AN ABSTRACT OF THE THESIS OF

Oliva Núñez for the degree of Master of Science in Toxicology presented on November 10, 1989.

Title: The Progression of Aflatoxin B_1 (AFB_1) Hepatocarcinogenesis in Rainbow Trout (Oncorhynchus mykiss): I. AFB_1 Metabolism and Cytotoxicity Affecting Hepatocarcinogenesis. II. Ultrastructure of Hepatocellular Neoplasms.

Abstract approved: Redacted for Privacy

The roles of metabolism in aflatoxin B_1 (AFB_1) cytotoxicity and of cytotoxicity in AFB_1 hepatocarcinogenesis in rainbow trout have been examined. Groups of rainbow trout fry were exposed to carcinogenic aqueous solutions of 0.05, 0.1, 0.25, or 0.5 ppm [^{3}H]-AFB_1 for 30 minutes. Another group of fry was fed 500 ppm B-naphthoflavone (BNF) for 1 week before exposure to 0.5 ppm [^{3}H]-AFB_1 for 30 minutes. Subsamples of fish were killed 24 hours and 2 weeks later for DNA-binding and histopathological analysis, respectively. Results indicated a linear dose-response in both DNA-binding and cytotoxicity. BNF treatment resulted in a decrease in both DNA-binding and cytotoxicity. These results suggest

that cytotoxicity, in common with carcinogenicity, is dependent on metabolism of AFB_1 to the electrophilic 8,9-epoxide that can react covalently with cellular macromolecules, and that cytotoxicity contributes to, but is not required for, hepatocarcinogenesis. At 24 hours prior to necropsy groups of fry were exposed to 0 or 0.5 ppm AFB_1 for 30 minutes and subsamples of fish were given [^{3}H]-thymidine ([^{3}H]-TdR) i.p. at a single dose of 5 μCi/g body weight at 0, 1, 3, 7, and 14 days following carcinogen exposure. Autoradiograms showed intense radioactivity in presumptive oval cells which were seen at 14 days after carcinogen exposure, but no labeling in degenerate, necrotic hepatocytes. These results suggest that biliary epithelial cells, presumptive oval cells, are responsible for liver regeneration.

The fine structure of hepatocellular neoplasms from aflatoxin B_1 (AFB_1)-initiated rainbow trout was studied by transmission electron microscopy. Large, usually uniform hepatic nuclei, large nucleoli, abundant, dilated rough-surfaced endoplasmic reticulum, and reduced glycogen storage were common findings in both hepatocellular adenomas and hepatocellular carcinomas. In addition, poorly developed microvilli, forming both the space of Disse and bile canaliculi, were seen in hepatocellular carcinomas. Few or no bile preductule cells were found in hepatocellular carcinomas. A striking

increase in intercellular spaces in hepatocellular carcinomas was also observed. The three latter characteristics of hepatocellular carcinomas suggest a loss of inter-relationships between individual hepatocytes, between hepatocytes and the biliary system, and between hepatocytes and the circulatory system (sinusoids). With respect to these parameters, adenomas were more similar to normal hepatocytes than carcinomas.

The Progression of Aflatoxin B_1 (AFB_1) Hepatocarcinogenesis in Rainbow trout (Oncorhynchus mykiss): I. AFB_1 Metabolism and Cytotoxicity Affecting Hepatocarcinogenesis. II. Ultrastructure of Hepatocellular Neoplasms.

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed November 10, 1989

Commencement June 1990

APPROVED:

Redacted for Privacy

Professor of Food Science and Technology in charge of major

Redacted for Privacy

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Date thesis is presented November 10, 1989

Typed by researcher for Oliva Núñez

"Dedico este trabajo a

mis padres, Antonio y Oliva".

ACKNOWLEDGMENTS

I want to express my gratitude to Dr. Jerry D. Hendricks, my major professor, for his guidance, understanding, and patience which helped me a great deal to complete my program at O.S.U. and to get more insights in fish histopathology.

I thank the other members of my graduate comittee Dr. Christophen Bayne, Dr. David Hinton, and Dr. David Williams, for their reviewing this thesis.

I am very grateful to Dr. Arthur Fong for his help, encouragement, and friendship.

I wish to thank Julie Duimstra for her invaluable help with the Electron Microscopy study.

My sincere gratitude to the members of the Food Toxicology and Nutrition Laboratory for their assistance with the trout and their friendship. Thanks to Daniel Arbogast, Jean Barnhill, Sheila Cleveland, Connie Owston, and Ted Will for making the lab such a comfortable and friendly place to work.

I am deeply grateful to my good friends Jerri, Susan and Pat, and Lani for their encouragment and friendship which helped me so much along the way. Thanks for the enjoyable moments we had together, in particular the "coffee-meetings".

Thanks to Dr. Richard and Mag Scanlan for their warm welcome into their family.

It would have been difficult for me to accomplish my program without the love and confidence of my family. Thanks to my parents, Antonio and Oliva, and to my sisters, Maria-Isabel and Maria-Antonia, for the enthusiasm and cheerfulness given to me.

I also thank faculty members and staff of both the Dept. of Food Science and Technology and the Toxicology Program at O.S.U..

I am grateful to the "INSTITUTO NACIONAL DE INVESTIGACIONES AGRARIAS" of Madrid (Spain) for their financial support.

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CONTRIBUTIONS OF AUTHORS

In addition to my major professor, Dr. Jerry Hendricks, the contributions of other authors of the manuscripts were as follows:

Chapter II: Dr. Arthur T. Fong helped with the experimental design and the isolation of DNA.

Chapter III: Julie R. Duimstra assisted with the preparation of tissues for Transmission Electron Microscopy.

THE PROGRESSION OF AFLATOXIN B_1 (AFB1) HEPATOCARCINOGENESIS IN RAINBOW TROUT (Oncorhynchus mykiss): I. AFB_1 METABOLISM AND CYTOTOXICITY AFFECTING HEPATOCARCINOGENESIS. II. ULTRASTRUCTURE OF HEPATOCELLULAR NEOPLASMS.

I. INTRODUCTION

The liver has been studied extensively as a target for chemical carcinogenesis because of the broad knowledge about its cellular and molecular biology and pathology (Farber 1980, Pitot et al. 1987).

Hepatocarcinogenesis is a multistep process in which a sequence of biochemical, cytological, and functional events lead to the development of hepatic neoplasms (Bannasch 1976, Farber & Cameron 1980, Bannasch et al. 1982, Bannasch & Zerban 1986). At least three stages have been characterized during hepatocarcinogenesis, although no definite boundaries can be discerned among them: a) initiation, an irreversible step in which a biochemical lesion to DNA is fixed into the genome by cell replication, b) promotion, a reversible stage in which the former initiated cells are cloned by cell proliferation, and c) progression, an irreversible step which is characterized by the development of a neoplasm

(Cameron et al. 1979, Farber 1984). Cytological and cytochemical changes of progression of hepatocarcinogenesis have been well described in rodents (Farber & Cameron 1980). However, the progression of hepatocarcinogenesis has received less attention in non-mammalian systems, e.g., teleosts (Stanton 1965, Couch & Courtney 1987, Hinton et al. 1988).

The use of fish in carcinogenesis can be viewed from three standpoints: 1) as a non-mammalian alternative animal model in cancer research, 2) as a biological indicator for monitoring environmental pollutants, and 3) as a low cost testing system for identifying potential human chemical carcinogens (Hawkins et al. 1985, Hawkins et al. 1988a, Hawkins et al. 1988b, Hawkins et al. 1988c, Hendricks et al. 1980b, Hendricks et al. 1985, Schoenhard et al. 1981, Sinnhuber et al. 1974). The trout model for cancer research provides several advantages, in appropiate facilities with high volumes of cold water, including the use of, 1) high numbers of animals at low cost as compared to rodents, 2) different routes of chemical exposure (i.e., water, diet, embryo microinjection), and 3) different life stages (embryo, sac-fry, fry, or adult) (Bailey et al. 1989).

The liver is the organ most susceptible to neoplastic change in bony fishes in general (Wellings

1969) and in salmonids in particular (Wood & Larson 1961). Plehn (1909) reported the first hepatic tumor in a brown trout, a multilocular cystadenoma of biliary origin, while Haddow and Blake (1933) reported the first hepatocellular tumor in a rainbow trout. Other reports of hepatic neoplasms in salmonids occurred in the 1950's (Scolari 1953, Nigrelli 1954, Cudkowicz & Scolari 1955, Nigrelli & Jakowska 1955, Honma & Shirai 1959, Levaditi et al. 1960), but in the early 1960's, hepatocellular neoplasia in hatchery-reared rainbow trout reached epizootic proportions in the United States (Ashley & Halver 1961, Ghittino 1961, Hueper & Payne 1961, Nigrelli & Jakowska 1961, Rucker et al. 1961, Snieszko 1961, Wood & Larson 1961). This epizootic was linked to dietary aflatoxin exposure, so that elimination of contaminated feed ingredients and diet surveillance have essentially eliminated this problem from salmonid culture.

Experimental carcinogenesis initiated by aflatoxins and other carcinogens has been studied extensively in rainbow trout (Hendricks 1982). Subsequent research in trout carcinogenesis has focused on the inhibition and/or promotion of carcinogenesis by food-borne or environmental chemicals and the mechanisms thereof (Grieco et al. 1978, Hendricks et al. 1980a, Nixon et al. 1984, Goeger et al. 1986, Shelton et al. 1986, Bailey et

al. 1987, Dashwood et al. 1988, Bailey et al. 1989, Dashwood et al. 1989). Nevertheless, the sequence of morphological events following exposure to chemical carcinogens and the biochemical bases for the morphological changes have not been systematically studied. In particular, the relationships among carcinogen dose, DNA binding, target cells, cell specific cytotoxicity, regeneration, and the development of neoplasia have not been defined.

The ultrastructure of normal trout liver has been well characterized (Hampton et al. 1985, Hampton et al. 1988a, Hampton et al. 1989), however, ultrastructural studies of experimentally initiated hepatic tumors in rainbow trout are conspicuously lacking. Only a single paper describes the ultrastructure of neoplastic hepatocytes from hatchery reared tumor-bearing trout (Scarpelli et al. 1963).

The aims of the present study were:

1) To investigate the inter-relationships among carcinogen dose, DNA-binding, target cells, cytotoxicity, and early regeneration during the initial two weeks, following AFB_1 exposure to fry.

2) To describe in detail the ultrastructural features and architectural changes from normal to hepatocellular neoplasms (both adenomas and carcinomas) in AFB_1-initiated rainbow trout.

II. Inter-relationships among aflatoxin B_1 (AFB_1) metabolism, DNA-binding, cytotoxicity, and hepatocarcinogenesis in rainbow trout (Oncorhynchus mykiss).

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ABSTRACT

The roles of metabolism in aflatoxin B_1 (AFB_1) cytotoxicity and of cytotoxicity in AFB_1 hepatocarcinogenesis in rainbow trout have been examined. Groups of rainbow trout fry were exposed to carcinogenic aqueous solutions of 0.05, 0.1, 0.25, or 0.5 ppm [^{3}H]-AFB_1 for 30 minutes. Another group of fry was fed 500 ppm B-naphthoflavone (BNF) for 1 week before exposure to 0.5 ppm [^{3}H]-AFB_1 for 30 minutes. Subsamples of fish were killed 24 hours and 2 weeks later for DNA-binding and histopathological analysis, respectively. Results indicated a linear dose-response in both DNA-binding and cytotoxicity. BNF treatment resulted in a decrease in both DNA-binding and cytotoxicity. These results suggest that cytotoxicity, in common with carcinogenicity, is dependent on metabolism of AFB_1 to the electrophilic 8,9-epoxide that can react covalently with cellular macromolecules, and that cytotoxicity contributes to, but is not required for, hepatocarcinogenesis. In a separate experiment, groups of fry were exposed to 0 or 0.5 ppm AFB_1 for 30 minutes and subsamples of fish were given [^{3}H]-thymidine ([^{3}H]-TdR) i.p. at a single dose of 5 μCi/g body weight at 0, 1, 3, 7, and 14 days following carcinogen exposure, 24 hours prior to necropsy.

Autoradiograms showed intense radioactivity in presumptive oval cells which were seen at 14 days after carcinogen exposure, but no labeling in degenerate, necrotic hepatocytes. These results suggest that presumptive oval cells are responsible for liver regeneration.

INTRODUCTION

Aflatoxin B_1 (AFB_1), produced by certain strains of Aspergillus flavus and A. parasiticus, is a potent hepatotoxin and hepatocarcinogen in rainbow trout (Halver 1967, Bauer et al. 1969, Sinnhuber et al. 1977). It has been established that in the trout AFB_1 is metabolized by cytochrome P-450-dependent enzyme systems (Williams & Buhler 1983) to produce the electrophilic AFB_1-8,9-epoxide, and the covalent binding of AFB_1-8,9-epoxide to DNA has been proposed to be a critical event in the initiation of AFB_1 carcinogenesis (Swenson et al. 1977).

In addition to AFB_1-8,9-epoxide, several other metabolites of AFB_1 including aflatoxicol (AFL), aflatoxin M_1 (AFM_1), and aflatoxicol M_1 (AFL-M_1) also are produced in control uninduced trout (Loveland et al. 1983). Glucuronides of AFL and AFL-M_1 are the major and minor biliary phase II conjugates, respectively, in the untreated trout (Loveland et al. 1984). In contrast to mammalian systems in which glutathione (GSH) conjugation of the AFB_1-8,9-epoxide is a major detoxification pathway (Degen & Neumann 1978), GSH conjugation does not contribute significantly to the detoxification of AFB_1 in trout (Valsta et al. 1988).

Modification of AFB_1 metabolism and carcinogenesis in rainbow trout by several compounds including flavonoids, indoles, and polychlorinated biphenyls has been extensively studied in our laboratory (Hendricks et al. 1982, Bailey et al. 1984, Loveland et al. 1984, Nixon et al. 1984, Goeger et al. 1986, Shelton et al. 1986, Dashwood et al. 1988, Dashwood et al. 1989). Pertinent to the objectives of this report are previous studies on the inhibitory effects of B-naphthoflavone (BNF) on AFB_1 carcinogenesis. Williams & Buhler (1984) reported on the induction of the cytochrome P-450 isozyme, LM_{4b}, by BNF. The induction of cytochrome P-450 LM_{4b} results in a shift of AFB_1 metabolism away from the formation of AFL, a highly carcinogenic metabolite, to the formation of AFM_1 and AFL-M_1, metabolites of much less carcinogenic potential (Loveland et al. 1983). Significantly higher levels of AFL-M_1 glucuronide and reduced DNA binding were observed in BNF-treated trout compared to control trout after AFB_1 exposure (Bailey et al. 1984, Loveland et al. 1984, Goeger et al. 1986). Therefore, the observed inhibition of AFB_1 carcinogenesis by BNF (Nixon et al. 1984, Goeger et al. 1986) appears to result from the altered metabolism of AFB_1 to less carcinogenic metabolites, increased conjugate formation, and reduced DNA binding.

