AN ABSTRACT OF THE THESIS OF

Samina Van Winkle for the degree of Master of Science in Food Science and Technology presented on August 11, 1988

Title: Diethylnitrosamine, Ethylnitrosourea, And Dimethylbenz(a)anthracene DNA Binding Studies In The Rainbow Trout.

Abstract approved: _____
George S. Bailey

Dimethylbenz(a)anthracene (DMBA), a carcinogen that requires metabolic activation to produce active metabolites capable of binding to DNA, has been studied in the trout and other fish. Polycyclic aromatic hydrocarbons (PAH) are of importance as they are ubiquitous in the environment and their carcinogenic effects in fish from contaminated waters are an important indication of the pollution risks to man. Since such pollution risk assessment presents the involvement of multiple agents, the study of the modulation of PAH-DNA binding produced by other agents is important. In this study the effect, of dietary pretreatment at 500 ppm, 100 ppm and 2000 ppm, using BNF, Aroclor 1254, or indole-3-carbinol (I3C) respectively on DMBA-DNA binding was examined. To study the effect of age on sensitivity to DMBA-DNA binding, adult trout and fry were used in two separate studies. The fish were fed treatment diet for at least two weeks. Fry

were then injected with [^{3}H] DMBA, at 22.4 μCi/3.9 x 10^{-2} μmole/fish and adult trout at 284 μCi/1.58 μmole/fish. Liver DNA was isolated, purified and binding of radioactivity to DNA was examined and computed as the covalent binding index (CBI). Mean CBI for control dietary group using adult trout was 1000 fold lower than for fry. Statistical analysis of covalent binding index for the treatment groups revealed that a statistically significant ($p < 0.05$) inhibition in DNA-DMBA binding response in adult trout and fry was produced by the BNF dietary treatment only.

Diethylnitrosamine (DENA), a potent hepatocarcinogen in several animal species belongs also to the class of compounds that require metabolic activation. Dietary treatment and continuous exposure of trout to the carcinogen in water, have produced hepatocellular carcinomas. The water exposure also produced a dose related DNA ethylation of the O^6 position of guanine, believed to be the promutagenic adduct produced after DENA exposure. This study examines two other routes of exposure to DENA, in vitro hepatocyte incubations and i.p. injection. Adult trout and fry were injected with [^{3}H] DENA. Adult fish received 3.3, 16.5, and 33 mg/kg DENA, and fry received 10, 50 and 100 mg/kg. Hepatocyte incubation was performed with doses up to 220 μM [^{3}H] DENA, or 1mM unlabelled DENA. DNA from fish livers and from hepatocyte pellets was isolated, purified and examined for radioactive binding of the DENA metabolites or in the case of the unlabelled DENA, was analyzed for O^6 and N7 adduct using an HPLC technique with fluorescence detection. O6-

EtG adduct after DENA exposure, in DNA of hepatocytes obtained from trout pretreated with beta-naphthoflavone (BNF, a known inducer of cytochrome P-450 dependent enzyme activities involved in the activation of xenobiotics) was below the limits of detection of the HPLC-fluorescence detection procedure used. To examine further if the lack of DNA binding and absence of the O^6-EtG adduct was due to rapid repair, the persistence of O6-EtG after exposure to 40 mM ethylnitrosourea (ENU, a direct ethylating agent) was studied in hepatocytes at 2, 4, and 5 hours after treatment. The activity of the alkyltransferase protein involved in the repair of alkylguanines also was determined using liver extracts from adult rainbow trout. The studies did not reveal a significantly high rate of repair. It is concluded that i.p.injection and in vitro hepatocyte incubations are not a good method for studying the kinetics of activation and DNA binding of DENA in the rainbow trout. The i.p. route may lead to substantial loss of the dose of the carcinogen administered and hepatocyte incubations are limited by the toxic effects of increasing carcinogen concentration. The reasons mentioned above, coupled with low levels of metabolism of nitrosamines in trout results in the inability to detect and study the appearance, persistence and repair of the O^6-EtG adduct.

Diethylnitrosamine, Ethylnitrosourea, And Dimethylbenz(a)anthracene DNA Binding Studies In The Rainbow Trout

by

Samina Van Winkle

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Master of Science

Completed August 11, 1988

Commencement June 1989

APPROVED:

Professor of Food Science & Technology in charge of major

Head of Department of Food Science & Technology

Dean of Graduate School

Date thesis is presented August 11, 1988

Typed by researcher for Samina Van Winkle

ACKNOWLEDGEMENT

For their meticulous academic guidance, I thank Dr. George S. Bailey and Dr. Arthur T. Fong. I gratefully acknowledge and thank Dr. Richard Scanlan for providing the chance to accept the challenge of graduate school and research. Foremost in my mind are the patient efforts of Tracy, which made it possible to launch this Thesis off the printer. I deliver my thanks to Dan Arbogast and Dr. Roderick Dashwood for their assistance in the collection of tissue samples and the numerous other varieties of help rendered. I thank Dr. David Williams and Dr. Arthur Fong for sharing their Data on nitrosamines. I will always remember with regard the solicitous support of Dr. Daniel Selivonchick and Dr. Antonio Torres. I thank Sandy Ernst for proof reading my Thesis. My most creative and educational experience in graduate school was working with Dr. Mike Penner as T.A. I thank him and also all other friends, faculty, staff at the Department of Food Science and Technology and all members of the FINL laboratory for their assistance in the research activities involved. For serving on my committee, I thank the members.

My very special regards go to my family, Saadia and Gary Van Winkle, for all those days when their support and good humor made the thought of tomorrow, a little easier.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Diethylnitrosamine, Ethylnitrosourea, And Dimethylbenz(a)anthracene DNA Binding Studies In The Rainbow Trout

FOREWORD

"Chirurgical observations relative to the cataracts, the polyps of the nose, the cancer of the scrotum, the different kinds of ruptures and mortification of the toes and feet" (Pott, 1775) can be classified in the annals of carcinogenesis as the study of the abnormal revealing the normal. Understanding cancer means understanding the normal cell, and the events involved in its transformation, survival, and perpetuation against the natural defenses present in the organism. Pott made the pioneering leap of correlating scrotal cancer with the occupation of chimney sweeping, thus providing the first evidence for a role of environmental agents in the etiology of cancer, the first clue to the role of soot in the genesis of the disease, and the first preventative measure recorded against this disease - to take daily baths in order to restrict dose of exposure.

Modern research efforts support Boveri's "Somatic Mutation Theory," which purports that neoplastic transformation occurs as a result of carcinogen interaction with DNA. The damage is fixed as a consequence of lack of effective repair, resulting in subsequent mutation on replication of the DNA. Evidence for this theory is presented in patients of xeroderma pigmentosa, known to have a deficient excision repair system for pyrimidine

dimers in DNA of cells exposed to ultraviolet radiation (Cleaver, 1986).

Research however has suggested that chemical induction may not be the only means of initiating neoplasia. This was demonstrated by studies with RNA viruses which produced cancer in animals, indicating a viral induction (Bishop, 1982). A symbiosis of the genetic and viral theories of cancer occurred when it was discovered that most of the known cancer-causing viruses in animals were not exclusively RNA viruses but could utilize viral reverse transcriptase to produce a DNA transcript of the viral RNA. It was postulated that elements of viral RNA could be attached to the genome of the cell and transmitted genetically to become activated at some future time and cause "spontaneous" cancer (Temin, 1972). In addition, retroviral molecular biology elucidated cellular genes, termed proto-oncogenes, that may be the critical targets for carcinogens. Pioneering work by investigators has revealed that human cells have proto-oncogenes that can be activated to oncogenes (Bishop, 1982, Weinberg, 1983). For example, a point mutation in the "ras" proto-oncogene at critical regions results in activation and subsequent neoplasia, identifying at least one target for DNA damage and mutation.

A fault in the regulation of some oncogenes which have roles crucial in the control of cell growth, differentiation and embryonic development can also be a cause of neoplastic transformation. Studies have indicated that over-expression of

the proteins of these oncogenes leads to cancer (Hunter, 1984). This can result from disturbances in the molecular mechanisms that normally control the expression of genes or from the amplification of such genes in DNA, so that their copy number is greatly increased. Evidence of such abnormal expression has been shown for the "myc" gene in a number of human cancers (Hunter, 1984).

Beyond the mechanisms involved at the level of the macromolecule lies the information at the level of the cell population. Although neoplasia can arise from the local proliferation of a single cell that escapes mutation repair, this early microtumor does not metastasize to violate other organs or sites. The critical event occurs when the tumor cells release tumor angiogenesis factor (TAF) to stimulate nearby blood vessels to supply the tumor tissue (Folkman, 1976). The inhibition or induction of TAF therefore is of therapeutic importance. Other polypeptides, identified as components of normal serum, also may influence growth and include polypeptides such as fibroblast growth factor, nerve growth factor, epidermal growth factor, and platelet derived growth factor. Possible involvement of platelet derived growth factor in both cell transformation and expansion of this malignant cell into a clone can be inferred from studies with the viral oncogene "sis." The proto-sis gene is the same as that which encodes platelet derived growth factor, and the "sis" oncogene is carried in the simian sarcoma virus (Hunter, 1984).

Investigators have also made the exciting discovery that a cancer cell need not be irreversibly committed. It has been shown that malignant stem cells of teratocarcinomas implanted into early, normal, mouse embryos results in the development of chimeric animals, containing normal cells that originated from the teratocarcinoma. These efforts have provided the first evidence of neoplastic reversion (Illmensee et al., 1979).

The information derived from these experimental studies can be amalgamated with the model for carcinogenesis that emerges from epidemiology. One theory postulates that each cell possesses several independent genes controlling growth, and that cancer develops when one or more of these genes has been inactivated by mutation. Since mutations are random events that accumulate with time, those individuals most conspicuously at risk are older people and the incidence of the disease rises steeply with age (Cairns, 1975). Based on this theory, the probability of any mutation occurring thus increases with age. For example death rates for cancer of the large intestine increase about a thousandfold between the ages of 20 and 80 (Cairns, 1975). Studies of migrant populations have revealed that the incidence of many common cancers is partly determined by our environment in youth. Therefore, it follows that the investigation of cause should not be confined to the patient's recent past. Information obtained from studies of immigrant populations shows that the incidence of cancer changes with environment, diet and habits such as smoking (Borek, 1986). This

presents the prospect that if the constituents of risk can be identified, they could be avoided. Thus cancer epidemiology tackles the prevention of the disease primarily by attempting to identify the causes of cancer. Experimental studies try to analyze mechanisms in the additional hope for a cure. As the mystery of the disease is still under intense research, the important step is to use the two disciplines to understand the cause and seek prevention.

One such measure involves the identification and restriction or the removal of chemical carcinogens from the environment. Biotransformation by host enzymes can convert many chemicals to reactive carcinogens, while others termed "direct acting" are carcinogenic in parent form and do not require metabolic activation. Chemical carcinogens belonging to the latter class include alkylating agents such as the nitrosoureas. Members of the former group, designated procarcinogens, include agents such as polycyclic aromatic hydrocarbons (PAH) and nitrosamines. Both types of carcinogens react covalently with cellular macromolecules including DNA. The fact that the neoplastic state is heritable at the cellular level implicates DNA as the crucial target for the genotoxic carcinogens mentioned above (Casarett and Doull, 1986).

This thesis presents research utilizing three such genotoxic compounds, i.e., ethylnitrosourea (ENU), diethylnitrosamine (DEN), and dimethylbenz(a)anthracene (DMBA).

CHAPTER I.

DIMETHYLBENZ(A)ANTHRACENE CARCINOGENESIS IN THE RAINBOW TROUT

INTRODUCTION

CHEMICAL CARCINOGENESIS

The induction of cancer by chemicals proceeds through a complex series of reactions. Experimental evidence indicates that a dynamic process occurs during cell transformation involving several stages broadly divided into the categories initiation, promotion and progression. Hence, tumor induction is evident only after a long latency period from initial exposure. The probability of transformation depends on cell type, species, reactivity of the carcinogen or its metabolite, and especially on dosage.

The initiation phase can be defined as a permanent change in the cell after exposure to an appropriate dose of the carcinogenic agent. This change may involve covalent binding of carcinogen metabolites to cellular DNA. It is believed that at least one cycle of cell replication must follow for the initiating event to be established (Farber, 1981). Initiation alone does not always result in tumor development. For example, it has been shown that no tumors are produced after painting mouse skin with a single dose of an initiating carcinogen such as DMBA, but subsequent, repetitive, sufficiently large doses of

phorbol esters will result in papillomas over the span of a year (Iversen et al., 1979). This post-initiation stimulation of initiated cells towards frank tumor development is termed promotion. Promoters like the phorbol esters can therefore be defined as agents that increase the tumorigenic response when applied after initiation by a subcarcinogenic dose of the carcinogen. Application of the promoting agent months, or even a year, after carcinogen exposure has been shown to be tumorigenic (Van Durren, 1978). The main conclusions from such studies are that promotion has no memory and is reversible during the early stages, whereas initiation, though irreversible, does not inevitably result in cancer (Pitot, 1983).

The irreversibility of initiation is demonstrated by the fact that multiple divided doses achieve the same result as a comparable total dose administered at one time (Casarett and Doull, 1986). It also has been stated that a chemical can be an initiator, a promoter, or both (Williams and Weisburger, 1986). Initiation-promotion studies have revealed that some chemicals in sufficiently large doses can induce tumors without the use of a promoter. It has been suggested that these compounds, termed "complete carcinogens", promote their own initiation. At low initiation levels, tumors do not result when promoter is applied prior to, rather than subsequent to the initiating agent. Examples of a few promoters of relevance to humans are phenols

(found in tobacco tars), phenobarbital, saccharin, cyclamates, and various hormones, notably estrogens (Borek, 1986).

The most extensively studied promoters, the phorbol esters, are not metabolically activated and do not bind covalently to cellular macromolecules (Pitot et al.,1983). These agents are known to increase cell replication, induce enzymes involved in growth and division or retardation of terminal differentiation, and modify membranes of cells growing in vitro (Blumberg, 1980). Several theories have been proposed, regarding their mechanism of action. A specific mechanism involving interaction with protein kinase C, a multifunctional enzyme that appears to play a central regulatory role in the control of cellular events, has been proposed. The phorbol esters can mimic the action of diacylglycerol, a product of inositol phospholipid hydrolysis in membranes, required in the activation of protein kinase C (Nishizuka, 1986).

A second theory proposes that promoters may activate cell replication processes. Since the action of many promoters is limited to specific cells and tissues, surface receptors on target cells are postulated to play a role in promotional mechanisms (Pitot et al., 1983). However, some promoting agents act in a nonspecific manner and their mechanism remains ambiguous.

The stage of promotion is followed by progression of the neoplasm from a benign to a malignant state. It is possible that this stage involves genes of an aberrant group of cells coding

for phenotypic properties which are more advantageous for the perpetuation of the tumor cell population (Folkman, 1976).

In summary, experimental evidence indicates initiation to be the decisive, though almost casually achieved step in chemical carcinogenesis. In contrast, promotion may be a more exacting step in experimentally induced neoplasia. This can be inferred from the fact that if modifiable laboratory conditions for promoting agents, such as dose, time, and frequency of exposure, are not met, tumors do not develop. The suggestion above, and the fact that promotion can be reversible, leads to the conclusion that this stage plays an important part in determining whether latent initiated cells become expressed. Since the possibility exists that such cells are present in all individuals, prevention of cancer may depend on obtaining more information about the mechanisms involved in this process, and the identification, restriction, and removal of tumor promoters as well as complete carcinogens from the environment (Cairns, 1986).

ENZYMATIC ACTIVATION

Procarcinogens lack biological activity until they are enzymatically converted to electrophilic species which can bind to nucleophilic macromolecules, DNA, RNA and proteins (Miller, 1978). In general, the enzyme systems that convert xenobiotics to reactive and toxic compounds also serve to detoxify them.

The mammalian microsomal cytochrome P-450 system can act in a mixed function oxidative (MFO) or reductive manner. Oxidation occurs with a great number of hydrophobic compounds. Reduction may generate radical species and involves the transfer of one electron from the heme protein. An example of oxidation is the formation of epoxides from polycyclic aromatic hydrocarbons. Reductive reactions catalysed by the P-450 enzyme complex include the reduction of quinones to semiquinone radicals (Conney, 1982).

Several other enzymes which normally detoxify chemicals can also be involved in the generation of electrophilic species. Gluthatione-S-transferase catalyses the formation of an episulfonium species from 1,2-dibromoethane which can alkylate DNA (Guengerich and Liebler, 1985). Another enzyme, epoxide hydrolase, forms 7,8-dihydroxy 7,8-dihydrobenzo(a)pyrene from the corresponding epoxide. This dihydrodiol is the precursor of a diol-epoxide, which is a putative proximate carcinogen (Conney, 1982). Thus it is possible for an original reactive metabolite to be converted to an even more reactive compound by further metabolism (Wood et al., 1976).

A detailed consideration of the biochemical properties of various enzyme systems involved in xenobiotic activation is important. Tissue localization plays an important role in metabolism, often providing a tissue-specific source of active metabolite and hence site of tumor formation. However, enzymatic studies have shown that the liver can be the source of reactive

metabolites for many extrahepatic sites (Boyd et al., 1983). Therefore activating enzymes in the target organs are at times not the only ones important in bioactivation of carcinogens.

The enzyme mainly implicated in xenobiotic bioactivation is the microsomal P-450 MFO system (Conney, 1982). P-450 enzymes have been found in almost all tissues studied thus far, with the exception of skeletal muscle and erythrocytes. Several different P-450 enzymes have been purified from human, rat, rabbit and hog (Guengerich and Liebler, 1985). They are sex specific and can be regulated by steroid hormones and numerous other xenobiotics. The isozymes studied from various animal sources display different rates of metabolism for substrates. Also single compounds can be metabolized in different ways by the different isozymes. For example, the procarcinogen aflatoxin B1 (AFB1) is converted to the proximate carcinogen AFB1 2,3-epoxide by cytochrome P-450, but to a much less potent 4-hydroxylated product aflatoxin M1, by cytochrome P-448 (Swenson et al., 1975). It is thus possible for a toxic chemical to be bioactivated by one isozyme and detoxified by another.

Various xenobiotics have been found to induce one or more different forms of P-450 enzymes, or decrease the level of one isozyme relative to another. These compounds include polychlorinated biphenyls (PCBs), beta-naphthoflavone (BNF), dioxins, phenobarbital and 3-methylcholanthrene. Where the mechanism of MFO induction is concerned, PAH are the best studied examples. They are known to enter cells, bind to

cytosolic receptors, and are translocated to the nucleus, thereby activating genes for transcription leading to increased levels of mRNAs coding for individual P-450s (Guengerich and Liebler, 1985). The degree of induction of individual P-450s by a single chemical can range from less than 2 to as much as 100 fold (Dannan et al., 1983).

The flavoprotein P-450-reductase delivers electrons to P-450, but can also reduce some chemicals directly. This enzyme has been purified in a number of laboratories. Evidence suggests that only one form of NADPH-P-450 reductase is present in each species (Guengerich and Liebler, 1985). The ratio of P-450 to NADPH reductase is 20:1 *in vivo* but the highest activity observed in *in vitro* reconstituted systems is with an equimolar mixture of purified P-450 and NADPH-P-450 reductase. The mode of interaction of the two enzymes in physiological situations is not completely understood.

Besides the P-450 enzyme complex, another mixed function oxidase that can activate chemicals is the microsomal flavin-containing monoxygenase system that oxygenates nitrogen and sulfur atoms. This enzyme system has been isolated from rat and hog liver, exists in a single form within each species, and the enzyme does not seem to be inducible by the classical inducing agents. In fact, in rodents, phenobarbital and beta-naphthoflavone actually produce enzyme attenuation (Guengerich and Liebler, 1985). In contrast to P-450 and NADPH-P-450

reductase, the levels of this enzyme in rodent lung and kidney are relatively higher compared with those in liver.

In addition to biochemical properties of activation enzymes, studies have also shown a genetic basis for variation in response to various xenobiotics. Individuals display different rates of metabolism for biotransformation reactions such as N-acetylation and oxidation (Idle et al., 1979). In studies with inbred mice it has been established that susceptibility to several types of PAH induced tumors is strongly associated with Ah receptor mediated inducibility of cytochrome P_1-450. It has been suggested that genetic differences in the inducibility of P_1-450 in humans might be important in determining susceptibility to lung cancer (Gelboin, 1983).

Complementary DNA for human cytochrome P_1-450 has been sequenced and shown to be highly homologous to mouse cytochrome P_1-450 (Jaiswal et al., 1985). This provides a clue to relating experimental models to human carcinogenesis. Evidence exists that within a family slow versus fast metabolizers may be present. The genetic model presents the involvement of a single allele, where homozygous recessive individuals comprise the slow metabolizers (Evans et al., 1983). A rat model also has been established for this system. (Al-Dabbagh et al., 1981). The hypothesis that slow metabolizers carry a decreased risk has been presented for aflatoxin B1. If the parent compound is activated to an electrophilic species, then it follows that a slow metabolizer could be at a decreased risk of carcinogenesis.

INFLUENCE OF CHEMICALS ON BIOACTIVATION OF CARCINOGENS

1. INDOLE-3-CARBINOL

The consumption of cruciferous vegetables such as broccoli, Brussels sprouts, and cauliflower has been associated with a reduced rate of cancer in several species (Haenszel et al., 1980). Indole-3-carbinol (I3C) found as a glucosinolate in such vegetables, in some experimental models can cause an increased activity of phase I enzymes, which are known to play a role in activating as well as inactivating chemical carcinogens (Wattenburg, 1978). An enhancement for some steps in phase II conjugation has been revealed in rats (Sparnins et al., 1982). Rats fed diets containing I3C at levels as low as 50 mg/kg diet for ten days display a six fold increase in benzopyrene hydroxylase (BPOH) activity. The no effect level was estimated to be 16 mg/kg to 25 mg/kg (Bradfield et al., 1984). Studies in rainbow trout, however, show no induction of MFO activities, though the tumor incidence was found to be 4% for fish fed I3C 1000 ppm compared with 38% in AFB1 positive controls (Nixon et al., 1984). While one phase II reaction, production of bile carcinogen-glucuronide, was shown to be enhanced by I3C *in vivo* in trout, no studies were conducted to examine induction of the appropriate enzymes (Goeger et al., 1986). Thus the relationship of MFO activities with inhibition of carcinogenesis by I3C is not clear.

As mentioned above, I3C is known to inhibit chemically induced carcinogenesis. However, administration during the initiation phase causes inhibition but post initiation intake actually enhances carcinogenesis produced by AFB1, the tumor enhancement being the result of an I3C dose of 2 mg/g diet of life long daily administration (Bailey et al., 1987). Vegetarians may consume a dose approaching that used experimentally, but usually, in man, the dose is likely to be much lower and ingestion more intermittent.

Where the enhancement of tumorigenicity is concerned, rats fed 1% I3C prior to, during, and after initiation by dimethylhydrazine showed increased incidence of adenomas of the intestine in comparison with rats fed control diets (Pence et al., 1986).

Further elucidation of dose response relationships is needed to understand the mechanism of action of this compound. A recent study has revealed that I3C inhibition is not highly dependent on route of AFB1 administration, and that trout pretreated with 0 ppm to 2000 ppm I3C show no alteration of type of DNA adducts formed, though there is a decrease in total DNA binding in a dose dependant manner (Dashwood et al., 1988). This type of carcinogen-inhibitor dose-response study has not been conducted with other compounds, but it is known that I3C provides protection against covalent binding of benzo(a)pyrene (BAP) and dimethylnitrosamine (DMN) (Shetzer et al., 1984). This information indicates a common mechanism mediated towards

protection against DNA damage produced by these compounds. It is not clear that the protection results from alteration in metabolism, as no relationship has been established regarding MFO activities or DNA binding and rate of metabolism after I3C treatment. Hence some investigators have proposed that I3C may protect against DNA binding by producing lipophilic-nucleophilic metabolites in the endoplasmic reticulum, the I3C metabolites then bind to the electrophiles produced by microsomal carcinogen metabolism. This hypothesis explains the decrease in covalent binding as proportional to the reactivity of each electrophile with the nucleophilic entity formed (Shetzer et al., 1984).

Before the information provided by these experimental studies can be applied in analyzing the significance to man, the role of I3C and its metabolites in modifying carcinogenesis needs to be examined further. The important conclusion is that the data portrays carcinogenesis as a multistage process, where dose and time of exposure to carcinogen and inhibitor are important modulatory factors.

2. BETA-NAPHTHOFLAVONE (BNF)

BNF, a known inducer of MFO activity in several animal models (Wattenburg, 1985) serves as an important tool in studying the mechanisms of modulation of chemical carcinogenesis. The induction of the MFO system can have implications in the initiation of carcinogenesis by producing

active metabolites, as well as the conversion of metabolic products to more hydrophilic compounds, thus facilitating excretion.

To determine the effects on the process of neoplasia involves identifying the types of MFO activities which are constitutive and those which can be induced by a chemical in an experimental model. In mammalian models, BNF stimulates P-450 mediated benzo(a)pyrene hydroxylase while inhibiting P-448 benzo(a)pyrene hydroxylase activity. The trout has been shown to possess P-450 activities much lower than those measured in rodent models. Statham et al. (Statham et al., 1978) administered BNF, 3MC, and 2,3- benzanthracene by the i.p. route to identify enzyme induction in rainbow trout. In this study the content of P-450 increased after treatment with all three compounds. BNF treatment <u>in vitro</u> caused an inhibition of the benzo(a)pyrene hydroxylase activity in control microsomes, suggesting that the enzyme activity induced was, in contrast to rodent models, of the P-448 type. No change in the ferrocytochrome-carbon monoxide complex was observed, indicating that induced and constitutive cytochromes were the same.

Other investigations have also shown P-448 activities in trout after BNF treatment. Elcombe et al. (Elcombe et al., 1979) showed that induced and control protein were similar in qualitative but not in quantitative properties. The suggestion was made that different subpopulations of P-450 existed in control and BNF treated trout. Later, Williams et al. (Williams

et al., 1984) succeeded in isolating five P-450 isozymes from rainbow trout after BNF induction (Williams et al. 1984). By contrast, trout are refractory to the inducing effects of the phenobarbital class of compounds which served to identify certain MFO activities in rodent models (Statham et al., 1978). The fact that trout respond to at least some inducers provides valuable data for comparison with mammalian species in considering the inhibition of carcinogenesis by BNF.

Dietary BNF has been shown to inhibit AFB1 carcinogenesis in the rainbow trout (Nixon et al., 1984). A pronounced change in in vitro AFB1 metabolism to less carcinogenic aflatoxin M1 and reduction in toxic aflatoxicol is observed after BNF treatment of trout. DNA adducts decreased by 60% and metabolism of AFB1 increased (Bailey et al., 1981).

