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Use of Small Fish Species in Carcinogenicity Testing

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Use of Small Fish Species in Carcinogenicity Testing

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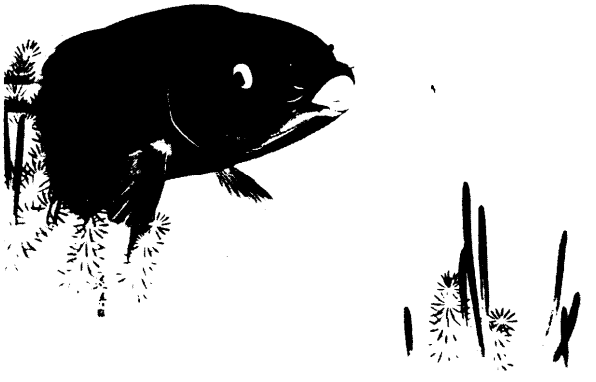

Proceedings

of a Symposium

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December 8–10, 1981

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National **Cancer institute**

Editor:

Karen L. Hoover, Sc.D.

TABLE OF CONTENTS

Session II. Alternative Testing Systems With the Use of Fish

Page

Session III. Maintenance of Fish in the laboratory

Session IV. Special Problems and Topics Related to the Use of Fish in Cancer Research

TABLE OF CONTENTS

Session V. Metabolic Handling of Xenobiotics by Fish

Histological Progression of Hepatic Neoplasia in Rain bow Trout (*Salmo gairdneri*) [1, 2, 3, 4]

Jerry D. Hendricks, Theodore R. Meyers, *and* Dennis W. Shelton[5, 6]

ABSTRACT—The histological progression of hepatic neoplasia has not been as systematically studied in rainbow trout as it has been in rodents. TWO **putative preneoplastic lesions have been identified, the eosinophilic focus and the basophilic focus, but whether these correspond to similar lesions in rodent livers is not known. Preneoplastic liver lesions in rodents have been extensively characterized kistochemically, but adaptation of these techniques to trout livers has not always been successful. Eosinophilic foci consist of hypertrophied cells, enlarged atypical nuclei, and dense glycogen-free cytoplasm. Mitotic figures are also occasionally seen. Usually, these foci have been infiltrated and at least partially destroyed by inflammatory cells, largely lymphocytes. In some liver sections, eosinophilic foci are intact and occasionally an eosinophilic-basophilic transformation can be seen. However, most often basophilic foci appear independently, surrounded by normal heptatocytes, with no indication of a prior eosinophilic stage. The cells of basophilic foci are similar to those of carcinomas: intensely basophilic, mitotically active, devoid of glycogen, and grouped into cords several cells in thickness.** These nodules **may appropriately be referred to as carcinomas in situ, because the only distinguishing characteristic is the size of the lesion. Attempts at differentiation between benign and malignant liver lesions appear arbitrary. We believe the best classification of the neoplastic liver lesion in trout is a hepatocellular carcinoma because the potential for malignant behavior always exists and. with sufficient time, can often be histologically demonstrated. We have also described our experience with the characteristics of other liver lesions associated with hepatocarcinogenesis. — Natl Cancer Inst Monogr 65: 321-336, 1984.**

The classification of proliferative liver lesions in rats resulting from exposure to hepatocarcinogens has received much attention from veterinary and human pathologists Accordingly, the literature contains numerous descriptions of the various sequential lesions encountered during hepatocarcinogenesis. Fundamental differences of opinion as to the nature and significance of various lesions as well as a lack of uniformity in semantics prompted several efforts toward standardization of a classification. The National Cancer Institute first sponsored a workshop in 1974 on the classification of hepatocellular tumors and related lesions that resulted in a highly useful report (*1*) In 1977, a subcommittee, established by the Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, began a series of meetings to establish a standardized classification of rat hepatic tumors. The end result of this extensive effort was the "Histologic Typing of Liver Tumors of the Rat." (*2*) which presently serves as the standard for researchers in this popular area of cancer research.

Unlike the welldefined classification scheme for experimental rat liver tumors, the classification of similarly induced liver tumors in trout is poorly understood and almost entirely lacking. Since the initial report of an epizootic of hepatoma in hatchery-reared rainbow trout (*3*), limited effort has been given to the development of this species or other fish species as cancer models. Consequently, even less effort has been expended in the characterization of the liver tumors themselves. Therefore, it is most encouraging to see the fruition of this Symposium that resulted from the present interest of the National Cancer Institute in fish species as possible model systems for cancer research.

In this paper, we describe our observations and those of others concerning the identification of hepatic tumors and other lesions resulting from exposure of trout to various hepatocarcinogens (4, 5). We will attempt to compare the lesions observed in trout with those described for rats (*1, 2*). but we do not imply that they are necessarily analogous.

ABBREVIATIONS: AFB$_1$ = aflatoxin B$_1$; CPFA = cyclopropenoid fatty acid(s); ppb = parts/ billion; ppm = parts/ million; H & E = hematoxylin and eosin; PAS = periodic acid-Schiff.

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[3] Animals were maintained under the guidelines set forth Oregon State University and the "Guide for the Care and Use of Laboratory Animals" the Institute of Laboratory Animal Resources, National Research Council.

[4] Technical Paper No. 6478, Oregon Agricultural Experiment Station, Oregon State University. Corvallis, Oregon 9733 I.

[5] Department of Food Science and Technology, Oregon State University. *Address reprint requests to* Dr. Jerry D. Hendricks.

[6] We thank Mr. John Casteel, and Mr. Theodore Will for their technical assistance.

GENERAL ASPECTS OF TROUT LIVER TUMORS

Spontaneous hepatic neoplasms in wild or cultured rainbow trout are extremely rare. When such tumors do appear in cultured trout, it is generally assumed that previous exposure to a carcinogen has occurred.

Carcinogens and Routes of Exposure

Among the number of chemical compounds proved to be hepatocarcinogens in rainbow trout, the foremost was AFB_1 (6). The rainbow trout was the first animal in which this now widely studied mycotoxin was shown to be a hepatocarcinogen. Subsequently, the following compounds have also proved to be- carcinogenic: aflatoxin G_1 (7), aflatoxin M_1 (8), aflatoxicol (9). aflatoxin Q_1 (10), sterigmatocystin (II), versicolorin A (II), CPFA (I.?, 13 dimethylnitrosamine (14, 15), diethylnitrosamine (unpublished results), nitrosopyrrolidine (unpublished results 2,6-dimethylnitrosomorpholine (unpublished results). nitrosomorpholine (unpublished results), methylazoxymethanol acetate (unpublished results), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (16), benzo[a]pyrene (17), and dichlorodiphenyltrichloroethane (5, 18). The list undoubtedly could be much longer if more compounds were tested. Interesting': the same types of liver tumors are produced by the action of each of these compounds, some of which vary greatly in their chemical structures. Whether this observation suggests the presence of an oncogene(s) involved in the

