AN ABSTRACT OF THE THESIS OF

Jack D. Kelly for the degree of Doctor of Philosophy in

Toxicology presented on   February 14, 1992

Title: Oxidative Stress and Carcinogenesis in the Trout

Redacted for Privacy

Abstract approved:

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The inhibitory effect of antioxidants in the multi-stage chemical carcinogenesis model indicates that free radicals are involved during neoplastic development. In chemical carcinogenesis initiation, the first event that causes mutational change, occurs by electrophile adduction to DNA. Many electrophiles are produced by cytochrome P450; however, a free-radical mediated pathway termed co-oxidation, has been shown to produce reactive electrophiles *in vitro*, but its significance *in vivo* remains to be elucidated. The promotion stage of carcinogenesis is often inhibited by antioxidants. The modified nucleoside 8-hydroxydeoxyguanosine (oh^{8}dG) is thought to be involved in promotion. The oh^{8}dG adduct is mutagenic *in vitro* and present in neoplastic tissue and presumed to activate cancer-causing genes. Metabolic activation via epoxidation and initiation with a polycyclic aromatic hydrocarbon derivative (7S-trans-7,8-dihydro-benzo[a]pyrene-7,8-diol (BP-7,8-DHD)) by cytochrome P450 and co-oxidation were investigated in the trout model using racemic

and pure BP-7,8-DHD enantiomers. Embryos and sac-fry were able to generate the ultimate carcinogen (BP-7,8-DHD-9,10-epoxide) under conditions characteristic of each pathway as assessed by DNA binding and tumor studies. In addition, the hepatocarcinogen (-)BP-7,8-DHD enantiomer is a more potent than (+)BP7,8-DHD. MNNG-initiated trout had significantly higher tumor incidences after chronic exposure to dietary hydrogen peroxide. The increase in tumor incidence correlated with increased hepatic levels of oh^{8}dG adducts. Since the presence of unrepaired adducts would increase the mutational activity, the repair rate of oh^{8}dG was examined. Dietary exposure to two free-radical generating compounds for 10 weeks did not result in higher oh^{8}dG levels in liver DNA. Intraperitoneal injection of hydrogen peroxide did not increase oh^{8}dG adduct levels. Longer exposure to prooxidants and higher doses for intraperitoneal experiments may be required to better assess oh^{8}dG repair rates.

OXIDATIVE STRESS AND CARCINOGENESIS IN TROUT

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirement for the
degree of

Doctor of Philosophy

Completed February 14, 1992

Commencement June 1992

APPROVED:

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Date of thesis presented _____ February 14, 1992

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ACKNOWLEDGEMENTS

Give thanks to the Lord, for he is good. His love endures forever. Psalm 136:1.

The fear of the Lord is the beginning of wisdom; all who follow his precepts have good understanding. To him belongs eternal praise. Psalm 111:10

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Mr. Mike Dutchuck and Dr. Hillary Carpenter: radiolabeled carcinogen exposure and DNA binding.

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OXIDATIVE STRESS AND CARCINOGENESIS IN THE TROUT

Chapter one

INTRODUCTION

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INTRODUCTION

Oxidative stress is characterized by an increase in the steady-state concentration of different reactive oxygen species (ROS), superoxide anion radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical ($HO^{\cdot}$) [1]. The hydroxyl radical is the most electrophilic of the ROS that cells are exposed to [2], reacting with cellular proteins, DNA and polyunsaturated fatty acids (PUFAs).

There is evidence that ROS are involved in chemical carcinogenesis [3-5] as antioxidants reduce neoplasm development . In addition, epidemiology data indicates that low plasma antioxidant levels in humans are associated with a higher risk of cancer mortality [6]. Chemical carcinogenesis is a multi-stage process that can be divided into four sequential stages: initiation, promotion, conversion and progression [7]. Initiation is an irreversible damage to DNA caused by highly reactive molecules such as electrophiles or free-radicals that leads to mutations of genes important in cell division and cell growth such as protooncogenes or supressor genes.

Initiation can be caused by direct attack of the parent chemical (direct carcinogen) or a reactive metabolite (indirect carcinogens). Direct carcinogens are genotoxic and require no further bioactivation by the cell. Indirect carcinogens or procarcinogens must be bioactivated by the cell

to be genotoxic; predominantly via cytochrome P450 monooxygenases (8).

In addition to cytochromes P450, another pathway referred to as co-oxidation (peroxidative pathways) has the potential to bioactivate procarcinogens such as polycyclic aromatic amines (PAHs) , nitrosamines and aromatic amines. Co-oxidation is the oxidation of a chemical by peroxyl radicals generated either non-enzymatically during lipid peroxidation, or by various peroxidases such as prostaglandin synthase (PHS) or lipoxygenases (9-11, and Figure 1.1).

Lipid peroxidation is non-enzymatic oxidation of PUFAs caused by oxidative stress. When the hydroxyl radical abstracts a hydrogen from a PUFA, the PUFA becomes a radical that reacts with diradical molecular oxygen, generating peroxyl radicals capable of epoxidizing carcinogenic PAH derivatives (12).

PHS (EC 1.14.99.1) is a bifuctional enzyme composed of two regions that catalyze two subsequent processes (cyclooxygenase and hydroperoxidase). Both reactions require heme as a prosthetic group for their activity. Initially, the cyclooxygenase of PHS uses arachidonic acid (AA) to form a hydroperoxide (PGG_2) that becomes a substrate for its hydroperoxidase action ultimately producing precursors (PGH_2) for prostaglandins. As PGG_2 interacts with the hydroperoxidase, a peroxyl radical is generated.

PHS may contribute significantly to initiation of

procarcinogens *in vivo* in extrahepatic tissues (lung, kidney, colon, prostate, stomach, bladder, and skin) where P450 specific content and activity is low (10). There is limited *in vivo* data showing conclusively that PHS is a significant pathway in initiation of carcinogenesis. However, the presence of tumors in extrahepatic tissues and their inhibition by aspirin (an inhibitor of cyclooxygenase) suggests that prostaglandin synthase may be responsible for initiation of procarcinogens (13).

Lipoxygenases are also bifunctional non-heme- containing enzymes that oxidize AA to form hydroperoxy fatty acids that serve as substrates for its hydroperoxidase to generate precursors of leukotrienes. Similar to PHS, the hydroperoxidase of lipoxygenase is capable of bioactivating procarcinogens (14), yet its significance *in vivo* as a potential pathway remains unknown.

The rainbow trout has been used as an animal model for chemical carcinogenesis for a variety of chemicals (15); however, the contribution of procarcinogens co-oxidation has not been assessed. Preliminary data in our lab suggested that co-oxidation may be an important pathway in trout. Therefore, one of our aims was to compare the bioactivating potential by co-oxidation (lipid peroxidation and PHS-dependent) with that of cytochromes P450.

Tumor promotion involves expansion of clonal cells that mutated during initiation. The mechanisms for clonal expansion

are not known. Because the promotion stage can be inhibited by antioxidants such as vitamins E and A, ROS may be involved in promoting cell proliferation of mutated cells.

Promoters typically generate ROS either directly or indirectly (16). Direct acting ROS promoters generate additional free radicals such as alkoxyl radicals. An example of this type of promoter is benzoyl peroxide, which has been shown to be a good promoter in the mouse skin carcinogenesis model (17,18). Indirect free-radical promoters such as the phorbol esters, cause an increase of endogenous sources of H_2O_2 that, in the presence of a transition metal (Fe^{+2}/Cu), give rise to hydroxyl radical (HO·) within the cell. When the hydroxyl radical attacks DNA, it causes a number of lesions including strand breaks and base modifications.

8-Hydroxy-2'-deoxyguanosine (oh[8]dG) is one of many modified bases produced by hydroxyl radical or singlet oxygen attack on DNA (19,20). oh[8]dG is mutagenic *in vitro* (21,22) and human breast cancer patients have significantly higher levels in mammary tumor DNA than in surrounding normal tissue (23). Exposure to initiators or promoters in other carcinogenic protocols also cause high levels of this adduct (24); however, its role in carcinogenesis remains to be determined.

Fish may be a potential model for the role of oh[8]dG and promotion during oxidative stress. Recently, Malins *et al.*, (25,26) found a significant increase in oh[8]dG content in DNA of neoplastic liver tissue of English sole. Therefore, studies

were designed to determine if the rainbow trout may be a good *in vivo* model for the relationship between oh^{8}dG, promotion and oxidative stress in carcinogenesis.

Figure 1.1 Epoxidation of (+) BP-7,8-DHD via P450 1A1 and

Cooxidation
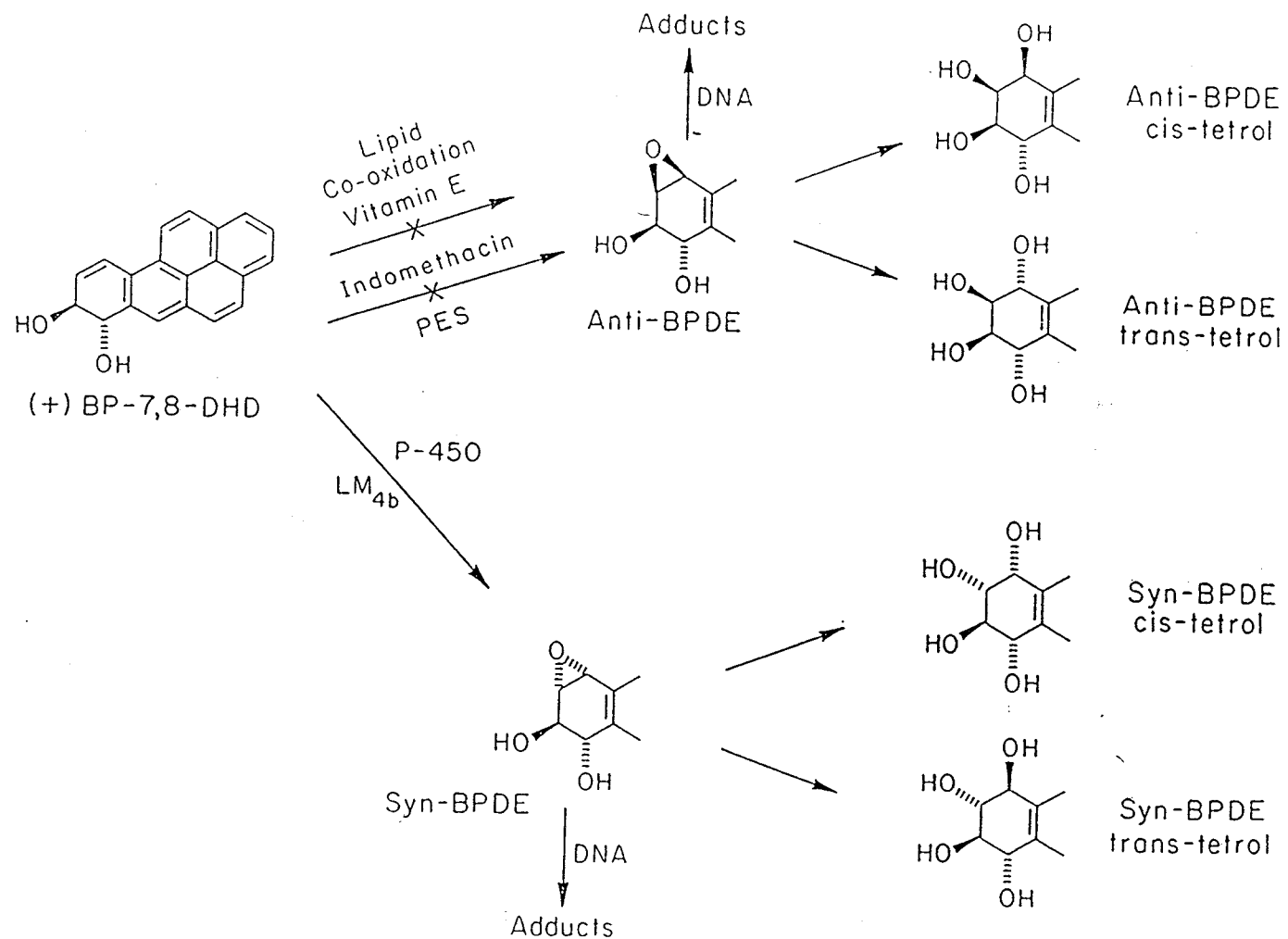
Figure 1.1

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Chapter two

COVALENT BINDING OF [^{14}C]-AND [^{3}H]-7S-TRANS-7,8-DIHYDRO-BENZO[A]PYRENE-7,8-DIOL AND [^{3}H]-7S-TRANS-7,8-DIHYDRO-BENZO[A]PYRENE-ANTI-9,10-EPOXIDE TO DNA OF TROUT EMBRYOS AND SAC-FRY

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COVALENT BINDING OF [14C]-AND [3H]-7S-TRANS-7,8-DIHYDRO-
BENZO[A]PYRENE-7,8-DIOL AND [3H]-7S-TRANS-7,8-DIHYDRO-
BENZO[A]PYRENE-ANTI-9,10-EPOXIDE TO DNA OF TROUT
EMBRYOS AND SAC-FRY

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ABSTRACT

Rainbow trout embryos, microinjected with 0.01-1.0 μg of [^{3}H]-(+)-7S-trans-7,8-dihydrobenzo[a]pyrene-7,8-diol-anti-9, 10-epoxide (anti-BPDE), exhibited a dose-dependent increase in DNA adduction. Subsequently, microinjection or bath exposure of trout embryos to [^{14}C](+)-7S-trans-7,8-dihydrobenzo[a] pyrene-7,8-diol ((+)-BP-7,8- DHD) also demonstrated a dose-dependent increase in DNA adduction, with the specific activity per μg injected almost the same as with anti-BPDE, suggesting that trout embryos are very efficient at bioactivation of (+)-BP-7,8-DHD. In order to determine the relative contribution of cytochrome P450-dependent versus peroxidase-dependent epoxidation of (+)-BP-7,8-DHD, trout embryos were co-injected with [^{14}C]-(+)-BP-7,8-DHD and either β- naphthoflavone (BNF) (to increase cytochrome P450-dependent epoxidation) or carbon tetrachloride (CCl_4,) (to enhance lipid peroxidation-dependent co-oxidation). Co-injection with BNF appeared to enhance covalent binding to DNA, but the difference was not statistically significant, due to high variability. An increase in binding with co-administration of BNF would be consistent with rapid induction of P450 1A1, as confirmed by western blotting. Co-injection with CCl_4, significantly increased covalent binding of [^{14}C]-(+)-BP-7,8-DHD to DNA, suggesting a contribution from radical-dependent pathways. Concentration- dependent increases in covalent binding of ^{3}H-(+)-BP-7,8-DHD to DNA of embryos exposed by

immersion was also observed, but the level of adduction was at least an order of magnitude lower than administration by injection. Experiments were also performed with newly hatched sac-fry. Injections of [^{3}H]-(+)-BP-7,8-DHD produced a dose-dependent increase in whole body DNA adduction. Co-injection of BNF or CCl$_4$, (10 μg) and ^{3}H-(+)-BP-7,8-DHD (1 μg) to sac-fry, significantly lowered DNA adduction compared to sac-fry injected with ^{3}H-(+)-BP-7,8-DHD alone. In contrast to the results with co-injections, sac-fry which had been pretreated as embryos with BNF, exhibited markedly higher levels of DNA adduction than embryos pretreated with vehicle, hydrogen peroxide or indomethacin. The enhanced binding in the BNF-treated group again correlated to enhanced levels of P450 1A1. These results confirm that trout embryos are capable of metabolically activating BP-DHD to the ultimate carcinogenic BPDE and that this bioactivation is at least partially dependent upon cytochrome P450 1A1.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHS) are by-products of combustion and human exposures occur through many routes, including air, water and diet (1) . In mammals, PAHs have been used as model carcinogens, primarily focusing on skin, lung and mammary tumors (2). Benzo[a]pyrene (B[a]P) is one of the most intensively studied PAHs and a great deal is known concerning metabolic routes of bioactivation, detoxication and DNA adduction (3). The predominant pathway for activation involves initial epoxidation by cytochrome P450 (predominantly by P450 1A1) at the 7,8-position followed by hydrolysis to yield the trans-BP-7,8-DHD. The BP-7,8-DHD is then epoxidized again to yield the ultimate carcinogenic metabolite, BPDE (3). This second epoxidation can be catalyzed by cytochromes P450 or by co-oxidation by peroxidases such as prostaglandin synthase or non-enzymatically during propagation of lipid peroxidation (4-7). Recent evidence suggests that these non-P450 peroxidative pathways may be important in bioactivation of PAHs, especially in nonhepatic target tissues where P450 contents are low, but peroxidase activity is high. Evidence in support of this comes from studies which exploit the stereochemical difference in epoxidation of (+)-BP-7,8-DHD by monooxygenases versus peroxidases (8,9). Epoxidation by P450 of (+)-BP-7,8-DHD yields predominantly the syn-BPDE, whereas, peroxidative pathways yield anti-BPDE.

Fish have been demonstrated to be responsive to tumor

initiation with B[a]P in the laboratory (10,11) and circum-stantial evidence has been presented linking high incidences of cancers in feral fish populations with environmental contamination by high levels of PAHs, predominantly in sediment (12).

The rainbow trout model offers a number of advantages as a cancer model, including low husbandry costs, low background tumor incidence and responsiveness to many mammalian carcinogens (13). Another advantage, which we attempt to exploit in this study, involves the sensitivity of trout embryos and sac-fry to carcinogen exposure either by bath immersion or microinjection (14-16). This sensitivity allows for conducting tumor studies with large numbers of animals and very rare, or expensive carcinogens. A goal of this laboratory was to utilize the trout to obtain quantitative estimates of the relative role of monooxygenases versus peroxidases in the metabolic activation of PAHs, using the pure (+)-BP-7,8-DHD enantiomer as an *in vitro* and *in vivo* probe.