Where mechanism of action of BNF as an inhibitor is concerned, its administration in various animal studies provokes the induction of multiple enzyme activities. Since several enzyme systems with coordinated activities are induced, reaction with a single receptor is postulated (Wattenberg, 1985). Inhibitors of this type, which modify procarcinogen activation, have been shown to be associated with inhibition of neoplasia in several experimental models (Wattenberg, 1985). Monooxygenase activities are shown to be enhanced, as are the conjugation enzymes, gluthathione S-transferase (GST) and uridine diphosphate glucuronosyltransferase (UDPG). GST activity has been shown to be enhanced in liver and small intestine of

female ICR/Ha mice, exposed to dietary BNF (Sparnins et al., 1982). This has important implications in the inhibition of chemical carcinogenesis, since one means of protection against cancer would be to induce or enhance the activity of detoxification systems (Sparnins et al., 1982).

3. POLYCHLORINATED BIPHENYLS

PCBs and their degradation products are environmental pollutants of concern as they are toxic, stable, lipophilic and tend to bioaccumulate. They occur in most human and animal adipose samples, milk sediment and numerous other matrices and their toxicity has led to the setting of workplace threshold limits.

The biological effects of PCBs upon the P-450 system are of particular importance due to the modifying effects produced on carcinogenesis. Both promoting and inhibiting effects on neoplasia, as well as induction of liver microsomal enzymes are known. Effects on fish populations are used as a measure of environmental pollution as well as a means of gaining information about their possible effects on human cancer (Varanasi et al., 1987).

In the rainbow trout, control fish show low monooxygenase activity compared to mammals, which may be a reflection of interspecies difference in substrate specificities. Studies by Elcombe et al. (Elcombe et al. 1979) have shown the existence

of several different forms of P-450s in fish. There was, however, a lack of induction of P-450-associated N-demethylation of ethyl morphine by Aroclor 1242. This is in contrast to mammalian species where both P-450 and P-448 activities can be induced (Goldstein et al., 1977).

Hendricks et al. (Hendricks et al., 1977) showed that response to toxic chemicals could be altered by feeding PCBs to rainbow trout. In this study Aroclor 1254 at 100 ppm fed for one year was not carcinogenic and reduced the carcinogenic effects of AFB1, and doses in the range of 100 ppm to 275 ppm Aroclor 1242 produced maximum induction of P-450. A later study (Voss et al., 1982) showed that dietary treatment of PCBs caused a marked increase in microsomal enzyme activity. Ethoxy resorufin-O-deethylase activity was found to increase 77-fold from control values. It was also found that tissue levels of PCBs did not increase after prolonged exposure to low dietary levels while enzyme induction reached steady state or began to decrease upon prolonged feeding.

In addition, Stott et al., 1978 (Stott et al., 1978) found that pretreatment of rainbow trout with various PCBs decreased the mutagen assay response (using AFB1 sensitive tester strain S. typhimurium TA 1538, and trout post mitochondrial fraction, producing an average of 5.2×10^4 revertant bacteria/μAFB1/g wet liver) to AFB1 in contrast to rat studies. Trout mutagen response to AFB1 also decreased with increase in chlorination of the PCBs used as pretreatment (Stott and Sinnhuber, 1978).

A study by Egaas and Varanasi (Egaas and Varanasi, 1982) using microsomes obtained from trout pretreated i.p with Aroclor 1254, showed that BAP binding to deproteinized salmon sperm DNA was significantly higher than controls. This however reveals only one portion of the effect of Aroclor 1254, its effect on BAP metabolic activation by phase I enzymes. In vivo phase II enzymatic effects cannot be accounted in this study. Thus dietary exposure of rainbow trout to PCB prior to and during AFB1 initiation results in reduction of carcinogenesis via MFO induction, promoting metabolism to less toxic aflatoxin M1. By comparison, exposure to PCB after initiation has no effect on tumor incidence (Shelton et al., 1986). This is not necessarily true of rodents where post initiation PCB effects resulted in significant inhibition of growth of Walker 256 carcinomas in rats (Kerkvliet et al., 1977).

It can be summarized from the discussion of the studies conducted on modulators of carcinogenesis, that tumor enhancers or promoters produce their impact on the process of neoplasia depending on the time of exposure, the type of carcinogen and the dose of modulator. The response to a carcinogen in turn, in rainbow trout, has been shown to be greatly influenced by dose of the particular carcinogen and the age of the fish. The extent of the resultant DNA alteration produced by the combination of the carcinogen and the modulator, will depend on the ultimate kinetics thus established between metabolism and repair.

POLYCYCLIC AROMATIC HYDROCARBONS

The astute observations regarding the carcinogenicity of soot made by Sir Percival Pott remained unexploited until Yamagiwa et al. (Yamagiwa et al., 1915) succeeded in inducing tumors on site in rabbits by repeated painting of the skin with coal tar. This was the first study of experimentally induced carcinogenesis. Methods were later refined to demonstrate that carcinogenic response was species specific and varied with dose applied. A quest for more specific information led to the extraction, isolation and identification of the PAHs, compounds found to be carcinogenic to animals and man (Kennaway, 1930).

Application of these compounds to the skin causes skin cancers, subcutaneous injection produces sarcomas, and the introduction into a particular organ results in local tumors. The carcinogenic potency of the PAH is in turn dependent on the proportion of active and inactive metabolites.

1. EXPOSURE AND RELEVANCE TO MAN

PAHs are components of tobacco tar, cigarette smoke and smog. Industrial use has demonstrated that a differential sensitivity to these agents exists in man. Occupational exposure via the lungs is of particular relevance. The PAHs may be deposited in the upper (nasopharyngeal area), middle

(tracheobronchial area), or lower (alveolar compartment) regions of the respiratory tract (IRPC Task Group, 1966).

Benzo(a)pyrene (BAP), a known mammalian carcinogen, in 1 to 2 um aerosol particles inhaled by rats, is deposited mainly in the upper respiratory tract (Mitchell, 1983). Metabolites can be detected in lung, liver and kidney, shortly after exposure. Unmetabolized BAP in the lung was found to be 20%, indicating that BAP was metabolized in the lung prior to clearance. A significant amount was bound to lung tissue and retained for a much longer time than the soluble metabolites. Studies indicate that mucocilliary clearance and subsequent swallowing of the material can result in high PAH levels in the gastrointestinal tract where they may be reabsorbed.

Direct contact with skin results in rapid absorption of these lipophilic compounds. In a topical application study, essentially all radioactive BAP solution applied was recovered within 16 days, indicating quantitative percutaneous absorption of BAP (Heildelberger et al., 1951). Maximum radioactivity was recovered from bile and faeces. It has also been shown that in rats and mice, unmetabolized BAP accumulates in the mammary gland and general body fat relative to other tissues after a single feeding of the carcinogen. In these animals, out of the 10 mg to 30 mg subcutaneously injected BAP, 70% to 75% was recovered in bile, while 4 to 10% was excreted unmetabolized via urine (Kotin et al., 1959).

2. METABOLISM IN FISH

PAHs exert carcinogenic action after metabolism to highly reactive metabolites which bind to DNA, RNA, and proteins. The P-450 enzyme complex abundant in the liver is involved in the oxidation of PAH compounds to active form as well as their detoxification in mammals (Conney, 1982).

The same process is suggested to occur in fish (Melius 1986). Both phase I and phase II enzymes have been identified in fish (Chambers et al., 1976). Williams et al. (Williams et al., 1984) have isolated five isozymes of liver P-450 from BNF induced trout. Treatment of fish between one and seven days with inducers such as Aroclor 1254, BNF or 3MC produces maximum P-450 content (James et al., 1980). The inducing agent has a great influence on the type of MFO activity present in the microsomal fraction of the animal.

The most widely used PAH in experimental studies of carcinogenesis is BAP. Tumor studies in trout exposed to 1000 ppm BAP via diet for 18 months have shown a 25% incidence of hepatocellular carcinoma in treated fish. Monthly i.p. injections of 1 mg BAP, produced a 50% incidence of hepatic neoplasms and MFO activity was also elevated (Hendricks et al., 1985).

The first step in the metabolic activation of PAHs by P-450 enzymes involves production of epoxides and phenols. A second enzyme, epoxide hydrolase (EH), converts epoxides into vicinal

diols (Melius, 1984). Biotransformed BAP, products in fish bind to DNA and proteins (Nishimoto et al., 1985). It is also evident from metabolic studies of BAP that incubation times of in vitro assays are important to distinguish the action of phase I from phase II enzymes, as at low substrate concentration and high incubation times the metabolism of this compound is complex and recycling of metabolites occurs (Melius, 1986).

Where metabolism in fish liver is concerned, the following sequence can occur:

BAP $\xrightarrow{\text{MFO}}$ 7,8 epoxide $\xrightarrow{\text{EH}}$ trans 7,8 dihydrodiol $\xrightarrow{\text{MFO}}$ dihydrodiol epoxide I and II $\longrightarrow$ DNA binding, mutation, cancer. Figure I.1 shows the scheme of metabolism of a typical polycyclic aromatic hydrocarbon, BAP, from studies of the activation, fate and identification of its biotransformation products in many species of mammals including explants of human cells from bronchus lung and colon (Conney, 1982, Casarett and Doull, 1986).

The bay region theory, first proposed by Jerina and Daly (Jerina et al., 1977) suggests that "bay region" (sterically hindered) diol epoxides of PAH are prime candidates for ultimate carcinogens. This is postulated on the basis of inhibition of carcinogenicity by inhibition of bay region diol epoxide formation, and higher chemical and mutagenic reactivity of PAHs having structures that favor bay region diol epoxide formation (Conney, 1982). BAP 7,8-diol 9,10-epoxide 2, believed to be the most potent carcinogenic metabolite of BAP binds covalently to

Figure I.1 Proposed Scheme For Benzo(a)pyrene Metabolism.

Reactions with P-450 MFO system yield epoxides at various positions. Also formed are a radical cation and a phenol at the 6-position, further oxidized to several quinones. Epoxides can react with gluthatione transferase to form water soluble conjugates. The epoxides can also rearrange to form phenols. Epoxide hydrases convert epoxides and phenols to diols which can be further oxidized or excreted as conjugates (Weisburger et al. 1980).

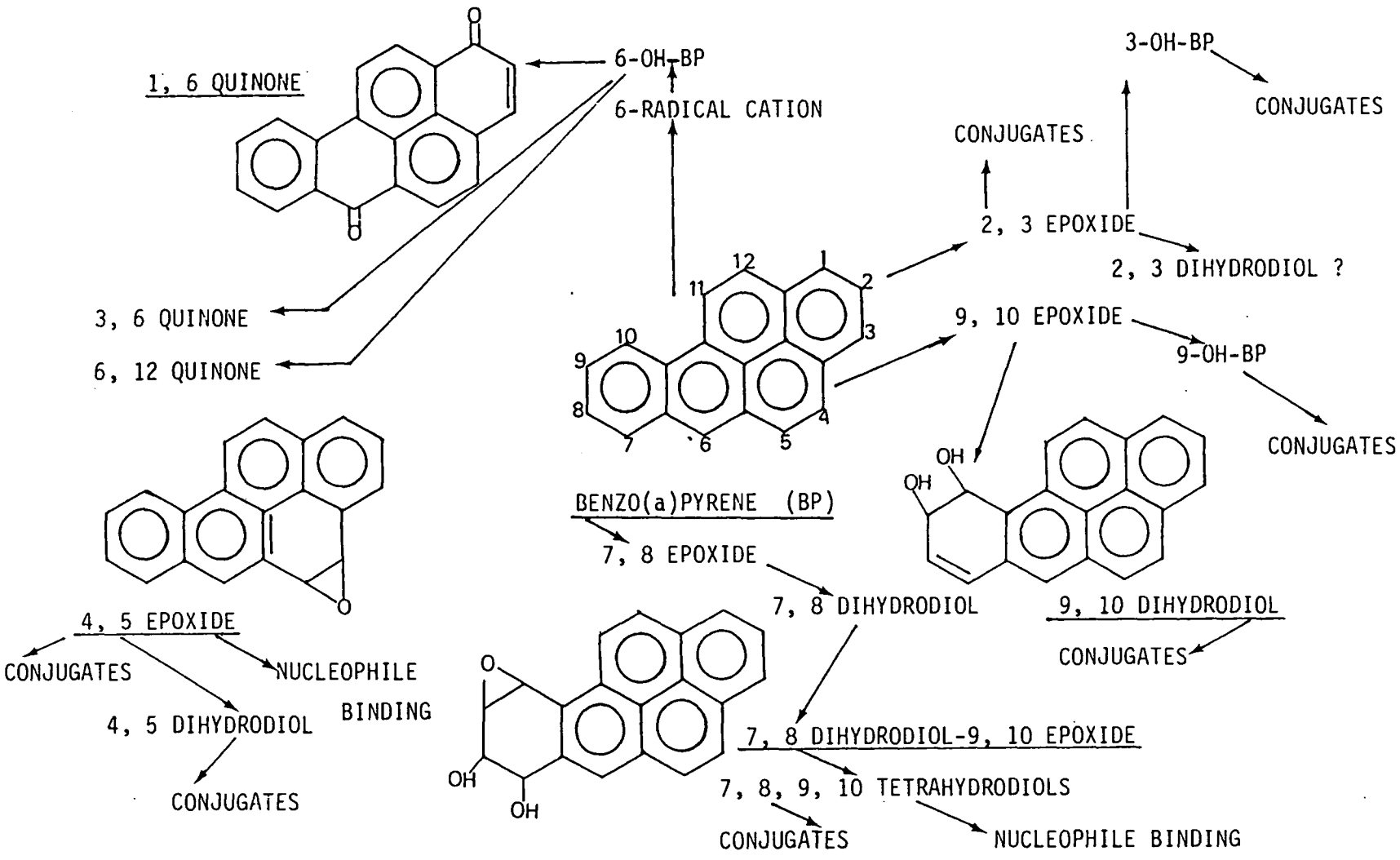
1, 6 QUINONE
6-OH-BP
6-RADICAL CATION
3-OH-BP
CONJUGATES
CONJUGATES
2, 3 EPOXIDE
2, 3 DIHYDRODIOL ?
3, 6 QUINONE
6, 12 QUINONE
9, 10 EPOXIDE
9-OH-BP
CONJUGATES
BENZO(a)PYRENE (BP)
7, 8 EPOXIDE
7, 8 DIHYDRODIOL
9, 10 DIHYDRODIOL
CONJUGATES
4, 5 EPOXIDE
CONJUGATES
NUCLEOPHILE BINDING
4, 5 DIHYDRODIOL
CONJUGATES
7, 8 DIHYDRODIOL-9, 10 EPOXIDE
7, 8, 9, 10 TETRAHYDRODIOLS
CONJUGATES
NUCLEOPHILE BINDING

Figure I.1

the amino groups of guanine to produce the major DNA adduct observed in mammalian systems (Figure I.2).

A consideration of the various metabolite profile of BAP is important as the various metabolites may display different abilities to bind to DNA. Microsomal preparations from fish (scup, winter flounder and killifish) have shown marked regioselective metabolism of total dose of BAP administered with 60% of BAP converted to 7,8-dihydrodiol, 60% to 9,10-dihydrodiol and with the K-region metabolite, BP 4,5-dihydrodiol being formed in undetectable amounts. Furthermore kidney, gill and liver from scup produced the two major metabolites of BAP in similar proportions as embryonic tissues of killifish and adult liver microsomes (Stegeman, 1981).

In mammals, epoxide hydrolases of different species have very different kinetics and can lead to activation or detoxification (Conney et al., 1982). Fish studies have indicated that epoxide hydrolases are also induced after exposure to treatment agents (Melius, 1984). However fish epoxide hydrolases may be ineffective modifying the K region of BAP, as BAP 4,5-oxide was found to be stable and could be isolated experimentally (Melius, 1984). BAP 7,8-oxide and 9,10-oxide were confirmed by the formation of their respective diols which indicates P-450 MFO and epoxide hydrolase activities in fish (Mass, 1979).

In many fish species BAP phenols at 1,2,5,7,8 positions are

Figure I.2 Formation Of Benzo(a)pyrene Adducts Of Guanine In DNA and RNA Of Mouse Skin.

Nucleic acid adducts of the BP 7,8-diol-9,10-epoxides formed from the cis and trans addition of the 2-amino group of guanine to the epoxide ring of the diol-epoxides are shown . The major adduct was from the trans addition of the 2-amino group of guanine across the epoxide group of (+)-BP 7,8-diol-9,10-epoxide-2 (Conney, 1982).

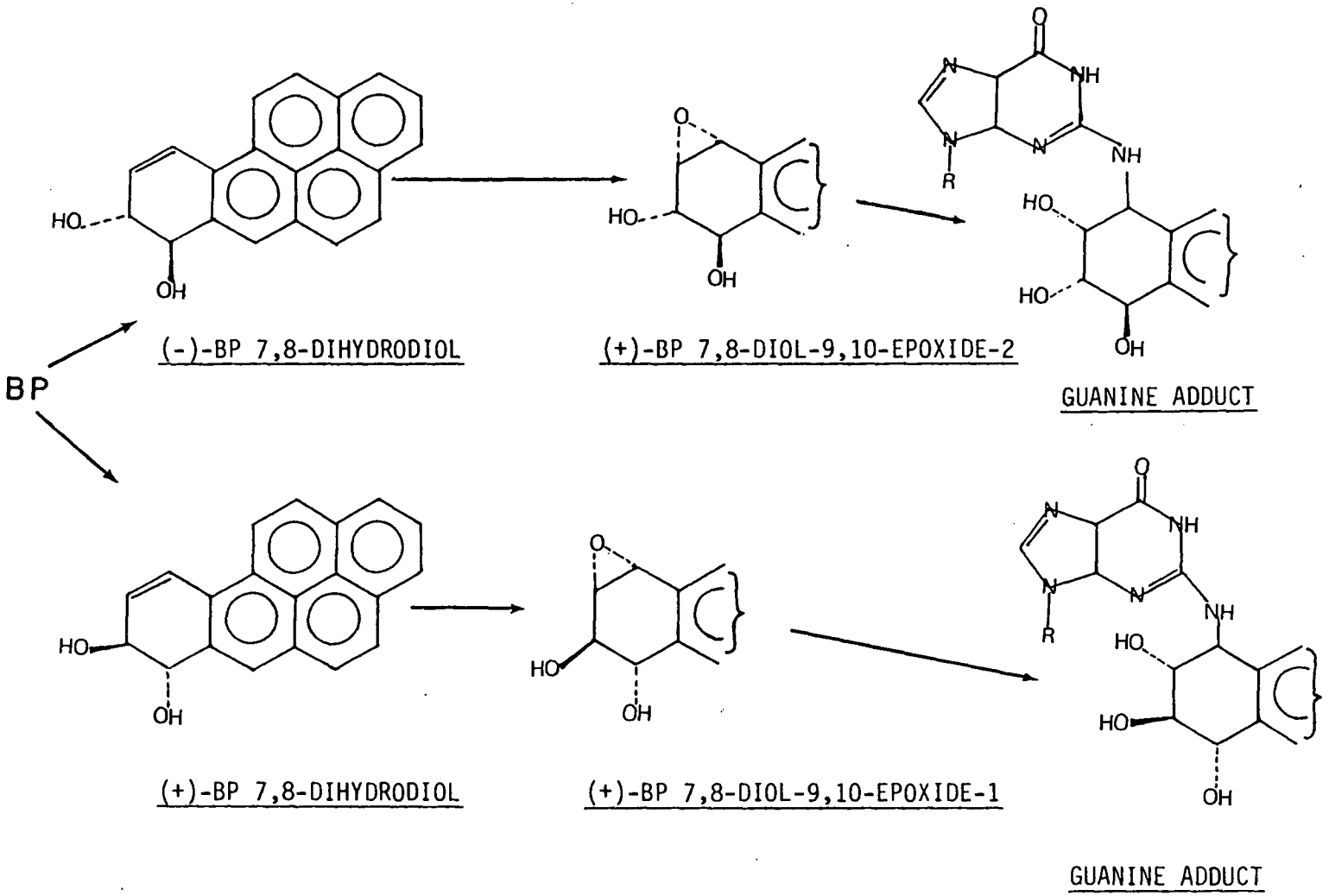
BP
(-)-BP 7,8-DIHYDRODIOL
(+)-BP 7,8-DIOL-9,10-EPOXIDE-2
GUANINE ADDUCT
(+)-BP 7,8-DIHYDRODIOL
(+)-BP 7,8-DIOL-9,10-EPOXIDE-1
GUANINE ADDUCT

Figure I.2

derived from isomerization of epoxides (Melius, 1984). BAP 3-phenol is produced in larger amounts than BAP 9-phenol. The production of BAP phenols are considered as detoxification steps because the phenols are converted to water soluble metabolites (Melius, 1986).

Oxidation of phenols produces quinones, which are known to be cytotoxic in rat and may also be important in terms of interaction with DNA. It is possible that their identification is made difficult by their ability to quench fluorescence. BAP quinones are not found in many fish or mammals as they are further metabolized to water soluble conjugates.

In evaluating the relative carcinogenic risk provided by active metabolites, detoxification and repair have to be taken into account. Therefore, lower risk of carcinogenesis in fish is not explained by rates of repair alone, as it is known that that fish cells have a very low rate of excision repair compared to rodents (Walton et al., 1984). The rate of detoxification and repair can vary among fish species as shown by the fact that liver microsomes from five different species of fish show similarities in activation, with BAP 7,8-diol constituting 20-30% of total metabolites for BAP but a wide range of covalent binding indices (CBI) (Varanasi et al., 1987). Studies have further shown the need for more extensive testing of PAH phase II metabolites. It has been shown that in mammals BAP 7,8-oxide and BPDE (benzo(a)pyrene dihydrodiol epoxide) are GST enzyme substrates in the order BPDE > BAP 7,8-oxide, and GSH activity

relates inversely with DNA binding of BAP metabolites (Jennstrom, 1985).

Where fish are concerned, it is known that the occurrence of hepatic neoplasms in starry flounder is less than in English sole from the same environment. Total GST activity in sole liver is 900+¯ 140 nmoles/mg protein/min compared with a flounder liver GST activity of 2500 +¯750 nmoles/mg protein/min (Varanasi, 1985). Biliary conjugates from English sole have been characterized and an inverse relationship between proportion of GSH conjugates and DNA binding of BAP metabolites to DNA has also been shown (Varanasi, 1983). This would indicate that sensitivity to develop carcinogenesis could be related to higher GST activity, but this concept cannot be generalized. Thus rat and skate liver GST show similar sterioselectivity in a racemic mixture of BAP 4,5-oxide (Hernandez, 1980), but in rat, K-region oxides which are more stable than bay region oxides are better substrates for GST enzymes (Jerina et al., 1976). Therefore, though studies support the conjecture that higher conjugation activities impart protection from covalent binding of metabolites to DNA, the effiency of GSH conjugation depends on substrate specificities of GST isozymes and cannot be predicted from total GST activity.

Phenols, quinones and dihydrodiols are converted to polar sulfate conjugates by cytosolic sulfotransferases, and to glucuronides by microsomal UDP glucoronyltransferases (Armstrong, 1987). Glucuronide formation has been shown

(Varanasi et al., 1987) for starry flounder and English sole. Conjugation, however, may not always present a path for detoxification. For example, the sulfate ester of 7-hydroxymethyl,12-methylbenz(a)anthracene is mutagenic to S. typhimirium strain TA 98. Glucuronide formation may also lead to similar activation of substrate. During enzymatic hydrolysis of the glucuronide of 3-hydroxy BAP, a BAP derivative is formed that binds to DNA to a far greater extent than does the 3-hydroxy-BAP or its glucuronide (Kinoshita and Gelboin, 1978).

3. DIMETHYLBENZ(A)ANTHRACENE (DMBA)

DMBA is a potent carcinogen, several times greater in activity than BAP. It produces skin cancer (Boyland and Sims, 1967), mammary tumors and leukemia (Huggins, 1979) and necrotic action on rat adrenals and testis (Hallberg and Rydstrom, 1987). It is the most potent ovarian chemical carcinogen in rodents (Moore et al., 1987). DMBA is also a photobiologically active carcinogen. The hydrophobic DMBA concentrates in the cytoplasm where binding to lysosomal membranes is initiated. DNA damage initiated by DMBA in the presence of UV light can be repaired but is followed by massive degradation of the DNA, suggesting extensive endonucleolytic attack by release of degradative enzymes (Utsumi, 1987)

In a recent study, Tagacuchi et al. (Tagacuchi et al., 1988) treated mice with 17 beta estradiol prior to gastric

intubations of 20 mg/kg DMBA once a week for 3 weeks. The incidence of ovarian tumors was observed to be much higher in DMBA + 17 beta estradiol group than DMBA positive controls (i.e., 14 of 18 and 5 of 15, respectively). Neonatal estrogen treatment induced a high degree of neoplasia and tumor development in ovaries of mice treated with DMBA. Moreover, the results strongly suggested a direct tumor initiation effect of DMBA on ovarian tissue, and ovarian carcinogenesis was proposed to be initiated by activation of ovarian aryl hydrocarbon hydroxylase (AHH) (Rydstrom, 1983).

Enzymatic activities to a great extent determine the metabolites formed. The metabolic profile of DMBA varies immensely in various tissues. P-450 epoxidation in skin (Boyland and Simms, 1967) and peroxidative mechanisms in mammary gland (Huggins, 1979) are believed to generate carcinogenic metabolites involved in the action of DMBA. Necrotic action in rat adrenal glands is coupled with a high rate of phenol and dihydrodiol production. Induction produces increased ring hydroxylated products in liver.

Classical cytochrome P-450 inducers do not induce adrenal AHH, rather the induction has been shown to be dependent upon adrenocorticotropic hormone (Hallberg and Rydstrom, 1987). Inhibitors of steroid hydroxylase and AHH, metyrapone and SU-9055, increase adrenal toxicity, while estradiol and testosterone increase necrosis (Hallberg and Rydstrom, 1987). Mutagenicity tests using S9 fractions from wild and laboratory

reared woodchucks have also suggested that natural inducers may be involved in liver microsomal enzyme activities for activation of DMBA (Rashid, 1988). Microsomal studies with Sudan III indicate a regioselective shift in metabolism of control and treated groups, leading to formation of K region diol epoxides which are better substrates for GST (O'Dowd and Burnett, 1988). Moreover, O' Dowd and Burnett found that though the absolute amount of P-450 did not increase, ECOD activity (associated with cytochrome P-450c) increased three-fold. UDPG activity was also found to be increased two-fold. Metabolic changes in liver were associated with decreased accumulation of metabolites of DMBA at other sites such as the bone marrow and also with prevention of leukemia in Long Evans rats treated with Sudan III.

Steriospecific metabolism of DMBA leads to the formation of optically active epoxides which may be hydrated by epoxide hydrolase to trans dihydrodiols (Conney, 1982). Both K region and bay region dihydrodiols and their hydroxy methyl derivatives have been identified using rat liver microsomes (Christou et al., 1986). 7,12-DMBA-trans-3,4-dihydrodiol-1,2-epoxide (bay region) is implicated as the carcinogenic metabolite and is produced by further monooxygenation of the 7,12-DMBA-trans-3,4-dihydrodiol (Figure I.3). Enantiomeric trans dihydrodiols of PAHs exhibit markedly different biological activities including mutagenicity and tumorigenicity (Conney, 1982).