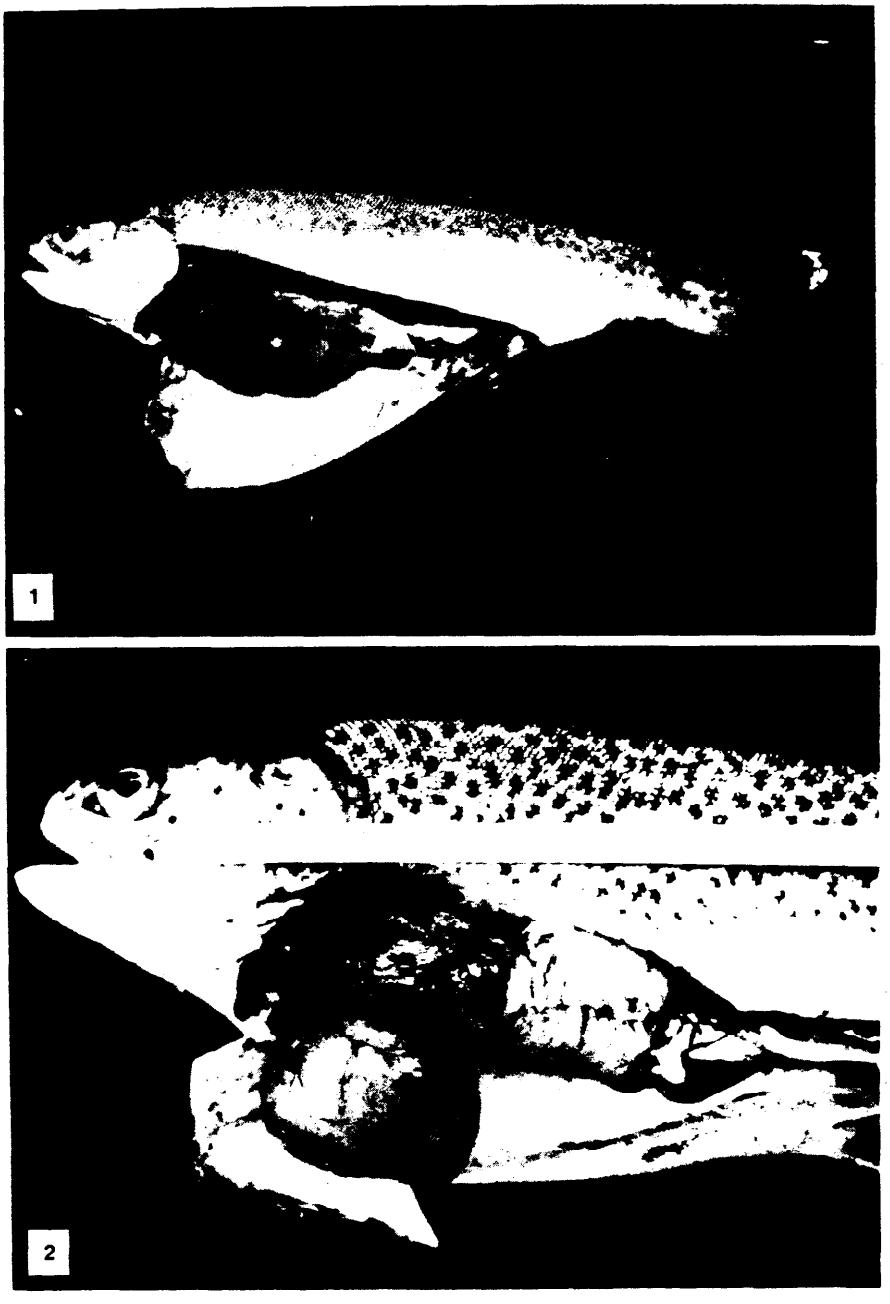


FIGURE I.-A rainbow trout with 2 large hepatocellular carcinomas resulting from consumption of a diet containing 2 ppb AFB_1 and 50 ppm CPFA for 9 mo.

FIGURE 2.—A rainbow trout with a single large hepatocellular carcinoma. This fish had been fed glandless cottonseed meal (containing CPFA) at 25% of the diet for I year. Figure is reproduced with permission of the publisher (13).

expression of hepatocellular neoplasia in trout is not known, but it is a hypothesis worthy of investigation.

Several routes of exposure have been effective in initiating cancer in rainbow trout including dietary (4, 5), ip injection (19), and static exposures of embryos or fry to aqueous solutions of carcinogens (20).

Gross Features of Trout Tumors

Trout exposed to high doses of strong carcinogens develop in 4-9 months large liver tumors which may be multiple (fig. 1) or single (fig. 2). Low doses of strong carcinogens or high doses of weak carcinogens will usually result in single, small tumors that require 12-18 months to appear. Grossly. small liver tumors are usually pale gray, tan. yellow. or white in color and centrally depressed. The larger tumors may be similar or mixed in color and are more nodular, often containing areas of hemorrhage, necrosis, or fibrosis. The trout liver, variable in shape and inconsistently organized into lobes, does not appear to have a particular region more susceptible to tumor formation. It does seem, however. that tumors appear more frequently on the dorsal convex surface rather than on the ventral concave surface. Generally, most hepatic tumors in trout can be observed grossly on the liver surface, but hand slicing and histological sections are required for one to detect the occasional tumors present internally. Surface tumors are first detectable when about 0.5 mm in diameter. Our usual laboratory procedure is to fix liver tissue in Bouin's solution for routine histology. We observed that fixation in Bouin's fortuitously provided a convenient means of gross tumor detection, particularly for internal growths. Bouin's fixative gives trout hepatic tumors a light yellow color, easily discernible against the darker mottled background of normal trout liver tissue (21). Hand slicing fixed livers into l-mm sections with a razor blade thus permits fairly accurate detection of hepatic tumors as small as 0.5 mm in diameter. Foci of altered cells smaller than 0.5 mm can only be observed histologically in prepared slides.

Metastasis

Metastasis of trout liver tumors occurs infrequently under most experimental conditions. Documented cases of metastasis are available in the literature (22, 23) and have been observed in our laboratory. These cases usually involve fish that are 3-6 years old. For instance. Ashley and Halver (22) reported that 30% of a group of 4-year-old. tumor-bearing rainbow trout had metastases to other organs. We often observe vascular invasion by neoplastic cells (fig. 3) or tumor emboli, or both (fig. 4) in 12- to

FIGURE 3.—Invasion of a large hepatic vein by a well-differentiated hepatocellular carcinoma in a rainbow trout fed 20 ppb AFB_1 for 4 wk and killed I yr later. Nore the protrusion of tumor tissue through the vessel wall into the lumen of the vein. H & E. Bar= 50 μm. × 320

FIGURE 4.—A tumor embolus in a large hepatic vein from a trout liver having several welldifferentiated hepatocellular carcinomas. The vein wall, a small hepatic artery, and normal liver tissue are present in the lower *left* of the photograph. The rainbow trout had been fed a diet containing 20 ppb AFB_1 for 4 wk and sampled I yr later H & E Bar = 50 μm × 512

18-month-old hepatic tumor-bearing rainbow trout that have been killed, which suggests the potential for metastases if the fish were allowed to live longer. It is not clear why hepatocellular carcinoma is slow to metastasize in the trout. The low temperatures at which these animals live delay overall cellular metabolism and possibly liver tumor growth, whereas the well-differentiated nature of most trout hepatic tumors suggests weakly malignant behavior rather than the invasiveness found in more anaplastic tumors. However, among the occasional cases of liver tumor metastasis in trout, most have involved well-differentiated tumors of apparent low malignancy.

Transplantation

To our knowledge, only one investigator attempted to demonstrate the transplantability of trout liver tumors. The obvious reason for this is that no inbred strains of rainbow trout have been available in which these experiments could have been performed. Halver (*18*), using a heterogeneous population, attempted to minimize the heterogeneity by using type sera and cross-matching as closely as possible donors with recipients. Using this technique, small fragments of tumor tissue were transplanted to the liver, stomach, spleen, pyloric cecal area, testes, ovaries, cardiac chamber, eye, muscle, and subcutaneous fat of recipient fish. After 90 days, transplanted tumor tissue was found growing in about 10% of the recipients. The tissues in which most transplants survived were the liver, musculature of the stomach, and the pyloric cecal area. Only one successful transplant occurred in a recipient fish which had not been serologically matched with its donor. These results indicated that trout liver tumors are transplantable, and, when syngeneic populations or possibly clones of rainbow trout are available, this characteristic and additional criterion for malignancy should be routinely demonstrable.

