consumed AFB₁. Other metabolites formed in tissues or under in vitro conditions with liver sub-cellular fractions include AFQ₁ and AFP₁. These metabolites are much less biologically active than AFB₁ and, as such, are considered to be detoxification products, although Sinnhuber et al. (1974) have reported that AFM₁ had some carcinogenic activity in rainbow trout. AFL is formed by a reduction mediated by cytosolic enzymes. Since this reaction is reversible, AFL has been proposed to provide an intracellular reservoir for AFB₁ (Patterson, 1974). AFL has also been shown to be highly carcinogenic in rats (Nixon et al., 1981) and in trout (Schoenhard et al., 1981). For these reasons, reduction of AFB₁ to AFL is a questionable detoxification step.

Since the supply of AF metabolites is quite limited, evaluation of their biological activity using methods that require only small amounts are desirable. The mutagen assay of Ames et al. (1975) utilizing Salmonella typhimurium strains has proved to be quite reliable in detecting most known carcinogens as bacterial mutagens (McCann et al., 1976). Wong and Hsieh (1976) demonstrated a good correlation between the relative mutagenic activity and in vivo carcinogenicity of AF metabolites using the frame shift detector S. typhimurium TA 98 in conjunction with rat S9. Ahokas et al. (1976) and others (Stott and Sinnhuber, 1978) have demonstrated that AFB₁ is converted to a bacterial mutagen using rainbow trout liver homogenates

and S. typhimurium. The present study was undertaken to identify optimal conditions for the use of trout liver post-mitochondrial fraction in the Ames assay and to compare the relative mutagenic activity of AF metabolites in this system to their carcinogenic activity in trout.

MATERIALS AND METHODS

Animals

Thirteen-month old rainbow trout (Salmo gairdneri), spawned and reared at the Food Toxicology and Nutrition Laboratory at Oregon State University, were used for this study. Fish were fed a semi-purified casein control diet (Sinnhuber et al., 1977).

Chemicals

AFB₁ and AFB₂ were obtained from Calbiochem Inc., Los Angeles, CA. AFM₁, AFP₁ and AFQ₁ were kindly provided by D.P.H. Hsieh of University of California, Davis, G. N. Wogan of M.I.T. and M. S. Masri of Western Regional Research Laboratory, Albany, CA, respectively. AFL (natural isomer) was synthesized and purified in our laboratory (Pawlowski et al., 1977). The identity and purity of all AFs were evaluated by TLC (benzene: ethyl acetate:acetone, 55:30:15) and UV spectrophotometric analysis. Purity evaluations of the AFs using between 150 to 400 ng revealed that there were no detectable fluorescent contaminants.* All AFs were dissolved in glass-distilled ethanol; no more than 100 μ l was added per plate, an amount which had no

*Since the lowest amount of AFB₁ detectable by these methods is approximately 0.1 ng, the maximum possible limit of AFB₁ contamination in any AF sample was 0.1/150 or 0.06%. At the highest AF dose level used, i.e., 9 μ g, this level of AFB₁ contamination would result in a nonsignificant increase of 3 revertants per plate.

detectable effect on the mutagenic response of the system. All biochemicals, histidine, glucose-6-phosphate, NADP⁺, biotin, were obtained from Sigma Chemical Co., St. Louis, MO.

Preparation of Postmitochondrial Fraction (PMF)

Fish were killed by a cranial blow, their livers removed, weighed, perfused with sterile, ice cold saline (0.9 percent) then sterilized by dipping several times into a beaker of cold 95% ethanol. Perfused livers were then minced with scissors, and homogenized in two volumes of 0.15 M KCl/0.01M KHP0₄ buffer (pH 7.4) in a Potter-Elvehjem apparatus by eight passes of the teflon pestle. Homogenates were then centrifuged at 20,000 xg for 10 min. and the supernatant (PMF) was collected and distributed in 5 ml portions in small plastic vials, then quickly frozen in liquid nitrogen, and stored at -80°C. All operations were carried out at 4°C using sterile labware and cold, sterile solutions. Protein content of the PMFs was determined by the Lowry method (Lowry et al., 1951).

Mutagen Assay

The bacterial tester strain, Salmonella typhimurium TA 98 was a gift from B. N. Ames, University of California, Berkeley. The microbial mutagen assays as well as the treatment and storage of the bacteria were carried out according to the method of Ames et al (1975) with modifications. Twelve to fourteen hour nutrient broth (Oxoid) cultures giving an inoculation level of about 10⁸

cells per plate were used in all experiments. A 0.1 ml portion of the tester strain was added to 2 ml of top agar at 45°C, followed by the AF, then 0.5 ml of the S20 mix containing 5 mg of PMF protein, NADP⁺, glucose-6-phosphate, salts and sodium phosphate buffer (pH 7.4). The contents were then quickly mixed on a vortex mixer and overlaid onto pre-poured Vogel-Bonner minimal glucose plates (Vogel and Bonner, 1965). Each dose level was performed in triplicate or quadruplicate, and each experiment was performed two or more times. Plates were preincubated approximately 18 h at 25°C, then 48 h at 37°C and revertants were counted using a Model C-110 electronic colony counter (New Brunswick Scientific, New Brunswick, NJ). All experimental procedures which included AFs were performed under subdued light. The mutagenic response of each AF was determined by calculating the slope of the linear, inclining portion of the dose-response curve generated by least squares regression analysis. In this way, it was assured that AF-mediated bacterial toxicity was not a significant factor in determining mutagenic potential. Each curve represented the combined data from two or more experiments. Student's t-test was used to determine the differences between mean values obtained in preliminary experiments.

RESULTS

Preliminary experiments were conducted using 0.80 μg of AFB_1 (within the predetermined linear dose response range) to determine optimal assay conditions. The effect of the level of S20 protein on AFB_1 mutagenesis is presented in Figure 1. In the absence of S20, there was no response above the spontaneous revertant value expected for strain TA 98. The spontaneous reversion rate of cells plus trout S20 averaged 45 ± 5.8 per plate. Approximately 5 mg protein per plate was found to give the optimal mutagenic response when compared with 0, 1, 3, 7 and 10 mg protein (Figure 1). The 20,000 xg supernatant (S20) was found to contain a significantly higher metabolic activity ($p < .01$) than the S9 and a (non-significantly) higher activity than the S30.

It was also shown that a preincubation period at 25°C increased the conversion of AFB_1 to active mutagens (Figure 2). The maximum mutagenic response was observed when plates were held at 25°C for 9 hours prior to the 37°C incubation. A slightly lower response was obtained at a 24 hour preincubation, while a significantly lower response ($p < .01$) was noted at a 48 hour preincubation. For convenience, plates were generally held at 25°C for about 18 h prior to a 48 h incubation at 37°C . Trout S20 was quite stable under storage conditions at -80°C ; aside from expected fluctuations due to the test system, the activity as measured by AFB_1 activation remained essentially constant for up to two months. Examination of the background lawns of each assay plate revealed no detectable bacteriocidal action.

The relative mutagenic potencies of AF and AF metabolites are shown in Table 1. They are presented in order of decreasing potency on the basis of the slope (revertants per μg) obtained from the regression line of the dose-response curve. Of the mold-produced AFs, AFB_1 was shown to be the most active followed by AFG_1 and AFB_2 . Among the animal metabolites of AFB_1 , AFL was by far the most mutagenic, followed by AFM_1 , AFP_1 , and AFQ_1 .

DISCUSSION

The data presented here clearly demonstrate the ability of the described rainbow trout liver PMF system to activate AFs to mutagens detectable by S. typhimurium TA 98. The trout system was optimized here in order to compare the mutagenic activities of AFs with their carcinogenicity in rainbow trout, considered to be the most sensitive animal to the carcinogenic effects of AFs (Hendricks et al., 1980). Unique problems previously encountered with trout PMF in the Ames assay such as a high spontaneous mutation rate (Ahokas et al., 1976) and a requirement for a special salt solution (Stott and Sinnhuber, 1978) were not seen here. This system, therefore, represents an improvement over other methods employing trout PMF.

One interesting finding was that AFB₁-induced mutagenesis decreased with protein levels above 5 mg (Figure 1). Two possible explanations are that either the cofactors present in the S20 preparation became limiting, or that increased competition for the binding of the activated form of AFB₁ occurred between bacterial DNA and nucleophilic components of the S20, such as protein and glutathione. It is also tenable that this apparent fall in mutagenesis is due in part to bacterial toxicity. Garner et al. (1972) reported that a reduction in survival of S. typhimurium TA 1538 occurred when the bacteria were incubated with AFB₁, rat liver S9 and a NADPH generating system. This effect was more marked with increasing liver S9 concentration.

However, significant toxicity effects were noted at AFB₁ levels much greater than those used in this study.

The necessity of a preincubation at 25⁰C (Figure 2) was not surprising since rainbow trout have an optimal temperature range of about 12-15⁰C, and in vitro experiments involving rainbow trout liver preparations are commonly performed at 20-25⁰C. This low-temperature requirement was also noted by Stott and Sinnhuber (1978) using an earlier trout PMF mutagen assay method.

The results show that the relative mutagenic potency of the AFs observed with this in vitro system generally correlates with in vivo carcinogenicity in rainbow trout (Table 2). AFQ₁ displayed little mutagenicity in this study with trout S20, and little (Wong and Hsieh, 1976) or no (Hsieh et al., 1974) mutagenicity with rodent S9. Similarly this compound is much less carcinogenic (100 X) than AFB₁ in trout feeding trials (Hendricks et al., 1980) and non-carcinogenic in a study employing a sensitive trout embryo exposure assay (Hendricks et al., 1980).

The most mutagenic metabolite of AFB₁, AFL, exhibited an activity 66% that of AFB₁ in this study. This compares with the in vivo carcinogenic activity demonstrated in trout (Schoenhard et al., 1981) and rats (Nixon et al., 1981) of 50% that of AFB₁. AFL has however been shown to have a mutagenic activity 23% that of AFB₁ using rat liver S9 (Wong and Hsieh, 1976). This suggests that liver microsomal preparations from trout may either be more

active in converting AFL back to AFB₁, or in converting AFL directly to an active mutagen compared to the rat.

With one exception, the order of relative potency shown in Table 1 matched that of a similar study by Wong and Hsieh (1976) of AF metabolites employing rat S9 and S. typhimurium TA 98. This suggests that mammalian and non-mammalian MFOs are qualitatively similar with respect to AF activation. AFQ₁, which was shown to be somewhat more mutagenic in the mammalian system, was placed ahead of AFB₂. That trout and rat MFO systems are often qualitatively similar was also substantiated by Ahokas et al. (1976) who showed that trout S9 not only activated AFB₁, but also benz(α)pyrene and 2-acetylaminofluorene to bacterial mutagens, in a manner similar to that of rat S9.

On the basis of revertants per μg AFB₁, it would appear that trout S20 is less active than rat S9 in the Ames assay (Ames et al., 1975; Wong and Hsieh, 1976). This ostensibly contradicts previous observations that relative activation in the Ames assay by hepatic enzymes strongly correlates with relative sensitivity to the carcinogenic effects of AFB₁ (Hsieh et al., 1977). However, AFB₁ activation in this system is mediated by uninduced S20, and is in fact either unchanged or decreased upon the administration of inducing agents such as phenobarbital or Aroclor 1254 (unpublished results). Hence a quantitative comparison to the Aroclor-induced rat S9 system may be misleading. Furthermore, species susceptibility to the carcinogenic effects of AFB₁ is not determined solely by the apparent rate of activation of AFB₁, but also by

processes such as detoxification and repair capabilities, proliferation and immunological recognition and removal of neoplastic cells.

Studies are currently in progress in our laboratory using this system to assess the effects of dietary modulators on AFB₁-induced carcinogenesis. For example, some compounds which were shown in our laboratory to inhibit AFB₁-induced carcinogenesis in trout in vivo also inhibit AFB₁-induced mutagenesis when added in vitro to extracts from control trout. Similar studies on AFB₁ mutagenesis using S20 prepared from trout fed these inhibitors are currently being conducted.

Fig. 1. Effect of the level of S20 protein on mutagenesis of 0.80 μg AFB₁ using S. typhimurium TA 98. Revertant values are the mean of five plates \pm 1 S.D.

