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There are striking associations between certain components of the human diet and reduced incidence of experimental cancer. Thus, implementing specific changes in diet may be an effective means to control human cancer, a disease responsible for about 25% of all deaths in the United States. "Chemoprevention" is a cancer control strategy entailing the deliberate administration of chemicals in order to prevent the occurrence of the disease. The micronutrient indole-3-carbinol (I3C) is a common dietary constituent shown to modulate tumor incidence in animals and is under consideration as a possible chemopreventive agent. Central to the implementation of this strategy is understanding the mechanisms by which "chemopreventives" exert their effects. Evidence is presented here that dietary I3C may inhibit tumor formation in the rat by a number of mechanisms, including induction of important carcinogen detoxifying enzymes such as cytochromes P-450 (CYP) and the glutathione *S*-transferases. For comparison, β-naphthoflavone, a synthetic flavonoid, was found to be a less potent inducer at doses approximately equally anticarcinogenic. The major role of CYP enzymes is to form or expose functional groups on lipophilic chemicals which enter the organism, thereby serving to enhance their excretion. Some lipophilic chemicals are acted on by CYPs to "bioactivate" them into

carcinogenic forms. Evidence is also presented that I3C metabolites and BNF will inhibit this enzymatic process, thus suggesting another mechanism of protection. Unlike I3C metabolites, BNF was found to enhance bioactivation under certain conditions *in vitro*. It is shown that concentrations of I3C metabolites that can inhibit CYP *in vitro* are present *in vivo* following an anticarcinogenic I3C dose. Aflatoxin B_1 was used as a model carcinogen throughout this study both because of its extreme potency and because it is a common contaminant of human foodstuffs, particularly in lesser developed countries.

Mechanisms of Inhibition of Chemical Carcinogenesis by Indole-3-Carbinol in the Rat

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PREFACE

This thesis is comprised of five parts consisting of an introduction, which gives an overview of indole-3-carbinol anticarcinogenesis and four chapters of original research written for publication. Chapters Two and Three have been accepted for publication in *Drug Metabolism and Disposition* and are scheduled to be published in Vol. 22, No. 3.

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MECHANISMS OF INHIBITION OF CHEMICAL CARCINOGENESIS BY INDOLE-3-CARBINOL IN THE RAT

Chapter 1

INTRODUCTION

Epidemiology studies have consistently found associations between the frequency or pattern of consumption of certain components of the diet and risk for certain cancers (1, 2). For example, the incidence of colorectal cancer is found to be elevated in populations consuming high amounts of fat (3), whereas a higher incidence of stomach cancer is associated with the consumption of salty foods, or a lack of intake of fruits and vegetables (4). The incidence of several cancers is reduced in populations that include a high proportion of fruits and vegetables in their diet (5). Epidemiology data, along with supporting laboratory studies in animals (6), have culminated in a series of dietary recommendations by several health authorities designed to lower risks for cancer (7, 8). In addition, these studies have given rise to the concept of "chemoprevention", a cancer control strategy involving the deliberate administration of purified chemicals (synthetic or naturally occurring) to humans with the intention of preventing or reversing the occurrence of the disease (9, 10). The concept has received considerable heed in recent years in part due to the fact that despite numerous medical advancements in the early detection and treatment of cancer, the five year survival rate of cancer patients in the United States was a dismaying 51% in 1992 (11). A prophylactic approach to cancer control thus appears to offer a hopeful alternative in decreasing overall morbidity and mortality in persons at risk for neoplasia. As part of their responsibilities, the Chemoprevention Branch of the Prevention Program, Division of Cancer Prevention and Control, National Cancer Institute identifies and characterizes potential candidate

chemopreventative agents (12). One compound that has received consideration is the naturally occurring indole, indole-3-carbinol[1] (I3C)[2].

In its naturally occurring form, I3C "exists" as the precursor glucobrassicin, the most widely occurring member of a class of compounds known as glucosinolates (reviewed in 13). Glucosinolates are found primarily in the family Cruciferae, which includes cabbage, broccoli, Brussels sprouts, kale and cauliflower as members. Upon maceration of plant tissue at neutral pH, glucobrassicin undergoes enzymatic hydrolysis, yielding glucose, sulfate, and presumably, by way of the intermediate 3-indolylmethyl isothiocyanate, thiocyanate ion and I3C (Fig. 1.1). Under conditions of lower pH, a second pathway will produce indole-3-acetonitrile, hydrogen sulfide and elemental sulfur. The catalyst in the initial hydrolysis in both pathways is the plant enzyme myrosinase, also known as thioglucoside glucohydrolase (E. C. 3.2.3.1). Once I3C is formed, it may condense with itself giving 3,3'-diindolylmethane (I33') or, in the presence of L-ascorbic acid, which is found at high levels in crucifers, ascorbigen. For experimental purposes, I3C is available commercially from Sigma Chemical Company (St. Louis, MO) or

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1 - synonyms include 3-indolylcarbinol, indole-3-carbinole, indol-3-ylcarbinol, indol-3-ylmethanol, indole-3-methanol, 3-indolemethanol and 3-hydroxymethylindole. Although, according to the International Union of Pure and Applied Chemistry *Nomenclature of Organic Chemistry* , section C (Butterworths, London, 1965), the use of the term 'carbinol' should be abandoned, 'indole-3-carbinol' remains widely used and for consistency with the literature, its use is propagated here.

2 - **Abbreviations**: I3C, indole-3-carbinol; I33', 3,3'-diindolylmethane; AHH, aryl hydrocarbon hydroxylase; DMBA, 7,12-dimethylbenzanthracene; BaP, benzo[*a*]pyrene; AFB_1, aflatoxin B_1; NNK, 4-(methylnitroamino)-1-(3-pyridyl)-1-butanone; DMH, dimethylhydrazine; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ICZ, indolo[3,2-*b*]carbazole; CYP, cytochrome P-450 (E.C.1.14.14.1); LT, [2-(indol-3-ylmethyl)-indol-3-yl]indol-3-ylmethane; CT, 5,6,11,12,17,18-hexahydrocyclonona[1,2-*b*:4,5-*b*':7,8-*b*'']triindole; EROD, ethoxyresorufin *O*-deethylase; GST, glutathione *S*-transferases (E.C. 2.5.1.18); UDPGT, UDP-glucuronosyl transferases (E.C. 2.4.1.17); EH, epoxide hydrolase (E.C. 3.3.2.3); QR, NAD(P)H:quinone oxidoreductase (1.6.99.2); DEN, diethylnitrosamine; IAA, indole-3-acetic acid.

Aldrich Chemical Company, Inc., (Milwaukee, WI). For the purposes of this thesis, discussion will be limited to I3C and its *in vitro* acid condensation products. The reader is referred to an excellent review by McDannell *et al.* (13) and references therein for an overview of the chemical and biological properties of indole glucosinolates

I3C as a Potential Chemopreventive Agent

Interest in I3C as a possible chemopreventative agent began as a result of the pioneering work initiated by Wattenberg, who found that when administered orally to rats, I3C could induce aryl hydrocarbon hydroxylase (AHH) activity in hepatic and intestinal tissue (14). Compounds increasing AHH activity had been previously found to inhibit polycyclic aromatic hydrocarbon-induced neoplasia. Subsequently, it was found that I3C could inhibit mammary tumor formation induced by 7,12-dimethylbenz[a]anthracene (DMBA) in female Sprague-Dawley rats and neoplasia of the forestomach induced by benzo[*a*]pyrene (BaP) in female ICR/Ha mice (15). Interest in I3C has remained high because of the demonstration of several characteristics desirable in candidate chemopreventative agents. *In vitro,* I3C does not exhibit obvious cytotoxicity (16) or mutagenicity (17, 18), and when given orally, I3C exhibits relatively low acute toxicity (19-21), and does not appear to be teratogenic (19). It has anticarcinogenic activity (in the form of inhibition of tumors or DNA-adduct formation) against several classes of environmentally relevant chemical carcinogens, including nitrosamines (22-29), polycyclic aromatic hydrocarbons (15, 26, 30-34), the mycotoxin, aflatoxin B_1 (AFB_1) (18, 35-42), and the nitroazarene, 4-nitroquinoline 1-oxide (43). Protection against estrogen related tumors has also been demonstrated (44-46), and short term studies in humans given I3C indicate a protective effect may occur against estrogen responsive breast cancer development (47, 48). The capacity to protect against many

carcinogens is highly desirable in chemopreventive agents because of the diverse etiology of human cancer (1, 2). Another desirable characteristic of I3C is the apparent lack of specificity in protection of target tissues. Studies thus far have shown chemopreventive effects in liver (18, 22, 25-31, 33-42), forestomach (15) or stomach (34), lung (23, 24, 31), mammary gland (15, 32, 45), larynx (44), tongue (43), nasal mucosa (24), swim bladder (34) and endometrium (46). Further, it appears that the protective effects of I3C is not species-delimited as I3C shows chemoprevention in mice (24, 44, 45), rats (25, 27, 42, 43), and trout (22, 34-37, 41). Results of mechanistic studies with hamsters (49), chick embryo (33) and monkey hepatocytes (50) suggest chemopreventive outcomes could occur in these species as well. Therefore, preliminary results in humans (47, 48) may be even more encouraging.

Adverse Effects of I3C

Although it appears I3C offers vast potential as cancer prophylactic agent, enthusiasm must be tempered by evidence indicating absence of protection or even adverse effects with I3C treatment. Jang *et al.* (51) found no effect of 0.5% dietary I3C on the incidence of pepsinogen 1-decreased pyloric glands, a putative preneoplastic marker of stomach tumorigenesis, when fed for 12 weeks following administration of N-methyl-N'-nitro-N-nitrosoguanidine. Morse *et al.* (23) found that rats given dietary I3C prior to intubation with the tobacco-specific nitrosamine 4-(methylnitroamino)-1-(3-pyridyl)-1-butanone (NNK) had reduced levels of 7-methylguanine adducts in lung and nasal mucosa DNA, however, adduct levels in hepatic DNA were significantly elevated. Although not kown to be mutagenic itself, I3C or its acid condensation products have been shown to be converted to mutagenic nitrosamines upon treatment with acid and

nitrite (52, 53) suggesting that under conditions which may occur in the stomach, I3C may indirectly particitipate in initiation events.

Besides possible adverse effects in the initiation step of carcinogenesis, I3C showed potential for enhancement or promotion of tumorigenesis, causing an elevation of 12-*O*-tetradecanoyl phorbol-13-acetate induction of ornithine decarboxylase activity (a marker of tumor promoting activity), in mouse epidermis (54). In some studies, dietary I3C actually increased the number of chemically induced tumors. When given to rats before, during, and after the colon-specific carcinogen dimethylhydrazine (DMH), I3C enhanced tumor incidence (55), possibly by enhancing binding of DMH to DNA in the presence of I3C (56). In addition, fecal extracts from rats given DMH and I3C were shown to be mutagenic in the Ames test, but mutagenicity did not appear to be related to metabolites of DMH (57). When I3C is given before and during administration of DMBA (34) or AFB_1 (35) to trout, a reduction in liver tumors is observed. When fed only after carcinogen exposure, a dramatic, dose-related increase in the number of tumors is found (34, 58). For AFB_1, the inhibitory and enhancing activity of I3C appears to be nearly equal over a range of I3C and AFB_1 doses (21). Promotion of hepatic tumors has also been found to occur in rats given I3C after administration of AFB_1 (42).

There is evidence that metabolites of I3C may mediate toxicity similar to that described for the well known toxic agent and tumor promoter 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)(59), through their capacity to bind to the *Ah* receptor. The most potent agonist described thus far, is indolo[3,2-*b*]carbazole (ICZ)(60, 61), which possesses a binding affinity rivaling that of TCDD and other toxic, carcinogenic and teratogenic *Ah* receptor ligands (62, 63). As with TCDD, immunosuppressive (64), estrogenic and anti-estrogenic (65) effects have been demonstrated. Other products formed from I3C have been shown to possess affinity for the *Ah* receptor (60, 61, 66, 67), and, while *Ah*

receptor binding may be associated with toxicity (68), this property of some I3C metabolites may be responsible, in part, for the chemopreventive effects of I3C.

Finally, intrinsic toxicity was reported by Shertzer and Sainsbury in the form of depletion of hepatic glutathione, elevated hepatic enzyme levels in plasma, and neurotoxicity, albeit at levels considerably higher than those required to elicit chemoprotection (20).

Mechanisms of I3C Anticarcinogenesis

As with any drug, essential in evaluating the risks or benefits of therapeutic use of I3C as a cancer prophylactic, is determining the precise mechanism(s) of action (10). This information will facilitate prediction of possible interactions with other dietary components or drug therapies. In a classification scheme developed by Wattenberg (69), chemopreventive agents can be categorized by the point in time during the multistep process of carcinogenesis when they exert their effects (Fig. 1.2). Some inhibitors act by preventing the formation of a carcinogen from precursor substances, such as ascorbic acid inhibition of carcinogenic nitroso compounds (70). Compounds inhibiting carcinogenesis by preventing the carcinogenic agents from reaching or reacting with critical target sites have been classified as 'blocking agents'. Inhibitors acting by preventing the manifestation of cancer when given subsequent to an otherwise carcinogenic dose of chemical are known as 'suppressing agents'. By far, the majority of chemopreventive agents fall into the latter two categories. I3C can be classified as a blocking agent, however, evidence exists that under some experimental conditions, I3C can act also as a suppressing agent (27, 43). Blocking agents can be further subdivided on the basis of mechanism of action. Possible mechanisms of blocking agents include (*i*) induction of biotransformation enzymes that could enhance excretion, (*ii*) inhibition or

inactivation of cytochrome(s) P-450 (CYP) that can bioactivate procarcinogens and (*iii*) physico-chemico interaction with carcinogens (i.e. nucleophilic trapping of electrophiles or complexing). Evidence exists that I3C or its metabolites may function within all three of these categories of blocking agents.

I) Induction of Biotransformation Enzymes

Induction of CYP

The CYP superfamily of proteins are of extreme interest because of their essential role in the biotransformation of numerous endo- and xenobiotics (71). In general, CYPs (a phase I enzyme system, defined in 72) oxidize substrates, resulting in the introduction or exposure of polar functional groups such as hydroxyl, amino, or epoxide moieties. The products of CYP reactions can serve as substrates for conjugation reactions catalyzed by phase II enzymes. However, CYPs sometimes "fail" at their role in detoxication and "bioactivate" some xenobiotics to a DNA-binding, carcinogenic form (73).

Because Wattenberg and coworkers have observed association of induction of (CYP1A1-mediated) AHH with inhibition of carcinogenesis, the mechanism of action of dietary I3C and I33' was ascribed to their ability to act as inducers of AHH activity (14, 15). Numerous investigators have since examined the CYP inductive properties of I3C, showing increases in associated activity (18, 20, 31, 45-50, 55, 61, 67, 74-91 [3]), isoform specific protein levels (18, 67, 83, 84, 88, 90), and gene transcription (92, 93). Induction of hepatic and small intestinal CYP is most often reported, and it appears intestinal monooxygenases are more sensitive to induction, responding to dietary levels as low as 50 ppm (86). Induction has also been reported in renal (75), lung (49), and colon

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3 - The data in reference 76 are identical to that in reference 77, but interpreted in a different manner. Only reference 76 is cited subsequently, based on publication date.

(92) tissue. The most commonly observed CYP isoform affected is CYP1A1 (61, 67, 92), a well characterized gene product that is part of the battery of enzymes induced in response to *Ah* receptor agonists (94). Other isoforms known to be affected are CYP1A2 (67, 83, 88, 92), CYP2B1 (31, 67, 83, 88, 93) and CYP3A (83, 88). It appears that I3C itself, is not responsible for the CYP inducing effects. Rather, induction may be attributed to components of a complex mixture of condensation products of I3C, formed in the presence of acid (31, 61, 85, 95). The identities of the individual components responsible for any CYP-specific induction have only been examined in some detail for CYP1A1. It has been suggested that ICZ, which binds with far greater affinity to the *Ah* receptor than any other I3C-derived acid condensation product, is highly important in the induction of this enzyme. Other acid condensation products, such as I33', [2-(indol-3-ylmethyl)-indol-3-yl]indol-3-ylmethane (LT), a linear trimer of I3C and 5,6,11,12,17,18-hexahydrocyclonona[1,2-*b*:4,5-*b*':7,8-*b*'']triindole (CT), a cyclic trimer of I3C, are known to have inductive properties themselves (18, 66, 83), and because of their expected higher yield (compared to ICZ) *in vivo* after oral I3C, may be collectively responsible for a significant portion of the total induction observed. Any inductive (or other biochemical) effects attributed to I3C seen *in vitro*, or when I3C is administered ip, should be interpreted with caution, because some acid condensation products (i.e. I33') may readily form in aqueous media at ambient temperature (96).

Induction of CYP is believed to represent the major I3C-mediated mechanism of protection against chemical carcinogenesis (10, 12). However, despite frequent association, there is surprisingly little direct evidence to support this hypothesis. On the contrary, more evidence exists that CYP induction is not a primary inhibitory mechanism. Inhibition of carcinogen-DNA binding or tumor incidence has been observed in the absence of detectable increases in hepatic AHH or other CYP activity in mice (26, 30) and

trout (40, 97), strongly suggesting that other mechanisms of protection are operative. Furthermore, hepatic induction of CYP1A1, seen most often with dietary I3C, is responsible for the bioactivation of a number of important dietary carcinogens (98), some of which I3C affords protection against. Under some conditions, there is good evidence that I3C induction of CYP is protective. When I3C or I3C acid condensation products were administered orally, but not ip, Park and Bjeldanes found protection against a subsequent challenge of [^{3}H]BaP given orally, in the form of reduced levels of [^{3}H]BaP binding to pulmonary DNA (31). Since intestinal ethoxyresorufin *O*-deethylase (EROD) was substantially induced only by oral administration, it was concluded that induction of intestinal CYP reduced the availability of BaP for bioactivation in the lung. Reduced binding of [^{3}H]BaP to hepatic DNA, however, was attributed to mechanisms other than CYP1A1 induction. In another study, the route of administration of AFB_1 (ip or po) did not affect levels of hepatic DNA binding in rats fed I3C despite induced levels of intestinal monooxgenases (39), suggesting protective effects of intestinal monooxygenase induction may be carcinogen specific. The effects of I3C, and, for comparison, the well-known CYP1A1 inducer ß-naphthoflavone, on the hepatic CYP induction and metabolism of AFB_1 are explored in Chapter Two. Fig 1.3 shows initial events thought to be important in determining the metabolic fate of AFB_1 in male rats and the important enzymes involved. The reader may find it helpful to refer to this diagram from time to time throughout the thesis.

Induction of Phase II enzymes

Enzymes that serve to conjugate endogenous ligands with functional groups introduced by phase I enzymes are known as phase II enzymes (72). These multimembered families of enzymes include the glutathione *S*-transferases (GST), UDP-

glucuronosyl transferases (UDPGT), sulfotransferases (E.C. 2.8.2.1) and epoxide hydrolases (EH). Although it does not conjugate substrates, NAD(P)H:quinone oxidoreductase or quinone reductase (QR) is categorized as a phase II enzyme because it does not introduce functional groups and exhibits coordinate induction with conjugation enzymes (99). Anticarcinogens that induce only phase II enzymes are preferred over those that induce both phase I and phase II enzymes because they are not carcinogen- or tissue-specific, and normally serve only to detoxify (10, 69). Of the phase II enzymes, those of particular interest are the GSTs, which serve to conjugate glutathione with carcinogenic electrophiles, EH, which conjugates water with reactive epoxides, and QR, which promotes obligatory two electron reduction of toxic quinone species, thereby preventing oxidative cycling and depletion of protective glutathione. Phase II enzymes induced by dietary I3C include QR (20, 81, 83, 84, 100), UDPGT (20, 33, 81), microsomal EH (76) and GST (20, 76, 81, 101-103). In most cases, hepatic enzyme activity was measured, however intestinal induction was obtained for GST and QR (as well as testosterone hydroxylase activity) in a detailed duration- and dose-response study by Wortelboer *et al.* (83). Only limited data are available on the induction of specific isoforms of the phase II enzymes by I3C (102), and, in most cases, activity measurements were determined with surrogate substrates. A drawback in using surrogate substrates is that they may be lacking in affinity for the isoform having high affinity for the compound of interest (i.e. 104), which, in animal chemopreventive studies, is the toxic or carcinogenic agent. In Chapter Three, evidence is presented for the first time that I3C induction of a single phase II enzyme (GST subunit Yc2) and its accompanying carcinogen-detoxifying activity, contribute to a reduction in carcinogenesis in mammals.

In some studies, I3C failed to induce QR (76), GST (40, 86, 105), UDPGT (20, 40, 76), or microsomal EH (86) when CYP activities were or were not induced. In the

trout model, it was shown that absence of induction of GST or UDPGT did not affect protection from either AFB_1, DEN or DMBA carcinogenesis (34, 35, 40, 105). Induction of QR would be of little consequence for AFB_1 and DEN since neither carcinogen is a substrate for this enzyme. It is not known what effect I3C has on the levels of microsomal or cytosolic EH in the trout. However, at least for the microsomal form, EH plays only a questionable role in detoxication of AFB_1 in mammals (106); and there are claims that epoxides of DMBA and some other PAHs may not be responsible for up to 99% of mammalian DNA adducts formed (107). Because in the trout model, dramatic protection occurs seemingly without a role for either phase I or phase II enzyme induction, it is apparent that still other mechanisms of protection must be operative.

Effects of I3C on the Regulation of Other Proteins

The effect of dietary I3C on other proteins that may influence metabolism and disposition has been reported. The cytosolic enzymes UDP-glucose dehydrogenase, essential in formation of UDP-glucuronic acid, a co-factor for UDPGT, was found to be elevated after administration to rats of 4100 ppm I3C in the diet for 10 days (76). In the same study, NADPH-cytochrome *c* - reductase (E.C. 1.6.2.4) and cytochrome b_5, proteins involved in electron transport during CYP catalysis, were also found to be elevated. Also elevated was glutathione reductase (EC 1.6.4.2), an effect subsequently corroborated by Shertzer and Sainsbury, who administered I3C by gavage to rats at a dose of 50 mg/kg for 10 days (81). This enzyme promotes the availability of reduced glutathione, the cofactor for the GSTs. Shertzer and Sainsbury also investigated the effects of I3C on other enzymes associated with protection against oxidative stress. No changes were observed in ascorbate synthase (EC 1.1.3.8), whereas a decrease was

observed in superoxide dismutase (EC 1.15.1.1) and glutathione peroxidase (EC 1.11.1.9) activities.

II) Inhibition or Inactivation of CYP Bioactivation

A decrease in CYP bioactivation of carcinogens appears to be the major mechanism of I3C protection in the trout model (18, 40, 41). By itself, I3C does not appear to be a strong inhibitor of CYP (38, 40, 41, 67) whereas much greater effects are seen with I3C acid reaction mixture or purified components thereof (18, 40, 41, 67, 108). Jongen *et al.* (33) reported that I3C was a potent inhibitor of chick embryo EROD activity, but it is not clear whether this was due to formation of more potent inhibitors (i.e., I33') that readily form from I3C in aqueous systems (63, 96). In Chapter 4, the effects of I33' on the inhibition of *in vitro* activity of trout, rat and human CYP isoforms, and on rat liver microsomal metabolism of AFB_1 is assessed. I33' is known to be a major *in vivo* derivative found after oral administration of I3C (84, 109, 110, see Chapter 5), thus the data strongly suggest inhibition of carcinogen bioactivation should occur *in vivo*. These data could account for the protection against hepato- and genotoxicity observed by Shertzer and his colleagues (26, 29, 30, 111, 112). Because of the observation that oral I3C could inhibit DMBA mammary tumorigenesis when given only four hours prior to DMBA, Wattenberg has recently classified I3C as a blocking agent acting by inhibiting carcinogen activation (32). This is in contrast to his original classification of I3C as an anticarcinogen operating by monooxygenase induction. Enzyme inhibitory effects are observed at similar potencies in trout and mammalian systems, and this mechanism of anticarcinogenesis should be a viable mechanism in all species, including humans. Central to establishing a role for inhibition of CYP *in vivo* is

answering the question of whether the *in vitro* inhibitors are bioavailable in target tissues and at what levels. This is addressed in Chapter 5.

III) Physico-Chemical Interactions with Carcinogens

Experiments with I3C or its acid condensation products have demonstrated chemopreventive effects that cannot be explained by effects on drug metabolizing enzymes. Acid condensation products, CT or I33' were shown by Takahashi (18) to be effective inhibitors of AFB_1 8,9-epoxide mutagenesis in the *Salmonella* assay in the absence of a trout S-20 cell fraction. However, a stronger inhibition was observed when AFB_1 8,9-epoxide was generated enzymatically by S-20 in the presence of AFB_1, suggesting enzyme inhibition plays the larger role. In another study, direct scavenging effects of AFB_1 8,9-Cl_2, an AFB_1 8,9-epoxide surrogate, was not demonstrated by either I3C or acid condensation products (40). Shertzer and his colleagues have extensively explored the antioxidant and electrophile scavenging properties of I3C and other indoles (26, 29, 30, 111, 112). Despite extensive and well obtained empirical evidence, most, if not all of the *in vivo* data can also be explained by enzyme inhibition of chemical bioactivation. Furthermore, although I3C readily reacts with itself under acid conditions (95), and with ascorbic acid under neutral conditions to form ascorbigen (13), no evidence has been obtained for an I3C-carcinogen adduct. Notwithstanding, it is clear that parent I3C *in vitro* or when administered ip, does possess antioxidant chemoprotective properties that are not easily explained by inductive or inhibiting effects on drug metabolizing enzymes. Unfortunately, there is only data in trout showing the existence of I3C *in vivo* after oral administration of I3C and, from a clinical standpoint, clearly oral administration is highly desirable. de Kruif *et al.* (84) found no evidence that

parent I3C could withstand the acid conditions in the stomach and similar evidence is presented in Chapter 5.

Other Mechanisms

Other potential mechanisms for I3C anticarcinogenesis may exist (113), and some have been explored to a limited extent. Morse *et al.* (23) examined the effect of 4410 ppm I3C in the diet for two weeks on the activity of the DNA repair enzyme, O^6-mGua-DNA-transmethylase, in mice. Tissue extracts form lung, liver or nasal mucosa exhibited no significant increase over controls in the capacity to remove O^6-mGua from methylated calf thymus DNA. An interesting mechanism that may apply to I3C or other anticarcinogens is the capacity of I3C acid condensation products to inhibit mouse cytosolic steroid-binding activity (102). The GST enzymes, in addition to their role in GSH conjugation with toxic electrophiles, are known to function as intracellular transport proteins that might be instrumental in the intranuclear localization of steroids or carcinogens. Interference in this putative transport process could therefore abrogate genotoxicity or detrimental gene expression. No inhibition of mouse liver cytosol-mediated GSH conjugation of AFB_1 8,9-epoxide was observed with up to 100 μM I33' (Chapter 4), suggesting that acid condensation products might only inhibit non-catalytic binding. The mechanism by which I3C acts as a suppressing agent (27, 43) is not well understood.

Other Biological Properties of I3C

In addition to the well documented anticarcinogenic properties of I3C, Dunn and LeBlanc (114) recently reported the ability of I3C to lower serum low density lipoprotein and very low density lipoprotein levels in mice. This effect was attributed to the

inhibition of acyl-CoA:cholesterol acyltransferase by purified I3C acid condensation products I33', CT and LT.

The I3C dimer, I33', has been reported to be an enzymatic oxidation product of the important plant growth hormone, indole-3-acetic acid (IAA) (115, 116) and is formed by autoclaving dimethylaminomethylindole (gramine)(115). Experiments by Grambow *et al.* (117) showed that I33' was a potent growth stimulator of the rust fungi *Puccinia graminis* f. sp. *tritici.*, and it was thus proposed that products of IAA metabolism might contribute to growth control of rust fungi *in vivo*. In contrast, I33' inhibited the growth of *Cochliobolus miyabeanus* and *Xanthomonas campestris* pv. *oryzae*, which are responsible for bacterial leaf blight of rice and *Helminthosporium* leaf spot, respectively (118, 119). In addition, the incidence of 'rice blast disease', caused by *Pyricularia oryzae mycelia*, was found to be reduced by spraying a 500-1000 ppm (approximately 2-4 mM) solution of I33' and was effective against *Pyricularia oryzae mycelia* growth *in vitro* at only one ppm (4 μM)(120).

Conclusions and Future Directions

In conclusion, it appears there are several mechanisms by which I3C inhibits tumorigenesis. Inhibition of CYP bioactivation, induction of CYP detoxication, and induction of phase II enzymes appear to be the most relevant mechanisms, however, evidence exists for electrophile or radical scavenging and inhibition of intracellular steroid transport as ancillary mechanisms. It is apparent that I3C is a non-specific protector with respect to carcinogen, species, or target tissue. However, the same may not be said about the mechanisms of protection against carcinogenesis, with the possible exception of inhibition of CYP bioactivation. Consequently, I3C-inhibition of CYP bioactivation may offer the most promise as a mechanism in its potential development as a chemopreventive.

Since 90% of known carcinogens require metabolic activation to become biologically active, even slight inhibition should result in protection. Only two studies with I3C have been conducted in humans (47, 48), and these demonstrate that oral I3C is an inducer of estradiol 2-hydroxylase, probably as a result of induced levels of CYP1A2. While this offers hope as prophylactic against breast cancer in women at high risk for the disease by decreasing metabolism of estradiol towards the toxic 16α-hydroxy metabolite, recent and convincing evidence shows that CYP1A2 is also the major CYP isoform responsible for bioactivating the potent carcinogens AFB_1 (121), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) (122) at, or near, dietary levels in humans. Administration of sub-CYP1A2 inducing levels might be expected to inhibit activation as well as inhibit 16α-hydroxylation of estrone if administered chronically. Phase II enzyme induction by I3C appears only at moderately high levels in mammals and other, more potent phase II inducers, such as oltipraz, are likely to offer more promise (123). It is hoped that the data in the succeeding chapters will assist not only in elucidating the anticarcinogenic mechanisms of I3C, but also of other potential chemopreventives.

As a final note, in this author's opinion, future studies with I3C *in vitro* (and perhaps *in vivo*) should be conducted not with I3C itself, but with its acid condensation products, in particular, I33'. This is because (*i*) there is little evidence to suggest that I3C *per se*, is responsible for any anticarcinogenic activity; (*ii*) there is no evidence in mammals that I3C can survive the acid conditions of the stomach, whereas I33' is more acid resistant and retains good stability in general; (*iii*) administration of I3C orally results in an extremely complex acid catalyzed reaction mixture whose composition varies with pH and starting concentration (95), both of which are expected to be variable *in vivo*; (*iv*)

I33' has been directly shown to possess anticarcinogenic activity and mechanisms have been explored for this compound, and (*v*), a facile method of synthesis from I3C has been published (124).

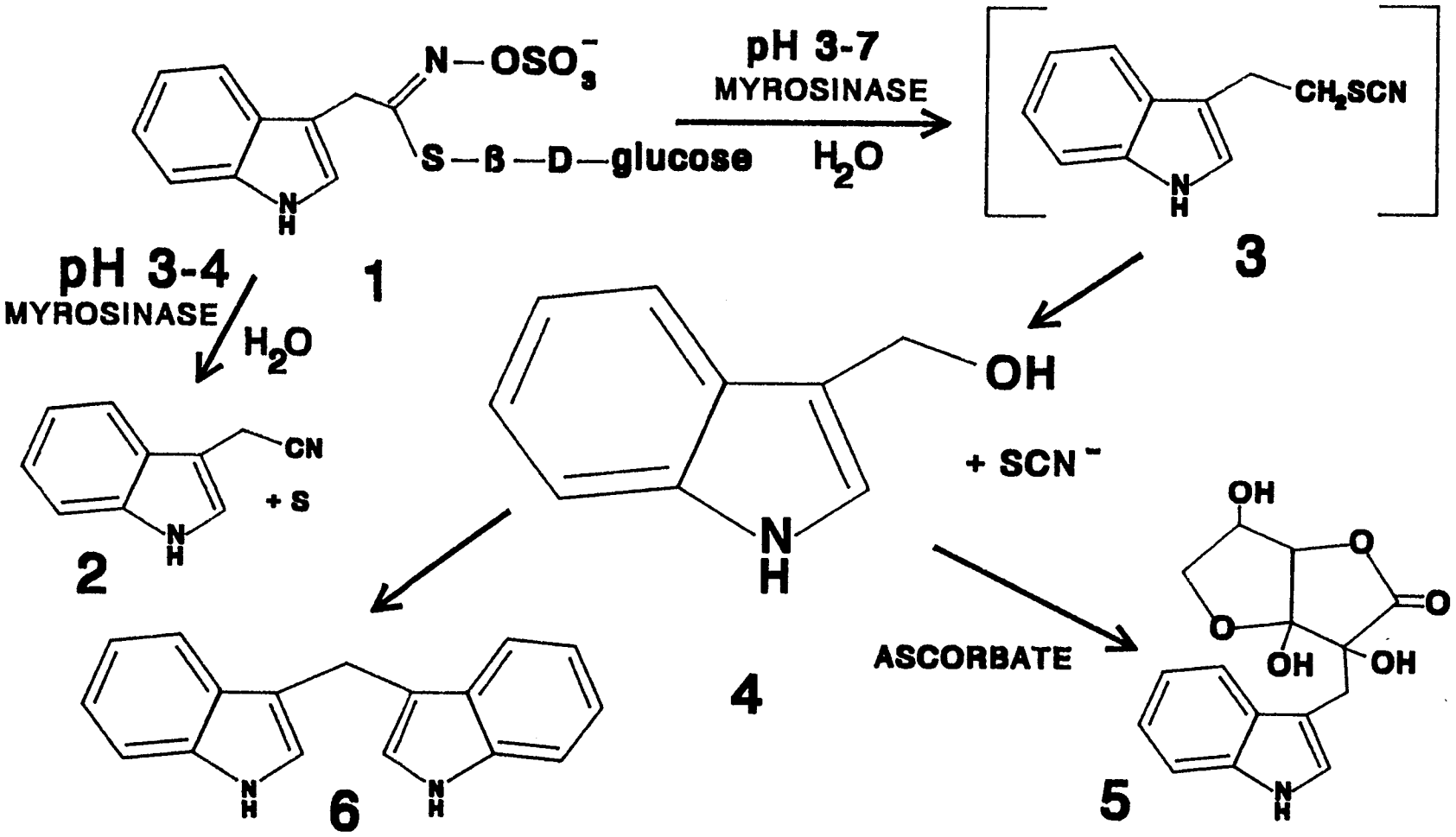

Figure 1.1. **Enzymatic hydrolysis of glucobrassicin, found in cruciferous vegetables, and formation of I3C. 1** = Glucobrassicin; **2** = Indole 3-acetonitrile; **3** = 3-Indolylmethyl isothiocyanate; **4** = I3C; **5** = Ascorbigen; **6** = 3,3'-Diindolylmethane.

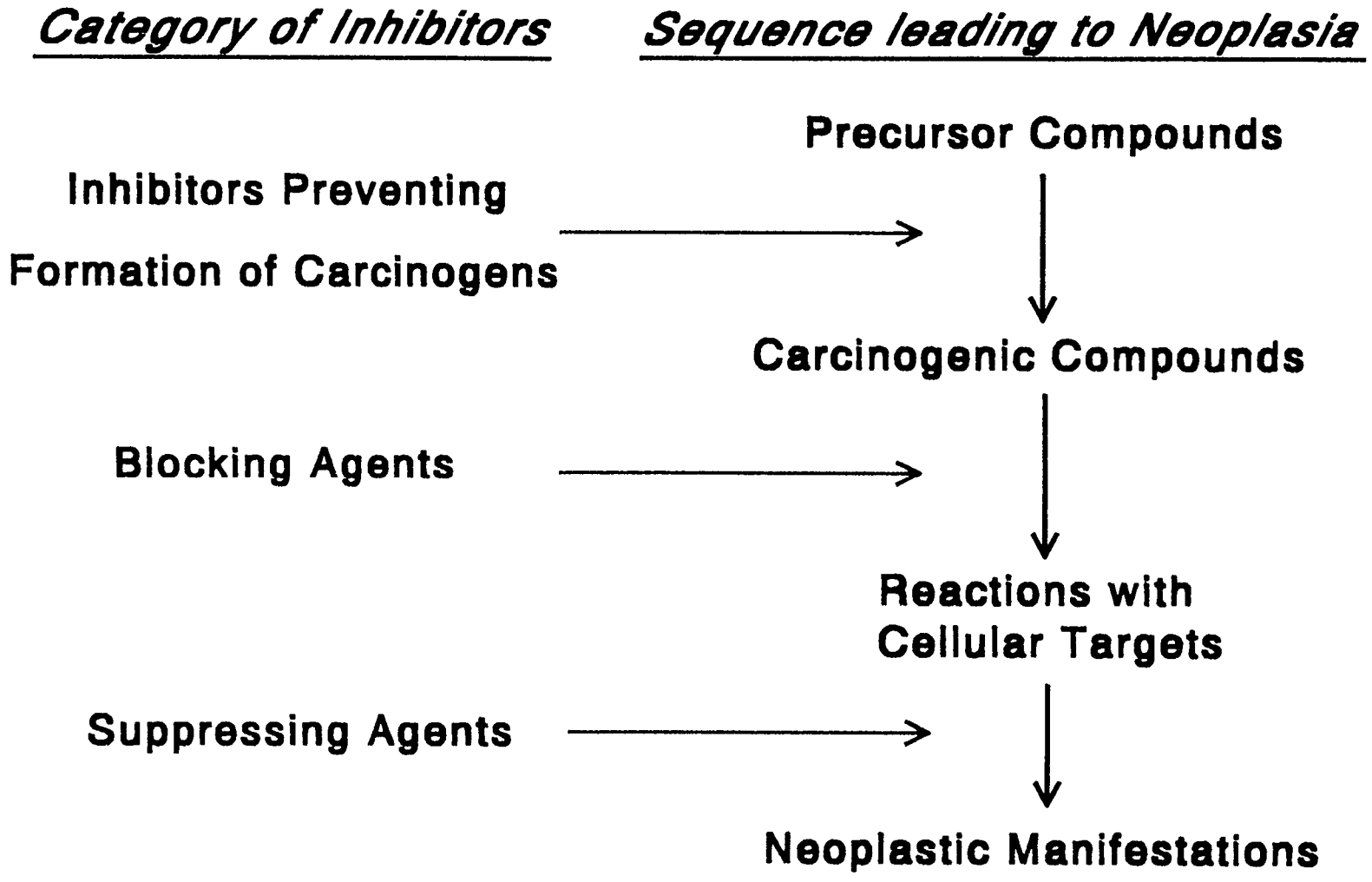

Figure 1.2. Classification of chemopreventive agents according to Wattenberg (69).