MATERIALS AND METHODS

Chemicals:

[7-^{14}C]- and [1,3-^{3}H]-(+)-trans-7,8-dihydrobenzo[a]
pyrene-7,8- diol ((+)-BP-7,8-DHD) and (-)-7S-r-7,t-8-dihy-
droxy-t-9,10-epoxy- 7,8,9,10-tetrahydro-[1,3-^{3}H]-benzo[a]
pyrene (anti-BPDE) were obtained from Chemsyn Sci. Labs. (NCI
Radiochemical Carcinogen Repository, Lenexa, KS). Proteinase
K, RNase A and RNase T1 were purchased from Sigma Chem. Co.
(St. Louis, MO). Tris-saturated phenol was purchased from
AMRESCO (Solon, OH). The reagents used for sodium dodecyl
sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were
from Bio-Rad (Richmond, CA). ^{125}I-Protein A was purchased from
ICN Radiochemicals (Irvine, CA). The isolation of trout
cytochrome P450 1A1 and production of polyclonal antibodies in
rabbit has been previously described (17).

Animals and Treatment:

Rainbow trout embryos (23 days post-fertilization) were
micro- injected with 1 μl of either vehicle (DMSO:ethanol
(1:2)) or radiolabelled chemical at doses of 0.01-1.0 μg.
Newly hatched sac-fry were injected utilizing the same
procedure (injections were into the yolk sac). Seventy-two or
96 hours post-treatment, the embryos or sac-fry were frozen in
liquid nitrogen and stored at -90° C for 1-2 weeks. The
embryos were isolated from the yolk, homogenized in 0.1 M
NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0 and the total DNA
extracted as described previously (18). The specific activity

of the DNA was determined as pmol radiolabel bound (liquid scintillation) per mg of DNA (assayed by the Burton method (19)).

The yolk-sac was removed from the sac-fry and DNA isolated from the whole body using the same procedure as with the embryos. In studies utilizing co-injection of embryos or sac-fry, BNF or CCl_4, was dissolved along with the radiolabelled B[a]P-metabolite and administered in the same injection. In some experiments, embryos were pretreated with BNF, hydrogen peroxide or indomethacin and the radiolabelled B[a]P-metabolite was administered 1-2 weeks later to the sac-fry as described above. In studies to examine levels of P450 1A1, trout embryos were treated with BNF only.

Immunoquantitation:

Embryos injected with BNF or sac-fry which had been pretreated as embryos with BNF were frozen in liquid nitrogen and stored at -90°C until assayed. The trout were homogenized (sac-fry first had the yolk-sac removed) in buffer as described above for embryos and protein determined by the method of Lowry et al., (20), utilizing bovine serum albumin (Sigma) as standard. The embryo and sac-fry homogenates were loaded onto SDS-PAGE mini-slab gels (21) and the resolved proteins electrophoretically transferred to nitrocellulose (22) using equipment purchased from Idea Sci. (Corvallis, OR). The detection and quantitation of trout P450 1A1 was performed as previously described (23,24).

RESULTS

Microinjection of the ^{3}H-labelled anti-BPDE derived from the (+)-enantiomer of BP-7,8-DHD, resulted in a dose - dependent increase in DNA adduction 72 hr post-treatment (Fig. 2.1). The specific activity of whole embryo DNA at the 1 μg dose was approximately 300 pmol/mg DNA. The anti-BPDE is apparently stable enough under these conditions to reach the target DNA within cells of the embryo. The half-life of BPDE in aqueous solutions is approximately 4 minutes at 22°C and 1.5 minutes at 37°C (25). The stability is markedly enhanced in the presence of protein and/or lipid. The embryo injections were done at 12°C.

The anti-BPDE should be capable of binding directly to DNA, without the need for bioactivation. In order to test if the embryo was capable of epoxidation of (+)-BP-7,8-DHD, radiolabelled compound was administered to embryos under the same dose regimen as the anti-BPDE. Again, a dose-dependent increase in whole embryo DNA adduction was observed (Fig. 2.2) and the specific activity per μg injected was comparable to anti-BPDE, suggesting that trout embryos are very efficient at bioactivation of (+)-BP-7,8-DHD, presumably through epoxidation at the 9,10-position. Co-injection of embryos with 1 μg ^{14}C-(+)-BP-7,8-DHD and 10 μg BNF appeared to enhance covalent binding, but the results were not significant due to the high variability (Fig. 2.2). Co-injection of ^{14}C-(+)-BP-7,8-DHD and 10 μg CCl$_4$, demonstrated significantly

enhanced covalent binding (Fig.2.2). Enhancement of DNA binding at 72 hr after co-treatment with BNF would be consistent with the elevation in cytochrome P450 1A1 levels, as demonstrated by immunoquantitation (Fig. 2.3). The enhancement obtained with CCl_4, is presumed to be due to enhanced lipid peroxidation-dependent co-oxidation of (+)-BP- 7,8-DHD.

Immersion is also a route of exposure for carcinogen administration to trout embryos. Exposure of embryos to 1 or 10 ppm ^{3}H-(+)-BP-7,8-DHD for 30 min, resulted in covalent binding to DNA after 72 hr of about 4 and 16 pmol/ mg DNA, respectively (Fig. 2.4). The specific activity achieved under these conditions is greater than 10-fold lower than by microinjection. Co-immersion with 70 ppm BNF or CCl_4 did not significantly effect the level of binding to DNA. Therefore, immersion was not as effective in producing DNA adducts with (+)-BP-7,8-DHD as direct injection, probably due to solubility limitations.

Although injections of radiolabelled (+)-BP-7,8-DHD to embryos resulted in high levels of DNA covalent binding, which could be modulated by induction of monooxygenase or peroxidase systems, we observed significant (>50% at the higher doses) mortalities. Previously, Hendricks et al., (10) observed similar high mortality rates in embryos injected with B[a]P. These high mortality rates would not be suitable for tumor studies, so we initiated studies with sac-fry to determine their response and mortality rates.

Injection of ^{3}H-(+)-BP-7,8-DHD into the yolk-sac of sac-fry resulted in a dose-dependent increase in DNA adduction after 72 hr (Fig. 2.5). Co-injection of 1 μg ^{3}H-(+)-BP-7,8-DHD with 10 μg BNF or CCl$_4$, (Fig. 5) resulted in significant reductions in DNA binding, just the opposite of the co-injection studies with embryos (Fig.2.2). In a separate experiment, when sac-fry, which had been pretreated as embryos with either vehicle or 1 μg of hydrogen peroxide, indomethacin or BNF, were injected with ^{3}H-(+)-BP-7,8- DHD, only the embryos injected with BNF displayed significantly higher levels of DNA adduction (Fig. 2.6). Again, the increased DNA binding correlated with enhanced levels of P450 1A1 (Fig. 2.7).

DISCUSSION

Rainbow trout have previously been shown to be responsive to hepatocarcinogenesis induction by PAHs (10). Indirect evidence has linked high incidences of hepatocellular carcinomas to environmental PAHs in feral fish populations (12). B[a]P is hepatocarcinogenic in trout, but only with prolonged dietary exposure at high levels or by multiple i.p. injections of older trout (10). Microinjection of embryos resulted in unacceptably high mortalities. In this study, we examined the potential of (+)- BP-7,8-DHD to covalently bind to DNA after administration to trout embryos or sac-fry. The ultimate aim of this work is to use this PAH as an *in vivo* probe of the relative contribution of P450- dependent monooxygenation to peroxidation-dependent co-oxidation of procarcinogens.

Microinjection of trout embryos with 0.01-1.0 μg of the anti- BPDE derived from (+)-BP-7,8-DHD produced a dose - dependent increase in covalent binding to DNA. The ability of embryos to carry out the activation of (+)-BP-7,8-DHD *in vivo* was demonstrated by microinjections of 0.01- 1.0 μg of the pure enantiomer. The potency of the precursor ((+)-BP-7,8-DHD) was almost as great as the anti-BPDE, suggesting efficient bioactivation by these embryos due to either little detoxification by conjugating enzymes or high expression of P450 1A1 at the embryo stage. A possible role for both P450 1A1 and lipid peroxidation-dependent co-oxidation was obtained

in experiments in which either BNF or CCl$_4$,, respectively was co-administered with (+)-BP-7,8-DHD.

Enhanced levels of P450 1A1 in trout embryos injected with BNF was confirmed by immunoquantitation. Dose-dependent increases in covalent adduction of embryo DNA was also observed when embryos were exposed to ^{3}H- (+)-BP-7,8-DHD by immersion, however, the degree of DNA binding was much lower.

As is the case with B[a]P, embryo injections resulted in mortality rates too high for future tumor studies, so we examined the responsiveness of newly hatched sac-fry to injection with ^{3}H- (+)-BP-7,8-DHD. Administration of 0.1-1.0 μg produced a dose- dependent increase in covalent DNA binding. Interestingly, unlike the case with embryos, co-injection with BNF or CCl$_4$, resulted in markedly reduced DNA adduction. Perhaps in the sac-fry, co- administration of BNF or CCl$_4$, (each at 10 μg) alters the pharmacokinetics of ^{3}H-(+)-BP-7,8-DHD or directly inhibits P450- dependent bioactivation of (+)-BP-7,8-DHD or production of CCl$_3$·, respectively. Pretreatment of embryos with BNF at one-tenth the dose produced a significant increase in DNA adduction in sac-fry injected with 0.5 μg ^{3}H-(+)-BP-7,8-DHD; conversely, hydrogen peroxide or indomethacin pretreatment of embryos had no effect. The increased binding observed in sac-fry which had been pretreated as embryos with BNF, again correlated with induction of P450 1A1 levels, observed by western blotting.

In summary, rainbow trout embryos and sac-fry have been

demonstrated to effectively catalyze the bioactivation of (+)-BP-7,8-DHD to DNA binding metabolites, presumably BPDE. The epoxidation may be catalyzed by both P450-dependent and co- oxidation-dependent pathways, as demonstrated by the increases observed upon co-administration of BNF or CCl_4. Future studies will attempt to analyze the ratio of syn- to anti- BPDE derived from (+)-BP-DHD in order to ascertain the relative contribution of each pathway. In addition, studies on the tumorigenicity of (+)- BP-7,8-DHD and its modulation by BNF and CCl_4, are currently underway.

Figure 2.1. Covalent binding of ^{3}H-anti-BPDE to embryo DNA
after microinjection. Three pools of 20 embryos
(23 days post- fertilization) were microinjected
with 0.01, 0.1, 0.5 or 1.0 μg of ^{3}H-anti-BPDE in
1 μl of solvent (DMSO/ethanol, 1:2). After 72 hr,
the embryos were frozen in liquid nitrogen and
stored at -90°C until the DNA could be purified
and the specific activity determined as described
in Materials and Methods. The results shown are
the mean of duplicate assays of 3 pools of 20
embryos; the bars depict the standard error of the
mean.
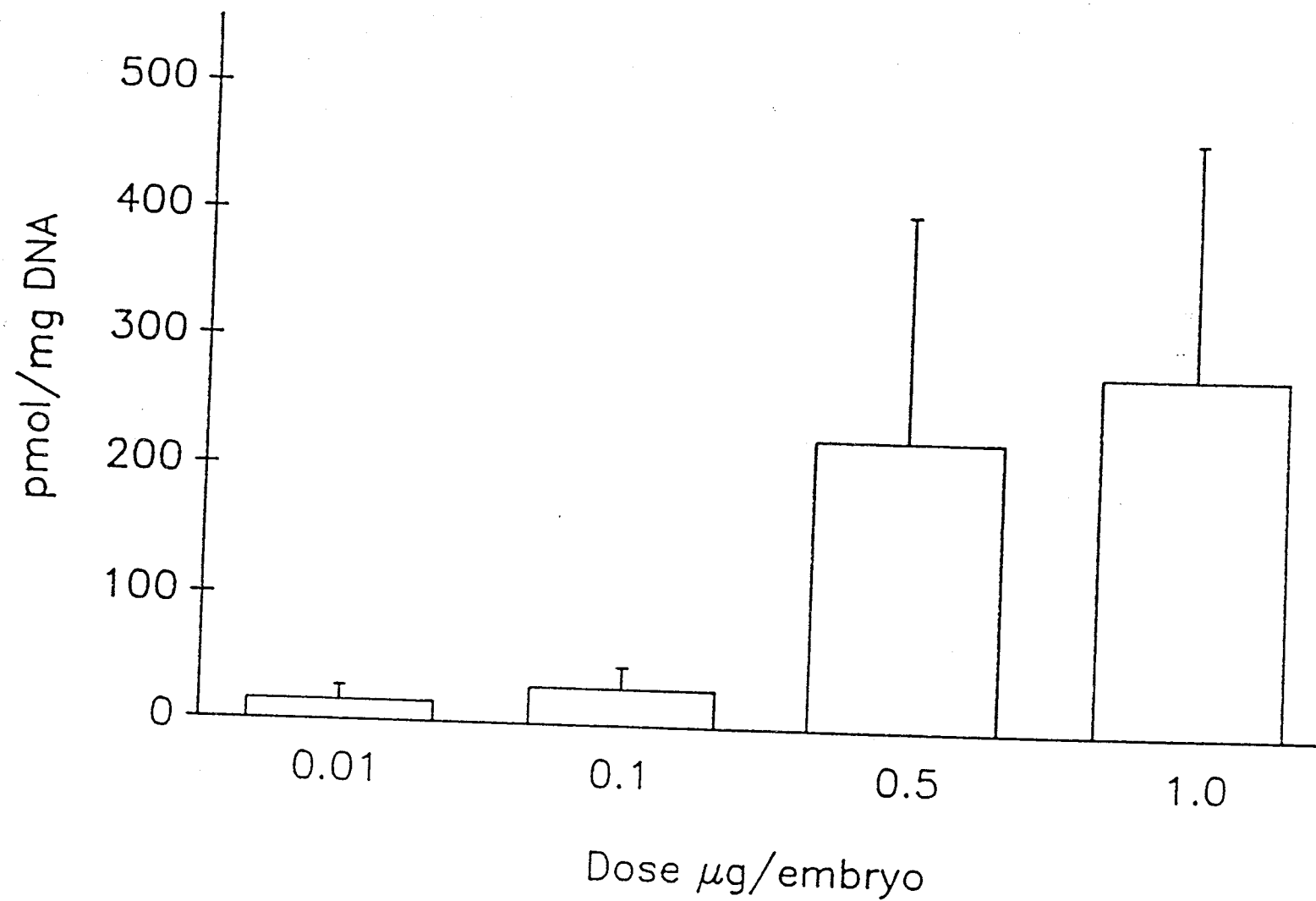
Figure 2.1

Figure 2.2. Covalent binding of ^{14}C-(+)-BP-7,8-DHD to embryo
DNA following microinjection. Embryo
microinjections were performed as described for
Figure 1. Co-injections at the highest dose of 1
μg were done with either BNF or CCl$_4$, (10 μg). The
bars indicate the mean of duplicate samples of 3
pools of 20 embryos. The * indicates significantly
different from controls (1 μg of ^{14}C-(+)-BP-
7,8-DHD alone) at p < 0.01 (2-way ANOVA).
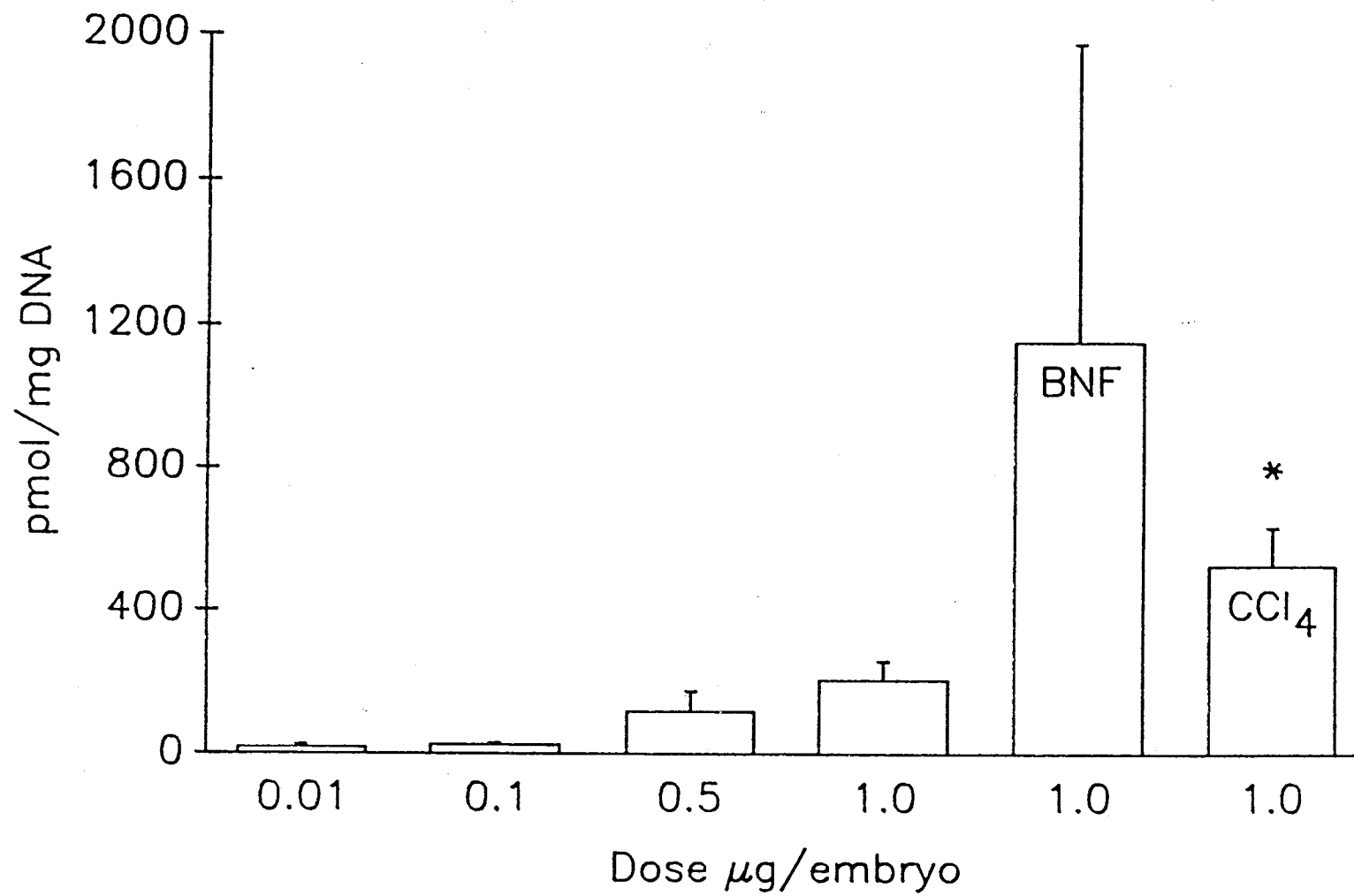
Figure 2.2.