Where metabolism of DMBA in fish is concerned, Smolarek et al. (Smolarek, 1987) showed that rainbow trout embryonic gonad

Figure I.3 Proposed Scheme For Dimethylbenz(a)anthracene Metabolism.

The hydrocarbon is metabolized by hydroxylation of one or both methyl groups to yield the 7-hydroxymethyl-12-methyl (7HOMMBA), 12-hydroxymethyl-7-methyl (12HOMMBA) and 7,12-dihydroxymethyl (not shown) derivatives. These derivatives, and the parent dimethylbenz(a)anthracene (DMBA), can be oxidized by the P-450 MFO system to the corresponding 3,4-epoxides. Epoxide hydrolases (EH) catalyse the formation of dihydrodiols from epoxides, and the 3,4-dihydrodiols can be oxidized further by P-450 to 3,4-diols-1,2-epoxides. All epoxides above are proposed to be able to bind to macromolecules, including DNA.

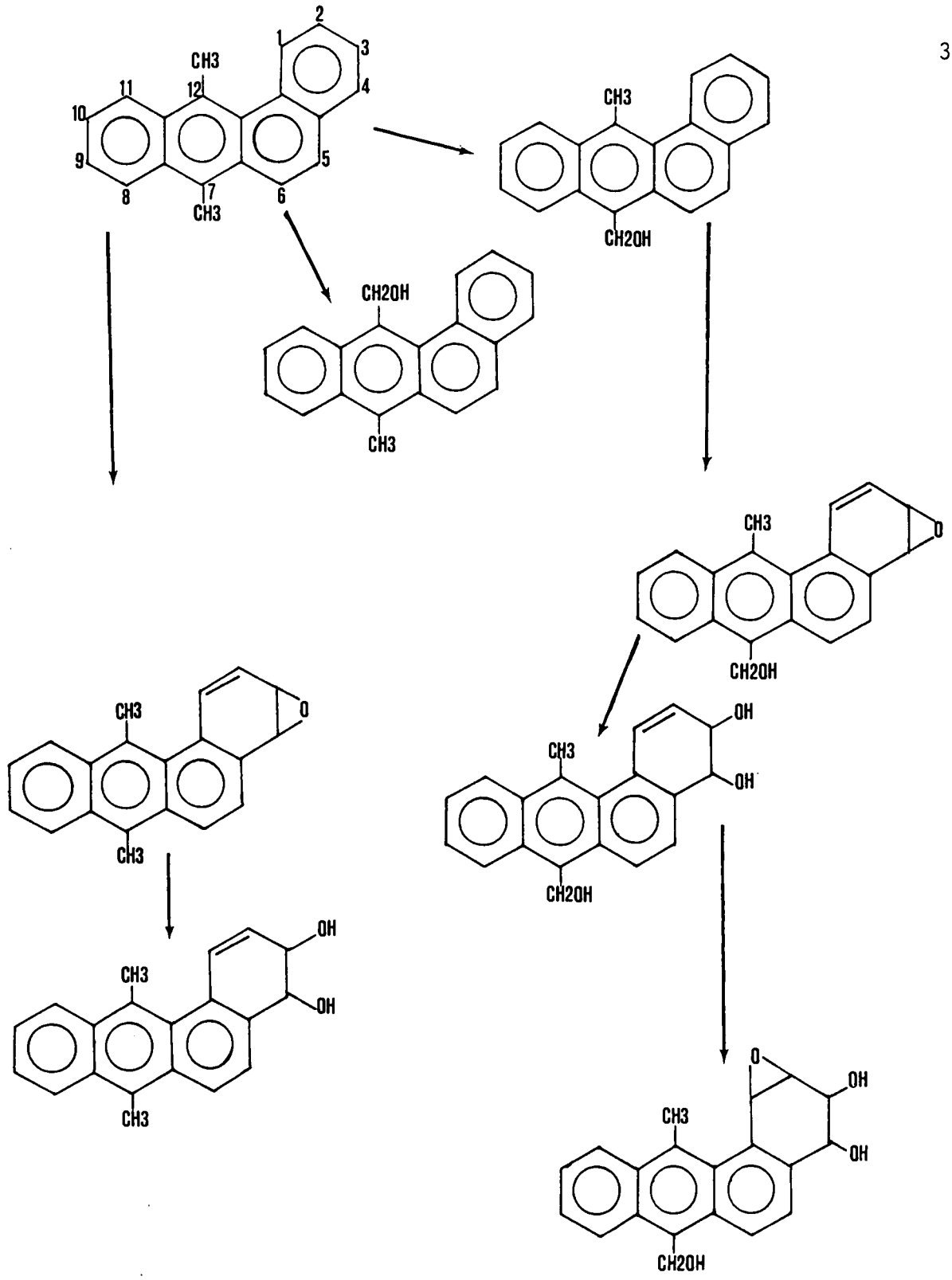

Figure I.3

cell cultures posses high metabolizing ability (97%), blue gill fry cell cultures possesses lowest (47%) and brown bull cells have intermediate ability (78%). In all cell types, 60% of the compound is converted to water soluble metabolites within 24 hours. However, the relative abilities of these cells to metabolize BAP is different from those of DMBA. Metabolism varied with the type of PAH (DMBA and BAP) and the different fish cells metabolized the same PAH at different rates. Hence oxidation to bay region diol epoxides cultures was a minor pathway of metabolism for DMBA in blue gill fry cells but significant in brown bull cell cultures. The fish cells showed similar metabolism rates as hamster embryo and Wistar rat embryo cell lines (Baird et al., 1979). Water soluble metabolites in fish cell cultures and rodent embryo cell culture were both $\geq$ 80% after 24 hours of exposure. Thornton et al. (Thornton et al., 1982) showed that fish converted metabolites to glucuronides and glutathione conjugates, trout cell lines forming 50% glutathione conjugates.

Metabolic competence is not the only factor involved. Products revealing phase II enzyme activity constitute important data. For example it has been shown that, in contrast to rodent embryo cells, most of the BP-7,8-diol added to the blue gill fry cells and the rainbow trout cell cultures was converted to glucuronides and glutathione conjugates, which may reveal a lower risk of DNA adduction for fish compared to rodents. Similarly, the hamster shows relative resistance to

carcinogenesis by DMBA and BAP. Though the presence of glucuronic acid conjugates and DNA bound products reveal the metabolic competence of the animal, sulfate conjugates in hamster epidermal cells or mouse epidermal cells have not been found (Digiovanni et al., 1983).

Total DNA binding observed in fish cell lines is in the order BAP > DMBA, and BAP shows higher values in rat embryo cells. Lower DNA binding in fish cells however is not due to low metabolism but due to high rates of conjugation. However, high proportions of bay region diols by fish cell lines could result if conjugating enzymes were inhibited.

DNA adducts of DMBA have been identified by Dipple et al. (Dipple et al., 1984) and studies suggest that ring hydroxylation in positions 1,2,3,4 is essential. Elution profiles of the DNA adducts shows 4 distinct peaks (Vigny, 1981). Studies conducted with rat and human mammary cultured cells by Moore et al. (Moore, 1987) have presented an interspecies difference in their mutagenicity (Gould, 1980), DNA binding and adduct formation upon BAP and DMBA treatment. Moore et al. found that DMBA treatment shows similar proportions of adducts but higher total amount in rat than in human cell cultures. This is coupled with higher mutagenicity mediated by rat cultured mammary cells after treatment with DMBA and BAP.

Consequently, analysis of the generation of the active metabolites of various PAHs, the DNA binding produced, together with a knowledge of the reactions of various conjugation

enzymes, may help to explain inter- and intra-species susceptibility to tumor formation (Smolarek et al., 1987; Varanasi et al., 1987).

OBJECTIVES AND RATIONALE

PAHs are of human concern because they are ubiquitous environmental pollutants and have been implicated in human cancer (Bjorseth and Becher, 1986). Though they have been the chemicals most extensively studied in investigating the molecular events involved in the process of neoplasia, several aspects of their structure-activity relationships, metabolism and biotransformation remain unclear.

The review of existing information suggests avenues for further exploration. For example, the study of the PAH BAP has revealed a variation in tumor response within mammalian species, the hamster showing relatively high resistance while others (e.g., rat, mouse, fish) are more sensitive. At the cellular level mammalian species vary from fish, the cells from these models showing differences in response such as DNA binding, production of various metabolites and rates of metabolism and detoxification of BAP. The metabolic profile and the carcinogenic potential further changes with the PAH used. It is therefore essential for the calculation of risks to humans and also for understanding the basis for resistance and sensitivity to the disease of cancer to improve the database concerning the response of various PAHs such as BAP and DMBA in several different models, including fish. The modulation of this response by various chemicals studied in mammalian species would further facilitate the extrapolation of data to humans. The

study in a fish species such as rainbow trout also provides a model for the estimation of environmental pollution effects of the PAHs.

The objectives of this study were to ascertain the modulation produced by dietary intake of three compounds, Aroclor 1254, BNF, and I3C, on the DNA binding produced by i.p. DMBA exposure in rainbow trout. To study inter-individual variation and examine the effect of age on the dietary treatment, the experiment was conducted using groups of fish where DNA binding was studied per fish and then repeated with fingerling trout where samples from ten fish were pooled together in each DNA analysis.

MATERIALS AND METHODS

SAFETY PROCEDURES FOR HANDLING OF CARCINOGENS

Refer to Appendix I for details.

CHEMICALS

All chemicals were obtained from Sigma Chemical Company and were of the highest grade available. Tritiated DMBA (specific activity, 29-50 Ci/mmole) obtained from Amersham was checked for purity by TLC using hexane as mobile phase. Unlabelled DMBA was obtained from Aldrich Chemical Company.

ANIMALS AND DIET

Rainbow trout, Salmo gairdneri, were spawned and raised at the Oregon State University Toxicology And Nutrition Laboratory. Controls were fed Oregon test diet #7, a semipurified caesin diet (Lee et al., in press). The modulators BNF, Aroclor 1254, or I3C were included in the diet at levels of 500 ppm, 100 ppm, and 2000 ppm, respectively.

EXPERIMENT 1

In experiment 1, ten month old trout, 129-248g, were fed for four weeks. One group of 10 fish/treatment (3 treatments, 1 control, 40 fish total) was used. Livers from each fish in the group were analyzed independently. Feeding was stopped 24 hours prior to i.p. injections. Tritiated DMBA was injected at 284 μCi/1.58 μmole/fish. Fish were sacrificed by severing spinal cords 24 hours after injection, livers were removed, frozen under liquid nitrogen and stored at -80° C.

To prepare the sample for DNA extraction, nuclei were isolated from livers in experiment 1 by the procedure detailed by Goeger et al. (Goeger et al., 1985). Briefly, livers were weighed, homogenized in sucrose buffer (0.25 M sucrose, 3 mM $MgCl_2$, 10 mM Tris, pH 7.4) and centrifuged. The pellet was washed twice with above buffer, resuspended in buffer plus Triton x-100 (0.5%), washed again and the nuclear pellet stored at -80° for DNA isolation.

DNA was isolated and purified, as detailed by Dashwood et al. (Dashwood et al., 1988). In summary, the nuclear pellet was suspended in Tris buffer (0.1 M NaCl, 10 mM Tris, 1 mM EDTA; pH 7.4) and the mixture added to an equal volume of 1% sodium dodecyl sulphate in buffer. This was followed by treatment with RNase A plus 200 U/ml RNase T_1. Samples were then incubated with proteinase K and extracted with solvents in the following order: Tris saturated phenol, chloroform, Tris saturated phenol and lastly chloroform:isoamyl alcohol (24:1). The aqueous layer was mixed with 2.5 volumes of isopropanol and the precipitated

DNA washed with the same. Purity was routinely estimated by DNA spectrophometric scans (210 nm to 320 nm) using 260/280 and 260/230 ratios (Maniatis et al.,1982). DNA was quantitated by Burton's assay (Burton, 1956) and radioactivity was determined by using a Beckman LS 7500 liquid scintillation counter and standard procedure. DMBA binding was calculated using the specific activity of the compound.

EXPERIMENT 2

In this experiment, three month old trout were fed control and modulator diets for two weeks. Trout weighed 5-7g and ten fish/group, four groups/treatment were used (three treatments, one control, 160 fish total). DMBA was injected at 22.4 μCi/3.9 x 10^{-2} μmole/fish. The same procedure as experiment 1 was followed except frozen livers (ten/group) were crushed, and powdered in a mortar and pestle using small quantities of liquid nitrogen. The liver samples were transferred to polypropylene tubes and suspended in Tris buffer. DNA was extracted and analyzed as mentioned for experiment 1.

OTHER ASSAYS

NCS tissue solubilizer (Amersham) was used to solubilize liver, bile, kidneys and intestine tissues to determine DMBA distribution after injection. About 0.2-0.5 g of tissues were

accurately weighed, minced in vials and 2 ml NCS was added. Gall bladders were snipped, washed with known volume of water and contents plus wash used in the analysis. The vials were heated at 50° C for 20 minutes. Digests were treated with 0.4 ml of benzoyl peroxide solution (1 g/5 ml toluene) at 50° C for 20 minutes. The samples were diluted to 20 ml with scintillation fluor and counted to determine radioactivity.

STATISTICAL ANALYSIS

All statistical tests were performed using the Statgraphics statistical graphics system (Statistical Graphics Corporation, user's guide, 1986). Log values were employed for covalent binding indices (CBIs) computed as {μmoles DMBA/mole nucleotide} /{millimole DMBA injected/Kg fish}. One way analysis of variance was used to examine if mean log CBI was different for the control and the treatment groups, using the Tukey range test, 95% confidence interval. The Kruskall-Wallis nonparametric method of one way analysis of variance also was used to compare the medians of all the groups. Nonparametric comparison of two samples was then employed to compare each treatment group with control group (Roxy and Peck, 1986).

RESULTS

DMBA, a potent mammalian carcinogen, was tested in trout to examine the extent of DNA binding response and to determine the effect of various dietary pretreatments on this value. Two different experiments were conducted using ten month and three month old trout to examine the effect of age on DNA binding. Further, fish liver samples were analyzed individually (experiment 1) and in pooled samples of ten livers/group (experiment 2) to reveal the extent of variation in DNA binding among individual fish. Fish livers were removed, samples prepared and DNA analyzed as described in Materials and Methods. Values obtained for DNA binding were used to compute the covalent binding indices (CBI) as follows: {μmoles DMBA/mole nucleotide (average molecular weight = 309)}/{millimoles DMBA injected/Kg fish}. The CBIs constituted the " response," and log values of response were used for statistical analysis to determine whether dietary I3C, Aroclor 1254, or BNF (described under animals and diets in the Materials and Methods section) groups differed from group fed control diet in each experiment.

STATISTICAL ANALYSIS

1. EXPERIMENT 1

Data for DNA binding from individual livers (experiment 1) are presented in Table I.1a, I.1b, Figure I.4, Figure I.5, and Table I.2. Fish in experiment 1 were analysed individually in each treatment group (three treatment and one control group) and Table I.1a lists the individual fish weight and liver weight recorded. The BNF dietary group shows the least amount of variation in body weight. There was no significant difference between the liver weight/body weight ratios.

Where the DNA binding response (computed as CBI) is concerned, one way analysis of variance was performed to determine if there was more variation between treatment group means than there was within the treatment group. The collected or pooled variation within groups was therefore used as a standard of comparison, as it measured the inherent observational variability in the data. In this way values of log response (log CBI) were analysed using the Tukey range test, 95% confidence level (Statgraphics, software). Table I.1b gives the means obtained experimentally for the log of the response variable (CBI) for control, I3C, Aroclor 1254 and BNF groups using the Tukey range test and confidence level described above. The null hypothesis tested in this test, i.e. that all treatment responses were the same, was rejected indicating that there was a statistically significant difference in at least one response ($p = 0.001$).

Figure I.4 presents the means for each factor level (control, I3C, Aroclor 1254, and BNF) and the 95% confidence

Table I.1a. Table Of Trout Liver And Body Weights For Treatment And Control Groups In Experiment 1.

CONTROL		I3C		AR1254		BNF	
WEIGHT (g)	LIVER	WEIGHT (g)	LIVER	WEIGHT (g)	LIVER	WEIGHT (g)	LIVER
129	1.087	133	2.283	141	1.532	166	2.176
177	1.968	131	1.850	149	1.250	180	1.977
181	1.525	144	1.777	156	1.149	141	1.510
124	1.298	152	2.168	164	1.480	145	1.374
152	1.544	155	1.935	130	1.173	152	1.971
138	1.880	182	2.610	146	1.315	138	1.576
147	1.073	185	2.701	153	1.301	169	2.371
163	1.960	165	2.025	179	1.906	176	1.884
151	1.703	153	2.269	130	1.132	141	1.577
170	1.734	176	2.335	175	1.833	161	1.989
Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
153.2	1.6	157.6	2.2	152.3	1.4	156.9	1.84
S.D	S.D	S.D	S.D	S.D	S.D	S.D	S.D
19.5	0.3	19.1	0.3	16.8	0.3	15.5	0.3

Table I.1b Table of Means For Log Response By Treatment. Experiment 1.

DIET	COUNT	[a]MEANS	STANDARD ERROR
1. CONTROL	10	4.44	0.33
2. I3C	10	3.54	0.34
3. AR1254	10	4.34	0.39
4. BNF	10	2.27	0.48

[a]Log Response = Log Covalent Binding Index (CBI).

CBI = $\frac{\mu\text{moles DMBA/mole nucleotide (Av. molec. wt.= 309)}}{\text{mmoles DMBA injected/kg fish.}}$

Range test: Tukey, confidence level: 95, One-Way Analysis of Variance.

intervals for the means using the Tukey. Considerable overlap can be observed between control interval and that for I3C and Aroclor 1254, such that the means were not significantly different. The range of the BNF group interval is distinct from the control group but some overlap of the BNF group occurs with that of the I3C range.

Figure I.5, shows a multiple box and whisker plot of the observations at each factor level. In this graphic representation the central box covers the middle 50% of the data values, between the lower and upper quartiles. The whiskers extend to the extremes (minimum and maximum values), while the central line is at the median. Unusual values are plotted as separate points. The whiskers extend only to the points that are within 1.5 times the interquartile range (box length).
It can be seen in Figure I.5, as in Figure I.4, that the median for all dietary treatment groups is below controls. Control and I3C show two extreme values, well above the bulk of the values. Log response values for I3C show the least amount of variation (smaller box and least whiskers) while AR1254 and BNF values show a much greater variation (larger box and whiskers). It is probable that medians as computed in the box and whisker plot may provide a better representation of the data than the means as the medians are not sensitive to extreme values.

A multiple range Tukey analysis was performed after the one way analysis of variation, which revealed that a difference in

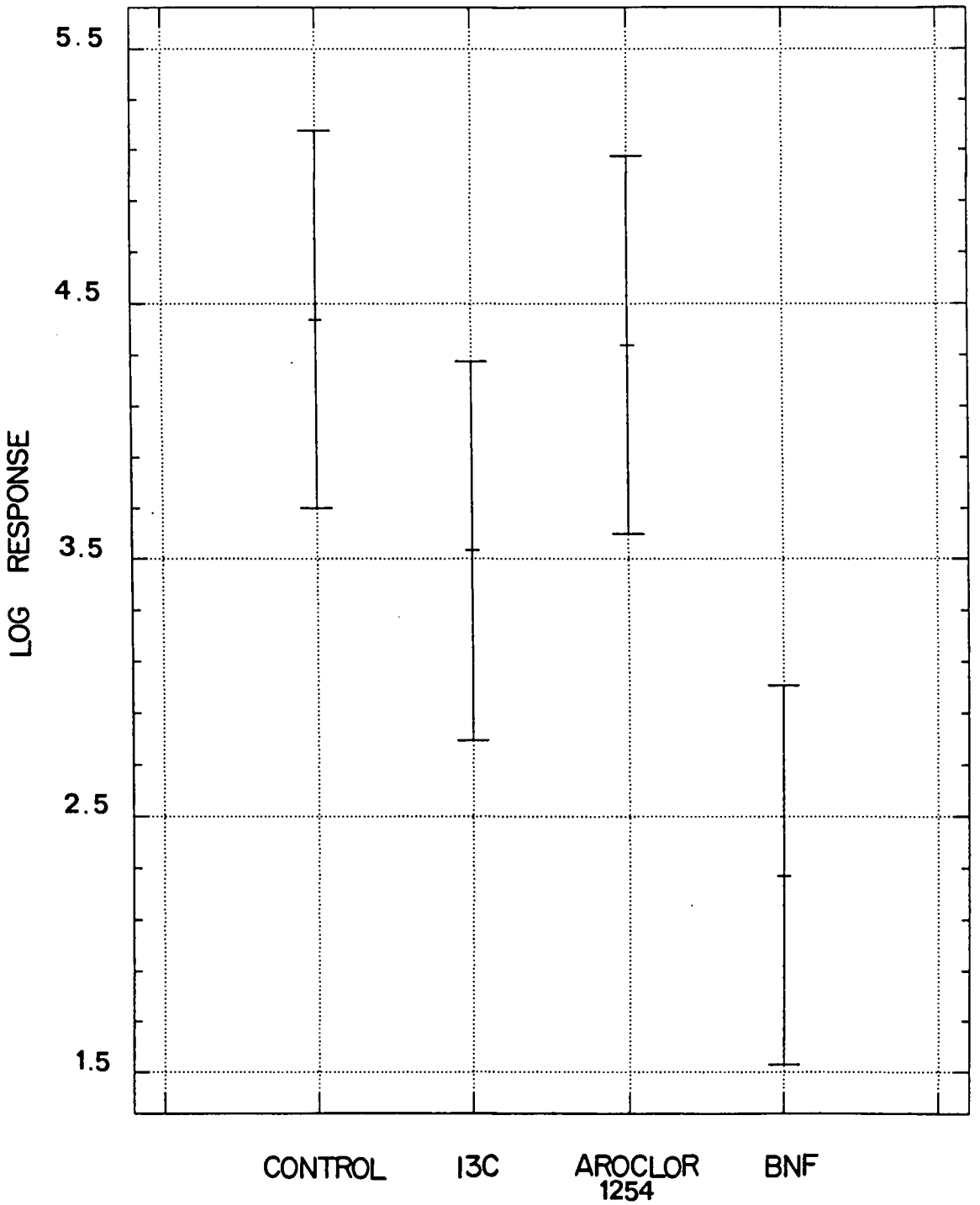

Figure I.4 95% Tukey HSD Intervals For Factor Means.
Experiment 1.

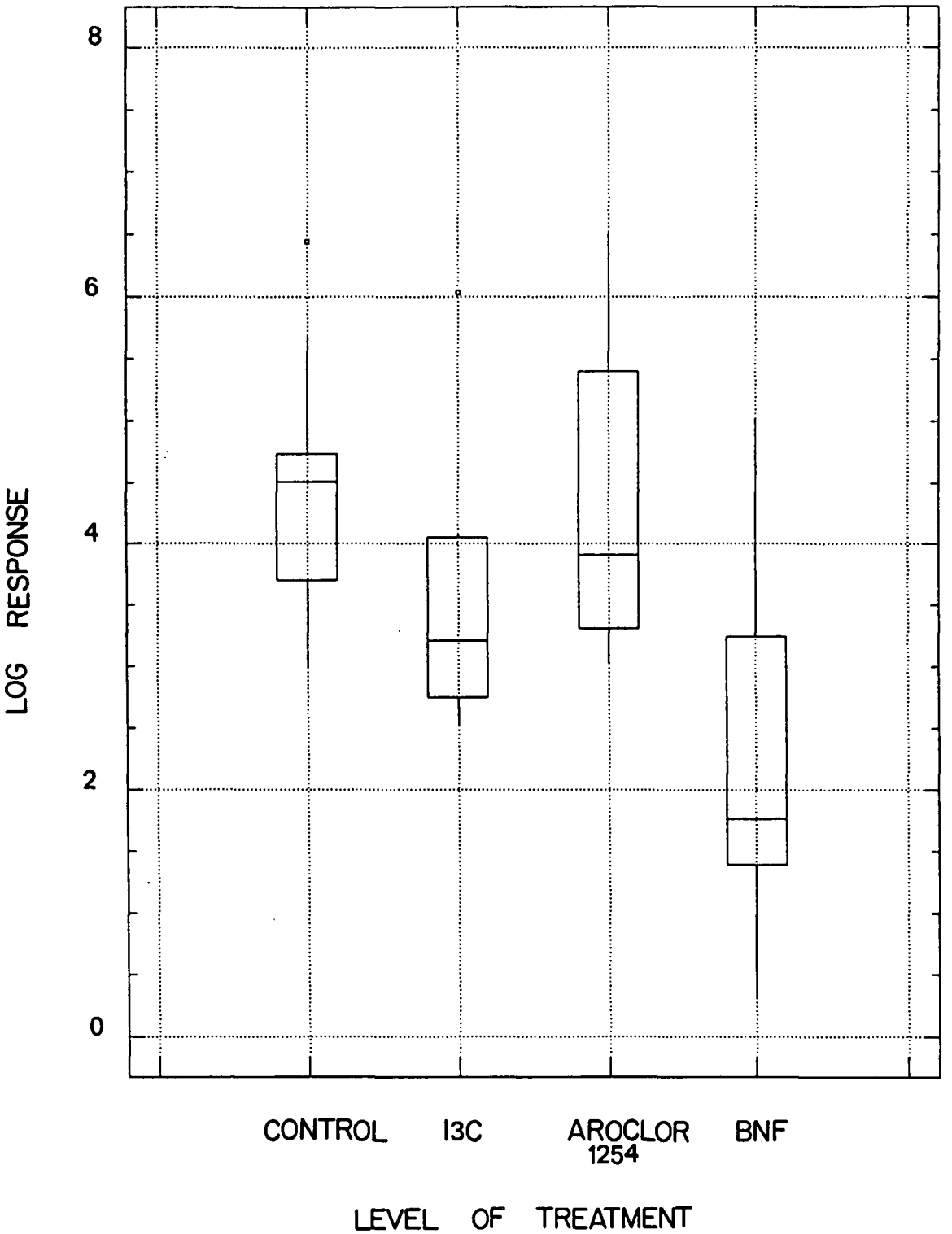

Figure I.5 Box And Whisker Plots For Factor Level Data. Experiment 1.

response from control value existed only for the BNF dietary treatment group.

