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Indole-3-carbinol (I3C) and butylated hydroxyanisole (BHA), anti-carcinogens present in the human diet, were tested for their *in vivo* and *in vitro* effect on aflatoxin B_1 (AFB_1) metabolism, and DNA adduct formation in the rainbow trout. Dietary BHA at either 0.3 or 0.03% had no effect on the hepatic tumor incidence of trout exposed to a 0.5 ppm AFB_1 solution as embryos, or when fed prior to and during dietary exposure to 10 ppb AFB_1. Previous studies have shown 0.1% I3C to inhibit AFB_1-induced hepatomas in trout. When fed at 0.2%, I3C produced a 70% reduction in average *in vivo* hepatic DNA binding of injected AFB_1 over a 21 day period compared to controls. A similar study with 0.3% BHA had no effect on AFB_1-DNA binding over a 7 day period. One hr incubations of AFB_1 with freshly isolated hepatocytes from either BHA-, I3C- or control-fed trout showed no differences in AFB_1 metabolism or DNA binding between BHA hepatocytes and controls. However, I3C hepatocytes had 20% less DNA binding with a 2-fold increase in aflatoxin M_1 production. Additions of 0, 1, 10 or 100 uM BHA or I3C to hepatocytes isolated from trout fed a control diet had no effect on AFB_1-DNA adduct formation except for a 20% decrease in the 100 uM BHA hepatocytes. A 24 hr distribution study of injected

[^{3}H]-AFB_1 in trout fed 0.3% I3C showed less total radioactivity in the blood and liver at all times examined, compared to controls. These reductions were accountable primarily as reduced levels of AFB_1 bound to red blood cell DNA, reduced plasma levels of the metabolite aflatoxicol (AFL), and decreased levels of AFB_1 and polar metabolites in the liver of I3C trout. Total radioactivity was significantly elevated in the bile of I3C fish resulting from a 7-fold increase in aflatoxicol-M_1 glucuronide levels over controls. AFL glucuronide levels were similar between treatments. Total radioactivity remaining in the carcasses of I3C or control trout was similar.

These data indicate that I3C inhibits AFB_1 hepatocarcinogenesis in trout through changes in carcinogen distribution, metabolism and elimination leading to reduced initial DNA damage. BHA does not appear to alter enzymes responsible for AFB_1 metabolism, and though it may have a weak direct affect on AFB_1-DNA adduct formation, this does not appear to be of importance <u>in vivo</u> since BHA had no effect on AFB_1-induced carcinogenesis.

MECHANISMS OF ACTION BY SOME INHIBITORS OF AFLATOXIN B_1 CARCINOGENESIS IN RAINBOW TROUT

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CONTRIBUTIONS OF AUTHORS

The coauthors of the manuscripts presented here have made the following contributions. Dennis Shelton quantitated total aflatoxin B_1 and metabolites present in the whole blood, and aflatoxin B_1-DNA adducts in liver and red blood cells of trout used in the 24 hr *in vivo* AFB_1 metabolism and distribution portion of the indole-3-carbinol study. He also helped with injecting, terminating and tissue collection from these same trout, and aided in interpretation of results. Jerry Hendricks helped with injecting, terminating and tissue collection from trout used in the 24 hr *in vivo* AFB_1 metabolism and distribution portion of the indole-3-carbinol study. He also conducted the *in vivo* studies on pre- and post-initiation effects of butylated hydroxyanisole on aflatoxin B_1 tumorigenesis in trout, and helped in writing that portion of the manuscript. George Bailey contributed to experimental design and interpretation of results from both studies and in preparation and review of the manuscripts.

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CHAPTER I

INTRODUCTION

The influence of diet on health has been the subject of many investigations. During the first half of the 20th century, many of these studies were concerned with anomalies resulting from nutrient deficiencies. As a result of these studies, most of the essential vitamins, minerals, fatty acids and amino acids have been identified as well as the disorders which occur when they are not consumed at adequate levels. Consequently, the longevity that is characteristic of people in developed countries is due, in part, to improvements in nutrition. More recently, studies concerned with diet and health have been focused more on diseases that are associated with aging, such as cancer and heart disease. Although progress has been made in these areas, considerable work is needed to fully explain such relationships.

Various forms of cancer have been shown to be inhibited by certain components found in the diet. These inhibitors may be present as either natural constituents or as food additives. Two such inhibitors are indole-3-carbinol, which is naturally occurring in certain vegetables, and butylated hydroxyanisole, a commonly used food preservative. Although these compounds inhibit certain chemically induced cancers, the exact mechanisms through which they operate

remain unclear. It is important that such mechanisms of action be determined since they could also provide valuable information on the processes of carcinogenesis itself. The use of inhibitors in preventing cancer would be a viable alternative to prevention by eliminating exposure since the ubiquitous occurrence of carcinogens in the environment would make the latter method all but impossible.

The following studies were conducted to investigate the effect of indole-3-carbinol and butylated hydroxyanisole on the metabolism of aflatoxin B_1, a food borne carcinogen. Both *in vivo* and *in vitro* techniques were employed using the rainbow trout as the experimental model. The trout is extremely sensitive to the carcinogenic effect of aflatoxin B_1 and other chemical carcinogens. Results obtained on the mechanisms of anti-carcinogenesis in species other than the more traditional laboratory animals are valuable for comparative purposes. The more consistent these mechanisms are between various species, the more likely they are to operate in a similar manner in humans.

CHAPTER II

LITERATURE REVIEW

The Relation Between Diet and Cancer

Through both experimental and epidemiological studies, the relationship between diet and certain forms of cancer has become evident (Stich, 1982; Wynder et al., 1983). One reason for such a relationship is the presence of carcinogens in food as well as their precursors which, under proper conditions, can form carcinogenic compounds. For example, nitrites are found naturally in foods and are also used as food additives. In aqueous solutions, the nitrite ion exists in equilibrium in various chemical forms, one of which is nitrous anhydride (Scanlan, 1975). Secondary amines which are also natural constituents of foods can, under proper conditions of pH, react with nitrous anhydride to produce nitrosamines. These compounds are not only carcinogenic but are toxic, teratogenic and mutagenic in a wide variety of lab animals. Another example of carcinogens that are produced from precursors in foods are heterocyclic amines. These compounds are formed in proteinaceous foods as a result of the pyrolysis of certain amino acids (Sugimura, 1982). The heterocyclic amines have been shown to be mutagenic to Salmonella typhimurium and are carcinogenic when fed to mice. Carcinogenic polycyclic aromatic hydrocarbons, including benzo[a]pyrene (BP) have also been found in meat after charcoal broiling (Lijinsky and Subik, 1964). Some carcinogens which are present as natural constituents in food include

safrole found in certain spices, hydrazines present in some edible mushrooms, flavonoids and quinones which are widespread in the diet and cyclopropenoid fatty acids found in oils of certain plants in the order Malvales (Ames, 1983). Mold metabolites, produced during improper storage or processing of foods, are also important sources of dietary carcinogens (Stoloff, 1976). One such metabolite, aflatoxin B_1 (AFB_1), is produced by Aspergillus flavus and A. parasiticus, and is one of the most potent naturally occurring carcinogens known (Bailey et al., 1982b). AFB_1 is metabolized by the mixed function oxidase enzymes (MFO) to metabolites with varying degrees of carcinogenicity. This will be discussed in greater detail in the following section.

Although it would appear that the diet is an unavoidable source of carcinogen exposure, it is also a source of compounds that are capable of inhibiting carcinogenesis (Ames, 1983; Fiala et al., 1985; Wattenberg, 1983). Like some of the dietary carcinogens, a large portion of these anti-carcinogens exist as natural components of foods. For example, retinoids, which are also the source of vitamin A, have been shown to prevent chemically induced mammary and bladder cancers. Presumably, the retinoids act at the promotional stages of carcinogenesis (Moon et al., 1983; Reddy, 1984). Green coffee beans, cabbage, cauliflower and orange oil are capable of inhibiting dimethylbenz[a]anthracene (DMBA) induced neoplasias in rats even when fed one week following carcinogen exposure (Wattenberg, 1983). Although the specific compounds responsible for this action have not been identified, these foods do contain indoles and isothiocyanates

which have been shown to prevent carcinogenesis when administered prior to initiation. Phenolic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene, although not natural dietary constituents, are commonly used food additives and have been shown to inhibit chemical carcinogenesis (Wattenberg, 1983).

The mechanisms by which anti-carcinogens work are numerous and many remain only speculative. One such mechanism may operate by preventing the formation of the carcinogen from precursor compounds (Wattenberg, 1983). An example of this is demonstrated by ascorbic acid and the tocopherols which inhibit nitrosamine formation (Mirvish, 1983). These anti-carcinogens reduce nitrous anhydride to nitric oxide thereby decreasing the level of the nitrosating agent available for nitrosamine formation. Another possible mode of action for anti-carcinogens would be by preventing the carcinogen from reaching the "target" tissue where initiation occurs (Wattenberg, 1983). There are several ways by which this may be brought about. First, the inhibitor may prevent the enzymatic formation of the electrophilic ultimate carcinogen. Second, they may act by scavenging, or increasing the level of compounds capable of scavenging, the ultimate carcinogen once formed. Finally, they could increase the activity or level of enzymes that detoxify the carcinogen. Another point in the carcinogenic process where an inhibitor might act is during the final stages where expression of the transformed or initiated cell is inhibited. This latter group of inhibitors is different from the previous ones in that they are effective when administered after exposure to the carcinogen.

Anti-carcinogens offer an appealing method to reduce human cancer

risks. Ideally, these compounds could be added to the diet to eliminate the adverse effects of carcinogens present which could not otherwise easily be removed. Studies on the mechanisms of action of anti-carcinogens can also provide information on the processes of carcinogenesis, and provide information on compounds with potential for therapeutic treatment of various cancers. Due to the abundance of information on inhibitors of chemical carcinogenesis, the ones to be discussed in this review will be limited to indole-3-carbinol (I3C) and BHA. Both compounds are common constituents of the daily human diet.

Aflatoxin B_1 Carcinogenesis and Metabolism

To better evaluate the mechanism of action of an anti-carcinogen, it is important to first understand the metabolism of the carcinogen that is being inhibited. In the present studies, the carcinogenic mycotoxin AFB_1 (Fig. II-1.) is the chemical carcinogen used since it constitutes a major health risk. AFB_1 is often incorporated in foods and feedstuffs as a result of the inadvertent use of mold-contaminated grains. The ingestion of AFB_1 by food producing animals can also result in accumulation of metabolites in edible tissues (Rodricks and Stoloff, 1977). AFB_1 is carcinogenic in a variety of animals (Newberne and Butler, 1969) and epidemiological studies have implicated it as the etiologic agent in the induction of hepatomas in man (Linsell and Peers, 1977; Shank, 1977a).

AFB_1 itself is not carcinogenic, but is converted by the MFO to the electrophilic 2,3-epoxide (Swenson *et al.*, 1974; Swenson *et al.*, 1977). This intermediate can then bind covalently to DNA or other cellular nucleophiles producing its toxic effect. AFB_1 is mutagenic to *Salmonella typhimurium* only when incubated in the presence of liver homogenate fractions and not when incubated alone (Shank, 1977b; Wogan, 1977). The formation of this mutagenic factor was found to be dependent on the liver microsomal fraction, oxygen and NADPH, and its formation was inhibited by carbon monoxide. All these factors indicate MFO involvement.

The principal *in vivo* AFB_1 adduct formed in the liver DNA of rats is 2,3-dihydro-2-(N^7-guanyl)-3-hydroxy-aflatoxin B_1 (Croy *et al.*, 1978), and it is chromatographically identical to the primary adduct

formed in rainbow trout (Croy et al., 1980). Two hours after IP injection of AFB_1, 80% of the AFB_1 associated with rat liver nuclear chromatin is bound to DNA (Groopman et al., 1980). Of the remaining 20%, half can be removed by dialysis and the remainder is bound to the dialyzed chromatin protein fraction. Studies on the distribution of AFB_1 adducts in trout liver DNA showed that AFB_1 is approximately five times more likely, on a nucleotide basis, to bind to the DNA located between nucleosomes as it is to bind to the nucleosomal core DNA (Bailey et al., 1980). Although the ultimate genomic damage responsible for the initiation of AFB_1 carcinogenesis is unknown, a good correlation does exist between AFB_1-DNA binding and mutagenesis in Salmonella typhimurium (Gurtoo et al., 1978) as well as carcinogenesis in the trout (Whitham et al., 1982). Thus, DNA adduct formation is often used as an indicator of initiation damage preceding carcinogenesis.

The MFO and certain cytosolic reductases may act on AFB_1 either singularly or together to produce a wide variety of metabolites (Campbell and Hayes, 1976). The toxicity of AFB_1 in a given species appears to be determined by its metabolism in a dynamic system of competing enzymatic pathways. Using the S10 liver fraction from six species, along with an NADPH generating system, Hsieh et al. (1977) found a distinct species variation in the metabolism of AFB_1 as determined by type, quantity and ratio of metabolites produced. Some of these AFB_1 metabolites include aflatoxin M_1 (AFM_1), aflatoxin P_1 (AFP_1), aflatoxin Q_1 (AFQ_1), aflatoxicol (AFL), aflatoxicol H_1 ($AFL\text{-}H_1$) and aflatoxicol M_1 ($AFL\text{-}M_1$) (Fig. II-1.).

AFL is the only metabolite of AFB_1 that is not dependent on MFO involvement. A soluble enzyme is present in the cytosol which catalyzes a reversible reduction of the carbonyl group in the cyclopentenone ring of AFB_1 (Shank, 1977b). Since this is a reversible reaction, it is thought that AFL acts as a reservoir of AFB_1 (Campbell and Hayes, 1976) and therefore may be why AFL has similar carcinogenicity as AFB_1 (Hendricks et al., 1980; Schoenhard et al., 1981). AFL is the major in vitro AFB_1 metabolite produced by rainbow trout liver fractions (Loveland et al., 1977; Schoenhard et al., 1976). Since this route of primary AFB_1 metabolism by trout does not result in detoxification, this may be one reason for its extreme sensitivity to AFB_1-induced hepatocellular carcinomas (Sinnhuber et al., 1977a).

AFM_1 was the first AFB_1 metabolite identified when it was isolated from the milk of cows fed AFB_1 (Campbell and Hayes, 1976). In other species, it has also been found in the urine and feces (Shank, 1977b) and it is produced primarily in animals that have been dosed orally with AFB_1 (Campbell and Hayes, 1976). The carcinogenicity and mutagenicity of AFM_1 are considerably less than that of AFB_1 (Campbell and Hayes, 1976; Hendricks et al., 1980; Sinnhuber et al., 1974). However, the acute toxicity, as determined by the day-old duckling assay, is similar to AFB_1 which suggests different mechanisms for carcinogenicity and acute toxicity (Shank, 1977b). The in vitro production of AFM_1 is increased in liver preparations from animals pretreated with phenobarbital (PB), 3-methylcholanthrene (3-MC) or beta-naphthoflavone (BNF), but inhibited

by treatment with SKF-525A (Campbell and Hayes, 1976; Gurtoo and Dahms, 1979). PB, 3-MC and BNF are all inducers of the MFO while SKF-525A is an inhibitor.

AFP_1 was initially overlooked in metabolic studies of AFB_1 since it was present in the aqueous phase of chloroform extracts of urine while the organic phase contained AFB_1 and most of its metabolites (Shank, 1977b). This is due to the presence of a hydroxyl group, produced by O-demethylation of the C^{17}-methoxy position, which increases its hydrophilicity. This position is then conjugated with either glucuronic acid or sulfate (Campbell and Hayes, 1976). Some AFP_1 may also be excreted as the free phenol. O-demethylation appears to be the reaction responsible for AFP_1 formation since 25% of the ^{14}C label of [^{14}C]methoxy AFB_1 appears in the respired CO_2 of rats 24 hours after dosing. AFP_1 formation also requires the NADPH-dependent MFO and its production <u>in vitro</u> is inhibited by SKF-525A. Due to its hydrophilic nature, AFP_1 is readily excreted and its toxicity is therefore less than AFB_1.

AFQ_1 is another hydroxy metabolite of AFB_1 and it represents approximately 30% of the metabolites produced when AFB_1 is incubated <u>in vitro</u> with human and rhesus monkey liver preparations (Salhab and Hsieh, 1975). This metabolite is not found in the urine presumably because little is conjugated (Shank, 1977b). A lesser metabolite produced by man and rhesus monkey is $AFL\text{-}H_1$. This may be viewed as either a reduced form of AFQ_1 or an oxidized form of AFL (Salhab and Hsieh, 1975). $AFL\text{-}H_1$ formation is therefore dependent on two enzyme systems located in different cellular compartments. One, in the

microsomal fraction, is responsible for the NADPH-dependent hydroxylation of AFB_1 to form AFQ_1 and the other, a reductase located in the S105 supernatant reduces AFQ_1 to $AFL\text{-}H_1$.

Another metabolite of AFB_1 which is produced by the action of both the MFO and a soluble reductase is $AFL\text{-}M_1$. This metabolite was first identified in in vitro incubations of AFB_1 and dog S12 liver fractions (Salhab et al., 1977). It was later identified as a major AFB_1 metabolite formed in liver fractions from rainbow trout fed BNF (Loveland et al., 1983). In the trout, $AFL\text{-}M_1$ may be produced either by a reduction of AFM_1 or by an oxidation of AFL. Although the mutagenicity of $AFL\text{-}M_1$ is only 5% of that found with AFB_1 or AFL (Coulombe et al., 1982), its carcinogenicity remains to be determined.

Indole-3-Carbinol as an Inhibitor of Chemical Carcinogenesis

An increasing amount of research in recent years is concerned with I3C and its mechanisms of inhibiting chemical carcinogenesis. I3C is one of a group of indole compounds produced from the enzymatic degradation of the glucosinolate 3-indolylmethylglucosinolate (Searle et al., 1984; Van Etten et al., 1969). The glucosinolate is hydrolyzed to the aglycon by the endogenous enzyme myrosinase (or thioglucosidase) when the wet, unheated plant material is crushed. Various physical conditions such as pH and metal ion concentration may contribute to the formation of either I3C or other indole analogs (Searle et al., 1984).