Figure 2.3. Immunoquantitation of P450 1A1 in trout embryos.
Trout embryos were microinjected with 0.1 μg of
BNF and the levels of P450 1A1 determined in whole
homogenates (after yolk removal) 1, 3, and 10 days
post-injection. Twenty-five μg of homogenate
protein was loaded onto an SDS-PAGE gel and the
proteins resolved and blotted onto nitrocellulose
as described in Materials and Methods.
Autoradiograms of western blots were scanned with
a laser densitometer and the results expressed as
pmol/mg protein using the purified trout P450 1A1
as the standard.
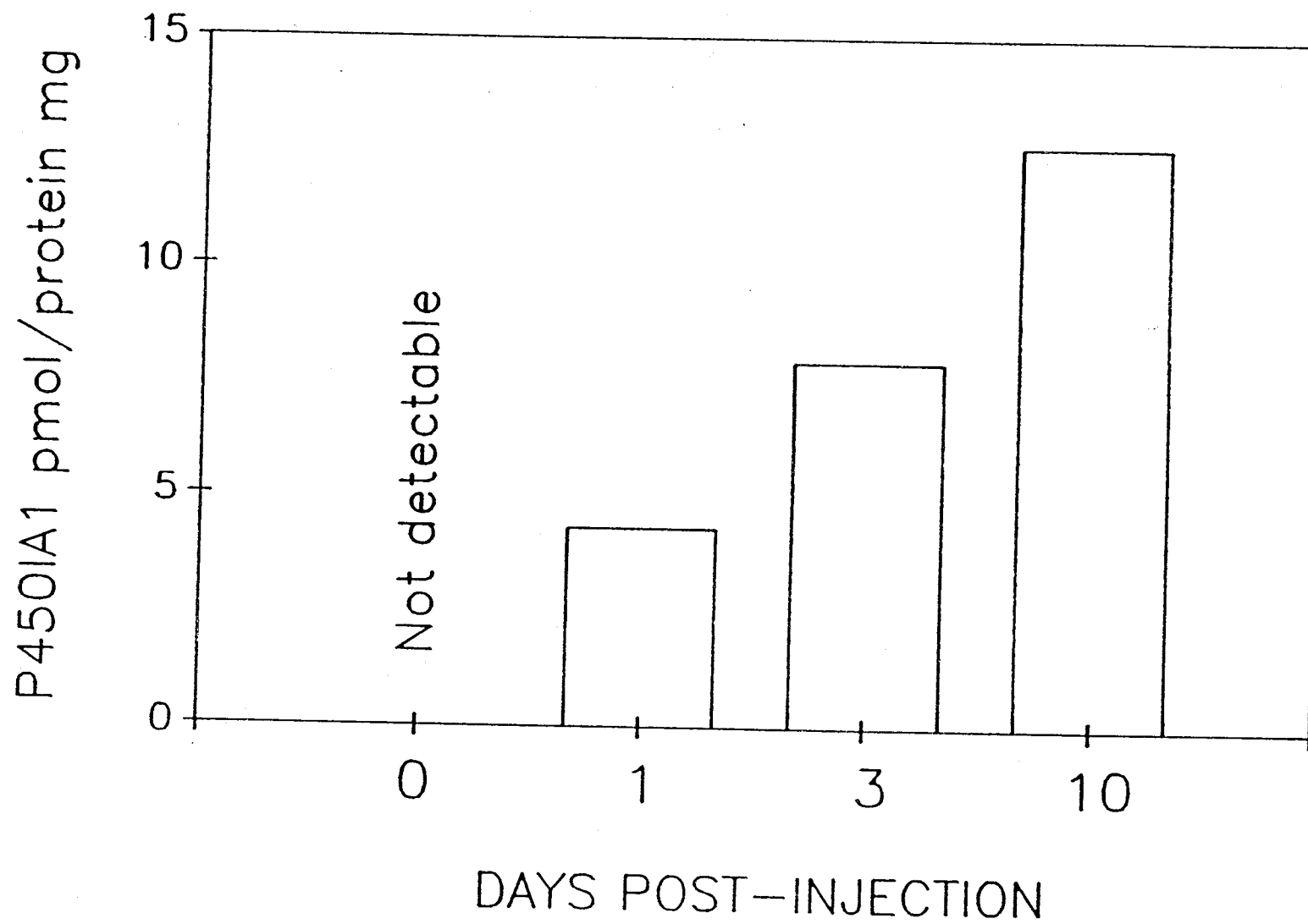
Figure 2.3.

Figure 2.4. Covalent binding of ^{3}H-(+)-BP-7,8-DHD to embryo

DNA after immersion exposure. Trout embryos were

exposed for 30 min to 1 or 10 ppm ^{3}H-(+)-BP-7,8-

DHD in water (12°C). After 72 hr in clean water,

the embryos were frozen in liquid nitrogen and the

specific activity of the DNA determined as

described for Figure 1. Co-immersion with BNF or

CCl_4, was done at levels of 70 ppm. The results

are the mean (bars are S.E.) of duplicate assays

from 3 pools of 10 embryos.
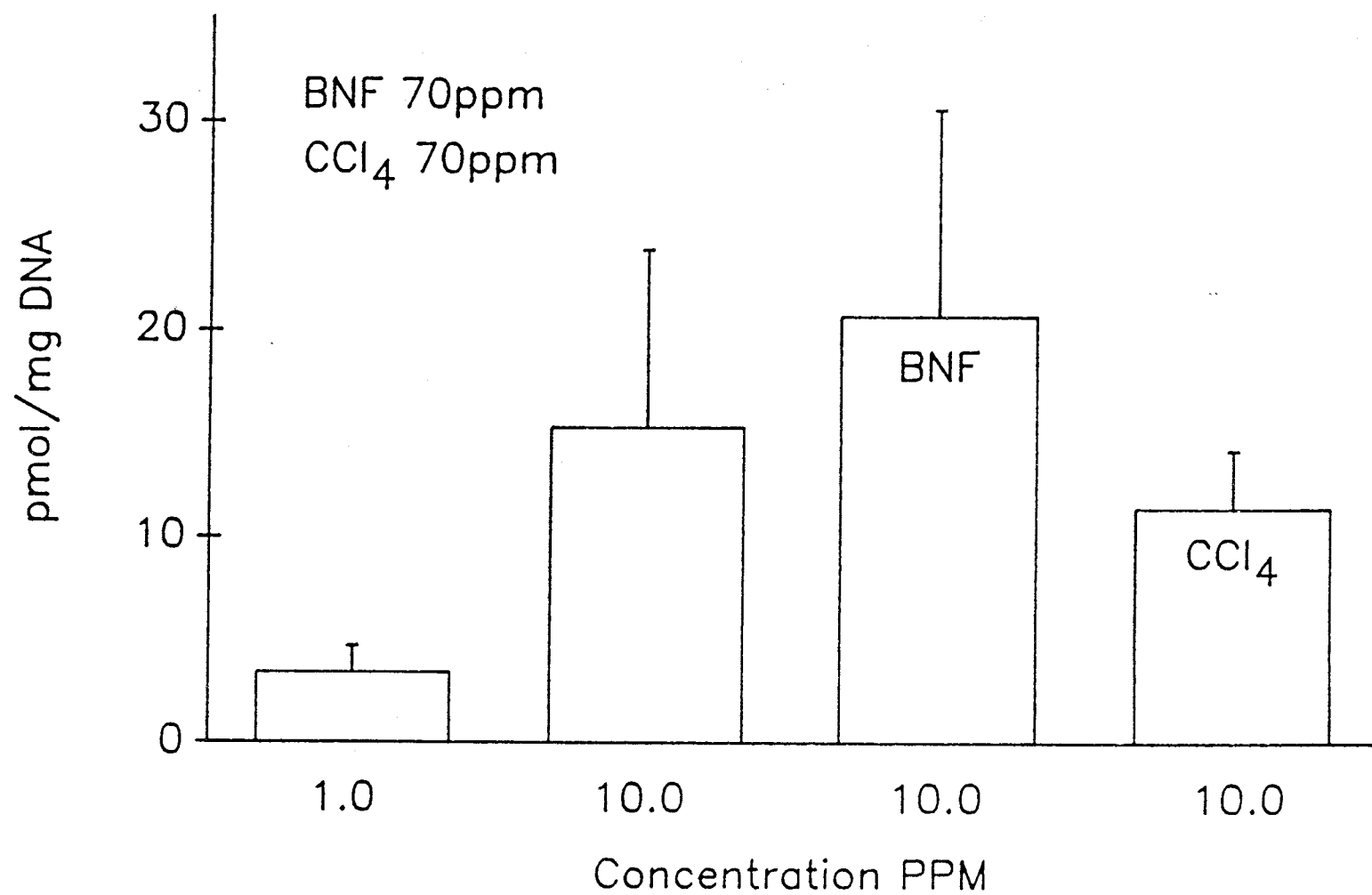
Figure 2.4.

Figure 2.5. Covalent binding of ^{3}H-(+)-BP-7,8-DHD to DNA after microinjection of sac-fry. Trout sac-fry were microinjected with 0.1, 0.5 or 1.0 μg of ^{3}H-(+)-BP-7,8-DHD and the DNA isolated 72 hr later as described in Figure,1. Co-injections with BNF or CCl$_4$, were performed with 10 μg of compound. The results represent the mean + SE (error bars) from duplicate samples of 3 pools of 3 sac- fry each. The * indicates significantly different from controls (1 μg of ^{3}H-BP-7,8-DHD alone) at $p < 0.01$ (2-way ANOVA).
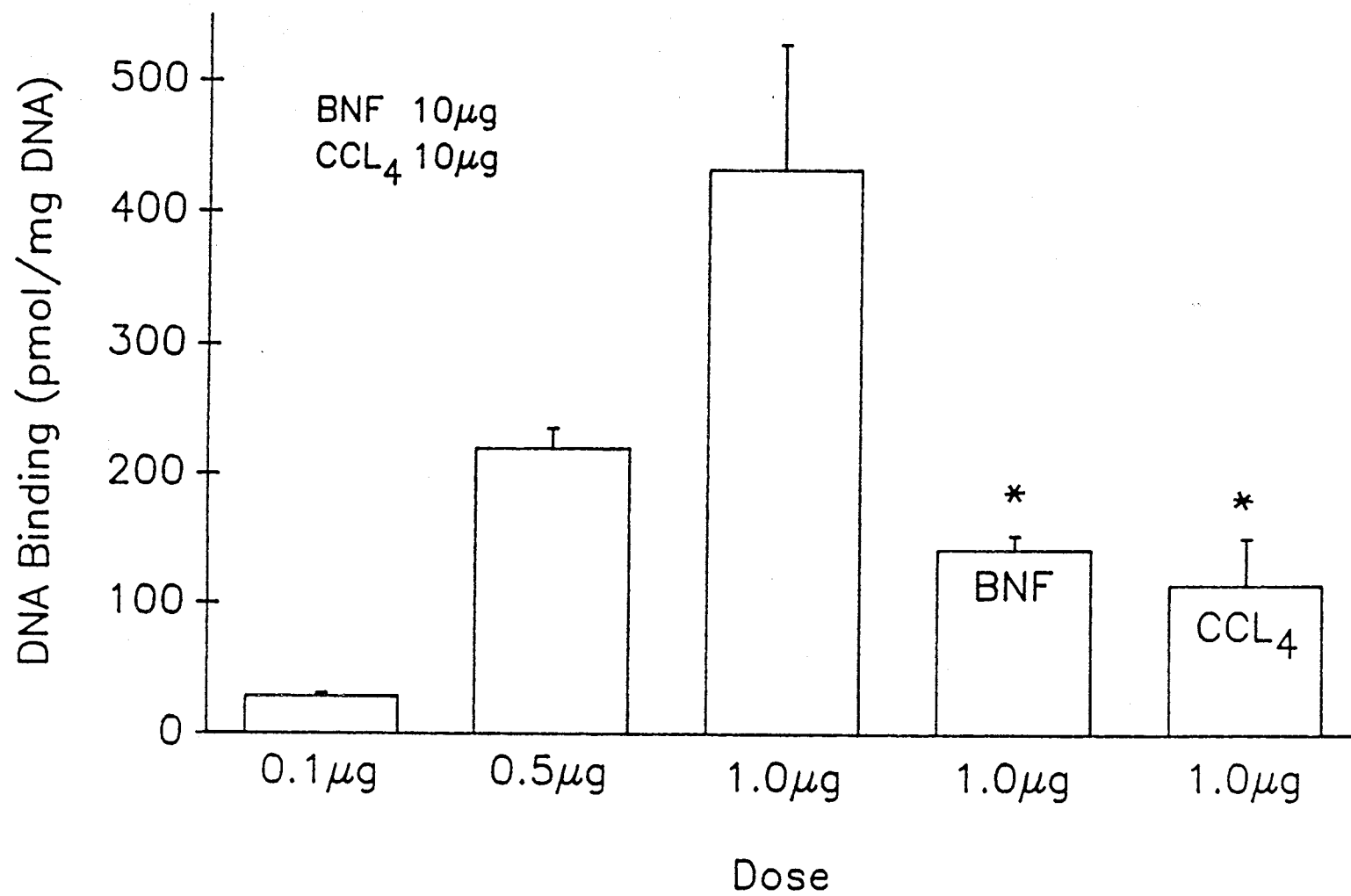
Figure 2.5.

Figure 2.6. Covalent binding of ^{3}H-(+)-BP-7,8-DHD to DNA
after microinjection of sac-fry which had been
pretreated as embryos. Embryos (4 pools of 10
each) were microinjected with either vehicle, or
1 μg of H_2O_2, indomethacin or BNF and then injected
again as sac-fry with 0.5 μg of ^{3}H-(+)-BP-7,8-DHD.
After 96 hr, the sac-fry DNA was isolated and the
specific activity determined as described in
Figure 2.1. The * indicates significantly
different from controls at $p < 0.01$ (2-way ANOVA).
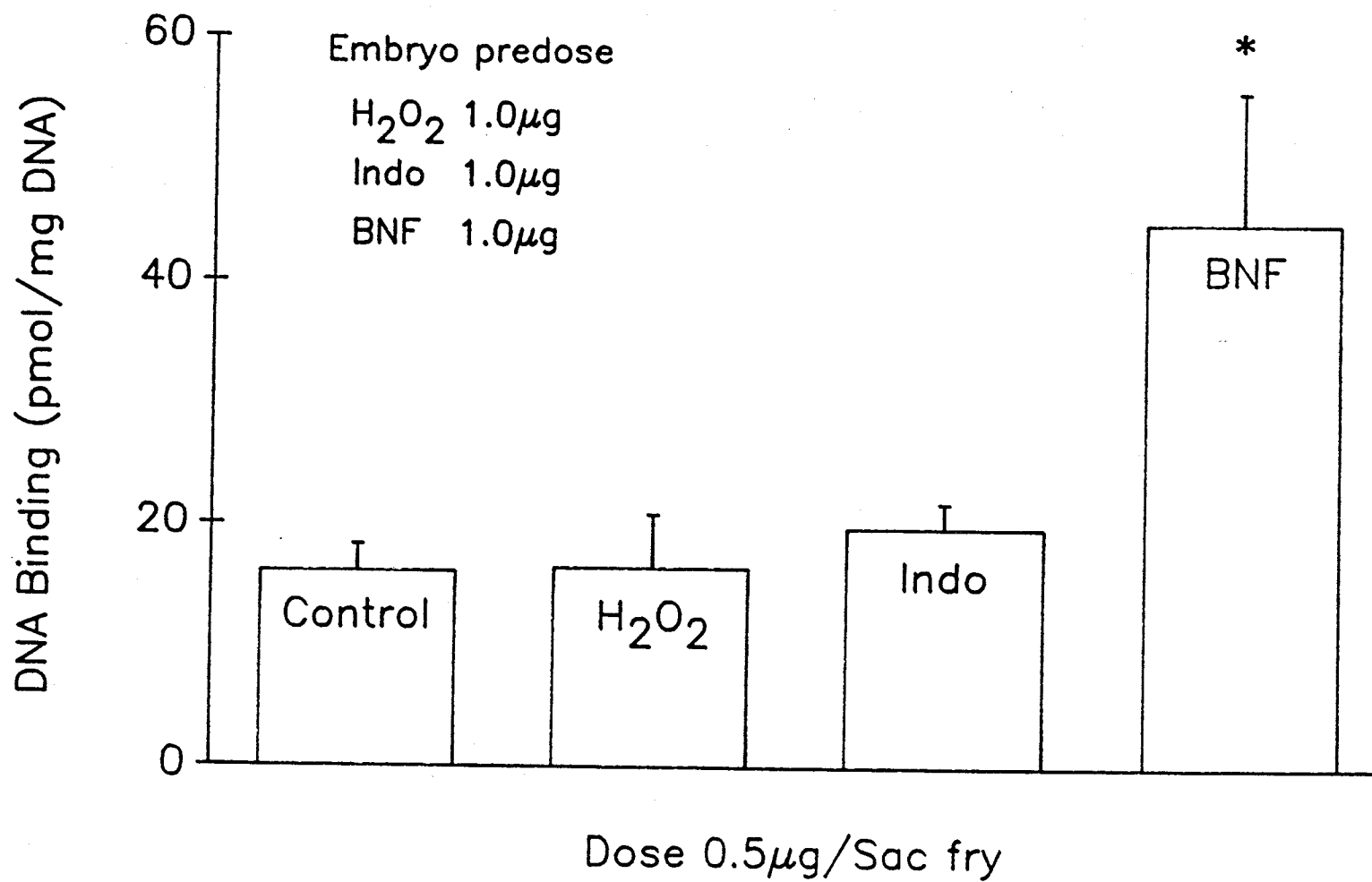
Figure 2.6.