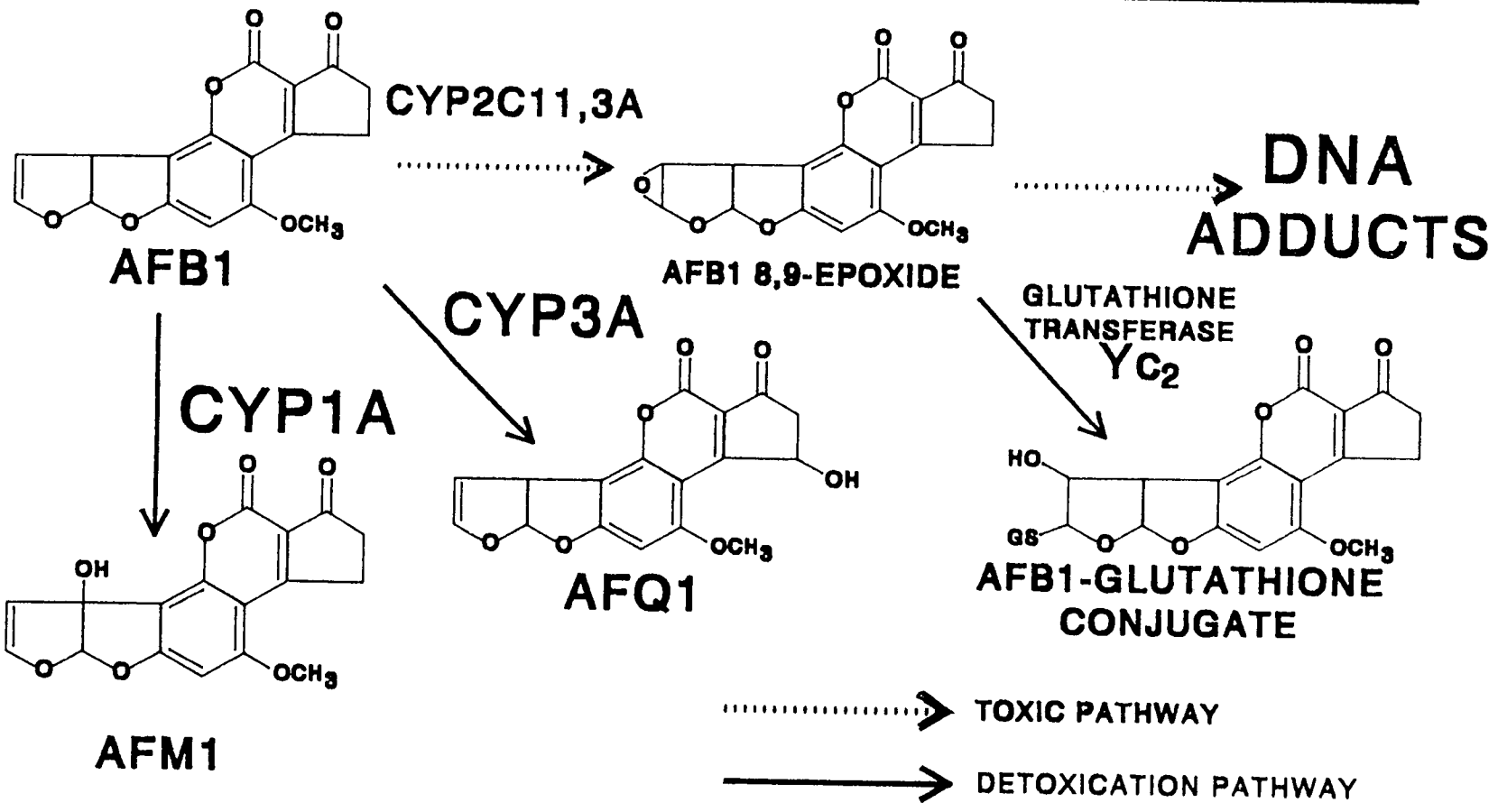

Figure 1.3. **Initial pathways of AFB_1 metabolism in male rats.** It is hypothesized that administration of I3C to rats will enhance overall metabolism towards the non-toxic pathway and inhibit metabolism towards the toxic pathway.

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Chapter 2

INDOLE-3-CARBINOL AND ß-NAPHTHOFLAVONE INDUCTION OF AFLATOXIN B_1 METABOLISM AND CYTOCHROMES P-450 ASSOCIATED WITH BIOACTIVATION AND DETOXICATION OF AFLATOXIN B_1 IN THE RAT

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ABSTRACT

Aflatoxin B_1 (AFB_1) is a highly hepatotoxic and hepatocarcinogenic secondary metabolite of the grain mold *Aspergillus flavus* and related fungi. Indole-3-carbinol (I3C), found in cruciferous vegetables, can both inhibit and promote AFB_1-induced carcinogenesis. We have examined the influence of dietary treatment with I3C and the well known Ah receptor agonist β-naphthoflavone (BNF) on the relative levels of different cytochrome P-450 isoforms known to metabolize AFB_1 in male Fischer 344 rats. After 7 days of feeding 0.2% I3C or 0.04% BNF, alone or in combination, the relative levels of hepatic CYP1A1, 1A2, 2B1/2, 2C11 and 3A were assessed by laser densitometry of western blots. Both diets containing I3C markedly increased band densities of CYP1A1 (up to 24-fold), 1A2 (3.1-fold), and 3A1/2 (3.8-fold), and had lesser effects on the levels of 2B1/2 (1.8-fold) and no effect on CYP2C11. BNF also strongly increased band densities of CYP1A1 (12-fold) and 1A2 (2.7-fold), but had no effect on the levels of CYP2B1/2 or 3A1/2 band densities, and repressed those of CYP2C11 (2-fold). In addition, we examined the *in vitro* hepatic microsomal metabolism of AFB_1 at 16, 124 and 512 μM substrate levels. Diets containing I3C elevated initial rates of AFM_1 (a detoxication product) production 18.6-19.2-fold over control at 16 μM AFB_1, which declined to 7.8-9.5-fold at 512 μM AFB_1. The BNF-only diet gave similar, but less dramatic effects (5.9-fold at 16 μM AFB_1, 3.5-fold at 512 μM AFB_1). I3C-containing diets also increased aflatoxin Q_1 (AFQ_1)(also a detoxication product) formation 2.7-2.9-fold at 16 μM AFB_1 and plateaued to 5.3-6.2-fold at 124 and 512 μM AFB_1. Limited effects on AFQ_1 production were observed with the diet containing only BNF (1.0 at 16 μM AFB_1 increasing to 1.4-1.5 at 124 and 512 μM AFB_1). I3C-containing diets increased the production of AFB_1 8,9-epoxide, the ultimate carcinogenic

metabolite 1.7-1.8-fold over control at 16 μM and plateaued at 1.8-2.2-fold at 124 and 512 μM AFB_1. No change in AFB_1 8,9-epoxide production was observed by feeding the diets containing only BNF (0.95-1.0-fold over the same ranges of substrate). When expressed as percent of initial rate of the three major metabolites formed, diets containing I3C decreased AFB_1 8,9-epoxide formation by 55-58%, 43-48%, and 33% at 16, 124 and 512 μM AFB_1 levels, respectively. The diet containing only BNF decreased percent AFB_1 8,9-epoxide formation in the same manner by 40%, 32% and 22% at the same AFB_1 levels. Our results suggest that BNF inhibits AFB_1 carcinogenesis, in part, by enhancing net production of less toxic, hydroxylated metabolites of AFB_1 as a result of elevated levels of P-450, and that I3C may share this mechanism. However other mechanisms, such as direct inhibition of P-450 bioactivation by I3C oligomers or induction of phase II enzymes also appear to contribute.

INTRODUCTION

Aflatoxin B_1 (AFB_1)[1], a metabolite of the grain mold *Aspergillus flavus*, is a potent hepatocarcinogen in animals and a probable human hepatocarcinogen (1, 2). AFB_1 is thought to be a primary etiologic factor in the development of liver tumors in certain regions of the world where contamination of foodstuffs with AFB_1 is common (3). Indole-3-carbinol (I3C) is a naturally occurring component of the human diet found in high concentrations in plants of the family Cruciferae (4). When incorporated into diets, I3C may show protection or enhancement of chemically-induced tumors depending on the sequence of administration of I3C and carcinogen (5-9). In most cases, inhibition is observed when I3C is fed prior to and during carcinogen administration and enhancement is observed when fed after. The synthetic flavonoid and well known Ah receptor agonist, ß-naphthoflavone (BNF), has similar tumor modulatory properties (7, 10, 11). In rodents, we have recently shown that diets containing I3C or BNF can inhibit AFB_1-induced toxicity and preneoplastic lesions when fed prior to and during carcinogen exposure, but, as seen in trout, show promotional activity when fed after [2].

While the promotional mechanisms remain poorly understood, the mechanisms by which I3C, BNF and most other chemopreventative agents inhibit tumorigenesis are believed to be related to their ability to alter expression of phase I and/or phase II drug metabolizing enzymes (reviewed in 12-14). Phase I enzymes of major importance to

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1 - **Abbreviations:** AFB_1, aflatoxin B_1; AFG_1, aflatoxin G_1; AFM_1, aflatoxin M_1; AFQ_1, aflatoxin Q_1; AFP_1, aflatoxin P_1; BNF, ß-naphthoflavone; I3C, indole-3-carbinol; BHA, butylated hydroxyanisole; DMSO, dimethyl sulfoxide; PMSF, phenylmethylsulfonyl fluoride; GST, glutathione *S*-transferase (E.C. 2.5.1.18); CYP, cytochrome P-450 (E.C.1.14.14.1); SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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carcinogen metabolism are certain members of the superfamily (primarily within families 1-3) of cytochromes P-450 (CYP). In general, CYP enzymes catalyze the formation of more polar, non-toxic products, however, bioactivation is sometimes a sequelae (15, 16). The phase II enzymes of primary importance are the glutathione *S*-transferases (GST), which catalyze conjugation of potentially toxic electrophiles to the tripeptide glutathione, generally rendering them non-toxic (17).

For AFB_1, the major rodent metabolic pathways and the GST and CYP isoforms involved have been substantially characterized (18-22). In a collaborative effort, we have recently shown that diets supplemented with 0.2% I3C and 0.04% BNF alone or in combination, induce the GST subunit Yc2 (subunit 10) and cytosolic activity towards AFB_1 8,9-*exo*-epoxide (23). This isoform possesses unusually high activity towards AFB_1 8,9-*exo*-epoxide (21), and is probably the predominant catalyst of this important detoxifying reaction in induced animals (24). For AFB_1 bioactivation reactions, non-CYP-dependent metabolism has been demonstrated and these may be quantitatively important under certain conditions. For example, prostaglandin H synthase, in the presence of arachidonic acid, can co-oxidize AFB_1 to mutagenic metabolites (25). This activity, while accounting for 1-2% of that for CYP in guinea pig liver microsomes, was approximately equivalent in guinea pig kidney microsomes. Lipoxygenase from guinea pig tissues, while exhibiting substantially lower capacity to oxidize AFB_1 to DNA-binding metabolites relative to CYP, reaches half-maximal rate of DNA binding at much lower AFB_1 concentrations (26). In the present study, we sought to examine the influence of dietary I3C and BNF on the *in vitro* hepatic microsomal metabolism of AFB_1 as well as their influence on the relative levels of the specific phase I enzymes (CYP1A1, CYP1A2, CYP3A1/2, CYP2C11, CYP2B1/2) thought to be the primary catalysts of

AFB_1 oxidative metabolism. The possible effects of these modulators on lipoxygenase and cyclooxygenase activities were not examined in this study. The results presented here show that pleiotropic induction of CYP enzymes and large increases in AFB_1 oxidative metabolism occur as a result of dietary exposure to I3C and BNF, and may contribute to the anticarcinogenic protection against AFB_1 exhibited by these compounds. Although I3C enhanced the formation of the ultimate carcinogenic metabolite, AFB_1 8,9-epoxide, the net increases in the combined amount of the detoxified products AFM_1 and AFQ_1 exceeded the net increase in AFB_1 8,9-epoxide, suggesting that an enhanced overall metabolism towards detoxified products may occur *in vivo*.

MATERIALS AND METHODS

Chemicals

AFB_1, aflatoxin G_1 (AFG_1), aflatoxin M_1 (AFM_1), aflatoxin Q_1 (AFQ_1), butylated hydroxyanisole (BHA) and BNF were purchased from Sigma Chemical company (St. Louis, MO). I3C was purchased from Aldrich Chemical company (Milwaukee, WI). Dimethyl sulfoxide (DMSO) and tetrahydrofuran were obtained from J.T. Baker (Phillipsburg, NJ). HPLC grade Methanol was obtained from Mallinckrodt (Paris, KY). All other chemicals were purchased from Sigma.

Animals and Diets

Twelve 4-week old Male Fischer rats were obtained from Simonsens (Gilroy, CA) and acclimatized for seven days on AIN-76A powdered semipurified diet formulated without preservatives (U.S. Biochemical, Cleveland, OH). The animals were then fed the same diet containing 0.2% I3C or 0.04% BNF alone or in combination for seven additional days. The levels of test compounds were similar to levels previously shown to inhibit hepatic preneoplastic lesions in the rat [2]. The following day, animals were injected ip with [^{3}H]AFB_1 (0.5 mg/kg, 480 μCi/kg) in 50-75 μl DMSO. The animals were killed by CO_2 asphyxiation 2 hours later and their livers were removed, blotted, weighed, frozen in liquid nitrogen and stored at -80°C until analysis. Swiss-Webster mice were obtained from Simonsens. These animals were fed a AIN-76A powdered semipurified diet containing 0.75% BHA (to induce GST levels for use in the AFB_1 metabolism assay) for ten days. Animals were killed by CO_2 asphyxiation and their livers were removed and frozen in liquid nitrogen and stored at -80° C.

Preparation of Hepatic Microsomes and Cytosol

Livers from rats fed the experimental diets were homogenized in 4 volumes of ice cold 0.1 M potassium phosphate buffer (pH 7.4) containing 0.15 M KCl, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF). Microsomes were prepared by differential centrifugation by the method of Guengerich (27). The supernatant from a 10,000 g centrifugation of liver homogenate was subjected to centrifugation at 100,000 g. The resulting pellet was washed with potassium pyrophosphate buffer (0.1 M, pH 7.4, 1 mM EDTA, 1 mM PMSF), centrifuged at 100,000 g, and the microsomes resuspended in homogenization buffer containing 20% glycerol to obtain microsomes. Microsomes were aliquotted in 500 µl portions and stored at -80°C. Cytosol was prepared from mice fed BHA according to Guengerich (27), except the post-100,000 g supernatant was subjected to an additional 100,000 centrifugation step to ensure the absence of membrane associated enzymes. Protein determinations on microsomes were made according to the method of Lowry *et al.*, (28).

AFB_1 Metabolism Assay

Metabolism of AFB_1 *in vitro* was determined essentially as described by Monroe and Eaton (29). This assay allows simultaneous quantification of hydroxylated metabolites and AFB_1 8,9-epoxide by trapping the latter as a stable glutathione conjugate. In the presence of mouse cytosol, trapping efficiency of the epoxide as the conjugate has been reported to be greater than 99% (29). Briefly, the final reaction mixture included 1 mg/ml test microsomal protein, 3 mg/ml BHA-induced mouse cytosolic protein, 16, 124 or 512 µM AFB_1, 5 mM GSH, 1 U/ml glucose-6-phosphate dehydrogenase, 5 mM

glucose-6-phosphate and 1 mM $NADP^+$ in a buffer containing 190 mM sucrose, 60 mM potassium phosphate, 80 mM Tris, 15 mM NaCl, 5 mM KCl and 4 mM $MgCl_2$, pH 7.6. The reaction mixture was preincubated at 37°C for 10 min and was initiated by the addition of cofactors (G6P, G6PDH, $NADP^+$, GSH) to give a final volume of 250 µl. After 10 min, the reaction was terminated by the addition of 50 µl 2 M acetic acid and 10 µl methanol containing the internal standard. The mixture was then frozen for at least two hours. The frozen mixture was then subjected to centrifugation at 14,000 g for 4 min at ambient temperature to thaw the mixture and pellet precipitated protein. Metabolites were resolved by HPLC on a 4.6×250 mm, C_{18} Econosphere cartridge column (Alltech Associates, Deerfield, Il) and detected by UV absorption at 362 nm. Retention times were 13.4 min for the AFB_1 8,9-epoxide glutathione conjugate, 19.0 min for AFQ_1, 19.5 min for AFM_1, 20.3 for the internal standard AFG_1 and 21.3 min for the parent compound. A representative HPLC chromatogram showing separation of the major metabolites is depicted in Figure 2.1. Quantification of metabolites was achieved with a Shimadzu Chromatopac integrator (Kyoto, Japan) using an AFB_1 standard curve and AFG_1 as an internal standard to correct for recovery. The mobile phase consisted of a combination of 0.1% ammonium phosphate, pH 3.5 (solvent A) and 95:5 methanol:THF (solvent B). From 0 to 2 minutes, the concentration of B was increased from 10% to 24%, then further increased to 38% B at 13 minutes. The concentration of B was further increased to 60% at 16 minutes and 90% at 17 minutes. At 20 minutes, the mobile phase was returned to starting conditions over a period of 5 minutes. All changes in the composition of the mobile phase were accomplished using a linear gradient. The flow rate was kept constant at 1.0 ml/min. The concentration of the AFB_1 stock solution (prepared in DMSO) was determined by UV absorbance spectrophotometry using an

extinction coefficient of 21.8 $M^{-1} cm^{-1}$. The final concentration of AFB_1 in the assay was calculated by adding a known amount of a AFB_1 stock solution (final concentration of DMSO in the assay was 4% v/v). Detectable levels of impurities that co-eluted with AFQ_1 and AFM_1 were corrected for using a control incubation in the absence of enzymes. When mouse cytosol was included in the absence of microsomes, the formation of AFB_1 8,9-epoxide as the GSH conjugate, was also detected, but represented < 8% of that formed by the least active microsomal preparations (i.e. control animals). This activity was corrected for in the values reported herein.

Other Assays

The ethoxyresorufin O-deethylase (EROD) assay was conducted as described by Pohl and Fouts (30).

SDS-PAGE and Immunoblotting

Microsomal proteins were separated on the basis of size by SDS-PAGE (31) on 8% acrylamide gels. Proteins were transferred electrophoretically to nitrocellulose paper (32) using a Buchler semi-dry blotter (Lenexa, KS) and fixed by heating at 70° C for 20 minutes. The blots were then probed with rabbit polyclonal antibodies to rat CYP1A1, 1A2, 2C11, 2B1/2 or 3A1/2. Antibodies to CYP1A1 and 3A1/2 were obtained from Oxygene (Dallas, TX). Antibodies against CYP1A2 were a gift of Dr. Paul Thomas (Rutgers University). Antibodies prepared against CYP2B1/2 and 2C11 were obtained from the laboratory of Dr. Donald Buhler at Oregon State University. The blots were subsequently probed with a goat-anti-rabbit secondary antibody conjugated to horseradish peroxidase (BioRad, Richmond, CA). CYP protein was then detected indirectly by

chemiluminescence using an ECL western blotting detection kit (Amersham Corp., Arlington Heights, IL). The relative densities of the individual bands were determined using a Zeineh SL densitometer (Biomed Instruments, Fullerton, CA). Results from our laboratory (not shown) indicate that increases in band density in the 1-20 μg microsomal protein range are less than linear (i.e. sublinear) with respect to the amount of protein applied to the gel. Therefore, we report our results operationally as fold increases in band densities which, at the 10-15 μg protein loading used in our experiments, provide a minimum, conservative estimate of CYP protein induction compared to controls. Because the response is less than linear, it follows that statistical significance in band density increases must correspond to equal or even greater significance in actual protein increases.

Statistics

Differences between groups were determined using one-way analysis of variance on the original or log transformed data, followed by the least significant difference multiple comparison test using the statistical software package, Statgraphics (version 5.0), (Statistical Graphics Corporation, Princeton, New Jersey). The decision whether to use log transformed data in the test was based on visual examination of residuals versus means plots. A probability value of less than 0.05 was considered significant.

RESULTS

Western blots of hepatic microsomal protein probed with polyclonal antibodies against various CYP enzymes are shown in Figure 2.2. Relative band densities were then assessed by scanning laser densitometry (Table 2.1). Experiments from our laboratory have shown that the increase in band density under these conditions represents a minimum measure of the level of induction of the actual protein (see Materials and Methods). Relative band densities of CYP1A1 were increased 24- and 19-fold in rats fed diets containing I3C or I3C and BNF combined, respectively. This protein was barely detectable in control animals. When BNF was fed at 0.04% in the diet, CYP1A1 band densities were also greatly increased (about 12-fold) as expected, but to a lesser extent than the I3C diets. The magnitude of induction and difference between the diets containing I3C and the diet containing only BNF is further illustrated in Figure 2.3, which shows EROD activity associated with the hepatic microsomal preparations. This assay is widely used to detect increases in primarily CYP1A1 activity (33). Induction of CYP1A2 was observed across all three treatments, with all diets increasing band densities approximately 3-fold (Table 2.1). The band densities of CYP2B1/2 were elevated 1.7-fold in rats fed I3C alone and 1.8-fold in rats fed I3C in combination with BNF, but no change was observed in rats fed only BNF. The I3C-only diet did not elevate band densities representing CYP2C11 (1.2-fold, not significant) whereas a 2-fold decrease in relative band densities representing CYP2C11 was observed in animals fed the diet containing only BNF. Animals on the combined diets were found to be significantly different from animals on the I3C-only diet and the BNF-only diet, but not the control diet. A potentially important finding was the observed induction of CYP3A1/2 by diets containing I3C. This would be a rare example of induction of this isoform by a naturally occurring component of the human diet. Two proteins were recognized by this antibody.

More prominent recognition of an unidentified protein possessing a lower molecular weight (denoted by "X") was observed in control and BNF treated animals. This suggests that concomitant with the induction by dietary I3C of the protein possessing higher molecular weight (3A1/2), was an apparent repression of the lower molecular weight protein.

As shown in Figure 2.4A, it was observed that only diets containing I3C increased AFB_1 8,9-epoxide formation. The same holds true for AFQ_1 formation (Fig. 2.4B) and a saturation effect becomes evident. In contrast, all three experimental diets increased rates of AFM_1 formation (Fig. 2.4C). The statistical significance of these data are outlined in Table 2.2. Figure 2.5 depicts the fold increase in rate of each metabolite formed as concentration of substrate increases. Diets containing I3C increased the rate of production of AFB_1 8,9-epoxide 1.7-1.8-fold over control at 16 μM and plateaued at 1.8-2.2-fold at 124 and 512 μM AFB_1. No change in rate of AFB_1 8,9-epoxide production was observed by feeding the diet containing only BNF (0.95-1.0-fold over the same ranges of substrate). This diet increased rate of AFQ_1 formation 2.7-2.9-fold at 16 μM AFB_1 and plateaued to 5.3-6.2-fold at 124 and 512 μM AFB_1, whereas the BNF-only diet induced no significant changes in AFQ_1. In contrast, diets containing I3C induced the rate of AFM_1 production 18.6-19.2-fold over control at 16 μM AFB_1, which gradually declined to 7.8-9.5-fold at 512 μM AFB_1. The BNF only diet produced similar, but less dramatic effects (5.9-fold at 16 μM AFB_1, 3.5-fold at 512 μM AFB_1).

When expressed as percent of the total amount of AFB_1 8,9-epoxide, AFM_1 and AFQ_1 formed (Fig. 2.6), AFB_1 8,9-epoxide formation was decreased by diets containing I3C by 55-58%, 43-48%, and 33% at 16, 124 and 512 μM AFB_1 levels, respectively. The diet containing only BNF decreased percent AFB_1 8,9-epoxide formation in the same

manner by 40%, 32% and 22% at the same AFB_1 levels.

Throughout these studies, AFP_1, the 4-hydroxy, demethylated metabolite of AFB_1, was detected, but could not be reliably quantified. Its production appeared to be increased with dietary I3C, in a manner kinetically similar to AFQ_1, but still accounted for less than 5% of the total production of microsomal oxidative metabolites.

DISCUSSION

Induction of Hepatic CYP Isoforms

The induction of several isoforms across three families of CYP, shown here with I3C, would be an unusual finding for any single compound. However, because of its rapid oligomerization into a complex mixture catalyzed by the acid conditions in the stomach (34), at least 24 different I3C derived compounds are found in ethyl acetate extracts of rat liver homogenates after oral gavage (see Chapter 5). Each component of this mixture may have varying biological effects and it appears oral administration of I3C is necessary for at least its CYP1A1 inducing effects (35). Induction of the latter enzyme may be largely due to one component found in the gastrointestinal tract, 3,2-*b*-indolocarbazole, which binds with far higher affinity to the Ah receptor than any other I3C-derived acid condensation product and induces CYP1A-mediated EROD activity in a murine hepatoma cell line (36). However, other components, which are present in the reaction mixture at higher yield, are also known to induce CYP1A1 in primary cultures of rat and monkey hepatocytes (37). These components may contribute substantially to the CYP1A1 and 1A2 induction observed in the present study.

We observed a small but significant induction in the band densities of CYP2B1/2, another isoform commonly induced by some xenobiotics. Recent investigations in other laboratories have reported similar findings (38, 39). No significant changes were observed in the band densities of the major constitutive, male-specific isoform, CYP2C11. This is consistent with the findings of Wortelboer *et al.*, who observed no changes in CYP2C11-mediated testosterone 2α-hydroxylase activity in the livers of rats fed up to 0.05% I3C in the diet (39). Dietary BNF repressed expression of this isoform as is normally observed (40), but not in the presence of I3C. An interesting finding,

which expands on the low-dose studies reported by Wortelboer *et al* (39), was the observation of a 3.8-fold increase in immunochemically detected CYP3A1/2 band densities in rats fed the diet containing 0.2% I3C alone and a 2.8-fold increase by diets containing both I3C and BNF. With the antibody described herein, we were unable to discern which of two major isoforms in the 3A subfamily (CYP3A1, 3A2) was induced by I3C. Both forms have identical mobilities using SDS-PAGE and 89% sequence similarity and only recently has it been possible to distinguish between the two forms using highly specific monoclonal antibodies (41). The appearance of a lower molecular weight protein cross reacting with the antibody in the present study, thus is suggestive of a member of the CYP3A subfamily other than CYP3A1 or 3A2. This protein(s) may be identical to the 50 kDa protein(s) described by Gemzik *et al.* (42), recognized by antibodies raised against CYP3A1. This protein was repressed by treatment with a known CYP1A and CYP2B inducer, Aroclor 1254, concurrent with induction of CYP3A1/2, similar to the results observed here with dietary I3C alone or in combination with BNF. The I3C derivative(s) responsible for CYP2B1/2 and 3A1/2 induction have not yet been identified.

I3C and BNF Dose Effects

The levels of I3C and BNF employed in this study (approximately 130 and 26 mg/kg body weight/day, respectively) were chosen to mimic levels found to be anticarcinogenic in rats and thus the mechanisms of anticarcinogenesis are examined at these doses. While this daily intake of I3C exceeds those normally encountered in the human diet (approximately 0.1 mg/kg/day in the U.K. (43)), Michnovicz and Bradlow have recently shown that humans given 6-7 mg/kg body weight/day (which is estimated to be equivalent to 300-500 g of cabbage/day) exhibited significant increases in estradiol

2-hydroxylation (44), which is apparently mediated by CYP1A2 and CYP3A in humans (45). A detailed examination of the effects of I3C dose on AFB_1-DNA adduction and hepatocarcinogenesis has been carried out with trout (46), but such data are not available for the rat model.

Induction of AFB_1 Metabolism

The pleiotropic induction of the different CYP isoforms described above is consistent with the substantial alterations in *in vitro* metabolism observed with dietary treatment with I3C alone or in combination with BNF. In rats and mice, an increased rate of AFM_1 production has been documented to be associated with exposure to Ah receptor ligands (47). At least in mice and humans, CYP1A2 appears to be the major catalyst converting AFB_1 to AFM_1 (48, 49) and may also be the major catalyst of this reaction in untreated rats. CYP1A1, which is essentially not expressed in untreated animals, also can catalyze this reaction (50), and quantitatively may be the major AFB_1 9a-hydroxylase (AFM_1) in induced animals expressing high levels of this enzyme. This latter hypothesis appears to be true for this species since rates of AFM_1 production were highly elevated concomitant with a substantial increase in CYP1A1 by diets containing I3C alone or in combination with BNF, but only moderately elevated in rats fed diets containing only BNF despite similarly induced levels of CYP1A2 across all dietary treatments. The extent of CYP1A1 induction by I3C-containing diets over that by BNF alone in the hepatic microsomes of these animals, is further illustrated by the EROD assay, a widely used marker of CYP1A1 activity (33). Production of AFQ_1 was also highly induced by diets containing I3C alone or in combination with BNF. In rats, this metabolite is increased in response to known CYP3A inducers including phenobarbital (51), pregnenolone-

16α-carbonitrile, dexamethasone and triacetyloleandomycin (18). The increase in AFQ_1 formation in the present study maybe consistent with the increase in CYP3A1/2 observed. CYP3A4 is known to catalyze this reaction in humans (49, 52). We observed AFP_1 formation in small quantities relative to the other metabolites as expected (53)(< 5% of the total in general, when quantified), and this activity appeared to be induced by I3C-containing diets in a manner similar to AFQ_1.

Diets containing I3C also increased production of AFB_1 8,9-epoxide, as determined indirectly by trapping as the glutathione conjugate in the presence of mouse cytosol. This occurred probably as a result of elevated levels of CYP3A1/2 (20) and possibly CYP1A2 (54, 55) which have been shown directly or indirectly to have the capacity to metabolize AFB_1 to mutagenic products. CYP2C11, perhaps the major catalyst of the bioactivating pathway in male rats (20, 56) was not directly responsible for the increase in AFB_1 8,9-epoxide formation since the levels of this isoform were unchanged by dietary treatment. There does not appear to be a significant role for CYP2B1/2 in the bioactivation of AFB_1 (20, 56), although a V79 Chinese hamster cell line expressing rat CYP2B1, has been shown to metabolize AFB_1 to mutagenic metabolites (57).

Kinetic Differences

We observed profound kinetic differences in metabolite formation *in vitro* that was dependent on initial substrate concentration. Specifically, with I3C-containing diets, the apparently CYP3A-mediated increases in AFQ_1 formation were greatest at high substrate levels, whereas with all experimental diets, the apparently CYP1A-mediated increases in AFM_1 formation were greatest at low substrate levels. These observations

implicate AFB_1 9a-hydroxylase (CYP1A, which forms AFM_1) as a low K_m enzyme and AFB_1 3-hydroxylase (apparently CYP 3A, which forms AFQ_1) as a high K_m enzyme, consistent with results observed with human liver microsomes or cDNA-expressed CYP1A2 and CYP3A4, respectively (49). Further, there was a general trend toward increased net formation of detoxified metabolites as initial substrate concentration decreased for all experimental diets.

Mechanisms of Protection against AFB_1-DNA Adduction and Tumorigenicity

The major factors determining susceptibility to AFB_1 carcinogenesis are believed to be related to the capacity of a given species to biotransform AFB_1 to non-toxic products (58). In this regard, the role of GST-mediated AFB_1 conjugation with glutathione is highly important (59). In a companion study to the present one, using the same animals described here, we showed that all three experimental diets significantly inhibited *in vivo* AFB_1 adduction to liver DNA at 2 h after administration of [^{3}H]AFB_1 (23). We also found that a GST subunit (Yc2), possessing unusually high activity towards AFB_1 8,9-*exo*-epoxide, was markedly induced in hepatic cytosol by both diets containing I3C, concomitant with an elevation in cytosolic activity towards AFB_1 8,9-*exo*- and *endo*-epoxide [the latter of which is produced only in small amounts by rat and human liver microsomes (60), and is considered relatively non-mutagenic [3]]. Based on these results, we believe that induction of the GST subunit by I3C contributes substantially to its protective effect against AFB_1, and that this is a less significant inhibitory mechanism for BNF.

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3 - T. Harris, personal communication.

Humans and non-human primates reportedly possess extremely limited capacity to conjugate AFB_1 with glutathione (22), and consequently may be relatively susceptible to AFB_1. High rates of CYP-mediated production of AFQ_1 may therefore be a primary mechanism of protection for these species (49, 53). Similarly, enhanced CYP mediated formation of AFM_1 in rats given BNF has been suggested by Gurtoo and coworkers as the major mechanism of protection conferred by this compound (10). Although AFM_1 and AFQ_1 are themselves mutagenic in the presence of hepatic S9 cell fraction, they are considered detoxication products since their mutagenicity is about 2% that of AFB_1 under similar conditions (61). Moreover, AFQ_1 was recently demonstrated to be a very poor substrate, relative to AFB_1, for epoxidation by human liver microsomes and that synthetic AFQ_1-epoxide produced only low levels of adduct formation with calf thymus DNA (62). In the present study, we show that anticarcinogenic dietary levels of I3C and/or BNF substantially induce levels of certain CYP isoforms and enhance initial rates of AFM_1 and AFQ_1 production *in vitro*. However, interpretation is complicated by the finding that initial rates of AFB_1 8,9-epoxide production *in vitro* are also increased, at all substrate levels tested, using microsomes from I3C-treated but not BNF-treated animals. Thus the effect of I3C exposure may conceivably be a bias in phase I metabolism favoring greater initial AFB_1 DNA damage as a result of the increased in AFB_1 8,9-epoxide production. Overall, however, I3C-mediated CYP induction is at least suggestive of a net protective effect because the relative rate of epoxide formation is decreased (22-58%) at all substrate levels tested and thus at the time of substrate exhaustion following a given AFB_1 bolus exposure *in vivo*, the total amount of epoxide formed for possible DNA adduction may be less. For BNF the effect is less ambiguous; induction of a competing phase I detoxication enzyme in the absence of enhanced epoxide production is likely to lead to

more rapid AFB_1 depletion and hence a net reduction in AFB_1 8,9-epoxide production *in vivo*.

Finally, it should be stressed that specific or non-specific inhibition of these various oxidative enzymic pathways by BNF or I3C oligomers *in vivo* may also be important (see Chapter 4 and refs. 63 and 64) and a balance between induction and inhibition will ultimately determine the CYP mediated production of DNA binding species by these modulators in rats.

ACKNOWLEDGMENTS

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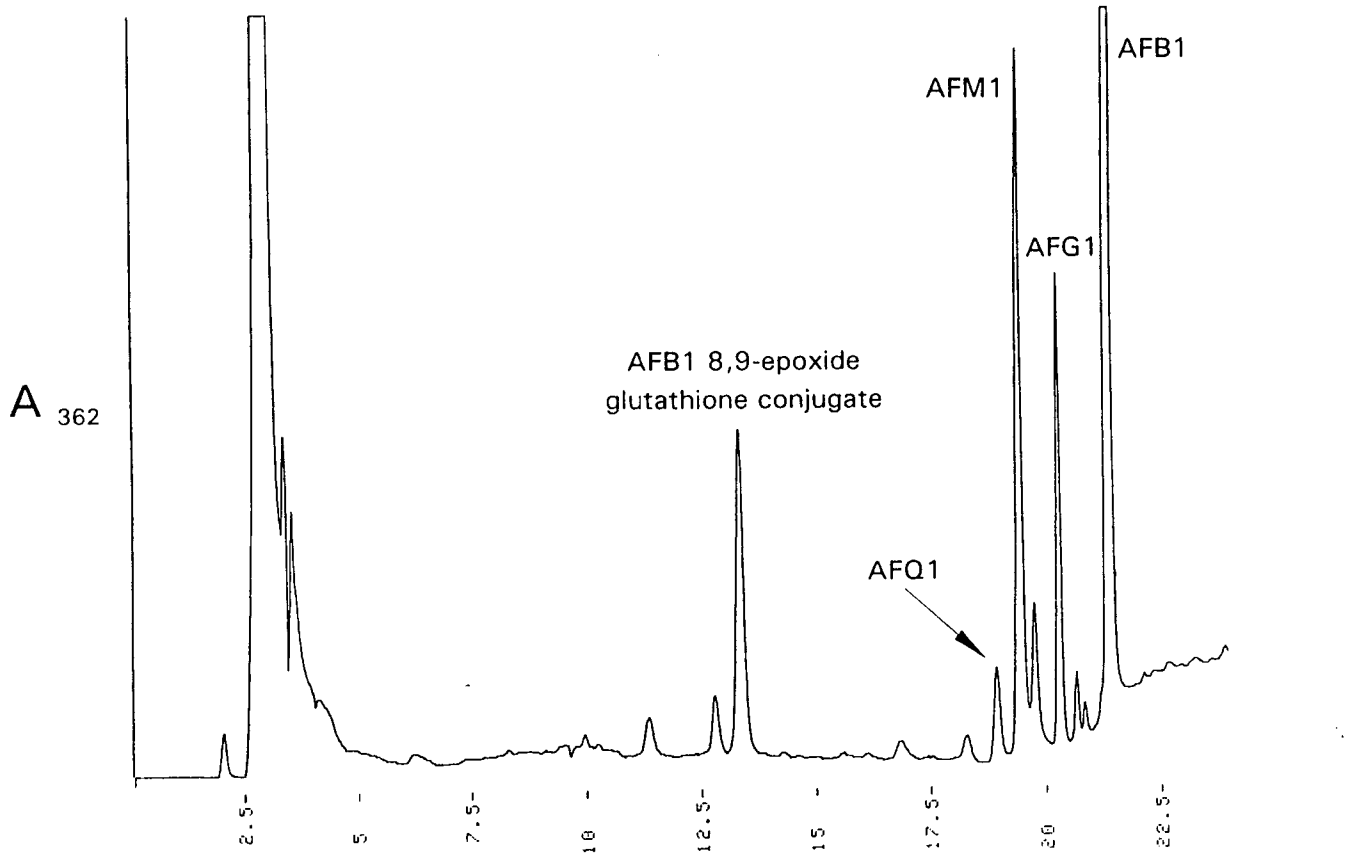

Figure 2.1. **Representative HPLC chromatogram showing separation of the three major AFB_1 oxidative metabolites, AFB_1 8,9-epoxide (trapped as the glutathione conjugate), AFM_1 , AFQ_1 and the internal standard AFG_1.** The incubation included 16 μM AFB_1 and hepatic microsomes from a rat fed the combined I3C and BNF diet.

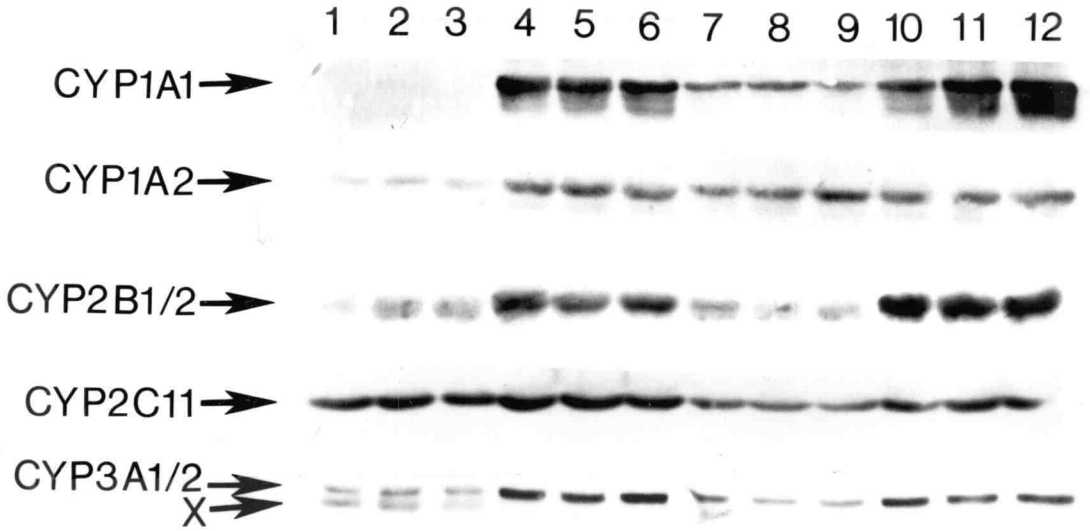

Figure 2.2. **Western blots of hepatic microsomal protein probed with antibodies against the enzymes indicated.** The band marked by "X" is an unknown protein of lower molecular weight than CYP3A1/2. Lanes assignments and the diets fed were as follows: 1-3, semi-purified diet; 4-6, 0.2% I3C; 7-9, 0.04% BNF, 10-12, 0.2% I3C + 0.04% BNF. Each lane represents 10 μg (blots for CYP1A2, 2B1/2 and 2C11) or 15 μg (blots for CYP1A1, 3A1/2) microsomal protein.