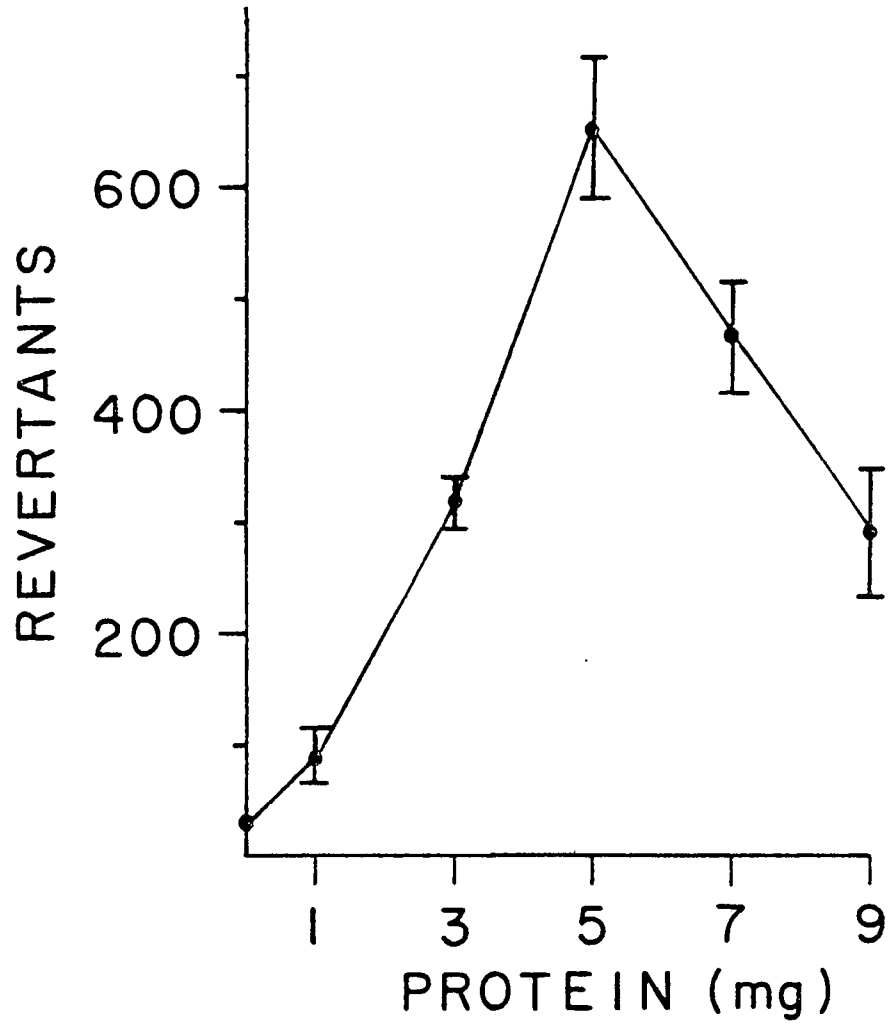


Figure 1

Fig. 2. Effect of metabolic preincubation at 25⁰C on mutagenesis of 0.80 µg AFB₁ using S. typhimurium TA 98. The response obtained at a 9 h preincubation was significantly higher (p <.05) than the 3 h response, and (nonsignificantly) higher than the 24 h preincubation. The response obtained at 48 h was significantly lower (p <.01) than the 24 h value. Plates were incubated at 37⁰C for 48 h after the preincubation interval. Revertant values are the mean of triplicate or duplicate plates ± 1 S.D.

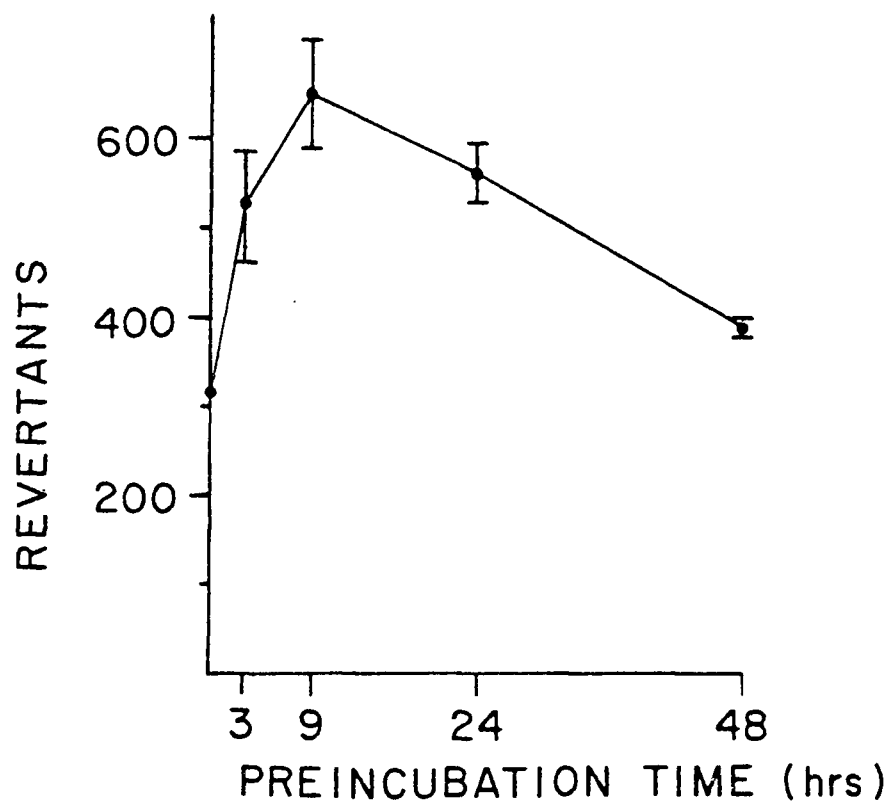
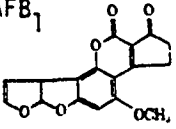
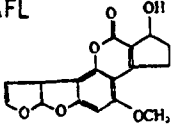
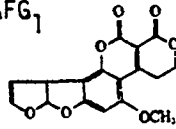
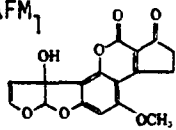
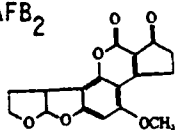
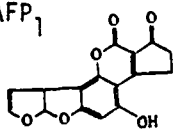
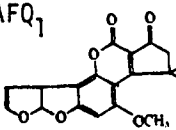


Figure 2

Table 1				
Relative mutagenicity of AFs and metabolites to <i>S. typhimurium</i> TA 98 using trout postmitochondrial fraction				
compound	dose range in μg (no. of expts.)	revertants per μg^{a}	R value ^b	potency relative to AFB ₁
AFB ₁ 	0.2-1.0 (4)	560.5	.981	1.000
AFL 	0.6-1.4 (2)	370.0	.999	.660
AFG ₁ 	0.5-5.0 (2)	35.9	.991	.064
AFM ₁ 	1.0-7.0 (3)	8.7	.879	.016
AFB ₂ 	1.0-9.0 (2)	2.6	.875	.005
AFP ₁ 	1.5-7.0 (4)	2.5	.953	.005
AFQ ₁ 	2.0-9.0 (4)	1.4	.957	.003

^aSlope of the regression line of the dose-response relationships from at least two determinations.

^bCorrelation coefficient of regression line.

Table 2-Correlation of *S. typhimurium* mutagenicity of aflatoxins to their carcinogenicity in rainbow trout via dietary or embryo exposure

Aflatoxin	relative mutagenicity ^a (%)	relative carcinogenicity via 12 month dietary exposure (ref)	relative carcinogenicity via embryo exposure fish sampled at 12 months (Hendricks <u>et al.</u> , 1980)
AFB ₁	100.0	most potent hepatocarcinogen (Sinnhuber <u>et al.</u> , 1977)	
AFL	66.0	appx. 50% as potent as AFB ₁ (Schoenhard <u>et al.</u> , 1981) ¹	equally potent
AFG ₁	6.4	considerably less potent than AFB ₁ (Ayres <u>et al.</u> , 1971)	mildly tumorigenic
AFM ₁	1.6	appx. 30% as potent as AFB ₁ (Sinnhuber <u>et al.</u> , 1974)	non-tumorigenic
AFB ₂	0.5	non-tumorigenic (Butler <u>et al.</u> , 1969)	not tested
AFP ₁	0.5	not tested	non-tumorigenic ^b
AFQ ₁	0.3	apx. 100x less potent than AFB ₁ . (Hendricks <u>et al.</u> , 1980)	non-tumorigenic

^aThis study

^bJ. D. Hendricks, personal communication

Comparative Activation of Aflatoxin B₁
to Mutagens by Isolated Hepatocytes from
Rainbow Trout (Salmo gairdneri) and Coho
Salmon (Oncorhynchus kisutch)

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ABSTRACT

Isolated hepatocytes from rainbow trout readily activate aflatoxin B₁ (AFB₁) to mutagens detectable by S. typhimurium TA 98. Characterization studies demonstrated that activation efficiency is linear with respect to hepatocyte concentration ($5 \times 10^5 - 2 \times 10^7$ cells/ml) and AFB₁ dose (0-10 µg/ml). This system was employed to assess possible differences in AFB₁ activation in hepatocytes from rainbow trout and coho salmon, two species which have been shown in in vivo studies to differ widely in sensitivity to AFB₁ carcinogenesis. Activation efficiency was approximately three times greater in trout hepatocytes compared to salmon hepatocytes. This difference was more marked when S20 liver fractions from the two species were used. Analysis of unbound [³H] AFB₁ metabolites performed on supernatants of hepatocyte incubations revealed that trout hepatocytes metabolized [³H] AFB₁ to a greater extent, and more aflatoxicol (AFL), aflatoxin M₁ (AFM₁) and polar conjugates were formed relative to salmon. In total, the results accurately reflect in vivo carcinogenesis trends in salmonid fish.

INTRODUCTION

AFB₁^{*}, a secondary metabolite produced by strains of Aspergillus flavus and parasiticus, is a potent hepatocarcinogen in a wide number of species (Sinnhuber et al., 1977; Wogan and Newberne, 1967; Carnaghan, 1965). AFB₁ is metabolically converted to a variety of metabolites, which include AFL, AFM₁ and AFQ₁ in vivo, or in vitro when incubated with a NADPH-generating system and hepatic preparations. Metabolic activation is a requisite step in the formation of the putative active intermediate, the AFB₁-2,3 epoxide (Swenson et al., 1977), reportedly responsible for DNA binding (Essigman et al., 1977), cytotoxicity (Garner et al., 1972), and bacterial mutagenicity (Garner and Wright, 1973).

Considerable variation exists in specie's responsiveness to the carcinogenic effects of AFB₁. For example, dietary AFB₁ levels as low as 4 ppb produced a 60% incidence of hepatic tumors in rainbow trout (Lee et al., 1968), the most sensitive animal to AFB₁ reported so far (Sinnhuber et al., 1977). In rats, however, 5 ppb AFB₁ resulted in a tumor incidence of only 4.5% when fed for a comparable time period (Wogan et al., 1974). In addition, it has been established that responsiveness to AFB₁ is modified by age (Wogan, 1966), sex (Newberne and Wogan, 1968), dietary promoters (Lee et al., 1968), xenobiotics (McLean and Marshall,

*Abbreviations: AFB₁, aflatoxin B₁; AFL, aflatoxicol; AFM₁, aflatoxin M₁; AFs, aflatoxins.

1971), and vegetables from the family Cruciferae (Stoewsand et al., 1978). The mechanisms which underlie such observed differences are currently the object of intense investigation and may lead to improved understanding of the human response to chemical carcinogenesis.

Evidence from these studies indicates that relative sensitivity to AFB₁ is in part dependent upon its overall metabolic fate in several competing pathways present in target tissues (Hsieh et al., 1977; Campbell and Hayes, 1977). In in vitro studies utilizing cell homogenates, Edwards et al. (1975) reported that sensitivity to AFB₁ is related to the proportion of AFB₁ reduced to AFL, while Hsieh et al. (1977) revealed that relative production of AFQ₁ and conjugated metabolites, as well as AFL, were important factors in species sensitivity.

Recent studies indicate that in vitro bacterial mutagenesis generally appear to accurately reflect in vivo carcinogenic trends. A positive correlation was found between the mutagenic activation of AFB₁ and species sensitivity in studies comparing rat, mouse and monkey liver preparations (Hsieh et al., 1977). It has been recognized, however, that hepatocytes appear to metabolize carcinogens in a manner more closely reflecting in vivo situations, presumably due to cellular organization and spatial orientation of enzymes not lost during cell fractionation (Bigger et al., 1980). Glatt et al. (1981) reported an improvement of the correlation of bacterial mutagenicity with carcinogenicity of benzo(α)pyrene and four of its major metabolites by activation

with rat hepatocytes compared to rat liver S9 fractions. Hepatocyte activation of n-nitroso compounds (Raineri et al., 1981; Jones et al., 1981) and AFB₁ (Gayda and Pariza, 1981) has also been described.

The use of trout hepatocytes in the study of AFB₁ metabolism has been described (Bailey et al., 1982a). The objectives of this study were to further develop and characterize fish hepatocytes to examine possible differences in activation of AFB₁ to bacterial mutagens by hepatocytes from rainbow trout (Salmo gairdneri) and coho salmon (Oncorhynchus kisutch), two species which are known to differ markedly in sensitivity to carcinogenic effects of AFB₁.

MATERIALS AND METHODS

Animals

Eighteen month-old Mt. Shasta strain rainbow trout (S. gairdneri) and coho salmon (O. kisutch), reared at the Food Toxicology and Nutrition Laboratory at Oregon State University, were used for this study. Trout were fed a semi-purified casein diet (Sinnhuber et al., 1977), and salmon were fed Oregon Brood Trout Ration*. For hepatocyte studies, fish were generally 400-700 g.