Figure 2.7. Western Blot of P450 1A1 in trout sac-fry

microinjected as embryos with BNF. Three pools of

10 embryos each were microinjected with vehicle

(controls) or 1 g of BNF and the presence of P450

1A1 determined after hatching (1-2 weeks). The

western blot was performed as described for Figure

2.3. No P450 1A1 was detected in the controls

under these conditions.
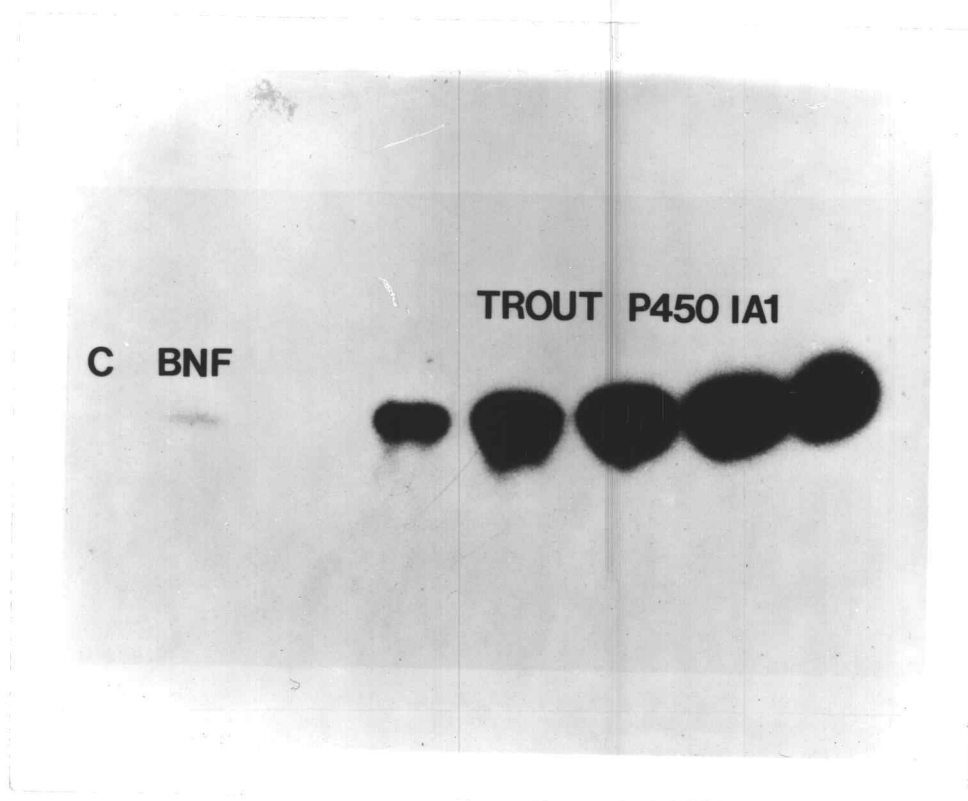
Figure 2.7

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Chapter three

HEPATOCARCINOGENIC POTENCY OF MIXED AND PURE ENANTIOMERS OF
TRANS-7,8-DIHYDROBENZO[A]PYRENE-7,8-DIOL IN TROUT

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HEPATOCARCINOGENIC POTENCY OF MIXED AND PURE ENANTIOMERS OF TRANS-7,8-DIHYDROBENZO[A]PYRENE-7,8-DIOL IN TROUT

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ABSTRACT

Rainbow trout embryos microinjected with (+/-)-trans-7,8-dihydrobenzo[a]pyrene-7,8-diol developed liver tumors 9 months after hatching. The tumor response increased with dose from 0.05- 1.0 µg (0-12.5%) and was enhanced by co-injected with β-naphthoflavone. However, the former exposure protocol resulted in high mortalities. Immersion exposure of trout embryos to (+/-) -trans-7,8-dihydrobenzo[a]pyrene at concentrations of 0.1-1.0 ppm for 30 min was ineffective at inducing hepatocarcinogenesis (0-2% incidence). Prolonging the exposure time to 5 hr, or the co-administration of β-naphthoflavone or carbon tetrachloride did not enhance the response. Microinjection of newly hatched sac-fry with 0.01-1.0 µg of (+/-)-trans-7,8-dihydrobenzo[a]pyrene-7,8-diol resulted in a dose-dependent production of liver tumors (0-13%) which resembled the results with embryos but without the problem of high mortalities. Co-injection of sac-fry with (+/-)-trans-7,8-dihydrobenzo[a]pyrene-7,8-diol and either β-naphthoflavone or carbon tetrachloride significantly enhanced the tumor response (approximately three-fold). As was the case with embryos, immersion exposure of sac-fry to (+/-)-trans-7,8-benzo[a]pyrene-7,8-diol at 0.01-1.0 ppm for 30 min or 5 hr resulted in tumor incidence only as high as 3%. The relative carcinogenic potencies of the pure (+) and (-) enantiomers of trans-7,8-benzo[a]pyrene-7,8-diol were evaluated by microinjection into sac-fry at doses of 0.5-5.0

μg. The results demonstrated that the (-) enantiomer was 4 - 18 times more potent than the (+). These results demonstrate that trout embryos and sac-fry are both responsive to hepatocarcinogenesis initiation by injection, but not immersion, with trans-7,8-dihydrobenzo[a]pyrene. The marked enhancement seen with co-injection of sac-fry with β-naphthoflavone or carbon tetrachloride suggests that both cytochrome P450-dependent and lipid peroxidation-dependent pathways could be involved in bioactivation of this compound, presumably through epoxidation at the 9,10-position. As is the case with mammals, the (-) enantiomer of trans-7,8-dihydrobenzo[a]pyrene-7,8-diol is a more potent carcinogen than the (+) enantiomer.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a class of environmental carcinogens found in smoke, water and diet, especially from charcoal broiled foods (1). Benzo[a]pyrene (B[a]P) is one of the most extensively studied PAHs. B[a]P is bioactivated in vivo first by a cytochrome P450-dependent epoxidation to yield B[a]P-7,8-epoxide which is then hydrolyzed by epoxide hydrolase to trans-7,8-dihydrobenzo[a]pyrene-7,8-diol (BP-7,8-diol). A second epoxidation at the 9,10-position yields what is thought to be the predominant carcinogenic metabolite, BP-7,8-diol-9,10-epoxide (BPDE) (2). This second epoxidation can be catalyzed by P450 or alternatively by co-oxidation catalyzed by peroxidases, including prostaglandin synthase, lipoxygenases or during propagation of lipid peroxidation (3-6). As two stereoisomers of trans-BP-7,8-diol are possible (designated as - and +) and the 9,10-epoxide can be produced in either the anti or syn configuration (relative to the benzylic hydroxyl), four different BPDEs can be formed from B[a]P (7).

Cytochrome P450 1A1 exhibits the highest activity toward formation of BP-7,8-diol, preferentially forming the (-)-BP-7,8-diol (8). Epoxidation of (-)-BP-7,8-diol yields predominantly the anti-BPDE, regardless of whether or not the catalyst involved monooxygenation or peroxidation. This anti-BPDE, derived from (-)-BP-7,8-diol has been shown to be the most effective mutagen and carcinogen of the 4 possible

stereoisomers of BPDE (7). The (+)-BP-7,8-diol enantiomer is selectively epoxidized to the syn- and anti-BPDE by monooxygenation and peroxidation, respectively (3). This enantioselectivity has made (+)-BP-7,8-diol a useful probe in vitro and in the mouse skin tumor model in vivo, for the relative contribution of P450 versus peroxidation in the bioactivation of a PAH carcinogen (9,10).

Fish are responsive to induction of carcinogenesis by PAHs, and the liver is the primary target organ (11). High incidences of hepatocarcinogenesis in feral fish populations have been correlated with elevated levels of PAHs in the environment (12,13).

Rainbow trout have been used as hepatocarcinogenesis models for different carcinogens, including B[a]P (14,15). One of the major metabolites in B[a]P-treated trout is (+) anti-BPDE (Nishimoto et al. unpublished results), presumably formed via trout P450 1A1 (16). Since P450s and peroxidation pathways bioactivate (-) BP-7,8-DHD to (+) anti-BPDE there is a need to determine their contribution in the trout carcinogenesis model by enhancing conditions characteristic of each pathway.

It is imperative to determine the carcinogenic potential of individual enantiomers and the contribution of the peroxyl-dependent and monooxygenase systems in the initiation process. Therefore, the objectives in this study were: i) to compare the tumorigenicity potency of pure (+ or -) enantiomers of BP-

7,8-DHD compared to the racemic mixture, and ii) to compare the effect of lipid peroxidation and P450 1A1 on tumor formation in trout exposed to BP-7,8-DHD.

MATERIALS AND METHODS

Chemicals

Unlabeled (+)-trans-7,8-dihydrobenzo[a]pyrene-7,8- diol ((+)-BP-7,8-DHD), (-)-trans-7,8-dihydrobenzo[a]pyrene-7,8-diol ((-)-BP-7,8-DHD), and (±)-trans-7,8-dihydrobenzo [a]pyrene-7,8- diol ((±)-BP-7,8-DHD) were obtained from Chemsyn Sci. Labs. (NCI Radiochemical Carcinogen Repository, Lenexa, KS).

Animals and treatments

Trout embryos (23 day old), or newly hatched sac-fry were microinjected with graded doses of (0.05 - 1.0 μg) or (0.01 - 1.0 μg) of (±)-BP-7,8-DHD, respectively. In the study of the tumorigenicity potency of the pure enantiomers, embryos were microinjected with 2 doses of 0.05 or 0.1 μg (+)-BP-7,8-DHD, and sac-fry were injected with 3 doses (0.5, 2.0 and 5.0 μg) of either (+) or (-) BP-7,8-DHD. All injections used DMSO/EtOH (1:10) as vehicle. Another set of embryos and sac-fry were co-injected with 1.0 μg of racemic (±)BP-7,8-DHD and 1.0 μg of either vitamin E, carbon tetrachloride (CCl$_4$) or β-naphthoflavone (BNF).

For the immersion experiment, embryos and sac-fry were immersed in (±)-BP-7,8-DHD-containing water (0.01 - 1.0 ppm) for 30 minutes or 5 hours.

Tumor detection

All trout were killed at nine month old and their livers were grossly and histologically examined. Classification of

tumors was done as described previously (17).

RESULTS

Embryos and sac-fry were exposed to racemic BP-7,8-DHD and pure enantiomers by immersion or microinjection. In some experiments they were co-injected with vitamin E, CCl_4 (to alter lipid peroxidation) or BNF (to enhance P450 1A1).

The tumor response of embryos and sac-fry when exposed to racemic BP-7,8-DHD by immersion was very low (Table 3.1). However, a dose-dependent increase in liver tumor incidence was observed in embryos and sac-fry after microinjected with (±)-BP-7,8-DHD (Figs. 3.1, 3.2). Embryos co-injected with vitamin E seemed to have a higher percentage of liver tumors than controls, but co-injection with CCl_4 did not have any effect on tumor formation (Fig. 3.1). No statistical analysis was done due to high mortality of embryos across all treatments.

Sac-fry co-injected with either BNF or CCl_4 had almost a 3- fold increase ($P < 0.005$) in liver tumor incidence over control (Fig. 3.2). Vitamin E co-injection did not show any effect in sac-fry.

A dose-dependent increase in hepatocarcinogenesis was observed when sac-fry were injected with (-)BP-7,8-DHD (Fig. 3.3). The (+)BP-7,8-DHD enantiomer, at the highest dose given, resulted in tumors similar to the lowest dose of (-)BP-7,8-DHD (Fig. 3.3).

Mixed carcinomas were the most prevalent tumor types (Table 3.2).

DISCUSSION

Exposure of embryos or sac-fry to racemic BP-7,8-DHD was less efficient via immersion than microinjection as assessed by liver tumor incidence. This difference could be attributed to low water solubility.

Racemic BP-7,8-DHD increased tumor incidence in a dose-dependent manner when trout were injected as embryos. Co-injection with vit E appeared to increase, and CCl_4 appeared to slightly decrease, tumor yields compared to controls. However, there was a high mortality rate in injected-initiated embryos which may have skewed the results. Because of the high mortality, subsequent experiments was conducted with sac-fry.

Sac-fry also exhibited a dose-dependent response to the racemic BP-7,8-DHD by injection. Co-injection with vit E seemed to offer a slight protective effect, whereas CCl_4 increased tumor formation. This findings suggest that lipid peroxidation *in vivo* is involved in initiation presumably via co-oxidation. Co-injection with BNF increased tumor yields, thus indicating that P450 1A1-dependent monooxygenase was also active. The fact that vitamin E slightly reduced tumor yields may indicate that under uninduced P450 1A1 conditions, the peroxyl-dependent pathway is more important than the P450 1A1 system. Pruess-Schwartz *et al.*, (10) showed that peroxyl-dependent bioactivation of (+)BP-7,8-DHD predominates in uninduced mouse skin *in vivo*.

To determine which BP-7,8-DHD enantiomer was more potent

in the racemic mixture for the hepatocarcinogenic response observed in embryos and sac-fry, sac-fry were microinjected with pure (+) or (-) BP-7,8-DHD. The results clearly demonstrate that the (-) enantiomer was more carcinogenic. The maximum dose given of the racemic mixture was 1 μg. When 2 μg was given of (+) BP-7,8-DHD, only 1 tumor out of 123 trout was observed which may be attributed to backgroud, however when injected with 0.5 μg of the (-) BP-7,8-DHD sac-fry developed tumors (5 tumors out of 135 trout).

Mixed carcinomas were the most prevalent tumor type in trout injected with either enantiomer. Mixed carcinomas is also prevalent in aflatoxin-initiated trout (18).

In the mouse skin carcinogenesis model, (-) BP-7,8-DHD is about 5-10 times more potent than (+) BP-7,8-DHD (19). Here, we show that in trout the (-) is between 4 - 18.5 times more potent than the (+) BP-7,8-DHD.

In conclusion, we have presented data that indicates that trout embryos and sac-fry are responsive to μg doses of racemic and pure enantiomers of BP-7,8-DHD. In addition lipid peroxidation and P450-dependent pathways are likely involved in initiation. Therefore, the trout may be a good model for further studies on the significance of co-oxidation and monooxygenase dependent pathways of metabolic activation of procarcinogens.

Table 3.1. Liver tumors in trout initiated as embryos or sac-fry by immersion for 30 minutes or 5 hours. Data represents one tank/treatment with 100 fish each.

Table 3.1.