FIGURE 5.—A section of trout liver stained histochemically for glucose-6-phosphatase activity. Four small, probable hepatocellular carcinomas are present and show a marked deficiency of this enzyme compared with that in surrounding normal tissue. Bar= 100 μm. X 80

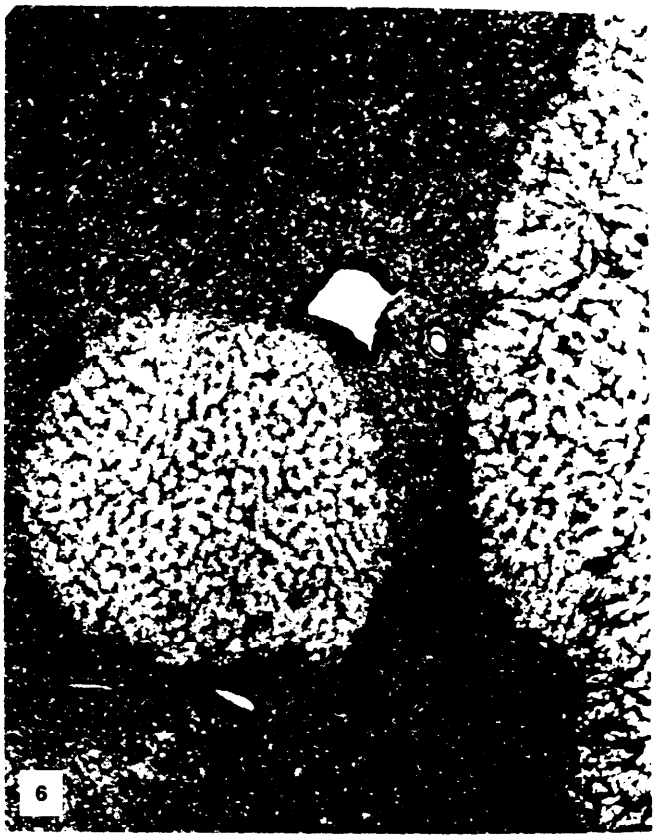


FIGURE 6.-A small glycogen storage nodule and a portion of a larger one in the liver of a rainbow trout fed 405 ppm methyl sterculate (a CPFA) for 11 mo and sampled 7 mo later. Hepatocyte nuclei are normal, but the cytoplasm is engorged with glycogen. Slight compression of surrounding tissue is evident. H & E. Bar= 100 μm. X 80

FOCI AND AREAS OF CELLULAR ALTERATIONS

In rats, 3 distinctive cellular lesions appear in the livers of animals exposed to hepatocarcinogens: the clear, the acidophilic, and the basophilic lesions (I, 2). In addition to their descriptive tinctorial characteristics with H & E staining, they also demonstrate a number of functional anomalies when histochemical techniques are used. Some of these include deficiencies of glucose-6-phosphatase and adenosine triphosphatase, abnormal glycogen storage, increased y-glutamyl transpeptidase activity, and an inability to store iron under siderotic conditions. These lesion types

may occur singly, in twos, or all three may be present in the same liver (*1*, *2*). They are considered to be preliminary stages in the development of hepatocellular carcinoma and may also coexist with fully developed liver tumors. If these lesions are smaller than the size of a lobule, they are referred to as foci rather than areas which occupy portions of 2 or more lobules (2).

In rainbow trout, lesions similar to those types in rats have been observed but are much less characterized for lack of functional studies. The only histochemical staining that we have successfully attempted on trout liver tumors at this time is with glucose-6-phosphatase using the method of Wachstein and Meisel(24). Liver lesions large enough to be hepatocellular carcinomas have consistently been deficient in this enzyme compared with normal tissue (fig. 5), although we have not identified the lesions histologically in serial sections. At present, we have no information on the histochemical characteristics of altered liver foci in trout.

In the descriptions that follow, most of the lesions that we have observed are from trout exposed to AFB_1, although similar lesions occur with other carcinogens. Those lesions which appear unique to certain compounds will be noted. We have arbitrarily designated basophilic lesions less than 0.5 mm as foci and greater than 0.5 mm as carcinomas. Eosinophilic lesions are invariably small and classified as foci. Other lesions that are larger than 0.5 mm but not neoplastic are termed nodules.

Clear Cell Focus

A clear cell focus in rats is an obvious lesion due to the apparent emptiness of cell cytoplasm resulting from excessive glycogen storage. A counterpart for this lesion does not occur in rainbow trout when exposed to most classical liver carcinogens. Glycogen-laden cells, which form large nodules that may become larger but do not appear to be associated with neoplastic development, occur in trout exposed to high dietary levels of CPFA ($\geq$200 ppm) for extended periods (fig. 6). The hepatocytes are normal except for the excessive glycogen storage. These nodules are obviously expansive lesions evidenced by compression of surrounding tissue (fig. 6); however, mitotic figures are almost never seen. The significance of this lesion is not known, but we believe it is not comparable to the clear cell focus of rats.

Acidophilic Cell Foci

Foci of parenchymal cells, characterized by intense eosinophilia, are frequently observed in the livers of trout exposed to various carcinogens (4, 5, 23, 25, 26). Hepato-

FIGURE 7.—A small focus of eosinophilic hepatocytes in the liver of a rainbow trout fed 20 ppb AFB_1 for 4 wk and sampled 9 mo later. *Note* the hypertrophied cells, enlarged, abnormally shaped nuclei, and lack of glycogen storage compared with surrounding normal tissue. Some cords of eosinophilic cells are continuous with cords of normal ceils. H & E. Bar= 50 μm. × 512

FIGURE 8.—A larger focus of eosinophilic hepatocytes similar to those described in figure 7. Normal liver tissue is present *above* and *to the right* of the lesion and around the bile ducts at lower *left*. The trout was fed 20 ppb AFB_1 for I mo and sampled I yr later. H & E. Bar = 50 μm. X 512

cytes within these foci are hypertrophied and have enlarged often atypical nuclei and dense eosinophilic glycogen-free cytoplasm with a ground-glass appearance (figs. 7, 8). These lesions are generally small (<0.5 mm in diameter) having the characteristic 2-cell-wide hepatic tubulocords which are larger than normal due to individual cell hypertrophy. These cords are continuous with adjacent normal cords and cause no compression. Mitotic figures, though rare, can be observed. Ultrastructural observations of these foci have shown that the intense cytoplasmic eosinophilia is due to an abundance of smooth endoplasmic reticulum (26). As mentioned. no data are available on the histochemical properties of these foci, and their significance is uncertain. Nonetheless at least two interesting observations have been made. First and most important is that these foci are often infiltrated and largely destroyed by cells of the immune system, predominantly lymphocytes (figs. 9. 10). Although only speculative, it would appear that these eosinophilic cells are recognized as antigenically foreign by the immune system; thus they are destroyed by cytotoxic lymphocytes. Secondly, because of the above described phenomenon, it is only rarely observed that an eosinophilic-basophilic transformation occurs in trout liver (figs. 11, 12). More commonly, we see the appearance of basophilic foci without any indication of a prior eosinophilic stage.

Basophilic Cell Foci

These lesions are usually small and distinguishable at a size of only a few cells in diameter, but they present a continuum of sizes through Iarge hepatocellular carcinomas (3, 4, 5, 23, *25-32*). They consist of small, unencapsulated, basophilic, glycogen-deficient cells with enlarged nuclei and prominent nucleoli. Small basophilic foci generally have cords 2 cells in thickness but the presence of multicell cords may also be observed. Mitotic figures are often present, but compression of surrounding tissue has not yet occurred (fig. 13). Ultrastructurally, the extreme basophilia is the result of extensive granular endoplasmic reticulum and free ribosomes (26). As previously mentioned, these foci usually appear directly in the midst of otherwise normal cells, and only rarely is an eosinophilic-basophilic transformation seen. Unlike eosinophilic foci. basophilic foci rarely elicit an immune response (*33*).