Chemicals

AFB₁ (Calbiochem) and [³H] AFB₁ (Moravek) were assessed for purity as described elsewhere (Coulombe et al., 1982; Bailey et al., 1982b). Purity evaluations revealed no detectable fluorescent contaminants.

Hepatocyte Preparation

Isolated hepatocytes were prepared by a two-step collagenase perfusion procedure, similar to the method of Hazel and Prosser (1979). 560 u heparin (Sigma H 7005; in 0.2 ml in 0.9% NaCl) were injected into the heart. After 2 min., the fish were then sacrificed with a sharp cranial blow and the liver exposed. The hepatic artery was then cannulated with a blunt needle, and

*Oregon brood trout ration: herring meal, 45.0%; crab meal, 2.0%; oat meal, 6.0%; tapioca starch, 3.0%; or vitamin premix, 1.5%; pacific shrimp, 33%; choline chloride (70% liquid), 0.5%; herring oil, 7.0%; antioxidant (0.04% BHA:BHT; 1:1).

secured with ligatures. After severing the hepatic portal and hepatic veins, the liver was perfused in situ with 70 ml of calcium-free Krebs-Ringer phosphate buffer (110 mM NaCl; 2 mM KCl; 0.1 mM $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$; 8 mM NaHCO_3 ; and 0.1 M NaHPO_4 buffer, pH 7.4) also containing 25 u heparin, at a flow rate of ca. 7 ml/min. In the second step, the liver was perfused with the Krebs-Ringer phosphate buffer containing 2.5 mM CaCl_2 , 0.5 mg/ml collagenase (Sigma type II) and 1 mg/ml hyaluronidase (Sigma type I-S) at a slightly higher flow rate. Both buffers were continually gassed with 95/5 O_2 - CO_2 and kept at 23°C; neither were recirculated. Upon completion of the perfusion (30-40 min.), the liver was gently removed and placed in a petri dish containing a small volume of cold, gassed, wash buffer (110 mM NaCl; 2 mM KCl; 0.1 mM $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$; 8 mM NaHCO_3 ; 0.1 M imidazole; 1 M CaCl_2 ; pH 7.4). The liver was then teased with a glass rod and forceps, filtered through cheesecloth, washed with ca. 100 ml wash buffer and centrifuged for 4 min. (34 xg). The cell pellet was then washed twice before resuspension in an incubation buffer consisting of 2% bovine serum albumin (Sigma fraction IV) in wash buffer. Cells were then enumerated and viability assessed in a hemocytometer after a 1:20 dilution into trypan blue (0.5% in incubation buffer). All labware was sterilized prior to use. Buffers were sterilized prior to the addition of enzyme and CaCl_2 ; incubation buffer was filter sterilized and stored at -70°C.

Mutagenesis Experiments with Hepatocytes

For mutagenesis testing, Salmonella typhimurium TA 98 a gift from B. N. Ames, Berkeley, CA was used. Incubations consisted of 3×10^6 hepatocytes/ml, 1 ml of an overnight bacterial culture (ca. 2×10^8 cells/ml, grown in Oxoid nutrient broth), and 0-10 $\mu\text{g/ml}$ AFB₁ dissolved in DMSO. The contents of each 25 ml erlenmeyer flask were brought to 4 ml with incubation buffer. DMSO was added so that the solvent concentration in all flasks was 0.5%, a level shown to have no significant effects on viability and AFB₁ activation in hepatocytes (data not shown). Each flask was continually gassed, and incubated 4 hours at 20°C on a metabolic shaking bath. At the end of the incubation period, a 1 ml aliquot from each flask was aseptically removed and centrifuged for 1 min. (Beckman microfuge B). The pellet was then resuspended in 1 ml of sterile phosphate buffered saline (137 mM NaCl; 2.7 mM KCl; 8.1 mM Na₂HPO₄; 1.5 mM KH₂PO₄) and an aliquot (0.2 ml) was added to 2 ml top agar (0.6% agar, 0.5% NaCl; 45°C) with 10% of a histidine biotin solution (0.5 mM histidine; 0.5 mM biotin), which was then plated on minimal agar (Vogel and Bonner, 1965). Spontaneous revertants were determined from flasks containing hepatocytes, bacteria and no AFB₁. Plates were incubated in the dark for 48 hours at 37°C, and colonies enumerated from triplicate plates using an electronic colony counter (New Brunswick Model C-110). Differences between mean values in comparative experiments were evaluated using Student's t-test.

Analysis of Unbound AFB₁ Metabolites

Incubations were carried out as described above except that bacteria were omitted, and [³H] AFB₁ (3.2 μCi; sp. act. 1 μCi/μg AFB₁) was added in 8 μl ethanol. At the end of 1 hour incubation, the hepatocytes were pelleted, and the supernatant was acidified with 20 μl acetic acid, frozen in liquid N₂ then stored at -80°C until analysis. The supernatants were made 10% in methanol and passed through a Sep-Pak C₁₈ mini-column (Waters Associates) which had been previously rinsed with 100% then 10% methanol in a 20 μM potassium acetate buffer, pH 5. Ten ml of 10% buffered methanol was then passed through the column to wash out unbound salts, protein, and any exchanged tritium (Bailey *et al.*, 1982a). AFB₁ and AFB₁ metabolites (AFL and conjugates) were eluted from the column by washing with 10 ml of 60% buffered methanol. The flow rate from the column was adjusted to ca. 2-3 drops/sec. Recovery of isotope from the Sep-Pak columns was between 91-100%. The 60% elutants were concentrated by evaporation and analyzed by HPLC (Waters Associates system) equipped with a Schoeffel spectroflow SPF 770 uv detector. The column was a μBondpak C₁₈ (3.9 mm x 30 cm, Waters), and the solvent system used was a modification of that described by Trucksess and Stoloff (1981): 0.02 M KOAc, pH 5.0; acetonitrile:methanol:tetrahydrofuran (70:15:20:3). The flow rate was 1.0 ml/min. and AFs were detected by uv (λ = 345 nm). Labelled samples were collected in 30 drop fractions and absolute counting rates

were determined by liquid scintillation (Beckman Model 7500) in ACS scintillation cocktail (Amersham).

Mutagenesis Assay with Liver Postmitochondrial Fraction

Liver S20 postmitochondrial fractions were prepared from rainbow trout and coho salmon, and mutagenesis assays conducted as previously described (Coulombe et al., 1982). AFB₁ was dissolved in ethanol, and no more than 50 μ l was delivered per plate, a level which had no detectable effect on the mutagenic response of the system. The hepatic S20 activation mix contained 5 mg of S20 protein, NADP⁺, glucose-6-phosphate, salts and sodium phosphate buffer, pH 7.4. Plates were preincubated 18 hours at 25^oC then 48 h at 37^oC and revertant colonies were enumerated.

RESULTS

Characterization experiments were conducted with rainbow trout hepatocytes to examine the effect of hepatocyte density and incubation time on hepatocyte-mediated mutagenesis, thereby identifying optimal experimental conditions. Figure 4 shows the effect of incubation time on mutagenesis of 4 $\mu\text{g/ml}$ AFB₁ using a range of hepatocyte densities. The mutagenic response increased from 1 to 4 hours in all cases, then either tapered off from 4 to 6 hours at the lower hepatocyte densities ($5 \times 10^5 - 1 \times 10^6$ cells/ml) or fell abruptly at the higher densities ($3 \times 10^6 - 2 \times 10^7$ cells/ml). Subsequent experiments were thus incubated 4 hours.

Four-hour data points from Figure 4 were used to demonstrate the relationship between hepatocyte density and mutagenic response (Figure 5). The data show that mutagenesis is a linear function of hepatocyte density from $5 \times 10^5 - 2 \times 10^7$ cells/ml ($R = .986$), indicating that activation efficiency of AFB₁ is constant over this range. For comparative experiments, it was therefore important to use identical cell concentrations since variations would exert a marked effect in mutagenic response. An intermediate concentration was chosen (3×10^6 cells/ml); this produced an adequate mutagenic response and, in addition, insured that several incubation flasks could be run using cells from an average liver.

Hepatocyte mediated mutagenesis is shown in Figure 6 to be related to dose over the range of 0-10 $\mu\text{g/ml}$ AFB₁ using cells

from either trout or salmon. The results also show a marked difference in activation in these species: expressed as revertant colonies, AFB₁ activation was significantly higher ($p < .025$) in rainbow trout hepatocytes compared to coho salmon. Slopes calculated from least squares regression analysis of dose-response relationships indicate trout hepatocytes (15.2 revertants/ μg AFB₁; $R = .956 \pm .009$) were approximately three times more efficient in conversion of AFB₁ to bacterial mutagens than salmon (5.28 revertants/ $\mu\text{g}/\text{ml}$; $R = .992 \pm .018$) in this system.

For comparison, an Ames mutagen assay using rainbow trout and coho salmon liver S20 was conducted. These experiments were performed with an 18 hour metabolic preincubation at 25⁰C, and 5 mg S20 protein per plate (Coulombe *et al.*, 1982). The results obtained here (Figure 7) were similar to those in hepatocyte experiments, except that the differences in AFB₁ activation in the two species were more dramatic. When the slopes of the dose response relationships were calculated, trout liver S20 (950 revertants/ μg AFB₁; $R = .939 \pm .019$) was shown to be over five times more active than coho salmon S20 (174 revertants/ μg AFB₁; $R = .912 \pm .021$).

It was of interest to determine if the observed differences in AFB₁ activation may be reflected in patterns of [³H] AFB₁ metabolites in hepatocytes. In preliminary experiments, it appears that this is indeed the case (Table 3). As expected, AFB₁ was metabolized by hepatocytes from both species, although much more is converted in trout as evidenced by a lower recovery

of [^3H] AFB_1 from the supernatants. Two recognized metabolites seen in uninduced trout, AFL and AFM_1 (Bailey et al., 1982a) were also seen, but in varying degrees. AFL was the major metabolite in trout, and comparatively greater amounts of AFM_1 and AF conjugates were formed. By comparison, AFL appears to be a minor metabolite in coho salmon. That coho salmon metabolize AFB_1 more slowly than trout was demonstrated in an additional experiment when salmon hepatocytes were incubated 4 hours instead of 1 hour under identical conditions. Analyses performed on the supernatants showed metabolite levels very similar to those found in the 1 hour experiment (AFB_1 , $72.1\% \pm 2.8$; AFL, $1.2\% \pm 0.0$; AFM_1 , $1.8\% \pm 0.1$; AF conjugates, $5.2\% \pm 0.1$).

DISCUSSION

The results of this study demonstrate the feasibility of using isolated trout hepatocytes to activate AFB₁ to mutagens detectable by S. typhimurium. However, relative to experiments using trout liver S20 as the activation system, the response is much weaker with respect to AFB₁ dose. A similar observation was seen by Glatt et al. (1981) who studied mutagenicity of benzo(α)pyrene and benzo(α)pyrene metabolites in isolated hepatocytes and homogenates from rats. Several possible explanations exist for the observed difference in mutagenic response. Pharmacokinetic aspects, such as comparative distances between the sites of generation of the active metabolite and the target, as well as the presence of intact cellular membranes (which may impede migration of the metabolite), are likely to be important. This hypothesis is supported by Kuroki and Drevon (1978) who demonstrated that direct or proximal contact between cells and metabolic activation systems is a requisite for mutagenesis. It is also tenable that these differences may be due to dilution or loss of enzymes or associated cofactors involved in detoxification of AFB₁ in the cell homogenates.

Data from characterization experiments demonstrate that efficiency of mutagenesis is a linear function with respect to hepatocyte density and incubation time to 4 hours. Gayda and Pariza (1981) noted that rat hepatocyte concentrations greater than 1.5×10^6 /ml decreased hepatocyte-mediated mutagenesis. The authors speculated that increasing hepatocyte-hepatocyte contacts

may affect either intracellular concentration of AFB₁ available for activation, or the activity of microsomal enzymes involved in AFB₁ activation. If this is the case, it is reasonable to conclude that trout hepatocytes are not susceptible to such density effects, since the mutagenic response of the system was essentially linear to a concentration as high as 2×10^7 hepatocytes/ml (Figure 5). The observed downturn of bacterial revertant colonies after 4 hours (Figure 4) is most likely due to cytotoxic effects in previously mutated bacteria. Cell death occurring in hepatocytes or nonmutated bacteria could not account for the decreased response since neither would cause a loss of reversion of bacteria already mutated. Experiments testing this hypothesis, however, were not conducted.

Rainbow trout exposed to dietary AFB₁ or to AFB₁ solutions as embryos are much more sensitive to hepatocarcinogenesis than coho salmon (J. D. Hendricks, unpublished data). In addition, Whitham et al. (1982) recently demonstrated a twentyfold higher binding of AFB₁ to liver DNA in vivo in rainbow trout relative to the AFB₁ binding observed in coho salmon. In general, the results of the present study are consistent with these findings. Activation of AFB₁ to bacterial mutagens was much higher in either hepatocytes or liver cell homogenates from trout than from salmon. Since mutagenesis and DNA binding presumably measure events related to tumor initiation, it is reasonable to assume that differences in AFB₁-mediated initiation may in large part

account for the observed differences in sensitivity to AFB₁ hepatocarcinogenesis in these two species.