Depending on the dose of AFB_1 administered, cytotoxicity often occurs as a concurrent lesion during experimental carcinogenesis. However, it is unclear whether or not cytotoxicity is causally related to carcinogenesis. Studies by Hoel et al. (1988) showed that cytotoxicity was not required for carcinogenesis but when it occurred, a promotional mechanism due to subsequent cell proliferation was postulated. Many hepatotoxins that cause cytotoxicity require metabolism to active metabolites which react covalently with tissue macromolecules (Zimmerman 1978). Nevertheless, the relationship between covalent DNA binding and cytotoxicity remains unclear.

In rainbow trout, the sequence of morphological events following exposure to chemical carcinogens and the biochemical bases for the morphological changes have not been systematically studied. In particular the relationships among carcinogen dose, DNA binding, cell specific cytotoxicity, regeneration, and the development of neoplasia have not been defined. In this report we have focused on the initial two weeks following AFB_1 exposure in order to define the inter-relationships among carcinogen dose, DNA-binding, target cells, cytotoxicity, and early regeneration.

Thymidine is incorporated exclusively into DNA. Therefore, tritiated thymidine ([^{3}H]-TdR) incorporation has been used as an effective technique for studying physiological processes such as proliferation, maturation, and migration of cells under natural or pathological situations (Hughes et al. 1958). In the present study, autoradiography was used after i.p. administration of [^{3}H]-TdR in order to evaluate which cell population was undergoing proliferation in the early cytotoxic/regenerative step of hepatocarcinogenesis.

MATERIALS AND METHODS

Experiment 1:

Protocol.- Groups of 55 fry (3 weeks after swim-up) reared at the Food Toxicology and Nutrition Laboratory, Oregon State University, were exposed to static aqueous solutions of carcinogenic doses of 0.05, 0.1, 0.25, or 0.5 ppm [^{3}H]-AFB_1 (250 µCi/l H_2O) for 30 minutes. A separate group of fry was fed 500 ppm BNF for 1 week before exposure to 0.5 ppm [^{3}H]-AFB_1 for 30 minutes. Following carcinogen exposure, the fish were transferred to fresh water without AFB_1 and triplicate groups of 15 fish each were killed 24 hours after carcinogen exposure. Livers were removed, pooled, and frozen in liquid N_2 before storing at -70° C.

Isolation of DNA.- Frozen pellets of 15 pooled livers each were crushed and powdered in a mortar and pestle, in liquid N_2, and transferred to 12 ml polypropylene tubes, to which 1.5 ml Tris-buffer (100 mM NaCl, 10 mM Tris, 10 mM EDTA; pH 7.4) containing 1 % sodium dodecyl sulfate (SDS) was added. The liver samples were treated with RNase A (500 µg in 50 µl buffer) for 30 minutes at 37° C, and then incubated overnight with proteinase K

(250 µg in 50 µl buffer) at 37° C. After adding 1.6 ml Tris-saturated phenol to each tube, the samples were mixed by inversion for 30 minutes at room temperature. The aqueous layer was separated from the phenol layer by centrifugation (3000 x g, 30 minutes). The resulting upper aqueous layer was transferred to new tubes, and then extracted once with Tris-saturated phenol and three times with isoamyl alcohol:chloroform (1:24) (Dashwood et al. 1988).

The DNA was precipitated with 160 µl 4 M Sodium acetate (NaOAC) and 3.2 ml ice-cold ethanol, and collected by centrifugation (5000 x g, 5 minutes). The precipitate was washed three times with ethanol to remove unbound AFB_1, and dried under a gentle stream of N_2. The DNA was dissolved in 1.0 ml Tris-buffer, an aliquot of which was taken for DNA determination by the fluorometric method of Cesarone et al. (1979) and a further aliquot was assayed for radioactivity by liquid scintillation counting.

Data from [^{3}H]-AFB_1-DNA binding were analysed by weighed linear regression analysis (Steel & Torrie 1980).

Histopathology.- Groups of 10 fish were sampled 2 weeks after carcinogen exposure. Livers were removed and fixed in Bouin's solution for 48 hours. Livers were processed by routine methods (Luna 1960), embedded in

paraffin, sectioned at 4 μm and stained with Gill's hematoxylin and eosin (H & E) for histologic examination.

Experiment 2:

Protocol.- Groups of 30 fry (3.2 ± 0.72 g) were exposed to static aqueous solutions of 0 (control) or 0.5 ppm AFB_1 for 30 minutes. The fish were transferred to fresh water without AFB_1 after carcinogen exposure. Subsamples of 5 fish from each group were each given ([^{3}H]-TdR) (specific activity = 55 Ci/mmole) i.p. as a dose of 5 μCi/g body weight (5 μl/g body weight, in sterile water) at 0, 1, 3, 7, and 14 days following carcinogen exposure. The fish were killed 24 hours after [^{3}H]-TdR injection, livers were rapidly removed, cut in two slices with an alcohol-cleaned razor blade, and fixed in 1% glutaraldehyde-1.5% formaldehyde-0.01% picric acid in 0.1 M phosphate buffer pH 7.2, for 48 hours at 4°C. Fixed livers were dehydrated in graded alcohols. These alcohols were checked for residual radioactivity levels by liquid scintillation counting. Tissues were embedded in paraffin, sectioned at 4 μm, and placed on alcohol-cleaned glass slides.

Autoradiography.- Sections were coated with Kodak NTB2 emulsion (diluted 1:1 with water) in a darkroom under

safelight conditions (Kodak filter NO. 2), placed in small black boxes containing drierite, sealed with Scotch No. 33 photographic tape, and slides were kept for 6 weeks at 4°C.

Autoradiograms were developed under safelight conditions in Kodak Dektol developer (1:1) for 2 minutes, rinsed in distilled water for 10 seconds, fixed in Kodak fixer for 5 minutes, and washed in distilled water for 5 minutes (Williams 1985). Slides were counterstained with H & E. In non-overlapping fields, a minimum of 100 hepatocytes were analyzed under high power (100 X). Similarly, the percentage of 100 biliary epithelial cells showing label was determined. A nucleus with 5 or more grains was considered positive.

Chemicals:

[^{3}H(G)] AFB_1 (Moravek Biochemicals, Inc., Brea, CA) was checked for purity by UV spectrometry and TLC (Loveland et al. 1983). [^{3}H]-TdR (55 Ci/mmole) was purchased from ICN Radiochemicals, Irvine CA. Phenol was purchased from Clontech Labs (Palo Alto, CA), and all other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

RESULTS

Biochemical analysis:

Results from *in vivo* [^{3}H]-AFB_1-DNA binding analysis are presented in Figure II.1. Linearity in [^{3}H]-AFB_1-DNA binding dose-response curve and a significant ($P < 0.001$) decrease in DNA binding in fish induced by BNF are shown. These results were found 24 hours after carcinogen exposure at which time [^{3}H]-AFB_1-DNA binding has been reported to be maximun following i.p. administration (Goeger *et al.* 1986). It was also observed that BNF pretreatment resulted in a decrease in DNA binding to a level comparable with the 0.1 ppm [^{3}H]-AFB_1 dose treatment in the control fish.

Histopathology:

Hepatocellular alterations from rainbow trout 2 weeks after exposure to [^{3}H]-AFB_1 are shown in Table 1. Livers, in which cytotoxic damage was seen microscopically had a striking white color when examined macroscopically. Early cytotoxic changes including nuclear and cytoplasmic swelling were observable 1 week after [^{3}H]-AFB_1 exposure, but by 2 weeks, cytotoxicity was well advanced revealing increasing severity with increasing dose (Table 1).

Normal trout liver is organized in a tubular pattern (Hampton et al. 1985). Polygonal hepatocytes were organized as tubules 2 cells wide in longitudinal section but 5 to 8 cells formed the tubules in cross section (Fig. II.2). In the present study cytotoxicity was classified into three categories according to its severity (Table 1). Fish exposed to 0.5 ppm [^{3}H]-AFB_1 presented severe cytotoxicity which was generalized throughout the liver. The normal tubular architecture was no longer discernible due to hepatocyte swelling and necrosis. Surviving hepatocytes exhibited foamy cytoplasm and pleomorphic nuclei (Fig. II.3). Small basophilic cells with scanty cytoplasm were seen interspersed among degenerating hepatocytes. In many cases, they appeared to originate from centrotubular regions, the location of bile ductules or presumptive oval cells. In contrast with rodent oval cells (bile ductule cells) of the canals of Hering, which are localized in periportal regions only, presumptive oval cells (bile ductule cells) in rainbow trout are localized throughout the liver in centrotubular (bile canalicular) locations (Hampton et al. 1988). Their size varied from very small with oval-shaped nuclei to larger polygonal cells with more rounded or irregularly shaped nuclei. The cells are presumed to be of similar origin, so variability in size and shape may reflect

age and degree of differentiation. Some of the cells occupied normal-appearing hepatic tubules, some formed ductules, while others had not yet acquired any organizational substructure. Mitoses were observed frequently and cell proliferation was not localized to any particular region of the liver (Fig. II.3). In spite of the severe liver damage no mortalities were found in fish exposed to 0.5 ppm [^{3}H]-AFB$_1$.

Focal cytotoxicity was seen mostly in livers from fish exposed to 0.25 ppm [^{3}H]-AFB$_1$ (Table 1). Degenerative changes and necrosis in the focal areas were comparable with the severe cytotoxicity observed in the 0.5 ppm [^{3}H]-AFB$_1$ group. Swollen hepatocytes with foamy cytoplasm disrupted normal architecture. Other hepatocytes, though non-necrotic, showed toxic effects such as swelling, atypical nuclei, loss of glycogen, and accentuation of intercellular spaces (Fig. II.4). Proliferation of presumptive oval cells was common in cytotoxic areas but rare in non-necrotic parenchyma (Fig. II.4).

Mild cytotoxicity was also seen in some livers from fish exposed to 0.25 ppm [^{3}H]-AFB$_1$ (Table 1). Livers in this category retained tubular architecture but degenerating hepatocytes were found scattered throughout the liver. In addition, surviving hepatocytes revealed glycogen depletion and nuclear atypia. Proliferation of

presumptive oval cells was also evident although to a lesser extent than in cases of severe or focal cytotoxicity (Fig. II.5).

Neither 0.1 ppm nor 0.05 ppm [^{3}H]-AFB_1 caused obvious cytotoxic effects in livers (Fig. II.6) except that atypical nuclei (pleomorphism in both size and shape) were observed occasionally. Since these observations were not quantitated they are not mentioned in Table 1. Glycogen levels were comparable to controls.

Fish fed 500 ppm BNF for 1 week prior to exposure to 0.5 ppm [^{3}H]-AFB_1 showed a significant ($P < 0.001$) decrease in cytotoxic response (Table 1). Normal liver architecture (Fig. II.7) was found in all but one liver that showed focal cytotoxicity (Table 1).

Autoradiography:

Labeled hepatocytes from control livers were located predominantly at the periphery of the liver (Fig. II.8). Fish exposed to 0.5 ppm [^{3}H]-AFB_1 showed severe cytotoxicity at 2 weeks, as was described in experiment 1. No labeling of hepatocytes was observed, however, presumptive oval cells, which were proliferating intermixed with degenerating hepatocytes, were strongly labeled (Fig. II.9). Percentages of labeled presumptive oval cells ranged from 9.3% to 29.6% in the fish analized 2 weeks after AFB_1 exposure.

DISCUSSION

Metabolism of AFB_1 to the electrophilic 8,9-epoxide, which binds covalently to macromolecules such as DNA, is required for AFB_1 carcinogenicity (Miller 1978). Consequently, DNA adduct formation can be used as an indicator of the carcinogenic potential of AFB_1 (Dashwood et al. 1989). Results from the present study showed a linear dose-response in [^{3}H]-AFB_1-DNA binding following exposure of fry to static solutions of [^{3}H]-AFB_1 (Fig. II.1). Even though none of these fish were maintained until tumors development, the present [^{3}H]-AFB_1 treatments would be carcinogenic for the following reasons. 1) A recent experiment in our laboratory tested the carcinogenicity of a broader but similar range of AFB_1 doses (0.01, 0.025, 0.05, 0.1, 0.25, 0.5 ppm) in trout embryos and showed a dose-responsive carcinogenicity beginning at even the 0.01 ppm level (unpublished results). Other experiments have shown repeatedly a greater sensitivity of fry than embryos to a given dose of carcinogen (unpublished results). 2) The levels of [^{3}H]-AFB_1-DNA binding found in the present study are in the range of DNA adduction that results in a significant final tumor response (Dashwood et al. 1989). Thus, each of the doses used in the present short-term study would

be expected to have a significant carcinogenic effect.

The relationship between cytotoxicity and carcinogenicity is of considerable interest. Hoel et al. (1988) concluded, after comparing 2-year rodent studies involving 99 chemicals, that a relatively small percentage of chemical carcinogens may act through a secondary mechanism such as cytotoxicity rather than a direct interaction with cellular DNA. In the present study, [^{3}H]-AFB_1 cytotoxicity also showed a dose-dependent response (Table 1). A relationship between the metabolic activation of [^{3}H]-AFB_1 and AFB_1 cytotoxicity was observed. Furthermore, the results from BNF-induced fish, which showed a significant reduction in cytotoxic effects, and reduced DNA binding (which may indicate reduced 8,9-epoxide formation) support the suggested relationship between AFB_1 activation and cytotoxicity. Although [^{3}H]-AFB_1-DNA binding, which was used in this study as an indirect measure of the production of reactive AFB_1 metabolites, correlates well with cytotoxicity, it is unlikely that DNA binding is directly responsible for cytotoxicity. At least two sets of evidence support this conclusion. First, as stated earlier, each of the doses administered in the present study resulted in DNA binding but only the 0.25 and 0.5 ppm doses caused observable cytotoxicity. Therefore,

binding of the 8,9-epoxide to other cellular macromolecules at high doses may be necessary for cytotoxicity. Appleton et al. (1982) investigated AFB_1 binding in rat liver and determined the relative levels of binding in RNA, DNA, and protein. Consistently the levels of binding were greater in RNA, intermediate in DNA and lowest in protein. Doses of AFB_1 used were low and no cytotoxic effects were mentioned. Second, cytotoxicity induced by other hepatotoxins such as bromobenzene (Reid & Krishna 1973) and acetaminophen (Jollow et al. 1973) seems to be linked primarily to cytoplasmic protein binding with only minimal nuclear binding. Thus at present we do not have definitive evidence on the critical macromolecular target for AFB_1 binding or possible other mechanisms involved in cytotoxicity.

