AN ABSTRACT OF THE THESIS OF

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Title: Effects of the Anticarcinogen Indole-3-carbinol on Xenobiotic Metabolizing Enzymes in the Rainbow Trout

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Indole-3-carbinol (I3C) inhibits chemically induced tumor formation in rodents and rainbow trout. This study examines the effect of I3C and its analog, indole-3-acetonitrile (I3N) on xenobiotic-metabolizing enzyme systems. The modulation of these enzyme systems have been shown to have significant effects on the interaction of chemical carcinogens and cellular constituents. Rainbow trout were fed 500, 1000 and 2000 ppm dietary levels of I3C and 50, 500 and 1000 ppm dietary levels of I3N for 8 days. β -napthoflavone (BNF), which is also an effective anticarcinogen in the trout, was fed at a 500 ppm dietary level and was used as a positive LM4b (a cytochrome P-450 isozyme) inducing control. Enzyme activities assayed were: ethoxyresorufin-O-deethylase (EROD), ethoxycoumarin-O-deethylase (ECOD), glutathione S-transferase (GST), and uridine diphosphoglucuronosyl transferase (UDPGT). Total cytochrome P-450 content was determined spectrophotometrically by the CO reduced method. The specific P-450 isozymes, LM2 and LM4b, were detected quantitatively using the western blot method. The BNF diet induced

EROD and ECOD activities by an average of 17 fold and 5.5 fold, respectively. Total P-450 content was increased 2-fold; the P-450 isozyme LM4b was induced more than 5-fold, but LM2 content remained unchanged. This diet increased UDPGT activity 1.5-2-fold, but GST activity was not induced by dietary BNF. Neither I3C nor I3N induced the activity levels of the enzymes assayed at any administered dietary levels, which have previously shown to inhibit tumor formation and reduce formation of carcinogen-DNA adducts. Thus, the anticarcinogenic mechanism of I3C may proceed in trout by mechanisms other than enzyme induction. Further experiments on the effect of I3C and I3C acid condensation products (RXN) on in vitro AFB1-DNA binding resulted in a 40% and 48% inhibition of AFB1-DNA binding by I3C and RXN, respectively. Additions of RXN at levels much lower than those estimated to exist in vivo in hepatic tissue resulted in a significant reduction in AFB1-DNA formation suggesting that even small levels of RXN offers protection against the genotoxic effect of AFB1. However, in vitro additions of neither I3C nor RXN had an effect on DNA binding using AFB1-Cl_2, an aflatoxin analog that does not require enzymatic activation. These results suggest that the primary mechanism for I3C inhibition of AFB1 induced carcinogenesis may proceed by inhibiton of formation of the ultimate electrophile, i.e. by reversible inhibition of cytochrome P-450.

Effects of the Anticarcinogen Indole-3-Carbinol on Xenobiotic Metabolizing Enzymes in Rainbow Trout

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INTRODUCTION

I. MECHANISMS OF CARCINOGENESIS

Initiation, promotion and progression

To understand the theories of anticarcinogenesis one must first be aware of the basic mechanisms of chemical carcinogenesis (See Ref. 1-4 for review). Cancer development is thought to be a multi-stage process consisting of initiation, promotion, and progression.

Initiation is defined as a permanent change in the genetic content (or genome) of the cell. For many chemical carcinogens, intiation results from the interaction of the reactive form of the carcinogen with cellular DNA. In most cases, the carcinogen will covalently bind to DNA nucleotides forming DNA adducts, which may lead to altered base pairing or changes in replication properties. Unless this damage is repaired, daughter cells may contain various types of mutations including deletions, insertions and point mutations. The point mutation is a specific alteration in the DNA that occurs during replication. It is important to recognize that this type of initiation event involves only a somatic mutation and thus is not inheritable. Promotion is the next stage in the carcinogenic process, in which the initiated cell proliferates into nodules or tumours. Progression is the process in which the benign tumour develops into a malignant tumour.

Genotoxic and epigenetic carcinogens

Two classes of carcinogens exist: genotoxic and epigenetic. Genotoxic carcinogens interact with cellular DNA and are divided into direct acting agents and agents that require metabolic activation. The direct acting carcinogens are chemically reactive. They undergo chemical decomposition to form an electrophile that is capable of covalently binding to the DNA molecule. An example of this type is ethylnitrosourea, which can rapidly form an electrophilic ethyl ion in vivo. Some procarcinogens must be enzymatically activated, which is usually an oxidation or hydroxylation reaction. Aflatoxin B1 and benzo[a]pyrene belong to this class. The monooxygenase enzyme system involved in the activation of procarcinogens incorporates one atom of molecular oxygen into the procarcinogen resulting in the formation of unstable epoxides. In the case of the carcinogen aflatoxin B1, the reactive metabolite, 8,9-dihydro-8,9-epoxy-aflatoxin B1, covalently binds to the nucleophilic N7 site of DNA guanyl residues.

Certain chemicals may intercalate into the strands of DNA. For example, intercalation by Adriamycin may result in the formation of frame-shift mutations, but the DNA complex formed does not appear to involve covalent interaction (1).

Epigenetic carcinogens are not genotoxic. By definition they do not operate by a direct carcinogen-DNA adduct type mechanism but proceed through other biological effects not well understood. These carcinogens include compounds which act as promoters such as phenobarbital which enhances the rate or effect of "spontaneous"

mutational events (5). In short, these compounds allow the proliferation of an event that has already occurred. Also included in this category are compounds that act as cocarcinogens, which upon co-exposure with the initiating agent increase the carcinogenic effect of the initiating agent. The cocarcinogenic effect may result from an increase in the level of carcinogen-DNA interaction or a promotion of the growth of the intiated cell. An example of this type is catechol which increased the multiplicity of esophageal tumors when given simultaneously with the carcinogen methyl-n-amylnitrosamine (6).

Phase I and phase II metabolism

Carcinogen metabolism involves two phases. Phase I metabolism involves oxidation, reduction, or hydrolysis in which the carcinogen is converted to a more polar compound. The primary oxidizing enzyme system is the cytochrome P-450 isozymes. Phase I metabolism of a carcinogen such as AFB1 involves competing pathways that either activate or detoxify the compound, the end result depending on which pathways are enzymatically favored at the time. Activation may be the formation of epoxides from aromatic compounds whereas deactivation may be the formation of more water soluble compounds such as hydroxylated products. However, hydroxylation may also act as an activation pathway. For example, available studies indicate that N-hydroxylation is a critical step in the activation of carcinogenic aromatic amines, amides and nitro compounds.

Phase II metabolism involves the conjugation of the now more

polar carcinogen with endogenous substrates. This step enables the compound to be more readily excreted into the bile or urine. The endogenous substrates include uridine diphosphoglucuronic acid, phosphoadenosyl phosphosulfate and glutathione.

An increase in the rate of bioactivation of the procarcinogen to the ultimate carcinogen may result in a corresponding increase in the carcinogenic effect. Bioactivation rates vary with species, environmental and dietary factors, and other parameters which influence the ratio of activated carcinogen to detoxified metabolites.

II. SIGNIFICANCE AND EFFECT OF ENZYME INDUCTION

Induction of both phase I and phase II enzymes has been studied for its effect on carcinogen bioactivation. (See Ref. 7 for review.) For example, when 3-methylcholanthrene (3MC), a carcinogenic polycyclic aromatic hydrocarbon, was fed with aminoazo dyes, the formation of hepatic tumours was greatly reduced (7). It was later discovered that 3MC enhanced microsomal enzyme activity and thus decreased the plasma half lives of the dyes (7). A large number of compounds, including the hydrocarbons, elevate enzyme activity possibly by stimulating the de novo synthesis of such enzymes. Although induction of microsomal enzymes may in some cases have a protective effect, in other instances the potential carcinogen may be activated by this process, resulting in an increase in carcinogenic response.

The most commonly studied induced enzyme system is that of the

monooxygenase system, the cytochromes P-450. This enzyme system is composed of isozymes, each a distinct gene product with characteristic but overlapping substrate specificities. Phenobarbital induces the cytochrome P-450 isozyme characterized by an increase in activity toward substrates such as benzphetamine. 3MC and the synthetic β-naphthoflavone (BNF) induce a cytochrome P-448 isozyme which is characterized by an increase towards substrates such as aryl hydrocarbons and ethoxyresorufin (8). The isozyme of interest is often termed by the substrate assayed, e.g. aryl hydrocarbon hydroxylase (AHH) and 7- ethoxyresorufin deethylase (EROD). However, metabolism of some xenobiotics, e.g., ethoxycoumarin, is thought to involve both cytochrome P-448 and P-450 isozymes (9). The enzyme activity toward this substrate is termed ethoxycoumarin deethylase (ECOD).

Cytochrome P-448, whose induction follows binding of the inducer with the Ah receptor protein in mammals, catalyzes the hydroxylation of many types of procarcinogens including benzo[a]pyrene and aflatoxin B1. Studies indicate that induction of cytochrome P-448 gene transcription is dependent on an interaction between the Ah receptor-ligand complex and an as yet unknown nuclear substance (See ref. 10 for review). Typical inducers of cytochrome P-448 include the polycyclic aromatic hydrocarbons such as 3MC. Although the cytochrome P-450 isozyme can be induced by phenobarbital and polychlorinated biphenyls in rats, no receptor has yet been discovered for this isozyme.

As mentioned previously, microsomal enzyme induction may increase or decrease the formation of the ultimate carcinogen, depending on the chemical carcinogen in question and the enzymes induced. BNF induces cytochrome P-448 activity and generally functions as an inhibitor, inhibiting the carcinogenicity of aflatoxin B1 and 7,12-dimethylbenz[a]anthrene (7).

Induction of phase II enzymes, such as sulfotransferase, glutathione S-transferases and UDP glucuronosyl transferases is a significant factor in carcinogen detoxification. Dietary BNF also induces the activities of glutathione S-transferase and UDP glucuronosyl transferase. Glutathione S-transferase activity was induced in trout (11) using styrene oxide as the substrate and in mice (12) using 1-chloro-2,4-nitrobenzene as the substrate. UDP glucuronosyl transferase activity was induced in rabbits (13), rats (14) and rainbow trout (15) using the substrate 1-napthol. In most cases, a compound that stimulates phase II conjugation tends to decrease the carcinogenic response. There are a number of compounds, however, that form DNA adducts after conjugation. For example, it was observed that DNA binding of 1,2-dibromo-[1,2-^{14}C]ethane was dependent on the presence of glutathione (16). 7, 12- dihydroxymethylbenz[a]-anthracene (DHBA) also appears to be activated following conjugate formation. Sulfotransferase mediates the formation of DHBA 7-sulfate which may then bind to either N1 of adenine residues or the N3 of guanine residues of DNA (17).

III. ANTICARCINOGENESIS

Three main catagories

Anticarcinogenesis may be defined as inhibition of any step of the carcinogenic process. Anticarcinogens may exert their effect when fed prior to or concomitantly with the administered carcinogen. They may also have an inhibitory effect when fed subsequent to carcinogen administration, during the promotion phase. For example, d-limonene increased the time to tumor formation when fed after 7,12-dimethylbenz[a]anthracene administration in rats (18). Inhibitors are a diverse set of compounds, many of which are normally encountered in the diet. This is exemplified by almost daily exposure to butylated hydroxyanisole (BHA) in bread, ascorbic acid in citrus fruits, and indole-3-carbinol in Brassica vegetables.

The current knowledge of anticarcinogenesis (inhibition) is incomplete. Wattenberg (19) has classified inhibitors according to the time in which they affect the carcinogenic process. There are three categories. The first consists of compounds that prevent the formation of the active carcinogen. An example is ascorbic acid which prevents the formation of N-nitrosamine compounds from precursors *in vivo* (20). Ascorbic acid acts as a competitor with endogenous amides or amines for reaction with available nitrosating agents. The second category involves compounds that prevent the electrophilic species from binding to cellular macromolecules. These compounds are aptly termed blocking agents. An example of this type is (BHA) which is widely used as an antioxidant in foods. The mechanism by which BHA

produces its inhibitory effects appears to be related its ability to induce phase II conjugation, especially induction of the glutathione transferases (21). The final category is the compounds that suppress the formation of neoplasia after the initiating species has reached its target site. Retenoids and protease inhibitors are included in this category.

Blocking agents

Blocking agents contain three groups of compounds also classified by their mechanism of action. The first group inhibits the formation of the ultimate carcinogen from its precursor, and therefore, are most effective against those carcinogens requiring metabolic activation. This is a direct blocking of enzymatic activation, such as inhibiting the oxidation by cytochrome P-450. The second group favors the detoxification pathway. This result is achieved by either suppressing the activation of the carcinogen from its precursor or enhancing a detoxification reaction such as phase II conjugation. For example, if the cytochrome P-450 pathway favored the formation of the ultimate carcinogen while the cytochrome P-448 catalyzed pathway favored formation of the least reactive metabolite, BNF would act as a blocking agent since it induces the cytochrome P-448 activity. In mammals, this enzyme induction seems to simultaneously result in a decrease in the levels of the other, presumably constitutive isozymes (22). Many blocking agents induce both phase I and phase II metabolizing enzyme systems. This action has been demonstrated by

administering BHA prior to BaP exposure. The resulting inhibition was due to a decrease in epoxide formation and an increase in the 3-hydroxy product, a detoxification metabolite (23). The third group scavenges the active carcinogen. Often it is the cellular constituent glutathione that acts as a nucleophile (24).

The primary mechanism of action of a blocking agent is often a modulation effect on xenobiotic metabolism which may include induction of enzyme activities. This induction may decrease the half-life of the carcinogen or the quantity of the ultimate carcinogen formed, thus the amount of metabolite available to bind to DNA is decreased.

Ideally, enzyme induction is detected by an in vivo determination of changes in the pattern and amount of metabolism of the carcinogen to be studied. However, these studies are often technically difficult. The most common alternative methods include an in vitro enzyme assay using the carcinogen as a substrate or, if this is not possible, by using a pseudo substrate that structurally resembles the carcinogen. Since many of the xenobiotic metabolizing enzymes are a system of isozymes with different substrate specificities, it may become necessary to examine several different pseudo substrates.

IV. INHIBITION BY INDOLE-3-CARBINOL

Inhibition of tumor formation

Indole-3-carbinol has been classified as a blocking agent as it acts to prevent the carcinogen from reaching the target site when fed prior to or during carcinogen administration. One of the modulating effects of indole-3-carbinol and its analogs is the ability to induce the monoxygenase enzyme system (25). It has been shown to inhibit formation of tumours initiated by a diverse set of compounds, many of which require metabolic activation.

I3C inhibited formation of mammary tumours induced by 7,12-dimethylbenz[a]anthracene when the inhibitor was administered by gavage and in the diet (26). Dietary administration of I3C also inhibited benzo[a]pyrene initated tumour formation in the forestomach (26) and pulmonary adenoma formation (23) in mice. In trout, I3C inhibited AFB1 induced liver tumours (27).

Effect on xenobiotic-metabolizing enzymes

I3C is a dietary constituent of the Brassica vegetable family. This family includes cauliflower, broccoli, cabbage, and Brussels sprouts and many dietary studies have examined the effect of these vegetables on xenobiotic metabolism. I3C and its breakdown products are shown in Figure 1.

An early study on the effect of dietary Brussels sprouts and dried cabbage (28) showed that these vegetables increased the metabolism of hexobarbital, phenacetin, 7-ethoxycoumarin and benzo[a]pyrene *in vitro*. These compounds are commonly used substrates

Figure 1. Indole-3-carbinol formation in plants. Breakdown of glucobrassicin by the enzyme myrosinase results in the formation of indole-3-carbinol (I3C) under basic conditions, but under acidic conditions the major product formed is indole-3-acetonitrile (I3N), an analog of I3C. I3C may be converted to indole or undergo condensation to form dimers or tetramers. GLB, glucobrassin; I3N, indole-3-acetonitrile; IMC, indole-methylisothiocyanate; I3C, indole-3-carbinol; ASB, ascorbigen; 3MI, 3-methyl-1-4-indole; I33, 3,3' diindolymethane; DIM, di-indolyl methine salts; I3A, indole-3-aldehyde; IAA, indole-3-acetic acid; IND, indole.

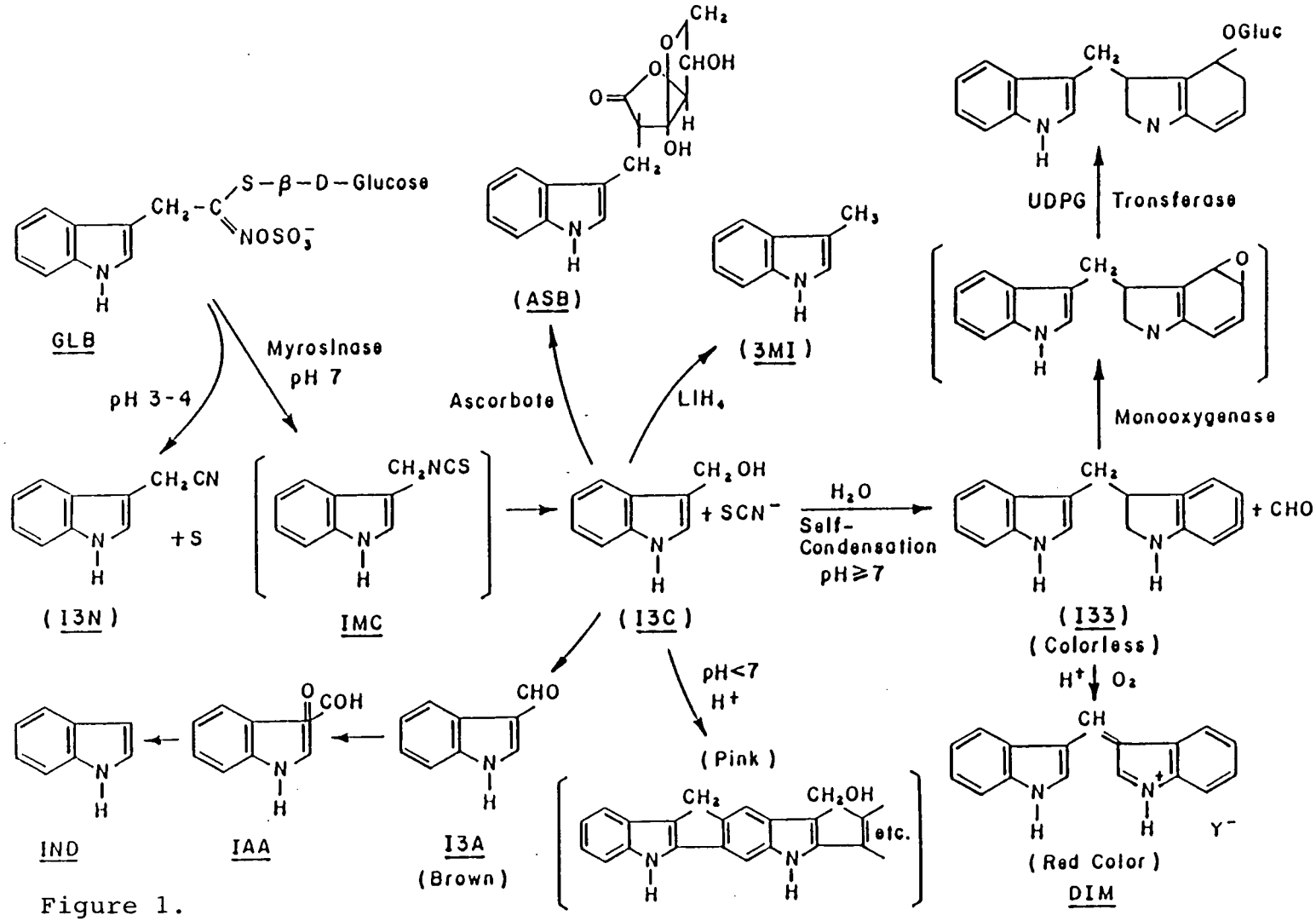

GLB
Myrosinase
pH 7
pH 3-4
(I3N)
+S
IMC
(ASB)
Ascorbate
LiH4
(3MI)
(I3C)
+ SCN⁻
H2O
Self-
Condensation
pH≥7
(I33)
(Colorless)
+ CHO
UDPG
Transferase
OGluc
Monooxygenase
H+ O2
(Red Color)
DIM
Y⁻
pH<7
H+
(Pink)
etc.
I3A
(Brown)
IAA
IND

Figure 1.

to assay the activity of cytochrome P-448 type enzymes. Dietary broccoli (25%) also was found to stimulate benzo[a]pyrene metabolism in rats (29). An increase in not only hepatic monoxygenase activity but also phase II activity, glutathione S-transferase (GST) and epoxide hydrase, was observed. Dietary cabbage (25%) did not increase ECOD or AHH activity in the liver but did stimulate activities in the intestine of rats (30). Hepatic and intestinal glutathione S-transferase and epoxide hydrase activities were induced. These increases in enzyme activities correlated with a decrease in AFB1 binding to DNA by 87% compared to the control group.