Stage	Exposure time	Enantiomer	Dose (ppm)	% liver tumors
Embryo	30 minutes	+BP-7,8-DHD	0.01	0
			0.10	1
			0.50	1
			1.00	2
	5 hours		0.50	2
Sac-fry	30 minutes	±BP-7,8-DHD	0.01	0
			0.10	1
			0.50	1
			1.00	3
	5 hours		0.50	3

Figure 3.1. Incidence of liver tumors in trout initiated as embryos by microinjection with (±)-BP-7,8-DHD alone or co-injected with 10 μg of either Vit E or CCl_4. Each data point represents one tank containing between 22 - 61 trout.
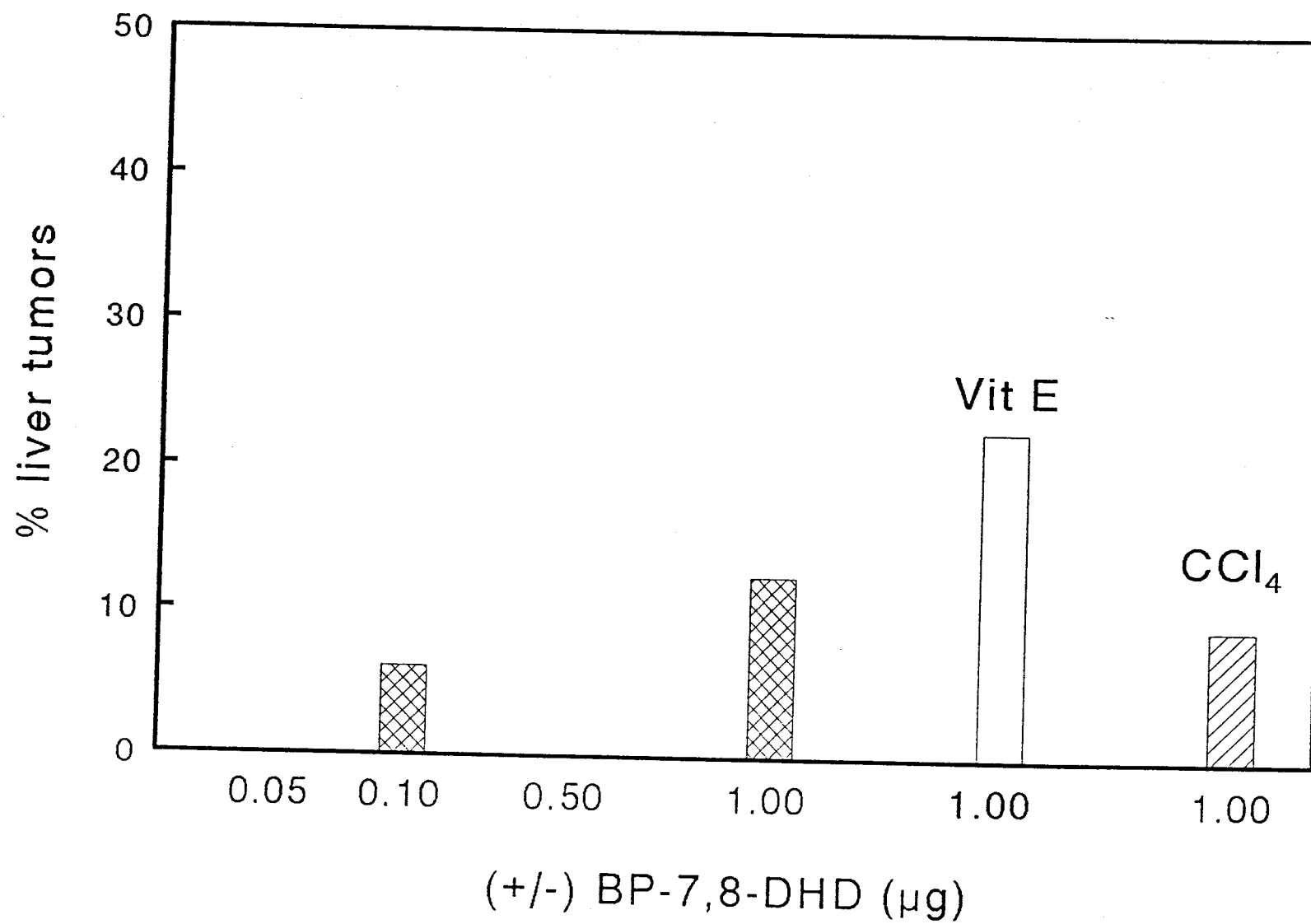
Figure 3.1.

Figure 3.2. Incidence of liver tumors in trout initiated as sac-fry by microinjection with (±)-BP-7,8-DHD alone or co-injected with 10 μg of either Vit E, CCl_4 or BNF. Each data point represents one tank containing between 55 - 100 trout.

* represents significantly different from control (1 μg of racemic diol) at $P < 0.005$ by Chi-square test.
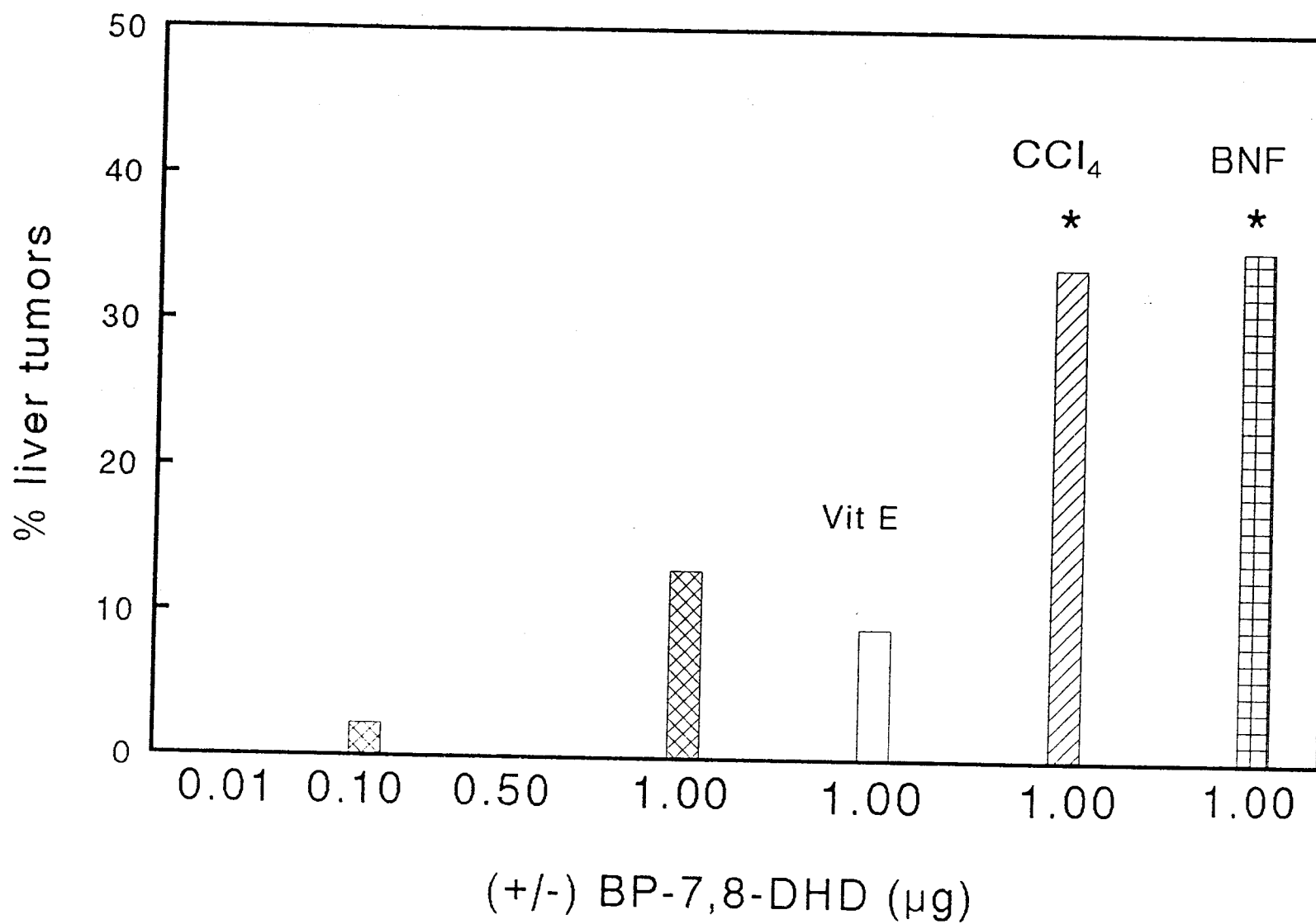
Figure 3.2.

Figure 3.3. Hepatocarcinogenic potency of pure (+) vs (-)
BP-7,8-DHD. Trout were initiated as sac-fry
by microinjection. Values represent the mean
percentage of two tanks with their respective
standard deviations. ** and * denotes
significantly higher (P < 0.005 and < 0.025
respectively by Chi-square test) than (+)
enantiomer at the same dose.
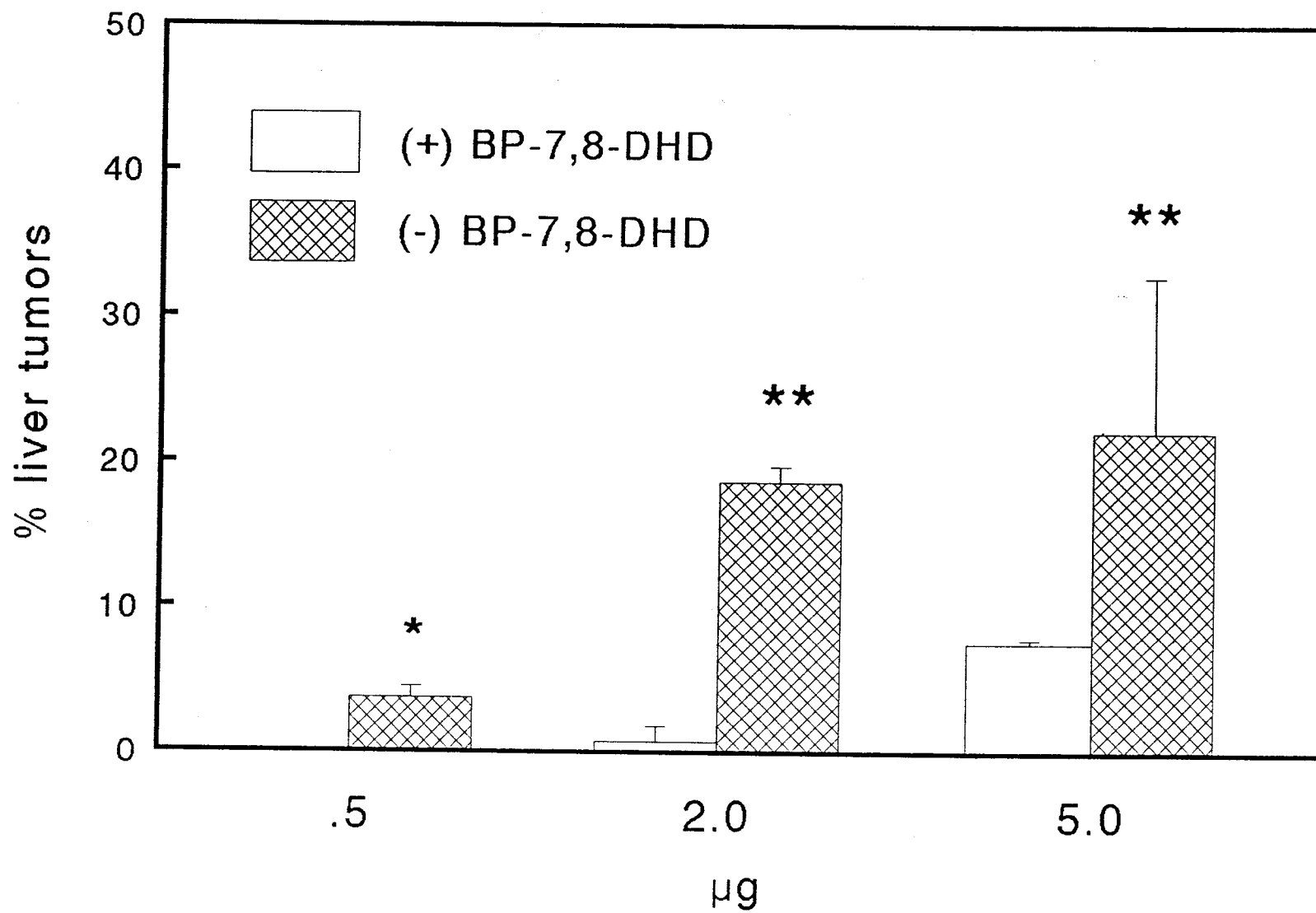
Figure 3.3.

Table 3.2. Classification of liver tumors from trout
initiated as sac-fry with pure (+) or (-) BP-
7,8-DHD. Numbers under classification of
tumors based on percentage from tumor
incidence.

Table 3.2.

Treatment	Tumor Incidence	Classification of Tumors (% of total tumors)				
		BF	MC	HCC	CCC	Ch
Sham	0/46					
0.5 μg (+)	0/88					
0.5 μg	0/73					
2.0 μg	0/56					
2.0 μg	1/67		100.0			
5.0 μg	6/78	16.7	50.0		33.3	
5.0 μg	5/69		60.0	20.0	20.0	
0.5 μg (-)	2/65		100.0			
0.5 μg	3/70		33.3	33.3		33.3
2.0 μg	13/73		53.8	30.8		15.4
2.0 μg	12/62		66.7	25.0	8.3	
5.0 μg	11/76	9.1	81.8	9.1		
5.0 μg	19/64		78.9	21.1		

BF= Basophilic focus MC= mixed carcinoma,
HCC= hepatocellular carcinoma, HCA= hepatocellular adenoma,
CCC= Cholangiocellular carcinomas,
Ch= Cholangioma.

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Chapter Four

DIETARY HYDROGEN PEROXIDE ENHANCES HEPATOCARCINOGENESIS IN
TROUT: CORRELATION WITH 8-HYDROXY-2'-DEOXYGUANOSINE
LEVELS IN LIVER DNA

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Dietary Hydrogen Peroxide Enhances Hepatocarcinogenesis in
Trout: Correlation with 8-Hydroxy-2'-deoxyguanosine
Levels in Liver DNA.

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ABSTRACT

The tumor enhancing effect of hydrogen peroxide (H_2O_2) on N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-initiated rainbow trout hepatocarcinogenesis was investigated and correlated with the levels of the mutagenic DNA adduct, 8-hydroxy-2'-deoxyguanosine (oh^8dG). In addition, the protective role of vitamin E was examined in relation to tumor enhancement and oh^8dG levels in liver DNA. Trout were fed diets containing two levels of vitamin E (1000 or 20 mg/Kg wet wt), each of which was made up to contain three levels of H_2O_2 (0, 600 or 3000 ppm). Dietary vitamin E levels had no significant effect on tumor incidence or levels of oh^8dG in liver DNA. On the other hand, dietary H_2O_2 enhanced liver tumors in a dose-dependent manner. Liver tumor incidences correlated significantly with the mean level of liver DNA oh^8dG content (r= 0.87). We conclude that the H_2O_2 tumor enhancing effect coincides with higher levels of oh8dG in the trout liver genome. Thus, rainbow trout may be a useful model for the study of the relationship of oh^8dG levels *in vivo* to enhancement or promotion of carcinogenesis and its modulation by dietary enhancers and inhibitors of oxidative stress.

INTRODUCTION

The mechanisms by which tumor promoters exert their effect on proliferation of a mutated cell are not yet known. However, it is known that many promoters generate high levels of reactive oxygen species (ROS) (1,2) and that promotion can be inhibited by antioxidants such as vitamins E and C (1). The major intracellular ROS are superoxide anion radical (O_2-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$). H_2O_2, in the presence of a transition metal, such as Fe^{2+} or Cu^+, produces $\cdot OH$ via the Fenton reaction (3,4). The $\cdot OH$ radical is the most electrophilic radical that DNA is exposed to (5), causing a large number of lesions (6) that include strand breaks and oxidized bases, making it difficult to identify which lesions are involved in carcinogenesis.

Among the many oxidized bases (7), 8-hydroxy-2'-deoxyguanosine (oh^8dG) is extensively used as a biomarker for oxidative DNA damage due to the development of a highly selective and sensitive assay, utilizing HPLC with electro-chemical detection (8,9). The oh^8dG adduct is mutagenic and has been shown *in vitro* to mispair its complementary template causing G→T transversions (10). This mutation has recently been confirmed *in vivo* (11) by site-directed mutagenesis. Therefore, the presence of unrepaired oh^8dG in genomic DNA has the potential to activate oncogenes or deactivate suppresor genes that are involved in promotion. Circumstantial evidence for such a role includes observations

that oh^{8}dG is found in high levels in target tissues utilizing different carcinogenesis protocols (12-17).

Recently, Malins *et al.*, (18,19) have shown that DNA from neoplastic liver tissue of English sole, exposed to creosote hydrocarbons, contained significantly higher levels of oh8dG (11- fold) than normal liver from non-contaminated fish. 2,6-Diamino-4-hydroxy-5-formamido pyrimidine (FapyGua), a product of oh^{8}dG, was only detected in fish from contaminated areas. The presence of FapyGua and oh^{8}dG in these fish is indicative of oxidative damage to DNA which may be involved in neoplastic changes during promotion.

There are limited data among experimental fish models regarding the possible promoting role of oxidative stress, specifically the role of oh^{8}dG. The trout model has provided valuable and significant mechanistic information regarding initiation and promotion stages of chemical carcinogenesis (20) and their dietary modulation. It is thus possible that the trout model may provide further mechanistic information about oh8dG and its possible role in tumor enhancement. Therefore, the objectives of the present study were: i) to determine the tumor enhancing effect of dietary H_2O_2 in MNNG-initiated trout, ii) to correlate tumor enhancement with oh^{8}dG content of the target tissue and iii) to examine the protective role of vitamin E on tumor incidence and oh^{8}dG formation.

MATERIALS AND METHODS

Chemicals

MNNG was obtained from Sigma Chem. Co. (St. Louis,MO).
Phenol was obtained from Baker (Phillipsburg, NJ) and AMRESCO
(Solon, OH). Acetic acid, NAOH and HCl were purchased from
Aldrich (Milwaukee,WI); citric acid was from High-Purity
Chemical (Portland, OR). Authentic oh^{8}dG was obtained from
Dr. Robert Floyd (Oklahoma Med. Center). All other chemicals
and reagents were purchased from Sigma.

Animals and diets

Shasta rainbow trout (Oncorhynchus mykiss) were spawned
and raised in this laboratory. Trout were initiated as 23 day
old embryos by immersion in water containing 25 ppm MNNG for
30 min. After hatching embryos were fed the Oregon Test Diet
(OTD) (21) for 4 weeks and then divided into six groups of
120 and switched to the experimental diets for 8 months. The
diets contained two levels of vitamin E, high (OTD, 1000
ppm), and low (OTD-modified, 20 ppm), to which H$_2$O$_2$ was
incorporated at three different levels (0, 600 or 3000 ppm).
Diets were made in 260 g batches and kept at -20°C for up to
6 weeks. Dietary H$_2$O$_2$ was analyzed as previously described
(22) and malondialdehyde (MDA) analysis was determined by the
thiobarbutiric acid assay (23).