Initiation of liver target cells is currently thought to require at least 2 steps (Columbano et al. 1981). These are covalent binding of an electrophile to DNA, and subsequent fixation of the DNA lesion into the genome by cell replication. Cytotoxicity may be linked to increased carcinogenicity through compensatory cell proliferation (Figs. II.2 & II.3). Questions logically arise as to which cell type(s) in the liver is(are) initiated by chemical carcinogens. At least 3

possibilities exist: 1) primitive stem cells, 2) hepatocytes or 3) bile ductule (oval) cells. Lombardi (1982) concluded that there is no direct evidence for the existence of stem cells in the liver. It is generally accepted, at the present time, that hepatocytes are the critical target cells since they possess the highest activity of mixed function oxidase (MFO) enzymes and metabolize procarcinogens to electrophilic species (Farber et al. 1977). Initiation is thought to produce a few altered hepatocytes which become resistant to the cytotoxic and cytostatic actions of chemical carcinogens and can proliferate under the compensatory growth stimulus of necrosis or partial hepatectomy (Farber 1981). Such is the hypothesis explaining the rapid emergence of gamma-glutamyl transpeptidase (GGT) positive foci in rat liver after carcinogen exposure, partial hepatectomy and dietary exposure to the cytostatic chemical 2-acetylaminofluorene (2-AAF) (Solt et al. 1977). An alternative hypothesis is that the GGT (+) foci originate from oval cells, which inheritantly have traits similar to the proposed altered hepatocytes, i.e. GGT positivity and resistance to the cytotoxic/cytostatic properties of many carcinogens (Lombardi 1982). In an effort to determine which cells in the liver are initiated by carcinogens, Sell et al. (1981) fed rats a

diet containing 2-AAF for 7 days along with multiple doses of [^{3}H]-TdR from days 4 through 6. Rats were killed on the 7th day and livers, stained for GGT, were processed for autoradiography. There was no labeling of hepatocytes, but GGT (+) cells in the portal triad regions were heavily labeled and displayed frequent mitotic figures. These results show that oval cells were not only resistant to the cytotoxic action of carcinogens but appeared to be stimulated to replicate their DNA and proliferate. On the other hand, hepatocytes underwent regressive changes resulting in cell necrosis.

Although oval cells have low monooxygenase activity (about 1 order of magnitude less than hepatocytes) (Lombardi 1982), and would be expected to produce a corresponding lower rate of DNA-adduction, their ability to survive and proliferate in the toxic environment of carcinogen exposure may make them more likely than hepatocytes to be the critical target cells of carcinogens. Since there is evidence that oval cells can differentiate into hepatocytes as well as bile ducts (Grisham 1980), they could be the progenitors of both hepatocellular and cholangiocellular tumors of the liver (Hampton et al. 1988). Previous studies in our laboratory have shown that mixed carcinomas are the predominant tumor type in rainbow trout experimental carcinogenesis

(Lee et al. 1989a, Nunez et al., in press). In the present study, autoradiographs showed labeling of presumptive oval cells intermixed with degenerating hepatocytes which were not labeled (Fig. II.9). These results are in agreement with those cited from rat studies and support the hypothesis that regeneration following cytotoxicity most likely originates with oval cells.

The hypothesis that oval cells are the target cells for carcinogen initiation and liver regeneration has greatest credibility when cytotoxic carcinogen doses are administered. Such doses are both cytotoxic and cytostatic to hepatocytes whereas oval cells may survive and proliferate either due to the compensatory demand created by hepatocyte loss or possibly by some unknown direct action of the carcinogen. The hypothesis has less credibility, however, when we consider non-cytotoxic doses of carcinogen. Lower doses of carcinogens would result in less DNA-binding to both hepatocytes and oval cells. Since there would be no hepatocyte loss, there would be no compensatory demand for proliferation. If "initiated" hepatocytes are capable of mitosis, they would seem to be better candidates as target cells than oval cells under these conditions. The problem is that we know very little about the life span, differentiation

and mitotic activity of normal, mature hepatocytes in fish. The source of normal liver growth or hepatocyte replacement is not well known. Whether new cells originate from mature hepatocytes, immature hepatocytes, bile ductule (oval) cells or stem-like cells has not been determined. We do not know if hepatocytes become post-mitotic and if so whether or not that condition is reversible. As a result, we do not know whether mature, differentiated, but "initiated" (by DNA adduction) hepatocytes are capable of mitotic activity. Since trout liver is not arranged into lobules, as in mammals, it has been assumed that the trout liver is homogeneous throughout (Hampton et al. 1985, Hampton et al. 1988). Based on iron staining and other histochemical stains, Lee et al. (1989b) suggested that a peripheral zone of more immature hepatocytes exists in rainbow trout. Tritiated thymidine incorporation in normal livers in the current study was noticeably concentrated at the periphery of the livers as well. This may indicate that peripheral hepatocytes are more immature and mitotically active than more centrally located hepatocytes. If so, this may relate to the observation that the vast majority of hepatocellular tumors in rainbow trout originate at or near the surface of the liver (Hendricks et al. 1984). We acknowledge that little is known about presumptive

oval cells in fish (Couch & Courtney 1987, Hampton et al. 1988, Hinton et al. 1988). Morphologically, there appear to be cells that resemble mammalian oval cells, but the lack of positive zonation for identification and specific experiments to determine their properties causes their occurrence and role in carcinogenesis to remain speculative at the present time. Additional research in the areas of hepatocyte and oval cell biology in fish are needed to answer the questions generated by this work.

Table 1. Hepatocellular alterations in rainbow trout fry exposed 2 weeks earlier to [^{3}H]-AFB_1.

Dose (ppm)	Cytotoxicity		
	Severe	Focal	Mild
0.5	8 /8	0 /8	0 /8
0.25	4 /11	4 /11	3 /11
0.1	0 /10	0 /10	0 /10
0.05	0 /8	0 /8	0 /8
0.5[1]	0 /11	1 /11	0 /11

[1] Fry were fed 500 ppm BNF for 1 week prior to exposure.

Figure II.1 In vivo DNA-binding dose-response curve for AFB_1 in rainbow trout fry. Data are means ± SD from three pools of 15 animals each; correlation coefficient = 0.913; (-0-) BNF pretreated trout, P-value < 0.001 from Student's t test (Steel & Torrie 1980).

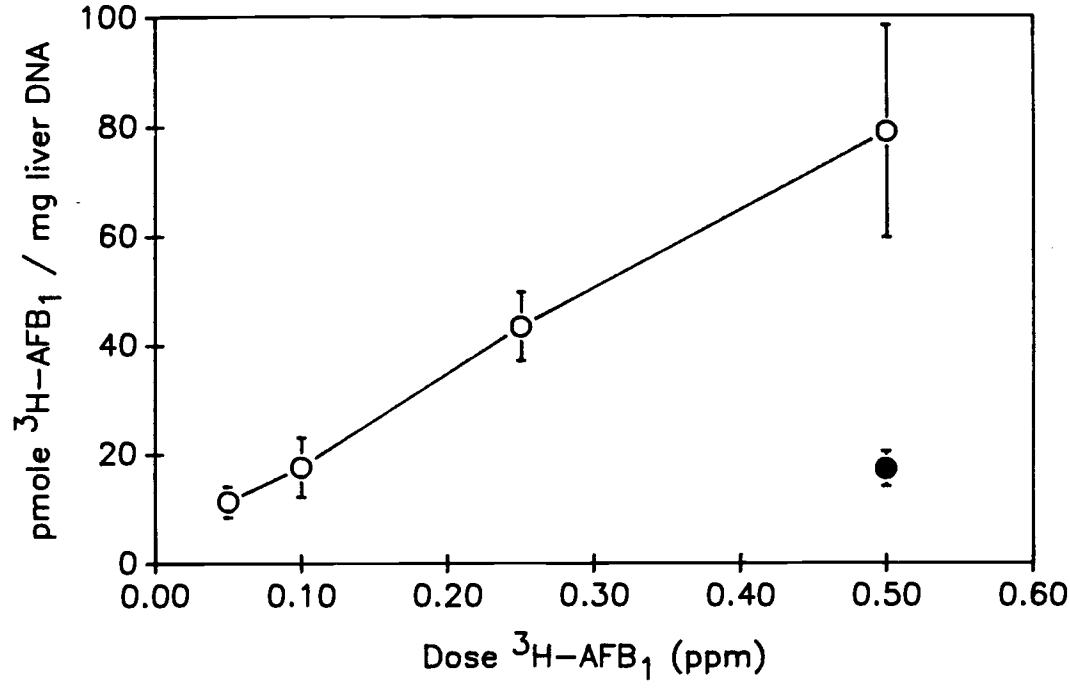
100
80
60
40
20
0
pmole ^{3}H–AFB$_1$ / mg liver DNA
0.00
0.10
0.20
0.30
0.40
0.50
0.60
Dose ^{3}H–AFB$_1$ (ppm)

Figure II.1

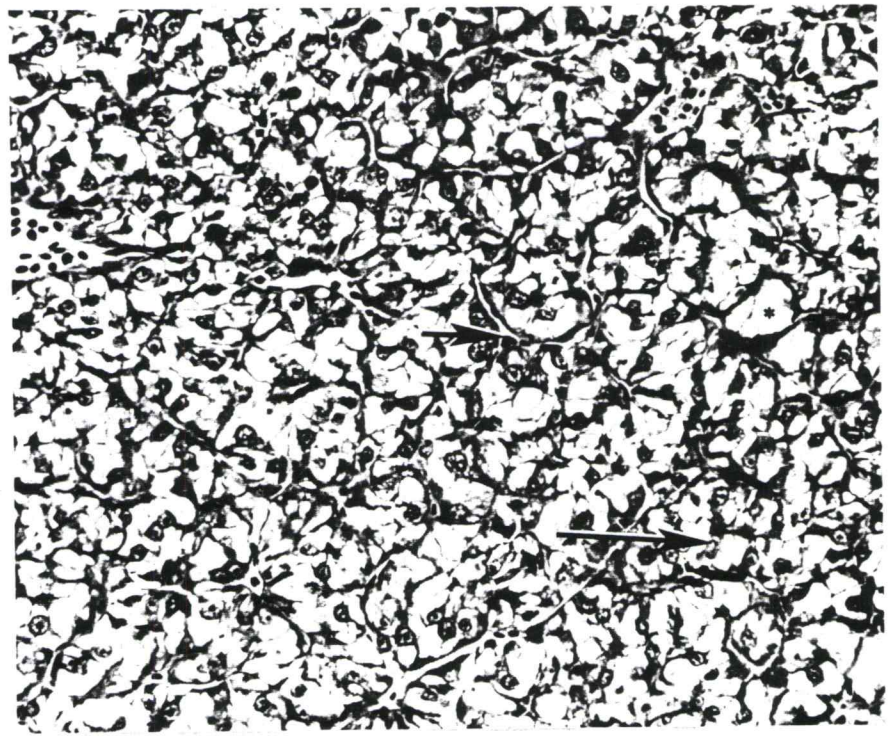

Figure II.2 Normal trout liver. Hepatocytes are organized in a tubular architectural pattern, interspersed with sinusoids (small arrow). Tubules are 2 cells-thick in longitudinal section (large arrow). High glycogen (*) is a normal feature in young trout livers. X 375. H & E.

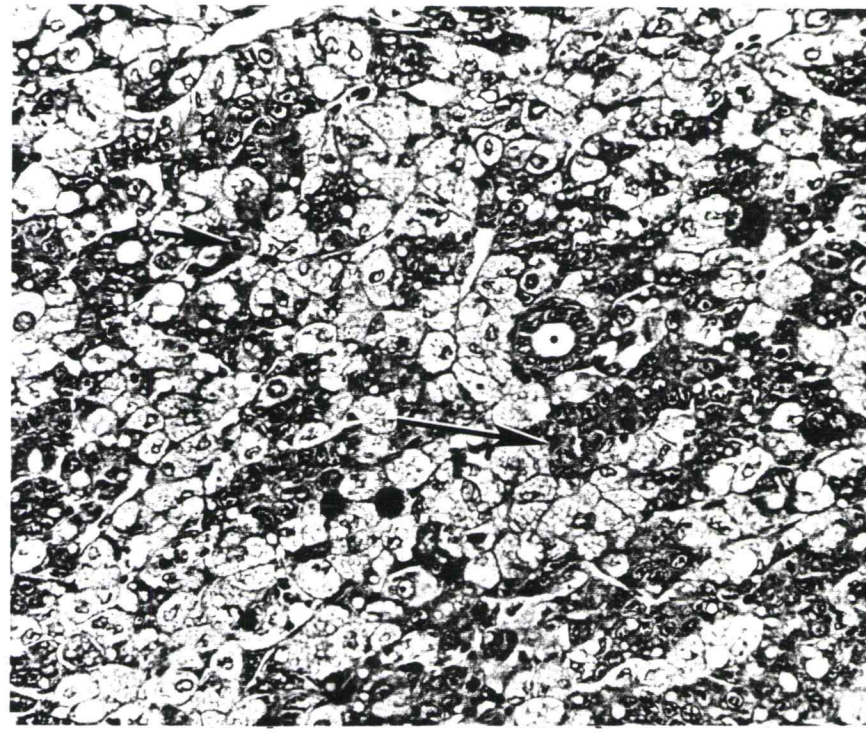

Figure II.3 Severe cytotoxicity in a fish exposed 2 weeks earlier to 0.5 ppm [^{3}H]-AFB_1 for 30 minutes. Liver architecture is disrupted, sinusoids are occluded due to dilation of hepatocytes which show foamy cytoplasm. Strongly basophilic cells, presumptive oval cells, (large arrow) are seen interspersed with degenerating or necrotic hepatocytes. Mitotic figures are frequent among basophilic cells (small arrow). Note the presence of an atypical ductule (*). X 375. H & E.