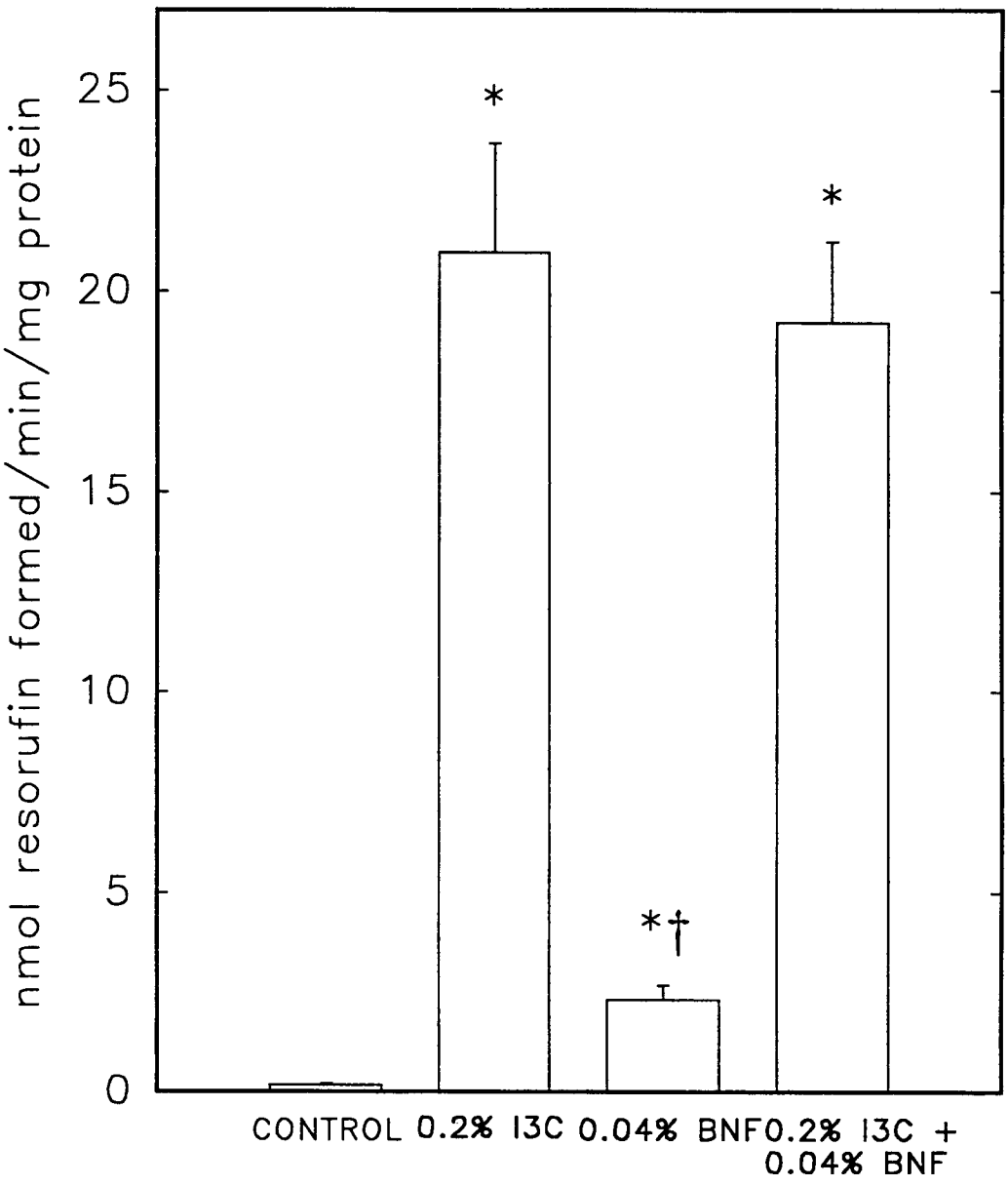

Figure 2.3. **EROD activity associated with hepatic microsomal preparations from I3C and BNF treated rats.** Activities were determined for the four groups at saturating substrate concentrations (5 μM). Error bars represent the standard error of the mean (n = 3 rats per group). * = significantly different from control at $P < 0.01$. † = significantly different from the I3C group and the combined I3C and BNF group at $P < 0.05$.

Figure 2.4. **Effect of dietary I3C and BNF, alone or in combination, on the *in vitro* hepatic microsome-mediated production of AFB_1 8,9-epoxide, AFQ_1 and AFM_1.** AFB_1 8,9-epoxide was trapped as the GSH conjugate, a reaction mediated by highly efficient GST isoforms presence in hepatic cytosol that was added to the reaction. This method of 8,9-epoxide quantification has been reported be >99% efficient (29). Metabolites were resolved by HPLC, monitored at 362 nm and quantified by integration of peak areas. Error bars represent the standard error of the mean (n = 3 rats). Duplicate or triplicate determinations were made for each rat. Table 2.2 gives the statistical significance of these data.

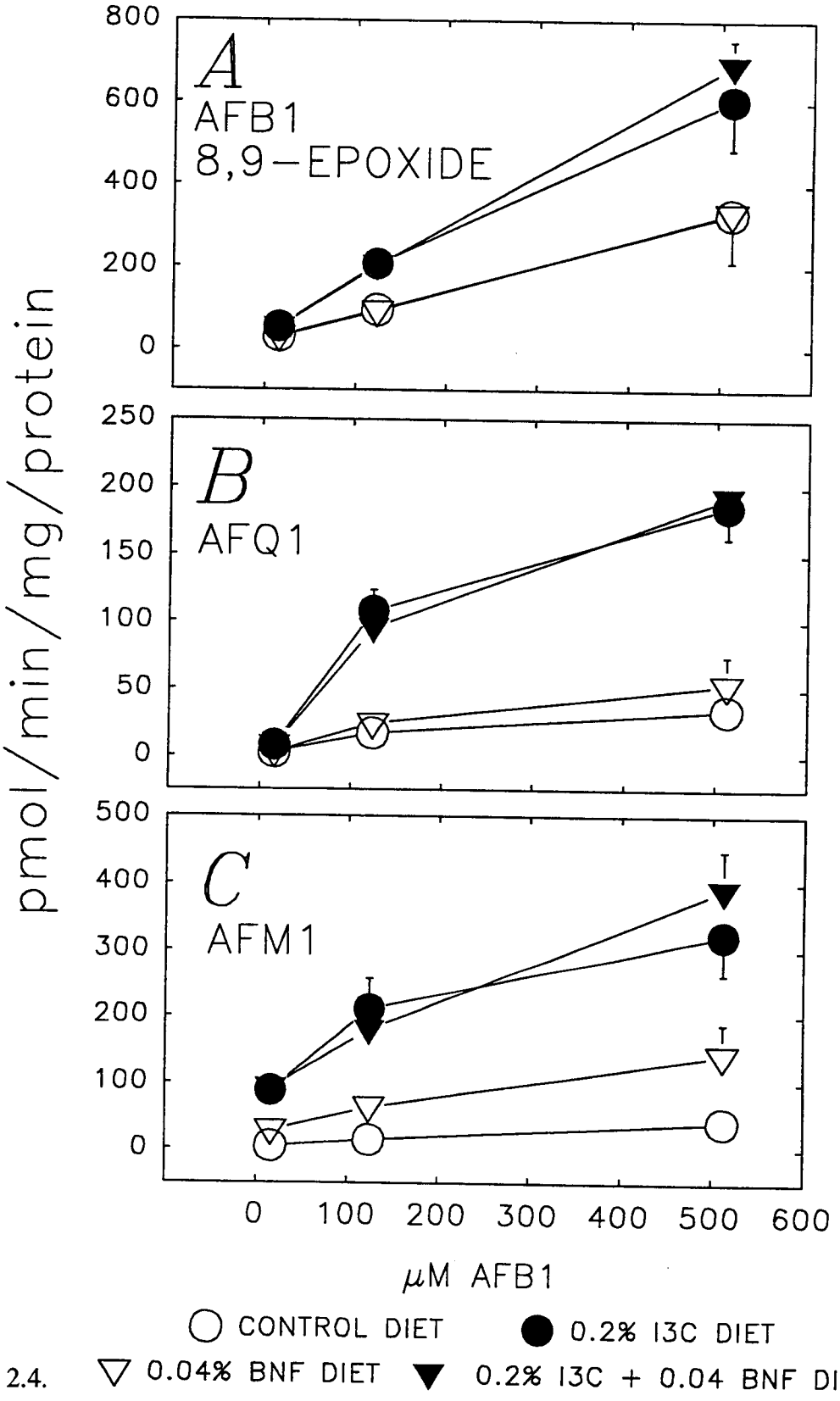
A
AFB1
8,9-EPOXIDE
B
AFQ1
C
AFM1
pmol/min/mg/protein
800
600
400
200
0
250
200
150
100
50
0
500
400
300
200
100
0
0
100
200
300
400
500
600
μM AFB1
CONTROL DIET
0.2% I3C DIET
0.04% BNF DIET
0.2% I3C + 0.04 BNF DIET

Figure 2.4.

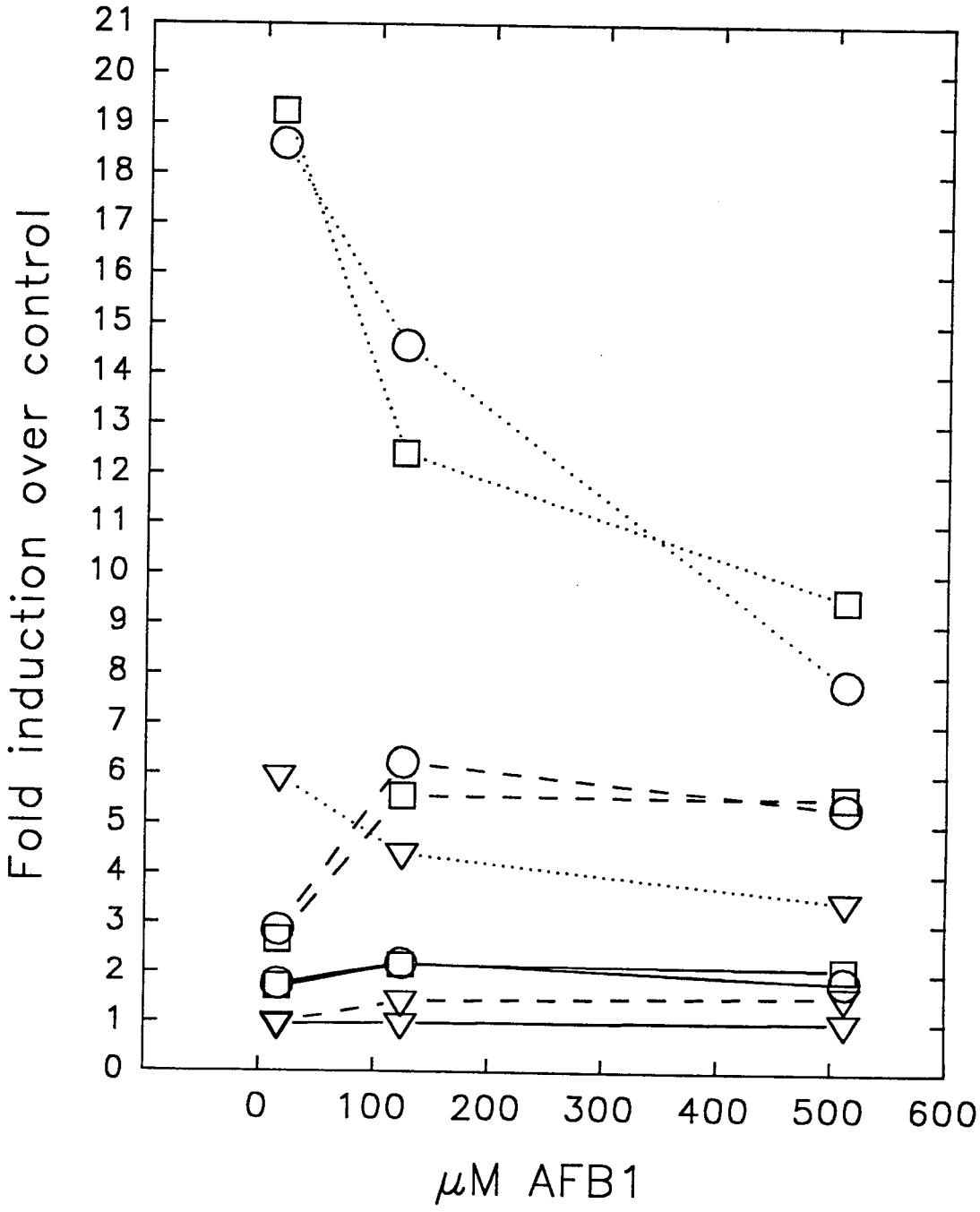

Figure 2.5. **Fold induction of metabolite over control at three AFB_1 substrate levels.** ▽ = BNF diet; O = I3C diet; □ = combination diet. ····· = AFM_1 formation; ------ = AFQ_1 formation; ____ = AFB_1 8,9-epoxide formation.

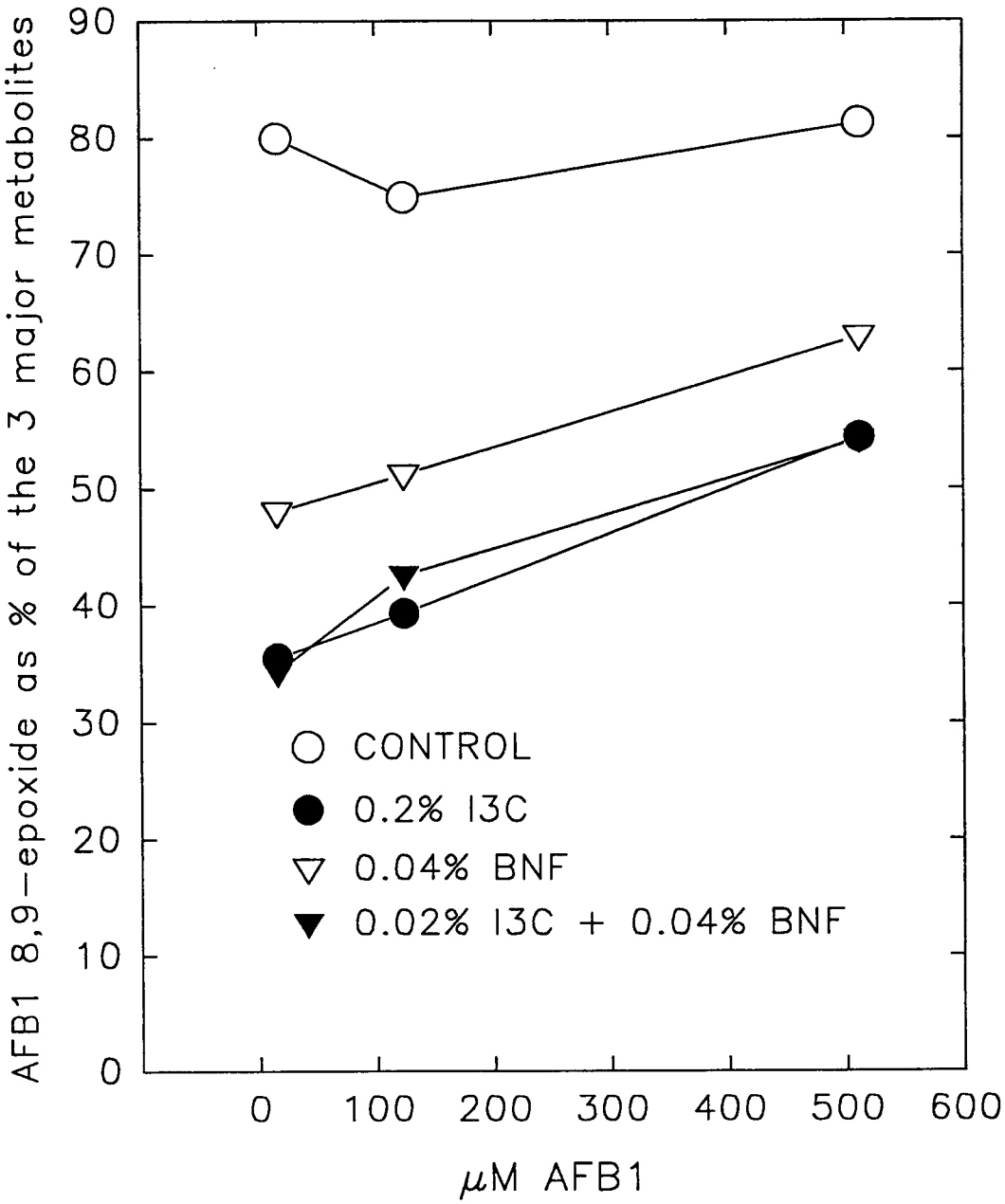

Figure 2.6. **Effect of experimental diets and initial AFB_1 substrate concentration on initial rates of microsomal AFB_1 8,9-epoxide formation expressed as percent of the three major AFB_1 oxidative metabolites, AFB_1 8,9-epoxide, AFM_1 and AFQ_1.**

Table 2.1. Relative band density analysis of western blots of microsomal CYP1A1, 1A2, 2B1/2, 2C11 and 3A1/2

Diets:	Control	0.2% I3C		0.04% BNF		0.2% I3C + 0.04% BNF	
	Mean band Density [a]	Mean band Density	fold increase	Mean band Density	fold increase	Mean band Density	fold increase
CYP1A1	0.31 ± 0.04	7.43 ± 0.70	24 [b]	3.86 ± 0.62	12 [b]	5.84 ± 0.14	19 [b]
CYP1A2	3.17 ± 0.27	9.76 ± 0.65	3.1 [b]	8.54 ± 1.56	2.7 [b]	8.69 ± 1.10	2.7 [b]
CYP2B1/2	10.1 ± 0.63	17.0 ± 1.94	1.7 [b]	7.91 ± 0.55	0.78	18.2 ± 1.28	1.8 [b]
CYP2C11	8.19 ± 0.30	10.2 ± 1.35	1.2	4.10 ± 0.27	0.50 [b]	7.13 ± 0.32	0.87 [c]
CYP3A1/2	1.81 ± 0.23	6.94 ± 0.57	3.8 [b]	2.53 ± 0.18	1.4	5.09 ± 0.59	2.8 [b]

[a] values are means ± SE of three animals (arbitrary units).

[b] significantly different from controls at $P < 0.01$.

[c] significantly different from group fed 0.2% I3C alone and group fed 0.04% BNF alone at $P < 0.05$.

Table 2.2. Statistical analysis of AFB1 microsomal metabolism

		AFB1 8,9-epoxide		AFQ1		AFM1	
AFB1 concentration	Group	ANOVA P value[a]	Homogeneous groups[b]	ANOVA P value	Homogeneous groups	ANOVA P value	Homogeneous groups
16 μM	control	0.0401	*	<0.0001	*	0.0002	*
	0.2% I3C		*		*		*
	0.04% BNF		*		*		*
	0.2% I3C + 0.04% BNF		*		*		*
124 μM	control	0.0106	*	0.0003	*	<0.0001	*
	0.2% I3C		*		*		*
	0.04% BNF		*		*		*
	0.2% I3C + 0.04% BNF		*		*		*
512 μM	control	0.0487	*	0.0002	*	0.0006	*
	0.2% I3C		* *		*		*
	0.04% BNF		*		*		*
	0.2% I3C + 0.04% BNF		*		*		*

[a] P values determined by ANOVA on the original or log-transformed data. This test shows that differences exist among the means, but does not identify them.

[b] Homogeneous groups are identified by vertical alignment of the asterisks within each concentration grouping. Significance was determined at alpha = 0.05 with the least significant difference test.

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Chapter 3

INDOLE-3-CARBINOL INDUCES A RAT LIVER GLUTATHIONE *S*-TRANSFERASE SUBUNIT (YC2) WITH HIGH ACTIVITY TOWARD AFLATOXIN B_1 *EXO*-EPOXIDE: ASSOCIATION WITH REDUCED LEVELS OF HEPATIC AFLATOXIN-DNA ADDUCTS *IN VIVO*.

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ABSTRACT

Aflatoxin B_1 (AFB_1), a metabolite of the grain mold *Aspergillus flavus*, is a potent hepatocarcinogen and widespread contaminant of human food supplies. AFB_1-induced tumors or preneoplastic lesions in experimental animals can be inhibited by co-treatment with several compounds, including indole-3-carbinol (I3C), a component of cruciferous vegetables, and the well known Ah receptor agonist ß-naphthoflavone (BNF). This report examines the influence of these two agents on the AFB_1-glutathione detoxication pathway and AFB_1-DNA adduction in rat liver. After 7 days of feeding approximately equally inhibitory doses of I3C (0.2%) or BNF (0.04%) alone or in combination, male Fischer 344 rats were administered [^{3}H]AFB_1 (0.5 mg/kg, 480 µCi/kg) intraperitoneally and killed 2 h later. All three experimental diets inhibited *in vivo* AFB_1-DNA adduction (BNF, 46%; I3C, 68%; combined, 51%). Based on western blots using antibodies specific for the glutathione *S*-transferase (GST) subunit Yc2 (subunit 10) appeared to be substantially elevated by the diets containing I3C (I3C diet, 4.0 fold increase in band density; combined diet, 2.8 fold). The BNF diet appeared to elevate Yc2 to a lesser extent (2.2 fold increase in band density). This isozyme has previously been shown to possess unusually high activity over other rat GSTs towards the genotoxic metabolic intermediate, AFB_1 *exo*-epoxide. An improved HPLC method for separation of the two isomeric forms of AFB_1 8,9-epoxide-glutathione conjugate was developed, using a chiral analytical column. Using this method, both I3C diets were shown to strongly induce GST activities towards AFB_1 *exo*-epoxide (I3C diet, 4.9 fold; combined diet, 4.0 fold), whereas BNF alone induced activity weakly (1.7 fold). A similar overall pattern of changes appeared for AFB_1 *endo*-epoxide (a lesser occurring metabolite lacking in genotoxicity) conjugation, but no elevation was observed with the BNF diet (BNF

diet, 0.8 fold; I3C diet, 2.4; combined diet, 2.6 fold). A good correlation was observed between Yc2 band density and cytosolic GST activity towards AFB_1 *exo*-epoxide (R = 0.88), but not AFB_1 *endo*-epoxide (R = 0.55) indicating Yc2 specifity for the *exo* isomer. The data suggest that enhanced detoxication of AFB_1, via increased glutathione conjugation efficiency as a result of elevated levels of the Yc2 GST subunit is one mechanism that contributes to the protective effect of I3C against AFB_1-induced preneoplastic lesions in the rat, and that this mechanism also participates to a lesser degree in protection by BNF.

INTRODUCTION

Aflatoxin B_1 (AFB_1)[2], a metabolite of the grain mold *Aspergillus flavus*, is a widespread contaminant of human food supplies (1). The hepatotoxic and hepatocarcinogenic effects of AFB_1 in several experimental animal species, as well as humans, have been intensely studied. In animals, AFB_1-induced toxicity and carcinogenicity can be altered by feeding diets containing various natural or synthetic compounds including ethoxyquin, butylated hydroxytoluene, butylated hydroxyanisole, ß-naphthoflavone (BNF), oltipraz, and γ- or α-hexachlorocyclohexane (summarized in 2). The mechanism(s) by which these compounds confer resistance to AFB_1-induced carcinogenesis is thought to be related to their ability to alter expression of phase I and/or phase II drug metabolizing enzymes (3), thereby enhancing the rate of metabolism towards non-toxic products (reviewed in 4-6).

Indole-3-carbinol (I3C), a component of cruciferous vegetables, also induces phase I and phase II enzymes in some species and organs. Phase I enzymes induced by dietary I3C include CYP1A1, CYP1A2, CYP2B1/2 and CYP3A1/2 (7-10). Phase II enzymes induced by dietary I3C include NAD(P)H:quinone oxidoreductase (8, 11, 12), UDP-glucuronosyl transferase (8, 11), and glutathione *S*-transferase (GST) (8, 11, 13, 14). Although phase I and phase II induction by I3C is associated with protection against *in vivo* AFB_1-DNA adduction and AFB_1-induced preneoplastic lesions [3], protection can occur in the absence of induction (15).

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2 - **Abrreviations**: AFB_1, aflatoxin B_1; ß-naphthoflavone, BNF; I3C, Indole-3-carbinol; CYP, cytochrome P-450 (E.C.1.14.14.1); GST, glutathione *S*-transferase (E.C. 2.5.1.18); GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; DMSO, dimethylsulfoxide;

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Although several biotransformation enzymes are inducible by dietary I3C, those that are relevant to AFB_1 detoxication appear to be limited to specific members of the superfamilies of cytochromes P-450 (16) and GSTs. Recently, two GSTs from the class α (for nomenclature, please refer to 17 and 18) with high activity towards AFB_1 8,9-epoxide were purified from male Fischer rats fed diets containing ethoxyquin (19). The high AFB_1 8,9-epoxide conjugating ability of these GSTs was associated with subunit Yc2 (subunit 10), whose expression is repressed in mature animals (19, 20). The majority of AFB_1 *exo*-epoxide-GSH conjugating activity in hepatic cytosol in that study, and in a recent study employing 1,2-dithiole-3-thione as an inducing agent (21), was attributed to the Yc2 subunit.

In this report, we examined the influence of dietary I3C on hepatic AFB_1-DNA adduction *in vivo* two hours after ip administration of AFB_1, and its association with the induction of the GST Yc2 subunit previously suggested to protect against AFB_1-induced hepatocarcinogenesis in the rat (21). In addition, the effect of the synthetic flavonoid and well known Ah receptor agonist BNF (alone or in combination with I3C) is assessed.

MATERIALS AND METHODS

Chemicals

[^{3}H]AFB_1 (20 Ci/mmol) was obtained from Moravek Chemicals (Brea, CA). Unlabeled AFB_1 and BNF were purchased from Sigma Chemical Co. (St. Louis, MO). AFB_1 8,9-epoxide (a 10:1 mixture of the *exo-* and *endo-* forms respectively) (22) and AFB_1 *exo*-epoxide-GSH conjugate (23) were chemically synthesized as described. I3C was purchased from Aldrich Chemical Co. (Milwaukee, WI). 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Eastman Kodak Co. (Rochester, NY). Dimethyl sulfoxide (DMSO) was obtained from J.T. Baker (Phillipsburg, NJ). All other chemicals were obtained from Sigma.

Animals and Diets

Twelve 4-week old male Fischer rats were obtained from Simonsens (Gilroy, CA) and acclimated for seven days on AIN-76A powdered semipurified diet (formulated without preservatives)(US Biochemical Corp, Cleveland, OH). The animals were then fed the same diet containing 0.2% I3C or 0.04% BNF, (alone or in combination) for seven additional days. These levels, though differing in weight percentage, were chosen because they have been shown to be approximately equally effective at inhibiting AFB_1-induced preneoplastic lesions in male Fischer rats [4]. The following day, animals were injected ip with [^{3}H]AFB_1 (0.5 mg/kg, 480 μCi/kg) in 50-75 μl DMSO. The animals were killed by CO_2 asphyxiation 2 hours later (when adduct levels are maximum in liver

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from untreated rats (24, 25)) and their livers removed, blotted, weighed, frozen in liquid nitrogen and stored at -80° C until analysis.

Preparation of Liver Homogenates and Analysis of Radioactivity

Livers were homogenized in 4 volumes of ice cold 0.1 M potassium phosphate buffer (pH 7.4) containing 0.15 M potassium chloride, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. Homogenates (125 µl) were digested with NCS tissue solubilizer (Amersham Corp., Arlington Heights, IL), decolorized with 200 µl 30% H_2O_2, and analyzed for radioactivity by scintillation counting.

Preparation of Cytosol

Cytosol was prepared by differential centrifugation by the method of Guengerich (26). Liver homogenates were centrifuged at 10,000 g to obtain supernatant that was subjected to further centrifugation at 100,000 g. The resulting supernatant (cytosol) was stored at -80° C in 500 µl aliquots. Protein determinations on cytosol were made according to the method of Lowry *et al.*, (27).

In Vivo Hepatic [^{3}H]AFB$_1$-DNA Adduction

Hepatic DNA was isolated by phenol and chloroform extraction as described in detail by Dashwood *et al.*, (28). Preparations of DNA were checked for purity by measuring the ratio of UV absorbance at 260 and 280 nm. The method of Burton (29) was used to determine concentrations of DNA. Aliquots of the hydrolysates used for the Burton assay were analyzed for the presence of radioactivity by scintillation counting using ACS scintillation fluid (Amersham).

Enzyme Assays

Cytosolic GST activity towards CDNB was determined as described by Habig *et al.*, (30). Cytosolic activity towards AFB_1 8,9-epoxide (10:1 *exo:endo*) was determined by a modification of the method previously described by Raney *et al.*, (23). Briefly, test cytosol (3 mg/ml) was added to 0.1 M potassium phosphate buffer (pH 7.4) containing 0.15 M potassium chloride, 1 mM EDTA and 5 mM GSH. AFB_1 8,9-epoxide in 8 µl acetone was added while mixing gently so that the final concentration was 46 µM and 4.6 µM AFB_1 *exo-* and *endo*-epoxide, respectively, in a total volume of 250 µl. Acetic acid (50 µl of a 2 M solution) was added to the mixture after 15 seconds to precipitate the protein and the mixture was frozen at -20° C for at least 2 hours. Mixtures were then thawed and centrifuged to pellet the protein. The supernatant (typically 75 µl) was injected onto a Pirkle covalently linked D-phenylglycine analytical column (4.6 × 250 mm) (Regis Chemical Co., Morton Grove,IL) equipped with a C_{18} guard column (Alltech Associates, Deerfield,IL). The AFB_1 *exo-* and *endo*-epoxide GSH conjugates were resolved under isocratic conditions with a mobile phase of 40% methanol in 20 mM potassium phosphate buffer (pH 4.0) at a flow rate of 1 ml/min and monitored at 360 nm. The column temperature was held at 30°C. Typical retention times were 12.0 min, 13.6 min and 21.7 min for the *exo*-epoxide GSH conjugate, *endo*-epoxide GSH conjugate and AFB_1 8,9-dihydrodiol, respectively. Under the conditions of this assay, the production of AFB_1-GSH conjugates is protein dependent (at the extreme, zero protein controls show approximately 22% background *exo-* conjugate and 5% background *endo-* conjugate formation compared to uninduced, control rat cytosol) but, because of spontaneous substrate hydrolysis to the 8,9-dihydrodiol, this is not a time-linear assay (23). Figure 3.1 depicts a chromatogram showing the resolved AFB_1 *exo-* and *endo-*

epoxide glutathione conjugates. We were unable to achieve adequate separation of the conjugates using the HPLC conditions described by Raney *et al.* (23). We found that use of the chiral stationary phase greatly improved resolution and reduced analysis times. Metabolites were quantified with a Shimadzu Chromatopac integrator (Kyoto, Japan) using a AFB_1 8,9-dihydrodiol standard curve after correction for non-enzymatic conjugation.

Identification of AFB_1 8,9-epoxide GSH conjugates

The AFB_1 *exo*-epoxide GSH conjugate was identified by coelution with standards. AFB_1 *endo*-epoxide GSH conjugate was identified by a different approach. Using the method of Raney *et al.*, (23), we obtained two incompletely resolved peaks that had similar retention times to those ascribed to the two GSH conjugate isomers. These peaks were collected, concentrated using a C_{18} sep-pak cartridge (Waters Associates, Milford, MA) and injected onto the chiral phase Pirkle D-phenylglycine column under the conditions described above or a high efficiency, non-chiral Beckman Ultrasphere ODS (4.6×250 mm) analytical column (San Ramon, CA). Baseline resolution of the two peaks was observed with the Pirkle column, whereas a single peak with a retention time of 14.8 min was observed with the Ultrasphere column using a linear gradient over 15 min of 20% to 40% methanol in 20 mM potassium phosphate, pH 4.0, indicating a complete lack of chiral interaction between the analytes and the stationary phase as expected. The identity of the AFB_1 *endo*-epoxide GSH conjugate was deduced from the following: 1) chromatography relative to the *exo*-epoxide GSH conjugate on the Raney *et al.* (23) system was as expected, 2) the peak was dependent on the inclusion of AFB_1

8,9-epoxide and GSH in the incubation, and 3) the yield of this peak substantially increased in the presence of liver cytosol.

SDS-PAGE and Immunoblotting

Cytosolic proteins were separated on the basis of size by SDS-PAGE (31). Cytosolic protein containing GSTs was electrophoretically transferred to nitrocellulose as described (32), probed with antibodies raised toward mouse Yc (thought to represent the murine ortholog of rat Yc2) and detected immunochemically using a Bio-Rad (Richmond, CA) goat-anti-rabbit-horseradish peroxidase conjugate immunoblot assay kit according to the manufacturers instructions. The antibodies react only with the Yc2 subunit in the rat and their preparation by affinity chromatography is described by McLellan *et al.*, (33). The densities of the individual bands were determined using a Zeineh SL laser densitometer (Biomed Instruments, Fullerton, CA). However, linearity of response against purified Yc2 subunit was not established in these experiments, and our data do not provide quantitative information as changes in Yc2 protein, only in changes in band densities.

Statistics

Differences between groups were determined using one-way analysis of variance on the original or log transformed data followed by least significant difference multiple comparison test using the statistical software package, Statgraphics (version 5.0), (Statistical Graphics Corporation, Princeton, New Jersey). The decision whether to use log transformed data in the test was based on visual examination of residuals plots. A probability value of less than 0.05 was considered statistically significant.

RESULTS

All three experimental diets were found to significantly inhibit *in vivo* AFB_1 adduction to liver DNA (Fig. 3.2). The diet containing 0.2% I3C inhibited adduction to the greatest extent (32% of control) while diet containing 0.04% BNF inhibited the least (54% of control). Combining I3C and BNF at the same doses produced intermediate inhibition (43% of control). No significant differences between the treated groups were observed. The extent of AFB_1-derived radioactivity present in liver homogenates at the time of sacrifice was also reduced by all three diets (Fig. 3.3).

Two assays were used to examine induction of GST activity in hepatic cytosol. Figure 3.4 shows conjugation activity towards CDNB, a substrate of several GST isoforms. Only diets containing I3C alone or in combination with BNF significantly induced activity above control levels. The combined diet showed significantly less activity than the I3C diet. In a second assay, using the more relevant activated form of AFB_1, AFB_1 8,9-epoxide (10:1 mixture of the *exo-* and *endo-* forms, respectively), significant increases in conjugating activity towards AFB_1 *exo*-epoxide were observed with all three experimental diets (Fig. 3.5a), with diets containing I3C inducing up to 4.9 fold. The overall pattern of induction appears to be similar with AFB_1 *endo*-epoxide as substrate (Fig. 3.5b), though no induction of activity with the BNF diet was detected. Note that the activity towards AFB_1 *endo*-epoxide was approximately twice that of AFB_1 *exo*-epoxide despite the 10 fold lesser amount of substrate, consistent with the observations of Raney *et al.* (23).

Figure 3.6 shows a western blot probed with antibodies to the mouse Yc subunit after cytosolic protein had been separated by SDS-PAGE and transferred to nitrocellulose paper. These antibodies are specific for the Yc2 subunit in the rat (33). All experimental diets increased band densities (BNF diet, 2.2 fold, I3C diet, 4.0 fold; combined diet, 2.8

fold) which we interpret to reflect an increase in Yc2 specific content (Fig. 3.7). However, since immunoblot assays are not always linear with applied protein, our analyses are of changes in band densities per unit applied protein, not in absolute amounts of Yc2. As an indication of the specificity of Yc2 towards AFB_1 *exo*-epoxide, densities of the bands were shown to correlate well with AFB_1 *exo*-epoxide-GSH conjugating activity ($R = 0.88$, $p = 0.00016$), but not AFB_1 *endo*-epoxide-GSH conjugating activity ($R = 0.55$, $p = 0.06315$) (Fig. 3.8). Expression of the Yc2 subunit is developmentally regulated and is repressed in adult rats (19, 20). The presence of detectable Yc2 protein in our control animals could be explained in that these animals were 6 weeks old at the time of sacrifice and had not yet reached maturity.

DISCUSSION

Inhibition of In Vivo AFB_1-DNA Adduction

In this study we have demonstrated that diets containing 0.2% I3C and 0.04% BNF alone or in combination can inhibit *in vivo* hepatic AFB_1-DNA adduction when animals are killed 2 hours after ip injection of AFB_1. Both compounds are known to inhibit AFB_1-induced hepatocarcinogenesis or preneoplastic lesions in the male Fischer rat with approximately equal efficiency at or near these dietary levels (34) [4] and thus these observations are consistent with these inhibitors acting via anti-initiation mechanisms, as previously described for I3C and BNF in rainbow trout (35, 36). Previous studies by Salbe and Bjeldanes (37) using much lower I3C doses (0.025%) found a small, nonsignificant reduction in hepatic AFB_1-DNA binding in the rat. Lotlikar indicated that pretreatment of rats with BNF inhibited hepatic AFB_1-DNA adduction *in vivo*, though the levels of inhibition were not reported (38). In trout, Nixon *et al.*, showed a 45% and 55% reduction in hepatic AFB_1-DNA binding in animals fed 0.1% I3C or 0.05% BNF, respectively (39). So far as we know, these are the first studies of the combined effects of these two agents on AFB_1-DNA adduction. We currently have no explanation for the absence of an additive effects of combining the inhibitors in the present study. One interpretation might be that these inhibitors are acting via identical mechanisms and that inhibition of binding was maximal for each individually at the dose tested, thus, combining the inhibitors in the diet would show no additivity. However, the evidence reported here and summarized in the following sections, indicates that distinct mechanisms exist.

Mechanisms of Inhibition of DNA-Adduction

Compounds inhibiting carcinogenesis by preventing the carcinogenic agents from reaching or reacting with critical target sites have been classified according to Wattenberg as 'blocking agents' (4). Such compounds may operate by various mechanisms. These include (i) induction of biotransformation enzymes that could enhance excretion, (ii) inhibition of cytochrome(s) P-450 that can bioactivate procarcinogens and (iii) physico-chemical interaction with carcinogens (i.e. nucleophilic trapping of electrophiles or complexing). In this study, we have examined a mechanism within category (i), namely induction of a GST subunit with high activity towards the DNA-binding form of AFB_1, AFB_1 *exo*-epoxide (24). Efficient conjugation of AFB_1 *exo*-epoxide with GSH, mediated by GSTs, would be expected to reduce the formation of DNA adducts, as the AFB_1 8,9-epoxide-GSH conjugate is the major biliary metabolite of AFB_1 in rats (40) and GSH conjugation is presumably directly competing with DNA adduction. In general, the contribution of microsomal epoxide hydrolase, which may catalyze the hydrolysis of AFB_1 8,9-epoxide to the corresponding dihydrodiol, is not believed to be a significant detoxifying pathway in the liver (41). To our knowledge, the contribution of cytosolic epoxide hydrolase to the detoxication of AFB_1 8,9-epoxide has not yet been determined, and we do not exclude the possibility that I3C induction of this enzyme might also contribute to enhanced AFB_1 detoxication. However, numerous studies have revealed glutathione conjugation with the 8,9-epoxide to be a predominant mechanism in AFB_1 detoxication in hepatic tissue (42). Any increase in hepatic GSH content as a result of dietary exposure to I3C or BNF would not be expected to be a major factor in reduction of DNA adduction both because of the unusually low K_m values for GSH (approximately

0.1 mM) observed for alpha, mu and pi class GSTs (43), and the observation that the 8,9-epoxide is a poor substrate for non-enzymatic conjugation (present results, 23, 44).

The chemical induction of GST activity by other agents has been shown by several investigators to be associated with protection against AFB_1-DNA binding or AFB_1-induced tumors (45-48). However, the inducing effects of the chemicals were often measured by surrogate substrates (primarily CDNB) not specific for the inducible GST subunit (Yc2, subunit 10) that possesses higher activity toward AFB_1 8,9-epoxide over other GSTs by 1-2 orders of magnitude (19, 49). Thus, the small but significant induction of activity towards AFB_1 *exo*-epoxide shown here with BNF (1.7 fold) was not detected by measuring activity towards CDNB. The assay measuring activity towards AFB_1 *exo*-epoxide, along with immunodetection with antibodies that specifically recognize the rat Yc2 subunit, thus represent specific measures of anticarcinogen-mediated increases in an important AFB_1 detoxifying enzyme.

The total contribution of Yc2 to conjugating activity towards AFB_1 *exo*-epoxide has been estimated to account for 68% of the total in 1,2-dithiole-3-thione induced male Sprague-Dawley rats when induced approximately 4-fold, with other forms accounting for 13% or less on an individual basis (21). The specificity of Yc2 towards the *exo* isomer is supported here by the observation of an excellent correlation between immunoblot band density and AFB_1 *exo*-epoxide-GSH conjugating activity, but not AFB_1 *endo*-epoxide-GSH conjugating activity. The Yc2 subunit is apparently orthologous to the constitutively expressed mouse Yc subunit (50, 51), which is believed responsible for the observed dramatic resistance to AFB_1 hepatocarcinogenicity in the mouse relative to the rat (52). The data presented here thus suggest that the induction of the Yc2 subunit by I3C at these dietary levels should be a major contributor to protection

against AFB_1-induced hepatocarcinogenicity. However, our study does not formally rule out the possibility that an additional, as yet uncharacterized GST isozyme also induced by I3C, but not induced by ethoxyquin or 1,2-dithiole-3-thiones, may also contribute to AFB_1 epoxide conjugation.

The immunoblot data indicated that the BNF diet also gave a modest increase in Yc2 band density. This observation is in agreement with that of Parola *et al.* (53), who reported a 2.9 fold, nonsignificant, elevation in Yc2 levels in hepatocytes isolated from rats given BNF ip. These data, together with the BNF-mediated increases in AFB_1 8,9-*exo*-epoxide glutathione conjugating activity, suggest that increased Yc2 may also contribute to BNF anticarcinogenesis. This protection would occur in addition to BNF induction of aryl hydrocarbon hydroxylase (CYP1A)(34), which is associated with the formation of aflatoxin M_1, the much less toxic 9a-hydroxy metabolite of AFB_1. Although the I3C- and BNF-induced GST activity towards AFB_1 *exo*-epoxide measured *in vitro* strongly suggests this would apply *in vivo*, actual enhanced biliary excretion of the AFB_1 *exo*-epoxide-GSH conjugate has yet to be shown.