DNA and oh^{8}dG isolation

DNA from 3 pools (2 fish/pool) per treatment was isolated
from non-neoplastic liver tissues as described by Dashwood et

al., (24)   with the slight modification of adding 1 mM

L-histidine to all buffers as a trap for any •OH generated

during the isolation procedure. DNA was resuspended in 20 mM

Na-acetate, 1 mM histidine   (pH 4.8). High purity phenol

(AMRESCO) was used to avoid any   increased background of

oh^{8}dG. The DNA concentration was determined   spectropho-

tometrically (20 U A$_{260}$= 1 mg). DNA (3 U/ 200 μl of

resuspending buffer) was hydrolyzed with 20 μg of nuclease P1

in a 95 % ETOH-washed microcentrifuge tube and incubated at

37°C for 30 min. Then, 20 μl of 1 M Tris-HCl (pH 7.3) was

added to the mixture, followed by alkaline phosphatase (1.1

U) and incubated at 37°C for 60 min.

oh^{8}dG and 2'deoxyguanosine (dG) detection

Proteins from the hydrolyzate were precipitated with 10

μl of   dilute glacial acetic acid (2:1) and the solution

filtered (0.45 μ). The filtered hydrolyzate (60-80 μl) was

injected onto HPLC,   (ODS1 column-SpherisORB 5 μ, 25 x 0.46

cm) . A Shimadzu UV detector(SPD-6AV) at 290 nm was used to

detect dG and an electrochemical detector (Shimadzu L-ECD--

6A), with an applied potential of +600 mV, to detect oh^{8}dG.

Mobile phase consisted of 12.5 mM citric acid, 25 mM sodium

acetate, 30 mM NaOH, 1mM EDTA and 10 mM acetic acid in 15 %

methanol (pH 5.1). Quantitation of nucleosides was done by a

standard curve, using the peak heights of authentic dG and

oh^{8}dG.

Tumor detection

Animals were killed at 10 months, while still sexually immature. Livers were examined grossly, fixed in Bouins solution and tumors confirmed by hand slicing and histologic examinations. Tumor classification was done according to Hendricks *et al.*, (25).

RESULTS

Dietary H_2O_2, significantly enhanced the incidence of liver tumors in trout in a dose-dependent manner (Figure 4.1). Assays of the actual levels of H_2O_2 demonstrated some loss either during preparation or with storage. The levels were found to be 2700 ± 195 and 2400 ± 360 ppm (3000 ppm added) in the high and low vitamin E/high H_2O_2 diets, respectively and 20 ± 5 and 42 ± 5 ppm (600 ppm added), in the high and low vitamin E-low H_2O_2 diets, respectively. The enhancement of tumor yield by dietary H_2O_2 was probably not due to increased levels of peroxidized lipids in the diets, as there were no significant differences in MDA levels between diets containing different H_2O_2 levels (data not shown). The apparent decreased yield of tumors in trout fed the high H_2O_2/low vitamin-E diet was probably the result of the poor growth and relatively high mortality (Table 4.1) of this treatment. Due to the high mortality in this group, statistical analysis for tumor incidence excluded this diet.

Mixed hepatocellular and cholangiocellular carcinomas were the most prevalent tumor types regardless of the H_2O_2 level (Table 4.2). The percentage of total tumors that were mixed carcinomas was consistently between 50-60 in all groups except the low vitamin E/Low H_2O_2, which was higher. Overall, the number of cholangiocellular carcinomas was inexplicably higher than normally seen.

Tumor size (Table 4.1) tended to be higher for trout fed high vitamin E compared to low vitamin E, and the H_2O_2 fed fish appeared to have slightly larger tumors. These results were not statistically significant due to the high S.E. of the measurements (tumor size measured in increments of 0.5 mm). Multiple tumors were significantly greater in H_2O_2-fed trout in the high vitamin E group (p=0.1) (Figure 4.2). Because of the high mortality in the low vitamin E-high H2O2 fed fish, multiple tumor statistical analysis in the low vitamin E group were not compared.

The high H_2O_2 diets depressed growth in trout, and this effect was magnified in the low vitamin E diet (Table 4.1). These fish were presumably more susceptible to H_2O_2-induced oxidative stress because of antioxidant depletion, resulting in poor growth and high mortality. The DNA adduct, oh^{8}dG, was significantly higher in trout fed high H_2O_2 regardless of the dietary vitamin E levels (p < 0.08) (Figures 4.3, 4.4). There was no signifi-cant difference between trout fed control and low H_2O_2, though a trend was observed. When oh8dG mean data from all treatments (except low vitamin E\high H_2O_2) were pooled, there was a significant correlation (0.87 at P=0.0551, Figure 4.5) between liver tumor incidence and average liver DNA oh^{8}dG content.

DISCUSSION

The promoting effect of H_2O_2 by topical application has been previously reported in the hamster model (26, 27). We report herein that dietary H_2O_2 enhances hepatocarcinogenesis using MNNG-initiated trout as the animal model. Analysis of H_2O_2 in the diets demonstrated that the actual levels were lower than the nominal levels. This was especially true for the low H_2O_2 diets (20 and 42 ppm measured versus 600 nominal). The measured levels in the high H_2O_2 diets (2400 and 2700 ppm) were within 10-20% of the nominal (3000 ppm) values. This loss in H_2O_2 from the diet tended to increase with storage time, but was also evident in freshly prepared diet. MDA levels on the average were 5-fold higher in the low vitamin-E than high vitamin-E diets (152 and 30 $\mu mol/g$ diet, respectively) yet tumor incidence was unaffected by vitamin-E levels. In addition, MDA levels were not significantly different at any level of H_2O_2 in the diet (data not shown), indicating that the enhancement effect observed was not due to high levels of peroxidized dietary lipids.

The slight protective role of vitamin-E against enhancement was not significant, but it reduced mortality significantly in trout fed high H_2O_2. The RDA for trout is 12 mg/Kg diet (wet wt.) (28). Both of the dietary vitamin E levels fed met this requirement based on our calculations. Under oxidative stress conditions, the tumor incidence was not inhibited even at high levels of vitamin E. This lack of

protection against enhancement by vitamin E indicates that either a higher level of vitamin E may be required by trout for protection against tumor enhancement by oxidative stress or inhibition by vitamin E has been maximized at 20 ppm. In addition to the higher incidence of liver tumors, we observed that H_2O_2-fed trout tended to have a greater tumor size/body weight ratio (Table 4.1), thus suggesting that proliferation of cells may occur to a greater extent in H_2O_2-fedtrout. The increase of multiple tumors per liver of tumor-bearing trout under oxidative stress shown in our experiment has recently been reported in trout treated with carbon tetrachloride as a prooxidantpromoting agent (29). These findings suggest that oxidative stress may lead to multiple tumors.

Mixed carcinomas were the most prevalent type of tumor in all treatments, as previously reported for aflatoxin carcinogenesis in rainbow trout (30). The results did not show any pattern that would indicate that H_2O_2may alter tumor type, although one of the groups (Low vitamin E/Low H2O2) had a much higher incidence, of mixed tumors than any other group.

The protooncogenes c-fos, c-myc, c-jun and H-ras have been shown to be induced under oxidative stress in different cell lines and animal models (31-33), yet the mechanism by which induction occurs remains unknown. It is thought that oh^8dG may have the potential to activate oncogenes or deactivate suppressor genes. Evidence for this hypothesis has

come from *in vitro* studies (10,34) and site-directed mutagenesis (11) indicating that there is misincorporation of base opposite to the oh8dG adduct, forming a oh8dG-(syn)*dA(anti) stable base pair as assessed by NMR (35) with the eventual mutation involving a G→T transversion. Our data demonstrate that the mean levels of liver DNA-oh8dG increased in the H_2O_2-fed trout. The oh8dG mean levels positively correlated liver tumor incidence.

From the present study, we can not rule out the possibility that H_2O_2 could be an initiator and/or a complete carcinogen in trout. In subsequent studies (unpublished) we have preliminary evidence that dietary H_2O_2 is not a complete carcinogen in trout.

In conclusion, our data demonstrate that post-initiation treatment with dietary H_2O_2 enhances tumor development in trout and the levels of the modified base, oh8dG in the liver genome, correlates with hepatic tumor enhancement. Thus, rainbow trout may be a good model for the study of the relationship of oh8dG levels *in vivo* to carcinogenesis and its dietary modulation.

ACKNOWLEDGEMENTS

Figure 4.1. Incidence of liver tumors at 10 months in
trout fed the different diets. Values
represent one tank/treatment. Each tank
contained between 52 and 93 fish (see Table
4.1.). * Significantly different ($P < 0.005$,
Chi-squared likelihood ratio) as compared
with controls.
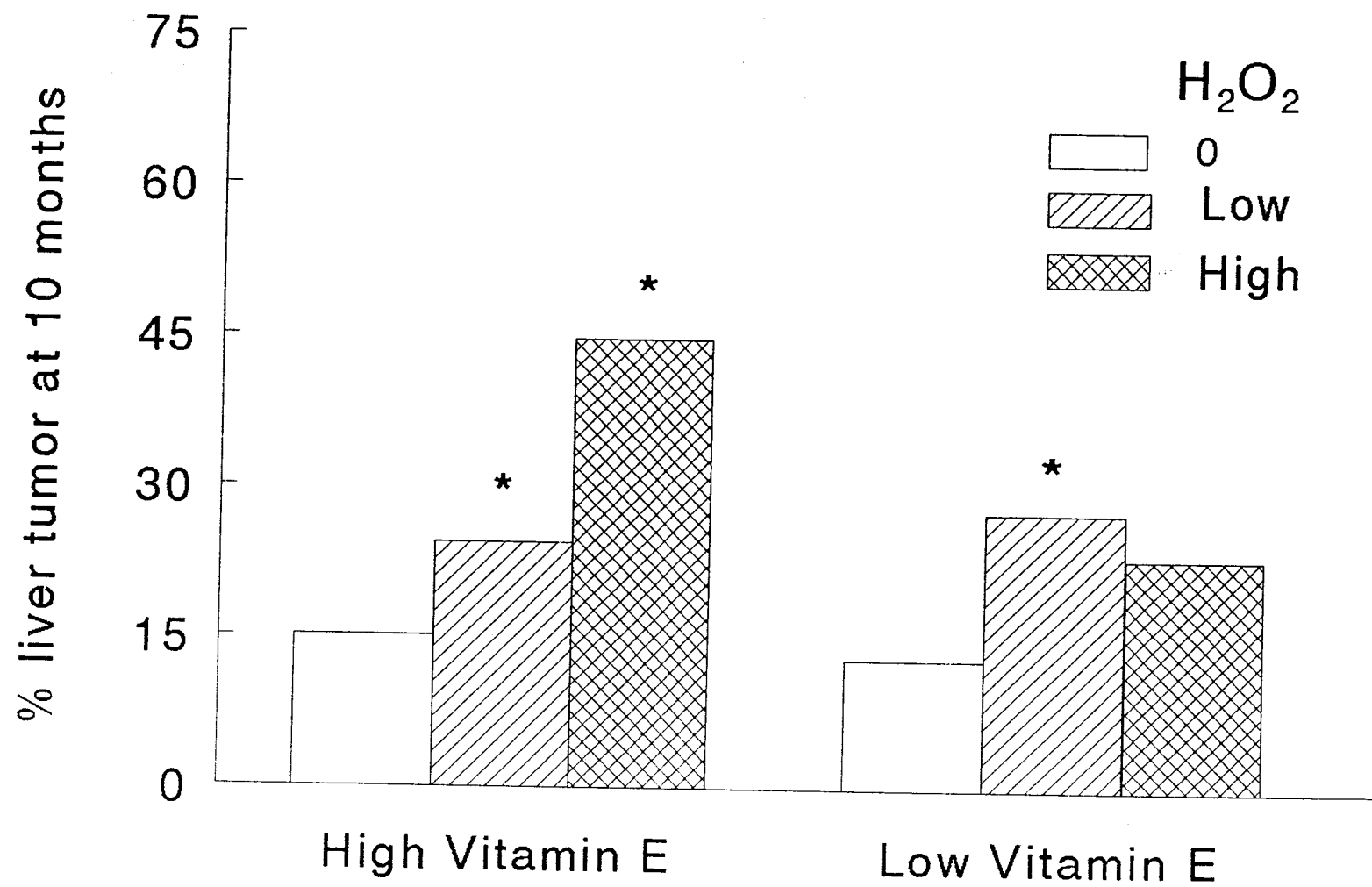
Figure 4.1.

Table 4.1. Effect of dietary hydrogen peroxide on body weight, mortality, tumor size and tumor size:body wt. ratio. Data taken at 10 month. Values (Means and standard errors) bearing different superscripts in the same column are statistically significant ($P < 0.05$) by 2-way ANOVA.

Table 4.1.

Diets	n	body wt. (g)	Mortality %	tumor size(mm)	tumor size/ body wt.
High Vit-E	93	20.9 ± 0.7^a	9	2.1	0.10
+low H_2O_2	90	19.3 ± 0.8^{ab}	12	2.5	0.13
+high H_2O_2	85	14.0 ± 0.5^d	17	2.5	0.18
Low Vit-E	85	16.7 ± 1.1^c	17	1.7	0.10
+low H_2O_2	87	18.5 ± 0.8^{bc}	15	1.8	0.10
+high H_2O_2	52	7.6 ± 0.3^e	49	1.9	0.25

Table 4.2. Classification of liver tumors from trout
enhanced by dietary H_2O_2. Tumor types are
expressed as percentage of tumors present per
treatment. See number of trout/treatment in
table 4.1.

Table 4.2.

Diets	tumor incidence	% tumors	Tumor classification (% of total tumors)						
			MC	HCC	HCA	CCC	Ch	MA	BF
High Vit-E	14/93	15.1	57.1	14.3	14.3	14.3	-	-	-
+low H_2O_2	22/90	24.4	59.1	4.5	4.5	31.9	-	-	-
+high H_2O_2	38/85	44.7	55.2	7.9	-	23.7	5.3	7.9	-
Low Vit-E	11/85	12.9	54.4	18.2	-	9.1	9.1	-	9.1
+low H_2O_2	24/87	27.6	87.5	4.2	-	8.3	-	-	-
+high H_2O_2	12/52	23.1	50.0	16.7	-	33.3	-	-	-

MC= mixed carcinoma, HCC= hepatocellular carcinoma, HCA= hepatocellular adenoma
CCC= cholangiocellular carcinoma, Ch= cholangioma, MA= mixed adenoma,
BF= basophilic focus.

Figure 4.2. Tumor multiplicity of trout fed high vitamin
E with either none, low or high hydrogen
peroxide at 10 months. H_2O_2-fed trout had
higher incidence of multiple tumors than
control (P= 0.10 by Chi-square test).
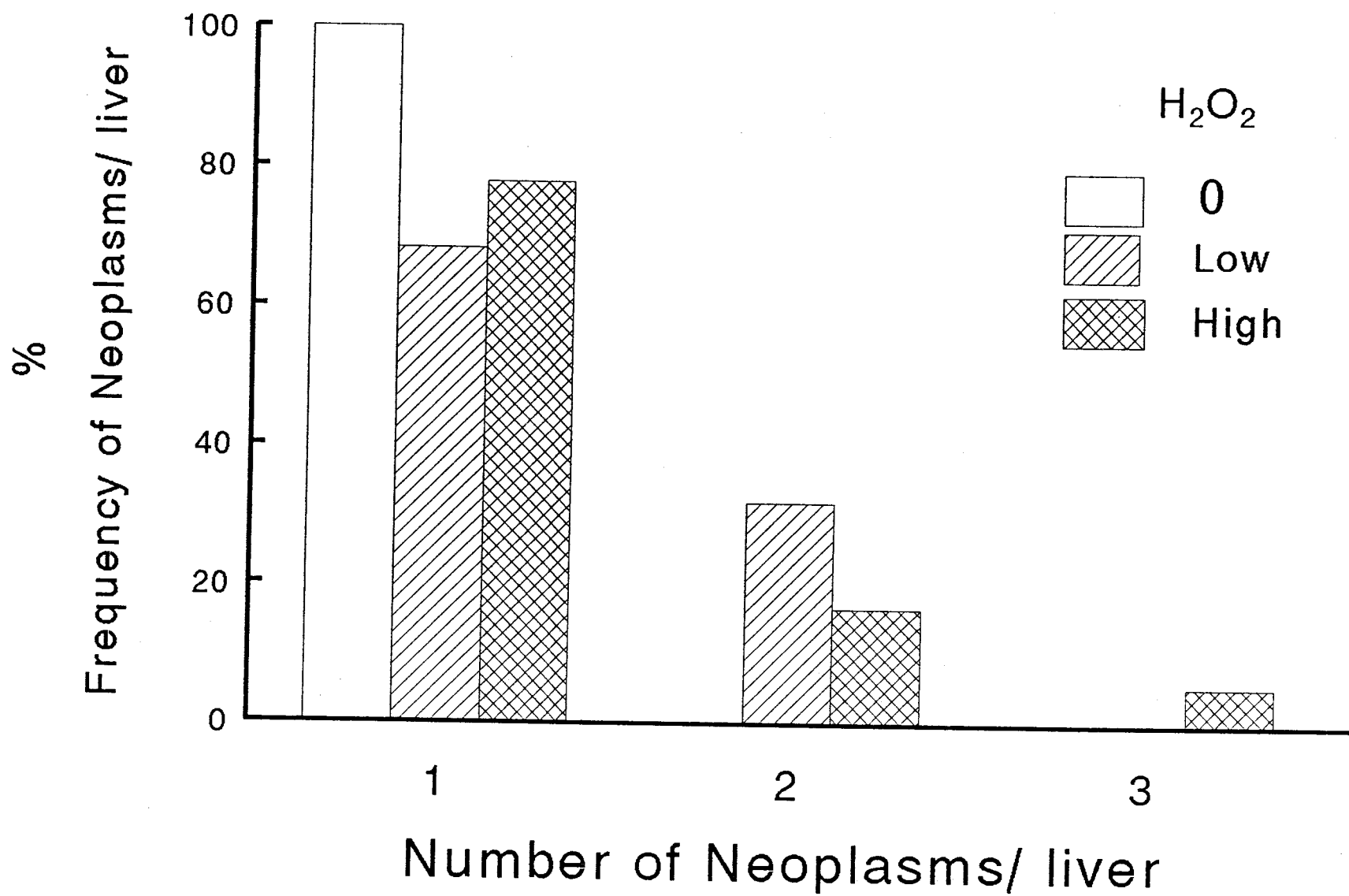
Figure 4.2.