The first set of analysis, Tukey, assumed a normal data distribution and hence the one way analysis of variance was repeated by the non parametric method of Kruskal-Wallis, in which each response value was assigned ranks as shown in Table I.2. The rationale was that if the data did not follow a Gaussian distribution, the results would only be an approximation and could be misleading. This nonparametric method provides the advantage of a greater range of applicability as it assumes that observations occur at random after respective treatment and that no tendency for the larger values to come first exists. However the disadvantage is that this technique disregards the actual scale of measurement and substitutes ranks to compare relative magnitude, resulting in the loss of power of sensitivity. The Kruskal-Wallis one way analysis of ranks revealed a statistically significant difference in response was present for at least one of the groups ($p = 3.9 \times 10^{-3}$).

Nonparametric comparison of paired samples was also tested in which each diet treatment group response value was compared to control group response value. This test revealed that the median for the I3C treatment group was significantly different from control ($p = 0.05$), Aroclor 1254 was not significantly different ($p = 0.7$) and BNF was significantly different ($p = 5.7 \times 10^{-3}$). The ultimate result of statistical analysis

Table I.2 Kruskall Wallis Analysis Of Response By Treatment Experiment 1

LEVEL	SAMPLE SIZE	AVERAGE RANK
1. Control	10	27.4
2. I3C	10	18.2
3. Aroclor 1254	10	25.9
4. BNF	10	10.5

was that only the BNF group was significantly different from the control.

2. EXPERIMENT 2

In experiment 2 statistical tests were performed in the same manner as experiment 1, though four groups of ten, three month old trout were used per treatment group. Table I.3a, Table I.3b, Figure I.6 and Table I.4 present the data from experiment 2 and are discussed in the above sequence.

Table I.3a gives body weight and liver weight data for groups of ten fish each. Control groups consistently showed highest mean weights and standard deviations, and Aroclor 1254 and BNF group body weights show least standard deviation. The differences among groups in mean weight do not directly influence the experiment, since the final test statistic, CBI is expressed in dosage (Materials And Methods). Table I.3b gives a table of means of log response values for treatment and control groups using the range test Tukey as described for experiment 1. Pooling of samples in groups of ten reduces the variation within groups as can be noted by comparing the spread of the box plots from each experiment, however values for relative error in each experiment are not easily computed and compared as log values of the CBIs were used in each experiment, and the mean CBI of control groups differs considerably between experiment 1 and 2, introducing a confounding variable.

Table I.3a. Table Of Trout Liver And Body Weights For Treatment And Control Groups In Experiment 2.

CONTROL		13C		AR1254		BNF	
WEIGHT (g)	LIVER	WEIGHT (g)	LIVER	WEIGHT (g)	LIVER	WEIGHT (g)	LIVER
63	0.017	62	0.011	48	0.015	48	0.016
62	0.014	52	0.013	50	0.014	51	0.020
65	0.012	48	0.012	51	0.012	54	0.016
87	0.009	53	0.013	50	0.016	50	0.019
Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
69.3	0.01	53.8	0.01	49.8	0.01	50.8	0.2
S.D	S.D	S.D	S.D	S.D	S.D	S.D	S.D
11.9	0.0	5.9	0.0	1.26	0.0	2.5	0.0

Each value for liver and body weight represents 10 individuals.

Table I.3b. Table Of Means For Log Response By Treatment. Experiment 2.

DIET	COUNT[b]	MEANS[a]	STANDARD ERROR
1. CONTROL	4	2.84	0.12
2. I3C	4	2.14	0.35
3. AR1254	4	2.09	0.25
4. BNF	4	0.78	0.27

[a]MEANS = (Log CBI)x 10^4
[b]COUNT = 4 groups/treatment, ten livers/group.

Figure I.6 gives a plot of the means for log response of each treatment group and the 95% confidence intervals by the Tukey test as in experiment 1. Pooling of samples did appear to reduce overlapping intervals for BNF group values with I3C group (as observed in experiment 1) resulting in this group being distinctly different from all other groups. I3C and Aroclor 1254 values present identical spread of interval, being partially overlapped by the control values.

Figure I.7 presents the multiple box and whisker plot for the three treatments and the control group. The amount of variation in values seen for the groups is in the order control < Aroclor 1254 < BNF < I3C, denoted by the size of the box plots and the whiskers. Medians for all treatments are below that of control. The BNF group emerges distinctly different from all other groups, the spread of the box values showing no overlap with any other treatment box. In comparison, pooling in experiment 1 eliminated the presence of individual outlying data points in these plots. As elaborated for experiment 1, Table I.4 presents the Kruskall Wallis nonparametric analysis of response in experiment 2 as computed by the statgraphics statistical software.

DISTRIBUTION OF RADIOACTIVITY IN TISSUES

Table I.5 gives the in vivo distribution of radioactivity in selected tissues of rainbow trout 24 hours after i.p. and i.c.

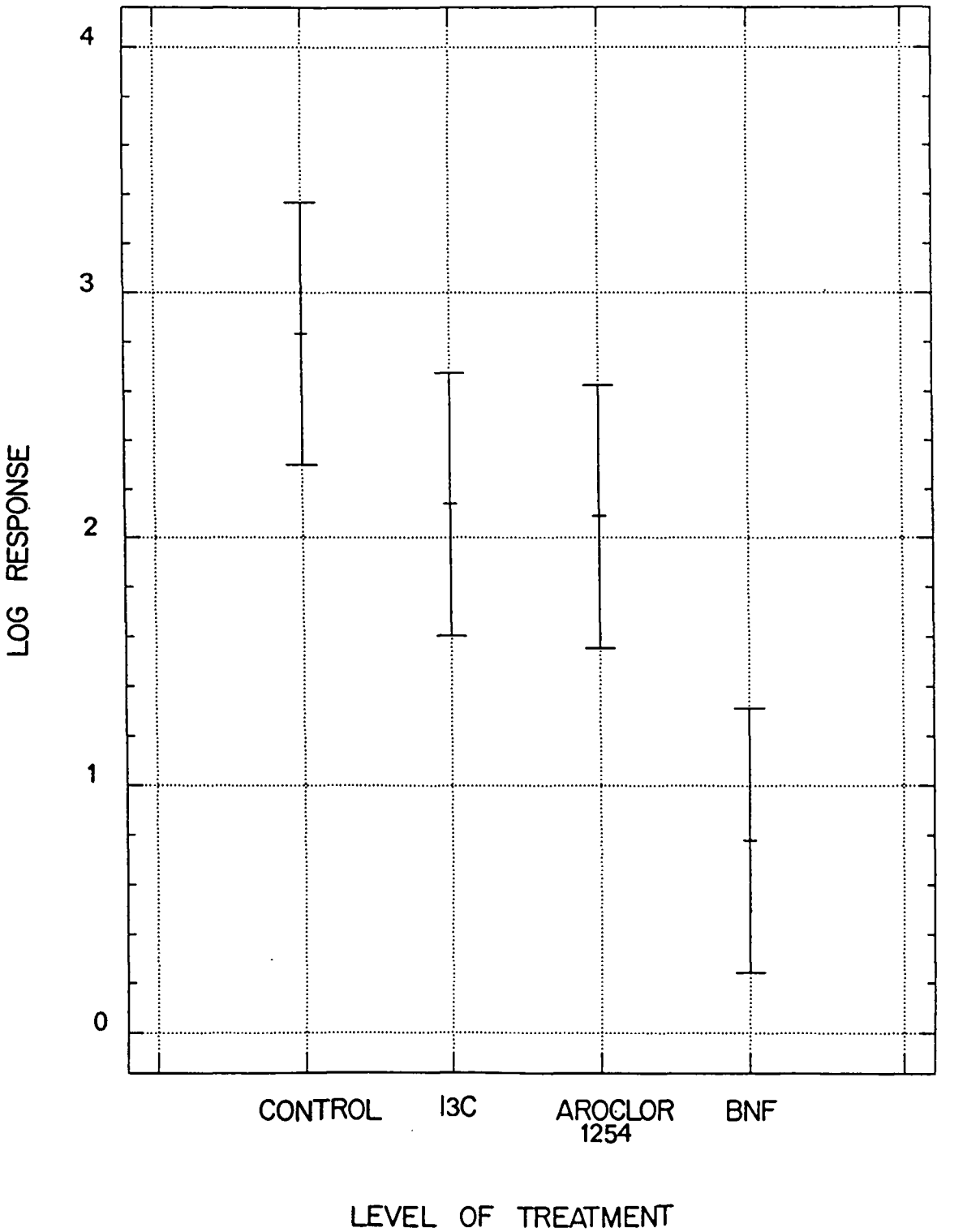

Figure I.6 95% Tukey HSD Intervals For Factor Means. Experiment 2.

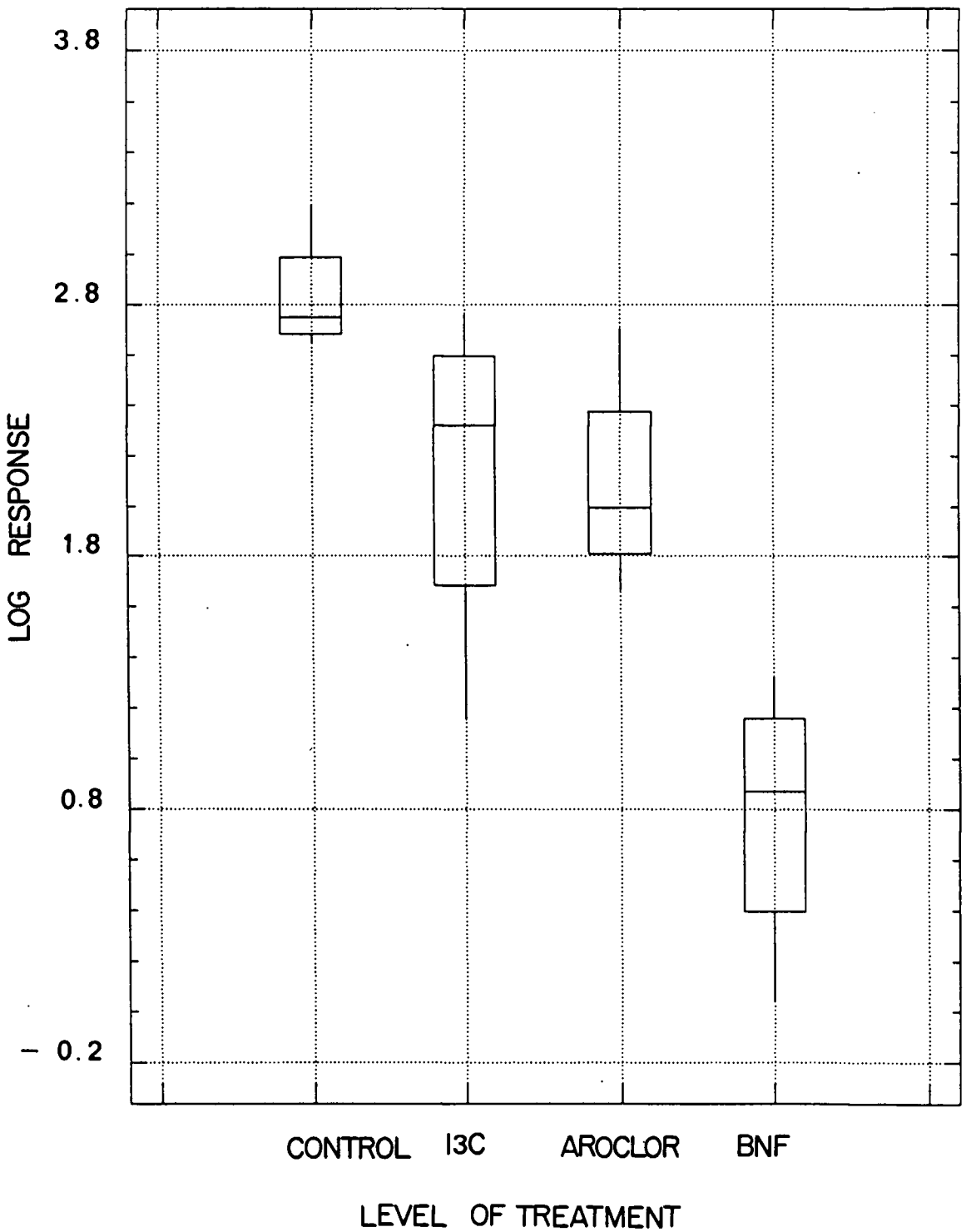

Figure I.7 Box And Whisker Plot For Factor Level Data. Experiment 2.

Table I.4 Kruskall Wallis Analysis Of Response By Treatment. Experiment 2.

[a]LEVEL	[b]SAMPLE SIZE	AVERAGE RANK
1. Control	4	13.75
2. I3C	4	9.25
3. Aroclor 1254	4	8.25
4. BNF	4	2.75

a Dietary treatment groups
b ten fish/group, four groups/treatment.

Table I.5 DMBA Distribution In Tissues Of Rainbow Trout Expressed As Radioactivity % Total Dose Given.

BODY WT. (G)	KIDNEY	LIVER	GALL BLADDER	INTESTINE
1. 62.40	1.05	3.88	49.50	1.05
2. 76.15	0.55	2.72	19.92	0.55
3. 74.07	0.45	2.36	19.93	0.45
4. 76.15	0.69	2.93	19.8	0.68

Data Given Is For 4 Individual Fish.

#1 fish injected i.c.
2, 3, 4, i.p. injection.
Distribution Measured 24 Hours After Injection, Solvent acetone.

injections (intracardial). Binding values obtained after i.c. injection show about double the amount of total binding in all tissues tested (kidney, liver, gall bladder and intestine) compared with those obtained from i.p. injections. Gall bladder shows the highest amount of radioactivity followed by the liver, while values for kidney and intestine were approximately the same.

VARIATION IN DNA BINDING WITH AGE

CBIs show marked variation with age of fish. The three month old trout show average control CBI of 1.7×10^{5} in experiment 2 and ten month trout have average value of 1.44×10^{2}, an almost 1000 fold difference in 7 months.

DISCUSSION

Results show BNF dietary treatment of 500 ppm, in rainbow trout (three months and ten months of age), produces a statistically significant inhibition of *in vivo* DMBA binding to DNA, when the carcinogen is administered by i.p injection.

Effects of the other two modulators are not as clear. Both I3C and Aroclor 1254 have lower medians for groups than control group. This difference however, does not register as statistically significant due to overlap of values within groups especially evident in experiment 1 (Figure I.5), where individual fish DNA was analyzed to generate the data for CBI. The spread of values in Figure 5, as denoted by the size of the box plots is considerable, especially for Aroclor 1254. The situation changes in experiment 2 (Figure I.7), with group values of I3C showing most variation. This further confounds any deductions that can be made from the data regarding effect of the diets.

BNF DIETARY MODULATION

The results obtained in this study are consistent with those from other experimental observations that BNF can inhibit the carcinogenic response. It has been shown that BNF is able to significantly inhibit tumor initiation by polycyclic aromatic hydrocarbons such as DMBA in mice, its protective effects on

tumor initiation being extended to the prenatal organism (NMRI mice) (Goerttler et al., 1986). In a 58 week tumor study of rainbow trout where carcinogenesis was initiated by AFB1, the tumor incidence was 6% for fish treated with 500 ppm BNF, and 18% for 50 ppm BNF compared to 38% for AFB1 positive controls (Nixon et al., 1984).

Treatment with BNF is known to cause a dramatic increase in monooxygenase activity, the cytochrome P-448 parts of which are known to generate bay region diol epoxides of DMBA. BNF induced activity of trout microsomes has been shown to be of the P-448 type (Williams and Buhler, 1984).

Trout cells have been shown to possess ability to metabolize DMBA (Smolarek et al., 1987). One possibility is that the inducing effect of BNF on monooxygenase activities can produce a regioselective shift in metabolism of DMBA to less reactive metabolites or one with a higher affinity for an isozyme catalyzed reaction, resulting in lower binding to DNA. Christou et al. (Christou et al., 1986) showed that in studies using 3MC induced rat microsomes, reorientation of metabolic activation can occur via hydroxylation of the parent DMBA. Constitutive rat enzyme P-450a favors direct activation of the parent compound, while 3MC induced rat microsomes contain P-448c. The hydroxylated derivatives and the parent compound compete for metabolism by P-448c, with the hydroxylated derivatives exhibiting higher affinities. Christou et al. however found

7-hydroxymethyl,12-methylbenz(a)anthracene to inhibit its own metabolism. Consequently, ratios of individual P-450s may be crucial in determining characteristics of DNA modification. Since in trout BNF induces P-448c, it is possible that hydroxylation is favored, resulting in less activation of the parent compound.

Williams et al. (Williams et al., 1983) have shown that a major cytochrome P-448 purified from BNF induced trout, produced a significant increase in dihydrodiol formation from AFB1. As mentioned above, studies have already suggested that PAH metabolism is dependent on P-448 activities. Thus a second possibility is that BNF induction of enzymatic activities may increase the overall rate of metabolism and detoxification, decreasing transit time of the active metabolite of DMBA and hence DNA binding. In support of this conjecture, it is known that the overall rate of metabolism for AFB1 is enhanced in studies with trout hepatocytes obtained from BNF pretreated trout and that DNA binding is reduced in such cells (Bailey et al., 1981). Studies here indicate that DMBA metabolism and DNA binding values for DMBA are also altered in trout by BNF dietary intake, suggesting that this compound may inhibit DMBA hepatocarcinogenesis in trout.

Studies with rainbow trout cells in culture have shown an inherent ability to metabolize PAH coupled with a high ability to conjugate metabolites, resulting in lower DNA binding values than rodent cells (Smolarek et al., 1987). Moreover, BNF is

known to increase the activity of phase II enzymes in mice (Sparnins et al., 1982). It is possible that similar induction occurs in trout. If increasing conjugating activities with BNF treatment is possible the result would be escalation of detoxification of DMBA, as a mechanism underlying reduced DNA binding.

In assessing the role of BNF modulation in PAH carcinogenesis, information is confounded by factors such as in vitro vs in vivo studies, age, time of exposure of modulator and carcinogen, dose of both compounds and species used. Many enzyme studies have been conducted in vitro and may serve the purpose of identifying some metabolites formed. However, in vitro studies are lacking in presenting the complex array of events occurring in an in vivo situation, of one chemical modifying the effects of another. For example phase II enzyme effects are ignored in many in vitro investigations, thus presenting possibly erroneous conclusions. Also relative amounts of protein and substrate are important for kinetic models and incubation times can further influence results.

In vivo, the role of BNF dietary effect in trout on PAH carcinogenesis needs to be further defined by describing the organic and water soluble metabolites formed. Also tumor incidence varies with the concentration of BNF dietary exposure (Nixon et al., 1984), and the need exists of correlating tumor studies with DNA binding and identification of metabolites formed in vivo to give an accurate estimate of the outcome of

phase I and phase II enzyme activities or detoxification. BNF is known to induce both phase I and II enzymes in mammals and such an effect can enhance as well as decrease neoplasia when influence of another substrate is also present. It is of interest to note that BNF has an inhibitory effect on AFB1 tumor response as well as that of a PAH like BAP when the modulator is introduced before or during exposure to the carcinogen.

INDOLE-3-CARBINOL

As observed for BNF, I3C administered in diet prior to DMBA exposure consistently reduces mean DNA binding of this carcinogen, but the reduction is not significant. It is possible that the level of modulator in the diet, 2000 ppm, is insufficient to produce a statistically significant inhibition in DNA binding. The need exists to verify the effects of the binding of DMBA to DNA with various levels of I3C dietary exposure, to reveal kinetic patterns of I3C dietary modulation as shown by the study of Dashwood et al. for AFB1 (Dashwood et al., 1988).

AROCLOR 1254

In the present study, dietary pretreatment of rainbow trout with 100 ppm Aroclor 1254 showed a mean for DNA binding response (CBI) lower than control group but the difference in response

was not statistically significant. Comparison of these results with other experimental studies is made difficult as PCBs are known to produce variable response depending upon the carcinogen used in trout studies.

PCB treatment in other studies is known to induce MFO activities. Voss et al., (Voss et al., 1982) in a study where trout were exposed to Aroclor, showed that ethoxyresorufin-O-deethylase (EROD) activity increased until at week 15 there was an increase of 77-fold. This was noted after 100 ppm dietary exposure to AR1254. EROD activity is characteristic of P448, implicated in PAH metabolism and responsible for generating dihydrodiol epoxides of DMBA, which are the carcinogenic species. It is possible therefore that PCBs could actually enhance DMBA tumor response in trout, depending on phase II effects.

Tumor response studies in trout have indicated that cofeeding of PCB enhances diethylnitrosamine carcinogenesis (Shelton et al., 1984) though it reduces AFB1 carcinogenicity when PCB is fed before and during AFB1 initiation. Hence fish studies with PCB treatment have shown that depending on the dose and the carcinogen used, the tumor response can be an enhancement or an inhibition (Bailey et al., 1988).

Using microsomal incubations, Egass and Varanasi (Egass and Varnasi, 1982) have demonstrated a substantial increase in PCB induced trout microsomes of binding to deproteinized salmon sperm DNA. The Egass study however is in vitro and cannot

account for detoxification and repair manifested in an in vivo situation. Furthermore, Varanasi et al. (Varanasi et al., 1987) have seen an increase in radioactive bile conjugates glucuronide and sulfate and GSH, demonstrating induction of conjugation enzymes in English sole and starry flounder.

In summary Aroclor 1254, a mixture of PCB congeners, possesses the ability to induce MFO and phase II enzyme activity in rainbow trout, hence the extent of DNA binding may depend on the outcome of competing metabolic pathways determining the amount of active metabolite produced for DMBA. Tumor studies with DMBA and the modulation of the tumor response by PCBs is essential to comprehend how this modulator can influence DMBA carcinogenesis, particularly since PAH and PCBs may be involved simultaneously as environmental pollutants. It is also possible that a higher dietary treatment dose of Aroclor 1254 may reduce DNA binding by statistically significant levels from control.

VARIATION WITHIN TREATMENT GROUPS

In experiment 1, biological variation was considerable which made computation of modulator effects difficult. Although certain fish species, e.g. Poleciliopsis lucida show no difference in dose responsiveness between different strains, rainbow trout demonstrate considerable tumor response variation (11%-64%) for AFB1 between strains (Bailey et al., 1988), and perhaps within the Mount Shasta strain.

Variation between individuals in experiment 1 was from 13% to 432% of mean value of CBI for controls. In experiment 2 where pools of ten fish were used, various control pools showed 81 to 139.6% of mean values of CBI. The variation manifested at an individual level can thus be decreased by pooling in some cases, but the large difference between each fish suggests a genetic influence on vulnerability to the effects of DMBA-DNA binding. Such effects between genera are known to exist (Somalarek et al., 1987, Bailey et al., 1988) but have not been explored at the level of individual fish.

SITES OF ACCUMULATION OF ADMINISTERED DOSE

It is important to define DMBA metabolism to known carcinogenic metabolites and the extent to which they are produced in tissues other than liver. Stegeman et al. (Stegeman et al., 1981) provided the information that extrahepatic tissue microsomes of kidney and gill form BP metabolites remarkably similar in profile to liver samples. Percent of total BAP metabolized during incubations was 8.5%, 2.0%, and 0.5% for liver, kidney, and gill respectively, during microsomal incubations using scup microsomes in this study. It is interesting that a similar order of magnitude exists for liver and kidney in Table I.5 where % distribution of total radioactive dose of DMBA in trout is considered.

Detoxification can also be measured in terms of conjugated metabolites of PAH released into the gall bladder (Varanasi et al., 1983). Varanasi et al., on analysis of BAP bile fraction found no residual unconverted BAP or unconjugated BAP metabolites 24 hours after oral exposure to tritiated compound in English sole. One to two per cent of the administered radiolabelled dose appeared in liver, and concentration in bile was ten times higher, concentrations similar to those obtained for DMBA accumulation in tissues of trout (Table I.5). With this distribution BAP conjugates in English sole contained a high proportion of glucuronide and GSH conjugates. Trout cell culture studies have revealed that the cells form more glutathione conjugates than glucuronides for BAP treatment (Somalarek et al., 1987). DMBA water soluble metabolites were more than 68% when 96.5% of the DMBA was metabolized. Thus if binding to tissues is a reflection of processes of activation and detoxification of DMBA, the profile exhibited is similar to that revealed for BAP tissue binding in English sole. The fact that trout cells show a high ability to metabolize DMBA to water soluble metabolites shows the ability to decrease carcinogen induced damage to DNA. Exact conjugates for DMBA in trout remain to be identified.

CHAPTER II

DIETHYLNITROSAMINE AND ETHYLNITROSOUREA CARCINOGENESIS IN THE RAINBOW TROUT.

INTRODUCTION.

IN VITRO MODELS

In many animal species the liver is an important site of primary tumor formation. It posseses the latent capacity to proliferate and is the seat of many enzymes of physiological importance in the activation and detoxification of xenobiotics. The growth of the liver can be induced by pregnancy, tissue loss, surgery, physical damage and chemical injury. In vitro tests involving the use of cultured hepatocytes, isolated hepatocyte suspensions and liver slices are limited by the physiological changes that may have occurred in the cells, following removal from the liver. However they provide comparison to evaluate processes at the organ and cell level.

HEPATOCYTES

It is difficult to interpret from in vivo studies whether a chemical has a direct effect on the liver or whether the

primary effects occur in some other tissue or organ. Isolated hepatocytes provide the ability to investigate and reveal complex biotransformation events in a controlled *in vitro* situation. By this method chemical substances can be studied singly and their effects on the hepatocytes evaluated. Dose response relationships such as inhibitor action on biotransformation of drugs can be studied (Tomera et al., 1984). The major advantage is that interrelationships between hepatic processes can be examined without the influence of complicating extrahepatic factors and investigations can be carried out using specific treatment conditions which would cause major problems to the health of the animal. Morphological and biochemical changes can be observed and used in the final analysis of carcinogen action. However the cells are not arranged in the same way as in the whole organ and chemicals with effects on bile production or which affect specific zones in the liver cannot be identified. This identification is of importance as hepatotoxins damage specific regions in the liver and hence indicate metabolic heterogeneity in the organ. This heterogeneity is manifested in the multiple factors governing the regulation of conjugation reactions, limiting the usefulness of *in vitro* hepatocyte studies.

Therefore the need exists to characterize the cells metabolically before they can be used routinely. For example, studies have indicated that treatments with inducers of microsomal enzymes have resulted in differing rates of

glucuronidation in periportal (an area exposed to blood rich in oxygen, substrates and hormones) and pericentral areas (low in oxygen substrates and hormones) of the rat liver. Fasting was shown to decrease the rate of glucuronidation by 50% in both regions (Tonada et al., 1983). Mixtures of hepatic cells obtained from liver perfusions can be divided into parenchymal and non parenchymal cells. The parenchymal cells, containing the bulk of liver P-450 activities, are the hepatocytes. The endothelial cells and the kupffer cells comprise the nonparenchymal cell fraction and carry a small proportion of the P-450 system. A knowledge of the distribution of the P-450 enzyme system in the hepatocyte preparation is important, especially where studies with activation dependent carcinogens are concerned. A major limitation of this model is the loss of the P-450 activities that occurs on prolonged incubation of the cells.