Basophilic foci similar to the focus shown in figure 13 appear as discrete foci, not continuous with the surrounding cords of normal cells. Some basophilic foci (fig. 14) appear less malignant and have cords of basophilic cells that are continuous with normal tissue, fewer or no observable mitoses, and more normal nuclei. The significance of these less malignant foci is not known at this time

Vacuolated Ceil Foci

Bannasch (34) described hepatic cells that contained numerous lipid droplets as components of neoplastic nodules in rats. Although we have not obsened lipid-containing hepatocytes as a regular component of actual liver neoplasia in trout, we often see foci of lipid-engorged hepatocytes (fig. 15) as a common lesion in liver from trout receiving dietary or embryo exposures of strong carcinogens such as AFB_1, dimethylnitrosamine, diethy lnitrosamine, or N-methyl-N'-nitro-i-nitrosoguanidine. These foci of lipid-filled hepatocytes do not appear to be involved in any type of stepwise progression toward neoplasia but represent another toxic effect of these compounds on trout hepatocytes. These lesions usually precede the appearance of visible liver tumors, but they are persistent and later occur with overt hepatic neoplasia. Their presence in a trout liver provides good evidence that hepatocellular carcinomas will eventually develop. They may be more closely related to the lesion of fatty change described by Stewart et al. (2).

PRIMARY NEOPLASMS

Our understanding of the neoplastic process in trout. as in rats (34). is that the appearance of the basophilic cell type. described above for basophilic foci, signifies that neoplastic transformation is complete. We distinguish between basophilic foci and hepatocellular carcinoma only on the basis of lesion size and compression of surrounding tissue. Because the same glycogen-poor basophilic cells comprise both lesions, we realize this distinction is arbitrary. We might more appropriately call these basophilic foci microcarcinomas or carcinomas in situ. Inasmuch as we have evidence that they develop into hepatocellular carcinomas.

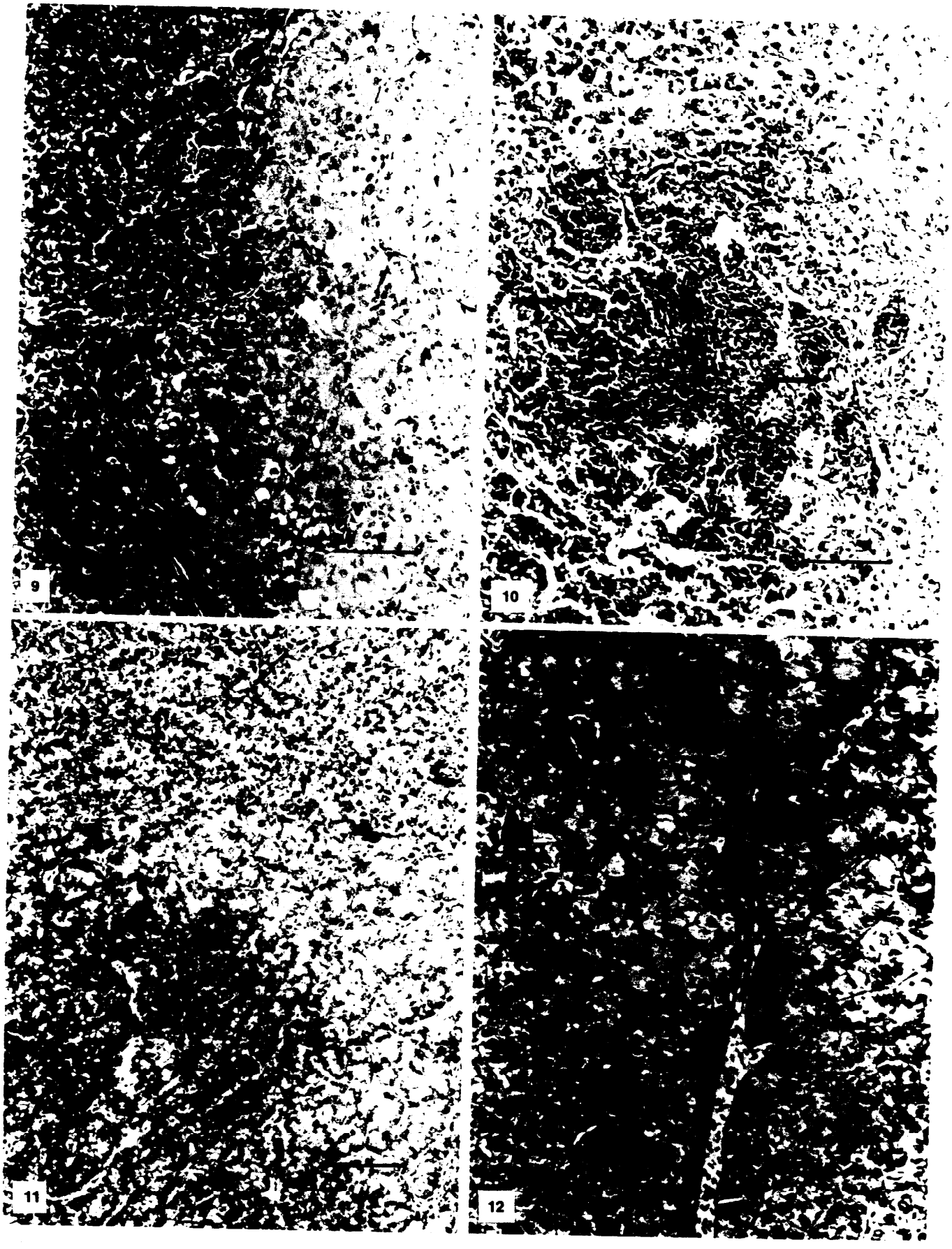
No lesion in trout is comparable with the neoplastic nodule of rats that is proliferative and may be composed of a combination of clear, eosinophilic, basophilic. or vacuolated ceil types (2. 34). It is considered neoplastic. as its name implies, and is an integral part in the rat hepatocarcinogenesis process. Because the most common pathway to neoplasia in trout is directly through the basophilic cell type present in basophilic foci and hepatocellular carcinomas. the designation of a stage, within that continuum of lesions, as a neoplastic nodule, is unwarranted.

FIGURE 9.-A larger focus of eosinophilic hepatocytes with extreme cellular hypertrophy, large. irregular nuclei with multiple nucleoli. and beginning invasion of lymphocytes along the *left* edge of lesion. Carcinogen exposure was dietary AFB_1 at 20 ppb for 4 wk. H & E. Bar= 50 μm. X 320

FIGURE 10.—A more advanced stage of lymphocytic invasion and destruction of an eosinophilic focus in the liver of a rainbow trout fed AFB_1 at 20 ppb for 4 wk. Only a few sequestered groups of eosinophilic cells remain (arrows). H & E. Bar= 50 μm. x 320

FIGURE 11.-A neoplastic lesion in the liver of a rainbow trout fed AFB_1. At the top of the photo are cords of normal hepatocytes. In the *middle* and *on the right* are cells of the eosinophilic type that merge directly with deeply basophilic cells at lower *left*. This is a rare observation. H & E. Bar= 50 μm. x 320