The anti-carcinogenic properties of cruciferous vegetables were first indicated when it was shown that feeding the dried plant material to rats induced certain MFO enzymes in the liver, small intestine and lung (Babish and Stoewsand, 1975; Wattenberg, 1972a). However, this is a rather nonspecific change since commercial rat diets had similar affects when compared to semipurified diets because of the presence of exogenous inducers. When various chemical extracts of dried brussels sprouts, cabbage or cauliflower were given by gavage to 5-week-old female rats, it was found that 60% of the increased aryl hydrocarbon hydroxylase (AHH) activity associated with these plants was accounted for by the ethyl acetate-soluble compounds (Loub et al., 1975). The major component of this fraction was determined to be indole derivatives. Further examination of these indoles showed I3C to be the most potent inducer of AHH activity in the small intestine of rats. Further evidence for the inductive capabilities of dietary

cruciferous vegetables on xenobiotic metabolism was subsequently shown by others (Bradfield and Bjeldanes, 1984; Pantuck et al., 1976). Pantuck et al. (1976) showed that diets containing either cabbage or brussels sprouts increased the in vitro intestinal metabolism of hexobarbital, phenacetin, 7-ethoxycoumarin and BP in the rat. Similar results were also obtained when pure indole compounds, including I3C, were given by gavage. When incorporated into semipurified diets, I3C was shown to induce both renal and hepatic AHH activity as well as hepatic N- and O-demethylase activity in rats but only at levels of 5000 and 7500 ppm (Babish and Stoewsand, 1978). The induction of intestinal AHH activity was seen only at the 7500 ppm level with no enzymatic alterations being detected at 50 or 500 ppm. The 5000 and 7500 ppm I3C diets also increased urinary glucuronide levels which indicate the induction of detoxification enzymes in vivo. Although Babish and Stoewsand (1978) determined these dietary levels of I3C to be far in excess of those that would be expected in vegetable containing diets and therefore irrelevant, Bradfield and Bjeldanes (1984) found enhanced intestinal MFO activities in rats with dietary I3C levels as low as 50 ppm. From dose curves, they calculated a no-effect level for dietary I3C induction of intestinal MFO activity to be approximately 25 ppm. This is interesting since they found that dietary I3C up to 500 ppm had no effect on similar hepatic enzymes.

Although the data support I3C as a MFO inducer, enhanced activity of enzymes responsible for carcinogen metabolism does not necessarily indicate anti-carcinogenesis. Most chemical carcinogens require enzymatic activation prior to initiation damage, so if these enzymes

are preferentially induced over detoxification enzymes, then the opposite would be expected. However, studies have shown that dietary I3C as well as cruciferous vegetables do inhibit chemically induced neoplasias (Boyd et al., 1982; Nixon et al., 1984; Wattenberg and Loub, 1978; Wattenberg, 1983). I3C, when given by PO intubation (0.1 mMol) or fed at 0.4% in a purified diet was shown to inhibit DMBA-induced mammary tumors in rats (Wattenberg and Loub, 1978). Dietary I3C at 0.9% also reduced the total number of forestomach tumors in mice treated with BP. DMBA-induced mammary tumors in rats were also inhibited by dietary cabbage and cauliflower (Wattenberg, 1983). The protective effects of I3C on the AFB_1-induced hepatocellular carcinoma incidence in trout was also demonstrated (Nixon et al., 1984). Feeding 0.1% I3C to trout resulted in a 4% hepatic neoplasm incidence 58 weeks after a 10 day exposure to 20 ppb dietary AFB_1. Trout receiving only AFB_1 had a 38% neoplasm incidence. When rats were fed diets containing 1 ppm AFB_1 for 26 weeks, the inclusion of 25% cabbage resulted in a significant decrease in the number of surface tumors on the liver of rats (Boyd et al., 1982).

Recent experimental data suggest that the apparent relationship between MFO induction and I3C inhibition of chemical carcinogenesis may only be incidental. Using the in vitro binding of BP to DNA and protein as an indicator of BP initiation in mice, Shertzer (1983) found the level of binding to reach a minimum two hours after I3C gavage. However, AHH activity increased after a lag period of 8-12 hours. The increased AHH activity was nearly double 24 hours after dosing at which time BP binding was near the zero-time value. Similar

results were obtained with *in vivo* binding. In the trout, I3C produces a dramatic reduction in the AFB_1 hepatic neoplasm incidence as well as *in vivo* binding of AFB_1 to liver DNA (Nixon *et al.*, 1984), but fails to increase liver MFO activity (Eisele *et al.*, 1983a). By contrast, a BNF diet (0.05%) which produces similar decreases in AFB_1 neoplasm incidence and hepatic DNA adduct formation in the trout as 0.1% I3C (Nixon *et al.*, 1984), has a dramatic effect on MFO levels and *in vitro* AFB_1 metabolism. The most significant changes consist of increases in cytochrome P-448 and in the production of AFM_1 and of glucuronide conjugates, both of which are AFB_1 detoxification products (Bailey *et al.*, 1984; Loveland *et al.*, 1984; Nixon *et al.*, 1984).

It is possible that I3C induced biochemical changes, other than those seen in the MFO, may be responsible for the anti-carcinogenic action of I3C. Sparnins *et al.* (1982) have shown that I3C enhances glutathione (GSH) S-transferase activity as do other inhibitors of chemical carcinogenesis. A direct interaction of I3C, or a metabolite of I3C, with the carcinogen may also be possible (Shertzer, 1983).

Butylated Hydroxyanisole as an Inhibitor of Chemical Carcinogenesis

BHA is an antioxidant that is commonly added to foods to prevent oxidative damage. It acts by donating a hydrogen to free radicals produced thereby preventing their propagation which can lead to oxidation of susceptible molecules (Gunstone and Norris, 1983; Branen, 1975). In 1948, the FDA approved the use of BHA in foods at levels up to 0.02%. Because of its widespread use, it has been extensively evaluated for its safety using both chronic and acute protocols (Branen, 1975).

Like I3C, BHA has been shown to inhibit chemical carcinogenesis as well as mutagenesis and toxicities in a wide range of animals and test systems (Josephy et al., 1985; Rao et al., 1984; Rosenbaum et al., 1984; Tsuda et al., 1984; Wattenberg, 1972b; Wattenberg, 1973). However, BHA does have the dubious distinction of also being carcinogenic and being capable of promoting carcinogenesis under certain experimental protocols (Imaida et al., 1983; Ito et al., 1983; Tsuda et al., 1984). For example Ito et al. (1983) found a dose-response relationship between dietary BHA and the incidence of forestomach papillomas and squamous cell carcinomas in male and female rats. The incidence of papillomas was greater than 95% with one third of the rats developing squamous cell carcinomas after consuming 1% BHA diets for 104 weeks. Rats fed 0.25% BHA for the same period of time had only a 2% incidence of papillomas but no animals developed squamous cell carcinomas. Tsuda et al. (1984) found a slight, although non-significant, promotional effect in the development of preneoplastic lesions in the kidneys of rats fed 2% BHA for 29 weeks

after exposure to N-ethyl-N-hydroxyethylnitrosamine. However, the incidences of pre-neoplastic lesions in liver, and hepatocellular carcinoma incidences were significantly decreased in these same rats. Similarly, a difference in organ response to the promotional effects of BHA has been demonstrated by Imaida et al. (1983). They found that when BHA was fed at levels of 1 or 2% for 32 weeks after N-butyl-(4-hydroxybutyl)-nitrosamine exposure, there was an increase in the numbers of urinary bladder tumors in rats. However, a six week treatment with the same levels of BHA following diethylnitrosamine exposure decreased the number of pre-neoplastic gamma-glutamyl transpeptidase positive foci in the livers. Although these studies do demonstrate the carcinogenic and promotional potential for dietary BHA, these are evident only after high doses given for extended lengths of time.

Many of the anti-carcinogenic studies with BHA deal with its ability to induce enzymes involved in xenobiotic detoxification. In particular, BHA appears to be very effective in inducing GSH S-transferase activity in various tissues of rats and mice (Aspry and Bjeldanes, 1983; Benson et al., 1979; Sparnins et al., 1982). Liver cytosols from rats and mice fed BHA were capable of decreasing by 75% the mutagenicity of urine from mice injected with BP if GSH was included in the incubations (Benson et al., 1978). These decreases were found to be dependent on the addition of both cytosol and GSH, and the cytosols from BHA-treated animals were much more effective than cytosols from control animals. BHA at 0.75% in the diets of rats and mice increased GSH S-transferase activity as determined by using

several substrates. The mouse cytosols, which had a 5- to 11-fold increase in GSH S-transferase activity over controls compared to a 2-fold increase found in rat liver cytosols, were much more effective in decreasing the mutagenicity of the mouse urine. Although BP and GSH conjugation were not directly assayed in these studies, Dock et al. (1984) showed directly that hepatic cytosols of BHA-fed mice conjugated twice as much of the ultimate BP carcinogen with GSH as did cytosols from mice fed a standard diet. A similar comparison made using 1-chloro-2,4-dinitrobenzene as a substrate showed a 14-fold increase in conjugating capacity with BHA-treated mice cytosols. However, these researchers hypothesized that the increased capacity for GSH conjugation may not be the primary mechanism for inhibiting BP carcinogenesis. Since control mouse liver cytosols could produce 5 nMol BP-GSH conjugates/min/mg protein, this level of activity is unlikely to be saturated by in vivo production of the ultimate BP carcinogen. Therefore, they concluded that an increase in GSH S-transferase would probably not increase detoxification for a pathway that was not otherwise overburdened.

Dietary BHA induction of GSH S-transferase has also been shown to reduce the binding of AFB_1 to rat liver DNA both in vivo and in vitro (Kensler et al., 1985; Lotlikar et al., 1984). A correlation was demonstrated in vivo between the level of GSH S-transferase induction by various dietary antioxidants, including BHA, and the amount of inhibition of AFB_1-DNA adduct formation (Kensler et al., 1985). In vitro microsomal mediated AFB_1-DNA adduct formation is not affected by dietary BHA, but addition of liver cytosols from BHA-treated rats to

these incubations produces a several-fold reduction in adduct formation when compared to cytosols from control rats (Lotlikar et al., 1984). The cytosols from the BHA-treated rats had significantly higher GSH S-transferase activity compared to controls.

Other xenobiotic detoxification enzymes induced by BHA include microsomal UDP-glucuronyltransferases and epoxide hydrolase (Aspry and Bjeldanes, 1983; Benson et al., 1979; Cha et al., 1978; Hjelle et al., 1985; Kensler et al., 1985). Glucuronide conjugates are the major BHA metabolites excreted by rodents (Cha and Heine, 1982). Glucuronides of BHA also represent a major metabolite in humans since 20% of an orally administrated dose was shown to be excreted in the urine as the glucuronide conjugate 24 hours after dosing (Castelli et al., 1984). It is therefore not surprising that BHA increases the activity of this transferase as well as other enzymes and cofactors needed for glucuronide conjugation since these play a major role in BHA metabolism. For example, hepatocytes isolated from mice fed BHA have a 5-fold increase in the glucuronidation rate of the drugs harmol and paracetamol (Moldeus et al., 1982). When BHA is fed to rats and mice, there is an increase in UDP-glucuronic acid levels as well as an increase in the level of the enzyme UDP-glucose dehydrogenase which is responsible for UDP-glucuronic acid production (Cha and Heine, 1982; Hjelle et al., 1985). The rate of glucuronidation has been shown to be dependent on levels of the cofactor UDP-glucuronic acid (Aw and Jones, 1984; Singh and Schwarz, 1981). Increased glucuronide conjugating activity is thought to play a role in BHA anti-carcinogenesis since it would aide in detoxification and elimination

of either the procarcinogens or reactive metabolites produced during their activation.

The increase in activity of epoxide hydrase from BHA pretreatment is more difficult to correlate with anti-carcinogenesis since this enzyme may participate in the enzymatic activation of some carcinogens. For instance, epoxide hydrase is needed in one step during the production of the ultimate BP carcinogen, the diol-epoxide (Benson et al., 1982). However, this carcinogen as well as the ultimate carcinogen produced from other polycyclic aromatic hydrocarbons as well as AFB_1 are thought to be epoxides which can be converted to less reactive diols by the action of epoxide hydrases (Oesch, 1972). Increases in the activity of this enzyme do occur in tissues of both rats and mice which include the stomach and colon, organs most responsive to the anti-carcinogenic action of BHA (Benson et al., 1982).

BHA also produces changes in the MFO that can lead to altered metabolism of carcinogens. These changes appear to be selective in nature since BHA can alter enzymatic activities without increasing the total level of P-450s. When BP is incubated with microsomes from BHA-fed mice the metabolites produced are different than those produced by control microsomes (Sydor et al., 1983). There is an increase in the production of more polar diols with a decrease in formation of the electrophilic diol epoxide. The overall rate of BP metabolism is also increased in the microsomes from BHA-fed mice. Such changes in BP metabolism would account for the decreased formation of in vivo BP-DNA adducts found in liver, lung and forestomach of BHA-fed mice (Anderson

et al., 1981; Ioannou et al., 1982). These regioselective changes in BP metabolism occurred without a significant increase in P-450 levels (Sydor et al., 1983). There are, however, changes in SDS-polyacrylamide gel electrophoresis patterns of the microsomal proteins and changes in kinetic parameters of ethoxycoumarin O-deethylase. Such changes suggest that BHA pretreatment produces alterations in specific P-450 isozymes present in the microsomes. Cha and Heine (1982) also found increases in liver MFO activity of mice fed BHA without any changes in total P-450 content. The inverse was found with rats where liver microsomal P-450 content was increased in BHA-fed animals without any change in MFO activity as determined by several substrates. Another species-dependent alteration in BHA-mediated metabolism was demonstrated using liver microsomes from trout fed BHA (Eisele et al., 1983b) where increases in certain MFO activities were detected with a significant decrease in P-450 levels. Other studies have also shown significant qualitative changes in MFO metabolism of xenobiotics using microsomes from BHA-treated animals (Lam and Wattenberg, 1977; Speier and Wattenberg, 1975; Rosenbaum et al., 1984). If such changes in microsomal metabolism would result in production of less carcinogenic metabolites, then this would be one way in which BHA could inhibit carcinogenesis.

BHA could also inhibit carcinogenesis through direct interaction at some point in the carcinogenic process. This would not require a pretreatment period which is required for inducing enzymes involved in detoxification but only that BHA be administered with or just prior to carcinogen exposure. One point for direct interaction would be by

direct inhibition of the enzymatic conversion of a procarcinogen to the ultimate carcinogen. When BHA is added to mouse liver microsomes at 30 uM along with BP, there is a 30% reduction in total BP metabolized (Sydor et al., 1983). Levine (1984) also showed a BHA concentration-dependent reduction in metabolism of the hepatocarcinogen N,N-dimethyl-4-aminoazobenzene by liver microsomes from rats. BHA was also found to be an inhibitor of AHH activity in microsomes from several organs of both mice and rats (Yang et al., 1981). It does not appear that all MFO enzymes are inhibited to the same extent by BHA. Using reconstituted hepatic P-450 systems purified from rats pretreated with MFO inducers, it was shown that the 3-MC and 2,3,7,8-tetrachlorodibenzo-p-dioxin inducible forms are less susceptible to inhibition by BHA than the P-450 present in control or phenobarbital-induced rats. BHA does form a complex with P-450 since it does produce a typical type I binding spectra with P-450 isolated from rat livers (Yang et al., 1974).

Another possible direct anti-carcinogenic mechanism for BHA was presented by Cummings and Prough (1983). They determined that a metabolite of BHA was capable of reacting with NADPH-cytochrome c reductase which resulted in the direct reduction of molecular oxygen, producing hydrogen peroxide as at least one product. Concurrent with this apparent uncoupling of the MFO electron transport system was an inhibition of benzphetamine N-demethylase activity; both effects were found to be dependent on BHA concentration.

A final mechanism of anti-carcinogenesis involving the direct action of BHA is through interaction with the carcinogen. It has been

shown that dietary BHA inhibits the formation of liver tumors in rats exposed to ciprofibrate (Rao et al., 1984). The carcinogenesis of this compound is believed to be mediated through free radical damage caused by hydrogen peroxide-produced active oxygen. Since BHA is a stable free radical acceptor, it could eliminate the free radicals produced and prevent such damage. Working by a similar mechanism, BHA has been shown to inhibit benzidine mutagenesis in the Ames test (Josephy et al., 1985). Benzidine is oxidized by peroxidases to a free radical intermediate that can bind to proteins and DNA (Josephy and Damme, 1984). This reactive intermediate can be chemically trapped by BHA thereby protecting vital cellular nucleophiles.

Although many mechanisms have been proposed to explain the anti-carcinogenic action of BHA, it is doubtful whether any one acts alone. Although one may contribute more to such activity than another, relative contributions would be dependent on the chemical carcinogen in question, the "target" tissue or organ affected, the timing relative to carcinogen exposure, and the species of animal. The most probable situation is that several mechanisms acting together are responsible for this activity.

Isolated Hepatocytes in Drug Metabolism Studies

The use of freshly isolated hepatocytes for *in vitro* studies of cellular metabolism is becoming more widespread. The fact that sufficient cells can be isolated from one animal to permit several parallel tests or treatments to be conducted helps to overcome some of the limitations of biological variability that exist between individuals. The extracellular environment can also be changed rapidly and uniformly in respect to nutrients, dissolved gases and xenobiotics (Goethals *et al.*, 1984). More importantly, isolated hepatocytes maintain, at least temporarily, the qualitative and quantitative characteristics of the intact liver (Cornell, 1985). This is imperative for many metabolic studies since compartmentation of enzymes plays a significant role in the integration and control of various metabolic pathways (Cornell *et al.*, 1985). For example, as pointed out earlier on AFB_1 metabolism, there are enzymes present in both the cytosol and the endoplasmic reticulum (microsomes) which may act singularly or together to produce various AFB_1 metabolites. Once produced, these metabolites may be conjugated by either cytosolic transferases, such as GSH S-transferase, or by glucuronyl transferase which is found in the microsomes. All these enzymatic reactions are dependent on cofactors that are generated in the cytosol. In order to study the *in vitro* metabolism of AFB_1 as well as other compounds metabolized by multiple enzyme systems, it is necessary to maintain cell compartmentation such that it approaches that found *in vivo*. Isolated hepatocytes therefore have a distinct advantage over more traditional broken cell preparations in conducting these types of

studies.