Besides these factors causing variation in response at the level of cell population, percent viability of the cells is also an important factor to insure quality of the hepatocyte preparation. Cell death can be denoted by the loss of integrity of the plasma membrane which commonly is assayed by using trypan blue exclusion as an indicator of membrane integrity. Its convenience makes it the choice though leakage of cytosolic enzymes such as lactate dehydrogenase (LDH) is quantitatively more precise. The incapability to retain intracellular enzymes is generally taken to be a sign of irreversible cell damage.

A very useful feature of hepatocyte in vitro studies is that they permit a critical evaluation of risks to humans. Human hepatocytes can be isolated in large numbers, with a high viability, and are used to evaluate DNA damage, repair, mutagenesis and xenobiotic metabolism. Qualitative similarities between results obtained from experimental animals and human cells, provide better surrogates for humans in carcinogen testing (Rauckman et al., 1987).

Rainbow trout hepatocytes have also been used in carcinogenesis studies, providing valuable data for comparative analysis with mammalian models and hence insights into risk assessment for humans. A knowledge of the biochemical, physiological and repair characteristics of trout hepatocytes is essential before such preparations can be used in carcinogen studies. Trout hepatocytes possess an initial viability of about 95% when isolated. However, unlike rat hepatocytes, trout hepatocytes do not attach to culture substrate. Their survival in suspension depends on the added serum concentration and incubation temperature. It has been demonstrated that in this model trout cells rarely divide or undergo DNA replication (Klauning et al., 1985). Klauning et al., showed that the level of cytochrome P-450 decreases steadily for the first four days of suspension type trout hepatocyte culture. With time, events related to cell aging and cell death appear such as clumping, nuclear elongation, increase in autophagic cell vacuoles and occurrence of bundles of filaments in the cytoplasm. As the

liver is the primary detoxification organ, it is of value to study the effects of various carcinogens using hepatocyte incubations.

NITROSAMINES: RELEVANCE TO CARCINOGENESIS

Nitrosamines have been shown to be carcinogenic in 45 animal species, producing tumors in a wide variety of organs (Magee et al., 1967). However each individual nitrosamine demonstrates pronounced organotrophy in its carcinogenic action. The toxic properties of nitrosamines have been described by many investigators (Herron and Shank, 1980; Magee and Barnes, 1967; Archer and Labuc, 1985) and doses of 20-40 mg/kg produce acute liver necrosis and death in several species.

It is known that man is exposed to nitrosamines via occupational exposure, consumption of certain food and tobacco products, and ingestion of precursors of nitrosamines in the gastrointestinal tract (Archer et al., 1985; Speilgelhalder and Preussman, 1985). The nitrosamines are tissue specific and susceptibility to a large extent depends upon the chemical structure (Appel et al., 1979). Owing to the relative simplicity of their structures, products of their interaction with cellular components can be easily identified and the nitrosamines provide an interesting and valuable series of compounds with which to study mechanisms of the chemical induction of cancer (Myrnes et al., 1983). They provide a valuable base for validation of

short term screening tests for carcinogenesis such as in vitro mutagenesis assays because a large number of these compounds have been tested in experimental animals. It is known from a case of human poisoning that DNA adducts formed by dimethylnitrosamine (DMN) in human liver (Herron and Shank, 1980) are the same as those found in the livers of experimental animals (Pegg, 1984).

DMN and diethylnitrosamine (DENA) are the two nitrosamines most extensively tested, and their carcinogenic effect is well documented by many investigators (Magee, 1967; Singer, 1986; Singer, 1981; Reznik-Schuller and Hague, 1981)). Changes in their structure lead to major changes in their mutagenic effects. This provides a means for understanding carcinogenic initiation. Information regarding their biotransformation and interactions can be elucidated by comparing the reactions of the activation dependent nitrosamines with direct acting nitroso compounds such as ethylnitrosourea (ENU). The nitroso compounds therefore are among the most useful models used in elucidating biological events in carcinogenesis.

METABOLISM

Most nitrosamines undergo oxidation of the alpha carbon moiety (Magee, 1967). The alpha-hydroxylation reaction, usually termed nitrosamine dealkylase activity is catalyzed by a P-450 dependent enzyme system (Jarman et al., 1986). The role of

cytochrome P-450 dependent enzymes in nitrosamine activation has been supported by studies using purified hepatic cytochrome P-450 isozymes. Of the isozymes tested, only cytochrome P-450j purified from ethanol and isoniazid treated rats had high catalytic activity towards N-demethylation of DENA at physiologically relevant nitrosamine concentrations (Levin et al., 1986). Dealkylase activity changes in rats with age and sex, and nitrosamine carcinogenicity usually correlates with changes in dealkylase activities (Lotikar et al., 1975). Evidence also exists for formation of nitrite from cytochrome P-450 mediated reductive denitrosation of nitrosamines, suggesting an alternative detoxification pathway for nitrosamines (Appel et al., 1985).

DENA is thought to extend its carcinogenic action following metabolic conversion to a reactive ethylating agent (Appel et al., 1979,). The exact chemical identity of the ultimate alkylating agent has not been determined, but it is believed that the alkylhydroxyalkylnitrosamine decomposes to an aldehyde and a much shorter lived ultimate metabolite which is probably an alkyl diazonium ion or an alkyl carbonium ion (Figure II.1). Once formed, the agent rapidly reacts with all nucleophiles, including water which yields the corresponding alcohol. Reactions with macromolecules lead to alkylated amino acids in proteins and alkylated bases in nucleic acids (Lawley et al., 1976, Pegg et al., 1982; Umbenhauer and Pegg, 1985).

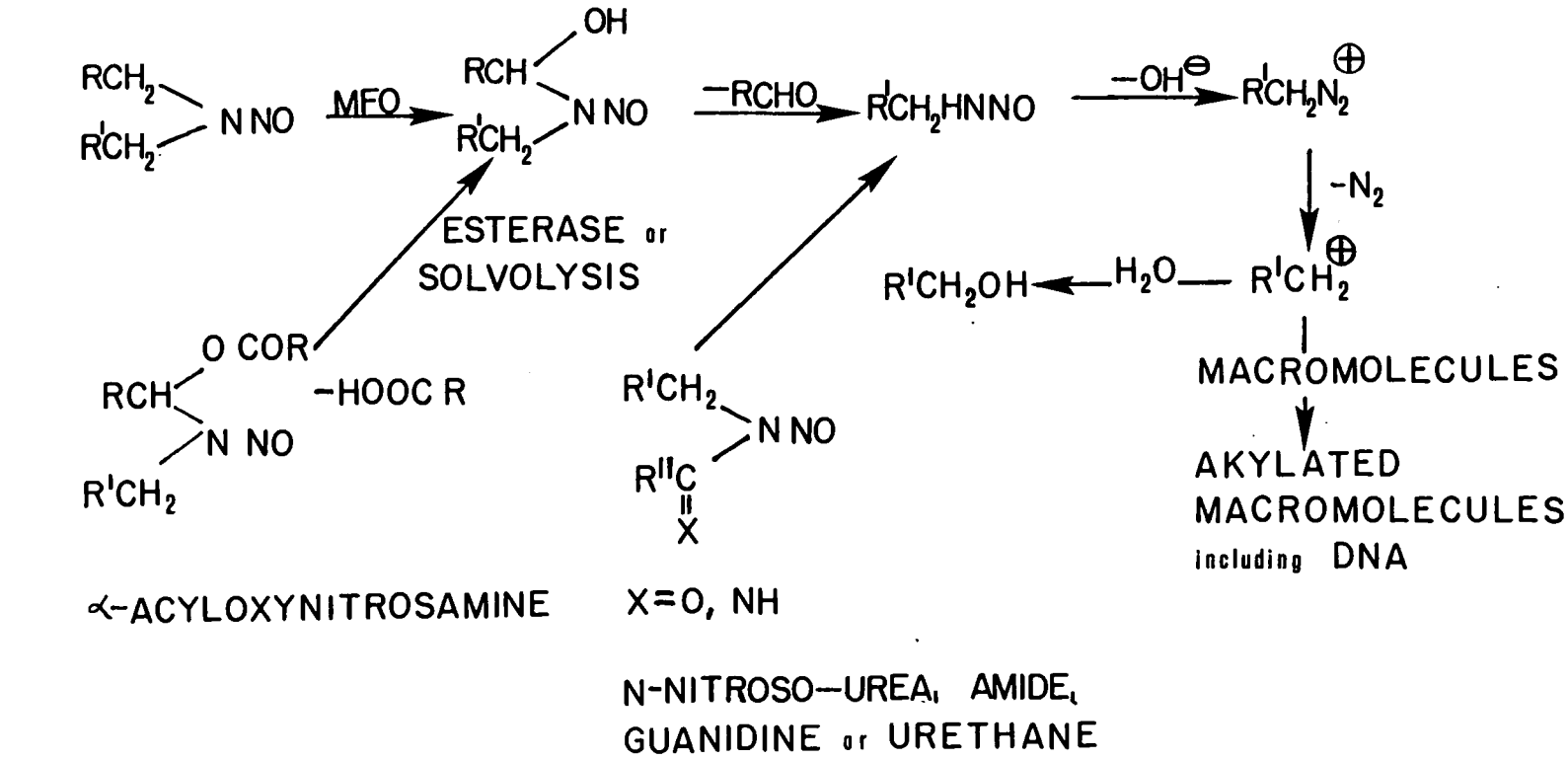

Figure II.1 Pathways For Metabolism And Decomposition Of N-nitroso Compounds (Guttenplan, 1987).

Studies have indicated that organ susceptibility is usually determined by nitrosamine chemical structure but a preexisting capacity for proliferation is also important. This can be seen in the case of single dose treatments of DMN and methylnitrosourea, which do not produce liver tumors in adult rats but do produce tumors when given to neonatal rats or rats in which the liver is regenerating after partial hepatectomy (Singer, 1984). Susceptibility correlates directly with levels of tissue metabolism, though this concept is not to be generalized (Arcos et al., 1975). For example, administration of DMN to adult rats produces kidney tumors, even though very low levels of enzymatic activity are found in this organ (Nicoll et al., 1975).

As nitrosamines require metabolic activation, the site of action of these compounds varies from direct acting alkylating agents such as ENU. In vitro, the reactions of direct acting compounds such as ENU, are difficult to compare with activation dependant compounds such as DENA, as the half life of the former in most buffers is on the order of a few minutes, and they give rise to a much greater amount of ultimate mutagen (Guttenplan, 1987). The nitrosoureas, thus are unstable and damage cells in any organ that they penetrate (Swann and Magee, 1968). The major interest in the carcinogenic activity of ENU has centered on the induction of tumors of the brain and nerve (Goth and Rajewsky, 1974).

Increased production of active oxygen, organic peroxides and radicals which also have been suggested to be involved in carcinogenesis have not received much attention in the metabolic action of nitrosamines. Ahotupa et al., (Ahotupa et al., 1987) demonstrated that chronic treatment of rats with DENA or DMN increases lipid peroxidation in vivo. This may play an important part in the carcinogenic action of these compounds. Lipid peroxidation was not found to relate directly with the relative acute toxicities, the mutagenic potential, or the DNA binding ability of the various nitrosamines. Its role in the carcinogenic process, initiated by these compounds is ambiguous.

Recent research has focused on identifying the critical lesions in DNA which may be related to carcinogenesis by alkylating agents such as the nitrosamines (Hoffe et al., 1986). At least 12 sites in DNA are targets for alkylation by N-nitroso compounds under physiological conditions (Singer, 1975; Engelese et al., 1985). On balance, the available evidence strongly favors the O^6 position of guanine as the critical target in the initiation of tumors (Pegg, 1984). In addition to O^6-alkylguanine ^{O4}-alkylthymine, O^2-alkylthymidine, and O^2-alkylcytosine are presumed to be miscoding lesions and may lead to initiation of carcinogenesis by the nitrosamines. Other lesions may cause mutation by yielding apurinic sites (Engelese et al., 1985).

Some investigators used the theory of hard and soft acids and bases to explain DNA adduct formation (Ho, 1977). The hard and

soft acids and bases principle is thought to characterize the alkylation reactions of DNA as acid base reactions, where the strong base reacts preferentially with a strong acid, and a weak base with a weak acid (Pearson, 1966). The acidity of carbonium ions increases from methyl, ethyl, isopropyl to 2-butyl. Since N7 is a weaker base than O^6 in guanine, stronger acids prefer reactions with O^6 position. Oxygens in thymidines and in phosphate diesters are classified as strong bases. They prefer reaction with branched carbonium ions (Coleo, 1985).

REPAIR

Presumably, failure to correctly repair certain carcinogen-induced DNA lesions prior to DNA replication leads to somatic mutation. Thus tissue and cell differences in the rates of repair of critical DNA lesions may play a central role in determining the site of tumor formation (Goth and Rajewsky, 1974). O^6-alkylguanine is removed from DNA by the action of an alkyltransferase protein which mediates the transfer of the alkyl group (Pegg, 1982). The alkyl group is transferred to a cysteine residue on the alkyl acceptor protein. The transfer reaction appears to be irreversible, hence the capacity of the repair system is limited by the level of the alkyltransferase present in the cell. The repair activity is both species and organ specific, but does not appear to be selective for a methyl or ethyl group on guanine. In E.coli, rat and fish the repair

activity can be induced by the same agents that produce the adduct (Nakatsuru et al., 1987). Thus rates of repair and capacity for DNA replication are factors involved in carcinogenesis.

N-nitrosoethyl compounds produce a much greater proportion of adducts on the oxygen atoms than N-nitrosomethyl compounds, and ethylating agents have proven to be more effective than methylating agents in producing hepatocellular carcinomas in rats. Differential repair of adducts may contribute to the superior ability of ethylating agents versus methylating agents to accomplish this tumor response. Differing rates of removal of ethylated purines and pyrimidines from various organs indicate that mammals possess multiple independent repair systems (Singer et al. 1986). Thus when examining the role of DNA repair, the relative risks of carcinogenesis must be determined for a particular model rather than extrapolated from data on other organisms. Repair of alkylpyrimidines has been studied and compared with that of alkylguanines (Richardson et al., 1985).

Most nitrosamine carcinogenesis studies have been conducted using rats, mice and hamsters. Several fish species have been shown to be sensitive to the carcinogenic effects of nitrosamines (Greico et al., 1978; Bailey et al., 1987; Ashley et al., 1968). Fish are of particular interest as many fish species show little capacity for alkyltransferase type repairs (Nakatsuru et al., 1987) or excision type repairs (Walton et al., 1984). The rainbow trout has been shown to be highly

sensitive to the carcinogenic effects of DMN and DENA (Bailey et al., 1987) and this model can serve to provide comparative information which is important in defining mechanisms of action and eventual extrapolation of results to man.

OBJECTIVES AND RATIONALE

Experimental studies of chemical carcinogenesis usually involve two steps:

1. Tumor studies are performed to establish the carcinogenicity of the compound, a process which usually takes several months or more than a year.
2. After tumorigenicity has been established, *in vivo* and *in vitro* biochemical studies are conducted to relate carcinogenicity to particular metabolites, and to the enzyme activities which produce and detoxify the metabolites and DNA adduct formation. Those adducts that present the prospect of miscoding can then be identified.

DENA, a potent mammalian carcinogen, is tumorigenic in several other classes of animals including fish. Dietary exposure has been shown to result in hepatocellular carcinomas in rainbow trout (Shelton et al., 1984). However the processes of N-nitroso DNA alkylation and subsequent adduct repair are not well understood in the trout model. Studies with nitrosoureas, which spontaneously decompose in vitro at neutral pH, have shown that O^6-alkylguanine formed may be a critical product in the initiation of cancer. Most of the alkylation occurs at N-7 of guanine and the ratio of formation of O^6-alkylguanine to ^{7}-alkylguanine varies with the alkylating species. The discovery that this ratio varied directly with dose of the nitrosamine indicated the existence of a saturable repair system for the

alkylguanine adduct (Pegg, 1982). Later studies confirmed that cell extracts could mediate the removal of O^6-methylguanine (Naktasuru, 1987).

Studies of this nature need to be conducted for nitrosamines in the trout model to strenghten its usefulness in comparative carcinogenesis. Although the trout is sensitive to DMN as well as DENA-initiated carcinogenesis, methylation of liver DNA was not detected following i.p. DMN injection (Kruger et al., 1970). The kinetics of guanine adduct formation and persistence can reveal information regarding metabolic activation and mechanism of nitrosamine carcinogenic action (Becker and Shank, 1985). Recent results in this laboratory also indicate that DENA carcinogenesis in trout can be strongly influenced by several dietary modulators (Hendricks et al., unpublished results) which may reflect modulator mediated alterations in DNA alkylation or repair.

Therefore the aim of this study was to determine whether carcinogenicity in trout was related to alkylation of guanine in DNA following exposure to DENA. The specific objectives were:

1. The determination of the formation of ethylguanines in the liver DNA of rainbow trout treated with i.p administration of diethylnitrosamine.
2. The investigation of the persistence of these adducts in trout hepatocytes after exposure to DENA and ENU.

MATERIALS AND METHODS

SAFETY PROCEDURES FOR HANDLING OF CARCINOGENS

Refer to Appendix I, for details.

CHEMICALS

[^{3}H] DENA (specific activity, 248 mCi/mmole, stored in ether) was custom synthesized by Amersham (Arlington heights, IL). Purity of the DENA solution was tested using reverse phase HPLC by Dr. Arthur Fong and the DENA was assessed to be of > 95% radiopurity. Unlabelled DENA was obtained from Kodak Products (Rochester, NY). Solutions for i.p. injection and for incubations with hepatocyte preparations were prepared by removing a desired aliquot of the ^{3}H DENA stock solution, immediately before use. The ether was evaporated under a gentle stream of nitrogen, and the [^{3}H] DENA was reconstituted with millipore water and unlabelled DENA to obtain the desired specific activity.

All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise mentioned and were of the highest grade available. ENU was obtained in sealed vials of one gram each, and dissolved in dimethylsulfoxide (dried) before use. All surplus solutions were decontaminated and discarded each time. Solutions for DENA and ENU were protected from light

during incubations, storage and transport. Fetal calf serum was obtained from Aldrich Chemical Company (Milwaukee, Wis). Authentic standards for O^6-EtG (Calbiochemicals, La Jolla, Ca) and N7-EtG were assayed for purity by HPLC and U.V. spectroscopy.

ANIMALS AND DIET

Rainbow trout, Salmo gairdneri, were spawned, raised and fed control diet as stated in Chapter I, Materials And Methods. Fish used in hepatocyte preparations were from 200-600 g in body weight. The BNF dietary treatment was 500 ppm (six weeks) for fish used in hepatocyte studies.

IN VIVO STUDIES

[^{3}H] DEN was administered i.p. and fish were killed by severing the spinal cords. The livers and gall bladders were removed and the livers were washed with ice-cold normal saline solution and frozen at -80°C. Nuclei were isolated from livers and DNA extracted from nuclear pellets. The procedures for preparation of nuclei, DNA isolation, purification and Burton's assay for DNA quantitation were detailed in Materials And Methods, Chapter I. DNA was quantitated using one, or a combination of the following three procedures, indicated where applicable in the Results section.

a. For routine check of purity, absorbance ratios of 260 nm to 280 nm and 260 nm to 240 nm were used. Amount of DNA was estimated using one absorbance unit = 50 μg/ml DNA.
b. Burton's assay was used where mentioned in the Results section (refer to Chapter I, Materials And Methods).
c. A microfluorometric method of DNA determination using a TKO 100 Mini Fluorometer and the procedure detailed in the operating instructions for TKO 100 also was used. Briefly, appropriate dilutions of standard calf thymus DNA and sample trout DNA were treated with a dye, Hoechst 33258 and fluorescence of the DNA-dye complex determined. The excitation wavelength used was 365 nm and emission wavelength at 492 nM.

DETERMINATION OF DNA BINDING

Purified DNA samples (from nuclear pellet or from hepatocyte pellet, were dissolved in 1-2 ml millipore water. Equal volume of 1 M perchloric acid (500 μl each) and sample DNA were mixed, heated at 70°C for 20 minutes and then cooled to room temperature. Duplicate aliquots of 200 μl were mixed with 7 ml of scintillation fluor and counted using a Beckman LS 7500 liquid scintillation counter and standard procedure.

HEPATOCYTE ISOLATION

A two step perfusion method was used for the isolation of hepatocytes from trout using the procedure described by Bailey et al. (Bailey et al., 1982). The steps are summarized and alterations made are mentioned where applicable. The fish was stunned with a blow to the head and heparin was injected into the heart. The body cavity was exposed and the liver cannulated with a blunt needle. The sinus venosus was severed to allow drainage during the in situ perfusion. The liver was perfused with calcium free buffer containing heparin to remove blood cells. A second buffer containing collagenase and hyaluronidase was used for the next perfusion. The liver was removed with care to avoid contact with the contents of the gall bladder, and the tissue dispersed in pH 7.4 wash buffer. The cells were filtered through cheese cloth, washed and centrifuged. The pellet was washed twice, and the cells suspended in PBS buffer (KCl 0.2, KH_2PO4 0.2, NaCl 8.0, $Na_2HPO4.7H_2O$ 2.16, g/L) washed again and resuspended in 4 ml of the PBS buffer. An aliquot of cells was diluted 1:1 with trypan blue and the viability and total number of cells determined.

STANDARD HEPATOCYTE INCUBATIONS

For incubations with ENU and DENA, hepatocytes were incubated in Dulbecco's Modified Eagles Medium with 2% fetal calf serum.

Total number of cells/incubation was 2.9 x 10^6-30 x 10^6 in the final incubation volume of 5ml. Solutions of ENU and DENA, prepared in dimethylsulfoxide and water respectively, were added to each incubation flask. Each flask was gassed continuously with 95% O_2/5% CO_2, and incubation was conducted at 20°C, on a metabolic shaker. Aliquots of 100 µl from each incubation were saved for determination of viablity by dilution with trypan blue. Incubations were stopped by pelleting of the cells which were washed with PBS, and rapid freezing in liquid nitrogen. The cellular pellets were stored at - 80°C until DNA extraction.

For study of repair of O^6-EtG, the cells were exposed to ENU or to AFB1, which served as a positive control, for 30 minutes and then washed and centrifuged at top speed in a table top clinical centrifuge, for 30 seconds. The cells were either frozen immediately after pelleting (zero time point) or resuspended in 5 ml fresh incubation buffer. The incubation flasks were then returned to the metabolic shaker and incubation continued for two, four and five hours at 20°C. At the end of the incubation periods the cells were pelleted, washed and frozen as mentioned for standard incubations.

DNA from hepatocyte pellets obtained from incubations with ENU and DENA, was extracted and purified as mentioned in the Materials And Methods Section, Chapter I. The DNA pellet was hydrolyzed and analyzed on the HPLC for guanine content and the O^6 adduct formed was expressed/mole guanine.

DETERMINATION OF GUANINE ADDUCTS BY CHROMATOGRAPHIC ANALYSIS OF DNA HYDROLYSATES

1. MILD ACID HYDROLYSATE -ISOCRATIC ANALYSIS

In this method the DNA was hydrolysed in 0.1 N HCl (5mg DNA/ml) at 70°C for 45 minutes to release purine bases. Separation of released bases was achieved by strong cation exchange HPLC (Whatman Partisil 10-SCX column, 25-0.45 cm) and quantitation achieved by fluorescence spectrophotometry (286 nm excitation wavelength with a 360 nM emission filter), as described by Becker and Shank (Becker and Shank, 1985). Isocratic elution employed 75 mM $NH_4H_2PO_4$ buffer, pH 2.0/methanol (6-14%). Ethylguanine concentrations were normalized to the concentration of guanine in each sample. Quantitation of guanines was achieved by injection of authentic standards, calculation of peak area (peak height x width of peak at 1/2 height) and linear regression analysis.

2. MILD ACID HYDROLYSATE-GRADIENT ANALYSIS

The hydrolysate obtained as described in part 1, was eluted from the column using a linear gradient of 5-20% methanol over 15 minutes.

3. NEUTRAL THERMAL HYDROLYSATE- MILD ACID HYDROLYSATE-ISOCRATIC

ANALYSIS

The DNA pellet was hydrolyzed and guanines analyzed in three steps. First, the sample was hydrolyzed by neutral thermal hydrolysis at 100°C for one hour, using 5 mg DNA/ml 10 mM sodium cacodylate to preferentially release N7-EtG. The partially apurinic DNA was precipitated by addition of 1 M HCl, 5 mg DNA/ml and the precipitate hydrolyzed in 0.1 M HCl at 70°C for 45 minutes (mild acid hydrolysis) to release the remaining purines including O^6-EtG as free bases. Neutral thermal hydrolysates (N7-EtG) were evaporated under reduced pressure, redissolved in 0.1M HCl, and analyzed by isocratic analysis using 5% methanol in the same manner as mentioned in part 1. Mild acid hydrolysates (O^6-EtG) were examined by HPLC (refer to part 1), using buffer/14% methanol, isocratic elution. Aliquots of 10-30 μl of the mild acid hydrolysate were fractionated by strong cation-exchange HPLC to detect guanine by U.V. absorbance at 274 nm and guanine concentration quantitated by the same procedure as described in part 1.

MICROSOMAL INCUBATIONS TO STUDY PROTEIN BINDING

[^{3}H] DENA, 5.13 μCi/μmole was incubated with 5 mg of microsomal proteins in a final volume of 1 ml/incubation. The

microsomes (obtained from H. Swanson) were spun at 37 K for one hour in the ultracentrifuge to remove the glycerol buffer. The super was discarded and the microsomes suspended in KCl buffer, homogenized, and aliquots of the homogenate incubated with the DENA solution. The incubations were performed in triplicate for 30 or 60 minutes, in a metabolic shaker at 25°C. The reaction was stopped by adding 5 ml aliquots of 10% ice-cold trichloroacetic acid, the precipitate washed with the same, and the washing repeated with 80% methanol. When the methanol supernatant showed no radioactivity, the samples were dissolved in 1 ml of 1 M NaOH and duplicate aliquots of 200 μl were counted. In each aliquot, protein was determined by the method of Bradford (Bradford, 1976). Binding was expressed as pico mole bound/mg protein.

ALKYLTRANSFERASE ASSAY

Assay for the *in vitro* removal of O^6-alkylguanine was the same as that described in detail by Gerson et al., and Fong et al. (Gerson et al., 1986; Fong et al., in press). In summary trout liver extracts were prepared by pooling livers, mincing and homogenizing the tissue, and sonicating the homogenate. The homogenate was centrifuged and the protein in the supernatant was determined by the Bradford method (Bradford, 1976).