A 25% dietary Brussels sprouts administration induced monooxygenase and GST activities in mouse livers (31) and in rat intestines (32). In the rat intestine, epoxide hydrase activity was also induced, and in vitro metabolism of BaP resulted in a reduction of active carcinogen formation. This was demonstrated by an increase in the conversion of BaP to the more polar metabolites. These results indicate that components of the brassica vegetables can act as inhibitors by preventing the formation of the ultimate carcinogen, a definition of a blocking agent.

Sparnins et al. (12) examined the ability of two constituent compounds of the brassica vegetables, I3C and indole-3-acetonitrile (I3N), for their ability to induce GST activity in the small intestine and liver of mice. Comparison of both compounds with a known GST inducer and inhibitor, BNF, and dietary Brussels sprouts showed that all of these dietary administrations significantly induced enzyme

activities in both organs.

I3C is a hydrolysis product of indole glucosinolate and glucobrassin. The reaction is catalyzed by the endogenous enzyme myrosinase. Since the previously mentioned studies did not consider relative glucobrassin content when studying the effect of dietary vegetables, McDannell et al. (33) examined the correlation of glucobrassin levels in cabbage with enzyme induction. A 25% dietary administration of cabbage induced EROD activities in the intestine and liver of rats. Boiling the cabbage decreased the level of intact glucosinolate and resulted in a corresponding decrease in the level of enzyme activity induced. When glucobrassin had been totally hydrolyzed to I3C and ascorbigen, considerable induction of the monooxygenase enzyme activities occurred in both the intestine and the liver. However, I3C had a more significant effect on the activities in the liver while ascorbigen had a greater effect in the intestine. This is in agreement with the previous finding of Loub et al. (34).

Babish and Stoewsand (35) observed hepatic monooxygenase enzyme induction in rat at only the 5000 and 7500 ppm dietary levels. Since these levels far exceed that which naturally occurs in the brassica vegetables, they concluded that I3C was not the primary inducing compound. In contrast, a later study (36) determined that a dietary level as low as 50 ppm I3C induced monooxygenase activity in intestine and GST activites in both liver and intestine in the rat. However, hepatic monooxygenase activity was not induced at any dietary level. These results also led to the suggestion that I3C was not the primary

constituent responsible for the induction of monoxygenase activity by cruciferea vegetables.

A study of the interaction of indole analogs with the Ah receptor was done by Gillner et al. (37) to determine the relationship between the structural features and the inducing abilites of these compounds. All of the 3-substituted indoles (including I3C and I3N) bound weakly to the receptor, leading Gillner to suggest that although I3C induces the cytochrome P-448 isozyme, it does so without strong interaction with the Ah receptor. Perhaps a weak interaction of I3C with the Ah receptor explains why high dietary levels of this compound must be administered before an induction effect can be detected.

Acid products of indole-3-carbinol

Bradfield and Bjeldenes (36) noted that some of the differences observed in enzyme induction by I3C could be due to its instability. They observed that I3C is susceptible to oxidation, resulting in a color change, and also decomposes during separation by thin layer chromatography. This instability was also demonstrated during autolysis of the parent vegetable. Although I3C was the major compound isolated from Brassica vegetables, 84% of the I3C isolate had decomposed within 24 hours (38).

In dried vegetables, the amount of I3C present could be less than 50 ug/gram vegetable. Since most studies on the effects of dietary vegetables administered 25% dried vegetable, the amount of I3C present is less than 50 ppm and less than the reported no effect level of I3C for enzyme induction. In rat intestine, the most

sensitive organ to I3C induction, I3C (250 ppm) induced ECOD and AHH activities almost 20 times whereas the 25% cauliflower had no effect (39). A 250 ppm dietary level of I3C did not induce ECOD activity in the mouse liver, but a 25% cauliflower diet increased the activity two fold. A time course study (39) revealed that induction by 25% cauliflower reached a maximum value after 7 days of feeding and began to recede by day 10.

Shertzer (40) provided evidence that I3C administration by gavage did induce rat hepatic monooxygenase activity (EROD) but did not induce enzyme activity when administered by intraperitoneal (i.p.) injection. The levels administered were as high as 1/10 and 1/3 the LD50 values of gavage and i.p. doses respectively. This led to the hypothesis that I3C must first be metabolized by passing thru the acidity of the stomach. Thus, the inducer may be an acid reaction product of I3C, not I3C itself. In the same study, both the mouse and the rat showed definite enzyme induction that appeared to be the cytochrome P-448 type, since the induction response closely paralleled that of BNF. Rabbits, however showed no sign of enzyme induction by either mode of administration, an indication that the effect of I3C is species specific. I.p. injection of I3C resulted in a slight increase in EROD enzyme activities. Indole-3-acetonitrile appeared to be less effective in inducing these enzyme activities than I3C.

Bradfield and Bjeldanes (41) then suggested that the activity of indole compounds (including I3C) is directly related to the acid sensitivity of the compounds. They demonstrated that I3C was quite

unstable in acidic media. I3C showed the greatest potency as an inducer towards EROD activity in comparison to other indole analogs after acute oral intubation. On the other hand, I3N appeared to be more stable in an acidic environment and did not significantly alter EROD acivity. This study supported Shertzer's metabolite theory (40) by demonstrating that the acid reaction products induced EROD activity after i.p. injection while I3C did not.

Some uncertainties of monooxygenase enzyme induction by I3C remain. In the studies previously cited, differences in the dosing regimens, i.e., dietary level, length of feeding, and method of administration (gavage, dietary, or i.p. injection) may account for some of the apparent discrepancies. Another consideration is the chemical instability of I3C which, may not survive diet preparation and storage protocols.

Effect on DNA binding

I3C inhibits DNA binding of BaP and N-nitrosodimethylamine (NDMA) metabolites in mice (42,43). From the data previously cited, it seems reasonable to assume that the level of monooxygenase activity induced would correspond to a decrease in DNA binding. However, this study demonstrated that the level of AHH enzyme induction by I3C did not correlate with the ability of I3C to protect against DNA binding of BaP metabolites. Although pretreatment with I3C did not result in a change in the rate of BaP oxidation catalyzed by mouse hepatic microsomes in vitro, it did result in a 80% decrease in DNA binding

in vivo. I3C decreased DNA binding of NDMA by 70-90% *in vitro* and caused a similiar decrease *in vivo*. Again, I3C did not have an effect on oxidation of NDMA (as determined by analysis of NDMA demethylase activity).

However, Chung *et al*.(44,45) found that pretreatment of rats with 4000 ppm dietary I3C actually stimulated the alpha-hydroxylation of nitrosamines, the activating step of N-nitrosopyrrolidine, N'-nitrosonornicotine, N-nitrosodimethylamine and 4-(methylnitrosamino-1-(3-pyridyl)-1-butanone. Although cytochrome P-450 is thought to be involved in this metabolism, this has not yet been proven.

In conclusion, I3C inhibits tumour formation initiated by a number of carcinogens. This inhibitory action has been linked to its ability to induce xenobiotic metabolizing enzymes, i.e., cytochrome P-448, glutathione S-transferase, UDP-glucuronosyl transferase, and epoxide hydrase. However, since protection against DNA binding by I3C is possible without evidence of enzyme induction, I3C inhibition may proceed by other mechanisms. Several possibilities exist: 1) I3C may be metabolized to nucleophilic products in the endoplasmic recticulum where they may be in close proximity to an electrophilic ultimate carcinogen and may therefore trap the electrophile, preventing it from binding to the DNA molecule (43). 2) I3C condensation products may modify carcinogensis by direct inhibition of the monooxygenases responsible for carcinogen bioactivation (41). 3) I3C may alter carcinogen uptake and transport systems.

V. AFB1 METABOLISM

Aflatoxin B1 is the most potent carcinogenic mycotoxin known to man. It is produced by Aspergillus flavis, a mold commonly associated with foods such as corn and peanuts. Thus, varying amounts of this carcinogen are present in our daily food supply. Dietary levels of AFB1 which range from 23 to 500 ppb in peanut oil and corn meal, or an average of 58 ug AFB1 intake/person/day, have been reported in the Fushui county of the People's Republic of China (46). The high dietary AFB1 level is correlated with an average incidence of liver cancer of 55.8/100,000 persons, which is one of the highest in the world (47). Human metabolism of AFB1 results in the formation of a significant amount of the same major DNA adduct as that found in laboratory animals. For example, 7-10 ng of 8,9-dihydro-8-(N7-guanyl)-9-hydroxy-aflatoxin was found in the urine of an individual who had consumed 87 ug of AFB1 the previous day (48).

AFB1 has shown varying potency for induction of hepatocellular carcinoma among various laboratory animal models. In Fischer rats, a carcinogenic dose response was seen after dietary levels of 1 to 50 ppb AFB1 (49) whereas the Swiss mouse was resistant to levels up to 150 ppm (50). Rainbow trout prove to be a very sensitive animal model; a dietary dose of 6 ppb can lead to a 70% tumor incidence (51).

Oxidative (phase I) metabolism of AFB1

The oxidative metabolism of AFB1 proceeds by a competing set of pathways. In mammals, at least three different isozymes of the

monooxygenase system are involved (52). The first isozyme catalyzes the conversion of AFB1 to the less carcinogenic AFM1. This enzyme is associated with the cytochrome P-448 system and genetic regulation involving the Ah locus. However, a negative correlation between metabolism of AFB1 and AHH or BaP metabolism indicates that it is distinct from the AHH enzyme (52). The other two enzymes metabolizing AFB1 are cytochrome P-450-linked systems. One catalyzes the conversion of AFB1 to AFQ1, and the other transforms AFB1 to the AFB 8,9-oxide, an unstable precursor to the ultimate carcinogen (metabolic activation). The involvement of the cytochrome P-450 isozymes has been examined using specific cytochrome P-450 and cytochrome P-448 inducers. Phenobarbital, a cytochrome P-450 inducer, enhanced *in vitro* AFQ1 production, whereas 3-methylcholanthrene, a cytochrome P-448 inducer, enhanced the production of AFM1. AFM1 may be considered a detoxification product as it is less carcinogenic than AFB1 in rats *in vivo* (53) and rainbow trout (54).

Oxidation at the 8,9 position of AFB1, AFM1, and AFP1 can lead to formation of electrophiles that react with water forming dihydrodiols or with cellular nucleophiles such as DNA forming covalent adducts (55).

Conjugative (phase II) metabolism of AFB1

After the epoxide is formed, three pathways compete for this highly reactive species. The epoxide may be hydrolyzed spontaneously or by epoxide hydrolase mentioned above, to form the AFB1-dihydrodiol. It may undergo conjugation with cellular GSH by a glutathione S-

transferase pathway, or it may react with cellular macromolecules (56). The epoxide hydrolase pathway is not considered of primary importance as inhibition of this pathway did not increase AFB1 mutagenicity (57) or AFB1-DNA binding (58). The importance of conjugation of AFB1 metabolites with sulfate or UDP-glucuronic acid in rodents has not yet been determined.

Degan and Neuman (59) have shown that of the three pathways listed above, glutathione conjugation is the dominant, enzymatically controlled inactivation step in rodents. The glutathione conjugate accounted for about 10% of the i.p. administered AFB1 dose in mice. This observation led to studies comparing the differences in AFB1 metabolism between sensitive and resistant species. These same researchers (56) correlated the lower sensitivity of mice as compared to rats to the more efficient inactivation of AFB1-epoxide by conjugation with glutathione. In the rat, pretreatment with the cytochrome P-450 and P-448 modulators, phenobarbital and Aroclor 1254, respectively, increased the overall rate of AFB1 metabolism but did not increase the amount of epoxide formed. More importantly, the glutathione conjugate level was twice that found in the control. This suggests that one of the protective effects of these modulators is by induction of formation of the glutathione conjugate.

The importance of the role of glutathione conjugation in mammals can also be illustrated by the effects of ethoxyquin on AFB1 carcinogensis (60). A dietary coadministration of ethoxyquin and AFB1 resulted in a 5-fold increase in multiple molecular forms of GST and a

corresponding 18-fold decrease in in vivo DNA-AFB1 binding.

Raj et al. (61) continued work on species comparison by examining differences in AFB1-glutathione formation between rat and hamster. In hamster, which is resistant to AFB1 carcinogenesis (62), the ratio of AFB1-GSH to AFB1-DNA binding is 10 to 15 times higher than that of the less resistant rat (63). Hamster cytosolic glutathione S-transferases catalyzed the formation of AFB1-GSH which may divert the AFB1-epoxide from binding to other cellular nucleophiles (61). Comparison between the GSH S-transferase activities in the livers of rat and hamster indicated that the hamster has a greater capability to inactivate the reactive metabolite of AFB1 (64). Three of the seven solubilized GSH S-transferases isolated from rat liver by Coles and Meyer et al. (65) had high activities towards the AFB1-8,9, oxide. The conjugation reaction with AFB1 is thought to mediated by the isozyme known as glutathione S-epoxide transferase and results in the formation of 8-(S-glutathionyl-9-hydroxy-8,9-dihydro AFB1). In the rat, glutathione-S-epoxide transferase activity was induced by both PB and 3-MC, but BNF had no effect (66).

Carcinogenicities of aflatoxins in rainbow trout

As mentioned previously, rainbow trout are extremely sensitive to AFB1 carcinogensis. In trout, fewer oxidative metabolites are formed, but the ultimate electrophile again results from the AFB1-8,9-epoxide. The relative mutagenic potencies of the metabolites formed in trout and shown in Figure 2 are:AFB1>AFL>AFLM1>AFM1 (67,68). AFL is almost

equally as potent as AFB1 (67) whereas AFM1 is only one-third as potent as AFB1 (68) in trout. Aflatoxicol formation, a cytosolic process, is possibily in equilibrium with AFB1 and may act as a reservoir for AFB1 (68).

Aflatoxin metabolizing enzymes in rainbow trout

In trout, the major phase I aflatoxin metabolizing enzymes are cytochrome P-448- and P-450-dependent systems. The major phase II enzyme system involved appears to be only the UDPG transferases, as the study of Valsta indicated that glutathione conjugation with AFB1 is not a major phase II metabolism pathway (69). Although some cytochrome P-450 isozymes have been isolated and characterized in trout (70), the glutathione S-transferases and UDPG transferases have not been fully characterized.

Trout enzymes in general tend to have a lower temperature optimum and a lower enzyme content and activity than mammalian systems (71). In trout, phase I microsomal enzyme activities such as EROD activities have a temperature optimum of 20-25 ° C while that of the rat is 40 ° C (72). The optimal incubation temperature for trout phase II enzyme activities such as UDPGA transferase, is also 25 ° C (73).

Phase I metabolism of aflatoxin B1 in rainbow trout

Both cytochrome P-450- and P-448-dependent reactions are thought to be involved in the phase I metabolism of AFB1. Five constitutive isozymes have been purified and characterized from untreated trout (74). The major consitutive form of cytochrome P-450 is LM2, but

Figure 2. Metabolism of aflatoxin B1 (AFB1) in the rainbow trout. Metabolites include: aflatoxin M1 (AFM1), aflatoxicol (AFL) and aflatoxicol M1 (AFL-M1). The ultimate carcinogen, 2,3 epoxide, may also be referred to as AFB1-8,9-epoxide.