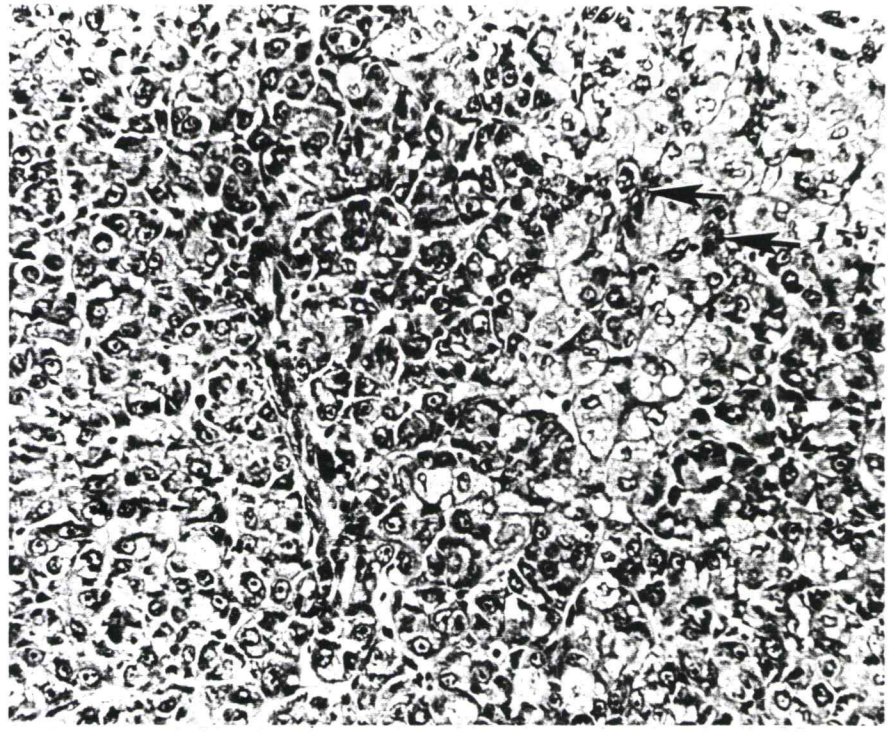

Figure II.4 Focal cytotoxicity in a fish exposed 2 weeks earlier to 0.25 ppm [^{3}H]-AFB_1 for 30 minutes. Degenerating hepatocytes with foamy cytoplasm are interspersed with presumptive oval cells (arrows). Non-necrotic hepatocytes on the left have greatly reduced glycogen content, and some nuclear atypia. X 375. H & E.

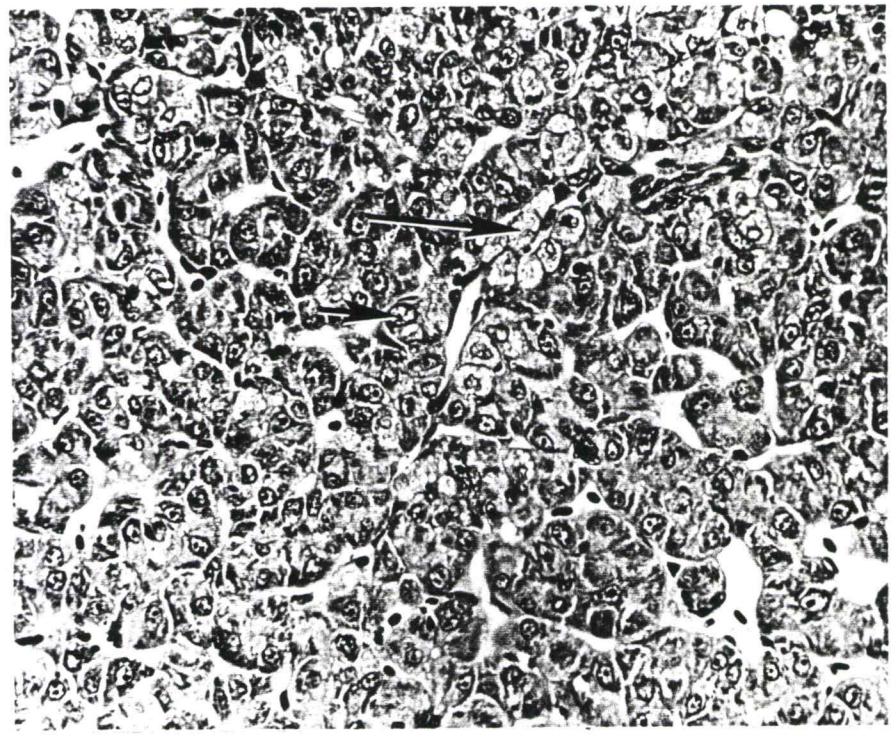

Figure II.5 Mild cytotoxicity in a fish exposed to 0.25 ppm [^{3}H]-AFB_1 for 30 minutes. Swollen hepatocytes with foamy cytoplasm (large arrow) are seen scattered throughout the parenchyma. Nuclear atypia (small arrow) and reduced glycogen are also observed. X 375. H & E.

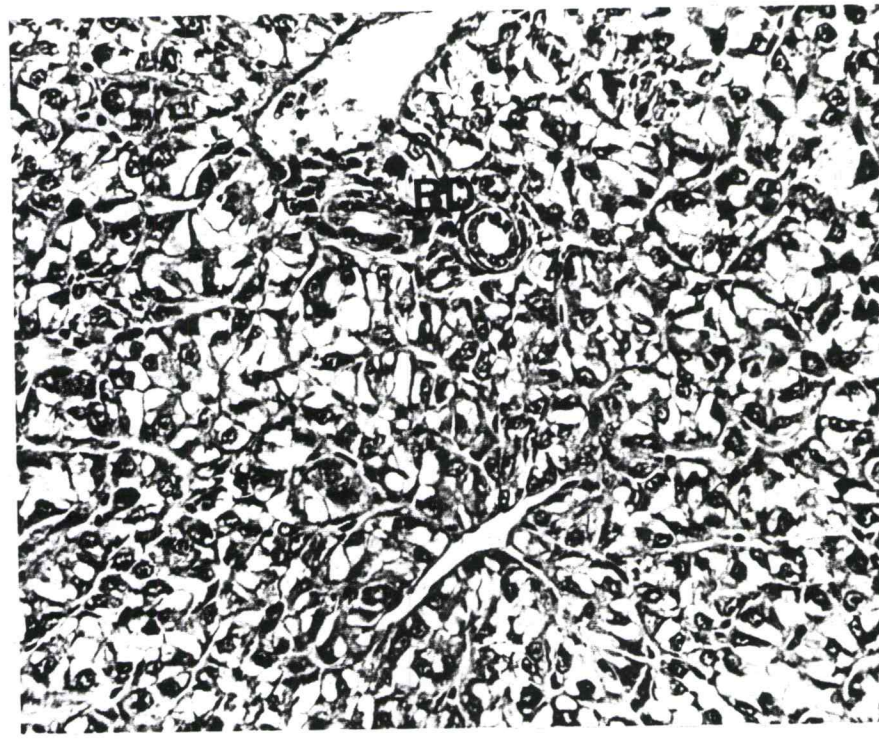

Figure II.6 A normal liver in a fish exposed 2 weeks earlier to 0.05 ppm [^{3}H]-AFB_1 for 30 minutes. Hepatocytes are organized in a tubular pattern, with glycogen storage at normal levels. No signs of cytotoxicity are seen. A blood vessel and two bile ducts (BD) are present. X 375. H & E.

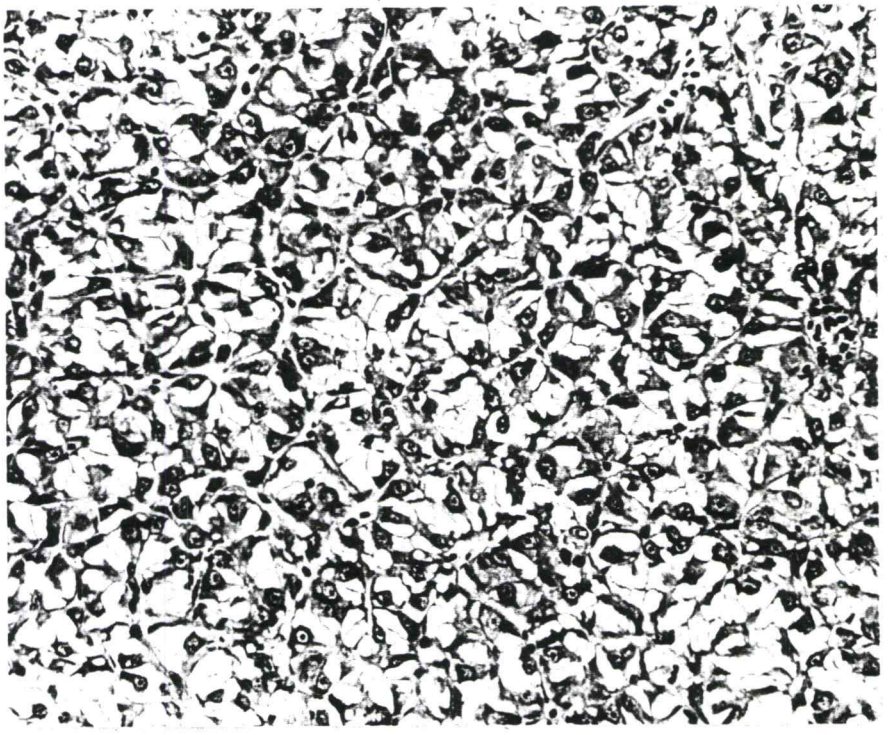

Figure II.7 Trout liver from a fish fed 500 ppm BNF for 1 week prior to exposure to 0.5 ppm [^{3}H]-AFB_1 for 30 minutes and sacrificed 2 weeks later. Lack of cytotoxicity is striking. X 375. H & E.

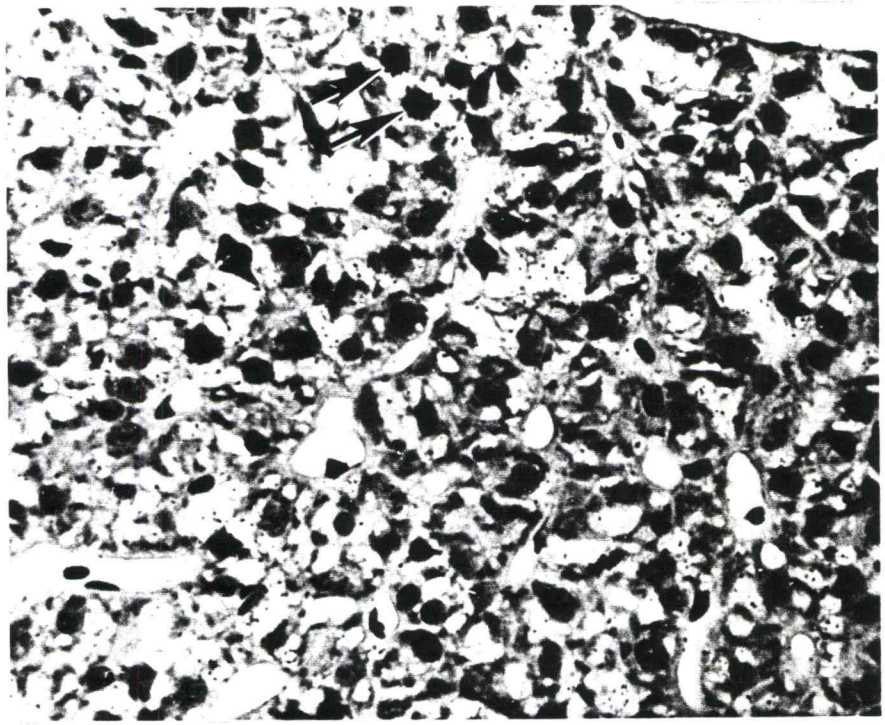

Figure II.8 Control liver from a trout 24 hours after i.p. injection of [^{3}H]-TdR. Strongly labeled hepatocytes (arrows) are seen at the periphery of the liver. X 600. H & E.

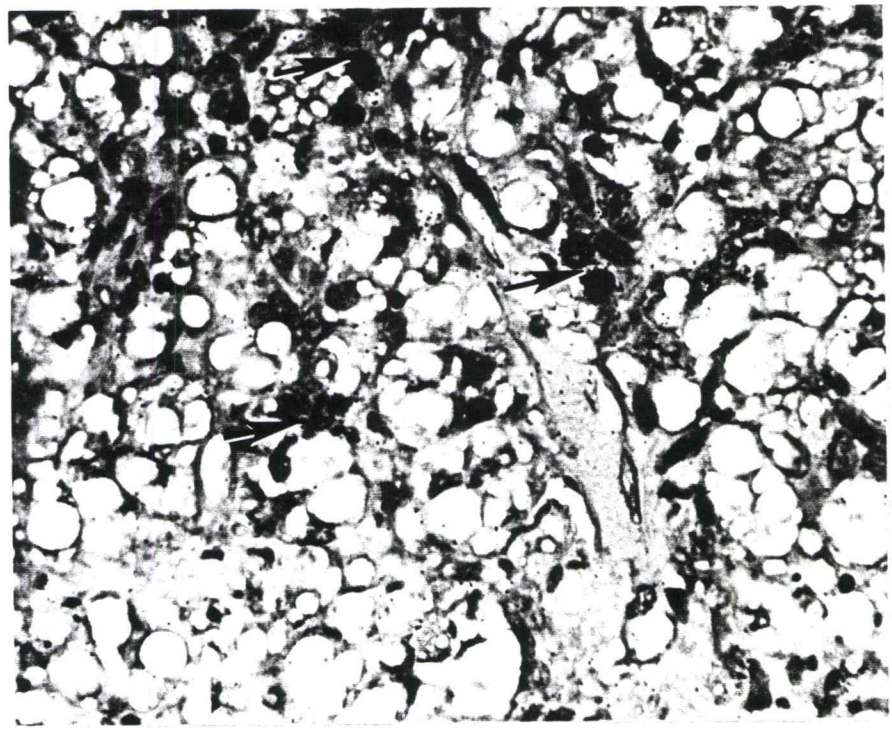

Figure II.9 Liver from a trout exposed to 0.5 ppm AFB_1 for 30 minutes 2 weeks before i.p. injection of [^{3}H]-TdR, and sacrificed 24 hr later. Severe cytotoxicity is evident. Strongly labeled presumptive oval cells (arrows) are seen interspersed among degenerating hepatocytes. X 600. H & E.