Figure 4.3. HPLC-EC traces of oh8dG analysis from 48 μg

of hydrolyzed trout liver DNA (A_{260nm}). Refer

to Materials and Methods for details. A)

Liver DNA from trout fed high vitamin E/ no

H_2O_2. B) Liver DNA from trout fed high

vitamin E/ high H_2O_2.
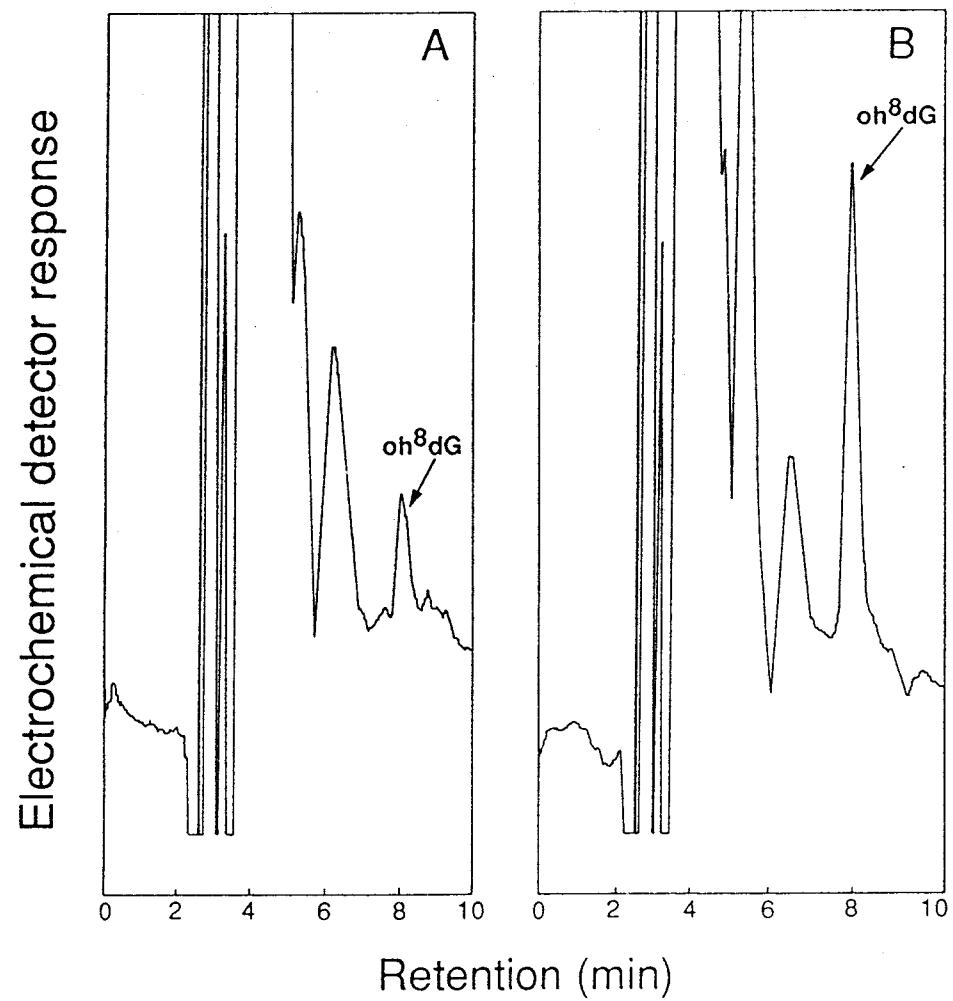
Figure 4.3.

Figure 4.4. Trout liver oh8dG content from DNA of trout
fed different diets. Values represent the
mean and standard error of duplicate
injections from 3 pools/diet as described in
Materials and Methods. * significantly
different (P < 0.08 by 2-way ANOVA) from
controls.
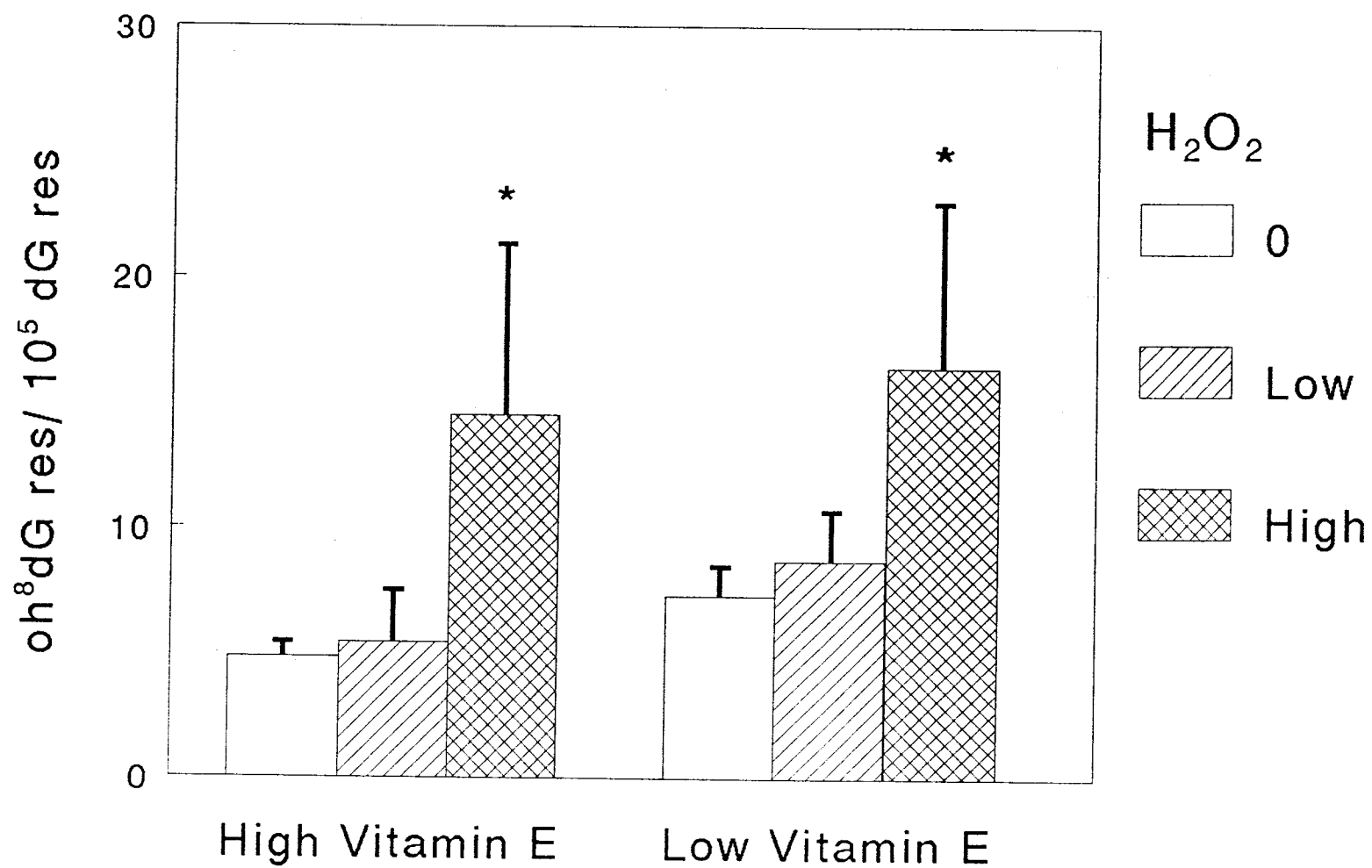
Figure 4.4.

Figure 4.5. Correlation between incidence of liver tumors and mean liver DNA oh8dG content. Low Vit-E/ high H_2O_2 was not included due to high mortality.
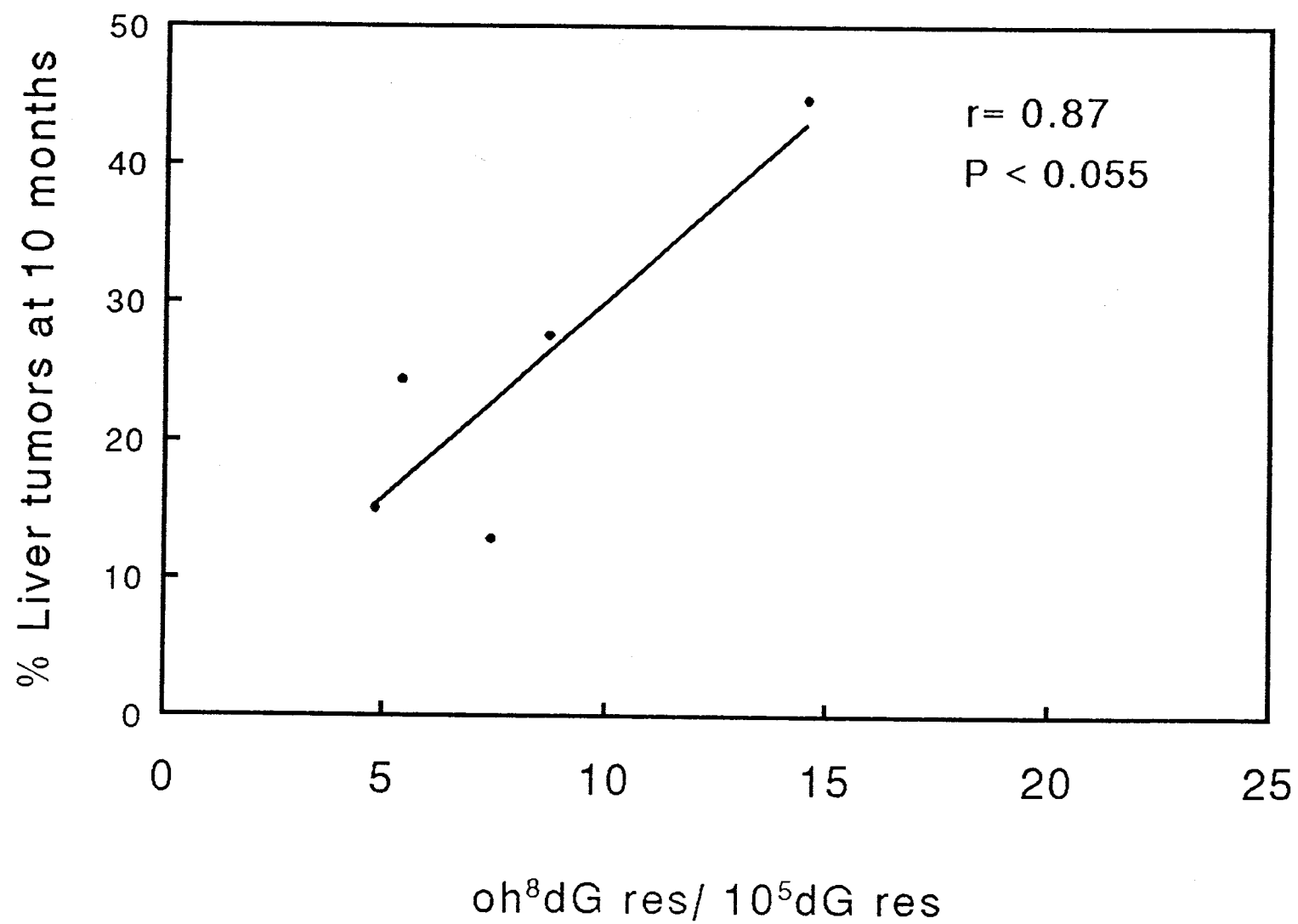
Figure 4.5.

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CONCLUSIONS

Rainbow trout embryos and sac-fry bioactivate (±) BP-7,8-DHD to presumably form the ultimate carcinogen, BPDE. The presumed pathways to 9,10-epoxidation are P450 monooxygenase and peroxyl radical-dependent, because modulation of both pathways increased DNA binding and liver tumor incidence. The peroxyl-dependent pathway seems to be the predominant pathway in uninduced P450 trout, and the P450 pathway in induced trout. To confirm these findings, experiments are underway using (+) BP-7,8-DHD as a probe to assess the relative contribution of both pathways to formation of ANTI and SYN-derived 9,10-epoxide-DNA adducts by HPLC-^{32}P postlabelling techniques.

Sac-fry are a better model for carcinogenic experiments due to a lower mortality rate than embryos after initiation by microinjection. Microinjection of sac-fry with pure (+) or (-) BP-7,8-DHD enatiomers demonstrated that the (-) enatiomer is 4-18 times more potent in forming liver tumors than the (+), as also is the case in the mouse skin carcinogenesis model (5-10).

Under oxidative stress conditions, liver trout DNA contained high levels of the mutagenic DNA adduct, oh^{8}dG, that positively correlated with liver tumor enhacement. The repair rate of this adduct may regulate mutational activity of cancer-causing genes. The lack of oh^{8}dG liver DNA adducts in sac-fry fed hydrogen peroxide for 10 weeks suggests that a

longer exposure time may be required to better assess repair rate.

We have presented data in this thesis, indicating that trout are a good model for the relationship between oxidative stress and carcinogenesis.

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APPENDICES

Appendix one

HYDROGEN PEROXIDE AND BENZOYL PEROXIDE MAY NOT BE TRUE PROMOTERS OR COMPLETE CARCINOGENS IN THE TROUT MODEL

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Hydrogen peroxide and benzoyl peroxide may not be true promoters or complete carcinogens in the trout model

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ABSTRACT

The promoting effect of two free radical generating compounds (hydrogen peroxide and benzoyl peroxide) by dietary means was examined in trout. The 8-hydroxydeoxy-guanosine (oh^{8}dG) liver adduct content was measured as an indicator of oxidative stress. Sac-fry were exposed to 1 ppm dimethylbenzo[a]anthracene (DMBA) for 7 hours, and a day later fed experimental diets for 8 months. The diets were: Oregon Test Diet (OTD), hydrogen peroxide (7500 ppm on a dry wt. basis), and benzoyl peroxide (7500 ppm on a dry wt. basis). A 10 % liver tumor incidence was expected in the OTD-fed trout; however, no tumors were observed. There were no tumors observed in the benzoyl peroxide-fed trout. There was only one liver tumor in the hydrogen peroxide-fed trout which may be attributed to background tumor rates in trout. The oh^{8}dG adduct levels in liver of trout fed high-peroxide did not differ from OTD-fed trout. The lack of tumors in OTD-fed trout may indicate that either trout may not have absorbed DMBA for initiation or may have been exposed to a subcarcinogenic dose of DMBA. Since no tumors were observed in the control-fed trout, conclusions can not be made. However, we suggest that neither hydrogen peroxide nor benzoyl peroxide are true promoters or complete carcinogens when fed at this levels.

INTRODUCTION

Chemical carcinogenesis is a multistage event that requires four sequential processes: initiation, promotion, conversion and progression (1).

The mechanism(s) of promoters have not been elucidated, but it has been noted that, in many cases, antioxidants are inhibitory. This suggests that free radicals (oxidative stress) are involved in neoplastic development.

Tumor promoters generate free radicals by two pathways (2,3): those metabolized within the cell to form primary radical intermediates (direct) and others that stimulate endogenous sources of reactive oxygen species (ROS) (indirect).

Benzoyl peroxide is a direct free radical promoter that is extensively used in the plastics and rubber industry, and is found as a food additive, as well as, in non-prescription drugs (4). This compound is metabolized to form the radical intermediate benzoyloxyl radical (5) and is a mouse skin tumor promoter (6). On the other hand, 12-O-tetradeconylphorbol-13 acetate (TPA), is an indirect free radical promoter which stimulates the formation of endogenous ROS including hydrogen peroxide ($H2O_2$) which, in the presence of a transition metal (Fe^{+2}/Cu), produces hydroxyl radical (OH$\cdot$) via the Fenton reaction.

The hydroxyl radical reacts with DNA resulting in a large number of base modifications which may be involved in

neoplastic development. One of these lesions is the formation of 8-hydroxydeoxyguanosine (oh^{8}dG). oh^{8}dG causes G→T transversions (7,8) and is highly mutagenic (9). Consequently, oh^{8}dG has the potential to activate protooncogenes or inactivate tumor suppressor genes during promotion. Different ROS-generating protocols result in enhanced expression of the protooncogenes c-jun, c-fos and c-myc (10,11). Elevated oh^{8}dG levels are found under various carcinogenesis regimes (12), and human breast carcinomas express a 10 fold increase of oh^{8}dG over noncancerous tissue (13).

Even though the promoting mechanism of benzoyl peroxide and TPA may be different, both cause greater expression of Ha-ras protooncogene in DMBA-initiated mouse skin (14). This enhanced expression could be mediated by the oh^{8}dG adduct. However, there is no information in the literature regarding the effect of benzoyl peroxide on oh^{8}dG adduct formation and association with neoplastic development.

Fish may be good animal models for the study of the relationship between oh^{8}dG and carcinogenesis. Neoplatic liver tissue of feral English sole, exposed to high environmental levels of aromatic hydrocarbon carcinogens, have significantly higher oh^{8}dG content than fish from non-contaminated sites (15).

The trout model has contributed significantly to the understanding of chemical carcinogenesis (16), however the role of oxidative stress during carcinogenesis has remained

largely unexplored. Using exogenous H_2O_2 as a model prooxidant for oxidative stress, we recently showed that dietary hydrogen peroxide enhanced liver tumor incidence in trout in a dose-dependent manner. We demonstrated that liver tumor incidence in trout significantly correlated (r= 0.87) with the average oh^8dG content in liver DNA. Previous study (17) also showed that topical application of H_2O_2 to hamsters promoted liver tumor incidence induced by tobacco-specific nitrosamines.