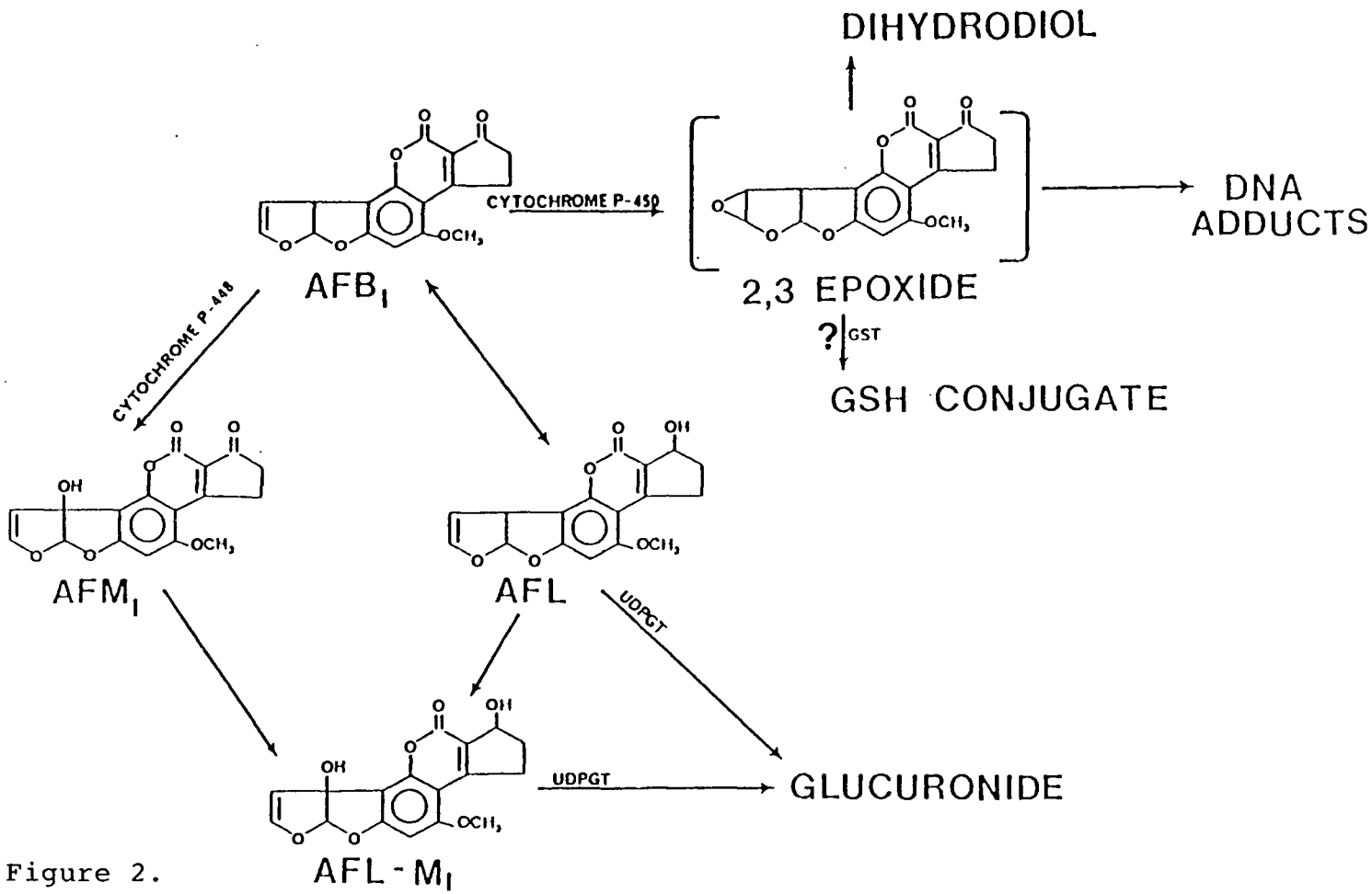

DIHYDRODIOL
CYTOCHROME P-450
DNA ADDUCTS
AFB1
2,3 EPOXIDE
?GST
GSH CONJUGATE
CYTOCHROME P-448
AFM1
AFL
UDPGT
UDPGT
GLUCURONIDE
AFL-M1

Figure 2.

LM4b is the major BNF inducible form and exhibits high benzo[a]pyrene hydroxylase activity (75). LM2 appears to be similiar to rat cytochrome P-450 in the activation of AFB1, i.e., trout cytochrome P-450 (LM2) efficiently converts the parent AFB1 to the AFB1-8,9-diol. Trout LM2 catalyzed the formation of over 13-fold more AFB1-8,9-diol than the similiar rat cytochrome P-450 and resulted in 22-fold greater DNA binding (70). This may account in part for the greater sensitivity of the trout to the carcinogenic action of aflatoxin B1. However, the trout appear to unresponsive to PB-type cytochrome P-450 induction (76); PB pretreatment had no effect on LM4b, LM4a or LM2 activities.

In contrast to the effect of PB, BNF pretreatment greatly increased the levels of LM4a/LM4b (76). Untreated trout have very low levels of LM4a/LM4b, but this is greatly enhanced after pretreatment with BNF and PCB. Trout LM4a and 4b are similiar to rat cytochrome P-448 with the LM4b comprising the majority of the two forms. These LM4a and LM4b isozymes have the same molecular weight and with the available techniques are immunologically identical, but certain physical properties such as their differences in resolution on DEAE-Sepharose amino acid composition indicate that they are discrete isozymes (75). Quantitation by most immunochemical techniques then, will detect both.

Phase II metabolism of aflatoxin B1 in rainbow trout

Glutathione-S-epoxide transferases and UDPG glucuronosyl

transferases are the primary enzymatic activities involved in AFB1 conjugate reactions. Both of these enzyme systems are comprised of multiple isozymes. Although glutathione conjugation is the primary phase II pathway of AFB1 conjugation in mammals, this is not the case in trout (69). The major phase II AFB1 metabolite is the glucuronide of AFL, but dietary BNF pretreatment results in the formation of AFL-M1-glucuronide as the major product. Small amounts of sulfate conjugates of AFB1 metabolites are produced (77).

Rainbow trout liver, which has at least seven soluble glutathione S-transferases (78), contains more than 90% of this enzyme activity in the cytosol fraction (79) and about 2% of the total glutathione S-transferase activity in microsomal fraction. Although mammalian glutathione S-transferases exhibit broad substrate specificities, trout do not have the same capabilities. For example, the trout enzymes appear to have little or no activity towards the substrates bromosulphophthalein, 1,2-dichloro-4-nitrobenzene and trans-4-phenyl-3-buten-2-one. This lack of substrate binding may be a result of the absence of the protein, termed ligandin, that is present in rat and binds organic ions and ligands such as bromosulphophthalein (80).

The substrate specificities of GST isozymes towards carcinogens have not been characterized in trout. Ramage and Nimmo (78) have grouped the isozymes as anionic or cationic depending on their chromatofocusing behavior. The anionic group differs from the cationic group in that it is the only one that recognizes a substrate such as delta 5-androstene-3,17-dione.

In rat, glutathione-S-epoxide activity was induced by pretreatment with PB and 3MC by 40-60% (66). BNF, however, did not have an effect. In contrast, pretreatment with BNF in trout elevated GSH transferase activity towards the same substrate, styrene-oxide (11).

The UDP-glucuronosyl transferase enzyme (UDPGT) exists in several forms that are substrate specific (see Ref 81 for review). In mammals, the two functionally distinct forms are classified as GT1 and GT2. These two forms have not been distinguished in trout. Since trout UDPGTs have not been fully characterized, much of the following discussion will compare properties of the UDPGT in mammal with what is known in trout.

GT1 activity is induced by compounds such as 3MC and the isozyme catalyzes conjugation with substrates such as 1-napthol which are normally planar phenolic aglycones. This form is also called the late-fetal group since it develops after birth. GT2 activity is induced by PB and the isozyme conjugates steroids and other compounds that are bulky, such as morphine. This neonatal group is present at birth.

Andersson et al. (15) examined UDPGT induction by BNF pretreatment in trout and found that the enzyme activities towards both types of substrates, i.e., 1-napthol and testosterone were induced. UDPGT activity retained the maximum levels towards 1-napthol four to six weeks after BNF injection whereas the activity towards testosterone rapidly declined after reaching a peak value at one week

following injection. Since the two types showed different patterns of induction, they suggested that trout contain functionally distinct UDPGT forms.

In mammals, GT1 is located in the endoplasmic reticulum of the centrolobular part of the liver. In contrast, GT2 is widely distributed throughout the hepatocyte. Significant levels of GT2 activities have been found in the golgi body and the plasma membrane.

UDPGTs have been termed latent enzymes in both mammals and fish because they do not reach their fully activated state in vitro until treated with an activator, usually a detergent. This property may result from the location of the enzyme, which is embedded deep in the membrane phospholipid bilayer. Detergent type and optimum concentration vary with the type of substrate used (82). In rats, surfactants can increase the activity as much as 12-fold, but in trout, activation is normally 2-fold (15). A comparison between trout and mammalian species showed that trout had a much higher UDPGT activity than the mammals assayed (rat, mouse, guinia pig) toward testosterone, but the activites towards other substrates, e.g., 1-napthol, were barely detectable in the control animals (71). Activities toward hydroxylated carcinogen metabolites have not been reported in trout.

Effect of BNF on AFB1 Metabolism

BNF inhibits AFB1-induced carcinogensis in both rats (83) and trout (27). In trout, pretreatment with dietary 500 and 50 ppm BNF resulted in a 6% and 15% tumor incidence, respectively, as compared to

the 38% incidence of the AFB1 group with no pretreatment (27).

BNF significantly induced trout cytochrome P-448 (indicated by an induction of EROD activity) by a dietary level as low as 50 ppm (27). Microsomes isolated from BNF-treated trout incubated with AFB1 produced an increase in the formation of AFM1 while reducing the formation of the AFB1-8,9-diol when compared to the control (70). Associated with cytochrome P-448 induction, dietary BNF reduced AFB1-DNA binding *in vivo* (27) and increased the formation of the less carcinogenic AFM1 (84) and AFL-M1 glucuronide (77).

In summary, BNF seems to exert its protective effect towards AFB1-induced carcinogenesis by modulating both phase I and phase II metabolism of this carcinogen. Induction of phase I metabolism (ie. the cytochrome P-448 isozyme) favors the formation of the less carcinogenic AFM1 metabolite, whereas induction of phase II metabolism favors the formation of the AFL-M1 glucuronide.

VI. EFFECT OF I3C ON AFB1 METABOLISM IN TROUT

Dietary I3C was previously examined for its ability to induce trout monooxygenase enzyme activities, which were determined by total cytochrome P-450 content, ECOD, EROD, NADPH-cytochrome c reductase and benzphetamine-N-demethylase enzyme assays (85). The effect on in vitro aflatoxin metabolism was also studied. I3C and its analogs (indole-3-ethanol, indole-3-aldehyde, and indole-3-acetic acid) did not significantly alter the assayed enzyme levels. The dietary pretreatments also did not change the pattern of aflatoxin metabolism. This study used a dietary level of 500 ppm I3C and not the level used for the DNA binding and tumor inhibition studies, 2000 ppm. Since previous studies in the mouse had found induced enzyme levels only at high doses of I3C, perhaps this dose was simply to low to show detectable induction.

AFB1-SG conjugation appears not to be a major conjugation reaction in vitro in the rainbow trout. Furthermore, pretreatment with either 500 ppm BNF or 2000 ppm I3C did not increase the formation of the AFB1 glutathione conjugate (69). However, the activity of glutathione S-epoxide transferase has not been carefully studied.

Some indication of dietary I3C induction of phase II conjugation in trout has been observed by use of tritated AFB1. I3C increased the level of total radioactivity in the bile and decreased total radioactivity in the blood and liver, signaling a change in the pharmokinetics of AFB1 (86). Pretreatment of I3C at a dietary level

of 2000 ppm reduced DNA binding in vivo by 70%. A 7-fold increase in aflatoxicol-M1 glucuronide over the control level in bile was also found. However, it was not determined whether the increase in glucuronides was a result of UDP-glucuronosyl transferase induction or an increase in the availability of the substrate, aflatoxicol-M1.

I3C inhibits tumour formation initiated by AFBI in rat and rainbow trout. It protects against DNA binding of AFB1 metabolites in trout in a dose-dependent manner in vivo (87). The concomitant increase in dietary exposure of I3C with constant level of AFB1 results in a corresponding decrease in formation of the AFB1- DNA adduct. HPLC analysis of the DNA adducts indicated that the major adduct consistently formed was the 8,9-dihydro-8-(N7-guanyl)-9-hydroxy aflatoxin regardless of dietary manipulations. This study also indicated the absence of a threshold below which I3C would offer no protection against DNA binding.

VII. OBJECTIVES AND RATIONALE

The objectives of this study are to examine the mechanisms for the inhibitory effects of I3C against aflatoxin B1 hepatocarcinogensis by determining the effect of an anticarcinogenic dose of I3C on the aflatoxin metabolizing enzymes in rainbow trout, specifically: Cytochromes P-450 and P-448 (LM2 and LM4b), glutathione-S-epoxide transferase and UDP glucuronosyl transferase. An attempt must be made in determining the enzyme activities towards the actual carcinogen precursor, eg. UDP glucuronosyl transferase towards AFL, as most trout enzyme activities have been determined using psuedo substrates. I3C and its cyano-analog, I3N will be fed for eight days at various dietary levels to determine if the effects are dose-responsive and if any differences in induction occurs between the two analogs. I3N was chosen for study as its CN- moiety is more nucleophilic than the OH- moiety of I3C. This may of importance if a metabolite of I3C is involved. I3N has not been studied for its effect on enzyme induction in trout. BNF will serve as a positive control and will be fed for the same duration at a 500 ppm dietary level. BNF induces the activities of most of the enzymes to be studied, which is associated with its protective effect against AFB1 induced carcinogenesis. Similiar induction by I3C may give an indication of its mechanisms of action.

Since 2000 ppm pretreatment with I3C reduced AFB1-DNA binding and inhibited AFB1-initiated carcinogenesis in trout, this study may also indicate whether a correlation exists between enzyme induction

and these two parameters. Other mechanisms of action, such as direct enzyme inhibition or electrophile trapping, are considered in Appendix III.

MATERIALS AND METHODS

I. CHEMICALS

[7(n)-3H] styrene oxide (specific activity 250 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). [Glucuronyl-U-^{14}C] uridine diphosphate glucuronic acid (specific activity 233 mCi/mmol and [^{125}I] labeled protein A (specific activity 30 uCi/ug) were purchased from ICN (Irvine, CA).

Resorufin, indole-3-carbinol and indole-3-acetonitrile were obtained from Aldrich Chemical Company (Milwaukee, WI). Premixed acrylamide (29:1) was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Nitrocellulose membrane (.45 um), blot absorbant filter paper, DEAE sepharose and hydroxyapatite were obtained from BioRad (Richmond, CA). Highly purified Lubrol PX was purchased from Pierce Chemical Company (Rockford, IL). [^{3}H] aflatoxicol and aflatoxicol M1 glucuronide standards were supplied by Pat Loveland, (Department of Food Science, Oregon State University). Purified LM2 trout isozyme, LM2-IgG and LM4b-IgG were supplied by Dr. David Williams, (Department of Food Science, Oregon State University). Trout LM4b was purified from trout pretreated with BNF (see Appendix I). All other chemicals were of the highest grade possible and were obtained from Sigma Chemical Co. (St. Louis, MO).

II. ANIMALS AND DIETS

Yearling rainbow trout (Salmo gairdneri) reared at Oregon State University Food Toxicology and Nutrition Laboratory, were fed semi-purified control diets (88) followed by an eight day exposure to either control or diet containing varying levels of inhibitors. The fish given control diet were the negative control group and the fish given the 500 ppm BNF diet were the positive control group. I3C was fed at 500, 1000 and 2000 ppm and I3N at 50, 500 and 1000 ppm of the dietary level. The experiments were conducted throughout a period of eleven weeks in which the average body weight of control trout increased from 90.7 to 208.1 grams. At the time of the enzyme assays, the average weights of treated animals were similiar to that of the control. The fish were starved two days prior to liver and intestine removal to make certain that no diet was present in the intestine. Each dietary group consisted of three pools of 10 fish. For the UDPG assay, fish were administered control, 500 ppm BNF or 2000 ppm I3C for two weeks and were not starved prior to liver removal.

III. MICROSOMAL ISOLATION

Livers and intestines were removed and rinsed with ice cold buffer (0.1 M Tris acetate, pH 7.4, 0.1 M KCl, 1 mM EDTA, 0.1 mM PMSF and 20 uM BHT). The livers were pooled and microsomes were isolated the same day. The livers were minced and homogenized in a volume of buffer equal to 4 times the weight of the pooled livers. The homogenate was centrifuged at 2000 g for 30 minutes and the supernatent removed and centrifuged at 18,800 g for 30 minutes. The

supernatent was removed and the microsomal pellet was obtained from the 18,000 g supernatant after centrifugation at 100,000 g for 1 hour. The lipid layer was removed from the supernatent, and the remaining liquid fraction (the cytosolic fraction) was immediately frozen and stored at -80° C until assayed for GST activity the following day. The pellet was resuspended in a volume of buffer (0.1 M potassium phosphate, pH 7.25, 1 mM EDTA and 20% glycerol) 2 times the pooled liver weights. The intestines were immediately frozen and stored at -80° C. Intestinal microsomes were isolated after 24 hours of storage. Microsomal preparation and cytosolic recovery was identical to the previous procedure, except that the buffer volume used for homogenization was equal to the intestinal weight. Hepatic microsomes were assayed for enzyme activities (EROD, ECOD and total P-450 content) immediately after isolation. An aliquot of the microsome suspension was frozen with liquid nitrogen and stored at -80° C until assayed for LM2 and LM4b content. Microsomes for the UDPG assay were freshly isolated from a separate group of animals. Unfortunately, intestinal microsomes did not have any detectable LM2 or LM4b content (as determined by the western blot method) due to enzyme degradation during storage and were not used for further analysis. Protein concentration was determined by the method of Lowry et al. (89).

IV. TOTAL P-450 DETERMINATION

Total cytochrome P-450 content was determined by the method of Estabrook et al.(90) using a Varian DMS 100 spectrophotometer. Microsomal suspensions were diluted to approximately 1 mg/ml with the

resuspension buffer. The samples were gassed with CO for three minutes, then placed into two cuvettes and a baseline of equal light absorbance was recorded. The sample cuvette was reduced with the addition of a few crystals of sodium dithionite. The difference spectrum was then recorded (absorbance of sample = absorbance at 450 nm - absorbance at 490) which represents nmol cytochrome P-450 reduced/mg protein.

V. ECOD ANALYSIS

Cytochrome P-448 and P-450 activity towards the substrate ethoxycoumarin was determined by a modification of the fluorescence ECOD method described by Srivastava et al. (91) with a LS5-Perkin Elmer fluorometer (model C653000). The incubation mixture consisted of 0.1 M HEPES, pH 7.8, 5 mM glucose 6-phosphate, 5 mM magnesium chloride, 0.45 mM ethoxycoumarin, 2 IU/ml glucose-6-phosphate dehydrogenase and 1-2 mg of microsomal protein in a total volume of 1.6 ml. The mixture was preincubated for 2 minutes at 25° C, and the reaction was initiated by the addition of 0.6 umole of NADPH. After a four minute incubation, the reaction was terminated with the addition of 2.5 ml of 100% methanol. A blank was produced from the above incubation mixture and addition of the stopping reagent prior to addition of NADPH. Protein precipitate was removed by centrifugation at 2000 g for 10 minutes. Product formation was detected by fluorescence at an excitation wavelength of 380 nm and emission wavelength of 460 nm.

VI. EROD ANALYSIS

Cytochrome P-448 activity toward the substrate resorufin was determined by the fluorometric EROD method of Burke et al. (92) as follows. The incubation mixture consisted of 82.5 mM Tris-HCl, pH 7.4, 61 uM resorufin and 2-5 mg/ml microsomal protein in a total volume of 2.45 ml. The mixture was preincubated at 25°C for 2 minutes. The reaction was initiated with the addition of 50 mM NADPH. Product formation was detected by flouresence at an excitation wavelength of 530 nm and emission wavelength of 586 nm. Enzyme activity was determined from the slope of the reaction curve recorded for 2 minutes.

VII. GST ANALYSIS

GST activities of both the liver and the intestinal cytosols were determined as described by James et al. (93) with [^{3}H] styrene oxide as the substrate. The reaction mixture contained 160 umoles HEPES, pH 7.6, 7.5 umoles glutathione and 1-2 mg cytosolic protein in a total volume of 1.45 ml. The reaction mixture was preincubated for 2 minutes and intiated by the addition of 1.5 umoles styrene oxide (1.5×10^{5} dpm). After 30 minutes the reaction was terminated by adding 4 ml ethyl acetate and mixing with a vortex. The unreacted substrate was removed by three extractions by 4 ml of ethyl acetate. After the organic layer was removed, the aqueous layer was transferred to a scintillation vial. The reaction vial was rinsed and washed with four 2.5 ml aliquots of ASC scintillation fluor (Amersham,

Arlington Heights, IL) which were combined with the aqueous layer. The samples were counted with a Beckman LS 7500 liquid scintillation counter.