Acknowledgments: We thank Daniel Arbogast, Sheila Cleveland and Ted Will for their assistance with the trout.

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III. ULTRASTRUCTURE OF HEPATOCELLULAR TUMORS IN AFLATOXIN B_1 (AFB_1)-INITIATED RAINBOW TROUT (*Oncorhynchus mykiss*).

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ABSTRACT

The fine structure of hepatocellular neoplasms from aflatoxin B_1 (AFB_1)-initiated rainbow trout was studied by transmission electron microscopy. Large, usually uniform hepatic nuclei, large nucleoli, abundant, dilated rough-surfaced endoplasmic reticulum, and reduced glycogen storage were common findings in both hepatocellular adenomas and hepatocellular carcinomas. In addition, poorly developed microvilli, located in the space of Disse and in the bile canaliculi, were seen in hepatocellular carcinomas. Few or no bile preductule cells were found in hepatocellular carcinomas. A striking increase in intercellular spaces in hepatocellular carcinomas was also observed. The three latter characteristics of hepatocellular carcinomas suggest a loss of inter-relationships between individual hepatocytes, between hepatocytes and the biliary system, and between hepatocytes and the circulatory system (sinusoids). With respect to these parameters, adenomas were more similar to normal hepatocytes than carcinomas.

INTRODUCTION

The liver is the organ most susceptible to neoplastic change in bony fishes in general and in salmonids in particular (Wellings 1969). Plehn (1909) was the first to report a hepatic tumor in a brown trout, a multilocular cystadenoma of biliary origin, while Haddow & Blake (1933) first reported a hepatocellular tumor in a rainbow trout. Other reports of hepatic neoplasms in salmonids occurred in the 1950's (Scolari 1953, Nigrelli 1954, Nigrelli & Jakowska 1955, Cudkowicz & Scolari 1955, Honma & Shirai 1959, Levaditi et al. 1960) but in the early 1960's, hepatocellular neoplasia in hatchery-reared rainbow trout reached epizootic proportions in the United States (Ashley & Halver 1961, Ghittino 1961, Hueper & Payne 1961, Nigrelli & Jakowska 1961, Rucker et al. 1961, Snieszko 1961, Wood & Larson 1961). This epizootic was linked to dietary aflatoxin exposure, but elimination of contaminated feed ingredients and diet surveillance have essentially eliminated this problem from salmonid culture. Experimental carcinogenesis initiated by aflatoxin and other carcinogens, however, has been studied extensively in rainbow trout (Hendricks 1982).

Hepatic neoplasia in non-salmonid feral fish is usually associated with environmental pollution. The first report was by Dawe et al. (1964) in white suckers and brown bullhead from Deep Creek Lake in Maryland. Over the last two decades the number of epizootic outbreaks of liver neoplasia, particularly in polluted marine or estuarine environments, has increased sharply (Baumann 1989, Black et al. 1982, Malins et al. 1985a, Malins et al. 1985b, Malins et al. 1987a, Malins et al. 1987b, May et al. 1987, Murchelano & Wolke 1985, Myers et al. 1987, Smith et al. 1979). Experimental liver carcinogenesis in non-salmonid fishes has been investigated primarily in small aquarium fish. Since the first report of Stanton (1965), the use of aquarium fish to study experimental hepatocarcinogenesis has increased dramatically (Aoki & Matsudaira 1977, Fournie et al. 1987, Harada et al. 1988, Hatanaka et al. 1982, Hawkins et al. 1985, Hawkins et al. 1986, Hawkins et al. 1988a, Hawkins et al. 1988b, Matsushima & Sugimura 1976, Schultz & Schultz 1982a, 1982b, 1988, Schultz et al. 1989, Thiyagarajah & Grizzle 1986).

The light microscopy of hepatic neoplasms has been described in salmonids (Hendricks 1982, Hendricks et al. 1984) and non-salmonids (Couch & Courtney 1987, Myers et al. 1987, Hawkins et al. 1988b, Schultz & Schultz 1982b).

The ultrastructure of non-salmonid hepatic tumors has also been documented for feral English sole (Parophrys vetulus) from Puget Sound (Stehr et al. 1988) and from experimental exposures of sheepshead minnow (Cyprinodon variegatus) to N-nitrosodiethylamine (DENA) (Couch & Courtney 1987), brown bullhead (Ictalurus nebulosus) to DENA (Hampton et al. 1988b), and medaka (Oryzias latipes) to methylazoxymethanol-acetate (MAMA) (Harada et al. 1988, Hinton et al. 1984).

The ultrastructure of normal trout liver has been well characterized (Hampton et al. 1988a, Hampton et al. 1988b, Hampton et al. 1989), however, ultrastructural studies of experimentally initiated hepatic tumors in rainbow trout are conspicuously lacking. Only a single paper describes the ultrastructure of neoplastic hepatocytes from hatchery reared, tumor-bearing trout (Scarpelli et al. 1963).

The aims of this study were to describe in detail the ultracytological features and architectural changes of hepatocellular neoplasms (both hepatocellular adenomas and carcinomas) in AFB_1-induced rainbow trout.

MATERIALS AND METHODS

Protocol:

Groups of 150 fry (8 weeks after swim-up), reared at the Food Toxicology and Nutrition Laboratory, Oregon State University, were exposed to aqueous solutions of 0 or 0.5 ppm AFB_1 for 30 minutes. Following carcinogen exposure, fry were transferred to fresh water without AFB_1 (Calbiochem, San Diego, CA) and were fed Oregon Test Diet (OTD) (Hendricks 1982) for the remainder of the experiment.

No tumors were observed macroscopically six months after exposure to AFB_1. Only preneoplastic lesions, e.g., basophilic foci and bile duct proliferation, were observed under microscopical examination. Therefore, fish were held three more months to allow for continued tumor development. At 9 months, tumor-bearing fish (N=4) and controls (N=4) were sampled for both light microscopy (LM) and transmission electron microscopy (TEM). Fish were killed by severing the spinal cord, tumor-bearing livers were rapidly removed, and tumors cut in half for processing, one half for LM, the other for TEM.

Light microscopy:

Samples for LM were fixed in Bouin's solution and processed by routine histological methods (Luna 1960). Tissues were embedded in paraffin, sections were cut at 4 μm and stained with H & E.

Transmission electron microscopy:

Samples for TEM were sliced and minced (1 mm^3) in ice chilled fixative (2.5 % glutaraldehyde in 0.1 M Sorenson's PO_4 buffer, pH 7.35), pooled, and fixed in fresh fixative solution for 6 hours at 4 °C. Tissues were washed in Sorenson's buffer solution for 30 minutes at 4 °C, post-fixed in 1 % OsO_4 for 30 minutes at 4 °C, and washed in buffer for 30 minutes. Samples were dehydrated in a graded series of acetone and infiltrated in resin (Medcast-Araldite 502, Ted Pella, Inc., Redding, CA). Semithin (1-2 μm) sections from randomly selected blocks of each liver were cut and stained with Methylene Blue-Azure II-Basic Fuchsin (Hayat 1986). Paraffin and semithin sections from each liver were examined for diagnostic interpretation. Thin sections (600-700 A) from not less than three selected blocks were cut, mounted on copper grids, and stained with aqueous uranyl acetate and either Reynolds' lead citrate (Reynolds 1963) or bismuth subnitrate (Hayat 1963). Stained sections were examined with a Zeiss EM 10A TEM at 60 KV.

RESULTS

Normal liver

Light microscopy:

Control trout liver has already been described in detail (Hampton et al. 1985, Hampton et al. 1988a, Hampton et al. 1989). Polygonal hepatocytes were organized as tubules which appeared as plates 2 cells-wide in longitudinal section, but 5 to 8 cells formed the tubules in cross section. Their apices were oriented towards a common, central bile canaliculus or preductule while their bases faced sinusoids from which they were separated by the space of Disse. The tubules branched randomly and were usually separated from each other by sinusoids (Fig. III.1).

Electron microscopy:

Hepatocytes forming tubules were arranged around a central bile canaliculus occupied by microvillar projections from the plasma membranes of the hepatocytes (Fig. III.2). Adjacent hepatocytes were joined in the canalicular area by tight junctions (Fig. III.2). Individual bile preductule cells appeared between hepatocytes (Fig. III.1), adjacent to the canaliculus and increased in number distally until they formed bile ductules (Fig. III.2). Bile ductule cells contained large

nuclei relative to hepatocytes with electron-dense, peripheral heterochromatin, and scanty cytoplasm with few organelles (Fig. III.2). Bile ductule cells were joined to each other and to adjacent hepatocytes by tight junctions (Fig. III.2).

Sinusoids were separated from hepatocytes by the Space of Disse (Fig. III.3). Microvilli from the basal surface of the hepatocytes occupied the space of Disse where fat-storing or Ito cells were often seen (Fig. III.4).

Hepatocytes contained a slightly indented, oblong to round, centrally located nucleus with one or two nucleoli. Heterochromatin was distributed as a peripheral band as well as randomly throughout the nucleoplasm (Fig. III.3). Mitochondria were rounded, oval or elongated with cristae of uniform size but irregular orientation depending on the plane of sectioning. Mitochondria were located predominantly in a perinuclear location or peripheral to the parallel profiles of the rough-surfaced endoplasmic reticulum (RER). Profiles of RER, heavily studded with ribosomes, were abundant and uniformly organized in parallel laminar arrays about the nucleus. Vesicles of smooth endoplasmic reticulum (SER) were scattered in more peripheral regions of the cytoplasm in close association with "rosettes" of glycogen (Fig.

III.5). The Golgi complex was formed by a few slightly curved flattened stacks of sacs and vesicles adjacent to the cell membrane (Figs. III.2 & III.3). Several functional stages or morphological forms of lysosomes were observed in control livers containing electron-dense material (Figs. III.2 & III.3), multivesicular bodies (Fig. III.2), or lipofuchsin granules (Figs. III.3 & III.5). Electron-lucent liposomes were rarely found in the cytoplasm of the hepatocytes but were seen in fat-storing cells (Fig. III.4).

Hepatic neoplasms

Hepatocellular adenomas (HCA) and hepatocellular carcinomas (HCC) were the only types of neoplasms found in trout 9 months after AFB_1 exposure.

Light microscopy:

Three HCA were examined. Macroscopically, they were nonencapsulated, irregular nodules 0.5 to 1 mm in diameter. They were composed of basophilic hepatocytes organized in tubules usually 2 cells in width in longitudinal section, although tubules with more than 2 cells were sometimes seen (Fig. III.6). Some glycogen vacuolation was present in tumor cells but it was clearly reduced from the level of glycogen storage in surrounding normal hepatocytes. Neither invasion nor compression of adjacent tissue was manifested (Fig. III.6).

Two well-differentiated trabecular HCC were studied. Macroscopically, they appeared as nodules 2 to 4 mm in diameter. They were composed of basophilic hepatocytes organized as expanded trabeculae, 5 to 6 or more cells wide in longitudinal section (Fig. III.7). Glycogen storage was similar to that observed in Figure III.6 but HCC often displayed no observable glycogen vacuoles. Invasion and compression of normal adjacent tissue was observed (Fig. III.7).

Electron microscopy:

Hepatocellular adenomas

The three HCA examined were similar in architectural and subcellular detail. Normal liver architecture was conserved in HCA, nevertheless, prominent disorganization of hepatocyte subcellular organelles was observed. Pleomorphic nuclei, large nucleoli, dilated RER, reduced glycogen storage, and increased numbers of lysosomes, compared to controls, characterized neoplastic hepatocytes (Fig. III.8). RER, mitochondria, SER, and glycogen were more randomly distributed and lacked the regular organization described for control hepatocytes. Well developed bile canaliculi, similar to controls, and bile preductules were observed. Bile preductule cells were morphologically similar to those found in normal

liver, i.e., large nuclei with electron-dense heterochromatin, and scanty cytoplasm (Fig. III.8).

Hepatocellular carcinomas

Neoplastic hepatocytes from both hepatocellular carcinomas were uniform and densely packed in broad chords or trabeculae. They were characterized by a prominent, central, electron-dense nucleolus, and a greater nuclear/ cytoplasmic ratio than in normal hepatocytes (Fig. III.9). Proliferation of RER, which accounted for the increase in basophilia under LM, was striking. Profiles of RER were dilated, disorganized, and occupied the majority of the cytoplasmic volume (Figs. III.9-III.11). Mitochondria were randomly distributed throughout the RER but they showed no remarkable morphological changes from those in control hepatocytes (Fig. III.9). Glycogen deposits were few but occasional hepatocytes contained glycogen surrounded by RER. Unlike control hepatocytes there was rarely any association between SER and glycogen, and SER vesicles were scarce throughout the cells (Figs. III.9-III.11). Lysosomes were variable in morphology and number from cell to cell, but no obvious departure from control conditions was observed (Figs. III.9-III.12). Electron-lucent liposomes (Figs. III.9 & III.12), well developed Golgi complexes (Fig. III.10), and binucleated

hepatocytes (Fig. III.11) were also observed. Macrophages were seen often between the hepatocytes or in proximity to the space of Disse (Figs. III.10 & III.12). They had an irregularly shaped nucleus with highly condensed heterochromatin, and a light cytoplasm filled with few mitochondria, moderate amounts of SER vesicles, and lysosomes (Fig. III.12).

Reduced vascularization, due to cellular proliferation without concomitant expansion of the sinusoidal system, resulted in broad chords or tubules of cells in which only the peripheral hepatocytes bordered sinusoids rather than all hepatocytes as in controls (Fig. III.9). In addition, microvilli which occupied the space of Disse were less developed and more irregular in size and shape than in controls (Figs. III.10 & III.12). Bile ducts were absent in HCC and both preductule and ductule cells were seen rarely (Fig. III.12). Bile canaliculi were poorly developed (Fig. III.12) or absent (Fig. III.11), although tight junctions were seen often in intercellular areas where bile canaliculi would be normally located (Fig. III.9). Microvilli, occupying bile canaliculi, were few in number, elongated, and failed to fill the lumen as in controls (Fig. III.11). Intercellular spaces were widened (Fig. III.11).