Berry and Friend (1969) were the first to develop a technique for producing a high-yield of hepatocytes from rat liver. This procedure employed a continuously recirculated perfusate consisting of a Ca^{+2}-free buffer containing collagenase and hyaluronidase. Later it was found that an initial Ca^{+2}-free perfusion followed by the perfusate containing the degradative enzymes with Ca^{+2} resulted in an increased recovery of cells (Seglen, 1973). This effect was thought to be due to removal of the Ca^{+2}-dependent adhesion factor which aids in holding the cells together. Through both biochemical and morphological studies, it has been shown that hepatocytes isolated under such conditions are comparable to normal hepatic parenchymal cells. Some of the routine assays which have been used to determine hepatocyte viability and functional integrity include: measuring the rate of gluconeogenesis, determining leakage of cytosolic enzymes into the incubation media, the ability of the cell to exclude certain dyes such as trypan blue, "normal" microscopic appearance and measuring respiration rate by monitoring either O_2 uptake or NADH oxidation (Berry and Friend, 1969; Moldeus et al., 1978; Seglen, 1973).

Through various modifications of the isolation procedure and incubation conditions, liver cells have been successfully isolated from species other than the rat and used in numerous metabolic studies. For example, Hazel and Prosser (1979) used hepatocytes isolated from rainbow trout acclimated to temperatures of either 5 or 20°C to study lipogenesis. These cells were incubated with [^{14}C]-acetate and the rate of its incorporation into specific fatty acids

and sterols was determined. In another study, rainbow trout hepatocytes were incubated with labeled lactate, pyruvate and alanine to study gluconeogenesis (Walton and Cowey, 1979). It was shown that glucose was produced from all three substrates, and that its production could be inhibited by addition to the incubation media of various inhibitors of enzymes involved in gluconeogenesis. Glucose production was stimulated by addition of the gluconeogenic hormone, glucagon. Hepatocytes have been isolated from over 15 animal species and used in numerous studies which include glycolysis, protein metabolism, membrane transport and others (Cornell, 1985; Moldeus, 1978).

Hepatocytes have been extremely useful in studying xenobiotic metabolism. When properly isolated, the hepatocyte P-450 content and its enzymatic activities appear stable during incubations of an hour or more (Goethals et al., 1984; Moldeus et al., 1978). Goethals et al. (1984) found that the P-450 content of isolated hepatocytes was equivalent to levels found in liver, when expressed on a per gram liver basis, and that the hepatocytes retained this level during the four hour incubations. Aldrin monooxygenase activity, which is totally dependent on P-450 content, also remained stable during the same period. The metabolism of Alprenolol, BP and 7-ethoxycoumarin are linear for at least one hour without the addition of any cofactors (Moldeus et al., 1978). The metabolism of these compounds is also dependent on P-450. Another indication of intact functional P-450 is that a P-450-drug complex is formed when various drugs are added to hepatocyte suspensions and these complexes exhibit typical type I

spectral characteristics as are produced by similar complexes in microsomes.

Phase II conjugating enzyme systems also remain operational in isolated hepatocytes with different conjugates being produced in one preparation (Parker et al., 1981; Zaleski et al., 1983). Using hepatocytes isolated from two inbred strains of mice, Zaleski et al. (1983) found sulfate, glucuronide and GSH conjugates as the major metabolites produced from BP metabolism. There were at least three different sulfate conjugates formed and two each for glucuronide and GSH. Total conjugate formation correlated with the amount of hepatocytes used in the incubations and their formation could be decreased by the addition of the inhibitors D-galactosamine, diethyl maleate and salicylamide. The glucuronidation rate for some substrates in hepatocytes is dependent on the intercellular concentration of the cofactor UDP-glucuronic acid (Aw and Jones, 1984; Singh and Schwarz, 1981). These levels can be manipulated by either fasting the animals prior to hepatocyte isolation or by the addition of D-galactosamine, an inhibitor of UDP-glucuronic acid formation.

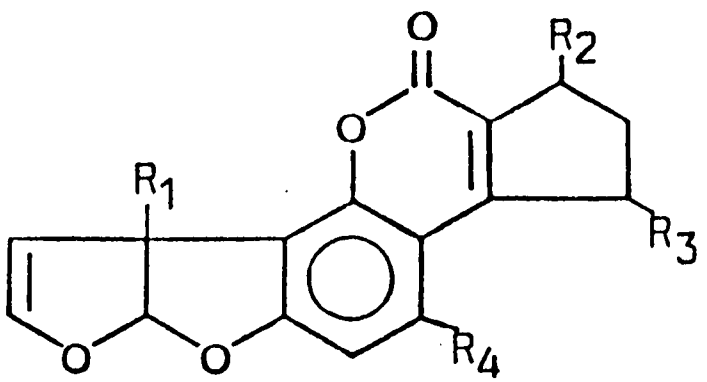
The use of isolated hepatocytes in AFB_1 metabolism studies has provided valuable information in helping to understand the mechanisms leading to its carcinogenic and toxic action. For example, comparison of AFB_1 metabolism in rat and mouse hepatocytes reveals differences which are consistent with their differences in in vivo toxic responses (Hanigan and Laishes, 1984). Mouse hepatocytes have a slower rate of AFB_1 uptake and produce much lower levels of AFB_1 adducts with cellular macromolecules than is seen with rat

hepatocytes. The LC_{50} of AFB_1 in mouse hepatocytes is 1,000-times higher than the LC_{50} in rat hepatocytes. These results indicate that rats would be more sensitive to AFB_1 toxicity, and indeed the *in vivo* LD_{50} is several-fold higher in mice than in rats. In rats, the binding of AFB_1 to cellular proteins and DNA is proportional to AFB_1 concentration and incubation time (Ch'ih *et al.*, 1983; Hanigan and Laishes, 1984). Similar results have been found with rainbow trout hepatocytes where AFB_1-DNA adduct formation is linear with dose up to 19 uM (Bailey *et al.*, 1982a). *In vivo* treatment of animals with MFO inducers prior to hepatocyte isolation can significantly affect AFB_1 metabolism (Bailey *et al.*, 1984; Metcalfe *et al.*, 1981). BNF pre-feeding of rainbow trout, which results in a reduction in the hepatocellular carcinoma incidence from AFB_1 exposure (Nixon *et al.*, 1984), results in reduced binding of AFB_1 to DNA in their isolated hepatocytes (Bailey *et al.*, 1984). These same hepatocytes have a much faster rate of AFB_1 metabolism with significantly more of the less carcinogenic glucuronide conjugates and AFM_1 metabolites being produced and a decreased production of the carcinogenic AFL metabolite compared to control hepatocytes. Presumably, the decrease in the hepatocellular carcinoma incidence *in vivo* results from similar metabolic changes.

In view of the past studies, the use of isolated hepatocytes would be a satisfactory *in vitro* model for studying AFB_1 metabolism in rainbow trout and factors which may alter its metabolism. This system has previously been well characterized (Bailey *et al.*, 1982a) and trout hepatocytes have been successfully employed in other

toxicological studies (Coulombe et al., 1984; Parker et al., 1981).

Fig. II-1. Structure of AFB_1 and major metabolites.



Aflatoxins	R_1	R_2	R_3	R_4
aflatoxin B_1 (AFB_1)	H	O	H	OCH_3
" M_1 (AFM_1)	OH	O	H	OCH_3
" P_1 (AFP_1)	H	O	H	OH
" Q_1 (AFQ_1)	H	O	OH	OCH_3
aflatoxicol (AFL)	H	OH	H	OCH_3
" H_1 ($AFL\text{-}H_1$)	H	OH	OH	OCH_3
" M_1 ($AFL\text{-}M_1$)	OH	OH	H	OCH_3

CHAPTER III

Mechanisms of Anti-Carcinogenesis by Indole-3-Carbinol: Effect on the Distribution and Metabolism of Aflatoxin B_1 in Rainbow Trout

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ABSTRACT

Indole-3-carbinol (I3C), a component of cruciferous vegetables, was previously shown to inhibit aflatoxin B_1 (AFB_1) carcinogenesis in trout. The purpose of this study was to examine the effect of I3C on AFB_1 metabolism and hepatic DNA adduct formation *in vivo* and *in vitro*. When fed at 0.2%, I3C produced a 70% reduction in average *in vivo* hepatic DNA binding of injected AFB_1 over a 21 day period when compared to controls. There was no apparent effect on the rate of loss of these AFB_1-DNA adducts. A 24 hr distribution study of injected tritiated AFB_1 in I3C fish showed less total radioactivity in the blood and liver at all times examined, compared to controls. These reductions were due primarily to reduced levels of AFB_1 bound to red blood cell DNA, reduced plasma levels of the primary metabolite aflatoxicol (AFL), and decreased levels of AFB_1 and polar metabolites present in the liver of I3C fish. In contrast to blood, total radioactivity was significantly elevated in the bile of I3C fish resulting from a 7-fold increase in aflatoxicol-M_1 glucuronide levels over controls. No difference was observed in concentration of AFL glucuronide, the primary conjugate present in control fish. There was no difference in total radioactivity remaining in the carcass of I3C or control fish. AFB_1 metabolism in freshly isolated hepatocytes from I3C fish showed 20% less DNA binding in a 1-hr assay, with a 2-fold increase in aflatoxin M_1 production. Addition of I3C to control hepatocytes at levels of 1, 10 or 100 uM had no effect on AFB_1 DNA binding. These findings indicate that I3C inhibition of AFB_1 hepatocarcinogenesis in trout involves substantial changes in the pharmaco-

kinetics of carcinogen distribution, metabolism and elimination, leading to significantly reduced initial hepatic-nuclear DNA damage *in vivo*.

INTRODUCTION

The human diet is an unavoidable source of exposure not only to carcinogens (Ames, 1983; Sugimura, 1982), but also to a variety of compounds which can inhibit experimental carcinogenesis (Fiala et al., 1985; Wattenberg, 1983). Examples of some inhibitors of carcinogenesis present in foods are retinoids which appear to act at the promotion stage to reduce chemically induced mammary and bladder cancers (Moon et al., 1983; Sporn and Roberts, 1984), naturally occurring flavones and indole compounds in fruits and vegetables which act at the initiation stage (Wattenberg et al., 1976) and selenium, phenolic antioxidants and ethoxyquin which may act at either the initiation or promotion stage (Griffin, 1982; Reddy and Maeura, 1984; Wattenberg, 1972b). One indole, indole-3-carbinol (I3C), found in cruciferous plants has been shown to inhibit chemically induced neoplasias (Bailey et al., 1982b; Nixon et al., 1984; Wattenberg and Loub, 1978). The mechanism of action of this compound as an anticarcinogen is not well understood. In some rodent species, I3C has been shown to produce changes in the mixed function oxidase (MFO) and other drug metabolizing enzymes (Babish and Stoewsand, 1978; Bradfield and Bjeldanes, 1984; Pantuck et al., 1976; Shertzer, 1982; Sparnins et al., 1982). While these findings suggest that enhanced production of less carcinogenic metabolites or metabolites which are more readily excreted may thus be one mechanism of I3C action, this has not been demonstrated in vivo for any carcinogen.

The purpose of the present study was to investigate the effect of dietary I3C on the in vitro and in vivo metabolism, distribution and

DNA binding of aflatoxin B_1 (AFB_1) in rainbow trout. This animal is particularly sensitive to the hepatocarcinogenic effects of AFB_1 (Sinnhuber et al., 1977a) and responds to the inhibitory effect of I3C on chemically induced neoplasias (Bailey et al., 1982b; Nixon et al., 1984). As a non-rodent, it can also provide important comparative information on mechanisms of anti-carcinogenesis.

MATERIALS AND METHODS

Tritiated-AFB_1 ($[^3H]$-AFB_1) (Moravek Biochemicals, Inc., Brea, CA) was checked for purity by UV spectrometry and thin-layer chromatography (Loveland et al., 1983). Collagenase (Type I), hyaluronidase (Type I-S), heparin, bovine serum albumin (BSA) (Fraction V), calf thymus DNA (Type I), ribonuclease T1 (Grade IV) and A (Type II-A) were obtained from Sigma Chemical Co., St. Louis, MO. BME vitamin, BME amino acid and L-glutamine solutions were from KC Biological, Lenexa, KS, and Pronase from Calbiochem, San Diego, CA. All chemicals were reagent grade.

Animals and diets

Mt. Shasta strain rainbow trout (Salmo gairdneri) were used for in vivo and in vitro studies. These were spawned and raised at the Oregon State University Food Toxicology and Nutrition Laboratory under conditions previously described (Sinnhuber et al., 1977a), and were fed a dextrose and casein-gelatin based semipurified diet (Sinnhuber et al., 1977b). The I3C was dissolved in the aqueous portion of the diet to give a final concentration of 0.2% on a dry matter basis. The food was divided in 500 gm portions, frozen and thawed just before feeding.

Hepatocyte preparation and incubation

Hepatocytes were isolated by a two-step perfusion procedure modified from those of Bailey et al. (1982a) and Hazel and Prosser

(1979). Fish, weighing 250 gm or more, were stunned by a blow to the head and the body cavity exposed ventrally by an incision anterior to the cloaca, terminating at the lower jaw. The liver was further exposed by incisions to the lateral line posterior to the pectoral fins. Heparin (560 U in 0.2 ml of 0.9% NaCl) was injected into the heart and the liver cannulated via the hepatic vein with a blunt 20 gauge needle, fitted to a peristaltic pump, and ligated in place. The portal vein and hepatic artery were severed to allow drainage during the in situ perfusions which were conducted at 3-5 ml/min. The first perfusate contained 280 U/ml heparin, 0.11 M NaCl, 2 mM KCl, 0.1 mM $MgSO_4$, 8 mM $NaHCO_3$, 10 mM glucose and 0.1 M phosphate buffer (pH 7.4). The perfusion continued approximately 10 min or until the liver was well cleared of blood. The second perfusate was similar to the first but without heparin, and contained 1 mg/ml hyaluronidase, 0.5 mg/ml collagenase and 2.5 mM $CaCl_2$. Both perfusates were continually gassed with 20:1 O_2:CO_2 and maintained at room temperature. When the liver appeared well digested (20-25 min) it was removed and gallbladder carefully cut away, avoiding bile contamination, and placed in 20 ml ice-cold wash buffer (0.11 M NaCl, 2 mM KCl, 0.1 mM $MgSO_4$, 8 mM $NaHCO_3$, 10 mM glucose, 2.5 mM $CaCl_2$ and 40 mM Hepes at pH 7.4). The liver was teased with glass rods, the cell suspension filtered through four layers of cheese cloth, brought up to 30 ml with wash buffer and centrifuged for 4 min (35 x g). The cell pellet was rewashed and suspended in incubation buffer (wash buffer with 2% BSA) to a cell concentration of approximately 15-20 x 10^6 cells/ml. The cell washing procedure was carried out at 4°C.

Cells were counted and viability approximated with a hemacytometer after dilution (1:20) with 0.5% trypan blue (w/v) in incubation buffer. Appropriate volumes of the cell suspension and incubation buffer were added to 25 ml Erlenmeyer flasks to give a final volume of 5 ml with 5×10^6 cells/ml. Fifty ul each of a 100x stock BME amino acid, BME vitamin and L-glutamine solution were added to each flask. Incubations were initiated by addition of 10 ul of [^{3}H]-AFB_1 (0.6-1.0 uMol/ml, and approximately 500 uCi/uMol) in 95% ethanol. Each flask was continuously gassed on a metabolic shaker at 20°C (±1°C). Incubations were stopped by pelleting the cells at high speed for 1 min with a clinical centrifuge and washing once with 4.0 ml ice-cold wash buffer. The supernatant and wash from each flask were combined and acidified with 50 ul acetic acid, to reduce tritium exchange, and stored at -60°C for AFB_1 metabolite analysis. The cells were resuspended in 0.75 ml 0.1 M NaCl, 50 mM EDTA (pH 7.2) and lysed with 0.75 ml lysis buffer (2% sodium dodecyl-sulfate, 12% sodium-4-aminosalicylate, 1% NaCl and 12% 2-butanol). At this point, the cell lysate could be conveniently stored at -60°C before further DNA purification if desired.

Preparation of nuclei

Trout were killed by a blow to the head, their livers removed and immediately frozen in liquid nitrogen for storage at -60°C. Nuclei were isolated by homogenizing in 9 volumes ice-cold 0.25 M sucrose, 3 mM $MgCl_2$, 10 mM Tris (pH 7.2) buffer using a glass-Teflon homogenizer. The homogenate was filtered through 4 layers of cheesecloth and

centrifuged at 500 x g for 5 min. The resulting pellet was suspended in 10 ml of the homogenation buffer containing 0.5% Triton X-100. After recentrifugation, the pellet was washed once with 25 ml of the original buffer, centrifuged, and the final nuclear pellet brought up in a volume of sucrose buffer equal to the original liver weight and frozen at -60°C for further DNA purification. All isolation procedures were performed at 4°C.

DNA was purified from a 0.2-0.5 ml portion of the nuclear suspension. This was centrifuged for 1 min at high speed in a clinical centrifuge and the pellet suspended in 0.75 ml 0.1 M NaCl, 50 mM EDTA (pH 7.2). An equal volume of the hepatocyte lysis buffer was added and mixed; final purification is described below.

DNA purification

DNA was purified from the nuclear and hepatocyte lysates by a modified Marmur procedure (Irving et al., 1968). To 0.75 ml of lysate, 0.75 ml of 0.1 M Tris-saturated phenol, 0.75 ml of chloroform with 4% isoamyl alcohol (IAC) and 0.4 ml 5 M $NaClO_4$ were added and shaken on a rotary shaker for 1 hr at 4°C. After centrifugation at 3,000 x g for 20 min, the upper layer was transferred to a new tube and extracted 2 times with equal volumes of IAC. DNA was precipitated with 2 volumes of ice-cold 95% ethanol, washed once, dried under a stream of nitrogen and redissolved in 1.5 ml 0.1 M NaCl and 50 mM EDTA (pH 7.2). One hundred ul of ribonuclease (300 U/ml RNase A and 5,000 U/ml RNase T1) were added and incubated at 37°C for 1 hr followed by 95 ul Pronase (560 PUK/ml in 0.1 M NaCl, 0.1 M Tris and 10 mM EDTA at

pH 8.0) and incubated at 37°C for 2 hr. Samples were extracted again with Tris-saturated phenol, IAC and $NaClO_4$ as described above. After centrifugation at 3,000 x g for 5 min, the upper layer was transferred to a new tube and extracted twice with IAC, and DNA was precipitated with 2 volumes of ice-cold 95% ethanol. The DNA was washed twice with 95% ethanol, dried under a stream of nitrogen and redissolved in 1-2 ml distilled water.