VII. WESTERN BLOT

The specific content of LM2 and LM4b enzymes were detected by the western blot method according to Burnette (94). This method involves separating the proteins by gel electrophoresis, transferring the proteins to nitrocellulose and detecting the specific enzymes by polyclonal antibody recognition. The antibody-enzyme complex is then labeled with [^{125} I] protein A which then can be detected by exposure to X-ray film. Electrophoretic equipment was from Hofer Scientific Instruments (San Francisco, CA) and transfer equipment from BioRad (Richmond, CA). Samples of 10 ug microsomal protein and either LM2 or LM4b standards (applied in a range of 0.2-1.6 pmole enzyme; 5-40 ug protein) were loaded onto a 1.5 mm x 10 cm x 14 cm sodium dodecyl sulfate (SDS) polyacrylamide gel consisting of a 3% polyacrylamide stacking gel and a 8% polyacrylamide running gel. Sample buffer consisted of 60 mM Tris-HCl, pH 6.8, 12.8% (w/v) glycerol, 1.25% SDS, 1.25% 2-mercaptoethanol and 0.125% (w/v) bromophenol blue. This was diluted 1:25 with the sample. Gels were run at 40 V per gel at 4 °C in running buffer that contained 25 mM Tris base, pH 8.3, 1.44% (w/v) glycine and 0.4% sodium lauryl sulfate until the tracking dye reached 1 cm from the bottom (approximately 4 hours). The gels were removed and equilibrated in transfer buffer (20 mM Tris base, pH 8.3, 1.08% (w/v) glycine and 25% methanol) for 15

minutes. The proteins were then transferred from the gel to the nitrocellulose by the following method. A sandwich in the transfer frame was made consisting of the following seven layers: 1) a fiber pad, 2) and 3) two sheets of blot absorbent filter paper, 4) the SDS gel, 5) a sheet of nitrocellulose paper (the absorbent paper and the nitrocellulose paper was equilibrated with water by immersing the paper in deionized water and shaking for 15 minutes), 6) a third sheet of blot absorbent filter paper, and 7) a second fiber pad. The assembled frame was then placed in a transfer chamber filled with transfer buffer. Transfer was completed after applying 30 V for 12 hours at 4 ° C.

After transfer, the nitrocellulose was removed and dried at 70° C for 30 minutes. The nitrocellulose was then incubated at room temperature for one hour with constant shaking in Buffer A (20 mM potassium phosphate, pH 7.4, 0.8 % NaCl and 2% bovine serum albumin) to block the nonspecific binding sites. The buffer A was poured off and replaced with buffer A with 0.05% Triton X-100 and 20 ug IgG/ml (the antibody used was either IgG-LM2 or IgG-LM 4b). This was incubated for 1.5 hours at room temperature. After incubation, the nitrocellulose was washed with the following solutions, each for 5 minutes with constant shaking: 1) 100 ml PBS buffer (20 mM potassium phosphate pH 7.4 and 0.8% NaCl), 2) 100 ml PBS buffer with 0.05% Triton X-100, 3) 100 ml PBS buffer with 0.05 % Triton X-100, 4) 100 ml PBS buffer. The nitrocellulose was incubated for 1 hour in buffer A containing [125] I-Protein A at 2 x 10^5 dpm /ml at room temperature

with constant shaking. The wash sequence listed above was repeated. The nitrocellulose was blotted between paper towels and heated at 70° C for 30 minutes. The nitrocellulose was then wrapped in plastic wrap and analyzed by autoradiography after exposure at - 80° C for 4 hours. The autoradiographs were analyzed with a scanning densitometer model SLR-5040-XL (Fullerton, CA).

VIII. UDPGA TRANSFERASE ASSAY

UDPGA transferase activity was measured by the method of Cougtrie et al. (95) with the following modifications. The glucuronide products were detected by HPLC and were verified by cleaving with β - glucuronidase and by using glucuronide standards (aflatoxicol M1-glucuronide, 1-napthol α-D-glucuronide or testosterone-α-D-glucuronide). The incubation mixture contained 50 mM Tris-maleate, pH 7.4, 10 mM $MgCl_2$, 0.8% digitonin, 2.7 mM UDPGA (0.25uCi) and 1mM 1-napthol or testosterone in a total volume of 150 ul. The reaction was initated by the addition of 1.5 to 2 mg of microsomal protein. After an incubation period of 30 minutes at 25° C, the reaction was terminated by the addition of 475 ul of cold ETOH. The samples were centrifuged at 16,000 x g for 3 minutes in a Hermle Z229 microcentrifuge (National Labnet Co., Woodbridge, NJ). The supernatant was filtered through a 0.45 um filter (Acro LC 13, Gelman Sciences, Ann Arbor, MI). The samples were immediately frozen in liquid nitrogen and stored at -80°C until HPLC analysis. The samples were analyzed within 1 week.

This assay was attempted using aflatoxicol as the substrate, but significant quantities of the aflatoxicol-glucuronide could not be detected (see Appendix II).

β-Glucuronidase treatment

Aliquots of the above samples were taken for the hydrolysis of the glucuronides prior to freezing. Two aliquots of 150 ul were removed from the samples after the centrifugation step and evaporated to near dryness. To the first aliquot, 100 ul of β-glucuronidase solution (25,000 units/ml in 0.2 M acetate buffer, pH 4.8) was added. The second mixture served as a control and consisted of the second aliquot and 100 ul of the acetate buffer. After incubation for 4 hour at 37 °C, the reaction was terminated with the addition of 400 ul of cold ETOH. The tubes were centrifuged at 15,000 g for 5 minutes, filtered with 0.45 um filters, and immediately frozen until analysis.

HPLC analysis

A Shimadzu LC-6A high pressure liquid chromatograph (HPLC) with a C18 reverse phase column, a SPD-6AV UV Spectrophotomer detector and a SCL-6A system controller (Shimadzu Corporation, Kyoto, Japan) were used for the analysis of the glucuronide conjugates. The 1-napthol glucuronide was detected at a wavelength of 310 nm and the testosterone glucuronide was detected at a wavelength of 238 nm. Aliquots of 90 ul of sample were injected. HPLC conditions were similiar to those used by Loveland (77). The mobile phase consisted of a isocratic gradient of 76% 0.01 M KOAc buffer, pH 5, and 24% acetronitrile:methanol:tetrahydrofuran (20:15:1) for eight minutes,

followed by a linear gradient of 76% to 24% KOAc buffer in acetonitrile:methanol:tetrahydrofuran for 20 minutes. This was held for 15 minutes. The system was returned to the original conditions and the next sample was injected after equilibration was achieved. Radioactivity was detected with an on-line Beckman 171 radioisotope detector (Beckman Instruments, Redmond, WA) with a flow rate of 2 ml/min of ACS scintillant fluid. Counting efficiency for the [14 C] was determined as 60%.

RESULTS

I. DETERMINATION OF FEEDING DURATION

The optimal duration for detection of enzyme induction was determined by dietary administration of BNF, a known monooxygenase inducer, at 150 and 500 ppm for 21 days. Three pools of six fish were sampled from both treatment groups at 0, 2, 7, 14 and 21 days. EROD and ECOD activities were analyzed and compared to the control values. As illustrated in Figure 3, EROD activity was induced approximately 43 times the control value after two days of treatment with 500 ppm BNF and appeared to have reached a plateau thereafter. Induction of EROD activity by 150 ppm BNF was similiar to that demonstrated by the 500 ppm level and was also significantly greater than control values at all time points studied.

A significant induction of ECOD activity also occurred, both treatment groups induced the activity to approximately the same level, at least 9 times the control value after two days of treatment (Figure 4). Induction of ECOD activity reached a plateau after 7 days of treatment of both 150 and 500 ppm BNF.

Evaluation of these data and of EROD and ECOD induction by I3C as demonstrated by Bradfield and Bjeldanes (34) suggests that an eight day feeding duration should be sufficient for detectable

Figure 3. Determination of feeding duration for optimal enzyme induction. EROD activity was assayed after 0, 2, 7, 14 and 21 days of 150 and 500 ppm dietary BNF administration. Values represent means of three pools of ten animals ± SD. (Some SD values were within the size of the corresponding symbols.)

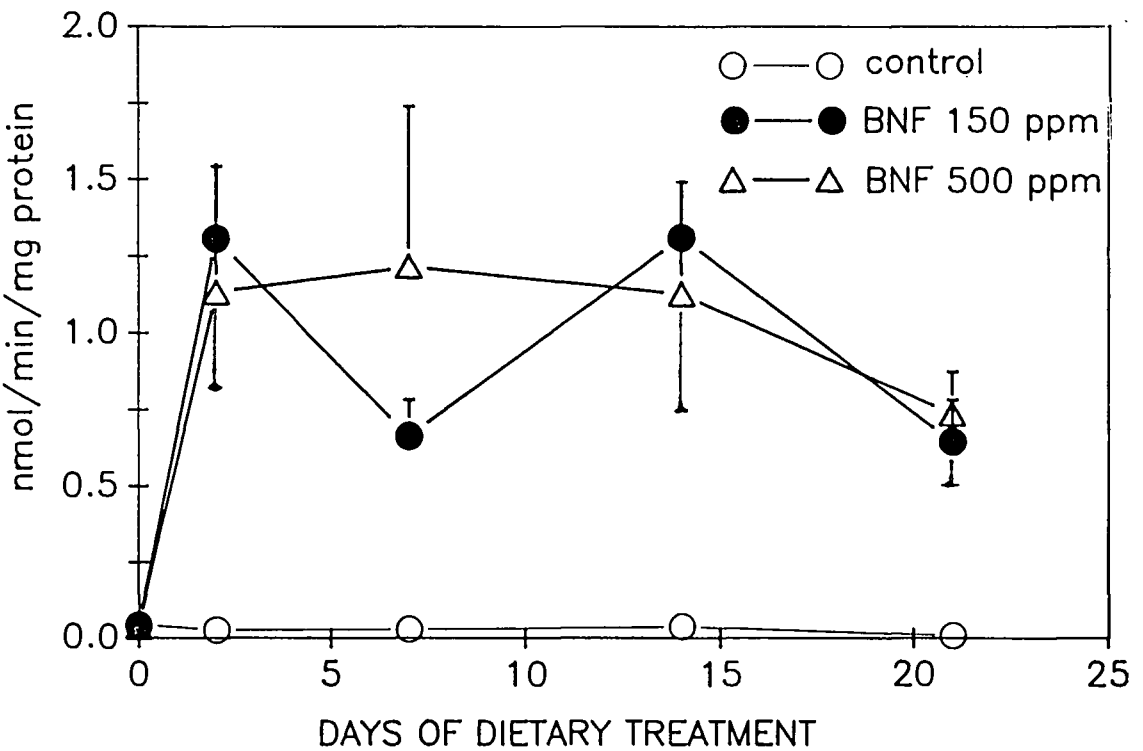
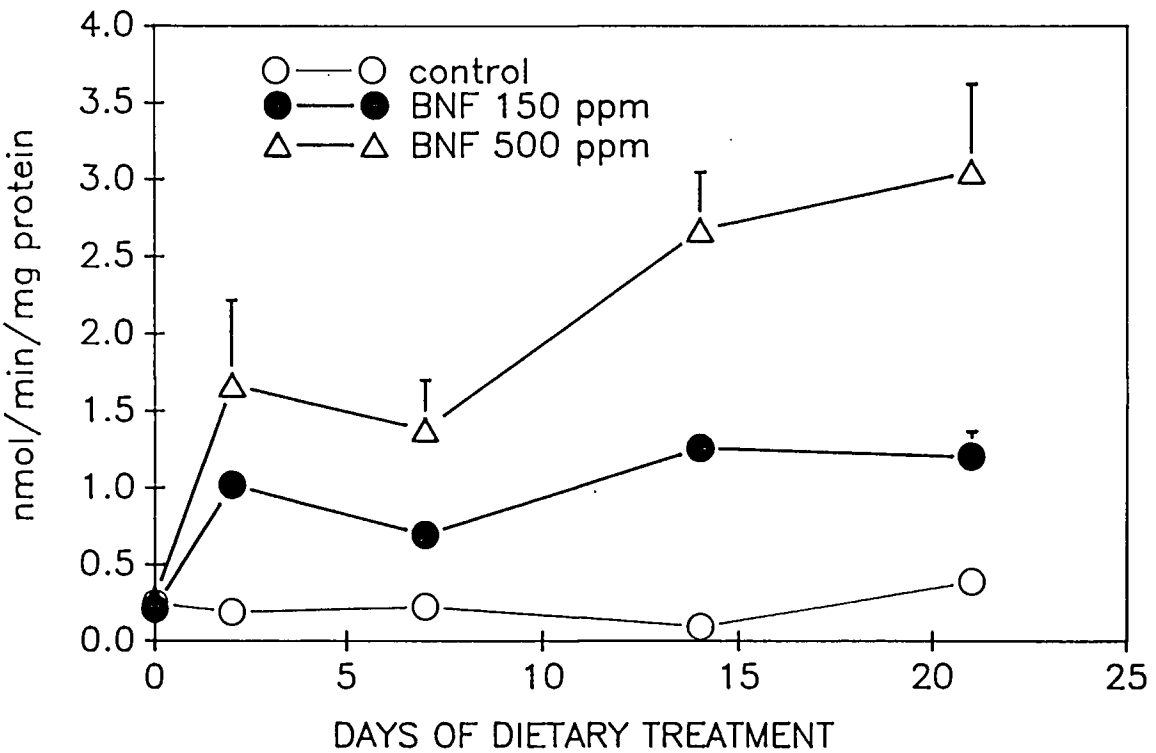


Figure 4. Determination of feeding duration for optimum enzyme induction. ECOD activity was assayed after 0, 2, 7, 14 and 21 days of 150 and 500 ppm dietary administration. Values represent means of three pools of ten animals ± SD.



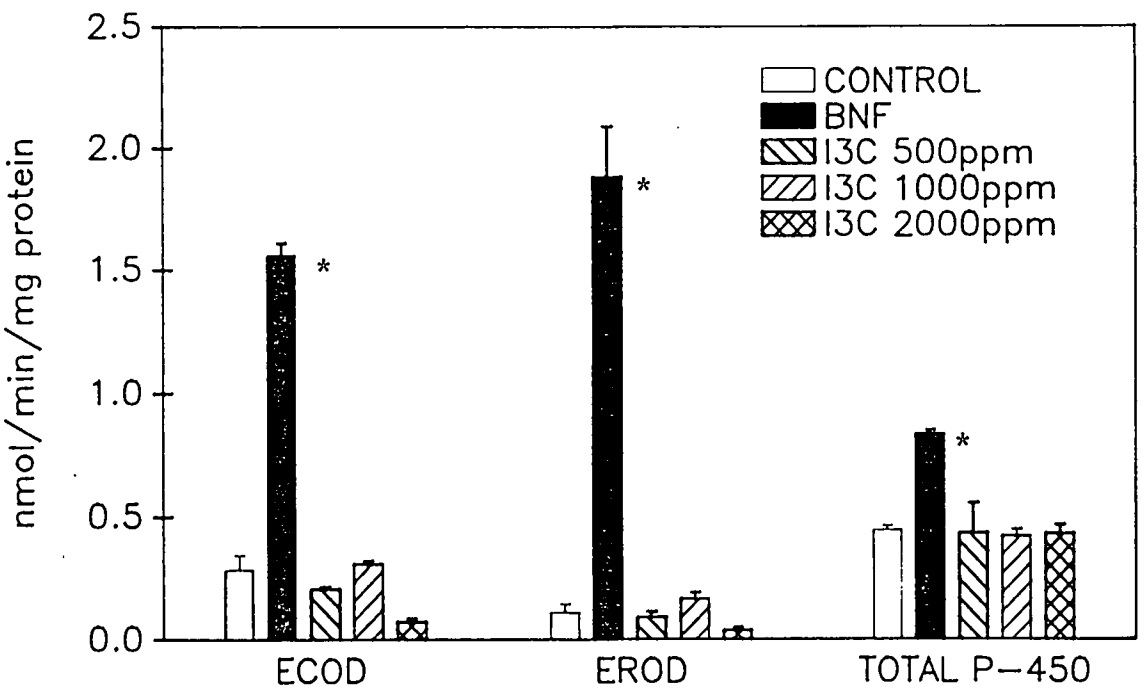
enzyme induction. This is also supported by the study of Andersson et al. (15) who found that induction of trout liver UDPG activity using testosterone as the test substrate reached a maximum level after seven days of treatment with BNF, after which this level rapidly decreased. However, induction of UDPG activity using the test substrate 1-napthol did not decrease after 1 week of treatment.

II. EFFECT OF I3C AND I3N ON HEPATIC PHASE I METABOLIZING ENZYMES

Although BNF significantly induced trout hepatic phase I enzyme activities (Figure 5), dietary I3C treatment at 500, 1000, or 2000 had no detectable effect on these enzyme activities, under the present experimental conditions. Induction by 500 ppm BNF is clearly demonstrated by an increase of nearly 17 times the control value of EROD activity. Similarly, dietary I3N treatment did not significantly affect the assayed enzymes (Figure 6). It may appear that the EROD activity was slightly depressed by dietary treatment with I3N, but this effect was not statistically significant.

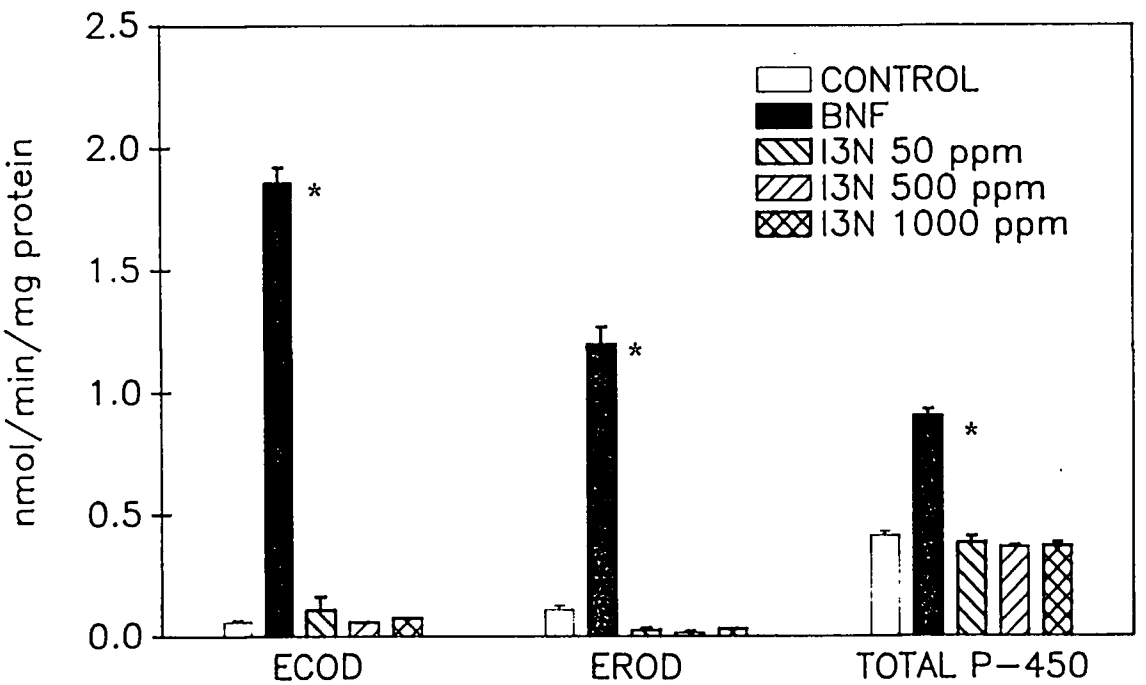
Analysis of LM2 content (as detected by the western blot method) confirmed the null effect of both I3C and I3N at all dietary level. Figure 7 shows only the effects of I3C at 2000 ppm and of I3N at 1000 ppm, the highest administered levels of each treatment; the effects of all lower dietary levels were similiar to that shown. As expected, from previous studies (75) dietary treatment of BNF did not affect the LM2 levels. The LM4b content (Figure 8) was significantly increased by treatment with 500 ppm BNF, but only unquantifiable trace amounts of this isozyme were detected in microsomes of control, I3C and I3N

Figure 5. Effect of dietary administration of 500 ppm BNF and various levels of treatment with I3C on Phase I metabolizing enzyme activities. Values represent means of three pools of ten animals ± SD.