DISCUSSION

The finding that our collection of hepatic tumors resulted in only HCA and HCC was unusual. Histopathological evaluation of tumor types from most experiments at our laboratory reveals that mixed hepatocellular-cholangiocellular carcinomas are equally or more common than HCA and HCC (Nunez et al. in press). Ultrastructural studies of these tumor types also need to be conducted.

The differences between HCA and HCC appear to be more on the organizational rather than on the subcellular level, implying that HCA may be merely a transitional stage leading to HCC, rather than an endpoint. Features of Figure III.6 support this view, since most of the tubules, composing the tumor, have the 2-cells wide configuration of normal hepatic tissue. At the upper left (Fig. III.6), however, there is a tubule of cells that is no longer 2-cells-wide but rather 5 or 6 cells in width and may indicate that the tumor would have soon become a HCC.

Proliferation and dilation of RER were the most striking feature described in neoplastic hepatocytes from hatchery-reared rainbow trout (Scarpelli et al. 1963). These authors (Scarpelli et al. 1963) also reported

elevated serum proteins in tumor-bearing trout, indicating that the ribosome-rich RER of these cells was highly active in protein synthesis warranting their classification as functional tumors. In the present study, hepatocytes from both HCA and HCC in AFB_1-initiated rainbow trout also displayed a high degree of proliferation and dilation of RER (Figs. III.8 & III.10). Similarly, RER proliferation in HCC's hepatocytes has been reported by other authors (Hampton et al. 1988b, Harada et al. 1988, Stehr et al. 1988). This characteristic supports the nature of these tumors as being relatively well differentiated, functional tumors capable of altered or elevated protein synthesis within the RER (Ghadially 1982). In spite of the RER proliferation and dilation, accumulation of proteinaceous material in the RER cisternae was not observed in the present study as it has been described in other hepatic tumors (Scarpelli et al. 1963, Stehr et al. 1988).

Glycogen depletion was observed in both HCA (Fig. III. 8) and HCC (Fig. III.10), nevertheless, it was more pronounced in HCC (Fig. III.10). Elevation of glycolysis and pentose-phosphate cycle activity which enhance synthesis of proteins and RNA, respectively, (Hirota & Yokoyama 1985) could result in the lack of glycogen and dilation of RER, the two major common findings in

neoplastic hepatocytes.

The presence of liposomes has been documented in hepatic neoplasms from feral fish (Harada et al. 1988). In the present study, liposomes were occasionally seen in the cytoplasm of hepatocytes containing electron-lucent material (Figs. III.8, III.9, III.12). Disturbance of membrane transport could explain the presence of lipid deposits, which were rarely found in normal trout hepatocytes.

Intercellular spaces were wider in HCC compared to controls indicating loss of intercellular communication. There was also a noticeably poor development of microvilli forming the surface specializations for both the space of Disse and bile canaliculi. This could result in reduced efficiency in both absoption and secretion mechanisms, respectively.

The uncontrolled proliferation of carcinoma cells within the hepatic tubule results in a large number of neoplastic hepatocytes that have neither a basal sinusoidal nor an apical canalicular association, as normal hepatocytes would have. Their apparent random placement, lack of functional orientation with respect to sinusoidal or canalicular structures or other neoplastic hepatocytes, lack of functional bile conducting system and the failure to observe evidence of

bile stasis at either the light or electron microscopic level, suggest that bile production by hepatocellular carcinoma cells is very limited or lacking altogether. This would constitute a major loss of function by these cells, but this has not been discussed in other TEM studies of fish hepatic tumors.

As previously mentioned, another neoplasm, not described here but normally seen at an equal or even greater frequency than HCC in rainbow trout hepatocarcinogenesis, is the mixed hepatocholangiocellular carcinoma (Nunez et al. in press) in which neoplastic cells of both hepatocyte and biliary origin comprise the tumor. Conceptually, mixed tumors would seem to contradict the hypothesis we have submitted, i.e. that HCC produce little or no bile and have no functional biliary conducting system. However, in mixed tumors, there does not appear to be any functional relationship between the neoplastic biliary and hepatocellular components and at least hepatocytes still exist in expanded, engorged tubules as in HCC. At the LM level, neoplastic bile ducts appear to develop independently, not as outgrowths of biliary cells from the expanded hepatic tubules. Ultrastructural studies on these tumors are obviously needed to further our understanding of trout hepatocarcinogenesis.

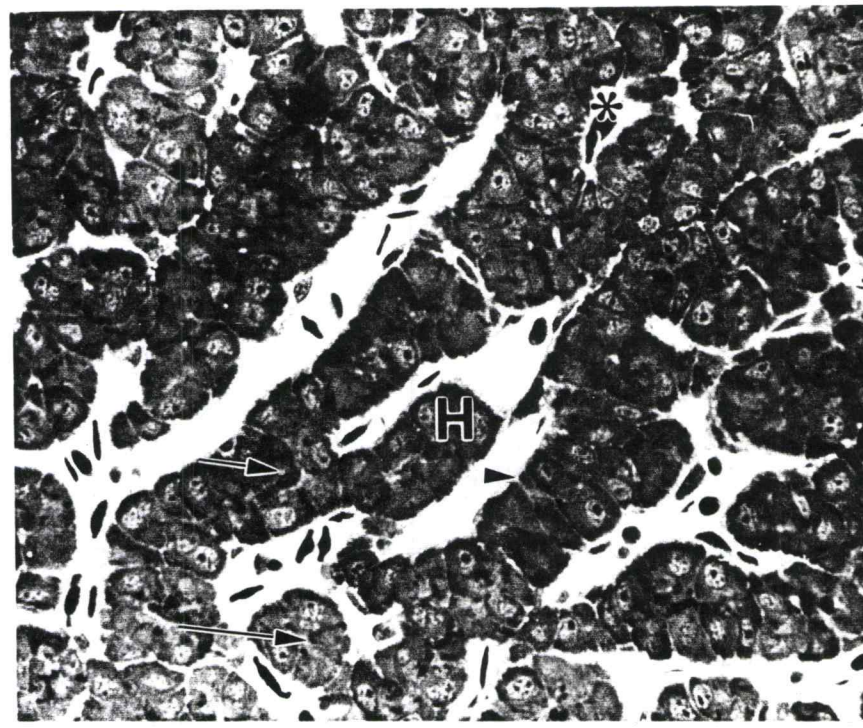

Figure III.1 Light micrograph of a normal liver, plastic section. Hepatocytes (H) are arranged as tubules and oriented, centrally, towards a bile canaliculus (large arrow). Biliary epithelial cells (small arrow) are common. Sinusoids (*) are interspersed throughout the parenchyma and separated from the hepatocytes by the Space of Disse (arrowhead). Methylene Blue-Azure II-Basic fuchsin. X 540.

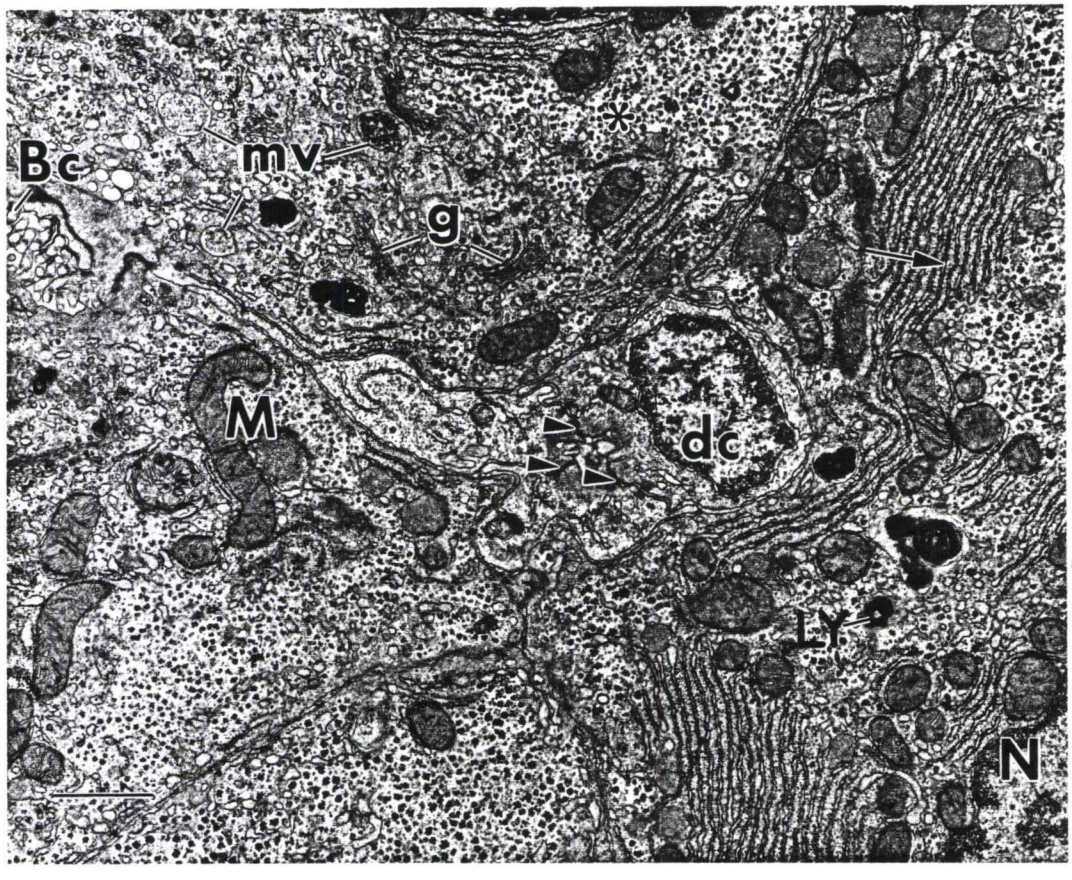

Figure III.2 An electron micrograph of a control liver. Ductule cells (**dc**) joined by tight junctions (arrowheads) are observed. A bile canaliculus (**Bc**) is observed on the left. Glycogen (*); golgi complex (**g**); lysosomes (**LY**); mitochondria (**M**); multivesicular bodies (**mv**); hepatocyte nucleus (**N**); RER (arrow); bar= 1 μm. Uranyl acetate-Bismuth subnitrate. X 11,900.

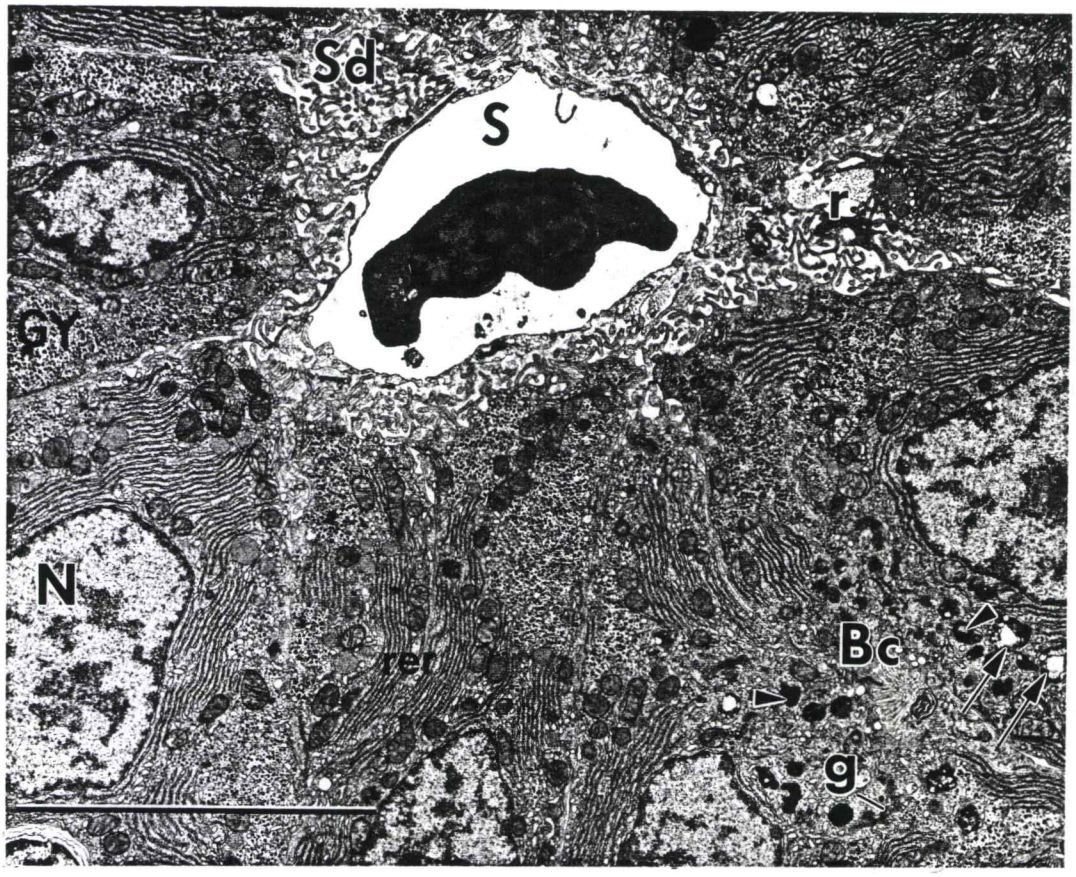

Figure III.3 An electron micrograph of a control liver showing a sinusoid (**S**), several hepatocytes, and a bile canaliculus (**Bc**). The sinusoid is separated from the hepatocytes by the space of Disse (**Sd**) which is occupied by numerous microvilli and reticular fibers (**r**). Golgi complex (**g**); glycogen (**Gy**); lysosomes (arrowheads); hepatocyte nucleus (**N**); RER (**rer**); residual bodies (arrows); bar= 10 µm. Uranyl acetate-Bismuth subnitrate. X 4,684.