Quantitation of AFB_1-DNA binding

DNA was quantitated by the method of Burton (1956). Radioactivity was determined on the same hydrolysates used for the Burton assay using a Beckman LS 7500 liquid scintillation counter and standard procedures. With the known specific activity of the [^{3}H]-AFB_1, total binding of AFB_1 and metabolites could be determined. Since no corrections were made for tritium exchange, adduct formation was not absolute but comparisons between paired treatments could be made. Previous studies have indicated a maximum of 15% tritium exchanged under these conditions.

Analysis of AFB_1 metabolites

C_{18} Sep-Pak minicolumns (Waters Associates, MA) were used to extract AFB_1 and metabolites from aqueous samples. The minicolumns were first wetted with 2.5 ml methanol and equilibrated with 2.5 ml distilled water (more recent procedures use 5 ml each methanol and water). Unless otherwise noted, samples were applied without methanol addition. All columns were washed with 10 ml 10% buffered methanol

(10 mM potassium acetate (KOAc), pH 5.0) and metabolites eluted with 4-5 ml 60% buffered methanol. A flow rate of approximately 2 ml/min was used. The metabolite solution was filtered with a Millipore 0.2 um filter just prior to high-performance liquid chromatography (HPLC) analysis.

Analysis of bile samples for conjugates from fish injected with [^{3}H]-AFB_1 was done according to the procedure of Loveland et al. (1984) with modifications. Bile volumes were measured prior to dilution with 3-4 volumes of 10 mM KOAc buffer at pH 5.0. The solution was adjusted to a final pH of 5.0 and made 10% in methanol prior to loading on Sep-Pak minicolumns. The columns were washed with successive 10 ml volumes of 10, 37 and 50% methanol in 10 mM KOAc. The 37% methanolic fraction which contained most of the radioactivity, was filtered (Millipore 0.2 um filter), reduced in volume under vacuum and analyzed by HPLC.

Reverse phase HPLC was performed with a Waters Associates instrument equipped with a uBondapack C_{18} column. For analysis of conjugates from bile, a 20 min linear gradient of 20 to 30% acetonitrile in 10 mM KOAc (v/v) was used. All other analyses used an isocratic mobile phase consisting of 10 mM KOAc-acetonitrile-methanol-tetrahydrofuran (70:15:20:3, by vol) using conditions similar to Trucksess and Stoloff (1981) as described by Loveland et al. (1983).

In vitro studies

To test the effect of I3C pretreatment on hepatocyte metabolism of AFB_1, 1 to 2 yr-old trout were fed either a control diet or the

control diet plus 0.2% I3C for 7 wk. Fish were killed and hepatocytes prepared and incubated with 10 nMol [^{3}H]-AFB_1 (0.44 uCi/nMol) for 1 hr at 20°C. Metabolite profiles and total binding of AFB_1 and metabolites to DNA were determined. Hepatocytes prepared from trout fed a control diet only were used to study the effect of direct addition of I3C on AFB_1 binding to DNA. Cells from 2 fish were pooled and divided into flasks to be incubated with either 100, 10, 1 or 0 uM I3C. The I3C was added in a volume of 5 ul dimethylsulfoxide (DMSO) to the 5.15 ml cell suspensions. DMSO only was added to control flasks and 6.7 nMol [^{3}H]-AFB_1 (0.47 uCi/nMol) was added to all flasks. DNA was isolated and total binding of AFB_1 and metabolites determined.

In vivo studies

The influence of dietary I3C on long term in vivo AFB_1 hepatic DNA binding was determined with 50 trout weighting approximately 400 gm each. The fish were randomly divided into two groups. One group was fed a control diet containing 0.2% I3C and the other the control diet for three wk followed by a two day fast. Due to their large size, the fish were lightly anesthetized in a solution of tricane methane sulfonate (MS-222) dissolved in water, weighed and injected IP with [^{3}H]-AFB_1 in 95% ethanol (1.0 uMol/ml and 468 uCi/uMol AFB_1) at the rate of 0.1 uMol AFB_1/kg body weight. We assume that this brief exposure to MS-222 would have little if any effect on AFB_1-DNA binding and no differential effect between groups. The fish were killed and livers removed for determination of AFB_1 hepatic DNA binding at 1, 2, 7 and 21 days after AFB_1 injection.

A 24 hr *in vivo* AFB_1 metabolism and distribution study was conducted using 72 fish 9 months of age. These were randomly divided into two groups, one receiving a control diet containing 0.2% I3C and the other the control diet only for 12 wk. All fish were injected IP with 25 ul of 95% ethanol containing [^{3}H]-AFB_1 (0.32 uMol/ml and 480 uCi/uMol AFB_1). At 5, 10, 16 and 24 hr after AFB_1 exposure, 9 fish from each group were selected and anesthetized in a solution of MS-222. Blood was quickly drawn from the tail vein of which 0.2 ml was kept for determination of total radioactivity and 0.5 ml pooled from all fish within a treatment at each time point. The pooled blood was centrifuged at high speed in a clinical centrifuge for six min to separate the red blood cells (RBC) and plasma. Clotting was prevented with heparin. AFB_1 binding to RBC DNA was determined using the hepatocyte procedure. Total radioactivity as well as the AFB_1 metabolite profile were determined on the plasma. Three fish from each time point were combined to give three groups of three pooled livers, bile and residual carcasses for each treatment. Samples were quickly removed after sacrifice, frozen in liquid nitrogen, and stored at -60°C until analysis. Total radioactivity was determined on liver homogenates prepared during nuclei isolation for AFB_1-DNA adduct quantitation. The 100,000 x g supernatants from these same homogenates were analyzed for AFB_1 metabolites. Total radioactivity was determined on carcasses after they were diced and ground to homogeneity with an equal volume of water using a Waring blender. Tissues were digested with NCS tissue solubilizer (Amersham, IL), following the manufacturer's procedures, and counted in OCS

scintillation solution (Amersham, IL).

Data analysis and statistics

All data for the 24 hr *in vivo* AFB_1 study was normalized to 100 gm body weight [i.e. normalized value = measured value x (body weight/100 gm)] to correct for differences in dosage resulting from variations in individual body weights. In the case of pooled samples, average body weight was used. All time-response studies were analyzed by analysis of variance (ANOVA) after first transforming the measured quantities to their natural logarithms. All other data were analyzed with Student's t test (Steel and Torrie, 1980).

RESULTS

Effects of I3C on AFB_1 hepatic-DNA adduct formation *in vivo*

The results of the *in vivo* effect of dietary I3C on the long term binding of AFB_1 to hepatic DNA are shown in Figure III-1. Fish pre-fed a diet containing 0.2% I3C for 3 wk prior to IP injection of AFB_1 had significantly less total binding over time ($P<.01$, ANOVA) when compared to controls. AFB_1 binding was greatest one day after exposure, followed by a slow elimination. This is in agreement with previous studies using rainbow trout (Whitham *et al.*, 1982) and trout embryos (Croy *et al.*, 1980). AFB_1 adducts in control trout have previously been shown to consist primarily of the 2,3-dihydro-2-(N^7-guanyl)-3-hydroxy aflatoxin adduct (Croy *et al.*, 1980), with a time-dependent conversion to the expected ring-opened species (Bailey, unpublished results). The specific AFB_1-DNA adducts formed by I3C-fed fish were not determined in the present study. However, I3C induces only minor changes in AFB_1 phase I metabolism (see below) and little if any detectable qualitative changes in adduct formation would be expected.

Effects of I3C on AFB_1 pharmacokinetics *in vivo*

Fish fed 0.2% I3C for 12 wk prior to [^{3}H]-AFB_1 IP injection had significantly ($P<.01$, Student's t) smaller body weights and larger relative liver weights compared to controls. In previous studies using rainbow trout, 0.1% I3C fed for 12 wk did not affect growth rate (Nixon *et al.*, 1984), but an increase in liver weight was found when

0.05% was fed for 3 wk (Eisele et al., 1983a). When I3C was fed at 0.5 and 0.75% for 3 wk to weanling rats, Babish and Stoewsand (1978) found lower body weights and increased relative liver weights when compared to controls. Body weights in the present 24 hr study averaged 68 and 53 gm while livers were 1.0 and 1.4% of total body weight for control and I3C fish, respectively.

Figure III-2.shows the total radioactivity present in blood, liver, bile and remaining carcass at different times after AFB_1 exposure in these same fish. Comparison of the 5-24 hr curves by ANOVA showed that I3C fish had significantly less ($P<.01$) radioactivity in the blood and liver, and more in the bile compared to controls. Although radioactivity was higher in I3C fish carcasses at 5 hr and lower at 24 hr, there was no significant overall difference in tritium content of carcasses between treatments. The concentration of AFB_1 metabolites was higher in bile than any other tissue examined in the present study being approximately 5-fold over that found in liver, and 125- and 150-fold greater than levels found in blood and carcass, respectively. These data support previous indications that bile is the primary means of eliminating certain AFB_1 metabolites as conjugates (Loveland et al., 1984).

Table III-1. shows more detailed distribution of AFB_1 and metabolites in the blood. Binding of AFB_1 to RBC DNA was significantly less ($P<.01$) in I3C fish compared to controls. In both groups, binding continued to increase 24 hr after AFB_1 exposure, a pattern similar to that seen in liver. The source of the activated AFB_1 metabolite, whether from the RBC themselves, liver or from other

tissue, is not known. There was no significant difference between I3C and control fish for total radioactivity in plasma. Since there was a higher total tritium concentration in the whole blood of control fish, this difference would be due to tritium associated with the RBC fraction. The level of AFB_1 bound to control RBC DNA was approximately twice that of the I3C fish. There was a small but significant decrease ($P<.05$) in aflatoxicol (AFL) found in the plasma of I3C fish when compared to controls but no significant differences were found between treatments for levels of AFB_1, aflatoxin M_1 (AFM_1) or polar metabolites of AFB_1.

Short term binding of AFB_1 to hepatic DNA <u>in vivo</u> in fish fed control or 0.2% I3C diets prior to AFB_1 IP injection is shown in Figure III-3. Binding was significantly reduced ($P<.01$) in I3C fish over controls. These data are consistent with the long term hepatic DNA binding results obtained with fish under similar conditions (Figure III-1.). In both cases, binding in I3C fish was less than 50% of control values. Table III-2. shows the levels of AFB_1 and metabolites found in the 100,000 x g supernatant of liver homogenates from these same fish. There was significantly less ($P<.01$) total radioisotope in the I3C fish compared to controls. Part of this decrease is attributable to a significant decrease ($P<.05$) in unmetabolized AFB_1 found in the I3C fish, together with decreased levels of tritium which failed to bind to the Sep-Pak. This material appears to be associated with the soluble protein fraction. Levels of polar metabolites were also significantly less ($P<.01$) in the I3C fish whereas AFM_1 and AFL levels appeared to be similar between the two

treatments.

Figure III-4. shows the levels of glucuronides present in the bile of fish fed control or 0.2% I3C diets 12 wk prior to AFB_1 IP injection. These consisted predominantly of glucuronides of aflatoxicol M_1 ($AFL\text{-}M_1$) and AFL. There was no significant difference between treatments in the level of AFL glucuronide but there was significantly more ($P<.01$) $AFL\text{-}M_1$ conjugate in the I3C-fed fish. This level was 7-fold greater than that found in controls, and in both treatments maximum levels appeared to be reached at 16 hr post injection with leveling off at 24 hr.

Not all possible effects of I3C on AFB_1 pharmacokinetics were examined in this study. For instance, the loss of AFB_1 by urine and feces and by diffusion through gills was not determined. However, the trout were fasted two days prior to AFB_1 exposure so fecal elimination would be minimal and the loss of AFB_1 through the gills of trout has been determined to be negligible (Ayres, 1969). Techniques for collecting urine from trout are currently being developed in our lab which will be invaluable in determining what part, if any, this plays in I3C enhanced elimination of AFB_1.

Effects of I3C on AFB_1 metabolism and DNA binding in isolated hepatocytes

Table III-3. shows AFB_1-DNA binding and AFB_1 metabolites present in media of hepatocytes prepared from trout pre-fed control or 0.2% I3C diets and incubated with 10 nMol $[^3H]$-AFB_1 for 1 hr. The I3C hepatocytes had 25% less DNA adduct formation and approximately twice

as much AFM_1 produced compared to controls. These differences, though not large, were both statistically significant ($P<.05$). The recoveries of polar metabolites, AFL and unreacted AFB_1 were similar in both treatments. The percent of added tritium found in the media at the end of the incubation period averaged 77 and 75%, respectively, for I3C and control hepatocytes, indicating similar rates of overall AFB_1 reactions. The remainder would consist of AFB_1 bound to cellular macromolecules and that which adhered to the incubation flasks and was not extracted. Of the total radioactivity in the media subjected to Sep-Pak isolation, 96% was recovered in the fraction analyzed by HPLC and 3% eluted through during the loading and subsequent washing steps. That portion lost was probably due to tritium exchange and AFB_1 which was associated with the soluble proteins.

Addition of I3C directly to hepatocytes prepared from control fish had no significant effect of AFB_1 binding to DNA at I3C concentrations of either 100, 10 or 1 uM. Binding in treated cells averaged (±SEM) 94 (±6), 94 (±8) and 101 (±7) percent of controls for 100, 10 and 1 uM added I3C, respectively, as determined in 4 replicates for each treatment. Hepatocyte viability was 95% or better prior to incubations as determined by dye exclusion. It would appear from these results that I3C itself does not inhibit AFB_1 binding at the cellular level through a direct mechanism.

DISCUSSION

Compounds could conceivably inhibit carcinogenesis by acting at any point in the multistage process. Inhibition would occur if a compound prevented the formation of carcinogens or procarcinogens from precursors, inhibited the ultimate carcinogen from reaching the "target site" or suppressed the phenotypic expression of transformed cells (Wattenberg, 1983). In the case of I3C, it would appear from present results that it is primarily the second mechanism that reduces the hepatocarcinogenicity of AFB_1 in rainbow trout. Evidence supporting this comes from the fact that pre-feeding trout I3C resulted in decreased *in vivo* AFB_1 binding to hepatic DNA (Figures III-1. and III-3.) as well as RBC DNA (Table III-1.). There is a positive correlation between hepatic DNA binding of AFB_1 and hepatocellular carcinoma incidence in trout (Whitham *et al.*, 1982). The first mechanism does not apply in these studies, since formation of the procarcinogen AFB_1 was not required. Nor does I3C appear to inhibit carcinogenesis in trout by suppressing phenotypic expression. In fact, I3C has been shown to promote AFB_1 hepatocarcinogenesis in trout if fed post-initiation (Bailey *et al.*, submitted).

Wattenberg (1983) has further subdivided the class of inhibitors which act by preventing the carcinogen from reaching the target site based on their mechanism of action. They may work by inhibiting the production of the ultimate carcinogen, by inducing enzymatic systems responsible for detoxification of the carcinogen or by sequestering the ultimate carcinogen once produced. Since the ultimate carcinogen

of AFB_1 has not been isolated, but only indicated to be the highly reactive 2,3-epoxide from acid hydrolysis products of rRNA-AFB_1 adducts (Swenson et al., 1974), its rate of production in vivo cannot presently be determined. The fact that there is a decrease in AFB_1 binding to DNA in I3C-treated fish could indicate that I3C reduces the formation of the 2,3-epoxide. However, reduced adduct formation could also occur if I3C enhanced primary metabolism and detoxification of AFB_1 (resulting in decreased substrate availability for the epoxide production), if I3C reacted directly with the epoxide, or if I3C enhanced enzymatic inactivation of the epoxide, thereby preventing its interaction with DNA. Inhibition could result from a combination of the above mechanisms or through the action of a metabolite of I3C.

The possibility that I3C acts by means of inducing detoxification pathways is partially supported by the data showing increased AFM_1 levels compared to controls in hepatocytes prepared from fish fed I3C and incubated with AFB_1 (Table III-3.). AFM_1 production is known to be increased in trout (Bailey et al., 1982b; Bailey et al., 1984; Loveland et al., 1983; Williams and Buhler, 1983) and rats (Gurtoo et al., 1985) treated with the P-448 isozyme inducer beta-naphthoflavone (BNF). Since AFM_1 has much lower carcinogenicity than AFB_1 when tested in trout (Hendricks et al., 1980; Sinnhuber et al., 1974), this must be regarded as a detoxification step. Studies have also been conducted that show I3C to possess MFO induction capabilities in other animal models (Aspry and Bjeldanes, 1983; Shertzer, 1982; Shertzer, 1983). Although I3C does not appear to be a hepatic MFO inducer in trout when fed at 0.05 or 0.1% (Eisele et al., 1983a; Haight et al.,

1983), its induction has not been examined at the 0.2% dietary level used in the present study. The small enhancement of AFM_1 production in isolated hepatocytes may reflect a modest potential for trout liver P-448 induction by 0.2% I3C.

An increased level of AFL-M_1 glucuronide in the bile of I3C-fed fish exposed to AFB_1 (Figures III-2. and III-4.) also indicates a mechanism of inhibition by increased detoxification. It has not yet determined if this reflects hepatic UDP-glucuronyltransferase induction or increased production of the substrate, AFL-M_1. An extra-hepatic origin of bile conjugates must also be considered. I3C enhances glutathione (GSH) S-transferase activity in the small intestine of mice (Sparnins et al., 1982). It was also shown in the present study that I3C did produce slightly elevated levels of polar AFB_1 metabolites in the plasma of I3C-fed fish (Table III-1.). Moreover, decreased glucuronide conjugate levels in the 100,000 x g supernatant of liver homogenates from these same fish was also observed (Table III-2.).

Although an induction of detoxification systems is indicated by these data as one mode of action for I3C inhibition, it is unclear if this is the sole operative mechanism. For example, 0.05% dietary BNF produces a similar decrease in AFB_1-induced hepatocellular carcinoma incidence as 0.1% I3C (Bailey et al., 1982b), and decreases AFB_1-DNA adduct formation in isolated hepatocytes (Bailey et al., 1984) to approximately the same extent as 0.2% I3C in this study. However, BNF produces alterations in AFB_1 metabolism in vivo and in vitro which are far more dramatic than those reported here for I3C. For example, BNF

hepatocytes produced only one third as much AFL as control while I3C had no effect. Similarly, BNF hepatocytes produced a 15-fold increase over controls in the hourly rate of AFM_1 production, which was far greater than the 2-fold enhancement observed here with I3C. In vivo, AFL-M_1 glucuronide conjugate accumulation in the bile of BNF fish 24 hr after AFB_1 injection was 15-fold higher than controls (Shelton et al., in preparation), compared to the 7-fold stimulation in I3C-fed fish shown here. Thus, the relative extent to which these doses of I3C and BNF enhanced the detoxification pathways assessed here correlates only imperfectly with reduction of AFB_1-DNA adduct formation.