The increase in activity towards AFB_1 *endo*-epoxide upon dietary treatment with I3C is indicative of an increase in the μ class of GSTs (23), specifically subunits Yb2 (subunit 4) and/or Yn1 (subunit 6). This activity is probably less important in detoxifying AFB_1, since the *endo*-epoxide is formed in much lesser amounts by both rat and human liver microsomes (54) and possesses much lower mutagenicity relative to the *exo* isomer [5]. The HPLC method described here, which allows a more consistent and rapid quantification of the two AFB_1 8,9-epoxide-GSH conjugates, should be useful in

[5]T. Harris *et al*, unpublished results

quantifying the production of these two metabolites (i.e. AFB_1 *exo*- and *endo*-epoxide) by various species.

These results indicate that induction of the GST Yc2 subunit can be a significant mechanism for protection against AFB_1-induced tumors in some species. However, other properties of I3C, such as its CYP inducing activity (7-10), CYP inhibitory activity (15, 55), anti-oxidant capabilities (56) and inhibitory effects on steroid-hormone-binding GSTs (14) may also contribute to the overall anticarcinogenic mechanism against AFB_1 or other carcinogens. A human ortholog to the rat Yc2, possessing similar catalytic activity toward AFB_1 *exo*-epoxide, is not known to exist. The discovery of such a GST, if found to be responsive to induction by chemopreventive agents as are other α class GSTs in primary human hepatocyte cultures (57), would be of major significance since hepatic cytosol from humans reportedly possesses an extremely limited capacity to conjugate AFB_1 *exo*-epoxide (23).

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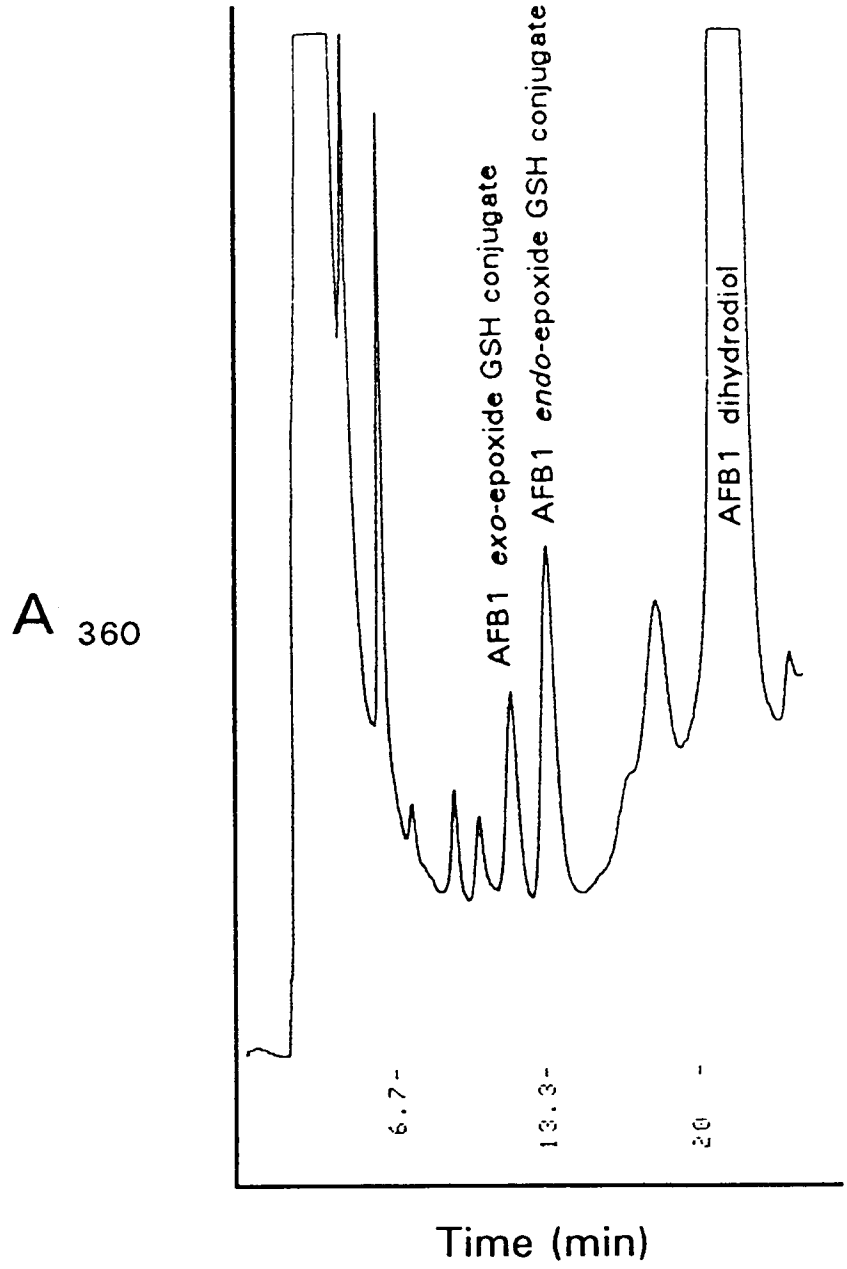

Figure 3.1. **HPLC chromatogram showing separation of AFB_1 *exo*- and *endo*-epoxide GSH conjugates.** Conjugates were formed by addition of a 10:1 mixture of AFB_1 *exo*- and *endo*-epoxide in the presence of GSH and hepatic cytosol from a rat fed 0.2% I3C and 0.04% BNF for 7 days. Separation of the conjugates was achieved using a chiral stationary phase (covalently linked D-phenylglycine) and a mobile phase of 40% methanol in 20 mM potassium phosphate (pH 4.0) at a flow rate of 1 ml/min. Products were monitored by UV absorbance at 360 nm. The full scale absorbance was 0.0025.

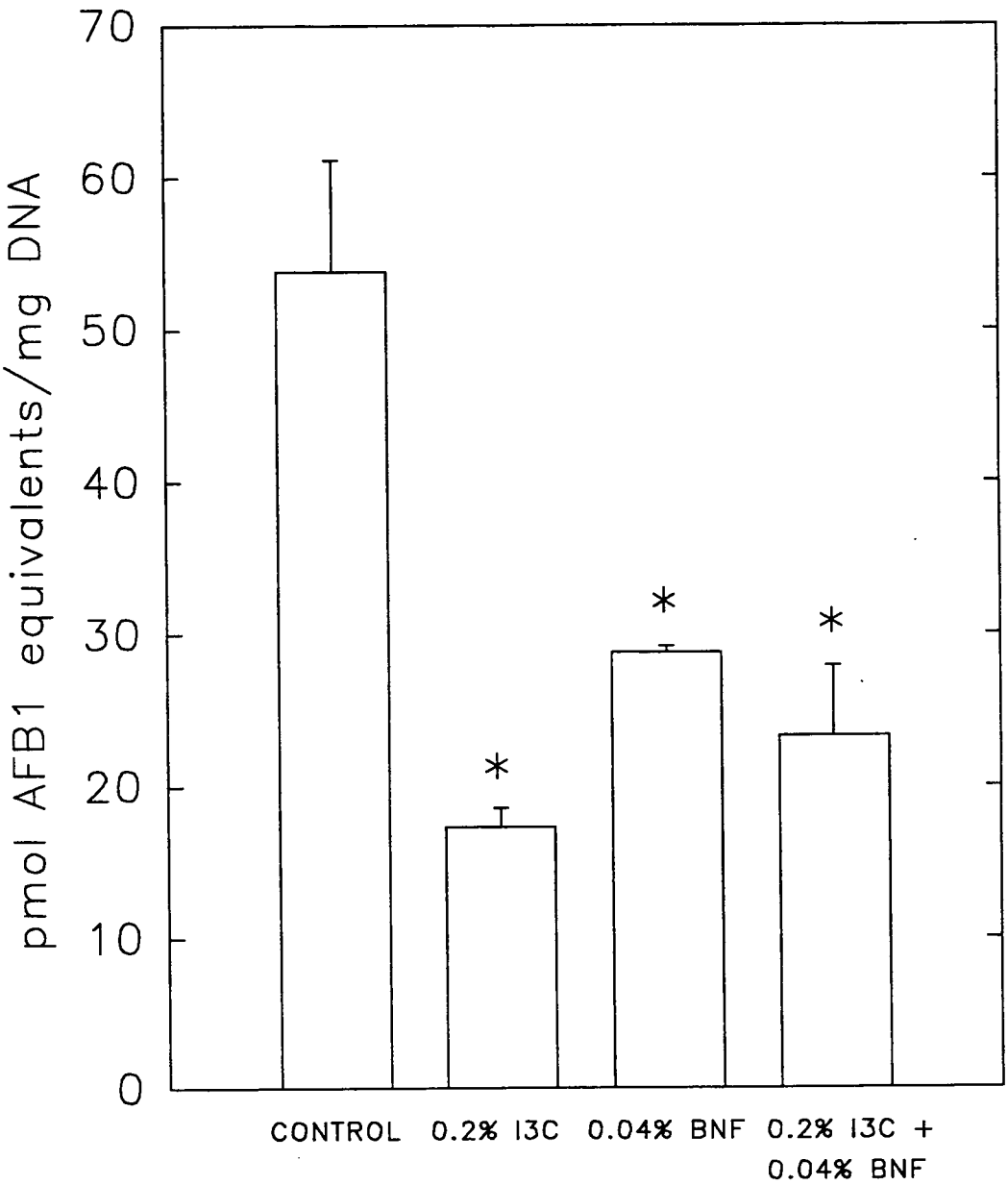

Figure 3.2. **The effect of feeding experimental diets containing 0.2% I3C or 0.04% BNF, alone or in combination, on *in vivo* hepatic [^{3}H]-AFB$_1$-DNA adduction.** Error bars represent the standard error of the mean (n = 3 rats) except for the I3C diet where the data are expressed as the mean and range of two samples. * = significantly different from control, $p < 0.05$.

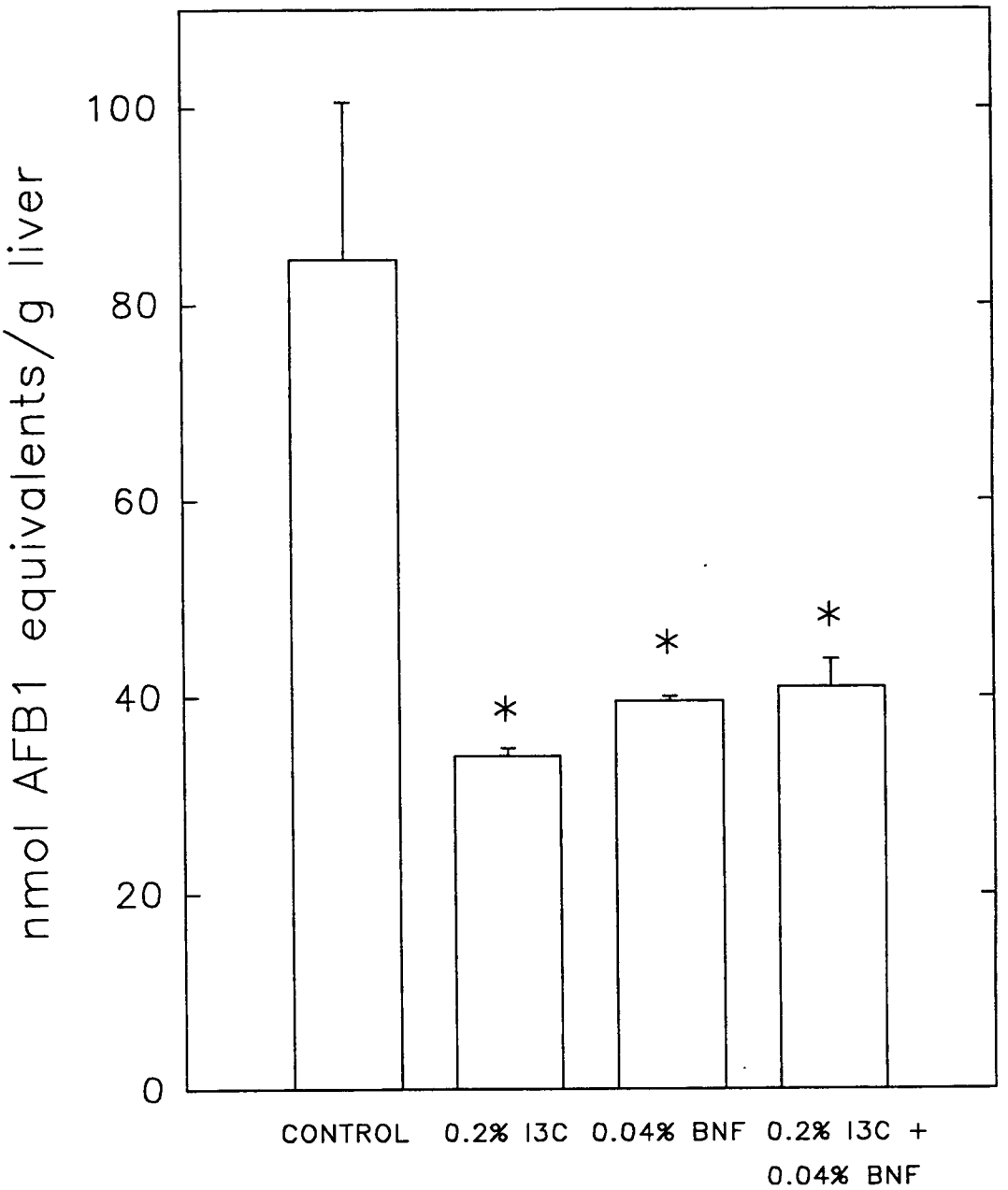

Figure 3.3. **Radioactivity in liver homogenate 2 hr after ip injection of [^{3}H]-AFB$_1$ (0.5 mg/kg, 480 μCi/kg) to rats fed control or 0.2% I3C and 0.04% BNF, alone or in combination, for 7 days.** Error bars represent the standard error of the mean (n = 3 rats). * = significantly different from control, $p < 0.05$.

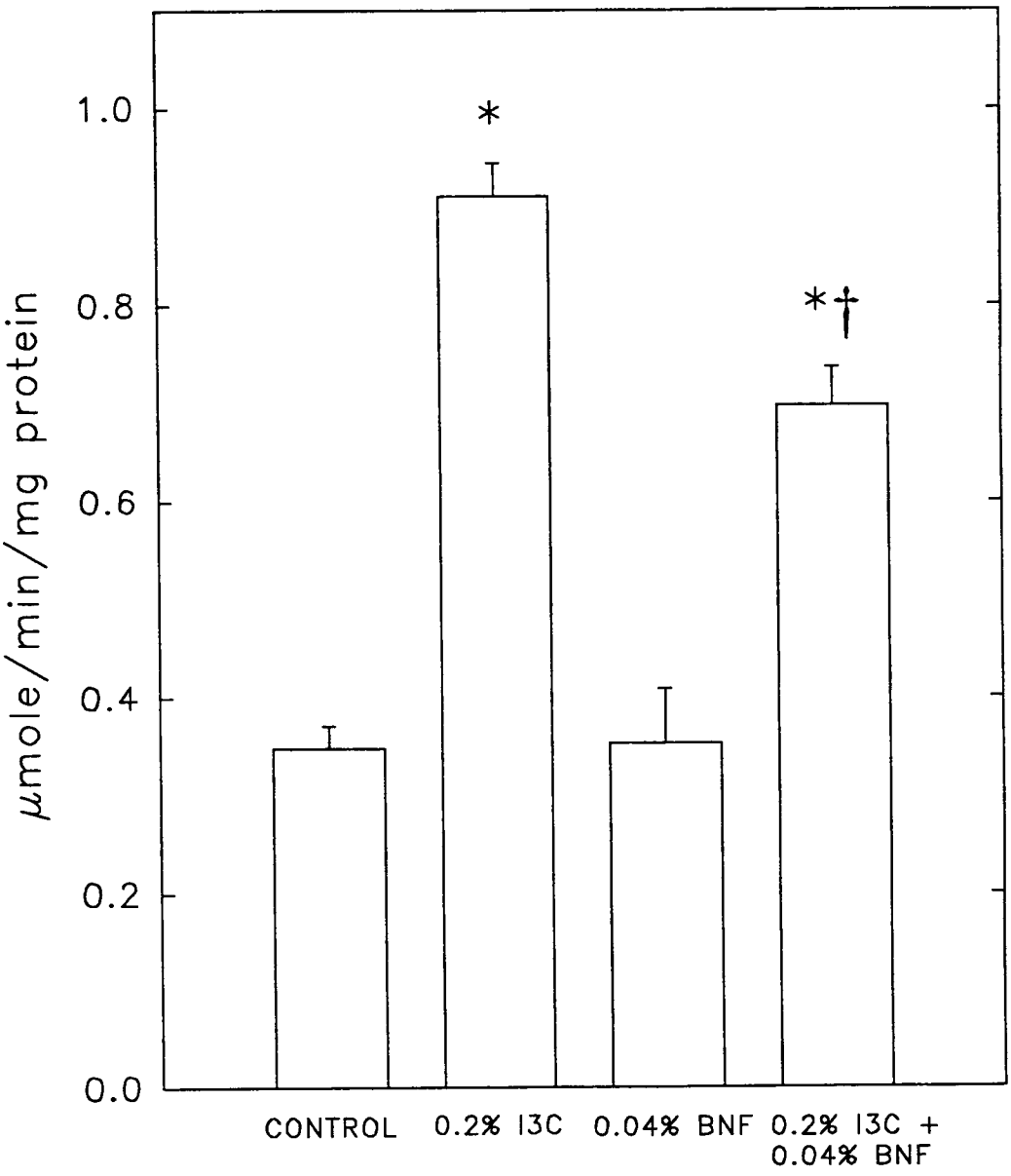

Figure 3.4. **GST activity toward CDNB in cytosol of rats fed control or 0.2% I3C and 0.04% BNF alone or in combination for 7 days.** The final concentration of protein in the assay was 12.5 μg/ml. Error bars represent the standard error of the mean (n = 3 rats). * = significantly different from control, $p < 0.05$; † = significantly different from the 0.2% I3C group, $p < 0.05$.

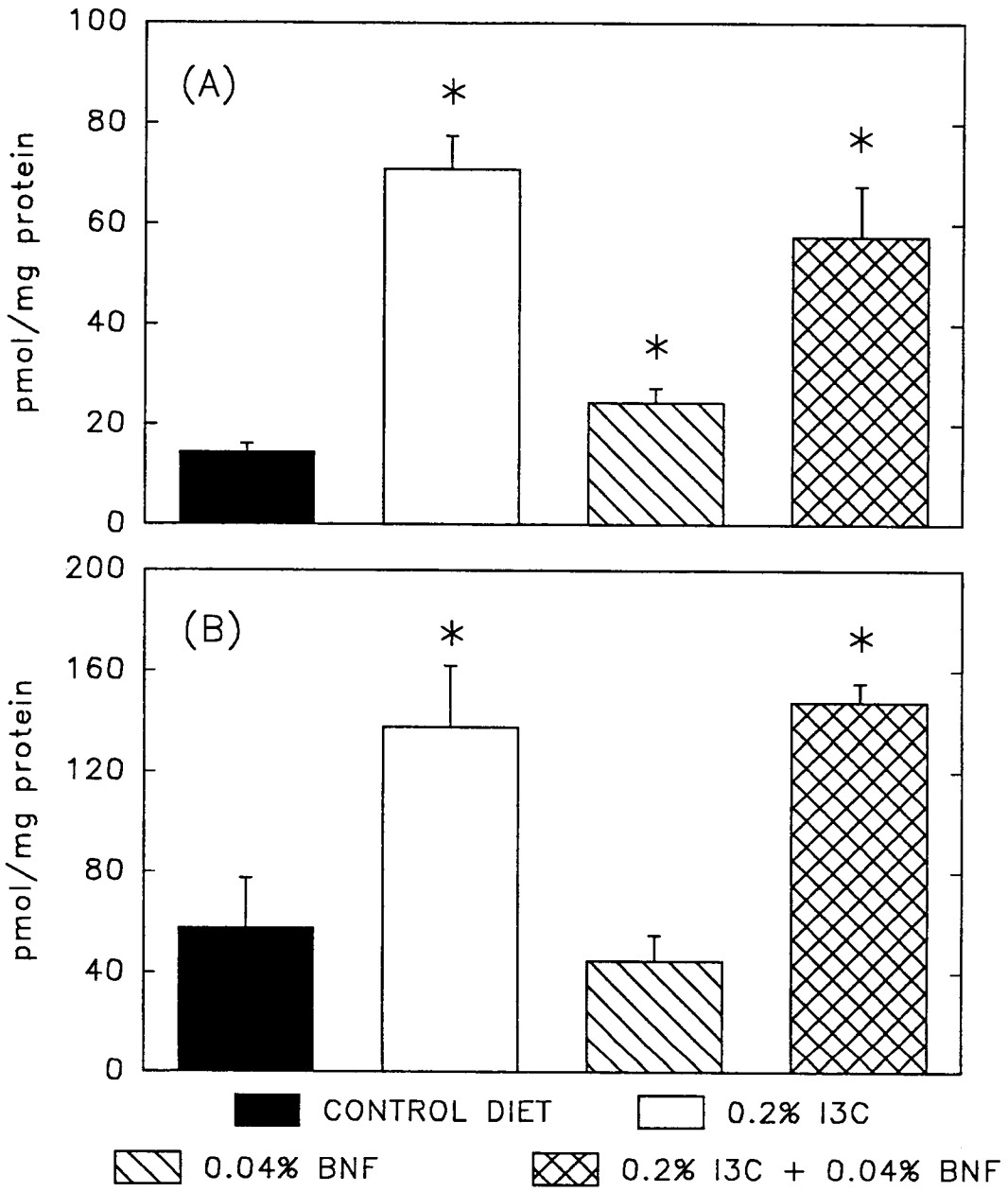

Figure 3.5. **Cytosolic GST mediated GSH conjugating activity towards AFB_1 *exo*-epoxide (A) or AFB_1 *endo*-epoxide (B) from rats fed control or 0.2% I3C or 0.04% BNF, alone or in combination, for 7 days.** Activities were determined simultaneously using a 10:1 mixture of the *exo*- and *endo*- forms respectively as substrates. The final concentration of cytosolic protein in the assay was 3 mg/ml. Error bars represent the standard error of the mean (n = 3 rats). * = significantly different from control, $p < 0.05$.

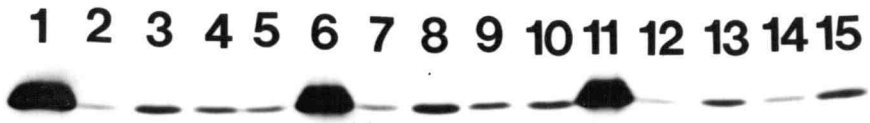

Figure 3.6. **The effect of dietary I3C and BNF on hepatic concentrations of the GST subunit Yc2.** Levels of the Yc2 GST subunit in the livers of male Fischer rats that had been fed experimental diets were examined by western blotting with affinity-purified antisera raised to the mouse Yc subunit. Cytosolic protein (30 μg) from the treated rats was applied to SDS-PAGE gels as follows: lanes 2, 7 and 12, hepatic cytosol from rats fed control diets; lanes 3, 8 and 13, hepatic cytosol from rats fed I3C; lanes 4, 9 and 14, hepatic cytosol from rats fed BNF-containing diets; lanes 5, 10 and 15, hepatic cytosol from rats fed I3C- plus BNF-containing diets. Lanes 1, 6 and 11 contained purified mouse Yc (1 μg) as standard. After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose paper and probed for cross-reactivity with the antisera.

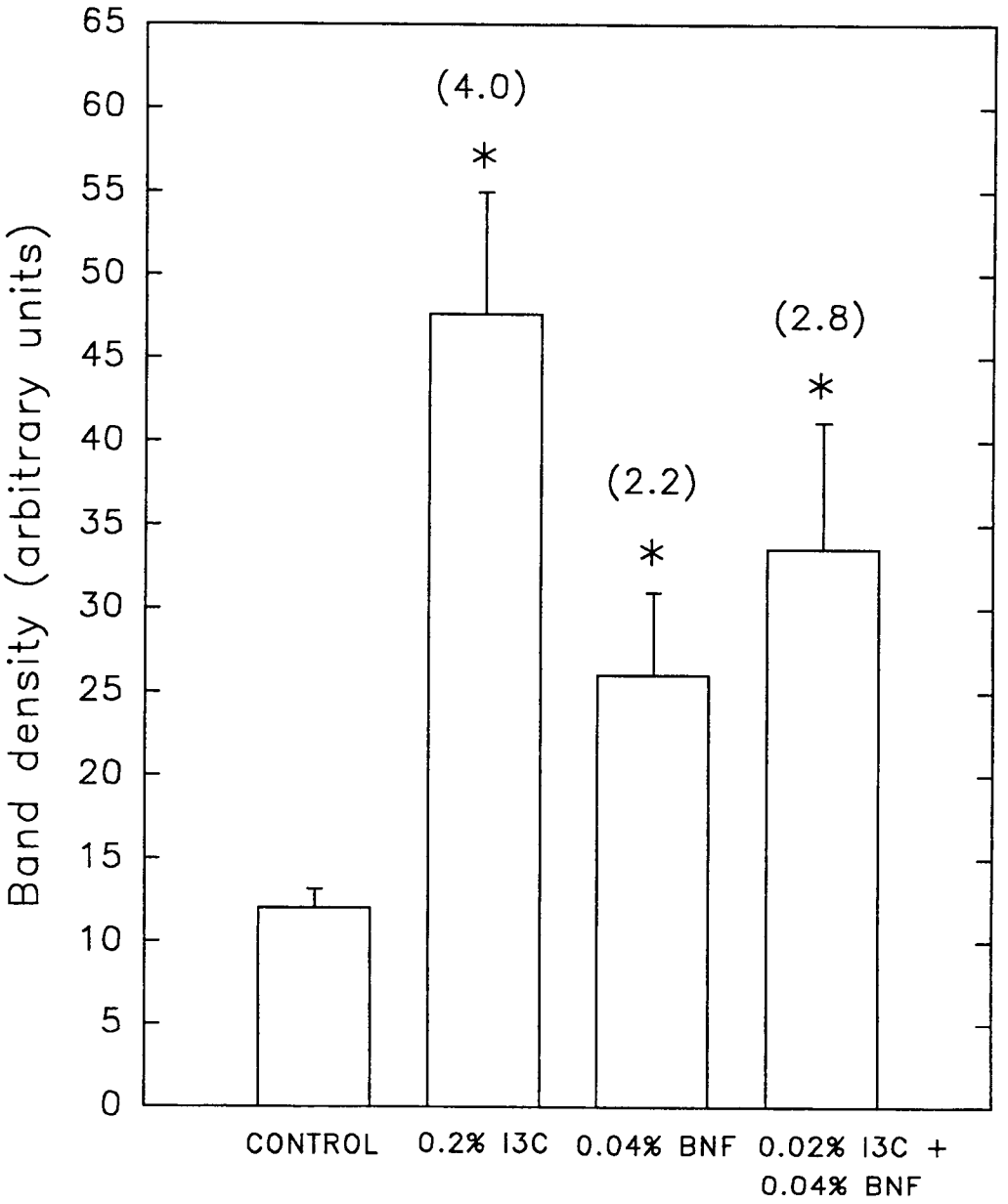

Figure 3.7. **Band densities of the western blot shown in Fig. 3.6 as determined by laser densitometry.** Error bars represent the standard error of the mean (n = 3 rats). Values in () indicate fold increase in band density relative to control (* = significantly different from control, $p < 0.05$). The true magnitude of Yc2 induction is not certain, as immunochemical blots do not always exhibit a linear response.

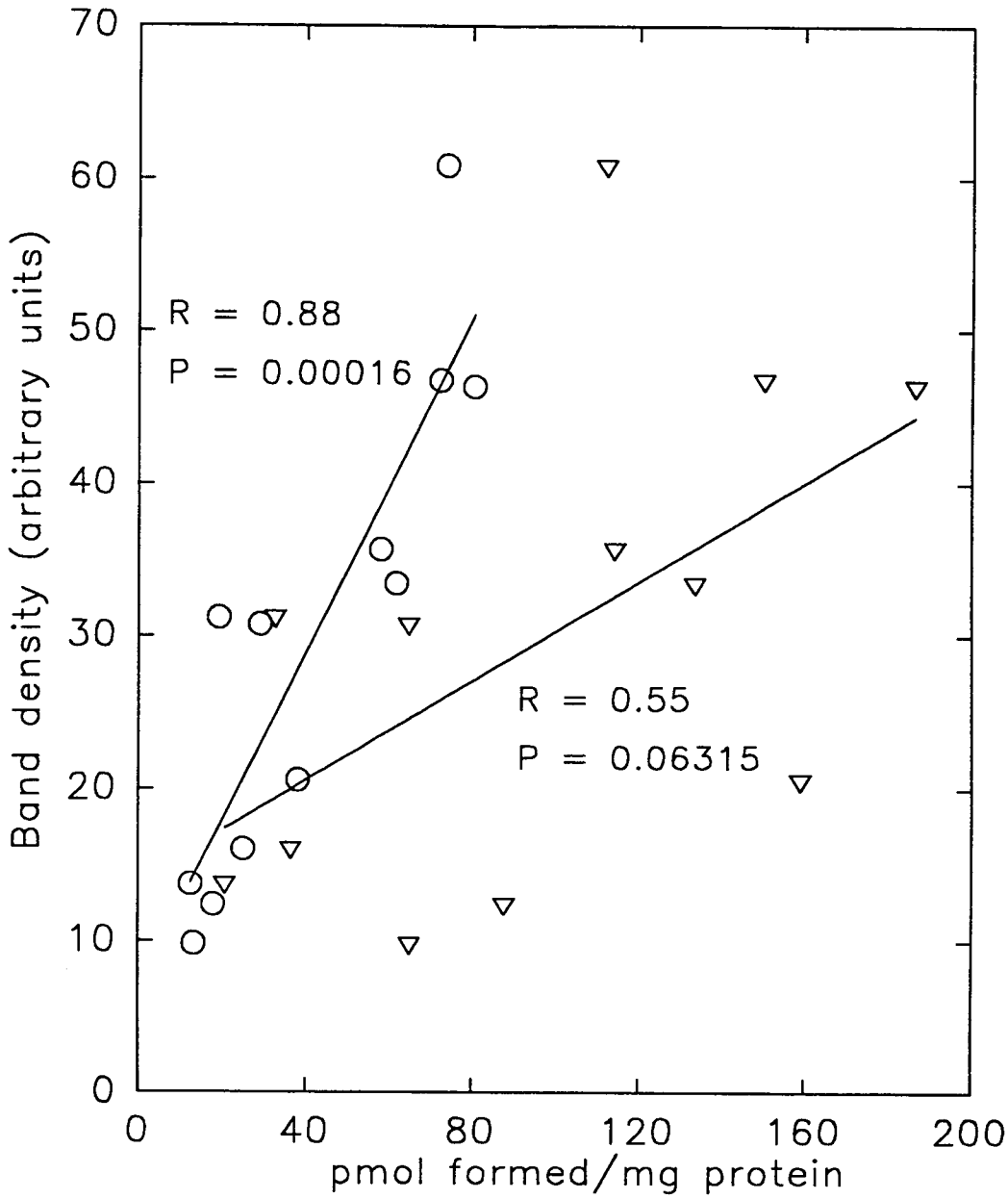

Figure 3.8. **Correlation of cytosolic GST activity towards AFB_1 *exo*-epoxide (O) or AFB_1 *endo*-epoxide (∇) and Yc2 band density, as measured by scanning laser densitometry, of a western blot of cytosolic protein probed with affinity-purified antisera raised to the mouse Yc subunit.** Activities towards each isomer were determined simultaneously by HPLC and represent the mean of 2-4 determinations per rat.

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Chapter 4

THE ANTICARCINOGEN 3,3'-DIINDOLYLMETHANE IS A NON-SPECIFIC INHIBITOR OF CYTOCHROMES P-450

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ABSTRACT

Indole-3-carbinol (I3C), when given orally, inhibits carcinogenesis in rats, mice and trout. Several mechanisms of inhibition may exist. We have found that 3,3'-diindolylmethane (I33'), a principal I3C degradation product found *in vivo* after oral administration of I3C, is a potent non-specific inhibitor of reactions catalyzed by trout, rat or human cytochromes P-450 (CYP). In the ethoxyresorufin *O*-deethylase assay (CYP1A specific), I33' inhibited trout, rat and human sources of the enzyme with K_i values ranging from 2-14 μM I33'. Noncompetitive inhibition was observed in the trout and human enzyme, whereas competitive inhibition was observed with the rat. For rat liver microsomal pentoxyresorufin *O*-depentylase (CYP2B1 associated), inhibition occurred in a noncompetitive manner. Constants were determined to be $K_{is} = 0.62 \pm 0.08$ μM I33' and $K_{ii} = 1.2 \pm 0.60$ μM I33'. A K_i value of 7.6 ± 4.1 μM I33' was determined for human CYP1A2-catalyzed acetanilide 4-hydroxylase, based on an apparently competitive mode of inhibition. I33' also inhibited *in vitro* CYP metabolism of the ubiquitous food contaminant and potent hepatocarcinogen, aflatoxin B_1 (AFB_1). At an initial concentration of 16 μM AFB_1, representative of subsaturating conditions, CYP1A-mediated aflatoxin M_1 production was inhibited 14, 32 and 46% at 10, 50 and 100 μM I33' respectively. Inhibition of AFB_1 bioactivation to the 8,9-epoxide, a reaction catalyzed by several CYP isoforms in the rat, occurred in a nearly identical concentration dependent manner (9%, 31% and 47% inhibition). At 124 μM AFB_1 initial concentration, representative of saturating conditions, I33' inhibited AFM_1 production by 11%, 14%, and 29% and AFB_1 8,9-epoxide formation by 15%, 36%, and 58%. The CYP3A-mediated metabolism toward AFQ_1, was inhibited 17%, 42% and 54%. Calculated noncompetitive inhibition constants for AFB_1 8,9-epoxidation were $K_{is} = 137$

$\pm$ 43 μM I33', K_{ii} = 58 $\pm$ 11 μM. Competitive inhibition was apparent with AFM_1 formation and the calculated K_i value was 128 $\pm$ 24 μM I33'. Inhibition of cytochrome *c* reductase was not observed. We also tentatively identified a mono-hydroxylated rat microsomal metabolite of I33' and found that [^{3}H]I33' exhibited protein and NADPH dependent binding to microsomal protein and DNA. These observations indicate that I33' can inhibit the catalytic activities of a range of CYP isoforms from lower and higher vertebrates *in vitro*. This broadly-based inhibition of CYP-mediated bioactivation of procarcinogens may be an I3C anticarcinogenic mechanism applicable to all species, including humans.

INTRODUCTION

Indole glucosinolates are found in high concentrations in the Cruciferae family of vegetables (1), whose members include cabbage, Brussels sprouts, broccoli and cauliflower. Glucobrassicin is the most abundant of all glucosinolates and, after enzymatic hydrolysis by the enzyme myrosinase, yields indole-3-carbinol (I3C)[1], glucose and isothiocyanate anion. When fed to experimental animals, I3C has been shown to possess potent tumor modulating properties, in most cases inhibiting tumor incidence (2-4), but with some protocols, I3C enhances tumor incidence (5, 6). The primary breakdown product of I3C in aqueous solution is a dimer of I3C, 3,3'-diindolylmethane (I33')(Fig. 4.1)(7), and I33' can be prepared in high yield by simply refluxing I3C in neutral solution (8). Under acidic conditions, as found in the stomach, I3C will quickly and irreversibly condense with itself to yield, in addition to I33', several other oligomeric derivatives as major products (9, 10). Thus, I33' has been found as a major product present *in vivo* after oral administration of I3C, whereas I3C was not detected (see Chapter 5 and ref. 11) or was found in lesser amounts (12). When aflatoxin B_1 (AFB_1) was co-injected with I33' or I3C into fertilized rainbow trout embryos, a profound reduction in hepatic AFB_1-DNA binding and hepatic tumor incidence was observed (13), but only in animals given I33'. Wattenberg and Loub (14) observed that oral administration of I3C or I33' to rats inhibited mammary tumor formation induced by 7,12-dimethylbenzanthracene in female Sprague-Dawley rats and neoplasia of the

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[1] - **Abbreviations**: I3C, indole-3-carbinol; I33', 3,3'-diindolylmethane; AFB_1, aflatoxin B_1; CYP, cytochrome P-450; QR, quinone reductase; UDPGT, uridine diphosphate glucuronyl transferase; GST, glutathione *S*-transferase; PB, phenobarbital; BNF, ß-naphthoflavone; AFM_1, aflatoxin M_1; AFG_1,aflatoxin G_1; AFM_1, aflatoxin M_1; AFQ_1, aflatoxin Q_1; AFP_1, aflatoxin P_1; PROD, pentoxyresorufin *O*-depentylase; EROD, ethoxyresorufin *O*-deethylase; NNK 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; EI, electron impact.

forestomach induced by benzo[*a*]pyrene in female ICR/Ha mice. These data show that I33' itself can be anticarcinogenic, and that I33' may be a major factor in the anticarcinogenicity observed with orally administered I3C.

Several anticarcinogenic mechanisms may exist for I33'. Previous studies have shown that oral (15, 16) or ip (17) administration of I33' can induce hepatic CYP1A1 (a phase I enzyme) or its associated activity in rats. CYP1A1 protein or activity can also be induced in trout embryos micro-injected with I33' (18), or in primary cultures of rat and monkey hepatocytes exposed to I33' in the culture media (19, 20). It has been suggested that this induction is responsible for altered carcinogen metabolism leading to reduced tumor incidence (21). In addition, administration of I33' to rats was found to induce CYP1A2 and microsomal estradiol 2-hydroxylation when given ip (17) or 4-androstenedione metabolism, when given ip or po (22). Enhancement of both metabolic pathways are suggested as mechanisms responsible for the anti-estrogenic effects of I3C given orally (17, 22). Induction of phase II enzymes, such as quinone reductase (QR), uridine diphosphate glucuronyl transferase (UDPGT) and glutathione *S*-transferase (GST), is an established mechanism of protection against carcinogenesis (23, 24). Treatment of rat or monkey hepatocytes in primary cell culture with I33' resulted in induction of QR and UDPGT (11, 20), but not GST (19, 20). However, administration of I3C orally (and therefore also I33' and other I3C oligomers) to trout or mice failed to induce monooxygenases (25-27), GST or UDPGT activity (26, 28) yet protection against carcinogen-DNA binding or tumorigenicity was observed. It was recently observed that I33' could inhibit mutagenesis induced by authentic AFB_1 8,9-epoxide in the *Salmonella* mutagenesis assay, suggesting a role for direct electrophile trapping as a means of protection (18).

CYP consist of a superfamily of enzymes which have various endo- and xenobiotics as substrates (29). In general, CYPs oxidize xenobiotics to more polar, non-toxic products, however, bioactivation to carcinogenic metabolites is sometimes a sequelae (30). When tested *in vitro*, I33' exhibited inhibition of CYP activity (18, 22, 31). To further characterize the effects of I33' as an *in vitro* inhibitor, we examined inhibition of CYP activities from trout, rat and human sources of the enzyme. In addition, we examined the effect on the *in vitro* oxidative metabolism of the ubiquitous food contaminant and potent hepatocarcinogen AFB_1 (32). Limited studies on *in vitro* microsomal metabolism and macromolecule binding of [^{3}H]I33' were also performed. Our results show that I33' is a non-specific *in vitro* inhibitor of several trout, rat or human CYP activities, with inhibition constants near I33' levels found *in vivo* after administration of an anticarcinogenic dose of I3C to rat or trout. Thus, I33'-inhibition of CYP mediated bioactivation may be an additional mechanism of anticarcinogenesis that could apply to all species, including humans.

MATERIALS AND METHODS

Animals

Male Fischer rats were obtained as weanlings from Simonsen's (Gilroy, CA) and housed at the Laboratory Animal Resource Center at Oregon State University. Rats were maintained on a AIN-76A semi-purified diet (U.S. Biochem. Corp, Cleveland, OH) and received diet and drinking water *ad libitum*. To induce CYP1A, rats received BNF (40 mg/kg) suspended in approximately 0.5 ml of corn oil, by ip injection for 4 consecutive days, were starved on day 4, and sacrificed on day 5. To induce CYP2B1, rats received drinking water containing 0.1% PB for seven consecutive days, were starved on day 7 and sacrificed on day 8. Animals were sacrificed by CO_2 asphyxiation.

Rainbow trout (200-300 g) were maintained in flowing well water (12°C) at the Food Toxicology and Nutrition Laboratory at Oregon State University and fed Oregon Test Diet (33) containing 700 ppm BNF for 7 days. The animals were sacrificed by electroshock on the following day.