FIGURE 12.—A portion of an eosinophilic focus from a rainbow trout liver (exposed to AFB_1) within which individual cells are undergoing basophilic change (arrows), This observation is also rarely seen. H & E. Bar= 50 μm. X 5 12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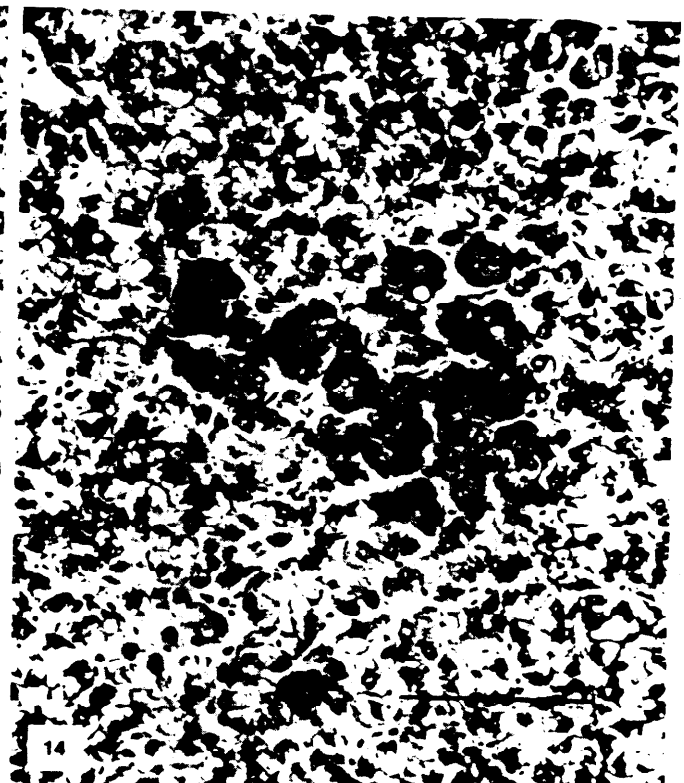
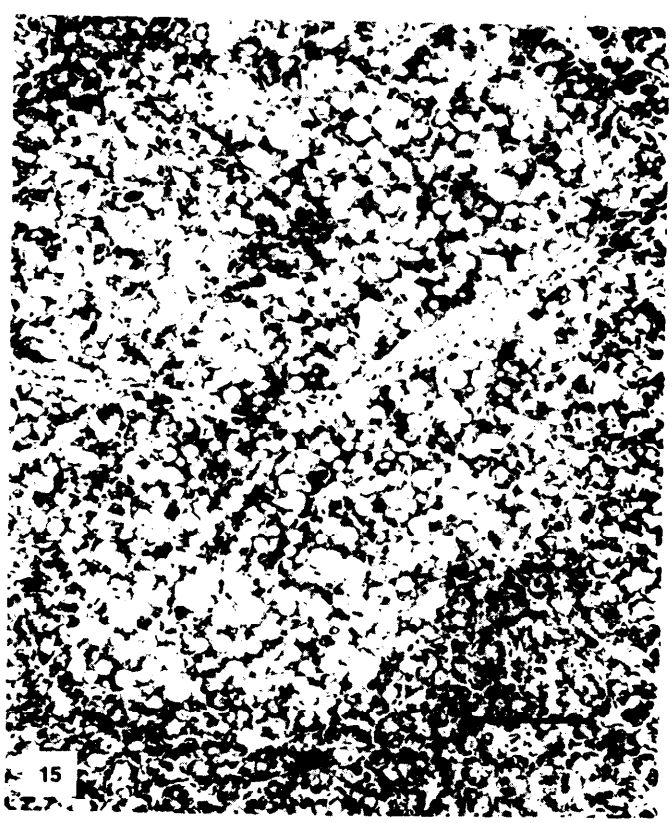


FIGURE 13.-A focus of basophilic cells in the midst of normal appearing hepatocytes with no indication of a prior eosinophilic stage. The trout was fed 6 ppb AFB_1 continuously for 6 mo. *Note* the small deeply basophilic, glycogen-poor cells, large nuclei. and mitotic figure. **H & E.** Bar= 50 μm. X 5 12. Figure is reproduced with permission of the publisher (4).

FIGURE 14.-A focus of basophilic cells that are less intensely basophilic and more normal in appearance. Some cords of these cells are continuous with cords of normal cells. The trout was fed 6 ppb AFB_1 for 6 mo. H & E. Bar= 50 μm. X 5 I2



Trabecular Carcinoma

Trabecular carcinoma is the most frequently observed form of carcinoma in rainbow trout liver. Described by many authors (*3-23, 25-33*), it is composed of broad, multicell cords of basophilic hepatocytes alternating with endothelium-lined sinusoids. The cords of the tumors are discontinuous with those of adjacent liver tissue and lie perpendicular to the compressed cords and sinusoids of the surrounding normal liver tissue (fig. 16). The cells are similar to those described for basophilic foci, but mitotic figures are usually more numerous (fig. J 7)

As a variant of this general trabecular pattern, tumors are often observed that have peripheral trabecular components and centrally located hyperplastic bile ducts and supporting fibrous connective tissue stroma (fig. IX) We do not believe the bile duct component is always neoplastic because the bile ducts are mostly normal in structures with few mitotic figures. The low columnar to cuboidal duct cells stain lightly with eosin and have round to oval nuclei. The eosinophilic staining is in marked contrast to the basophilic hepatocellular tumor cells (fig. 19). We have

FIGURE 15.—A focus of lipid engorged hepatocytes in the liver of a trout fed AFB_1 at 20 ppb for 4 wk and sampled I2 mo later. Surrounding normal hepatocytes have little or no demonstrable lipid H & E. Bar= 50 μm. X 320

FIGURE 16.—A well-differentiated trabecular hepatocellular carcinoma (*below*) from a trout exposed to 0.5 ppm aqueous AF B_1 as an embryo and killed 12 mo later. Nore the broad cords of basophilic tumor cells, large vesiculated nuclei with prominent nucleoli, compression of adjacent normal tissue (above), and mitotic figures. H & E. Bar = 50 μm. X 320. Figure is reproduced from (17)

FIGURE 17.—A portion of a well-differentiated, trabecular hepatocellular carcinoma illustrating cellular detail and frequent mitotic figures. Trout was exposed to 20 ppb dietary AFB_1 and killed 12 mo later. H & E. Bar= 50 μm. X 512

demonstrated that normal bile ducts in trout are commonly PAS positive in the apical portions of the biliary epithelium (fig. 20). Such staining is also present in many of the hyperplastic ducts within these tumors, which indicates they are of true bile duct origin (fig. 21). Bile duct hyperplasia probably results when an expanding trabecular carcinoma envelops a portal region with its bile duct(s). Among the classical causes of hyperplasia (nutritional deficiencies, endocrine imbalance, irritation), chronic irritation resulting from the mechanical growth of tumor cells or perhaps from a product of tumor cell metabolism (33) is most likely the stimulus for proliferation of bile ducts and supportive connective tissue. Sometimes, the bile duct and connective tissue components actually appear to overgrow and replace the hepatocellular component of a tumor (33) as is illustrated in figure 22.

Adenocarcinoma

Unlike the tumors having the benign bile ductular hyperplasia described above, other liver tumors of trout have a glandular or acinar component which appears to be neoplastic (fig. 23). This is in addition to the neoplastic hepatocellular elements already present. These glandular areas are composed of duct-like structures with irregularly shaped lumina, some having papilliferous projections and adjacent solid nests of the same cell type. These neoplastic cells have a high mitotic index and are basophilic (fig 23). The question arises as to whether these neoplastic cells are of hepatocyte or bile duct origin. In rats, researchers disagree on whether biliary epithelium gives rise to adenomatous tumors (2). Until this controversy is resolved, it is suggested that these tumors be classified as adenocarcinomas (2). Ashley and Halver (14) argued that the glandular component was derived from hepatocytes rather than bile ducts and called the tumors adenocarcinomas. At least two histological features of the glandular structures suggest to us that they are not of hepatocyte origin. First there is the presence of connective tissue that invariably surrounds the glands (ducts); in trout liver, this is a normal feature of bile ducts but not of hepatocytes. Secondly, although the staining of the neoplastic liver cells and the glandular cells appears more similar to figure 23 than it does in figure 19, the nuclei are distinctly different from those of neoplastic liver cells. They are more irregular in shape and lack the

prominent nucleolus of neoplastic hepatocytes. At this time, we believe that the ductlike structures more nearly resemble neoplastic bile ducts rather than glands derived from hepatocytes. Thus a classification of mixed carcinoma or hepatocholangiocarcinoma would appear appropriate in these uncommon instances. More research on the nature of these structures is obviously needed.

Cholangioma

Occasionally, we have noted proliferative lesions composed entirely of ductular elements and supportive stroma especially in feeding trials with dichlorodiphenyltrichloroethane (figs. 24, 25). The duct cells are columnar in shape, stain lightly with eosin, and have round-to-oval nuclei that stain lightly with hematoxylin; the ducts are often multilocular and mitotic figures are common. These are clearly bile duct tumors with no accompanying hepatocellular component.