Other detoxification reactions of possible importance include epoxide hydrase and GSH S-transferase inactivation of the 2,3-epoxide intermediate. We have recently demonstrated that neither BNF or I3C enhance GSH conjugation of AFB_1 in trout (Valsta et al., in preparation). We have not investigated the impact of I3C on AFB_1 dihydrodiol formation by epoxide hydrase. This is clearly important if we are to further understand the mechanisms of I3C anti-initiation at the cellular level.

An additional possible mechanism of action is that I3C may react directly with the ultimate carcinogen before it can produce cellular damage. However, we observed no effect on binding of AFB1 to DNA in isolated hepatocytes by direct addition of I3C at concentrations of 1, 10 or 100 uM. Although Shertzer (1983) observed a decrease in benzo[a]pyrene-DNA binding with the addition of 200 and 300 uM I3C to mice 14S liver fractions, such concentrations are of questionable in

vivo relevance, and we do not suggest direct action of I3C as an important mechanism. It may be possible that a metabolite of I3C is required to produce this effect, but that it might not be produced at high enough concentrations in 1 hr incubations of isolated hepatocytes to show an effect of added I3C. The possibility of an active compound other than I3C itself has previously been suggested (Shertzer, 1983). Studies of I3C metabolites would be important for examining this question.

The relation between liver enlargement and tumor initiation should also be considered. In our 21 day study of adduct formation *in vivo* (Figure III-1.), the fish pre-fed I3C had significantly less DNA adducts per cell compared to controls (25-60% of controls at various times after exposure). However, I3C fish also had somewhat enlarged livers (1.2% body weight) compared to controls (1.0%). Schwarz *et al.* (1985) also observed that some rat liver MFO inducers reduced alkylation of total cellular DNA by dimethylnitrosamine, but elevated total liver DNA content, so that the overall effect of modifiers on total DNA damage per organ was in some cases negated. Although the relative contributions of hyperplasia and hypertrophy to I3C-induced liver enlargement were not determined, overall damage to liver DNA would in any event be substantially inhibited by dietary I3C. Even if I3C enlargement were due exclusively to increased cell numbers per liver, total hepatic nuclear DNA adduct formation in I3C fish would be 30-72% of controls (e.g. 25% x 1.2/1.0). Similarly, cellular adduct formation for the first 24 hr period (Figure III-3.) in I3C fish was only 35-50% of control, at various times after exposure. In this

experiment, I3C-fed fish had a 1.4% liver:body weight ratio, but the control fish were 1.0%. A similar correction as above would result in total adduct formation in I3C livers of 49-70% that of controls. These results indicate clearly that reduced initiation at the organ as well as cellular level is an important mechanism for I3C inhibition. Further studies are needed to characterize hypertrophic and hyperplastic effects of I3C in the trout system.

It is clear from this study and others that I3C reduces the binding of chemical carcinogens to hepatic DNA (Nixon et al., 1984; Shertzer, 1983) as one mechanism for inhibiting chemically induced neoplasias (Bailey et al., 1982b; Nixon et al., 1984; Wattenberg and Loub, 1978). The precise mechanisms through which this anti-initiation is achieved are incompletely understood. Our results indicate that in trout, an enhancement of biliary elimination of AFB_1 as glucuronide conjugated metabolites is important. The possibilities for enhanced epoxide hydrase detoxification, induction of a P-450 with lowered efficiency for epoxide formation and for direct epoxide interaction with an I3C metabolite remain to be investigated in the trout.

Table III-1. AFB_1 and metabolites in blood of I3C-fed or control trout at various times after AFB_1 exposure.[a]

Treatment	hr after AFB_1	nMol AFB_1/ gm RBC DNA	Concentration of AFB_1 and metabolites in plasma (nM)				
			Total	Polar	AFM_1	AFB_1	AFL
Control	5	.705	29.1	1.4	0.6	19.7	2.2
	10	.692	19.1	0.5	1.1	8.8	0.8
	16	.679	10.2	0.2	0.7	5.9	0.4
	24	.910	7.9	0.3	1.0	3.8	0.2
I3C	5	.247	31.6	1.5	4.2	16.9	1.6
	10	.327	18.5	1.2	3.0	7.6	0.6
	16	.404	10.2	0.7	1.2	4.0	0.3
	24	.481	7.7	0.5	0.2	2.7	0.1

[a] Fish were fed either a control or 0.2% I3C diet for 12 wk prior to [^{3}H]-AFB_1 IP injection. Each sample represented equal volumes of plasma pooled from nine fish for each time point and resolved by HPLC after Sep-Pak treatment as described in Materials and Methods. AFB_1 binding to RBC DNA was determined on pooled RBC from the same fish used for plasma analysis. There was significantly less ($P<.01$) binding of AFB_1 to RBC DNA and less ($P<.05$) AFL in plasma of I3C fish compared to controls. ANOVA was used after transformation of data to natural log.

Table III-2. AFB_1 and metabolites in the 100,000 x g supernatant of liver homogenates from trout fed 0.2% I3C or control diets and injected with [^{3}H]-AFB_1.[a]

Treatment	hr after AFB_1	pMol metabolites/gm liver				
		Total[b]	Polar[c]	AFM_1	AFB_1	AFL
Control	5	392	78	4	139	34
	10	274	58	3	102	17
	16	207	52	3	50	19
	24	188	32	0	46	13
I3C	5	290	71	4	104	31
	10	189	47	3	72	15
	16	178	41	2	46	19
	24	133	26	1	33	10

[a] Fish were fed either a control or 0.2% I3C diet for 12 wk prior to [^{3}H]-AFB_1 IP injection. Liver homogenates were combined from 3 groups of 3 fish pooled at each time point after AFB_1 exposure. The AFB_1 and metabolites extracted from the 100,000 x g supernatant using Sep-Paks, and analyzed by HPLC as described in Materials and Methods. There was significantly less ($P<.01$) combined AFB_1 and metabolites in I3C-treated fish over controls which was due, in part, to the decreased ($P<.05$) level of AFB_1 and decreased ($P<.01$) level of polar metabolites.

[b] Includes total soluble AFB_1 and metabolites as well as that portion associated with soluble macromolecules present in the sample prior to Sep-Pak extraction.

[c] It was found that this fraction from polychlorinated biphenyl-fed fish can be resolved into AFL and AFL-M_1 glucuronides by HPLC.

Table III-3. Metabolism of AFB_1 in freshly isolated hepatocytes prepared from rainbow trout pre-fed 0.2% I3C or control diets.[a]

	Percent of total aflatoxins recovered[b]				
Diet	Polar Metabolites	AFM_1	AFB_1	AFL	DNA Adducts[c]
Control	2.6 ± 0.4	1.8* ± 0.2	86 ± 1	8.1 ± 0.6	0.86* ± .06
0.2% I3C	2.6 ± 0.3	3.8* ± 0.9	85 ± 1	7.8 ± 0.5	0.67* ± .03

[a] Metabolites were extracted from the incubation media made 10% in methanol using Sep-Paks equilibrated with 10% buffered methanol (10mM KOAc, pH 5.0).

[b] Mean ± SEM of percent total radiolabel recovered from HPLC after 1 hr reaction of [^{3}H]-AFB_1 with hepatocytes. No significant differences were found in tritium remaining in media of treatments after incubations. N=5 for each dietary treatment with viability 95% or better using dye exclusion.

[c] Mean ± SEM uMol AFB_1 bound/gm DNA; N=9 for each dietary treatment.

* Denotes means that are different ($P<.05$) from controls using Student's t test.

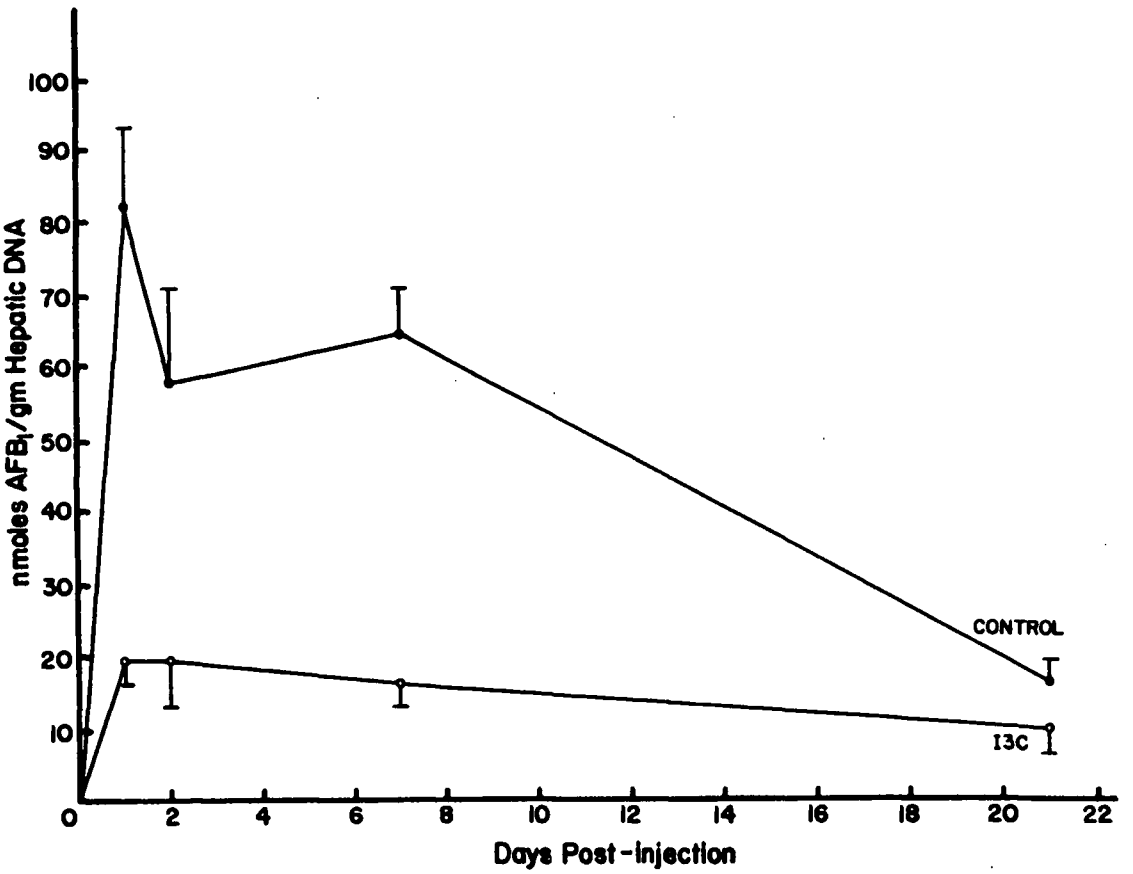

Fig. III-1. Influence of dietary I3C on long term hepatic DNA binding of AFB_1 in trout. Fish were pre-fed control (—•—) or 0.2% I3C (—○—) diets for 3 wk and killed at various times up to 21 days after IP injection of [^{3}H]-AFB_1. Liver DNA was isolated and specific activity determined as in Materials and Methods. Points are means of 5-8 fish with error bars representing SEM. There was significantly less ($P<.01$) binding in the I3C fish compared to controls, when the 1-21 day curves are compared by ANOVA.

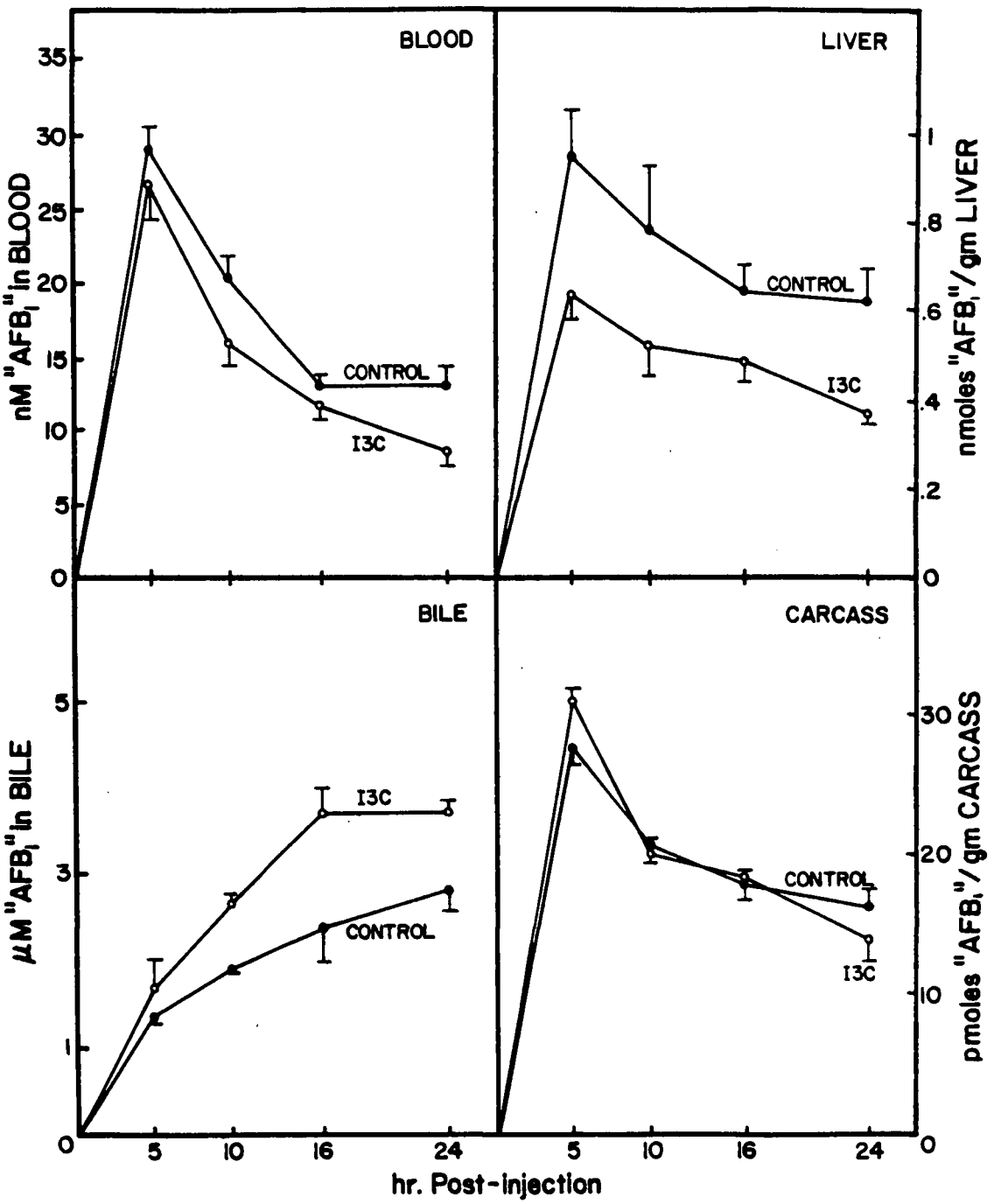

Fig. III-2. Total radioactivity in tissues from trout fed control (—•—) or 0.2% $I3C_3$ (—○—) diets for 12 wk and killed at various times up to 24 hr after [^{3}H]-AFB_1 IP injection. Values are means with error bars representing SEM of 9 individual fish for blood, or 3 groups of 3 pooled fish for liver, bile and residual carcass. I3C fish had significantly less ($P<.01$) radioactivity in the blood and liver but more in bile compared to controls.

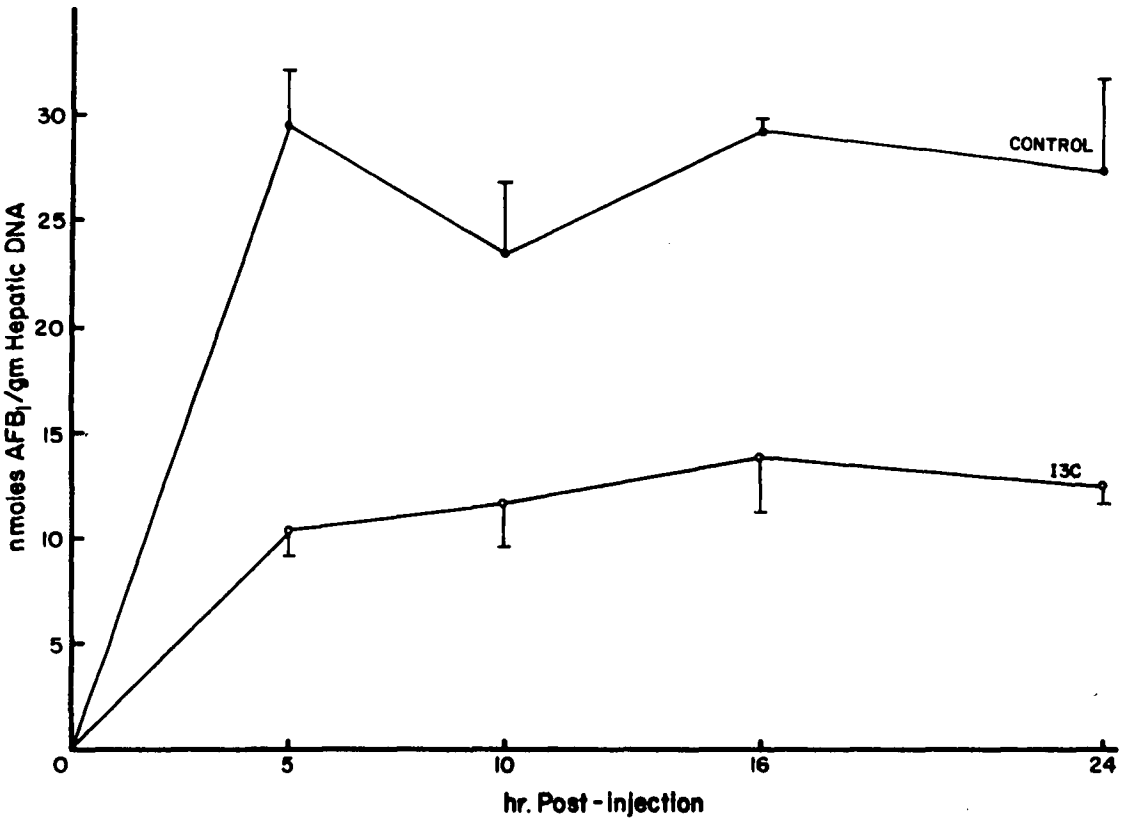

Fig. III-3. Influence of dietary I3C on short term binding of AFB_1 to hepatic DNA in trout pre-fed control (—•—) or I3C (—○—) diets as described in Fig. III-2. Liver DNa was isolated and specific activity determined. Points are means of 3 groups of 3 pooled fish with error bars representing SEM. There was significantly less ($P<.01$) binding in I3C fish compared to controls.