Preliminary evidence also indicates that the rate of metabolism of AFB₁ is higher in trout than in salmon hepatocytes (Table 3). In support of this finding, other work in our laboratory has demonstrated that in vitro metabolism of AFB₁ and activities of certain MFO enzymes are generally lower in salmon than in trout (Loveland, P. M., Nixon, J. E., Hendricks, J. D., Sinnhuber, R. O.: unpublished data). Similarly, AFL was found in higher levels in supernatants from trout and hepatocyte preparations (Table 3). AFL has been shown to be a potent carcinogen in trout (Schoenhard et al., 1981) and in rats (Nixon et al., 1981) as well as a powerful bacterial mutagen (Coulombe et al., 1982; Wong and Hsieh, 1976). For these reasons, AFL is not considered to represent a detoxification product of AFB₁. It is therefore possible that a higher conversion of AFB₁ to AFL in trout hepatocytes and cell homogenates contributed to the higher mutagenic responses.

The results indicate that overall susceptibility to AFB₁ carcinogenesis is reflected in relative efficiency in mutagenic activation of AFB₁ as well as AFB₁ metabolism in isolated hepatocytes. Species susceptibility, however, is likely to also be determined by other factors. Experiments are currently being conducted in our laboratory to examine possible differences in AFB₁-DNA adduct profiles as well as enzymatic removal of bound AFB₁ in DNA from trout and salmon hepatocytes. It is hoped that

results from these studies in addition to the present findings may reveal quantitative differences in the initiation events associated with relative sensitivity to AFB₁ hepatocarcinogenesis.

Figure 4. Effect of incubation time and hepatocyte density on activation of AFB₁. Flasks contained varying amounts of trout hepatocytes (5×10^5 - 2×10^7 cells/ml), with AFB₁ ($4 \mu\text{g/ml}$) and bacteria in a 5 ml volume with incubation buffer. Suspensions were then incubated at 20°C. One ml aliquots were aseptically removed from each incubation flask at 1, 2, 4 and 6 hours, washed in 1 ml phosphate buffered saline and 0.2 ml was plated on minimal glucose plates. Values are the mean of triplicate plates from one flask at each hepatocyte level. The spontaneous reversion rate of bacteria, hepatocytes (5×10^6 cells/ml) and no AFB₁ was 16 ± 4.2 . S. typhimurium TA 98 was the tester strain used in all experiments. Two such experiments were conducted, of which these results are representative.

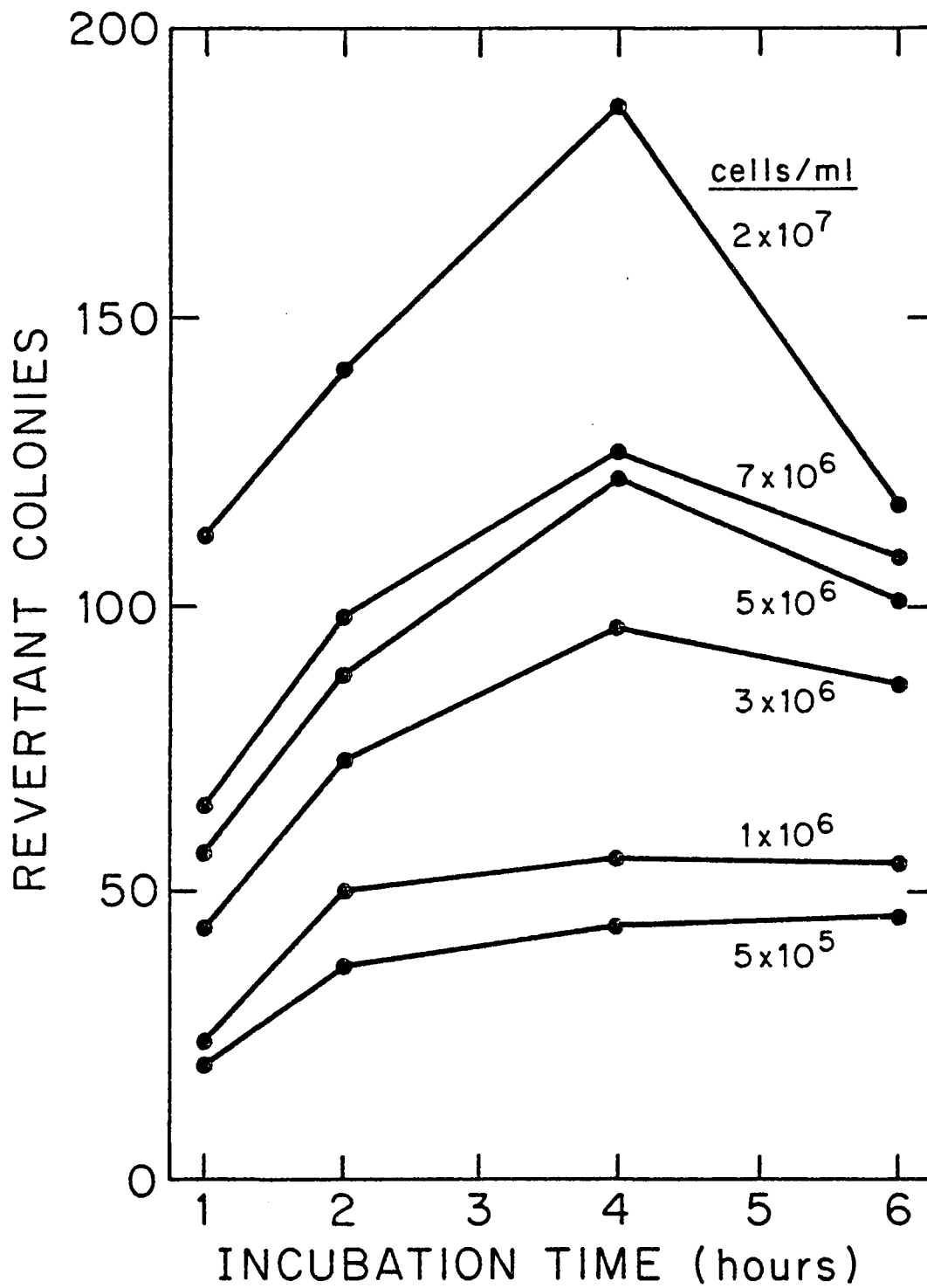


Figure 4

Figure 5. Revertant values at 4 hr. incubation at each hepatocyte level in Figure 1 were used in this figure to demonstrate the relationship between hepatocyte concentration on activation of AFB₁.

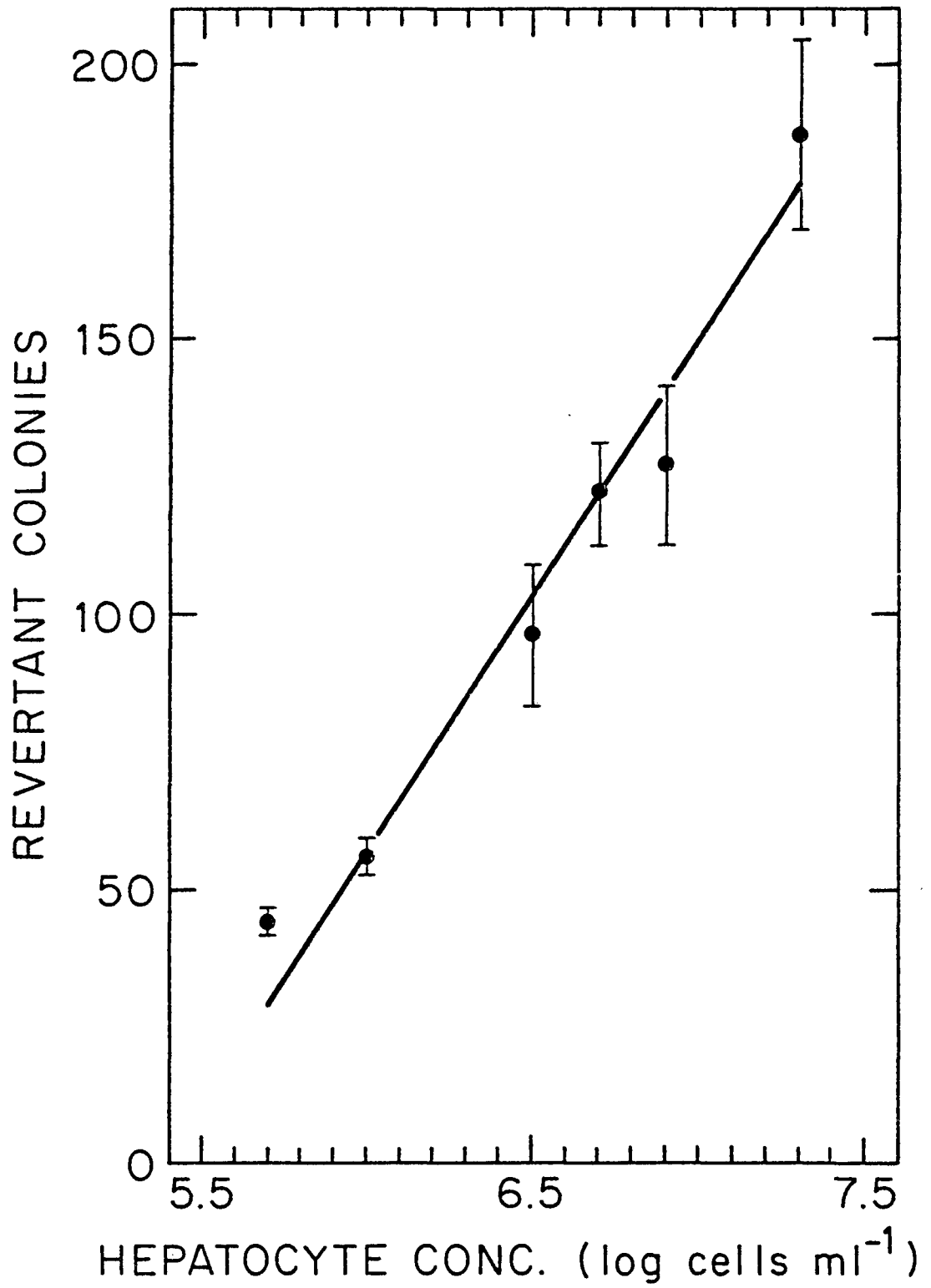


Figure 5

Figure 6. Comparative activation efficiencies of hepatocytes from rainbow trout and coho salmon. Values represent mean (± 1 S.D.) from three experiments with salmon and two with trout, performed on different days. Flasks containing approximately 3×10^6 hepatocytes/ml., were incubated 4 hrs. at 20°C . Reversion responses were significantly greater ($p < .025$) in each level of AFB_1 -treated incubations. The spontaneous reversion rate of bacteria, hepatocytes and no AFB_1 was 17 ± 2.8 and 15 ± 5.1 for trout and salmon, respectively.