Poorly Differentiated Carcinomas

The degree of anaplasia in trout liver tumors is usually quite restricted, although some tumors do vary from the usual well-differentiated type with its cells of uniform size, shape, and staining characteristics (fig. 26). The more anaplastic tumors have cells that are irregular in size and shape, not organized into well-defined trabeculae. mitotically active, and that may have spindle-like cells. Giant multinucleated cells are rare (fig. 26). Although most trout liver tumors are benign in appearance, the majority of these tumors eventually exhibit malignant behavior. Thus we feel it is speculative to distinguish between malignant and benign liver tumors only on the basis of histological detail. As in rats (*1, 2*), mature benign neoplasms of hepatic origin in trout cannot be determined with certainty.

ASSOCIATED LESIONS

Ceroid Deposits

Ceroid, a polymerized lipid pigment insoluble in organic solvents is both acid-fast and PAS positive. During hepatocarcinogenesis in trout, variable amounts of this pigment are observed in macrophages within melanomacrophage centers surrounding portal tracts of the trout liver (fig. 27).

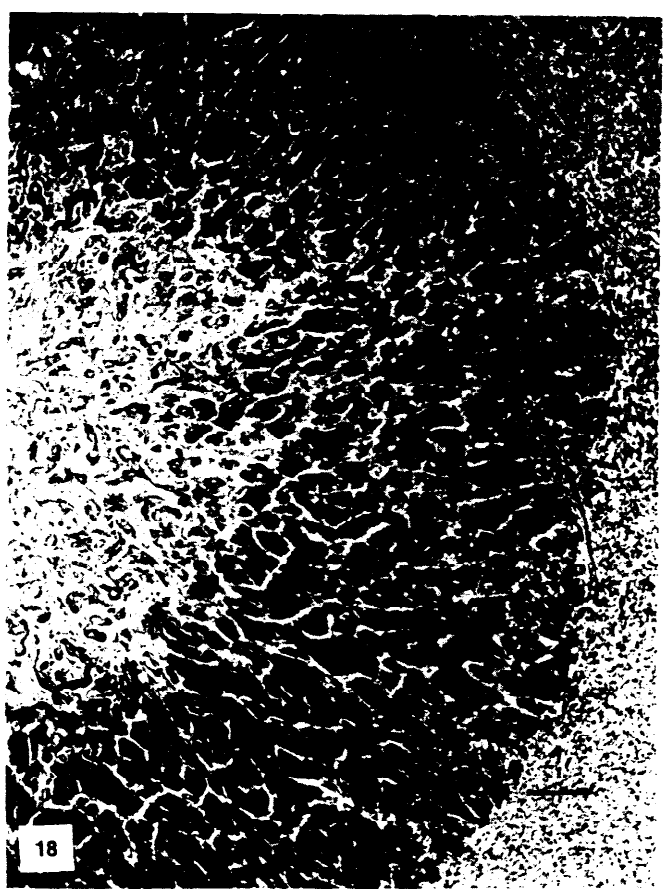
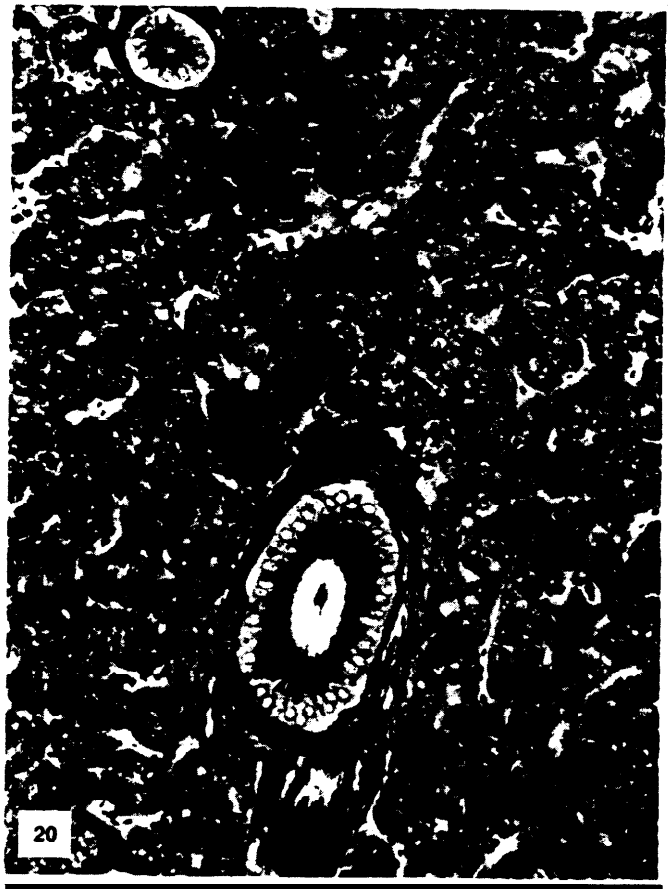


FIGURE 18.—A hepatocellular carcinoma with peripheral basophilic trabecular components and centrally located acidophilic bile ducts and fibrous connective tissue. The trout was exposed to 0.5 ppm sterigmatocystin for I hr as an embryo and killed I2 mo later H & E. Bar= 100 μm. X 80. Figure is reproduced from (II).

FIGURE 19.—Central region of a hepatocellular carcinoma initiated by embryo exposure to AFB_1. Photo illustrates the distinct staining differences between the hepatocellular and bile duct components. Trabecular tumor tissue has been sequestered rnto small islands by the bile ducts and connective tissue. H & E. Bar= 100 μm. × 320. Figure IS reproduced from (II).



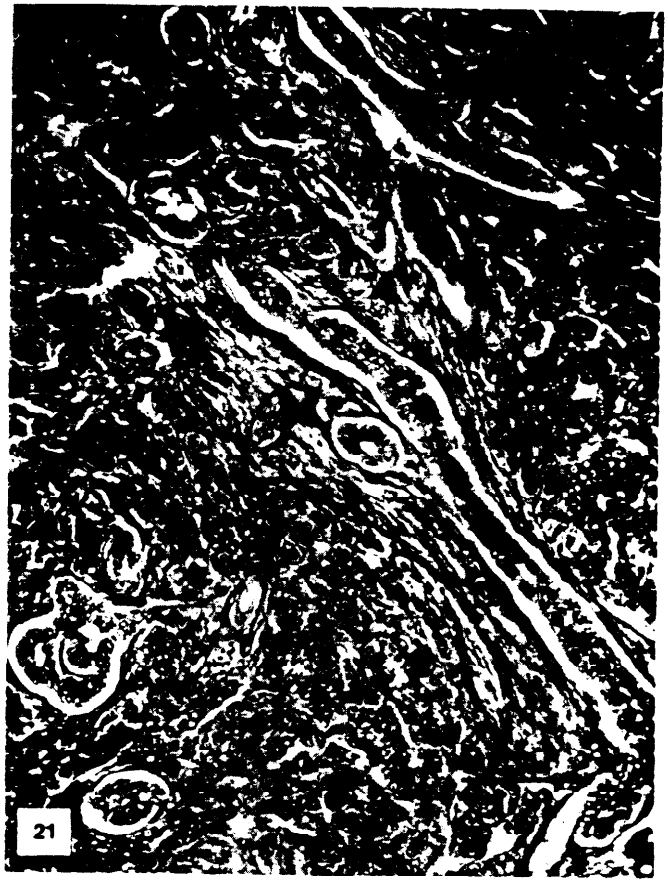


FIGURE 20.—A control trout liver containing a large and small bile duct. *Note* the intense PAS positive staining at the luminal border of the large bile duct cells and the weaker, sporadic staining of the cells in the smaller bile duct. PAS Bar = 50 μm. × 512

FIGURE 21.—Central region of a hepatocellular carcinoma illustrating the PAS-positive nature of the luminal surface of the bile duct cells. Staining intensity is similar to that in the small bile duct of figure 20. PAS. Bar= 50 μm. × 320

FIGURE 22.—A tumor-like lesion composed entirely of connective tissue and some sparsely located bile ducts. Whether this lesion started as a hepatocellular carcinoma is not known, but the bile duct and connective tissue elements are similar to those seen in other tumors such as shown in figure 18. Trout was exposed to 20 ppb dietary AFB_1 and sampled 12 mo later H & E Bar = 100 μm. × 80