Chemicals

Ethoxyresorufin, pentoxyresorufin and resorufin were obtained from Molecular Probes Inc. (Eugene, OR). Aflatoxins B_1, Q_1, M_1, and G_1, cytochrome *c*, NADPH and BNF were obtained from Sigma Chemical Co. Ltd (St. Louis, MO). AFB_1 8,9-epoxide-glutathione conjugate was a gift of Dr. David Eaton of the University of Washington. Aflatoxin B_1 8,9-epoxide was gift of Dr. Thomas Harris of Vanderbilt University. I33' was synthesized according to the method of Leete and Marion (8) in a neutral solution and shown to be pure by HPLC. I3C, acetanilide, 3-OH acetanilide and 4-OH acetanilide were purchased from Aldrich Chemical Co, (Milwaukee, WI). All other chemicals were

purchased from Sigma. The method of Dashwood *et al.*, (34) was used to tritium-label I3C at the 5 position. The [^{3}H]I3C was diluted with cold carrier to an approximate specific activity of 270 mCi/mmol. An acid reaction mixture of products originating from [^{3}H]I3C was generated according to the method of Bjeldanes *et al.* (35). We isolated [^{3}H]I33' from this mixture by HPLC using a Beckman ODS 5μ 4.6 × 250 mm analytical column (Palo Alto, CA). Starting solvent concentrations were 20% acetonitrile (Solvent A) and 80% Milli-Q water (Millipore Corp., Bedford, MA)(Solvent B). These conditions were held for 30 sec, before changing to 15% B over the next 29.5 min (linear gradient). This ratio was held for 5 min, then changed to 0% B over the following 5 min, held for an additional 5 min, then returned to starting conditions over the next 10 minutes. The total flow rate was 1 ml/min. Metabolites were monitored by UV absorbance at 280 nm using a Shimadzu SPD-6AV spectrophotometer detector (Kyoto, Japan). The elution time for I33' in this system is routinely 24.4 min.

Preparation of Microsomal and Cytosolic Enzymes

Microsomes were prepared from the livers of trout or rats by differential centrifugation according to the method of Guengerich (36). Cytosol from BHA-induced mice, used to quantify production of AFB_1 8,9-epoxide, was a gift of Dr. David Eaton. Protein concentration of cytosol and microsomes was determined according the method of Lowry *et al.*, (37). Microsomes from a human lymphoblastoid cell line containing human CYP1A1 or human CYP1A2 were obtained from Gentest (Woburn, MA). This cell line has only a small amount of natively expressed CYP activity (CYP1A1), which is responsive to induction by PAHs. Microsomes used for ethoxyresorufin *O*-deethylase (EROD) assay were prepared from the PAH pretreated (CYP1A1 induced) cell line. The

microsomes containing CYP1A2 were prepared from the same cell line, untreated, which had been transfected with the human *CYP1A2* cDNA. Microsomes from the native, untreated cell line were used as controls for the CYP1A2-mediated acetanilide 4-hydroxylase assay.

Enzyme Assays

The EROD assay was conducted as modified according to Pohl and Fouts (38) using liver microsomes from BNF treated trout or rat, or microsomes from a PAH induced human lymphoblastoid cell line. The assay was conducted at 25°C (trout) or 37°C (rat and human).

The PROD assay was conducted according to the method of Burke *et al.*, (39), at 37°C using microsomes from PB treated rats. For both EROD and PROD, the slight quenching of fluorescence by I33' was compensated for by including the inhibitor in the standard curve solutions at the concentrations employed in the assay.

Cytochrome *c* reductase activity was determined according to the method of Yasukochi and Masters (40).

Metabolism of AFB_1 was determined essentially as described by Monroe and Eaton (41) using liver microsomes from BNF treated rats. This assay allows simultaneous quantification of hydroxylated metabolites and AFB_1 8,9-epoxide by trapping the latter as a stable glutathione conjugate. In the presence of mouse cytosol, trapping efficiency of the epoxide as the conjugate has been reported to be greater than 99% (41). I33' was added to the reaction mixture at final concentrations of 10, 50 or 100 μM in DMSO (final DMSO concentration was 2% v/v) and preincubated at 37°C for 5 minutes before initiating the reaction with AFB_1. The final reaction mixture included 1 mg/ml microsomal protein, 3 mg/ml BHA-induced mouse cytosolic protein, 16 or 124 μM AFB_1, 5 mM GSH, 1 U/ml glucose-6-phosphate dehydrogenase, 5 mM glucose-6-

phosphate and 1 mM $NADP^+$ in a buffer containing 190 mM sucrose, 60 mM potassium phosphate, 80 mM Tris, 15 mM NaCl, 5 mM KCl and 4 mM $MgCl_2$, pH 7.6 in a reaction volume of 250 µl. After 10 min [within the linear range for metabolite production (42)], the reaction was terminated by the addition of 50 µl 2 M acetic acid and 10 µl methanol containing the internal standard. The mixture was then frozen for at least two hours. The frozen mixture was then subjected to centrifugation at 14,000 g for 4 min at ambient temperature to thaw the mixture and pellet precipitated protein. Metabolites were resolved by HPLC on a 4.6×250 mm, C_{18} Econosphere cartridge column (Alltech Associates, Deerfield, Il) and detected by UV absorption at 362 nm. Quantification of metabolites was achieved with a Shimadzu Chromatopac integrator (Kyoto, Japan) using an AFB_1 standard curve and AFG_1 as an internal standard to correct for recovery. The mobile phase consisted of a combination of 0.1% ammonium phosphate, pH 3.5 (solvent A) and 95:5 methanol:THF (solvent B). From 0 to 2 minutes, the concentration of solvent B was increased from 10% to 24%, then further increased to 38% solvent B at 13 minutes, 60% at 16 minutes and 90% at 17 minutes. At 20 minutes, the mobile phase was returned to starting conditions over a period of 5 minutes. All changes in the composition of the mobile phase were accomplished using a linear gradient. The flow rate was kept constant at 1.0 ml/min. The concentration of the AFB_1 stock solution (prepared in DMSO) was determined by UV absorbance spectrophotometry using an extinction coefficient of 21.8 M^{-1} cm^{-1}. The final concentration of AFB_1 in the assay was calculated by adding a known amount of a AFB_1 stock solution (final concentration of DMSO in the assay was 4% v/v). When mouse cytosol was included in the absence of microsomes, the formation of AFB_1 8,9-epoxide as the GSH conjugate, was also

detected, but represented < 8% of that formed by the least active microsomal preparations (i.e. control animals). This activity was corrected for in the values reported herein.

CYP1A2 mediated 4-hydroxylation of acetanilide was conducted essentially as described by Liu *et al.*, (43). The final reaction mixture contained 0.1 to 3.0 mM acetanilide, 0 to 100 μM I33' delivered in DMSO (final concentration DMSO 1% v/v), 1 U/ml glucose-6-phosphate dehydrogenase, 10 mM glucose-6-phosphate, 0.5 mM NADPH, 50 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA and 0.5 mg/ml microsomal protein in a volume of 250 μl. All components, excepting microsomes, were preincubated for two minutes before initiating the reaction with microsomes. Control incubations excluded either the NADPH regenerating system or microsomes, or used microsomes containing only the slight amounts of CYP 1A1 activity natively expressed in the untreated, nontransfected cell line. The reaction was terminated at 45 minutes with the addition of 0.1 μg 3-OH acetanilide (internal standard) in acetone and 0.3 ml ice-cold ethyl acetate. The ethyl acetate extract (3 × 0.3 ml) was evaporated under a stream of N_2 and resuspended in 50 μl of HPLC mobile phase (initial conditions). The 4-hydroxy metabolite was quantified by HPLC (40 μl injection volume) with a Shimadzu Chromatopac integrator using 3-OH acetanilide as an internal standard to correct for recovery and a 4-OH acetanilide standard curve. Metabolites were separated using a binary gradient consisting of 5:17 acetonitrile:methanol (solvent A) and water (solvent B). Initial conditions were 22% solvent A: 78% solvent B. This solvent ratio was changed linearly to 23.5% solvent A: 76.5 % solvent B over three minutes, then changed to 45% solvent A: 55% solvent B over the next two minutes. This solvent ratio was held for 5 minutes before returning to initial conditions over the following 2 minutes. The metabolites were detected at a wavelength of 254 nm using a Shimadzu SPD-6AV

detector. The column used was an Econosil (Alltech Assoc., Deerfield, IL) C_{18} 5 μ 250 × 4.6 mm held at 40°C. Flow rate was constant at 1 ml/min.

We used BNF treated rat microsomes to examine the microsomal metabolism of [^{3}H]I33'. Tritium-labeled I33' (0.5 μCi) was added in ethanol to microcentrifuge tubes and reduced to dryness under a stream of argon. Unlabeled I33' prepared in DMSO, was added so that the final concentration was 100 μM. Other components were added so that their final concentrations were 1 U/ml glucose-6-phosphate dehydrogenase, 10 mM glucose-6-phosphate, 0.5 mM NADPH, 10 mM potassium phosphate (pH 7.4), 1 mM $MgCl_2$, 0.2 mM EDTA. The mixture was then incubated at 37°C for two min prior to the addition of 1.0 mg/ml microsomal protein in a volume of 100 μl. Incubations in the absence of microsomes, in the presence of heat inactivated microsomes, or lacking an NADPH regenerating system, were run concurrently as controls. After 30 min, the reaction was terminated by the addition of 100 μl cold acetonitrile and the mixture was cooled on ice for 30 min prior to centrifugation at 10,000 g for 10 min at 4°C. The mixture was frozen until analysis of the supernatant by HPLC under the conditions described above for the isolation of [^{3}H]I33'. Metabolites were monitored at a wavelength of 280 nm and by radioisotope detection using an on-line Beckman M171 radioisotope detector and a liquid scintillation cell and a scintillation cocktail flow rate of 2 ml/min.

Mass Spectrometry

Low resolution electron impact (EI) mass spectral analysis was performed on a Finnigan model 4023 quadrupole mass spectrometer upgraded with a model 4500 source and a Varian model 3400 gas chromatograph at the Environmental Health Sciences Center

at Oregon State University. The mass spectrometer is controlled by a Galaxy 2000 data system (LGC Co., San Jose, CA). Chromatography was performed using an Alltech (Deerfield, Il) SE-54 column (10 m × 0.25 mm i.d. with a 0.25 μm coating) operated at injector pressures of 3 psi. EI mass spectra were obtained at an electron energy of 70 eV at a source temperature of 140°C. We found that silylation of active hydrogens on the indole or alcohol of the putative hydroxylated metabolite of I33' conferred excellent thermal stability, thus facilitating GC/MS analysis.

Covalent Binding of [^{3}H]I33' to Microsomal Protein and DNA

Microsomal pellets from the [^{3}H]I33' metabolism assay were washed successively with hot (50°C) methanol until no radioactivity could be extracted. The pellet was dissolved overnight in 1 N NaOH at 37°C prior to determination of protein (37) and associated radioactivity by liquid scintillation counting. Covalent binding to DNA was assessed by performing an experiment similar to the [^{3}H]I33' metabolism assay. The incubation included 0.5 mg/ml calf thymus DNA and the concentration of microsomal protein was reduced to 0.8 mg/ml in a final volume of 250 μl. The incubation was terminated with the addition of 50 μl of 10% sodium dodecyl sulfate at 60 min and DNA was isolated by phenol and chloroform extraction as described in detail by Dashwood *et al.*, (44). Preparations of DNA were checked for purity by measuring the ratio of UV absorbance at 260 and 280 nm. The method of Burton (45) was used to determine concentrations of DNA. Aliquots of the hydrolysates used for the Burton assay were analyzed for the presence of radioactivity by scintillation counting using ACS scintillation fluid (Amersham). We used liver microsomes from untreated rats, PB treated rats, or BNF treated rats or trout. Control incubations were performed in the absence of

NADPH or with heat treated microsomes. Assays were performed at 37°C except when using trout microsomes where the reaction temperature was 29°C.

Kinetic analysis

K_{ii} and K_{is} values were determined by nonlinear regression using computer analysis (46). Data were fitted to a competitive or noncompetitive [nomenclature of Cleland (47)] inhibition model based on visual examination of Lineweaver-Burke plots. The correct model choice was not unequivocal since computer drawn regression curves reflect experimental error.

Statistics

Data for inhibition of AFB_1 metabolite production and inhibition of cytochrome *c* reductase were analyzed by one-way analysis of variance and differences between specific means were compared using least significant difference. Differences with $p \leq 0.05$ were considered significant.

RESULTS

In Vitro Inhibition of EROD and PROD

Figure 4.2 shows a double reciprocal plot of the inhibition of trout liver microsomal EROD at three different concentrations of I33'. A K_{is} value of 2.7 ± 0.5 μM I33' and a K_{ii} value of 14 ± 2.2 μM I33' were calculated assuming noncompetitive inhibition. With rat microsomes (Fig. 4.3), inhibition appeared competitive and a K_i value of 2.2 ± 0.2 μM I33' was determined. Ethoxyresorufin *O*-deethylase has high specificity for CYP1A1 activity both in trout (48) and rat (49) when animals have been induced by BNF or similar inducer. I3C (100 μM) had a much weaker though measurable inhibitory effect on resorufin production (data not shown), however inhibition due to I33', formed from decomposition of I3C in an aqueous medium, could not be ruled out and no attempt was made to determine K_i values for this compound.

Figure 4.4 shows I33' inhibition of human CYP1A1 mediated EROD. The activity of these microsomes was relatively low compared to BNF induced animals [V_{max} = 114 pmol/min/mg protein versus 600 pmol/min/mg protein (trout) and 20,300 pmol/min/mg (rat)]. Though these data are somewhat imprecise, the fitted lines are more compatible with non-competitive inhibition, which yields a K_{is} value of 7.4 ± 2.0 μM I33' and a K_{ii} value of 13 ± 2.7 μM I33'.

Figure 4.5 depicts I33' inhibition of rat liver microsomal PROD, an activity associated with CYP2B1. Assuming a noncompetitive mechanism, kinetic constants were determined to be K_{is} = 0.62 ± 0.08 μM I33' and K_{ii} = 1.2 ± 0.60 μM I33'. This was the strongest inhibition observed in all the *in vitro* assays performed in this study.

Inhibition of Acetanilide 4-Hydroxylase

Figure 4.6 shows the inhibition of human CYP1A2-mediated acetanilide 4-hydroxylase at concentrations of 10 and 50 μM I33'. While there is clear evidence for inhibition, the data intersects are inadequate t o suggest inhibitory mechanism unequivocally. Competitive inhibition was judged to best fit the data as determined by sum of squares analysis, and a K_i value of 7.6 ± 4.1 μM I33' was determined. No acetanilide 4-hydroxylase activity was observed in incubations containing control microsomes that express only a low level of CYP1A1 activity (see Materials and Methods).

Inhibition of In Vitro AFB_1 Metabolism

Figures 4.7 and 4.8 depict the results of an experiment assessing inhibition of *in vitro* metabolism of AFB_1 by I33' using BNF-induced rat liver microsomes. Inhibition was examined at two different levels (16 and 124 μM) of AFB_1 and three different levels (10, 50 and 100 μM) of I33'. At the low concentration of AFB_1, the velocity of CYP1A-catalyzed conversion of AFB_1 to AFM_1 (50) was inhibited 14, 32 and 46 % at the three concentrations of inhibitor tested (Fig. 4.7). Inhibition of AFB_1 8,9-epoxide-glutathione conjugate (i.e. AFB_1 8,9-epoxide) formation occurred in a nearly identical concentration dependent manner (9, 31 and 47% inhibition)(Fig. 4.7).

At the high concentration of AFB_1 used (124 μM), I33' inhibition of AFM_1 production was not as strong as that seen in the low concentration employed. Inhibition was 11, 14 and 29% at 10, 50 and 100 μM concentrations of I33', respectively (Fig. 4.8). In contrast to the findings at the low concentration of AFB_1, the degree of inhibition of AFB_1 8,9-epoxide-glutathione conjugate production at the high

concentration was nearly twice that of AFM_1 (Fig. 4.8). Calculated noncompetitive inhibition constants for AFB_1 8,9-epoxidation were K_{is} = 137 ± 43 μM I33', K_{ii} = 58 ± 11 μM. Competitive inhibition was apparent with AFM_1 formation and the calculated K_i value was 128 ± 24 μM I33'. At this concentration of AFB_1, we were able to accurately quantify production of AFQ_1. Its production, mediated by CYP3A, was inhibited 17, 42 and 54 % at the three concentrations of I33' used (Fig. 4.8). Other peaks, representing AFP_1 and two unidentified metabolites, also showed inhibition, but peak areas could not be reliably quantified. Inhibition of AFB_1 8,9-epoxide-glutathione conjugate formation by I33' was not observed when AFB_1 8,9-epoxide was added in the presence of increasing concentrations of mouse cytosol (data not shown).

I33' had no apparent effect on the ability of PB induced rat liver microsomes to reduce cytochrome *c* (data not shown).

Metabolism of [^{3}H]I33'

When microsomes from BNF-treated rats were incubated with 100 μM [^{3}H]I33' in the presence of NADPH for 30 minutes, a major metabolite with an HPLC elution time of 18.7 min was produced at the rate of 49 ± 13 pmol/min/mg protein. Electron impact mass spectral analysis of this peak revealed a compound possessing an *m/z* ratio of 478, and a fragmentation pattern consistent with a trimethylsilated derivative of monohydroxylated I33' (Fig. 4.9). Mass spectral data for the *N, N, N* -trimethylsilyl derivative of the putative hydroxylated I33': *m/z* 479 (86), 478 (100, M+), 405 (15), 333 (1.4), 290 (4.2), 202 (8.3). We have not determined the position of the -OH moiety.

Covalent Binding of [^{3}H]-I33' to Microsomal Protein and DNA

Microsomal protein from the [^{3}H]I33' metabolism experiment was found to contain bound [^{3}H]I33' equivalents that was dependent on the presence of functional microsomal enzymes and NADPH. We observed 3.78 ± 0.19 nmol [^{3}H]I33' equivalents bound/min/mg microsomal protein when all components of the metabolizing system are present, but only 0.64 and 1.30 nmol [^{3}H]I33' equivalents bound when we used heat-inactivated microsomes or when the incubation occurred in the absence of NADPH, respectively (Fig. 4.10). Based on these data, we examined the possibility that [^{3}H]I33' could be activated to DNA binding metabolites. Using microsomes from untreated rats or rats pretreated with phenobarbital or BNF, we observed covalent binding to calf thymus DNA that was dependent on functional microsomes and NADPH (Fig. 4.11). We could not consistently inhibit binding by inclusion of 1-10 mM GSH in the assay (data not shown). When untreated rat liver microsomes were bubbled with carbon monoxide, only a slight decrease in binding was observed relative to heat treated microsomes or when the incubation was performed in the absence of NADPH. Microsomes from BNF pretreated trout failed to produce DNA binding metabolites.

DISCUSSION

Inhibition of CYP Isoform-Specific Reactions

According to Wattenberg, chemopreventive agents may be classified on the basis of mechanism of action (23). One category is inhibitors of enzymes, such as CYP, which bioactivate procarcinogens. Results presented here show that I33', a non-enzymatic product of I3C, is a potent inhibitor of reactions catalyzed preferentially or exclusively by CYP1A1, CYP1A2 (trout, rat or human) CYP2B1 and CYP3A (rat), enzymes known to bioactivate carcinogens. In BNF-induced rat microsomes, EROD activity is attributed primarily to CYP1A1, with a minor contribution from CYP1A2. CYP1A1 is also believed to be responsible for EROD activity in trout, however, Berndtson and Chen (51) recently found that two *CYP1* genes are expressed in trout and it is possible, based on precedent in mammalian systems, that this second *CYP1* gene product may contribute to EROD activity. Human CYP1A1 is the only CYP enzyme present in the microsomes from the lymphoblastoid cell line and therefore the inhibition of EROD by I33' is exclusively due to inhibition of CYP1A1. We also found I33' to strongly inhibit the activity of human CYP1A2. Expression of CYP1A2 is apparently restricted to hepatic tissue in humans (52), whereas expression of CYP1A1 appears to be expressed only in extrahepatic tissues (52-54). I33' inhibition of CYP1A proteins in these tissues would be expected to protect against carcinogens that are CYP1A bioactivated including PAHs, heterocyclic amines (30, 55) and the mycotoxin, AFB_1 (56).

In hepatic microsomes from PB induced rat, CYP2B1 is probably responsible for the high level of PROD activity observed (37). This isoform can activate a number of carcinogens (30) including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)(57).

Though present only in low levels in rat liver, it is constitutively expressed in rat as well as mouse lung (58, 59). Potent inhibition of CYP2B1 should therefore contribute to the I3C inhibition of NNK induced lung tumorigenesis in mice (3).

We also examined the ability of I33' to inhibit activation of the hepatocarcinogen AFB_1, a common contaminant of human food supplies. As shown previously, the contributions of the various CYP enzymes to AFB_1 microsomal metabolism vary with initial substrate concentration, particularly in animals pretreated by an inducer of CYP (42, 56, 60). However, in the rat it appears that primarily CYP2C11 (61, 62) and CYP3A (62) contribute to bioactivation reactions. CYP1A and 3A are associated with the detoxication reactions catalyzing formation of AFM_1 (63) and AFQ_1 (64), respectively.

Failure of I33' to inhibit NADPH-cytochrome P-450 reductase at up to 100 μM I33' is consistent with inhibition of catalytic activity via interaction with the hemoprotein component of CYP. Indeed, we observed that I33' is a substrate of a microsomal and NADPH-dependent enzyme, forming a putative mono-hydroxylated form of I33', consistent with a typical CYP product. It has recently been found that I33' can act as a suicide inhibitor of trout EROD (18), consistent with our data showing the formation of protein or DNA binding metabolites as shown here for the rat. Failure of CO to substantially inhibit binding could indicate that catalysis by a non-heme containing protein, perhaps flavin-containing monooxygenase or prostaglandin H-synthase. However, in contrast to the rat, hepatic microsomes from BNF treated trout did not activate [^{3}H]I33' to a DNA binding species. The significance of [^{3}H]I33' binding to DNA in the context of anticarcinogenesis is not known, but these data show that anticarcinogenic chemicals are capable of forming electrophilic metabolites. Garg and Gupta (65) recently found that broccoli, which is associated with an anticancer effect, contains a compound capable of binding to DNA both *in vitro* and *in vivo* .

I33' Inhibition of CYP as a Mechanism of Protection Against AFB_1 Carcinogenesis

Dietary I3C is a potent inhibitor of AFB_1 carcinogenesis in the trout (2) and rat (66). A reduction in hepatic *in vivo* AFB_1-DNA binding is associated with induction of specific phase I and phase II AFB_1 metabolizing enzymes by I3C in the rat (60, 67), but not in trout (26), suggesting other mechanisms apply for the latter species. Takahashi *et al.*(18), have recently shown inhibition of trout microsome catalyzed AFB_1-DNA binding by I33' *in vitro* and we extend those result to the rat by an alternate method, showing inhibition of formation of the major genotoxic AFB_1 metabolite, AFB_1 8,9-epoxide. Although we cannot rule out the possibility that I33' is acting as a nucleophilic trapping agent in this system, which may be the anti-mutagenic mechanism shown with I33' and synthetic AFB_1 8,9-epoxide in the *Salmonella typhimurium* assay (18), this seems unlikely given the trapping efficiency of mouse cytosol in the presence of glutathione (41). I33' should inhibit *in vivo* CYP activation of AFB_1 in the rat, as was concluded for the trout model (13). This mechanism of protection would occur in addition to induction of GST (67). However, I33' also inhibits known phase I detoxication pathways (i.e. AFM_1 and AFQ_1 formation), in addition to inhibiting the bioactivation pathway, and, under conditions of sustained feeding, I3C (and therefore, possibly I33') enhances *in vitro* production of AFB_1 8,9-epoxide, as well as AFM_1 and AFQ_1 (60). It was recently shown that a CYP1A2 inhibitor (furafylline) inhibited *in vivo* activation of heterocyclic amines in humans (55). Thus, the human CYP1A2 inhibitor I33' may similarly offer a means of inhibiting the CYP1A2 activation of AFB_1 (56). However, dietary I3C also induces CYP1A2 and 3A associated estradiol 2-hydroxylation in humans (68), suggesting the possibility of enhancement of AFB_1 bioactivation. *In vivo* studies are

necessary to fully comprehend the balance between the I33' inhibition or induction of both AFB_1 bioactivation and detoxication pathways.

Although hitherto we have not discussed the bioavailability of I33', it is known to be a major component of both trout and rat liver extract following oral gavage or dietary administration (see Chapter 5 and refs. 11 and 12). Six hours after oral gavage of an anticarcinogenic dose of 1.0 mmol I3C/kg body weight, I33' liver concentrations were estimated to be on the order of 2-4 μM, whereas levels found in trout 48 hours after oral gavage of an anticarcinogenic dose of 0.27 mmol I3C/kg body weight, were determined to be approximately 70 μM. These levels are in the range of K_i values calculated for inhibition of EROD, PROD, or acetanilide 4-hydroxylase employed in this study, but are below that calculated for AFB_1 oxidation reactions, despite, in some cases similarity in enzymes (i.e. rat CYP1A1 for both AFB_1 9a-hydroxylase and EROD). One explanation for this apparent anomaly is the fact that, in contrast to most enzymes, CYPs exhibit broad substrate specificity. Thus, affinity of ethoxyresorufin and AFB_1 for the CYP1A1 catalytic site may be substantially different, resulting in unequal K_i values calculated for the same inhibitor, I33'. Nevertheless, significant inhibition of AFB_1 8,9-epoxidation is observed with only 10 μM I33' and any inhibition of procarcinogen activation would be expected to protect *in vivo*.

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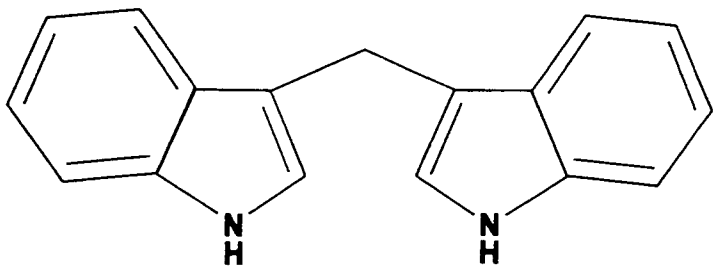

Figure 4.1. **Structure of 3,3'-diindolylmethane, a primary acid condensation product of I3C.**

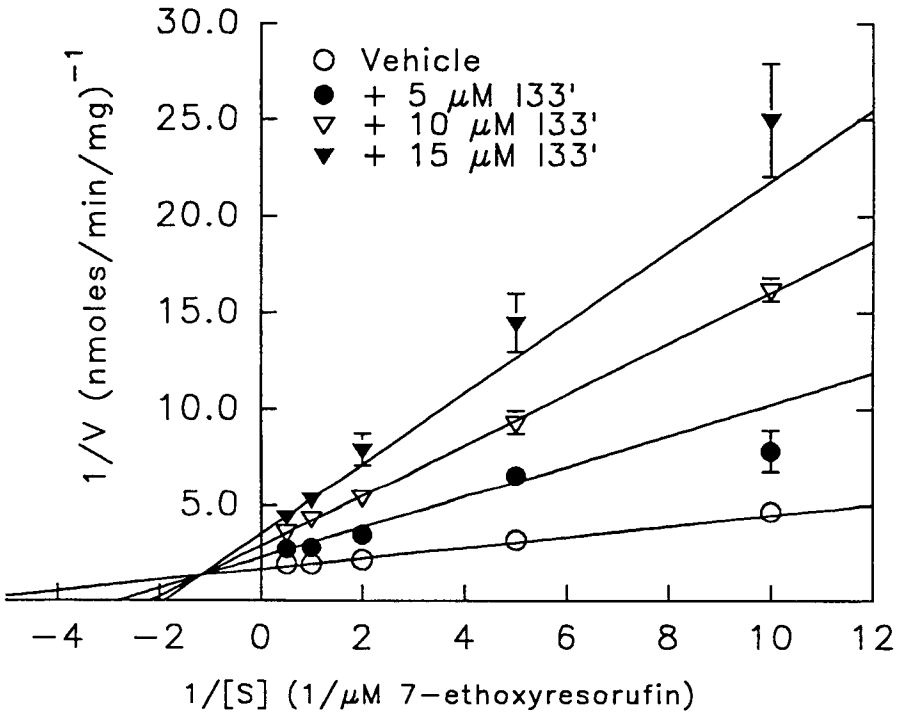

Figure 4.2. **Lineweaver-Burke plot of inhibition of trout liver microsomal EROD by I33'.** Incubations were performed as described in Materials and Methods and included 0.1 mg protein/ml. Points represent means of 3-6 determinations ± SE or means of 2 determinations ± range. V_{max} = 600 ± 25 pmol/min/mg protein; K_m = 168 ± 23 nM; K_{is} = 2.7 ± 0.5 μM I33'; K_{ii} = 14 ± 2.2 μM I33'.

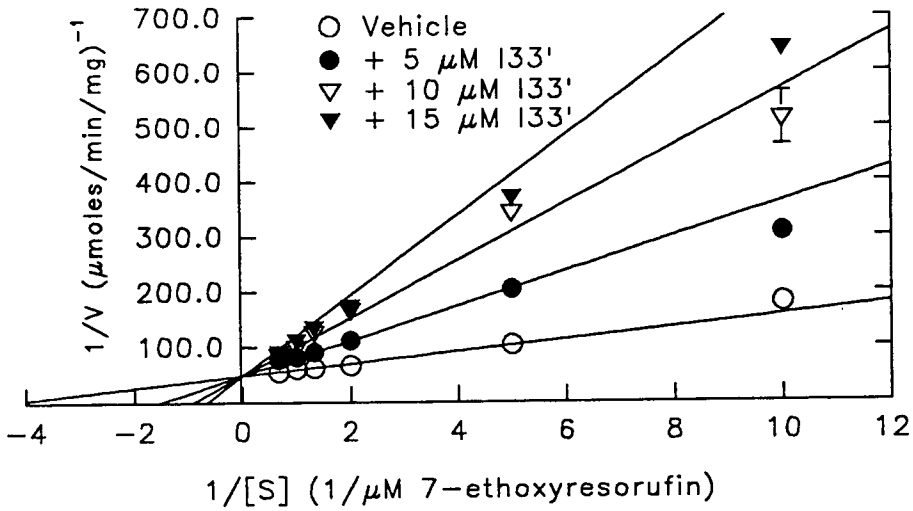

Figure 4.3. **Lineweaver-Burke plot of inhibition of rat liver microsomal EROD by I33'.** Incubations were performed as described in Materials and Methods and included 5 μg/ml protein. Points represent the means of 3 determinations ± SE. V_{max} = 20.3 ± 6.0 nmol/min/mg protein; K_m = 205 ± 24 nM; K_i = 2.2 ± 0.2 μM I33'.

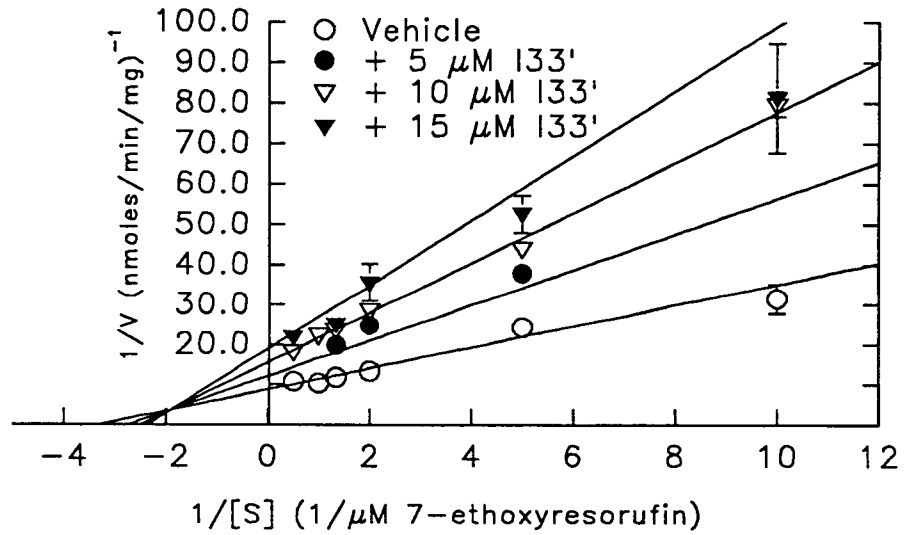

Figure 4.4. **Lineweaver-Burke plot of inhibition of human microsomal CYP1A1 catalyzed EROD by I33'.** Incubations were performed as described in Materials and Methods and included 50 μg protein/ml. Points represent means of 3 determinations ± SE or means of 2 determinations ± range. V_{max} = 114 ± 7.0 pmol/min/mg protein; K_m = 301 ± 50 nM; K_{is} = 7.4 ± 2.0 μM I33'; K_{ii} = 13 ± 2.7 μM I33'.

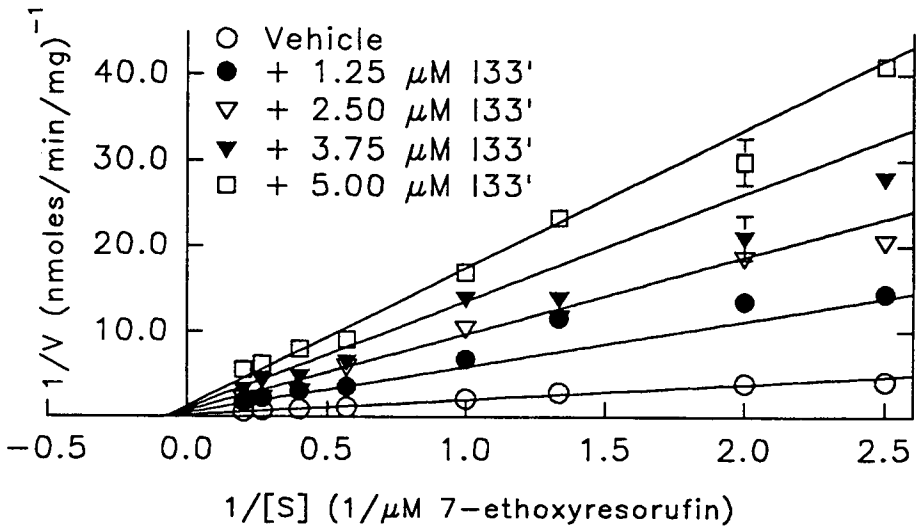

Figure 4.5. **Lineweaver-Burke plot of inhibition of rat liver microsome catalyzed PROD by I33'.** Incubations were performed as described in Materials and Methods and included 75 μg/ml protein. Points represent means of 3-5 determinations ± SE or means of 2 determinations ± range. In limited instances, only one data point was obtained. V_{max} = 4.06 ± 0.68 nmol/min/mg protein; K_m = 7.3 ± 1.6 μM; K_{is} = 0.62 ± 0.08 μM; K_{ii} = 1.2 ± 0.60 μM I33'.

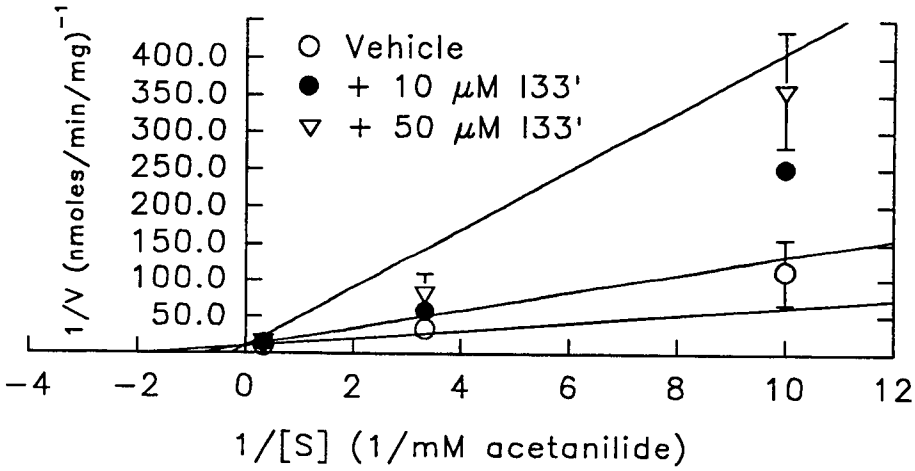

Figure 4.6. **Lineweaver-Burke plot of inhibition of human microsomal CYP1A2 catalyzed 4-hydroxylation of acetanilide by I33'.** Incubations were performed as described in Materials and Methods and included 0.5 mg/ml protein. Points represent the means of 2 determinations ± range. V_{max} = 95 ± 19 pmol/min/mg protein; K_m = 500 ± 208 μM; K_i = 7.6 ± 4.1 μM I33'.

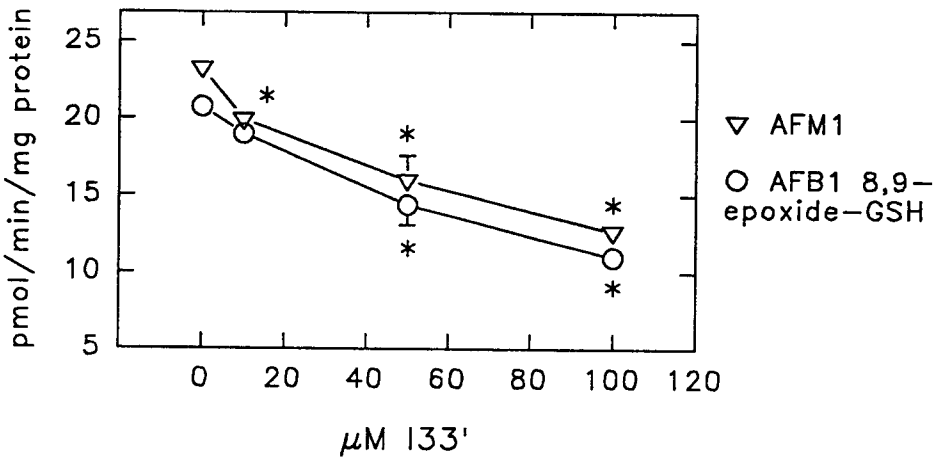

Figure 4.7. **Inhibition of BNF induced rat microsomal AFM_1 formation and AFB_1 8,9-epoxide-GSH conjugate formation by I33' at an AFB_1 concentration of 16 μM.** Control activity was 23.2 ± 0.6 pmol AFB_1 8,9-epoxide-GSH conjugate/min/mg protein and 20.8 ± 0.5 pmol AFM_1/min/mg protein. Values represent the means of 3 determinations ± SE. Bars marked by * are significantly different than control ($p \leq 0.05$).

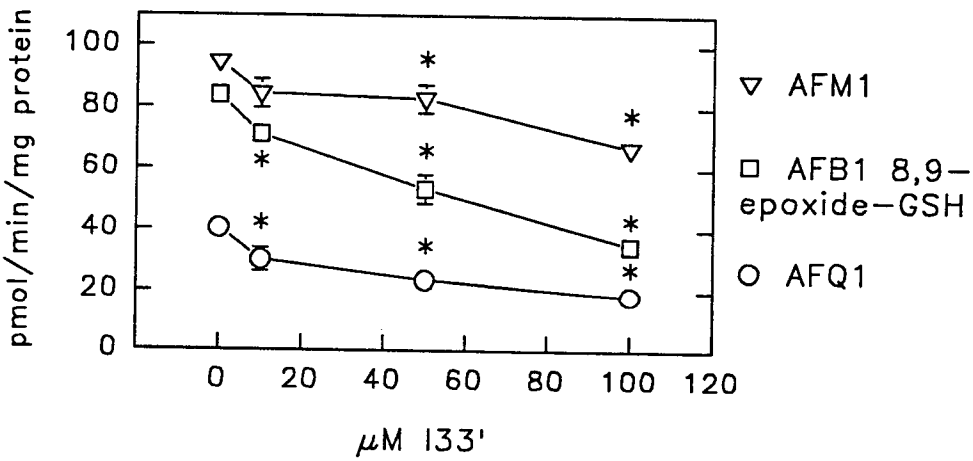

Figure 4.8. **Inhibition of BNF induced rat microsomal AFM_1 formation, AFB_1 8,9-epoxide-GSH conjugate formation, and AFQ_1 formation by I33' at an AFB_1 concentration of 124 μM.** Control activity was 94.6 ± 1.0 pmol AFM_1/min/mg protein, 84.0± 2.5 pmol AFB_1 8,9-epoxide-GSH conjugate/min/mg protein and 40.5 ± 1.0 pmol AFQ_1/min/mg protein. Values represent the means of 3 determinations ± SE. Bars marked by * are significantly different that control ($p \leq 0.05$).