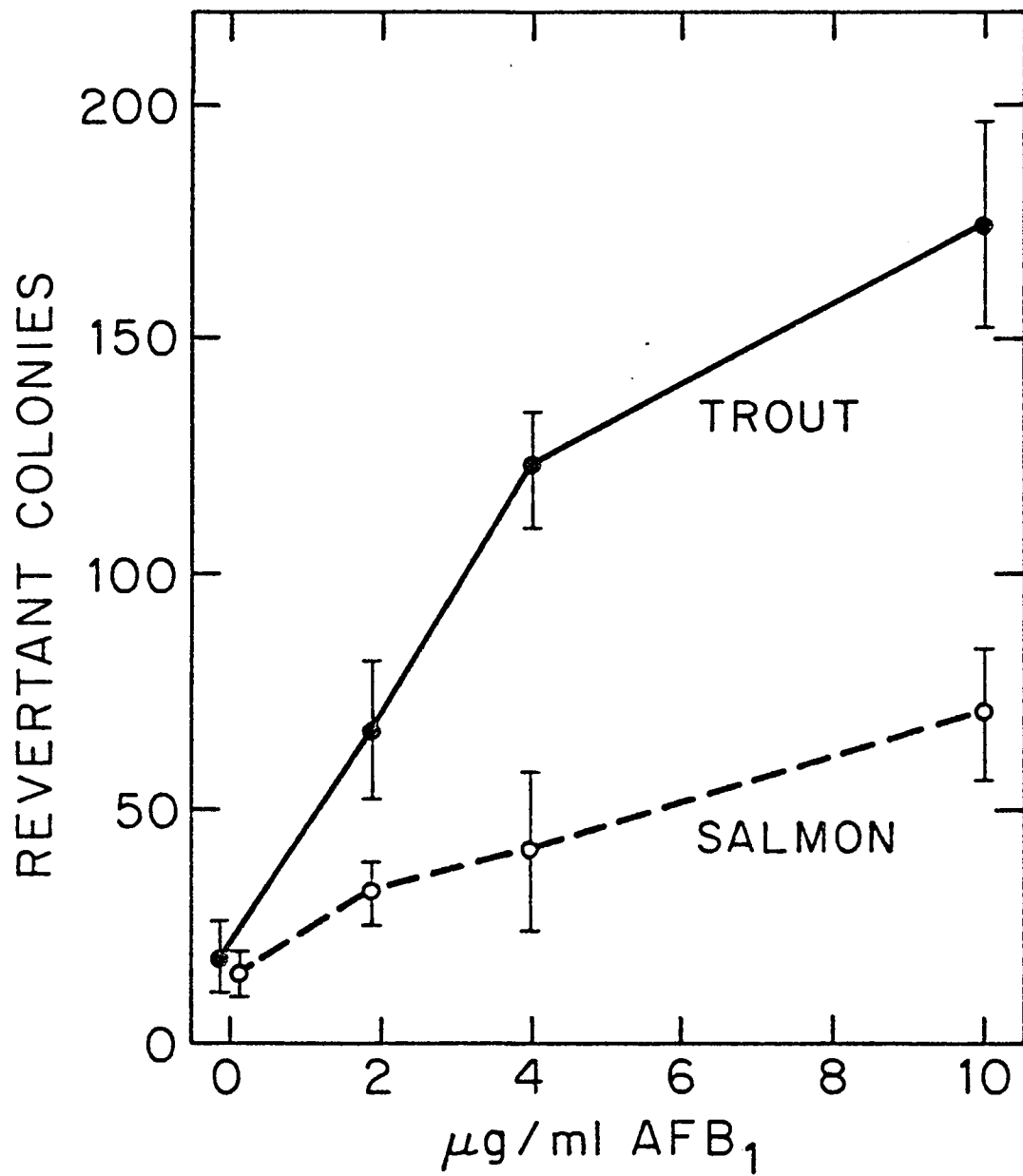


Figure 6

Figure 7. Comparative activation efficiencies of S20 liver fractions from trout and salmon in a standard bacterial mutagenesis assay. Each plate received 5 mg S20 protein. Values represent the mean (± 1 S.D.) results from two groups of three trout and salmon each. Reversion responses were significantly greater ($p < .01$) at each dose of AFB₁ using trout S20 relative to salmon. The spontaneous reversion values of cells, S20 and no AFB₁ was 29 ± 1.4 and 33 ± 6.4 for trout and salmon, respectively.

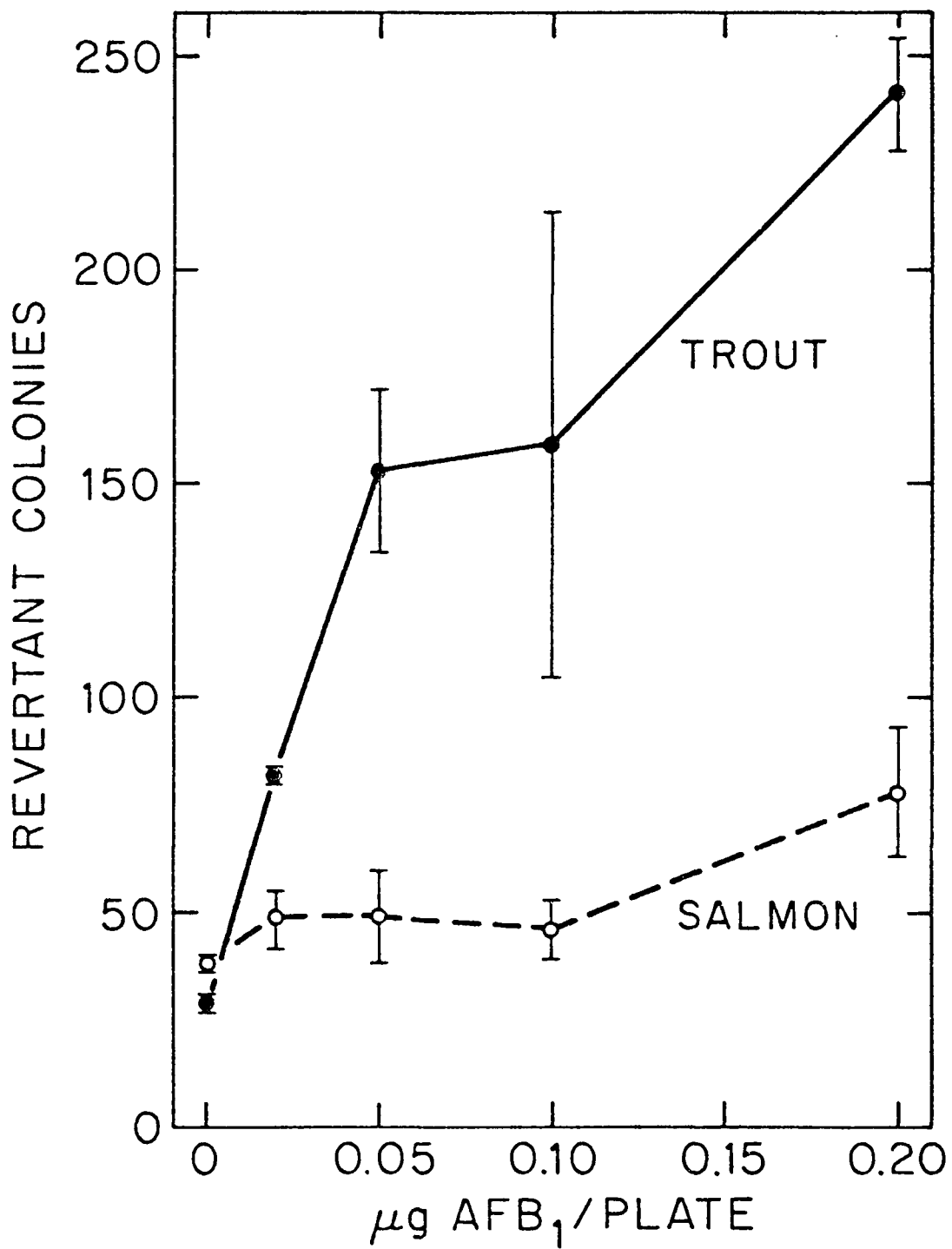


Figure 7

TABLE 3 - Comparative metabolism of [³H] AFB₁ in hepatocytes from rainbow trout and coho salmon

Species	Distribution of AFB ₁ and unbound metabolites ^{a,c}			
	as AFB ₁ (%)	as AFL (%)	as AFM ₁ (%)	as conjugates ^b (%)
Trout	51.1 (1.1) ^d	21.3 (1.1) ^d	1.2 (2.1)	7.8 (0.8)
Salmon	68.0 (2.9)	3.9 (0.5)	0.4 (0.7)	9.8 (3.6)

^aFrom HPLC analysis of Sep-Pak treated supernatants. Values expressed as % of total label injected into HPLC.

^bConjugates were defined as the complex peak(s) of polar radiolabeled compounds emerging between the solvent front and AFM₁ (see Bailey *et al.*, 1982a).

^cValues represent the mean (\pm 1 S.D.) of triplicate determinations.

^dMetabolite levels significantly different ($p < .01$) compared to in salmon.

REFERENCES

- Ahokas, J.T. (1977), Ph.D. Thesis, University of Oulu, Finland.
- Ahokas, J., Paakonen, R., Ronnholm, K., Rannio, V., and Pelkonen, O. (1976), Oxidative metabolism of carcinogens by trout liver resulting in protein binding and mutagenicity, *Z. Physiol. Chem.*, 357, 1019.
- Ames, B.N., Durston, W.E., Yamaski, E., and Lee, F.D. (1973), Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection, *Proc. Natl. Acad. Sci. USA*, 70, 2281-2285.
- Ames, B.N., Lee, F.D., Durston, W.E. (1973), An improved bacterial test system for the detection and classification of mutagens and carcinogens, *Proc. Natl. Acad. Sci. USA*, 70, 782-786.
- Ames, B.N., McCann, J., and Yamaski, E. (1975), Methods for detecting carcinogens and mutagens with the salmonella/mammalian-microsome mutagenicity test, *Mutation Res.*, 31, 347-364.
- Ayres, J.L., Lee, D.J., Wales, J.H., and Sinnhuber, R.O. (1971), Aflatoxin structure and hepatocarcinogenicity in rainbow trout, *J. Natl. Cancer Inst.*, 46, 561-564.
- Bailey, G.S., Taylor, M.J., and Selivonchick, D.P. (1982), Aflatoxin B₁ metabolism and DNA binding in isolated hepatocytes from rainbow trout (*Salmo gairdneri*), *Carcinogenesis*, 3, 511-518.
- Bailey, G., Taylor, M., Selivonchick, D., Eisele, T., Hendricks, J., Nixon, J., Pawlowski, N., and Sinnhuber, R. (1982), Mechanisms of dietary modification of aflatoxin B₁ carcinogenesis, in Hollander, A., (ed.), *Genetic Toxicology, An Agricultural Perspective* (in press).
- Bend, J.R. (1977), General discussion, in Kraybill, H.F., Dawe, C.J., Harshberger, T.C., and Tardiff, R.C. (eds.), *Aquatic Pollutants and Biological Effects with Emphasis on Neoplasia*, *Ann. NY Acad. Sci.*, 298, 463-481.
- Bend, J.R., and James, M.O. (1979), Xenobiotic metabolism in marine and freshwater species, *Biochem. Biophys. Perspect. Mar. Biol.*, 125-188.

- Benson, C.E., and Gofs, J. (1975), Genetic modification of substrate specificity of hypoxanthine phosphoribosyl transferase in Salmonella typhimurium, J. Bacteriol., 121, 77-82.
- Benzer, S. (1961), On the topography of genetic fine structure, Proc. Natl. Acad. Sci. USA, 47, 403-415.
- Berenblum, I. (1954), Carcinogenesis and tumor pathogenesis, Adv. Cancer Res., 2, 129.
- Blumberg, P.M. (1981), In vitro studies on the mode of action of the phorbol esters, potent tumor promoters: Part 2, CRC Crit. Revs. Toxicol., 9, 199.
- Blumberg, P.M. (1980), In vitro studies on the mode of action of the phorbol esters, potent tumor promoters: Part I, CRC Crit. Revs. Toxicol., 8, 153.
- Booth, S.C., Bosenberg, H., Garner, R.C., Hertzog, P.J., and Norpoth, K. (1981), The activation of aflatoxin B₁ in liver slices and in bacterial mutagenicity assays using livers from different species including man, Carcinogenesis 2, 1063-1068.
- Boutwell, R.R., and Verma, A.K. (1979), Effects of vitamin A and related retinoids on the biochemical processes linked to carcinogenesis, Pure Appl. Chem., 51, 857-866.
- Boyd, J.N., Babish, J.G., and Stoewsand, G.S. (1982), Modification by beet and cabbage diets of aflatoxin B₁-induced rat plasma α -fetoprotein elevation, hepatic tumorigenesis and mutagenicity of urine, Fd. Chem. Toxicol., 20, 47-52.
- Boyd, J.N., and Stoewsand, G.S. (1981), Blood α -fetoprotein changes in rats fed aflatoxin B₁ and various levels of cabbage, J. Food Sci., 46, 1923-1926.
- Brodie, B.B., and Maickel, R.P. (1962), Comparative biochemistry of drug metabolism, Proc. 1st Int. Pharmacol. Meet., 6, 299-324.
- Buening, M.K., Chang, R.L., Huang, M.T., Fortner, J.G., Wood, A.W., and Conney, A.H. (1981), Activation and inhibition of benzo(α)pyrene and aflatoxin B₁ metabolism in human liver microsomes by naturally occurring flavonoids, Cancer Res., 41, 67-72.
- Burdette, W.J. (1955), The significance of mutation in relation to the origin of tumors: A review, Cancer Res., 15-201-226.
- Butler, W.H., Greenblatt, M., and Lijinski, W. (1969), Carcinogenesis in rats by aflatoxins B₁, G₁ and B₂, Cancer Res., 29, 2206-2211.