DNA substrate was provided by Dr. Fong prepared by the method of Lindamood et al. (Lindamood et al., 1984). The [^{3}H] methyl-N-

nitrosourea calf thymus DNA, 0.25 mg (containing 1.33 picomoles O^6 [3]H methylguanine) was incubated with 2.5 mg of liver extract trout protein, in triplicate. Control incubations were performed with 2.5 mg of calf thymus DNA at 20°C. After one hour, the protein was precipitated, by the addition of 1 N $HClO_4$. DNA was hydrolyzed in 0.1N HCl (0.5 ml/sample) at 70°C for 45 minutes, to yield free purine bases. Purine bases were separated by strong cation exchange HPLC as described before. Fractions were collected, and radioactivity determined by liquid scintillation counting to measure the loss of O^6 methylguanine from the DNA substrate.

RESULTS

REACTION OF ENU WITH PURIFIED DNA

An experiment was conducted to determine if N7-EtG and O^6-EtG adducts could be detected under the conditions of hydrolysis and HPLC resolution used in the present study. For this study purified calf thymus DNA was incubated with two different concentrations of ENU (20 or 40 mM). After incubation, DNA was purified and subjected to either mild acid hydrolysis, or to neutral thermal hydrolysis prior to mild acid hydrolysis. DNA hydrolysates were separated by strong cation exchange HPLC eluted either isocratically with 6-14% methanol in 75 mM ammonium phosphate, or using a linear gradient of 5-20% methanol in ammonium phosphate as described in Materials and Methods. Figure II.2 presents the elution profile of calf thymus DNA hydrolysates showing the presence of O^6-EtG. Analysis of DNA from control incubations (which received the same treatment as sample incubations except that the volume of ENU solution was replaced by an equal volume of incubation buffer) did not show similar O^6-EtG fluorescing peaks. Approximately 200-300 μmol O^6-EtG/mole guanine were detected after 30 minutes ENU 20 mM exposures (Figure II.3, this Figure also shows the O^6-EtG/mole guanine detected for DNA obtained from trout hepatocytes, after incubation with ENU and is discussed later). The small N7-ETG peak did not resolve completely from the large

Figure II.2 Analysis Of Hydrolysed DNA Using A Linear Gradient Solvent System.

DNA hydrolysates from control incubation or 20 mM ENU incubation (a) were analysed by fluorescence detection. Control DNA hydrolysate showed no peaks that eluted with authentic N7-EtG, or O^6-EtG standards. Control DNA hydrolysate spiked with O^6-EtG and N7-EtG standards is shown in chromatogram b. Py, pyrimidine oligonucleotides; G, Guanine; A, adenine.

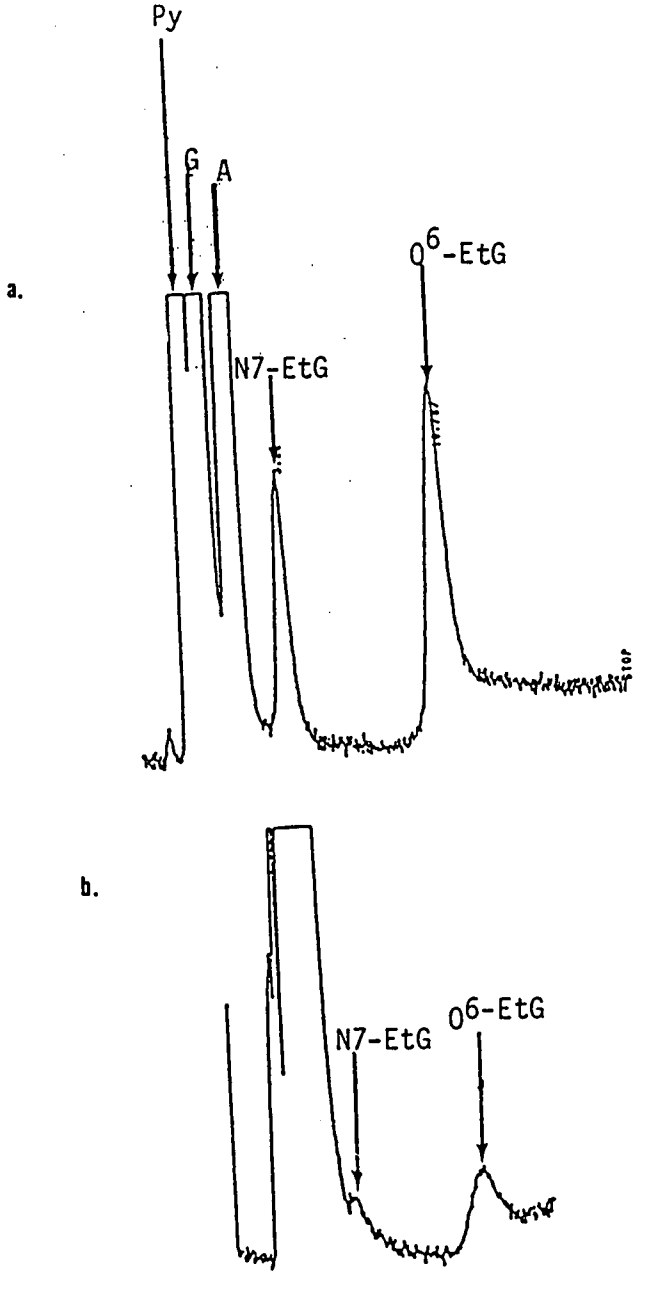

Figure II.2

Figure II.3 O^6-ethylguanine Formation In Rainbow Trout Hepatocyte DNA Or Calf Thymus DNA, After Treatment With ENU.

This figure contains data from two different studies. The first study was the reaction of ENU with purified DNA (Calf Thymus DNA) to ascertain if O^6-EtG and N7-EtG could be detected and quantitated by the HPLC fluorescence detection method used. A solution of the calf thymus DNA was prepared in the incubation buffer and incubated with ENU 20 mM and 40mM using the standard incubation conditions.

The second study was performed to establish standardized conditions for the detection and quantitation of ethyl-guanines from trout hepatocytes, incubated with the direct alkylating agent ENU. Trout hepatocytes were incubated with concentrations of ENU, 10-40 mM, while a volume of incubation buffer was substituted in control incubations. At the end of the incubation cells were pelleted, washed, and frozen. DNA isolated from the cells (trout DNA) was analysed for guanine adducts by HPLC fluorescence detection.

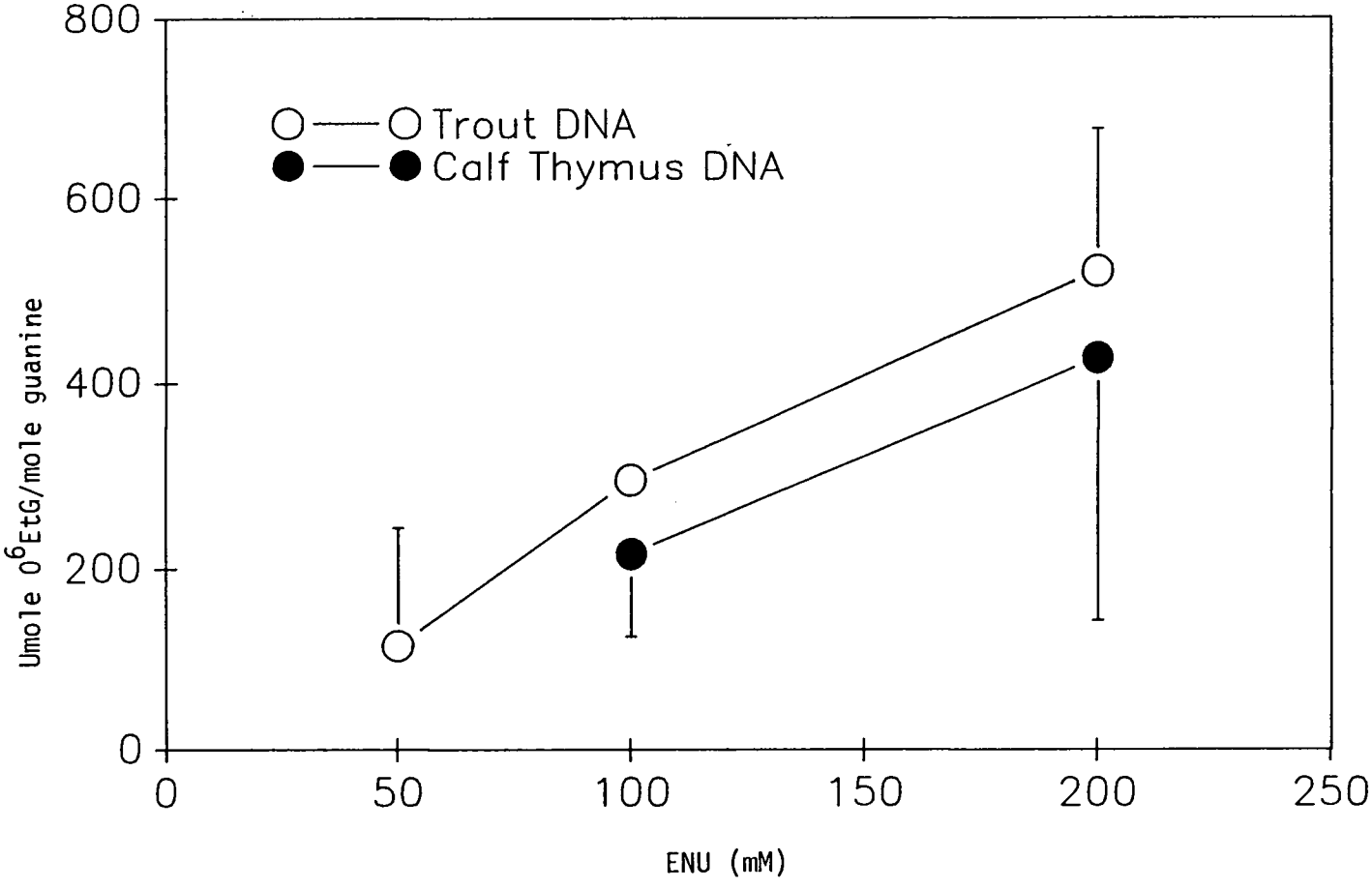
800
600
400
200
0
Trout DNA
Calf Thymus DNA
Umole O^6EtG/mole guanine
0
50
100
150
200
250
ENU (mM)

Figure II.3

adenine peak. Though no N7-EtG was detected in the DNA from trout hepatocytes incubated with ENU, authentic standard for this adduct and hydrolysed DNA samples spiked with N-7 EtG standard (as shown in Figure II.2) showed that conditions used in the present study were suitable for the detection and the quantitation of this DNA adduct. Hence both guanine adducts O6-EtG and N7-EtG could be determined if formed by alkylating agents, within the limits of detection of the chromatographic procedure of this study. The lowest level at which ethyl adduct authentic standards could be quantitated by this method was 8 picomoles O^6-EtG and 140 picomole of N7-EtG.

DETECTION AND QUANTITTATION OF ETHYL-GUANINE DNA ADDUCTS FORMED IN ISOLATED HEPATOCYTES

The purpose of the next study was to establish standardized conditions for detection and quantitation of specific DNA adducts induced by ethylating agents in hepatocyte preparations. Isolated trout hepatocytes were incubated with the direct alkylating agent ENU, under specified conditions, *in vitro* (see Materials And Methods). The alkylated DNA was purified, hydrolysed to release O^6-EtG and N7-EtG as the free bases, and chromatographic analysis was performed for detection and quantitation of O^6-EtG and N7-EtG as described for reaction of ENU with purified calf thymus DNA.

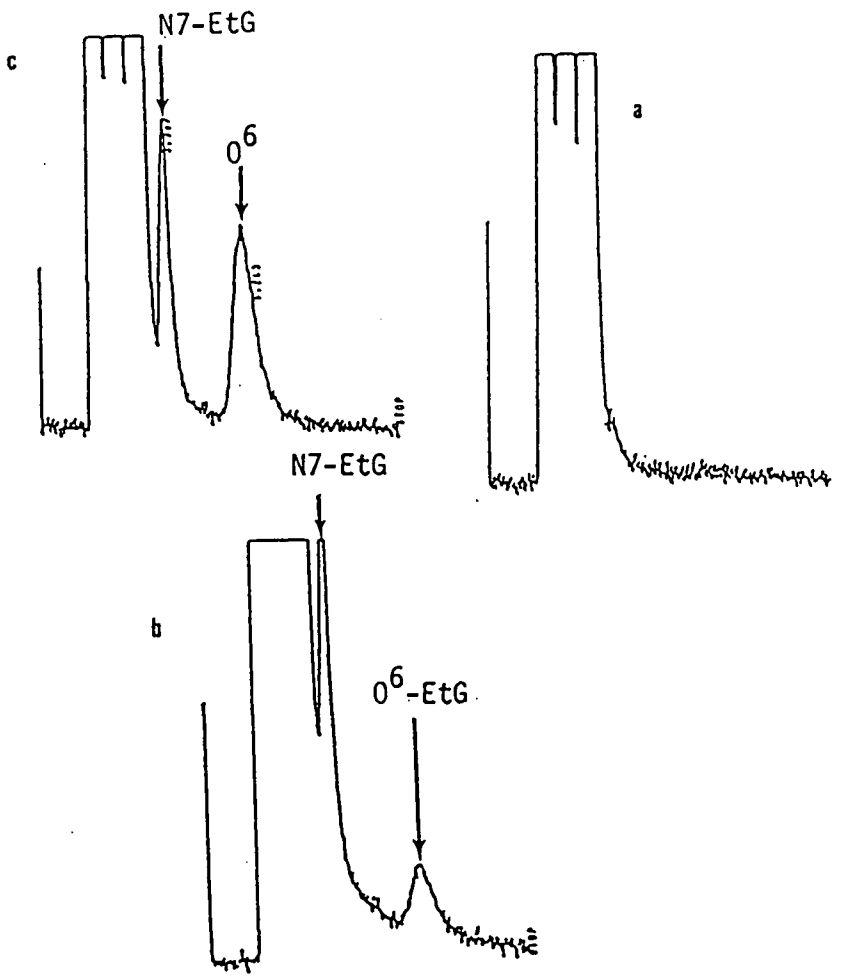

Figure II.4 Analysis Of Hydrolysed DNA Using An Isocratic Solvent System.

Hepatocyte DNA hydrolysates from control incubation (a) or 5 mM ENU incubation (b) were analysed by fluorescence detection. Control DNA hydrolysate spiked with O^6-EtG and N7-EtG standards is shown in chromatogram c.

Analysis of DNA hydrolysates by HPLC (Figure II.4) showed that exposure to ENU at 5 mM resulted in quantifiable O6-EtG and N7-EtG peaks. However the ethyl guanine adducts were not reliably detected in all DNA samples from hepatocytes exposed to 5 mM ENU. Therefore, higher concentrations of ENU were tested (Figure II.3). An increase in O^6-EtG level was found with increase in ENU concentrations between 10 and 40 mM ($R = 0.99$). Increasing the ENU concentration above 40 mM led to greater degrees of alkylation, but hepatocyte viability, as determined by trypan blue exclusion dropped to < 43% at 120 mM ENU (Figure II.5). Hence the extent of effective DNA alkylation was limited by the effect of higher ENU doses on hepatocyte viability. Furthermore, cell viability decreased with time of incubation, a 50% viability was found in control incubations after 20 hours. Therefore hepatocyte incubations did not exceed 8-10 hours at which time cell viability was about 90%.

STUDY OF DENA METABOLISM AND DNA BINDING BY I.P INJECTION AND IN HEPATOCYTES FROM CONTROL OR BNF-INDUCED TROUT.

After conditions for detection of ethylated bases were established, experiments were conducted to determine alkylation of trout liver DNA following i.p. [^{3}H] DENA treatment. Adult trout (average body weight 303.7g) and fry (average body 10 g) were injected with 1 μCi/mg DENA solution. Adults received 3.3, 16.5, and 33 mg DENA/kg doses and fry received 10, 50, and 100

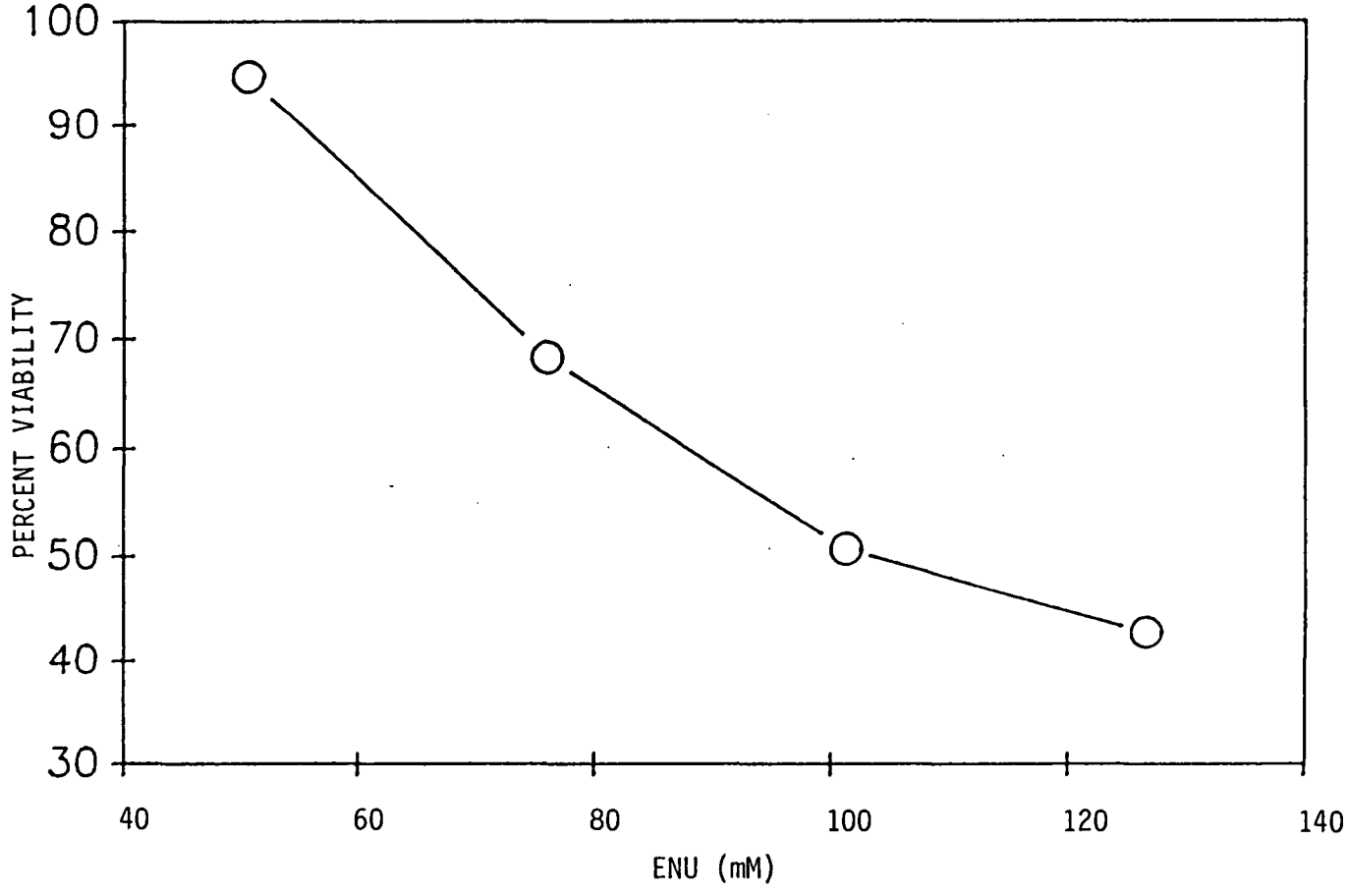

Figure II.2 Viability Of Hepatocytes After 30 Minutes Treatment With ENU.

mg DENA/kg. No covalently bound radioactivity was detected in purified liver DNA from fry or adult trout.

DENA exposure in a short term (1-5 hour) hepatocyte incubation was then performed. AFB1 was included as a positive control in these studies, as its metabolism and DNA binding, have been well characterized in trout hepatocytes (Bailey et al., 1981). Hepatocytes were exposed (as described in Materials And Methods) to [^{3}H] DENA (5.09 μM to 20μM) and DNA purified and analysed for covalently bound radioactivity. Both Burton's assay and microfluorometric analysis of DNA revealed sufficient quantities of purified DNA to be present in each hepatocyte sample. However no covalently bound radioactivity was detected in DNA from hepatocytes exposed to [^{3}H] DENA. In contrast significant levels of radioactivity (23.8 μg AFB1/g DNA, 3.172 μCi/μg AFB1) was found in DNA from hepatocytes exposed to ^{3}H AFB1. Furthermore, hepatocytes incubated with 1 mM, 125 μM, 250 μM and 500 μM solutions of unlabelled DENA using fluorescence detection analysis, resulted in inconsistent appearance of O^6-EtG peaks that were not quantifiable. Therefore the possible loss of the DENA because of its volatility, was examined. Test under hepatocyte incubation conditions using [^{3}H] DENA showed 95-100% of the radioactive DENA dose was present in the incubation solution after one hour, with or without oxygen.

The ability of hepatocytes from trout treated with BNF, a known inducer of cytochrome P-450, to activate DENA to a DNA binding metabolite also was examined. Incubations with

hepatocytes using high radioactive dose ([^{3}H] DENA 4.5 to 53.8 μCi/5ml) 18 μM to 220μM, for 60 minutes were performed. Distribution of radioactivity in the supernatant the pellet, and the wash solution was analysed, by collecting total volume and counting aliquots. The first phenol and chloroform fractions during the subsequent DNA extraction were collected and analysed similarly. The results given in Table II.1 show that, while 59-65% of the radioactive dose was lost in the wash solution, 32% was associated with the pellet still, no DNA binding was observed for these samples after DNA was isolated, hydrolysed and counted. HPLC analysis of DNA hydrolysate from hepatocyte incubations, using BNF induced fish, showed minute peaks for O^6-EtG at 1mM concentration of DENA (chromatograms in Figure II.6) for 30 minutes, 60 minutes and 120 minutes. Spiked samples showed that O^6-EtG and N7-EtG could be detected under the present chromatographic conditions.

Results from these studies indicate that in the two methods of exposure to DENA (i.p in vivo, hepatocyte incubation in vitro) under the most extreme conditions examined, DNA alkylation by DENA was insufficient to permit detection of specific adducts, or overall DNA binding.

To investigate further the lack of DNA binding seen in the i.p. and hepatocyte exposure treatments to DENA mentioned above, an experiment was designed to examine the ability of isolated trout liver microsomes to activate DENA to a metabolite capable of binding to the microsomal proteins. Microsomal incubations

Table II.1 Distribution Of Radioactivity In Rainbow Trout Hepatocyte Cell Suspensions, Exposed To Diethylnitrosamine

TOTAL (UCi)	PER CENT RADIOACTIVITY IN FRACTION[a]				
	Phenol	Chloroform	Supernatant	Pellet	Wash[b]
4.5	0.4	0.1	3.6	32.3	64.2
14.2	1.5	0.1	4.2	37.3	58.6
26.3	1.8	0.3	18.5	19.4	62.1
23.9	1.4	0.1	4.5	33.5	61.9
37.5	1.1	0.1	3.9	30.7	65.4
53.8	1.1	0.2	3.8	31.6	64.6

[a]Fractions obtained from 6 separate DNA extraction were collected and counted.

[b]Pellet washed with 4 ml wash buffer 2x.

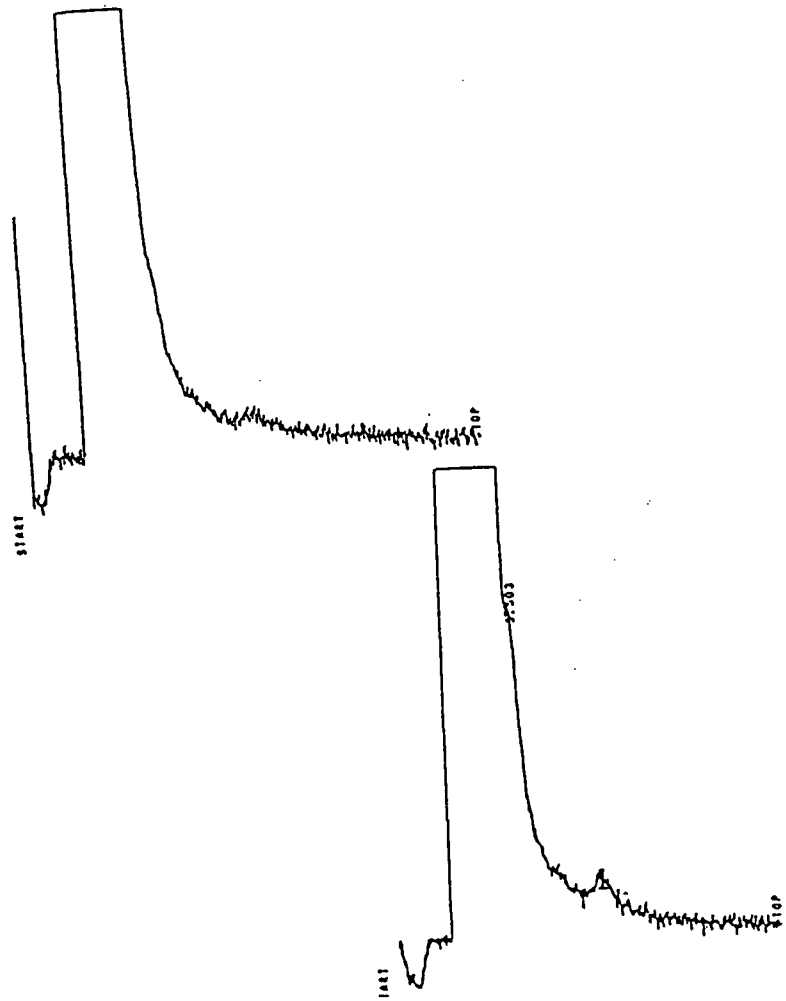

Figure II.6 Analysis Of DNA Hydrolysates From BNF-induced Hepatocytes.

Hepatocytes were exposed to incubation buffer for control (a) treatment, or to 1 mM DEN for 2 Hours (b).

with [^{3}H] DENA were performed for 30 and 60 minutes, proteins precipitated, and hydrolysed. Aliquots of the precipitated protein were used for protein quantitation and scintillation counting as described in Materials And Methods. Levels of binding were low i.e. 92 and 79 picomoles DENA/mg protein detected at 30 and 60 minutes respectively.

DETERMINATION OF THE ETHYL-GUANINE REMOVAL ACTIVITY IN TROUT HEPATOCYTES.

It was of interest to know the capacity of trout hepatocytes for removal of ethyl-guanine adducts. For example, failure to detect such adducts in some assays could be due to very rapid removal. To examine this question, hepatocytes were incubated with ENU. Incubations were performed using 40 mM ENU for 30 minutes. After ENU treatment, hepatocytes were transferred to fresh incubation medium, and the levels of O^6-EtG found at 2, 4 and 5 hours after the 30 minute incubation were determined. AFB1 was included as positive control. No significant changes in O^6-EtG levels were found at four or five hours post treatment (Figure 6). The graph shows linear increase for each time point studied, for AFB1. O^6-EtG/guanine ratio shows small decrease between four and five hour values. AFB1 ratios show 256% increase from two to four hours, and 31.8% increase from four to five hours. O^6-EtG ratios show 1.51% decrease from two to four hours and a 12.8% decrease between four to five hours.