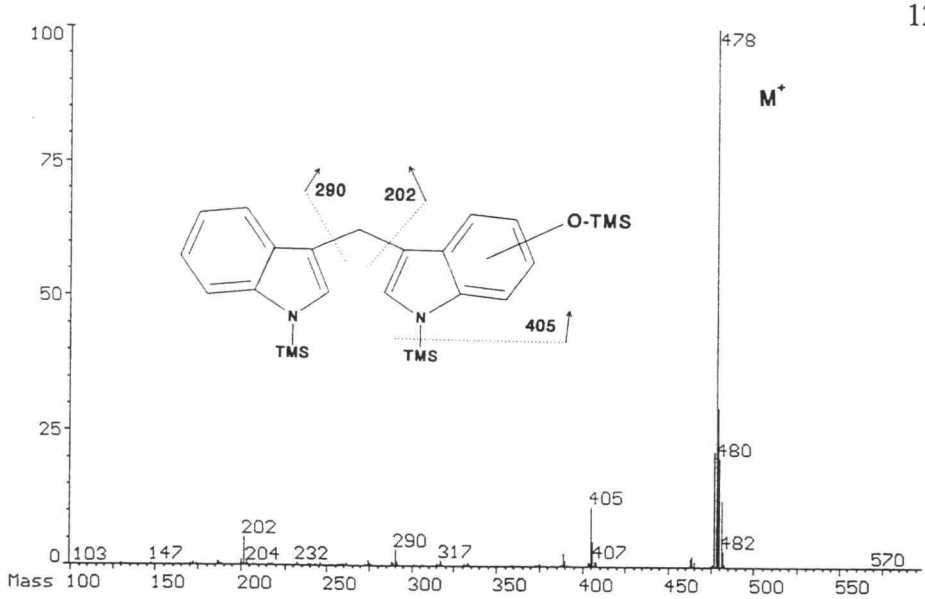

Figure 4.9. **Mass spectra of an I33' metabolite isolated by HPLC from a 30 min incubation of rat liver microsomes in the presence of an NADPH regenerating system and 100 μM [^{3}H]I33'.** The *m/z* ratio is consistent with a trimethylsilated derivative of monohydroxylated I33'.

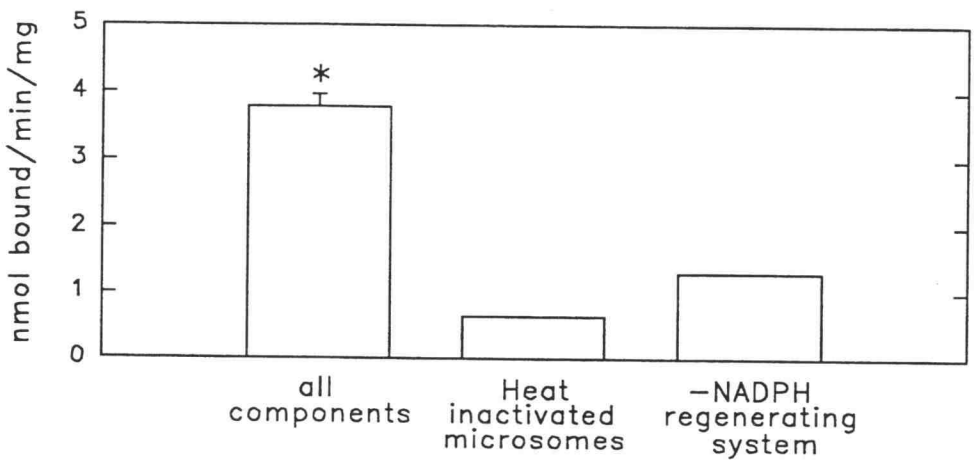

Figure 4.10. **Covalent binding of [^{3}H]I33' equivalents to liver microsomal protein from BNF pretreated rats.** The results are the mean ± SE of 3 samples containing all components of an intact microsomal metabolizing system. Single control incubations were performed concurrently with heat treated microsomes or the with the complete system in the absence of NADPH. * = significantly different from the combined controls at $p < 0.05$.

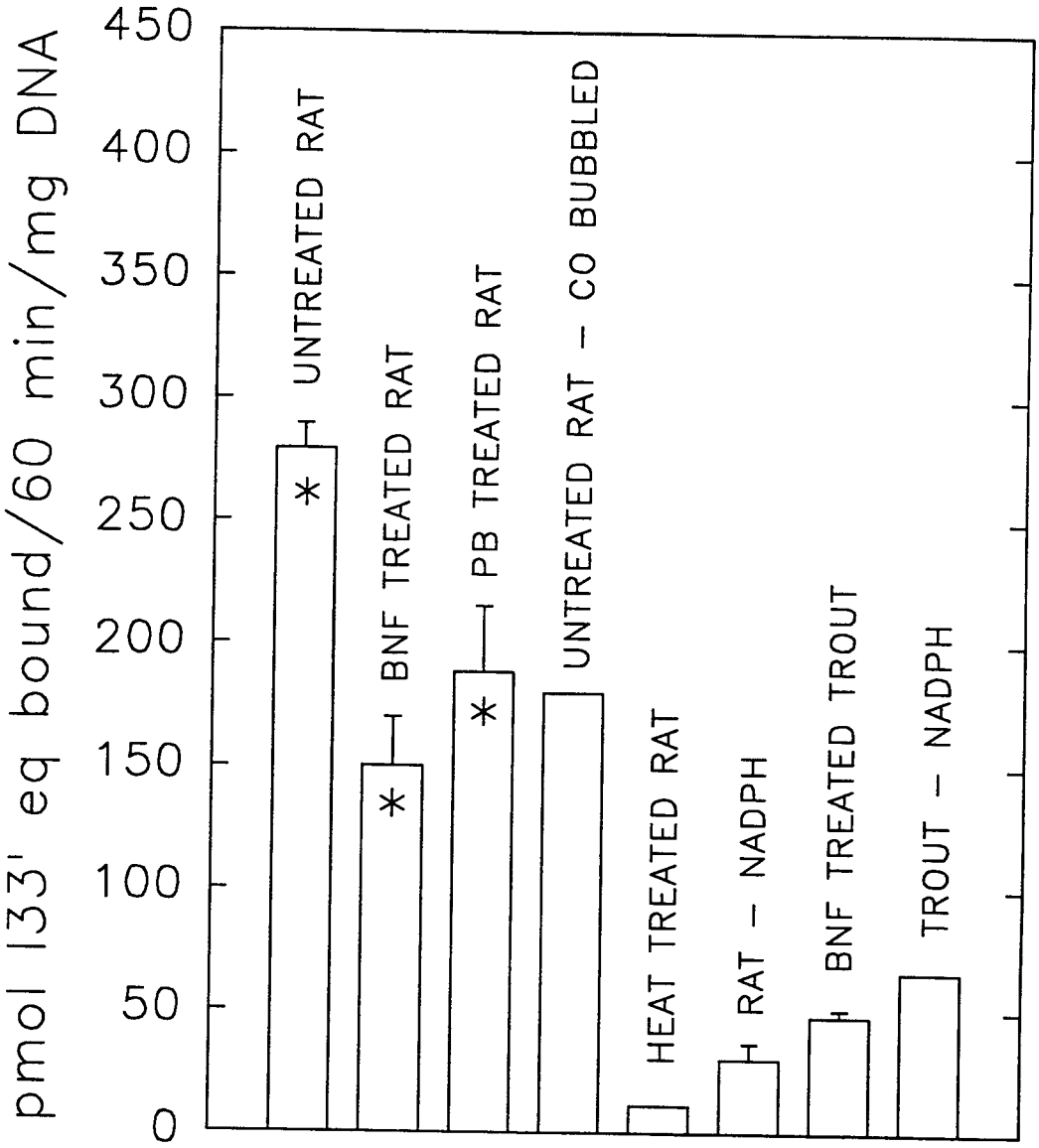

Figure 4.11. **Covalent binding of [^{3}H]I33' equivalents to calf thymus DNA.** Results are expressed as the mean ± SE of 3-5 samples, except for the PB treated group which is expressed as the mean ± the range of two samples and untreated rat-CO, heat inactivated rat microsomes, and BNF treated trout microsomes for which one sample was obtained. The experiment was performed as described in the Materials and Methods. Incubations were terminated at 60 min and performed at 37°C for rat samples or 29°C for trout samples. * = significantly different from rat microsomal controls lacking NADPH at $p < 0.05$. Statistical comparisons were not made with controls for which a single data point was obtained.

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Chapter 5

DISPOSITION AND EXCRETION OF [3-H]INDOLE-3-CARBINOL IN MALE FISCHER 344 RATS

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ABSTRACT

The naturally occurring indole, indole-3-carbinol (I3C), is a potent inhibitor and promoter of experimental carcinogenesis. However, little detailed information is available regarding the biodistribution of I3C-derived products into target tissues. To better understand its mechanism of tumor modulation, we have examined the disposition and excretion of [^{3}H]I3C in male Fisher 344 rats by continuous feeding and by bolus administration. Steady state urinary excretion was attained 40 hours after the start of dietary administration (0.32 % [^{3}H]I3C diet for 1 d; 0.20% [^{3}H]I3C for the next 6 d) whereas fecal elimination reached steady state levels at 112 hours and, under steady state conditions, 77% of the recovered label is found in the feces. After 7 d on the diet, some rats were euthanized to determine steady state tissue levels or were returned to the semipurified diet and were euthanized 24 or 48 hr later. A mean of 1154 μM I3C equivalents were in the liver at 0 hr and this decreased to 643 and 411 μM 24 and 48 hr later, respectively. Mean levels in lung decreased from 436 to 219 μM and blood levels decreased from 320 to 208 μM over the same 48 hr period. When given a bolus of 1 mmol/kg [^{3}H]I3C (a comparable daily dose as in the feeding study), mean liver levels were 257, 283 and 541 μM at 1.5, 3 and 6 hr after dosing and these levels represented 0.97%, 1.34% and 2.45% of the total. Mean levels in kidney and blood were highest at 6 hr as well, but concentration in tongue and lung tissue and in urine were highest at 1.5 hr. HPLC analysis of ethyl acetate liver extracts from rats given a bolus dose revealed 24 [^{3}H]I3C-derived peaks that were, in general, more non-polar than the parent compound. Two major peaks were observed to be more polar than I3C. Three of the predominant non-polar peaks were identified as 3,3' diindolylmethane (a linear dimer of I3C) and [2-(indol-3-ylmethyl)-indol-3-yl]indol-3-ylmethane (a linear trimer), which are present in an *in vitro* acid-catalyzed I3C reaction mixture (RXM). The identity of a novel I3C

metabolite, present in the liver and in RXM was determined to be 1-(3-hydroxymethyl)-indolyl-3-indolylmethane (HI-IM). A major non-polar peak in the liver extracts, not present in RXM, was not identified. In general, levels of these products were between 0.5 and 6.1 μM at all three time points and maximum levels were reached at 6 hr post-dosing, except HI-IM, which was highest at 1.5 hr post-dosing. We have previously shown RXM or I33' to be inhibitors of rat cytochromes P-450 *in vitro*, with K_i values at or near the *in vivo* concentrations determined here. Thus, the presence of these oligomers in chemical carcinogenesis target tissues suggest the possibility that they may exert their anticarcinogenic effects, in part, by means of inhibition of cytochromes P-450 bioactivation of carcinogens.

INTRODUCTION

Indole-3-carbinol (I3C)[1] is found at high levels in cruciferous vegetables such as broccoli, Brussels sprouts and cabbage. Administration of I3C to experimental animals can inhibit hormonal or chemical carcinogenesis in mammals or fish and can provide protection at several target organs (1-7). Under some experimental conditions, I3C can promote carcinogenesis (8, 9), causing concern over its potential use as a chemoprophylactic in humans (10). Consumption of cruciferous vegetables is associated with reduced cancer incidence in humans (11, 12), but, besides indoles, numerous other tumor modulators such as isothiocyanates and dithiothiones, are found in crucifers. Further information regarding the mechanism(s) of tumor inhibition or tumor promotion is needed to better understand the risks or benefits for human consumption of purified I3C. An essential step towards elucidating these mechanisms is understanding the tissue distribution, metabolism and excretion in the experimental model. These data are available for the rainbow trout (13), and limited studies have been done in mammals (14-17). In this study, we have obtained quantal data of [^{3}H]I3C distribution after 7 d feeding or bolus administration. In addition, by means of EI-MS and ^{1}H-NMR, we have elucidated the structure of a novel I3C metabolite, 1-(3-hydroxymethyl)indolyl-3-indolylmethane (HI-IM), found as a major component in liver extract *in vivo* and in an I3C acid catalyzed reaction mixture generated *in vitro*. The structure of HI-IM and other

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1 - **Abbreviations**: I3C, indole-3-carbinol; HI-IM, 1-(3-hydroxymethyl)indolyl-3-indolylmethane; I3A, indole-3-carboxaldehyde; I3CA, indole-3-carboxylic acid; I33', 3,3'-diindolylmethane; RXM, acid-catalyzed I3C reaction mixture; LT, [2-(indol-3-ylmethyl)-indol-3-yl]indol-3-ylmethane; CT, 5,6,11,12,17,18-hexahydroclonal[1,2-*b*:4,5-*b*':7,8-*b*]:triindole; ICZ, 3,2-*b*-indolocarbazole; DMSO, dimethylsulfoxide; BHT, butylated hydroxytoluene; MSTFA, N-methyltrimethylsilyltrifluoroacetamide; BSTFA, N, O-bis(trimethylsilyl)trifluoroacetamide; MTBSTFA, N-methyl-N-(t-butyldimethylsilyl)trifluoroacetamide; TMSIM, trimethylsilylimidazole; MBTFA, N-methyl-N-bis(trifluoroacetamide); NMR, nuclear magnetic resonance; nOe, nuclear overhauser effect; EI, electron impact; CI, chemical ionization; 2D-COSY, two dimensional correlated spectroscopy.

metabolites of I3C found in liver extracts after oral administration of I3C is shown in Figure 5.1.

MATERIALS AND METHODS

Chemicals.

The method of Dashwood *et al.*, (18) was used to tritium-label I3C at the 5 position. [^{3}H]I3C was diluted with unlabeled I3C carrier to an approximate specific activity of 125 mCi/mmol. The radiochemical purity of this compound was found to be approximately 90-95% based on HPLC analysis using a Beckman M171 Radioisotope detector/110B solvent delivery module (Palo Alto, CA). Unlabelled I3C, indole-3-carboxaldehyde (I3A), and indole-3-carboxylic acid (I3CA) were obtained from Aldrich (Milwaukee, WI). 3,3'-Diindolylmethane (I33') was synthesized according to the method of Leete and Marion (19) in a neutral solution and shown to be > 95% pure by HPLC analysis using UV detection at 280 nm. An acid-catalyzed reaction mixture (RXM) of products originating from I3C or [^{3}H]I3C was generated according to the method of Bjeldanes *et al.*, (14). The RXM contains I33' and [2-(indol-3-ylmethyl)-indol-3-yl]indol-3-ylmethane, a linear trimer (LT) of I3C, as major components, 5,6,11,12,17,18-hexahydroclonal[1,2-*b*:4,5-*b*':7,8-*b*]:triindole, a cyclic trimer (CT) of I3C, and 3,2-*b*-indolocarbazole (ICZ) as a very minor component. Identification of LT and CT in RXM was confirmed by co-elution with the authentic compounds. LT, CT and ICZ were provided by Dr. Leonard F. Bjeldanes of the University of California, Berkeley. To identify peaks representing I3C-derived products present in HPLC chromatograms of liver extracts, we used RXM, I33' and ICZ as standards.

Animals and Treatment.

Disposition of radiolabel was determined after continuous feeding and after a single oral bolus. For the feeding study, 10 male Fischer 344 rats (181-207 g)

(Simonsens, Gilroy, CA) were housed individually in stainless steel metabolism cages with free access to food and water. Rats were maintained under controlled conditions of temperature (21 ± 1°C) and humidity (50 ± 10%) and a light/dark cycle of 12 hr. Following a one week adjustment period, animals were fed a powdered AIN76 semipurified rat-mouse diet (US Biochemical Corp, Cleveland, OH) diet containing 0.2% [^{3}H]I3C (sp. activity 10 μCi/mg) for six days following one day at 0.32% [^{3}H]I3C. Animals received diets prepared fresh just prior to the dark cycle. Based on the amount of diet consumed, rats received a mean dose of 0.88 mmol ± 0.074 mmol I3C/kg body weight/24 hr period (n=10) during the last six days. Of this, 71.3% ± 14.8 (n=10) was consumed within the first 10 hr and 94.9 % ± 9.4% within 16 hr.

I3C is well known to be an unstable compound (13, 20). When prepared by adding an ethanolic solution of [^{3}H]-I3C directly to the diet, as here, I3C reacts over time to form three prominent ethyl acetate extractable compounds - an unidentified compound, I33' and, to a lesser extent, LT. It was found that extractable I3C in the diet was approximately 85% radiochemically pure at 0.67 hr and purity declined to 40% after 24 hr at ambient temperature. The efficiency of [^{3}H]I3C equivalents extraction declines over the same time period (94% at 0.67 hr to 76% at 24 hr), demonstrating the formation of product(s) not extractable by ethyl acetate. The ethyl acetate extractable compounds are present in RXM and in stomach contents after oral administration of 90-95% pure I3C (14) [2]. By dietary treatment or by oral gavage, I3C undergoes extensive polymerization in the acid conditions of the stomach prior to absorption and subsequent biodistribution (14) [2]. Thus, for the feeding study, our data reflect the disposition of [^{3}H]I3C that had

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2 - D. M. Stresser, unpublished observations.

already undergone partial polymerization, which would be typical of any animal (or human) dietary treatment with initially pure I3C.

After the treatment period of seven days, four animals were anesthetized with CO_2 and their livers perfused with 0.9% (w/v) NaCl solution containing 10 USP units heparin/ml via the portal vein. The remaining animals were returned to semi-purified diet without I3C and killed 24 or 48 hr later. All animals were killed by CO_2 asphyxiation. The livers and lungs were excised, rinsed with saline and stored at -80°C until analysis. Blood was collected from the vena cava in a syringe pre-rinsed in heparin solution (1000 USP units/ml) and stored at 4°C until analysis. Urine and feces were collected throughout the experiment and stored at -20°C until analysis.

For the gavage study, 16 male Fischer 344 rats (152-183 g)(Simonsens) were housed individually in stainless steel metabolism cages and had free access to food (AIN76 semipurified rat-mouse diet) and water. Rats were maintained under identical conditions as in the feeding study. Following a one week adjustment period, treatment animals received unlabeled I3C dissolved in DMSO, corn oil suspension (1.25% DMSO v/v; 0.1 mmol I3C/ml vehicle) at dosage of 1 mmol/kg body weight by gavage for six days. On day seven, treated animals received [5-^{3}H]I3C (3.25 mCi/kg body weight) at the same dosage. Control animals received vehicle only during the seven day period. All gavage solutions were prepared fresh the night before administration and stored at 4°C until use. Animals were anesthetized with sodium pentobarbital (nominally 90 mg/kg, ip) and their livers perfused with a 0.9% w/v NaCl solution containing heparin. Animals were killed (pneumothorax) under anesthesia on four successive days at 1.5, 3 and 6 hr after the radiolabelled dose was given. A 500 µl aliquot of blood was taken from the vena cava. Livers, lungs and kidneys were excised, rinsed with saline and stored at -80°C until analysis. The stomachs were ligated at the distal opening prior to excision.

Contents and a 5 ml wash with 0.9% w/v NaCl solution were collected after cutting just above the ligation by pressing the material through the resultant opening. At this time, the pH of the content plus washing was recorded. The proximal 20 cm of small intestine was excised and the contents were carefully collected by pressing the tissue between the fingers, followed by a 5 ml wash (also collected) with 0.9% w/v NaCl. The tongues were excised by making an incision approximately 3 cm distal to the tip and at the point of attachment at the base of the organ. Finally, an aliquot of urine was collected by puncture of the bladder wall. Abnormally high levels of [^{3}H]I3C equivalents were subsequently found in the lung tissue of one rat killed at the 1.5 hr time point, together with abnormally low levels in other tissues. This suggests the possibility of aspiration of gavage media and consequently the data from this animal were not included herein.

Quantification of Radioactivity in Tissues, Gastrointestinal Contents, Urine and Feces

Tissues were homogenized in three (liver and kidney), five (lung) or twenty (tongue) volumes 0.1 M KPO_4, 0.25 M sucrose, 1 mM EDTA, 0.1% BHT, pH 7.4. Stomach and intestinal contents were thoroughly mixed at the highest speed on a Vortexer II just prior to sampling. Aliquots of stomach and intestinal contents (10 µl) and homogenate or blood (25-75 µl) were digested in BTS-450 tissue solubilizer (Beckman) according to the manufacturer's instructions and color reduced (except gastrointestinal contents) with an appropriate volume (usually 200 µl) of 30% hydrogen peroxide. After samples attained ambient temperature, 15 ml of 3a70B liquid scintillation cocktail (Research Products International, Mount Prospect, IL) were added. Feces were homogenized in 10 volumes of water and 100 µl aliquots were digested in 500 µl NCS tissue solubilizer overnight at 45 °C prior to addition of 200 µl of 30% hydrogen peroxide. DPMs in urine (5 µl) were determined without treatment. All samples were

stored in the dark for 24 hr prior to quantifying radioactivity using a Beckman LS7500 liquid scintillation counter.

HPLC Analysis of I3C and Metabolites

For routine analysis of I3C, RXM or components of RXM, we used a Beckman ODS 5μ 4.6 × 250 mm column. Starting conditions were 20% acetonitrile (Solvent A) and 80% Milli-Q water (Millipore Corp., Bedford, MA)(Solvent B). These conditions were held for 30 sec, before changing to 15% solvent B over the next 29.5 min (linear gradient). This ratio was held for 5 min, then programmed to 0% solvent B over the following 5 min, held for an additional 5 min, then returned to starting conditions over the next 10 minutes. The total flow rate was 1 ml/min. Metabolites were monitored by UV absorbance at 280 nm using a Shimadzu SPD-6AV spectrophotometer detector (Kyoto, Japan), by radioactivity using a Beckman M171 radioisotope detector/110B solvent delivery module and liquid flow cell (scintillation cocktail flow rate was 2 ml/min) or by fraction collection using a Gilson model 203 fraction collector (Middleton, WI) followed by liquid scintillation counting. Retention times for I3C and products were: **M1**, 5.7 min; **M2**, 6.3 min; I3C, 8.3 min; I3CA, 9.6 min; I3A, 10.4 min; HI-IM, 21.7 min; **M3**, 24.0 min; I33', 24.4 min; ICZ, 25.0 min; CT, 27.0 min; LT, 28.7 min. To characterize and quantify individual components present in hepatic tissue following gavage of [^{3}H]I3C, liver homogenates (about 10 ml) were extracted three times in three volumes ice cold ethyl acetate containing 0.001% BHT. Using this method, extraction efficiency of [^{3}H]RXM from untreated rat liver homogenate was greater than 95%. Liver extracts from treated rats were evaporated under vacuum on an ice bath. The yellow-green residue was centrifuged at 14,000 g for 30 sec in a TOMY-Seiko MC-150

microcentrifuge (Tokyo, Japan) and 20 µl was injected directly onto a Beckman ODS 5µ 4.6 × 250 mm analytical column and detected as described above. To remove adhered lipids, the column was washed sequentially between samples with acetonitrile:isopropanol, acetonitrile:chloroform and acetonitrile:isopropanol, using a 20 min gradient from 100% acetonitrile to 50% acetonitrile to 100% acetonitrile for each of the three solvent pairs. Recovery of radioactivity applied to the column was an estimated 79.8 ± 5.9% prior to washing and this was not corrected for in the final analysis. Radioactivity associated with individual peaks were quantified by fraction collection followed by scintillation counting, with correction for background. When mass spectrometry was used to identify metabolites present in liver extracts, we collected fractions and evaporated the acetonitrile-water solvent directly under a stream of nitrogen gas, or evaporated the acetonitrile portion and extracted the aqueous fraction by ethyl acetate, followed by solvent removal under a stream of nitrogen gas. The dried samples were stored for 24 hr under argon in a 400 µl glass insert (Alltech) within a 1 ml vial containing desiccant prior to GC/MS.

Mass Spectrometry Instrumentation and Sample Derivatization

Low resolution electron impact (EI) and chemical ionization (CI) mass spectral analysis were performed on a Finnigan model 4023 quadrupole mass spectrometer upgraded with a model 4500 source and a Varian model 3400 gas chromatograph at the Environmental Health Sciences Center at Oregon State University. The mass spectrometer is controlled by a Galaxy 2000 data system (LGC Co., San Jose, CA). EI and CI mass spectra were obtained at an electron energy of 70 eV at source temperatures of 140°C and 120°C, respectively. Samples were applied to the column using splitless injection, with an injector temperature of 275°C and a transfer temperature of 300°C. The

carrier gas was purified oxygen-free helium. The initial column temperature was 60°C and was increased at a rate of 20 degrees/min to 300°C. This temperature was held for 10 min. Chromatography was performed using either a J&W (Rancho Cordova, CA) DB-1 column (6 m × 0.25 mm i.d., with a 0.1 μm coating) or an Alltech (Deerfield, Il) SE-54 column (10 m × 0.25 mm i.d., with a 0.25 μm coating) operated at injector pressures of 3 psi and 4 psi, respectively. We found that methylation, trimethylsilylation or t-butyldimethylsilylation of active hydrogens on the indole, as well as the alcohols in the various compounds, confer excellent thermal stability, thus facilitating GC/MS analysis. Of several derivatizing agents tried, optimal results were obtained with N-methyltrimethylsilyltrifluoroacetamide (MSTFA), which was the more aggressive and volatile reagent. Other reagents used with good success were MethElute (0.2 M trimethylanilinium hydroxide in methanol), (Pierce Biochemical Co., Rockfield, IL), N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and N-methyl-N-(t-butyldimethylsilyl)trifluoroacetamide(MTBSTFA). The latter reagent confers resistance to hydrolysis, permitting sample manipulation and longer storage times, but higher temperatures are required to elute these high molecular weight derivatives, making the mass spectrometry somewhat more difficult. Poor results were obtained using trimethylsilylimidazole (TMSIM). Because of its expected low levels *in vivo*, special consideration was given to ICZ to optimize sensitivity. High sensitivity was achieved by making the trifluoroacetyl derivatives using N-methyl-N-bis(trifluoroacetamide)(MBTFA) as a derivatizing agent, and using electron capture negative chemical ionization mass spectrometry. MBTFA can also selectively derivatize trimethylsilyl derivatives at the amine nitrogens, leaving *O*-derivatives intact. We were thus able to dual derivatize HI-IM, which helped confirm its structure. Derivatization was achieved by dissolving the

water-free sample in 20 µl of anhydrous pyridine followed by 20 µl of the selected agent. The septum topped vial was heated at 60°C for 0.5 hr prior to injecting nominally 4 µl of sample into the GC.

Nuclear Magnetic Resonance Instrumentation

1D-proton NMR, nOe, and 2D-COSY spectra were obtained on a Bruker AM400 spectrometer at the Department of Chemistry at Oregon State University. Analysis was performed in deuterated methanol using tetramethylsilane as the internal standard. Chemical shifts are reported in ppm relative to the internal standard.

Isolation and NMR and MS data for a Novel I3C Acid Condensation Product

A major [^{3}H]I3C derived product found in the livers of rats given [^{3}H]I3C by oral gavage was synthesized *in vitro* using I3C as a starting material. I3C (5.0 g) was refluxed in 1 liter of Milli-Q water for 5.25 hr and the mixture cooled overnight at ambient temperature. Crude I33' was vacuum filtered and twice recrystallized from benzene to obtain >95% pure I33' in 22% yield. In an attempt to obtain previously uncharacterized I3C oligomers found in liver extracts in concentrated yield for subsequent purification by HPLC, the benzene supernatant from the initial I33' recrystallization was boiled to obtain a reddish residue. Ethanol was added (10 ml) to dilute the mixture to an estimated 114 mg/ml and the entire solution was filtered through a 0.45 µm nylon filter. Upon HPLC analysis, it was observed that a peak with a R_t of 21.7 min was obtained in high yield (3-4% of total at A_{280}) and was well resolved from all other I3C-derived components, even at high concentrations of up to 1.1 mg/injection. Repeated injections and collection, followed by removal of acetonitrile under a stream of nitrogen, ethyl acetate extraction, and solvent removal under vacuum, afforded enough material to perform ^{1}H-NMR.

Mass spectra of the *O*-trimethyl silyl, *N*-trifluoroacetyl derivative: *m/z* 445(16), 444(49, M+), 414(9.5), 355(4.9), 257(4.4), 226(7.9), 103(39), 73(100). NMR: [methanol-d_4, 25°C] δ 4.17 (s, 2H, CH_2), 5.48 (s, 2H,CH_2OH), 6.93 (dd, J = 7.8 Hz, 1H, H-5'), 6.96 (s, 1H, H-2), 6.97 (s, 1H, H-2'), 7.01 (dd, J = 7.4, 1H, H-6) 7.05 (dd, J = 7.4, 1H, H6'), 7.14 (ddd, J = 7.6, 7.6, 1.0, 1H, H-5), 7.31 (d, J = 8.0, 1H, H-7'), 7.45 (d, J = 8.8, 1H, H-4), 7.49 (d, J = 8.0, 1H, H-4'), 7.53 (d, J = 7.2, 1H, H-7').

Other Mass Spectrometry Data

***N, O*-Trimethylsilyl derivative of HI-IM**: *m/z*, 421(46), 420 (100, M+), 419(19), 390(3.0), 389(5.4), 331(4.9), 330(4.4), 329(5.7), 318(9.2), 317(33), 290(2.0), 257(3.3), 202(21), 103(7.7), 73(99).

***N, N*-Trimethylsilyl derivative of I33'**: *m/z*, 391(41), 390(100, M+), 389(49), 375(2.3), 318(4.3), 317(14), 301(2.6), 246(1.4) 245(1.4), 202(7.5), 73(54).

***N, N*-Trimethylsilyl derivative of LT**: *m/z*, 592(3.2), 591(3.8, M+), 403(43), 402(100), 401(12), 387(2.2), 329 (4.3), 313(3.9), 247(1.6), 202(2.6), 189(2.6), 147(7.1), 117(3.5). Data for masses below 100 were not obtained.

***N, N*-Trifluoroacetyl derivative of ICZ**: *m/z*, 449(24), 448(100, M+), 352(35), 351(27), 208(15). Data for masses below 150 were not obtained.

Experiment to Assess Possible Tritium Exchange

To assess any tritium exchange of the 5-label, or exchange of non-specific label at the nitrogen or other positions, 10 µl of 0.034 µCi [^{3}H]I33' in ethanol was counted directly in triplicate or after removal of ethanol followed by resuspension in homogenate buffer and storage of 0, 1, 2, 10 and 44 days. After these time periods, the buffer was

removed under a stream of nitrogen gas and the radioactivity determined by scintillation counting.

RESULTS

Elimination of I3C Equivalents

Figure 5.2 shows rates of fecal and urinary elimination of I3C eq in rats fed semi-purified diets containing 0.2% [^{3}H]I3C. We did not identify fecal elimination processes therefore fecal elimination may include processes such as biliary excretion or simple passage of I3C equivalents not absorbed. Steady state urinary excretion was observed by 40 hr after the start of the diet whereas fecal elimination was not at steady state levels until 112 hr. During steady state, 21.3 mg I3C equivalents/d could be recovered in urine and feces, accounting for 75% of the dose based on mg I3C consumed per day. During steady state, a mean of 77% of the recovered label was eliminated in the feces while a mean of 23% was excreted in the urine. Elimination rates had fallen to 53% (averaged over the previous 24 hr) and 24% (averaged over the previous 14 hr) of steady state levels for feces and urine respectively by 48 hours after animals were returned to the control diet in the absence of I3C. In rats given [^{3}H]I3C by gavage, concentrations of [^{3}H]I3C equivalents in urine were 23.1 $\pm$ 3.6 μM, 10.4 $\pm$ 6.1 μM and 9.4 $\pm$ 3.4 μM at 1.5, 3 and 6 hr after administration, respectively.

Stomach Emptying and Blood and Organ Disposition

Figure 5.3 shows concentrations of [^{3}H]I3C equivalents present in liver, lung and blood after rats had been fed a 0.2% [^{3}H]I3C diet for seven days or seven days 0.2% [^{3}H]I3C diet followed by one or two days feeding of control diets. Based on the amount of diet consumed, rats received an estimated 0.88 mmol/kg body weight per day (n=10) during the last six days. A mean of 1154 μM I3C equivalents were in the liver at the end of seven days and this decreased to 643 and 411 μM after one and two days,

respectively. Mean levels in lung decreased from 436 to 219 μM and blood levels decreased from 320 to 208 μM over the same two day period. Figure 5.4 depicts the levels of μmol [^{3}H]I3C equivalents in the stomach contents and intestinal contents (proximal 20 cm) at 1.5, 3 and 6 hr when different animals are given a bolus of 1 mmol/kg [^{3}H]I3C (a comparable daily dose as in the feeding study). The stomach contained mean levels of 351, 443 and 281 μmol [^{3}H]I3C/kg body weight at the three time points, representing 35%, 44% and 28% of the dose, respectively. Corresponding mean levels in the intestinal contents were 33, 31 and 12 μmol [^{3}H]I3C/kg body weight, representing 3.3%, 3.1% and 1.2% of the dose respectively. These data indicate that appreciable stomach emptying has occurred by 1.5 hr with approximately 72% cleared by 6 hr. The pH of stomach contents was estimated to be 4.26 ± 0.30 (mean of 15 animals ± SD) and did not appear to be influenced by the presence of I3C or the time at which the animals were killed. Figure 5.5 depicts μM equivalents I3C found in liver, kidney, lung, tongue and blood at 1.5, 3 and 6 hr following gavage of [5-^{3}H]I3C. Mean levels were highest at 6 hr after dosing, except in the tongue and lung, where maximal levels were observed at 1.5 hr. Mean liver levels were 257, 283 and 541 μM at 1.5, 3 and 6 hr after dosing and these levels represented 0.97%, 1.34% and 2.45% of the total dose given. Corresponding percentage data for other tissues were 0.20%, 0.17%, and 0.20% (kidney), 0.061%, 0.024% and 0.054% (lung) and 0.0075%, 0.0023%, and 0.0035% (tongue). When [^{3}H]I3C-derived radiolabelled products were extracted from hepatic tissue of rats given [^{3}H]I3C by gavage, it was observed that the percentage of extractable radioactivity by ethyl acetate declined over time from 46% ± 6% to 35% ± 5% and 31% ± 5% at the 1.5, 3 and 6 hr time points respectively. This occurred despite an overall increase in extractable radioactivity and [^{3}H]I3C derived products. This decreasing trend

in extraction efficiency may be consistent with low efficiency of extraction of liver homogenates from rats killed after seven days of dietary [^{3}H]I3C (16.92% ± 4.11%). However, extraction efficiency of [^{3}H]RXM from untreated liver homogenates was greater than 95%, thus we believe that [^{3}H]I3C derived products not extracted by ethyl acetate in treated animals represent compounds other than those found in [^{3}H]RXM.

Quantification of I3C Acid Condensation Product In Vivo

Figure 5.6 shows a comparison of HPLC chromatograms of, RXM generated from I3C *in vitro*, and an ethyl acetate extract of a liver taken from a rat killed three hr after oral gavage of 1 mmol [^{3}H]I3C/kg body weight, monitored at a wavelength of 280 nm. The lower chromatogram in the bottom panel shows the radioactivity associated with some of the peaks in the liver extract. Preliminary analysis using an online radioisotope detector indicated that the major radioactive peaks fall in the selected windows. It was found that fraction collection was necessary to achieve adequate sensitivity and resolution of radiolabelled peaks. Using the online detector, it was observed that at least 24 radiolabelled peaks are present in liver extracts of animals killed six hr after dosing. Many of these peaks have identical R_t of peaks present in RXM. HPLC analysis of control liver extract revealed the presence of endogenous compounds absorbing at 280 nm with prominent peaks eluting at approximately 4.6, 11.4, 14.8, and 22.4 min. In animals given [^{3}H]I3C by gavage, the 22.4 min peak was not resolved from a labeled peak, suggesting the possibility that an I3C-derived structure may be identical to a compound produced endogenously, for example as a tryptophan metabolite. In addition, other smaller peaks in control extract appeared to have R_t similar to I33', HI-IM and LT, though we do not propose identity.

The six most consistently abundant radiolabelled HPLC peaks were quantified as shown in Figure 5.7. Three peaks, **M1**, **M2**, and **M3**, were not identified, whereas the other three were identified as I33', LT and HI-IM by mass spectrometry and/or co-elution with authentic standards. Mean levels of the six metabolites ranged from 0.5 to 6.1 μM I3C equivalents (nmol/g liver) and were highest at the 6 hr time point with the exception of HI-IM, which was most abundant at the 1.5 hr time point. The sum of the six metabolites accounted for 24%, 20%, and 24% of radiolabel recoverable from the HPLC effluent at 1.5, 3 and 6 hr after dosing. Metabolites were marked by considerable quantitative variability among animals but were qualitatively consistent. It was observed that **M1** and **M2** eluted earlier than co-injected [^{3}H]I3C. In contrast to **M3**, I33', LT and HI-IM, the peak shapes of **M1** and **M2** were quite broad and R_t were more variable. **M1** and **M2** exhibited nominal R_t of 5.1 and 6.0 min respectively, earlier than either I3A (10.3 min) or I3CA (9.6 min). The latter has been previously reported to be a major plasma component after oral administration of I3C (21). A labeled and UV absorbing compound with the R_t of I3A was found to be the largest peak detected in one rat killed at the 1.5 hr time point, but in most samples this peak was not detected or present only at very low levels. No obvious peak was observed at the R_t of I3CA or I3C nor could we detect the presence of I3C by MS analysis of corresponding fractions. When [^{3}H]I3C is extracted from untreated rat liver homogenate, 95% of the radiolabel is recovered. There is only a slight degradation of I3C during the extraction process (95.5% pure prior to extraction, 86.6% pure after extraction). Thus it appears that I3C itself is not a major product *in vivo* after oral administration of I3C. Attempts to collect **M3** for mass spectrometry analysis were unsuccessful as it appeared to be quite unstable. Preparation of RXM using a 10-fold excess of 3-methyl indole, which may be present in the GI tract, yielded a single major peak (besides 3-methyl indole) with an HPLC R_t of 27.9 min, but

no peak was observed with the R_t of **M3**. Preparation of "RXM" using L-tryptophan in place of I3C as a starting material similarly failed to yield a peak corresponding to **M3**. However, several small peaks with identical R_t as HI-IM, I33' and LT were observed. This latter result may explain the presence of peaks with these R_t in control liver extracts.

An I3C derived compound of major interest is ICZ, a high affinity ligand for the *Ah* receptor (14). This compound elutes at 25.0 min on our HPLC system, but no obvious radiolabelled peak at this R_t was detected in liver extracts from rats given [^{3}H]I3C by gavage. This was not unexpected because of the previous observation of its low yield *in vivo* (14). We attempted to quantify ICZ in rat liver extract by collecting HPLC fractions between 24.7 and 25.3 min (R_t of authentic ICZ = 25.0 min) from the combined extracts of livers (equivalent to a total of 2.8 g of tissue) from three rats killed at the 6 hr time point. By derivatizing the HPLC isolate with MBTFA and using negative CI MS in multiple ion detection mode, we detected a compound with ions and a GC R_t consistent with that of authentic trifluoroacetylated ICZ (Fig. 5.8A). ICZ was monitored at masses of 351, 352, 448, and 449 (Fig. 5.8B). Using an ICZ standard curve obtained from a stock ICZ solution quantified by UV spectroscopy ($\log \varepsilon = 4.6$), we estimated a minimum *in vivo* liver content of ICZ from a single determination to be 439 pg/g tissue or ~ 0.000015% of the total dose given.

Assessment of Tritium Exchange

Only the nitrogen protons of [5-^{3}H]I3C or [5-^{3}H]I3C oligomers would be expected to undergo appreciable proton exchange with those available in the surrounding matrix. A small but significant 8% loss of label was observed after [^{3}H]I33' was resuspended in homogenization buffer followed by removal of buffer by a stream of

nitrogen and subsequent scintillation counting. There was no significant change when stored for 0, 1, 2, 10 and 44 days at -80°C. Thus, we interpret these data to indicate that minimal loss of tritium may occur between the time of administration of [^{3}H]I3C and quantification of label in tissues and excreta. We did not apply an 8% correction of values reported herein.