- Campbell, T.C., and Hayes, J.R., (1975), Liver microsomal system and mycotoxin metabolism in Sehlessenger, D. (ed.), Microbiology, Am. Soc. Microbiol., Washington, DC.
- Carnaghan, R.S. (1965), Hepatic tumors in ducks fed at a low level of toxic groundnut meal, *Nature*, 208, 308.
- Coulombe, R.A., Shelton, D.W., Sinnhuber, R.O., and Nixon, J.E. (1982), Comparative mutagenicity of aflatoxins using a Salmonella/trout hepatic activation system, *Carcinogenesis* (in press).
- Croy, R.G., Essigman, J.M., Reinhold, V.N., and Wogan, G.N. (1978), Identification of the principal aflatoxin B₁-DNA adduct formed in vivo in rat liver, *Proc. Natl. Acad. Sci. USA*, 75, 1745-1749.
- Croy, R.G., Nixon, J.E., Sinnhuber, R.O., and Wogan, G.N. (1980), Investigation of covalent aflatoxin B₁-DNA adducts formed in vivo in rainbow trout (Salmo gairdneri) embryos and liver, *Carcinogenesis*, 1, 903-909.
- Cuthbertson, W.F., Laursen, A.C., and Pratt, D.A.H. (1967), Effect of groundnut meal containing aflatoxins on cynomolgus monkeys, *Brit. J. Nutr.*, 21, 893-908.
- DeMatteis, F. (1974), Covalent binding of sulfur to microsomes and loss of cytochrome P450 during oxidative desulfuration of several chemicals, *Mol. Pharmacol.*, 10, 849-854.
- Edwards, G.S., Rintel, T.D., and Parker, C.M. (1975), Aflatoxicol as a possible predictor for species sensitivity to aflatoxin B₁ (AFB₁), *AACR Abstracts*, San Diego, CA, p. 133.
- Eisele, T.A., Coulombe, R.A., Williams, J.L., Shelton, D.W., and Nixon, J.E. (1982), Time and dose dependent effects of dietary CPFA on the hepatic microsomal MFO system of rainbow trout (manuscript in preparation).
- Eisele, T.A., Nixon, J.E., Pawlowski, N.E., and Sinnhuber, R.O. (1978), Effects of dietary cyclopropene fatty acids on the mixed function oxidase system of the rainbow trout (Salmo gairdneri), *J. Environ. Pathol., Toxicol.*, 1, 773-778.
- Elcombe, C.R., Franklin, R.B., and Lech, J.J. (1979), Induction of hepatic microsomal enzymes in rainbow trout in Khan, M.A.Q., Lech, J.J., and Menn, J.J. (eds.), *Pesticide and xenobiotic metabolism in aquatic organisms*, Amer. Chem. Soc. Symposium Series No. 99.

- Emeh, J.K., Niranjan, B.G., Bhut, N.K., and Avadhani, N.G. (1981), Modulation of hepatic transcription during early stages of aflatoxin B₁ carcinogenesis, *Carcinogenesis*, 2, 373-378.
- Essigman, J.M., Croy, R.G., Nadzan, A.M., Busby, W.F., Reinhold, V.N., Buchi, G., and Wogan, G.N. (1977), Structural identification of the major DNA adduct formed by aflatoxin B₁ in vitro, *Proc. Natl. Acad. Sci. USA*, 74, 1870-1874.
- Estabrook, R.W., Cooper, D.Y., and Rosenthal, O. (1963), Light-reversible carbon monoxide inhibition of the steroid C-21 hydroxylase system of the adrenal cortex, *Biochem.*, 2, 338, 741-755.
- Franklin, R.B., Elcombe, C.R., Vodicknik, M.J., and Lech, J.J. (1980), Comparative aspects of the disposition and metabolism of xenobiotics in fish and mammals, *Fed. Proc.*, 39, 3144-3149.
- Garner, R.C., Miller, E.C., and Miller, J.A. (1972), Liver microsomal metabolism of aflatoxin B₁ to a reactive derivative toxic to Salmonella typhimurium TA 1530, *Cancer Res.*, 32, 2058-2066.
- Garner, R.C., Miller, E.C., Miller, J.A., Garner, J.V., and Henson, R.S. (1971), Formation of a factor lethal for S. typhimurium TA 1530 and TA 1531 on incubation of aflatoxin B₁ with rat liver microsomes, *Biochem. Biophys. Res. Commun.*, 45, 774-780.
- Garner, R.C., and Wright, C.M. (1973), Induction of mutations in DNA-repair deficient bacteria by a liver microsomal metabolite of aflatoxin B₁, *Brit. J. Cancer*, 28, 544-551.
- Gaudin, D., Gregg, R.S., and Yielding, K.L. (1972), Inhibition of DNA repair by cocarcinogens, *Biochem. Biophys. Res. Commun.*, 48, 945.
- Gayda, D.P., and Pariza, M.W. (1981), Activation of aflatoxin B₁ by primary cultures of adult rat hepatocytes: effects of hepatocyte density, *Chem. Biol. Inter.*, 35, 255-265.
- Gelboin, H.V., Wiebel, F., and Diamond, L. (1970), Dimethylbenzanthracene tumorogenesis and aryl hydroxylase in mouse skin: inhibition by 7,8 benzoflavone, *Science*, 170, 169-171.
- Glatt, H.R., Billings, R., Platt, K.L., and Oesch, O. (1981), Improvement of the correlation of bacterial mutagenicity with carcinogenicity of benzo(α)pyrene and four of its major metabolites by activation with intact liver cells instead of cell homogenates, *Cancer Res.*, 41, 270-277.

- Groopman, J.D., Croy, R.G., and Wogan, G.N. (1981), In vitro reactions of aflatoxin B₁-DNA, Proc. Natl. Acad. Sci. USA 78, 5445-5449.
- Gurtoo, H.L., and Dahms, R.P. (1979), Effects of inducers and inhibitors on the metabolism of aflatoxin B₁ by rat and mouse, Biochem. Pharmacology, 28, 3441-3449.
- Halver, J.E., Ashley, L.M., Smith, C.E., and Wogan, G.N. (1967), Early acute aflatoxicosis stimulates rainbow trout hepatoma-genesis, Tox. Appl. Pharmacol., 10, 398.
- Hazel, J.R., and Prosser, C.L. (1979), Incorporation of 1-¹⁴C-acetate into fatty acids and sterols by isolated hepatocytes of thermally acclimated rainbow trout, J. Comp. Physiol., 134, 321-329.
- Heidelberger, C. (1973), Current trends in chemical carcinogenesis, Fed. Proc., 32, 2154-2161.
- Hendricks, J.D. (1982), Chemical carcinogenesis in fish, in Weber, L. (ed.), Aquatic Toxicology, Raven Press, NY.
- Hendricks, J.D. (1981), The use of rainbow trout (Salmo gairdneri) in carcinogen bioassay, with special emphasis on embryonic exposure, in Dawe, C.J. (ed.), Japan Sci. Soc. Press, Tokyo, pp. 227-240.
- Hendricks, J.D., Putnam, T.P., Bills, D.D., and Sinnhuber, R.O. (1977), Inhibitory effect of a polychlorinated biphenyl (Arochlor 1254) on aflatoxin B₁ carcinogenesis in rainbow trout, J. Natl. Cancer Inst., 59, 1545-1551.
- Hendricks, J.D., Sinnhuber, R.O., Loveland, P.M., Pawlowski, N.E., and Nixon, J.E. (1980), Hepatocarcinogenicity of glandless cottonseeds and cottonseed oil to rainbow trout (Salmo gairdneri), Science, 208, 309-311.
- Hendricks, J.D., Sinnhuber, R.O., Nixon, J.E., Wales, J.H., Masri, M.S., and Hsieh, D.P.H. (1980), Carcinogenic response of rainbow trout (Salmo gairdneri) to aflatoxin Q₁ and synergistic effect of cyclopropene fatty acids, J. Natl. Cancer Inst., 64, 523-527.
- Hendricks, J.D., Wales, J.H., Sinnhuber, R.O., Nixon, J.E., Loveland, P.M., and Scanlan, R.A. (1980), Rainbow trout (Salmo gairdneri) embryos: a sensitive animal model for experimental carcinogenesis, Fed. Proc., 39, 3222-3229.
- Hsieh, D.P.H., Salhab, A.S., Wong, J.J., and Yang, S.L. (1974), Toxicity of aflatoxin Q₁ as evaluated with the chicken embryo and bacterial auxotrophs, Tox. Appl. Pharmacol., 30, 237-242.

- Hsieh, D.P.H., Wong, Z.A., Wong, J.J., Michas, C., and Ruebner, B.H. (1977), Comparative metabolism of aflatoxin, in Rodricks, J.V., Hesseltine, C.W., and Mehlman, M.A. (eds.), *Mycotoxins in Human and Animal Health*, Pathotox Publ. Inc., Park Forest South, IL, pp. 37-50.
- Hunter, A.L., and Neal, R.A. (1975), Inhibition of hepatic mixed function oxidase activity in vitro and in vivo by various thiono-sulfur-containing compounds, *Biochem. Pharmacol.*, 24, 2199-2205.
- Isono, K., and Yourno, J. (1974), Chemical carcinogens as frameshift mutagens: Salmonella DNA sequence sensitive to mutagenesis by polycyclic carcinogens, *Proc. Natl. Acad. Sci. USA*, 71, 1612-1617.
- Kaufman, E.R., and Davidson, R.L. (1979), Bromodeoxyuridine mutagenesis in mammalian cells is stimulated by purine deoxyribonucleosides, *Somat. Cell Genet.*, 5, 653-663.
- Kerkvliet, N.I., and Kimeldorf, D.J. (1977), Antitumor activity of a polychlorinated biphenyl mixture, Arochlor 1254, in rats inoculated with Walker 256 carcinosarcoma cells, *J. Natl. Cancer Inst.*, 59, 951-955.
- Klingenberg, M. (1958), Pigments of rat liver microsomes, *Arch. Biochem. Biophys.*, 75, 376-386.
- Kuroki, T., and Drevon, C. (1978), Direct or proximal contact between cells and metabolic activation systems is required for mutagenesis, *Nature*, 271, 368-370.
- Lam, L.K., Spornins, V.L., Hochalter, J.B., and Wattenberg, L.W. (1981), Effects of 2- and 3-tert-butyl-4-hydroxyanisole on glutathione-S-transferase and epoxide hydrolase activities and sulfhydryl levels in liver and forestomach of mice, *Cancer Res.*, 41, 3940-3943.
- Lech, J.J., and Bend, J.R. (1980), The relationship between biotransformation and the toxicity and fate of xenobiotic chemicals in fish, *Environ. Health Perspect.*, 34, 115-131.
- Lee, D.J., Sinnhuber, R.O., Wales, J.H., and Putnam, G.B. (1978). Effect of dietary protein on the response of rainbow trout (Salmo gairdneri) to aflatoxin B₁, *J. Natl. Cancer Inst.*, 60, 317-320.
- Lee, D.J., Wales, J.H., and Sinnhuber, R.O. (1969), Hepatoma and renal tubule adenoma in rats fed aflatoxin and cyclopropenoid fatty acids, *J. Natl. Cancer Inst.*, 43, 1037-1044.

- Lin, J., Miller, J.A., and Miller, E.C. (1977), 2,3-dihydro-2-(guan-7-yl)-3-hydroxy aflatoxin B₁-DNA or ribosomal RNA adducts formed in hepatic microsome-mediated reactions and rat liver in vivo, *Cancer Res.*, 37, 4430-4438.
- Loveland, P.M., Nixon, J.E., Pawlowski, N.E., Eisele, T.A., Libbey, L.M., and Sinnhuber, R.O. (1979), Aflatoxin B₁ and aflatoxicol metabolism in rainbow trout (Salmo gairdneri) and the effects of dietary cyclopropene, *J. Environ. Pathol. Toxicol.*, 2, 707-718.
- McCann, J., Choi, E., Yamaski, E., and Ames, B.N. (1976), Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals, *Proc. Natl. Acad. Sci. USA*, 72, 5135-5139.
- McCann, J., Springarn, N.E., Kobori, J., and Ames, B.N. (1975), Detection of carcinogens as mutagens: bacterial tester strains with R factor plasmids, *Proc. Natl. Acad. Sci. USA*, 72, 979-983.
- McLean, A.E., and Marshall, A. (1971), Reduced carcinogenic effects of aflatoxin in rats given phenobarbitone, *Br. J. Exp. Pathol.*, 52, 322-329.
- Miller, J.A. (1970), Carcinogenesis by chemicals: An overview, *Cancer Res.*, 30, 559-576.
- Muller, D., Nelles, J., Deperade, E., and Arni, P. (1980), Activity of S9 liver fractions from seven species in the Salmonella/mammalian microsome mutagenicity test, *Mutation Res.*, 70, 279-300.
- Newberne, P.M., and Butler, W.H. (1969), Acute and chronic effects of aflatoxin on the liver of domestic and laboratory animals: A review, *Cancer Res.*, 29, 236-250.
- Newberne, P.M., and Rogers, A.E. (1971), Aflatoxin carcinogenesis in rats: dietary effects, in Purchase, I.F. (ed.), *Mycotoxins in Human Health*, McMillan, New York, pp. 195-208.
- Nixon, J.E., Eisele, T.A., Wales, J.H., and Sinnhuber, R.O. (1974), Effects of subacute toxic levels of dietary cyclopropene fatty acids upon membrane function and fatty acid composition in the rat, *Lipids*, 9, 314-321.
- Nixon, J.E., Hendricks, J.D., Pawlowski, N.E., Loveland, P.M., and Sinnhuber, R.O. (1981), Carcinogenicity of aflatoxicol in Fischer 344 rats, *J. Natl. Cancer Inst.*, 66, 1159-1163.