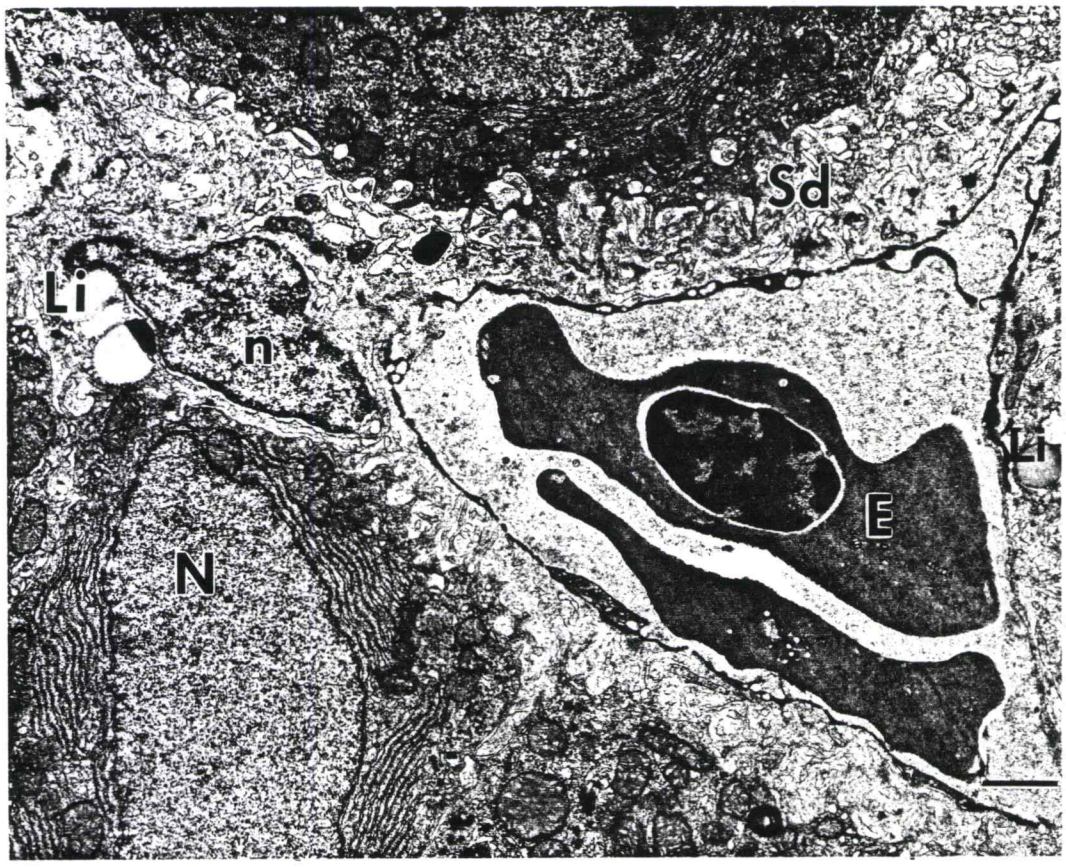

Figure III.4 Electron micrograph of a control liver showing an Ito cell containing lipid droplets (**Li**). Erythrocyte (**E**); hepatocyte nucleus (**N**); perisinusoidal cell nucleus (**n**); space of Disse (**Sd**); bar= 1 μm. Uranyl acetate-Bismuth subnitrate. X 8,125.

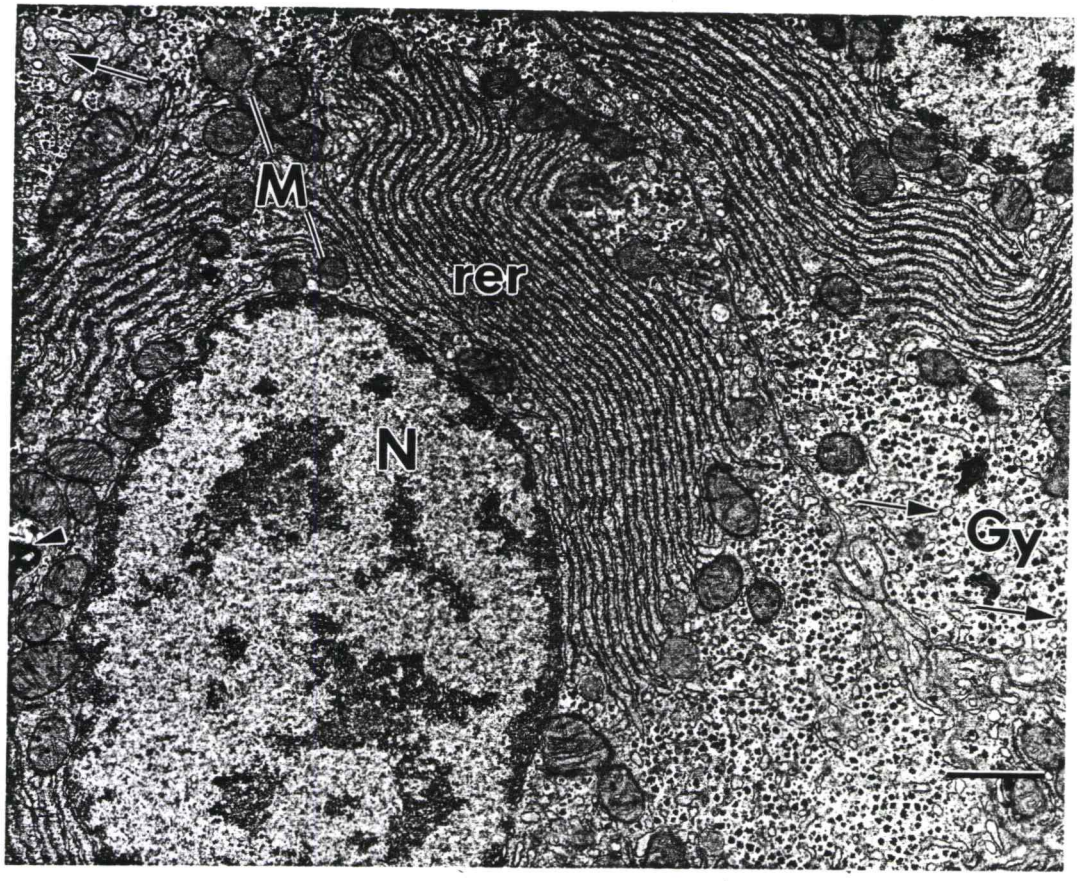

Figure III.5 High magnification electron micrograph illustrating two normal hepatocytes. Mitochondria (**M**) are located in a perinuclear location or peripheral to the RER (**rer**). SER vesicles (arrows) are seen in association with glycogen (**Gy**). Hepatocyte nucleus (**N**); residual body (arrowhead); bar= 1 μm. Uranyl acetate-Bismuth subnitrate. X 13,020.

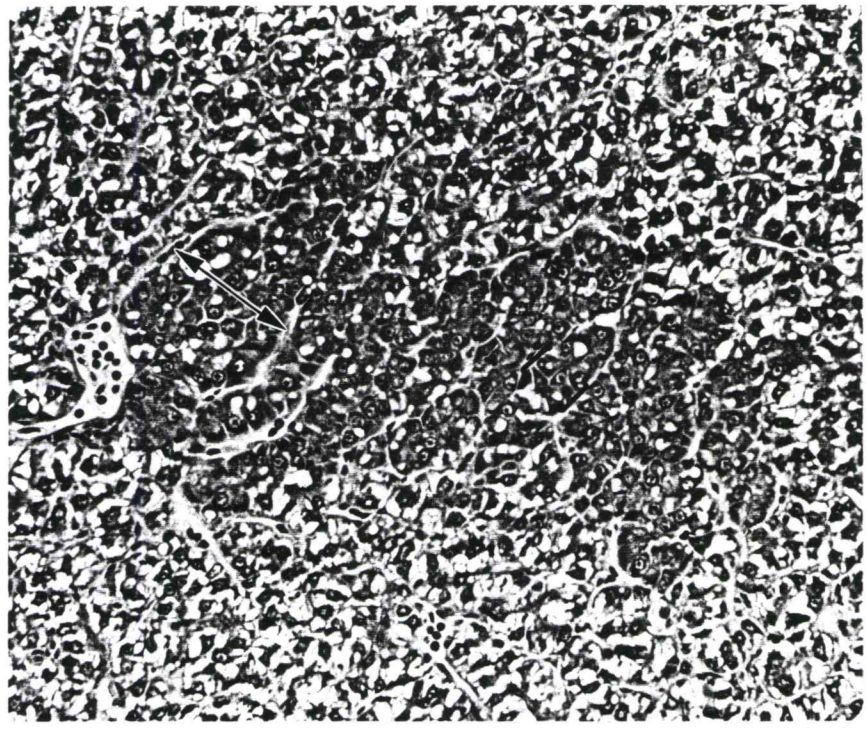

Figure III.6 Light micrograph of a hepatocellular adenoma (HCA) at 9 months from a trout exposed to 0.5 ppm AFB_1. Tubules are composed of basophilic hepatocytes usually 2 cells in width ([]). Note a chord with more than 2 cells in width at the upper left (double arrow). Note the high amount of glycogen (clear areas) in normal parenchyma. Paraffin section, H & E. X 280.

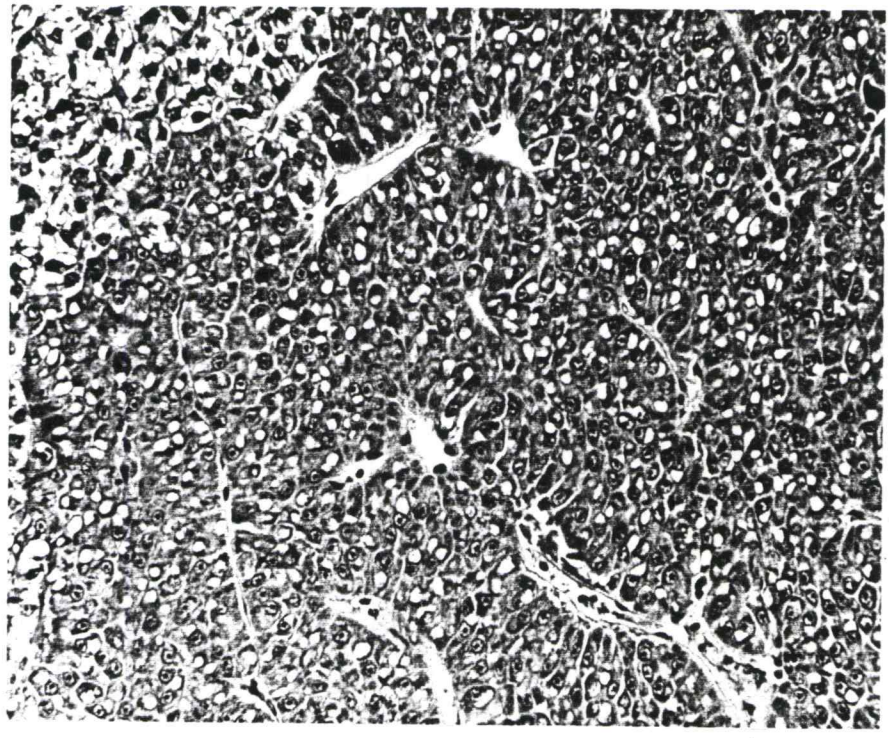

Figure III.7 Light micrograph of a hepatocellular carcinoma (HCC) at 9 months from a trout exposed to 0.5 ppm AFB_1. Wide chords composed of basophilic hepatocytes, compression, and invasion of adjacent normal tissue (upper left) are observed. Paraffin section, H & E. X 280.

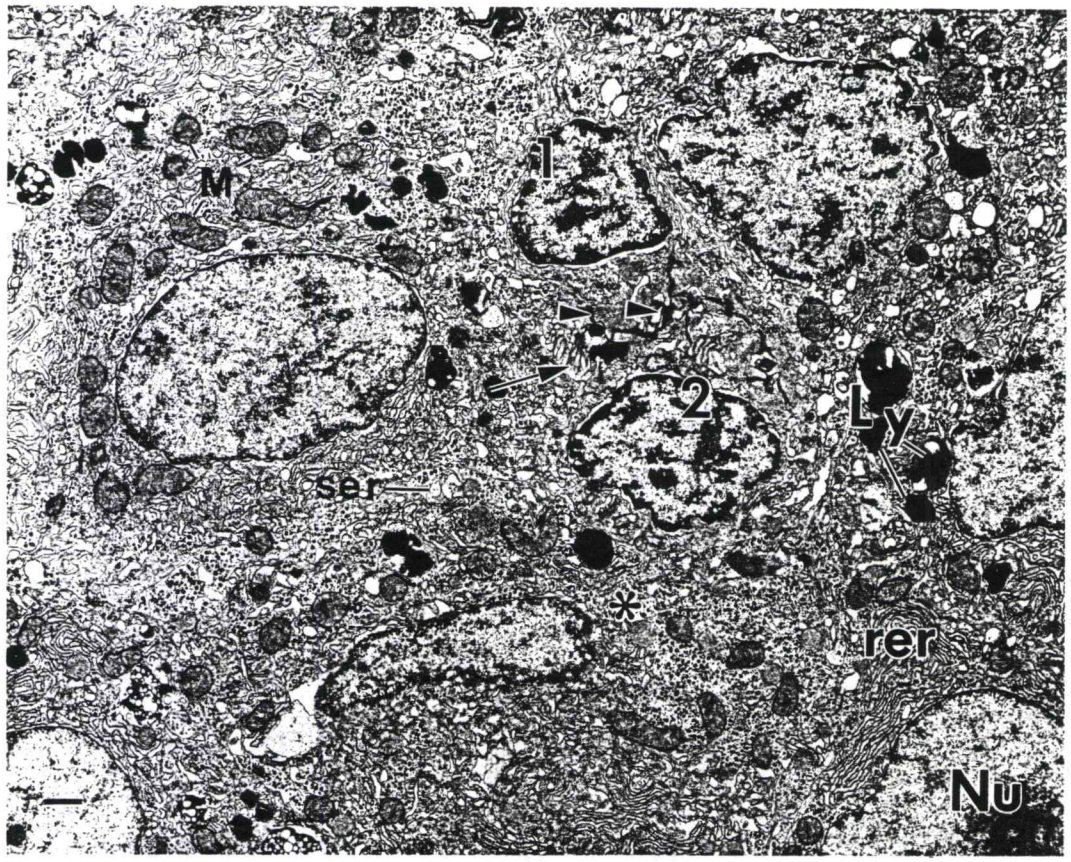

Figure III.8 Electron micrograph of a hepatocellular adenoma. Neoplastic hepatocytes have pleomorphic nuclei, large nucleoli (**Nu**), and dilated RER (**rer**). Two bile preductule cells (**1,2**) joined by tight junctions (arrowheads) are seen. Glycogen deposits (*) are randomly distributed. Bile canaliculus (arrow); lysosomes (**Ly**); mitochondria (**M**); SER (**ser**); bar= 1 µm. Uranyl acetate-Bismuth subnitrate. X 5,250.