FIGURE 23.-A field from a trout liver carcinoma having **both** neoplastic liver cells and glandular (ductular) **structures.** Neoplastic hepatocytes are slightly more basophilic **than the** glandular cells. Nore the irregular lumina of the **ducts,** papilhferous projections, solid nests of cells, mitotic figures, and supportive connective tissue. Trout was fed a diet containing 800 ppm dimethylnitrosamine for 12 mo **and** killed **after** 18 mo. H & E. Bar = 50 μm. × 512

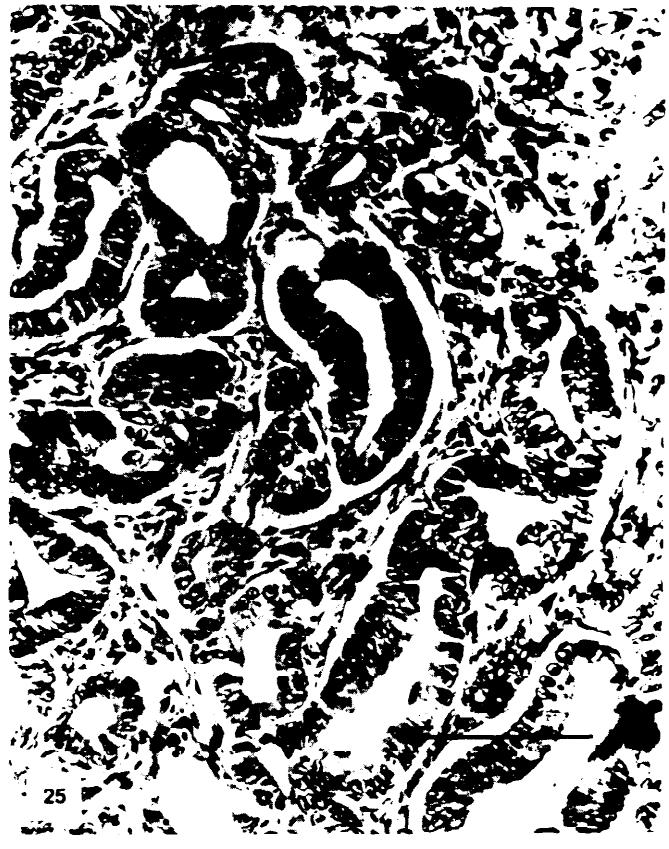
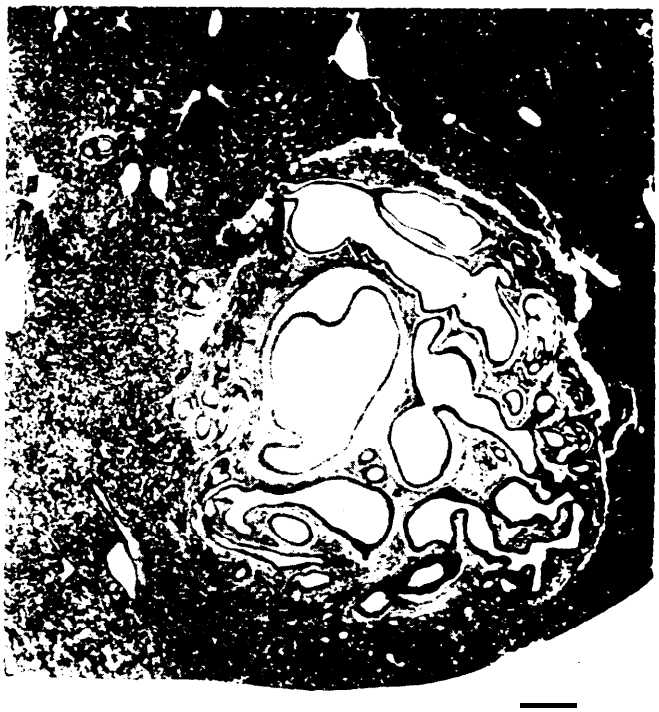


FIGURE 24.-A small cholangioma in the liver of a trout fed 25 ppm dichlorodiphenyltrichloroethane for 18 mo. **Lesion** is **composed** entirely of neoplastic bile ducts and supportive connective tissue. **H & E. Bar= 100 μm. X 80**

FIGURE 25.-Detail of **figure** 24 showing neoplastic bile ducts. *Note* the multilocular nature of the ducts, frequent mitotic **figures.** and absence of neoplastic hepatocytes. H & E. Bar = **50 μm. X** 5 12

FIGURE 26.-A portion of a more anaplastic hepatocellular carcinoma from a trout exposed to dietary dimethylnitrosamine. Hepatocytes are irregular in size and shape, not organized into well-defined trabeculae, possible spindle cells are present, and mitoses are common. H & E. Bar = 50 μm. X 5 12

FIGURE 27.-Ceroid deposits within a melano-macrophage center surrounding a portal area in the liver of a trout fed 50 ppm CPFA for 12 mo. H & E. Bar= 50 μm. X 512



28

FIGURE 28.-A multilocular cyst from the liver of a trout exposed to a 0.5 ppm AFB_1 as an embryo. The cuboidal epithelium of the larger cysts is detached from the stroma. H & E. Bar = 100 μm × 80

cysts

Multilocular cysts often occur in the livers of trout treated with hepatocarcinogens. They are often on the surface and are composed of lightly staining cuboidal cells lining empty lumina (fig. 28). Their origin is not clear.

Adenofibrosis

This lesion, occupying large areas of the liver, is composed of immature bile ducts and an abundant connective tissue stroma (fig. 29). Particularly prevalent in trout subjected to prolonged dietary administration of CPFA, it is a rare lesion when trout are exposed to other carcinogens.

Pancreatic Cell Metaplasia

This lesion has been observed in trout fed CPFA or AFB_1. In the CPFA exposure, pancreatic cell metaplasia appeared in otherwise normal liver parenchyma as well as within hepatocellular carcinomas. In AFB_1-treated trout, it has only appeared within the tumor mass of large hepatocellular carcinomas. Special stains have not been applied to

FIGURE 29.—**Adenofibrosis** in the liver of a trout fed AFB_1-contaminated milk. *Note* the immature bile ductule-like structures, abundant connective tissue, and a moderate number of inflammatory cells. H & E. Bar= 50 μm. × 320

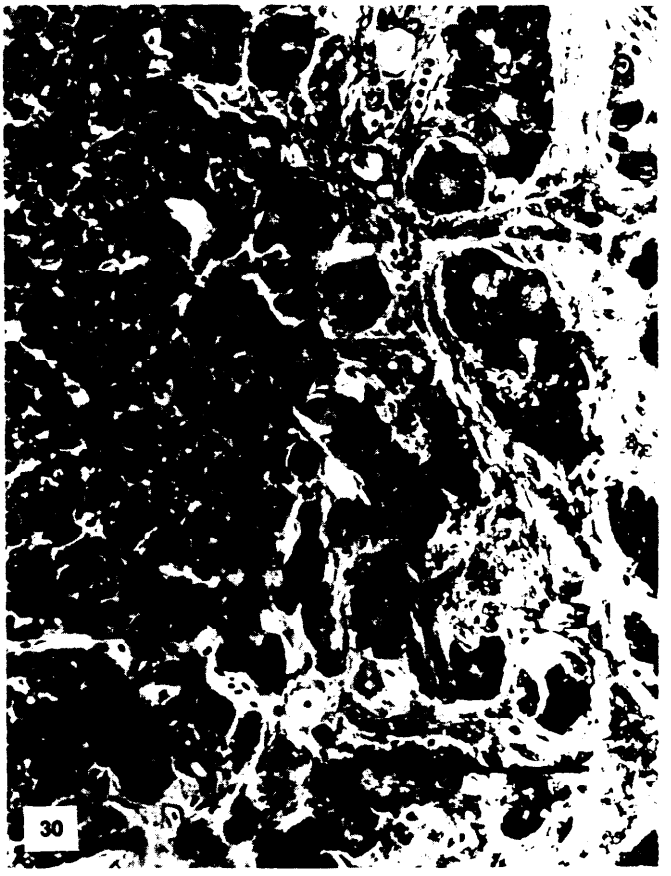


FIGURE 30.-Portion of a hepatocellular carcinoma with **both** neoplastic hepatocytes *(right)* and pancreatic **cell** metaplasia (*left*). Nore the **apically** located zymogen **granule\ and large** nucleoli in many of the pancreatic acinar **cells.** Fish were **fed** AFB_1 at 20 ppb for 4 wk. H & E. Bar=50 μm. × 512

these lesions, but, histologically, the cells are similar to exocrine pancreas, complete with zymogen granules and a large prominent nucleolus (fig. 30).