- Okada, Y., Streisinger, G., Owen, J., Newton, J., Tsugita, A., and Inouye, M. (1972), Molecular basis of a mutational hot spot in the lysozyme gene of bacteriophage T4, *Nature*, 236, 338-341.
- Omura, T., and Sato, R. (1964), Carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hepoprotein nature, *J. Biol. Chem.*, 239, 2370-2378.
- Omura, T., Sato, R., Cooper, D.Y., Rosenthal, O., and Estabrook, R.W. (1965), Function of cytochrome P-450 in microsomes, *Fed. Proc.*, 24, 1181-1189.
- Patterson, D.S. (1974), Metabolism as a factor in determining the toxic action of the aflatoxins in different animal species, *Food Cosmet. Toxicol.*, 11, 287-294.
- Pawlowski, N.E., Schoenhard, G.L., Lee, D.J., Libbey, L.M., Loveland, P.M., and Sinnhuber, R.O. (1977), Reduction of aflatoxin B₁ to aflatoxicol, *J. Agric. Food Chem.*, 25, 437-438.
- Peers, F.G., and Lindsell, C.A. (1973), Dietary aflatoxins and liver cancer - a population based study in Kenya, *Brit. J. Cancer*, 27, 473-484.
- Peraino, C., Fry, R.J.M., and Staffeldt, E. (1971), Reduction and enhancement by phenobarbital of hepatocarcinogenesis induced in the rat by 2-acetylaminofluorene, *Cancer Res.*, 31, 1506-1512.
- Peraino, C., Fry, R.J.M., Staffeldt, E., and Christopher, J.P. (1975), Comparative enhancing effect of phenobarbital, amobarbital, diphenylhydantoin, and dichlorodiphenyl-trichloroethane on 2-acetylaminofluorene-induced hepatic tumorigenesis in the rat, *Cancer Res.*, 35, 2884-2890.
- Pitot, H.C., Barsness, L., Goldsworthy, T., and Kitawaga, T. (1978), Biochemical characterization of stages of hepatocarcinogenesis after a single dose of diethylnitrosamine, *Nature*, 271, 456-458.
- Poland, A., and Glover, E. (1977), Chlorinated biphenyl induction of arylhydrocarbon hydroxylase activity: a study of the structure-activity relationship, *Mol. Pharmacol.*, 13, 924-938.
- Raineri, R., Poiley, J.A., Andrews, A.W., Pienta, R.J., and Linjinski, W. (1981), Greater effectiveness of hepatocyte and liver S9 preparations from hamsters than rat preparations in activating N-nitroso compounds to metabolites mutagenic to Salmonella, *J. Natl. Cancer Inst.*, 67, 1117-1122.

- Rice, D.W., Hamilton, P.B., and Hsieh, D.P.H. (1981), In vitro metabolism of aflatoxin B₁ by resistant and susceptible lines of Japanese quail, The Toxicologist, 1, 322.
- Rucker, R.R., Yasutake, W.T., and Wolf, H. (1961), Trout hepatoma - a preliminary report, Prog. Fish-Cult., 23, 3-7.
- Salocks, C.B., Hsieh, D.P.H., and Byard, J.L. (1981), Butylated hydroxytoluene pretreatment protects against cytotoxicity and reduces covalent binding of aflatoxin B₁ in primary hepatocyte cultures, Toxicol. Appl. Pharmacol., 59, 331-345.
- Schoenhard, G.L., Hendricks, J.D., Nixon, J.E., Lee, D.J., Wales, J.H., Sinnhuber, R.O., and Pawlowski, N.E. (1981), Aflatoxin-induced hepatocellular carcinoma in rainbow trout (Salmo gairdneri) and the synergistic effects of cyclopropenoid fatty acids, Cancer Res., 41, 1011-1014.
- Schoental, R. (1970), Hepatotoxic activity of retrorsine, senkirkine and hydroxysenkirkine in newborn rats, and the role of epoxides in carcinogenesis by pyrrolizidine alkaloids and aflatoxins, Nature, 227, 401-402.
- Selivonchick, D.P., Williams, J.L., and Schaup, H.W. (1981), Alteration of liver microsomal proteins from rainbow trout (Salmo gairdneri) fed cyclopropenoid fatty acids, Lipids, 16, 211-214.
- Shamberger, R.J. (1970), Relationship of selenium to cancer. I. Inhibitory effect of selenium on carcinogenesis, J. Natl. Cancer Inst., 44, 931-936.
- Shelton, D.W., Coulombe, R.A., Pereira, C.B., Casteel, J.L., and Hendricks, J.D. (1982), Inhibitory effect of Aroclor 1254 on aflatoxin-initiated carcinogenesis in rainbow trout and mutagenesis using a Salmonella/trout hepatic activation system (submitted for publication).
- Singer, B. (1975), The chemical effects of nucleic acid alkylation and their relation to mutagenesis and carcinogenesis, in Cohn, W.E. (ed.), Progress in Nucleic Acid Research and Molecular Biology, Academic Press, New York, pp. 219-280.
- 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.
- Sinnhuber, R.O., Lee, D.J., Wales, J.H., Landers, M.K., and Keyl, A.C. (1974), Hepatic carcinogenesis of aflatoxin M₁ in rainbow trout (Salmo gairdneri) and its enhancement by cyclopropene fatty acids, J. Natl. Cancer Inst., 53, 1285-1288.

- Sporn, M.B., Dunlop, N.M., and Newton, D.L. (1976), Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids), *Fed. Proc.*, 35, 1332-1338.
- Stark, A.A. (1980), Mutagenicity and carcinogenicity of mycotoxins: DNA binding as a possible mode of action, *Ann. Rev. Microbiol.*, 34, 235-262.
- Stark, A.A., Essigman, J.M., Demain, A.L., Skopek, T.R., and Wogan, G.N. (1979), Aflatoxin B₁ mutagenesis, DNA binding, and adduct formation in Salmonella typhimurium, *Proc. Natl. Acad. Sci. USA*, 76, 1343-1347.
- Stoewsand, G.S., Babish, J.B., and Wimberly, H.C. (1978), Inhibition of hepatic toxicities from polybrominated biphenyls and aflatoxin B₁ in rats fed cauliflower, *J. Environ. Path. Toxicol.*, 2, 399.
- Stott, W.T., and Sinnhuber, R.O. (1978), Trout hepatic enzyme activation of aflatoxin B₁ in a mutagen assay system and the inhibitory effect of PCBs, *Bull. Env. Contam. Toxicol.*, 19, 35-41.
- Swenson, D.H., Lin, J-K, Miller, E.C., and Miller, J.A. (1977), Aflatoxin B₁ - 2,3-oxide as a probable intermediate in covalent binding of aflatoxin B₁ and B₂ to rat liver DNA and ribosomal RNA in vivo, *Cancer Res.*, 37, 172-181.
- Swenson, D.H., Miller, J.A., and Miller, E.C. (1973), 2,3 dihydro-2,3-dihydroxy-aflatoxin B₁: An acid hydrolysis product of an RNA-aflatoxin B₁ adduct formed by hamster and rat liver microsomes in vitro, *Biochem. Biophys. Res. Commun.*, 53, 1260-1267.
- Thilly, W.G., and Liber, H.L. (1980), Genetic toxicology, in Doull, J., Klaassen, C.D., and Amdur, M.O. (eds.), *Casarett and Doull's Toxicology*, 2nd ed., MacMillan Pub. Co., New York, p. 151.
- Truckess, M.W., and Stoloff, L. (1981), High performance liquid chromatographic determination of aflatoxin in milk, blood, and liver, *J. Assoc. Off. Anal. Chem.*, 64, 1083-1087.
- Umbenhauer, D.R., and Pegg, A.E. (1981), Alkylation of intracellular and extracellular DNA by dimethylnitrosamine following activation by isolated rat hepatocytes, *Cancer Res.*, 41, 3471-3474.
- Vogel, H.J., and Bonner, D.M. (1965), Acetylornitinase of E. coli: partial purification and some properties, *J. Biol. Chem.*, 218, 97-106.

- Vose, C.W., Coombs, M.M., and Bhatt, T.S. (1981), Cocarcinogenicity of promoting agents, *Carcinogenesis*, 2, 687-689.
- Wattenberg, L.W. (1981), Inhibition of carcinogen-induced neoplasia by sodium cyanate, tert-butyl isocyanate, and benzyl isothiocyanate administered subsequent to carcinogen exposure, *Cancer Res.*, 41, 2991-2994.
- Wattenberg, L.W. (1978), Inhibition of chemical carcinogenesis, *J. Natl. Cancer Inst.*, 60, 11-18.
- Wattenberg, L.W. (1977), Inhibition of carcinogenic effects of polycyclic aromatic hydrocarbons by benzyl isothiocyanate and related compounds, *J. Natl. Cancer Inst.*, 58, 395-398.
- Wattenberg, L.W. (1973), Inhibition of chemical carcinogen-induced pulmonary neoplasia by butylated hydroxyanisole, *J. Natl. Cancer Inst.*, 48, 1425-1430.
- Wattenberg, L.W. (1970), Inhibition of the carcinogenic action of benzo(α)pyrene by flavones, *Cancer Res.*, 30, 1922-1925.
- Weeks, C.E., and Slaga, T.J. (1979), Inhibition of phorbol ester-induced polyamine accumulation in mouse epidermis by anti-inflammatory steroid, *Biochem. Biophys. Res. Commun.*, 91, 1488-1496.
- Whitham, M., Nixon, J.E., and Sinnhuber, R.O. (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.
- Williams, G.M. (1981), Liver carcinogenesis: the role for some chemicals of an epigenetic mechanism of liver tumor promotion involving modification of the cell membrane, *Fd. Cosmet. Toxicol.*, 19, 577-583.
- Williams, G.M., Ohmori, T., Katayama, S., and Rice, J.M. (1980), Alteration by phenobarbital of membrane associated enzymes including gamma glutamyl transpeptidase in mouse liver neoplasms, *Carcinogenesis*, 1, 813.
- Wogan, G.N., Edward, G.S., and Newberne, P.M. (1971), Structure activity relationships in toxicity and carcinogenicity of aflatoxins and analogs, *Cancer Res.*, 31, 1936-1942.
- Wogan, G.N., and Newberne, P.M. (1967), Dose response characteristics of AFP₁ carcinogenesis in the rat, *Cancer Res.*, 27, 2370-2376.

- Wogan, G.N., Paglialunga, S., and Newberne, P.M. (1974),
Carcinogenic effects of low dietary levels of aflatoxin B₁
in rats, *Fd. Cosmet. Toxicol.*, 12, 681-685.
- Wong, J.J., and Hsieh, D.P.H. (1976), Mutagenicity of aflatoxins
related to their metabolism and carcinogenic potential, *Proc.
Natl. Acad. Sci. USA*, 13, 2241-2244.

APPENDICES

Much of the work conducted in the fulfillment of the Ph.D degree has been in cooperation with other workers in the department, and thus does not appear in the first two manuscripts. The purpose of this section is to report results from experiments investigating the effects of in vivo modulators of AFB₁ carcinogenesis on AFB₁ mutagenesis in vitro, and on the isolation, purification and mutagenic potency of a new trout metabolite, AFL-M₁. Much of this work is included in manuscripts submitted for publication or in preparation.

APPENDIX I

Dietary Modification of AFB₁ metabolism by β -naphthoflavone (β -NF) in hepatic microsomes from rainbow trout

1. A new trout metabolite, 4-hydroxyaflatoxicol (AFL-M₁) was formed in small amounts in hepatic microsomes from β -NF fed trout, using AFL as a substrate. AFL-M₁ was undetected in control microsomes.
2. AFL-M₁ was isolated by reverse phase HPLC (μ Bondpak C₁₈; 0.2M KOAC, pH5, acetonitrile, methanol, tetrahydrofuran, 70:15:20:3) and identity compared to published descriptions by UV, MS and NMR analysis.
3. Mutagenesis experiments (Figure I) demonstrated AFL-M₁ had an activity 4.1% that of AFB₁, 6.1% of AFL and 400% that of AFM₁.
4. AFL-M₁ is therefore predicted to represent a detoxification product of AFB₁ or AFL on the basis of mutagenic activity and thus is expected to be less carcinogenic.

Reference: Loveland, P.M., Coulombe, R.A., Libbey, L.M., Pawlowski, NE., Sinnhuber, R.O., and Nixon, J.E. (1982), Identification and mutagenicity of aflatoxicol-M₁ produced by metabolism of aflatoxin B₁ and aflatoxicol by liver fractions from rainbow trout (*Salmo gairdneri*) fed β -naphthoflavone (manuscript in preparation).

Figure I. Modified Ames mutagen assay of AFL-M₁. Values on the ordinate represent the mean number of revertants (± 1 S.D.) of triplicate plates from three experiments. The slope at the regression line from 0-2 μ g AFL-M₁ was 23.2 ± 1.4 revertants/ μ g AFL-M₁ ($R = .874 \pm .016$).