Structural Characterization of 1-(3-Hydroxymethyl)indolyl-3-indolylmethane (HI-IM)

Figure 5.9 shows a ^{1}H-NMR spectrum of a compound isolated by HPLC from RXM as described in the Materials and Methods section. The appearance of singlets at 4.17, 5.48, 6.96 and 6.97 ppm indicate the presence of individual protons or proton groups not coupled to other hydrogens. The downfield signals at 6.96, 6.97 and other signals in the 6.8-7.6 ppm region are typical of aromatic protons. nOe's were observed at the singlet at 6.96 ppm and at the doublet at 7.45 ppm upon irradiation at 5.48 ppm, indicating that the 5.48 ppm signal represents a proton or proton group nearby in space. Irradiation at 4.17 ppm gave an nOe at both the 6.96 and 6.97 ppm aromatic singlets as well as the aromatic doublets at 7.49 and 7.53. These data, along with coupling data obtained from a 2D ^{1}H-^{1}H correlated spectrum (homonuclear COSY) of the aromatic protons (Fig. 5.10) allowed for the proton assignments. The assignments at each of the pairs 6.93 and 7.05 ppm, and 7.01 and 7.14 ppm, could be reversed, but do not affect proof of structure. The EI mass spectrum for the trimethylsilyl and trifluoroacetyl derivative of HI-IM and a plausible fragmentation pattern are shown in Figure 5.11. Because MBTFA can selectively derivatize trimethylsilyl derivatives at the amine nitrogens, leaving *O*-derivatives intact, we could determine the existence of one oxygen and one nitrogen atom available for derivatization. The mass spectrum of a *N,N*-trimethylsilyl derivative of a compound found in liver extracts (Fig. 5.12) is consistent

with the authentic *N, N*-trimethylsilyl derivative of HI-IM (see data in Materials and Methods section).

DISCUSSION

The purpose of these experiments was to quantify tissue levels and elimination rates of the naturally occurring tumor modulator I3C in male Fischer 344 rats after continuous feeding or after oral administration of a single bolus. Because I3C is under consideration as a potential chemopreventive agent (22), an assessment of the disposition of this compound after oral administration will aid in understanding the mechanism(s) by which I3C can modulate carcinogenesis. Levels of I3C administered in the feeding (23) and gavage (1) protocols have previously been shown to be capable of modulating tumor incidence.

After continuous administration of partially polymerized [^{3}H]I3C in the diet to male Fischer rats, we found the major route of elimination was initially urinary, but by 40 hr, fecal elimination became the major route, and by 110 hr was about three times that of urinary excretion. This would indicate that a substantial portion of the administered dose is either not absorbed from the GI tract or that biliary excretion is a major route. Dashwood *et al.* (12), indicated that biliary excretion is a significant excretory route in rainbow trout given [^{3}H]I3C by diet or oral gavage. Our data are in contrast to the results obtained by Potchoiba *et al.* (17), who found that approximately 80% of the label was recovered in the urine of goats given a 2 hr infusion of approximately 0.27 mmol [^{3}H]I3C/kg body weight by 72 hr. Because administration by this route bypasses the acid conditions in the stomach, the formation of higher molecular weight I3C oligomers may be suppressed, thus excretion pathways may be substantially different. Approximately 25% of the estimated daily intake of [^{3}H]I3C was not accounted for by urinary or fecal elimination during steady state elimination in the present study. Possible explanations for this observation include deposition of residual material on cages,

exhalation of volatile, labeled species, or uptake and incorporation of the label into tissues.

In the study described above, Potchoiba *et al.* (17), indicated approximately 1% of the total dose of [G^{3}H]I3C was accounted for by tritium exchange with body water and we observed only an 8% loss of label attributable to tritium exchange even after long term storage of [5-^{3}H]I33' in buffer. Thus, body water should not account for a substantial portion of disposition of the label in the present study. After 7 d on labeled diet, liver levels of [^{3}H]I3C equivalents exceeded that of lung and blood by about 2.6 and 3.6-fold, respectively. Most of the 1.15 mM [^{3}H]I3C equivalents was probably present in the form of water soluble products or possibly covalent adducts (see Chapter 4) since less than 20% was typically extractable by ethyl acetate, using a procedure that recovers >95% of RXM from liver homogenates.

For the gavage study, we chose to administer a dose (1 mmol I3C/kg body weight) known to be anticarcinogenic in the rat (1). In general, maximum tissue levels were observed at 6 hr after gavage. The rise in tissue levels between 1.5 and 6 hr was accompanied by a concurrent decrease in levels found in stomach contents. Because no plateau was indicated and because a substantial portion of the dose (28%) remained in the stomach after 6 h, it is probable that maximal tissue levels, particularly liver, were not yet attained. In mice, Shertzer *et al.* (16), found liver tissue maxima of 121 μM at only 0.5 hr after oral gavage of 0.34 mmol I3C/kg body weight. It was not clear whether the animals in the latter study were starved prior to administration, which may result in accelerated absorption. To mimic dietary conditions, we gavaged our animals nominally at 8:00 AM, when animals' stomachs contained a large proportion of the food consumed in the previous night. This is important because the profile of RXM products is known to vary with pH (15, 24), which rises in response to the buffering effect of proteins in food.

In particular, a major component of some RXM preparations (see refs 15, 24, 25) CT, becomes absent at pH >4.5 (15). This is consistent with our observations of only small amounts (less than the other six metabolites described here) of a peak with a R_t of authentic CT in liver extracts when stomach contents pH were approximately 4.26. de Kruif *et al.*(15), detected no CT in any rat tissues examined when pH of stomach contents was 4.5 to 5.5.

In animals given [^{3}H]I3C by gavage, we obtained quantal data for 6 metabolites present in liver extracts. Consistent with the results of Dashwood *et al.*(13) and de Kruif *et al.*(15), we found that I33' was a major metabolite present. Whereas Dashwood *et al.*(13) found that I33' comprised up to 40% of the total hepatic radiolabel in trout after 48 hr, we observed only 0.6% of the total hepatic radiolabel in the form of I33' at the 6 hr time point. The reasons for the apparent disparity are unknown. However, in contrast to the trout, the most abundant metabolites in rat liver appear to be associated with the aqueous fraction, suggesting the mammalian liver may efficiently metabolize and excrete RXM components and other ethyl acetate extractable metabolites. I33' is known to be a substrate for a rat liver microsomal and NADPH-dependent enzyme (see Chapter 4). In apparent contrast to Dashwood *et al.*(12), and de Kruif *et al.*(15), we found a major metabolite, **M3**, which eluted 0.4 min prior to I33'. This compound, unlike HI-IM, I33' and LT, does not appear to be present in RXM (See Fig. 5.6), or stomach or intestinal contents extract (data not shown), suggesting that it may be formed by a post-absorbtion process.

The presence of HI-IM in RXM or as an *in vivo* metabolite of I3C has not been previously reported. This may be identical to the compound reported by de Kruif *et al.* (15) to have an HPLC R_t of 32.5 min.

ICZ has been reported to possess *Ah* receptor binding affinity similar to that of the potent carcinogen and tumor promoter 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (14, 26). This suggests that ICZ may exert similar *Ah* receptor-mediated toxic effects. When I3C is given to rats, Bjeldanes *et al.*(14), estimated the pre-absorption, *in vivo* conversion to ICZ was on the order of 0.01%. We obtained an estimate that at least 0.000015% of the dosage of 1 mmol I3C/kg given to rats is converted to ICZ and is present in liver at 6 hr after oral gavage. Because approximately 2.45% of the total [^{3}H]I3C dose is present in the liver at this time, this corresponds to a minimum 0.0006% of total liver [^{3}H]I3C equivalents present as ICZ. The reason for the apparent discrepancy between ours and the results of Bjeldanes *et al.* is unclear. Because we did not use an internal standard, our estimate represents a minimum amount of ICZ present in liver. Another possibility is that other I3C equivalents may be preferentially absorbed or ICZ preferentially metabolized.

In conclusion, the data presented herein should be useful in elucidating the mechanisms by which I3C inhibits or promotes chemical or hormonal carcinogenesis in rats. Several mechanisms may exist for the chemopreventive action of agents such as I3C (27). Potential mechanisms by which I3C may inhibit tumors is induction of CYP enzymes (28) or glutathione transferases (29) that enhance the rate of elimination of carcinogens. Although I3C itself does not appear to be present *in vivo* after oral administration to rats, I3C has been shown to possess antioxidant properties which may influence tumorigenesis (16). Another possible mechanism is inhibition of CYP bioactivation of carcinogens. I33' has been shown to possess potent inhibitory activities toward CYP-dependent activities including inhibition of conversion of the hepatocarcinogen aflatoxin B_1 to its DNA binding 8,9-epoxide form (see Chapter 4). Although *in vivo* hepatic levels of I33' after oral gavage (2-4 μM) are below K_i values estimated for *in vitro* P-450 inhibition (K_{ii} - 52 μM, K_{is} - 138 μM), the total levels of

[^{3}H]I3C equivalents are well in excess of this value. The combined inhibition by the numerous I3C oligomers known to be present may be quantitatively important. The mechanism(s) of tumor promotion by chronic post-initiation I3C treatment, and the role of the individual I3C derivatives quantified herein, remain to be determined.

ACKNOWLEDGMENTS

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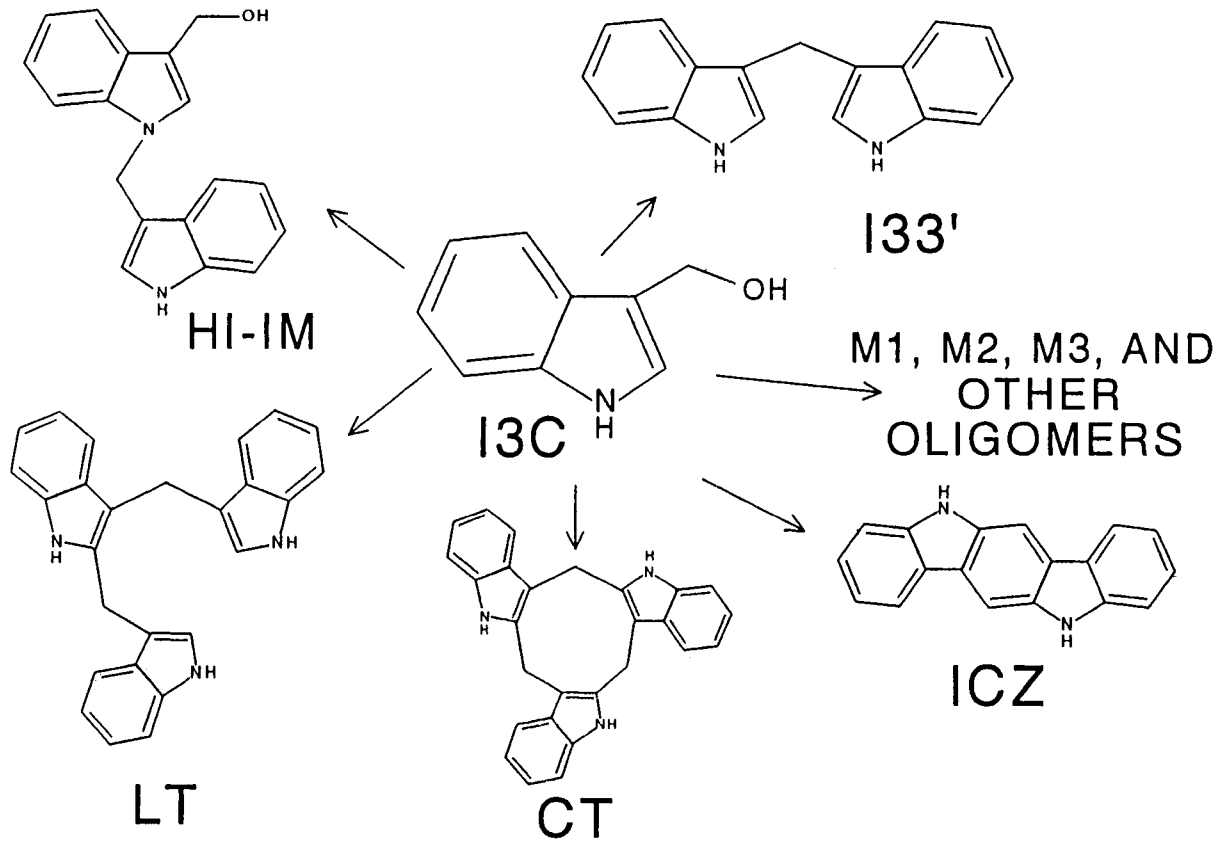

Figure 5.1. **Structures of I3C acid condensation products found in liver extracts of rats given I3C orally.** I3C itself was not detected in liver extracts. I33, 3,3'- diindolylmethane; LT, [2-(indol-3-ylmethyl)-indol-3-yl]indol-3-ylmethane; HI-IM, 1-(3-hydroxymethyl)indolyl- 3-indolylmethane; CT, 5,6,11,12,17,18-hexahydroclonal[1,2-*b*:4,5-*b*':7,8-*b*]:triindole; ICZ,3,2-*b*-indolocarbazole. **M1**, **M2**, and **M3** are also found in significant quantities, but their structures have not yet been elucidated.

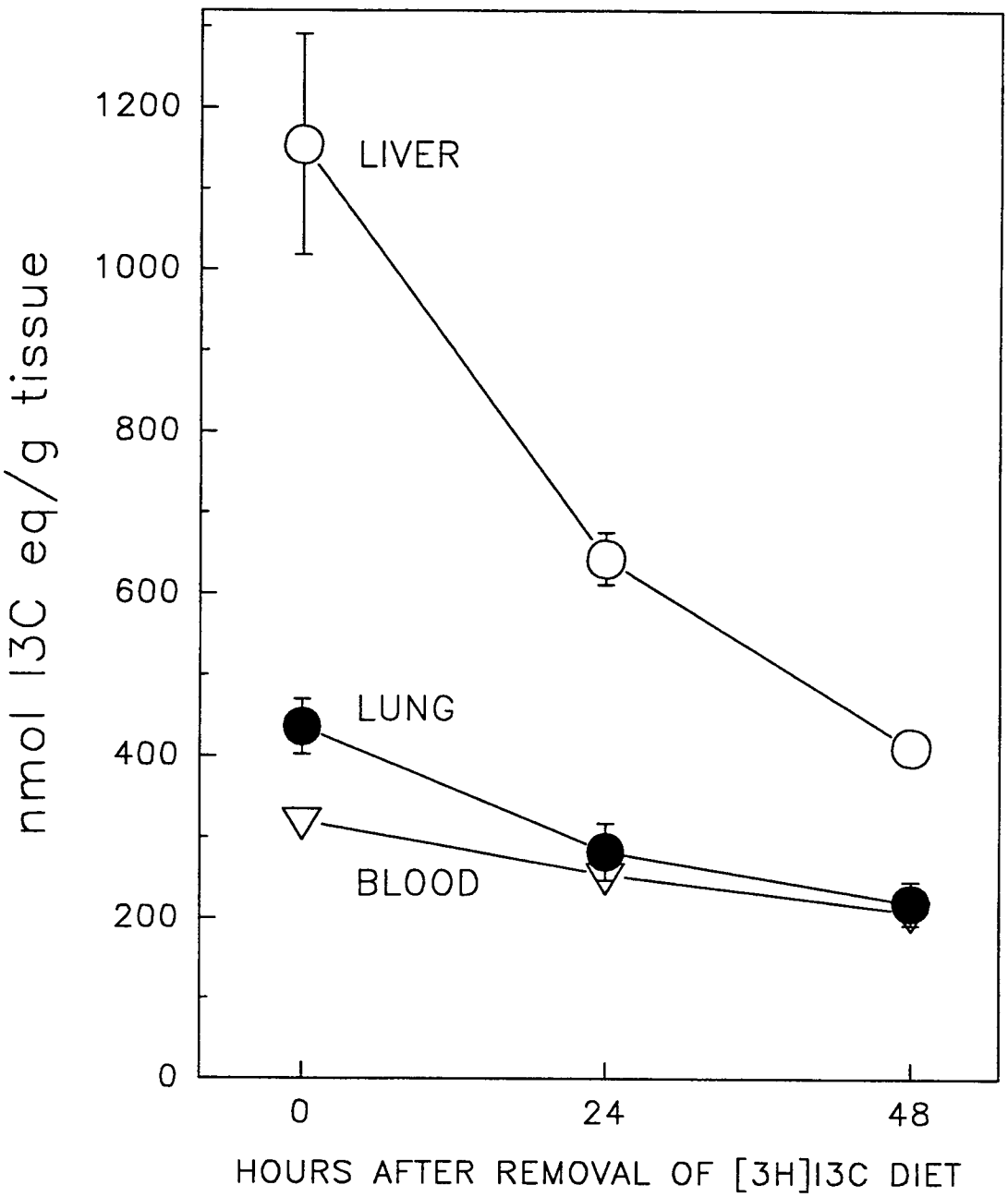

Figure 5.2. **Rates of fecal and urinary elimination of I3C eq in rats fed semi-purified diets containing 0.2% [^{3}H]I3C.** Values represent the mean ± SD of 3-10 rats.

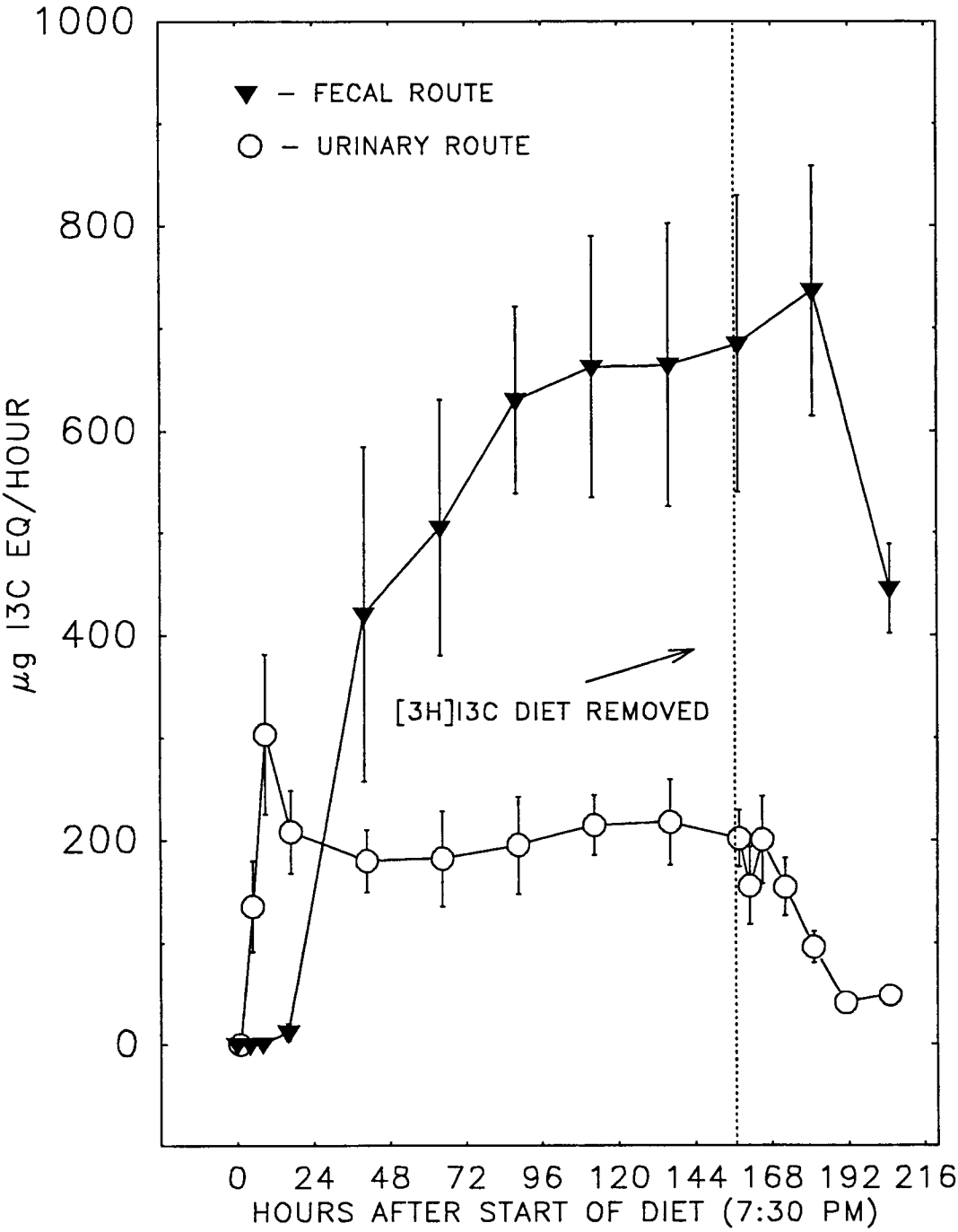

Figure 5.3. **Concentration of I3C equivalents in liver, lung and blood at steady state levels, 24 hr, and 48 hr after removal of the [^{3}H]I3C diet.** Rats received a mean daily dose of 0.88 mmol/kg during the last six days of the [^{3}H]I3C diet. Values represent the mean ± SD of 3-4 rats.

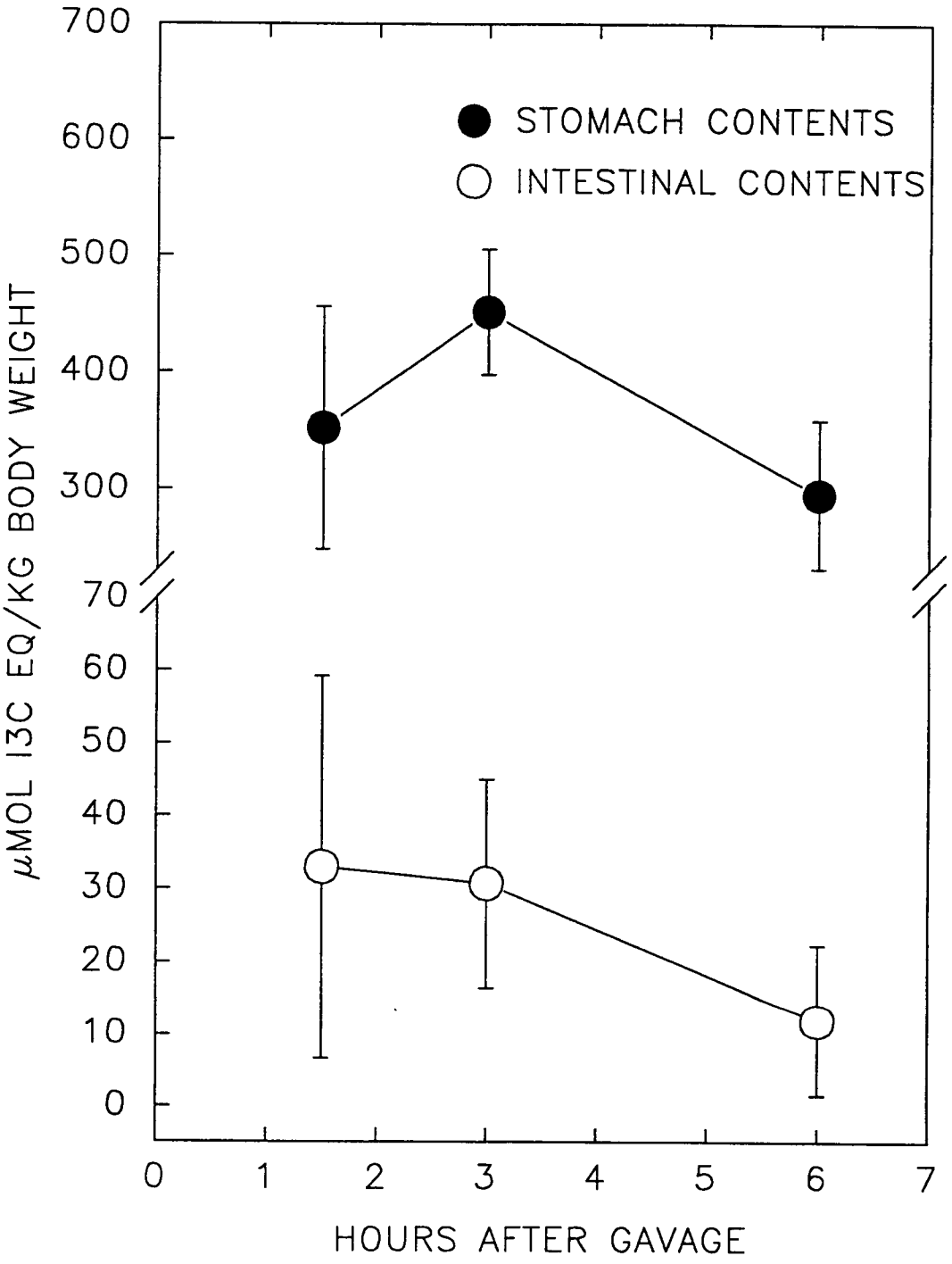

Figure 5.4. **Levels of μmol [^{3}H]I3C equivalents in the stomach contents and intestinal contents (proximal 20 cm), 1.5, 3 and 6 hr after administration of 1 mmol [^{3}H]I3C/kg body weight to male Fischer rats.** Values represent the mean ± SD.

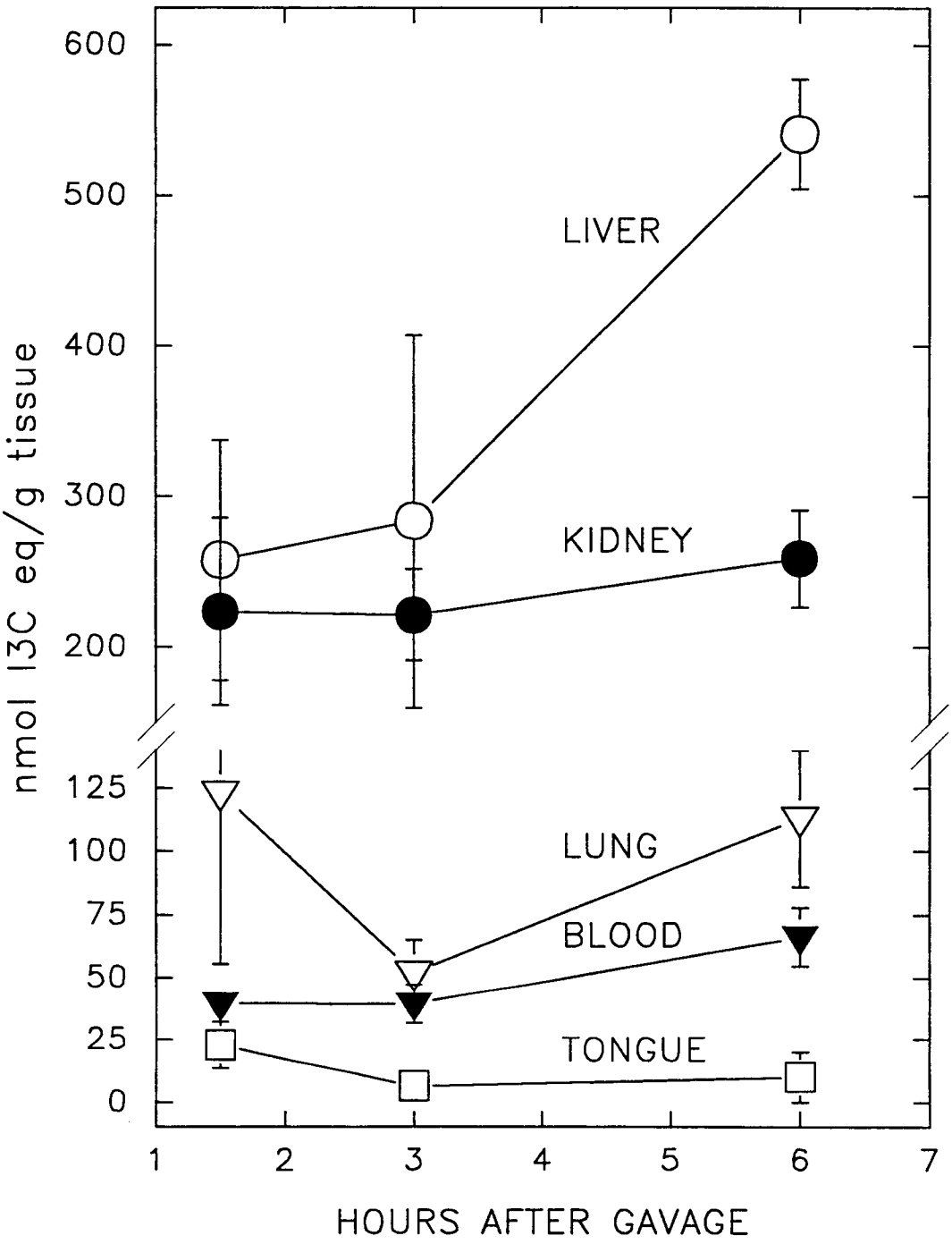

Figure 5.5. **Levels in μM equivalents of [^{3}H]I3C present in liver, kidney, lung, tongue and blood at 1.5, 3 and 6 hr after oral administration of 1 mmol [^{3}H]I3C/kg body weight to male Fischer rats.** Values represent the mean ± SD of 3-4 rats.

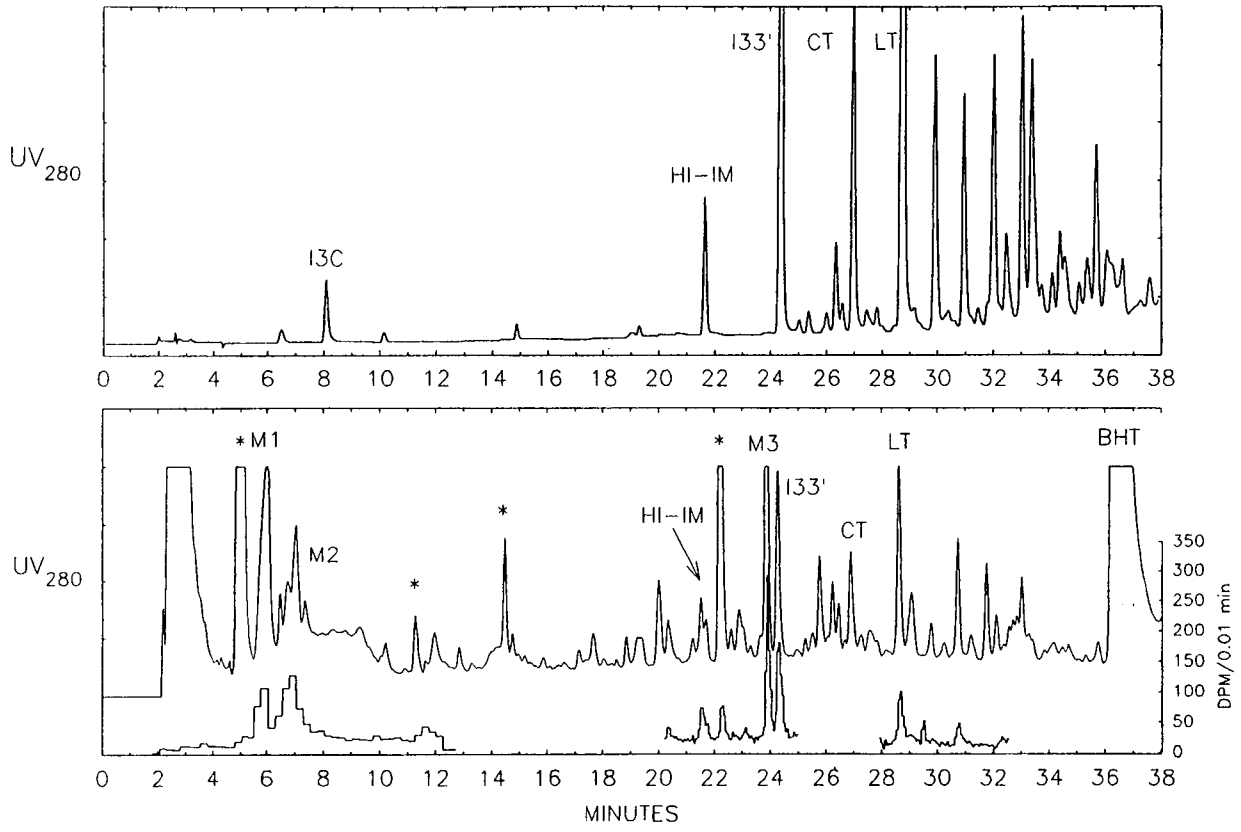

Figure 5.6. **HPLC chromatograms of RXM generated from I3C *in vitro* (top), and an ethyl acetate extract of a liver taken from a rat killed three hours after oral gavage of 1 mmol [^{3}H]I3C/kg body weight (bottom), monitored at a wavelength of 280 nm.** The lower chromatogram in the bottom panel shows radioactivity associated with some of these peaks in liver extracts. Fractions were collected every 15, 3.6 or 4.2 sec in the three windows, respectively. In order to compare the fractions on the same scale, values were normalized to DPMs/ 0.01 min. Peaks marked by (*) are prominent compounds also found in liver extracts from rats given only the DMSO-corn oil vehicle.

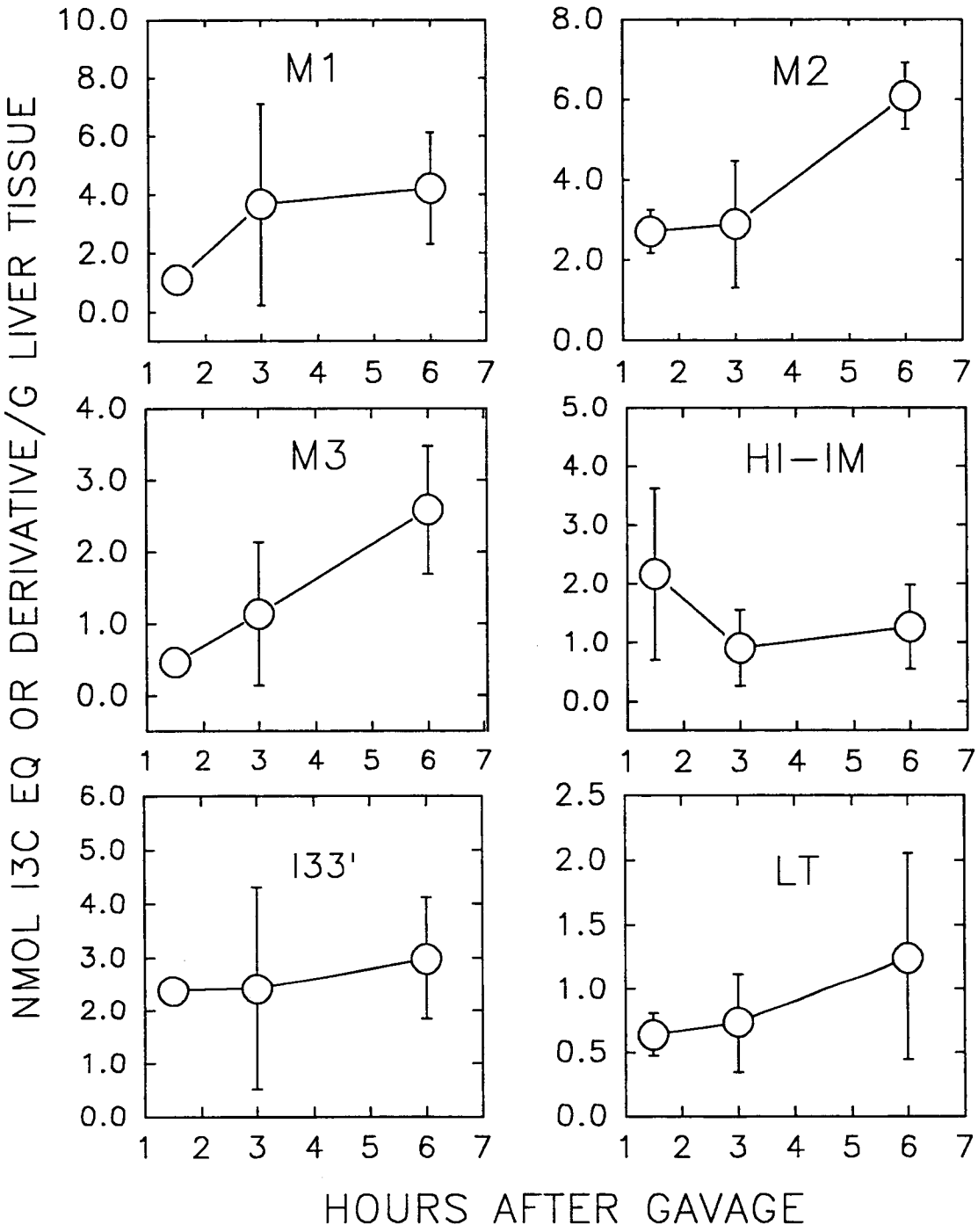

Figure 5.7. **Levels in μM [^{3}H]I3C equivalents of the six most abundant I3C metabolites present in liver extracts at 1.5, 3 and 6 hr after oral gavage of 1 mmol [^{3}H]I3C/kg body weight.** Values represent the mean ± SD.

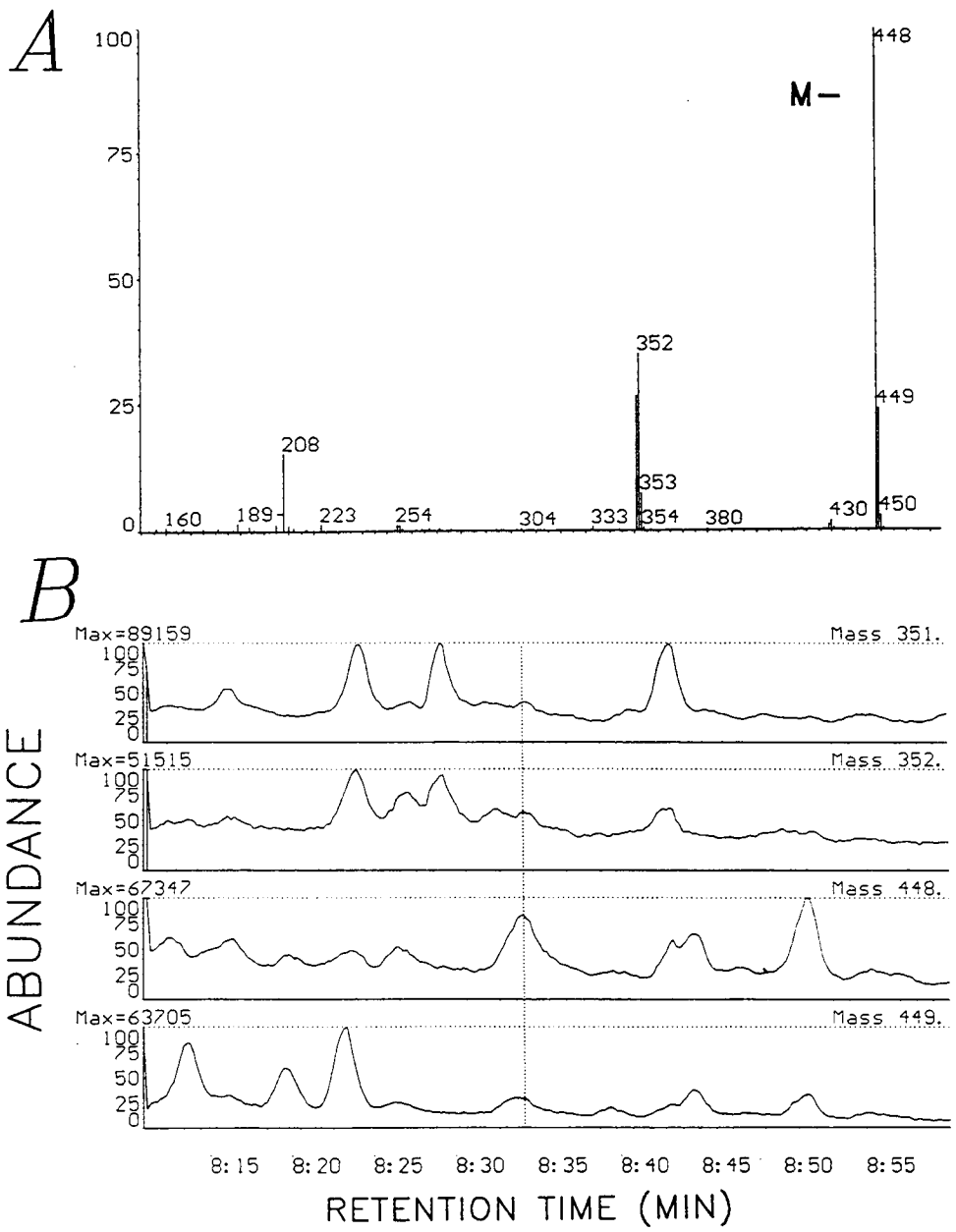

Figure 5.8. **A) Negative CI Mass spectrum of authentic *N*, *N*-trifluoroacetylated ICZ. B) GC Chromatogram with detection by multiple ion monitoring at masses of 351, 352, 448 and 449.** Ethyl acetate extract of 2.8 g of liver from 3 rats given I3C by oral gavage and killed after 6 hr was fractionated by HPLC. The fraction eluting at the R_t of authentic ICZ was derivitized and analyzed by GC/MS. The vertical dotted line represents the GC R_t of putative ICZ in the sample (8:34.3). The R_t of authentic ICZ was found to be 8:36.5. The Y-axis scale was normalized to the most abundant peak in the window.