CONCLUSIONS

This discussion has illustrated the statements made earlier, that although we have made a number of observations concerning cancer of the trout liver, a great deal of effort is still needed for better descriptions and classifications of these neoplastic lesions. From this effort, a better understanding of the neoplastic process may evolve. We hope that these observations will prove useful and encourage others to assist in fitting together the unknown pieces of the puzzle, so that a complete picture of liver neoplasia **in** trout can be formed.

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DISCUSSION

J. Klaunig: Have you seen any correlation between the number of focal lesions, the basophilic and eosinophilic lesions, that appear early and the number of tumors that are produced? For example, in the rodent livers, there is usually an excess number of focal lesions, and only a few tumors produced.

J. D. Hendricks: That is something we really have not studied. so I cannot give you a good answer. Although I do know that. particularly with feeding trials, with aflatoxin and other carcinogens, we do see a multiple tumor situation, rather than just I or 2. Generally speaking, the norm is many tumors per liver, so it indicates that a number of them do progress on to carcinomas.

J. Rice: I have a question for you about the capacity of these lesions to metastasize. Is it not true that in the trout hepatocellular carcinomas metastases may form grossly apparent lesions at sites such as the gills, and if so. how far advanced do the primary lesions have to appear for this to be demonstrable?

Hendricks: Metastases definitely do occur, but generally only late in life. During the duration of most of our experiments, which are usually I year in length, we do not see metastases. However, the metastases that have been observed have usually occurred in fish that are 2. 3, or 4 years old; they have been found in various tissues, e.g.. gill, the kidney.

Rice: Do you think it is possible that they are found so late in life largely because of a lack of really detailed, systematic histological examinations for micrometastases that might be demonstrable earlier in life?

Hendricks: That is possible because, at least up until just the past few years, most of our histopathology has dealt just with the liver. and so, really, we have not investigated further. I think that is a good possibility.

H. C. Chen: Have you ever seen vascular changes associated with what you saw in the liver tumors or in degenerative changes in the liver, such as peliosis hepatis?

Hendricks: It is not a common occurrence. Another interesting point is that in rodents hemangiosarcomas are fairly common, but I have never seen one in trout liver.

C. **J. Dawe:** I know that Dr. Hendricks is aware of it but did not mention the fact that the pancreatic metaplasia lesion that he has shown has also been seen in rat livers in experiments in carcinogenesis. I think Kimbrough was

probably the first one to describe that. They are similar, and they are a great mystery, at least to me.

We know that the pancreatic rudiments, the anlage for pancreas and that for a liver, both originate close together as diverticulae from the gut. It seems possible, at least, that if these carcinogens are either mutagenic or epigenetic or have epigenetic influences on cells that are transformed, that, rarely, it is possible to get a switchover from liver expression to pancreas expression in some of these lesions. That is merely a rationalistic way of looking at it, and I believe we know nothing about the details.

Hendricks: Recently, Dr. Scarpelli showed the reverse situation in pancreas, a tissue in which he saw differentiation into hepatocytes, so that is just another flip-flop of that idea.

K. Hoover: Recently, investigators at the National Toxicology Program completed testing the compound 2,6-dichloro-p-phenylenediamine, and the pathologists there also observed ectopic hepatocytes adjacent to the islets of Langerhans in the pancreases of some treated rats. My questions have been progressively answered, but I would like to know more details about the carcinogenesis of dichlorodiphenyltrichloroethane and benzo[*a*]pyrene in trout.

Hendricks: Dichlorodiphenyltrichloroethane, surprisingly, is quite carcinogenic in the rainbow trout. A dietary level of only 25 ppm induced tumors of the liver in approximately 50% of the animals after 18 months, and it was interesting in that it was definitely sex related. The number of females with tumors was up nearly 75%, and the number of males was down about 20%; it is something that needs to be studied in greater detail.

The report on benzo[a]pyrene is some work that we have just recently completed. We have not even looked at all the slides yet, histologically, but we can say for sure that, without any promoting compounds involved, benzo[*a*]pyrene, by dietary exposure, does produce tumors in the rainbow trout.

Hoover: In what concentration?

Hendricks: It was a high one: 1,000 ppm.

J. C. Harshbarger: Some of the tom cod lesions, i.e., those tumors that Mr. Charlie Smith worked with from the Hudson River, are metaplastic pancreatic tissue also. As a matter of fact, quite a lot are. In those tumors that he studied, the hepatocytes were usually grossly hypertrophied. The tumors had large static pools of blood and serum.

Did you ever see the hypertrophied cells and the pools of blood in the trout?

Hendricks: Within the tumor itself?

Harshbarger: Yes.

Hendricks: Not usually. They are pretty uniform as you can see there in the slides.

D. E. **Hinton:** Have you any ideas about how you might design a study with tumor-laden fish to investigate reversibility of lesions?

Hendricks: Perhaps the best way to answer that is with our embryo exposure, which is a one-time exposure for a brief period and early in life; we can hold these fish for years and those tumors persist. I do not really know what to do beyond that. It is obvious that the tumor continues to develop in the absence of the carcinogen, and it does not regress.

M. Sigel: You may have already broached one of my questions. How does your basophilic cell relate to Dr. Hinton's cell with the increased rough endoplasmic reticulum? Is this the same cell to which he was referring?

Hendricks: Well, certainly, our basophilic cell has an increased rough endoplasmic reticulum. It is really packed with that particular organelle.

Sigel: Would you both agree that this is maybe the essence of a transformed cell in the early stages of carcinogenesis? Thus far, there has been no consensus about anything. is this the beginning of a consensus perhaps, in two parts of the country?

Hendricks: Yes, I believe that the basophilic cell is the first neoplastic stage that we see. What do you think. Dr. Hinton?

Hinton: Well, I am trying to think. The compound that produced the proliferation, if not carcinogenic, has been shown to be a promoter at least, and it certainly serves as a warning sign.

Sigel: I have a second question, if I may. regarding metastases. Where were the metastases located? Would you care to speculate whether metastases occur when the tumor develops late in life, or whether the fish gets an early tumor. manages to survive for a long time, e.g., for I or 2 years, despite a gross lesion in the liver, and then develops metastases? Which is your guess or speculation in this case?

Hendricks: First of all, metastases, like I said before, can be found in a number of different organs. I would say the gill is probably one of the first places; the liver may be a second if it is an embolism-type transfer through the heart, but the first place it is going to lodge is the gill.

I believe that a tumor has to be there a long time before it does metastasize. Generally speaking, our fish are much more sensitive, as young individuals or as frv or embryos, than they are as older adult fish. I think it is a situation in which the tumor has been present for a considerable period before it does metastasize.

J. Stegeman: What was the purity of the dichlorodiphenyltrichloroethane that you used?

Hendricks: It was para para prime form. It was not a pesticide-type formulation.

J. Couch: Going back to the question that Dr. Sigel raised concerning the proliferation of rough endoplasmic reticulum, we have to remember that in pretransformed cells you can have the increase in rough endoplasmic reticulum in a basophilic cell resulting from induction or enhancement of the proliferation of reticulum. In the transformed cell, which may be the authentic neoplastic cell, you may have rough endoplasmic reticulum for different functional reasons. This would not negate what you said because you thought that the transformed cells that were neoplastic cells also had the rough endoplasmic reticulum. I think we have to realize that both the nontransformed and transformed cell may have this characteristic for different functional reasons.