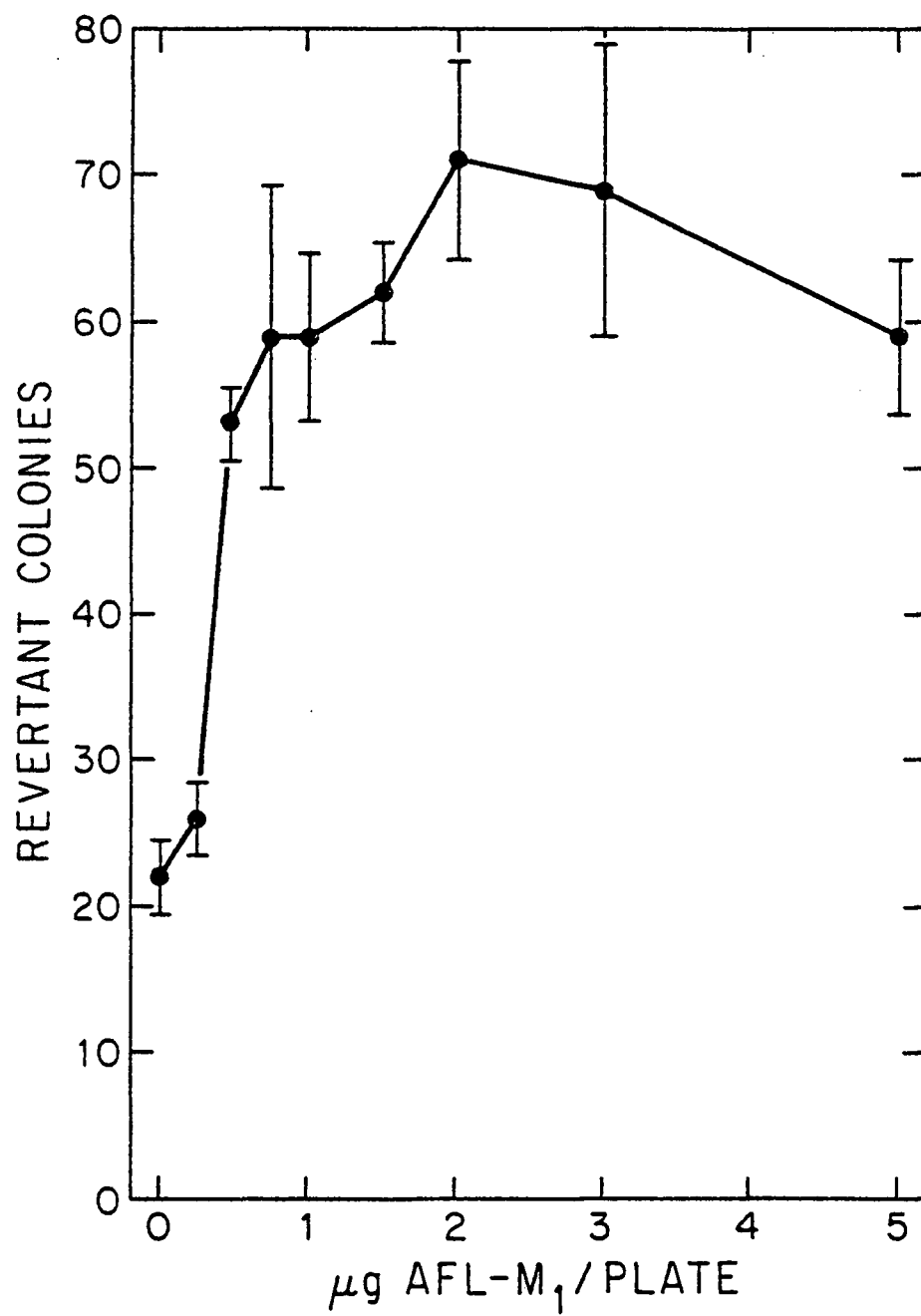


Figure I

APPENDIX II

Null effect of dietary β -naphthoflavone (β -NF) pretreatment
on mutagenesis of AFB₁ using hepatocytes and liver S20
from rainbow trout

1. Preliminary experiments demonstrated that dietary administration of β -NF markedly altered in vitro metabolism of AFB₁. For example, AFL production was significantly decreased, whereas production of AFM₁ was significantly increased in β -NF microsomes compared to controls. It was therefore of interest to evaluate possible effects of dietary β -NF on in vitro activation of AFB₁ by hepatocytes and trout liver S20.

2. Results from mutagenesis experiments showed that activation efficiencies of hepatocytes and liver S20 were unchanged in β -NF groups relative to controls (Tables IIa and IIb).

3. These findings do not reflect the demonstrated effect of β -NF in reducing AFB₁-DNA adducts in vivo (Whitham et al., 1982), as well as β -NF mediated reduction of hepatocellular carcinomas in trout in vivo (Bailey et al., 1982b).

4. Investigations of the relationship between DNA adduct formation and mutagenesis in S. typhimurium are in progress, in an attempt to explain the ostensible discrepancy in DNA binding and mutagenesis.

Table IIa. Null effect of dietary β -naphthoflavone on hepatocyte-mediated activation of AFB₁ in S. typhimurium.

Conc AFB ₁ (μ g/ml)	Revertant colonies ^{a,b,c}	
	Control	β -NF treated
0	12 \pm 2.1	18 \pm 4.9
1	17 \pm 2.0	21 \pm 4.9
2	44 \pm 0.7	42 \pm 1.4
4	79 \pm 12.7	86 \pm 21.2
10	204 \pm 30.4	198 \pm 55.2

^a revertant values represent average results \pm 1 S.D. from two paired (control and β -NF trout) experiments conducted on different days.

^b hepatocyte density: 5×10^6 cells/ml

^c no significant difference ($p < .05$) between control and β -NF treatment using students "t-test".

Table IIb. Null effect of dietary β -NF on liver S20 mediated mutagenesis of AFB₁ in S. typhimurium.

dose AFB ₁ /plate (μ g)	Revertant colonies ^{a,b}	
	Control	β -NF treated
0	41 \pm 0.0	43 \pm 2.6
20	117 \pm 20.3	97 \pm 17.6
50	161 \pm 23.5	125 \pm 21.9
100	177 \pm 43.6	147 \pm 8.1
200	374 \pm 61.4	330 \pm 37.5

^a revertant values represent mean \pm 1 S.D. of triplicate plates from three groups of five treated and one group of ten control trout.

^b no significant difference ($p < .05$) between control and β -NF treatment using students "t-test".

APPENDIX III

Effect of PCB on activation of AFB₁. Experiments were conducted to evaluate possible effects of dietary PCB as well as PCB added in vitro on mutagenesis of AFB₁.

1. In vitro experiments showed a PCB dose-related inhibition, which was high as 67% at the highest dose tested (500 µg Arochlor 1254/plate) (Table III).

2. Dietary PCB (100 ppm) was shown to decrease in vitro mutagenesis of AFB₁ (Figure III) which appears to reflect its in vivo inhibitory effect of AFB₁ carcinogenesis seen in trout.

Table III. The in vitro inhibitory effect of PCB on AFB₁ mutagenesis using Salmonella typhimurium strain TA 98.

Arochlor 1254 (µg/plate)	revertants per plate a,d	% inhibition
0 ^b	407 ± 66	0
50 ^c	282 ± 58	30.7
200	207 ± 54	49.1
350	194 ± 46	52.3
500	136 ± 35	66.6

^a mean ± S.D. of four plates per dose level.

^b control plates contained 100 µl DMSO, the maximum amount contained in test plates.

^c plates contained 0.80 µg AFB₁ in 80 µl ethanol.

reference: Shelton, Coulombe et al., 1982

Figure III. Effect of dietary Aroclor 1254 on in vitro mutagenesis of AFB₁. Values on the graph represent mean responses (± 1 S.D.) of triplicate plates from two groups of five PCB-fed trout and one group of six control trout.

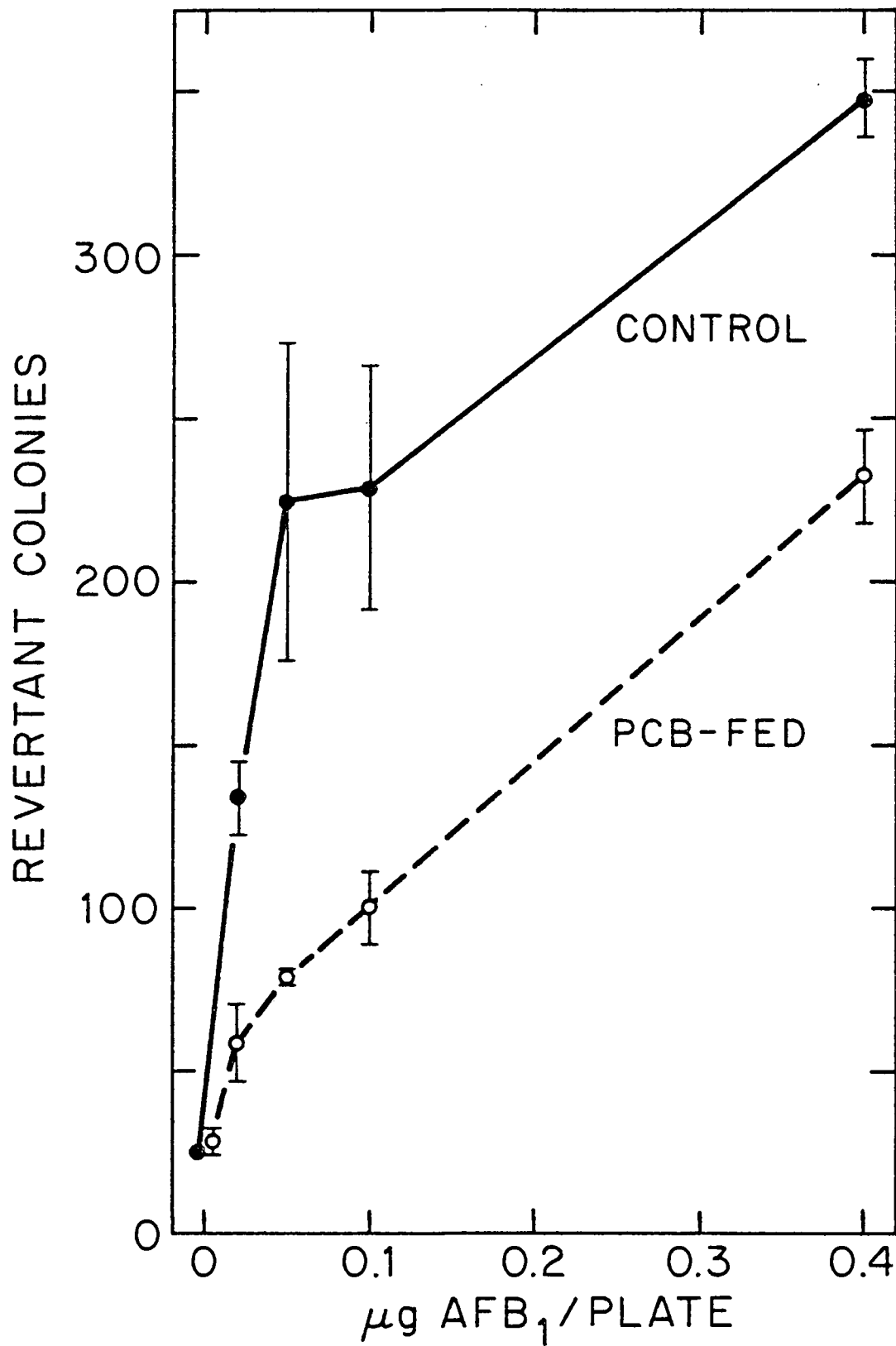


Figure III

APPENDIX IV

Null effect of dietary CPFAs on the mutagenic activity of AFB₁ in S. typhimurium TA 98.

1. In a cooperative study, the effect of dietary CPFAs on the hepatic mixed function oxidase system of rainbow trout was investigated. In addition to measuring cyt P-450, and in vitro oxidation of non-AFB₁ substrates, AFB₁ activation to bacterial mutagens was measured in CPFA and control groups.

2. Consistent with proposed mechanisms of many promoters, CPFA appear to have no effect on metabolic activation of AFB₁.

Table IV. Effect of dietary CPFAs on in vitro mutagenesis.

Dietary treatment (ppm CPFAs)	revertant colonies ^a
0	272 ± 70 ^{b,c}
50	255 ± 78
150	282 ± 75
300	264 ± 35
600	313 ± 52

^amean ± standard deviation of triplicate plates from three groups of five fish per treatment.

^bno significant difference ($p < .05$) between control and each diet containing CPFAs using students "t-test".

^c0.40 µg AFB₁ per plate.

3. In addition, CPFAs (as triglycerides, Me-esters or sodium salts) were not mutagenic to S. typhimurium when added in vitro.

Reference: Eisele, T.A., Coulombe, R.A., Williams, J.L., Shelton, D.W., and Nixon, J.E. (1982) Time and dose dependent effects of dietary cyclopropenoid fatty acids on the hepatic mixed function oxidase system of rainbow trout (manuscript in preparation).