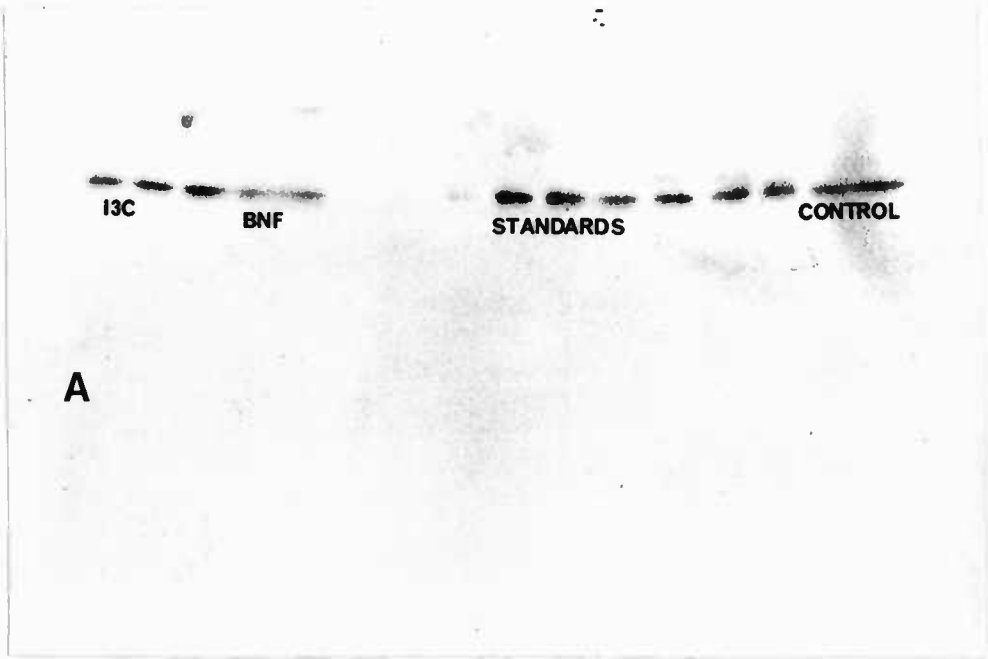
*significantly different from control: P > .05 (ANOVA)

Figure 6. Effect of dietary administration of 500 ppm BNF and various levels of treatment with I3N on Phase I metabolizing enzyme activities. Values represent means of three pools of ten animals ± SD.



*significantly different from control; P > .05 (ANOVA)

Figure 7. Western blot of microsomes isolated from animals fed control, 500 ppm BNF and 2000 ppm I3C (A) or 1000 ppm I3N (B) dietary administrations and immunostained for P-450 LM2. All lanes contain 10 ug of microsomal protein from pools containing ten animals unless specified otherwise. In the top photograph (A), lanes 1, 2 and 3 contain microsomal protein from trout I3C-treated trout; lanes 4 and 5 contain microsomal protein from BNF-treated trout; lanes 6, 7, 8, 9 and 10 contain 1.6, 1.2, 0.8, 0.4 and 0.2 nmole purified LM2 standard; Lanes 11 and 12 contain 5 ug microsomal protein from control (untreated) trout; lanes and 13 and 14 contain 10 ug microsomal protein from control trout. In the bottom photograph (B), lanes 1, 2 and 3 contain microsomes from control trout; lanes 4, 5, 6 , 7, and 8 contain 0.2, 0.4, 0.8, 1.2 and 1.6 purified LM2 standards; lanes 9, 10 and 11 contain microsomes from BNF-treated trout; lanes 12, 13, 14, 15 and 16 contain microsomes from I3N-treated trout. (Cross-reaction of the IgG-LM2 antibody with the LM4b isozyme resulted in detection of the LM4b isozyme as seen in the band directly above the LM2 isozyme.)



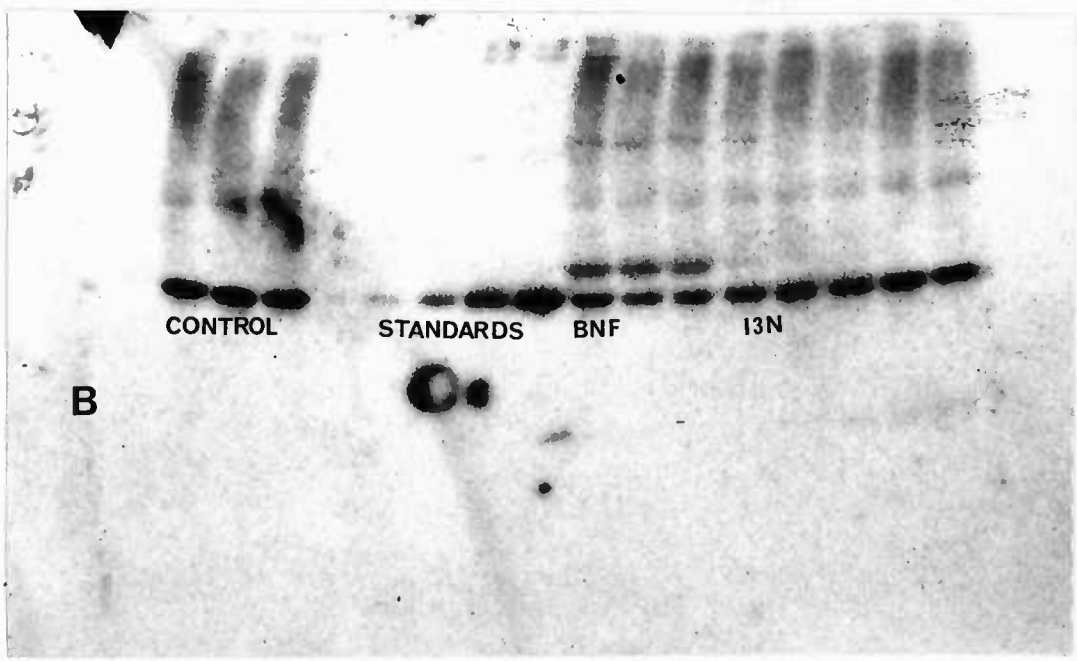
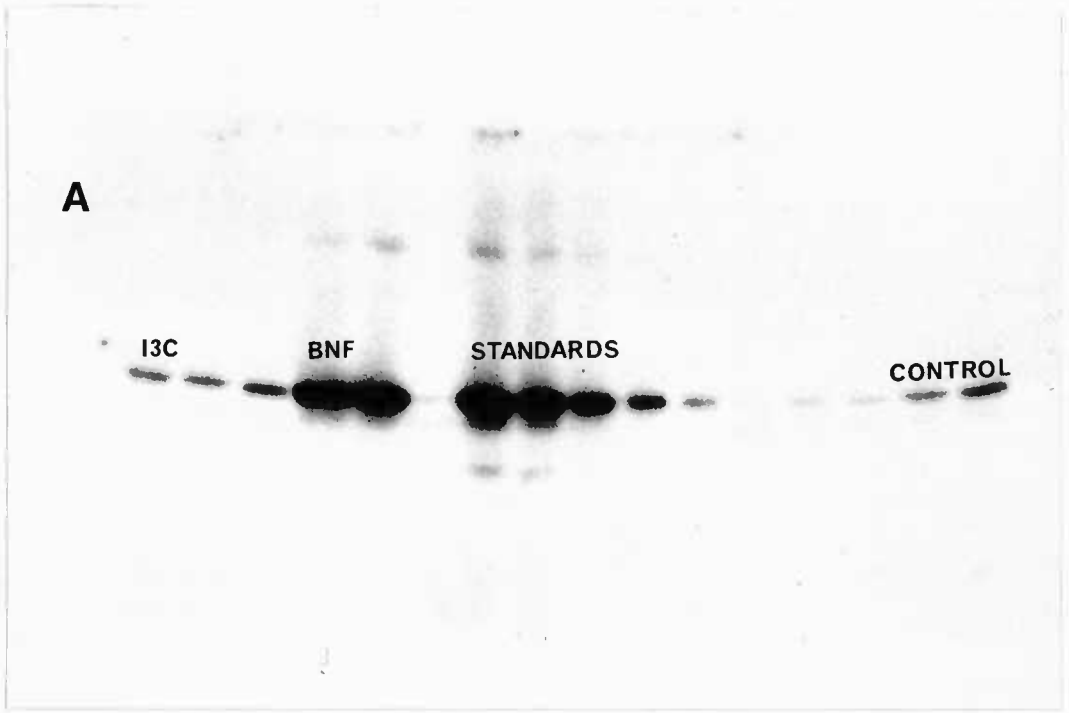


FIGURE 7

Figure 8. Western blot of microsomes from trout treated with control, 500 ppm BNF and 2000 ppm I3C (A) or 1000 ppm I3N (B) and immunostained for P-450 LM 4b. Lanes contained the same samples as described in Figure 7 except the standards used in this figure were purified LM4b instead of purified LM2.



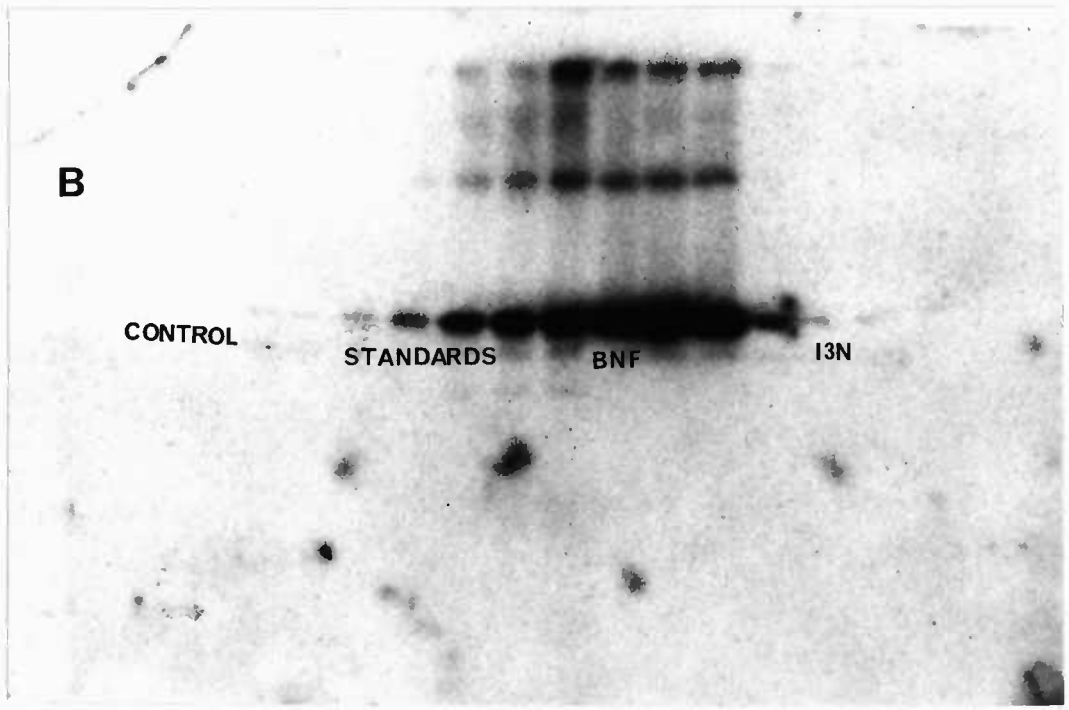


FIGURE 8

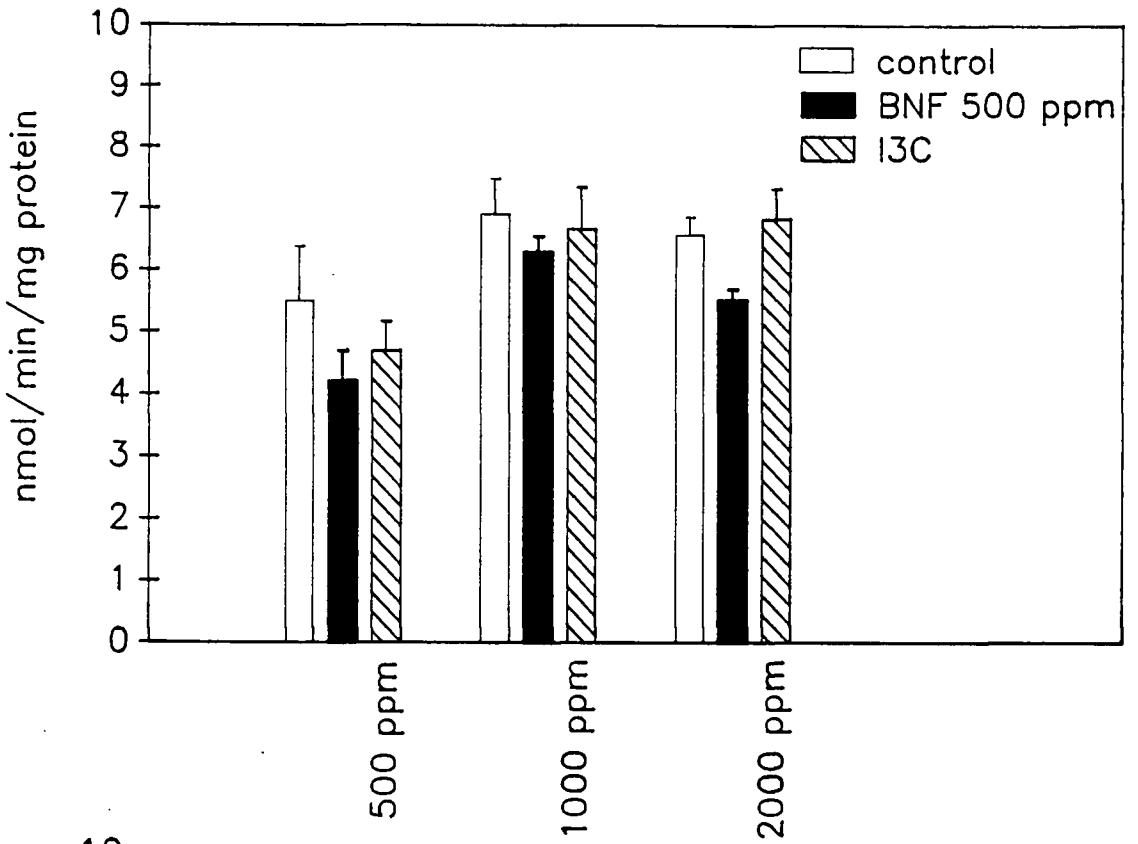
treated trout. I3N treated fish were barely detectable (Figure 8 shows only the effects of I3C and I3N at the highest treatment levels; again the effects of all lower dietary levels were similiar).

III. EFFECT OF I3C AND I3N ON HEPATIC AND INTESTINAL GST ACTIVITY

Neither BNF, I3C or I3N appeared to induce GST activity in the liver as illustrated in Figures 9a and 9b. A small but significant difference was observed between the BNF group and the group fed 2000 ppm I3C. Although the variability in the control values makes intrepretation difficult BNF and I3N appear to suppress liver GST activity while I3C had no detectable effect.

Dietary treatments of BNF, I3C and I3N did not have the same effects on intestinal as on hepatic GST activity (Figures 10a and 10b). Intestinal GST activity was slightly, but significantly increased in groups given 500 ppm I3C compared to control groups, but activities of 1000 ppm and 2000 ppm I3C groups were similar to that of controls. Of the three similarly BNF-treated positive control groups, intestinal GST activity was significantly increased in the BNF positive control group that was examined along with the 500 ppm I3C group. However, an inductive effect of BNF was not detected in BNF-treated groups that served as positive controls for the 1000 ppm and 2000 ppm I3C groups and for the I3N treated groups. The most reasonable interpretation of these data is that the control group in Figure 10a, 500 ppm group was anomalously low and that there is no convincing evidence for BNF or I3C induction.