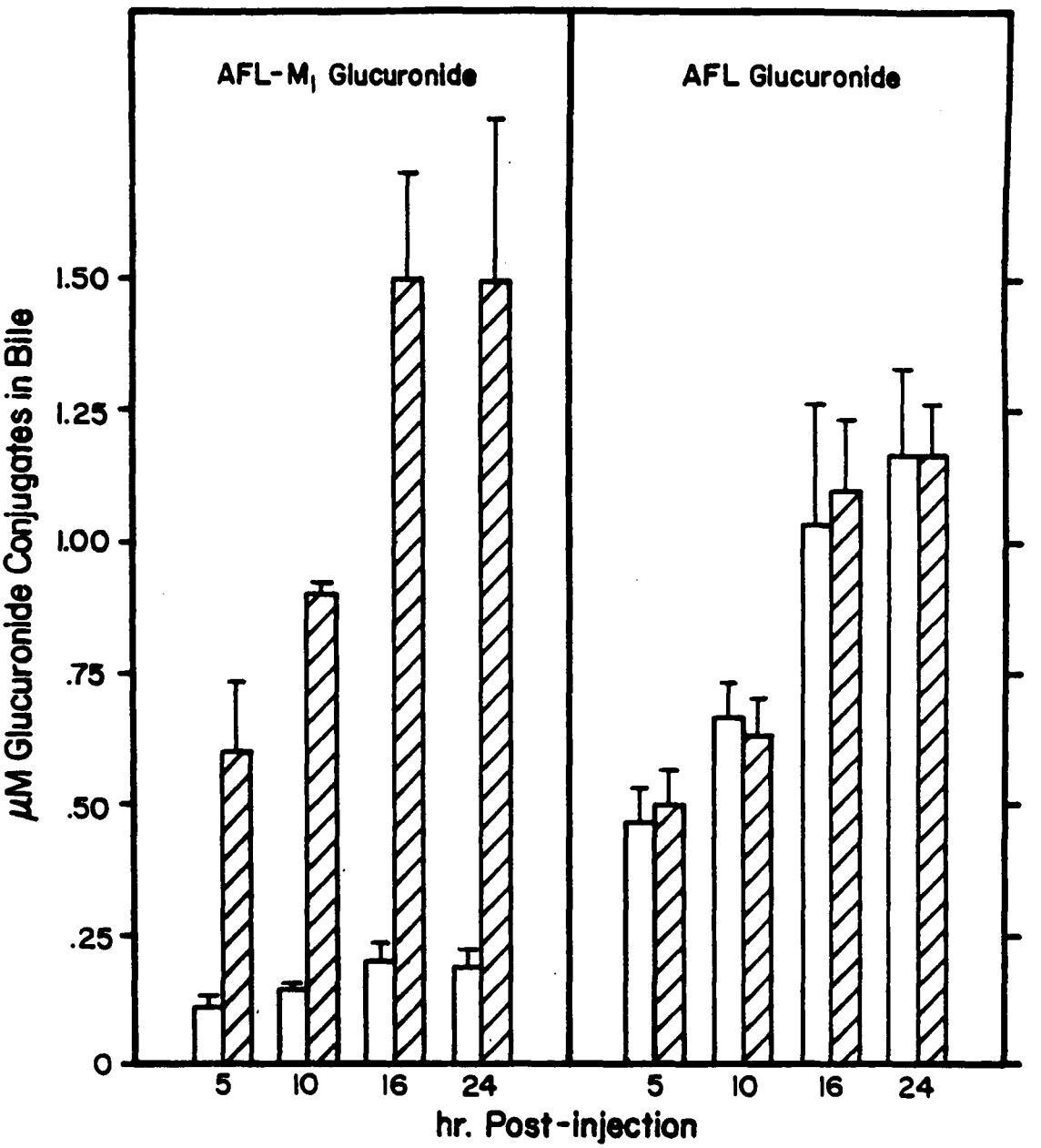

Fig. III-4. Glucuronide conjugate levels of AFB_1 metabolites found in bile of control (open bars) or I3C-fed (hatched bars) fish after IP injection of AFB_1 as described in Fig. III-2. Bile samples were diluted, made 10% in methanol and aflatoxins isolated using Sep-Paks equilibrated with 10% buffered methanol (10 mM potassium acetate, pH 5.0) as described in Materials and Methods. There was no significant difference in AFL glucuronide levels between treatments but $AFL\text{-}M_1$ glucuronide levels were significantly greater ($P<.01$) in I3C-fed fish.

CHAPTER IV

Null Effect of Butylated Hydroxyanisole on Aflatoxin B_1 *In Vivo* Carcinogenesis, Metabolism and DNA Adduct Formation in Rainbow Trout

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ABSTRACT

Butylated hydroxyanisole (BHA), an antioxidant with anti-carcinogenic properties in some experimental animals, was tested for its effect on aflatoxin B_1 (AFB_1) metabolism, hepatic DNA adduct formation and tumorigenesis in the rainbow trout. When fed at either 0.03 or 0.3%, BHA had no effect on the 52 wk hepatic tumor incidence of trout exposed to a 0.5 ppm AFB_1 solution as embryos. These diets were fed for an 8 wk period starting at swim-up. The same dietary levels of BHA had no effect on the hepatic tumor incidence when fed 4 wk prior to and during a 4 wk dietary exposure of 10 ppb AFB_1. However, there was a significant increase in relative liver weight of trout consuming the diets containing AFB_1, with or without BHA, when compared to trout consuming the control diet only. A 3 wk pretreatment with 0.3% dietary BHA had no effect on nuclear AFB_1-DNA adduct formation in liver at 0.5, 1, 2 and 7 days after AFB_1 IP injection, when compared to controls. One hr incubations of AFB_1 with freshly isolated hepatocytes from BHA-fed fish produced no differences in AFB_1-DNA adduct formation or ratios of aflatoxin M_1, aflatoxicol or polar metabolites produced when compared to hepatocytes isolated from fish fed a control diet only. BHA at 1 or 10 uM added directly to hepatocytes isolated from fish fed a control diet only had no effect on AFB_1-DNA adduct formation, but addition of 100 uM BHA reduced adduct formation by 20% compared to hepatocytes treated with vehicle only. These data indicate that BHA does not alter enzymes responsible for AFB_1 initiation as determined by DNA adduct formation. BHA does

have a weak direct affect on AFB_1-DNA adduct formation either by competing for metabolism *in vitro*, or by direct trapping of the electrophilic AFB_1 2,3-epoxide but this does not appear to be of importance *in vivo* since dietary BHA had no effect on AFB_1 induced carcinogenesis.

INTRODUCTION

Butylated hydroxyanisole (BHA), an antioxidant commonly added to foods to prevent oxidative spoilage, has been shown to inhibit chemically induced neoplasias (Reddy and Maeura, 1984; Wattenberg, 1972b; Wattenberg, 1973; Wattenberg and Sparnins, 1979). Maximal effect is achieved when BHA is fed prior to or during carcinogen exposure. Although the exact mechanisms for BHA mediated anti-carcinogenesis have not been determined, studies indicate that changes in enzymes responsible for xenobiotic metabolism may be involved. For example, dietary BHA enhances glutathione (GSH) S-transferase and UDP-glucuronyl transferase activity in mice and rats (Benson et al., 1979; Cha and Bueding, 1979; Hjelle et al., 1985; Sparnins et al., 1982), induces enzymes of the mixed-function oxidase system (MFO) (Aspry and Bjeldanes, 1983; Eisele et al., 1983b; Kensler et al., 1985; Sydor et al., 1983) and induces epoxide hydrolase activity (Aspry and Bjeldanes, 1983; Cha et al., 1978). Such changes in enzymatic activities could result in the production of less carcinogenic metabolites or compounds more readily excreted and thus decrease chemical initiation leading to carcinogenesis. Direct mechanisms of anti-carcinogenesis by BHA are possible since in vitro studies show that direct addition of BHA inhibits microsomal metabolism of some carcinogens (Levine, 1984; Sydor et al., 1983) which could prevent the production of the ultimate carcinogen in vivo. The antioxidative activity of BHA may also protect against free radical mediated lipid peroxidative damage in the promotional phase of carcinogenesis (Rao et

al., 1984).

Currently, there are no studies on the effect of 8HA on AFB_1 carcinogenesis in any animal, although the antioxidant ethoxyquin has been shown to inhibit AFB_1 induced hepatocellular carcinomas in rats (Cabral and Neal, 1983). 8HA does reduce the binding of AFB_1 to hepatic DNA in rats (Kensler et al., 1985) and similar results have been obtained with primary hepatocyte cultures prepared from rats pre-fed the phenolic antioxidant butylated hydroxytoluene (Salocks et al., 1984). These results appear to be due to enzyme induction with increased AFB_1 detoxification and not to the antioxidant potential of these compounds. The rainbow trout has shown to be very sensitive to the carcinogenic effect of AFB_1 (Sinnhuber et al., 1977a; Sinnhuber et al., 1977b). Previous studies have also shown that the trout is responsive to some inhibitors of carcinogenesis which include flavone and indole compounds (Bailey et al., 1982b; Goeger et al., 1985; Nixon et al., 1984) and Aroclor 1254 (PC8) (Shelton et al., 1982; Shelton et al., 1984; Shelton et al., 1985). Reported here are the effects of 8HA on $AF8_1$ carcinogenesis and the underlying mechanisms.

MATERIALS AND METHODS

AFB_1 (Calbiochem, San Diego, CA) and [^{3}H]-AFB_1 (Moravek Biochemicals, Inc., Brea, CA) were checked for purity by UV spectrometry and thin-layer chromatography (Loveland et al., 1983). BHA, Collagenase (Type I), hyaluronidase (Type I-S), heparin, bovine serum albumin (Fraction V), calf thymus DNA (Type I), ribonuclease TI (Grade IV) and A (Type II-A) were from Sigma Chemical Co., St. Louis, MO. BME vitamin, BME amino acid and L-glutamine solutions were from KC Biological, Lenexa, KS and Pronase from Calbiochem. All other chemicals were reagent grade.

Animals and diets

Mt. Shasta strain rainbow trout (Salmo gairdneri) were used for in vivo and in vitro studies. Rearing conditions and facilities have previously been described (Sinnhuber et al., 1977a). BHA was dissolved in salmon oil used in formulating the dextrose and casein-gelatin based semipurified diet (Sinnhuber et al., 1977b) so that the final concentration was 0.3% on a dry matter basis. The food was divided into 500 gm portions, frozen and thawed just before feeding.

Hepatocyte preparation and incubation

Hepatocytes were isolated by a two-step perfusion procedure modified from those of Bailey et al. (1982a) and Hazel and Prosser (1979) which has been described in detail (Goeger et al., 1985). Cell viability was determined by trypan blue exclusion and was consistently

greater than 95% before incubation. Incubations consisted of 5 ml total volume containing 5 x 10^6 cells/ml and were conducted at 20°C. Reactions were started by addition of 10 ul [^{3}H]-AFB_1 in 95% ethanol and stopped by pelleting the cells at high speed in a clinical centrifuge. The supernatant was analyzed for AFB_1 and metabolites. Total cellular DNA was isolated and purified from the cell pellet and AFB_1-DNA binding quantitated as reported elsewhere (Goeger et al., 1985).

DNA purification and AFB_1 adduct quantitation

Preparation of nuclei from liver following in vivo AFB_1 exposure, DNA purification and AFB_1 adduct quantitation are detailed elsewhere (Goeger et al., 1985). The DNA was purified by a modified Marmur procedure (Irving et al., 1968) and quantitated by the method of Burton (1956) using calf thymus DNA as a standard. Radioactivity was determined on the same DNA hydrolysates used for the Burton assay using a Beckman LS 7500 liquid scintillation counter and standard procedures. With the known specific activity of the [^{3}H]-AFB_1, total binding of AFB_1 and metabolites could be determined.

Analysis of AFB_1 metabolites

AFB_1 and its metabolites were extracted from the cell-free hepatocyte incubation media with C_{18} Sep-Pak minicolumns (Waters Associates, MA). The minicolumns were first wetted with 5.0 ml methanol (MeOH) and equilibrated with 5 ml 10% MeOH in 10 mM potassium acetate (KOAc) buffer at pH 5.0. Samples containing 10% MeOH were

applied, columns "washed" with 10 ml 10% buffered MeOH and metabolites eluted with 10 ml 60% buffered MeOH. Samples were reduced in volume under vacuum, and filtered with a Millipore 0.2 um filter just prior to high performance liquid chromatography (HPLC) analysis. Reverse phase HPLC was performed with a Waters Associates instrument equipped with a uBondapack C_{18} column using an isocratic mobile phase of 10 mM KOAc at pH 5.0-acetonitrile-methanol-tetrahydrofuran (70:15:20:3, by vol.) and conditions similar to Trucksess and Stoloff (1981) as described by Loveland et al. (1983).

In vivo tumorigenesis studies

The effect of BHA on AFB_1 carcinogenesis in rainbow trout was assessed in two studies. The first study examined the post-initiation effects of BHA on AFB_1 carcinogenesis. This involved the use of 6 groups of 200 rainbow trout embryos which were exposed to 0.5 ppm solutions of $AF8_1$ for 30 min on day 21 of incubation at 12°C. The embryos were handled as previously described until swim-up (Hendricks et al., 1984). At swim-up, 3 duplicate groups of 100 vigorous fry were placed in 3 foot diameter fiberglass tanks receiving well water at 3 gpm. Duplicate groups were pair-fed either the Oregon Test Diet (OTD), OTD plus 0.03% BHA or OTD plus 0.3% BHA for 8 wk. After this period, all groups were fed the OTD only until the experiment was terminated 1 year after embryo exposure. The second study investigated the effects of BHA on $AF8_1$ carcinogenesis when fed prior to and during $AF8_1$ exposure. This employed the use of 4 duplicate groups of 100 control swim-up fry. These were placed in tanks as

described above with each group being pair-fed either OTD, OTD plus 0.03% BHA or OTD plus 0.3% BHA for 4 wk. At the end of 4 wk, 10 ppb AFB_1 was added to each of these 3 diets and pair-fed for 4 additional wk. During the AFB_1 exposure period, each group of fish was fed at the rate of 1.75% their body weight per day. The fish were weighed weekly to compute an appropriate amount of diet to feed. After the 4 wk AFB_1 exposure period, all groups were returned to the OTD for 12 months at which time the experiment was terminated. Previous feeding studies have shown that BHA fed at these levels for 8 wk is noncarcinogenic (data not shown). A fourth group of fish was fed the OTD only throughout the entire experimental period to serve as a negative control.

At termination, the fish were anesthetized with tricane methane sulfonate, weighed and livers removed. The livers were then weighed, examined under a binocular microscope for tumors and fixed in Bouins solution. After fixation, the livers were sliced into 1 mm slices to find internal tumors. Representative tumors from each positive fish were embedded in paraffin and processed by routine histological methods.

In vivo DNA binding studies

The influence of BHA on in vivo binding of AFB_1 to hepatic-nuclear DNA was determined with 48 trout weighing approximately 150 gm each. The fish were randomly divided into two groups. One group was fed a control diet and the other the control diet plus 0.3% BHA for 3 wk. The trout were injected IP with 25 ul of $[^3H]$-AFB_1 in 25%

ethanol (1.0 uMol/ml and 620 uCi/uMol) and 6 fish from each group killed at 0.5, 1, 2 and 7 days after AFB_1 injection. The livers were quickly removed and frozen with liquid nitrogen and stored at -60°C until nuclei were isolated for determination of AFB_1-DNA binding.

Often in studies involving <u>in vivo</u> metabolism of injected xenobiotics in trout, the fish are fasted prior to exposure so that bile accumulates for metabolite analysis. A study was conducted to investigate the effect of fasting on the binding of injected AFB_1 to hepatic DNA and the effect that dietary BHA pretreatment may have. Twenty-four trout averaging 150 gm each were divided into 2 equal groups. One group received a control diet plus 0.3% BHA and the other the control diet only for 3 wk. Three days prior to $[^3H]$-AFB_1 IP injection, six trout from each group were restricted from feeding while the remainder continued with their respective feeding regime and diets. Two days after $[^3H]$-AFB_1 injection (25 ul of 1.0 uMol/ml $[^3H]$-AFB_1 in 95% ethanol, 620 uCi/uMol) all fish were killed and their livers removed for isolation of nuclei and determination of AFB_1-DNA binding.

<u>In vitro</u> hepatocyte studies

The effect of BHA pretreatment on hepatocyte metabolism of AFB_1 was examined using 1 to 2 yr-old trout fed either a control diet or the same diet plus 0.3% BHA for 3 wk. Fish were killed, hepatocytes prepared and incubated with 9 nMol $[^3H]$-AFB_1 (0.9 uMol/ml and 440 uCi/uMol) for 1 hr at 20°C. Total binding of AFB_1 and metabolites to DNA as well as metabolite profiles were determined.

The influence that the direct addition of BHA had on AFB_1 binding to DNA was determined by using isolated hepatocytes from fish fed a control diet only. Cells from 2 fish were pooled and divided into flasks to be incubated with either 100, 10, 1 or 0 uM BHA. The BHA was added in a volume of 5 ul dimethylsulfoxide (DMSO). DMSO only was added to control flasks and 7 nMol [^{3}H]-AFB_1 (0.7 uMol/ml and 470 uCi/uMol) was added to all flasks. DNA was purified and total binding of AFB_1 and metabolites determined.

Data analysis and statistics

Significance of the in vivo tumor incidence data was determined using the Fisher Exact Test (Brownlee, 1965). Analysis of variance (ANOVA) and standard multiple comparisons of the means were used to determine differences in body weights, liver to body weight ratios and in vivo DNA binding. The in vitro BHA addition study was analyzed by ANOVA using a randomized block design. Student's t test was used on all other comparisons (Steel and Torrie, 1980).