Hydrogen peroxide may also be a complete carcinogen. Mice developed duodenal tumors after oral administration of H_2O_2 (18). In our previous experiment, trout developed multiple tumors when fed H_2O_2. While H_2O_2 may be a promoter, there is the possibility that new tumors developed independent of carcinogen exposure. In addition, the number of oh^8dG adducts from trout fed the high H_2O_2 diet was almost 2-fold higher than that found in ciprofibrate-fed rats (a peroxisome proliferator and complete hepatocarcinogen) (19) suggesting that the high H_2O_2 diet fed to trout could act as a complete carcinogen as do peroxisome proliferators. Therefore, the objectives of this investigation were: i) to determine if H_2O_2 and benzoyl peroxide are true hepatic promoters, ii) to assess the complete carcinogen potential of H_2O_2 and iii) to determine if the promoting effect of benzoyl peroxide is related to the oh^8dG liver content in the trout model.

MATERIALS AND METHODS

Chemicals

Phenol was obtained from AMRESCO (Solon, OH). Acetic acid, NaOH and HCl were purchased from Aldrich (Milwaukee,WI); citric acid was from High-Purity Chemical (Portland, OR). Authentic oh[8]dG was obtained from Dr. Robert Floyd (Oklahoma Med. Center). Alkaline phosphatase was obtained from Pharmacia (Menasha,WI). DMBA and other chemicals were purchased from Sigma Chem. Co. (St. Louis, MO).

Animals and diets

Shasta rainbow trout (Oncorhynchus mykiss) were spawned and raised in this laboratory. Trout were initiated as sac-fry by immersion in water containing 1 ppm DMBA for 7 hours. Trout were fed either Oregon Test Diet (OTD) (20), or OTD plus H_2O_2 or benzoyl peroxide at two levels (1500 or 7500 ppm) for 8 months. Diets for both experiments were made in small batches and kept at -20°C for up to 6 weeks.

DNA and oh[8]dG isolation

DNA from 3 pools (2 fish/pool) per treatment was isolated from non-neoplastic liver tissues as described by Dashwood et al., (21) with the slight modification of adding 1 mM L-histidine to all buffers as a trap for any ·OH generated during the isolation procedure. DNA was resuspended in 20 mM Na-acetate, 1 mM histidine (pH 4.8). High purity phenol (AMRESCO) was used to avoid any increased background of

oh^{8}dG. The DNA concentration was determined by the Burton assay (22). DNA (100 μg/ 200 μl of resuspension buffer) was hydrolyzed with 20 μg of nuclease Pl in a 95 % ETOH-washed microcentrifuge tube and incubated at 37°C for 30 min. Then, 20 μl of 1 M Tris-HCl (pH 7.3) was added to the mixture, followed by alkaline phosphatase (1.5 U) and incubated at 37°C for 60 min.

oh^{8}dG and 2'deoxyguanosine (dG) detection

Volume between 50-80 μl of the hydrolyzate was injected onto HPLC, (ODS1 column-SpherisORB 5 μ, 25 x 0.46 cm) . A Beckman UV detector at 290 nm was used to detect dG and an electrochemical detector (Shimadzu L-ECD-6A), with an applied potential of +600 mV, to detect oh^{8}dG. The mobile phase consisted of 12.5 mM citric acid, 25 mM sodium acetate, 30 mM NAOH, 1 mM EDTA and 10 mM acetic acid in 15 % methanol (pH 5.1). Nucleosides were quantitated by the peak heights of authentic dG and oh^{8}dG.

Tumor detection

Animals were killed at 10 months, while still sexually immature. Livers were examined grossly, and a sample of ten livers were fixed in Bouins solution for histologic examinations.

RESULTS

The tumor incidence in liver of DMBA-initiated trout was negative for all treatments (Table 1). Histological examination of a sample of ten fish showed no apparent lesions as a result of either dietary H_2O_2 or benzoyl peroxide. The effect of the free-radical generating compounds on oh[8]dG liver DNA content was not altered compared to OTD-fed trout (Table A.1).

DISCUSSION

The results obtained from DMBA-initiated trout were unexpected for several reasons. The dose and exposure time used for initiation was determined previously in our laboratory. The expected liver incidence of OTD-fed trout was 10 % . However our OTD-fed trout did not have any tumors. It is possible that fish did not uptake the procarcinogen. On at least one other occasion DMBA immersion has failed to cause any tumors. These discrepancies may be as a result of components in the water (probably season-dependent) that may be complexing with DMBA.

Because no tumors were observed in the controls, we have to make one of two assumptions. One, trout were probably exposed to a low dose, or two, trout were never exposed. Promoters can be classified as true promoters or enhancers. A true promoter increases tumor yields at doses too low to result in tumors with initiator alone. On the other hand, an enhancer increases tumor formation in animals initiated with a dose high enough for control animals to have tumors.

Since no tumors were observed in our control fish, then we can assume that any increase in tumors would have been a true promoting effect. Thus, neither H_2O_2 nor benzoyl peroxide were true promoters. Benzoyl peroxide is a true promoter in the mouse skin carcinogenesis model (5). The lack of promoting effect in our study may have been due to a lack of initiation events.

If trout were never exposed to DMBA, then any tumor observed would have been due to the chronic peroxide exposure. The enhacing effect of H_2O_2 on MNNG-initiated trout results in multiple tumors (chapter 4). Although, multiple tumors is a parameter often used for promotion, we could not rule out the possibility that their formation was due to peroxide exposure. However, the lack of tumors in H_2O_2-fed trout in this study indicates that H_2O_2 is not an initiator.

oh^8dG adducts are used as a marker for oxidative stress (23,24). The oh^8dG content in liver DNA of H_2O_2-fed trout did not differ from controls. OTD-fed trout have consistently shown to weigh more than H_2O_2-fed trout, indicating that H_2O_2-fed trout might have been under oxidative stress. Previously, we showed that trout fed high levels of H_2O_2 contained significantly higher levels of oh^8dG. However, in this study, liver from H_2O_2-fed and OTD-fed trout contained the same oh^8dG adduct levels. The levels reported here fall in the range of some mammalian species under normal conditions. This would suggest that perhaps these trout were not under intensive oxidative stress.

The promoting effect of benzoyl peroxide is unknown, but recent evidence suggests that it may generate singlet oxygen (25) which also produces oh^8dG *in vitro* (26). There is no evidence in the literature that benzoyl peroxide increases oh^8dG *in vivo*. Our data demonstrate that benzoyl peroxide did not generate oh^8dG adduct in liver DNA, yet fish seem to be

under oxidative stress as reflected by their lower body weight (Table 5.1).

In summary, the lack of tumor formation in peroxide-fed trout indicates that these compounds are not complete carcinogens in the trout. The lack of tumor formation in the DMBA-exposed controls does not allow definitive conclusions to be made but suggests the possibility that these compounds may not be true promoters.

Table A.1. The effect of two free-radical generating compounds on liver tumors, liver oh^{8}dG adduct content and body weight of DMBA-exposed sac-fry. Values bearing different supercripts in the same column are significantly different from OTD-fed trout by T'test (P < 0.05)

Table A.1.

Diets	n	Body wt. g. mean± s.d	No. tumors	oh^8dG res/ 10^5 dG res mean± s.d
OTD	83	30 ±11.2[a]	0	1.7 ± 0.4
+ H_2O_2	59	22 ± 5.4[b]	0	1.6 ± 0.1
	75	20 ± 5.3	1	
+ Benzoyl peroxide	64	13 ± 6.3[b]	0	1.8 ± 0.4
	58	18 ± 5.8	0	

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Appendix two

REPAIR OF THE MUTAGENIC 8-HYDROXY-2'-DEOXYGUANOSINE (oh^{8}dG) ADDUCT IN TROUT LIVER UNDER OXIDATIVE STRESS CONDITIONS

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Repair of the mutagenic 8-hydroxy-2'deoxyguanosine (8OHdG) adduct in trout liver under oxidative stress conditions

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ABSTRACT

The 8-hydroxy-2'-deoxyguanosine (oh^8dG) DNA adduct is formed during oxidative stress resulting in G→T transversion mutations in the genome, and, thus, has the potential to activate neoplastic events. The repair rate of this adduct *in vivo* may be an important factor in determining the potential mutational activity. We have attempted to examine the disappearance of liver oh^8dG in trout after exposure to free radical-generating compounds (hydrogen peroxide or benzoyl peroxide) by diet or intraperitoneal administration in time-course experiments. After exposure by dietary means, the number of adduct did not change in the peroxide-fed trout. Intraperitoneal injection of hydrogen peroxide did not increase the adduct formation. Longer dietary exposure or higher doses of hydrogen peroxide by i.p. may be required to assess the repair rate in the sac-fry trout model.

INTRODUCTION

8-Hydroxy-2'-deoxyguanosine (oh^{8}dG) is currently used as an index of oxidative damage to DNA (1,2) and because of its mutagenicity *in vitro* (3,4) and transfecting techniques (5), it may activate protooncogenes or deactivate suppresor genes during carcinogenesis. oh^{8}dG induces G→T transversions (5). Nickel, a carcinogenic metal, enhances oh^{8}dG formation *in vitro* (6), and most of the point mutations in K-ras caused by this metal in mammalian cells are G→T transversions (7). Different oxidative stress protocols enhance expression of c-fos, c-jun, c-myc and Ha-ras oncogenes (8-10), and elevated levels of oh^{8}dG are observed in different carcinogenesis regimens (11), including ductal carcinomas in breast cancer in females (12) where H-ras is overexpressed in 70 % of the breast carcinomas (13). The role of the oh^{8}dG adduct either as an initiator or a promoter during neoplastic development remains to be elucidated (14).

oh^{8}dG is excreted in the urine (2,15), indicating that there is a repair mechanism within cells to prevent potential mutations. Evidence of repair *in vivo* has come from the time-course disappearance of the adduct in mouse liver and rat kidney (16,17).

Recently, an enzyme that removes oh^{8}dG adducts from ds DNA has been purified from *E. coli* (18) and a similar enzyme in human polymorphonuclear neutrophils catalyzes the same reaction (19).

oh^8dG may increase the number of mutations if it is unrepaired, and the rate of its removal may be a significant factor in carcinogenesis. This becomes more critical when extensive oxidative damage overwhelms the repair mechanism and amplified when oxidative stress itself inhibits the repair of other DNA lesions (20). Thus, oxidative stress may inhibit the repair of oh^8dG and other adducts, increasing the mutation frequency in the genome.

Trout are very sensitive to aflatoxin B_1 (21) due, in part, to their inability to effectively repair the aflatoxin-DNA bulky adducts (22). Trout also repair alkylated base adducts (O^6 ethyldG) at a slower rate compared to mammals (23,24). This relatively inefficient repair may also include the oh^8dG adduct. A significant amount of oh^8dG is found in neoplastic livers of feral English sole exposed to high levels of aromatic hydrocarbon carcinogens (25) and the amount is comparable to those found in breast cancer patients (12). Thus, fish may be a good model for assessing the role of oh^8dG in human cancer.

We recently demonstrated a significant positive correlation between enhancement of hepatocarcinogenesis and liver oh^8dG content in trout as a result of chronic exposure to dietary hydrogen peroxide. To understand the correlation with respect to DNA repair mechanisms in the trout model, it is important to determine if trout can effectively repair oh^8dG under oxidative stress conditions. The aim of this

research was to determine i) the rate of repair of oh^8dG, and ii) the effect that oxidative stress has on the adduct repair rate in the trout model.

MATERIALS AND METHODS

Chemicals

Phenol was obtained from AMRESCO (Solon, OH). Acetic acid, NaOH and HCl were purchased from Aldrich (Milwaukee,WI) and citric acid from High Purity Chemical (Portland, OR). Authentic oh^8dG was obtained from Dr. Robert Floyd (Oklahoma Med. Res. Foundation, Oklahoma City, OK). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals and diets

Rainbow trout (*Oncorhynchus mykiss*), initially weighing between 0.6-0.65 g, were fed one of the following diets: Oregon Test Diet (OTD) (26), OTD plus low or high hydrogen peroxide (1500 or 7500 ppm) and OTD plus low or high benzoyl peroxide (1500 or 7500 ppm) for 10 weeks. At the end of the tenth week, all fish were fed OTD for 7 days. A sample of fish from each treatment (4 replicates of at least 4 fish each) were killed at 0, 1, 2, 3 and 7 day post-experimental feeding, the liver excised and immediately immersed in liquid nitrogen.

For intraperitoneal exposure, trout were injected with hydrogen peroxide at two doses: low (37.5 mg/Kg) or high (75.0 mg/Kg). Control fish were injected with vehicle (0.9 % saline) at the same volume required per body weight as hydrogen-peroxide treated trout. Fish were killed at 0, 1, 3, 7 after injection.

DNA and oh^8dG isolation

DNA was isolated from liver as described previously (27)

with the slight modification of adding 1 mM L-histidine to all buffers. DNA was resuspended in 20 mM Na-acetate, 1 mM histidine (pH 4.8), and assayed by the Burton method (26). DNA (35-220 μg/ 200 μl of resuspending buffer) was hydrolyzed with 20 μg of nuclease P_1 in a 95 % ETOH-washed microcentrifuge tube and incubated at 37°C for 30 min. Then, 20-40 μl of 1 M Tris-HCl (pH 7.3) was added to the mixture, followed by alkaline phosphatase (1.1 U), and incubated at 37°C for 60 min.

oh^{8}dG and 2'deoxyguanosine (dG) detection

Volume between 50-80 μl of hydrozylate was injected onto HPLC; flow rate 1 ml/min, ODS1 column (SpherisORB 5 μ, 25 x 0.46 cm). An electrochemical detector (Shimadzu L-ECD-6A), with an applied potential of +600 mV, was used to selectively detect oh^{8}dG. The mobile phase consisted of 12.5 mM citric acid, 25 mM sodium acetate, 30 mM NaOH, 1 mM EDTA and 10 mM acetic acid in 15 % metanol (pH 5.1). Quantitation of nucleosides was done by peak height of authentic oh^{8}dG.

RESULTS

Trout were fed free-radical generating compounds for ten weeks and liver oh^{8}dG adduct levels were detected for the subsequent seven days in a time-course experiment.

OTD-fed trout had significantly higher oh^{8}dG liver adducts at 0 time than trout fed the high hydrogen peroxide diet (Table A.2.1.). However, the benzoyl peroxide-fed group was not different from controls at any level. oh^{8}dG levels did not differ at any other day between controls and either peroxide-fed group. Intraperitoneal injection at the doses given did not seem to affect oh^{8}dG liver levels (Table A.2.2.).

DISCUSSION

We made an attempt to determine the rate of repair of the mutagenic DNA adduct, oh[8]dG, in liver tissue by two routes of exposure- diet and intraperitoneal injection- of two free radical generating compounds.

The time of dietary exposure was arbitrarily chosen, and the levels observed in the peroxide-fed trout at the end of exposure were equal or lower than control. A longer dietary exposure to peroxides may increase the level of the adduct and repair rate could be better assessed. We have previously demonstrated that after 8 months of hydrogen peroxide intake, trout had higher incidences of liver tumors which correlated with higher levels of liver DNA oh[8]dG adducts.

There are no data in the literature indicating that benzoyl peroxide increases oh[8]dG adduct content *in vivo*. However, benzoyl peroxide may generate singlet oxygen (29) which has been shown to produce oh[8]dG *in vitro* (30). Our results, though not conclusive, indicate that benzoyl peroxide does not cause increased levels of oh[8]dG in the liver.

The i.p dose given was chosen after a preliminary experiment indicated that no apparent toxicity was observed after seven days. However, the results suggest that liver oh[8]dG adduct contents were low. Even though the doses injected may have been too low to observe any significant changes, we can not rule out the possibility that sac-fry may have a system that can remove the adduct effectively at low degrees

of oxidative stress.

In summary, the repair rate of liver oh^{8}dG adduct was not successfuly assessed under our oxidative stress conditions.

Table A.2.1. Mean oh^{8}dG liver content after
dietary exposure to two free-radical
generating compounds. Values represent 3-4
pools/treatment/period. Each pool had at
least 4 fish. Values are given as fmol/μg
DNA and their standard error ($\pm$).

Table A.2.1

Diets		Post-feeding days				
		0	1	2	3	7
Control		$30.2^a \pm 5.2$	9.6 ± 4.3	16.8 ± 2.6	14.2 ± 7.5	14.8 ± 3.7
H_2O_2	Low	$19.2^a \pm 3.4$	6.8 ± 1.2	10.5 ± 2.2	7.9 ± 0.6	19.8 ± 3.4
	High	$11.2^b \pm 1.4$	8.9 ± 1.9	10.8 ± 0.8	12.8 ± 2.8	16.6 ± 3.3
Benzoyl	Low	21.3 ± 5.5	8.1 ± 4.0	10.7 ± 1.3	13.2 ± 1.7	12.6 ± 1.9
	High	18.3 ± 2.8	8.8 ± 0.9	12.6 ± 3.8	10.8 ± 2.0	16.2 ± 4.3

Table A.2.2. Mean oh[8]dG liver content after i.p
injection of two doses of hydrogen
peroxide. Values represent at least two
HPLC injections from at least 2 pools/
treatment with 2 fish/pool. Values are
given as fmol/μg DNA and their standard
deviations ($\pm$).

Table A.2.2

Treatment	days			
	0	1	3	7
Sham low	16.1 ±10.0	4.6 ±4.7	6.2 ±6.7	11.4 ±3.4
H_2O_2 low		11.3 ±6.7	2.2 ±3.2	11.7 ±9.4
Sham high	9.4 ±1.6	12.3 ±4.7		11.4 ±5.4
H_2O_2 high		14.7 ±4.1	14.4 ±3.6	9.9 ±3.1

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