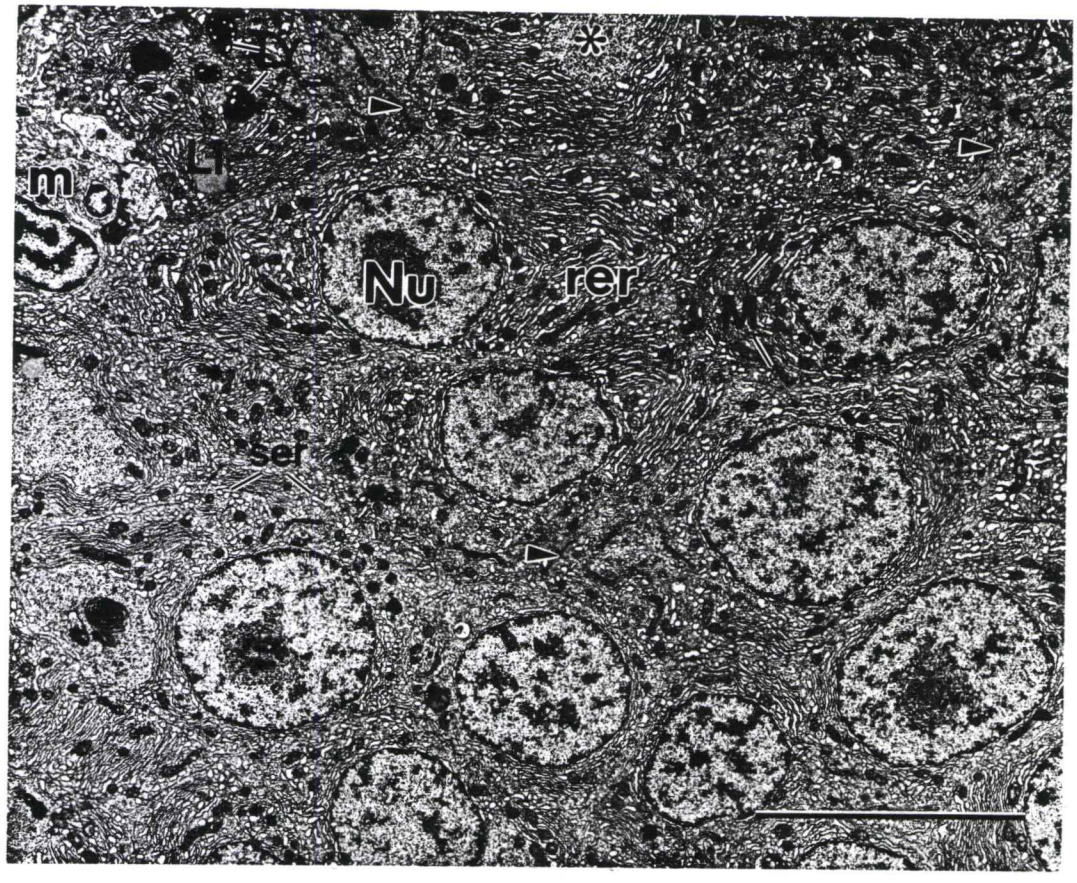

Figure III.9 Low magnification TEM of a hepatocellular carcinoma. Neoplastic hepatocytes have central, round nuclei, large nucleoli (**Nu**), and dilated RER (**rer**). Note decrease of glycogen (*) and lack of biliary components. Lipid (**Li**); lysosomes (**LY**); macrophage (**m**); mitochondria (**M**); SER (**ser**); tight junctions (arrowheads); bar= 10 μm. Uranyl acetate-Bismuth subnitrate. X 3,536.

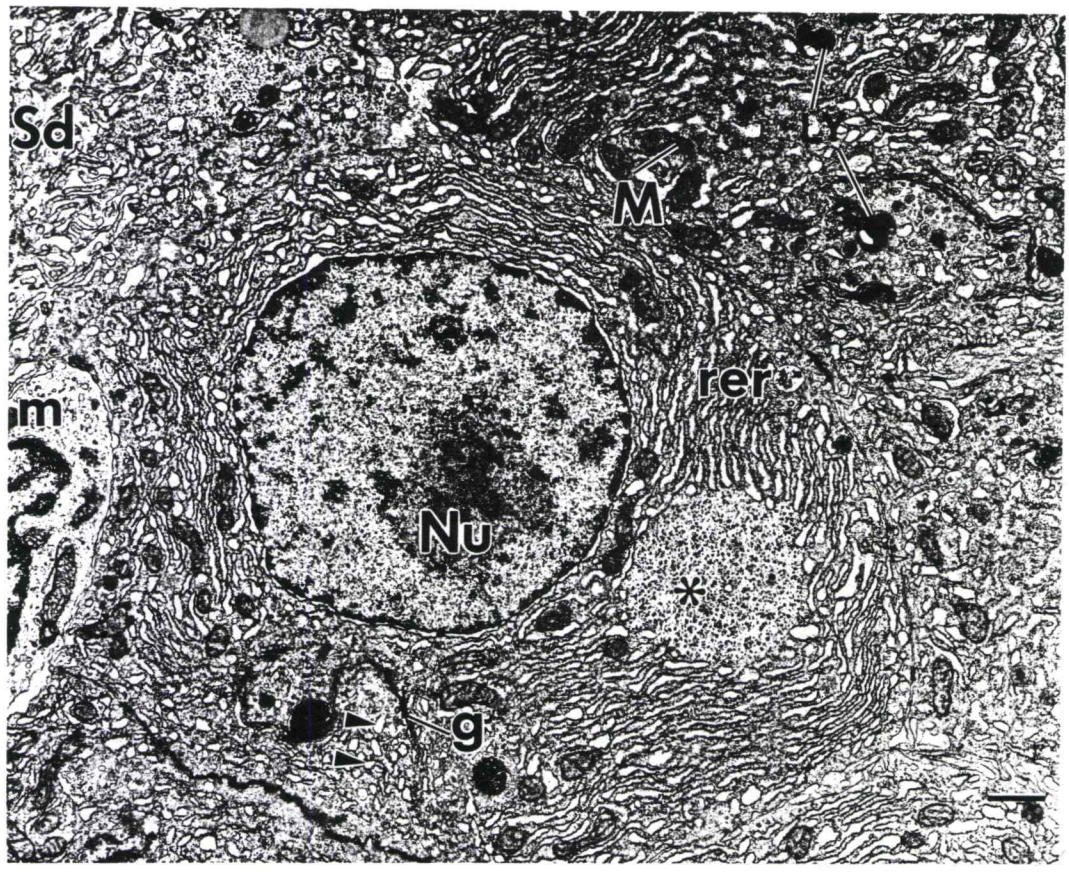

Figure III.10 High magnification of a HCC showing a neoplastic hepatocyte. Glycogen (*); golgi complex (g); lysosomes (LY); macrophage (m); mitochondria (M); hepatocyte nucleolus (Nu); RER (rer); SER (arrowheads); space of Disse (Sd); bar= 1 μm. Uranyl acetate-Bismuth subnitrate. X 7,555.

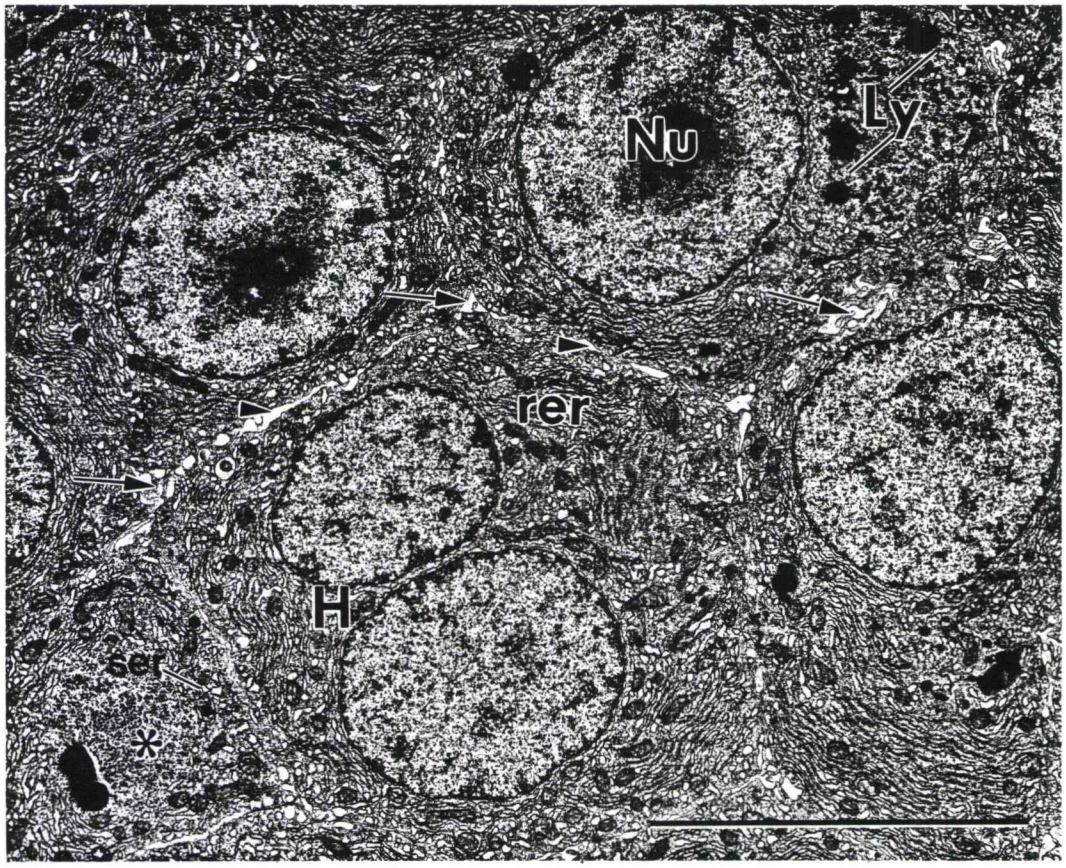

Figure III.11 TEM of a HCC. A binucleated hepatocyte (**H**) is seen. Microvilli (arrows) are less developed than in controls. Note increase in intercellular spaces (arrowheads). Glycogen (*); Lysosomes (**Ly**); nucleolus (**Nu**); RER (**rer**); SER (**ser**) bar= 10 µm. Uranyl acetate-Bismuth subnitrate. X 4,942.

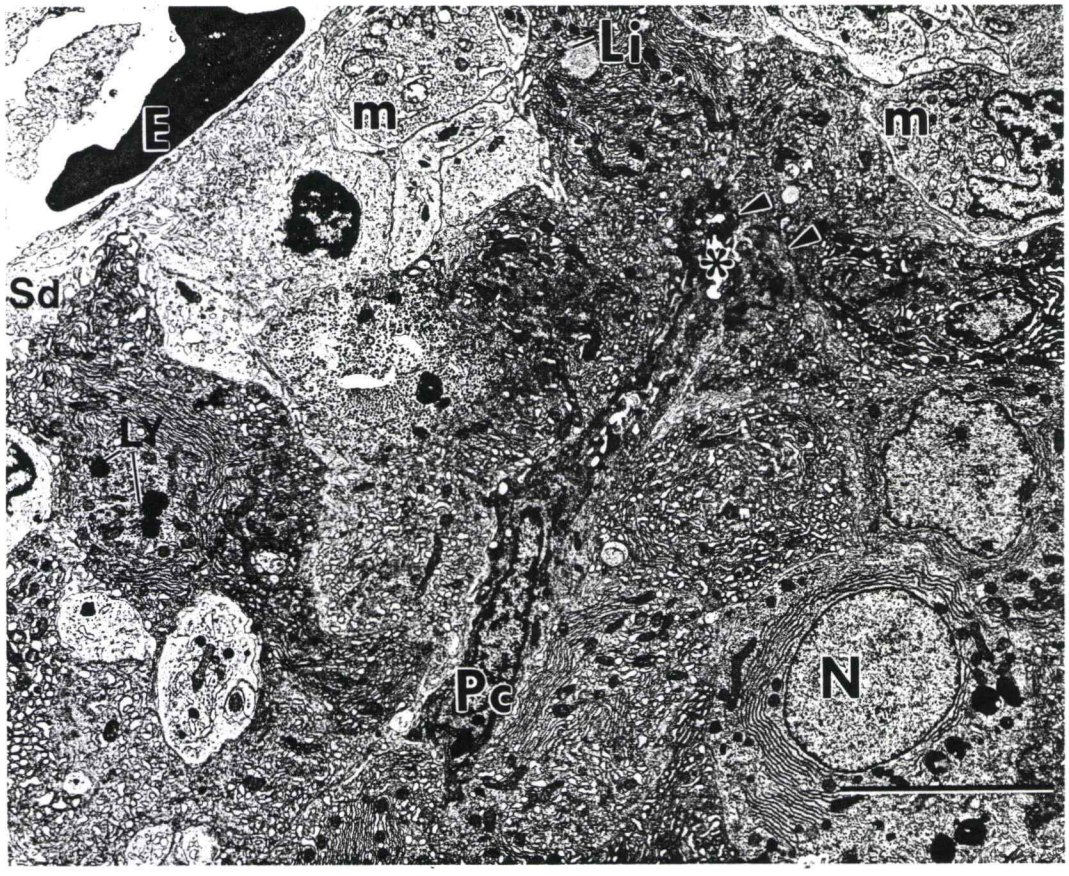

Figure III.12 Low magnification of a HCC showing a bile preductule cell (**Pc**). A poorly developed bile canaliculus is also observed (*). Several macrophages (**m**) are present. Erythrocyte (**E**); lipid (**Li**); lysosomes (**LY**); hepatocyte nucleus (**N**); space of Disse (**Sd**); tight junctions (arrowheads); bar= 10 µm. Uranyl acetate-Bismuth subnitrate. X 2,800.

Acknowledgments: We thank Daniel Arbogast, Sheila Cleveland, and Ted Will for their assistance with the trout.

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