RESULTS

In vivo tumorigenesis

Results of the effects of dietary BHA on trout exposed to AFB_1 as either embryos or fry are presented in Tables IV-1. and IV-2., respectively. Addition of 0.03 or 0.3% BHA to the diets of fingerlings after exposure to 0.5 ppm AFB_1 as embryos had no effect on either body weight or relative liver weight (Table IV-1.). Although there was a slight increase in the hepatic tumor incidence in the high dietary BHA group compared to the other two groups, there was no significant difference between any dietary treatment in tumor response. Dietary AFB_1 by itself and in combination with 0.03 or 0.3% BHA increased relative liver weight when compared to negative controls (Table IV-2.). Trout receiving the 0.3% BHA diet also had significantly higher relative liver weights than trout fed 0.03% BHA. However, there were no differences between treatments in body weight or liver carcinoma incidence.

In vivo AFB_1 hepatic DNA binding

Results of the in vivo effect of dietary BHA on AFB_1 binding to hepatic nuclear DNA is shown in Figure IV-1. Pre-feeding 0.3% BHA for 3 wk prior to AFB_1 IP injection had no significant effect on total AFB_1-DNA adduct formation over the period studied. Maximal levels of binding were obtained early in the study in both treatments followed by a slow elimination. BHA pretreatment had no effect on the maximal level of adducts formed or the rate of adduct elimination.

Results of the effect of fasting on the binding of AFB_1 to hepatic DNA in BHA or control fed fish are given in Table IV-3. There was no significant difference due to the main effect of diet composition or to interaction between treatments as determined by two factor ANOVA. However, there was a significant difference due to feeding schedule at the $P<.10$ level. If the BHA and control non-fasted trout are combined and compared to the combined BHA and control fasted trout, there is a significant increase ($P<.05$) in AFB_1-DNA binding in the fasted trout. This increase is approximately 50% over binding in the non-fasted trout.

AFB_1 metabolism and DNA binding in isolated hepatocytes

Table IV-4. shows AFB_1-DNA binding and AFB_1 metabolites produced by hepatocytes isolated from trout fed either control or 0.3% BHA diets and incubated with 9 nMol [^{3}H]-AFB_1 for 1 hr. There were no significant differences in total adduct formation between treatments or in the pattern of AFB_1 metabolites produced. Of the total tritium added, 80% was recovered from the media of both control and BHA hepatocytes at the end of the 1 hr incubations. The remaining 20% consisted of AFB_1 bound to cellular macromolecules and that which adhered to the incubation flasks and was not extracted. Sep-Pak recovery of the soluble metabolites for HPLC analysis exceeded 96%, with 3% eluting through during the loading and subsequent washing steps. HPLC analysis of soluble metabolites revealed no differences in the production of polar conjugates, AFL or AFM, or the amount of unreacted AFB_1 recovered (Table IV-4.).

BHA addition to control hepatocytes

Table IV-5. shows the effect of direct addition of BHA in DMSO on AFB_1-DNA binding in isolated hepatocytes from trout fed a control diet only. There was a significant decrease ($P<.05$) in total DNA adducts formed only at the highest BHA concentration of 100 uM. This represented an 18% reduction compared to adducts formed in hepatocytes treated with DMSO only (0.77±0.2 uMol AFB_1/gm DNA). There was no significant effect on adduct formation at BHA concentrations of 1 or 10 uM.

DISCUSSION

Although results of the present study show that BHA has no effect on AFB_1 carcinogenesis and metabolism in the rainbow trout, these data are consistent with other studies on AFB_1 metabolism in trout, and they do support some proposed mechanisms of BHA anti-carcinogenesis. For instance, GSH S-transferase induction is one possible mechanism of action for BHA. Sparnins *et al.* (1982) have shown that BHA as well as other unrelated inhibitors of chemical carcinogenesis enhance GSH S-transferase activity in the liver and mucosa of the small intestine. The binding of AFB_1 to DNA has also been shown to be inversely related to AFB_1-GSH conjugates formed *in vitro* (Raj *et al.*, 1984). However, GSH conjugation does not appear to be important in AFB_1 metabolism in trout since this conjugate was not found in *in vitro* subcellular incubations or in the bile of fish injected with AFB_1 (Valsta, 1985). Furthermore, GSH S-transferase activity was not induced in trout by dietary indole-3-carbinol, an inducer of this enzyme in mice (Sparnins *et al.*, 1982). Acid-soluble sulphydryl groups were found depressed in livers of trout fed 0.1% BHA (Eisele *et al.*, 1983b). Although GSH levels, GSH S-transferase activity or other phase II conjugating enzymes were not directly assayed in the present study, the lack of BHA mediated anti-carcinogenesis *in vivo* and lack of effect on AFB_1-DNA binding and metabolism *in vivo* and *in vitro* (Figure IV-1.; Tables IV-1., IV-2. and IV-4.) indicate that any such changes are not sufficient to affect AFB_1 carcinogenesis in the trout.

BHA has been shown to induce MFO enzymes in mice and rats (Aspry

and Bjeldanes, 1983; Kensler et al., 1985; Sydor et al., 1983). Eisele et al. (1983b) found that dietary BHA as low as 0.1% induced ethoxycoumarin-O-deethylase and benzo[a]pyrene hydroxylase activity in the trout while depressing p-nitroanisole-O-demethylase activity. If enzymes responsible for detoxification are preferentially induced over those responsible for carcinogen activation, then this would likely have a positive effect on anti-carcinogenesis. However, such general MFO assays do not appear to reliably predict metabolic changes involving specific isozymes and substrates. Results from the present study indicate that any changes in the MFO produced by BHA do not result in alterations of AFB_1 metabolism. For example, hepatocytes isolated from BHA-fed fish had the same metabolite profile, and rate of AFB_1 metabolism as hepatocytes from control fish (Table IV-4.). By contrast, aflatoxin M_1 (AFM_1), which is less carcinogenic than AFB_1 (Hendricks et al., 1980; Sinnhuber et al., 1974), is elevated several-fold in hepatocytes isolated from Aroclor 1254 (PCB) or beta-naphthoflavone (BNF) fed fish. These hepatocytes also metabolize AFB_1 at a much faster rate than controls (Shelton et al., 1985; Bailey et al., 1984). PCB and BNF also inhibit AFB_1 carcinogenesis in trout and reduce hepatic DNA binding *in vivo*, apparently through enhanced detoxification mechanisms.

Although results obtained from the direct addition of BHA to hepatocytes indicate that very high levels of BHA might inhibit AFB_1 carcinogenesis through a direct mechanism (Table IV-5.), this is not evident *in vivo*. BHA has been shown to produce a P-450 type I binding spectrum and inhibit rat liver MFO activity to various substrates

(Sydor et al., 1983; Yang et al., 1985). Therefore, it is possible that BHA competes with AFB_1 for metabolism by an enzyme that is responsible for production of the ultimate carcinogen, the 2,3-epoxide. A decrease in level of epoxide production would reduce the total adducts formed. However, the 100 uM concentration necessary to elicit this response in vitro may not be produced in vivo since total DNA adduct formation was unchanged between 0.3% BHA and control fish (Figure IV-1.). Other direct mechanisms of anti-carcinogenesis by BHA would be through its antioxidant properties by direct trapping of the AFB_1 2,3-epoxide, or by scavenging free radicals generated during carcinogen metabolism that could lead to peroxidative mediated carcinogenesis (Rao et al., 1984). Since in vivo tumorigenesis was unaffected by 0.03 and 0.3% dietary BHA (Table IV-1. and IV-2.), this would indicate that the mechanism of unpromoted AFB_1 carcinogenesis in the rainbow trout is not through free radical damage, and that the putative epoxide intermediate is not susceptible to appreciable BHA trapping in vivo.

The underlying mechanism for the increase in AFB_1-DNA binding in fasted trout injected with AFB_1 is not known. One possible explanation for this increase could result from decreased glucuronide conjugation. For example, glucuronidation represents one of the primary means of detoxifying AFB_1 and other xenobiotics in the rainbow trout (Loveland, et al., 1984; Parker et al., 1981). Aw and Jones (1984) have shown that the glucuronidation rate of acetaminophen in rat isolated hepatocytes was proportional to UDP-glucuronic acid concentration and that both decreased when the rats were fasted for 24

hr prior to hepatocyte isolation. Therefore, fasting the trout could hinder AFB_1 detoxification by depleting UDP-glucuronic acid levels and reducing the rate of glucuronidation. The presence of a food bolus in the gut of non-fasted trout also may have an effect by absorbing some of the AFB_1 dose thereby decreasing the internal concentration through non-enzymatic means. The slow release of this portion of the AFB_1 dose during digestion may be at a rate more suitable for detoxification and elimination thus resulting in decreased adduct formation.

Nonetheless, the increase in AFB_1-DNA adduct formation from fasting trout does not necessarily warrant its elimination as a comparative procedure in studying AFB_1 metabolism since it is much easier and less traumatic to the animal than bile duct cannulation for studying bile metabolites. However, it is important to be aware of such changes so they are not attributed to other experimental treatments.

In conclusion, the mechanisms responsible for BHA mediated anti-carcinogenesis in other animal models do not appear to be operative for AFB_1 carcinogenesis in the rainbow trout. Whether this is true for other carcinogens remains to be determined. The lack of responsiveness of the trout to enzymatic induction by BHA may provide a useful experimental model for investigating mechanisms of anti-carcinogenesis through direct interaction of BHA and carcinogens, or for examining the antioxidant effects of BHA on two-stage carcinogenesis in the absence of inductive, anti-initiating effects.

Table IV-1. Effects of dietary BHA on rainbow trout exposed to AFB_1 as embryos.[a]

Diet, wk 1-8	Body weight (gm)[b]	Liver weight (% BW)	% hepatoma incidence[c]
OTD	122 ± 3	1.09 ± 0.03	40 (N=191)
OTD + 0.03% BHA	117 ± 3	0.98 ± 0.03	40 (N=197)
OTD + 0.3% BHA	118 ± 3	0.93 ± 0.05	47 (N=192)

[a] Trout embryos were exposed to 0.5 ppm AFB_1 solution for 30 min at day 21 of incubation. Diets commenced at swim-up. Duplicate groups for each treatment were combined for reporting results.

[b] Values are means ± SEM. There were no significant differences in body weight or relative liver weight.

[c] There was no significant difference between treatments in tumor incidence 52 wk after AFB_1 exposure.

Table IV-2. Effects of BHA on rainbow trout when fed prior to and during dietary AFB_1 exposure.[a]

Dietary additions				
Wk 1-4	Wk 5-8	Body weight (gm)[b]	Liver weight (% BW)[b]	% hepatoma incidence[c]
none	10 ppb AFB_1	198 ± 7	0.67 ± 0.01[cd]	13 (N=173)
0.03% BHA	0.03% BHA + 10 ppb AFB_1	198 ± 8	0.66 ± 0.01[c]	8 (N=169)
0.3% BHA	0.3% BHA + 10 ppb AFB_1	202 ± 7	0.73 ± 0.02[cd]	11 (N=174)
none	none	190 ± 7	0.61 ± 0.01	0 (N=179)

[a] Dietary additions were made to the OTD. Duplicate groups for each treatment were combined for reporting results.

[b] Values are means ± SEM. Means with different superscripts are significantly different ($P<.01$).

[c] There was no significant difference between AFB_1 treatments in tumor incidence 52 wk after exposure.

Table IV-3. Effect of fasting on AFB_1 binding to hepatic DNA in trout fed control or BHA diets.[a]

Diet	uMol AFB_1 bound/gm DNA ± SEM	
	Non-fasted	Fasted
Control	0.11 ± 0.03 (N=6)	0.18 ± 0.04 (N=5)
BHA	0.13 ± 0.02 (N=6)	0.18 ± 0.04 (N=6)
Combined	0.12 ± 0.02[b] (N=12)	0.18 ± 0.03[b] (N=11)

[a] Trout were fed either 0.3% BHA or control diets for 3 wk. Three days prior to AFB_1 IP injection, half the trout in each group were fasted and all trout killed and hepatic AFB_1-DNA adducts determined 2 days after injection. Two factor analysis of variance showed no significant interaction or main effect differences due to diet. Fasting resulted in a significant ($P<.10$) increase in AFB_1-DNA binding.

[b] If Student's t test is used to compare AFB_1-DNA binding in all non-fasted trout to all fasted trout, there is significantly ($P<.05$) more binding with the fasted treatment.

Table IV-4. Metabolism of AFB_1 in freshly isolated hepatocytes prepared from rainbow trout pre-fed control or 0.3% BHA diets.

	Percentage of total aflatoxins recovered[a]				
Diet	Polar metabolites	AFM_1	AFB_1	AFL	DNA adducts[b]
Control	10 ± 2	5 ± 1	74 ± 3	1.9 ± 0.3	1.15 ± .19
0.3% BHA	12 ± 2	4 ± 1	72 ± 4	2.5 ± 0.3	0.96 ± .10

[a] Mean ± SEM of percent total tritium recovered from HPLC analysis of incubation media from 1 hr reactions of $[^3H]$-AFB_1 with hepatocytes. There were no differences in rate of AFB_1 metabolism (data not shown) or relative changes in metabolite profiles between treatments. N=5 for each dietary treatment with viability 95% or better using dye exclusion.

[b] Mean ± SEM uMol AFB_1 bound/gm DNA; N=10 for each dietary treatment. There was no significant difference in total adduct formation between treatments.

Table IV-5. Effect of direct addition of BHA on AFB_1-DNA adduct formation in freshly isolated hepatocytes.

	uM BHA concentration		
	1	10	100
Relative percent adducts formed[a]	105 ± 5	99 ± 4	82* ± 5

[a] Percent ± SEM adducts formed in BHA treated hepatocytes of adducts formed in hepatocytes treated with vehicle only after 1 hr incubation. Hepatocytes treated with vehicle only averaged 0.77 ± 0.2 (±SEM) uMol AFB_1/gm DNA.

* Indicates significant ($P<.05$) reduction in adducts formed using ANOVA of a random block design. Blocks consisted of hepatocytes pooled from two trout for the four treatments (N=4).

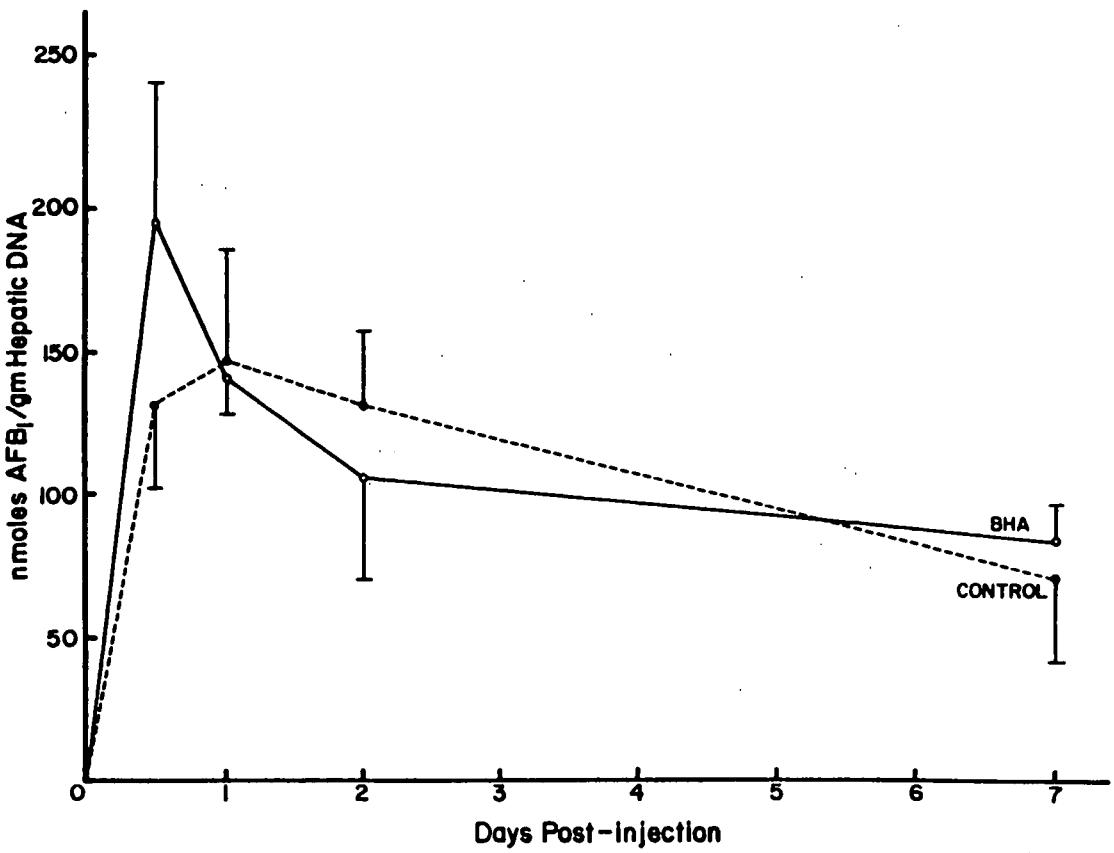

Figure IV-1. Influence of dietary BHA on hepatic DNA binding of AFB_1 in trout. Fish were pre-fed control (-•-) or 0.3% BHA (-○-) for 3 wk and killed at 0.5, 1, 2 and 7 days after IP injection of [^{3}H]-AFB_1. Liver nuclear DNA was isolated and total AFB_1 and metabolite adducts determined. Points are means of six fish with error bars representing SEM. There was no difference in total adducts between treatments.

CHAPTER V

CONCLUSIONS

The following conclusions may be derived from results of the previous studies.

1) Dietary indole-3-carbinol appears to inhibit aflatoxin B_1-induced hepatocellular carcinomas in rainbow trout through mechanisms which decrease initial DNA damage. The direct involvement of indole-3-carbinol with the carcinogen does not appear to be responsible for such action, however, indole-3-carbinol may induce enzymes involved in aflatoxin B_1 metabolism and detoxification resulting in less DNA adduct formation.

2) Butylated hydroxyanisole is not an inhibitor of aflatoxin B_1-induced hepatocellular carcinomas in rainbow trout under the conditions and dietary levels employed. Unlike indole-3-carbinol, dietary butylated hydroxyanisole does not affect the *in vitro* or *in vivo* metabolism of aflatoxin B_1 as determined by metabolite and DNA adduct formation.

3) A correlation exists between the anti-carcinogenic potential of dietary indole-3-carbinol and butylated hydroxyanisole, and their effects on *in vivo* hepatic nuclear DNA adduct formation of the hepatocarcinogen aflatoxin B_1 in the rainbow trout.