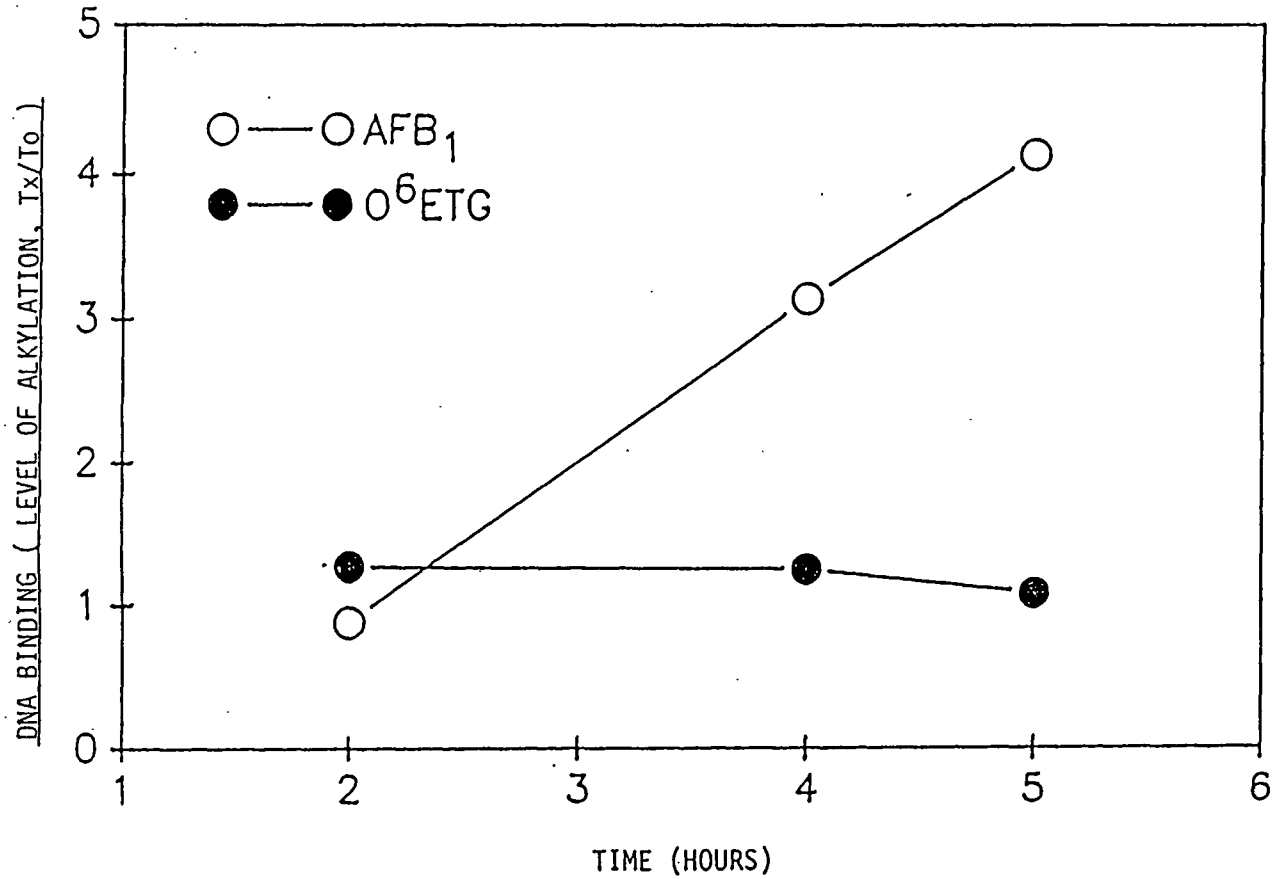

Figure II.7 Levels Of DNA Alkylation In Hepatocytes At Various Times (Tx).

T0, time at 30 minute carcinogen exposure. Values are expressed as the level of alkylation found at Tx relative to the level found at T0.

Table II 2. O^6 Alkyl Guanine Alkyl Transferase Activity In Rainbow Trout.

f MOLES O^6-METHYLGUANINE REMOVED/MG PROTEIN.			
TROUT[a] (ADULT)	TROUT[b] (FRY)	SALMON[c]	RAT[d]
33.1	16	18.1	27.3

[a]Average body weight, 200g. Values are calculated from 3 pools of 3 livers.

[bc]Average body weight, 20-30g. values are calculated from 3 pools of 10 livers (Fong et al., in press).

[d]Average body weight 120-140g. Values are calculated from 3 individual livers (Fong et al., in press).

An additional measure of repair capacity involved the assay of the alkyltransferase repair protein itself. This study was a collaborative effort with Dr. Arthur Fong. The activity of O^6-alkylguanine alkyltransferase in cell extracts was measured as the removal of the [^{3}H] methyl adduct from the O^6 position of guanine in [^{3}H] methyl-DNA, obtained by the reaction of calf thymus DNA with with ^{3}H N-methylnitrosourea (refer to Materials And Methods). As shown in Table II.2, the amount of repair protein in trout liver ranged from 16-33 fmoles O^6-methylguanine removed/mg protein, depending on the age of the fish. By comparison rat liver extract contained activity equivalent to 27 fmoles O^6- methylguanine removed/mg protein.

INCUBATION OF TROUT HEPATOCYTES WITH DIMETHYLNITROSAMINE, A POTENT ALKYLATOR

Dimethylnitrosamine treatment in mammalian systems is known to produce greater amount of guanine adducts than treatment with DENA. Therefore, higher levels of DNA alkylation may be expected following exposure of trout hepatocytes to DMN. This possibility was examined by analyzing DNA from monolayer cultures of trout hepatocytes treated with 1.5 mM, 20 hours, 80-100g trout (Fong, personal communication, Figure II.8). Resulting chromatograms show high guanine content but very little O^6-methylguanine (Figure II.8).

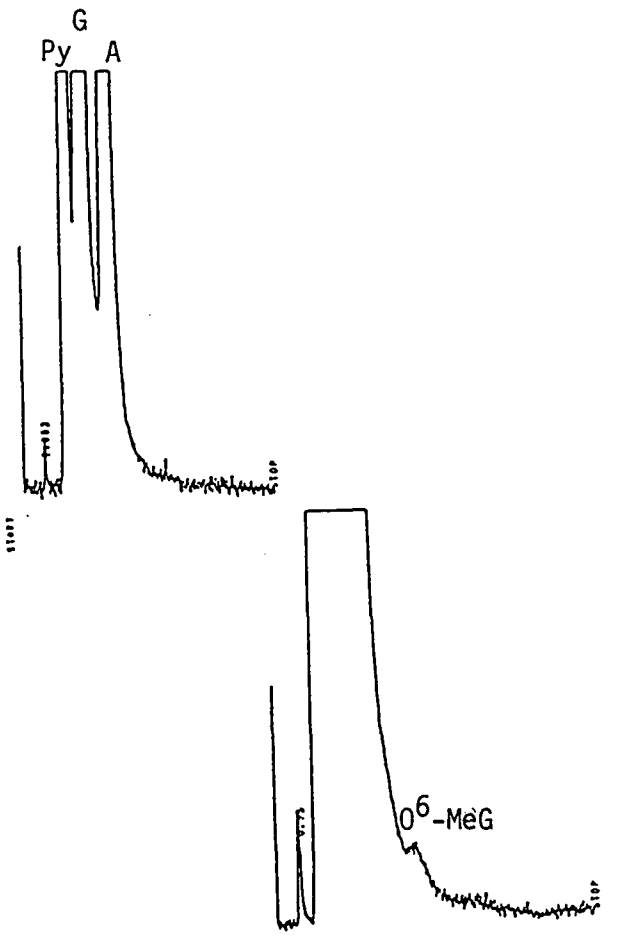

Figure II.8 Analysis Of DNA Hydrolysates From Hepatocytes.

Hepatocytes were exposed to 1.5mM DMN for 20 hours, and HPLC analysis was performed using fluorescence detection. Py, pyrimidine oligonucleotides; A, adenine; O^6-MeG, O^6-methylguanine; G, guanine.

DISCUSSION

IN VITRO STUDIES

In this study exposure of freshly prepared trout hepatocytes to DENA at concentrations up to 1 mM resulted in no detectable binding to DNA. Furthermore, only unquantifiable, trace amounts of O6-EtG were found following DENA exposure of hepatocytes isolated from BNF pretreated trout. The absence of quantifiable DNA alkylation found under the present experimental conditions appears not to be a problem with transport of DENA into the hepatocytes, because the hepatocyte pellets, which had been rinsed with 4 ml of buffer twice, after incubations, contained 19-30% of the radioactive dose. Also, detectable levels of radioactivity 0.4-1.8% added to the incubation mixture were found in the phenol fraction collected during DNA isolation, indicating the presence of DENA in the hepatocytes.

The results of the present study are in agreement with the *in vitro* study of Montesano et al. (Montesano, et al., 1973) which compared the capacity of trout and rat liver slices to metabolically activate DMN. Significantly less DNA alkylation, measured as the production of N7-methylguanine, was found in trout liver slices compared to rat liver slices following incubation with DMN.

Other studies have indicated that the capacity to activate nitrosamines to a reactive metabolite is extremely low in trout.

Recent studies were conducted on the in vitro metabolism of [^{3}H] DENA by Williams (Williams, 1987, personal communication), using microsomes isolated from control and BNF-pretreated trout. The microsomal oxidative dealkylation of DENA was measured by the production of aldehyde, formed when the activated intermediate spontaneously decomposed to an alkylating species. Liver microsomes from BNF-pretreated trout produced 0.032-0.025 nmoles of aldehyde/min/mg protein while similar incubations for rat liver showed 3.5 nmoles of aldehyde/minute/mg protein (Farrelly, 1980).

P-450 activity levels have been shown to decline in isolated mouse hepatocytes incubated more than 6 hours, such that only 35% and 24% of the initial activity remained after 16 and 21 hours, respectively (Raukman et al., 1987). Since all hepatocyte incubations in this study lasted less than 6 hours, depletion of P-450 activity may not have been the major cause for the low DNA alkylation observed. It has been observed that after exposure to the same amount of carcinogen, the extent of alkylation of DNA is an order of magnitude less when rat hepatocytes are incubated with DMN, compared to the DNA alkylation produced by in vivo exposure to DMN (Umbenhauer and Pegg, 1981). Umbenhauer and Pegg found that extensive reaction of the alkylating species generated from DMN could occur with extracellular DNA used in the hepatocyte incubations. In the hepatocytes, which are diluted with incubation buffer to ensure oxygenation, a significant amount of the alkylating species can

diffuse out of the cell and react with water or other nucleophiles present. Further support in agreement with the observations of Umbenhauer and Pegg is provided by the induction of mutation in cells which do not metabolize DMN, when these are cultured with isolated hepatocytes and DMN (Lagenbach et al., 1978).

Renton and Peterson (Renton and Peterson, 1983) have demonstrated that metabolism of xenobiotics by isolated hepatocytes may be influenced by the milieu of the cell population. When a mixed cell suspension of liver cells (hepatocytes and Kuppfer cells) was used and incubated with dextran sulfate or latex beads, cytochrome P-450-dependent enzyme activities and drug biotransformation were depressed in the cell mixture compared to incubations containing hepatocytes alone. The depressed activity suggests that effects of Kuppfer cells in hepatocyte incubation studies could primarily affect hepatocyte function. It has been postulated that subpopulations rather than whole organs are the targets for transformation, therefore depending on the cell crop of hepatocytes, the metabolism and the biotransformation may differ for a particular carcinogen, from one hepatocyte preparation to another. Of particular importance to the present study is the finding that the level of cytochrome P-450 in rat nonparenchymal cells is only about 15% of that found in parenchymal cells (Lafranconi et al., 1986). The nonparencymal cells i.e. cells located along the sinusoids and endothelial cells, may be more

likely released from the liver by the perfusion method employed in the current study, the low and variable levels of DNA binding found in this study may be related to the selective but variable concentration of nonparenchymal cells in the different trout hepatocyte preparations.

ENU also was shown to produce low and inconsistent alkylation in trout hepatocytes, in the present study. ENU, unlike DENA is a direct alkylating agent. Hence low levels of DNA alkylation found after ENU are not directly related to the low nitrosamine metabolizing activity in trout hepatocytes. ENU produces the same alkylating species as DENA (Guttenplan, 1987) and the half life of ENU in most buffers is about 7 minutes, the major product being the corresponding alcohol. This alcohol is formed from the reaction of the alkylating species with water, which is a weak nucleophile. The levels of DNA binding will depend upon the amount of the reactive metabolite reaching the DNA target. To study the effect of decreasing the volume, trout hepatocyte incubations were performed in a total volume of 0.5 ml in contrast to the 5 ml used for standard hepatocyte incubations. This treatment produced a increase in the amount of O^6-EtG adduct seen but also resulted in the appearance of this adduct in DNA from control incubation, presenting a toxic manifestation.

IN VIVO STUDIES

The absence of DNA alkylation found after i.p. injection of DEN in trout in this study, extends the observations of Kruger et al. (Kruger et al., 1970) who found no methyl guanine adducts after i.p. administration of DMN to trout. Trout have been shown to be resistant to the acute toxicity of DMN though dietary exposure leads to tumor occurrence (Greico et al., 1978). DENA at 1110 ppm in the diet, for 1 year has been shown to produce hepatocarcinomas in trout (Shelton et al., 1984). These tumor studies show that trout display some sensitivity to the carcinogenicity of the nitrosamines, but this response is inconsistent with the undetectably low DNA alkylation after i.p. exposure. Montesano et al.(Montesano et al., 1973) have investigated the excretion of an i.p. dose of DMN from goldfish. The recovery in water after injection of 12.5 mg dose of DMN, determined by polarography, reached 20, 46, 68.8, 88.5, and 97.4% at 15, 30, 60, 120, and 180 minutes respectively. At 180 minutes only 3.7% of the dose was found in the body of the fish. This rapid elimination as shown by Montesano et al., is probably one of the reasons why no ethylation of DNA was observed in the trout in the present study, and why prolonged dietary exposures are needed to induce tumors in the trout by DEN or DMN.

The most profound effects on DNA alkylation have been produced by a single 24 hour exposure to DEN via water. This

method produced an 86% incidence of hepatic neoplasms (Fong et al., in press). This method of exposure has been used successfully with other fish. In the fish Rivulus ocellatus marmoratus, single and continuous exposures as low as 9mg/L DEN have revealed pancreatic neoplasms (Thiyagarajah and Grizzle, 1986). Exposure of trout by this method also has resulted in a linear dose dependent liver DNA ethylation in doses as low as 62.5 mg/L of DEN, detected by the same HPLC methods used in the present study (Fong et al., in press). The ultimate dose of the nitrosamine delivered by this route may therefore be much higher than that expected from the single pulse i.p. injection.

REPAIR

DNA damage resulting from the action of genotoxic carcinogens can be repaired by several mechanisms. Tissues and cells with low repair capacities usually are sensitive to the actions of these carcinogens (Goth and Rajewsky, 1974). The probability of initiation is related to the level of DNA damage, the rate of DNA replication, and the amount of DNA at risk (Swenberg et al., 1984). The rainbow trout is very sensitive to AFB1 initiated carcinogenesis, and AFB1-DNA adducts persist in trout liver DNA (Bailey et al., 1987). Trout appear to be deficient in excision type repair (Collier, 1988). Results of the present study indicate that the trout contains O^6-methylguanine removal activity, and that the activity is age dependent.

Extracts from trout liver examined for O6-methylguanine removal from DNA were shown in Table II.3. Age is an important variable in carcinogen studies with neonatal carcinogen exposure usually producing a higher tumor response. Some carcinogens produce tumors in young animals while older ones become refractory to them. This is especially true of liver tumors, as rapidly growing liver tissue has a much greater chance of producing a lesion. It may also be a function of repair capability of the organism. Thiagarajah and Grizzle (Thiagarajah and Grizzle, 1986) have shown that when larvae and juvenille fish were exposed to DEN through water, benign neoplasms were produced in the fish exposed when juvenile as opposed to adenocarcinomas in fish exposed when larvae.

Results obtained for older trout, in the present study showing higher O^6-methylguanine removal activity than younger trout, are consistent with the observation of age related decrease to carcinogen sensitivity.

Different patterns of formation and persistence in DNA alkylation were found in hepatocytes exposed to AFB1 and ENU (Figure II.7). While O^6-EtG level decreased after ENU treatment, the level of AFB1 DNA binding increased. Removal of O6-EtG, Figure II.7, shows that O6-ETG decreases only very slightly with time in ENU treated hepatocytes between two and five hours. Older fish have been shown to have substantial alkyltransferase, yet hepatocyte studies show slow rate of removal. This is consistent with the knowledge that the alkyltransferase protein

is inactivated by the irreversible binding of the alkyl group to a cystiene residue of the enzyme. Since the incubation with ENU was performed for 30 minutes (T0) resulting in the initial alkylation of guanine, most of the enzyme would be already utilized and the slow removal seen may represent a resynthesis of the protein by the hepatocytes. Furthermore, calculation of O^6-EtG/g liver at time 30 minutes (T0) and alkyltransferase activity/g liver reveal that alkylation exceeds the enzyme activity by > 100 times. This supports the theory that the enzyme is consumed during the initial 30 minutes of incubation with the carcinogen and therefore very small decrease in O^6-EtG is observed between two and five hours.

AFB1 shows an increase in DNA binding, as the compound being lipophilic, remains in the cellular pellet, being activated to produce the metabolite capable of binding to DNA. Also the repair of AFB1-DNA adducts has been shown to be poor in trout (Bailey et al., 1987). Hence the ENU curve results from removal of the adduct only, in contrast the AFB1 DNA adducts are repaired slowly, and the major factor in determining the levels of DNA binding in this study is the activation of AFB1.

In summary, it is suggested that initial low alkylation of DNA in trout may cause the determination of kinetics of carcinogen-DNA binding response to be difficult for N-nitroso compounds. The two routes of exposure tested in this study, i.p. injection and incubation with hepatocytes, did not allow a sufficient exposure dose of the DENA to study DNA binding.

BIBLIOGRAPHY

1. Ahotopa, M., Bussacchini-Griot, V, Bereziat, J., Camus, A. and Bartsch, H., 1987. Rapid oxidative stress induced by N-Nitrosamines. Biochem. Biophys. Res. Commun., 146, 1047-1054.

2. All-Dabbagh, S. G., Idle, J. R., Smith, R. L., 1981. Animal modelling of human polymorphic drug oxidation the metabolism of Debrisoquine and phenacetin in rat inbred strains. J. Pharm. Pharmacol., 33, 161.

3. Appel K. E., Richart, R, Schwarz, M. and Kunz, W., 1979. Influence of drugs on activation and inactivation of hepatocarcinogenic nitrosamines. Arch. Toxicol., 2, 471-477.

4. Appel, K. E., Christian, S. R. and Hildebrandt, A. G., 1985. Oxidative dealkylation and reductive denitrosation of nitrosomethylamine in vitro, Chem. Biol. Interact., 69-76.

5. Archer, M. C. and Labuc, G. E., 1985. In Bioactivation of Foreign Compounds. M. W. Baders, Eds., 403-431.

6. Arcos, J. C., Bryant, G. M., Verkatesan, N., and Argus, M.F., 1975. Repression of dimethylnitrosamine demethylase by

typical inducers of microsomal mixed function oxidase. Biol. Chem. Pharmacol., 24, 1544-1547.

7. Armstrong, R., N., 1987. Enzyme catalyzed detoxification reactions: Mechanisms and steriochemistry, CRC, biochem., 22, 1.

8. Ashley, L. M. and Halven, J. E., 1968. Dimethylnitrosamine induced hepatic cell carcinoma in rainbow trout. J. Natl. Cancer Inst., 41, 531-552.

9. Bailey, G. S., Taylor, M. J., Loveland, P., Wilcox, J., Sinnhuber, R. and Selivonchick, D. P., 1981. Dietary modification of AFB1 carcinogenesis: Mechanism studies with isolated hepatocytes from rainbow trout. Natl. Cancer Inst. Monogr., 65.

10. Bailey, G. S., Taylor, M. J., Selivonchick, D. P., 1982. Aflatoxin B1 metabolism and DNA binding in isolated hepatocytes, from rainbow trout (Salmo gairdneri). Carcinogenesis 3, 511-518.

11. Bailey, G., Selivonchick, D., Hendricks, J., 1987. Initiation, promotion, and inhibition of carcinogenesis in rainbow trout. Environ. Health. Perspec., 71, 147-153.

12. Bailey, G. S., Hendricks, J. D., Shelton, D. W., Nixon, J. E., and Pawlowski, N. E., 1987. Enhancement of carcinogenesis by natural anticarcinogen indole-3-carbinol. J. Natl. Cancer Inst., 78, 931-936.

13. Baird, W. M., Chemery, S. R., Erikson, A. A., Cherr, C. J. and Diamond, L., 1979. In Polynuclear Aromatic Hydrocarbons, Differences in pathway of polycyclic aromatic hydrocarbon metabolism as detected by analysis of the conjugates formed. Jones, P. W. and Laber P. Eds., Science:Ann Arbor, 507-516.

14. Bartsh, H., Malaveille, C., Barbina, A. and Planche, G., 1979a. Mutagenic alkylating metabolites of halo-ethylenes, chlorobutadienes, and dichlorobutenes produced by rodent or human liver tissues: Evidence for oxirane formation by P450-linked microsomal mono-oxygenases, Arch. Toxicol., 41, 249-277.

15. Becker, R. A. and Shank, R. C., 1985. Kinetics of formation and persistence of ethylguanines in DNA of rats and hamsters treated with dimethylnitrosamine. Cancer Res., 45, 2078-2084.

16. Bishop, M. J., 1982. In Cancer Biology. Readings from Scientific American, 1986 Ed., W. H. Freeman: N.Y.

17. Bjorseth, A., Becher, G., 1986. In PAH in work atmospheres: occurrence and determination, CRC:Boca Raton, Florida.

18. Blumberg, P. M., 1980. In vitro studies on the model of action of phorbol esters, potent tumor promoters, CRC, Toxicol., 8, 153.

19. Borek, C., 1986. In Antimutagenesis and anticarcinogenesis, hormones and dietary factors controlling gene activation and expression. Plenum:NY, London.

20. Boyd, M. R and Statham, C. N., 1983. The effect of hepatic metabolism on the production and toxicity of reactive metabolites in extra hepatic organs. Drug. Metab. Rev., 14, 35.

21. Boyland E. and Sims, P., 1967. The carcinogen activities in mice of compounds related to benz(a)anthracene. Intl. J. Cancer, 2, 500-504.

23. Bradfield, C. A., and Bjeldanes, L. F., 1984. Effect of dietary indole-3-carbinol on intestinal and hepatic monooxygenase, gluthathione S-transferase and epoxide hydrolase activities in rat. Fd. Chem. Toxic., 22, 977.

24. Bradfield, C. A., Bjeldanes, L. F., 1987. Structure activity relationships of dietary indoles: A proposed mechanism of action as modifiers of xenobiotic metabolism. J. Toxicol. Environm. Health., 21, 311-323.

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

26. Cairns, 1986. In Antimutagenesis and anticarcinogenesis, A summary and a look ahead, Plenum:NY, London.

27. Cairins, J., 1975. In Cancer Biology, Readings from Scientific American, 1986, Ed., W. H. Freeman:N. Y.

28. Casarett, J. L. and Doull, J., 1986. Toxicology: The basic science of poisons, 3rd Ed., Macmillian.

29. Chambers, J. E., and Yarbrough J. D., 1976. Xenobiotic biotransformation systems in fishes, Comp. Biochem. Physiol., 55c, 77-84.

30. Christou, M., Marcus, C., Jefcoate, R. C., 1986. Selective interactions of cytochromes P-450 with the hydroxymethyl derivatives of 7,12, dimethylbenz(a)anthracene. Carcinogenesis, 7, 871-877.

31. Coleo, B., 1985. Effects of modifying structure on electrophilic reactions with biological nucleophiles. Drug Metab. Rev., 15, 1307-1334.

32. Cleaver, J. E., 1986. In Antimutagenesis and anticarcinogenesis, DNA repair and replication in xeroderma pigmentosum and related disorders. Plenum:NY, London.

33. Collier, J. M., 1988. A study of the DNA excision repair capabilities of rainbow trout (Salmo gairdneri) exposed to dietary cyclopropenoid fatty acids. M. S. thesis, Dept. Food Science and Tech., O.S.U., Corvallis, OR.

34. Conney, A. H., 1982. Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons, G. H. A. Clowes memorial lecture. Cancer Res. 42, 4875-4917.

35. Dannan, G. A., Guengerich, F. P., Kamisky, L. S., and Aust, S. D., 1983. Regulation of cytochrome P450: immunochemical quantitation of eight isozymes in liver microsomes of rats treated with polybrominated biphenyl congeners. J. Biol. Chem., 258, 1282-1290.

36. Dashwood, R. H., Arbogast, D. N., Fong, A. T., Hendricks, J. D., Bailey G. S., 1988. Mechanisms of anti-carcinogenesis by indole-3-carbinol: detailed in vivo DNA binding dose response studies after dietary administration with aflatoxin B1 carcinogenesis. Carcinogenesis, 9, 427-432.

37. Devore, J. and Peck, R., 1986. In Statistics, The Exploration Of Data. West:MN.

38. Di Giovanni, J., Siva, J. F, Ashurst, S. W., Singer, J. M., and Diamond, L., 1983. Benzoapyrene and 7, 12-dimethylbenz(a)anthracene metabolism and DNA adduct formation in primary cultures of hamster epidermal cells. Cancer Res., 43, 163-170.

39. Egaas, E., Varanasi, U., 1982. Effects of polychlorinated biphenyls and enviornmental temperature on in vitro formation of benzo(a)pyrene metabolites by liver of trout (Salmo gairdneri), Biochem. Pharmacol., 4, 561-566.

40. Elcombe, C. R., Lech, J. J, 1979. Induction and characterization of hemproteins P-450 and monooxygenation in rainbow trout (Salmo gairdneri). Toxicol. Appl. Pharmacol., 49, 437-450.

41. Engelse L. D., Menkveld, J. G., Debrij, J. R., 1985. Formation and stability of alkylated pyrimidines and purines, Carcinogenesis, 3, 393-403.

42. Evans, D. A. P., Harmer, D., Downham, D. Y., Whibley, E. J., Idle, J. R., Ritchie, J. and Smith, R. L., 1983. The genetic

control of debrisoquine metabolism in man with new methods of analyzing bimodal distributions. J. Med. Genet., 20, 321-326.