Figure 9. Effect of BNF, I3C (A) and I3N (B) on hepatic glutathione S-transferase activity using styrene oxide as the substrate. Variances in control values reflect different experimental groups. Values represent means of three pools of ten animals $\pm$ SD. *significantly different from control: $P > .05$ (ANOVA)


nmol/min/mg protein
10
9
8
7
6
5
4
3
2
1
0
control
BNF 500 ppm
I3C
500 ppm
1000 ppm
2000 ppm

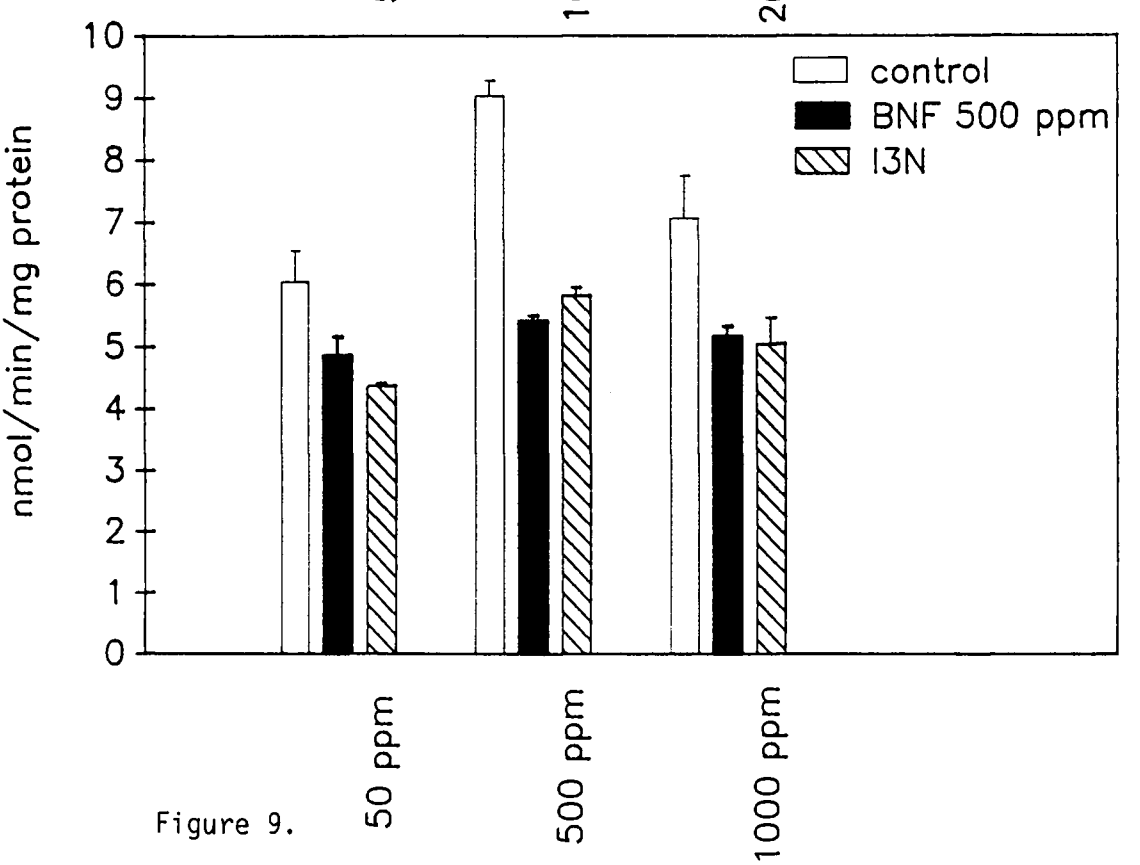
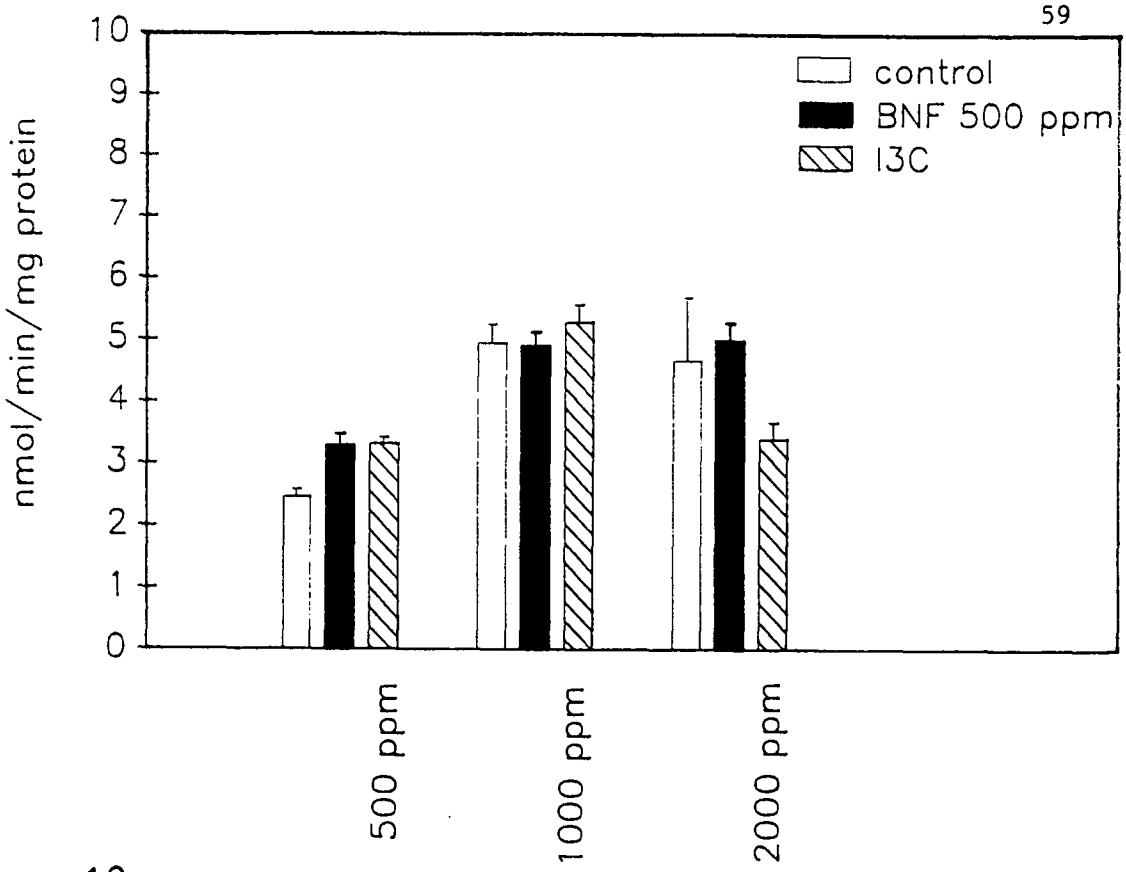

nmol/min/mg protein
10
9
8
7
6
5
4
3
2
1
0
control
BNF 500 ppm
I3N
50 ppm
500 ppm
1000 ppm

Figure 9.

Figure 10. Effect of BNF, I3C (A) and I3N (B) on intestinal glutathione S-transferase activity using styrene oxide as the substrate. Values represent means of three pools of ten animals $\pm$ SD. * significantly different from control: $P > .05$ (ANOVA)


nmol/min/mg protein
10
9
8
7
6
5
4
3
2
1
0
control
BNF 500 ppm
I3C
500 ppm
1000 ppm
2000 ppm

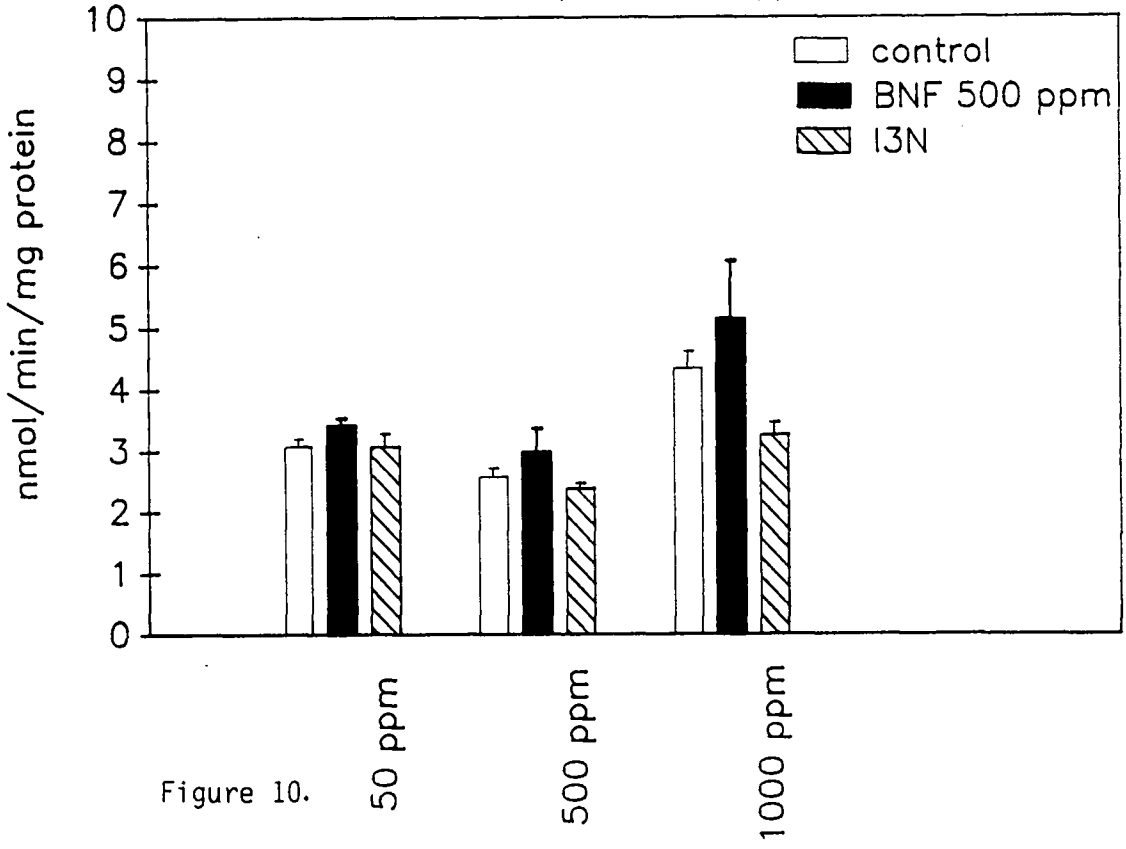

nmol/min/mg protein
10
9
8
7
6
5
4
3
2
1
0
control
BNF 500 ppm
I3N
50 ppm
500 ppm
1000 ppm

Figure 10.

IV. EFFECT OF I3C ON HEPATIC UDPGT ACTIVITY

UDPGT activity, measured using the substrate 1-napthol, was increased two fold by BNF treatment (500 ppm) but appeared to be slightly decreased by I3C treatment (2000 ppm) as shown in Figure 11 and Table 1. However, this slight decrease was not statistically significant. When testosterone was used as the substrate, a 45 % increase was found in the BNF-treated samples , but this induction was not statistically significant (Table 1). Again, I3C at 2000 ppm did not have an inductive effect on enzyme activity but did appear to have a slight inhibitory effect that was not statistically significant. It is important to note that the detection of product formation of the testosterone glucuronide from the I3C treatment group approached the limits of detection by this method.

Figure 11. Detection of 1-napthol by HPLC. —— denotes before glucuronidase treatment, ---- denotes after glucuronidase treatment. Peak 1 = 1-napthol glucuronide (product), peak 2 = 1-napthol (substrate).

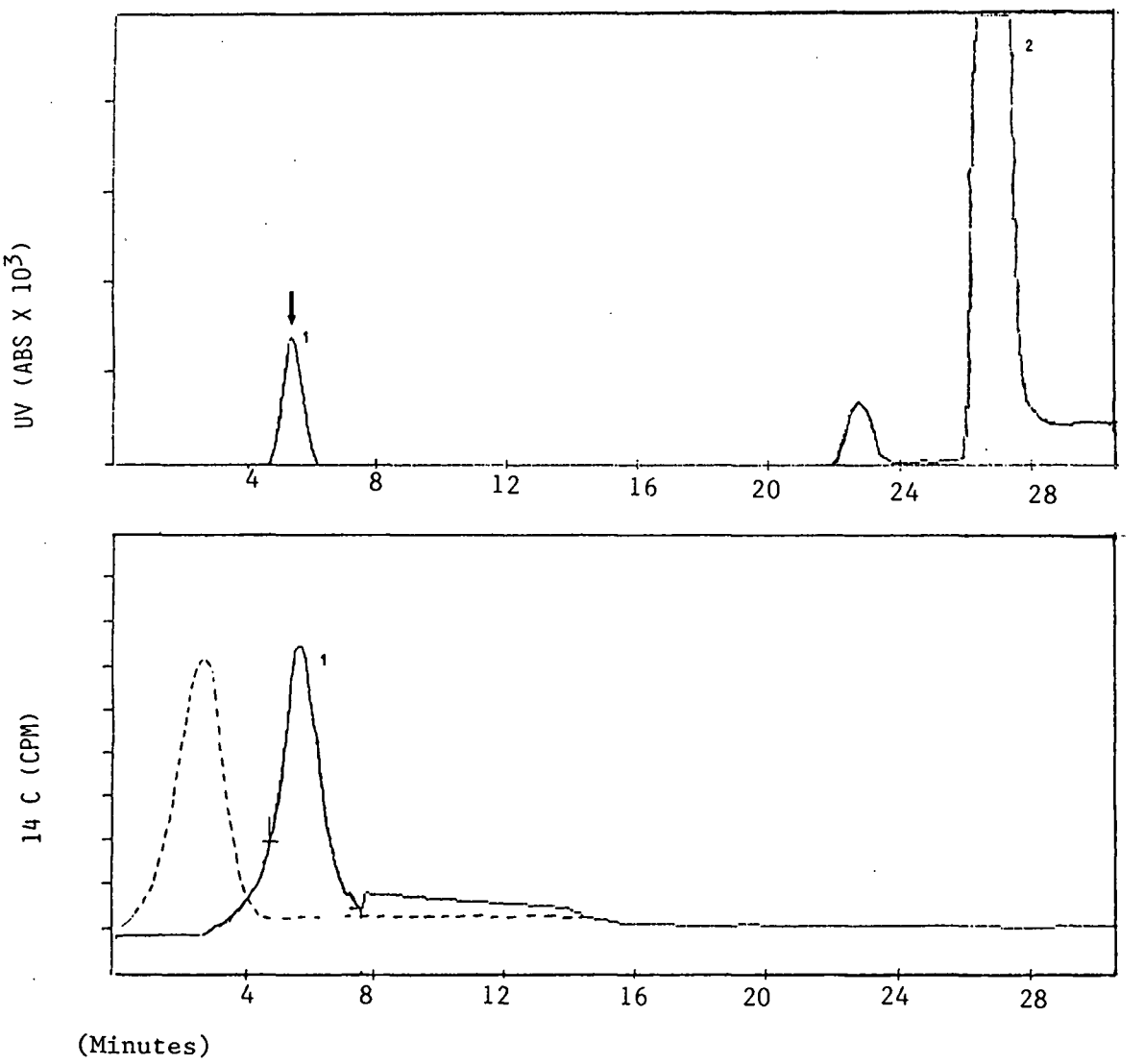


Table 1. Effect of dietary administration of 500 ppm BNF and 2000 ppm I3C on uridine glucuronosyl transferase (UDPGT) activity with 1-napthol and testosterone as substrates.

in vivo treatment	1-Napthol (nmol/min/mg protein)	Testosterone (nmol/min/mg protein)
Control	0.5253 ±.079	0.1349 ±.004
BNF	1.2178 ±.079 a)	0.1964 ±.004 a)
I3C (2000 ppm)	0.3658 ±.063	0.0906 ±.029

a)significantly different from control: $P > .05$ (ANOVA)

DISCUSSION

The anticarcinogenic action of I3C results at least in part from its ability to reduce binding of chemical carcinogens to cellular DNA. However, only recently have the precise mechanisms by which the reduction is effected been critically examined.

The results presented in this study indicate that I3C and I3N do not induce hepatic phase I metabolizing enzyme systems in the rainbow trout at the dietary levels which have been shown to be effective in protecting against the carcinogenicity of AFB1. The lack of induction of phase I enzyme systems is consistent with that reported by Eisele et al. (85) who found that I3C and its analogs (not including I3N) did not induce EROD, ECOD or benzphetamine N-demethylase activities in trout at a dietary level of 500 ppm. This is in contrast to the induction of hepatic cytochrome P-448 type enzyme systems by I3C and I3N in rats (35,36).

The species-specific nature of induction by I3C and its analogs has been indicated by previous studies. For example, Shertzer (40) found that rabbits were not responsive to cytochrome P-448 induction by I3C and I3N administered by gavage at 1/10 the lethal dose. Rats and mice, however, did show an induction response under identical conditions. In the present study, cytochrome P-450 levels were not affected even by 2000 ppm I3C, a strongly anticarcinogenic and near-toxic dose in the trout.

Other studies (34) have found that low pH acid condensation products of I3C produced in vitro, such as 3,3'-diindolymethane, are more effective at inducing cytochrome P-448 enzyme activities (as detected by the EROD assay) than the parent I3C and these products bind to the Ah receptor with different degrees of affinities depending on the structural features of the compound (37). An example of differential binding affinities is the relatively high affinity of indole[3,2-b]carbazole, a possible chemical derivative of 3,3'-diindolylmethane (96), and lower affinities of 3,3'diindoylmethane and indole-3-carbinol (37)

Although it is not known what acid condensation products are formed in vivo either in the rat or trout, it is possible that acid condensation products are formed in the stomach of the rat where the pH conditions are quite low (normally at pH < 2) and may be different than those formed in the trout where pH conditions are slightly higher (pH ranges from 3.5 - 4.5 (97)). The condensation products formed in the trout may have less affinities for Ah receptor binding and therefore may not have as great of an ability to induce EROD activity. It has been reported that the type of I3C condensation products formed is pH dependent (98). At low pH conditions (eg. reaction with concentrated HCl), I3C is converted to an oxygen-free polymer, while at higher pH (pH > 6) self-condensation yields the dimer 3,3' - diindolylmethane and its diindolylmethine salts (urorosein).

I3C and I3N do not appear to have an inducing effect on phase II metabolizing enzyme systems. Neither compound increased hepatic or

intestinal GST activity towards the substrate styrene oxide, except for a possible small inductive effect of the 500 ppm I3C administration in the intestine. The lack of apparent induction in this study is consistent with the lack of effect of dietary treatments of 500 ppm and 2000 ppm I3C on the in vivo formation of aflatoxin-glutathione (69). BNF appeared to suppress hepatic GST activity in these studies. In contrast, an induction of activity towards styrene oxide following dietary administration of BNF was observed by Bailey et al (11). We presently have no explanation for this discrepancy.

The lack of phase II induction by I3C and apparent suppression by I3N is in contrast to that which has been reported for mice. Dietary I3C (5000 ppm) induced hepatic and intestinal GST to approximately 2.5 and 3 times, respectively, the control value. Dietary I3N (2000 ppm) induced hepatic and intestinal GST activity to approximately 3.6 and 1.8 times, respectively, the control value (12). In rats, I3C did not induce intestinal and hepatic GST at a dietary level of 500 ppm (36). There is currently no data available for dietary levels greater than 500 ppm in rats.

Goeger et al. (86) had found that dietary administration of I3C at 2000 ppm increased the amount of AFL-M1 glucuronide formed in vivo. This could result from increased formation of the glucuronidation substrate AFL-M1 or from induction of the UDPGT isozyme. In this study, no induction of UDPGT activity towards the substrates 1-napthol or testosterone was found after the same dose of I3C was administered. The control enzyme activity level detected in this

study was twice that found by Andersson et al.(15) towards 1-napthol and testosterone. The induction by BNF in this study was 2 times and 1.5 times the control value towards 1-napthol and testosterone, respectively, and since the present HPLC method used was specific for the glucuronide products formed, it confirms the previously reported induction effect by dietary BNF. There is, therefore, no evidence for I3C induction of UDPGT activity towards the psuedo substrates 1-napthol or testosterone.

The UDPGT isozymes are not well characterized and are latent enzymes that must be activated to achieve their maximum level of activities (99). Often the activating agent is a detergent which varies with substrate to be assayed. The assay conditions used in this experiment were optimized for the substrates 1-napthol and testosterone. Because these were not the real substrate of interest, an attempt was made to derive an in vitro assay for UDPGT activity using AFL, and eventually AFL-M1. However, significant product formation of the aflatoxicol glucuronide could not be detected with these experimental conditions (See Appendix II). These conditions may not be sufficient for activation of the aflatoxicol glucuronosyl transferase and the HPLC method used may not be sensitive enough for detection of the unactivated form. Variability in degree of activation has been demonstrated by the activation of Triton X-100 on p-nitrophenol glucuronosyl transferase and the lack of activation of o-aminophenol with the same detergent and experimental conditions (100).