4) In vitro metabolism and DNA adduct formation of aflatoxin B_1 in freshly isolated hepatocytes from rainbow trout fed either indole-3-carbinol or butylated hydroxyanisole parallel results obtained with in vivo metabolism of aflatoxin B_1 in trout fed the same diets.

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APPENDICES

Appendix Introduction

The following section consists of experimental results which by themselves did not warrant independent publication but were nonetheless significant observations. These consist of preliminary investigative studies, or studies which supplemented results of experiments conducted by others and have been submitted for publication. Only methods which were not described in the accompanying manuscripts will be detailed in this section.

APPENDIX I

Effect of incubation temperature on AFB_1 metabolism and binding in isolated trout hepatocytes.

The water temperature in which the trout at the Food Toxicology and Nutrition Laboratory are raised is 12°C. However, AFB_1 metabolism studies using isolated hepatocytes are conducted at 20°C. The purpose of this study was to determine what effect such a temperature change would have on AFB_1 metabolism in isolated hepatocytes.

Hepatocytes were isolated from a single fish and divided into 4 flasks, 2 of which were incubated at 12 ± 1°C and 2 at 22 ± 1°C for 1 hr with 9 nMol AFB_1. DNA was purified from the cell pellet and total binding of AFB_1 and metabolites determined as well as the specific adducts formed. Metabolite production was determined on the cell-free incubation media. Hydrolysis of AFB_1-DNA adducts was done by acid and enzymatic digestion (Croy and Wogan, 1981). DNA was dissolved in 5.0 ml of 0.1 M HCl, heated at 90°C for 10 min followed by rapid cooling in an ice bath. To this, 0.5 ml of 0.5 M potassium acetate buffer (KOAc), pH 5.0, was added and the final pH adjusted to 5.0 using 1 M KOH. Enzymatic digestion was accomplished by addition of 0.05 ml of a 1 mg/ml nuclease P1 (Sigma) solution in 0.05 M KOAc and incubated at 37°C for 2 hr. A C_{18} Sep-Pak minicolumn was used to isolate adducts prior to separation by reverse phase HPLC. Conditions were similar to those of Croy and Wogan (1981), except for the use of an isocratic mobile phase of 18% ethanol in 0.01 M KOAc and monitoring of absorbance at 365 nm. Identification of peaks was accomplished by

retention time using authentic standards obtained from Croy and Wogan.

Hepatocytes incubated at 22°C had significantly more binding ($P<.01$) of AFB_1 to DNA and more metabolites produced than hepatocytes incubated at 12°C (Table AI-1.). In all cases, these increases were approximately 2-fold. Although there was a significant difference in total metabolites produced between treatments, there was no difference in the percent that each metabolite represented of the total metabolite pool (Table AI-1.). Likewise, although there were more total AFB_1-DNA adducts formed at the higher incubation temperature, the N^7-guanine adduct was the predominant adduct formed and similar ratios existed between treatments in the level of this adduct and its derivatives, peaks I and II (Table AI-2.). The latter adducts are chemical transformation products of the N^7-guanine adduct resulting from hydrolysis of the positively charged imidazole guanyl ring (Croy and Wogan, 1981). In other studies, the parent N^7-guanine adduct represents approximately 80% of the total DNA adducts formed *in vivo* in trout and rats (Irvin and Wogan, 1985; Croy *et al.*, 1980), and *in vitro* in isolated trout hepatocytes (Bailey *et al.*, 1982). Since this adduct is unstable and the guanine ring is easily hydrolyzed by alkaline conditions and heating (Irvin and Wogan, 1985), DNA isolation conditions employed in the present study may have produced uncharacteristicly high levels of the N^7-guanine-AFB_1 derivatives. Nonetheless, AFB_1-DNA adduct formation is significantly increased at the higher incubation temperature.

Although there are significant quantitative differences in AFB_1 metabolism by isolated trout hepatocytes incubated at 12 and 22°C,

there does not appear to be any significant qualitative change in metabolites or DNA adducts produced. Therefore, incubations carried out at the higher temperature would have the advantage of decreasing relative incubation time.

Table AI-1. Effect of incubation temperature on metabolism of AFB_1 by freshly isolated trout hepatocytes.

	Percent of total aflatoxin recovered[a]				
Incubation temperature	Polar metabolites	AFM_1	AFB_1	AFL	DNA adducts[b]
12 (± 1°C)	7.4* ± 1.2	1.2** ± .2	76** ± 3	10** ± 1	.59** ± .07
22 (± 1°C)	12.2 ± 0.6	2.6 ± .3	61 ± 2	18 ± 2	1.13 ± .15

	Percent of metabolite in total pool[c]		
	Polar metabolites	AFM_1	AFL
12 (± 1°C)	39 ± 7	6 ± 1	55 ± 8
22 (± 1°C)	37 ± 3	8 ± 1	56 ± 6

[a] Mean ± SEM of percent total radiolabel recovered from HPLC analysis of 1 hr incubations of [^{3}H]-AFB_1 with hepatocytes. Viability 95% or better using dye exclusion; N=5 for each treatment.

[b] Mean ± SEM uMol AFB_1 bound/gm DNA; N=5 for each treatment.

[c] Mean ± SEM of percent each metabolite contributes to total metabolite pool (i.e. AFB_1 excluded).

* Denotes means that are different ($P<.05$) using analysis of variance of a random block design.

** Denotes means that are different ($P<.01$) using analysis of variance of a random block design.

Table AI-2. Effect of incubation temperature on ratios of major AFB_1-DNA adducts produced in freshly isolated trout hepatocytes.

Incubation temperature	Percent of total adducts recovered[a]		
	Peak I	Peak II	N^7-guanine AFB_1
12 (± 1°C)	8.0 ± .4	33 ± 2	55 ± 2
22 (± 1°C)	7.8 ± .8	32 ± 3	57 ± 3

[a] Mean ± SEM of percent radiolabel recovered from HPLC analysis of hydrolyzed DNA. Peaks I and II are chemical derivatives of the N^7-guanine-AFB_1 adduct; N=3 for each treatment.

APPENDIX II

Metabolism of AFB_1 by freshly isolated hepatocytes from trout fed PCB

Aroclor 1254 (PCB) has previously been shown to inhibit AFB_1 carcinogenesis and reduce *in vivo* hepatic AFB_1-DNA adduct formation in the rainbow trout (Shelton *et al.*, 1983; Shelton *et al.*, 1984; Shelton *et al.*, 1985). The purpose of this study was to investigate the metabolism of AFB_1 in hepatocytes isolated from trout fed PCB. Some of these data have been submitted for publication (Shelton *et al.*, 1985).

Hepatocytes were isolated from fish fed 100 ppm PCB for 3 months and incubated with 10 nMol [^{3}H]-AFB_1. DNA was purified and total AFB_1 adducts determined as well as AFB_1 metabolites produced. Hepatocytes were also isolated from fish fed either 100 ppm PCB or control diets for 6 wk and used to examine AFB_1-DNA adduct formation when incubated with AFB_1 at concentrations of 5.4, 4.2, 2.9 and 1.9 nMol. Hepatocytes from one fish in each dietary treatment were used for all AFB_1 levels in an individual trial. The effect of direct addition of PCB, at concentrations of 73, 7.3 and .73 uM, on AFB_1-DNA adduct formation in hepatocytes isolated from trout fed a control diet only was determined. This was compared to additions of beta-naphthoflavone (BNF) at 100, 10 or 1 uM concentrations since this is also an inhibitor of AFB_1 carcinogenesis in the trout and appears to work through a similar mechanism as PCB. Both PCB and BNF are inducers of the MFO which could result in more rapid metabolism and elimination of

AFB_1 thus decreasing initiation damage.

Results of AFB_1 metabolism by hepatocytes isolated from trout fed PCB and control diets are shown in table AII-1. There were significantly more polar metabolites ($P<.05$) and less AFL ($P<.01$) produced in the PCB hepatocytes compared to controls. There was also a 17-fold increase ($P<.01$) in the level of AFM_1 produced and 35% more AFB_1 metabolized ($P<.01$) in the PCB hepatocytes. Although there were significant changes in the rate of AFB_1 metabolism and ratio of metabolites produced in the PCB hepatocytes, there was no significant difference in total AFB_1-DNA adducts formed. This would indicate that although enzymes in hepatocytes responsible for metabolism of AFB_1 to AFL, AFM_1 and polar metabolites are altered from dietary PCB pretreatment, the isozyme responsible for production of the adduct forming 2,3-epoxide remains unchanged and, in a 1 hr assay, the rate of production of adducts was similar between treatments. However, when adduct formation was expressed in terms of adducts formed per uMol AFB_1 metabolized, there was a significant ($P<.05$) decrease in adducts formed in the PCB hepatocytes compared to controls (Table AII-1.).

In the above study, it was calculated that the PCB hepatocytes metabolized 6.1 nMol of AFB_1 in 1 hr versus 4.2 nMol for control hepatocytes. A smaller dose than the 10 nMol AFB_1 used may have resulted in decreased AFB_1-DNA adduct formation in the PCB hepatocytes since the increase in activity of enzymes associated with AFB_1 detoxification could deplete the level of AFB_1 available for epoxide formation. Figure AII-1. shows that there was no significant

difference between treatments in adducts formed at AFB_1 doses of 5.4, 4.2, 2.9 or 1.9 nMol. However, results of this study may have been compromised since it was observed that all of the males used were sexually mature and that the DNA adducts formed in these trout were significantly higher ($P<.05$) than those produced in the remaining immature females. Regression analysis of male and female response to changes in AFB_1 dosage showed a significantly greater slope ($P<.01$) of the male response curve (Figure AII-1.). Although a broader range of AFB_1 dosages would have been more informative, it would appear that at the doses studied, the 2,3-epoxide forming isozyme of the female trout was near saturation since there were small changes with increase in AFB_1. However, if the same isozyme was present at higher levels in mature male trout, then a larger increase in response would be expected at increasing AFB_1 doses. Increases in cytochrome P-450 levels and changes in MFO activities have been shown to occur in spawning trout (Koivusaari, et al., 1984). The slight increase in DNA adducts formed in control hepatocytes (Figure AII-1.) over those formed in the PCB hepatocytes was probably due to the higher ratio of male to female trout (3 of 5) in the control group compared to the PCB group (1 of 5). This experiment needs to be repeated using a wider range of AFB_1 doses including a lower level which would be completely metabolized in 1 hr. Sexually mature fish would need to be eliminated so changes in hepatocyte metabolism due to dietary pretreatment could be determined.

Table AII-2. shows the effect of direct addition of PCB and BNF on AFB_1-DNA adduct formation in hepatocytes isolated from fish fed a

control diet only. There was no significant difference in adduct formation at 73, 7.3 or .73 uMol concentrations of PCB when compared to hepatocytes treated with vehicle (DMSO) only. This would indicate that inhibition of AFB_1 carcinogenesis and hepatic DNA adduct formation *in vivo* are not as a result of direct influence of PCB on AFB_1 initiation. In hepatocytes treated with BNF, there was an increase in AFB_1-DNA adduct formation at 100 uM BNF when compared to DMSO treated hepatocytes. However, this change was significant only at the $P<.10$ level. The reason for this increase is not known, but the *in vivo* significance of this increase is questionable since 500 ppm dietary BNF inhibits AFB_1 carcinogenesis and decreases the binding of AFB_1 to hepatic DNA in trout (Bailey *et al.*, 1982b; Nixon *et al.*, 1984).

Results indicate that PCB inhibits AFB_1 carcinogenesis in rainbow trout through enhanced detoxification of the carcinogen. Although AFB_1-DNA adduct formation was unaffected in a 1 hr assay in the isolated hepatocytes, the amount of adduct formed per unit AFB_1 metabolized was lower in hepatocytes isolated form PCB-fed fish. In addition, *in vivo* factors such as distribution and elimination of AFB_1, which may also aid in reducing DNA adduct formation, would not be operative in the present *in vitro* system.

Table AII-1. Metabolism of AFB_1 in isolated hepatocytes from rainbow trout pre-fed control or 100 ppm PCB diets.

	Percent of total aflatoxin recovered[a]				DNA adducts[b]	
Diet	Polar metabolites	AFM_1	AFB_1	AFL	uMol AFB_1/gm DNA	uMol AFB_1/gm DNA/ug AFB_1 metabolized
Control	3.6 ± 0.6	1.6 ± 0.3	84 ± 2	11.0 ± 1.0	1.4 ± 0.3 (N=9)	0.64 ± .19 (N=5)
100 ppm PCB	5.8* ± 0.5	27.0** ± 3.0	62** ± 3	5.2** ± 0.9	1.4 ± 0.2 (N=9)	1.12 ± .13 (N=5)

[a] Mean ± SEM of percent radiolabel recovered from HPLC analysis of 1 hr incubations of [^{3}H]-AFB_1 with hepatocytes; N=5 for each dietary treatment.

[b] Each value represents mean ± SEM.

* Denotes means that are significantly different from controls ($P<.05$) using Student's t test.

** Denotes means that are significantly different from controls ($P<.01$) using Student's t test.

Table AII-2. Effect of direct addition of PCB and BNF, inhibitors of AFB_1 carcinogenesis in rainbow trout, on AFB_1-DNA adduct formation[a] in freshly isolated trout hepatocytes.

Inhibitor	Inhibitor concentration[b]		
	Low	Medium	High
PCB (N=4)	96 ± 16	99 ± 10	76 ± 11
BNF (N=5)	110 ± 7	110 ± 30	180* ± 30

[a] Values are percent ± SEM of adducts formed in treated hepatocytes of adducts formed in hepatocytes treated with vehicle (DMSO) only after 1 hr incubations. Inhibitors were added in 5 ul DMSO to the 5.15 total incubation volume; adducts formed in DMSO treated cells were 0.46 and 0.60 uMol AFB_1/gm DNA for PCB and BNF controls respectively.

[b] Final inhibitor concentrations were .73, 7.3 and 73 uM for PCB and 1, 10 and 100 uM for BNF at low, medium and high concentrations respectively.

* Indicates means that are significantly different ($P<.10$) from controls using analysis of variance of a random block design. Blocks consisted of hepatocytes pooled from two trout for the individual treatments.

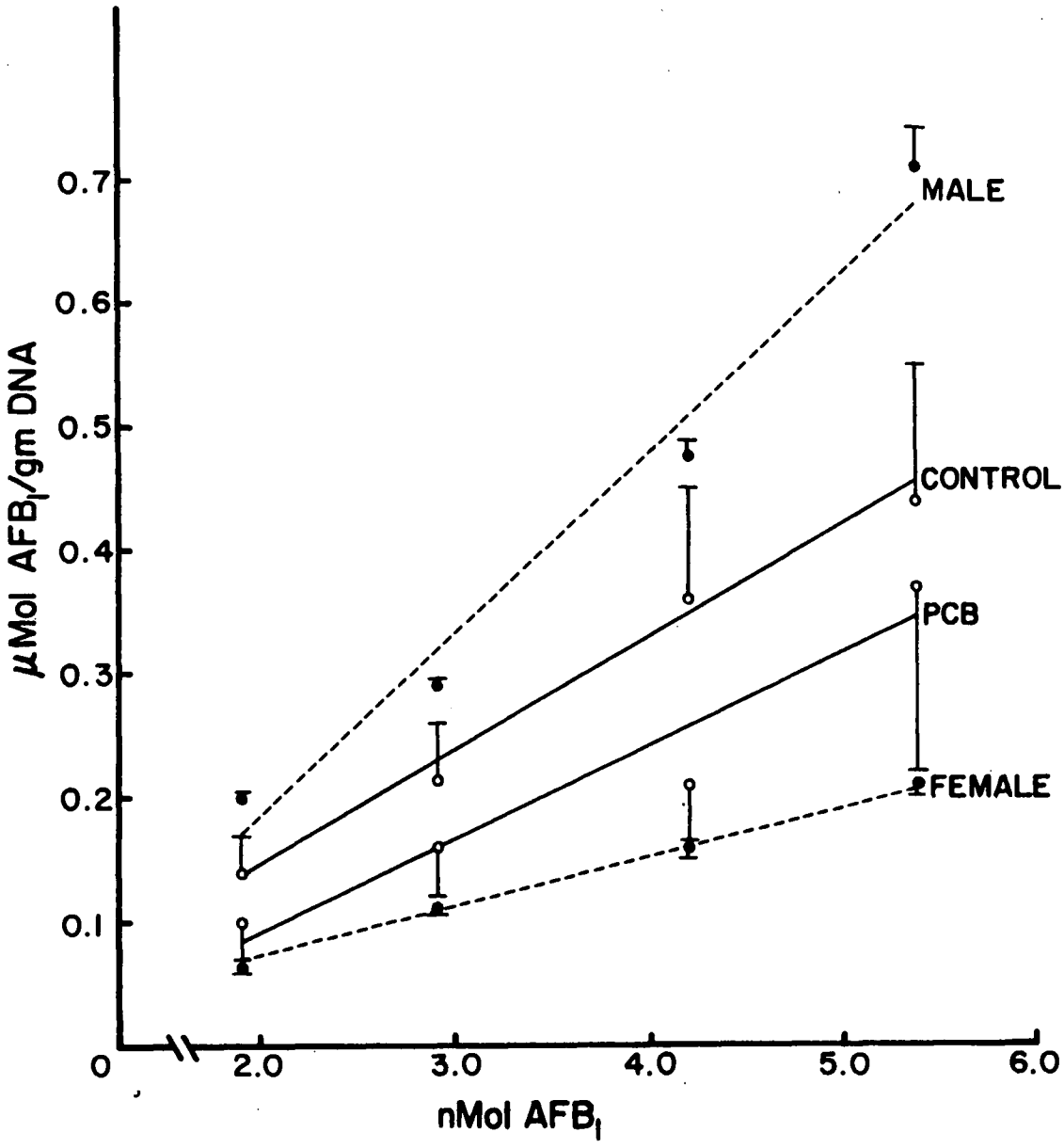

Figure AII-1. Effect of AFB_1 incubation dose on total adducts formed in hepatocytes isolated from trout fed either a control or PCB diet. There was no significant difference in adducts formed as a result of dietary pretreatment (—o—). However, if dietary treatments are ignored and trout are grouped according to sex (-•-), male trout had significnatly more ($P<.05$) total adducts formed per dose of AFB_1 and the slope of their response curve was significantly greater ($P<.01$) than female trout when analyzed by regression analysis. Variability of the mean response to dose was less when trout were grouped according to sex.