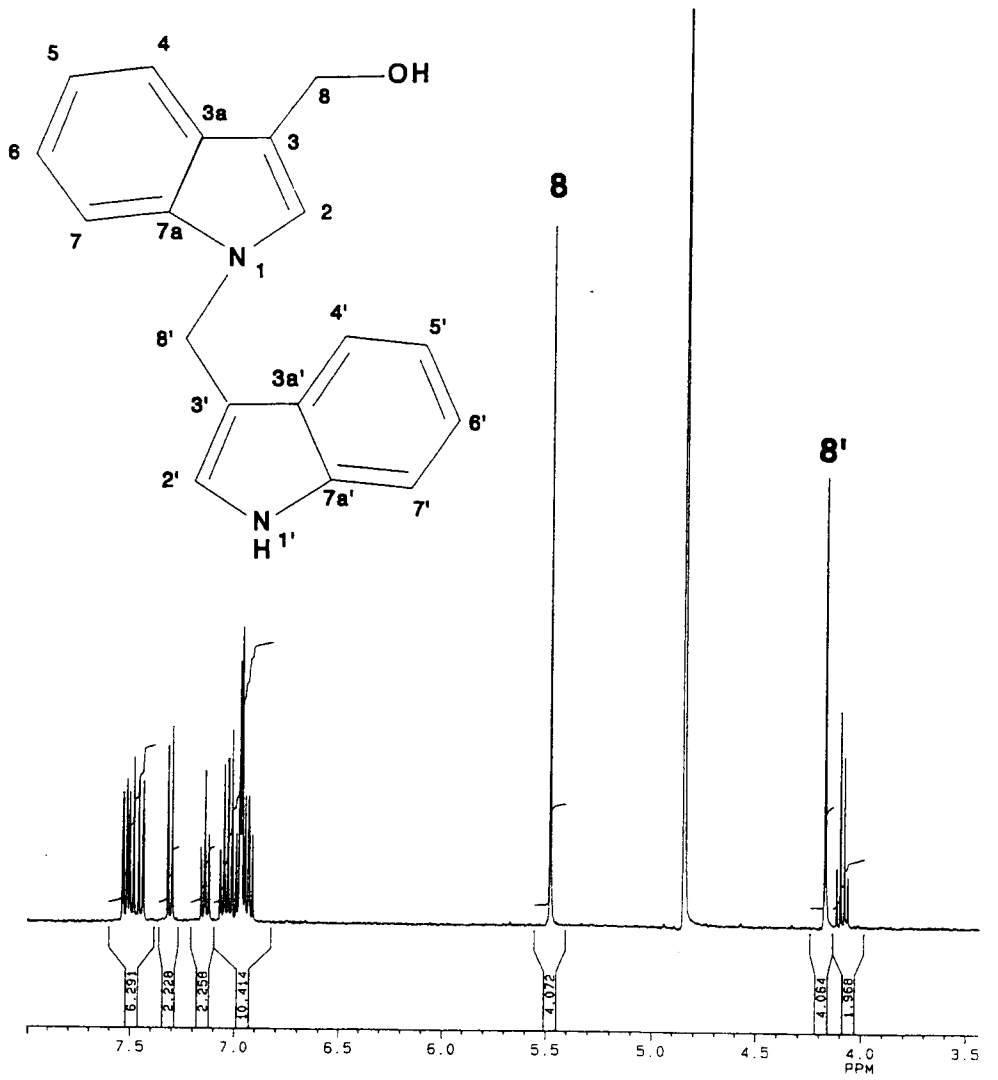

Figure 5.9. **^{1}H-NMR spectrum for HI-IM, which was isolated by HPLC from RXM as described in the Materials and Methods section.**

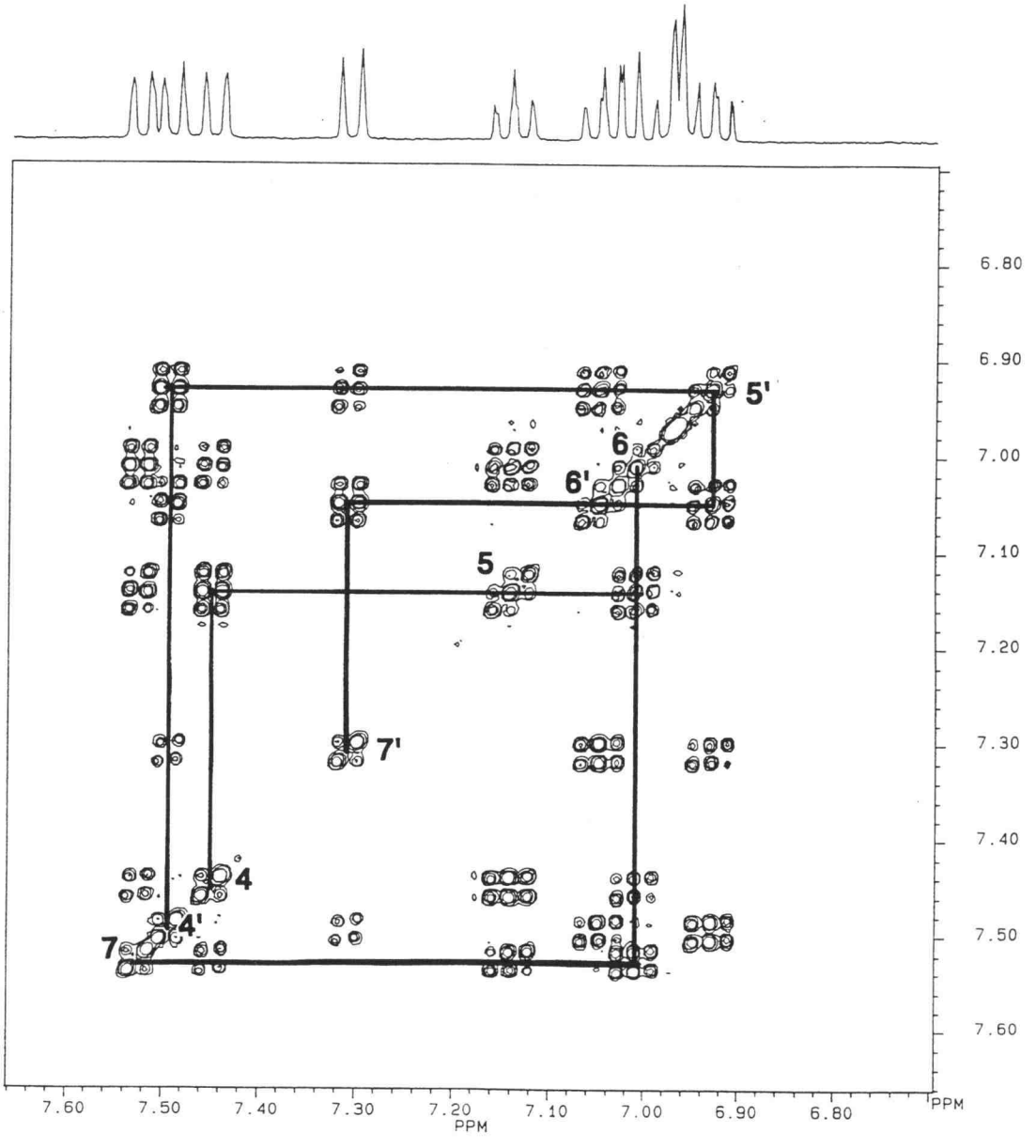

Figure 5.10. **2D ^{1}H-^{1}H correlated spectrum (homonuclear COSY) of the aromatic protons shown in the downfield portion of the spectra in Figure 5.9.**

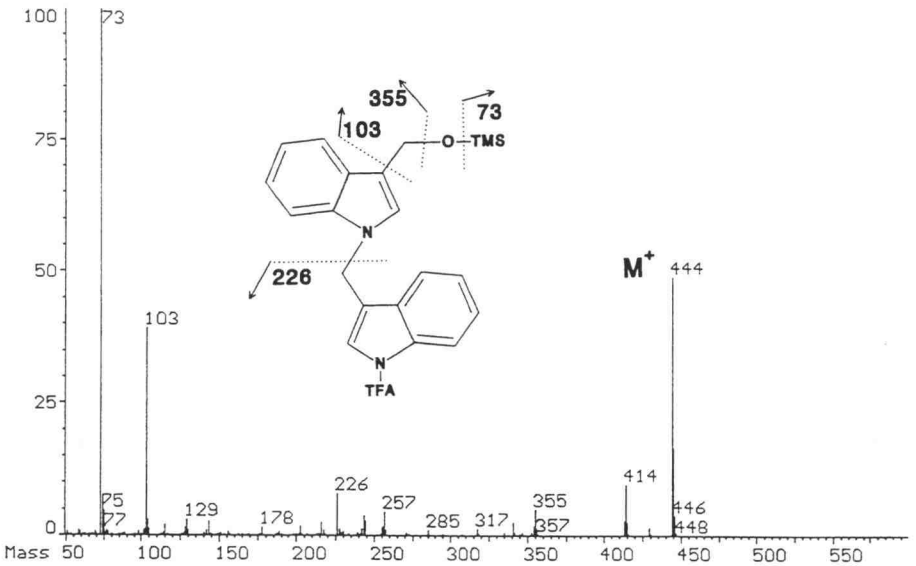

Figure 5.11. **EI mass spectrum for the *O*-trimethylsilyl and *N*-trifluoroacetyl derivative of HI-IM and a plausible fragmentation pattern.**

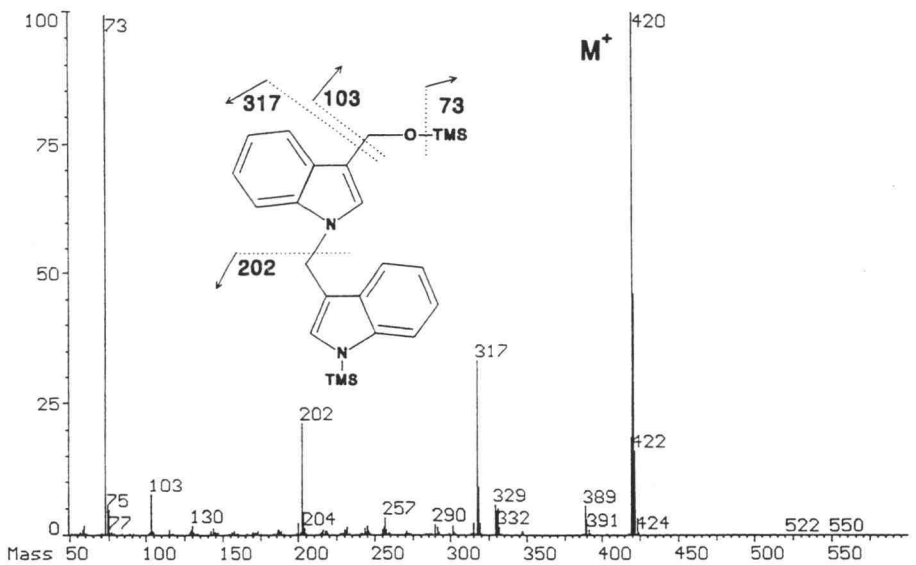

Figure 5.12. **EI mass spectrum of the *N,N*-trimethylsilyl derivative of a compound found in liver extracts consistent with the *N, N*-trimethylsilyl derivative of authentic HI-IM.**

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Chapter 6

ß-NAPHTHOFLAVONE, AN AFLATOXIN B_1 CARCINOGENESIS INHIBITOR, ENHANCES AND INHIBITS MICROSOMAL ACTIVATION OF AFLATOXIN B_1 IN THE RAT

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ABSTRACT

ß-Naphthoflavone (BNF) is a synthetic flavonoid and model *Ah* receptor agonist that has been previously shown to inhibit aflatoxin B_1 (AFB_1)-induced hepatocarcinogenesis in both trout and rats. The mechanism of anti-carcinogenesis has been ascribed to the induction of the cytochrome P-4501A subfamily of enzymes that enhances AFB_1 metabolism toward the less carcinogenic 9a-hydroxylated metabolite of AFB_1, aflatoxin M_1. Recent evidence from our laboratories indicate that BNF is a potent inhibitor of AFB_1 activation by trout liver microsomes as determined *in vitro* by trapping AFB_1 8,9-epoxide, the ultimate carcinogenic metabolite, as an adduct of DNA. In this study we examined the effect of BNF against AFB_1 activation by rat microsomes using the AFB_1-DNA binding assay and by HPLC. By HPLC, it is also possible to quantify the other major rat metabolites thought to be detoxication products, aflatoxin M_1, aflatoxin Q_1 and aflatoxin P_1. Similar to results obtained in the trout, we found that BNF inhibits activation, but only at less than 25 µM. In contrast to the trout, when BNF is present *in vitro* at 25-200 µM, activation is enhanced up to 2-fold. At all concentrations of BNF, we found inhibition in formation of the detoxication metabolites. These results suggest that under some conditions of BNF modulation of AFB_1 carcinogenesis in the rat, conditions may exist where enhancement of carcinogenesis at the initiation step may occur. However, the possibility that inhibition of CYP bioactivation, at least at low exposures to BNF, may constitute a mechanism of inhibition of AFB_1 carcinogenesis in the rat is also suggested.

INTRODUCTION

ß-Naphthoflavone (BNF)[1] is a synthetic flavonoid and model *Ah* receptor agonist that has been previously shown to inhibit aflatoxin B_1 (AFB_1)-induced hepatocarcinogenesis in both trout (1) and rats (2). The mechanism by which BNF inhibits carcinogenesis has been attributed to the induction of the cytochrome P-450 (CYP) 1A family, associated with AFB_1 9a-hydroxylase, the enzyme which forms aflatoxin M_1 (AFM_1). This reaction is considered a detoxication step because AFM_1 is a less potent carcinogen in both species (3, 4). However, recent results from our laboratories (5), show that dietary BNF can strongly inhibit AFB_1-DNA binding *in vivo* in trout at low doses of BNF that do not induce CYP1A1. These data strongly suggested that CYP1A1 induction is not the primary mechanism of inhibition for this species. It was subsequently shown that BNF was a potent inhibitor of AFB_1-DNA binding catalyzed by trout liver microsomes *in vitro* (K_i ~3 μM)(6). Thus it was hypothesized that inhibition of CYP-activation of AFB_1 to its DNA-binding form may be the primary inhibitory means at all levels of dietary BNF. It was therefore of interest to us to determine if BNF could inhibit the *in vitro* activation of AFB_1 in the rat as it does in the trout. Our results show that, like the trout, BNF inhibits activation at concentrations approaching the nanomolar range. In contrast to the trout however, at higher concentrations, activation to the DNA-binding 8,9-epoxide is enhanced.

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1 - **Abbreviations**: BNF, ß-naphthoflavone; AFB_1, aflatoxin B_1; CYP, cytochrome P-450; AFM_1, aflatoxin M_1; AFG_1,aflatoxin G_1; AFM_1, aflatoxin M_1; AFQ_1, aflatoxin Q_1; AFP_1, aflatoxin P_1; BHA, butylated hydroxyanisole; GSH, glutathione; DMSO, dimethyl sulfoxide; PMSF, phenylmethylsulfonyl fluoride; ANF, α-naphthoflavone.

MATERIALS AND METHODS

Chemicals

AFB_1, aflatoxin G_1 (AFG_1), AFM_1, aflatoxin Q_1 (AFQ_1), aflatoxin P_1 (AFP_1) butylated hydroxyanisole (BHA), glutathione (GSH) and BNF were purchased from Sigma Chemical company (St. Louis, MO). Dimethyl sulfoxide (DMSO) and tetrahydrofuran were obtained from J.T. Baker (Phillipsburg, NJ). HPLC grade methanol was obtained from Mallinckrodt (Paris, KY). All other chemicals were purchased from Sigma.

Animals

Weanling male Fischer 344 rats were obtained from Simonsens (Gilroy, CA). After an acclimation period of 7 days, the animals were killed by CO_2 asphyxiation and their livers were removed, blotted, weighed, frozen in liquid nitrogen and stored at -80° C until preparation of microsomes. Swiss-Webster mice (Simonsens) were fed a AIN-76A powdered semipurified diet containing 0.75% BHA (to induce GST levels for use in the AFB_1 metabolism assay) for ten days. Animals were killed by CO_2 asphyxiation and their livers were removed and frozen in liquid nitrogen and stored at -80° C until preparation of cytosol.

Preparation of Hepatic Microsomes and Cytosol

Livers from rats were homogenized in 4 volumes of ice cold 0.1 M potassium phosphate buffer (pH 7.4) containing 0.15 M KCl, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF). Microsomes were prepared by differential

centrifugation by the method of Guengerich (7). The supernatant from a 10,000 g centrifugation of liver homogenate was subjected to centrifugation at 100,000 g. The resulting pellet was washed with potassium pyrophosphate buffer (0.1 M, pH 7.4, 1 mM EDTA, 1 mM PMSF), centrifuged at 100,000 g, and the microsomes resuspended in homogenization buffer containing 20% glycerol to obtain microsomes. Microsomes were stored in aliquots of 500 µl and stored at -80°C. Cytosol was prepared from mice fed BHA according to Guengerich (7), except the post-100,000 g supernatant was subjected to an additional 100,000 centrifugation step to ensure the absence of membrane associated enzymes. Protein concentrations were determined on the sub-cellular fractions according to the method of Lowry *et al.*, (8).

AFB_1 Metabolism Assay

Metabolism of AFB_1 *in vitro* was determined essentially as described by Monroe and Eaton (9) and modified as described elsewhere (10). This assay allows simultaneous quantification of hydroxylated metabolites and AFB_1 8,9-epoxide by trapping the latter as a stable GSH conjugate. In the presence of mouse cytosol, trapping efficiency of the epoxide as the conjugate has been reported to be greater than 99% (9). When present in the assay, BNF was added in a DMSO solution so that final concentrations were 1-200 µM. The final concentration of AFB_1 was 64 µM. After buffer, AFB_1, BNF, microsomes (1 mg/ml) and cytosol (3 mg/ml) were added, the reaction was initiated by addition of cofactors (5 mM GSH, 1 U/ml glucose-6-phosphate dehydrogenase, 5 mM glucose-6-phosphate and 1 mM NADP+). The reaction was terminated after 10 min by the addition of 50 µl 2 M acetic acid. Controls were run in the absence of NADP+, microsomes, or microsomes and cytosol.

In Vitro AFB_1-DNA Binding Assay

An *in vitro* [^{3}H]AFB_1-DNA binding assay was conducted as previously described (11). The final reaction mixture volume was 250 µl which contained 150 µg calf thymus DNA, 250 µg microsomal protein, 0.2 mM EDTA, 30 µM $MgCl_2$, 0.1 M Tris-HCl (pH 8.0), and 16 or 124 µM [^{3}H]AFB_1 (1.0 µCi/sample, in 5 µl DMSO) with 0-100 µM BNF (in 5 µl DMSO). The reaction was initiated by adding NADPH to 1 mM and incubated in the dark at 37°C for 10 min. The reaction was terminated by adding 50 µl of 10% sodium dodecyl sulfate. DNA was extracted by Tris-saturated phenol, chloroform and chloroform-isoamyl alcohol (24:1 v/v). DNA was precipitated in isopropanol and sedimented with a bench-top centrifuge and resuspended in a Tris-EDTA buffer (0.1 M Tris-HCl (pH 8.0), 10 mM EDTA). The final concentrations of DNA were measured by the method of Burton (12), and [^{3}H]AFB_1 adducts to DNA were quantified by scintillation counting. As a control, only vehicle (DMSO) was added to the incubation in place of inhibitor.

RESULTS

Figure 6.1 shows the effect of 1, 2, 5 and 10 μM BNF on the *in vitro* metabolism of 64 μM AFB_1. Enzymes catalyzing the formation of the AFB_1 8,9-epoxide and the mono-hydroxylated metabolites AFQ_1, AFM_1 and AFP_1 were inhibited in a concentration dependent manner. AFB_1 8,9-epoxide production was inhibited to the greatest extent and clear inhibition was observed at concentrations of 1 μM BNF. In contrast, as shown in Figure 6.2, when BNF was included in the reaction at concentrations ranging from 25-200 μM, an enhancement of AFB_1 8,9-epoxidation is observed. The rate of epoxidation was more than doubled at 25 μM BNF, increased slightly and appeared to plateau at 50 μM. Similar to the results observed using the low range of BNF concentrations, production of the hydroxylated metabolites was inhibited, with the exception of AFP_1, which appeared to follow the same relative pattern as that for AFB_1 8,9-epoxide. In order to confirm the apparent bi-modal effect of BNF on AFB_1 activation, we used concentrations of BNF which spanned those at which an inhibitory and enhancing effect was observed. In addition, we used an alternative method to quantify epoxidation, the [^{3}H]AFB_1-DNA binding assay, identical to that used previously with trout microsomes (6). The results of this experiment are shown in Figure 6.3. At the low concentration of AFB_1 (16 μM) representative of subsaturating concentrations (13), a steep decline in activation is observed initially at 5 μM BNF. Activation begins at 10 μM and plateaus at 50-100 μM BNF. A similar pattern is observed at the high concentration of AFB_1 (124 μM), representative of saturating conditions. At this concentration, the absolute level of inhibition and activation is higher, but the relative level is less than that observed at 16 μM AFB_1.

DISCUSSION

In this study, we have shown that BNF can stimulate the microsomal metabolism of AFB_1 to its activated form by two different assays which measure AFB_1 activation. However, the effect was only observed beginning at the 10-25 μM range and increased to apparent maximal values at 50-100 μM. At 10 μM and below, an inhibitory effect was observed. Both effect were demonstrated across a range of AFB_1 concentration representative of saturating and subsaturating levels (13). The phenomenon of flavone-mediated activation of CYP catalysis has been described previously on numerous instances (14-17), primarily with the BNF isomer, 7,8-naphthoflavone or α-naphthoflavone (ANF). Although ANF stimulated AFB_1 activation to mutagens using human liver microsomes (14, 17), it had a slight inhibitory effect using liver microsomes from immature Long-Evans rats (14). The inhibitory effect of ANF on *in vitro* AFB_1-DNA binding was also observed using liver microsomes from phenobarbital treated rats (18). These results suggest that for ANF, stimulation of AFB_1 activation may be specific to human sources of enzyme. In addition, it was shown that inclusion of BNF at 100 μM had essentially no effect on AFB_1-DNA binding, but at 500 μM BNF, 65% inhibition was observed (18). This is in contrast to our results that show 100 μM BNF had a clear stimulatory effect, using microsomes from untreated rats. Gurtoo and Dahms (19) also found a 51-69% inhibition of AFB_1 activation by 500 μM BNF using hepatic microsomes from control rats or rats pretreated with phenobarbital or 3-methylcholanthrene. We did not test for the effect of BNF at concentrations beyond 200 μM and, at 200 μM, we observed a non-significant decline in activation. Because AFB_1 activation is known to be catalyzed by several CYP isoforms in the rat (20-22), one interpretation of these data is that BNF is exhibiting differential binding affinities for the

different isoforms. Thus, BNF may preferentially inhibit activation in the 1-10 μM, stimulate in the 25-200 μM range and inhibit beyond 200 μM.

Pretreatment of both trout and rats with BNF induces CYP1A (AFB_1 9a-hydroxylase), an AFB_1 detoxifying enzyme. Thus, CYP1A induction has been suggested as the primary mechanism by which BNF inhibits AFB_1 carcinogenesis in both species (1, 2). However, recent results from our laboratories (5) have shown that BNF can inhibit AFB_1-DNA binding at sub-CYP1A inducing dietary levels. In addition, similar to the results observed here for the rat, BNF was shown to inhibit *in vitro* AFB_1-DNA binding at low micromolar ranges (6). These data, along with the observation that BNF fails to enhance AFB_1 activation at any concentration up to 500 μM in the trout, strongly suggest that BNF inhibition of AFB_1 activation constitutes the primary mechanism of anticarcinogenesis for this species. For the rat, this mechanism of AFB_1 anticarcinogenesis may also be true for low exposures to BNF. However, because BNF stimulates AFB_1 activation at higher concentrations, interpretation is equivocal.

In summary, these results suggest that BNF could have a beneficial or detrimental effect on AFB_1 genotoxicity and carcinogenicity in rats that may be dependent on the concentration of BNF in BNF-AFB_1 tumor studies. These data also suggest the possibility, as indicated for the trout model, that induction of CYP1A may not be the primary mechanism of BNF inhibition of AFB_1 carcinogenesis in the rat. Rather, inhibition of CYP bioactivation, at least at low exposures to BNF, may constitute an important mechanism of inhibition.

ACKNOWLEDGMENTS

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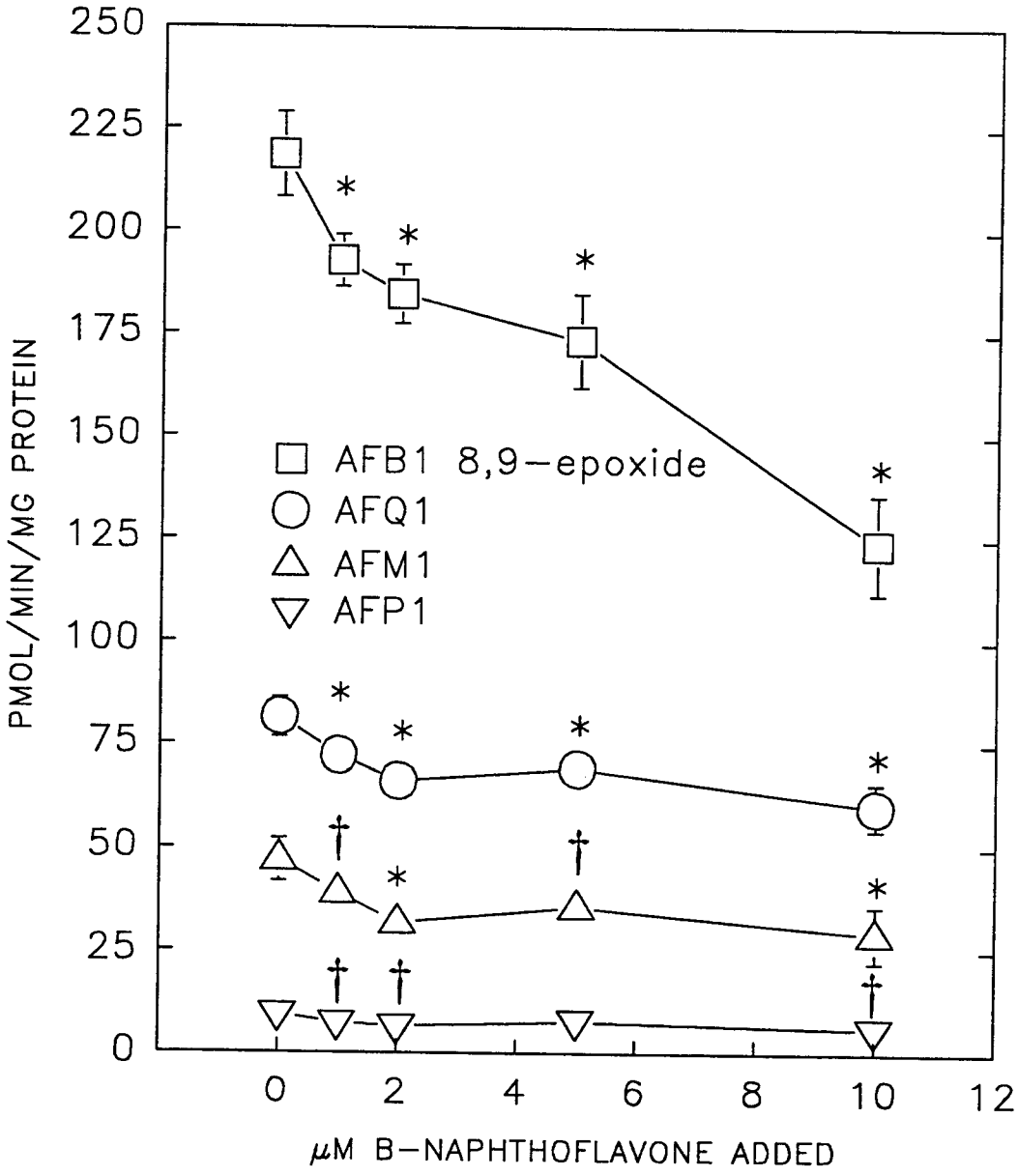

Figure 6.1. **Inhibition of rat hepatic microsomal metabolism of 64 μM AFB_1 by 1-10 μM BNF.** The 8,9-epoxide was trapped as a stable GSH conjugate in the presence of GSH and hepatic cytosol from BHA-treated mice. Values represent the mean of triplicate determinations ± SD, except for values for AFP_1, which, because of integration difficulties on poorly shaped peaks, represent the mean ± SD of 2-3 determinations. Metabolites were monitored at a wavelength of 362 nm and quantified using an AFB_1 standard curve. Asterisks and crosses indicate significantly different from the no BNF control as determined by analysis of variance, followed by Newman-Keuls multiple range test (* = $P < 0.01$, † = $P < 0.05$).

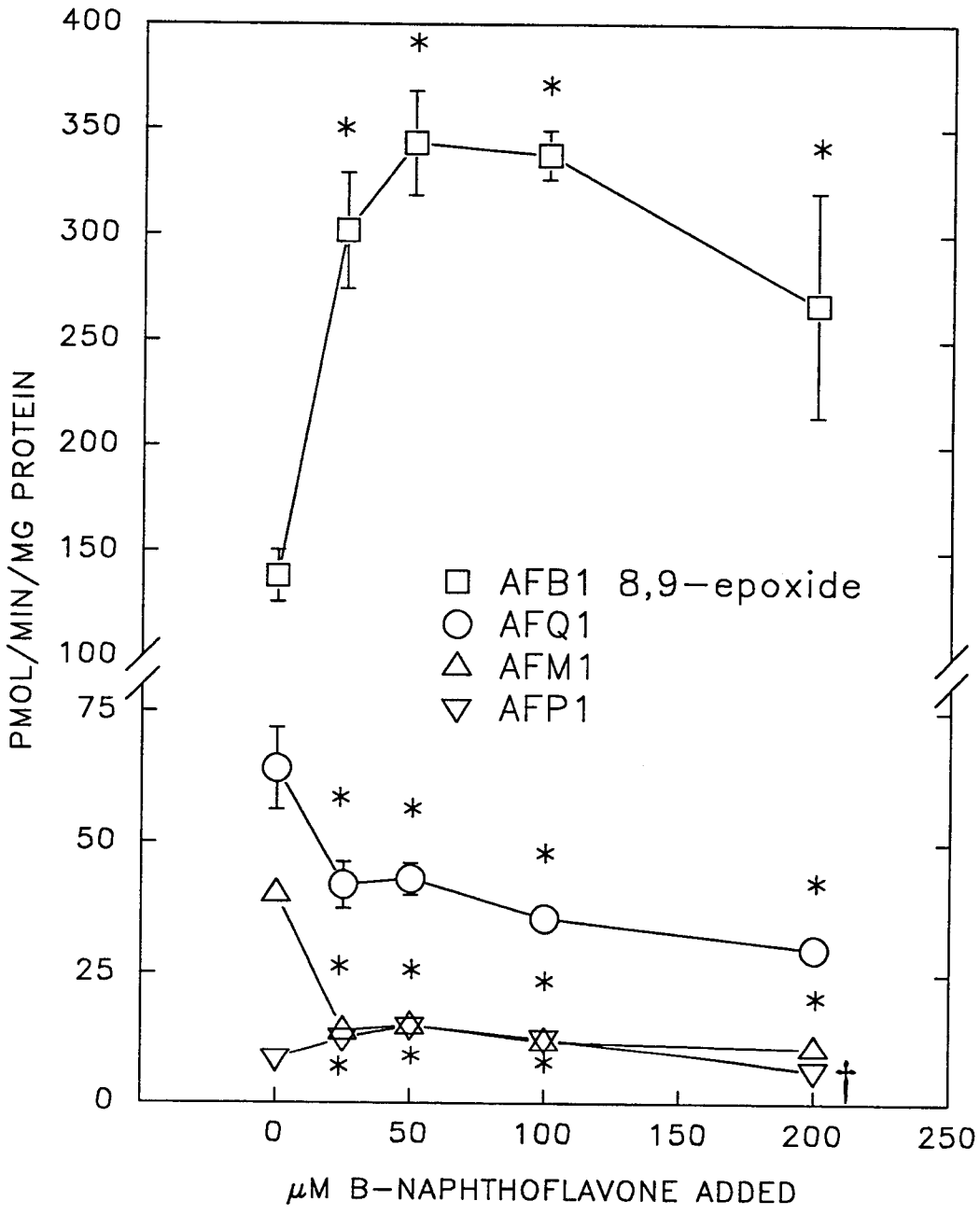

Figure 6.2. **Inhibition of rat hepatic microsomal metabolism of 64 μM AFB_1 by 25-200 μM BNF.** Values represent the mean of triplicate determinations ± SD, except for the values for AFP_1, which represent the mean ± range or SD of 2 and 3 determinations, respectively. Metabolites were monitored at a wavelength of 362 nm and quantified using an AFB_1 standard curve. Asterisks and crosses indicate significantly different from the no BNF control as determined by analysis of variance, followed by Newman-Keuls multiple range test (* = $P < 0.01$, † = $P < 0.05$).

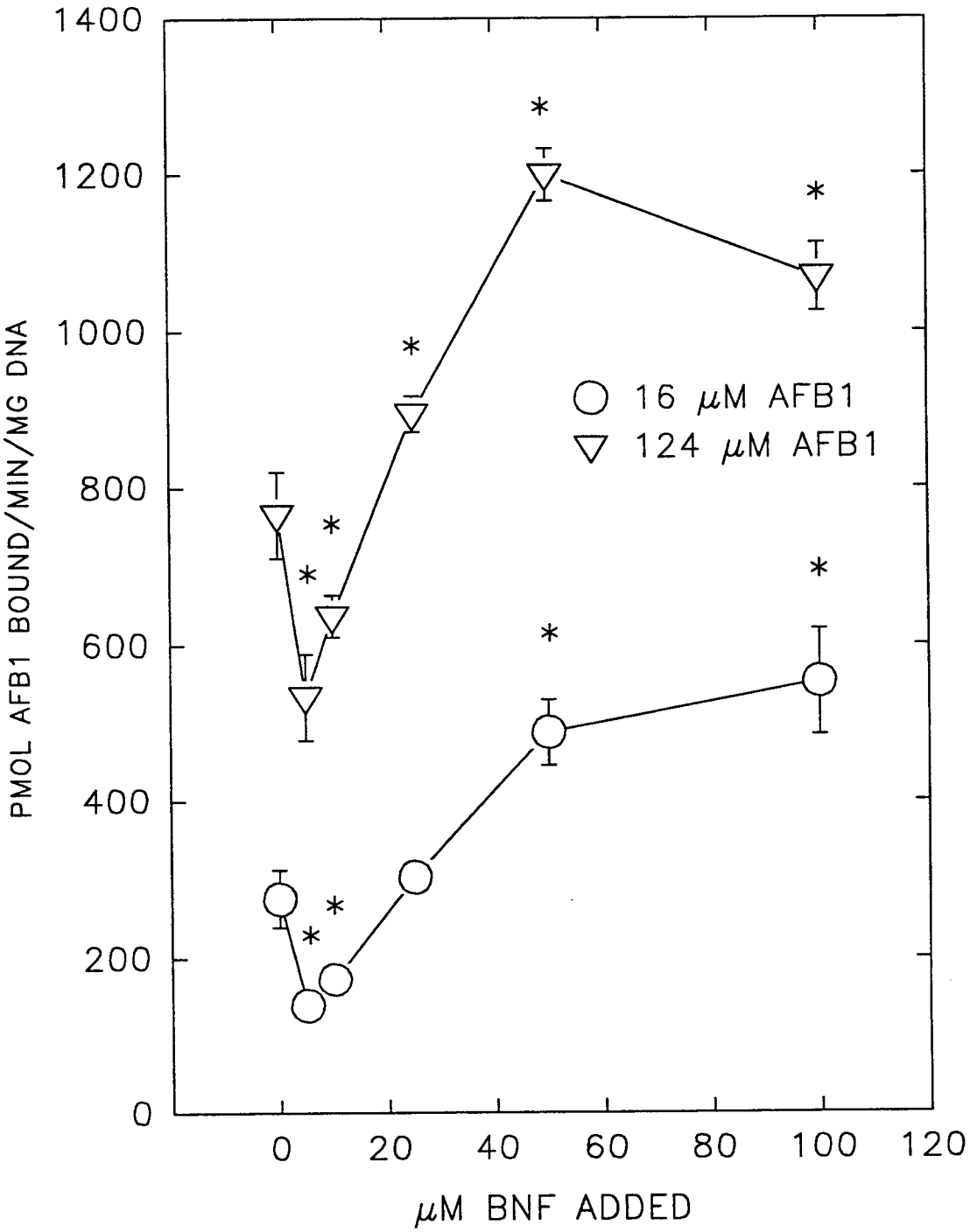

Figure 6.3. **Inhibition of rat hepatic microsomal metabolism of 16 and 124 μM AFB_1 to DNA-binding metabolites by 5-100 μM BNF.** Values represent the mean of triplicate determinations ± SD. Asterisks indicate significantly different from the no BNF control as determined by analysis of variance, followed by Newman-Keuls multiple range test (* = $P < 0.01$).

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Chapter 7

CONCLUSIONS

There are several mechanisms by which I3C may inhibit tumors. I have explored three mechanisms that should be important mechanisms of protection against various chemical carcinogens, in particular, AFB_1. Induction of CYP isoforms important in both the bioactivation and detoxication of AFB_1 was demonstrated in rats fed I3C. The transient increases in hepatic microsomally generated AFB_1 8,9-epoxide after 7 days on the diets might be expected to increase toxicity and carcinogenicity. However, a reduction in AFB_1-DNA binding and the previous demonstration of decreased incidence in tumors indicate that the increase in epoxide production was compensated for, probably by concomitant increases in the less toxic AFM_1 and AFQ_1 or increases in the GST isoform Yc2. ß-naphthoflavone, a synthetic flavonoid and model *Ah* receptor agonist was found to be less potent inducer of AFM_1 production at dietary levels approximately equally anticarcinogenic. No increases were seen in AFQ_1 and AFB_1 8,9-epoxide production with the BNF only diets. When both I3C and BNF were combined at the same levels as when fed on an individual basis, the response of all biochemical parameters tested approximated that observed in rats given only I3C and no additive or synergistic effect was indicated. A major I3C derivative, I33', was found to be a potent inhibitor of several CYP isoforms in trout, rat and humans. Inhibition of enzymatic carcinogen bioactivation thus may be one mechanism that is applicable to all species including humans. Unlike some I3C derivatives, BNF was found to enhance bioactivation under certain conditions *in vitro*. It is shown that concentrations of I3C

metabolites that can inhibit CYP *in vitro* are present *in vivo* following an anticarcinogenic I3C dose.

Inhibition of carcinogenicity appears to be highly dependent on route of administration of I3C. Previously, it has been demonstrated that acidic conditions such as those found in the stomach catalyze the conversion of I3C to several distinct products, most of which appear to be higher molecular weight, oligomeric derivatives of I3C. The structure of one of these derivatives, 1-(3-hydroxymethyl)-indolyl-3-indolylmethane, was determined herein. Central in determining the mechanisms of action of I3C is an understanding of its disposition and excretion. By administering anticarcinogenic levels of I3C in the diet or by gavage, it was determined that I3C derivatives were available in target organs at concentrations that are CYP-inhibiting *in vitro*. This strongly suggests that enzyme inhibition should occur *in vivo* as well, but we have only suggestive evidence that this occurs. This information should help in evaluating the potential for I3C as a chemopreventive agent for use in humans.

In conclusion, there is potential for I3C for use in humans as a chemopreventive agent. However, there are serious concerns regarding its safety as it has previously been shown to be a potent tumor promoter under some conditions that mimic human dietary patterns. The I3C derivatives responsible for tumor promotion have not yet been identified. However, one candidate is ICZ, an I3C derivative that binds with high affinity to the *Ah* receptor as does other potent tumor promoters 2,3,7,8-tetrachlorodibenzo*p*-dioxin and some PCBs. Although, in general, epidemiology studies suggest that consumption of I3C-containing cruciferous vegetables is associated with a protective effect, these vegetables are known to contain several other factors that may be protective including phytochemicals and antioxidants, some of them known anticarcinogens themselves. Thus, it cannot be inferred that the safety of consumption of I3C in purified

form is tantamount to consumption of the cruciferous vegetable that contains an equivalent amount of I3C. Further study is needed to fully determine the risks versus benefits of consumption of I3C in its purified form.

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