43. Farber, E., 1987. Hepatocarcinogenesis: A Dynamic cellular perspective. Lab. Investig., 56, 4-22.

44. Folkman, J. 1976. In Cancer Biology, Readings from Scientific American, The vascularization of tumors, 1986 Ed., W. H. Freeman:N. Y.

45. Fong, A. T., Hendricks, J. D., Dashwood R. D., Van Winkle, S. and Bailey, G. S., (in press). Formation and persistence of ethylguanines in liver DNA of rainbow trout (Salmo gairdneri) treated with diethylnitrosamine by water exposure.

46. Gelboin, H. V., 1983. Carcinogens, drugs and cytochromes P-450. N. Eng. J. Med., 309, 105-107.

47. Gerson, L. S., Trey, J. E., Miller, K. and Berger, N. A., 1986. Comparison of O^6-alkylguanine-DNA alkyltransferase activity based on cellular DNA content in human, rat and mouse tissues. Carcinogenesis, 5, 745-749.

48. Goeger, D. E., Shelton, D. W., Hendricks, J. D., Bailey, G. S., 1986. Mechanisms of anticarcinogenesis by indole-3-carbinol:

effect on the distribution and metabolism of aflatoxin B1, in rainbow trout. Carcinogenesis, 7, 2025-2031.

49. Goerrttler, K., and Loehrke. H., 1986. Inhibition of the prenatal DMBA induced tumor initiation in mice by prior administration of 7,8, benzoflavone. Carcinogenesis, 7, 1187-1189.

50. Goldstein, J. A., Hickman, P., Bergman, H., McKinney, J. D. and Walker, M. P., 1977. Separation of pure PCB isomers into 2 types of inducers on the basis of induction of P-450 or P-448. Chem. Biol. Interact. 17, 69-78.

51. Goth, R. and Rajewsky, M. F., 1974. Persistence of O^6-ethylguanine in rat brain DNA: Correlation with nervous system specific carcinogenesis by ethylnitrosourea, Proc. Natl. Acad. Sci. U.S.A., 71, 639-643.

52. Gould, M. N., 1980. Mammary gland cell-mediated mutagenesis of mammalian cells by organ-specific carcinogens. Cancer Res. 40, 1836-1841.

53. Greico, M. P, Hendricks, J. D., Scanlan, R. A., Sinnhuber, R. O., Pierce, D. A., 1978. Carcinogenecity and acute toxicity of DMN in rainbow trout (Salmo gairdneri), J. Natl. Cancer. Inst., 5, 1127-1131.

54. Guengerich, P. F. and Liebler, D. C., 1985. Enzymatic activation of chemicals to toxic metabolites, CRC. Toxicol., CRC:Boca Raton, Florida.

55. Guttenplan, B. J. 1987. N-Nitrosamines, bacterial mutagenesis and in vitro metabolism. Mutat. Res., 186, 81-134.

56. Haenszel, W., Locke, F. B. and Segi, M., 1980. A case control study of large bowel cancer in Japan. J. Natl. Cancer Inst., 64, 17-22.

57. Hallberg, E. and Rydstrom, J., 1987. Toxicity of 7,12-dimethylbenz(a)anthracene and 7-hydroxymethyl-12-methylbenzanthracene and its prevention in altered rat adrenal cells. Evidence for peroxidation mechanism of action. Toxicol., 47, 259-275.

58. Heidelberger, C. and Weiss, S. M., 1951. The distribution of radioactivity in mice following the administration of 3,4-benzopyrene 5-C14 and 1,2,5,6-dibenzanthracene 9,10-C14. Cancer Res. 11, 885-890.

59. Hendricks, J. D., Stott, W. T., Putnam, T. P. and Sinnhuber, R. O., 1977. The inhibitory effect of a PCB (Aroclor

1254) on aflatoxin B1 carcinogenesis in rainbow trout. J. Natl. Cancer Inst., 59, 1545-1551.

60. Hendricks, J. D., Myers, T. R. , Shelton, D. W., Casteel, J. L.and Bailey, G. S., 1985. Hepatocarcinogenicity of benzoapyrene to rainbow trout by dietary exposure to and intraperitoneal injection. J. Natl. Cancer Inst., 47, 839-851.

61. Hernandez, O., Walker, M., Cox, R. H., Foreman, G. L., Smith, B. and Bend, J., 1980. Regiospecificity and steriospecificity in the enzymatic conjugation of gluthathione with +- benzoapyrene 4,5-oxide. Biochem. Biophys. Res. Commun. 96, 1494-1502.

62. Herron, D. C., and Shank, R. C., 1980. Methylated purines in liver DNA after probable dimethylnitrosamine poisoning. Cancer Res. 40, 3116-3117.

63. Ho, T., Ho, H. G., Hamilton, L. D., 1978. Biochemical significance of the hard and soft acids and bases principle. Chem. Biol. Interact., 23, 65-84.

64. Hofe, E., Kleihues, P., Keefer, K., 1986. Extent of DNA 2-hydroxyethylation by N-nitrosomethylethylamine and N-nitrosodiethylamine in vivo. Carcinogenesis, 8, 1335-1337.

65. Huggins, C. B., 1979. In Experimental Leukaemia and Mmammary Cancer, University of Chicago:Chicago, IL.

66. Hunter, T., 1982. In Cancer Biology, Readings from Scientific American, The proteins of oncogenes. 1986 Ed., W. H. Freeman:N. Y.

67. Idle, J. R.and Smith, R. L., 1979. Polymorphism of oxidation at carbon centers of drugs and their clinical significance. Drug. Metab. Rev. 9, 301-320.

68. Illmensee, K., and Stevens, L. C., 1979. Teratomas and chimeras, In Cancer Biology, Readings from Scientific American, W. H. Freeman: N. Y., 1986 edition.

69. International Radiological Protection Commission, Task Group on Lung Dynamics, 1966. Deposition of retention models for internal dosimetry of the human respiratory tract, Health. Phys. 12, 173-178.

70. Iversen, U. M. and Iversen, O. H., 1979. The carcinogenic effects of TPA (12-O-tetradecanoyl phorbol-13-acetate) when applied to the skin of hairless mice, Virchows. Arch. B, 30, 33-40.

71. Jaiswal, A. K., Gonzalez, F. J. and Nebert, D. W., 1985. Human dioxin-inducible cytochrome P-450 complementary DNA and amino acid sequence. Science, 228, 80-83.

72. James M. O., Bend, J. R., 1980. Polycyclic aromatic hydrocarbons induction of cytochrome P-450 dependent mixed function oxidases in marine fish. Toxicol. Appl. Pharmcol. 54, 117-133.

73. Jarman, M. and Manson, D., 1986. The metabolism of N-nitrosomorpholine by rat liver microsomes and its oxidation by the fenton system. Carcinogenesis, 4 , 559-565.

74. Jerina, D. M., 1976. In Glutathione: Metabolism and Function, Raven:NY, Arias, I. M. and Jakoby W. B. Eds.

75. Jernstrom, B., Martinez, M., Meyer, D. J. and Kettener, B., 1985. Glutathione conjugation of the carcinogenic and mutagenic electrophile (+-)-7B, 8 alpha dihydroxy 9 alpha 10 oxy 7,8,9,10 tetrahydro-benzoapyrene catalyzed by purified rat liver gluthathione transferase. Carcinogenesis, 6, 85-89.

76. Kennaway, E. L. and Hieger, I., 1930. Carcinogenic substances and their fluorescence spectra. Br. Med. J., 1044-1049.

77. Kinoshita, N. and Gelboin H. V., 1978. Beta-glucuronidase-catalyzed hydrolysis of benzo(a)pyrene-3-glucuronide and binding to DNA. Science, 199, 307-309.

78. Kerkvliet, N. I. and Kimenldorf. D. J., 1977. Antitumor activity of a polychlorinated biphenyl mixture, Aroclor 1254, in rats innoculated with Walker 256 carcinoma cells. J. Natl. Cancer Inst., 59, 951-955.

79. Klauning, J. E., Ruch, J. R. and Goldblatt, P. J., 1985. Trout hepatocyte culture isolation and primary culture. In Vitro Cell. and Devel. Biol., 4, 221-228.

80. Kotin, P., Falk, H. L., Busser, R., 1959. Distribution retention and elimination of C14 3-4, benzopyrene after administration to mice and rats. J. Natl. Cancer Inst., 3, 541-545.

81. Kruger F. W., Walker, G., Wiessler, M., 1970. Carcinogenic action of DMN in trout not related to methylation of nucleic acids and protein in vivo. Experientia, 26, 520-522.

82. Lagenbach, R., Freed, H. J. and Huberman, E., 1978. Liver cell-mediated mutagenesis of mammalian cells by liver carcinogens. Proc. Natl. Acad. Sci. U.S.A., 75, 2864-2867.

83. Lawley, P. D., 1976. Carcinogenesis by alkylating agents. ACS. Monogr., 173, 83-244.

84. Levin, W., Thomas, N. O., Oldfield, N. and Ryan, D. E., 1986. N-Demethylation of N-Nitrosodimethylamine catalyzed by purified rat hepatic microsomal cytochrome P-450: isozyme specificity and role of cytochrome b5. Arch. Biochem. Biophys., 248, 158-165.

85. Lindamood, C.III, Bedell, M. A., Billings, K. C., Dyroll, M. C. and Swenberg, J. A., 1984. Dose response for DNA alkylation, [^{3}H]thymidine uptake into DNA, and O^{6}-methyltransferase activity in hepatocytes of rats and mice continuously exposed to dimethylnitrosamine. Cancer Res., 44, 196-200.

86. Lotikar, P. D., Baldy W. J., and Dwyer E. N., 1975. Dimethylnitrosamine demethylation by reconstituted liver microsomal cytochrome P-450 enzyme system. Biochem. J., 152, 705-708.

87. Magee, P. N. and Barnes, J. M., 1967. Carcinogenic nitroso compounds. Adv. Cancer Res., 10, 163-246.

88. Maniatis, T., and Fritsch, E. F., 1982. In Molecular Cloning a Laboratory Manual, Cold Spring Harbor.

89. Mass, M. J. and Kaufman D. G., 1979. Benzoapyrene quinones metabolism in tracheal organ cultures, Biochem. Biophys. Res. Commun., 89, 885-892.

90. Melius, P., 1984. Comparative benzopyrene metabolite patterns in fish and rodents. Natl. Cancer Inst. Monogr. 65, 387-390.

91. Melius, P. and Tan, B., 1986. Polynuclear aromatic hydrocarbon metabolism in fishes. Commun. Biochem. Physiol., 83c, 217-224.

92. Miller, E. C., 1978. Some current perspectives on chemical carcinogenesis in humans and experimental animals: presidential address. Cancer Res. 38, 1479-1496.

94. Montesano, R., Ingram A. J. and Magee, P. N., 1967. Metabolism of dimethylnitrosamine by amphibians and fish in vitro. Experientia, 29, 599-565.

95. Mitchell, C. E., 1983. The metabolic fate of benzo(a)pyrene in rats after inhalation. Toxicol., 28, 65.

96. Moore, C. J., Pruess-Schwartz, D., Mauthe, R. J., Gould, M. N. and Baird, W. M., 1987. Inter-species differences in the major adducts formed from BAP but not 7,12-

dimethylbenz(a)anthracene in rat and mammary cell cultures. Cancer Res., 47, 4402-4406.

97. Myrnes, B., Giercksky, K. E. and Kokrann, H., 1983. Interindividual variation in the activity of O^6-methylguanine DNA-methyltransferase and uracil DNA glycosylase in human organs. Carcinogenesis, 4, 1565-1568.

98. Naktasuru, Y., Nemoto, N., Nakagawa, K., Mashahito P. and Ishikawa, T., 1987. O^6-Methylguanine DNA methyltransferase activity in liver from various fish species. Carcinogenesis 8, 1123-1127.

99. Nicoll, J. W., Swann, P. F., Pegg, A. E., 1975. Effect of dimethylnitrosamine on persistence of methylated guanines in rat liver and kidney DNA. Nature, London, 254-262.

100. Nishimoto, M. Varanasi, U., 1985. Benzopyrene metabolism and DNA adduct formation mediated by English sole liver enzyme, Biochem. Pharmacol. 34, 263-268.

101. Nishizuka, Y., 1986. Studies and perspectives of protein kinase C, Science, 305-311.

102. Nixon, J. E., Hendricks, J. D., Pawlowski, N. E., Pereira, C. B., Sinnhuber, R. O. and Bailey, G. S, 1984. Inhibition of

Aflatoxin B1 carcinogenesis in rainbow trout by flavone and indole compounds. Carcinogenesis, 5, 615-619.

103. O'Dowd, J. J. and Burnett, A. K., 1988. The distribution of DMBA and its dihydrodiols in tissues of control and Sudan III treated Long Evans rats after the injection (i.v.) in vivo of a leukaemogenic dose of the hydrocarbon. Carcinogenesis 9, 29-35.

104. Pearson, R. G., 1966. Acids and bases. Science, 151, 172-177.

105. Pegg, A. E., 1984. Methylation of the O^6 position of guanine in DNA is the most likely initiating event in carcinogenesis by methylating agents. Cancer Invest., 3, 223-231.

106. Pegg, A. E., Roberfroid, M., von Bahr, C., Foote, R. S., Mitra, S., Bresil, H., Likhachev, A., and Montesano, R., 1982. Removal of O^6 methylguanine from DNA by human liver fractions. Proc. Natl. Acad. Sci. U.S.A. 79, 5162-5165.

107. Pence, B. C., Buddingh, F. and Yang, S. P., 1986. Multiple dietary factors in the enhancement of dimethylhydrazine carcinogenesis main effect of indole-3-carbinol. J. Natl. Cancer Inst. 77, 269-275.

108. Pitot, H. C., 1983. Chemicals and cancer. Hosp. Prac., 7, 101-113.

109. Pott, P., 1775. Chirurgical observations relative to the polyps of the nose the cancer of the scrotum the different kinds of ruptures and mortification of the toes and feet. In Cancer Biology, Readings from Scientific American. 1986, Ed., W. H. Freeman:New York.

110. Richardson et al., 1985. Differential repair of O4 alkylthymidine following exposure to methylating and ethylating hepatocarcinogens. Carcinogenesis, 4, 625-629.

111. Rashid, K. A., Babish, J. G. and Mumma, R. O., 1988. In vitro activation assays with hepatic S9 preparation from wild and laboratory reared woodchucks in the Salmonella mutagenicity test. Toxicol., 48, 53-59.

112. Raukman, E. J. and Padilla G.M., 1987. The isolated hepatocyte: use in toxicology and xenobiotic biotransformations. Academic: London.

113. Regan, J. D., Snyder, R. D., Francis A. A., and Olla, B. L., 1983. Excision repair of ultraviolet and chemically induced

damage in the DNA of fibroblasts derived from two closely related species of marine fish. Aquatic. Toxicol. 4, 181-188.

114. Renton, K. W., 1983. In Biological Basis of Detoxication. J. Caldwell and W. B. Jakoby Eds. 307-324, Academic:New York.

115. Reznik-Schuller and Hague, 1981. Autoradiographic study of the distribution of bound radioactivity in the respiratory tract of the Syrian hamster given N-3H-nitrosodiethylamine. Cancer Res., 41, 2147-2150.

116. Ritchie, J. C. and Idle, J. R., 1982. Population studies of polymorphism in drug oxidation and its relevance to carcinogenesis, host factors in human carcinogenesis. Bartsch, H., Armstrong, B., Eds., International agency for research on cancer, Publ. 37, Lyon, France 381.

117. Rydstrom, J., Bengtsson, M., 1983. Regulation of carcinogen metabolism in the rat ovary by the estrous cycle and gonadotropin, Science, 219, 1437-1438.

118. Shelton, D. W, Hendricks J. D., Bailey G. S., 1984. The hepatocarcinogenicity of diethylnitrosamine to rainbow trout and its enhancement by Aroclor 1242 and 1254. Toxicol. Lett., 22, 27-31.

119. Shelton, D. W., Goeger, D. E., Hendricks, J. D. and Bailey G. S., 1986. Mechanisms of anticarcinogenesis: the distribution and metabolism of aflatoxin B1 in rainbow trout fed Aroclor 1254. Carcinogenesis, 7, 1065-1071.

120. Shetzer, H. G., 1984. Indole-3-carbinol protects against covalent binding of benzoapyrene and N-Nitrosodimethylamine metabolites to mouse liver macromolecules. Chem. Biol. Interact., 48, 81-90.

121. Singer, B., 1984. Alkylation of the O^6 of guanine is only one of many chemical events that may initiate carcinogenesis. Cancer Investi., 3, 233-238.

122. Singer, B., Spengler, S. J., Fraenkel, C. H. and Kusmierek, J. T., 1986. O^4 methyl-ethyl or isopropyl substituents on thymidine in poly (da-dt) all lead to transitions upon replication. Proc. Natl. Acad. Sci. U.S.A., 83, 28-32.

123. Singer, B., Spengler, S., and Bodell, W. J., 1981. Tissue dependent enzyme mediated repair on removal of O-ethyl pyrimidines and ethyl purines in carcinogen treated rats. Carcinogenesis 10, 1069-1073.

124. Somalarek, T. A., Morgan S. L., Monihan G. C., Lee, H., Harvey, R. G., and Baird, W. M., 1987. Metabolism and DNA adduct

formation of Benzo(a)pyrene and 7,12-DMBA in fish cell lines in culture. Carcinogenesis, 10, 1501-1509.

125. Sparnins, V. L., Venegas, P. L, Wattenberg, W., 1982. Glutathione-s-transferase activity: enhancement by compounds inhibiting chemical carcinogenesis and by dietary constituents. J. Natl. Cancer Inst., 68, 493-496.

126. Spielgelhalder, B. and Preussmann, R., 1985. In vivo nitrosation of amidopyrine in humans: Use of ethanol effect for biological monitoring of N-nitrosodimethylamine in urine. Carcinogenesis, 4, 545-548.

127. Statgraphics, 1987. User's guide, Statistical graphics system by statistical graphics corporation.

128. Statham, C. N., Elcombe, C. R., Szyka, S. P. and Lech, J. J., 1978. Effect of PAHs on hepatic microsomal enzymes and disposition of methyl naphthalene in rainbow trout in vivo. Xenobiotica, 8, 65-71.

129. Stegeman, J., Woodin, B. R., Binder, R. L., 1981. Patterns of benzo(a)pyrene metabolism by varied species, organs, and developmental stages of fish. Natl. Cancer Inst. Monograph, 65.

130. Stott, W. T. and Sinnhuber, R. O. 1978. Trout hepatic enzyme activation of aflatoxin B1 in a mutagen assay system and the inhibitory effects of PCBs. Bulletin Environm. Contamn. Toxicol. 35, 41-48.

131. Swann, P. F. and Magee, P.N., 1968. Nitrosamine induced carcinogens. The alkylation of nucleic acids of the rat by N-methyl-n nitrosourea, dimethylnitrosamine, dimethyl sulfate, and methylmethanesulfonate. Biochem. J., 110,39-47.

132. Swenberg, J. A., Dyroff, C. M., Bedell, M. A., Popp, J. A., Huh, N., Kirsteyi, U. and Rajewsky, M. F., 1984. O^4-ethyl deoxythymidine, but not O^6-ethyldeoxyguanosine accumulates in hepatocyte DNA of rats exposed continuously to diethylnitrosamine. Proc. Natl. Acad. Sci. U.S.A., 81, 1692-1695.

133. Swenson D. H., Miller, J. A. and Miller, E. C., 1975. The reactivity and the carcinogenicity of aflatoxin B1 2,3-dichloride and a model for the putative 2, 3-oxide metabolite of aflatoxin B1. Cancer Res., 35, 3811-3823.

134. Taguchi, O., Michael, S. D, Nishizuka, Y., 1988. Rapid induction of ovarian granulosa cell tumors by 7,12-DMBA in neonatally estrogenized mice, Cancer Research, 48, 425-429.

135. Temin, H. M., 1972. In Cancer Biology, Readings from Scientific American, RNA-directed DNA synthesis. 1986 Ed., W. H. Freeman:N.Y.

136. Thiyagarajah, A. and Grizzle, J. M., 1986. Diethylnitrosamine induced pancreatic neoplasms in the fish, Rivulus ocellatus marmonatus. J. Natl. Cancer Inst., 1, 141-147.

137. Thornton, S. C., Diamond, L., and Baird W. M., 1982. Metabolism of BAP by fish cells in culture. J. Toxicol. Environ. Health, 10, 157-167.

138. Tomera, J. F., Skipper, P. L., Wishnok, J. S., Tannenbaum, S. R. and Brunengraber, H., 1984. Inhibition of N-nitrosodimethylamine metabolism by ethanol and other inhibitors in the isolated perfused rat liver. Carcinogenesis, 1, 113-116.

139. Tonada, K. and Hirata, M., 1983. Glucuronidation and sulfation of P-nitrophenol in isolated rat hepatocyte subpopulations. Effects of phenobarbital and 3-methylcholanthrene pretreatment. Chem. Biol. Interact., 47, 277-287.

140. Umenbauer, D. R. and Pegg, A. E., 1981. Alkylation of intracellular and extracellular DNA by dimethylnitrosamine following activation by isolated rat hepatocytes. Cancer Res. 4, 3471-3474.

141. Umbenhauer, D. R. and Pegg, A. E., 1985. Extent of formation of O^4-methylthymidine in calf thymus DNA methylated by N-methyl-N-nitrosourea and lack of repair of this product by rat liver O^6 alkylguanine DNA alkyltransferase. Carcinogenesis, 11, 1611-1614.

142. Utsumi, H., Kitani, H., Chang-Liu, C., Lkind, M. M., 1987. 7,12-DMBA plus near-U.V. light initiates DNA damage and repair in Chinese hamster cells. Carcinogenesis, 8, 1439-1444.

143. Van Durren, B. L. 1978. Effect of aging in two stage carcinogenesis on mouse skin with phorbol myristate acetate as promoting agent. Cancer Res., 38, 865-870.

144. Varanasi, U. Nishimoto, M. and Stover, J., 1983. Analysis of biliary conjugates and hepatic DNA binding in benzopyrene exposed English sole. PAH 8th International Symposium on Mechanisms Methods, Metabolism, Battelle:Columbus Ohio.

145. Varanasi, U., Nishimoto, M., Reichert, W. L. and Eberhart, L. B., 1985. Comparative metabolism of benzopyrene and covalent

binding to hepatic DNA in English sole, starry flounder and rat. Cancer Res., 46, 3817-3824.

146. Varanasi, U., Stein, J. E., Nishimoto, M., Reichert, W. L. Collier, T. K. 1987. Chemical carcinogenesis in feral fish: uptake, activation and detoxification of organic xenobiotics, Environm. Health Perspec., 71, 155-170.

147. Vigny, P., Kindts, M., Cooper, C. S., Grover, P. L. and Sims, P., 1981. Fluorescence spectra of nucleoside-hydrocarbon adducts formed in mouse skin treated with 7,12-dimethylbenzanthracene. Carcinogenesis, 2, 115-119.

148. Walton, D., Acton, A. B., and Stich, H. F., 1984. DNA repair synthesis following exposure to chemical mutagens in primary liver, stomach, and intestinal cells isolated from rainbow trout. Cancer Res., 44, 1120-1121.

149. Watabe, T., Ishizuka, T., Isobe, M., Ozowa, N., 1982. A 7-hydroxymethyl sulphate ester as an active metabolite of 7,12-DMBA. Science 215, 403-404.

150. Wattenberg, L. W., Loub, W. D., 1978. Inhibition of polycyclic aromatic hydrocarbon induced neoplasia by naturally occurring Indoles. Cancer Res., 38, 1410- 1413.

151. Wattenberg, L. W., 1985. Chemoprevention of cancer, Cancer Res. 45, 1-8.

152. Weinberg, R, A., 1983. In Cancer Biology, Readings from Scientific American, A molecular basis of cancer. 1986 Ed., W. H. Freeman:N.Y.

153. Wiessler, M., 1986. DNA adducts by N-nitroso compounds, J. Cancer Res., 2, 81-84.

154. Williams, D. E., Buhler, D. R., 1984. Benzo(a)pyrene-hydroxylase catalyzed by purified isozymes of cytochrome P-450 from beta-naphthoflavone fed rainbow trout. Biochem. Pharmacol. 33, 3743-3753.

155. Williams, G. M. and Weisburger J. H., 1986. In Cassarett and Doull's Toxicology the Basic Science of Poisons, Chemical Carcinogens. 3rd Ed., 1986.

156. Wood, A. W., Levin, W., Lu, A. Y. H., Yagi, H., Hernandez, O., Jerina, D. M., Conney, A. H., 1976. Metabolism of benzopyrene and benzopyrene derivatives to mutagenic products by highly purified hepatic microsomal enzymes. J. Biol. Chem., 251, 4882-4890.

APPENDICES

APPENDIX I

SAFETY PROCEDURE FOR HANDLING OF CARCINOGENS

Guidelines established by the OSU Safety Committee in the Chemical Carcinogen Safety Manual, were followed depending upon the specified class of the carcinogen being used. In general, a laboratory coat was worn at all times, all carcinogen solution preparations were conducted in a hood and were restricted to a designated hazard area, clearly marked with warning labels. Protective eyewear was worn at all stages of carcinogen handling and use. All stock solutions were transported in sealed secondary containers, with appropriate labels declaring amount, date of preparation, storage, and radioactive strength. For decontamination, the procedures outlined in the International Agency For Research On Cancer publications number 37, 43, 55, 49, for Aflatoxin B1, N-nitrosamines, N-nitrosamides, and Polycyclic aromatic hydrocarbons respectively, were followed.

REFERENCES

1. Castegnaro, M., Grimmer, G., Hutzinger, O., Karcher, W., Kunte, H., Lafontaine, M., Sansone, E. B., Telling, G., and Tucker S. P., 1983. Laboratory decontamination of carcinogens in laboratory wastes: Some polycyclic aromatic hydrocarbons, publication, 49. IARC:Lyon.

2. Castegnaro, M., Hunt D. C., Snasone E. B., Schuller, P. L., Siriwardana, M. G., Telling, H. P., Egmond V. H. P., and Walker E. A., 1980. Laboratory decontamination and destruction of aflatoxins B1, B2, G1, G2, publication 37, IARC:Lyon.

3. Castegnaro, M., Eisenbrand, G., Ellen G., Keefer, L., Klein, D., Sansone, E. B., Spincer, D., Telling G. and Webb, K., 1982. Laboratory decontamination of carcinogens in laboratory wastes: Some N-Nitrosamines, publication 43, IARC:Lyon.

4. OSU Chemical Safety Committee, 1986. Chemical Carcinogen Safety Manual, Oregon State University, Corvallis, Oregon.

4. Castegnaro, M., Bernard, M., Broekhoven, V. L. W., Fine, D., Massey, R., Sansone, E. B., Smith, P. L. R., Spielgelhalder, B., Stacchini, A., Telling, G., Vallon J. J., 1983. Laboratory

decontamination and destruction of carcinogens in laboratory wastes: Some N-Nitrosamides, publication 55, IARC:Lyon.