Wattenberg has classified I3C as a blocking agent (19). By this definition the primary mechanism of its inhibition is by 1) induction of activities of the enzyme systems that catalyze either the formation of the less carcinogenic species or the formation of a conjugate of the carcinogen that is more readily excreted, 2) inhibition of the activities of enzyme systems in rodents that catalyze the activation of the ultimate carcinogen or 3) direct nucleophilic trapping action. In the case of metabolism of aflatoxin B1, the enzyme activities whose induction would lead to inhibition of carcinogenesis are cytochrome P-448, glutathione S-transferase and UDPG transferase. This study indicates that, with the possible exception of AFL-M1-UDPG transferase, none of these enzyme activities is induced in the rainbow trout by dietary treatment of I3C, and therefore enzyme induction does not appear to be the primary mechanism leading to the inhibition of I3C towards AFB1-induced carcinogensis in rainbow trout. The induction of enzyme systems may be a secondary response to an as yet undetermined primary mechanism for inhibition of I3C.

It may be possible that I3C or its metabolites act to inhibit the activation pathway. In the case of AFB1 metabolism in the rainbow trout this inhibition would involve direct inhibition of the cytochrome P-450 isozyme. Alternatively, I3C may be metabolized to a nucleophilic species that traps the electrophilic carcinogen, preventing it from interacting with DNA. This hypothesis is supported by the studies of Shertzer (43), who reported that although I3C and I3N did inhibit DNA covalent binding of metabolites of benzo[a]pyrene

and NDMA in mouse liver, this inhibition was not accompanied by a concommitant induction of phase I metabolizing enzyme systems. He therefore concluded that enzyme induction was not the primary mechanism for inhibition by I3C. Shertzer suggested that perhaps I3C or a metabolite of I3C is sequestered within the endoplasmic recticulum in close proximity to the electrophilic ultimate carcinogen. The compound may then act as a nucleophilic trapping agent and prevent the electrophile from covalently binding to DNA.

The experiments presented in Appendix III support the hypothesis that the inhibition mechanism proceeds by an inhibition of the activation of AFB1, ie. inhibition of cytochrome P-450. It appears that even low concentrations (1/300 x the in vitro equivalent of a 2000 ppm I3C orally administrated dose in trout) of indole-3-carbinol acid products (RXN) resulted in a significant reduction in in vitro AFB1-DNA adduct formation. The reduction in in vitro AFB1-DNA adduct formation appears to be the result of the inhibition of the activation process and not a direct nucleophilic trapping mechanism. This is demonstrated by the lack of inhibition of AFB1-DNA adduct formation using AFB1-dichloride as the alkylating agent, which does not require enzymatic activation. The data presented in appendix III gives evidence that RXN inhibits in vitro DNA binding to a greater degree than the parent compound I3C.

In a recent study, Shertzer et al. (101) has shown that oral administration of I3C to mice resulted in a 63% decrease in carbon tetrachloride-induced centrolobular necrosis. This protective effect

was correlated to the *in vitro* inhibition of I3C on carbon tetrachloride mediated lipid peroxidation (an indication of cell membrane damage mediated by free radicals). Although I3C was not as effective an antioxidant as BHT, it did protect against oxidative damage in a dose-dependent manner. These findings give further evidence that inhibitory effect of I3C may proceed through mechanisms that do not involve enzyme induction.

Comparison of the relative inhibitory potencies of BNF (the positive control used in the present study) and I3C with their effects on xenobiotic metabolizing enzymes may indicate whether the primary mechanisms of inhibition are similiar. BNF is an effective anticarcinogen in both rodents and trout when administered in the diet. In rats, pretreatment of 0.015% dietary BNF resulted in a 25% hepatic tumor incidence as compared to a AFB1 control level of 100% (83). In a separate study a dietary pretreatment of 0.05% BNF resulted in a complete inhibition of hepatic tumors as compared to the control incidence of 20% (102). In rainbow trout, dietary pretreatment with 0.05% (500 ppm) BNF resulted in a 6% hepatic tumor incidence as compared to a 38% incidence of the AFB1 positive control (27). In comparison, dietary administration of 0.2% I3C to rats decreased the incidence of hepatocellular tumors from the AFB1 control level of 20% to 0% (102). In rainbow trout, 0.1% (1000 ppm) dietary administration of I3C resulted in a 4% hepatic tumor incidence compared to 38% for the AFB1-positive control (27). Inhibition of I3C is also demonstrated by a 95% reduction in AFB1-DNA binding at a dietary level

of 0.4%. I3C protection of AFB1-DNA binding occurs in a linear fashion at dietary levels of 0.1%-0.2% (87). These studies clearly indicate that dietary administration of both BNF and I3C can act as effective anticarcinogens when administered to both rodents and rainbow trout.

In rats, there is strong evidence that the anticarcinogenic mechanism of BNF proceeds primarily by the induction of cytochrome P-448 (AFB1-4-hydroxylase), possibly leading to an increase in formation of the less carcinogenic M1 (83) and by induction of glutatione S-transferase leading to increased formation of AFB1-glutathione (64). Similiarly, in trout, pretreatment with BNF leads to in vitro production of more AFM1, less AFL and as a result, a 50% decrease in AFB1-DNA binding (84). In trout, dietary pretreatment of BNF also significantly increased the formation of the AFL-M1 glucuronide as compared to the untreated controls exposed to AFB1 (77).

The mechanisms of inhibition of I3C in rodents have also been associated with changes in xenobiotic metabolism via enzyme induction (19). However, at least in the trout, the induction of xenobiotic metabolizing enzymes cannot be correlated with its protective effects against tumor formation or DNA binding (36,37,38). No induction of cytochrome P-448, glutathione epoxide S-transferase, UDP-glucuronosyl transferase activities or changes in the levels of cytochrome P-450 (LM2) or cytochrome P-448 (LM4b) were found in the present studies. This confirms the lack of correlation of the inhibitory properties of I3C with enzyme induction and suggests that unlike the mechanisms of BNF, I3C inhibition of carcinogenesis is not necessarily mediated by

enzyme induction in trout.

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APPENDICES

APPENDIX I

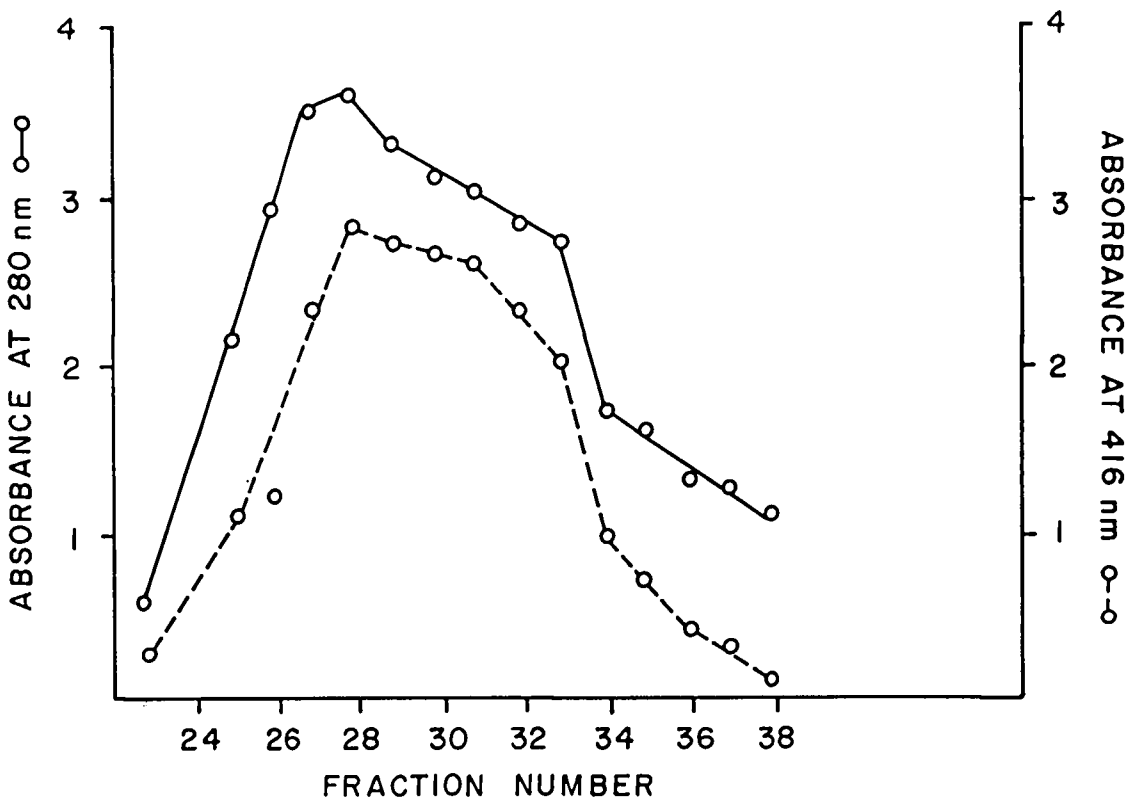
PURIFICATION OF LM4b

Isolation and purification of LM4b isozyme from trout was done according to the procedure by C.L.Miranda and D.Williams (personal communication).

Twenty fish were administered 500 ppm dietary BNF for two weeks prior to liver removal. Microsomes were prepared and protein content was determined by the method of Lowry et al. (1). Protein concentration was adjusted to 8 mg/ml by the addition of Buffer C (0.1 M potassium phosphate, pH 7.25, 20% glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). One ml was reserved for analysis by SDS PAGE, total P-450 and protein content. One gram microsomal protein (125 ml of solution) was solubilized by the dropwise addition of 10% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) in Buffer A. After 10 minutes, 20% sodium cholate (pH 7.5) was added. Final concentration of CHAPS and sodium cholate were 1% and 0.2% respectively. Final protein concentration was less than 2 mg/ml. A 50% (w/v) stock solution of polyethylene glycol (PEG) 8000 (in buffer A) was added incrementally to the soluble fraction and the solution was stirred for thirty minutes. The pellet began to precipitate between 1% and 18% PEG concentrations. This pellet was recovered by centrifugation at 100,000 g for two hours.

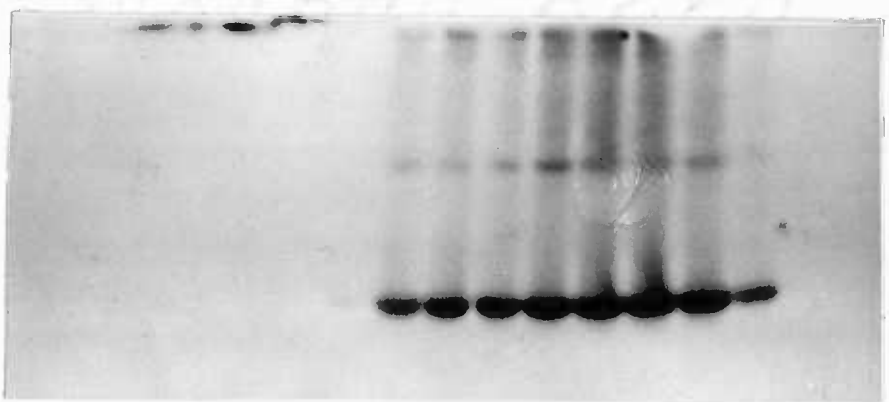
The pellet was dissolved by homogenization in 25 ml of buffer D (10 mM potassium phosphate, pH 7.5, 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, 0.2% cholate, 0.1% Lubrol PX and 0.1 mM PMSF). A one ml aliquot was removed and was assayed for total P-450 and protein content and for purity by SDS-PAGE. The solution was centrifuged at 45,000 g for 1 hour and the supernatent recovered. The sample was then degassed and loaded onto an equilibrated DEAE-sepharose column (1.6 X 40 cm) not exceeding 5 nmole P-450/ml resin. The column was washed with 200 ml buffer B until no visible color due to hemoglobin could be detected. No P-450 was detected in the wash. The P-450 was eluted with a linear gradient of 400 ml of 0-0.5 M KCl in buffer B. The flow rate was maintained at 25-30 ml/hr. Five ml fractions were collected and analyzed for absorbance at 416 nm and 280 nm and for total P-450 content (absorbance of cytochrome P-450 occurs at 416 nm and that of protein at 280 nm). Ten fractions containing the highest 416 nm/280 nm ratios were pooled and dialyzed overnight in 20% glycerol, 0.1 mM EDTA, 0.1 mM PMSF, 0.1 mM DTT and 10 mM potassium phosphate. The dialyzed sample was then loaded onto another equilibrated DEAE sepharose column (0.9 X 60 cm) and eluted with the same gradient as stated previously, the P-450 containing fractions were pooled and dialyzed overnight. After dialysis, the sample was loaded onto a hydroxyapatite column (1.2 X 10 cm) equilibrated with buffer D, washed with the same buffer and eluted with a linear gradient of 10-125 mM potassium phosphate in buffer D. Fractions were again examined for maximum 416/280 nm ratios (see Figure I.1). Fractions 27-35 were

Figure I.1. Spectrometric detection of LM4b isozyme. Fractions yielding the greatest 416:280 ratios were collected for analysis by western blot for further analysis.



determined to have acceptable LM4b concentrations and purities by SDS-page and the western blot as shown in Figure I.2. These fractions were pooled and dialyzed for two hours in 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT and 0.1 mM PMSF. The sample was concentrated to 15 ml by ultrafiltration. It was then dialyzed overnight in 10 mM potassium phosphate, pH 7.7, 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF, 0.2% cholate, and 0.1% lubrol. This was loaded onto an equilibrated 20 ml DEAE column and washed with two volumes of buffer used for the dialysis. The P-450 was eluted stepwise with the equilibration buffer by increasing concentrations of potassium phosphate. The first step consisted of a five column volume wash with 40 mM potassium phosphate buffer, the second, a five column volume wash with 80 mM potassium phosphate buffer and finally, a five column volume wash with 160 mM potassium phosphate buffer. Fractions with the highest 416/ 280 nm absorbance ratios were pooled and dialyzed for two hours in 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF and 0.1% cholate to remove the lubrol. This was next loaded onto a small hydroxyaptite column (bed volume of 4 ml) that was equilibrated with 5 mM potassium phosphate, pH 7.4, 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF and 0.1% cholate. The column was washed with 50 ml of the buffer. The P-450 was eluted with a buffer consisting of 0.3 M potassium phosphate buffer pH 7.4, 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF and 0.1 % cholate. The sample was dialyzed overnight with a 50 mM Tris-acetate buffer, pH 7.4, 20% glycerol, 0.1 mM EDTA and 0.1 mM PMSF to remove the cholate. The sample was then divided

Figure I.2. Analysis of fractions 27-35 by western blot confirmed that these fractions contained sufficient LM4b isozyme but indicates that these fractions require further purification.



into aliquots and stored at -80 ° C .

Figure I.3 shows the western blot of the final LM4b isozyme. Two bands above the LM4b isozyme can be seen and consist of higher molecular weight dimers and tetramers. These bands are typically seen in western blots of purified isozymes especially when the sample is applied at high protein levels (2).

Figure I.3. Analysis of final fractions by western blot. These fractions were used as standards for detection of specific content of LM 4b isozyme in isolated microsomes as described in the Materials and Methods section.

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APPENDIX II

DETERMINATION OF OPTIMAL CONDITIONS FOR UDPG TRANSFERASE ACTIVITY TOWARDS THE SUBSTRATE AFL

The method of Coughtrie et al. (1) using rat microsomes (orginal incubation mixture consisted of: 50 mM Tris-maleate, pH 7.4, 10 mM $MgCl_2$, UDP[^{14}C]GA (0.25uCi), 2.7 mM UDPGA, 1 mM substrate, 0.1-0.5 mg microsomal protein and Lubrol PX in a 0.25 detergent:protein ratio) was modified for trout as described below. All of the following modifications were determined using microsomes from BNF pretreated trout.

Optimum pH with the substrate 1-napthol was determined as pH 7.4 (data not shown) and was consistent with that reported by Castren and Oikari (2) who found that enzyme activity remained relatively constant at a pH range of 6.5-8.0. Optimum temperature was determined as 25 $^{\circ}$ C (data not shown) and was also consistent with that of Castren and Oikari.

Detergent type and concentration have an important effect on maximum enzyme activity as shown by Hanninen (3). Trout and rat microsomes show significant differences in their responses to detergent activation, but a detergent such as digitonin will sufficiently activate both. The assay used by Hanninen (a modification of Bock and White (4)) was used to determine if the same results could be obtained. Detergent types and concentrations

assayed were: Triton X-100 (0.01-0.5%), lubrol (0.01-0.5%) and digitonin (0.1-0.5%). Although the microsomes treated with Triton X-100 and lubrol reached maximum activities towards the substrate 1-napthol at 0.1%, this rapidly decreased with an increase in detergent concentration. However, digitonin-treated microsomes had the same level of activity as the Triton X-100 and lubrol treated microsomes at the 0.1% detergent concentration and this level remained constant up to a detergent concentrations of 0.5%, the highest concentration tested. In a separate experiment, Brij 58 was used as the activating agent but was found to suppress all activity towards the substrate 1-napthol. Optimum detergent was determined to be digitonin at a concentration of 0.5-0.8%.

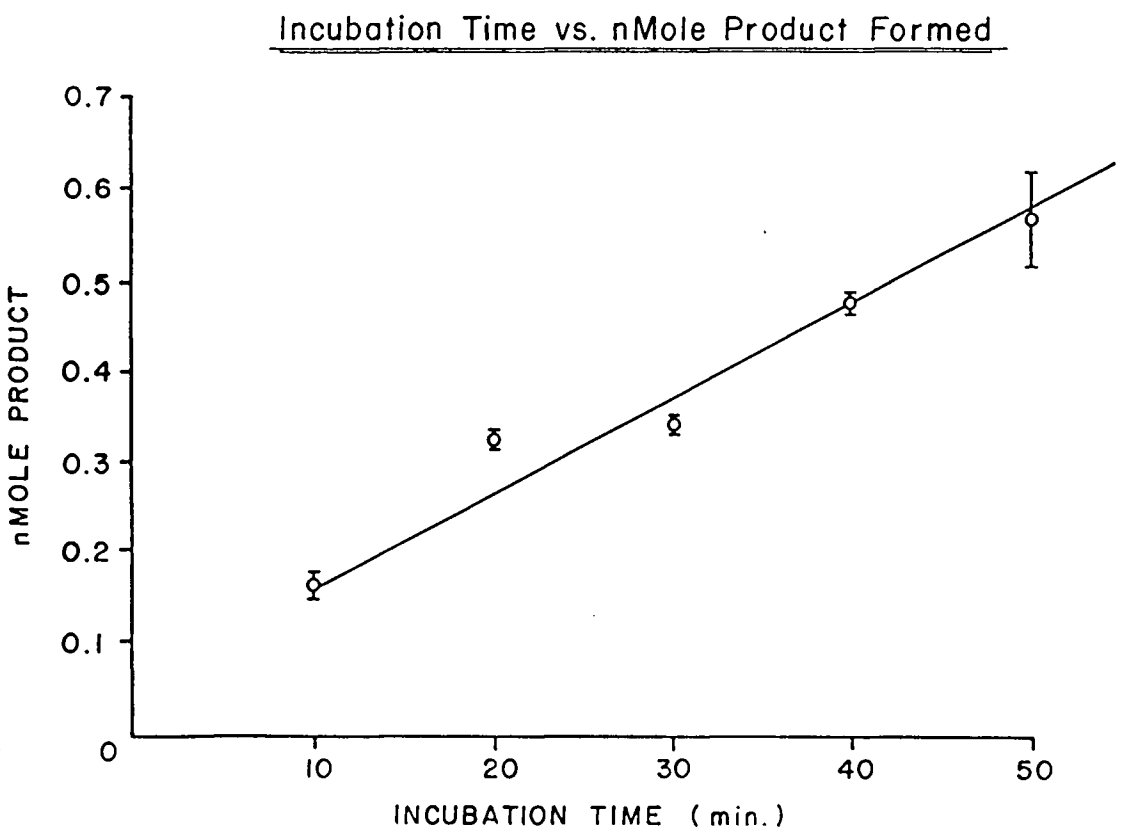
Microsomal protein concentration was increased to the highest obtainable at 2 mg per incubation mixture.

Reaction time course was determined as illustrated in Figure II.1. The curve remained linear through out the time points tested. Therefore, 30 minutes appears to be a sufficient time peroid for incubation.

Using these conditions (50 mM Tris-maleate, pH 7.4, 10 mM $MgCl_2$, 2.7 mM UDPGA (0.25 uCi), 0.8% digitonin concentration, 2 mg microsomal protein, pH 7.4, at 25° C for 30 minutes) a suitable substrate concentration for AFL was investigated. The substrate 1-napthol (1 mM) was used as a positive control for each set of incubations. Although Cougtrie recommended a substrate concentration of 1 mM, this is not possibile for AFL since its limit of solubility was approached

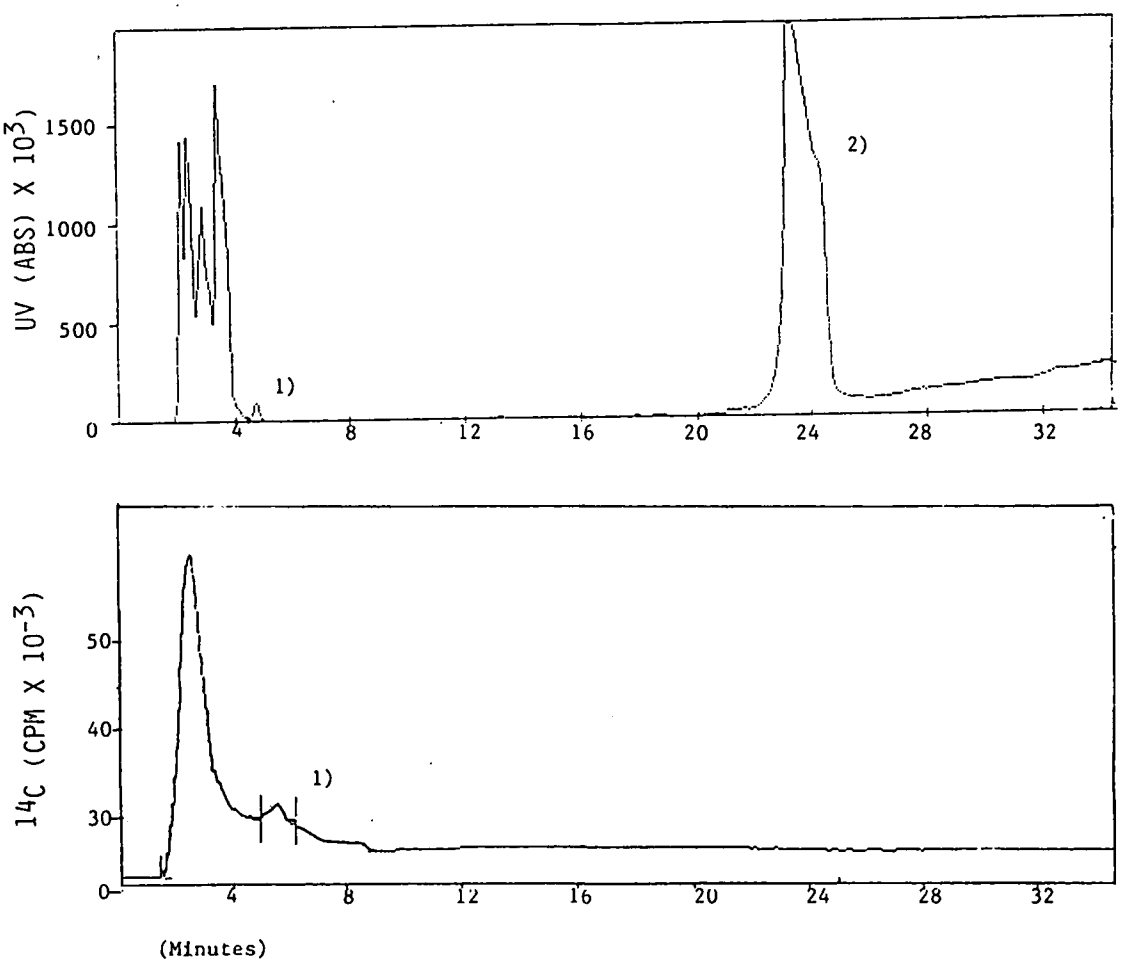
at 0.06-0.1mM. Concentrations of AFL ranging from 0.02-0.24 mM were assayed without detection of any AFL-glucuronide product formation. UDPG transferase activity towards 1-napthol was detected using a substrate concentration as low as 0.03 mM.

Figure II.1. Determination of optimal incubation period for UDPG transferase using 1-napthol as the substrate. The incubation mixture consisted of 50 mM Tris-maleate, pH 7.4, 10 mM $MgCl_2$, 0.8% digitonin, 2 mg microsomal protein, 2.7 mM UDPGA (0.25 uCi) and 1 mM 1-napthol. This was incubated at 25 ° C and terminated at the indicated time points by the addition of 475 ul ice-cold ethanol.



Microsomal protein was replaced with post mitochondrial fraction (S-20 or PMSF) to determine if addition of cytosol would result in AFL-glucuronide formation. The following incubation mixture was used: 50 mM Tris-maleate, pH 7.4, 10 mM $MgCl_2$, 2.7 mM UDPGA (0.25uCi), 0.8% digitonin, 0.18 mM AFL and 2 mg protein. This was incubated for 30 minutes at 25 °C. A small amount of AFL-glucuronide could be detected as shown in Figure II.2 and corresponds to .0853 nmole/mg/min. As this product was formed using PMSF fraction of BNF pretreated trout, it would be expected that the PMSF protein obtained from control trout would form even less of the AFL-glucuronide product. In this case, product formation would be beyond the limits of detection of the HPLC conditions used in this experiment.

Figure II.2. Detection of the product AFL-glucuronide formed following incubation of the substrate, 0.18 mM aflatoxicol, in 50 mM Tris-maleate, pH 7.4, 10 mM $MgCl_2$, 2.7 mM UDPGA (0.25 uCi) and 0.8% digitonin containing 2 mg PMSF protein at 25 ° C for 30 minutes. Peak 1, aflatoxicol glucuronide (product), peak 2, aflatoxicol (substrate)



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APPENDIX III

INHIBITION OF IN VITRO DNA BINDING BY I3C AND I3C ACID CONDENSATION PRODUCTS

The objective of the experiments presented in this section is to examine if I3C or its acid products (RXN) inhibit AFB1-DNA via a nucleophilic trapping mechanism. The inhibitory action of I3C or RXN may result from the trapping of the electrophilic AFB1 metabolite by I3C or RXN, or from a direct inhibition of AFB1 activating cytochrome P-450 isozymes.

I. MATERIALS AND METHODS

[^{3}H] Aflatoxin B1 (specific activity, 24 Ci/mmol) was obtained from Moravak, Biochemicals, Inc. (Brea, CA) . [^{3}H] Aflatoxin B1-dichloride was synthesized by reaction of Cl dissolved in methylene chloride with AFB1 as described by Swenson et al. (1) and purified by HPLC (1). Indole-3-carbinol acid reaction products (RXN) were generated with the addition of .05 N HCL to I3C as described by Bradfield and Bjeldanes (2) and were analyzed by TLC.

II. EXPERIMENT I:DIRECT NUCLEOPHILIC TRAPPING

The incubation mixture consisted of: 1 mg/ml calf thymus DNA, 100 uM EDTA, 50 uM Na_2PO_4, pH 7.4, and 0.35 mM I3C or RXN. After a preincubation period of 2 minutes at 25°C, 0.06 pmol AFB1-Cl_2 was added. After incubation for one hour, reaction mixtures were frozen by immersion of the reaction tubes into liquid nitrogen. DNA was isolated by phenolic extraction (3). One ml of 0.1 M Tris-saturated

one ml of chloroform with 4% isoamyl alcohol (IAC) were added to the thawed incubation mixture and shaken on a rotary shaker for 1 hour at 4 ° C. After centrifugation at 3,000 x g for 20 minutes, the upper layer was transferred to a new tube and extracted three times with equal volumes of IAC. The DNA was precipitated with 2 volumes ice-cold 100% ethanol, washed three times with 100% ethanol, dried under a stream of nitrogen and redissolved in 2 ml water. Aliquots were removed for counting. Absorbance at 260 nm was determined and DNA content calculated. The 260/280 ratio was $\geq$ 1.8.

III. EXPERIMENT II: INHIBITION OF ENZYMATIC MEDIATED AFB1-DNA BINDING

Microsomes were isolated from forty rainbow trout (average body weight was 285.1 $\pm$ 44.7) as described previously. The incubation mixture contained 3 mg of microsomal protein, 100 uM EDTA, 50 uM Na_2PO_4, pH 7.4, 1 mg calf thymus DNA, 2 nmol [3H] AFB1. Following a preincubation of two minutes at 25 ° C, 0.35 mM I3C or 0.35mM, 0.117 mM, 0.035 mM, 0.0035 mM or 0.0012 mM RXN was added, and the reaction initiated by the addition of 1 mg NADPH. The reaction was terminated after one hour by immersion of the reaction tubes into liquid nitrogen. DNA was purified and specific binding determined as described above. The 0.35 mM concentration of I3C and RXN was extrapolated from *in vivo* experiments in trout fed 2000 ppm [^{3}H] I3C (4) to an *in vitro* equivalent dose.

VI.RESULTS AND DISCUSSION

The appropriate incubation period was experimentally determined using [^{3}H] AFB1 and microsomal activation to be one hour as this time point was on the linear portion of the curve (amount DNA adduct formed versus time, not shown). The effect of RXN and I3C on AFB1-DNA binding using AFB1-Cl_2 as the substrate is shown in Figure III.1. Neither RXN nor I3C significantly reduced AFB1-DNA adduct formation, at the *in vivo* equivalent dose. The decomposition of AFB1-Cl_2 to reactive species does not require metabolic activation and likely goes to completion after approximately ten minutes under the present experimental conditions.

Figure III.2 illustrates the effect of I3C and RXN on AFB1-DNA adduct formation catalyzed by microsomes. Although all *in vitro* additions significantly inhibited DNA adduct formation, the *in vivo* equivalent dose of RXN (0.35 mM) had the largest inhibitory effect of 48%. Concentrations of 1/3, 1/10 and 1/100 of the equivalent dose (0.117, 0.035 and 0.0035 mM, respectively) and 0.35 mM I3C also inhibited the formation of DNA adducts, but the effects were significantly less than that of 0.35 mM RXN. The inhibition observed at 0.117 mM RXN was not significantly altered until the concentration of RXN added was reduced to 0.0012 mM RXN.

The results shown in Figure III.1 suggest that the inhibitory effect of I3C or RXN does not proceed by a direct nucleophilic trapping mechanism because addition of either compound did not decrease the amount of AFB1-DNA adduct formed. The mechanism is more likely one that is mediated enzymatically as indicated in Figure

Figure III.1. Lack of inhibition of *in vitro* AFB1-DNA binding following direct addition of 0.35 mM RXN (indole-3-carbinol acid treatment products) and 0.35 mM I3C to the incubation mixture. [^{3}H] Aflatoxin B1-dichloride, which does not require metabolic activation to produce a reactive species, was used as the electrophilic source.

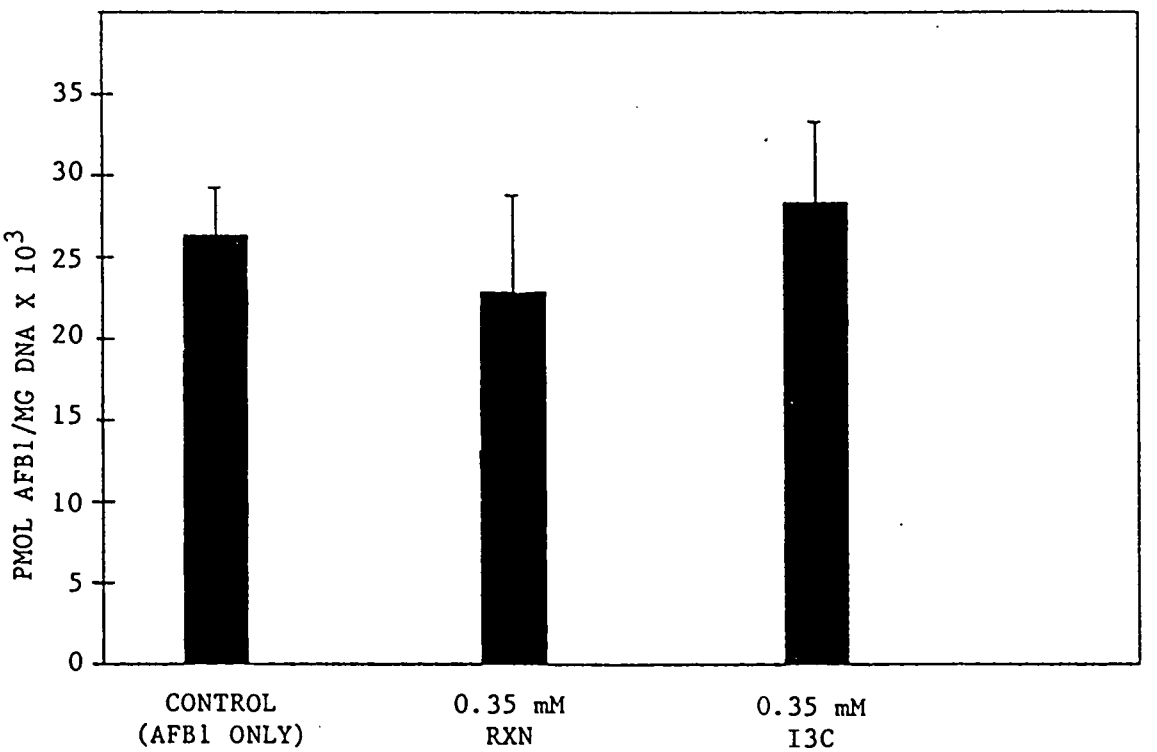
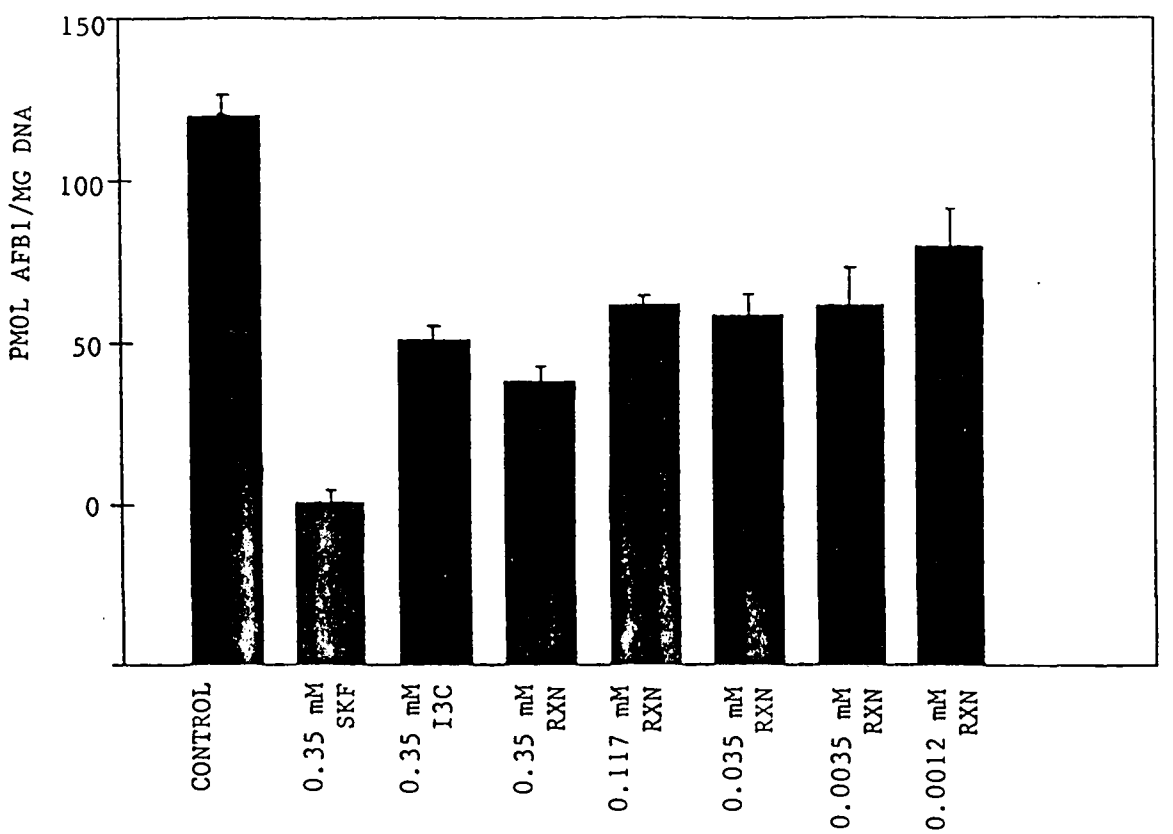


Figure III.2. Inhibition of in vitro AFB1-DNA binding following addition of RXN (indole-3-carbinol acid treatment products) at several concentrations and I3C at 0.35 mM to the incubation mixtures. SKF-525A, an inhibitor of cytochrome P-450 activity was added at 0.35 mM and served as a positive control. [^{3}H] Aflatoxin B1 was used as the electrophillic source and 3 mg of microsomal protein was used as the activating system.(All inhibitory effects were significantly different from the control value.)



III.2.

Since I3C had no effect (that is, neither induction nor suppression) on enzyme activities assayed in previous experiments, it is possible that the anticarcinogenic action of I3C proceeds by reversible not irreversible inhibition of the AFB1 activating cytochrome P-450. The parent compound significantly reduced AFB1-DNA adduct formation as did all concentrations of RXN used in this experiment. This effect at very low concentrations (0.0012 mM) gives evidence that I3C or its acid condensation products may act as anticarcinogens in liver at very low levels.

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